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Responsible structural features for cytotoxic and other kinds of activity of *neo*-clerodane diterpenes from genus *Scutellaria*

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Twelve natural neo-clerodane diterpenes, isolated from three *Scutellaria* (*Labiatae*) species, were tested for cytotoxicity on two cell lines, from human tumors of the lung designated as H1299 and normal cells from a navel string (HUVEC), using the MTT (3-/4,5-dimethylthiazol-2-yl/-2,5-diphenyltetrazolium bromide) method. The cytotoxic activity was evaluated as rate of IC_{50} (such concentration of the compound in μM by which half of the cells die). Three compounds, scutalpins A, E and F, exhibited moderate cytotoxic properties on both cell lines. Among all tested compounds the highest activity was detected for scutalpin A, with IC_{50} values of 21.35 and 23.9. Some significant aspects of the relationship structure-activity are discussed.

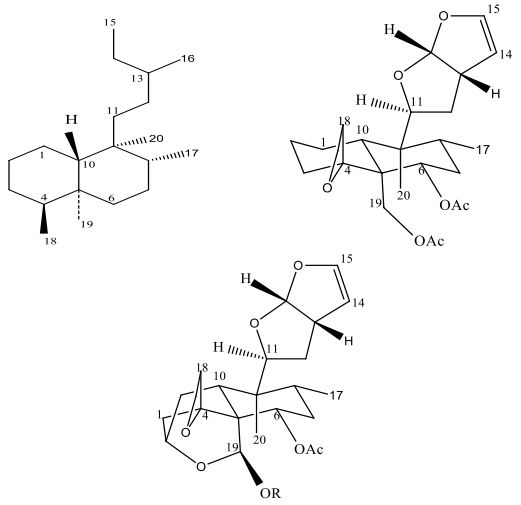
Key words: *Scutellaria*; Diterpenes; Cytotoxic, Antifeedant, Antifungal activities.

INTRODUCTION

The natural *neo*-clerodane diterpenoids, isolated from *Scutellaria* species, draw attention because of their varied biological activities especially as potent insect antifeedants [1-4] and antifungal [5] agents against plant pathogenic fungi.

All diterpenoids isolated from the species of genus *Scutellaria* (Labiatae) are with *neo*-clerodane skeleton (**1**, Fig. 1) which Bruno *et al.* divided in two part substructures bearing different substitutes [1]. The first substructure, including the carbon atoms from C-1 to C-10, is always a *trans*-bound bicyclic system - decalin core. The second one covers the carbon atoms C-11– C-16 and consists of different fragments: tetra- (**2a,** Fig. 2) or hexahydrofurofuran system (**2b)**; α,β-unsaturated-γ-lactone (**3**) junked between C-13 – C-16; γ-lactone 13-spiro, bound up with cyclic ether inclusive C-8 and forming two epimers with C-13S (**4**) and C-13R (**5**) configurations. Finally, in the C-11 – C-16 substructure may be formed a lactol ring that comprises carbons C-11, C-12, C-13, C-16 (**6**) or carbons C-13 – C-16 (**7**).

Clerodanes, obtained from *Scutellaria* plants, displaying the above activities, predominantly possess a decalin ring with C-4-C-18 *spiro*-epoxide and two acetate groups at C-6 and C-19 positions and **2a** or **2b** moiety in the C-11 – C-16 fragment. The compounds clerodin (**8)**, jodrellin A (**9)** and jodrellin B (**10)** exhibit strong antifeedant activity. Particulary **10** was reported to be the most potent antifeedant known to date [4]. These three compounds have been assayed for antifungal activity against the plant pathogenic fungi *Fusarium oxysporum f.* sp. *lycopersici* and *Verticillium tricorpus*. Spore germination of *V. tricorpus* was delayed by clerodin and jodrellin B.



1, 8, 9: R = Ac; **10:** R = CO^{i}Pr

Figure 1. *Neo*-clerodane skeleton (**1)**, clerodin (**8)**, jodrellin A (**9)** and jodrellin B (**10)**

Growth of *F. oxysporum* and *V. tricorpus* was inhibited by the clerodanes in a dose-dependent manner [5].

To the best of our knowledge the *neo*-clerodane diterpenoids from *Scutellaria* species have not been studied for other biological properties. In continuation with our research on this topic we had examined *neo*-clerodane diterpenoids, isolated in our laboratory, for antifeedant activity against *Leptinotarsa decemlineata* (Say) [6,7] and that study confirmed results achieved in previous works. Subsequently, we tested these compounds for antioxidant effects but none of the diterpenes

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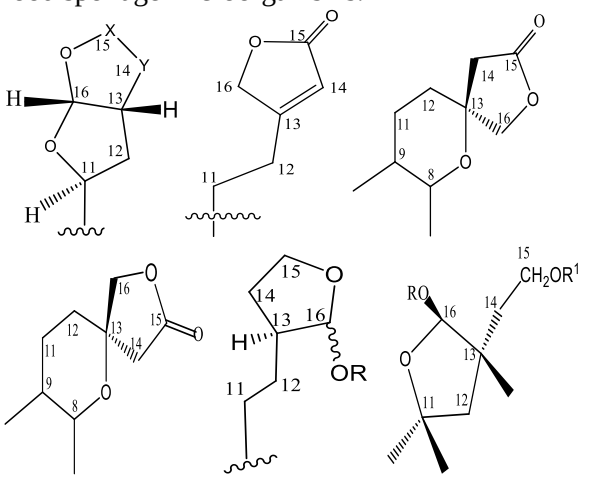
showed such activity. The next step was to evaluate the influence of these clerodanes on pathogenic and food spoilage microorganisms.



X-Y **3** **4**

5 **6** **7**

2a: H**C=CH** R = H; Me R = Alkyl

2b: H_2C—CH_2 R^1 = Acyl

Figure 2. C-11 – C-16 moiety

We concluded that the compounds, containing fragment **4** or **5** in their structures, exhibit antimicrobial activity while the compounds possessing the other kind of C-11 – C-16 moiety are inactive [8].

Herein we report the results from the bioassay of natural *neo*-clerodane diterpenoids, isolated from the acetone extracts of the aerial parts of three *Scutellaria* species, for cytotoxic activity on H1299 and HUVEC cell lines.

EXPERIMENTAL

Plant material: The stems of all plants were collected during their blossoming as follows: *scutellaria alpina* in August 1991, at Pirin Mountains, near Bansko, Bulgaria; *Scutellaria galericulata* in June 2012, near Lovech and Pleven, Bulgaria; *Scutellaria altissima* in June 2011, in Bachkovo, near Assenovgrad, Bulgaria.

Structural data: ^{1}H NMR spectra were recorded on Bruker DRX-250, Varian Mercury-400 or Bruker Avance II+ spectrometers, operating at 250.13 MHz, 400.13 or 600.130 MHz, respectively. ^{13}C NMR spectra were recorded at 100.61 and 150.903 MHz, respectively, on the corresponding spectrometers. TMS was used as internal standard and $CDCl_3$ as solvent. Chemical shifts (δ) are expressed in ppm and coupling constants (J) in Hertz. The IR spectra were registered in KBr pellets on a Perkin-Elmer 1750 FT-IR spectrometer from 4000 cm^{-1} to 450 cm^{-1} at resolution 4 cm^{-1} with 9 scans. MS and HRMS spectra were registered on Accela quaternary UHPLC pump with Accela autosampler and HRMS "Q-Exative" detector (Thermo Fisher Scientific, Waltham, MA, USA) with heated electro spray (H-ESI) interphase.

Extraction and isolation: Dried and finely powdered aerial parts of the plants were extracted with Me_2CO threefold at room temperature for a week. After filtration, the solvent was evaporated to dryness under reduced pressure and low temperature (<40 °C) yielding a gum, which was dissolved in aq. Me_2CO (40 % H_2O, v/v, 100 mL). This solution was cooled to 4 °C for 24 h and filtered. The filtrate was extracted with $CHCl_3$ and the organic layer was dried (Na_2SO_4) and evaporated in vacuum to afford a residue (bitter fraction). This residue was subjected to CC (silica gel Merck n. 7734, deactivated with 10% H_2O, w/w). Pure petroleum ether, followed by a gradient of petroleum ether - EtOAc mixtures (10:1 to 4:1) and dichloromethane were used first as eluting solvents. The diterpene fractions (TLC monitoring) were eluted with 1% to 3% methanol in DCM. Rechromatography or repeated prep TLC of these fractions (2% methanol in DCM or EtOAc as eluent) afforded crude compounds. Crystallization and recrystallization from acetone yielded pure diterpenes.

Test compounds: All tested neo-clerodane diterpenoids (Fig. 3) were available from previous investigations. 14,15-dihydrojodrellin T (**10**), neoajugapyrin A (**11**), scutegalerin A (**12**), scuecolumnin C (**13**) and scutegalin D (**20**) were retrieved from *Scutellaria galericulata L.* as described above [10, 11]. Scutecyprol A (**14**), scupolin H (**15**), clerodin (**16**) and scutaltisin G (**21**) were isolated from *Scutellaria altissima L.* [12, 13]. Diterpenoids scutalpin A (**17**), scutalpin F (**18**) and scutalpin E (**19**) were obtained from *Scutellaria alpina L.* [14, 15]. The molecular structures of the compounds were established by spectroscopic means: IR spectroscopy, MS, 1D (^{1}H, ^{13}C including Dept 125) and 2D (HSQC, HMBC, ^{1}H-^{1}H COSY, NOESY) NMR experiments. Absolute configuration of scutalpin A was determined by X-ray diffraction analysis.

Diterpenoids have been kept in the refrigerator at 4°C. Before doing the cytotoxicity bioassays we measured and compared with the literature the melting points and the IR spectra of the tested compounds. The purity of the compounds was checked by TLC with different solvents (diethyl ether, ethyl acetate, from 1% to 3% methanol in DCM).

Cytotoxicity bioassays. Summary. Cell lines: For the *in-vitro* cytotoxic activity screening, two cell lines were selected: from human tumors of the lung, designated as H1299, and normal cell lines HUVEC (cells from a navel string). The cell lines

were obtained from BPS Bioscience, Recombinant Cell Lines & Assay Kits. The bought cells were sustained in the artificial medium (DMEM – HUVEC; RPMI – H1299) and often resewed. Stock solutions: The working probes were prepared by dissolution of 1 mg of diterpenoides in 50 μL of DMSO (dimethylsulfoxide). To obtain the tests solutions two or three μL from the DMSO solutions of the compounds were diluted in 1 mL culture medium as the concentration of DMSO in the tests solutions became 0.2 – 0.3 %.. For bioassays we took so much quantity of the tests solutions that after subsequent diluting in the culture medium, to ensure a concentration in 125 μL of 300, 150, 75, 37.5 and 19 μM. In such conditions the diterpenoids remained well soluble and did not react with the solvent DMSO and the components of the culture medium, based on the TLC monitoring. Pure water was used for the control probe. Four repetitions of assays were made for each concentration. The influence of different concentrations of DMSO on the cytotoxic activity on the cell lines was studied. This step is important because the terpenoids have only poor water solubility and usage of compatible organic solvents such as DMSO is required. Cytotoxic effect for the cells was observed in the mixture of DMSO/water with w/w of DMSO of 1%. Concentration of DMSO in the working probes ranged between 0.1 and 0.007%.

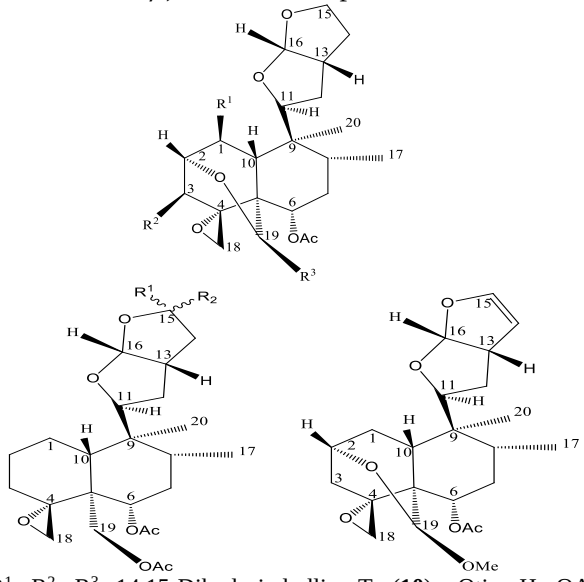
MTT assay: Cells ($0.5 \times 10^4 - 1 \times 10^4$) were plated in 125 μL of medium/well in a 96–well plate. After seventy-two hours we threw the old nutritive medium together with the compound and added a new medium with the dissolved MTT. The cell mitochondrial respiratory chain should include MTT and if the cell is alive and breaths its color changes from yellow to purple. After four hours the medium + MTT was thrown and the remains, that were bottom purple crystals MTT (phurmazanic), were dissolved with 2% solutions of formic acid in *iso*-propanol. The intensity of the purple tinge was measured with the device, ELISA reader. The data were processed with the program GraphPadPrism. Activity was evaluated as rate of IC_{50}.

RESULTS AND DISCUSSIONS

Twelve natural neo-clerodane diterpenes (Fig. 3), isolated from *Scutellaria galericulata* L., *Scutellaria alpina* L. and *Scutellaria altissima* L., growing in Bulgaria, were tested for cytotoxicity on two cell lines, from human tumors of the lung designated as H1299 and normal cells from a navel string (HUVEC), using the MTT method. The cytotoxic activity was evaluated as rate of IC_{50}.

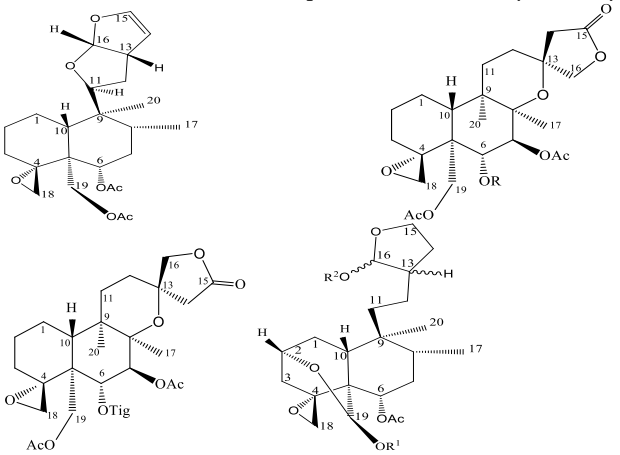
Characteristic signals for the neo-clerodane skeleton in all tested diterpenoids were easily determined at δ_H in the region 1.10-1.20 s (Me-20), 0.78-0.95 d (Me-17) and 1.75-2.25 d or dd (H-10β). The decalin moiety contains some constant functional features: the decalin junction is always trans; methyl groups C-17 and C-20 are always α-orientated; α-hydroxy or α-acyloxy groups are always present on carbons C-6 and C-19; while carbon C-10 is never functionalized; bearing C-4–C-18 *spiro*-epoxide. The two doublets from the AB quartet corresponding to the C-18 two hydrogens appeared at the region of δ_H 2.40-2.50 for H-18A and 2.95-3.05 for the H-18B, respectively. In compounds having substitute at third position the signal for 18A is replaced at δ_H 2.85-2.92.

In accordance with the aim of this investigation the compounds were separated into four groups **I-IV** on the basis of the presented C-11 – C-16 substructures (Fig. 3). Members of the **I** (diterpenoids from **10** to **14**) have hexahydrofurofuran moiety (**2b**) (1H, δ_H 2.80-2.95 br tt, H-13; 1H, δ_H 5.60-5.70 d, H-16) and display a 2α,19-hemiacetal or acetal function (1H, δ_H 3.98-4.10 ddd, H-2β) with the exception of **14**.



R^1 R^2 R^3 14,15-Dihydrojodrellin T (**10**): Otig H OAc Scutecyprol A (**14**): R^1, R^2 = H, OH Scupolin H (**15**) Neoajugapyrin A (**11**): H OH Otig (R and S form) Scutegalerin A (**12**): OH H Otig Scutecolumnin C (**13**):H OH

Separate compounds differ from one another by the substitutes at C-1, C-3 or C-19 position. In the second group (**II**) are included two clerodanes **15** and **16** which contain the fragment **2a** (1620 cm^{-2} vinyl ether; 1H, δ_H 4.81 t, H-14 / δ_C 102.0 d; 1H, δ_H 6.45 t, H-15 / δC 146.7 d). The series **III** was constructed by three diterpenoids, scutalpins A, E and F, as the configuration of the carbon C-13 in



R R^1 R^2 Clerodin (**16**) Scutalpin A (**17**): MeBu Scutalpin E (**19**) Scutegalin D (**20**): Tig H Scutalpin F (**18**): Ac Scutaltisin G (**21**): H CH_3

Figure 3. Molecular structures of the tested *neo*-clerodane diterpenoids

scutalpin E (1H, 2.48 d, H-14*α*; 1H, 3.12 d, H-14*β* / δ_C 44.3 t, C-14; 1H, 4.30 d, H-16*α*; 1H, 4.10 d, H-16*β* / δ_C 79.4 t, C-16 and δ_C 173.1 s, C-15)0 is opposed to that in the scutalpins A and F (1H, 2.57 d, H-14*α*; 1H, 2.73 d, H-14*β* / δ_C 42.6 t, C-14; 1H, 4.13 d, H-16*α*; 1H, 4.35 d, H-16*β* / δ_C 79.6 t, C-16 and δ_C 174.6 s, C-15). The representatives **20** and **21**, possessing the lactol ring **6** (δ_H 3.86 td, 1H, H-15A; 3.93 td, 1H, H-15B; 4.64 d, 1H, H-16), form the last group **IV**.

Scutalpins A, E and F, exhibited moderate cytotoxic properties on both cell lines (Table 1), but the rest of the compounds were inactive within the studied concentration range. Based on these results, it could be concluded that the tetra- or hexahydrofurofuran substructures are not the responsible moiety for cytotoxic action, which is in discrepancy with the affirmation from Kojima and Kato, that these structural features of the molecules are accountable conditions for the significant antifeedant activity [15].

Table 1. Cytotoxic activity of neo-clerodane diterpenes on H1299 and HUVEC cell lines

Compounds	IC_{50} values		Compounds	IC_{50} values	
	H1299	HUVEC		H1299	HUVEC
14,15-Dihydrojodrellin T (**10**)	242,21	244,43	Clerodin (**16**)	236,35	236,84
Neoajugapyrin A (**11**)	288,65	287,59	Scutalpin A (**17**)	21,35	23,89
Scutegalerin A (**12**)	452,01	458,00	Scutalpin F (**18**)	26,62	31,28
Scutecolumnin C (**13**)	667,44	665,79	Scutalpin E (**19**)	34,24	32,48
Scutecyprol A (**14**)	335,76	365,32	Scutegalin D (**20**)	888,35	883,47
Scupolin H (**15**)	578,71	574,55	Scutaltisin G (**21**)	892,02	892,22

Presumably, there is no correlation between the two kinds of biological activities, cytotoxic and antifeedant. All three compounds with cytotoxic effect, have 13-spiro connected *γ*-lactone as the configuration of the asymmetric center C-13 is S in **17** and **18** and R in **19,** respectively. Diterpenoids differ from one another by the substitute at carbon C-6. The most active among them scutalpin A contains 2-methylbutyrate, while the less active scutalpin F and scutalpin E are presented with acetyl or (E)-2-methyl-2-butenoyl ester. It is uncertain that only changing of the 2-methylbutyrate function with the tiglate one causes the bigger decrease in the action of **19**, because the C-11 – C-16 substructure is not with 13S configuration like in scutalpin A, but it is with the other possible orientation - 13R.

CONCLUSION

The achieved results by the series of bioassays on antifeedant activity confirm the previously reported, by Blaney *et al*. [16]; Houghgoldstein & Whalen [17], dependence of activity on the presence in the molecule of tetra- or hexahydrofurofuran ring. Neo-clerodane diterpenoids do not display antioxidant activity. Compounds which exhibit cytotoxic and antimicrobial activity possess 13-*spiro*-α,β-unsaturated-γ-lactone. Thus, it be concluded that the higher activity depends on the characteristic features of the whole molecular structure. Responsible structural features for biological activity vary for the separate kinds of properties.

REFERENCES

1. M. Bruno, F. Piozzi, S. Rosselli, *Natural Product Reports*, **19** (3), 357 (2002).
2. M. Bruno, F. Piozzi, A. Maggio, M. S. J. Simmonds, *Biochemical Systematics and Ecology*, **30** (8), 793 (2002).
3. E. A. Klein-Gebbinck, B. J. M. Jansen, A. de Groot, *Phytochemistry*, **61** (7), 737 (2002).
4. J. C. Anderson, W. M. Blaney, M. D. Cole, L. L. Fellows, S. V. Ley, R. N. Sheppard, M. S. J. Simmonds, *Tetrahedron Letters*, **30** (35), 4737 (1989).
5. M. D. Cole, P. D. Bridge, J. E. Dellar, L. E. Fellows, N. C. Cornish, J. C. Anderson, *Phytochemistry*, **30** (4), 1125 (1991).

6. P. I. Bozov, T. A. Vasileva, I.N. Iliev, *Chemistry of Natural Compounds*, **50** (4), 762 (2014)
7. P. I. Bozov, K. H. Nikolova, V. B. Bivolarski, T. A. Vasileva, *Journal of BioScience and Biotechnology*, SE/ONLINE. (2014).
8. P. I. Bozova, T. Girova, N. Prisadova, Y. Hristova, V. Gochev, *Natural Product Communications*, **10** (11), 1797 (2015).
9. P. I. Bozov, P. N. Penchev, T. V. Vasileva, I. N. Iliev, *Chemistry of Natural Compounds*, **50** (3), 554 (2014).
10. P. I. Bozov, P. N. Penchev, J. Coll, *Natural Product Communications*, **9** (3), 347 (2014).
11. Y. P. Georgieva, R. D. Mladenov, P. I. Bozov, *Chemistry of Natural Compounds*, **50** (6), 1146 (2014).
12. P. I. Bozov, J. Coll, *Natural Product Communications*, **10** (1), 13 (2015).
13. P. Bozov, P. Malakov, G. Papanov, M. de La Torre, B. Rodriguez, A. Perales, *Phytochemistry*, **34** (2), 453 (1993).
14. P. I. Bozov, G. Y. Papanov, P. Y. Malakov, *Phytochemistry*, **35** (5), 1285 (1994).
15. Y. Kojima, N. Kato, *Tetrahedron*, **37** (15), 2527 (1981).
16. W. M. Blaney, M. S. Simmonds, S. V. Ley, P. S. Jones, *Entomol. Exp. Appl*., **46**, 267 (1988).
17. J. Houghgoldstein, J. Whalen, *Biol. Control*, **3**, 343 (1993).

НЯКОИ СТРУКТУРНИ ОСОБЕНОСТИ, ОПРЕДЕЛЯЩИ ЦИТОТОКСИЧНОСТТА И ДРУГИ ВИДОВЕ АКТИВНОСТИ НА НЕО-КЛЕРОДАНОВИ ДИТЕРПЕНИ ОТ РОДА *SCUTELLARIA*

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(Резюме)

Дванадесет природни нео-клероданови дитерпени, изолирани от три вида на рода *Scutellaria* (Labiatae), са тествани за цитотоксичност на две клетъчни линии, от тумори на човешки бял дроб, означени с H1299 и нормални клетки от пъпна връв (HUVEC) с помощта на MTT (3-/4,5-диметилтиазол-2-ил/-2,5-дифенилтетразолиев бромид) метод. Цитотоксичната активност е изчислена като IC_{50} стойност (такава концентрация на съединението в μM, при която половината от клетките умират). Три съединения, скуталпини A, E и F проявяват умерени цитотоксични свойства за двете клетъчни линии. От всички тествани съединения най-висока активност е отчетена за скуталпин A, със стойности на IC_{50} съответно 21,35 и 23,9.

Обсъдени са някои значими аспекти на взаимовръзката структура - активност.

SUPPLEMENTARY DATA

In this section the GraphPadPrism experimental results for the cytotoxic active compounds are shown.

Scutalpin A (**17**)

Table format: XY	X	A			
	concentration	h1299 2			
	X	A:Y1	A:Y2	A:Y3	A:Y4
1	Title	0.01	0.316	0.295	0.290	0.276
2	Title	19.00	0.113	0.160	0.144	0.149
3	Title	37.50	0.107	0.133	0.139	0.117
4	Title	75.00	0.030	0.077	0.077	0.063
5	Title	150.00	0.006	0.007	0.006	0.005
6	Title	300.00	0.008	0.009	0.008	0.011

Nonlin fit	A
	h1299 2
	Y
1 log(inhibitor) vs. normalized response -- Va	
2 Best-fit values	
3 LOGIC50	1.329
4 HILLSLOPE	-1.144
5 IC50	21.35
6 Std. Error	
7 LOGIC50	0.04359
8 HILLSLOPE	0.1406
9 95% Confidence Intervals	
10 LOGIC50	1.239 to 1.420
11 HILLSLOPE	-1.436 to -0.8527
12 IC50	17.33 to 26.29
13 Goodness of Fit	
14 Degrees of Freedom	22
15 R?	0.9604
16 Absolute Sum of Squares	1089
17 Sy.x	7.035

Table format: XY	X	A			
	concentration	huvec 2			
	X	A:Y1	A:Y2	A:Y3	A:Y4
1	Title	0.01	0.243	0.232	0.229	0.241
2	Title	19.00	0.109	0.117	0.104	0.153
3	Title	37.50	0.085	0.116	0.120	0.120
4	Title	75.00	0.018	0.033	0.042	0.055
5	Title	150.00	0.006	0.006	0.007	0.006
6	Title	300.00	0.009	0.010	0.010	0.008

Nonlin fit	A
	huvec 2
	Y
1 log(inhibitor) vs. normalized response -- Va	
2 Best-fit values	
3 LOGIC50	1.378
4 HILLSLOPE	-1.275
5 IC50	23.89
6 Std. Error	
7 LOGIC50	0.04098
8 HILLSLOPE	0.1669
9 95% Confidence Intervals	
10 LOGIC50	1.293 to 1.463
11 HILLSLOPE	-1.621 to -0.9285
12 IC50	19.64 to 29.06
13 Goodness of Fit	
14 Degrees of Freedom	22
15 R?	0.9520
16 Absolute Sum of Squares	1372
17 Sy.x	7.897

Scutalpin F (**18**)

Table format: XY	X	A			
	concentration	huvec 1			
	X	A:Y1	A:Y2	A:Y3	A:Y4
1 Title	0.01	0.243	0.232	0.229	0.241
2 Title	19.00	0.112	0.151	0.142	0.141
3 Title	37.50	0.064	0.136	0.124	0.120
4 Title	75.00	0.073	0.101	0.099	0.110
5 Title	150.00	0.004	0.005	0.008	0.008
6 Title	300.00	0.004	0.006	0.006	0.006

Nonlin fit	A
	huvec 1
	Y
1 log(inhibitor) vs. normalized response -- Va	
2 Best-fit values	
3 LOGIC50	1.495
4 HILLSLOPE	-1.127
5 IC50	31.28
6 Std. Error	
7 LOGIC50	0.05258
8 HILLSLOPE	0.1742
9 95% Confidence Intervals	
10 LOGIC50	1.386 to 1.604
11 HILLSLOPE	-1.488 to -0.7654
12 IC50	24.34 to 40.21
13 Goodness of Fit	
14 Degrees of Freedom	22
15 R?	0.9138
16 Absolute Sum of Squares	2413
17 Sy.x	10.47

Table format: XY	X	A			
	concentration	h1299 1			
	X	A:Y1	A:Y2	A:Y3	A:Y4
1 Title	0.01	0.316	0.295	0.290	0.276
2 Title	19.00	0.160	0.158	0.154	0.137
3 Title	37.50	0.129	0.133	0.134	0.157
4 Title	75.00	0.064	0.124	0.117	0.110
5 Title	150.00	0.007	0.016	0.018	0.016
6 Title	300.00	0.009	0.014	0.008	0.013

Nonlin fit	A
	h1299 1
	Y
1 log(inhibitor) vs. normalized response -- Va	
2 Best-fit values	
3 LOGIC50	1.425
4 HILLSLOPE	-1.015
5 IC50	26.62
6 Std. Error	
7 LOGIC50	0.05046
8 HILLSLOPE	0.1364
9 95% Confidence Intervals	
10 LOGIC50	1.320 to 1.530
11 HILLSLOPE	-1.297 to -0.7319
12 IC50	20.92 to 33.87
13 Goodness of Fit	
14 Degrees of Freedom	22
15 R?	0.9393
16 Absolute Sum of Squares	1575
17 Sy.x	8.461
18 Number of points	

Scutalpin E (**19)**

Table format: XY	X	A			
	concentration	huvec 3			
	X	A:Y1	A:Y2	A:Y3	A:Y4
1 Title	0.01	0.2430	0.232	0.229	0.241
2 Title	19.00	0.1460	0.132	0.151	0.125
3 Title	37.50	0.1240	0.117	0.121	0.094
4 Title	75.00	0.1070	0.092	0.099	0.087
5 Title	150.00	0.0090	0.010	0.009	0.007
6 Title	300.00	0.0070	0.006	0.005	0.007

Nonlin fit	A
	huvec 3
	Y
1 log(inhibitor) vs. normalized response -- Va	
2 Best-fit values	
3 LOGIC50	1.512
4 HILLSLOPE	-1.130
5 IC50	32.48
6 Std. Error	
7 LOGIC50	0.04314
8 HILLSLOPE	0.1438
9 95% Confidence Intervals	
10 LOGIC50	1.422 to 1.601
11 HILLSLOPE	-1.428 to -0.8313
12 IC50	26.43 to 39.91
13 Goodness of Fit	
14 Degrees of Freedom	22
15 R?	0.9380
16 Absolute Sum of Squares	1680
17 Sy.x	8.738

Table format: XY	X	A			
	concentration	h1299 3			
	X	A:Y1	A:Y2	A:Y3	A:Y4
1 Title	0.01	0.316	0.295	0.290	0.276
2 Title	19.00	0.185	0.182	0.183	0.183
3 Title	37.50	0.145	0.168	0.189	0.139
4 Title	75.00	0.092	0.095	0.088	0.045
5 Title	150.00	0.020	0.020	0.021	0.011
6 Title	300.00	0.010	0.010	0.011	0.008

Nonlin fit	A
	h1299 3
	Y
1 log(inhibitor) vs. normalized response -- Va	
2 Best-fit values	
3 LOGIC50	1.535
4 HILLSLOPE	-1.336
5 IC50	34.24
6 Std. Error	
7 LOGIC50	0.02905
8 HILLSLOPE	0.1294
9 95% Confidence Intervals	
10 LOGIC50	1.474 to 1.595
11 HILLSLOPE	-1.604 to -1.067
12 IC50	29.80 to 39.33
13 Goodness of Fit	
14 Degrees of Freedom	22
15 R?	0.9633
16 Absolute Sum of Squares	1037
17 Sy.x	6.864

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Rose oil isolated from oil-bearing *Rosa damascena* Mill. as a protector against ionizing radiation-induced oxidative disorders

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Essential oils are derived from different natural plant materials such as leaves and flowers. They are commercially important and have been widely used in cosmetics, household products and medicines because of pharmacologically active components. Essential oil as antioxidant is able to prevent oxidative processes and to inhibit the oxidation reaction effect caused by radiation-induced oxygen/nitrogen free radicals. Ionizing radiation is a recognized method of maintaining the quality of aromatic herbs, spices and vegetables for a long time. The present study focused on identifying the radioprotective efficacy of rose oil against oxidative damage of molecules by ionizing radiation in *in vitro* models.

Keywords: *Rosa damascena* Mill., DPPH scavenger, Radiomodulation

INTRODUCTION

The available data indicate that the effect of ionizing radiation leads to changes in biological systems and to an increase in the level of free radicals [1, 2]. Consequently, the evaluation of the pharmacological effect of essential oils after ultraviolet (UV) and gamma (γ) radiation is of considerable interest because of their supposed antioxidant and therapeutic effect, as well as the overall composition. Essential oils as antioxidants are able to prevent oxidative processes [3] and to inhibit the oxidation reaction effect caused by radiation-induced oxygen/nitrogen free radicals [4]. Ionizing radiation is a recognized method of maintaining the quality of aromatic herbs, spices and vegetables for a long time [5]. Increased antioxidant activity was observed in oils obtained from previously irradiated leaves and fruits [6, 7]. *In vitro* and *in vivo* systems serve as models for preliminary observations in assessing pharmacological activity, changes in chemical composition and various forms of the spectrum *versus* time and T°C [7, 8] oils as protective antioxidants that can effectively involve radiation-induced oxidative changes [9, 10]. The Western European *Rose Damascus* Mill. (*R. damascena*) as a plant species of Europe was used in homeopathic and pharmaceutical preparations [11]. Rose oil was characterized by antibacterial, neuropharmacological, anti-inflammatory and stable antioxidant action [12-15]. More than 300 components referring to terpenic and non-terpenic hydrocarbons, glycosides, flavonoids, citronellol, geraniol, farnesol, alcohols, nerol, linalool and esters, have been recognized in the oil structure [16, 17]. The present study focused on identifying the radioprotective efficacy of rose oil against oxidative damage of molecules by ionizing radiation in *in vitro* models.

EXPERIMENTAL

Isolation and characterization of Bulgarian rose oil

Rose flowers (stage IV-V) were collected on May 26, 2009, between 6 and 10 am at a relative humidity of 86-93% and temperature of 13.5-14.8ºC from 2 plantations located at IREMC, Kazanlak, Bulgaria. Isolation and characterization of the main components of rose oil were carried out using Clevenger water vapor distillation and gas chromatography (GC) (chromatograms obtained for the determination of the representative and distinctive ingredients given in BS ISO 9842-2004), as described in previous reports [19, 20]. The quality of the Bulgarian rose oil corresponds to the

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standard [21] for rose concrete and rose absolute grown from dry leaves [17].

Irradiation

Experimental samples (in a de-aerated capillary) were exposed to UV-B (UV-vis Transilluminator-4000, Bulgaria; 290-320 nm; two lamps; 220V ~ 50 Hz; microwave power 7.70VA; for 2 h rate; humidity- 40 %) and to ^{60}Co radiation at doses of 2.5, 5, 10, 20, 30, 50 Gy using γ- chamber Gamma Cell 5000 (dose rate of 1.4 Gy/h, Board of Radiation and Isotope Technology, India). Dosimetry was carried out using Baldwin Farmer's secondary dosimeter and Fricke's chemical method and all the radiation safety measures were strictly followed during experimentation.

Electron donation potential estimation assay

The electron donation potential of UV and γ-irradiated samples and oil alone, was determined by the Oyaizu [22] method. A range of concentrations (1 - 500 μg/ml) before and after gamma irradiation (2.5 Gy) was firstly tested to determine the concentration at which oil exhibited maximal donation potential. Further, this concentration (50 μg/ml) was UV and γ- irradiated with doses ranging from 5 Gy to 50 Gy and the electron potential was determined both immediately and 24 h post irradiation. The reaction mixture was left for 10 min at room temperature and the absorbance was measured at 700 nm. An increased absorbance of the reaction mixture indicates increased reducing power:

$$\% \text{ Inhibition} = [(OD_{control} - OD_{test}) / OD_{control}] \times 100$$

2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical-scavenging activity

Radical scavenging activity of γ-irradiated samples and oil alone against the stable DPPH radical was determined according to Cuendet *et al*. [23]. Briefly, 1.0 ml of DPPH (100 μM) was added to 500 μl of different volume concentrations of the studied samples. Mixtures were incubated in the dark for 10 min and their absorbance at 517 nm was measured. Quercetin was used as a positive control. The percentage of DPPH radicals scavenged was calculated according to the equation:

$$\% \text{ Inhibition} = (A_{control} - A_{test}) / A_{control} \times 100$$

2,2-Azinobis (3-ethylbenzothiazoline–6–sulfonic acid) ($ABTS^{\bullet+}$) radical-scavenging assay

$ABTS^{\bullet+}$ radical scavenging assay of γ- irradiated samples and oil alone was performed according to Re *et al*. [24] with slight modifications. The reaction mixtures were incubated at 24^{0}C for 30 min and the intensity of chromogen was measured at 734 nm. Antiradical activity of the examined sample was presented as the percentage of $ABTS^{\bullet+}$ radical scavenging and calculated according to the equation:

$$\% \text{ Inhibition} = (A_{control} - A_{test}) / A_{control} \times 10$$

Nitric oxide (NO^-) ion scavenging assay

The NO^- potential of γ-irradiated samples and oil alone samples (0.1–250 μg/ml) was determined according to the method described by Shirwaikar *et al*. [25]. The scavenging potential was evaluated as the decrease in percent absorbance of the chromogen formed by diazotisation of nitrite with sulfanilamide and subsequent coupling with naphthyl ethylenediamine recorded at 546 nm.

Protection of membrane against radiation damage (membrane protection index)

Soya lecithin and cholesterol (1:1 molar ratio) were suspended in an appropriate amount of chloroform. A thin film was developed by complete evaporation of chloroform in a rotary evaporator (Buchi, Newcastle, USA) at 40ºC. The film was subjected to hydration in (0.1 M, pH 7.4) PBS and was incubated in a water bath (40ºC) for 4 h. The stock solution was diluted with PBS to the final concentration in terms of phospholipid content, cf. Lasic&Papahadjopoulous [26]. Different treated oil samples, liposome only (untreated), radiation only (2.5Gy), liposome + rose oil and liposome + rose oil+2.5 Gy were evaluated for the levels of malondialdehyde, the final product of membrane degeneration. A radiation dose of 2.5 Gy at a dose rate of1.4 kGy/h was used and after exposure the samples were immediately incubated for 1 h at 37ºC. 10 % TCA and 0.5% thiobarbituric acid, 1:1 ratio and 0.025 M NaOH were added. The mixture was heated in a water bath (80ºC) for 1 h and absorbance was measured at 535 nm [27].

Statistical analysis

Statistical analysis was performed with Statistica 6.1, StaSoft Inc., and results were expressed as means ± standard error (SE). Statistical significance was determined by Student's *t*-test. Value of $p < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Ionizing radiation (IR) induces oxidative disturbances by the accumulation of reactive oxygen species (ROS) as singlet oxygen ($^1O_2$), nitrogen oxide ($NO^{\bullet}$), hydrogen peroxide (H_2O_2), macromolecular degradation and lipid peroxidation

[28, 29]. Oxidation of lipids and other vital macromolecules leads to persistent oxidative disturbances and obstruction of intracellular processes. Therefore, in order to protect biological macromolecules from free radicals of ionizing radiation, it is necessary to shield them by an effective radiation-protective agent.

Thus, the oils of the bearing plants and plant extracts were evaluated for stable antioxidant activity *in vitro* and *in vivo*, scavenging of ROS / RNS and radiation protection property [30-32]. Donation potential is usually associated with the presence of reducing agents that exert an antioxidant effect by breaking down the radical chains by donating a hydrogen atom [33]. The maximum reduction ability for non-irradiated oil (Fig. 1a) and 2.5 Gy samples (Fig. 1b) with respect to the Fe^{3+} complex was observed at a concentration of 50 μg / ml. Two hours after UV irradiation, the oil samples showed a greater donation potential compared to non-irradiated oil (0.664 ± 0.016 *vs*. 0.472 ± 0.005). *R. damascena* displayed a good restoring ability to the complex of Fe^{3+} after UV in both treatment groups up to 5Gy (Fig. 1).

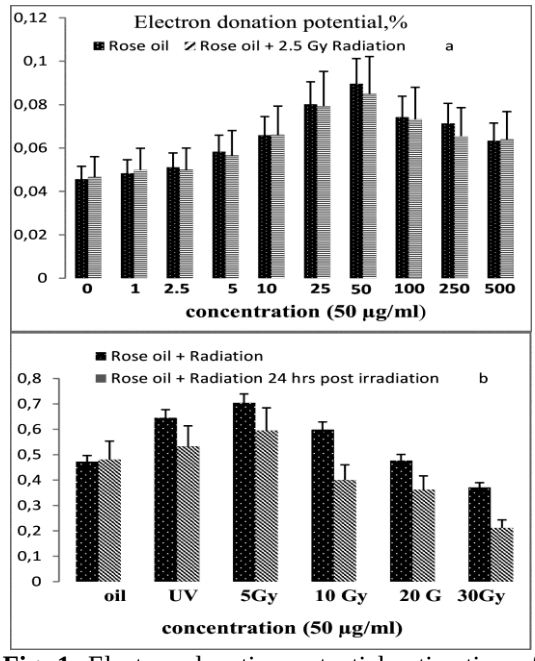

Fig. 1. Electron donation potential estimation of 50 μg/ml oil alone/ after 2.5Gy irradiation (a) /after UV and γ-irradiation (immediately and 24 h post irradiation, (b)).

Donation potential of the oil was tested at different time intervals, immediately and 24 h after treatment. Therefore, the results of this study clearly indicate that the samples after irradiation showed a significant reduction in the donor potential in comparison with non-irradiated oil and with those that were measured immediately after irradiation, but with the same dependence. Radiation exposure increases the load of iron in the cellular environment and leads to hemolysis [34]. It can be assumed that the reduction of Fe^{3+} to the less dangerous Fe^{2+} is a possible mode of action of *R. damascena*, which showed antioxidant protection to overcome oxidative disorders caused by radiation *in vivo*.

The efficiency of DPPH scavenging activity of *R. damascena*, non-irradiated and γ-irradiated (2.5 Gy) oil is shown in Fig. 2. The maximum DPPH - scavenging activity of 2.5 Gy oil (74.4 ± 0.97%) was detected at 50 μg / ml compared to non-irradiated oil (63.4 ± 1.12%, 500 μg / ml). In two ranges of increasing concentrations, namely 2.5 to 25 μg / ml and 50 to 500 μg / ml, a statistically insignificant increase in the DPPH scavenging activity was observed in both non-irradiated oil and γ-irradiated samples.

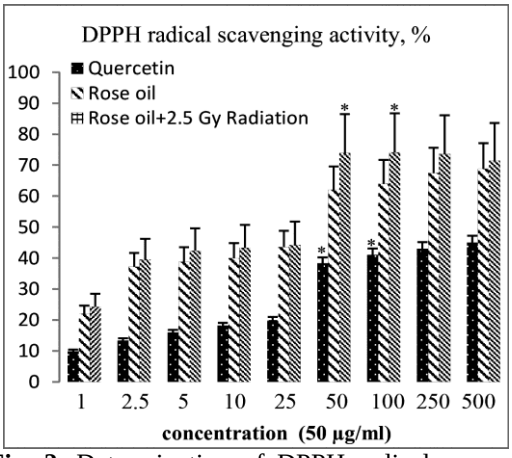

Fig. 2. Determination of DPPH radical scavenging activity of γ-irradiated (2.5 Gy) and oil alone. Quercetin was used as a standard.

For the same concentration ranges, an insignificant increase in DPPH activity of the γ-irradiated sample was also observed in comparison with the non-irradiated oil. It should be noted that irradiated and non-irradiated oil in all studied concentrations demonstrates a statistically higher ability of DPPH scavenging compared to quercetin used as a positive control. The efficacy in the scavenging abilities of the stable DPPH radicals of geraniol [35] and citronellol and the good inhibitory effect on UV irradiation [36] are consistent with our results. A significant reduction in the radical scavenging ability of oil at higher doses (> 5 Gy) of IR irradiation can be explained by the significant structural changes of some constituents in the form of geraniol, citronellol and eugenol, which probably determined its antioxidant activity.

The ABTS analysis is excellent for determining the total antioxidant activity of hydrogen-donating antioxidants (scavenging aqueous-phase radicals) and chain breaking antioxidants (scavenging lipid-

peroxyl radicals) [37]. The present study used an improved $ABTS^{\bullet+}$ decolorization assay [24], which is applicable to lipophilic and hydrophilic antioxidants. In the range of 10-500 μg/ml, both γ-irradiated and non-irradiated oil samples (Fig. 3) demonstrated an increase in scavenging abilities towards $ABTS^{\bullet+}$ in a concentration-dependent manner. As indicated at 500 μg/ml, the $ABTS^{\bullet+}$ scavenging ability percentage was 70.36 ± 1.91% for non-irradiated oil and 72.38 ± 1.08% for 2.5 Gy samples. Only at 25 μg/ml and 50 μg/ ml was a statistically significant increase in $ABTS^{\bullet+}$ activity of 2.5 Gy oil compared to non-irradiated oil. With the exception of 500 μg/ml, all other oils alone and 2.5 Gy irradiated concentrations showed better $ABTS^{\bullet+}$ scavenging activity than the standard.

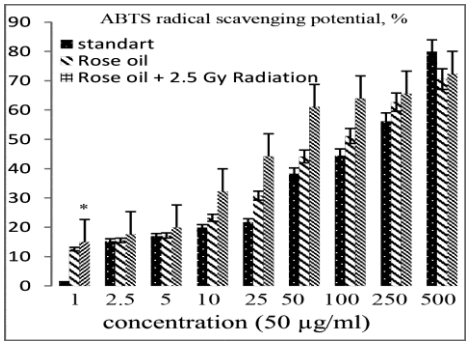

Fig. 3. ABTS radical scavenging potential of γ-irradiated (2.5 Gy) and oil alone in concentration range (1 – 500 μg/ml).

Nitric oxide ($NO^{\bullet}$) is an important messenger in a variety of normal physiological processes. In addition to its physiological actions, the overload of NO• can lead to cell damage by changing the function of the protein by nitrosylation, promote glutamate exutotoxicity, inhibit mitochondrial respiratory complexes, participate in fragmentation of organelles and mobilize zinc from internal stores [38]. Oxidative stress induced by IR generates $NO^{\bullet}$ and plays a critical role in the initiation and progression of oxidative damage [39], the function and dysfunction of the nervous system, [38]. The scavenging ability of NO• (Fig. 4) increases with an increase in the concentration of 2.5 Gy and samples with non-irradiated oil. At 250 μg/ml maximum NO• scavenging activities were 34.07± 1.1% and 32.2 ± 3.01%, respectively.

In *in vitro* non-irradiated oil samples and ionizing radiation samples, $NO^{\bullet}$ production was markedly reduced, indicating that the oil contains hydrophilic antioxidants that have $NO^{\bullet}$ scavenging ability. In addition, for each test concentration of 2.5 Gy of oil, a higher scavenging activity was shown compared to non-irradiated oil, suggesting that additional structures are involved in reducing $NO^{\bullet}$ levels. Pathologically, NO• radicals reacted with $O_2^{\bullet-}$ anion to peroxynitrite ($ONOO^-$) and led to serious toxic reactions with proteins and lipids [40, 41]. In view of the fact that *R. damascene* exhibits a pronounced scavenging activity against NO•, as before/after IR makes the oil a potential antioxidant, it can be used in IR-induced pathological situations associated with excessive production of NO•[41]. Moreover, γ-irradiation induces changes in fatty acids of membrane phospholipids forming unstable and highly reactive peroxy radicals that decompose into alcohols, ketones and malondialdehyde [42]. The artificial membrane system (liposomes) was used to assess the ability of rose oil to protect the lipids of

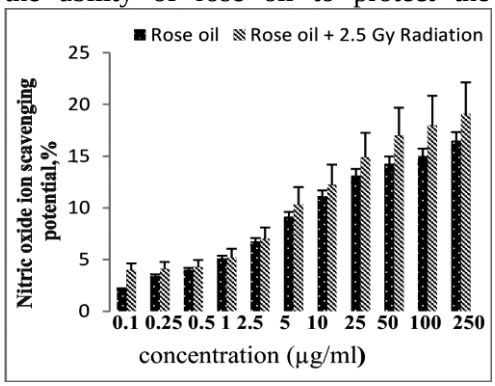

Fig. 4. Nitric oxide scavenging potential of γ-irradiated (2.5 Gy) and oil alone in the concentration range (0.1 – 250 μg/ml).

liposomes against lipid peroxidation 2.5 Gy (dose rate = 1.24 KGy / h). The most effective dose for inhibition of peroxidation in the tested liposome system was 100 μg/ml. Rose oil exhibited significantly higher membrane protection at 150 μg/ml (0.062 ± 1.04%; $p<0.05$). Maximal activity of 2.5 Gy was at 100-150 μl/ml (Fig. 5). The samples with 2.5 Gy indicated two times higher anti-lipid peroxidation, compared to oil alone. It might be assumed that the higher membrane protection of the 2.5Gy samples was due to chelation of transition metal ions [43] by filling the aqua-coordination sites of the hydrophilic rose oil substances exhibiting radical scavenging abilities, which indirectly demonstrated protective properties and possible reduction of pathophysiological consequences [44]. Good ability of geraniol to inhibit lipid peroxidation in egg-liposomal suspension is in agreement with our reported results [35].

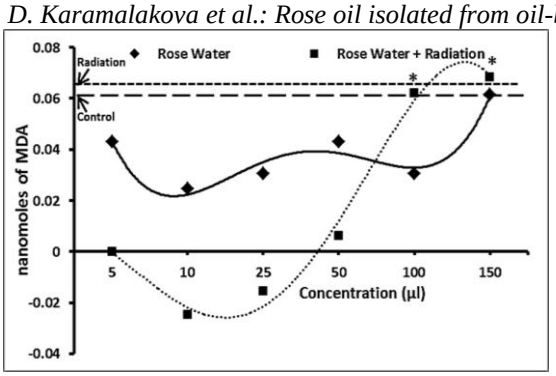

Fig.5. Analysis of the membrane-protecting ability of non-irradiated and γ- irradiated (2.5 Gy) rose oil utilizing an artificial membrane system (liposome). A significant ($p < 0.05$) decrease in the formation of malondialdehyde (MDA) with increasing concentration of γ- irradiated oil (50 – 150 µg/ml) was recorded. Effect of different concentrations of non-irradiated and γ- irradiated (2.5 Gy) rose oil on radiation (1.24KGy/h)- mediated lipid peroxidation evaluated in erythrocytes. Each experiment was performed in triplicate. The lipid peroxidation activity is expressed as nanomoles of MDA formed.

CONCLUSION

Rose oil after irradiation showed a significant reduction in the donor potential in comparison with non-irradiated oil and with those that were measured immediately after irradiation, but with the same dependence. Moreover, irradiated and non-irradiated oil in all studied concentrations demonstrates a statistically higher ability of DPPH compared to quercetin used as a positive control.

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REFERENCES

1. G.C. Jagetia, *J. Clin. Biochem. Nutrition*, **40**, 74 (2007).
2. A. Wei, T. Shibamoto, *J. Agric. Food Chem*., **58**(12),7218 (2010).
3. F. Liu, Q. Xu, R. Dai, Y. Ni, *Acta Sci. Pol. Technol. Aliment*. **14**, 37 (2015).
4. B. Poljsak, D. Šuput, I. Milisav, *Oxidat. Med. and Cell. Long*., **29**, 11 (2013).
5. European Directive 1999/3/EC of the Europ. Parliam. and of the Council of 22 Febr. 1999 on the Establishment of a Community list of foods and food ingredients treated with ionizing radiation, *Offic. J. Europ Comm*. L66, 13.3., 1999, p. 24.
6. M. Suhaj, J. Horvathova, *J. Food and Nutrition Res*., **46**(3), 112 (2008).
7. Y. Karamalakova, J. Sharma, G. Nikolova, S. Stanev, R. Arora, V. Gadjeva, A. Zheleva, *Biotechnol. & Biotechnolog. Equipm*., *27*(3), 3861 (2013).
8. G. J. Troup, L. Navarini, F. S. Liverani, S.C. Drew, *PloS ONE*, **10**, e0122834 (2015).
9. R. Arora, CABI Publishing, Wallingford, Oxon, UK, 2008, p. 332.
10. P. Paul, K. Unnikrishnan, N. Nagappa, *Ind. J. Nat. Prod. Res*.., **2**, 137 (2011).
11. P. T. Beales, W. Cairns, W. Duncan, K. Grant, P. Grapes, K. Harkness, J. H. Mattock, D. Ruston, Randomhouse Australia Pty. Ltd., Sydney, 1998.
12. E. Basim, H. Basim, *Fitoterapia*, **74,** 394 (2003).
13. M. A. B. Nyeem, M. A. Alam, M. A. Awal, M. Mostofa, S. J. Uddin, N. Islam, R. Rouf, *Iran. J. Pharmaceut. Res*., **5**, 171 (2007).
14. O. Sagdiç, G. Özkan, N.G. Baydar, H. Baydar, *Revista de Agroquimica y Tecnologia de Alimentos*, **10**(4), 277 (2004).
15. V. Hajhashemi, A. Ghannadi, M. Hajiloo, *Iran. J. Pharm.Res*. **9**, 163 (2010).
16. N. Kumar, B. Singh, V. K. Kaul, *Nat. Prod. Comm*., **1**(8), 623 (2006).
17. K. Rusanov, N. Kovacheva, A. Atanassov, I. Atanassov, *Floricult. Ornament. Biotech*.. **3**, 14 (2009).
18. M. H. Boskabady, M. N. Shafei, Z. Saberi, S. Amini, *Iran J. Basic Med. Sci.*, **14**(4), 295 (2011).
19. G. Diakov, A. Balinova, Rastenievudni nauki. Plant Science. Bulgaria, 1975.
20. ISO 9842:2003. Oil of rose (Rosa × damascena Miller). 2003. International Standards for Business, Government and Society. Available online: <www.iso.org>.
21. N. Kovacheva, K. Rusanov, I. Atanassov, *Direct and Chall*., **24**, 2, (2010).
22. M.Oyaizu, *Jap. J. Nutr*., **44**: 307 (1986).
23. M. Cuendet, K. Hostettmann, O. Potterat, W. Dyatmiko, *Helvet. Chim. Acta*, **80**, 1144 (1997).
24. R. Re, N. Pellegrini, A. Proteggente, A. Pannala, M. Yang, C. Rice-Evans, *Free Radical Biology and Medicine*, **26**(9), 1231 (1999).
25. A. Shirwaikar, K. Prabhu, R. Punitha, *Ind. J. Experim. Biol*., **44**, 993 (2006).
26. D.D. Lasic, D. Papahadjopoulos, editors. Medical applications of liposomes, *Elsevier* (1998).
27. H. Ohkawa, N. Ohishi, K. Yagi, *Anal. Biochem*. **95**, 351 (1979).
28. B.D. Lawenda, K.M. Kelly, E.J. Ladas, S.M. Sagar, A. Vickers, J.B. Blumberg, *J of the Nat. Cancer Inst.* **100**(11), 773 (2008).
29. G. Barrera, *Oncology*, **2012**, 137289 (2012).
30. D.M. Maestri, V. Nepote, A.L. Lamarque, J.A. Zygadlo, *Res. Signopost*, Kerala, India. 105 (2006).
31. M.S. Baliga, S. Rao, *J. Cancer Research and Therapeutics*, **6**, 255 (2010*).*

32. M.G. Miguel, *Molecules*, **15**, 9252 (2010).
33. Rahman, M.M., Islam, M.B., Biswas, M., Alam, A.H.K. *BMC Research Notes*. 8, 621. (2015).
34. L. Giambarresi, A. Jacobs, *J. Acad. Press*, London, 1987, p. 265.
35. S. Cavar, M. Maksimovic, *Food Contr*. **23**(1): 263 (2012).
36. M. Mileva, I. Nikolova, N. Nikolova, *Acta Microbiol. Bulg*., **31,** 48 (2015).
37. S. Kaviarasan, H. Naik, R. Gangabhagirathi, V. Anuradha, I. Priyadarsini, *Food Chem*., **103**, 31 (2007).
38. A.B. Knott, E. Bossy-Wetzel, *Antioxid. Redox. Sign*. **11**(3), 541 (2010).
39. A. Bhattacharyya, R. Chattopadhyay, S. Mitra, S.E. Crowe, *Physiol. Rev*., **94**, 329 (2014).
40. V. Yermilov, J. Rubio, M. Becchi, M.D. Friesen, B. Pig-Natelli, H. Ohshima, *Carcinogenesis*, **16**, 2045 (1995).
41. A. Rahal, A. Kumar, V. Singh, B. Yadav, R. Tiwari, S. Chakraborty, K. Dhama, *BioMed Res. Int.* Article ID 761264, 19 pages (2014). Doi:10.1155/2014/761264
42. R. Kumar, D. D. Bansal, D. D. Patel, S. Mishra, Y. Karamalakova, A. Zheleva, V. Gadjeva, R. K. Sharma, *Mol. and Cell. Bioch*. **349**(1-2), 57 (2011).
43. P.I. Kumar, H.C. Goel, *Ind. J. Exp. Biol*., **38,** 1003 (2000).
44. R.M. Samarth, M. Samarth, Y. Matsumoto, *Drug Design, Devel. and Ther*., **9,** 5355 (2015).

РОЗОВО МАСЛО, ИЗОЛИРАНО ОТ МАСЛОДАЙНАТА *Rosa damascena* MILL. КАТО ЗАЩИТНО СРЕДСТВО СРЕЩУ РАДИАЦИОННО ПРЕДИЗВИКАНИ ОКСИДАТИВНИ НАРУШЕНИЯ

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(Резюме)

Есенциални масла се извличат от различни природни растителни материали като листа и цветове. Те са важни от търговска гледна точка и се използват широко в козметиката, домакинските продукти и лекарствата поради фармакологично активните си компоненти. Като антиоксиданти есенциалните масла могат да предотвратят окислителни процеси и да подтиснат влиянието на окислителната реакция, предизвикана от радиационно-индуцирани свободни радикали на кислород/азот. Йонизиращата радиация е признат метод за запазване качеството на ароматни билки, подправки и зеленчуци за дълго време. Целта на настоящото изследване е да се идентифицира радиозащитната ефективност на розовото масло срещу оксидативни нарушения на молекулите, дължащи се на йонизиращото лъчение, с използване на *in vitro* модели.

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New mechanisms in preventive effect of ellagic acid on cognition in mice with Alzheimer's disease type dementia

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Antioxidant mechanisms in protective effects of some natural compounds on progression of Alzheimer's disease (AD) were reported during last years. Our previous data revealed significant improving effect of a natural polyphenol Ellagic Acid (EA) on rodent cognitive functions. The goal of this study was to evaluate the effect of EA on cognition of mice with chemically induced dementia from AD type. This animal model was produced via Scopolamine treatment of male Albino mice and was verified by cognitive and biochemical methods. After 5-days treatment with EA both the changes in the cognitive functions of animals and biochemical correlates were evaluated. Significant preventive effect of EA on the processes of learning and memory (Step-through test) of dement animals was established. The high percent (50%) of memory prevention by EA was accompanied by significant antioxidant effect (decreased lipid peroxidation) and inhibited activity of acetylcholine esterase in the brains of EA-treated animals. An increase of dopamine uptake in the brains of EA-treated dement animals was also found. Our results reveal some of the complex mechanisms underlying the EA preventive effect on the cognition in mouse model of AD-dementia.

Key words: Ellagic acid, Alzheimer's disease, Memory, Antioxidants, Acetylcholine esterase, Dopamine

INTRODUCTION

Alzheimer disease (AD) is the most common dementia with yet disputable etiology [1]. Along with the leading Aβ-amyloid hypothesis, the degeneration in the cholinergic system and the brain oxidative stress are other important players in the AD pathogenesis [2].

The cholinergic system plays a crucial role in learning and memory [3] and therefore treatment with AChE-inhibitors relieves some of the key AD symptoms [4]. Recent studies show that along with the cholinergic system the dopaminergic system can also be affected in AD in a complex way. Changes in the dopamine (DA) levels, expression of the DA-receptors and DA transporter (DAT) are often observed in the course of AD [5].

The brain oxidative stress is another key player in AD pathology. The increased accumulation of free radicals gradually leads to depletion of the antioxidant system of the brain cells which leads to damage of the lipid membrane constituents [6, 7].

Protective effects of some polyphenol compounds on the progression of AD were reported during last years [8]. Our previous unpublished data revealed a significant improving effect of the natural polyphenol ellagic acid (EA) on the cognitive functions of rodents.

EA is a dimeric derivative of gallic acid, which spontaneously forms a dilactone [9]. Due to the unique chemical structure of EA its carbon and oxygen atoms form a planar complex extended π-electron system [10, 11], which allows EA to serve as an efficient free radical scavenger [12], as well as a chelating agent to some polyvalent metal ions [13, 14].

The goal of this study was to study some mechanisms of the protective effect of EA on the cognition of mice with chemically induced dementia of AD type.

EXPERIMENTAL

Experimental animals and treatment scheme

Male albino ICR mice (18-20 g) were used, divided in the following experimental groups: Control (saline); Sco-treated (Scopolamine-treated); EA+Sco-treated.

The animal model of AD was induced *via* treatment with daily i.p. injection of 1 mg/kg scopolamine for 11 days. The method was verified by cognitive and biochemical tools and markers, i.e. learning and memory tasks, lipid peroxidation and acetylcholine esterase (AChE) activity in brain.

Control animals received saline in the same volume and way of treatment for 11 days.

The possible preventive effect of EA was studied by treating the animals from the EA+Sco-group

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with scopolamine (1mg/kg b.w. i.p.) for 11 days and 400 mg/kg dose of the natural polyphenol (p. o.) for five consecutive days.

Behavioral and biochemical analysis

On the 24th hour after the last treatment all groups were tested behaviorally and afterwards decapitated and the brains were removed on ice and collected for biochemical evaluation.

The behavioral changes were estimated with the Step-through test (for estimation of the changes in the emotional learning and memory of animals at the end of treatment) by Jarvik and Kopp [15]. The step-through task is a one-trial emotional memory task combining fear conditioning with an instrumental response [16] in a special apparatus [15].

Neuromuscular coordination and the effect of learning new locomotor skills and memory was tested on Rot-a-Rod set up [17, 18]. Initially the animals were trained on new motor skills on the Rot-a-Rod apparatus and after the treatment period were retested again.

Next biochemical parameters were evaluated in a 10% supernatant of brain homogenates in 0.1 M potassium phosphate buffer with pH 7.4:

Acetylcholinesterase activity (AChE) was estimated by the colorimetric reaction of the products of the AChE catalyzed decomposition of acetylthiocholine according to Ellman's method [19].

For the evaluation of antioxidant activity of EA, the lipid peroxidation was measured by determination of the TBARS (thiobarbituric acid reactive substances) through the color products from their reaction with thiobarbituric acid according to the protocol by Buege and Aust [20].

The DA uptake was measured with the protocol by Nicklas *et al.* [21]. In brief, brain synaptosomal fractions were incubated in Krebs-Ringer medium with radioactive DA. At suitable intervals portions of the suspension were filtered, afterward the filters were washed and the washings' radioactivity was measured to determine the DA uptake.

Statistical analysis

Experimental data were analyzed statistically by Student's t-test. Results were expressed as means ± SEM. Differences were considered significant at $P < 0.05$.

RESULTS AND DISCUSSION

After 5-days treatment of Sco- mice with EA both the changes in the cognitive functions of animals and the biochemical parameters were evaluated in all groups.

Significant preventive effect of EA on the processes of learning and memory was found *via* the step-through test. A very high percent (50%) difference in avoidances between Sco and EA+Sco groups was observed signifying significant memory prevention by EA ($P < 0.001$) (Fig. 1).

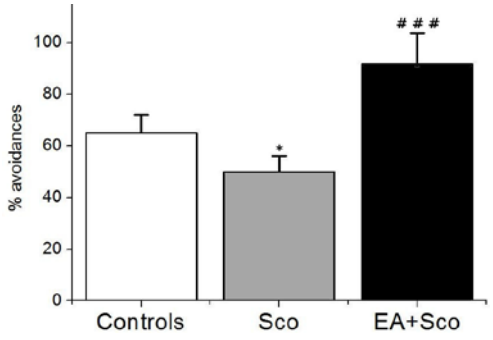

Fig. 1. Step-through test performance (avoidances) of the control, Sco-treated (Sco) and EA+Sco treated (EA+Sco) animals (*P<0.05, *vs* controls; ###P<0.001, *vs* Sco-treated).

The preventive effect of EA was also available in the second parameter of the Step-through test – the latency time – which is comparable with the saline-treated controls (Fig. 2).

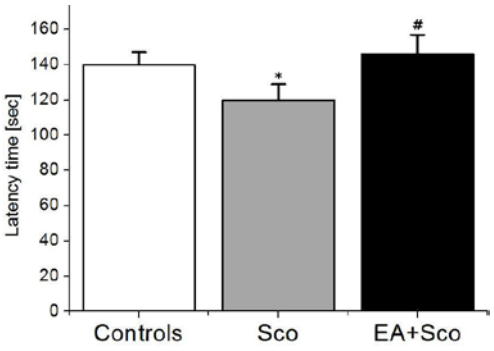

Fig. 2. Step through test performance (latency) of the control, Sco-treated (Sco) and EA+Sco-treated (EA+Sco) animals (*P<0.05, vs controls; #P<0.05, vs Sco-treated).

Preventive effect of EA after the last treatment on the motor learning and memory and neuromuscular coordination of dement animals also was established via Rot-a-Rod test (Fig. 3), where the motor learning effect appears as the elongated falling latency in the test performance [18]. There is over 30% increase in the latency time in EA+Sco-treated over Sco-treated group.

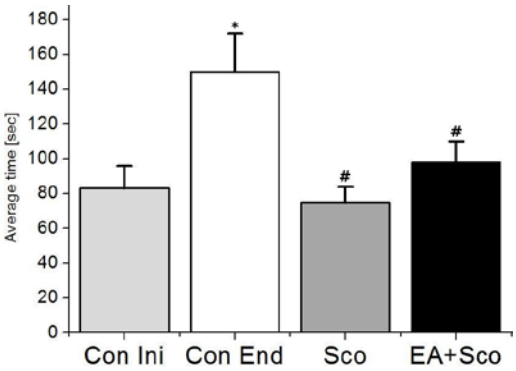

Fig. 3. Rot-a-Rod test performance: initial state of controls (Con Ini) and the effect of learning in the controls at the end of treatment (Con End), Sco-treated (Sco) and EA+Sco-treated (EA+Sco) animals (*P<0.05, *vs* Con Ini; #P<0.05, *vs* Con End).

The treatment with EA of Sco-animals also showed beneficiary effects upon the measured biochemical parameters. The measured AChE activity in the brain was reduced by over 50% in the EA+Sco-treated group compared to the Sco-treated group (Fig. 4).

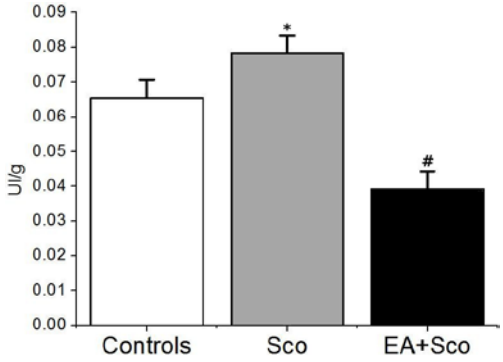

Fig 4. Brain AChE activity in control, scopolamine-treated (Sco) and EA+Sco-treated (EA+Sco) animals (*P<0.05, *vs* controls; #P<0.05, *vs* Sco-treated).

Similar trend was established for the measured products of the lipid peroxidation (Fig. 5), showing significant antioxidant activity of EA reducing the level of TBARS by 13% compared to the Sco-treated group. A decrease in DA uptake in brains of EA+Sco-treated AD animals was also found (Fig. 6). The DA uptake was reduced by over 17% in the EA+Sco-treated animals compared to the Sco-treated group.

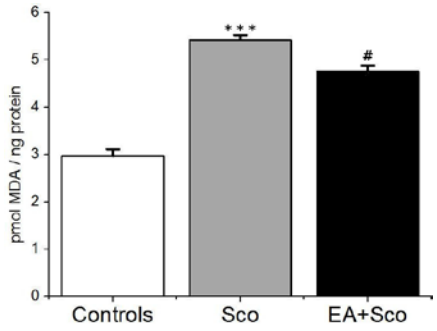

Fig. 5. TBARS in the brain in control, Sco-treated (Sco) and EA+Sco-treated (EA+Sco) animals (***P<0.001, *vs* controls; #P<0.05, *vs* Sco-treated).

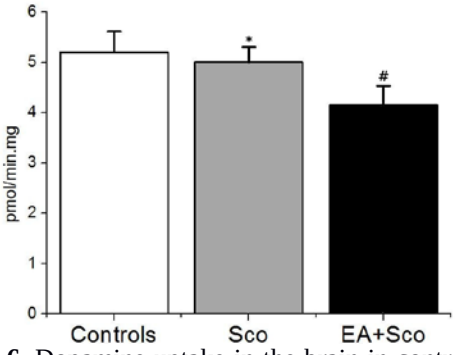

Fig. 6. Dopamine uptake in the brain in control, Sco-treated (Sco) and EA+Sco-treated (EA+Sco) animals (*P<0.05, *vs* controls; #P<0.05, *vs* Sco-treated).

Our results revealed some of the complex mechanisms underlying the EA preventive effect on the cognitive disorders in mouse model of dementia.

The high percent (50%) of prevention by EA on the emotional **learning and memory** which relies on cholinergic neurons rich pathways as the thalamo-amygdalo-cortical one [22, 23], as shown on the step through test, was accompanied by decreased lipid peroxidation which precedes amyloid formation leading to neurons death [24]. Also there is good preventive effect of EA on the motor learning and memory as shown on the Rot-a-rod test performance, which in the recent years has also been shown to be affected in AD [25]. This means that EA may impact not only on the commonly evaluated in AD rich in cholinergic neurons learning memory pathways but also on the motor learning related cerebellar-thalamic-cortical networks poorer in such neurons [26]. However, unlike the results in emotional learning where EA acts as a rather strong memory enhancer/protector whose effect may be attributed to the protective effect on the cholinergic system, here EA has less pronounced capacity to restore the evaluated impaired motor-cognitive functions. This may be due to the complexity of the underlying networks which have, receive and project terminals to structures with varying degree of impair in AD, where the greatly benefiting from EA-treatment

cholinergic neurons [27, 28] is a minor portion of it such as the basal ganglia [29]. Portion of the cholinergic basal ganglia acts as map "reorganizer" in the motor learning processes [29] and it seems that once impaired, their complex dynamic functionality is hard to be repaired sufficiently within the duration of the study.

EA can decrease **lipid peroxidation** in either direct or indirect routes in AD. EA being amphipathic molecule and having better solubility in lipids than in water [30] may bind non-covalently to the lipid membranes or embed in them [31]. AD has pronounced oxidative stress caused damage of lipid membranes and membrane-related cell and biochemical processes [32, 33]. A possible direct mechanism for EA membrane protection is that EA "shields" the membranes from the undesired oxidation and peroxidation processes related to the oxidative stress. Being both radical scavenger and chelating agent *via* indirect route EA can inhibit NADPH and ADP-Fe^{3+}-dependent lipid peroxidation [34].

EA also inhibited the activity of **acetylcholine esterase** in the brains of animals. AChE is a group of enzymes with complex molecular polymorphism of quaternary structure. Although the enzyme forms display similar catalytic activity, they differ in their hydrodynamic parameters and ionic or hydrophobic interactions; for example some of the forms with a hydrophobic terminal can bind selectively to amyloid plaques in AD [27].

A recent study using enzyme assay for AChE (from electric eel) reports that EA can act as AChE inhibitor at *in vitro* conditions with IC_{50}=45.63 μM [35]. However, in the complex environment of the body's cells and fluids the correlation between EA and AChE activity may not be that straightforward, since EA can also act as an antioxidant reducing some of the triggering factors for AChE overexpression like stress and Aβ-amyloid formation processes [27]. Also EA can reduce the formation of Aβ oligomers by inhibiting BACE1 and by promoting the Aβ fibrilization [36]. It is known that the most common form of AChE G4 can aggregate and co-localize with Aβ oligomers and fibrils [27]. In this way EA can also reduce the AChE availability and activity.

A decrease of **dopamine uptake** in the brains of EA-treated dement animals was also found. EA significantly reduces the levels of the DA uptake in the brain. DA is uptaken mostly by the DA transporter (DAT), however, another monoamine or other less specific organic ion transporters can also participate in its membrane transport [37]. In EA-treated animals there is only 17% decrease in the DA uptake which probably means that EA acts upon this parameter by some indirect mechanism or by inhibiting the secondary uptake routes. A recent study reports that EA is a very potent inhibitor of some of the organic anion transporters in the brain like OAT1. It was found that EA has IC_{50}=207 nM for this particular transporter [38]. These transporters are involved in the transport of the metabolites from the DA pathway and along with kidney are specifically expressed in the brain [39]. So the reduction of the DA uptake in EA-treated animals may be attributed to inhibition of some of the secondary routes for DA transport.

This reduced DA uptake is related to the improvement in the motor memory according to a recent study [18], where inhibition of DA transport enhances motor learning.

CONCLUSIONS

The present study reveals the complex mechanisms of EA preventing effect on the memory of dement mice, namely antoxidant activity, AChE inhibition and DA modulation in the brain. Further studies will elucidate new details in the established complex effects of EA on the learning and memory of animals with scopolamine-induced dementia.

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REFERENCES

1. K. G. Yiannopoulou, S. G. Papageorgiou, *Ther. Adv. Neurol. Disord.*, **6**, 19 (2013).
2. J.C. de la Torre, Alzheimer's Turning Point. Springer, Cham. 2016, p. 49.
3. M.E. Hasselmo, *Curr. Opin. Neurobiol.*, **16**, 710 (2006).
4. A.M. Tata, L. Velluto, C. D'angelo, M. Reale. *CNS Neurol. Disord. Drug Targets*, **13**, 1294 (2014).
5. A. Martorana, G. Koch, *Front. Aging Neurosci.*, **6**, 252 (2014).
6. E. Ferreiro, I. Baldeiras, I.L. Ferreira, R.O. Costa, A.C. Rego, C.F. Pereira, C.R. Oliveira, *Int. J. Cell Biol*., 735206, (2012).
7. I. Baldeiras, M.T. Santana, R. Garrucho, A. Pascoal, D. Rodrigues, D. Duro, C.R. Oliveira. *J. Alzheimers Dis*., **15**, 117 (2008).
8. R. E. Hartman, A. Shah, A. M. Fagan, K. E. Schwetye, M. Parsadanian, R. N. Schulman, M. B. Finn, D. M. Holtzman, *Neurobiol. Dis.*, **24**, 506 (2006).
9. G. Skrede, R. E. Wrolstad, in: Functional Foods: Biochemical and Processing Aspects, vol. 2, G. Mazza, M. Le Maguer & J. Shi (eds.), CRC Press, Lancaster, 2002, p. 80.
10. A. McL Mathieson, B. J. Poppleton, *Acta Cryst.* **B24,** 1456, (1968).
11. W. Vermerris, R. Nicholson, Phenolic Compound Biochemistry. Springer, Dordrecht, 2008.

12. J. Zhang, Y. Xiong, B. Peng, H. Gao, Z. Zhou, *Comput. Theor. Chem.*, **963**, 148 (2011).
13. S. R. Przewloka, B. J. Shearer, *Holzforschung*, **56**, 13 (2002).
14. I. Kilic, Y. Yeşiloğlu, Y. Bayrak, *Spectrochim. Acta Mol. Biomol. Spectrosc.*, **130**, 447 (2014).
15. M. E. Jarvik, R. Kopp, *Psychol. Rep.* **21**, 221 (1967).
16. S. O. Ogren, O. Stiedl., in: Encyclopedia of Psychopharmacology, vol. 2 (L-Z), I. P. Stolerman (ed.), Springer Science+Business Media, New York, 2010, p.960.
17. N. W. Dunham, T. S. Miya, *J. Am. Pharm. Assoc. Am. Pharm. Assoc*., **46,** 208 (1957).
18. H. Shiotsuki, K. Yoshimi, Y. Shimo, M. Funayama, Y. Takamatsu, K. Ikeda, R. Takahashi, S. Kitazawa, N. Hattori, *J. Neurosci. Methods*, **189**, 180 (2010).
19. G. L. Ellman, K. D. Courtney, V. Andres, R. M. Featherstone, *Biochem. Pharmacol.*, **7**, 88 (1961).
20. J. A. Buege, S. D. Aust, *Methods Enzymol.*, *52*, 302 (1978).
21. W. J. Nicklas, S. Puszkin, S. Berl, *J. Neurochem.*, **20**, 109 (1973).
22. J. E. LeDoux, *Behav. Brain Res.*, **58**, 69 (1993).
23. M. H. Schäfer, L. E. Eiden, E. Weihe, *Neuroscience*, **84**, 331 (1998).
24. D. Praticò, K. Uryu, S. Leight, J. Q. Trojanoswki, V. Lee, *J. Neurosci.*, **21**, 4183 (2001).
25. I. A. van Halteren-van Tilborg, E. J. Scherder, W. Hulstijn, *Neuropsychol. Rev.*, **17**, 203 (2007).
26. F. Mawase, S. Bar-Haim, L. Shmuelof, *J. Neurosci.*, **37**, 349 (2017).
27. V. N. Talesa, *Mech. Ageing Dev.*, **122,** 1961 (2001).
28. R. Kaur, S. Mehan, D. Khanna, S. Kalra, *Int. J. Neurology Res.*, **1**, 141 (2015).
29. J. M. Conner, M. Kulczycki, M. H. Tuszynski, *Cereb. Cortex*, **20**, 2739 (2010).
30. Z. Marković, D. Milenković, J. Đorović, J. M. D. Marković, B. Lučić, D. Amić, *Monatsh. Chem.* **144**, 803 (2013).
31. D. A. Vattem, K. Shetty, *J. Food Biochem.*, **29**, 234 (2005).
32. P. H. Axelsen, H. Komatsu, I. V. Murray, *Physiology*, **26**, 54 (2011).
33. D. Praticò, *Trends Pharmacol. Sci.*, **29**, 609 (2008).
34. K. L. Khanduja, A. Bhardwaj, *Indian J. Biochem. Biophys.*, **40**, 416 (2003).
35. G, Nag, D. Bratati, *Int. J. Pharm. Pharm. Sci.*, **3**, 121 (2011).
36. Y. Feng, S. G. Yang, X. T. Du, X. Zhang, X. X. Sun, M. Zhao, G.Y. Sun, R. T. Liu, *Biochem. Biophys. Res. Commun.*, **390**, 1250 (2009).
37. H. Nishijima, M. Tomiyama, *Front. Neurosci.*, **10**, 575 (2016).
38. S. A. Hussain, A. A. Sulaiman, H. Alhaddad, Q. Alhadidi, *J. Intercult. Ethnopharmacol.*, **5**, 97 (2016).
39. N. Anzai, Y. Kanai, H. Endou. *J. Pharmacol. Sci.*, **100**, 411 (2006).

НОВИ МЕХАНИЗМИ В ПРЕВАНТИВНИЯ ЕФЕКТ НА ЕЛАГОВАТА КИСЕЛИНА ВЪРХУ КОГНИТИВНИТЕ ФУНКЦИИ ПРИ МИШКИ С МОДЕЛ НА БОЛЕСТТА НА АЛЦХАЙМЕР

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(Резюме)

През последните години бяха съобщени различни антиоксидантни механизми в защитните ефекти на някои природни съединения върху прогреса на болестта на Алцхаймер (БА). Наши предишни данни разкриха значително подобряващ ефект на естествения полифенол - елаговата киселина (ЕК) - върху когнитивните функции на гризачите. Целта на това изследване е да се оцени ефектът на ЕК върху когнитивните способности на мишки с химически индуцирана деменция от тип БА. Животинският модел на БА бе предизвикан чрез третиране със скополамин на мъжки мишки и бе потвърден с поведенчески и биохимични методи. След 5-дневно третиране с ЕК бяха оценени както промените в когнитивните функции на животните, така и биохимичните корелати. Беше установен значителен превантивен ефект на ЕК върху процесите на учене и паметта на животни с деменция. Високият процент (50%) на превенция върху паметта чрез ЕК е придружен от значителен антиоксидантен ефект (понижена липидна пероксидация) и инхибиране на активността на ацетилхолинестеразата в мозъците на третирани с ЕК животни. Установено е също така увеличение на поемането на допамин в мозъка на животни, третирани с ЕК. Нашите резултати разкриват някои от сложните механизми, които стоят в основата на превантивния ефект на ЕК върху когнитивните функции в модел на деменция тип БА при мишки.

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Interaction of natural thiols and catecholamines with reactive oxygen species

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Natural thiols (TSH) cysteine, glutathione, and homocysteine, as well as catecholamines (CA) dopamine, noradrenaline and adrenaline are known as multifunctional biologically active compounds with antioxidant potential, i.e. bio-antioxidants, which play an important role in the regulation of the redox status and free radical formation – utilization in living organisms. The kinetic characteristics of interaction of TSH and CA with peroxyl radicals, RO_2•, formed from the azo-initiator AAPH in aqueous solutions at 37°C by the method of competing reactions were determined. The kinetics of radical formation in the reactions of TSH with H_2O_2 was studied by the inhibitors method. The polymethine dye (A, pyridine salt of 3,3'-di-ϒ-sulphopropyl-9-methylthia-carbocyanine betaine) was used as a radical scavenger. CA demonstrated the highest antiradical activity ($k_i > 10^6 (M \cdot s)^{-1}$, whereas TSH possess moderate activity ($k_i \sim 10^5 (M \cdot s)^{-1}$).

Keywords: Free radical generation**,** Thiols, Catecholamines, Caffeic acid, Kinetics

INTRODUCTION

Oxidative stress is characterized by an increased content of reactive oxygen species (ROS) and reflects an imbalance between the rates of formation of ROS and their utilization [1-4].The generation of free radicals by biochemical redox reactions is part of the normal cellular metabolism and cells have evolved a variety of mechanisms for scavenging them. Natural thiols (TSH) cysteine, CSH, [5-7], glutathione, GSH, [8-10], and homocysteine, HSH, [11-15], as well as catecholamines (CA) dopamine, DA, norepinephrine, NE, and adrenaline, epinephrine EN, [16-20] are known as multifunctional biologically active compounds with antioxidant potential, i.e. bio-antioxidants, which play an important role in the regulation of the redox status and free radical formation – utilization in living organisms. Catecholamines (CA) compose the group of biogenic amines containing 3,4-dihydroxyphenyl (catechol) as a common structural fragment, which exhibits antioxidant properties in free radical oxidation reactions. Both TSH and CA are water-soluble compounds. Catecholamines are neurotransmitters and neurohormones in animals and humans, and they also function as endogenous antioxidants in the nervous system. In a number of studies, TSH and CA are considered together as compounds, which affect the redox situation in the nerve cells and have potential relevance to age-related diseases [21-23]. In [21] the antioxidant and pro-oxidant capacity of catecholamines (CA) and related compounds were analyzed using the oxygen radical absorbance capacity (ORAC) assay, in which 2,2-azobis (2-amidino-propane) dihydrochloride (AAPH) was a peroxyl radical generator. The antioxidant effects of CA and glutathione (GSH) were in the order: dopamine (DA)> norepinephrine (NE)>> GSH. The comparative assay of antioxidant potential of TSH made in [24] showed that their antiradical activity decreased in the order: CSH> HSH > GSH, and the recovery of hydrogen peroxide by thiols was found to be accompanied by a low yield of radicals [24, 26].

The first goal of this study was to evaluate and to compare the antioxidant and pro-oxidant nature of CA and TSH in the presence of AAPH using the method of competing reactions with the polymethine dye A (pyridine salt of 3,3'-di-ϒ-sulphopropyl-9-methylthia-carbocyanine betaine) in aqueous solution. The second goal was to estimate the effect of H_2O_2 on the GSH behavior towards the phenol antioxidants resveratrol and caffeic acid known as having immune-modulatory, anti-inflammatory activity and inhibitory effect on cancer cell proliferation [27-30] and having in the molecule a double bond conjugated with the phenolic ring.

EXPERIMENTAL

Commercially available natural thiols glutathione (GSH), homocysteine (HSH) and cysteine (CSH), catecholamines epinephrine (EN), norepinephrine (NE), and caffeic acid (AC) (Sigma-Aldrich), trans-resveratrol, RVT (abcr GmbH), hydrogen peroxide (Usolehimprom), dopamine (DA) (Fluka) (Fig. 1), azo-initiator AAPH (2,2′-azobis (2-methylpropionamidine dihydrochloride, Fluka) were used as purchased. The polymethine dye (A, pyridine salt of 3,3'-di-ϒ-sulphopropyl-9-methylthia-

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carbocyanine betaine) (Fig. 1) was used in the method of competing reactions as a reference free radical scavenger with known spectral-kinetic characteristics: ε = $0.77 \cdot 10^5$ $L \cdot mol^{-1} \cdot cm^{-1}$ atλ_{max} = 546 nm; the rate constant of the reaction of A with peroxyl radicals derived from AAPH is equal to $5{,}4 \cdot 10^4$ $L \cdot mol^{-1} \cdot s^{-1}$and the stoichiometric coefficient f =1 at 37ºC [25]. The concentration of A, RVT and AC was determined spectrophotometrically. All reactions were carried out in redistilled water at the physiological temperature of 37 °C directly in a temperature-controlled cell (*l* = 1 cm) of Ultraspec 1100. The determination error of the kinetic characteristics was about/less than 10%.

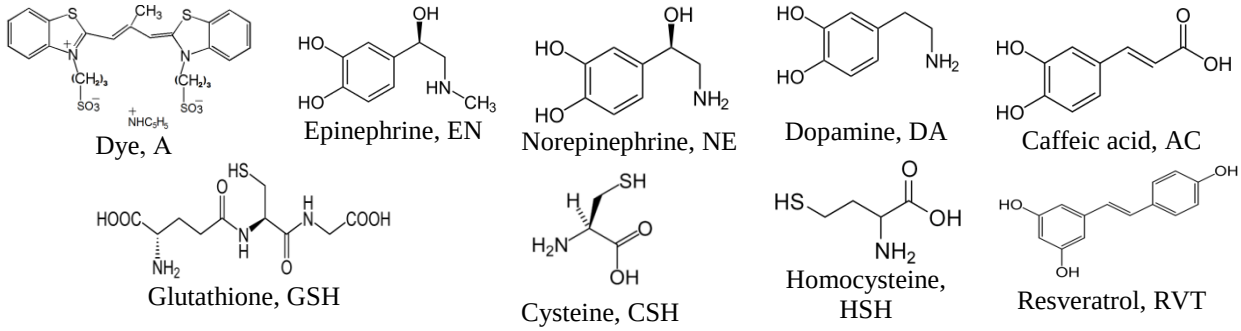
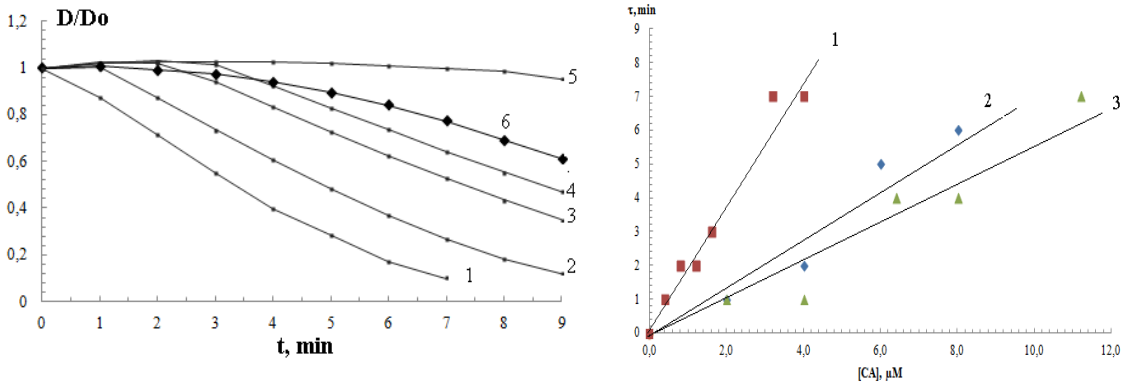


Figure 1. Structural formulas of the dye (A), catecholamines, natural thiols, resveratrol and caffeic acid

RESULTS AND DISCUSSION

Antiradical activity of catecholamines and natural thiols

Figure 2a shows that small additives of DA, more than an order of magnitude less than the concentration of dye (A) leads to dose-depending induction period (τ) in A consumption in the reaction with peroxyl radicals (RO_2•) generated byAAPH. After the end of the induction period, the dye is consumed with a rate of noninhibited reaction.



a b

Figure 2. a) Kinetic curves of the consumption of the 10 μM dye (A) in reaction with peroxyl radicals with additives of DA and GSH; [DA] μM: 1 – 0; 2 – 0.8; 3 –1.6; 4 – 2; 5 – 4; 6 - 4 μM GSH; [AAPH] 18 mM; b) Dependences of the induction periods observed in consumption of dye (A) on the concentration of catecholamines: 1-DA; 2-EN, and 3-NE. [AAPH] 18 mM; [A] 10μM.

Induction periods are observed in the presence of EN and NE as well, but they are shorter than τ in the case of DA (Fig. 2b).The main kinetic characteristics of antiradical activity of a radical scavenger (X) are the rate constant for its reaction with radicals (k_X) and the stoichiometric coefficient f, which largely determines the duration of the induction period with the known rate of radical initiation (W_i) [31-34]:

$$\tau = f\,[X]_0/W_i \qquad (1)$$

In the case of dopamine, the dependence of the induction period observed in dye (A) consumption at $W_i = 1.8 \cdot 10^{-8}$ M/s (Fig. 2b, curve 1) is linear and the stoichiometric coefficient f = 2, calculated according to eqn. (1).

Table 1. Kinetic characteristics of antiradical activity of catecholamines, thiols and resveratrol in aqueous solution

Kinetic Characteristic	DA	EN	NE	GSH [24]	HSH [24]	CSH [24]	RVT [35]
f	2	0.8	0.6	1	1	1	2
$k_{ROO\bullet}\cdot10^{-5}M^{-1}\cdot s^{-1}$	>10	>10	>10	0.84	2.2	4.4	1.1
$\varpi\cdot10^{3}$, $M^{-1}\cdot s^{-1}$	-	-	-	0.07	0.07	2.1	-

The induction periods for adrenaline (curve 2) and noradrenaline (curve 3), whose molecules in contrast to DA contain a hydroxyl group in α-position to the aromatic ring, are significantly shorter. Evaluation of stoichiometric coefficients, calculated for the catecholamines according to Fig. 2b and eqn. (1), gives values of 0.8 and 0.6 for EN and NE, accordingly (Table 1).

It is known from the literature that catecholamines are highly active in reactions with different radicals. In [36], by means of the stop-flow method, the reaction rate constants were determined for the reaction of DA, EN and NE with low-reactive tocopheroxyl ($k_{Toc\bullet}\sim10^{2}M^{-1}\cdot s^{-1}$) radicals in 2-propanol/water medium. In [37], by pulsed laser photolysis method, the rate constant for the reaction of CA with cumyloxyl radicals ($k_{RO\bullet}\sim 10^{7}\ M^{-1}\cdot s^{-1}$) was determined in alcohol media. It must be noted that the stoichiometric coefficients for DA, EN and NE in [36, 37] are equal to those determined in this work (table 1). The rate constants in reactions with various radicals including phenoxyl and alkoxyl radicals decrease in the order: DA> EN > NE. Antioxidant properties of CA were tested in [19] in the PC liposomes oxidation initiated by AAPH. It was found that for DA, EN, and NE f = 2 in phosphate buffer of pH 7.2. In aqueous solution f = 2 for DA and NE, but f = 4 for EN and in aqueous solution EN oxidizes to pink adrenochrome. May be, these results can be explained by interactions of CA with negatively charged model bio-membranes, found in [38, 39].

Contrary to CA, the additive of GSH (Fig. 2a curve 6) does not show an induction period, but it results in the measured decrease of A consumption rate. In [24], the kinetics of A consumption in the presence of competitive radical scavenger (X) in respect to thiols was described by the following reactions:

$AAPH \rightarrow RO_2\bullet$, $W_i = k_i\,[AAPH]$ is the rate of initiation;

$RO_2\bullet + A \rightarrow A\bullet$, W_A is the rate of consumption of A;

$RO_2\bullet + X \rightarrow X^* + RO_2H$, W_X is the rate of consumption of X;

$A\bullet + A\bullet \rightarrow$ Products,

$X\bullet + X\bullet \rightarrow$ Products,

$A\bullet + X\bullet \rightarrow$ Products.

The latter three reactions of recombination/ disproportionation of radicals A• formed from the dye and recombination of thiyl radicals X• formed from thiols proceed with high rates [24, 40] and provide stoichiometric coefficients f = 1 for dye (A) and thiols in the reactions with peroxyl radicals. At sufficient concentrations of the scavengers under steady-state conditions, i.e. $W_i = W_A + W_X$ the rate of dye consumption upon addition of X is equal to $W_A = k_A[A][RO_2\bullet] = (k_A[A]W_i)/\{k_A[A] + k_X[X]\}$. To analyze the experimental data, eqn. (1) was transformed into the form (2):

$$1/W_A = [1 + (k_X/k_A)\bullet([X]/[A])]/W_i \qquad (2)$$

From the slope of the dependence of $1/W_A$ on the concentration ratio [X]/[A], the rate constants for all the natural thiols were determined (table 1). This approach [24] is suitable for determining rate constants for reactions with peroxyl radicals in aqueous media for inhibitors of medium strength ($10^{3}<k_{ROO\bullet}<10^{6}\ M^{-1}\cdot s^{-1}$). It was applied in [35] to determine $k_{ROO\bullet}$ and f for resveratrol (table 1) which characterized RVT as a moderate scavenger of free radicals.

Free radical formation in the reaction of thiols with H_2O_2

The polymethine dye A was used as the radical scavenger to study the free radical generation upon the reaction of thiols with H_2O_2 [24]. The specific rates of radical generation equal to $\varpi_i = W_i/([TSH][H_2O_2])$ are presented in table 1. The yield of radicals in the reaction of TSH with H_2O_2 is very small <1%, however, this value of free radical formation may be sufficient to initiate chain processes, especially in multiphase systems. We have established [35] that the well-known antioxidant resveratrol (RVT), which has in the molecule a double bond activated by conjugation with two phenolic rings, interacts with glutathione (GSH) and cysteine (CSH) in aqueous solutions at 37ºC. Reaction of RVT with thiols (thiol-ene reaction) proceeds by a chain mechanism and is accelerated in the presence of H_2O_2. In this study, we investigated the interaction of caffeic acid (AC) with

glutathione. The reactions were carried out in aqueous solution at 37ºC.

The behavior of AC was controlled *via* the UV absorption spectra. When GSH and H_2O_2 were added separately, the AC spectrum did not change (Fig. 3b, curves 1 and 2). However, when GSH and H_2O_2 were added together, AC consumption was observed (Fig. 3a and curve 3 Fig. 3b) with a rate equal to $W_{AC} = 4.9 \cdot 10^{-8}$ $M \cdot s^{-1}$. The rate of radical initiation in the reaction GSH +H_2O_2 can be calculated as follows:

$W_i = \varpi[GSH][H_2O_2] = 0.07 \cdot 10^{-3} \times 5 \cdot 10^{-3} \times 8.6 \cdot 10^{-3} = 3 \cdot 10^{-9}$ $M \cdot s^{-1}$.

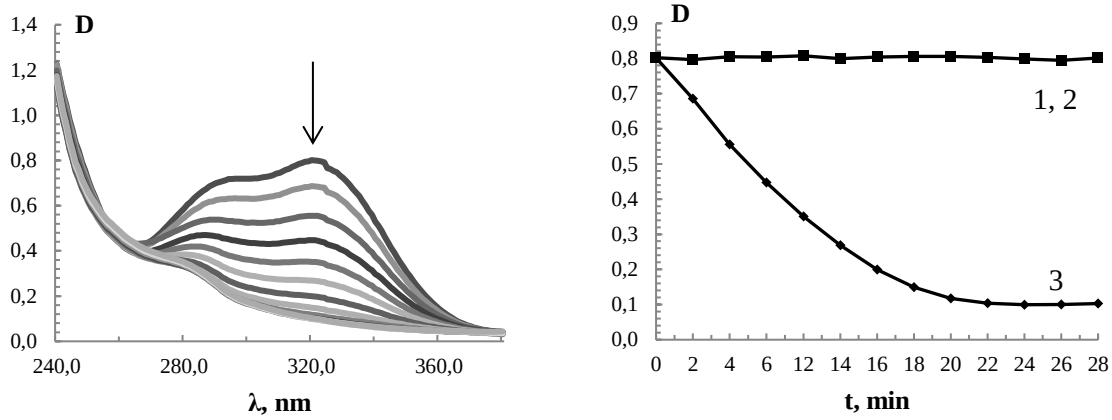


Figure 3a) Change of the UV absorption spectra of caffeic acid in the reaction with glutathione initiated by H_2O_2: [GSH] 5 mM; [H_2O_2] 8.6 mM; [AC] 0.053 mM; b) Changes of optical density of caffeic acid at 321 nm in reaction with GSH and H_2O_2. 1: [AC] 0.053 mM, [GSH] 5 mM; 2: [AC] 0.053 mM, [H_2O_2] 8.6 mM; 3: [AC] 0.053 mM, [GSH] 5 mM, [H_2O_2] 8.6 mM.

The comparison of the rates W_{AC} and W_i reveals that similar to the reaction of resveratrol with GSH [35], the reaction of caffeic acid with GSH in the presence of H_2O_2 proceeds by a chain mechanism with rather long chain length equal to $\nu = W_{AC} / W_i = 16$.

$2GSH + H_2O_2 \rightarrow [2GSH \ldots H_2O_2] \rightarrow G\text{-}S\text{-}S\text{-}G + 2H_2O$

$\downarrow$

Radicals ($GS^{\bullet}$)	Chain initiation
$GS^{\bullet} + AC \rightarrow AC^{*\bullet}$	Chain propagation
$AC^{*\bullet} + GSH \rightarrow GS\bullet + AC^{*}H$	
$GS^{\bullet} + GS^{\bullet} \rightarrow G\text{-}S\text{-}S\text{-}G$	Chain termination

Here, $AC^{*\bullet}$ is alkyl radical resulted from $GS^{\bullet}$ addition to the double bond of AC.

CONCLUSION

Using the polymethine dye (A, pyridine salt of 3,3'-di-ϒ-sulphopropyl-9-methylthia-carbocyanine betaine) as the reference radical scavenger, the kinetic characteristics of interaction of natural thiols (TSH) and catecholamines (CA) with peroxyl radicals, $RO_2^{\bullet}$, formed from azo-initiator AAPH in aqueous solutions at 37°C by the method of competing reactions were determined. CA have demonstrated the highest antiradical activity ($k_{ROO} > 10^6 (M \cdot s)^{-1}$, whereas TSH possess moderate activity ($k_{ROO} \sim 10^5 (M \cdot s)^{-1}$). The kinetics of radical formation in the reactions of TSH with H_2O_2 was studied by the inhibitors method and specific rates of these reactions were determined. Due to radical formation in the reaction of GSH with H_2O_2, we have established for the first time that the reaction of caffeic acid with glutathione in the presence of H_2O_2 proceeds by a chain mechanism with rather long chain length.

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REFERENCES

1. H. Sies, in: Oxidative Stress, H. Sies (ed.), Academic Press, London, 1985.
2. H. Sies, D. P. Jones, in Encyclopedia of Stress, ed. G. Fink, vol. **3**, Elsevier, San Diego, CA, 2007.
3. E. Schnitzer, I. Pinchuk, D. Lichtenberg, *Eur. Biophys. J.*, **36**, 499 (2007).
4. N.A. Porter, *J. Org. Chem.*, **78**, 3511 (2013).
5. C.S. Sevier, C.A. Kaiser, *Nat. Rev. Mol. Cell Biol.*, **3**, 836 (2002).
6. N. Nagano, M. Ota,K. Nishikawa, *FEBS Lett.*, **458**, 69 (1999).
7. C. D. Pereira, N. Minamino, T. Takao, *Analytical Chemistry*, **87**, 10785 (2015).
8. A.Guttman, H.G.Gao, R.Haas, *Clinical Chemistry*, **47**, 1467 (2001).
9. B. G.Hill, A. Bhatnagar, *IUBMB Life*, **59**, 21 (2007).
10. B.G. Harbrecht, M.D. Silvio, V. Chough, Y.-M. Kim, R.L. Simmons, T.R. Billiar, *Ann. Surg.*, **225**, 76 (1997).

11. K.S.McCully, *Comprehensive Physiology*, **6**, 471 (2016).
12. M.Cattaneo, *Thrombosis and Haemostasis*, **81**, *165 (*1999).
13. M.S. Morris, *Lancet Neurology*, **2**, *425 (*2003).
14. A.Dietrich-Muszalska, J.Malinowska, B.Olas, R.Głowacki, E.Bald, B.Wachowicz, J.Rabe-Jabłońska, *Neurochemical Research*, **37,**1057 (2012).
15. MA. Smach, N. Jacob, J.L. Golmard, B. Charfeddine, T. Lammouchi, L.B. Othman, H.Dridi, S. Bennamou, K. Limem, *European Neurology*, **65**, 270 (2011).
16. L. Giilfin, *Chemico-Biological Interactions*, **179**, 71 (2009).
17. V. A. Roginsky, *Arch. Biochem. Biophys.*, **414**, 261 (2003).
18. B. Frei, R. Stocker, B. N. Ames, *Proc. Natl. Acad. Sci. USA*, **85**, 9748 (1988).
19. Mengele E.A., Plashchina I.G., Kasaikina O.T. *Colloid J.*, **73**, 701 (2011).
20. V. D. Kancheva, O.T. Kasaikina, *Current Medicinal Chemistry*, **20**, 4784 (2013).
21. E. Sofic, N. Denisova, K. Youdim, V. Vatrenjak-Velagic, C. De Filippo, A. Mehmedagic, A. Causevic, G. Cao, J. A. Joseph, R. L. Prior. *J. Neural Transm.*, **108**, 541 (2001).
22. M.B. Spina, G. Cohen, *Proc. Natl. Acad. Sci. USA*, **88**, 1398 (1989).
23. Z. Spolarics, J. X.Wu, *Am. J. Physiol.*, **273,** 1304 (1997).
24. K.M. Zinatullina, N.P. Khrameeva, O.T. Kasaikina, B.I. Shapiro, V.A. Kuzmin, *Russ. Chem. Bull.*, **7** (2017) in press.
25. K.M. Zinatullina, O.T. Kasaikina, V.A. Kuzmin, N.P. Khrameeva, B.I. Shapiro, *Russ. Chem. Bull*., **12**, 2825 (2016).
26. E.A. Mengele, D.A Krugovov, O. T. Kasaikina, *Russ. Chem. Bull.*, **64**, 846 (2015).
27. L. Fremont, *Life Sci.*, **66**, 663 (2000).
28. A. Plauth, A. Geikowski, S. Cichon, S.J. Wowro, L. Liedgens, M. Rousseau, C. Weidner, L. Fuhr, M. Kliem, G. Jenkins, S. Lotito, L.J. Wainwright, S. Sauer, *Free Radical Biology and Medicine*, **99**, 608 (2016).
29. N.R. Prasad, A. Karthikeyan, S. Karthikeyan, B. V. Reddy, *Mol. Cell Biochem*., **349**, 11(2011).
30. M. Hirose, Y. Takesada, H. Tanaka, S. Tamano, T. Kato, T. Shirai, *Carcinogenesis*, **19**, 207 (1998).
31. N. Emanuel, D. Gal, Modelling of Oxidation Processes. Prototype: The Oxidation of Ethylbenzene, Budapest, Akademiai Kiado, **91**, 681 (1986).
32. E.T. Denisov, I.B. Afanas'ev, Oxidation and Antioxidants in Organic Chemistry and Biology, Boca Raton, Fla., CRC, 2005.
33. E.N.Frankel, Lipid Oxidation, Dundee, Scotland, Oily Press, 2nd ed., 2005.
34. A. Kamal-Eldin (ed.), Lipid Oxidation Pathways, Champaign, Ill., AOCS, 2003.
35. K.M.Zinatullina, N.P. Khrameeva, O.T. Kasaikina, B.I.Shapiro ,V.A. Kuzmin, *Rus. Chem. Bull*. (2017), in press.
36. K.Mukai, K.Nagai, Y.Egawa, A.Ouchi, S.Nagaoka, *J. Phys. Chem. B,* **120**, 7088 (2016).
37. T.Kawashima, K.Ohkubo, S.Fukuzumi, *J. Phys. Chem. B,* **1**, 114, (2010).
38. K.Jodko-Piórecka, G. Litwinienko, *ACS Chem. Neurosci*, **4**, 1114 (2013**).**
39**.** K.Jodko-Piórecka, G. Litwinienko, *Free Radical Biology and Medicine*, **83**, 1 (2015).
40. Landolt-Bernstein: Numerical Data and Functional Relationships in Science and Technology, H. Fischer (ed.), vol. 13**,** Springer-Verlag, Berlin, 1983, p. 308.

ВЗАИМОДЕЙСТВИЕ НА ПРИРОДНИ ТИОЛИ И КАТЕХОЛАМИНИ С РЕАКТИВНИ ФОРМИ НА КИСЛОРОДА

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(Резюме)

Природните тиоли (TSH) цистеин, глутатион и хомоцистеин, както и катехоламините (CA) допамин, норадреналин и адреналин са известни като мултифункционални биологично активни съединения с антиоксидантен потенциал, т.е. био-антиоксиданти, които играят важна роля за регулиране на редокс статуса и образуването и използването на свободни радикали в живите организми. Определени са кинетичните характеристики на взаимодействието на TSH и CA с пероксилните радикали RO_2•, образувани от азоинициатора AAPH във водни разтвори при 37°C по метода на конкурентните реакции. Кинетиката на радикалообразуването при реакциите на TSH с H_2O_2 е изследвана по инхибиторния метод. Полиметиновото багрило (A, пиридинова сол на 3,3'-ди-ϒ -сулфопропил-9-метилтиа-карбоцианин бетаин) е използвано като радикалоуловител. CA проявяват висока антирадикалова активност ($k_i > 10^6 (M \cdot s)^{-1}$, докато активността на TSH е умерена ($k_i \sim 10^5 (M \cdot s)^{-1}$).

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Oxidative stress and related diseases. Part 1: Bronchial asthma

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Oxidative stress (OS) plays a significant role in the pathogenesis of a number of human diseases such as ischemia/reperfusion injury, atherosclerosis, cancer, neurodegenerative diseases, allergy, etc. Bronchial asthma (BA) is a chronic inflammatory disease of the lungs, resulting in a restriction of airflow, hyperactivity, and airway remodeling. Clinical and experimental data in recent years show that increased OS and destructive effects of free radical oxidation can be an important cause of chronic pathological processes in the lungs. The aim of the present study was to investigate and compare the level of oxidative stress in blood of asthmatic patients that differ in the degree of disease control. In the study were included 30 patients with BA and 24 age-matched healthy volunteers. Patients were diagnosed with BA with allergic component longer than one year. For this purpose, the ROS and RNS products, the final products of lipid and protein were explored, the relationship between oxidative stress parameters and C-reactive protein (CRP) as a marker of inflammation degree was also studied. By using the EPR spin trapping technique, ongoing real time oxidative processes were confirmed in blood samples isolated from asthmatic patients differing in the degree of disease control. Moreover, positive correlation was found between the levels of studied OS biomarkers and CRP as a marker of inflammation degree. In BA patients, oxidative processes in real time were demonstrated. The results of correlation analysis confirmed that the development and maintenance of inflammatory processes in respiratory tract are associated with the oxidative and nitrosative stress.

Keywords: ROS, RNS, Bronchial asthma, CRP, MDA

INTRODUCTION

Bronchial asthma (BA) is a chronic inflammatory disease of the lungs, leading to the limitation of airflow, hyperactivity and airway remodeling. This disease is a global medical and social problem [1]. The problems of diagnosis and treatment of asthma are associated with complex clinical disease polymorphism [2,3]. Characteristic because of poor prognosis is severe therapy of resistant asthma, which is associated with uncontrolled inflammation. Clinical and experimental data in recent years show that chronic inflammation of the respiratory tract and oxidative stress (OS) play a key role in respiratory diseases [4]. Increased OS and destructive effects of free radical oxidation can be an important cause of chronic pathological process in the lungs. OS can cause hyperreactivity of the respiratory tract, and free radicals can remove stimulating signals as a critical intracellular second messenger, occurring in the modulation of immune responses [5]. OS is a major feature of asthma, so one of the goals of therapy is to fight the disease by preventing, reducing pulmonary insufficiency and the risks associated with it. According to Taylor [6], achieving control and progression of the disease generally affects the decision to treat. Similarly, the degree of asthma symptoms that are eliminated or reduced by treatment is called "asthma control". The most recent studies have shown [7] that reactive species of nitrogen (RNS) are critical for the progress of asthma. It is known that the molecule of nitric oxide (NO•) participates in the development of asthma by the direct and indirect contribution of allergic inflammation. Nitrosative stress is one of the leading mechanisms of inflammation of the allergic respiratory tract in asthma.

Our goal was to study and compare the OS in the blood of patients with asthma, which differ in the degree of disease control. To achieve this goal, we studied: 1) the levels of some products of ROS and RNS as parameters of OS in real time; 2) levels of end products of oxidation of lipids and proteins, measured as the content of MDA and carbonyl protein (PCC), respectively; 3) the relationship between C-reactive protein (CRP) as a marker of the degree of inflammation and levels of OS parameters.

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EXPERIMENTAL

In this study, 46 patients with asthma from the "Stoyan Kirkovich" University Hospital, Stara Zagora, Bulgaria and 24 age-appropriate healthy volunteers were included. All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Patients were diagnosed with BA with an allergic component for more than one year. The diagnosis BA was made in accordance with *GINA* [1] with positive bronchodilator performed between 15 and 30 min after inhalation of 400 μg Ventolin pMDI without a spacer. The spirometry test was performed on the Master Screen spirometer, eRT, Germany, using ATS/ERS standards 2006. The control of the disease was assessed with thr questionnaire Asthma Control Test. Result of 25 points is considered as well-controlled asthma, partially controlled - 20-24 points; uncontrolled less than 20 points. Patients were divided into three groups depending on the control of asthma: well-controlled (n = 15), partially controlled (n = 14) and uncontrolled (n = 17) asthma, according to GINA [8]. All groups were treated with inhaled corticosteroid (ICS) and/or without leukotriene receptor antagonists (LTRA). The control group consisted of people without a family history of asthma.

Fasting samples of venous blood were collected in the morning from 8.00 to 10.00. Blood to determine lipid peroxidation was collected in tubes containing 10% EDTA (ethylenediaminetetraacetic acid). To measure ROS products, NO• and PCC, whole blood was collected in a closed test tube (no anticoagulant). All samples from each subject were separated and executed in triplicate.

Determination of products of lipid peroxidation

The total number of lipid peroxidation products in plasma was assessed using thiobarbituric acid (TBARs) [9], by measuring reactive malondialdehyde products (MDA) at 532 nm, and the results were expressed in μmol/L.

Electron paramagnetic resonance (EPR)

EPR measurements were carried out on an X-band EMX^{micro}, Bruker spectrometer, Germany, equipped with a standard resonator. The spectral processing was performed using the programs Bruker WIN-EPR and Sinfonia.

EPR ex vivo estimates of serum ROS levels

The ROS levels were determined according to Shi *et al.* [10] with some modification. Experimental spectroscopy of erythrocytes with EPR expression using N-tert-Butyl-α-phenylnitrone (PBN) spin-trap was used to study real-time formation of active forms of oxygen (ROS) in the serum of asthmatic patients and controls.

Evaluation of EPR ex vivo levels of NO•

Based on the methods published by Yoshioka *et al.* [11] and Yokoyama *et al.* [12], we developed and adapted the EPR method to estimate the levels of NO• radicals in serum.

The content of carbonic protein (PCC)

The PCC was measured using a commercial OxiSelect™ Protein Carbonyl ELISA Kit (Cell biolabs, incorporation) kit in accordance with the manufacturer's instructions.

CRP measurement

CRPs were measured by immunoturbidimetric method (Tina-quant CRP detection method; Roche Diagnostics) performed on an automated Hitachi 717 analyser with a detection limit of 0.1 mg / L and an extended measurement range of 0.1-240 mg / L (with repeats). CV between analyses was 2.6% at 4.65 mg / L CRP [13].

Statistical analysis

Unpaired t-test was used to compare the results of healthy control subjects with the results of patients with asthma. Biochemical parameters were compared in patients with different disease control using one-way ANOVA. The relationship between the various parameters of the study and the degree of airway obstruction was assessed according to Student's t-test. The value of $p \leq 0.05$ was considered statistically significant.

RESULTS

Determination of lipid peroxidation products

Plasma levels of lipid peroxidation products measured as MDA (Fig. 1), in all asthma groups compared to control subjects, were statistically significantly increased: for well-controlled group 2.45 ± 0.05 μmol/l *vs* 1.85 ±0.1μmol/l, $p < 0.003$, t-test; for partially controlled 2.61 ± 0.1 μmol/l vs 1.85±0.1μmol/l, $p < 0.000$, t-test; and for uncontrolled BA 2.99±0.1μmol/l *vs* 1.85±0.1 μmol/l, $p < 0.000$, t-test. There was no statistically significant difference between asthma groups.

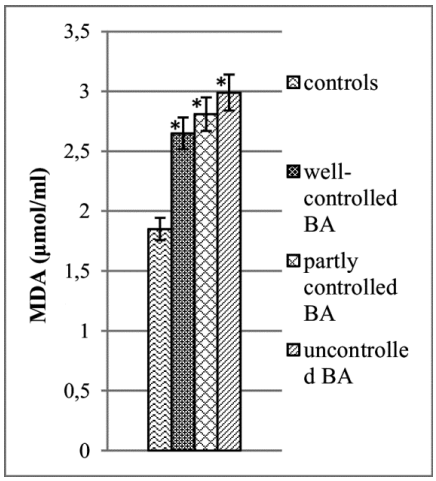

Fig. 1. Lipid peroxidation in plasma, expressed as micromoles of TBARS per litter in controls, well-controlled asthma group, partly controlled and uncontrolled asthma, p < 0.0001 (*) statistically significant compared to controls.

Protein carbonyl content (PCC)

PCC (Fig. 2) measured in serum was statistically significant increased in all groups compare with healthy controls: for well-controlled group mean 10.11 ± 2.3 nmol/mg, *vs* mean 1.72 ± 0.2 nmol/mg, p = 0.000, t-test; for partially controlled mean 7.61 ± 0.8 nmol/mg, *vs* mean 1.72 ± 0.2 nmol/mg, p = 0.000, t-test; for uncontrolled mean 9.11 ± 0.9 nmol/mg, *vs* mean 1.72 ± 0.2 nmol/mg, p = 0.000, t-test. There was no statistically significant difference between asthma groups, p = 0.3, t-test.

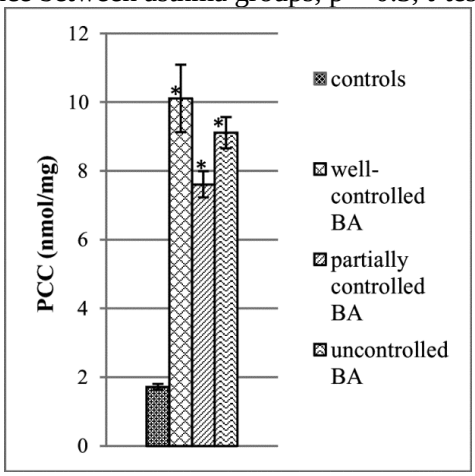

Fig. 2. Protein carbonyl content measured in nanomoles per milligram of protein, (*) p <0.000 statistically significant compared to controls. (**) p < 0.000 statistically significant compared to uncontrolled asthma.

ROS levels measurement

ROS levels (Fig. 3) measured in serum of patients with uncontrolled asthma were statistically significantly higher than the control subjects (mean 2.77 ± 0.3 *vs* mean 0.33 ± 0.1, p <0.000, t-test). In patients with well-asthma control (mean 1.35 ± 0.3 *vs* mean 0.33 ± 0.1, p <0.000, t-test) and partially controlled asthma (mean 1.07 ± 0.4 *vs* mean 0.33 ± 0.1, p <0.04, t-test), the ROS levels were also statistically significant higher compared to the controls. Statistically significant difference were observed between the ROS levels in well-controlled and uncontrolled BA group, (p ≥ 0.06), also statistically significant difference were shown in partly controlled asthma group vs uncontrolled, (p ≥ 0.00).

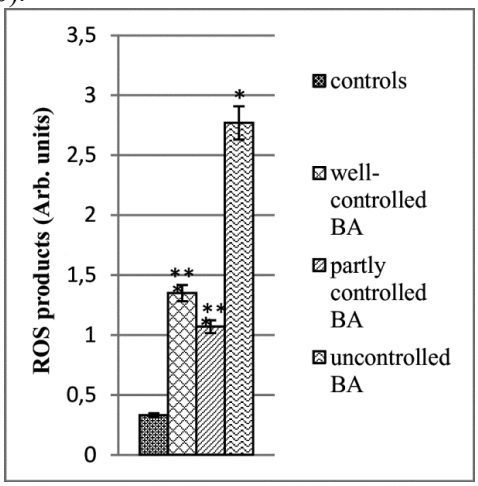

Fig. 3. The ROS levels are expressed in arbitrary units in controls, well-controlled asthma group, partly controlled and uncontrolled asthma, (*) p <0.000 statistically significant compared to controls. (**) p <0.000 statistically significant.

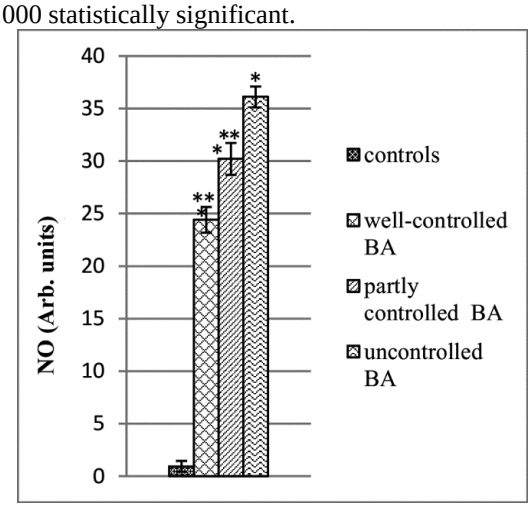

Fig. 4. The NO• radicals measured in arb. units in serum of healthy controls, well-controlled asthma group, partly controlled and uncontrolled asthma, (*) p <0.000 statistically significant compared to controls. (**) p <0.000 statistically.

Ex vivo evaluation the NO• levels

The levels of NO• radicals (Fig. 4) in patients with uncontrolled asthma were statistically significant increased compare to the healthy controls (mean 36.1 ± 1.1, *vs* mean 0.95 ± 0.1, p > 0.00, t-test). The similar increase was observed in the well-controlled group compared to the controls (mean 24.4 ± 2.7, *vs* mean 0.95 ± 0.1, p ≥ 0.00, t-

test), and partially controlled group (mean 30.2 ± 1.63, *vs* mean 0.95±0.1, p ≥ 0.00, t-test). Statistically significant difference in NO• levels was also observed in both asthma groups, with well-controlled and partially controlled, compared to uncontrolled patients (p ≥ 0.00).

Correlations between levels of CRP and OS parameters

MDA plasma levels showed positive correlation with reactive C protein (MDA *vs* CRP r = 0.29; p = 0.056). PCC in serum samples showed very high positive correlation with reactive C protein (PCC *vs* CRP r= 0.59; p=0.00). NO• levels significantly correlated with reactive C protein (NO• *vs* CRP, r = 0.54, p = 0.000). ROS levels showed positive correlation with reactive C protein (r= 0.39, p = 0.006).

Positive correlation and statistically significant difference were also observed between biochemical parameters, MDA *vs* NO• r = 0.74, P = 0.000; MDA *vs* ROS products r = 0.53, P = 0.000; MDA vs PCC r = 0.56, P = 0.000; ROS vs PCC r = 0.42; P = 0.003.

DISCUSSION

At this stage, numerous studies have found that the OS and in particular lipid peroxidation contributes to the pathophysiology of asthma. However, still systematic and comprehensive characterization of OS of asthmatics has not been conducted primarily due to the lack of suitable OS biomarkers [14]. In the current research using different techniques we have investigated and compared levels of ROS and NO• measured in real time, MDA and PCC measured as end products of lipids and proteins oxidation and CRP in blood of asthmatic patients divided in three groups differing in the degree of disease control - well-controlled disease, partially controlled and uncontrolled disease. Chronic airway inflammation and OS play a key role in the pathogenesis and progression of respiratory diseases including asthma [14] and may be a final common pathway leading to tissue damage. Inflammatory cells after activation respond with a “respiratory burst”, which involves the uptake of oxygen and subsequent release of ROS into surrounding cells. During “respiratory burst”, the inflammatory cells generate high concentrations of $^{\bullet}O_2^-$, •OH, HOCl, and H_2O_2 that may penetrate into surrounding cells causing increased quantities of free radicals in airway tissues. Moreover, the inflammatory cells of asthmatics possess an increased ability to generate free radicals in comparison with controls, which further contributes to elevation of ROS concentrations [15, 16]. Asthmatics may also produce an excess of reactive nitrogen species (RNS) such as NO• [17, 18]. The latter can react with $^{\bullet}O_2^-$, to form peroxynitrite that has many damaging effects, including lipid oxidation [19]. On the other hand, NO• can be converted to nitrite which can oxidise proteins. Thus, excess amounts of ROS and RNS accumulated in asthmatics can overcome the body's antioxidant defenses and cause OS. Since ROS and RNS are extremely unstable structures they can be measured only by EPR spectroscopy [20]. PBN was selected as a spin trap to evaluate the levels of ROS products in serum of asthmatics and healthy controls. Whether the radical trapped by PBN was oxygen-centered (PBN/•OR) or carbon-centered (PBN/•R) it can be determined by calculating the values of the hyperfine splitting constants aN and aH_β from the EPR spectrum of the registered spin adduct. According to literature data about the values of both splitting constants (aN = 13.88 G and aHβ = 2.35 G) the radical species trapped were identified as secondary oxygen centered alkoxy radicals (PBN/•OR), which resulted from the attack of the primary oxygen-centered radicals to membrane phospholipids [21]. Statistically higher levels of ROS products observed in the three groups of patients in comparison with the controls, means that oxidative processes are realized in all asthmatics at the time of the study. Moreover, the fact that both groups of partially and well-controlled asthmatics expressed significantly lower levels of ROS compared with uncontrolled group, shows that in those two groups the oxidative processes are partially reduced, which is apparently due to the successfully implemented therapy. Increased ROS production found in all asthmatics was also additionally confirmed with the increased levels of MDA registered in their plasma (see Fig. 1). Due to the highest production of ROS in the uncontrolled group the highest level of MDA was observed. It is well-known that lipid peroxidation is induced by ROS and a good correlation exists between the degree of ongoing lipid peroxidation processes and the amount of formed MDA reactive substances which are considered as a specific biomarker of OS [14]. The lipid peroxidation and the breakage of lipids with the formation of reactive substances can lead to changes in the permeability and fluidity of the membrane lipid bilayer and can dramatically alter cell integrity [21, 22]. LPO products are characterized by carbohydrate chains of different length, reactive aldehyde groups and double bonds, which make these molecules reactive to nucleic

acids, proteins and cellular thiols. Modifications of proteins with LPO products may regulate cellular processes like apoptosis, cell signaling and senescence [23]. On the other hand, ROS can promote protein carbonylation - a process by which reactive aldehydes or ketones are incorporated into proteins by oxidation [24]. Protein carbonylation is a result of the direct metal-catalyzed oxidation of amino acid side chains (primary protein carbonylation) or the addition of reactive aldehydes to amino acid side chains (secondary protein carbonylation) [25]. Oxidative modifications of proteins by reactive species, especially ROS are implicated in etiology or progression of a wide range of disorders and diseases. The level of oxidatively modified proteins can be quantified by measuring the PCC [26].

In the current study statistically significant increased levels of PCC found in all groups comparing to healthy controls were in accordance with elevated ROS established for the three groups of asthmatic patients. It was interesting that protein carbonyls levels in the well-controlled group were higher than those of the other two groups while the level of ROS products of the uncontrolled group was significantly higher than the well-controlled and partially controlled asthmatics. Based on this finding we assume that PCC registered in well-and partially controlled groups is mainly a result of the direct oxidation by ROS (primary protein carbonylation) while in the uncontrolled group predominates indirect oxidation by reaction with secondary by-products (reactive aldehydes) of OS (secondary protein carbonylation). This assumption is additionally supported by the highest level of malondialdehyde end products of lipid peroxidation found in uncontrolled asthmatics comparing to the other two groups.

The development and maintenance of allergic inflammation in the respiratory tract with bronchial asthma is associated with the implementation of oxidative and nitrosative stress. Nitrosative stress is characterized by an increase in the levels of $NO^{\bullet}$ radicals and ROS products measured in serum. OS is characterized by an increase in the MDA level and protein carbonyl content, depending on the control of the disease. The intensity of lipid peroxidation, proteins oxidation and levels of generated free radicals in our study positively correlate with the level of reactive C protein.

CONCLUSION

For the first time using the EPR spin trapping method, oxidative processes in real time were demonstrated in patients with asthma. It is important to emphasize that the group with uncontrolled asthma showed the highest level of oxidative stress, which was confirmed by both higher levels of real-time OS parameters and higher levels of the end products of oxidation of lipids and proteins compared to the other two groups of asthmatics. The results of correlation analysis confirm that the development and maintenance of inflammatory processes in the respiratory tract is associated with the oxidative and nitrosative stress present in asthmatic patients.

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REFERENCES

1. Global Initiative for Asthma (GINA) (2006) revision www.ginasthma.com
2. D. Dimov, T. Tacheva, V. Ilieva, A. Koychev, G. Prakova, Z. Andreev, *Allergy, Asthma and Immunophys.: From Basic Sc. to Clin. Manag.*, **17,** 4 (2013).
3. D. Dimov, M. Kurzawski, T. Tacheva, V. Ilieva, A. Koychev, G. Prakova, M. Kurzawski. V. Dimitrov, M. Drozdzik, T.Vlaykova, *Allergy, Asthma and Immunophys.: From Basic Sc. to Clin. Manag*., **17**, 3 (2013).
4. A.M. Cantin, D. Hartl, M.W. Konstan, J.F. Chmiel, *J. Cystic Fibr.*,**14**, 419 (2015).
5. Y.S. Cho, H.B. Moon, *Allergy, Asthma and Imm. Res.*, **2**(3), 183 (2010).
6. D.R. Taylor, *Thieme Med Publish*., **33**, 620 (2012)
7. A. Nadeem, S.K. Chhabra, A Masood, *J Allergy and Clin.Immunol*., **111,** 72 (2003)
8. Global Initiative for Asthma Global Strategy for Asthma Management and Prevention. Vancouver, WA, USA:GINA, (2014) www.ginasthma.org
9. Z.A. Plaser, L.L. Cushman, B.C. Jonson, *Anal. Biochem.*, **16,** 359 (1966).
10. H. Shi, Y. Sui, X. Wang, Yi. Luo, L. Ji, *Comp. Biochem. and Physiol. Part C: Toxicol. & Pharmacol.*, **140**, 115 (2005).
11. T. Yoshioka, N. Iwamoto, K. Lto, *J. Am. Soci. Nephrol.*, **7,** 961 (1996).
12. K. Yokoyama, K. Hashiba, H. Wakabayashi, *Anticanc. Res.*, **24,** 3971 (2004).
13. N. Khuseyinova, A. Imhof, G, Trischler, *Clin. Chem.*, **49**, 1691 (2003).
14. L.G. Wood, P.G. Gibson, M.L. Garg, *Eur. Respir. J*., **21,** 177 (2003),
15. L. Zuo, N.P. Otenbaker, B.A. Rose, K.S. Salisbury, *Molec. Immunol.*, **56,** 57 (2013).
16. P. Kirkham, I. Rahman, *Pharmacol. Ther.*, **11** (2006).
17. S.A. Kharitonov, D. Yates, R.A. Robbins, R. Logan-Sinclair, E.A. Shinebourne, P.J. Barnes, *Lancet*, **343,** 133 (1994).
18. L. Zuo, M.S. Koozechian, L.L. Chen, *Ann. Allergy Asthma Immunol.*, **112,** 18 (2014).

19. I. Spasojević, M. Mojović, A. Ignjatović, G. Bačić, *J. Serb. Chem. Soc.*, **76,** 647 (2011).
20. B. Halliwell, J.M. Gutteridge, Oxford Univ. Press, USA, 2015.
21. T.A. Dix, J. Aikens, *Chem. Res. in Toxicol*., **6**, 2 (1993).
22. G. Barrera, Review Article. Intern Scholarly Res Network ISRN Oncology, Article ID 137289, 21pages doi:10.5402/2012/137289 (2012).
23. A. Winczura, B. Tudek, *Free Rad. Res.*, **46,** 442 (2012).
24. Y.J. Suzuki, M. Carini, D.A. Butterfield, Antioxid. Redox Signal, **12,** 620 (2010).
25. Ch.M. Wong, G. Bansal, L. Marcocci, Y.J. Suzuki, *Redox. Rep*., **17**, 90 (2012).
26. E.R. Stadtman, R.L. Levine, Ann. N.Y. Acad. Sci., **899**, 191 (2000).

ОКСИДАТИВЕН СТРЕС И СВЪРЗАНИ С НЕГО БОЛЕСТИ. ЧАСТ 1: БРОНХИАЛНА АСТМА

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(Резюме)

Оксидативният стрес (OS) играе важна роля в патогенезата на голям брой болести при човека, като исхемична болест/реперфузионно увреждане, атеросклероза, рак, невродегенеративни болести, алергии и др. Бронхиалната астма (BA) е хронично възпалително заболяване на дробовете, което ограничава притока на въздух, води до хиперактивност и ремоделиране на дихателните пътища. Клинични и експериментални данни от последните години показват, че повишеният OS и деструктивните ефекти от окислението на свободните радикали може да са важни причини за хроничен патологичен процес в дробовете. Целта на настоящата работа е да се изследва и сравни нивото на оксидативния стрес в кръвта на пациенти с астма, които се различават по степента на контрола на заболяването. В изследването са включени 30 пациенти с BA и 24 подходящи по възраст здрави доброволци. Пациентите са диагностицирани с BA с алергичен компонент по-дълго от една година. За целта са изследвани продуктите на ROS и RNS, крайните продукти на липидите и протеините, проследена е и връзката между параметрите на оксидативния стрес и С-реактивния протеин (CRP) като маркер на степента на възпаление. С помощта на EPR спин-улавяща техника са потвърдени протичащите в реално време процеси в кръвни проби, изолирани от астматични пациенти с различен контрол на заболяването. Установена е позитивна корелация между нивата на изследваните OS биомаркери и CRP като маркер на степента на възпаление. Оксидативните процеси в реално време са демонстрирани при пациентите с BA. Резултатите от корелационния анализ потвърждават, че развитието и поддържането на възпалителните процеси в дихателния тракт са свързани с оксидативния и нитрозативния стрес.

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Oxidative stress and related diseases. Part 2: Parkinson' disease

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Parkinson' disease is a progressive neurodegenerative movement disorder associated with a selective loss of neurons in the midbrain area called the substantia nigra pars compacta. Pharmacological treatment of PD traditionally has centered on administrering medications that restore dopaminergic influence in the basal ganglia. During the early stage of disease, Levodopa admistration can produce significant improvements in parkinsonian symptoms moreover, the effects of Levodopa, often diminish after only a few years of continuous use, and this drug often is ineffective in the advanced stages of PD. The aim of current research was to evaluate and compare oxidative status in blood of PD patients treated with Madopar, alone and with a combination of Madopar and vitamin C by several analytical techniques. To realize the aim were investigated: 1) levels of ROS products, ascorbate (Asc•) and NO• radicals as real time oxidative stress biomarkers using Electron paramagnetic resonance (EPR) spectroscopy; 2) the oxidative end products of lipids, proteins and DNA, levels of malondialdehyde (MDA) measured by visible spectrophotometry, protein carbonyl content (PCC) and also 8-hydroxy-2′-deoxyguanosine (8-OHdG) determined by ELISA; and 3) erythrocyte SOD activity by visible spectrophotometry in blood of PD patients.

Keywords: Oxidative stress, Parkinson' disease, vitamin C.

INTRODUCTION

Parkinson's disease (PD), also known as idiopathic or primary parkinsonism/*paralysis agitans*/ is a neurodegenerative disorder (ND) of the central nervous system. Pathologically, the disease is characterized by the proteinaceous cytoplasmic inclusions presence known as Lewi bodies. Contemporary treatment is more efficient during the initial stages of the motor disorders mainly, by the treatment with Levodopa and Dopamine agonists. The Levodopa effects often diminish after few years' continuous use, and in the advanced PD stages, the drug is ineffective. More importantly, the Levodopa administration seems not to delay the PD progression. Simple increase of dopaminergic influence in the basal ganglia does not prevent the deterioration of neurons in the *substantia nigra*. Recent efforts, therefore, have been focused on identifying the factors responsible for neuronal degeneration and for developing medications that might be able to delay nigral cell death [1]. Consequently, with the progress of the disease and neuronal loss, Levodopa becomes ineffective, yielding complications such as dyskinesias. Motor symptoms of PD disease result in dopamine-generating cells loss in the *substantia nigra pars compacta*. The reason for this cell death is unknown, but a number of studies [2, 3] consider the role of free radicals and implicated oxidative stress (OS) that leads to cellular dysfunction and demise.

OS can cause cellular damage and subsequent cell death because the reactive oxygen species (ROS) oxidize vital cellular components [4]. exhibit increased levels of oxidized lipids [5], proteins [6], and DNA [7]. OS occurs when an imbalance is formed between production of ROS and cellular antioxidant defense mechanisms that include removal of O_2, scavenging of reactive oxygen/nitrogen species or their precursors, inhibition of ROS formation, binding of metal ions needed for the catalysis of ROS generation and up-regulation of endogenous antioxidant defenses. Various antioxidant therapies have been proposed for reducing oxidative stress in neurodegenerative diseases including PD, but the results with regard to the efficacy of the antioxidant treatments remain controversial [8]. Substances that delay, prevent, or remove oxidative damages to a target molecule are considered as antioxidants. One of the vital roles of the classic antioxidant vitamin C is to protect cellular components from free radical damage. Ascorbic acid has been shown to scavenge free radicals directly in the aqueous phases of cells and the circulatory system [9]. The acid has also been proven to protect membrane and other hydrophobic compartments from such damage by regenerating the antioxidant form of vitamin E. Mechanism of vitamin C antioxidant function, myriad of

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pathologies resulting from its clinical deficiency, and the many health benefits it provides, are reviewed [7].

In the current study, we explored the influence of vitamin C on the oxidant/antioxidant status of PD patients undergoing prolonged therapy with Madopar whose main ingredient is Levodopa. To achieve the aim of the present research the levels of ROS products formed in real time and end products from oxidation of lipids, proteins and nucleic acid in blood samples isolated from PD patients after therapy with Madopar (Levodopa) alone and in combination with vitamin C were examined and compared.

MATERIALS AND METHODS

All chemicals used in this study were of analytically grade and purchased from Sigma-Aldrich Chemie GmbH (Germany). Spin-traps N-*tert*-butyl-α-phenylnitrone (PBN) and 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazole-1-oxyl-3-oxide (Carboxy-PTIO.K), were purchased from Sigma Chemical Co, St. Louis, USA. The ELISA kits for PCC and Oxidative DNA Damage (8-OHdG) measurement were purchased from Cell Biolabs Inc., USA. In our study were included 22 patients hospitalized in the Neurological Clinic of the University Hospital, Stara Zagora, Bulgaria. All studied parameters were compared with those of the 20 control healthy individuals. For PD diagnosing were used the national consensus for PD (medical history and pathological criteria in the neurological condition such as tremor, muscular rigidity, bradykinesia and postural instability), which is in compliance with the PD society brain bank clinical diagnostic criteria. The group of selected patients is homogenous according to the clinical features in parallel with the disorders of the cognition, mood, behavior, and mentation, etc. The Unified Parkinson Disease Rating Scale (UPDRS) was used for assessment of the PD and the level of pathological changes was assessed with the modified scale of Hoehn and Yahr. The patient capability for everyday activity was assessed according to the Schwab and England scale.

The PD patients group were treated only with Madopar (250 mg), containing –Levodopa 200 mg and Benserazide 50 mg from 2 to 4 years. Afterwards, the same patients have received both Madopar (250 mg) and vitamin C (1000 mg) for 2 months. Informed consent was obtained from all PD patients and healthy volunteers enrolled in this study, according to the ethical guidelines of the Helsinki Declaration (1964). Fasting samples of venous blood were collected in the morning between 8.00 and 10.00 a.m. Blood for determination of MDA, NO• and ROS products was collected in tubes containing 10% EDTA (ethylenediaminetetraacetic acid). Whole blood in a covered test tube (without anticoagulant) was collected for determination of PCC, 8-OHdG and erythrocyte SOD activity. All samples from each subject were split and run in triplicate.

Ex vivo electron paramagnetic resonance (EPR) study

EPR measurements were performed at 22^0C temperature on an X-band EMX^{micro}, spectrometer Bruker, Germany. The experiments were carried out in triplicate and repeated thrice. Spectral processing was performed using Bruker WIN-EPR and *Sinfonia* software.

Ex vivo evaluation of the levels of ROS products

The ROS levels were determined according to [10] with modification. To investigate in real time formation of ROS in the sera of PD patients and controls *ex vivo* EPR spectroscopy combined with PBN as a spin-trapping agent was used.

Ex vivo evaluation of the levels of Asc•

Endogenic ascorbic acid can be oxidized by ROS to a stable ascorbate radical and the latter can be detected by direct EPR method which does not interfere with the biochemical processes. The levels of Asc• were studied according to Bailey *et al.* [11] with some modification.

Ex vivo evaluation of the levels of •NO radicals

Based on the methods published by Yoshioka *et al*. [12] and Yokoyama *et al*. [13], we developed and adapted the EPR method for estimation of the levels of •NO radicals in serum.

Determination of SOD activity in erythrocytes

CuZn-SOD activity was determined as described by Sun *et al*. [14] with minor modifications. Results were expressed as units per g hemoglobin (U/gHb).

Determination of lipid-peroxidation products (MDA)

Total amount of lipid peroxidation in the plasma of healthy volunteers and patients was estimated using the thiobarbituric acid (TBA) method of [15], by measuring the malondialdehyde (MDA) reactive products at 532 nm. Results were expressed in μmol/l.

Protein carbonyl content

PCC was measured by using a commercial ELISA kit following manufacturer's instructions.

Quantity of 8-hydroxy-2'-deoxyguanosine

The measurement of 8-OHdG was carried out using a commercial ELISA kit, following manufacturer's instructions. The kit has an 8-OHdG detection sensitivity range of 100 pg/mL - 20 ng/mL.

RESULTS

The ROS products (Fig.1) in the sera of PD patients treated with Madopar was unsignificantly higher compared to controls (mean 3.47 ± 1.17 *vs* mean 3.09 ± 0.41, p > 0.05, t-test). The ROS products in PD patients treated with a combination of Madopar + vitamin C were less than in the controls, (average 2.81 ± 0.3 *versus* mean 3.09 ± 0.4, p> 0.05 t-test). However, there was a statistically significant difference between PD group treated with the combination of Madopar and vitamin C and PD treated only with Madopar (mean 2.81 ± 0.3 *vs*. mean 3.47 ± 1.17, p = 0.001, t-test).

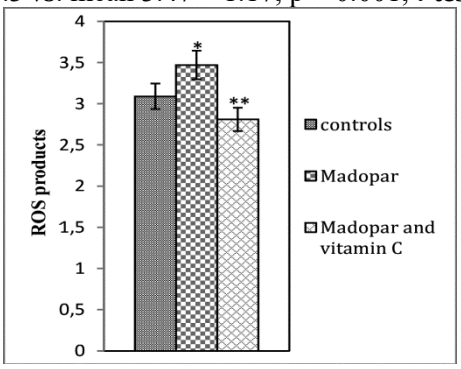

Fig. 1. Levels of ROS products expressed in arbitrary units in sera of controls, PD patients with Madopar therapy and PD patients with combined therapy – Madopar +vitamin C. (*) p < 0.001 – PD group treated with Madopar *vs* controls; (**) p < 0.001 –PD group treated only with Madopar *vs* group treated with Madopar + vitamin C.

Statistically significant increased Asc• levels (Fig. 2) were observed in PD patients treated with Madopar compared to controls (mean 2.49 $\times 10^6$ ± 0.35, *vs* mean 0.86 $\times 10^6$ ± 0.02, p < 0.001, t-test). Moreover, the Asc• levels in PD patients treated with Madopar +vitamin C did not show statistically significant difference compared to the controls (mean 0.99 × 10^6 ± 0.03, *vs* mean 0.86 × 10^6 ± 0.02, p > 0.05, t-test), but there was a statistically significant difference comparing to patients treated with Madopar alone (mean 0.99 × 10^6 ± 0.03, *vs* mean 2.49 $\times 10^6$ ± 0.35, p = 0.001, t-test).

Statistically significant higher NO• levels (Fig. 3) were measured in Madopar-treated patients compared to controls (mean 4.85 ± 0.4, *vs* mean 26.74 ± 1.2, p=0.00, t-test). It should be noted that after treatment with Madopar + vitamin C, statistically significantly lower NO• levels were found compared to those measured in the same patients prior to inclusion of vitamin C in the therapy (mean 26.74 ± 1.2, *vs* mean 14.86 ± 0.4, p=0.00, t-test).

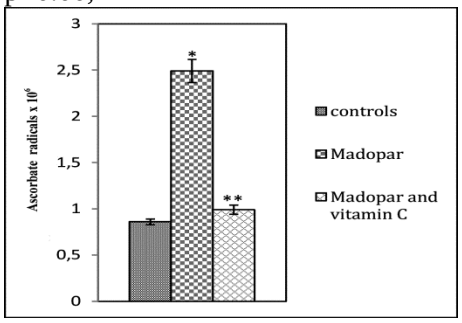

Fig. 2. Levels of Asc radicals expressed in arb. units in sera of controls, PD patients with Madopar therapy and PD patients with combined therapy –Madopar + vitamin C. (*) p < 0.001 – PD group treated with Madopar *vs* controls; (**) p < 0.001 – Madopar treated PD group *vs* PD group treated with Madopar + vitamin C.

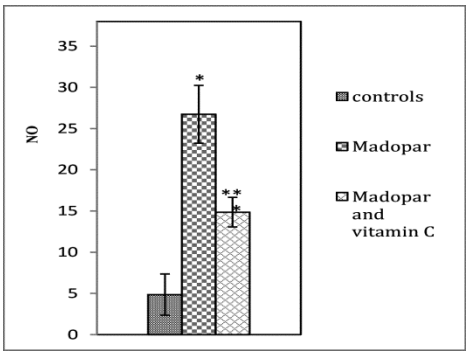

Fig. 3. Levels of NO radicals expressed in arb. units in sera of controls, PD patients with Madopar therapy and PD patients with combined therapy –Madopar and vitamin C. (*) p < 0.001 – PD group treated with Madopar vs controls; (**) p < 0.001 –PD group treated only with Madopar vs PD group treated with Madopar + vitamin C.

SOD activity (Fig. 4) in PD group treated with Madopar was statistically lower compared to controls (mean 13.4 $\times 10^2$± 1.34 U/gHb *vs* 14.94 × 10^2 ± 1.45 U/gHb, p<0.05, Student's t-test), as well as compared to the SOD measured in the same patients after inclusion of vitamin C (mean 13.4 $\times 10^2$± 1.34 U/gHb *vs* 15.75 $\times 10^2$ ± 1.57 U/gHb). SOD in PD patients measured after inclusion of vitamin C was close to that of the controls (mean 15.75 $\times 10^2$ ± 1.57 U/gHb *vs* 14.94 × 10^2 ± 1.45 U/gHb, p > 0.05, Student's t-test).

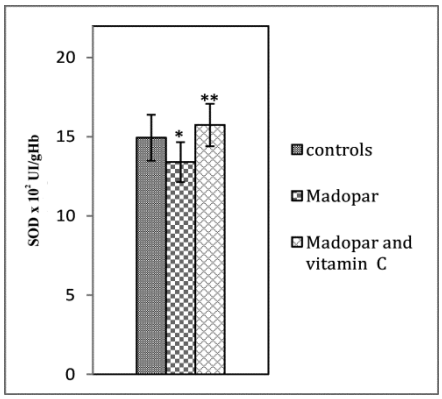

Fig. 4. SOD activity in controls, PD patients with Madopar therapy and PD patients with combined therapy –Madopar +vitamin C. (*) p <0.0001 PD group treated only with Madopar vs controls; (**) p < 0.0001 - Madopar treated PD group vs PD group treated with combination of Madopar + vitamin C.

MDA levels (Fig. 5) measured in plasma of PD patients treated with Madopar alone was lower than the controls (mean 2.45 μmol/l ± 0.09 *vs* mean 2.65 μmol/l ± 0.07, p>0.05, Student's t-test). MDA measured in PD patients treated with Madopar + vitamin C compared to controls (mean 1.94 μmol/l ±0.07, *vs* 2.65 ± 0.07 μmol/l p < 0.0001) and to PD patients treated only with Madopar (mean 1.94 μmol/l ±0.07, *vs* 2.45 μmol/l ± 0.09, p< 0.001, Student's t-test, respectively) were significantly reduced.

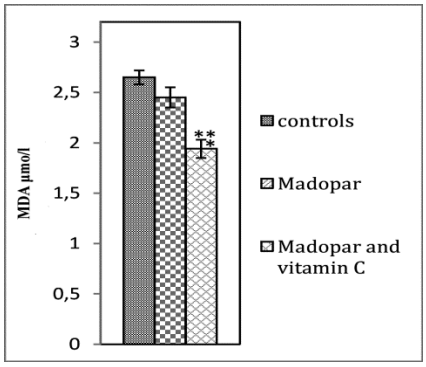

Fig. 5. MDA plasma levels in controls, PD patients with Madopar therapy and PD patients with combined therapy –Madopar + vitamin C. (*) p <0.0001 - PD group treated with Madopar + vitamin C. (**) p < 0.0001 - PD group treated with combination Madopar + vitamin C *vs* Madopar treated PD group.

PD patients treated with Madopar showed statistically higher PCC (Fig. 6) compared to controls (mean 8.02 nmol/mg ± 0.7, *vs* 1.26 nmol/mg ± 0.13, p = 0.0001, Student's t-test). Statistically significant difference was also measured in PD patients treated with Madopar +vitamin C compared to control (mean 5.42 nmol/mg ± 0.43, vs 1.26 nmol/mg ± 0.13, p = 0.001, Student's t-test). The patients, undergoing combined therapy, expressed significantly lower PCC levels in comparison with Madopar treated (mean 5.42 nmol/mg ± 0.43, *vs* 8.02 nmol/mg ± 0.13, p = 0.0001, Student's t-test).

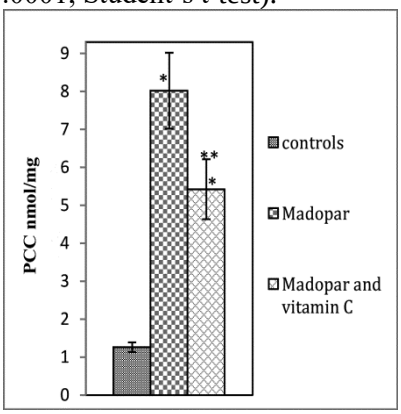

Fig. 6. PCC in controls, PD patients with Madopar therapy and PD patients with combined therapy – Madopar + vitamin C. (*) p < 0.001–*vs* controls; (**) p < 0.0001 Madopar treated PD group *vs* PD group treated with combination of Madopar + vitamin C.

Significant increase of 8-OHdG (Fig.7.) levels was found in PD patients treated with Madopar, compared to the controls (mean 18.91ng/ml ± 0.9 *vs* 11.03 ng/ml ± 0.6, p < 0.0001, Student's t-test). PD patients treated with Madopar + vitamin C were in contrast compared to controls (mean 16.04 ng/ml ± 0.8 vs 11.03 ng/ml ± 0.6, p < 0.001, Student's t-test). The amount of 8-OHdG measured in PD patients treated with Madopar + vitamin C was significantly reduced in comparison to Madopar treated PD patients (mean 16.04 ng/ml ± 0.8 *vs* 18.91 ng/ml ± 0.9, p < 0.001, Student's t-test).

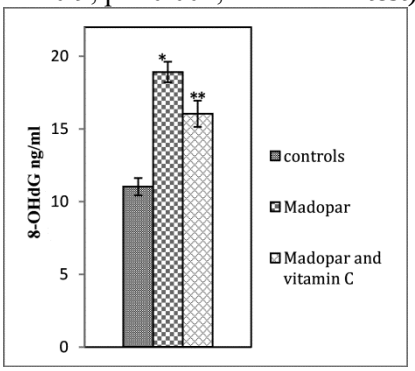

Fig. 7. DNA damage, measured as 8-OHdG (ng/ml) in controls, PD patients with Madopar therapy and PD patients with combined therapy –Madopar and vitamin C. (*) p < 0.001–*vs* controls; (**) p < 0.0001 Madopar treated PD group *vs* PD group treated with combination of Madopar + vitamin C.

DISCUSSION

Investments in drug research and development have increased in recent decades, but the annual number of truly innovative new medicines has not increased accordingly, which creates a problem in

the pharmaceutical industry to replace the loss of revenues due to patent expiration [16]. Therapy with Madopar is still the most efficient method of PD treatment [17]. Despite evidence that OS has played a significant role in PD, a good target for pharmacological management has to be determined. ROS should be a therapeutic target in PD, this is necessary to recognize that an antioxidant in chemical systems may be not an efficient agent in biological ones. The effectiveness of antioxidants is probably limited by their bioavailability and the fact that they would have to be present in high concentrations to be able to compete with endogenous targets. Therefore, the current study was undertaken to clarify the protective effect of vitamin C against oxidative toxicity of Madopar in PD patients. The most susceptible biological macromolecules to oxidative injuries are membrane lipid, proteins, and DNA. Moreover, in biosystems quantification of final products of oxidative damage constitutes indirect evidence of OS as a result of increased generation of ROS and RNS. The above mentioned facts prompted us using proper techniques to explore selected OS parameters such as Asc•, ROS products, NO•, SOD activity and levels of final products of lipids, proteins and DNA oxidation in blood of PD patients subjected to prolonged treatment with Madopar and to evaluate the effect of vitamin C on the levels of these parameters after inclusion in the therapy of the same PD patients. In the course of lipid peroxidation a variety of unstable radical species are formed that can be measured [18]. Short-lived radicals such as ROS formed during *in vivo* oxidation can be proved only by EPR spectroscopy using proper spin traps or spin probes [19,20]. By using spin probe CMH (1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine) a quantitative EPR method was developed to monitor ROS production in physiological and pathological conditions. It was found that increased ROS production corresponded to increased plasma TBARS and protein carbonyl concentrations in patients with neurodegenerative disorders such as Mild Cognitive Impairment, and Amyotrophic Lateral Sclerosis, comparing to controls [20]. All changes indicated increased OS are directly related to an increase in ROS production [20]. The higher ROS levels in Madopar treated patients in comparison with controls means that oxidative processes [21] are available in real time. At present, Asc• is used as an endogenous marker for OS in biological systems. From the thermodynamic point of view, the ascorbate ranks at the end of oxidative radicals series. Obviously, oxidative species as hydroxyl (•OH); alkylperoxyl (ROO•), lipidperoxyl (LOO•), tocoperoxyl (•TO) radicals and peroxynitrite ($ONOO^-$) have a higher redox potential and can be reduced, thereby generating Asc• radical [22]. In biological systems the endogenic ascorbic acid, oxidized by ROS to stable Asc• radical possessing comparatively long half-life, which in aqueous solution and at room temperature can be readily detected [23]. Significantly increased Asc• levels after prolonged Madopar treatment compared to controls indicate the presence of ongoing oxidative processes in PD patients. Considering the above-mentioned thermodynamic order of oxidative species, it is obvious that the four-fold higher levels of Asc• found in the PD patients *versus* the controls was a result of increased OS due to increased generation of oxidative species having a higher redox potential than the ascorbate. The consideration was supported by the higher levels of ROS products and NO• measured in the patient's group compared to controls.

The increase in the OS due to the low activity of antioxidant enzymes might cause many secondary complications and may contribute to the neurodegeneration in PD [24]. SOD is a scavenging enzyme and is considered as the first line defense against ROS overproducing. Various research groups have investigated OS in blood cells of PD patients and reported controversial results related to erythrocyte activities of SOD, CAT and possible correlation with age, duration and stage of PD [25, 26]. The erythrocyte SOD was decreased only in treated patients and non-treated PD patients [27]. Our study demonstrated a significant decrease in erythrocyte SOD activity in Madopar treated PD patients that corresponds to the increased levels of ROS in the same group. Moreover, in PD patients was also established increase in the NO• levels. The administration of therapeutic agents results in a greater degree of OS than that induced by the disease itself [28-30]. The prolonged drug treatment could lead to further OS and imbalance between production and elimination of ROS, and could contribute to different complications during the course of the PD [16]. Further, to explore how the increased production of ROS and NO reflects on the OS in PD patients subjected to prolonged treatment with Madopar we studied end products of lipids, proteins and DNA oxidation. Well known is that oxidative modification of proteins causes in the greatest extent formation of protein carbonyl groups, therefore the level of the latter has become the most commonly used marker for protein oxidation during OS, aging and neurodegenerative diseases [31]. In fact, DNA also cannot escape oxidative attacks and typical example is

deoxyguanosine that is converted by ROS to 8-hydroxydeoxyguanosine (8-OHdG), a biomarker of oxidative DNA damage [32]. MDA level in plasma of PD patients was insignificantly lower than that of the controls. Lots of studies reported that at early stages of Parkinson, plasma MDA level peaked, while at the late stages of the disease this level even fell below the controls [33, 34], and established negative correlation between plasma lipid peroxidation and intake of levodopa dose. Formerly, evidence was provided that levodopa treatment may cause decrease in plasma lipid peroxidation. Current result provides additional evidence that prolonged Madopar (levodopa) treatment may decrease plasma lipid peroxidation. Another indirect proof for involvement of ROS in drug-induced toxicity is the overcome of the OS by adding typical antioxidants [17, 35]. It is known that antioxidants are needed to prevent the formation and oppose the actions of free radicals. There is evidence that a higher intake of vitamin C and other antioxidants is associated with reduced risk of degenerative or chronic diseases, probably through antioxidant mechanisms [29,36]. Improved antioxidant status helps to minimize oxidative damage, and thus can delay or prevent pathological changes. This suggests the possible utility of antioxidant-based dietary strategies for lowering the risk of chronic age-related, free radical-induced diseases, and their complications. Vitamin C is the first line of the non -enzymatic antioxidant defense against oxygen radicals [37]. Overproduction of free radicals accompanies all neurodegenerative diseases, and ascorbic acid may block some processes associated with ROS overproduction [38]. Water-soluble vitamin C acts as a chain-breaking antioxidant and in biological systems scavenges free radicals such as ROS by donating electrons and thus may prevent other biological molecules from being oxidized [36]. To check the possibility ROS to be involved in drug-induced oxidative toxicity vitamin C was added to Madopar therapy of the same patients at a dose of 1000 mg per day for 2 months. We assumed that the significant decline in ROS levels and bringing them close to the control group after inclusion of vitamin C was due to the classic antioxidant that largely overcomes the ongoing oxidative processes in PD patients subjected to prolonged treatment with Madopar. This assumption was additionally confirmed by the significant decrease in PCC and 8-OHdG sera levels measured in PD patients after inclusion of vitamin C in their therapy. It should be emphasized that vitamin C in the tested dosage and duration of administration successfully overcomes lipid oxidative toxicity induced by both Levodopa long-term treatment and the disease, itself.

CONCLUSION

Regardless of a lot of clinical studies. suitable antioxidants by which effectively to treat neurodegenerative diseases including PD are not yet found. We consider that taken together, recently published results of the present and other authors, as well as the present survey, launched a promising opportunity and a new direction in search of relationship between different oxidative stress biomarkers in brain tissue and blood of PD patients, which further might help in developing efficient antioxidant schemes to find application in treatment of PD.

Conflict of interests: *No conflict of interests to declare.*

REFERENCES

1. D. Ciccone, *Phys. Therapy*, **78**(7), 313 (1998).
2. D. Jolanta, K. D. Wojciech, in: A.Q. Rana (ed.), Etiology and Pathophys. of Parkinson's Dis., InTech, Chapter 16, 2011.
3. O. Hwang, *Experim. Neurobiol*. **22**(1),11 (2013).
4. D.A. Bosco, D.M. Fowler, Q. Zhang, J. Nieva, E.T. Powers, P. Jr. Wentworth, R.A. Lerner, J.W. Kelly, *Nature Chem. Biol*., **2**(5), 249 (2006).
5. E. R. Stadtman, B. S. Berlett, *Drug Metab. Reviews* **30**(2), 225 (1998).
6. Y. Nakabeppu, D. Tsuchimoto, H. Yamaguchi, K. Sakumi, *J. Neurosci. Res*., **85**(5), 919 (2007).
7. M. Carocho, I. C. Ferreira, *Food and Chem. Toxicol.*, **51**,15 (2013).
8. H. Kim, J.E. Kim, S.J. Rhie, S. Yoon, *Exp. Neurobiol*., **24**(4), 325 (2015).
9. M. Krajčovičová-Kudláčková, M. Dušinská, M. Valachovičová, P. Blažíček, V. Pauková, *Physiolog. Res*., **55** (1), 227 (2006).
10. H. Shi, Y. Sui, X. Wang, Yi. Luo, L. Ji, *Comp. Biochem. and Physiol. Part C*: *Toxicol. and Pharmacol.*, **140**(1), 115 (2005).
11. D.M. Bailey, K.A. Evans, P.E. James, J. McEneny, I.S. Young, L. Fall, M. Gutowski, E. Kewley, J.M. McCord, K. Møller, P.N. Ainslie, *The J. of Physiol*. **587,**73 (2009).
12. T. Yoshioka, N. Iwamoto, K. Lto, *J. Am. Soc. Nephrol*. **7,** 961 (1996).
13. K. Yokoyama, K. Hashiba, H. Wakabayashi, *Anticanc. Res*., **24**,3917 (2004).
14. Y Sun, L.W. Oberley, Y. Li, *Clin Chem*., **34**, 497-500 (1988)
15. Z.A. Plaser, L.L. Cushman, B.C. Jonson, *Analyt. Biochem*., **16**(2), 359 (1966).
16. D.C. Swinney, J. Anthony, *Nature Reviews Drug Discov*., **10** (7), 507 (2011).
17. A.Melo, L.Monteiro, R. M. Lima, D.de Oliveira, M.D. de Cerqueira, R.S. El-Bachá, *Oxidative*

medicine and cellular longevity, Article ID 467180, 14 p. doi:10.1155/2011/46 7180 (2011).
18. E.N. Frankel, Anti-oxidants in food and biology,Elsevier, (2014).
19. S. Mrakic-Sposta, M. *Cell. Longev.,* Article ID 306179, 10. (2014).
20. K.K. Griendling, R.M. Touyz, J.L. Zweier, S. Dikalov, W. Chilian, Y.R. Chen, D.G. Harrison, A. Bhatnagar, *Circular Res*., **119**(5),39 (2016).
21. T.H. Togashi, H. Shinzawa, T. Matsuo, Y. Takeda, T. Takahashi, M. Aoyoma, K. Oikawa, H. Kamada, *Free Radic. Biol. Med*., **28**(6), 846 (2000).
22. G.R. Buettner, SFRBM Workshop: Rigorous Detection and Identification of Free Radicals in Biology and Medicine, 2005.
23. D. Armstrong, *Humana Press Ins. Totowa*, NJ, USA. 108, (1999).
24. S. Abraham, C.C. Soundararajan S. Vivekanandhan, M. Behari, *Ind. J. Med. Res*., **121**, 111 (2005).
25. Y.M. Ihara, S. Chuda, T. Kuroda, Hayabara, *J. Neurol. Sci*.,**170**, 90 (1999).
26. K. Sudha, A.V. Rao, S. Rao, A.Rao, *Neurol India*, **51**, 60 (2003).
27. E.M. Gatto, M.C. Carreras, G.A. Pargament, N.A. Riobo, C. Reides, M. Repetto, M. Pardal, S. Lesuy, J.J. Poderoso, *Mov. Disord*. **11**, 261 (1996).
28. J.S. Beckman, J.P. Crow, *Biochem. Soc. Trans*. **21**, 30 (1993).
29. M.M. Goldenberg, *Pharm. and Therapeut*. **33**(10), 590 (2008).
30. V. Dias, E. Junn, M. M. Mouradian, *J. Parkinson's Dis.,* **3**(4), 461 (2013).
31. R. L. Levine, E. R. Stadtman, *Experiment. Gerontol*., **36**(9), 1495 (2001).
32. C. Zhou, Y. Huang, S. Przedborski, *Ann. NY Acad. Sci*., **11**(47), 93 (2008).
33. P.L. Chan, J.G.,Nutt, N.H. Holford, *Pharm. Res.* **24**, 791 (2007).
34. A. Agil, R. Durán, F. Barrero, B. Morales, M. Araúzo, F. Alba, M.T. Miranda, I. Prieto, M. Ramírez, F. Vives, *J. Neurol. Sci*., **240,** 31 (2006).
35. Z. I. Alam, A. Jenner, E. Daniel, A.J. Lees, N. Cairns, D. Marsden, P. Jenner, B. Halliwell, *J. Neurochem*., **69**,1196 (1997).
36. S.J. Padayatty, A. Katz, Y. Wang, P. Eck, O. Kwon, J.H. Lee, S. Chen, C. Corpe, A. Dutta, S. Dutta, M. Levine, *J. Am. College Nutrit*. **22**, 18 (2003).
37. B. Uttara, A.V. Singh, P. Zamboni, R. Mahajan, *Current Neuropharmacol*., **7**(1), 65 (2009).

ОКСИДАТИВЕН СТРЕС И СВЪРЗАНИ С НЕГО БОЛЕСТИ. ЧАСТ 2. БОЛЕСТ НА ПАРКИНСОН

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(Резюме)

Болестта на Паркинсон (PD) е прогресивно невродегенеративно разстройство на движението, свързано със селективна загуба на неврони в областта на средния мозък, наречена *substantia nigra pars compacta*. Фармакологичното лечение на PD традиционно се състои в прилагане на лекарства, възстановяващи допаминергичното влияние в базалните ганглии. През време на началния етап на болестта, прилагането на Levodopa може да доведе до значително подобрение на симптомите на Паркинсоновата болест, но влиянието на Levodopa често отслабва след няколко години непрекъсната употреба и това лекарство често е неефективно в напредналите стадии на PD. Целта на настоящото изследване е да се оцени и сравни оксидативният статус в кръвта на пациенти с PD, третирани само с Madopar, и с комбинация от Madopar и витамин C с използване на различни аналитични техники. За целта са изследвани: 1) нивата на продуктите на реактивни кислородни видове ROS, аскорбатни (Asc•) и NO• радикали като биомаркери на оксидативния стрес в реално време с използване на EPR спектроскопия; 2) оксидативните крайни продукти на липиди, протеини и DNA, нивата на малондиалдехид (MDA), измерени чрез спектрофотометрия във видимата област, съдържанието на протеинов карбонил (PCC), а също и на 8-хидрокси-2′-деоксигуанозин (8-OHdG), определени с ELISA тест; 3) еритроцитната SOD активност чрез спектрофотометрия във видимата област на кръв от пациенти с PD.

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Exhausted antioxidant defense in SSUV-exposed skin of hypothyroid rats

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The decrease of the overall antioxidant defense due to solar simulated ultraviolet irradiation or hypothyroidism has been reported both in humans and animal models. Using a rat model, we aimed to investigate how the combination of UV radiation and hypothyroidism affects the antioxidant defense in the photo-exposed skin. The antioxidant resistance of the rat skin was characterized directly by the radical scavenging (RSA) activity toward stable 2,2-diphenyl-1-picryl-hydrazyl radical (DPPH•), and indirectly by the formation of free radicals due to Fenton reaction-induced oxidative stress. Four groups of male Wistar albino rats, named C (controls), SSUV (irradiated), PTU (hypothyroid), and PTU+SSUV, were used in this experiment. Drug-induced hypothyroidism was developed by the addition of 0.01% (w/w) 6-n-propyl-2-thiouracil (PTU) for 5 weeks in the *ad libitum* consumed drinking water. Then SSUV and PTU+SSUV groups were irradiated for 7 days. The results showed drastically lower antioxidant activity of the H-donors in PTU+SSUV skin than that in the skin of healthy controls. In PTU and SSUV groups a lower antioxidant activity than controls was found as well, the decrease being in the order: *C>PTU≥SSUV>>(PTU+SSUV).* Free radical accumulation was many times higher in SSUV-treated euthyroid skin compared to non-irradiated skin of controls.The Fenton reaction in the PTU group resulted in the formation of very few free radicals in the skin that might be related with the better RSA and slowed the metabolism of the hypothyroid rats. In conclusion, the combination of chronic sun exposure and hypothyroidism could be a risky and harmful factor leading to exhausted antioxidant defense and possible skin damage.

Keywords: Hypothyroidism, UV radiation, SSUV, Antioxidant defense, Skin, DPPH•

INTRODUCTION

Skin - the external body organ is chronically exposed to sunlight, where the ultraviolet (UV) radiation is one of the most harmful exogenous factors with negative biological effects [1]. UV radiation is a well-recognized generator of reactive oxygen species (ROS) and reactive nitrogen species (RNS) which play a key role in mediating its biological effects. Under the control of endogenous antioxidants, these species participate in redox-dependent regulation of cell metabolism in response to UV stress, but if unbalanced, they induce oxidative damage. Their accumulation is considered as a risk factor in photoaging, photoimmunosuppression and photocancerogenesis.

Thyroid hormones have a strong impact on oxidative status of the body [2]. Long lasting hypothyroidism substantially affects multiple organs and systems [3-5], leading to increased level of oxidative stress (OS) [3,6-8]. Recently, it was observed that the mean basal serum total antioxidant status (TAS) was lower, while serum total oxidant status (TOS) and OS index were significantly higher in the blood of hypothyroid patients. TOS has been positively correlated with free levothyroxine (fT4) and negatively correlated with the thyroid stimulating hormone (TSH) [9]. Moreover, hypothyroidism was found to decrease the antioxidant enzyme activity of superoxide dismutase, catalase, glutathione peroxidase, glutathione-S-transferase, and reduced glutathione [10]. Thyroxine application, alone or in combination with apelin, demonstrated a beneficial effect of lowering lipid peroxidation and increasing antioxidant enzyme activity in humans and rodents [11,12].

The decrease of the overall antioxidant defense due to solar simulated ultraviolet (SSUV) exposure [13-16] or hypothyroidism [17-19] has been reported in both humans and animal models.

No data have been published so far on the mutual effects of the overt hypothyroidism and prolonged sunlight exposure on antioxidant defense of the skin. Therefore, using animal model, we aimed to investigate how the UV radiation together with hypothyroidism affect the antioxidant defense in the photo-exposed rat skin.

The antioxidant resistance of the rat skin was characterized directly by the radical scavenging activity (RSA) toward stable 2,2-diphenyl-1-picryl-hydrazyl radical (DPPH•), and indirectly by the formation of free radicals due to Fenton reaction-induced oxidative stress.

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MATERIALS AND METHODS

All chemicals used in this study were of the highest grade available (Sigma-Aldrich).

Animal model

36 male Wistar albino rats of body weight (BW) 135±5 g were assigned to 4 groups: C (control), SSUV [euthyroid rats exposed to solar simulated ultraviolet (SSUV) radiation], PTU (hypothyroid rats) and PTU+SSUV (SSUV treated hypothyroid rats), all housed in transparent standard containers. The animals were kept at room temperature (25±0.5 °C), standard humidity (60±1 %) and a light/dark (12/12 h) cycle. All animals were treated in agreement with the general regulations for treatment of experimental animals, established by the Ethics Committee of the Medical University of Sofia, in agreement with EU Directive 2010/63/EU on the protection of animals used for scientific purposes.

After one week of adaptation, hypothyroidism was induced in the PTU and PTU+SSUV groups by administration of 0.01% (w/w) 6-n-propyl-2-thiouracil (PTU) for five weeks in the *ad libitum* consumed drinking water. The BW of the rats was measured on a daily basis. Average weekly BW gain and average daily BW of each animal for the week were calculated for every group. The average daily dose of PTU consumed by the model animals was determined on the basis of the average daily consumption of PTU solution, and was found to be 16±3 mg/kg BW. At the end of the fourth week of the experiment, thyroid hormones were measured for each group. During the final week, euthyroid (SSUV group) and hypothyroid rats (PTU+SSUV group) received ultraviolet radiation, using a SSUV lamp (type "Helios",UV-125W/IR-175W, IBORA, Bulgaria). The lamp combined UV (180 – 400 nm) and IR sources that were adjusted to mimic sunlight. The SSUV source was positioned at a distance of one meter from the animals' cage. The two groups were irradiated for 15 min four times per day for seven days with periods of 15 min rest between sessions (UV-45 mJ/sm^2; IR-63 mJ/sm^2). Our UV irradiation model was modified from Erden Inal *et al.* [20] to avoid radiation-inflicted burns and to mimic low-dose daily sunlight.

After the seventh day of SSUV- exposure, skin samples were taken from half of the animals in each group and used to analyze the antioxidant potential of the skin.

Preparation of the supernatant

Skin tissue was stored and homogenized in a sonified ice-cold PBS (50 mM, pH 7.45) solution of 0.04% 3,5-di-*tert*-4-butylhydroxytoluene (BHT) (to prevent autoxidation) [21-23]. After centrifugation at 4°C and 7500 rpm for 15 min, the supernatant was collected and stored in an ice-cold bath. The amount of proteins (in mg/ml) in the supernatant was determined as described by Stoscheck [24].

Assay for RSA toward DPPH•

The relative decrease in absorption of the signal at 517 nm (characteristic band for DPPH•) was monitored for 10 min using the kinetic software of the apparatus. The absorption at 517 nm was recorded every minute. RSA (%) was determined using the formula:

$$RSA = \frac{A_{blank} - (A_{sam.} - A_{contr.})}{A_{blank}} * 100,$$

A_{blank} being the absorption due to the presence of the sample's solvent in the DPPH• solution (2 ml DPPH• solution and 0.02 ml PBS), A_{contr} is the absorption due to the sample alone (0.02 ml sample solution in 2 ml ethanol), and A_{sam} is the absorption due to interaction of the sample with DPPH• (2 ml DPPH•solution and 0.02 ml sample in PBS). RSA is presented as a percentage of the value obtained for the control group.

Fe(II)-induced free radical accumulation (FRA) assay

FRA in the supernatant was initiated by the Fe(II)/H_2O_2/EDTA/ascorbate model system in PBS medium, and the formation of MTT-formazan from MTT was used as a marker. The relative increase of the intensity at 578 nm (characteristic for MTT-formazan) was monitored each minute, for 10 min. FRA was evaluated using the formula:

$$FRA = \frac{\Delta A_{blank} - (\Delta A_{sam.} - \Delta A_{contr.})}{\Delta A_{blank}} * 100,$$

ΔA being the relative change of the absorption at 578 nm for 10 min. ΔA_{blank} corresponds to ΔA in the presence of the OH•-producing model system alone (0.05 ml Fe(II)/H_2O_2/EDTA, 0.05 ml ascorbate, 0.2 ml MTT, and PBS to 2 ml), ΔA_{contr} describes the relative change of A (578 nm) in the presence of supernatant alone (0.2 ml MTT, supernatant containing 1 mg/ml proteins and PBS to 2.0 ml), and ΔA_{sam} shows the relative change of the 578 nm signal due to interaction between the model system and the supernatant (0.05 ml Fe(II)/H_2O_2/EDTA, 0.05 ml ascorbate, supernatant containing 1mg/ml proteins, 0.2 ml MTT, and PBS to 2 ml). Data are presented as a percentage of data for the controls.

Statistical analysis

The standard statistical software package was applied for statistical evaluation of data. The

significance in differences among standard deviations was verified using Bartlett test. One way ANOVA test was performed, followed by Bonferroni post-test.

RESULTS

Similar to our previous research [8], here we also found a negative impact of hypothyroidism on the growth and weight gain, and slowed metabolism. The total antioxidant capacity of all hydrogen donors in the SSUV-treated skin of hypothyroid rats was determined using discoloration of DPPH$^{\bullet}$ (517 nm), as presented in Fig. 1.

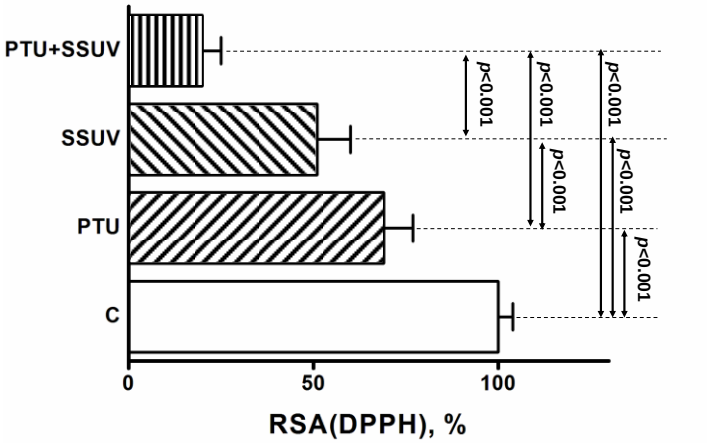

Fig. 1. Total antioxidant capacity toward DPPH$^{\bullet}$. Control group (C), SSUV-exposed group (SSUV); propylthiouracil-induced hypothyroid group (PTU), and propylthiouracil-induced hypothyroid group exposed to SSUV radiation (PTU+SSUV).

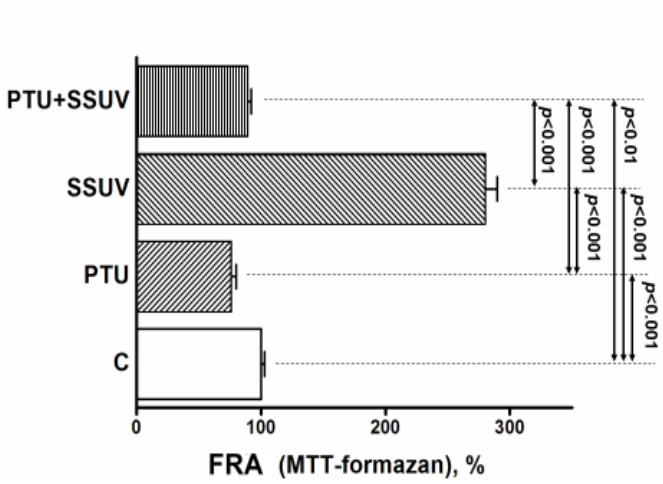

Fig. 2. FRA in a model system generating OH$^{\bullet}$(Fe^{2+}/H_2O_2/EDTA/ascorbate). Control group (C), SSUV-exposed group (SSUV); propylthiouracil-induced hypothyroid group (PTU), and propylthiouracil-induced hypothyroid group exposed to SSUV radiation (PTU+SSUV).

The results showed drastically lower antioxidant activity of the H-donors in PTU+SSUV rat skin than that in the skin of healthy controls. In PTU and SSUV groups a lower antioxidant activity than controls was found too, the decrease being in the order: *C>PTU≥SSUV>>(PTU+SSUV).*

To further monitor the antioxidant activity of photo-exposed hypothyroid rat skin, Fenton reaction was initiated by Fe(II)/H_2O_2/EDTA/ ascorbate model system (Fig. 2). FRA was many times higher in SSUV-treated euthyroid skin compared to the non-irradiated skin of controls. Hypothyroid skin demonstrated a decrease ($p<0.001$) and after irradiation a slight increase ($p<0.01$) of FRA, both statistically significant, in comparison with FRA in healthy skin of controls.

DISCUSSION

The current study provided important data about the impact of the combination of chronic sun exposure and hypothyroidism on the antioxidant defense of the skin.

In addition to other research findings about decreased antioxidant defense measured in the blood of hypothyroid patients [9,10], we found a reduced antioxidant protection in the skin of our PTU-induced model. This approach allowed us to observe that SSUV exhausted more hydrogen (H) donating antioxidants than the hypothyroidism, while the combination of the two factors led to a very strong decrease of the RSA.

The Fenton reaction resulted in the formation of very few free radicals in the skin of PTU compared to the SSUV group. The lower FRA might be related with the better RSA and slowed metabolism of the PTU rats. These results are in agreement with other studies in the literature showing a decrease in specific and total oxidative capacity in many hypothyroid tissues with active metabolism, such as liver, heart, and brown adipose tissue [25-27]. Evidently, the metabolic rates play a decisive role in the accumulation of free radicals in SSUV-irradiated skin.

Antioxidants with radical scavenging properties are promising therapeutics against oxidative stress in hypothyroid patients being chronically exposed to UV radiation.

CONCLUSIONS

1. SSUV exposure of hypothyroid rats resulted in compromised RSA in the skin, compared to irradiated euthyroid rats.

2. Combination of chronic sun exposure and hypothyroidism could be a risky and harmful factor leading to exhausted antioxidant defense and possible skin damage.

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REFERENCES

1. T. Bald, T. Quast T, J. Landsberg,M. Rogava, N. Glodde, D. Lopez-Ramo, J. Kohlmeyer, S. Riesenberg, D. van den Boorn-Konijnenberg, C. Hömig-Hölzel,R. Reuten, B. Schadow, H. Weighardt, D. Wenzel, I. Helfrich, D. Schadendorf, W. Bloch, M. E. Bianchi, C. Lugassy, R.L. Barnhill, M. Koch, B.K. Fleischmann, I. Förster, W. Kastenmüller, W. Kolanus, M. Hölzel, E. Gaffal, T. Tüting, *Nature*, **507**, 109 (2014).
2. H. Erdamar, H. Demirci, H. Yaman, M.K. Erbil, T. Yakar, B. Sancak, G. Biberoğlu, I. Yetkin, *Clin. Chem. Lab. Med*., **46**, 1004 (2008).
3. A. Santi, M.M. Duarte, R.N. Moresco, C. Menezes, M.D. Bagatiini, M.R. Schetinger, V.L. Loro, *Clin. Chem. Lab. Med*., **48**, 1635 (2010).
4. A. Monnereau, S.L. Glaser, C.W. Schupp, K. Ekström Smedby,S. de Sanjosé, E. Kane, M. Melbye, L. Forétova, M. Maynadié, A. Staines, N. Becker, A. Nieters, P. Brennan, P. Boffetta, P. Cocco, I. Glimelius, J. Clavel, H. Hjalgrim, E.T. Chang, *Blood*, **122**, 3492 (2013).
5. M. Obradovic, Z. Gluvic, E. Sudar-Milovanovic, A. Panic, J. Trebaljevac, V. Bajic, M. Zarkovic, E. R. Isenovic, *Curr. Vasc. Pharmacol*., **14**, 266 (2016).
6. A. N. Torun, S. Kulaksizoglu, M. Kulaksizoglu, B. Q. Pamuk, E. Isbilen, N. B.Tutunku, *Clin. Endocrinol. (Oxf)*, **70**, 469 (2009).
7. M. Messarah, A. Boumendjel, A. Chouabia, F. Klibet, C. Abdennour, M. S. Boulakoud, A. E. Feki, *Exp. Toxicol. Pathol*., **62**, 301 (2010).
8. M. L. Valcheva-Traykova, G. S. Bocheva, *Bulg. Chem. Commun*., **3**, 384 (2016).
9. I. Ates, M. Altay, F. M. Yilmaz,C. Topcuoglu, N. Yilmaz, D. Berker, S. Guler, *Eur. J. Endocrinol.*, **174**, 727 (2016).
10. P. Pasupathi, R. Latha, *Thyroid Sci.*, **3**, CLS1-6 (2008).
11. F. Faraji Shahrivar, M. Badavi, M. Dianat, A. Mard, A. Ahangarpour, A. Samarbaf-Zadeh, *Braz. Arch. Biol. Technol*., **59,** e16150585 (2016).
12. G. Baskol, H. Atmaca, F. Tanriverdi, M. Baskol, D. Kocer, F. Bayram, *Exp. Clin. Endocrinol. Diabetes*, **115**, 522 (2007).
13. S. B. Lohan, R. Müller, S. Albrecht, K. Mink, K. Tscherch, F. Ismaeel, J. Lademann, S. Rohn, M. C. Meinke, *Exp. Dermatol*., **25**, 380 (2016).
14. M. C. Meinke, R. Müller, A. Bechtel, F. Haag, M. E. Darvin, S. B. Lohan, F. Ismaeel, J. Lademann, *Exp. Dermatol*., **24**, 194 (2015).
15. S. Arndt, S. F. Haag, A. Kleemann, J. Lademann, M. C. Meinke, *Exp. Dermatol*., **22**, 354 (2013).
16. L. Zastrow, N. Groth, F. Klein, D. Kockott, J. Lademann, L. Ferrero, *Hautarzt*, **60**, 310 (2009).
17. V. S. Reddy, S. Gouroju, M. M. Suchitra, V. Suresh, A. Sachan, P. V. Srinivasa Rao, A. R. Bitla, *Horm. Metab. Res*., **45**, 754 (2013).
18. D. Cattani, P. B. Goulart, V.L. Cavalli, E. Winkelmann-Duarte, A.Q. Dos Santos, P. Pierozan, D.F. de Souza, V. M. Woehl, M. C. Fernandes, F. R. Silva, C. A. Gonçalves, R. Pessoa-Pureur, A. Zamoner, *Mol. Cell Endocrinol*., **375**, 14 (2013).
19. M. S. Dzhazaérli, V. V. Davydov, *Ukr. Biokhim. Zh.* **78**, 132 (2006).
20. M. Erden Inal, A. Kahraman, T. Köken, *Clin. Exp. Dermatol*., **26**, 536 (2001).
21. Y. J. Garcia, A. J. Rodríguez-Malaver, Peñaloza N, *J. Neurosci. Methods*, **144**, 127 (2005).
22. S. Kwiecieñ, T. Brzozowski, P. Ch. Konturek, S. J. Konturek, *J.Physiol. Pharmacol*., **53**:761 (2002).
23. L. Horáková, O. Ondrejicková, K. Bachratá, M. Vajdová, *Gen. Physiol. Biophys*., **19**, 195 (2000).
24. C. M. Stoscheck, *Methods Enzymol.*, **182**, 50 (1990).

25. Y. J. Chang, C. M. Hwu, C. C. Yeh, P. S. Wang, S. W. Wang,*Molecules*,**19**, 11178 (2014).
26. V. Welch-White, N. Dawkins, T. Graham, R. Pace, *Lipids Health. Dis*.,**12**, 100 (2013).
27. M. Moreno, A. Lanni, A. Lombardi, F. Goglia, *J. Physiol*., **505**, 529 (1997).

ИЗТОЩЕНА АНТИОКСИДАНТНА ЗАЩИТА В КОЖАТА НА ХИПОТИРОИДНИ ПЛЪХОВЕ СЛЕД ОБЛЪЧВАНЕ СЪС СИМУЛИРАНА СЛЪНЧЕВА СВЕТЛИНА

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(Резюме)

Намаляване на общата антиоксидантна защита след облъчване със слънчева светлина или при хипотиреоидизъм е съобщавано както при хора, така и при животински модели. Използвайки плъхове като модел, си поставихме за цел да изследваме как комбинацията от ултравиолетова радиация и хипотиреоидизъм действа върху антиоксидантната защита на фото-експозирана кожа. Антиоксидантната резистентност на кожата беше охарактеризирана директно чрез радикал-извличаща активност (RSA) към стабилен 2,2-diphenyl-1-picryl-hydrazyl радикал (DPPH$^{\bullet}$) и индиректно чрез образуване на свободни радикали при индуциран оксидативен стрес в резултат на Fenton реакция. За експеримента бяха използвани 4 групи мъжки плъхове, порода Wistar albino, означени като: C (контрола), SSUV (облъчени), PTU (хипотиреоидни), и PTU+SSUV. Лекарствено-индуцираният хипотиреоидизъм беше получен чрез 0.01% 6-n-propyl-2-thiouracil (PTU) във водата им за пиене за период от 5 седмици. След този период, групите SSUV и PTU+SSUV бяха облъчени за 7 дни. Резултатите показаха драстично по-ниска антиоксидантна активност на донорите на водород в кожата на групата PTU+SSUV, отколкото в кожата на здравите контроли. При PTU и SSUV групите беше намерена също по-ниска антиоксидантна активност, в сравнение с контролата: *C>PTU≥SSUV>>(PTU+SSUV).* Натрупването на свободни радикали беше многократно по-голямо при облъчената еутиреоидна кожа, в сравнение с необлъчената кожа на контролите. Реакцията на Fenton при PTU групата доведе до образуване на незначително количество свободни радикали в кожата, което би могло да се свърже с по-добрата RSA и забавения метаболизъм на хипотиреоидните плъхове. В заключение, комбинацията от хронично слънчево облъчване и хипотиреоидизъм би могла да бъде рисков и увреждаш фактор, водещ до изтощена антиоксидантна защита и възможно увреждане на кожата.

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Effect of *Melissa officinalis* L. on the level of induced lipid peroxidation in mouse liver

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The aim of this study was to evaluate the effect of *Melissa officinalis* L. aqueous extract on the level of induced lipid peroxidation in mouse liver homogenate. Samples were prepared from homogenized BALB/c mice liver, and subsequently incubated with one of the following lipid peroxidation inducing agents: 0.5 mM H_2O_2; 0.1 mM $FeCl_3$ +ascorbate or H_2O_2 +$FeCl_3$+ascorbate (Fenton reaction), in the presence or absence of extract. *M. officinalis* aqueous extract was prepared by extraction with boiling deionized water in 1:10 ratio (w/v). In the experiments were used two-fold dilutions of the extract containing phenolics equivalent of 21.4 to 1.32 mg gallic acid following preliminary determination of the total phenolic content by Folin-Ciocalteu assay. The levels of lipid peroxidation in mouse liver homogenate, caused by all of the oxidative agents were significantly reduced by all tested dilutions of the extract. *M. officinalis* aqueous extracts could be effective for protection of liver cells from induced lipid peroxidation.

Keywords: Phenols, Mice, Antioxidant, Aqueous extract, *M. officinalis*

INTRODUCTION

Lipid peroxidation is a process in which different types of oxidants (free radicals or non-radical species) deteriorate lipids especially those containing polyunsaturated fatty acids in cells, tissues and organs. The variety of metabolic products (i.e. malondialdehyde and 4-hydroxynonenal) produced in this process are associated with development of several major pathological processes as cancer, diabetes, liver and cardiovascular diseases, etc. (for review see Fruhwirth *et al*. [1], Ayala *et al.* [2]; Griffiths *et al.* [3] and references therein). Modern stressful lifestyle and polluted ecosystems are a rich source of free radicals and reactive oxygen species (ROS) whose harmful effect has to be mitigated using different approaches. Therefore usage of phytochemicals is seen as one of most promising strategies as they could be used not only in drug preparations, foods and beverages but also as packaging materials [4, 5].

Lemon balm (*Melissa officinalis* L.) belongs to the family Lamiaceae and it is well known in phytotherapy (both traditional and conventional), cosmetics and culinary. The species is traditionally used as different infusions in Europe and Asia, however nowadays it is in cultivation worldwide and being sold in different forms (extracts, essential oil, and herbal teas). Along with many other properties (i.e. antiviral, antimicrobial, sedative, digestive, antispasmodic, etc.) the aqueous extracts were reported to have antioxidant activity that could be useful in drug and food industry [6-9]. High levels of phenolic acids found in *M. officinalis*, mainly rosmarinic acid, as well as caffeic acid, protocatechuic, ferulic and syringic acids, contribute to the beneficial effects of its extracts [10-12].

Current work reports on the protective effect of *M. officinalis* L. aqueous extract (MAE) against induced lipid peroxidation in mouse liver homogenate.

EXPERIMENTAL

Chemicals

All reagents were obtained from Sigma–Aldrich (Germany).

Plant material and preparation of extract

M. officinalis L. was obtained from the collection of the Institute of Biodiversity and Ecosystem Research, Bulgarian Academy of Sciences at vegetative stage. Herbage was dried in an oven at 40 ° C and finely powdered. Extract from lemon balm was prepared in 1:10 ratio (w/v) with deionized water through microwave-assisted

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extraction at p=100W for 5 min. Resulting extract was filtered through filter paper and centrifuged for 10 minutes at 6,000 × g. Supernatant was filtered through PES Millipore 0.22 μm filter (Millipore, USA).

Total phenolics determination

Total phenolic content (TPC) of the extract was determined by Folin-Ciocalteu assay [13]. Briefly, 150 μL samples were introduced into disposable test tubes together with 750 μL of Folin-Ciocalteu's reagent (diluted 1:10 with deionized water) and 600 μL of 7.5% sodium carbonate solution. The tubes were incubated in a water bath at 50 °C for 10 min. Absorbance was measured at 760 nm. TPC was expressed as milligram gallic acid equivalents (GAE) per gram plant material.

Animals

White BALB/c mice with a weight of 30-40 grams were used. At the time of sacrifice, the mice were anesthetized with ether. The experiments were performed according to the "Principles of Laboratory Animal Care" (NIH publication No. 85-23) and the rules of the Ethics Committee of the Institute of Neurobiology, Bulgarian Academy of Sciences (registration FWA 00003059 by the US Department of Health and Human Services).

Preparation of liver homogenate

The liver was perfused *in situ* with ice-cold 0.15 M KCl. The gall bladder was removed and the liver was isolated, washed, cut with scissors and again washed with ice-cold 0.15 M KCl. The resulting biological material was filtered through gauze and 0.15 M KCl was added to a final volume of 10 ml. The liver was homogenized with a teflon pestle homogenizer and subsequently centrifuged at 3000 × g at 0-4°C for 10 min. After filtration through gauze, the amount of protein of the resulting supernatant from the non-nuclear homogenate was measured.

The protein quantification was performed by the method of Lowry *et al.* [14].

Measurement of lipid peroxidation

Lipid peroxidation (LPO) was determined by the amount of the thiobarbituric acid reactive substances (TBARS), formed in fresh preparations, according to the method of Hunter *et al.* [15]. The hepatic homogenate was diluted with 0.05 M potassium phosphate buffer, pH 7.4, to a final amount of 1 mg protein / ml solution. For each tested concentration of the MAE, 4 samples were prepared containing 500 μl of MAE and 500 μl of liver homogenate. After incubation for 1 hour in a water bath in a shaker at 37 °C, the samples from each dilution were divided into 4 groups: only with extract; with extract and H_2O_2 (10 μl of 0.5 mM H_2O_2); with extract and Fe^{3+} (10 μl of 0.1 mM $FeCl_3$) and ascorbate (50 μl of 0.5 mM ascorbate); with extract and Fe^{3+} (10 μl of 0.1 mM $FeCl_3$), ascorbate (50 μl of 0.5 mM ascorbate) and H_2O_2 (10 μl of 0.5 mM H_2O_2). Additionally, double samples of the homogenate alone, as well as of the pro-oxidants with hepatic homogenate were also prepared, and used for comparison with the samples treated with the extracts. After incubation on a water bath in a shaker at 37 °C for 30 min, 0.6 ml of 40% trichloroacetic acid / 5N HCl / 2% thiobarbituric acid in a 2:1:2 ratio was added to each sample. The so prepared samples were boiled in a water bath for 15 min. After cooling and centrifuging at 3000 × g. for 5 min, the samples were measured spectrophotometrically at wavelengths of 532 nm and 600 nm. The amount of thiobarbituric acid reactive substances was expressed as nmol malondialdehyde (MDA) / mg protein and was calculated using a molar extinction coefficient of 1.56×10^5 M^{-1} cm^{-1}. The experiment was performed three times with double replicates of all samples.

Statistics

Obtained data for the lipid peroxidation in the different induction systems was tested for variance and results from different extract treatments were compared to controls using Dunnett's t test (p=0.05).

RESULTS AND DISCUSSION

The polyphenolic compounds are considered as the main antioxidants in plants [16]. Pereira *et al.* [17] made a comparison between the antioxidant effect of quercetin, gallic acid, quercitrin and rutin, which are some of the most common phenolic compounds found in plant extracts, including *M. officinalis*. They found that highest antioxidant activity is exhibited by quercetin followed by gallic acid, quercitrin and rutin.

Since the polyphenols are considered the compounds directly linked to the antioxidant activity in plants, in our study we measured the total phenolic content in the MAE. The total phenolic content in the aqueous extract of *M. officinalis* was 169.11 ± 0.3 mg GAE / per gram plant material. In the experiments were used two-fold dilutions of the MAE containing phenolics ranging from 21.4 to 1.32 mg GAE.

On Fig. 1 it can be seen that the level of naturally occurred LPO in mouse liver homogenate is reduced around three times after treatment with

any of the subsequent dilutions of the MAE. There was no significant difference in the degree of effectiveness in the tested concentrations of the MAE.

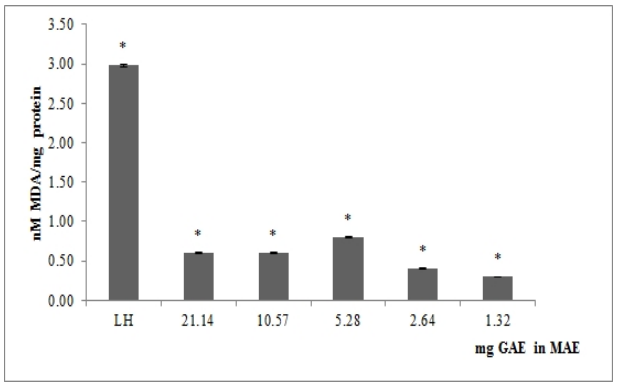

Fig. 1. Effect of *Melissa officinalis* aqueous extract (MAE) on naturally occurred LPO in mouse liver homogenate. LH - liver homogenate, *- $p<0.05$ (n=3)

The experiments with pro-oxidants showed that the highest level of LPO in mouse liver homogenate, presented as amount of produced MDA, is obtained after treatment with $FeCl_3$+ascorbate (Fig. 3) or H_2O_2 + $FeCl_3$ + ascorbate (Fenton reaction) (Fig. 4), while H_2O_2 alone does not produce such a strong effect (Fig. 2).

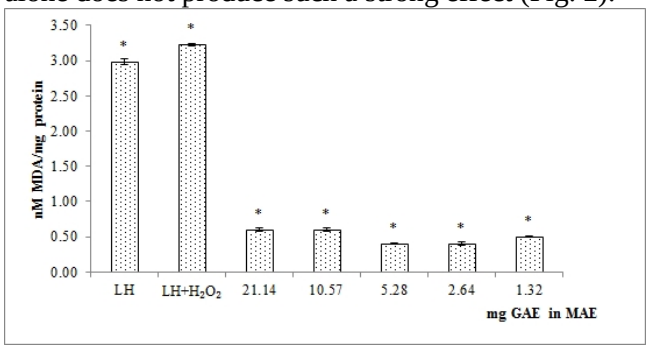

Fig. 2. Effect of *Melissa officinalis* aqueous extract (MAE) on LPO in mouse liver homogenate induced by H_2O_2. LH - liver homogenate, *- $p<0.05$ (n=3).

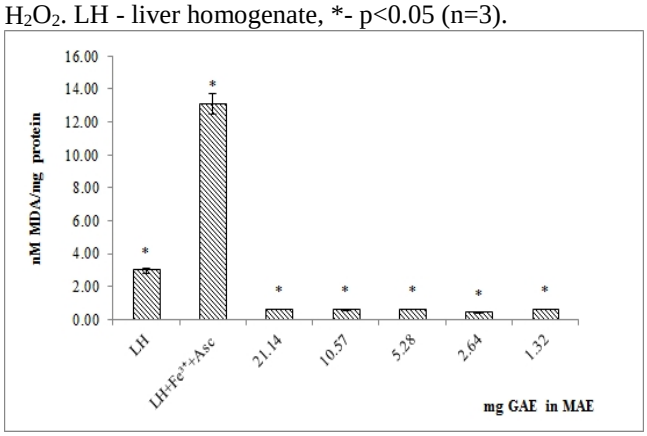

Fig. 3. Effect of *Melissa officinalis* aqueous extract (MAE) on LPO in mouse liver homogenate induced by Fe^{3+} and ascorbate. LH - liver homogenate, *- $p<0.05$ (n=3).

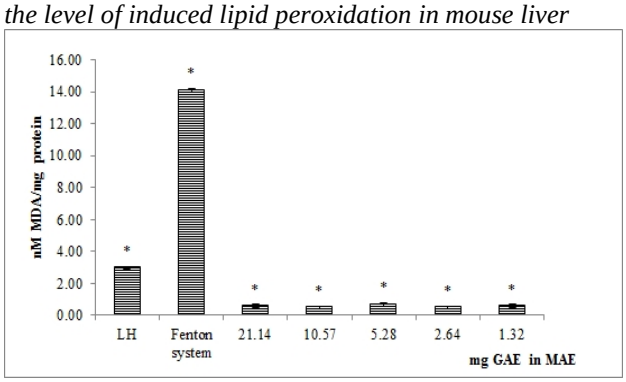

Fig. 4. Effect of *Melissa officinalis* aqueous extract (MAE) on LPO in mouse liver homogenate induced by Fenton's reaction. LH - liver homogenate, *- $p<0.05$ (n=3).

In the treatments with the different pro-oxidants, all tested concentrations of the extract significantly reduced the amount of MDA to levels even below the control of liver homogenate ($p<0.05$). The strongest effect was observed in the experiment with Fenton reaction where the decrease of MDA in the samples treated with the MAE extract had thirteen times lower MDA levels than those treated with the pro-oxidant alone. There were no significant differences between the antioxidant activities of the different concentrations of MAE, which suggested than even in very low concentration, the aqueous extract of *M. officinalis* can protect the liver cells from lipid peroxidation. Similar results were registered by Mimica-Dukic *et al.* [18] who found that treatment with *M. officinalis* essential oil possesses very strong inhibition of LPO, particularly in the Fenton system of induction.

Hohmann *et al.* [19] found that aqueous methanol extract of *M. officinalis* has considerable concentration-dependent inhibition of lipid peroxidation enzyme-dependent and enzyme-independent lipid peroxidation systems.

In an experiment with induced cerebral lipid peroxidation by iron sulfate (10 μM), sodium nitroprusside (5 μM) or 3-nitropropionic acid (2 mM), Pereira *et al.* [17] found that the aqueous extract of *M. officinals* has a strong effect on TBARS production induced by all tested pro-oxidants. Moreover, the effect of the aqueous extract was superior to those of methanolic and ethanolic extracts of the plant.

Our results suggested that the aqueous extract of *M. officials* protects the liver cells, both from naturally occurred and induced by pro-oxidants lipid peroxidation. The effect is very strong even in very low amounts of polyphenols (1.32 mg GAE), which are considered the main substances responsible for the antioxidative effect of the plant.

CONCLUSION

It can be concluded that the aqueous extract of *M. officinalis* effectively protects the liver cells from artificially induced and naturally occurred lipid peroxidation *in vitro*.

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REFERENCES

1. G. O. Fruhwirth, A. Loidl, A. Hermetter, *Biochimica et Biophysica Acta: Molecular Basis of Disease*, **1772**, 718 (2007).
2. A. Ayala, M. F. Muñoz, S. Argüelles, *Oxid. Med. Cell. Longev.*, (2014). http://dx.doi.org/10.1155/ 2014/360438
3. K. Griffiths, B. B. Aggarwal, R. B. Singh, H. S Buttar, D. Wilson, F. De Meester, *Diseases*, **4**, 28 (2016).
4. A. Sanches-Silva, D. Costa, T. G. Albuquerque, G. G. Buonocore, F. Ramos, M. C. Castilho, A. V. Machado, H. S. Costa, *Food Addit. Contam.: Part A*, **3**, 374 (2014).
5. M. C. T. Duarte, M. Rai, in: Therapeutic Medicinal Plants: From Lab to the Market, CRC Press, Taylor & Francis Group, Boca Raton, 2015.
6. N. G. Bisset, M. Wichtl, in: Herbal Drugs and Phytopharmaceuticals. Medpharm Scientific Publishers, Stuttgart, 1994.
7. D. Ivanova, D. Gerova, T. Chervenkov, T. Yankova, *J. Ethnopharm.*, **96**, 145 (2005).
8. K. Triantaphyllou, G. Blekas, D. Boskou, *Int. J. Food Sci. Nutr.*, 52, **313** (2001).
9. A. Akarpat, S. Turhan, N. S. Ustun, *J. Food Proc. Pres.*, **32**, 117 (2008).
10. I. Fecka, S. Turek, *J. Agric. Food Chem.*, **55**, 10908 (2007).
11. A. Arceusz, M: Wesolowski, *J. Pharm. Biomed. Anal.*, **83**, 215 (2013).
12. M. Katsarova, S. Dimitrova, L. Lukanov, F. Sadakov, P. Denev, E. Plotnikov, I. Kandilarov, I. Kostadinova, *Bulg. Chem. Comm.*, **49G**, 93 (2017).
13. S. McDonald, P. D. Prenzler, M. Autolovich, K. Robards, *Food Chem.*, **73**, 73 (2001).
14. O. H. Lowry, N. J. Rosebrough, A. L. Farr, R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
15. F. E. Hunter, J. M. Gebicki, P. E. Hoffsten, J. Weinstein, A. Scotta, *J. Biol. Chem.*, **238**, 828 (1963).
16. I. B. Pandey, S. I. Rizvi, *Oxid. Med. Cell. Longev.*, **2**, 270 (2009).
17. R. P. Pereira, R. Fachinetto, A. de Souza Prestes, R. L. Puntel, G. N. Santos da Silva, B. M. Heinzmann, T. K. Boschetti, M. L. Athayde, M. E. Bürger, A. F. Morel, V. M. Morsch, J. B. Rocha, *Neurochem. Res.*, **34**, 973 (2009).
18. N. Mimica-Dukic, B. Bozin, M. Sokovic, N. Simin, *J. Agric. Food. Chem.*, **52**, 2485 (2004).
19. I. Hohmann, I. Zupkó, D. Rédei, M. Csányi, G. Falkay, I. Máthé, G. Janicsák, *Planta Med.*, **65**, 576 (1999).

ЕФЕКТ НА *Melissa officinalis* L. ВЪРХУ НИВОТО НА ИНДУЦИРАНА ЛИПИДНА ПЕРОКСИДАЦИЯ В ЧЕРЕН ДРОБ НА МИШКИ

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(Резюме)

Целта на това изследване е да се оцени ефектът на воден екстракт от *Melissa officinalis* L. върху нивото на индуцирана пероксидация на липидите в хомогенат от миши черен дроб. Пробите са приготвени от хомогенизиран BALB/c миши черен дроб, който в последствие е инкубиран с един от следните агенти, индуциращи липидна пероксидация: 0.5 mM H_2O_2; 0.1 mM $FeCl_3$ + аскорбат или H_2O_2 + $FeCl_3$ + аскорбат (реакция на Фентън) в присъствие или отсъствие на екстракт. Воден екстракт от *M. officinalis* L. е приготвен чрез екстракция с кипяща дейонизирана вода в съотношение 1:10 (w/v). В експериментите е използвано двукратно разреждане на екстракта, съдържащ фенолен еквивалент от 1.4 до 1.32 mg галова киселина след предварително определяне на общото фенолно съдържание по метода на Folin-Ciocalteu. Нивата на липидната пероксидация на хомогената от миши черен дроб, предизвикана от всички оксидативни агенти, са понижени съществено при всички разреждания на екстракта. Водните екстракти на *M. officinalis* могат да са ефективни за протекция на чернодробните клетки от индуцирана липидна пероксидация.

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Antioxidant mechanisms in neuroprotective action of lipoic acid on learning and memory of rats with experimental dementia

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Alzheimer's disease (AD) is one of the most common dementia affecting about 36 million people and without effective cure. Oxidative stress is one of many hypotheses for the AD mechanisms. Possible preventive AD effects of some antioxidants continue to be the object of clinic and experimental research. The aim of this study was to evaluate the antioxidant mechanism in the neuroprotective effect of lipoic acid (LA) on the cognitive functions in experimental dementia. Alzheimer's disease type dementia was produced *via* scopolamine treatment (Sco, 1 mg/kg i.p., 11 days) on male Wistar rats. Lipoic acid (LA, 30 mg/kg, i.p.) was applied for the same period. Learning and memory performance of the rats were evaluated using passive avoidance learning test (Step through test). At the 24th hour after the last treatment the brain frontal cortex, hippocampus, and striatum were isolated and homogenized. The homogenates were used for determination of malondialdehyde (MDA), total glutathione (tGSH), and activities of superoxide dismutase (SOD), glutathione peroxidase and catalase (CAT). The dementia model was verified by the cognitive tests used. In brain structures of the Sco-group increased MDA, and decreased tGSH levels, as well as activated antioxidant enzymes were observed. LA significantly improved cognitive functions and oxidative status damaged by Sco by increased tGSH level, restored CAT and SOD activities. Thus LA significantly protects memory impairments of dement animals due to its antioxidant capacity and could be used in prevention and therapy of AD.

Key words: Alzheimer's disease, Lipoic Acid, Oxidative stress, Scopolamine

INTRODUCTION

Alzheimer's disease (AD) is the major senile type of dementia. According to The World Alzheimer Report (2016) in 2015 AD has affected about 47 million people. It is considered that this number will reach more than 130 million in 2050. Various hypotheses try to explain the mechanisms of AD, including the oxidative stress (OS). Many studies have shown increased lipid peroxidation, protein and DNA oxidation in the AD brain [1-3]. Since OS is involved in the pathogenesis of the disease it could be assumed that antioxidants would have a beneficial effect. There are evidences that higher intake of vitamin E [4] and vitamin C [5] may reduce the risk of dementia and AD.

Alpha lipoic acid (LA) is an endogenous organosulfur compound that demonstrates considerable antioxidant properties. Lipoate, or its reduced form, dihydrolipoate, reacts with reactive oxygen species such as superoxide radicals, hydroxyl radicals, hypochlorous acid, peroxyl radicals, and singlet oxygen [6]. In addition it can interact with vitamin C and glutathione, which may recycle vitamin E. Moreover it is able to chelate transition metal ions, in particular iron, preventing hydroxyl radicals' generation *via* Fenton reaction [7]. A positive effect of LA was shown in a number of pathological conditions with OS ethology [8-11].The effect of LA has been investigated also in some neurodegenerative disorders. Li *et al*. [12] demonstrated a protective effect of LA on dopaminergic neurons in a model of Parkinson disease through inhibition of intercellular ROS levels and mitochondrial transmembrane permeability. The intraperitoneal administration of LA (30 mg/kg body weight/day) into aged rats for 14 days reduced lipid peroxidation and protein oxidation in various brain regions [13]. These results demonstrate that LA is a potent antioxidant for neuronal cells against age associated oxidative damage.

The goal of this work was to study the possible neuroprotective effect of lipoic acid on scopolamine-induced dementia and to evaluate its antioxidant capacity in some brain structures of rats.

EXPERIMENTAL

Materials

The reagents (2-thiobarbituric acid, $NADP^+$, NADPH, reduced and oxidized glutathione, riboflavine, methionin) were obtained from Sigma-

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Aldrich (Germany). Scopolamine was purchased from ACROS Organics and α-lipoic acid from Solupharm GmbH & Co. KG (Germany) (as Thiogamma Turbo-Set solution for injection 600 mg, 50 ml). All other chemicals were of the highest commercially available purity.

Animals

Male Wistar rats (180-200 g) were housed at 22°-25°C with free access to food and water and a natural day/night light cycle. The rats were divided in 3 groups (each with 6 animals) and animals were treated for 11 days with saline (control group); scopolamine (Sco group) (1 mg/kg i.p.) and the combination lipoic acid (30 mg/kg i.p) and scopolamine (1 mg/kg i.p.) - (LA+ Sco group). Scopolamine was dissolved *ex tempore* in distilled water. Lipoic acid was dissolved in a saline solution. Drugs were administered intraperitoneally at a volume of 0.10 ml/100 g b.w. Fresh drug solutions were prepared on each day of the experiment. Control groups obtained saline injections of the same volume and *via* the same route of administration.

All experiments were performed according to the "Principles of Laboratory Animal Care" (NIH publication No. 85-23, revised 1985), and the rules of the Ethics Committee of the Institute of Neurobiology, Bulgarian Academy of Sciences (registration FWA 00003059 by the US Department of Health and Human Services).

Methods

Behavioral test

Learning and memory performance of the rats were evaluated using passive avoidance learning test (Step through test) [14]. The apparatus consisted of two separate chambers connected through a guillotine door. One chamber was illuminated, while the other was dark. The floor of the dark chamber consisted of steel grids for delivering electric shocks by an isolated stimulator. For the record of initial latency (acquisition latency time) rats were individually placed in the illuminated chamber. After a habituation period (5 min), the guillotine door was opened and after the rat entered the dark chamber, the door was closed and an inescapable scrambled electric shock (0.5 mA, 1 s once) was delivered. The time of entrance into the dark chamber was recorded and rats with initial latency (IL) >60 s were excluded from the study.

Twenty-four hours (for short-term memory) later, before obtaining the treatment and on the 12th day (for long term memory) each rat was placed in the illuminated chamber for retention trial (step-through latency). The interval between the placement in the illuminated chamber and the entry into the dark chamber was measured as step-through latency (STL up to a maximum of 180 s as cut-off). Behavioral observations were carried out from 9 a.m. to 12 a.m.

Tissue preparations

At the 24th hour after the last treatment (on the 12th day) the animals were decapitated. Brains were quickly removed on ice and the next brain structures, related to learning and memory: cerebral cortex, hippocampus and striatum, were dissected by the method of Valzelli and Garattini [15]. A 10%-homogenate of each structure was obtained by a Potter-Elvehjem glass homogenizer with a Teflon pestle. The homogenates were centrifuged for 10 min at 3000 rpm, and a post nuclear fraction was obtained. This preparation was used for quantitative measurement of the levels of total glutathione and lipid peroxidation. Part of the post nuclear homogenate was centrifuged for 20 min at 12,000 rpm (temperature control, between 0° and +4°C). The resulting postmitochondrial supernatant was used for measuring the antioxidant enzyme activities.

Analytical methods

Protein content was measured by the method of Lowry *et al.* [16].

Lipid peroxidation (LP) was determined by the amount of thiobarbituric acid reactive substances (TBARs) formed in fresh biological preparations [17]. The postnuclear homogenates of the brain structures (mg protein/ml) in 0.15 M KCl-10 mM potassium phosphate buffer, pH 7.4, were heated for 15 min at 100°C in the presence of 2.8% trichloroacetic acid + 5N HCl + 2% thiobarbituric acid in 50 mM NaOH (2:1:1 v/v) for color developing. The absorbance was read at 532 nm against appropriate blank. The values were expressed in nmoles malondialdehyde (MDA) per mg protein, with a molar extinction coefficient of $1.56 \times 10^5 M^{-1}cm^{-1}$.

Total glutathione (tGSH) level was measured according to Tietze [18] and was expressed in ng/mg protein, with glutathione oxidized (GSSG) as a reference standard.

Catalase (CAT) activity was determined according to Aebi [19]; the enzyme activity was expressed as ΔE_{240}/min/mg protein.

Cu,Zn-superoxide dismutase (SOD) activity, determined according to Beauchamp and Fridovich [20], was expressed in U/mg protein (one unit of SOD activity is the amount of the enzyme producing

a 50% inhibition of Nitroblue tetrazolium reduction).

Glutathione peroxidase (GPx) activity was measured by the method of Gunzler *et al.* [21] and was expressed in nmoles NADPH oxidized per minute per mg protein, with a molar extinction coefficient of $6.22\times10^{6}M^{-1}cm^{-1}$.

Statistics

The results were statistically analyzed by one-way ANOVA (Dunnett post-test), with $p<0.05$ accepted as the minimum level of statistical significance of the established differences.

RESULTS

Effect of LA on learning and memory of dement animals

Our results exhibited significant damage of learning and memory of Sco treated animals. Step through latency (STL) of passive avoidance response in Sco treated group was decreased significantly. The established decrease was by 53% at the 24th hour (for short-term memory) and by 50% at the 12th day (for long-term memory) in comparison to healthy controls ($p<0.05$, Fig. 1).

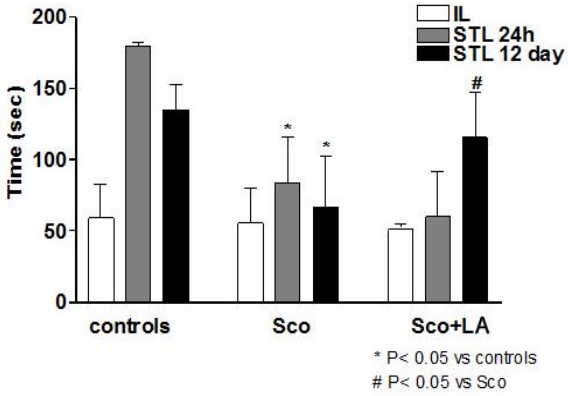

Fig. 1. Effect of LA (on the 24th hour and 12th day) on learning and memory (step-through latency - STL) in rats with scopolamine model of dementia (mean±SEM).

LA administration demonstrated significant improving effect on the learning and memory of animals treated simultaneously with Sco. STL in the group with combination LA+Sco increased significantly by 42% in comparison to Sco treated group (Fig. 1).

Effects of LA administration on OS markers in brain structures of dement animals

An increase of LP was observed in the brain tissues of Sco-treated animals in comparison to the control rats (Fig. 2). The administration of scopolamine led to elevation of TBARs content in cortex by 14.2%, in hippocampus by 51.6% and in striatum by 31.4%. In cortex, the LA reduced the Sco-induced elevation of TBARs by 16.5% as compared to Sco-group. However, in the others structures striatum and hippocampus the LA did not significantly reduce the Sco-induced elevation of TBARs (Fig. 2).

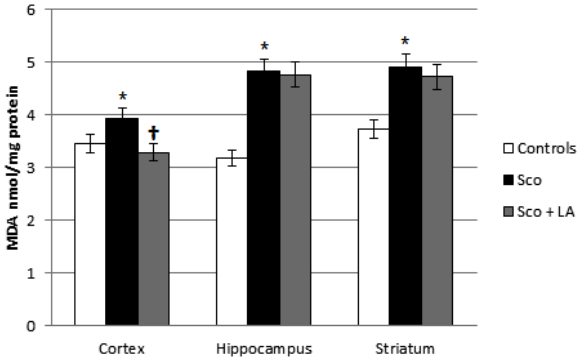

Fig. 2. Effect of LA on lipid peroxidation in brain structures (cortex, hippocampus and striatum) of rats with scopolamine model of dementia (mean±SEM); *p<0.05 *vs.* control group, †p<0.05 *vs.* scopolamine group.

In comparison to the control group the treatment of the animals with Sco led to decrease of tGSH level by 4% in the cortex, 26.7% in hippocampus and 22.3% in striatum (Fig. 3). The LA had a positive effect preventing the Sco-induced reduction of tGSH level. In the Sco+LA group the levels of tGSH in all brain structures were similar to those in the control group (Fig. 3).

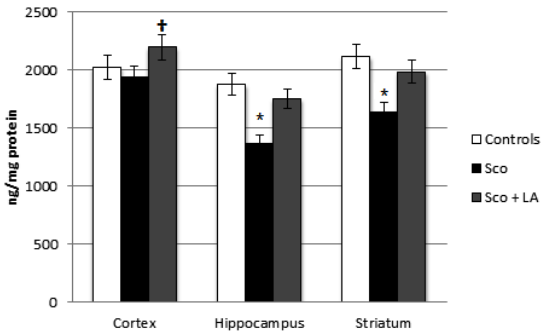

Fig. 3. Effect of LA on total glutathione levels in brain structures (cortex, hippocampus and striatum) of rats with scopolamine model of dementia (mean±SEM); *p<0.05 *vs* control group, †p<0.05 *vs* scopolamine group.

In the Sco-group there was a significant activation of antioxidant enzymes in different brain regions: CAT activity was increased by 30.1% in the cortex, 31.3% in hippocampus and 56.3% in striatum (Fig. 4); SOD activity was increased by 73.6% in the cortex, 62.0% in hippocampus and 90.6% in striatum (Fig. 5); GPx activity was increased by 5.3% in the cortex, 6.0% in hippocampus and 8.9% in striatum (Fig. 6) compared to healthy control.

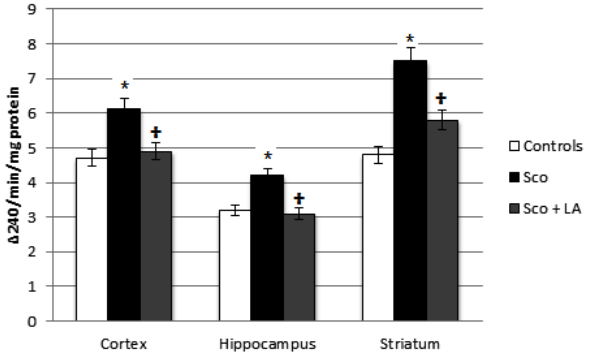

Fig. 4. Effect of LA on CAT activities in different brain structures in rats with scopolamine model of dementia (mean±SEM);*p<0.05 *vs* control group, †p<0.05 *vs* scopolamine group.

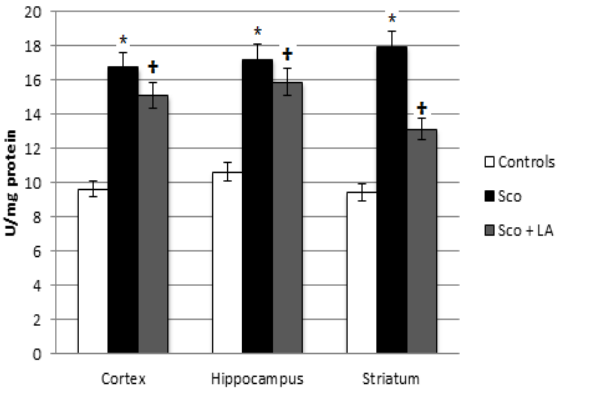

Fig. 5. Effect of LA on SOD activities in different brain structures in rats with scopolamine model of dementia (mean±SEM);*p<0.05 *vs* control group, †p<0.05 *vs* scopolamine group.

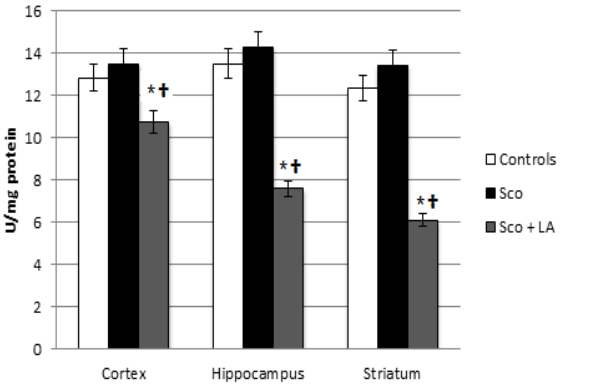

Fig. 6. Effect of LA on GPx activities in different brain structures in rats with scopolamine model of dementia (mean±SEM); *p<0.05 *vs* control group, †p<0.05 *vs* scopolamine group.

From the detected increase of antioxidant enzymes' activities, that of the GPx was the lowest, less than 10%. The LA administration jointly with Sco restored the CAT activities and slightly decreased the Sco-induced increase of SOD activities. The GPx activities were strongly inhibited in the Sco+LA group.

DISCUSSION

It is believed that oxidative stress is a critical factor in AD and scopolamine treatment of animals increases oxidative stress in the whole brain, as well as in the brain structures associated with memory and learning [23]. In our study Sco-treatment of rats significantly increased TBARs concentration and decreased tGSH level. Our data confirmed the ability of scopolamine to produce similar to AD type dementia accompanied by oxidative stress. This effect was established by many other studies in the literature [24-27]. In the present study we also observed an increase in antioxidant enzyme activities after Sco treatment of the rats. Similar to our findings El-Sherbiny *et al.* [26] observed an increase in brain GPx activity in rats after acute administration of scopolamine (1.4 mg/kg, i.p.). Literature data related to antioxidant enzyme activities after animal treatment with scopolamine are controversial. Nade *et al.* [28] found a decrease in SOD activity after injection of young mice (8 weeks age) with Sco (1 mg/kg, i.p.). Budzynska *et al.* [24] noticed a significant decrease in GPx activities in whole brain, prefrontal cortex and hippocampus, as well as a significant decrease in both glutathione reductase and SOD activities in prefrontal cortex and hippocampus in scopolamine treated mice (1 mg/kg, i.p.) in comparison to saline-treated group. The research of Tabari *et al.* [29] showed little changes in brain SOD and GPx in tissue in animals which had received scopolamine. The differences in obtained data may be due to differences in experimental design rather than to the difference in animals' species used. In regard to our results, it could be assumed that the activation of antioxidant enzymes in response to Sco-induced OS is a cellular protective mechanism. It has been found that the chronic stress induces an increase in oxidative enzyme (Mg-SOD, Cu,Zn-SOD, and CAT) activities in rat hippocampus [30]. The negligible activation of GPx, detected in our study, is probably related to the reduction of the tGSH that is a substrate for the enzyme.

Our results showed that the coadministration of LA with Sco led to a decrease of Sco-induced OS, demonstrated in decreased LPO, increased GSH and restored levels of antioxidant enzymes SOD and CAT to the baseline values. Moreover, an improvement in the learning and memory processes was established after 11 days of administration of LA despite the simultaneous administration of Sco. Although these results could not provide clear understanding of the mechanism of action of the LA, it could be hypothesized that the antioxidant properties of LA are able to affect positively the

impaired brain cognitive functions. It has been suggested that nutritional LA does not act as a direct antioxidant, but it stimulates important stress response pathways in cell affecting endogenous cellular antioxidant levels and diminishing the pro-inflammatory processes [31]. Regardless of whether LA acts directly or indirectly, it has been demonstrated that the administration of 600 mg of LA/daily along with the standard treatment with choline-esterase inhibitors to patients with AD for a period of 12 months led to a stabilization of cognitive functions [32]. The extended study up to 48 months showed lower progression of the disease in patients with additive LA supplementation compared to patients with standard therapy [33].

Therefore, our results, in agreement with some literature data, indicate a significant protective effect of LA on brain structures against scopolamine-induced memory impairment and oxidative damage. In conclusion, our study suggests that LA as a powerful antioxidant may provide a successful approach in prophylactics and therapy of AD.

REFERENCES

1. W.R. Markesbery, *Free Radic. Biol. Med.,* **23**, 134 (1997).
2. S. Varadarajan, S. Yatin, M. Aksenova, D. A. Butterfield, *J. Struct. Biol.*, **130**, 184 (2000).
3. S. Bennett, M. M. Grant. S. Aldred, *J Alzheimers Dis.,* **17**, 245 (2009).
4. E.E. Devore, F. Grodstein, F.J. VanRooij, A. Hofman, M.J. Stampfer J.C. Witteman M.M. Breteler, *Arch. Neurol.,* **67**, 819 (2010).
5. M.J. Engelhart, M.I. Geerlings, A. Ruitenberg, J.C. Van Swieten, A. Hofman, J.C. Witteman, M.M. Breteler. *JAMA*, **287**, 3223 (2002).
6. L. Packer, E.H. Witt, H.J. Tritschler, *Free Radic. Biol. Med.,* **19**, 227 (1995).
7. Y.F. Ali, S.O. Desouky, N.S. Selim, Kh.M. Ereiba, *J. Rad. Res. Appl. Sci*., **8,** 26 (2015).
8. N. Ambrosi, V. Arrosagaray, D. Guerrieri, P.D. Uva J. Petroni, M.B. Herrera, J. L. Iovanna, L.León, C. Incardona, H.E.Chuluyan, D.H. Casadei, *Transplant.*, **4**, 908 (2016).
9. U. Singh, I. Jialal, *Nutrition Reviews*, **66**, 646 (2008).
10. E. Kan, E. Kiliçkan, A. Ayar, R. Çolak, *Int. Ophthalmol.*, **35**, 115 (2014).
11. K. Steliou, D.V. Faller, C.A. Pinkert, M.H. Irwin, W.H. Moos, *Drug Dev. Res.*, **76**, 167 (2015).
12. D.W. Li, G.R Li, Y. Lu, Z.Q. Liu, M.Chang, M.Yao, W.Cheng, L.S.Hu. *Int. J. Mol.Med.,* **32**, 108 (2013).
13. P. Arivazhagan, T. Thilakavathy, K. Ramanathan, S. Kumaran, C. Panneerselvam, *J. Nutrit. Biochem.*, **13**, 619 (2002).
14. M.E. Jarvik, R. Kopp, *Psychol. Rep*., **21**, 221 (1967).
15. L. Valzelli, S. Garattini, *J. Neurochem.*, **15**, 259 (1968).
16. O.H. Lowry, N.J. Rosenbrough, A.L. Farr, R.J. Randal, *J. Biol. Chem.*, **193,** 265 (1951).
17. F. Hunter, J. Gebinski, P. Hoffstein, J. Weinstein, A. Scott, *J. Biol. Chem*., **238**, 828 (1963).
18. F. Tietze, *Anal Biochem.*, **27**, 502 (1969).
19. H. Aebi, *Methods Enzymol*., **105**, 121 (1984).
20. C. Beauchamp, I. Fridovich, *Anal. Biochem*., **44**, 276 (1971).
21. W.A. Günzler, H. Vergin, I. Müller, L. Flohé, *Hoppe-Seyler's Z. Physiol. Chemie*, **353**, 1001 (1972).
22. D. Pachauri, S. Tota, K. Khandelwal, P.R. Verma, C. Nath, K. Hanif, R. Shukla, J.K. Saxena, A.K. Dwivedi, *J. Ethnopharmacol.*,**139**, 34 (2012).
23. D.G. Smith, R. Cappai, K.J. Barnham, *Biochim. Biophys. Acta*, **768**, 1976 (2007).
24. B. Budzynska, A. Boguszewska-Czubara, M. Kruk-Slomka, K. Skalicka-Wozniak, A. Michalak, I. Musik, G. Biala, *Psychopharmacol.*, **232**, 931 (2015).
25. M.F. El-Khadragy, E.M. Al-Olayan, A.E. Abdel Moneim, *CNS Neurol. Disord. Drug Targets*, **13**, 684 (2014).
26. D.A. El-Sherbiny, A.E. Khalifa, A.S. Attia, E.D. Eldenshary, *Pharmacol. Biochem. Behav*., **76**, 525 (2003).
27. H. F. Zaki, A. May. A. E. Fattah, S. Amina, *Bull. Fac. Pharm., Cairo University*, **52**, 15 (2014).
28. V.S. Nade, S.V. Kanhere, L.A. Kawale, A.V.Yadav, *Indian J. Pharmacol.* **43**, 137 (2011).
29. S. S. Tabari, S. Babri, F.Mirzaie, F.Farajdokht, G.Mohaddes, *Acta Cir. Bras.*,**31**, 520 (2016).
30. V. Stojiljković, A. Todorović, J. Kasapović, S. Pejić, S.B. Pajović, *Ann. N. Y. Acad. Sci.*,**1048**, 373 (2005).
31. S.K.P. Shay, R.F. Moreau, E.J. Smith, T.M. Hagen, *UBMB Life*, **60**, 362 (2008).
32. K. Hager, M. Kenklies, J. McAfoose, J. Engel, G. Münch, *J. Neural. Transm. Suppl.*, **72**, 189 (2007).
33. K. Hager, A. Marahrens, M. Kenklies, P. Riederer, G. Münch, *Erratum in Arch. Gerontol. Geriatr.*, **51**, 110 (2010).

АНТИОКСИДАНТНИ МЕХАНИЗМИ НА НЕВРОПРОТЕКТИВНИЯ ЕФЕКТ НА ЛИПОЕВА КИСЕЛИНА ВЪРХУ ОБУЧЕНИЕТО И ПАМЕТТА НА ПЛЪХОВЕ С ЕКСПЕРИМЕНТАЛНО ПРЕДИЗВИКАНА ДЕМЕНЦИЯ

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(Резюме)

Болестта на Алцхаймер (AD) е една от най-често срещаните форми на деменция, която засяга около 36 милиона души и няма ефективно лечение. Оксидативният стрес е една от предполагаемите причини за механизма на действие на AD. Възможните превантивни ефекти на някои антиоксиданти продължават да са предмет на клинични и експериментални изследвания. Целта на това изследване е да се оцени антиоксидантният механизъм на невропротективния ефект на липоевата киселина (LA) върху когнитивните функции при експериментално предизвикана деменция. Деменция от Алцхаймеров тип е предизвикана чрез прилагане на скополамин (Sco, 1 mg/kg интраперитонеално, i.p.) на мъжки Wistar плъхове в продължение на 11 дни. Липоева киселина (LA, 30 mg/kg i.p.) е прилагана през същия период. Обучението и паметта на плъховете са оценени с помощта на пасивен тест за обучение за избягване на неприятни ситуации (Step through test). На 24-ия час след последното прилагане фронталната мозъчна кора, хипокампусът и стриатумът са изолирани и хомогенизирани. Хомогенатите са използвани за определяне на малонов диалдехид (MDA), общ глутатион (tGSH) и активностите на супероксид дисмутазата (SOD), глутатион пероксидазата и каталазата (CAT). Моделът на деменцията е проверен с помощта на когнитивни тестове. В мозъчните структури на Sco-групата MDA се повишава, tGSH се понижава и се наблюдават активирани антиоксидантни ензими. LA значително подобрява когнитивните функции и оксидативния статус, влошени от Sco, чрез повишеното ниво на tGSH, възстановените CAT и SOD активности. По този начин LA значително подобрява влошената памет на животни с деменция посредством антиоксидантния си капацитет и би могла да се използва за превенция и терапия на AD.

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Comparative analysis of real-time oxidative stress biomarkers measured in mussels (*Mytilus galloprovincialis*) and veined rapa whelks (*Rapana venosa*) in relation to two seasons - An electron paramagnetic resonance study

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The aim of this study was to elucidate the radical mechanisms for protection and survival of mussels (*Mytilus galloprovincialis*) and veined rapa whelks (*Rapana venosa*) during spring and summer season by following out levels of some real-time oxidative stress (OS) biomarkers. Thirty specimens of each species were analyzed by using electron paramagnetic resonance (EPR) spectroscopy. During spring, statistically higher levels of ROS products were found in *R. venosa* compared to *M. galloprovincialis*. During summer, statistically significant higher levels of ROS products were found in both *R. venosa* and *M. galloprovincialis*, compared to the same groups during spring. NO radicals in *R. venosa* were higher, although not statistically significant than those in *M. galloprovincialis* during both spring and summer periods. During summer, statistically significant higher levels of ascorbate radicals (Asc•) were found in both *R. venosa* and *M. galloprovincialis*, compared to the same groups during spring. However, during the summer the levels of ascorbate radicals measured in *R. venosa* were significantly higher compared to *M. galloprovincialis*. Our results showed that changes in oxidative/antioxidant status may reflect the gradient of contamination confirming the rational use of biomarkers of oxidative stress in biomonitoring of contamination. *R. venosa* has effective biochemical mechanisms of protection and survival, in particular a strong antioxidant system that provides this type of high adaptability and survival against oxidative stress.

Keywords: *Mytilus galloprovincialis*, *Rapana venosa*, Oxidative stress biomarkers, ROS, NO• and Asc• radicals

INTRODUCTION

Reactive oxygen species (ROS) and reactive nitrogen species (RNS, e.g. nitric oxide, NO) are well recognised for playing a dual role as both deleterious and beneficial species. ROS and RNS are normally generated by tightly regulated enzymes, such as NO synthase (NOS) and NAD(P)H oxidase isoforms, respectively. The high levels of ROS/RNS and their inadequate elimination by cellular defence mechanisms lead to oxidative/nitrosative stress [1]. Main consequences of the stress are damages of nucleic bases, lipids and proteins, which can seriously compromise the viability of the cell or induce different cellular responses through the generation of secondary reactive species, and ultimately lead to cell death by necrosis or apoptosis [2, 3]. Increased oxidative/ nitrosative stress is usually described as a condition in which the cellular protective antioxidants are insufficient to inactivate ROS/RNS. Under such a condition, organisms, that have elevated antioxidant protection systems, are more adaptive to oxidative stress (OS). Study of the physiological behaviour of marine organisms has shown that it is a valuable approach to assessing biological responses to environmental stress [4, 5]. Various animals and their chemical components or molecular, biochemical and/or physiological properties are used as bioindicators of marine pollution [6-8]. In recent years, mussels such as *Mytilus galloprovincialis* are commonly used as bioindicators for environmental monitoring [9, 10]. These mussels are known to accumulate high levels of trace metals and organic compounds in their tissues, providing a time-integrated indication of environmental contamination with observable cellular and physiological responses [11]. They have a number of properties which make them useful sentinels for chemical pollution: they have a wide geographical distribution, are easy to collect and are abundant in estuarine waters, which are submitted to high contamination levels [12]. Moreover, mussels are sedentary, euryhaline and normally the dominant species in their habitats [13]. Although there have been numerous investigations on mussels (*M. galloprovincialis*) [6, 14, 15], there are few available

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studies of *R. venosa*, mainly due to its capacity to accumulate heavy metals. *R. venosa* is a sea snail with high ecological potential due to its high fertility, rapid growth rate and resistance to low salinity, high and low temperatures, water pollution and oxygen deficiency [16]. In fact, *R. venosa* is a very cruel predator with impact on both native and cultivated populations of oysters, clams and other molluscs which causes significant negative ecosystem changes [17]. Therefore, *R. venosa* has effective mechanisms of protection and survival but the biochemical characteristics of these processes are not well studied [18]. As a result of changes in the functioning of biochemical regulatory systems usually accompanied by the activation of active oxygen generation (ROS) processes or the reduction of antioxidant activity, the risk of oxidative stress is increased in marine organisms [19]. Adaptation of hydrobionts is facilitated by their high polymorphism, as well as a well-developed antioxidant system that can serve as an indicator of the physiological state of marine organisms. Certain properties of the marine life antioxidant system have been investigated with pelagic fish and bivalve molluscs near the bottom, whereas gastropod molluscs, including *R. venosa*, have not been studied in this regard.

The aim of this research is to elucidate the radical mechanisms for protection and survival of *R. venosa* and *M. galloprovincialis* during spring and summer by tracking levels of some real-time oxidative stress biomarkers using EPR spectroscopy.

EXPERIMENTAL

Sample collection and preparation

Mussels (*Mytilus galloprovincialis*) and veined rapa whelks (*Rapana venosa*) were collected in Varna Bay in the Bulgarian Black Sea coast by divers during the spring and summer periods in 2016. After immediate transportation to the laboratory, the shells of the organisms were measured for appropriate size selection, then carefully removed and the whole of the soft tissue from 30 specimens of each species was immediately washed in cold saline, homogenates were prepared and centrifuged at 3000 rpm for 15 min. After centrifugation, the samples were studied by EPR spectroscopy for their radical scavenging abilities.

Electron paramagnetic resonance (EPR) studies

EPR measurements were performed at room temperature on an X-band EMX^{micro}, Bruker, Germany, equipped with standard Resonator. The experiments were carried out in triplicate. Spectral processing was performed using Bruker *WIN-EPR* and *Simfonia* software.

EPR ex vivo evaluation of the levels of ROS products

The levels of ROS were determined according to [20] with modification by [21]. To investigate real-time formation of reactive oxygen species (ROS) in the samples EPR spectroscopy combined with *ex vivo* PBN spin trapping was used. The spin-trap PBN, upon reaction with unstable radicals forms a relatively stable spin adduct that can be subsequently detected by EPR spectroscopy. Briefly, to 100 μl sample 900 μl of 50 mM PBN dissolved in DMSO was added and after centrifugation at 4000 rpm for 10 min at 4 °C, the spectrum of the supernatant was recorded. The levels of ROS products were calculated as double integrated plots of spectra and results were expressed in arbitrary units. EPR settings were as follows: 3503.73 G center field, 20.00 mW microwave power, 5G modulation amplitude, 50 G sweep width, 1×10^5 gain, 81.92 ms time constant, 125.95 s sweep time, 5 scans per sample.

EPR ex vivo evaluation of the levels of •NO radicals

Based on the methods published in [22, 23] we developed and adapted the EPR method for evaluation the levels of •NO radicals in the sample. Briefly, a 50 μM solution of Carboxy.PTIO.K was dissolved in a mixture of 50 mM Tris (pH 7.5) and DMSO in a ratio of 9:1. To 100 μl sample was added 900 μl Tris buffer dissolved in DMSO (9:1) after that the mixture was centrifuged at 4000 rpm for 10 min at 4 ºC. 100 μL of sample and 100 μL of 50 mM solution of Carboxy.PTIO were mixed and spectra of spin-adduct formed between the spin trap Carboxy.PTIO and the generated •NO radicals were recorded. The levels of •NO radicals were calculated as double integrated plots of spectra and results were expressed in arbitrary units. The EPR settings were as follows: 3505 G centerfield, 6.42mW microwave power, 5G modulation amplitude, 75 G sweep width, 2.5×10^2 gain, 40.96 ms time constant, 60.42 s sweep time, 1 scan per sample.

EPR ex vivo evaluation of the levels of Asc•

Endogenic ascorbic acid can be oxidized by ROS to a stable Asc• and the latter can be detected by direct EPR spectroscopy which is the only method not interfering with the biochemical processes. The levels of Asc• were studied according to [24] with modification. Briefly, the homogenates were prepared in DMSO in a ratio of 1:3. After centrifugation at 4000 rpm for 10 min at 4°C the

supernatants were collected and immediately transferred into quartz tubes and placed in EPR cavity. The levels of Asc• were calculated as double integrated plots of EPR spectra and results were expressed in arbitrary units. EPR settings were as follows: 3505.00 G center field, 20.00 mW microwave power, 1.00 G modulation amplitude, 15 G sweep width, 1×10^5 gain, 40.96 ms time constant, 60.42 s sweep time, 10 scans per sample.

Statistical analysis

Statistical analysis was performed with Statistica 7, StaSoft, Inc. The results were expressed as means ± S.E. Statistical analysis was performed with Student's t-test. $p\leq0.05$ was considered statistically significant.

RESULS AND DISCUSSION

It is known that mollusks and other aquatic invertebrates are characterized by metabolic rhythm transformations induced by biotic and abiotic factors. Depending on the availability of nutrients, reproductive status, growth rate related with season and other factors, (temperature, salinity, oxygen, chemical pollution, etc.) the activity of antioxidant defense enzymes and other biomarkers fluctuates significantly throughout the year [9]. The annual cycle of *R. venosa* is divided into four periods: spring - pre-spawning period, summer - reproductive period, autumn - sexual rest period and wintering rest period. For each period of the annual cycle *R. venosa* is characterized by a different intensity and direction. In the current study, we explored and compared for the first time to our knowledge the activity of some real-time biomarkers of OS, namely ROS products, NO products and Asc radicals during two seasons – spring and summer, using EPR technique.

Results from the study of seasonal ROS products in *R. venosa* compared to mussels *M. galloprovincialis* are shown in Fig. 1. As seen, during spring there are statistically higher levels of ROS products found in *R. venosa* compared to mussels *M. galloprovincialis* (mean 1.38± 0.01 *vs.* 0.967± 0.02, $p<0.05$; t-test). During summer, in both *R. venosa* and mussels *M. galloprovincialis* statistically significant higher levels of ROS products were found compared to the same groups during spring (for Rapana: mean 1.77±0.05 *vs.* 1.38±0.04, $p<0.05$; and mean 1.497±0.01 *vs.* 0.967±0.02, for mussels *M. galloprovincialis*, respectively, $p<0.07$; t-test).

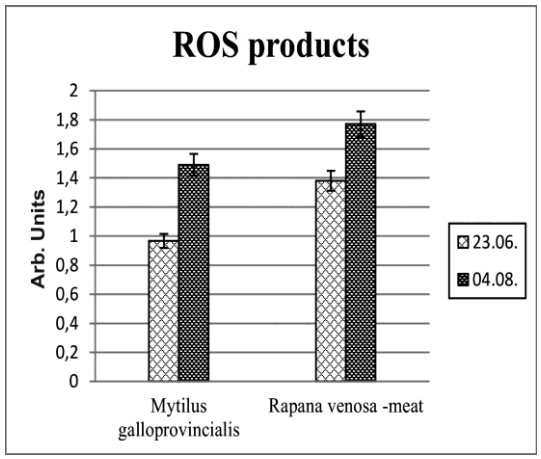

Figure 1. Levels of ROS products expressed in arbitrary units

The obtained result indicates significantly higher OS during the summer for both *R. venosa* and mussels *M. galloprovincialis* that is probably due to the reproductive cycle, increased in this period before spawning and furthermore oxidative processes available in the tested animals at the time of the experiment. We report about 20% increase in ROS production in *R. venosa* for the reproductive period compared to the same group before the reproductive period, which means, *R. venosa* were about 20% more sensitive to OS. Moreover, for both seasons the levels of ROS products in *R. venosa* were significantly higher compared to mussels *M. galloprovincialis*. Hence, the first evidence of the greater sensitivity to oxidative stress of *R. venosa* are the statistically higher levels of ROS products found in *R. venosa* compared to *M. galloprovincialis* during the summer.

Further confirmation that oxidative processes take place in real-time are the results obtained for the levels of seasonal NO measured in *R. venosa* compared to mussels *M. galloprovincialis* for both seasons which were similar to those for ROS radicals (Fig. 2). As is seen, NO radicals in *R. venosa* are higher, although not statistically significant, than those of *M. galloprovincialis* during both spring and summer periods (for spring: mean 18.53±1.94 *vs.* 17.28±0.98, $p<0.00$; for summer: mean 19.99±2.05 vs. mean 19.58±1.57, $p<0.7$; t-test). NO radicals in *R. venosa* are higher, although not statistically significant. than those of *M. galloprovincialis* during both spring and summer periods.

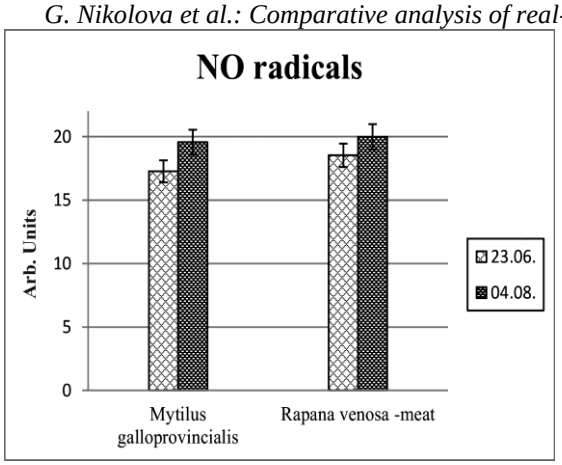

Figure 2. Levels of •NO expressed in arbitrary units

It is known that under OS, ascorbate is a much more effective antioxidant than the protein thiols, a-tocopherol, bilirubin, etc. [25]. In biological systems ascorbic acid acts as a chain breaking antioxidant delivering electrons to neutralize the excess of toxic radical species such as ROS. Pathologically, the generated ROS are able to oxidize endogenic ascorbic acid to a stable radical structure (Asc•) which can be detected by direct EPR, the only method that does not interfere with the biochemical processes [26]. The relatively long lifespan of Asc• makes it a suitable natural indicator for assessing oxidative stress in living organisms in real time [27-29]. In the present study, the elevated levels of Asc radicals established in both groups *R. venosa* and *M. galloprovincialis* during the summer were in accordance with the elevated levels of ROS products measured in the same groups. Since Asc radicals and ROS products are OS biomarkers and moreover, they are radical structures registered by EPR in real-time, it is evident that during the summer oxidative processes still take place in both groups - *R. venosa and M. galloprovincialis* throughout the study. Moreover, during summer, statistically higher level of Asc radicals were measured in *R. venosa* compared to *M. galloprovincialis*. This result is in agreement with the result for ROS production.

Seasonal measurement of Asc• in both tested groups *R. venosa* and *M. galloprovincialis* during summer as compared to the same groups during spring is shown in Fig. 3. Moreover, we report about 30% increase in radicals Asc• in *R. venosa* for the reproductive period compared to *M. galloprovincialis* which means that *R. venosa* are about 30% more adaptive to OS. During the summer, in both *R. venosa* and *M. galloprovincialis* statistically significant higher levels of Asc• were found compared to the same groups during spring (mean 0.32±0.03 *vs.* 0.162± 0.04, for *R. venosa* and mean 0.23±0.01 vs. 0.16 ± 0.02, for *M. galloprovincialis*, correspondingly, P < 0.001, t-test). However, during the summer, the levels of ascorbate radicals measured in *R. venosa* were significantly higher compared to *M. galloprovincialis* (p<0,00).

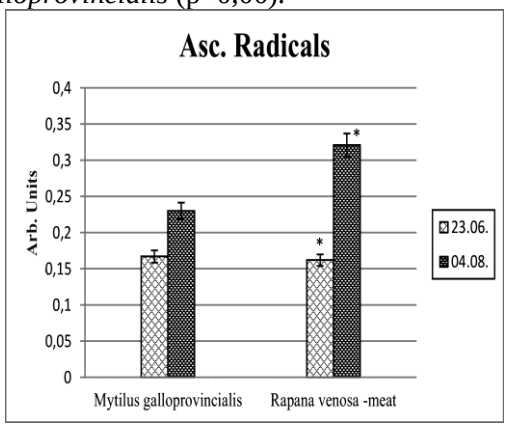

Figure 3. Levels of Asc• radicals expressed in arbitrary units

Since Asc• measured in *R. venosa* during summer is about 1.5 times higher compared to the same during spring, one might suggest that *R. venosa* are more adaptive to OS than *M. galloprovincialis* and the reproductive period could play an important role in the development of adaptive response to OS. The Gulf of Varna, the second largest bay on the Bulgarian Black Sea coast, is subject to many anthropogenic loads (chemical industry, shipping, tourism, fishing, urban pressure, etc.), resulting in a serious deterioration in the ecological quality of the area [5]. Chemical differences between *R. venosa* and mussels *M. galloprovincialis* from polluted and nonpolluted sites of the Bulgarian Black Sea coast were studied in [5], in order to use them as additional bioindicators of environmental ecological quality.

The authors obtained that antioxidant values for mussels from polluted area were significantly higher than for nonpolluted samples, as found in a number of previous reports [3, 4, 17, 20]. *R. venosa* samples react in the same way: the antioxidant characteristics in polluted samples and overall antioxidant activities were significantly higher than in non-polluted samples ($p < 0.05$). Comparison of the changes seen in two animals from polluted and non-polluted areas showed that mussels were more sensitive to pollution.

These results demonstrated that alterations in antioxidant enzymes reflected the gradient of contamination, confirming the rational use of biomarkers of oxidative stress in biomonitoring aquatic metal pollution. Despite its widespread use, the lack of detailed knowledge about variability in species-specific responses to different pollutants is still a limitation of the biomarker approach.

Differences in the sensitivity of various organisms are expected because no single species could be the most suitable ones for detecting all possible pollutants [7]. Bioindicators are properties of living organisms and as such may be affected by periodic changes in environmental factors (such as light, temperature, dissolved oxygen and pollutants) and changes in biological functions (e.g., metabolic rate or reproduction cycles, environmental changes).

In conclusion, our results showed that changes in oxidative/antioxidant status may reflect the gradient of contamination, confirming the rational use of biomarkers of oxidative stress in biomonitoring of contamination. *R. venosa* has effective biochemical mechanisms of protection and survival, in particular a strong antioxidant system that provides this type of high adaptability and survival against OS.

CONCLUSION

Our results show that changes in oxidative/antioxidant status may reflect the gradient of contamination, confirming the rational use of biomarkers of oxidative stress in biomonitoring of contamination. *R. venosa* has effective biochemical mechanisms of protection and survival, in particular a strong antioxidant system that provides this type of high adaptability and survival against OS.

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REFERENCES

1. M. Valko, C.J Rhodesb, J. Moncola, M. Izakovica, M. Mazura, *Chem-Biolog. Interact*., **160**, 1 (2006).
2. V. Gadjeva, Academic Publ. "Trakia University", *Stara Zagora* (2007).
3. E. Birben, U.M. Sahiner, C. Sackesen, S. Erzurum, O. Kalayci, *World Allergy Organ. J.* **5**, 9 (2012).
4. R.J. Orth, T.J. Carruthers, W.C. Dennison, C.M. Duarte, J.W. Fourqurean, K.L. Heck, A.R. Hughes, G.A. Kendrick, W. J. Kenworthy, S. Olyarnik, F.T. Short, *AIBS Bulletin,* **56**, 987 (2006).
5. S. Moncheva, J. Namiesnik, R. Apak, P. Arancibia-Avila, F. Toledo, S.G. Kang, S.T. Jung, S. Gorinstein, *Chem. Ecol*., **27**, 31 (2011).
6. S. Gorinstein, P. Arancibia-Avila, S. Moncheva, F. Toledo, S. Trakhtenberg, A. Gorinstein, I. Goshev, J. Namiesnik, *Environ. Int.*, **32**, 95 (2006).
7. Q. Zhou, J. Zhang, J. Fu, J. Shi, G. Jiang, *Anal. Chim. Acta*, **606**, 135 (2008).
8. A. Merdzhanova, D.A. Dobreva, M. Stancheva, L. Makedonski, *Analele Universitatii "Ovidius" Constanta-Seria Chimie,* **25**, 15 (2014).
9. T. Vlahogianni, M. Dassenakis, M.J. Scoullos, A. Valavanidis, *Marine Poll. Bull.,* **54**, 1361 (2007).
10. A. Hamza-Chaffai, *Int. J. Biotech. Well. Ind.,* **3**, 19 (2014).
11. A.A. Lachance, R. Hennebicq, B. Myrand, J.M. Sévigny, E. Kraffe, Y. Marty, I. Marcotte, R. Tremblay, *Aqu. Living Res*., **24**, 283 (2011).
12. G. Frenzilli, R. Bocchetti, M. Pagliarecci, M.Nigro, F. Annarumma, V. Scarcelli, D. Fattorini, F.Regoli, *Mar. Environ. Res.,* **58**, 609 (2004).
13. D. Sheehan, A. Power, *Comp. Biochem. Physiol. C.,* **123**, 193 (1999).
14. B. Laffon, T. Rábade, E. Pásaro, J. Méndez, *Environ. Int.,* **32**, 342 (2006).
15. J. Namiesnik, S. Moncheva, Y.S. Park, K.S. Ham, B.G. Heo, Z. Tashma, E. Katrich, S. Gorinstein, *Chemosphere,* **73**, 938 (2008).
16. J.M. Harding, R. Mann, P. Moeller, M.S. Hsia, *J. Shellfish Res*., **28**, 363-367 (2009).
17. I.P. Bondarev, *Int. J. Mar. Sci.,* **4**, 42 (2014).
18. T. Catherine, M. Vanessa, S. Evangelia, C. Valentina, R. Andreja, A.A. Rana, C. Susana, F. Serena, K. Alisa, L. Yiota, H. Ioannis, *Environ. Sci. Poll. Res.,* **23**, 1789 (2016).
19. D. Wilhelm-Filho, Cg. Fraga, A. Boveris, *Comp. Biochem. Physiol. C. Toxicol. Pharmacol.,* **199**, 90-97 (2017).
20. H. Shi, Y. Sui, X. Wang, Y. Luo, L. Ji, *Physiol. C. Toxicol. Pharmacol*., **140**, 115 (2005).
21. A. Zheleva, Y. Karamalakova, G. Nikolova, R. Kumar, R. Sharma, V. Gadjeva, *Biotechnol. Biotechnol. Equip*., **26** (Suppl.1), 146 (2012).
22. K. Yokoyama, K. Hashiba, H. Wakabayashi, *Anticanc. Res*., **24**, 3917 (2004).
23. T. Yoshioka, N. Iwamoto, K. Lto, *J. Am. Soc. Nephrol*., **7**, 961 (1996).
24. D.M. Bailey, K.A. J. Evans, P.E. Mceneny, J. Young, I.S. Fall, L. Gutowski, M. Kewley, E. Mccord, J.M. Møller, K. Ainslie, *J. Physiol.,* **587**, 73 (2009).
25. B. Frei, L. England, B.N. Ames, *Proceedings of the National Academy of Sciences,* **86**, 6377 (1989).
26. B. Halliwell, M. Whiteman, *Brit. J. Pharmacol*., **142**, 231 (2004).
27. H.,Loertzer, S. Bauer, W. Morke, P. Fornara, H.J. Bromme, *Transplant. Proc*., **38**, 674 (2006).
28. I. Spasojevic, M. Mojovich, A. Ignatovic, G. Bacic, *J. Serb. Chem. Soc.,* **76**, 647 (2011).
29. G. Nikolova, D. Ivanova, A. Zheleva, V. Gadjeva, *J. Chem. Biol. Phys. Sci. Sect. B: Biol. Sci.* **4**, 2294 (2014).

СРАВНИТЕЛЕН АНАЛИЗ НА БИОМАРКЕРИТЕ НА ОКСИДАТИВНИЯ СТРЕС В РЕАЛНО ВРЕМЕ, ИЗМЕРЕНИ ПРИ МИДИ (*Mytilus galloprovincialis*) И ЖИТНИ РАПАНИ (*Rapana venosa*) ПРЕЗ ДВА СЕЗОНА - ПРОУЧВАНЕ С ЕЛЕКТРОНЕН ПАРАМАГНИТЕН РЕЗОНАНС

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(Резюме)

Целта на изследването е да се изяснят радикаловите механизми за защита и оцеляване на миди (*Mytilus galloprovincialis*) и житни рапани (*Rapana venosa*) през пролетния и летния сезон, като се следят нивата на някои биомаркери на оксидативния стрес (ОС) в реално време. Тридесет екземпляра от всеки вид са анализирани чрез EPR спектроскопия. През пролетта има статистически по-високи нива на ROS продукти, намерени в *R. venosa* в сравнение с *M. galloprovincialis*. През лятото, както в *R. venosa*, така и в *M. galloprovincialis*, статистически значими са по-високите нива на ROS продукти в сравнение със същите групи през пролетта. NO радикалите в *R. venosa* са по-високи, въпреки че не са статистически значими спрямо тези на *M. galloprovincialis* през пролетния и летния период. През лятото в *R. Venosa и в M. galloprovincialis* статистически значими по-високи нива на аскорбатни радикали (Asc •) са установени в сравнение със същите групи през пролетта. През лятото обаче нивата на Asc•, измерени при *R. venosa*, са значително по-високи в сравнение с *M. galloprovincialis*. Нашите резултати показват, че промените в оксидативния/антиоксидантния статус могат да отразяват градиента на замърсяването, потвърждавайки рационалното използване на биомаркерите на оксидативния стрес за биомониторинг на замърсяването. *R. venosa* има ефективни биохимични механизми за защита и оцеляване, по-специално силна антиоксидантна система, която осигурява висока адаптивност и оцеляване срещу оксидативен стрес.

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Real time oxidative stress markers of patients with post-stroke depression: EPR study

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Depression is a common consequence of stroke. In the last few years, oxidative stress has been seen as one of the contributing factors in the pathogenesis of depression. Lately it has been discussed also as an accompanying factor in many chronic neurodegenerative pathologies, as well as in acute cerebrovascular disorders like stroke. The aim of our study is to investigate the role of oxidative stress in the etiopathogenesis of depressive disorders in post-stroke patients in order to optimize diagnostic, therapeutic and medical-social approaches. To achieve evaluating the level of oxidative stress in post-stroke depression we investigated the levels of ROS products, ascorbate (Asc•) and NO• radicals as real time oxidative stress biomarkers using electron paramagnetic resonance (EPR) spectroscopy.

Keywords: Oxidative stress, Post -stroke depression, ROS, RNS, Ascorbate radicals

INTRODUCTION

Approximately one third of stroke survivors experience significant symptoms of depression [1]. Post-stroke depression may have aetiology with many factors including organic and reactive components. Oxidative stress (OS) is involved in the pathogenesis of various diseases and may be a common pathogenetic mechanism responsible for many mental illnesses as the brain is more vulnerable to oxidative damage. In the last few years, oxidative stress is considered to be one of the contributing factors in the pathogenesis of post-stroke depression.

Disrupting the balance between the processes producing ROS and antioxidant defence systems, leads to the emergence of oxidative stress causing cellular damage and direct inhibition of enzyme proteins [2]. Oxidative stress is an imbalance between the biochemical processes that generate ROS and the ability of a biological system to neutralize them. As a result, ROS are formed faster than the ability of cellular defense systems to remove them [3]. The development of oxidative stress and its consequences depend on the ability of the organism alone, or with external help, to restore the physiological balance between pro-oxidants and antioxidants [4]. Certain neurological disorders, including stroke, are associated with oxidative and nitric modification of specific proteins and the accumulation of oxidative damage. There is some evidence indicating the involvement of oxidative and nitrate stress in the pathophysiology of depression [5]. Several studies have shown increased levels of reactive oxygen and nitrogen species in depression, mainly peroxide [5] nitric oxide [6], and lowered levels of antioxidants, such as glutathione (GSH), in the brain of deceased patients with depression [7]. This is why oxidative and nitrogen mechanisms have been proposed as targets for new antidepressants [8]. The aim of the present study was to evaluate the oxidative/antioxidant status in post-stroke depression through the levels of ROS products, ascorbate (Asc•) and NO• radicals as real time oxidative stress biomarkers using electron paramagnetic resonance (EPR) spectroscopy.

MATERIALS AND METHODS

In our study were included 93 patients hospitalized in the Neurological Clinic, diagnosed with a stroke, according to the criteria of ICD10, in the first three days after the initial diagnosis was recorded in the medical documentation of the disease and neuro-imaging study (CAT or NMR). All studied parameters were compared with those of 32 controls healthy individuals. Informed consent was obtained from all post-stroke patients and healthy volunteers enrolled in this study, according to the ethical guidelines of the Helsinki Declaration (1964). To assess the severity of depressive disorder according to criteria of ICD10 we used the Hamilton Depression Rating Scale (HAM-D-17; Hamilton, 1960). The study was conducted in the form of an interview with the patient. Nine of the symptoms

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(low mood, feeling of guilt, suicide, retardation, activity and work, agitation, mental anxiety, somatic anxiety, hypochondriacity) are given from 0 to 4 points. Other eight symptoms (sleep disorders, disorders of sleep continuity, early awakening, gastrointestinal somatic symptoms, genital symptoms, general somatic symptoms, loss of weight, disease awareness) are given 0 to 2 points. The total score on the scale varies from 0 to 54 points, with 0 to 7 points objectively meaning lack of depression, from 8 to 13 points meaning mild depression, 14 to 18 points - medium depression, 19 to 22 - moderate depression and 23 to 54 points - severe depression. Fasting samples of venous blood were collected in the morning between 8.00 and 10.00 a.m. Blood for determination of NO• and ROS products was collected in tubes containing 10% EDTA (ethylenediaminetetraacetic acid). All samples from each subject were split and run in triplicate.

Ex vivo electron paramagnetic resonance (EPR) study

EPR measurements were performed at a temperature of 22°C on an X-band EMX^{micro}, spectrometer Bruker, Germany. The experiments were carried out in triplicate. Spectral processing was performed using Bruker WIN-EPR and *Sinfonia* software.

Ex vivo evaluation of the levels of ROS products

The ROS levels were determined according to [9] with modification. To investigate in real time formation of ROS in the sera of PD patients and controls *ex vivo* EPR spectroscopy was used combined with PBN as a spin-trapping agent.

Ex vivo evaluation the levels of Asc•

Endogenic ascorbic acid can be oxidized by ROS to a stable ascorbate radical and the latter can be detected by direct EPR method which does not interfere with the biochemical processes. The levels of Asc• were studied according to Bailey *et al.* [10], with some modification.

Ex vivo evaluation of the levels of •NO radicals

Based on the method published by Yoshioka *et al*. [11] the EPR method for estimation of the levels of •NO radicals in serum was developed and adapted.

RESULTS AND DISCUSSION

The development of post-stroke depression is often a mood disorder affecting about a third of the patients with stroke. In our study, depressive disorder was reported by two-thirds of the patients with stroke. Previous studies have established links between post-stroke depression and severe functional impairment [12]. In our results, the depressive disorder was concomitant in 60 (64.5%) of the patients with stroke.

The brain with its high oxygen requirements and lipid-saturated environment is considered to be highly sensitive to oxidative stress or redox imbalances. The oxidative stress is prone to occur in many mental disorders, including depression. Oxidative stress is a disturbance in the balance between the biochemical processes generating ROS and the capability of a biological system to deal with them.

The results from the levels of ROS in patients with stroke are given in Figure 1. We have discovered a statistically significant increase of ROS values in post-stroke patients with severe post-stroke depression (3.75±0.19 arb. units) compared to controls (0,62±0.11 arb. units), compared to non-depressed patients (2.44±0.2 arb. units) and compared to patients with moderate level of depression (2.56±0.2 arb. units), ($p<0.05$). There is also statistically significant increase in patients with moderate level of depression (3.19±0.2 arb. units) compared to the control group, post-stroke non-depressed patients and patients with mild depression ($p<0.05$). As a result, ROS products formed faster than their removal by the cellular defense systems [3]. Although some studies have established a link between oxidative stress and mental disorders, the causal relationship between them is not fully defined. Several mechanisms have been proposed, including genetic predisposition, disturbances in traditional signal transduction pathways, and oxidative stress participating in the pathogenesis of depression theory [13]. It has been proven that depression in somatically healthy patients is associated with elevated concentrations of blood cytokine [14] and increased levels of ROS and RNS [6, 7, 15]. Mechanisms associated with post-stroke depression may include an imbalance between pro-inflammatory and anti-inflammatory activity that is responsible for increasing oxidative stress and may in turn weaken cognitive sensitivity. Wei *et al*. [16] have reported an increase in ROS levels in a major depressive episode, causing immune dysregulation by suppressing T-cell responses.

The latest trend is that antidepressants have a therapeutic effect by suppressing the production of inflammatory cytokines, ROS and RNS, or even increasing antioxidant protection [9].

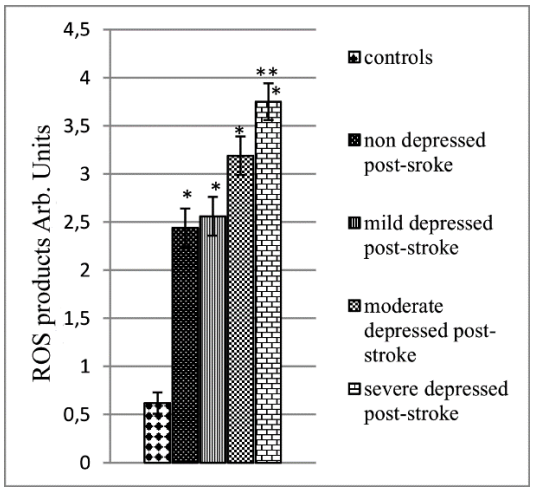

Fig. 1. Levels of reactive oxygen species in patients with stroke

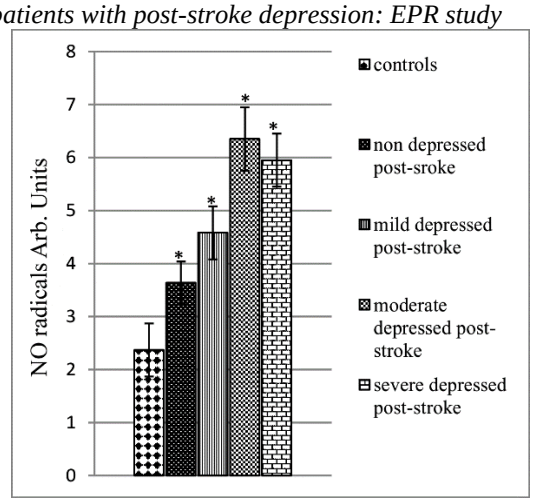

Fig. 2. Nitric oxide levels in patients with stroke

Accordingly, these studies place particular emphasis on the consideration of biochemical parameters that may have a direct impact on improving the functionality of patients in compliance with the severity of their depression.

Brain ischemia unlocks a complex cascade of metabolic events, most of which involve the formation of nitrogen and oxygen free radicals. Oxidative stress associated with inflammation or post-cerebral reperfusion may lead to oxidative nitrate modifications of the protein structure, resulting in changes in their functional properties. It can also lead to the accumulation of modified protein products that have been observed under various conditions such as aging, cell differentiation and apoptosis [17].

Nitric oxide levels in patients with stroke are given in Figure 2. The results show a statistically significant increase of levels of NO in patients with moderate depression (6.35±0.6 arb. units) compared to the control group (2.37±0.9 arb. units), non-depressed post-stroke patients (3.64±0.5 arb. units) and patients with mild depression (4.58±0.48 arb. units), ($p<0.05$), (Fig. 2). Statistically significant increase was detected also in patients with severe depression (5.95±0.5 arb. units) compared to the control group and patients with mild level of depression. ($p<0.05$).

In our study, we observed increases in NO levels in patients with post-stroke depressive disorder, with the highest levels in moderate and severe depressive disorders. According to research of Kudlow *et al*. [18] low concentrations of nitric oxide are neuroprotective and mediate physiological stimulation, whereas high concentrations mediate neuroinflammatory reactions and are neurotoxic.

They also point out that the increased concentrations of nitric oxide increase the synthesis of reactive nitrogen species (RNS) and reactive oxygen species (ROS). Although the clinical benefit of NO-synthase polymorphisms has not yet been determined, there is a possibility of treating a major depressive disorder with pharmacological agents by reducing NO levels. Evidence suggests that many current antidepressants, initially thought to primarily act on neurotransmitters, can actually be mediated by normalizing NO levels by affecting several interconnected pathways (e.g., microglial activity [19] or phosphodiesterase enzyme activity [20]. Perhaps the three-pronged approach by which traditional antidepressants are used in combination with pharmacological agents aimed at normalizing NO and inflammatory signaling pathways will turn out to be the most appropriate one. It should be emphasized that despite polymorphisms in the enzyme NO synthase can identify significant subpopulation; these variants are unlikely to be a sufficient or necessary condition for the induction of a major depressive disorder. Rather, some polymorphisms in NO synthase enzymes may be involved in a larger model of vulnerability. Vulnerability may include multiple factors (e.g., genetic, epigenetic, environmental factors) that contribute to a comprehensive patho-etiological model explaining why some people are more susceptible and others less prone to depressive symptoms [21].

Statistically significant increase of ascorbate radicals can be seen in non-depressed patients (0.44±0.09 arb. units) and patients with mild depression (0.46±0.2 arb. units) compared to the control group (0.31±0.04 arb. units), ($p<0.05$), (Figure 3). On the other hand, with the increase in the severity of depression, levels of ascorbate radicals decline. Statistically significant decrease compared to the control group, non-depressed

patients and patients with mild depression, can be seen in patients with severe depression (0.21±0.09 arb. units), ($p<0.05$). Patients with moderate depression (0.28±0.05 arb units) display a statistically significant decrease compared to non-depressed and mildly depressed patients ($p<0.05$).

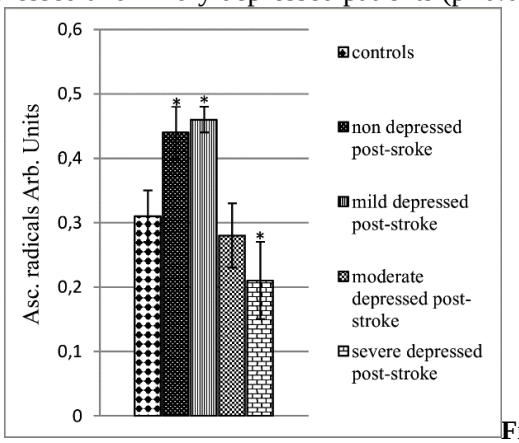

Fig. 3. Levels of ascorbate radicals in patients with stroke

There are reports that the major depressive disorder is associated with decreased levels of ascorbate radicals [22]. This is confirmed by a study of vitamin C concentration and symptoms of depression in adult patients (over 65 years of age) [28]. We observed that in heavier degrees of depression the level of ascorbate radicals is lower than in lower degrees of depression, therefore is the lowest possible level of oxidative stress compensation. For this reason, the complex therapy of depression recommends an intake of vitamin C. Ascorbate radicals and ROS products have been studied in patients with ischemic stroke using EPR [23]. The use of vitamin C as a substitution therapy has been studied by many authors, but the results are contradictory. Sahranian *et al.* [24], in a double-blind study found that adding vitamin C to citalopram did not increase its effectiveness in a major depressive episode. Conversely, a study of [25] shows positive results from the addition of vitamin C to fluoxetine during a major depressive episode in infant ages. More extensive studies are needed to expand the evidence of oxidative stress in post-stroke depression and to establish a potential therapeutic approach.

CONCLUSION

The levels of reactive oxygen species (ROS) and nitric oxide (NO) generation in post-stroke patients are dependent on the presence of a depressive disorder and its severity. The smallest opportunity to cope with oxidative stress is found in heavier degrees of depression due to low levels of ascorbate radicals. Because of these results, anti-depressant antioxidants such as vitamin C, coenzyme Q, omega 3, etc., should also be involved in the complex treatment of depression with antidepressants.

REFERENCES

1. M. Hackett, C. Yapa, V. Parag, C. Anderson, *Stroke*, **36**, 1330 (2005).
2. S. Chopra, H.M. Wallace, *Biochem. Pharmacol.*, **55**, 1119 (1998).
3. V. Gadjeva, Monography, *University Press, Stara Zagora*, 2007.
4. J. Himmelfarb, R. Hakim, *Curr. Opin. Nephrol. Hypertens*., **12**, 593 (2003).
5. M. Maes, P. Galecki, Y. Chang, M. Berk. *Progress in Neuro-Psychopharmacology and Biological Psychiatry*, **35**, 676 (2011).
6. H. Suzuki, M. Colasanti, *BioFactors*, **15**, 123 (2001).
7. J.W. Gawryluk, J.F. Wang, A.C. Andreazza, L. Shao, L.T. Young, *Int. J. Neuropsychopharm.*, **14**, 123 (2011).
8. S.Y. Lee, S.J. Lee, C. Han, A.A. Patkar, P.S. Masand, C.U. Pae, *Progress NeuroPsychopharm. Biol. Psychiatry*, **46**, 224 (2013).
9. H. Shi, Y. Sui, X. Wang, Yi. Luo, L. Ji, *Comp. Biochem. Physiol. Part C: Toxicol. Pharmacol.*, **140**, 115 (2005).
10. D.M. Bailey, K.A. Evans, P.E. James, J. McEneny, I.S. Young, L. Fall, M. Gutowski, E. Kewley, J. McCord, K. Møller, P. Ainslie, *J. Physiol.*, **587**, 73 (2009).
11. T. Yoshioka, N. Iwamoto, K. Lto, *J. Am. Soc. Nephrol.*, **7**, 961 (1996).
12. M. Kauhanen, J. Korpelainen, P. Hiltunen, E. Brusin, H. Mononen, R. Määttä, P. Nieminen, K. Sotaniemi, V. Myllylä, *Stroke*, **30**,1875 (1999).
13. F. Ng, M. Berk, O. Dean, AI. Bush, *Int. J. Neuropsychopharm.*, **11**, 851 (2008).
14. Y. Dowlati, N. Herrmann, W. Swardfager, H. Liu, L. Sham, E. Reim, K.L. Lanctôt, *Biol. Psychiatry*, **67**, 446 (2010).
15. M. Maes, *Prog. Neuropsychopharmacol. Biol. Psychiatry*, **35**, 784 (2011).
16. J. Wei, M. Zhang, J. Zhou, *Psychiatry Res.*, **228**, 695 (2015).
17. K. Uchida, E.R. Stadtman, J. *Biol. Chem.*, **268**, 6388 (1993).
18. P. Kudlow, D. Cha, A. Carvalho, R. McIntyre, *Curr Mol. Med.*, **16**, 206 (2016).
19. D. Liu, Z. Wang, S. Liu, F. Wang, S. Zhao, A. Hao, *Neuropharmacology*, **61**, 592 (2011).
20. G. Reierson, S. Guo, C. Mastronardi, J. Licinio, M. Wong, *Curr. Neuropharmacol.*, **9**, 715 (2011).
21. P. Kudlow, D Cha, R.W. Lam, R.S. McIntyre, *Sleep Med.*, **14**, 943 (2013).
22. S.D. Khanzode, G.N. Dakhale, S.S. Khanzode, A. Saoji, R. Palasodkar, *Redox Rep.*, **8**, 365 (2003).
23. S. Gariballa, *Int. J. Vitam. Nutr. Res.*, **84**, 12 (2014).
24. A. Sahraian, A. Ghanizadeh, F. Kazemeini, *Trials*, **16,** 94, 2015.
25. M. Amr, A. El-Mogy, T. Shams, K. Vieira, S.E. Lakhan, *Nutr. J.* **12**, 31 (2013).

МАРКЕРИ В РЕАЛНО ВРЕМЕ ЗА ОКСИДАТИВЕН СТРЕС ПРИ ПАЦИЕНТИ С ПОСТ-ИНСУЛТНА ДЕПРЕСИЯ

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(Резюме)

Депресията е обичайно следствие при инсулт. През последните години оксидативният стрес се счита за един от факторите, допринасящи към патогенезата на депресията. Напоследък се дискутира, че той е придружаващ фактор при много навродегенеративни патологии, както и при акутни мозъчно-съдови нарушения, например инсулт. Целта на нашето изследване е да се изучи ролята на оксидативния стрес в етиопатогенезата на депресивни нарушения при пациенти след инсулт с оглед да се оптимизират диагностичните, терапевтичните и медико-социалните подходи. За оценка на нивото на оксидативния стрес при след-инсултна депресия са изследвани нивата на продуктите на реактивни кислородни форми (ROS), аскорбатни (Asc•) и NO• радикали като биомаркери на оксидативния стрес в реално време с използване на EPR спектроскопия.

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A semiquinone glucoside derivative (SQGD) isolated from *Bacillus sp.* INM-1 as a provider of antioxidant protection to male mice against CCNU-induced oxidative toxicity

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Present investigation was focused on evaluation of a semiquinone glucoside derivative (SQGD) isolated from radioresistant bacterium *Bacillus sp. INM-1* for its activity against CCNU- induced oxidative stress in healthy mice. Mice were divided into four groups, i.e., (I) untreated controls; (II) SQGD treated (40 mg/kg b. wt. i.p.) mice; (III) CCNU (40 mg/kg b.wt., i.p.); and (IV) SQGD (40 mg/kg b.wt., i.p.) administered 1 h prior to CCNU-administration (40 mg/kg b.wt., i.p.). Following treatment, liver homogenates and blood serum of the treated animals were subjected to ascorbate radical levels estimation and ROS production. Results indicated that SQGD+CCNU administration significantly ($p< 0.05$) reduced ascorbate radicals and ROS products in the liver and blood serum of mice as compared with CCNU-treated group. Reduction in oxidative disorders was observed in healthy mice which were treated with SQGD only, compared with controls. Further, the maximal concentration of free SQGD (a.u.) in the blood-flow was established at 30 min after i.p., and completely reduced after 240 min. The pharmacokinetic profile of free SQGD showed significant selective accumulation, mostly in liver and lungs (60 min), brain (90 min), followed by kidney, pancreas, spleen, blood and testicles. Thus, it can be concluded that SQGD treatment alone and in combination SQGD+CCNU neutralized oxidative toxicity caused by medicines not only by reducing lipid peroxidation but also by improving antioxidant status of organs and blood, and this effect may emphasize SQGD as a strong radical-scavenger and excellent natural protector.

Keywords: SQGD, CCNU, Pharmacokinetic profile, Lipid peroxidation, Protection

INTRODUCTION

Lomustine (CCNU) is a cytotoxic chemotherapeutic antitumor drug with an alkylating effect. Its mechanism of action involves the alkylation of DNA and RNA matrices, as well as the inhibition of key enzyme processes by altering the structure and function of many proteins and enzymes [1]. The main drawback limiting the clinical use of CCNU is the damaging effect of the gastrointestinal tract and the delayed cumulative dose-dependent hepatotoxicity [2]. Metabolism of CCNU results in the production of nitric oxide (NO•), hydrogen peroxide (H_2O_2), hydroxyl radicals (OH•) or peroxynitrite ($ONOO^-$), thus increasing oxidative toxicity in the body by generating reactive oxygen species (ROS) [3-5]. Excessive use of medicines increases the generation of OH• and leads to a rapid initiation of lipid peroxidation, overproduction of lipids (L•), lipid peroxyl (LOO•) and hydroperoxides (LOOH•) [6].

A series of radical reactions changes the functionality of biomembranes and generates potential toxic products of lipid peroxidation [7]. Therefore, it is important to look for protective agents [8] that can overcome the toxicity caused by drugs and prevent lipid peroxidation in models *in vivo* and *in vitro*. From this point of view, we hypothesized that radioresistant microbial species that survive in an extreme radiation environment can synthesize new secondary metabolites with better adaptability in the presence of recorded oxidative disorders and be used as suitable protectors [8,9], neutralizing CCNU-toxicity. For this purpose, the new semiquinone glucoside derivative SQGD (deposited in NCBI gene bank with accession number EU 240544.1), isolated from the fermented broth of *Bacillus Sp. INM-1* [10], was evaluated for its protective activity against the modulation of oxidative lesions in liver and blood serum in male RCS/b mice. A number of authors commented on the antioxidant [10,11] and immuno-suppressive properties [12], as well as the radio protective activity of SQGD to the reproductive, gastrointestinal [8,13], and renal systems [14].

The present study focuses on blood clearance/ serum resorption and organ bio-distribution of the natural protector SQGD investigated by electron paramagnetic resonance (EPR) assays. Oxidative toxicity in the body and ROS generation induced by

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CCNU treatment and their redox modulation after combination with SQGD were also analyzed by examining levels of ascorbate radicals and residual lipid peroxide products in *ex vivo* models.

EXPERIMENTAL

Materials and instrumentation

N-cyclohexyl-N-(2-chloroethyl)-N-nitrosourea (CCNU); 0.1M phosphate buffer saline; ярin-trap *n-tert*-butyl-alpha-phenylnitrone (PBN), dimethylsulfoxide (DMSO) were purchased from Sigma Chemical Co, St. Louis, USA. Ethanol was purchased from Haymann Chemicals, Essex, England.

EPR measurements were performed on an X-band EMX^{micro}, spectrometer (Bruker, Germany), equipped with a standard resonator. Experiments were carried out in triplicate. The EPR spectrum was immediately registered at room temperature (305 K) in the organs and blood plasma/ serum (expressed in arbitrary units) with following settings: 3505 g centerfield, 6.42 mw microwave power, 5-10 G modulated amplitude, 1-5 scans. All experiments were made in triplicate and results were calculated by double integration.

Characterization of SQGD

Characterization (strong, single symmetrical signal, g= 2.0056 ± 0.0002 registered in powder) of SQGD was carried out using EPR spectroscopy [15] and other extraction procedures described previously [10, 12]. The type strain (SQGD) (*Bacillus* sp. INM-1, MTCC No. 1026, IBG-21) was deposited at the Institute of Microbial Technology, Chandigarh and INMAS, Delhi, India as a reference.

Animals

Non-inbred 6 weeks old (26–30 g of body weight, specific pathogen-free, second line) male RCS/b mice maintained at the Vivarium, Medical Faculty, Stara Zagora, Bulgaria were used in the study. Mice were maintained under standard conditions (20-22ºC and 12 h light/12 h dark) on animal feed, water *ad libitum* and housed in groups of 6 in polypropylene cages at standard humidity of 40–60%. The regulations and rules of Research Ethics Commission of the Medical Faculty, Trakia University and the European directive 210/63/EU from 22.09.2010, were strictly followed during the experimental process.

Treatment groups

Mice were divided into the following four groups: *Group I* (n = 6): Control mice treated with 300 μL of cold saline intraperitoneal (i.p.); *Group II* (n = 6): SQGD (40 mg/kg b.wt., i.p.); *Group III* (n = 6): CCNU (40 mg/kg b.wt., i.p.); *Group IV* (n = 6): SQGD (40 mg/kg b.wt., i.p.) administered 1 h prior to CCNU-administration (40 mg/kg b.wt., i.p.). Upon completion of i.p. injection with SQGD, the different groups of animals were dissected after 10, 30, 60, 90, 240, 1440 min and subjected to EPR blood clearance and organ/ blood biodistribution assays. The complete set of experiments was repeated three times. The presented data are averaged by three independent experiments.

EPR blood clearance experiments

Blood resorption and SQGD toxicity were assessed after 10, 30, 60, 90, 240, 1440 min in blood samples taken from free-flowing blood and collected in heparinized tubes containing cold PBS (pH= 7-7.4). The presence of SQGD (determined in arbitrary units) was calculated by double integration of the plot of the registered spectrum and the g-factors were measured.

EPR ex vivo organ/tissue and blood distribution

Different groups of animals were decapitated at appropriate time points following injection (10, 30, 60, 90, 240, 1440 min) and dissected. 80-100 mg samples of lungs, liver, spleen, brain, kidneys, pancreas, testicles and blood were collected and kept in 0.1M phosphate buffer saline (10% w/v) and homogenized immediately using a hand-held homogenizer. The tissue homogenate was centrifuged at 2000×g for 15 min. Supernatant separated from tissue and blood was used for analysis. Biodistribution of SQGD (40 mg/kg; i.p.) was evaluated by EPR spectroscopy as described previously [16].

Ex vivo evaluation of ROS production

Liver tissue 100 mg (100 μl plasma) were homogenized with 900 μl of 50 mM spin-trap *n-tert*-butyl-alpha-phenylnitrone (PBN) dissolved in DMSO using a sonicator at one cycle for 1 min. After 5 min of incubation in ice, the suspension was centrifuged at 4000 rpm for 10 min at 4ºC. Supernatants were transferred into Eppendorf tubes and immediately analyzed. The real time formation of ROS products in the supernatant was estimated as described earlier [17] with some modifications [15].

Ex vivo evaluation of ascorbate radical levels

The method [18] was used to evaluate the ascorbate radical levels (Asc$^{\bullet}$) and the protection against CCNU-induced damage by SQGD treatment. In brief, 200 mg liver samples and 100 μl

plasma were homogenized in cold DMSO (10% w/v) and centrifuged at 4000 × g at 4°C for 10 min. Supernatants were transferred into Eppendorf tubes and immediately analyzed. The spin-adduct formed between DMSO and generated Asc• radicals was recorded in real time.

Statistical analysis

The data were presented as means ± standard error (SE) of three independent experiments. Statistical analysis was performed with Statistica 8.0, *Stasoft, Inc*. Level of significance $p < 0.05$ was considered as statistically significant.

RESULTS AND DISCUSSION

Oxidation-reduction processes involving oxygen, peroxides, hydroperoxides, etc., as well as many biochemical oxidation-reduction reactions in the body, form ROS that can exhaust the antioxidant protective system and lead to excessive damage to cellular membrane lipid structures, important enzymes, proteins and DNA. Redox processes are also involved in the metabolism of a number of drugs, which can also produce ROS and reactive nitrogen species (RNS). Redox processes also participate in the metabolism of a number of drugs that can also produce ROS and RNS. In order to protect biological molecules against drug toxicity, there is an increasing interest in the search for new natural antioxidants and combinations thereof for the purpose of disposing of ROS and RNS produced by drug therapy or radiation [19, 20].

In this study, a comprehensive assessment of SQGD was conducted against the potential for practical significance and application, the extent of diffusion and exclusion from different tissues and blood flow, reduction of oxidative stress levels, lipid peroxidation and protective properties against CCNU-induced toxicity. The results of the *ex vivo* blood clearance study of SQGD in mice are presented on Fig. 1. The maximum concentration of free SQGD (*arbitrary units*) in the blood serum was established 30 min after i.p. injection, and was almost completely reduced after 240 min. Significantly, the bacterial fraction has a short half-life and is rapidly and permanently absorbed by the bloodstream. In accordance with our results, Mishra *et al*. [21] found rapid serum resorption of SQGD, as well as significantly improved expression of G-CSF in sera from mice treated with the antioxidant alone.

Based on data demonstrating the selective accumulation of TEMPO and spin-labeled synthetic agents [16, 22] in certain organs in animals, we investigated and compared the distribution and the pharmacokinetic profile of SQGD by the EPR method.

The results (Fig. 2) showed that SQGD accumulates selectively, mostly in liver and lungs (60 min), brain (90 min), followed by kidney, pancreas, spleen and blood. It is obvious that SQGD, as a fraction isolated from bacteria surviving in extreme conditions, has a high antioxidant activity due to the radical structure of o-semiquinone, as evidenced by our group [10,15]. A stable radical function is also a probable cause of selective accumulation in the liver and brain, rapid elimination in the blood and suggests a low total toxicity [21] of the metabolite. Other authors [23] commented on the accumulation of SQGD in the liver as therapeutically acceptable, because it is a common metabolic organ.

Ascorbic acid undergoes long-lasting ROS modifications in the body, which leads to the formation of stable structures - ascorbate radicals (•Asc) [18]. They are identified as a good endogenous marker to prove the generation of toxic reactive radicals in the body. To confirm the efficacy of SQGD in neutralizing oxidative toxicity generated by drug agents such as CCNU, ascorbate radical levels in liver and blood serum were evaluated. The results clearly demonstrate the immunosuppressive toxic effects of CCNU: 2 hours after injection an almost four-fold increase of ascorbate radicals levels in liver was registered (mean 1.108 ± 0.093 *vs* mean 0.308 ± 0.06, *$p<0.005$), and serum (mean 0.769 ± 0.08 vs mean 0.179 ± 0.006; *$p<0.05$) relative to the controls, (see Fig. 3). In contrast, the oxidative status in mice treated with SQGD alone in the liver (0.291 ± 0.007, *$p<0.00001$) and in blood serum (0.0997 ± 0.0006, *$p<0.001$) was almost commensurate with that of the controls. The combination of SQGD + CCNU resulted in a 2.4-fold decrease in Asc• values in both studied organs (**$p<0.00003$). It was reported the increased cellular antioxidant activity of SQGD in the liver and the ability of the metabolite to overcome oxidative disorders generated by toxic agents such as H_2O_2 [23]. Additional *ex vivo* studies [21] have shown that SQGD as a potent M-CSF inducer exerts immune-stimulatory effects and protects not only liver oxidative disorders but also similar in blood serum.

Ex vivo demonstration of short-living free radicals is accomplished by the use of suitable spin-accelerators that react with radical structures to form stable products (spin-adducts). The latter are suitable for EPR analyses [24]. In the present study, PBN was used as a spin-trap dissolved in DMSO to investigate the lipid peroxidation levels (ROS products in aerobic conditions), simultaneously in

liver and serum (Fig. 4). The results revealed a statistical increase in ROS products in the liver (mean 1.683 ± 0.017 *vs* 0.697 ± 0.06, *p <0.005) and serum (1.844 ± 0.11 *vs* 0.821 ± 0.02, *p <0.05) in the CCNU treated groups, compared to the controls. The study showed that SQGD treatment inhibited lipid peroxidation (~58%, p> 0.05) in serum.

The combination of SQGD + CCNU (40mg/kg + 40mg/kg) demonstrated a statistically comparable reduction in lipid peroxidation in both cases against animals treated only with CCNU. Similar results have been reported in our previous studies, in both agents at a ratio of 20mg/kg / 80mg/kg [15]. Inhibition of lipid peroxidation by SQGD in both liposomes and irradiated hepatic and brain homogenates is also reported by other groups [14]. It is speculated, that highly toxic CCNU *in vivo* generates NO• radicals, and contributes to the overproduction of $ONOO^-$ and OH• in tissues [3].

Therefore, SQGD, as a bacterial metabolite and antioxidant, possibly containing non-enzymatic moieties (tocopherols, phenolic acids, ascorbic acid, flavonoids), exhibits a better reducing effect on toxic oxidative processes initiated by CCNU. Probably, SQGD inhibits and completely neutralizes the overproduction of ROS and in particular OH• by a series of electron-donor reactions [25, 26] and thus significantly modulates the influence of free radicals on bio-molecules [14].

Finally, the present study demonstrates that SQGD alone or in combination provides a protective effect against induced oxidative damages and thus can be used in the future as an excellent inhibitor against drug/or radiation induced toxicity.

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REFERENCES

1. R.L.Comis, S.K. Carter, *Cancer Treat. Rev.*, **1**, 221 (1974).
2. O.Kristal, K.M.Rassnick, J.M.Gliatto, N.C.Northrup, J.D.Chretin, K. Morrison-Collister, S. M.Cotter, A. S. Moore, *J Vet Intern Med.***, 18**, 75 (2004).
3. A.M.Zheleva, and V.G. Gadjeva, *Int. J. Pharm.* **212**, 2 (2001).
4. V.Gadjeva, G.Lazarova, A.Zheleva, *Toxicol. Lett.*, **144**, 3 (2003).
5. A.Zheleva, *Academic Ed. "Thrakia University" Stara Zagora*, (2012).
6. A.Catala, *Int. J. Biochem. Cell Biol.,* **38,** 1482 (2006).
7. M.E.Greenberg, X.M.Li, B.G.Gugiu, X.Gu, J.Qin, R.G.Salomon, S.L. Hazen, *J. Biol. Chem.*, **283,** 2835 (2008).
8. D.D.Patel, D.D.Bansal, S.Misha, R.Arora, R.K.Sharma, S.K.Jain, R. Kumar, *Environ. Toxicol.*,(2012). DOI: 10.1002/tox.21781.
9. R.Kumar, D.Patel, D.D.Bansal, S.Mishra, A.Mohammed, R.Arora, A.Sharma, R.K.Sharma, R.P. Tripathi, Extremophiles: Sustainable Resource of Natural Compounds-Extremolytes. The Netherlands: Springer, 279(2010).
10. R.Kumar, D.D.Bansal, D.D.Patel, S.Misha, Y.Karamalakova, A.Zheleva, V. Gadjeva, *Mol. Cell. Biochem.*, **349,** 57 (2011).
11. Y.Karamalakova, J.Sharma, R.Sharma, V.Gadjeva, R.Kumar, A. Zheleva, *Biotechnol & Biotechnolog Equipm*., **26**, 1 (2012).
12. R.Kumar, R.K.Sharma, D.D.Bansal, D.D.Patel, S.Misha, L.Miteva, Z.Dobreva, V.Gadjeva, S. Stanilova, *Cell. Immunol.*, **267**, 314, (2011).
13. 13. D.D.Patel, D.D.Bansal, S.Misha, R.Arora, A.Sharma, S.K.Jain, R. Kumar, *Mol. Cell. Biochem.*, **370,** 115, (2012).
14. S.Mishra, D.S.Reddy, V.S.Jamwal, D.D. Bansal, D.D.Patel, P. Malhotra, A.K. Gupta, P.K. Singh, S.Jawed, R. Kumar, *Mol. Cell. Biochem*., **379**, 1 (2013).
15. A.Zheleva, Y.Karamalakova, G.Nikolova, R.Kumar, R.Sharma, and V.Gadjeva, *Biotechnol & Biotechnolog Equipm*., **26**, 1 (2012).
16. V.Gadzheva, R. Koldamova, *Anti-cancer Drug Design.*, **16**, 247 (2001).
17. H.H.Shi, Y.X.Sui, X.R.Wang, Y.Luo, L.Ji, *Comp Biochem Physiol C Toxicol Pharmacol.,* **140**, 1 (2005).
18. G.R.Buettner, and B.A. Jurkiewicz, *Free Radic Bio Med*., **14**, 1 (1993).
19. N.Nakatani, *J Jpn Soc Nut Food Sci.*, **56**, 6 (2003).
20. M. S. Brewer, *Compreh Rev in Food Sciand Food Safety*, **10**, 4 (2011).
21. S.Mishra, P. Malhotra, A. K.Gupta, P. K.Singh, S.Javed, R.and Kumar, *J. Immunotoxicol.*, **12**,1 (2015).
22. M.Simeonova, T. Ivanova, Z.Raikov, H.Konstantinov, *Acta Physiol. Pharmacol.,. Bulg.*, **20**, 77 (1994).
23. S.Mishra, D.D.Bansal, P.Malhotra, K. Reddy, D. Sudheer, V.S.Jamwal, R.Kumar, *Environm toxicol.***, 29**, 1471 (2014).
24. N.Khan, H. Swartz, *Mol. Cell. Biochem*., **234,** 341 (2002).
25. A. Michalaк, *Polish J.of Environ.Stud.*,**15**, 4 (2006).
26. M. M Posmyka, C. Bailly, K. Szafrańskaa, K. M. Janas, F. Corbineau, *J. Plant Physiol* .**162**, 403 (2005).

СЕМИХИНОН ГЛЮКОЗИДЕН ДЕРИВАТ (SQGD), ИЗОЛИРАН ОТ *BACILLUS SP*. INM-1, ОСИГУРЯВА АНТИОКСИДАНТНА ЗАЩИТА СРЕЩУ CCNU-ИНДУЦИРАНА ОКСИДАТИВНА ТОКСИЧНОСТ ПРИ МЪЖКИ МИШКИ

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(Резюме)

Настоящото изследване е фокусирано върху оценката на семихинон глюкозидния дериват (SQGD), изолиран от радиоустойчивата бактерия *Bacillus sp.* INM-1 и неутрализирането на CCNU-индуциран оксидативен стрес при здрави мишки. Мишките се разделят на четири групи, т.е. (I) нетретирани контроли; (II) мишки лекувани с SQGD (40 mg/ kg телесно тегло, интраперитонеално); (III) мишки, третирани с CCNU (40 mg/ kg телесно тегло, интраперитонеално); и (IV) мишки, третирани с SQGD (40 mg/kg телесно тегло, интраперитонеално), приложен 1 час преди прилагане на CCNU (40 mg/ kg телесно тегло, интраперитонеално). След провеждане на изследвабето са оценени нивата на аскорбатни радикали и продуцирането на ROS в чернодробни хомогенати и кръвен серум от третираните животни. Резултатът показва, че приложението на SQGD + CCNU значително намалява ($p < 0,05$) нивата на аскорбатни радикали и ROS продукти в черния дроб и серума при мишките в сравнение с групата, третирана единствено с CCNU. При третираните с SQGD на здрави мишки е регистрирано намаляване на оксидативните нарушения, спрямо контролите. Освен това максималната акумулация на SQGD (a.u.) в кръвния поток се установява 30 минути след интраперитонеално прилагане и е напълно редуцирана след 240 минути. Фармакокинетичният профил на SQGD показва значително селективно натрупване, максимално в черния дроб и белите дробове (60 минути), мозъка (90 минути), последвано от бъбреци, панкреас, далак, кръв и тестиси. По този начин може да се заключи, че третирането с SQGD самостоятелно и в комбинация SQGD + CCNU неутрализира оксидативната токсичност, причинена от лекарства, не само чрез намаляване на липидното перокисление, но и чрез подобряване на антиоксидантния статус в органи и кръв. Това подчертава ефекта на SQGD като силен радикало-уловител и отличен природен протектор.

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Green extracts of grape seed oil - potential source of fatty acids and health benefits

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Supercritical CO_2 extraction of oil from grape seed samples obtained from a Portuguese industry without any previous treatment was carried out at temperatures from (313 to 333) K, pressures up to 40.0 MPa and different $scCO_2$ flow rates.

The qualitative analysis of the crude oil was carried out by NMR. The fatty acids were analyzed by GC-FID with reference to the parameters in Annex I to European Commission Regulation.

The results show similar content of triacylglycerols and diacylglycerols both in the *n*-hexane and $scCO_2$ extracts, but the latter have higher content of polyunsaturated fatty acids and lower content of saturated fatty acids, and hence are more beneficial for human health and wellbeing.

K**eywords:** Grape (*Vitis vinifera* L.) seed oil; supercritical CO_2 extraction; triacylglycerols; fatty acids.

INTRODUCTION

Seed biomass from *Vitis vinifera* L. contains typically (8–15) % (w/w) of oil which is rich in long chain polyunsaturated fatty acids (PUFAs) and antioxidants [1]. PUFAs are possibly less degradable than other fatty acids under particular conditions, and hence their presence in the extracts can increase the value of the oil obtained.

Development and implementation of sustainable processing concepts promotes reuse of residues of biomass. The biomass generated by the wine industry represents about 20-25 % of the total residues, and hence its recycling and reuse, grape seeds in particular, is of great importance since seed oil is beneficial for human health and wellbeing due to its high content of unsaturated fatty acids and antioxidant compounds [2, 3, 4].

Extraction of the pressed grape seeds with *n*-hexane is the current technique typically applied in an attempt to reuse the seeds biomass. However, a viable, green alternative extraction technique, applying supercritical CO_2 ($scCO_2$) as the solvent, can improve and reduce the environmental footprint. Supercritical fluids (especially $scCO_2$) possess a gas-like viscosity and diffusivity, and liquid-like density and solvating power [5, 6, 7], and have been applied and accepted as future industrial solvents, mainly in the field of thermolabile high value-added products.

Supercritical extraction (SCE) of oil from grape seeds from different cultivars as well as SCE of different high-added value substances from grape seed oil have been studied and reported by other research groups, the following references [4], [8, 9, 10, 11] are just few examples of the most recent contributions in the respective field. However, data about the yield and characterization of grape seed oil obtained by SCE of industrial, without any previous treatment, samples are scarce. Hence, further investigation that will provide new experimental data, analyses results and determine appropriate operating conditions will contribute to the knowledge of how to improve the quality of the valuable green extracts and products obtained from grape seed biomass.

In view of the above, the aims of our work are to: *i*. Obtain oil extracts from grape seeds, supplied by a Portuguese industry, applying SCE and *n*-hexane extraction; *ii*. Compare the influence of the extraction method on the yield, and on the lipids and fatty acids composition of the extracts, with the view to determine which is more beneficial for human health.

MATERIALS AND METHODS

Grape seeds from the center of Portugal, separated and milled in advance, were provided by a Portuguese wine industry. The biomass was drying for a period of 48 h at 343 K, which resulted in a decrease of the mass within (9.0±0.6) %. Thermogravimetric process in an electronic balance was used to determinate moisture content in a (Kern MRS 120-3), at a temperature of 378 K. The result of a triplicate analysis was (2.43±0.21) %.

Supercritical CO_2 and organic solvent extraction

Supercritical CO_2 extraction was performed in a laboratory apparatus, shown in Fig. 1, equipped with a 50 cm^3 internal volume vessel, built from AISI 316 stainless steel tubing (32 cm long and an internal

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diameter of 1.41 cm). Liquid CO_2 from the cylinder (G) is compressed (C) to the required pressure, then passes via the heater system and after that through the extraction vessel (E), in which CO_2 flows through the matrix sample before expansion in the micrometer valve (MM).

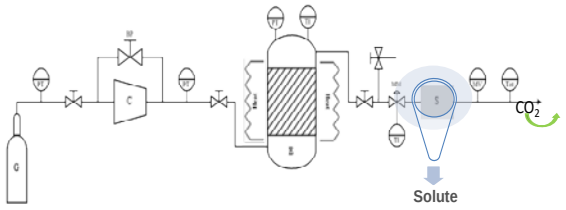

Fig. 1. Diagram of the supercritical fluid extraction apparatus. G, CO_2; C, compressor; E, extractor; S, separator; BP, back-pressure regulator; MM, micrometer valve; MV, flow meter; Tot, totalizer; TI, temperature indicator, PT, pressure indicator.

In our experiments the extracts were collected in a U tube (S), at atmospheric pressure and at a temperature controlled with a refrigerated bath. The amount of extract obtained was assessed gravimetrically with an uncertainty of ±0.1 mg. The total volume of CO_2 was determined with a mass flow meter and a totalizer from Alicat Scientific (USA), model M-5 SLPM-D (MV and Tot) [12, 13, 14]. CO_2 (purity 99.99 %) was supplied by GASIN - Air Products, Portugal.

The SCE was carried out using samples of 17 g of the grape seeds with particle size of (0.62±0.04) mm. Conditions of extraction were: CO2 flow rates of 0.11 kg/h, pressures up to 40 MPa, and temperatures up to 333 K. The conditions were controlled during all experiments, within (130-230) min time span, until no longer any significant mass of oil was recovered, which was considered as an indication that the extraction process was completed.

The Soxhlet extractions of the grape seeds to isolate the oil, were carried out with 250 mL of *n*-hexane (Sigma Aldrich) and 20 g of seeds samples, for 4 h at the solvent boiling point. The extract was filtered and the solvent removed by reduced pressure evaporation (rotavapor) to constant weight. The extraction yield was 12.28±0.35 % (w/w). The oil extract was kept at 253 K until analyzed.

Analysis of the crude oil extracts by ^1H NMR

The ^{1}H-NMR spectra of the crude oil extracts were obtained on a Bruker Avance 400 MHz NMR spectrometer (Bruker Inc., Bremen, Germany), with a 5 mm PABBO BB-1H probe using standard Bruker routines (90° proton pulse length of 11.8 µs and a delay time between acquisitions of 30 s). All spectra were taken at 298 K in $CDCl_3$ (500 µL, 75–100 mM grape seed oil) and the residual signal of the solvent was used as the internal reference. Chemical shifts (δ) were assigned based on previous reports [4], [15, 16].

Quantitative analysis of fatty acids (FAs)

Transesterification was carried out in a methanol solution of KOH (2M), following the necessary procedures as established in the Annex I to Commission Regulation (EEC) No 2568/91(1), CELEX_01991R2568 published 04.12.2016, with the necessary adaptations.

Gas chromatography of the fatty acid methyl esters (FAMEs) was performed using a fused-silica capillary column SP-2380, 60 m length, 0.25 mm of internal diameter, 0.20 µm film thickness, with helium as the carrier gas at a constant flow rate of 1.0 mL/ min.

The oven temperature in the GC was 438 K for 25 min; programmed heating from (438 to 483) K at 5 K/min and subsequent holding at 483 K for 10 min. The temperatures of the injector and detector were kept constant at (523 and 553) K, respectively. FAMEs were identified by comparing their retention times with those of a reference standard solution (supplied by Sigma-Aldrich) at the same condition.

RESULTS AND DISCUSSION

Supercritical fluid vs n-hexane extraction – oil yield and time

An evaluation of the effect of SCE operating parameters - pressure and temperature – on the extraction yields, as well as a comparison with the *n*-hexane extraction yield, can be deduced from Table 1.

Table 1. SCE times and oil yields, as a function of the operating conditions, at $scCO_2$ flow rate F = 0.11 kg·h^{-1}, as compared to *n*-hexane extraction results.

Extraction Method		Oil Yield (%)	Time (min)
	Hexane	12.28±0.35[a]	240
SCE conditions: *p*(MPa)/*T* (K)	20/313	7.20±0.50[b]	227
	30/313	11.96±0.60[a]	191
	40/313	12.07±0.55[a]	148
	30/333	12.17±0.38[a]	214
	40/333	12.83±0.56[a]	134

[a,b] In column two, the values with different letters are significantly different (p≤0.05), according to Tukey HSD test

One-way ANOVA with post-hoc Tukey HSD calculator was performed to determine differences between oil yields obtained by *n*-hexane and by SCE at the operating conditions of the experiment.

The maximum oil yields achieved by the SCE were in the range (12.0-12.8) % and were attained at the extraction conditions applied, the lower pressure of 20.0 MPa being an exception. The *n*-hexane extraction oil yield was 12.3 %. Moreover, the SCE times to obtain the maximum yields, compared with *n*-hexane extraction, were shorter - around 140 min for the higher pressure (40 MPa) and 200 min – for the case when the pressure was 30 MPa (Table 1).

The experimental SCE yields obtained are in agreement with the results of other authors [4], [9], [17, 18]. Yet, slight differences can be found in the literature [18, 19] regarding the overall evolution of the extraction of the oil, which can be attributed to a number of factors such as the origin of the vegetable matrices, pretreatment at industrial scale, particle size of the plant material, and the moisture content.

Quantitative analysis of the crude oil extracts

The crude grape seeds oil extracts were quantitatively analyzed by ^{1}H NMR. Fig. 2 shows the ^{1}H NMR spectrum of the grape seeds oil obtained by $scCO_2$ extraction as an example, and the relevant NMR signals used for determination of the chemical composition. The results of the ^{1}H-NMR quantitative analyses are shown on Fig. 3a, 3b and Fig. 4, which display a comparison of the lipids composition of the grape seed oils obtained by *n*-hexane and $scCO_2$ extraction, and of the fatty acids groups in the lipids, respectively.

The presence of 1,2-DAGs in the oil samples was determined by the signal at δ_H 3.72 ppm attributed to the glyceryl methylene protons at sn-3 position, as well as the oxidized lipids (linolenic hydroperoxides), by the characteristic olefinic protons of the conjugated diene system in the region 6.60-5.70 ppm. Fig. 3a shows that the lipid composition of both the SCE and *n*-hexane extracts is largely dominated by triacylglycerols (TAGs, 95-98 %). Other compounds like of 1,2-diacylglycerols (1.6–3.5 %) and oxidized lipids (0.4–1.8 %) represent only a minor contribution to the overall composition of the oil extracts as displayed on Fig. 3b. It should be noted, that the results obtained by us are dependent on the extraction conditions and on the vegetable matrix origin [4, 18], and are in a good agreement with literature data.

The fatty acids content of the grape seeds oils can also be evaluated by the relative integration of the ^{1}H-NMR signals attributed to the hydrocarbon chains with different number of unsaturations. The signal at δ_H 2.30 ppm (**a**) attributed to the methylene group adjacent to the carbonyl group and present in all the fatty ester derivatives was chosen to determine and normalize the integrations of the other NMR signals. The relative integration determines the distribution between the monounsaturated (MUFA) (signal **b**) and the diunsaturated (DUFA) (signal **c**) acyl chains on the glycerol backbone. The abundance of the saturated (SFA) chains is obtained as the difference between the total fatty acids (FA) and all the unsaturated (MUFA + DUFA) chains.

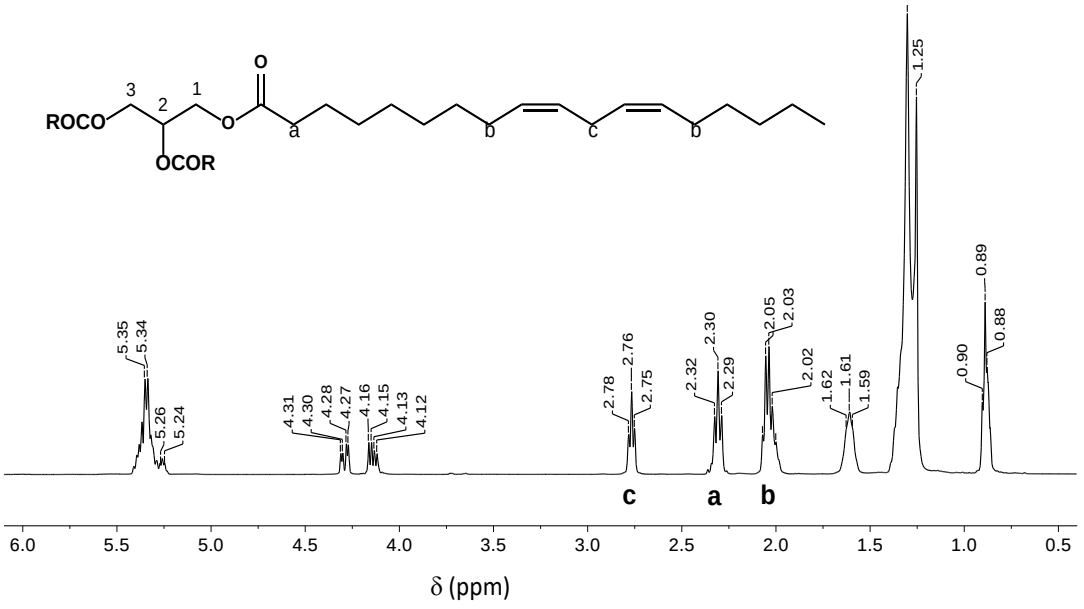

Fig. 2. ^{1}H-NMR spectrum of grape seed oil obtained by $scCO_2$ in $CDCl_3$ showing the attribution of the signals to specific protons in the linoleic acyl chain.

Fig. 3a **Fig. 3b**

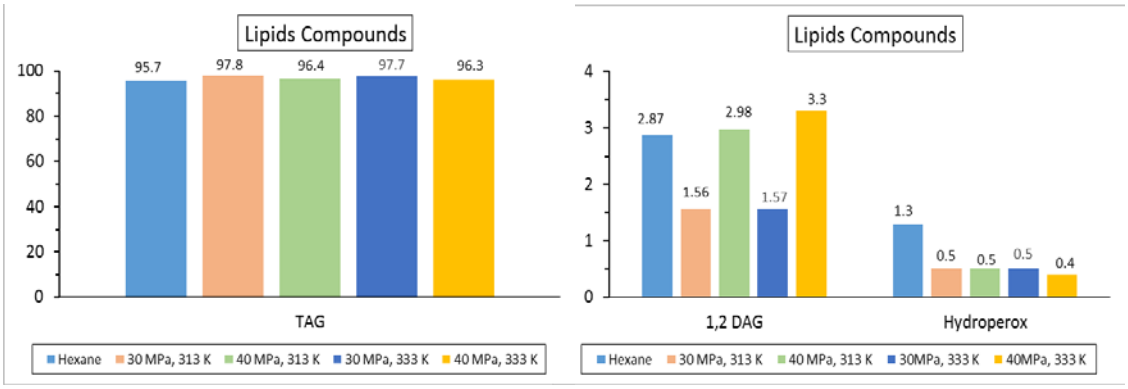

Fig. 3. Lipids composition of grape seed oils obtained by hexane and $scCO_2$ extraction at a flow rate of $scCO_2$ F = 0.11 $kg \cdot h^{-1}$, as established by ^{1}H-NMR quantitative analysis. All values represent % molar fractions.

Table 2. Fatty acid composition (% of total fatty acids) from FAME GC-FID analysis of grape seed oils obtained by hexane and $scCO_2$ extraction at $scCO_2$ flow rate F = 0.11 $kg \cdot h^{-1}$.

Fatty acid	Hexane	$scCO_2$ conditions: p(MPa)/T (K)			
		30/313	40/313	30/333	40/333
C14:0 - myristic	0.06	0.05	0.05	0.05	0.06
C16:0 - palmitic	8.13	7.53	7.48	7.59	7.38
C16:1 - palmitoleic	0.12	0.12	0.11	0.12	0.11
C17:0 - margaric	0.06	0.06	0.06	0.06	0.06
C18:0 - stearic	5.61	4.91	5.02	4.85	5.04
C18:1 - oleic	20.64	19.22	19.24	19.18	19.27
C18:2 - linoleic	64.52	67.30	67.17	67.37	67.23
C18:3 - linolenic	0.30	0.34	0.34	0.32	0.33
C20:0 – arachidic	0.23	0.18	0.2	0.17	0.21
C20:1 – gadoleic	0.19	0.17	0.18	0.15	0.17
C22:0 – behenic	0.04	0.04	0.04	0.05	0.04
C24:0 - lignoceric	0.07	0.04	0.04	0.04	0.04

Uncertainties in the values of composition (x) are: 0.003<x< 0.1 ±0.03; 0.1<x<1 ±0.05; 1<x<10 ±0.13 and ≥10±0.52

The unsaturation index (UI), defined as UI = (2×DUFA % molar fraction + MUFA % molar fraction)/100 is an important parameter which defines the ratio of these compounds. All supercritical oil extracts obtained contain higher percentages of DUFAs and similar MUFAs, when compared to the hexane extract, and hence possess higher values of UI.

Quantitative analysis of fatty acids (FAs)

Table 2 shows the fatty acid composition (% of total fatty acids) analysis of grape seed oils obtained by *n*-hexane and $scCO_2$ extraction. These results are in a good agreement (see Fig. 4) with those obtained by NMR analysis, with the exception of the hexane extract for which a minor disagreement with regard to the DUFA represented by C18:2 – linoleic can be found. The major fatty acids are the C18:2 – linoleic (64.5-67.47 %); C18:1 – oleic (19.18-20.64 %); C16:0 – palmitic (7.38-8.22 %) and C18:0 – stearic (4.33-5.61 %). The results in Table 2 confirm that DUFAs are the principal fatty acids present in the grape seeds oils, followed by the MUFAs and SFAs.

As suggested by Garavaglia *et al*. [20], high content of MUFAs in foods and diets is very important, because, for example, MUFAs may help lower the risk of heart disease by lowering the total and low-density lipoprotein (LDL) cholesterol levels while maintaining high-density lipoprotein (HDL) cholesterol level.

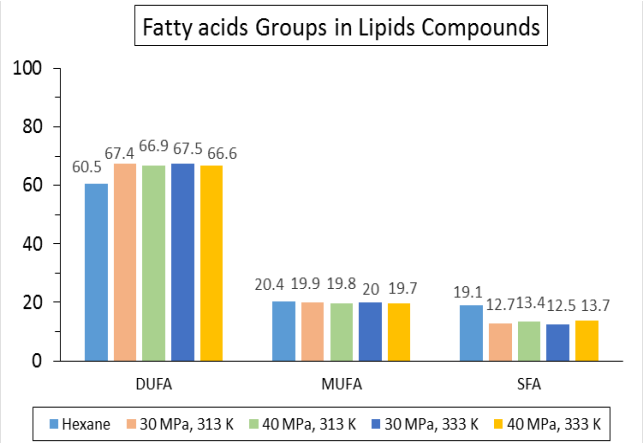

Fig. 4. Fatty acids groups in lipids composition of grape seed oils obtained by hexane and $scCO_2$ extraction at a flow rate of $scCO_2$ F = 0.11 $kg \cdot h^{-1}$. All values represent % molar fractions.

CONCLUSIONS

The influence of SCE operating parameters – temperature and pressure - on the yield and the fatty acid profile of the oil extracted from industrial grape seeds biomass were analyzed in detail and reported.

The highest oil yields achieved by the SCE were in the range 12.0-12.7 %, as compared to 12.3 % obtained by a conventional *n*-hexane extraction. However, in the former case, not only a free solvent extract can be obtained, but also the extraction times are lower. The main fatty acids present in the $scCO_2$ oil extracts are linoleic and oleic acids, with an average percentage of (67 and 20) %, respectively.

Taking into consideration the more favorable unsaturation index (UI) of, and the higher linoleic acid content in, the $scCO_2$ oil extracts, as compared to those obtained by the conventional *n*-hexane extraction, it can be concluded that SCE is the appropriate environmentally benign process to achieve a high quality grape seed oil extracts that can be used as an excellent food and diet supplement.

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REFERENCES

1. S. Bail, G. Stuebiger, S. Krist, H. Unterweger, G. Buchbauer, *Food Chem*., **108**, 1122 (2008).
2. D. Richard, K. Kefi, U. Barbe, P. Bausero, F. Visioli, *Pharmacol. Res*., **57**, 451 (2008).
.3. F. Temelli, *J. Supercrit.* Fluids, **47**, 583 (2009).
4. L. Fiori, V. Lavelli, K. S. Duba, P. S. C. Sri Harsha, H. Ben Mohamed, G. Guella, *J. Supercrit. Fluids*, **94**, 71 (2014).
5. G. Bru*nner, Annu. Rev. Chem. Biomol. Eng*., **1**, 321 (2010).
6. A. Tabernero, S. A. B. Vieira De Melo, R. Mammucari, E. M. Martín Del Valle, N. R. Foster, *J. Supercrit. Fluids*, **93**, 91 (2014).
7. J. P. Coelho, A. F. Palavra, in: High Pressure Fluid Technology for Green Food Processing, T. Fornari, R.P. Stateva (eds), Springer, 2015, p. 357.
8. A. Molero Gómez, C. Pereyra López, E. Martinez de la Ossa, *Chem. Eng. J. Biochem. Eng. J*., **61**, 227 (1996).
9. L. Fiori, *J. Supercrit. F*luids, **43**, 43 (2007).
10. K. S. Duba, L. Fiori, *J. Chem. Thermodyn*., **100**, 44 (2016).
11. C. Da *Porto,* A. Natolino, *J. Supercrit. Fluids*, **130**, 239 (2017).
12. J. P. Coelho, K. Bernotaityte, M. A. Miraldes, A. F. Mendonça, R. P. Stateva, *J. Chem. Eng. Data*, **54**, 2546 (2009).
13. J. P. Coelho, A. F. Mendonça, A. F. Palavra, R. P. Stateva, *Ind. Eng. Chem. Res*., **50**, 4618 (2011).
14. J. P. Coelho, G. P. Naydenov, D. S. Yankov, R. P. Stateva, *J. Chem. Eng. Data*, **58**, 2110 (2013).
15. E. Hatzakis, A. Agiomyrgianaki, S. Kostidis, P. Dais, *J. Am. Oil Chem. Soc*., **88**, 1695 (2011).
16. D. F. Andrade, J. L. Mazzei, C. R. Kaiser, L. A. D'Avila, *J. Am. Oil Chem. Soc*., **89**, 619 (2012).
17. C. P. Passos, R. M. Silva, F. A. Da Silva, M. A. Coimbra, C. M. Silva, *J. Supercrit. Fluids*, **48**, 225 (2009).
18. C. P. Passos, R. M. Silva, F. A. Da Silva, M. A. Coimbra, C. M. Silva, *Chem. Eng. J.*, **160**, 634 (2010).
19. J. M. Prado, I. Dalmolin, N.D.D. Carareto, R.C. Basso, A. J.A. Meirelles, J.V. Oliveira, E.A.C. Batista, M.A.A.Meireles, *J. Food Eng*., **109**, 249 (2012).
20. J. Garavaglia, M. M. Markoski, A. Oliveira, A. Marcadenti, *Nutr. Metab. Insights*, **9**, 59 (2016).

МАСЛО ОТ ГРОЗДОВИ СЕМКИ ПОЛУЧЕНО СЪС СВРЪХКРИТИЧНА ЕКСТРАКЦИЯ - ИЗТОЧНИК НА МАСТНИ КИСЕЛИНИ И ПОЛЗИ ЗА ЗДРАВЕТО

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(Резюме)

В настоящата работа са проведени екстракции със свръхкритичен въглероден диоксид (СКФ) на масло от гроздови семки. Пробите са получени директно от португалска индустрия без предварителна обработка. Експериментите са проведени при температури от (313 до 333) K, налягания до 40.0 MPa и различни скорости на потока на СКФ. Качественият анализ на суровото масло бе извършен чрез NMR. Мастните киселини се анализираха с GC-FID по отношение на параметрите в приложение I към Регламента на Европейската комисия. Получените резултатите показаха, че съдържанието на триацилглицероли и диацилглицероли в екстрактите получени с n-хексан е подобно на това в екстрактите получени със СКФ. Последните, обаче, имат по-високо съдържание на полиненаситени мастни киселини и по-ниско съдържание на наситени мастни киселини и следователно са по-полезни за човешкото здраве и благополучие.

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Optimization of the extraction of natural antioxidants from avocado seeds

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Food supplements of plant origin are widespread for dietary use. Most often they are in the form of extracts rich in natural antioxidants with high radical-scavenging action. Avocado (*Persea americana*) is a fruit widely cultivated throughout the world. There are many studies that attest to its health benefits. Avocado peels and seeds are considered as waste. However, there are indications that avocado seeds, which represent about 23 % of fruit weight, have even higher antioxidant activity than that of its flesh. So, the aim of this work is to study the influence of process parameters on the extraction of avocado seeds in order to determine the optimal conditions for obtaining extracts containing maximum amount of bioactive substances and having high antioxidant activity. An experimental approach is developed, which allows the optimal process parameters to be found by a reduced number of experiments. As a result, the following important process parameters are determined: selection of appropriate "green" solvent, which dissolves more antioxidant compounds; minimum solvent-to-solid ratio and minimum process duration necessary for complete extraction, which minimizes process costs; process temperature and ensures an acceptable compromise between higher solubility and thermal stability of the antioxidant compounds. The results obtained are useful for development of technological schemes for production of antioxidant extracts for use as functional supplements obtained from bio-wastes.

Keywords: Avocado seeds, Extraction, Polyphenols, Optimization, Antioxidant activity

INTRODUCTION

Avocado fruit is widely cultivated throughout the world. In 2014, world production of avocados was 5 million tons, with Mexico alone accounting for 30% (1.52 million tons) of the total [1].

The avocado fruit is valued not only for its flavor and nutritional profile, but also for its pharmacological properties. One important medicinal property of avocado fruit is its cancer-preventive effect due to combination of nutrients and phytochemicals (alkanols, terpenoid glycosides, various furan ring-containing derivatives, flavonoids and coumarins) [2]. Many polyphenol compounds can be found in avocado, but in the greatest quantity are lutein and zeaxanthin. Avocado fruit is also rich in several B vitamins and vitamin K, with good content of vitamin C, vitamin E and potassium [3]. The pulp of the avocados is rich of monounsaturated lipids (as oleic acid (67% of total lipids), polyunsaturated (linoleic acid), and saturated lipids (palmitic acid). Nutrient-rich avocado oil has diverse uses for salads or cooking and in cosmetics and soap products [4].

Polyphenols are known for their antioxidant properties. They are secondary metabolites of plants and are generally involved in defense against ultraviolet radiation or aggression by pathogens [5]. In general, they are attracting the attention, due to their role in the plant's immune response, their influence in the oxidative stability and organoleptic characteristics of foods, and the wide variety of health-promoting effects attributed to them [6]. In food, polyphenols may contribute to the bitterness, astringency, color, flavor, odor and oxidative stability. Towards the end of 20th century, epidemiological studies and associated meta-analyses strongly suggested that long-term consumption of diets rich in plant polyphenols offered some protection against development of cancers, cardiovascular diseases, diabetes, osteoporosis and neurodegenerative diseases [7-9].

The growing interest in the replacement of synthetic food antioxidants has led to multiple investigations in the field of naturally-sourced antioxidants. The search for cost-efficient natural antioxidants has led to the exploration of raw materials of residual origin [10]. Recently, fruit waste has become one of the main sources of municipal solid wastes, which have been an increasingly tough environmental issue. One of the solutions to this problem is to use fruit wastes as a source of valuable compounds - the bioactive constituents, especially phenolic compounds, and use them in the food, pharmaceutical, as well as cosmetics industry. Thus, utilization may be of considerable economic benefits and has become increasingly attractive [11]. Vegetables and some

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fruits yield between 25% and 30% of nonedible products [4, 5]. The by-products of fruits and vegetables are made up of skins and seeds of different shapes and sizes that normally have no further usage and are commonly wasted or discarded [12]. Avocado seeds represent about 20% from its mass and are considered as waste at a volume more than one million tons per year. This waste has a significant environmental impact due to the organic charge. It also requires additional costs for handling and storage [13]. There are a number of studies that find use of seeds as activated carbon [14], natural food dye (it produces orange color) [15], and as protection of oils and fats from oxidation [13].

Extraction is the first step in the studies of medicinal plants, because it is necessary to extract the desired chemical components from the plant materials for further separation and characterization [16]. Many factors, such as solvent composition, time of extraction, temperature, pH, solid-to-liquid ratio and particle size, may significantly influence the solid–liquid extraction. The big difference in the polarity of polyphenols requires the use of solvents with different polarity (water, acetone, methanol, ethanol, or their mixtures with water). Water/ethanol mixtures are possibly the most suitable solvent systems for the extraction of polyphenols due to the different polarities of the bioactive constituents, and the acceptability of this solvent system for human consumption. There is a growing interest in efficient and environmentally acceptable extraction methods. The desirable features of 'green' extraction methods are low solvent consumption, short extraction time and high extraction yield. Attention is now being directed to the extraction techniques that rely on solvents that are not hazardous to human health [17-19].

The aim of this work is to study the influence of process parameters on the extraction of avocado seeds in order to determine the optimal conditions for obtaining extracts containing maximum amount of bioactive (polyphenol) substances and having high antioxidant activity. An experimental approach is developed, which allows the optimal process parameters to be found by a reduced number of experiments.

EXPERIMENTAL

Raw material

Avocado fruits were purchased from the local market. The seeds were manually separated from the fruits. Before air drying they were cut and ground to particle size of 1 mm by using a chopper. Finally, the ground and dried seeds were stored in dark until use.

Chemicals and reagents

Analyses for total content of polyphenols were made using Folin-Ciocalteu phenol reagent (2N), gallic acid and anhydrous Na_2CO_3, 2,2-diphenyl-1-picrylhydrazyl (DPPH) and methanol were obtained from Sigma. Ethanol-water solvents were prepared by using 96% ethanol obtained from Valerus.

Extraction procedure

Extraction from the plants is an empirical exercise in which different solvents are utilized under a variety of conditions such as time, liquid/solid ratio and temperature of extraction. The success of the extraction process depends on the most appropriate assay [20].

In order to simplify the extraction process and save time and costly reagents, we have developed a four-step experimental procedure for finding the conditions for obtaining maximally enriched extracts. In general, a sample of ground raw material (10 g) was mixed in a flask with corresponding amount of solvent (depending on solvent-to-solid ratio). The extractions were carried out in a thermostated water bath shaker (Gyrotory Water Bath Shaker, model G76, New Brunswick Scientific, USA) at 160 rpm. After extraction, the mixture was filtered, and the filtrate was collected and stored at 4^0C for analyses. Each test was repeated in duplicate or in triplicate in case of bigger difference between two analyses. Mean values were used.

Analyses

Analyses for total polyphenols and antioxidant activity of the extracts were made.

Determination of total phenolic content. Total polyphenolic content (TPC) of the extracts was determined by the Folin–Ciocalteu method [21,22] using UV-VIS spectrophotometer (UNICAM®-Helios β). The absorbance of samples was measured at 765 nm. TPC was expressed as mg of gallic acid equivalent (GAE) per 1 gram of dry extract (mg GAE/g de).

Determination of in vitro antioxidant capacity (AOC). AOC was determined by DPPH method [23,24]. The method is based on a color reaction between the nitrogen atom (from DPPH) and the hydrogen atom of a hydroxyl group of the antioxidant compound. 1 ml extract was mixed with 4 ml solution of DPPH in methanol (0.004%). After keeping the sample in dark at room temperature for 60 min, the absorbance was measured at 517 nm. AOC was expressed as IC50 (quantity of extract neutralizing 50% of DPPH amount).

Graphically antioxidant capacity is expressed as mg DPPH which is neutralized by the corresponding amount (grams) of dry extract.

Statistical treatment. One-way Analysis-of-Variances software (ANOVA, Microsoft) with significance level 0.05 was applied to the treatment of experimental data in order to distinguish statistically equal mean results as opposed to statistically different ones.

RESULTS AND DISCUSSION

As a result of the four-step experimental procedure, the following important process parameters were determined:

Step 1: Selection of appropriate concentration of a "green" solvent, which dissolves maximum amount of antioxidant compounds (polyphenols).

Usually, solvents as methanol, ethanol, acetone, ethyl acetate, etc., at different volume fractions in water have commonly been used for extraction of polyphenols from different plants [25-27]. In this research water, ethanol, and aqueous solutions of ethanol (30, 48, 70%) were used for the extraction of avocado polyphenols. In order to achieve complete extraction, high solvent-to-solid ratio (20:1), as well as high temperature and long contact time (70ºC and 120 min respectively), were applied.

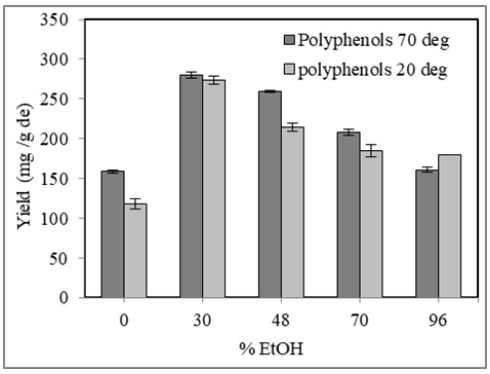

Fig. 1. Extraction yield at different solvent concentrations

From the results shown in Fig. 1, it is evident that an increase in the volume fraction of ethanol in the aqueous solutions does not have positive influence on the extraction efficiency. The yield of TPC has a maximum at 30 % ethanol and decreases afterwards with increasing percentage of ethanol. The results of ANOVA test confirm this conclusion.

Step 2: Determination of minimum solvent-to-solid material (hydromodule).

In determining the impact of the basic parameters on the extraction of target components at atmospheric pressure, the variation of hydromodule was investigated [28]. The aim was to minimize the amount of solvent, at which maximum amount of target components is derived. Experiments were performed at different ratios of solvent/solid phase: from 5/1 to 20/1. Our experience based on results of previous studies on plant materials extraction has shown that two hours of phase contact usually are more than sufficient to achieve equilibrium and to complete the mass-exchange process. For this reason, the extraction was conducted for two hours. The results are shown in Figure 2. According to them, at a ratio of 5/1 the amount of solvent is not sufficient for dissolving of all active components, while at a ratio of 8, 10, 12.5 and 20:1 the yield of extracted polyphenols is approximately equal – about 290 mg/g de. ANOVA test has also shown that hydromodules in the range 8 – 20 produce statistically equal yield. Consequently, from economic point of view, the most advantageous liquid-to-solid ratio is 8:1, which ensures high extraction at a lesser solvent quantity. Therefore, the following studies were conducted with this hydromodule.

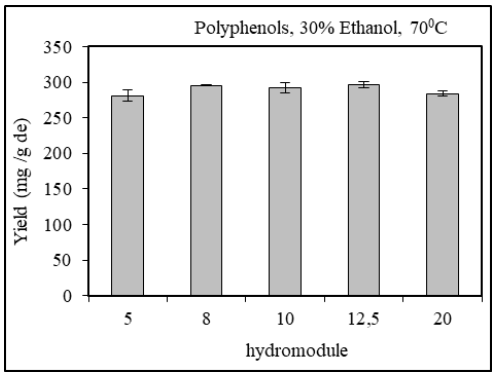

Fig. 2. Extraction yield at different hydromodules

Step 3: Determination of the process temperature:

Knowing in advance that higher temperature will probably lead to better solubility of the solid matter, our aim here was to check how the temperature affects the thermal stability of extracted antioxidant compounds [29,30]. The influence of temperature on the yield of polyphenols is shown in Figures 1 and 3. Three temperatures below the boiling temperature of the solvent were examined (20, 45 and 70ºC). From Fig. 1 it is seen that the yield at 70ºC is always higher than at 20ºC except for 96% ethanol. However, regarding the details in Fig. 3, which represents the temperature dependence of the yield for the optimal solvent concentration, it can be seen that at 70ºC the yield of the extracted polyphenols is significantly higher than that at 45ºC (14.5 %).

It is a sign for no temperature degradation of polyphenols. For this reason 70ºC has to be chosen as an appropriate process temperature. ANOVA

analysis has shown that the results for 20 and 40ºC are statistically equal, while the yield at 70ºC is different from that obtained at other tested temperatures.

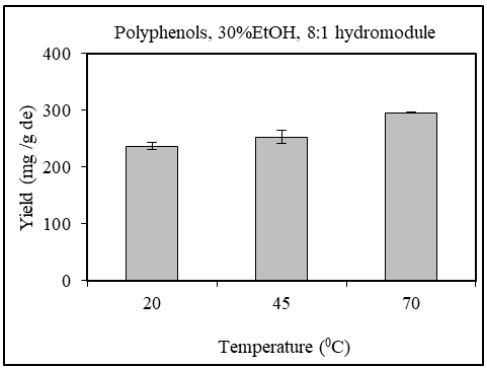

Fig. 3. Extraction yield under different temperature conditions

Step 4: Determination of minimum process duration necessary for extraction completion:

A) Kinetics of polyphenols extraction

The duration of the process necessary for maximizing the polyphenols yield was determined by a kinetic study tracking the evolution of the process over time. The results are presented in Figure 4. It is observed that after about 60 minutes, the extraction curve seems to reach a plateau, and no significant additional quantities are extracted afterwards (281.8 mg/g de for 60 min against 295.4 mg/g de for 120 min or 4.6 % more). This observation was confirmed by ANOVA test, which determined the group of results in the interval 60 – 120 min as statistically equal. Consequently, 60 minutes process duration can be selected as sufficient and optimal extraction time.

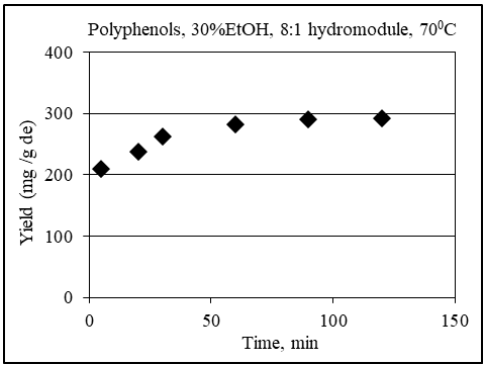

Fig. 4. Kinetics of polyphenols extraction

B) *In vitro* antioxidant capacity (AOC)

The phenolic compounds have an antioxidant effect due to the delivery of a hydrogen atom or an electron that stabilizes and neutralizes the free radicals, thus preventing their damaging oxidative action [31].

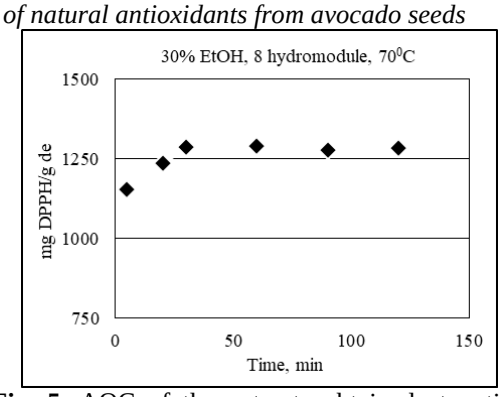

Fig. 5. AOC of the extracts obtained at optimized conditions

Figure 5 shows the time evolution of the antioxidant capacity (AOC) of the extracts obtained at optimal process parameters. According to ANOVA test, AOC values keep statistical equality after 30 minutes. However, in view of the fact that more polyphenols are extracted for 60 min than for 30 min, it is recommended to apply 60 minutes of extraction.

CONCLUSION

Four-step experimental procedure is developed for optimizing the extraction of bioactive substances from vegetables. It gives results with a low number of experiments (about 15). The method is applied to the extraction of antioxidant polyphenolic compounds from avocado waste (seeds), and the optimal processing conditions are determined as follows: 30% ethanol, 70^{0}C, solvent-to-solid material ratio 8, process duration 60 min. Generally, there is correlation between AOC values and the amount of extracted polyphenols. Extracts with higher polyphenols concentration possess higher antioxidant capacity.

The results are useful for development of technological schemes for production of antioxidant extracts for use as functional supplements obtained from bio-wastes.

REFERENCES

1. http://www.fao.org/faostat/en/#data/QC/visualize.
2. O.B.O. Ashton, M. Wong, T.K. McGhie, R. Vather, Y. Wang, C. Requejo-Jackman, P. Ramankutty, A.B. Woolf, *J. Agric. Food Chem.*, **54**, 10151 (2006).
3. M.L. Dreher, A.J. Davenport, *Crit. Rev. Food Sci. Nutr.*, **53**, 738 (2013).
4. http://nutritiondata.self.com/facts/fruits-and-fruit-juices/1843/2
5. C.H. Beckman, *Physiol. Mol. Plant Pathol.*, **57,** 101 (2000).
6. E. Hurtado-Fernandez, T. Pacchiarotta, M. Gomez-Romero, B. Schoenmaker, R. Derks, A. M. Deelder, O. A. Mayboroda, A. Carrasco-Pancorbo, A. Fernandez-Gutierrez *J. Chromatogr., A***, 1218,** 7723 (2011).

7. B. A. Graf, P. E. Milbury, J. B. Blumberg. *J. Med. Food*, **8**, 281 (2005).
8. I. C. W. Arts, P. C. H. Hollman, *Am. J. Clin. Nutr.*, **81**, 317S (2005).
9. K. B. Pandey, S. I. Rizvi, *Oxid. Med. Cell. Longev*., **2**, 270 (2009).
10. X. Tian, *Adv. Food Technol. Nutr. Sci. Open J*.; **S7**. (2016).
11. D. Gui-Fang, S. Chen, X. Xiang-Rong, K. Ru-Dan, G. Ya-Jun, Z. Li-Shan, G. Li-Li, L. Xi, X Jie-Feng, X. En-Qin, L. Sha, W. Shan, C. Feng, L. Wen-Hua, L. Hua-Bin, *Int. J. Mol. Sci.*, **13**, 8308 (2012).
12. T. Varzakas, G. Zakynthinos, F. Verpoort, Foods, **5**, 88 (2016).
13. F.S. Gómez, S.P. Sánchez, M.G.G. Iradi, N.A.M. Azman, M.P. Almajano**,** *Antioxidants*, **3**, 439 (2014).
14. L. A. Rodrigues, M. L. C. P. da Silva, M. O. Alvarez-Mendes, A. dos Reis Coutinho, G. P. Thim, *Chem. Eng. J.*, **174**, 49 (2011).
15. A. A. Arlene, A. K. Prima, L. Utama, A. S. Anggirini, *Procedia Chem.*, **16**, 334 (2015).
16. S. Sasidharan1, Y. Chen, D. Saravanan, K.M. Sundram, L.Y. Latha, *Afr. J. Tradit. Complem.*, **8**, 1 (2011).
17. M. Dent, V. Dragovič-Uzelac, M. Penič, M. Brnčič, T. Bosiljkov, B. Levaj, *Food Technol. Biotechnol*., **51**, 84 (2013).
18. H. Wang, G.J. Provan, K. Helliwell, *Food Chem*., **87,** 307 (2004).
19. N. E. Durling, O. J. Catchpole, J. B. Grey, R. F. Webby, K. A. Mitchell, L. Y. Foo, N. B. Perry, *Food Chem*., **101**, 1417 (2007).
20. J. H. Doughari, Phytochemicals - A Global Perspective of Their Role in Nutrition and Health, Chapter 1: Phytochemicals: Extraction Methods, Basic Structures and Mode of Action as Potential Chemotherapeutic Agents, InTech, 2012.
21. V. L. Singleton, R. Orthofer, R. M. Lamuela-Raventos, *Meth. Enzymol*., **299,** 152 (1999).
22. A. L. Waterhouse, *Curr. Protoc. Food Analyt. Chem.*, I1.1.1 (2001).
23. C. Sanchez-Moreno, *Food Sci. Technol. Intern.*, **8**, 121 (2002).
24. J. Perez-Jimenez, S. Arranz, M. Tabernero, M. Diaz-Rubio, J. Serrano, I. Gono, F. Saura-Calixto, *Food Res. Int.*, **41**, 274 (2008).
25. W. Wang, T. R. Bostic, L. Gu , *Food Chem*., **122**, 1193 (2010).
26. J. G. Rodríguez-Carpena, D. Morcuende, M. J. Andrade, P. Kylli, M. Estévez, *J. Agric. Food Chem.*, **59**, 5625 (2011).
27. O. A. Folasade, R. A. Olaide, T. A. Olufemi, *J. Adv. Food Sci. Technol*.**,** 3, 101 (2016).
28. M. Pinelo , M. Rubilar , M. Jerez, J. Sineiro , M.J. Núñez, *J. Agric. Food Chem.*, **53** (6), 2111 (2005).
29. F. J. Segovia, J. J. Corral-Pérez, M. P. Almajan, *Ind. Crops Prod*., **85**, 213 **(**2016).
30. A. Bucic-Kojic, M. Planinic, S. Tomas, L. Jakobek, M. Seruga, *Int. J. Food Sci. Tech***., 44**, 2394 (2009).
31. J. M. R. da Silva., N. Darmon, Y. Fenandez, S. Mitjavila, *J.n. Agric. Food Chem.*, **39**, 1549 (1991).

ОПТИМИЗИРАНЕ НА ЕКСТРАКЦИЯТА НА ПРИРОДНИ АНТИОКСИДАНТИ ОТ КОСТИЛКИ НА АВОКАДО

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(Резюме)

Хранителни добавки от растителен произход са широко разпространени за диетична употреба. Най-често те са под формата на екстракти, богати на природни антиоксиданти със силно радикал-прихващащо действие. Авокадото (*Persea americana*) е широко култивиран плод в целия свят. Има много изследвания, доказващи неговите ползи за здравето. Люспите и костилките на авокадото са отпадък. Има обаче индикации, че костилките на авокадото, които представляват около 23 % от теглото на плода, имат дори по-силно антиоксидантно действие от месестата му част. Целта на настоящата работа е да се изследва влиянието на параметрите на процеса върху екстракцията на костилки от авокадо с оглед да се определят оптималните условия за получаване на екстракти, съдържащи максимално количество биоактивни вещества и имащи висока антиоксидантна активност. Разработен е експериментален подход, даващ възможност за намиране на оптималните параметри на процеса посредством намален брой експерименти. В резултат са определени следните важни параметри на процеса: избор на подходящ „зелен“ разтворител, който да разтваря по-голям брой антиоксидантни съединения с минимално съотношение разтворител:твърдо вещество и минимална продължителност на процеса, необходима за пълна екстракция при минимална температура, с което минимизира разходите за процеса и осигурява приемлив компромис между по-високата разтворимост и термичната стабилност на антиоксидантните съединения. Получените резултати могат да се използват за разработване на технологични схеми за производство на антиоксидантни екстракти, които да се използват като функционални добавки, получени от био-отпадъци.

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Characterization of polyphenol content and antioxidant capacity of spent coffee grounds

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In this study spent coffee grounds were investigated as a source of natural antioxidants. The polyphenolic content and antioxidant capacity of the extracts from fresh roasted coffee was determined and compared to those of spent coffee grounds (after a common espresso preparation). An optimized water-ethanol solvent was used. The extracts were analyzed for the total polyphenol content by Folin-Chiocalteau phenol reagent, by UV spectrophotometry for the content of chlorogenic acid. The antioxidant activity was investigated using the DPPH assay. It was found that the coffee remains still contain significant amount of polyphenols and show high antioxidant capacity. In our case study, spent coffee grounds (SCG) contain 17 mg polyphenols/g SCG, 15.8 mg chlorogenic acid/g SCG, and its antioxidant capacity was 86 mg DPPH/g SCG. The remaining polyphenolic content, chlorogenic acid and antioxidant capacity of spent coffee grounds represent 28%, 31% and 32.5%, respectively, as compared to untreated fresh coffee. In conclusion, spent coffee is far from being exhausted by a simple hot water extraction, and it can be considered as a rich, valuable and widely available source of useful natural bioactive substances with antioxidant activity.

Keywords: Spent coffee, Extraction, Polyphenols, Antioxidant capacity

INTRODUCTION

Coffee has been for decades the most commercialized food product and most widely consumed beverage in the world, taking second place after the water. 85% of human population drinks coffee. The world production in 2016 was about 9 million tons, the biggest producer being Brazil with 3 million tons. The other major producers are Vietnam, Colombia, Indonesia, and Ethiopia. Europe, especially northern European Scandinavian countries take first place in consumption led by Finland - 12 kg per year per capita. The consumption in Balkan countries is less, although significant (Greece – 5.5 kg, Bulgaria – 3.5 kg, Romania – 2.5 kg) [1].

Coffee production and consumption create a huge amount of wastes. There are research studies aimed at valorization of this waste. It has been investigated for biodiesel production [2,3], as sorbent [4,5], as source of sugars [6]. Spent coffee grounds are often used at small scale, mainly for composting and fertilizing [7,8] as they are known to slowly release nitrogen in the soil. The major amount is going to waste.

Normal biological functions naturally produce highly reactive molecules called free radicals or reactive oxygen species. They have toxic and damaging effects in the body, which are counteracted by antioxidants produced by the body or taken with foods. When there is insufficient antioxidant capacity to balance the effect of free radicals, the result is oxidative stress. Scientists now believe that many of the disease processes in the body involve oxidative stress as a common pathway [9,10].

Coffee contains more than 500 different compounds. Besides its refreshing effect due to caffeine, it contains many healthy constituents. Among them, coffee phenolics with the main representative chlorogenic acid (CGA) have attracted great attention by the scientific and medical communities due to their strong antioxidant properties that have positive influence against the oxidative stress [11,12].

A common practice in making a beverage is by shortly contacting milled roasted coffee with hot water. It seems doubtful that this simple treatment with hot water can fully extract the useful substances. So, it might be expected that coffee grounds still contain non-extracted heathy compounds.

The aim of this work was to study the remains after coffee extraction with hot pressurized water (espresso preparation) in order to establish whether they still contain antioxidant substances (chlorogenic acid and other polyphenols) in a quantity that may deserve additional treatment in view of obtaining enriched extracts with antioxidant activity from largely available wastes.

EXPERIMENTAL

Materials

Roasted and milled coffee (called fresh coffee hereafter) and spent coffee grounds after expresso

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preparation were collected from a local coffee shop. Spent coffee grounds were dried and kept refrigerated at dark along with the fresh coffee. Sieve analysis was made and the following fractions were found: the major quantity (about 80%) is of size 0.2-0.5 mm, about 20% is of size 0.5 mm or bigger, negligible quantity (0.6%) is less than 0.2 mm.

Chemicals

The following chemicals were used for the extracts preparation and for analyses: 96% ethanol of food quality, methanol 99.9% (from Lab Scan), Folin-Ciocalteu reagent (2N solution), DPPH, gallic acid, chlorogenic acid, all from Sigma, anhydrous Na_2C0_3 (from Valerus).

Extraction procedure

CGA and the majority of other polyphenols in the coffee are well soluble in methanol, ethanol and their mixtures with water [13]. Although studies using methanol as solvent exist [14], in this study ethanol-water mixtures were used for the reason of lower toxicity of the solvent.

Extraction was made with non-fractionated solid material and a corresponding solvent. As two-hour and one-hour extraction results were equal, one hour of process duration was applied. In order to eliminate solubility limitations, a big solvent-to-solute ratio (hydromodule) of 20 was chosen.

5 g of milled fresh coffee or dry spent coffee grounds were mixed in a flask with 100 ml of solvent with different ethanol content (from 0 to 96%). The suspension was vortexed for one hour at 70^{o}C in a thermostated water bath shaker Gyrotory G76, New Brunswick Scientific Co. Then the solid-liquid system was filtered, a sample of the liquid phase (liquid extract) was taken for analyses, another liquid sample was dried in order to determine the yield of extraction. All experiments were made in double, and good reproducibility was observed.

Analyses

Analysis for chlorogenic acid. This analysis was made according to the method described in [15,16] using UV-Vis spectrophotometer UV-1600PC, VWR international. The UV spectrum, 260-400 nm, was registered from ethanol extract after suitable dilution with ethanol against the reference cuvette with solvent. The concentration of chlorogenic acid was determined from the absorption maximum at 324-328 nm using a calibration curve prepared with pure chlorogenic acid.

Analysis of total phenolic content. The total phenolic content was determined spectrophotometrically with the Folin-Ciocalteu reagent [17,18]. 0.02 mL of the extract was mixed with 0.1 mL of 2N Folin-Ciocalteu reagent and 0.3 mL of Na_2CO_3 (20 % w/v), all diluted to 2 mL with distilled water. The resulting mixture was incubated at room temperature for 2 hours for color development. The absorbance of the samples was measured at 765 nm using double beam UV/VIS spectrophotometer UV-1600PC, VWR international. Calibration curve with gallic acid was made, and the total phenolic content was expressed as gallic acid equivalents. The reference cuvette contained all reagents except the sample extract.

Antioxidant capacity (AOC). AOC was determined by the DPPH method, which is largely used because of its simplicity and reproducible results [19,20]. This method is based on the reaction of antioxidant substances with methanol solution of DPPH, resulting in neutralization of free radicals emitted by DPPH. The latter absorbs at 517 nm, but upon reduction by an antioxidant the absorption decreases, and the color changes from deep violet to yellow. The absorption was measured spectrophotometrically. The analytical protocol was as follows:

The blank sample was adjusted by measuring a mixture of 1 mL solvent and 4 mL methanol solution of DPPH against methanol (A_0). The analyzed sample was obtained by mixing 1 mL of plant extract with 4 mL of 0.004 % (0.1 mM) solution of DPPH in methanol. After 60 min incubation in dark, the light absorbance of the sample was measured (A_e) against methanol at 517 nm. The scavenging concentration (SC) of the sample was calculated by the expression:

$$SC\ [\%] = (1 - A_e/A_0) \times 100 \qquad (1)$$

The antioxidant capacity was expressed as SC50 value, which represents the concentration of a sample that inhibits 50% of the free radicals added to the system. SC50 value can be determined from the chart that expresses SC as a function of the extract concentration C_e.

The graphical relationship $SC = f(C_e)$ for an extract was obtained by measuring the absorption of a series of samples containing different amounts of this extract added to the solvent [mL/L]. Appropriate dilution of the samples is necessary in order to fall in the linear part of the graph in IC interval 0 to above 50 %. The extract concentration reducing 50% of free radicals can be calculated from the linear equation by setting SC=50, or determined from the chart as the abscissa of the intersection point of the horizontal line from the

50% SC ordinate and the data line. A smaller value of *C* corresponds to higher AOC, i.e. a smaller quantity of this extract is needed for neutralization of 50 % of the free radicals. SC50 concentration can be transformed and expressed as mg DPPH neutralized by 1 g of raw material (rm) [mg DPPH/g rm] or by 1 g of dry extract (de) [mg DPPH/g de]. In this case the representation is more logic, because higher values correspond to higher AOC.

Extraction yield. After extraction, 10 mL samples of the liquid extract were dried at 80°C until constant weight was reached (henceforth referred to as dry extract - de). Laboratory analytical balance Sartorius with 0.1 mg accuracy was used.

RESULTS AND DISCUSSION

Optimizing the solvent composition

For determination of the solvent composition, at which maximum quantity of target bioactive components is recovered, the extractions were carried out with different concentrations of ethanol in the solvent (0, 24, 40, 48, 60, 72, 96%).

The results for spent coffee grounds are shown in Fig. 1 as mg extracted substance (TPC or CGA) per gram of raw material (mg/g_{rm}).

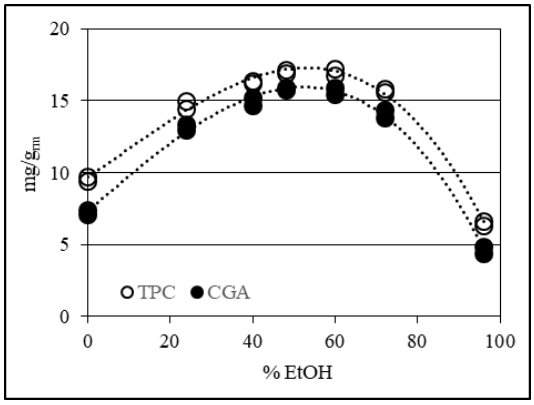

Fig. 1. Effect of ethanol concentration on the quantity of extracted substances from the raw material

It is seen that ethanol concentration is most efficient and equally suitable between 48 and 60%. From economic point of view it is advisable to choose the less concentrated solvent containing 48% ethanol. Also, water is a better solvent than concentrated ethanol (96%).

The quantitative result for the highest content of the studied bioactive compounds in the spent coffee, as taken from Fig. 1, is 17 mg TPC and 15.8 mg CGA per gram of solid matter. Accounting for the fact that chlorogenic acid is phenolic compound, it might be concluded that CGA is the main phenolic representative in the waste (93%), while the other phenolics altogether represent 7% of TPC.

The picture becomes slightly different when considering the obtained dry extract (see Fig. 2). The extract obtained with 60% ethanol seems to be more concentrated. However, one-way analysis of variance (ANOVA) with significance level 0.05 (made by Microsoft software) has shown that concentrations of TPC between 24 and 72% ethanol are statistically equal, the same being valid for 48 and 60% ethanol extracts of CGA. So, the solvent concentration selected initially (48%) still appears to be appropriate for the optimal extraction of the target substances. Again, water is a better extractant than concentrated ethanol. It is worth mentioning that after extraction the content of TPC in the raw material (17 mg/g_{rm}) has raised to 265 mg/g_{de} in the extract, i.e. the extract is 16 times more concentrated. The same is valid for CGA (concentration increased from 15.8 to 245 mg/g).

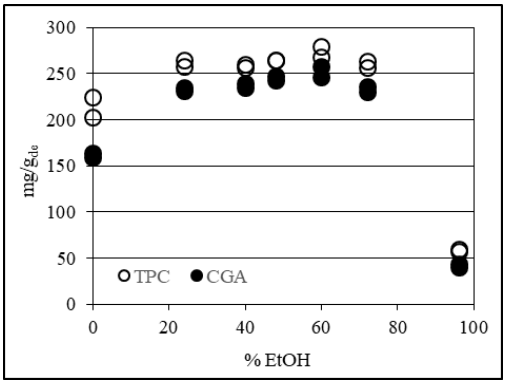

Fig. 2. Influence of solvent composition on the concentration of total polyphenols (TPC) and chlorogenic acid (CGA) in the dry extract

Fig. 3 depicts the total quantity of solid matter (de) extracted from the raw material. The tendency is similar to that shown in Figs. 1 and 2, namely maximum yield is obtained with a solvent containing 40-60% ethanol. Confronting the maximum values (obtained with 48% ethanol) for total extract (65 mg/g_{rm}) to these for TPC (17 mg/g_{rm}), it follows that our target antioxidant compounds represent about 26% of the total dry matter extracted from spent coffee grounds.

Concerning a study using methanol as solvent [14], some different results are obtained there. Pure methanol is reported to be a better solvent than water, and the optimal solvent concentration is 60% methanol.

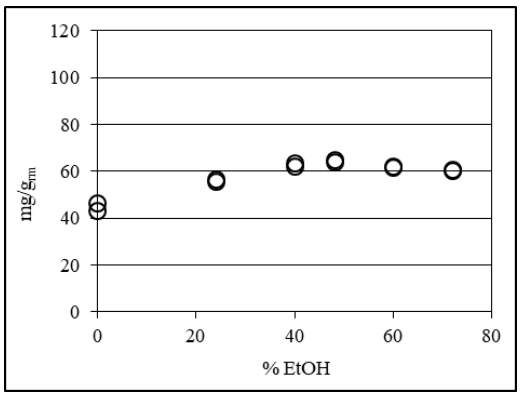

Fig. 3. Effect of ethanol concentration on the yield of extracted matter

Although not far from these data, our result regarding the solvent concentration states that less concentrated and less expensive 48% ethanol is slightly better than a more concentrated solvent. However, we have obtained more concentrated phenolic extracts with water than with ethanol. Additionally, as compared to methanol, we use a non-toxic solvent still extracting similar quantity of phenolic compounds as in [14].

The antioxidant capacity of extracts from spent coffee grounds obtained at different solvent composition is shown in Fig. 4. Clear tendency of higher AOC corresponding to higher phenolic concentration is seen (cf. Fig. 1 and Fig. 4). The solvent containing 48% ethanol produces extracts with the highest antioxidant capacity.

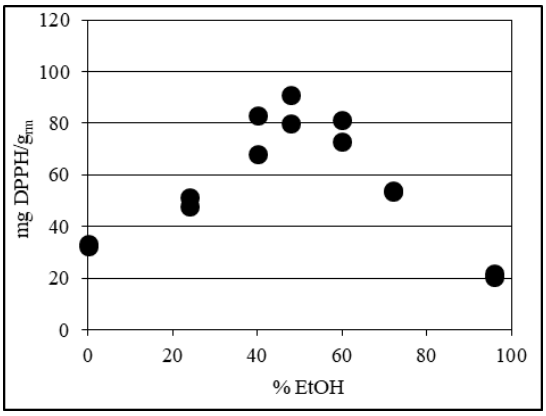

Fig. 4. Effect of ethanol concentration on the AOC of raw material

Table 1. Comparison of results for fresh and spent coffee

	1	2	3	4	5
	Fresh coffee	Spent coffee	Espresso drink (2-1)	Spent/Fresh (2:1) %	Spent/Espresso (2:3) %
TPC [mg/g_{rm}]	61± 0.68	17± 0.08	44		
CGA [mg/g_{rm}]	51± 0.68	15.8± 0.05	35.2		
AOC [mg_{DPPH}/g_{rm}]	265± 9.36	86± 5.45	-		

Regarding Table 1, it is seen that about 1/3 of the bioactive substances in fresh coffee have not been extracted during the espresso preparation and remain in the spent coffee grounds (see column 4, lines 1 and 2). The result for antioxidant capacity closely corresponds to the phenolic content, i.e. spent coffee possesses 1/3 of fresh coffee AOC (column 4, line 3) and contains around 40% of the phenolics found in espresso drink (column 5). So, fresh coffee is far from being exhausted by coffee preparation, and its remains contain significant amount of useful antioxidants.

CONCLUSION

This study presents comparative results for the content of polyphenols in roasted and milled coffee beans and in spent coffee grounds obtained after espresso preparation. The extraction is made with non-toxic water-ethanol mixtures. Optimal solvent concentration of 48% ethanol is found to extract maximum polyphenols. The results attest that significant quantity of useful healthy antioxidant substances remain in the spent coffee grounds after short treatment of milled coffee with hot pressurized water (espresso preparation). Nearly 30% of initial content of polyphenolic compounds remain non-extracted. Concerning the antioxidant activity of spent coffee, it represents about 1/3 of the AOC of fresh coffee. The dry extract obtained from spent coffee contains about 25% of polyphenols.

Based on the quantitative results of this study, it might be concluded that spent coffee grounds can be considered as a rich potential source of useful natural antioxidant compounds, which can be obtained from largely available wastes.

Comparison of results for fresh and spent coffee

Table 1 reports the results for the content of target components and antioxidant capacity in fresh coffee along with spent coffee. All results are obtained at identical extraction conditions using the optimized solvent concentration.

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REFERENCES

1. http://www.ico.org/documents/cy2016-17/annual-review-2015-16-e.pdf

2. N. Caetano, V. Silva, T. Mata, *Chem. Eng. Trans.*, **26**, 267 (2012).
3. S. Phimsen, W. Kiatkittipong, H. Yamada, T. Tagawa, K. Kiatkittipong, N. Laosiripojana, S. Assabumrungrat, *Energy Convers. Manage.*, **126**, 1028 (2016)
4. N. Fiol, C. Escudero, I. I. Villaescusa, *J. Sep. Sci. Tech.*, **43**, (3), 582 (2008).
5. K. Kante, J. Rangel-Mendez, T. Bandosz, *J. Hazard. Mater.* **201-202**,141 (2012).
6. S. Mussato, L. Carneiro, J. Silva, I. Roberto, J. Teixeira, *Carbohyd. Polym.*, **83**, 368 (2011).
7. D. Preethu, B. Bhanu Prakash, C. Srinivasamurthy, B. Vasanthi, Maturity Indices as an Index to Evaluate the Quality of Compost of Coffee Waste Blended with Other Organic Wastes, (Proc. Intern. Conf. on Sustainable Solid Waste Management), Chennai, India, (2007), p. 270.
8. R. Cruz, E. Mendes, A. Torrinha, S. Morais, J. Pereira, P. Baptista, S. Casal, *Food Res. Int.*, **73**, 190 (2015).
9. R. Linkie, M. Hum, Diabetes defeated: 97 Most Powerful Secrets For Controlling Blood Sugar Levels Naturally, Agora Health, (2013).
10. D. Pitocco, F. Zaccardi, E. Stasio, F. Romitelli, S. Santini, C. Zuppi, G. Ghirlanda, *Rev. Diabet. Stud.*, **7**, 15 (2010).
11. A. Farah, Coffee constituents, in Coffee: Emerging Health Effects and Disease Prevention, Yi-Fang Chu (Ed.), First Edition, John Wiley & Sons, Inc. (2012).
12. J. Dai, R. Mumper, *Molecules*, **15,** 7313 (2010).
13. M. Pinelo, A. G. Tress, M. Pedersen, A. Amous, A. S. Meyer, *Amer. J. Food Technol*., **2**, 641 (2007).
14. S. Mussato, L. Ballesteros, S. Martins, J. Teixeira, *Separ. Purif. Technol.*, **83**, 173 (2011).
15. L. Dao, M. Friedman, *J. Agric. Food Chem.*, **40**, 2152 (1992).
16. A. Belay, A. Gholap, *Afr. J. Pure Appl. Chem.*, **3**, 234 (2009).
17. V. L. Singleton, R. Orthofer, R. M. Lamuela-Raventos, *Meth. Enzymol*., **299,** 152 (1999).
18. A. L. Waterhouse, *Curr. Protoc. Food Analyt. Chem.*, I1.1.1 (2001).
19. C. Sanchez-Moreno, *Food Sci. and Technol. Intern.*, **8**, 121 (2002).
20. J. Perez-Jimenez, S. Arranz, M. Tabernero, M. Diaz-Rubio, J. Serrano, I. Gono, F. Saura-Calixto, *Food Res. Int.*, **41**, 274 (2008)..

ОПРЕДЕЛЯНЕ НА СЪДЪРЖАНИЕТО НА ПОЛИФЕНОЛИ И АНТИОКСИДАНТНИЯ КАПАЦИТЕТ НА УТАЙКА ОТ КАФЕ

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(Резюме)

В настоящата работа е изследвана утайка от кафе като източник на природни антиоксиданти. Съдържанието на полифеноли и антиоксидантният капацитет на екстракти от прясно изпечено кафе са определени и сравнени с тези на утайка от кафе (след приготвяне на обикновено еспресо). Използван е оптимизиран разтворител вода-етанол. Екстрактите са анализирани за тотално съдържание на полифеноли с фенолния реагент на Folin-Chiocalteau, а за съдържание на хлорогенна киселина – с УВ спектрофотометрия. Антиоксидантната активност е изследвана с помощта на DPPH метода. Установено е, че остатъците от кафе съдържат значителни количества полифеноли и проявяват висок антиоксидантен капацитет. В конкретния случай утайката от кафе (УК) съдържа 17 mg полифеноли/g УК, 15.8 mg хлорогенна киселина acid/g УК и антиоксидантният й капацитет е 86 mg DPPH/g УК. Съдържанието на полифеноли и хлорогенна киселина, както и антиоксидантният капацитет на утайката от кафе са съответно 28%, 31% и 32.5% от тези на необработено прясно кафе. В заключение следва, че полезните вещества в кафето далеч не са изразходвани чрез обикновена екстракция с гореща вода и утайката от кафе представлява богат, ценен и широко разпространен източник на полезни природни биоактивни вещества с антиоксидантен капацитет.

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Flavonoid content and antioxidant activity of *Betonica bulgarica* Degen et Neič

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The Bulgarian endemic *Betonica bulgarica* Degen et Neič (syn. *Stachys bulgarica* Hayek) is a protected plant by the Biological Diversity Act and it is included in the Red Data Book of Bulgaria under the category "endangered". The aim of this study was to determine the flavonoid content and antioxidant activity of different plant organs of this species (leaves, flowers, roots, stems and seeds), from four populations. Three flavonoids were found in significant amounts: rutin, quercetin and hispidulin. Rutin was in the largest quantity, followed by quercetin and hispidulin. The largest total flavonoid content was measured in leaves, followed by roots and flowers. The antioxidant activity of methanol extracts was tested by DPPH-method. The total polyphenol was also assayed. The correlation between flavonoid content and antioxidant activity of the studied plant organs was established.

Key words: Flavonoids; Polyphenols; Antioxidant activity; *Betonica bulgarica*

INTRODUCTION

The Bulgarian endemic *Betonica bulgarica* Degen et Neič (syn. *Stachys bulgarica* Hayek) is a protected plant by the Biological Diversity Act (2002) [1], and it is included in the Red Data Book of Bulgaria under the category "endangered" [2]. *Betonica* and *Stachys* species are widely used in folk medicine as anti-inflammatory [3, 4], antibacterial [5, 6], anti-cancer [7, 8] and antioxidant agents [9-11]. Recently, they were officially applied in homeopathic medicine [12, 13]. Previous studies of *B. officinalis* showed presence of bioactive compounds with proven antioxidant activity like phenolic compounds, flavonoids and essential oils [14-20]. Nevertheless, the literature data about antioxidant activities of Bulgarian endemic species are missing and little is known about chemical components with antioxidant activity, like flavonoids and polyphenols. The quantification of three major flavonoids: rutin (RU), quercetin (QU) and hispidulin (HI) and total polyphenols, their distribution in different plant parts (leaves, flowers, roots, stems and seeds) of these endemic species from four populations was the aim of this work, in order to study the natural variability of *B. bulgarica*. The relation of this content to the antioxidant activity was also investigated.

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MATERIALS AND METHODS

Plant material and extract preparation

Aerial parts of *Betonica bulgarica* were harvested from July to September 2016 in four locations from naturally growing populations in Bulgaria (Table 1). The roots were collected at the end of the vegetative period. The voucher specimens from the studied populations are kept in the herbarium of the Agricultural university in Plovdiv (SOA). Plant material was airdried in shade at room temperature and ground in a mechanical grinder (final powder size less than 400 μm). The samples were stored in dark and cool rooms at 16 – 18 ° C prior to the analysis.

The target compounds were extracted by ultrasonication of 1 g of powdered plant material in 10 ml of methanol for 30 min at 40 ° C in triplicate. Ultrasonic extraction is convenient and straightforward and was selected because of the high rate of extraction of flavonoids and polar bioactive compounds [21].

Flavonoids determination

The flavonoids levels in methanolic extracts were determined by HPLC analysis developed and validated by Ashokkumar *et al*. [22]. The extract of each sample was filtered through a 0.45 μm membrane and the volume was adjusted to 25 ml with methanol. The solutions were stored overnight at -12 ° C prior to the HPLC analysis. A small quantity of each extract was transferred into a screw-capped vial and placed in the HPLC system autosampler.

Table 1. Basic characteristics of the populations from where the plant materials of *Betonica bulgarica* were collected

Population No	Location, voucher number	North	East	Elev. m a.s.l.	Ecological conditions
1	Balkan Foothill Region, Lovnidol village, Pashova Livada area (SOA 062252)	42°59.079’	25°15.846’	368	Soil type – Cambisols (WRBSR, 2006). Herbaceous community dominated by *Festuca pratensis.* The terrain is slightly sloped (4 ° – 5 °), non-eroded, facing south-west.
2	Balkan Foothill Region, Lovnidol village, Above Avdjiiski trap area (SOA 062253)	43°01.327’	25°15.154’	503	Soil type – Cambisols (WRBSR, 2006). Herbaceous community dominated by *Trifolium pratense* L. The terrain is very slightly sloped (2 ° – 3 °), non-eroded, facing north-east.
3	Eastern Stara planina (the Balkan), Sinite kamani Natural; park, Karandilska poliana area (SOA 062254)	42°71.688’	26°36.872’	972	Open meadow of the clifs northwest. Herbaceous community dominated by *Betonica bulgarica.* The terrain is slightly sloped (4 ° – 5 °), non-eroded, facing north-east.
4	Eastern Stara planina (the Balkan), Sinite kamani Natural; park, Ablanovo area (SOA 062255)	42°42.638’	26°17.262’	540	Soil type – Chromic Luvisols (WRBSR, 2006); Open meadow on the edge of a mixed deciduous forest comprising *Carpinus betulus L., Quercus robur L., Ulmus minor Mill., Fraxinus ornus L. and Crataegus monogyna Jacq.* The herbaceous community is dominated by *B. bulgarica*. The terrain is very slightly sloped (3 ° – 4 °), non-eroded, facing south-east.

Analytical HPLC was performed with a C18 column Hypersil Gold (5 μm; 150 mm × 4.6 mm) on a Thermo system composed of a Surveyor LC Pump Plus, Surveyor Autosampler Plus, and Surveyor photodiode array detector PDA Plus. Quantitative analysis was performed in a 6-min run, isocratic mode, with methanol/acetonitrile/ water/acetic acid (40+20+39+1, v/v/v/v) at a flow rate of 0.8 ml.min^{-1}. The flavonoids were simultaneously identified using UV absorbance at 350 nm for hispidulin (HI), and 254 nm for rutin (RU) and quercetin (QU). The external calibration was carried out using five concentration levels (0.05, 0.5, 1.0, 2.0 and 5.0 mg.l^{-1}) of reference materials - rutin hydrate (min 94 %, HPLC), quercetin (min 98 %, HPLC) and hispidulin (min 98 %, HPLC), purchased from Sigma-Aldrich (St. Louis, MO). Each calibration standard was run in triplicate. The squared correlation coefficients (r^2) obtained by linear regression (0.9990 for RU, 1.000 for QU and 0.9995 for HI) demonstrated an excellent relationship between peak area and concentration according to the International Conference on Harmonization (ICH) guidelines [23]. Figure 1 illustrates a typical chromatogram of a standard solution containing 1 mg.l^{-1} rutin, quercetin and hispidulin. The retention times were ca 2.2 min for RU, ca 3.3 min for QU, and ca 4.9 min for HI.

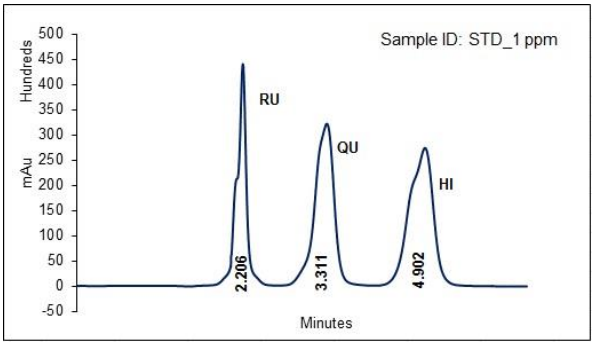

Figure 1. Typical chromatogram of a standard solution containing 1 mg.l^{-1} rutin, quercetin and hispidulin

Polyphenol determination

The collected methanol extracts were concentrated to a final volume of ca 7 ml by a rotary evaporator under vacuum at 30 ° C and transferred to a 10-ml volumetric flask. The dry matter of these methanol extracts was determined gravimetrically by drying 1 ml of each extract at 120 ° C for 6 hours. The experimental procedure described by Anesini *et al.* [24] was applied for determination of total polyphenol content (TPC). Briefly, 1 ml of the methanol plant extract with concentration of 0.2

mg.ml^{-1} or 1 ml of standard solution were mixed in separate tubes with 5.0 ml of Folin-Ciocalteu's reagent (1/10 dilution with water of the commercial reagent). Then, 4 ml of Na_2CO_3 in water (7.5 % w/v) was added and the tubes were left at room temperature for one hour. The absorbance at 765 nm was measured against water. Each sample was analyzed in triplicate. Gallic acid (Sigma-Aldrich, St. Louis, MO) solutions in methanol ranging from 0.1 to 10 μg.ml^{-1} were used for the calibration curve (R^2 = 0.998). TPC of each sample was expressed as mmol GAE in 1 kg dm of starting plant material.

Determination of radical scavenging activity by DPPH method

1,1'-diphenyl-2-picrylhydrazyl-radical (DPPH) was purchased from Sigma-Aldrich (St. Louis, MO). This substance has a single electron on the nitrogen atom and its solution in methanol has an absorption maximum at λ = 517 nm. The mechanism of the DPPH-method is based on the reaction between the test compound and DPPH-radical, wherein the potential free radical scavengers reduce the DPPH-radical (violet solution) to a yellow colored 1,1'-diphenyl-2-(2,4,6-trinitrorhenyl) hydrazine by donating a hydrogen atom.

The method described by Serpen *et al.* [25] was applied to measure the radical-scavenging potential of methanolic extracts obtained from different plant parts of *B. bulgarica*. Briefly, to 2 ml of a 60 μM solution of DPPH in methanol was added 20 μl of methanol extract (1 mg.ml^{-1}). Two parallel samples of each extract were analyzed. Absorption at 517 nm was measured 30 min later. Since the composition of the extracts is complex, the results for their radical-binding capacity were compared with that of Trolox (water-soluble analogue of Vitamin E) and calculated by regression analysis from the linear dependence between concentration of Trolox and absorption at 517 nm. The results were expressed as μmol Trolox equivalent in 1 kg dm of plant material.

Statistical data analysis

All analytical assays were carried out in duplicate or triplicate as specified above and the data are mean values ± standard deviation (SD). The Pearson correlation coefficients were determined using SPSS Statistics for Windows, Version 17.0 SPSS Inc. 2008, Chicago.

RESULTS AND DISCUSSION

A number of research teams, reviewed by Tundis *et al.* [26], have studied the polyphenol incl. flavonoid profile of *Stachys* species in relation to chemotaxonomy. Looking for new structures, they forgot the main and well-known ones, like rutin, quercetin, and hispidulin, which are widely spread in the plant world, including species from the genus *Stachys* [27]. These antioxidants were found in the object of the present study, Bulgarian endemic *B. bulgarica*. The identification and quantification of RU, QU and HI were carried out by HPLC-PDA method, described in the section Materials and Methods, by comparison with external standards of reference materials.

Five different plant parts from four populations of *B. bulgarica* were analyzed. The flavonoid content differences between organs in the populations were significant due to the specific growth conditions. In all of them, the rutin was in the largest quantity. The second one was quercetin except in the leaves, were the amount of hispidulin was higher than that of quercetin (Table 2). The obtained results clearly show significant differences in the flavonoid content between organs and locations.

The largest total content of RU, QU and HI in the tested plant was found in the leaves of *B. bulgarica* from two populations: Pashova livada and Above Avdjiiski trap, followed by seeds and roots, whose content was 22% and 15%, respectively, less than that in the leaves. The flavonoid content in the leaves of the other two populations (Karandilska poliana and Ablanovo) was 20% and 25%, respectively, and was lower than that measured in leaves from the first two populations. The highest flavonoid content was found in the endemic species from the population Above Avdjiiski trap, followed by Pashova Livada, Karandilska poliana and Ablanovo. Hajdari *et al.* [18] established the total flavonoid content in leaves and roots of *Betonica officinalis* L. from Kosovo. They found no significant total flavonoids differences between the localities for both organs and an about 3-fold higher content of flavonoids and polyphenols in leaves than in roots.

Table 2. Flavonoid content in different plant parts of *Betonica bulgarica* Degen et Neič from four studied populations (n=3 for organs in populations; n=20 for organs average)

Population No	Organ	Content, mean ± SD*, mg.kg^{-1} dm		
		Rutin	Quercetin	Hispidulin
1 Pashova Livada	Leaves	3789.3 ± 274.8	94.5 ± 7.6	202.5 ±14.1
	Flowers	1165.8 ± 90.4	431.0 ± 32.7	15.5 ±1.2
	Seeds	2638.4 ± 164.6	261.7 ± 19.8	255.7 ± 17.4
	Stems	209.9 ± 18.9	291.9 ± 23.6	143.9 ± 9.5
	Roots	3478.6 ± 306.4	36.2 ± 3.6	8.9 ± 0.7
2 Above Avdjiiski trap	Leaves	4941.7 ± 345.1	279.1 ± 21.2	412.7 ± 30.8
	Flowers	2324.2 ± 139.4	376.2 ± 26.9	42.9 ± 3.9
	Seeds	3702.6 ± 201.5	291.6 ± 19.5	304.0 ± 19.6
	Stems	277.6 ± 20.7	334.9 ± 24.3	158.4 ± 9.7
	Roots	4576.1 ± 364.3	104.7 ± 8.1	21.5 ± 1.7
3 Karandilska poliana	Leaves	1842.1 ± 128.3	174.3 ± 13.2	428.5 ± 34.9
	Flowers	981.9 ± 78.5	472.9 ± 32.4	22.4 ± 2.1
	Seeds	1322.4 ± 95.3	237.6 ± 14.8	408.8 ± 20.6
	Stems	123.0 ± 10.2	133.9 ± 9.3	104.6 ± 4.5
	Roots	1693.5 ± 98.8	67.3 ± 5.1	28.9 ± 1.8
4 Ablanovo	Leaves	1602.9 ± 109.4	103.2 ± 8.1	355.8 ± 24.0
	Flowers	713.6 ±59.2	328.8 ±23.4	14.1 ± 0.9
	Seeds	1168.3 ± 84.3	154.3 ± 11.3	319.5 ± 19.2
	Stems	104.6 ± 8.4	198.0 ± 15.5	112.1 ± 4.7
	Roots	1492.8 ± 119.7	40.2 ± 3.0	7.9 ± 0.7

*SD- Standard Deviation

Table 3. Factor influence on distribution of *Betonica bulgarica* Degen et Neič populations

Population	Factor 1	Factor 2	Factor 3
1 Pashova Livada	-0.03692	0.387644	-0.02283
2 Above Avdjiiski trap	-2.36332	-0.10301	0.016024
3 Karandilska poljana	0.82401	-0.29183	-0.03496
4 Ablanovo	1.57623	0.007197	0.041765

Principle component analyses for distributions of *B. bulgarica* population depending on the content of rutin, quercetin and hispidulin by plant parts demonstrate that three main factors can be defined (Figure 2). Eigen value for these factors was F1- 2.917; F2 - 0.082: F3 - 0.001. Factor 1 has the greatest influence describing 97.22 % of the variations, while Factor 2 describes 2.73 % and Factor 3 – 0.042%. Distribution of the population shows that *B. bulgarica* originated from Karandilska poliana is positive for F1 describing 97.22 % of the variations, but is negative by F 2 (Table 3). Population from Ablanovo is positive by the two factors, while population above Avdjiiski trap is negative by the two factors (Figure 2).

The methanolic extracts of *B. bulgarica* were also tested for total phenolic content (TPC) and radical scavenging activity by the DPPH method. The results obtained are shown in Table 4. The GAE-equivalents between organs and localities were significant different. The antioxidative activity of root extracts (Trolox equivalents) did not show any significant difference between the localities.

The highest total phenolic content was found in leaves, followed by flowers, seeds, roots and stems. This distribution was valid for all populations. The largest TPC was found in the species of *B. bulgarica* from the population Above Avdjiiski trap, followed by Pashova livada, Karandilska

poliana and Ablanovo, the same population ranking as for flavonoid content.

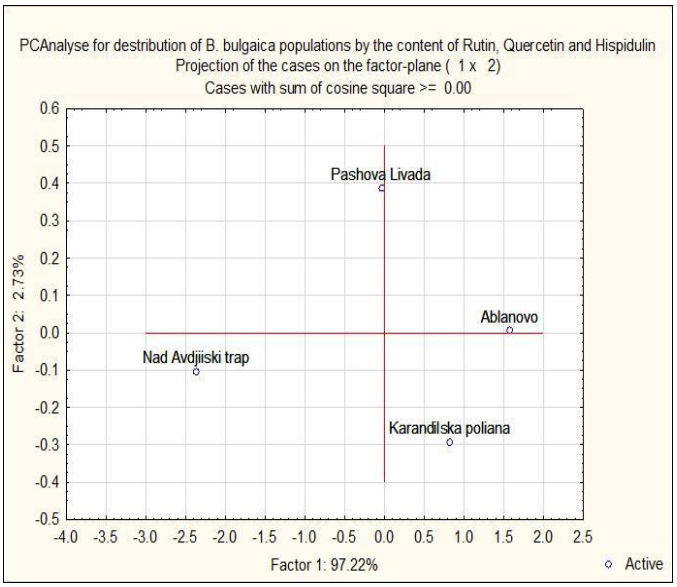

Figure 2. Component analysis for distribution of the four studied populations of *Betonica bulgarica* Degen et Neič

The radical scavenging activity of the *B. bulgarica* from the population Above Avdjiiski trap was the highest one, followed by Pashova livada, Karandilska polyana and Ablanovo. The most potent radical scavenging capacity had the methanolic extracts obtained from leaves followed by seeds, flowers, roots and stems. Within one and the same population the results for antioxidant potential of the seeds, flowers, and roots were similar.

Table 4. TPC and antioxidant activity in different organs of *Betonica bulgarica* Degen et Neič from four populations

Population	Organ	mmol GAE eq. kg^{-1} dm	μmol Trolox eq. kg^{-1} dm
1	Leaves	80.64 ± 5.23	52.73 ± 2.80
Pashova	Flowers	71.02 ±4.12	37.28 ± 1.53
Livada	Seeds	62.92 ± 4.25	43.41 ± 2.09
	Stems	28.21 ± 1.91	8.16 ± 0.35
	Roots	59.13 ± 3.55	38.49 ± 1.97
2	Leaves	122.43 ± 6.77	94.19 ± 5.92
Above	Flowers	91.32 ± 5.49	72.45 ± 3.61
Avdjiiski	Seeds	83.03 ± 3.74	60.64 ±3.07
trap	Stems	32.22 ± 1.87	20.76 ± 1.05
	Roots	72.69 ± 3.58	51.44 ± 2.49
3	Leaves	91.83 ± 4.33	63.25 ± 3.06
Karandilska	Flowers	87.02 ± 3.89	52.29 ± 2.21
poliana	Seeds	72.17 ± 4.06	60.64 ± 2.97
	Stems	25.06 ± 1.66	9.16 ± 0.44
	Roots	79.91 ± 4.01	50.26 ± 2.69
4	Leaves	90.84 ± 4.63	52.26 ± 2.76
Ablanovo	Flowers	68.64 ± 3.47	55.71 ± 3.11
	Seeds	69.39 ± 3.75	39.76 ± 2.18
	Stems	26.87 ± 1.37	12.66 ± 0.76
	Roots	44.81 ± 2.22	26.12 ± 1.83

All indicators were normally distributed by the One-Sample Kolmogorov-Smirnov test. The methanolic extracts of *B. bulgarica* showed a similar TPC and DPPH radical scavenging capacity compared with other species of the *Lamiaceae*, such as the leaves of *Stachys sylvatica* and leaves of *Betonica officinalis* [18, 28].

Consistent with most polyphenolic antioxidants, both the configuration and the total number of hydroxyl groups in flavonoids structure substantially influence their antioxidant activity. Free radical scavenging capacity is primarily attributed to the high reactivity of phenol group that participates in the following reaction:

$$\text{Flav-OH} + \text{R}^{\cdot} \longrightarrow \text{Flav-O}^{\cdot} + \text{RH}$$

Single electron delocalization makes this reaction thermodynamically favorable and the free radical formed may further react with a second radical; a reaction that turns the phenolic group into a stable quinone structure.

Correlation between TPC and antioxidant activity, tested by the DPPH method, of *Stachys* species was found by a number of research teams [17, 18, 26]. Several possible mechanisms of the demonstrated antioxidant properties of flavonoids have been proposed [29]; among them are direct scavenging of reactive oxygen species and metal chelating properties. In our *in vitro* study a good Pearson correlation between radical scavenging activity and TPC at significance level $p \leq 0.01$ was found (Figure 3) underlying the importance of polyphenol moiety in flavonoid structure for radical scavenging potency.

Comparison between mmol gallic acid equivalents and μmol Trolox equivalents in kg dry plant material, and concentration of the three quantified flavonoids in different plant parts on the other hand also showed good correlations (Figure 4, panel A and panel B). The Pearson correlation was established and showed positive dependence.

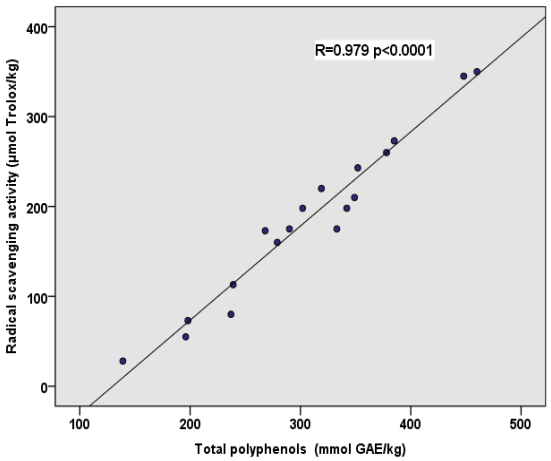

Figure 3. Pearson correlation between TPC and antioxidant activity by significant level, $P \leq 0.01$ (2-tailed)

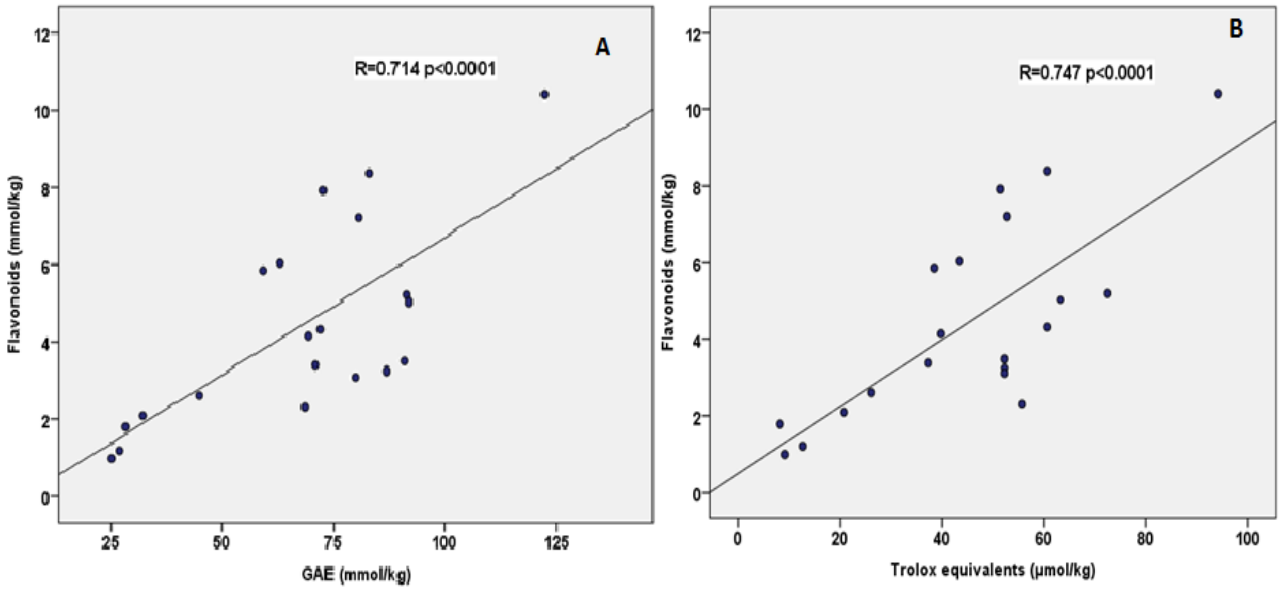

Figure 4. Pearson correlation between flavonoid content and TPC (panel A) and flavonoid content and antioxidant activity (panel B), $P \leq 0.01$ (2-tailed)

The correlation coefficients were lower: 0.714 and 0.746, respectively. Most likely the phenolic compounds including flavonoids present in the leaves are responsible for the high antioxidative capacity of these parts of plants. In roots and seeds, antioxidant capacities assayed by the two methods were not so well correlated with flavonoid content indicating that different substances aside from flavonoids might be responsible for the specific antioxidant effects.

Based on the results obtained, the main conclusions that can be drawn are:

- The Bulgarian endemic *B. bulgarica* contains three major flavonoids: rutin, quercetin and hispidulin, in good quantities;
- *B. bulgarica* has lower antioxidant activity than the other studied *Stachys* species [11, 17, 18, 30];
- The correlation between flavonoid content and TPC and flavonoid content and antioxidant activity is very high.
- The highest flavonoid and polyphenol concentrations were found in the leaves;

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REFERENCES

1. Biological diversity act, Republic of Bulgaria, National Assembly, Promulgated, State Gazette No 77/9.08.2002.
2. E. Genova, Betonica bulgarica Deg. et Neic. in: Plants & Fungi, Red Data Book of the Republic of Bulgaria, Peev D (eds), vol. 1, 2011.
3. M. Sharifzadeh, K. Sharifzadeh, M. Khanavi, A. Hadjiakhoondi, A. Shafiee, *Int. J. Pharmacol.*, **1**, 132 (2005).
4. J. Kukik, S. Dobric, S. Petrovic S., *Pharm. Biol.*, **45**, 560 (2007).
5. S. Grujic-Jovanovic, H. D. Skaltsa, P. Marin, M. Sokovic, *Flavour Frag. J.*, **19,** 139 (2004).
6. A. Sonboli, P. Salehi, S. N. Ebrahimi, *Chem. Nat. Comp.*, **41**,171 (2005).
7. Z. Amirghofran, M. Bahmani, A. Azadmehr, K. Javidnia, *Neoplasma*, **53,** 428 (2006).
8. Z. Amirghofran, M. Bahmani, A. Azadmehr, K. Javidnia, *Med. Sci. Monit.* **13**, 145 (2007).
9. A. Aydin, B. Sener, I. Cakici, N. N. Turan, N. Erdemoglu, *Phytother. Res.*, **20**, 91 (2006).
10. 10. J. Kukik, S. Petrovic, M. Niketic,*Biol. Pharm. Bull.*,**29**, 725 (2006).
11. A. Matkowski, M. Piotrowska, *Fitoterapia*, **77**, 346 (2006).
12. M. Wiesenauer, Homöopathie für Apotheker und Ärzte, Deutscher Apotheker Verlag, Stuttgart, 2000, p. 184.
13. W. Blaschek, S. Ebel, E. Hackenthal, U. Holzgrabe, K. Keller, J. Reichling, V. Schulz, Hagers Enzyklopädie der Arzneistoffe und Drogen, Wissenschaftliche Verlagsgesellschaft GmbH, Stuttgart, 2007, p. 829
14. V. Bankova, J. Koeva-Todorovska, T. Stambolijska, M. D. Ignatova-Groceva, D. Todorova, S. Popov, *Z. Naturforsch.*, **54c**, 876 (1999).
15. I. Dimitrova-Dyulgerova, P. Merdzhanov, K. Todorov, D. Seymenska, P. Stoyanov, R. Mladenov, A. Stoyanova, *Comptes rendus de l'Academie bulgare des Sciences*, **68(8)**, 991 (2015).
16. V. Bilušić-Vundać, A. H. Brantner, M. Plazibat, *Food Chem.*, **104** 1277 (2007).
17. M. Khanavi, M. Hajimahmoodi, M. Cheraghi-Niroomand, Z. Kargar, Y. Ajani, A. Hadjiakhoondi, M. R. Oveisi, *African J. Biotech.*, **8(6)**, 1143 (2009).
18. A. Hajdari, B. Mustafa, Ch. Franz, J. Novak, Total Flavonoids, *Acta Horticult.*, **860**, 75 (2010).
19. I. Sliumpaitea, R. P. Venskutonisa, M. Murkovicb, O. Ragazinskiene, *Ind. Crops and Products*, **50**, 715 (2013).
20. K. Bączek, O. Kosakowska, J. L. Przybył, Z. Węglarz, *Herba Polonica*, **62(2)**, 7 (2016).
21. H. Hajimehdipoor, B. M. Kondori, G. R. Amin, N. Adib, H. Rastegar, M. Shekarchi, *J. Pharm. Sci.*, **20**, 57 (2012).
22. K. Ashokkumar, K. Selvaraj, S. K. M. Devi, *J. Med. Plants Res.*, **7(47)**, 3393 (2013).
23. Validation of Analytical Procedures: Methodology. ICH, Q2B, FDA, Federal Register, 1997.
24. C. Anesini, G. E. Ferraro, R. Filip, *J. Agr. Food Chem.*, **56(19)**, 9225 (2008).
25. A. Serpen, E. Capuano, V. Fogliano, V. Gökmen, *J. Agr. Food Chem*., **55(19)**, 7676 (2007).
26. R. Tundis, L. Peruzzi, F. Menichini, *Phytochemistry*, **102**, 7 (2014).
27. V. Vundać, Z. Maleš, M. Plazibat, P. Golja, B. Cetina-Čižmek, *J. Planer Chromatography*, **18**, 104 (2007).
28. A. Hajdari, B. Mustafa, A. Kaçiku, X. Mala, B. Lukas, A. Ibraliu, G. Stefkov, J. Novak, Chemical Composition of the Essential Oil, Total Phenolics, *Rec. Nat. Prod.*, **10(6)**, 750 (2016).
29. D. Procházková, I. Boušová, N. Wilhelmová, *Fitoterapia*. **82(4)**: 513 (2011).
30. G. Miliauskas, P. R. Venskutonis, T. A. Van Beek, *Food Chem.*, **85**, 231 (2004).

СЪДЪРЖАНИЕ НА ФЛАВОНОИДИ И АНТИОКСИДАНТНА АКТИВНОСТ НА *BETONICA BULGARICA* DEGEN ET NEIČ

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(Резюме)

Българският ендемит *Betonica bulgarica* Degen et Neič е защитен вид от Закона за биологичното разнообразие и е включен в Червената книга на България под категорията "застрашени". Целта на това изследване е да се определи съдържанието на флавоноиди, както и антиоксидантната активност на различни органи на растението (листа, цветове, корени, стебла и семена) от четири популации. В значителни количества са определени три флавоноида: рутин, кверцетин и хиспидулин. В най-голямо количество е рутинът, последван от кверцетина и хиспидулина. С най-високо флавоноидно съдържание се отличават листата, след което се нареждат корените и цветовете. Атиоксидантната активност е тествана чрез DPPH-метод. Определено е също така и общото полифенолно съдържание. Установена е положителна корелация между флавоноидното съдържание и антиоксидантната активност на изследваните органи на растението.

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In vivo evaluation of the antioxidant potential of dicaffeoylquinic acid isolated from *Geigeria alata*

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Geigeria alata (DC) Oliv. & Hiern. (Asteraceae) is an aromatic medicinal plant used in traditional Sudanese medicine, with a number of biological activities including suggested antidiabetic potential. The objective of this study was to evaluate the antioxidant properties of 3,5-dicaffeoylquinic acid (diCQA), the major compound isolated from *Geigeria alata* roots extract in an experimental model of streptozotocin-induced type 2 diabetes in male Wistar rats. Diabetes results in severe organ pathology which main pathophysiological mechanisms are related to oxidative stress, discerned by increased production of malondialdehyde (MDA) and disturbance in both non-enzymatic (GSH) and enzymatic (GPx, GR, GST) antioxidant defense. DiCQA (5 mg/kg/po) administered for 21 days to control and diabetic Wistar rats ameliorated the activity of the antioxidant enzymes and the levels of the cellular protector GSH, as well as reduced the production of MDA. It also exerts antidiabetic effect in diabetic rats. On the basis of these results, as well as knowing that formation of ROS is considered to be one of the mechanisms in the pathogenesis of diabetes we concluded that diCQA isolated from *Geigeria alata* possesses antioxidant properties which most probably determined its *in vivo* antidiabetic activity.

Keywords: 3,5-Dicaffeoylquinic acid, Diabetes, Oxidative stress, Rats

INTRODUCTION

Oxidative stress now appears to be one of the fundamental mechanisms underlying a number of human disorders, like neurological, endocrine and others. In relation to this knowledge, antioxidants, preferably from natural sources, are used both to prevent the development of such disorders or to support their conventional treatment. Among the perspective biologically active compounds with antioxidant potential are carotenoids, phenolic acids, flavonoids, phenolic diterpenes and others [1]. Acylquinic acids, often called chlorogenic acids, are a group of esters formed between trans-cinnamic acids and (-)-quinic acid. Multiple acylquinic acid isomers usually co-exist in plants, most notably in some species from the Asteraceae family [2]. They demonstrate a variety of biological activities: enhance the accumulation of bile and reinforce the secretion of pancreatic enzymes, slow the aging process, regulate the lipid metabolism and weight gain, have anti-inflammatory and high antioxidant potential [3].

Geigeria alata (DC) Oliv. & Hiern is an aromatic plant belonging to the Asteraceae family found in northern and central Sudan. The roots and leaves are reputed in Sudanese folk medicine to be effective against epilepsy, pneumonia, and rheumatism. In addition, aerial parts are used for the treatment of cough and intestinal complaints. *G. alata* also showed α-glucosidase inhibitory potential with which the antidiabetic effect of an aqueous-methanol roots extract in streptozotocin-induced diabetic rats was explained [4].

As a part of our ongoing investigation of Sudanese medicinal plants, we reported the isolation of acylquinic acids from *G. alata* roots and their contribution to antioxidant and antimicrobial plant capacity [5]. Our experiments, using high-resolution mass spectrometry (LC-HRMS) revealed that the main compounds in *G. alata* roots extract belong to the group of phenolic and cafeoilquinic acids (mono-, di- and tricaffeoylquinic acids, *p*-coumaroylquinic, caffeoylsinapoylquinic, caffeoylferuloylquinic and feruloylquinic acids). 3,5-dicaffeoylquinic acid was the most abundant acylquinic acid in the hroots, being present at 25.96 ± 2.08 mg/g dry weight. The *in vitro* free radical scavenging potential of 3,5-diCQA was investigated using antiradical scavenging activity against (2,2-diphenyl-1-picrylhydrazyl) radical, ABTS and ferric reducing antioxidant power (FRAP) methods. The 3,5-diCQA demonstrated strong radical-scavenging

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activity, higher than that of 5-chlorogenic acid (5-CQA) [5].

On the basis of this information the aim of the current study was to investigate the *in vivo* antioxidant potential of 3,5-diCQA in an experimental model of streptozotocin-induced type 2 diabetes in rats.

MATERIALS AND METHODS

Plant material and isolation of the 3,5-dicaffeoylquinic acid (diCQA)

Geigeria alata roots were collected in July 2011 from west Kordofan (Sudan). Botanical identification was performed by Dr. Wail El Sadig, and a voucher specimen № 41935/HNC was deposited in the herbarium of Botany Department, Faculty of Sciences, University of Khartoum, Sudan.

Air-dried roots were stored at room temperature and protected from the light. Powdered dried roots (300 g) of *G. alata* were extracted with aqueous methanol (80 %, v/v) by ultrasound assisted extraction (2×15 min). Sample-solvent ratio was 1:10 (w/v). The combined extracts were concentrated under vacuum at 40°C. The crude extract was purified by solid-phase extraction (SPE) on Vac Elut 10 vacuum manifold (Varian, Walnut Creek, CA, USA). Aliquots of the crude extract (1 g) were fractionated on cartridges Strata C18-E, 10 g/60 ml (Phenomenex, USA). After loading samples on previously conditioned cartridges, and washing with 10 ml of water, the elution step was accomplished consequently with 30%, 70% and 100% methanol. Eluates obtained with methanol concentration of 30% were purified by repeated low-pressure liquid chromatography as described earlier to yield 0.950 g of 3,5-dicaffeoylquinic acid (diCQA) [5].

Liquid chromatography–mass spectrometry (LC-MS)

The identity of diCQA was verified by ultrahigh-performance liquid chromatography – high resolution mass spectrometry (UHPLC-HRMS). LC-MS analysis was performed on Q Exactive mass spectrometer (ThermoScientific Co, Waltham, MA, USA) equipped with heated electrospray ionization module IonMax® (ThermoScientific Co, Waltham, MA, USA) and TurboFlow Ultra High Performance Liquid Chromatography (UHPLC) system (ThermoScientific Co, Waltham, MA, USA).

The chromatographic analysis was carried out by Syncronis® C18 column (2.1 mm × 50 mm i.d., 1.7 μm) using as eluents: (A) 0.1% formic acid in water and (B) 0.1% formic acid in acetonitrile at a flow rate of 300 μl/min. The following binary gradient was used: 10% B for 1 min; 10-60% B for 8.0 min; 60-100% B for 2.0 min; 100% B for 1 min and 100-10% B for 2.0 min. Spray voltage at 4.2 kV, sheath gas flow rate 35 AU, auxiliary gas flow 8 AU, capillary temperature 320º C, probe heater temperature 300º C and S-lens level 50 were adjusted for the interface. Full-scan mass spectra over the *m/z* range 150-1800 were acquired in negative ion mode at resolution settings of 140 000. Targeted MS^2 mode at resolution settings of 17 500 and 1.0 amu isolation window of precursor ions was used for structural elucidation study. Data were processed using XCalibur® (ThermoScientific Co, Waltham, MA, USA) instrument control/data handling software.

Deprotonated molecule $[M-H]^-$ was observed at *m/z* 515.12. The MS/MS spectrum of diCQA gave the fragment ions as follows: 191.06 (100% relative abundance), 179.03 (80.04), 135.04 (14.89), 353.09 (8.58), 173.04 (6.50), 161.02 (4.70), 155.03 (1.56), 335.08 (0.85).

Animals

Male Wistar rats (body weight 200–250 g) were used. The rats were housed in plexiglass cages (3 per cage) in a 12/12 light/dark cycle, under standard laboratory conditions (ambient temperature 20°C ± 2°C and humidity 72% ± 4%) with free access to water and standard pelleted rat food 53-3, produced according to ISO 9001:2008. The animals were purchased from the National Breeding Center, Sofia, Bulgaria. Seven days acclimatization was allowed before the commencement of the study and a veterinary physician monitored the health of the animals regularly. Vivarium (certificate of registration of farm № 0072/01.08.2007) was inspected by the Bulgarian Drug Agency in order to check the husbandry conditions (№A-11-1081/03.11.2011). All performed procedures were approved by the Bulgarian Food Safety Agency (BFSA) and the principles stated in the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (ETS 123) [6] were strictly followed during the experiment.

Chemicals

All the reagents used were of analytical grade. Streptozotocine, beta-nicotinamide adenine dinucleotide 2-phosphate reduced tetrasodium salt (NADPH), ethylenediaminetetraacetic acid (EDTA), bovine serum albumin (fraction V), 2,2-dinitro-5,5-dithiodibenzoic acid (DTNB) were obtained from Merck (Darmstadt, Germany).

Reduced glutathione (GSH), oxidized glutathione (GSSG), glutathione reductase (GR), and cumene hydroperoxide were purchased from Sigma Chemical Co. (Taufkirchen, Germany).

Induction of diabetes

Prior to induction of diabetes, the rats were fasted for at least 16 hours. Diabetes was induced in rats by intraperitoneal injection of streptozotocine (STZ) (40 mg/kg body weight) [7], dissolved in 0.1M citrate buffer, pH 4.5. Another group of rats which served as control was injected with citrate buffer alone without STZ. Forty eight hours after STZ injection, diabetes was confirmed by measuring blood glucose concentrations (using an Accu-Chek Glucometer, Roche, Germany) in blood samples taken from tail vein. Rats with blood glucose levels of 9 mmol/L or more were considered to be diabetic and included in the study.

Design of the experiment

The animals were divided into eight groups (n=6) as follows:

Group 1 (C): Control animals, treated with the saline vehicle, administered by gavage at 5 mL/kg bw/day for 21 days. On day 7 of the experiment the animals received an i.p. injection with citrate buffer.

*Group 2 (*CQA*):* Animals treated with the positive control chlorogenic acid (5 mg/kg bw/day, oral-gavage) for 21 days.

*Group 3 (*3,5-diCQA*):* Animals treated with 3,5-dicaffeoylquinic acid at 5 mg/kg bw/day, oral-gavage for 21 days.

Group 4 (DM): Animals challenged with 40mg/kg bw, i.p. streptozotocin (STZ) dissolved in 0.1M citrate buffer, pH 4.4.

*Groups 5 (DM+CQA) and 6 (DM+*3,5-diCQA*):* Up to day 7th the animals were treated the same way as groups 2 and 3. On day 7th the animals were challenged with STZ (40 mg/kg bw, i.p.) and after that continued to be treated with chlorogenic acid at 5 mg/kg bw/day (group 5) and 3,5-diCQA at 5 mg/kg bw/day (group 6) for additional 14 days.

On the 22nd day of the experiment, blood has been collected from the tail vain of all animals and the glucose levels have been measured. After that, the animals in all groups were sacrificed and the livers were taken to assess the oxidative stress biomarkers – MDA, GSH, and the antioxidant enzymes GR, GPx and GST. For all following experiments the excised livers were perfused with cold saline solution (0.9% NaCl), blotted dry, weighed, and homogenized with appropriate buffers.

Preparation of liver homogenate for MDA assessment

Lipid peroxidation was determined by measuring the rate of production of thiobarbituric acid reactive substances (TBARS) (expressed as malondialdehyde (MDA) equivalents) described by Polizio and Peña [8] with slight modifications. One volume of homogenate was mixed with 1 mL of 25% trichloroacetic acid (TCA) and 1 mL of 0.67% thiobarbituric acid (TBA). Samples were then mixed thoroughly, heated for 20 min in a boiling water bath, cooled and centrifuged at 4000 rpm for 20 min. The absorbance of supernatant was measured at 535 nm against a blank that contained all the reagents except the tissue homogenate. MDA concentration was calculated using a molar extinction coefficient of $1.56\ 10^5\ M^{-1}\ cm^{-1}$ and expressed in nmol/g wet tissue.

Preparation of liver homogenate for GSH assessment

GSH was assessed by measuring non-protein sulfhydryls after precipitation of proteins with TCA, using the method described by Bump *et al.* [9]. Tissues were homogenized in 5% trichloroacetic acid (TCA) and centrifuged for 20 min at 4 000 ×g. The reaction mixture contained 0.05 mL supernatant, 3 mL 0.05 M phosphate buffer (pH = 8), and 0.02 mL DTNB reagent. The absorbance was determined at 412 nm and the results expressed as nmol/g wet tissue.

Assessment of antioxidant enzymes activity

The antioxidant enzymes activity was measured in the supernatant of 10% homogenates, prepared in 0.05M phosphate buffer (pH=7.4). The protein content of liver homogenate was measured by the method of Lowry [10]. Glutathione peroxidase activity (GPx) was assessed by NADPH oxidation, using a coupled reaction system consisting of glutathione, GR, and cumene hydroperoxide [11]. Glutathione reductase activity (GR) was measured spectrophotometrically at 340 nm according to the method of Pinto *et al*. [12] by following NADPH oxidation. GST was measured using CDNB as substrate [13].

Statistical analysis

Statistical analysis was performed using statistical programme 'MEDCALC'. Results are expressed as mean ± SEM for six rats in each group. The significance of the data was assessed using the non-parametric Mann–Whitney *U* test. Values of $P \leq 0.05$ were considered statistically significant.

RESULTS

Changes in blood glucose and body weight

All animals survived until the end of the treatment period. During treatment, there were no observed changes in behavior or in food and water consumption among the animals in either the control or treated groups. The initial and final body weights, as well as the body weight changes observed during treatment are presented in Table 1. The animals in the DM group had statistically significantly lower final body weight when compared to the control animals. In all other groups, the mean body weight was comparable to the control. Streptozotocin administration (DM group) resulted in increased blood glucose levels by 77 % ($p<0.05$). The results are presented in Table 1.

Table 1. Mean body weights and blood glucose levels – the effect of 3,5-dicafeoilquinic acid on control and diabetic rats.

Group	Mean body weight (g)			Blood glucose (mmol/L)
	Initial	Final	Change	
Control	205 ± 3	255 ± 5	50	5.2 ± 0.46
CQA	190 ± 3	258 ± 4	68	5.8 ± 0.40
3,5-diCQA	210 ± 4	260 ± 4	50	5.4 ± 0.39
DM	215 ± 3	235 ± 3*	20*	9.2 ± 0.69*
DM+CQA	205 ± 3	262 ± 6^{+}	57^{+}	5.9 ± 0.50^{+}
DM+ 3,5-diCQA	212 ± 3	258 ± 4^{+}	46^{+}	6.0 ± 0.49^{+}

Data are expressed as mean ± SEM of six rats (n = 6). For comparison between groups Mann—Whitney U test was performed. *$p<0.05$ *vs* control, $^{+}p<0.05$ *vs* DM

It is interesting to note that the treatment of the diabetic rats with both 3,5-diCQA and CQA resulted in a statistically significant decrease in blood glucose levels by around 35 % ($p<0.05$) when compared to the DM group.

Markers of oxidative stress

The quantities of the oxidative stress marker MDA and the levels of GSH are presented in Table 2.

Table 2. Effect of 3,5-dicafeoilquinic acid on MDA quantities and GSH levels in control and diabetic rats

Group	MDA (μmol/g wet tissue)	GSH (μmol/g wet tissue)
Control	1.27 ± 0.36	7.32 ± 0.36
CQA	1.35 ± 0.15	7.29 ± 0.54
3,5-diCQA	1.30 ± 0.16	7.05 ± 0.64
DM	4.80 ± 0.57*	4.57 ± 0.28*
DM + CQA	2.65 ± 0.45^{+}	5.35 ± 0.35^{+}
DM + 3,5-diCQA	2.70 ± 0.43^{+}	6.01 ± 0.38^{+}

Data are expressed as mean ± SEM of six rats (n = 6). For comparison between groups Mann—Whitney U test was performed. *$p<0.05$ *vs* control, $^{+}p<0.05$ *vs* DM

In the DM group rats, the MDA production was nearly three times higher and GSH levels were reduced by 37 % ($p<0.05$) when compared to the control. 3,5-dCQA administered to diabetic rats normalizes the levels of both parameters to those of the control. Compared to diabetic rats, 3,5-diCQA decreased the production of MDA by 44 % ($p<0.05$) and increased the GSH levels by 31 % ($p<0.05$). The effect of 3,5-diCQA was comparable to that of CQA, used as positive control.

Changes in antioxidant enzymes

In this study the activity of the antioxidant enzymes related to GSH turnover was measured. The results are shown in Table 3. Compared to control animals, the activity of GPx, GR and GST was statistically significantly ($p<0.05$) decreased by 26 %, by 38 % and by 18 %, respectively, in the rats from the DM group. Compared to the DM group, 3,5-diCQA treatment significantly restored the enzymatic activity as follows: GPx activity was increased by 21 % ($p<0.05$), GR activity – by 25 % ($p<0.05$) and GST activity – by 14 % ($p<0.05$). The effect was commensurable with that of CQA.

Table 3. Effect of 3,5-dicafeoilquinic acid on the activity of the antioxidant enzymes: GR, GPx and GST in control and diabetic rats

Group	GR (μmol/mg/min)	GPx (μmol/mg/min)	GST (μmol/mg/min)
Control	0.26 ± 0.031	0.46 ± 0.03	1.53 ± 0.088
CQA	0.27 ± 0.016	0.48 ± 0.02	1.56 ± 0.056
3,5-diCQA	0.25 ± 0.033	0.43 ± 0.05	1.54 ± 0.102
DM	0.16 ± 0.022*	0.34 ± 0.03*	1.25 ± 0.062*
DM + CQA	0.20 ± 0.015^{+}	0.42 ± 0.02^{+}	1.48 ± 0.035^{+}
DM + 3,5-diCQA	0.19 ± 0.015^{+}	0.41 ± 0.01^{+}	1.43 ± 0.070^{+}

Data are expressed as mean ± SEM of six rats (n = 6). For comparison between groups Mann—Whitney U test was performed. *p<0.05 *vs* control, $^{+}$p<0.05 *vs* DM

DISCUSSION

Type 2 diabetes is characterized by insulin resistance and inability of the beta cell to sufficiently compensate. One of the discussed mechanisms in the pathogenesis of the secondary complications of diabetes is oxidative stress. The evidence for oxidative damage in diabetic patients has been reported as far back as 1979 by Sato *et al.* [14] who reported that the average level of lipid peroxide in plasma is higher in diabetic patients than in healthy controls. It is proved that hyperglycemia generates reactive oxygen species (ROS) by several mechanisms. Giacco and Brownlee [15] showed that persistent hyperglycemia can enhance the oxidative stress by increasing glucose auto-oxidation, nonenzymatic protein glycation, and activation of polyol pathway. The oxidative cell damage ultimately results in vascular complications as a secondary damage in diabetes.

Along with the conventional drug therapy of diabetes, recently, attention has been directed towards nutraceuticals originating from plants that are rich in antidiabetic phyto-constituents and antioxidants. In this context, the effect of antioxidant supplementation on oxidative stress in diabetes has been extensively studied [16]. In this study, our attention was focused on *Geigeria alata*, Asteraceae, a Sudanese plant, used for the management of diabetes in Sudanese traditional medicine. Its effective use was supported by the observed antidiabetic effect of an aqueous-methanol roots extract in streptozotocin-induced diabetic rats [4]. *Geigeria alata* roots extract is rich in phenolic acids, mainly cafeoilquinic acids. 3,5-diCQA is the major component for which a potent free radical scavenging potential *in vitro* has been proven [5]. In order to extend our studies on the antioxidant activity of 3,5-diCQA, the aim of the current study was to investigate its antioxidant potential, applying a model of streptozotocin-induced diabetes in rats.

In experimental toxicology the induction of type 2 diabetes in rodents is an appropriate model used to investigate the effects of the diabetes alone, as well as to test drugs and therapies which main mechanism is related either to decrease the insulin resistance or to ameliorate some of the secondary mechanisms involved in the complications of this disease, such as the ROS formation. Streptozotocin is a common chemical of choice for inducing diabetes in experimental animals due to its irreversible damage to pancreatic β-cells [18]. In the scientific literature a dose range from 20 up to 200 mg/kg bw has been reported. In our study we clearly demonstrated that streptozotocin at a dose of 40 mg/kg was able to induce a sustained hyperglycemia in rats, discerned by significant increase of the blood glucose levels (see Table 1). The induced hyperglycemia was accompanied by oxidative stress, judged by increased production of MDA and depletion of GSH levels (see Table 2). Our results are in good agreement with the effects of diabetes reported in the scientific literature. Bandeira *et al*. [19] reported an increased MDA quantity in plasma, serum, and many tissues in diabetic patients. Decreased antioxidant defense, enzymatic and non-enzymatic alike is also reported as one of the main characteristics of diabetes [20-22]. In our study, STZ administration resulted in decreased activity of antioxidant enzymes: GR, GPx and GST (see Table 3). The treatment of the diabetic rats with 3,5-diCQA resulted in decreased production of MDA and normalized levels of GSH and antioxidant enzymes. These results proved the antioxidant potential of the studied compound. What is interesting to be noted is that 3,5-diCQA exerted an antidiabetic effect, discerned by decreased level of plasma glucose in STZ-induced diabetic rats (see Table 1). The effect was statistically significant, compared to the diabetic group and was commensurable with that of the chlorogenic acid, for which experimental data about its hypoglycemic activity are available [23, 24].

CONCLUSION

In the current study the *in vivo* antioxidant activity and possible antidiabetic potential of 3,5-

diCQA, the major compound isolated from *Geigeria alata* roots extract were investigated using a model of streptozotocin-induced type 2 diabetes in rats. Under the conditions of this study we could conclude that 3,5-diCQA showed potent *in vivo* antioxidant potential which confirms and supports our previous studies on the *in vitro* free radical scavenging activity of 3,5-diCQA. The tested compound also exerted a hypoglycemic activity, which is most probably due to its antioxidant properties. This study provides support for the use of natural antioxidants, in our case 3,5-diCQA, isolated from *G. alata*, in the supplementation therapy for reducing the level of oxidative stress and slowing or preventing the development of complications associated with diabetes.

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REFERENCES

1. P. Pietta, P. Simonetti, P. Mauri, *J. Agric. Food Chem.*, **46,** 4487 (1998).
2. A. Plazonić, F. Bucar, Ž. Maleš, A. Morna, B. Nigović, N. Kujundžić, *Molecules*, **14**, 2466 (2009).
3. H. Morishita, M. Ohnishi, *Stud. Nat. Prod. Chem.*, **25,** 919 (2001).
4. R. Hafizur, R. Babiker, S. Yagi, S. Chishti, N. Kabir, M. Choudhary, *J. Endocrinol.*, **214,** 329 (2012).
5. D. Zheleva-Dimitrova, R. Gevrenova, M. M. Zaharieva, H. Najdenski, S. Ruseva, V. Lozanov, V. Balabanova, S. Yagi, G. Momekov, V. Mitev, published on-line in Wiley Online Library, *Phytochem. Anal.*, **28**, 176 (2017).
6. Council of Europe. European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes. CETS No. 123, 1991, displayed 30 May 2007
7. V.A.V. Costa, L.M. Vianna, *Braz. Arch. Biol. Technol.*, **51,** 43 (2008).
8. A.H. Polizio, C. Peña, *Regul. Pept.*, **128**, 1 (2005).
9. E.A. Bump, Y.C. Taylor, J.M. Brown, *Cancer Res.*, **43**, 997 (1983).
10. O.H. Lowry, N.J. Rosebrough, A.L. Farr, R.J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
11. A.L. Tappel, *Method. Enzymol.*, **52,** 506 (1978).
12. M.C. Pinto, A.M. Mata, J. Lopez-Barea, *Arch. Biochem. Biophys.*, **228,** 1 (1984).
13. W.H. Habig, M.J. Pabst, W.B. Jakoby, *J. Biol. Chem.*, **249,** 7130 (1974).
14. Y. Sato, N. Hotta, N. Sakamoto, S. Matsuoka, N. Ohishi, K. Yagi, *Biochem. Med*., **21,** 104 (1979).
15. F. Giacco, M. Brownlee, *Circ. Res.*, **107**, 1058 (2010).
16. Y. Srivastava, H. Venkatakrishna-Bhatt, Y. Verma, K. Venkaiah, B.H. Raval, *Phytother. Res.*, **7**, 285 (1993).
17. H. H. EL-Kamali, M. Y EL-amir, *Curr. Res. J. Biol. Sci*., **2,** 143 (2010).
18. N. Rakieten, M.L. Rakieten, M.V. Nadkarni, *Cancer Chemother. Rep.* **29**, 91 (1963).
19. S. de M. Bandeira, G. da S. Guedes, L. J. S. da Fonseca, A. S. Pires, D. P. Gelain, J. C. Moreira, *Oxid. Med. Cell. Longev*., Article ID 819310, 13 pages, (2012).
20. M. Haluzik, J. Nedvidkova, *Physiol. Res.*, **49**, 37 (2000).
21. B.K. Tiwari, K.B. Pandey, A.B. Abidi, S.I. Rizvi, *J. Biomarkers*, 2013, Article ID 378790, (2013).
22. A. Ullah, A. Khan, I. Khan, *Saudi Pharm. J.*, **24,** 547 (2016).
23. A. Hunyadi, A. Martins, T.-J. Hsieh, A. Seres, I. Zupkó, *PLoS ONE*, **7,** e50619 (2012).
24. J. Santana-Gálvez, L. Cisneros-Zevallos, D. A. Jacobo-Velázquez, *Molecules*, **22,** 358 (2017).

In vivo ОЦЕНКА НА АНТИОКСИДАНТНИЯ ПОТЕНЦИАЛ НА ДИКАФЕОИЛХИНОВА КИСЕЛИНА, ИЗОЛИРАНА ОТ *Geigeria alata*

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(Резюме)

Geigeria alata (DC) Oliv. & Hiern. (Asteraceae) е ароматно медицинско растение, използвано в традиционната суданска медицина с широк кръг от биоактивности, включително предполагаем антидиабетен потенциал. Целта на настоящото изследване е да се оценят антиоксидантните свойства на 3,5-дикафеоилхиновата киселина (diCQA), която е основният компонент, изолиран от екстракт от корените на *Geigeria alata* в експериментален модел на диабет тип 2, индуциран посредством стрептозотоцин в мъжки Wistar плъхове. Диабетът причинява тежка органна патология, чиито основни патофизиологични механизми са свързани с оксидативен стрес, характеризиращ се с повишено производство на малонов дианхидрид (MDA) и нарушение както на неензимната (GSH), така и на ензимната (GPx, GR, GST) антиоксидантна защита. DiCQA (5 mg/kg/po), прилагана в продължение на 21 дни на контролните и на диабетните плъхове, подобрява активността на антиоксидантните ензими и нивата на клетъчния протектор GSH, както и намалява производството на MDA. DiCQA има също антидиабетно действие при диабетни плъхове. На основата на тези резултати и имайки пред вид, че образуването на реактивни форми на кислорода (ROS) е един от механизмите на патогенезата на диабета, ние правим извода, че diCQA, изолирана от *Geigeria alata*, притежава антиоксидантни свойства, които най-вероятно определят нейната *in vivo* антидиабетна активност.

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Evaluation of the antioxidant potential of defatted extract from *Astragalus spruneri* in spontaneously hypertensive rats (SHRs)

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The aim of this study was to evaluate the antioxidant potential of *Astragalus spruneri* (Fabaceae) in spontaneously hypertensive rats (SHR). Hypertension is a non-communicable disease and oxidative stress is regarded as one of the main pathophysiological mechanisms. Defatted extract of *A. spruneri* (EAS) was administered at a dose of 100 mg/kg bw (1/20 LD_{50}) for 14 days. At the end of the treatment period the animals were euthanized and the activities of catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPx) as well as the levels of non-enzyme cell protector reduced glutathione (GSH) were assessed in the brain, liver, kidney and spleen of SHR. In comparison to normotensive Wistar rats, in control, non-treated SHRs the GSH level and the activity of GPx were decreased in all organs, while the activity of CAT and SOD was decreased in brain, liver and kidney, and unchanged in spleen. Compared to the control SHRs *A. spruneri* exerted antioxidant activity, discerned by statistically significant increased activities of CAT and SOD in liver and kidney, of GPx and GSH in liver, kidney and spleen. It is worth to be noted that the extract did not exert any effect in the brain. This might be due to the fact that it cannot penetrate the blood brain barrier. Based on the results of our study we could conclude that the lyophilized extract of *A. spruneri* showed antioxidant potential in spontaneously hypertensive rats – a model of essential hypertension in humans.

Keywords: *Astragalus spruneri*, Oxidative stress, SHR, Antioxidant enzymes

INTRODUCTION

Recently it has been shown that oxidative stress is a key player in the pathogenesis of hypertension. A reduction in superoxide dismutase (SOD) and glutathione peroxidase (GPx) activity has been observed in newly diagnosed and untreated hypertensive subjects, which are inversely correlated with blood pressure [1]. Hypertension (HTN) is the most important cardiovascular risk factor, contributing to coronary heart disease and cerebrovascular diseases [2].

Oxidative stress is an independent risk factor in the development of hypertension in experimental animal models, as spontaneously hypertensive rats (SHRs) [3].

If oxidative stress is indeed a cause of hypertension, then, antioxidants should have beneficial effects on hypertension control and reduction of oxidative damage should result in a reduction in blood pressure. A significant body of experimental [4] and clinical trial data [5-7] suggest that diets known to contain significant concentrations of naturally occurring antioxidants appear to reduce blood pressure and may reduce cardiovascular risk.

In relation to this information, dietary and plant-derived antioxidants may have beneficial effects on hypertension and cardiovascular risk factors [8]

Astragalus is a large genus of herbs and small shrubs, belonging to the legume family Fabaceae. In the folk medicine *Astragalus* plants are used for treatment of common cold, upper respiratory infections, allergies, fibromyalgia, anemia, and to regulate the immune system. It is also used for chronic fatigue syndrome (CFS), kidney disease, diabetes, and high blood pressure [9]. The species have been proved to accumulate three main groups of pharmacologically significant metabolites – saponins, flavonoids and polysaccharides. Many flavonoids isolated from some *Astragalus* species have been proved to exert antioxidant effects that inhibit free radical production and act as their scavengers and cytoprotectors. In the body, free

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radicals damage cells and are linked to many health problems including hypertension [10].

Astragalus spruneri Boiss. (Spruner's Milk-vetch) is a clump-forming perennial plant endemic for the Balkan Peninsula and Turkey [11]. The total and differential flavonoid content of the species has been recently evaluated [12]. Up to date there is no information present on its pharmacological action.

In light of these data, we aimed to evaluate the antioxidant and antihypertensive potential of defatted extract from *A. spruneri* in spontaneously hypertensive rats (SHRs), a model of essential hypertension in humans.

MATERIALS AND METHODS

Plant material and preparation of defatted extract

The overground parts of *A. spruneri* were collected during flowering period from Kozhuh Mountain, Rupite area, Bulgaria, in April, 2014. The species was identified by Dr. D. Pavlova from the Faculty of Biology, Sofia University, Bulgaria, where a voucher specimen was deposited (SO-107625).

Air-dried plant material (120 g) was defatted with dichloromethane (800 ml, 24 h) and then extracted with 100% (200 ml) and 80% methanol (1000 ml) *via* percolation for 72 h. The methanol extract was concentrated on a rotary evaporator in order to eliminate the solvent and lyophilized. There were 12 main flavonoids in the extract and the total flavonoid content was 14 mg/g dry weight. Three main saponins were proved as well with total quantity of 8 mg/g dry weight. Quantitative analysis of the extract was performed by a HPLC method (RP C18 column 250 × 4.6 mm, linear gradient of MeOH and 0.1% H_3PO_4 in water as described in [12].

Animals

Experiments were performed with 12 male SHRs (initial body weight 180-230 g) and 12 male NTRs (initial body weight 200-250 g), obtained from Charles River Laboratories (Sülzfeld, Germany). The animals were housed in Plexiglas cages (3 per cage) at 20 ± 2 °C and 12/12 h light/dark cycle. Food and water were provided *ad libitum*. All procedures were approved by the Bulgarian Agency of Food Safety (№ of permission 169) and performed strictly following the principles stated in the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (ETS 123).

Chemicals

All the reagents used were of analytical grade. Streptozotocin, as well as other chemicals, bovine serum albumin (fraction V), beta–nicotinamide, adenine dinucleotide 2`-phosphate reduced tetrasodium salt (NADPH), reduced glutathione (GSH), oxidized glutathione (GSSG), glutathione reductase (GR) enzyme, and cumene hydroperoxide were purchased from Sigma Chemical Co. (Taufkirchen, Germany). 2,2- Dinitro-5,5 dithiodibenzoic acid (DTNB) was obtained from Merck (Darmstadt, Germany).

Blood pressure measurement

Blood pressure was measured in conscious animals using an automated tail-cuff device (CODA non-invasive blood pressure system, Kent Scientific Corporation, USA). Before the experimental period, the rats were conditioned to the restraining cylinders. Rats were pre-warmed for 10 min using a temperature-controlled warming holder (37 °C) to facilitate tail blood flow before their blood pressure was measured. The mean of three tail-cuff readings was used as the systolic and diastolic blood pressure value. SHRs with highest blood pressure values were taken for the *in vivo* experiment.

Design of the in vivo experiment

The rats were randomly divided into four groups (n=6) as follows:

Group 1: control NTRs, treated with the saline vehicle, administered by gavage at 5 mL/kg bw/day, 14 days.

Group 2: NTRs treated with EAS alone at 100 mg/kg bw/day, 14 days [13].

Group 3: control SHRs, treated with the saline vehicle, administered by gavage at 5 mL/kg bw/day, 14 days.

Group 4: SHRs treated with EAS alone at 100 mg/kg bw/day, 14 days.

The animals in all groups were sacrificed on the 15^{th} day from the beginning of the experiment. Brains, livers, kidneys and spleens from SHRs and NTRs were taken for assessment of parameters of antioxidant status. For all following experiments the excised organs were perfused with cold saline solution (0.9% NaCl), blotted dry, weighed, and homogenized with corresponding buffers (see *Markers of oxidative stress* below).

Markers of oxidative stress

Reduced glutathione (GSH) was assessed by measuring non-protein sulfhydryls after precipitation of proteins with 5% trichloroacetic acid (TCA), using the method described by Bump *et al.* (1983) [14]. A total of 10% homogenates were prepared in 0.05M phosphate buffer (pH 7.4) and centrifuged at 7 000 × g and the supernatant was used for antioxidant enzymes assay.

Glutathione peroxidase (GPx) was measured by NADPH oxidation, using a coupled reaction system consisting of GSH, GR, and cumene hydroperoxide (Tappel, 1978) [15]. Catalase (CAT) activity was determined by measuring the decrease in absorbance at 240 nm of a reaction mixture consisting of H_2O_2 in phosphate buffer, pH 7.0, and requisite volume of supernatant sample. The molar extinction coefficient of 43.6 M cm^{-1} was used to determine catalase activity. The specific activity was calculated and was expressed as μmol/min/mg of total protein (Aebi, 1974) [16]. Superoxide dismutase activity (SOD) was measured according to the method of Misura and Fridovich (1972) [17], following spectrophotometrically the autoxidation of epinephrine at pH=10.4, 30 °C, using the molar extinction coefficient of 4.02 mM^{-1} cm^{-1}.

Histopathological examination

For light microscopic evaluation, pre-sectioned brain, liver, kidney and spleen samples (n=10 per group) were fixed in 10% buffered formalin and thin sections (4 μm) were subsequently stained with hematoxylin/eosin (HE) for general histological features determination [18]. Sections were studied under light microscope Leica DM 500 (DMR + 550, Leica, Wetzlar, Germany)

Statistical analysis

Statistical programme 'MEDCALC' was used. The results were expressed as mean ± SEM for six rats in each group. The significance of the data was assessed using the nonparametric Mann–Whitney test. For both statistical methods, values of $P \leq 0.05$ were considered statistically significant.

RESULTS

Blood pressure

Blood presure values are shown in Table 1. SHRs have higher sistolic blood pressure (SBP) by 45% ($p<0.05$) and higher diastolic blood pressure (DBP) by 44% ($p<0.05$), compared with the normotensive rats. EAS did not change the blood pressure in NTRs. In SHRs, however, the EAS decreased SBP by 18% ($p<0.05$) and DBP by 23% ($p<0.05$).

Table 1. Blood pressure values measured in conscius NTR and SHR rats and in those treated with defatted EAS.

Blood pressure	NTR	SHR	NTR+EAS	SHR + EAS
SBP	136.6 ± 3.4	198 ± 9.5*	128.6 ± 7.2	162.2 ± 9.1+
DBP	78 ± 3.6	112.2 ± 5.2*	82.5 ± 2.1	86.6 ± 6.7+

*p< 0.05 *vs* NTR control; +p<0.05 *vs* SHR control

GSH levels and activity of antioxidant enzymes

The differences in the GSH level and activity of antioxidant enzymes between normotensive and hypertensive rats are presented in Table 2. The results showed that SHRs have lower GSH level and antioxidant capacity in all investigated organs than the respective NTR control group. GSH level was statistically significantly ($p<0.05$) lower in the liver by 22%, in the kidney by 21%, in the brain by 34% and in the spleen by 26 %. CAT activity was significantly ($p<0.05$) lower in the liver, kidney and brain of SHR by 20%, 21% and 37%, respectively. SOD activity was lower in the liver by 18% ($p<0.05$), in the kidney by 22% ($p<0.05$) and in the brain – by 31% ($p<0.05$). GPx activity was lower in the liver by 15% ($p<0.05$), in the kidney and brain by 21% ($p<0.05$), and in the spleen – by 23% ($p<0.05$).

Table 2. GSH levels, antioxidant enzymes GPx, CAT and SOD activity

Parameters	GSH[a]		GPx[b]		CAT[c]		SOD[d]	
Controls	NTR	SHR	NTR	SHR	NTR	SHR	NTR	SHR
Liver	6.62±0.38	5.22±0.15*	0.33±0.01	0.28±0.02*	21.2±0.87	16.9±1.3*	0.28±0.01	0.23±0.01*
Kidney	4.46±0.29	3.55±0.21*	0.24±0.01	0.19±0.01*	16.3±1.02	12.9±0.5*	0.23±0.01	0.18±0.02*
Brain	1.69±0.17	1.11±0.1*	0.43±0.01	0.34±0.02*	42.1±1.1	26.8±1.26*	0.32±0.02	0.22±0.02*
Spleen	3.23±0.24	2.39±0.23*	0.31±0.02	0.27±0.01*	18.9±1.1	16.8±0.7	0.33±0.02	0.35±0.01

*p<0.05 *vs* control NTR; [a]nmol/g tissue; [b]nmol/min/mg protein; [c]μmol /min/mg protein

The effects of the defatted extract from *A. spruneri* (EAS) on the antioxidant capacity of SHR are shown in Fig. 1. Fourteen-day treatment with the EAS increased the level of GSH statistically significantly ($p<0.05$) in the liver, kidney and spleen of SHR by 27%, 26% and 35%, respectively, when compared to control SHR group. CAT activity was increased in the liver and kidney by 25% ($p<0.05$). SOD activity was also increased by the EAS in the liver and kidney in statistically significant manner ($p<0.05$) by 22% and 28%, respectively. GPx activity was increased significantly in the same organs, in the liver by 18% ($p<0.05$) and in the kidney by 26% ($p<0.05$).

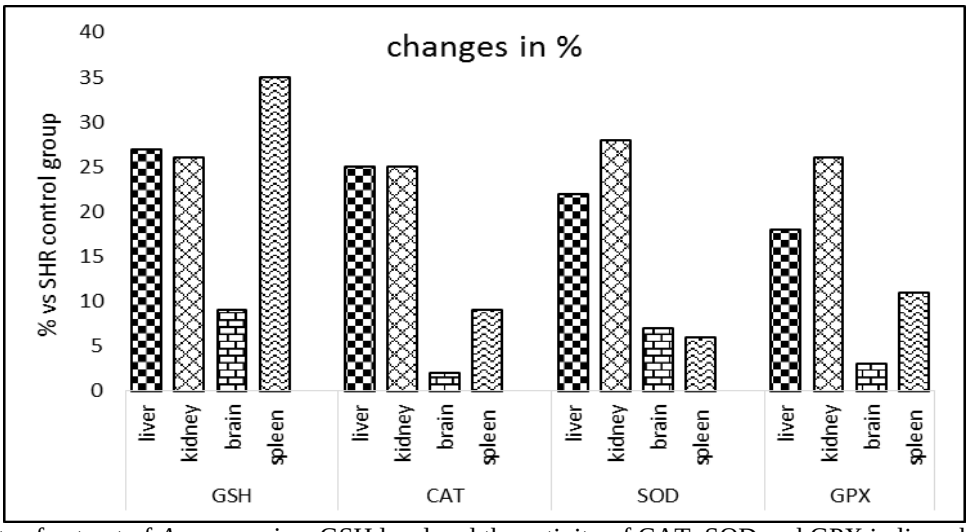

Figure 1. Effects of extract of *A. spruneri* on GSH level and the activity of CAT, SOD and GPX in liver, kidney, brain and spleen from SHR, compared to the control SHR.

Histopathological evaluation

The results from the histopathological examination are shown in Fig. 2. It is visible that EAS did not affect the investigated organs and did not change their morphological structure.

DISCUSSION

In both humans and animals, essential hypertension acts as a risk factor for subclinical organ damages [19] but mechanisms that correlate hypertension and organ damages have not been elucidated extensively. Several experimental and clinical data prove that hypertension and oxidative stress are closely related [20, 21], although it is unclear whether oxidative stress is a cause or an effect of hypertension [22, 23]. The important pathophysiological role of ROS in hypertension development is due, in a large part, to oxygen excess and decreased NO bioavailability in vasculature [24]. Increased oxidative stress has been revealed in genetic and experimental models of hypertension although the effectiveness of antioxidant treatments in reducing blood pressure has been not fully verified [25]. Oxidative stress seems to be a salient feature also in human hypertension. There is evidence that hypertensive patients show both increased oxidative stress and reduced antioxidant capacity [26].

In the current study, we used a genetic model of hypertension, the spontaneously hypertensive rats (SHRs), derived from the normotensive Wistar Kyoto rats (WKY) [27]. We hypothesized that the increased oxidative stress in genetic models of hypertension may be attenuated by natural antioxidants contained in *A. spruneri.*

On the basis of this assumption, the aim of the current study was to investigate the possible antihypertensive and antioxidant activity of the defatted extract from *A. spruneri* on SHR, a model of essential hypertension in humans.

EAS exerted a pronounced antihypertensive effect in SHRs, probably due to flavonoids in this extract. Evidence exists to support several potential mechanisms whereby flavonoids might decrease blood pressure and decrease the severity of hypertension in animals and humans. These mechanisms are: decrease in oxidative stress, interference with the renin-angiotensin-aldosterone system (RAAS), and/or improving vascular function in an endothelium-dependent or independent manner [28].

Our results clearly show a significant depletion in the efficiency of antioxidant enzymes in SHR. In comparison to normotensive Wistar rats, in control, non-treated SHRs the GSH level and the activity of GPx were decreased in all investigated organs, e.g. liver, kidney, brain and spleen by approximately 25%.

The activity of CAT and SOD was decreased in brain, liver and kidney by around 30%, and unchanged in spleen. Our results confirmed literature reports in animal models [24].

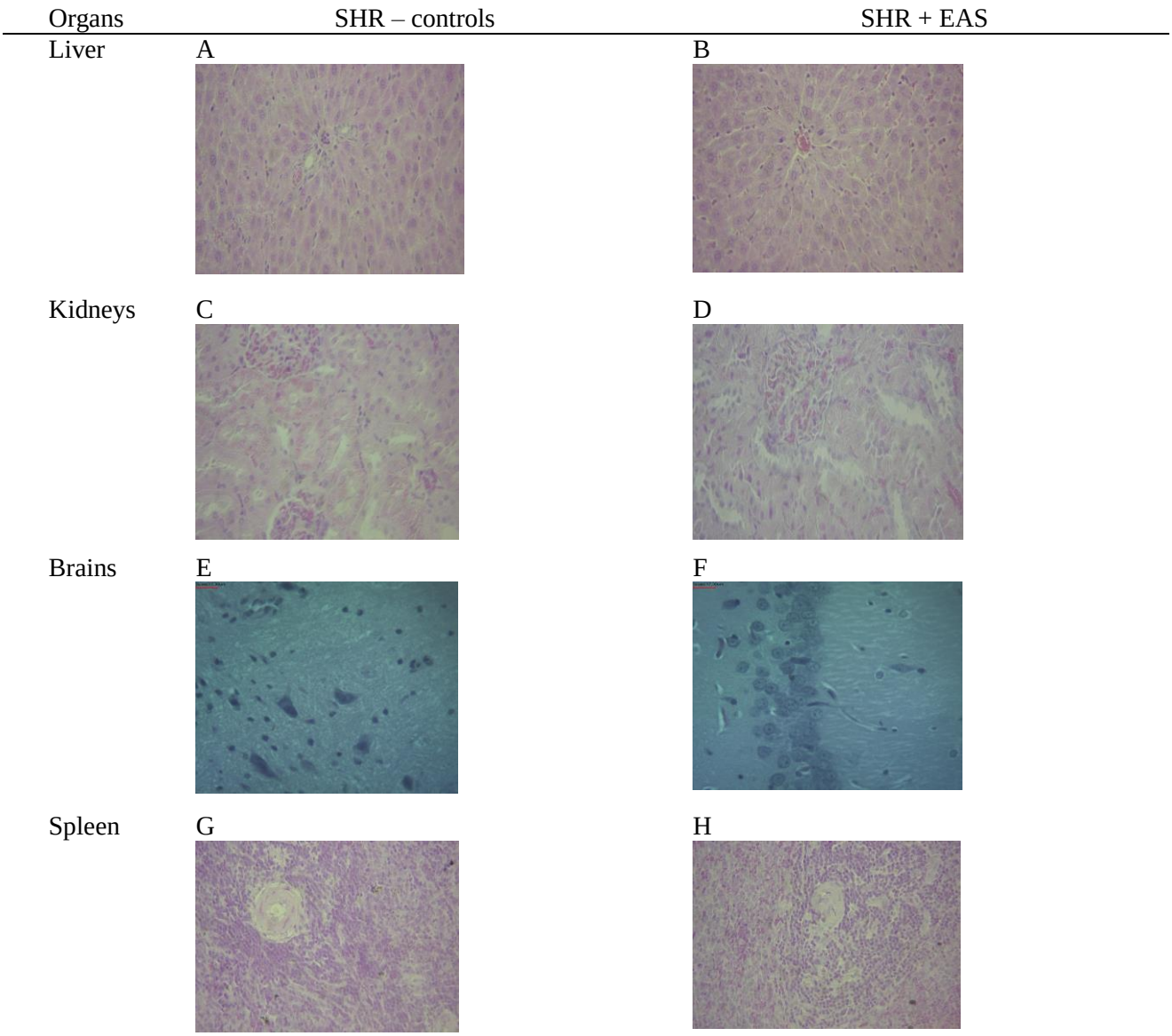

Figure 2. Histological examination of liver, kidney, brain and spleen of control and EAS gavaged SHR.

Compared to the control SHRs *A. spruneri* exerted antioxidant activity, discerned by statistically significant increased activities of CAT and SOD in liver and kidney, of GPx and GSH in liver, kidney and spleen (see Fig. 1). The antioxidant effects observed in our study can be attributed to many phytochemicals in the EAS as they are reported to possess the antioxidant activity. Among these, flavonoids have been very frequently correlated with the antioxidant potential of any plant extract. It has been proposed by Ye *et al.* [29] that flavonoids have a very strong capacity to eliminate free radicals in the blood and promote the activities of antioxidant enzymes such as SOD, GPx, and CAT. These actions of flavonoids are also dose-dependent. This information could explain the observed increase in the activity of the antioxidant enzymes in our study. The extract did not exert any effect in the brain, which might be due to the fact that it cannot penetrate the blood brain barrier. EAS did not affect the activity of antioxidant enzymes in the spleen neither, which might be due to affected blood circulation in this organ in SHR.

Hypertension causes target organ damage (TOD) that involves vasculature, heart, brain and kidneys. Complex biochemical, hormonal and hemodynamic mechanisms are involved in the pathogenesis of TOD. Common to all these processes is an increased bioavailability of reactive oxygen species (ROS). Both *in vitro* and *in vivo* studies explored the role of mitochondrial oxidative stress as a mechanism involved in the pathogenesis of TOD in hypertension, especially focusing on atherosclerosis, heart disease, renal failure, cerebrovascular disease [30]. The SHRs, besides being the most widely used model for essential hypertension, are also an excellent model for studying the development of TOD in the context of

human hypertension. We utilized kidney and brain as target organs and liver and spleen as control tissues, since these organs do not appear particularly susceptible to hypertensive damage [31].

Our results from the histopathological evaluation did not show morphological changes in the investigated organs neither in control SHR nor in EAS treat SHR (fig. 2).

Based on the results of our study we could conclude that under the conditions of our study the defatted extract of *A. spruneri* showed antihypertensive effect and antioxidant potential in the liver and kidney from SHRs – a model of essential hypertension in humans.

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REFERENCES

1. E. Schulz, T. Gori, T. Münzel, *Hypertens. Res.,* **34**, 665 (2011).
2. J. A. Whitworth, *J. Hypertens*., **21**, 1983 (2003).
3. S. I. Dikalov, Z. Ungvari, *Am. J. Physiol. Heart Circ. Physiol.,* **305,** 1417 (2013).
4. R. Del Pino-García, M. D. Rivero-Pérez, M. L. González-San José, K. D. Croft, P. Muñiz, *Food Funct.*, **7**, (2017).
5. T. J. Moore, W. M. Vollmer, L. J. Appel, F. M. Sacks, L. P. Svetkey, T. M. Vogt, P. R. Conlin, D. G. Simons-Morton, L. Carter-Edwards, D. W. Harsha, *Hypertension,* **34,** 472 1999.
6. P. R. Conlin, D. Chow, E. R. Miller, L. P. Svetkey, P. H. Lin, D. W. Harsha, T. J. Moore, F. M. Sacks, L. J. Appel, *Am J Hypertens*., **13,** 949 (2000).
7. J. H. John, S. Ziebland, P. Yudkin, L. S. Roe, H. A. Neil, *Lancet*, **359**, 1969 (2002).
8. A. A. Javkhedkar, M. F. Lokhandwala, A. A. Banday, *Am. J. Physiol. Renal. Physiol.*, **302,** 47 (2012).
9. I. N. Krasteva, A. M. Shkondrov, I. I. Ionkova, P. K. Zdraveva, *Phytochem. Rev*., **15,** 567 (2016).
10. V. M. Bratkov, A. M. Shkondrov, P. K. Zdraveva, I. N. Krasteva, *Phcog. Rev.*, **10,** 11 (2016).
11. S. S. Valev, Astragalus L., in: D. Yordanov (ed.) Flora Republicae Popularis Bulgaricae. Aedibus Academiae Scientiarum Bulgaricae, Sofia, **6,** 167 (1976).
12. A. M. Shkondrov, I. N. Krasteva, D. K. Pavlova, P. K. Zdraveva, *C. R. Acad. Bulg. Sci.* **70**, 363 (2017).
13. R. L. Simeonova, I. N. Krasteva, M. S. Kondeva-Burdina, N. Y. Benbassat, *Int. J. Pharm. Bio. Sci.* **4**, 179 (2013).
14. E.A. Bump, Y.C. Taylor, J.M. Brown, *Cancer Res.*, **43**, 997 (1983).
15. A.L. Tappel, *Methods Enzymol.* **52,** 506 (1978).
16. H. Aebi, In: Bergrenyer, H.U. (Ed.) Methods of enzymatic analysis. 2nd ed. Academic Press, New York, p. 673 (1974).
17. H.P. Misura, I. Fridovich, *J. Biol. Chem.* **247**, 3170 (1972).
18. J. D. Bancroft, M. Gamble, Theory and practice of histological techniques 5th ed., Churchill Livingstone Publications, Edinburgh, UK, 2002.
19. J. Zhang, M.K. Fallahzadeh, P.A. McCullough, *Cardiorenal Med.*, **7**, 1 (2016).
20. M. Majzunova, I. Dovinova, M. J.Y. Barancik, *J. Biomed. Sci.,* **20** (2013).
21. J.F. Ndisang, A. Vannacci, S. Rastogi, *Oxid. Med. Cell. Longev.* **2014,** (2014).
22. R. Loperena, D.G. Harrison, *Med. Clin. North Am.* **101,** 169 (2017).
23. N. C. Ward, K. D. Croft, *Clin. Exp. Pharmacol. Physiol.,* **33,** 872 (2006).
24. R. Rodrigo, M. Libuy, F. Feliú, D. Hasson, *Dis. Markers.* **35**, 773 (2013).
25. E. Grossman, *Diabetes Care,* **31**, 185 (2008).
26. M. Mihalj, R. Tadzic, A. Vcev, S. Rucevic, I. Drenjancevic, *Kidney Blood Press. Res.*, **41,** 721 (2016).
27. K. Okamoto, K. Aoki, *Jpn. Circ. J.*, **27**, 282 (1963).
28. A. J. Larson, J. D. Symons, T. Jalili, *Adv. Nutr*., **3**, 39 (2012).
29. Y. Ye, Y. Guo, Y. T. Luo, *Int J Mol Sci*., **13**, 12401 (2012).
30. S. Rubattu, B. Pagliaro, G. Pierelli, C. Santolamazza, S.D. Castro, S. Mennuni, M. Volpe, *Int. J. Mol. Sci.* **16**, 823 (2014).
31. Y. Sun, T. Zhang, L. Li, J. *Kidney Blood Press. Res.*, **40,** 41 (2015).

ОЦЕНКА НА АНТИОКСИДАНТНИЯ ПОТЕНЦИАЛ НА ОБЕЗМАСЛЕН ЕКСТРАКТ ОТ *Astragalus spruneri* В СПОНТАННО ХИПЕРТОНИЧНИ ПЛЪХОВЕ

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(Резюме)

Целта на това изследване е да се оцени антиоксидантният потенциал на *Astragalus spruneri* (Fabaceae) в спонтанно хипертонични плъхове (SHR). Хипертонията е незаразна болест и оксидативният стрес се счита за един от основните патофизиологични механизми. Обезмаслен екстракт от *A. spruneri* (EAS) е приложен в доза от 100 mg/kg bw (1/20 LD_{50}) в продължение на 14 дни. В края на този период животните са евтаназирани и активностите на каталаза (CAT), супероксид дисмутаза (SOD) и глутатион пероксидаза (GPx), както и нивата на неензимния клетъчен протектор редуциран глутатион (GSH) са оценени в мозъка, черния дроб, бъбреците и далака на SHR. В сравнение с Wistar плъхове с нормално кръвно налягане, в контролните нетретирани SHR нивото на GSH и активността на GPx са понижени във всички органи, докато активността на CAT и SOD е понижена в мозъка, черния дроб и бъбреците, но е непроменена в далака. В сравнение с контролните SHR, *A. spruneri* проявява антиоксидантна активност, което личи от статистически значимо нарасналите активности на CAT и SOD в черния дроб и бъбреците, и на GPx и GSH в черния дроб, бъбреците и далака. Трябва да се отбележи, че екстрактът няма ефект върху мозъка. Това може да се дължи на факта, че екстрактът не може да проникне през кръвно-мозъчната бариера. От получените резултати може да се заключи, че лиофилизираният екстракт от *A. spruneri* проявява антиоксидантен ефект в спонтанно-хипертонични плъхове – модел за есенциална хипертония при хора.

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Development of lipid damage of pumpkin seed oil stabilized with different antioxidants during long-term storage

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Oxidative stability of pumpkin seed oil during long-term storage at different conditions was investigated. The examinations were carried out with pumpkin seed oil (as a control sample) and stabilized oil with different natural antioxidants such as caffeic acid, ethyl gallate and combination of both. Long-term storage for all samples was led for 6 months at room temperature (25°C) and in a dark place at 10°C. Indicators that define oxidative stability of the oils (acid and peroxide value, fatty acid composition and total tocopherol content) were monitored during the long-term storage. Significant increase in the peroxide value was detected in all samples during the whole period, especially in those that were kept at room temperature (25°C), while in acid value and fatty acid composition the deterioration was minor. Same tendency was observed in total tocopherol content where the latter decreased insignificantly in samples, stored at 10°C in a dark place. Generally, it could be considered that pumpkin seed oil was more stable when was stabilized with ethyl gallate and stored at 10°C.

Keywords: Pumpkin seed oil, Antioxidants, Long-term storage, Fatty acids, Tocopherols

INTRODUCTION

The oxidation of lipids is an issue with a great importance for the food industry. It is related with deterioration of fats and oils which can lead to lowering of the nutritive value of the food [1]. This provokes searching of different ways to minimize the process of oxidation and improve oxidative stability of lipid products [2]. Therefore, adding antioxidants to the food play an important role in food processing and storage because they have the ability to inhibit the oxidation [1].

Pumpkins are annual plants which belong to Cucurbitaceae family. Their seeds are by-products by food industry but they exhibit a high oil content (37.8-50.0%) which make them a rich source of lipids. The main fatty acid in the lipid fraction is linoleic acid (42.0-68.5%) followed by oleic (20.0-38.0%) and the content of palmitic and stearic acid is much lower (13.0 and 6.0%, respectively). The amount of unsaturated fatty acids is about 80.0% but the quantity of saturated fatty acids is relatively lower (about 19.0%) [3–9]. This is the reason pumpkin seed oil to be susceptible to oxidation process. The quality of vegetable oils is mainly based on their fatty acid composition, the content of natural antioxidants, as well as their ability to be stable during long-term storage. Linoleic acid, which is predominant in pumpkin seed oil, is easily susceptible to oxidation processes under the influence of light and oxygen, which may cause deterioration of the oils. It is established that during the refining process some of the tocopherols which are the major antioxidants in vegetable oils are also removed [10]. Irrespectively of the type of the oil, this leads to significantly decrease of its stability. Pumpkin seed oil that is available on the market is cold pressed, so it has higher stability. According to Vidrih *et al*. (2010) [11] the induction period of the oil at 110°C is 12.8-25.7 h which is higher than those of olive oil (9.1-23.2 h), sunflower oil-high oleic type (11.5-15.0 h), rape seed oil (7.4-10.5 h), soybean oil (6.2-8.8 h) and sunflower oil (5.3-7.8 h). Poiana *et al*. (2009) [12] examined the influence of the storage conditions on the oxidative stability and antioxidant properties of sunflower and pumpkin oil and established that during the period of 120 days the pumpkin seed oil showed higher oxidative stability than sunflower oil. They also proved that the oxidation processes were induced at a greater extent by the impact of the daylight than by the temperature.

Tocmo (2012) [13] examined the effect of packaging, light and storage temperature on the oxidative stability of pumpkin oil obtained by cold pressing of seeds of the species *Cucurbita pepo* and *Cucurbita moschata*. The oils were found to be stable for at least 20 weeks when stored in brown bottles at temperatures below 30°C, whereas from the accelerated deterioration of the oxidative stability of both oils the suggested shelf life was 15 months.

There is no information about the development of lipid damage of pumpkin seed oil produced in

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Bulgaria. Therefore, the aim of the present study was to monitor the changes of peroxide, acid value, tocopherol content and fatty acid composition of non-stabilized and stabilized pumpkin seed oil with different antioxidants during long-term storage.

MATERIALS AND METHODS

The examinations were carried out with pumpkin seed oil (as a control sample) and stabilized oil with different antioxidants such as caffeic acid, ethyl gallate and combination of both. Antioxidants were diluted in ethanol; after that they were added to the oil at a concentration of 0.05% if only one was used and 0.025% with more than one antioxidant used. Long-term storage for all samples was led for 6 months at 25°C and at 10°C. Indicators that defined oxidative stability of the oils (acid and peroxide value, total tocopherol content and fatty acid composition) were monitored during the long-term storage – on the 30th, 60th, 90th, 120th, 150th and 180th day of storage.

Peroxide and acid value

Peroxide and acid values were determined titrimetrically by procedures of ISO [14, 15].

Fatty acids

The fatty acid composition was determined by gas chromatography (GC) after transmethylation of the sample with 2% H_2SO_4 in absolute CH_3OH [16]. Determination of fatty acid methyl esters was performed on an HP 5890 series II (Hewlett Packard GesmbH, Vienna, Austria) gas chromatograph equipped with a 75 m × 0.18 mm (I.D.) × 25 μm (film thickness) capillary column Supelco and a flame ionization detector. The column temperature was programmed from 140°C (5 min), at 4°C/min to 240°C (3 min); injector and detector temperatures were kept at 250°C. Hydrogen was the carrier gas at a flow rate of 0.8 mL/min. Fatty acids methyl esters were identified and quantified relative to the Supelco 37 component FAME mix (Supelco, USA) which was subjected to GC under identical experimental conditions [17].

Tocopherols

Tocopherols were determined directly in the oil by high performance liquid chromatography (HPLC) on a Merck-Hitachi (Merck, Darmstadt, Germany) instrument equipped with 250 mm × 4 mm Nucleosil Si 50-5 column (Merck, Darmstadt, Germany) and fluorescent detector Merck-Hitachi F 1000. The operating conditions were as follows: mobile phase n-hexane:dioxan, 96:4 (v/v), flow rate 1.0 mL/min, excitation 295 nm, emission 330 nm. 20 μL 2% solution of oil were injected. Tocopherols were identified by comparing the retention times with those of authentic individual tocopherols. The tocopherol content was calculated on the base of tocopherol peak areas in the sample *vs*. tocopherol peak area of standard tocopherol solution [18]. α-, γ- and δ-tocopherols were identified in the examined pumpkin seed oils. γ-Tocopherol predominated in all samples (over 90.0%).

RESULTS AND DISCUSSION

Data about changes of acid and peroxide value of control sample and stabilized pumpkin seed oil with different antioxidants stored at 10°C and 25°C during the whole period is shown in Figures 1 and 2.

There were insignificant changes of acid value in all samples during the storage, which varied between 8.1-9.6 mg KOH/g. Overall, there was no significant hydrolysis process that occurred in the oil during the storage at both conditions. This could be explained by the lack of moisture in the oils which caused hydrolysis.

The peroxide value of pumpkin seed oil considerably increased during the storage at 25°C. The highest value was observed in the control sample on the 120th day (81.4 meq active oxygen/kg). The best results were noticed in the stabilized oils with ethyl gallate (up to 70.2 meq active oxygen/kg on the 120th day) and combination of ethyl gallate and caffeic acid (up to 60.2 meq active oxygen/kg). The peroxide value of the stabilized pumpkin seed oil with caffeic acid, ethyl gallate and a combination of the two antioxidants met the standards for unrefined vegetable oils (PV ≤ 20 meq active oxygen/kg) [19] until the 30th day of storage at room temperature. Though it was relatively lower in the first month, this indicator was 25 times higher on the 60th day of storage in comparison with the value at the starting point of the examination and finally doubled on the 90th day. After that the peroxide value slightly increased on the 120th day.

The peroxide value of the oil that was kept at 10°C was almost the same on the 30th day (1.5-2.5 meq active oxygen/kg), but slightly increased up to 4.6-6.1 meq active oxygen/kg on the 60th day. The change was minor in the oil that was stabilized with caffeic acid (up to 4.6 meq active oxygen/kg in the second month). Then, the peroxide value doubled on the 90th day of storage (11.0-13.7 meq active oxygen/kg) and reached the value of 20.5-27.9 meq active oxygen/kg on the 120th day.

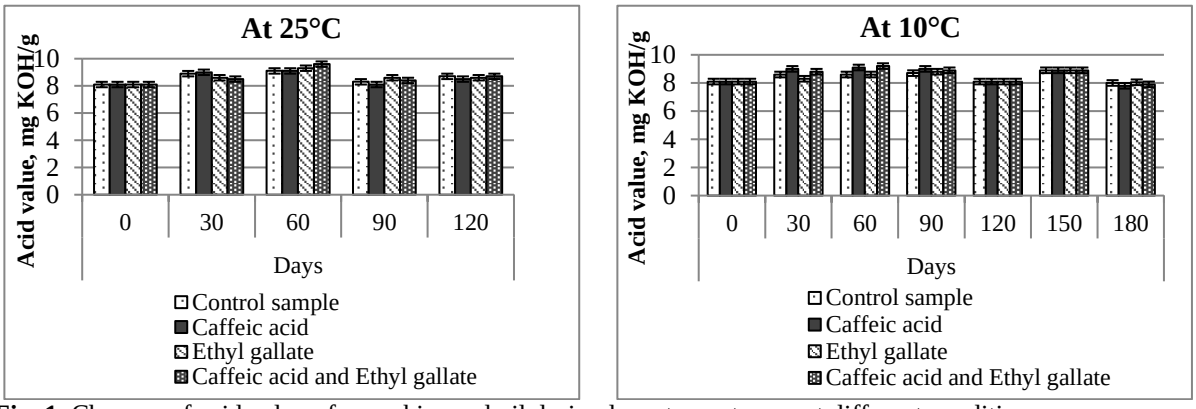

Fig. 1. Changes of acid value of pumpkin seed oil during long-term storage at different conditions

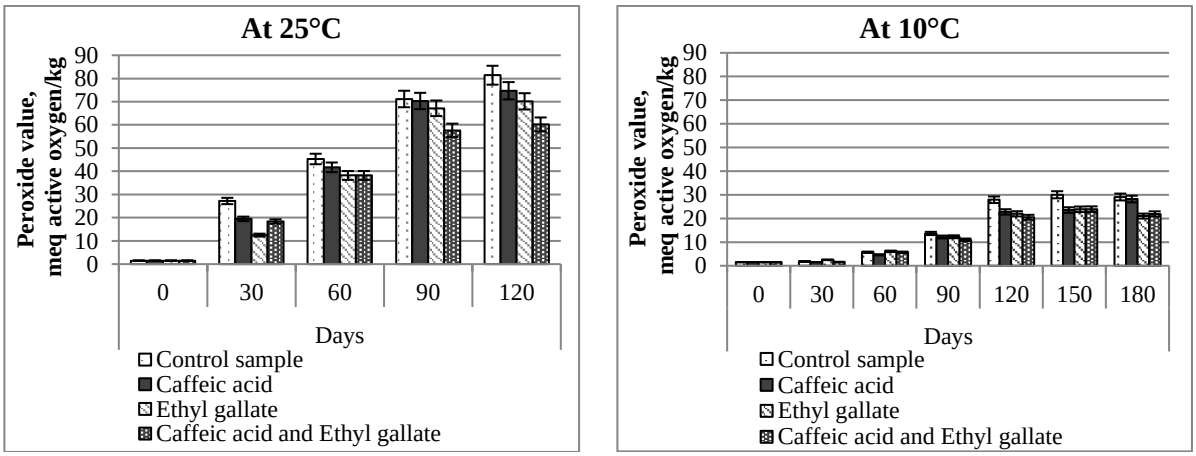

Fig. 2. Changes of peroxide value of pumpkin seed oil during long-term storage at different conditions

After that this indicator slightly increased up to 21.0-29.1 meq active oxygen/kg on the 180th day. Generally, the control sample of pumpkin seed oil that was kept at 10°C had the highest peroxide value at the final stage of examination, while those of the oil stabilized with ethyl gallate was relatively lower (21.0 meq active oxygen/kg). Therefore, ethyl gallate was the most efficient antioxidant of pumpkin seed oil stored at lower temperature.

According to the results it could be considered that pumpkin seed oil, kept at these conditions, was appropriate for human consumption no more than 120 days after its production.

Changes of total tocopherol content of non-stabilized and stabilized pumpkin seed oils with different antioxidants during long-term storage were also monitored. The results are presented in Figure 3.

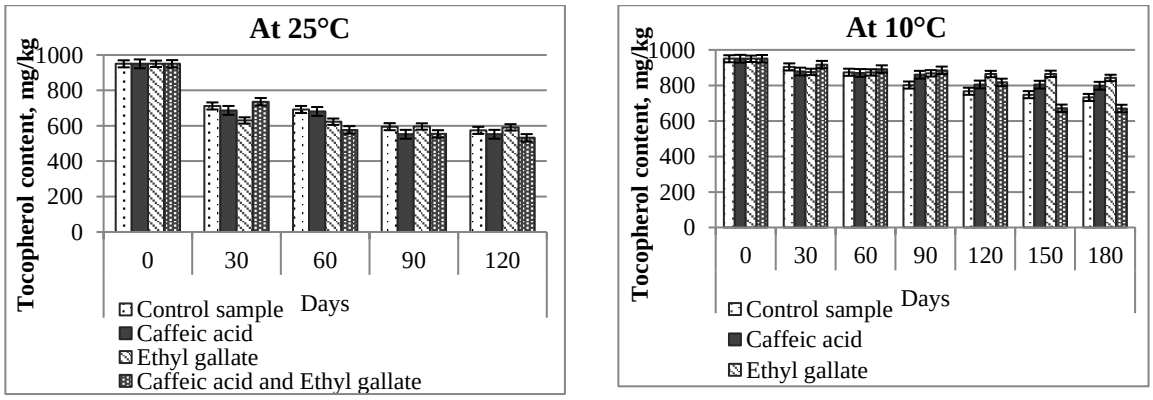

Fig. 3. Changes of tocopherol content of pumpkin seed oil during long-term storage at different conditions

Total tocopherol content of the control sample kept at room temperature (25°C) gradually decreased from 950 to 712 mg/kg on the 30th day and after that continued to decrease up to 574 mg/kg on the 120th day. Total tocopherols in the sample that

was stored at 10°C slightly decreased from 904 mg/kg (30th day) to 732 mg/kg (180th day).

It was observed that tocopherol content of oil stabilized with caffeic acid and stored at 25°C decreased drastically in the final days of examination (552 mg/kg) and its amount was lower than those of non-stabilized oil. On the other hand, its quantity decreases slightly in the pumpkin seed oil stabilized with the same antioxidant and stored at 10°C – it was 878 mg/kg on the 30th day and reached the value of 798 mg/kg on the 180th day.

The tocopherol content of the oil stabilized with ethyl gallate and stored at 25°C decreased sharply from 950 to 630 mg/kg on the 30th day and then remained almost constant (591 mg/kg on the 120th day). When this oil was stored at 10°C, tocopherols were reduced smoothly with slight differences in their amount during long-term storage (876 mg/kg on the 30th day and 842 mg/kg on the 180th day).

The content of tocopherols decreased drastically in pumpkin seed oil stabilized with caffeic acid and ethyl gallate and stored at 25°C (from 950 to 577 mg/kg on the 60th day), but after that they reduced gradually to 532 mg/kg on the 120th day. Their amount slightly decreased from 950 to 817 mg/kg on the 120th day in the oil kept at 10°C and reached up to 671 mg/kg on the 150th day. After that, total tocopherol content remained almost the same in the final period of examination.

It could be concluded that the most significant change in tocopherol content was observed in the control sample and in the oil stabilized with both caffeic acid and ethyl gallate and the reducing of the tocopherols was noticed to a lesser extent in the oil stabilized with ethyl gallate. Long-term storage of pumpkin seed oil at a lower temperature (10°C) did not affect significantly the total tocopherol content, whereas its amount decreased to a greater degree at room temperature and when the oil was exposed to daylight. The two factors (higher temperature and light) initiated oxidation of the oil which could lead to reduction of the amount of tocopherols because of their binding to the free radicals.

Table 1. Changes of fatty acid composition of pumpkin seed oil during long-term storage at 25°C

At 25°C						
Stabilized with:	Fatty acids, %	Days				
		0	30	60	90	120
Control sample	$C_{16:0}$ Palmitic	21.6±0.4	17.3±0.3	20.3±0.5	23.6±0.6	21.1±0.1
	$C_{18:0}$ Stearic	7.4±0.2	6.9±0.5	2.5±0.1	4.7±0.4	3.5±0.2
	$C_{18:1}$ Oleic	47.2±0.6	40.8±0.5	46.8±0.7	42.6±0.2	41.3±0.3
	$C_{18:2}$ Linoleic	22.2±0.2	33.1±0.1	28.0±0.4	27.4±0.5	33.4±0.3
Caffeic acid	$C_{16:0}$ Palmitic	21.6±0.6	20.0±0.4	17.8±0.2	17.5±0.5	17.1±0.1
	$C_{18:0}$ Stearic	7.4±0.2	7.6±0.4	5.1±0.3	0.9±0.1	0.9±0.1
	$C_{18:1}$ Oleic	47.2±0.4	41.1±0.2	45.2±0.4	40.9±0.5	39.1±0.2
	$C_{18:2}$ Linoleic	22.2±0.3	29.2±0.5	29.4±0.4	39.8±0.5	42.4±0.4
Ethyl gallate	$C_{16:0}$ Palmitic	21.6±0.2	18.0±0.4	17.2±0.3	21.6±0.5	21.7±0.2
	$C_{18:0}$ Stearic	7.4±0.1	7.0±0.2	4.9±0.2	4.3±0.1	3.5±0.1
	$C_{18:1}$ Oleic	47.2±0.5	41.4±0.6	41.4±0.5	44.8±0.4	41.3±0.3
	$C_{18:2}$ Linoleic	22.2±0.2	31.8±0.5	34.8±0.3	27.6±0.4	32.9±0.4
Caffeic acid and Ethyl gallate	$C_{16:0}$ Palmitic	21.6±0.2	18.3±0.3	19.1±0.1	19.8±0.4	17.1±0.2
	$C_{18:0}$ Stearic	7.4±0.3	7.0±0.1	2.1±0.2	1.0±0.1	1.0±0.1
	$C_{18:1}$ Oleic	47.2±0.3	40.7±0.5	46.6±0.4	41.5±0.5	37.8±0.2
	$C_{18:2}$ Linoleic	22.2±0.3	32.2±0.2	30.3±0.5	36.4±0.4	43.3±0.3

The results about changes of fatty acid composition of the examined oils are shown in Tables 1 and 2. The amount of oleic acid decreased during the first month of examination of non-stabilized oil and oil stabilized with caffeic acid, and combination of caffeic acid and ethyl gallate stored at 25°C, after that slightly increase of its quantity in the second month was observed. Then, the content of the same acid decreased again up to the 120th day of storage.

Table 2. Changes of fatty acid composition of pumpkin seed oil during long-term storage at 10°C

At 10°C								
Stabilized with	Fatty acids, %	Days						
		0	30	60	90	120	150	180
Control sample	$C_{16:0}$ Palmitic	21.6± 0.1	22.1±0.4	16.3±0.3	16.9±0.5	18.4±0.4	15.7±0.7	23.7±0.5
	$C_{18:0}$ Stearic	7.4±0.2	6.8±0.5	5.3±0.3	6.3±0.2	1.6±0.1	1.6±0.1	6.4±0.4
	$C_{18:1}$ Oleic	47.2±0.5	44.2±0.4	43.1±0.4	38.7±0.3	37.7±0.5	38.2±0.4	49.6±0.6
	$C_{18:2}$ Linoleic	22.2±0.2	25.0±0.2	33.5±0.4	36.2±0.5	40.8±0.6	43.6±0.5	19.4±0.2
Caffeic acid	$C_{16:0}$ Palmitic	21.6±0.3	21.5±0.5	20.3±0.5	15.1±0.4	18.9±0.2	17.0±0.2	18.7±0.3
	$C_{18:0}$ Stearic	7.4±0.1	6.7±0.2	4.6±0.2	5.8±0.2	1.1±0.1	2.4±0.1	4.6±0.3
	$C_{18:1}$ Oleic	47.2±0.5	44.3±0.5	43.4±0.4	37.4±0.4	37.6±0.3	39.9±0.2	41.5±0.5
	$C_{18:2}$ Linoleic	22.2±0.3	25.8±0.4	30.1±0.4	40.1±0.4	40.8±0.3	38.6±0.3	34.5±0.4
Ethyl gallate	$C_{16:0}$ Palmitic	21.6±0.2	21.2±0.3	16.8±0.2	15.9±0.2	17.7±0.4	17.4±0.3	21.9±0.5
	$C_{18:0}$ Stearic	7.4±0.1	6.1±0.2	1.9±0.1	5.9±0.2	1.5±0.1	2.3±0.1	5.4±0.1
	$C_{18:1}$ Oleic	47.2±0.4	44.4±0.4	40.6±0.5	37.6±0.3	37.5±0.2	40.2±0.5	46.9±0.5
	$C_{18:2}$ Linoleic	22.2±0.2	26.2±0.3	39.3±0.4	38.8±0.5	41.9±0.4	38.8±0.6	24.8±0.4
Caffeic acid and Ethyl gallate	$C_{16:0}$ Palmitic	21.6±0.3	20.8±0.3	18.3±0.2	17.8±0.3	18.8±0.3	16.3±0.3	19.3±0.4
	$C_{18:0}$ Stearic	7.4±0.2	2.1±0.1	5.0±0.2	6.7±0.3	1.6±0.1	1.8±0.1	4.4±0.2
	$C_{18:1}$ Oleic	47.2±0.5	45.1±0.4	41.8±0.5	39.9±0.3	39.3±0.4	39.3±0.4	40.7±0.5
	$C_{18:2}$ Linoleic	22.2±0.3	30.2±0.3	33.3±0.4	33.7±0.3	39.0±0.2	41.6±0.5	34.7±0.4

On the other hand, it was observed inconsiderably reducing of the amount of oleic acid in the oil stabilized with ethyl gallate which was almost the same until the 60th day. After that its quantity gradually increased on the 90th day and decreased up to the 120th day. The amount of linoleic acid increased during long-term storage of the oil stabilized with caffeic acid and the mixture of the antioxidants. On the other hand, the results about the same acid differed in the stabilized oil with ethyl gallate. The content of linoleic acid slightly increased during the second month of storage, after that decreased on the 90th day and finally gradually increased up to the 120th day. Its quantity increased in non-stabilized pumpkin seed oil during the first month and again decreased in the second month of storage. The amount of linoleic acid remained almost the same during the third month and finally increased after the fourth month. Changes of fatty acid composition of the examined pumpkin seed oils at 10°C were also established and the content of oleic acid gradually decreased on the 90th day in non-stabilized and stabilized oil with both antioxidants. Then its amount remained almost the same up to the 150th day. After that the quantity of the same fatty acid increased sharply in the control sample and gradually in the stabilized oil. The content of oleic acid in the rest of the samples decreased on the 90th day and its amount remained the same up to the 120th day. The quantity of the latter acid increased gradually in the oil stabilized with caffeic acid, but the increase was sharp in the oil with addition of ethyl gallate.

The amount of linoleic acid increased gradually in the control sample and in the oil stabilized with both antioxidants during five months of storage, but after that it decreased. The content of this fatty acid in the stabilized pumpkin seed oil with caffeic acid increased on the 90th day and remained almost the same up to the 120th day and finally decreased

gradually on the 180th day. The amount of linoleic acid in the sample stabilized with ethyl gallate smoothly increased up to the second month, remained the same up to the 150th day and sharply decreased on the 180th day.

Finally, changes of fatty acids of the oils stored at 10°C were less than those kept at 25°C and exposed to a day light.

CONCLUSION

According to the obtained results pumpkin seed oil was more stable when was stored at 10°C in a dark place. On the other hand, even stabilized oils deteriorated for 120 days at 25°C. Peroxide value increased to a higher level in the control sample and in stabilized oils kept at 25°C. The changes in acid value and fatty acid composition were minor but the content of tocopherols decreased significantly during storage. Overall, ethyl gallate could be considered as the most appropriate antioxidant for preserving pumpkin seed oil during long-term storage.

REFERENCES

1. I. Klimczak, M. Malecka, B. Pacholek, *Nahrung/Food*, **46**, 184 (2002).
2. F. Shahidi, Y. Zhong, *Chem. Soc. Rev.*, **39**, 4067 (2010).
3. M. B. Achu, E. Fokou, C. Tchiegang, M. Fotso, F. M. Tchouanguep, *Afr. J. Biotechnol.*, **4**, 1329 (2005).
4. M. B. Achu, E. Fokou, C. Tchiegang, M. Fotso, F. M. Tchouanguep, International Union of Food Science and Technology, 13th World Congress of Food Science and Technology, 2006, p. 1249.
5. A. G. Ardabili, R. Farhoosh, M. H. H. Khodaparast, *J. Agr. Sci. Tech.*, **13**, 1053 (2011).
6. E. Fokou, M. B. Achu, G. Kansci, R. Ponka, M. Fotso, C. Tchiegang, F. M. Tchouanguep, *Pak. J. Nutr.*, **8**, 1325 (2009).
7. E. Fokou, M. B. Achu, M. F. Tchouanguep, *Afr. J. Food Agric. Nutr. Dev.*, **4**, 11 (2004).
8. F. W. Martin, Cucurbit seeds as possible oil and protein sources, Echo Technical Note, ECHO, North Ft. Myers, Florida, USA, 1998.
9. J. Tsaknis, St. Lalas, E. Lazos, *Grasas y Aceites*, **48**, 267 (1997).
10. N. V. Yanishlieva, A. Kamal-Eldin, E. M. Marinova, A. G. Toneva, *Eur. J. Lipid Sci. Technol.*, **104** (5), 262 (2002).
11. R. Vidrih, S. Vidakovič, H. Abramovič, *Czech J. Food Sci.*, **28**, 376 (2010).
12. M. A. Poiana, E. Alexa, D. Moigradean, M. Popa, 44th Croatian & 4th International Symposium on Agriculture, 2009, p. 449.
13. R. Tocmo, M.S. Thesis of Food Science and Technology, Cornell University, 2012.
14. ISO 3960:2017. Animal and vegetable fats and oils. Determination of Peroxide value. Iodometric (visual) endpoint determination (2017).
15. ISO 660:2009. Animal and vegetable fats and oils. Determination of acid value and acidity (2009). Revised 2014.
16. ISO 12966-2:2011. Animal and vegetable fat and oils. Gas chromatography of fatty acid methyl esters – Part 2: Preparation of methyl esters of fatty acids (2011).
17. ISO 12966-1:2014. Animal and vegetable fats and oils. Gas chromatography of fatty acid methyl esters – Part 1: Guidelines on modern gas chromatography of fatty acid methyl esters (2014).
18. ISO 9936:2016. Animal and vegetable fats and oils. Determination of tocopherol and tocotrienol contents by high-performance liquid chromatography (2016).
19. Codex standard for named vegetable oils, Codex-Stan 210 – 1999. Revisions 2001, 2003, 2009, Amendment 2005, 2011.

ПРОМЕНИ ПРИ ОКИСЛЕНИЕ НА ТИКВЕНО МАСЛО, СТАБИЛИЗИРАНО С РАЗЛИЧНИ АНТИОКСИДАНТИ ПРИ ДЪЛГОСРОЧНО СЪХРАНЕНИЕ

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(Резюме)

Проследени са промените, протичащи при окислението на масло от семена на тиква при дългосрочно съхранение и различни условия. Изследванията са проведени с тиквено масло и такова, стабилизирано с различни антиоксиданти (кафеена киселина, етилгалат и комбинация от двата антиоксиданта). Дългосрочното съхранение е проведено за период от шест месеца при стайна температура (25°C) и на тъмно при 10°C. На определени интервали от време са проследени важни индикатори, които определят оксидантната стабилност на маслата, като киселинно и пероксидно число, мастнокиселинен състав и общо съдържание на токофероли. Значителни промени са установени в стойностите на пероксидното число при всички проби за целия период на изследването, като по-големи са в тези, съхранявани при стайна температура. Промените в стойностите на киселинното число и мастнокиселинния състав са незначителни. Общото съдържание на токофероли намалява при всички изследвани масла, като промяната е изразена в по-малка степен при тези, съхранявани при по-ниска температура (10°C). Установено е, че най-стабилно е тиквено масло с добавка от етил-галат и съхранявано при 10°C.

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Antioxidant and antimicrobial behaviour of alga *Gracilaria gracilis* extracts during hake (*Merluccius merluccius*) chilled storage

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The present research is a first attempt for the use of alga *Gracilaria gracilis* as a source of preservative compounds to be applied during the chilled storage of fish. For it, a combination of ethanolic and aqueous extracts of this edible seaweed were included in the icing medium employed for the chilling storage of hake (*Merluccius merluccius*). Chemical and sensory analyses related to quality loss were carried out in fish throughout 9-day storage. Three different alga concentrations were tested and compared to an icing control (traditional ice). As a result, an inhibitory effect ($p<0.05$) on lipid oxidation development (tertiary oxidation compounds) and microbial activity (trimethylamine formation) was observed in fish corresponding to batches including the two most concentrated alga conditions. However, a definite effect on lipid hydrolysis development (free fatty acids formation) could not be implied ($p>0.05$). Concerning sensory analysis, samples from the same two batches revealed a higher acceptance ($p<0.05$) than control; this difference was based on the evaluation of various descriptors such as skin, external odour, raw flesh odour and cooked flesh odour. On the basis of the presence of antioxidant and antimicrobial molecules, a profitable effect on quality retention of chilled hake is concluded by including *G. gracilis* extracts in the icing system.

Keywords: *Gracilaria gracilis*; *Merluccius merluccius*; Chilling; Antioxidant; Antimicrobial; Shelf life

INTRODUCTION

Marine species commercialised in the fresh state represent the highest proportion in seafood production and human consumption. However, aquatic food products deteriorate rapidly after the death of the animals they derive from due to the effects of a variety of biochemical and microbial degradation mechanisms [1]. In agreement with nowadays consumer demand for high-quality fresh products, food technologists and fish trade have focussed on the search for new and advanced strategies to maintain the original quality of marine species [2]. Among them, the inclusion of natural preservative compounds in the icing system employed for the chilling storage has been developed. Thus, previous research accounts for the employment of vegetable extracts [3, 4], low-molecular weight organic acids [5, 6] and edible seaweed [7].

Gracilaria gracilis is a red alga (Rhodophyta) widely distributed in different parts of the world. Most previous research on this species has been focused on its employment as a source of agar [8, 9] and as indicator of contamination [10, 11]. However, research focused on its chemical composition has shown that this species can be considered as a multi products source for biotechnological, nutraceutical and pharmaceutical applications [12-14] in agreement with its high contents of total polyphenolic compounds, microelements, polysaccharides, etc.; additionally, a high antioxidant and radical scavenging activity was proved [12, 13].

The present research is a first attempt for the use of *G. gracilis* as a source of preservative compounds to be applied during the chilled storage of fish. For it, ethanolic and aqueous extracts of this edible seaweed were included in the icing medium employed for the chilling storage of hake (*Merluccius merluccius*). Chemical and sensory properties related to quality loss were analysed in fish throughout 9-day storage.

MATERIALS AND METHODS

Preparation of G. gracilis extracts and icing systems

The lyophilised alga *G. gracilis* was provided by Porto-Muiños (Cerceda, A Coruña, Spain). Three different concentrations were tested in the present study. For the lowest one, 1 g alga was mixed with absolute ethanol (2 × 120 mL), stirred for 30 s and

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centrifuged at 3,500 rpm for 10 min at 4 ºC. Then, the supernatant was recovered and filled to 80 mL with absolute ethanol. Additionally, the remaining lyophilised alga was mixed with distilled water (2 × 120 mL), stirred for 30 s and centrifuged at 3,500 rpm for 10 min at 4 ºC. Then, the supernatants were recovered, pooled together with the previously obtained ethanolic extract and the mixture was diluted to 6 L with distilled water (0.17 g lyophilised alga L^{-1} aqueous solution). This solution was packaged in polyethylene bags, kept frozen at –18 ºC and later used as icing medium (G-1 condition).

Likewise, 4 and 15 g of lyophilised alga were extracted successively with ethanol and water, and finally diluted to 6 L to obtain 0.67 and 2.50 g lyophilised alga L^{-1} aqueous solutions, respectively. These solutions were also packaged, kept frozen and further employed as G-2 and G-3 icing conditions, respectively. Besides, 80 mL of absolute ethanol were diluted in 6 L of distilled water; the solution was packaged and kept frozen in the same way as the three other ices and further employed as Control batch (G-0 condition). Before addition to individual fish specimens, the different icing systems were ground to obtain ice flakes.

Fish material, processing and sampling

Fresh European hake (*Merluccius merluccius*) (78 individuals) were caught near the Galician Atlantic coast (north-western Spain) and transported on ice to the laboratory ten hours after catching. The length and weight of the fish specimens were in the following ranges: 30 to 34 cm and 160 to 200 g, respectively.

Upon arrival in the laboratory, six individual fishes were separated and analysed as starting raw fish (day 0); for it, three different groups (two individuals per group) were analysed independently to perform statistical analysis (n=3). The remaining fish were divided into four batches (18 individuals in each batch), placed in boxes and directly surrounded by the four kinds of ices previously mentioned (G-0, G-1, G-2 and G-3 conditions), respectively; a 1:1 fish-to-ice ratio was employed. All batches were placed in a refrigerated room (4 ºC). Boxes employed allowed draining and ice was renewed when required. Fish samples from all batches were taken for analysis on days 2, 6 and 9. At each sampling time, six individuals of each batch were taken for analysis, being considered into three groups (two individuals in each group) that were studied independently (n=3).

Sensory analysis was carried out on the whole fish; chemical analyses were carried out on the white muscle.

Chemical analyses

All solvents and chemical reagents used were of reagent grade (Merck, Darmstadt, Germany).

Total polyphenols content of lyophilised alga was assessed by means of the Folin-Ciocalteu colorimetric method (Cary 3E UV–Visible spectrophotometer, Varian; Mulgrave, Victoria, Australia) as described previously [15]. Measurements were made in triplicate. Gallic acid (GA) was used as standard. Results were expressed as mg GAE g^{-1} lyophilised alga.

Lipids were extracted from the fish white muscle by the Bligh and Dyer [16] method, which employs a single-phase solubilisation of the lipids using a chloroform-methanol (1:1) mixture. The results were calculated as g lipid kg^{-1} muscle.

Free fatty acid (FFA) content was determined in the lipid extract of the fish muscle by the Lowry and Tinsley [17] method based on complex formation with cupric acetate-pyridine followed by spectrophotometric (715 nm) assessment. Results were calculated as mg oleic acid kg^{-1} muscle and expressed as mg FFA kg^{-1} muscle.

The peroxide value (PV) was determined spectrophotometrically (Beckman Coulter, DU 640; London, UK) using the lipid extract *via* previous peroxide reduction with ferric thiocyanate according to the Chapman and McKay [18] method. The results were expressed as meq active oxygen kg^{-1} lipids.

The thiobarbituric acid index (TBA-i) was determined according to Vyncke [19]. This method is based on the reaction between a trichloroacetic acid extract of the fish muscle and thiobarbituric acid. Content of thiobarbituric acid reactive substances (TBARS) was spectrophotometrically measured at 532 nm and calculated from a standard curve using 1,1,3,3-tetraethoxy-propane (TEP); results were expressed as mg malondialdehyde kg^{-1} muscle.

The formation of fluorescent compounds (Fluorimeter LS 45; Perkin Elmer España; Tres Cantos, Madrid, Spain) was determined by measurements at 393/463 nm and 327/415 nm as described by Losada *et al*. [20]. The relative fluorescence (RF) was calculated as follows: $RF = F/F_{st}$, where F is the fluorescence measured at each excitation/emission maximum and F_{st} is the fluorescence intensity of a quinine sulphate solution (1 μg mL^{-1} in 0.05 M H_2SO_4) at the corresponding wavelength. The fluorescence ratio (FR) was calculated as the ratio between the two RF values: $FR = RF_{393/463\ nm}/RF_{327/415\ nm}$. The FR value was determined using the aqueous phase that resulted from the lipid extraction of the fish muscle [16].

Trimethylamine-nitrogen (TMA-N) values were determined using the picrate colorimetric (Beckman Coulter, DU 640; London, UK) method, as previously described by Tozawa *et al*. [21]. This method involves the preparation of a 5% trichloroacetic acid extract of fish muscle (10 g/25 mL). The results were expressed as mg TMA -N kg^{-1} muscle.

Sensory analysis

Sensory analysis was conducted by a sensory panel that consisted of five experienced judges who adhered to traditional guidelines concerning fresh and refrigerated fish, which were adapted to hake [22]. Before carrying out the present experiment, the judges received training on refrigerated hake. Special attention was paid to the evolution of the sensory descriptors that were found as limiting factors for the shelf life.

For the sensory acceptance, four categories were ranked [22]: highest quality (E), good quality (A), fair quality (B) and unacceptable quality (C). Sensory assessment of the fish included the following descriptors: skin and mucus development, eyes, external odour, gills appearance and odour, consistency, flesh (raw and cooked) odour and flesh taste. At each sampling time, whole fish specimens were coded with 3-digit random numbers and presented to the panellists in individual trays, which were scored individually.

Statistical analysis

Data obtained from the different chemical analyses were subjected to the ANOVA method to explore differences resulting from the effect of the chilling condition; the comparison of means was performed using the least-squares difference (LSD) method. Data obtained from the sensory evaluation were analysed by the non-parametric Kruskal-Wallis test. In all cases, analyses were carried out using the PASW Statistics 18 software for Windows (SPSS Inc., Chicago, IL, USA); differences between batches were considered significant for a confidence interval at the 95 % level ($p<0.05$) in all cases.

RESULTS AND DISCUSSION

Assessment of quality loss by chemical indices

Lipid content of hake fish ranged between 5.0 and 5.7 g kg^{-1} muscle. Lipid hydrolysis development was measured by the FFA determination (Fig. 1). A marked increase was observed in all kinds of samples at the end of the storage when compared with the initial fish.

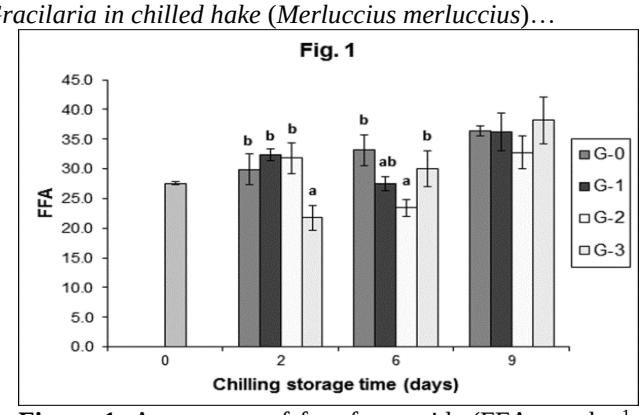

Figure 1. Assessment of free fatty acids (FFA; mg kg^{-1} muscle) formation* in chilled hake muscle stored under various icing conditions**

* Average values of three replicates (*n*=3). Standard deviations are indicated by bars. Average values accompanied by different letters (a, b) denote significant differences ($p<0.05$). No letters are included when significant differences were not found ($p>0.05$).

** Abbreviations of icing conditions as expressed in Table 1.

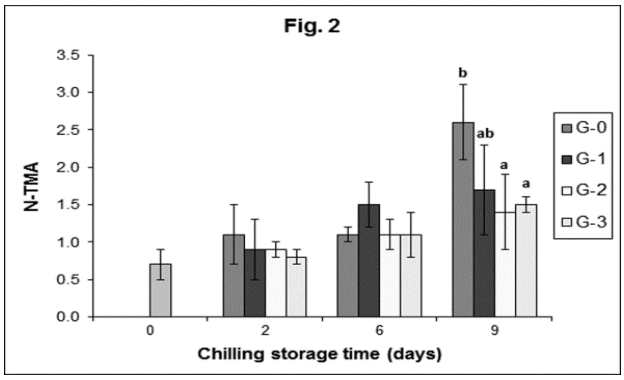

Figure 2. Assessment of trimethylamine-nitrogen (TMA-N; mg kg^{-1} muscle)* in chilled hake muscle stored under various icing conditions**

* Average values of three replicates (*n*=3). Standard deviations are indicated by bars. Average values accompanied by different letters (a, b) denote significant differences ($p<0.05$). No letters are included when significant differences were not found ($p>0.05$).

** Abbreviations of icing conditions as expressed in Table 1.

A definite effect of *G. gracilis* extracts in the chilling system on the lipid hydrolysis development could not be proved; however, at day 2, the lowest FFA formation ($p<0.05$) was found in samples corresponding to G-3 condition, while the lowest average values in the 6-9-day period corresponded to the G-2 batch.

Previous related research showed opposite conclusions. In agreement with the present results, the presence of citric, lactic and ascorbic acids in the icing system did not lead to a marked inhibitory effect on FFA formation in chilled horse mackerel [5]; the same conclusion was obtained by Özyurt *et al*. [4] by employing a rosemary extract during the chilling storage of sardine. Contrary, an inhibitory

effect on fish FFA formation could be observed by applying an ethanolic-aqueous extract of *Fucus spiralis* during the chilled storage of hake [7]; a similar result was attained by Quitral *et al*. [3] by employing ice including rosemary or oregano extracts in chilled Chilean jack mackerel.

Lipid oxidation development was measured by different and complementary indices. Primary compounds assessment (PV) (Table 1) showed a low peroxide formation in all kinds of samples; values were in all cases below 3.1. Scarce differences could be observed between samples, so that a definite effect of alga presence in the ice could not be proved ($p>0.05$) for the peroxide content in chilled hake.

Secondary lipid oxidation development was measured by means of the TBA-i (Table 1). This index showed a marked increase in all samples after a 9-day chilled storage. However, values obtained can be considered low in all cases, being all scores lower than 0.22. Consequently, differences between samples can be considered scarce. Interestingly, lower average values were observed in fish corresponding to the highest *G. gracilis* presence (G-3 condition) throughout the whole experiment; however, differences with the control were not found significant ($p>0.05$).

Interaction compounds formation was measured by the FR (Table 1). A progressive formation of such compounds could be observed for all samples throughout the whole chilling storage.

At the end of the experiment, all kinds of samples including *G. gracilis* extracts in the icing system showed a lower FR ($p<0.05$) than the control. Consequently, an inhibitory effect on tertiary lipid oxidation compounds could be concluded.

Table 1. Lipid oxidation development* in chilled hake muscle stored under various icing conditions**

Quality index	Chilling time (days)	Icing condition			
		G-0	G-1	G-2	G-3
Peroxide value (meq active oxygen kg^{-1} lipids)	0	1.84 (0.13)			
	2	2.23 b (0.04)	1.83 ab (0.45)	1.72 a (0.01)	1.81 ab (0.73)
	6	2.96 (0.01)	2.57 (0.37)	2.67 (0.47)	2.57 (0.41)
	9	1.72 ab (0.32)	1.09 a (0.40)	2.49 ab (0.92)	3.00 b (0.28)
Thiobarbituric acid index (mg malondialdehyde kg^{-1} muscle)	0	0.09 (0.01)			
	2	0.07 (0.02)	0.12 (0.06)	0.05 (0.04)	0.02 (0.00)
	6	0.06 (0.02)	0.08 (0.01)	0.06 (0.04)	0.03 (0.02)
	9	0.18 ab (0.01)	0.15 a (0.03)	0.22 b (0.01)	0.12 a (0.05)
Fluorescence ratio	0	2.72 (0.65)			
	2	2.61 b (0.09)	2.19 ab (0.66)	2.02 a (0.19)	2.26 ab (0.53)
	6	3.31 (0.77)	3.02 (0.11)	3.03 (0.15)	2.98 (1.18)
	9	4.18 b (0.15)	3.24 a (0.16)	3.18 a (0.50)	3.11 a (0.23)

* Average values of three replicates (n=3); standard deviations are indicated in brackets. Average values followed by different letters (a, b) denote significant ($p<0.05$) differences as a result of the icing medium. No letters are indicated when differences were not found ($p>0.05$). ** Icing conditions: G-1, G-2 and G-3 (ices prepared from 0.17, 0.67 and 2.50 g L^{-1} aqueous solution of *G. gracilis* extract, respectively); G-0 (Control; without alga extract in the icing system).

This result can be explained on the basis that previous research on *G. gracilis* has shown a marked radical-scavenging ability and antioxidant behaviour [12, 13]. Interestingly, a polyphenol content of 3.1±0.8 mg GAE g^{-1} lyophilised alga was obtained in the current study. This polyphenol

content can be considered very similar to the one described by Heffernan *et al*. [13] (2.79-5.36 mg GAE g^{-1}) but lower than the one reported by Francavilla *et al*. [12] (2.3-65.0 mg GAE g^{-1}).

In a closely related research [7], the inclusion in the icing medium of alga *F. spiralis* led to a lower FR in hake muscle during the chilled storage. Previous research related to other natural sources of antioxidant compounds has shown a preservative effect when present in the icing medium.

This accounts for Chilean jack mackerel as a result of including an oregano or rosemary extract in the icing system [3], horse mackerel by the presence of an organic acid mixture (citric, lactic and ascorbic acids) as ice system [5] and sardine by including a rosemary extract in the chilling medium [4].

Table 2. Sensory acceptance* of chilled hake stored under various icing conditions**

Descriptor	Chilling time (days)	Icing condition			
		G-0	G-1	G-2	G-3
Skin	0		E		
	2		A		
	6		A		
	9	C^y		B^z	
Eyes	0		E		
	2		A		
	6		A		
	9		B		
External odour	0		E		
	2		A		
	6	B^y		A^z	
	9	C^y		B^z	
Gills	0		E		
	2		A		
	6		B		
	9		B		
Consistency	0		E		
	2		A		
	6		B		
	9		B		
Raw flesh odour	0		E		
	2		A		
	6		A		
	9	C^y		B^z	
Cooked flesh odour	0		E		
	2		A		
	6		A		
	9	C^y		B^z	
Flesh taste	0		E		
	2		A		
	6		A		
	9		B		

* Scores as expressed in the material and methods section. For each descriptor, scores with different superscripts (z, y) indicate significant ($p<0.05$) differences as a result of the icing condition. No superscripts are included when differences were not found ($p>0.05$).

** Abbreviations of icing conditions as expressed in Table 1.

Microbial activity development was measured by the TMA detection (Fig. 2). Thus, a progressive formation in all kinds of samples with chilling was obtained for this quality parameter. Comparison between samples showed lower values ($p<0.05$) in samples corresponding to G-2 and G-3 batches

when compared with the control. Accordingly, an inhibitory effect on TMA formation was implied by the presence of *G. gracilis* in the icing system. Interestingly, none of the samples surpassed the legal limit established for this species (5 mg kg^{-1}) [23].

Volatile amine compounds such as TMA have been reported to be produced mostly as a result of microbial development during fish chilled storage [1]. The inhibitory effect of *G. gracilis* on microbial activity obtained in the present study can be explained on the basis of previous research pointing out the possibility of using this alga species as a multi-product source for nutraceutical and pharmacological application [12, 14]. The antimicrobial effect of algae in general has been attributed especially to the presence of terpenes, polyphenols and oligomeric phlorotannins [24, 25].

Previous research shows the inhibitory effect on TMA content as a result of including a wide range of natural preservative compounds in the icing system. Thus, the presence of citric, lactic and ascorbic acids led to a lower TMA formation in chilled mackerel [6]. Microbial activity inhibition was also detected by Özyurt *et al*. [4] by the presence of rosemary extract in the ice during sardine chilling; thus, a lower formation of histamine and putrescine was detected in fish samples corresponding to batches including the plant extract. Contents on microbial counts of various bacteria groups showed to decrease as a result of including wild-thyme hydrosol in the icing medium during Transcaucasian barb chilled storage [26]. Microbial activity inhibition was also observed in previous related research [7]; thus, lower counts of aerobe, psychrotroph, proteolytic and lipolytic bacteria were implied in chilled hake by employing ice including ethanolic-aqueous extracts of alga *F. spiralis*.

Assessment of sensory acceptance

Sensory acceptance was evaluated by analysis of different descriptors. Results obtained are expressed in Table 2. For all kinds of samples, all descriptors showed a sensory quality decrease with chilling time. Fish corresponding to control and G-1 batches were found rejectable at the end of the experiment. Comparison among icing conditions showed higher scores ($p<0.05$) for fish corresponding to G-2 and G-3 batches when compared with their counterparts from G-1 and G-0; such differences were obtained for skin, external odour, raw flesh odour and cooked flesh odour. Consequently, a preservative effect was concluded by the presence of *G. gracilis* extracts in the icing medium. This effect is in agreement with the above-mentioned results on antioxidant and antimicrobial properties found in the current alga extracts.

An increased shelf life time has also been observed as a result of including preservative compounds in the icing system. This is the case of a rosemary extract during chilled storage of sardine [4], an acid mixture (citric, lactic and ascorbic) during the chilled storage of horse mackerel [5] and wild-thyme hydrosol in chilled transcaucasian barb [26].

CONCLUSIONS

G. gracilis extracts were tested as a source of natural preservative compounds to be included in the icing system employed during the chilled storage of hake. As a result, an inhibitory effect on lipid oxidation development (tertiary oxidation compounds) and microbial activity (trimethylamine formation) was observed in fish corresponding to batches including the two most concentrated alga conditions. However, a definite effect on lipid hydrolysis development could not be implied ($p>0.05$). Concerning sensory analysis, samples from the same two batches revealed a higher acceptance ($p<0.05$) than control; this difference was based on the evaluation of various descriptors such as skin, external odour, raw flesh odour and cooked flesh odour. On the basis of the presence of antioxidant and antimicrobial compounds, a profitable effect on quality retention of chilled hake is achieved by including *G. gracilis* extracts in the icing system.

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REFERENCES

1. I. Ashie, J. Smith, B. Simpson, *Crit. Rev. Food Sci. Nutrit.*, **36**, 87 (1996).
2. C. Campos, M. Gliemmo, S. P. Aubourg, J. Barros-Velázquez, in: Novel Technologies in Food Science, A. McElhatton, P. Amaral Sobral (eds.), Chapter 13, Springer, New York, USA, 2012, p. 299.
3. V. Quitral, MªL. Donoso, J. Ortiz, MªV. Herrera, H. Araya, S. P. Aubourg, *LWT-Food Sci. Technol.*, **42**, 1450 (2009).
4. G. Özyurt, E. Kuley, E. Balikçi, Ç. Kaçar, S. Gökdogan, M. Etyemez, F. Özogul, *Food Bioproc. Technol.*, **5**, 2777 (2012).
5. M. Sanjuás-Rey, J. Barros-Velázquez, S.P. Aubourg, *Grasas Aceites*, **62**, 436 (2011).
6. M. Sanjuás-Rey, J. M. Gallardo, J. Barros-Velázquez, S. P. Aubourg, *J. Food Sci.*, **77**, M264 (2012).

7. J. Barros-Velázquez, J. Miranda, M. Ezquerra-Brauer, S. P. Aubourg, *Int. J. Food Sci. Technol.,* **51**, 2081 (2016).
8. E. Marinho-Soriano, E. Bourret, *Biores. Technol*., **90**, 329 (2003).
9. M. Rodríguez, M. Matulewicz, M. Noseda, D. Ducatti, P. Leonardi, *Biores. Technol*., **100**, 1435 (2009).
10. A. Smit, D. Robertson-Andersson, S. Peall, J. Bolton, *Aquaculture*, **269**, 377 (2007).
11. B. Henriques, L. Rocha, C. Lopes, P. Figueira, R. Monteiro, A. Duarte, M. Pardal, E. Pereira, *J. Chem. Eng.*, **281**, 759 (2015).
12. M. Francavilla, M. Franchi, M. Monteleone, C. Caroppo, *Mar. Drugs*, **11**, 3754 (2013).
13. N. Heffernan, T. Smyth, R. FitzGerald, A. Soler-Villa, N. Brunton, *Int. J. Food Sci. Technol*., **49**, 1765 (2014).
14. D. Rodrigues, A. Freitas, L. Pereira, T. Rocha-Santos, M. Vasconcelos, M. Roriz, L. Rodríguez-Alcalá, A. Gomes, A. Duarte, *Food Chem*., **183**, 197 (2015).
15. A. Rodríguez-Bernaldo de Quirós, S. Frecha-Ferreiro, A. Vidal-Pérez, J. López-Hernández, *Eur. Food Res. Technol.*, **231**, 495 (2010).
16. E. Bligh, W. Dyer, *Can. J. Biochem. Physiol.*, **37**, 911 (1959).
17. R. Lowry, I. Tinsley, *J. Amer. Oil Chem. Soc.*, **53**, 470 (1976).
18. R. Chapman, J. McKay, *J. Amer. Oil Chem. Soc.*, **26**, 360 (1949).
19. W. Vyncke. *Fette Seifen Anstrichm.*, **72**, 1084 (1970).
20. V. Losada, J. Barros-Velázquez, J. M. Gallardo, S. P. Aubourg, *Eur. J. Lipid Sci. Technol.*, **106**, 844 (2004).
21. H. Tozawa, K. Erokibara, K. Amano, in: Fish Inspection and Quality Control, R. Kreuzer (ed.), Fishing News Books Ltd., London, UK, 1971, p. 187.
22. European Council Regulation, European Community (EC), No 2406/96, 26 November 1996. *Off. J. Eur. Comm.*, L-334/2:23.12 (1996).
23. S. Baixas-Nogueras, S. Bover-Cid, T. Veciana-Nogués, MªL. Nunes, C. Vidal-Carou, *J. Food Sci.*, **68**, 1067 (2003).
24. E. Sandsdalen, T. Haug, K. Stensvag, O. Styrvold, *World J. Microb. Biotechnol.*, **19**, 777 (2003).
25. C. De Almeida, H. de S. Falcão, G. de M. Lima, C. de A. Montenegro, N. Lira, P. de Athayde-Filho, L. Rodrigues, M. de Sousa, J. Barbosa-Filho, L. Batista, *Int. J. Mol. Sci.*, **12**, 4550 (2011).
26. N. Oral, M. Gülmez, L. Vatansever, A. Güven, *J. Food Prot.*, **71**, 218 (2008).

АНТИОКСИДАНТНИ И АНТИМИКРОБНИ СВОЙСТВА НА ЕКСТРАКТИ ОТ ВОДОРАСЛОТО *Gracilaria gracilis* ПРИ СЪХРАНЕНИЕ НА ХЕК (*Merluccius merluccius*) В ОХЛАДЕНО СЪСТОЯНИЕ

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(Резюме)

Настоящото изследване е първи опит за използване на водораслото *Gracilaria gracilis* като източник на консервиращи съединения, които да се използват при съхранение на риба в охладено състояние. За целта, комбинация от етанолов и воден екстракт от ядивното морско водорасло е добавена към замразяващата среда, използвана за съхраняване на хек (*Merluccius merluccius*) в охладено състояние. За установяване на евентуално влошаване на качеството са проведени химически и сензорни анализи на рибата в продължение на 9-дневно съхранение. Изследвани са три концентрации на водораслото и са сравнени с контролна проба (традиционно охлаждане с лед). Установен е инхибиторен ефект ($p<0.05$) върху окислението на липидите (образуване на третични окислени съединения) и микробната активност (образуване на триметиламин) в сериите от риба, съдържащи двете най-високи концентрации от водораслото. Не е установен обаче ефект ($p>0.05$) върху хидролизата на липидите (образуване на свободни мастни киселини). Сензорният анализ на проби от същите две серии показва по-добро отнасяне ($p<0.05$) в сравнение с контролата. Разликата се основава на оценката на различни дескриптори като кожа, външен мирис, мирис на суровото месо и мирис на свареното месо. Направен е изводът, че включването на екстракт от *G. gracilis* в охладителната система има положителен ефект върху запазването на качеството на охладения хек като следствие от присъствието на антиоксидантни и антимикробни молекули.

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Optical characteristics and antioxidant activity of lingonberry (*Vaccinium vitis-idaea*) fruit juice

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This research is focused on juices of wild berries and more specifically on lingonberry juice. The kinetics of color parameters of the samples with inulin or lactulose during the storage is investigated. The content of anthocyanins, phenolic content, antioxidant activity and fluorescence spectra has been measured. The dependences between chemical parameters, color characteristics and ratio between intensity of emission and intensity of excitation for exciting wavelength 275 nm have been found. The dependence between antioxidant activity and total phenolic content also exists. Excitation in the UV region is suitable for distinguishing the phenolic content and antioxidant compounds. The most suitable wavelengths found to be 265 nm and 275 nm.

Keywords: Lingonberry (*Vaccinium vitis-idaea*), Antioxidant activity, Total phenolic content, Fluorescence in UV region**,** fruit juice

INTRODUCTION

Fruit juices contain a large amount of vitamins and minerals and are often recommended by nutritionists and medics for their healing effect. They contain alkali-acid equilibrium of cells, influence nutrition of organs, change positively or negatively the reactivity of the body.

Recently an increased interest has been demonstrated towards wild blueberries, blackberries and raspberries. The fruit of cranberry exhibits antiseptic, anti-putrefactive and anti-inflammatory effects. They contain bioactive compounds such as phenolic compounds [1-3], anthocyanins, omega-3 fatty acids, vitamins [4]. These compounds have nutritional and medical application – they stimulate the immune system, modulate hormone metabolism and possess antibacterial and antiviral action [5-7]. In numerous studies authors reported that the foods from lingonberry can reduce the incidence of cancer, cataracts, macular degeneration and cardiovascular disease [8,9]. They are also used in case of avitaminoses, scurvy, or as generally strengthening beverages to improve appetite. Extracts of these berries are used with nephrolithiasis, pielitis, cystitis, joint rheumatism and gout.

The aim of the present research is to demonstrate the capability of fluorescence spectroscopy for quality detection of some biologically active substances and antioxidants in fruit juice from lingonberry with additional substances such as lactulose or inulin, and to investigate the changes in the color characteristics during the storage period.

MATERIALS AND METHODS

Samples and technologies

The investigation used wild fruits of *Vaccinium vitis-idaea* L. harvested in 2015 in the region of Velingrad. The juice from lingonberry was obtained by using a technological scheme. The pasteurized juices with added lactulose or inulin were investigated.

The samples were produced by crushing and squeezing of fruits after which the juice was filtered and poured in glass bottles with metal caps. The samples were enriched with lactulose at concentrations of 0.5 ml, 1 ml and 1.5 ml and with inulin at concentrations of 1g, 2g, 3g, respectively.

Content of polyphenols and anthocyanins

0.5 - 1 ml of fresh plant material was triturated with quartz sand and 2-5 ml of 70% methanol in a mortar, quantitatively transferred to a flask with a reflux refrigerator. It was extracted at 700ºC three times for 20 min each. Anthocyanin pigment concentration, expressed as cyanidin-3-glucose equivalent, was calculated. The contents of biologically active substances such as anthocyanin and phenolic components in the juice samples were measured spectrophotometrically.

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Ferric reducing antioxidant power (FRAP) assay was used for determining the antioxidant activity. The reaction was started by mixing 3.0 ml of FRAP reagent with 0.1 ml of the investigated juice. The reaction time was 10 min at 37 °C in darkness and the absorbance was measured at 593 nm.

Fluorescence spectra measurements

The sources used to measure the fluorescence spectra were 245 nm, 265 nm, 275 nm and 295 nm light-emitting diodes (LEDs). A fiber optic spectrometer (AvaSpec-2038, Avantes) with sensitivity in the (200-1100) nm range and a resolution of about 8 nm was used to measure the fluorescence spectra. The lingonberry juices were placed in a cuvette 10 mm × 10 mm and illuminated by LEDs.

Color measuring

The color characteristics in the CIELab colorimetric system were determined by the spectrophotometer Konica Minolta CR-400 / 410. They were determined by measuring the reflectance spectrum in a cuvette of 8 mm length. The color parameters a, b and the brightness L of the tested samples were measured. Parameter chroma C was defined as follows:

$$C = \sqrt{a^2 + b^2} \quad (1)$$

All measurements were carried out at room temperature and the average value was taken from 3 measurements.

RESULTS

The juice from lingonberry with different concentrations of lactulose and inulin was investigated for total content of anthocyanins and polyphenolic compounds. The results are presented in Table 1. By using FRAP method the data for antioxidant activities were obtained (Table 1).

Table 1. Chemical characteristics of juices from lingonberry

Sample	Anthocyanins, mg/L	Total polyphenolic content, mg GAE/L	Antioxidant activity, mMTE/L (FRAP method)
Juice+Lactulose 0.5 ml	161. 65	2607. 95	21554. 68
Juice+Lactulose 1 ml	131. 59	2002. 31	16175.56
Juice+Lactulose 1.5 ml	121. 23	1966. 47	15857.26
Inulin, 1 g	137. 9328	1763. 399	14053.61
Inulin, 2 g	140. 6046	1759. 815	14021.79
Inulin, 3 g	134. 76	1806. 403	14435.56

The juice from lingonberry with inulin has a lower phenolic content than that obtained from lingonberry with lactulose. The fluorescence signal is too weak. For this reason, the fluorescence spectra were obtained only for the juices from lingonberry with lactulose. There is a connection between the investigated chemical parameters (Table 1) and the fluorescence peaks in the visible region. The fluorescence spectra for excitation wavelengths 245 nm, 265 nm, 275 nm and 295 nm are presented on Figure 1. Color characteristics in CIE Lab colorimetric system were measured at the first, the 5th, and the 10th day (Figures 2 and 3) from the production of the juices. There is a correlation between some color parameters and storage time. The dependences between chemical parameters, color characteristics and ratio between intensity of emission and intensity of excitation for exciting wavelength 275 nm were found. There is also dependence between antioxidant activity and total phenolic content. The existing dependences and their correlation coefficients are presented in the discussion.

DISCUSSION

The research is focused on juices of wild berries and more specifically on cranberry juice.

The fruits of cranberry have very good nutritional and gustatory qualities, rich in vitamin C. They are widely used in traditional medicine. In the present study we established relations between the optical characteristics and the contents of biologically active substances, the influence of additions such as inulin and lactulose on the kinetics of color indicators, as well as the benefits for healthy nutrition.

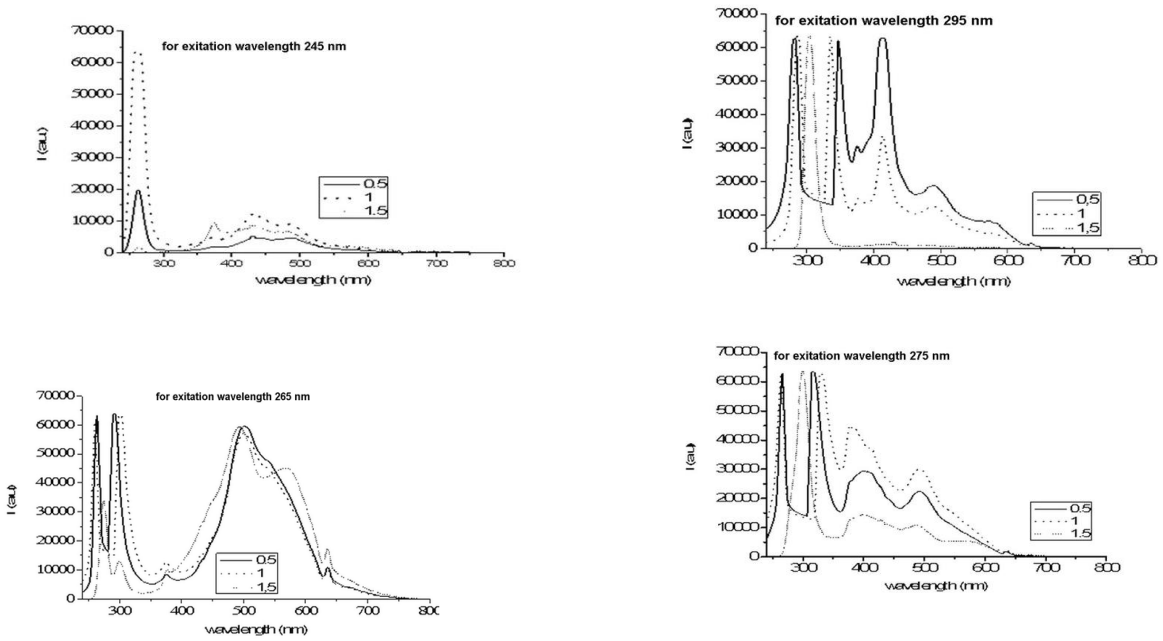

Figure 1. Fluorescence spectra of juices from lingonberry with different concentrations of lactulose

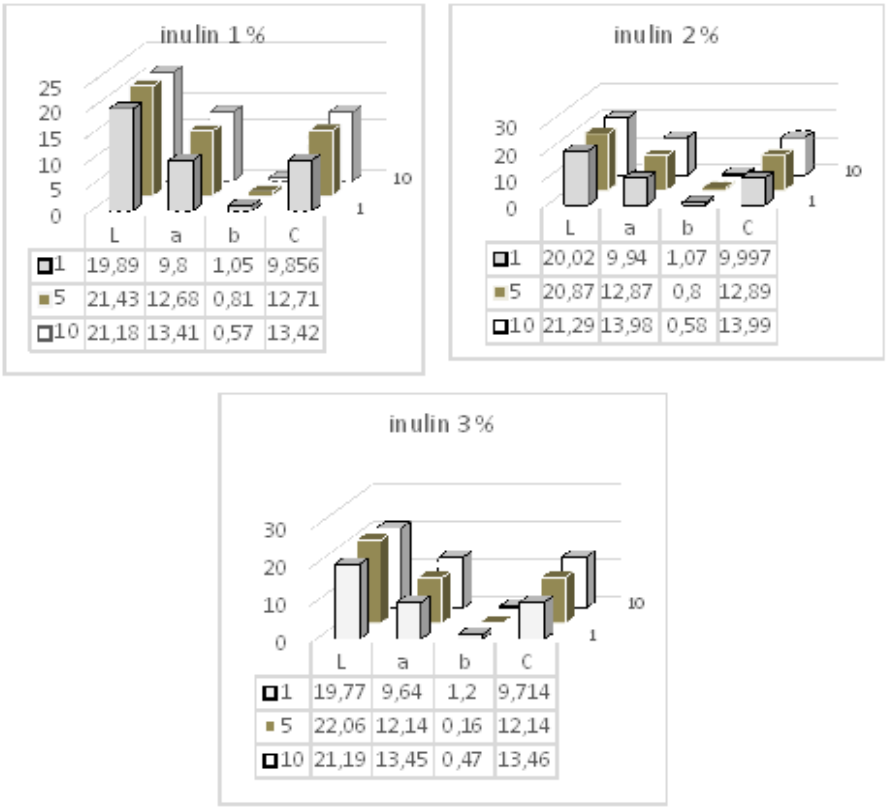

Figure 2. Kinetics of color parameters during the storage time of juice from lingonberry with inulin

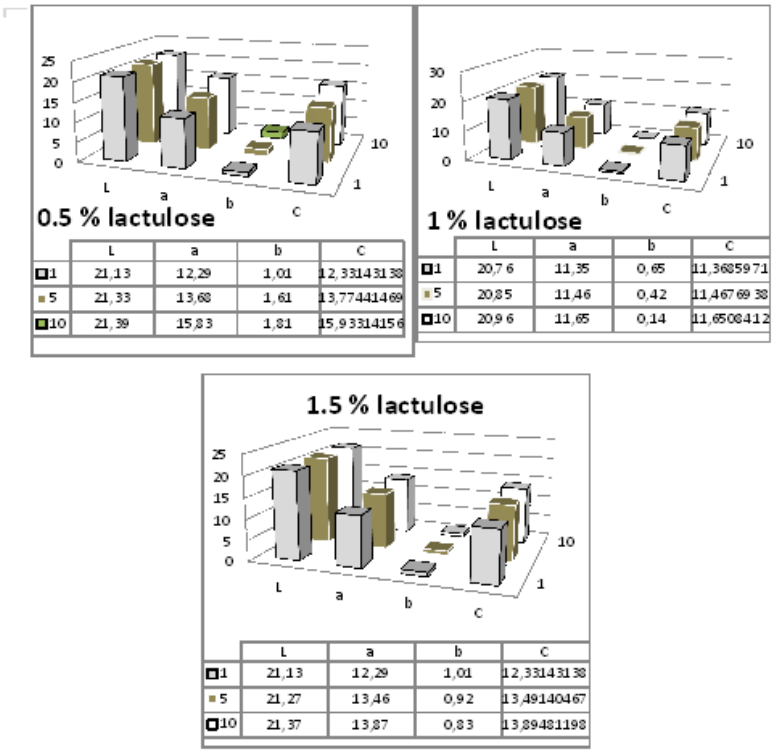

Figure 3. Kinetics of color parameters during the storage time of juice from lingonberry with lactulose

The inulin and lactulose added are often used as substitutions of sweeteners. Lactulose is a disaccharide formed by monosaccharides fructose and galactose connected through a glycosidic bond. Inulin is a reserve polysaccharide, accumulated in underground roots (roots, rhizomes, tubers) of plants such as elecampane, ground apple, dahlia, dandelion, etc. It has been proven that inulin has a number of effects beneficial for the body such as:

- Reduces toxic metabolites;
- Has anti-constipation effect;
- Reduces liver cholesterol and triglycerides;
- Reduces blood pressure;
- Normalizes blood sugar level;
- Improves lipid metabolism in patients with diabetes;
- Improves absorption of minerals in the organism.

Both the brightness L and the value of the red color component "a" increase in the lingonberry juices with concentration of inulin between 1 g and 2 g. Slight decrease was observed for the indicated components if the concentration of inulin increases up to 3g. No similar tendency was observed for samples with lactulose.

For inulin concentrations between 1 g and 2 g an increase of lightness and of the red component of the investigated samples was observed. An increase of the concentration to 3 g leads to a weak reduction of the indicated components. No similar tendency was observed for samples with lactulose.

For all concentrations of inulin and lactulose in cranberry juice an increase of lightness and chroma was observed from the first to the tenth day. For the same period a reduction of the yellow and an increase of the red component was observed.

The latter fact can be explained with the change in the quantity of anthocyanins. Probably, the course of chemical reactions leads to an increase in their contents during storage. The addition of even higher quantity of lactulose leads to their reduction (Table 1), while that of inulin leads to weak changes of the contents of anthocyanins.

An increase in brightness and chroma for all concentrations of inulin and lactulose in the lingonberry juice from the first to the 10^{th} day was observed. The yellow color component decreased and the red component increased during the same period. The latter fact can be explained with the change in the quantity of the anthocyanins. The possibility of chemical reactions to take place led to an increase in their content during juices storage. The addition of a quantity of lactulose lead to a decrease in the anthocyanins (Table 1), and the addition of inulin lead to slight changes in the content of anthocyanins.

The interest to anthocyanins is not dictated by their application as natural colorants alone [10], but by their potential for the development of pharmaceutical products. They exhibit a strong P-

vitamine activity, reduce the fragility and the permeability of the capillaries [11]. They are characterized by a strong anti-inflamatory, hypotonic and collagen stabilizing action [12]. The intake of anthocyanins leads to a normalization of the capillary permeability and improvement of eye sight. They play an important role in the cross-linking of collagen by inhibiting the enzymatic digestion during inflammatory processes [13].

Anthocyanins are applied successfully in the prophylaxis and treatment of glaucoma and other eye diseases [14]. In model systems anthocyanin extracts exhibit cardio-protective effect [15] and inhibit the growth of cancer cells. Some of the therapeutic effects of anthocyanin are attributed to their antioxidant properties [16].

It is seen from Table 1 that cranberry with inulin possesses a lower phenol content and a weaker antioxidant activity. This explains the considerably weaker fluorescence signal of samples containing lactulose. Therefore, in the present study we have studied and commented only fluorescence spectra from cranberries with lactulose.

It is evident from Figure 1 that the emission intensity has the lowest value for excitation wavelength of 245 nm. The fluorescence spectra are clearly distinguishable for 275 nm and the best ratio between excitation and emission intensities is for 265 nm excitation. The excitation/emission ranges in the fluorescence spectra can be connected with the following phenolic components and flavonoids:

- Chlorogenic acid: (245-250 nm)/ (430-440 nm). Similar results have been obtained by Mazina *et al*. [17] for apple juice;
- Caffeic acid: (230-350 nm)/ (405-470 nm) [18];
- Vanillic acid: (295-300 nm)/ (305 -355 nm);
- Tannins: (230-315 nm)/ (345-405 nm);
- Catechin and epicatechin: (275-280 nm)/ (320-335 nm) [19].

The fluorescence spectroscopy is an advanced technique for the rapid screening of lingonberry juices for total phenolic contents. A dependence was found between the ratio of intensity of fluorescence and intensity of excitation for wavelength 275 nm and total phenolic content. A positive correlation was obtained between phenols content and antioxidant activity in accordance with literature data for apple juice [20].

Linear dependences exist between:

- Total phenolic content (TPC) and anthocyanins (A): TPC=-132.66+16.83×A with correlation coefficient $R^2 = 0.96$;
- Antioxidant activity (AA) and total phenolic content (TPC): AA=8.88×TPC-1609.2, correlation coefficient $R^2 = 1$;
- Total phenolic content and the ratio $I_{emission}/I_{exitation}$ for exciting wavelength 275 nm: $TPC=1750+262.20\times I_{emission}/I_{excitation}$, correlation coefficient $R^2 = 0.99$;
- Lightness of the samples and the ratio $I_{emission}/I_{exitation}$ for exciting wavelength 275 nm: $L=-20.31+0.99\times I_{emission}/I_{excitation}$, correlation coefficient $R^2 = 0.937$.

CONCLUSIONS

From the obtained results it can be concluded that:

Front–face fluorescence spectroscopy gives the possibility of qualitative detection of phenolic components in lingonberry juice, which are important for nutrition hygiene. The proposed technique includes fast and cheap methods, without using chemical reagents.

The phenolic content and the antioxidant activity of the juice from lingonberry with lactulose are higher than those of the juice from lingonberry with inulin.

REFERENCES

1. P. Kylli, L. Nohynek, R. Puupponen-Pimia, B. Wasterlund-Wikstrom, T. Leppanen, J. Welling, E. Moilanen, M. Heinonen, *Journal of Agricultural and Food Chemistry*., **59**, 3373 (2011).
2. W. Kalt, S. MacKinnon, J. McDonald, M. Vingvist, C. Craft, A. Howell, *Journal of the Science of Food and Agriculture*., **88**, 68 (2008).
3. Jk. Hellstrom, Ar. Torronen, Ph. Mattila, *Journal of Agricultural and Food Chemistry*, **57,** 7899 (2009).
4. E. Bere, *European Journal of Clinical Nutrition*, **61**, 431(2007).
5. S. Vasconcelos, M. Goulart, J. Moura, V. Benfato, L. Kubota, *Sociedade Brasileira de Química, Química Nova*, **30,** 1323 (2007).
6. S. Ribeiro, J. Queiroz, M. Pelúzo, N. Costa, S. Matta, M. Queiroz, *Bioscience Journal*, **21**, 133 (2005).
7. A. Szajdek, E. Borowska, *Springer, Plant Foods for Human Nutrition*, **63**,147 (2008).
8. M. Fraser, A. Lee, C. Binns, *Expert Review Anticancer Therapy*, **5**, 847 (2005).
9. W. Stahl, H. Sies, Biochimica et Biophysica Acta - Molecular Basis of Disease Elsevier Academic Press, Chapter 2, p. 101, 2005.
10. P. Bridle, CF. Timberlake, *Food Chem.*, **58**, 103 (1997).
11. H. Wagner, Ann. Proc. *Phytochem. Soc. Europe*, **25**, 409 (1985).
12. M.N. Lila, *J. Biomed. Biotech*., **5**, 306 (2004).
13. M.C. Ronziere, D. Herbage, R. Garrone, J. Frey, *Biochem. Pharmacol.*, **30,** 1771 (1981).

14. D. Ghosh, T. Konishi, *Asia Pac. J. Clinic. Nutr*., **16**, 200 (2007).
15. D.R. Bell, K. Gochenaur, *J. Appl. Physiol*., **100,** 1164 (2006).
16. M. Kahkonen, M. Heinonen, *J. Agric. Food Chem*., **51**, 628 (2003) .
17. J. Mazina, M. Vaher, M. Kuhtinskaja, L. Poruvkina, M. Kaljurand, *Talanta*, **139**, 233 (2015).
18. I. Alexieva, V. Terzieva, Dr. Buhalova, M. Milev, T. Sapundzhieva, I. Milkova, Abstract Book&List of Participants, 2012 Oct 5; 43:99. ISBN 978-88-902152-6-1.
19. A. Rodrigez, G. Diaz, D. Meraz, P. Wold, *Journal of Agricultural and Food Chemistry*, **57**, 1711 (2009).
20. Z. Kobus, R. Nadulski, T. Guz, I. Kamińska, *Technical Sciences*, **17,** 67 (2014).

ОПТИЧНИ ХАРАКТЕРИСТИКИ И АНТИОКСИДАНТНА АКТИВНОСТ НА СОК ОТ ЧЕРВЕНА БОРОВИНКА (*Vaccinium vitis-idaea*)

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Постъпила на коригирана на

(Резюме)

Изследването е съсредоточено върху сокове от горски плодове, по-специално върху сок от червена боровинка (*Vaccinium vitis-idaea*). Изследвана е кинетиката на цветните параметри на пробите с добавен инулин или лактулоза по време на съхранението. Измерено е съдържанието на антоцианини, фенолното съдържание, антиоксидантната активност и флуоресцентните спектри. Открити са зависимостите между физико-химичните параметри, цветните характеристики и съотношението между интензитета на излъчване и интензитета при дължина на вълната на възбуждащата светлина (275 nm). Установена е зависимост между антиоксидантната активност и общото фенолно съдържание. Възбуждането в областта на ултравиолетовите лъчи е подходящо за качествено откриване на феноли и на антиоксиданти. Най-подходящите дължини на вълните са 265 nm и 275 nm.

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Preservative effect of jumbo squid (*Dosidicus gigas*) skin extract as glazing material during the frozen storage of Atlantic Chub mackerel (*Scomber colias*)

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The present research was focused on the quality loss of Atlantic Chub mackerel (*Scomber colias*) during the frozen storage. Its basic objective was to investigate the effect of including a lipophilic extract of jumbo squid (*Dosidicus gigas*) skin (JSS) in the glazing system applied previously to the frozen storage. For it, two different concentrations of skin extracts were tested and compared with two control treatments (water glazing and non-glazing conditions). Quality changes were monitored for a 8-month frozen storage by sensory (odour and taste) and chemical (lipid hydrolysis and oxidation development) evaluation. An inhibitory effect ($p<0.05$) of skin extracts on lipid hydrolysis (free fatty acid formation) evolution was observed; furthermore, lower average values for lipid oxidation indices (peroxide and fluorescent compounds formation) were observed in fish samples corresponding to the highest JSS presence in the glazing system. Some sensory quality enhancement was evident in mackerel as a result of including JSS extracts in the glazing medium, especially for raw-flesh and cooked-flesh odours; as for chemical indices, an increasing effect was implied by increasing the skin extract presence in the glazing medium. Under the conditions tested in the present study, the JSS extract has shown promising antioxidant properties that could be applied to enhance the seafood quality during the commercialisation in frozen conditions. Further research would be necessary to optimise its use in the glazing system.

Keywords: Frozen mackerel; Squid by-product; Glazing; Shelf life; Lipid damage

INTRODUCTION

Fatty fish are attracting considerable attention because of the positive role of marine lipids in human nutrition and health [1]. Nevertheless, during processing and storage, marine lipids are reported to undergo fast oxidation of highly unsaturated fatty acids, which is directly related to the production of off-flavours and odours and to decrease in the nutritional value [2, 3]. To extend the shelf life time, frozen storage has widely been employed to maintain fish properties before consumption or use in other technological processes. However, during frozen storage of fish, lipid hydrolysis and oxidation have been shown to occur and to influence fish acceptance [4, 5].

One particular established technology greatly used during freezing and frozen storage of seafood is the application of a layer of ice to the surface of a frozen product, referred as glazing [6, 7]. Thus, adequate glazing of fish fillets prior to frozen storage would protect the final product from dehydration, oxidation and quality loss. The amount of glaze applied would depend on various factors such as glazing time, seafood temperature, water temperature, product size and shape [8]. Previous research has shown profitable effects of glazing by inhibiting lipid hydrolysis [9] and oxidation [10-12] development and microbial activity [13,14].

To extend the shelf life time during the frozen storage of marine species, the employment of natural preservatives represents a relevant choice. In this sense, seafood by-products have been detected as a good source of antioxidant compounds. Among them, jumbo squid (*Dosidicus gigas*) skin (JSS) represents a promising possibility as being a rich source of pigments and other preserving molecules. Thus, marked antioxidant and antimicrobial effects have been proved on heated marine-oil systems [15] and chilled fish [16,17]. Moreover, no toxic effect was detected in such extracts [17].

The present research was focused on the quality loss of Atlantic Chub mackerel (*Scomber colias*) during the frozen storage. Its basic objective was to investigate the effect of including a lipophilic extract of JSS in the glazing system prior to the frozen storage. For it, two different concentrations of skin extracts were tested and compared with two control treatments (water glazing and non-glazing conditions). Quality changes were monitored for a 8-month frozen storage by sensory (odour and taste) and chemical (lipid hydrolysis and oxidation development) evaluation.

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MATERIAL AND METHODS

Preparation of squid skin extracts and glazing systems

Jumbo squid specimens were caught by local fishermen using the jigging fishing method at the Guaymas harbour (Sonora, Mexico; 8.75°N/112.25°W, 15–18 °C). The length and weight of the squid specimens ranged from 40 to 45 cm and from 2.0 to 3.0 kg, respectively. Squids were transported to the Seafood Laboratory at the University of Sonora 8 h after being captured. The skin was manually removed from the mantle and fins, cut into small pieces (about 15 cm length) and freeze-dried for two days (LABONCO Freeze Dry, Kansas City, MO, USA). The freeze-dried skin (100-mg portions) was placed in polyethylene-sealed bags, which were stored at –25 ± 2 °C.

Acetic acid-ethanol pigment extracts from freeze-dried skin were prepared according to the method developed previously [15]. Briefly, 8 g of freeze-dried skin were blended in 80 mL of a 0.5% acetic acid-ethanol solution (v/v) at 0 °C for 1 min using an Ultra-Turrax equipment (IKA-UltraTurrax T-25, Staufen, Germany). The blended mixture was then submitted to an ultrasound bath at room temperature (18-20 ºC) (Ultrasons, Selecta, Barcelona, Spain) for 3 min. Afterwards, the homogeneous mixture was centrifuged at 3,500 × g at 4 °C for 10 min, then the supernatant being recovered. This process was repeated three times, so that the supernatant recovered after the last centrifugation process was colourless. Extracts were pooled together and carried out to a 200-mL volume solution with the acetic acid-ethanol mixture.

Three glazing systems were prepared. For it, 0, 24 and 144 mL of the above-prepared JSS extract were diluted, respectively, to 11 L with distilled water and led to water glazing (WG; water control), low-concentrated glazing (LCG) and high-concentrated glazing (HCG) systems, respectively. To maintain the same quantity of the solvent mixture in each glazing medium, 144 and 120 mL of 0.5% acetic acid-ethanol solution were added during the preparation of the WG and LCG systems, respectively.

Fish material, glazing and sampling

Fresh Atlantic Chub mackerel (102 specimens) were caught near the Galician Atlantic coast (North-Western Spain) and transported to the laboratory. Throughout this process (10 h), the fish were maintained in ice. The length and weight of the fish specimens ranged from 23.0 to 27.0 cm and from 104 to 121 g, respectively.

Upon arrival to the laboratory, six specimens were separated and analysed as initial fish. These fish specimens were divided into three different groups (two individuals per group) that were analysed independently to perform the statistical analysis; n=3). The remaining fish specimens were divided into four batches (24 individuals in each batch) that were immediately frozen at –40 ºC.

After 48 hours at –40 ºC, the first batch was packaged in polyethylene bags (two pieces per bag) and stored at –18 ºC (blank control; non-glazed batch; NG batch). At the same time, the remaining batches were immersed in the WG, LCG and HCG systems, respectively. In all cases, specimens were immersed for 30 s at 0 ºC, allowed to drain for 15 s, packaged in polyethylene bags (two pieces per bag) and stored at –18 ºC.

Sampling was undertaken at months 2, 4, 6 and 8 of frozen storage at –18 ºC. At each time and for each condition, six individuals were taken, that were divided into three groups (two individuals per group) and studied separately. Analysis of frozen material was undertaken after thawing; thawing was carried out by overnight storage in a cool room (4 ºC).

Chemical analyses related to quality loss

All solvents and chemical reagents used were of reagent grade (Merck, Darmstadt, Germany). Chemical analyses related to fish quality were carried out on the white muscle of mackerel.

Lipids were extracted from mackerel muscle by single-phase solubilisation with chloroform-methanol (1:1), as described by Bligh and Dyer [18]. Results are expressed as g lipid kg^{-1} muscle.

The free fatty acid (FFA) concentration of the mackerel lipid extract was determined by colorimetric reaction with cupric acetate-pyridine and absorbance was measured at 715 nm, according to Lowry and Tinsley [19]. Results are expressed as mg FFA kg^{-1} muscle.

The peroxide value (PV) of the mackerel lipid extract was determined by peroxide reduction with ferric thiocyanate and absorbance was measured at 500 nm (Beckman Coulter, DU 640; London, UK), as described by Chapman and McKay [20]. Results are expressed as meq active oxygen kg^{-1} lipids.

The formation of fluorescent compounds (Fluorimeter LS 45; Perkin Elmer España, Tres Cantos, Madrid, Spain) was determined by measurements at 393/463 nm and 327/415 nm as described by Losada *et al*. [21]. The relative fluorescence (RF) was calculated as follows: $RF = F/F_{st}$, where F is the fluorescence measured at each excitation/emission maximum and F_{st} is the fluorescence intensity of a quinine sulphate solution (1 μg ml^{-1} in 0.05 M H_2SO_4) at the corresponding

wavelength. The fluorescence ratio (FR) was calculated as the ratio between the two RF values: $FR = RF_{393/463\ nm}/RF_{327/415\ nm}$. The FR value was determined using the aqueous phase that resulted from the lipid extraction of the fish muscle [18].

Determination of sensory acceptance

Sensory analysis was carried out by a sensory panel consisting of four to six experienced judges. Before carrying out the present experiment, the judges received special training on frozen mackerel, focused on the evaluation of specimens that exhibited different qualities. Special attention was paid to the evolution of the sensory descriptors that were found as limiting factors for the shelf life. Consequently, descriptors analysed were: external odour, raw flesh odour, cooked flesh odour and flesh taste. The different descriptors were evaluated on a scale from 7.0 (stage of highest quality) to 0.0 (stage of lowest quality) in agreement with Lehmann and Aubourg [22]. Four rang categories were considered [23]: 7.0-5.6 (excellent), 5.5-3.6 (good), 3.5-1.6 (fair) and 1.5-0.0 (rejectable).

At each sampling time, fish individuals from each batch were analysed. Evaluation began by the analysis of fish in the raw state and was followed by the analysis of samples in the cooked state. Cooking was accomplished at 95-100 ºC for 7 min in a pre-warmed oven with air circulation and then submitted to the panel. At each sampling time, whole fish specimens were coded with 3-digit random numbers and presented to the panellists in individual trays, which were scored individually. Each descriptor of each sample was scored a single time by each member of the panel. The panel members shared samples tested.

Statistical analysis

Data obtained (three replicates; n=3) from the different sensory and chemical analyses were subjected to the ANOVA method to explore differences resulting from the effect of the glazing system employed; the comparison of means was performed using the least-squares difference (LSD) method. Analyses were carried out using the PASW Statistics 18 software for Windows (SPSS Inc., Chicago, IL, USA); differences among batches were considered significant for a confidence interval at the 95% level ($p<0.05$) in all cases.

RESULTS AND DISCUSSION

Chemical analyses related to quality

Lipid content of mackerel fish ranged between 24.7 and 33.4 g kg^{-1} muscle. Results concerning the lipid hydrolysis development are depicted in Fig. 1. A marked FFA formation was obtained throughout the whole frozen storage period in all kinds of samples.

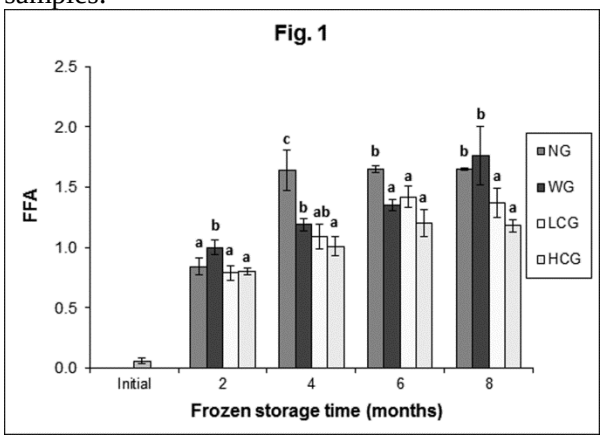

Figure 1. Assessment of free fatty acids (FFA; mg kg^{-1} muscle) formation* in frozen mackerel previously submitted to various glazing conditions**.

* Average values of three replicates (n=3). Standard deviations are indicated by bars. Average values accompanied by different letters (a, b, c) denote significant differences ($p<0.05$). No letters are included when no significant differences were found ($p>0.05$). ** Abbreviations of glazing conditions: NG (without glazing), WG (water glazing), LCG (low-concentrated squid skin glazing) and HCG (high-concentrated squid skin glazing) in agreement with the material and methods section.

Fish corresponding to the HCG system showed the lowest average values for the 4-8-month period; differences were found significant ($p<0.05$) at months 4 and 8 when compared with their counterparts from NG and WG conditions. Furthermore, an inhibitory effect ($p<0.05$) was also observed in mackerel corresponding to the LCG system at the end of the experiment. Consequently, an inhibitory effect on lipid hydrolysis development can be concluded by including the JSS extract in the glazing medium.

Lipid hydrolysis development is reported to be produced during the frozen storage as a result of lipase release from liposomes into the muscle, which then facilitates closer proximity between enzyme and substrate [4]. In a previous research [17], the JSS extract showed to inhibit the FFA formation during the chilled storage of hake; in this case, the JSS extract was included in the icing medium employed during the chilling process. In agreement with the current study, the employment of essential oils (sage, thyme and clove) extracts in the glazing system led to an inhibitory effect on the formation of FFA in frozen (6 months at –18 ºC) rainbow trout fillets [9]; additionally, an inhibitory effect was also observed in fish samples corresponding to water glazing when compared with samples not glazed.

Lipid oxidation development was determined by means of the peroxide and fluorescent compounds detection. Peroxide formation (Table 1) did not provide a definite trend throughout the storage for none of the fish batches; interestingly, the highest average value was obtained at month 4 in most kinds of samples.

Table 1. Peroxide value assessment* in frozen mackerel previously submitted to different glazing conditions**

Frozen storage time (months)	Glazing condition			
	NG	WG	LCG	HCG
Initial		2.09 (0.72)		
2	5.54 (2.25)	3.99 (0.61)	3.28 (1.73)	3.32 (2.38)
4	7.89 b (2.29)	4.69 ab (0.17)	5.14 ab (0.86)	3.61 a (1.28)
6	7.12 b (2.44)	4.12 ab (0.87)	2.30 a (0.49)	3.07 a (0.79)
8	1.57 a (0.34)	1.34 a (0.45)	7.93 b (1.55)	2.12 a (1.58)

* Average values of three replicates (n=3); standard deviations are indicated in brackets. Average values followed by different letters (a, b) denote significant differences ($p<0.05$). No letters are indicated when no differences are found ($p>0.05$). ** Abbreviations of glazing systems as expressed in Fig. 1.

Comparison among samples showed scarce significant differences; thus, significant differences could not be obtained ($p>0.05$) by comparing mackerel corresponding to any glazing system including the JSS extract with fish corresponding to NG and WG conditions. However, values of mackerel previously glazed under HCG system were included in a relatively low peroxide range (2.12-3.61).

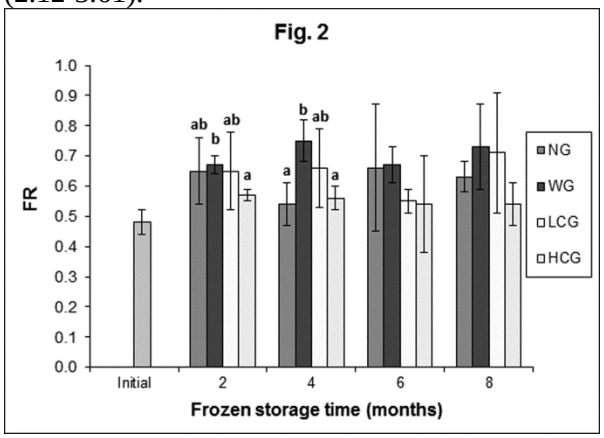

Figure 2. Assessment of the fluorescence ratio (FR)* in frozen mackerel previously submitted to various glazing conditions**.

* Average values of three replicates (*n*=3). Standard deviations are indicated by bars. Average values accompanied by different letters (a, b) denote significant differences ($p<0.05$). No letters are included when no significant differences are found ($p>0.05$). ** Abbreviations of glazing conditions as expressed in Fig. 1.

Scarce differences could be observed by assessing the tertiary lipid oxidation compounds (Fig. 2). When compared with initial values, a small increase in this quality parameter can be implied in all kinds of samples. Comparison among samples showed a lower ($p<0.05$) value in fish corresponding to the HCG batch than in their counterpart from the WG batch in the 2-4-month period. At most storage times, average values for fish from the HCG system were lower than from any other batch.

Frozen storage is known to be associated with fish lipid oxidation processes where different kinds of endogenous enzymes may be involved [5]. Partial inhibition of lipid oxidation found in the current research can be explained on the basis of previous research [15]. In it, marked radical scavenging activity (ABTS assay) and oxygen radical absorbance capacity (ORAC assay) were detected; additionally, inhibition of lipid oxidation in a marine-oil heated model system was evident. In agreement with various spectroscopic and chemical analyses, JSS molecules responsible for this antioxidant behavior were identified as belonging to the ommochrome family [15].

In agreement with the present study, previous related research accounts for lipid oxidation inhibition (formation of thiobarbituric acid reactive substances in all cases) as a result of including various preservative compounds in the glazing system. This accounts for chitosan nano particles combined with sodium tripolyphosphate nano particles in frozen (30 days at –21 ºC) shrimp [12], green tea and grape seed extracts in frozen (5 months at –18 ºC) bonito [7], green tea extract in frozen (180 days at –21 ºC) shrimp [11] and Pollock skin hydrolysates in frozen (4 months at –35 ºC) pink salmon [10].

Additionally, Çoban [9] studied the lipid oxidation development by assessing primary and secondary lipid oxidation compounds formation during the frozen storage (6 months at –18 ºC) of rainbow trout fillets. By including essential oils

(sage, thyme or clove) extracts in the glazing system, an inhibitory effect on the formation of both kinds of lipid oxidation compounds was implied. Furthermore, an inhibitory effect was observed in fish samples corresponding to water glazing when compared with those samples not glazed.

Determination of the sensory acceptance

Sensory analysis showed a progressive quality decrease throughout the storage time for all descriptors under study (Table 2).

Table 2. Sensory analysis* of frozen mackerel previously submitted to different glazing conditions**

Descriptor/frozen time (months)	Glazing condition			
	NG	WG	LCG	HCG
External odour				
2	3.5 (0.7)	4.0 (1.4)	5.0 (1.4)	4.5 (0.7)
4	4.0 a (0.0)	4.0 ab (1.4)	4.0 a (0.0)	5.5 b (0.7)
6	0.7 a (0.6)	2.0 ab (1.0)	3.3 b (0.6)	2.7 ab (1.5)
8	1.3 a (0.5)	2.0 ab (0.7)	3.0 b (1.0)	3.2 b (1.0)
Raw flesh odour				
2	4.0 (1.4)	4.5 (0.7)	4.5 (0.7)	5.0 (1.4)
4	3.5 a (0.7)	4.5 a (0.7)	6.0 b (0.0)	4.5 a (0.7)
6	0.7 a (0.6)	2.3 b (0.6)	3.3 b (0.6)	2.0 ab (1.0)
8	1.4 a (0.6)	2.7 ab (0.6)	2.3 ab (1.2)	3.7 b (0.6)
Cooked flesh odour				
2	4.5 a (0.7)	4.5 a (0.7)	5.5 a (0.7)	6.0 b (0.0)
4	4.5 (0.7)	4.5 (0.7)	5.0 (1.4)	4.5 (0.7)
6	1.0 a (0.0)	2.7 b (0.6)	3.7 b (0.6)	2.3 ab (1.2)
8	1.3 a (0.6)	2.3 ab (1.2)	2.7 ab (1.5)	3.7 b (0.6)
Flesh taste				
2	4.0 (0.0)	4.0 (1.4)	4.5 (0.7)	5.0 (1.4)
4	4.5 (0.7)	4.5 (0.7)	5.5 (0.7)	5.5 (0.7)
6	1.3 a (0.6)	2.0 ab (1.0)	3.0 ab (1.0)	3.3 b (0.5)
8	1.0 a (0.0)	2.0 ab (1.0)	2.0 ab (1.0)	2.7 b (1.3)

* Descriptors were evaluated on a scale from 7.0 (highest stage of quality) to 0.0 (lowest stage of quality) in agreement with the material and methods section. Values followed by different letters (a, b) indicate significant differences ($p<0.05$). No letters are included when no significant differences are found ($p>0.05$). Initial fish was assigned score 7.0 in all descriptors. ** Abbreviations of glazing systems as expressed in Fig. 1.

As a result, fish corresponding to NG system (blank control) was found rejectable at month 6, while the remaining samples were still acceptable at the end of the experiment. Such rejection was observed in all descriptors under study. Scarce differences could be proved as a result of the presence of the JSS extract in the glazing system. Thus, higher scores ($p<0.05$) were observed in LCG samples at month 4 (raw flesh odour) and in HCG fish at month 2 (cooked flesh odour) when compared with their counterparts fish from NG and WG conditions. Interestingly, higher average values were obtained in all descriptors at the end of the study for mackerel corresponding to the highest

concentration of JSS extract in the glazing system. A profitable effect of JSS extracts on fish sensory quality has already been observed during the chilled storage of mackerel [16] and hake [17]; in both cases, the inclusion of this extract in the icing system employed for the chilling storage led to a marked enhancement of sensory acceptance. Furthermore, a previous phosphate dipping to freezing and frozen (15 days at –25 ºC) storage led to a sensory quality enhancement in red shrimp [13]. Contrary, Çoban [9] included essential oils (sage, thyme or clove) extracts in the glazing system prior to the frozen storage (6 months at –18 ºC) of rainbow trout fillets; however, no differences in sensory quality were observed when compared with fish corresponding to control-water glazing.

CONCLUSIONS

A novel glazing system based on the inclusion of a lipophilic extract of JSS was applied to frozen Atlantic Chub mackerel. For it, quality changes were monitored for a 8-month frozen storage by sensory and chemical evaluation. As a result, an inhibitory effect ($p<0.05$) of skin extracts presence on lipid hydrolysis (free fatty acid formation) evolution was observed; furthermore, lower average values for lipid oxidation indices (peroxide and fluorescent compounds formation) were observed in samples corresponding to the highest JSS presence in the glazing system. Some sensory quality enhancement was evident in mackerel as a result of including JSS extracts in the glazing medium, especially for raw-flesh and cooked-fresh odours. As for chemical indices, an increasing effect was implied by increasing the skin extract presence in the glazing medium. Under the conditions tested in the present study, the JSS extract has shown promising antioxidant properties that could be applied to enhance the seafood quality during the commercialisation in frozen conditions. Further research would be necessary to optimise its use in the glazing system.

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REFERENCES

1. A. Simopoulos, in: Seafood from producer to consumer, integrated approach to quality, J. Luten, T. Börrensen, J. Oehlenschläger (eds.), Elsevier Science, London, UK, 1997, p. 589.
2. I. Medina, J. Gallardo, S. P. Aubourg, *Int. J. Food Sci. Tehnol.* **44**, 1467 (2009).
3. A. Kolakowska, in: Chemical and functional properties of food lipids, Z. Sikorski, A. Kolakowska (eds.), CRC Press, London, UK, 2003, p. 133.
4. R. Sista, M. Erickson, R. Shewfelt, in: Quality in frozen food, M. Erickson, Y-C. Hung (eds.), Chapman and Hall, New York, USA, 1997, p. 101.
5. Z. Sikorski, E. Kolakoski, in: Seafood enzymes, N. Haard, B. Simpson (eds.), Marcel Dekker, New York, USA, 2000, p. 451.
6. L. Vanhaecke, W. Verbeke, H. De Brabander, *Anal. Chim. Acta*, **672**, 40 (2010).
7. P. Yerlikaya, N. Gokoglu, *Int. J. Food Sci. Technol*., **45**, 252 (2010).
8. S. Jacobsen, K. Fossan, *J. Food Eng*., **40**, 21 (1999).
9. O. Çoban, *J. Food Proces. Preserv*., **37**, 759 (2013).
10. S. Sathivel, J. Huang, P. Bechtel, *J. Food Biochem*., **32**, 247 (2008).
11. S. Sundararajan, A. Prudente, D. Bankston, J. King, P. Wilson, S. Sathivel, *J. Food Sci*., **76**, E511 (2011).
12. K. Solval, L. Rodezno, M. Moncada, D. Bankston, S. Sathivel, *LWT-Food Sci. Technol*., **57**, 172 (2014).
13. A. Gonçalves, C. Gindri, *J. Food Eng*., **90**, 285 (2009).
14. N. Soares, T. Mendes, A. Vicente, *J. Food Eng*., **119**, 316 (2013).
15. S. P. Aubourg, W. Torres-Arreola, M. Trigo, J. M. Ezquerra-Brauer, *Eur. J. Lipid Sci. Technol*., **118**, 1293 (2016).
16. J. M. Ezquerra-Brauer, J. Miranda, A. Cepeda, J. Barros-Velázquez, S. P. Aubourg, *LWT-Food Sci. Technol*., **72**, 134 (2016).
17. J. M. Ezquerra-Brauer, J. Miranda, J. Chan-Higuera, J. Barros-Velázquez, S. P. Aubourg, *J. Sci. Food Agric*., **97**, 3412 (2017).
18. E. Bligh, W. Dyer, *Can. J. Biochem. Physiol.*, **37**, 911 (1959).
19. R. Lowry, I. Tinsley, *J. Amer. Oil Chem. Soc.*, **53**, 470 (1976).
20. R. Chapman, J. McKay, *J. Amer. Oil Chem. Soc.*, **26**, 360 (1949).
21. V. Losada, J. Barros-Velázquez, J. M. Gallardo, S. P. Aubourg, *Eur. J. Lipid Sci. Technol.*, **106**, 844 (2004).
22. I. Lehmann, S. P. Aubourg, *Int. J. Food Sci. Technol*., **43**, 270 (2008).
23. European Council Regulation, European Community (EC), No 2406/96, 26 November 1996. *Off. J. Eur. Comm.*, L-334/2:23.12 (1996).

КОНСЕРВИРАЩ ЕФЕКТ НА ЕКСТРАКТ ОТ КОЖА НА ГИГАНТСКИ КАЛМАРИ (*Dosidicus gigas*) КАТО ГЛАЗИРАЩ МАТЕРИАЛ ПРИ СЪХРАНЕНИЕ НА АТЛАНТИЧЕСКА СКУМРИЯ (*Scomber colias*) В ЗАМРАЗЕНО СЪСТОЯНИЕ

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(Резюме)

В настоящата работа са изследвани промените в качеството на атлантическа скумрия (*Scomber colias*) по време на съхранението ѝ в замразено състояние. Проследено е влиянието на липофилен екстракт от кожа на гигантски калмари (*Dosidicus gigas*) (JSS) в глазиращата система, приложена преди замразяване. Тествани са две концентрации от кожни екстракти и са сравнени с две контролни обработки (глазиране с вода и без глазиране). Промените в качеството са проследени в продължение на 8-месечно съхранение в замразено състояние чрез сензорна (мирис и вкус) и химическа (хидролиза и окисление на липидите) оценка. Установен е инхибиторен ефект ($p<0.05$) на JSS екстрактите върху хидролизата на липидите (образуване на свободни мастни киселини), като по-ниски средни стойности на липидните окислителни индекси (образуване на пероксидни и флуоресцентни съединения) са наблюдавани при рибните проби с по-висока концентрация на JSS в глазиращата система. Установено е повишение на сензорното качество на скумрията в резултат на включване на JSS екстракти в глазиращата система., особено по отношение на мириса на суровото и свареното месо. Установено е и повишение на химическите индекси с увеличаване концентрацията на JSS екстракта в глазиращата система. При изследваните условия, JSS екстрактът е демонстрирал перспективни антиоксидационни свойства, които биха намерили приложение за повишаване на качеството на морската храна при търговията в замразено състояние. Предстоят изследвания за оптимизиране на употребата на JSS в глазиращата система.

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Antioxidant potential of high molecular weight polyphenol fraction from green tea

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Green tea is one of the most popular beverages. Due to the high content of bioactive compounds it exhibits many health-promoting properties. Our study focused on analyzing the high molecular weight (HMW) fraction obtained from 13 green tea samples for their antioxidant capacity and chelating ability and comparing the results with those recorded for green tea extract (Ex) and its low molecular weight (LMW) fraction. HMW and LMW were obtained using Sephadex LH-20 column chromatography. Obtained fractions were characterized by total phenolic content, size-exclusion high performance liquid chromatography (SE-HPLC), 2,2-diphenyl-1-picrylhydrazyl radical (DPPH$^{\bullet}$) scavenging capacity, and ability to chelate Fe(II) ions. HMW exhibited the highest total phenolic content and scavenging activity. The results obtained for the chelating capacity showed that changes in UV-spectra after addition of ferric (II) chloride observed for HMW solutions were the most significant.

Keywords: Proanthocyanidins, Green tea, SE-HPLC, DPPH, Chelation, Ferric ions, Sephadex LH-20, UV spectrum analysis

INTRODUCTION

Green tea is a widely consumed beverage across the whole world. There are many health-beneficial aspects of regular drinking infusion prepared from dry leafs of *Camellia sinensis* [1-3]. For example, drinking green tea can help patients with obesity in reducing body mass [4]. Green tea is an abundant source of polyphenols [6] and is well known for its antioxidant properties [7, 8]. It was proven that antioxidants present in green tea leafs are responsible for many health-beneficial attributes, in particular EGCG which can be detected as over 50% of the total sum of polyphenols [9-11]. Mandel *et al.* [12] claimed that the ability of EGCG to chelate ferric ions might have protective role against neurodegenerative diseases connected to abnormal iron metabolism. Although proanthocyanidins obtained from green tea exhibit anti-inflammatory properties [13] it seems that the high molecular fraction of green tea, which is composed by proanthocyanidins [14] is still not well described. However, there are reports suggesting protective role of proanthocyanidins from other sources against neurodegenerative diseases when tested on cell lines or mice [15, 16], proanthocyanidins have limited absorption through the gut barrier. Therefore, one of the aims of our study was to test the ability of green tea extract and of its low (LMW) and high molecular weight (HMW) fractions to chelate ferric (II) ions.

In our study we focused on highlighting the antioxidant potential of green tea's high molecular fraction, compared with the extract and the low molecular fraction. Research was focused on characterizing HMW separated from different commercially available green teas and comparing it with corresponding Ex and LMW using total phenolic content, DPPH radical scavenging activity test, size-exclusion high performance liquid chromatography (SE-HPLC) and changes in UV spectrum of extract/fractions inducted by ferric (II) chloride addition.

EXPERIMENTAL

Chemicals

Methanol, acetone, ethanol, sodium carbonate and ferric (II) chloride 4-hydrate were purchased from POCH S.A. (Polskie Odczynniki Chemiczne). Folin-Ciocalteu reagent, EGCG, gallic acid, tannic acid, DPPH, acetonitrile and trifluoroacetic acid were purchased from Sigma–Aldrich, USA. Procyanidin B2 was purchased from Extrasynthese (Genay, France).

Samples and extraction

13 samples of green tea were purchased from local shops in Olsztyn, Poland. Before extraction the samples were powdered in a coffee mill (Bosh, Ljubljana, Slovenia) and then phenolics were extracted from the green tea by mixing 20 g of powdered leafs with 80 % acetone (v/v) in a solid:liquid ratio 1:10 at 70°C for 15 min in a water shaking bath (SW 22, Julabo GmbH, Seelbach, Germany). After cooling, the obtained extract was filtered through Whatman filters. The extraction procedure was repeated two times. Filtrates were

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combined, organic solvent was evaporated (Büchi, Rotavapor R-200, Büchi Labortechnik AG, Flawil, Switzerland) and the obtained aqueous concentrate was frozen and lyophilized for 72 h at -48°C (Freezone 6, model 77530, Labconco Co., Kansas City, MO, USA). Dry crude green tea extracts were stored at -20°C until further analysis.

Fractionation of crude extracts

Separation of two different molecular size fractions from green tea extract was conducted using the method of Naczk *et al*. [17]. Ex was dissolved in absolute ethanol and applied onto the column (5 × 40 cm) filled with Sephadex LH-20 gel in ethanol. Elution was performed with ethanol to obtain LMW fraction (approximately 1000 ml of eluate). Next, the mobile phase was changed for 50 % (v/v) acetone. HMW was eluted using 600 ml of the second eluent. Organic solvents were evaporated and the aqueous residue was lyophilized and stored under -4°C until further analysis.

Total phenolics assay

Total phenolics contetnt (TPC) was characterized using Folin-Ciocalteu's method adjusted for microplate readers and described by Horszwald and Andlauer [18]. Samples of Ex, LMW and HMW that were firstly dissolved in methanol (25 µl; 0.2 ml/ml) were placed in 96-well microplates. Next, 250 µl of Folin-Ciocalteu reagent was added (1:15; v/v) using built-in microplate reader's (TECAN Infinite M1000) injector. After 10 min of incubation 15 µl of 20% sodium carbonate was automatically added and incubated for 20 min. Process was conducted with plate shaking after each reagent addition. Absorbance was measured at λ=755 nm. Results were expressed as (-) epigallocatechingallate (EGCG) equivalent as mg per g of extract/fraction. For this purpose a calibration curve was prepared using a solution of EGCG (1 mg/ml) to prepare a series of dilutions. As reference sample pure methanol was used. Green tea extract and its fractions were analyzed in triplicate.

Antiradical scavenging activity against (2,2-diphenyl-1-picrylhydrazyl) radical (DPPH•)

For investigation of DPPH radical scavenging activity the microplate method was used [18]. 10 mg of DPPH powder was dissolved in 25 ml of methanol. DPPH• solution was diluted to obtain A~1.1 measured at λ=517 nm freshly before its injection. Each sample was dissolved in methanol (1 mg/ml), diluted (in the range 0.006-0.035 mg/ml) and placed inside the microplate (20 µl). Solution of DPPH• was automatically added (270 µl) and then the plate was shaken and incubated for 30 min in the dark with a cover on which was removed before absorbance measurement. Results are presented as the curve of absorbance (λ=517 nm) reduction, and % of radical reduction for final concentration of the sample calculated using the equation:

$$\%R = (A_0 - A_{fc}) \times 100\% / A_0$$

where %R is % of reduction of DPPH radical; A_0 is absorbance of blank; A_{fc} is absorbance of sample at final concentration.

Size exclusion-high performance liquid chromatography (SE-HPLC)

HMW fraction was dissolved in the mobile phase (0.5 mg/ml), filtered through Whatman 0.45 µm NYL w/GMF filters and injected (20 µl) into the HPLC system (LC-10AD pump, controller SCTL 10A and photodiode array detector SPD-M 10AQ). Samples were analyzed using TSK $G2000SW_{XL}$ column in isocratic mode where mobile phase was 45 % (v/v) acetonitrile with 0.1 % of trifluoroacetic acid and flow rate of 0.2 ml/min. Separation was monitored at λ=280 nm. Retention times of recorded peaks were compared with three standards: gallic acid, procyanidin B2 and tannic acid.

Iron (II) chelation

Method of Stookey [19] with modifications of Karamać and Pegg [20] was adjusted to microplate reader and UV spectrum analysis. Ex, LMW and HMW were dissolved in water (0.18 mg/ml). Samples (200 µl) were placed in quartz-bottom microplates and mixed with freshly prepared, automatically added ferric (II) chloride (0.4 mM $FeCl_2 \times 4H_2O$). Plates were incubated for 10 min and the UV spectrum was recorded in the wavelength range of 230-400 nm. The reference UV spectrum was recorded for Ex, LMW and HMW without ferric (II) chloride addition.

Statistical analysis

All analyses were conducted in triplicate. Statistical analysis was performed using STATISTICA 10, StatSoft. Differences between treatments were determined using ANOVA and Duncan's test.

RESULTS AND DISCUSSION

Results obtained for the ability of Ex, LMW and HMW to reduce Folin-Ciocalteu reagent are presented in Table 1.

Table 1. Total phenolics content (mg EGCG / g of extract or fraction) in 13 green teas.

Green tea	Ex				LMW				HMW			
1	599	±	35.9	fgh	494	±	10.30	h	588	±	40.5	cd
2	565	±	63.8	efg	466	±	9.31	gh	706	±	32.6	g
3	526	±	18.2	de	400	±	17.7	de	550	±	15.5	c
4	529	±	13.5	de	387	±	38.7	cd	421	±	7.38	b
5	615	±	23.8	gh	528	±	18.1	i	672	±	24.0	efg
6	406	±	22.1	b	423	±	17.2	ef	583	±	25.6	cd
7	312	±	4.38	a	286	±	27.5	a	341	±	8.64	a
8	463	±	42.1	c	367	±	7.16	bc	642	±	25.8	ef
9	548	±	17.0	ef	418	±	23.4	def	699	±	56.7	g
10	513	±	35.3	cde	416	±	7.42	def	626	±	23.8	de
11	473	±	16.0	c	355	±	14.1	b	689	±	38.1	fg
12	478	±	13.8	cd	447	±	9.57	fg	384	±	7.88	ab
13	643	±	4.70	h	480	±	4.94	h	718	±	37.3	g
mean	513	±	89.6		421	±	64.5		586	±	128	

Data are expressed as mean ± standard deviation (n=3); values in the same column having different letters differ significantly (P<0.05)

Total phenolics content was determined in the samples in a broad range. Mean value for HMW was 586 ± 128 mg/g and was the highest among all three groups (513 ± 89.6 mg/g for Ex and 421 ± 64.5 mg/g for LMW). The range for which results were obtained was 312 – 643 mg/g for Ex, 286 – 480 mg/g for LMW and 341 – 718 mg/g for HMW. Statistical analysis revealed that HMW was the most differentiated group, however all three groups showed moderate variability. What is interesting, the highest results were more than two times higher (for Ex and HMW) or nearly two times higher (in case of LMW) than the lowest ones. It presented the differences in ability to synthesize those compounds by different *Camellia sinensis* varieties and/or differences in the quality of final product. The range in which total phenolics content can be found in literature data is broad. There are findings that report TPC from 120-185 mg gallic acid equivalent (GAE) per g dry weight (DW) for infusions [7], 13.75-20.43% of DW as GAE [21] and up to 837.6 mg/g of extract in catechin equivalent [22]. Such variability of the results can be explained by different varieties, manufacturing processes and storage conditions. Other reports present that HMW from other sources that contain polymeric polyphenols exhibit higher values of absorbance [23, 24].

Typical results obtained after analyzing the scavenging activity of Ex, LMW fraction and HMW fraction from green tea against DPPH radical are depicted in Fig. 1.

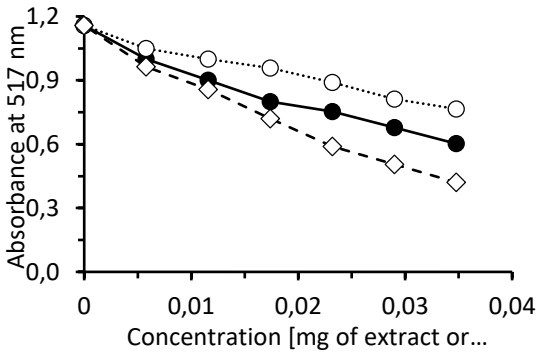

Figure 1. DPPH radical scavenging capacity for Ex, LMW and HMW (Black circles – Ex, white circles – LMW, white panes – HMW).

Curves for Ex, LMW and HMW are not linear. In case of each sample set (Ex, LMW and HMW) the most significant decrease of absorbance by solutions with different concentration of the samples was recorded for HMW. Ex with final concentration of 0.035 mg/ml was able to scavenge 48.00 % of DPPH$^{\bullet}$, LMW 33.88 % and HMW 63.72 %. DPPH assay presents results as the reduction of DPPH radicals measurable spectroscopically at 515nm. It can be observed as a loss of dark-purple color. Due to this method of expressing radical scavenging activity, the lowest results indicate that the sample which caused the strongest change in absorbance is the most active scavenger. Presented curves of decrease of absorbance in relation to concentration of the extract/ fractions clearly indicate that HMW was characterized by the highest free radical activity. Amarowicz *et al*. [23] reported that HMW fraction from red lentil exhibited few times higher activity against DPPH radical than Ex or LMW. In case of

LMW and HMW in our study, the difference was not as significant, because in both low and high molecular fractions compounds with high antioxidant potential could be detected. Nevertheless, differences in radical scavenging activity between Ex and LMW might suggest that compounds that can be separated to obtain HMW possess significant impact on antioxidant activity against DPPH$^{\bullet}$ of Ex.

Phenolic compounds extracted from green tea leafs were separated using SE-HPLC technique. Typical results are depicted in Fig. 2.

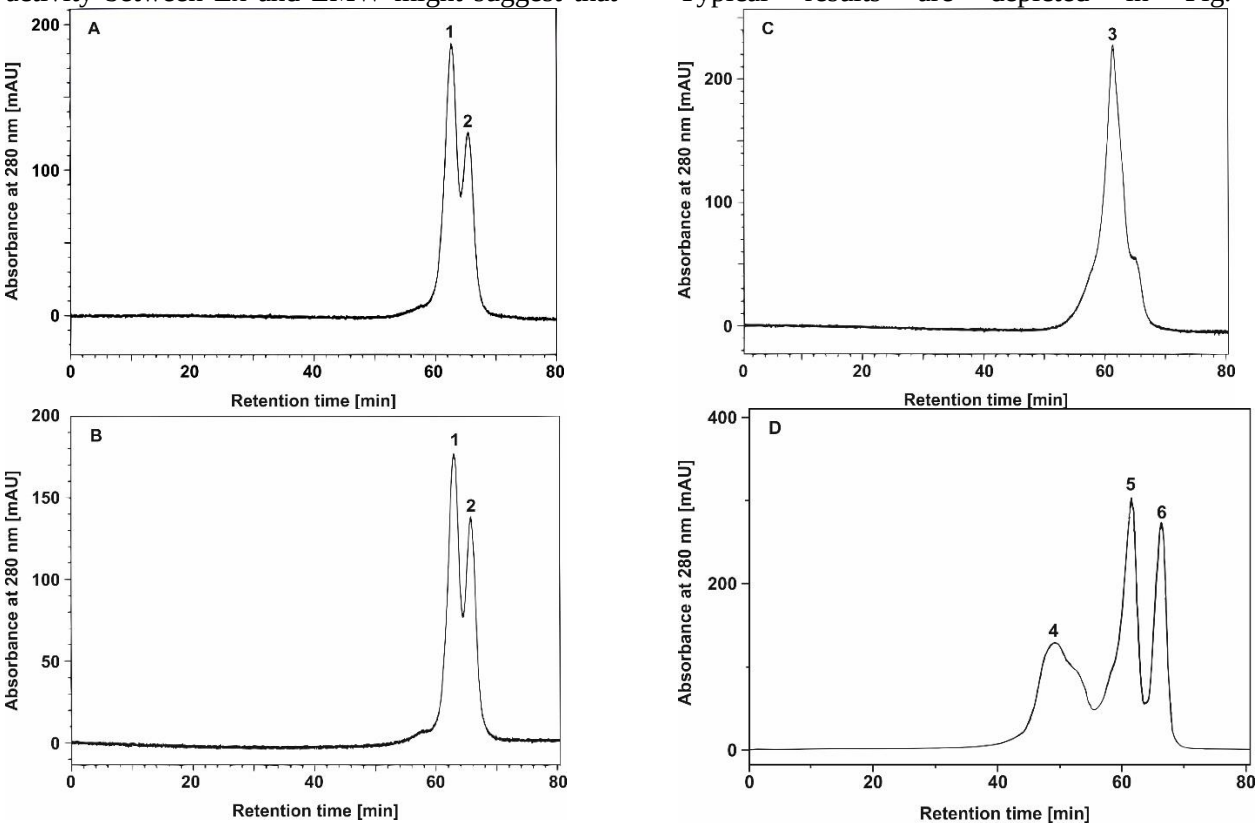

Figure 2. SE-HPLC chromatograms of Ex (A), LMW (B), HMW (C) obtained from green tea sample 11 and mix of standards (D): tannic acid (peak 4), procyanidin B2 (peak 5) and gallic acid (peak 6).

To compare the retention times of the separated compounds three standards with gradation of molecular weight were used. They were tannic acid (1701.19 g/mol), procyanidin B2 (578.52 g/mol) and gallic acid (170.12 g/mol). The results are presented in Fig. 2d. By using SE-HPLC technique some relatively small organic compounds like polyphenols extracted from different plant sources were investigated [24-27]. Using size exclusion column, compounds with higher molecular mass are eluted earlier than those with lower mass. As it can be noticed, peak 3 has similar retention time to peak 4, which corresponds to procyanidin B2. Peaks 1 and 2 were recorded for catechins so their retention times are shorter. Retention time of peak 2 is shorter than peak 6 recorded for gallic acid.

HMW fraction from green tea is dark, brownish and exhibit high values of absorbance around λ=562 nm. This wavelength was used in the colorimetric method with ferrozine [20]. There are also reports of other authors that assign changes of absorbance in the range of 546-600 nm to formation of complexes of polyphenol with metal ions [28, 29]. Due to those facts it was decided to present differences after ferric (II) chloride addition within the spectra in the UV wavelength range of Ex, LMW and HMW. Few other authors made successful attempts to compare UV spectra of pure polyphenolic compounds before and after metal ions addition [28, 31, 32]. Typical UV spectra obtained in this study before and after addition of Fe^{2+} are presented in Fig. 3.

It can be noticed that addition of ferric ions shifted areas around 250, 300 and 316 nm. It was previously reported that addition of metal ions to EGCG solution resulted in changes at similar areas, including shifting maximum of spectra to 312 nm [31]. Similar effect was noticed also after addition of Cu^{2+} to different flavonoids [30]. Addition of Al^{3+} to apigenin-7-O-glucopyranoside resulted in bathochromic shifting [32].

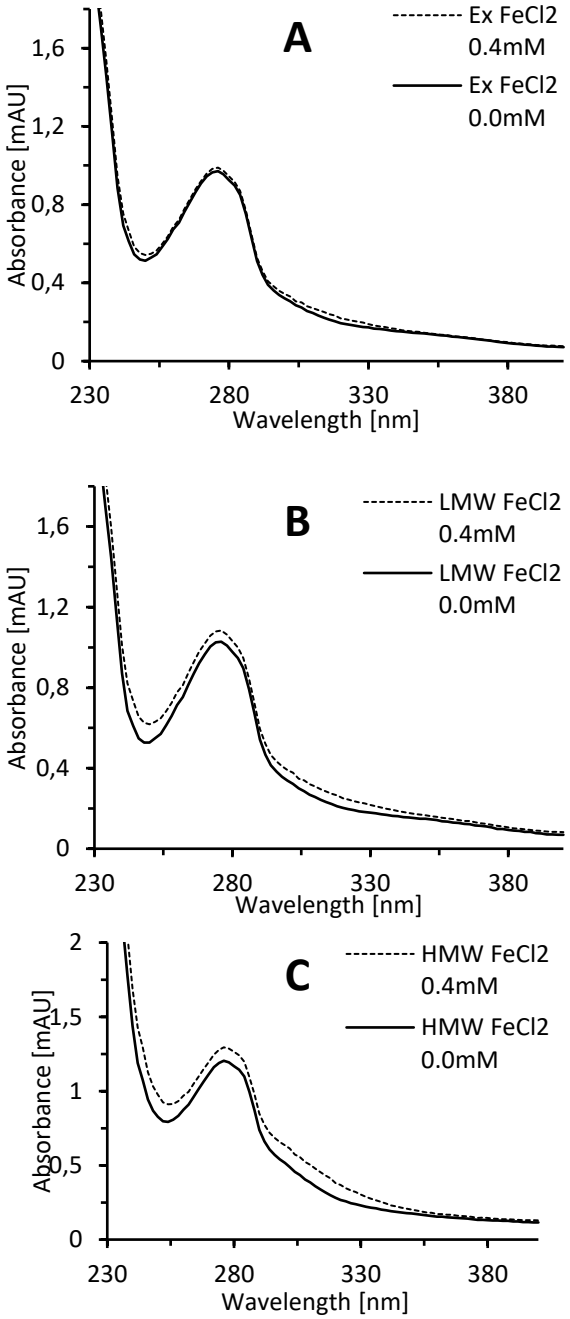

Figure 3. Chelation ability of Fe (II) by Ex (A), LMW (B) and HMW (C) obtained from green tea sample 11.

However, it was demonstrated that tannins from the same source bind Cu^{2+} in greater amounts than Fe^{2+} [20] which can lead to the conclusion that changes of spectrum after addition of Fe^{2+}might be less significant. In case of HMW, the strongest changes were within the area 316-320 nm of the UV spectrum. LMW exhibited weaker and Ex the weakest changes. This can be explained by the ability of tannins to bind metal ions at higher levels than monomeric polyphenols [20]. HMW as a stronger chelating agent than LMW might mitigate changes in spectrum of Ex in which condensed tannins are in minority, but can greatly participate in metal ions binding properties. Condensed tannins possess a significant number of hydroxyl groups which are potential places where metal ions can be bound to. UV spectrum of Ex is mostly a result of overlapping spectra of monomeric catechins which are in majority in Ex. Thus bigger changes of the spectrum of tannins that are also present in Ex are covered by the spectrum of monomers. Catechins in Ex bind less Fe^{2+} than catechins in LMW, because some amounts of ions were bonded to condensed tannins. Moreover [28], it was observed that after addition of a stronger chelator (EDTA) to a solution of a weaker chelator (phenolic acids), the spectra and λ_{max} of the polyphenol-metal ion complexes were changed. This suggests that a similar phenomenon can be found in case of natural mixtures of phenolics. It appears that priority in binding ferric ions can have stronger chelators, in case of Ex – proanthocyanidins. However, Ex, LMW and HMW are mixtures of many compounds what causes difficulties in presenting simple conclusions.

CONCLUSION

HMW fraction exhibits significant impact on green tea's antioxidant capacity. Especially, HMW was also the fraction which showed the highest values obtained for total phenolic content. The method of analyzing chelating ability of the colored plant extracts by observing changes in the UV-spectrum after addition of ferric (II) chloride seems to be a promising way that will help to analyze complexes between polyphenols and metal ions.

REFERENCES

1. H. Mukhtar, N. Ahmad, *Am. J. Clin. Nutr*. **71,** suppl**.,** 1698 (2000).
2. D.L. McKay, J.B. Blumberg, *J. Am. Coll. Nutr*., 1, 1 (2002).
3. D. Richard, K. Kefi, U. Barbe, A. Poli, P. Bausero, F. Visioli, *Pharmacol. Res*., **59**, 351 (2009).
4. W. Tang, S. Li, Y. Liu, M.-T. Huang, C.-T. Ho, *J. Funct. Foods*, **5**, 1784 (2013).
5. C. Snoussi, R. Ducroc, M.H. Hamadaoui, K. Dhaoudai, H. Abaidi, F. Cluzeaud, C. Nazaret, M. Le Gall, A. Bado, *J. Nutr. Biochem*., **25**, 557 (2014).
6. C.-T. Ho, C.W. Chen, U.N. Wanasundara, F. Shahidi, Natural antioxidants from tea. in: *Natural Antioxidants. Chemistry. Health Effects and Application* (Shahidi F., ed.). AOCS Press Champaign, Illinois. 213, 1997.
7. A. Rusaczonek, F. Świderski, B. Waszkiewicz-Robak, *Pol. J. Food. Nutr. Sci.*, **60**, 33 (2010).
8. A.K. Dutta, M.A. Siddiquee, S. Hossain, Y. Kabir, *Malay. J. Pharm. Sci*., **11**, 11 (2013).
9. H. Wang, G.J. Provan, K. Helliwell, *Trends Food. Sci. Technol*., **11**, 152 (2000).

10. G. Williamson, C. Manach, *Am. J. Clin. Nutr*. **81,** suppl., 243 (2005).
11. D.G. Nagle, D. Ferreira, Y-D. Zhou, *Phytochemistry*, **67**, 1849 (2006).
12. S. Mandel, G. Maor, M.B. Youdim , *J. Mol. Neurosci*. **24**, 401 (2004).
13. D.-X. Hou, S. Masuzaki, F. Hashimoto, T. Uto, S. Tanigawa, M. Fuji, Y. Sakata ,*Arch. Biochem. Biophys.*, **460**, 67 (2007).
14. A. Kiehne, C. Lakenbrink, U.H. Engelhardt, *Eur. Food Res. Technol*., **205**, 153 (1997).
15. K.E. Strathearn, G.G. Yousef, M.H. Grace, S.L. Roy, M.A. Lila, J.-C. Rochet, *Brain Res*., **1555**, 60 (2014).
16. Y.-S. Gong, J. Guo, K. Hu, Y.-Q. Gao, B.-J. Xie, Z.-D. Sun, E.-N. Yang, F.-L. Hou, *Exp. Gerontol*. , **74**, 21 (2016).
17. M. Naczk, R. Amarowicz, R. Zadernowski, F. Shahidi, *Food Chem*. **73**, 467 (2001).
18. Horszwald A., Andlauer W.*J. Berry Res*. **1**, 189 (2011).
19. L.L. Stookey, *Anal. Chem*., **42**, 779 (1970).
20. M. Karamać, R.B. Pegg, *J. Agric. Food Chem*., **57**, 6425 (2009).
21. C. Astill, M.R. Birch, C. Dacombe, P.G. Humphrey, P.T. Martin, *J. Agric. Food Chem*., **49**, 5340 (2001).
22. A. Gramza, K. Pawlak-Lemańska, J. Korczak, E. Wąsowicz, M. Rudzińska, *Pol. J. Environ. Stud*., **14**, 861 (2005).
23. R. Amarowicz, I. Estrella, T. Hernández, M. Dueńas, A. Troszyńska, A. Kosińska, R.B. Pegg, *Int. J. Mol. Sci*. **10**, 5513 (2009).
24. E. Pelvan Pelitli, M.A. Janiak, R. Amarowicz, C. Alasalvar , *Food Chem*., **218**, 584 (2017).
25. A. Yanagida, T. Shoji, T. Kanada, *Biosci. Biotechnol. Biochem*., **66**, 1972 (2002).
26. M. Karamać, A. Kosińska, A. Rybarczyk, R.B. Pegg, *Pol. J. Food Nutr. Sci.*, **57**, 87 (2007).
27. A Kosińska., A. Urbalewicz, K. Penkacik, M. Karamać, R. Amarowicz, *Pol. J. Food Nutr. Sci*., **61**, 263 (2011).
28. M. Andjelkocić, J. Van Camp, B. De Meulenaer, G. Depaemelaere, C. Socaciu, M. Verloo, R. Verhe , *Food Chem*., **98**, 23 (2009).
29. N.R. Perron, H.C. Wang, S.N. DeGuire, M. Jenkins, M. Lawson, J.L. Brumaghim, *Dalton Trans*. ,**39**, 9982 (2010)
30. J.E. Brown, H. Khodr, R.C. Hider, C.A. Rice-Evans, *Biochem. J*., **330**, 1173 (1998).
31. M. Kumamoto, T. Sonda, K. Nagayama, M. Tabata, *Biosci. Biotechnol. Biochem*., **65**, 126 (2001).
32. T.H. Costa Marques, C.H. Santos De Melo, R.B., Fonseca De Carvalho, L.M. Costa, A.A. De Souza, J.M. David, J. David, P. De Lima, R.M. De Freitas, *Biol. Res*., **46**, 231 (2013).

АНТИОКСИДАНТЕН ПОТЕНЦИАЛ НА ВИСОКОМОЛЕКУЛНА ПОЛИФЕНОЛНА ФРАКЦИЯ ОТ ЗЕЛЕН ЧАЙ

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(Резюме)

Зеленият чай е една от най-популярните напитки. Поради високото си съдържание на биоактивни съединения той проявява много стимулиращи здравето свойства. В настоящото изследване е анализирана високомолекулната част (HMW) от 13 проби зелен чай по отношение на антиоксидантния капацитет и хелатообразуващата способност, като резултатите са сравнени с тези, получени за екстракт от зелен чай и нискомолекулната му фракция (LMW). HMW и LMW фракции са получени чрез колонна хроматография с използване на Sephadex LH-20. Получените фракции са характеризирани чрез определяне на общото фенолно съдържание, високоефективна течна хроматография с изключване на размера (SE-HPLC), капацитет за улавяне на 2,2-дифенил-1-пикрилхидразил радикал (DPPH$^{\bullet}$) и способност за образуване на хелат с Fe(II) йони. HMW има най-високо фенолно съдържание и проявява най-висока радикал-улавяща активност. Резултатите за хелатообразуващата активност показват, че най-значителни промени в UV-спектрите след добавяне на железен (II) хлорид се наблюдават за HMW разтвори.

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Antioxidant and antiproliferative activity of *Juniperus* L. species of Bulgarian and foreign origin and their anticancer metabolite identification

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The genus *Juniperus* L. (Cupressaceae) comprises more than 50 species in the world. Widely distributed junipers are evergreen plants that are easy for cultivation, produce a considerable amount of biomass all the year round and are rich of biologically active compounds, including antioxidants and anticancer substances. This study is pointed out at a systematic investigation of Bulgarian *Juniperus* species in comparison with foreign representatives with the aim to select these having high antiproliferative and antioxidant activity. In the group of studied plant extracts, the best antiproliferative activity in NB4 acute promyelocytic leukemia cells was determined for *J. sabina* L., *J. virginiana* L. and *J. virginiana* 'Grey Owl' extracts. The best antioxidant activity was exhibited by *J. sibirica* and *J. excelsa* leaves extracts in the group of analyzed species. While efficient antioxidant activity is desired for cancer prevention, efficient antiproliferative agents are required in the anticancer chemotherapy. Using liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS/MS) we identified podophyllotoxin, a known anticancer drug precursor, in selected juniper extracts with antiproliferative activity. Identification of other plant metabolites (lignans, phenolic compounds etc.) is in progress. The identification of juniper extracts with efficient antiproliferative and antioxidant activity has potential application in clinical trials about combination therapy with other anticancer agents for treatment of resistance to conventional chemotherapy, disease relapse and reduction of the therapeutical doses of cytostatic drugs.

Keywords: *Juniperus* L., Antiproliferative activity, Antioxidant activity, Podophyllotoxin, HPLC/HRMS analysis

INTRODUCTION

Junipers are exquisite evergreen plants that appear in the nature as magnificent trees or small shrubs with trailing branches. The genus *Juniperus* L. (Cupressaceae) includes more than 50 juniper species, widely spread throughout the world [1]. Juniper representatives are currently under investigation as alternative sources of podophyllotoxin derivatives, known as precurors of antiviral and anticancer agents (etoposide, teniposide etc.) [2]. Podophyllotoxin is an aryltetralin lignan, currently isolated from the *Podophyllum peltatum* L. and *Podophyllum hexandrum* Royle, which are considered already as endangered species because of their intensive industrial exploitation and difficult cultivation. On the other side, junipers are widely distributed evergreen plants, easy for cultivation and producing big amount of biomass all the year.

A great diversity of cytotoxic compounds (podophyllotoxin derivatives, thuriferic acid, savinin, yatein, isocupressic acid, communic acid, arctiol; widdrol, etc. [3]) were identified in *J. communis* L., *J. chinensis* L., *J. sabina* L., *J. excelsa* M. Bieb., *J. taxifolia* Hook. et Arn., *J. brevifolia* (Seub.) Antoine, *J. phoenicea* L., *J. thurifera* L. etc. [4]. In addition, plenty of polyphenol compounds (quercetin, rutin, apigenin, luteolin, amentoflavone, chlorogenic acid, tannins, catechin, proanthocyanidin, etc.) with antioxidant properties were also detected in juniper extracts [5]. Efficient antioxidant activity in correlation with high total polyphenol content was found for extracts of *J. excelsa, J. sibirica, J. communis, J. sabina* and other juniper species of different origin [6]. Extracts with antioxidant properties were proposed to enhance the therapeutic potential of cytotoxic drugs (Vincristine) in combination with *J. excelsa* extract by treatment of leukemia cells [7]. Combination of cytostatic agents with antioxidants in future clinical trials is aimed at reduction of the therapeutic doses and side effects of the cytostatic drugs.

Cancer is a life-threatening disease, involving abnormal division of genetically modified malignant cells with potential to metastasise in

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different organs of the living beings. Activation of oncogenes is associated with complicated, including genetically inheritable, factors. In this respect, conversion of normal cells into cancer cells is often an oxidative-stress associated process, involving carcinogenic action of free radicals, produced by many metabolic pathways or by environmental pollution substances, penetrating through the living tissues.

We targeted our study at determination of the growth inhibitory activity of juniper extracts on NB4 APL t(15;17) (acute promyelocytic leukemia) cell line. APL t(15;17) is characterized by a balanced reciprocal chromosomal translocation that fuses the retinoic acid receptor alpha gene (RARα) with the promyelocytic leukemia gene (PML), leading to formation of a fusion oncogene. After therapy with all-*trans*-retinoic acid (ATRA) and anthracycline antibiotics, up to 80-90% of the PML-RARα positive patients achieve remission. However, about one quarter of the patients relapse, become resistant to ATRA and/or develop a life-threatening retinoic acid syndrome (fever, dyspnea, pulmonary infiltrates etc.).

Hence, in response to demands of the clinical practice in identification of new therapeutic agents for prevention and therapy of cancer we focused our research on the first systematic investigation of the antioxidant and antiproliferative properties of the extracts of Bulgarian juniper species in comparison with foreign juniper representatives.

EXPERIMENTAL

Materials

Chemicals and reagents: Podophyllotoxin, DPPH (2,2-diphenyl-1-picrylhydrazyl), MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazo-lium bromide], Folin-Ciocalteu's reagent (2N), gallic acid, formic acid, RPMI 1640 medium were purchased from Sigma-Aldrich Co. (Saint Louis, MO, USA). Fetal calf serum for cell culture was delivered from Biochrom GmbH (Berlin, Germany), DMSO was from Fluka Chemie AG (Buchs, Switzerland). LC grade ultra-pure water was prepared by a Millipore Direct-Q3 system (Bedford, MA, USA). LC-MS grade solvents were purchased from Ficher Scientific (USA) and Sigma-Aldrich (USA).

Plant material: J. virginiana 'Grey Owl' was received from the Arnold Arboretum, Harvard University, USA (specimen №00175599, accession №1136-61*A, 15.06.2017). *J. communis* L. was from the village Ognyanovo, Blagoevgrad Province, Rhodope Mountains (41°37'47.3" N; 23°47'14.5" E, 700 m a.s.l., 11.04.2017, SOM 174400); *J. sibirica* Burgsd. - from the Vitosha mountain, on the outskirts of Sofia (42°34'59.6" N; 23°17'28.6" E, 1803 m a.s.l., 13.04.2017, SOM 174401); *J. pigmaea* C. Koch - from the Smolyan Province, Mursalitsa region of the Rhodope Mountains (41°38'40.8" N; 24°29'58.5" E, 1898 m a.s.l., 13.05.2017, SOM 174402); *J. deltoides* R. P. Adams - from the village Ognyanovo, Blagoevgrad Province, Rhodope Mountains (41°37'46.6" N; 23°47'15.4" E, 695 m a.s.l., 11.04.2017, SOM 174403); *J. excelsa* M. Bieb. - from the reserve Tisata, on the riverside of Struma (41°44'01.6" N; 23°09'22.5" E, 199 m a.s.l., 09.04.2017, SOM 174404). *J. sabina* L. was collected from the resort Borovets, Sofia Province, Rila mountain (42°14'19.8" N; 23°32'33.6" E, 1182 m a.s.l., 14.05.2017, SOM 174405); *J. virginiana* L. was collected from the University of Forestry Arboretum, Sofia (42°39'08.7" N; 23°21'30.1" E, 612 m a.s.l., 13.04.2017, SOM 174406). Voucher specimens were deposited in the Herbarium of Institute of Biodiversity and Ecosystem Research, Bulgarian Academy of Sciences. *Juniperus* species were authenticated by A. Tashev (University of Forestry, Sofia) and according to R. P. Adams [1].

Extraction procedure

Fresh plant material was stored in a freezer (at -20ºC) in vacuum plastic bags until extraction. Then, the plant material (5 g) was ground and 80% (v/v) methanol (50 ml) was added. The suspension was placed in an Erlenmeyer flask with stopper and was stirred for 1.5 h in a shaker water bath at 20ºC (ambient temperature). The mixture was filtered and the extract was collected. The remaining solid material was subjected to a second extraction for 1.5 h with a new portion of 80% methanol (50 ml). After filtration, the solid mass was stirred again for 1.5 h in 80% methanol (25 ml). The combined extracts were concentrated by a vacuum evaporator. The remaining residue was freeze-dried (24 h, -50ºC, 0.2 mbar) and kept at -20ºC until analyses**.**

Folin-Ciocalteu method for determination of total phenol content

The total polyphenol content (TPC) of the corresponding juniper extract was determined by Folin-Ciocalteu method with minor modifications [8]. In brief, 20 µl of the extract [5 mg/ml in 80% (v/v) methanol] were mixed with distilled water (1.58 ml) and FC-reagent (100 µl) was added. The control sample contained the same reagents but without plant extract. After 3-5 min 300 µl of sodium carbonate (20% w/v) were added and the samples were kept at room temperature for 2 h. The

absorbance at 765 nm was registered on a spectrophotometer.

The calibration curve was obtained using gallic acid standard. The TPC of the extracts was expressed in GAE (Gallic Acid Equivalents) according to the formula: $C = c.V/m$,

where C is concentration of phenolic compounds in mg GAE per gram dry extract; c – gallic acid concentration [mg/ml] from the calibration curve; m – weight of plant extract [g]; V – volume of plant extract [ml]. The TPC of each extract was determined by 2 independent analyses and was given as an average value ± SD.

DPPH radical scavenging method for determination of antioxidant activity

The radical scavenging activity of the extracts was determined by the DPPH-method [9]. Half-maximum DPPH-scavenging concentration (SC_{50}) of the corresponding plant extract was determined as concentration of the extract in the test sample that decreased the DPPH-concentration by 50%. Briefly, stock solutions (10 mg/ml) of the corresponding plant extract in 80% (v/v) methanol were prepared by ultrasonication (2×5 min, 55ºC). Then, 1 ml of the extract (at different concentrations) was mixed with 4 ml of DPPH solution (0.004% w/v) in a test tube. The control sample was prepared with the same reagents but without plant extract. The blank sample contained 80% (v/v) methanol. The solutions were kept at room temperature for 1 h in the dark and then the absorption at 517 nm was measured on a spectrophotometer. The percentage of the DPPH-inhibition was calculated according to the formula:

$$\%\, inhibition = [(Ac - As)/Ac]x100,$$

where A_c is the absorbance of DPPH solution in the control sample without extract and A_s is the absorbance of DPPH in sample containing the corresponding plant extract.

A *UV-1600PC* spectrophotometer (*VWR int.*) was used for Folin-Ciocalteu and DPPH-assays.

Cell culture and MTT-test for determination of antiproliferative activity of plant extracts

NB-4 cells (DSMZ, Germany) were cultured in a humidified incubator (37ºC, 5% CO_2) in RPMI-1640 medium, supplemented with 10% fetal calf serum, glutamine (2 mM) and HEPES buffer (25 mM). Stock solutions of freeze-dried extracts (10 mg/ml in DMSO) were diluted with RPMI-1640 to obtain the desired concentrations. The solvent in the medium was less than 0.5% (v/v). Cells (3×10^5 cells/ml) were seeded into 96-well plates (100 µl/well) and were exposed to various extract concentrations for 72 h. Cell proliferation was determined by MTT-assay as it was described in the literature [10]. MTT-tests were carried out using a microplate reader (Labexim LMR1s).

Data processing and statistics

The MTT-assays were carried out in at least 4 separate experiments. The MTT data were fitted to sigmoidal concentration–response curves and the IC_{50} values were calculated using non-linear regression analysis (GraphPad Prism software). Statistical processing exploited Student's t-test with $p \leq 0.05$ set as the lowest level of statistical significance.

LC-ESI-MS/MS and UHPLC/HRMS for podophyllotoxin identification:

For liquid chromatography - electrospray ionization mass spectrometry analyses (LC-ESI-MS/MS) an Agilent 1200 HPLC system (USA) was connected to 3200 QTRAP Mass spectrometer (AB Sciex, USA). The QTRAP-MS system operated at positive-ion mode, capillary temperature 500°C and source voltage 4.5 kV. Nitrogen was used as curtain and collision gas. The optimum conditions of Multiple Reaction Mode monitoring were determined in the infusion mode. Eclipse XDB-C18 column (4.6 × 50 mm, 1.8 µm particle size; Agilent Technologies, USA) was maintained at 25°C. Mobile phase A (0.1% formic acid in water) and mobile phase B (acetonitrile with 0.1% formic acid) were used. The gradient program was as follows: 0-0.5 min 35% B, then a linear ramp to 53% B to 1 min, 1-4 min 53% B, next a linear ramp to 90% B to 5 min, and a hold at 90% B until 6 min. The total run time was 9 min, including 2 min equilibration. The injection volume was 5 µL with a mobile phase flow rate of 450 µL/min. Data acquisition and processing were carried out by *Analyst 1.5* software (AB Sciex, USA). Triplicate injections were made for each standard solution and sample. Prior to injection, samples were subjected to solid-phase purification by Sep-Pak C18 Cartridges (Waters, Ireland), prepared for sample loading using 60% (v/v) acetonitrile.

Ultra-high performance liquid chromatography analyses in tandem with high resolution mass spectrometry (UHPLC/HRMS) were performed on a Thermo Scientific Dionex Ultimate 3000 RSLC system (Germany), coupled to Thermo Scientific Q Exactive Plus mass spectrometer (Bremen, Germany) with heated electrospray probe HESI-II. The instrument operated at spray voltage 3.5 kV, while the ion transfer tube and HESI-II vaporizer temperatures were set at 320°C. Data acquisition

and processing were done using *Thermo Scientific Xcalibur* 3.0 software. A AkzoNobel Kromasil Externity XT-1.8-C18 (Bohus, Sweden) narrow-bore column (2.1×100 mm, 1.8 μm) with Phenomenex Security Guard ULTRA UHPLC EVO C18 (Torrance, USA) was used and maintained at 40°C. The mobile phase consisted of systems A (0.1% formic acid in water) and B (0.1% formic acid in acetonitrile). The following gradient was used: the mobile phase was held at 5% B for 0.5 min, gradually turned to 60% B over 22.5 min, kept at 60% B for 2 min, followed by a gradual increase to 85% B over 2.5 min, kept at 85% B over 2 min and the system was turned to the initial condition of 5% B in 0.5 min. The system was conditioned at 5% B for 4.5 min before injection. The flow rate and injection volume were 300 μL/min and 2 μL, resp.

RESULTS AND DISCUSSION

Selection of Juniperus species with best antiproliferative activity

In a preliminary screening we determined that *Juniperus virgiana* 'Grey Owl' extract showed best antiproliferative activity after treatment of a panel of cancer cells [11] with different medicinal plant extracts [12]. Thus, we directed our research at a first systematic analysis of the antiproliferative properties of extracts obtained from *Juniperus* species of Bulgarian origin in comparison with extracts of foreign juniper representatives. Considering the pharmaceutical interest in identification of plant extracts for prevention of the living cells from the carcinogenic action of free radicals, we analyzed also the antioxidant activity of the juniper extracts.

Several *Juniperus* species are distributed in Bulgaria [13]: *J. communis* L., *J. deltoides* R. P. Adams, *J. excelsa* M. Bieb., *J. sabina* L., *J. pigmaea* K. Koch, *J. sibirica* Burgsd., as well as junipers, native to North America, such as *J. virginiana* L. etc.

The study of the antiproliferative activity of extracts of junipers of Bulgarian and foreign origin was focused on NB4 APL cell line, bearing t(15;17) PML-RARA fusion oncogene. Efficient anticancer extracts provide a potential alternative treatment of APL in cases of resistance to conventional therapy, life-threatening ATRA-syndrome and disease relapse. The analysis of the dose-response curves by the MTT- assay revealed that all studied juniper extracts exhibited antiproliferative properties on NB4 cells, however *J. sabina*, *J. virginiana* and *J. virginiana* 'Grey Owl' extracts were selected as the best antiproliferative agents (Table 1).

Selection of Juniperus species with best antioxidant activity

Considering the interest in identification of plant extracts for prevention of the living cells from the carcinogenic action of free radicals, we analyzed also the antioxidant activity of the studied juniper extracts in correspondence with their total polyphenol content (TPC). The DPPH-radical scavenging activity of the extracts was evaluated by their SC_{50} value, calculated as concentration of the extract that decreased the initial DPPH concentration by 50%. Hence, lower SC_{50} values denote higher DPPH-radical scavenging activity. In general, the leaves extracts demonstrated better TPC and DPPH-SC_{50} values in comparison with the galbula extracts (with the exception of the *J. sabina* extract, where both galbula and leaves extracts exhibited similar antioxidant properties). Best antioxidant activity was determined for leaves extracts of *J. sibirica* and *J. excelsa* that corresponded to their highest total polyphenol content values (Table 1).

However, the different mechanism of action of the antioxidant and antiproliferative compounds does not point at a correlation between both activities. This conclusion was confirmed by our study of other plant extracts in order to compare their antioxidant and anticancer properties.

In this connection, we registered a low antiproliferative activity (IC_{50} 131±26 μg/ml on NB4 cells) of an efficient antioxidant *Rhodiola rosea* L. rhizome extract with high TPC value (374±17 mg GAE/g dry extract). *Rh. rosea* extract contained 0.8% of salidroside and 2.2% of rosavin as antioxidant compounds. By contrast, *I. helenium* root extract exhibited low TPC value (21±1 mg GAE/g dry extract), however it possessed efficient anticancer activity (IC_{50} 5±1 μg/ml on NB4 cells). Anticancer compounds alantolactone and isoalantolactone were identified in the *I. helenium* extract. In the case of juniper extracts, their antioxidant properties are feasible to render a preventive effect on the healthy tissues during intense chemotherapies.

Podophyllotoxin identification by LC-ESI-MS/MS and UHPLC/HRMS

Podophyllotoxin (PPT) acts as a suppressor of the mitotic-spindle microtubule assembly [14]. However, its derivatives (etoposide, teniposide) were supposed to have different mechanisms of action (inhibition of DNA topoisomerase II, DNA unwinding and replication), while other PPT derivatives have as yet unknown mechanisms of action [15].

Table 1. Comparison of the total polyphenol content (TPC), DPPH-radical scavenging (SC_{50}) and NB4-growth inhibitory half-maximum (IC_{50}) concentrations of the studied juniper extracts.

№	Name of *Juniperus* species	TPC [GAE mg/g]	DPPH-SC_{50} [μg/ml]	NB4-IC_{50} [μg/ml]
1A	*J. sabina* L.	79±1	394	0.5 ± 0.2
1B	*J. sabina* L.	87±2	246	0.5 ± 0.0
2A	*J. virginiana* L.	55±1	561	0.5 ± 0.1
3A	*J. virginiana* 'Grey Owl'	97±0	251	0.7 ± 0.1
3B	*J. virginiana* 'Grey Owl'	45±0.2	352	0.5 ± 0.2
4A	*J. communis* L.	132±4	154	1 ± 0.4
4B	*J. communis* L.	90±2	207	4 ± 2
5A	*J. sibirica* Burgsd.	182±18	104	3 ± 1
5B	*J. sibirica* Burgsd.	68±0.4	530	15 ± 3
6A	*J. pigmaea* K. Koch	138±4	140	5 ± 1
6B	*J. pigmaea* K. Koch	113±6	145	29 ± 7
7A	*J. deltoides* R. P. Adams	135±7	154	66 ± 8
7B	*J. deltoides* R. P. Adams	47±4	411	70 ± 5
8A	*J. excelsa* M. Bieb.	169±7	103	137 ± 12
8B	*J. excelsa* M. Bieb.	119±9	152	188 ± 55

Abbreviations: TPC - total polyphenol content in milligrams Gallic acid equivalents per gram dry extract; DPPH-SC_{50} – half-maximum DPPH-scavenging concentration of the plant extracts; NB4-IC_{50} – half-maximum growth-inhibitory concentration of the extracts on NB4 cells. MTT-test positive control: podophyllotoxin with NB4-IC_{50} 0.005±0.001 μg/ml. IC_{50} and SC_{50} values were given in micrograms dry extract per milliliter of solvent. Lower IC_{50} and SC_{50} values denote higher activity. All experiments were performed using leaves (A) and galbula (B) extracts;

Podophyllotoxin identification in *J. sabina*, *J. virginiana* and *J. virginiana* 'Grey Owl' extracts with best antiproliferative activities was performed with both LC-ESI-MS/MS and UHPLC/HRMS methods. This anticancer plant metabolite was identified by its protonated molecular ion at 415 $[M+H]^+$ *m/z* and fragment ions at 397 *m/z* and 247 *m/z* (mass-to-charge ratios), comparing their retention times (RT) and transitions with the corresponding values and transitions of the PPT standard tested under the same conditions (Table 2).

Table 2. LC-ESI-MS/MS parameters for podophyllotoxin identification.

Compound	RT (min)	Mol. weight	$[M+H]^+$ (*m/z*)	Fragment ions (*m/z*)	Collision energy (eV)
Podophyllotoxin	3.60	414	415	397	-46
				247	-60

Abbreviations: RT - retention time, *m/z* – mass-to-charge ratio, M- molecular mass.

Using UHPLC/HRMS we detected the exact mass of the protonated molecular ion of PPT at 415.1385 *m/z* (calculated for $C_{22}H_{23}O_8$ 415.1387 *m/z*). Identification of other anticancer metabolites in the studied extracts is in progress.

CONCLUSION

To our knowledge, we performed the first systematic investigation of the antiproliferative and antioxidant properties of total extracts of *Juniperus* species of Bulgarian origin and compared their activity with extracts of foreign juniper representatives. While efficient antioxidant activity is desired in the prevention of cancer, the powerful antiproliferative agents are required in the anticancer chemotherapy. We determined that in the group of studied plant species the leaves extracts of *J. sibirica* and *J. excelsa* exhibited best antioxidant activity in accordance with their best TPC values (Table 1). However, efficient antioxidants might not have high antiproliferative activity, while weak antioxidants might possess excellent antiproliferative properties due to the different mechanisms of both actions. Our study pointed out that most juniper representatives exhibited antiproliferative activity, however in the

group of studied junipers the extracts of *J. sabina*, *J. virginiana* and *J. virginiana* 'Grey Owl' were selected as the best antiproliferative extracts on NB4 cells (Table 1). These species possessed also antioxidant activity that is perspective in studies of prevention of the healthy cells from the harmful cytostatic drugs during anticancer chemotherapy.

Using liquid chromatography in tandem with electrospray ionization-mass spectrometry (LC-ESI-MS) we identified podophyllotoxin in *J. sabina*, *J. virginiana* and *J. virginiana* 'Grey Owl' extracts with best antiproliferative activities.

Our study set the pattern for further identification of other plant metabolites (lignans or polyphenolic compounds) in the analyzed juniper extracts, as well as it pointed out at a future comparative bioactivity-guided study of *Juniperus* species distributed in different habitats.

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REFERENCES AND NOTES

1. R. P. Adams, Junipers of the World: The Genus *Juniperus*, 4th Edition, Trafford Publishing Co., Bloomington, Indiana, USA, 2014.
2. C. Canel, R. M. Moraes, F. E. Dayan, D. Ferreira, *Phytochemistry*, **54**, 115 (2000).
3. a. I. Ionkova, *Mini Rev. Med. Chem.*, **11**, 843 (2011); b. A. San Feliciano, J.L. Lopez, M. Medarde, J.M. Miguel del Corral, B. de Pascual-Teresa, P. Puebla, *Tetrahedron*, **44**, 7255 (1988); c. K. W. Woo, S. U. Choi, J. C. Park, K. R. Lee, *Arch. Pharm. Res.*, **34**, 2043 (2011); d. A. San Feliciano, J. M. Miguel del Corral, M. Gordaliza, A. Castro, *Phytochemistry*, **30**, 3483 (1991); e. C. D. Carpenter, T. O'Neill, N. Picot, J. A. Johnson, G. A. Robichaud, D. Webster, C. A. Gray, *Journal Ethnopharmacol.*, **143,** 695 (2012); f. L. M. Moujir, A. M. L. Seca, L. Araujo, A. M. S. Silva, M. C. Barreto, *Fitoterapia* **82**, 225 (2011); g. M.R. Kang, S.-K. Park, Ch. W. Lee, I.J. Cho, Y. N. Jo, J.W. Yang, J.-A. Kim, J. Yun, K. H. Lee, H. J. Kwon, B. W. Kim, K. Lee, J. S. Kang, H. M. Kim, *Oncol. Rep.*, **27,** 1407 (2012).
4. a. S. van Slambrouck, A. L. Daniels, C. J. Hooten, S. L. Brock, A. R. Jenkins, M. A. Ogasawara, J. M. Baker, G. Adkins, E. M. Elias, V. J. Agustin, S. R. Constantine, M. J. Pullin, S. T. Shors, A. Kornienko, W. F. A. Steelant, *Oncol. Rep.*, **17,** 1487 (2007); b. S. Benzina, J. Harquail, S. Jean, A.-P. Beauregard, C.D. Colquhoun, M. Carroll, A. Bos, Ch. A. Gray, G. A. Robichaud, *Anti-Cancer Agents Med. Chem.*, **15**, 79 (2015); c. S. Georgieva, P. Hadjieva, C. Chanev, S. Arpadjan, C. R. Acad. Bulg. Sci., **67**, 635 (2014); d. A.M. Ali, I. Intan-Safinar, M.M. Mackeen, S.H. El-Sharkawy, K. Takahata, H. Kanzaki, K. Kawazu, *Nat. Prod. Sci.*, **4,** 180 (1998); e. T. Huyan, Q. Li, Y.-L. Wang, J. Li, J.-Y. Zhang, Y.-X. Liu, M. R., Shahid, H. Yang, H.-Q. Li. *J. Ethnopharmacol*., **185**, 289 (2016); f. G. Topçu, A. C. Gören, G. Bilsel, M. Bilsel, O. Çakmak, J. Schilling, D. G. I. Kingston, *Pharm. Biol*., **43**, 125 (2005); g. N. Muto, T. Tomokuni, M. Haramoto, H. Tatemoto, T. Nakanishi, Y. Inatomi, H. Murata, A. Inada, *Biosci. Biotechnol. Biochem*., **72**, 477 (2008); h. A. M. L. Seca, A. M. S. Silva. *Phytochem. Lett*., **3,** 126 (2010); i. A. F. Barrero, J. F. Q. del Moral, M. M. Herrador, M. Akssira, A. Bennamara, S. Akkad, M. Aitigri. *Phytochemistry*, **65**, 2507 (2004); j. A. San Feliciano, J. M. Miguel del Corral, J. L. Lopez, B. de Pascual-Teresa, *Phytochemistry*, **31**, 1719 (1992).
5. a. N. S. Ghaly, S. A. Mina, N. A. H. Younis, *J. Pharm. Sci. Res*., **8**, 128 (2016); b. M. M. Lesjak, I. N. Beara, D. Z. Orcic, K. N. Petar, N. D. Simin, S. D. Emilija, N. M. Mimica-Dukic, *J Funct. Foods*, **7,** 257 (2014); c. M. M. Lesjak, I. N. Beara, D. Z. Orcic, G. T. Anackov, K. J. Balog, M. M. Franciskovic, N. M. Mimica-Dukic. *Food Chem.* **124,** 850 (2011); d. N. Orhan, D. Orhan, A. Gokbulut, M. Aslan, F. Ergun, *Iranian J. Pharm. Res.*, **16**, 64 (2017); e. Q. X. Liu, D. W. Li, W. Wang, D. M. Wang, X. X. Meng, Y. T. Wang, *Chemistry & Biodiversity*, **13**, 1240 (2016); f. T. M. Chaouche, F. Haddouchi, F. Atik-Bekara, R. Ksouri, R. Azzi, Z. Boucherit, C. Tefiani, R. Larbat, *Ind. Crops Prod.*, **64,** 182 (2015).
6. a. S. K. Hosseinihashemi, A. Dadpour, A. Lashgari, *Nat. Prod. Res.*, **31,** 681 (2017); b. S. A. Emami, J. Asili, Z. Mohagheghi, M. K. Hassanzadeh, *Evid. Based Complement. Alternat. Med.*, **4,** 313 (2007); c. S. Moein, M. Moein, *Iranian J. Pharm. Sci.*, **6,** 133 (2010); d. A. Ved, A. Gupta, A. K. S. Rawat, *Pharmacogn. Mag.*, **13,** 108 (2017); e. A. Luis, F. Domingues, A. P. Duarte, *Nat. Prod. Commun.*, **6,** 1863 (2011); f. M. F. Taviano, A. Marino, A. Trovato, V. Bellinghieri, T. M. La Barbera, A. Guvenc, M. M. Hurkul, R. De Pasquale, N. Miceli, *Pharm. Biol.*, **49,** 1014 (2011); g. N. Miceli, A. Trovato, P. Drugo, F. Cacciola, P. Donato, A. Marino, V. Bellinghieri, T. M. La Barbera, A. Guvenc, M. F. Taviano, *J. Agric. Food Chem.*, **57,** 6570 (2009); h. M. Elmastas, I. Gulcin, S. Beydemir, O. I. Kufrevioglu, H. Y. Aboul-Enein, *Anal. Lett.*, **39,** 47 (2006); i. T. Nakanishi, N. Iida, Y. Inatomi, H. Murata, A. Inada, J. Murata, F. A. Lang, M. Iinuma, T. Tanaka, *Phytochemistry*, **65,** 207 (2004); j. N. Orhan, I. E. Orhan, F. Ergun, *Food Chem. Toxicol.* 49, 2305 (2011).

7. M. Darvishi, S. Esmaeili, N. Dehghan-Nayeri, P. Mashati, A. Gharehbaghian, *J. Appl. Biomed.*, **15,** 219 (2017).
8. a. O. Folin, V. Ciocalteu, *J. Biol. Chem.*, **27**, 627 (1927); b. V. L. Singleton, R. Orthofer, R. M. Lamuela-Raventos, *Meth. Enzymol.*, **299,** 152 (1999); c. A. L. Waterhouse, in: Current Protocols in Food Analytical Chemistry, R. E. Wrolstad, (ed.), vol. 1, Wiley, New York, 2001.
9. a. C. Sanchez-Moreno, *Food Sci. Technol. Int.*, **8,** 121 (2002); b. J. Perez-Jimenez, S. Arranz, M. Tabernero, M. Diaz-Rubio, J. Serrano, I. Gono, F. Saura-Calixto, *Food Res. Int.* **41,** 274 (2008).
10. a. T. Mosmann, *J Immunol Methods*, **65**, 55 (1983); b. S. M. Konstantinov, H. Eibl, M. R. Berger, *Br. J. Haematol.*, 107, 365 (1999).
11. HepG2 hepatocellular carcinoma, MDA-MB-231 ER^-, PR^-, $HER2^-$ triple negative breast carcinoma, MCF-7 ER^+, PR^+, $HER2^-$ breast carcinoma, HeLa cervical carcinoma, HT-29 colorectal carcinoma, HL-60 acute promyelocytic leukemia, NB4 APL t(15;17) acute promyelocytic leukemia.
12. *Inula helenium* L., *Betula pendula* Roth., *Buxus sempervirens* L., *Ruta graveolens* L., *Inula britannica* L., *Juniperus virginiana* L. 'Grey Owl', *Rhodiola rosea* L., *Clinopodium vulgare* L., *Achillea collina* (Becker ex. Rchb.f.) Heimerl, *Achillea thracica* Velen., *Sideritis scardica* Griseb., *Paeonia peregrina* Mill., *Thalictrum aquilegifolium* L., *Ononis arvensis* L., *Tribulus terrestris* L., *Smilax excelsa* L., *Physalis alkekengi* L., *Marrubium peregrinum* L., *Eryngium campestre* L., *Anemone pavonina* Lam., *Stachys recta* L., *Centaurea trinervia* Willd, *Centaurea finazzeri* Adamovic, *Cynara cardunculus var. scolimus* L. etc.
13. B. Assyov, A. Petrova, Conspectus of the Bulgarian vascular flora, 4th ed., Bulgarian Biodiversity Foundation, Sofia, 2012.
14. H. Ardalani, A. Avan, M. Ghayour-Mobarhan, Avicenna, *J. Phytomed.*, **7**, 285 (2017); C. Canel, R. M. Moraes, F. E. Dayan, D. Ferreira, *Phytochemistry*, ***54****:* 115 (2000).
15. M. Gordaliza, M. A. Castro, J. Miguel del Corral, A. San Feliciano, *Curr. Pharm. Design*, **6**, 1811 (2000).

АНТИОКСИДАНТНА И АНТИПРОЛИФЕРАТИВНА АКТИВНОСТ НА ВИДОВЕ ОТ РОД *JUNIPERUS* L. ОТ БЪЛГАРСКИ И ЧУЖДОЗЕМЕН ПРОИЗХОД И ИДЕНТИФИКАЦИЯ НА ТЕХНИ АНТИТУМОРНИ МЕТАБОЛИТИ

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(Резюме)

Род *Juniperus* L. (Cupressaceae) включва над 50 вида хвойна. Широко разпространените видове хвойна са вечнозелени растения, лесни за култивиране, които целогодишно произвеждат значително количество биомаса, богата на биологично-активни съединения, включително антиоксиданти и потенциални антитуморни вещества. В настоящето изследване е проведен систематичен сравнителен анализ на български видове хвойна по отношение на чуждоземни представители от този род с цел да се изберат тези, които притежават висока антипролиферативна и антиоксидантна активност. В групата на изследваните растителни екстракти най-висока антипролиферативна активност при NB4 APL t(15;17) клетки (остра промиелоцитна левквмия) е установена за екстракти на *J. sabina* L., *J. virginiana*' L. и *J. virginiana* 'Grey Owl'. Най-висока антиоксидантна активност в групата на изследваните видове проявяват екстрактите от листа на *J. sibirica* и *J. excelsa.* Ефективната антиоксидантна активност е необходима за превенция на рака, докато ефективни антипролиферативни вещества се изискват при хемотерапията на рака. Чрез метода на течна хроматография в тандем с масспектрометрия (LC-ESI-MS/MS) в екстрактите от хвойна с антипролиферативна активност е идентифициран подофилотоксин, който е известен прекурсор за синтез на антитуморни лекарства. Предстои идентификация и на други растителни метаболити (лигнани, фенолни съединения и др.) във видове *Juniperus* с различен произход. Идентифицирането на екстракти от хвойна с ефективна антиоксидантна и антипролиферативна активност има потенциално приложение при бъдещи клинични изследвания относно комбинирана терапия с други антитуморни средства в случаи на резистентност към конвенционалната химиотерапевтика, рецидиви на онкологични заболявания, както и за понижаване на терапевтичните дози на цитостатици при лечение на рака.

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Activity-guided extraction optimization of highly efficient antioxidant plant species: study of *Rhodiola rosea* L. (Golden root)

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The species of genus *Rhodiola* L. (Crassulaceae) are mainly distributed in different cold regions of the Northern Hemisphere of the world. The Golden root (*Rhodiola rosea* L.) extract is known in the traditional medicine as adaptogen to treat fatigue, depression and stress-associated diseases. The Golden root extract was selected for a comprehensive study because it exhibited superior antioxidant properties in a group of other efficient antioxidant plant species studied here. Consequently, we aimed our study at an antioxidant activity – guided optimization of *Rh. rosea* extraction by selection of experimental conditions leading to maximum total polyphenolic content and antioxidant activity of the extract. Using a set of variable parameters (solvent composition, temperature, ratio solvent-to-raw material, process duration), it was suggested that extraction in 25% ethanol, at 50ºC, using solvent-to-solid ratio of 15 (v/w) and process duration of 3-5 min are optimal extraction conditions for obtaining of Golden root extracts with maximum total phenolic content (TPC) and antioxidant activity. Metabolite identification in the extract with the best antioxidant activity was performed by both HPLC and UHPLC/HRMS methods. It was found that *Rh. rosea* extract, obtained at optimal conditions, contained 2.29±0.05% of rosavin and 0.80±0.02% of salidroside. The Golden root extract, obtained in this study at optimal conditions regarding its antioxidant activity, has potential application in the production of high-quality plant extracts for prevention of cancer and oxidative-stress associated diseases, including cardio-, neuro- and hepato-degenerative disorders.

Keywords*:* Antioxidant activity, Extraction optimization, *Rhodiola rosea* L., Rosavin, Salidroside, Total phenolic content

INTRODUCTION

Rhodiola rosea L. (Crassulaceae) is a perennial plant, known as Golden root, Roseroot, Arctic root. This plant species is found at high altitudes in cold regions of Europe, Asia and Nord America. The Golden root is known from centuries in the traditional medicine [1-4]. The plant is used as adaptogen, to treat depression, fatigue, psychological disorders, for improvement of memory and cognitive functions of the central nervous system [5]. *Rhodiola rosea* has very low toxicity [6, 7] and has demonstrated induction of no mutations in humans [8]. The antioxidant activity of *Rhodiola rosea* points to its potential application in the prevention of cancer, neuro-, cardio-, and hepato-degenerative diseases by protection of the cells from the harmful action of radicals obtained in different metabolic pathways.

The extraction of biologically active compounds from *Rhodiola rosea* is an object of increasing scientific and industrial interest [9]. Classical and ultrasonic extractions have been used for determination of appropriate conditions for isolation of bioactive compounds from different species of the genus *Rhodiola* L.: phenylethanoids (salidroside, *p*-tyrosol), phenylpropanoids (rosavin, rosarin, rosin), monoterpenes (rosiridin) [10], flavonoids [11] etc. Microwave-assisted [12] and supercritical fluid [13, 14] extractions have been also employed for recovery of bioactive compounds content in *Rhodiola* species. The effects of the origin, plant part, harvest season and processing of *Rhodiola rosea* cultivars have been studied in order to obtain high-quality extracts for medicinal use [15, 16]. HPLC with mass-spectrometry and rapid resolution liquid chromatography were applied for analyses of salidroside, rosavins and other bioactive compounds from the Golden root [17, 18]. However, the conclusions about the optimal extraction conditions in terms of solvent composition, solvent-to-solid ratio (v/w) and extraction duration differ considerably in the literature, depending on the method, experimental parameters or target compounds used for extraction optimization.

Considering the scientific and industrial interest in obtaining of high quality plant extracts, we studied the antioxidant properties of *Rh. rosea* rhizomes in a group of other efficient antioxidant

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species: *Smilax excelsa* L., *Sideritis scardica* Griseb., *Achillea collina* (Becker ex Rchb.f.) Heimerl, *Achillea thracica* Velen., *Inula helenium* L., *Clinopodium vulgare* L. In this comparative study we determined a superior antioxidant activity of the Golden root, which was the reason to initiate an activity-guided extraction optimization of this species complemented with antioxidant metabolite quantification of the extract obtained at optimal experimental conditions.

EXPERIMENTAL

Materials and methods

Rhodiola rosea rhizomes (Russian origin) were delivered from a pharmaceutical supplier '*Bilki*' Ltd., Sofia; *Sideritis scardica* and *Achillea thracica* were collected from the Botanical garden of the Institute of Biodiversity and Ecosystem Research, Bulgarian Academy of Sciences (IBER-BAS); *Inula helenium* was from experimental botanical field 'Beglica' (the Rhodope mountains); *Achillea collina* - from village Bistritsa (Vitosha mountain); *Clinopodium vulgare* – from village Zelenigrad (Province Pernik); *Smilax excelsa* – from village Belopolyane (Province Haskovo). The plant species were authenticated by a botanist Dr. Dessislava Sopotlieva (IBER, BAS).

Chemicals and reagents

Salidroside (98%), rosavin (98%), DPPH, Folin-Ciocalteu's (FC) reagent (2N), gallic acid were supplied from Sigma-Aldrich Co. (Saint Louis, MO, USA). LC-MS grade solvents were purchased from Fischer Scientific (Waltham, USA).

Extraction procedure

Dried ground *Rh. rosea* rhizomes (1.5 g) were mixed with the solvent (at different solvent/solid ratios). The extraction was carried out in a shaker water bath at various experimental conditions (Table 1). The mixture was filtered and antioxidant properties of the extracts were analyzed by Folin-Ciocalteu- and DPPH-assays.

Folin-Ciocalteu method for determination of total polyphenol content in different plant extracts

The total polyphenol content (TPC) of the extracts of different plant species was determined by the Folin-Ciocalteu (FC) method [19-21]. In brief, stock solutions of freeze-dried plant extracts, dissolved in the corresponding solvent at a concentration of 10 mg/ml were prepared by ultrasonication (2×5 min, 55ºC). The stock solution of *Rhodiola rosea* extract was 5-fold diluted before starting the spectrophotometric measurements because of its strong antioxidant activity. The stock solutions of the other plant extracts were 3-fold diluted. Then, 20 µl of the corresponding diluted extract were mixed with 1.58 ml of distilled water and 100 µl of FC-reagent were added. The control sample contained the same reagents without plant extract. After 3-5 min, 300 µl of sodium carbonate (20% w/v) were added and the samples were kept at room temperature for 2 h. The sample absorbance at 765 nm was registered on a spectrophotometer.

The calibration curve was generated using a gallic acid standard. The TPC was given in gallic acid equivalents (GAE), according to the formula $C = c.V/m$, where C is concentration of the TPC in mg GAE/g dry extract; c – gallic acid concentration [mg/ml], determined from the calibration curve; m – weight [g] of the plant extract; V – volume [ml] of the extract.

DPPH method for determination of antioxidant activity of different plant extract

IC_{50} values of the radical scavenging activity of extracts were determined by DPPH-method [22, 23]. In brief, 1 ml of the corresponding plant extract (10 mg/ml) was mixed with 4 ml of DPPH solution (0.004% w/v) in a test tube. The control sample was prepared with the same reagents excluding the plant extract. The blank sample contained only solvent. The solutions were kept at room temperature for 1 h in the dark and then decrease of the absorption was measured on a spectrophotometer at 517 nm. DPPH inhibition was calculated according to the formula:

$$\%\, inhibition = [(Ac - As)/Ac]x100,$$

where A_c is the absorbance of the DPPH in the control sample without extract and A_s is the absorbance of the DPPH in the sample with plant extract. The half-maximum inhibitory concentrations (IC_{50}) were determined as the concentration of the extract in the test sample that decreased the initial DPPH concentration by 50%.

An *UV-1600PC* spectrophotometer (*VWR int.*) was used for FC- and DPPH-assays.

Statistical analysis was done by single-factor analysis of variance (ANOVA) using Microsoft Excel software and p-value≤0.05 was set as the lowest level of statistical significance.

Antioxidant metabolite quantification of the extract with optimal antioxidant activity

UHPLC/HRMS analyses for the antioxidant metabolite identification in the Golden root extract were performed on a Thermo Scientific Dionex Ultimate 3000 RSLC (Germany), consisting of 6-

channel degasser SRD-3600, high pressure binary gradient pump HPG-3400RS, autosampler WPS-3000TRS and column compartment TCC-3000RS. The LC system was coupled to Thermo Scientific Q Exactive Plus mass-spectrometer (Germany) with heated electrospray probe HESI-II. Data acquisition and processing were done using Thermo Scientific Xcalibur 3.0 software. An Akzo Nobel Kromasil Externity XT-1.8-C18 (Bohus, Sweden) narrow-bore column (2.1×100 mm, 1.8 μm) with Phenomenex Security Guard ULTRA UHPLC EVO C18 (Torrance, USA) column was used and maintained at 40°C. The mobile phase consisted of systems A (0.1% formic acid in water) and B (0.1% formic acid in acetonitrile). The following gradient was employed: the mobile phase was held at 5% B for 1 min, gradually turned to 95% B over 27 min, kept at 95% B for 2 min and the system was turned to the initial condition of 5% B in 1 min. The system was conditioned at 5% B for 4.5 min before injection. The flow rate and the injection volume were set to 300 μL/min and 2 μL, respectively. The instrument was set at spray voltage 3.5 kV, ion transfer tube and HESI-II vaporizer temperatures at 320 °C.

HPLC analyses for quantitative determination of antioxidant metabolites in the Golden root extract were performed on a HP1100 system with a manual injector (Rheodyne, model 7725), fitted with a 20 μL sample loop and a diode–array detector (G1365B), controlled by ChemStation software (Rev. 04.03, Agilent Technologies). Analytical column ChromSep SS, Inertsil 5 ODS-2 (250 × 4.6 mm i.d., 5 μm particle size) with a ChromSep guard column (Varian, Palo Alto, CA) was used. The mobile phase was acetonitrile/water (5:95, v/v) containing 0.1% formic acid (pH 3) at a flow rate of 1.0 ml/min. The column compartment was kept at 30ºC. The detector signal was monitored at 205, 254 and 280 nm. The extract was filtered (PTFE, 0.22 μm) prior to the analysis.

Calibration curves: The absolute calibration method (external standard method) was used to establish the calibration curve and to quantify the analytes. The standard compounds (0.005 g) were diluted with acetonitrile in volumetric flasks. Five standard solutions with concentrations in the range of 0.2-1 mg/ml were prepared and analyzed in triplicate; the results were presented graphically (peak area versus concentration). The equations of the linear calibration curves are given below:

for salidroside: Y = 1436.5 X – 11.8; regression coefficient 0.992;

for rosavin: Y = 38016.0 X + 36.1; regression coefficient 0.999;

where Y is the DAD peak area and X is the compound concentration [mg/ml].

RESULTS AND DISCUSSION

Selection of plant species with superior antioxidant activity

The variety of experimental methods and target compounds (salidroside, rosavins), used in the literature for extraction optimization, led to different conclusions about the optimal extraction conditions of the Golden root. Due to the interest in identification of efficient antioxidants for prevention of oxidative stress-associated disorders, we performed an antioxidant activity-guided optimization of *Rh. rosea* rhizome extraction. We established the best antioxidant properties of *Rhodiola rosea* rhizomes extract (1) in a group of extracts of other efficient antioxidant plant species, such as: *Smilax excelsa*, aerial parts (2); *Sideritis scardica*, aerial parts (3); *Achillea thracica*, flower heads (4); *Achillea collina*, flower heads (5); *Inula helenium*, leaves (6); *Clinopodium vulgare*, aerial parts (7). The antioxidant activity of the extracts was analyzed by their DPPH-radical scavenging activity. The IC_{50} values of DPPH-inhibitory activity of the extracts were calculated as the concentration of the extract, required to decrease the initial amount of DPPH by 50%. A lower IC_{50} revealed higher antioxidant activity. The comparison of TPC (Fig. 1) with the DPPH-radical scavenging activity (Fig. 2) of the total extracts revealed superior TPC value (374±17 GAE mg/g dry extract) of the Golden root extract, which corresponded to its best DPPH-radical scavenging activity (IC_{50} 29±1 μg/ml). Hence, *Rhodiola rosea* rhizome extract was selected for extraction optimization experiments because it demonstrated the best antioxidant properties in the group of the studied efficient antioxidant plant species.

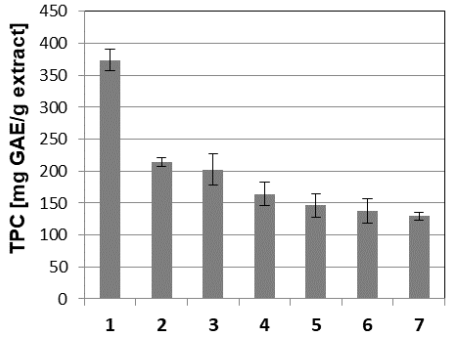

Fig. 1. Comparison of total polyphenolic content of different plant extracts. *Rhodiola rosea* rhizomes (1), *Smilax excelsa*, aerial parts (2), *Sideritis scardica*, aerial parts (3), *Achillea thracica*, flower heads (4), *Achillea collina*, flower heads (5), *Inula helenium*, leaves (6), *Clinopodium vulgare*, aerial parts (7). The extraction

was performed in 80% methanol, at solvent-to-solid ratio 10 (v/w) and 65°C for 1 h (3-fold).

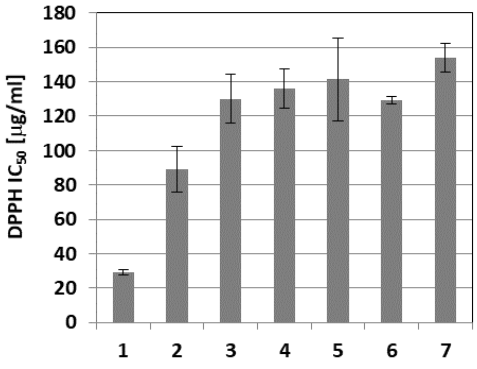

Fig. 2. Comparison of half-maximum DPPH-radical scavenging concentrations (IC_{50}) of plant extracts. The extracts numbers are the same as in Fig. 1. Lower IC_{50} values reveal higher antioxidant activity.

Antioxidant activity-guided optimization of Rhodiola rosea rhizome extraction

Effect of solvent, temperature, hydromodule and extraction duration on the antioxidant activity of Rhodiola rosea rhizome extract. Different conditions for *Rhodiola rosea* rhizome extraction were applied in order to determine their effect on the TPC, resp. on the DPPH-radical scavenging activity of the extracts. We started with determination of the influence of various concentrations of aqueous ethanol (0%, 25%, 50%, 80% and 96%), chosen as an ecological solvent, on the antioxidant activity of the Golden root extract. The TPC, DPPH-radical scavenging activity and the yields of the extracts increased using concentrations from 0% to 25% EtOH (Fig. 3), however further increase of the ethanol concentration did not improve the values of the analyzed experimental parameters. ANOVA test confirmed that the experimental parameter values in the range of 25-96% ethanol were statistically equal. Hence, 25% ethanol was selected as optimal solvent for *Rh. rosea* extraction.

Variation of the extraction temperature from 30°C to 70°C revealed that the half-maximum DPPH-inhibitory concentrations and the extraction yields were similar at all tested temperatures, while some higher TPC values were registered at 50°C (Table 1). Therefore, operational temperature of 50°C for *Rh. rosea* rhizome extraction in 25% ethanol was preferred for further process optimization.

The effect of hydromodule (HM, solvent-to-solid ratio v/w) on the antioxidant activity of the *Rh. rosea* extract was also examined (Table 1). The extraction yields significantly decreased at solvent-to-solid ratios less than 10 (v/w) due to insufficient quantity of the solvent needed for complete recovery of the extracted matter. The extractions at HM15 and HM20 showed the best and statistically similar values (confirmed by ANOVA) of the analyzed parameters (TPC, DPPH-IC_{50}, extraction yields); However, HM15 was chosen as economically more advantageous parameter over HM20 because of the lower solvent consumption at HM15. Consequently, 25% ethanol, temperature 50°C and solvent-to-solid ratio 15 (v/w) were selected as beneficial conditions for *Rh rosea* rhizome extraction.

Variation of the process duration (3, 5, 15, 30, 60 min) at the above selected conditions revealed that the antioxidant metabolite extraction of the Golden root proceeds very rapidly and only 3-5 min were sufficient to obtain *Rh. rosea* rhizome extract with optimal TPC, DPPH-radical scavenging activity and yields (Table 1).

In conclusion, analysis of the impact of experimental variables (solvent, temperature, solvent-to-solid ratio, process duration) on the antioxidant activity parameters, such as half-maximum DPPH-inhibitory concentrations in correspondence with total phenol content and process yields, revealed that 25% ethanol at temperature 50°C, solvent-to-solid ratio 15 (v/w) and process duration of 3-5 min were assumed as optimal conditions for Golden root extraction.

Antioxidant metabolite quantification in the extract, obtained at optimal conditions

Using UHPLC/HRMS we identified and quantified both rosavin and salidroside as efficient antioxidant metabolites in the Golden root extract, obtained at optimal extraction conditions in view of exhibiting optimal antioxidant activity.

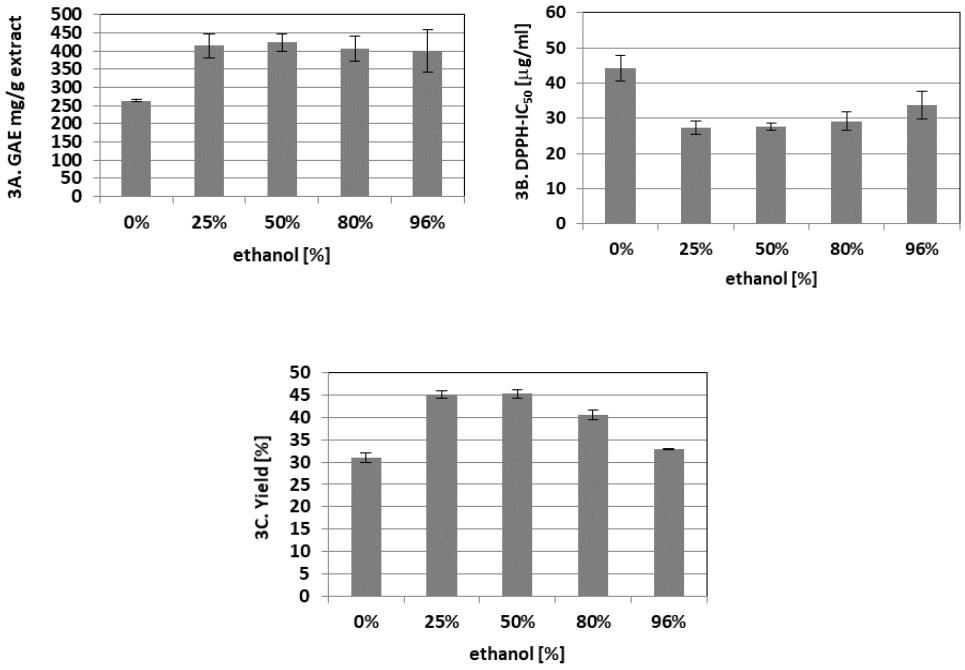

Fig. 3. Results for TPC (3A), DPPH-IC_{50} (3B) and yields (3C) of the Golden root extract depending on the solvent composition. **3A.** Total polyphenol content (TPC) is given in GAE mg/g dry extract, obtained at initial conditions – duration 2 h, temperature 70ºC, using HM 20 (v/w) and different solvent composition; 3**B.** IC_{50} [µg/ml] of DPPH-radical scavenging activity of the extracts, obtained at the same conditions, as in Fig. 3A. A lower IC_{50} revealed higher antioxidant activity; 3**C.** Total extracted matter [% of raw material], obtained at the same conditions, as in Fig. 3A.

Table 1. Effects of variation of the experimental conditions on total polyphenol content (TPC), DPPH-antioxidant activity and experimental yields of the Golden root extraction after selection of 25% ethanol as optimal solvent.

Exp. №	*Variable* experimental parameter	*Constant* experimental parameters	TPC [GAE mg/g]	DPPH-IC_{50} [µg/ml]	Extraction yield [%]
1a	70ºC	HM20, 1 h	384.7 ± 30.0	27 ± 5	43 ± 4
1b	50ºC	HM20, 1 h	420.6 ± 33.5	27 ± 3	42 ± 3
1c	30ºC	HM20, 1 h	396.0 ± 30.0	28 ± 1	40 ± 2
2a	HM 20	50ºC, 1 h	420.3 ± 23.5	27 ± 0	42 ± 3
2b	HM 15	50ºC, 1 h	406.7 ± 17.7	26 ± 3	37 ± 1
2c	HM 10	50ºC, 1 h	388.3 ± 13.5	29 ± 3	30 ± 1
2d	HM 7	50ºC, 1 h	362.0 ± 11.6	31 ± 1	21 ± 1
3a	3 min	50ºC, HM 15	370.4 ± 11.4	29 ± 1	38 ± 2
3b	5 min	50ºC, HM 15	380.0 ± 2.1	34 ± 1	37 ± 2
3c	15 min	50ºC, HM 15	389.6 ± 15.5	33 ± 0.4	37 ± 2
3d	30 min	50ºC, HM 15	377.7 ± 3.4	33 ± 1	38 ± 2
3e	60 min	50ºC, HM 15	387.3 ± 15.0	34 ± 2	35 ± 5

The values are given as an average of two independent experiments ± SD; TPC - total phenolic compounds, given in milligrams GAE (gallic acid equivalents) per gram dry extract; IC_{50} - concentration of the plant extract (in µg/ml), which decreases the initial DPPH concentration by 50%; HM – hydromodule [solvent-to-solid ratio (v/w)]. All experiments, described in the table, were performed in 25% ethanol, selected as optimal solvent for *Rh. rosea* extraction; *Experiment 1 (a-c)* set the parameters for optimization of the extraction temperature at a constant solvent-to-solid ratio; *Experiment 2 (a-d)* set the parameters for optimization of the solvent-to-solid ratio at a constant temperature, selected in the previous experiment 1; *Experiment 3 (a-e)* set the parameters for optimization of the extraction duration at constant temperature and solvent-to-solid ratio, selected in the previous experiments 1 and 2.

Exact mass of the protonated molecular ion of rosavin was found at 429.1751 *m/z* (mass-to-charge ratio, calculated for $C_{20}H_{29}O_{10}$ 429.1755 *m/z*). Exact mass of the protonated molecular ion of salidroside

was found at 301.1286 *m/z* (calculated for $C_{14}H_{21}O_7$ 301.1282 *m/z*). Quantitative HPLC-analysis determined that the chemical composition of the Golden root extract obtained at optimal experimental conditions (given above) corresponded to a content of 2.29±0.05% of rosavin and 0.80±0.02% of salidroside.

CONCLUSION

In this study we performed an activity-guided extraction optimization and antioxidant metabolite quantification of *Rhodiola rosea* rhizome extract, obtained at optimized extraction conditions. We found that extraction of the Golden root in 25% ethanol, at 50°C, solvent-to-solid ratio 15 (v/w) and extraction duration for 3-5 min creates the most beneficial conditions for obtaining of extracts with optimal TPC and DPPH-radical scavenging activity. The chemical composition of the Golden root extract, obtained at optimal extraction conditions for its antioxidant properties, corresponded to a content of 2.29±0.05% of rosavin and 0.80±0.02% of salidroside. Being a powerful antioxidant with no major toxicity, the Golden root extract with optimized antioxidant properties is a feasible pharmaceutical agent in the prevention of cancer and oxidative stress-associated neuro-, cardio-, hepato-degenerative disorders.

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REFERENCES

1. C. Linnaeus, in: Materia medica, vol. 1, Publisher: Laurentii Salvii, Stockholm, 1749.
2. Pharmacopée Francaise (9th ed.), Paris, 1976.
3. National Pharmacopoeia of the USSR (11th ed.), The USSR Ministry of Health, Moscow, 1990.
4. A. N. Shikov, O. N. Pozharitskaya, V. G. Makarov, H. Wagner, R. Verpoorte, M. Heinrich, *J. Ethnopharmacol.*, **154**, 481 (2014).
5. F. Khanum, A. S. Bawa, B. Singh, *Compr. Rev. Food Sci.*, **4**, 55 (2005).
6. S. K. Hung, R. Perry, E. Emst, *Phytomedicine*, **18**, 235 (2011);
7. R. P. Brown, P. L. Gerbarg, Z. Ramazanov, *Herbal Gram.*, **56**, 40 (2002).
8. A. S. Saratikov, E. A. Krasnov, in: *Rhodiola rosea* (Golden root), 4th ed., Tomsk State University Publishing House, Tomsk, 2004.
9. A. S. Marchev, A. T. Dinkova-Kostova, Zs. György, I. Mirmazloum, I. Aneva, M. Georgiev, *Rhytochem. Rev.*, **15,** 515 (2016).
10. Ch-Y. Ma, J. Tang, H. Wang, X. Gu, G-J. Tao, *Chromatographia*, **67**, 383 (2008).
11. J. Staneva, M. Todorova, N. Neykov, L. Evstatieva, *Nat. Prod. Commun.* **4**, 935 (2009).
12. Y. Mao, Y. Li, N. Yao, *J. Pharm. Biomed. Anal.*, **45**, 510 (2007).
13. P. Iheozor-Ejiofor, E. S. Dey, *J. Supercrit. Fluids*, **50**, 29 (2009).
14. Y-X. Wu, Q. Wang, B. Liu, M-Y. You, T. Jin, *J. Chinese Chem. Soc.*, **58**, 222 (2011).
15. W. Peschel, J. M. Prieto, C. Karkour, E. M. Williamson, *Phytochemistry*, **86**, 92 (2013).
16. W. Peschel, A. Kump, A. Horváth, D. Csupor, *Ind. Crops Prod.* **83**, 787 (2016).
17. I. A. Rodin, A. N. Stavrianidi, A. V. Braun, O. A. Shpigun, M. V. Popik, *J. Anal. Chem.*, **67**, 1026 (2012).
18. Y-C. Ma, X-Q. Wang, F. F. Hou, J. Ma, M. Luo, S. Lu, P. Jin, A. Chen, I. Xu, A. V. Patel, D. Gorecki, *J. Pharm. Biomed. Anal.*, **55**, 908 (2011).
19. O. Folin, V. Ciocalteu, *J. Biol. Chem.*, **27**, 627 (1927).
20. V. L. Singleton, R. Orthofer, R. M. Lamuela-Raventos, *Meth. Enzymol.*, **299**, 152 (1999).
21. A. L. Waterhouse, in: Current Protocols in Food Analytical Chemistry, R. E. Wrolstad (ed.), vol. 1, Wiley, New York, 2001
22. C. Sanchez-Moreno, *Food Sci. Technol. Int.*, **8,** 121 (2002).
23. J. Perez-Jimenez, S. Arranz, M. Tabernero, M. Diaz-Rubio, J. Serrano, I. Gono, F. Saura-Calixto, *Food Res. Int.* **41,** 274 (2008).

ОПТИМИЗИРАНЕ НА ЕКСТРАКЦИЯТА НА РАСТЕНИЯ С ВИСОКА АНТИОКСИДАНТНА АКТИВНОСТ: ИЗСЛЕДВАНЕ НА *Rhodiola rosea* L. (ЗЛАТЕН КОРЕН)

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Постъпила на коригирана на

(Резюме)

Растенията от род *Rhodiola* L. (Crassulaceae) са разпространени главно в различни студени райони на северното полукълбо. Екстрактът от златен корен (*Rhodiola rosea* L.) се използва в традиционната медицина като адаптоген за лечение на умора, депресия и болести, предизвикани от стрес. Екстрактът от Златен корен беше избран за задълбочено изследване поради превъзходните му антиоксидантни свойства сред групата от други ефективни антиоксидантни растителни видове, изследвани в настоящата работа. Екстракцията на *Rh. rosea* беше оптимизирана въз основа на активността на екстракта чрез подбор на експериментални условия, водещи до максимално тотално фенолно съдържание и максимална антиоксидантна активност. Чрез оптимизиране на променливи експериментални параметри бяха намерени следните оптимални условия на екстракция за получаване на екстракт от Златен корен с максимално съдържание на полифеноли и максимална антиоксидантна активност: разтворител 25% етанол, температура 50ºC, съотношение разтворител/суровина 15 (v/w) и продължителност на процеса 3-5 минути. Идентифицирането на метаболити в екстракта с най-добра антиоксидантна активност беше проведено чрез HPLC и UHPLC/HRMS методи. Установено беше, че екстрактът от *Rh. rosea*, получен при оптималните условия, съдържа 2.29±0.05% розавин и 0.80±0.02% салидрозид. Екстрактът от златен корен, получен в настоящето изследване при оптимални условия относно антиоксидантната му активност, има потенциално приложение при производство на висококачествени растителни екстракти за превенция на онкологични заболявания и болести, свързани с оксидативен стрес, вкл. кардио-, невро- и хепато-дегенеративни заболявания.

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Comparative study of antioxidant potential of curcumin and its degradation products– vanillin, ferulic acid and dehydrozingerone

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Curcumin is one of the most intensively studied biologically active antioxidants during the last decade. Its chemical instability and rapid degradation at physiological conditions (pH ≈ 7) was stated as the most important limitation for its potential applications. Numerous approaches have been undertaken to overcome the problem with the bioavailability of curcumin, including the use of such adjuvants as piperine, liposomes, system of nanoparticles, phospholipid complexes and design of new structural analogs of curcumin. On the other hand, degradation of a compound does not necessarily lead to loss of its activity. Whether and how the degradation and oxidation pathways contribute to the biological and antioxidant activities of curcumin has also been discussed. The aim of this study was to compare the antioxidant activity of curcumin with those of its degradation products (ferulic acid, vanillin and dehydrozingerone), when added in binary and ternary mixtures. Lipid autoxidation was used for assessing the chain-breaking antioxidant efficiency and reactivity of the phenols. The results obtained showed much stronger activity of curcumin than that of all the individual compounds and their binary and triple mixtures.

Keywords: Curcumin, Vanillin, Ferulic acid, Dehydrozingerone, Antioxidant potential, Double and triple mixtures

INTRODUCTION

The use of dry extract from the rhizomes of *Curcuma longa*, i.e. turmeric, in the folk (traditional) medicine in South Asia is extremely varied and many of its therapeutic effects are proven by scientific and medicinal researches. Most of the biological properties of curcumin (**Cu**), a main pigment and active component in turmeric, are found in various *in vitro* and *in vivo* model systems and are described in detail in the review of Prasad and Aggarwal [1]. There are various mechanisms by which **Cu** exhibits its antioxidant properties depending on the conditions and the medium being used. Direct hydrogen atom transfer (HAT) from the phenolic groups is considered to be the classical mechanism of its antioxidant action in a nonpolar environment [2-4]. Litwinienko and Ingold [5] proposed the concept of the so-called SPLET-mechanism (Sequential Proton Loss Electron Transfer) by referring to the effect of the solvent. Subsequently Foti *et al.* [6] proved π-complex formation between the enolate anion of **Cu** and the picryl part of DPPH˙ radical.

Despite its strong chemopreventive and chemotherapeutic potential, the use of **Cu** is limited because of its poor bioavailability due to poor absorption from the gastrointestinal tract and rapid degradation [1]. Numerous approaches have been undertaken to overcome the problem with the bioavailability of **Cu**, including the use of such adjuvants as piperine, liposomes, system of nanoparticles, phospholipid complexes and the design of new structural analogs of curcumin [7]. Angelova and Antonov [8] published a theoretical study on the structure of a complex between calixarene and **Cu** (host-guest system) in water and found that both tautomeric (diketo- and keto-enol) forms of **Cu** can enter into or leave the host cavity easily.

On the other hand, degradation of **Cu** does not necessarily lead to loss of its activity. Whether and how the degradation and oxidation pathways contribute to the biological and antioxidant activities of **Cu** has also been discussed by Schneider *et al.* [9]. There are several possible degradation pathways of **Cu** (Scheme 1), some of which including cleavage of its heptadienone carbon chain. Wang *et al.* [10] described *trans*-6-(4′-hydroxy-3′-methoxyphenyl)-2,4-dioxo-5-hexenal (**TDH**) to be the major degradation product of **Cu** in phosphate buffer ensuring pH around 7.0 and at temperature 37 °C (Scheme 1).

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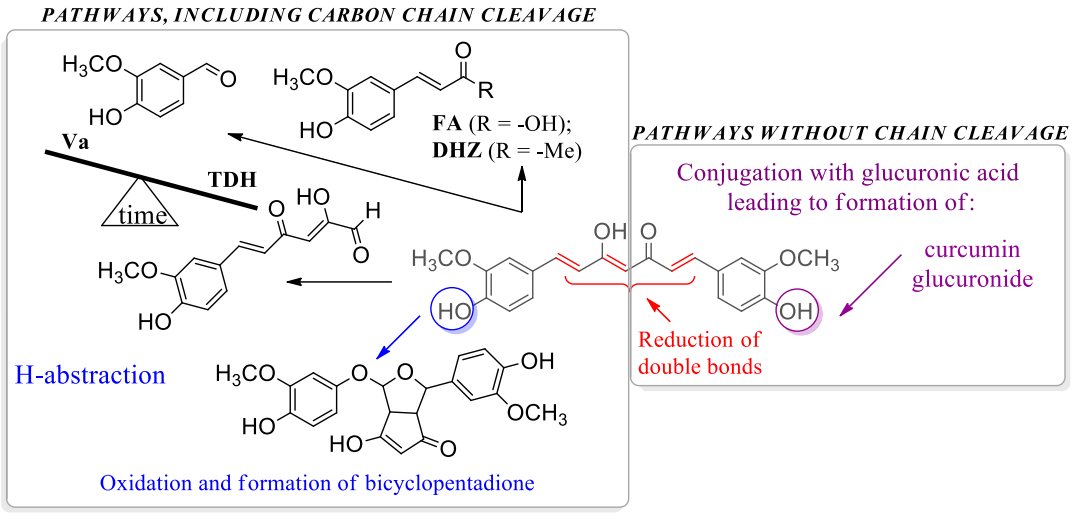


Scheme 1. Possible degradation pathways of curcumin.

Ferulic acid (**FA**), feruroylmethane or dehydrozingerone (**DHZ**) and vanillin (**Va**) are minor degradation products but it was proved that the amount of **Va** increases with incubation time at the expense of **TDH**. Despite the detailed studies of the antioxidant action of **Cu**, which has been well documented [2-13], according to Wang *et al.* [10] "it would be valuable and interesting to compare the potency of vanillin and curcumin on these aspects". Moreover, the important biological activities of **DHZ**, considered as a half **Cu** molecule [14], encourage investigations in this direction.

Considering the lipophilicity of **Cu** and the role that it exerts at the protein-lipid interface interfering on fluidity of the biological membrane [15, 16], it would be useful to study **Cu** degradation products in lipid systems.

The aim of this study was to compare the antioxidant activity of **Cu** with those of its degradation products: **FA**, **Va** and **DHZ** under bulk lipid autoxidation as individual compounds, as well as combined in binary and ternary antioxidant mixtures.

EXPERIMENTAL

All compounds used in the experimental study (Scheme 1), except **DHZ**, were purchased from Sigma-Aldrich. **DHZ** was synthesized starting from **Va** in acetone, using NaOH as the base. In the synthesis of the unsaturated compound **DHZ**, *trans*-configuration was exclusively obtained at the olefinic double bond and detected by NMR spectroscopy, as we described previously [17, 18].

Radical scavenging activity. TLC DPPH rapid test

The compounds were dissolved in acetone and spotted onto silica gel 60 F_{254} plates (E. Merck, Germany). The plates were air-dried and sprayed with 0.03% DPPH radical solution in methanol for detecting the compounds with rapid scavenging properties [19, 20]. The compounds that showed white or yellow spots onto a purple background were considered as active radical scavengers. Taking into account that the stability of DPPH radical is much higher in acetone solution than in methanol [21], the same concentration of DPPH radical in acetone was prepared and used to test the activity of the studied compounds. The effect of concentration (1mM and 10 mM), reaction time (1 min and 10 min) and solvent (acetone and methanol) were studied.

Chain-breaking antioxidant activity

Lipid samples: Triacylglycerols of commercially available sunflower oil (TGSO) were cleaned from pro- and antioxidants by adsorption chromatography and stored under nitrogen at a temperature of 20 ºC. Fatty acid composition of the lipid substrate was determined by GC analysis of the methyl esters: 10:0 (0.2%); 14:0 (0.2%); 16:0 (7.4%); 16:1 (0.3%); 18:0 (2.6%); 18:1 (29.1%); 18:2 (59.1%); 18:3 (0.7%); 20:0 (0.3%). The numbers x:y indicate the number of carbon atoms and double bonds in the fatty acid, respectively. Lipid samples containing various inhibitors were prepared directly before use. Aliquots of the antioxidant solutions in purified acetone were added to the lipid sample. Solvents were removed under a nitrogen flow. For more experimental details see reference [22].

Lipid autoxidation: The process was carried out in a thermostatic bath at (80±0.2)ºC by blowing air through the samples in special vessels. The oxidation process was monitored by withdrawing samples at measured time intervals and subjecting them to iodometric determination of the primary

products (lipid hydroxyperoxides, LOOH) concentration, i.e. the peroxide value (PV). All kinetic data were expressed as the average of two independent measurements which were processed using the computer programmes Origin 6.1 and Microsoft Excel 2010.

Determination of the main kinetic parameters of the studied compounds [22-25]: Protection factor (PF) was determined as the ratio between the induction period in the presence (IP_A) and in the absence (IP_C) of antioxidant, i.e. $PF = IP_A/IP_C$. It is a measure of antioxidant efficiency.

Inhibition degree (ID) is a measure of the antioxidant reactivity, e.g., how many times the antioxidant shortens the oxidation chain length, i.e. $ID = R_C/R_A$. The initial oxidation rates R_C in the absence and R_A in the presence of antioxidant were found from the tangent at the initial phase of the kinetic curves of hydroperoxides accumulation.

RESULTS AND DISCUSSION

Radical-scavenging activity towards DPPH•.

Rapid TLC DPPH-test is useful for preliminary selection of compounds as active or non-active towards free radicals, i.e. DPPH•. The yellowish-white spots on the purple background of DPPH• solutions in methanol and in acetone on the plates were observed (Fig. 1).

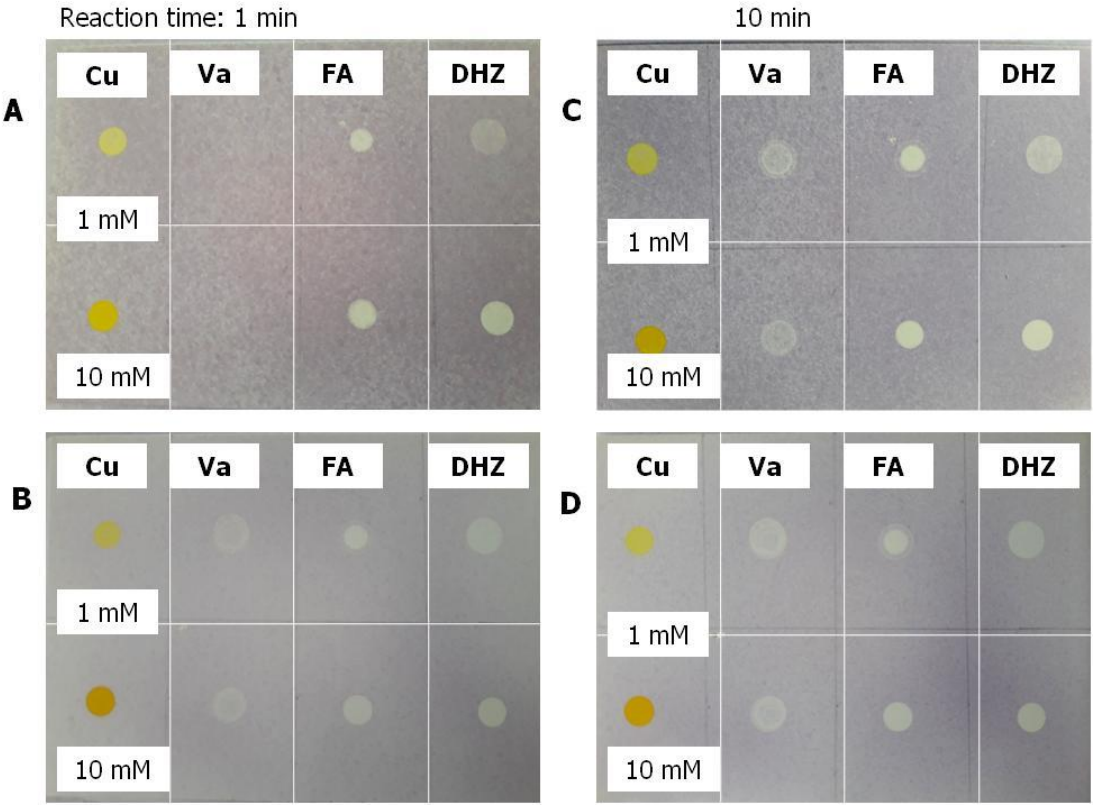


Figure 1. TLC DPPH rapid test: within 1 min after spraying the DPPH radical solution in acetone (A) and in methanol (B) and 10 min after spraying the solution of DPPH radical in acetone (C) and in methanol (D).

Effect of reaction time: Reaction time is one of the most important criteria in this measurement. We studied the activity of all compounds after 1 min and 10 min from spraying both solutions of the radical (DPPH•) - in acetone and in methanol, respectively. All compounds (**Cu**, **FA**, **Va** and **DHZ**) showed bright spots immediately after spraying with methanolic solution of DPPH• while in acetone solution, **Va** did not show a spot at both studied concentrations (1 mM and 10 mM) within the first minutes after spraying the plate. Surprisingly, after 10 min **Va** showed yellowish-white spots in acetone. The result obtained showed that **Va** exerts different activity in protic (methanol) and dipolar aprotic (acetone) solvents.

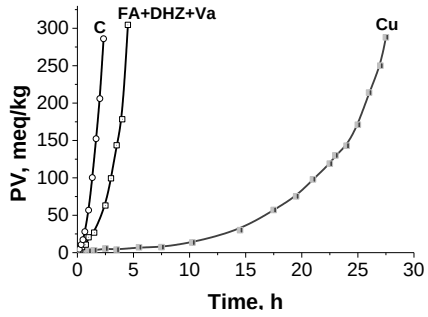
Effect of concentration: The intensity of the spots of **Cu** and its degradation products **FA** and **DHZ** was stronger at the higher concentration of 10 mM, for both reaction times and in both solutions. We did not observe any effect of concentration of **Va** immediately after spraying with acetone solution of DPPH• or even several minutes after spraying. Otherwise, the brightness of the spots of **Va** at 10 mM after 10 min was the same in both solvents (Fig. 1).

Chain-breaking antioxidant activity

Figure 2 presents the experimental results of TGSO autoxidation kinetics in presence of **Cu** and its degradation products: **FA**, **Va** and **DHZ** separately and in mixtures. The highest activity was obtained for **Cu** as individual component. In this study **Va** did not show any inhibitory activity, i.e. its behavior was analogous to that of the control sample. Usually, the lack of electron-donating group (EDG) in *ortho*- or *para*-position towards the phenolic group in the structures of some phenols, including phenol itself, is the reason for the absence of antioxidant properties. The presence of an electron-withdrawing group (EWG) in the **Va** structure, particularly in *para*-position to the phenolic OH-group, increases bond dissociation enthalpy (BDE) of the latter and also hampers the radical stabilization even though the presence of a guaiacyl unit (2-methoxyphenol) is generally considered beneficial for antioxidant activity. Our results, obtained for the antioxidant activity of **Va**, are in agreement with those obtained by other authors proving the fact that **Va** and vanillic acid do not exert high activity toward neutral radicals like DPPH$^{\bullet}$ or LOO$^{\bullet}$ in nonpolar medium [26-29]. According to Wang *et al.* [10] **Va** becomes the major degradation product with increasing the incubation time in buffer solution at 37°C. In fact, there are a number of studies in the literature reporting results for antioxidant activity of **Va** in hydrophilic or ionizing systems [29-33]. Probably, in such an environment, **Va** is involved in reactions with free radicals, different than the classical hydrogen atom transfer. The latter is assumed to be the main mechanism in autoxidation process in homogeneous lipid medium and the lack of activity of **Va** in TGSO oxidation is in accordance with what we have observed with TLC DPPH rapid test (after 1 min reaction time) in acetone (Fig. 1A).

FA and **DHZ** exerted weak or moderate antioxidant activity, as it is expected for monophenols, depending on their concentration being used [18, 34].

A



B

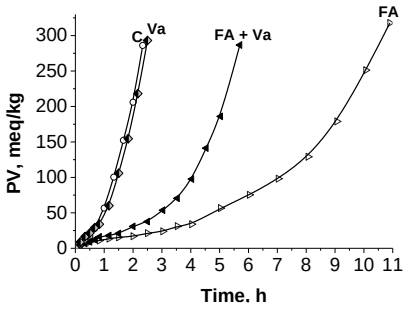


Figure 2. Kinetic curves of lipid peroxide accumulation during TGSO autoxidation at 80 ºC in absence (control, C) and in presence of studied compounds.

Table 1 presents the main kinetic parameters characterizing the TGSO autoxidation at 80 ºC in presence of the studied compounds. Then we compared the kinetic parameters of **Cu** with **FA**, **Va** and **DHZ** in equimolar ratio at 0.5 mM and 0.33 mM as binary (1:1) and ternary (1:1:1) mixtures, respectively.

Table 1. Kinetic parameters characterizing TGSO autoxidation at 80 ºC in presence of studied compounds.

Compound	Conc., M	IP_A, h	PF-	R_A, 10^{-6}M/s	ID	Activity
Curcumin (Cu)	1.0	22 ± 2.0	16.9	0.3 ± 0.02	15.7	Strong
Vanillin (Va)	1.0	1.3 ± 0.2	1.0	4.7 ± 0.5	1.0	No activity
Ferulic acid (FA)	1.0	7.5 ± 0.9	5.8	0.7 ± 0.2	12.6	Moderate
Va + FA (1:1)	0.5	4.2 ± 0.5	3.2	2.6 ± 0.3	1.8	Weak
Va + FA + DHZ (1:1:1)	0.33	3.5 ± 0.4	2.7	1.9 ± 0.3	2.5	Weak

*Kinetic parameters of **DHZ** characterizing its chain-breaking antioxidant activity was studied and described in our earlier publications [17, 18].

As an individual component **FA** inhibits the lipid oxidation process to a greater extent than in the cases when it is in mixtures. It could be seen from the results (Fig. 2, Table 1) that when **FA** is in a binary mixture with **Va** and in a ternary mixture with **DHZ** and **Va**, only weak activity was observed, much weaker than in the case of **Cu** at 1.0 mM as individual component.

However, the C2-symmetric dimer of **DHZ** prepared by us previously [18] demonstrated the same antioxidant activity as **Cu**. The latter evidence and the results obtained in the present study

encourage searching new dimeric structures in which the most important features of **Cu** or its metabolites are combined. Such compounds probably will be more active as individual components than their corresponding monomers when used in mixtures.

CONCLUSION

In this study a comparison between the antioxidant activity of **Cu** and that of its degradation products (**FA**, **Va**, and **DHZ**) at physiological conditions was assayed in bulk lipid autoxidation as individual components and in equimolar binary and ternary mixtures.

Cu showed the best antioxidant activity whereas weak activity was observed when its degradation products were studied in mixtures. **Va** did not show any activity, i.e. its behavior was analogous to that of the control sample. **FA** and **DHZ** binary mixture (1:1) showed very weak effect, weaker than that detected when both compounds were tested as individual components.

REFERENCES

1. S. Prasad, B. B. Aggarwal, Turmeric, The Golden Spice: from Traditional Medicine to Modern Medicine, in: Herbal Medicine: Biomolecular and Clinical Aspects, I. F. F. Benzil, S. Wachtel-Galor (eds.), Boca Raton (FL): CRC Press Taylor&Francis Group, 2011, p. 263.
2. R. L. C. Barclay, M. R. Vinqvist, K. Mukai, H. Goto, Y. Hashimoto, A. Tokunaga, H. Uno, *Org. Lett.*, **2**, 2841 (2000).
3. Y.-M.Sun, H.-Y. Zhang, D.-Z Chen, C.-B. Liu, *Org. Lett.*, **4**, 2909 (2002).
4. K. I. Priyadarsini, D. K. Maity, G. H. Naik, M. S. Kumar, M. K. Unnikrishnan, J. G. Satav, H. Mohan, *FreeRadic. Biol. Med.*, **35**, 475 (2003).
5. G. Litwinienko, K. U. Ingold, *J. Org. Chem.*, **69**, 5888 (2004).
6. M. Foti, A. Slavova-Kazakova, C. Rocco, V. D. Kancheva, *Org. & Biomol. Chem.*, 14, 8331 (2016).
7. B.B. Aggarwal, B. Sung, *Cell Press*, **30**, 85 (2009).
8. S. Angelova, L. Antonov, *Chem. Select.*, **2**, 9658 (2017).
9. C.Schneider, O. N. Gordon, R. L. Edwards, P. B. Luis, *J. Agric.Food Chem.*, **63**, 7606 (2015).
10. Y.-J. Wang, M.-H.Pan, A.-L.Chenq, L.-I.Lin, Y.-S.Ho, C.-Y.Hsieh, J.-K.Lin,*J. Pharm.Biomed. Anal.* **15**, 1867 (1997).
11. Y. Oda, *Mutat. Res*., **348**, 67 (1995).
12. Sreejayan, M. N. A. Rao, *J. Pharm. Pharmacol.*, **46**, 1013 (1994).
13. B. Joe, B. R. Lokesh, *Biochim. Biophys.Acta.*, **1224**, 255 (1994).
14. G. A. Hampannavar, R. Karpoormath, M. B. Palkar, M. S. Shaikh, *Bioorg. Med. Chem.*, **24**, 501 (2016).
15. Y. A. Mahmmoud, *Biochim. Biophys. Acta*, **1808**, 466 (2011).
16. A. V. Filippov, S. A. Kotenkov,B. V. Munavirov,A. V. Khaliullina, O. I. Gnezdilov, O. N. Antzutkin, *Mendeleev Commun.*, **26**, 109 (2016).
17. V. Kancheva, A. Slavova-Kazakova, D. Fabbri, M. A. Dettori, G. Delogu, M. Janiak, R. Amarowicz, *Food .*, **157**, 263 (2014).
18. A. K. Slavova-Kazakova, S. E. Angelova, T. L. Veprintsev, P. Denev, D. Fabbri, M. A. Dettori, M. Kratchanova, V. V. Naumov, A. V. Trofimov, R. F. Vasil'ev, G. Delogu, V. D. Kancheva, *Beilstein J. Org. Chem*, **11**, 1398 (2015).
19. V. D. Kancheva, L. Saso, P. V. Boranova, A. Khan, M. K. Saroj, M. K. Pandey, S. Malhotra, J. Z. Nechev, S. K. Sharma, A. K. Prasad, M. B. Georgieva, C. Joseph, A. L. DePass, R. C. Rastogi, V. S. Parmar, *Biochemie*, **92**, 1089 (2010).
20. K. R. Prabhakar, V. P. Veerapur, P. Bansal, V. K. Parihar, K. M. Reddy, A. Barik, B. Kumar, D. Reddy, P. Reddanna, K. I. Priyadarsini, M. K. Unnikrishnan, *Bioorg. Med. Chem.*, **14**, 7113 (2006).
21. N. D. Yordanov, *Appl. Magn. Reson.*, **10**, 339 (1996).
22. V. D. Kancheva, *Eur. J. Lipid Sci. Technol.*, **111**, 1072 (2009).
23. E. N. Frankel, Lipid oxidation. Dundee, Scotland: The Oily Press, 1998.
24. E N. V. Yanishlieva-Maslarova, Inhibiting oxidation, in: Antioxidants in food. Practical applications. J. Pokorny, N.Yanishlieva, M. Gordon (eds.), Boca Raton: CRC Press. Cambridge, UK, Woodhead Publishing Ltd, 2001, p. 22.
25. T. Denisov, T. G. Denisova, *Chem. Rev.*, **78**, 1129 (2009).
26. O. T. Kasaikina, V. D. Kortenska, E. M. Marinova, I. F. Rusina, N. V. Yanishlieva, *Russ. Chem. Bull.*, **46**, 1070 (1997).
27. W. Brand-Williams, M. E. Cuvelier, C. Berset, *Lebensm.-Wiss. Technol*., **28**, 25 (1995).
28. N. Nenadis, H.-Yu. Zhang, M.Z. Tsimidou, *J. Agric. Food Chem*., **51**, 1874 (2003).
29. A. Tai, T. Sawano, F. Yazama, H. Ito, *Biochim. Biophys. Acta* - General Subjects, **1810**, 170 (2011).
30. Y. Murakami, A. Hirata, S. Ito, M. Shoji, S. Tanaka, T. Yasui, M. Machino, S. Fujisawa, *Anticancer Res.*, **27**, 801 (2007).
31. E. J. Better, A. Davidson, *Oil and Soap*, **23**, 245, 283 (1946).
32. J. P. Kamat, A. Ghosh, T. P.A. Devasagayam, *Mol. Cell Biochem.*, **209**, 47 (2000).
33. K. S. Santosh, K. I. Priyadarsini, K. B. Sainis, *Redox. Rep.*, **7**, 35 (2002).
34. M. Karamac, L. Koleva, V. D. Kancheva, R. Amarowicz, *Molecules*, **22**, 527 (2017).

СРАВНИТЕЛНО ИЗСЛЕДВАНЕ НА АНТИОКСИДАНТНИЯ ПОТЕНЦИАЛ НА КУРКУМИН И НЕГОВИ РАЗПАДНИ ПРОДУКТИ – ВАНИЛИН, ФЕРУЛОВА КИСЕЛИНА И ДЕХИДРОЦИНГЕРОН

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(Резюме)

Куркуминът е един от най-интензивно изследваните биологично активни антиоксиданти през последното десетилетие. Неговата нестабилност и бързо разпадане при физиологични условия (pH ≈ 7) е едно от основните ограничения, свързани с потенциалните му приложения. Предприети са редица подходи за преодоляване на проблема с бионаличността на куркумина, включително използването на пиперин, липозоми, наночастици, фосфолипидни комплекси и дизайн на нови структурни аналози на куркумин. От друга страна, разграждането на съединението не води непременно до загуба на неговата активност. Дискутира се дали и как пътищата на разграждане и окисляване допринасят за биологичните и антиоксидантните активности на куркумина. Целта на това изследване е да се сравни антиоксидантната активност на куркумина с тази на неговите продукти на разграждане (ферулова киселина, ванилин и дехидроцингерон), когато те се добавят в би- и трикомпонентни (тройни) антиоксидантни смеси. Липидното автоокисление се използва за оценка на ефективността на антиоксиданта и на реактивността на фенолите. Получените резултати показват много по-силна активност на куркумина, в сравнение с тази на което и да е от разпадните съединения и техните двойни и тройни смеси.

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Phenolics content and antioxidant activity of beverages on the Bulgarian market – wines, juices and compotes

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In wine, natural juices and compotes the taste is heavily influenced by the presence of phenolics. They also contribute to the antioxidant activity of fruits and processed foods from them and have health-protecting effect. The phenolic content and antioxidant activity are not included in the standard documentation for food labeling and control. The aim of the present work was to analyze and to compare the content of total phenolics and anthocyanins, as well as the antioxidant potential of alcoholic and non-alcoholic fruit drinks on the Bulgarian market. Commercial natural fruit juices, compotes, red, rosé and white wines on the Bulgarian market were used for testing total phenolics, anthocyanins and antioxidant activity. Among the tested alcoholic drinks the red wines revealed significantly higher polyphenolic (567±33 mg/L) and AC (97.9±40.7 mg/L) content *vs* rosé (323±84 mg/L, $p<0.0001$; 9.9+8.2 mg/L, $p<0.01$) and white (281±42 mg/L, $p<0.0001$; 0.2±0.1 mg/L, $p<0.001$) wines. In the group of the red wines, the highest polyphenolic content (625±13 mg/L) was detected in Merlot wine, and the lowest one in Syrah (534±20 mg/L). The wine Malbec was found to be the richest one in anthocyanins (156.6±1.5 mg/L), while in Mavrud the anthocyanins content was the lowest one (45.2±1.0 mg/L). The tested red wines showed high antioxidant activity, especially strong in Aronia wine (45.55±0.35 mM uric acid equivalent, UAE). In the tested non-alcoholic drinks, the highest polyphenolic content was found in Aronia juice (592±9 mg/L) and compote (556±62 mg/L). The red wines and compotes have been an element of traditional nutrition in Bulgaria and nowadays their input in the healthy diet is reassessed because of their high phenolics content and strong antioxidant potential.

Keywords: Polyphenols, Anthocyanins, Antioxidant capacity, Wine, Compote, Juice

INTRODUCTION

Polyphenolics are the most abundant and widespread compounds in the plant kingdom. They are a product of the secondary plant metabolism and can be found in all plant parts located in the hydrophilic intracellular compartment and in the extracellular fluids. There are currently more than 8,000 phenolic structures, with a common feature - an aromatic ring bound to at least one hydroxyl group (-OH) [1] Phenolics are classified into three important groups: phenolic acids, flavonoids and tannins.

A sub-class of flavonoids are anthocyanins - water-soluble natural pigments responsible for the red, purple and blue colors of the fruits and their products [2]. Depending on acidity of the media, AC exist in various chemical forms with different coloration. They are stable under acidic conditions, and under normal processing and storage they can be transformed to colorless compounds and subsequently to insoluble brown pigments. In the plants AC are present mostly as glycosides, more stable than their non-glycosylated forms (anthocyanidins), which contributes to their resistance to destroying factors such as light, pH and oxidation [3].

Plant polyphenolics are redox-active substances possessing antioxidant activity. Numerous studies have proven causal links between the high polyphenolic content and antioxidant activity. [4, 5]. Due to their antioxidant properties it is believed that they have health -promoting properties including antibacterial, anti-mutagenic, anti-inflammatory and vasodilatory actions [6]. For example, AC, as very efficient antioxidants, play multiple biologic roles such as anti-inflammatory action, inhibition of blood platelet aggregation and antimicrobial activity, treatment of diabetic retinopathy and prevention of cholesterol-induced atherosclerosis [7, 8]. Flavonoids have also been shown to exhibit powerful antioxidant activities and health promoting effects. They act as free radical scavengers and transition metal chelators. Experimental studies have proven that due to their antioxidant effect they can lower oxidative products such as protein carbonyls, DNA base damage and malonaldehyde in blood and in tissues of experimental animals [9].

Wines and fruit juices represent an excellent source of dietary polyphenols. Red wines may contain from 1000 to 4000 mg/L, rosé 500-700 mg/L, and white wines from 200-300 mg/L [10], chokeberry fresh juices from 3002 to 6600 mg/L

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total phenolics [11]. The phenolic compounds present in wines and natural juices strongly contribute not only to their sensory characteristics but also to the antioxidant activities that they possess [10]. Wine phenolics react with free radicals, which makes them potent antioxidants both in wine and *in vivo*. Phenolics in wines are recently attributed to have protective activity against neurodegenerative diseases [12]. The "French Paradox" was also explained with high polyphenolic content and antioxidant activity of red wines.

Traditionally in Bulgaria fruits are used as dried food, in the form of juices, syrups, jams, compotes and for wine production. The compotes are traditional Bulgarian fruit drinks. To our knowledge there are no studies on the phenolic content and antioxidant properties of compotes.

Nowadays, with the rise of economy, commercial fruit juices have been proposed as substitutes of fresh ones due to their convenience. However, there are very few data about their phenolic levels and antioxidant properties.

Bulgarian local wines are produced from native and also from the so-called 'international' cultivars, including Cabernet Sauvignon, Merlot, Syrah, Grenache, etc. There is a considerable lack of information with regard to polyphenolic composition of Bulgarian red, white and rosé wines and as a result, their antioxidant capacity related to polyphenolic content.

The aim of the present study was to analyze and to compare the content of total phenolics and anthocyanins, as well as the antioxidant potential of alcoholic and non-alcoholic fruit drinks on the Bulgarian market.

EXPERIMENTAL

Selection of alcoholic and non-alcoholic fruit drinks

For this study we selected the most important and representative commercial table wines available on the Bulgarian market. Red, white and rosé wines produced from native cultivars, five different types from each, were randomly selected from the Bulgarian market. Six red, five white and five rosé wines were chosen. The red wines included the cultivars Merlot, Syrah, Mavrud, Zelas, Malbec, and Aronia. From the rosé wines were chosen Pinot Gris, Syrah, Cabernet Sauvignon, Mavrud, and Grenache. The white wines were Sauvignon blanc, Muskat, Traminer, Dimyat, Mavrud, and Chardonnay. Six different types of natural fruit juices were chosen: "Strawberry", "Blueberry", "Forest fruit", "Sour cherry", "Black currant", and "Aronia". Compotes from five different types of fruits (red currant, strawberry, aronia, blackberry, and sour cherry) were used in the study.

All experiments were carried out in triplicate.

Determination of total phenolics

Total phenolics content was determined by the method of Folin-Ciocalteau with modifications [13, 14]. In brief, 20 µl of sample (wine, fruit juice, compote) was mixed with 1580 µl of deionized water and 100 µl of Folin-Ciocalteu phenol reagent. The solution was incubated for 5 min at room temperature and 300 µl of 7.5% Na_2CO_3 were added. After 2 h incubation in a dark place the optical density was measured at λ=765 nm against blank. The quantitation was done by the method of external calibration using gallic acid as a standard. Working standard solutions (600, 500, 400, 300, 200, 100, 50, 20 µg/ml) were prepared by dilution of the stock gallic acid standard solution. The results were presented in mg/L.

Determination of total monomeric anthocyanin pigments

For the determination of the content of total monomeric anthocyanin pigments in the tested samples the pH-differential method was used. The assay is based on the different coloration of anthocyanins with a change in pH. The colored oxonium form predominates at pH 1.0 and the colorless hemiketal form at pH 4.5. The optical density of each sample was measured at two wavelengths, 510 nm and 700 nm and at two pH values, 1.0 and 4.5 [14]. The appropriate dilution factor was determined until the absorbance of the sample at the λvis-max (510 nm) was within the linear range of the absorbance. Two dilutions of the sample were prepared, one with potassium chloride buffer, pH 1.0, and the other with sodium acetate buffer, pH 4.5, equilibrated at room temperature for 15 min. The optical density was measured at the λ 510 nm and at λ 700 nm. The concentration of monomeric anthocyanin pigment was calculated using the molar absorptivity and molar mass of cyanidine-3-glycoside. The results were expressed in mg/L.

Determination of antioxidant activity

The antioxidant capacity of samples was determined by decolorization of the stable ABTS radical-cation [15, 16]. The method was based on the depletion of the pre-formed $ABTS^{\bullet+}$ radical in the presence of potassium persulfate. The sample (10 µl) was added to 1 ml of $ABTS^{\bullet+}$ solution in PBS (pH 7.4). The optical density was read at 734 nm on zero time and on the 6^{th} minute after adding the sample against PBS as a blank. The quantitation was done by the method of external calibration using uric acid

as a standard. The results were expressed in mM UAE. The percentage decrease of the absorbance at 734 nm was calculated by the formula:

$A = [(A_{sample\ t=0min.} A_{sample\ t=6min.}) - [(A_{blank\ t=0min.} A_{blank\ t=6min.})$

RESULTS AND DISCUSSION

Total polyphenolic content, anthocyanin concentration and antioxidant activity of the tested drinks are presented in Figs. 1 and 2.

Fig. 1. Total phenolic content of wines, natural fruit juices and compotes from the Bulgarian market. TP – total phenolics

Fig. 2. Total monomeric anthocyanin content of wines, natural fruit juices and compotes from the Bulgarian market. AC – total monomeric anthocyanins

The highest polyphenolic content was established for the red wines, followed by natural fruit juices, compotes, rosé wines, and white wines. Among the tested alcoholic drinks the red wines revealed significantly higher average polyphenolic content (567±32 mg/L) *vs* rosé (323±84 mg/L, $p<0.0001$) and white wines (281±42 mg/L, $p<0.0001$). The natural fruit juices and compotes revealed similar polyphenolic content 470±99 mg/L *vs* 421±139 mg/L, respectively (Fig. 1).

The highest total monomeric anthocyanin content was established in the red wines (97.9±40.7 mg/L), followed by compotes (47.4±42.2 mg/L), natural fruit juices (36.1±16.4 mg/L), rosé wines (9.9±8.2 mg/L). The white wines were with the lowest content of anthocyanins 0.2±0.1 mg/L (Fig. 2).

Regarding the antioxidant capacity of the tested drinks, the red wines represent the highest average antioxidant capacity (24.50±11.30 mM) followed by the compotes (6.30±1.40 mM UAE), natural juices (5.30±1.90 mM), white wines (4.00±0.40 mM), and rosé wines (3.50±0.80 mM) (Fig. 3).

Fig. 3. Antioxidant capacity of wines, natural fruit juices and compotes from the Bulgarian market. TAC – total antioxidant capacity

The polyphenolic content of the different wine cultivars, natural fruit juices and compotes is presented in Table 1.

In the group of the red wines, the highest polyphenolic content (625±13 mg/L) was detected for Merlot wine, and the lowest for Syrah (534±20 mg/L). The wine Malbec was found to be the richest one in anthocyanins (156.6±1.5 mg/L), while in Mavrud their concentration was the lowest one (45.2±1.0 mg/L). The tested red wines showed high antioxidant capacity, especially strong for aronia wine (45.54±0.35 mM UAE). Among the non-alcoholic drinks, the highest polyphenolic content was found for aronia natural juice (592±9 mg/L) and compote (556±62 mg/L)

The established high content of total polyphenols and anthocyanins found in aronia drinks is consistent with the results of Nvenuti *et al.* [17] who detected high polyphenolic and anthocyanin content in non-alcoholic aronia drinks.

Surprisingly, a weak correlation between the anthocyanin content and the antioxidant capacity was found in the red wines Mavrud and Malbec. This is probably due to the fact that the pH differential method used in our study is selective for monomeric anthocyanins only. According to Arnous *et al.* [18] polymeric anthocyanin pigments are likely to contribute to the overall antioxidant capacity of the drink.

Aronia compote, aronia juice and strawberry juice revealed low antioxidant capacity, while their polyphenolic content was high. At the same time all cultivars red wines showed the opposite relationships. Moreover, we found a strong positive correlation between anthocyanin content and antioxidant capacity for rosé wines ($r=0.965$, $p<0.01$). Possible explanation is that the technologies for producing wines, juices, and compotes are quite different. The wine does not undergo heat treatment while the juices and the compotes are pasteurized or sterilized at high temperature. Changes that occur during their thermal treatment and storage may lead to significant losses of anthocyanins and low antioxidant capacity [19-21]. In support of this assumption are also the results

for aronia wine, juice and compote. We found high total polyphenolic and anthocyanin content for these three drinks, but only the aronia wine showed high antioxidant capacity - 45.54 mM UAE *vs* 8.28 mM UAE for the juice and 6.00 mM UAE for the compote.

Table 1. Phenolic composition and antioxidant capacity of alcoholic and non-alcoholic fruit drinks from Bulgarian market

Drinks	Type	Total phenolics [mg/L]	Total monomeric anthocyanins [mg/L]	TAC [mM UAE]
Red wine	Merlot	625±13	103.4±7.9	24.37±0.14
	Syrah	534±20	130.5±1.4	20.33±0.22
	Mavrud	544±42	45.2±1.	24.77±0.10
	Zelas	551±16	72.8±2.8	12.01±0.12
	Malbec	571±2	156.6±1.5	20.23±0.13
	Aronia	576±9	79.2±1.3	45.54±0.35
Rosé wine	Pinot Gris	283±14	0.6±0.1	3.04±0.06
	Syrah	250±7	19.6±0.7	4.52±0.12
	Cabernet Sauvignon	391±29	17.3±0.3	4.18±0.12
	Mavrud	433±4	5.4±0.1	3.08±0.01
	Grenache	257±15	6.4±0.3	2.79±0.02
White wine	Sauvignon blanc	255±10	0.1±0.01	4.08±0.01
	Muskat	304±21	0.5±0.3	3.52±0.10
	Traminer	223±14	0.1±0.05	4.20±0.13
	Dimyat and Mavrud	330±5	0.3±0.2	4.42±0.02
	Chardonnay	291±13	0.2±0.1	3.72±0.01
Natural fruit juices	Strawberry	508±4	27.5±3.6	5.82±0.06
	Blueberry	326±11	15.5±1.3	2.76±0.04
	Forest fruit	553±18	35.6±2.2	6.04±0.07
	Sour cherry	414±15	29.6±1.1	3.64±0.06
	Black currant	428±3	62.8±2.6	5.48±0.68
	Aronia	592±9	45.7±3/3	8.28±0.03
Compotes	Red currant	239±21	2.8±0.2	6.60±0.06
	Strawberry	308±8	23.0±1.2	8.52±0.17
	Aronia	556±62	113.0±2.5	6.00±0.03
	Blackberry	519±6	39.0±0.9	4.80±0.07
	Sour cherry	485±15	62.0±1.0	5.64±0.08

CONCLUSION

The red wines and compotes have been an element of traditional Bulgarian cuisine and nowadays their input in the healthy diet is reassessed, because of their high phenolics content and strong antioxidant potential.

REFERENCES

1. J. B. Harbone, in: Encyclopedia of plant physiology, secondary plant products, E. A. Bell , B. V. Charlwood (eds.), vol. 8, New York: Springer-Verlag, Berlin, Heidelberg, 1980, p. 674.
2. C.Manach, A.Scalbert, C. Morand, C. Rémésy, L. Jime´nez, *American Journal of Clinical Nutrition*, **79**, 727 (2004).
3. C. L. Zhao, Z. J. Chen, X. S. Bai, C. Ding, T. J. Long, F. G. Wei, K. R. Miao, *Molecular Diversity*, **18**, 687 (2014)
4. M. Pasheva, M. Nashar, D. Pavlov, S. Slavova, D. Ivanov, D. Ivanova,. *Science and Technologies*, ***3***, 123 (2013).
5. D. Ivanova, D. Gerova, T. Chervenkov, T. Yankova , *Journal of Ethnopharmacology*, **96**, 145 (2005).
6. L. K. Beh, Z. Zakaria, B. K. Beh , W. Ho, S. K. Yeap, N. B. Alitheen, *Journal of Medicinal Plants Research*, **6**, 5857 (2012).
7. D. Arslan, *Agronomy Research*, **13**, 892 (2015).
8. J. W. McClure, Physiology of flavonoids in plants, in: Plant Flavonoids in Biology and Medicine: Biochemical, Pharmacological, and Structure-Activity Relationships. Alan R. Liss, Inc., New York, 1986 , p.614.
9. G. G. Duthie, S. J. Duthie, J. A. M Kyle, *Nutrition Research Reviews*, **13**, 79 (2000).
10. N. Paixao, R. Perestrelo, J. C. Marques, J. S. Camara, *Food Chemistry*, **105** , 204 (2007).

11. M. Tolić, I.L. Jurčević, I. P. Krbavčić, K. Marković, N. Vahčić, *Food Technology and Biotechnology*, **53,** 171 (2015).
12. R. C. Minussi, M. Rossi, L. Bologna., L. Cordi, D. Rotilio, G. M. Pastore, *Food Chemistry*, **82**, 409 (2003).
13. V. L. Singleton, J .A. Jr. Rossi, *American Journal of Enology and Viticulture*, **16**, 144 (1965).
14. J. Whitaker, Current Protocols in Food Analytical Chemistry, 2001, p.1199
15. R. Re, N. Pellegrini, A. Proteggente, A. Pannala, M. Yang, C. Rice-Evans, *Free Radical Biology and Medicine*, **26**, 1231 (1999).
16. T. Chervenkov, D. Ivanova, D. Gerova, T. Yankova, *Annals of Rousse University*, **40,** 203 (2003).
17. S. Nvenuti, F. Pellati, M. Melegari, D. Bertelli, *Journal of Food Science*, **69**, 164 (2004).
18. A. Arnous, D. P. Makris, P. Kefalas, *Journal of Food Composition and Analysis* **15**, 655 (2002).
19. D. Georgiev; D. Ludneva, Acta Hortic., 2009, p. 825
20. D. Fracassetti, C. Del Bo, P. Simonetti, C. Gardana, D. Klimis-Zacas, S. Ciappellano, *Journal of Agricultural and Food Chemistry*, **61**, 2999 (2013).
21. D. Arslan, *Agronomy Research*, **13**, 892 (2015).
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ФЕНОЛНО СЪДЪРЖАНИЕ И АНТИОКСИДАНТНА АКТИВНОСТ НА НАПИТКИ ОТ БЪЛГАРСКИЯ ПАЗАР – ВИНА, СОКОВЕ И КОМПОТИ

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(Резюме)

Вкусът на вината, натуралните сокове и компотите се влияе силно от присъствието на феноли. Последните допринасят и за антиоксидантната активност на плодовете и продуктите от тяхната преработка и имат здравословно действие. Фенолното съдържание и антиоксидантният ификт не са включени в стандартната документация за етикетиране и контрол на храните. Целта на настоящата работа е да определи и сравни съдържанието на общи феноли и антоцианини (АС), както и антиоксидантния ефект на алкохолни и безалкохолни плодови напитки от българския пазар. Анализирани са търговски натурални плодови сокове, компоти, червени, розе и бели вина от българския пазар. Сред изследваните алкохолни напитки червените вина имат по-високо съдържание на полифеноли (567±33 mg/L) и АС (97.9±40.7mg/L) в сравнение с розе (323±84 mg/L, $p<0.0001$; 9.9+8.2 mg/L, $p<0.01$) и белите вина (281±42 mg/L, $p<0.0001$; 0.2±0.1 mg/L, $p<0.001$). В групата на червените вина най-високо полифенолно съдържание има мерло (625±13 mg/L), а най-ниско –сира (534±20 mg/L). Виното малбек е най-богато на антоцианини (156.6±1.5 mg/L), докато в мавруд съдържанието на антоцианини е най-ниско (45.2±1.0 mg/L). Изследваните червени вина проявяват висока антиоксидантна активност, особено виното от арония (45.55±0.35 mM UAE). В изследваните безалкохолни напитки най-високо полифенолно съдържание е установено в сок и компот от арония (съответно 592±9 mg/L и 556±62 mg/L). Червените вина и компотите са били елемент от традиционната храна в България и понастоящем техният принос към здравословната диета се преоценява поради високото им съдържание на общи феноли и силния антиоксидантен потенциал.

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Glycine-rich peptides from *Cornu aspersum* snail with antibacterial activity

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Antimicrobial peptides are a unique and diverse group of molecules that have a great potential for use in new antimicrobial drugs, as many of them have a pronounced cytotoxicity to number of multi-drug resistant bacteria. We have been investigating different mucus extracts from the garden snail *Cornu aspersum* against the pathogen Gram-negative bacterial strain - *Escherichia coli* NBIMCC 878 and it has been found that the fraction below 10 kDa demonstrated strong antibacterial activity. Using tandem mass spectrometry we identified the primary structures of 9 novel antimicrobial peptides with molecular masses between 1000-3000 Da in this fraction. Most of them contain high level of glycine and leucine residues into the amino acid sequences.

Keywords: antimicrobial peptides, *Cornu aspersum*, HPLC, *Escherichia coli*, antibacterial activity, tandem mass spectrometry.

INTRODUCTION

In April 2014, the World Health Organization announced the beginning of a post-antibiotic era and declared antimicrobial resistance a public health priority demanding global action [1, 2]. New therapies to tackle multidrug resistant bacterial pathogens are urgently needed. This set the need of discovering novel molecules that could battle pathogen drug resistance. Antimicrobial peptides have proven to be a good natural alternative to chemical antibiotics [2, 3]. Antimicrobial peptides (AMPs) are important components of the nonspecific host defense or innate immune system in a variety of organisms. Besides direct antimicrobial activity, AMPs carry immunomodulatory properties [4, 5], which make them especially interesting compounds for the development of novel therapeutics. They have been found virtually in all organisms and they display a remarkable structural and functional diversity. So far more than 750 different antimicrobial peptides have been isolated and characterized from different sources – insects, plants and animals as well as humans [6 - 9]. Even bacteria themselves produce antimicrobial peptides – 50 AMPs have been isolated from different Gram (+) bacteria, especially those that produce lactic acid [10]. Most AMPs commonly consisting of 10–50 amino acids are amphipathic and hydrophobic α-helical peptides, with a positively charged domain. These properties are thought to be essential for their biological activity, with the amphipathic character causing disruption of the negatively charged bacterial membrane [11]. This property helps them enter the lipid bilayer and bring about the lysis of the attacked membranes [12]. Most AMPs have the ability to kill microbial pathogens directly, whereas others act indirectly by modulating the host defense systems. In general, peptide therapeutics are considered to have advantages from the safety perspective compared to small molecule drugs since their degradation products are natural amino acids and, because of their short half-life, few peptides accumulate in tissues. This reduces the safety risk and risk of complications caused by metabolites [2].

It has been proven that AMPs can be used to battle Gram (+), Gram (-) bacteria as well as viruses, fungi and even cancer cells [13]. Several peptides from the hemolymph of molluscs and arthropods exhibit a broad-spectrum of antimicrobial activity [14, 15]. Recently, a series of active peptides and glycopeptides with different physiological functions were extracted from marine molluscs [14]. Several novel proline-rich antimicrobial peptides with molecular masses between 3000 and 9500 Da from the hemolymph of *Rapana venosa snails* also were identified [16]. Some of them showed strong antimicrobial activities against *Staphylococcus aureus* (Gram+) and low activity against *Klebsiella pneumonia* (Gram-) [16]. Furthermore, it has been reported for several peptides from the hemolymph of the garden snail *H. lucorum* and *H. aspersa* that exhibit a broad spectrum of antimicrobial activity against *S.*

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aureus, S. epidermidis and *P. acnes* [17, 18]. The cysteine-rich peptide mytimacin-AF from the *Achatina fulica* snail, composed of 80 amino acid residues, and the mytilins A and B from the hemocytes and plasma of the bivalve mollusc *Mytilus galloprovincialis*, composed of 40 residues, exert the strongest antimicrobial activity against *S. aureus* [19].

We here report on the antibacterial properties of mucus extract below 10kDa containing AMPs, isolated from the mucus of *Cornu aspersum* snail against the Gram-negative bacteria *Escherichia coli*. We focused on *E.coli* because it has the ability to cause serious diseases and bacteremia is increasing worldwide. This is why the multidrug resistance in *E.coli* is becoming a serious concern in global healthcare. Using tandem mass spectrometry we have determined amino acid sequences of 9 new peptides in active fraction.

EXPERIMENTAL

Materials and Methods

Mucus collection and separation of different fractions. The mucus was collected and purified from *Cornu aspersum* snails, grown in Bulgarian farms using patented technology without suffering any snail [20]. The mucus extract was separated to different fractions using Milipore filters (10, 30 and 50 kDa). Three mainly fractions were obtained: Fraction 1 (masses between 0-10 kDa), Fraction 2 (masses between 30 and 50 kDa) and Fraction 3 (masses above 50 kDa). Fraction 1 (below 10 kDa) was then further separated using Milipore filters (3 and 5 kDa) into three fractions: Fraction A (masses below 3 kDa), Fraction B (masses between 3 and 5 kDa) and Fraction C (masses between 5 and 10 kDa).

HPLC isolation and purification of peptides from active fraction. Fraction 1 (below 10 kDa,) was lyophilized and then applied on a Nucleosil C18 column, equilibrated with 0.1% trifluoroacetic acid (TFA, v/v) (solution A). Elution was performed with a linear gradient formed by solutions A (0.1% TFA/water) and solution B (100% acetonitrile in 0.1% TFA (v/v)) at a flow rate of 1.0 ml/min, over 75 min. Ultraviolet absorption was monitored at 216 nm. The eluted fractions were collected and dried by vacuum concentration Speed-110 vac. The fractions were reconstituted in Milli Q water containing 0.10% TFA (v/v).

Mass spectrometry analysis. The molecular masses of isolated fractions were measured by an Autoflex™III, High-Performance MALDI-TOF & TOF/TOF System (Bruker Daltonics) which uses a 200 Hz frequency-tripled Nd–YAG laser operating at a wavelength of 355 nm. Some 50 pmol of the HPLC fractions were dissolved in 0.1% (v/v) TFA and applied to the target. Analysis was carried out using α-cyano-4-hydroxycinnamic acid as a matrix. A total of 3500 shots were acquired in the MS mode and a collision energy of 4200 was applied. A solution of protein standards was used to calibrate the mass scale. The mass values assigned to the amino acid residues are average masses. *De novo* sequencing of the peptides was performed by MS/MS in a 4700 proteomics analyzer with TOF-TOF optics (Applied Biosystems). The MS/MS spectra were carried out in reflector mode with external calibration, using the 4700 calibration mixture kit (Applied Biosystems). Peptide *de novo* sequencing was performed by precursor ion fragmentation.

Antibacterial assays of the peptides. The Gram-negative bacterial strain *Escherichia coli* NBIMCC 8785 was used in the antibacterial assays. *Escherichia coli* (Migula 1985) Castellani and Chalmers 1919 was representative of bacteria from family *Enterobacteriaceae* and was obtained from the National Bank of Industrial microorganisms and cell cultures.

The bacterial strain *E. coli* NBIMCC 8785 was initially grown in Nutrient Agar I to restore the lyophilized strain. After that the microbial culture was inoculated in nutrient broth and cultivated for 24 hours to obtain microbial suspension. The suspension was used to receive standardized suspension.

Five cm^3 standardized suspensions from the bacterial cultures (OD_{430}=0.600 abs) was inoculated in Nutrient agar, mixed, and poured in petri dishes layers with depth 2 mm. The petri dishes remained at room temperature (20°C) to solidify. The antibacterial tests were also performed in wells with no peptides added to serve as a negative control.

Fractions (A, B and C) were tested via the agar well diffusion method. Three wells (3 repetitions) were drilled using a punch, and then each hollow was filled with 50 µl of each fraction. Incubation for 24-72 hours at 37°C was then performed. The antibacterial effect was indicated in mm sterile zone around the wells.

The antibacterial tests were also performed on a medium with no peptides added to serve as a negative control. The fractions were also tested by a diffusion method of dripping a certain volume (10 µl) of each fraction on a certain spot on an inoculated Nutrient agar medium. Incubation for 24-72 hours at 37°C was then performed Results and discussion

RESULTS AND DISCUSSION

Antimicrobial activity

The rapidly increasing resistance towards conventional antibiotics suggests that, without urgent action, we are heading for a "post-antibiotic era," in which the previously effective therapeutic strategies are no longer relevant [2].

Mucus extract collected from the garden snails *Cornu aspersum* is a complex mixture of biochemically and pharmacologically active compounds. The mucus extract of *Cornu aspersum* was subdivided into four fractions, obtained after separation over Milipone filters with a cut-off of 10, 5 and 3 kDa, as described in section " Materials and methods".

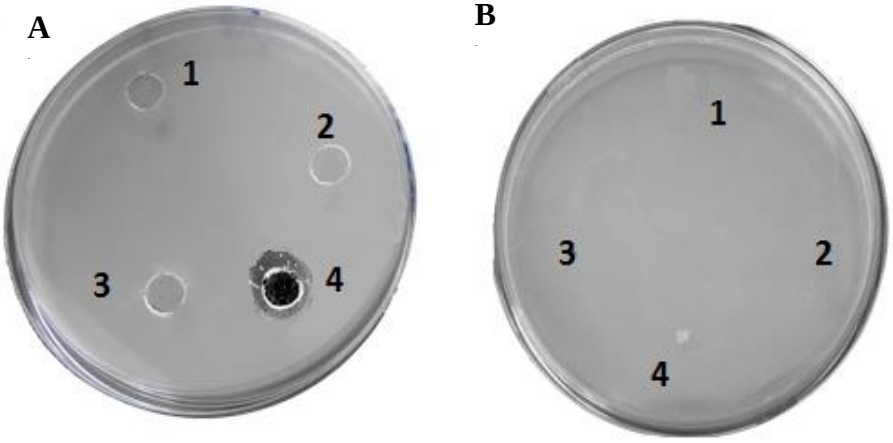

Fig. 1. A) Antimicrobial assays against Gram-negative *E.coli* of isolated fractions: A (spot 1), B (spots 2) and C (spots 3). Antimicrobial assays via agar well diffusion method against Gram-negative *E.coli* of isolated Fraction 1 (spot 4). Each fraction was applied on the agar medium in 50 µl of the peptide solution. **B)** No activity is shown in any of the fractions after dilutions (1/2, 1/4, 1/8 and 1/16) from the starting concentration.

The antimicrobial activity of fractions containing peptides, isolated from the mucus of garden snail *Cornu aspersum* were tested *in vivo*, against gram-negative bacterial strain *Escherichia coli* NBIMCC 8785. Upon testing their antimicrobial activity on agar medium after incubation for 24-72 hours at 37°C, in the agar well diffusion method only Fraction 1 appeared to generate a zone of 1,4 cm zone of inhibition of *E.coli* (Fig.1A spot 4).

The Gram-negative bacterial strain *Escherichia coli* NBIMCC 8785 was used in the antibacterial assays. It was chosen because it is a human pathogenic species and is commonly used in antimicrobial tests. To determine the minimal antimicrobial concentration of the active peptides, an aliquot of the *E.coli* strain was placed on Nutrient agar plates, mixed and allowed to solidify. Then the active fraction 1 (under 10 kDa) was tested by drilling a well in the agar using a punch and then each hollow was filled with 50µl of decreasing dilutions (1/2, 1/4, 1/8 and 1/8 from the starting concentration. Incubation for 24-48 hours at 37°C was then performed. It was found that the diluted peptide fraction 1 didn't show any activity meaning the initial concentration suppressed the growth of *E.coli* (Fig.1B).

These our results are preliminary testing the general effect of the isolated peptides. Further our efforts will be in direction to elucidate the mechanisms of the antibacterial effect in order to discover the best way for application of the peptides in pharmacy or cosmetics.

Characterization of peptides, containing in the fraction with antibacterial activity

Antibacterial active Fraction 1 (below10 kDa) was lyophilized and then applied on a Nucleosil C18 column, equilibrated with 0.10% trifluoroacetic acid (TFA, v/v) (solution A). Elution was performed with a linear gradient formed by solutions A (0.1% TFA/water) and B (100% acetonitrile in 0.1% TFA (v/v)) at a flow rate of 1.0 ml/min, over 75 min. Ultraviolet absorption was monitored at 216 nm. The eluted fractions were collected and dried by vacuum concentration. The fractions were reconstituted in Milli Q water. Additional purification of the isolated peptides was performed using the same equipment and conditions (Fig. 2). Mainly 23 sub-fractions were eluted and only a number of them were selected for further mass spectrometry analyses to determine their amino acid sequences in order to explain the antibacterial assessment results.

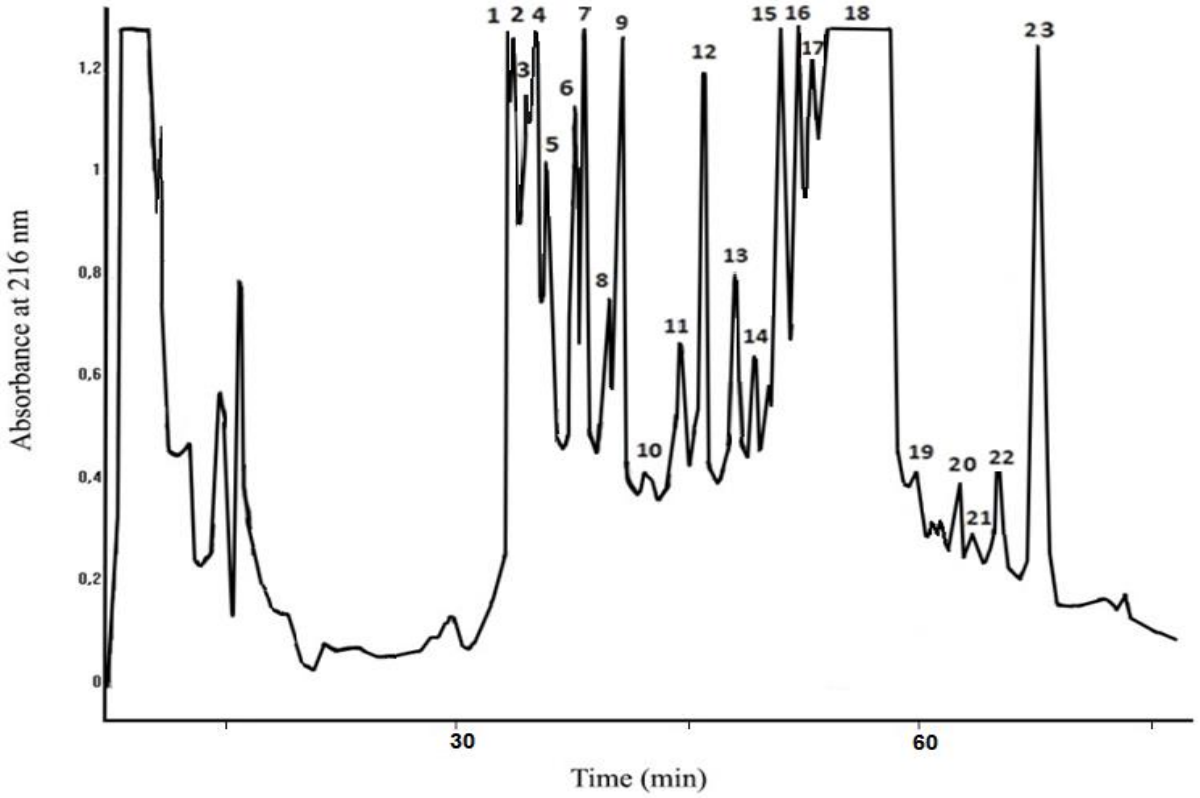

Fig. 2. RP-HPLC purification of the peptides with masses between 0-10 kDa residing in Fraction 1, to Nucleosil C18 RP-HPLC column (250 mm × 10 mm) with linear gradient of buffer A (0.1% TFA/water) and buffer B (100% acetonitrile in 0.1% TFA (v/v)) at a flow rate of 1.0 ml/min.

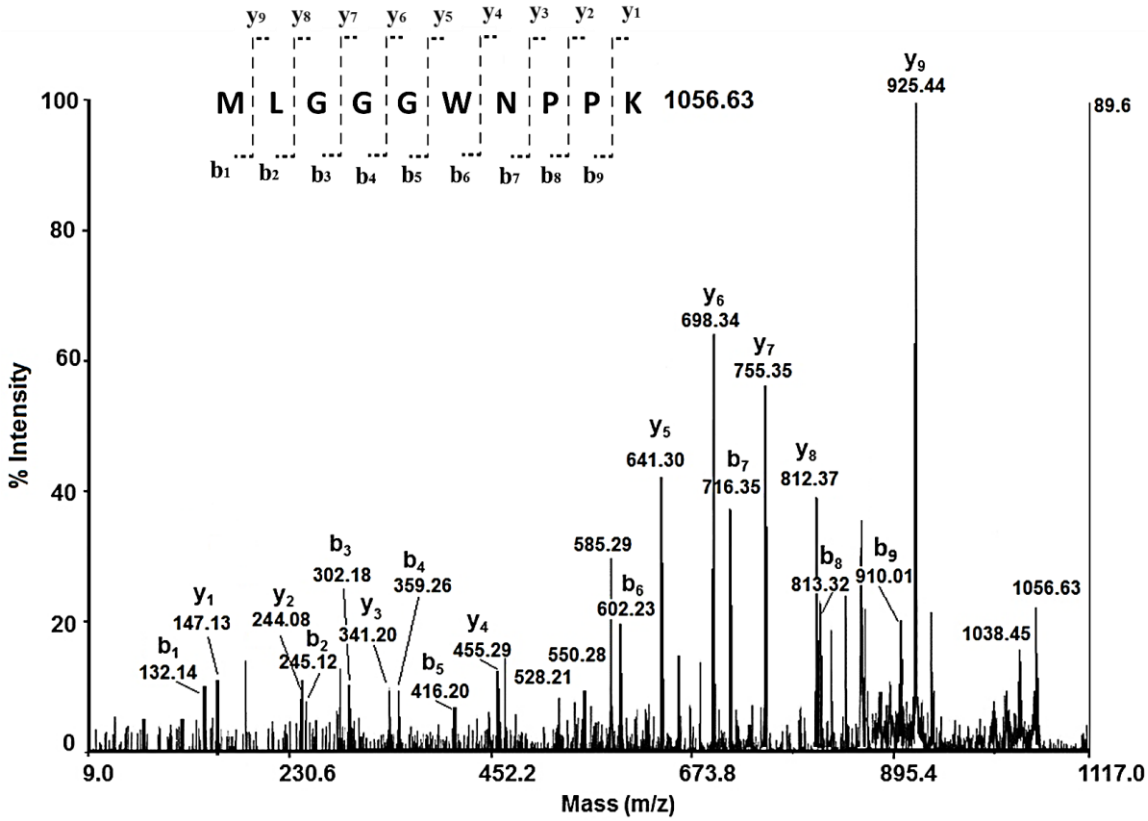

Fig. 3. MALDI-MS/MS spectrum of peptide at m/z 1056.63 Da (positive ion mode). Standard peptide solution was used to calibrate the mass scale of the AutoflexTM III, High Performance MALDI-TOF & TOF/TOF Systems (Bruker Daltonics).

Table 1. Amino acid sequences of selected peptides in Fraction 1, which shows antibacterial activity against negative bacterial strain *Escherichia coli* NBIMCC 8785.

№	Amino acid sequence of peptides	MALDI-MS $[M+H]^+$ (Da)	pI (calculated)	Mass peptide (Da) (calculated)
1	M**LGGGW**N**PPK**	1056.63	8.50	1055.52
2	ML**GGV****LGGGP**LK	1098.65	8.50	1097.63
3	M**GGLLGGGGV****GGG**SLVG**P**GA**P**	1666.98	5.28	1665.85
4	LF**GGH**Q**GGGL**V**GG**L**W**RK	1738.99	11.00	1737.94
5	MGG**W**GG**LGGG**HN**GGW**M**PPK**	1853.06	8.52	1851.83
6	M**P**K**GGGL**V**GGLL**GD**W**MGHK	1910.08	8.37	1908.97
7	MVNLALV**GGLLGG**KCLA**P**AR	1953.08	9.50	1952.01
8	EN**LGGGL**V**GGLL**GG**W**FL**H**D**P**K	2136.24	5.32	2135.12
9	**H**AFDVAV**GGLLGGGG**A**GGGGL**V**GGGGLGGGG**A	2478.39	5.08	2477.24

Matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF/MS) was used to determine the molecular mass and sequence of the purified peptide. In the fraction with antibacterial activity, we determined 9 novel peptides with molecular masses between 1000-3000 Da (Table 1). Each of the peptides is detected in MS spectrum as the protonated molecular ions $[M+H]^+$ ion at m/z, respectively. These ions were selected for transmission through the first analyser, then fragmented in the collision cell and their fragments analysed by the second analyser to produce the following MS/MS spectrum. So, using tandem mass spectrometry, the primary structure of the peptides were identified by de novo MALDI-TOF-MS/MS sequencing experiments of the protonated molecule ions $[M+H]^+$ (Table 1) The amino acid sequence of peptide detected et m/z 1056,63 Da $[M+H]^+$, is shown in Fig. 2. Following the series of y- and b-ions from MALDI-TOF/MS/MS spectrum the sequence MLGGGWNPPK was deduced. The isoelectric points (pI) of the peptides were predicted by the ExPASy MW/pI tool program, (Table 1) [21]. In fraction1 were found six cationic peptides (№ 1, 2, 4, 5, 6 and 7) and three anionic peptides (№ 3, 8, 9). These peptides had a wide variety of structural motifs, but they belonged to two mainly groups AMPs. The first group is of α-helical antimicrobial peptides that usually contain an abundance of helix stabilizing residues such as alanine (A), leucine (L), and lysine (K). Often these peptides are not strictly α-helices and may contain an internal kink [22, 23]. In aqueous solutions these peptides are often unstructured but assume their amphipathic α-helical conformations when associated with a cell membrane or in a membrane mimetic environment. The second group AMPs is flexible rich in specific amino acids as such as proline (P), tryptophan (W), histidine (H), arginine (R), and glycine (G). Proline-rich AMPs, with a high content of Pro (P) and Arg (R) residues, are an important group of AMPs predominantly active against Gram-negative bacteria [24]. Our results for amino acid sequence of the peptides (Table 1) show that peptides No 1 and 5, contain composition –PPK to C -terminal end. Furthermore, peptides No 2, 3, 7 and 8 contain one Pro residue inserted into the sequences of α-helical to C-terminal region, only peptide No 6 - to N-terminal region. Probably, the biological activity is carried by the C-terminal region. Previous studies have shown that when Pro residues are inserted into the sequences of α-helical AMPs, the ability of these peptides to permeabilize the bacterial cytoplasmic membrane decreases substantially as a function of the number of Pro residues incorporated [25], and this could explain our results. On the other hand, the amino acid sequence sequences shown on Table 1 reveal a high content of glycine residues, especially for peptides № 2, 3, 4, 8 and 9. They belong to the class of AMPs, named the glycine-rich peptides. The percentage of glycine residues in these peptides varies considerably, from 10–30% in some species to more than 60% [26]. They are able to inhibit the growth of fungi and have been isolated and characterized from different taxonomic groups, including plants amphibians, and arthropods [26]. Recently, Lorenzini et al., reported for three isoforms of a novel glycine-rich AMP, named ctenidins from the hemocytes of unchallenged tarantula spider *Acanthoscurria gomesiana* [26]. They are also active against *E. coli* [26]. Another Gly-rich antimicrobial peptide is leptoglycin, isolated from skin secretion of

Leptodactylus pentadactylus. Leptoglycin was able to inhibit the growth of Gram-negative bacteria *Pseudomonas aeruginosa*, *Escherichia coli* and *Citrobacter freundii* [27]. The amino acid sequence of leptoglycin with high level of glycine (GLLGGLLGPLLGGGGGGGGGLL) (59.1%) and leucine (36.4%) containing an unusual central proline suggests the existence of a new class of Gly/Leu-rich antimicrobial peptides. Similar to leptoglycin, the amino acid sequence of peptide No 9 shows high level of glycine and leucine into its amino acid sequence.

CONCLUSION

We determined by mass spectrometry the primary structures of 9 novel antimicrobial peptides with molecular masses between 1000 and 3000 Da which are contained in the fraction showed strong antibacterial activity against the Gram-negative bacterial strain - *E. coli* NBIMCC 878. Identified peptides contain high level of glycine and leucine as well as one or two proline residues inserted into the sequences of α-helical to C-terminal region, only one peptide – to N-terminal region. Probably this is important for their α-helical structures and antimicrobial activity. Our results may be considered as basic information for further investigations on bioactive peptides from *C. aspersum* and their potential applications in therapy.

In an era where we have run out of most 'off-the-shelf' antibiotics and are critically running out of last-resort options, it is imperative to continue to develop and accurately evaluate alternative antimicrobials [2]. We are persuaded that peptides have enormous potential as future therapeutics.

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REFERENCES

1. World Health Organization. Antimicrobial Resistance: Global Report on Surveillance, (2014).
2. M. Totsika, *Curr. Med. Chem.*, **6**, 30 (2016).
3. S.H. Marshall, G. Arenas, *Electron. J. Biotechnol*., vol. 6, n.3, Valparaíso dic. (2003)
4. C. D. Fjell, J. A. Hiss, R. E. Hancock, G. Schneider, *Nat. Rev. Drug. Discov*., **11**, 37 (2012).
5. M. Mahlapuu, J. Håkansson, L Ringstad, C. Björn, *Front Cell Infect Microbiol*., **6**, 194 (2016).
6. E. Mendez, A. Moreno, F. Colilla, F. Pelaez, G.G. Limas, R. Mendez, F. Soriano, M. Salinas, C. de Haro, Eur. *J. Biochem*., **194**, 533 (1990).
7. F.J. Colilla, A. Rocher, E. Mendez, *FEBS Letters*, **270**, 191 (1990).
8. W. Liu, J.N. Hansen, *Appl. Environ. Microbiol.*, **56**, 2551 (1990).
9. D. Schnapp, C.J. Reid, A. Harris, *J. Pathol*., **186**, 99 (1998).
10. T. Luders, G.A. Birkemo, G. Fimland, J. Nissen-Meyer, I.F. Nes, *Appl. Environ. Microbiol.*, **69**, 1797 (2003).
11. Y. Shai. *Biochim. Biophys. Acta*, **1462**, 55 (1999).
12. M. R. Yeaman, N. Y. Yount, *Pharmacol Rev.*, **55**, 27 (2003).
13. K.V. Reddy, R.D. Yedery, C. Aranha, *Int. J. Antimicrob. Agents*., **24**, 536 (2004).
14. S. Conti, G. Radicioni, T. Ciociola, R. Longhi, L. Polonelli, R. Gatti, T. Cabras, I. Messana, M., Castagnola A. Vitali, *BBA – Biomembranes*, **1828**, 1066 (2013).
15. D. Defer, F. Desriac, J. Henry, N. Bourgougnon, M. Baudy-Floc'h, B. Brillet, P. Le Chevalier, Y. Fleury, *Fish Shellfish Immunol.*, **34**, 1439 (2013).
16. P. Dolashka, V. Moshtanska, V. Borisova, A. Dolashki, S. Stevanovic, T. Dimanov, W. Voelter, *Peptides*, **32**, 1477 (2011).
17. P. Dolashka, A. Dolashki, S. Stevanovic, W. Voelter, J. Van Beeumen, I*nt. J. Curr. Microbiol. App. Sci.*, **4,** 1061 (2015).
18. P. Dolashka,a. Dolashki, L. Velkova, S. Stevanovic, L. Molin, P. Traldi, R. Velikova, W. Voelter, *J. BioSci. Biotechnol.*, SE/ONLINE: 147(2015).
19. J. Zhong, W. Wang, X. Yang, X. Yan, R. Liu, *Peptides*, **39**, 1 (2013).
20. P. Dolashka, *BG Useful model* 2097, (2015)
21. http://web.expasy.org/compute_pi/
22. T. Ebenhan, O. Gheysens, H. G. Kruger, J. R. Zeevaart, M. Sathekge, *Biomed. Res. Int.*, **2014**, 867381 (2014).
23. I. Zelezetsky, A. Tossi, *Biochim. Biophys. Acta*, **1758**, 1436 (2006).
24. V. S. Paulsen, H. M. Blencke, M. Benincasa, T. Haug, J. J. Eksteen, O. B. Styrvold, M. Scocchi, K. Stensvåg, *PLoS One*., **8**, e53326 (2013).
25. L. Zhang, R. Benz, R.E. Hancock, *Biochemistry*, **38**, 8102 (1999).
26. D. M. Lorenzini, P.I. Jr. da Silva, A. C. Fogaça, P. Bulet, S. Daffre, *Dev. Comp. Immunol.*, **27**, 781 (2003).
27. J.C. Sousa, B. R. Ferto, E. A. Gois, N.C. Fontenele-Cardi, J. E. Jr. Honório, K. Konno, M. Richardson, M. F. Rocha, A. A. Camargo, D. C. Pimenta, B. A. Cardi, K. M. Carvalho, *Toxicon.*, **54**, 23 (2009).

БОГАТИ НА ГЛИЦИН ПЕПТИДИ ОТ ОХЛЮВ *Cornu aspersum* С АНТИБАКТЕРИАЛНА АКТИВНОСТ

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(Резюме)

Антимикробните пептиди са уникална и разнообразна група от молекули, които имат голям потенциал за използване в нови антимикробни лекарствени средства, тъй като много от тях имат силно изразена цитотоксичност към редица лекарствено-резистентни бактерии. Изследвахме различни екстракти от слузта градински охлюв *Cornu aspersum* срещу патогенния Грам-отрицателен бактериален щам - *Escherichia coli* NBIMCC 878 и установихме, че фракцията под 10 kDa проявява значителна антибактериална активност. Използвайки тандем масспектрометрия, ние идентифицирахме първичните структури на 9 нови антимикробни пептиди с молекулни маси между 1000-3000 Da в активната фракция. Повечето от тях имат високо съдържане на глицинови и левцинови остатъци в аминокиселинните си последователности.

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Antioxidative screening of fractions from the mucus of garden snail *Cornu aspersum*

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Antioxidative peptides have been utilized by various species to combat pathogenic microorganisms and stress. In the present study, the antioxidant potential of peptide fractions obtained from the mucus of the garden snail *Cornu aspersum* was evaluated. Bioactive compounds from the mucus of the garden snail were separated into four fractions with different molecular weight (MW): Fraction 1 (compounds with MW<5kDa), Fraction 2 (compounds with MW<10kDa), Fraction 3 (compounds with MW<20kDa) and Fraction 4 - in the region 10-30kDa. Three complementary test methods were employed for preliminary antioxidative screening, including measurement of the radical scavenging activity on the 1,1-Diphenyl-2-picrylhydrazyl free radical (DPPH), total antioxidant activity (ABTS method) and the inhibition of nitro blue tetrazolium (NBT) reduction by photochemically generated superoxide radicals ($^{\bullet}O_2^-$). The results demonstrated that the lower MW fraction of <5kDa exhibited better antioxidant potential compared to the others.

Key words: *Cornu aspersum*, Peptides, Mucus, Antioxidant activity, Mass spectrometry

INTRODUCTION

During the organism's metabolism, reactive oxygen species (ROS) or free radicals are naturally produced by oxidation reactions through breathing. These ROS include the superoxide anion radical ($^{\bullet}O_2^-$), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($OH^{\bullet}$) and can cause peroxidation of lipids, oxidation of proteins, damage to nucleic acids, enzyme inhibition, activation of programmed cell death (PCD) pathway and ultimately lead to cell death [1]. The cell defense system includes enzyme components that directly detoxify ROS. The enzymes superoxide dismutase (SOD), catalase (CAT), peroxidase and glutathione peroxidase are among the most important protection agents [2, 3]. Non-enzymatic antioxidants are reported as well, most common of which are glutathione, thioredoxin, ubiquinones, carotenoids and ascorbic acid [4-6]. Prevention or inhibition of oxidative effects are key functions of the system [7, 8]. The aim is to avoid the imbalance between oxidants and antioxidants in the cell, since this could lead to „oxidative stress" [9,10].

Antioxidants from natural origins have been found to possess the ability to effectively prevent the damage caused by ROS [11] and hence, there is a growing interest towards the discovery of novel natural antioxidant compounds, especially among naturally derived peptides.

Naturally derived peptides with antioxidant (AO) properties are well-known for their contributions to human health improvement through the prevention and treatment of non-communicable chronic degenerative diseases [12]. Also, AO peptides exert effective metal ion (Fe^{2+}/Cu^{2+}) chelating and lipid peroxidation inhibitory abilities in addition to having very few side effects. These features render them with potential properties as food processing additives [13]. It has been proposed that the majority of nutrient-based AOs act as chain-breaking AOs by stopping or slowing down the oxidative process once it has begun [14]. AO peptides can behave as sacrificial peptides by reacting with a radical before vital biomolecules are damaged or as donor AOs in which they are preferentially oxidized to stable products that cease the propagation of the radical chain reaction (i.e., uric acid, vitamins C, E) [14].

Terrestrial slugs and snails produce mucus which performs a variety of functions, including facilitating movement along the ground, communication and a non-specific, defensive response to physical or chemical irritation [15]. The excreted biological fluid is a rich source of bioactive natural compounds and is being commercialized in many skin care compositions

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and other formulations for the treatment of a number of skin ailments, for example wounds, burns, scars, keratosis, psoriasis, acne, wrinkles and age and skin damage [15, 16]. Moreover, snail mucus is well known for its anti-aging properties and widely investigated for antibacterial potency [17, 18]. In general, both of these properties are bound to the ability of the organism to cope with the generation of free radicals. However, to this date, there are no sufficient data on the antioxidant potency of the compounds in snail species mucus, a fact that opens a variety of perspective investigations in this aspect.

Herein, we present an initial antioxidant screening of different fractions derived from the mucus of the garden snail *Cornu aspersum*. The purified mucus was subdivided by molecular weight cut-off filters into fractions. Then the total antioxidant potential of the tested fractions was assessed by the DPPH and ABTS radical scavenging activity methods and the nitro blue tetrazolium (NBT) reduction assay. Based on the obtained results SO and CAT activities were evaluated as well.

MATERIALS AND METHODS

Purification of the fractions from the mucus of garden snail Cornu aspersum

The snails *Cornu aspersum* were collected in Bulgaria and the mucus was purified. After that the mucus from the snails was subjected to ultrafiltration on Millipore filters (5, 10 and 30 kDa) to obtain fractions with different molecular mass: Fraction 1 (compounds with MW < 5kDa), Fraction 2 (compounds with MW <10kDa), Fraction 3 (compounds with MW < 20kDa) and Fraction 4 (compounds with MW between 10-30kDa).

DPPH assay

Each extract was evaluated for the ability for electrons (or hydrogen atoms) donation by using the bleaching level of purple-colored solution of 1,1-diphenyl-2-picrylhydrazyl (DPPH) – a stable radical used as a reagent. This spectrophotometric assay was done according to the method of Murthy *et al.*, [19] with small modifications. A volume of 0.5 ml from each of the three fractions was added to 1 ml of DPPH (100 μM) solution in ethanol. The absorbance was read against a blank at 517 nm, after 30 min of incubation period at 37 °C. Inhibition of free radical DPPH in percent was calculated according to the formula:

$$\text{Inhibition\%} = (A_{blank} - A_{sample} / A_{blank}) \times 100,$$

where A_{blank} is the absorbance of the control reaction (containing all the reagents except the test compound) and A_{sample} is the absorbance of the test compound.

ABTS radical scavenging activity

Total antioxidant activity was measured by ABTS radical cation ($ABTS^{\cdot+}$) assay as well, using the method of Re *et al.* [20] with some modifications. Shortly, 12 hours prior to use, $ABTS^{\bullet+}$ was generated in a mixture between 7 mM stock solution of ABTS and 2.45 mM potassium persulfate at room temperature. The stock $ABTS^{\bullet+}$ solution was diluted with methanol to achieve absorbance of 0.7 ± 0.01 at 734 nm. *Cornu aspersum* samples were added in 1 ml volume to 2 ml of the $ABTS^{\bullet+}$ solution. The final absorbance was measured after 1 min at 734 nm. Caffeic acid and ascorbic acid were used as reference compounds.

Superoxide anion scavenging activity

Determination of superoxide anion scavenging activity was done by inhibition of NBT reduction by photochemically generated superoxide ($^{\bullet}O_2^-$) [21]. The reaction mixture contained 56 μM NBT, 0.01 M methionine, 1.17 μM riboflavin, 20 μM NaCN and 0.05 M phosphate buffer with a pH of 7.8. Superoxide presence was evaluated by the increase in absorbance at 560 nm at 30°C after 6 min of incubation from the beginning of the illumination. Caffeic acid was used as a reference substance applied in a concentration of 5 μg/ml. The tested fractions and the caffeic acid were assayed by quantities of 0.02 ml in triplicate (n=3).

Enzyme activity determination

SOD activity in the tested fractions from *Cornu aspersum* was determined by NBT reduction [21]. One unit of SOD activity was assigned as the amount of SOD required for inhibition of the reduction of NBT by 50% (A560) and was expressed as units per mg protein (U/mg protein).

CAT activity was evaluated by the method of Beers *et al.* [22] in which the decomposition of hydrogen peroxide was analyzed spectrophotometrically at a wavelength of 240 nm. One unit of catalase activity was determined as the amount of enzyme that decomposes 1 mmol of hydrogen peroxide per min at an initial hydrogen peroxide concentration of 30 mmol/L, at pH = 7.0 and 25°C. The specific activity was represented in units per mg protein (U/mg protein).

Protein content was measured by the Lowry procedure [23], using crystalline bovine albumin as a standard.

Determination of peptides by mass spectrometry analysis

MS was performed on an LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) equipped with a nanoelectron spray ion source. MS analysis was run in data-dependent acquisition using a top five method (i. e., the five most intense ions with a positive charge between 2 and 4 analyzed during survey scan were selected for fragmentation during each scan cycle). Survey scans were performed in the Orbitrap at a resolution of 60,000 with a scan range of 450–650 m/z (450–750 m/z for absolute quantification). Peptides were fragmented using collision-induced dissociation (normalized collision energy, 35%; activation time, 30 ms; isolation width, 1.3 m/z) with resulting fragment ions (MS/MS scans) analyzed in the linear ion trap. Dynamic exclusion was enabled for all runs (maximal number of masses excluded at each time point [exclusion list size] 500; duration of exclusion for each mass: 40s).The mass spectra were analysed using Xcalibur Qual Browser (Thermo Scientific).

RESULTS AND DISCUSSION

Purification and mass spectrometric peptide sequencing

Four fractions were isolated after ultrafiltration of collected mucus from the snails on Millipore filters: Fraction 1 (with MW <5kDa), Fraction 2 (with MW <10kDa), Fraction 3 (with MW <20kDa) and Fraction 4 (with MW between 10-30kDa). Mass spectrometric peptide analysis was performed by LTQ Orbitrap XL mass spectrometer and several peptides were identified as shown in figure 1A. Figure 1B depicts the mass fragmentation spectrum of LLMGPEV following b- and y-ions in MS/MS spectrum of peptide with m/z 379.71 $[M+H]^{2+}$.

Most of the peptides appeared as z=2 and the amino acid sequences of some of them were confirmed by their MS/MS spectra. Following b- and y-ions in the MS/MS spectrum shown in figure 1C, the primary structure LPDSWEPGGGG of the peptide with m/z 1071.56 $[M+H]^{+}$ was determined. Peptide sequencing performed by MS/MS spectra showed that Pro (P), Gly (G), Tyr (Y) and Trp (W) – rich peptides are present in the mucus (Table 1).

Antioxidant screenings

The screenings of the antioxidant properties of the mucus from *C. aspersum* started with evaluation of the total antioxidant activity. At first, the free radical scavenging activity was measured by the DPPH method that is usually used for different natural extracts. The effects of antioxidants on DPPH radical scavenging are thought to be due to their hydrogen-donating ability. The decrease in absorbance of DPPH radical caused by antioxidants was compared to the control, because of the reaction between antioxidant molecules and radical progressed, results in the scavenging of the radicals by hydrogen donation [24]. Based on the data obtained, Fractions 2 and 3 of MW <10 kDa and 10-30 kDa, respectively, demonstrated scavenging activity (Fig. 2).

The tested fractions were able to reduce the stable free radical DPPH to the yellow-colored 1,1-diphenyl-2-picrylhydrazyl. Fraction 2 with MW <10 kDa exhibited better free radical DPPH reduction to the yellow-colored 1,1-diphenyl-2-picrylhydrazyl. Moreover, this fraction exhibited better free radical scavenging activity than the others with higher MW.

Similar results have been reported for peptides derived from meat muscles [25] and protein hydrolysates of blue mussel, salmon, grass carp skin, etc. [26]. Therefore, the present results suggest that fractions with MW < 10kDa and 10-30kDa are DPPH• inhibitors and primary antioxidants that react with free radicals.

The total antioxidant activity of the tested mucus fractions was calculated by another complementary method - the decolorization of $ABTS^{+}$ in the reaction mixture. The results were expressed as percentage inhibition of absorbance.

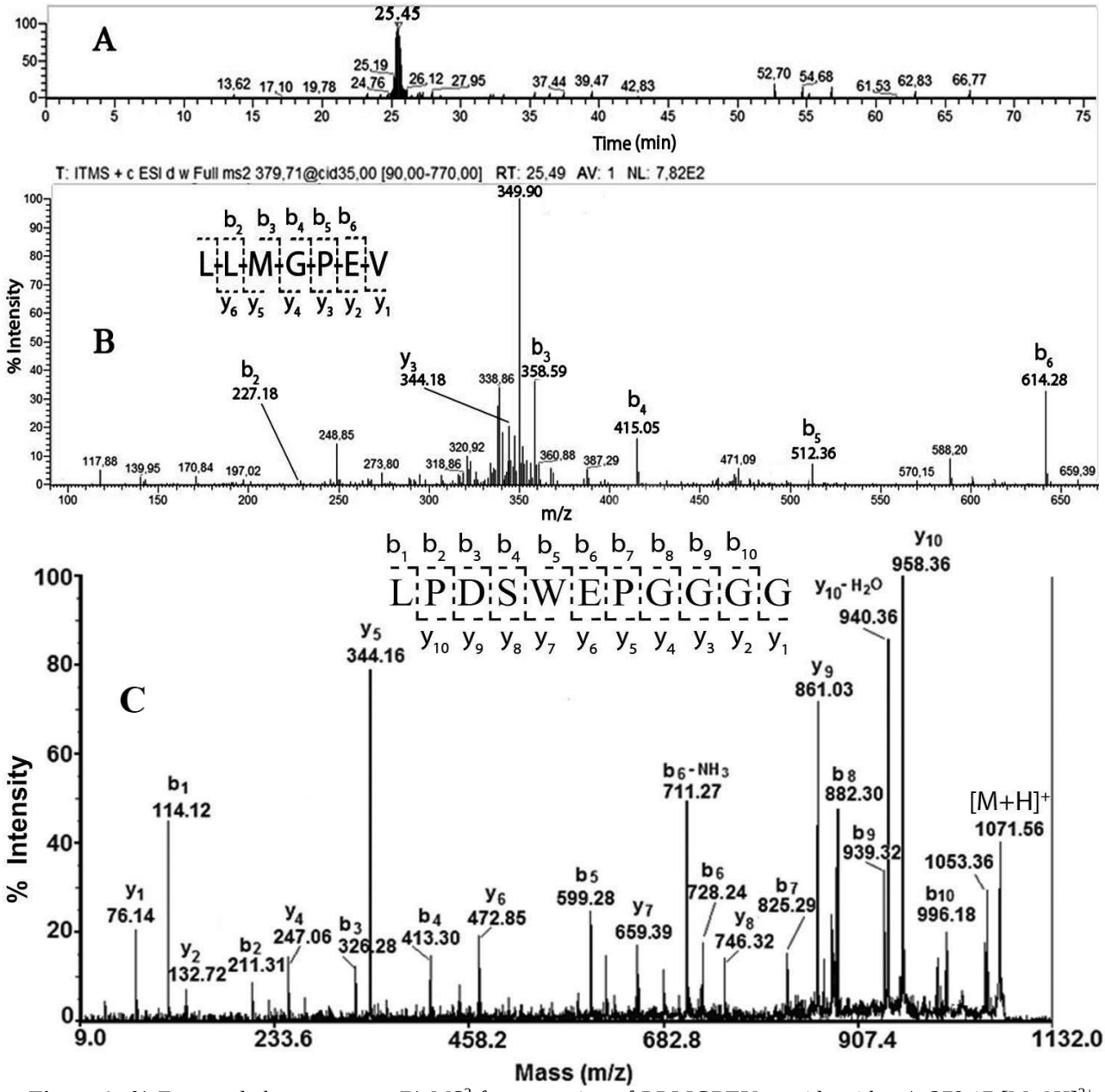

Figure 1. A) Extracted chromatogram, **B)** MS^2 fragmentation of **LLMGPEV** peptide with m/z 379.17 $[M+2H]^{2+}$, **C)** MALDI-MS spectrum (positive ion mode) of the peptide at m/z 1071.56$[M+H]^+$. Standard peptide solution was used to calibrate the mass scale of the AutoflexTM III, High Performance MALDI-TOF & TOF/TOF Systems (Bruker Daltonics).

Table 1. Some sequenced peptides from the mucus of *Cornu aspersum.*

Sequence	Modifications	Activation Type	Charge	m/z	$[M+H]^+$	ΔM [ppm]	RT [min]
LLMGPEV		CID	2	379.71	758.41	1.35	25.49
LmYQPP	M2(Oxidation)	CID	2	382.69	764.36	-0.28	5.22
GNGPTGLHmA	M9(Oxidation)	CID	2	485.72	970.44	-2.76	65.55
QSGKSPGFGL		CID	2	489.25	977.50	-2.63	45.50
LPDSWEPGGGG		CID	2	536.20	1071.56	-1.86	56.30
VGQGCDEmLQG	M8(Oxidation)	CID	3	384.83	1152.46	-1.74	79.64
YNGFRPGDCY		CID	3	397.83	1191.49	-1.94	69.68

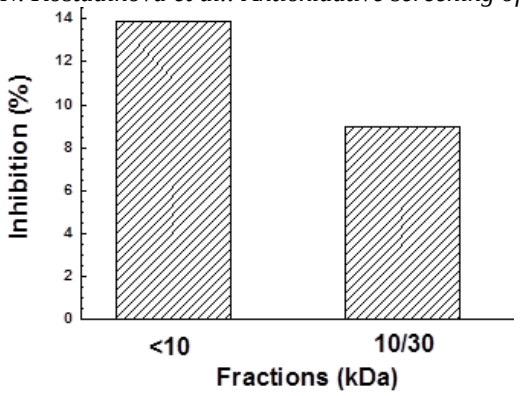

Fig. 2. DPPH scavenging power of Fraction 2 and Fraction 3 isolated from the extract from *C. aspersum*

The data in Fig. 3 clearly show the higher scavenging effect of the fraction with molecular mass below 20 kDa in comparison with the total extract. The values of the <20 kDa fraction were similar to those of the reference compounds vitamin C and caffeic acid. In addition, we evaluated the <5 kDa and 10-30 kDa fractions, which also expressed antioxidant activity by the $ABTS^{+}$ method. The results from Table 2 demonstrate two different dose-dependent trends. While the scavenging activity of the fraction of <5kDa slightly decreased with the increase in the protein concentration from 5 to 40 µg, the 10-30 kDa fraction demonstrated an opposite tendency. Concentration above 15 µg caused more than 20% increase in the antioxidant effect.

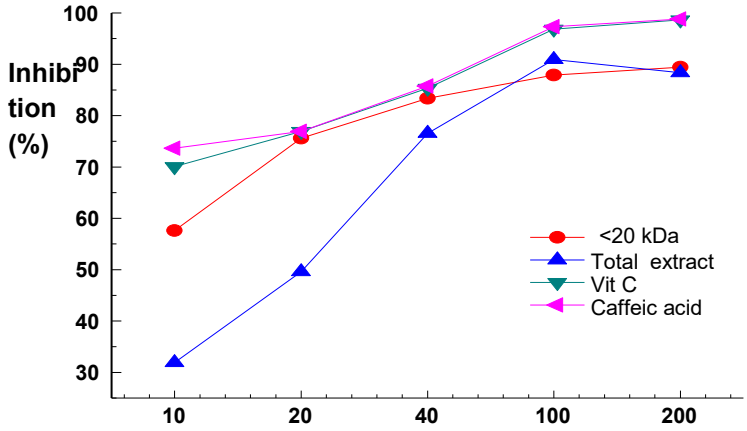

Fig. 3. Potential of the tested samples from *C. aspersum* for scavenging of ABTS radical.

Table. 2. Scavenging effect of *C. aspersum* according to the applied protein quantity.

Protein (µg)	Scavenging effect (%)	
	<5 kDa	10-30 kDa
5	82.42	70.00
10	75	72.92
15	67.55	83.96
20	67.27	84.,11
40	70.15	84.27

Moreover, the low-molecular fraction of <5 kDa was observed to have a more potent scavenging effect (82.42%) which was more strongly expressed at low concentrations than the others. Antioxidative peptides of <1 kDa from zein or *Struthio camelus* egg hydrolysate also displayed high ABTS activity [27, 28].

Further, the antioxidant effects of the *C. aspersum* snail extracts were evaluated by suppression of the superoxide anion radicals generated in a photochemical system in the presence of the test samples (NBT assay) as is shown in Fig. 4. The best $^{\bullet}O_2^-$ scavenging activity was shown by the fraction with MW below 5 kDa. These preparations inhibited the development of the color produced during the reaction of $^{\bullet}O_2^-$ with NBT by 57, 42, 40 and 39%, respectively. These radicals are known to be highly reactive, mainly because of their role in the formation of more powerful and dangerous hydroxyl radicals and singlet oxygen, both of which are involved in the appearance of oxidative stress events [29].

The reducing power of a natural compound may serve as a significant indicator of its antioxidant activity level. A study on *Giant African Snail (Achachatina maginata)* antioxidant activities reports that its hemolymph possesses antioxidant activity and significantly inhibits the acute liver toxicity induced by CCL4 in rats [30]. The authors suggest that the hepatoprotective activity of the

hemolymph may be due to its free radical scavenging and antioxidant activity, resulting from the presence of flavonoids and phenolic compounds in the hemolymph that enhance the regeneration ability of liver.

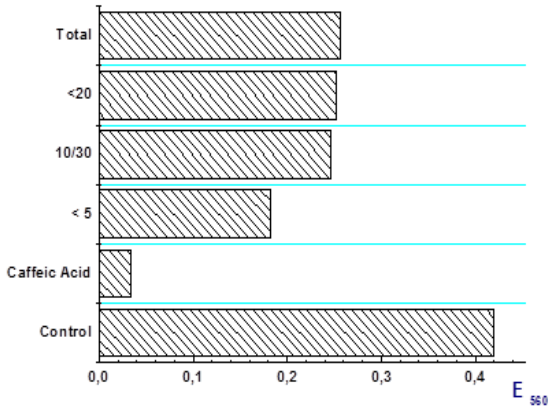

Fig. 4. Inhibitory effect of the fractions from *C. aspersum* on the reduction of NBT by photo-chemically generated superoxide anion radicals

The next step in the present investigation was the measurement of the potential SOD and CAT activities of the tested fractions. At first, SOD and CAT activity values were reported for the total mucus sample. These activities were low but they were detected in the total sample where high MW proteins exist (Fig. 5).

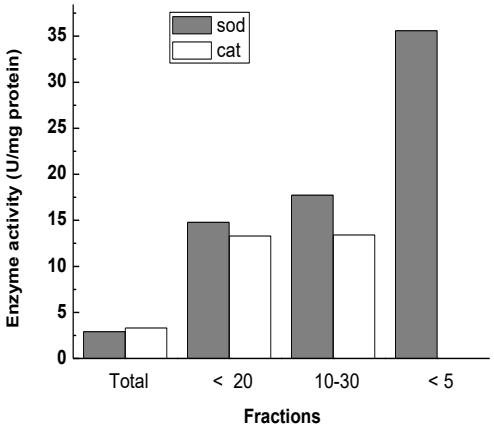

Fig. 5. SOD and CAT activities of the tested samples from ***C. aspersum***.

The fractions with MW <20 kDa and between 10-30 kDa demonstrated values similar to each other for both enzymes. The fraction of <5 kDa showed the highest SOD activity (of 35.6 U/mg protein), although there was no CAT activity detected. It is possible that the high SOD recorded in the latter fraction represents the so called "SOD-like activity" that could be displayed by some peptides with low molecular mass capable of superoxide scavenging. The real enzyme cannot be found in low-molecular fractions.

Over the last decade, various reports have been made for the possible use of snail slime in wound treatment. Brieva *et al.* [31] found that slime from *Cryptomphalus aspersa* (also known as *Helix aspersa* or the common garden snail) contains antioxidant superoxide dismutase and glutathione-s-transferase activity. As a whole, a few studies concentrate on the antioxidant enzymes in that type of natural products, which requires additional and complex investigation in that direction.

It has been reported that the snail mucus contains allantoin (0.3-0.5%), collagen (0.1-0.3%), glycolic acid (0.05-0.1%), lactic acid (0.05-0.1%), anti-protease (1.3-1.8%), vitamins, trace minerals and a high content of glycine, hydroxyproline, proline and glutamic acid [32]. Recently, several peptides [17] and glycoproteins [33] have been isolated from the mucus of *Helix aspersa* and *H. pomatia* snail species and high concentrations of Asp, Glu, Gly, Leu, Pro and Lys were also reported [17].

As seen from the results, the low-molecular weight fractions exhibited better antioxidant potential compared to the other tested fractions which is in accordance with other observations where low-MW peptide fractions have displayed in general better antioxidant activity compared to fractions with higher MW. As reported, around 70% of the identified antioxidant peptides had MW ranging from 400 to 650 Da [13]. In another study [34], the DPPH radical scavenging activity of a 3–5 kDa fraction was exerted mainly by the sub-fraction dominated by peptides with masses below 600 Da. Also, the activity of the <3 kDa fraction was attributed mainly to the radical scavenging activity of the sub-fractions with lower MW. The highest reducing power was found in a sub-fraction containing peptides rich in Arg, Tyr and Phe. The authors state that both free amino acids and low-MW peptides thus seemed to contribute to the antioxidative potential. It has been recognized that peptides are more reactive than proteins, due to their lower MW and bioactive peptides mostly contain less than 20 amino acid residues [35]. In an attempt to identify and isolate antioxidant peptides Najafian *et al.* [36] reported that the highest antioxidant potential was displayed by peptides with 8–12 amino acid residues. Analyzing the amino acid composition of 42 antioxidant peptides, Zou *et al.* [13] have calculated the percentage distribution of the individual amino acids in these peptides. Gly (G), Pro (P) both having approximately 12% each distribution account for the majority of the amino acids, followed by Leu (L) and Ala (A) with 9.8% and 6.3%, respectively. Moreover, the antioxidant potential of the mucus

can also be attributed to the presence of allantoin, which has been shown to possess antioxidant properties [37].

CONCLUSION

The present investigation reports initial data on the antioxidant potential of *C. aspersum* mucus. The results from the antioxidant screenings of *C. aspersum* mucus and its fractions show that this naturally derived product, specifically the low-molecular weight fractions, possess the properties to counteract the formation of reactive free radicals. From the mucus, several peptides having predominantly Pro (P), Gly (G), Tyr (Y) and Trp (W) were sequenced by mass spectrometry.

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REFERENCES

1. I. Fridovich, *J. Exp. Biol.*, **201**, 1203 (1998).
2. P.J. Pomposiello, B. Demple, *Trends in Biotechnol.*, **19**, 109 (2001).
3. J. Aguirre, M. Ríos-Momberg, D. Hewitt, W. Hansberg, *Trends in Microbiol.*, **13**, 111 (2005).
4. K. Kelly, C.M. Havrilla, T.C. Brady, K.H. Abramo, E.D. Levin, *Environ. Health Persp.*, **106**, 375 (1998).
5. L.A. del Río, L.M. Sandalio, D.A. Altomare, B.A. Zilinskas, *J. Exp. Bot.*, **54**, 923 (2003).
6. E. Kużniak, M. Skłodowska, *Planta*, **222**, 192 (2005).
7. I.F. Benzie, *Eur. J. Nutr*, **39**, 53 (2000).
8. P. Sharma, R. Dubey, *Plant Cell Rep.*, **26**, 2027 (2007).
9. J.M. Matés, F.M. Sánchez-Jiménez, *The Int. J. Biochem. & Cell Biol.*, **32**, 157 (2000).
10. V. Lushchak, *Biochemistry*, **72**, 809 (2007).
11. Y. Shi, J. Kovacs-Nolan, B. Jiang, R. Tsao, Y. Mine, *J. Funct. Food*, **11**, 571 (2014).
12. A. Montoya-Rodríguez, E.G. de Mejía, *Food Res. Int.*, **77**, 204 (2015).
13. T.-B. Zou, T.-P. He, H.-B. Li, H.-W. Tang, E.-Q. Xia, *Molecules*, **21**, 72 (2016).
14. O. Power, P. Jakeman, R. FitzGerald, *Amino Acids*, **44**, 797 (2013).
15. M.C. Milinsk, R. das Graças Padre, C. Hayashi, C.C. de Oliveira, J.V. Visentainer, N.E. de Souza, M. Matsushita, *Jo Food Comp. Analysis*, **19**, 212 (2006).
16. S. Pitt, M. Graham, C. Dedi, P. Taylor-Harris, A. Gunn, *Brit. J. Biomed. Sci.*, **72**, 174 (2015).
17. P. Dolashka, A. Dolashki, L. Velkova, S. Stevanovic, L. Molin, P. Traldi, R. Velikova, W. Voelter, *J. Biosci. and Biotechnol.*, SE/ONLINE, 147 (2015).
18. L.B. Etim, C. Aleruchi, G.A. Obande, *Brit. Microbiol. Res. J.*, **11**, 1 (2016).
19. N.K. Murthy, K. Pushpalatha, C.G. Joshi, *J. Chem. Pharm. Res.*, **3**, 218 (2011).
20. R. Re, N. Pellegrini, A. Proteggente, A. Pannala, M. Yang, C. Rice-Evans, *Free Radical Biol. Med.*, **26**, 1231 (1999).
21. C. Beauchamp, I. Fridovich, *Analyt. Biochem.*, **44**, 276 (1971).
22. R.F. Beers, I.W. Sizer, *J. Biol. Chem.*, **195**, 133 (1952).
23. O.H. Lowry, N.J. Rosebrough, A.L. Farr, R.J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
24. L. Aksoy, E. Kolay, Y. Ağılönü, Z. Aslan, M. Kargıoğlu, *Saudi J. Biol. Sci.*, **20**, 235 (2013).
25. R. Liu, L. Xing, Q. Fu, G.-h. Zhou, W.-g. Zhang, *Antioxidants*, **5**, 32 (2016).
26. M.Y. Hong, J. Lumibao, P. Mistry, R. Saleh, E. Hoh, *J. Nutr.*, **145**, 939 (2015).
27. X. Tang, Z. He, Y. Dai, Y.L. Xiong, M. Xie, J. Chen, *J. Agr. Food Chem.*, **58**, 587 (2009).
28. A. Asoodeh, M. Homayouni-Tabrizi, H. Shabestarian, S. Emtenani, S. Emtenani, *J. Food Drug Analysis*, **24**, 332 (2016).
29. N. Saeed, M.R. Khan, M. Shabbir, *BMC*, **12**, 221 (2012).
30. O.K. Shittu, B. Lawal, G.M. Haruna, E.B. Berinyuy, A.A. Yusuf, A.M. Ibrahim, *Eur. J. Biotechnol. Biosci.*, **3**, 12 (2015).
31. A. Brieva, N. Philips, R. Tejedor, A. Guerrero, J. Pivel, J. Alonso-Lebrero, S. Gonzalez, *Skin Pharmacol. Physiol.*, **21**, 15 (2008).
32. G. Penazzi, *Cosmetic Technology*, **13**, 25 (2010).
33. U.I. Gabriel, S. Mirela, J. Ionel, *Journal of Agroalimentary Processes and Technologies*, **17**, 410 (2011).
34. K.S. Farvin, L.L. Andersen, J. Otte, H.H. Nielsen, F. Jessen, C. Jacobsen, *Food Chem.*, **204**, 409 (2016).
35. H. Korhonen, A. Pihlanto, *Curr. Pharm. Design*, **9**, 1297 (2003).
36. L. Najafian, A.S. Babji, *J. Funct. Foods*, **9**, 280 (2014).
37. E. Guskov, M. Kletskii, I. Kornienko, L. Olekhnovich, V. Chistyakov, T. Shkurat, V. Prokofev, Y.A. Zhdanov, Allantoin as a free-radical scavenger, *Doklady Biochemistry and Biophysics*, **383,** 105 (2002).

СКРИНИНГ НА АНТИОКСИДАНТНАТА АКТИВНОСТ НА ФРАКЦИИ, ИЗОЛИРАНИ ОТ СЛУЗ НА ГРАДИНСКИ ОХЛЮВ *CORNU ASPERSUM*

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(Резюме)

Природните пептиди, получени от различни животински видове проявяват и антиоксидантно действие, като част от механизма за борба с патогенни микроорганизми и оксидативния стрес. Настоящото изследване проучва антиоксидантния потенциал на пептидни фракции, получени от слуз на градински охлюв *Cornu aspersum*. Биологично-активните вещества от слузта са разделени на четири фракции с различно молекулно тегло (MW): фракция 1 (съединения с MW<5kDa), фракция 2 (съединения с MW<10kDa), фракция 3 (съединения с MW<20kDa) и фракция 4 (MW между 10 30kDa). За определяне на антиоксидантната активност на получените фракции са използвани три допълващи се метода: обезвреждане на 1,1-дифенил-2-пикрилхидразил (DPPH) радикал; обща антиоксидантна активност по ABTS метод и инхибиране редукцията на nitro blue tetrazolium (NBT) чрез фотохимично генериране на супероксидни радикали ($^{\bullet}O_2^-$). Установено е, че фракцията с MW<5 kDa проявява по-добър антиоксидантен потенциал в сравнение с останалите фракции.

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Kinetic parameters of the copigmentation effect of caffeic acid and strawberry anthocyanins

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The effect of temperature on the stability of the copigmentation complex of strawberry anthocyanin extract as pigment and caffeic acid as copigment was investigated. The system was studied with a high concentration of caffeic acid 1:20 to 1:100 molar ratio. Different temperatures and copigment concentrations were used for the investigated pigment:copigment interaction and kinetic parameters such as activation energy (Ea), z – factor and degradation rates (k) were calculated. According to the calculated results, at high temperatures (50ºC) destruction of the complex was observed. Decreasing the temperature in the range of 20–30ºC did not lead to restoration of the complex, indicating irreversibility of the copigmentation process.

Key words: Copigmentation, Caffeic acid, Anthocyanins, Kinetic parameters

INTRODUCTION

Anthocyanins belong to the class of flavonoids and represent one on the most important and most widely spread groups of plant pigments [1]. They are known for their ability to form copigments with other phenolic compounds, thus affecting the colour appearance of plant material and anthocyanin-rich foods. There are many studies on the copigmentation process and the stability of copigment complexes in different foods. For example, spectrophotometric measurements ($\Delta\lambda$, ΔA) revealed that there is an interaction between a crude anthocyanin extract from Cabernet Sauvignon grape extracts and caffeic acid suggesting copigmentation in both model and yoghurt systems [2]. The addition of caffeic acid (1:1 w/v) significantly increased the stability of anthocyanins in both systems. Petrova *et al*. studied the interactions between strawberry anthocyanins as pigment and caffeic acid as copigment finding no reversibility of the copigmentation process [3]. Chang *et al*., investigated the copigmentation effects of exogenous caffeic acid and ferulic acid on anthocyanins stability in blackberry juice using visible absorption spectra and HPLC-DAD-MS [4]. The results showed that both caffeic acid and ferulic acid significantly increase the absorption intensity and wavelength maximum (λmax) of anthocyanins in blackberry juice. The copigmentation is an effective way to enhance the colour of anthocyanins [5]. For example, Sharara [6] investigated the possibility to increase the stability of anthocyanins in roselle extract during storage by the addition of some phenolic acids (ferulic, cinnamic and coumaric) as a natural alternative instead of harmful synthetic ones. Copigmentation effects of citric acid, DL-malic acid, tartaric acid, caffeic acid and ferulic acid on purple sweet potato anthocyanins were studied. Results showed that these five organic acids increased the absorption value of purple sweet potato anthocyanins, but did not change the anthocyanin composition. The half-life of anthocyanin degradation (15.3 h) was increased by citric acid, DL-malic acid and tartaric acid to 19.1, 19.0 and 16.9 h at 90ºC, but was reduced by caffeic acid and ferulic acid to 1.8 and 1.6 h, respectively, indicating an unfavorable effect on the thermal stability of purple sweet potato anthocyanins. Sari observed investigated the copigmentation effect of *Berberis crataegina* anthocyanins with phenolic acids (i.e., tannic, gallic, ferulic and caffeic acids) at 90ºC in various pigment:copigment molar ratios (1:10; 1:50; 1:100) [7]. Copigmentation increased the stability of anthocyanins, but the increase in pigment:copigment ratios resulted differently on each phenolic acid compound, i.e., as the molar ratio increased in gallic acid copigmented samples, the anthocyanin degradation decreased, but conversely, it increased in all the other acids used. Additionally, the compatibility of the degradation of copigmented anthocyanins to first order and Weibull distribution models was studied. New colorimetric variables have been defined in the

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uniform CIELAB colour space to assess the quantitative and qualitative colour changes induced by copigmentation and their incidence on visual perception [8]. The copigmentation process was assayed in a model solution between malvidin 3-glucoside and three phenolic compounds (catechin, epicatechin and caffeic asid) as a function of pH and the pigment:copigment molar ratio. Increasing copigment concentration induced perceptible colour changes at molar ratios higher than 1:2, consisting in a bluish and darkening effect of anthocyanin solutions. Among the different CIELAB attributes, hue difference was the best-correlated parameter with the increase of copigment concentration, providing the relevance of this physicochemical phenomenon on the qualitative changes of anthocyanin colour.

Patras *et al*. [9] investigated the effect of storage time and temperature on the degradation of bioactive compounds such as ascorbic acid, anthocyanins, total phenols, colour and total antioxidant capacity of strawberry jam. The results indicated that lightness (*L*) value decreased significantly over 28 days of storage at 4 and 15°C, with lower values measured at higher temperatures. Anthocyanins, ascorbic acid and colour degradation followed first-order kinetics where the rate constant increased with an increase in the temperature. Wang *et al.* [10] studied the thermal and storage stabilities of anthocyanins in blackberry juice and concentrate over the temperature ranges of 60–90°C and 5–37°C. Results indicate that the thermal degradation of anthocyanins followed first-order reaction kinetics. The temperature-dependent degradation was adequately modelled by the Arrhenius equation. The activation energy value for the degradation of blackberry anthocyanins during heating was 58.95 kJ/mol for the 8.90 Brix blackberry juice. During storage, antocyanins in the 65.0 Brix blackberry juice concentrate degraded more rapidly than that in 8.90 Brix blackberry juice, with activation energies of 65.06 kJ mol^{-1} and 75.5 kJ mol^{-1}, respectively.

The aim of this work was to calculate the kinetic parameters of the copigmentation effect between caffeic acid and strawberry anthocyanins, and to determine the stability of the system as a function of temperature and copigment concentration.

EXPERIMENTAL

Chemicals

Caffeic acid (98%) was purchased from Sigma-Aldrich, (Germany). Reagents (citric acid monohydrate and disodium hydrogen phosphate dodecahydrate) for the McIlvaine buffer (pH 3.4), were from Merck (Darmstadt, Germany). The adsorbent resin Amberlite XAD 16N was purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). All other reagents and solvents used were of analytical grade.

Strawberry anthocyanins extraction and isolation

Strawberry anthocyanins were extracted and purified *via* the following procedure: Frozen strawberries were thawed and manually squeezed in a beaker. The homogenized purée was extracted overnight at 4°C using methanol acidified with hydrochloric acid (1%, v/v) at a solvent/solid ratio of 2.5:1 (v/w). The extraction mixture was filtered and the organic solvent was evaporated under vacuum (30°C). To remove sugars, salts, and amino acids from the crude extracts, samples were purified using a column (465 × 30 mm i.d.) filled with adsorption resin Amberlite XAD 16N (Sigma Aldrich Co., St. Louis, MO, USA). Prior to sample application, the resin was conditioned and equilibrated by rinsing with 500 ml of methanol and 1000 ml of water, and acidified with trifluoroacetic acid (TFA, pH 2). Subsequently, 250 ml of the aqueous strawberry extract were applied and the column was rinsed with 1000 ml of acidified water (pH 2). For elution of the pigments at least 500 ml of a mixture of methanol and acidified water (TFA, pH 2) (95:5, v/v) was applied until the column was colorless. The organic solvent of the eluate was evaporated under vacuum (30°C). To separate anthocyanins from colorless polyphenols, further purification was performed by extracting the aqueous phase three times with the same volume of ethyl acetate. After evaporation and concentration under vacuum (30 °C), the residue was lyophilized for 72 h. Total monomeric anthocyanins were assessed by the pH-differential method.

Sample preparation

Stock solutions of strawberry extract with concentration 1×10^{-4} M (on the basis of total anthocyanins expressed as pelargonidin 3-glucoside equivalents) and caffeic acid in different concentrations (2.10^{-3}, 4.10^{-3}, 6.10^{-3}, 8.10^{-3}, 1.10^{-2}), were prepared in McIlvaine buffer (0.1 M, pH 3.4). Model solutions of strawberry anthocyanins and caffeic acid were obtained by mixing equal volumes (5 ml) of the corresponding stock solutions and were left for equilibration for 30 min at room temperature. Before spectrophotometric measurements, model solutions of samples (model solutions) were thermostated (VEB MLW Prüfgerätewerk Medingen, Sitz Freital, Germany)

at 20, 30, 40 or 50°C at heating and after that at cooling to 40, 30 and 20ºC, respectively.

Spectrophotometric measurements

Absorption from 380 to 780 nm was measured using a Helios Omega UV-Vis spectrophotometer equipped with VISION*lite* software (all from Thermo Fisher Scientific, Madison, WI, USA) using 1 cm path length cuvettes.

Modelling of kinetic parameters

For calculation of kinetic parameters two models were chosen.

– The first is a conventional chemical kinetic model, Arrhenius model [11, 12]. There is a linear relationship between ln K and 1/T:

$$k = k_0 e^{-\frac{E_a}{RT}} \quad (1)$$

where: Ea is activation energy (kJ mol^{-1}); R is the universal gas constant (R = 8.314 J K^{-1} mol^{-1}), T is the absolute temperature (K).

– The second model is Ball's model [11. 13], which defines a decimal reduction time which is related to temperature *via* a factor z.

$$D = \ln 10 k \quad (2)$$

$$D = D_0 10^{-\frac{T}{z}} \quad (3)$$

where: D is decimal reduction time at temperature T (s); D_0 - value of D extrapolated at 0°C; z is expressed in °C.

The model's parameters were identified using linear regression on the logarithmic curves of experimental data. The z value could be estimated from Ea using the relationship:

$$z = \ln(10) \frac{RT^2}{E_a} \quad (4)$$

Statistical analysis

The results reported in the present study are the mean values of at least two determinations and the coefficients of variation were found to be below 2.5 % in all cases. Linear regression analysis was performed using the statistical package of Microsoft Excel®.

RESULTS AND DISCUSSION

The kinetic investigation of the copigmentation system in our study was done using the following kinetic parameters: degradation rate (k), activation energy (Ea) and z-factor [11]. According to the results and especially the high value of the correlation coefficient (R), the interaction between pigment and copigment could be described as first order reaction (Table 1). These results are in compliance with the results of Shikov *et al*. [14] and Gonnet [15] and the correlation coefficient (R) > 0.9 in all cases confirms that the degradation of visual colour follows a first order reaction at all temperatures [18]. Anthocyanin degradation is connected with the half-life ($t_{1/2}$) and degradation rates (k), but Ea is connected with the z-factor.

Table 1. Kinetic parameters for thermal investigation of pigment:copigment interactions between strawberry anthocyanin and caffeic acid following the Arrhenius and Ball models.

System	t, ºC	k, s^{-1}	R^2	$t_{1/2}$, s	Ea, kJ mol^{-1}	R^2	z, ºC
Anthocyanins :caffeic acid 1:20/1:100	20	0.00463	0.941	149.709	20.533	0.927	88.561
	30	0.00521	0.917	133.042			
	40	0.00695	0.931	99.733			
	50	0.00834	0.921	83.111			
	50/40	0.00927	0.924	74.773	15.801	0.841	111.319
	40/30	0.00758	0.912	91.444			
	30/20	0.00731	0.873	94.822			

The constant k was obtained for the corresponding system when changing the concentration for a specified time. Then the rate constants for the different temperatures were calculated. The increase in anthocyanin half-life degradation time ($t_{1/2}$) means decreasing the rate of their degradation [16], whereas low values of the constant are related to low degradation rate of the reaction and high stability of the system. During heating, the lowest rate constant was at 20°C, which proves system stability at this temperature. The high values of the degradation rate during cooling is an evidence for the destruction of the complex and the irreversibility of the copigmentation process.

The anthocyanin degradation rate constants obtained for each model solution were plotted as a function of heating temperature and linear dependence was observed (Figure 1). The obtained results are in agreement with previous studies including strawberry anthocyanins [14, 15, 17] Using linear regression, the degradation rate was analyzed using Eq. (1) to determine the overall

order and rate constant (k) for the degradation reaction.

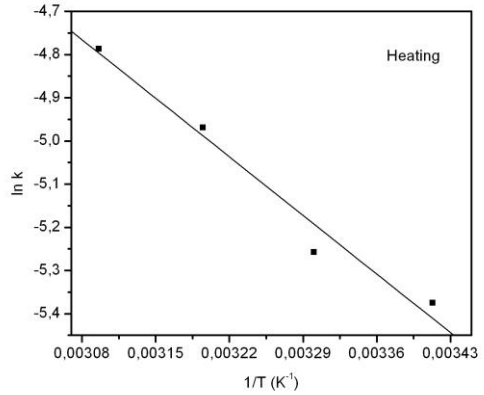

Fig.1. Arrhenius plots for degradation of anthocyanins in strawberry fruits extracts at heating.

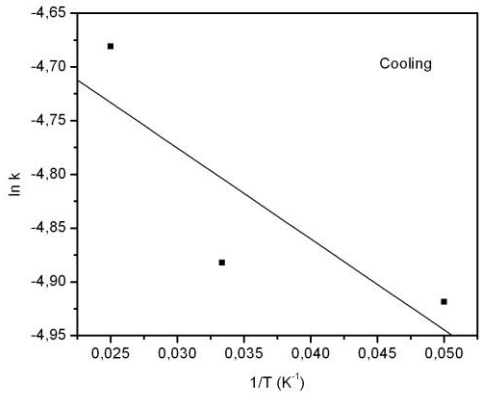

Fig. 2. Arrhenius plots for degradation of anthocyanins in strawberry fruits extracts at cooling.

Figure 2 shows a similar dependence of degradation rates (k) *versus* 1/T at cooling. There is some deviation of the points from the straight line, which is connected with the destruction of the copigment complex without subsequent restoration after cooling.

Based on the experimental results, the activation energy (Ea) and z-factor were calculated. It was observed that this system is characterized with low Ea and high z–factor. These results are connected with the low stability of the system and the irreversibility of the copigmentation process during cooling. The activation energies (Ea) ranged between 20 kJ mol^{-1} at heating to 15 kJ mol^{-1} at cooling and z-factor ranged from 88 to 111°C. According to these results, it could be concluded that higher activation energy implies lower rate of degradation and higher stability of the system. Similar Ea were reported for colour degradation of green vegetable purées. Activation energies of 28.55, 41.15 and 34.01 kJ mol^{-1} for spinach purée, mustard leaves and a mixed purée, respectively, were reported by Ahmed *et al*. [18].

Besides the kinetic parameters, we determined the colour parameters of the system at all temperatures (Table 2). Some authors connect these parameters with the kinetics of the process [12, 13]. These parameters are: *L* for lightness, *a* for redness and *b* for yellowness. According to the colorimetric investigation, the decrease of *L* values was related to the copigmentation process. We observed that lightness (L) decreased at 20ºC and pigment:copigment ratio of 1:100, which is an evidence for a copigmentation effect. Increasing the temperature to 50ºC led to elevation of L values, but the subsequent cooling did not decrease L values. This is a confirmation for the irreversible destruction of the complex at higher temperatures, which is not restored at lower temperature.

Table 2. CIELab colour parameters of strawberry anthocyanin:caffeic acid complex at heating and cooling.

Molar ratio of pigment:copigment	λmax.	Amax.	L	a	b
t = 20ºC					
1:0	501	0.575	84.9	28.4	21.5
1:20	504	0.647	81.9	33.2	20.9
1:40	504	0.697	80.2	36.7	20.9
1:60	506	0.756	78.2	39.9	21.1
1:80	509	0.773	74.0	40.1	20.1
1:100	510	0.779	73.2	41.9	20.1
t = 30ºC					
1:0	501	0.526	85.8	26.6	19.6
1:20	503	0.587	83.6	30.6	19.9
1:40	505	0.640	81.5	33.9	19.6
1:60	506	0.704	79.5	37.5	20.4
1:80	507	0.706	79.1	37.8	19.0
1:100	508	0.722	78.0	37.0	20.6
t = 40ºC					
1:0	501	0.535	84.1	25.5	19.4
1:20	503	0.562	83.9	29.3	19.1

1:40	504	0.607	81.9	31.6	18.9
1:60	506	0.684	79.2	34.2	20.4
1:80	504	0.680	81.5	34.0	18.3
1:100	504	0.626	81.0	34.5	18.2
t = 50ºC					
1:0	500	0.530	84.6	25.7	19.8
1:20	502	0.571	83.7	29.6	19.4
1:40	504	0.626	81.1	32.0	19.0
1:60	506	0.961	61.7	30.1	17.4
1:80	507	0.637	80.3	34.2	18.4
1:100	507	0.703	77.8	35.0	21.0
t = 50/40ºC					
1:0	500	0.790	81.9	33.8	29.9
1:20	501	0.891	78.2	39.0	30.1
1:40	503	0.913	77.9	41.2	29.7
1:60	506	0.924	77.5	42.0	30.1
1:80	507	0.918	77.1	41.3	30.2
1:100	504	0.940	76.8	41.4	29.7
t = 40/30ºC					
1:0	500	0.793	81.7	33.9	30.0
1:20	502	0.884	78.8	39.3	30.2
1:40	504	0.913	77.4	40.4	29.6
1:60	506	0.918	77.5	41.0	29.8
1:80	506	0.953	76.4	41.8	29.8
1:100	508	0.954	76.1	41.3	28.9
t = 30/20ºC					
1:0	500	0.806	81.5	34.1	30.4
1:20	504	0.897	78.5	40.2	30.1
1:40	505	0.923	77.4	40.8	29.6
1:60	505	0.926	77.4	41.3	29.6
1:80	507	0.956	76.3	41.1	29.7
1:100	507	0.960	76.2	41.7	29.9

CONCLUSIONS

The thermal stability of strawberry anthocyanin:caffeic acid (1:20 to 1:100) complex was proved first by heating the system to 50ºC and then cooling to 20ºC. At high temperatures (around 40-50ºC) destruction was observed and there was not restoration of the complex at 20ºC. This was confirmed by the calculated kinetic parameters and the colorimetric investigation of the system. On the basis of the calculated kinetic parameters it could be concluded that the copigmentation process is possible only at a
temperature of 30ºC or lower.

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REFERENCES

1. R. Boulton, *Am. J. Enol. Vitic.*, **52**, 67(2001).
2. E.F. Gris, E.A. Ferreira, L.D. Falcão, M.T. Bordignon-Luiz, *Food Chem.*, **100**, 1289, (2007).
3. I. J. Petrova, V. T. Shikov, V.D. Gandova, K. M.Mihalev, D. I. Dimitrov, *Sci. works UFT-Plovdiv.*, **63**, 64 (2016).
4. P. Chang-an, L. Feng-bo, Y.Ye, T. Xiao-yuan, H. Yong-bin, *J. Nat. Prod.,* **24**, 94 (2012).
5. W. Meng, L. Xiao-ding, L.Shuo, Z. Shao-hua, J. Hong, *J. Food Sci.*, **35**, 119 (2014).
6. M. S. Sharara, *Am.* J. *Food Sci. Tech.,* **5**, 45 (2017).
7. F. Sari, *J. Food Process. Pres.*, **40**, 422 (2016**).**
8. B. Gordillo, F. J. Rodríguez-Pulido, M. L.Escudero-Gilete, M. L. González-Miret, F. J. Heredia, *J. Agric. Food Chem.*, **60,** 2896 (2012).
9. A. Patras, N. P. Brunton, B. K. Tiwari, F. Butler, *Food Bioproc. Tech.*, **4**, 1245 (2011).
10. W. Wang, S. Xu, *J. Food Eng.*, **82**, 271 (2007).
11. S. Zozio, D. Pallet, M. Dornier, *Fruits*, **66**, 203 (2011).
12. M. Cisse, F. Vaillant, O. Acosta, C. Dhuique- Mayer, M. Dornier, *J. Agric. Food Chem.*, **57**, 6285 (2009).
13. M. Kopjar, K. Jaksic, V. Pilizota, *J. Food Process.*, **36**, 545 (2012).
14. V. Shikov, D. Kammerer, K. Mihalev, P. Mollov, R. Carle, *Food Res. Int.*, **46**, 552 (2012).
15. J. Gonnet, *Food Chem.*, **66**, 87 (1999).
16. V.T. Shikov, PhD Thesis, UFT, Plovdiv, 2011.

17. B. Moldovan, L. David, C. Chişbora, C. Cimpoiu, *Molecules*, **17**, 11655 (2012).
18. O. Ezekiel, R. Mustafa, T. Olur, *Fresh Produce*, **5**, 39 (2011).
19. J. Ahmed, A. Kaur, U. Shivhare, *J. Food Sci.*, **67**, 1088 (2002).

КИНЕТИЧНИ ПАРАМЕТРИ НА КОПИГМЕНТАЦИОННИТЕ РЕАКЦИИ МЕЖДУ КАФЕЕНА КИСЕЛИНА И АНТОЦИАНИ ОТ ЯГОДИ

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(Резюме)

Изследван е ефектът на температурата върху стабилността на комплекса между антоциани от ягоди като пигмент и кафеена киселина като копигмент. Системата е изследвана при високи концентрации на кафеената киселина с моларно съотношение от 1:20 до 1:100. Използвани са различни температури и концентрации на копигмента за изследване на системата пигмент:копигмент, изчислени са кинетични параметри като активираща енергия (Ea), z - фактор и скоростна константа (k). Според изчислените резултати, при високи температури (50 °C) се наблюдава разрушаване на комплекса. Понижаването на температурата в диапазона 20-30 °C не доведе до възстановяване на комплекса, което доказва необратимост на процеса на копигментация.

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Preparation of quercetin delivery systems on the basis of amino-modified KIL-2 mesoporous silica

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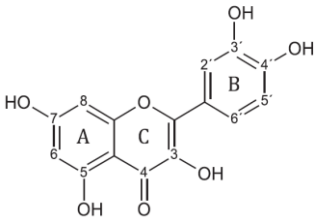
KIL-2 silica with textural mesoporosity was synthesized and modified with amino groups by post-synthesis method. Quercetin was successfully loaded on amino-modified KIL-2 by incipient wetness impregnation or solid-state method. Quercetin-loaded KIL-2NH_2 particles were coated by a polyelectrolyte polymer complex containing κ-carrageenan-chitosan-κ-carrageenan. The parent, amino-modified and quercetin loaded samples were characterized by XRD, N_2 physisorption, thermal gravimetric analysis and ATR-FT-IR spectroscopy. *In-vitro* release of quercetin from quercetin loaded formulations was studied in two acceptor media resembling physiological pH GIT (pH=1.2 and pH=6.8). The *in-vitro* release study showed slower quercetin release from polymer coated quercetin-loaded KIL-2NH_2 samples prepared by both methods compared to the uncoated ones.

Key words: Quercetin delivery, *In-vitro* release, Modified mesoporous silica KIL-2NH_2, Oral administration

INTRODUCTION

The interest in the recent years towards application of mesoporous silica materials as potential carriers for drugs is based on the advantages of these materials, such as biocompability, high specific surface and pore volume, tunable pore size, controlled particle size, morphology and possibility for surface functionalization [1-5]. Mesoporous silica carriers can overcome the problems associated with poor aqueous solubility, low bioavailability and chemical stability of the loaded bioactive substance. The appropriate surface modification of the silica matrix with organic functional groups can optimize the loading efficacy and the release kinetics of the drug [2, 4, 5].

In the recent years. natural flavonoids have attracted research interest due to their pleiotropic therapeutic potential [6-10]. Quercetin (2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4H-chromen-4-one) is one of the most common flavonols present in nature, a strong antioxidant and a major dietary flavonoid. It has been extensively studied because of its broad-spectrum pharmacological activities such as anticancer, antiviral, antimutagenic and lipid peroxidation inhibitory effects. Initially these effects have been attributed to the strong antioxidant properties of quercetin, because of its ability to scavenge free radicals and influence the intracellular redox status.



Scheme 1. Molecular structure of quercetin

It was found that quercetin can regulate cell cycle by modulating several molecular targets, including p21, cyclin B, p27, and topoisomerase [11]. In addition, quercetin displays specific inhibitory effects in various groups of kinases, including Janus kinases (JAK) and especially JAK-3 kinase, which is a non-receptor tyrosine kinase predominantly expressed in haematopoetic cells [12]. Quercetin is also known as an antiviral, anti-ageing, antimutagenic, anti-inflammatory, antiallergic and anti-psoriatic agent.

Unfortunately, the clinical realization of quercetin's therapeutic potential is greatly hampered due to unfavorable physicochemical and pharmacokinetic properties. Quercetin is characterized with very low aqueous solubility and with chemical instability, which respond in low bioavailability. To overcome these unfavorable characteristics of the drug a possible approach is

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the elaboration of carriers for optimized oral, systemic, site-specific or topical delivery of quercetin. Quercetin has been formulated in liposomes [13], nanoparticles [14], and metal ion complexes [15].

In the present study a novel approach to optimized oral delivery of quercetin is suggested. It is based on its encapsulation in amino-modified mesoporous silica materials. Two methods for deposition of quercetin have been applied, in particular loading from solution or by solid-state reaction into the pores of nanosized NH_2-modified KIL-2 mesoporous silica. Coating by a polymer complex of quercetin loaded mesoporous silica nanoparticles was used in order to modify the release properties. The adsorption capacity and the rate of release of the encapsulated quercetin in these delivery systems were assessed.

MATERIALS AND METHODS

Synthesis of KIl-2 silica material

Mesoporous disordered silicate KIL-2 was prepared by two-step synthesis in presence of the following compounds in a molar ratio of: 1 TEOS : 0.5 TEA : 0.1 TEAOH : 11 H_2O [16]. The template was removed by calcination at 773 K for 10 h in air flow.

Functionalization of KIL-2 with aminopropyl groups

Modification of the KIL-2 material with aminopropyl groups was accomplished by reaction with 3-aminopropyltriethoxysilane (APTES) in toluene (60^{o}C, 24 h) [17]. After completing the reaction, the samples were washed with several portions of toluene, methanol and finally water. 20 ml of APTES in 100 ml of toluene was applied to 1 g of silica. The APTES modified samples were designated as KIL-2NH_2.

Quercetin loading

The KIL-2NH_2 sample was loaded with quercetin by two different methods: deposition by incipient wetness impregnation and by solid-state reaction. KI-2NH_2 and quercetin in a weight ratio of 1.0:0.5 were stirred in 1 mL of ethanol till the total evaporation of the solvent. Then the powdered products were washed 3 times with 5 mL water, and dried at 40 °C overnight. For the solid-state method, the same loading ratio of quercetin to the KIL-2NH_2 material was used. Quercetin was mixed with the carrier at room temperature in a DDR-GM 9458 type vibrational ball mill mixer with a holder (ø = 24 mm) and one ball (ø = 9.5 mm), for 3 min without adding any additive for wetting. The obtained preparations by the two methods were designated as KIL-2NH_2/Qu(IW) and KIL-2NH_2/Qu(SS).

Coating by polyelectrolyte polymer complex

Solutions of chitosan (2 mg/ml, pH 5) and k-carrageenan (2 mg/ml, pH 5) were prepared. A "layer-by-layer" technique was applied to form a three-layer polymer coating around the particles. It involved alternating deposition of the oppositely charged polyelectrolytes starting with carrageenan. At each step the particles were dried at room temperature under reduced pressure. Coated samples were designated as KIL-2NH_2/Qu(IW)P and KIL-2NH_2/Qu(SS)P.

Characterization

X-ray powder diffraction patterns were recorded by a Philips PW 1810/3710 diffractometer with Bragg-Brentano parafocusing geometry applying monochromatized CuK_α (λ=0.15418 nm) radiation (40 kV, 35 mA) and proportional counter.

Nitrogen physisorption measurements were carried out at -196°C using Tristar 3000 Micromeritics volumetric adsorption analyzer. Before the adsorption analysis, the silica sample was outgassed under vacuum for 2 h at 200°C, while modified and drug-loaded samples were pretreated at 80°C for 5 h.

Thermogravimetric measurements were performed with a Setaram TG92 instrument with a heating rate of 5°C/min in air flow.

Attenuated total reflection infrared (ATR-FT-IR) spectra were recorded by means of a Varian Scimitar 2000 FT-IR spectrometer equipped with a MCT (mercury-cadmium-tellur) detector and a single reflection ATR unit (SPECAC “Golden Gate”) with diamond ATR element. In general, 128 scans and 4 cm^{-1} resolution were applied. For all spectra ATR-correction was performed (Varian ResPro 4.0 software).

In vitro release study

An *in vitro* quercetin release study was performed in buffers with pH = 1.2 and 6.8 at 37°C. The quercetin-loaded particles (2 mg) were incubated in 100 ml of 0.1N HCl (pH=1.2) and phosphate buffer (pH=6.8) at 37°C under stirring (100 rpm). At appropriate time intervals, 3-ml samples were withdrawn from the release medium and analyzed with UV-Vis spectroscopy at a wavelength of 367 nm. The concentration of the released quercetin was calculated according to the standard curves prepared in pH=1.2 and 6.8 solutions (r>0.9993).

RESULTS AND DISCUSSION

Material characterization

The low-angle powder XRD patterns of KIL-2 indicate that a mesoporous structure with textural mesoporosity was synthesized (not shown). For the amino-modified and quercetin-loaded silica carriers decreased intensity and some broadened reflections were observed which were indication of partial pore filling. XRD patterns at higher angles of quercetin-loaded amino-modified samples (Fig. 1) show the presence of crystalline quercetin which is more pronounced for the sample prepared by solid-state reaction.

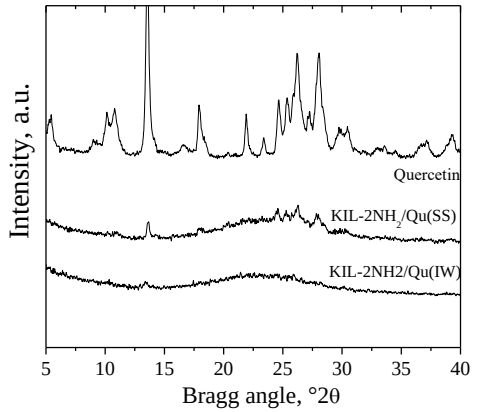

Figure 1. XRD patterns of quercetin-loaded parent and amino modified KIL-2 formulations compared to pure quercetin

This is un evidence that a part of quercetin can be found on the outer surface of the silica nanoparticles or in the voids among the particles.

The nitrogen physisorption isotherms of parent, and quercetin loaded amino-modified KIL-2 samples are presented in Fig. 2. Textural parameters are summarized in Table 1.

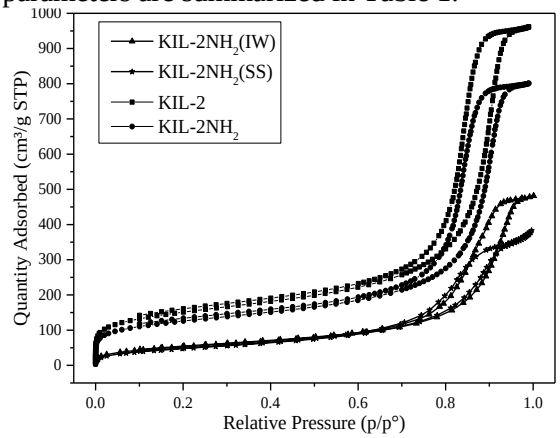

Figure 2. Nitrogen adsorption and desorption isotherms of the parent, amino-functionalized and quercetin-loaded samples.

Significant decrease of the textural parameters, such as surface area and total pore volume of the quercetin-loaded samples indicate pore filling by quercetin. This effect is similar for both quercetin-loaded formulations. Despite the smaller amount of loaded quercetin on the KIL-2NH_2/Qu(SS) sample, its partial deposition on the external surface leads to similar decrease in surface area and pore volume as that obtained for the KIL-2NH_2/Qu(IW) sample.

Table 1. Textural properties of the parent, amino-functionalized and quercetin-loaded mesoporous silicas

Samples	BET (m^2/g)	Pore volume (cm^3/g)	PD[a] (nm)	Quercetin amount (wt.%)
KIL-2	660	1.20	15.3	-
KIL-2NH_2	530	1.00	13.2	-
KIL-2NH_2/Qu(SS)	202	0.41	8.2	19.8
KIL-2NH_2/Qu(IW)	195	0.40	7.9	30.9

For clarification of the interaction between the quercetin molecule and the amino groups of the mesoporous silica carrier the quercetin-loaded samples were characterized by the ATR FT-IR method (Fig. 3). Pure quercetin shows characteristic IR bands of stretching vibrations of aryl ketonic carbonyl ($n_{C=O}$ at 1660 cm^{-1}) and of aromatic ring C=C (at 1605, 1555, 1511 and 1457 cm^{-1}). Band at 1350 cm^{-1} belongs to OH bending vibration of the phenols and the band around 1309 cm^{-1} can be assigned as in-plane bending vibration of aromatic C-H [18]. The modification of KIL-2 sample by APTES results in the appearance of the bands at 2929 cm^{-1} and at 1540 cm^{-1} (not shown) which are attributed to C-H stretching and N-H scissoring vibrations of aminopropyl residues anchored on the surface of the mesoporous support [19, 20]. In the ATR-FTIR spectra for quercetin-loaded KIL-2NH_2 a shift of $\nu_{C=O}$ at 1660 to 1668 cm^{-1} is registered, while the bands of aromatic ring C=C vibrations show up-shift. The band at 1350 cm^{-1} (belonging to OH bending of phenols) is shifted to higher wavenumbers.

The registered changes in the ATR-FTIR spectra are evidence for interaction between the NH_2 groups of the KIL-2 matrix and the phenolic OH groups of quercetin, which leads to a conjunction

loss among the aromatic and the pyrone ring in the quercetin molecule.

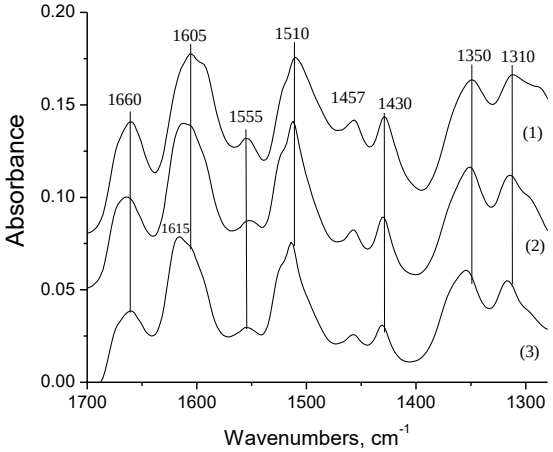

Figure 3. ATR FT-IR spectra of quercetin (1), KIL-$2NH_2$/Qu(SS) (2) and KIL-$2NH_2$/Qu(IW) (3) samples.

Quercetin loading and in vitro release

The amount of the functional aminopropyl groups, loaded quercetin and polymer coatings in the prepared samples were investigated by thermogravimetric method. The calculated amount of aminopropyl groups connected on the surface of KIL-$2NH_2$ is 6.5 wt.%. The TG analysis determined the actual amount of quercetin in the carriers after correcting the curves by water and aminopropyl content for KIL-$2NH_2$. TG data show that the loading of quercetin on KIL-$2NH_2$ samples by solid state and incipient wetness impregnation is around 19.8 and 30.2 wt.%, respectively (Table 1). The higher amount of loaded quercetin by the impregnation method can be explained by easier penetration of dissolved quercetin molecules in the pores of the support. Moreover, the quercetin loaded by solid state reaction is partially deposited on the external surface of KIL-$2NH_2$ as can be seen from XRD data. The calculated weight losses from TG analysis due to the decomposition of polyelectrolyte complex containing k-carrageenan – chitosan-k-carrageenan were 9.5 wt% for KIL-$2NH_2$/Qu(SS) and 8.8 wt% for KIL-$2NH_2$/Qu(IW).

Faster release of quercetin was registered for the KIL-$2NH_2$/Qu(IW) sample in both buffers compared to its analogue prepared by solid state reaction. The quercetin-loaded KIL-$2NH_2$ formulations show slower release in buffer with pH=6.8 than that in buffer with pH=1.2. Total quercetin release for KIL-$2NH_2$/Qu(IW) at pH=1.2 was achieved in 3 h whereas in buffer with pH=6.8 the maximum release of quercetin for the same sample reached 92 % in 4 h. This result can be explained by protonation of aminopropyl groups in acidic buffer facilitating the release of quercetin molecule as a result of the competitive adsorption between quercetin and water molecules. Faster release of quercetin from the KIL-$2NH_2$/Qu(IW) sample can be explained with the amorphization of quercetin during its penetration into the pore system of the carrier in comparison to the KIL-$2NH_2$/Qu(SS) material, in which the presence of crystalline quercetin was seen by XRD. The coating with a k-carrageenan –chitosan-k-carrageenan complex leads to decrease of the release rate of quercetin in both buffers for the KIL-$2NH_2$/Qu(IW)P and KIL-$2NH_2$/Qu(SS)P materials as seen in Fig.4.

From the comparison of the release profiles of the uncoated and coated particles it can be calculated that in the first 30 min the latter system releases twice less amount of drug. In conclusion, by the applied formulation method an efficient delivery system could be developed, providing controlled release of quercetin.

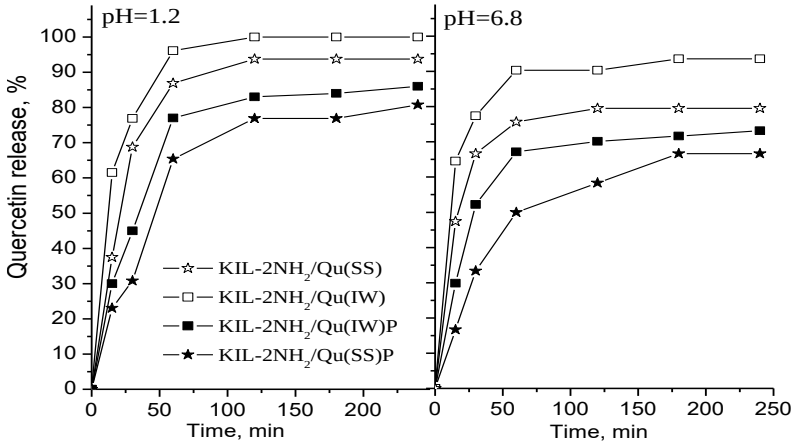

Figure 4. *In-vitro* release profiles of quercetin loaded amino modified KIL-2 samples at pH=1.2 and 6.8.

CONCLUSIONS

Mesoporous KIL-2 silica type was synthesized and modified with aminopropyl groups by a post-synthesis method. Incipient wetness impregnation and solid-state reaction methods were used for quercetin loading on the amino-modified KIL-2 samples. High loading capacity (20-30 wt. %) was registered on the KIL-2NH_2 by both methods. The *in-vitro* release process at pH=1.2 and 6.8 showed faster quercetin release from KIL-2NH_2 sample prepared by impregnation in comparison to that prepared by solid state reaction. It was shown that the release of quercetin loaded on the mesoporous nanocarrier can be additionally controlled by formation of a polyelectrolyte polymer complex.

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REFERENCES

1. M. Vallet-Regi, A. Ramila, R.P. del Real, J. Perez-Pariente, *Chem. Mater.*, **13,** 308 (2001).
2. P. Horcajada, C. Marquez-Alvarez, A. Ramila, J. Perez-Pariente, M. Vallet-Regi, *Solid-State Science* **8**, 1459 (2006).
3. Sh. Wang, *Micropor. Mesopor. Mater.* **117**, 1 (2009).
4. I. Slowing, B. Trewyn, S. Giri, V. Lin, *Adv. Funct. Mater.*, **17-8**, 1225 (2007).
5. I. Slowing, J. Vivero-Escoto, C-W. Wu, V. Lin, *Adv. Drug Deliv. Rev.*, **60**, 1278 (2008).
6. A. Ahmedova, K. Paradowska, I. Wawer, *J. Inorg. Biochem.*, **110**, 27 (2012).
7. G.R. Beecher, B.A. Warden, H. Merken, *Proc. Soc. Exp. Biol. Med.*, **220**, 267 (1999).
8. R. Boly, T. Gras, T. Lamkami, P. Guissou, D. Serteyn, R. Kiss, J. Dubois, Quercetin inhibits a large panel of kinases implicated in cancer cell biology. *Int. J. Oncol.*, **38**, 833 (2011).
9. W. Bors, C. Michel, M. Saran, *Oxyg. Radic. Biol. Syst.*, **D 234**, 420 (1994).
10. M. Catauro, F. Papale, F. Bollino, S. Piccolella, S. Marciano, P. Nocera, S. Pacifico, *Sci. Technol. Adv. Mater.* **16**, 035001 (2015).
11. J.-H. Yang, T.-C. Hsia, H.-M. Kuo, *Drug Metab. Dispos.*, **34(2),** 296 (2006).
12. D.X. Hou, T. Kumamoto, *Antioxid. Redox. Signal.*, **13,** 691 (2010).
13. A. P. Landi-Librandi, T. N. Chrysostomo, A. E. C. S. Azzolini, C.M. de Oliveira C. A. Marzocchi-Machado, Y. M. Lucisano-Valim, *J. Lipos. Res.*, **22**, 89 (2012).
14. A. Kumari, S.K. Yadav, Y.B. Pakade, B. Singh, S.C. Yadav, *Colloids Surf.*, **B 80**, 184 (2010).
15. M. Y. Moridani, J. Pourahmad, H. Bui, A. Siraki, P. J. O'Brien, *Free Rad. Biol. & Med.*, **34(2),** 243 (2003).
16. N. Novak Tušar, A. Ristić, G. Mali, M. Mazaj, I Arčon, D. Arčon, V. Kaučič, N. Zabukovec Logar, *Chem. Eur. J.*, **16**, 5783 (2010).
17. A. Szegedi, M. Popova, I. Goshev, *J. Solid State Chem.*, **184**, 120(2011).
18. M. Heneczkowski, M. Kopacz, D. Nowak, A. Kuzniar, *Acta. Pol. Pharm.*, **58**, 415 (2001).
19. Y.-J. Yang, X. Tao, Q. Hou, J.-F. Chen, *Acta Biomaterialia*, **5**, 3488 (2009).
20. Sh. Xing Wang, Y. Zhou, W. Guan, B. Ding, *Nanoscale Res. Lett.*, **3**, 289 (2008).

ПРИГОТВЯНЕ НА СИСТЕМИ ЗА ДОСТАВЯНЕ НА КВЕРЦЕТИН НА ОСНОВАТА НА АМИНО-МОДИФИЦИРАН KIL-2 МЕЗОПОРЕСТ СИЛИКАТ

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KIL-2 силикат с текстурална мезопористост е синтезиран и модифициран с аминогрупи чрез следсинтезен метод. Кверцетин е нанесен върху амино-модифицирания KIL-2 чрез импрегниране с омокряне или чрез твърдо-фазна реакция. Частиците от KIL-2NH_2 с нанесен кверцетин са обвити с полиелектролитен полимерен комплекс, съдържащ к-карагенан-хитозан-к-карагенан. Изходните, амино-модифицираните и кверцетин-съдържащите образци са охарактеризирани чрез XRD, азотна физисорбция, термогравиметричен анализ и ATR-FT-IR спектроскопия. *In-vitro* освобождаването на кверцетин от образците е изследвано в две физиологични среди с pH GIT (pH=1.2 and pH=6.8). Резултатите от *in-vitro* освобождаването показа, че кверцетина се освобождава по-бавно от образци, получени по двата метода и обвити с полимер, в сравнение с необвитите.

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Structure and antibacterial activity of isolated peptides from the mucus of garden snail *Cornu aspersum*

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The world provides a rich source of peptides with antimicrobial, antiviral and antitumor activity. Peptides and glycopeptides are an expanding group of structurally complex anti Gram positive antibacterial agents, which are used in human and veterinary medicine. Quite a series of proline-rich peptides, isolated from arthropods and molluscs, were considered to be promising candidates for the treatment of microbial infections and suppression of microbial resistance. In the present study, we report the primary structure and antimicrobial activity of peptides produced by the mucus of garden snail *Cornu aspersum* in comparison to similar peptides isolated from molluscs. Several peptides with molecular masses between 1 and 4 κDa measured by mass spectrometric analysis were identified in the mucus. Their amino acid sequences were determined by MS/MS analysis as is shown for peptide at m/z 1438.87 $[M+H]^+$ (ML/INVAVNQ/KGEVKH). The fraction with peptides with molecular masses below 3 kDa exhibited antibacterial activity against Gram-negative *Pseudomonas aeruginosa* AP9 and Gram-positive *Brevibacillus laterosporus* BT271 bacteria and the inhibition effects of the peptides can be explained with the amino acid residues. The *de novo* sequence of six peptides revealed that most of them contain glycine, proline, tryptophan and valine which are typical for peptides with antimicrobial activity.

Keywords: Antimicrobial peptides, *Pseudomonas aeruginosa*, *Brevibacillus laterosporus*, *Helix lucorum*, *Rapana venosa*, *Cornu aspersum*, Mass spectrometry

INTRODUCTION

Most living organisms are constantly exposed to potentially harmful pathogens *via* direct contact, inhaling and intake [1]. Their survival in an environment with an enhanced presence of microorganisms depends on a multicomponent mechanism system for protection. On one side there are the T and B cells against different antigens [2, 3]. In contrast to this primary defense mechanism are endogenous peptides which are constitutively expressed or induced in some cases and provide a rapid and effective protection against pathogens. Nowadays the number of pathogens that are resistant to the antibiotics used in treating them is rapidly increasing. This set the need of discovering novel molecules that could overcome pathogen drug resistance. Antimicrobial peptides have proven to be a good natural alternative to chemical antibiotics [4]. So far more than 750 different antimicrobial peptides have been isolated and characterized from different sources – insects, plants and animals, as well as humans [5-8]. Many of these active peptides have demonstrated antibacterial effects against a wide range of Gram-positive and Gram-negative bacteria [9, 10].

The antimicrobial peptides composed of less than 10 amino acid residues, comprising two groups of amino acid sequence as: group 1) arginine, lysine, valine, or isoleucine and group 2) tryptophan, phenylalanine or proline, were identified as new antibiotic peptides, useful in treating microbial or viral infections or in inactivating Gram-positive and -negative bacteria, protozoa, fungi and viruses [11].

Our group identified several peptides from the hemolymph of the garden snail *Helix lucorum* and marine snail *Rapana venosa* that exhibit a broad spectrum of antimicrobial activity against *Staphylococcus aureus, Staphylococcus epidermidis, Escherichia coli, Helicobacter pylori, Propionibacterium acnes* and others [12-14].

We here report on the antibacterial properties of new antimicrobial peptides isolated from the mucus of the snail *Cornu aspersum* against the Gram (-) bacteria *Pseudomonas aeruginosa* AP 9 and the Gram (+) *Brevibacillus laterosporus BT-271*.

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Pseudomonas bacteria are a serious problem in hospital environments since the infections species are the second-most common infectors in hospitalized patients. This might be because of the wide range of secreted proteins and their ability to cause serious diseases and bacteremia is increasing worldwide [14].

EXPERIMENTS

Purification of the fractions from the mucus of garden snail Cornu aspersum

The snails *Cornu aspersum* were collected in Bulgaria and the mucus was purified. The crude extract was separated using Millipore filters (10, 30 and 50 kDa) into three fractions: Fraction 1 (compounds with Mw between 0-10 kDa), Fraction 2 (Mw 30 - 50 kDa) and Fraction 3 (Mw above 50 kDa). Fraction 1 was then further separated using Millipore filters of 3 kDa into two fractions: Fraction A (compounds with Mw <3 kDa) and Fraction B (compounds with Mw > 3 kDa).

Molecular mass analysis and de novo sequencing of low molecular weight peptides

Isolated fraction A was lyophilized and analyzed by MALDI-TOF-TOF mass spectrometry on an AutoflexTM III, High Performance MALDI-TOF & TOF/TOF System (Bruker Daltonics) which uses a 200 Hz frequency-tripled Nd–YAG laser operating at a wavelength of 355 nm. Samples were prepared by mixing 2.0 μl of the sample with 2.0 μl of matrix solution (7 mg/ml of α-cyano-4-hydroxycinnamic acid (CHCA) in 50% ACN containing 0.1% TFA) and 1 μl of the mixture was spotted on a stainless steel 192-well target plate. They were dried at room temperature and subjected to mass analysis. A total of 3500 shots were acquired in the MS mode and collision energy of 4200 was applied. A solution of protein standards was used to calibrate the mass scale. The mass spectrometer was externally calibrated with a mixture of angiotensin I, Glu-fibrino-peptide B, ACTH (1–17), and ACTH (18–39). For MS/MS experiments, the instrument was externally calibrated with fragments of Glu-fibrino-peptide B. The mass values assigned to the amino acid residues are average masses.

Antibacterial assays of the peptides

The Gram-negative bacterial strain *Pseudomonas aeruginosa* AP9 and the Gram-positive *Brevibacillus laterosporus* BT271 were used in the antibacterial assays. They were chosen because of their antibiotic resistance. Beside that they were chosen as model pathogenic bacteria from different essential Gram-negative and Gram-positive groups, with specific relations towards antibiotics and xenobiotics, as well as specific permeability of their cell wall.

The strain *Pseudomonas aeruginosa* AP9 was isolated by Topalova (1989) and was characterized as resistant towards aryl-containing xenobiotics and aryl-containing antibiotics, possessing the ability to degrade these compounds.

The strain *Brevibacillus laterosporus* BT-271 was isolated and characterized by Topalova, 1982, as resistant against aryl-containing xenobiotics and antibiotics. The two bacteria were used as model Gram-negative and Gram-positive bacteria, with a potential to resist and to degrade aryl-containing antibiotics. Five cm^3 of standardized suspensions of bacterial cultures (OD_{430} = 0.600 abs were inoculated in Nutrient agar, mixed, and poured in petri dishes layers with depth of 2 mm. Petri dishes were left at room temperature (20ºC) to solidify. Fraction 1, Fraction A and Fraction B in concentrations 13.1, 6,9 and 5.3 mg/ml, respectively, determined by Lowry method, were tested *via* the agar well diffusion method. Three wells (3 repetitions) were drilled using a punch, and then each hollow was filled with 50 μl of each fraction and incubated for 24-72 hours at 37°C. The antibacterial effect was indicated in mm sterile zone around the wells. The antibacterial tests were also performed in a medium with no peptides added, to serve as a negative control.

RESULTS AND DISCUSSION

Several bioactive compounds with antimicrobial and antiviral activities were isolated from molluscs. It was found that the isolated fractions, containing compounds with different molecular masses from the mucus of garden snail *Cornu aspersum*, had a strong antibacterial effect against several strains of *Pseudomonas aeruginosa* and a weak effect against *Staphylococcus aureus*. It was published that the inhibition effect of separated fractions from *Helix aspersa* on the growth of bacteria was not caused by bacteriophages; the experiments indicated that the substance with Mw between 30 and 100 kDa in *H. aspersa* has antimicrobial activity [16].

We have analyzed the biochemically active peptides in the mucus of *Cornu aspersum* with molecular weights ranging from 1 to 10 kDa (Fraction 1) in a concentration of 13.1 mg/ml. This fraction was additionally subdivided into two fractions, Fraction A (Mw<3 kDa and concentration of 6,9 mg/ml) and Fraction B (Mw>3 kDa and concentration of 5.3 mg/ml), obtained after separation over Millipore filters with a cut-off of 3

kDa. Upon testing their antimicrobial activity in agar medium after incubation for 24-72 hours at 37°C, no inhibition effect was observed of isolated Fraction A (Fig. 1A, spot 1) and Fraction B (Fig. 1B, spot 3) against *Pseudomonas aeruginosa* AP9 and *Brevibacillus laterosporus* BT-271. However, applying agar well diffusion method, only Fraction 1 in concentration of 13.1 mg/ml appeared to generate a zone of 1.5 and 1.2 cm, respectively, inhibition of both *Pseudomonas aeruginosa* AP9 (Fig. 1A, spot 4) and *Brevibacillus laterosporus* BT271 (Fig. 1B, spot 4).

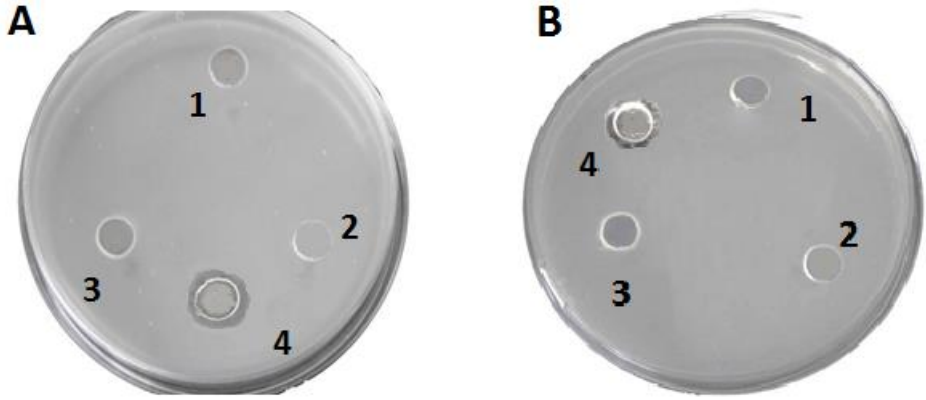

Fig.1. A) Antimicrobial assays against *Pseudomonas aeruginosa* AP9 and B) *Brevibacillus laterosporus* BT-271 of isolated Fraction A (spot 1) and Fraction B (spot 3). Antimicrobial assays via agar well diffusion method against *Pseudomonas aeruginosa* AP9 of isolated Fraction 1 for *Pseudomonas* and for *Brevibacillus*) (spot 4). The results were compared with control without any peptide fraction (spot 2). Each fraction was applied on the agar medium in 50 µl of the peptide solution. No activity is shown in any of the fractions.

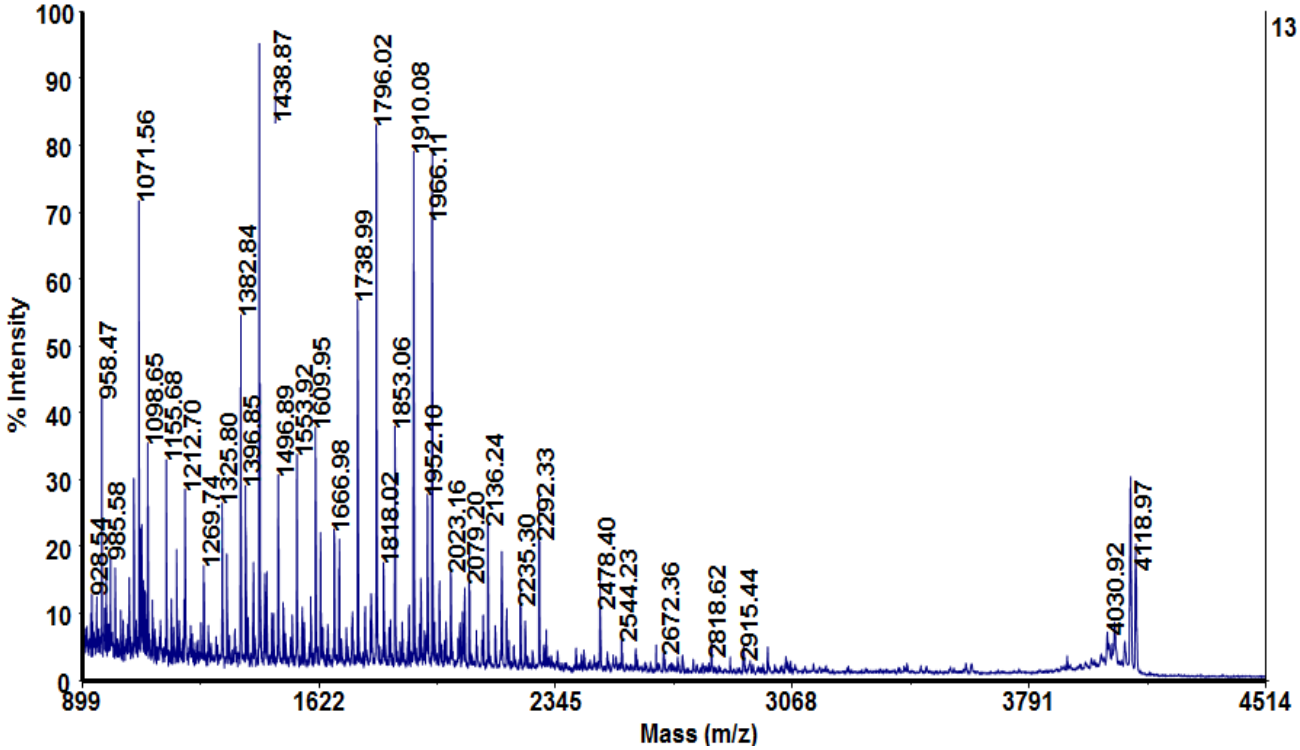

Fig. 2. MALDI-MS spectrum of Fraction A containing peptides with molecular masses between 1-5 kDa. The sample was measured by MALDI-TOF Ultraflex II (Bruker Daltonics, Bremen, Germany).

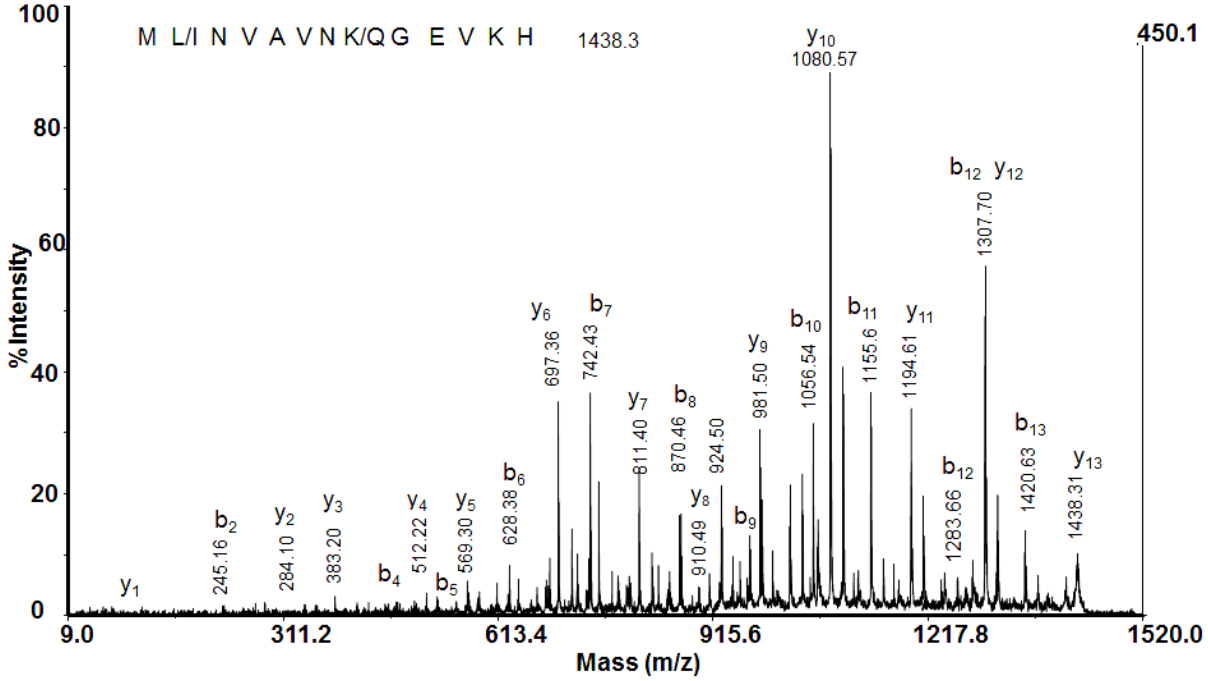

Fig. 3. *De novo* sequence by MALDI-MS spectrum (positive ion mode) of the peptide at m/z 1438.87. Standard peptide solution was used to calibrate the mass scale of the AutoflexTM III, High Performance MALDI-TOF & TOF/TOF Systems (Bruker Daltonics). All labeled fragment ions are y-ions (y13) and other fragment ions are indicated (b-ions).

To determine the minimal antimicrobial concentration of the active peptides the above described method was applied and the effect of Fraction 1 with 50 µl of decreasing dilutions (1/2, 1/4 and 1/8 from the starting concentration) was tested. The obtained results didn't show any activity for diluted peptide fractions against both bacteria. Only the initial concentration of Fraction 1 (13.9 mg/ml) suppressed the growth of the bacteria. To explain the mechanism of antimicrobial activity of mucus against *P. aeruginosa* AP9 and *B. laterosporus* BT271 Fraction 1 was analyzed by mass spectrometry. Several peptides were identified by their MS spectra (Fig. 2) with molecular masses determined by MALDI/MS (Table 1). The primary structure of six peptides with m/z at 1155.68, 1325.8, 1438.87, 1496.89, 1796.02 and 2292.33 Da were analyzed by their MALDI-MS/MS spectra. *De novo* sequence was derived for the antimicrobial peptide generated by *de-novo* explorer with the highest score value (b ion values and y ion values). Following the series of y- and b-ions the sequence ML/INVAVNQ/KGEVKH was deduced for Peptide 3 (Fig.3). The primary structures of six peptides from Fraction 1, shown in Table 1, were analyzed by their MALDI-MS/MS spectra.

The amino acid sequence of the peptides determined by MS/MS analysis showed that they are rich in glycine (Gly), proline (Pro), tryptophan (Trp) and isoleucine/leucine (Leu). A number of antibacterial and antifungal peptides rich in Cys, Pro, Ser or Gly residues have been isolated and characterized in the past from other mollusks like *Helix lucorum* [18]. Amino acid sequence comparisons of peptides with molecular masses between 1 and 3 kDa of the mucus of garden snail *C. aspersum* with peptides from the extract of *H. lucorum* determined by MS/MS analyses and peptides from the hemolymph of the marine snail *R. venosa* determined by Edman degradation show homology with this category of peptides. They are rich in Pro, Val, Trp and Gly residues.

Eleven proline-rich peptides exhibiting high antimicrobial activity against *S. aureus* and low activity against *K. pneumoniae* with molecular weights ranging from 3000 and 9500 Da were isolated from the hemolymph of *R. venosa* snails [18]. Antimicrobial activity exhibit also cysteine-rich peptides identified in the *Achatina fulica* snail and bivalve mollusc *Mytilus galloprovincialis* [17].

Table. 1 Amino acid sequence comparisons of peptides from Fraction 1 of the mucus of garden snail *Cornu aspersum* and extract of *H. lucorum* determined by MS/MS analyses with peptides from the hemolymph of the marine snail *Rapana venosa* determined by Edman degradation. Proline, valine, tryprophane and glycine residues are in boldface.

	Amino acid sequence of peptides	MALDI_MS $[M+H]^+$(Da)	pI	Mass (Da) calculated
CaP1	M **G** V **G** A V W N G H K	1155.68	8.52	1154.59
CaP2	M **L G G G** V **N** S **L** R **P P** K	1325.80	11.00	1324.73
CaP 3	M **L N** V **A** V **N K** G E V **K H**	1438.87	8.37	1437.78
CaP 4	N **L** V G G **L** S **G G G** R **G G A P G G G G**	1496.89	9.75	1495.75
CaP 5	**L L L** D G F **G G G L** L V E **H** D **P** G S	1796.02	4.02	1794.92
CaP6	M **P K** R A L **G G G** L V G G L L **G G G G** E **G** L L **V N**	2292.33	8.50	2291.24
HlP 1	V **P** K A R V M T S **G** K K K			
HlP2	R T V **P** F **G G** A E E E L L D L **G** V **G**			
HlP 3	**G** S **G G** A D D **G** C L **P** V Y R R F **P** A N M L			
Pep 2	L **G G** KS **P P** N Q P **S** I M T F D Y A K T N K			
Pep 4	S L **P P** T L E E E F N M K K M **G**			
Pept5	S **P P P G** E **S** K V D M **S** F N Y A L S N P A Q			
Pep 6	S **P P S** E Q L G K **S** F N F			
Pep7	A **P P P G** L **S** A G V			
Pep 8	A **P P P G** Y A M E S D **S** F S			
Pep 9	F **P P P G** E S A V D M S F F Y A L S N P			

CONCLUSIONS

This study showed that the mucus from the common brown garden snail *C. aspersum* contains bioactive compounds exhibiting a demonstrable antimicrobial activity against bacterial strains of *P. aeruginosa* AP9 and *B. laterosporus* BT271. The obtained results show higher inhibiting effect of the peptides with molecular weight between 1000 and 10000 Da on the growth of the bacterial strain *P. aeruginosa* AP9 (1.5 cm zone) than of *B. laterosporus* BT271 (1.2 cm zone). The effect may be explained with the amino acid sequence comparisons of peptides from Fraction A which are rich in proline, valine, tryprophane and glycine residues.

These results are preliminary testing of the general effect of the isolated peptides. Further, our efforts will be in direction to elucidate the mechanisms of the antibacterial effect in order to discover the best way for application of the peptides in pharmacy or cosmetics.

Understanding the function and mechanism of action of antibacterial peptides can contribute to the development of new anti-bacterial therapeutics.

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REFERENCES

1. World Health Organization. Antimicrobial Resistance: Global Report on Surveillance, (2014).
2. M. Totsika, *Curr. Med. Chem.*, **6(1)**, 30 (2016).
3. S.H. Marshall, G. Arenas, *Electron. J. Biotechnol*., vol. 6, n.3, Valparaíso dic. (2003)
4. C. D. Fjell, J. A. Hiss, R. E. Hancock, G. Schneider, *Nat. Rev. Drug. Discov*., **11**, 37 (2012).
5. M. Mahlapuu, J. Håkansson, L Ringstad, C. Björn, *Front Cell Infect Microbiol*., **6**, 194 (2016).
6. E. Mendez, A. Moreno, F. Colilla, F. Pelaez, G.G. Limas, R. Mendez, F. Soriano, M. Salinas, C. de Haro, Eur. *J. Biochem*., **194(2)**, 533 (1990).
7. F.J. Colilla, A. Rocher, E. Mendez, *FEBS Letters*, **270**, 191 (1990).
8. W. Liu, J.N. Hansen, *Appl. Environ. Microbiol.*, **56**, 2551 (1990).
9. D. Schnapp, C.J. Reid, A. Harris, *J. Pathol*., **186(1)**, 99 (1998).

10. T. Luders, G.A. Birkemo, G. Fimland, J. Nissen-Meyer, I.F. Nes, *Appl. Environ. Microbiol.*, **69(3)**, 1797 (2003).
11. Y. Shai, *Biochim. Biophys. Acta*, **1462**, 55 (1999).
12. M. R. Yeaman, N. Y. Yount, *Pharmacol. Rev.*, **55(1)**, 27 (2003).
13. K.V. Reddy, R.D. Yedery, C. Aranha, *Int. J. Antimicrob. Agents*, **24(6)**, 536 (2004).
14. S. Conti, G. Radicioni, T. Ciociola, R. Longhi, L. Polonelli, R. Gatti, T. Cabras, I. Messana, M. Castagnola, A. Vitali, *BBA – Biomembranes*, **1828(3)**, 1066 (2013).
15. D. Defer, F. Desriac, J. Henry, N. Bourgougnon, M. Baudy-Floc'h, B. Brillet, P. Le Chevalier, Y. Fleury, *Fish Shellfish Immunol.*, **34(6)**, 1439 (2013).
16. P. Dolashka, V. Moshtanska, V. Borisova, A. Dolashki, S. Stevanovic, T. Dimanov, W. Voelter, *Peptides*, **32(7)**, 1477 (2011).
17. P. Dolashka, A. Dolashki, S. Stevanovic, W. Voelter, J. Van Beeumen, I*nt. J. Curr. Microbiol. App. Sci.*, **4(4),** 1061 (2015).
18. P. Dolashka, A. Dolashki, L. Velkova, S. Stevanovic, L. Molin, P. Traldi, R. Velikova, W. Voelter, *J. BioSci. Biotechnol.*, SE/ONLINE: 147(2015).
19. J. Zhong, W. Wang, X. Yang, X. Yan, R. Liu, *Peptides*, **39**, 1 (2013).
20. P. Dolashka, *BG Useful model* 2097, (2015)
21. http://web.expasy.org/compute_pi/
22. T. Ebenhan, O. Gheysens, H. G. Kruger, J. R. Zeevaart, M. Sathekge, *Biomed. Res. Int.*, **2014**, 867381 (2014).
23. I. Zelezetsky, A. Tossi, *Biochim. Biophys. Acta*, **1758(9)**, 1436 (2006).
24. V. S. Paulsen, H. M. Blencke, M. Benincasa, T. Haug, J. J. Eksteen, O. B. Styrvold, M. Scocchi, K. Stensvåg, *PLoS One*., **8(1)**, e53326 (2013).
25. L. Zhang, R. Benz, R.E. Hancock, *Biochemistry*, **38(25)**, 8102 (1999).
26. D. M. Lorenzini, P.I. Jr. da Silva, A. C. Fogaça, P. Bulet, S. Daffre, *Dev. Comp. Immunol.*, **27(9)**, 781 (2003).
27. J.C. Sousa, B. R. Ferto, E. A. Gois, N.C. Fontenele-Cardi, J. E. Jr. Honório, K. Konno, M. Richardson, M. F. Rocha, A. A. Camargo, D. C. Pimenta, B. A. Cardi, K. M. Carvalho, *Toxicon.,* **54(1)**, 23 (2009).

СТРУКТУРА И АНТИБАКТЕРИАЛНА АКТИВНОСТ НА ПЕПТИДИ, ИЗОЛИРАНИ ОТ СЛУЗТА НА ГРАДИНСКИ ОХЛЮВ *Cornu aspersum*

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(Резюме)

Светът осигурява богат източник на пептиди с антимикробна, антивирусна и антитуморна активност. Пептидите и гликопептидите са разширяваща се група от структурно сложни антибактериални пептиди с ефект срещу Грам-положителни бактерии, които се използват в медицината, както за лечение на хора, така и на животни. Голям брой богати на пролин пептиди, изолирани от артроподи и мекотели, се считат за обещаващи кандидати за лечение на микробни инфекции и потискане на микробната резистентност. В представените изследвания ние докладваме за първичната структура и антимикробната активност на пептиди, изолирани от слузта на градинския охлюв *Cornu aspersum* в сравнение със сходни пептиди, изолирани от мекотели. Няколко пептида, с молекулни маси между 1 и 4 kDa, измерени чрез масспектрометричен анализ, бяха идентифицирани в слузта. Аминокиселинните им последователности са определени чрез MS/MS анализи, както представения пептид при m/z 1438.87 [M+H] (ML/INVAVNQ/KGEVKH). Фракцията, която съдържа пептиди с молекулни маси под 3 kDa, проявява антибактериална активност срещу Грам-отрицателен *Pseudomonas aeruginosa* AP9 и Грам-положителните *Brevibacillus laterosporus* BT271 бактериални щамове, като инхибиращият ефект на тези пептиди може да бъде обяснен с аминокиселинните остатъци. De novo фрагментирането на шест пептида разкри, че повечето от тях съдържат глицин, пролин, триптофан и валин, които са типични за пептиди с антимикробна активност.

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Effect of Pr(III) nitrate and Pr(III) complex on the accumulation of free radicals in rat blood serum

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The success of the cancer treatment depends on the good control over the oxidative stress (OS). Lanthanides and their compounds are promising anticancer agents due to the prooxidant activity of the Ln(III) cations. A complex formed by coordinatively bonding lanthanide ion with antioxidant ligand is promising strategy in the search of efficient anticancer medications. The 5-aminoorotic acid (HAOA) proved to be antioxidant at homeostatic pH. In this investigation, the effect of HAOA, Pr(III), and their complex, PrAOA, on the free radicals accumulation in rat blood serum was estimated. Pr(III) exhibited prooxidant properties. Below concentrations of 10^{-6} M both HAOA and PrAOA did not influence the $OH^{\bullet}$-initiated OS in the serum. Above this concentration, both compounds were antioxidants, the complex being weaker than the ligand. It was proposed that the antioxidant effect of PrAOA resulted from both antioxidant properties of the ligands and prooxidant properties of Pr(III).

Keywords: Praseodymium cation, 5-Aminoorotic acid, Praseodymium complex, Antioxidant properties, Free radicals accumulation, Rat blood serum.

INTRODUCTION

5-Aminoorotic acid (HAOA) is the amino-derivative of naturally occurring orotic acid (vitamin B_{13}), latter being intermediate in the biosynthesis of the pyrimidine nucleotides of DNA and RNA. Orotic acid (HOA) and its metal complexes have attracted growing attention in medicine [1-5], their structural and spectroscopic properties being comprehensively studied [6,7]. The HOA molecule is related to the molecules of uracil or thymine. Various theoretical studies on these types of molecules have been performed [8-10] and these results are very helpful in the characterization of newly synthesised complexes of HOA and HAOA. We have reported promising results on the significant cytotoxic activity of such types of Ln(III) complexes in different human cell lines [11-15]. The present work can be regarded as a continuation of our efforts in the bioinorganic chemistry of Ln(III) complexes with a number of biologically active ligands.

The coordination chemistry of lanthanides, relevant to the biological, biochemical and medical aspects, makes a significant contribution to understanding the basis of application of lanthanides, particularly in biological and medical systems. The lability of lanthanide complexes, strong oxyphilicity, very fast water exchange reaction, no directionality of lanthanide ligand bond and varying coordination number, all contribute towards lanthanide interaction with biomolecules. The ionic size of Ln(III) varies from one lanthanide to another; in addition, the ionic size of a particular lanthanide also varies significantly with the coordination number. Smaller size of chelating biologically active ligand can even suit larger lanthanides with lowered coordination number. Similarly small lanthanides can expand their coordination number and can form stable chelates with larger biomolecules. This can explain the different coordinating potential and biological behavior of different lanthanides under various physiological conditions. It has been reported by us earlier that Ln(III) ions attack cancer cells and induce apoptosis, considered as the core of the lanthanide potential as anticancer activity [11-15]. Along with apoptosis, there are several synergic related effects, ROS scavenging, cell protection, cytoskeleton stabilization and also immunologic enhancement [16-24].

Reactive oxygen species (ROS) are involved in the development of many diseases and the antioxidants are used to limit the ROS overproduction. Recently the antioxidant activity of Ln(III) complexes was determined by DPPH radical scavenging method. Some of these complexes exhibited more effective antioxidant activity than the respective ligands [17,18,23,24]. Their considerable radicals scavenging activity was explained by the chelation of organic molecules to rare earth ions. Ln(III) ions such as La(III), Sm(III), Eu(III) and Dy(III) exerted differential and

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selective effects on scavenging radicals of the biological system. Therefore, the studied Ln(III) complexes of biologically active derivatives of HAOA deserve to be further explored.

The aim of this work was to synthesize and characterize a new praseodymium(III) complex of HAOA and to evaluate its antioxidant activity. HAOA, $Pr(NO_3)_3.6H_2O$, and their complex (PrAOA) were investigated for possible involvement in the free radicals homeostasis, using rat blood serum as a model system. The effect of Pr(III) was estimated by subtracting the effect of the NO_3^- from this of $Pr(NO_3)_3.6H_2O$. The $OH^\bullet$-induced oxidative stress (OS) in the blood serum was initiated by the $Fe(II)/H_2O_2$/EDTA/ascorbate model system. The free radicals accumulation (FRA) was estimated spectrophotometrically, using the transformation of the yellow marker molecule 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) into the purple MTT formazan (λ=576 nm in phosphate buffer). The solvent effect on the compounds investigated was illustrated on the example of UV spectra in water and phosphate buffer.

MATERIALS AND METHODS

Chemicals and solutions: The compounds used for preparing the solutions for the synthesis were Sigma-Aldrich products, p.a. grade: $Pr(NO_3)_3.6H_2O$ and 5-aminoorotic acid. Latter was used as a ligand for the preparation of the metal complex.

The carbon, hydrogen and nitrogen contents of the compound were determined by elemental analysis.

Stock solutions of 10^{-3} M $NaNO_3$, $Pr(NO_3)_3.6H_2O$, HAOA and PrAOA in distilled water or PBS were prepared. Before the experiment they were diluted further to desired concentrations. Ice-cold aqueous solution of 3 mM $FeCl_2$, 3 mM H_2O_2, 0.3 mM EDTA and ice-cold aqueous solution of 4 mg/ml ascorbate were prepared and stored in ice bath. MTT was dissolved in distilled water to concentration of 3mg/ml.

Infrared spectra: The solid-state infrared spectra of the ligand and its Pr(III) complex were recorded in KBr in the 4000-400 cm^{-1} frequency range by FT-IR 113V Bruker spectrometer.

Raman spectra: The Raman spectra of HAOA and PrAOA were recorded with a Dilor microspectrometer (Horiba-Jobin-Yvon, model LabRam) equipped with 1800 grooves/mm holographic grating. The 514.5 nm line of an argon ion laser (Spectra Physics, model 2016) was used for the probes excitation. The spectra were collected in a backscattering geometry with a confocal Raman microscope equipped with an Olympus LMPlanFL 50× objective and with a resolution of 2 cm^{-1}. The detection of Raman signal was carried out with a Peltier-cooled CCD camera. Laser power of 100 mW was used in our measurements.

UV-spectra: UV spectra were recorded in quartz cuvette, using Shimadzu 1600 apparatus equipped with software package, within 400-200 nm, at very low speed (step of 0.5 nm). The instrumental errors were eliminated by recording the spectrum of solvent (water or PBS) against solvent and subtracting this spectrum from the experimental spectrum of the corresponding solution. By scanning of the same spectrum three times we found that the experimental error for λ was ±1.0 nm in position and ±0.001 a.u. in intensity.

Blood serum preparation: The serum was separated from rat total blood as described in [25,26] and stored at -86°C for biochemical analysis. The proteins concentration in the serum was measured [27]. Serum containing 1 mg/ml protein was left in contact with solution investigated (10^{-7}, 10^{-6}, 10^{-5} or 10^{-4} M) or pure solvent (0 M, controls). Five test tubes with identical content were prepared for each concentration. All flasks were kept at 4°C for 30 min. Before FRA assay, the samples were conditioned to room temperature.

FRA assay: The content of each flask was transferred in a quartz cuvette, where MTT was introduced and $OH^\bullet$-induced OS was generated. The relative change of the absorption at 576 nm was measured for 10 minutes, using the kinetics software of the Shimadzu 1600, against solvent.

For FRA in the serum alone: one ml of the sample cuvette contained serum corresponding to 1 mg proteins, 0.10 ml MTT, 0.1 ml $Fe(II)/H_2O_2$/EDTA/ascorbate, and PBS to 1 ml; the control measurement was performed in absence of $Fe(II)/H_2O_2$/EDTA/ascorbate; for the background the serum was omitted.

For FRA in the serum, in presence of a solution, in 1 ml of the sample cuvette: serum corresponding to 1 mg proteins, 0.10 ml MTT, 0.1 ml of the solution of desirable concentration, 0.10 ml $Fe(II)/H_2O_2$/EDTA/ascorbate, and PBS to 1 ml; the control measurement was done in absence of the solution of the compound investigated; for the background measurement the serum was omitted.

To estimate the effect of Pr(III) FRA of the serum was measured in presence of $NaNO_3$ in concentrations adjusted to have the same NO_3^- content, as these in the corresponding $Pr(NO_3)_3.6H_2O$ solutions.

The $OH^\bullet$ initiated free radicals accumulation in the blood serum alone, and in the presence of the

compounds investigated, was assessed using the formula:

$$FRA = \frac{\Delta A_{sample} - \Delta A_{control}}{\Delta A_{background}} * 100,$$

where FRA - free radicals accumulation in %; ΔA - the relative change of the absorption at 576 nm for 10 min for the control, sample and background.

Data management: Average values and standard deviations of FRA at each concentration of the compounds investigated were calculated. Concentration effect was statistically verified using One Way ANOVA. To evaluate the Pr(III) effect, samples of nitrates containing and not containing Pr(III) were compared. The effects of Pr(III) and PrAOA on the FRA of the blood serum were statistically verified by considering different standard deviations and two-tailed P distribution (non-parametric *t*-test with Welch's correction). The Pr(III) effect was verified by comparing data for praseodymium and sodium nitrates solutions with the same amounts of NO_3^-. The effect of PrAOA was verified by comparing FRA of the complex with this of HAOA for each concentration.

RESULTS

The complex was synthesized by reaction of Pr(III) salt and the ligand, in amounts equal to metal: ligand molar ratio of 1:3. The synthesis was made in different ratios (1:1, 1:2, 1:3) but in all the cases the final product was with the composition 1:3. The complex was prepared by adding an aqueous solution of Pr(III) salt to an aqueous solution of the ligand, subsequently raising the pH of the mixture gradually to ca. 5.0 by adding dilute solution of sodium hydroxide. The reaction mixture was stirred with an electromagnetic stirrer at 25 °C for one hour. At the moment of mixing of the solutions, precipitate was obtained. The precipitate was filtered (pH of the filtrate was 5.0), washed several times with water and dried in a desiccator to constant weight. The obtained complex was insoluble in water, methanol and ethanol, but well soluble in DMSO.

Reaction of Pr(III) and 5-aminoorotic acid afforded a complex which was found to be quite stable both in solid state and in solution. The new Pr(III) complex was characterized by elemental analysis. The content of the metal ion was determined after mineralization. The used spectral analyses confirmed the nature of the complex.

The data of the elemental analysis of the Pr(III) complex serve as a basis for the determination of its empirical formula and the results are presented below. The elemental content of the Pr(III) complex of HAOA ($Pr(AOA)_3.H_2O$) is shown as % calculated/found: C= 26.90/26.64; H= 2.09/2.29; N= 18.83/19.00; H_2O= 2.69/2.25; Pr= 21.07/20.76, where HAOA= $C_5N_3O_4H_5$ and AOA= $C_5N_3O_4H_4^-$.

In our previous work the geometry of 5-aminoorotic acid was computed and optimized with the Gaussian 03 program employing the B3PW91 and B3LYP methods with the 6-311++G** and LANL2DZ basis sets [29]. In the present study the binding mode of the HAOA ligand to Pr(III) ions was elucidated by recording the IR and Raman spectra.

The UV spectra of 10^{-4} M solutions in water and PBS (pH 7.45) of the compounds investigated are seen in Fig.1.

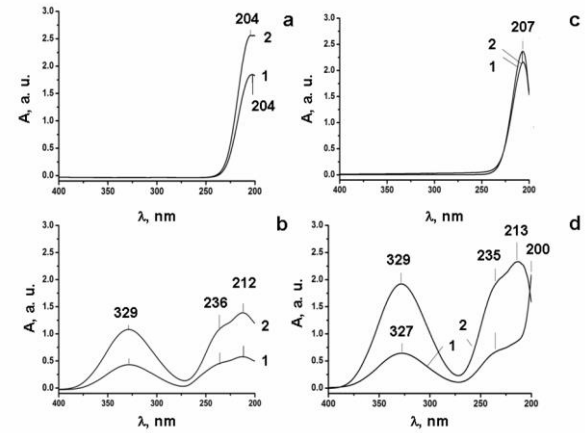

Fig. 1. UV spectra of aqueous (a,b) and PBS (c,d) solutions of: 3×10^{-4} M $NaNO_3$ (a,c-1), and 10^{-4} M solutions of $Pr(NO_3)_3.6H_2O$ (a,c-2), HAOA (b,d-1) and PrAOA (b,d-2).

Spectra were resolved based on literature data [4-9]. The solvent effects on UV spectra of the compounds were observed by comparing aqueous (Fig. 1a,b) with PBS (Fig. 1c,d) solutions. Spectra of nitrates (Fig.1a,c) indicated PBS as a solvent diminishing the relative differences within effects of Na(I) and Pr(III) on NO_3^-. Therefore, in PBS (pH 7.45) it might be expected similar reactivity of NO_3^- of both praseodymium and sodium nitrates toward free radicals produced in blood serum. As Na(I) does not affect the free radicals accumulation, any difference within FRA of sodium compared to praseodymium nitrate might be associated with Pr(III) ions. The intensities of UV spectra of PBS solutions of HAOA and PrAOA (Fig.1d) were significantly higher than these of the corresponding aqueous solutions (Fig.2c), this effect being stronger for the PrAOA spectrum (spectra 2) than for the HAOA spectrum (spectra 1), with no significant shift in positions of λ. The UV spectra of HAOA in water and PBS differed in structure too (Fig.1c,d, spectra 1). A new very intensive and sharp band at about 200 nm was observed in the

PBS solution. This, along with the smaller relative increase in intensities of the rest of the characteristic bands for 5-aminoorotic acid, might be an indication of dissociation of some HAOA molecules in PBS.

The Gauss deconvolution of the experimental spectra is presented in Fig.2.

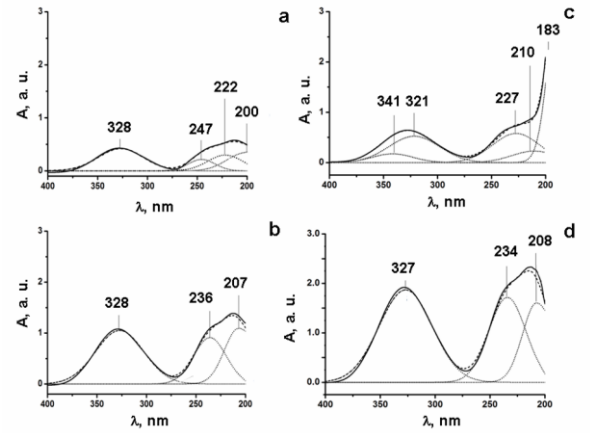

Fig. 2. Gauss deconvolution of the UV spectra of 10^{-4} M aqueous (a, b) and PBS (c, d) solutions of: a- 5-aminootoric acid (HAOA, a, c), and b- Pr complex (PrAOA, b, d). The Gauss components of the spectra are drawn as dotted lines; spectra reproduced the real spectrum with R^2>0.996.

The UV spectrum of 0.1 mM aqueous solution of HAOA (Fig. 2a) was presented as superposition of 4 components, associated with the characteristic bands of 5-aminoorotic acid [4-9]. The Gauss deconvolution of the spectrum seen in Fig. 2c revealed two new components (183 nm and 341 nm). The strong and sharp component at 183 nm was typical for the C=O vibration in the ionized COO^- group, while the component at 341 nm might result from a shift in the $\pi \rightarrow \pi^*$ transition in the aryl ring, due to the ionization:

$$HAOA + H_2PO_4^- \rightarrow AOA^- + H_3PO_4$$

Yet, bands typical for non-dissociated molecules are present in the spectrum (321, 227 and 210 nm).

The effects of $Pr(NO_3)_3$, $NaNO_3$, HAOA and PrAOA on the $OH^\bullet$ induced free radicals accumulation in rat blood serum are illustrated in Fig.3.

In Fig. 3a, curve 1 shows that in the presence of NO_3^- FRA slowly but gradually increases with increasing of the concentration. The effect of Pr(III) is observed in the relatively higher FRA than this in the presence of Na(I) at any given concentration (p=0.0019, 0.0006, 0.0002 and <0.0001 for 10^{-7}, 10^{-6}, 10^{-5} and 10^{-4} M, respectively). In presence of HAOA and PrAOA FRA in the serum decreased with concentration (Fig. 3b). The relative differences between HAOA and PrAOA were significant at concentrations above 10^{-5} M (p= 0.5000 and 0.3081 for 10^{-7} and 10^{-6} M, and <0.0001 for 10^{-5} M and 10^{-4} M, respectively).

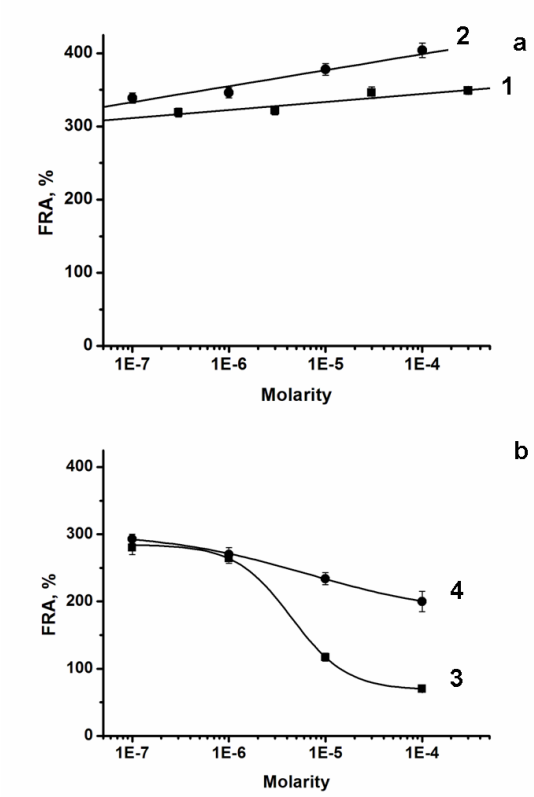

Fig. 3. Effect of nitrates (a) and HAOA containing solutions (b) on the $OH^\bullet$-induced free radicals accumulation (FRA) in rat blood serum: 1- $NaNO_3$ solutions, 2- $Pr(NO_3)_3.6H_2O$ solutions, 3- HAOA solutions, 4- PrAOA solutions

DISCUSSION

Pr(III) accelerated the $OH^\bullet$ induced free radicals accumulation in rat blood serum, while the 5-aminoorotic acid and its Pr(III) complex exhibited antioxidant effect in the same model system, this effect being stronger in presence of HAOA than in presence of PrAOA. The diminishing FRA in the blood serum in the presence of a compound is an indication of its antioxidant activity. From this viewpoint, the NO_3^- and Pr(III) acted as prooxidants, while HAOA and PrAOA showed antioxidant properties.

The UV spectra of the compounds indicated that the medium influenced the state of the dissolved molecules. HAOA partially dissociated, and the energy of some electron transitions in the rest of the undissociated molecules increased. This could increase the chance for interaction with free radicals, resulting in antioxidant activity of the molecule in homeostatic conditions. The Pr(AOA) complex exhibited weaker antioxidant activity than HAOA. The UV spectra of the compound indicated that this complex is stable in homeostatic medium. As no dissociation of the complex was observed in

the UV spectra of PrAOA, the lower antioxidant activity of the complex compared with this of HAOA alone, may be related with the effect of Pr(III) on the partial charges distribution and dipole moment in the ligands.

CONCLUSIONS

1. The complex of Pr(III) with 5-aminoorotic acid has been synthesized and characterized by elemental, UV-VIS and vibrational spectral analyses, including IR and Raman spectra.

2. $Pr(NO_3)_3.6H_2O$ and $NaNO_3$ with same content of nitrate ions increased *in vitro* the free radicals accumulation in rat blood serum, in which the oxidative stress was induced by Fe(II)/H_2O_2/EDTA/ascorbate model system. The stronger effect of Pr(III) than this of the Na(I) nitrate was related with prooxidant effect of Pr(III).

3. 5-aminoorotic acid and its complex with Pr(III) decreased *in vitro* the free radicals accumulation in rat blood serum, in which the oxidative stress was induced by the Fe(II)/H_2O_2/EDTA/ascorbate model system.

3. Different solvent effects of water and PBS (pH 7.45) on the UV spectra of the compounds investigated, were detected, and related with their *in vitro* antioxidant properties.

REFERENCES

1. H. Van der Meersch, *J. Pharm. Belg.,* **4,** 97 (2006).
2. F.L. Rosenfeldt, *Cardiovasc. Drugs Ther.*, **12,** 147 (1998).
3. M.T. MacCann, M.M. Thompson, I.C. Gueron, M. Tuchman, *Clin. Chem.,* **41,** 739 (1995).
4. NPL Kale&Laby, Table of physical and chemical constants. Chapter 3: Chemistry, Section 3.8. Molecular spectroscopy; Subsection 3.8.7. UV-Spectroscopy (2015). http://www.kayelaby.npl.co.uk/chemistry/3_8/3_8_7.html.
5. F.-X. Shmidt, Encyclopedia of Life Science, MacMillan Publishers Ltd, Nature Publishing Group, (2001).
6. I. Kostova, M. L. Valcheva-Traykova, *Appl. Organomet. Chem.*, **29**, 815 (2015).
7. I. Kostova, M. L. Valcheva-Traykova, *J. Coord. Chem*., **68**, 4082 (2015).
8. I. Kostova, M. L. Valcheva-Traykova, S. Balkansky, *J. Iran. Chem. Soc.*, **13**, 891 (2016).
9. M. Valcheva-Traykova, I. Kostova, *Int. J. Chem.*, **5**, 1 (2016).
10. D. Michalska, K. Hernik, R. Wysokinski, B. Morzyk-Ociepa, A. Pietraszko, *Polyhedron*, **26,** 4303 (2007).
11. P. Ruasmadiedo, J.C. Badagancedo, E. Fernandez Garcia, D.G. Dellano, C.G. de los Reyes Gavilan, *J. Food Protect.,* **59,** 502 (1996).
12. I. Kostova, N. Peica, W. Kiefer, *Vibr. Spectrosc.*, **44,** 209 (2007).
13. I. Kostova, S. Balkansky, J. Mojzis, *Int. J. Curr. Chem.,* **1,** 271 (2010).
14. W.B. Person, K. Szczepaniak, In: *Vibrational Spectra and Structure*, J.R. Durig (ed.), p. 239, Elsevier, Amsterdam (1993).
15. H. Rostkowska, K. Szczepaniak, M.J. Nowak, J. Leszczynski, K. Kubulat, W.B. Person, *J. Am. Chem. Soc.*, **112,** 2147 (1990).
16. I.R. Gould, I.H. Hillier, *J. Chem. Soc. Perkin Trans*, **2,** 329 (1990).
17. I. Kostova, T. Stefanova, *J. Rare Earths*, **28,** 40 (2010).
18. I. Kostova, Ts. Stefanova, *J. Trace Elem. Med. Biol.*, **24,** 7 (2010).
19. I. Kostova, M. Traykova, *Med. Chem.*, **2,** 463 (2006).
20. I. Kostova, G. Momekov. *J. Coord. Chem.*, **61,** 3776 (2008).
21. I. Kostova, Ts. Stefanova. *J. Coord. Chem.*, **62,** 3187 (2009).
22. J. Kaur, Y. Tsvetkova, K. Arroub, S. Sahnoun, F. Kiessling, S. Mathur, *Chem. Biol. Drug Design*, **89,** 269 (2017).
23. A.M. Ajlouni, Z.A. Taha, K.A. Al-Hassan, A.M. Anzeh, *J. Luminesc*., **132**, 1357 (2012).
24. P. X. Xi, Z. H. Xu, X. H. Liu, F. J. Chen, L. Huang, Z. Z. Zeng, *Chem. Pharmac. Bull*., **56**, 541 (2008).
25. Y. Peng, J. X. Hu, H. Lu, R. M. Wilson, M. Motevalli, I. Hernández, W. P. Gillin, P. B. Wyatt, H. Q. Ye, *RSC Advances*, **7,** 128 (2017).
26. Z. Ahmed, R.E. Aderne, J. Kai, J.A. Resende, M. Cremona, *Polyhedron*, **119,** 412 (2016).
27. M. Karbowiak, C. Rudowicz, T. Nakamura, R. Murakami, T. Ishida, *Chem. Phys. Lett*., **662,** 163 (2016).
28. E.V. Cherkasova, E.V. Peresypkina, A.V. Virovets, T.G. Cherkasova, *Russ. J. Inorg. Chem*., **61**, 174 (2016).
29. M.N. Gueye, M. Dieng, I.E. Thiam, D. Lo, A.H. Barry, M. Gaye, P. Retailleau, *South Afr. J. Chem*., **70**, 8 (2017).
30. T.A. Antonenko, D.B. Shpakovsky, Y.A. Gracheva, T.V. Balashova, A.P. Pushkarev, M.N. Bochkarev, E.R. Milaeva, *Inorg. Chim. Acta*, **455**, 276 (2017).
31. Collection of serum from blood, in: http://vlab.amrita.edu/?sub=3&brch=69&sim=196&cnt=2
32. M. K. Tuck, D. W. Chan, D. Chia, A. K. Godwin, W. E. Grizzle, K. E. Krueger, W. Rom, M. Sanda, L. Sorbara, S. Stass, W. Wang, D. E. Brenner, Standard Operating Procedures for Serum and Plasma Collection: Early Detection Research Network Consensus Statement Standard Operating Procedure Integration Working Group. *J. Proteome Res.*, **8**(1), 113 (2009). doi: 10.1021/pr800545q
33. How are extinction coefficients determined for Proteins? in: http://www.biosyn.com/tew/How-is-Extinction-Coefficient-Determined-for-Proteins.aspx
34. I. Kostova, N. Peica, W. Kiefer, *Chem. Phys.*, **327,** 494 (2006).

ЕФЕКТИ НА PR((III) НИТРАТ И PR(III) КОМПЛЕКС ВЪРХУ НАТРУПВАНЕТО НА СВОБОДНИ РАДИКАЛИ В КРЪВЕН СЕРУМ НА ПЛЪХ

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Постъпила на коригирана на

(Резюме)

Успехът при терапия на рака зависи от качеството на контрола върху оксидативния стрес (ОС). Поради прооксидантната активност на лантанидните йони, лантанидите и техните съединения са перспективни антиракови агенти. Синтезът на La(III) комплекси с антиоксидантни лиганди е атрактивна стратегия при търсенето на антиракови лекарства. 5-Аминооротовата киселина (HAOA) е доказан антиоксидант при хомеостатично pH. В настоящата работа е показано влиянието на HAOA, Pr(III) и техния комплекс, PrAOA, върху натрупването на свободни радикали в кръвен серум на плъх. Pr(III) проявява прооксидантни свойства. В концентрации под 10^{-6} M, HAOA и PrAOA не променят нивото на $OH^{\bullet}$-индуцирания ОС в серума. Над тази концентрация двете съединения са антиоксиданти, с по-слаб ефект при комплекса в сравнение с този на лиганда. Предположено е, че антиоксидантният ефект на PrAOA е кумулативен резултат на прооксидантните свойства на Pr(III) и антиоксидантните свойства на HAOA.

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Radical scavenging activity toward 2,2-diphenyl-1-picrylhydrazyl and hydroxyl radicals of 5-aminoorotic acid and its Ga(III) complex

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Along with anti-tumor activity, flexible control over oxidative stress (OS) levels is a desirable quality of any anticancer drug. Radicals scavenging activity (RSA) toward 2,2-diphenyl-1-picrylhydrazyl radical (DPPH$^{\bullet}$) is widely used to evaluate the ability to eliminate free radicals by donating hydrogen. 5-aminoorotic acid (HAOA) is known to have antioxidant properties and has been used as a ligand in lanthanide(III) complexes possessing anticancer activity in cell cultures. Ga(III) salts are known for their anticancer activity. Thus, the Ga(III) complex with HAOA, GaAOA, might be a promising anticancer agent with antioxidant properties that have not been explored so far. In the present work, the UV spectra and RSA of HAOA and GaAOA toward DPPH$^{\bullet}$ and OH$^{\bullet}$ were evaluated and discussed. The stereochemistry of HAOA and its Ga(III) complex was evaluated, and compared by means of IR, Raman IR and Raman spectral data. Two factors affected the UV spectra of the molecules: their arrangement (steric properties) and their interaction with the solvent. As far as the RSA was determined in absolute ethanol (for DPPH$^{\bullet}$) and in water (for OH$^{\bullet}$), the UV spectra of the molecules in water and ethanol were compared and discussed. The hypochromicity in the UV spectra of GaAOA, compared to the expected intensities, indicated an arrangement of the ligands that diminished the dipole moment. The RSA of HAOA and GaAOA towards both radicals was concentration-dependent. GaAOA, at the lowest concentration in ethanol, exhibits signs of dissociation, manifested in an anomalous RSA increase. That demonstrates the potential of GaAOA for a controlled release of the antioxidant ligands.

Keywords: Ga(III) complex with 5-aminoorotic acid, Antioxidant activity, DPPH radical, OH radical, 5-Aminoorotic acid.

INTRODUCTION

The role of the reactive oxygen species (ROS) and oxidative stress (OS) in carcinogenesis [1-3] and cancer therapy [4,5] is very complex and intensively investigated. OS is involved in carcinogenesis *via* several pathways, but it is also able to kill malignant cells by altering their redox homeostasis. Disturbance of the redox homeostasis of the cancerous cells by using metal complexes is a promising approach in cancer therapy [5]. Metallodrugs based on Ga(III) are intensively investigated as promising anticancer agents [6,7], due to strong analogy between Ga(III) and Fe(III) in terms of ionic radius, electron affinity, electronegativity, coordination geometry, and Lewis base affinity. Ga(III) does not change its valent state in physiological conditions, unlike Fe(III). As the malignant cells have a greater requirement for iron than normal cells do [8], strategies to disrupt the iron-dependent metabolic pathways in malignant cells by introduction of Ga(III) are promising in cancer treatment. Lanthanide(III) complexes of 5-aminoorotic acid (HAOA) showed both antioxidant and anticancer activities [9-11]. Thus, the Ga(III) complex with HAOA might be a promising anticancer agent with antioxidant properties, that have not been explored so far. The hydroxyl radical, OH$^{\bullet}$, is the most reactive among ROS. It is formed as a result of interaction between H_2O_2 and free metal ions with variable valent states *via* the Fenton reaction [12]. The OH$^{\bullet}$ radical is easily recombined by molecules capable of donating hydrogen. The ability of GaAOA to donate hydrogen and react with OH$^{\bullet}$ has not been explored so far. The hydrogen-donor's total antioxidant capacity is often estimated by monitoring the radicals scavenging activity (RSA) toward the stable 2,2-diphenyl-1-picrylhydrazyl radical (DPPH$^{\bullet}$) [13-15], while the interaction with hydroxyl radicals may be estimated in the presence of various OH$^{\bullet}$ generating model systems [16-22].

In the present work, the ability to donate hydrogen and the interaction with hydroxyl radicals of HAOA and GaAOA were estimated by measuring the Radical Scavenging Activities toward DPPH$^{\bullet}$ and OH$^{\bullet}$. The solvent effects of H_2O and C_2H_5OH on the investigated molecules were observed by recording the UV spectra of the solutions in both media. The interactions of HAOA and GaAOA with solvent molecules were visualized by steric energy minimization in

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presence of one molecule of solvent per ligand, using MM2 subroutine of a ChemOffice program package.

EXPERIMENTAL PART

The compounds used for preparing the solutions in this investigation were of finest purity (Sigma-Aldrich products), including $Ga(NO_3)_3$ and 5-aminoorotic acid. The latter was used as a ligand for the preparation of the metal complex.

The carbon, hydrogen and nitrogen contents of the compound were determined by elemental analysis.

The solid-state infrared spectra of the ligand and its Ga(III) complex were recorded in KBr in the 4000-400 cm^{-1} frequency range by FT-IR 113V Bruker spectrometer.

The Raman spectra of HAOA and its new Ga(III) complex were recorded with a Dilor microspectrometer (Horiba-Jobin-Yvon, model LabRam) equipped with a 1800 grooves/mm holographic grating. The 514.5 nm line of an argon ion laser (Spectra Physics, model 2016) was used for the probes excitation. The spectra were collected in a backscattering geometry with a confocal Raman microscope equipped with an Olympus LMPlanFL 50× objective and with a resolution of 2 cm^{-1}. The detection of Raman signal was carried out with a Peltier-cooled CCD camera. Laser power of 100 mW was used in our measurements.

Bi-distilled water and 96% ethanol were used as solvents and reaction media. Standard 10^{-3} M aqueous and ethanol solutions of both HAOA and GaAOA were prepared, and for the purpose of the experiment were further diluted to concentrations of 10^{-4} M, 10^{-5} M, 10^{-6} M and 10^{-7} M. Aqueous solutions, one of them containing 3 mM $FeCl_2$, 3 mM H_2O_2, and 0.3 mM EDTA, and another containing 4 mg/ml ascorbate, were prepared prior to the experiment and kept in ice bath. Standard 0.05 M $DPPH^{\bullet}$ solution was prepared in pure ethanol, covered with aluminum foil and kept at -25°C in a freezer. Before each experiment, this solution was diluted with 96% ethanol to give absorption between 0.7 and 0.9 a.u. at 517 nm.

All spectrophotometric measurements were performed using Shimadzu 1600 UV-VIS spectrophotometer (quartz cuvette) equipped with software, connected to a PC.

Assay for RSA toward $DPPH^{\bullet}$: The relative decrease in intensity of the signal at 517 nm (characteristic band for $DPPH^{\bullet}$) was monitored for 30 min, using the kinetics software of the apparatus. The absorption at 517 nm was recorded every 5 min. RSA (%) was determined using the formula:

$$RSA = \frac{A_{blank} - (A_{sam.} - A_{contr.})}{A_{blank}} * 100,$$

A_{blank} being the absorbance due to the presence of the sample's solvent in $DPPH^{\bullet}$ solution (2 ml $DPPH^{\bullet}$ solution and 0.02 ml sample's solvent), $A_{contr.}$ is the absorbance due to the sample alone (0.02 ml sample solution in 2 ml ethanol), and $A_{sam.}$ is the absorbance due to interaction of the sample with $DPPH^{\bullet}$ (2 ml $DPPH^{\bullet}$ solution and 0.02 ml sample solution). Data are presented as RSA (%) *vs* time. For further simplification, "RSA($DPPH^{\bullet}$)" will be used instead of "RSA toward $DPPH^{\bullet}$".

RSA toward Fe(II)-induced $OH^{\bullet}$ assay: $OH^{\bullet}$ was produced by the model system Fe(II)/H_2O_2/EDTA/ascorbate, in aqueous medium. MTT transformation into formazan was used as marker for the free radicals accumulation in the solution. The relative increase of the intensity at 578 nm (characteristic for the MTT formazan) was monitored each minute, for 10 minutes. RSA was evaluated using the formula:

$$RSA = \frac{\Delta A_{blank} - (\Delta A_{sam.} - \Delta A_{contr.})}{\Delta A_{blank}} * 100,$$

ΔA being the relative change of the absorbance at 578 nm for 10 min. ΔA_{blank} corresponded to ΔA in the presence of the $OH^{\bullet}$ - producing model system alone (0.05 ml Fe(II)/H_2O_2/EDTA, 0.05 ml ascorbate, 0.2 ml MTT, and H_2O to 2 ml), ΔA_{contr} describes the relative change of A(578 nm) in the presence of the sample solution and MTT (0.2 ml MTT, 0.2 ml sample solution and H_2O to 2.0 ml), and $\Delta A_{sam.}$ is the relative change of the 578 signal due to interaction between the free radicals in the model system and the sample solution (0.05 ml Fe(II)/H_2O_2/EDTA, 0.05 ml ascorbate, 0.2 ml sample solution, 0.2 ml MTT, and H_2O to 2.00 ml). For simplification in the text "RSA($OH^{\bullet}$)" will be used instead of "RSA toward $OH^{\bullet}$".

UV-spectral analysis: The UV-spectra were recorded within 400-200 nm, at very slow speed (λ_{step}= 0.5 nm) after base correction for the spectrum of the solvent in the cuvette. The instrumental errors were evaluated by scanning the spectrum of the solvent, with solvent base correction. The experimental error limits in position and absorbance of λ in the UV spectra were estimated by recording each spectrum for three times. These were found to be within ± 1 nm for λ position and ±0.001 a.u. for absorption.

Data management and presentation: For each concentration of each compound, RSA were

calculated based on 5 parallel measurements. Average values and standard deviations were calculated. Relative changes within the experimental error limits were not discussed. The concentration effects on RSA of the solutions of HAOA and GaAOA were statistically verified using One-way ANOVA, followed by Bonferoni post-test. The Bartlett test verified that all standard deviations belong to the same population. Differences due to the chemical composition at same concentration of solutions were statistically verified using non-parametric *t*-test with Welch correction.

ChemOffice program package v. 3.01 was used to build molecule models of the compounds investigated, as well as to illustrate their interactions with solvent molecules. The solvent effect on the molecular geometry was illustrated by presenting interaction of one solvent molecule per one HAOA or AOA ligand.

RESULTS

The complex was synthesized by reaction of Ga(III) salt and the ligand, in amounts equal to metal: ligand molar ratio of 1:3. The synthesis was made in different ratios (1:1, 1:2, 1:3) but in all the cases the final product was with the composition 1:3. The complex was prepared by adding an aqueous solution of Ga(III) to an aqueous solution of the ligand subsequently raising the pH of the mixture gradually to ca. 5.0 by adding dilute solution of sodium hydroxide. The reaction mixture was stirred with an electromagnetic stirrer at 25 °C for one hour. At the moment of mixing of the solutions, precipitate was obtained. The precipitate was filtered (pH of the filtrate was 5.0), washed several times with water and dried in a desiccator to constant weight. The obtained complex was insoluble in water, methanol and ethanol, but well soluble in DMSO.

Reaction of Ga(III) and 5-aminoorotic acid afforded a complex which was found to be quite stable both in solid state and in solution. The new Ga(III) complex was characterized by elemental analysis. The content of the metal ion was determined after mineralization. The used spectral analyses confirmed the nature of the complex.

The data of the elemental analysis of the Ga(III) complex serve as a basis for the determination of its empirical formula and the results are presented below. The elemental content of the Ga(III) complex of HAOA ($Ga(AOA)_3.H_2O$) is shown as % calculated/found: C= 30.10/30.04; H= 2,.34/2.55; N= 21.07/21.16; H_2O= 3.01/3.28; Ga= 11.66/11.19, where HAOA= $C_5N_3O_4H_5$ and AOA= $C_5N_3O_4H_4^-$.

In our previous work the geometry of 5-aminoorotic acid was computed and optimized with the Gaussian 03 program employing the B3PW91 and B3LYP methods with the 6-311++G** and LANL2DZ basis sets [23]. In the present study the binding mode of the HAOA ligand to Ga(III) ions was elucidated by recording the IR and Raman spectra.

The stability of HAOA and GaAOA, dissolved in water and ethanol, was evaluated by recording the spectra of their solutions. HAOA was stable in both solvents. All aqueous solutions, and ethanol solutions of GaAOA above 10^{-6} M, were stable too. UV spectra were resolved according to data in existing literature [24-28]. Characteristic bands for 5-aminoorotic acid (individual, and as a ligand) are seen in all the spectra, as illustrated in Fig. 1 for the 10^{-4} M concentrations.

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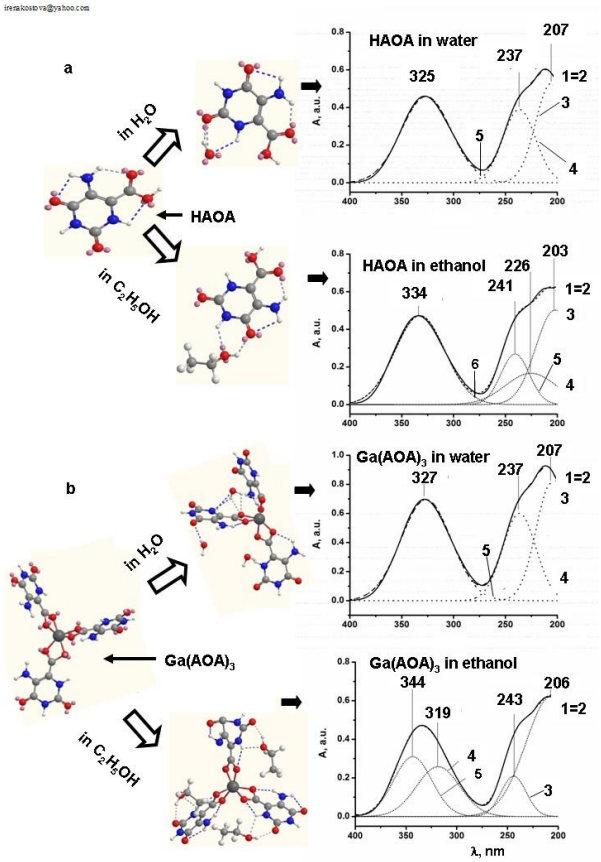

Figure 1. Solvent effects of water and ethanol on the geometry and UV-spectra of HAOA (a) and GaAOA (b).

In the UV spectrum of 10^{-6} M GaAOA in ethanol some bands indicating ionization (Fig. 2) were observed.

After subtraction of the HAOA spectrum (Fig. 2, spectrum 1) from this of GaAOA (Fig. 2, spectrum 2), a new component appeared (spectrum 3), with a sharp, intensive maximum at about 206 nm, and broad, low-intensive band at 376 nm. In agreement with literature, these new bands may be associated

with the appearance of ionized AOA^- in the solution.

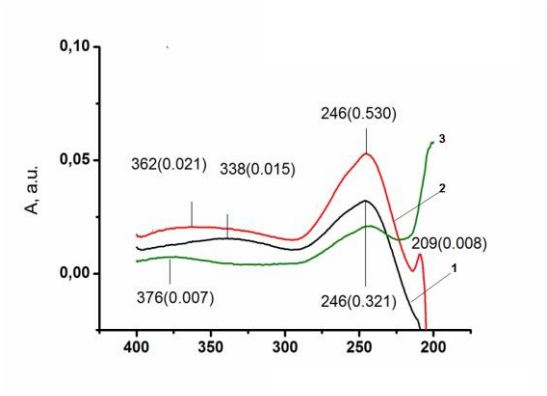

Figure 2. UV spectra of 10^{-6} M ethanol solutions of HAOA (1), GaAOA (2) and the result of the subtraction (3) of (1) from (2), in the interval of 400-200 nm.

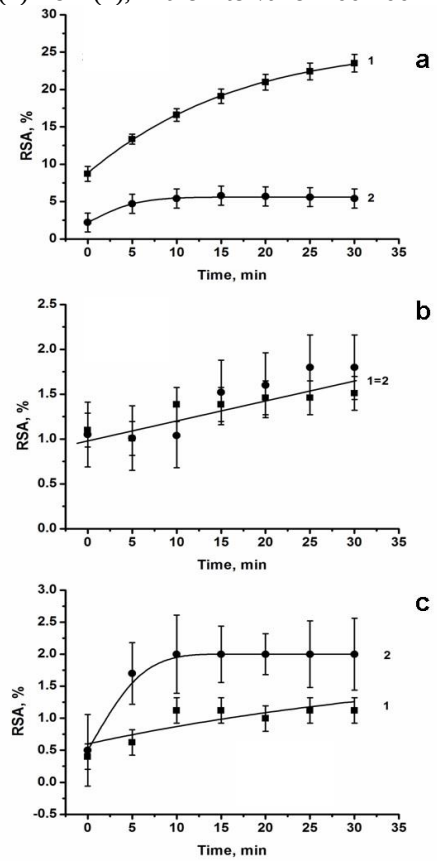

Figure 3. Radicals scavenging activity of 10^{-4} M (a), 10^{-5} M (b), and 10^{-6} M (c) solutions of HAOA (1) and GaAOA (2).

The RSA($DPPH^•$) of HAOA (1) and GaAOA (2) at different concentrations are seen in Fig. 3. The 10^{-7} M solutions of HAOA and GaAOA did not show any significant RSA($DPPH^•$). In Fig. 3 a-c it is seen that the RSA($DPPH^•$) of HAOA decreased in the order: 10^{-4} M>10^{-5} M (p<0.001)=10^{-6} M (p>0.05). RSA($DPPH^•$) in the presence of GaAOA decreased in the order: 10^{-4} M>10^{-6} M (p<0.001)>10^{-5} M (p<0.05). The RSA($DPPH^•$) of 10^{-4} M HAOA was much higher than this of GaAOA of the same concentration (Fig. 3a). The 10^{-5} M solutions of both HAOA and GaAOA exhibited the same RSA($DPPH^•$) (p>0.05, Fig. 3b), while this of the 10^{-6} M solution of GaAOA was slightly, but noticeably higher than this of 10^{-6} M HAOA (Fig. 3c).

The RSA($OH^•$) of HAOA and GaAOA are presented in Fig. 4b and compared with RSA($DPPH^•$) for the same time period (Fig. 4a). RSA toward both radicals of HAOA was higher than this of its Ga(III) complex. RSA($DPPH^•$) was significantly lower than RSA($OH^•$) for each compound at any given concentration (in all comparisons p was less than 0.01).

DISCUSSION

The UV spectra of HAOA and GaAOA showed that in aqueous medium both compounds were stable. The Gauss deconvolution of the spectra revealed components typical for the 5-aminoorotic acid, similarly to UV spectra of HAOA complexes with lanthanide ions [26-28]. The band at 330-340 nm was assigned to $\pi \rightarrow \pi^*$ transitions in the ring structure of 5-aminoorotic acid. The band at about 230-240 nm may be associated with $\pi \rightarrow \pi^*$ transitions of the triple-conjugated double bond system in AOA and $–NH_2$. The 207 nm band was related to possible E2-type ($\pi \rightarrow \pi^*$) band of the C=O and C=C in the molecule. The band around 201 nm might be a $\pi \rightarrow \pi^*$ transition of isolated C(OH)=O groups. In general, the solvent effects of H_2O and C_2H_5OH on the UV spectra of both molecules were consistent with the higher polarity of water compared to ethanol, and specificities in location and hydrogen bonding of the solvent toward solute. This is illustrated on the simple molecular models shown in Fig. 1. In Fig. 1a it is seen that the attachment of C_2H_5OH to HAOA affected mainly the $\pi \rightarrow \pi^*$ transitions of the triple-conjugated double bond system in AOA and $–NH_2$, the band related to possible E2-type ($\pi \rightarrow \pi^*$) band of the C=O and C=C in the molecule, and the $\pi \rightarrow \pi^*$ transition of isolated C(OH)=O groups. The UV spectra of aqueous and ethanol solutions of GaAOA (Fig. 1b) were much less intensive than expected for a compound containing three AOA ligands. This might be related with solvents' effect on the ligands orientation in the complex, as illustrated by the molecule models. The specific geometry of one AOA^- ligand in the ethanol solution of $Ga(AOA)_3.H_2O$ might be the reason for the appearance of two components in the characteristic band for the $\pi \rightarrow \pi^*$ ring transitions in the UV

spectrum. The appearance of new components in the spectrum of 10^{-6} M ethanol solution of GaAOA (Fig. 2) might be related with some dissociation of GaAOA in this medium. If true, this will result in higher RSA(DPPH$^{\bullet}$) of the 10^{-6} M GaAOA ethanol solution than this of 10^{-6} M HAOA. (Fig. 3c).

In presence of 10^{-4} M solutions, in which the intact Ga(III) complex dominated (Fig. 1b - spectrum in ethanol) RSA(DPPH$^{\bullet}$) decreased in the order HAOA>GaAOA (p<0.0001) (Fig. 3a). The smaller size and less complicated geometry of HAOA in comparison with these of GaAOA suggested an increased probability for the formation of the transition state needed for the hydrogen transfer to DPPH$^{\bullet}$. In the presence of 10^{-5} M solutions (Fig. 3b) HAOA and GaAOA exhibited the same (p>0.05) radical scavenging activity, while in the presence of 10^{-6} M solutions (Fig. 3c) the latter decreased in the order GaAOA>HAOA (p<0.01). Data in Figs. 2 and 3b indicated that a small amount of the Ga(III) complex might dissociate in ethanol, thus leading to higher RSA(DPPH$^{\bullet}$) than expected at a concentration of 10^{-6} M. Based on data in Figs. 1b, 2 and 3 it was proposed that the simpler the geometry and the higher the stability of the compound in ethanol environment, the higher RSA(DPPH$^{\bullet}$) would be. Comparisons between radicals scavenging activities of HAOA and GaAOA (Fig. 4) indicated that in presence of a given free radical and environment, the geometry and the size of the radical scavenger may influence the radical scavenging effectiveness. Data about RSA of HAOA in Fig. 4a,b suggested that the size and the geometry of the free radical, as well as the solvent effect on the scavenger also may play a role regarding the effectiveness.

The anticancer activity of Ga(III) [8], in combination with the antioxidant activity of the AOA ligands, as well as the weak instability of the GaAOA complex in ethanol environment suggest that the Ga(III) complex with 5-aminoorotic acid might be a promising anticancer agent.

CONCLUSIONS

1. The complex of Ga(III) with 5-aminoorotic acid has been synthesized and characterized by elemental, UV-VIS and vibrational spectral analyses, including IR and Raman spectra.

2. The 5-aminoorotic acid alone and as a ligand in the complex with Ga(III) exhibited hydrogen donor activity toward DPPH$^{\bullet}$ and OH$^{\bullet}$.

3. The better radicals scavenging activity of HAOA than this of GaAOA at concentrations above 10^{-5} M toward DPPH$^{\bullet}$ might be related with the smaller size and simpler geometry of the individual compound than those of the complex. Below this concentration, the effect of the complex was stronger than this of the individual compound, probably due to some dissociation in ethanol environment.

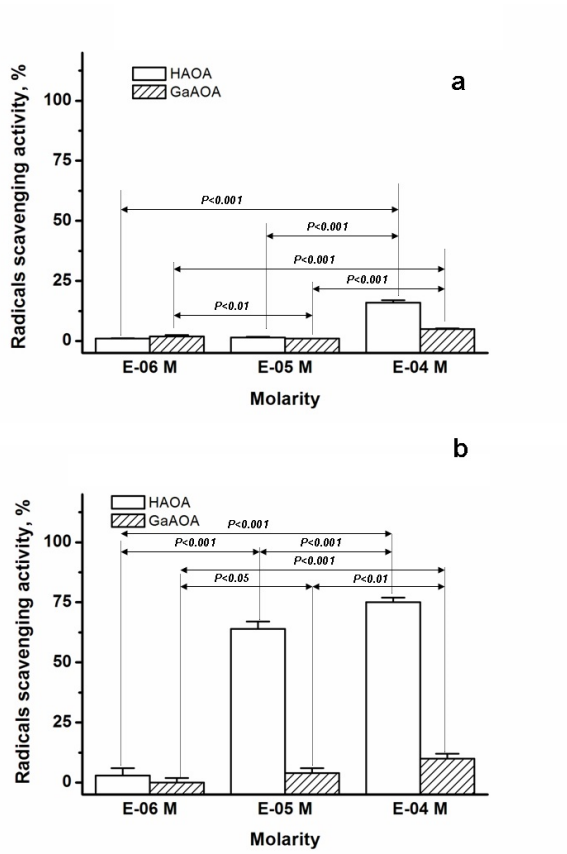

Figure 4. Radicals scavenging activity (RSA, %) of HAOA and GaAOA toward DPPH$^{\bullet}$ and OH$^{\bullet}$ radicals; reaction time – 10 min.

4. The better radicals scavenging activity of each compound toward OH$^{\bullet}$ than this toward DPPH$^{\bullet}$ might result from the smaller size, higher chemical reactivity and much simpler geometry of the hydroxyl radical than these of the stable and large DPPH$^{\bullet}$.

5. The combination of anticancer activity of Ga(III) and antioxidant activity of 5-aminoorotic acid, along with the instability of the complex depending on the environment suggest that GaAOA might be a promising anticancer agent.

REFERENCES

1. S. Prasad, S. C. Gupta, A. K. Tyagi, *Cancer Letters*, **387,** 95 (2017).
2. A. A. Dayem, H.-Y. Choi, J.-H. Kim, S.-G. Cho, *Cancers (Basel)*, **2,** 859 (2010).
3. M. Tafani, L. Sansone, F. Limana, T. Arcangeli, E. De Santis, M. Polese, M. Fini, M. A. Russo, *Oxid.*

Med. Cell. Longlivity, **2016,** Article ID 3907147, 18 pages (2016).
4. L. Markovic, N. Zarkovic, L. Saso, *Redox Biology*, **12**, 727 (2017).
5. U. Jungwrith, C. R. Cowol, B. K. Keppler, Ch. G. Hartinger, W. Berger, P. Heffer, *Antiox. Redox. Signal.*, **15**, 1085 (2011).
6. Y. Allaoui, S. Parashar, and S. Gómez-Ruiz, *Inorganics*, **5**, doi: 103390/organics 501004 (2017).
7. C. R. Chitambar, *Future Med. Chem.*, **4,** 1257 (2012).
8. C. R. Chitambar, W. E. Antholine, *Antiox. Redox Signaling*, **18,** 956 (2012).
9. I. Kostova, V. K. Rastogi, W. Kiefer, A. Kostovski, *Appl. Organomet. Chem.*, **20,** 483 (2006).
10. I. Kostova, V. K. Rastogi, W. Kiefer, A. Kostovski, *Arch. Pharm.*, **339,** 598 (2006).
11. J. Martin, P. Mladěnka, L. Sasso, I. Kostova, *Redox Report*, **21,** 84 (2016).
12. B. Halliwell, J. M. Gutteridge, *Biochem. J*., **219**, 1 (1983).
13. S. B. Kedare, R. P. Singh, *J. Food Sci. Technol*., **48,** 412 (2011).
14. P. Molyneux, *J. Sci. Technol*., **26,** 211 (2004).
15. J. Chrzczanowicz, A. Gawron, A. Zwolinska, J. de Graft-Johnson, W. Krajewski, M. Krol, J. Markowski, T. Kostka, D. Nowak, *Clin. Chem. Lab. Med.*, **46,** 342 (2008).
16. T. D. De Oliveira, W. S. Martini, M. D. R. Santos, M. A. C. Matos, L. L. da Rocha, *J. Braz. Chem. Soc.*, **26**, 178 (2015).
17. P. Cañizaers, R. Pas, C. Záez, M. A. Rodrigo, *Electrochim. Acta*, **53,** 2144 (2008).
18. S. Huang, S. G. Huling, S. Ko, *Chemosphere*, **78,** 563 (2010).
19. J. De Laat, G. T. Le, *Environm. Sci. Technol.*, **39**, 1811 (2005).
20. Y. Deng, E. Rosario-Muniz, X. Ma, *Waste Manage. Res.*, **30**, 12 (2012).
21. E. Neyens, and J. Bayerns, *J. Hazard. Mater.*, **98**, 33 (2003).
22. J. de Laat, G. Leand, B. Leube, *Chemosphere*, **55**, 715 (2004).
23. I. Kostova, N. Peica, W. Kiefer, *Chem. Phys.*, **327,** 494 (2006).
24. NPL Kale&Laby, Table of physical and chemical constants. Chapter 3: Chemistry, Section 3.8. Molecular spectroscopy; Subsection 3.8.7. UV-Spectroscopy (2015), http://www.kayelaby.npl.co.uk/chemistry/3_8/3_8_7.html.
25. F.-X. Shmidt, Encyclopedia of Life Science, MacMillan Publishers Ltd, Nature Publishing Group, (2001)
26. I. Kostova, M. L. Valcheva-Traykova, *Appl. Organomet. Chem.*, **29**, 815 (2015).
27. I. Kostova, M. L. Valcheva-Traykova, *J. Coord. Chem*., **68**, 4082 (2015).
28. I. Kostova, M. L. Valcheva-Traykova, S. Balkansky, *J. Iran. Chem. Soc.*, **13**, 891 (2016).

РАДИКАЛОПРИХВАЩАЩ ЕФЕКТ НА 5-АМИНООРОТОВА КИСЕЛИНА И НЕЙНИЯ Ga(III) КОМПЛЕКС СПРЯМО 2,2-ДИФЕНИЛ-1-ПИКРИЛХИДРАЗИЛОВ И ХИДРОКСИЛЕН РАДИКАЛ

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(Резюме)

Освен противотуморна активност, гъвкав контрол върху нивата на оксидативен стрес е желано качество на всяко противораково лекарство. Радикалоприхващащият ефект (RSA) спрямо 2,2-дифенил-1-пикрилхидразилов радикал (DPPH$^{\bullet}$) е широко използван за преценка на способността да се елиминират свободни радикали чрез отдаване на водород. 5-аминооротовата киселина (AOA) притежава антиоксидантни свойства и е използвана като лиганд в лантанидни(III) комплекси, които проявяват противоракови свойства в клетъчни култури. Солите на Ga(III) са известни със своята противоракова активност. По тази причина комплексът на Ga(III) с AOA (GaAOA) може да бъде обещаващо противораково съединение с антиоксидантни свойства, които не са проучвани до този момент. В настоящата работа са изследвани и анализирани УВ спектрите и RSA на HAOA и GaAOA спрямо DPPH$^{\bullet}$ и хидроксилен радикал (OH$^{\bullet}$). Стереохимията на HAOA и нейния Ga(III) комплекс са изследвани и сравнени с ИЧ, Раманови ИЧ и Раманови спектрални данни. Два фактора влияят върху УВ спектрите на молекулите: тяхното подреждане (стерични свойства) и тяхното взаимодействие с разтворителя. Тъй като RSA е определен в абсолютен алкохол (DPPH$^{\bullet}$) и вода (OH$^{\bullet}$), УВ спектрите на съединенията във вода и етанол са изследвани и анализирани. Хипохромното отместване на УВ спектрите на GaAOA, в сравнение с очакваните интензитети, свидетелства за подреждане на лигандите, намаляващо диполния момент. RSA на HAOA и GaAOA спрямо двата радикала е концентрационно-зависимо. GaAOA в най-ниската концентрация в етанол дава признаци за дисоциация, изразени чрез аномално нарастване на RSA Това показва потенциала на GaAOA за контролирано освобождаване на антиоксидантни лиганди.

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Effects of orotic and 5-aminoorotic acids on the free radicals accumulation in rat blood serum

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Orotic (HOA) and 5-aminoorotic (HAOA) acids are ligands in metal complexes with *in vitro* antioxidant and anticancer activities. Dietary orotic acid *in vivo* increases the free radicals formation in the liver by diminishing both activity and mRNA level of Cu,Zn-SOD. It seems that HOA may act as antioxidant by scavenging free radicals, but as prooxidant by diminishing the efficacy of Cu,Zn-SOD. The effect of orotic acid on the accumulation of free radicals (FRA) in the blood serum is still not assessed. In this investigation free radicals formation in rat blood serum was achieved by adding small amount of xanthine or Fe(II)/H_2O_2/ascorbate solution, thus superoxide or hydroxyl radical - induced free radicals formation was provoked. The effects of HOA and HAOA (within concentrations of 10^{-4} and 10^{-6} M) on the accumulation of free radicals in the blood serum were monitored using spectrophotometric method. FRA decreased with increasing of concentration of both compounds was observed, the effect being stronger for HAOA, and for the $OH^{\bullet}$ generating model system. The weaker antioxidant effect of HOA compared to HAOA on the free radicals accumulation in rat blood serum might be a result of negative influence on Cu,Zn-SOD, along with radicals scavenging activity of the molecule. It was assumed that at concentrations below 0.1mM, the antioxidant effect of HOA and HAOA in the blood serum prevailed. More detailed investigations are under way.

Keywords: Orotic acid, 5-Aminoorotic acid, antioxidant properties, Superoxide radical, Free radicals accumulation.

INTRODUCTION

Orotic acid is essential for the synthesis of the building blocks of the nucleic acids (RNA, DNA) for the transformations of the digestive lipids in the liver increases the utilization of fatty acids by the heart, increases the activity of lipoprotein lipases, the hepatic levels of uracil nucleotides, the expression of peroxisome proliferator- activated receptor α and its affected enzymes; metal orotates help the supplementation of the body with essential minerals [1]. Ca(II) orotate is used in the treatment of multiple sclerosis, while Mg(II) orotate reduces the severity of the chronic myocardial dysfunction and structural damage of cardiomyopathy in animal models. It also improved the exercise tolerance in patients with coronary artery disease and in trained athletes. The delicate balance within accumulation and elimination of orotic acid (HOA) is closely related with health status [2-5]. The deficiency of orotic acid leads to cell degeneration, heart problems, premature ageing, mental retardation, anemia, depressed immunity, crystals in the urine, skin problems and liver disorders. The excess of orotic acid was associated with Cu,Zn-SOD depletion, non-alcoholic fatty liver, liver steatosis, and in animal models - cancerogenesis. Hence, HOA may act as *in vivo* prooxidant *via* onset of diseases and pathologies due to its excess or deficiency [6-9], or by affecting the elements of antioxidant defense [10,11]. Orotic acid is synthesized in the body and supplied by exogenous sources (food, dietary supplements and medications) [12-15]. As *in vivo* source of free radicals, dietary HOA is potential hazard for onset of oxidative stress (OS) - induced cancer [16].

The use of metal complexes to disturb the redox-balance in cancer cells is promising approach in cancer treatment [17]. Fenton reaction [18] and superoxide accumulation [19] are among the major factors in development of ROS- induced oxidative stress in tissues and biological fluids. As the involvement of free radicals (especially of the reactive oxygen species, ROS) in cancerogenesis and cancer therapy is very complex and still not enough elucidated [20-23], the interactions with free radicals from the biological environment is important for the medicinal application of metal complexes with promising anticancer activity. Along with the anticancer action, possible involvement of a prospective drug in onset and development of OS in the living body is associated with manifestation of toxicity and undesirable side effects.

Metal complexes of both orotic (HOA) and 5-aminoorotic (HAOA) acids were found to be promising *in vitro* anticancer agents [24], some of them possessing antioxidant activity [25]. The effect of the ligands alone on the free radicals accumulation in the blood plasma was not enough

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elucidated. In this investigation, the influence of HOA and HAOA on the free radicals accumulation in biological environment was estimated, using rat blood serum as model system. The free radicals accumulation was initiated by introduction of small amount of xanthine or Fe(II)/H_2O_2/ascorbate solution, thus $O_2^{\bullet-}$ or $OH^{\bullet}$ - induced OS was provoked. The effects of HOA and HAOA on the accumulation of free radicals was monitored by measuring the transformation of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) into MTT formazan.

MATERIALS AND METHODS

All chemicals were of finest grade (Sigma-Aldrich). Distilled water was used as solvent. The reaction medium was 50 mM Na,K-phosphate buffer (PBS) of pH=7.45. Stock 1 mM aqueous solutions of HOA and HAOA were prepared and diluted to 10^{-4}, 10^{-5}, and 10^{-6} molar concentrations, prior to experiment. For the model system generating 3mM aqueous solution of xanthine was prepared, to generate $O_2^{\bullet-}$ in the serum. Two solutions were used for the $OH^{\bullet}$ generating model system: aqueous solution of 3mM $FeCl_2$, 3 mM H_2O_2, and 0.4 mM EDTA, and aqueous solution of ascorbate (4 mg/ml). Ice-cold water was used in the preparation, and during the experiment they were kept in ice bath. The free radicals accumulation in the samples was monitored by measuring the transformation of the yellow MTT to purple MTT formazan (λ=576 nm in PBS) for 10 min, using Shimadzu 1600 spectrophotometer.

Blood serum was isolated as described elsewhere [26]. Briefly, the total blood was left at room temperature to coagulate and centrifuged at 4000 rpm in a refrigerated centrifuge (Zamezki K-24) for 10 min. The serum was transferred in plastic test tubes and kept at -25°C (freezer) to the next day, when the biochemical analysis was performed. Before the experiment, the amount of proteins in the serum was determined [27].

EXPERIMENTAL PART

Samples preparation: Serum with proteins content of 1mg/ml was allowed to interact with a solution of desired concentration for 15 min in ice bath, then conditioned to room temperature and used for determination of the free radicals accumulation. For the control measurement the HOA and HAOA solutions were omitted.

Xanthine/xanthine oxidase assay: The free radicals accumulation was initiated by a small amount of xanthine solution, which produced superoxide radicals by reacting with the xanthine oxidase in the blood serum. One ml of the sample cuvette contained blood serum corresponding to 1 mg proteins, 0.01 ml xanthine, 0.10 ml MTT, 0.10 ml sample solution, and PBS to 1 ml. For the control measurement, the sample solution was omitted, and for the blank measurement the serum was omitted. The relative change of the intensity at 576 nm was measured for 10 min.

Fe(II)/H_2O_2/EDTA/ascorbate assay: The free radicals accumulation was initiated by initiating Fenton reaction in the serum. For the sample measurement one ml of the cuvette contained serum corresponding to 1 mg proteins, 0.10 ml MTT, 0.10 ml of the sample solution, 0.025 ml Fe(II)/H_2O_2/EDTA, 0.025 ml ascorbate, and PBS to 1 ml. In the control measurement the sample solution was omitted, while for the blank measurement the serum was omitted.

Data management and processing: The free radicals accumulation (FRA) was calculated using the formula:

$$FRA = \frac{\Delta A_{sample} - \Delta A_{blank}}{\Delta A_{control} - \Delta A_{blank}} * 100 ,$$

where ΔA is the relative change of the absorption at 576 nm for the sample, control or blank measurement, as indicated in the subscript.

The effect on the FRA of each compound at each concentration was measured 5 times. Average values and standard deviations were used for the comparisons. The statistical verification of the concentration effect was performed using One Way ANOVA. The relative differences within solutions of HOA and HAOA at any given concentration were statistically evaluated by using non parametric *t*-test with Welch correction (two-tailed P and SDs belonging to different populations were assumed).

The options of ChemOffice were used to understand the solvent effect and pH on both molecules investigated. The effect of the basic PBS was presented by one $H_2PO_4^-$ anion, the effect of basicity itself was illustrated using one OH^-, while solvent effect was being simulated by introduction of one water molecule to each HOA, HAOA and $H_2PO_4^-$.

RESULTS AND DISCUSSION

It is seen that the free radicals accumulation in rat blood serum in presence of HOA and HAOA decreased in a concentration-dependent manner (Fig.1). For each compound investigated, if initiated by $O_2^{\bullet-}$, FRA was significantly higher (Fig.1,a) than if initiated by Fenton reaction (Fig. 1,b).

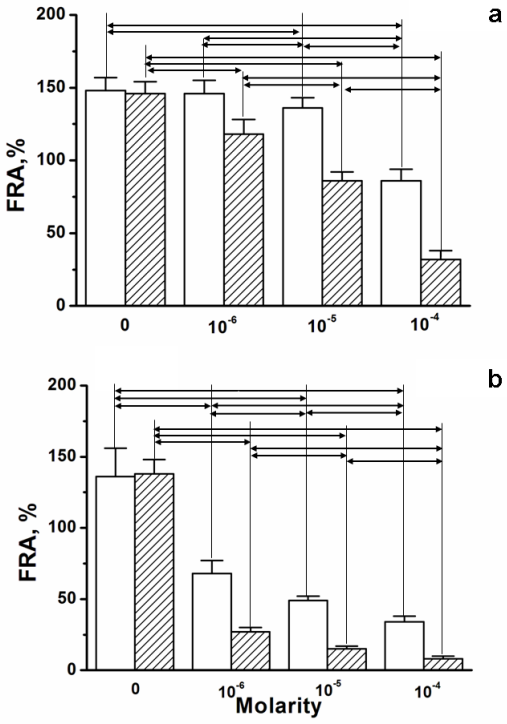
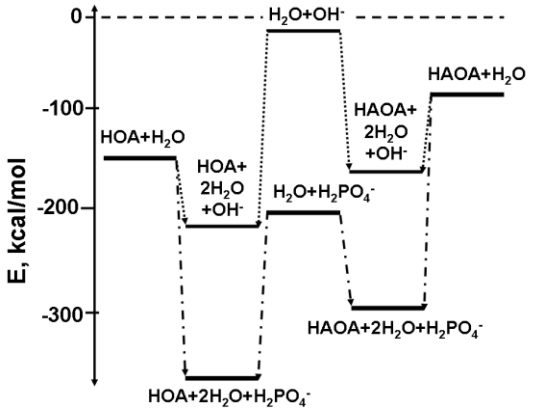


Figure 1. Free radicals accumulation (FRA, %) of HOA(□) and HAOA (▨) in rat blood serum, in the presence of model systems, creating $O_2^{-\bullet}$ (a) and $OH^{\bullet}$ (b). Statistically significant differences are marked with (↔). One Way ANOVA P<0.0001, all Bonferoni *p* are smaller than 0.05 (p<0.01, or p<0.001).

FRA in the presence of HAOA was lower than this in the presence of HOA (for each given concentration p<0.0001) in both ROS- generating model systems. It is well known that the higher the FRA, the lower the antioxidant activity is. Therefore, based on Fig 1 it was proposed that the molecules investigated were better antioxidants in the serum if the oxidative stress was initiated by Fenton reaction than if it was provoked by superoxide radical. This might be related with different types of interactions of HOA and HAOA with different model systems generating oxidative stress in blood serum. Large variety of interactions and/or reaction conditions might affect the potential energy of a molecule in a reaction medium, resulting in overall lower or higher minimum potential energy, this way either facilitating or obstructing the free radicals scavenging. In our experiment the reaction medium consisted of water as a solvent and PBS with physiological pH. Association of HOA and HAOA with solvent and influence of the pH on the potential energies of the solutes was possible. As lower than zero the potential energy, as stable the molecule in the reaction medium would be, as lower the probability to participate in free radical scavenging, and as low the antioxidant activity would be expected.

The possible effects of the pH if $H_2PO_4^-$ was carrier of the negative charge on the potential energy of aqueous associates of HOA and HAOA were estimated using a Molecular Mechanics modeling (Scheme 1). The effect of the solvent interaction with solutes on the minimum potential energy was illustrated by the effect of one water molecule associated with one HOA, HAOA, OH^- or $H_2PO_4^-$.



Scheme 1. Minimum potential energy (E, kcal/mol) of orotic (HOA) and 5-amino orotic (HAOA) acids in the presence of one OH^- or $H_2PO_4^-$, in aqueous medium.

The minimum potential energy of hydrated HAOA or HOA decreased if interacting with a hydrated hydroxyl anion. This indicated that the basicity of the medium and solvent interactions might affect the minimum potential energy of a molecule in a solution, this way affecting possible involvement in further chemical interactions. If hydroxyl anion was the carrier of basicity, the minimum potential energy level of the hydrated HAOA was higher than this of the hydrated HOA. If interacting with a free radical, the hydrated 5-aminoorotic acid might more easily undergo the energetic barrier than the hydrated orotic acid. If $H_2PO_4^-$ was carrier of basicity, the relative difference within minimum potential energy levels of hydrated HAOA and HOA was larger than this in basic environment created by OH^-. The relative difference within antioxidant activities of HOA and HAOA in PBS might be higher than the same relative difference in presence of OH^-. This proposition will be examined in future investigations. On the basis of this, higher reactivity of the HAOA than this of HOA might be expected in the PBS environment.

As the free radicals accumulation decreases in presence of antioxidants, the data in Fig. 1 suggested that both compounds investigated are antioxidants in rat blood serum, in PBS (pH 7.45), HAOA being better than HOA. The antioxidant behavior of these molecules might be a cumulative

result of their chemical properties and the effect of the environment. The relative difference in potential energies of both compounds investigated (Scheme 1) suggested that in PBS of homeostatic pH HAOA should be more reactive than HOA. This was in agreement with the data presented in Fig. 1. The relative difference within antioxidant activities in the presence of serum, in different ROS-producing model systems might be related with some interactions of HOA and HAOA with components of the antioxidant defense and/or with the components of the free radicals producing model systems. Orotic acid decreased *in vivo* the activity and gene expression of the Cu,Zn-SOD in rat liver [10,28]. The involvement of orotic and 5-aminoorotic acids in the redox homeostasis is still not enough elucidated. Possible interactions of HOA and HAOA with $O_2^{\bullet-}$ and $OH^{\bullet}$ might occur too. This hypothesis is under experimental evaluation right now.

On the basis of the present investigation and previous data [29-31] it may be proposed that both orotic and 5-aminoorotic acids may act as *in vitro* antioxidants in blood serum. This effect was stronger if OS was initiated by Fenton reaction than if it was provoked by superoxide resulting from enzymatic transformation of xanthine to uric acid *via* interaction with xanthine oxidase. The high antioxidant activity toward $OH^{\bullet}$- induced OS in blood serum is a favorable quality for a ligand in the metal complex that is prospective anticancer agent. To better understand the involvement of orotic and 5-aminoorotic acids in the free radicals homeostasis more investigations are needed.

CONCLUSIONS

1. Orotic and 5-aminoorotic acids diminished the free radicals formation in rat blood serum. It was proposed that both exhibited antioxidant properties at concentrations above 10^{-6} M.

2. The higher stability in PBS of pH 7.45 of the orotic acid compared with its 5-amino derivative may result in lower reactivity toward free radicals of HOA compared to this of HAOA.

3. The effect of the compounds investigated on the free radicals accumulation in rat blood serum might depend on the type of the free radical which initiated the OS.

4. In the case of orotic acid, the involvement in the free radicals homeostasis might be related with both chemical reactivity and influence on the Cu, Zn-SOD activity and expression.

REFERENCES

1. The vitamins: Fundamental aspects in nutrition and health (G. F. Combs, Jr., J. P. McChing, eds.). Academic press, N.Y., p. 494, 2016.
2. J. Zschocke, *Chapter:* Disorders of the Biosynthesis and Breakdown of Complex Molecules., in Inherited Metabolic Diseases (G.Hoffmann, J. Zschocke, W. Nyhan, eds.). Springer, Berlin, Heidelberg, 9 (2017).
3. U. Lichter-Konecki, Chapter 5 in Metabolic diseases: foundations of clinical management, genetics and pathology (E. Gilbert-Barness, I. A. Barness, P. M. Farrell, eds.), IOS Press, STM House, Amsterdam, p. 137, 2017.
4. L. Wang, P. Bell, H. Motizone, Zh. He, E. Pumbo, H. Yu, J. White, M. L. Batashaw, J. M. Wilson, *Mol. Gen. Metab.*, **120**, 299 (2017).
5. Orotic acid: Human health effects. *TOXNET:* Toxicology Data Network. https://toxnet.nlm.nih.gov/cgi-bin/sis/search /a?dbs+hsdb:@term+@DOCNO+6377.
6. J. Zschocke, *Chapter:* Disorders of the Biosynthesis and Breakdown of Complex Molecules, in Inherited Metabolic Diseases (G. Hoffmann, J. Zschocke, W. Nyhan, eds.), Springer, Berlin, Heidelberg, 9 (2017).
7. U. Lichter-Konecki, Chapter 5 in Metabolic diseases: foundations of clinical management, genetics and pathology (E. Gilbert-Barness, I. A. Barness, P. M. Farrell, eds.), IOS Press, STM House, Amsterdam, p. 137, 2017.
8. L. Wang, P. Bell, H. Motizone, Zh. He, E. Pumbo, H. Yu, J. White, M. L. Batashaw, J. M. Wilson, *Mol. Gen. Metab.*, **120**, 299 (2017).
9. Orotic acid: Human health effects. *TOXNET:* Toxicology Data Network. https://toxnet.nlm.nih.gov/cgi-bin/sis/search /a?dbs+hsdb:@term+@DOCNO+6377.
10. Y. Aoyama, and M. Morifuji, *J. Nutr. Sci. Vitaminol.*, **48**, 40 (2002).
11. M. Morifuji, and Y. Aoyama, *J. Nutr. Biochemistry*, **13** 403 (2002).
12. M Löffler, E. A. Carrey, and E. Zameitat, *J. Gen. Genomics*, **42**, 207 (2015).
13. M Löffler, E. A. Carrey, and E. Zameitat, *Nuceloz. Nucleot. Nucl. Acids*, **35**, 566 (2016).
14. A. F. G. Cicero, and A. Colleti, *Curr. Pharm. Design*, **23**, 1265 (2017).
15. Orotic acid: *Drug Information System.* http://www.druginfosys.com/drug.aspx?drugcode=1597&type=1.
16. https://www.efsa.europa.eu/en/efsajournal/pub/1187
17. U. Jungwrith, C. R. Cowol, B. K. Keppler, Ch. G. Hartinger, W. Berger, P. Heffer, *Antiox. Redox. Signal.*, **15**, 1085 (2011).
18. J. Prousek, *Pure Appl. Chem.*, **79**, 2325 (2007).
19. E. Birben, U. M. Sahiner, C. Sackesen, S. Esurum, O. Kalayci, *World Allergy J.*, **5**, 9 (2012).
20. M. Valko, C. J. Rhodes, J. Moncol, M. Izakovic, M. Mazur, *Chem.-Biol. Interact.*, **160**, 1 (2006).
21. D. Dreher, A. F. Junod, *Eur. J. Cancer*, **32**, 30 (1996).

22. P. Poprac, K. Jomova, M. Simunkova, V. Kollar, C. J. Rhodes, M. Valko, *Trends Pharm*., **38**, 598 (2017).
23. X. Gào, B. Schőttker, Oncotarget, Advance Publication, (2017), www.impactjournals.com/ oncotarget/
24. M. Nath, M. Vats, P. Roy, *Eur. J. Med. Chem.*, **59**, 310 (2013).
25. M. L Valcheva-Traykova, L. Saso, I. P. Kostova, *Curr. Top. Med. Chem.*, **14**, 2508 (2017).
26. M. L. Valcheva-Traykova, G. Bocheva, *Compt. Rend. Acad. Bulg. Sci*., **69**, 1503 (2016).
27. http://www.ruf.rice.edu/~bioslabs/methods/ protein/abs280.html.
28. M. .Morifuji, Y. Aoyama. *J. Nutr. Biochem.*, **13** 403 (2002).
29. I. Kostova, M. Valcheva-Traykova, *J. Coord. Chem.*, **68**, 4082 (2015).
30. I. Kostova, M. Valcheva-Traykova, *Appl. Organomet. Chem*., **29**, 815 (2015).
31. I. Kostova, M. Valcheva-Traykova, S. Balkansky, *J. Iran. Chem. Soc*., **13**, 891 (2016).

ЕФЕКТИ НА ОРОТОВАТА И 5-АМИНО ОРОТОВАТА КИСЕЛИНИ ВЪРХУ НАТРУПВАНЕТО НА СВОБОДНИ РАДИКАЛИ В КРЪВНА ПЛАЗМА НА ПЛЪХ

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(Резюме)

Оротовата (HOA) и 5-амино оротовата (HAOA) киселини са лиганди в метални комплекси с *in vitro* антиоксидантна и антиракова активност. Погълната с храната и лекарствата, оротовата киселина *in vivo* ускорява образуването на свободни радикали в черния дроб чрез понижаване едновременно на активността и m-РНК експресията на Cu, Zn-супероксид дисмутазата (Cu,Zn-SOD). Изглежда HOA може да се проявява като антиоксидант, но понижавайки активността и експресията на Cu,Zn-SOD, може да проявява прооксидантна активност. Ефектът на оротовата киселина върху натрупването на свободни радикали (FRA) в кръвен серум все още не е проучен. В това изследване натрупването на свободни радикали в кръвен серум на плъх бе постигнато чрез добавяне на малко количество ксантин, или в присъствие на моделна система Fe(II)/H_2O_2/аскорбат, при което оксидативният стрес се генерира от супероксиден или хидроксилен радикал. Ефектите на HOA и HAOA (в концентрационни граници между 10^{-4} и 10^{-6} M) върху натрупването на свободни радикали в кръвния серум бяха изследвани с помощта на спектрофотометрични методи. Натрупването на свободни радикали намаляваше с нарастване на концентрациите на двете изследвани съединения, като ефектът бе по-силен при HAOA отколкото при HOA и в присъствие на $OH^{\bullet}$-формиращата моделна система в сравнение с $O_2^{\bullet-}$-генериращата. По-слабият антиоксидантен ефект на HOA в сравнение с HAOA би могъл да бъде свързан с негативното влияние на първата молекула върху Cu,Zn-SOD, съпътстващо радикал-отнемащия ефект на съединението. Бе направено заключението, че при концентрации под 0.1 mM в кръвния серум антиоксидантният ефект на HOA и HAOA доминира. По-детайлни изследвания по тези въпроси са в ход.

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Study on the cytoprotective and antioxidant *in vitro* activity of Pr(III) complex of 5-aminoorotic acid

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This study investigates the possible cytoprotective and antioxidant potential of Pr(III) complex of 5-aminoorotic acid (PrAOA), at a concentration of 100 μM, on sub-cellular (isolated rat liver microsomes) and cellular (isolated rat hepatocytes) level. The Pr(III) complex of 5-aminoorotic acid (PrAOA) was synthesized by reaction of the respective inorganic salt in amounts equal to ligand molar ratio of 1: 3. The newly synthesized complex was characterized by means of elemental analysis, FTIR and FTRaman spectroscopies. The effects of PrAOA were evaluated on two toxicity models: non-enzyme lipid peroxidation and *tert*-butyl hydroperoxide (*t*-BuOOH) and compared to those of 5-aminoorotic acid and quercetin. On isolated rat liver microsomes, in conditions of non-enzyme lipid peroxidation, PrAOA complex revealed good statistically significant antioxidant activity (decreasing malondialdehyde (MDA) production – marker for lipid peroxidation), closer to that of quercetin and stronger than that of 5-aminoorotic acid (AOA). On isolated rat hepatocytes, we determined the main parameters of the hepatocytes' functional and metabolic status: cell viability (measured by trypan blue exclusion), levels of lactate dehydrogenase (LDH), reduced glutathione (GSH) and MDA. In *t*-BuOOH-induced oxidative stress, PrAOA complex showed statistically significant cytoprotective and antioxidant activities, closer to those of quercetin and stronger than those of AOA. The complex prevented the loss of cell viability and GSH depletion, decreased LDH leakage and MDA production. The stronger hepatoprotective and antioxidant activity of PrAOA than that of AOA on both *in vitro* toxicity models, might be due to the presence of Pr(III) ions in the complex.

Keywords: Praseodymium(III), 5-Aminoorotic acid, Rat microsomes, Rat hepatocytes, Cytoprotection, Antioxidant

INTRODUCTION

Over the last decades lanthanide complexes have attracted much attention not only for being valuable catalysts in organic synthesis but also for their wide applications in material and biological sciences mainly due to their vary narrow emission bands, long excited-state lifetimes, large Stokes shifts, etc. About the biological activity of these complexes there is no enough information. Some authors found anti-cancer activity of lanthanide complexes. There is an information about the role of lanthanum in suppressing tumor growth as: inhibits the uptake of iron; inhibits ROS (Reactive oxygen species) formation by connecting with hydro-peroxides; masks free radicals by magnetic interaction. Lanthanide ions are of great interest to scientists. They separately, as well as coumarins, exhibit anti-tumor properties. Therefore their complexes have such activity [1-3]. Through experiments using the HL-60 (human leukocytoma) cells it has been found that complexes of Ce(III), La(III) and Nd(III) with 3,3'-ortho-pyridine methylene di(4-hydroxy coumarin) were potent cytotoxic agents, although significant differences in the IC_{50}-values were not detected. Cerium complex showed the highest activity, and neodymium the lowest one [2, 4].

Sm(III), Gd(III), and Dy(III) complexes with coumarin-3-carboxylic acid were tested for antiproliferative activity on K-562 cell line (derivatives of chronic myeloid leukemia). Samarium salt showed a lower cytotoxic activity on these cells. This was in contrast, however, with the Sm(III) complex, which had a very strong activity [5].

There are literature data about the antioxidant effect of some lanthanide complexes in blood plasma. Complexes of cerium, lanthanum and neodymium with 5-aminoorotic acid (AOA) exerted a strong antioxidant effect on the formation of radicals released in the blood plasma of Wistar rats [4].

According to this information, in this study we investigated a possible cytoprotective and antioxidant activity of Pr(III) complex of 5-aminoorotic acid (PrAOA) on sub-cellular (isolated

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rat liver microsomes) and cellular (isolated rat hepatocytes) level.

MATERIAL AND METHODS

Synthesis of the of Pr(III) complex of 5-aminoorotic acid

The compounds used for preparing the solutions for the synthesis were Sigma-Aldrich products, p.a. grade: $Pr(NO_3)_3.6H_2O$ and 5-aminoorotic acid. 5-Aminoorotic acid (Fig. 1) was used as a ligand for the preparation of the metal complex.

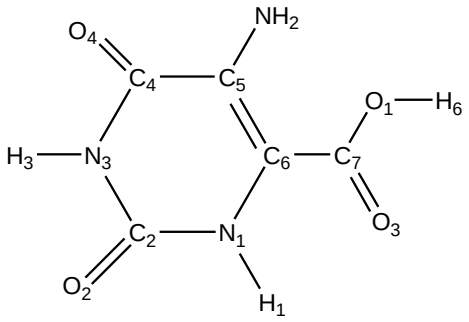

Fig. 1. The structure of the ligand 5-aminoorotic acid (AOA)

The carbon, hydrogen and nitrogen contents of the compound were determined by elemental analysis.

The solid-state infrared spectra of the ligand and its Pr(III) complex were recorded in KBr in the 4000-400 cm^{-1} frequency range on a FT-IR 113V Bruker spectrometer.

The Raman spectra of AOA and its new Pr(III) complex were recorded with a Dilor microspectrometer (Horiba-Jobin-Yvon, model LabRam) equipped with a 1800 grooves/mm holographic grating. The 514.5 nm line of an argon ion laser (Spectra Physics, model 2016) was used for the probes excitation. The spectra were collected in a backscattering geometry with a confocal Raman microscope equipped with an Olympus LMPlanFL 50× objective and with a resolution of 2 cm^{-1}. The detection of Raman signal was carried out with a Peltier-cooled CCD camera. Laser power of 100 mW was used in our measurements.

Chemicals

The chemicals used in the biological experiments were: pentobarbital sodium (Sanofi, France), HEPES (Sigma Aldrich, Germany), NaCl (Merck, Germany), KCl (Merck), D-glucose (Merck), $NaHCO_3$ (Merck), KH_2PO_4 (Scharlau Chemie SA, Spain), $CaCl_2.2H_2O$ (Merck), $MgSO_4.7H_2O$ (Fluka AG, Germany), collagenase from *Clostridium histolyticum* type IV (Sigma Aldrich), albumin, bovine serum fraction V, minimum 98 % (Sigma Aldrich), EDTA (Sigma Aldrich), 2-thiobarbituric acid (4,6-dihydroxypyrimidine-2-thiol; TBA) (Sigma Aldrich), trichloroacetic acid (TCA) (Valerus, Bulgaria), 2,2'-dinitro-5,5'-dithiodibenzoic acid (DTNB) (Merck), lactate dehydrogenase (LDH) kit (Randox, UK) and *tert*-butyl hydroperoxide (Sigma Aldrich).

Animals

Animals were purchased from the National Breeding Center, Sofia, Bulgaria. At least 7 days of acclimatization was allowed before the commencement of the study. The health was monitored regularly by a veterinary physician. The vivarium (certificate of registration of farm № 0072/01.08.2007) was inspected by the Bulgarian Drug Agency in order to check the husbandry conditions (№ A-11-1081/03.11.2011). All performed procedures were approved by the Institutional Animal Care Committee and made according Ordinance № 15/2006 for humaneness behavior to experimental animals.

Isolation of liver microsomes

Liver was perfused with 1.15 % KCl and homogenized with four volumes of ice-cold 0.1 M potassium phosphate buffer, pH=7.4. The liver homogenate was centrifuged at 9 000 × g for 30 min at 4°C and the resulting post-mitochondrial fraction (S9) was centrifuged again at 105 000 × g for 60 min at 4°C. The microsomal pellets were re-suspended in 0.1 M potassium phosphate buffer, pH=7.4, containing 20 % glycerol. Aliquots of liver microsomes were stored at -70°C until use [6]. The content of microsomal protein was determined according to the method of Lowry and co-workers, using bovine serum albumin as a standard [7].

$FeSO_4$/ascorbic acid-induced lipid peroxidation in vitro

As a system, in which metabolic activation may not be required in the production of lipid peroxide, 20 μM $FeSO_4$ and 500 μM ascorbic acid were added directly into rat liver microsomes and incubated for 20 min at 37°C [8].

As microsomes are a sub-cellular fraction, which includes only parts of endoplasmatic reticulum, and whole cells – hepatocytes, we used different methods for measuring the MDA production.

Lipid peroxidation in microsomes

After incubation of microsomes (1 mg/ml) with the compounds, we added to the microsomes 1 ml

of 20 % (w/v) trichloroacetic acid (TCA) and 1 ml of 0.67 % 2-thiobarbituric acid (TBA). The mixture was heated at 100 °C for 20 min. The absorbance was measured at 535 nm, and the amount of MDA was calculated using a molar extinction coefficient of 1.56×10^5 $M^{-1}cm^{-1}$ [9].

Isolation and incubation of hepatocytes

An optimized i*n situ* liver perfusion using less reagents and shorter time of cell isolation was performed [10]. The method resulted in a higher amount of live and metabolically active hepatocytes.

Rats were anesthetized with sodium pentobarbital (0.2 ml/100 g). After portal catheterization, the liver was perfused with HEPES buffer (pH = 7.85) + 0.6 mM EDTA (pH = 7.85), followed by HEPES buffer (pH = 7.85) and finally HEPES buffer containing collagenase type IV (50 mg/200 ml) and 7 mM $CaCl_2$ (pH = 7.85). The liver was excised, minced into small pieces, and hepatocytes were dispersed in Krebs-Ringer-bicarbonate (KRB) buffer (pH = 7.35) + 1 % bovine serum albumin.

Cells were diluted with KRB to make a suspension of about 3×10^6 hepatocytes/ml. Incubations were carried out in flasks containing 3 ml of the cell suspension (i.e. 9×10^6 hepatocytes) and were performed in a 5 % CO_2 + 95 % O_2 atmosphere.

The rat liver microsomes and hepatocytes were incubated with a concentration of 100 μM of the complex PrAOA, 5-amino-orotic acid (AOA) and quercetin (Q) [11].

The cell viability was measured by using Trypan blue (0.05 %) as reagent [10]. Initial viability averaged 89-90 %.

Lactate dehydrogenase (LDH) release

LDH release in isolated rat hepatocytes was measured spectrophotometrically using a LDH kit [10].

Reduced glutathione (GSH) depletion

At the end of the incubation, isolated rat hepatocytes were recovered by centrifugation at 4°C, and used to measure intracellular GSH, which was assessed by measuring non-protein sulfhydryls after precipitation of proteins with trichloroacetic acid (TCA), followed by measurement of thiols in the supernatant with DTNB. The absorbance was measured at 412 nm [10].

Malondialdehyde (MDA) assay

Hepatocyte suspension (1 ml) was taken and added to 0.67 ml of 20 % (w/v) TCA. After centrifugation, 1 ml of the supernatant was added to 0.33 ml of 0.67 % (w/v) 2-thiobarbituric acid (TBA) and heated at 100°C for 30 min. The absorbance was measured at 535 nm, and the amount of TBA-reactants was calculated using a molar extinction coefficient of MDA 1.56×10^5 $M^{-1}cm^{-1}$ [10].

Statistical analysis

Statistical analysis was performed using statistical programme 'MEDCALC'. Results are expressed as mean ± SEM for 6 experiments. The significance of the data was assessed using the nonparametric Mann–Whitney test. Values of $P \leq 0.05$; $P \leq 0.01$ and $P \leq 0.001$ were considered statistically significant. Three parallel samples were used.

RESULTS

The complex was synthesized by reaction of Pr(III) salt and the ligand, in amounts equal to metal: ligand molar ratio of 1: 3. The synthesis was made in different ratios (1:1, 1:2, 1:3) but in all the cases the final product was with the composition 1:3. The complex was prepared by adding an aqueous solution of Pr(III) salt to an aqueous solution of the ligand subsequently raising the pH of the mixture gradually to ca. 5.0 by adding a dilute solution of sodium hydroxide. The reaction mixture was stirred with an electromagnetic stirrer at 25 °C for one hour. At the moment of mixing of the solutions, precipitate was obtained. The precipitate was filtered (pH of the filtrate was 5.0), washed several times with water and dried in a desiccator to constant weight. The obtained complex was insoluble in water, methanol and ethanol, but well soluble in DMSO.

Reaction of Pr(III) and 5-aminoorotic acid afforded a complex which was found to be quite stable both in solid state and in solution. The new Pr(III) complex was characterized by elemental analysis. The content of the metal ion was determined after mineralization. The used spectral analyses confirmed the nature of the complex.

The data of the elemental analysis of the Pr(III) complex served as a basis for the determination of its empirical formula and the results are presented below. The found elemental analysis of Pr(III) complex of AOA ($Pr(AOA)_3.H_2O$) is shown as % calculated/found: C= 26,90/26,64; H= 2,09/2,29; N=18,83/19,00; H_2O= 2,69/2,25; Pr= 21,07/20,76, where HAOA= $C_5N_3O_4H_5$ and AOA^-= $C_5N_3O_4H_4^-$.

In our previous work the geometry of 5-aminoorotic acid was computed and optimized with the Gaussian 03 program employing the B3PW91 and B3LYP methods with the 6-311++G** and

LANL2DZ basis sets [12]. In the present study the binding mode of the AOA ligand to Pr(III) ions was elucidated by recording the IR and Raman spectra.

In the well-defined high-frequency field present in the IR and Raman spectra, extreme intensity changes were observed in going from the acid to the complex. In the spectral region 3500–2000 cm^{-1} the O–H, N–H, and C–H stretches give rise to intense IR bands. The involvement of these groups in hydrogen bonds produces a relevant band broadening in the IR and Raman spectra.

The double bond stretching vibrations ν(C=O) and ν(C=C) are the internal coordinates that dominate in the modes with fundamentals in the 1800–1600 cm^{-1} spectral range. One very strong band can be observed at 1691 cm^{-1} in the IR spectrum of the ligand assigned to the symmetrical stretching mode of C=O of the heterocyclic molecule. Opposite to the IR spectra, in this region of the Raman spectra only a medium band at 1699 cm^{-1} for the free ligand was observed. These bands (broad and relatively strong in the IR spectrum) shifted in the spectra of the title complex. The same shifts were observed for the strong IR band at 1667 cm^{-1} tentatively assigned to the ν(C=O) mode of the carboxylic group and for the experimental Raman band at 1341 cm^{-1} assigned to the stretching ν(C–O) mode as a medium signal from the spectra of the free ligand. It has to be mentioned that strong H-bonds are expected through the carboxylic group. Different stretches of the uracil ring contributed to the bands in the 1600–900 cm^{-1} region slightly shifted in the spectra of the title complex.

The metal affects the carboxylate anion, as well as the ring structure. The spectra in the frequency region below 600 cm^{-1} are particularly interesting, since they provide information about the metal-ligand vibrations. The new bands in the 600–500 cm^{-1} region present only in the IR spectrum of the complex can be due to the Pr-O interactions. The Raman spectra are particularly useful in studying the metal-oxygen stretching vibrations, since these vibrations give rise to medium intensity bands in Raman, but are weak in the infrared spectra. The observed bands and their assignments are in accordance with the literature data for similar coordination compounds [12].

Effect of PrAOA on MDA activity in isolated microsomes

Microsomes incubation with Fe^{2+}/AA, resulted in statistically significant increase of the amount of malondialdehyde (MDA) with 191 % *vs* control (non-treated microsomes).

In the non-enzyme-induced lipid peroxidation model, pre-treatment with PrAOA and AOA (at concentration 100 μM) significantly reduced lipid damage by 71 % and 44 % respectively, as compared to the toxic agent (Fe^{2+}/AA) (Fig. 2). At the same concentration quercetin, used as a control, lowered MDA formation by 73 %.

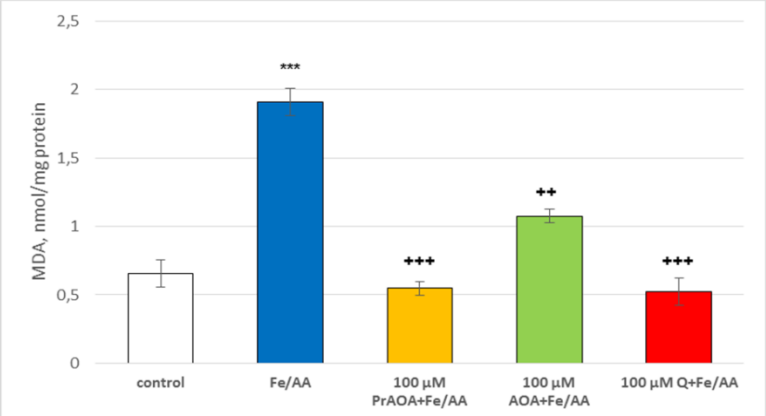

Fig. 2. Effects of PrAOA, AOA and quercetin on MDA production in conditions of non-enzyme lipid peroxidation on isolated rat liver microsomes. *** P < 0.001 *vs* control (non-treated microsomes). ++ P < 0.01; +++ P < 0.001 *vs* toxic agent (Fe^{2+}/AA)

Effects of PrAOA on parameters, characterizing the functional-metabolic status of isolated rat hepatocytes

One of the most useful models of oxidative stress is *t*-BuOOH (at concentration 75 μM) [13]. Administered alone, *t*-BuOOH leads to significant reduction of cell viability by 77 %, increased LDH activity by 150 %, depletion of cell GSH by 65 % and increased lipid peroxidation by 210 %,

compared to the control (non-treated hepatocytes) (Figs. 3 and 4).

In this model of oxidative stress, pre-treatment with PrAOA and AOA (at concentration 100 μM) significantly preserved cell viability by 183 % and 117 %, respectively, as compared to the toxic agent (*t*-BuOOH) (Fig. 3). At the same concentration quercetin, used as a control, preserved cell viability by 273 %.

Increased LDH leakage is a sign for cellular damage. Pre-treatment with PrAOA and AOA significantly decreased LDH leakage by 100 % and 70 % respectively, as compared to the toxic agent (*t*-BuOOH) (Fig. 3). At the same concentration quercetin, used as a control, decreased LDH leakage by 150 %.

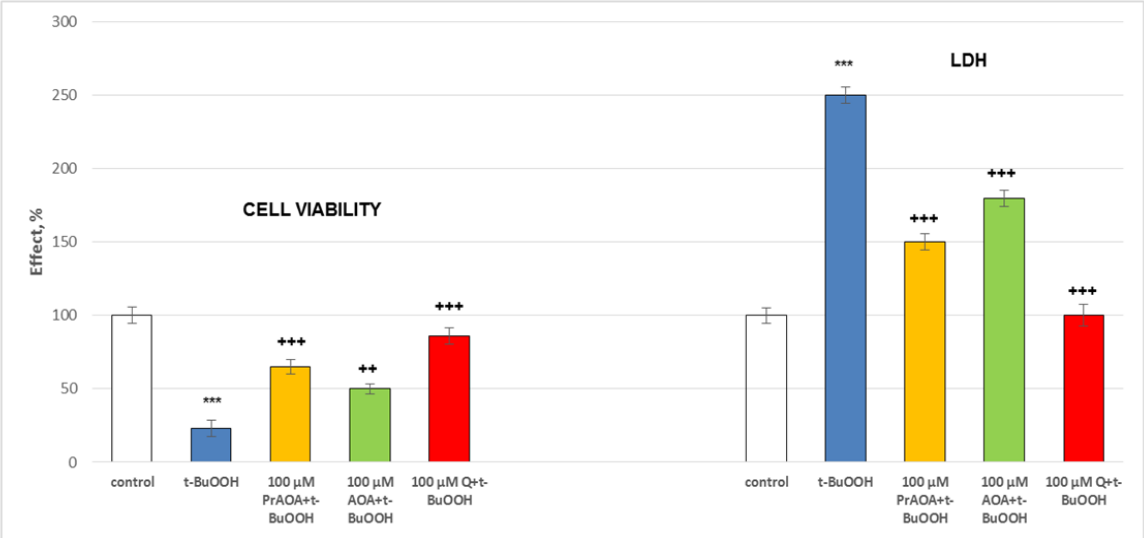

Fig. 3. Effects of PrAOA, AOA and quercetin on cell viability and LDH leakage in conditions of *t*-BuOOH-induced oxidative stress on isolated rat hepatocytes. *** P < 0.001 *vs* control (non-treated hepatocytes) ++ P < 0.01; +++ P < 0.001 *vs* toxic agent (*t*-BuOOH).

Reduced glutathione (GSH) is one of the main hepatic protectors. It is a nucleophile, which binds electrophiles, like ROS. Decreased level of GSH is a sign for cellular damage. Pre-treatment with PrAOA and AOA significantly preserved GSH level by 60 % and 37 % respectively, as compared to the toxic agent (*t*-BuOOH) (Fig. 4). At the same concentration quercetin, used as a control, preserved GSH level by 137 %.

The main marker for lipid peroxidation is the high production of malondialdehyde (MDA). Pre-treatment with PrAOA and AOA significantly decreased MDA production by 60 % and 30 % respectively, as compared to the toxic agent (*t*-BuOOH) (Fig. 4). At the same concentration quercetin, used as a control, decreased MDA production by 130 %.

DISCUSSION

In experimental toxicology, the *in vitro* systems are widely used for the investigation of the xenobiotics biotransformation, and for revealing the possible mechanisms of toxic stress and its prevention.

Some of the most suitable sub-cellular *in vitro* systems for investigation of drug metabolism are isolated microsomes. Normally in the cells, the microsomes do not exist. They are prepared artificially by differential centrifugation, and represent fragments from endoplasmic reticulum. Microsomes preserve the enzyme activity, mostly cytochrome P450 enzymes and can also be used as a model of lipid membrane in experiments, related to the processes of lipid peroxidation [14].

Here, we show that the complex PrAOA revealed a statistically significant antioxidant effect, stronger than that of AOA and similar to that of the classical hepatoprotector quercetin, in non-enzyme-induced lipid peroxidation at isolated microsomes. Our results demonstrated that MDA level in the samples treated with PrAOA, was markedly decreased.

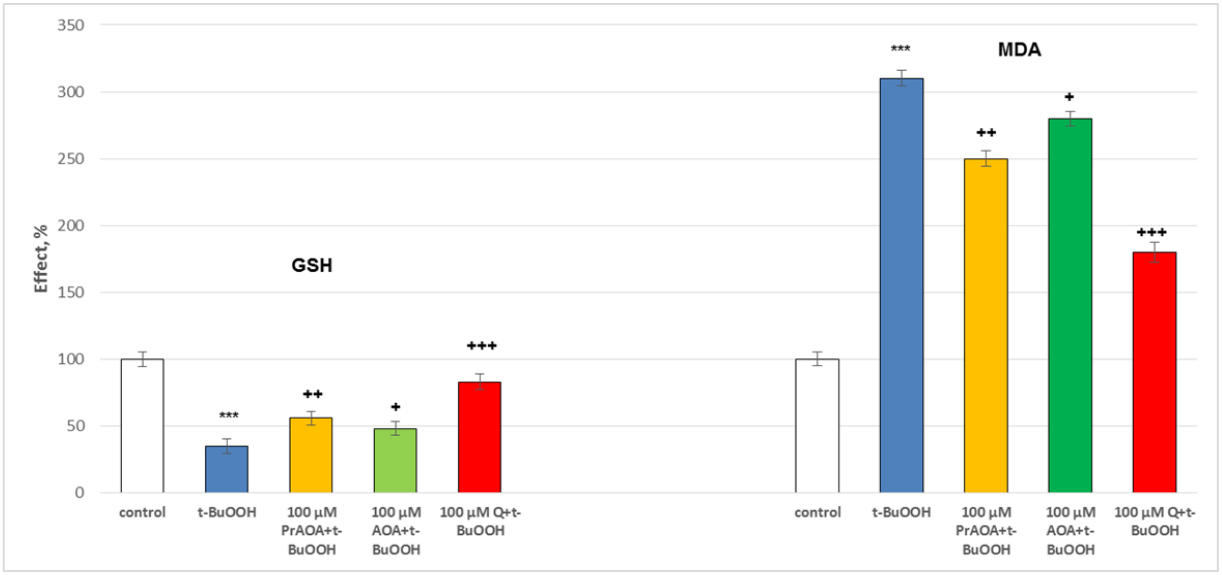

Fig. 4. Effects of PrAOA, AOA and quercetin on GSH level and MDA production in conditions of *t*-BuOOH-induced oxidative stress on isolated rat hepatocytes. *** P < 0.001 vs control (non-treated hepatocytes) + P < 0.05; ++ P < 0.01; +++ P < 0.001 vs toxic agent (*t*-BuOOH)

Isolated liver cells are a convenient model system for evaluation of the cytoprotective effects of some promising biologically active compounds, both newly synthesized and derived from plants.

One of the classical models of oxidative stress is *tert*-butyl hydroperoxide (*t*-BuOOH). Two mechanisms for *t*-BuOOH action were proposed: depletion of GSH cellular stores and oxidation of functionally important SH groups on mitochondrial enzymes, and/or changes of mitochondrial membrane integrity induced by peroxidation of membrane lipids [13].

The results from the present study showed that in the model of *t*-BuOOH-induced oxidative stress, PrAOA had statistically significant cytoprotective and antioxidant activities, stronger than those of AOA and similar to those of quercetin.

The results from this study correlate with previous results about the antioxidant effect of some lanthanide complexes in blood plasma. Complexes of cerium, lanthanum and neodymium with 5-aminoorotic acid exerted a strong antioxidant effect on the formation of radicals released in the blood plasma of Wistar rats [4].

Lanthanides are considered of high potential because of their inherent antioxidant properties. There are other literature data, which support that in conditions of *tert*-butyl hydroperoxide-induced oxidative stress, Ln(III) lost reactivity of produced peroxides, when they were bound to membrane. The lanthanide inhibiting ROS involves strong oxyphilicity inherent to lanthanides, because of the availability of oxygen sites on these free radicals, making them excellent targets for Ln(III) coordination (attack). This causes lanthanide to play the role of scavenger of reactive oxygen species, therefore presenting good potential for lanthanide as a future drug for a number of degenerative diseases due to ROS. Ln(III) very easily interacts with either free radicals or peroxides but is not transformed as radicals [15].

We suggest that these antioxidant effects of PrAOA might be due to possible mechanism as those of other Ln(III) complexes.

CONCLUSION

The present study provides novel and important data on the *in vitro* cytoprotective and antioxidant activity of the newly synthesised Pr(III) complex of 5-aminoorotic acid (PrAOA) on different toxicity models on sub-cellular (isolated rat liver microsomes) and cellular level (isolated rat hepatocytes). The complex was characterized by elemental and vibrational spectral analyses, including IR and Raman spectra. The complex PrAOA revealed statistically significant antioxidant and cytoprotective effects, stronger than those of 5-aminoorotic acid and similar to those of classical hepatoprotector quercetin.

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REFERENCES

1. K.F. Devienne, A.F. Carlgaro-Helena, D.J. Dorta, I.M. Prado, M.S. Raddi, W. Vilegas, S.A. Uyemura, A.C. Santos, C. Curti, *Phytochemistry*, **68**, 1075 (2007).
2. B. Thati, A. Noble A, B.S. Creaven, M. Walsh, M. McCann, K. Kavanagh K, M. Devereux, D.A. Egan, *Cancer Lett.*, **248**, 321 (2007).
3. Y. Yu, L. Wang, R.L. Walzem, E.G. Miller, L.M. Pike, B.S. Patil, *J. Agric. Food. Chem.*, **53**, 2009 (2005).
4. I. Kostova, M. Traykova, V. K. Rastogi, *Med. Chem.*, **4**, 371 (2008).
5. I. Kostova, G. Momekov, P. Stancheva, *Met. Based Drugs*, 15925 (2007).
6. R.L. Simeonova, V.B. Vitcheva, M.S. Kondeva-Burdina, I.N. Krasteva, S.D. Nikolov, M.K. Mitcheva, *Phytomedicine*, **17**, 346 (2010).
7. O.H. Lowry, N.J. Rosebrough, A.L. Farr, R.J. Randal, *J. Biol. Chem.*, **193,** 265 (1951).
8. H.J. Kim, Y.J. Chun, J.D. Park, S.I. Kim, J.K. Roh, T.C. Jeong, *Planta Med.*, **63**, 415 (1997).
9. E.D. Wills, *Biochem. J.*, **99**, 667 (1966).
10. M. Mitcheva, M. Kondeva, V. Vitcheva, P. Nedialkov, G. Kitanov, *Redox Rep.*, **11**, 1 (2006).
11. K.Y. Oh, V.H. Roberts, M.C. Schabel, K.L. Grove, M. Woods, A.E. Frians, *Radiology*, **276**, 110 (2015).
12. I. Kostova, N. Peica, W. Kiefer, *Chem. Phys.*, **327,** 494 (2006).
13. V. O'Donell, M.J. Burkit, *Biochem. J.*, **304**, 707 (1994).
14. I. Zaidi, R. Agarwal, G. Eichler, B.D. Rihter, M.E. Kenney, H. Mukhtar, *Photochem. Photobiol.*, **58**, 204 (1993).
15. S.N. Misra, M.A. Gagnani, I.M. Devi, R.S. Shukla, *Bioinorg. Chem. Allp.*, **2**, 155 (2004).

ИЗСЛЕДВАНЕ НА ЦИТОПРОТЕКТИВНАТА И АНТИОКСИДАНТНА *in vitro* АКТИВНОСТ НА PR(III) КОМПЛЕКС С 5-АМИНООРОТОВА КИСЕЛИНА

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(Резюме)

В настоящата работа е изследван възможният цитопротективен и антиоксидантен потенциал на комплекса на Pr(III) с 5-аминооротовата киселина (PrAOA) с концентрация100 μM, на субклетъчно (изолирани микрозоми от черен дроб на плъх) и клетъчно (изолирани хепатоцити от плъх) ниво. Комплексът на Pr(III) с 5-аминооротовата киселина (PrAOA) е синтезиран чрез реакция на съответната неорганична сол в количество, отговарящо на моларно съотношение с лиганда от 1: 3. Новосинтезираният комплекс е характеризиран чрез елементен анализ, FTIR и FTRaman спектроскопия. Влиянието на PrAOA е изследвано с помощта на два модела токсичност: неензимна липидна пероксидация и трет.-бутилов хидропероксид (*t*-BuOOH) и е сравнено с тези на 5-аминооротовата киселина и кверцетина. Върху изолирани микрозоми на черен дроб от плъх при условия на неензимна липидна пероксидация, PrAOA комплексът проявява добра статистически значима антиоксидантна активност (намалявайки продукцията на малонов дианхидрид (MDA)– маркер за липидна пероксидация), близка до тази на кверцетина и по-силна от тази на 5-аминооротовата киселина (AOA). Върху изолирани хепатоцити са определени основните параметри на функционалния и метаболитния статус на хепатоцитите: клетъчна жизнеспособност (измерена чрез ексклузия на трипаново синьо), нивата на лактат дехидрогеназа (LDH), редуциран глутатион (GSH) и MDA. При *t*-BuOOH-индуциран оксидативен стрес, PrAOA комплексът проявява статистически значима цитопротективна и антиоксидантна активност, близка до тази на кверцетина и по-силна от тази на AOA. Комплексът предотвратява загубата на клетъчна жизнеспособност и изчерпването на GSH, намалява загубата на LDH и производството на MDA. По-силната хепатопротективна и антиоксидантна активност на PrAOA в двата *in vitro* модела на токсичност от тази на AOA би могла да се дължи на присъствието на Pr(III) йони в комплекса.

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Antioxidant effect of green synthesized silver nanoparticles on moderate local heat burn injury

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By penetrating the damaged blood vessels walls, the free radicals formed in the heat burn wound field lead to oxidative stress-induced distant organs failure, latter being the most serious consequences of the burn injury. The oxidative stress (OS) may be successfully diminished by administering scavengers of free radicals along with substances preventing bacterial superposing. Silver is a well known highly efficient antimicrobial agent against antibiotic-resistant bacterial strains, with no registered allergic reactions. Recently silver nanoparticles on carriers (AgNPs) are preferred in wound treatment. In the present work, glycerol was used as a medium for AgNPs synthesis from $AgNO_3$ and green tea extract. The resulting AgNPs suspension was applied on local moderate rat's heat burn wound for 1 to 5 days. The OS in the rat blood serum was monitored for 5 days. The topical treatment with green synthesized AgNPs resulted in a decreased OS level in the serum than those for the untreated burned animals.

Keywords: Heat burn, Systemic circulation, Oxidative stress, Silver nanoparticles, Green nanosynthesis, Rat's blood serum.

INTRODUCTION

The heat burn injury results in local oxidative stress (OS) and immediate and long lasting inflammatory response, producing toxins, inflammatory cytokines and free radicals which penetrate through the damaged blood vessels walls [1-3]. Once in the systemic circulation, they simultaneously induce OS and generalized inflammatory response in which the OS is a component, resulting in distant organs damage [4]. Under strict and efficient control by the antioxidant defense of the patient, the local OS is involved in wound healing [1,5,6], but if this control fails, the systemic inflammation and generalized OS prevail [1,3]. The heat burn-inflicted OS is clinically controlled by co-treatment with antioxidants and silver-containing bactericidal compositions [1,7-9].

Most of the topical compositions for burn treatment contain silver salts or silver nanoparticles (AgNPs) [10-12], the biological action of the latter being intensively investigated [13-15]. Despite the controversial data (mostly *in vitro*) about the toxicity [16,17] and efficacy of AgNPs in wound healing [10,18], the green synthesized AgNPs have been found to be promising for possible application in patients [19-24].

The bactericidal properties of glycerol [25,26], green tea extract [27,28] and green tea-synthesized AgNPs [29,30] grounded our attempt to produce green tea-synthesized AgNPs in glycerol medium and to investigate their effect on heat burn-induced OS in the systemic circulation in a rat model of moderate local heat burn injury.

MATERIALS AND METHODS

Materials and solutions

All chemicals were of finest grade, (Sigma-Aldrich). Distilled water, 96% ethanol and pure glycerol were used as solvents. Dried green tea leafs (Ahmad Tea, London) were used in this investigation. Several aqueous solutions were prepared at room temperature: 50 mM K,Na-phosphate buffer of pH 7.45 (PBS), 3 mM xanthine (X), and 3 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). The following ice-cold aqueous solutions were prepared immediately before being used: one containing 3mM $FeCl_2$, 3 mM H_2O_2, and 0.4 mM EDTA; one containing 4 mg/ml ascorbate; one of 3 mM $FeCl_2$ and one of 3 mM H_2O_2. Glycerol was used to prepare 1 mM $AgNO_3$ solution.

Preparation of the green tea extract

Dried green tea leafs were kept in glycerol (100 mg dried leafs/ml) at 60°C for 10 min, followed by filtration using yellow and blue filters.

Preparation of the AgNPs

One ml of 1mM $AgNO_3$ was vigorously mixed for 15 min (Vortex) with 0.250 ml of the green tea extract, then sonified for 15 min and left at room

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temperature for 1 h. After 24 h rest in dark at room temperature, it was used for topical wound treatment in a rat model of moderate local heat burn. The UV spectra and transmission electron microscopy (TEM) micrographs proved that this was AgNPs suspension.

Animal models

21 male Wistar albino rats (200±30 g) were housed in individual standard cages, kept at room temperature (25±0.5 °C), standard humidity (60±1 %) and light/dark (12/12 h) cycle, receiving standard rodents food, and tub water *ad libitum*. All animals were treated in agreement with the general regulations for treatment of experimental animals, established by the Ethics Committee of the Medical University of Sofia, in agreement with EU Directive 2010/63/EU on the protection of animals used for scientific purposes.

After 4 days of adaptation, the rats were separated into 7 groups (n=3). The negative control group (Control) wasn't injured or treated. All other animals were anesthetized by injecting 1mg/100g BW xylasine and 7.8 mg/ 100g BW ketamine, and shaved on the back. Area of 1 cm^2 was burned with stainless steel rod heated to 100°C as described by Cai *et al.* [31]. Half of the injured rats were separated in groups named "1 day", "3 days" and "5 days", depending on the number of days after infliction of the heat burn injury. The wounds of the rest ones were treated locally with 0.04 ml/cm^2 of AgNPs suspension once per day for 1, 3 or 5 days after the injury, forming groups "1 day+AgNPs", "3 days+AgNPs" and "5 days+AgNPs". After 1, 3 or 5 days, the corresponding groups were exterminated under anesthesia (100 mg/ 100g BW of ketamine).

Blood serum isolation

Briefly, the total blood was left at room temperature to coagulate and further centrifuged at 4000 rpm at 297K for 10 min (Zamezki K-24) [32]. The amount of proteins in the serum was determined [33].

UV spectral and spectrophotometric measurements

UV spectral and spectrophotometric measurements were performed using Shimadzu 1600 UV-VIS spectrophotometer equipped with software program package and connected to a PC.

Free radicals accumulation (FRA)-assay

FRA in the serum was initiated by the Fe(II)/H_2O_2/EDTA/ascorbate model system, in PBS medium. The formation of MTT-formazan from MTT was used as a marker. The relative increase of the absorption at 578 nm (characteristic for MTT-formazan) was monitored for 10 min using the kinetic software of the spectrophotometer. FRA was evaluated using the formula:

$$FRA = \frac{\Delta A_{blank} - (\Delta A_{sam.} - \Delta A_{contr.})}{\Delta A_{blank}} * 100 .$$

For the blank measurement the cuvette contained 0.05 ml Fe(II)/H_2O_2/EDTA, 0.05 ml ascorbate, 0.2 ml MTT, and PBS to 2 ml., for the control - 0.2 ml MTT, serum containing 1 mg/ml proteins and PBS to 2.0 ml, and for the sample measurement - 0.05 ml Fe(II)/H_2O_2/EDTA, 0.05 ml ascorbate, serum containing 1mg/ml proteins, 0.2 ml MTT, and PBS to 2.00 ml. The FRA of a group was presented as a percentage of that for group C.

Xanthine oxidase (XO) activity assay

Small amount of 3mM xanthine (X) introduced in the serum produced uric acid (UA) by reacting with the endogenous XO. The sample cuvette contained serum corresponding to 1 mg/ml proteins, 0.01 ml X, and PBS to 2 ml. For the control measurement the X was omitted, and for the blank measurement the serum was omitted. The relative change of the absorbance at 293 nm was measured for 10 min. One unit of XO activity was defined as the amount of XO needed to transform 1 μm of X to UA for 1 min. The XO activity for each group was presented as a percentage of that for group C.

Assay for MDA

The MDA accumulation in the serum was estimated by measuring the relative increase in absorption of the MDA characteristic band, A(245 nm), due to $OH^{\bullet}$-initiated lipid peroxidation in presence of the Fe(II)/H_2O_2 model system. The MDA accumulation was estimated using the formula:

$$MDA = \frac{\Delta A_{sample} - (\Delta A_{control} - \Delta A_{blank})}{\Delta A_{blank}},$$

ΔA being the relative change of the A(245 nm) for 10 min. The index "sample" corresponded to ΔA in presence of serum containing 1mg/ml proteins, 0.05 ml $FeCl_2$, 0.01 ml H_2O_2 and PBS to 2 ml. For the "control" measurement the $FeCl_2$ was omitted, and for the "blank" measurement the serum was omitted. The results for MDA were presented as percentage of the MDA content in the serum of group C.

UV spectra

The UV spectra were collected in the range of 600- 200 nm in a quartz cuvette at a very low speed

against glycerol. The Shimadzu instrumental limits for absorption detection were ±3.999 a.u. If the absorption of a band in a spectrum of a compound exceeded ±3.999 a.u, the compound was diluted until its spectrum appeared within the instrumental limits. The instrumental error was estimated by recording the spectrum of solvent against solvent and was found to be ± 2 nm in position, and ± 0.005 a.u. in absorption.

TEM

A drop of AgNPs suspension was fixed on a standard Cu grid covered with amorphous carbon and dried at room temperature in pure atmosphere for 24 h. Then the grid was mounted on the holder and introduced in the microscope for visualization of the particles morphology with TEM bright field mode. The TEM study of AgNPs particles was performed on the transmission electron microscope JEOL JEM 2100 at 200 kV accelerating voltage. The particle-size distribution was determined by measuring the linear diameter D of particles at different magnifications, and was based on a general population of 576 measured particles.

Data management

Each experimental point in the biochemical experiments was presented by average value and standard deviation of 5 parallel measurements. The statistical analysis was performed using One way ANOVA and Bonferoni post-test.

RESULTS

Fig.1a displays the UV-VIS spectra of the reactants (1 and 2) and that of the reaction product (3), recorded between 600 and 200 nm against glycerol. Below 300 nm the absorption in the spectrum of the green tea extract (Fig.1a, spectrum 1) was above 3.999 a.u.. The green tea extract was diluted 10 times in glycerol and its spectrum is presented in Fig.1b. The UV spectra were resolved in agreement with literature data for UV spectra of green tea [34-36] and AgNPs [16,17,37-43]. In the UV spectra of the green tea extract (Fig.1a, spectrum 1, Fig.1b) there are characteristic bands resulting from superposition of characteristic bands for epigallocatechin (EGC) at 340 and 240 nm, and those for epigallocatechin gallate (EGCG) at 272 and 212 nm.

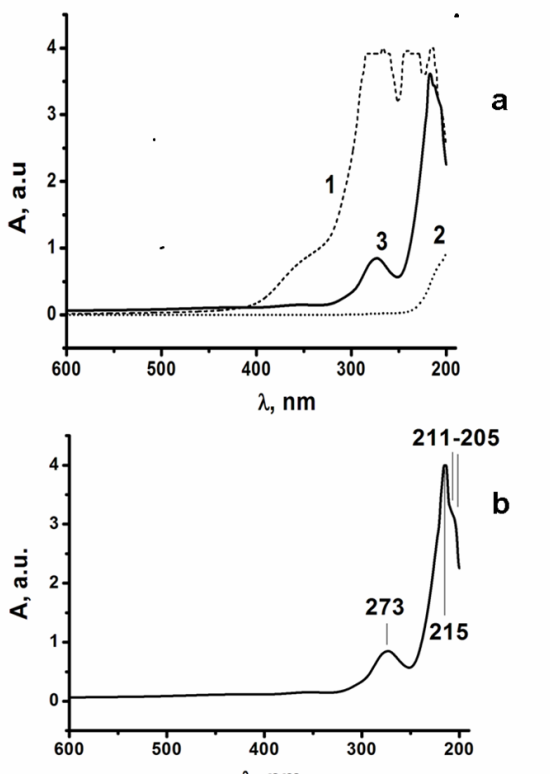

Figure 1. UV spectra of: (a) the reactants and the reaction mixture after 24 h, and (b) of green tea alone, diluted with glycerol 1:10 (v/v): a,1- 0.25 ml green tea extract in glycerol and 1 ml glycerol; a,2- 1.00 ml $AgNO_3$ solution in glycerol and 0.25 ml glycerol, a,3- 0.25 ml glycerol extract of green tea and 1.00 ml 1mM glycerol solution of $AgNO_3$.

The spectrum of the AgNPs suspension (Fig.1a, spectrum 3) was much more similar by intensity and bands to that of the 10-fold diluted (Fig. 1b), than to the spectrum of the undiluted green tea reactant (Fig.1a, spectrum 1). This might be explained with a decrease in concentration of the green tea extract in the suspension. The UV spectrum of the AgNPs suspension against green tea extract is shown in Fig. 2a. Similar to other studies [44-46], the characteristic band at 222 nm may be associated with $\pi \rightarrow \pi^*$ transitions of isolated C(OH)=O groups, while that at 248 nm is associated with $\pi \rightarrow \pi^*$ transitions in conjugated double bond systems in the organic molecules, or/and of phenolic O-H bond vibrations strongly affected by the solvent. The wide and intensive band with maximum at 431 nm is typical for the plasmonium resonance spectrum of AgNPs. The existence of AgNPs in the suspension was proved by the TEM micrograph (Fig. 1b). The size distribution of the AgNPs (Fig. 1b) showed monodispersity with average D=4.74±0.13 nm.

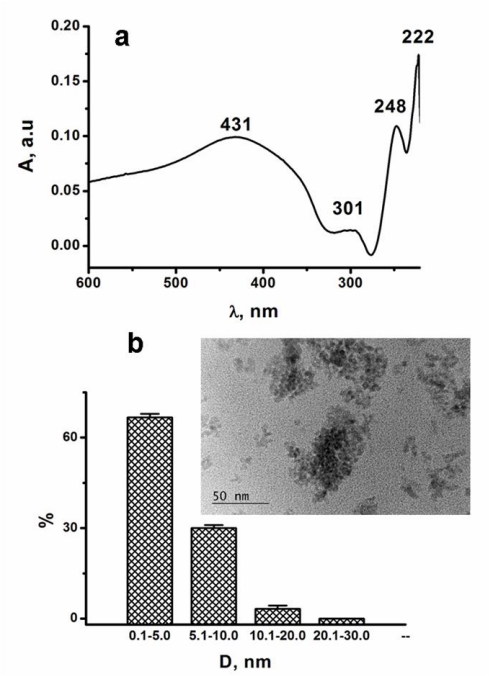

Figure 2. UV (a) and TEM (b) characteristics of Ag in the reaction mixture 24 h after the reaction.

Data in Fig. 2 confirmed the synthesis of AgNPs from green tea and $AgNO_3$ in glycerol, while Fig. 1 suggested a possible involvement of the green tea extract in capping of the AgNPs.

The effect of the moderate local heat burn injury on the OS level in the rat systemic circulation is illustrated in Fig. 3.

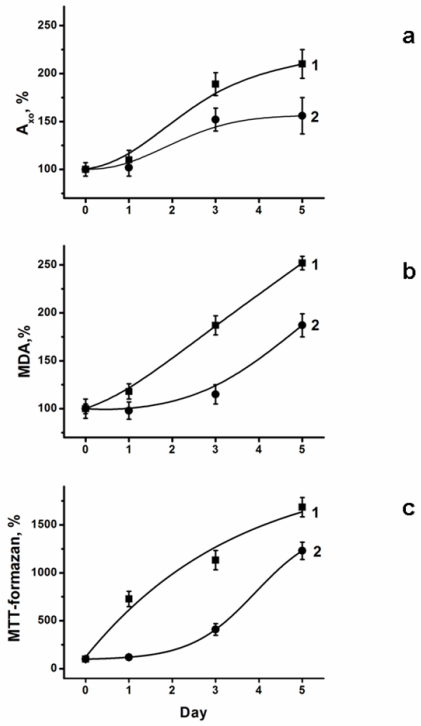

Figure 3. OS level in the blood serum of the untreated (curves 1) and treated (curves 2) positive controls as a percentage of that for the negative control group: (a) activity of XO, (b) MDA- accumulation, (c) free radicals accumulation.

All OS markers increased with time after the burn, in agreement with other studies [1-4]. The XO activity (Fig. 3a), the MDA levels (Fig. 3b) and FRA (Fig. 3c) were higher in the serum of the untreated (curves 1) than in the serum of the corresponding treated (curves 2) burned groups.

DISCUSSION

We experimentally proved the green synthesis of small AgNPs (4.74±0.13 nm) in glycerol. The UV spectra suggested a possible involvement of the green tea extract in capping of the AgNPs. Topical wound treatment with AgNPs suspension led to a decreased blood serum OS level in a rat model of moderate local heat burn injury. This allowed to suppose that the newly synthesized AgNPs suspension could diminish the risk of burn-induced OS in the systemic circulation, in this way decreasing the rate of OS-induced distant organs damage. In agreement with the literature data on the effects of any of our reactants and glycerol medium, and based on our research it might be assumed that the good control over the OS level in the rat blood serum resulted from the combined effects of the green tea extract, glycerol and AgNPs.

CONCLUSIONS

1. Small AgNPs (4.74±0.13 nm) were synthesized using green tea extract in glycerol medium.
2. Topical application of the AgNPs suspension significantly diminished the OS in the blood serum in a rat model of moderate local heat burn injury, in this way decreasing the risk of systemic OS.

REFERENCES

1. A. Parihar, M. S. Parihar, S. Milner, S. Bhat, *Burns*, **34**, 6 (2008).
2. N. Bhatia, G. Kaur, V. Soni, J. Kataria, R. K. Dhawan, *Burns&Trauma*, **17**, 53 (2016).
3. B. Cacir, B. C. Yegen, *Turk. J. Med. Sci*., **34**, 215 (2004).
4. R. F. Edlich, D. B. Drtake, W. B. Long III, Thermal Burns. MedScape, Drugs&Diseases, 2016, http://emedicine.medscape.com/article/1278244-overview.
5. C, K. Sen, S. Roy, *Biochim. Biophys. Acta*, **1780**, 1348 (2008).
6. M. Shafer, S. Werner, *Pharmacological Res*., **58**, 165 (2008).
7. E. Barbosa, J. Faintuch, E. A. M. Moreira, V. R. C. da Silva, M. J. L. Pereima, R. L. M. Fagundes, D. W. Filho, *J. Burn Care*, **30**, 859 (2009).
8. B. S. Atiyeh, M. Costagliola, N. Shady, S. N. Hayek, S. A. Dibo, *Burns*, **33**, 139 (2007).

9. M. P. Rowan, L. C. Cancio, L. C. A. Elster, D. M. Burnmeister, S. Natesan, R. K. Chan, R. J. Christy, K. K. Chung, *Crit. Care*, **19**, 243 (2015).
10. C. Rigo, L. Ferroni, I. Tocco, M. Roman, I. Munivrana, C. Gardin, R. L. Cairns, V. Vindigni, B. Azzena, C. Barbante, B. Zavan. *Int. J. Mol. Sci*., **14**, 4817 (2013).
11. A. Adhya, J. Brain, O. Ray, A. Hazra, S. Adhikari, G. Dutta, S. Ray, B. K. Majumdar, *J. Basic Clin. Pharm*. **6**, 29 (2015).
12. T. Dai, Y.-Y. Huang, S. K. Sharma, J. T. Hashmi, D. B. Kurup, M. R. Hambin, *Res. Pat. Antiinfect. Drug Discov*., **5**, 124 (2010).
13. A. Panacek, L. Kvitek, R. Prucek, M. Kolar, R. Vecelova, R. Sboril, *J. Phys. Chem*., **110**, 16248 (2006).
14. S. S. Birla, V. V. Tiwari, A. K. Gade, A. P. Ingle, A. P. Yadav, M. K. Rai, *Lett. Appl. Microbiol.* **48**, 173 (2009).
15. Y. Ionue, M. Uota, T. Torikai, T. Watari, I. Noda, T. Hotokebuchi, *J. Biomed. Mat. Res*., **92A**, 1171 (2010).
16. A. Rónavári, D. Kovàcs, N. Igas, C. Vágvölgyi, I. M. Boros, Z. Kónya, I. Pfeiffer, M. Kiricsi, *Int. J. Nanomed.*, **12**, 871 (2017).
17. M. C. Moulton, L. K. Braydich-Stolle, M. N. Nadagouda, S. Kunzelman, S. M. Hussain, R. S. Varma, *Nanoscale*, **2**, 763 (2010).
18. Z. M. Rashaan, P. Krijnen, R. R. Klamer, O. M. Dekkers, R. S. Breederveldt, *Wound Repair. Reg*. **22**, 473 (2014).
19. S. Iravani, *Green Chem*., **13**, 2638 (2011).
20. N. Savithramma, M. L. Rao, K. Rukmini, P. Suvarnalathe Devi, *Int. J. ChemTech. Res*., **3**, 1394 (2011).
21. S. C. Forester, J. D. Lambert, *Mol. Nutr. Food Res*., **55**, 844 (2011).
22. I. Al-Ogaidi, M. I. Salman, F. I. Mohammad, Z. Aguilar, M. Al-Ogaidi, Y. A. Hadi, R. M. A. Al-Rhman, *World J. Exper. Biosci*., **5**, 39 (2017).
23. A. Moosa, A. M. Ridha, M. H. Allawi, *Int. J. of Curr. Eng.&Technol.*, **5**, 3233 (2015).
24. S. Babu, M. O. Claville, K. Ghebreyessus, *J. Exper. Nanosci*., **10**, 1242 (2015).
25. E. I. Stout, A. McKessor, *Adv. Wound Care*, **1**, 48 (2012).
26. M. D. Gianeti, D. G. Mercurio, P. M. Campos, *Dermatol. Ther*. **26**, 267 (2013).
27. A. Zink, C. Traidi-Hoffmann, *J. Dtsch. Dermatol. Ges*. **13**, 768 (2015).
28. M. P. Morin, T. B. Bertan, J. Fournier-Larente, B. Haas, J. Azelmat, D. Grenier, *BMC Complement. Altern. Med*., **48**, 2015, doi: 10.1186/S 12906-015-0557-z
29. A. M. Dar, A. Ingle, M. Rai, *Nanomedicine: Nanotechnol. Biol. Med.*, **9**, 105 (2013).
30. T. Gunasekaran, T. Nigusse, D. D. Nharanaraju, *J. Am. College of Clin. Wound Specialists*, **3**, 82, (2012).
31. E. C. Cai, C. H. Ang, A. Raju, K. B. Tan, E. C. H. Hing, Y. Loo, Y. C. Wong, H. Lee, J. Lim, S. M. Moochhala, C. A. E. Hauser, T. C. Lim, *Arch. Plast. Surg*., **41**, 294 (2014).
32. M. L. Valcheva-Traykova, G. Bocheva, *Comt. Rend. Acad. Bulg. Sci*., **69**, 1503 (2016).
33. http://www.ruf.rice.edu/~bioslabs/methods/protein/abs280.html.
34. T. Atomssa, A. V. Gholap, *J. Eng. Technol. Res*., **7**, 22 (2015).
35. S. P. Mitra, *Ind. J. Chem*., **53B**, 1255 (2014).
36. S. Suteerapataranon, J. Butsoongren, P. Punturat, W. Jorpalit, C. Thanomsilp, *Food Chem.*, **114**, 1335 (2009).
37. V. Dhand, L. Soumya, S. S. Bharadwaj, S. Chakra, D. Bhatt, B. Sreedhar, *Mat. Sci. Eng. C: Mater. Biol. Appl*., **58**, 36 (2016).
38. Y. Y. Loo, B. W. Chieng, M. Nishibuchi, S. Radu, *Int. J. Medicine*, **7**, 4263 (2017).
39. J. Baharara, F. Namvar, T. Ramezani, M. Musavi, R. Mohamad, *Molecules*, **20**, 2693 (2015).
40. M. Ndikan, N. M. Noah, D. M. Andala, E. Masika, *Int. J. Anal. Chem*., **2017**, article ID: 8108504, 9 pages, https://doi.org/10.1155/2017/8108504.
41. P. Logeswari, S. Silambarasan, J. Abraham, *J. Saudi Chem. Soc*. **19**, 311 (2015).
42. P. Prema, in "Progress in Molecular and environmental bioengineering - from analysis and modeling to technology applications" Chapter 6, A. Capri (ed.), open access book, published in August 2011, under CC BY-\NC-SA 3.0 licence; ISBN 978-953-307-268-5.
43. K. Saware, B. Sawle, B. Salimath, K. Jayanthi, V. Abbaraju, *TJET*, **3**, 867 (2014).
44. NPL Kale&Laby, *Table of physical and chemical constants.* Chapter 3: Chemistry; Section 3.8. Molecular spectroscopy; Subsection 3.8.7. UV-Spectroscopy (2015). http://www.kayelaby.npl.co.uk/chemistry/3_8/3_8_7.html. Accessed 24 May 2015.
45. I. Kostova, M. L. Valcheva-Traykova, *Appl. Organomet. Chem.*, **29**, 815 (2015).
46. I. Kostova, M. L. Valcheva-Traykova, *J. Coord. Chem*., **68**, 4082 (2015).
47.

АНТИОКСИДАНТЕН ЕФЕКТ НА СРЕБЪРНИ НАНОЧАСТИЦИ, ПОЛУЧЕНИ ЧРЕЗ ЗЕЛЕН СИНТЕЗ, ПРИ ЛОКАЛНО ИЗГАРЯНЕ ОТ СРЕДНА СТЕПЕН

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(Резюме)

Проникващи в системното кръвообращение през увредените кръвоносни съдове, свободните радикали от раната при термично изгаряне достигат до отдалечени органи и предизвикват там оксидативни увреди. Това е едно от най-тежките усложнения при изгарянията. Оксидативният стрес може да бъде понижен при едновременното прилагане на радикалови скевинджъри и антибактериални препарати. Среброто е добре познат антибактериален агент с висока ефективност по отношение на резистентни на антибиотици щамове, без регистрирани алергични реакции към него. Напоследък при лечението на рани все по-предпочитани са материали и консумативи, съдържащи сребърни наночастици (СНЧ). В настоящето изследване СНЧ са синтезирани от екстракт на зелен чай и $AgNO_3$ в среда от глицерол. Получената суспензия на СНЧ бе приложена за третиране на термично кожно изгаряне при модел на плъх, за период от 1 до 5 дни. Бе оценено нивото на оксидативния стрес в кръвния серум. Третирането на раната със суспензията от СНЧ доведе до намаляване на оксидативния стрес в серума на третираните в сравнение с този при нетретираните животни с рани от изгаряне.

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Role of some styryl-heterocycles in the control of ochratoxin A biosynthesis

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Ochratoxin A (OTA) is a mycotoxin whose dangers have been sufficient for many countries to regulate its presence in various foods. In different countries, the black Aspergilli group, in particular *Aspergillus carbonarius*, causes the highest OTA contamination in fruit. Here we describe the effects of different styryl-heterocyclic compounds on the prevention of OTA biosynthesis by *A. carbonarius* cultured in a conducive liquid medium. The most effective and long-lasting control of OTA biosynthesis was achieved with (*E*)-3,5-dimethoxy-(2-thienyl) styrene (**10b**) and (*E*)-3,4,5-trimethoxy-(3-thienyl) styrene (**11d**). In fungal cultures treated with these compounds at 50 ppm, OTA biosynthesis decreased by 65% and 90%, respectively, after 8 days of incubation. A lower reactivity enhances the inhibition of OTA biosynthesis, in particular long-term. This study underlines the greater effectiveness of the phenyl ring substitution model as compared with that of the thiophene substitution model. Natural compounds present in edible plants having a styryl-heterocyclic scaffold may be effective inhibitors of OTA biosynthesis.

Keywords: Ochratoxin A, Thienyl-styrenes, Control effect.

INTRODUCTION

Ochratoxin A (OTA) is a mycotoxin well known for causing severe health hazards for humans and animals [1]. In particular, OTA interferes with tRNA and inhibits protein synthesis, resulting mainly in kidney pathologies [2]. This mycotoxin is synthesized by several different widespread fungal species, some of which (e.g., *Penicillium viridicatum*) are particularly adapted for cold environments, whereas others (e.g., *Aspergillus carbonarius*) are adapted for temperate or warm environments. Despite the huge diversity among OTA-producing fungi, it has been shown that oxidative stress in the cellular environment is commonly associated with this mycotoxin's biosynthesis [3]. Taking this correlation into account, different studies have been conducted for testing molecules whose antioxidant activity is used to control the biosynthesis of some mycotoxins [4, 5]. Furthermore, the antioxidant activity of the *cis* and *trans* isomers of several analogues of resveratrol and pterostilbene was investigated [6].

Considering these studies, we investigated the biological evaluation of various synthetic *trans*-styryl-heterocycle derivatives in order to preliminarily correlate their structure–activity relationships, and find a molecule with a significant and long-lasting inhibitive effect on OTA biosynthesis that could also serve as a new lead for chemical optimization**.** Monoatomic five-membered heteroaromatic compounds such as furan, thiophene or pyrrole display higher reactivity in oxidative conditions compared to the phenyl ring. The replacement of the phenyl ring with a heteroaromatic one with a stilbene pattern generates styryl-heterocycles with potentially strong biological activity [7]. The styryl-heterocycles can inhibit the production of nitric oxide [8], HIV-1 integrase [9], cell viability [10], cyclooxygenase and lipoxygenase activity [11-13], the growth of *Mycobacterium tuberculosis* [14] and *Botrytis cinerea* [15], and ochratoxin A biosynthesis by *Aspergillus carbonarius* [7, 16]. The type and efficacy of biological effects of styryl-heterocycles depend on several factors such as the number and nature of heteroatoms in the heterocycle (oxygen, nitrogen, or sulfur), the heterocycle ring's dimensions (5- or 6-membered), and the functions present on the phenyl ring.

In this study, *Aspergillus carbonarius* was used as OTA-producing fungus because this fungal species is widely diffused (particularly in temperate/warm environments) and most *A. carbonarius* strains can produce large quantities of OTA. Our aim was to investigate the effects of some styryl-heterocyclic compounds on the biosynthesis of OTA.

EXPERIMENTAL

Chemicals

The solvents (HPLC grade) and the reagents used were purchased from Sigma-Aldrich S.r.l. (Milan, Italy). Flash column chromatography was conducted on silica gel 230-400 mesh (Merck S.p.A., Milan). Reactions were monitored by TLC using Merck silica gel 60F-254 plates with UV indicator and/or visualized with phosphomolybdic acid (10% sol in EtOH). HPLC analyses were performed by using an

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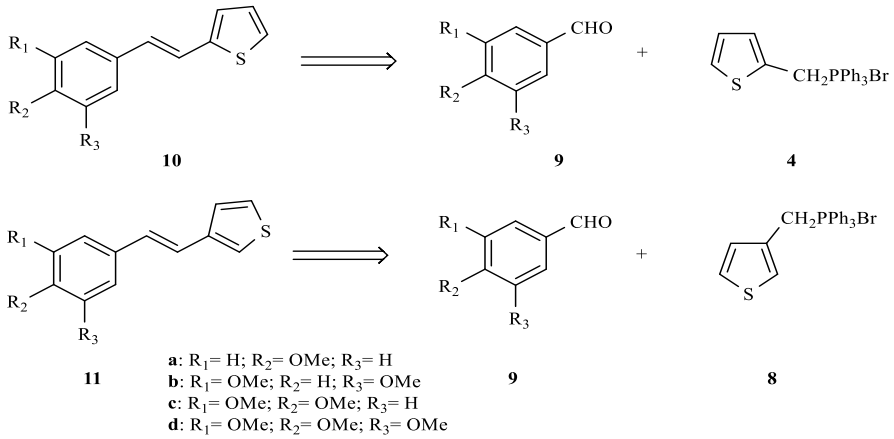
Agilent 1260 Liquid Chromatograph equipped with a Diode Array Detector (DAD). The ^{1}H and ^{13}C spectra were recorded with a Varian Mercury 3000 spectrometer at 300Mhz and 75MHz, in $CDCl_3$ as solvent. ^{1}H-NMR chemical shifts (δ) are expressed in parts per million (ppm), coupling constants (*J*) were measured in Hertz (Hz), and coupling patterns are indicated as s (singlet), d (doublet), t (triplet), q (quartet), bs (broad singlet), bd (broad doublet), m (multiplet). ^{13}C-NMR results (in ppm) were measured in correlation to $CDCl_3$ (δ=77.0 ppm for centerline).

All the experiments were performed using an *Aspergillus carbonarius* strain able to synthesize high concentrations of OTA isolated from wine grapes grown in Manduria (TA, Italy) in 2010. The fungus was maintained in Czapek-Dox (CD) Agar slants at 25°C, and 10-day cultures were used for conidia inoculum in liquid medium. The experiments to assess the effect of the synthesized molecules were performed using Czapek-Dox Yeast (CDY) 0.5%. Fungal growth was evaluated by weighing the mycelial part of the *A. carbonarius* culture after drying at 80°C for 48 h.

*Retrosynthetic analysis of 2-thienyl-styrenes **10** and 3-thienyl-styrenes **11** (Scheme 1).*

The stilbenoid structure was achieved by means of the Wittig reaction between the appropriate aromatic aldehydes **9** and the suitable thienyl-methylene ylides, which were generated *in situ* from the corresponding phosphonium salts **4** and **8**.



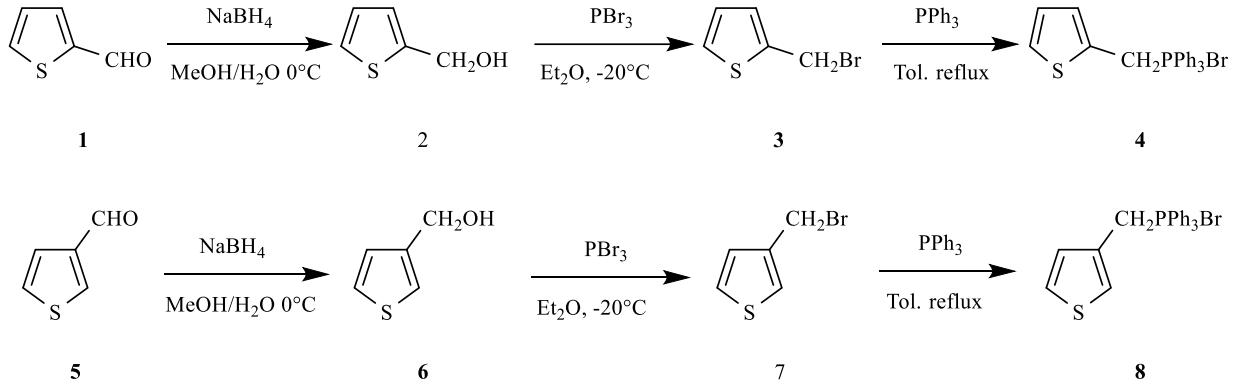
Scheme 1. Retrosynthetic analyses of the 2- and 3-thienyl-styrenes **10** and **11**.

*Synthesis of the phosphonium salts: 2-thienyl-methylen-phosphonium bromide **4** and 3-thienyl-methylene-phosphonium bromide **8** (Scheme 2)*

To perform this synthesis, $NaBH_4$ (10 mmol) was added portionwise to a solution of 2-formyl-thiophene **1** (10 mmol) in methanol (10 mL) at 0°C. After 30 min, the aldehyde **1** was consumed and the reaction mixtures were concentrated under reduced pressure. The material was dissolved in ethyl ether and washed with brine. The organic solution was dried over anhydrous Na_2SO_4 and, after filtration, the solvent was evaporated *in vacuum* to obtain alcohol 2-(hydroxymethyl) thiophene **2** (9.5 mmol) as pale yellow liquid. The alcohol **2** was dissolved in anhydrous diethyl ether (15 mL) at 0°C, and PBr_3 (19.2 mmol) was slowly added to the well-stirred solution. After 1 h the alcohol completely reacted (TLC: hexane/diethyl ether 95:5 v/v) and cold water was slowly added. The extraction process was accomplished by using diethyl ether (3×30mL). The organic layer was washed with water, a solution of $NaHCO_3$ 2M, and brine; then it was dried over anhydrous Na_2SO_4. Filtration and evaporation of diethyl ether *in vacuum* at room temperature resulted in 2-(bromomethyl) thiophene **3** (7.6 mmol) as brownish oil. The bromide **3** was dissolved into 40 mL of toluene, and triphenylphosphine (10.1 mmol) was added portionwise at room temperature. The reaction was conducted at reflux and the total consumption of the bromide **3** was monitored by TLC (hexane/diethyl ether 98:2 v/v). The suspension was filtered through a Büchner funnel, and the solid part was washed with cold toluene and dried *in vacuum*. This resulted in solid 2-thienyl-methylen-triphenyl-phosphonium bromide **4** (6.94 mmol). The overall yield from **1** is 69%. ^{1}H-NMR (300 MHz, $CDCl_3$), δ (ppm): 7.80-7.60 (m, 15H), 7.38 (bd, 1H *J* = 4.9 Hz), 7.12 (d, 1H, *J* = 5.1 Hz), 6.71 (m, 1H), 5.57 (d, 2H, *J* = 13.7 Hz). Anal. Calcd for $C_{23}H_{20}BrPS$: C, 62.88; H, 4.59; S, 7.30. Found: C, 62.96; H, 4.67; S, 7.33.

The 3-thienyl-triphenyl-phosphonium bromide **8** was synthesized from 3-formyl-thiophene **5** in the same way, the overall yield was 68%. ^{1}H-NMR (300 MHz, $CDCl_3$), δ (ppm): 7.80-7.60 (m, 15H), 7.35

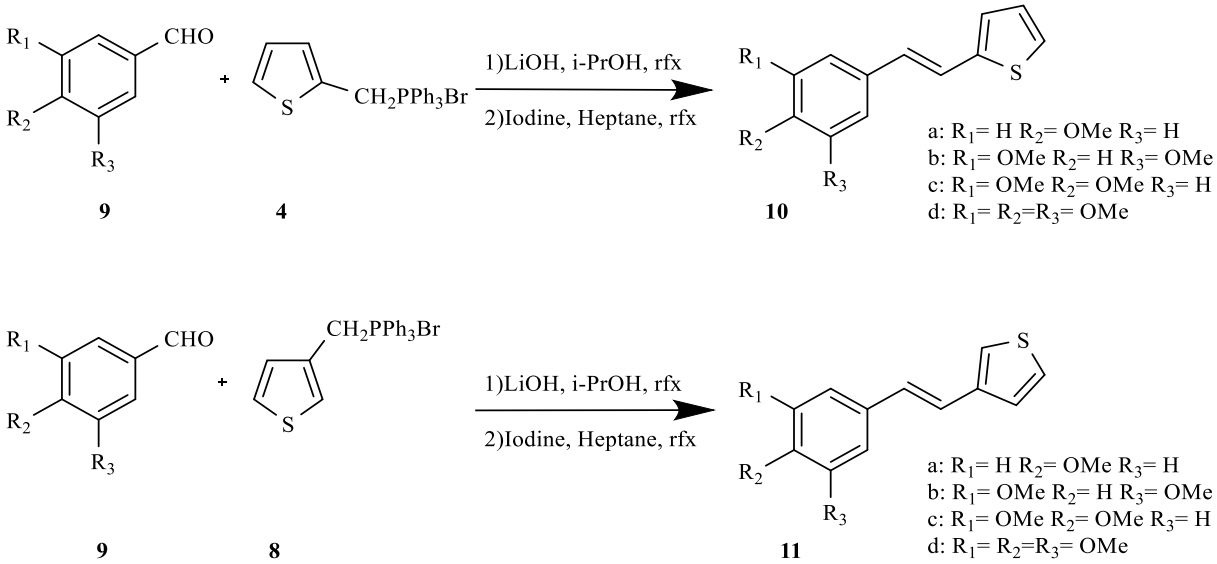
(bs, 1H), 7.12 (d, 1H, *J* = 4.9 Hz), 6.69 (d, 1H, *J* = 5.0 Hz), 5.61 (d, 2H, *J* = 13.9 Hz). Anal. Calcd for $C_{23}H_{20}BrPS$: C, 62.88; H, 4.59; S, 7.30. Found: C, 62.98; H, 4.64; S, 7.35.



Scheme 2. Synthesis of the phosphonium salts (**4**) and (**8**) used in the Wittig reaction.

Synthesis of 2-thienyl-methoxy-styrenes and 3-thienyl-methoxy-styrenes (Scheme 3).

An amount of $LiOH \cdot H_2O$ (4 mmol) was added to a stirred solution of phosphonium salt **4** or **8** (3 mmol) in isopropyl alcohol (10 mL). After 15 min the benzaldehyde **9** (3 mmol) was added to the mixture, the reaction was refluxed until complete consumption of **9** (TLC n-hexane/ethyl acetate 9:1 v/v), and the solution was brought to 25°C. The solvent was evaporated *in vacuum* and the crude product was purified by flash chromatography by eluting with a mixture of hexane/diethyl ether 9:1 (v/v). The residue was dissolved in 20 mL of CH_2Cl_2; the solution was washed with brine (3×10 mL) and dried over $MgSO_4$. The solvent was removed *in vacuum* to yield the desired 2-thienyl-methoxy-styrenes **10** and 3-thienyl-methoxy-styrenes **11** (ca. 2.7–2.8 mmol) as a mixture of *E*-and *Z*-isomers (*Z*/*E* ratio from 1:1 to 5:1). The *cis/trans* isomers were separated by silica gel column chromatography (hexane/ether), or the *Z*/*E* mixtures were converted to the *E*-isomers by heating with catalytic amounts of iodine in refluxing heptane. The *Z*-isomers or mixtures of two stereoisomers *E* and *Z* (1 mmol) were dissolved in heptane (10 mL). A catalytic amount of iodine (ca. 1-3 mg) was added to this solution and heated at reflux for 12 h. The reaction mixture was diluted with 20 mL of diethyl ether and washed with saturated aqueous $Na_2S_2O_3$ (10 mL) and brine (2×10 mL). The organic layer was dried over $MgSO_4$ and concentrated *in vacuum* to provide the desired *E*-isomers. Although the synthesized compounds are already known, the synthetic procedure conducted in this study presents elements of originality with respect to those reported in the literature [8, 16, 17]. The isolated compounds were characterized by ^{1}H-NMR and ^{13}C-NMR spectroscopy.



Scheme 3. Synthesis of 2-thienyl-styrenes (**10)** and 3-thienyl-styrenes (**11**) by Wittig reaction

OTA extraction and analysis

To extract OTA, 25 mL of $CHCl_3$ were added to the same volume of the fungal cultural filtrate acidified by 1% of 0.1M H_3PO_4 and mixed for 1 min; after 5 min the organic phase was collected and the extraction was repeated three times. The collected extracts were concentrated in a rotary evaporator, redissolved in the mobile phase used for HPLC, and analyzed. An isocratic mixture of CH_3CN:H_2O:formic acid 99:99:2 v/v/v was used as mobile phase, and an Agilent YMC column (150 × 2 mm; 2.1 nm) equipped with a pre-column filter as stationary phase. The spectra were collected at 333 nm (maximum of OTA absorbance). The quantification of OTA was performed using the external standard method by comparing the peak values obtained by the pure standard with those having the same retention time obtained by analyzing the samples. The amount of OTA is expressed in ppm., with 1 ppm corresponding to 1 μg of OTA per mL of culture medium (CDY 0.5%). The evaluation of peak purity by DAD was also performed. A stock solution of OTA was prepared by dissolving 5 mg of OTA in 2 mL of toluene acidified by 1% formic acid and stored at -20°C. Working solutions were prepared by serial dilutions of the stock solution and stored at 4°C for 7 days.

Each experiment was repeated twice, with three replicates of each measure. The data were statistically analysed using Statistica software (7.0 StatSoft). Mean values were evaluated using ANOVA and compared using Fisher's Protected LSD Test (P =0.05).

RESULTS AND DISCUSSION

The described compounds were synthesized by the Wittig reaction using the commercially available benzaldehydes **9** carrying methoxy groups in different positions, and the 2-phosphonium salt **4** or the 3-phosphonium salt **8** (Scheme 1).

The synthesis of phosphonium salts **4** and **8** starts from 2-formyl-thiophene **1** and 3-formyl-thiophene **5** through 3 steps: 1) reduction, 2) bromination, and 3) phosphorylation, as shown in Scheme 2.

The Wittig reaction was accomplished by using lithium hydroxide because this compound is an efficient base in this reaction [17] due to its ability to extract, in *iso*-propyl alcohol, the proton from the phosphonium bromides **4** and **8**, thereby yielding, *in situ*, the corresponding ylides. The reaction of these ylides with the methoxy-benzaldehydes **9a-d** provides thienyl-styrene derivatives **10** and **11** in high yields (Scheme 3).

The final olefin products **10a-d** and **11a-d** were obtained as *cis*/*trans* isomers. Their structures were determined from the characteristic ^{1}H-NMR spectra and the geometries of the double bonds were established by comparing the ^{1}H-NMR spectra of the isomeric hydrogen pairs.

The olefin protons of the *Z*-isomers were at 0.3-0.4 ppm higher field than those of the olefin protons of *E*-isomers. The coupling constant of the vinyl protons of the *E*-isomers was about 16 Hz, whereas the *Z*-isomers coupling constant was 12 Hz. The *Z*/*E* mixtures were converted to the *E*-isomers by heating with catalytic amounts of iodine in refluxing heptane [18] when the separation of the *Z*/*E*-isomers was not possible by chromatography.

Among the 2-thienyl-styrenes assayed (compounds **10a-d**), compound **10b** (*E*-3,5-dimethoxy-(2-thienyl) styrene) was the most effective at inhibiting OTA biosynthesis (Fig. 1 a,b).

It is worth noting that Caruso *et al.* [15], testing 2-furyl styrenes with aromatic substitutions models equal to those of the molecules **10a**, **10b** and **10d**, showed that the *E*-3,5-dimethoxy-(2-furyl) styrene is the most effective at inhibiting the mycelial growth of *Botrytis cinerea*, while still taking into account the differences in the reactivity between furyl and thienyl moiety and in the biological activity considered.

Moreover, the *E*-3,4,5-trimethoxy-(2-thienyl) styrene **10d** also showed a significant ability to control OTA biosynthesis.

Among the 3-thienyl-styrenes assayed (compounds **11a-d**), compounds **11b** (*E*-3,5-dimethoxy-(3-thienyl) styrene) and **11d** (*E*-3,4,5-trimethoxy-(3-thienyl) styrene) displayed the strongest biological activity.

Compounds **10a** and **10b,** as well as **11a** and **11b,** have been selected for their similarity to resveratrol's structure (3,5,4'-trihydroxy stilbene) concerning the substitution pattern of the phenyl ring. Compounds **10c** and **10d,** as well as **11c** and **11d,** have been selected because they present at least two substituents in cathecolic position. Several studies report that such a substitution pattern leads to greater biological activity compared to that fostered by other substitution patterns [4, 19, 20].

In our experiments, the 3,5-dimethoxy and the 3,4,5-trimethoxy derivatives yielded the best results in controlling OTA biosynthesis, while 3,4-dimethoxy compounds resulted in significantly lower inhibition.

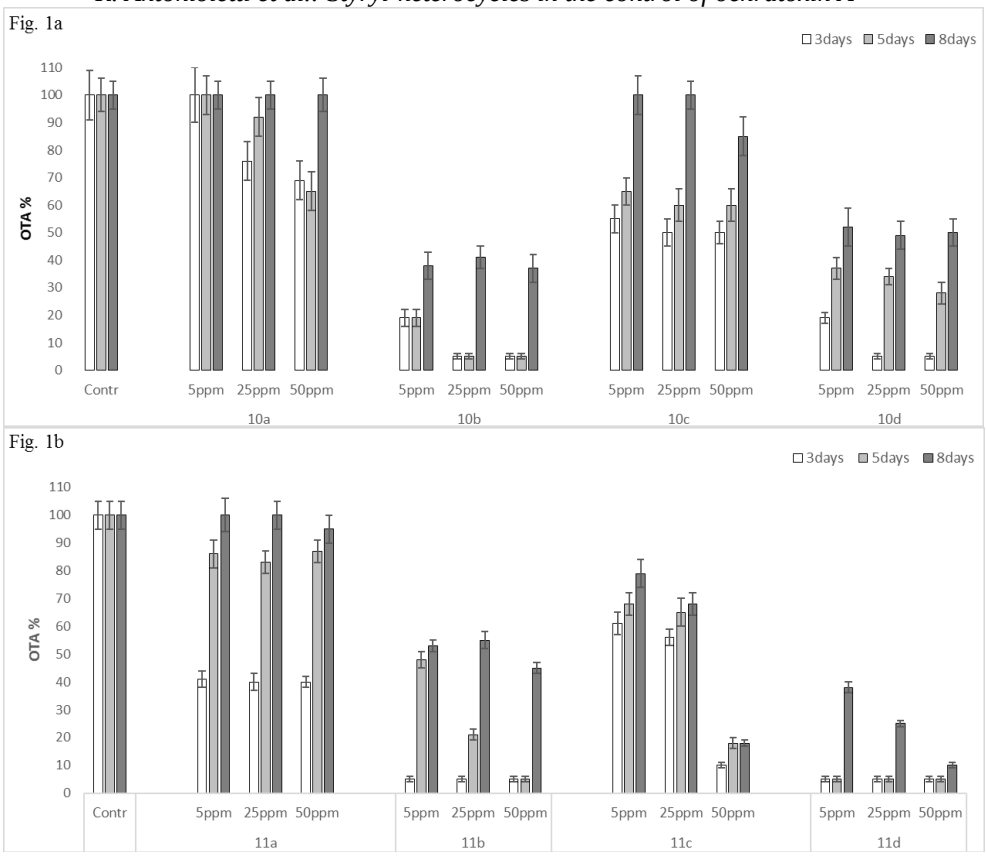


Fig. 1 a,b. Effect of the assayed 2-thienyl (a) and 3-thienyl (b) compounds on OTA biosynthesis. Means marked by the same letter are not significantly different (Fisher's Protected LSD Test, P=0.05).

Note that when the *para* position is occupied, the 3-thienyl-styrenes **11a**, **11c**, and **11d** yielded better results than those of the 2-thienyl-styrenes **10a**, **10c**, and **10d**, whereas, when the *para* position is unsubstituted, the 2-thienyl-styrene **10b** was better at inhibiting OTA production than was the 3-thienyl-styrene **11b (**Fig. 1 a,b**).** In particular, in the case of molecules having unsubstituted *para* position (**10b** and **11b**), due to the presence of 2 free α positions in the heterocycle, the 3-thienyl derivative (**11b**) displays a greater reactivity than the corresponding 2-thienyl derivative (**10b**). This may explain the shorter duration of the **11b** compound, which was able to significantly inhibit OTA production through the 5th day of the experiment but was not effective on the 8th day of incubation. On the other hand, molecule **10b** is able to maintain the ability to inhibit OTA biosynthesis over time (Fig. 1). These results are consistent with those obtained by Lee [8], who tested the inhibiting activity on NO production by LPS-activated RAW264.7 macrophage cells, although that biological system responds more readily.

CONCLUSION

This study highlights the importance of the phenyl ring's substitution pattern in determining the ability of the molecule to control OTA biosynthesis by *Aspergillus carbonarius*. However, the substitution pattern of the heterocyclic ring also was influential, albeit to a lesser extent. It is also important to highlight that the molecules **10a** and **11a**, both of which are mono substituted in *para* position, showed the lowest control effect on OTA biosynthesis.

(E)-4-methoxy-(2-thienyl) styrene 10a:

Yield: 87%. ^{1}H-NMR, δ: 3.81 (s, 3H); 6.88 (d, 1H, *J* = 16.1 Hz); 6.90 (d, 2H, *J* = 8.8 Hz); 6.96-7.06 (m, 2H); 7.10 (d, 1H, *J* = 16.1 Hz); 7.15 (d, 1H, *J* = 5.4 Hz); 7.40 (d, 2H, *J* = 8.8 Hz). ^{13}C-NMR, δ: 159.2, 143.2, 129.7, 128.0, 127.5, 127.4, 125.3, 123.7, 119.7, 114.1, 55.2. Anal. Calcd for $C_{13}H_{12}OS$: C, 72.19; H, 5.59; S, 14.82. Found: C, 72.26; H, 5.65; S, 14.80.

(E)-3,5-dimethoxy-(2-thienyl) styrene 10b:

Yield: 95%. **^{1}**H NMR, δ: 3.75 (s, 6H); 6.31 (t, *J* = 4 Hz, 1H); 6.55 (m, 2H); 6.78 (d, *J* = 16.2 Hz, 1H); 6.93 (dd, *J* = 3.3, 5.1 Hz, 1H); 7.00 (bd, *J* = 3.0 Hz, 1H); 7.13 (d, *J* = 16 Hz, 1H); 7.21 (d, *J* = 5.0 Hz, 1H). ^{13}C NMR, δ: 160.9, 142.6, 138.9, 128.2, 127.6, 126.3, 124.5, 122.3, 104.3, 100.0, 55.3. Anal. Calcd for $C_{14}H_{14}O_2S$: C, 68.26; H, 5.73; S, 13.02. Found: C, 68.32; H, 5.81; S, 13.06.

(E)-3,4-dimethoxy-(2-thienyl) styrene 10c:

Yield: 91%. ^{1}H-NMR, δ: 3.90 (s, 3H); 3.94 (s, 3H); 6.85 (d, 1H, *J*= 8.8 Hz); 6.88 (d, 1H, *J* = 16.0

Hz); 6.98–7.04 (m, 4H); 7.11 (d, 1H, *J* = 16.0 Hz); 7.17 (d, 1H, *J* = 4.6 Hz). ^{13}C-NMR, δ:149.3, 149.1, 138.2, 130.2, 128.3, 127.7, 125.6, 124.0, 120.2, 119.8, 111.4, 108.7, 56.1, 56.0. Anal. Calcd for $C_{14}H_{14}O_2S$: C, 68.26; H, 5.73; S, 13.02. Foud: C, 68.31; H, 5.79; S, 12.98.

(E)-3,4,5-trimethoxy-(2-thienyl) styrene 10d:

Yield: 93%. **^{1}**H NMR, δ: 3.87 (3H, s), 3.88 (s, 6H), 6.68 (bd, *J* = 4 Hz, 2H); 6.72 (d, *J* = 16 Hz, 1H); 7.00 (dd, *J* = 3.1, 5.0 Hz, 1H); 7.06 (bd, *J* = 5.1 Hz, 1H); 7.14 (d, *J* = 15.98 Hz, 1H); 7.17 (d, *J* = 4.75 Hz, 1H). ^{13}C NMR, δ: 153.6, 142.9, 135.8, 132.9, 128.6, 128.5, 127.8, 126.2, 124.5, 124.4, 121.5, 103.6, 61.1, 56.3. Anal. Calcd for $C_{15}H_{16}O_3S$: C, 65.19; H, 5.84; S, 11.60. Found: C, 65.25; H, 5.92; S, 11.64.

(E)-4-methoxy-(3-thienyl) styrene 11a:

Yield: 83%. ^{1}H-NMR, δ: 3.81 (s, 3H); 6.92 (d, *J* = 16.2 Hz, 1H); 6.95 (m, 1H); 7.00 (d, *J* = 8.6 Hz, 2H); 6.99 (d, *J* = 16.3 Hz, 1H); 7.20 (dd, *J* = 2.8, 1.3 Hz, 1H); 7.33-7.28 (m, 1H); 7.40 (d, *J* = 8.6 Hz, 2H). ^{13}C-NMR, δ: 159.6, 140.8, 130.6, 128.7, 127.9, 126.4, 125.3, 121.9, 121.4, 114.6, 55.7. Anal. Calcd for $C_{13}H_{12}OS$: C, 72.19; H, 5.59; S, 14.82. Found: C, 72.29; H, 5.64; S, 14.85.

(E)-3,5-dimethoxy-(3-thienyl) styrene 11b:

Yield: 87%. **^{1}**H NMR, δ: 3.82 (s, 6H); 6.38 (t, *J* = 2Hz, 1H); 6.61 (d, *J* = 2Hz, 2H); 6.65 (m, 1H); 6.74 (d, *J* = 16.2 Hz, 1H); 6.95 (d, *J* = 16.1Hz, 1H); 7.4 (m, 1H); 7.53 (s, 1H). ^{13}C-NMR, δ: 160.1, 136.5, 135.1, 131.5, 126.1, 125.8, 124.1, 120.1, 104.3, 56.9. Anal. Calcd for $C_{14}H_{14}O_2S$: C, 68.26; H, 5.73; S, 13.02. Found: C, 68.35; H, 5.79; S, 13.05.

(E)-3,4-dimethoxy-(3-thienyl) styrene 11c:

Yield: 95%. ^{1}H-NMR, δ: 3.90 (s, 3H); 3.94 (s, 3H); 6.85 (d, 1H, *J* = 7.9 Hz); 6.93 (d, 1H, *J* = 16.1 Hz); 7.03 (m, 2H); 7.09 (d, 1H, *J* = 16.1 Hz); 7.21-7.33 (m, 3H). ^{13}C-NMR, δ: 148.8, 148.5, 140.0, 130.2, 128.2, 125.9, 124.6, 121.5, 120.8, 119.4, 110.9, 108.2, 55.6, 55.5. Anal. Calcd for $C_{14}H_{14}O_2S$: C, 68.26; H, 5.73; S, 13.02. Found: C, 68.36; H, 5.82; S, 13.07.

(E)-3,4,5-trimethoxy-(3-thienyl) styrene 11d:

Yield: 88%. **^{1}**H NMR, δ: 3.87 (s, 3H), 3.94 (s, 6H); 6.70 (s, 2H); 6.88 (d, *J* = 16.2 Hz, 1H); 7.04 (d, *J*= 16.2 Hz, 1H); 7.21-7.33 (m, 3H). ^{13}C-NMR, δ: 152.7, 139.7, 136.1, 133.9, 131.2, 129.2, 128.5, 126.2, 120.3, 102.5, 59.8, 56.3, 56.1. Anal. Calcd for $C_{15}H_{16}O_3S$: C, 65.19; H, 5.84; S, 11.60. Found: C, 65.27; H, 5.90; S, 11.57.

REFERENCES

1. WHO (2001) Safety evaluation of certain mycotoxins in food. Fifty sixth report of the joint FAO/WHO expert committee on food additives. WHO Food Additives Series 47. World Health Organization, Geneva, p 700.
2. S. Stoev, *Vet. Hum. Toxicol.*, **40**, 352 (1998).
3. J. Aguirre,W. Hansberg, R. Navarro, *Med. Mycol.*, **44**, 101 (2006).
4. M. Reverberi, A. Ricelli, S. Zjalic, A. A. Fabbri, C. Fanelli, *Appl. Microbiol. Biotechnol.*, **87**, 3, 899 (2010).
5. X. S. Qin, M. J. Cao, F. N. Lai, F. Yang, W. Ge, X. F. Zhang, S. F. Cheng, X. F. Sun, G. Q. Qin, W. Shen, L. Li, *PlosOne*. **10**, 6, e0127551 (2015).
6. R. Amorati, M. Lucarini, V. Mugnaini, G. Pedulli, M. Roberti, D. Pizzirani, *J. Org. Chem.*, **69**, 7101 (2004).
7. R. Antonioletti, A. Viglianti, S. Cristofoli, A. Ricelli, *Curr. Org. Chem.*, **20**, 2029 (2016).
8. S. K. Lee, H. Y. Min, S. K. Huh, E. Y. Kim, E. Lee, S. Song, S. Kim, *Bioorg. Med. Chem. Lett.*, **13**, 21 3689 (2003).
9. E. Deprez, S. Barbe, M. Kolaski, H. Leh, F. Zouhiri, C. Auclair, J. C. Brochon, M. Le Bret, J. F. Mouscadet, *Mol. Pharmacol.*, **65**, 85 (2004).
10. A. V. Lipeeva, E. E. Shults, M. M. Shakirov, M. A. Pokrovsky, A. G. Pokrovsky, *Molecules*, **19**, 6, 7881 (2014).
11. D. L. Flynn, T. R. Belliotti, A. M. Boctor, D. T. Connor, C. R. Kostlan, D. E. Nies, D. F. Ortwine, D. J. Schrier, J. C. Sircar, *J. Med. Chem.*, **34**, 518 (1991).
12. S. K. Lee, E. J. Park, E. Lee, H. Y. Min, E. Y. Kim, T. Lee, S. Kim, *Bioorg. Med. Chem. Lett.*, **14**, 2105 (2004).
13. C. Lim, M. Lee, E. J. Park, R. Cho, H. J. Park, S. J. Lee, H. Cho, S. K. Lee, S. Kim, *Bioorg. Med. Chem. Lett.*, **20**, 6938 (2010).
14. D. Veau, S. Kykun, G. Mori, B. S. Orena, M. R. Pasca, C. Frongia, V. Lobjois, S. Chassaing, C. Lherbet, M. Baltas, *Chem. Med. Chem.*, **11**, 1078 (2016).
15. F. Caruso, L. Mendoza, P. Castro, M. Cotoras, M. Aguirre, B. Matsuhiro, M. Isaacs, M. Rossi, A. Viglianti, R. Antonioletti, *PlosOne*, **6**, 10, e25421 (2011).
16. R. Antonioletti, G. Righi, A. Ricelli, I. Rossetti, A. Viglianti, C. Frezza, F. Marino-Merlo, B. Macchi. *Curr. Org. Chem.* **21**, 939 (2017).
17. R. Antonioletti, F. Bonadies, A. Ciammaichella, A. Viglianti, *Tetrahedron*, **64**, 4644 (2008).
18. J. T. Zhang, W. Dai, R. G. Harvey, *J. Org. Chem.*, **63**, 8125 (1998).
19. M. Murias, W. Jager, N. Handler, T. Erker, Z. Horvath, T. Szekeres, H. Nohl, L. Gille, *Biochem. Pharmacol.*, **69**, 903 (2005).
20. J. C. Cheng, J. G. Fang, W. F. Chen, B. Zhou, L. Yang, Z. L. Liu, *Bioorg. Chem.*, **34**, 142 (2006).

РОЛЯ НА НЯКОИ СТИРИЛОВИ ХЕТЕРОЦИКЛИ ЗА КОНТРОЛ НА БИОСИНТЕЗА НА ОКРАТОКСИН А

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(Резюме)

Ократоксин А (ОТА) е микотоксин, чието опасно действие е причина много страни да регулират присъствието му в различни храни. Черната Aspergilli група, и по-специално *Aspergillus carbonarius*, причинява най-голямо замърсяване с ОТА в плодовете. В настоящата работа е описано влиянието на различни стирил-хетероциклени съединения върху превенцията на биосинтеза на ОТА от *A. carbonarius,* отглеждана в проводима течна среда. Най-ефективен и продължителен контрол на биосинтеза на ОТА се постига с (*E*)-3,5-диметокси-(2-тиенил) стирен (**10b**) и (*E*)-3,4,5-триметокси-(3-тиенил) стирен (**11d**). В гъбични култури, третирани с тези съединения на ниво 50 ppm, синтезът на ОТА намалява съответно с 65% и 90% след инкубация от 8 дни. По-ниската реактивоспособност повишава инхибирането на биосинтеза на ОТА особено в дългосрочен план. Установено е, че моделът на заместване на фенолния пръстен е от голямо значение за заместителния модел на тиофена. Природните съединения, присъстващи в ядливите растения, които имат стирилов хетероциклен скелет, може да са ефективни инхибитори на биосинтеза на ОТА.

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Antioxidant activity of selected *o*-methoxyphenols and biphenols: theoretical and experimental studies

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A combination of theoretical and experimental approaches was applied to study and to explain the structure – antioxidant activity relationship for selected *ortho*-methoxyphenols (natural and natural-like phenols). The corresponding dimers (biphenols) possessing guaiacyl moiety were handpicked in order to study the influence of the conformation and substituents in the aromatic ring on the antioxidant activity. Chain-breaking antioxidant activities of the compounds under study were determined from the kinetic curves of bulk lipid autoxidation. Full geometry optimization of neutral molecules and their corresponding phenoxyl radicals for all compounds under study were obtained by using DFT (B3LYP/6-31+G**) calculations. Good correlation between experimental and predicted activity was achieved which is helpful for the structure-activity relationship explanation.

Keywords: Antioxidants, Protective effect, Bulk lipid autoxidation, Natural phenols, Hydroxylated biphenyls, DFT calculations

INTRODUCTION

Antioxidants act as chain-breaking agents by transferring a hydrogen atom to peroxyl radicals at a rate higher than the propagation reaction, as a result, conversion of peroxyl radicals into non-radical products occurs [1,2]. Currently there is a great interest in friendly antioxidants as a replacement for the toxic ones (such as butylated hydroxytoluene, BHT) commonly used in foods, cosmetics and fragrances [3]. The reactions of peroxyl radicals with the phenol OH group of an effective phenolic antioxidant are much faster than those with C-H bond [4]. Naturally occurring phenols are a valuable source of bioantioxidants [5, 6] which are associated with a vast array of useful biological activities, especially antimicrobial and anti-inflammatory properties [7, 8]. Nowadays, there is a strong interest in drugs and food additives. Food supplements in combination with drugs having two or more actions (e.g. antioxidant and anti-inflammatory), targeted at multiple etiologies of the same disease, may offer a great therapeutic benefit.

2-Methoxy phenol is one of the common classes of secondary metabolites in medicinal plants [9, 10]. When these phenols, characterized by a guaiacyl unit, are substituted at position 4 with an electron donating group (EDG), the guaiacyl unit makes the compound a potential antioxidant. This is due to the favoured stabilization of the generated phenoxyl radical by forming a five-membered ring with the methoxyl group and the EDG group in *para* position to the phenol OH group [11]. The antioxidant properties of several natural phenols, like eugenol (**Eu**), creosol (**Cr**), apocynin (**Apo**) and isoeugenol (***iso*Eu**), have already been reported [12-14] and the protective effect in different oxidant systems and biosystems has been assessed [15, 16]. They are constituents of spices, flavours and fragrances. Eugenol (**Eu**) efficiently inhibits the metal-mediated low density lipoprotein (LDL) oxidation acting as a physiological antioxidant *in vivo* [15] and apocynin (**Apo**) is an efficient inhibitor of the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complex [16].

Hydroxylated biphenyls are widely present in nature and conversely to their corresponding natural monomeric phenols, they represent an important source of bioactive compounds that has not been well investigated [17]. Structurally they are dimers of phenols where two aromatic rings are C-C single bond bridged. The presence of hydroxylated functionalities in the biphenyl structure provides interesting features in terms of bioavailability, interactions with proteins and antioxidant activities [18, 19]. Compared to phenols, often, hydroxylated biphenyls are less toxic than the corresponding phenolic monomer [20] from which they are produced by oxidative coupling reaction. Some of the hydroxylated biphenyls have been already isolated from many plants and characterized [21], others are still undiscovered. Dehydrodieugenol (**DEu**) has been isolated from the buds of clove (*Syzygium*

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aromaticum) and, structurally, it represents a C_2-symmetrical dimer of **Eu** [22]. **DEu** manifests interesting antimutagenic activity, compared to its monomer it is less toxic and possesses higher antioxidant activity on lipid peroxidation [23]. Similarly, the dimer of apocynin (**DApo**), detected in biological systems treated with apocynin (**Apo**), manifests higher anti-inflammatory and antioxidant activities in comparison to its corresponding monomer **Apo** [24]. Considering the increased lipophilicity of the dimers compared to monomers and the role they can play in delaying the cellular damage due to membrane lipid peroxidation, an investigation of natural phenols (monomers and dimers) on bulk lipid oxidation appears useful and strategic for further studies on the effect of natural bioactive phenols in biological systems.

The aim of this work was to prepare a small collection of hydroxylated biphenyls starting from the corresponding natural phenol monomers, commercially available, and to study their effect during bulk lipid autoxidation in comparison with their corresponding monomers (Figure 1). We selected 2-methoxy phenol monomers as creosol (**Cr**), vanillin (**Va**), apocynin (**Apo**), eugenol (**Eu**) and their corresponding dimers **DCr**, **DVa**, **DApo**, **DEu**, respectively (Figures 1a and 1b). Antioxidant activity of the monomers was also compared with that of isoeugenol (***iso*Eu**), a 2-methoxy phenol known for the high antioxidant activity [15]. The structure-activity relationship was investigated by Density Functional Theory (DFT) calculation of monomers and dimers with the aim to identify structural parameters able to decrease the bond dissociation enthalpy (BDE) of the phenol OH bond and to stabilize the phenoxyl radical.

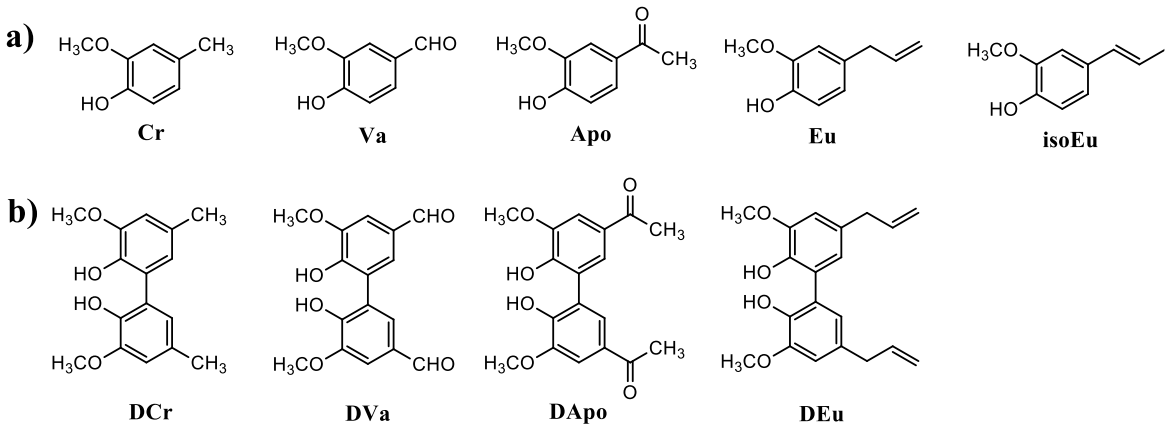


Figure 1. Structures of the studied compounds: a) 2-methoxy phenol monomers; b) hydroxylated biphenyls.

EXPERIMENTAL AND COMPUTATIONAL DETAILS

Experimental details

All ^{1}H NMR and ^{13}C NMR spectra were recorded on a spectrometer Varian Mercury Plus operating at 399.93 MHz and 100.57 MHz, respectively. Chemical shifts are given in ppm (δ) and coupling constants in Hertz. $CDCl_3$, acetone-d_6, were used as solvents as indicated below. Shifts are given in ppm relative to the remaining protons of the deuterated solvents used as internal standards (^{1}H, ^{13}C). All reagents were of commercial quality and used as purchased from various producers (Sigma-Aldrich, Merck). Flash chromatography was carried out with silica gel 60 (230-400 mesh, Kiesgel, EM Reagents) eluting with an appropriate solution in the stated v:v proportions. Analytical thin-layer chromatography (TLC) was performed with 0.25 mm thick silica gel plates (Polygram® Sil G/UV$_{254}$, Macherey-Nagel). The purity of all new compounds was judged to be >98% by ^{1}H-NMR spectral determination. Biphenyls **DEu**, **DVa** and **DApo** were prepared as previously described by us [25-27]. Solvents were used without additional purification or drying, unless otherwise noted.

2,2-Dihydroxy-3,3-dimethoxy-5,5-dimethyl-1,1-biphenyl (dehydrodicreosol, **DCr***)*

To a solution of 2-methoxy-4-methylphenol (creosol **Cr**) (0.5 g, 3.6 mmol) in dry dichloromethane (25 mL), was added dropwise a solution of methyl-*t*-butyl ammonium permanganate (MTBAP) (0.57 g, 1.8 mmol) in dry dichloromethane (15 mL) under nitrogen at 0°C. The solution was stirred for 30 min at 0 °C. Aqueous $Na_2S_2O_5$ was added to the mixture, the organic layer was dried over Na_2SO_4 and removed under reduced pressure to obtain a brown solid. The product obtained was washed twice with pentane (2×50 mL) to yield 0.42 g (85%) of the dimer **DCr** as a light yellow solid. Mp 133-135 °C (Lit. [28] 132-134 °C); ^{1}H NMR ($CDCl_3$) δ 2.34 (s, 6H, CH_3), 3.91 (s, 6H, OCH_3), 6.0 (bs, 2H, OH), 6.72-6.74 (m, 4H, ArH); ^{13}C NMR ($CDCl_3$) δ 21.10, 56.06, 111.31, 123.43, 124.40, 129.64, 140.33, 147.09;

Anal. Calcd for $C_{16}H_{18}O_4$: C, 70.06; H, 6.61; Found: C, 70.10; H, 6.66.

Biphenyls **DEu**, **DCr**, **DVa**, and **DApo** were prepared starting from the corresponding monomers **Eu**, **Cr**, **Va**, and **Apo**, respectively, following known coupling reaction procedures with slight modifications carried out by us to improve yields and make the methodology more straightforward. Although all monomers belong to the family of 2-methoxy phenols, substitution at *para* position to phenol-OH group required different oxidative conditions and reagents. **Eu** was treated with a solution of NH_4OH and $K_3Fe(CN)_6$ in acetone-water at room temperature in open air. **DEu** was obtained as a colorless solid in 95% yield after recrystallization from absolute ethanol [25]. Regioselective dimerization of **Va** in the presence of the oxidant mixture $K_2S_2O_8/FeSO_4$ in water-acetone at 50 °C, gave **DVa** by precipitation. The solid, after dissolution in aqueous NaOH and further acidification, was collected in 95% yield [26]. **Apo** was treated with a stoichiometric amount of $K_2S_2O_8/FeSO_4$ at room temperature in open air using a mixture of water/acetone as solvent. **DApo** was obtained in 80% yield without further purification [27]. **DCr** was obtained in good yield following a known procedure by reaction of **Cr** with methyl-*t*-butyl ammonium permanganate in dichloromethane at room temperature [28].

Chain-breaking antioxidant activity: Triacylglycerols of commercially available sunflower oil (TGSO) were cleaned from pro- and antioxidants by adsorption chromatography and stored under nitrogen at temperature 20 °C. Fatty acid composition of the lipid substrate was determined by GC analysis of the methyl esters: 16:0 (6.7%); 18:0 (3.6%); 18:1 (25.1%); 18:2 (63.7%); 20:0 (0.2%); 22:0 (0.7%); the numbers x:y indicate the number of carbon atoms and double bonds in the fatty acid, respectively. Lipid samples containing various inhibitors were prepared directly before use. Aliquots of the antioxidant solutions in purified acetone were added to the lipid sample. Solvents were removed under a nitrogen flow.

Lipid autoxidation: The process was carried out in a thermostatic bath at 80±0.2 °C by blowing air through the samples in special vessels. The oxidation process was monitored by withdrawing samples at measured time intervals and subjecting them to iodometric determination of the primary products (lipid hydroxyperoxides, LOOH) concentration, i.e. the peroxide value (PV). All compounds were subjected to lipid autoxidation at 80 °C at two concentrations, 0.1 and 1.0 mM, respectively. All kinetic data are expressed as the average of two independent measurements which were processed using the computer programmes Origin 6.1 and Microsoft Excel 2010. The basic kinetic scheme of lipid autoxidation is published elsewhere [29].

Determination of the main kinetic parameters of the studied compounds [30-32]:

Protection factor (PF) is a measure for the antioxidant efficiency i.e. $PF = IP_A/IP_C$ and means how many times the oxidation stability of a lipid substrate increased in presence of an antioxidant. IP_C and IP_A are the induction periods of control sample and in presence of an inhibitor.

Inhibition degree (ID) is a measure of the antioxidant reactivity, e.g. how many times the antioxidant shortens the oxidation chain length, i.e. $ID = R_C/R_A$. The initial oxidation rates R_C in the absence and R_A in the presence of antioxidant were found from the tangent at the initial phase of the kinetic curves of hydroperoxides accumulation.

Antioxidant capacity (Rm) is a measure of the consumption of the antioxidant during the induction period.

Radical scavenging activity: the capacity of studied compounds to scavenge free radicals was estimated by the DPPH radical test in acetone solution. Experimental details are previously presented [33]. The main kinetic parameters of the process are radical scavenging activity (%RSA), rate constants of radical reactions between DPPH radical and studied compounds (k_{RSA}) and stoichiometric coefficient (n) that shows how many radicals are trapped by one molecule of antioxidant. All these kinetic parameters were determined and compared.

Computational details

Unrestricted open-shell approach UB3LYP [34] and 6-31+G(d,p) [35, 36] basis set were used to optimize the geometry of compounds studied and their radicals without symmetry constraints with the default convergence criteria using the Gaussian 09 program [37]. Frequency calculations for each optimized structure were performed at the same level of theory. No imaginary frequency was found for the lowest energy configurations of any of the optimized structures. Unscaled thermal corrections to enthalpy were added to the total energy values. The BDEs for the generation of the respective radicals from the parent compounds were calculated by the formula:

$$BDE = H_{298}(AO^{\bullet}) + E_T(H^{\bullet}) - H_{298}(AOH) \quad (1)$$

where $H_{298}(AO^{\bullet})$ and $H_{298}(AOH)$ are enthalpies calculated at 298 K for radical species, $AO^{\bullet}$ and

neutral molecule AOH, respectively, $E_T(H^{\bullet})$ (calculated total energy of $H^{\bullet}$) is -313.93 kcal mol^{-1}. Solvation effects were accounted for by employing the polarizable continuum model (PCM) [38] as implemented in the Gaussian 09 suite of programs: all structures were optimized in acetone and DMSO surrounding environments. PyMOL molecular graphics system was used for generation of the molecular graphics images [39].

RESULTS AND DISCUSSION

Chain breaking antioxidant activity

The effect of monomers' concentration during bulk lipid autoxidation was studied at 0.1 and 1.0 mM, respectively, as depicted in Figures 2 and 3.

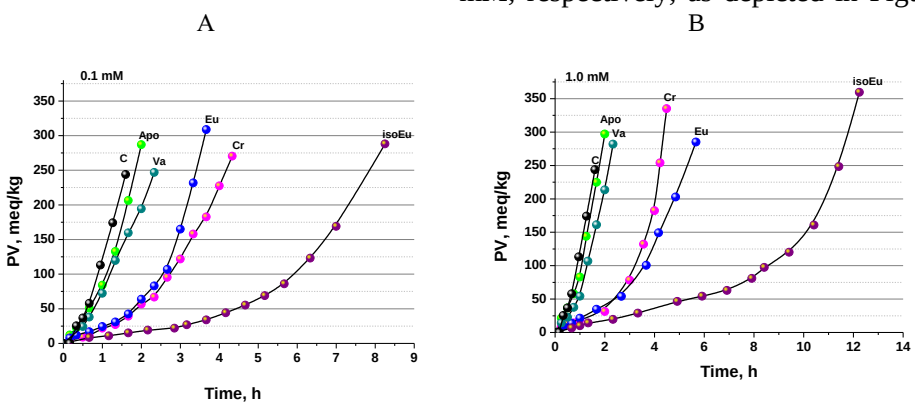


Figure 2. Kinetics of TGSO autoxidation at 80° C in absence (control sample, C) and in presence of A) 0.1 mM and B) 1.0 mM of the studied monomers.

The main kinetic parameters (PF, ID, Rm) are reported for all studied monomers (Table 1) evidencing the highest chain-breaking antioxidant efficiency and reactivity of ***iso*Eu** at both concentrations. New orders of antioxidant efficiency (PF), reactivity (ID) and capacity (Rm) were obtained for both studied concentrations:

Low concentration (0.1 mM)

PF: isoEu (5.7) > Cr (2.7) ≥ Eu (2.5) > Va (1.1) = Apo (1.1)

ID: isoEu (5.2) > Cr (3.3) > Eu (2.4) > Va (1.3) > Apo (0.9)

Rm 10^{-8}: isoEu (0.5) < Cr (1.0) < Eu (1.1) < Va (2.5) = Apo (2.5)

High concentration (1.0 mM)

PF: isoEu (10.1) > Cr (3.6) > Eu (3.2) > Va (1.3) = Apo (1.3)

ID: isoEu (6.9) > Cr (3.5) > Eu (2.9) > Va (1.3) > Apo (0.7)

Rm 10^{-8}, M/s: isoEu (2.7) < Cr (7.7) < Eu (8.7) < Va (21.4) = Apo (21.4)

From the main kinetic parameters the antioxidant activity of the studied compounds was evaluated. The highest antioxidant activity was observed for ***iso*Eu**. Moderate activities for **Cr** and **Eu** were deduced. **Va** and **Apo** have no activity in our model system of lipid autoxidation and this can be explained with their structures. The presence of electron-withdrawing group (EWD) in the latter two monomers hampers the formation of radical species and their stabilization. Moreover, their participation in side reactions with LOOH should not be ruled out.

*Iso*Eugenol is the most effective antioxidant. This can be explained with the presence of a vinyl double bond in the side chain favoring an extended conjugation with the aromatic ring when a radical methide is generated. **Cr** and **Eu** are effective chain-breaking antioxidants as a result of their participation in "homo-disproportionation" reactions (Schemes 1 and 2) allowing regeneration of the antioxidant.

Both **DVa** and **DApo** dimers are completely insoluble in acetone (our reference solvent for the monomers) and in triglycerides, so they could not be tested in our system. Due to the partial solubility of **DEu** in acetone, it was dissolved at the minimum concentration of DMSO (49 mM) and then assayed in bulk lipid autoxidation as shown in Figure 4. **DCr** is soluble in acetone, but its activity in the same acetone/DMSO mixture was tested in order to be compared with that of **DEu.** It was shown that in presence of DMSO the capacity of both studied dimers to inhibit bulk lipid autoxidation decreases. The effect is more pronounces for the dimeric structures in comparison to the monomeric ones.

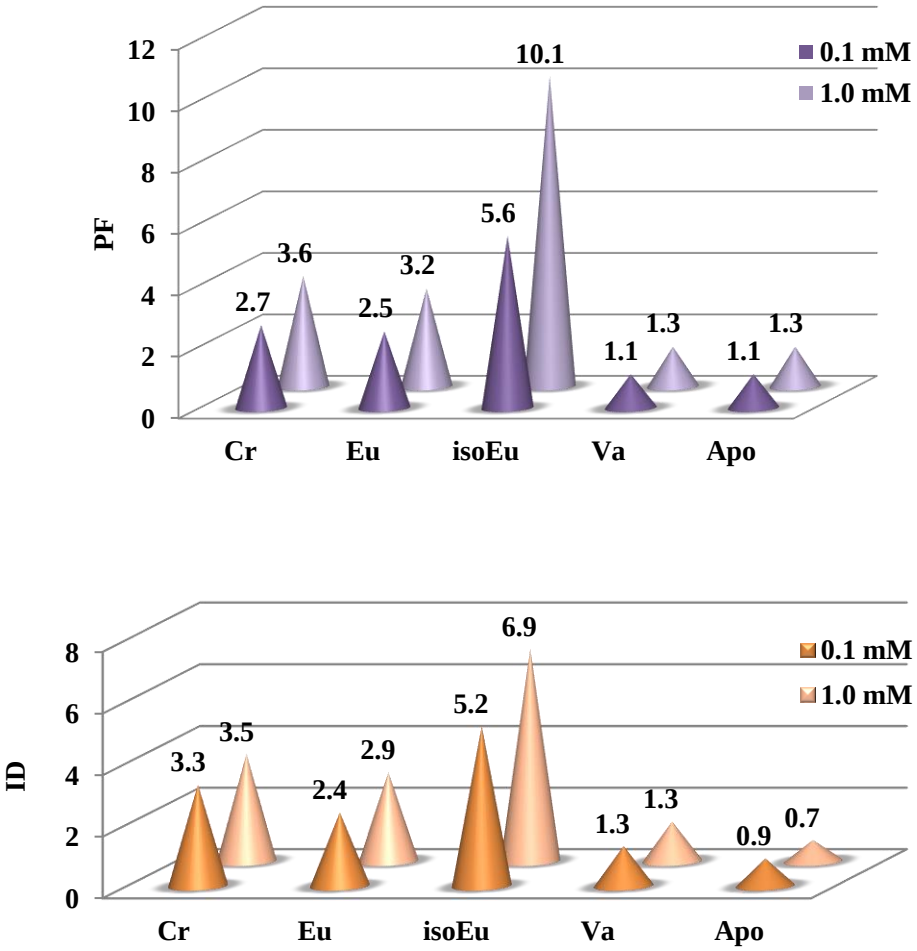
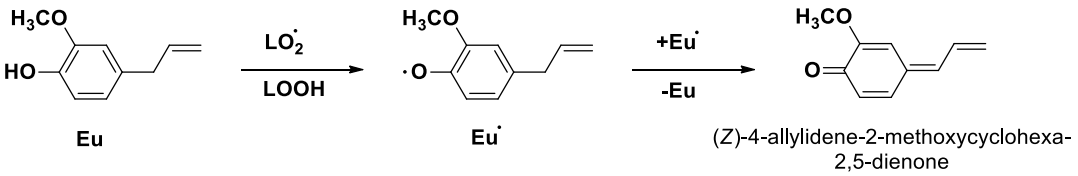


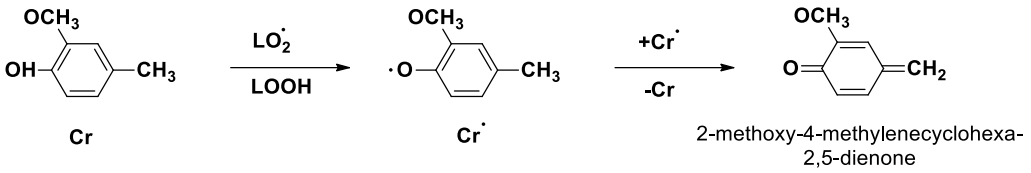
Figure 3. Protection factor (**PF**) and inhibition degree (**ID**) of studied monomers for both concentrations (0.1 mM and 1.0 mM).

Table 1. Main kinetic parameters, characterizing TGSO autoxidation at 80˚C in presence of 0.1 mM and 1.0 mM of the test compound. For control sample: IP_C = (1.0 ± 0.2) h, $R_C = 8.3\times10^{-6}$ M /s.

Compd	Concentration	IP_A, h	PF -	R_A 10^{-6}, M/s	ID -	Rm 10^{-8}, M/s	RRm 10^{-3}, -	Activity
Cr	0.1	2.7±0.2	2.7	2.5±0.2	3.3	1.0±0.2	4.0	Moderate
	1.0	3.6±0.3	3.6	2.4±0.2	3.5	7.7±0.4	32.1	moderate
Eu	0.1	2.5 ±0.2	2.5	3.4±0,3	2.4	1.1±0.2	3.2	moderate
	1.0	3.2±0.3	3.2	2.9±0.3	2.9	8.7±0.4	30.0	moderate
isoEu	0.1	5.6±0.5	5.6	1.6±0.3	5.2	0.5±0.03	3.1	strong
	1.0	10.1±0.8	10.1	1.2±0.2	6.9	2.7±0.2	22.5	strong
Va	0.1	1.1±0.2	1.1	6.4±0.5	1.3	2.5±0.2	3.9	no activity
	1.0	1.3±0.2	1.3	6.5±0.5	1.3	21.4±2.0	32.9	no activity
Apo	0.1	1.1±0.1	1.1	9.1±0.5	0.9	2.5±0.2	2.8	no activity
	1.0	1.3 ±0.2	1.3	11.6±0.9	0.7	21.4±2.0	18.4	no activity



Scheme 1. Reaction mechanism of eugenol.



Scheme 2. Reaction mechanism of creosol.

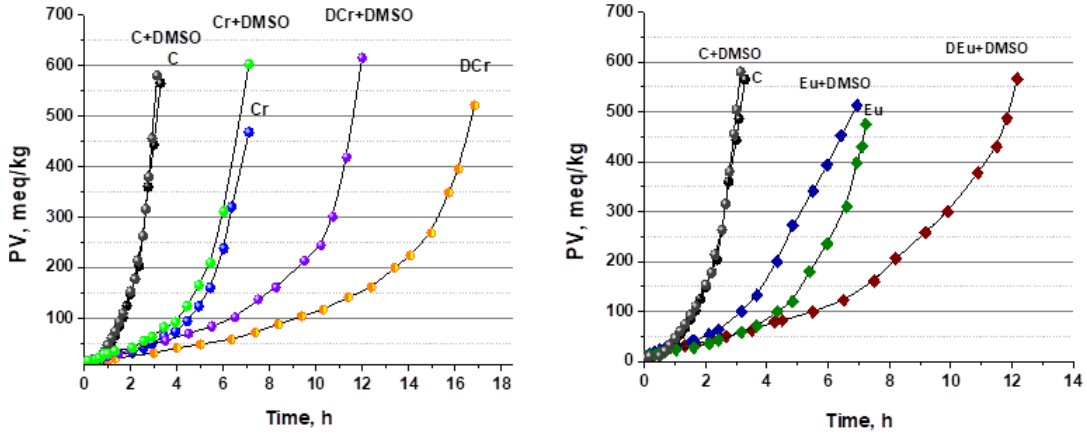


Figure 4. Kinetic curves of lipid hydroperoxides accumulation during TGSO autoxidation at 80 °C in absence (control sample, C) and in presence of 1.0 mM of studied couples: **Cr**/**DCr**, **Eu**/**DEu**, in absence and in presence of 49 mM DMSO.

Table 2. Main kinetic parameters in the presence of 1.0 mM of **Cr**, **Eu** and their corresponding dimers in 49 mM DMSO (effect of solvent) under TGSO autoxidation at 80° C. For control sample: IP_C=2.0 h, R_C=3.4 10^{-6} M/s, IP_{C+DMSO}=2.0 h, R_{C+DMSO}=2.9 10^{-6} M/s.

Compounds	IP_A, h	PF-	R_A 10^{-6}, M/s	ID -	Rm 10^{-8}, M/s	RRm 10^{-3}, -	Activity
Cr	5.2±0.5	2.6	1.5±0.2	2.27	5.37±0.3	35.87	moderate
Cr + DMSO	5.1±0.5	2.6	2.7±0.1	1.07	5.42±0.3	20.07	moderate
DCr	14.3±0.8	7.2	1.1±0.1	3.21	1.94±0.2	18.3	strong
DCr + DMSO	10.3±0.6	5.2	1.4±0.2	2.09	2.70±0.2	19.4	strong
Eu	5.5±0.5	2.8	1.9±0.2	1.83	5.05±0.3	27.15	moderate
Eu + DMSO	3.3±0.2	1.7	3.4±0.5	0.86	8.42±0.4	24.9	moderate
DEu + DMSO	10.2±0.6	5.1	2.8±0.5	1.04	2.71±0.2	9.7	strong

The results presented in Table 3 show higher radical scavenging activity towards DPPH radical for all dimers than that for the corresponding monomers of the studied compounds. This finding is in agreement with the chain-breaking antioxidant activity during bulk lipid autoxidation.

DFT calculations

The geometries of all parent compounds and possible phenoxyl radical species were optimized at UB3LYP/6-31+G(d,p) level. The optimized geometries only of the thermodynamically preferred rotamers of the parent compounds are presented in Figure 5. Complete geometrical parameters of all investigated systems are available on request. Theoretically calculated parameters characterizing the parent compounds (monomers and dimers) and the radical species (radicals and biradicals) are collected in Table 4. The BDEs derived from the respective enthalpy values are also presented graphically in Figure 5. The BDE values in gas phase, acetone and DMSO are compared.

Table 3. Main kinetic parameters of radical scavenging activity for studied compounds.

Compound	Time, min	Concentration, μM					
		25			39		
		RSA, %	n	k_{RSA} $M^{-1}s^{-1}$	RSA, %	n	k_{RSA} $M^{-1}s^{-1}$
Eu	<1 min			1.2			1.6
	2 min	0.37	0.01		0.74	0.02	
	20 min	2.03	0.08		3.58	0.09	
DEu	<1 min			49.6			49.7
	2 min	10.35	0.40		16.2	0.39	
	20 min	28.36	1.08		41.7	0.98	
Cr	<1 min			4.4			3.3
	2 min	1.10	0.05		1.31	0.03	
	20 min	4.01	0.16		5.28	0.14	
DCr	<1 min			19.1			31.8
	2 min	4.94	0.20		12.4	0.31	
	20 min	17.33	0.68		39.5	0.97	
Apo	<1 min			0.6			1.0
	2 min	0.26	0.01		0.52	0.01	
	20 min	0.63	0.03		1.18	0.03	
DApo	<1 min			3.4			3.4
	2 min	0.87	0.04		1.38	0.04	
	20 min	2.06	0.08		2.93	0.08	

BDE calculations confirmed the results obtained during bulk lipid autoxidation of monomers. For the series of the monomeric species the BDE values range between 77.41 and 83.69 kcal/mol in the gas phase, 73.28 and 81.47 kcal/mol in acetone medium, 73.70 and 81.71 kcal/mol in DMSO. **IsoEu** is characterized with the lowest BDE values both in the gas phase and in solvents confirming the best antioxidant activity. The high **Apo** (r) and **Va** (r) BDEs evidence the inability of the compounds to generate and stabilize a radical. A similar BDEs trend was estimated for radical **DApo** (r) and **DVa** (r), both unable to generate stable radicals. All C-C bridged dimers were characterized with lower BDE for radical species generation than the respective monomers, the lowest values were calculated for **DEu** and **DCr** (79.36 and 79.05 kcal/mol in the gas phase, respectively). The BDEs calculated for the biradical species (**br**) generation are higher than those for the first H-atom abstraction. It is known that in an *ortho-ortho* biphenol structure the pKa of the second phenol OH group is higher compared to the first one, thus, it is reasonable to expect a similar behavior also in the biradicals species formation (higher BDE values). C_2-symmetric dimer originating from ***iso*Eu** dimer was not considered because it does not exist in nature [40]. The spin density values at the oxygen atom (**O•**) from the hydroxyl group (listed also in Table 4) are not characteristic for the antioxidant activity.

A conclusion can be drawn (from the theoretical calculations) that **Eu**, **IsoEu**, **Cr** and their dimers are expected to be better antioxidants than **Apo** and **Va**. All dimeric structures have lower BDEs in comparison to the respective monomeric species.

CONCLUSIONS

A set of natural phenols known for their valuable biological activity was assayed in bulk lipid autoxidation and their chain-breaking antioxidant efficiency and reactivity were compared with those for the corresponding C_2-symmetric dimers soluble in acetone or DMSO. The dimers showed stronger antioxidant effectiveness, in particular **DCr** (that possesses a methyl group in *para* position to the guaiacyl unit). BDEs were calculated for all compounds and a good correlation with the experimental data was found. The results presented herein can motivate a deeper study of C_2-symmetric dimers of natural phenols in a combinatory drug therapy where the antioxidant activity plays an important role.

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Table 4. UB3LYP/6-31+G(d,p) calculated enthalpies (H_{298}) at 298 K (Hartree) in the gas phase, BDE (kcal/mol) and spin density (e^-).

Compound	H_{298}	BDE			Spin density at O$^\bullet$
		gas phase	acetone	DMSO	
		Monomers			
Apo	-574.496591				
Apo (r)	-573.863719	83.21	80.64	80.92	0.33
Eu	-538.531134				
Eu (r)	-537.903146	80.14	76.41	76.79	0.36
IsoEu	-538.541134				
IsoEu (r)	-537.917493	77.41	73.28	73.70	0.31
Cr	-461.169137				
Cr (r)	-460.541662	79.82	75.86	76.27	0.36
Va	-535.200736				
Va (r)	-534.567087	83.69	81.47	81.71	0.32
		Dimers			
DApo	-1147.810582				
DApo (r)	-1147.178797	82.53	80.20	80.49	0.31
DApo (br)	-1146.545817	83.27	80.61	80.95	0.31
DEu	-1075.878887				
DEu (r)	-1075.252146	79.36	75.93	76.26	0.34
DEu (br)	-1074.624177	80.13	76.29	76.71	0.34
DCr	-921.154892				
DCr (r)	-920.528641	79.05	75.40	75.81	0.35
DCr (br)	-919.901109	79.86	75.81	76.27	0.35
DVa	-1069.218675				
DVa (r)	-1068.586389	82.84	80.88	81.13	0.30
DVa (br)	-1067.952627	83.77	81.37	81.69	0.30

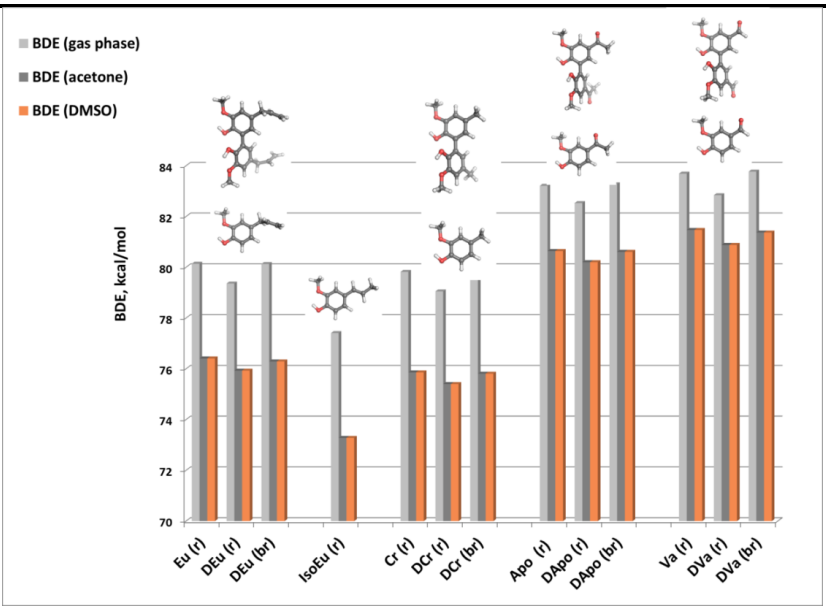

Figure 5. BDEs (in kcal/mol) in gas phase (light grey), acetone (grey) and DMSO (orange).

REFERENCES

1. K. U. Ingold, D. A. Prelog, *Chem. Rev.*, **114**, 9022 (2014).
2. V. Kancheva, *Eur. J. Lipid Sci. Technol.*, **111**, 1072 (2009).
3. R. Amorati, M. C. Foti, L. Valgimigli, *J. Agric. Food Chem.*, **61**, 10835 (2013).
4. M. Foti, *J. Pharm. Pharmacol.*, **59**, 1673 (2007).
5. A. Altemimi, N. Lakhssassi, A. Baharlouei, D. G. Watson, D. A. Lighfoot, *Plants*, **6**, 42 (2017).
6. N. K. Zenkov, A. V. Chechushkov, P. M. Kozhin, N. V. Kandalintseva, G. G. Martinovich, and E. B. Menshchikova, *Biochemistry (Moscow)*, **81**, 297 (2016).
7. H. Gurer-Orhan, E. Ince, D. Konyar, L. Saso, *Curr. Med. Chem.* (2017), doi: 10.2174/0929867324666170711114336.
8. A. Kerimi, G. Williamson, Mol. *Nutr. Food Res.* **60**, 1770 (2016).
9. B. Ahmad, M. U. Rehman, I. Amin, A. Arif, S. Rasool, S. A. Bhat, I. Afzal, I. Hussain, S. Bilal, M. Mir, *Scient. World J.*, **2015**, 816364 (2015).

10. T. Esatbeyoglu, P. Huebbe I. M. A. Ernst, D. Chin, A.E. Wagner, G. Rimbach, *Angew. Chem. Int. Ed.*, **51**, 5308 (2012).
11. A. Slavova-Kazakova, S. Angelova, T. L. Veprintsev, P. Denev, D. Fabbri, M. A. Dettori, M. Kratchanova, V. V. Naumov, A. V. Trofimow, R. F. Vasil'ev, G. Delogu, Beil. *J. Org. Chem.* **11**, 1398 (2015).
12. S. Fujisawa, T. Atsumi, Y. Kadoma, H. Sakagami, *Toxicology*, **177**, 39 (2002).
13. J. Stefanska, R. Pawliczak, *Mediators Inflam.*, **106507**, 1-10 (2008) doi:10.1155/2008/106507.
14. M. Ito, K. Murakami, M. Yoshino, *Food Chem. Toxicol*. **43**, 461 (2005).
15. T. Atsumi, S. Fujisawa, K. Tonosaki, *Toxicol. In Vitro*, **19**, 1025 (2005).
16. J. Y. Kim, X. N. Tang, M. A. Yenari, *Open Drug Discov. J*. **2**, 187 (2010).
17. S. Quideau, D. Deffieux, C. Douat-Casassus, L. Pouységu, *Angew. Chem. Int. Ed.*, **50**, 586 (2011).
18. G. Bérubé, *Curr. Med. Chem.*, **13**, 131 (2006).
19. V. Kancheva, A. Slavova-Kazakova, D.Fabbri, M.A. Dettori, G. Delogu, M. Janiak, R. Amarowicz, *Food Chem.*, **157**, 263 (2014).
20. S. Fujisawa, T. Atsumi, Y. Murakami, Y. Kadoma, *Arch. Immunol. Ther. Exp.*, **53**, 28 (2005).
21. G. Bringmann, T. Gulder, T.A.M. Gulder, M. Breuning, *Chem. Rev.*, **111** 563 (2011).
22. M. Miyazawa, M. Hisama, *J. Agric. Food Chem.*, **51**, 6413 (2003).
23. M. Ogata, M. Hoshi, S. Urano, T. Endo, *Chem. Pharm. Bull.*, **48**, 1467 (2000).
24. M. P. P. Kanegae, A. Condino-Neto, L. A. Pedroza, A. C. de Almeida, J. Rehder, L. M. da Fonseca, V. F. Ximenes, *Biochem. Biophys. Res. Commun.*, **393**, 551 (2010).
25. G. Calia, P. Monti, S. Marceddu, M. A. Dettori, D. Fabbri, S. Jaoua, R. O'Neil, P. A. Serra, G. Delogu, Q. Migheli, *Analyst*, **140**, 3607, (2015).
26. A. Marchiani, S. Mammi, G. Siligardi, R. Hussain, I. Tessari, L. Bubacco, G. Delogu, D. Fabbri, M. A. Dettori, D. Sanna, S. Dedola, P. A. Serra, P. Ruzza, *Amino Acids*, **45**, 327 (2013).
27. G. Delogu, M. A. Dettori, A. Patti, S. Pedotti, A. Forni, G. Casalone, *Tetrahedron: Asymmetry*, **14**, 2467 (2003).
28. J. Qing, S. Wenbing, T. Mi, T. Jie, G. Cancheng, *Eur. J. Org. Chem.*, **10**, 1861 (2013).
29. V.D. Kancheva, A.K. Slavova-Kazakova, S.E. Angelova, S.K. Singh, S. Malhotra, B.K. Singh, L. Saso, A.K. Prasad, V.S. Parmar, *Biochimie*, **140** 133 (2017).
30. I. Tichonov, V. Roginsky, E. Pliss, *Eur. J. Lipid Sci. Technol.* **112**, 887 (2010).
31. E. Denisov, T. , T. Denisova, G., *Russ. Chem. Rev.*, **78**, 1047 (2009).
32. E. T. Denisov, T. G. Denisova, Handbook of Antioxidants. Bond Dissociation Energies, Rate Constants, Activation Energies and Enthalpies of Reactions. CRC Press, New York, 2001.
33. V. D. Kancheva, L. Saso, S. E. Angelova, M. C. Foti, A. Slavova-Kasakova, C. Daquino, V. Enchev, O. Firuzi, J. Nechev, *Biochimie*, **94**, 403 (2012).
34. A. D. Becke, *J. Chem. Phys.*, **98**, 5648 (1993).
35. R. Ditchfield, W. J. Hehre, J. A. Pople, *J. Chem. Phys.*, **54**, 724 (1971).
36. T. Clark, J. Chandrasekhar, G.W. Spitznagel, P.V.R. Schleyer, *J. Comput. Chem.*, **4**, 294 (1983).
37. M. J. Frisch et al., Gaussian 09, Revision D.01. Gaussian, Inc., Wallingford CT, 2013.
38. S. Miertuš, E. Scrocco, J. Tomasi, *Chem. Phys.*, **55**, 117 (1981).
39. The PyMOL Molecular Graphics System, Version 1.7.6.6, Schrödinger, LLC.
40. T. Shiba, L. Xiao, T. Miyakoshi, C.-L. Chen, *J. Mol. Cat. B:Enzymatic*, **10**, 605 (2000).

АНТИОКСИДАНТНА АКТИВНОСТ НА ПОДБРАНИ *О*-МЕТОКСИФЕНОЛИ И БИФЕНОЛИ: ТЕОРЕТИЧНО И ЕКСПЕРИМЕНТАЛНО ИЗСЛЕДВАНЕ

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(Резюме)

Използвана е комбинация от теоретични и експериментални подходи за изследване и обяснение на зависимостта структура - антиоксидантна активност за избрани *орто*-метоксифеноли (природни и синтетични аналози). Съответните им димери (бифеноли) с гваяколови фрагменти са така подбрани, че да може се изследва влиянието на конформацията и заместителите в ароматния пръстен върху антиоксидантната активност. Антиоксидантната активност на изследваните съединения е определена от кинетичните криви на липидно автоокисление в хомогенна среда. На B3LYP/6-31+G** теоретично ниво са оптимизирани геометриите на всички молекули на изследваните съединения и на съответните им феноксилни радикали. Постигната е добра корелация между експериментално определената и теоретично предвидената активности, което е предпоставка за обяснение на зависимостта структура-активност.

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Antioxidant activity of 3-hydroxyphenol, 2,2'-biphenol, 4,4'-biphenol and 2,2',6,6'-biphenyltetrol**:** theoretical and experimental studies

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A set of selected phenolic compounds (phenol, 3-hydroxyphenol (resorcinol), 2,2'-biphenol, 4,4'-biphenol and 2,2',6,6'-biphenyltetrol) is designed in order to study the structure – antioxidant activity relationship for the compounds with one benzene ring and two C-C bridged benzene rings. The corresponding "dimeric" structures (biphenols and biphenyltetrol) of phenol and resorcinol are handpicked in order to study the influence of the number and mutual position of the substituents (OH group(s) in the aromatic ring) on the antioxidant activity. A combination of theoretical and experimental approaches is applied. Chain-breaking antioxidant activities of compounds under study are determined from the main kinetic parameters of bulk lipid autoxidation. Full geometry optimization of neutral molecules and their corresponding phenoxyl radicals for all compounds under study are obtained by using DFT (B3LYP/6-31+G**) calculations. Good correlation between experimental and predicted activity is achieved.

Keywords: Antioxidants, Protective effect, Bulk lipid autoxidation, Natural phenols, Hydroxylated biphenyls, DFT calculations

INTRODUCTION

Phenols, (ArOH), a major group of antioxidant phytochemicals, are of great importance due to their biological and free radical scavenging activities. They are found in all plants as secondary metabolites produced during the normal cycle of the plant and overproduced under biotic and abiotic stress conditions [1,2]. Generally, phenols are able to control the oxidation of organic compounds by transferring H atom from the phenol OH group(s) to the chain-carrying radicals (ROO•).

In this paper, our study focused on the relationship between antioxidant structure and activity of: phenol (**PhOH**), 3-hydroxyphenol (resorcinol, **Res**), 2,2'-biphenol (***o*-DHB**), 4,4'-biphenol (***p*-DHB**) and 2,2',6,6'-biphenyltetrol (**DRes**) (Figure 1). Biphenols and biphenyltetrol are C_2-symmetrical C-C bridged dimers of phenol and resorcinol, respectively. A combination of theoretical (Density Functional Theory, DFT, calculations) and experimental approaches (bulk lipid autoxidation) is applied.

EXPERIMENTAL AND COMPUTATIONAL DETAILS

Experimental details

All ^{1}H NMR and ^{13}C NMR spectra were recorded on spectrometer Varian Mercury Plus operating at 399.93 MHz and 100.57 MHz, respectively.

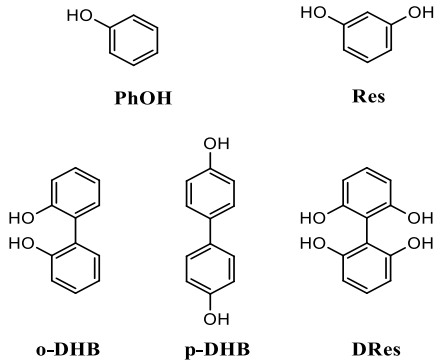

Figure 1. Structures of the studied phenolic compounds.

Chemical shifts are given in ppm (δ) and coupling constants in Hertz; multiplicities are indicated by s (singlet), d (doublet), t (triplet). $CDCl_3$ and acetone-d_6, were used as solvents as indicated below. Shifts are given in ppm relative to the remaining protons of the deuterated solvents used as internal standard (^{1}H, ^{13}C). All reagents were of commercial quality and used as purchased from various producers (Sigma-Aldrich, Merck). Flash chromatography was carried out with silica gel 60 (230-400 mesh, Kiesgel, EM Reagents) eluting with appropriate solution in the stated v:v proportions. Analytical thin-layer chromatography (TLC) was performed with 0.25 mm thick silica gel plates (Polygram® Sil G/UV_{254}, Macherey-Nagel). The purity of all new compounds was judged to be >98% by ^{1}H-NMR spectral determination. **Res** and biphenyls ***o*-DHB** and ***p*-DHB** were purchased from Sigma-Aldrich. **DRes** was prepared as previously described by us [3]. The solvents were used without

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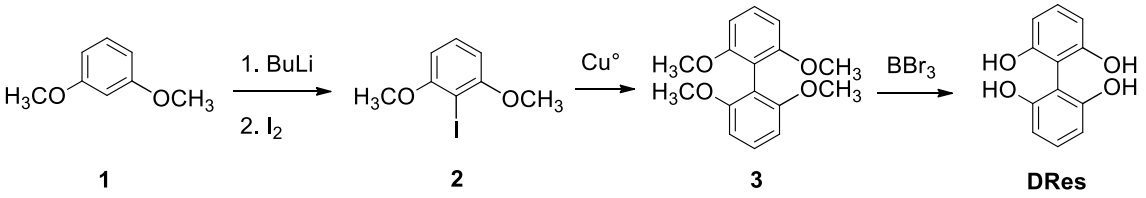
additional purification or drying, unless otherwise noted. The melting points of the newly synthesized compounds are uncorrected.

DRes was obtained in three steps starting from resorcinol dimethyl protected compound **1**, quenching of radical with iodine, followed by coupling reaction in presence of Cu(0) and demethylation by BBr_3 in anhydrous dichloromethane (Scheme 1) [3].



Scheme 1. Synthesis of **DRes**.

2-Iodo-1,3-dimethoxybenzene **(2).** To a solution of 1,3-dimethoxybenzene **1** (25 g, 180 mmol) in dry diethyl ether (150 mL) was slowly added butyllithium (112.5 mL of 1.6 M solution in hexanes, 180 mmol) under nitrogen at r.t. The reaction was stirred at r.t for 30 h and then cooled to -35°C. Iodine (45.7 g, 180 mmol) was added and the reaction was stirred for 24 h at 20°C and then poured into 10% chloridric acid (60 mL). The aqueous phase was separated and extracted with ethyl acetate (2 × 60 mL) and the combined organic extracts washed with saturated aqueous sodium thiosulfate (60 mL), brine (60 mL), dried over sodium sulfate, filtered and concentrated in *vacuo*. The product was purified by crystallization (diethyl ether) to give **2** (34 g, 71%) as a white solid; mp 105 -106 °C (Lit.104 °C) [4]; ^{1}H NMR ($CDCl_3$): δ 3.80 (s, 6H), 6.43 (d, *J* = 8.4, 2H), 7.17 (t, *J* = 8.4, 1H); ^{13}C NMR ($CDCl_3$): δ 56.6, 76.3, 103.9, 129.8, 159.5; Anal. Calcd for $C_8H_9IO_2$: C, 36.39; H, 3.44; Found: C, 36.40; H, 3.46.

2,2',6,6'-Tetramethoxybiphenyl **(3)**. In a crucible was placed a mixture of 16 g of 2-iodo-1,3-dimethoxybenzene **2** and 30 g of copper bronze. The mixture was covered with a layer (15 g) of copper bronze. The crucible was heated in an oven at 200°C for 2 h. After cooling, the reaction mixture was extracted in a Soxhlet apparatus with acetone. The product was purified by recrystallization from acetone to give **3** as white solid (6.6 g, 85%); mp 175-176 °C (Lit.175-176 °C) [5]; ^{1}H NMR ($CDCl_3$): δ 3.75 (s, 12H), 6.68 (d, *J* = 8 Hz, 4H), 7.32 (t, *J* = 8 Hz, 2H); ^{13}C NMR ($CDCl_3$): δ 56.1, 104.4, 112.5, 128.7, 158.4; Anal. Calcd for $C_{16}H_{18}O_4$: C, 70.06; H, 6.61. Found: C, 70.09; H, 6.62.

2,2',6,6'-Tetrahydroxybiphenyl (**DRes**). 2,2',6,6'-Tetramethoxybiphenyl **3** (4.7 g, 16.7 mmol) was dissolved in dry dichloromethane (110 mL) and cooled to -78 °C. A solution of boron tribromide (6.3 mL, 66.9 mmol) in dichlorom ethane (23 mL) was added dropwise under nitrogen. The solution was allowed to reach room temperature during 5 h. Water was carefully added to the reaction mixture. The solution was extracted several times with diethyl ether. The combined organic solutions were dried and evaporated. The residue was crystallized from ethanol to obtain *2,2',6,6'*-tetrahydroxybiphenyl **DRes** (2.84 g, 78%; mp 193-194 °C (Lit. 191-192 °C) [6]; ^{1}H NMR (acetone-d_6): δ 6.47 (d, *J* = 8 Hz, 4H), 7.02 (t, *J* = 8 Hz, 2H); ^{13}C NMR (acetone-d_6): δ 107.28, 129.04, 156.52, 205.51; Anal. Calcd for $C_{12}H_{10}O_4$: C, 66.05; H, 4.62. Found: C, 66.08; H, 4.65.

Chain-breaking antioxidant activity

Triacylglycerols of commercially available sunflower oil (TGSO) were cleaned from pro- and antioxidants by adsorption chromatography and stored under nitrogen at temperature 20 °C. Fatty acid composition of the lipid substrate was determined by GC analysis of the methyl esters: 16:0 (6.7%); 18:0 (3.6%); 18:1 (25.1%); 18:2 (63.7%); 20:0 (0.2%); 22:0 (0.7%); the numbers x:y indicate, respectively, the number of carbon atoms and double bonds in the fatty acid. Lipid samples containing various inhibitors were prepared directly before use. Aliquots of the antioxidant solutions in purified acetone were added to the lipid sample. Solvents were removed under a nitrogen flow. Lipid autoxidation was carried out in a thermostatic bath at (80±0.2) °C by blowing air through the samples in special vessels. The oxidation process was monitored by withdrawing samples at measured time intervals and subjecting them to iodometric determination of the primary products (lipid hydroxyperoxides, LOOH) concentration, i.e. the peroxide value (PV). All compounds were subjected to lipid autoxidation at 80 °C at two concentrations, 0.1 and 1.0 mM, respectively. All kinetic data are expressed as the average of two independent measurements which were processed using the computer program Origin 6.1 and Microsoft Excel 2010. The basic kinetic scheme of lipid autoxidation is published elsewhere [7].

Determination of the main kinetic parameters of the studied compounds [8-10]

Protection factor (PF) is a measure for the antioxidant efficiency i.e. $PF = IP_A/IP_C$ and means how many times the oxidation stability of lipid substrate increased in presence of an antioxidant. IP_C and IP_A are the induction periods of control sample and in presence of an inhibitor.

Inhibition degree (ID) is a measure of the antioxidant reactivity, e.g. how many times the antioxidant shortens the oxidation chain length, i.e. $ID = R_C/R_A$. The initial oxidation rates R_C in the absence and R_A in the presence of antioxidant were found from the tangents at the initial phase of the kinetic curves of hydroperoxides accumulation.

Antioxidant capacity (Rm) is a measure of the consumption of the antioxidant during the induction period.

Radical scavenging activity: the capacity of studied compounds to scavenge free radicals was estimated by DPPH radical test in acetone solution. Experimental details are previously presented [11]. The main kinetic parameters of the process are radical scavenging activity (%RSA) and stoichiometric coefficient (n) that shows how many radicals are trapped by one molecule of antioxidants. All these kinetic parameters are determined and compared.

Computational details

Unrestricted open-shell approach (Becke three-parameter hybrid functional B3LYP [12] and 6-31+G(d,p) [13,14] basis set) was used to optimize the geometry of compounds studied and their radicals without symmetry constraints with the default convergence criteria using the Gaussian 09 program [15]. Frequency calculations for each optimized structure are performed at the same level of theory. No imaginary frequency is found for the lowest energy configurations of any of the optimized structures. Unscaled thermal corrections to enthalpy are added to the total energy values. The BDEs for the generation of the respective radicals from the parent compounds are calculated by the formula

$$BDE = H_{298}(AO^{\bullet}) + E_T(H^{\bullet}) - H_{298}(AOH) \quad (1)$$

where $H_{298}(AO^{\bullet})$ and $H_{298}(AOH)$ are enthalpies calculated at 298 K for radical species, $AO^{\bullet}$ and neutral molecule AOH, respectively, and $E_T(H^{\bullet})$ (calculated total energy of $H^{\bullet}$) is -313.93 kcal mol^{-1}.

Solvation effects are accounted for by employing the polarizable continuum model [16] (PCM) as implemented in the Gaussian 09 suite of programs: all structures are optimized in acetone surrounding environment. PyMOL molecular graphics system was used for generation of the molecular graphics images [17].

RESULTS AND DISCUSSION

Chain-breaking antioxidant activity

Figure 2 (a-d) presents the kinetics of TGSO autoxidation at 80ºC in absence and in presence of **Res** and **DRes** at concentrations 0.1 mM and 1.0 mM. The main kinetic parameters determined are shown in Table 1.

Res manifested no antioxidant effect (PF) at lower concentration (0.1 mM) where a small pro-oxidant effect appears. At higher concentration (1.0 mM) **Res** showed the same activity as the control lipid (TGSO) sample, however ID increased 2-fold and Rm 10-fold growing the concentration. These data suggest a significant role of the side reactions with participation of **Res**. The lack of antioxidant activity was expected because of the *meta* positions of phenol OH groups and more difficult H-atom abstraction from **Res** to the lipid peroxide radicals.

DRes also demonstrated pro-oxidant effect at lower concentration and no effect at higher concentration. Its antioxidant reactivity (ID) shows low values at both concentrations, however the antioxidant capacity (Rm) grows significantly (10-fold) at higher concentration.

Protection factor of ***o*-DHB** increased 2.4-fold at higher concentration, however inhibition degree does not change, and the main rate of antioxidant consumption (Rm) increased 4.6-fold (Figure 3 and Table 1). These data confirm the participation of ***o*-DHB** in side reactions, leading to a decrease in its antioxidant capacity. Effect of the positions of phenolic OH groups was studied for ***o*-DHB** and ***p*-DHB**. A comparison of ***o*-DHB** and ***p*-DHB** demonstrates 2-fold higher antioxidant efficiency (PF) and reactivity (ID) for ***o*-DHB** at lower concentration (0.1 mM). Rm does not demonstrate significant differences between ***o*-DHB** and ***p*-DHB**.

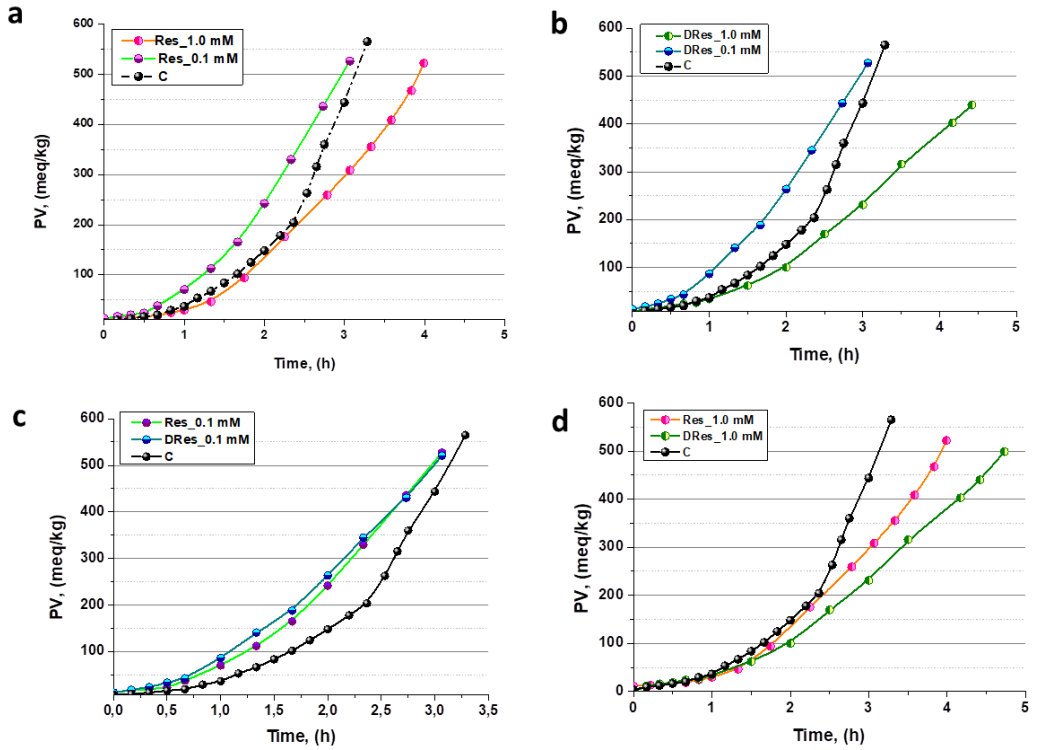


Figure 2. Kinetics of TGSO autoxidation at 80° C in absence (C) and in presence of 0.1 mM and 1.0 mM of **Res** and **DRes.**

Table 1. The main kinetic parameters, characterizing TGSO autoxidation at 80°C in presence of 0.1 mM and 1.0 mM of the tested compounds.

Compd.	Conc.	IP_A, h	PF -	R_A 10^{-6}, M/s	ID -	Rm 10^{-8}, M/s	RRm 10^3 -	Activity
Res[a]	0.1 1.0	1.3±0.2 2.3±0.3	0.6 1.1	2.9±0.4 1.5±0.2	1.2 2.3	2.1±0.2 12.1±1.2	7.24 80.7	prooxidant no activity
DRes[a]	0.1 1.0	1.3±0.2 1.7±0.2	0.7 0.8	4.7±0.5 3.3±0.5	0.7 1.0	2.1±0.2 16.3±1.5	4.5 49.4	prooxidant no activity
o-DHB[b]	0.1 1.0	2.5±0.2 6.0±0.5	2.5 6.0	4.6±0.6 4.2±0.3	1.8 2.0	1.1±0.2 4.6±0.3	2.4 10.9	weak moderate
p-DHB[b]	0.1	1.1±0.1	1.1	7.5±2.0	1.1	2.5±0.2	3.3	no activity

Control sample: [a]IP_C=(2.0±0.3) h, R_C =3.4×10^{-6} M/s.; [b]IP_C=(1.0±0.2) h, R_C=8.3×10^{-6} M/s

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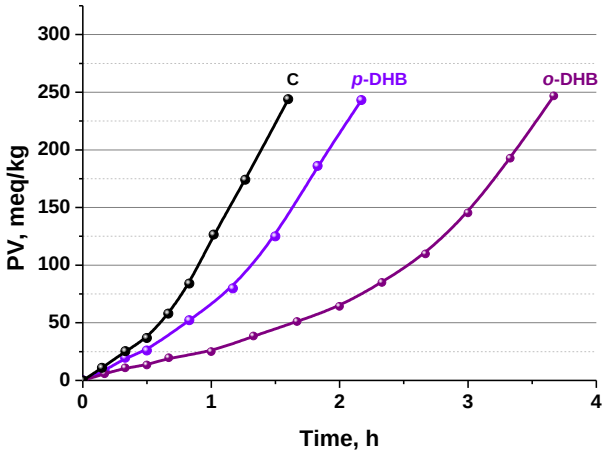

Figure 3. Kinetics of TGSO autoxidation at 80° C in absence (C) and in presence of 0.1 mM of ***o*-DHB** and ***p*-DHB**.

Figure 2 and Table 1 present the kinetic data of TGSO in presence of **Res** and **DRes**. **Res** and **DRes** at low concentration (0.1 mM) demonstrate similar pro-oxidant activity (PF), however ID and Rm values for **DRes** are lower in comparison to the values for **Res** (almost 2-fold).

At higher concentration (1.0 mM) **DRes** is less active than **Res** (PF), 2-fold lower for **DRes** for Rm. Interestingly, the increase of the number of phenol OH-groups in *ortho* positions to the C-C single bond (**DRes**) does not lead to a proportional increase in the antioxidant activity in comparison to the dimer with two phenol OH groups (***o*-DHB**). This result may be is due to the orthogonal position of the two aromatic rings in **DRes** that excludes a hypothetical conjugation between the two aromatic

rings. In fact the kinetic parameters for **DRes** are comparable with these for **Res** (having two phenol-OH groups in *meta*-position).

Radical scavenging activity

The results presented in Table 2 show a weak radical scavenging activity of studied compounds towards DPPH radical. A possible explanation is formation of inactive (to scavenge free radicals) complexes between the solute and acetone molecules.

Table 2. Radical scavenging activity towards DPPH radical in acetone solution.

Compound	Reaction time, min	Concentration, μM			
		25		39	
		RSA, %	n	RSA, %	n
DRes	2 min (fast kinetics)	3.21	0.13	4.42	0.11
	20 min (total kinetics)	17.49	0.70	23.03	0.57
o-DHB	2 min (fast kinetics)	0.21	0.01	0.9	0.02
	20 min (total kinetics)	2.33	0.10	4.28	0.10
p-DHB	2 min (fast kinetics)	0.70	0.03	0.63	0.02
	20 min (total kinetics)	1.48	0.06	1.50	0.04

DFT calculations

The geometries of the parent compounds and possible phenoxyl radical species are optimized at UB3LYP/6-31+G(d,p) level. The optimized geometries only of the thermodynamically preferred rotamers of the parent compounds are presented in Figure 4. The calculated enthalpies, H_{298}, for the parent compounds and radical species (radicals and biradicals) are given in Table 3. The BDEs derived from the respective enthalpy values are also listed in Table 3 and presented in Figure 4. The values in gas phase and in acetone are compared.

Phenol and resorcinol: in the gas phase they are characterized by consistently high BDE values for **PhOH (r)** and **Res (r)** radical species generation (81.76 kcal/mol and 82.77 kcal/mol, respectively); the biradical generation from **Res (r)** is characterized with lower BDE value – 81.54 kcal/mol. In acetone medium the BDEs for **PhOH (r)** and **Res (r)** decrease, while that for the **Res (br)** generation increases.

Biphenols: the Ar-Ar dihedral angles in the parent biphenols ***o*-DHB** (with H bond between the OH groups) and ***p*-DHB** are 50° and 40°, respectively. In the radical species the Ar-Ar dihedral angles angles decrease (31° and 29°, respectively), but for the biradicals species an increase (to 64° and 48°, respectively) is observed. In the gas phase ***o***- and ***p*-DHB** are characterized by lower BDE values (in comparison to phenol) for radical species generation (75.31 and 78.61 kcal/mol, respectively), while the BDEs for the biradical species (**br**) generation are higher than those for the first H-atom abstraction (generation of **PhOH (r)**, ***o*-DHB (r)** and ***p*-DHB (r)**). In acetone medium the BDE for ***o*-DHB (r)** increases (ΔBDE= 1.48 kcal/mol), for ***o*-DHB (br)** decreases noticeably (ΔBDE= 4.09 kcal/mol). In acetone medium the BDE for ***p*-DHB (r)** decreases (ΔBDE= 1.79 kcal/mol), while for ***p*-DHB (br)** the BDE changes slightly (ΔBDE= 0.58 kcal/mol).

Biphenyltetrol (**DRes**): the benzene rings of **DRes** lie in perpendicular planes and the BDE for the first H-atom abstraction from **DRes** has almost the same value (82.72 kcal/mol) as from "monomeric" **Res** (82.77 kcal/mol), while for **DRes (r)** the angle between the distinct planes of the benzene rings is ~53°. Two possible biradicals can be generated from **DRes (r)**: **DRes (br1)** (formed after H-atom abstraction from the same ring, with 36° angle between the planes) and **DRes (br2)** (formed after H-atom abstraction from the second ring, 40° angle). The BDEs for the second H-atom abstraction are lower than for the first one both in the gas phase and in acetone medium. The second H-atom abstraction from the second ring is characterized by lower BDEs in the gas phase and in acetone medium.

CONCLUSIONS

Although ***o*-DHB** manifests a weak/moderate antioxidant activity it shows higher antioxidant efficiency than ***p*-DHB.** The latest is a result of the lower BDE (75.31 kcal/mol) than that of ***p*-DHB** (78.61 kcal/mol). There is agreement between the theoretically predicted and experimentally observed antioxidant properties for these compounds. Bond dissociation enthalpies calculated for **Res** and **DRes** are of the same order, i.e. their antioxidant activity is expected to be similar. There is an excellent agreement between the theoretically calculated BDEs and experimental data (**Res** and **DRes** show the same antioxidant efficiency at low concentration). The discrepancy between the

theoretical BDE values and experimental results at higher concentrations can be explained by the side reactions that take place to a greater extend and that are not accounted for in the calculations.

Table 3. UB3LYP/6-31+G(d,p) calculated enthalpies (H_{298}) at 298 K (Hartree) in the gas phase and BDEs (kcal/mol).

Structure	H_{298}		BDE	
	gas phase	acetone	gas phase	acetone
PhOH	-307.383018	-307.389653		
PhOH (r)	-306.752447	-306.760335	81.76	80.97
o-DHB				
o-DHB (r)	-612.968012	-612.976369	75.31	76.79
o-DHB (br)	-612.323554	-612.338417	90.48	86.39
p-DHB				
p-DHB (r)	-612.962677	-612.977519	78.61	76.82
p-DHB (br)	-612.325169	-612.339322	86.12	86.54
Res				
Res (r)	-381.971644	-381.984213	82.77	81.20
Res (br)	-381.341432	-381.351743	81.54	82.95
DRes				
DRes (r)	-763.404066	-763.417064	82.72	80.28
DRes (br1)	-762.782027	-762.793415	76.41	77.41
DRes (br2)	-762.783847	-762.794883	75.27	76.49

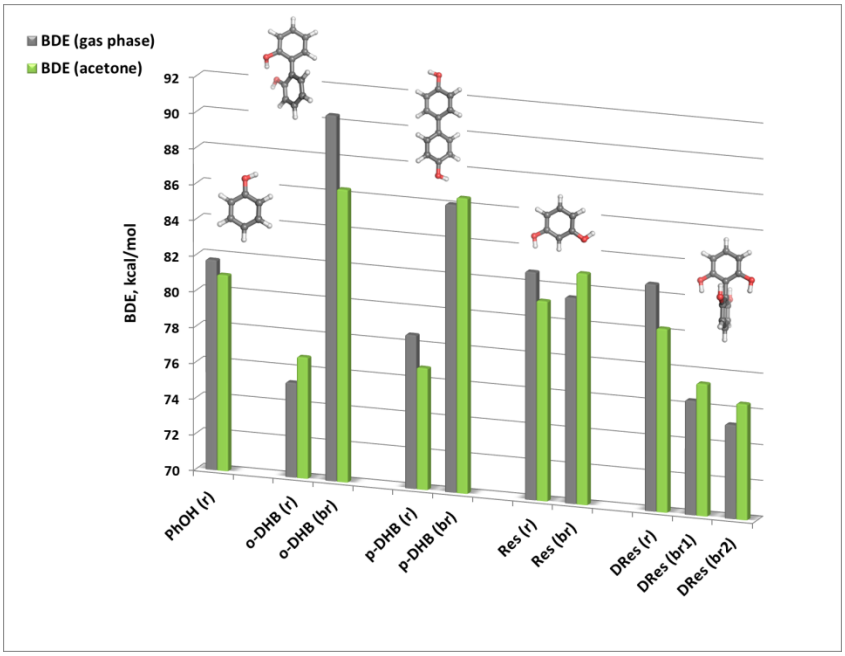

Figure 4. BDEs (in kcal/mol) in gas phase (grey) and in acetone (green).

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REFERENCES

1. C. Rice-Evans, N. Miller, G. Paganga, *Trends in Plant Science*, **2**, 152 (1997).
2. N.F. Gomes-Rochette, M. da Silveira Vasconcelos, S.M. Nabavi, E.F. Mota, D.C.S. Nunes-Pinheiro, M. Daglia, D.F. de Melo, *Current Pharmaceutical Biotechnology*, **17,** 986 (2016).
3. P. Ruzza, P.A. Serra, D. Fabbri, M.A. Dettori, G. Rocchitta, G. Delogu, *European Journal of Medicinal Chemistry*, **126**, 1034 (2017).
4. J. C. Anderson, D. J. Pearson, Journal of the Chemical Society, *Perkin Transactions*, **1**, 2023 (1998).

5. A. M. VanArendonk, M.E. Cupery, R. Adams, *Journal of the American Chemical Society*, **55**, 4225 (1933).
6. M. Frantsi, G. Lindsten, O. Wennerström, *Acta Chem. Scand. B*, **36,** 135 (1982).
7. V. D. Kancheva, A. K. Slavova-Kazakova, S. E. Angelova, S. K. Singh, S. Malhotra, B. K. Singh, L. Saso, A. K. Prasad, V. S. Parmar, *Biochimie*, **140**, 133 (2017).
8. I. Tichonov, V. Roginsky, E. Pliss, *European Journal of Lipid Science and Technology*,**112,** 887 (2010).
9. E. Denisov, T. T. Denisova, G., *Russian Chemical Reviews*, **78**,1047 (2009).
10. E. T. Denisov, T. G. Denisova, Handbook of Antioxidants. Bond Dissociation Energies, Rate Constants, Activation Energies and Enthalpies of Reactions. , CRC Press, New York, 2001.
11. V. D. Kancheva, L. Saso, S. E. Angelova, M. C. Foti, A. Slavova-Kasakova, C. Daquino, V. Enchev, O. Firuzi, J. Nechev, *Biochimie*, **94**, 403 (2012).
12. A. D. Becke, *The Journal of Chemical Physics*, **98**, 5648 (1993).
13. R. Ditchfield, W.J. Hehre, J. A. Pople, *The Journal of Chemical Physics*, **54**, 724 (1971).
14. T. Clark, J. Chandrasekhar, G.W. Spitznagel, P.V.R. Schleyer, *Journal of Computational Chemistry* **4**, 294 (1983).
15. M. J. Frisch et al., Gaussian 09, Revision D.01. Gaussian, Inc., Wallingford CT, 2013.
16. S. Miertuš, E. Scrocco, J. Tomasi, *Chemical Physics*, **55**, 117 (1981).
17. The PyMOL Molecular Graphics System, Version 1.7.6.6,. Schrödinger, LLC.

АНТИОКСИДАНТНА АКТИВНОСТ НА 3-ХИДРОКСИФЕНОЛ, 2,2'-БИФЕНОЛ, 4,4'-БИФЕНОЛ И 2,2',6,6'-БИФЕНИЛТЕТРОЛ**:** ТЕОРЕТИЧНО И ЕКСПЕРИМЕНТАЛНО ИЗСЛЕДВАНЕ

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(Резюме)

Изследвана е зависимостта структура - антиоксидантна активност за избрани фенолни съединения с едно бензеново ядро и две С-С свързани бензенови ядра (фенол, 3-хидроксифенол (резорцинол), 2,2'-бифенол, 4,4'-бифенол и 2,2',6,6'-бифенилтетрол). Съответните "димерни" структури (бифеноли и бифенилтетрол) на фенол и резорцин са така подбрани, че да може да се изследва влиянието на броя и взаимното положение на заместителите (ОН група/и в ароматния пръстен) върху антиоксидантната активност. Използвана е комбинация от теоретични и експериментални подходи. Антиоксидантната активност на изследваните съединения е определена от основните кинетичните параметри на липидното автоокисление в хомогенна среда. Молекулите на всички изследвани съединения и техните съответни феноксилни радикали са оптимизирани на теоретично ниво B3LYP/6-31+G**. Постигната е добра корелация между теоретично предсказаната и експериментално определената антиоксидантна активност.

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Mutual influence of lipid-antioxidant-surfactant in microheterogeneous systems

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This short review focuses specifically on the current understanding of the role of microheterogeneous reaction medium and surfactants in particular, in preventing and inhibiting lipid oxidation, and on the influence of surfactants on the behavior of known natural and synthetic antioxidants (AO). The mutual influence of the components and the conditions of occurrence of synergism and antagonism in a complex system (lipid – antioxidant – surfactant) are discussed as well.

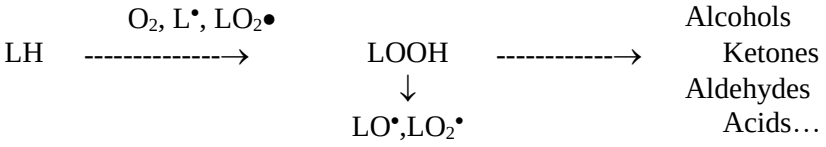
Key words: Free radicals, Hydroperoxides, Antioxidants, Surfactants, Synergism, Antagonism

INTRODUCTION

After Millennium, there is a steady trend in the use of natural products in innovative technologies for the creation of healthy and healing foods, cosmetics, and medicines containing essential unsaturated lipids and natural antioxidants (AO) because of consumer demand for natural ingredients [1-3]. Every drugstore sells a large set of individual antioxidants and their mixtures in the free market. Antioxidants are classified into two broad divisions, depending on whether they are soluble in water (hydrophilic) or in lipids (lipophilic). Multicomponent microheterogeneous systems usually contain surface-active substances (surfactants, S), which may have an impact not only on the structure and on the distribution of components in the system, but also on the reactivity and chemical behavior of key components. It must be noted that living organisms as a whole and individual cells can be considered as an open biochemical reactor of full displacement with multicomponent microheterogeneous medium as well. In recent years, a number of studies have produced evidence that for the development of innovative antioxidant technologies that prolong the quality of lipid containing products, for properly using of antioxidant drugs, multiphase and boundary effects in microheterogeneous systems containing surfactant, and mutual influence of lipids-antioxidants - surfactants have to be taken into account [4-8]. This short review focuses specifically on the current understanding of the role of microheterogeneous reaction medium and surfactants in particular, in preventing and inhibiting lipid oxidation, and the influence of surfactants on the behavior of known natural and synthetic antioxidants (AO). The mutual influence of the components and the conditions of occurrence of synergism and antagonism in a complex system of (lipid–antioxidant–surfactant) are discussed as well.

Pro- and antioxidant effect of surfactant on lipid and hydrocarbon oxidation

For a long time (all the last century) hydrocarbon and lipid (LH) oxidation by oxygen was considered as a free radical chain branching process [8-13].



Scheme 1.

The rate of the chain process (W_{O2}) is the product of the initiation rate (W_i) on the chain length (ν):

$$W_{O2} = W_i \cdot \nu \qquad (1)$$

Hydroperoxides (LOOH) – the primary oxidation products play the key role in the radical initiation. The chain initiation rate can be described by eqn. (2):

$$W_i = W_0 + e\, k_d [LOOH] \qquad (2)$$

where, W_0 is the initiation rate without LOOH participation, k_d – apparent rate constant and e – the so called „radical escape“ during LOOH decomposition. So, the additives which affect the LOOH decomposition can regulate the whole oxidation rate. For example, transient metals, which catalyze LOOH decomposition into free radicals, accelerate oxidation. The additives, which reduce

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LOOH into molecular products without radical formation, decrease oxidation rate.

In multicomponent microheterogeneous systems such significant factors as the uneven distribution of reagents within the scope and the influence of environment polarity on their reactivity, can greatly affect the oxidation rate and the product composition [4-7,15-17]. It is important that hydroperoxides and surfactants (S) form mixed micelles in microheterogeneous systems, direct micelles in water solutions (O/W) and reverse ones (W/O) in lipid medium:

$$mLOOH + nS \Leftrightarrow (mLOOH\ldots nS) \quad (3)$$

The formation of mixed micelles was studied and confirmed by different methods such as tensiometry, NMR, DLS [15,17,18]. The charge of surfactants is currently considered as one of the main factors in the oxidative stability of O/W systems because it governs attractive or repulsive forces (the Coulomb interaction of charges) between metal ion in water and interfaces [6,19,20], where LOOH and other amphiphilic compounds are concentrated. By this reason, O/W lipid systems stabilized by cationic surfactants (S^+) were found to be more oxidative stable than systems stabilized with anionic (S^-) surfactants [19,21].

In the reverse systems W/O, the influence of surfactant's charges is weakened in oil medium and reverse dependence on the nature of the surfactant is observed. Cationic surfactants (S^+) were found to be pro-oxidants because they accelerate the oxidation of hydrocarbons and lipids [17,18, 22-24]. The key reaction, which causes the acceleration, is the catalytic decomposition of LOOH into free radicals in mixed micelles ($mLOOH...nS^+$) [17,18,22]. In these micelles, the peroxide bond is evidently located in a strong electrical field of the electrical double layer with strength of $\sim 10^5$—10^7 $V \cdot m^{-1}$, weakening bond –O-O- and stimulating the homolytic decomposition of LOOH. The activation energy of the LOOH thermal decomposition is 90—120 kJ mol^{-1}, whereas in micelles with cationic surfactants the activation energy decreases to 40—60 kJ mol^{-1}. The polar metal compounds concentrate in the reversed micelles and further accelerate the homolysis of LOOH with the formation of radicals. Synergism between cationic surfactants and transient metals was observed in accelerated oxidation of ethylbenzene and limonene [23, 24].

The influence of anionic surfactants (S^-) in the systems W/O strongly depends on the hydroperoxide nature and on the structure of the polar head of the surfactant. Bright mutual influence of "lipid - surfactant" resulted in strong antioxidant effect that was discovered in the system "alkylaromatic hydrocarbons – sodium dodecyl sulfate (SDS) or alkylphosphates". SDS completely suppresses the oxidation of ethylbenzene and other alkylaromatic hydrocarbons [17, 25], but it does not affect unsaturated lipid oxidation [25]. The decomposition of ethylbenzene and cumene hydroperoxides in the presence of SDS occurs without free radical generation and results in the formation of phenol and corresponding carbonyl compound. Very low rate of radical initiation (only *via* chain origin in thermal reactions of O_2 and LOOH) and the resulting phenol together provide effective inhibition of the oxidation of alkylaromatic hydrocarbons. It is shown in [26] that synergism of the inhibiting action of surfactants SDS and trialkyl phosphates and alkylaromatic hydrocarbons is observed in the mixtures of decane and about 10% of alkylaromatic ethybenzene and cumene.

Nonionic and zwitter-ionic surfactants and some proteins have been generally found to better protect the lipid phase against oxidation in both O/W [20, 26-28] and W/O systems [26,29,30] due to their ability to form thick layers at the interface and to separate the hydrophilic initiator and the lipid substrate. Popular synthetic nonionic surfactants Triton X-100, Tweens and Pluronics along with zwitter-ionic lecithins are widely used in food and medical industry because they are non-toxic and non-expensive. They form direct micelles in water solution. Because they include a hydrophilic polyethylene oxide chain, these surfactants can be oxidized by a chain mechanism in the presence of radical initiators [28]. Under equal conditions, the surfactant activity in the chain radical oxidation decreases in the order: PC > TX-100 > F-68 >Tween-65 [28]. Here PC is egg phosphatidylcholine. A less reactive TX-100 acts as an "antioxidant" in the oxidation of phosphatidylcholine. In the case of a mixture of PC and TX-100, the rate of oxidation is less than the rates of individual components. Measurements of micellar sizes showed that TX-100 coats multilamellar liposomes of PC and thus protects PC from oxidation initiated in water phase. Fig. 1 shows the possibility of egg phosphatidylcholine (PC) to inhibit lipid oxidation in the model experiment of limonene oxidation, catalyzed by hydrophilic colloidal catalyst on the base of Fe(III) oxide [30,31]. This catalyst facilitates limonene hydroperoxide decomposition into free radicals and thereby it accelerates limonene oxidation which occurs *via* free radical chain mechanism and can be stopped by a chain- breaking inhibitor (Fig. 1, curve 1).

The addition of PC results in the decrease of O_2 uptake rate. However, the double addition of the same inhibitor does not affect the oxidation rate. It

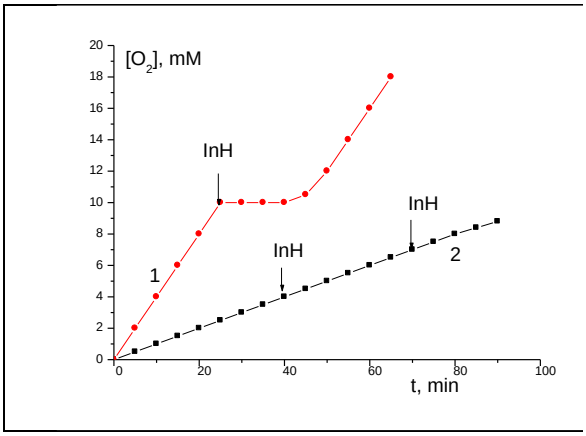


Figure 1. Kinetic curves of O_2 uptake during catalytic oxidation of (*1*) limonene and (*2*) limonene containing lecithin (PC) at the following compositions of reaction mixtures. (*1*) 4 mL of 1 M limonene solution in chlorobenzene and 32 mM LOOH + 21 μL catalyst suspension; (*2*) the same + 0.132 g lecithin; 60°C.The overall concentration of Fe^{3+} in both experiments is 1×10^{-4} M. The moments of addition of the inhibitor InH (0.023 mM) are shown by arrows.

means that PC solubilizes all the hydrophilic colloid catalyst which affects only PC capsule oxidation and the escape of radicals into bulk limonene is hampered. The similar shielding effect of PC on the escape of radicals generated by a hydrophilic micellar system (acetylcholine – hydroperoxide) in n-decane solution is described in [29].

The synergism of PC with α-tocopherol (vitamin E) in inhibiting the peroxidation of edible lipids was observed and investigated by many authors in various lipids and model systems [33-36]. It must be noted that the mechanism of synergism for phospholipids and α-tocopherol includes a colloid protective factor as well. The reactions of chain transfer by radicals derived from the inhibitor are known as a kinetic property of unhindered phenoxyl radicals, tocopheryl-radicals, in particular [9,13]. The chain transfer reactions to a marked degree decrease the antioxidant efficacy [37]. Cooperation of amphiphilic tocopherols and tocopheryl-radicals with PC can hamper the chain transfer and increase oxidative stability of oil. May be by this reason, lipids extracted from Antarctic Crill containing high content of PC and α-tocopherol demonstrated the highest antioxidant activity in β-carotene oxidation as compared with sunflower oil, lard and individual α-tocopherol taken in the equal quantity under the same conditions [26].

We have considered a few mutual synergistic effects in the system "S-LH-AO" resulted in more pronounced inhibition of oxidation. But there are antagonistic effects in lipid-antioxidant-surfactant mixtures which have to be detected and taken into account. First of all, antagonism of cationic surfactants and AO in the lipid W/O systems. The key reaction is the accelerated decomposition of LOOH into free radicals in the mixed micelles ($mLOOH \ldots nS^+$). In inhibited oxidation, pro-oxidant action of cationic surfactants may increase due to direct oxidation of amphiphilic AO by a hydroperoxide, activated in the mixed micelles [38]. The mixture of PC with $CaCl_2$ appeared to be antagonistic to β-carotene in limonene oxidation. Calcium is not a transient metal, and Ca^{2+} does not affect LOOH decay. However, the mixture PC + $CaCl_2$ accelerates β-carotene consumption because of the release of choline from the zwitter-ionic polar head of PC and transformation of PC into a cationic surfactant, which catalyzes LOOH decay into free radicals [39].

Mutual influence of (thiol-lipid- other AO) on oxidative stability

Special attention has to be paid to the peculiar pro- and antioxidant properties of thiols such as important endogenous glutathione, cysteine and homocysteine, as well as a number of SH-containing substances used in food, cosmetics and drug production.

Thiols (RSH) are known as preventive antioxidants reducing hydroperoxides and H_2O_2 into molecular products [8-13, 40]. Natural thiols cysteine and especially glutathione (GSH) which concentration in living cells is rather high (several mM) are of great importance for living organisms and are considered as bioantioxidants. However, thiols are a potential source of thiyl radicals, which are known to catalyze *cis-trans*- isomerization of double bonds [41-43]. Molecules of unsaturated fatty acid present in the living organisms and high-quality natural oils adopt the *cis*-configuration. *Trans*-isomers appear in the course of hydrogenation and high-temperature treatment of natural fats and oils. In living organisms, *trans*-lipids incorporate into cell membranes and thus violate the balance of exchange processes [44]. The rate of c*is—trans*-isomerization caused by thiyl radicals decreases in the presence of oxygen, and phenolic antioxidants inhibit isomerization through chain termination [45]. In the last two decades, along with *cis-trans* isomerization, the thiol-ene reactions attract much attention as means of synthesis of hetero-chain-compounds. These reactions occur *via* radical-chain mechanism and are accelerated by light and initiators [46-49]. Recently [50], the simplest thiol mercaptoethanol (RSH) was found to accelerate the oxidation of hydrocarbons and methyl linoleate due

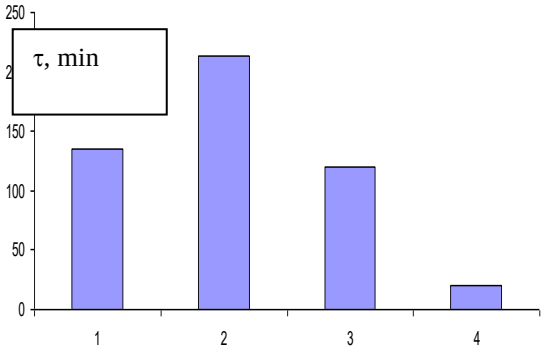
Table 1. Rates of O_2 uptake (W_{O2}) and results of the analysis of the content of trans-isomers in 1 hour of oxidation of methyl linoleate (0.2M), initiated by AIBN (5mM) at 50°C in n-decane solution in the presence of mercaptoethanol, RSH, (50mM) and diphenylamine, DA (5mM) and in parallel experiments conducted under nitrogen atmosphere.

No	System	W_{O2} 10^9, mol·(L·s)$^{-1}$	Percentage of *trans*-isomers in 1 h under O_2, %	Percentage of *trans*-isomers in 1 h under N_2, %
1	ML	6.1	0	0
2	ML+DA	2.1	0.2	0.3
3	ML+RSH	7.7	7.1	14.2
4	ML+DA+RSH	9,7	11.5	15.8

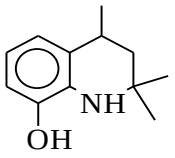
to the interaction of RSH with hydroperoxides resulting in a low yield of free radicals. In combination with phenolic AO, mercatoethanol shows synergism of the antioxidant action, whereas with the aromatic amines RSH exhibits antagonism.

The data of Table 1 demonstrate the antagonistic effect of mercaptoethanol additives (50 mM) on the rate of methyl linoleate (ML) oxidation, initiated by 5 mM of AIBN (azobisisobutyronitrile) and inhibited by diphenylamine (5mM). It is seen that RSH alone gently accelerates O_2 uptake and stimulates *cis-trans*-isomerization, which decelerates under oxygen atmosphere. DA added alone inhibits the oxidation. However, when DA and RSH are added together, it results in the increase of both the rates of ML oxidation and *cis-trans*-isomerization.

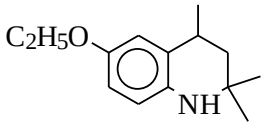
The differences in the behavior of RSH towards phenolic AO and aromatic amines can be brightly illustrated by the comparison of the induction periods caused by a mixture of RSH with two hydrogenated quinolines HQ1 and HQ2 (Figure 2). These compounds are chain-breaking AO of great efficacy [51,52]. The rate constants for the reaction of HQ1 and HQ2 with peroxyl radicals are higher than 10^6 L(mol s)$^{-1}$.



(a)



HQ1 (b)



HQ2 (c)

Figure 2. (a) Induction periods in the oxidation of methyl linoleate ([ML] = 0.2 M, [AIBN] = 4 mM) in decane at 50°C in the presence of $3 \cdot 10^{-5}$M hydrogenated quinolines HQ1 (1,2) and HQ2 (3,4) without (1,3) and with 50 mM mercaptoethanol (2,4); (b) and (c) are the structures of hydrogenated quinolines HQ1 and HQ2.

However, HQ1 having 8-hydroxy-substituent reacts with radicals as a phenol resulting in phenoxyl radical and next quinone formation, whereas HQ2 reacts with radicals as an amine to produce aminyl- and next nitroxyl radicals [51,52]. Figure 2 shows that the mixture HQ1 + RSH demonstrates synergism in oxidative stabilization of methyl linoleate contrary to the antagonistic mixture HQ2+RSH. Synergistic effect can be explained by the ability of thiols to reduce quinones into phenoxyl radicals and phenols and thereby regenerate a strong inhibitor:

$$Q + 2\ RSH \rightarrow QH_2 + RS\text{-}SR.$$

The antagonistic effect of mixtures of aromatic amines (>NH) with thiols in the oxidation of unsaturated methyl linoleate (LH) can be explained by fast reactions of thiyl radicals (RS*) with double bonds, the addition of oxygen to the alkyl radical (RS-LH*) formed, and fast reaction of aminyl radicals with thiols:

$$L'O_2^* + >NH \rightarrow LO_2H + >N^*$$
$$>N^* + RSH \rightarrow >NH + RS^*$$
$$RS^* + \text{cis- LH} \Leftrightarrow RS\text{-}LH^* \Leftrightarrow \text{trans-LH} + RS^*$$
$$RS\text{-}LH^* + O_2 \rightarrow RS\text{-}LHO_2^*\ (L'O_2^*)$$

Water soluble endogenous thiols glutathione and cysteine do not as a rule affect lipid oxidation and efficacy of lipophilic AO. However, in water medium they generate radicals in the reaction with hydrogen peroxide [53]. The yield of radicals is low but it can be enough to initiate thiol-ene reaction with compounds containing double bonds such as resveratrol or caffeic acid [54].

The data presented in this short review may be useful to understanding of physiological role of thiols in the overall oxidative process. It can be expected that further studies of the behavior of thiols in microheterogeneous systems will reveal new reactions and open new opportunities for regulation of red-ox reactions and overcome stressful conditions.

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REFERENCES

1. D. S. Berson, *J. Drugs Dermatol.*, **7** (7), 7 (2008).
2. M. S. Brewer, *Comprehensive Reviews in Food Science and Food Safety*, **10**, 221 (2011).
3. N. Al-Waili, H. Al-Waili, T. Al-Waili, K. Salom, *Redox Rep.* 22(3), 99 (2017).
4. M. Laguerre, C. Bayrasy, A. Panya, J. Weiss, D. J. McClements, j. Leconte, E. A. Decker, P. Villeneuve, *Crit. Rev. Food Sci. Nutr.*, **55**(2), 183 (2014).
5. O. S. Maldonado, R. Lucas, F. Comelles, M. J. Gonzalez, J. L. Parra, I. Medina, J. C. Morales, *Tetrahedron*, **67**, 7268 (2011).
6. W. Chaiyasit, R. J. Elias, D, J. McClements, E. A. Decker, *Crit. Rev. Food Sci. Nutr.*, **47**, 299 (2007).
7. K. Kittipongpittaya, A. Panya, L. Cui, D. J. McClements, E. A. Decker, *J. Am. Oil Chem. Soc.*, **91**(11), 1955 (2014).
8. N. M. Emanuel, Yu. N. Lyaskovskaya, *Inhibition of Lipid Oxidation*, Moscow, Pishcheproducty, 1961.
9. N. M. Emanuel, E. T. Denisov, Z. N. Maizus, *Chain reactions of hydrocarbons oxidation in liquid phase*, Moscow, Nauka, 1965.
10. G. Scott, *Atmospheric Oxidation and Antioxidation*, Elsevier, Amsterdam, 1965.
11. E. N. Frankel, *Lipid Oxidation*, Glasgow, The Oily Press, 2005.
12. *Lipid Oxidation Pathway*, A. Kamal_Eldin, (ed.), Champaign, IL: AOCS Press, 2003.
13. E. T. Denisov, I. F. Afanas'ev, *Oxidation and Antioxidants in Organic Chemistry and Biology*; CRC Press Taylor & Francis Group, 2005.
14. E. S. Budilarto, A.Kamal-Eldin, *Eur. J. Lipid Sci. Technol.* (2015), doi: [10.1002/ejlt.201400200].
15. V. D. Kancheva, O. T. Kasaikina, *Curr. Med. Chem.*, **20** (37), 4784 (2013).
16. E. N. Frankel, S. W. Huang, J. Kenner, J. B. German, *J. Agric. Food Chem*. **42**, 1054 (1994).
17. O. T. Kasaikina, Z. S. Kartasheva, L. M. Pisarenko, *Russ. J. Gen. Chem*., **78**(8), 1533 (2008).
18. O. T. Kasaikina, A. A. Golyavin, D. A. Krugovov, Z. S. Kartasheva, L. M. Pisarenko, *Mosc. Univ. Chem. Bull.*, **65,** 206 (2010).
19. I.Y. Mei, D. J. McClements, J. N. Wu, E. A. Decker, *Food Chem.*, **61** (3), 307 (1998).
20. C. Berton, M.-H. Ropers, M. Viau, C. Genot, *J. Agric. Food Chem.*, **59**, 5052 (2011).
21. J. R. Mancuso, D. J. McClements, E. A. Decker, *J. Agric. Food Chem.*, **47**(10), 5052 (1999).
22. N. A. Trunova, Z. S. Kartasheva, T. V. Maximova, Yu. G. Bogdanova, O. T. Kasaikina, *Colloid J*. **69**(5), 697 (2007).
23. D. A. Krugovov, L. M. Pisarenko, V. G. Kondratovich, *Petr. Chem*., **49**(2),120 (2009).
24. O. T. Kasaikina, N. V. Potapova, D. A. Krugovov, L. M. Pisarenko, *Kinetics and Catalysis*, **58**(5), 556 (2017).
25. O. T. Kasaikina, V. D. Kancheva, T. V. Maximova, Z. S. Kartasheva, N. V. Yanishlieva, V. G. Kondratovich, I. R. Totseva, *Oxid. Comm.*, **3**, 574 (2006).
26. O. T. Kasaikina, D. A. Krugovov, E. A. Mengele, *Eur. J. Lipid Sci. Technol*., **119**, 1600286 (2017).
27. A. M. Haahr, C. Jacobsen, *Eur. J. Lipid Sci. Technol*., **110** (10), 949 (2008).
28. O. T. Kasaikina, E. A. Mengele, I. G. Plashchina, *Colloid J*., **78** (6), 767 (2016).
29. N.V. Potapova, D.A. Krugovov, O.T. Kasaikina, *Bulg. Chem. Comm.*, this issue (2018).
30. O. T. Kasaikina, L. M. Pisarenko, V. I. Lesin, *Colloid J.*, **74** (4), 483 (2012).
31. O. T. Kasaikina, V. I. Lesin, L. M. Pisarenko, *Catal. Sustain. Energy*, **1**, 21 (2014).
32. L. M. Pisarenko, O. T. Kasaikina, *Russ. Chem. Bull.*, **51**(3), 449 (2002).
33. A. Judde, P. Villeneuve, A. Rossignol-Castera, A. le Guillou, *J. Am. Oil Chem. Soc.*, **80,** 1209 (2003).
34. T. Oshima, Y. Fujita, C. Koizumi, *J. Am. Oil Chem. Soc.*, **70**, 269 (1993).
35. P. Lambelet, F. Saucy, J. Loliger, *Free Radical Res.*, **20**, 1 (1994).
36. T. Koga, J. Terao, *J. Agric. Food Chem.*, **43**, 1450 (1995).
37. O. T. Kasaikina, V. D. Kortenska, N. V. Yanishlieva, *Russ. Chem. Bull.*, **10**, 1915 (1999).
38. L. M. Pisarenko, T. V. Maximova, O. T. Kasaikina, *Russ. Chem. Bull. (Int. ed.)*, 1419 (2003).
39. E. A. Mengele, Z. S. Kartasheva, I. G. Plashchina, O. T. Kasaikina, *Colloid J.*, **70**, 753 (2008).
40. G. H. Denison, *Ind. Eng. Chem*., **36**, 477 (1944).
41. C. Chatgilialoglu, A. Altieri, H. Fischer, *J. Am. Chem. Soc*., **124**, 12818 (2002).
42. C. Chatgilialoglu, A. Samadi, M. Guerra, H. Fischer, *ChemPhysChem*, **6**, 286 (2005).
43. C. Chatgilialoglu, C. Ferreri, *Acc. Chem. Res*., **36**, 441 (2005).
44. J. L. Sebedio, W. W. Christie, *Trans Fatty Acids in Human Nutrition*, The Oily Press, Dundee, 1998.

45. E. A. Mengele, C. Ferreri, C. Chatgilialoglu, O. T. Kasaikina, *Moscow Univ. Chem. Bull., Ser. Khim.* (*Engl. Transl.*), **65**, 210 (2010).
46. C. E. Hoyle, C. N. Bowman, *Angew. Chem., Int. Ed*., **49**, 1540 (2010).
47. M. J. Kade, D. J. Burke, C. J. Hawker, *J. Polymer Sci., Part A: Polym. Chem.*, **48**, 743 (2010).
48. O. Turunk, M. A. R. Meier, *Macromol. Rapid Commun*., **31**, 1822 (2010).
49. U. Biermann, W. Butte, R. Koch, P. A. Fokou, O. Turunk, M. A. R. Meier, J. O. Metzger, *Eur. J. Lipid Sci. Technol*., **18**, 8201 (2012).
.
50. E. A. Mengele, D. A. Krugovov, O. T. Kasaikina, *Russ. Chem. Bull.* (*Int. ed.*), 4, (2015)
51. O. T. Kasaikina, A. M. Kashkay, T. V. Maximova, *Oxid. Comm.* **23** (3), 383 (2000).
52. V. G. Kondratovich, T. V. Lobanova, I. F. Rusina, Yu. A. Ivanov, E. N. Khodot, O. T. Kasaikina, *Petr. Chem*., **44** (3), 226 (2004).
53. K. M. Zinatullina, N. P. Khrameeva, O. T. Kasaikina, B. I. Shapiro, V. A. Kuzmin, *Russ. Chem. Bull. (Int. Ed.)*, , No.7, (2017)
54. K. M. Zinatullina, N.P. Khrameeva, O.T. Kasaikina, V.A. Kuzmin, *Bulg. Chem. Commun.*, 50, 25 (2018).

ВЗАИМНО ВЛИЯНИЕ МЕЖДУ ЛИПИД-АНТИОКСИДАНТ-ПОВЪРХНОСТНО АКТИВНО ВЕЩЕСТВО В МИКРОХЕТЕРОГЕННИ СИСТЕМИ

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(Резюме)

Този кратък обзор е фокусиран върху съвременното разбиране на ролята на микрохетерогенната реакционна система и по-специално на повърхностно активните вещества (ПАВ) за предотвратяване и инхибиране на окислението на липидите, както и върху влиянието на ПАВ върху отнасянията на познати природни и синтетични антиоксиданти. Дискутирано е и взаимното влияние на компонентите и условията за протичане на синергизъм или антагонизъм в комплексна система (липид-антиоксидант-ПАВ).

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Effects of pH and surfactant concentration on the local concentrations of antioxidants in binary oil-water mixtures and in oil-in-water emulsions

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Antioxidants (AOs) react with radicals to minimize their harmful effects. The rates of their reactions depend on both the rate constant for the particular reaction and the concentrations of reactants at the reaction site. Thus, knowledge on their concentrations is very useful and desirable to interpret their reactivity (e.g., efficiency) because AOs with high rate constants may have low concentrations at the reaction site (i.e., their bioavailabity is low). Here we analyze the effects of surfactant concentration and acidity on the local concentrations of a model phenolic acid antioxidant in binary oil-water mixtures and, for the sake of comparisons, in corn oil-in-water emulsions. Local concentrations in emulsions can be up to ~160 times higher than the stoichiometric concentrations, thus having a significant effect in AO efficiencies.

Keywords: Antioxidants, emulsions, interfacial concentration

INTRODUCTION

Phenolic and some non-phenolic weak acids (e.g., ascorbic, sorbic, acetic, citric, etc.) are important in the prevention and control of the oxidative stability of lipids and because of their activities as food preservatives and metal chelators[1,2]. Polyphenols function as antioxidants (AOs) because they are excellent hydrogen donors that are accepted by reactive radicals to yield much less reactive radical and non-radical species[3].

Antioxidants minimize the oxidation of bioorganic material through several pathways, which can be simultaneous [3]. Whatever is the exact mechanism of action, an efficient antioxidant can be defined as that molecule whose rate of trapping radicals is equal to, or higher than, the rate of radical production. Chemical kinetics shows us that the rate of any reaction depends on both the rate constant for the particular reaction and the concentration of reactants at the reaction site. The rate of the reaction of AOS with radicals depends on the structure of the AO and on the properties of the reaction site (e.g., its polarity). In general, it is found that AOs with only one –OH group in their aromatic ring are less efficient than those with two –OH (catecholics) or three –OH (gallates) groups [1, 4]. In addition, the chemical structure of AOs strongly affects their bioavailability (concentration) at the reaction site, so that their hydrophilic-lipophilic balance, HLB, controls their location in the water or in less polar regions (e.g., oils, lipid bilayers, interfacial regions of emulsions, etc.) [5, 6]. For instance, we recently demonstrated that the HLB of an AO does not correlate with its fraction in the interfacial region of emulsions [7, 8] and that an increase in the HLB of a series of AOs bearing the same reactive moiety promotes their incorporation into the interfacial regions of oil-in-water emulsions, but only up to a point because a further increase in their HLB may make them to be more soluble in the oil region than in the interfacial one [5, 6, 9, 10].

Many studies focused on the structure-activity relationships of phenols and phenolic acids [1, 11, 12], however, there are only a few experimental contributions on the effects of acidity on their partitioning in food emulsions [13-16]. Partitioning of AOs is of great importance in predicting many of their biological aspects, mainly because of the large pH range experienced by food during its way through the digestive tract, and recent works demonstrated that there is a direct relationship between the AO efficiency and its concentration at the reaction site.[5, 6, 10, 17] The acidity of the medium plays an important role in AOs behavior because their antioxidant activity is largely affected by their degree of dissociation [9, 15]. For instance, the ionized forms of phenolic acids (phenolates) are much more soluble in the water regions than in the interfacial and oil ones.

Here we have investigated the effects of acidity on the partitioning of caffeic acid in binary corn oil-water mixtures and in corn oil-in-water emulsions. We chose caffeic acid (CA) because it is a major representative of hydroxycinnamic and phenolic acids [17-20]. Other phenolic and non-phenolic acids such as Trolox, gallic acid and ascorbic acids also constitute examples of antioxidants that can also be partially ionized in food systems [15] but their behavior in binary oil-water and oil-in-water

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emulsions should be very similar to that of CA because the acidity constants pK_a(1) of their carboxylic groups are all within the same range, 3.9 - 4.5 [21, 22] and thus, they can be present either as neutral or partially ionized molecules at the typical acidities (2-6) of foods [23]. The degree to which this partitioning may occur depends on their chemical structure (which affects their solubility) but is also pH-dependent, and changes in the acidity of the medium change their distribution between the oil, interfacial and water regions of the emulsions, as well as their antioxidant efficiency[8, 10, 24].

EXPERIMENTAL SECTION

Materials

Caffeic acid (CA), stripped corn oil, (Acros Organics, d = 0.918 g/mL) and polyoxyethylene (20) sorbitan monolaurate (Tween 20, Fluka) were of maximum purity available and used as received. The acidity of the aqueous phase was measured potentiometrically and controlled using citric acid buffers (CB, 0.04 M). Other chemicals were from Scharlau or Panreac. Aqueous solutions were prepared by using Milli-Q grade water. Emulsions of 1:9 (v:v) oil to water ratio were prepared by mixing 1 mL of corn oil and 9 mL of acidic (CB buffer) water. CA was solubilized in water (≈ 4.2 mM) and a weighed amount of non-ionic surfactant was added. The volume fraction of surfactant, Φ_I, (defined hereafter as $\Phi_I = V_{surf}/V_{emulsion}$) varied from $\Phi_I = 0.005$ up to $\Phi_I = 0.04$. The mixture was stirred with a high-speed rotor (Polytron PT 1600 E) for 1 min. The emulsion was transferred to a continuously stirred thermostated cell and the stability of the emulsions was checked visually.

Methods

Determining the partition constant P_W^O in binary oil/water mixtures and in oil-in-water emulsions

Hereafter, the term "apparent" partition constant, P_W^O(app), refers to the experimentally determined partition constant of CA between the oil and water phases at any pH (CA may be partially ionized), meanwhile the true partition constant P_W^O refers to the partition constant of the neutral form of CA.

P_W^O(app) values in binary oil-water systems were determined at the different acidities by employing a shake-flask method [25]. CA was dissolved in 9 mL of water and mixed with 1 mL of corn oil, stirred with a high-speed rotor for 1 min and allowed to stay for 30 min to permit phase separation and thermal equilibrium. The phases were then separated by centrifugation and the AO concentrations in the aqueous and oil phases were determined by UV spectrometry with the aid of previously prepared calibration curves. Results were obtained in duplicate or triplicate with deviations less than 5%. P_W^O values were calculated by employing Eqn. 1, where V_W and V_O are the aqueous and oil region volumes, respectively, and the magnitude in parentheses ( ) means concentrations in moles per liter of the oil (o) and aqueous (w) regions.

$$P_W^O(\text{app}) = \frac{(\text{AO}_O)}{(\text{AO}_W)} = \frac{\%\text{AO}_o}{\%\text{AO}_W} \times \frac{V_W}{V_O} \qquad (1)$$

The percentages of AOs in the aqueous phase, $\%AO_W$, were determined, at different pH values, by interpolation of the absorbance of aliquots (30 μL) of the aqueous phase of the binary corn oil/acidic water mixtures diluted with buffer solutions of the same pH and the concentration of the AO was determined by using previously prepared calibration curves at each pH.

Determining partition constants in intact oil-in-water emulsions

Determining partition constants in emulsions is a difficult task because methods used in binary oil/water systems cannot be employed because of the physical impossibility of separating the interfacial from the oil and water regions. This means that the partition constants of the AOs need to be assessed in the intact emulsions and for the purpose, a well-established chemical kinetic method was employed. The method exploits the reaction between a hydrophobic arenediazonium ion, 16-ArN_2^+, and the antioxidants. The 16 carbon tail and the aromatic ring of the arenediazonium ion makes 16-ArN_2^+ to be insoluble in the water region, and also in the oil region because of its cationic $-N_2^+$ headgroup, thus its concentration in the aqueous and oil regions is effectively zero and there is no reaction between the antioxidant and 16-ArN_2^+ in these regions. Mathematical relationships (not shown here) between the partition constants and the observed rate constants k_{obs} have been derived on the grounds of the pseudophase kinetic model. Details of the method are described elsewhere in detail [5-7, 17, 26].

RESULTS

Hereafter, magnitudes between parentheses, e.g. (AO), mean concentrations in moles per liter of the volume of a given region, while those between brackets, e.g., [AO] stand for concentrations in moles per liter of the total volume of the system.

Effects of acidity on the partition constant, P_W^O, and on the distribution of caffeic acid in binary corn oil-water mixtures

The results in Table 1 show that the experimentally determined P_W^O(app) values are pH dependent, decreasing upon decreasing the acidity. This is a consequence of the carboxylic groups on CA: ionized phenolic acids are much more soluble in water than the undissociated acids. Results in Table 1 show that P_W^O(app) values for CA are very low at any pH, $P_W^O < 0.1$, in keeping with the high solubility of the AO in water, $S_{CA} \approx 2.9$ g dm^{-3} at T = 298 K [27]. The P_W^O(app) value of 0.025 at pH ~ 4 is very similar to that found by Huang *et al*. [28] in an unbuffered 1:9 corn oil-water binary mixture P_W^O(app) = 0.028 [29]. Results in Table 1 indicate that more than 99% of the phenolic acid is in the aqueous phase at pH < 2.5, and that this percentage increases upon decreasing the acidity of the medium because of the increase in the fraction of ionized antioxidants. Hence, in emulsified systems, phenolic acids are expected to be mainly distributed between the aqueous and interfacial regions with negligible amounts in the oil phase.

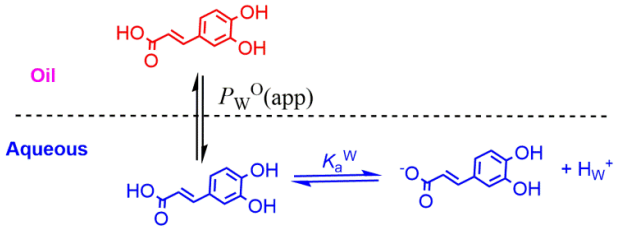
Thermodynamics show us that when a neutral molecule (e.g., the weak acid) partitions between two phases (e.g., oil and water) at equilibrium, the partition constant P_W^O between those phases is defined by Eqn. 2, which assumes that the activities of the molecule in each phase are equal or very close to unity. This approximation usually holds because the concentration of the molecule in each phase is low.

Caffeic acid may both dissociate in the aqueous phase and partition into the oil phase and both, ionized and neutral species are in equilibrium in the oil and water phases. However, ionization constants of acids in oils (O) are usually 5-6 orders of magnitude smaller than in aqueous phases [30], (i.e., $pK_a(O) >>> pK_a(W)$) and, for the sake of simplicity, we can assume that the ionization of the weak acids in the oil phase is negligible, and Scheme 1 applies, from where the apparent (measured) partition coefficient defined by equation 3 can be derived.

Table 1. Values of the partition constant P_W^O for the distribution of CA in binary corn oil-water systems at different acidities, determined at T = 25 ºC.

pH	2.50	2.88	3.05	3.35	3.39	3.53	3.67	3.70	3.97	4.01	4.10
P_W^O (app)	0.086	0.079	0.073	---	0.065	0.050	---	0.046	---	0.026	---
P_W^I (app)	603	556	476	351	---	---	278		194	---	102

The dissociation of caffeic acid in water is characterized by the dissociation constant K_a, Eqn. 4. Substitution of Eqn. 4 into Eqn. 2 leads to Eqn. 5, which sets the relationship between the apparent partition constant and the acidity.



Scheme 1. Partitioning of caffeic acid between the oil and water phases of binary mixtures. Ionization is assumed to take place exclusively in water.

$$P_W^O = \frac{[CA_{oil}]}{[CA_{water}]} \quad (2)$$

$$P_W^O(app) = \frac{[CA_{oil}]}{[CA_{water}] + [CA_W^-]} \quad (3)$$

$$K_a = \frac{(CA_w^-)(H^+)}{(CA_W)} \quad (4)$$

$$P_W^O(app) = \frac{P_W^O}{1 + \frac{K_a}{[H^+]}} \quad (5)$$

$$\frac{1}{P_W^O(app)} = \frac{1}{P_W^O} + \frac{K_a}{P_W^O}\frac{1}{[H^+]} \quad (6)$$

Equation 5 predicts a sigmoidal variation of the apparent (measured) partition constant value with acidity. Note that when pH <<< p*K*a, P_W^O (app) = P_W^O, that is, there exists an upper limit in the experimentally measured partition constants which corresponds to that of the neutral molecule. The reverse of equation 5 is equation 6, which predicts a linear variation of 1/ P_W^O (app) with 1/[H^+], Figure 1A, from where a value of $P_W^O = 0.087 \pm 0.003$ can be determined for the partition constant P_W^O of neutral CA. The local concentration of CA in the water phase can be obtained by bearing in mind the corresponding mass balance for the concentration of the acid in terms of the total volume of the system gives Eqn. 7.

$$[CA_T]V_T = (CA_{oil})V_{oil} + (CA_W)V_W + (CA_W^-)V_W \quad (7)$$

where brackets [ ] mean concentration in moles per liter of the total volume. Defining the volume fraction of the oil phase as $\Phi_O = V_{oil}/(V_o+V_w)$, Eqn. 8 can be derived.

$$\frac{(CA_W)}{[CA_T]} = \frac{1}{\Phi_W + P_W^O \Phi_O + \frac{K_a}{[H^+]}} \qquad (8)$$

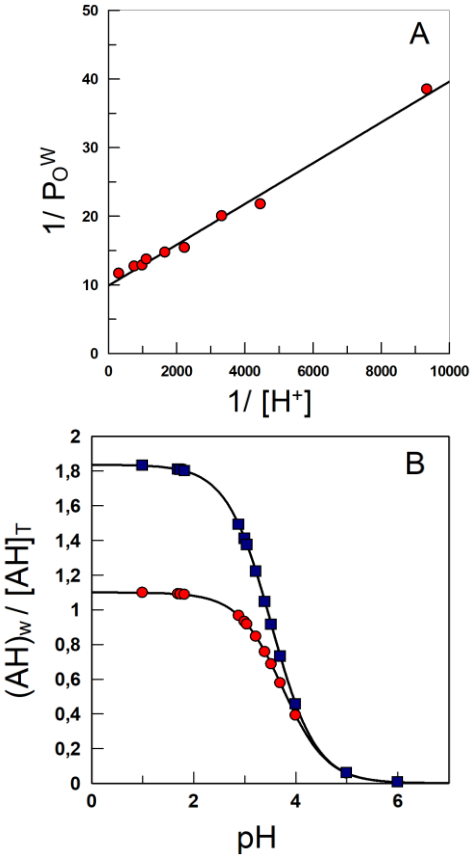


Figure 1. A) Variation of the measured partition constant in oil/water mixtures, P_W^O (app), with pH. B) Variation with pH of the ratio between the local concentration of the AO in the aqueous region and the total or stoichiometric concentration.

Eqn. 8 is a modification of the Henderson-Hasselbach equation and gives the concentration of the neutral weak acid in a two-phase system relative to the total (stoichiometric) concentration as a function of the acid concentration, the volume fractions of each phase, the partition constant of the neutral molecule and the ionization constant. Figure 1B simulates the variation of the concentration of CA in the water phase at two o:w ratios. It becomes apparent that at low pH, the concentration of CA in the water phase of oil-water mixtures with a high content of water (e.g., 1:9 o:w) is very similar to the stoichiometric concentration, while in 1:1 oil:water mixtures, the concentration in the water phase is almost twice that of the stoichiometric concentration. As expected, the ratio $(CA_w)/[CA_T]$ decreases upon increasing pH as a consequence of the ionization of CA. The results, though somehow expected, illustrate the fact that the local concentrations of AOs may be quite different from the stoichiometric concentrations and constitute an important remark because when AOs partition in multiphasic systems, their interfacial concentration may be by orders of magnitude higher than the stoichiometric concentration (see below).

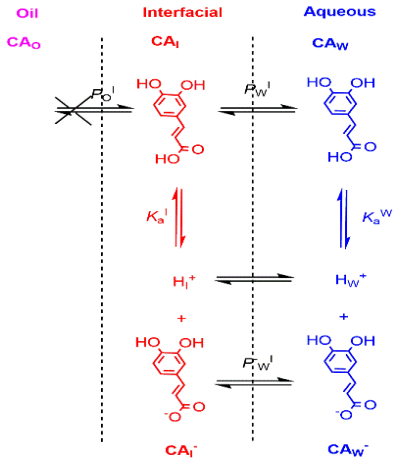
Effects of acidity on the partitioning and on interfacial concentrations of CA in oil-in-water emulsions

Lipids are typically present in foods in the form of oil-in-water emulsions (butter, mayonnaise, dressings, etc.) The interfacial region of the emulsions is highly anisotropic and contains a mixture of oil, water and surfactant, and, therefore, can solubilize both hydrophobic and hydrophilic molecules. Antioxidants added to minimize the oxidation of the lipids distribute between the oil, water and interfacial regions, and their efficiency strongly depends on their partitioning.

In general, the distribution of AOs in emulsions can be described by two partition constants, Scheme 2, that between the oil and interfacial, P_O^I, and that between the water and interfacial, P_W^I, regions, but simplifications can be made when AOs are very hydrophobic or very hydrophilic. For instance, CA is a hydrophilic AO whose solubility in oils is expected to be very low because the measured partition constants between water and corn oil, P_W^O (see above) in corn oil/water mixtures were very low. Thus, one can safely assume that the concentration of CA in the oil region of the emulsions is negligible and, thus, CA will mainly partition between the aqueous (w) and interfacial (I) regions of oil-in-water emulsions and only one partition constant (P_w^I) is needed to describe its distribution, Scheme 2. In general, phenolic AOs containing weakly acidic functional groups, e.g, a carboxylic acid moiety, have very low oil solubility. However, their solubilities in water can be modified by changing the acidity of the system because at the typical acidities (2-6) of emulsified foods [23], phenolic acids may be neutral or partially ionized and a change in emulsion acidity changes their distributions between the interfacial and aqueous regions.

Note that the ionic forms of the AOs are generally oil insoluble, but they are much more water soluble than the neutral forms. The P_W^I values displayed in Table 2 were determined as described elsewhere by

employing a kinetic method in the intact emulsions [6, 7, 26, 31].



Scheme 2. Equilibria for the distribution of the hydrophilic neutral and anionic form of an AO, e.g., Scheme 1, between the oil, interfacial and aqueous regions of the emulsion

Results in Table 1 show that P_W^I values decrease by a factor of ~ 4 upon increasing pH from ~2.5 to ~4.2 but, at any acidity, $P_W^I > 1$, indicating that the Gibbs free energy of transfer from the aqueous to the interfacial region is negative, that is, CA has a natural tendency to incorporate into the interfacial region.

Figure 3A shows their variation with pH. CA may ionize in both the aqueous and interfacial regions and the apparent partition constant P_W^I (app) is defined by Eqn. 9.

The pK_a values of weak acids within interfacial regions of nonionic association colloids and emulsions are somewhat different – but not too much - from their values in aqueous solution because the polarity of the interfacial region is less than that of water[32, 33].

$$P_W^I(app) = \frac{((AO_I) + (AO_I^-))}{((AO_W) + (AO_W^-))} \qquad (9)$$

$$P_W^I(app) = \frac{P_W^I + \frac{P^{-I}{}_W K_a}{[H^+]}}{1 + \frac{K_a}{[H^+]}} \qquad (10)$$

Nonionic micellar solutions usually have small effects on the apparent pK_a values of weak acids, unlike cationic and anionic micelles that are known to shift substantially the pK_a values of weak acids compared to water in opposite directions[34, 35]. For example, Jaiswal *et al*. [21] found that the pK_a of ascorbic acid increases by less than 0.3 units in Triton X micelles compared to water. We expect similar behavior between the aqueous and interfacial regions of emulsions and micelles prepared with nonionic surfactants, and we assume that p$K_a(_W)$ ≈ p$K_a(_I)$, so that the percentage of phenolic acids in their anionic form will be about the same in the interfacial region of Tween 20 emulsions as in the bulk aqueous phase. Using the equations defining the ionization (eq. 4) and partition (eq. 9) constants and bearing in mind Scheme 2, Eqn. 10 can be derived, where $P^{-I}{}_W$ stands for the partition constant of the ionized form of CA between the aqueous and interfacial regions.

Eqn. 10 predicts that the variation of P_W^I with pH follows a sigmoidal-shaped curve as can be seen in Figure 2 (solid line). Eqn. 10 predicts that at high acidities (pH << pK_a), the measured P_W^I (app) value is equal numerically to P_W^I, that is, the partition constant of the neutral CA, since at low pH, all CA molecules are neutral and the P_W^I (app) values at such acidities should be independent of pH, as shown in Figure 2. At pH >> pK_a, the measured P_W^I (app) value decreases and approaches that of $P^-{}_W^I$ (depending on the magnitude of $P^-{}_W^I$ and K_a) because CA is fully deprotonated and the carboxylate has low solubility in the interfacial region of the emulsions as demonstrated by Huang *et al*. [22], who found that at pH = 6, more than 95% of the total added Trolox is located in the aqueous region of corn oil/Tween 20 (Φ_I = 0.01) emulsions and at pH = 7, the percentage of Trolox found in the aqueous region was ~100%. Hence, values of P_W^I (app) are expected to be close to zero at pH > 6, Figure 2.

Bearing in mind the corresponding mass balances, the concentrations of the antioxidant in the interfacial and aqueous regions of the emulsion relative to the stoichiometric concentration can be obtained by means of Eqns. 11 and 12, respectively.

$$\frac{(AO_I)}{[AO_T]} = \frac{P_W^I}{\Phi_W + P_W^I \Phi_I} \qquad (11)$$

$$\frac{(AO_W)}{[AO_T]} = \frac{1}{\Phi_W + \Phi_I P_W^I} \qquad (12)$$

Figure 4 illustrates the variations of the local concentrations of CA in the interfacial and aqueous regions relative to the stoichiometric CA concentration. At the lowest surfactant volume fraction employed, Φ_I = 0.005, the interfacial concentration of CA is as much as ~130 times higher than the stoichiometric concentration (pH = 2). On the contrary, the local concentration in the aqueous region is much lower than the stoichiometric concentration.

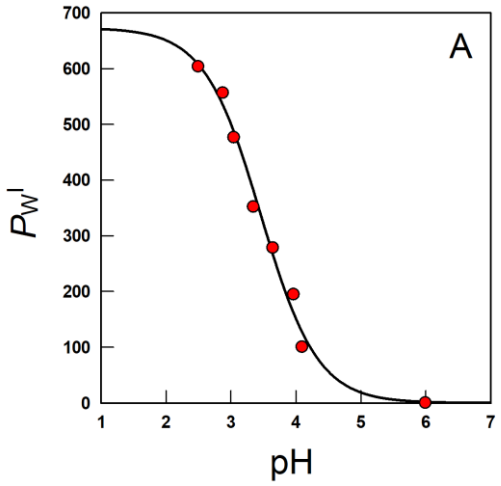


Figure 3A. Effects of acidity on the partition constant P_W^I of caffeic acid in emulsions.

At a constant acidity, the interfacial and aqueous concentrations decrease to almost zero upon increasing the surfactant volume fraction from Φ_I = 0.005 to Φ_I = 0.045, highlighting the enormous impact of the surfactant concentration on the local concentrations of antioxidants. Note that the largest variations in the local concentrations are achieved for Φ_I values ranging 0.005 to $\Phi_I \approx 0.015$. Further increases in Φ_I do not change substantially the local concentrations. Alternatively, at constant Φ_I, the interfacial concentration decreases upon decreasing the acidity by a factor of ~2 on going from pH = 2 to pH ≈ 4 because of the ionization of CA. Note that the local aqueous concentrations increase upon increasing pH, but still is a fraction of the stoichiometric concentration. Eventually, at high pH (>6), the local aqueous concentration will approach the stoichiometric concentration.

CONCLUSIONS

Antioxidant efficiency depends on several factors including the rates of the reaction between the AO and the radicals (which depends on the physical properties of the AO) and its concentration at the reaction site. It is currently accepted that the oxidation of the oil starts in the interfacial region of the emulsion [17, 36] and we recently showed [10, 37] that there exists a direct correlation between the mole fraction of antioxidants in the interfacial region of emulsions and their antioxidant efficiency. Interfacial concentrations are much higher than stoichiometric concentrations and this is part of the reason why only small amounts of AOs are needed to minimize the oxidation of lipids.

The results obtained here are important to understand the relative efficiency of AOs in emulsified systems. They show, among others: 1) the partition constants in binary oil-water systems cannot be employed to predict the relative distribution of AOs in multiphasic systems. 2) In binary oil-water mixtures, local AO concentrations

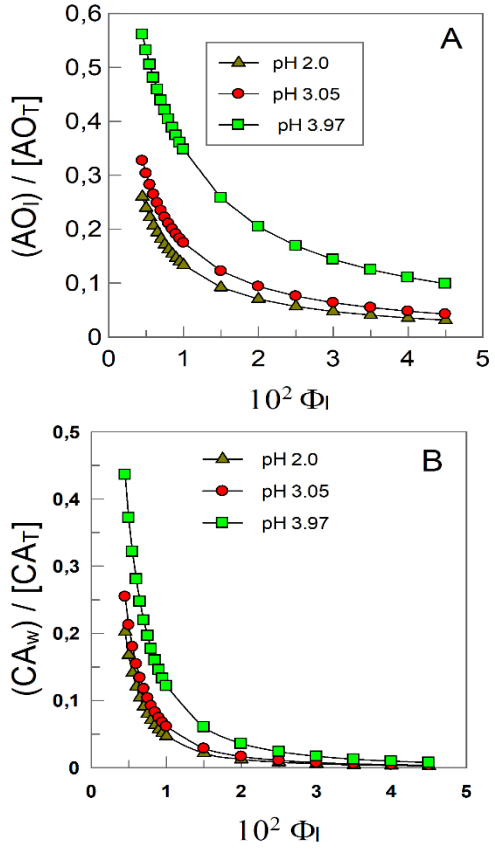


Figure 4. Variation of the interfacial (A) and aqueous (B) concentrations of CA in corn oil emulsions with the surfactant volume fraction at selected acidities. The values at pH= 2 were determined theoretically by employing equations 11 and 12 and the P_w^I value determined by fitting the experimental data in Figure 2 to equation 10 (P_w^I = 650).

increase modestly with respect to the stoichiometric concentrations. 3) In emulsions, the interfacial region has a significant role because it contains concentrated AOs so that interfacial AO concentrations are by orders of magnitude higher than the stoichiometric concentrations. 4) The acidity plays a significant role in the behavior of phenolic acids, which upon ionization, decrease significantly their interfacial concentration and therefore a decrease in their efficiency upon increasing pH is expected.

Indeed, the molecular structure of phenolic AOs (position and number of hydroxyl or other substituents on their aromatic ring) has a considerable effect on their antioxidant properties, which in turn also affects their partitioning between the different regions of the emulsions [2, 38]. However, chemical modifications of the reactive

moieties to modulate their efficiency are not always possible because of the inherent experimental difficulties and because of the regulatory status of AO_S cannot be ignored when selecting antioxidants for use in particular foods. [39, 40] Thus, strategies exploiting changes in their relative solubility leading to changes in their local concentrations (e.g., the interfacial concentration in emulsions) are needed to improve their efficiency while maintaining their antioxidant properties. Therefore, improvement of strategies to modulate interfacial concentrations deserve further investigations.

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REFERENCES

1. F. Shahidi, Handbook of Antioxidants for Food Preservation, 1st Edition, Woodhead Pub., 2015.
2. E.N. Frankel, Lipid Oxidation, The Oily Press, PJ Barnes & Associates, Bridgwater, England, 2005.
3. S. Losada-Barreiro, C. Bravo-Díaz, *Eur. J. Med. Chem*., **133**, 379 (2017).
4. S.B. Nimse, D. Palb, *RSC Advances*, **5**, 27986 (2015).
5. M. Costa, S. Losada-Barreiro, F. Paiva-Martins, C. Bravo-Diaz, *RSC Advances*, **6**, 91483 (2016).
6. J. Almeida, S. Losada-Barreiro, M. Costa, F. Paiva-Martins, C. Bravo-Díaz, L.S. Romsted, J. *Agric. Food Chem*., **64,** 5274 (2016).
7. C. Bravo-Díaz, L.S. Romsted, C. Liu, S. Losada-Barreiro, M.J. Pastoriza-Gallego, X. Gao, Q. Gu, G. Krishnan, V. Sánchez-Paz, Y. Zhang, A. Ahmad-Dar, *Langmuir*, **31**, 8961 (2015).
8. S. Losada-Barreiro, V. Sánchez Paz, C. Bravo-Díaz, *J. Colloid. Interface Sci.*, **389**, 1 (2013).
9. S. Losada-Barreiro, C. Bravo-Díaz, L.S. Romsted, *Eur. J. Lipid Sci. Technol*., **117**, 1801 (2015).
10. S. Losada-Barreiro, C. Bravo Díaz, F. Paiva Martins, L.S. Romsted, *J. Agric. Food Chem.*, **61,** 6533 (2013).
11. Y. Chen, H. Xiao, J. Zheng, G. Liang, Structure-thermodynamics-antioxidant activity relationships of selected natural phenolic acids and derivatives: an experimental and theoretical evaluation, in: PLoS One, 2015, p. e0121276.
12. D. Kahveci, M. Laguerre, P. Villeneuve, 7 - Phenolipids as New Antioxidants: Production, Activity, and Potential Applications, in: Polar Lipids, Elsevier, 2015, p. 185-214.
13. H. Stöckman, K. Schwarz, *Langmuir*, **15,** 6142 (1999).
14. S.S. Pekkarinen, H. Stöckman, K. Schwarz, I.M. Heinoen, A.I. Hopia, *J. Agric. Food Chem.*, **47,** 3036 (1999).
15. A. Galan, S. Losada-Barreiro, C. Bravo-Díaz, *ChemPhysChem*., **17,** 296 (2016).
16. M. Costa, S. Losada-Barreiro, F. Paiva-Martins, C. Bravo-Díaz, *J. Am. Oil Chem. Soc*., **90**, 1629 (2013).
17. M. Costa, S. Losada-Barreiro, F. Paiva-Martins, C. Bravo-Díaz, L.S. Romsted, *Food Chem.*, **175,** 233 (2015).
18. Y. Sueishi, M. Hori, M. Ishikawa, K. Matsuura, E. Kamogawa, Y. Honda, M. Kita, K. Ohara, *J. Clinic. Biochem. Nutr*, **54,** 67 (2014)..
19. I. Medina, I. Undeland, K. Larsson, I. Storr, R. T., C. Jacobsen, V. Kristinova, *Food Chem*., **131** 730 (2012).
20. M. Touaibia, J. Jean-François, J. Doiron, *Mini Rev. Med. Chem.*, **11**, 1 (2011).
21. P.V. Jaiswal, A.K. Srivastava, *Colloids and Surfaces B: Biointerfaces*, **46,** 45 (2005).
22. S. Huang, E.N. Frankel, K. Schwarz, J.B. German, *Agric. Food Chem*., **44**, 2496 (1996).
23. CRC, Handbook of Chemistry and Physics, 78 ed., CRC Press., Boca Raton, 1997.
24. P. Lisete-Torres, S. Losada-Barreiro, H. Albuquerque, V. Sánchez-Paz, F. Paiva-Martins, C. Bravo-Díaz, *J. Agric. Food Chem*., **60**, 7318 (2012).
25. A. Berthod, S. Carda-Bosch, *J. Chromatog.* A, **1037,** 3 (2004).
26. L.S. Romsted, C. Bravo-Díaz, *Curr. Opin. Colloid Interface Sci.*, **18,** 3 (2013).
27. F.L. Mota, A.J. Queimada, S. Pinho, E. Macedo, *Ind. Eng. Chem. Res*., **47**, 5182 (2008).
28. S.W. Huang, E.N. Frankel, R. Aeschbach, J.B. German, *J. Agric. Food Chem*., **45,** 1991 (1997).
29. K. Schwarz, E.N. Frankel, J.B. German, *Fett/Lipid*, **98**, 115 (1996).
30. J.A. Dean, Lange´s Handbook of Chemistry in, McGraw-Hill, Inc., New York, 1992.
31. S. Losada-Barreiro, V. Sánchez-Paz, C. Bravo-Díaz, *Org. Biomol. Chem*., **6**, 4004 (2008), **13** 876 (2015).
32. L.S. Romsted, Introduction to Surfactant Self-Assembly, in: P.A. Gale, Steed, J. W. (Ed.) Supramolecular Chemistry: From Molecules to Nanomaterials, J. Wiley & Sons Ltd., NY, 2012, p. 181.
33. R. Shoemaker, K. Holmberg, Reactions in organized surfactant systems, in: S. Stubenrauch (ed.) Microemulsions: Background, New Concepts, Applications, Perspectives, Blackwell Publishing, Oxford, 2009, p. 148.
34. L.S. Romsted, Micellar Effects on Reaction Rates and Equilibria, in "Surfactants in Solution", K.L. Mittal, J. Lindman, (eds.), Plenum Press, NY, 1984.
35. O. El Seoud, *Adv. Colloid. Interf. Sci.*, **30**, 1 (1989).
36. C.C. Berton-Carabin, M.-H. Ropers, C. Genot, *Compr. Rev. Food Sci. Food Safety*, **13,** 945 (2014).
37. M. Costa, S. Losada-Barreiro, F. Paiva-Martins, C. Bravo-Díaz, L.S. Romsted, *Food Chem*., 175, 233 (2015).

38. F. Shahidi, Y. Zhong, *Chem. Soc. Rev.*, **39,** 4067 (2010).
39. J.W. Finley, A. Kong, K.J. Hintze, E.H. Jeffery, L.L. Ji, X.G. Lei, *J. Agric. Food Chem*., **59**, 6837 (2011).
40. M.S. Brewer, *Compr. Rev. Food Sci. Food Saf*., **10** 221 (2011).

ВЛИЯНИЕ НА РН И КОНЦЕНТРАЦИЯТА НА ПОВЪРХНОСТНО АКТИВНО ВЕЩЕСТВО ВЪРХУ ЛОКАЛНИТЕ КОНЦЕНТРАЦИИ НА АНТИОКСИДАНТИ В БИНЕРНИ СМЕСИ ОЛИО-ВОДА И ЕМУЛСИИ НА ОЛИО ВЪВ ВОДА

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(Резюме)

Антиоксидантите (АО) реагират с радикалите за минимизиране на вредното им влияние. Скоростта на реакциите зависи от скоростната константа на съответната реакция и от концентрациите на реактантите на мястото на реакцията. Познаването на техните концентрации на мястото на реакцията е много полезно за интерпретиране на реактивоспособността им (например, ефективността), тъй като АО с високи скоростни константи може да са с ниски концентрации на мястото на реакцията (т.е., тяхната бионаличност е ниска). В настоящата работа е изследвано влиянието на киселинността и концентрацията на повърхностно активно вещество върху локалните концентрации на моделен антиоксидант фенолна киселина в бинерни смеси олио-вода и за сравнение в емулсии на царевично олио във вода. Локалните концентрации в емулсиите може да са до ~160 пъти по-високи от стехиометричните концентрации, което има значителен ефект върху ефективността на АО.

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Expanding the antioxidant activity into higher temperatures – fullerene C_{60} conjugated with α-tocopherol analogue as a hybrid antioxidant in saturated lipid systems

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Oxidative stability of two fatty acids: stearic acid (STA) as a model of saturated lipid and linolenic acid (LNA) as model of polyunsaturated lipid containing C_{60} derivative with covalently bonded hydroxychromanyl moiety as analogue of α-tocopherol (F-1) was monitored by differential scanning calorimetry. The overall Arrhenius kinetic parameters (activation energy E_a, pre-exponential factor Z, and rate constants k) of non-isothermal oxidative decomposition indicate that in saturated hydrocarbons the hybrid C_{60}+hydroxychromane derivative is effective antioxidant acting at temperatures above 120°C, expressed as 9 kJ/mol increase of E_a and values of k twice smaller than for oxidation of non-inhibited pure STA. However, experiments with LNA oxidized at temperatures above 80°C indicated that the hybrid derivative did not improve the oxidative stability of polyunsaturated lipids at higher temperatures (E_a, Z and k's are almost the same as for oxidation of pure LNA). We suggest that C_{60} is able to inhibit STA autoxidation due to formation of thermally stable adducts with alkoxyl radicals whereas autoxidation LNA is mediated by peroxyl radicals that are not effectively scavenged by C_{60}.

Keywords: Fullerene, Tocopherol, Antioxidant, Lipids, Hydrocarbon oxidation, Oxidation kinetics

INTRODUCTION

Peroxidation (autoxidation) of lipids and hydrocarbons (LH) is a chain process mediated by alkyl and alkylperoxyl radicals ($L^\bullet$ and $LOO^\bullet$, respectively) where after the initiation, i. e., generation of primary radical species, a series of consecutive additions of molecular oxygen (1) and abstraction of hydrogen (2) form the kinetic propagation chain [1]:

$$L^\bullet + O_2 \xrightarrow{k_{p1}} LOO^\bullet \quad k_{p1}\sim 10^9\ M^1s^{-1} \qquad (1)$$

$$LOO^\bullet + LH \xrightarrow{k_{p2}} LOOH + L^\bullet \quad k_{p2}\sim 10\text{-}100\ M^{-1}s^{-1} \qquad (2)$$

For low partial pressures of oxygen not all radicals react with molecular O_2, moreover, at higher temperatures the products of propagation chain like hydroperoxides (LOOH) might undergo subsequent thermal or metal-induced decomposition, therefore, the autoxidation can be mediated by species other than alkylperoxyls. For example, when the process occurs at higher temperatures (in lubricants) or with limited access to oxygen (polymers) the propagation can be facilitated with alkyl and alkoxyl radicals [2, 3].

Application of chain-breaking antioxidants is one of the possible ways of protection of food, polymers and other hydrocarbons, as well as biomolecules being the components of living organisms. The role of chain-breaking antioxidants is to stop any of the propagation processes, mainly by reducing the propagating radicals ($Y^\bullet$= $LOO^\bullet$ or $LO^\bullet$) to relatively stable radicals or non-radical products, schematically shown as reactions 3 and 4, or by formation of non-reactive adducts (reaction 5).

$$Y^\bullet + Aox\text{-}H \rightarrow Y\text{-}H + Aox^\bullet \qquad (3)$$

$$Y^\bullet + Aox^\bullet \rightarrow Y\text{-}Aox \qquad (4)$$

$$Y^\bullet + antioxidant \rightarrow (Y\text{-}antioxidant)^\bullet \qquad (5)$$

The rates of scavenging (reactions 3-5) depend on the nature of the antioxidant including its structure, bond strengths, stereoelectronic effects, ionisation potential, localisation and other factors. Some kinds of molecules exhibit antioxidant action under specific conditions. For example, Burton and Ingold demonstrated that β-carotene and other carotenoids (hydrocarbons with conjugated double C=C bonds) are active chain-breaking antioxidants (reaction 5) in the systems with partial oxygen pressure below 15 kPa and carotenoids lost their activity at higher oxygen pressure [4].

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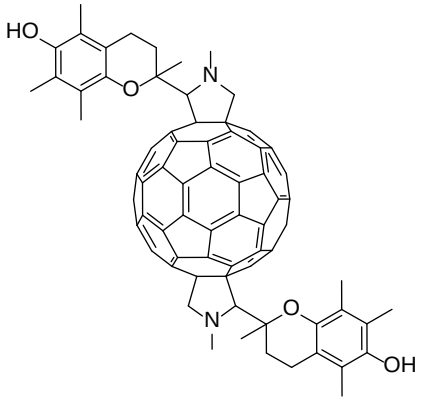
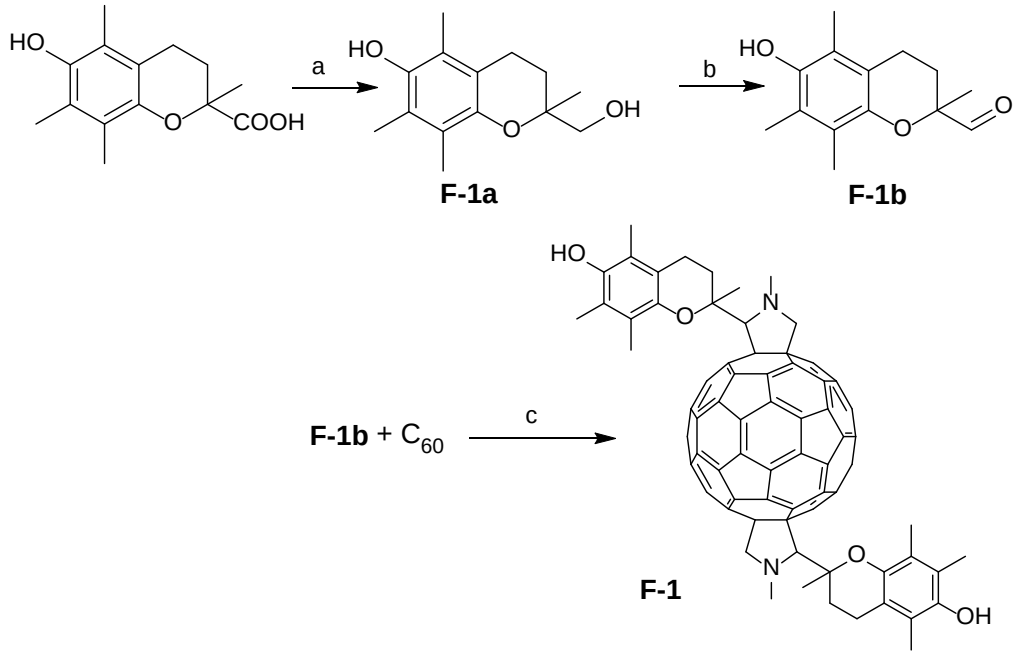
Fullerenes have been the subject of many studies, including the antiradical activity of C_{60} molecule, named the radical sponge, because of its ability to form adducts with unusually high number of benzyl, methyl and other alkyl radicals with a single C_{60} molecule [5-9]. Reactions of C_{60} with those radicals are fast and lead to the formation of stable radicals, with electrons delocalized over the whole sphere of fullerene. When passing from artificial radicals to the ones naturally occurring during autoxidation, the antiradical properties of C_{60} are not as evident - the review devoted to antioxidant properties of fullerene brings several examples of water soluble derivatives of C_{60} able to diminish the level of Reactive Oxygen Species in biologically relevant systems [10], but pristine C_{60} is claimed to be not a good chain-breaking antioxidant in a model system with styrene autoxidation [11], perhaps because the reaction with alkylperoxyl radicals is too slow to be competitive to reaction 2. On the other hand, fullerene conjugated with derivatives of phenolic antioxidant (like 2,6-di-*tert*-4-methylphenol, BHT) behaved as typical chain- breaking antioxidants. [11] Taking into account the results reported by various research teams and basing on our previous results indicating that pristine fullerene is an effective inhibitor of saturated hydrocarbon oxidation carried out at higher temperatures [12-14], we proposed a series of new hybrid antioxidants with phenolic moiety responsible for reaction with peroxyl radicals and C_{60} sphere responsible for scavenging of radical species generated at higher temperatures in oxygen-poor systems. In this work we are testing the antioxidant activity of C_{60} with a covalently bonded derivative of tocopherol, namely 6-hydroxy-2,5,7,8-tetramethylchromanyl group. Presumably, such hybrid antioxidant might connect the advantages of β-carotene (ability to scavenge radicals in the process of addition to a conjugated system of double bonds) with the advantages of α-tocopherol (low bond dissociation enthalpy facilitating the H atom transfer from phenolic O-H bond to a radical). We synthesized a derivative of C_{60} with covalently bonded chromanol moieties attached to the carbon sphere *via* N-methylpyrrolidine rings, compound **F-1**:



EXPERIMENTAL

Stearic acid, STA, (99%, Sigma-Aldrich) and linolenic acid, LNA, (POCH, 99%) were stored at 0°C in darkness. Fullerene C_{60} was of 99+% purity (MER Corporation, Tucson). 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox™), N-methylglycine (Sarcosine™), lithium aluminium hydride, ethyl acetate, hexane, manganese dioxide, dioxane, toluene, THF were purchased from Sigma-Aldrich. Toluene and THF were dried and distilled before use, other solvents were analytical grade reagents and were used as received. ^{1}H and ^{13}C NMR spectra were recorded using Varian 200 MHz instruments. Fourier transform infrared (FT-IR) spectra were obtained using Shimadzu FTIR-8400S spectrometer in the 4000-400 cm^{-1} range. Thermogravimetry was performed using TA Q50 instrument at a heating rate of 20 K/min in nitrogen (platinum vessels were used). Oxidation process of STA and LNA was monitored by differential scanning calorimetry (Du Pont 910 apparatus with Du Pont 9900 thermal analyzer and normal pressure, recently refurbished cell was used). Temperature and cell constant were calibrated with ultrapure indium standard. TA Instruments software (General V4.01) was used for collecting the data and for determination of temperatures from DSC curves. The oxidations were performed under oxygen flow of 6 dm^3/h. Samples (3.0-3.5 mg) were heated from 50 to 250°C in an open aluminium pan with a linear heating rate β (2.5; 5.0; 7.5; 10.0; 12.5; 15.0; 17.5; 20.0 K/min). As a reference material an empty aluminium pan was used. Temperatures of extrapolated start of oxidation, T_e, were determined from the plots of heat flow *versus* temperature dependence for each β. Multistep synthesis routes for derivative **F-1** are depicted in Scheme 1 and described in the next subsection, together with identification of final products by NMR and IR.



Scheme 1. Synthesis of fullerene derivative F-1.

Synthesis of F-1

Step a. Compound **F-1**a was obtained using the synthetic procedure reported by Huang *et al.* [15]. To a stirred solution of 0.78 g of $LiAlH_4$ (20 mmol) in dry THF (10 mL) at 0°C 300 mg (1.2 mmol) of 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid in 18 mL of dry THF was added dropwise. The solution was stirred at room temperature under nitrogen for another 6 h, and 4 mL of 0.25 M NaOH was added to stop the reaction. The mixture was stirred at room temperature for 0.5 h, filtered, and concentrated under reduced pressure. The resulting residue was purified by flash silica gel chromatography (ethyl acetate : hexane, 1:2 v/v) to afford 2-(hydroxymethyl)-2,5,7,8-tetramethylchroman-6-ol (**F-1a**) in 75% yield. Analysis: FT-IR (KBr disc 300 mg + 1 mg **F-1b**) [cm^{-1}]: 3550 (O-H), 2950 (str, C-H); 1250 (str, C-O). ^{13}C NMR (200 MHz, acetone-d_6) δ (ppm): 146.7, 146.2, 123.9, 120.8, 118.3, 113.9, 82.4, 76.5, 75.0, 68.9, 29.2, 22.0, 21.3, 13.0, 12.1, 11.3.

Step b. Compound **F-1b** was obtained using the synthetic procedure reported by Reynaud *et al.* [16]. The compound **F-1a** (100 mg, 0.42 mmol) was dissolved in ethyl acetate (6 mL), and after addition of MnO_2 (1.2 g, 12 mmol) the heterogeneous mixture was vigorously stirred for 1 h. Then the mixture was filtered through celite, dried over Na_2SO_4, and concentrated. The residue was purified by filtration through a silica gel column (hexane : ethyl acetate, 3:7, v/v) to give 6-hydroxy-2,5,7,8-tetramethylchromane-2-carbaldehyde (**F-1c**) in 80% yield. Analysis: FT-IR (KBr disc 300 mg+1 mg **F-1c**) [cm^{-1}] 3504 (O-H), 2950 (str C-H); 1680-1720 (str C=O).

Step c. The final fullerene derivative was obtained using the Prato method, described in our previous paper. [13] A mixture of C_{60} (70 mg, 0.14 mmol), sarcosine (43 mg, 0.69 mmol, 5 eq.), **F-1c** (25 mg, 0.14 mmol, 1 eq.) and 60 mL of dry toluene was stirred in reflux for 24 h in a 100-mL flask. The reaction mixture was cooled down and the solvent was removed under reduced pressure. The residue was purified by column chromatography (dioxane : toluene, 1:9, v/v) to give 35 mg prod*uct **F-1** as a brown solid (29% yield based on converted C_{60}). Analysis: ^1H NMR (200 MHz, $CDCl_3$, TMS) δ (ppm): 5,13 (s, 1H), 4.15-3.63 (s, 2H), 4.12-3.59 (s, 2H), 2.66 (s, 1H), 3,42-2,32 (s, 2H), 1.88-1.77 (s, 9H), 1.40 (s, 3H), 1.22 (s, 3H). ^{13}C NMR (200 MHz, $CDCl_3$) δ (ppm): 147.56; 134.66; 128.78; 128.56; 126.94; 81.01; 77.83; 76.56; 74.76; 35.91; 29.88; 22.78; 22.25; 13.01; 11.77; 10.51 The weight loss was 47% in the temperature range 250-600°C, which corresponds to two groups attached to the fullerene molecule.

RESULTS AND DISCUSSION

In our previous papers we reported the kinetic parameters of oxidation of several lipid and hydrocarbon matrices like saturated [17] and unsaturated [18] fatty acids and their esters, oils [19, 20], polyethylene [21]. Recently, we compared the antioxidant behaviour of four C_{60} adducts with

simple monohydroxyphenols [13] and we demonstrated that for high temperature oxidation of saturated hydrocarbons the conjugates of C_{60} with simple phenols are more active antioxidants than the building blocks (pristine C_{60} and phenols) used separately. In our present work we extended our search onto conjugates with analogue of α-tocopherol due to reasons explained in the Introduction section.

Differential scanning calorimetry is a very useful tool for monitoring the rate of oxidation of non-volatile materials like hydrocarbons and lipids. Typical DSC traces for non-isothermal oxidation of STA containing 2 mM of **F-1** are presented in Figure 1 with the way of determination of temperature of extrapolated start of oxidation, T_e, as a cross-section of tangents of baseline and thermal peak of oxidation.

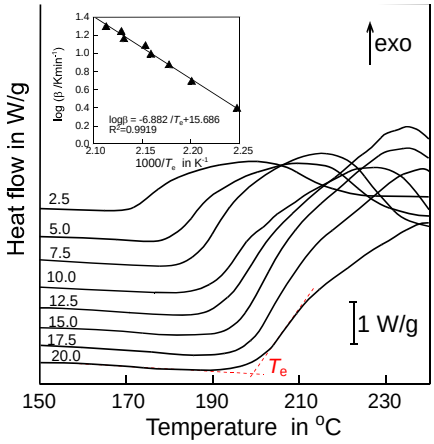


Figure 1. DSC curves of non-isothermal oxidative decomposition of STA containing **F-1** (C = 2.0 mM) recorded for linear heating rates β from 2.5 to 20.0 K/min, as indicated over each curve. Curves were shifted vertically for clarity of presentation. *Inset*: Plot of logβ versus 1000/T_e for oxidation of STA containing 2.0 mM **F-1**.

Within one series, each curve was recorded for a different linear heating rate (β) and one can observe that at higher β a higher T_e was determined (see Fig. 1). The values T_e recorded for the whole series of β's are listed in Table 1 (the presented values T_e are the mean of at least three measurements). For the same β value, the comparison of temperatures of start of oxidation of pure stearic acid with the T_e values measured for STA containing **F-1** clearly demonstrates the inhibiting effect of **F-1**, that always causes a prolongation (5-7°C) of the lag phase, defined here as extended range of temperatures without detectable thermal effect of spontaneous oxidation. Such shift of T_e to higher temperatures can be interpreted as antioxidant effect of the additive. Thus, non-isothermal oxidation monitored by DSC is an alternative method to other accelerated tests like Oxidative Stability Index or Rancimat Test [18-20].

Due to the simplicity of measurement and short time of each sample analysis, non-isothermal oxidation mode has an advantage over accelerated tests, because the changes of T_e recorded for several different β can be used for calculation of the overall kinetic parameters, activation energy (E_a) and pre-exponential factor Z for oxidation processes by the Ozawa-Flynn-Wall's method [12, 17, 19, 21], from the linear dependence:

$$\log \beta = a \times T_e^{-1} + b \qquad (6)$$

where the slope $a = -0.456\ E_a / R$ and intersection $b = -2.315 + \log(ZE_a / R)$, and R is the gas constant (8.314 [J mol^{-1}K^{-1}]).

Table 1 also contains the kinetic parameters measured and calculated for oxidation of pure STA (E_a = 116 ± 8 kJ/mol and Z = 1.34 × 10^{13} s^{-1}) being in reasonable agreement with the activation energy of isothermal oxidation of saturated fatty

Table 1. Temperatures of start of oxidation (T_e) obtained for different heating rates (β), statistical parameters of straight line equation $\log\beta = a/T_e + b$, and overall kinetic parameters E_a - activation energy, Z - pre-exponential factor, k - rate constants obtained for oxidation of pure STA and STA containing 2 mM derivative **F-1**. The errors of E_a were calculated from the standard error σ of the slope a calculated with confidence level 90% ($\sigma_{90\%}$).

STEARIC ACID (STA)			*STA with 2 mM **F-1***		
β [K/min]	T_e [K]	Statistical and kinetic parameters	β [K/min]	T_e [K]	Statistical and kinetic parameters
2.5	437	a = -6.36	2.5	444	a = -6.8819
5.0	447	b = 14.95	5.0	454	b = 15.8680
7.5	452	R^2 = 0.9982	7.5	457	R^2 = 0.9919
10.0	456	E_a = 116 ± 8 kJ/mol	10.0	463	E_a = 125 ± 9 kJ/mol
12.5	458	$Z = 1.34 \times 10^{13}$ min^{-1}	12.5	464	$Z = 1.01 \times 10^{14}$ min^{-1}
15.0	461	$k_{50°C} = 2.50 \times 10^{-6}$ min^{-1}	15.0	468	$k_{50°C} = 5.66 \times 10^{-7}$ min^{-1}
17.5	465	$k_{100°C} = 8.07 \times 10^{-4}$ min^{-1}	17.5	470	$k_{100°C} = 2.93 \times 10^{-4}$ min^{-1}
20.0	466	$k_{150°C} = 6.66 \times 10^{-2}$ min^{-1}	20.0	473	$k_{150°C} = 3.45 \times 10^{-2}$ min^{-1}
		$k_{200°C} = 2.16 \times 10^{0}$ min^{-1}			$k_{200°C} = 1.49 \times 10^{0}$ min^{-1}
		$k_{250°C} = 3.61 \times 10^{1}$ min^{-1}			$k_{250°C} = 3.12 \times 10^{1}$ min^{-1}

acids [17]. The comparison of activation parameters for oxidation of pure STA and STA with 2 mM **F-1** suggests ca. 9 kJ/mol increase of the overall activation barrier, but taking into account our former experience, the reaction rates are more reliable and trustful parameters than the E_a. This is because the so called accelerated tests of the oxidation of lipids and hydrocarbons are carried out at temperatures above 100°C, that is, at temperatures very close or even higher than the isokinetic temperature, T_{iso}, the temperature at which two different processes have the same rate constants. Above T_{iso} the process with higher E_a proceeds faster than the process characterized by lower E_a [14, 19], as a consequence of cross-section of exponential functions describing the Arrhenius rate constant:

$$k = Z \exp(-E_a/RT) \qquad (7)$$

originating from different sets of E_a and Z. This phenomenon is a source of a series of counterintuitive observations, for example, the inversion of oxidative stabilities or inversion of antioxidant effect into pro-oxidant effect when the results from lower temperatures are compared with the results of experiments performed at higher temperatures. Perhaps, this is also a possible explanation why α-tocopherol and its simple analogues are not good antioxidants in lipid systems oxidized at temperatures above 110°C as in OSI, Rancimat, or Oxipress test [22, 23]. In our system the values of k calculated from eqns. 7 for STA and STA containing **F-1** are also listed in Table 1 for temperatures 50-250°C. Indeed, we can observe that addition of **F-1** improves the oxidative stability of STA at temperatures below 200°C (at 250°C the values of k are close for both systems).

We also performed a series of experiments with LNA, chosen as a completely different model of polyunsaturated fatty acid to be oxidized, in the presence of **F-1** (see Fig. 2).

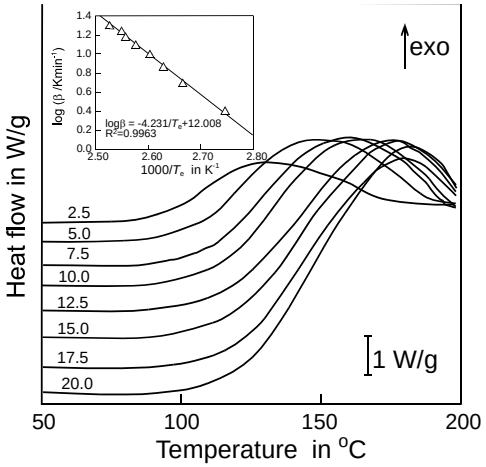


Figure 2. DSC curves of non-isothermal oxidative decomposition of LNA containing **F-1** (C = 2.0 mM) recorded for linear heating rates β from 2.5 to 20.0 K/min, as indicated over each curve. Curves were shifted vertically for clarity of presentation. Inset: Plot of logβ *versus* 1000/T_e for oxidation of LNA containing 2.0 mM **F-1**.

LNA reacts with oxygen ca. 170 times faster than STA and due to this reason LNA is often used for studies of antioxidant activity of phenolic antioxidants by the DSC method, with T_e values by about 100°C lower than for oxidation of STA. The results are presented in Table 2, where the data are compared with oxidation parameters for LNA without the additive (results taken from ref. [13]).

The kinetic data presented in Table 2, E_a, Z and k's are similar, that is, the addition of **F-1** to LNA did not cause any inhibiting effect This observation is in disagreement with the inhibiting effect of **F-1** in STA, but is a good example of the important role of the lipid/hydrocarbon matrix taken as model system for testing the antioxidant

Table 2. Results (T_e, statistical parameters of equation 1, E_a, Z, and k) obtained for oxidation of pure LNA and LNA containing **F-1** (C = 2 mM). Symbols are the same as explained in the heading to Table 1.

LINOLENIC ACID (LNA)			*LNA with 2 mM **F-1***		
β [K/min]	T_e [K]	Statistical and kinetic parameters	β [K/min]	T_e [K]	Statistical and kinetic parameters
2.5	366	a = -4.3015	2.5	364	a = -4.2231
5.0	375	b = 12.1509	5.0	375	b = 12.0076
7.5	383	R^2 = 0.9879	7.5	380	R^2 = 0.9963
10.0	385	E_a = 78 ± 6 kJ/mol	10.0	384	E_a = 77 ± 3 kJ/mol
12.5	391	$Z = 3.10 \times 10^{10}$ min^{-1}	12.5	388	$Z = 2.27 \times 10^{10}$ min^{-1}
15.0	392	$k_{50°C} = 6.81 \times 10^{-3}$ min^{-1}	15.0	391	$k_{50°C} = 8.04 \times 10^{-3}$ min^{-1}
17.5	393	$k_{100°C} = 3.38 \times 10^{-1}$ min^{-1}	17.5	392	$k_{100°C} = 3.74 \times 10^{-1}$ min^{-1}
20.0	396	$k_{150°C} = 6.68 \times 10^{0}$ min^{-1}	20.0	395	$k_{150°C} = 7.04 \times 10^{0}$ min^{-1}
		$k_{200°C} = 7.02 \times 10^{1}$ min^{-1}			$k_{200°C} = 7.11 \times 10^{1}$ min^{-1}
		$k_{250°C} = 4.70 \times 10^{2}$ min^{-1}			$k_{250°C} = 4.62 \times 10^{2}$ min^{-1}

activity of any compound. One of the possible explanations is that a clear inhibiting effect (lag phase) can be observed for sufficiently active chain-breaking antioxidants, when the rate constant of inhibition (reactions 3-5, depending on the mechanism) is by three orders of magnitude greater that the rate constant of propagation. Thus, the same molecule can act as antioxidant in the saturated hydrocarbons, where k_p = 0.00034 $M^{-1}s^{-1}$ (hexadecane at 30°C [24]) but it will be not active in LNA (k_p = 48 $M^{-1}s^{-1}$ at 37ºC [25]). This hypothesis would be valid for C_{60} conjugated with simple phenols and can, to some extent, explain the better activity of the C_{60} component in derivative **F-1**. Lack of clear inhibiting effect in the LNA matrix indicates almost complete deactivation of the tocopherol-like component (hydroxychromanyl part) in **F-1** at higher temperatures. This rather disappointing observation can be justified in another way, with the assumption taken that in polyunsaturated fatty acids the autoxidation is mediated by alkylperoxyl radicals. In this case the hydroxychromanyl site reacts with the radicals but at 90-120ºC the process of breaking the chain is not effective since the "tocopheroxyl" radical is still able to abstract H atom from a bis-allyl position of polyunsaturated LNA. Similar mechanism has been proposed by Liebler *et al.* [26] at lower temperatures (30ºC) in homogeneous solutions and also by Stocker *et al.* [27] and Ingold *et al.* [28] for LDL oxidation in the presence of α-tocopherol and was named tocopherol mediated peroxidation (TMP). Taking into account that the tocopheroxyl radical formed in reaction 3 is not reduced immediately (for example, in reaction 4) it is very plausible that at 90-120ºC this radical will attack the weakest C-H bond in LNA and reinitiate a new chain of propagation. Such mechanism of reinitiation is also probable in STA at temperatures much higher than for oxidation of LNA, however, as mentioned above, k_p for saturated hydrocarbons is by four orders of magnitude lower than k_p for LNA, moreover, autoxidation of saturated hydrocarbons at high temperatures with limited access to molecular oxygen proceeds with participation of radical species other than alkylperoxyls. As it was observed by other researchers and described in the Introduction, under such conditions C_{60} is able to efficiently scavenge alkyl and alkoxyl radicals, thus preventing the system against reinitiation or branching the kinetic chain of oxidation.

Concluding, we designed and prepared a C_{60} derivative with covalently bonded analogue of α-tocopherol (hydroxychromanyl moiety) and tested its antioxidant activity in two model lipid matrices: saturated (stearic acid) and polyunsaturated (linolenic acid) during the non-isothermal oxidation monitored by differential scanning calorimetry. The obtained kinetic parameters of oxidation (activation energy, pre-exponential factor and rate constants calculated for the overall oxidation process) indicate a clear antioxidant effect of the derivative in the saturated system but no antioxidant effect was detected during oxidation of linolenic acid. The presented results show the important role of the hydrocarbon used as model lipid for assessment of the antioxidant activity at higher temperatures in the range 90-180ºC, typical for accelerated tests of oxidative stability.

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REFERENCES

1. E. T. Denisov, I. B. Afanas'ev, in: Oxidation and Antioxidants in Organic Chemistry and Biology; CRC Press, 2005.
2. G. Gryn'ova, J. L. Hodgson, M. L. Coote, *Org. Biomol. Chem.*, **9**, 480 (2011).
3. R. Lee, G. Gryn'ova, K. U. Ingold, M. L. Coote, *Phys. Chem. Chem. Phys.*, **18**, 23673 (2016).
4. G. Burton, K. Ingold, *Science*, **224**, 569 (1984).
5. P. J. Krusic, E. Wasserman, P. N. Keizer, J. R. Morton, K. F. Preston, *Science*, **254**, 1183 (1991).
6. R. Klemt, E. Roduner, H. Fischer, *Chem. Phys. Lett.*, **229**, 524 (1994).
7. E. Schaffner, H. Fischer, *J. Phys. Chem.*, **97**, 13149 (1993).
8. M. Walbiner, H. Fischer, *J. Phys. Chem.*, **97**, 4880 (1993).
9. J. R. Morton, F. Negri, K. F. Preston, *Acc. Chem. Res.*, **31**, 63 (1998).
10. S. Bosi, T. Da Ros, G. Spalluto, M. Prato, *Eur. J. Med. Chem.*, **38**, 913 (2003).
11. R. F. Enes, A. C. Tomé, J. A. S. Cavaleiro, R. Amorati, M. G. Fumo, G. F. Pedulli, L. Valgimigli, *Chem. Eur. J.*, **12**, 4646 (2006).
12. R. Czochara, P. Ziaja, P. Piotrowski, R. Pokrop, G. Litwinienko, *Carbon*, **50**, 3943 (2012).
13. R. Czochara, J. Kusio, M. Symonowicz, G. Litwinienko, *Ind. Eng. Chem. Res.*, **55**, 9887 (2016).
14. R. Czochara, J. Kusio, G. Litwinienko, *RSC Advances*, **7**, 44021 (2017).
15. J.-W. Huang, C.-W. Shiau, J. Yang, D.-S. Wang, H.-C. Chiu, C.-Y. Chen, C.-S. Chen, *J. Med. Chem.*, **49**, 4684 (2006).
16. E. Reynaud, G. Aydemir,R. Rühl, O. Dangles, C. Caris-Veyrat, *J. Agric. Food. Chem.*, **59**, 1457 (2011).
17. G. Litwinienko, A. Daniluk, T. Kasprzycka-Guttman, *Ind. Eng. Chem. Res.*, **39**, 7 (2000).

18. G. Litwinienko, T. Kasprzycka-Guttman, *Ind. Eng. Chem. Res.*, **39**, 13 (2000).
19. G. Litwinienko, in: Analysis of Lipid Oxidation; AOCS Publishing, 2005.
20. G. Litwinienko, K. Jodko-Piórecka, in: Differential Scanning Calorimetry; CRC Press, 2014; pp 49.
21. P. Ziaja, K. Jodko-Piorecka, R. Kuzmicz, G. Litwinienko, *Polym. Chem.*, **3**, 93 (2012).
22. T. Verleyen, A. Kamal-Eldin, R. Mozuraityte, R. Verhé, K. Dewettinck, A. Huyghebaert, W. De Greyt, *Eur. J. Lipid Sci. Technol.*, **104**, 228 (2002).
23. Z. Réblová, *Eur. J. Lipid Sci. Technol.*, **108**, 858 (2006).
24. S. Korcek, J. H. B. Chenier, J. A. Howard, K. U. Ingold, *Can. J. Chem.*, **50**, 2285 (1972).
25. B. Katušin-Ražem, D. Ražem, *J. Phys. Chem. A,*
26. **104**, 1482 (2000).
27. D. C. Liebler, P. F. Baker, K. L. Kaysen, *J. Am. Chem. Soc.*, **112**, 6995 (1990).
28. R. Stocker, V. W. Bowry, B. Frei, *Proc. Natl. Acad. Sci. U. S. A.*, **88**, 1646 (1991).
29. K. U. Ingold, V. W. Bowry, R. Stocker, C. Walling, *Proc. Natl. Acad. Sci. U. S. A.*, **90**, 45 (1993).

РАЗШИРЯВАНЕ НА АНТИОКСИДАНТНАТА АКТИВНОСТ ПРИ ВИСОКИ ТЕМПЕРАТУРИ – ФУЛЕРЕН C_{60} СПРЕГНАТ С α-ТОКОФЕРОЛОВ АНАЛОГ КАТО ХИБРИДЕН АНТИОКСИДАНТ В НАСИТЕНИ ЛИПИДНИ СИСТЕМИ

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Постъпила на коригирана на

(Резюме)

Оксидативната стабилност на две мастни киселини - стеаринова киселина (STA) като модел на наситен липид и линоленова киселина (LNA) като модел на полиненаситен липид, съдържащ C_{60} производно с ковалентно свързана хидроксихроманилова част като аналог на α-токоферол (F-1) е проследена чрез диференциална сканираща калориметрия. Общите Арениусови кинетични параметри (активираща енергия E_a, пре-експоненциален фактор Z и скоростни константи k) на неизотермното оксидативно разлагане показват, че в наситени въглеводороди хибридното C_{60}+хидроксихроманово производно е ефективен антиоксидант, действащ при температури над 120°C, изразено като 9 kJ/mol нарастване на E_a и стойности на k два пъти по-малки отколкото при окисление на неинхибирана чиста STA. Опитите с LNA, окислена при температури над 80°C показват обаче, че това хибридно производно не подобрява оксидативната стабилност на полиненаситени липиди при високи температури (E_a, Z и k's са почти същите, както при окисление на чиста LNA). Ние предполагаме, че C_{60} може да инхибира автоокислението на STA поради образуване на термично стабилни адукти с алкоксилни радикали, докато в автоокислението на LNA участват пероксилни радикали, които не са ефективно уловени от C_{60}.

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Effect of some membrane lipids on radical generation in the system acetylcholine-hydroperoxide

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The influence of membrane lipids (0.001 - 0.1 mM) on the rate of β-carotene oxidation initiated by the lipophilic azoinitiator AMVN and by microaggregates formed by acetylcholine (ACh) and tert-butyl hydroperoxide (ROOH) in n-decane (37 ° C) was studied. Phosphatidylcholine, sphingomyelin and cholesterol were investigated as lipid components. In homogeneous systems, all the additives slightly decrease the consumption rate of β-carotene initiated by AMVN due to cross radical reactions. In the case of initiation by mixed microaggregates {mROOH…nACh} which generate peroxyl radicals $RO_2^{\bullet}$, a dose-dependent r etarding effect of phosphatidylcholine on the β-carotene consumption rate was observed whereas cholesterol additives caused acceleration of β-carotene oxidation. Sphingomyelin did not show any significant differences. It was found that the changes of radical initiation rate correlated with the changes of microaggregates sizes measured by dynamic light scattering.

Keywords: Acetylcholine chloride, Membrane lipids, Micellar catalysis, Cholesterol, Phosphatidylcholine

INTRODUCTION

The surfactant effect on lipid oxidation is determined by the ability of hydroperoxides (ROOH), the primary products of oxidation, to form mixed micelles-microaggregates with surfactants: mROOH + nS ⇔{mROOH…nS} [1-3]. The formation of mixed micelles that have a strong influence on the decomposition of ROOH was studied and confirmed by nuclear magnetic resonance (NMR) spectroscopy, dynamic light scattering (DLS), and tensiometry [2-4]. Depending on the chemical nature of the surfactant and oxidized substrate, catalysis of oxidation in the case of cationic surfactants (S+) [1-4], inhibition [5,6], or no effect at all [1,2] can take place. The key reaction of the catalytic mechanism of cationic surfactants (S^+) on the lipid oxidation is the accelerated decomposition of hydroperoxides into radicals in mixed micelles. With anionic and nonionic surfactants, hydroperoxides also form mixed micelles, but the radical decay is accelerated only in combination with S^+. In micelles with S^+, peroxide bond -O-O-, evidently, falls properly into a strong electric field of a double electric layer with a high voltage of ~ 10^5-10^7 V/m, which attenuates this bond and stimulates homolytic decay. Simple micellar effects on the rate of ROOH decomposition into free radicals due to the concentration of reagents in the micelle core and interface or due to the change in the polarity compared to that in bulk solution [6] do not explain the scale and selectivity of the effect inherent only to cationic surfactants [1-5]. The activation energy of the thermal decomposition of different ROOH is 90–120 kJ·mol^{-1} [7-9]; in reverse micelles {mROOH…nS$^+$}, it decreases to 40–60 kJ/mol [2, 3, 10,11]. As a result, the binary system S^+– ROOH can be applied as a lipophilic (reverse micelles) and hydrophilic (direct micelles) initiator of free radicals:

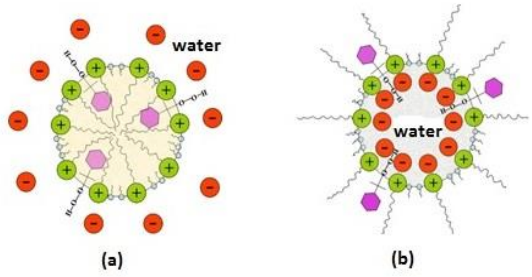

Figure 1. Direct (a) and reverse (b) micelles {mROOH…nS}

It was found that the known neurotransmitter acetylcholine chloride (ACh), forms mixed micelles with ROOH and accelerates the decomposition of ROOH into radicals and the oxidation of lipids similar to cationic surfactants [12,13]:

$$mROOH + nACh \Leftrightarrow \{mROOH…n\ ACh\} \rightarrow RO_2^{\bullet}$$

Oxidation of biomembrane lipids, such as phosphatidylcholine (PC) and cholesterol (Chol), has been recognized to be related to human diseases, such as atherosclerosis and cancer [14,15]. Along with sphingomyelin (SM), PC and Chol are constituents of rafts (“ruft-like lipids” [16]), whose role in the physiology of the cell is being intensively studied. Sphingomyelins are present in the plasma

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membranes of animal cells and are especially prominent in the brain substance and peripheral nervous system [17]. Phosphatidylcholines (lecithins), the most common molecules of cell membranes, are widely represented in the cells of various tissues; they perform both metabolic and structural functions in membranes. Cholesterol ensures the stability of cell membranes in a wide range of temperatures. Chol is necessary for the production of vitamin D, the production by adrenal glands of various steroid hormones, including cortisol, aldosterone, female sex hormones estrogen and progesterone, the male sex hormone testosterone, and according to recent data Chol plays an important role in the activity of brain synapses and the immune system, including protection from cancer.

In this work, we studied the effect of PC, Chol, and SM on the rate of β-carotene oxidation initiated by the binary system ACh and tert-butyl hydroperoxide (ROOH) in n-decane (37° C) as compared with the oxidation initiated by the lipophilic azoinitiator AMVN. β-Carotene (pro-vitamin A) is a polyunsaturated hydrocarbon of high activity in scavenging of various free radicals [18,19]. In micellar systems, hydrophobic β-carotene is localized in the organic phase and does not interact with surfactants. By this reason, β-carotene is a convenient free radical acceptor to use in the inhibitor's method for determination of the initiation rate.

EXPERIMENTAL

Acetylcholine chloride (ACh), tert-butyl hydroperoxide (ROOH), egg-phosphatidylcholine (PC) (all from Fluka), cholesterol (Chol), sphingomyelin (SM), β-carotene (A) and n-decane (all Sigma-Aldrich) were used as purchased.

The oxidation of β-carotene was carried out directly in a constant temperature-maintained quartz cell (1 cm) of an Ultrospec 1100 pro spectrophotometer at 37° C to determine the kinetics of β-carotene consumption. Its concentration was chosen in a way to intercept all the radicals escaped. ACh, dissolved in a mixture chloroform: methanol (2:1), was added to the solution of ROOH and a lipid component in n-decane. The mixture was immersed in an ultrasonic stirring bath (10 min); then 3 mL of the mixture were placed in a quartz cell, to which 9 μL of β-carotene stock solution in n-decane was added. The average size of microaggregates was determined by dynamic light scattering (DLS) using a Zetasizer Nano ZS analyzer (Malvern Instruments, United Kingdom) equipped with a laser operating at 633 nm. The particle sizes were measured in the range from 0.6 nm to 6 μm. The measurements were carried out at 25°C and a scattering angle of 173°.

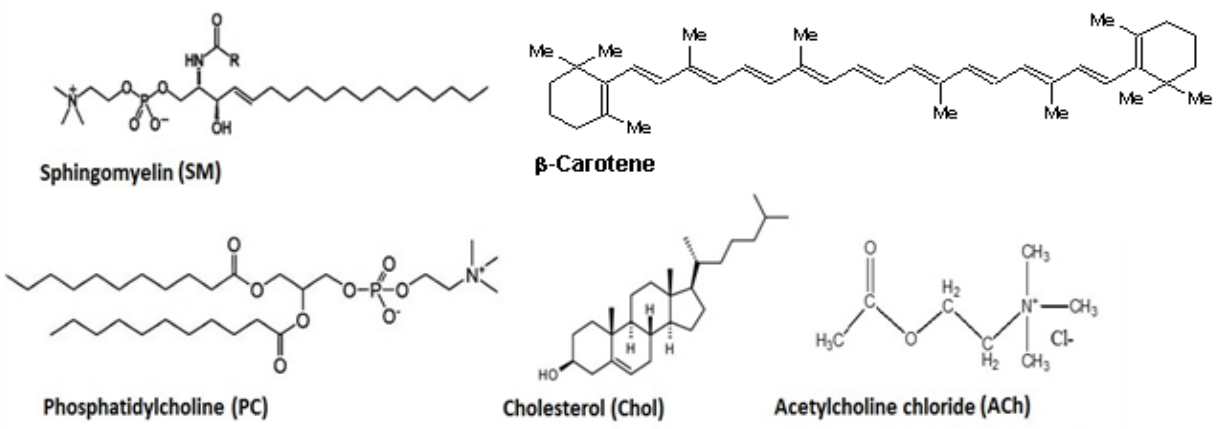

Figure 2. Structures of ACh, β-carotene and "ruft-like lipids" studied

RESULTS

There is a large number of studies on the inhibitory properties of phospholipids and cholesterol [20-24]. These compounds do not have the usual inhibitory groups to compete successfully with bulk lipids for radicals. However, SM and PC possess surface activity. Chol is known to affect the membrane structure and to contribute to the transformation of spherical direct micelles to vesicles [25]. We compared the effect of "ruft-like lipids" on β-carotene (A) consumption in homogeneous n-decane solution and in a microheterogeneous system, in which peroxyl radicals were generated in mixed micelles {mROOH…n ACh}.

Table 1. Effect of lipid additives (LH) on the rate of β-carotene (A) oxidation initiated by the azoinitiator $1.5\ 10^{-3}$ M AMVN in the presence of $1{\cdot}10^{-5}$ M lipid, [β-carotene] $=1 \cdot 10^{-5}$ M, 37ºC

Lipid additive (LH)	Without additive	Cholesterol	Phosphatidylcholine	Sphingomyelin
Rate of β-carotene consumption, $M{\cdot}s^{-1}$	$4.3{\cdot}10^{-9}$	$3.7{\cdot}10^{-9}$	$3.9{\cdot}10^{-9}$	$3.8{\cdot}10^{-9}$

Table 1 shows the influence of the additives of Chol, PC, and SM on the rate of β-carotene oxidation initiated by lipophilic AMVN in homogeneous medium. It can be seen that the additives did not lead to strong differences in rates and the decrease in the rate of β-carotene (A) consumption is probably associated with involvement of lipids in radical reactions and the increase in the rate of cross-termination (Scheme 1). In the case of radical initiation by the binary micellar system ACh-ROOH, we observed different effects of lipid additives (Table 2).

Chol additives demonstrated dose-dependent increase of β-carotene oxidation rate, whereas in the case of PC, a retardation effect was observed. Sphingomyelin demonstrated retarding influence, much weaker than PC (Table 2).

1) AMVN $\rightarrow$ $RO_2^{\bullet}$
2) A + $RO_2^{\bullet}$ $\rightarrow$ $AO_2^{\bullet}$
3) $AO_2^{\bullet}$ + $RO_2^{\bullet}$ $\rightarrow$ products
4) $AO_2^{\bullet}$ + $AO_2^{\bullet}$ $\rightarrow$ products
5) LH + $RO_2^{\bullet}$ $\rightarrow$ $LO_2^{\bullet}$ + RO_2H
6) LH + $AO_2^{\bullet}$ $\rightarrow$ $LO_2^{\bullet}$ + AO_2H
7) $LO_2^{\bullet}$ + $AO_2^{\bullet}$ $\rightarrow$ products
8) $LO_2^{\bullet}$ + $RO_2^{\bullet}$ $\rightarrow$ products

Scheme 1.

Table 2. Effect of lipid additives on the rate of β-carotene oxidation initiated by the binary system ACh-ROOH at initial concentrations: $1{\cdot}10^{-3}$ M ACh, $1{\cdot}10^{-2}$ M ROOH, $1{\cdot}10^{-5}$ M β-carotene, in n-decane, 37ºC

Concentration of additives, M	Rate of β-carotene consumption, $M{\cdot}s^{-1}$		
	Chol	SM	PC
Without additive	$1.15{\cdot}10^{-9}$		
$1{\cdot}10^{-6}$	$3.5{\cdot}10^{-9}$	$1.00{\cdot}10^{-9}$	$0.02{\cdot}10^{-9}$
$5{\cdot}10^{-6}$	$3.8{\cdot}10^{-9}$	$0.82{\cdot}10^{-9}$	$0.005{\cdot}10^{-9}$
$1{\cdot}10^{-5}$	$4.2{\cdot}10^{-9}$	$0.75{\cdot}10^{-9}$	$0.002{\cdot}10^{-9}$
$5{\cdot}10^{-5}$	$4.8{\cdot}10^{-9}$	$0.61{\cdot}10^{-9}$	$0.001{\cdot}10^{-9}$
$1{\cdot}10^{-4}$	$5.0{\cdot}10^{-9}$	$0.57{\cdot}10^{-9}$	0

It should be noted that hygroscopic acetylcholine is not soluble in organic solvents, but in combination with hydroperoxides, ACh forms relatively large mixed aggregates, with a size of ~350 nm (Fig. 3a). For comparison, a typical cationic surfactant cetyltrimethylammonium bromide (CTAB) under similar conditions forms together with tert-butyl hydroperoxide mixed reverse micelles with a size of 10 nm [13] and provides a higher rate of β-carotene consumption [2,3,5,13]. Because PC and SM are surface active substances and Chol as an essential structural component of all cell membranes, known to affect the structure of direct micelles and membranes [26,27], the influence of Chol and PC on the sizes of mixed micelles {mROOH…nACh} was studied.

In the presence of Chol, the time required to establish a stationary distribution for DLS measurement decreases, and the average size is reduced to ~300 nm (Fig. 3.b). Since size reduction is accompanied by an increase of the rate of radical generation, we can suggest that Chol promotes, to some extent, the integration of the –O-O- bond into a double electric layer, which leads to an increase in ROOH decay and radical initiation rate. PC is relatively well soluble in organic solvents to yield transparent or slightly opalescent (at [PC] > 20 mg/ml) solutions. According to the DLS data, at [PC] = 20–90 mg/ml in n-decane, reverse micelles with average hydrodynamic diameters of 6 nm are formed [28]. When PC is added to the micellar solution of {mROOH…nACh}, micelles of ~100

nm are formed along with large aggregates of 1500 nm (Fig. 3.c). Most likely, PC solubilizes the micelles {mROOH…nACh} or the individual ACh. This results in destruction of the inner structure of the {mROOH…nACh}, changing of space location of the –O-O- bond, and decrease of ROOH decay and rate of radical escape into bulk solution.

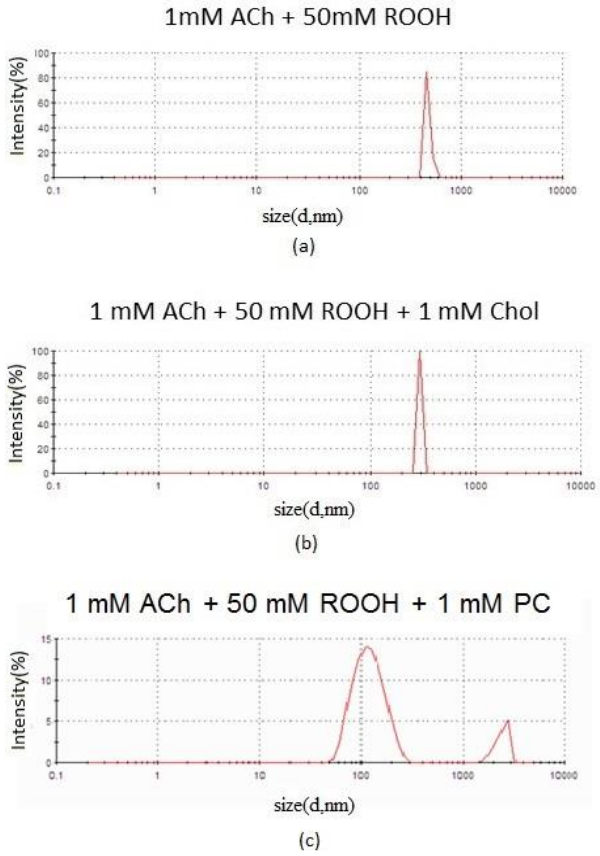

Figure 3. Effect of Chol (b) and PC (c) on the average size of microaggregates formed by the mixture of ACh with tert-butyl hydroperoxide (a) in n-decane solution.

CONCLUSIONS

Acetylcholine forms common micelles with hydroperoxides in the organic medium, which facilitates the breakdown of hydroperoxides into free radicals. The effect of the membrane lipids on the rate of free radical generation in microaggregates {mACh ... nROOH} was revealed.

Phosphatidylcholine (lecithin) solubilizes {mACh ... nROOH} in an organic medium, which results in a decrease of the radical initiation rate, and the yield of radicals into the volume is hampered. Cholesterol (Chol), on the contrary, stabilizes {mACh ... nROOH}, accompanied by an increase of the rate of radical generation.

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REFERENCES

1. O.T. Kasaikina, V.D. Kortenska, Z.S. Kartasheva, G.M. Kuznetsova, T.V. Maximova, T.V. Sirota, N.V.Yanishlieva, *Colloids Surf., A*, **149**(1-3), 29 (1999).
2. O.T. Kasaikina, Z.S. Kartasheva, L.M. Pisarenko, *Russ. J. Gen. Chem.*,**78**(8), 1533 (2008).
3. O.T. Kasaikina, A.A. Golyavin, D.A. Krugovov, Z.S. Kartasheva, L.M. Pisarenko, *Moscow Univ. Chem. Bull.*,**65**, 206 (2010).
4. N.A.Trunova, Z.S. Kartasheva, T.V. Maksimova, Y.P. Bogdanova, O.T. Kasaikina, *Colloid J.*,**69**, 697 (2007).
5. O. T. Kasaikina, N. V. Potapova, D. A. Krugovov, L. M. Pisarenko, *Kinetics and Catalysis*, **58**(5), 556 (2017).
6. G. La Sorella, G. Strukul, A. Scarso, *Green Chem.*,**17**, 644 (2015).
7. N. Emanuel, D. Gal, *Modelling of Oxidation Processes. Prototype: The Oxidation of Ethylbenzene*, Budapest, Akademiai Kiado, 1986.
8. E.T. Denisov, I.B. Afanas'ev, *Oxidation and Antioxidants in Organic Chemistry and Biology*, Boca Raton, Fla., CRC, 2005.
9. E.N. Frankel, *Lipid Oxidation*, Dundee, Scotland, Oily Press, 2nd ed. 2005.
10. D.A. Krugovov, L.M. Pisarenko, V.G. Kondratovich, A.N. Shchegolikhin, O.T. Kasaikina, *Pet. Chem.*, **49**, 120 (2009).
11. Z.S. Kartasheva, T.V. Maksimova, E.V. Koverzanova, O.T. Kasaikina, *Pet. Chem.*, **37**, 245 (1997).
12. O.T. Kasaikina, V.D.Kancheva, T.V.Maximova, Z.S.Kartasheva, N.V. Yanishlieva, V.G. Kondratovich, I.R. Totseva, *Oxid. Commun.*, **3**, 574 (2006).
13. D.A. Krugovov, E.A. Mengele, O.T. Kasaikina, *Russ. Chem. Bull.*, **63**(8), 1837 (2014).
14. E. Schnitzer, I. Pinchuk, D. Lichtenberg, *Eur. Biophys. J.*,**36**, 499, (2007).
15. U. Matt, O. Sharif, R. Martins, S. Knapp, *Cell Mol. Life Sci.*,**72**, 1059 (2015).
16. A.C. Caritá, B. Mattei, C. C. Domingues, E. de Paula, K.A. Riske, *Langmuir*, **33**(29), 7312 (2017), DOI: 10.1021/acs.langmuir.7b01134.
17. Y. Barenholz, T.E. Thompson. *Chem. Phys. Lipids*, **102**(1–2), 29 (1999).
18. O. Ozhogina, O.T. Kasaikina, *Free Rad. Biol. Med.*, **19**(5), 575 (1995).
19. V.D. Kancheva, O.T. Kasaikina, *Cur. Med. Chem.*, **20**(37), 4784 (2013).
20. M.F. King, L.C. Boyd, B.W. Sheldon, *J. Am. Oil Chem. Soc.*, **69**, 545 (1992).
21. A. Judde, P. Villeneuve, A. Rossignol-Castera, A. le Guillou, *J. Am. Oil Chem. Soc.*, **80**, 1209 (2003).
22. H. Sugino, M. Ishikawa, T. Nitoda, M. Koketsu, L.R. Juneja, M. Kim, T. Yamamoto, *J. Agric. Food Chem*., **45**, 551 (1997).
23. A.J. Neto, M. R. Cordeiro, *Biochim. Biophys. Acta*, **1858** (9), 2191 (2016).

24. P. Sarkar, A. Chattopadhyay, *Chem. Phys. Lipid*, **195**, 34 (2016).
25. M. Cano-Sarabia, A. Angelova, N. Ventosa, S. Lesieur, J. Veciana, *J. Colloid Interface Sci.*, **350**, 10 (2010).
26. S. Ghosh, J. Kuchlyan, S. Roychowdhury, D. Banik, N. Kundu, A. Roy, N. Sarkar, *J. Phys. Chem. B*, **118**, 9329 (2014), DOI: 10.1021/jp503938b.
27. M.A. Sedgwick, A. M. Trujillo, N. Hendricks, N. E. Levinger, D. C. Crans, *Langmuir*, **27**(3), 948 (2011).
28. E.A. Mengele, Z.S. Kartasheva, I.G. Plashchina, O.T. Kasaikina, *Colloid J.*, **70**(6), 753 **(**2008).

ЕФЕКТ НА НЯКОИ МЕМБРАННИ ЛИПИДИ ВЪРХУ ГЕНЕРИРАНЕТО НА РАДИКАЛИ В СИСТЕМАТА АЦЕТИЛХОЛИН-ХИДРОПЕРОКСИД

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(Резюме)

Влиянието на мембранни липиди (0.001 - 0.1 mM) върху скоростта на окисление на β-каротин, инициирано от липофилния азоинициатор AMVN и от микроагрегати, образувани от ацетилхолин (ACh) и трет.-бутилов хидропероксид (ROOH) е изследвано в n-декан при 37 °C. Фосфатидилхолин, сфингомиелин и холестерол са изследвани като липидни компоненти. В хомогенни системи всички добавки слабо понижават скоростта на изразходване на β-каротина, инициирано от AMVN, поради кръстосани радикалови реакции. В случая на инициране от смесени микроагрегати {mROOH…nACh}, които генерират пероксилни радикали $RO_2^{\bullet}$, се наблюдава концентрационно-зависим ефект на забавяне на консумацията на β-каротина от фосфатидилхолина, докато добавката от холестерол ускорява окислението на β-каротина. Сфингомиелинът не води до значими различия. Установено е, че промените в скоростта на радикалово инициране корелират с промените в размера на микроагрегатите, измерени чрез динамично разсейване на светлината.

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Essentiality of dietary amino acids for antioxidative defense

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The amino acids are not only building blocks for proteins synthesis. They also participate in many other biochemical processes and functions: cell signaling pathways, antioxidative reactions, acid-base balance, gene expression, regulation of immune function, and many others. The aim of this study was to present scientific data concerning the essentiality of dietary non-essential amino acids (NEAA) for antioxidative defense in the body, and to present data about their composition and quantity in Bulgarian foods. Information is provided on the content of amino acids with antioxidant activity (Cys,Glu, Gly, Met and total content of S-amino acids) in 27 Bulgarian food products.The data were obtained by the classical method for analysis of amino acids – ion exchange chromatography with post-column derivatization. The importance of NEAA for the antioxidant defense of the body through their participation in glutathione, taurine and hydrogen sulfide synthesis is clarified. Their role in activating the antioxidant capacity of human serum albumin – an important endogenous antioxidant is outlined. Examples are listed showing effective implementation of antioxidant amino acids at thermal processing of dietary fats substituting the synthetic additives.The current data clearly showed that non-essential amino acids, especially those containing extra thiol, thioether, or extra amine group, have strong antioxidant activities. On the basis of the essentiality of dietary non-essential amino acids for the antioxidative defense of the human body, their broad participation in preventive and healthy diets is strongly recommended.

Keywords: Amino acids, Antioxidation, Glutathione, Taurine, Human serum albumin, Additives

INTRODUCTION

The concept identifying amino acids (AA) as essential (EAA) and non-essential (NEAA) has been implemented by the nutritional science for more than a century. One of the basic topics of the scientific research in this period was dedicated to the assessment of dietary protein quality that cumulated a great amount of research energy. That was exactly the aspect by which the proteinogenic amino acids were classified in two major groups – essential (Ile, Leu, Val, Lys, Met, Thr, Phe, Trp,His) and non-essential (Ala, Arg, Asn, Asp, Cys, Glu,Gln, Gly, Pro, Ser, Tyr) and the ratio between their content was adopted as a marker of dietary protein quality[1-3].

Essential amino acids are not synthesized in human organisms thus determining their importance as essential ones that have to be supplied through the diet in order to realize the specific protein synthesis.All research was focused on those amino acids which availability determines the sufficiency of food protein. Studies on the role of replaceable amino acids also started during the last decades of the past century. The replaceable amino acids with their variable chemical composition and structure outline a broad spectrum of biochemical reactions specific for their particular involvement. The following more important processes could be marked among this variety: regulation of cell signalling pathways (Arg, Gln, Gly), antioxidant defense of the organism (Glu,Cys,Gly),maintaining of the acid-alkaline balance (Gln),involvement in immune function processes(Arg, Gln, Pro), and others[4, 5].

The research efforts to clarify the "diet-health" relationship established the "Antioxidant hypothesis", determining the important role and significance of dietary components with antioxidant activity, in the leading place. The introduction of the antioxidant hypothesis explaining the effects of food on human health triggered comprehensive studies searching for compounds substantiating the antioxidant power of the food. The variety of food compositions showed presence of nutrients and of a number of bioactive compounds with antioxidant activity, determining the importance of food for strengthening the antioxidant defense of the organism and for delaying the aging processes [6, 7].

The current studies on the antioxidant activity of non-essential amino acids are concentrated on two aspects. The first one is associated with the role of essential amino acids against oxidative stress in the organism, supplied through the diet [8, 9]. The second one surveys the options to use those amino acids in food technologies as antioxidant additives [5, 10, 11]. This broad spectrum of NEAA implementation in oxidation processes requires comprehensive knowledge on the amino acid

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composition of foods and on their activity in various biological media as well.

The aim of the current study was to present scientific data concerning the essentiality of dietary non-essential amino acids (NEAA) for the antioxidative defense in the body, and to present data about their composition and quantity in foods, typical for the Bulgarian diet.

MATERIAL AND METHODS

An analysis and assessment of recent scientific information from the last decade was conducted, clarifying in a new light the role and importance of NEAA.The survey covered a total of 27 food products typical for the Bulgarian diet. The results for the content of amino acids associated with the organism antioxidant defense were taken from our data base for the amino acid composition of Bulgarian food products that did not contain data about fruits and vegetables because of their poor protein content. The amino acid composition was determined after hydrochloric acid hydrolysis with 6M HCl at 110°C for 22 hours by the classic method of Speckman, Stein and Moor using ion exchange chromatography with post-column derivatization with ninhydrin and UV detection at 570nm (CLA-5, Hitachi). The total protein content in the samples was determined by Kjeldahl method where the content of studied amino acids was expressed on the basis of 100 g protein. The amino acids glutamine, cysteine, glycine, and the essential amino acid methionine as cysteine precursor and sulfur-containing amino acid were in the focus of the present study.Cystine and cysteine were determined as a total complying with the routine analytical recommendations after their oxidation with performic acid to cysteic acid [12].

RESULTS AND DISCUSSION

The results of this survey are presented in two parts. The first one includes analysis of current scientific data about the antioxidant properties of essential amino acids engaged in the antioxidant defense of the organism and in their implementation as antioxidant additives in food technologies. The second part presents the content of those amino acids in Bulgarian food products.

Engagement of NEAA in the antioxidant defense of the organism and their implementation as antioxidant additives

The engagement of essential amino acids in the antioxidant defense is performed through various biochemical pathways. Three of them were in the focus of the present survey: glutathione synthesis, taurine synthesis and involvement in the activation of the antioxidant capacity of human serum albumin (HSA).

Glutathione is an important endogenous antioxidant, part of a precisely coordinated antioxidant enzyme system of peroxidases, synthases and reductases. Glutathione synthesis (tripeptide – gamma-glutamyl-cysteinyl-glycine) requires the presence of the amino acids glycine, cysteine and glutamine (Gly, Cys, Glu). There are several proposed and described mechanisms for explanation of the antioxidative activity of those amino acids. They can take part in antioxidative defenses also through antioxidative enzymes (glutathione peroxidase, superoxide dismutase, and H_2O_2- peroxidase).NEAA have a role in disposal of ammonia, oxidants, and xenobiotics, and in anti-inflammation and regulation of cell's apoptosis.

Glutathione is one of the earliest compounds established as a redox-system involved in biological oxidation.In1889 it was stated that reduced glutathione (GSH) is the body's own master antioxidant. About 30 years ago the scientists began to understand its functions and discovered how to raise its levels. GSH is a small protein molecule produced in every cell of the human body performing many biological roles [13].

Glutathione in its oxidized form contains a disulfide bridge (linking two glutathione molecules) and in its reduced form – sulfhydryl groups. Being a good reductant together with NADPH it participates in the disposal of free radicals. It plays the role of a co-factor of glutathione peroxidase participating in the disposal of hydrogen peroxide and the free hydroxyl radical [14,15].Based on the data of Choe and Min [7]amino acids could be accepted as radical scavengers quenching singlet oxygen.The scientific publications have revealed many different data on synergistic interactions with tocopherols and other primary antioxidants, as well as evidence for antioxidant activity of secondary compounds, produced by reaction between AA and oxidized lipids [5]. Some studies supplied results about chelating (to metals) properties of amino acids and peptides that catalyze the breakdown of hydroperoxides into free radicals [16]. The antioxidative activity of GSH is based specifically on the presence of sulfur atom - crucial for scavenging peroxides (H_2O_2), and other free radicals. The master antioxidant role of GSH is the recycling of vitamin C, vitamin E,lipoic acid, and the regulation of caspases, controlling apoptotic cell death, by redox reactions through glutathione and thioredoxin. The thiol status is an important factor in the therapeutic control of different diseases [13, 17].

L-cysteine plays an important role in glutathione synthesis. It is a semi-essential amino acid which, besides being a glutathione precursor together with glutamate and glycine, participates also in protein synthesis.Besides from the diet, this amino acid could be supplied by trans-sulfuration pathway from methionine and through decomposition of proteins. Various studies using diet supplementation with amino acids building glutathione have shown elevated level of synthesized glutathione in the liver of test animals[18]. Other studies have presented even quantitative evaluations in a sense that moderate amino acid supplementation definitely brought about elevated glutathione level but exceeding the intake dose caused reduced glutathione concentration in the liver tissue – a fact that has not been fully explained yet. There are multiple studies on diet supplementation with cysteine at different diseases and almost all of them confirmed elevated glutathione levels and enhanced antioxidant capacity[19-22]. Certain studies on *per oral* glutathione import have not revealed changes in its serum and tissue level in the organism, thus suggesting and emphasizing the role of the amino acids, building it.Those data clearly showed the necessity of knowledge on the content of above mentioned amino acids in the foods constituting the diet in order to compile precise healthy dietary regimes providing the necessary glutathione synthesis in the organism.

The antioxidant activity of the amino acid cysteine is supported by the evidence for its involvement in production of taurine and hydrogen sulfide which possess antioxidant, anti-inflammatory and neuroprotective activity, thus enhancing the esteem of this amino acid as participant in the antioxidant defense of the organism[13]. Stipanuk's results[23, 24] proving that a diet rich in sulfur-containing amino acids caused the activation of the enzyme L-cysteine dioxygenase involved in taurine-formation process support this concept.

The major taurine precursor is cysteine-sulfinate. The enzymes cysteine dioxygenase(CDO), cysteinesulfinate decarboxylase(CSD), and cysteaminedioxygenase pathways [25] participate in taurine synthesis.There is evidence that low cysteine levels block CDO activity while its elevated concentration causes quick recovery and enhancement of the enzyme activity in the adipocytes and liver[24].This sensitivity of the enzyme to cysteine level is an important factor for taurine formation as it can be a regulating factor in its synthesis. Studies on the role of cysteine sulfinate have shown that 66% of it participated in taurine production and the remaining part – in sulfite production(34%)[24].Taurine, besides its antioxidant activity has also cytoprotective effects, participates in the regulation of intracellular Ca-concentration, in the movement of ions and neurotransmitters, etc.It is accepted as a protective factor against the effect of various environmental contaminants and variety of drug forms[26, 27].The increased production of H_2S is also a preventive mechanism against oxidative stress caused mainly in the brain by the elevated presence of glutamate and its accumulation into asynaptic cleft. Dysfunction of glutamate transporters is associated with various neurodegenerative diseases[28,29].Lu *et al*.demonstrated that H_2S has a potential therapeutic value for oxidative stress-induced brain damage[30].There is abundant information on food protein hydrolysates, amino acids residues and free amino acids with antioxidant activities, based on scavenging of free radicals or reactive species, but mechanisms of action have not been studied in details[30-32]. In this aspect many studies focus on the antioxidant properties of human serum albumin (HSA)[8, 33-35]. NEAA have particular relationship with the antioxidant activity of HSA that acts as antioxidant in biological systems to oxidative stress in aging and inflammation processes.Various studies have proven a relationship between the redox change in HSA and the oxidation of several amino acid residues. The antioxidant activity of HSA is strongly dependent on Cys34, and could serve as a bio marker for evaluation of systemic redox states, important for the efficacy of the therapy and disease progression[8, 34]. Quantitative studies on the role of cysteine 34 and methionine residue in the antioxidant activity of serum albumin have shown respectively 61% and 29% *versus* singlet oxygen; 68% and 61% *versus* hydrogen peroxide, thus clearly defining the more important role of cysteine [33].The analysis of the information explicitly identifies the role of amino acids in the antioxidant defense enhancing in this way the spectrum of compounds with antioxidant properties activating the antioxidant potential of the organism.

From practical point of view the implementation of amino acids as antioxidant additives in fats and oils undergoing thermal processing provokes certain interest. The data from scientific publications show that amino acids act as primary antioxidants but also as antioxidants synergic to alfa-tocopherol. Amino acids containing a thiol group or extra amine group, such as arginine and cysteine have high antioxidant activity while those with functional groups as amide, carboxyl acid

imidazole or phenol have relatively lower antioxidant activity. A study on the antioxidant activity of 20 amino acids added to soy oil undergoing thermal processing at 180°C showed significantly higher antioxidant activity of the amino acids compared to the synthetic antioxidant *tert*-butylhydroquinone (TBHQ) routinely implemented in technological processes [5].

Filipenko and Gribova [10] investigated the effect of amino acids on the processes of sunflower oil oxidation. The higher levels of antioxidant activity of certain amino acids is associated with the greater number of nitrogen atoms in their molecule. The authors have proven that both amino acids and their salts had antioxidant activity.Amino acids, as natural compounds, are recommendable for implementation instead of synthetic antioxidants that now have a long history of implementation in food technologies.

Content of AA with antioxidant potential in Bulgarian foods

Table 1 presents data about the content of amino acids with antioxidant potential in 27 Bulgarian foods. It lists data about glutathione-building NEAA – Cys, Glu, Gly and the essential amino acid Met as sulfur-containing and engaged in cysteine synthesis. Cysteine exists mainly in the form of cystine because of rapid oxidation that is why the table lists the total content of the two amino acids Data are provided about the total protein content of foods, and the content of each amino acid was calculated *versus* it.

Table 1. Content of AA with antioxidant potential in Bulgarian foods

№	Food Product	Protein (%)	AMINO ACIDS /g / 100 g protein				
			Cys + Cystine	Met	Glu	Gly	Total S-amino acids
GRAINS AND PULSES							
1.	Bread, wheat, white	6.73	1.39	1.50	34.83	3.26	2.89
2.	Bread, wheat, black	7.34	1.62	1.45	33.52	3.35	3.07
3.	Bread, rye	4.65	1.63	1.56	27.73	3.73	3.19
4.	Rice	6.46	1.00	2.25	18.94	3.92	3.25
5.	White beans	19.14	0.66	1.13	17.64	3.78	1.79
6.	Lentils	23.33	0.76	0.77	18.39	3.79	1.53
7.	Green peas	23.50	0.86	0.92	18.62	3.93	1.78
MILK AND DAIRY PRODUCTS							
8.	Fresh cow's milk	3.21	0.77	2.38	22.49	1.57	3.15
9.	Fresh sheep's milk	4.68	0.69	2.49	20.58	1.56	3.18
10	Yogurt - cow	3.24	0.71	2.38	22.10	1.63	3.09
11	White cow's cheese	16.41	0.61	2.36	21.36	1.61	2.97
12	White sheep's cheese	15.59	0.69	2.30	21.33	1.61	2.99
13	Yellow cheese "Vitosha"	24.60	0.73	2.19	22.16	1.48	2.92
14	Yellow cheese "Balkan"	24.02	0.80	2.17	22.13	1.49	2.97
15	"Cheddar" cheese	24.06	0.55	2.58	20.46	1.56	3.13
16	"Roquefort" cheese	21.18	0.69	2.50	20.89	1.60	3.19
MEAT AND MEAT PRODUCTS							
17	Veal	20.51	0.88	2.67	16.59	4.36	3.55
18	Beef	18.97	1.07	2.05	16.28	5.67	3.12
19	Pork	12.49	0.67	2.46	15.92	5.48	3.13
20	Pork filet	20.60	0.75	2.83	15.41	3.99	3.58
2.	Lamb	20.58	0.93	2.38	15.34	6.05	3.31
22	Chicken (white meat)	23.43	0.74	2.74	15.52	3.96	3.48
23	Chicken (red meat)	19.72	0.92	2.73	16.40	4.28	3.65
24	Egg (hen)	12.57	2.03	3.49	13.31	2.95	5.52
FISH							
25	Carp	18.51	0.67	2.92	15.99	4.39	3.59
26	Trout	22.16	0.67	3.34	14.66	4.71	4.01
27	Mackerel	17.37	0.73	3.17	14.63	4.36	4.0

The last column shows the total content of sulfur-containing amino acids based on published data on their role in a number of biochemical processes.

The table does not list data about the content of the investigated amino acids in fruits and vegetables because of the specificity of those foods, characterized by a broad spectrum of bioactive compounds, and very low protein content suggesting yet lower level of the investigated amino acids. Similar values would have greater theoretical importance rather than practical applicability. The data in the table could be used for more comprehensive characteristics of the particular foods and for elaboration of preventive diets.

CONCLUSION

The presented scientific information on the role of NEAA in the antioxidant defense of the organism enables the enhancement of the spectrum of nutrients with antioxidant potential and revision of the amino acids "essential – nonessential" classification framework because there is a lot of new evidence for amino acids which, although non-essential for the protein synthesis in the body are essential for certain biochemical processes.

The application of amino acids as additives is a new option to remove the synthetic antioxidants undesired by the consumers from various foodstuffs.

The presented data for amino acid amount in certain foods will facilitate the establishment of preventive and healthy nutrition of the Bulgarian population and will enrich the technologists' information about new food products.

REFERENCES

1. S. Damodaran, in: Food Chemistry, O.R. Fennema (ed.), vol. 25, Marcel Dekker, Inc. NewYork 1996, p. 322.
2. R. Rezaei, W.Wang, Z. Wu, Z. Dai, J.Wang, G.Wu, *Journal of Animal ScienceandBiotechnology* **4**,1(2013).
3. Y. Hou, Y. Yin, G. Wu, *Experimental Biology and Medicine*, **240**, 997(2015).
4. Y. Hou, G. Wu, *Adv. Nutr*. **8**, 137(2017).
5. H-S. Hwang, J. K.Winkler-Moser, *Food Chemistry*, **221,** 1168 (2017).
6. T.Finkel, N.J. Holbrook, *Nature* **408,** 239 (2000).
7. E. Choe, D. B. Min, *Comprehensive Reviews in Food Science and Food Safety*,**8**,345(2009).
8. M. Anraku, *YakugakuZasshi*, *(PubMed)*,**134**, 973 (2014).
9. S. Garibaldi, C. Barisione, B. Marengo, P. Ameri, C. Brunelli, M. Balbi, G.Ghigliotti, *Toxins*, **9**, 1 (2017).
10. T.A. Filippenko, N.Y. Gribova, *Pharmaceutical Chemistry Journa*, **45**, 296 (2011).
11. C. U. Chibuike, R. E. Aluko, *Int. J.Mol. Sci*., **12,** 3148 (2011).
12. S. M. Rutherfurd, Amino Acid Analysis of Plant Products. in: Amino Acids inHigherPlants, J.P.F.D'Mello (ed.), vol. 25, CABI, USA, 2015, p.481.
13. J.Yin, W. Ren., G. Yang, J. Duan, X. Huang, R. Fang, Ch. Li, T. Li, Y. Yin, Y.Hou,S.W.Kim, G. Wu, *Mol.Nut. Food Res.*, **60,** 134 (2016).
14. R. Njalsson, S. Norgren, *ActaPaediatr***., 94,** 132 (2005).
15. V. Ribas, C. Garcia-Ruiz, J. C. Fernandez-Checa, *Front Pharmacol*.,**5**, 1 (2014).
16. E.A. Decker, V. Ivanov, B.Z. Zhu, B. Frei, *J Agric Food Chem*., **49,** 511 (2001).
17. M. D. Coleman, *Environmental Toxicology and Pharmacology*, **10,** 139 (2001).
18. Y. Chen, H. Dong, D. C. Thompson, H. G. Shertzer, D. W. Nebert, V Vasiliou,*FoodChem. Toxicol.*,**60**, 38 (2013).
19. C. J. Kim, J. Kovacs-Nolan, C. Yang, T. Archbold, *Biochim. Biophys. Acta*, **1790**,1161 (2009).
20. G. Wu, *Amino Acids*, **45**, 407 (2013).
21. K. Vidal, D. Breuille, P. Serrant, P. Denis, F. Glomot, F.Béchereau, A. Papet, *Eur. J. Nutr.*, **53**, 963 (2014).
22. Y. Q. Hou, L. Wang, D. Yi, G. Wu, *Front. Biosci.*,**20**, 872 (2015).
23. M. H. Stipanuk. *Ann. Rev. Nutr.*, **24**, 539 (2004).
24. M. H. Stipanuk, I.Ueki, *J. Inher. Metab. Dis.*, **34**,17 (2011).
25. I. Ueki, M. H. Stipanuk, *J. Nutr.*, **139**, 207 (2009).
26. C. De la Puerta, F.J. Arrieta, J.A. Balsa, J.I. Botella-Carretero, I. Zamarrón,C.Vázquez, *Nutr. Hosp.*, **25,** 910 (2010).
27. H. Ripps, W. Shen, *Mol. Vis.*, 18, 2673 (2012).
28. X. L.Sun, X.N.Zeng, F.Zhou, C.P.Dai, J-H.Ding, G.Hu, *Neuropsychopharmacology*, **33**, 1336 (2008).
29. R. J. Elias, S. S. Kellerby, E. A. Decker, *Crit. Rev. Food Sci. Nutr.*,**48**, 430 (2008).
30. M. Lu, L. Hu, G. Hu, J. S. Bian, *Free Radic. Biol. Med.*, **45**,1705 (2008).
31. T. L. Pownall, C. C. Udenigwe, R. E. Aluko, *J. Agric. Food Chem.*,**58,** 4712(2010).
32. C. C. Udenigwe, R. E. Aluko, *Int. J. Mol. Sci.*,**12,** 3148 (2011).
33. Y. Iwao, Y. Ishima, J. Yamada, T. Noguchi, U. Kragh-Hansen, K. Mera, D. Honda,A. Suenaga, T. Maruyama, M. Otagiri, *IUBMB Life*, **64,** 450 (2012)
34. M. Anraku, V. T. Chuang, T. Maruyama, M. Otagiri, *Biochim. Biophys. Acta*,**1830**,5465 (2013).
35. M. Anraku, R. Shintomo, K. Taguchi, U. Kragh-Hansen, T. Kai, T. Maruyama, M.Otagiri,*Life Sci*., **134**, 36 (2015).

ЕСЕНЦИАЛНОСТ НА АМИНОКИСЕЛИНИТЕ В ХРАНАТА ЗА АНТИОКСИДАНТНАТА ЗАЩИТА

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(Резюме)

Аминокиселините са не само изграждащи единици за протеиновия синтез. Те участват също в много други биохимични процеси и функции: клетъчни сигнални пътища, антиоксидантни реакции, киселинно-основен баланс, генна експресия, регулиране на имунната функция и много други. Целта на това изследване е да представи научни данни за значението на неесенциалните аминокиселини (NEAA) в храната за антиоксидантната защита на организма и за техния състав и количество в български храни. Представена е информация за съдържанието на аминокиселини с антиоксидантна активност (Cys, Glu, Gly, Met и общо съдържание на S-аминокиселини) в 27 български хранителни продукти. Данните са получени по класическия метод за определяне на аминокиселини – йонообменна хроматография със следколонна дериватизация. Изяснено е значението на NEAA за антиоксидантната защита на организма чрез участието им в синтеза на глутатион, таурин и водороден сулфид. Подчертана е ролята им за активиране на антиоксидантната способност на човешкия серумен албумин– важен ендогенен антиоксидант.Приведени са примери за ефективно прилагане на антиоксидантни аминокиселини при термичната обработка на мазнини, заместващи синтетичните антиоксиданти. Настоящите данни ясно показват, че неесенциалните аминокиселини, особено тези, съдържащи допълнителна тиолова, тиоетерна или аминогрупа, имат висока антиоксидантна активност. Имайки пред вид значението на неесенциалните аминокиселини в храната за антиоксидантната защита на човешкия организъм се препоръчва използването им в превантивни и здравословни диети.

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The flavonoids composition of Bulgarian foods – comparison with USDA database

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Flavonoids are polyphenolic antioxidants of plant origin, comprising more than 5000 individual compounds. Their protective role to human health is associated with reduced risk of development of series of degenerative diseases. Therefore the quantitative knowledge of the flavonoids composition is of crucial importance for elaboration of healthy nutrition diets. The aim of the study was to estimate differences between flavonoids content of Bulgarian foods and the USDA Flavonoids Database. For achieving the set aim, the Bulgarian Flavonoids Food Composition Database have been translated and transferred in Excel format, according the unified Access scheme of the USDA Database. The fast track and comparison of the data was made with automotive program written on Visual Basic for Application (VBA). The original data for representatives of 3 major classes of flavonoids in Bulgarian foods are presented. The data for the flavonols – myricetin, quercetin and kaempferol, flavan-3-ols – (+)-catechin and (-)-epicatechin and flavones – luteolin and apigenin were determined by validated HPLC methods. The food samples were collected according to a precise sampling plan and the origin of all foods was documented. In the current study data for 15 fruits, 30 vegetables and vegetable products and 3 leafy green condiments are reported. Our data are compared with the pool of results, summarized in USDA Database for the Flavonoids Content of Foods, where data for 506 foods are presented, covering 308 scientific literature papers. The current study revealed a number of topics whose further study would advance the development of nutritional databases.

Keywords: Flavonoids, Flavonols, Flavan-3-ols, Flavons, Data base

INTRODUCTION

Food is a source of essential nutritional compounds and provides energy and a broad spectrum of biologically active compounds for living organisms. It plays a vital role for the human body throughout its living cycle, ensuring growth and development and sustaining its physical and mental activity. Therefore, the knowledge on food composition is one of the most important tasks of food and nutrition sciences.

Flavonoids are representatives of the large class of phenolic compounds, which are known as powerful antioxidants and the initial interest in those compounds was mainly due to their pigment properties in plants [1]. The interest in flavonoids began to raise in 1993, triggered by the publishing of the first epidemiological survey (‘‘Zutphen Elderly Study’’) that revealed a reverse relationship between the high intake of flavonoids (flavonols and flavones) and the risk of cardiovascular disease (CVD) [2]. This survey was supported by numerous studies outlining the protective role of flavonoids concerning CVD [3-5], cancer [6-8] and neurodegenerative diseases like Parkinson’s and Alzheimer’s diseases [9].

Flavonoids are the biggest and most abundant class of phenolic compounds found in the plant kingdom. Structurally they diverse and vary from single molecules to highly polymerized structures [1]. Flavonoids are important plant pigments and the study of their chemical structure and properties started half a century ago. The classification of the flavonoids depends on two major factors – the place of the second phenyl radical bond to the heterocyclic skeleton and the oxidation state of the same heterocyclic ring. Different classes of flavonoids are differing by the number and the position of substituents in the aromatic rings. The main functional groups in flavonoids` structure are the hydroxyl groups (OH-) that could be methylated or glycosylated.

Flavonoids could act either as chain-breaking or preventive antioxidants and exert their antioxidant activity by different mechanisms: scavenging species that initiate peroxidation, chelating metal ions, thus making them unable to generate reactive species, decompose lipid peroxides, quenching O_2-, breaking the auto-oxidation chain reactions, and/or reducing localized oxygen concentrations [10, 11].

The flavonoids – scavengers of free radicals

It has long been known that flavonoids act like scavengers of free radicals [12-14] and their antiradical activity in water and lipid media has been subjected to a thorough research [12, 15]. It is deemed that flavonoids’ activity as inhibitors of chain radical peroxidation is performed in two

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stages [11]. Initially one of the hydroxyl groups of the molecule (usually the 4'-OH group), is dissociated homolytically or heterolytically, thus leaving a flavonoid radical (aroxyl radical).

Homolytic dissociation of OH-group:

Flavo-OH → Flavo-O·+·H

Heterolytic dissociation of OH-group:

Flavo-OH → Flavo-O· + e^-+H^+

The resulting flavonoid radical is stabilized through resonance conjugation of the unpaired electron with the π-electron system of the benzene ring. The stability of the flavonoid radicals is very important for their antioxidant activity, since the generated radical must have high stability and low reactivity in order to prevent the initiation of new free radicals in the system. The resulting stable flavonoid radical intercepts the chain reaction, giving various molecular products as a result. This is the so-called stage of interruption of the chain radical peroxidation [11, 12].

R· + Flavo-O·→ stable non-radical products

The antioxIdant activity of flavonoids depends on two main factors: (1) the ability of the hydroxyl groups to bind to the aromatic ring and to yield hydrogen atom, which then binds the free radicals and (2) the fact that the system can sustain an unpaired electron through delocalization of the π-electron system.

The flavonoids – inhibitors of singlet oxygen

The oxygen molecule is in triplet state ($^3O_2$) and has two unpaired electrons (one for each of the constituting oxygen atoms), which are with parallel spins [16]. Therefore, the oxygen molecule is a biradical. Nevertheless, the oxygen molecule has low reactivity in biological systems, containing predominantly molecules with dominating covalent bonds [16]. The oxygen molecule can be activated in biological systems through monovalent reduction, which yields in succession $O_2^{-\cdot}$, H_2O_2, HO·and$^1O_2$ [17]. The singlet oxygen ($^1O_2$) is formed by the triplet oxygen in some photosensibilization processes [18] and unlike the triplet one has greater oxidation potential and readily engages in reactions with biomolecules. Flavonoids can inactivate singlet oxygen through its physical transformation to the more stable triplet form or through direct interception of its molecule (scavenging effect) [10, 19].

The flavonoids - chelating agents of metal ions

It is well known that ions of transition metals (Fe^{2+}/Fe^{3+}, Cu^+/Cu^{2+}) catalyse the formation of free radicals, such as hydroxyl or hydroperoxyl radicals [20, 21]. Therefore, binding these ions in stable chelate complexes decreases their role in the initiation of free radical-forming processes. Thus, chelating agents are acting as preventive antioxidants [20]. Stable chelate complexes of flavonoids and transition metals are mostly formed at the catechol group.

MATERIALS AND METHODS

In the present study original data for representatives of 3 major classes of flavonoids in Bulgarian foods are presented - the data for the flavonols – myricetin, quercetin and kaempferol, the flavan-3-ols – (+)-catechin and (-)-epicatechin and flavones – luteolin and apigenin .

The food samples are collected according precise sampling plan and the origin of all foods has been documented. In the current study data for 15 Bulgarian fruits, 30 Bulgarian vegetables and vegetable products and 3 leafy green condiments are reported.

The following apparatus and analytical methods are used in the analysis of Bulgarian foodstuffs:

Apparatus

Hewlett Packard Liquid Chromatograph with HP pump 1050; thermostat: HP 1100; UV detector: HP 1050; fluorescent detector; injector: Rheodyne 750; ChemStation Software for data handling. The chromatographic analysis was performed by using Alltima (100 × 4.6 mm, i.d. 3μm) C18 analytical column, connected to pre-column Alltima (4×4.6 mm, i.d. 3μm) C18, Alltech Association Inc. Isocratic elution with 53% MeOH in 2% acetic acid, with a flow rate of 0.8mL/min was applied.

HPLC analysis of flavonols and flavones: myricetin, quercetin, kaempferol, luteolin and apigenein were measured as free aglucones after acid hydrolysis and HPLC determination with UV detection.

HPLC analysis of catechins: (+)-catechin and (-)-epicatechin were determined in freeze-dried samples by RP-HPLC and fluorescence detection at λ_{EX}=280 nm and λ_{EM}=315 nm.

The used foods composition tables and data are:

- Our data for flavonoid composition and quantity in Bulgarian fruit and vegetables [22, 23].
- USDA Database for the Flavonoid Content of Selected Foods – Nutrient Data Laboratory, USDA [24]. (S. Bhagwat, D. Haytowitz, J. Holden, 2014)
- Release 1 (March 2003) – Flavonoid content of 225 foods items.
- Release 3.1 (December 2013) The database contains values for 506 food items, covering 308 scientific literature papers and for 26

predominant dietary flavonoids that belong to the five subclasses reported below:

Flavonols: isorhamnetin, kaempferol, myricetin, quercetin;

Flavones: apigenin, luteolin;

Flavanones: eriodictyol, hesperetin, naringenin;

Flavan-3-ols: (+)-catechin, (+)-gallocatechin, (-)-epicatechin, (-)-epigallocatechin, (-)-epicatechin 3-gallate, (-)-epigallocatechin 3-gallate, theaflavin, theaflavin 3-gallate, theaflavin 3'-gallate, theaflavin 3,3'-digallate, thearubigins;

Anthocyanidins: cyanidin, delphinidin, malvidin, pelargonidin, peonidin, petunidin

On figure 1 the database comparison algorithm is presented. The usage of MS Office product for American Database /MSACCESS/, and Bulgarian Database/ MSEXECEL/, allows us to use Visual Basic for Application as a programming language to compare information in both bases. The main moment in the process is the compliance test. We have three levels of comparison:

First Level – Identification of the foodstuffs by names. On this level the names of the individual products in the bases are characterized and compared. The VBA function for the text comparison is used. Below is provided a program code:

```
Dim LResultAs Integer
LResult = StrComp ("BG DB Food/Fl Name", "USDA DB Food/FL Name")
```

We checked the result from comparison: if the LResult was equal to 0 then the first level test was passed and we shifted to second level; if the LResult was not equal to 0 the first level test did not pass and we finished with this Food/Fl and selected the next Food/Fl from USDA database.

Second Level – Identification by biological family, spices variety. The comparison is manual by using additional information from different scientific sources.

Third Level – Parameter and value check – The third level is crucial for the comparison of the two databases. We established a difference between BG Data and USDA Data in mg/100 g.

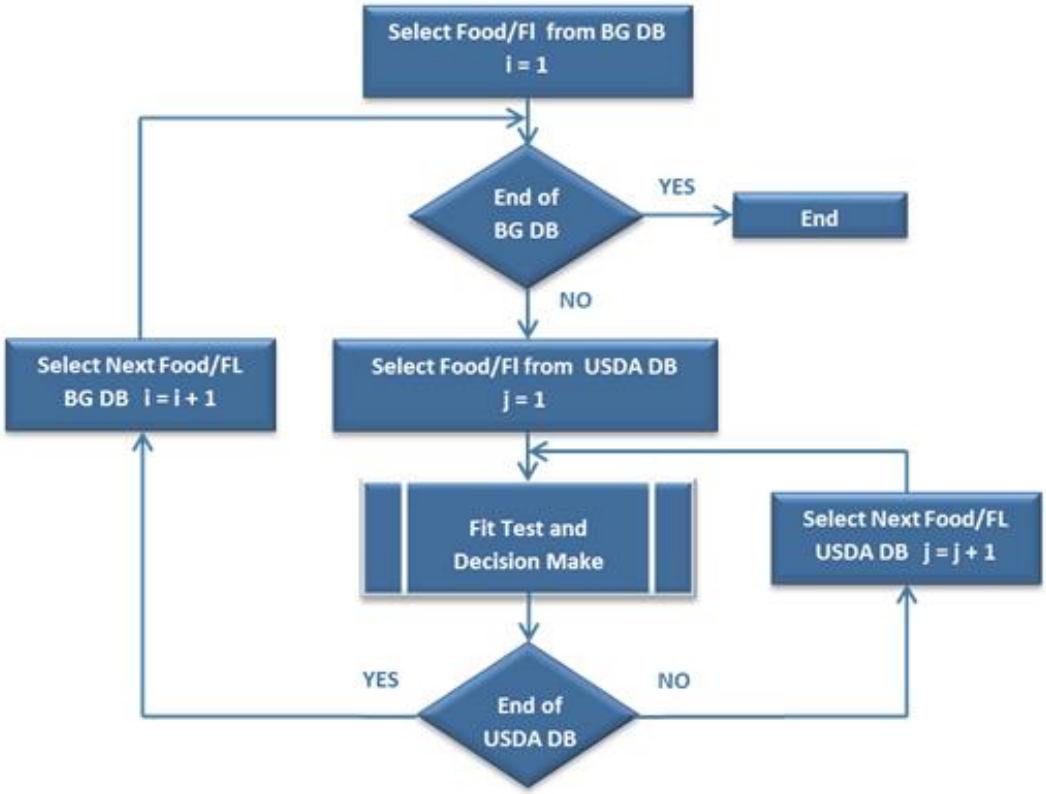

Figure 1. Database comparison scheme

RESULTS

The data of flavonoids composition in Bulgarian fruits are presented on Figure 2. The richest sources of flavonoids are blueberries. The myricetin, in significantly lower content was found only in blueberries. The comparison of our data and those, reported in USDA for the flavonoid content in fruits is presented on Figure 3.

Flavonoids in Bulgarian fruits

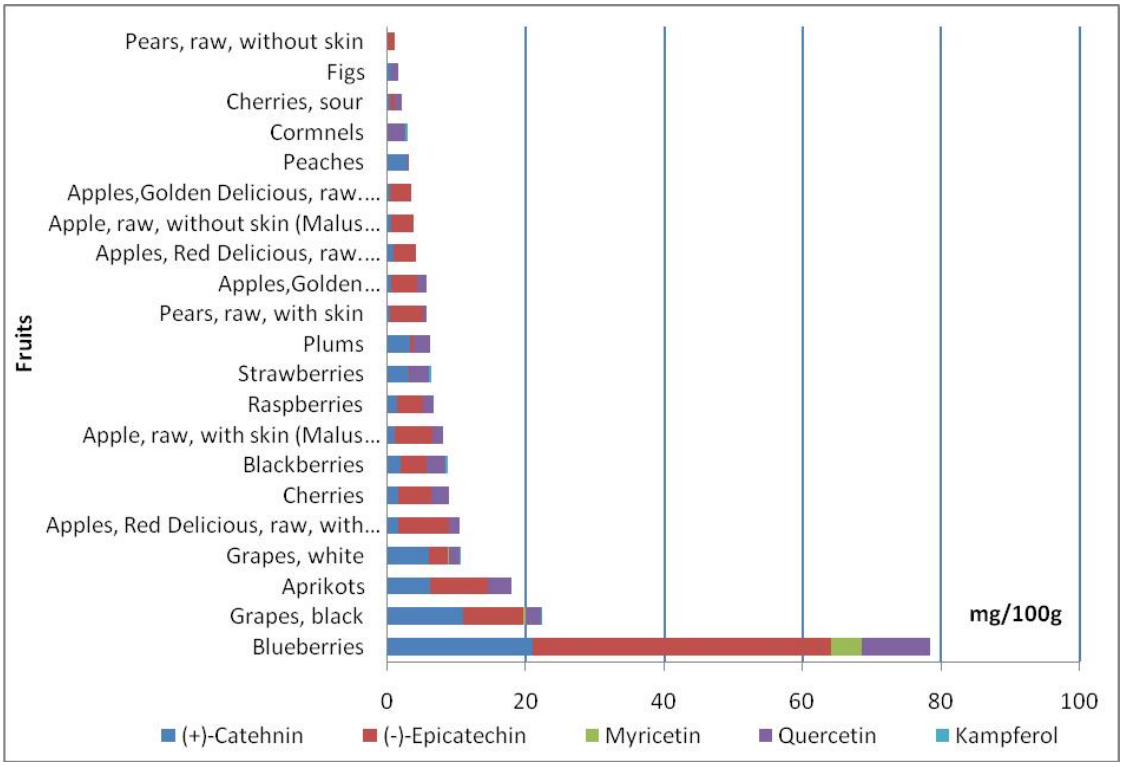

Figure 2. Flavonoids composition in Bulgarian fruits.

Flavonoids in Bulgarian vegetables

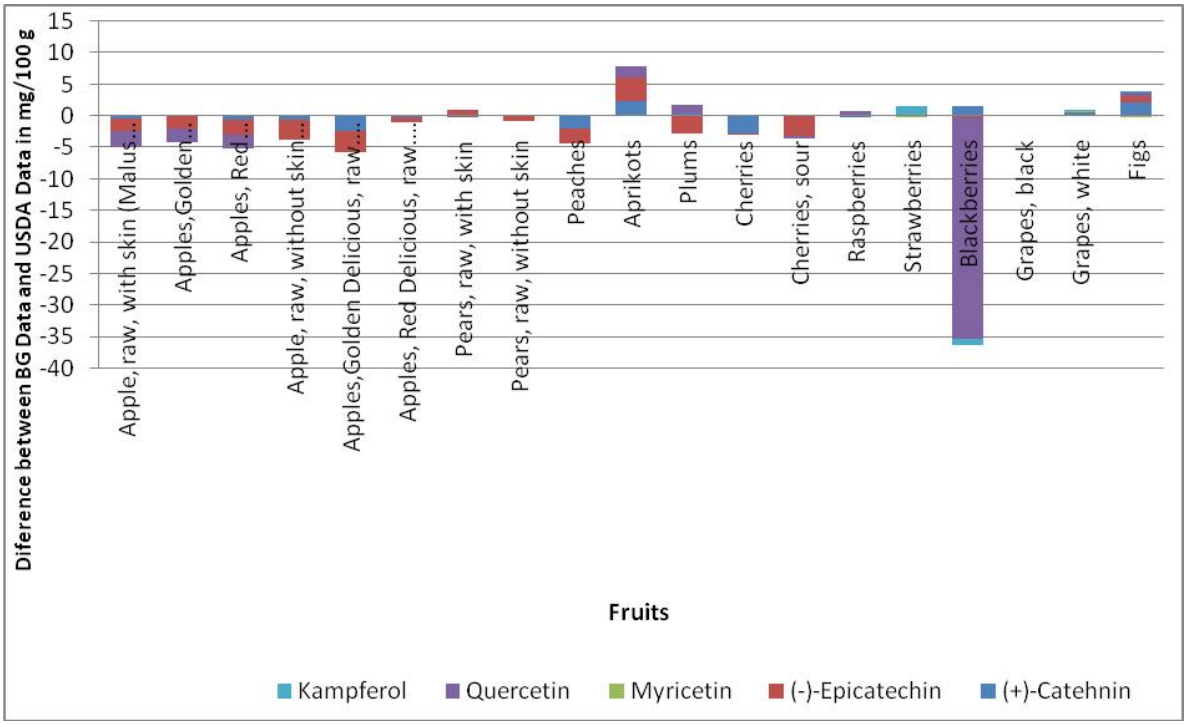

Figure 3. The comparison of Bulgarian and USDA data for the flavonoid content in fruits.

The results for flavonoids content in Bulgarian vegetables are shown in figure 4. We have to point out that the flavonol myricetin was not found in any of the vegetables analysed in our study, therefore data for myricetin were not included in figure 3. Very interesting results are the flavonoids data for parsley and dill. They show that parsley contains only one representative of flavonoids – apigenin, while dill is very rich in quercetin.

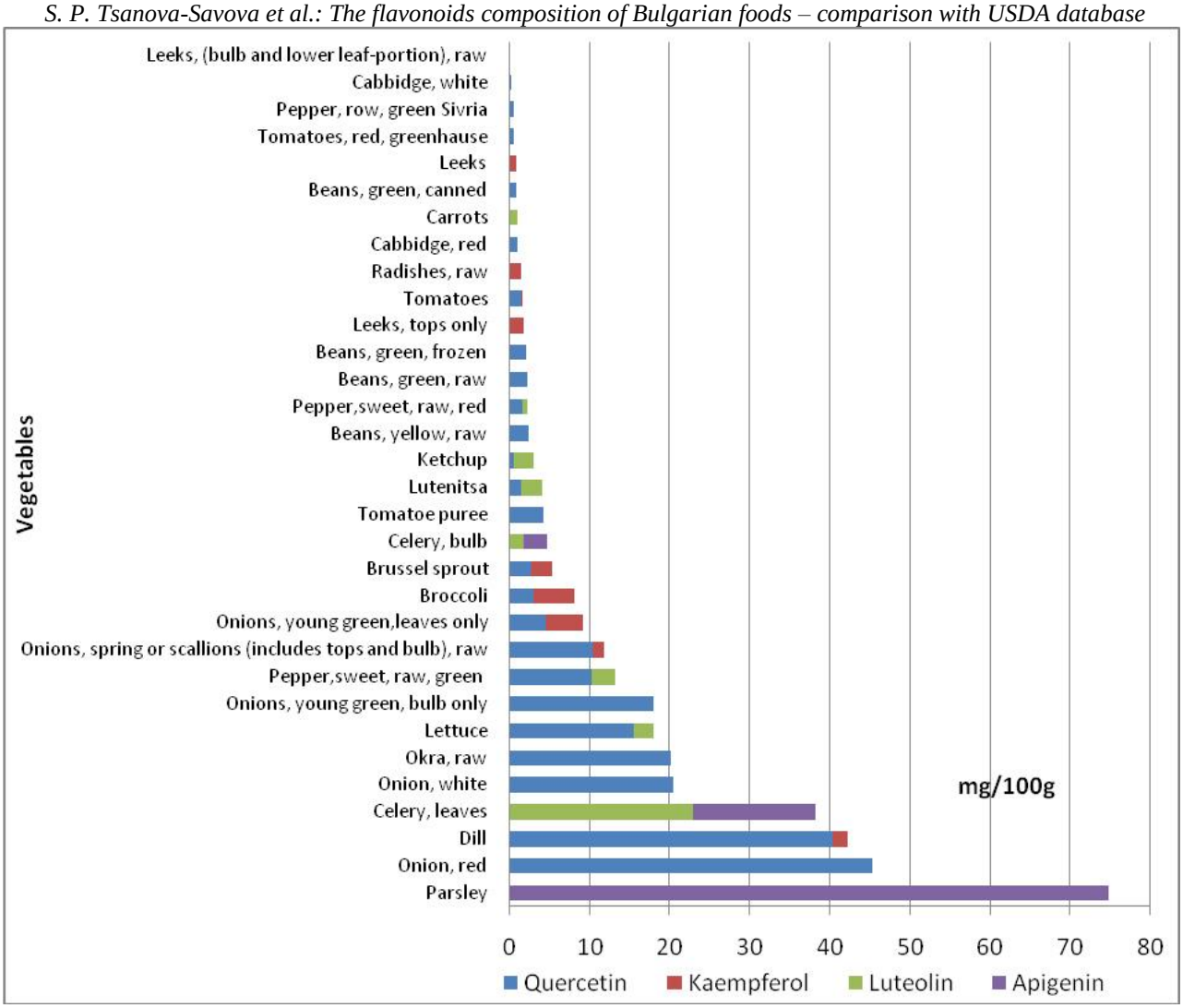

Figure 4. Flavonoids composition in Bulgarian vegetables..

The establishment of differences between our data for flavonoids in vegetables, and those in the USDA is presented in figure 5.

The determined differences between our data and those from USDA for flavonoid composition in fruits and vegetables can be due to various factors. In this aspect the biological variability of the plants products have the crucial role.

CONCLUSION

In the present study data of flavonoids content in Bulgarian fruits and vegetables, along with their comparison with the USDA Flavonoids Database are evaluated. Automation approach – VBA script for individual flavonoids' quantitative differences with three levels of data fit checks. The major differences between our data and the USDA ones were found for blackberries (- 35 mg/100 g) and parsley (-140 mg/100 g).

The current study revealed a number of topics whose further study would advance the development of nutritional databases. The validity of nutritional epidemiological studies depends on accurate food composition data. Nutritional database is an essential basic tool for virtually all quantitative nutritional research, dietary assessment, and development of food and nutritional policies. The nutritional data base programing should be an integral part of any national nutritional politic.

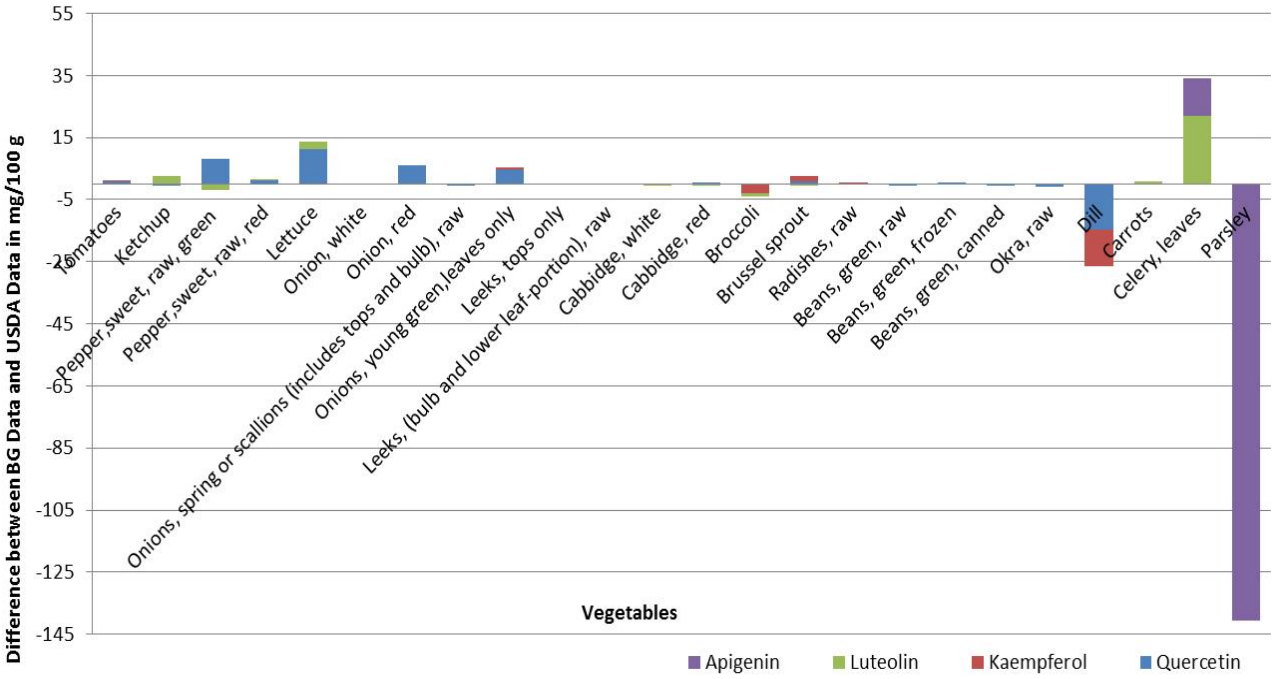

Figure 5. The comparison of Bulgarian and USDA data for the flavonoid content in vegetables

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REFERENCES

1. J. H. Von Elbe, J.S. Schwartz, Colorants in: Food Chemistry, O. R. Fennema, (ed.), Marcel Dekker, Inc., New York, 1996, p. 651.
2. M. G. L. Hertog, E. J. M. Fekens, P. C. H. Hollman, M. B. Katan, D. Kromhout, *Nutr. Cancer*, **22**, 175 (1994).
3. A. Cassidy, E. Rimm, E. O'Reilly, G. Logroscino, C. Kay, E. Stephanie, *Stroke*, **43**, 946 (2012).
4. A. Cassidy, K. J. Mukama, L. Lui, M. A. Franz, H. Eliassen, E. B. Rimm, *Circulation*, **127**, 188 (2013).
5. S. Rautiainen, S. Larsson, J. Virtamo, A. Wolk, *Stroke*., **43**, 335 (2011).
6. P. Knekt, R. Jarvinen, R. Seppanen, M. Heliovaara, L. Teppo, E. Pukkala, A. Arommaa, *Am. J. Epidemiol*., **146**, 223 (1997).
7. R. Garcia-Closar, C. A. Gonzales, A. Agudo, E. Riboli, *Cancer Causes Control*, **10,** 71 (1999).
8. R. Garcia-Closar, C. A. Gonzales, A. Agudo, E. Riboli, *Nutr. Cancer*, **35**, 212 (1999).
9. X. Gao, A. Cassidy, M. A. Schwarzschild, E. B. Rimm, A. Ascherio, *Neurology*, **78,** 1138, (2012).
10. S. Kumar, A. K. Pandey, *The Sci. W. J.*, **2013**, Art. ID 162750, (2013).
11. S. A. VanAsker, M. J. deGroot, D. J. vandenBerg, M. N. Tromp, G. Donne-OpdenKelder, W. vanderVijgh, A. Bast, *Chem. Res. Toxicol.*, **9**, 1305,(1996).
12. C. A. Rice-Evans, N. J. Miller, G. Paganga, *Free Radic. Biol. Med.*, **20**, 933 (1996).
13. Q. Meng, Z. Qian, X. Li, D. Li, W. Huang, J. Zhao, S. Li, *Acta Pharm. Sin. B.*, **2**, 246 (2012).
14. G. P. P. Lima, F. Vianello, C. P. Correa, R. A. da Silva Campos, M. G. Borguini, *Food Nutr. Sci.*, **5**, 1065 (2014).
15. Y. Y. Bilto, S. Suboh, T. Aburjai, S. Ablalla, *Natural Science*, **4**, 740 (2016).
16. J. A. Thomas, Oxidative stress, oxidation defense, and dietary constituents, in: M. E. Shils, J. A. Olson, M. Shike, W. Wilkins, (eds.), Modern Nutrition in Health and Disease, 8th ed., Lea &Febiger, 1994, p. 501.
17. C. Tournaire, S. Croux, M. T. Maurette, Y. Beck, M. Hocquaux, A. M. Braun, E. Oliveros, *J. Photochem. Protobiol. B.*, **19**, 205 (1993).
18. K. Huvaere, L. H. Skibsted, *J. Sci. Food Agric.*, **95**, 20 (2015).
19. N. Saewan, A. Jimtaisong, *J. Appl. Pharm. Sci.*, **3**, 129 (2013).
20. J. Terao, M. K. Piskula, Flavonoids as Inhibitors of Lipid Peroxidation in membranes, in: C. A. Rice-Evans, (Ed.), Flavonoids in Health and Disease. Lester Parker, University of California, California, 1998, p. 277.
21. A. Mirzaei, R. Khatami, *Bull. Env. Pharmacol. Life Sci.*, **2**, 27 (2013).
23. S. Tsanova-Savova, F. Ribarova, *Pol. J. Food Nutr.Sci*., **63**, 173 (2013).
22. S. Tsanova-Savova, F. Ribarova, M. Gerova, *J. Food Compos. Anal.*, **18**, 691 (2005).
24. S. Bhagwat, D.B. Haytowitz, J.M. Holden, 2014. USDA Database for the Flavonoid Content of Selected Foods, Release 3.1. U.S. Department of Agriculture, Agricultural Research Service. Nutrient Data Laboratory, http://www.ars.usda.gov /nutrientdata/flav

ФЛАВОНОИДЕН СЪСТАВ НА БЪЛГАРСКИ ХРАНИ - СРАВНЕНИЕ С БАЗАТА ДАННИ НА САЩ

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(Резюме)

Флавоноидите са полифенолни съединения с антиоксидантна активност от растителен произход, включващи повече от 5000 индивидуални съединения. Защитната им роля за човешкото здраве се изразява в намаляване на риска от развитие на редица дегенеративни заболявания. Количественото познаване на състава на флавоноидите в различните растителни храни е от решаващо значение за разработването на диети за здравословно хранене. Целта на изследването е да се оценят различията между съдържанието на флавоноиди в български храни и съдържанието на флавоноиди, предоставено в американската база данни. За постигането на поставената цел, българската база данни за флавоноиди в храни е преведена и прехвърлена в Excel формат, съгласно унифицираната схема за достъп до USDA. Сравнението на данните е автоматизирано, като се използва програма написана на Visual Basic for Application (VBA). Предоставени са оригиналните данни за представители на 3 основни класа флавоноиди в български храни. Данните за флавонолите - мирицетин, кверцетин и камферол, флаван-3-ол (+)-катехин и (-)-епикатехин и флавони - лутеолин и апигенин са определени чрез валидирани HPLC методи. Пробите са набирани съгласно прецизен план за вземане на проби и техният произход е документиран. В настоящото проучване са включени данни за 15 плода, 30 зеленчуци и зеленчукови продукти и 3 зелени подправки. Данните ни са сравнени с тези, предоставени в базата данни на USDA за съдържанието на флавоноиди в 506 храни, обхващащи 308 научни статии. Настоящото проучване разкри редица въпроси, чието по-нататъшно изучаване би подпомогнало изграждането на хранителните бази данни.

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Traditional Bulgarian food – source of antioxidant vitamins

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Many studies have shown that traditional foods are related to good health and longevity of population. The aim of the current study was to provide data on the content and biological importance of antioxidant vitamins: vitamin A (as beta-carotene), vitamin E and vitamin C in some traditional Bulgarian foods. 15 traditional Bulgarian foods from plant origin are included in the present study. The antioxidant vitamins were determined using HPLC methods. A comparative analysis of the obtained results with information from European and American databases was carried out. The highest values of the studied vitamins were determined in vegetables - red peppers and tomatoes, and in fruits - cherries and peaches, which are traditional for Bulgarian diet. Enhancement of antioxidant defenses through dietary antioxidant vitamins would seem to provide a more reasonable and practical approach for reducing the level of oxidative stress. The data provided on antioxidant vitamins levels in the studied products are a scientific evidence that traditional foods are sources of antioxidants, which through the diet contribute to Bulgarians health and longevity, by enhancing the body antioxidant defenses.

Keywords*:* Antioxidant; Traditional foods; Provitamin A; Vitamin E; Vitamin C.

INTRODUCTION

In recent decades science has provided a wide range of data concerning the role and importance of traditional foods for human health [1-4]. One of the main reasons for this attention to traditional foods is the powerful invasion of foreign cuisines today and new non-recognisable for the general population foods, which are available by the free movement of goods in the open global food market. We are witnessing a time when the every day nutrition list of Bulgarians is full of snacks, pizza, hamburgers and many others, but rarely includes traditional Bulgarian foods. This fact is a challenge for science of providing new data on the nutritional, historical and cultural significance of traditional nutrition and preserving their key position in the diet of Bulgarians.

Traditional foods are related to good health and longevity of population. These types of foods were the cause for the discovery of vitamin deficiencies in the early 20th century, and the pathogenesis of many diseases related to the diet of local populations from different regions of the world. In this aspect we are facing again a problem related to vitamin deficiencies, having a new form of impact on human health. There are numerous examples of vitamin D and vitamin B12 deficiencies which are considered as risk factors for the initiation of various diseases [5-8].

The scientific exploration of the link between food and health has led to the development of the *Antioxidant hypothesis* clarifying the importance of antioxidant ingredients in the diet for augmenting cellular defenses, and protecting components of the cell against oxidative damage. With regard to this concept, antioxidant vitamins have an important position in the defence mechanisms of the human body [9]. However, much more information about foods is needed including dietary antioxidants and other bioactive compounds. Initially, it may be necessary to study the food's antioxidants composition.

The aim of the current study was to provide data on the content and biological importance of antioxidant vitamins: vitamin A (as beta carotene), vitamin E (as alpha-tocopherol) and vitamin C in some traditional Bulgarian foods.

EXPERIMENTAL

15 traditional Bulgarian foods from plant origin are included in the present study. The food sample collection was performed on a randomized basis. The analytical samples were prepared by mixing three individual samples for each product. All requirements related to food sampling were covered. The antioxidant vitamins were determined using HPLC methods with a Perkin Elmer chromatographic system - Series 4. In the examined foods, vitamin A is mainly present as beta-carotene (provitamin A), determined under the following chromatographic conditions: reverse-phase C18 column; mobile phase - acetone:water (100:5, v/v); flow rate - 0.8 ml/min; temperature - 35 °C; UV/VIS detection at 450 nm. The parameters of the method are: detection limit (LOD): 0.6 μg/100g; limit of quantification (LOQ): 1.8 μg/g; analytical

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yield - 96%; relative standard deviation (RSD) - 3.2%.

Vitamin E was determined using a method for determination of both vitamins A (retinol) and E (alpha-tocopherol) under the following chromatographic conditions: reverse phase C18 column; mobile phase – 97 % methanol; flow rate – 1 ml/min; detection - fluorimetric: Ex = 285 nm, Em = 345 nm. The characteristics of the method are: detection limit (LOD) - 0.2 μg/100 g; limit of determination (LOQ) - 0.6 μg/100 g; analytical yield 98%; relative standard deviation (RSD) - 3.9%.

The following chromatographic conditions were used in the vitamin C determination: reverse phase C18 column; mobile phase - methanol/buffer (pH = 5.5) (35:65, v/v); flow rate - 0.8 ml/min; temperature - 35 °C; detection - UV-254 nm. The parameters of the method are: detection limit (LOD) - 0.02 mg/100 g; limit of determination (LOQ) - 0.05 mg/100 g; analytical yield – 92%; RSD - 1.9%.

A comparative analysis of the obtained results with information from European and American databases for food composition was carried out [10,11].

Table 1. Antioxidant vitamins in traditional Bulgarian foods and in different databases

Foods	Provitamin A, RE μg			Vitamin E (alpha-tocopherol), mg			Vitamin C, mg		
	BG	USA	Danish	BG	USA	Danish	BG	USA	Danish
Onions	0	0	30	0.43	0.02	0.07	11.7	7.4	8.19
Garlic	0	0	0	0.01	0.08	0.01	29.3	31.2	8.21
Tomato, red, ripe, raw	114	42	992	0.34	0.5	1.1	17.8	13	15
Pepper, sweet, green, raw	41	18	167	0.7	0.37	0.55	102	80.4	104
Pepper, sweet, red, raw	297	157	1270	2.94	1.58	2.9	143	127.7	191
Cabbage, white, raw	13,1	5	35	1,69	0,15	0.02	47.8	36.6	45.8
Beans, white	3.6	0	13	-	0	0.34	0	0	2.04
Lentils	10.3	2	60	-	0,49	0	0	4.5	0
Apple	5.05	2	25	0.61	0.18	0.55	5.8	4.6	7.7
Pears	2.03	1	65	0.71	0.12	0.7	4.1	4.3	4.9
Plums	45.3	17	80	0.55	0.26	0.4	13.4	9.5	5
Cherries, sour	142	64	69	0.14	0.07	0.2	11.1	10	10
Peaches, yellow	57.5	16	70	1.82	0	1.8	7.1	6.6	6.6
Grapes	6.3	3	33	0.4	0.19	0.4	9.7	3.2	10.8
Quince	4.4	2	24	0	0	0	16.5	15	15

RESULTS AND DISCUSSION

Table 1 presents the results from the analysis of the three antioxidant vitamins (vitamins A, E and C) in the tested Bulgarian traditional foods. Vitamin A is presented as provitamin A (beta-carotene). Corresponding data from Danish and USA databases are also presented [10,11]. The assessment of our results showed higher levels of beta-carotene in vegetables traditional for Bulgarian diet - red peppers and tomatoes, followed by green peppers and cabbage. Fruits with higher levels of beta-carotene are cherries and peaches, followed by prunes. The results for both groups of fruits and vegetables show closer values to the USA database, but they are significantly different from the Danish data. Beta-carotene can directly scavenge free radicals, which determines its antioxidant activity. It is recommendable to take beta-carotene in its natural form with foods and not as a synthetic product, since the *cis*-form contained in natural foods acts as an antioxidant, while the *trans*-form

has a prooxidant activity. Another important biological activity of beta-carotene is its role as a precursor to vitamin A [9].

Numerous studies have demonstrated the antioxidant activity of vitamin E in foods from plant origin. In the present study, higher levels of vitamin E in red peppers were found. This result shows that red peppers are a great source for vitamin E not only for the traditional Bulgarian diet, but also for Europeans and Americans. Antioxidant activity of vitamin E is related to its ability to quench free radicals and break oxidation chains, which determines its important role in the body's antioxidant protection.

Similar data for vitamin C are shown in the three groups according to the type of products. Traditional Bulgarian products - peppers and cabbage are with the highest level of vitamin C. Its primary role in antioxidant protection is the recovery of the activity of oxidized forms of vitamin E in the process of lipid peroxidation.

Enhancement of antioxidant defense through dietary antioxidant vitamins would seem to provide a more reasonable and practical approach for reducing the level of oxidative stress.

A number of factors, including genetics, and growing conditions can affect the spectrum and quantity of antioxidant vitamins in plant foods. These differences need to be considered in developing a database of food antioxidants. Due to the lack of a standard assay, it is difficult to compare our results with the data from different studies. However, it is of great interest to the food science experts and medical researchers to know the antioxidant composition and capacity of the local or national foods.

CONCLUSION

The established differences between our data and the data in both databases on the one hand and the differences between the two foreign databases on the other, show the necessity to build national food composition tables as they depend on the geographical origin, the impact of specific environmental conditions, harvesting and preservation of the crop, as well as on the way of production and analysis of the food products.

The data provided on antioxidant vitamins levels in the studied products are a scientific evidence that traditional foods are sources of antioxidants, which through the diet contribute to Bulgarians health and longevity, by enhancing the body antioxidant defense. The data can be used for building preventive and healthy nutrition.

REFERENCES

1. U. Spiekermann, *Forum of Nutrition*, **57**, 11 (2005).
2. F. G. P. Costa, S.A.N. Morais, E. T. Noguira, M. Kutschenko, C.C. Goulart, R. C. L. Neto, C. F. S. Oliveira, V. P. Rodrigues, M. R. Lima, *European Journal of Clinical Nutrition*, **73**, 5 (2010).
3. T. Sanches-Silva, G Albuquerque, P. Finglas, T. Ribeiro, A. Valente, E. Vasilopoulou, A. Trichopoulou, I. Alexieva, N. Boyko, C.-E. Costea, O. Hayran, M. Jorjadze, L. Kaprelyants, D. Karpenko, L. F. D'Antuono, H. S. Costa, *Journal of the Science of Food and Agriculture*, **93,** 3545 (2013).
4. F. Danesi, F. Pasini, M. F. Caboni, L. F. D' Antuono, A. Bodoni, *Journal of Food and Agriculture*, **93**, 3595 (2013).
5. F. O'Leary, S. Samman, *Nutrients*, 299, 2, (2010);
6. J. M. Sacheck, M. I. Van Rompay, V. R. Chomitz, C. D. Economos, M. Eliasziw, E. Goodman, C. M. Gordon, M. F. Holick , *J. Clin. Endocrinol. Metab.* **102**, 4496 (2017).
7. C. A. D'Amore, F. Marsico, A. Parente, S. Paolillo, F. De Martino, P. Gargiulo, F. Ferrazzano, A.M. De Roberto, L. La Mura, C. Marciano, S. Dellegrottaglie, B. Trimarco, P. Perrone Filardi, *Nutr. Metab. Cardiovasc. Dis.*, **27**, 837 (2017)
8. B. Stefanowski, A.Z. Antosik-Wójcińska, Ł. Święcicki, *Psychiatr. Pol.*, **51**, 437 (2017).
9. F. Ribarova, Foods and Vitamins, AEM AMS, Sofia, 2007.
10. USDA Food Composition Database, https://ndb.nal.usda.gov/ndb/search/list
11. The official Danish Food Composition Database, www.foodcomp.dk/fcdb_default.asp.

ТРАДИЦИОННИ БЪЛГАРСКИ ХРАНИ – ИЗТОЧНИЦИ НА АНТИОКСИДАНТНИ ВИТАМИНИ

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(Резюме)

Много проучвания показаха значението на традиционните храни за доброто здраве и дълголетието на популацията. Целта на настоящото проучване бе да предостави данни за съдържанието и биологичната значимост на антиоксидантните витамини: витамин А (като бета-каротен), витамин Е и витамин С в някои традиционни български храни. В настоящото проучване са включени 15 традиционни български храни с растителен произход. Количествата на антиоксидантните витамини са определени чрез използване на HPLC-методи. Получените резултати са сравнени с данните в Европейска и Американска база данни. Най-високи нива на изследваните витамини са установени при зеленчуците за червени чушки и домати, а при плодовете – за череши и праскови, които са типични традиционни храни в диетата на българина. Повишаването на антиоксидантната защита на организма чрез антиоксидантните витамини в храната е подходящ практичен подход за редукция на оксидативния стрес. Предоставените данни за антиоксидантните витамини в изследваните продукти са научно доказателство, че традиционните храни са източници на антиоксиданти, които чрез диетата на българина, допринасят за неговото здраве и дълголетие, повишавайки антиоксидантната защита на организма.

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The naturally derived "Stoletnik" dairy product as a model for nutraceutical traditional food

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Current tendency for improving quality of live led, among all, to a demand for novel food products with beneficial effect on human health. The key requirement is that these products are either of natural origin or produced through organic farming and with as little processing as possible. Major sources for such products are the traditional regional recipes – ethnic foods. These recipes are an extract of a long-term experience (decades or even centuries) and reflect local biodiversity as well. Sporadic research on their composition and properties revealed significant beneficial effects on human health, sometimes in unexpected areas. These preliminary studies place the traditional foods as a promising new source for high quality healthy food products. In this paper we made an attempt to outline the beneficial properties of a traditional dairy product "Stoletnik" using the latest scientific knowledge in the field. Its traditional recipe was successfully converted into an industry-grade technology and was implemented in a small-scale production.

Keywords: Lactobacillus, Functional food, Nutraceuticals, Yogurt, Cheese, Curd, Dairy product

INTRODUCTION

The extensive advance during the last three decades in the fields of biochemistry, molecular biology, genetics and human physiology brought to existence a new class of food products called 'functional foods'. A significant part of this newly formed class consists of local or ethnical food products – a direct result of culture interchange on a global scale.

There is no single definition of the term 'functional food'. Currently adopted definition, debated at the 9th International Conference on "Functional Foods and Chronic Diseases: Science and Practice" at the University of Nevada, Las Vegas on March 15-17, 2011 states that: "Functional Food is a natural or processed food that contains known biologically-active compounds which, when in defined quantitative and qualitative amounts, provides a clinically proven and documented health benefit, and thus, an important source in the prevention, management and treatment of chronic diseases of the modern age" [1].

The medicinal values of certain food ingredients allow such products to be qualified as nutraceuticals or food medicines [2]. The term covers a wide range of plants (vegetables, fruits, spices) as examples of nutraceuticals. These products have found their place not only in food industry but also in the pharmaceutical production.

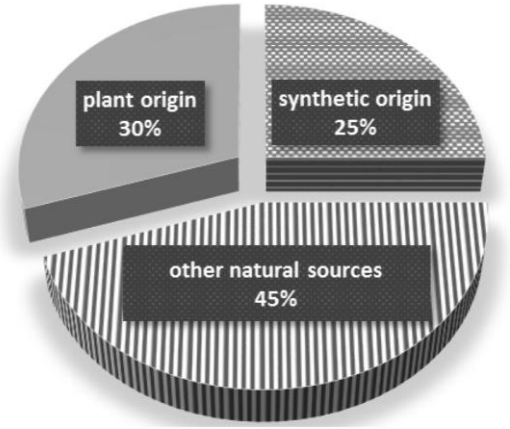

Figure 1. Ratio of natural and synthetic sources in the new chemical compounds [3].

Since 1981 to 2010 two thirds of the active agents in the new chemicals originate from natural sources (Figure 1) [3].

Origins of the "Stoletnik" dairy product

"Stoletnik" ("Centenarian") is a contemporary dairy product fulfilling the definition of functional food. It took nearly 30 years for its development by the late Prof. Hristo Chomakov on the basis of a traditional recipe from Rhodope Mountains region in Bulgaria. During the last five years the thorough descrip tion of the product resulted into small-scale industrial production by a dairy company located in the same region – "Rodopa Milk".

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"Stoletnik" is among the best examples of a food product, combining Bulgarian biodiversity – lactic acid bacteria as probiotics and plant additives as prebiotics. In 2017 "Stoletnik" was recognized as a Utility Model by the Patent Office of the Republic of Bulgaria (2693 from 13.09.2017).

Ingredients of the "Stoletnik" dairy product – brief description of their properties.

All "Stoletnik" ingredients are of natural origin. The main ingredients of "Stoletnik" are yogurt, white brined cheese, curd (1:1:1) along with small amounts of onions, ground red pepper and unrefined sunflower oil. Since this product relies on fermentation, another important component is its microflora. The microflora consists of viable and active cells of probiotic bacteria — *Lactobacillus delbrueckii* ssp. *bulgaricus (Lb. bulgaricus), Streptococcus thermophilus (Str. thermophiles), Lactobacillus casei (Lb. casei), Lactobacillus plantarum (Lb. plantarum)* at 10^8cfu.g^{-1}.

Bulgarian yoghurt is a biologically transformed dairy product resulting from the activity of *Lb. bulgaricus* and *Str. thermophilus*. The product contains not only a large number of lactic acid bacteria in the order of 10^8cfu.g^{-1}, but also a rich assortment of biologically active components. Recently it was found that viable cells of *Lb. bulcaricus* possess high cytotoxic activity against the cancer cell line HT-29, which reaches 81%, best expressed after 48 hours of fermentation [4]. Cytotoxic activity is also possessed by the peptides derived from the milk proteins, hydrolysed by *Lb. bulgaricus*. This cytotoxic activity varies between 0.96% and 41.25% in the case of the curd protein lactoferricin. Also, during lactic acid fermentation, *Lb. bulgaricus* hydrolyses x-casein, liberating a peptide with strong anti-oxidative properties [5]. In that way Bulgarian yogurt protects the individual from the every-day oxidative stress, closely associated with diseases such as cancer, atherosclerosis and diabetes [6].

The decomposition of viable cells of *Lb. bulgaricus* and *Sir. thermophilus* in the intestinal tract stimulates the activity of B-cells and the synthesis of IL-6, resulting in increased secretion of dimeric IgA. The production of IgA offers protection to the mucus layer of the intestinal tract, which is the most important health- promoting effect of Bulgarian yogurt. During lactic acid fermentation proteins are hydrolyzed into fragments, which strongly stimulate the growth of bifidobacteria [7]. The unhydrolysed milk sugar in yoghurt is another important bifidogenic factor [8]. Bifidobacteria, in turn, enhance the synthesis of anti-viral IgA and decrease the risk factors of carcinogenesis.

Ripe white brined cheese is the second basic ingredient of the product. White brined cheese is a fermented dairy product with high nutritional value determined by the components of milk and the metabolites formed during the ripening performed by lactic acid bacteria. White brined cheese has a number of health-beneficial effects on the organism. This product is a source of biologically active peptides with anti-hypertensive and anti-oxidative activity, as well as probiotic bacteria *Lb. casei* and *Lb. plantarum*. The product contains anti-carcinogenic compounds and a large quantity of biologically available calcium. Data demonstrating the stimulating effect of *Lb. plantarum* on the synthesis of a biologically active component TRAIL are of special interest with respect to the anti-tumor properties of white brined cheese [9]. TRAIL is a chromium-containing ligand and is endogenously synthesized as a membrane protein or as soluble protein in the lymphocytes [10]. TRAIL plays an essential role in the suppression of carcinogenesis since insufficient expression enhances the malignant character of tumors in mice [11]. *Lb. plantarum* induces TRAIL synthesis and increases the activity against cancer cells through induction of endogenous TRAIL synthesis by immunity cells. The synthesis of the TRAIL component stimulated by *Lb. plantarum* elevates the activity of the natural killer cells, which effectively attack malignant tumor cells both *in-vitro* and *in-vivo* [9]. The same study reveals a notable biological property of *Lb. plantarum* — the induction of TRAIL synthesis is not strain-specific, which is otherwise the case for the most of the activities of probiotic bacteria. The other representative of probiotic bacteria, *Lb. casei*, also has anti-tumor activity *in-vitro* and *in-vivo* [12].

Cheese is a rich source of biologically active peptides with various biological effects on the organism such as anti-oxidative, anti-microbial, anti-hypertensive, immunomodulating and anti-carcinogenic activities [13]. Special attention is drawn to the anti-oxidative properties of cheese with respect to the prevention and control of the oxidative stress on the organism. There is a strong correlation between the anti-oxidative activity and extent of cheese ripeness. The peptides formed during ripening are the anti-oxidative components in the cheese. *Lb. casei*, which is contained in the product, has the strongest anti-oxidative properties among all lactic acid bacteria and decreases and in particular cases eliminates the daily oxidative stress of the organism. At the same time *Lb. casei* reduces cholesterol levels, modulates the immune system and is a powerful antagonist of *Escherichia coli* [14]. The cheese is a rich source of biologically available calcium, which is absorbed better that calcium from

milk [15]. Calcium reduces the risk of colon cancer. The intake of substantial amounts of calcium with cheese and the presence in the product of potassium, magnesium and biologically active peptides has a very favorable effect on the lowering of blood pressure of the individual.

Cheese contains other anti-carcinogenic components such as conjugated linoleic acid (CLA), sphingolipids and biologically active peptides. Conjugated linoleic acid with its two biologically active forms cis-9 and trans-11 possesses well expressed anti-mutagenic properties. The two forms of CLA suppress carcinogenesis by modulating the proliferation of cells and the anti-oxidative mechanism [16]. The intake of linoleic acid results in lowering of serum cholesterol in blood, especially the low density cholesterol. At the same time the level of high density cholesterol is increased. The concentration of triglycerides in blood serum is lowered, which is related to reduction of the risk of cardio-vascular disease and heart attack. Conjugated linoleic acid in the organism can be increased by consuming dairy products including cheese with elevated content of CLA. In this respect milk obtained by animals grazing in mountain planes of over 1000 meters above sea level is of great interest. In the milk obtained from such animals the content of conjugated linoleic acid is two to three times higher than in the milk of animals grazing in lowlands. In France, Switzerland, Germany and Austria the milk of these animals is processed into yoghurt and different varieties of cheese. These products are offered to tourists, organized in so-called preventive vacations for two weeks in the respective region. The tourists consume these dairy products daily. In that way healthy nutrition is widely applied as preventive means against cardiovascular disease and cancer.

Sphingolipids are an interesting group of lipids related to cancer prevention. They express anti-carcinogenic properties upon consumption [17]. With respect to the anti-tumor properties of white brined cheese the application of a selected starter of *Lb. bulgaricus* and *Str. thermophilus* for the production of this product is of particular interest. Based on the cytotoxicity of *Lb. bulgaricus* on cancer cells we have enough evidence to suppose that cheese prepared with such a starter should possess enhanced anti-tumor properties compared to products from different lactic acid bacteria starters.

Curd and curd proteins are unique food ingredients. They possess not only high nutritional value, but also important biological properties with respect to improvement of health and protection of the organism from diseases [18]. Curd proteins comprise a-lactalbumin, 13-lactoglobulin, lactoferrin, immunoglobulins, serum albumin, lactoferrin and other micro-components. These proteins are hydrolyzed by the proteases in the stomach and the pancreatic juices — mainly trypsin /chymotrypsin/ and the microorganisms. The hydrolysis results in the formation of biologically active peptides which play essential physiological role in the organism [19]. Curd proteins increase the pH of the medium to 6.2-6.5 and facilitate the survival of yogurt microflora — *Lb. bulgaricus* and *Str. thermophilus* in the presence of pepsin and low pH in the stomach (pH 1.5- 3.0). The matrix of curd proteins increases the tolerance of *Lb. bulgaricus* and *Str. thermophilus* to bile salts and strongly influences their survival in the intestinal tract. The digestion of 13-lactoglobulin by trypsin liberates a peptide with strong anti-hypertensive activity [19]. Among curd proteins α-lactalbumin is a potent anti-oxidant and a natural anti-tumor component which causes apoptosis of tumor cells. Also, α -lactalbumin stimulates the synthesis of mucin and strengthens the defense of the intestinal epithelium against pathogenic microorganisms.

Lactoferrin is another biologically active component of milk and curd with anti-carcinogenic properties [20, 21]. Under the hydrolyzing activity of pepsin in the intestinal tract [22] the peptide lactoferricine is formed, possessing strong anti-carcinogenic activity [23, 24]. Lactoferricine treatment of 5 weeks of duration substantially decreases proliferation of cancer cells; lactoferricine prolongs the S-phase of the cancer cell division cycle, resulting in decrease of tumor cell numbers. This prolongation of the cell division cycle lowers cancer risk as cancer development depends on the speed of cancer cell proliferation. When cancer develops, food components can slow down the growth of tumor cells. Possibly the decrease in the speed of cancer cell proliferation is the basic mechanism of lowering cancer risk by milk consumption. Lactoferricine has also strong anti-microbial activity and well expressed cytotoxic effect on cancer cells, which reaches 41%.

Curd protein plays an essential role in bone growth in the organism. Curd proteins stimulate the osteoblasts, which produce osteocalcin, the only one bone-specific protein [25]. Curd protein increases bone density in the organism and facilitates fast healing of surgery wounds. In this respect curd protein can serve as a potential therapeutic product in osteoporosis treatment or as local aid for fast treatment of fractures.

Ingredients of plant origin of the "Stoletnik" are fresh onions, ground dried red pepper and unrefined sunflower oil. These three ingredients provide phytosterols, inulin, compounds with phytoncide

activity, and a number of secondary metabolites. These components act synergically with the main ingredients and improve health- beneficiary properties of the product.

Phytosterols are naturally occurring plant sterols, which are structural analogues of cholesterol. They decrease cholesterol absorption in the intestinal tract, and especially influence the levels of low-density serum cholesterol [26].

The native inulin, contained in the product, stimulates the growth of health-beneficial intestinal microbiota — bifidobacteria, *Lb. casei*, *Lb. plantarum* and facilitates the survival of *Lb. bulgaricus* and *Str. thermophilus* in the intestinal tract. Inulin increases calcium absorption and stimulates the functioning of the immune system. Unlike other fermented milk products, in which the number of lactic acid bacteria decreases during storage, in "Stoletnik" the number of lactic acid bacteria increases and they are preserved in active state.

The presence of phytoncides serves as a biological means for preservation of the quality of the product and increasing the defense strength of the organism. The added ground red pepper is a rich source of potassium, which together with calcium, magnesium and biologically active peptides has very favorable influence on high blood pressure values in the organism. The question of the synergism of the separate food components in the product on the physiological processes in the organism remains open. Up to now it was found that mixing casein with curd proteins strongly increases antibody production in the organism. Possibly it is the synergism between the product ingredients that is responsible for its very good effect on the endothelial function of blood vessels.

The processing of the components is presented in Figure 2 as a simplified technological map.

All steps can be performed in standard dairy industry environment and do not require specialized equipment or unusual procedures. Final product can be stored for 96 h at 4-6°C.

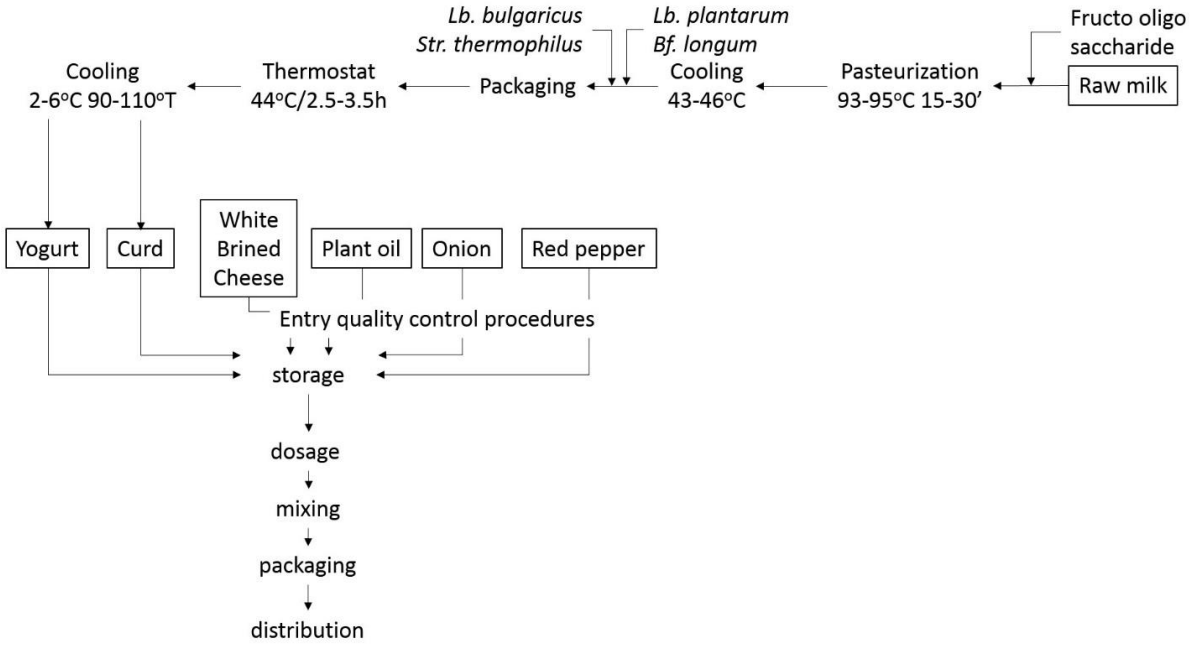

Figure 2. Simplified technological map of "Stoletnik" production.

CONCLUSION

The developed functional product "Stoletnik" contains a rich assortment of biologically active peptides with anti-hypertensive, anti-microbial, anti-tumor and immunomodulating properties The product optimizes the functioning of the immune system; increases IgA secretion in the intestinal tract; protects the individual from infections; makes the organism more resistant to diseases; contains large quantities of anti-oxidants and protects the organism from every-day oxidative stress; improves heart health; expresses high cytotoxicity to cancer cells. The product maintains the balance of intestinal microflora — a decisive condition for the health of the individual. Furthermore, the product facilitates the decrease of the total content of cholesterol, low-density cholesterol and triglycerides in blood serum; stimulates the cytotoxicity of immunocytes and activates the natural killer cells; improves bone density; enhances the healing of surgery wounds and fractures; improves the endothelial functions of the blood vessels. The product optimizes the physiological processes in the organism, improves

health, decreases the risk of diseases and ensures healthy aging.

"Stoletnik" is an example of successful conversion of a local home-made dairy product into an industrial technology. Present knowledge on its properties and the role of main ingredients is a good starting point for further research aiming at the development of novel variants with improved composition, properties and better preventive effect on human health. Current work on this topic will result in a set of modifications targeting different consumer groups.

REFERENCES

1. D.M. Martirosyan (ed.), Functional Foods and Chronic Diseases: Science and Practice. Food Science Publisher, 2011
2. V. Brower, *Nat. Biotechnol*., **16**, 728 (1998).
3. D.J. Newman, G.M. Cragg, *J. Nat. Prod.*, **75**, 311 (2012).
4. C. Remilland, L. Ozimek, *Milchwiss*., **63**, 424 (2008).
5. K. Igoshi, Y. Kondo, H Kobayashi, N. Rabata, H. Kawakami, *Milchwiss*., **63**, 424 (2008).
6. M. Yakovlieva, T. Tacheva, S. Mihaylova, R. Tropcheva, K. Trifonova, *Benef. Microbes*, **6**, 505 (2015).
7. T. Vasiljevich, P. Shah, *Int. Dairy J.*, **18**, 456 (2008).
8. G. Schaafsma, *Int. Dairy J.*, **18**, 714 (2008).
9. M. Horinaka, T. Yoshida, A. Kishi, K. Akatani, T. Yasuda, J. Kouhara, M. Wakada, T. Sakai, *FEBS Lett*., **584**, 577 (2010).
10. I. Monleon, M. J. Martínez-Lorenzo, L. Monteagudo, P. Lasierra, M. Taulés, M. Iturralde, A. Piñeiro, L. Larrad, M. A. Alava, J. Naval, A. Anel, *J. Immunol*., **167**, 6736 (2001).
11. N. Zerafa, J. A. Westwood, E. Cretney, S. Mitchell, P. Waring, M. Iezzi, M.J. Smyth, *J. Immunol*., **175**, 5586 (2005).
12. A. Takagi, T. Matsuzaki, M. Sato, K. Nomoto, M. Morotomi, T. Yokokura, *Med. Microbiol. Immunol*., **188**, 111 (1999).
13. B. Walther, A. Schmid, R. Sieber, K. Wehrmüller, *Dairy Sci. Technol.*, **88**, 389 (2008).
14. W. Ylcheng, Z. Guo, Q. Zhang, L. Yan, Y. Ohen, X. C. Xiao-Minglin, W. Chen, H.-P. Zhang, *Int. J. Dairy Technol.*, **63**, 105(2010).
15. K. Kato, Y. Takada, H. Matsuyama, Y. Kawasaki, S. Aoe, H. Yano, Y. Toba, *Biosci. Biotechnol. Biochem.*, **66**, 2342 (2002).
16. M. A. Belury, *J. Nutr.*, **132**, 2995 (2002).
17. H. Vesper, E. M. Schmelz, M. N. Nikolova-Karakashian, B. L. Dillehay, D. V. Lynd, A. H. Merrill, *J. Nutr*., **129**, 1239 (1999).
18. A. R. Madureira, C. I. Pereira, A. M. P. Gomes, M. E. Pintado, F. X. Malcata, *Food Res. Int.*, **40**. 1187 (2007).
19. A. R. Madureira, T. Tavares, A. M. Gomes, M. E. Pintado, F. X. Malcata, *J. Dairy Sci.*, **93**, 437 (2010).
20. N. van Belzen, *Sciences des Aliments*, **22**, 461 (2002).
21. S. Min, J. M. Krochta, *J. Food Sci.*, **70**, M87 (2005).
22. W. Bellamy, M. Takase, K. Yamauchi, H. Wakabayashi, K. Kawase, M. Tomita, *Biochim. Biophys. Acta*, **1121**, 130 (1992).
23. H. Wakabayashi, M. Takase, M. Tomita, *Curr. Pharm. Res.*, **9**, 1277 (2003).
24. J. S. Mader, A. Richardson, J. Salsman, D. Top, R. de Antueno, R. Duncan, D. W. Hoskin, *Exp. Cell Res.*, **313**, 2634 (2007).
25. R. Xu, *J. Dairy Sci.*, **92**, 3014 (2009).
26. S. B. Racette, X. Lin, M. Lefevre, C. A. Spearie, M. M. Most, L. Ma, R. E. Ostlund Jr., *Am. J. Clin. Nutr.*, **91**, 32 (2010).

ПРИРОДНИЯТ МЛЕЧЕН ПРОДУКТ „СТОЛЕТНИК" КАТО МОДЕЛ ЗА ТРАДИЦИОННА ЛЕЧЕБНА ХРАНА

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(Резюме)

Съвременната тенденция за подобряване качеството на живота доведе до необходимост от нови хранителни продукти с благоприятно влияние върху човешкото здраве. Основното изискване към тези продукти е да бъдат от естествен произход или да са произведени чрез органично земеделие и възможно малка обработка. Основните източници на такива продукти са традиционните местни рецепти – т. нар. етнохрани. Тези рецепти са плод на продължителен опит (десетилетия и дори столетия) и отразяват местното биоразнообразие. Спорадични изследвания върху състава и свойствата им са разкрили значителни благоприятни ефекти върху човешкото здраве, понякога в неочаквани области. Настоящите предварителни изследвания представят традиционните храни като обещаващ нов източник на висококачествени и здравословни хранителни продукти.В тази статия е направен опит за очертаване на благоприятните качества на традиционния млечен продукт „Столетник" с използване на най-новите научни знания в тази област. Традиционната рецепта е развита успешно в промишлена технология, адаптирана за микро- и малки предприятия.

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Phytochemical characteristics and *in vitro* antioxidant activity of fresh, dried and processed fruits of Cornelian cherries (*Cornus mas* L.)

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In the current study, chemical and technological properties and antioxidant activity of fresh, dried and processed Cornelian cherry fruits were evaluated. Several fruit characteristics, such as total titratable acidity (TA), pH, total phenolic compounds, total monomeric anthocyanin (TMA), total antioxidant activity and sugar composition were studied. Average fruit mass and flesh ratio were as follows: for fresh fruits (1.53 g and 77.16%), for dry fruits (0.86 g and 67.61%) and for compôte (1.68 g and 63.15%), respectively. In fruits of Cornelian cherry dry matter varied from 18.7 to 81.4%, ash content from 0.5 to 2% and TA from 1.5 to 3.4%. The highest content of TMA - 32.1 mg cyd-3-glu/100 g fresh weight (fw), as well as of total phenolic compounds - 4.56 mg GAE/g fw was found in fresh fruits. Fresh Cornelian cherry demonstrated the highest antioxidant potential - 36.5 mM TE/g fw (DPPH assay) and 29.6 mM TE/g fw (FRAP assay). The sugar composition in all Cornelian cherry products mainly consisted of sucrose, glucose and fructose, as sucrose was in low levels from 0.15 to 0.30% fw. The polyuronic content was in the range from 1.1 to 1.3%. The current study demonstrated nutritional characteristics of Cornelian cherry fruits suitable for direct consumption and various forms as infusions or compôte. The analyzed products were evaluated as natural foods with high antioxidant activity and potential health benefits.

Key words: Cornelian cherry, Phytochemical compounds, Antioxidant activity

INTRODUCTION

Cornelian cherry (*Cornus mas* L.) is a species of the genus Dogwood (*Cornus* L.) and belongs to the family Cornaceae [1]. In the recent decades the Cornelian cherry has been recognized as an important source of safefood, and breeding programs on this kind of fruit have been launched in several countries as Ukraine, Slovakia, Turkey, Serbia, the Czech Republic, Bulgaria and Austria [2].

Cornelian cherry fruits are widely used in traditional and modern medicine, pharmacy and food industry [2-4]. At the stage of technological maturity, Cornelian cherries acquire a sweet-sour taste [3]. They are not only consumed fresh but also used in the production of jams, jellies, marmalade, stewed fruit [1, 3], as seasoning for meat and fish, in the form of various processed products: wine, brandy, candies, compôte, syrup, fruit juice, fruit yogurts, etc. Fresh processed fruits of the Cornelian cherry can be safely recommended as food for children and persons with impaired health [2].

In Bulgaria, Cornelian cherry is typically consumed fresh, dried as decoct, like marmalade or as compôte [5]. In Turkey its fruits are used for pestil (a locally dried fruit-pulp product), pickled like olives or even in tarhana production [6]. In traditional medicine Cornelian cherry fruits have been used to treat diarrhea, hemorrhoids, diabetes, sore throat, digestion problems, measles, chicken pox, anemia, rickets, liver and kidney diseases [2, 6]. It was reported that fruits also possess astrigent, anti-inflammatory, antioxidative and antihelmintic activity [7-10]. In Turkey, the Cornelian cherry is used for its antiallergenic, antimicrobial and antihistamine properties [4].

Recent research suggests that Cornelian cherry contains significant phytochemical properties and antioxidant capacity [10]. Fresh Cornelian cherry fruits contain many nutritional and bioactive compounds. Furthermore, fruits contain high amount of vitamin C (106.3 mg/100 g), they are rich in sugars (7.2-8.5%), organic acids, cellulose (0.75-1.00%), pectin (0.5-1.12%) and tannin [5, 11]. Jaćimović *et al*. [12] found pectin content in the range of 1.03 to 2.47%. Many studies reported the presence of significant amounts of bioactive substances including anthocyanins in Cornelian cherry fruits [2, 10, 13]. The study of Yilmaz *et al*. [10] also demonstrated high total phenolic content (74.8 mg GAE/g fw), anthocyanins (115 mg cyd-3-glu/100 g fw) and high antioxidant activity [10]. Bjorøy *et al*. [14] reported that anthocyanins from Cornelian cherry fruits demonstrated anticancer,

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anti-inflammatory and antioxidant effects and showed promising results for treatment of diabetes mellitus-related disorders.

Many of the reports discussed the chemical composition of fresh fruits from different *Cornus mas* L. genotypes [2, 4, 13, 15, 16]. The reports for the chemical composition of dried and processed fruits of Cornelian cherry were absent or not available.

The aim of the current study was to evaluate the chemical and technological properties and the antioxidant activity of fresh, dried and processed Cornelian cherry fruits. Several fruit characteristics, such as total titratable acidity (TA), pH, total phenolic compounds, total monomeric anthocyanin (TMA), total antioxidant activity and sugar composition were studied. Phytochemical characteristics and *in vitro* antioxidant activity of fresh, dried and processed fruits of Cornelian cherries were investigated as well.

EXPERIMENTAL

Plant material

Fruits of *Cornus mas* L. were collected in August 2016 from the region of Plovdiv, Bulgaria. Part of the collected fruits was air-dried in darkness at room temperature (25 ºC) and then packed in close tight jars for further uses. Another part from the harvested fresh fruits was directly used. The last part of Cornelian cherry was processed for production of home-made drink with whole fruits, named 'compôte' without added sugar (250 g of Cornelian cherry and 750 ml of distilled water) and boiling for 30 min. All other solvents and chemicals were of analytical grade.

Fruit weight (g) and flesh ratio (%) were assessed. Fruit weight was measured by a digital balance with a sensitivity of 0.0001 g. Flesh ratio (%) was calculated considering fruit and seed weight [2, 4].

Moisture and ash content

Moisture content was determined by the weight difference after drying of the sample, following the official method of AOAC [17]. Ash content was determined according to the standard AOAC procedures [17].

Total acidity

The content of total acids was measured by potentiometric titration of aqueous fruit extract with sodium hydroxide to the pH value of 8.1 by using pH meter 7110 WTW (Germany). The obtained result was converted to the content of acids (expressed as maleic acid) [18].

pH determination

Fruit tissue (5 g) was homogenized with 25 ml of distilled H_2O. pH was determined using pH meter 7110 WTW (Germany) according to AOAC [17].

Fruit extraction

For the extraction of phytochemical compounds, fruits (5 g) were extracted with 70% (v/v) aqueous ethanol (solid to liquid ratio 1:20 (w/v)). The extraction procedure was performed in an ultrasonic bath (VWR, Malaysia, 45 kHz and 30 W) at 45 °C for 15 min [19]. Sugars were extracted from Cornelian cherry fruits with distilled H_2O under ultrasound-assisted irradiation at the above mentioned conditions.

Total soluble and reducing sugars

The total soluble sugars content in fruit samples was estimated by the phenol-sulfuric acid method [20]. The absorbance was measured at 490 nm against blank with distilled H_2O. The amount of present carbohydrates was determined from a calibration curve constructed with glucose [21]. The reducing sugars were evaluated by the PAHBAH method [22].

HPLC analysis of mono-and disaccharides

Chromatographic separation and determination of sugars present were performed on a HPLC instrument Elite Chrome Hitachi with refractive index detector (RID) Chromaster 5450. The separation was done on a Shodex® Sugar SP0810 (300 mm × 8.0 mm) with Pb^{2+} and a guard column Shodex SP - G (5 µm, 6 × 50 mm) operating at 85°C, mobile phase distilled H_2O with flow rate of 1.0 ml/min and injection volume of 20 µl [23].

Polyuronic acid content

The polyuronic acid content (PUC) in the plant materials was determined titrimetrically according to Owens *et al*. [24].

Total phenolic contents

Total phenolic contents were measured using a Folin-Ciocalteu reagent. Briefly, 1 ml Folin-Ciocalteu reagent diluted five times was mixed with 0.2 ml of sample and 0.8 ml of 7.5% Na_2CO_3. The reaction was performed at room temperature in darkness for 20 min. The absorbance was measured at 765 nm against blank. The results were expressed as mg equivalent of gallic acid (GAE) per g fresh weight (fw) [25].

Determination of total monomeric anthocyanins

Total anthocyanins content was determined according to the pH differential method [26]. Absorbance was measured at 520 and 700 nm. Data were reported as cyanidin-3-glycoside per 100 g of fw of fruit or 100 g of tissue for at least three replicates.

$$TMA = \frac{\mathrm{A} \times \mathrm{MW} \times \mathrm{DF} \times 1000}{\varepsilon \times 1}, \text{ mg/L}$$

where A=(A_{520nm} – A_{700nm})pH1.0 – (A_{520nm} – A_{700nm})pH4.5; MW (molecular weight) 449.2 g/mol cyanidin-3-glycoside (cyd-3-glu); DF – dilution factor, l = path length in cm; ε-molar coefficient of 26900 L × mol^{-1}× cm^{-1} for cyd-3-glu and factor 1000 for conversion from g to mg.

DPPH radical-scavenging ability

Cornelian cherry fruits ethanol extract (0.15 ml) was mixed with 2.85 ml of freshly prepared 0.1mM solution of DPPH in methanol. The sample was incubated at 37 °C in darkness for 15 min. The reduction of absorbance was measured at 517 nm against a blank containing methanol and % inhibition was calculated [25].

Ferric reducing antioxidant power (FRAP) assay

The assay was performed according to Benzie and Strain [27] with slight modification. The FRAP reagent was freshly prepared by mixing 10 parts 0.3 M acetate buffer (pH 3.6), 1 part of 10 mM 2,4,6-tripyridyl-s-triazine (TPTZ) in 40 mM HCl and 1 part of 20 mM $FeCl_3$×$6H_2O$ in distilled H_2O. The reaction was started by mixing 3.0 ml of FRAP reagent with 0.1 ml of investigated extract. The reaction time was 10 min at 37 °C in darkness and the absorbance was measured at 593 nm against a blank prepared with methanol. Antioxidant activity was expressed as mM Trolox® equivalents (TE) per g fresh weight (fw) [25].

Statistical analysis

All experimental measurements were carried out in triplicate and were expressed as average of three analyses± standard deviation.

RESULTS AND DISCUSSION

Physicochemical characteristics of fruits

The results for some physical parameters of Cornelian cherry fruits are summarized in Table 1. The average fruit weight was in the range of 0.86 to 1.68 g. Our results were lower than reported for Turkish and Serbian Cornelian cherry, where the average fruit weight was in range of 2.9 to 3.9 g [2, 3]. However, our results coincided with other reports for some Turkish and Slovakian Cornelian cherry fruit types (0.39-1.03 g) [13] and 0.5-3.4 g [28], respectively.

Table 1. Fruit characteristics of Cornelian cherry (*Cornus mas* L.)

Product	Number of fruits	Total fruit weight, g	Pulp, g	Stones, g	Fruit weight, g	Flesh/solid ratio, %
Fresh fruits	21	32.22	24.86	6.53	1.53	77
Dried fruits	21	17.97	11.97	5.99	0.86	67
Compôte fruits	29	48.76	30.79	17.97	1.68	63

Flesh/solid ratio varied from 77 to 63% in our study. The fresh Cornelian cherry fruits showed higher values of flesh ratio than processed fruits (Table 1). Many studies showed that fruit weight and fruit flesh ratio is the most variable characteristics of Cornelian cherries. Fruit weight and fruit flesh ratio of Cornelian cherry genotypes in Turkey were reported between 1.49-9.11 g [29-31] and 74-93% [10, 31]. For Serbian *Cornus mas* L. genotypes these values were in the range of 3.42 to 8.00 g and 79.32 and 88.55%, respectively [2, 16, 32]. The highest results for fruit weight and fruit flesh ratio were reported for Ukrainian *Cornus mas* L (5.0-8.0 g and 89-92%) [33]. The results in our study were near to Turkish Cornelian cherry genotypes, reported previously [29-31]. The contents of total dry matter in fruits (Table 2) varied in the range from 18.19% to 81.37%. Especially, fresh Cornelian cherry fruits had high dry matter content - 30.74%, which was near to the results reported for some Serbian genotypes [2, 16], but higher than previously reported values for Turkish genotypes [13, 18, 30, 31]. The reason for this increase could be explained by the different growing and environmental conditions, because the values of these parameters vary considerably not only as a result of the genotypes [30].

Ash content in pulp from fresh, dried and processed fruits of Cornelian cherry varied in the range of 0.49 to 2.83%. Our results coincided with previous reports for ash in fresh fruits of some Serbian and Turkish *Cornus mas* L. genotypes [2, 10, 16, 18].

In the present study the pH values of Cornelian cherry fruits were between 3.19 – 3.46 which was in agreement with other reports (2.5–3.53) [3, 10, 13, 18, 29].

Total acidity of Cornelian cherry fruits was found to be in the range of 0.95 to 1.49% (as maleic acid), while in the similar studies TA was between 1.10 to 4.69% [2-4, 10, 16, 30]. Our results demonstrated lower total acidity of Bulgarian fresh and dried Cornelian cherry fruits in comparison with fresh samples collected from territory of Serbia and Turkey [2, 16, 13].

Table 2. Physicochemical characteristics of Cornelian cherry (*Cornus mas* L.)

Product	Moisture content, %	Total dry matter, %	Ash content, %	pH	Total acidity, %
Fresh fruits	69.25±1.79	30.74±1.79	0.88±0.01	3.40	0.95±0.23
Dried fruits	18.62±0.22	81.37±0.22	2.83±0.35	3.46	1.05±0.20
Compôte fruits	81.81±0.74	18.19±0.75	0.49±0.01	3.19	1.49±0.22

Sugar content in the fruits of Cornelian cherry

The individual sugar contents present in aqueous extracts of fresh, dried and processed Cornelian cherry fruits were analyzed by HPLC-RID method. Only three sugars (sucrose, glucose and fructose) were detected (Fig. 1) in *Cornus mas* fruits.

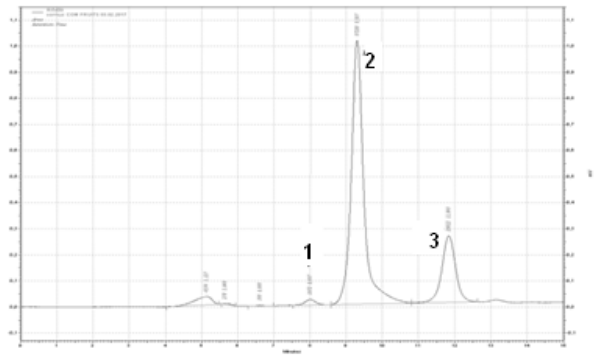

Fig. 1. HPLC-RID chromatograms of aqueous extracts from dry Cornelian cherry fruits, where 1 – sucrose, 2- glucose and 3-fructose

Glucose and fructose were the main monosaccharides detected in all fruits of Cornelian cherry. Their content was 12.26±0.36 and 1.90±0.21% dw, respectively. Sucrose content in fresh fruits was low - 0.17% fw. Sucrose content in dried fruits was 0.25% dw, whereas its content in fruits of compôte was 0.15% (Table 3).

The results reported by us were higher than the content of reducing sugars by Kalyoncu *et al.* [3] and near to some other Turkish genotypes [18]. The monosaccharides content in dry samples was near to reports for Bulgarian *Cornus mas* L. fruits [34]. Our results for total sugars were in agreement with Burmistrov [35], who reported that the content of sugars in fruits of wild genotypes varied between 9.4% and 17.4%. Cornelian cherry genotypes from Slovakia [28] contained from 6.5% to 15.1% of total sugars.

Table 3. Carbohydrate content in Cornelian cherry fruits

Product	Total soluble sugars, %	Reducing sugars, %	Sucrose, %	Glucose, %	Fructose, %	PUC %
Fresh fruits	16.88±0.54[b]	14.56±0.26[b]	0.17±0.09[b] 0.56±0.05[a]	12.26±0.36[b] 39.84±0.36[a]	1.90±0.21[b] 6.11±0.21[a]	1.3[a]
Dried fruits	15.50±0.36[b]	11.03±0.13[a]	0.25±0.07[a]	7.45±0.29a	2.63±0.64[a]	1.2[a]
Compôte fruits	13.78±0.66[b]	10.56±0.25[b]	0.15±0.09[b] 0.82±0.09[a]	8.43±0.53[b] 46.28±0.53a	0.88±0.25b 4.83±0.25a	1.1[a]

a – dry weight, b – fresh weight

The reducing sugars content for dry Cornelian cherry were similar to those in the reports of Didin *et al.* [36] and Kalyoncu *et al.* [3]. Cornelian cherry genotypes from Turkey had significantly lower contents of total and reducing sugars [10, 30] in relation to our results. Our results for total and reducing sugars coincided with some Serbian genotypes [2, 16], but sucrose levels in our research were lower than in previous studies [2, 16]. The explanation for the lower level of sucrose in Cornelian cherry fruit is that they are a rich source of acid and hydrolysis to glucose and fructose could be possible. Therefore, our study evaluated Cornelian cherry fruit as fruits with low sucrose content.

Cornus mas L. fruits were evaluated as a source of pectin as PUC content in fresh, dried and possessed fruits was in the range from 1.1 to 1.3% dw. The drying and heating caused a small decrease in pectin content to 1.1% that is due to enzyme or temperature partial hydrolysis of pectic polysaccharides. The well-proven properties of

pectin as soluble dietary fibers find significant application in healthy and dietary nutrition. The pectin content in Serbian genotypes varied from 0.37 to 1.57% expressed as Ca-pectate [2, 16]. Therefore, our data showed significantly higher content of pectin in *Cornus mas* fruits in comparison with some Serbian varieties.

Phenolic compounds and antioxidant activity

Cornelian cherry fruits were evaluated as a good source of phenols and anthocyanin; therefore they are a food with high antioxidant capacity (Table 4). Cornelian cherry fruits are characterized with a significant content of colored substances. The content of total phenols ranged from 10.44 to 5.78 mg GAE/g dw and from 4.35 to 1.90 mg GAE/g fw (Table 4). The lowest levels were detected in dried fruits, the highest values were found in fresh fruits of *Cornus mas* L. The obtained results for total phenols in *Cornus mas* L. were in accordance with reported 432 mg GAE/100 g fresh weight [37]. Close to our results were also the reports of Tural and Koca [13] who reported that the total phenolic contents of Cornelian cherries ranged between 2.81 mg/g and 5.79 mg/g. Our results coincide also with reports for total phenols, especially for Turkish Cornelian cherry in dark red stages of ripening (4162 μg GAE/g fw) [29]. Moreover, the various factors such as genotype, agronomic practices, maturity level at harvest, postharvest storage, climatic and geographical locations influence the total phenolic content of the plant [2, 4, 16]

Table 4. Total phenols, total monomeric anthocyanins and antioxidant activity of fruits of *Cornus mas* L.

Product	Total phenols mg GAE[1]/g	TMA, mg cyd-3-glu/100 g	DPPH, mM TE[2]/g	FRAP, mM TE/g
Fresh fruits	4.35±0.19[b]	48.04±1.16[b]	29.91±0.76[b]	29.12±0.26[b]
	14.14±0.19[a]	136.63±1.16[a]	97.21±0.76[a]	94.64±0.26[a]
Dried fruits	5.78±0.01[a]	3.55±0.16[a]	46.58±3.34[a]	48.53±0.32[a]
Compôte	1.90±0.48[b]	20.44±1.15[b]	14.63±5.04[b]	13.12±0.14[b]
fruits	10.44±0.48[a]	112.22±1.15[a]	80.32±5.04[a]	72.03±0.14[a]

a – dry weight, b – fresh weight, [1] GAE –gallic acid equivalents, [2] TE – Trolox equivalents

The content of total monomeric anthocyanins in Cornelian cherry varied from 136.63 to 112.22 mg cyd-3-glu/100 g fw (Table 4). Similar findings have been published for Cornelian cherry genotypes grown in Turkey, with anthocyanins values from 107 to 292 mg cyd-3-glu/100 g fw [13, 15]. Our results for TMA were similar to those for some Serbian genotypes [2, 16]. In compôte the content of TMA decreased approximately twice on fresh weight, while in dry Cornelian cherry it decreased more than 45 times. Therefore, TMA remained in compôte and the latter is a proper approach for processing fruits, better than drying process.

The antioxidant activity of extracts from fruits of C*ornus mas* was determined using DPPH and FRAP methods, based on different mechanisms. The highest antioxidant activity evaluated by both methods possessed fresh fruits of Cornelian cherry followed by processed ones as compôte. The lowest antioxidant activity showed dried fruits of *Cornus mas* L. - 46-48 mM TE/g dw. Therefore, the preservation of Cornelian cherry as compôte demonstrated a promising antioxidant potential of fruits and presented a beverage with high content of phenolic compounds.

CONCLUSION

Our study evaluated the nutritional and antioxidant potential of fresh and dried Cornelian cherry fruits, as well as compôte. The results demonstrated that the highest content of phytochemical compounds was found in fresh fruits of *Cornus mas* L. The conservation of this fruits as compôte showed significantly higher levels of TMA and antioxidant activity. The lowest content of biologically active substances was found in dried fruits. Therefore, the current study recommends consumption of Cornelian cherry fruits in fresh state or as compôte.

REFERENCES

1. M. S. Stankovic, M. Zia-Ul-Haq, B. M. Bojovic, M. D. Topuzovic, *Bulg. J. Agric. Sci*., **20**, 358, (2014).
2. S. M. Bijelić, B. R. Gološin, J. N. Todorović, S. Cerović**,** *J. Agr. Sci. Tech*., **5**, 310 (2011).
3. I. H. Kalyoncu, N. Ersoy, M. Yilmaz, *Asian J. Chem.*, **8,** 6555 (2009).
4. S. Ercisli, S. O. Yilmaz, J. Gadze, A. Dzubur, S. Hadziabulic, Y. Aliman, *Not. Bot. Hortic. Agrobot. Cluj-Napoca*., **39**, 255 (2011).
5. B. Michev, A. Naidenov, S. Chortanova, T. Malinov, Forest fruits – food and healing means. Zemizdat, Sofia, 1983.
6. F. Isık, İ. Çelik, Y. Yılmaz, *Akademik Gıda*, **12**, 34 (2014).
7. İ. Gülçin, Ş. Beydemir, I.G. Şat, Ö.İ. Küfrevioğlu, *Acta Alimentaria*, **34**, 193 (2005).
8. G. E. Pantelidis, M. Vasilakakis, G.A. Manganaris, Gr. Diamantidis, *Food Chem*., **102**, 777 (2007).

9. B. Paulovicsova, I. Turianica, T. Jurikova, M. Baloghova, J. Matuškovič, *Sci. Papers Animal Sci. Biotech*., **42**, 608 (2009).
10. K. U. Yilmaz, S. Ercisli, Y. Zengin, M. Sengul, E. Y. Kafkas, *Food Chem*., **114**, 408 (2009).
11. D. Maghradze, E. Abashidze, Z. Bobokashvili, R. Tchipashvili, E. Maghlakelidze, *Acta Hortic*., **818**, 65 (2009).
12. V. Jaćimović, D. Božović, S. Ercisli, Vl. Ognjanov, B. Bosančić, *Erwerbs-Obstbau*, **57**, 119, (2015).
13. S. Tural, I. Koca, Sci. *Hortic*., **116**, 362 (2008).
14. Ø. Bjorøy, T. Fossen, Ø. M. Andersen, *Phytochem*., **68**, 640 (2007).
15. H. Hassanpour, Y. Hamidoghli, J. Hajilo, M. Adlipour, *Sci. Hortic*., **129**, 459 (2011).
16. S. M. Bijelić, B. R. Gološin, J. I. N. Todorović, S. B. Cerović, B. M. Popović, *HortScience*, **46**, 849 (2011).
17. AOAC, Official methods of analysis, 18th ed. VA, USA, Association of Official Analytical Chemists, Arlington, 2007.
18. M. Sengul, Z. Eser, S. Ercısli, *Acta Sci. Pol., Hortorum Cultus*, **13**, 73 (2014).
19. N. Petkova, I. Ivanov, P. Denev, A. Pavlov, *Turk. J. Agric. Nat. Sci*., **1**, 1773 (2014).
20. M. Dubois, K. Gilles, J. Hamilton, P. Rebers, F. Smith, *Anal. Chem*., **28**, 350 (1956).
21. M. Dimitrova, N. Petkova, P. Denev, I. Aleksieva, *Int. J. Pharm. Phytochem. Res*., **7**, 621 (2015).
22. M. Lever, *Anal. Biochem*., **47**, 273 (1972).
23. N. Petkova, R. Vrancheva, P. Denev, I. Ivanov, A. Pavlov, *ASN*, **1**, 99 (2014).
24. H. S. Owens, R. M. McCready, A. D. Shepheral, T. H. Schultz, E. L. Pippen, N. A. Swenson, J. C. Miers, F. R. Erlander, W. D. Maclay, (Methods used at Western Regional Research Laboratory for extraction and analysis of pectic materials), U.S. Dept. Agr. Bull. AIC-340, 1952.
25. I. Ivanov, R. Vrancheva, A. Marchev, N. Petkova, I. Aneva, P. Denev, V. Georgiev, A. Pavlov, *Int. J. Curr. Microbiol. Appl. Sci.*, **3**, 296 (2014).
26. J. Lee, R. W. Durst, R. E. Wrolstad, *J. AOAC Int.*, **88**, 1269 (2005).
27. I. Benzie, J. Strain, *Anal. Biochem.*, **239**, 70 (1996).
28. P. Brindza, J. Brindza, D. Tóth, S. V. Klimenko, O. Grigorieva, *Acta Hortic*., **818**, 85 (2009).
29. K. Gunduz, O. Saracoglu, M. Özgen, S. Serce, *Acta Sci. Pol., Hortorum Cultus*, **12**, 59 (2013).
30. F. Demir, I. H. Kalyoncu, *J. Food Eng*., **60**, 335 (2003).
31. T. Karadeniz, H. Deligöz, M. S. Çorumlu, M. Şenyurt, T. Bak, *Acta Hort*., **825**, 83 (2009).
32. V. Ognjanov, S. Cerović, J. Todorović, V. Jaćimović, B. Gološin, S. Bijelić, B. Vračević, *Acta Hort*., **814**, 121 (2009).
33. S. Klimenko, *J. Fruit Ornam. Plant Res. (Spec. Ed.)*, **12**, 93 (2004).
34. G. Toncheva, K. Nikolova, D. Boyadzhiev, I. Alexieva, D. Georgieva, G. Antova, A. Aladjadjiyan, D. Buhalova, *Bulg. J. Agric. Sci.*, **22**, 857 (2016).
35. L. A. Burmistrov, WANATCA Yearbook West Australian Nut and Tree Crops Association, **18**, 3 (1994).
36. M. Didin, A. Kizilaslan, H. Fenercioglu, *Gida*, **25**, 435 (2000).
37. D. Marinova, F. Ribarova, M. Atanassova, *J. Univ. Chem. Tech. Metall.*, **40**, 255 (2005).

ФИЗИКОХИМИЧНА ХАРАКТЕРИСТИКА И *in vitro* АНТИОКСИДАНТНА АКТИВНОСТ НА СВЕЖИ, ИЗСУШЕНИ И ПРЕРАБОТЕНИ ПЛОДОВЕ ОТ ОБИКНОВЕН ДРЯН (*Cornus mas* L.)

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(Резюме)

В настоящето изследване са оценени химичните и технологичните свойства на свежи, изсушени и преработени плодове от обикновен дрян. Определени са редица характеристики на плода, като механичен състав, обща титруема киселинност, pH, общо полифенолно съдържание, общи мономерни антоциани, антиоксидантна активност и захарен състав. Средната маса на плода и процентът на месестата част са както следва: за свежи плодове (1.53 g и 77.16%), за изсушени плодове (0.86 g и 67.61%) и съответно за компот (1.68 g и 63.15%). Сухото вещество на плодовете варира от 18.7 до 81.4%, пепелно съдържание от 0.5 до 2%, а титруемата киселинност от 1.5 до 3.4%. Най-високо съдържание на общи антоциани и полифенолни компоненти беше намерено в свежите плодове – съответно 32.1 mg cyd-3-glu/100 g св. м. и 4.56 mg GAE/g св. м. Свежите плодове показаха най-висок антиоксидантен потенциал - 36.5 mM TE/g св. м. (DPPH метод) и 29.6 mM TE/g св. м. (FRAP метод). От захарите във всички плодове най-застъпени са захарозата, глюкозата и фруктозата, като захарозата е намерена в най-малки количества от 0.15 до 0.30% св. м. Полиуронидното съдържание се открива в границите от 1.1 до 1.3%. Настоящото изследване демонстрира хранителните характеристики на дренките, като те са подходящи за консумация в свежо състояние, а също така и под формата на запарка или компот. Анализираните продукти бяха оценени като естествена храна с висока антиоксидантна активност и потенциални здравни ползи.

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Different pathways of the *para*-O-H bond dissociation in di- and trihydroxyphenolic acids: a DFT investigation

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Phenols may inhibit but can also enhance oxidative damage processes in biomolecules. This ambivalent behavior heavily depends on their chemical structure. Phenolic acids with two and three hydroxyl groups and a different length and degree of saturation of the side chain are selected for this investigation. The radical-scavenging activity of the compounds was assessed, as well as the role of the different structural features on it. The most appropriate mechanism for the *para*-O-H bond breaking in an aqueous medium is determined. A high level DFT investigation was performed using the B3LYP functional with the 6-311++G(d,p) orbital basis; the solvent effects were evaluated by PCM.

Keywords: Phenols, DFT, Radical-scavenging, Reaction mechanisms

INTRODUCTION

The interest towards phenols is mainly determined by their antioxidant activity and by their involvement in the prevention of pathologies such as cancer [1,2], cardiovascular diseases [3-6] and inflammatory disorders [7]. The specific role of dietary and synthetic antioxidants in carcinogenesis is unclear [8]. The main proposed mechanism of this action is associated with the suppression of the harmful oxidative processes in the cells resulting from their radical-scavenging activity [6].

On the other hand, it has been found that some antioxidants, which may delay carcinogenesis, may also appear as accelerators of the tumor development in the second phase [9]. These compounds, depending on the concentration and type of active radicals, may also show pro-oxidative activity [10]. According to a suggested mechanism, the cytotoxicity of polyphenols may be due particularly to their prooxidant activity. Thus, depending on the structure, dose, target molecule, and environment, phenols may inhibit but also enhance oxidative damage processes in biomolecules [11], i.e. they can behave as anti- but also as pro-oxidants [10,12].

These controversial bioactivities of phenols depend heavily on their chemical structure [10,13,14]. It is believed that the prooxidant activity is proportional to the total number of hydroxyl groups in a flavonoid molecule [12]. Series of mono- and di-hydroxyflavonoids demonstrated no detectable prooxidant activity, while multiple hydroxyl groups, especially in the B-ring, significantly increased the production of hydroxyl radicals in the Fenton reaction [12,15].

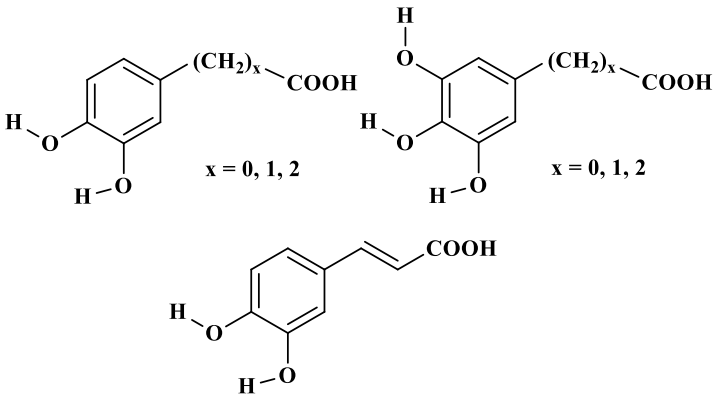

Figure 1. Structure of the investigated compounds

The target of this study is a series of phenolic acids (Figure 1) with: (i) two and three hydroxyl groups; (ii) different length and degree of saturation of the side chain [16]. What we will try to compare is the total reactivity of the phenolic compounds and specifically the reactivity toward radicals, related to the structural differences between them. Undoubtedly, the structural features have strong

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influence on the radical-scavenging activity [17]. Dissociation of the *para*-hydroxyl group *was* considered in the formation of phenoxyl radical presented in Figure 2.

The various phenolics react with active radicals following different mechanisms. This study should give an answer to the question which mechanism of the reaction between the investigated phenolic acids and radicals is preferred (Figure 2) [18-21]: (i) electron transfer from the antioxidant to the active radical, which produces a cation-radical and an anion; the electron transfer is followed by proton transfer from the cation-radical to the anion (SET-PT); (ii) direct hydrogen atom transfer between the antioxidant and the active radical (HAT); (iii) deprotonation of the antioxidant followed by electron transfer from the resulting anion to the active radical; the next step is protonation of the anion produced by the active radical (SPLET).

Some authors differentiate a fourth mechanism (iv) for the phenolic type antioxidants: proton-coupled electron-transfer (PCET) [19,22]. In the PCET mechanism, the radical (R•) possesses one or two lone pairs of electrons on the atom bearing the unpaired electron and the hydrogen transfer is mediated by an in-between formation of a temporary hydrogen bond which draws the O atom of PhA-OH and the radical center closer together, thus facilitating the proceeding of one of the above, e.g. the HAT, mechanism (see Figure 2). The mechanisms shown in Figure 2 address only the formation of the final stable radical PhA-O• and do not account for any subsequent transformations of this radical.

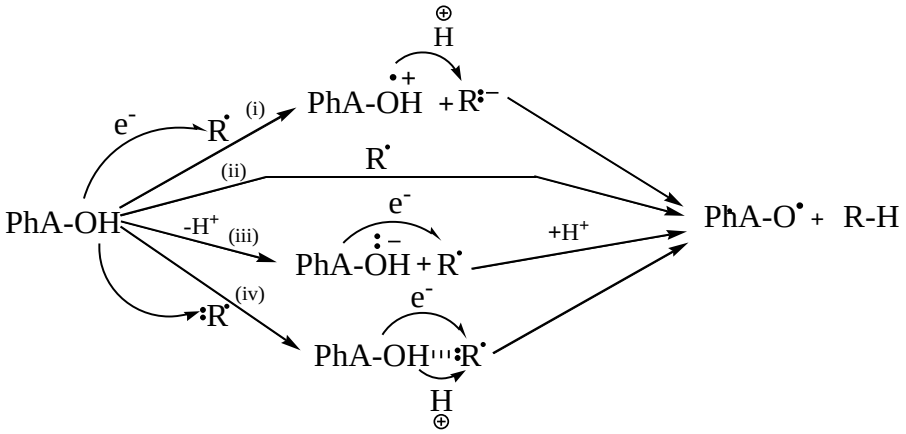

Figure 2. Mechanisms of O-H bond dissociation. PhA-OH stands for phenolic antioxidant.

The first step of mechanism (i) – Single Electron Transfer–Proton Transfer (SET–PT) can be described by the ionization potential (IP) or sometimes by the energy of the highest occupied molecular orbital according to Koopmans theorem [23,24]. As a rule, the second step is faster and can be quantified by the proton dissociation enthalpy (PDE) defined by equation 4. Mechanism (ii) Hydrogen Atom Transfer (HAT) dominates when the Bond Dissociation Enthalpy (BDE) is low (equation 2). Mechanism (iii): Sequential Proton Loss Electron Transfer (SPLET) is expected to occur only in antioxidants with an easily deprotonated functional group as the phenolics. SPLET is feasible when a hydroxyl group is acidic: the system features low proton affinity (PA) and low electron transfer enthalpy (ETE) as they are defined below (equations 5 and 6).

Normally, the free energy is the criterion for a thermodynamically preferred process. For the investigated reaction, Klein and co-authors have found that the absolute value of the entropic term ($T\Delta_r S$) is much smaller than the enthalpic term [25]. Therefore, a comparison of BDEs, IPs, PDEs, PAs and ETEs can show which mechanism is thermodynamically preferred. The utility of the values of enthalpy changes thus calculated is confirmed by their successful use in the QSAR analysis [26].

COMPUTATIONAL DETAILS

The calculations were carried out using the DFT, as implemented in the Gaussian09 program package [27]. The optimization of the geometry was performed with the Becke 3-parameter hybrid exchange functional combined with the Lee-Yang-Parr correlation functional (B3LYP) with the standard 6-311++G(d,p) basis set [28]. The optimization was achieved without any geometry constraints. For all structures the harmonic vibrational frequencies were computed to confirm the true minima on the calculated potential surface.

All possible intramolecular interactions were taken into account in the initial geometries.

Solvent effects on the calculated structures were investigated with the self-consistent reaction field

(SCRF) method, *via* the polarized continuum method (PCM) [29].

The total enthalpies of the species X are usually estimated from the equation:

$$H(X) = E_0 + ZPE + \Delta H_{trans} + \Delta H_{rot} + \Delta H_{vib} + RT \quad (1)$$

where E_0 is the calculated total energy in the Born-Oppenheimer approximation, ZPE stands for the zero-point energy, ΔH_{trans}, ΔH_{rot}, and ΔH_{vib} are the translational, rotational and vibrational contributions to the enthalpy. Finally, RT is the pV-work term added to convert the internal energy into enthalpy. The total enthalpies were calculated at T = 298 K. The ZPE values were not scaled.

All calculated enthalpy changes are defined by equations *1–5*. From the calculated total enthalpies, we have determined the following quantities:

$$PhA\text{-}OH \leftrightarrow PhA\text{-}O^{\cdot} + H^{\cdot}$$

$$BDE = H_{(PhA\text{-}O\cdot)} + H_{(H\cdot)} - H_{(PhA\text{-}OH)} \quad (2)$$

$$PhA\text{-}OH \leftrightarrow PhA\text{-}OH^{\cdot+} + e^{-}$$

$$IP = H_{(PhA\text{-}OH^{\cdot+})} + H_{(e^-)} - H_{(PhA\text{-}OH)} \quad (3)$$

$$PhA\text{-}OH^{\cdot+} \leftrightarrow PhA\text{-}O^{\cdot} + H^{+}$$

$$PDE = H_{(PhA\text{-}O\cdot)} + H_{(H^+)} - H_{(PhA\text{-}OH^{\cdot+})} \quad (4)$$

$$PhA\text{-}OH \leftrightarrow PhA\text{-}O^{-} + H^{+}$$

$$PA = H_{(PhA\text{-}O^-)} + H_{(H^+)} - H_{(PhA\text{-}OH)} \quad (5)$$

$$PhA\text{-}O^{-} \leftrightarrow PhA\text{-}O^{\cdot} + e^{-}$$

$$ETE = H_{(PhA\text{-}O\cdot)} + H_{(e^-)} + H_{(PhA\text{-}O^-)} \quad (6)$$

The calculated proton enthalpy ($\mathbf{H_{(H^+)}}$) is -1083.803 kJ.mol^{-1}; the enthalpy of an electron ($\mathbf{H_{(e^-)}}$) is -232.676 kJ.mol^{-1}, the enthalpy of a hydrogen atom ($\mathbf{H_{(H^\cdot)}}$) is –1307.291 kJ.mol^{-1} [30].

Table 1. Enthalpy changes (in kJ/mol) related to the different mechanisms of O-H bond dissociation.

Compounds*	BDE	IP	PDE	PA	ETE
DHB	331.341	372.021	-49.868	98.733	223.421
DHPE	314.318	338.431	-33.301	97.237	207.894
DHPP	314.168	335.091	-30.111	102.870	202.109
DHPPE	315.433	341.358	-35.113	103.779	202.466
THB	311.907	370.606	-67.887	97.342	205.378
THPE	298.323	337.559	-48.424	97.024	192.112
THPP	295.267	331.996	-45.917	102.807	183.271

*Full names: 3,4-dihydroxybenzoic acid **(DHB)**, 3,4-dihydroxyphenyl ethanoic acid **(DHPE)**, 3,4-dihydroxyphenyl propanoic acid **(DHPP)**, *trans*-3,4-dihydroxyphenyl propenoic acid **(DHPPE,** caffeic acid**)**, 3,4,5-trihydroxybenzoic acid **(THB,** gallic acid**)**, 3,4,5-trihydroxyphenyl ethanoic acid **(THPE)**, 3,4,5-trihydroxyphenyl propanoic acid **(THPP)**.

RESULTS AND DISCUSSIONS

O-H Bond Dissociation Enthalpies (BDE)

The O-H BDE is indicative of the aptitude of a substance to enter into radical reactions and, therefore, for the radical-scavenging potential. Here we will only look at the change in the thermodynamic function associated with the detachment of the first hydrogen atom from the compounds because this is the rate-determining step. Subsequent cleavage of a hydrogen atom from the second (or third) hydroxyl group will result in the formation of a stable ortho-quinone.

However, BDE shows the ability to react with radicals only by the bimolecular one-stage mechanism (Figure 2, ii). The lowest BDE (under 300 kJ/mol) in an aqueous medium have the hydroxyl groups in THPP (295.267 kJ/mol) and in THPE (298.323 kJ/mol). Four of the investigated compounds have higher BDE values: THB (311.907 kJ/mol), DHPP (314.168 kJ/mol), DHPE (314.318 kJ/mol) and DHPPE (315.433 kJ/mol). As can be seen from Table 1, the highest BDE value stands for DHB (331.341 kJ/mol).

BDE decreases starting from acids with two hydroxyl groups to trihydroxyl acids in line with gradual elongation of the side chain. In other words, with side chain lengthening the ability to react with radicals increases and the trihydroxyl acids react more easily with radicals in a homolytic manner. The number of hydroxyl groups has a stronger impact on BDE than the chain extension.

Only the DHPPE deviates from this rule. This can be explained by the fact that the side chain in it is not saturated and links the mesomeric electron-withdrawing carboxyl group with the π-electronic system of the phenolic ring and the dissociable hydroxyl group at *para*-position.

The lower the BDE, the more reactive the compounds are. Also, the longer the chain and the greater the number of hydroxyl groups in the phenyl ring, the higher the reactivity of the acids with respect to radicals.

Ionization potential (IP)

IP is indicative for the propensity of the investigated compounds to participate in the SET-PT mechanism (Figure 1, i).

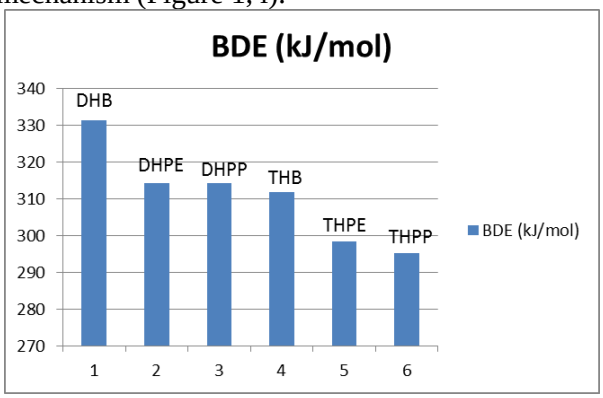

Chart 1. BDE dependence on the side chain length and the number of hydroxyl groups.

This is a two-stage mechanism that begins with an electron extraction from the phenolic acid and formation of a cation-radical followed by the cleavage of a proton. This mechanism (SET-PT), as well as the SPLET mechanism, is usually observed in the reactions between radical-scavengers and active radicals in polar solvents. Charged intermediates are formed in these mechanisms which are more stable in polar solvents.

BDE and IP have close values for every compound, but the latter are larger, which means that even in an aqueous environment the propensity to participate in a HAT mechanism is greater than in a SET-PT one.

The highest IP (and BDE) value possesses DHB (372.021 kJ / mol) and the lowest IP value has THPP (331.996 kJ / mol). Here, it should be noted again that the dihydroxyl derivatives have higher values than the trihydroxyl derivatives as it is with the BDE and again the positive inductive effect of the side-chain lowers the IP and increases the propensity of the compounds to react with the radicals in a SET-PT mechanism. Here, in contrast to BDE, the side chain length has a stronger impact on the IP than the number of hydroxyl groups.

The significantly higher IP values of the benzoic acid derivatives have to be explained separately. Generally speaking, the structural factors affecting the BDE affect the IP in a similar way. Only with THB and DHB the electron-withdrawing effect of the carboxyl group has a significantly stronger impact on the IP than on the BDE.

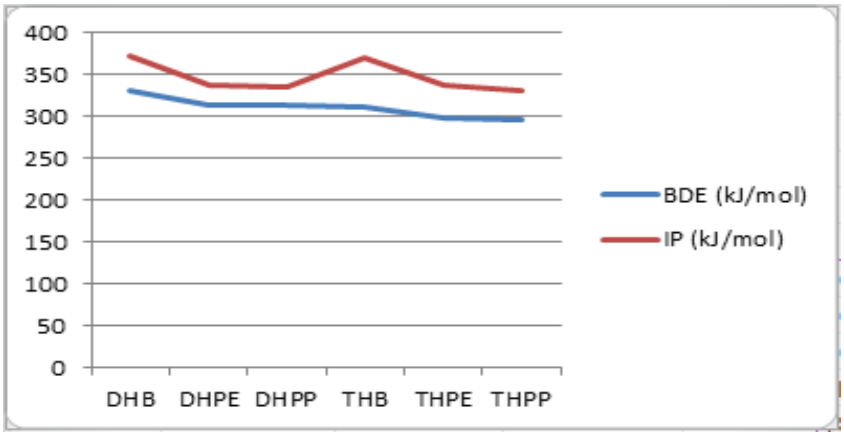

Chart 2. IP and BDE profiles.

Obviously, the direct bond between the phenyl ring and the carboxyl group allows the carboxyl group to have a negative mesomeric effect on the electron density in the phenyl ring, and this is the reason for the significantly higher enthalpy needed to detach an electron from benzoic acid derivatives. This is confirmed by the fact that the trihydroxyl derivatives (having one more electron-donating hydroxyl group) have a slightly lower ionization potential than the dihydroxyls (See Figure 1).

Proton dissociation enthalpies (PDE)

According to the SET-PT mechanism, an electron is initially torn off from the phenolic acids and a cation-radical is formed. Then a proton is cleaved from the cation-radical to form the corresponding radical, which is also produced by the other two mechanisms (Figure 2). PDE shows the propensity of the cation-radicals of phenolic acids to give up protons and become radicals [49]. PDE values indicate that proton cleavage is an exothermic process. This is an energy-efficient process and the rate of the reaction will depend solely on the electron detachment rate. The PDE can serve only as a measure of the acidity of the OH groups of the cation-radicals of the phenolic acids and for the acids themselves.

As can be seen, this is the only descriptor that has negative values. The most acidic is the hydroxyl

group at the para-position of the cation-radical of THB, followed by the para-hydroxyl group in the cation-radical of the DHA.

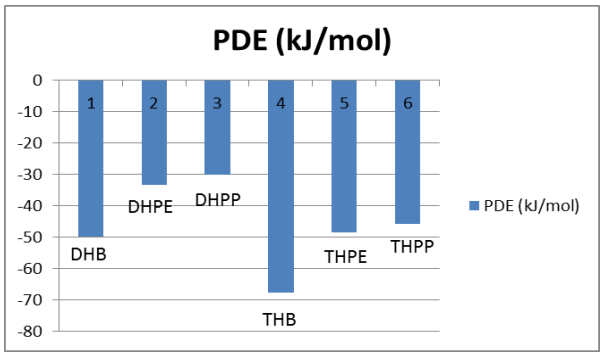

Chart 3. Acidity of the cation-radicals of the phenolic acids studied.

The least acidic are the hydroxyl groups in DHPP and DHPE. Again, DHPPE occupies a special place in the group of compounds under consideration. Here again, the negative mesomeric effect of the carboxyl group in DHB and THB, as well as the side chain induction effect in the other compounds influence the acidity. The rate of electron detachment will be determining for the total reaction rate in the SET-PT mechanism (Fig. 2 (i)).

Proton affinity (PA)

PA, the enthalpy of proton dissociation from the carboxyl group of the phenolic acids, is another measure of their acidity. It can be seen from the table that the highest acidity possesses THPE (97.024 kJ/mol) and immediately after it are DHPE (97.237 kJ/mol), THB (97.342 kJ/mol) and DHB (98.733 kJ/mol). Roughly, the remaining acids have close PA values: THPP (102.807 kJ/mol), DHPP (102.870 kJ/mol) and DHPPE (103.779 kJ/mol). The lowest acidity has DHPPE (103.779 kJ/mol).

Surely, the detachment of a proton from the acid is not a rate-determining step in the SPLET mechanism. ETE is indicative of the ability of the anion to release an electron. The removal of an electron from the anion of THPP (183.271kJ/mol) is the easiest because it requires the least amount of energy. The removal of an electron from the anion of DHB (223.421 kJ/mol) is the most difficult. This is not surprising. Generally, electron separation from more stable anions is more difficult. Therefore, the more difficult process in the SPLET-mechanism is the detachment of an electron.

Electron transfer enthalpies (ETE)

Ultimately, it turns out that the SPLET mechanism will be implemented in the aqueous environment. The rate that can be achieved with this mechanism will be significantly higher than the remaining mechanisms if the reaction occurs in an aqueous environment.

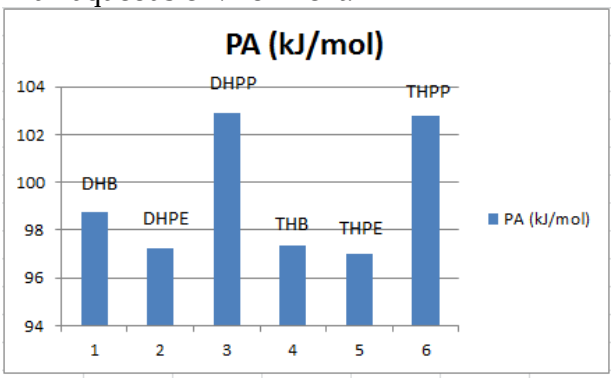

Chart 4. Proton affinity of the investigated phenolic acids.

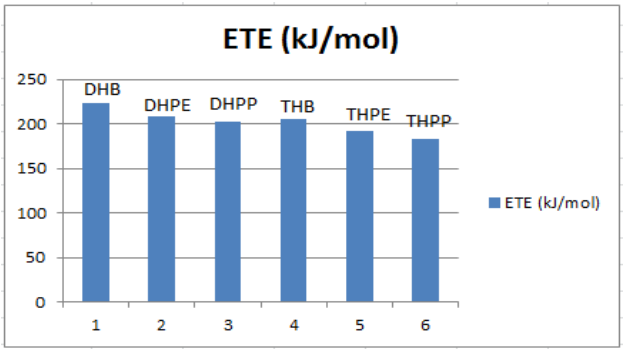

Chart 5. Enthalpy of the electron release from the phenolic anions.

CONCLUSION

- The study made it clear that the most active compound is THPP, followed by THPE, while DHB has the lowest activity against radicals.
- The trihydroxyl derivatives give away a hydrogen atom more easily than the dihydroxyl derivatives. The trihydroxyl derivatives, with the exception of THB, release an electron more easily. They are also stronger radical-scavengers than the dihydroxyl derivatives.
- The elongation of the hydrocarbon chain leads to an enhancement of the radical-scavenging activity. THB and DHB are among the least reactive compounds.
- The obtained results reveal that SPLET is the determining mechanism of O-H bond dissociation in the target molecules. It passes through the most energetically stable intermediates

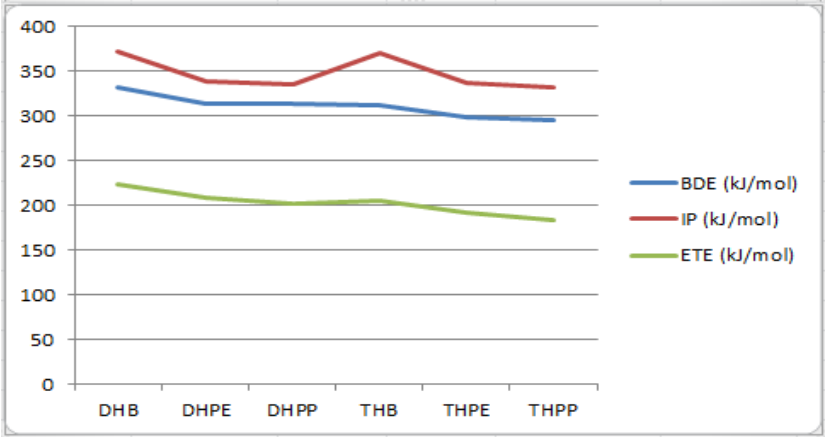

Chart 6. Comparison between BDE, IP, and ETE.

REFERENCES

1. T. Gao, Y. Ci, H. Jian, C. An, *Vib. Spec.*, **24**, 225 (2000), and refs. therein.
2. A. M. S. Silva, C. M. M. Santos, J. A. S. Cavaleiro, H. R. Tavares, F. Borges, M. Silva, Magnetic Resonance in Food Sciences. A View to the Next Century; Royal Society of Chemistry, London, United Kingdom, 2001.
3. P. Lubal, D. Siroky, D. Fetsch, J. Havel, *Talanta*, **47**, 401 (1998).
4. W. Kordel, M. Dassenakis, J. Lintelmann, S. Padberg, *Pure Appl. Chem.*, **69**, 1571 (1997).
5. L. Bravo, *Nutr. Rev.*, **56**, 317 (1998).
6. M. J. Gonzalez, D. Lopez, M. Argulies, N. H. Riordan, *Age*, **19**, 17 (1996).
7. M. Inoue, N. Sakaguchi, K. Isuzugawa, H. Tani, Y. Ogihara, *Biol. Pharm. Bull.*, **23**, 1153 (2000).
8. O. I. Aruoma, *Chem. Toxic.*, **32**, 671 (1994).
9. Y. Nakamura, K. Torikai, Y. Ohto, A. Murakami, T. Tanaka, H. Ohigashi, *Carcinogen.*, **21**, 1899 (2000).
10. E. Sergediene, K. Jonsson, H. Szymusiak, B. Tyrakowska, I. C. M. Rietjens, N. Cenas, *FEBS Lett.*, **462**, 392 (1999).
11. N. S. Khan, A. Ahmad, S. M. Hadi, *Chemico- Biol. Interact.*, **125**, 177 (2000).
12. G. Cao, E. Sofic, R. L., *Free Rad. Biol. Med.*, **22**, 749 (1997).
13. C. Castellucio, G. Paganga, N. Melikian, G. P. Bolwell, J. Pridham, J. Sampson, E. C. Rice, *FEBS Lett.*, **368**, 188 (1995).
14. S. Passi, M. Picardo, M. Nazarro-Porro, *Biochem. J.*, **245**, 537 (1987).
15. Y. Hanasaki, S. Ogawa, S. Fukui, *Free Rad. Biol. Med.*, **16**, 845 (1994).
16. C.A. Gomes, T. Gitao da Cruz, J. L. Andrade. N. Milhazes, F. Borges, and M. Paula M., *J. Med. Chem.*, **46**, 5395 (2003).
17. K. E. Heim, A.R. Tagliaferro, D. J. Bobilya, *J. Nutr. Biochem.*, **13,** 572 (2002).
18. C. H. Evans, J. C. Scaiano, K. U. Ingold, *J. Am. Chem. Soc.*, **114**, 4589 (1992).
19. K. Mukai, Y. Uemoto, M. Fukuhara, S. Nagoaka, K. Ishizu, *B. Chem. Soc. Jpn.*, **65**, 2016 (1992).
20. S.A.B.E. van Acker, D.-J van Den Berg, M.N.J.L. Tromp, D.H. Griffioen, W. P. Van Bennekom, W.J.F. van der Vijgh, A. Bast, *Free Radic. Biol. Med.*, **20,** 331 (1996).
21. M. A. Livrea, L. Tesoriere, D. X. Tan, R .J. Reiter, Radical and reactive intermediate scavenging properties of melatonin in pure chemical systems (chapter 29), in: E. Cadenas, L. Packer (Eds.). Handbook of Antioxidants, Second ed. Marcel Dekker, Inc., Los Angeles, 2002.
22. D. G. Truhlar, A. Ceulemans, M. T. Nguyen, *J. Am. Chem. Soc*., **130,** 7000 (2008).
23. T. Koopmans, *Physica*, **1**(1-6), 104 (1934).
24. M. Ernzerhof, *J. Chem. Theory Comput.*, **5**, 793 (2009).
25. J. Rimarcik, V. Lukes, E. Klein, M. Ilcin, *J. Mol. Struc.-Theochem.*, **952**, 25 (2010).
26. P. Alov, I. Tsakovska, I. Pajeva, *Curr. Top. Med. Chem.*, **15**, 85 (2015).
27. M. J. Frisch, G. W. Trucks, H. B. Schlegel, G. E. Scuseria, M. A. Robb, J. R. Cheeseman, G. Scalmani, V. Barone, B. Mennucci, G. A. Petersson, H. Nakatsuji, M. Caricato, X. Li, H. P. Hratchian, A. F. Izmaylov, J. Bloino, G. Zheng, J. L. Sonnenberg, M. Hada, M. Ehara, K.Toyota, R. Fukuda, J. Hasegawa, M. Ishida, T. Nakajima, Y. Honda, O. Kitao, H. Nakai, T. Vreven, J. A. M. Jr., J. E. Peralta, F. Ogliaro, M. Bearpark, J. J. Heyd, E. Brothers, K. N. Ku- din, V. N. Staroverov, R. Kobayashi, J. Normand, K. Raghavachari, A. Rendell, J. C. Burant, S. S. Lyengar, J. Tomasi, M. Coss, N. Rega, J. M. Millam, M. Klene, J. E. Knox, J. B. Cross, V. Bakken, C. Adamo, J. Jaramillo, R. Gomperts, R. E. Stratmann, O. Yazyev, A. J. Austin, R. Cammi, C. Pomelli, J. W. Ochterski, R. L. Martin, K. Morokuma, V. G. Zakrzewski, G. A. Voth, P. Salvador, J. J. Dannenberg, S. Dapprich, A. D. Daniels, O. Farkas, J. B.Foresman, J. V. Ortiz, J. Cioslowski and D. J. Fox, Gaussian, Inc.,Wallingford CT, 2009.
28. R. G. Parr, W. Yang, Density-Functional Theory of Atoms and Molecules, Oxford University Press. Oxford, 1989.
29. J. B. Foresman, T. A. Keith, K. B. Wiberg, J. Snoonian and M. J. Frisch, *J. Phys. Chem.*, **100**, 16098 (1996).
30. E. Klein, V. Lukes, M. Ilcin, *Chem. Phys.*, **336,** 51 (2007).

РАЗЛИЧНИ ПЪТИЩА НА ДИСОЦИАЦИЯТА НА ПАРА-О-Н ВРЪЗКАТА В ДИ- И ТРИ-ХИДРОКСИФЕНОЛНИ КИСЕЛИНИ: DFT ИЗСЛЕДВАНЕ

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(Резюме)

Фенолите могат както да инхибират, така и да засилят оксидативните нарушения в биомолекулите. Това двойствено отнасяне силно зависи от химичната им структура. Обект на това изследване са фенолни киселини с две и три хидроксилни групи и различна дължина и степен на насищане на страничната верига. Оценени са радикал-улавящата активност на съединенията, както и влиянието върху нея на различните структурни характеристики. Определен е най-подходящият механизъм за разкъсване на пара-О-Н връзката във водна среда. Проведено е DFT изследване на високо ниво с използване на B3LYP функционал в комбинация с 6-311++G(d,p) орбитален базис. Влиянието на разтворителя е оценено чрез PCM.

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A preliminary study on radical scavenging abilities of two dihydroxy-coumarins by electron paramagnetic resonance (EPR) spectroscopy

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In current research were evaluated and compared the radical scavenging abilities of two coumarins differing in positions of ortho-dihydroxyl groups in their aromatic rings. Scavenging abilities towards superoxide ($\cdot O_2^-$) and hydroxyl (·OH) radical of 7,8-dihydroxy-4-methyl-2H-chromen-2-one (**a$_1$**) and 6,7-dihydroxy-4-methyl-2H-chromen-2-one (**b$_1$**) were studied. To realize the aim of the study proper Electron Paramagnetic Resonance (EPR) spin trapping spectroscopy was applied the only technique that allows scavenging, detecting and distinguishing of short live radicals such as $\cdot O_2^-$ and ·OH species. In studied Fenton system was demonstrated higher inhibiting activity against •OH for **a$_1$** in comparison with **b$_1$**, while in hypoxanthine/xanthine oxidase system **b$_1$** exhibited better inhibiting ability against $\bullet O_2^-$ generation.

Keywords: Dihydroxy-coumarins, EPR spin trapping, Superoxide anion radical, Hydroxyl radical

INTRODUCTION

Antioxidants are important species possessing ability to protect the living organisms from damages caused by free radical-induced oxidative stress [1]. Antioxidant was defined as any substance that directly scavenges reactive oxygen species (ROS) or indirectly acts to up-regulate antioxidant defense or inhibit ROS production" [2]. In a number of studies, have been demonstrated different antioxidants exhibiting selective scavenging activity towards various ROS [3]. Unfortunately, under various pathophysiological conditions, human antioxidative defense system, fails to eliminate the excess of ROS. Therefore, there is continuous demand for exogenous antioxidants in order to prevent oxidative stress, representing a disequilibrium redox state in favor of oxidation. However, high doses of isolated compounds may be toxic, owing to pro-oxidative effects at high concentrations or their potential to react with beneficial concentrations of ROS normally present at physiological conditions that are required for optimal cellular functioning. Coumarins are an important class of oxygen heterocycles, widespread in nature occurring in a lot of green plants as well as in fungi and bacteria [4, 5]. They greatly attract the attention of researchers because possess diverse pharmacological properties [6, 7]. For coumarins have been reported a remarkable range of biological activities that include inhibition of xanthine oxidase and direct scavenging of harmful ROS produced by enzymes other than xanthine oxidase [8, 9]. Moreover, EPR spectroscopy is the only analytical technique for direct and indirect detection of stable and unstable free radicals in chemical, physical and biological systems. Methods, based on EPR spectroscopy, are widely used because can detect paramagnetic species without interference from the sample properties, including the phase of the sample (solid, liquid or gas) [10]. As far as we are informed EPR spectroscopy studies on scavenging activity against the superoxide and hydroxyl radicals of 7,8 and 6,7-dihydroxy 4-methyl coumarins are quite scarce. All above facts prompted us to investigate the antioxidant activity of 7,8-dihydroxy-4-methylcoumarin (**a$_1$**) and 6,7-dihydroxy-4-methylcoumarin (**b$_1$**) to scavenge hydroxyl and superoxide radicals using EPR spin trapping technique and evaluate the effect of different positioning of o-dihydroxyl groups in their aromatic rings on radical scavenging ability.

EXPERIMENTAL

Materials

Coumarins: 7,8-dihydroxy-4-methyl-2H-chromen-2-one (**a$_1$**) and 6,7-dihydroxy-4-methyl-2H-chromen-2-one (**b$_1$**) (Fig. 1) were synthesized and characterized at the Department of Chemistry, University of Delhi, Delhi as described formerly [11-13].

Spin trap 5-*tert*-butoxycarbonyl-5-methyl-1-pyrroline *N*-oxide (BMPO), diethylene triamine pentaacetic acid (DTPA), hypoxanthine, xanthine oxidase, iron (II) sulfate heptahydrate, hydrogen

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peroxide and ethanol were purchased from Sigma-Aldrich Chemical Co, St. Louis, USA. All other chemicals used in this study were of analytical grade. Deionized and distilled water was used for all experiments.

Methods

In vitro EPR spectroscopy experiments

EPR measurements of studied coumarins were performed at room temperature (18-23°C) on an X-band EMX^{micro}, spectrometer Bruker, Germany, equipped with a standard resonator. Quartz capillaries were used as sample tubes. The capillary tubes were sealed and placed inside a standard EPR quartz tube (i.d. 3 mm) that was fixed in the EPR cavity. Due to insufficient coumarins quantity we had, it was not possible to determine their IC_{50} – the concentration inhibiting 50 percent of the generated superoxide ($\cdot O_2^-$**)** or hydroxyl (·OH) radicals. EPR experiments were carried out in triplicate. All results presented are based on averaging of three independent EPR measurements. Spectral processing was performed using Bruker WIN-EPR and SimFonia software.

Coumarins **a$_1$** and **b$_1$** were dissolved in ethanol at increasing concentrations as indicated in Tables 1 and 2.

EPR study on in vitro generated superoxide anion radicals

Superoxide anion scavenging activity was determined by the EPR method and hypoxanthine/xanthine oxidase system was used to generate $\cdot O_2^-$ [14]. Reaction was initiated by adding of xanthine oxidase (XO) and 5-*tert*-butoxycarbonyl 5-methyl-1-pyrroline *N* – oxide (BMPO) used as a specific spin trap for the superoxide anion radicals [15]. Final volume of reaction mixture was 200 µl and contained: 50 µl of 1mM of hypoxanthine dissolved in 50 mM phosphate buffer (pH 7.4, containing 50 µM of DTPA as a transition metal chelator), 20 µl of 100 mM BMPO in phosphate buffer, 50 µl of the studied coumarin concentration, 30 µl of phosphate buffer and 50 µl of xanthine oxidase 1 U/ml dissolved in phosphate buffer. Control sample contained 80 µl of phosphate buffer instead of 30 µl. EPR spectra were recorded at the 5th min after the reaction starts. The effect of **a$_1$** and **b$_1$** on *in vitro* generated superoxide anion radicals was evaluated according to the equation:

% O_2^- radicals scavenged by BMPO = [I/Io] × 100 %

where: Io - double integrated plot of the EPR spectrum of BMPO/·OOH adduct registered in the control sample; I - double integrated plot of the EPR spectrum of BMPO/·OOH spin adduct registered after addition of the tested sample.

EPR settings were as follows: center field 3505 G, sweep width 100 G, microwave power 12.62 mW, modulation amplitude 10 G, receiver gain 2.52×10^4, time constant 40.96 ms, sweep time 40.96 s, 1 scan per sample.

EPR study on in vitro generated hydroxyl radicals (·OH)

To evaluate the effect on *in vitro* gen*erated ·OH both extracts were examined by the EPR method described by Wang *et al.* [16] with modifications. The reaction mixture contained 40 µl of 20 mM BMPO, 40 µl of 0.2 mM $FeSO_4$ freshly prepared, 80 µl of the studied coumarin concentration and 100 µl of 2mM H_2O_2. Control sample contained 80 µl of distilled water instead of coumarin tested. The EPR spectra were recorded 5 min after the start of reaction. The effects of both extracts on *in vitro* generated hydroxyl radicals were evaluated according to the equation:

% ·OH radicals scavenged by BMPO = [I / Io] × 100 %

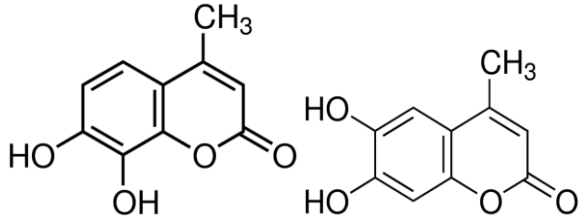
where: Io – double integrated plot of the EPR spectrum of BMPO/·OH spin adduct registered in the control sample; I - double integrated plot of the EPR spectrum of BMPO spin adduct registered after addition of the tested sample containing the corresponding coumarin concentration.

EPR settings were as follows: center field 3505 G, sweep width 100 G, microwave power 8.02 mW, gain 2.52×10^4, modulation amplitude 5 G, time constant 163.84 ms, sweep time 81.92 s, 5 scans per sample.

RESULTS AND DISCUSSION

It is well known that coumarins as natural products found in plants possess remarkable bioactivities including XO inhibition [17]. It should be noted that unlike most other XO inhibitors they act as radical scavengers against ROS produced by enzymes other than XO [18]. The superoxide anion radical ($\cdot O_2^-$), called the "primary" ROS is considered the most important physiologically generated radical [3,19,20]. *In vivo* overproduced $\cdot O_2^-$ directly interacts with other molecules, as well as through enzyme- or metal-catalyzed processes and causes generation of the "secondary" ROS such as H_2O_2, peroxyl (ROO^-),·OH. In general, the least ambiguous technique for detecting and characterizing free radicals at *in vitro* and *in vivo* conditions is EPR spectroscopy. However, direct detection of short lived radicals (e.g. superoxide

and hydroxyl radicals) is very difficult or impossible at room temperature. EPR spin trapping technique is the only one that allows scavenging, detecting and distinguishing of such species. Spin traps used in EPR spectroscopy are not radicals but can form stable radical adducts with short-lived radicals, generated *in vitro* or *in vivo*. In the present research BMPO was selected for *in vitro* evaluation of the radical scavenging abilities of the studied coumarins, because it can form stable and distinguishable spin adducts with superoxide and hydroxyl radicals [15]. In the system generating $\cdot O_2^-$ containing only BMPO (control sample) an EPR spectrum consisting of four spectral lines with equal intensity was recorded and identified as a BMPO/·OOH spin adduct (*conformer I*, Fig. 2A) [15]. The effect of **a₁** and **b₁** on the levels of generated $\cdot O_2^-$ in hypoxanthine/xanthine oxidase system is given in Table 1.



a₁ **b₁**

Figure 1. 7,8-dihydroxy-4-methyl-2H-chromen-2-one (**a₁**) and 6,7-dihydroxy-4-methyl-2H-chromen-2-one (**b₁**).

As can be seen, with an increase in **a₁** or **b₁** concentration, the percentage of $\cdot O_2^-$ radicals scavenged by BMPO decreases, which means that coumarins ability to scavenge $\cdot O_2^-$ also increases. Paya and co-workers [4], using a spectrophotometric technique, studied the reduction of ferricytochrome ***c*** to ferrocytochrome ***c*** depending on the amount of $\cdot O_2^-$ radicals generated in a hypoxanthine/xanthine oxidase system. They found **a₁** and **b₁** at a concentration of 100 μM and lower than 10 μM were not capable to scavenge $\cdot O_2^-$. Other research groups using systems containing hypoxanthine or xanthine and xanthine oxidase also demonstrated the abilities of *ortho*-dihydroxy coumarins to inhibit $\cdot O_2^-$ generation [8, 9]. By *in vitro* assays Hofmann and co-workers [8] investigated the inhibitory potencies against isolated XO of 18 coumarins differing in number and position of the hydroxyl groups at C6, C7, C8 and substituents at C4. They found that esculetin possessing a hydroxyl group at C6 and C7 exhibited the highest XO inhibitory potency comparing to the rest coumarins including those possessing OH groups at C7 and C8. Using computational ligand docking the same authors demonstrated snugly esculetin accommodation to the binding (active) site of XO due to formation of a hydrogen bond between the hydroxyl group at esculetin's C6 and the side chain of Glu802 whereas a second one involved the carbonyl oxygen and the guanidine group of Arg880 in enzyme [8]. Šeršeň and Lácová [9] reported very good scavenging against $\cdot O_2^-$ either for 7,8-dihydroxylated or 6,7- dihydroxylated coumarins but in contrary found almost twice higher activity for **a₁** comparing to esculetin. Current research shows that **a₁** and **b₁** can inhibit superoxide generation at a concentration of 10 μM and lower. Based on the other authors' and current results, we accept that two mechanisms can be involved through which both coumarins reduce superoxide generation: a) direct scavenging of $\cdot O_2^-$ and b) XO inhibition by binding the corresponding coumarin to enzyme active side. We believe that higher IC_{50} of the two coumarins (> 10μM) reported by the other authors [8, 9] are due to the use of spectrophotometric techniques whose sensitivity is considerably lower comparing to EPR spectroscopy used in current research. The fact that **b₁** exhibits higher inhibiting activity comparing to **a₁** at every studied concentration (see Table 1) we can explain by more effective XO inhibition due to C6 hydroxyl group presents in **b₁** structure like as in esculetin [8].

Table 1 Percent scavenged $\cdot O_2^-$ radicals by BMPO in presence of studied coumarins.

Concentration (μmol/ml)	**a₁*** (% scavenged O_2^- by BMPO)	**b₁*** (% scavenged O_2^- by BMPO)
2.5	62.71	27.99
5	54.85	13.68
10	45.05	7.3

***Averaged value of three independent measurements with ±SD ≤ 5 %

Despite the reaction with •OH is not as specific as that with DPPH and $\cdot O_2^-$, it is used in many studies to assess the antioxidant activity of natural extracts, fractions and substances [21-25]. In the present research after addition of BMPO to the system generating •OH the typical EPR spectrum of a spin adduct between hydroxyl radical and the spin trap BMPO was registered (Fig. 2**B**) and identified as BMPO/•OH *conformer II* [15, 26].

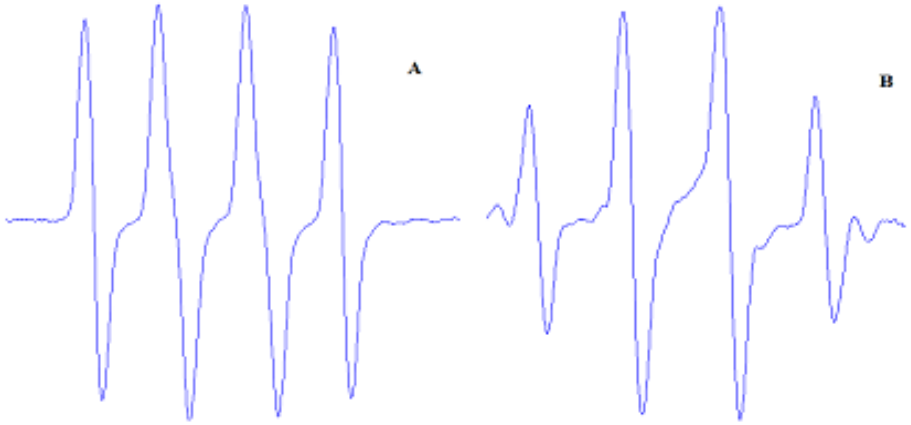


Figure 2. **A)** EPR spectrum of BMPO/•OOH spin adduct (*conformer I*) and **B)** EPR spectrum of BMPO/•OH spin adduct (*conformer II*)

As is seen in Table 2, when $\mathbf{a_l}$ and $\mathbf{b_1}$ concentrations added to the control sample increased, the amount of hydroxyl radicals scavenged by BMPO decreased, demonstrating the antioxidant behavior of the studied coumarins.

Table 2. Percent scavenged·OH radicals by BMPO in presence of studied coumarins.

Concentration (μmol/ml)	$\mathbf{a_1}$* % scavenged ·OH by BMPO	$\mathbf{b_1}$* % scavenged ·OH by BMPO
2.5	41.68	85.24
5	34.58	45.97
10	32.25	19.67

*Averaged value of three independent measurements with ±SD ≤ 5 %

Although IC_{50} value was not determined, obviously, it is lower than 2.5 μM for $\mathbf{a_1}$ and is between 2.5 μM and 5 μM for $\mathbf{b_1}$ (see Table 2). Moreover, $\mathbf{a_1}$ manifested itself as a more active compound in the studied Fenton system. This result is supported by findings of other research teams using EPR spin trapping technique and a Fenton system with the same composition as ours [9] or spectrophotometric technique and a Fenton system with different constituents [4]. In several studies well expressed metal chelating abilities towards ferric [4] or ferrous ions [28] were found for coumarins possessing ortho-dihydroxy phenol structures. Using the same Fenton system and spin trapping EPR spectroscopy, Šeršeň and Lácová [9] determined 9.18 μM IC_{50} for $\mathbf{a_1}$ and 57.04 μM for esculetin (6,7-dihydroxycoumarin) values higher than ours. It should be noted that this research group used DMPO spin trap for ·OH scavenging instead of BMPO. As was mentioned above, spin trap BMPO is the most suitable one for the specific *in vivo* or *in vitro* detection of superoxide and hydroxyl radicals by EPR spin trapping spectroscopy. The well-established fact that the DMPO spin trap does not easily distinguish superoxide and hydroxyl radical because of spontaneous decay of the DMPO-superoxide adduct into DMPO-hydroxyl adduct [15, 29] made us believe that the results obtained using BMPO are more reliable than those of Šeršeň and co-workers [9]. Paya et al. [4], by spectrophotometric technique using two systems based on Fenton chemistry, evaluated interaction of coumarins with •OH generated in slow rate ($FeCl_3$-EDTA and H_2O_2) and rapid rate ($FeCl_3$-ascorbate and H_2O_2) system. In the first system they found accelerated hydroxyl radical formation (pro-oxidant activity) for all 6,7- and 7,8-dihydroxy coumarins including $\mathbf{a_1}$ and $\mathbf{b_1}$. Contrary, in the second (rapid rate) system containing $FeCl_3$-ascorbate and H_2O_2, all 6.7- and 7,8-*ortho*-dihydroxyl coumarins exhibit striking inhibition of site-specific deoxyribose degradation induced by iron ions similar to desferrioxamine - a typical iron chelator [4]. These authors explain the ·OH inhibiting activity of ortho-dihydroxy coumarins with their ability to chelate ferric (Fe^{3+}) ions. On the other hand, many studies in relation with metal chelating ability of different natural products and compounds demonstrated that their ferrous (Fe^{2+}) ions chelating ability was due to the presence of ortho-dihydroxy phenol structures. [29,30]. In relation to the mechanism that was involved in $\mathbf{a_1}$ and $\mathbf{b_1}$ abilities to reduce the amount of ·OH radicals generated we accepted that both coumarins were able to scavenge either directly·OH (confirmed by EPR spectroscopy) or through chelation of ferrous ions present in the system by their ortho-dihydroxy phenol structure. We also assumed that the higher inhibiting potency against •OH generation demonstrated by $\mathbf{a_1}$ was due to its higher chelating abilities towards Fe^{2+} ions. The significantly low concentrations at which both coumarins exhibited inhibitory effect against hydroxyl radical generation can be explained on

one hand with the high sensitivity of the EPR spectroscopic technique and on the other with the use of BMPO as a spin trapping agent.

CONCLUSION

For the first time, by EPR spectroscopy combined with an appropriate spin trap, it was demonstrated that at concentrations lower than 10 μM, the two coumarins **a₁** and **b₁** showed *in vitro* a well expressed inhibitory effect on superoxide and hydroxyl radical generation. Results obtained show that **a₁** exhibits a higher scavenging activity against $^{\bullet}OH$ than **b₁** in the Fenton system whereas in the hypoxanthine/xanthine oxidase system **b₁** exhibits a higher ability to inhibit formation of $^{\bullet}O_2^-$. Given the various biological effects reported for coumarins, we have planned further detailed EPR studies with **a₁** and **b₁** to determine their IC_{50} values, to investigate the exact mechanisms of interaction with various ROS and also to evaluate their abilities for reducing oxidative damages at *in vivo* conditions.

REFERENCES

1. J.M. Čanadanović-Brunet, S.M. Djilas, G.S. Cetkovic, *J. Sci. Food. Agric*. **85**, 265 (2005).
2. A.I. Khlebnikov, I.A. Schepetkin, N.G. Domina, L.N. Kirpotina, M.T. Quinn, *Bioorg. Med. Chem.* **15**: 1749 (2007).
3. M. Wojcik, I. Burzynska-Pedziwiatr, L.A .Wozniak, *Curr. Med. Chem.*, **17**, 3262, (2010).
4. M. Paya, B. Halliwell, R.S. Hiult, *Biochem. Pharmacol*., **44**(**2**)**,** 205, (1992).
5. F. Borges, F. Roleira, N. Milhazes, L. Santana, E. Uriarte, *Curr. Med. Chem*. **12**, 887 (2005).
6. J.R. Hoult, M. Paya, *Gen. Pharmacol*. **27,** 713 (1996).
7. V.D.Kancheva, A.K. Slavova-Kazakova, S.E. Angelova, S.K. Singh, S. Malhotra, B.K. Singh, L. Saso, A.K. Prasad, V.S. Parmar, *Biochimie*, **140,** 130 (2017).
8. E. Hoffman, J. Webster, T. Kidd, R. Kline, M. Jayasinghe, S. Paula, *Int. J. Biosci. Biochem. Bioinfor*., **4**(4)**,** 234 (2014).
9. F. Šeršeň, M. Lácová, *Acta Fac. Pharm. Univ. Comen., LXII*, (Suppl. IX), 41 (2015).
10. M. Kohno, *J. Clin. Biochem. Nutr*., **47**: 1 (2010).
31.
11. V. Kumar, S. Tomar, R. Patel, A. Yousaf, V.S. Parmar, S.V. Malhorta, *Synth. Commun*., **38**, 2646 (2008).
12. S. Tomar, PhD Thesis, Department of Chemistry, Universuity of Delhi, 2010.
13. S.S. Bahekar, D. B. Shinde, *Tetrahedron Lett*., **45**, 7999 (2004).
14. N. Noda, M. Kohno, A. Mori, L. Packer, *Methods Enzymol.*, **299**, 28 (1999).
15. H. Zhao, J. Joseph, H. Zhang, H. Karoui, B. Kalyanaraman, *Free Radic. Biol. Med.*, **31(5)**, 599 (2001).
16. C.Y. Wang, S.Y. Wang, J-J. Yin, J. Parry, L.L. Yu, *J. Agric. Food Chem*., **55**, 6527 (2007).
17. W. S. Chang, H. C. Chiang, *Anticancer Res*., **15**, 1969 (1995).
18. H.C. Lin, S.H. Tsai, C.S. Chen, Y.C. Chang, C.M. Lee, Z.Y. Lai, C.M. Lin, *Biochem. Pharmacol*., **75(6),** 1416 (2008).
19. I. Fridovich, *Arch. Biochem. Biophys*., **247**, 1 (1986).
20. M. Valko, D. Leibfritz, J. Moncol, M.T. Cronin, M. Mazur, J. Telser, *Int. J. Biochem. Cell Biol.*, **39**, 44 (2007).
21. P. Valentao, E. Fernandes, F. Carvalho, P.B. Andrade, R.M. Seabra, M.L**.** Bastos, *Biol. Pharm. Bull*., **25**(10), 1320 (2002).
22. F.A. Hashen, *J. Med. Sci*., **7**(6), 1027 (2007).
23. Ch. Li, Y. Li, Ch. Yao, W. Wang, *J. Med. Plants Res*., **6(11**), 2224 (2012).
24. R. Indumathy, A. Aruna, *Int. J. Pharm. Pharmaceut. Sci*., **5**(4), 634 (2013).
25. D.A. Raj, T.A. Malarvili, S. Velavan, *Int. J. Res. Biochem. Biophys*., **3**(4), 29 (2013).
26. S.S. Eaton, G.R. Eaton, L.J. Berliner, *Biomedical EPR*, **23**, 80 (2005).
27. T**.** Filipsky, M. Riha, K. Macakova, E. Anzenbacherova, J. Karlickova, P. Mladenka, *Curr. Top. Med. Chem.*, **15**(5), 415 (2015).
28. N. Khan, C.M. Wilmot, G.M. Rosen, E. Demidenko, J. Sun, J. Joseph, J. O'Hara, B. Kalyanaraman, H.M. Swartz, *Free Radic. Biol. Med*., **34**, 1473 (2003).
29. S. Khokhar, R.K.O. Apenten, *Food Chem.*, **81,** 133 (2003).
30. M. Andjelković, J.V. Camp , B.D. Meulenaer, G. Depaemelaere, C. Socaciu, M. Verloo, R. Verhe, *Food Chem*., **98**(**1**), 23 (2006).

ПРЕДВАРИТЕЛНО ПРОУЧВАНЕ НА РАДИКАЛ-УЛАВЯЩИТЕ СПОСОБНОСТИ НА ДВА ДИХИДРОКСИ-КУМАРИНА ЧРЕЗ EPR СПЕКТРОСКОПИЯ

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(Резюме)

В настоящото изследване са оценени и сравнени възможностите за радикал-улавящата способност на два кумарина, които се различават в позициите на орто-дихидроксилните групи в техните ароматни пръстени. Изследвана е способността на 7,8-дихидрокси-4-метил-2H-хромен-2-он (**а$_1$**) и 6,7-дихидрокси-4-метил-2H-хромен-2-он (**б$_1$**) да улавят супероксидния (·O_2^-**)** и хидроксилния (·OH) радикал. За да се осъществи целта на това изследване е използвана подходяща спин-улавяща EPR спектроскопия техника, която е единствената техника, позволяваща улавяне, откриване и разграничаване на краткоживущи радикали, като например: ·O_2^- и ·OH видове. Получените резултати показват, че **а$_1$** проявява по-висока радикал-улавяща активност срещу ·OH в сравнение с **б$_1$** във Fenton системата, докато в хипоксантино / ксантин оксидазна система **б$_1$** проявява по-висока способност да инхибира образуването на·O_2^-.

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Near-infrared spectroscopy as a tool for rapid estimation of the antioxidant capacity of red wines

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The present investigation evaluates the feasibility of using near-infrared (NIR) spectroscopy as an accurate, fast and non-destructive analytical tool for estimation of the antioxidant properties of red wines. The evaluation of the antioxidant properties was conducted using ABTS and DPPH assays for total antioxidant activity determination. NIR measurements were performed by NIRQuest 512 spectrophotometer in the region 900-1700 nm using transmittance mode. Partial least-square regression with internal cross-validation was used for calibration models development for determination of tested parameters and SIMCA for creation of model for classification on the base of the spectral data. All the investigated wines have demonstrated better antioxidant properties in the ABTS model system compared to the DPPH one. Although the observed activity highly varied, the wines containing varieties Syrah and Malbec presented better antioxidant effect in both model systems. The Merlot wines produced from the vineyard situated on the southern slopes of Sakar Mountain in the time interval of 2012 – 2016 denoted a tendency of decreasing SV_{50} values from year to year. In both model systems Merlot 2016 demonstrated the best antioxidant effect correlating to the lowest SV_{50} values. The determination of the antioxidant activity of the tested wines on the basis of their spectra in the NIR region revealed a high degree of accuracy of estimation. This indicates that NIR spectroscopy could be a promising technique in quantitative determination of antioxidant activity and building classification models for discrimination of wines according to their antioxidant properties.

Keywords: Wine, Antioxidant activity, ABTS, DPPH, NIR spectroscopy

INTRODUCTION

During the years the customers' taste and preferences have changed in response to health concerns. The fact that the increased consumption of products rich of natural polyphenols like grapes, cherries and berries is associated with diminished rick of cardiovascular disease has attached particular attention to red wine products due to the abundant content of biologically active compounds in them [1, 2].

The phenolic compounds (anthocyanidins, flavanols, flavones, flavanones, cynnamic acids and stilbenes) which have important impact on the organoleptic characteristics of the wine product are some of its most important quality parameters. There are literature data revealing their multiple biological activities associated with anti-carcinogenic, antiviral, antibacterial and anti-inflammatory effects mainly attributed to their antiradical activity and powerful antioxidant properties [3, 4].

The customers' desire for quality products possessing health benefits and the increasing number of available new wines on the market has created the necessity of the development of a fast and easy way to operate, accurate analytical tool for the quantitative determination of these bioactive compounds.

During the years several methods have been used for screening of the chemical composition and food quality determination and prediction – high-performance liquid chromatography (HPLC), near-infrared spectroscopy (NIR), mass spectrometry (MS), capillary electrophoresis in combination with different electrochemical (EC) detectors [5]. From all of them NIR spectroscopy has proven itself as a perfect technology for routine analysis in the food industry due to the speed of the performed analysis and its low cost [6]. It is a non-destructive method, requiring minimal or no sample preparation which has shown good prediction abilities in the determination of the content of flavanols, anthocyanins and other phenolic compounds [7-9].

The objective of the present investigation was to prove the applicability of the NIR spectroscopy as an analytical tool for estimation of the antioxidant properties of red wines and to verify its effectiveness in building classification models for

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discrimination of wine according to their antioxidant properties.

EXPERIMENTAL

Wine samples

The analysis was carried out using a total of 35 wine samples – 33 of them were Bulgarian red wines. All samples were from commercially available wines produced from a wide range of different varieties. Details for the origin and the principal grape variety are presented in Table 1. Ten wines (numbered from 1 to 10) were supplied from a winery vineyard situated on the southern slopes of Sakar Mountain – all of them produced using the same wine making technology. Five of the samples (from 1 to 5) were selected to present variation in the age (years ranging from 2012 to 2016) and six of them – grape varieties (from 5 to 10).

Spectrophotometric model systems

To determine the antioxidant properties of the wine samples we have used test systems containing stable ABTS radical cations and DPPH radicals. Despite the fact that both radicals are foreign to the living organisms the combination of these methods is the most commonly used approach for *in vitro* assessment of the antioxidant activity (AOA) of complex samples. The methods are fast, reliable and reproducible. The scavenge of ABTS radical cation and DPPH radical has a different mechanism (SET for $ABTS^{\bullet+}$ and HAT for $DPPH^{\bullet}$). The simultaneous use of both methods provides more accurate evaluation and interpretation of the character of the observed antioxidant properties.

Both methods are based on the ability of the tested compounds to scavenge the pre-formed $ABTS^{\bullet+}$ or $DPPH^{\bullet}$ radicals, which is resulting in decolorization (decrease of the absorbance) of the sample solution proportional to the extent of the radical reduction. The latter is used as a determinant of the antioxidant activity. Stronger antioxidant properties of the tested compounds are associated with higher extent of suppression of the absorbance of the solution.

ABTS method – the experimental part was accomplished according to Re *et al.* [10]. The assay utilizes the free mono-cation radical of 2'2-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid), generated by the oxidation of ABTS with potassium persulfate. The working solution was obtained by diluting the preformed $ABTS^{\bullet+}$ in buffer solution - K_2HPO_4/KH_2PO_4, pH 7.4 to produce a final solution with absorbance of 0.700 ± 0.002 at 734 nm. The reduction of the absorbance of 2 ml of the $ABTS^{\bullet+}$ by different volumes of a 2% solution of the bottled wine product was measured at 734 nm after 1 hour incubation. Fresh radical solution was prepared for each experiment.

DPPH method – the procedure was performed according to Goupy *et al.* [11]. A working purple colored solution of the DPPH radical in ethanol with absorbance of 0.900 ± 0.003 at 517 nm was prepared. Different volumes of a 2% solution of the bottled wine product were mixed with 2 ml of it. After 60 min incubation the absorbance of the probes was measured at 517 nm.

All analyses were performed in triplicate. The obtained results were presented as means ± S.D. The AOA of the wine samples was estimated using the equation:

$$AOA\% = \frac{A_{control} - A_{sample}}{A_{control}} \bullet 100\%$$

Considering the observed results for AOA, the volume of bottled wine product, which reduces the quantity of free radicals in the final reaction volume of the system by 50% was calculated – SV_{50} (scavenge volume 50) The smaller the SV_{50} the higher is the antioxidant potential of the tested wine.

NIR spectral measurements and data analysis

The near-infrared analysis was performed using NIRQuest 512 spectrometer (Ocean Optics, Inc) in the region of 900-1700 nm using transmittance mode and 10 mm cuvette.

A commercial software program – Pirouette Version 4.5 (Infometrix, Woodinville, WA) was used to process the obtained data. PLS regression was used for quantitative determination of SV_{50} on the base of spectral data. The calibration equations for each parameter were developed and validated with leave-one-out cross validation. Leave-one-out method is recommended when a few samples are used to build the calibration equations. The leave-one-out cross-validation routine works by omitting one observation once a time, recalculating the equation using the remaining data, and then predicting the omitted observation. This routine is repeated until each observation in the dataset is used once as validation data. Finally, any validation errors generated are combined into a standard error of cross-validation SECV.

The prediction capacity of each calibration equation was evaluated using R – multiple correlation coefficients between reference values and NIR spectra, SEC – standard error of calibration, SECV – standard errors of cross validation and the ratio performance deviation

(RPD) parameter, which can be defined as the relationship between the standard deviation of the chemical method (SD) and the standard error of cross-validation SECV in the NIR model. The RPD evaluated the prediction errors in light of the standard deviation of the reference data and thus enables comparison between models for constituents with different variation ranges. The RPD values showed levels of prediction accuracy as follows: RPD between 2.0 and 2.5 indicates good prediction; RPD between 2.5 and 3.0 indicates very good prediction; and RPD > 3 indicates excellent prediction.

Soft independent modeling of class analogy (SIMCA) was performed to classify samples according to their antioxidant capacity. SIMCA is a supervised classification method and develops models for each initially determined group of samples (class) based on principal components analysis (PCA). SIMCA first centers and then compresses raw data by means of PCA, and a multidimensional space is constructed containing the scores corresponding to each class. Each class model treats new samples separately, and an assessment of cluster membership is made on the basis of the distance of any given sample to the center of the cluster.

Samples were divided into two classes with low and high antioxidant capacity according to SV_{50} values, estimated by DPPT method. The threshold value was set to be 3.0 μl.

RESULTS AND DISCUSSION

The radical scavenging activity of the red wine samples was tested in model systems containing stable free radicals – ABTS and DPPH. The results from the performed experiments and additional information for the wines (brand, varieties and vintage) are presented in Table 1. The examined wines showed antioxidant activity in both model systems.

Comparing the results from both model systems presented in Table 1 it is evident that all samples exhibit significant differences in their reduction activities against the ABTS and DPPH radicals. The wines demonstrate changeable antioxidant activity in each model system, which is due to the different wine varieties, vintages and differences in the production technologies. All wine samples denote almost twice higher efficiency against the ABTS radical (SV_{50} from 0.919 μl to 2.154 μl) in comparison to the DPPH radical (SV_{50} from 2.170 μl to 4.850 μl). Although the observed activity highly varies the wines containing varieties Syrah and Malbec presented better antioxidant effect in both model systems.

Due to the fact that the different wine making techniques are an important factor affecting the phenolic levels of the obtained final product and respectively its antioxidant properties we have included in our investigation eleven red Bulgarian wines purchased from winery which vineyard is situated on the southern slopes of Sakar Mountain. The fact that all wines were produced using the same wine making technology will give us the possibility to compare the antioxidant properties of wines produced from diverse varieties and vintages from this region.

The comparison of the data from the Merlot wines produced from the same vineyard in the time interval of 2012 – 2016 denoted a tendency of decreasing SV_{50} values from year to year (Table 1 lines 1-5). In both model systems Merlot 2016 demonstrated the best antioxidant effect corresponding to the lowest SV_{50} value.

The NIR spectra of the tested red wine samples are presented in Figure 1a. The NIR spectrum of wine in the 900-1700 nm region is dominated by a large absorption band around 1400 nm that corresponds to OH bonds of water.

Second derivatives of the spectra of measured wine samples are presented in Figure 1b. The second derivative technique is often used approach in processing NIR data. It gives the possibility to separate the overlapping absorbance bands, to remove the baseline shifts and to increase the apparent spectral resolution. The broad absorption maximum around 1400 nm is composed from two separate maxima at 1349 and 1395 nm, connected with C-H and O-H bonds. The other intensive maxima at 976 and 1196 nm are connected with second overtone of O-H and C-H bonds, respectively. The biggest variations in the spectral data are observed between 1300 and 1400 nm.

Statistical parameters from the calibration procedure for NIRS evaluation of antioxidant activity of examined red wine samples are presented in Table 2.

Table 1. Details of red wines analyzed for antioxidant properties and SV_{50} values

	Year	Origin	Principal grapes	SV_{50} [μl] ABTS	SV_{50} [μl] DPPH
1	2012	Harmanly	Merlot	1.11	2.71
2	2013	Harmanly	Merlot	1.10	2.84
3	2014	Harmanly	Merlot	1.07	2.71
4	2015	Harmanly	Merlot	1.01	2.51
5	2016	Harmanly	Merlot	1.01	2.33
6	2016	Harmanly	Cabernet franc	1.01	2.84
7	2016	Harmanly	Cabernet sauvignon	0.92	2.17
8	2016	Harmanly	Syrah	1.00	2.30
9	2016	Harmanly	Malbec	0.96	2.28
10	2016	Harmanly	Cabernet sauvignon, Merlot, Syrah, Pamid	1.16	2.54
11	2014	Suhindol	Cabernet sauvignon	1.30	3.03
12	2013	Stara Zagora Thracian Plain	Cabernet sauvignon	1.08	2.45
13	2013	Stara Zagora Thracian Plain	Cabernet sauvignon, Merlot & Syrah,	1.05	2.45
14	2013	Pleven	Merlot	1.37	2.95
15	2013	Thracian Plain	Merlot	1.22	2.76
16	2013	Vidin	Cabernet sauvignon & Syrah	1.35	3.04
17	2013	Yambol	Merlot	1.32	3.55
18	2014	Thracian Plain	Merlot & Maller	1.19	3.07
19	2013	Asenovgrad	Merlot	1.52	3.72
20	2013	Stara Zagora	Merlot	1.45	3.59
21	2013	South Africa	Merlot	1.54	3.37
22	2014	Chilean Central Valley	Merlot	1.18	3.25
23	2013	Sakar Mountain	Cabernet sauvignon & Syrah	1.12	2.83
24	2014	Svilengrad	Merlot & Malbec	1.11	3.10
25	2015	Thracian Plain	Beaujolais	1.30	3.92
26	2013	Thracian Plain	Cabernet sauvignon	1.43	3.26
27	2013	Montana	Cabernet sauvignon	2.15	4.58
28	2013	Montana	Cabernet sauvignon & Merlot	1.30	2.97
29	2013	Veliki Preslav	Merlot	1.33	3.82
30	2013	Thracian Plain	Cabernet sauvignon	1.19	3.42
31	2013	Stara Zagora	Cabernet franc	1.26	3.49
32	2013	Thracian Plain	Cabernet sauvignon	1.15	3.01
33	2013	Danube plain	Merlot	1.21	3.47
34	2013	Italy	Barbera d'Asti	1.78	4.85
35	2013	Sakar Mountain	Cabernet sauvignon, Merlot & Syrah	0.99	2.50

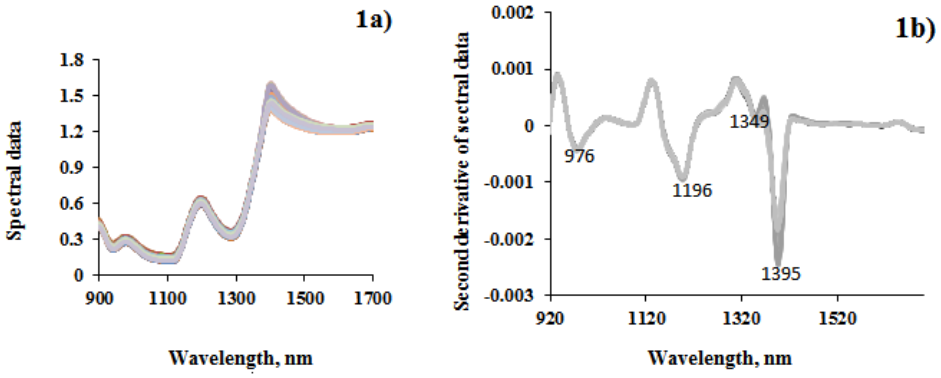

Figure 1. Near-IR spectra of wine samples (1a) and their second derivative transformation (1b).

Table 2. Statistical data of the calibration equations for NIRS determination of AOA of the red wine samples.

Method	SECV	Rcv	SEC	Rcal	RPD
DPPH	0.154	0.97	0.152	0.97	4.08
ABTS	0.079	0.94	0.077	0.95	3.06

R – multiple correlation coefficients, SEC – standard error of calibration, SECV – standard errors of cross validation, RPD – relationship between the standard deviation of the chemical method (SD) and the standard error of cross-validation SECV in the NIR model.

The obtained multiple correlation coefficients R between reference values and NIR spectra were bigger than 0.94 and the ratio performance deviation RPD parameter bigger than 3, which showed excellent prediction abilities. Graphical illustration of the accuracy of determination of tested parameters is presented in Figure 2.

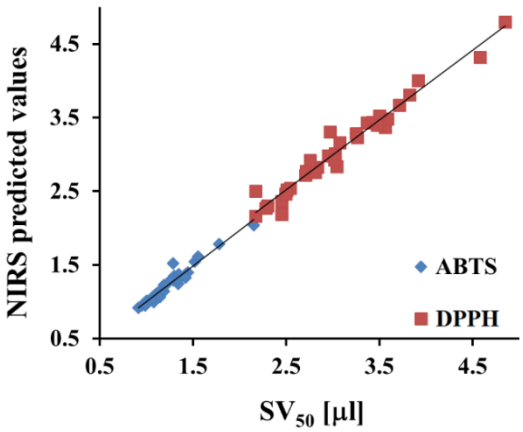

Figure 2. Scatter plots of laboratory measured and NIRS predicted values of SV_{50} determined using the ABTS and the DPPH model systems.

The SIMCA models for discrimination of wine samples with low and high antioxidant capacity were developed. The SIMCA models were compared in terms of class distance values and number of misclassified samples. The best models were obtained using smoothing and second-derivative transformation of spectral data, which correctly classified 100 % of the samples from the calibration set (Figure 3). The value of the parameter „Interclass distance“, which describes the distance from the centre of the classes, was 4.26. Large class distances imply well-separated classes. A distance of more than 3 is an indication of good SIMCA class separation and that the models are really different. The obtained value of interclass distance in our investigation indicates very good possibilities of NIRS for classification of the tested wines in different classes according to their antioxidant activity.

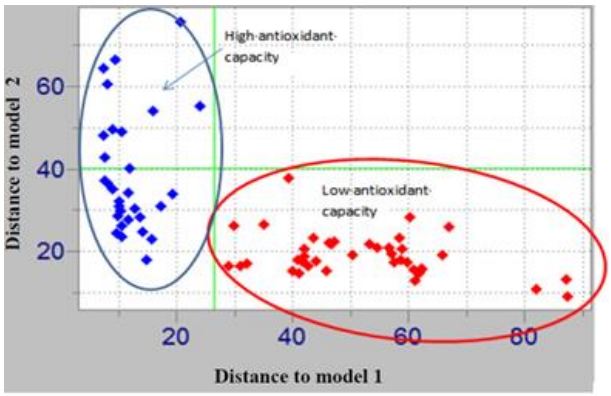

Figure 3. SIMCA classification of wine samples according to their antioxidant capacity**.**

CONCLUSION

All the investigated wines demonstrated better antioxidant properties in the ABTS model system compared to the DPPH one. The comparison of the results obtained for the wines purchased from the vineyards situated on the southern slopes of Sakar Mountain revealed that for the Merlot wines produced in the time range of 2012 – 2016 denote a tendency of decreasing SV_{50} values from year to

year. According to the data obtained from both methods Merlot 2016 demonstrated the best antioxidant effect correlating to the lowest SV_{50} values. The evaluation of the antioxidant activity of all tested wines on the basis of their spectra in the near-infrared region revealed the high degree of accuracy of estimation. According to the obtained results NIR spectroscopy could be a promising technique in quantitative determination of antioxidant activity and building classification models for discrimination of red wines according to their antioxidant properties.

REFERENCES

1. R. Videnov, *Lozarstvo & Vinarstvo*, **3**, 24 (2016).
2. T. S. Saleem, S. D Basha, *J. Cardiovasc. Dis. Res.*, **1,** 171 (2010).
3. H. H. Orak, *Sci. Hortic*., **111,** 235 (2007).
4. M. M. Markoski, J. Garavaglia, A. Oliveira, J Olivaes, A. Marcadenti, *Nutr. Metab. Insights,* **9**, 51 (2016).
5. R. J. McGorrin, in: Introduction. Encyclopedia of Analytical Chemistry (2006).
6. J. H.Qu, D. Liu, J. H. Cheng, D. W. Sun, J. Ma, H. Pu, X. A. Zeng, *Crit. Rev. Food Sci. Nutr.*, **55,** 1939 (2015).
7. V. Di Egidio, N. Sinelli, G. Giovanelli, A. Moles, E. Casiraghi, *Eur. Food Res. Technol.*, **230,** 947 (2010).
8. D. Cozzolino, *Molecules*, **20,** 726 (2015).
9. S. Fragoso, L. Acena, J. Guasch, M. Mestres, O. Busto, *J. Agric. Food Chem.*, **59,** 10795 (2011).
10. R. Re, N. Pellegrini, A. Proteggente, A. Pannala, M. Yang M, C. Rice-Evans, *Free Radic. Biol. Med.*, **26,** 1231 (1999).
11. P. Goupy, C. Dufour, M. Loonis, O. Dangles, *J. Agric. Food Chem.*, **51**, 615 (2003).

СПЕКТРАЛЕН АНАЛИЗ В БЛИЗКАТА ИНФРАЧЕРВЕНА ОБЛАСТ КАТО СПОСОБ ЗА БЪРЗО ОПРЕДЕЛЯНЕ НА АНТИОКСИДАНТНАТА АКТИВНОСТ НА ЧЕРВЕНИ ВИНА

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(Резюме)

Целта на настоящото изследване е да се оцени възможността за прилагане на спектралния анализ в близката инфрачервена област (NIR спектроскопия) като способ за бързо, точно и недеструктивно определяне на антиоксидантните свойства на червени вина. За оценка на антиоксидантните свойства са използвани моделни системи, съдържащи стабилни свободни радикали (ABTS и DPPH), позволяващи определяне на тоталната антиоксидантна активност. Спектралното измерване е проведено посредством измерване на пропускливостта на пробите вино чрез спектралния апарат NIRQuest 512, работещ в диапазона 900-1700 nm. За количествен анализ е използвана Partial least-square regression (PLS). За класификация на пробите на базата на техните спектри е използван методът SIMCA (Soft Independent Modeling of Class Analogy). Всички изследвани вина проявиха по-добър антиоксидантен потенциал в моделната система съдържаща ABTS радикал в сравнение с тази с DPPH. Въпреки че е отчетено значително вариране в изследваните свойства между отделните тествани продукти, и в двете моделни системи винята съдържащи винен сорт Сира и Малбек показаха по-добър антиоксидантен ефект. При пробите вино, произведени от един и същи лозов масив от сорта Мерло от винарната, разположена на южните склонове на Сакар планина в периода 2012-2016 г., се наблюдава тенденция за нарастване на стойността на SV_{50} със стареенето на виното. И при двата изследвани радикала стойностите са най-ниски за виното от 2016 г. Точността на определяне на антиоксидантната активност на изследваните вина на базата на спектрите им в близката инфрачервена област е много добра. Това показва, че NIR спектроскопията е подходящ метод за количествен анализ на антиоксидантния потенциал и информацията от спектрите на винята позволява създаването на модели за класификация им в зависимост от антиоксидантния им капацитет.

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Antioxidant properties of extracts from Daizo silkworm cocoons and relationship with near infrared spectra of intact cocoons

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The aim of this study is to investigate antioxidant properties of water-soluble extracts from silkworm (*Bombyx mori*) cocoons, breed Daizo and their relation to near-infrared (NIR) spectra of intact cocoons. The process of extraction with water was carried out in two stages: an ultrasonic extraction for 30 min and subsequent incubation at 60°C for 1 h. The antioxidant activity (AOA) was determined after each stage by ABTS and DPPH test. It was found that the extract exhibits radical-scavenging properties in both model systems. The incubation at 60°C increases twice the yield of substances with antioxidant properties. The measurement of the standard sericin in the same conditions shows that the AOA of the obtained extracts is not only due to the sericin, but to the other extracted components, which contributes to the observed biological effect. Comparison between the AOA of the extracts with NIR spectra of intact cocoons shows a good correlation between these properties and spectral characteristics – obtained errors of estimation were low and correlation coefficients higher than 0.96.

Key words: *Bombyx mori*, Daizo, Antioxidant activity, ABTS, DPPH, NIR spectroscopy

INTRODUCTION

Silkworm (*Bombyx mori*) is an insect grown by people for production of silk, extracted from its cocoon, which is part of its lifecycle. The cocoon shell is composed from 70% silk fibroin fibers, 25% sericin and 5% non-sericin components. The sericin and non-sericin components are concentrated in a layer surrounding the silk fibroin and acting like a glue to stick the fibers together. Non-sericin components consist of carbohydrates, salt, wax, flavonoids and flavonoid derivatives and vary according to the silkworm strain [1]. The sericin, which is considered as waste product in the production of raw silk, is highly hydrophilic with a molecular weight that ranges from 20 to 400 kDa. It consists of 18 amino acids, including essential ones, and structurally it has a globular structure. In hot water above 50-60°C the protein adopts its soluble form.

Recent studies related to the extracts of the cocoons have proved the different beneficial effects associated with the protein sericin and have shown its potential use in the field of polymers, biomaterials, cosmetics, food industry [2], cryopreservation, wound healing and as a vehicle designed for drug delivery [3]. Sericin has many bioactivities, including antioxidant [4] and antitumor [5, 6] properties, skin care [7], UV protection [8] and other. The dietary sericin can protect mice against diabetic complication [9] and it is reported to have beneficial effects on lipid and carbohydrate metabolism of rats on a high-fat diet [10].

Especially for the medical applications of the sericin it is important to have information about antioxidant properties of its water extracts obtained from the cocoons, their dependence from the conditions of extraction and the presence of other water-soluble components. For silk production, the cocoons are boiled at 100°C and our preliminary tests showed very low radical scavenging activity (RSA) of such extract. We have studied two different mild methods – ultrasonic extraction and incubation at 60 °C, having better antioxidant properties [11].

In this paper we also investigated the correlation between the antioxidant properties and the near-infrared (NIR) spectra of the intact cocoons. Such correlations are used for example for fast identification of silkworm gender inside the cocoon [12]. As the main contribution to antioxidant properties comes from sericin, a non-destructive and fast method like NIR spectroscopy (NIRS) can be used in the breeding process in order to produce cocoons with higher content of this protein, like the one reported by Mase *et al.* [13].

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EXPERIMENTAL

Cocoon samples

The analysis was carried out using cocoons from breed Daizo. It is a polyvoltine race, introduced from Japan in 1998. The cocoons are green yellow in color and spindle-shaped. Feeding and rearing of silkworms was done according to the requirement for highly productive breeds in the Experimental centre at the section of Sericulture at the Faculty of Agriculture of Trakia University. Its only food is mulberry leaves. Each of the cocoons was tested separately.

Each of the cocoons was cut into small pieces and distilled water was added to it to obtain a concentration of 30 mg dry material per ml. The process of extraction with water was carried out in two stages: ultrasonic (US) extraction at a power of 80 W for 30 min and subsequent incubation at 60°C for 1 h. After each stage, a sample was taken for measurement of radical scavenging activity.

Using the same conditions, a solution of pure sericin, produced from Seiren Co., Ltd., in distilled water in a concentration of 10 mg/ml was prepared.

Total antioxidant activity evaluation

To determine the total antioxidant capacity we tested the extracts in two model systems with stable ABTS radical cation [14] and DPPH radical [15]. Both methods are widely used together and are complementary to each other because of the different mechanisms of interaction of the radicals with complex samples – SET and HAT type antioxidant activity (AOA), respectively.

They are based on the measurement, at a specific wavelength, of decolorization of the initial solution of the radical, due to the interaction between the radical and the substances with AOA in the investigated sample. We have observed linear dependence between the absorbance and the amount of radical in the solution. The observed reduction of the absorbance is proportional to the radical scavenging activity of the sample added to the radical and can be used to evaluate it. The $ABTS^{\bullet+}$ solution was prepared with initial absorbance of 0.700 ± 0.002 at 734 nm. The $DPPH^{\bullet}$ solution had initial absorbance of 0.900 ± 0.003 at 517 nm.

The absorption spectra of the extracts were measured with UV-Vis spectrophotometer in the range of 190-1100 nm to check that there are no absorption lines around the above wavelengths.

In both methods, different amounts of extracts were added to 2 ml of initial radical solution. The absorption was measured at the given wavelength after 1 h incubation at room temperature. Three samples of each concentration of the investigated extracts were measured and the mean value and the standard deviation (SD) were calculated.

The radical scavenging activity is a linear function of the concentration of the extracts so for evaluation of antioxidant scavenge capacity we can use SC_{50}, which we define as amount of cocoon, the extract of which reduces the absorbance (the amount of free radical) by half. Smaller amount of SC_{50} means higher antioxidant activity.

NIR spectral measurements and data analysis

Diffuse reflection spectra from the intact cocoon surfaces were obtained with a scanning spectrophotometer NIRQuest 512 (Ocean Optics) in the range 900-1700 nm. For each cocoon, nondestructive measurements were made at 3-4 different points on its surface and then averaged.

The NIR spectral data of intact cocoons were used for development of calibration equations for estimation of antioxidant activity of the water extracts of cocoons. The spectral data were processed by software program Pirouette 4.5 (Infometrix, Inc., Woodinville, WA, USA). Partial Least Square Regression (PLS) was used for quantitative analysis using spectral data transformed as second derivatives. The calibration equations for each parameter were validated with leave-one-out cross validation. Leave-one-out method is recommended when a few samples are used to build the calibration equations. The leave-one-out cross-validation procedure works by omitting one observation, recalculating the equation using the remaining data, and then predicting the omitted observation. This routine is repeated until each observation in the data set is used once as validation data. Finally, the generated validation errors are combined into a standard error of cross-validation SECV.

The prediction capacity of each calibration equation was evaluated using R – multiple correlation coefficients between reference values and NIR spectra, SEC – standard error of calibration, SECV – standard errors of cross validation and the ratio performance deviation (RPD) parameter, which can be defined as the relationship between the standard deviation of the values of the investigated parameter, determined by chemical method (SD) and the standard error of cross-validation SECV in the NIR model. The RPD evaluated the prediction errors considering the standard deviation of the reference data and thus enabled comparison between models for constituents with different variation ranges. The RPD values showed levels of prediction accuracy as follows: RPD between 2.0 and 2.5 indicates

good prediction; RPD between 2.5 and 3.0 indicates very good prediction; and RPD >3 indicates excellent prediction.

RESULTS AND DISCUSSION

The absorption spectrum in the UV region of one of the extracts (the rest have the same behavior) and the spectrum of a solution of pure sericin are shown on Fig.1. In all spectra, two characteristic peaks around 210 nm and 280 nm are observed due to the presence of sericin. No absorption is observed in the spectra of the extracts at wavelengths above 450 nm and in the sericin spectrum above 300 nm. The observed difference in the spectra is the low intensity band between 250-450 nm, seen in the extracts spectra, which can be due to dissolved non-sericin components.

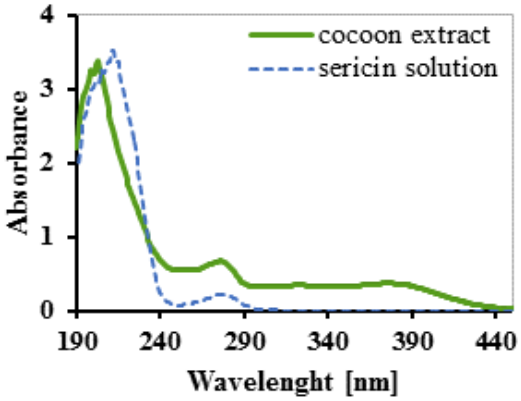

Fig. 1. UV absorption spectra of one of the cocoon extracts and of pure sericin solution.

Table 1. Average values and standard deviations (SD) for SC_{50}[mg/ml] for all measured cocoons and for pure sericin solution. The smaller the SC_{50} the higher is the AOA.

Methods	SC_{50}(US)	SC_{50}(60°C)	SC_{50}(US):SC_{50}(60°C)	SC_{50}(sericin)
$SC_{50(ABTS)}$	0.59± 0.16	0.32± 0.13	1:2	0.029± 0.003
$SC_{50(DPPH)}$	3.71± 1.69	1.93± 0.21	1:2	0.053± 0.005
$SC_{50(ABTS)}$:$SC_{50(DPPH)}$	1:6	1:6		1:2

The extracts exhibit radical scavenging properties in both ABTS and DPPH model systems. The distribution of values, obtained by both methods, for individual cocoons can be seen on the x-axis in Figs. 2 and 3, respectively. The relatively big SC_{50} differences are due to the individual characteristics of the cocoons and lead to higher standard deviation of the average values.

In Table 1 we summarize the average values for SC_{50} for two types of extractions and for pure sericin, obtained by ABTS and DPPH methods. On average, the incubation at 60°C increases twice the yield of substances with antioxidant properties. The ratios between SC_{50} from both radical tests per each method of extraction are approximately equal (1:6), but are quite different from the same ratio for pure sericin (1:2), which shows that AOA of our extracts is not only due to the sericin, but there is contribution from dissolved non-sericin components. The difference in the ratios shows that predominant components in the extracts exhibit greater SET activity.

We observe variations in obtained NIR spectra of intact cocoons, which could be related to differences in their chemical composition. It can be expected that differences in the amount of sericin affect cocoons spectra, as sericin is one of the two major components in cocoon shells. This allows us to investigate the correlation between the NIR spectra and the antioxidant activity of the extracts of the cocoon shells.

The results from NIR spectral data analysis described in the previous paragraph are presented in Table 2. We show statistical parameters from the calibration procedure for quantitative determination of antioxidant activity of the extracts of the cocoons.

The obtained multiple correlation coefficients R between reference values and NIR spectra were bigger than 0.96 and the ratio performance deviation RPD parameter bigger than 3, which showed excellent prediction abilities of the model. Graphical illustrations of the accuracy of determination of the tested parameters are presented in Figs. 2 and 3. The similar graphs are obtained for extracts after incubation at 60°C. Very high correlation between AOA of the investigated extracts and measured NIR spectra of cocoons and low error of estimation confirm the possibilities for fast and nondestructive evaluation of the properties of silkworm cocoons by using NIR spectroscopy.

Table 2. Statistical data of the calibration equations for NIRS for each method of extraction and radical model. R is multiple correlation coefficient, SEC – standard error of calibration, SECV – standard error of cross validation, RPD – relationship between the standard deviation of the chemical method (SD) and the standard error of cross-validation SECV in the NIR model.

Radical – extraction method	SECV	Rcv	SEC	Rcal	RPD
DPPH – US	0.464	0.961	0.458	0.962	3.62
DPPH – 60°C	0.060	0.962	0.060	0.968	3.61
ABTS – US	0.038	0.972	0.038	0.973	4.15
ABTS – 60°C	0.035	0.968	0.034	0.968	3.96

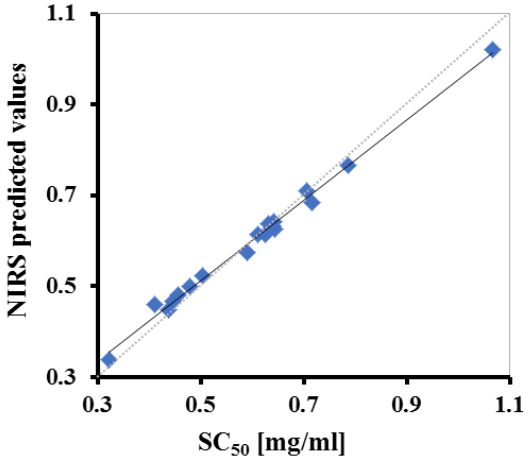

Fig. 2. Scatter plots of laboratory measured and near-infrared spectroscopy (NIRS) predicted values of SC_{50} determined using the ABTS model system for ultrasound extraction.

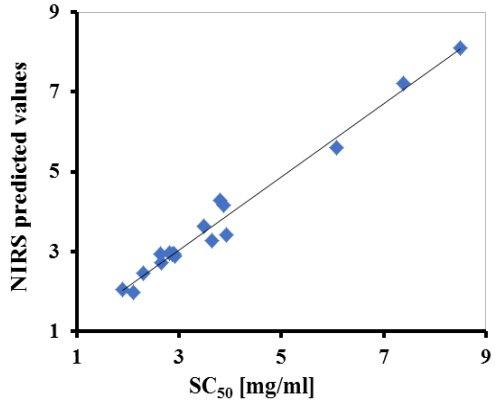

Fig. 3. Scatter plots of laboratory measured and near-infrared spectroscopy (NIRS) predicted values of SC_{50} determined using the DPPH model system for ultrasound extraction.

CONCLUSION

The extract of Daizo cocoons exhibits radical-scavenging properties in both ABTS and DPPH model systems. Incubation at 60°C increases twice the yield of substances with antioxidant properties. Comparison with measurements of the standard sericin at the same conditions shows that the AOA of the obtained extracts is not only due to sericin, but to other extracted components which contribute to the observed biological effect.

Very good correlation between AO of the investigated extracts and measured NIR spectral characteristics of intact cocoons was found – obtained errors of estimation are low and correlation coefficients are higher than 0.96. This shows the potential of the NIR spectroscopy method for estimation of the quality of the cocoons.

REFERENCES

1. H.Y.Wang, Y.J. Wang, L.X. Zhou, L. Zhu,Y.Q. Zhang, *Food Funct.*, **3**, 150 (2012).
2. R. I. Kunz, R. M. C.Brancalhão, L. F. C. Ribeiro, M. R. M. Natali, *Biomed. Res. Int.*, 8175701 (2016).
3. L. Lamboni, M. Gauthier, G. Yang, Q. Wang, *Biotechnol. Adv.*, **33**, 1855 (2015).
4. M. Sasaki, N. Kato, H. Watanabe, *Oncol. Rep.*, **7**, 1049 (2000).
5. M. Sasaki, N. Kato, H. Watanabe, H. Yamada, *Oncol. Rep.*, **5**, 1049 (2000).
6. S. Zhaorigetu, M. Sasaki, H. Watanabe, N. Kato, *Biosci. Biotechnol. Biochem.*, **65**, 2181 (2001).
7. H. Yamade, M. Fuwannomura, *Eur. Patent* 0841065A2 (1998).
8. R. Dash, M. Mandal, S.C. Kundu, *Mol. Cell Biochem.*, **311**, 111 (2008).
9. C. J. Song, Z. J. Yang, M. R. Zhong, Z. H. Chen, *Neural. Regen. Res.*, **8**, 506 (2013).
10. Y. Okazaki, S. Kakehi, Y. Xu, K. Tsujimoto, M. Sasaki, H. Ogawa, N. Kato, *Biosci. Biotechnol.Biochem.*, **74**, 1534 (2010).
11. V. Mihaylova, Y. Evtimova, L. Atanasova, N. Hristova-Avakumova, M. Panayotov, V. Hadjimitova, In: Proc. 3rd Bulg. Nat. Congr. Phys. Sci., Sofia, 2016, Heron Press, Sofia, 2016, S08.43.
12. T. Jin, L. Liu, X. Tang, H. Chen, *J. Near Infrared Spectrosc.*, **3**, 89 (1995).
13. K. Mase, T. Iizuka, E. Okada, T. Miyajima, T. Yamamoto, *J. Insect Biotech. Sericol.*, **75**, 85 (2006).
14. R. Re, N. Pellegrini, A. Proteggente, A. Pannala, M. Yang, C. Rice-Evans, *Free Rad. Biol. Med.*, **26**, 1231 (1999).
15. P. Goupy, C. Dufour, M. Loonis, O. Dangles, *J. Agric. Food Chem.*, **51**, 615 (2003).

АНТИОКСИДАНТНИ СВОЙСТВА НА ЕКСТРАКТИ ОТ ПАШКУЛИ НА КОПРИНЕНИ БУБИ Daizo И ОПРЕДЕЛЯНЕТО ИМ ЧРЕЗ СПЕКТРИТЕ НА ПАШКУЛИТЕ В БЛИЗКАТА ИНФРАЧЕРВЕНА ОБЛАСТ

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(Резюме)

Целта на това изследване е да се определят антиоксидантните свойства на водоразтворими екстракти, получени от пашкули на копринена буба (*Bombyx mori*), порода Daizo и зависимостта на тези свойства от спектрите на пашкулите, измерени в близката инфрачервена област (NIR). Екстракцията е проведена на два етапа: ултразвукова екстракция за 30 минути и последваща инкубация при температура 60 °C за 1 час. Антиоксидантната активност (АОА) е определяна след всеки етап на екстракция чрез ABTS и DPPH тестове. Установено е, че получените екстракти проявяват радикал-улавящи свойства и по двата използвани метода. Инкубацията при 60 °C увеличава два пъти добива на вещества с антиоксидантни свойства. Измерването на стандарта серицин при същите условия показва, че АОА на получените екстракти се дължи не само на серицина, а и на извлечените заедно с него други компоненти, притежаващи антиоксидантни свойства. Сравнението между АОА на екстрактите със спектрите на пашкулите, от които са получени, в NIR област показва добра корелация между тези свойства и спектралните им характеристики. Получените уравнения за определянето на АОА показват много малки грешки на определяне и корелационни коефициенти по-големи от 0,96.

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4-Methoxy aroylhydrazones – promising agents protecting biologically relevant molecules from free radical damage

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The concomitant toxicity of iron observed in many disorders associated with excess of this essential trace element is mainly due to its ability to promote and participate in free radical generation reactions and the subsequent oxidative damage. The dietary recommendations for some of the patients and the restriction for supplementation with essential antioxidants have led to the necessity of developing novel high-efficiency pharmacologically active substances with multipotent antioxidant properties. The present investigation aimed to determine the protection effect against oxidative stress of three structurally characterized derivatives of the active chelator salicylaldehyde benzoylhydrazone (SBH). The capability of the tested compounds 4-methoxysalicylaldehyde benzoylhydrazone (4mSBH), 4-methoxysalicylaldehyde-4-hydroxybenzoylhydrazone (4mShBH) and 4-methoxysalicylaldehyde isonicotinoylhydrazone (4mSIH)) to preserve biologically relevant molecules from oxidative damage was evaluated in *in vitro* spectrophotometric model systems with diverse mechanisms of free radical generation containing deoxyribose and egg yolk homogenate. Additional experiments have been performed in chemiluminescent systems containing different type of reactive oxygen species. The obtained results indicated that the incorporation of methoxy group at 4th position in the aldehyde part of the molecule ameliorates the evaluated properties. The extent of the observed improvement depends on the subsequent modifications in the hydrazide moiety. Greatest protection effect against the oxidative damage of the biologically important molecules was observed in the samples containing hydroxyl bearing compound (4mShBH). This hydrazone also demonstrated higher activity against $OH^{\bullet}$ and $O_2^{\bullet -}$ in the chemiluminescent model systems. Comparing the C_{50} values from the different model systems we suggest that the observed protection effect is associated with antioxidant activity different from Fe-chelation.

Keywords: Hydrazones, Antioxidant properties, Chemiluminescence

INTRODUCTION

Iron is an essential trace element which plays a crucial role in many cellular processes, e.g., oxygen transport, energy generation, DNA synthesis, etc. This transition metal acts as a cofactor in the active center of many enzymes involved in fundamental biochemical pathways [1]. Although its biological importance the excess of iron has pathological consequences which are mainly due to the fact that it easily participates in oxidation-reduction processes implicated in free radical generation like Haber-Weiss and Fenton reactions [2]. The produced hydroxyl radicals are capable to initiate chemical reactions with the main molecular components resulting in their oxidative damage and cell death [3]. Beside this, free iron is able to interact with unsaturated lipid molecules which results in peroxyl and alkoxyl radical production.

In long-term the increased generation of reactive oxygen species associated with disorders which pathophysiology relates to elevated iron levels such as β-thalassemia, coronary heart disease, neurodegenerative disease, etc., is accompanied by helplessness of the organism to maintain the vital cellular oxidation/reduction status [4]. This relates with disturbance of the protection of the living organisms against pathological deviation of this parameter. The redox homeostasis is troubled due to the impossibility of the enzymatic, non - enzymatic and daily consumed organic, inorganic and natural compounds enhancing food nutritional qualities to prevent free radical oxidative damage [5, 6].

The dietary recommendations for those patients and the restriction for supplementation with essential antioxidants have led to the necessity of developing novel high-efficiency pharmacologically active substances with multipotent antioxidant properties [7]. The numerous performed investigations indicate that particular attention has been paid to the chelating agents and the possibility to develop on the base on their chemical structure pharmacologically active molecules possessing antioxidant properties [8, 9].

The aim of the present investigation is to estimate the protection effect of three novel

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derivatives of the active chelator salicylaldehyde benzoylhydrazone (SBH) against the damaging effect of different types of ROS and iron-induced oxidation of biologically important molecules. The investigated compounds - 4-methoxysalicylaldehyde benzoylhydrazone (4mSBH), 4-methoxysalicylaldehyde-4-hydroxybenzoylhydrazone (4mShBH) and 4-methoxysalicylaldehyde isonicotinoylhydrazone (4mSIH) have been synthesized and structurally characterized in the Faculty of Pharmacy of the Medical University of Sofia [10].

Our scientific interest associated with the exploration and the evaluation of the protection effect and the antioxidant properties of these SBH derivatives is due to the information available in the scientific literature proving their relatively easy technology of synthesis, chemical stability and variety of biological activities.

EXPERIMENTAL

Chemiluminescent model systems

The evaluation of the chemiluminescent response was done using LKB 1251 luminometer (BioOrbit, Turku, Finland) provided with an automatic injector. The apparatus was connected *via* AT-serial interface with an AT-type computer and the MultiUse program ver. 10.8 (BioOrbit, Turku, Finland) was used for the collection and the processing of the experimental data. In both used assays the chemiluminescent response was measured using the "flash assay" option of the software and calculated by determination of the area under the chemiluminescent curve. To evaluate the scavenging properties we have used the chemiluminescent scavenging index (CL-SI) – the CL ratio in the presence and absence of the investigated 4-methoxy hydrazone in percentage. Both used chemiluminescent model systems have proven themselves as reliable in our previous experiments concerning the evaluation of the effect of hydrazones with similar structure [11].

Luminol-dependent chemiluminescence in a system of iron-dependent hydroxyl radical formation - The experiment was performed using 1 ml samples of phosphate saline buffer (PBS), containing 0.1 mmol/L luminol, 0.1 mmol/L Fe^{3+}, 0.1 mmol/L EDTA, 0.1 mmol/L ascorbate, 1 mmol/L H_2O_2 and the tested compound. In the control samples, the studied compounds were omitted. The chemiluminescence was registered for 1 min. every 50 milliseconds after the addition of hydrogen peroxide.

Luminol-dependent chemiluminescence in a system of KO_2 produced $O_2^{-\bullet}$ - One milliliter samples of 50 mmol/L K_2HPO_4/KH_2PO_4 buffer, pH 7.4, containing 0.1 mmol/L luminol and the tested 4-methoxy SBH derivatives were prepared. Due to the fast release of superoxide, the CL response was measured instantly after the addition of 20 µl of KO_2 dissolved in DMSO. The chemiluminescence was registered for 1 min. every 50 milliseconds.

Spectrophotometric model systems

Protection effect in a lipid containing model system – a colorimetric method was used for the quantitative determination of the thiobarbituric acid reactive substances. The degree of the oxidative molecular damage was presented as percentage of the untreated control. The experiment of iron-induced peroxidation was performed in model systems of egg yolk homogenate. The concentration of the oxidisable substrate was 1 mg/ml. The initiation of the lipid peroxidation was achieved by the addition of 0.1 mmol/L $FeCl_2$. All samples were incubated for 30 min at 37˚C, which was followed by the addition of 0.5 ml of 2.8% trichloroacetic acid and 0.5 ml of 1% thiobarbituric acid. Second 20 min incubation at 100˚C was performed and the absorbance was measured at 532 nm.

UV-induced deoxyribose damage – the experiment was performed according to Halliwell *et al*. [12], with some modifications. The investigated hydrazones and the 2' deoxyribose [0.6 mmol/L] were diluted in 50 mmol/L K_2HPO_4/KH_2PO_4, pH 7.4. Control samples in which hydrazones were omitted were prepared. All probes were UV irradiated for 30 min (UV 220-400 nm). To 1 ml of each irradiated sample solution were added 0.6 ml of 1% trichloroacetic acid and 0.6 ml of 0.6% thiobarbituric acid. The obtained mixture was heated for 20 min at 100˚C and the absorbance of the samples was measured at 532 nm. Results were presented as percentage of the untreated control and reflect the "damage of 2-deoxyribose"

Determination of C_{50} values – the concentration of the investigated derivatives decreasing by 50% the chemiluminescent response was named C_{50}. The methodology of determination requires fitting of the obtained experimental data to the "sigmoidal model". Using the same formula we have determined the hydrazone concentration providing 50% AOA in the spectrophotometric model systems.

RESULTS AND DISCUSSION

For the chemiluminescent evaluation of the effect of the 4-methoxy derivatives as inhibitors and/or scavengers we have chosen a model system containing the superoxide anion radical and an assay of hydroxyl radical generation *via* Fenton

reaction. Choosing these model systems we wanted to provide information concerning: (i) the opportunity the studied by us compounds to possess the capability of tackling the formation of one of the most reactive and potent ROS - the hydroxyl radical. One of its major targets is the DNA molecule and the possible resulting oxidative damages are associated with structural changes of its sugar (deoxyribose); (ii) the capability of the tested hydrazones to reduce the formation of the superoxide anion radical which is the basic step responsible for a cascade of reactions initiating the production of other ROS, which subsequently interact and prompt oxidative damages in biologically important molecules and disturb their functionality.

The evaluation of the radical scavenging activity against $O_2^{\bullet-}$ has been performed in the concentration range from 3 to 100 μmol/L. The compounds have demonstrated diverse degree of activity and this effect is more prominent with the increase of the hydrazone concentration in the samples. Comparing these results with previously performed experiments concerning the evaluation of the CL-SI index of the initial compound SBH we have seen that the 4-methoxy derivatives demonstrate better scavenging activity – CL-SI%$_{SBH}$ = 74.09 % at 100 μmol/L [13]. The obtained lower values of the CL-SI index for the samples containing the 4-methoxy derivatives compared to that of the initial compound SBH, proved the favorable effect of the structural modification associated with incorporation of methoxy group at 4^{-th} position in the aldehyde part of the molecule on the superoxide anion scavenging activity in this system. 4mSBH demonstrated the lowest inhibition effect from the 4-methoxy derivatives – corresponding to the highest values of the CL-SI% index. The incorporation of hydroxyl group (4mShBH) and substitution with hetero atom (4mSIH) had beneficial effect on the studied properties. The observed diminishment at the highest tested concentration of 100 μmol/L of the CL-SI is 55.53 % (for 4mShBH) and 74.25 % (for 4mSIH) from that of 4mSBH, which denotes the additional favorable effect of these subsequent structural changes on $O_2^{\bullet-}$ scavenging activity. The investigation of the ability of the studied 4-methoxy derivatives to suppress the chemiluminescent response in the hydroxyl radical generating model system indicated that at the lowest tested concentration none of the compounds induced statistically significant diminishment of the CL-SI index compared to the untreated control.

The subsequent increases of the concentration of the hydrazones in the sample induced concentration dependent inhibition effect on the chemiluminescent response. This correlation is less expressed for the SBH (CL-SI%$_{SBH}$ = 62.77 %) [13].

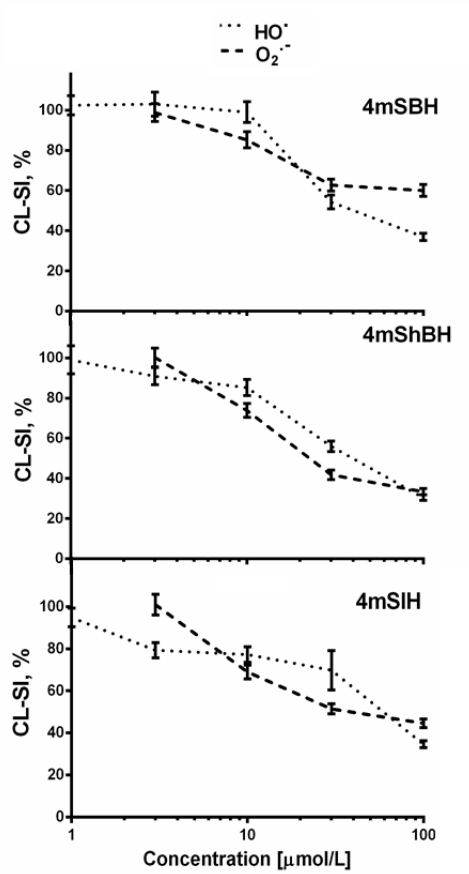

Figure 1. 4-methoxy aroylhydrazones derivatives induced diminishment of the chemiluminescent scavenging index in: (HO•) – system of iron-dependent hydroxyl radical formation; ($O_2^{-\bullet}$) – system of KO_2 produced superoxide.

Compared to the initial compound the 4-methoxy derivatives demonstrate higher degree of inhibition of the chemiluminescent lightening. At the highest tested concentration 100 μmol/L the observed effects of 4-methoxy derivatives are similar but statistically different and the CL-SI values are approximately 1/3 of that of the untreated control.

To make possible the comparison of the effect of the investigated compounds we have calculated the hydrazone concentration inducing 50% inhibition of the chemiluminescent scavenging index (C_{50}) using the data presented in Figure 1. The results are shown in Figure 2. The C_{50} values for SBH were calculated from previously performed experiments [13]. In the $O_2^{\bullet-}$ containing model system the 4-methoxy derivatives exhibited different extent of radical scavenging activity. 4mSBH had the lowest inhibitory effect among the investigated compounds and its C_{50} value was estimated using extrapolation.

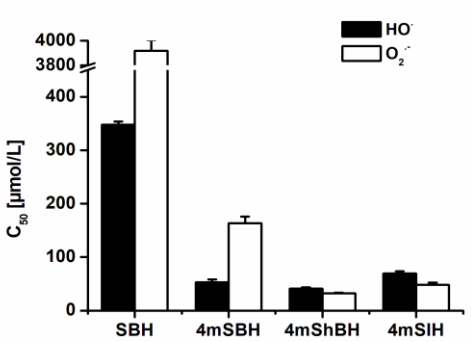

Figure 2. Comparison between the concentrations of hydrazones leading to 50% reduction of the chemiluminescent response (C_{50}) in the chemiluminescent model system containing hydroxyl and superoxide radicals.

The subsequent structural modifications of this compound including incorporation of hydroxyl group (4mShBH) or substitution with hetero atom (4mSIH) reveal the positive effect of the structural changes – which can be seen from the decrease of the C_{50} values. They are respectively 1/5 for 4mShBH and 1/3 for 4mSIH from the C_{50} value of 4mSBH.

The 4-methoxy derivatives express alike behavior in the hydroxyl radical containing model system. The C_{50} values are in the concentration interval from 41.09 to 69.28 μmol/L. The incorporation of hydroxyl group in the hydrazide part of the molecule of 4mSBH increases the investigated in this system properties $C_{50\ 4mShBH}$ = 41.09 μmol/L, whereas the presence of hetero atom is associated with a slight decrease of the activity.

Figure 2 reveals the comparable behavior of 4mShBH and 4mSIH in the chemiluminescent model systems. Both of them presented themselves as potent inhibitors of the luminol-dependent chemiluminescence created by the ROS generated in the systems, which is a measure for their scavenger activity. The obtained values of C_{50} indicate that the observed effect is more pronounced in the $O_2^{\bullet -}$ containing model system compared to the one where iron dependent hydroxyl radical formation is expected. The lower C_{50} values of these two compounds suggest that they possess the potential to influence the free radical processes at the initial step of the cascade of reactions, i.e. the $O_2^{\bullet -}$ generation.

The studied hydrazones display protection effect in both spectrophotometric systems despite the different mechanisms of induction of free radical damage and the use of different oxidisable substrates.

In the system of Fe-induced lipid peroxidation the 4-methoxy derivatives expressed concentration dependent protection of the egg yolk homogenate (Table 1). With the increase of the hydrazone concentration in the samples the percentage of the oxidized molecules decreases. This effect is more pronounced in the samples containing 4mSBH where we have observed a five-fold decrease of the damages when the concentration of the compound was changed from 80 μmol/L to 160 μmol/L. For 4mShBH this decrease is 1.8-fold and for 4mSIH approximately 1.2-fold. Again at the lowest tested concentration of 80 μmol/L, 4mShBH demonstrated the highest protection activity.

Table 1. Degree of damage in percentage of the studied 4-methoxy SBH derivatives during iron- induced peroxidation in egg yolk homogenate containing model system. Control damage value was 100 %.

Compound	Hydrazone concentration 80 [μmol/L]	Hydrazone concentration 160 [μmol/L]
4mSBH	49.115 ± 3.97	9.786 ± 2.906
4mShBH	34.637 ± 2.967	19.367 ± 4.945
4mSIH	41.516 ± 5.363	35.547 ± 2.005

In order to explain more accurately the observed antioxidant effect of the investigated compounds and their interaction with ROS we have evaluated their ability to prevent free radical damage in iron-free spectrophotometric systems where we have used UV-irradiation to generate free radical damage. The method of estimation of ROS generation includes the quantitative determination of the 2'-deoxyribose oxidative product.

The results presented in Figure 3 reveal that the 4-methoxy derivatives possess notable activity in protecting the 2'-deoxyribose molecules. The degree of the oxidative 2-deoxyribose molecules damage was presented as percentage of the control. Their effectiveness was studied in the concentration interval from 0 to 100 μmol/L and at the highest tested concentration all of them decreased the degree of the damaged molecules by more than 60 %.

Comparing the observed effect with the antioxidant efficacy of classical hydroxyl radical scavengers (DMSO – 43.13% ± 0.61) and antioxidants (Trolox 34.42% ± 0.38) at a concentration of 100 μmol/L we can conclude that they possess equivalent concentration range of effectiveness in the studied aqueous solutions.

To make a suggestion about the type of the revealed antioxidant effect we have compared the C_{50} values from two model systems where we have used different mechanism for registration of the evaluated properties and for the ROS generation – the chemiluminescent assay of iron-dependent hydroxyl radical formation and the spectrophomet-

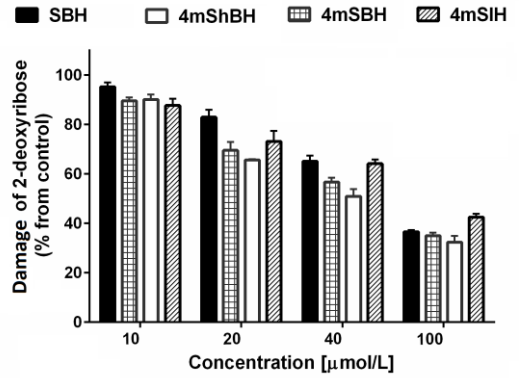

Figure 3. Effect of the chelator SBH and its 4-methoxy derivatives on the UV induced "damage of 2-deoxyribose".

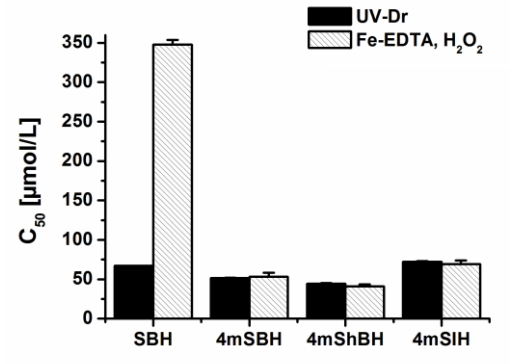

Figure 4. Comparison between the C_{50} values obtained from the chemiluminescent system of Fe-induced hydroxyl radical formation and from the spectrophotometric assay of UV induced 2-deoxyribose damage.

ric system of UV induced deoxyribose damage (Figure 4). For these experiments we have used as reference compound salicylaldehyde benzoylhydrazone SBH with proved chelation activity.

The obtained results for the C_{50} concentration in both model systems indicate that the observed effect of the 4-methoxy derivatives in both of them is due to direct antioxidant activity using a similar mechanism of action - different from the Fe-chelation. These data are in accordance with previously published by us results concerning the antioxidant effect of 3-methoxy derivatives of SBH [11]. They exclude the possibility of quenching effect which could be one possible reason for diminishment of the chemiluminescent response and support our statement that the chemiluminescent methods are suitable for the evaluation of the antioxidant properties of this group of compounds.

CONCLUSION

The performed by us experiment indicates that all the studied derivatives of SBH possess radical scavenging activity against $OH^{\bullet}$ and $O_2^{\bullet -}$ and demonstrate a protection effect against free radical damage of biologically relevant molecules. Comparison with previously performed experiments indicates that the performed structural modification, i.e., incorporation of methoxy group at 4th position in the aldehyde part of the molecule ameliorates the scavenging properties. The extent of the observed improvement depends on the subsequent structural modifications in the hydrazide moiety. The hydroxyl bearing compound (4mShBH) demonstrated higher activity against $OH^{\bullet}$ and $O_2^{\bullet -}$ and better protection effect in all model systems. Comparing the C_{50} values from model systems where we have used different mechanisms for registration of the evaluated properties and for the ROS generation we suggest that the observed effect is associated with antioxidant activity different from Fe-chelation.

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REFERENCES

1. N. Abbaspour, R. Hurrell, R. Kelishadi, *J. Res. Med. Sci.,* **19**, 164 (2014).
2. Y. Kohgo, K. Ikuta, T. Ohtake, Y. Torimoto, J. Kato, *IJH,* **88**, 7 (2008).
3. R. Britton, G. Ramm, J. Olynyk, R. Singh, R. O'Neill, B. Bacon, *Adv. Exp. Med. Biol*., **356,** 239 (1994).
4. M. Valko, D. Leibfritz, J Moncola, M. Cronin, M. Mazura, J. Telser, *Int. J. Biochem. Cell Biol.*, **39**, 44 (2007).
5. L. Pham-Huy, He Hua, C. Pham-Huy, *Int. J. Biomed. Sci.,* **4**, 89 (2008).
6. A. Rahal, A. Kumar, V. Singh, B. Yadav, R. Tiwari, S. Chakraborty, K. Dhama, *Biomed Res Int*.; **2014**, 761264 (2014).
7. N. Anastassova, A. Ts. Mavrova, D. Yancheva, M. Kondeva-Burdina, V. Tzankova, S. Stoyanov, B. Shivachev, R. Nikolova, *Arab. J. Chem., in press*
8. H. Zhang, D. Yang, G. Tang, *Drug Discov. Today*, **15-16**, 749 (2006).
9. H. Schulman, M. Hermes-Lima, E. Wang, P. Ponka, *Redox Rep.*, **5,** 373 (1995).
10. B. Nikolova-Mladenova, N. Halachev, R. Iankova, G. Momekov, D. Ivanov, *Arzneimittel For/ Drug Res.*, **61,** 714 (2011).
11. N. Hristova-Avakumova, K. Yoncheva, B. Nikolova-Mladenova, T. Traykov, G. Momekov, V. Hadjimitova, *Redox Rep.*, **10,** 1 (2017).
12. B. Halliwell, J. M. Gutteridge, O. I. Aruoma, *Anal. Biochem.*, **165,** 215 (1987).
13. N. Hristova-Avakumova, B. Nikolova-Mladenova, V. Hadjimitova, *Bulg. Chem. Comm.*, **47,** 1053 (2015).

4-МЕТОКСИ АРОИЛХИДРАЗОНИ – СЪЕДИНЕНИЯ, ПОНИЖАВАЩИ СТЕПЕНТА НА ОКСИДАТИВНО УВРЕЖДАНЕ НА БИОЛОГИЧНО ЗНАЧИМИ МОЛЕКУЛИ

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(Резюме)

При редица заболявания се наблюдава повишаване на плазмените нива на желязото, което води до токсични ефекти. Това се дължи на способността му да инициира свободно-радикални процеси и произтичащото от тях оксидативно клетъчно увреждане. Затова е важно разработването на фармакологично активни вещества, притежаващи наред с хелатиращите си свойства и многостранно проявяваща се антиоксидантна активност. Това би подобрило качеството на живот на пациентите, които трябва да спазват определен хранителен режим и които имат противопоказания за провеждане на съпътстваща терапия с хранителни добавки, съдържащи есенциални антиоксиданти. Настоящото проучване цели да се изследва протективния ефект на три структурно охарактеризирани структурни аналога на активния хелатор салицилалдехидбензоилхидразон (SBH). Способността на тестваните съединения 4-метоксисалицилалдехидбензоилхидразон (4mSBH), 4-метоксисалицилалдехид-4-хидрокси бензоилхидразон (4mShBH) и 4-метоксисалицилалдехидизоникотиноилхидразон (4mSIH)) да понижават степента на оксидативно увреждане на биологично релевантни молекули беше изследвана *в in vitro* спектрофотометрични моделни системи с различен механизъм на инициране на оксидативното увреждане, съдържащи дезоксирибоза и жълтъчен хомогенат. Бяха проведени съпътстващи експерименти в хемилуминесцентни системи, доказващи радикалулавящи свойства спрямо различни активни форми на кислорода. Получените резултати показват, че заместването с метокси група в алдехидното ядро на изходното съединение SBH подобрява изследваните в тези системи свойства. Степента на подобряване на протективния ефект зависи и от последващите структурни изменения в хидразидната част на молекулата. Оксидативното увреждане на биологично важните молекули се понижава най-силно от хидроксил съдържащия структурен аналог (4mShBH). Това съединение показа и най-силно изразена активност спрямо $OH^{\bullet}$ и $O_2^{\bullet-}$ в хемилуминесцентните системи. Сравняването на изчислените C_{50} стойности показва че наблюдаваният ефект на понижение на степента на оксидативно увреждане е свързан с директна антиоксидантна активност, а не с хелатиращи свойства.

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DPPH radical-scavenging activity of Galantamine hydrobromide and Pymadine alone and in combination

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The overproduction of reactive oxygen species and the weakness of the antioxidant defense mechanisms in the human body are the main reasons for the oxidative stress, which underlies the development of neurodegenerative Alzheimer's disease. Alkaloid Galantamine is nonselective acetylcholinesterase inhibitor with antioxidant activity. Pymadine is non-depolarizing potassium channel blocker having a synergistic effect with Galantamine on the symptomatic treatment of Alzheimer's disease. The aim of the current study was the evaluation of the radical-scavenging activity (RSA) of Galantamine hydrobromide, Pymadine and the combination Galantamine hydrobromide/Pymadine towards 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical. The decrease in the absorbance of 0.05 mM methanol solution of DPPH at λ = 516 nm in presence of methanol solution of: 1 mM Butylhydroxytoluene (BHT) (standard); 1 mM ÷ 5 mM Galantamine hydrobromide; 1 mM ÷ 5 mM Pymadine and 5 mM Galantamine hydrobromide/5 mM Pymadine was monitored by spectrophotometry in equal intervals of 5 s for a total period of 30 min. The regression equations were used for calculation of the RSC_{50}: RSA (Galantamine hydrobromide) = $3.419.e^{0.293}$.c, RSC_{50} (Galantamine hydrobromide) = 9.16 mM; RSA(Pymadine) = $0.460.e^{0.411}$.c, RSC_{50} (Pymadine) = 11.41 mM. RSA of the investigated compounds was compared with the effect of standard BHT and the relative radical-scavenging activity (RRSA) and relative decrease of radical-scavenging activity (RDRSA) were calculated. The experimental results showed that the combination of 5 mM Galantamine hydrobromide/5 mM Pymadine has a higher RSA (20.19 %), compared to 5 mM Galantamine hydrobromide (15.44 %) and 5 mM Pymadine (2.48 %) itself.

Keywords: Galantamine hydrobromide, Pymadine, Combination, DPPH

INTRODUCTION

The brain is particularly sensitive to the influence of free radicals due to high oxygen consumption, unsaturated fatty acids and decreased activity of oxidative sensitive endogenous antioxidant systems. In Alzheimer's disease, oxidative stress arises as a result of disturbing the balance between endogenous or exogenous overproduction of reactive free radicals and the reduction of antioxidant protective mechanisms. Oxidative disorders are one of the initial pathological changes in Alzheimer's disease and occur selectively in brain areas responsible for the regulation of memory functions [1]. Oxidative stress plays an important role in the pathogenesis of neuronal degeneration [2], because the formation of free radicals leads to inflammatory processes [3], cell membrane dysfunction [1], activation of programmed nerve cell death (apoptosis) by oxidation of proteins, lipids and nucleic acids (DNA, RNA) [4] and impaired glucose metabolism [5].

Reactive oxygen species include charged and neutral species such as: superoxide anion (O_2^{-}•), peroxide radical (O_2^{2-}•), hydrogen peroxide (H_2O_2), hydroxyl radical (HO•), singlet oxygen ($^1O_2$), alkoxyl radicals (RO•) and peroxyl radicals (ROO•) [6]. One of the therapeutic approaches of Alzheimer's disease is related to decreasing neuronal degeneration by antioxidants [7-9].

The widely used in clinical practice alkaloid Galantamine acts as a nonselective acetylcholinesterase inhibitor [10], but it was recently found that it also displays a considerable antioxidant activity. On the other hand, Pymadine, as a non-depolarizing muscle-relaxant antagonist (potassium channel blocker) [11], was discovered also to have a synergistic effect with the Galantamine on the symptomatic treatment of this widespread disease. The ability to pass through the blood-brain barrier and the different routes of administration (oral, intravenous, intramuscular, subcutaneous, intraocular, electrophoretic) determine the following Galantamine applications: Alzheimer's disease, Alzheimer's with cerebrovascular syndrome, vascular dementia [12-14].

The antioxidant activity of Galantamine hydrobromide was investigated *in vitro* by a luminol-dependent chemiluminescent method. The antioxidant action is bound to the enol group and disappears upon conversion of the enol group (Galantamine and Galantamine hydrobromide) to a

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carbonyl group (Narwedine, Narwedine hydrobromide). Changing Galantamine in Galantamine hydrobromide is accompanied by an increase in antioxidant activity due to the quaternary nitrogen atom [15]. Galantamine has a neuroprotective effect by reduction of oxidative neuronal damage by binding free radicals: superoxide, peroxide, hydroxyl and alkoxy [16]. The optimal dose for the activation of α7-subtype nicotinic acetylcholine receptors, whereby Galantamine protects neurons from the influence of superoxide radicals, is 1.5 mg/kg - 5.0 mg/kg [17].

Methods for examination of radical-scavenging activity are [18]: A) electron transfer based methods, in which the increase in the radical-scavenging activity of the test compounds is directly proportional to: I) the decrease of the absorbance of a solution of: (i) 1,1-diphenyl-2-(picrylhydrazyl) (λ=516 nm): DPPH method; (ii) 2,2-azinobis- (3-ethylbenzothiazoline-6-sulfonic acid (λ=734 nm): ABTS method; (iii) hydrogen peroxide (λ=230 nm): peroxidic radical-binding method; (iv) nitroblue tetrazolium: superoxide-radical-scavenging method; II) increasing of the absorption of a solution of: (i) Cu^{+}-(neocuproine) (λ=450 nm), obtained by reduction of Cu^{2+}-(neocuproine): CUPRAC method; (ii) N,N-dimethyl-p-phenylenediamine (λ=505 nm): DMPD peroxide-radical method; (iii) Fe^{2+}-(2,4,6-tripyridyltriazine) (λ=595 nm), obtained by reduction of Fe^{2+}-(2,4,6-tripyridyltriazine): FRAP method; (iv) Prussian blue (λ = 700 nm), obtained by reaction of potassium ferrocyanide with ferric chloride: Prussian blue method; (v) Mo^{5+} (λ = 765 nm), obtained by reduction of Mo^{6+}: Folin-Ciocalteu method; B) proton based methods, where the increase in the radical-scvenging activity of the test compounds is directly proportional to the lower rate of reduction of: I) the absorption of a solution of β-carotene (λ = 450 nm); II) the fluorescence of: (i) 4,4-difluoro-5-(4-phenyl-1,3-butadienyl) 4-bora-2-amidinopropane) hydrochloride: a method of inhibiting lipid peroxidation; (ii) 2.7-dichlorofluorescein ($\lambda_{excitation}$ = 350 nm, $\lambda_{emission}$ = 540 nm), fluorescein ($\lambda_{excitation}$ = 493 nm, $\lambda_{emission}$ = 518 nm) under the influence of AAPH [2,2'-azobis(2-amidinopropane) – ORAC method, HORAC method); (iii) the product (λ=425 nm) of the reaction between hydrogen peroxide and luminol.

The DPPH method is a rapid, simple, accurate and inexpensive assay for measuring the radical scavenging activity of flavonoids [19, 20], coumarins [21, 22] and their synthetic analogues [22] from some medical plants, to determine antioxidant capacity of wines [23, 24], beer [25], tea infusions [26] and antioxidant activity of plant foods, oils, beverages [27] and extracts from *Citrus* [28], *Crataegus oxyacantha L.* [29], *Ocimum basilicum L.* [30].

The aim of the current study was the evaluation of the radical-scavenging activity (RSA) of Galantamine hydrobromide, Pymadine and the combination Galantamine hydrobromide/ Pymadine towards 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical by measuring the decrease in the absorbance of 0.05 mM methanol solution of DPPH radical at λ = 516 nm by using standard 1 mM methanol solution of butylhydroxytoluene (BHT).

Materials

Test compounds: Galantamine hydrobromide, 4-aminopyridine (Pymadine). Reagents with pharmacopoeia purity: 1.1′-diphenyl-2-picrylhydrazyl (DPPH) (99 %), (Sigma Aldrich, N: STBD 4145 V), butylhydroxytoluene (BHT) (99%) (Sigma-Aldrich, N: BCBL 8166 V), methanol (99.9 %) (Sigma-Aldrich, N: SZBD 063 AV UN 1230).

Methods

DPPH-method for in vitro study of the radical-binding activity. Accurately measured quantities: 0.0441 g of BHT (M = 220.35) and 0.0039 g of DPPH (M = 394.32) were dissolved in methanol to 100.0 ml to obtain solutions with concentrations of 2 mM BHT and 0.1 mM DPPH.

Precisely measured amounts of Galantamine hydrobromide (M = 368.27): 0.0737 g, 0.1473 g, 0.2210 g, 0.2946 g, 0.3683 g and 4-aminopyridine (Pymadine) (M = 94.12): 0.0188 g, 0.0376 g, 0.0565 g, 0.0753 g, 0.0941 g were dissolved separately in methanol to 100.0 ml for obtaining solutions with concentrations of 2 mM, 4 mM, 6 mM, 8 mM, and 10 mM.

RESULTS AND DISCUSSION

The solution of DPPH (1,1'-diphenyl-2-picrylhydrazyl) radical has an absorption maximum at λ = 516 nm. The mechanism of the DPPH method is based on the reduction of the DPPH-radical (violet solution) to the yellow colored 1,1'-diphenyl-2-(2,4,6-trinitrophenyl) hydrazine by free radicals capture compounds.

The experimental results for the change of absorption (A) of DPPH-radical solution and for the radical scavenging activity RSA [%] for 30 min of 1 mM BHT (standard), 1 mM÷5 mM Galantamine hydrobromide and 1 mM÷5 mM Pymadine are summarised in Table 1.

The decrease of the absorbance (A) of 0.05 mM solution of DPPH-radical at λ = 516 nm under the

effect of the test compounds in every 5 s for 30 min is shown on Figure 1 (1 mM BHT, 1 mM÷5 mM Galantamine hydrobromide, 5 mM Galantamine hydrobromide/5 mM Pymadine.

Table 1. DPPH-radical scavenging activity of 1 mM BHT (standard), 1 mM ÷ 5 mM Galantamine hydrobromide and 1 mM ÷ 5 mM Pymadine for 30 min.

	1 mM BHT					
t [min]	A	RSA [%]	t [min]	A	RSA [%]	
0	0.4862	0.00	20	0.3168	34.84	
5	0.4373	10.06	25	0.2847	41.44	
10	0.3925	19.27	30	0.2559	47.37	
15	0.3526	27.48				

1 mM ÷ 5 mM Galantamine hydrobromide										
t [min]	1 mM		2 mM		3 mM		4 mM		5 mM	
	A	RSA [%]	A	RSA [%]	A	RSA [%]	A	RSA [%]	A	RSA [%]
0	0.4880	0.00	0.5059	0.00	0.4845	0.00	0.4933	0.00	0.5037	0.00
5	0.4834	0.94	0.4967	1.82	0.4720	2.58	0.4782	3.06	0.4799	4.73
10	0.4794	1.76	0.4900	3.4	0.4632	4.40	0.4681	5.11	0.4654	7.60
15	0.4760	2.46	0.4843	4.27	0.4569	5.70	0.4601	6.73	0.4537	9.93
20	0.4730	3.07	0.4792	5.28	0.4522	6.67	0.4535	8.07	0.4438	11.89
25	0.4700	3.69	0.4745	6.21	04484	7.45	0.4479	9.20	0.4349	13.66
30	0.4673	4.24	0.4702	7.06	0.4451	8.13	0.4430	10.20	0.4268	15.27

1 mM ÷ 5 mM Pymadine										
t[min]	A	RSA [%]	A	RSA [%]	A	RSA [%]	A	RSA [%]	A	RSA [%]
0	0.5615	0.00	0.5671	0.00	0.5828	0.00	0.5812	0.00	0.5856	0.00
5	0.5564	0.91	0.5623	0.85	0.5775	0.91	0.5770	0.72	0.5822	0.58
10	0.5550	1.16	0.5600	1.25	0.5743	1.46	0.5753	1.02	0.5786	1.20
15	0.5554	1.09	0.5591	1.41	0.5717	1.90	0.5735	1.32	0.5760	1.64
20	0.5565	0.89	0.5588	1.46	0.5706	2.09	0.5720	1.58	0.5740	1.98
25	0.5575	0.71	0.5596	1.32	0.5715	1.94	0.5711	1.74	0.5723	2.27
30	0.5578	0.66	0.5610	1.08	0.5726	1.75	0.5707	1.81	0.5709	2.51

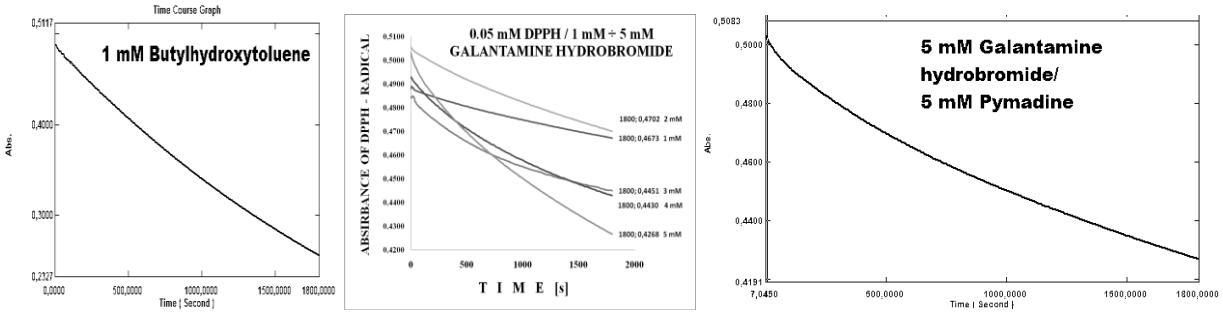


Figure 1. Effect of 1 mM Butylhydroxytoluene, 1 mM ÷ 5 mM Galantamine hydrobromide, 5 mM Galantamine hydrobromide/5 mM Pymadine on the absorbance of 0.05 mM solution of DPPH-radical for a period of 30 min.

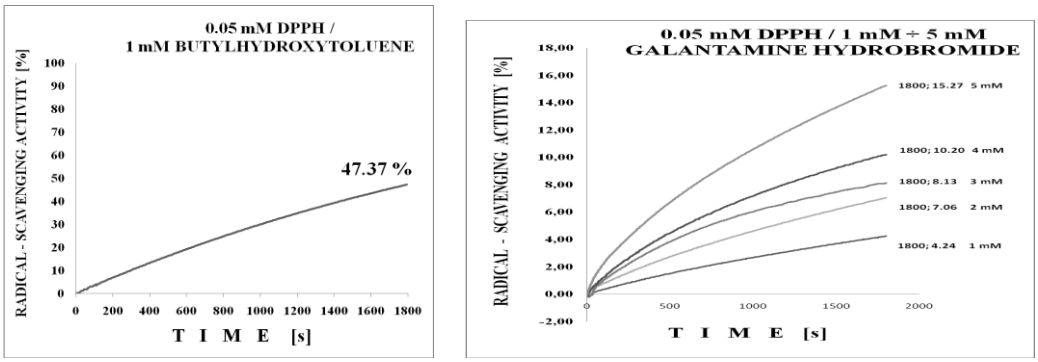


Figure 2. Kinetics of scavenging of 0.05 mM DPPH-radical from 1 mM BHT and 1 mM ÷ 5 mM Galantamine hydrobromide for a period of 30 min.

The experimental kinetic curves for scavenging of DPPH-radical for 30 min by 1 mM BHT and 1 mM÷5 mM Galantamine hydrobromide are illustrated in Figure 2.

The increase of the radical binding effect with an increase of the concentration of Galantamine hydrobromide and Pymadine is illustrated on Figure 3.

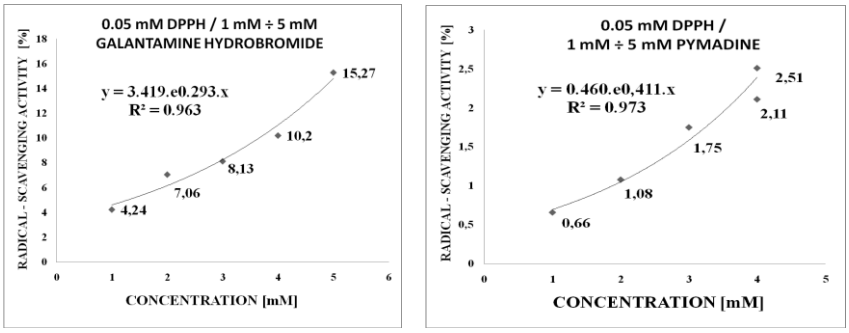
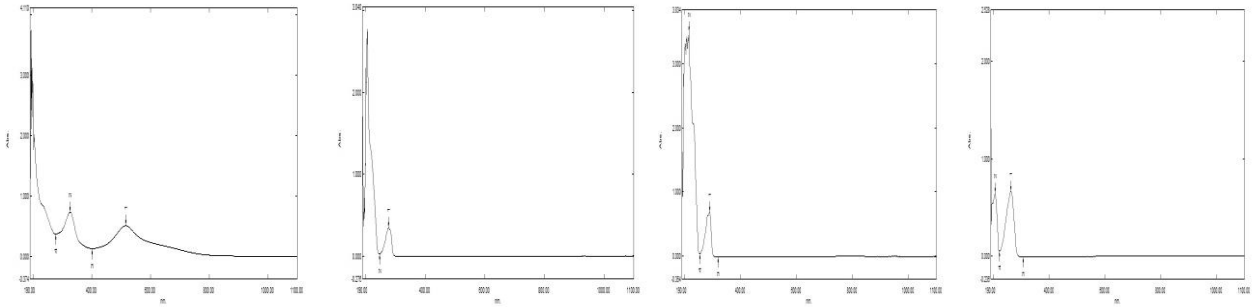


Figure 3. Relationship between the radical binding activity and concentration of Galantamine hydrobromide and Pymadine.



DPPH BHT Galantamine hydrdogromide Pymadine

Figure 4. Spectra of DPPH, BHT, Galantamine hydrobromide and Pymadine

The method is applicable for the study of radical scavenging activity of the test compounds, because they do not possess a measurable absorbance at the absorption maximum λ = 516 nm, where the DPPH-test is carried out. Spectra in methanol of DPPH, BHT, Galantamine hydrobromide and Pymadine are illustrated on Figure 4.

Table 2. Radical-binding activity of 1 mM BHT and 5 mM Galantamine hydrobromide/5 mM Pymadine after 30 min reaction with 0.05 mM solution of DPPH-radical.

N:	1 mM BHT			5 mM Galantamine hydrobromide/5 mM Pymadine				
	A	RSA[%]	UA	A	RSA[%]	UA	RRSA [%]	RDRSA [%]
1.	0.36263	47.47	0.84	0.54881	20.50	0.71	43.19	56.81
2.	0.36066	47.76	0.15	0.54971	20.37	0.41	42.65	57.35
3.	0.35957	47.92	0.69	0.55431	19.71	1.12	41.13	58.87
$\overline{X}$	0.36095	47.72		0.55094	20.19		42.32	57.68
SD	0.002	0.23		0.003	0.42		1.07	2.07
RSD [%]	0.55	0.48		0.54	2.08		2.53	1.86

Table 3. Radical-binding activity of 5 mM Galantamine hydrobromide and 5 mM Pymadine after 30 min reaction with a 0.05 mM solution of DPPH-radical.

N:	5 mM Galantamine hydrobromide					5 mM Pymadine				
	A	RSA [%]	UA	RRSA [%]	RDRSA [%]	A	RSA [%]	UA	RRSA [%]	RDRSA [%]
1.	0.58649	15.05	1.36	31.70	68.30	0.67228	2.53	1.0	5.33	94.67
2.	0.58273	15.59	0.52	32.64	67.36	0.67342	2.46	0.35	5.15	94.85
3.	0.58208	15.69	0.85	32.74	67.26	0.67354	2.44	0.65	5.09	94.91
$\overline{X}$	0.58377	15.44		32.36	67.64	0.67328	2.48		5.19	94.81
SD	0.002	0.34		0.57	0.57	0.0004	0.05		0.12	0.12
RSD [%]	0.34	2.20		1.76	0.84	0.06	2.02		2.31	0.13

The results of DPPH-radical binding activity for a period of 30 min for 1 mM BHT, 1÷5 mM Galantamine hydrobromide and 1 ÷ 5 mM Pymadine (Table 1), were calculated by the equation:

$$\text{RSA [\%]} = \frac{Acontrol - Asample}{Acontrol}.100$$

$A_{control}$ - absorption of the solution of DPPH-radical before interaction with the investigated compound; A_{sample} - absorption of the solution of DPPH-radical after reacting with the investigated compound.

Experimental results show that with increasing of the concentration from 1 mM to 5 mM for both compounds was observed an increase in radical-scavenging effect (Figure 3.). The equations:

$y = 3.419.e^{0.293.x}$ (Galantamine hydrobromide), $y = 0.460.e^{0.411.x}$ (Pymadine)

were used for calculatation of $[RSC_{50}]$ – the concentration which achieves 50 % binding of DPPH-radical. For Galantamine hydrobromide: $[RSC_{50}]$ = 9.16 mM and for Pymadine: [RSC50] = 11.41 mM.

For the calculation of radical binding activity under identical conditions, 2 ml of 0.1 mM of DPPH ($A_{0\ min.}$ = 0.69037) (Figure 5.), were added to 2 ml of 2 mM BHT, 2 ml of the model mixture containing 10 mM Galantamine hydrobromide/10 mM Pymadine. The spectra of the solutions after 30 min. reaction with DPPH-radical are presented in Figure 5. (1 mM BHT and 5 mM Galantamine hydrobromide/5 mM Pymadine) and Figure 6. (5 mM Galantamine hydrobromide and 5 mM Pymadine).

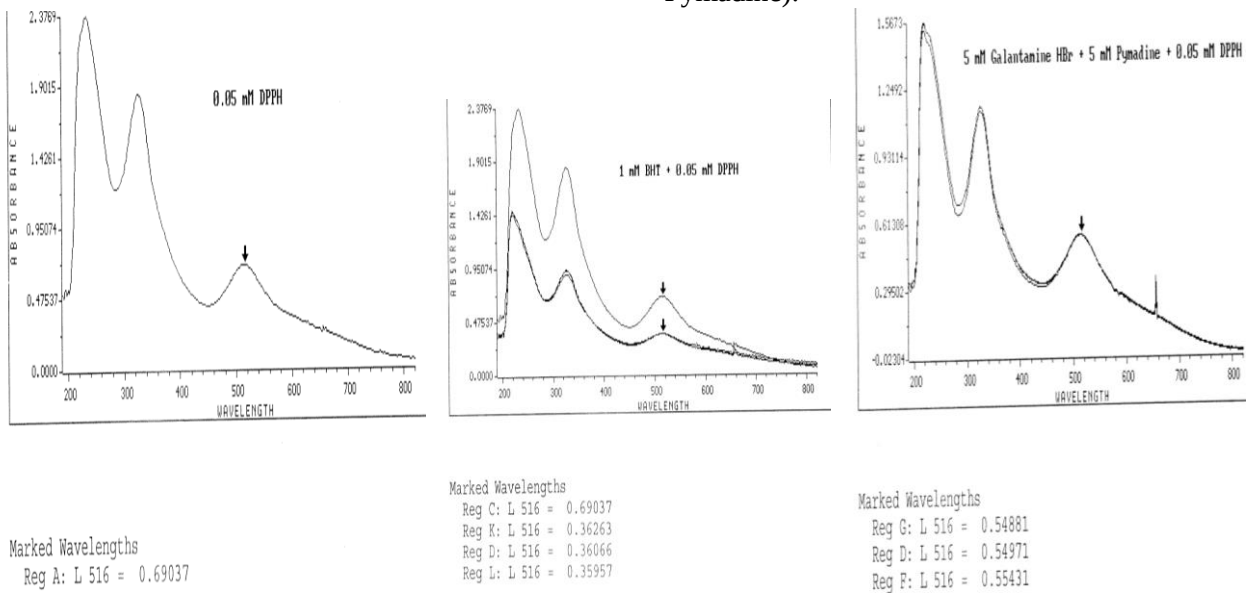


Figure 5. Spectra of 0.05 mM solution of DPPH-radical: A 0 min. = 0.69037 and after 30 min reaction with 1 mM BHT and 5 mM Galantamine hydrobromide/5 mM Pymadine.

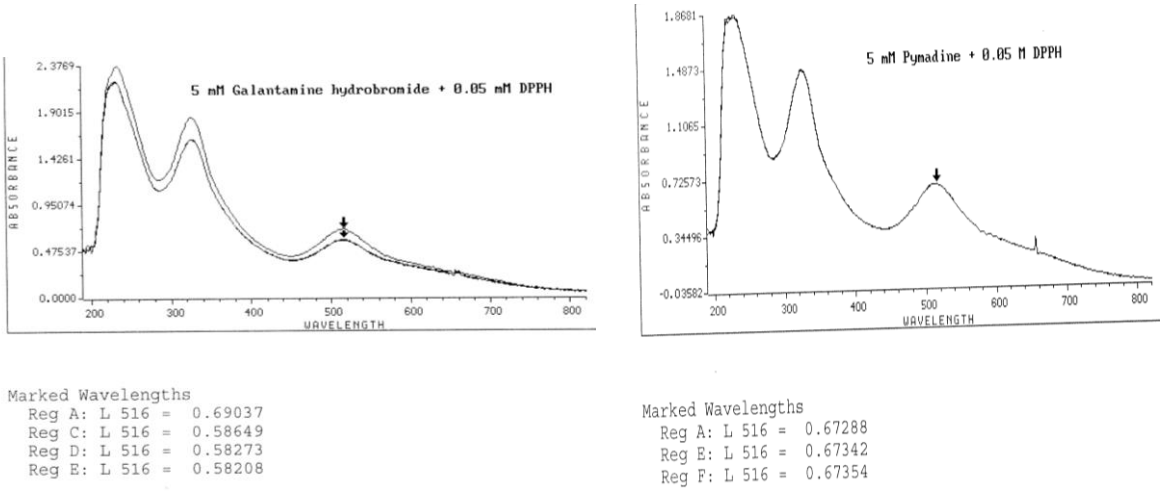


Figure 6. Spectra of 0.05 mM solution of DPPH-radical after 30 min. interaction with 5 mM Galantamine hydrobromide and 5 mM Pymadine**.**

The radical-scavenging activity of the test compounds was compared to the activity of the standard butylhydroxytoluene. The relative radical scavenging activity (RRSA, [%]) and the relative decrease in radical scavenging activity (RDRSA, [%]) were calculated and were compared to the standard butylhydroxytoluene.

$$\text{RRSA [\%]} = \frac{RSA\,sample}{RSA\,BHT}.100$$

$$\text{RDRSA [\%]} = \frac{RSA\,BHT - RSA\,sample}{RSA\,BHT}.100$$

RSA_{sample} - radical binding activity of the test compounds; RSA_{BHT} - radical binding activity of the standard butylhidroxytoluene.

The results for the antiradical activity of the test compound after 30 min interraction with DPPH-radical are shown in Table 2 (1 mM BHT and 5 mM Galantamine hydrobromide/5 mM Pymadine), Table 3 (5 mM Galantamine hydrobromide, 5 mM Pymadine).

CONCLUSIONS

The following results for the radical scavenging activity were obtained: 1 mM÷5 mM Galantamine hydrobromide (4.24 %÷15.27 %) and 1 mM÷5 mM Pymadine (0.66 %÷2.51 %). The results show that the combination 5 mM Galantamine hydrobromide/5 mM Pymadine (20.19 %) has a stronger radical scavenging activity than 5 mM Galantamine hydrobromide (15.44 %).

REFERENCES

1. P. H. Axelsen, H. Komatsu, I. V. J. Murray, *Physiol.*, **26**(1), 54 (2011).
2. M. A. Ansari, S. W. Scheff, *J. Neuropathol. Exp. Neurol.*, **69**(2), 155 (2010).
3. P. Agostinho, R. A. Cunha, C. Oliveira, *Curr. Pharm. Des.*, **16**(25), 2766 (2010).
4. W. R. Markesbery, M. A. Lovell, *Arch. Neurol.*, **64**(7), 954 (2007).
5. G. Münch, B. Westcott, T. Menini, A. Gugliucci, *Amino Acids*, **42**(4), 1221 (2012).
6. S. Losada-Barreiro, C. Bravo-Díaz, *Eur. J. Med. Chem*., **133**, 379 (2017).
7. F. Massoud, G. C. Léger, *Can. J. Psychiatry*, **56**(10), 579 (2011).
8. O. Firuzi, R. Miri, M. Tavakkoli, L. Saso, *Curr. Med. Chem.*, **18**(25), 3871 (2011).
9. J. Teixeira, T. Silva, P. B. Andrade, F. Borges, *Curr. Med. Chem*., **20**(24), 2939 (2013).
10. Y. Ago, K. Koda, K. Takuma, T. Matsuda, *J. Pharmacol. Sci*., **116**(1), 6 (2011).
11. M. Markov, N. Danchev, P. Uzunov, H. Higashino, A. Suzuki, *Acta Med. Kinki. Univ.*, **19**(2), 119 (1994).
12. M. Gaudig, U. Richarz, J. Han, B. Van Baelen, B. Schäuble, *Curr. Alzheim. Res.*, **8**(7), 771 (2011).
13. B. Seltzer, *Clin. Interv. Aging*, **5**(1), 1 (2010).
14. D. Prvulovic, H. Hampel, J. Pantel, *Expert Opin. Drug Metab. Toxicol.*, **6**(3), 345 (2010).
15. M. Traykova, T Traykov, V. Hadjimitova, K. Krikorian, N. Bojadgieva, *Z. Naturforsch. C.*, **58**(5-6), 361 (2003).
16. M. J. M. Ezoulin, J.-E. Ombetta, H. Dutertre-Catella, J. - M. Warnet, F. Massicot, *Neurotoxicol.*, **29**(2), 270 (2008).
17. H. Geerts, *Brain Res. Bull.*, **64**(6), 519 (2005).
18. G. Marinova, V. Batchvarov, *Bulg. Journal Agricult. Sci.*, **17**(1), 11 (2011).
19. E. Lombardo, C. Sabellico, J. Hájek, V. Staňková, T. Filipský, V. Balducci, P. De Vito, S. Leone, E. I. Bavavea, I. P. Silvestri, G. Richi, P. Luly, L. Saso, P. Bovicelli, J. Z. Pedersen, S. Incerpi, *PLoS ONE*, **8**(4), 1 (2013).
20. M. Okawa, J. Kinjo, T. Nohara, M. Ono, *Biol. Pharm. Bull.*, **24**(10), 1202 (2001).
21. J. Z. Pedersen, C. Oliveira, S. Incerpi, V. Kumar, A. M. Fiore, P. De Vito, A. K.

Prasad, S. V. Malhotra, V. S. Parmar, L. Saso, *J. Pharm. Pharmacol.*, **59**(12), 1721 (2007).

22. Z. Rehakova, V. Koleckar, F. Cervenka, L. Jahodar, L. Saso, L. Opletal, D. Jun, K. Kuca, *Toxicol. Mech. Methods.*, **18**(5), 413 (2008).
23. Y. Y. Belisario-Sánchez, A. Teboada-Rodríges, F. Marín-Iniesta, A. López-Gómez, *J. Agricult. Food Chem.*, **57**(15), 6770 (2009).
24. B. Buttari, E. Profumo, F. Facchiano, E. I. Ozturk, L. Segoni, L. Saso, R. Riganò, *Oxid. Med. Cell Longev.*, **2013**(1), 574029 (2013).
25. G. L. Freitas, E. M. Kuskoski, L. Gonzaga, R. Fett, *Alimentos e Nutrição,* **17**(3), 303 (2006).
26. M. K. Roy, M. Koide, T. P. Rao, T. Okubo, Y. Ogasawara, L. L. Juneja, *Int. J. Food Sci. Nutr.*, **61**(2), 109 (2010).
27. J. Pérez-Jiménez, S. Arranz., M. Tabernero, M. E. Diaz-Rubio, J. Serrano, I. Goñi, F. Saura-Calixto, *Food Res. Int.*, **41**(3), 274 (2008).
28. R. Tundis, M. R. Loizzo, M. Bonesi, F. Menichini, V. Mastellone, C. Colica, F. Menichini, *J. Food. Sci.*, **77**(1), 40 (2012)
29. D. A. Kostić, J. M. Velicković, S. S. Mitić, M. N. Mitić, S. S Randelović, *Trop. J. Pharm. Res.*, **11**(1), 117 (2012)
30. M. Kiendrebeogo, A. Y. Coulibaly, R. C. H. Nebie, B. Zeba, C.E. Lamien, A. Lamien-Meda, O. G. Nacoulma, *Braz. J. Pharmacogn.*, **21**(1), 63 (2011)

DPPH РАДИКАЛ-СВЪРЗВАЩА АКТИВНОСТ НА ГАЛАНТАМИН ХИДРОБРОМИД И ПИМАДИН САМОСТОЯТЕЛНО И В КОМБИНАЦИЯ

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(Резюме)

Свръхпроизводството на реактивни кислородни видове и отслабването на антиоксидантните защитни механизми в човешкото тяло са основната причина за оксидативния стрес, който е в основата на развитието на невродегенеративната болест на Алцхаймер. Алкалоидът галантамин е неселективен инхибитор на ацетилхолинестеразата с антиоксидантна активност. Пимадин е недеполяризиращ блокер на калиевите канали и оказва синергичен ефект с галантамин върху симптоматичното лечение на болестта на Алцхаймер.

Целта на настоящото изследване е оценката на радикал-свързващата активност (RSA) на галантамин хидробромид, пимадин и комбинацията галантамин хидробромид/пимадин по отношение на 2,2-дифенил-1-пикрилхидразил (DPPH) радикала. Намалението на абсорбцията на 0.05 mM метанолен разтвор на DPPH при λ = 516 nm в присъствието на метанолни разтвори на: 1 mM бутилхидрокситолуен (стандарт), 1 mM ÷ 5 mM галантамин хидробромид, 1 mM ÷ 5 mM пимадин и 5 mM галантамин хидробромид/5 mM пимадин се наблюдава през равни интервали от 5 s за общ период от 30 min чрез спектрофотометричен метод. Регресионни уравнения се използват за изчисляване на стойностите на RSC_{50}: RSA (галантамин хидробромид) = $3.419.e^{0.293}.c$, RSC_{50} (галантамин хидробромид) = 9.16 mM; RSA (пимадин) = $0.460.e^{0.411}.c$, RSC_{50} (пимадин) = 11.41 mM. RSA на изследваните съединения се сравнява с ефекта на стандартния BHT и се изчисляват относителната радикал-свързваща активност (RRSA) и относителното намаляване на радикал-свързващата активност (RDRSA). Експерименталните резултати показват, че комбинацията от 5 mM галантамин хидробромид/5 mM пимадин има по-висока RSA (20.19 %) в сравнение с 5 mM галантамин хидробромид (15.44 %) и 5 mM пимадин (2.48 %).

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The equations are written using “Equation Editor” and chemical reaction schemes are written using ISIS Draw or ChemDraw programme.

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REFERENCES

1. D. S. Newsome, *Catal. Rev.–Sci. Eng*., **21**, 275 (1980).
2. C.-H. Lin, C.-Y. Hsu, *J. Chem. Soc. Chem. Commun*., 1479 (1992).
3. R. G. Parr, W. Yang, Density Functional Theory of Atoms and Molecules, Oxford Univ. Press, New York, 1989.
4. V. Ponec, G. C. Bond, Catalysis by Metals and Alloys (Stud. Surf. Sci. Catal., vol. 95), Elsevier, Amsterdam, 1995.
5. G. Kadinov, S. Todorova, A. Palazov, in: New Frontiers in Catalysis (Proc. 10th Int. Congr. Catal., Budapest,(1992), L. Guczi, F. Solymosi, P. Tetenyi (eds.), Akademiai Kiado, Budapest, 1993, Part C, p. 2817.
6. G. L. C. Maire, F. Garin, in: Catalysis. Science and Technology, J. R. Anderson, M. Boudart (eds), vol. 6, SpringerVerlag, Berlin, 1984, p. 161.
7. D. Pocknell, *GB Patent* 2 207 355 (1949).
8. G. Angelov, PhD Thesis, UCTM, Sofia, 2001, pp. 121-126.
9 JCPDS International Center for Diffraction Data, Power Diffraction File, Swarthmore, PA, 1991.
10. CA **127**, 184 762q (1998).
11. P. Hou, H. Wise, *J. Catal.*, in press.
12. M. Sinev, private communication.
13. http://www.chemweb.com/alchem/articles/1051611477211.html.

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