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Isolation and identification of flavonoids from *Thymus longidens* var. *lanicaulis* and *T. longidens* var. *dassareticus* (*Lamiaceae*)

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Two different taxa of the genus *Thymus* L. (*Thymus longidens* var. *lanicaulis* and *T. longidens* var. *dassareticus*) growing wild in the Republic of Macedonia, were subjected to the flavone aglycones examination. Two flavones (luteolin and apigenin) and two flavanones (eriodyctiol and naringenin) were isolated from the diethylether extract of var. *lanicaulis*, whereas from the other taxa, var. *dassareticus*, instead of luteolin diosmetin was isolated. The isolated compounds were identified by the UV spectroscopy, TLC and HPLC-DAD analyses. The related phenolic acids, rosmarinic and caffeic, were detected by HPLC-DAD.

Keywords: *Thymus* L., *Thymus longidens* var. *lanicaulis*, *Thymus longidens* var. *dassareticus*, flavonoids, isolation, identification, HPLC analysis

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A lot of wildly growing species of genus *Thymus* L. are used in folk medicine for treating respiratory infections, abdominal throes and other diseases in the Republic of Macedonia (1). Officially *Serpylli herba* is used as a substitute of *Thymi herba* although its biological origin is not specified clearly and many different Macedonian *Thymus* species are covered by that name. The majority of *Thymus* taxa that appear in Macedonian flora belong to the Sect. *Marginati* (A. Kerner) A. Kerner (2). Most of the taxa are characteristic for the Balkan peninsula representing certain endemisams. *Thymus longidens* var. *lanicaulis* Ronn. is spread widely throughout the whole territory of Macedonia, whereas *T. longidens* var. *dassareticus* Ronn. is rarely found (2).

In order to investigate possible pharmacological interest of the Macedonian *Thymus* taxa, we have started an extensive study of their chemical constituents. In relation to the

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first part of our examination considering essential oils composition, certain data have been recently reported (3). The second part of our program is connected with the examination of polyphenols. The aim of the present study is the isolation and identification of free flavonoid aglycones.

EXPERIMENTAL

Plant material

The upper parts of the plants were collected in flowering stage, and air-dried. The taxa were botanically identified by V. Matevski, Department for Botany, Faculty of Science, Skopje, Republic of Macedonia, as *Thymus longidens* var. *lanicaulis* Ronn. as well as *T. longidens* var. *dassareticus* Ronn. Voucher specimens were deposited at the Herbarium of Department for Botany, Faculty of Science, Skopje.

Instruments and chemicals

A Perkin-Elmer Lambda 16 UV-VIS spectrophotometer with 1 cm quartz cells was used under following operating conditions: scan speed 60 nm min^{-1}, scan range 200–500 nm, slit width 2 nm. HP 1090 M liquid chromatograph with diode array detector for HPLC analysis was used.

All reagents used were of analytical grade. Authentic samples of apigenin, luteolin, diosmetin, naringenin, eriodyctiol (Extrasinthese, Lyon, France), rosmarinic acid and caffeic acid (Roth, Karlsruhe, Germany), Kiesel gel GF 254 Alufolien and microcrystalline cellulose – Avicel (Merck, Darmstadt, Germany) for TLC and Silicagel G 60 (Merck) (70–230 mesh) for CC were used.

Extraction, separation and purification

Dried plant material was cut into small pieces and extracted with ethanol-water (7:3), at room temperature, mixed periodically for 24 hours. The extract was evaporated under reduced pressure until only water remained. As-obtained dense aqueous concentrate was then extracted with diethylether. The last extract was evaporated to small volume and submitted to CC on silicagel column, 50 × 2 cm. The mixture of $CHCl_3$–EtOAc–MeOH with an increasing ratio of EtOAc and then MeOH, was used for separation. Fractions were tested by TLC. Total purification was obtained by preparative TLC.

Identification of isolated components

UV/VIS spectroscopy. – UV-spectra of isolated flavonoids were recorded in methanol and after an addition of classical shift reagents according to the guide for systematic identification of flavonoids (4). Data were compared to authentic samples.

TLC. – Silicagel (S) and cellulose (CL) plates with the following solvent systems were used: S-1: toluene : EtOAc : HCOOH (58 : 33 : 9); S-2: $CHCl_3$: MeOH (93 : 7); S-3:

C_6H_6 : dioxane : AcOH (90 : 25 : 4); S-4: C_6H_6 : EtOAc : HCOOH (40 : 10 : 5); S-5: C_6H_6 : AcOH (4 : 1); S-6: toluene : MeEtCO : AcOH (18 : 5 : 1); CL-1: 30% AcOH; detection: UV light (λ = 254 nm and λ = 366 nm), 5% methanolic solution of $AlCl_3$.

HPLC analysis. – A C-18 reversed-phase column (250 × 4.6 mm) with 5 μm particle size was used. The separation of different components was performed using water-HCOOH (99.90 : 0.10) solvent A/acetonitrile solvent B, at the flow rate of 0.8 mL min^{-1}, starting with 10% B for the first 5 min, followed by a gradient to 35% of B after 10 min. UV detection was carried out at λ = 254, 286 and 360 nm. The volume of 20 μL of the solution was injected. Data were compared with authentic samples.

RESULTS AND DISSCUSION

Column chromatography followed by preparative TLC was used to isolate five flavonoid aglycones from diethylether extracts of two different varieties of *Thymus longidens:* var. *lanicaulis* and var. *dassareticus*. The isolated compounds were flavones (apigenin, luteolin and diosmetin) and flavanones (naringenin and eriodyctiol). They were identified on the basis of the coincidence between the data from the UV spectra measurements with usual reagents shifts (Table I), TLC comparison with authentic samples as well as HPLC-DAD analysis (Table II). Related phenolic acids (rosmarinic and caffeic) were identified in diethylether extracts by HPLC-DAD (Table II).

The two taxa of the genus *Thymus* in the flora of the Republic of Macedonia contained the common flavonoid aglycones. Apigenin was identified in both taxa. Luteolin was present as principal flavone in var. *lanicaulis* whereas for var. *dassareticus* the principal flavone was diosmetin.

Apigenin and luteolin were identified in almost all the *Thymus* taxa investigated until now (5–8). Diosmetin was rarely present and up to now it was identified only in *T. hirtus* (9). Isolation and identification of diosmetin in the taxa of Sect. *Marginati* of genus *Thymus* in the flora of Macedonia represent important data for distribution of diosmetin in genus *Thymus*. The flavanones naringenin and eriodyctiol were present in both taxa. Up to now, the two flavanones have been simultaneously present in a few Iberian *Thymus* taxa (5, 8, 10) whereas some data point out that naringenin commonly occurs as flavanone in genus *Thymus* (11, 12). The occurrence of eriodyctiol has been rarely noticed, *e.g.* in *T. moroderi* (13).

In relation to the free flavonoid aglycones, the two taxa of genus *Thymus* in Macedonian flora were similar to Iberian *Thymus* taxa, *T. webbianus* (10). On the other hand, many more differences have been found due to the lack of methylated flavones in those Macedonian *Thymus* taxa. Highly methylated flavonoids have been found to be very important components responsible for certain spasmolitic effects of genus *Thymus* (14). These components were important from the chemotaxonomic point of view as well (8). Some of them are characteristic for particular section of genus *Thymus* (11). Since we have examined flavonoids in diethylether extracts only, we could affirm that for these extracts the lack of methylated flavonoids was characteristic for both varieties of *T. longidens*.

Table I. UV spectrophotometric data of isolated flavone aglycones in methanol and after addition of classical shift reagents

	Components	Band II (λ_{max}, nm)	Band I (λ_{max}, nm)
A (naringenin)	MeOH	286	326 sh.
	NaOMe	323 (+37 nm)	
	$AlCl_3$	312 (+26 nm)	375
	$AlCl_3$/HCl	311 (+25 nm)	371
	NaOAc	323 (+37 nm)	
	NaOAc/H_3BO_3	290	332 sh.
B (apigenin)	MeOH	268	333
	NaOMe	275	389 (+56 nm)
	$AlCl_3$	276a, 301b	348, 386 (+53 nm)
	$AlCl_3$/HCl	276a, 300b	340, 380 (+47 nm)
	NaOAc	274a,300b (+6 nm)	372
	NaOAc/H_3BO_3	268a, 300b	335
C (eriodyctiol)	MeOH	286	320 sh.
	NaOMe	246, 324 (+38 nm)	
	$AlCl_3$	310 (+24 nm)	378 (+58 nm)
	$AlCl_3$/HCl	310 (+24 nm)	373 (+53 nm)
	NaOAc	289, 325 (+39 nm)	
	NaOAc/H_3BO_3	289	330 (+10 nm)
D (luteolin)	MeOH	254, 268, 290 sh.	347
	NaOMe	270, 329 sh.	406 (+59 nm)
	$AlCl_3$	274, 300 sh.	328,420 (+73 nm)
	$AlCl_3$/HCl	260 pr., 275, 300	355, 385 (+38 nm)
	NaOAc	270, 326 (+16 nm)	384 (+37 nm)
	NaOAc/H_3BO_3	260	375 (+28 nm)
E (diosmetin)	MeOH	240, 252, 267	344
	NaOMe	270, 302	386 (+42 nm)
	$AlCl_3$	273, 298	360, 390 (+46 nm)
	$AlCl_3$/HCl	276, 295	352, 385
	NaOAc	275, 322 (+23 nm)	367
	NaOAc/H_3BO_3	253, 267	348

Table II. TLC and HPLC data of flavone aglycones analyzed

Common name	R_f (TLC)[a]							t_r (HPLC) (min)
	S-1	S-2	S-3	S-4	S-5	S-6	CL-1	
Rosmarinic acid	–	–	–	–	–	–	–	11.6
Caffeic acid	–	–	–	–	–	–	–	29.6
Eriodyctiol	0.56	0.35	0.44	0.47	0.28	0.13	0.63	34.9
Luteolin	0.38	0.26	0.31	0.30	0.11	0.17	0.15	40.6
Naringenin	0.60	0.46	0.63	0.61	0.46	0.30	0.70	42.4
Apigenin	0.58	0.40	0.47	0.50	0.16	0.32	0.31	47.3
Diosmetin	0.52	0.34	0.51	0.46	–	–	0.16	49.6

[a] Systems used in TLC – see experimental.

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SAŽETAK

Izolacija i identifikacija flavonoida iz biljaka *Thymus longidens* var. *lanicaulis* and *T. longidens* var. *dassareticus (Lamiaceae)*

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Ispitivani su flavonski aglikoni u dvije različite podvrste roda *Thymus* L. (*Thymus longidens* var. *lanicaulis* and *T. longidens* var. *dassareticus*) koje u Republici Makedoniji rastu samoniklo. Ekstrakcijom dietileterom iz var. *lanicaulis* izolirana su dva flavona (luteolin i apigenin) i dva flavanona (eriodiktiol i naringenin), dok je iz var. *dassareticus umjesto luteolina izoliran diosmetin. Izolirani spojevi identificirani su UV spektroskopijom, TLC i HPLC-DAD kromatografijama. Fenolne kiseline, ružmarinska i kofeinska, detektirane su HPLC-DAD analizom.*

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