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Structure elucidation of phenolic acids, flavonoids and hypocholesterolemic activity of *Nepeta septemcrenata* and *Otostegia fruticosa*

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ABSTRACT

The flavonoids of both Nepeta septemcrenata and Otostegia fruticosa (Lamiaceae family) were isolated from the chloroform and ethyl acetate fractions of the aqueous methanolic extract . The flavonoids of N.septemcrenata were identified as: genkwanin, thymusinin addition to caffeic acid and ferulic acid, (from chloroform fraction), vecinin-2 and thymusin-6-O-glucoside(from ethyl acetate fraction). O.fruticosaaffordedferulic acid, Pectolinarigenin, 6,5'dihydroxy diosmetin(from chloroform fraction) and chrysoeriol-7-O-glucoside(from ethyl acetate fraction). Identification of the isolated compounds was carried out using different chromatographic techniques and spectroscopic measurements(UV, MS,^{1}*H-nmr). The acute toxicity study of N.septemcrenata alc. extract proved that, it has a wide margin of safety(LD_{50}= 421.8 mg/kg b. wt.) and the i.p. injection 1/5LD_{50}(84.5 mg/kg b. wt.) of the extract to rats produces a reduction in the cholesterol and triglycerides levels.*

Key words : *Lamiaceae*, *Nepeta septemcrenata*, *Otostegia fruticosa*, phenolics, flavonoids and hypocholestrolemic activity.

INTRODUCTION

Lamiaceae family is one of the largest plant families and known to be rich in medicinal plants[1]. Two plants of this family known as *Nepeta septemcrenata*and *Otostegia fruticosa*were chosen to study their phenolic, flavonoidal constituents and investigation of the hypocholestrolemic activity. The two plants are growing in South Saini, Egypt and used by the native Bedouins as antipyretic, sedative, protection against myocardial infarction, treatment of children's gum, and in some types of ophthalmia[2-5].The previous studies on both species revealed the existence of many chemical classes like volatile oils di- and triterpenes, phenylpropanoids, flavonoids and others. Ziba et al in 2003 [(6)] studied the external flavonoids of 38 nepeta species they identified 8-hydroxycirsiliol, 5,4-dihydroxy-6,7-dimethoxyflavone, isothymusin and genkwanin, Pervykh et al[7] isolated apigenin, luteolin, cosmosiin and cynaroside from *Nepetavelutina*. Luteolin 7-*O*-glucuronide, luteolin 7-O-glucurono-(1→6)-glucoside,apigenin 7-*O*-glucuronide , luteolin and apigen in addition to phenolic acids p-coumaric, caffeic and rosmarinc acids were isolated from *Nepetacataria* Var. *citriodora*[8].4'-*O*-methylisoscutellarein-7-*O*-[β-D-allopyranosyl-(1→2)-6"-*O*-acetyl-β-D-gluco-pyranoside and Isoscutellarein-7-*O*-[β-D-allopyranosyl-(1→2)-6"-*O*-acetyl-β-D-glucopyranoside] are the main flavonoids in *Nepetacadmea*[9]. It was found that, the essential oils and methanolic extract of *Nepeta* species exhibited many biological activities like anti-inflammatory, hypoglycemic, hepatoprotective, antibacterial, antiviral and antifungal [10].

Khan et al in 2009[(11)]identified four flavonoids from the roots of *O. limbata*as: keampferol-3-β-D-glucosyl-(1→ 2)-[β-D-glucosyl-(1 → 4)]-[6-*O*-[(2E)-3-(4-hydroxy-phenyl) prop-2-enoyl]-β-D-glucosyl-(1→ 3)]-α-L-rhamnoside, keampferol-7-[(α-L-rhamnopyranosyl)-3- [6-*O*-[(2E)-3-(4-hydro-xyphenyl)prop-2-enoyl]-β-D-glucosyl-(1→2)]-[β-D-glucopyranosyl-(1→4)]-[6-*O*-[(2E)-3-(4-hydro-xyphenyl)prop-2-enoyl]-β-D-gluco-syl-(1→3)]-α-L-rhamnoside,

cirsimaritin and 3′-*O*-methyleupatorin.Ahmed, et al and Rashid et al[12-13] proved the potential antioxidant activity of both *O. limbata*and *O. aucheri* respectively. While Amin et al [14] stated that, the methanolic extract of *O. persica*exhibited significant healing activitywhen topically applied on rats.

This paper aimed to investigate the phenolic acids and flavonoids of both *N. septemcrenata* and *O. fruticosa* in addition to evaluate the hypocholesrolemic effect of methanolic extract of *N. septemcrenata* .

MATERIALS AND METHODS

Plant material: Both *N. septemcrenata* and *O. fruticose* were collected from St. Catherine region(Southern Sinai), both plants were collected in May during the flowering stage, dried in shade and grinding to fine powder. the plants were kindly, identified by Dr. Elgebally M. prof. of taxonomy at Botany dept., Faculty of Sci., Cairo univ., and the voucher specimens were deposited at NRC herbarium.

Extraction and isolation ofphenolic acids and flavonoids :
Instruments:-
1-UV-Vis.spectrophotometer pc. 2401Schimadzu.
2-Bruker NMR spectrometer operating at 500 MHz for ^{1}H NMR .
3-Mass spectra were recorded on a Finnigan MAT SSQ 700 system in EI mode.

Extraction and isolation of flavonoids:
About 1.0kg of air dried powdered herb of both *N. septemcrenata* and *O. fruticosa* were defatted, separately, with petroleum ether(br. 40 –60 °C) in a soxhlet . The defatted powders were extracted with methanol (80%, 4x2L) . the combined methanol extract of each plant was evaporated *in vacuo*at50ºC till free from methanol and diluted with hot distilled water(600 ml), left in the refrigerator overnight and filtered the. The aqueous filtrates were partitioned with chloroform (400 ml x 3) and then with ethyl acetate(500mlx4). Each combined solvents were dried over anhydrous sodium sulfate and evaporated till dryness.

About 2.7 g of the chloroform fraction of *N. septemcrenata* were applied to a polyamide column (6S Riedel-deHaen, 90x4cm) eluted with water (100%) followed by water/methanol gradient up to methanol (100%) and fraction 1Leach were collected and afforded eight fractions(A-H). the fraction- E was found to contain compound **N-I** and **N-II** as main compounds which were isolated by preparative paper chromatography (PPC, Whatmann 3MM) using 15% acetic acid as a solvent system. Two main bands**N-I** and **N-II** were eluted and further purified over SephadexLH-20 column eluted with 95% methanol to afford compounds **N-I** and N-II (5mg and 8 mg respectively). While the fraction –G was found to contain compounds **N-III** and **N-IV** which were separated in pure form using preparative TLC with polyamide plates developed with ethyl acetate: chloroform :methanol,(2: 3: two drops) to give 2mg and 9 mg respectively .

Two grams of the ethyl acetate fraction of were subjected to PPC using B.A.W.(3:1:1) as an irrigating solvent. Two main bands **N-V**and **N-VI** were eluted with 80%methanol and both of them were further purified over Sephadex LH-20 column which gave compounds **N-V** and **N-VI** in pure form as a yellowish powder (7mg and 4mg respectively).

About 3g of the chloroform fraction of *O. fruticose* were subjected to PPC using B.A.W.(3:1:1) as a developing solvent. Three main bands were eluted with 95%methanol and further purified over Sephadex LH-20 column which gave compounds **O-I**, **O-II** (as a white powder) and **O-III**(as a yellowish solid) in pure form(8mg,9mg and 10mg respectively).

the ethyl acetate fraction of *O. fruticosa* was found to contain one flavovonoidal spot which was isolated by passing over Sephadex LH-20 column eluted with 90% aqueous methanol. The fractions containing compound **O-IV** in pure form were polled together and evaporated under reduced pressure to afford a yellowish powder (13mg).

pharmacotoxicity studies:
a- 70% ethanolic extract of *N. septemcrenata* herb.
b-Animals: spraquedawly rats and mice were used. The animals were housed under hygienic conditions and maintained on a commercial balanced formula diet and were allowed free access to normal food and fresh water.
c- Diagnostic kits: commercial diagnostic kits from Biomerieux, laboratory reagents and products, Marcy L'Etoile 169260charbonnieres Les Bains, France, were used for the determination of activities of alanine transaminase(ALT)[(14)], aspartate transaminase(AST)[(14)], alkaline phosphatase(AP)[(15)], serum cholesterol[(16)] and triglycerides[(17)].

d- Acute toxicity study of the plant alcoholic extract was determined by measuring LD_{50} according to Behrens and Karber[18] by intraperitoneal injection of different groups(8 mouse each of the same age and weigh) with different doses of the plant alc. ext. .
e- Sub-acute toxicity study was carried out using two equal groups of rats (6 rats each), the 1st group was injected (IP) with saline as control and the 2nd group was injected with $1/5LD_{50}$(84.38 mg / kg b.wt.) the two groups were administered daily for successive 15 days. Blood samples were collected from retro-orbitals venous after 5.10 and 15 days and centrifuged for serum preparation according to Helperin et al[19].
f- Statistical analysis: significance of the results were evaluated by using student's 't' test[20] .

RESULTS AND DISCUSSION

The phenolic acids and flavonoids were obtained from the alcoholic extracts of both plants by partition between chloroform and ethyl acetate respectively, the isolated compounds were identified as follow:

N-I: *caffeic acid*, this compound was obtained as a white powder and appears as a bluish spot under UV light and have the same R_f values in different solvents and ^{1}H-nmr, MS data of an authentic sample[21].

N-II and O-I: *ferulic acid*, these two compounds were found the same and isolated from both plants. The EI-MS of the compound exhibited M^+ at m/z (rel. int.) =194(100), 179(17)[M^+ - CH_3], 163(21) [M^+ - OCH_3] and 133(18) [M^+ - CH=CH-COOH]. The ^{1}H-nmr spectrum(DMSO) showed signals at □□in ppm 7.5 and 6.25 as two proton doublets with *J*= 15 Hz proved the presence of two olefenic protons H-2' and H-1' in the side chain respectively, the aromatic protons appeared at 7.25(1H,s, H-3) , 7.1 (d, *J*=8 Hz, H-5), 6.6.8 (d; *J*=9 Hz, H-6) and the methoxy protons group at 3.75 (s). these data were in accordance with that reported by Sayed et al[22].

N-III: *genkwanin*, the compound was isolated from the chloroform fraction as a yellowish powder and it is an aglycone in nature (through it's chromatographic behavior on paper chromatog.). it is a flavone type where UV spectra gave band-I at 333nm in methanol, while no bathochromic shift with in band-II with sodium acetate which indicate the absence of a free OH group at C-7. EI-MS showed the [M^+]at m/z(rel. int.)= 284(52) corresponding to the molecular formula $C_{16}H_{12}O_5$. The fragments at 256(13)[M^+ - CO], 166(12)[$A_1^{+\cdot}$] and118(9) [$B_1^{+\cdot}$] confirm the structure[23].^{1}H-nmr spectrum (270MHz, DMSO, δ ppm): 3.75(3H,s,OMe), 5.9 (1H,d,J = 2 Hz, H-6), 6.2(1H, d, *J* = 2Hz, H-8), 6.55 (1H, s, 1H, H-3), 6.78, 7.15 (2H, d,d, *J* = 8.5, H-3´,5´) and 7.8 (2H, d, *J* =8.2 Hz, H-2´,6´). These data are coincided with that reported for genkwanin by Seyyed et al in 2009[24].

N-IV: *thymusin*, the compound was isolated from the chloroform fraction as a yellow powder in a small amount and an aglycone in nature. It's UV spectrum in methanol proved the flavone nature with a monosubstituted B ring (band-I 335 nm), and a highly substituted A ring with free hydroxyls at C-5 and C-6 (band- II 296 nm) [10]. The EI-MS of the compound exhibited a molecular ion peak at m/z 330 (69 %), in accord with a flavone containing three hydroxyls and twomethoxy groups, add all the data are agree with that reported by Federico et al[25].

N-V:*vicenin-2*, this compound was obtained from the ethyl acetate fraction, it's a glycosedic in nature and resist the hydrolysis under normal conditions, so, it is a *C*-glycoside not *O*-glycoside. The UV absorption spectrum in methanol displayed band-I at 332nm which prove the flavone structure of the compound and on addition of NaOMe shifted band-I to397nm with high intensity indicating the presence of a free OH group at C-4'[23]. The other data of uv spectra proved the existence of free OH groups at C-5 and C-7. The EI-MS showed the M^+at m/z=594 which fit to the molecular formula $C_{27}H_{32}O_{15}$ proving the presence of an aglycone as apigenin (m/z=270) with two hexose moieties. The presence of $A_1^{+\cdot}$ Fragment at m/z =177 confirm the presence of the two sugar moieties in ring A at C-6 and C-8 as *C*- glycoside[23]. The ^{1}H-nmr spectrum showed signals at 7.92 (2H, d,d, *J*= 8.7 Hz, H-2', H-6'), 6.91 (2H, d,d, *J*= 8.5 Hz, H-3', H-5'), 6.59 (1H, s, H-3), the two anomeric protons of the sugar moieties are appeared at 4.76 (d, *J*= 9.8 Hz, H-1"),4.88 (d, *J*= 9.8 Hz, H-1'") which were assigned as glucose and the rest of the two glucose moieties appeared as a complex signals at, 3.30 -3.86 ppm. The absence of the signal due to H-6 and H-8 confirmed the presence of two sugars at these positions, so, comparing these data with that reported [26-27] resulted in identification of the compound as vicenin-2.

N-VI: *thymusin-6-O-glucoside*, it isolated as a yellow powder and gave the same data in UVwith different shift reagents as compound **N-IV** (thymusin) but differ on paper chromatography where it's $R_f$in different solvent proved that it is a flavone glycoside(R_f=0.35, 15% acetic acid and 0.45 in BAW3:1:1). The ^{1}H-nmr spectrum (DMSO, □ in ppm) exhibited signals at 3.9 and 3.85(3H, 3H, s,s 2-OCH_3) , 4.9 (d, *J*= 8.7 Hz, H-1") due to the anomeric proton of the glucose moiety, 6.55 (1H, s, H-3), 6.87 (2H, d,d, *J*= 9.1 Hz, H-3', H-5') and 7.75 (2H, d,d, *J*= 8.7 Hz, H-2', H-6'). The acid hydrolysis revealed the presence of thymusin as an aglycone(gave the same UV and MS of compound **N-IV**) and glucose as a sugar, accordingly the compound could be identified asthymusin-

6-*O*-glucosid.

O-II: *Pectolinarigenin* , this compound was isolated as an off white powder from the chloroform fraction, it is an a flavone aglycone in nature where it displayed band-I in UV spectrum with methanol at λ_{max}= 336nmandthe other shift reagent proved the presence of free OH groups at C-4, C-7 and substituted at C-4'. The molecular weight was found to be at m/z= 314 correspond to $C_{17}H_{14}O_6$.The nmr data were agree with the literature [28-29]. **O-III:***5,6,7,3',5'-pentahydroxy-4'-methoxy flavone***,**this compound was isolated as a yellowish powder and a flavone aglycone in nature. The uv spectra in methanol exhibited band –I at λ_{max}= 329 nm which bathochormically shifted with decrease in intensity upon addition of NaOMe indicating the presence of the methoxy group at C-4'. Also EI-MS displayed M^+at m/z= 332 and [$A1^{+\cdot}$] atm/z= 186 indicates the presence of three OH groups on ring –A[23]. The ^{1}H-nmr spectrum (DMSO, δin ppm) exhibited signals at 7.38 (2H, s, H-2',6'), 6,46 (1H,s, H-8) and 3.99 (3H, s, OCH_3), these data closely related to that reported by Anderson et al[30].

O-IV:*chrysoeriol-7-O-glucoside*, it separated from the ethyl acetate fraction as a yellowish solid and it is a flavone glycoside. The UV spectra proved the presence of a free OH group at C-4'and the sugar moiety at C-7 which was confirmed through the acid hydrolysis where it gave glucose as a sugar moiety and chrysoeriol as an aglycone. The EI-MS spectrum showed M^+ - 1 at m/z= 461, 446[M^+ - CH_3], and 299[M^+ - glucose] due to the aglycone. The other data are fitted with the literature data[31].

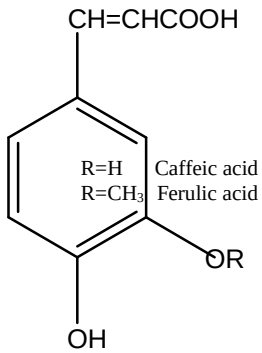
The acute toxicity study of the alc. Ext. of *N. septemcrenata* revealed that, the extract has a wide margin of safety where, it's LD_{50}= 421.8 mg/kg b. wt. (i.p. injection).

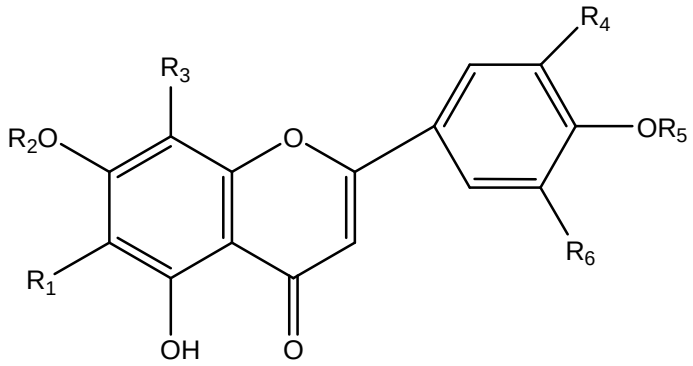
The i.p. injection of 1/5LD_{50} (84.5mg/kg b. wt.) into rats resulted in a reduction in the cholesterol and triglycerides levels in the tested group of animals as shown in table-1. This decrease was started from the 5th day to the end of the experiment. The results are in agreement with those of Agrwal et al[32], where they reported that, the alc. Ext. of *N. hindostana* lowered serum cholesterol and *beta* lipoprotein levels in normal and high fat fed pigs. Also, it was found that some flavones reduce cholesterol, *beta* lipoprotein and phospholipid levels in blood[33].so, it appears that, the effect of the alc. Ext. of the plant may be due to the presence of flavones in the extract.

Table (1): effect of alc. ext. of *N. septemcrenata* on different biochemical parameters after ipinjection (84.5mg/kg b.wt.) for 15 days

Time/day	5 days		10 days		15 days	
Group parameters	control	Plant ext.	control	Plant ext.	control	Plant ext.
AST(IU/ml)	125.16±2.12	129.83±3.82	126.17±2.63	124.83±2.21	124.17±2.43	122.00±1.79
Calculated t	-1.069		0.38833		0.71846	
ALT(IU/ml)	62.83±3.0	60.50±3.42	61.88±3.62	58.80±3.15	62.50±3.58	56.33±1.94
Calculated t	0.5123		1.08278		1.51532	
AP(K.K./ml)	15.33±1.56	18.17±1.56	16.33±1.33	15.83±1.013	15.50±1.12	13.67±1.26
Calculated t	-1.2836		0.29851		1.09023	
Cholesterol (mg/dl)	51.17±2.91	41.50±3.30	52.33±2.08	**36.67±1.87	54.00±2.82	**32.17±1.94
Calculated t	2.19408		5.60159		6.38455	
Triglycerides (mg/dl)	64.83±2.14	**48.17±1.76	66.83±3.00	**44.83±2.30	67.17±2.01	**42.67±2.11
Calculated t	6.15925		5.81323		8.4172	

***: significantly different from the control at p≤ 0.01*
Critical ''t'' was t=2.228 at p ≤ 0.05, t=3.169 at p ≤ 0.01





R_1, R_3, R_4, R_5, R_6 = H, R_2=CH_3	genkwanin
R_1=OH, R_2=CH_3 R_3=OCH_3, R_4, R_5,R_6 =H,	thymusin
R_1, R_3 = *C*-gluco, R_2,R_4,R_5,R_6=H	vicenin-2
R_1= *O*-gluco, R_2=CH_3, R_3=OCH_3, R_4,R_5,R_6=H	thymusin-6-*O*-glucoside
R_1=OH, R_2=CH_3, R_3, R_4, R_6=H, R_5=CH_3	Pectolinarigenin
R_1,R_2,R_4,R_6=OH, R_3=H, R_5=CH_3	6,5'-dihydroxy diosmetin
R_1,R_3,R_5,R_6=H, R_2=gluco. R_4=CH_3	chrysoeriol-7-*O*-glucoside

CONCLUSION

four flavonoids and two phenolic acids were isolated from *N. septemcrenata* while *O. fruticosa* was found to contain three flavonoids and one phenolic acid. The acute toxicity study of alc. extract of *N. septemcrenata* has a wide margin of safety and produces a reduction in the cholesterol and triglycerides levels in experimental rats.

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