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Research Paper

Essential oil and iridoide glycosides of *Nepeta septemcrenata* Erenb

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ABSTRACT

The essential oil of the aerial parts of *Nepeta septemcrenata* Erenb was analyzed by GC/MS. Thirty nine compounds, accounting for 98.3% of the total oil, were identified, where the main constituents were 4aα, 7α, 7aβ-nepetalactone (24.2%), 4aα, 7α, 7aα-nepetalactone (19.9%), elemol (13.8%), 1, 8-cineole (8.5%), linalool (5.6%), β -bisbolene (3.7%), Terpine-4-ol (2.7%) and α-terpinol (2.5%). The antimicrobial activity of the essential oil of *N. septemcrenata* was tested against six gram-negative or gram-positive bacteria and six fungi, where the results of the bioassays showed interesting antimicrobial activity, in which *Bacillus subtilis, Staphylococcus aureus* and *Escherichia coli* were the most sensitive to the oil. Meanwhile the oil exhibited a remarkable antifungal activity against all the tested fungi. The iridoid glycosides (ajugol, ajugoside and aucubin) were separated and identified from the plant.

Keywords: *Nepeta septemcrenata*; Essential oil; Iridoid glycosides; Antimicrobial activity.

Abbreviation: a = Axial for discussing the stereochemistry structure of the compound.

INTRODUCTION

About 250 species of *Nepeta* were reported (Evans, et al., 1996) but only one species of this genus was recorded in Egypt and this species (*Nepta septemcrenata*) is endemic for Sinai (Tackholm, 1974). *Nepeta* species are widely used in folk medicine for their antispasmodic, expectorant, diuretic, antiseptic, antitussive, antiasthmatic and febrifuge activities (Newall, et al., 1996; Zargari, 1990; Baser, et al., 2000).

Meanwhile, *Nepeta cataria* L. was used in traditional medicine and as food additive (Tucker and Tucker 1988). Nepetalactone, a primary component of catnip oil, was reported as cockroaches (Peterson, et al., 2002) and mosquitoes repellant (Peterson 2001).

The analysis of the aerial parts of *Nepeta sintenisii* Bornm using GC/MS revealed the presence of forty constituents (96.5% of the total oil), where 4aβ, 7α, 7aβ-nepetalactone (23.4%), elemol (16.1%), E-β-farnesene (9.5%), 1, 8-cineole (8.2%), cis-sabinene hydrate (6.5%), β- bisabolene (4.2%) and germacrene-D (3.5%) were identified as main components (Sajjadi**,** 2005).

On the other hand iridoid glycosides were found in many medicinal plants of family *Lamiaceae* and responsible for their pharmaceutical activities, where the isolated iridoid exhibit a wide range of bioactivities including cardiovascular, antipheptotoxic, chlorectic, hypoglycemic, anti-inflammatory, antispasmodic, antitumor, antiviral, immunomodulator and purgative activities (Didna, 2007), while Yang et al., (1983) detected aucubin in *Aucuba japonica* and protected against liver damage induced by carbon tetrachloride or alpha-amanitin in mice and rats when 80 mg/kg was dosed intraperitoneally. Hence we choice *Nepeta septemcrenata* for studying its main components as the previous literature did not included any reports about its active constituents of volatile oil or its iridoid glycosides.

MATERIALS AND METHODS

***Plant material*:** Aerial parts of *Nepeta septemcrenata* were collected at full flowering stage from Kathrine [(Wadi Cherage, as identified by local Bedouin) in March 2006.

***Isolation of the essential oils*:** Fresh aerial parts of *Nepeta septemcrenata* **(**100g) were hydrodistilled in a cleveger-type apparatus (Denny, 1989). After 3h of distillation, the essential oils were removed from the surface of the water. The oils were dried over anhydrous sodium sulphate, stored in sealed dark glass vials and kept in refrigerator for further analysis.

***GC-MS analysis*:** GC-MS analysis was carried out on a Hewlett-Packard 6890 gas chromatograph fitted with a fused silica HP-5MS capillary column (30 m × 0.25 mm; film thickness 0.25 µm). The oven temperature was programmed from 60ºC at 3ºC/min. Helium was used as carrier gas at a flow rate of 2 ml/min. The gas chromatograph was coupled to a Hewlett-Packard 6890 mass selective detector. The MS operating parameters were: ionization voltage, 70 eV; ion source temperature 200ºC.

Identification of components of the volatile oil was based on retention indices relative to *n*-alkanes and computer matching with the WILEY 275. L liberary, as well as by comparison of the fragmentation patterns of the mass spectra with those reported in the literature (Adams, 1995; Swigar and Silverstein, 1981).

***Isolation of iridoid glycosides*:** About 1kg of the air dried parts of *Nepeta septemcrenata* were extracted with methanol-water mixture (80%). The combined alcoholic extracts were evaporated in vacuum at 40ºC, where the alcoholic extract was mixed with celite, dried, packed into a glass column and successively eluted with petroleum ether 40-60ºC (fraction a), methylenechloride (fraction b), methylenechloride-methanol (1:1) (fraction c) and finally with methanol (fraction d). Each fraction was evaporated and tested for iridoid glycosides. Only one fraction which was eluted with methylenechloride-methanol (1:1) gave a positive test for iridoid glycosides (Trim and Hill reagent) according to Trim and Hill (1952).

***Thin layer chromatography of fraction ©*:** Thin layer chromatography of iridoid glycosides containing fraction © using silica gel plate 60 DF254 (Riedel De Haen) and the solvent system chloroform- methanol- water (80:18:2), where the sprayed reagent was 2N sulphuric acid, followed by heating at 110ºC for 5 min.

***Column chromatography of fraction ©*:** Fraction © was subjected to column chromatographic fractionation using silica gel (0.063-0.2 mm) Merck, packed into a glass column and eluted with methylenechloride-methanol-water (80:18:2). Fraction 200 ml each were collected and monitored by thin layer chromatography, where similar fractions were collected together and tested for iridoid glycosides (Trim and Hill reagent). The positive test was re-chromatographed on silica gel (0.01-0.04 mm) column Merck and elution was performed with chloroform-methanol (95:5), followed

by increasing amounts of methanol in the ratios of (90:10), (80:20), (70:30), (50:50) and finally with methanol. Also similar fractions were collected together and tested for iridoid glycosides (Trim and Hill reagent) and the positive test was preparative on thin layer chromatography using the solvent system chloroform- methanol- water (80:18:2) and each band was eluted with methanol 90%, where it was contained a single spot and three compounds were separated and purified from the eluted bands.

***Ultraviolet spectrophotometric analysis (UV)*:** Chromatographically pure materials were dissolved in pure methanol and subjected to ultraviolet spectrophotometric investigation using Shimadzu UV visible recording spectrophotometer UV-240.

Mass spectrometric analysis (MS): The mass spectrum was conducted using finnigan SSQ 7000 and MM 7070 E.

***^{1}H-Nuclear Magnetic Resonance Analysis (^{1}H-NMR)*:** The NMR measurements were carried out on A JEOL EX-270 NMR spectrometer apparatus (270MHz).

Antimicrobial activity: The antimicrobial activity of essential oil of *N. septemcrenata* was tested against six gram-negative or gram-positive bacteria (Bacterial strains: *Bacillus subtilis, Staphylococcus aureus, Micrococcus leteus, Escherichia coli, Salmonella* sp. and *Pseudomonas* sp.) and seven fungi (*Aspergillus niger, Aspergillus flavus, Fusarium solani, Fusarium oxysporium, Microsporum fulvum, Alternaria tennius* and Yeast).

The microorganisms were obtained from Plant Pathology and Microbiology Department, the National Research Center, Cairo, Egypt. It was checked for purity and identity and regenerated to obtain active microorganisms. The cultures were stored in refrigerator at 5ºC and reactivated on the media suitable for each microorganism.

***Preparation of the Spore Suspension*:** Bacteria inocula, consisted of 18 hours cultures grown in nutrient broth at 37ºC, where serial dilutions were made to prepare a suspension containing 105 cells/ml. Fungi were grown on slants of potato-dextrose agar (PDA) media and incubated for 7 days at 25ºC + 2ºC. Spores were harvested by adding sterilizing solution (between 80: 0.42 v/v) and filtered through several layers of cheese cloth. The number of conidia was estimated by hemocytometer and the suspension was adjusted to contain approximately 105 spores/ml (Padwal, et al., 1976).

***Agar diffusion method*:** Agar diffusion method was used to check the presence or absence of antimicrobial agents by using the agar diffusion method as described by Booth (1972). The plates were separately inoculated on to the surface of appropriate nutrient media (nutrient agar for bacteria and PDA) for fungi. After inoculation of the organism in each plate, four cups of one centimeter diameter were achieved in each plate by using a sterilized cork borer, three for the extract and one for the control solution of 1 %, in sterile water or the selected solvent. By sterile pipettes 4 drops of the vegetative parts and legumes extracts (250, 500, 1000 and 2000 ppm) were transferred into the cups. All steps of the experiment were done under sterile conditions. Nutrient agar plates were incubated at 37 ºC for 24 hours, while fungi were incubated at 28ºC + 2ºC for 5 days. The zone of inhibition was measured at least in two diameters (in mm).

RESULTS

Identification of the essential oils: The aerial parts of *Nepeta septemcrenata* yielded 0.4% of clear yellowish oil, where thirty nine components, representing 98.3% of the total oil were detected and their percentages and shown at table 1. The major

separated constituents were 4aα, 7α, 7aβ-nepetalactone (24.2%), 4aα, 7α, 7aα-nepetalactone (19.9%), elemol (13.8%), 1, 8-cineole (8.5%), linalool (5.6%).

Identification of iridoid (1): UV spectral data (λ_{max}, nm) of iridoid glycoside in MeOH was 220. Its mass spectrum showed molecular ion peak (M^+) at m/z 348 (calc. for $C_{15}H_{24}O_9$). ^{1}H-NMR spectral data of the compound illustrated at table (2) showed a comparison of the compound with authentic ajugol. The illustrated data of iridoid (1) was found to be identical with ajugol.

Identification of iridoid (2): UV spectral data (λ_{max}, nm) of iridoid glycoside in MeOH was 224. Its mass spectrum showed molecular ion peak (M^+) at m/z 390 (calc. for $C_{17}H_{26}O_{10}$). ^{1}H-NMR spectral data of the compound illustrated at table (3) showed a comparison of the compound with authentic ajugoside. The illustrated data of iridoid (2) was found to be identical with ajugoside.

Identification of iridoid (3): UV spectral data (λ_{max}, nm) of iridoid glycoside in MeOH was 200. Its mass spectrum showed molecular ion peak (M^+) at m/z 346 (calc. for $C_{15}H_{22}O_9$). ^{1}H-NMR spectral data of the compound illustrated at table (4) showed a comparison of the compound with authentic aucubin. The illustrated data of iridoid (3) was found to be identical with aucubin.

The iridoid glycosides (ajugol, ajugoside and aucubin) were separated for first time from *N. septemcrenata.*

Antimicrobial activity*:* The antimicrobial activity was tested for the essential oil of *N. septemcrenata* and illustrated at table (5), where the best effect of the essential oil was detected when treated on *Bacillus subtilis, Staphylococcus aureus* and *Escherichia coli.* Meanwhile, the oil exhibited a remarkable antifungal activity against all the tested fungi.

DISCUSSION

The analysis of the volatile oil of the aerial parts of *N. septemcrenata* revealed that it contained 39 components, where Kalpoutzakis, (2001) declared that each of the aerial parts of *Nepeta camphorata* and *Nepeta argolica ssp. dirphya* contained 52 components. On the other hand the detected major volatile oil components of *N. septemcrenata* were 4aα, 7α, 7aβ-nepetalactone (24.2%), 4aα, 7α, 7aα-nepetalactone (19.9%), elemol (13.8%), 1, 8-cineole (8.5%), linalool (5.6%), while the detected major volatile oil components of each of the aerial parts of *Nepeta camphorata* and *Nepeta argolica ssp. dirphya* were 1, 8-Cineol and two nepetalactones (Kalpoutzakis, 2001). Kokdil (1996) stated that the main constituents of the *Nepeta* species oil are diastereomeric nepetalactones, which these are responsible for their feline attractant or insect repellant properties.

The essential oil of *N. septemcrenata* showed best antimicrobial effect on *Bacillus subtilis, Staphylococcus aureus* and *Escherichia coli,* beside exhibited a remarkable antifungal activity against all the tested fungi. These results may be suggested to be related to the high concentration of 4aα, 7α, 7aβ-nepetalactone (24.2%), 4aα, 7α, 7aα-nepetalactone (19.9%), elemol (13.8%) and 1, 8-cineole (8.5%), while the essential oil of *Nepeta crispa* Willd. showed best antimicrobial effect on *Bacillus subtilis* and *Staphylococcus aureus*, beside a strong activity against the teseted fungi (Sonbolia, et al., 2004).

The separated iridoid glycosides of *N. septemcrenata* were identified as ajugol**,** ajugoside and aucubin, which were reported for the first time from *N. septemcrenata.* Meanwhile Akkol et al., (2007) detected ajugol and picroside, beside ilwensisaponin A and C in the flowers of Verbascum pterocalycinum var. Mutense, On the other hand Pianaro et al., (2007) separated ajugol and two phenolic derivatives

(p-hydroxy-benzoic acid and methyl p-hydroxy-benzoate) from *Spathodea campanulata* roots, while Yang et al., (1983) detected aucubin in *Aucuba japonica* Shim et al., (2007) stated that aucubin is an iridoid glycosidewith a variety of pharmacological effects, such as antimicrobial and anti- inflammatory, whilst also promoting dermal wound healing.

CONCLUSION

Thus present investigation revealed that essential oil of *Nepeta septemcrenata* Erenb. aerial parts contains; 4aα, 7α, 7aβ-nepetalactone (24.2%), 4aα, 7α, 7aα-nepetalactone (19.9%), elemol (13.8%), 1, 8-cineole (8.5%), linalool (5.6%), which may be responsible for its effect on *Bacillus subtilis, Staphylococcus aureus* and *Escherichia coli,* beside the oil exhibited a remarkable antifungal activity against the tested fungi. These results related to the high concentration of 4aα, 7α, 7aβ-nepetalactone (24.2%), 4aα, 7α, 7aα-nepetalactone (19.9%), elemol (13.8%) and 1, 8-cineole (8.5%). On the other hand *N. septemcrenata* contained the iridoid glycosides (ajugol, ajugoside and aucubin) which were separated for first time from the plant and has antimicrobial, anti- inflammatory, cardiovascular, antipheptotoxic, chlorectic, hypoglycemic, antispasmodic, antitumor, antiviral, immunomodulator and purgative activities, where they evaluate the economic values of *N. septemcrenata* as new resources for natural products.

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Table-1: Composition of the essential oil of *Nepeta septemcrenata*.

Peak	Component	Percentage	RI
1	α-pinene	0.3	936
2	Sabinene	0.9	970
3	B- pinene	0.6	975
4	butan, 1-chloro-2-methyl	0.2	983
5	Myrcene	0.3	987
6	α –terpinene	0.5	1012
7	P-cymene	1.1	1021
8	Limonene	0.6	1030
9	1, 8-cineole	8.5	1033
10	Ocimene	0.1	1036
11	Γ-terpinene	0.7	1056
12	Linalool	5.6	1006
13	Terpinolene	0.3	1085
14	B-phelladrene	0.8	1090
15	Trans-sabinene hydrate	0.1	1096
16	Camphor	0.2	1101
17	Cis-ρ-menth-2-en-1-ol	0.2	1118
18	Geijerene	0.1	1140
19	Trans-α-dihydroterpineol	0.3	1157
20	Terpine-4-ol		1173
21	α-terpinol	2.5	1191
22	4aα, 7α, 7aα-nepetalactone	19.9	1332
23	4aα, 7α, 7aβ-nepetalactone	24.2	1342
24	α-copaene	0.1	1371
25	β-caryophyllene	0.6	1415
26	β-gurjunene	0.7	1428
27	δ-cadinene	1.2	1456
28	Zingiberene	0.5	1491
29	β –bisbolene	3.7	1506
30	γ –cadinene	0.2	1511
31	β-sesquiphellandrene	1.9	1522
32	Elemol	13.8	1550
33	Guaiol	0.4	1575
34	Caryophyllene oxide	0.7	1580
35	γ –eudesmol	0.9	1627
36	α -epi-cadinol	1.5	1638
37	β-eudesmol	0.9	1647
38	α –eudesmol	0.4	1650
39	n-heptadecane	0.1	1701

- Retention indices on HP-5 capillary column

Table-2: ^{1}H-NMR spectral data of iridoid (1) comparison with authentic ajugol.

Carbon No.	^{1}H of iridoid 1 ppm in D_2O	^{1}H of ajugol ppm in D_2O
1	5.62 (1H, d, J= 1.96 Hz)	5.45 (1H, d, J= 2.1Hz)
3	6.45 (1H,dd,J- 6.17, 1.96 Hz)	6.14 (1H, dd, J= 6.0, 2.1 Hz)
4	5.22 (1H, dd, J= 6.18, 3.2 Hz)	5.05 (1H, dd, J= 6.15, 3Hz)
5	3.3 (1H.m)	2.6 (1H.m)
6	4.22 (1H, m, J= 5.15, 3Hz)	3.76 (1H, m, J= 5.2, 3Hz
7	2.38 (dd, J= 13.5, 5.9 Hz)	2.11 (dd, J= 13.5, 5.5 Hz)
9	2.82(d, J= 9.67 Hz)	2.54(br d, J= 9.5 Hz)
10 CH_3	1.55 (3H, s)	1.25 (3H, s)
1' Glucose	5.10 (1H, d, J= 7.5 Hz)	4.86 (1H, d, J= 7.0 Hz)
6' A Glucose	4.21 (dd, J= 12.2, 2.6 Hz)	4.0 (dd, J= 12.9, 2.3 Hz)
6' B Glucose	4.12 (dd, J= 12.2, 6 Hz)	3.66 (dd, J= 12.9, 6 Hz)

Table-3: ^{1}H-NMR spectral data of iridoid (2) comparison with authentic ajugoside.

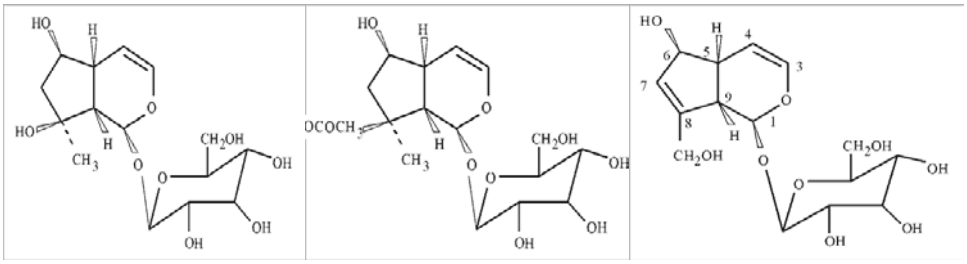
Carbon No.	^{1}H of iridoid 1 ppm in D_2O	^{1}H of ajugoside ppm in D_2O
1	6.61 (1H, s)	6.68 (1H, s)
3	6.31 (1H,d,J- 6.41 Hz)	6.24 (1H,d,J- 6.37Hz)
4	4.15 (1H, d, J= 6.41 Hz)	4.08 (1H, d, J= 6.38 Hz)
5	2.69 (1H, m)	2.60 (1H.m)
6	3.94 (1H, d, J= 7.65 Hz)	3.84 (1H, d, J= 7.65 Hz)
7	2.16 (dd, J= 5.5, 15.09 Hz)	2.10 (dd, J= 5.6, 15.05 Hz)
9	2.82(d, J= 9.67 Hz)	2.54(br d, J= 9.5 Hz)
10 $OCOCH_3$	1.55 (1H, s)	1.48 (1H, m)
1' Glucose	4.85 (1H, d, J= 7.10 Hz)	4.46 (1H, d, J= 7.5 Hz)
6' A Glucose	3.57 (dd, J= 5.9, 12.3 Hz)	3.46 (dd, J= 3.1. 12.01 Hz)
6' B Glucose	3.77 (d, J= 12.3Hz)	3.68(d, J= 12.01 Hz)

Table-4: ^{1}H-NMR spectral data of iridoid (3) comparison with authentic aucubin.

Carbon No.	^{1}H of iridoid 2 ppm in D_2O	^{1}H of aucubin ppm in D_2O
1	5.30 (1H, d, J= 5.25 Hz)	5.26 (1H, d, J= 5.14Hz)
3	6.37 (1H,dd,J- 6, 1.62 Hz)	6.14 (1H, dd, J= 6, 1.62 Hz)
4	5.12 (1H,dd,J- 6, 3 Hz)	5.14 (1H,dd,J- 6, 3.4 Hz)
5	2.84 (m)	2.81 (m)
6	4.59 (m)	4.61(m)
7	5.77 (m)	5.74 (m)
9	3.15 (m)	3.11 (m)
10	4.25 (dd)	4.30 (dd)
1' Glucose	4.82 (1H, d, J= 7.6 Hz)	4.77 (1H, d, J= 7.5 Hz)
6' A Glucose	3.88 (dd, J= 12.2, 2.6 Hz) 3.69 (dd, J= 12.2, 4.2 Hz) 4.77, 3.95, 3.74 ppm are consistent with that of glucose	3.88 (dd, J= 12.6, 2.6 Hz) 3.69 (dd, J= 12.6, 4.2 Hz) 4.81, 3.98, 3.76 ppm are consistent with that of glucose

Table-5: The antimicrobial activity of essential oil of *N. septemcrenata.*

	Inhibition zone (mm)					
Bacteria	*Bacillus subtilis* + ve	*Micrococcus leteus* +ve	*Staphylococcus aureus* + ve	*Pseudomonas sp.* -ve	*Escherichia coli* - ve	*Salmonella spp.* -ve
Essential oil effect	62.4	28.8	51.2	20.7	57.5	24.6
Fungi	*Asperigllus niger*	*Asperigllus flavus*	*Fusarium oxysporium*	*Fusarium solani*	*Alternaria tennius*	*Microsporum fulvum*
Essential oil effect	51.3	42.5	50.7	45.9	43.6	46.1



Iridoid (1): Ajugol **Iridoid (2): Ajugoside** **Iridoid (3): Aucubin**

Figure-1: Structures of the iridoid glycosides (1; 2 and 3), which were separated for the 1st time from this plant.

