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Investigation of flavonoids and antimicrobial activity of *Ballota andreuzziana*

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ABSTRACT

Investigation on *Ballota andreuzziana* (Family *Labiatae*) for flavonoids led to isolation of two aglycones from ethyl acetate fraction which were identified as 7-methoxy luteolin and 6,7-dimethoxy scutellarein, in addition to three glycosides known as luteolin-7-*O*-glucoside, 6, 4'-dimethoxy scutellarein-7-*O*-glucoside and quercetin-7-*O*-rhamno-glucoside from butanol fraction. Antimicrobial studies revealed that, butanol extract exhibited the marked activity against *M. Phlei*, *S. aureus* and *C. albicans* (I.Z. =16.3, 11.3, and 10.7mm, Conc.=150mg/ml) respectively. While, chloroform extract also showed the better activity against *B. subtilis* (I.Z. =11.0mm, Conc.=150mg/ml). Furthermore aqueous extracts exhibited no effects at all against all tested organisms at all concentrations also, all tested Gram negative bacteria and the fungus *A. niger* are resistance to all concentrations of tested extracts.

Keywords: *Ballota andreuzziana, Lamiaceae (Labiatae),* Volatile oil, Flavonoids, Antimicrobial activity.

INTRODUCTION

The family *Labiatae* contains a wide variety of chemicals and volatile oils which are common to many members of the family. It was found to contain variety of mono, sesqui, diterpenoids, iridiods and phenolic compounds (1–3). Flavonoids also occur in the *Labiatae* in a variety of structure forms including flavones, flavonols, flavanones, dihydroflavonols and chalcones (4). *Ballota andreuzziana* belonging to *Labiatae* family is an endemic Libyan plant growing in Al Gabel Alakhder region (Wadi El-Husaien) (5). Several compounds have been isolated and identified from *Ballota* genus include: volatile oils, diterpenoids and various polyphenols including phenylpropanoid derivatives and natural phenolics (flavonoids and phenolic acids) (6,7). Plants of Ballota species have been used traditionally and in modern medicine for treatment of wounds, burns, suppress coughs, upper respiratory system inflammation, neurosedative, antiulcer, antispasmodic, diuretic, antihemorrhoidal, nausea, vomiting, nervous dyspepsia, specifically for vomiting of central origin and also are used as antiemetic, antioxidant, antimicrobial, anti-inflammatory and as hepatoprotective (8,12). Literature revealed a single report on the diterpenoids of *B. andreuzziana* (13) concerning their chemical structures and their biological activity. Hence, it was aimed to study on investigation of the flavonoidal constituents along with its antimicrobial activity

MATERIALS AND METHODS

Plant Material

The plant was collected from Wadi El-Husaien, along the coastal of Ras El-Hilal to Shahat city road, Gabel

Alakhder city, in April 2005 during the flowering stage. The plant was kindly identified by Dr. Mohamed Alsharif at Botany department, Faculty of Science, Garyounis University. A voucher specimen was deposited at the Herbarium of Biology department, Faculty of Science, Altahadi University, Sirte, Libya.

Instruments Used

UV viewing lamp at the long wave length.
UV- Vis. spectrophotometer 2401Shimadzu.
UVIKON 931 double beam UV- vis. Spectrophotometer.
All measurements in region of 200–500 nm.
Bruker NMR spectrometer operating at 300 MHz for ^{1}H and 75MHz for ^{13}C NMR in DMSO and acetone.
Jeol- Ex- 270 MHz and Jeol 500 MHz.
Mass spectrometer finnigan mat SSQ 7000.

Extraction of flavonoids

About 700g of the air dried powdered defatted plant material was macerated with methanol (70%) until exhaustion. The alcoholic extract was evaporated in *vacuo* at about $50^{0}C$ (25 g), dissolved in hot distilled water (300 ml), left overnight in refrigerator and filtered. The aqueous filtrate was partitioned by successive portions of ethyl acetate (4×500 ml) followed by butanol (5×100ml). The filtrates were dried, separately, over anhydrous sodium sulphate and evaporated in *vacuo* at about $45^{0}C$. The ethyl acetate and butanol fraction were investigated by Planar Chromatography (PC), preparative paper chromatography (PPC) and Coloumn Chromatography (CC) using different solvent systems.

About 2.5g of the ethyl acetate extract was subjected to column chromatography on Sephadex LH-20 (80×4 cm). Elution was done using methanol: water with decreasing the polarity. The course of chromatographic fractionation was followed using TLC in 20 % acetic acid as a developing solvent. The isolated fractions were re-chromatographed using a small column of Cellulose eluted with acetic acid: water (5:95). The isolated flavonoidal components were further purified by preparative paper chromatography (Whatman 3MM) (20% acetic acid and butanol: acetic acid: water 3:1:1) and BAW (3:1:1) to afford compound 1 and 2.

About 10g of butanol extract was dissolved in methanol and loaded into silica gel column (5×80 cm, 60–120 mesh BDH). Elution started with $CHCl_3$: MeOH (50:50), then the polarity was gradually increased by addition of methanol and water. The fractions containing compounds 3, 4 and 5 were collected and further purified. The compounds were identified by their color under UV light (nm) before and after spraying with the $AlCl_3$ reagent, in addition to their R_f values in different solvent systems.

Biological activity Study

About 100 g of the air dried powdered plant was extracted (defatted) with pet. ether in a Soxhlet for 24 hours to obtain pet. ether extract. The defatted plant material was dried and macerated in acetone (500 ml) for overnight, filtered and further extracted with methanol (70 %) and fractionated to give chloroform, ethyl acetate, butanol and mother liquor (M.L) extracts. Three concentrations were prepared from each extract as a, b and c (50, 100 and 150 mg/ml respectively).

Antimicrobial activity

The antimicrobial activity was determined using the sensitivity disk method of Kirby-Bauer and determination of inhibitory zone (I.Z.) (14–15).

Tested extracts:

Pet. ether, Ethyl acetate, Aqueous, Methanol, Acetone, Chloroform, Butanol, and mother liquor extract.

Microorganisms:

Staphylococcus aureus, Bacillus subtilis, Escherichia coli, Mycobacterium phlei, Enterobacter cloacae, Aspergillus niger and *Candida albicans* (Table 2) were obtained from the stock culture of Chemistry of Microbial and Natural Products Dept., National Research Center, Cairo, Egypt.
Standards: Standard Tetracycline and Miconazole (250 mg)

RESULTS AND DISCUSSION

Identification of flavonoidal compounds:

Compound-1: *7- methoxy luteolin*, the UV absorption spectra in methanol and different shift reagents indicatd as a flavone type with free OH groups at C-5, 3', 4'. The EI-MS showed a molecular ion peak at m/z= 300 (M^{+}; 16%) which corresponds to the molecular formula $C_{16}H_{12}O_{6}$. Another important peaks at m/z= 286 (M^{+}- CH_2; 31%), 285 (M^{+} - CH_3; 20%) and 242(M^{+} -[CO + HCHO]; 22%). The fragmentation pathway of compound-1 undergoes Retero Diel's Alder reaction (RDAR) leading to fragments at m/z= 166 (A^{+}_{1}; 22%), 137(B^{+}_{1}; 16%) and 134 (B^{+}_{2}; 39%) which confirm the presence of a methoxy group at C-7. Finally, the chromatographic and the available spectroscopic data substantiated that compound-1 is *7-methoxy luteolin* (5, 3', 4'-trihydroxy-7-methoxy flavone).

Table 2: Antimicrobial activity of different extracts of B. andreuzziana

Extract	Conc.	Diameter of inhibition zone (mm) GZ. +ve bacteria *S. aureus*	*B. subtillis*	*My. phlei*	Fungi *C. albicans*
Pet. Ether	a	NI	NI	11.3±1.2	NI
	b	NI	NI	13.3±1.2	NI
	c	NI	NI	14.7±0.6	NI
Methanol	a	NI	NI	11.3±0.6	NI
	b	NI	7.3±0.6	12.7±0.6	NI
	c	NI	8.0±0.0	13.3±0.6	NI
Chloro-form	a	NI	NI	NI	NI
	b	NI	8.0±0.0	NI	NI
	c	NI	11.0±1.0	NI	NI
Ethyl acetate	a	NI	8.0±0.0	11.3±0.6	NI
	b	9.0±0.0	9.0±0.0	13.0±1.0	NI
	c	10.0±0.0	10.0±0.0	14.3±0.6	NI
Butanol	a	8.7±1.5	NI	13.0±4.4	NI
	b	9.3±1.2	NI	15.0±4.4	NI
	c	11.3±1.2	NI	16.3±4.9	10.7±0.6
M. L	a	NI	NI	11.3±0.6	NI
	b	NI	NI	13.0±1.0	NI
	c	NI	NI	14.3±0.6	9.7±0.6
Aqueous	a	NI	NI	NI	NI
	b	NI	NI	NI	NI
	c	NI	NI	NI	NI
Acetone	a	NI	NI	10.0±1.0	NI
	b	NI	7.7±0.6	11.7±1.2	NI
	c	NI	8.7±0.6	14.7±2.5	NI

a= 50mg/ml, b= 100mg/ml, c= 150mg/ml, NI= No Inhibition ; Standard Tetracycline: I.Z. = 19 mm. Standard Miconazole: I.Z. = 26 mm.

These data was in accordance to Siciliano *et. al.* (16) showed isolation of 7,3' dimethoxy luteolin from *B. undulata*.

Compound-2: *6,7-dimethoxy scutellarein*, showed a band-I at 340 nm in addition to a bathochromic shift in band-I with NaOMe from 340 nm to 396 nm indicated the presence of a free OH group at C-4'. No hypsochromic shift occured in band-I in $AlCl_3$/HCl spectrum relative to $AlCl_3$ spectrum which confirms the absence of an *ortho*-dihydroxy system and furtherassociated with NaOAc/ H_3BO_3 spectrum. The NaOAc spectrum showed no bathochromic shift in band-II relative to methanol spectrum confirming absence of a free OH group at C-7. The EI-mass spectrum showed a molecular ion peak at m/z= 314 (M^+; 17%) which corresponds to the molecular formula $C_{17}H_{14}O_6$. The most important peaks at m/z= 313 (M^+ -1; 4.3%), 286 (M^+ - CO; 23.4%), 284(M^+- OCH_3; 21.7%), 269(M^+ -[CH_3 + CH_2O]; 17%) and 241(M^+ - [CO + CH_3 + CH_2O]; 13%). The fragmentation pathway of compound-2 undergoes (RDAR) lead to fragments at m/z 196 (A^+_1; 23.4%), 118(B^+_1; 25.5) and (B^+_2; 36%) confirm the presence of two methoxy groups at ring-A. The ^{1}H-NMR spectrum of compound-2 in acetone showed signals at δ in ppm 7.52 (2H, J=8.5 Hz, d, H-2',6'), 7.03 (2H, d, J =9.0 Hz, H-3',5'), 6.63 (1H, s, H-3), 6.85 (1H, s,H-8), 3.99(3H, s, C-7-OCH_3) and 3.79 (3H, s, C-6-OCH_3). The ^{13}C-NMR spectrum of compound-2 displayed the most important peaks for 5, 4'-dihydroxy-6, 7-dimethoxy flavone in addition to the carbonyl carbon at δ= 178 pp. The resulted data was in accordance to 5, 4'-dihydroxy-6, 7-dimethoxy flavones (17). Hence, compound-2 is identified as *6,7-dimethoxy scutellarein*. A similar compound known as 4', 7- dimethoxy scutellarein was previously isolated from *B. acetabulosa* by Mericli *et. al.* (18).

Compound-3: *luteolin-7-O-glucoside*, the chromatographic pattern of this compound indicated as a glycoside in nature. It showed $\lambda_{max\ (MeOH)}$ at 346nm which proved the flavone nature of this compound with a free OH group at C-4'. The presence of an *ortho*-dihydroxy system was also confirmed, there is a hypsochromic shift (37 nm) in band-I of $AlCl_3$/HCl spectrum relative to $AlCl_3$ spectrum. The absence of a free OH group at C-7 was confirmed due to absence of bathochromic shift in band-II of NaOAc spectrum. The EI-mass spectrum displayed a molecular ion peak M^+ at m/z= 449(M^+ +1; 30%) calculated for the molecular formula ($C_{21}H_{20}O_{11}$ +1), the peaks at m/z= 286 (M^+ - hexose moiety; 18%) and 258 (M^+ - [CO + hexose

moiety]; 22%), 21.7%) confirms the presence of hexose moiety. The fragmentation pathway of compound-3 undergoes (RDAR) giving rise to fragments at m/z 153 (A^{+}_{1} + 1; 20 %) and 134 (B^{+}_{1}; 15%) The ^{1}H- NMR spectrum of compound-3 (DMSO) showed signals at δ in ppm at 7.41(2H, d, H-2', H-6'), 6.85 (1H, dd-H-5'), 6.78(2H, d, H-6, H-8), in addition to one anomeric protons for glucose moiety at C-7, 5.1(1H, d, H-1''). Based on the above data and acid hydrolysis compound-3 is identified as *luteolin-7-O-glucoside*. Also, the literature survey revealed that this compound was also isolated from other *Ballota* species (19).

Compound-4: *6, 4'-dimethoxy scutellarein-7-O-gluco*side, the compound showed absorbance $\lambda_{max\ (MeoH)}$ at 325 nm (flavone type) in addition to a bathochromic shift band-I with NaOMe (52nm) with decrease in intensity which indicate the absence of a free OH group at C- 4'. The other data with the other shift reagent revealed the absence of ortho-dihydroxy system and absence of free OH group at C-7. The EI-mass spectrum showed a molecular ion peak M^{+} at m/z= 475(M^{+} −1; 4%), which constituted with the molecular formula ($C_{23}H_{24}O_{11}$). Other important peaks at m/z= 314 (M^{+} -hexose moeity; 3%), 280 (M^{+} - [H_2O + CH_3 + hexose]; 10%) and 245 (M^{+} - [CHO+CO+CH_2 + hexose]; 6%) proves the aglucone is 6, 4'-dimethoxy scutellarein with a hexose moiety (M^{+}- glycoside; 4%). The fragmentation pathway of compound-4 undergoes (RDAR) giving rise of fragments at m/z 182 (A^{+}_{1}; 5%), 131(B^{+}_{1}; 12%) and 135(B^{+}_{2}; 10%). The fragmentation pattern confirmed the presence of the methoxy group at ring-A.

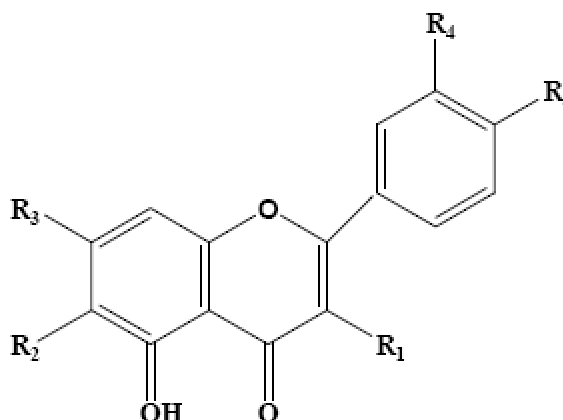
The ^{1}HNMRspectrum (DMSO) displayed signals at δ = 8.09 (d, 2H, J= 7Hz, H2', 6'), 7.12 (S, 2H, H- 3,8), 6,98 (d, 2H, J= 8.5Hz, H-3', 5'). The anomeric proton of glucose appears as a sharp signal at δ= 5.12 (d, 1H). The two methoxy group protons at C-4' and C-6 appears as two sharp singlet at δ=3.93 and 3.74ppm respectively. The ^{13}C NMRspectrum (DMSO) showed the characteristic signals for methoxylated flavone glycoside where C_4 appears at δ= 182.84 ppm, also the presence of two methoxy carbons at δ= 60.59 and 57.02ppm at C-4' and C-6 respectively and the chemical shift of C-3 at 104.19 ppm confirms the flavone nature of the compound. The anomeric carbon C-1'' of glucose moiety appears at δ= 100.39 ppm (Table 1).

The data was in agreement with the results reported by Chari et. al.(20). Finally, these data substantiated that compound-4 is 6, 4'-dimethoxy scutellarein-7-*O*-glucoside.

Table 1: ^{13}C- NMR data of compound- 2 and 4

Carbon No.	δ (ppm)(2)	δ (ppm)(4)
2	165.2	163.96
3	103.4	104.19
4	178.5	182.84
5	150.1	152.56
6	132.2	132.49
7	165.2	153.23
8	91.8	92.24
9	146.2	159.28
10	113.8	105.72
1′	129.2	124.42
2′	116.1	128.74
3′	141.5	117.15
4′	149.4	160.91
5′	119.4	117.15
6′	129.5	128.74
C-4-OCH_3	–	57.02
C-6-OCH_3	56.1	60.59
C-7-OCH_3	59.8	–
1″	–	100.39
2″	–	73.72
3″	–	77.08
4″	–	70.24
5″	–	77.72
6″	–	61.20

Compound-5: *quercetin-3-O-rhamno-glucoside*, the UV absorption spectra in methanol showed band-I at 350 nm which proves the flavonol nature of the compound in addition to a bathchromic shift in band-I with NaOMe (50nm) with increasing in intensity indicating the presence of a free OH group at C-4'. The $AlCl_3$ spectrum showed a bathochromic shift in band-I (68 nm) indicating the presence of a free OH group at C-5. Moreover, the $AlCl_3$/HCl spectrum exhibited a hypsochromic shift (40 nm) in band-I relative to $AlCl_3$ spectrum indicating the presence of an *ortho*-dihydroxy system in ring-B, further, it was also confirmed in NaOAc/H_3BO_3 spectrum, where there is a bathochromic shift (21 nm) in band-I relative to methanol spectrum. The NaOAc spectrum showed that a bathochromic shift (15nm) in band-II related to methanol spectrum indicating the presence of a free OH at C-7. The EI-mass spectrum showed a molecular ion peak at m/z = 610 (M^{+}; 8%) which constituted with the molecular formula ($C_{27}H_{30}O_{16}$) and other peaks at m/z= 302(M^{+}- [Deoxyhexose + hexose]; 12%), and 284 [(M^{+}- Deoxyhexose + hexose- CO); 17%). The fragmentation pathway undergoes (RDAR) yielded fragments at m/ z= 151($A^{.+}_{1}$; 13%), 137(B^{+}_{1}; 22) and 134($B^{.+}_{2}$; 3%). The ^{1}H NMR spectrum of compound-5 (DMSO) displayed signals at δ= 7.7 (1H, d,J= 7.0 Hz, H-6'), 7.6 (1H, d, J= 7.0 Hz, H-2'), 6.9 (1H, d, J= 8.0 Hz, H-5'), 6.3 (1H, d, J= 4.5Hz, H- 8) and 6.2 (1H, d, J= 4.5Hz, H-6) in addition to two anomeric protons as doublet at 5.3 and 5.1 ppm for glucose and rhamnose respectively. Finally, the CH_3 protons of rhamnose moiety appeared as a sharp doublet at 1.19 ppm. Hence, compound-5 could be identified as quercetin-3-*O*-rhamnoglucoside as Radwan *et. al.* (21) previously isolated it from *B. undulata*. It is interesting to



1: R_1, R_2 =H; R_4, R_5=OH; R_3=OCH_3

2: R_1, R_4 =H; R_2, R_3= OCH_3; R_5=OH

3: R_1, R_2=H; R_3 = O-gluc.; R_4, R_5=OH

4: R_1, R_4 =H; R_5, R_2= OCH_3; R_3= O-gluc.

5: R_2=H; R_3, R_4, R_5=OH; R_1= O-rhamno-gluc.

report the flavonoids present this species for the first time and all identified compounds were isolated for the first time from this plant.

The antimicrobial activity of different extracts with different concentrations (a=50mg/ml, b=100g/ml, c=150mg/ml) isolated from *B. andreuzziana* showed a great variation on tested microorganisms (bacteria and fungi) (Table 2). Results showed that butanol extract exhibited the highest activity against *My. Phlei*, *S. aureus* and *C. albicans* (I.Z. =16.3, 11.3, and 10.7 mm, Conc. =150 mg/ml) respectively. The pet. ether extract showed varied effects only against *M. phlei* (I.Z.=14.7 mm, Conc.=150 mg/ml). The methanol and acetone extracts showed different effects only against *M. phlei* and *B. subtilis*) (I.Z. =13.3 & 8.0mm and 14.7.0 & 8.7mm, Conc. =150 mg/ml) respectively. The chloroform extract showed moderate inhibition effect only against *B. subtilis* (I.Z.=11.0 mm, Conc.=150 mg/ml).

The ethyl acetate extract showed different effects against only Gram positive bacteria tested (*M. Phlei*, *S. aureus* and *B. subtilis*), (I.Z. =14.3, 10.0 and 10.0mm, Conc. =150mg/ml) respectively. The mother liquor (M.L) extract showed an activity only against *M. phlei* and *C. albicans* (I.Z. =14.33 and 9.67 mm, Conc. =150 mg/ml) respectively. The results of the present study proved that all tested gram negative bacteria and the fungus *A. niger* are resistance to all type of extracts tested.

These data are in accordance with that reported by Citoglu *et. al* (23–24) showed that some extracts and some flavonoidal compounds isolated from some *Ballota* species growing in Turkey have antimicrobial activity, Also these activities may be due to detected diterpenoidal compounds in chloroform and ethyl acetate extracts (9–10).

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