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Antibacterial Activity Of Two New Monoterpene Coumarins From *Ethulia Conyzoides*

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ABSTRACT

Re-investigation of the methylene chloride : Methanol extract of the aerial parts of *Ethiolia conyzoides* gave two new monoterpene coumarins, named 9-hydroxyethuliacoumarin (1) and 1',2'- epoxyethuliacoumarin (2) . The structures elucidation were determined by MS, ^{1}H- ^{13}C- 1D and 2D NMR spectral data. The antibacterial activity of isolated compounds. was determined against Gram-negative strains (*Serratia* sp., *Pseudomonas sp., Escherichia coli*) and Gram-positive bacteria (*Bacillus cereus, Staphylococcus aureus*).

Key words: *Ethulia conyzoides*, family Asteraceae; monoterpene coumarins

INTRODUCTION

The genus Ethulia (Tribe Vernonieae), Family Compositae, with ca 19 species is widely distributed in tropical Africa and less commonly in tropical Asia (1) . The characteristic metabolites are terpenoid 5-methyl coumarins . *Ethulia conyzoides* and *Ethulia vernonioides* are only the two species from that genus have been studied chemically. Among this genus, *Ethulia conyzoides L* var. gracillis Asch and scheweinf, awild growing Egyptian plant, is used in folklore medicine as an antihelminthic for round worms and for abdominal disorder (2,3). Several structurally close relatives terpenoid 5-methyl coumarin were isolated from *Ethulia conyzoides* (4-8) and some flavanoid glycosides were isolated (9). In continuation of my studies on the chemical constituents of the genus Ethulia (10) I have reinvestigated the aerial parts of the Egyptian species *Ethulia conyzoides L.*

MATERIALS AND METHODS

The aerial parts of *Ethulia conyzoides* were collected on the delta area, Elmansoura University area in June 2005, the plant identified by Prof. Dr. E. M. Mashally, Department of botany, Faculty of science, Elmansoura University, Egypt .

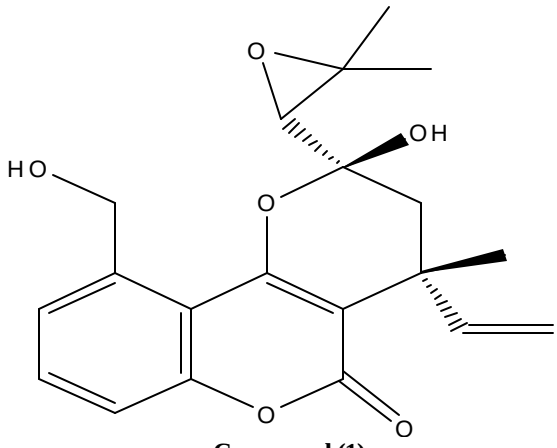
The air-dried plant materials (3 Kg) were ground and extracted at room temperature with CH_2Cl_2-MeOH (1:1). The extracts were concentrated in vacuum to obtain residues of (200 g). The extract was separated by flash column, silica gel, using n-hexane, increasing the degree of polarity by addition of CH_2Cl_2. The extract was prefractionated by CC (6 ×120 cm) on silica gel eluting with n-hexane followed by a gradient of n-hexane-CH_2Cl_2 up to 100% CH_2Cl_2 and CH_2Cl_2-MeOH up to 15 % MeOH. The 100% CH_2Cl_2 fraction was further purified by CC (2×40 cm), on Sephadex LH-20 eluted with *n*-hexane-CH_2Cl_2-MeOH (6: 4: 1) to give a mixture of **1** (14 mg) and **2** (11 mg). The mixture was purified by HPLC (MeOH-H_2O, 75: 25, R_t= 5.6 and 6.0 min).

RESULTS

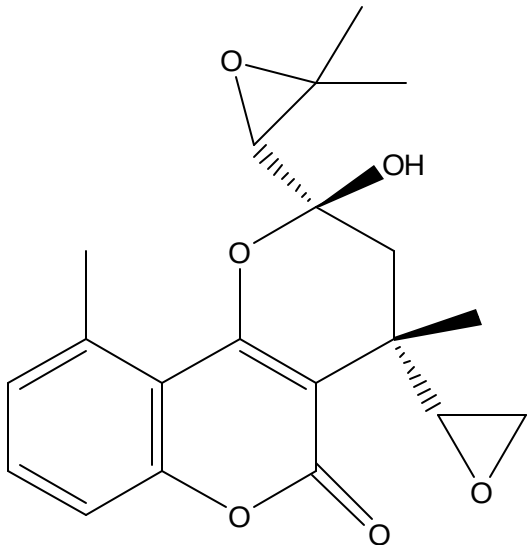
In my work, two new monoterpene coumarins: 9-hydroxyethulia coumarin (1) and 1',2'-epoxyethuliacoumarin (2) were isolated from methelene chloride : methanole (1:1) extract of the dried aerial parts of *Ethulia conyzoides* . By using column chromatography on silica gel, the extract was fractionated, then purified by further column chromatography on sephadex LH-20 and finaly purification by HPLC with solvent system Methanol:water (75:25) to afford two new monoterpene coumarins 1 and 2 .

DISCUSSION

EIMS of 1 showed a molecular ion peak $[m]^+$ at m/z 358 in according with the molecular formula $C_{20}H_{22}O_6$.The IR spectrum showed absorption bands at 3475 cm-1 (OH); 1675 cm-1 (C=O); 1620,1580 cm-1 (aromatic) . The structure of 1 was established from analysis of the ^{1}H-NMR spectra table (1) and ^{13}C-NMR spectra table (2) .



Compound (1)



Compound (2)

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Table 1 : ^{1}H-NMR spectral data (500 MHz- $CDCl_3$) of compounds 1 and 2

No.	d_H of 1	d_H of 2
6	7.02 (d, *J* = 8 Hz)	6.98 (d, *J* = 8 Hz)
7	7.33 (dd, *J* = 8,8 Hz)	7.35 (dd, *J* = 8,8 Hz)
8	6.99 (d, *J* = 8 Hz)	6.93(d, *J* = 8 Hz)
9_a	1.98 (d, *J* = 14 Hz)	2.62 (s)
9_b	1.95(d, *J* = 14 Hz)	
1'	5.07 (d, *J* = 17 Hz)	3.30 (d, *J* = 11 Hz)
$1'_a$	5.12 (d, *J* = 10 Hz)	3.35 (d, *J* = 8 Hz)
2'	6.12 (dd, *J* = 17,10 Hz)	4.35 (dd, *J* = 11,8 Hz)
4'	2.24 (d, *J* = 14 Hz)	2.21 (d, *J* = 14 Hz)
$4'_a$	2.18 (d, *J* = 14 Hz)	2.19 (d, *J* = 14 Hz)
6'	3.13 (s)	2.99 (s)
8'	1.49 (s)	1.29 (s)
9'	1.37 (s)	1.13 (s)
10'	1.70 (s)	1.61 (s)

d: doublet, s: singlet, dd: double of doublet

Table 2 : ^{13}C-NMR spectral data of compounds 1 and 2 (500 MHz – $CDCl_3$)

No.	d_C of 1	d_C of 2
2	158.5 (s)	157.6 (s)
3	106.5 (s)	106.1 (s)
4	160.2 (s)	161.0 (s)
4_a	118.4 (s)	118.1 (s)
5	137.8 (s)	137.0 (s)
6	127.8 (d)	128.0 (d)
7	133.1 (d)	133.5 (d)
8	117.7 (d)	117.9 (d)
8_a	153.9 (s)	154.5 (s)
9	53.8 (t)	23.6 (q)
1'	115.2 (t)	61.4 (t)
2'	140.9 (d)	67.0 (d)
3'	36.1 (s)	35.7 (s)
4'	41.9 (t)	42.1 (t)
5'	97.5 (s)	97.2 (s)
6'	66.8 (d)	66.0 (d)
7'	56.3 (d)	56.8 (d)
8'	25.2 (q)	26.5 (q)
9'	16.9 (q)	17.2 (q)
10'	27.5(q)	28.0 (q)

Multiplicity was determined by DEPT experiments, Assignments by ^{1}H-NMR, ^{13}C-NMR and NOESY experiments; s= quaternary, d= methine, t= methylene, q= methyl.

Table 3 : Antimicrobial activities of compounds 1 and 2 (Dry DMSO as solvent)

Test organism	1[c]	2[b]	Ampicillin[d]	Amoxillin[d]
Gram- Positive Strain				
Bacillus cereus	12[a]	N[a]	10[a]	N[a]
	19[b]	N[b]		N[a]
Staphylococcus aureus	N[a]	N[a]	8[a]	
	6[b]	N[b]		
Gram-Negative Strain				
Serratia sp				**13[a]**
Pseudomonas sp.	12[a]	15[a]	11[a]	13[a]
	19[b]	19[b]		
Escherichia coli	13[a]	15[a]	11[a]	
	19[b]	19[b]		13[a]
	12[a]	14[a]	11[a]	
	18[b]	19[b]		

[a] Values show the zone of inhibition in mm.; conc. of the samples was 200 mg/ml,[b] Values show the zone of inhibition in mm.; conc. of the samples was 400mg/ml,[c] Data are the mean of five measurements with neglected standard errors,[D]Refference antibiotics were carried out at 200 mg/ml only, N = No effect.

The multiplicities of the carbons were determined by the DEPT and HMQC as following : nine quaternary carbons, three tertiary carbons, three secondary carbons and five primary carbons . The ^{1}H-NMR spectrum showed three proton aromatic signals at δ_H 7.02 (d, *J*= 8 Hz, H-6), 7.33 (dd, *J*= 8,8 Hz, H-7) and 6.99 (d, *J*= 8 Hz, H-8) and two proton doublets at δ_H 1.98 (*J*= 14 Hz, H-9_a) and 1.95 (*J*= 14 Hz, H-9_b), which assignable to 5-hydroxymethyl coumarin moiety. The presence of 5-hydroxymethylene group in position 9 obvious from the two doublet signals at δ_H 1.98, 1.95 which showed long range coupling with doublet signal at δ_H 7.02 (H-6) which assigned for H-9 in HNBC spectrum . The 13C-NMR spectrum exhibited twenty carbon signals which correspond to the molecular formula composed of 5-hydroxymethyl coumarin moiety in the skeleton from the signals at δ_C 158.5 (s, C-2), 106.5 (s, C-3), 160.2 (s, C-4), 118.4 (s, C-4_a), 137.8 (s, C-5), 127.8 (d, C-6), 133.1 (d, C-7), 117.7 (d, C-8), 153.9 (s, C-8_a) and 53.8 (t, C-9) . In addition to monoterpene moiety which showed the characteristic two signals assigned to the exomethylene protons at δ_H 5.07 (d, *J*= 17 Hz, H-$1'_{trans}$) and 5.12 (d, *J*= 10 Hz, H-$1'_{cis}$) these two protons showed a coupling with a dd signal at δ_H 6.12 (*J*= 17, 10 Hz, H-2',δ_C 140.9) in the ^{1}H-^{1}H COSY spectrum .

Another two doublet signals observed at δ_H 2.24 and 2.18 which assigned for H-$4'_a$ and H-$4'_b$(δ_C 41.9), furthermore, one oxygen – bearing methin proton singlet signal at ä$_H$ 3.13 assigned for H-6' which correlated with a carbon signal at ä$_C$ 66.8 in the 1H-13C COSY spectrum, morever, the three singlet signals at ä$_H$ 1.49, 1.37 and 1.70 which assigned for the three methyl protons at positions 8',9' and 10' respectively . The other carbon signals for monoterpene moiety, see table (2) . The stereochemistry of 1 was confirmed by NOE experiment where, irradiation of H-6' inhanced H-2' which confirmed α-orientation of these two protons . All these data proved that 1 is 5-hydroxyethulia coumarin .

Compound 2 is very close to 1 . ^{1}H-NMR spectrum showed three aromatic signals at δ_H 6.98 (d, *J*= 8 Hz, H-6), 7.35(dd, *J*= 8,8 Hz, H-7) and 6.93 (d, *J*= 8 Hz, H-8) . Also, one-proton singlet atδ_H 2.62 (s, H-9) which assignable to hydrogen of 5-methylcoumarin moiety. Monoterpen moiety signals showed some differences, upfield of the two doublets at δ_H 3.30 (d, *J*= 11 Hz) and 3.35 (d, *J*= 8 Hz) which assigned for H-$1'_a$ and H-$1'_b$. Morever, upfield of double of doublet signal at δ_H 4.35 (*J*= 11, 8 Hz) which assigned for H-2' . ^{13}C-NMR spectrum confirmed this change from upfield of the three signals at δ_C 23.6, 61.4 and 67.0 which assigned for C-9, C-1' and C-2' respectively . Also, DEPT experiment showed clear change which indicated four tertiary carbons and two secondary carbons, as well as, nine quaternary carbons and five primary carbons .This means epoxidation between C-1' and C-2' which confirmed by EIMS showed a molecular ion peak at m/z 358 corresponding to $C_{20}H_{22}O_6$. Another ^{1}H-NMR and ^{13}C-NMR data, see table (1,2) .

Antibacterial activity

In vitro, screening experiments for antibacterial activities of compounds 1,2 was subjected to biological testing . To substantiate the antibacterial results, we screened compounds 1,2 against an assortment of two Gram-positive bacteria (*Bacillus cereus, Staphylococcus aureus*) and Gram-negative bacteria (*Serratia Sp., Pseudomonos*

Sp., Escherichia coli) using ampicillin and amoxillin as a reference standard . The minimum inhibitory concentrations (MICs,µg/ml) were determined using standard agar dilution method (11) . The MIC value is summarized in Table 3.

From the obtained data, it is clear that compound 1 posses high activity against Gram-positive strain, particulerly *Bacillus cereus.* Also, posses high activity against Gram-negative strains. On the contrary, compound 2 posses higher activity against Gram-negative strains and not affected at tested concentrations against Gram-positive strain as shown in Table 3. Our results are in agreement with those reported earliear by Joklik et al. (1992) (12), they reported that some antibiotics such as ampicillin and amoxillin have been developed as inhibitors of cell wall synthesis of bacterial cell . So, compound 1 and 2 have the common structural feature of penicillins exhibit antibacterial activities .

EXPERIMENTAL

^{1}H-NMR (500 MHz, $CDCl_3$). ^{13}C-NMR (500 MHz, $CDCl_3$) and 2D spectra were recorded on a JEOL 500 MHz, Lambda spectrometer, TLC: precoated silica gel type 60 (Merck). HPLC was performed in the reversed phase mode on Knauer pump 64 and different refractometer (column: RP-8, 250 × 25 mm, flow = 17 mL/min, elution with MeOH-H_2O, mixtures, refractive index), optical rotation were measured in $CHCl_3$ with a perkin-elemer 2435 polarimeter, IR spectra were recorded on a JASCO FT/IR-5300 spectrometer, EIMS were recorded on a JEOL SX102A mass spectrometer .

Bioassay

The antibacterial activity of compounds 1 and 2 were determined against Gram-negative strains (*Serratia* sp., *Pseudomonas sp.*, *Escherichia coli*) and Gram-positive bacteria (*Bacillus cereus*, *Staphylococcus aureus*), obtained from culture collection of Bacteriological Laboratory, Department of Botany, Faculty of Science, El-Minia University, Egypt, using Whatman filter paper No. 1, 1 cm. Diameter, disc diffusion assay methods. Five replicates were performed for the compound with two concentrations (200 mg/mL and 400 mg/mL) of each compound were done . Discs were soaked in the test compound for 30 sec, evaporated, then overload on the surface of the nutrient agar media cultured with the tested bacterium. All plates were incubated at 30°C for 48 hours . Ampicillin (purchased from ADWIC Comp., Egypt) and amoxillin (purchased from ADCO Comp., Egypt) were used as a reference compounds.

CONCLUSION

9-hydroxyethuliacoumarin (**1**), white powder; IR ν^{KBR}_{max} cm^{-1}: 3475 (OH), 1675 (C=O), 1620, 1580 (aromatic); EIMS, m/z (rel. int.) : 358 $[M]^+$ ($C_{20}H_{22}O_6$) (6), 340 $[M-H_2O]^+$ (3), 300 $[M-Me_2C=O]^+$ (4), 286 $[M-Me_2C=CHOH]^+$ (40), 244 [286- $C_2H_2O]^+$ (55) ; $[\alpha]^{25}_D$ + 35 (c. 0.1, $CHCl_3$) .

1',2'-epoxyethuliacoumarin (**2**), white powder; IR ν^{KBR}_{max} cm^{-1}: 3465 (OH), 1685 (C=O), 1625, 1575 (aromatic); EIMS, m/z (rel. int.) : 358 $[M]^+$ ($C_{20}H_{22}O_6$) (5), 340 $[M-H_2O]^+$ (3), 300 $[M-Me_2C=O]^+$ (5), 286 $[M-Me_2C=CHOH]^+$ (50), 244 [286- $C_2H_2O]^+$ (60) ; $[\alpha]^{25}_D$ + 37 (c. 0.125, $CHCl_3$).

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