MAIN PHENOLIC COMPOUND OF PETALS OF *ECHIUM AMOENUM* FISCH. AND C.A. MEY., A FAMOUS MEDICINAL PLANT OF IRAN

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ABSTRACT

Echium amoenum Fisch. and C.A. Mey. (Boraginaceae) is an indigenous Iranian plant, that its dry violet–blue petals (Gol-e-Gavzaban) have long been used in traditional medicine of Iran. In this study concentrated metanolic extract of the grounded dried petals of *E. amoenum* was fractionated by column chromatography and the fractions were purified by preparative HPLC. The structure of main pure component which was characterized by UV, IR, one and two dimensional ^{1}H and ^{13}C-NMR and Mass spectroscopy was found to be rosmarinic acid which is widespread in the plants of the Lamiaceae and Boraginaceae families in insignificant quantities and has antimicrobial, antiviral, and anti-inflammatory effects.

Keywords: *Echium amoenum*; Rosmarinic acid; HPLC; NMR

INTRODUCTION

Dried violet–blue petals of *Echium amoenum* Fish. and C.A. Mey. (Boraginaceae) has long been used as a tonic, tranquillizer, diaphoretic and as a remedy for cough, sore throat and pneumonia is known in traditional medicine of Iran as Gol-e-Gavzaban (1, 2). *E. amoenum* is a biennial or perennial herb indigenous to the narrow zone of northern part of Iran and Caucasus, where it grows at an altitude ranging from 60-2200 m (3). *Echium* genus has 4 species in Iran (4) and only *E. amoenum* has medicinal uses (1, 5). A search through the literature revealed that the petals of *E. amoenum* have anthocyanidine (13%), flavonoid aglycons (0.15%) and trace amount of alkaloids (5, 6) , a clear lemon-yellow volatile oil (0.05%) with δ-cadinene (24.25%) as the major component (7). *E. amoenum* has anxiolytic effect in mice (8, 9) and has the capacity to increase the cellular immune response (10). Because the decoct of its dry petals are used in folk medicine, we tried to identify the major phenolic compounds that may define some interesting biological activities of this plant.

MATERIALS AND METHODS

Plant Material:

Petals of *E. amoenum* were collected from a farm in 80 km north of Ghazvin in June 2000. Voucher specimens (No. 1001) were authenticated and then deposited in Herbarium of the Department of Pharmacognosy, Faculty of Pharmacy, Kerman University of Medical Sciences, Kerman, Iran.

Extraction and isolation:

Dried ground petals of *E. amoenum* were exhaustively extracted with methanol in a Soxhlet apparatus under reduced pressure at 30 ºC. The resulting methanolic extract was filtrated and concentrated in vacuum and after addition of water was washed successively with hexane and chloroform and was then extracted with ethyl acetate. After evaporation of the solvent, the residue was subjected to column chromatography.

Phytochemical analyses:

For primary purification and separation of components of extract, column chromatography on silica gel (mesh size 70-230 Merck) with 1,2-dichloroethane- methanol- acetic acid- water (54:28:11:7) as eluent was used and the fraction that had main compound (checked by TLC using the same eluent) was applied for HPLC analysis.

High performance liquid chromatography (HPLC) analysis:

A Waters HPLC system, equipped with prep LC-4000, UV-Vis dual λ 2487 spectrophotometric detector, was used for analytical and preparative HPLC analyses of the isolated fraction by column

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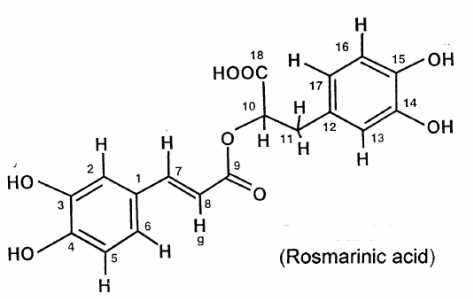

Figure 1. Structure of rosmarinic acid (11).

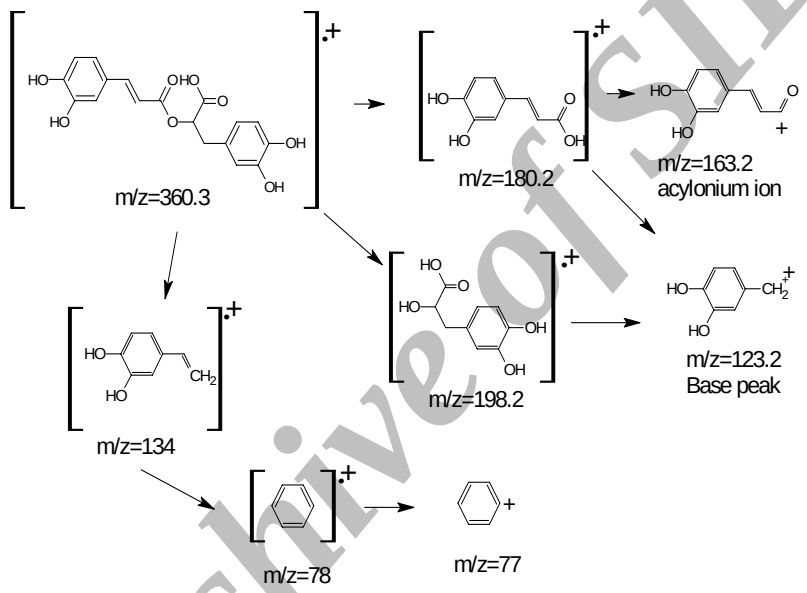

Figure 2. The main Mass fragmentation profile of rosmarinic acid

m/z=360.3 ($C_{18}H_{16}O_8^{+\cdot}$), radical ion of rosmarinic acid; m/z= 198.2 ($C_9H_{10}O_5^{+\cdot}$), radical ion of 3,4-dihydroxy phenyl lactic acid; m/z= 180.2 ($C_9H_8O_4^{+}$), radical ion of caffeic acid; m/z= 163.2 ($C_9H_7O_3^{+}$) ; m/z= 134 ($C_8H_6O_2^{+\cdot}$); **m/z=123.2 ($C_7H_7O_2^{+}$) base peak;** m/z=78 ($C_6H_6^{+\cdot}$); m/z= 77 ($C_6H_5^{+}$)

Table 1. IR spectrum data of rosmarinic acid

ν $_{(cm^{-1})}$	Groups and bonds	Comment
3520,3460,3400,3370	O-H	Stretching of free OH as a sharp peak
3180	O-H	Stretching of OH with intramolcular hydrogen bond
2920	C-H	Aliphatic stretching
1740	C=O	Non conjugated acidic bond
1720	C=O	Esteric bond
1650	C=C	Double bond with trans stereochemistry (weak)
1615,1540,1465	C=C	Aromatic rings
1350	C-O	Acid
1280,1260	C-O	Ester (stretching symmetric and asymmetric)
1230-1075	C-O , C-C	Aromatic rings and alkene group
900-690	C-H	Bending extra planer of =C-H (Aromatic rings and alkene)

Table 2. Electron impact mass fragmentation data of rosmarinic acid

m/z	%Intensity (45 eV)	%Intensity (70 eV)	Fragment ion
360	0.2	-	$C_{18}H_{16}O_8^{+\cdot}$
300	1	-	$C_{17}H_{16}O_5^{+\cdot}$
299	1	-	$C_{17}H_{15}O_5^{+}$
198	10	8	$C_9H_{10}O_5^{+\cdot}$
197	8	-	$C_9H_9O_5^{+}$
181	1	2	$C_9H_9O_4^{+\cdot}$
180	15	15	$C_9H_8O_4^{+}$
179	8	4	$C_9H_7O_4^{+\cdot}$
164	1	2	$C_9H_8O_3^{+\cdot}$
163	6	5	$C_9H_7O_3^{+}$
162	2	1.5	$C_9H_6O_3^{+\cdot}$
137	8	2	$C_8H_9O_2^{+\cdot}$
136	7	5	$C_8H_8O_2^{+\cdot}$
135	4	3	$C_8H_7O_2^{+}$
134	10	5	$C_8H_6O_2^{+\cdot}$
124	9	8	$C_7H_8O_2^{+}$
123	**100**	**75**	$\mathbf{C_7H_7O_2^{+}}$
122	20	2	$C_7H_6O_2^{+\cdot}$
110	2	2	$C_6H_6O_2^{+}$
109	2	1.5	$C_6H_5O_2^{+\cdot}$
78	5	5	$C_6H_6^{+\cdot}$
77	10	20	$C_6H_5^{+}$
44	**5**	**100**	$\mathbf{CO_2^{+}}$

Table 3. Proton and ^{13}C assignments for rosmarinic acid (in d_6- DMSO).

Proton number	^{1}H-NMR (at 500 MHz, in d_6- DMSO)			^{13}C-NMR $\delta_{(C)}$ ppm*
	[a] Number of H	[a] J (Hz)	$\delta_{(H)}$ ppm	
1	-	-	-	126.2 s
2	1	-	7.06(s, b)	115.74 d
3	OH (phenolic)	-	8.81(s, vb)	144.85 s
4	OH (phenolic)	-	9.68(s, vb)	145.79 s
5	1	$8_{(5,6)}$	6.77(d)	116.64 d
6	1	$8_{(6,5)}$	7.01(d)	122.45 d
7	1	$16_{(7,8)}$	7.47(d)	146.73 d
8	1	$16_{(8,7)}$	6.24(d)	114.18 d
9	-	-	-	166.81 s
10	1	$8.6_{(17,16)}$, $4_{(17,16)}$	5.03(dd)	73.84 d
11	1	$14_{(16,16)}$, $4_{(16,17)}$	2.99(dd)	37.01 t
	2 = +			
	1	$14_{(16,16)}$, $8.6_{(16,17)}$	2.91(dd)	
12	-	-	-	128.28 s
13	1	-	6.69(s, b)	117.55 d
14	OH (phenolic)	-	8.75(s, vb)	146.47 s
15	OH (phenolic)	-	9.20(s, vb)	149.49 s
16	1	$7.6_{(14,15)}$	6.64(d)	116.25 d
17	1	$7.6_{(15,14)}$	6.53(d)	120.90 d
18	OH (acidic)	-	12.90(s, vvb)	171.84 s

* Multiplicities were determined by DEPT135°. s = singlet (no hydrogen bond to carbon), d = doublet (one hydrogen bond to carbon), t = triplet (two hydrogen bond to carbon)

[a] According to ^{1}H-NMR, ^{1}H-^{1}H (COSY) and ^{1}H-^{13}C (HETEROCOSY), (multiplicities of hydrogen: s = singlet, d = doublet, dd = doublet doublet, b = broad , vb = very broad, vvb= very very broad, t = triplet) .

chromatography. An analytical µBondapack C_{18} (10 µm) stainless steel column (4.6×250 mm) was used for this purpose. Flow rate was 1ml/min and the injected volume was 20 µl. The column used for preparative HPLC was a Radialpak µBondapack C_{18} (10 µm) (25×200 mm). The injected volume was 1000 µl and flow rate was 5 ml/min. Mobile phase was methanol (A) and 5% formic acid (B) , and a linear gradient from 100% B to 45% A in 175 min was applied. The detector was preset at 280 nm and 350 nm. The major compound was collected by Waters II fraction collector and after removal of the solvent; the residue was subjected to spectroscopic analyses.

The UV absorption spectra (220-400 nm) of the purified compound (in methanol) were recorded using a Secomam S-1000 UV/Vis spectrophotometer.

The IR spectrum of purified compound (KBr) was recorded using a Perkin-Elmer 650 IR spectrophotometer.

Mass spectra of the purified compound was recorded by an electron impact (EI) mode at 45 and 70 eV in Finnigan-mat TQS 70EI and Shimadzu Qp 1100EX EI quadruple mass respectively. The source, probe and scanning temperatures, which were used in this study were 200, 100-300 and 25-30 ˚C, respectively.

Nuclear magnetic resonance (NMR) spectroscopy:

The NMR spectra were recorded using a Bruker DRX 500 Avence spectrometer. ^{1}H-NMR (at 500 MHz) and ^{1}H-^{1}H (COSY) and ^{1}H-^{13}C (HETEROCOSY) correlation , DEPT 135˚ and ^{13}C-NMR(125 MHz) spectroscopic data were collected at room temperature in d_6- DMSO. Chemical shifts (δ, ppm) were reported relative to tetramethylsilane (TMS) as an internal standard.

RESULTS

The results of the spectroscopic data in comparison with references (11-14) suggest that the major phenolic compound of the ethyl acetate extract of petals of *E. amoenum* is rosmarinc acid (figure 1). The compound had a UV absorbance maximum at 330 nm and a shoulder at 290 nm. The IR spectrum, electron impact mass fragmentation data, proton and carbon assignments of the compound according to ^{1}H-NMR, ^{1}H-^{1}H (COSY), ^{1}H-^{13}C (HETEROCOSY), DEPT 135˚ and ^{13}C-NMR are shown in Tables 1-3. The main fragmentation profiles of electron impact mass spectrum are shown in Figure 2.

DISCUSSION

The UV absorbance maximum at 330 nm and a shoulder at 290 nm could be due to a phenolic acid with two aromatic rings (11). The IR spectrum showed that compound has OH, esteric C=O and acidic C=O groups, and aromatic rings. The mass fragment profile at 45 eV showed a very small molecular radical ion ($M^{+\cdot}$) at *m/z* of 360.2 (intensity = 0.1%) that was not present at 70 eV. Although there is not any information about NMR spectroscopy of rosmarinc acid in d_6- DMSO, but application of other spectroscopic methods was very useful for structural elucidation of this compound. NMR spectroscopy of rosmarinc acid in d_6- DMSO, in comparison to CD_3OD resulted in shift of all signals to upper field and appearance of the protons of phenolic and acidic groups.

Rosmarinic acid has antimicrobial, antiviral, antioxidant and anti-inflammatory activities, which makes it a valuable component for pharmaceutical, food and cosmetic industries (15). The presence of rosmarinic acid in medicinal plants such as *E. amoenum* has beneficial and health promoting effects.

Rosmarinic acid is an ester of caffeic acid and 3, 4-dihydroxyphenyl lactic acid and is widespread in the plants of Lamiaceae and Boraginaceae families in significant quantities (15, 16).

In the previous report; roots of *E. vulgare,* which is another species of *Echium* genus was shown to have high concentrations of rosmarinic acid and it was suggested that this plant is a good source for this compound and rosmarinic acid may play an important role in pharmacological properties of this plant (16, 17) and also *E. amoenum*. This is the first report about the presence of rosmarinic acid in petals of *Echium* species.

The genus *Echium* is especially rich in pyrrolizidine alkaloids and the previous phytochemical examinations of *Echium* were focused on their alkaloid constituents..

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