TOXIC PYRROLIZIDINE ALKALOIDS OF *ECHIUM AMOENUM* FISCH. & MEY.

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ABSTRACT

Toxic pyrrolizidine alkaloids are present in some species of *Echium* (Boraginaceae). In this study petals of *Echium amoenum* Fisch. & Mey. (Gol-e-Gavzaban) as a popular herbal medicine in Iran, were investigated for pyrrolizidine alkaloids (PAs). The alkaloids were separated and purified by preparative TLC and characterized by IR, one and two dimensional ^{1}H and ^{13}C-NMR and Mass spectroscopy. Four toxic alkaloids namely: echimidine I, echimidine isomer II, 7-angeloyl retronecine III and 7-tigloyl retronecine IV were identified.

Keywords: *Echium amoenum,* Pyrrolizidine alkaloids, Echimidine , Echimidine isomer , 7-angeloyl retronecine, 7-tigloyl retronecine

INTRODUCTION

Pyrrolizidine alkaloids have been isolated from several plant families such as Boraginaceae including *Echium* genus (1). The chemistry of these alkaloids in relation to their toxic and therapeutic effects has been the subject of several investigations (1, 2). Petals of *Echium amoenum* Fisch. & Mey. (Gol-e-Gavzaban) a popular herbal medicine in Iran, has long been used as a tonic, tranquillizer, diaphoretic and as a remedy for cough, sore throat and pneumonia (3, 4). *Echium* genus has 4 species in Iran (5) of which only *E. amoenum* has been used as medicinal (3, 4). *E. amoenum* is indigenous to the narrow zone of northern part of Iran and Caucasus (6). Literature review showed the petals of *E. amoenum* have anthocyanidine, flavonoid aglycons, traces of alkaloids (7, 8), volatile oils (0.05%) (9) and rosmarinic acid (11). *E. amoenum* has shown to increase the cellular immune responses (10) and has anxiolytic effects (12, 13). Toxic pyrrolizidine alkaloids are present in some species of *Echium.* The decoct of dry petals of *E. amoenum* are used in Iranian folk medicine as a popular herbal tea. Some chemical constituents of this petals reported in previous work (11). Isolation of its pyrrolizidine alkaloids was investigated in this study.

MATERIALS AND METHODS

General experimental procedures:

The IR spectra were recorded using a Perkin-Elmer 650 IR spectrophotometer. Mass spectra were recorded using an electron impact (EI) mode at 45 in Finnigan-mat TQS 70 EI quadruple mass. The source, probe and scanning temperatures were 200, 100-300, and 25-30˚C, respectively. The NMR spectra were recorded using a Bruker DRX 500 Avence spectrometer. ^{1}H-NMR (500 MHz) and ^{1}H-^{1}H (COSY) and ^{1}H-^{13}C (HETEROCOSY) correlation and ^{13}C-NMR(125 MHz) spectroscopic data were collected at room temperature in $CDCl_3$. Chemical shifts (δ, ppm) were reported relative to tetramethylsilane (TMS) as an internal standard. TLC aluminium sheets (silicagel 60 F_{254} 20 × 20) was used for TLC, and TLC silicagel 60 GF_{254} was used for thick layer chromatography (thickness: 1 mm). All chemicals were purchased from Merck Chemical Company, Germany.

Plant Material:

Petals of *E. amoenum* were collected from a farm at 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* (5kg) were exhaustively extracted with methanol in a Soxhlet apparatus under reduced pressure at 30˚C. The resulting methanolic extract was filtrated and concentrated in vacuum and after addition of 2N HCl for adjustment of pH to 4, it was washed first with hexane and then with chloroform.

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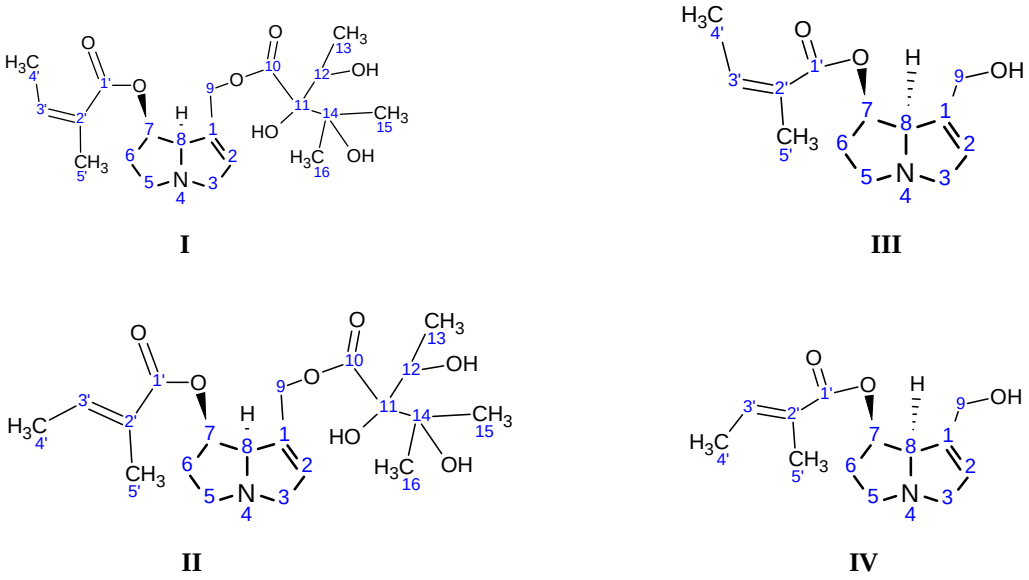

Figure 1. Structures of compounds I-IV.
I = Echimidine; II = Echimidine isomer; III = 7-Angeloyl retronecine; IV = 7-Tigloyl retronecine

The pH of the aqueous phase was adjusted to 2 by addition of 2N HCl and then for each 15 ml of the solution 0.5 g NaCl was added. After 24 h, the gummy non-alkaloid residue was filtered and divided to two equal parts and named "portion of A" and "portion of B" respectively. The pH of the "portion of A" was adjusted to 10 by addition of 25% NH_4OH, the mixture was extracted with $CHCl_3$ (10×300 ml) and the solvent was evaporated to give the crude alkaloids (part A*). To investigate the presence of pyrrolizidine alkaloids N-oxide, the pH of the aqueous layer of "portion of B" was adjusted to 2 by addition of 6N H_2SO_4. After addition of Zn dust (10 g/l) to the solution, the mixture was stirred for 48 h, filtered and the filtrate was made alkaline and treated as above (14).

Phytochemical analysis:
In order to compare A* and B* portions for the presence of pyrrolizidine alkaloids N-oxide, 50 µl of solution of 100mg of each extract in 10ml of methanol were subjected to TLC using CH_2Cl_2 : MeOH : NH_4OH (85 : 15 : 1) as the mobile phase (14). TLC plates were sprayed by Dragendorff or Erlich reagent (15). Since there was no differences between the portions of A* and B*, they were mixed and subjected to thick layer chromatography using the same solvent system for TLC. Fractions were extracted by methanol and after evaporation of the solvent; the residues were analyzed by spectroscopic methods.

RESULTS

The total alkaloid content of dried petals of *E. amoenum* was 0.01%. In this petals, structures of four pyrrolizidine alkaloids namely: echimidine I, echimidine isomer II, 7-angeloyl retronecine III and 7-tigloyl retronecine IV (figure 1) were identified by NMR (Table 1), IR and mass spectral data. The structures of compounds I–IV were identified by comparison of their spectral data with those reported in the literature (16-27).

Echimidine I and Echimidine isomer II: gummy, R_f = 0.7, IR (film) $\nu_{cm^{-1}}$: 3400 (OH); 1725, 1715 (ester C=O); 1650, 1610 (C=C); 1255, 1230 (ester C-O); 1160, 1000 (alcohol C-O).
EIMS m/z (% relative intensity at 45 eV): 397 (M^+, 0.2), 298(0.5), 297(4), 296(5), 237(4), 220(100), 219(90), 141(10), 137(9), 136(36), 135(17), 120(60), 106(5), 94(15), 93(25), 82(12), 80(5), 55(7).

7-angeloyl retronecine III and 7-tigloyl retronecine IV: gummy. R_f = 0.5. IR (film) $\nu_{cm^{-1}}$:3380 (OH); 1715 (ester C=O); 1650, 1600 (C=C); 1260, 1235 (ester C-O); 1150, 1000 (alcohol C-O).
EIMS m/z (% relative intensity at 45 eV): 237 (M^+, 12), 220(10), 219(12), 206(1), 193(5), 154(20), 139(8), 138(48), 137(91), 135(31), 124(40), 120(18), 111(58), 106(54), 93(88), 80 (100).

Table 1. ^{1}H-NMR an d ^{13}C-NMR assignments of compounds I-IV.

Situation *	^{1}H-NMR (at 500 MHz, in $CDCl_3$) [a]								^{13}C-NMR(125 MHz, in $CDCl_3$) $\delta_{(C)}$ ppm			
	I		II		III		IV		I	II	III	IV
	$\delta_{(H)}$ ppm	J Hz	$\delta_{(H)}$ ppm	J Hz	$\delta_{(H)}$ ppm	J Hz	$\delta_{(H)}$ ppm	J Hz				
1’	-	-	-	-	-	-	-	-	167.32	167.51	167.78	167.93
2’	-	-	-	-	-	-	-	-	127.80	128.85	124.45	125.31
3’	6.05(1H)q	$7_{(3',4')}$	6.72(1H)q	$7_{(3',4')}$	6.10(1H)q	$7.5_{(4',3')}$	6.63(1H)q	$7.5_{(4',3')}$	139.65	138.21	138.20	138.94
4’	1.91(3H)d	$7_{(4',3')}$	1.73(3H)d	$7_{(4',3')}$	1.97(3H)dd	$7.5_{(4',3')}$, 0.8	1.80(3H)d	$7.5_{(4',3')}$	16.16	14.80	16.20	14.90
5’	1.76(3H)s	-	1.72(3H)s	-	1.83(3H)s	-	1.79(3H)s	-	20.19	12.30	21.00	12.40
1	-	-	-	-	-	-	-	-	133.42	133.34	139.50	139.60
2	5.81(1H)bs	-	5.81(1H)bs	-	5.66(1H)s	-	5.88(1H)s	-	129.40	128.89	127.98	127.97
3	3.34(1H)d	$15_{(3,3)}$	3.32(1H)d	$15_{(3,3)}$	3.48(1H)m	-	3.46(1H)m	-	63.16	63.24	63.62	63.74
	3.83(1H)d	$15_{(3,3)}$	3.83(1H)d	$15_{(3,3)}$	3.94(1H)dq	$14.8_{(3,3)}$, 1.8	3.94(1H)dd	$14.8_{(3,3)}$, 1.8				
5	2.60(1H)t	$9_{(5,6)}$	2.62(1H)t	$9_{(5,6)}$	2.71(1H)m	-	2.73(1H)m	-	54.13	54.13	53.96	53.92
	3.27(1H)m	-	3.27(1H)m	-	3.36(1H)m	-	3.36(1H)m	-				
6	2.05(2H)m	-	2.05(2H)m	-	2.14(2H)m	-	2.14(2H)m	-	34.84	34.75	35.05	34.97
7	5.37(1H)bs	-	5.33(1H)bs	-	5.42(1H)d	$2.5_{(7,6)}$	5.38(1H)d	$2.5_{(7,6)}$	74.03	74.18	74.64	74.85
8	4.32(1H)bs	-	4.32(1H)bs	-	4.36(1H)bs	-	4.78(1H)bs	-	76.06	75.99	76.30	76.26
9	4.60(1H)d	$13.5_{(9,9)}$	4.58(1H)d	$13.5_{(9,9)}$	4.20(2H)d	$8_{(9,9)}$	4.19(2H)d	$8_{(9,9)}$	62.83	62.87	60.50	60.50
	4.86(1H)d	$13.5_{(9,9)}$	4.83(1H)d	$13.5_{(9,9)}$								
10	-	-	-	-					174.66	174.66		
11	-	-	-	-					83.63	83.65		
12	4.12(1H)q	$6.5_{(12,13)}$	4.13(1H)q	$6.5_{(12,13)}$					69.96	69.96		
13	1.21(3H)d	$6.5_{(13,12)}$	1.21(3H)d	$6.5_{(13,12)}$					18.86	18.88		
14	-	-	-	-					73.84	73.84		
15	1.17(3H)s	-	1.17(3H)s	-					26.45	26.45		
16	1.26(3H)s	-	1.25(3H)s	-					25.19	25.19		

* According to fig.1

[a] According to ^{1}H-NMR, ^{1}H-^{1}H (COSY) and ^{1}H-^{13}C (HETEROCOSY)

Multiplicities of hydrogen: s = singlet, d = doublet, m = multiplet, q = quartet, t = triplet, bs = broad singlet, dd = doublet doublet

I = Echimidine; II = Echimidine isomer; III = 7-Angeloyl retronecine; IV = 7-Tigloyl retronecine

Table2. Reported pyrrolizidine alkaloids in *Echium* genus (16-19, 28-30).

Alkaloids \ Plants	*E. horridum*	*E. humile*	*E .pininana*	*E. plantagineum*	*E. rauwolfii*	*E. setosum*	*E. vulgare*
Echimidine	+	+	+	+	+	+	+
Echimidine isomer (tigloyl)	+				+	+	+
12-Acetyl echimidine						+	+
Echihumiline		+				+	+
Echihumiline N-oxide		+					
Echiupinine			+				
Echiupinine N-oxide			+				
Echiumine				+			
Echinatine							+
Myoscorpine			+				
Myoscorpine N-oxide			+				
Hydroxy myoscorpine			+				
Uplandicine	+				+	+	+
Intermidine				+			
7-Acetyl intermidine			+				
Lycopsamine	+	+		+	+		
7-Acetyl lycopsamine	+	+			+		
7-Tigloyl lycopsamine	+				+		
7-Angelyl lycopsamine	+				+		
7-Senecioyl lycopsamine		+					
Retronecine						+	+
7-Angelyl retronecine	+				+	+	+
9-Angelyl retronecine				+		+	+
7-Tigloyl retronecine	+				+	+	+
9-Tigloyl retronecine						+	+
7-Senecioyl retronecine		+					
9-Senecioyl retronecine		+				+	+
7- Angelyl 9-(2-methyl butyryl) retronecine	+				+		
7- Tigloyl 9-(2-methyl butyryl) retronecine	+				+		
7- Angelyl 9-(2,3-dihydroxy butyryl) retronecine	+				+	+	+
7- Tigloyl 9-(2,3-dihydroxy butyryl) retronecine	+				+	+	+
7-(2-Methyl butyryl) 9-(2,3-dihydroxy butyryl) retronecine		+				+	+
7-(2-Methyl butyryl) 9-echimidinyl retronecine		+					
7-(2-Methyl butyryl) retronecine		+				+	+
9-(2-Methyl butyryl) retronecine						+	+
References	19	17	16	28, 29	19	18	18, 30

DISCUSSION

Echimidine (cis isomer), echimidine isomer (trans isomer), 7-angeloyl retronecine (cis isomer) and 7-tigloyl retronecine (trans isomer) are common pyrrolizidine alkaloids in another *Echium* species (16-19, 28-30). In Table 2, pyrrolizidine alkaloids which are reported in literatures for different *Echium* species are shown. This is the first report on the isolation of pyrrolizidine alkaloids from *E. amoenum*. Since the cis and trans isomers could not be separated by TLC (21, 27), the structural elucidation of isomers were based on chemical shifts of H-3' that in cis isomers appeared in up field (I at δ = 6.05ppm and III at δ = 6.72ppm) and in trans isomers appeared in down field (II at δ = 6.10ppm and IV at δ = 6.63ppm) (21, 25).

Echimidine and echimidine isomer were obtained as a mixture. They differ only in having Z and E configuration respectively, about of the C-3'. ^{1}H-NMR spectrum of the mixture resembled that of echimidine, except that the quartet δ 6.05 (H-3') was equivalent to only half a proton, and an additional quartet, also equivalent to a half proton, was present at 6.72. This is consistent with an approximately 1: 1 mixture of equivalent to a half proton of 7- angeloyl and 7- tigloyl esters. Also 7-angeloyl retronecine and 7-tigloyl retronecine were obtained as a mixture like above, except that the quartet δ 6.10 (H-3') was equivalent to 3/4 proton, and an additional quartet, also equivalent to 1/4 proton, was present at 6.63 (H-3'). A similar result has been reported about symphytine and symlandine in *Symphytum* x *uplandicum* (27).

Pyrrolizidine alkaloids present in the *Echium* and especially the compounds I-IV which were found in *E. amoenum*, have hepatotoxic effects similar to those of di-ester of unsaturated pyrrolizidin alkaloids. However this study does not provide enough evidences for forbidding the usages of this popular herbal medicine in Iran. Further investigations including in vitro and in vivo toxicological studies are required to confirm these points.

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