Pyrrolizidine Alkaloids from *Echium rauwolfii* and *Echium horridum* (Boraginaceae)

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Echimidine was isolated from *Echium rauwolfii* and *Echium horridum* and identified by MS, ^{1}H- and ^{13}C NMR as a major alkaloid. In addition, structures of 12 minor alkaloids were inferred from GLC and GLC-MS analyses: 7-angeloylretronecine, 7-tigloylretronecine, lycopsamine, 7-acetyllycopsamine, uplandicine, 7-angeloyllycopsamine, 7-tigloyllycopsamine, tigloyl isomer of echimidine, 7-angeloyl-9-(2-methylbutyryl)retronecine, 7-tigloyl-9-(2-methylbutyryl)retronecine, 7-angeloyl-9-(2,3-dihydroxybutyryl)retronecine, and 7-tigloyl-9-(2,3-dihydroxybutyryl)retronecine. Both species had similar alkaloid profiles. Alkaloid extracts exhibited antibacterial effects with a MIC of 1.7 mg/ml in *E. coli*. Microscopic examination of *E. coli* treated with different subtoxic alkaloid concentrations (13–52 μg/ml) revealed extensive filamentation.

Introduction

Pyrrolizidine alkaloids (PAs) occur wide-spread in the plant families Boraginaceae (all tribes), Compositae (tribes Senecioneae and Eupatorieae), and Leguminosae (tribe Crotalarieae) (Robins, 1982; Mattocks, 1986; Rizk, 1990; Hartmann and Witte, 1995). PAs mainly function as defence compounds against herbivores and exhibit toxic and carcinogenic properties in life stock and humans (reviews in Mattocks, 1986; Hartmann and Witte, 1995; Röder, 1995; Schmeller *et al.*, 1997; Roberts and Wink, 1998).

About 40 species of *Echium* are known that are mainly distributed in the Mediterranean region, Southern Europe and Western Asia (Feinbrun-Dothan, 1978; Jafri and El-Gadi, 1979). In Egypt the genus is represented by about 7 species (Täckholm, 1974). *Echium rauwolfii* Del. is an erect annual herb with narrow leaves and a 12–15 mm long, white- or flesh-coloured corolla. *Echium horridum* Batt., is a rare annual herb similar to the former one, but the corolla is 20 mm long and violet (Täckholm, 1974). Alkaloid composition of *E. rauwolfii* and *E. horridum* has not been reported previously. We have analyzed the PA profiles of both taxa by GLC and GLC-mass spectrometry. Thirteen or 12 PAs were detected in the reduced alkaloid extract of *E. horridum*, and *E. rauwolfii*, respectively. The major alkaloid in both species, echimidine, was identified by MS, ^{1}H-NMR, and ^{13}C-NMR analysis. Isolated alkaloid extracts were analysed for antimicrobial activity.

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Material and Methods

Plant materials

Plants of *Echium rauwolfii* and *E. horridum* were cultivated in the Botanical garden of the Faculty of Pharmacy, Zagazig University, Egypt. In the flowering stage the plants were collected in April 1996. Identification of these plants was confirmed by Dr. A. El-Hadidi, Faculty of Science, Cairo University. Voucher specimen are deposited at the Herbarium of the Department of Pharmacognosy, Faculty of Pharmacy, Zagazig University.

Alkaloid extraction and identification

The dried aerial plant material (100 g) was extracted with 200 ml 0.5 N HCl. After defatting of the acidic extract, half of the extract was made alkaline with ammonia (pH 9) and extracted with

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1000 ml CH_2Cl_2 to obtain the tertiary (3ry) pyrrolizidine alkaloids (free PA bases without PA N-oxides) which amounted to 0.018% and 0.008% dry weight for *E. horridum* and *E. rauwolfii*, respectively. The other half of the acified extract was stirred with zinc dust overnight, filtered and extracted with 1000 ml CH_2Cl_2 to yield the total alkaloids (3ry bases plus reduced N-oxides). The difference between the total yield (after reduction) and the free 3ry alkaloids (before reduction) is attributed to the N-oxides. Total alkaloid yields of *E. horridum* and *E. rauwolfii* were 0.1% and 0.07% dry weight, respectively.

PTLC [Si gel F_{254}, CH_2Cl_2, MeOH, NH_4OH (25%), 85:15:2 v/v] of the reduced extract of *E. horridum* and *E. rauwolfii* yielded alkaloid **12** with Rf 0.43. The isolated alkaloid was identified by MS, ^{1}H- and ^{13}C NMR. Echimidine (**12**); colourless oil, GLC-**EI**MS, *m/z* (rel. int.%) [M^+] $C_{20}H_{31}NO_7$ 397 (0.2), 382 (0.4), 352 (0.1), 338 (0.3), 321 (0.1), 297 (3), 221 (25), 220 (100), 219 (7), 141 (13), 138 (6), 137 (6), 136 (55), 121 (25), 120 (70), 119 (26), 106 (5), 94 (35), 93 (63), 83 (44), 80 (13), 67 (10), 59 (12), 55 (39). GLC-**CI**MS, *m/z* (rel. int.%) [M^++1] 398 (60), 352 (10), 336 (27), 322 (100), 238 (40), 222 (35), 120 (25), 118 (25). ^{1}H- and ^{13}C NMR spectra were recorded on a AC Bruker Instrument in $CDCl_3$ at 300 and 75 MHz, respectively (Table I).

GLC and GLC-MS were carried out as reported in earlier studies (El-Shazly *et al.*, 1996a, b, 1998).

Microorganisms for antimicrobial testing

The following microorganisms were obtained from stock cultures of the Department of Microbiology, Faculty of Pharmacy, Zagazig University. Gram negative bacteria: *Escherichia coli* and *Klebsiella*. Gram-positive bacteria: *Bacillus subtilis* and *Staphylococcus aureus*; fungi: *Candida albicans* and *Aspergillus flavus*. Antimicrobial effects were determined with the agar-diffusion test (cup test). Each cup was filled with 50 µl of 25 mg extract dissolved in 1 ml dimethylformamide (DMF) with pure DMF as a control. The plates were incubated overnight at 37 °C in case of bacteria and 30 °C for fungi. The diameter of inhibition zones was measured (in mm) using tetracycline, gramicidine, penicillin, and chloramphenicol as standard.

Table I. NMR spectral data of echimidine isolated from *Echium horridum* and *E. rauwolfii*.

Position	^{1}H NMR ($CDCl_3$, 300 MHz) δ (H)	^{13}C NMR ($CDCl_3$, 75 MHz) δ (C)*
1	–	132.91, s
2	5.86 (1H, m)	127.26, d
3	4.05 (1H, dm, *J* = 15.4 MHz, 3-Hα) 3.59 (1H, m, 3-Hu)	62.45, t
5	2.75 (1H, m, 5-Hα) 3.43 (1H, m, 5-Hu)	53.81, t
6	2.15 (2H, m)	34.40, t
7	5.48 (1H, m)	73.29, d
8	4.56 (1H, m)	76.14, d
9	4.92 (1H, dm, *J* = 13.2 MHz, 9-Hα) 4.65 (1H, dm, *J* = 13.2 MHz, 9-Hu)	62.14, t
10	–	174.24, s
11	–	83.13, s
12	4.19 (1H, q, *J* = 7.2 MHz)	69.80, d
13	1.25 (3H, d, *J* = 6.4 MHz)	18.52, q
14	–	73.67, s
15	1.22 (3H, s)	25.97, q
16	1.29 (3H, s)	24.86, q
17	–	166.71, s
18	–	127.19, s
19	6.10 (1H, qq, *J* = 1.5, 7.2 MHz)	139.71, d
20	1.95 (3H, dq, *J* = 1.5, 7.2 MHz)	15.77, q
21	1.79 (3H, quin., *J* = 1.5 MHz)	20.43, q

* Multiplicities were determined by APT.

Results and Discussion

The major alkaloid of both species was isolated by preparative layer-chromatography. MS, ^{1}H- and ^{13}C NMR spectra for this compound **12** (Table I) were identical with those reported for echimidine (Roeder *et al.*, 1991; Sarg *et al.*, 1992; El-Shazly *et al.*, 1996).

The reduced alkaloid extracts were analysed by capillary GLC and GLC mass spectrometry. Besides echimidine, twelve minor alkaloids were detected in *E. horridum*; six of which could be unequivocally identified by direct comparison of their retention indices (RI), mass spectra or authentic material (El-Shazly *et al.*, 1996, 1998; Witte *et al.*, 1993) (Tables II, III). These alkaloids were: 7-angeloylretronecine, 7-tigloylretronecine, lycopsamine, 7-acetyllycopsamine, and uplandicine. The remaining alkaloids were tentatively identified on the base of mass fragmentation as 7-angeloyl-9-(2,3-dihydroxybutyryl)retronecine, 7-tigloyl-9-(2,3-dihydroxybutyryl)retronecine, 7-tigloyllycopsamine, and tigloyl isomer of echimidine. 7-angeloyl-9-(2-methylbutyryl)retronecine and its tigloyl isomer were probably artefacts (El-Shazly *et al.*, 1996). Alkaloid **11** (RI 2473) was identified as 7-tigloyllycopsamine because of the close similarities of its mass spectrum with those of 7-angeloyllycopsamine (or its isomer). Stelljes *et al.* (1991) reported that the tigloyl esters are delayed in GLC relative to angeloyl esters. This is due to the trans configuration of the carbonyl and methyl groups of the tigloyl esters versus the cis configuration on the angeloyl esters. According to this logic compound **11** should be the C-7 tigloyl isomer of lycopsamine.

The alkaloid profile of *E. rauwolfii* seems to be very similar to that of *E. horridum* except for the absence of lycopsamine and some quantitative variation (Tables II, III).

Alkaloid yields before and after reduction revealed that PA N-oxide level is nearly 10 times higher than that of the corresponding 3ry bases as found in many other plants (Hartmann and Witte, 1995). Echimidine was the major PA in the alkaloid extract of *E. horridum* after and before reduction, with 65% and 45% of total alkaloids, respectively. Whereas echimidine dominated the alkaloid

Table II. Profile of pyrrolizidine alkaloids in extracts of *Echium horridum* and *E. rauwolfii* as determined by capillary gas-liquid chromatography (% of total alkaloid).

No. Alkaloid	*E. horridum*		*E. rauwolfii*	
	3ry alkaloid*	Total alkaloid**	3ry alkaloid*	total alkaloid**
1 7-Angeloylretronecine	–	3.17	11.76	2.17
2 7-Tigloylretronecine	–	tr	12.49	tr
3 Lycopsamine	–	0.86	–	–
4 7-Angeloy-9-(2-methylbutyryl)-retronecine	–	15.85	12.52	12.23
5 7-Tigloy-9-(2-methylbutyryl)-retronecine	–	2.08	tr	2.72
6 7-Acetyllycopsamine	–	1.44	–	1.90
7 Uplandicine	–	tr	–	tr
8 7-Angeloyl-9-(2,3-dihydroxy-butyryl)retronecine	–	4.99	tr	3.80
9 7-Tigloyl-9-(2,3-dihydroxy-butyryl)retronecine	–	7.64	tr	6.25
10 7-Angeloyllycopsamine	14.68	5.76	29.03	12.23
11 7-Tigloyllycopsamine	9.51	3.46	9.56	5.71
12 Echimidine	65.39	44.67	24.64	42.12
13 Echimidine isomer (tigloyl)	10.42	10.08	tr	10.86
Total alkaloids***	0.018	0.1	0.008	0.07

tr = trace amounts < 0.5%.
– = not detected.
* = before reduction.
** = free base + N-oxides.
*** = dry weight %.

Table III. Identification of pyrrolizidine alkaloids from *Echium horridum* and *E. rauwolfii* by GLC-MS.

Alkaloid	*E. horridum*	*E. rauwolfii*	RI	EI-MS [M^+] Base peak	CI-MS [$M^+ +1$]	Ref.
1 7-Angeloylretronecine	+	+	1787	237 80	238	1, 2, 3
2 7-Tigloylretronecine	+	+	1816	237 80	238	1, 2
3 Lycopsamine	+	–	2145	299 138	300	4, 5
4 7-Angeloy-9-(2-methylbutyryl)-retronecine*	+	+	2155	221 220	222	3
5 7-Tigloyl-9-(2-methylbutyryl)-retronecine*	+	+	2170	221 220	222	3
6 7-Acetyllycopsamine	+	+	2230	341 180	342	6
7 Uplandicine	+	+	2305	357 180	358	7
8 7-Angeloyl-9-(2,3-dihydroxy-butyryl)retronecine*	+	+	2315	339 136	340	3
9 7-Tigloyl-9-(2,3-dihydroxy-butyryl)retronecine*	+	+	2325	339 93	340	3
10 7-Angeloyllycopsamine	+	+	2460	381 220	382	8
11 7-Tigloyllycopsamine*	+	+	2473	381 220	382	
12 Echimidine	+	+	2560	397 220	398	2, 3
13 Echimidine isomer (tigloyl)*	+	+	2580	397 220	398	3

* Identification based on MS fragmentation pattern; 1 = Witte *et al.* (1993); 2 = Roeder *et al.* (1992); 3 = El-Shazly *et al.* (1996a); 4 = Roeder and Bourauel (1992); 5 = El-Shazly *et al.* (1996b); 6 = Kelley and Seiber (1992); 7 = Culvenor *et al.* (1980); 8 = El-Shazly *et al.* (1998).

extract after reduction (42%), 7-angeloyllycopsamine and echimidine were the major components before reduction with 29 and 25%, respectively.

Our results are in agreement with a recent examination of other *Echium* species (El-Shazly *et al.*, 1996a; 1996b) which showed that all pyrrolizidine alkaloids in *Echium* derive from the retronecine base. All the toxic PAs are esters of 1,2-unsaturated pyrrolizidine nucleus (necines) (Bull *et al.*, 1968; Mattocks, 1986; Hartmann and Witte, 1995; Röder, 1995). Although not studied in detail yet, PAs in *E. horridum* and *E. rauwolfii* are suspected to be hepatotoxic, pneumotoxic, mutagenic and carcinogenic.

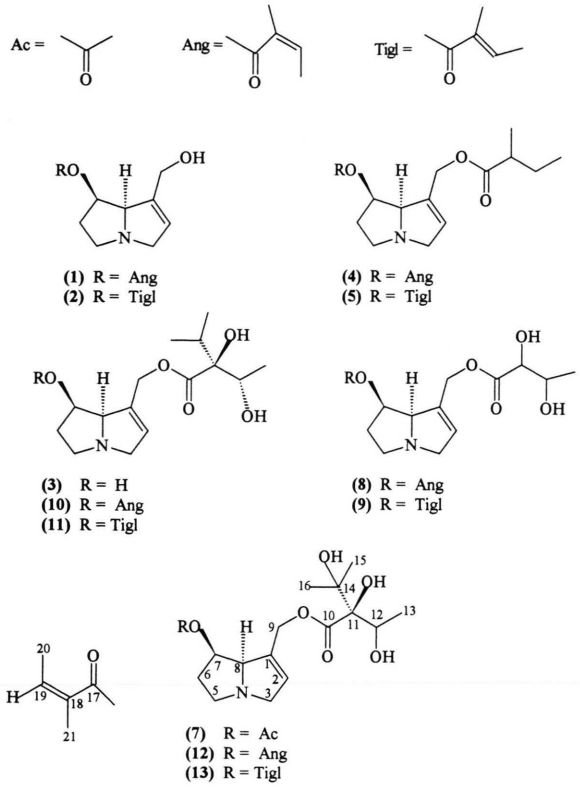
We have analyzed whether PA extract exhibit any antibacterial or antifungal activity. The antimicrobial activity of the total methanolic and of alkaloid extracts was determined by the agar diffusion method (Table IV). The alkaloid extract of *Echium rauwolfii* showed significant effects on *S. aureus* and *B. subtilis*, whereas total methanolic extracts were less active.

The MIC values were defined as the lowest concentration at which no visible growth occurred. It

Table IV. Results of antimicrobial screening of the total extracts and alkaloid extracts of *Echium rauwolfii* and *E. horridum*. 50 µl were applied in each assay.

Material	Diameter of inhibition zone [in mm]					
	Gram-neg. bacteria		Gram-pos. bacteria		Fungi	
	E. coli	*Klebsiella*	*S. aureus*	*B. subtilis*	*C. albicans*	*A. flavus*
Total methanolic extract of *E. rauwolfii**	10	10	–	–	13	–
Total methanolic extract of *E. horridum**	8	7	–	–	7	–
Alkaloid extract of *E. rauwolfii**	10	8	15	17	16	–
Tetracycline 30 µg/disc	–	9	8	16	–	–
Chloramphenicol 30 µg/disc	15	15	20	15	–	–
Penicillin 10 µg/disc	–	–	5	–	–	–
Gramicidin 10 µg/disc	–	18	12	25	–	–

– = No zone of inhibition.
DMF was used as a solvent.
* Concentration: 25 mg extract/ml DMF.



Structures of pyrrolizidine alkaloids in *Echium horridum* and *E. rauwolfi.* Numbering is according to Table II and III.

was estimated by two fold serial dilution method. The alkaloid extract of *E. rauwolfii* showed a MIC in *E. coli* at 1.66 mg/ml. Microscopic examination of *E. coli* treated with different subtoxic concentrations (13–52 μg/ml) revealed extensive filamentation. At higher concentrations (830–104 μg/ml) the cells appeared elongated but not as filaments. The effect may be due to inhibition of DNA synthesis, cell division, and mutations, as filamentation was observed in bacteria treated with the antibiotic ciprofloxacin (Diver and Wise, 1986). The exact mechanism of antibacterial effects by PAs will be a challenging topic for future studies and whether the effect is caused by an individual PA (*e.g.*, echimidine) or all PAs. Quite a number of alkaloids are known which exhibit multiple allelochemical activities, ranging from effects in animals, to plants and microbes (Wink, 1993; Wink *et al.*, 1998; Roberts and Wink, 1998).

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Bull L. B., Culvenor C. C. J. and Dick A. T. (1968), The Pyrrolizidine Alkaloids. North- Holland Publishing Co., Amsterdam.

Culvenor C. C. J., Edgar J. A., Frahn J. L. and Smith L. W. (1980), The alkaloids of *Symphytum* × *uplandicum* (Russian comfrey). Aust. J. Chem. **33**, 1105–1113.

Diver J. M. and Wise R. (1986), Morphological and biochemical changes in *Escherichia coli* after exposure to ciprofloxacin. J. Antimicrob. Chemother. **18** (suppl. D) 31–41.

El-Shazly A., Sarg T., Ateya A., Abdel Aziz E., El-Dahmy S., Witte L. and Wink M. (1996a), Pyrrolizidine alkaloids from *Echium setosum* and *Echium vulgare* . J. Nat. Prod. **59**, 310–313.

El-Shazly A., Sarg T., Ateya A., Abdel Aziz E., El-Dahmy S., Witte L. and Wink M.(1996b), Pyrrolizidine and tetrahydroisoquinoline alkaloids from *Echium humile.* Phytochemistry **42**, 225–230.

El-Shazly A., El-Domiaty M., Witte L. and Wink M. (1998), Pyrrolizidine alkaloids in members of the Boraginaceae from Sinia (Egypt). Biochem. Syst. Ecology **26,** 619–636.

Feinbrun-Dothan N. (1978), Flora Palaestina, The Israel Academy of Sciences and Humanities. Vol. **3**, pp. 74–77.

Hartmann T. and Witte L. (1995), Chemistry, biology and chemecology of pyrrolizidine alkaloids. In: Alkaloids: Chemical and Biological Perspectives (Pelletier, S. W., ed.). Pergamon, Oxford, Vol. **9**, pp. 155–233.

Jafri S. M. H. and El-Gadi A. (1979), Flora of Libya, Al Faateh University, Tripoli.Vol. **68**, pp. 33–49.

Kelley R. B. and Seiber J. N. (1992), Pyrrolizidine alkaloid chemosystematics in *Amsinckia.* Phytochemistry **31**, 2369–2387.

Mattocks A. R. (1986), Chemistry and Toxicology of Pyrrolizidine Alkaloids. Academic Press London.

McLean E. K. (1970), The toxic action of pyrrolizidine (*Senecio*) alkaloids. Pharmacol. Rev. **22**, 429–475.

Rizk A. M. (1990), Naturally occurring pyrrolizidine alkaloids. CRC Press, Boca Raton.

Roberts M. F. and Wink M. (1998), Alkaloids- Biochemistry, ecology and medicinal applications. Plenum, New York.

Robins D. J. (1982), The pyrrolizidine alkaloids. Fortschr. Chem. Org. Naturstoffe. **41**, 115–203.

Roeder E. (1995), Medicinal plants in Europe containing pyrrolizidine alkaloids. Pharmazie **50,** 83–98.

Roeder R., Liu K. and Bourauel T. (1991), Pyrrolizidine alkaloids from *Echium pininana.* Phytochemistry **30**, 3107–3110.

Roeder E. and Bourauel T. (1992), Pyrrolizidine alkaloids from *Neatostema apulum*. Phytochemistry **31**, 3613–3615.

Roeder E., Sarg T., El-Dahmy S. and Abdel Ghani A. (1992), Pyrrolizidine alkaloids from *Alkanna orientalis.* Fitoterapia **63**, 405–408.

Sarg T., El-Dahmy S., Abdel Aziz E., Abdel Ghani A. and Roeder E. (1992), Pyrrolizidine alkaloids from *Echium angustifolium*. Fitoterapia **63**, 466–468.

Schmeller T., El-Shazly A. and Wink M. (1997), Allelochemical activities of pyrrolizidine alkaloids: Interactions with neuroreceptors and acetylcholine related enzymes. J. Chem. Ecology **23**, 399–416.

Smith L. W. and Culvenor C. C. J. (1981), Plant sources of hepatotoxic pyrrolizidine alkaloids J. Nat. Prod. **44**, 129–152.

Stelljes M. E., Kelley R. B., Molyneux R. J. and Seiber J. N. (1991), GC-MS determination of pyrrolizidine alkaloids in four *Senecio* species. J. Nat. Prod. **54**, 759–773.

Täckholm V. (1974), Students Flora of Egypt, Cairo University, Cooperative Printing Co. Beirut. 2nd. Ed., pp. 450–451.

Wink M. (1993), Allelochemical properties and the raison d'être of alkaloids. In: The Alkaloids (G. Cordell, ed.). Academic Press, San Diego, Vol. **43**, 1–118.

Wink M., Schmeller, T. and Latz-Brüning, B. (1998), Modes of action of allelochemical alkaloids: Interaction with neuroreceptors, DNA and other molecular targets. J. Chemical Ecology **24**, 1881–1937.

Witte L., Rubiolo P., Bicchi C. and Hartmann T. (1993), Comparative analysis of pyrrolizidine alkaloids from natural sources by gas chromatography-mass spectrometry. Phytochemistry **32**, 187–196.