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Fatty Acid Pattern and Alkaloids of Echium Rauwolfii

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Abstract: The GC/MS analysis of hexane extract revealed the presence of palmitic acid as saturated fatty acid (1.05%), versus oleic acid (2.18%), linoleic acid (1.13%), cis-8,11,14-eicosatrienoic acid (2.12%) as unsaturated fatty acids. On the other hand, CH_2Cl_2 extract contained palmitic acid methyl ester (3.55%), and methyl isostearate (1.17%) as saturated fatty acids, versus linoleic acid methyl ester (3.57%) and linolenic acid methyl ester (10.01%) as unsaturated fatty acids. The GC/MS analysis of the alkaloid-rich fraction indicated the presence of the pyrazolidine alkaloids petranine (2.97%), 7-angeloyl-9-(2-methylbutyryl) retronecine (4.22%), 7-angeloylretronecine (0.59%) and 9-angeloylretronecine (0.47%).

The butanol extract showed the heights DPPH radical scavenging activity (IC_{50} = 14.3 µg),. while ethyl acetate extract was very weak in activity (IC_{50} = 432.3 µg) and no activity with hexane and methylene chloride extract.

The antimicrobial potentials of *E. rauwolfii* extracts were examined. The inhibition of the fungi species by ethyl acetate extract exert was comparable to Amphotericin B. The inhibition zone of the butanol extract against *Streptococcus pneumonia* was comparable to Ampicillin, against *Pseudomonas aeruginosa* was comparable to Gentamicin and *Escherichia coli* was comparable to Gentamicin.

The cytotoxicity against HePG-2 of ethyl acetate extract and butanol extract were "very strong", and that of hexane extract and methylene chloride extract were "moderate", against MCF-7 of ethyl acetate extract and butanol extract were "strong", that of methylene chloride extract was "moderate", and that of hexane extract was "weak" and against HCT-116 of butanol extract was "very strong", of ethyl acetate extract was *"strong", of methylene* chloride extract and hexane extract were "moderate".

Keywords: fatty acids, pyrrolizidine alkaloids, *Echium rauwolfii*, Boraginaceae

1. INTRODUCTION

Echium rauwolfii Delile (Boraginaceae) is an erect or ascending hispid-setose annual winter herb with branched stems [1]. About 40 species of Echium are known that are mainly distributed in the Mediterranean region, Western Asia and Southern Europe [2, 3]. The genus is represented by about 7 species in Egypt, including E. rauwolfii [1].

Echium seeds contain specialty oil; it has many potential uses in the pharmaceutical industry for treatment of eczema, acne, and other skin disorders and in the cosmetic and personal care products industry. Echium oil is applied topically to reduce skin wrinkles and protects and moisturizes the skin from sun exposure [4]. As a chemical class, pyrrolizidine alkaloids are some of the leading plant-based toxins associated with harmful effects in both humans and animals [5, 6]. However, the antioxidant, antimicrobial and anti cancer (Hep-G2, HCT-116 and MCF-7) activities, that we will introduce here, do not confirm this situation, so that, we have interested here to introduce the results of inspection of both the fatty acid and the pyrrolizidine alkaloid patterns.

2. EXPERMINTAL

2.1 GC/MS

The GC/MS analysis was performed at Agriculture Research Center, National Research Center (NRC), Dokki, Cairo, Egypt, using a Thermo Scientific, Trace GC Ultra / ISQ Single Quadrupole MS, TG-5MS fused silica capillary column (30 m, 0.251 mm, 0.1 mm film thickness). For GC/MS detection, an electron ionization system with ionization energy of 70 ev was used, Helium gas was used as the carrier gas at a constant flow rate of 1mL/min. The

injector and MS transfer line temperature was set at 280°C. The oven temperature was programmed at an initial temperature 40°C (hold 3 min) to 280°C as a final temperature at an increasing rate of 5°C /min (hold 5 min). The quantification of all the identified components was investigated using a percent relative peak area. A tentative identification of the compounds was performed based on the comparison of their relative retention time and mass spectra with those of the NIST, WILLY library data of the GC/MS system.

2.2 Solvents

Hexane (60-80°C) was obtained from Alpha Chemika; methylene chloride was obtained from SDFCL sd fine-chem limited; ethyl acetate, methanol, butanol and acetone were obtained from Adowic.

2.3 Cell lines

Hepatocellular carcinoma HePG-2, mammary gland breast cancer MCF-7 and colorectal carcinoma HCT-116. The cell lines were obtained from ATCC via Holding company for biological products and vaccines (VACSERA), Cairo, Egypt.

2.4 Chemical reagents

The reagents RPMI-1640 medium, MTT, DMSO and 5-fluorouracil (sigma co., St. Louis, USA), Fetal Bovine serum (GIBCO, UK). 5-fluorouracil was used as a standard anticancer drug for comparison.

2.5 Plant material

Echium rauwolfii Delile was collected in March 2014 at Hibis Temple from arable land-side of cultivation in EL Kharga Oasis which, Western Desert, Egypt. It was identified by the 4th author according to [1, 7] and [8]. A herbarium specimen was deposited in the Herbarium of Botany Department, Faculty of Science, Cairo University.

2.6 Processing of plant material

The collected plant material was dried in shade and grinded at room temperature to give (181.760 g) of dried powder material. The plant material was extracted by soxhlet extractor with hexane, followed by methylene chloride, then ethyl acetate, and finally butanol to give extracts (5.780, 2.200, 1.49, 58.21g, respectively).

A sample of hexane extract was analyzed using GC/MS technique to give: 1-hexadecanol (Rt 31.09 min, 2.26%), 1-octadecanol (Rt 36.51 min, 4.18%), nonadecane (R_t 39.18 min, 0.91%), 1-eicosanol (R_t 41.43 min, 3.27%), eicosane (R_t 41.57 min, 1.15%), palmitic acid (R_t 45.67 min, 1.05%), oleic acid (R_t 45.93 min, 2.18%), linoleic acid (R_t 48.16 min, 1.13%), cis-8,11,14–eicosatrienoic acid (R_t 48.32 min, 2.12%), pentacosane (R_t 52.12 min, 2.70%), heptacosane (R_t 55.79 min, 1.81%), nonacosane (R_t 59.21 min, 1.96%), nonacosanol (R_t 61.12 min, 1.19%), hentriacontane (R_t 62.42 min, 2.97%).

A sample of CH_2Cl_2 extract was analyzed by GC/MS technique to give: hexadecane (R_t 31.29 min, 1.31%), octadecane (R_t 36.68 min, 0.94%), palmitic acid methyl ester (R_t 39.91 min, 3.55%), eicosane (R_t 41.57 min, 0.60%), linoleic acid methyl ester (R_t 43.82 min, 3.57%), linolenic acid methyl ester (R_t 43.97 min, 10.01%), methyl isostearate (R_t 44.54 min, 1.17%), docosane (R_t 46.04 min, 0.45%), tetracosane (R_t 50.16 min, 0.47%), pentacosane (R_t 52.09 min, 0.86%), hexacosane (R_t 53.97 min, 0.90%), heptacosane (R_t 55.77 min, 0.72%).

A sample of plant material (135.000 g) was soaked in methanol (1L) for 24 h filtered, and the filtrate was evaporated to 1/4 the initial volume. The extract was diluted with water and acidified with hydrochloric acid, then extracted by CH_2Cl_2. The aqueous acidic layer was basified to pH 9 by adding ammonium hydroxide and then extracted again by CH_2Cl_2 to give the alkaloid-rich fraction (22.610 g).

A sample from the alkaloid-rich fraction gave by GC/MS: trans 3-pinanone (R_t 19.80 min, 3.90%), petranine (R_t 21.12 min, 2.97%), endobornyl acetate (R_t 23.78 min, 1.90%), 7-angeloyl-9-(2-methylbutyryl)retronecine (R_t 27.50 min, 4.22%), 7-angeloylretronecine (R_t 28.22 min, 0.59%), 9-angeloylretronecine (R_t 29.28 min, 0.47%), 14-methylpentadecanoic acid methyl ester (R_t 42.26 min, 0.40%), eicosane (R_t 43.98 min, 0.58%), heneicosane (R_t 46.30 min, 0.87%), docosane (R_t 48.52 min, 1.50%), tricosane (R_t 50.66 min, 2.42%), hexanedioic acid, dioctyl ester (R_t 52.72 min, 24.04%), pentacosane (R_t 54.68 min, 4.48%), hexacosane (R_t 56.57 min, 4.16%), heptacosane (R_t 58.41 min, 2.75%), octacosane (R_t 60.17 min, 1.69%).

2.7 Evalution of biological activity of the plant extracts

2.7.1 Antioxidant activity

The antioxidant activity of extract was determined at the Regional Center for Mycology and Biotechnology (RCMB) at Al- Azhar University *by* the DPPH free radical scavenging assay in triplicate and average values were considered. Freshly prepared (0.004% w/v) methanol solution of 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical was prepared and stored at 10°C in the dark. A methanol solution of the test compound was prepared. A 40ml aliquot of the methanol solution was added to 3ml of DPPH solution. Absorbance measurements were recorded immediately with a UV-visible spectrophotometer (Milton Roy, Spectronic 1201). The decrease in absorbance at 515 nm was determined continuously, with data being recorded at 1 min intervals until the absorbance stabilized (16 min). The absorbance of the DPPH radical without antioxidant (control) and the reference compound ascorbic acid were also measured. All the determinations were performed in three replicates and

averaged. The percentage inhibition (PI) of the DPPH radical was calculated according to the formula:

PI = [{(*AC*- *AT*)/ *AC*} x 100].

Where *AC* = Absorbance of the control at t = 0 min and
AT = absorbance of the sample+DPPH at t = 16 min [9].

2.7.2 Antimicrobial activity assessment

Extracts were individually tested against a panel of Gram positive (*Staphylococcus aureus*), Gram negative bacteria (*Escherichia coli*) and fungi (*Candida albicans*). Each of the extracts was dissolved in DMSO (1 mg /ml). Whitman filter paper discs were cut with standard size (5 mm) and sterilized in an autoclave. The paper discs were soaked in the desired concentration of the extracts and placed aseptically in the Petri dishes containing nutrient agar media (agar 20 g + beef extract 3 g + peptone 5 g) and seeded with *Staphylococcus aureus*, *Escherichia coli* and *Candida albicans*. The Petri dishes were incubated at 36°C and the inhibition zones were recorded after 24 h of incubation. Each treatment was replicated three times. The antibacterial activity of a common standard antibiotic Ampicillin, Gentamicin and antifungal Amphotericin B was also recorded using the same procedure as above at the same concentration and solvents. The % activity index for the extract was calculated by the formula:

$$\%\ Activity\ Index = \frac{Zone\ of\ inhibition\ of\ test\ extract\ (diametre)}{Zone\ of\ inhibition\ by\ standard\ (diametre) x\ 100}$$

2.7.3 Cytotoxicity MTT assay [10, 11]

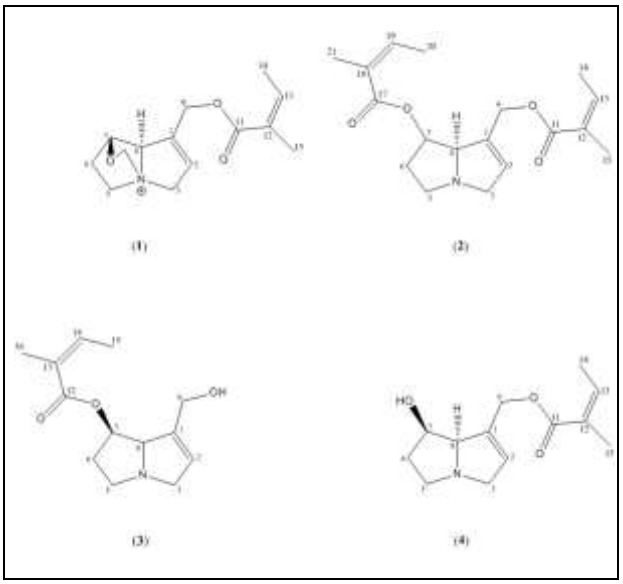
The cell lines HePG-2, MCF-7 and HCT-116 were used to determine the inhibitory effects of extracts on cell growth using the MTT assay. This colorimetric assay is based on the conversion of the yellow tetrazolium bromide (MTT) to a purple formazan derivative by mitochondrial succinate dehydrogenase in viable cells. The cells were cultured in RPMI-1640 medium with 10% fetal bovine serum. Antibiotics added were 100 units/ml penicillin and 100 µg/ml streptomycin at 37oC in a 5% CO_2 incubator. The cells were seeded in a 96-well plate at a density of 1.0 x 104 cells/well [12]. At 37oC for 48 h under 5% CO2. After incubation the cells were treated with different concentrations of extracts and incubated for 24 h. After 24 h of treatment, 20 µl of MTT solution at 5 mg/ml was added and incubated for 4 h. Dimethyl sulfoxide (DMSO) in volume of 100 µl is added into each well to dissolve the purple formazan formed. The colorimetric assay is measured and recorded at absorbance of 570 nm using a plate reader (EXL 800). The relative cell viability in percentage was calculated as (A570 of treated samples/A570 of untreated sample) X 100.

3. RESULT AND DISCUSSION

3.1 Fatty acid and alkaloid pattern

Many *Echium* species are characterized by the presence of polyunsaturated fatty acids [13]. The GC/MS analysis of the volatile fractions (hexane extract and methylene chloride extract) revealed the presence of palmitic acid as saturated fatty acid (1.05%), versus oleic acid (2.18%), linoleic acid (1.13%), cis-8, 11, 14-eicosatrienoic acid (2.12%) as unsaturated fatty acids in the hexane extract. On the other hand, the GC/MS analysis of CH_2Cl_2 extract gave palmitic acid methyl ester (3.55%) and methyl isostearate (1.17%) as saturated fatty acids, versus linoleic acid methyl ester (3.57%) and linolenic acid methyl ester (10.01%) as unsaturated fatty acids.

The GC/MS analysis of the alkaloid-rich fraction indicated the presence of the pyrazolidine alkaloids petranine **1** (2.97%), 7-angeloyl-9-(2-methylbutyryl) retronecine **2** (4.22%), 7-angeloylretronecine **3** (0.59%) and 9-angeloylretronecine **4** (0.47%). **1** and **4** was identified previously from *Echium glomeratum* [14], **2** and **3** was identified previously from *Echium rauwolfii* and *horridum* [15]. The structures as the following figures:



3.2 Antioxidant activity evaluation

The free radical of 2, 2-diphenyl-1-picrylhydrazyl (DPPH) is used for detection of the antioxidant activity of the extracts [8]. The butanol extract showed the heights scavenging activity (IC_{50} = 14.3 µg, Table 1), while ethyl acetate extract was very weak in activity (IC_{50} = 432.3 µg, Table 1) and no activity with hexane and methylene chloride extract when tasted at concentration ranged from (10- 5000 µg/ml).

Table 1: DPPH radical scavenging activity (IC_{50} µg/ml)

Sample code	DPPH radical scavenging activity (IC_{50} µg/ml)
Hexane extract, Er1	-ve
Methylene chloride extract, Er2	-ve
Ethyle acetate extract, Er3	432.3
Butanol extract, Er4	14.3
Ascorbic acid	14.2

-ve: no activity even when tasted at concentration ranged from (10- 5000 µg/ml).

3.3 Antimicrobial activity assessment

The antimicrobial potentials of *E. rauwolfii* extracts were examined by the disc diffusion assay method, using eight pathogenic microbial species; *Aspergillus fumigates, Syncephalastrum racemosum, Geotricum candidum, Candida albicans,* represent pathogenic fungal species. *Streptococcus pneumonia, Bacillis subtillis,* represents Gram positive bacteria, *Pseudomonas aeruginosa, Escherichia coli,* represent Gram negative bacteria. The data were presented in Table 2. The results showed that there were remarkable inhibitions of the microbial growth against the tested extracts. The inhibition zones of ethyl acetate extract (Er3) against the fungi species were comparable to Amphotericin B. The inhibition zone against the Gram-positive bacteria *Streptococcus pneumonia* of the butanol extract (Er4) was comparable to *Ampicillin*. The inhibition zones of the butanol extract (Er4) against the Gram-negative bacteria *Pseudomonas aeruginosa* were comparable to Gentamicin. The inhibition zones of the ethyl acetate extract (Er3) and the butanol extract (Er4) against the Gram-negative bacteria *Escherichia coli* were comparable to Gentamicin.

3.4 Cytotoxicity assessment

Table 3 indicated the relative viability of cells (%) and table 4 indicated the in-vitro cytotoxicity IC50 (µg/ml). The cytotoxicity against HePG-2 of ethyl acetate extract (Er3) and butanol extract (Er4) were "very strong", and that of hexane extract (Er1) and methylene chloride extract (Er2) were "moderate", against MCF-7 of ethyl acetate extract (Er3) and butanol extract (Er4) were "strong", that of methylene chloride extract (Er2) was "moderate", and that of hexane extract (Er1) was "weak" and against HCT-116 of butanol extract (Er4) was "very strong", of ethyl acetate extract (Er3) was "strong", of methylene chloride extract (Er2) and hexane extract (Er1) were "moderate".

Table 2: The inhibition zone in mm of extracts of *E. rauwolfii* compared to standard antibiotics.

Extract / Tested microorganism	Hexane, Er1	CH_2Cl_2, Er2	ethyl acetate, Er3	Butanol, Er4	Standard antibiotic
Fungi					**Amphotericin B**
Aspergillus fumigate (RCMB 02568)	NA	NA	19.2 ±0.72	NA	23.7 ± 0.1
Syncephalastrum racemosum (RCMB 05922)	NA	NA	18.3 ±0.58	NA	19.7 ± 0.2
Geotricum candidum (RCMB 05097)	NA	NA	21.6 ±0.63	NA	28.7 ± 0.2
Candida albicans (RCMB 05036)	NA	NA	20.2 ±1.2	NA	25.4 ± 0.1
Gram positive bacteria					**Ampicillin**
Streptococcus pneumonia (RCMB 010010)	NA	16.3 ±0.58	18.6 ±0.63	20.3 ±0.58	23.8 ± 0.2
Bacillis subtillis (RCMB 010067)	NA	17.6 ±1.2	21.6 ±1.2	23.4 ±1.2	32.4 ± 0.3
Gram negative bacteria					**Gentamicin**
Pseudomonas aeruginosa (RCMB 010043)	NA	15.2 ±1.2	16.8 ±1.2	18.2 ±0.72	20.6 ± 0.58
Escherichia coli (RCMB 010052)	NA	13.7 ±0.72	19.2 ±0.58	20.6 ±0.58	19.9 ± 0.3

NA: No activity.

Table 3: Relative viability of cells (%) of *E. rauwolfii* extracts against human tumor cell lines HePG-2, MCF-7 and HCT-116

Concentration	HePG-2	MCF-7	HCT-116
5-FU			
100 µg/ml	8.6	7.9	7.4
50 µg/ml	17.1	14.8	12.1
25 µg/ml	24.0	21.0	19.8
12.5 µg/ml	33.1	34.5	31.4
6.25 µg/ml	56.8	47.3	49.9
3.125 µg/ml	70.6	58.2	60.5
1.56 µg/ml	88.7	76.0	73.6
Hexane extract, Er1			
100 µg/ml	31.7	44.1	34.0
50 µg/ml	42.6	56.3	45.2
25 µg/ml	55.3	67.5	57.6
12.5 µg/ml	68.2	78.6	69.8
6.25 µg/ml	87.4	95.2	93.9
3.125 µg/ml	100	100	100
1.56 µg/ml	100	100	100
Methylene chloride extract, Er2			
100 µg/ml	24.5	33.7	20.8
50 µg/ml	35.4	45.1	27.4
25 µg/ml	46.8	58.6	38.5
12.5 µg/ml	57.2	69.3	52.3
6.25 µg/ml	71.7	90.5	73.6
3.125 µg/ml	92.6	100	91.2
1.56 µg/ml	100	100	100
Ethyl acetate extract, Er3			
100 µg/ml	9.3	17.6	13.4
50 µg/ml	17.2	25.0	20.2
25 µg/ml	24.7	33.5	27.1
12.5 µg/ml	36.4	45.3	38.7
6.25 µg/ml	55.1	70.7	67.9
3.125 µg/ml	73.8	86.1	75.6
1.56 µg/ml	95.6	100	96.5
Butanol extract, Er4			
100 µg/ml	8.0	18.8	8.8
50 µg/ml	15.8	26.3	15.9
25 µg/ml	23.4	35.7	24.7
12.5 µg/ml	31.9	47.2	35.2
6.25 µg/ml	54.6	68.9	58.1
3.125 µg/ml	67.1	89.4	74.0
1.56 µg/ml	85.3	100	92.3

Table 4: Cytotoxicity assessment of *E. rauwolfii* extracts against human tumor cell lines HePG-2, MCF-7 and HCT-116

Extract	In vitro Cytotoxicity IC50 (µg/ml)*		
	HePG-2	**MCF-7**	**HCT-116**
5-FU*	7.9±0.25	5.4±0.18	5.3±0.33
Hexane, Er1	36.4±2.81	67.3±3.73	41.2±2.64
Methylene chloride, Er2	22.9±1.34	40.9±3.12	17.6±1.35
Ethyl acetate, Er3	8.6±0.36	14.2±0.97	10.7±1.10
Butanol, Er4	7.1±0.30	15.1±1.06	8.5±0.78

IC50 (µg/ml)*: 1 – 10 (very strong). 11 – 20 (strong). 21 – 50 (moderate). 51 – 100 (weak) and above 100 (non-cytotoxic); **5-FU***= 5-fluorouracil, a standard cytotoxic.

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1. REFERENCES

1. Täckholm, V. 1974. Students Flora of Egypt, Cairo University, Cooperative Printing Co. Beirut. 2nd. Ed; pp. 450-451.
2. Feinbrun-Dothane, N. 1978. Flora Palaestina, The Israel Academy of sciences and Humanities. Vol. 3, pp. 74-77.
3. Jafri, S. M. H. and El-Gadi, A.1979. Flora of Libya, Al Faateh University, Tripoli. Vol. 68, pp. 33-49.
4. Nicholls, P.,2000.Viper's bugloss and related species Echium spp. John King & Sons Ltd. www.dweckdata.com/Published_papers/Echium.pdf
5. Schmeller T., El-Shazly A., Wink M., 1979 Allelochemical activities of pyrrolizidine alkaloids: interactions with neuroreceptors and acetylcholine related enzymes. J. Chem. Ecol. 23, 399–416.
6. Stegelmeier B.L., Edgar J.A., Colegate S.M., Gardner D.R., Schoch T.K., Coulombe R.A. and Molyneux R.J. 1999. Pyrrolizidine alkaloid plants, metabolism and toxicity. J. Nat. Toxins 8, 95–116.
7. Boulos L. 2000. Flora of Egypt. Al Hadara Publishing, Cairo, Egypt. Volume 2 pp. 303,302.
8. Boulos L. 2009. Flora of Egypt (Checklist). Revised annotated addition, Al Hadara Publishing, Cairo, Egypt. pp.190.
9. Yen G.C. and Duh P.D. 1994. Scavenging effect of methanolic extracts of peanut hulls on free radical and active oxygen species, J Agric Food Chem, 42: 629-632.
10. Mosmann T. 1983. Immunol. J. Methods 65, 55-63.

11. Denizot F. 1986. Lang. R.; Immunol. J. Methods 22, 271-277.
12. MauceriHJ, HannaNN, BeckettMA, GorskiDH, StabaMJ, StellatoKA, BigelowK, Heimann R, Gately S, Dhanabal M, SoffGA, Sukhatme VP, Kufe DW, Weichselbaum RR Nature 1998. Combined effects of agiostatin and ionizing radiation in antitumour therapy. 394:287-291.
13. Sakineh Abbaszadeh S., Radjabian T., Taghizadeh M., Fazeli F. and Salmaki Y**.** September 2011**.** Characterization of fatty acids in different organs of some Iranian *Echium* plants. Vol. 5(19), pp. 4814-4821, 23.
14. Alali F.Q., Tahboub, Y.R., Ibrahim, E.S., Qandil, A., Tawaha, K., Burgess, J.P, Sy, A., Nakanishi, Y., Kroll, D.J., Oberlies, N.H. 2008. Pyrrolizidine alkaloids from *Echium glomeratum (Boraginaceae).* Phytochemistry. 69, 2341–2346.
15. El-Shazly A., Abdel-All M., Tei, A., Wink, M. 1999. Pyrrolizidine alkaloids from Echium rauwolfii and Echium horridum (Boraginaceae). Z. Naturforsch. 54c, 295–300.