Lipophylic Compounds from *Euphorbia peplis* L. – a Halophytic Plant from the Bulgarian Black Sea Coast

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The chemical composition of the lipophylic fraction from the halophytic plant *Euphorbia peplis* L. was investigated. Compared to other terrestrial higher plants an increase of triacylglycerols and especially of glycolipids was observed. The main phospholipid was phosphatidyl choline, followed by almost equal concentrations of phosphatidyl ethanolamine and phosphatidyl glycerol. A relatively high concentration of phosphatidic acids (6.5% of the total phospholipids) was found. The main sterol appeared to be sitosterol and significant amounts of tetracyclic triterpene alcohols were found. The composition of the volatile compounds is relatively simple and only one chlorinated compound, identified as 2,2-diethoxy-1-chloroethane, was found. There was a strong toxicity of the total lipophylic extract towards *Artemia salina.*

Key words: Euphorbia peplis, Lipids, Secondary Metabolites

Introduction

Approximately one third of the world land surface is arid or semi-arid. These soils are able to support only minimal agricultural development. Although Bulgaria is situated in a moderate climatic zone, there are some areas along the Black Sea where the soil may contain up to 700–900 mg salts in 100 g soil. Only few plants, mainly from the families Chenopodiaceae and Euphorbiaceae can grow under these extreme conditions (salinity and temperature up to 36 °C). The genus *Euphorbia* consists of more than 1600 species growing in nearly all types of climates throughout the world. They can be found as herbs, shrubs and trees. In the search for new plants with a high potential for the production of chemicals and liquid fuels as alternative energy sources, several *Euphorbia* species have been previously examined for possible economic utilisation (Zarrouk and Cherif, 1983; Hemmers and Gülz, 1986; Villalobos and Correal, 1992).

In some *Euphorbia* species Khafady *et al.* (1975) identified sitosterol and flavonoid monosaccharides. Cholesterol, campesterol, stigmasterol, sitosterol, isofucosterol and Δ^7-isofucostanol have been identified in the sterol fraction from *Euphorbia peplus* (Ferreira *et al.*, 1993).

The yield of the waxes from leaves of five *Euphorbia* species, growing in Europe (Hemmers and Gülz, 1986) ranged from 0.6 to 2.1% per dry mass. The waxes contained hydrocarbons (4–17%), wax esters (8–18%), aldehydes (2–6%), free fatty acids (1–8%) and free primary alcohols (13–39%). It was observed that the major carbon chain lengths ranged from C_{19} to C_{37} in hydrocarbons, from C_{36} to C_{52} in wax esters, from C_{24} to C_{36} in aldehydes, from C_{12} to C_{30} in acids and from C_{24} to C_{28} in alcohols. Saturated compounds dominated.

Diterpenes (Gotta *et al.*, 1984), triterpenes (Rizk *et al.*, 1980) and flavonoids (Rizk *et al.*, 1980) were identified in different *Euphorbia* sp. Triterpenoids from the cycloartenol type are characteristic for these plants. They were found in *E. cyparissias*, *E. clarkeana* (Conolly and Hill, 1996), *E. nivulia* and other *Euphorbia* species (Conolly *et al.*, 1994; Ravikanth *et al.*, 2002). Pentacyclic triterpenoids from the oleanane type were found in *E. supina* (Conolly *et al.*, 1994), *E. chamaesyce*, *E.*

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tortilis (Conolly and Hill, 1996), as well as in other *Euphorbia* species.

Euphorbia peplis L. is endangered for extinction, growing in the sands very close to the sea and irrigated mainly from the seawater in some regions near Black Sea, Caspian and Mediterranean Sea, Canary and Azores Islands. To the best of our knowledge, there is just one investigation on the chemical composition of this plant. The digalactosyl diacylglycerol (2*S*)-2-O-(9,12,15-octadecatrienoyl)-glyceryl-6-O-(α-D-galactopyranosyl)-β-D-galactopyranoside, exhibiting an anti-inflammatory activity, was isolated from it (Cateni *et al.*, 2000).

Materials and Methods

Collection of samples

The sample of *Euphorbia peplis* was collected at the Bulgarian Black Sea shore near the town of Pomorie in August, 1999. The plant was growing on the beach at 5–6 m from the sea. The soil salinity was about 800 mg in 100 g soil. Voucher-specimen was determined by Assoc. Prof. L. Evstatieva and deposited in the Institute of Botany, Bulgarian Academy of Sciences, Sofia 1113, Bulgaria.

Isolation of the lipophylic extract

The plant material (50–60 g fresh wt) was homogenised with 100 ml of methanol and refluxed for 5 min for inactivation of the enzymes. The total lipophylic extract was obtained according to Bligh and Dyer (1959). The yield of the total lipophylic extract was 5.9% of the dry weight of the plant.

Separation of the main lipid classes

A part of the total lipophylic extract was separated by two-dimensional thin layer chromatography (TLC) on silica gel G Merck plates with mobile phases (I) $CHCl_3/MeOH/H_2O$ (65:25:4 v/v/v) and (II) $CHCl_3/MeOH/NH_4OH$/isopropylamine (65:35:5:0.5 v/v/v/v). The separated zones of triacylglycerols (TAG), monogalactosyl diacylglycerols (MGDG), digalactosyl diacylglycerols (DGDG), sulphoquinovosyl diacylglycerols (SQDG) and phospholipids (PL) were visualized by iodine vapors. The main phospholipid classes were separated on a TLC plate using as a first mobile phase hexane/ethyl ether/AcOH (80:20:1 v/v/v). The chromatogram was developed in the second direction with $CHCl_3/MeOH/NH_4OH$/isopropylamine (65:35:5:0.5 v/v/v/v). The spots of the main phospholipids – phosphatidyl choline (PC), phosphatidyl ethanolamine (PE), phosphatidyl glycerol (PG) and phosphatidic acid (PA) – were visualised by iodine vapours.

Transesterification and gas chromatographic (GC) analysis of the fatty acid methyl esters (FAME)

The lipid spots from the two-dimensional TLC were scrapped off from the chromatogram. The fatty acids were transesterified with 3% sulphuric acid in absolute methanol (Christie,1989). The resulting FAME were analysed on a Hewlett-Packard Series II-5890 gas chromatograph equipped with a fused silica capillary column (30 m × 0.32 mm) with SUPELCOWAX 10 at temperature gradient from 185 °C to 230 °C at 11 $°C \cdot min^{-1}$.

Isolation and analysis of sterols and triterpene alcohols

A part of the lipophylic extract was evaporated under reduced pressure at a temperature of 40 °C. The dry residue (2 g) was subjected to column chromatography on 100 g of silica gel (1:50). Petroleum ether, followed by petroleum ether/acetone mixtures, chloroform and chloroform/methanol mixtures in ascending polarity were used as eluents.

The fractions containing sterols (eluted with petroleum ether/acetone 10:1, chloroform, and chloroform/methanol 99:1) were combined and purified by preparative thin layer chromatography (TLC) on silica gel and a mobile phase hexane/acetone 10:1 v/v.

The fractions containing triterpene alcohols (eluted with petroleum ether/acetone 15:1) were combined and purified by TLC and a mobile phase hexane/acetone 15:1 v/v.

The total sterol and triterpene mixtures were investigated by gas chromatography (GC) and gas chromatography-mass spectrometry (GC/MS).

GC: Pye Unicam 304, equipped with a flame ionization detector (FID) and a capillary column SPB-50 (30 m × 0.32 mm, 0.25 μm film thickness). Temperature programme: 230 °C–300 °C at 4 $°C \cdot min^{-1}$ and a 10-min hold at 300 °C. Injector temperature 300 °C, detector temperature 320 °C. Carrier gas: nitrogen.

GC/MS: Hewlett-Packard 6890 + MS 5973 with a capillary column SPB-50 (30 m × 0.32 mm, 0.25 μm film thickness). Carrier gas: helium. Temperature programme: 270 °C–290 °C at 4 $°C \cdot min^{-1}$ and a 20-min hold at 290 °C. The ion source was set at 250 °C and the ionisation voltage was 70 eV.

The total triterpene fraction was also analyzed by ^{1}H NMR spectroscopy on BRUKER DRX 250; $CDCl_3$ as a solvent and TMS as an internal standard were used.

Isolation and analysis of volatile compounds

A part of the lipophylic extract (400 mg) was subjected to a four-hour distillation-extraction in a Lickens-Nickerson apparatus (Hendriks *et al.*, 1981). The volatile compounds were extracted from the distillate with diethyl ether (yield: 130 mg, 32.5% of the total chloroform extract). They were investigated by GC/MS. A GC/MS investigation of the pure solvent was also performed.

GC/MS: Hewlett-Packard 6890 + MS 5973, HP5-MS capillary column was used (30 m × 0.25 mm, 0.25 μm film thickness). Temperature programme: 40 °C – 280 °C at 6 $°C \cdot min^{-1}$; carrier gas: helium.

Cytotoxic activity

The toxicity towards *Artemia salina* L., Arthropoda, Crustacea (Solis *et al.*, 1993) was determined using caffeic acid phenethyl ester (CAPE) as an active reference substance, whose LC_{50} (μg/ml) is 0.45 ± 0.05. 50 mg of the chloroform extract were dissolved in 1 ml DMSO. For the concentration of 1000 μg/ml 0.1 ml of the above mentioned solution were placed in a vessel, containing 10 *A. salina* nauplii in 5 ml sea water. In order to obtain concentrations of 100, 10 and 1 μg/ml the initial solution was consequently diluted. The experiment was made in triplicate. A control experiment was also made.

Results and Discussion

Analysis of lipids

The concentrations of the main lipid classes in *E. peplis* are presented in Table I. The main lipid class appears to be MGDG, followed by PL. The content of Triacylglycerols (TAG) is relatively high. TAG are not membrane constituents, although it is known that the relative content of TAG increases in some desiccation resistant plants at water stress (Stefanov *et al.*, 1992).

Table I. Lipid and fatty acid composition of the lipid classes in *Euphorbia peplis* L.

	Lipids[a,b]		Fatty acid composition (wt% of total)[c]									
Class	DM [$mg \cdot g^{-1}$]	% of total	14:0	16:0	16:1[d]	18:0	18:1[d]	18:2	18:3	20:0	20:1	22:0
Triacylglycerols	26.7 ± 2.1	8.9	2.1	22.1	5.1	5.5	29.5	7.9	21.7	2.4	3.7	–
Monogalactosyl diacylglycerols	99.6 ± 8.0	33.3	0.4	3.5	1.4	–	–	2.0	91.6	–	1.1	–
Digalactosyl diacylglycerols	60.2 ± 4.8	20.2	0.3	21.4	2.0	2.5	2.5	1.0	68.1	–	2.7	–
Sulphoquinovosyl diacylglycerols	36.9 ± 3.0	12.4	2.4	43.9	6.0	5.1	9.6	3.3	22.0	–	7.6	–
Phospholipids:												
Phosphatidyl choline	29.4 ± 2.4	9.8	2.2	42.9	5.8	4.6	11.7	8.7	15.1	2.2	4.7	2.0
Phosphatidyl ethanolamine	12.1 ± 1.0	4.0	4.5	25.3	11.6	4.6	17.6	10.2	11.8	–	14.4	–
Phosphatidyl glycerol	14.5 ± 1.2	4.8	3.6	35.5	23.8	4.1	11.5	4.1	6.1	–	11.2	–
Phosphatidic acids	19.3 ± 1.6	6.5	2.6	35.1	8.2	4.2	14.6	10.3	16.5	0.7	7.8	–
Total phospholipids	75.3 ± 6.0	25.1										

[a] Mean values ± SD from three determinations.
[b] Calculated using conversion factors (Elenkov *et al.*, 1993).
[c] Mean values with a range of < 5% for major peaks (10% FA and more) and < 10% for the others.
[d] More than one isomer presented.

The main PL is PC, followed by almost equal concentrations of PE and PG. The PA is 6.5% of total PL. Such high concentration is not typical for the leaves of the higher plants. The PA are precursors in the PL biosynthesis, but usually their concentrations in plants are low.

A high content of glycolipids (65% of total lipophylic extract) is found. The same was observed in the halophytic plant *Suaeda maritima* (Chenopodiaceae) inhabiting the same region of Bulgaria (Ivanova *et al.*, 2000). This is unusual since the increase of glycolipids and especially of MGDG leads to an increased fluidity of the cell membranes (Kuiper, 1984). Exactly the opposite was the effect of the salt for two other halophytic plants from family Chenopodiaceae – *Bassia hirsuta* and *Salicornia europaea.* With the increase of the soil salinity the total lipid content and especially the amounts of glycolipids in these plants decreased (Ivanova *et al.*, 2000). Similar lipid changes were observed during drought stress (Ivanova *et al.*, 1998) and herbicide treatment of bean plants (Ivanova *et al.*, 1999).

The fatty acid composition of the main lipid classes (Table I) is similar to those of the other higher plants except the high content of 20:1 acid in the PE and PG. The role of this acid as a precursor in the biosynthesis of polyunsaturated fatty acids was investigated but its role in the phospholipid membranes is still obscure (Gurr and James, 1975).

Analysis of sterols and triterpenoids

The data of the composition of the two groups of compounds are summarized in Table II. The sterol composition is very simple. The main sterol appears to be sitosterol (89% from the total sterols; Table II). The sterol composition is similar to that found in other *Euphorbia* sp. (Ferreira *et al.*, 1993).

The ^{1}H NMR of the total triterpenoid fraction shows that this fraction contains triterpenoids with two methyl groups at C-4 (H-3 at 3.27 ppm). There are two geminal protons from a cyclopropan ring (two doublets at 0.32 ppm and 0.55 ppm and a coupling constant 4.12 Hz). This is an indication for the presence of a cyclopropane ring, which is characteristic for the tetracyclic triterpenes of cycloartenol type. This is confirmed by the GC/MS and GC investigations, which show that the main component of the triterpene mixture is 24-methylene-9,19-cyclolanostan-3β-ol, followed by 9,19-cyclolanost-24-en-3β-ol. They both comprise 85.8% from the total triterpene fraction.

Table II. Sterol and triterpene composition of *E. peplis.*

Sterols	%
Cholesterol	1.5
24-Methyl-cholesta-5,22-dien-3β-ol	1.4
24-Methyl-cholest-5-en-3β-ol	6.1
24-Ethyl-cholesta-5,22-dien-3β-ol	2.0
24-Ethyl-cholest-5-en-3β-ol	89.0
Triterpene alcohols	**%**
Lanosterol	3.2
9,19-Cyclolanost-24-en-3β-ol	23.7
24-Methylene-9,19-cyclolanostan-3β-ol	62.1
α-Amyrine	4.5
Taraxasterol	6.5

Values obtained from three parallel analyses. The standard deviation was ± 1.5 for all compounds.

The presence of the 24-methylene group in the triterpene mixture is confirmed by the two peaks at 4.66 ppm and at 4.71 ppm. The presence of 9,19-cyclolanost-24-en-3β-ol is confirmed by the signals at 5.09 ppm and 1.54 ppm. The triterpene alcohols in *E. peplis* are found to be mainly tetracyclic ones (Table II). They also predominate in other investigated *Euphorbia* sp. (Conolly *et al.*, 1994). The main triterpenoid 24-methylene-9,19-cyclolanostan-3β-ol was found earlier in *E. soongarica* (Conolly *et al.*, 1994), but 9,19-cyclolanost-24-en-3β-ol is found for the first time in *Euphorbia* sp.

The pentacyclic triterpenes α-amyrine and taraxasterol are found in low concentrations (Table II). The pentacyclic triterpenes from the ursane group are characteristic for Euphorbiaceae (Mahato *et al.*, 1992; Conolly *et al.*, 1994).

Analysis of the volatiles

The data on the analysis of the volatiles are summarised in Table III. It is evident that the diversity and the concentrations of the volatiles are relatively low. Compared to the volatiles of other non-essential oil higher plants (Campeol *et al.*, 2001), the composition of the volatiles of *E. peplis* is pretty simple. In *E. peplis* almost half of the hydrocarbons contained a double bond, always lo-

Table III. Composition of the volatile fraction* (% of the total volatiles).

Compound	%
Hydrocarbons	*1.9*
Pentadecane	0.4
1-Hexadecene	0.4
Hexadecane	0.5
1-Octadecene	0.3
Octadecane	0.3
Acids	*6.2*
Acetic acid	0.9
Myristic acid	2.9
Palmitic acid	2.4
Aromatic compounds	*2.6*
Benzaldehyde	0.3
2-Octylbenzoate	2.3
Terpenes	*0.3*
Dihydroactinidiolide	0.3
Cl-containing compounds	*1.9*
2,2-Diethoxy-1-chloroethane	1.9

* The ion current generated depends on the characteristics of the compound and is not a true quantification.

cated at C-1. An unusual characteristic of the investigated plant is that in the hydrocarbon fraction predominated hydrocarbons with even number of carbon atoms.

As in the volatiles of other higher plants, free fatty acids were also found. They possess defensive functions and often serve as sex pheromones and allelopathic substances (Yasumoto *et al.*, 2000; Ramsewak *et al.*, 2001). They are active metabolites (Ringbom *et al.*, 2001) and are an important energetic substrate for the cells. Defensive functions might also possess 2-octylbenzoate as well as benzaldehyde.

Dihydroactinidiolide, which is a metabolite of β-carotene (Sakan *et al.*, 1967), was the only monoterpenoid identified.

The investigated volatiles contained only one halogenated compound, identified as 2,2-diethoxy-1-chloroethane. Such compounds possess variable biological activities, including antibacterial and antifungal activities and could possess defensive functions.

The identified compounds with defensive functions could explain the strong toxicity of the total lipophylic extract towards *Artemia salina* (LC$_{50}$ 6.53 ± 4.31 μg/ml; Table IV).

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Table IV. Toxicity assay of the chloroform extract from *Euphorbia peplis* on nauplius of *Artemia salina*.

Sample	deaths (%) at 1000 μg/ml	deaths (%) at 100 μg/ml	deaths (%) at 10 μg/ml	deaths (%) at 1 μg/ml	LC$_{50}$[a] + SD[b] [μg/ml]
Chloroform extract	100	90	53	23	6.53 ± 4.31
CAPE	100	100	83	63	0.45 ± 0.05

[a] Lethal concentration for 50% of the *Arthemia salina* nauplii.

[b] Mean of three measurements (10 nauplii per concentration plus control in one measurement; dead nauplii were counted).

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