Chemistry of Natural Compounds, Vol. 48, No. 1, March, 2012 [Russian original No. 1, January-February, 2012]

NEUTRAL LIPIDS, PHOSPHOLIPIDS, AND BIOLOGICAL ACTIVITY OF EXTRACTS FROM *Zygophyllum oxianum*

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UDC 547.958:665.37:576.8:57.085.2

The chemical composition of lipid extracts from aerial parts of Zygophyllum oxianum *was determined by GC and TLC. The yield of total lipids was 0.75% in leaves, 0.33% in stems, and 0.49% in fruit calculated for fresh plant mass. Phospholipids were represented by nine classes, of which phosphatidylcholine dominated (55.8% in leaves, 56.4% in stems, 63.7% in fruit of total PL). The n-BuOH extract from the plant aerial part exhibited noticeable antifungal activity. The $CHCl_3$:MeOH (2:1) extract from leaves and stems exhibited pronounced in vitro cytotoxicity against human bladder carcinoma cell line 5637 with IC_{50} 6.2 μg/mL.*

Keywords: *Zygophyllum oxianum*, neutral lipids, phospholipids, fatty-acid composition, antibacterial activity and cytotoxicity.

The plant *Zygophyllum oxianum* Boriss. (Zygophyllaceae) is widely distributed in Uzbekistan and is used in folk medicine as an agent against rheumatism and diabetes.

In continuation of our research on the chemical composition of plants of this genus [1, 2], we studied for the first time the lipid composition including neutral lipids (NLs), phospholipids (PLs), and fatty acids of total lipids from aerial organs of *Z. oxianum*. Several extracts were checked for antibacterial activity and cytotoxicity.

The aerial part of the plant was collected during mass fruiting. Table 1 presents the yield of organs from the aerial part and their moisture content.

Leaves, stems, and fruit of freshly collected plants were frozen by liquid N_2 and ground in that form in order to preserve native substances [3, 4]. Frozen and ground material was treated with acetone in order to neutralize phospholipase D and dehydrate the plant organs. Dehydrated material was worked up by the Folch method [5]. Table 1 presents the yield of pulp per organ after work up by all solvents.

The acetone and $CHCl_3$:MeOH extracts were evaporated and combined (Σ_{ext}). The combined extracts were washed with H_2O to afford the total lipids (Σ_l), which were separated over a column of SiO_2 into NL, glycolipids (GL), and PL by the literature method [6] (Table 2).

The content of NL in leaves was 0.37%, which was nine times greater than in stems and four times greater than in fruit (Table 2).

The NL contained 10 classes of compounds, 6 of which were identified by qualitative reactions, chromatographic mobility, and literature data [6, 7] (Table 3).

Hydrocarbons, triterpenol and sterol esters, triacylglycerols, free fatty acids, and sterols were common to all extracts. Chlorophyll pigments (37.3% of total NLs), free fatty acids (24.9%), triterpenol and sterol esters (16.6%), and sterols (12.4%) dominated in the leaves. Hydrocarbons, triterpenol and sterol esters, and free fatty acids dominated in stems (85.5% total sum).

Fruit was dominated by triterpenol and sterol esters, triacylglycerols, free fatty acids, hydrocarbons, and sterols (96.5% total sum) (Table 3).

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0009-3130/12/4801-0011

TABLE 1. Moisture and Yield of Organs and Pulp from the Aerial Part of *Zygophyllum oxianum*

Organ	Yield of organ from aer. part, %	Moisture of organ, %	Yield of pulp per organ, %
Leaves	45.2	87.2	11.7
Stems	39.2	77.3	22.2
Fruit	15.6	92.5	6.9

TABLE 2. Yield of Extracts and Separate Lipid Classes from Aerial Organs of *Zygophyllum oxianum*

Organ	Yield per native organ, %					Yield of lipids, % of organ total lipids		
	Σ_{ext}	Σ_l	NL	GL	PL	NL	GL	PL
Leaves	1.06	0.77	0.37	0.35	0.05	49.3	46.7	4.0
Stems	0.54	0.33	0.04	0.22	0.07	12.1	66.7	21.2
Fruit	0.63	0.49	0.09	0.32	0.08	18.4	65.3	16.3

TABLE 3. Composition of Neutral Lipids from *Zygophyllum oxianum*, %

Neutral lipids	R_f (system 1)	Leaves	Stems	Fruit
Hydrocarbons	0.95	3.4	47.0	16.1
Triterpenol and sterol esters	0.90	16.6	25.6	26.8
Unident.	0.81	3.1	–	–
Unident.	0.65	0.4	–	–
Unident.	0.53	0.2	–	–
Triacylglycerols	0.38	1.7	0.8	21.5
Unident.	0.21	Tr.	0.9	2.4
Free fatty acids	0.18	24.9	12.9	21.4
Sterols	0.09	12.4	8.5	10.7
Chlorophylls and associated substances	0.05	37.3	4.3	1.1

TABLE 4. Composition of Phospholipids in Organs of *Zygophyllum oxianum*, %

Phospholipids	R_f, system		Leaves	Stems	Fruit
	3	4			
Phosphatidylcholine	0.44	0.27	55.8	56.4	63.7
Phosphatidylinositol	0.25	0.22	7.5	8.2	8.4
Phosphatidylethanolamine	0.53	0.53	6.0	28.0	23.1
Phosphatidylglycerol	0.46	0.49	20.5	1.2	2.1
Phosphatidic acid	0.22	0.45	1.2	6.2	0.9
Diphosphatidylglycerol	0.55	0.74	4.7	Tr.	1.4
Unident.	0.29	0.47	2.0	–	–
Unident.	0.44	0.50	1.5	–	–
Unident.	0.48	0.49	0.8	–	–

Leaves contained 0.13% chlorophylls and associated substances per raw mass; stems and fruit, 0.01% and 0.005%, respectively.

GLs represented the principal part of total lipids in stems (66.7%) and fruit (65.3%). Their content in leaves (46.7%) was almost equal to that of NLs. Monogalactosyldiglycerides, digalactosyldiglycerides, sterolglycosides, and their esters were observed in organs of *Z. oxianum* according to qualitative reactions, chromatographic mobility, and literature data [8, 9].

PLs made up 21.2% of total lipids in stems; 16.3%, in fruit; 4.0%, in leaves (Table 2). Nine classes of PLs, six of which were identified by qualitative reactions, chromatographic mobility, and literature data [6, 10] were observed in the extracts (Table 4).

TABLE 5. Composition of Fatty Acids of Total Lipids from *Zygophyllum oxianum*, %, GC

Acid	Leaves	Stems	Fruit	RT (vs. 16:0)	Acid	Leaves	Stems	Fruit	RT (vs. 16:0)
12:0	1.2	0.2	1.0	0.250	18:2	6.5	5.3	31.0	2.875
14:0	2.9	1.1	1.4	0.563	Unident.	3.3	2.8	0.8	3.500
16:0	56.4	49.1	19.8	1	18:3	7.8	–	11.9	4.000
16:1	5.3	Tr.	0.8	1.188	$\sum_{sat}$	66.9	67.4	27.8	
17:0	–	–	2.4	1.500	$\sum_{unsat}$	29.8	29.8	71.4	
18:0	6.4	17.0	3.2	1.875	$\sum_{unident}$	3.3	2.8	0.8	
18:1	10.2	24.5	27.7	2.250					

RT is relative retention time. Tr.: traces.

TABLE 6. Biological Activity of Various Extracts from *Zygophyllum oxianum*

Sample	Diameter of inhibition zone, mm				IC_{50} of 5637 cells, μg/mL
	Staphylococcus aureus	*Bacillus subtilis*	*Escherichia coli*	*Candida maltosa*	
Extract 1	8	6	–	–	6.2
Extract 2	–	–	–	–	37.6
Extract 3	–	5	4	20	47.8
Ampicillin	26	28			
Gentamicin			15		
Nystatin				25	
Etoposide					0.31 (0.54 μM)

Dash (–) means that the sample was inactive.

The principal PLs in leaves were phosphatidylcholine (55.8%) and phosphatidylglycerol (20.5%). The main ones in stems and fruit were phosphatidylcholine and phosphatidylethanolamine, the totals of which were 84.4% in stems and 86.8% in fruit. The phosphatidylinositol contents in the organs were similar to each other.

The fatty-acid composition of total lipids from *Z. oxianum* was determined (Table 5).

The saturated fatty-acid contents in stems and leaves were more than two times greater than that in fruit. The principal saturated fatty acid was palmitic acid. The principal unsaturated fatty acids of fruit were linoleic, oleic, and linolenic acids (total 70.6%).

Several studies collected in a handbook [11] reported small quantities of margaric ($C_{17:0}$) and hexadecadienoic ($C_{16:2}$) acids. The retention times of hexadecadienoic and margaric acids under our conditions and according to the literature [12] are similar to each other. The Kaufman method [13] in which the activity of bromine is insufficient to displace H was used to brominate the double bonds of methyl esters of fatty acids obtained from total lipids of fruit. The methyl esters of saturated fatty acids were separated from the bromo derivatives using TLC. GC analysis showed that the methyl ester of the fatty acid with retention time 1.500 remained unchanged. Therefore, total lipids of *Z. oxianum* fruit contained margaric acid ($C_{17:0}$).

Biological tests of the $CHCl_3$:MeOH (2:1) extract from leaves and stems (extract 1) found pronounced cytotoxicity against human bladder carcinoma cell line 5637 with IC_{50} 6.2 μg/mL but weak antibacterial and antifungal activity. The $CHCl_3$:MeOH (2:1) extract from fruit (extract 2) was less cytotoxic (IC_{50} 37.6 μg/mL) and did not exhibit antibacterial and antifungal activity. The *n*-BuOH extract from whole air-dried aerial organs (extract 3) was used for a comparative study. Its IC_{50} value was 47.8 μg/mL. It displayed high antifungal activity against *Candida maltosa* but weak antibacterial properties against the studied strains (Table 6).

In summary, it is noteworthy that the contents of NLs in leaves, stems, and fruit and their qualitative and quantitative compositions were studied in *Z. oxianum* during mass fruiting. Leaves contained nine times more NLs than stems and four times more than fruit. Hydrocarbons, triterpenol and sterol esters, triacylglycerols, free fatty acids, and sterols were common to all NLs from aerial organs. Chlorophyll pigments dominated the leaves (37.3% of total NLs and 0.13% of raw leaf mass).

The yield of PLs from stems was 21.2% of total lipids; 16.3%, from fruit; 4.0%, from leaves. The principal PLs in leaves were phosphatidylcholine (55.8%) and phosphatidylglycerol (20.5%); in stems and fruit, phosphatidylcholine and phosphatidylethanolamine, the totals of which were 84.4% and 86.8%, respectively.

The fatty-acid compositions of total lipids were determined. Fruit contained over two times more unsaturated fatty acids, the principal ones of which were linoleic, oleic, and linolenic acids. Saturated acids dominated in leaves and stems mainly due to palmitic acid.

Biological screening results found pronounced antifungal activity and cytotoxicity for separate extracts from the plant aerial organs.

EXPERIMENTAL

Plant Material and Extraction. Leaves, stems, and fruit were collected in July 2009 in the vicinity of Khalkabad (Tashkent Oblast, Rep. Uzb.) during mass fruiting. The plant was identified taxonomically by Cand. Biol. Sci. A. M. Nigmatullaev. A specimen (No. 1063) is preserved in the Herbarium of the Institute of the Chemistry of Plant Substances, AS, RU. Organs of *Z. oxianum* were worked up with liquid N_2 and ground while frozen. Cold ground material was transferred into a vessel with acetone in a 1:3 ratio (v/v). The first extraction with acetone was carried out for 15 min; the second, 30 min, both times with periodic agitation. An aliquot was taken from the acetone extract and evaporated at 40°C in vacuo in a rotary evaporator. The aqueous phase was separated and worked up with $CHCl_3$ (3×) in a separatory funnel. The $CHCl_3$ solution was returned to the flask with the acetone residual. The aqueous phase was discarded. The pulp was extracted with $CHCl_3$:MeOH (2:1, 3×) in a 1:3 ratio (v/v) for 1 h with periodic shaking.

An aliquot of the $CHCl_3$:MeOH extract was taken. Aliquots of the acetone and $CHCl_3$:MeOH extracts were combined and evaporated to dryness in order to determine the yields of total extracted substances. The $CHCl_3$:MeOH extract was evaporated. The aqueous phase was separated and worked up the same as for the acetone extract. The acetone and $CHCl_3$:MeOH extracts in $CHCl_3$ were combined and washed (3×) with distilled H_2O to remove water-soluble compounds and produce the total lipids. Total lipids from each organ were placed onto separate columns of SiO_2. NLs were eluted with $CHCl_3$; GLs, acetone; PLs, MeOH by the literature method [6].

Air-dried aerial plant organs were extracted with MeOH (5×). The resulting extracts were combined, evaporated to a syrupy consistency, and diluted with H_2O. The aqueous part was extracted with *n*-BuOH (5×). The yield of the BuOH extract was 5.1% (relative to air-dried raw material) (extract 3).

Lipid Analysis. The qualitative composition of NLs in total lipids was determined on Silufol plates using hexane:Et_2O:HOAc (90:10:1) (1) and 80:20:1 (2). Plates were sprayed with phosphotungstic acid in EtOH and with H_2SO_4 (50%) [14]. Standards of sunflower oil triacylglycerols, free fatty acids, β-sitosterol, paraffin, methyl esters of fatty acids, and total carrot carotinoids were used. The quantitative composition was determined gravimetrically after preparative TLC on KSK SiO_2 using systems 1 and 2.

The qualitative composition of PLs was established on KSK SiO_2 plates after two-dimensional chromatography [14–16] using systems $CHCl_3$:MeOH:NH_4OH (10:5:2, 3) and $CHCl_3$:MeOH:AcOH:H_2O (14:5:1:1, 4). Four detectors were used sequentially for accurate localization and identification of PLs. These were ninhydrin (free amine), Dragendorff's reagent (quaternary N), Vaskovsky–Svetashev reagent (PLs), and H_2SO_4 (50%, general detector) [17]. The quantitative composition of PLs was determined gravimetrically after preparative TLC using the same solvent systems; the fatty-acid composition, using GC of methyl esters after alkaline hydrolysis. The completeness of the methylation of fatty acids was monitored using TLC. The chromatography conditions were Chrom-4, glass column (2.5 m × 3 mm), packed with Reoplex 5% on Inerton support (0.16–0.20 mesh), column temperature 190°C, vaporizer 280°C, N_2 carrier gas, flame-ionization detector. The standards were methyl esters of fatty acids 12:0, 14:0, 16:0, 17:0, 18:0, 20:0 (3.75, relative retention time), and 22:0 (7.625).

Double bonds of fatty acid methyl esters were brominated using the Kaufman method [13]. Bromo derivatives of fatty acid methyl esters were separated from saturated fatty acid methyl esters on TLC using systems 1 and 2.

Determination of Antibacterial Activity. Activity against *Staphylococcus aureus* (ATCC 29213), *Bacillus subtilis* (ATCC 6059), *Escherichia coli* (ATCC 25922), and one fungal strain *Candida maltosa* (SBUG 17) was determined using a modified agar-diffusion method [18]. The SBUG strain was obtained from a collection at the Institute of Microbiology (SBUG), University of Greifswald, Germany.

A suspension of bacterial cells was prepared from a 1-day subculture of the corresponding strain with 1×10^6 colonies per 1 mL. Sterile nutrient agar (Immunpraparate, Berlin, Germany, 25 g agar/L distilled H_2O) was inoculated with bacterial cells (200 μL of bacterial cells in 2 mL of 0.9% NaCl suspension and 20 mL of medium) and poured into Petri dishes to produce the solid phase. *C. maltosa* (1×10^5 CFU/mL) was inoculated into sterile Mueller–Hinton agar (Becton Dickinson,

Heidelberg) according to DIN E 58940-3 for agar disk-diffusion methods [19]. Test materials (40 μL, equivalent to 2 mg for extracts and/or 0.2 mg of pure compound) were dissolved in solvents that were used for the extraction and placed on sterile paper disks (6-mm diameter, Schleicher and Schuell, Germany, ref. No. 321860). Ampicillin, gentamicin sulphate, and nystatin were used as positive controls; solvents, as negative controls. Solvents were evaporated in a stream of air at room temperature. Disks were deposited on the surface of inoculated agar dishes. Then, the dishes were stored for 3 h in a refrigerator for pre-diffusion of the compounds in the agar. Dishes with bacteria were incubated at 37°C for 24 h; with fungi, for 48 h at 26°C. The inhibition zone (including the disk diameter) was measured and recorded after the incubation time. Average inhibition values were calculated after repetitions in triplicate.

Determination of Cytotoxic Activity against Human Tumor Cells. Cytotoxicity was measured by the neutral-red method [20] using human bladder carcinoma cell line 5637. Only viable cells were capable of reacting and accumulating neutral-red. Human bladder carcinoma cell line 5637 (ATCC HTB-9) was cultivated as a monolayer on RPMI medium (Lonza, Verviers, Belgium) containing fetal bovine serum (10%, Sigma, Deisenhofen, Germany) and penicillin/streptomycin solution (1%, Penicillin 10,000 IE/mL; Streptomycin 10,000 μg/mL, Biochrom AG, Berlin, Germany). Cells were grown at 37°C in 95% humidity and 5% CO_2 and were subcultured twice per week using trypsin/EDTA (0.05%/0.02%, Lonza).

Cells (5637) were seeded at density 3×10^4 cells/mL in 96-well plates (TPP, Trasadingen, Switzerland) for the analysis. After 24 h, nutrient medium was replaced by a medium containing a certain amount of tested extracts and a toxic control (Etoposide). Plates were stored at 37°C for 72 h, rinsed twice with HBSS, and incubated in freshly prepared neutral-red in RPMI (3.3 μg/mL) in an incubator for 3 h. The supernatant was removed. The wells were thoroughly rinsed. Neutral-red was dissolved in acidic EtOH and shaken for 45 min. Absorption was determined at 540 nm. Etoposide was used as a toxic control; cell-culture medium, as a non-toxic control. Results were recalculated in percent of the control. All experiments were performed twice with six repetitions for each checked concentration.

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