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LIPIDS OF SOME *Lycopodiophyta* SPECIES

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The fractional composition of neutral and polar lipids from four Lycopodiophyta *species was studied. It has been established that the neutral lipids consist of esters of glycerin, alcohols, fatty acids, sterols, waxes, and hydrocarbons. The polar lipids contain glycolipids, phospholipids, and the betaine lipid of DGTS.*

Key words: *Lycopodiophyta*, neutral and polar lipids, phospholipids, glycolipids, fatty acids, hydrocarbons, sterols.

Lycopodiales include some of the most ancient higher plants [1]. They flourished during the late Paleozoic era and are now represented by a comparatively small number of genera and species. However, they play an important role in the origin and evolution of currently existing flora [2, 3]. Club mosses can be found in the modern pharmacopoeia of many countries. They are used to cure various human and animal illnesses [4]. The chemical composition of club mosses is rather well known [5-9]. However, the lipids require further study.

We investigated lipids of several representatives of this ancient plant group because the new data can provide the key to understanding the biochemical processes that occurred in the past and can unravel the evolutionary paths on the chemical and biochemical levels.

We investigated lipids of four lycopodiale species from different geographical regions of Russia: *Diphasiastrum complanatum* (L.) Holub, *Huperzia selago* (L.) Bernh. ex Schranket, *Lycopodium annotinum* L., and *Selaginella selaginoides* (L.) Link.

Samples of *H. selago* and *L. annotinum* were collected in Odintsovo region of Moscow district in June 1999; *L. annotinum*, in Beloretsk region of Bashkiria in September 2000; and *L. complanatum*, *H. selago*, and *S. selaginoides*, in Loukh region of Karelia in August 1999. For *H. selago*, we collected separately vegetative runners and runners with unopened sporangia; for *D. complanatum*, vegetative runners and runners with spikelets; for *L. annotinum*, vegetative runners, runners with spikelets, and spikelets; for *S. selaginoides*, vegetative runners and runners with spikelets. The content of total lipids and the qualitative and quantitative composition of neutral and polar lipids were determined in the studied samples.

The content of total lipids (TL) is 4.0-20.0% of the dry mass (Table 1). Lipids were separated into separate classes using column chromatography over silica gel. A large part of the TL consisted of neutral lipids (NL), with the exception of *D. complanatum*, in which the content of NL was 43.5% of the TL.

Polar and neutral lipids were identified separately. The NL fraction gave 10 spots, which we denoted 1-10 and identified using TLC, physicochemical analysis, and chemical transformations (Fig. 1). Thus, saponifiable components (spots 1-3, 7, 8, and 9) are found after mild alkaline hydrolysis. These spots were absent in the mixture of NL after hydrolysis. The IR spectra of each of the products corresponding to these spots contained absorption bands at 1735 and 1160 cm^{-1} (ester carboxyl). Spots 1-3 were assigned to mono- and disubstituted glycerin esters (the IR spectrum of these products contains an absorption band at 3620 cm^{-1}, corresponding to an associated hydroxyl, in addition to the ester); spot 7, to triacylglycerins. The mobility of spot 7 (R_f 0.83) corresponds to that of tripalmitoglycerin (R_f 0.85). For the free fatty acids (spot 6), the R_f value (0.73) coincides with that of stearic acid (0.76). The IR spectrum of this fraction contains an absorption band at 1750 cm^{-1} (carbonyl). The R_f value of spot 5 (0.59) corresponds to that of heptadecanoic alcohol (0.59). The IR spectrum of the compounds in this region of the chromatogram exhibits an absorption band at 3620 cm^{-1}, which is consistent with the presence of a hydroxyl group. The compounds from spot 5 were isolated, purified twice, and analyzed by chromatography—mass spectrometry to give β-sitosterol and campasterol in a 2:1 ratio. This is consistent with the literature [5].

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TABLE 1. Total Lipid Content and Ratio of Lipid Classes of *Lycopodiophyta* (% of Total Lipids)

No.	Species	Total lipids, mg/g	Neutral lipids	Glycolipids	Phospholipids	DGTS
1*	*Diphasiastrum complanatum* (vegetative runners)	39.9+0.3	43.5	28.8	23.5	4.1
2**	*D. complanatum* (runners with spikelets)	87.2+0.1	62.4	15.1	17.7	4.8
3*	*Huperzia selago* (vegetative runners*)	204.2+0.6	53.3	26.5	16.8	3.4
4**	*H. selago* (vegetative runners**)	89.8+0.4	69.2	14.4	12.5	3.9
5*	*H. selago* (runners with sporangia)	87.4+0.1	55.4	23.8	17.4	3.4
6*	*Lycopodium annotinum* (runners with spikelets)	122.6+8.0	68.1	18.2	12.5	1.2
7***	*L. annotinum* (vegetative runners)	72.5+0.8	75.1	13.3	7.4	3.7
8***	*L. annotinum* (spikelets)	202.8+10.6	76.5	9.9	8.4	5.2
9**	*Selaginella selaginoides* (vegetative runners)	67.1+13.9	61.8	17.0	16.2	5.0
10**	*S. selaginoides* (runners with spikelets)	140.9+14.0	72.9	13.6	10.7	2.8

Samples were collected: *in Zvenigorod region, Moscow district; **in Loukh region, Karelia; ***in Beloretsk region, Bashkiria.

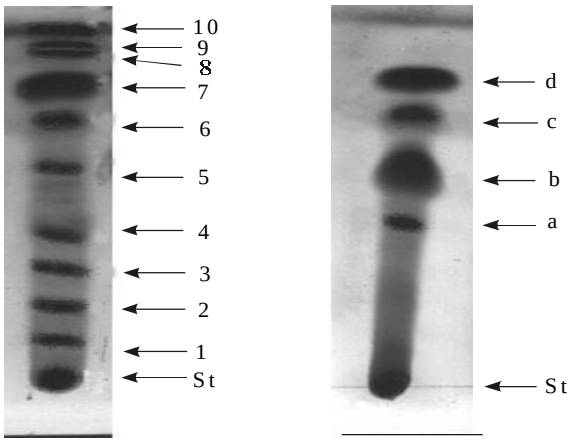

Fig. 1. One-dimensional thin-layer chromatogram of neutral lipids isolated from *Lycopodium complanatum*. Line **A**: Origin (St); monoacylglycerols (1), diacylglycerols (2, 3), free sterols (4), alcohols (5), free fatty acids (6), triacylglycerols (7), sterol esters (8), waxes (9), hydrocarbons (10). Line **B**: sitosterol (a), heptadecanoic alcohol (b), stearic acid (c), tripalmitoglycerol (d).

The results indicate that NL of *Lycopodiophyta* samples collected in various habitats contain hydrocarbons, waxes, alcohols, sterols and their esters, free fatty acids, higher alcohols, and mono-, di-, and trisubstituted esters of glycerin. The ratios of these components are listed in Table 2.

TABLE 2. Neutral Lipid Composition of *Lycopodiophyta* (% of Total Neutral Lipids)

No.	Species	MAG	DAG	TAG	FFA	FS	SE	AL	W	HC
1*	*Diphasiastrum complanatum* (vegetative runners)	13.8	9.4	20.7	Tr.	9.2	12.2	11.8	12.2	10.7
2**	*D. complanatum* (runners with spikelets)	6.5	18.2	35.4	13.2	5.7	5.0	9.3	4.0	2.6
3*	*Huperzia selago* (vegetative runners*)	18.4	29.4	14.6	12.8	5.0	7.6	4.6	1.0	6.6
4**	*H. selago* (vegetative runners**)	9.1	20.4	15.8	15.3	5.5	12.6	13.2	6.5	1.6
5*	*H. selago* (runners with sporangia)	7.1	20.4	18.7	11.5	6.1	15.0	15.2	2.5	3.4
6*	*Lycopodium annotinum* (runners with spikelets*)	1.8	11.1	24.6	18.2	8.3	12.1	11.0	9.6	3.3
7***	*L. annotinum* (vegetative runners)	3.1	10.3	21.5	19.9	7.2	5.6	10.3	10.8	11.3
8***	*L. annotinum* (spikelets)	2.1	10.5	30.8	22.8	10.0	Tr.	7.3	7.3	9.1
9**	*Selaginella selaginoides* (vegetative runners)	4.9	11.4	30.6	15.7	6.8	12.6	6.0	7.4	4.5
10**	*S. selaginoides* (runners with spikelets)	6.4	12.5	30.9	12.6	11.3	1.5	12.6	8.2	4.0

Samples were collected: *in Zvenigorod region, Moscow district; **in Loukh region, Karelia; ***in Beloretsk region, Bashkiria. MAG, monoacylglycerol; DAG, diacylglycerol; TAG, triacylglycerol; FFA, free fatty acids; FS, free sterols; SE, sterol esters; AL, alcohols; W, waxes; HC, hydrocarbons.

The total content of glycerin esters varies from 37.5 (*L. annotinum*) to 62.3% (*H. selago*) of the total NL. The content of fatty acids is rather high (11.5 to 22.8%) in certain species. However, only traces of fatty acids were found in *D. complanatum*. Sterols occur in the studied samples as both free (FS) and esterified (SE) forms.

The amount of NL and the ratio between individual components vary markedly and depend on the species and habitat (Table 2). This can be seen using mono-, di-, and trisubstituted glycerin esters as examples. Thus, the total amount of glycerides in vegetative runners of *H. selago* is 62.4% of the TL for samples collected in Moscow district (3) and 45.3% for those from Karelia (4). In both these samples, diglycerides represent a large fraction (20.4-29.4%) of the glycerin esters. However, *L. annotinum* has a noticeably lower total amount of glycerides (36.7-34.9%), in which triglycerides dominate.

The polar lipids (PL) consist of 11 components (Fig. 2). Three of these are galactolipids (spots 7, 8, and 10): monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG), and sulfoquinovosyldiacylglycerol (SQDG). The P-containing lipids include phosphatidylglycerol (PG), phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidic acid (PA), and diphosphatidylglycerol (DPG) (spots 1-6). Table 3 lists the ratios between phospholipids (PhL) measured from the P content in them. The lipid that lacks P but contains a trimethylammonium group, like PC, is the betaine lipid diacylglyceryl-N,N,N-trimethylhomoserine (DGTS, spot 11). Its chemical structure was established after isolation and purification. The IR spectrum contains the following bands: 3000, 2960, 2920, 1390 (CH_3- and CH_2-groups); 1740, 1450 (CH_3–COO); 1580 (COO–); 1370, 1030 (C–N); and 960 cm^{-1} [N–$(CH_3)_3$]. The PMR spectrum has signals at 0.87-0.89 (CH_3), 1.23-1.34 (–CH_2–), 1.62-1.82 (–CH_2–C–O–), 3.2 (CH_3–N), 3.72-3.75 (CH_2–O), 4.29 (CH–O–), and 5.31 ppm (N–CH–COO). These properties are consistent with the DGTS structure being 1,2-diacylglycero-O-(N,N,N-trimethyl)homoserine.

The total PL make up 24.4-56.5% of the TL (Table 3). The glycolipid (GL) content is rather low compared with many higher plants [10], although it is higher than that of PhL in both vegetative and spore-containing runners. In all studied samples, MGDG dominates among the GL, except for *S. selaginoides*. SQDG is present in a smaller amount and is rather widely distributed in photosynthetic organisms.

TABLE 3. Composition of Glyco- and Phospholipids of *Lycopodiophyta* (% of Total Polar Lipids)

No.	Species	MGDG	DGDG	SQDG	PC	PE	PG	PI	PA	DPG
1*	*Diphasiastrum complanatum* (vegetative runners)	43.8	33.2	22.9	41.5	22.9	16.6	14.5	3.9	0.6
2**	*D. complanatum* (runners with spikelets)	44.5	27.8	27.7	45.5	11.7	14.7	20.5	4.4	3.2
3*	*Huperzia selago* (vegetative runners*)	48.5	36.4	15.1	28.6	14.7	20.0	21.2	15.5	Tr.
4**	*H. selago* (vegetative runners**)	45.1	39.0	15.9	29.2	13.5	15.5	22.7	9.8	9.2
5*	*H. selago* (runners with sporangia)	43.0	33.0	24.0	26.6	15.7	17.5	26.6	9.5	4.1
6*	*Lycopodium annotinum* (runners with spikelets)	42.2	35.8	21.9	30.9	15.7	25.3	19.7	8.4	Tr.
7***	*L. annotinum* (vegetative runners)	42.4	35.2	22.4	29.2	14.5	26.1	19.0	11.2	Tr.
8***	*L. annotinum* (spikelets)	53.6	24.5	21.9	44.8	10.1	14.0	16.5	14.6	Tr.
9**	*Selaginella selaginoides* (vegetative runners)	39.3	45.0	15.7	39.8	22.4	11.0	14.2	6.1	6.5
10*	*S. selaginoides* (runners with spikelets)	32.9	47.0	20.1	55.6	14.4	12.5	11.5	2.1	3.8

MGDG, monogalactosyldiacylglycerol; DGDG, digalactosyldiacylglycerol; SQDG, sulfoquinovosyldiacylglycerol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PA, phosphatidic acid; DPG, diphosphatidylglycerol.

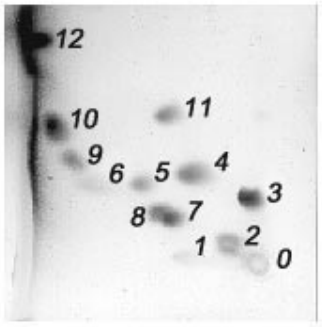

Fig. 2. Two-dimensional thin-layer chromatogram of polar lipids isolated from *Lycopodium complanatum*. Origin (O); phosphatidic acid (1), phosphatidylinositol (2), phosphatidylcholine (3), phosphatidylethanolamine (4), phosphatidylglycerol (5), diphosphatidylglycerol (6), sulfaquinovosyldiacylglycerol (7), digalactosyldiacylglycerol (8), unidentified lipid (9), monogalactosyldiacylglycerol (10), diacylglyceryl-N,N,N-trimethylhomoserine (11), neutral lipids (12).

PC makes the largest contribution to the PhL. This trend persists in vegetative runners collected in various regions

and in spore-containing runners. However, the PC content in *H. selago* is slightly lower (28.6-29.2%) compared with the other plant samples.

The betaine lipid DGTS was observed in all studied plants. This lipid is exclusively of plant origin but is observed in a limited number of specimens [11]. In particular, it was observed in lower plants, including algae, and cryptogams. However, it was not isolated in seeds and flowers of higher plants [12, 13]. The maximal content in the lycopodiales that we studied occurred in vegetative runners of *S. selaginoides* (5% of the TL) and in spikelets of *L. annotinum* (5.2% of the TL) (Table 1). Various types of cells in a single plant are capable of synthesizing DGTS. Thus, the DGTS content in vegetative runners and spikelets of *L. annotinum* collected at the start of summer in Moscow district (sample 6) was 1.2% of the TL. A separate analysis for DGTS content in vegetative runners and spikelets on runners from this same species collected in Bashkiria at the end of summer gave 3.7% (sample 7) and 5.2% (sample 8), respectively, of the TL. This is more than three times the content of this lipid in sample 3. These results indicate that the amount of DGTS varies over wide ranges depending on the plant species, collection time, and habitat.

Thus, data on the lipid composition of Lycopodiales was obtained. The qualitative composition of NL, GL, and PhL of the studied club mosses is typical of photosynthetic plants. The presence of DGTS is characteristic of this plant group. Its content differs quantitatively between species and within the same species.

EXPERIMENTAL

IR spectra were recorded on a Specord M-82 IR spectrophotometer (Germany) as a thin layer on KBr plates. ^{1}H NMR were recorded on a Bruker AM-300 instrument (Germany) at working frequencies 300 and 75.47 MHz with TMS internal standard and $CDCl_3$ solvent.

GC of sterols was carried out on a Chrom-5 chromatograph (DIP, column 1.2 m × 3 mm, 5% SE-20 on Inerton Supper, temperature programmed 200-300°C, He carrier gas, 40 mL min^{-1}).

Extraction and Chromatography of Lipids. Collected samples were treated with $CHCl_3$—CH_3OH (1:1), tightly stoppered, and transported to the analysis site. Samples were ground under laboratory conditions using a high-speed homogenizer. Lipids were extracted as before [14]. The lipid extracts were separated by column chromatography over silica gel (Chemapol, Czech Rep.) (100-160 mesh) by the literature method [15] into NL, GL, and PhL. The lipids were separated into individual classes using TLC on microplates (6×6 cm or 10×10 cm) with a fixed layer of silica gel (Haapsalu, Estonia). NL were separated using one-dimensional chromatography with successive use of the solvent systems: toluene—hexane—formic acid (70:30:0.5) (1) and hexane—diethylether—formic acid (60:40:1) (2). GL were separated using acetone—benzene—water (91:30:8); PhL, chloroform—methanol—benzene—ammonia (130:60:20:12) in the first direction and chloroform—methanol—benzene—acetone—acetic acid (140:60:20:10:8) in the second.

Lipid Analysis. NL were identified using TLC data by comparing R_f values with the corresponding values of standards (Fig. 1, lines **A** and **B**) (stearic acid, tripalmitoglycerol, β-sitosterol, and heptadecanoic alcohol were obtained from Sigma).

Mild alkaline hydrolysis was performed by reacting lipids (10-20 mg) with aqueous KOH (10%) [15].

PL were identified using known methods and specific reagents (Fig. 2). Thus, molybdenum blue was used to identify P-containing lipids (spots 1-6); Dragendorff's solution, lipids containing the trimethylammonium group (spots 3 and 11). Spot 4 gave a characteristic color with ninhydrin; spots 7, 8, and 10, a positive reaction with an anthrone reagent.

The amount of NL was determined by the method of Kabara and Chen [16]; of GL, from the galactose content with anthrone reagent [17]; PL, by the Vaskovsky method [18].

The amount of DGTS was calculated spectrophotometrically after ashing in H_2SO_4. A calibration curve was constructed using known amounts of isolated and purified DGTS.

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