Phytochemical and pharmacological assessment of Hungarian bryophytes, with special focus on *Paraleucobryum longifolium*

Summary of PhD. Thesis

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Szeged, Hungary

2023

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Szeged
2023

1 INTRODUCTION

From the plant kingdom, vascular plants are the most thoroughly explored taxon from a phytochemical and pharmacological point of view, but bryophytes, taxonomically placed between the algae and the pteridophytes and belonging to the non-vascular plants, are less well studied. The fact that bryophytes are not damaged by insects, microorganisms, slugs, snails and mammals suggests that these plants contain bioactive secondary metabolites that have toxic or repellent effect against other species. However, the pharmacological profiles of the majority of species are undisclosed, intensive chemical and pharmacological studies have been performed only in the last few decades.

More than 20,000 species belong to bryophytes, comprising Marchantiophyta (liverworts, ~6000 species), Bryophyta (mosses, ~14,000 species), and Anthocerotophyta (hornworts, ~300 species), can be found everywhere in the world except in the sea. The Hungarian flora contains 659 species, with the predominance of mosses (2 hornworts, 146 liverworts, 511 mosses).

From phytochemical and pharmacological point of view, bryophytes are poorly explored because of the difficulties of their collection. Still high numbers of new compounds were discovered from mosses, including more than 40 new carbon skeletons of terpenoids and phenolic compounds. Mono-, sesqui-, di-, and triterpenes, flavonoids, bibenzyls, acetogenins are the most common types of secondary metabolites of bryophytes. These compounds show interesting biological activities, such as insecticide, insect antifeedant, cytotoxic, pesticide, muscle relaxant, plant growth regulator, anti-HIV, DNA polymerase β inhibitor, anti-obesity, neurotrophic, antioxidant, NO production inhibitor, antimicrobial, and antifungal activity.

Form a therapeutic point of view, the most perspective bioactivities of bryophytes are the anticancer and antibacterial effects. Several bryophyte crude extracts and isolated compounds were tested for cytotoxic activity on various cancer cell lines. Terpenoids and bibenzyls seems to be the most potent cytotoxic compounds as they may induce apoptosis by activating a number of genes and enzymes, however, the exact mechanism of action is still unknown. DNA fragmentation, nuclear condensation, activation of caspases, inhibition of anti-apoptotic nuclear transcriptional factor-kappaB, activation of p38 mitogen-activated protein kinase may play a role in apoptotic mechanism.

Liverworts are chemically different from mosses and hornworts because of the lack of oil bodies in the last two classes. In liverworts, the most common secondary metabolites are terpenoids, especially sesqui- and diterpenoids, from which more than 1600 compounds have

been isolated over 40 years, however such compounds can be found in some mosses, including *Mnium, Plagiomnium, Homalia, Plagiothecium* and *Taxiphyllum* species and in hornworts includes *Anthoceros* species.

The first report of the antimicrobial effect of bryophytes was in published in 1942. In 1952 Madsen and Potes reported the antimicrobial effect of *Sphagnum portoricense*, *Sphagnum strictum, Conocephalum conicum* and *Dumortiera hirsuta*. In 1959, an examination where 12 species of bryophytes were tested showed the remarkable antibacterial effect of *Anomodon rostratus*, *Orthotrichum rupestre* and *Mnium cuspidatum*. A comprehensive study was published in 1979, where 52 species of bryophytes were tested on 8 bacterial strains; 56% of the tested species were active against at least one of the test bacteria.

However, most of the Hungarian bryophytes have not been examined phytochemically and pharmacologically. These species might be considered as an undiscovered and so far neglected corner of the (phyto)chemical space.

2 AIM OF THE STUDY

Despite the widespread etnomedicial use of bryophytes, poor pharmacological and chemical datas are presented in the literature. Our aim was to discover the chemical composition and pharmacological properties of selected bryophytes from the Carpathian Basin. To fulfill this aim, the following tasks were performed:

- phytochemical screening of selected bryophytes to identify the ones with the most potent antiproliferative and antimicrobial effects,
- the identification of possible secondary metabolites by HRESIMS of the most potent species from screening,
- isolation of compounds from *Paraleucobryum longifolium* using various chromatographic technics (CC, VLC, GFC, FC, PLC, HPLC),
- elucidation the structures of the isolated compounds by different spectrometric methods,
- investigation the pharmacological effect of the isolated compounds.

3 MATERIALS AND METHODS

Bryophyte species for phytochemical screening were collected in the Northern Medium Mountains (Hungary) in September and October of 2014 and were identified by Dr. Péter Szűcs. *Paraleucobryum longifolium* was collected in Hungary, Mátrafüred and was identified by Dr. Péter Szűcs.

Samples for phytochemical screening were prepared by the methanolic extraction of powdered, dried plant materials, which were further separated by liquid-liquid chromatography using various polarities of solvents.

Isolation of compounds from *Paraleucobryum longifolium* was carried out by multiple steps of various chromatographic methods such as open column chromatography (CC), vacuum liquid chromatography (VLC), gel filtration chromatography (GFC), flash chromatography (FC), preparative layer chromatography (PLC) and high pressure liquid chromatography (HPLC) using both normal and reversed phase.

The identification of possible compounds through phytochemical screening was conducted by LC-MS screening, structure elucidation of purified compounds was mainly done by NMR measurements altogether with the acquisition and elucidation of ECD and ESI-HRMS spectrums. To determinate the structure of isolated compounds from *P. longifolium* 1D and 2D NMR technics were used, which included $^{1}H–^{1}H$ COSY, HSQC, HMBC, and EASY-ROESY experiments. HRESIMS technic were used to measure the exact molecular weight of isolated compounds and to obtain information about the structure of possible compounds via phytochemical screening. ECD measurements and sTDA ECD calculations were used to determine the axial chirality of the compounds, and the central chirality elements were assigned by TDDFT-SOR calculations.

Investigation of antiproliferative activity of bryophyte extracts and purified compounds were carried out by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay on human cervical (HeLa and SiHa), ovarian (A2780) and breast (T47D and MDA-MB-231) cancer cell lines. Cell cultures were grown on Minimum Essential Medium (MEM) supplemented with 10% fetal bovine serum, 1% non-essential amino acids, and an antibiotic–antimycotic mixture. Cisplatin was used as a reference agent.

Antimicrobial activity of bryophyte extracts were investigated on 11 bacteria strains determined by the disc-diffusion method. The standard Gram-positive strains were B*acillus subtilis* (ATCC 6633), *Staphylococcus aureus* (ATCC 29213), *Staphylococcus epidermidis* (ATCC 12228), Streptococcus agalactiae (ATCC 13813), Streptococcus pneumoniae (ATCC

49619), *Streptococcus pyogenes* (ATCC 19615), and methicillin-resistant *Staphylococcus aureus* (ATCC 43300). The standard Gram-negative strains were *Escherichia coli* (ATCC 35218), *Klebsiella pneumoniae* (ATCC 700603), and *Moraxella catarrhalis* (ATCC 43617). Positive activity was accepted above 6 mm of inhibition zone diameter.

The pharmacological examinations were performed in a cooperation with the Institute of Pharmacodynamics and Biopharmacy (University of Szeged, Faculty of Pharmacy) and Department of Medical Microbiology Educational and Research Centre (University of Szeged, Albert Szent-Györgyi Medical School).

4 RESULTS

4.1 Phytochemical screening of Hungarian moss species

From 42 bryophyte species belonging to 35 genera and 20 families, altogether 168 extracts were prepared with n-hexane (A), $CHCl_3$ (B), aqueous MeOH (C), and H_2O (D) (**Figure 1).**

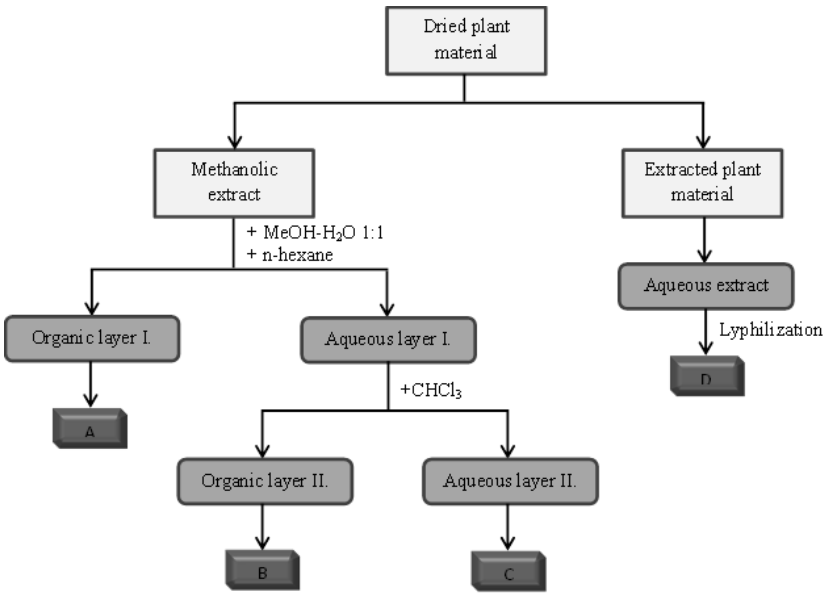


Figure 1. Extraction of bryophyte samples

4.2 Pharmacological activity of selected bryopytes via phytochemical screening

4.2.1 Antiproliferative assay

From 41 species totally 98 extracts showed >25% inhibition of proliferation of at least one of the cell lines at 10 g/mL, and from 17 species 25 extract showed more than 50% inhibition on at least one of the cell lines at 10 g/mL concentration. Results are presented in **Table 1**. Chloroformic fractions (B extracts) exhibited the most potent activity in most cases. Six

species, namely, *Brachythecium rutabulum*, *Climacium dendroides*, *Encalypta streptocarpa*, *Pleurozium schreberi*, *Neckera besseri*, and *Pseudoleskeella nervosa* have more than one active extracts. Chloroformic extract of *Paraleucobryum longifolium* possessed not only the highest inhibition (78.54% inhibition on HeLa at 10 g/mL), but it showed activity on each of the tested cell lines with slight difference between 10 g/mL and 30 g/mL concentrations.

From the tested families, *Brachytheciaceae* (with *Brachythecium rutabulum*, *Homalothecium philippeanum*, and *Pseudoscleropodium purum*) and *Amblystegiaceae* (with *Amblystegium serpens* and *Hygroamblystegium tenax*) provided the highest numbers of active extracts.

Table 1. The results of the antiproliferative assays. Extracts exerting less than 25% inhibition of cancer cell growth were considered inactive (red) values, exceeding 50% inhibition are coloured from yellow to green.

Species	Extract A						Extract B					
	HeLa		A2780		T47D		HeLa		A2780		T47D	
	10 µg/mL	30 µg/mL	10 µg/mL	30 µg/mL	10 µg/mL	30 µg/mL	10 µg/mL	30 µg/mL	10 µg/mL	30 µg/mL	10 µg/mL	30 µg/mL
Abietinella abietina	<25	<25	<25	<25	<25	32.26	30.00	38.62	<25	<25	42.99	48.97
Amblystegium serpens	<25	46.13	29.58	49.94	49.15	70.15	61.93	70.78	53.46	65.35	70.15	74.76
Anomodon viticulosus	26.96	50.72	<25	<25	<25	27.81	27.04	49.35	32.35	53.87	<25	36.32
Atrichum undulatum	<25	<25	<25	<25	<25	41.66	59.93	76.26	37.78	64.28	64.11	65.26
Barbula unguiculata	45.74	63.27	<25	<25	<25	34.14	65.46	75.11	<25	47.47	44.20	53.16
Brachytheciastrum velutinum	31.92	64.96	<25	35.58	<25	41.01	34.43	55.09	<25	61.29	34.43	51.51
Brachythecium rutabulum	53.49	61.64	25.04	34.93	45.40	55.36	51.95	53.89	<25	35.30	46.79	54.92
Bryum argenteum	47.79	80.09	<25	<25	<25	<25	36.11	54.52	<25	<25	35.95	41.26
Bryum caespiticium	30.37	57.84	<25	<25	<25	48.80	48.64	59.57	<25	<25	28.58	48.17
Bryum moravicum	<25	38.27	<25	<25	<25	29.73	46.72	62.09	27.34	48.22	40.64	59.69
Calliergonella cuspidata	<25	<25	<25	<25	<25	<25	<25	32.79	<25	<25	39.02	49.49
Ceratodon purpureus	<25	26.67	<25	32.49	<25	28.88	30.67	42.00	<25	35.18	<25	28.48
Cirriphyllum piliferum	51.34	67.39	<25	42.24	<25	31.19	<25	28.32	<25	<25	<25	<25
Climacium dendroides	52.79	63.79	<25	32.63	<25	<25	56.79	64.89	<25	<25	55.46	57.16
Dicranum tauricum	<25	<25	<25	28.55	<25	31.60	33.14	51.60	<25	48.94	35.38	54.93
Encalypta streptocarpa	76.66	61.32	34.04	87.90	25.72	44.08	54.46	72.90	73.72	80.12	33.22	33.27
Funaria hygrometrica	<25	39.84	<25	<25	<25	36.21	48.44	62.88	25.66	51.06	46.44	53.18
Homalothecium lutescens	<25	37.79	<25	31.59	<25	30.29	<25	<25	<25	<25	28.74	30.64
Homalothecium philippeanum	38.34	63.66	<25	40.77	37.39	48.19	46.93	73.77	33.60	74.93	63.90	62.51
Hygroamblystegium tenax	<25	51.08	<25	26.53	28.34	43.75	36.99	43.86	<25	<25	49.19	55.28
Leskea polycarpa	<25	29.00	<25	37.18	<25	25.71	<25	31.32	<25	35.02	<25	37.07
Leucodon sciuroides	26.00	43.88	<25	<25	<25	34.43	42.48	61.74	<25	<25	28.88	39.63
Neckera besseri	54.29	68.98	<25	<25	33.72	38.33	69.13	83.28	<25	76.48	50.07	68.26
Orthotrichum diaphanum	<25	28.22	<25	<25	<25	<25	40.19	51.75	25.04	50.65	35.43	40.61
Oxyrrhynchium hians	<25	50.41	<25	42.22	26.03	46.46	25.65	39.79	<25	28.61	34.01	46.64
Paraleucobryum longifolium	<25	27.34	<25	<25	<25	<25	78.54	83.93	63.23	78.03	46.84	56.87
Plagiomnium affine	<25	41.79	<25	<25	<25	<25	42.41	55.53	<25	42.11	42.49	56.05
Plagiomnium cuspidatum	39.13	39.44	26.49	97.60	<25	86.33	<25	<25	<25	56.15	<25	36.11
Plagiomnium rostratum	26.01	60.72	<25	44.99	28.68	36.26	46.52	60.22	43.23	67.06	45.56	54.59
Plagiomnium undulatum	35.77	33.42	<25	26.07	29.36	43.49	<25	<25	<25	33.21	<25	32.10
Pleurozium schreberi	61.85	93.41	41.15	37.25	<25	31.65	60.49	74.30	<25	36.89	29.26	43.95
Pohlia nutans	<25	<25	<25	<25	<25	<25	29.51	49.60	<25	<25	<25	<25
Polytrichastrum formosum	<25	34.69	<25	<25	<25	28.85	<25	34.18	<25	<25	<25	<25
Porella platyphylla	31.89	79.22	48.22	83.33	48.94	64.37	35.69	47.36	<25	41.93	29.33	47.86

Species	Extract A						Extract B					
	HeLa		A2780		T47D		HeLa		A2780		T47D	
	10 µg/mL	30 µg/mL	10 µg/mL	30 µg/mL	10 µg/mL	30 µg/mL	10 µg/mL	30 µg/mL	10 µg/mL	30 µg/mL	10 µg/mL	30 µg/mL
Pseudoleskeella nervosa	68.43	75.64	<25	<25	38.94	43.69	61.71	71.88	<25	36.28	42.77	45.50
Pseudoscleropodium purum	<25	34.16	<25	<25	<25	<25	62.06	70.27	<25	28.01	53.88	54.58
Rhytidiadelphus squarrosus	<25	<25	<25	<25	<25	<25	43.99	53.66	<25	<25	40.31	51.65
Rhytidium rugosum	32.34	56.50	<25	36.29	<25	25.80	30.20	39.48	<25	<25	<25	27.52
Schistidium crassipilum	<25	33.32	<25	<25	<25	<25	27.52	53.09	<25	72.36	<25	38.36
Syntrichia ruralis	<25	<25	<25	<25	<25	<25	27.05	33.02	<25	<25	30.49	39.25
Thamnobryum alopecurum	29.98	57.12	<25	26.75	<25	<25	34.35	53.87	<25	51.91	<25	<25
Thuidium assimile	<25	<25	<25	29.18	<25	<25	43.36	57.09	34.62	58.86	65.70	56.12

Species	Extract C						Extract D					
	HeLa		A2780		T47D		HeLa		A2780		T47D	
	10 µg/mL	30 µg/mL	10 µg/mL	30 µg/mL	10 µg/mL	30 µg/mL	10 µg/mL	30 µg/mL	10 µg/mL	30 µg/mL	10 µg/mL	30 µg/mL
Abietinella abietina	35.25	52.48	<25	<25	38.22	49.01	<25	<25	<25	<25	<25	30.04
Amblystegium serpens	33.19	44.83	<25	26.71	48.58	58.34	<25	<25	<25	<25	27.91	35.73
Anomodon viticulosus	<25	34.02	<25	<25	<25	27.47	<25	<25	<25	<25	<25	<25
Atrichum undulatum	29.14	41.65	<25	46.14	<25	37.95	33.37	36.14	<25	<25	<25	<25
Barbula unguiculata	29.68	35.91	<25	<25	<25	27.68	27.92	27.4	<25	<25	<25	<25
Brachytheciastrum velutinum	<25	32.09	<25	<25	27.26	34.7	34.9	34.68	<25	<25	38.53	38.46
Brachythecium rutabulum	<25	34.26	<25	<25	<25	34.81	<25	<25	<25	<25	<25	<25
Bryum argenteum	<25	<25	<25	<25	<25	<25	<25	<25	<25	<25	<25	<25
Bryum caespiticium	<25	35.57	<25	<25	<25	<25	<25	<25	<25	<25	<25	<25
Bryum moravicum	<25	37.94	<25	<25	<25	26.51	<25	<25	<25	<25	<25	<25
Calliergonella cuspidata	<25	<25	<25	<25	<25	<25	<25	<25	<25	<25	<25	<25
Ceratodon purpureus	<25	<25	<25	<25	<25	31.86	<25	<25	<25	<25	<25	<25
Cirriphyllum piliferum	28.18	42.07	<25	<25	<25	26.85	<25	32.27	<25	<25	<25	<25
Climacium dendroides	<25	<25	<25	<25	<25	27.68	<25	27.42	<25	<25	27.38	37.52
Dicranum tauricum	<25	28.29	<25	<25	33.52	49.97	29.75	37.11	<25	<25	45.31	47.21
Encalypta streptocarpa	28.01	39.61	<25	<25	<25	32.5	27.05	<25	<25	<25	<25	<25
Funaria hygrometrica	<25	<25	<25	<25	42.27	48.22	25.11	38.47	<25	<25	35.4	45.16
Homalothecium lutescens	<25	<25	<25	<25	<25	<25	<25	<25	<25	<25	<25	27.6
Homalothecium philippeanum	<25	33.51	<25	28.04	43.32	51	<25	<25	<25	<25	33.68	41.04
Hygroamblystegium tenax	26.66	38.22	<25	<25	52.69	55.03	<25	31.71	<25	<25	37.34	40.38
Leskea polycarpa	25.62	31.09	<25	<25	<25	34.12	<25	<25	<25	<25	<25	<25
Leucodon sciuroides	<25	29.98	<25	<25	<25	<25	<25	<25	<25	<25	<25	<25
Neckera besseri	37.13	41.25	<25	<25	54.5	55.63	<25	<25	<25	<25	32.28	43.28
Orthotrichum diaphanum	<25	40.79	<25	<25	<25	28.2	<25	<25	<25	<25	<25	<25
Oxyrrhynchium hians	<25	<25	<25	<25	<25	29.53	<25	<25	<25	<25	<25	<25
Paraleucobryum longifolium	<25	<25	<25	<25	<25	<25	<25	<25	<25	<25	<25	<25
Plagiomnium affine	42.04	50.67	<25	26.86	53.3	57.53	<25	<25	<25	<25	<25	<25
Plagiomnium cuspidatum	35.49	46.35	<25	<25	33.53	45.9	<25	<25	<25	<25	<25	27.79
Plagiomnium rostratum	40.44	51.65	<25	42.42	33.23	45.84	<25	<25	<25	<25	<25	<25
Plagiomnium undulatum	<25	<25	<25	<25	<25	<25	<25	<25	<25	<25	<25	<25
Pleurozium schreberi	<25	32.99	<25	<25	<25	<25	<25	<25	<25	<25	<25	<25
Pohlia nutans	<25	32.63	<25	<25	<25	<25	<25	<25	<25	<25	<25	<25
Polytrichastrum formosum	<25	<25	<25	<25	<25	<25	<25	<25	<25	<25	<25	<25
Porella platyphylla	<25	41.69	<25	<25	<25	27.66	<25	<25	<25	<25	<25	<25
Pseudoleskeella nervosa	60.51	65.03	<25	26.27	49.89	54.5	<25	<25	<25	<25	<25	25.43
Pseudoscleropodium purum	<25	28.06	<25	<25	<25	31.29	<25	29.89	<25	<25	32.22	40.05
Rhytidiadelphus squarrosus	<25	26.56	<25	<25	<25	33.78	<25	<25	<25	<25	<25	<25
Rhytidium rugosum	<25	33.67	<25	<25	<25	<25	<25	<25	<25	<25	<25	<25
Schistidium crassipilum	<25	<25	<25	<25	<25	<25	<25	<25	<25	<25	<25	<25
Syntrichia ruralis	<25	<25	<25	<25	<25	<25	30.83	41.17	<25	<25	57.42	59.35
Thamnobryum alopecurum	<25	31.9	<25	<25	<25	<25	26.26	40.68	<25	<25	<25	<25
Thuidium assimile	<25	<25	<25	34.23	29.78	43.24	<25	<25	<25	<25	32.28	44.58

4.2.2 Antimicrobial assay

Only 19 samples of 15 taxa showed moderate antibacterial activity (**Table 2**). None of the extracts were active on *Pseudomonas aeruginosa* ATCC 27853, *Escherichia coli* ATCC 35218, and *Klebsiella pneumoniae* ATCC 700603. Methicillin-resistant *Staphylococcus aureus* ATCC 43300 and *Staphylococcus aureus* ATCC 29213 were the most susceptible strains to the examined extracts. Among the fractions with different polarities, the relatively apolar n-hexane and chloroform extracts demonstrated antibacterial activities. The aqueous and MeOH fractions were inactive. *Plagiomnium cuspidatum* was active on eight tested strains.

Amblystegium serpens, *Brachythecium rutabulum*, *Cirriphyllum piliferum*, *Climacium dendroides*, *Paraleucobryum longifolium*, *Plagiomnium affine*, and *Pseudoscleropodium purum* were active in both assays.

Table 2. Antibacterial activities of moss extracts (inhibition zones in millimetres).

Species	Extract	MRSA	*S. aureus*	*S. epidermidis*	*B. subtilis*	*S. pyogenes*	*S. pneumoniae*	*S. agalactiae*	*M. catarrhalis*
		ATCC 43300	ATCC 29213	ATCC 12228	ATCC 6633	ATCC 19615	ATCC 49619	ATCC 13813	ATCC 43617
Amblystegium serpens	**B**	—	—	—	—	—	—	—	**9.0**
Brachythecium rutabulum	**B**	**9.0**	**9.0**	—	—	—	—	—	—
Calliergonella cuspidata	**A**	—	**7.3**	—	—	—	—	—	—
	B	—	**7.0**	—	—	—	—	—	—
Cirriphyllum piliferum	**B**	—	—	—	—	—	**7.0**	—	—
Climacium dendroides	**A**	—	**7.3**	—	—	—	—	—	—
Dicranum tauricum	**B**	—	—	—	—	—	**8.0**	—	—
Oxyrrhynchium hians	**A**	**8.6**	**8.6**	—	—	—	—	—	—
	B	—	**8.0**	—	—	—	—	—	—
Paraleucobryum longifolium	**B**	**9.6**	**9.6**	—	—	**11.6**	—	—	—
Plagiomnium affine	**B**	—	—	—	**8.0**	—	**8.5**	—	—
Plagiomnium cuspidatum	**A**	**11.3**	**10.7**	**9.0**	**9.0**	**10.0**	**12.0**	**10.0**	**10.0**
	B	**7.6**	**7.6**	—	—	—	—	—	—
Plagiomnium undulatum	**A**	**7.0**	**8.0**	—	—	—	—	—	—
	B	—	**8.0**	—	—	—	—	—	—
Pseudoscleropodium purum	**A**	—	**7.3**	—	—	—	—	—	—
Rhytidium rugosum	**B**	—	—	—	**7.5**	—	**8.0**	—	**7.5**
Schistidium crassipilum	**B**	**8.0**	**7.0**	—	**9.0**	—	**11.5**	—	**7.7**

4.2.3 MS analysis

We aimed to identify characteristic compounds from the most active extracts. The acquired MS^2 peak lists were converted to a text file by using the msConvert tool, and the top 100 MS survey scan peaks were chosen for MS^2 identification against KEGG's small-molecule database, using the MetFrag online search tool. The hits were filtered manually using an 80% matched peak result when the number of MS^2 fragment peaks was at least five. From 8 species 22 compounds were identified.

4.3 Isolation of secondary metabolites

4.3.1 Isolation of secondary metabolites from *P. longifolium*

Due to highly potent effects showed in both antiproliferative and antimicrobial assay, *Paraleucombryum longifolium* was selected for further investigation. Plant material was extracted in a percolator with MeOH in room temperature and evaporated. Using multistep chromatographic methods, such CC, VLC, GFC, FC, PLC and HPLC (**Figure 2**), one known flavonoid glycoside, namely diosmetin 7-*O*-[2,4-di-*O*-(α-L-rhamnopyranosyl)]-β-D-glucopyranoside (compound **6**) has been isolated, altogether with five new 9,10-Phenanthrenequinone dimers, namely leucobryn A (compound **1**), leucobryn B (compound **2**), leucobryn C (compound **3**), leucobryn D (compound **4**), and leucobryn E (compound **5**), which are firstly described in the literature.

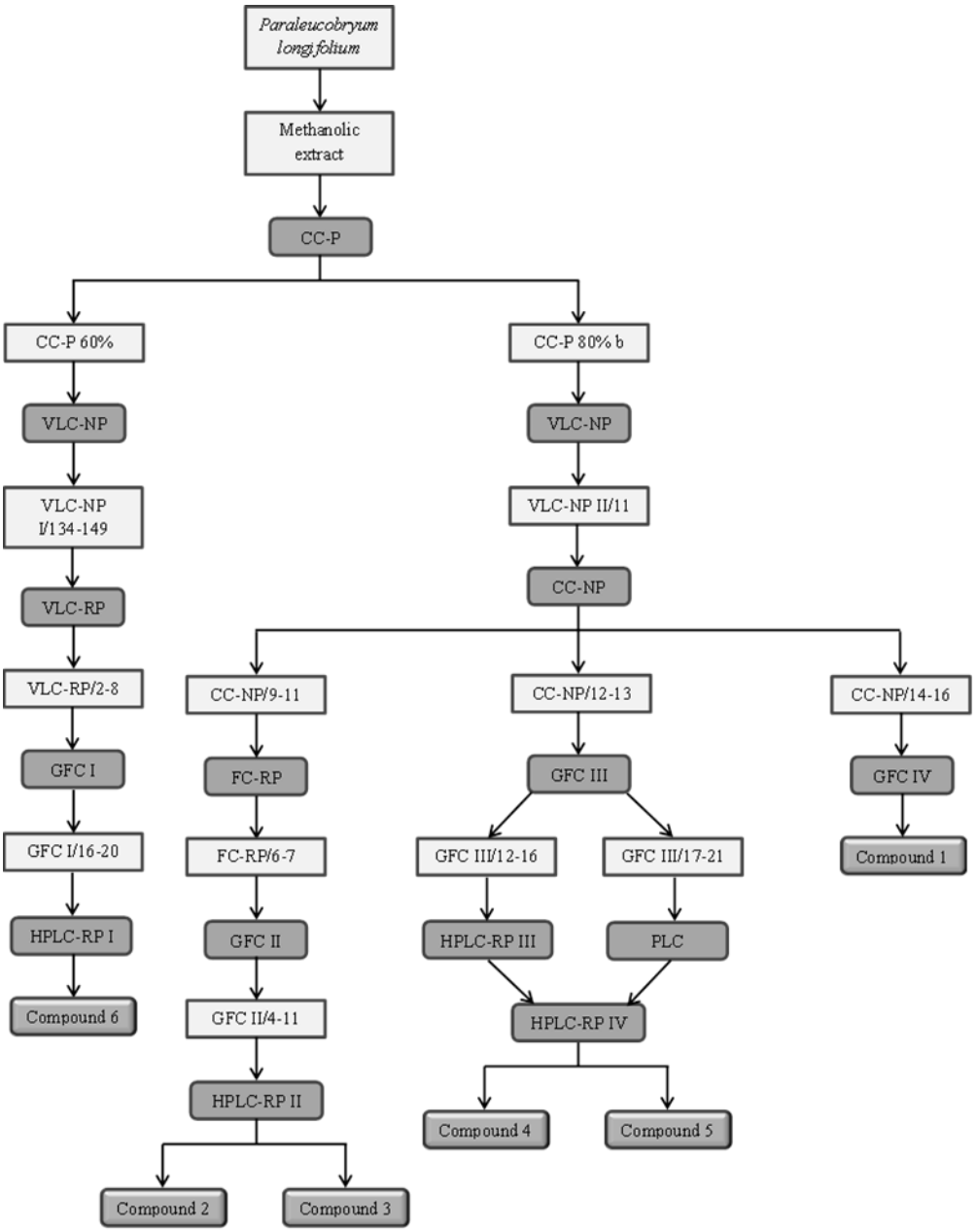


Figure 2. Isolation of compounds from *P. longifolium*

4.4 Pharmacological activity of isolated compounds from *P. longifolium*

The antiproliferative action of the isolated natural products (leucobryn A-E) was determined against four human cancer cell lines of gynecological origin at two relative high concentrations (30 and 60 μM). Leucobryn A exerted close to 70% or higher inhibition of cell growth at 60 μM and the calculated IC_{50} values were considerable high (40-50 μM). Leucobryn B elicited even weaker cancer cell growth inhibitory action. All the other compounds had no relevant action on the cancer cells up to 60 μM (**Table 3**). Since frequently utilized reference agents (e.g. cisplatin) elicits substantially more pronounced antiproliferative

actions with low micromolar IC_{50} values this property of the currently presented natural products is considered moderate (leucobryn A and B) or negligible (leucobryn C, D and E).

Table 3. The antiproliferative action of leucobryns against human cancer cell lines

Compound	Conc.	Inhibition (%) ± SEM [calculated IC_{50} (μM)]			
		SiHa	**HeLa**	**A2780**	**MDA-MB-231**
Leucobryn A	30 μM	22.47 ± 1.56	25.69 ± 1.88	–*	–
	60 μM	69.34 ± 1.14	80.41 ± 2.20	86.43 ± 0.46	76.04 ± 0.61
		[45.56]	[40.40]	[46.34]	[50.81]
Leucobryn B	30 μM	–	10.72 ± 2.85	–	–
	60 μM	22.44 ± 0.69	37.48 ± 2.03	16.38 ± 2.44	59.80 ± 0.71
Leucobryn C	30 μM	–	–	–	–
	60 μM	–	31.63 ± 1.36	–	–
Cisplatin	10 μM	88.64 ± 0.50	42.61 ± 2.33	83.57 ± 1.21	67.51 ± 1.01
	30 μM	90.18 ± 7.78	99.93 ± 0.26	95.02 ± 0.28	87.75 ± 1.10
		[7.84]	[12.43]	[1.30]	[3.74]

4.5 Structure elucidation of isolated compounds

The molecular formula of compound **1** was established as $C_{40}H_{38}O_{12}$ based on HRESIMS data, which showed the molecule ion $[M+Na]^+$ at m/z 733.2252. The ^{13}C JMOD spectrum indicated that compound **1** is a symmetric dimer. HMBC correlations disclosed that compound **1** contains a 9,10-phenanthrenedione parent system. The structure of compound **1**, named leucobryn A, could be established as a dimer in which the monomers are connected via their C-8 atoms.

Compound **2** (leucobryn B) possessed a molecular formula $C_{50}H_{54}O_{12}$ as determined by the HRESIMS ion at *m/z* 869.3505 $[M+Na]^+$. The ^1H NMR and ^{13}C JMOD spectra suggested a symmetric dimeric structure, similarly to compound **1**. The ^1H NMR and ^{13}C JMOD spectra of **2** also resembled those of **1**. In particular, the ^1H and ^{13}C chemical shifts of the 9,10-phenanthrenedione part were close to those of **1**. The main differences were found in the C_5 prenyl side chain, which was changed to a C_{10} moiety in **2**.

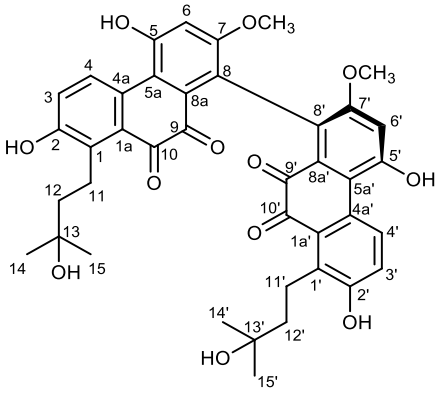
Compound **3** (leucobryn C) possesses the same molecular formula of $C_{50}H_{54}O_{12}$ as that of compound **2,** with the molecula ion at *m/z* 869.3507 $[M+Na]^+$. 1D and 2D $^1H/^{13}C$ NMR

characteristics of **2** and **3** were similar, with only slight differences. These data suggest that the structural differences are explicable in terms of the stereochemistry of the compounds.

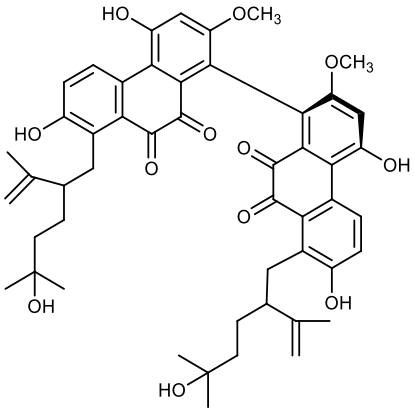
HRESIMS data of compound **4** (leucobryn D) revealed a molecular formula of $C_{45}H_{46}O_{12}$ according to the sodium adduct ion at *m/z* 801.2876 $[M+Na]^+$. ^1H NMR and ^{13}C JMOD data contained signals similar to the resonances of both compounds **1** and **2**, indicating that compound **4** is a heterodimer constructed by the monomeric moieties of **1** and **2**. As in compounds **1** and **2**, the monomeric constituents of **4** are connected via their C-8 atoms. 2D NMR studies provided further evidence to confirm the structure of this compound as depicted in structural formula **4**.

In the case of compound **5** (leucobryn E) all HRESIMS and NMR characteristics were highly similar to those of **4**, suggesting a molecular pair with the same 2D structure, but with different chirality.

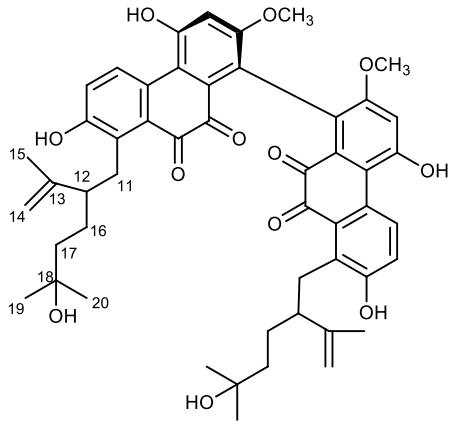
Compound **6** was identified as diosmetin 7-*O*-[2,4-di-*O*-(*α*-L-rhamnopyranosyl)]-*β*-D-glucopyranoside.



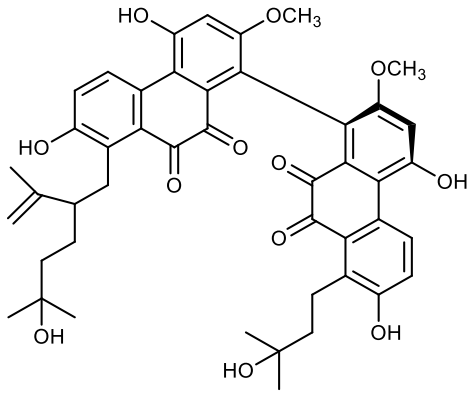
Leucobryn A



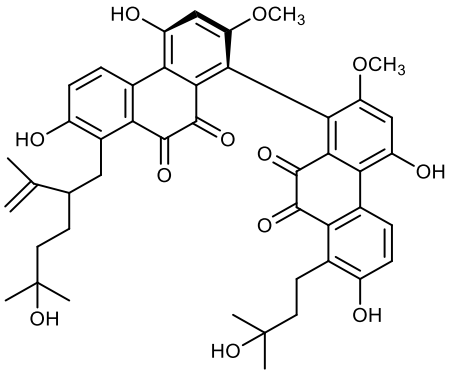
Leucobryn B



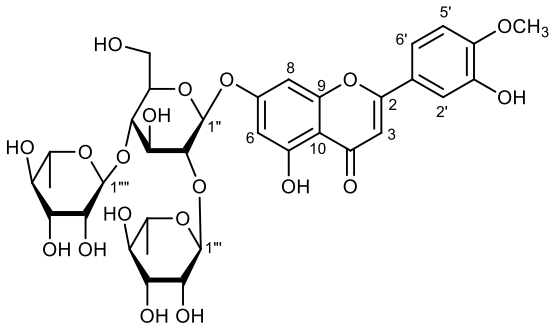
Leucobryn C



Leucobryn D



Leucobryn E



diosmetin 7-*O*-[2,4-di-*O*-(*α*-L-rhamnopyranosyl)]-*β*-D-glucopyranoside

5 DISCUSSION AND SUMMARY

The aim of this study involved the screening of bryophytes from the Carpathian Basin from chemical and pharmacological point of view and the further analysis of *P. longifolium* by different chromatographic methods for the identification of active compounds.

Antiproliferative MTT assays were performed on human cervical (HeLa and SiHa), ovarian (A2780) and breast (T47D and MDA-MB-231) cancer cell lines. The antimicrobial activity was determined by disc-diffusion method on either Gram-positive and Gram-negative strains. The results show that from 168 extract of 42 family of bryophytes, collected in the Carpathian Basin, 98 extract derived from 41 species exerted at least 25% inhibition of proliferation of at least one of the cancer cell lines, and from 17 species 25 extract showed more than 50% inhibition on at least one of the cell lines at 10 g/mL concentration. The highest inhibition was observed by the chloroform extract of *P. longifolium*. In the disc-diffusion assay only 15 taxa showed moderate antibacterial activity, among them *Plagiomnium cuspidatum* showed the most abundant activity with the inhibition on eight

strains. *Amblystegium serpens*, *Brachythecium rutabulum, Cirriphyllum piliferum, Climacium dendroides, Paraleucobryum longifolium, Plagiomnium affine*, and *Pseudoscleropodium purum* were active in both assays.

Based on the pharmacological screening experiments, *Paraleucobryum longifolium* was selected for detailed phytochemical analysis. Six compounds were isolated from the extract of *P. longifolium* by using CC, VLC, GFC, PLC, HPLC technics. The identification and structure determination was performed by 1D and 2D NMR (COSY, HSQC, HMBC, and EASY-ROESY experiments), HRMS and ECD.

This was the first time to describe complex secondary metabolites from *P. longifolium*. Altogether 5 previously unknown prenyl-substituted 8,8'-linked 9,10-phenanthrenequinone dimers (leucobryns A-E) and 1 flavonoid-glycoside (diosmetin 7-O-[2,4-di-O-(□-L-rhamnopyranosyl)]-β-D-glucopyranoside, which was previously described *from Dicranum scoparium*) were isolated from this species. Although phentanthrenes are the specific pigments of bryophytes, leucobryns are the first natural occurring 9,10-phenanthrenequinone dimers wherein monomers are connected via C-8 linkage. Even 9,10-phenanthrenequinone monomers are rarely found in nature, which possess antibacterial and antiviral activities.

The antiproliferative activities of leucobryns were tested *in vitro*. Leucobryn A and B showed moderate inhibition, while leucobryns C-E showed negligible inhibition. These very distinct molecules deserve extensive pharmacological examination.

6 ACKNOWLEDGEMENTS

I would like to express my greatest gratitude to Dr. Dezső Csupor for leading my PhD work with his invaluable professional knowledge and guiding me through the path of scientific life.

I am deeply indebted to Prof. Judit Hohmann as former head of the Department of Pharmacognosy for providing me all the opportunities to perform experimental work.

I am thankful to Dr. Péter Szűcs and Dr. Marianna Marschall for plant materials identification and gathering, to Prof. Judit Hohmann and Dr. Norbert Kúsz for NMR measurements, to Dr. Katalin Kövér E. and Attila Csorba for LC-MS measurements, to Dr. Boglárka Csupor-Löffler for antibacterial tests, to Prof. István Zupkó and Seyyed Ashkan Senobar Tahaei for antiproliferative tests and to Prof. Tibor Kurtán and Dr. Attila Mándi for ECD measurements.

I am also grateful to Dr. Dóra Stefkó for supporting me during this period of my life.

My thanks are likewise due to all my colleagues in the Department of Pharmacognosy. I am especially grateful to Attila Horváth, Dr. Zoltán Zomborszki, Dr. Tivadar Kiss, Horváth-Boros Klára, Dr. Diána Kerekes, Balázs Kovács and Dr. Javad Mottaghipisheh for friendship and supporting my academical work. I would like to extend my special thanks to my parents, to my brother, relatives and friends. I could not have carried out this work without their support.

The thesis is based on the following publications:

I. **Martin Vollár**, András Gyovai, Péter Szűcs, István Zupkó, Marianna Marschall, Boglárka Csupor-Löffler, Péter Bérdi, Anikó Vecsernyés, Attila Csorba, Erika Liktor-Busa, Edit Urbán, Dezső Csupor

Antiproliferative and Antimicrobial Activities of Selected Bryophytes

Molecules, 2018, 23(7): 1520 **IF: 3.06**

II. Dezső Csupor, Tibor Kurtán, **Martin Vollár**, Norbert Kúsz, Katalin Kövér E., Attila Mándi, Péter Szűcs, Marianna Marschall, Seyyed Ashkan Senobar Tahaei, István Zupkó, Judit Hohmann

Pigments of the Moss Paraleucobryum longifolium: Isolation and Structure Elucidation of Prenyl-substituted 8,8'-linked 9,10-Phenanthrenequinone Dimers

Journal of Natural Products, 2020, 83(2): 268-276 **IF: 4.05**

Further publucation:

III. **Martin Vollár**, Gábor Feigl, Dóra Oláh, Attila Horváth, Árpád Molnár, Norbert Kúsz, Attila Ördög, Dezső Csupor, Zsuzsanna Kolbert

Nitro-Oleic Acid in Seeds and Differently Developed Seedlings of Brassica napus L.

Plants (Basel), 2020, 9(3): 406

IV. Ferencz, Elek, Spengler, Gabriella, Zupkó, István, **Vollár, Martin**, Zomborszki, Zoltán Péter, Kúsz, Norbert, Hohmann, Judit, Kovács, Balázs, Csupor, Dezső, Laczkó-Zöld, Eszter; Csupor-Löffler, Boglárka

Isolation of compounds from the roots of Ambrosia artemisiifolia *and their effects on human cancer cell lines*

Zeitschrift für Naturforschung C, 2023, 78(7-8):299-305