HUNGARIAN BRYOPHYTES AS POTENTIAL SOURCES

FOR PHARMACOLOGICALLY ACTIVE SUBSTANCES

FINAL RESEARCH REPORT

1. COLLECTION OF PLANT MATERIAL:

During field trips in Hungary, the search for bryophyte species was intensive. 57 bryophyte species for screening for biological activity were collected, taxonomic identity was checked by light microscopy and stereomicroscopy. The description and determination of the life-strategy and the morphology of bryophyte's cushions or mats were carried out. Potential relationship between the bioactive substance content and the type of life strategy was analyzed.

A register of the collected species, including the place (GPS coordinates) and time of collection and ecological characteristics (vegetation, habitat and substrate preference of the collected bryophyte) was established. Taxonomic identification was carried out by microscopy. The collected samples were purified and dried for further analysis. The list of the collected species and places of collection is the following:

	Species	**Place of collection**
1	Syntrichia ruralis (Hedw.) F.Weber & D.Mohr	Bükkalja
2	Climacium dendroides (Hedw.) F.Weber & D.Mohr	Bükkalja
3	Pseudoscleropodium purum (Hedw.) M.Fleisch.	Bükkalja
4	Homalothecium lutescens (Hedw.) H.Rob.	Bükkalja
5	Calliergonella cuspidata (Hedw.) Loeske	Bükkalja
6	Rhytidiadelphus squarrosus (Hedw.) Warnst.	Bükkalja
7	Plagiomnium undulatum (Hedw.) T.J.Kop.	Bükkalja
8	Abietinella abietina (Hedw.) M.Fleisch.	Bükkalja
9	Plagiomnium cuspidatum (Hedw.) T.J.Kop.	Bükkalja
10	Hypnum cupressiforme Hedw.	Bükkalja
11	Oxyrrhynchium hians (Hedw.) Loeske	Bükkalja
12	Ceratodon purpureus (Hedw.) Brid.	Bükkalja
13	Brachythecium rutabulum (Hedw.) Schimp.	Gyöngyösi-sík
14	Orthotrichum diaphanum Schrad. ex Brid.	Gyöngyösi-sík
15	Leskea polycarpa Hedw.	Gyöngyösi-sík
16	Tortula muralis Hedw.	Gyöngyösi-sík
17	Grimmia pulvinata (Hedw.) Sm.	Bükkalja
18	Schistidium crassipilum H.H.Blom	Bükkalja

19	Brachytheciastrum velutinum (Hedw.) Ignatov & Huttunen	Bükk-hg.
20	Paraleucobryum longifolium (Hedw.) Loeske	Bükk-hg.
21	Encalypta streptocarpa Hedw.	Bükk-hg.
22	Leucodon sciuroides (Hedw.) Schwägr.	Bükk-hg.
23	Polytrichastrum formosum (Hedw.) G.L.Sm.	Bükk-hg.
24	Anomodon viticulosus (Hedw.) Hook. & Taylor	Bükk-hg.
25	Porella platyphylla (L.) Pfeiff.	Bükk-hg.
26	Pterigynandrum filiforme Hedw.	Bükk-hg.
27	Eurhynchium angustirete (Broth.) T.J.Kop.	Bükk-hg.
28	Homalothecium sericeum (Hedw.) Schimp.	Bükk-hg.
29	Rhytidiadelphus triquetrus (Hedw.) Warnst.	Bükk-hg.
30	Dicranum scoparium Hedw.	Bükk-hg.
31	Plagiochila porelloides (Nees) Lindenb	Bükk-hg.
32	Ctenidium molluscum (Hedw.) Mitt.	Bükk-hg.
33	Isothecium alopecuroides (Lam. ex Dubois) Isov.	Bükk-hg.
34	Pseudoleskeella nervosa (Brid.) Nyholm	Bükk-hg.
35	Neckera besseri (Lobarz.) Jur.	Bükk-hg.
36	Hygroamblystegium tenax (Hedw.) Jenn.	Bükk-hg.
37	Homalothecium philippeanum (Spruce) Schimp.	Bükk-hg.
38	Atrichum undulatum (Hedw.) P.Beauv.	Bükk-hg.
39	Plagiomnium rostratum (Schrad.) T.J.Kop.	Bükk-hg.
40	Thuidium assimile (Mitt.) A.Jaeger	Bükk-hg.
41	Polytrichum piliferum Hedw.	Bükk-hg.
42	Anomodon attenuatus (Hedw.) Huebener	Bükk-hg.
43	Tortella bambergeri (Schimp.) Broth.	Bükk-hg.
44	Cirriphyllum piliferum (Hedw.) Grout	Mátraalja
45	Pleurozium schreberi (Willd. ex Brid.) Mitt.	Nagy-Eged
46	Rhytidium rugosum (Hedw.) Kindb.	Nagy-Eged
47	Bryum argenteum Hedw.	Bükkalja
48	Amblystegium serpens (Hedw.) Schimp.	Mátraalja
49	Bryum caespiticium Hedw.	Bükkalja
50	Barbula unguiculata Hedw.	Bükkalja
51	Bryum moravicum Podp.	Mátra
52	Dicranum tauricum Sapjegin	Mátra
53	Funaria hygrometrica Hedw.	Gyöngyösi-sík
54	Plagiomnium affine (Blandow ex Funck) T.J.Kop.	Mátra
55	Pohlia nutans (Hedw.) Lindb.	Mátra
56	Schistidium sp. (pontosabb azonosításra vár)	Mátra
57	Thamnobryum alopecurum (Hedw.) Gangulee	Mátra

Based on the results of pharmacological screening, three species (active at least in one of the pharmacological screening experiments) were collected in bigger quantities for preparative phytochemical experiments:

Paraleucobryum longifolium (Hedw.) Loeske 4 kg: collected on 03/09/2016, in Borsod-Abaúj-Zemplén County, Zemplén mountains, in the area of Hollóháza, Bodogány Hill, acidophilus beech forest, nort-eastern exposure, 440 m, GPS coordinates: N 48°31'28.2", E 21°25'12.6" and on 08/09/2016, in Heves county, Mátra Mountain, area of Gyöngyös-Mátrafüred, Csatorna-valley, Menyecske Hill, acidophilus oak forest), north-western exposure, 490 m, GPS coordinates: N 47°50'31.9", E 19°58'52.6"

Pseudoscleropodium purum (Hedw.) M.Fleisch 3 kg: collected on 07/09/2016 in Heves county, Mátra Mountains, area of Mátraháza, southern exposure, enyhe déli kitettség, antrhropogenic habitat, 670 m, GPS coordinates: N 47°51'48.4", E 19°58'39.3"

Climacium dendroides (Hedw.) F.Weber & D.Mohr 0.5 kg: 07/09/2016 in Heves county, Mátra Mountains, area of Mátraháza, southern exposure, enyhe déli kitettség, antrhropogenic habitat, 680 m, GPS coordinates: N 47°51'53.5", E 19°58'29.3"

2. PREPARATION OF EXTRACTS:

Extracts of different polarity (4 per sample) were prepared from the samples. All extracts were prepared from 5 g of plant material comminuted with an electric grinder. Samples were extracted with 3x50 ml of MeOH using a VWR ultrasonic bed at room temperature. After filtration, solvents were evaporated to dryness. The residues were dissolved in a mixture of MeOH–H_2O 1:1 (25 ml) and were subjected to solvent-solvent partitioning between *n*-hexane (3×25 ml) and $CHCl_3$ (3×25 ml). The *n*-hexane-soluble and the $CHCl_3$-soluble fractions were evaporated to dryness to yield extracts marked with A and B, respectively. After evaporation, the remnant aqueous methanolic phases gave extracts C. The residual plant materials were dried and extracted with 30 ml of boiling H_2O for 15 minutes, using a multiple water bath. The filtered extracts were freeze-dried by means of a Hetosicc liophilizator, affording extracts D. Altogether, from 57 plants 228 extracts were prepared for testing.

3. SCREENING FOR BIOACTIVITY:

The extracts were tested for antiproliferative effect on 3 cancer cell lines (HeLa – human cervical carcinoma, T47D – human breast carcinoma and A2780 – human ovarian carcinoma) using MTT assay. the species with remarkable antiproliferative effect (at least one extract from A, B C and D on at leas one cell line exerting >50% inhibition were the following:

Climacium dendroides (Hedw.) F.Weber & D.Mohr
Pseudoscleropodium purum (Hedw.) M.Fleisch.
Plagiomnium cuspidatum (Hedw.) T.J.Kop.
Brachytheciastrum velutinum (Hedw.) Ignatov & Huttunen
Paraleucobryum longifolium (Hedw.) Loeske
Encalypta streptocarpa Hedw.
Leucodon sciuroides (Hedw.) Schwägr.
Porella platyphylla (L.) Pfeiff.
Pterigynandrum filiforme Hedw.
Plagiochila porelloides (Nees) Lindenb

The extracts were tested for antibacterial effect on 10 strains by disc-diffusion method. In the first experiments, the standard strains are *Acinetobacter baumannii* (ATCC 83048), *Enterococcus faecium* (QC/2008/11/9), *Klebsiella pneumoniae* (ATCC 700603), *Pseudomonas aeruginosa* (ATCC 27853) and *Staphylococcus aureus* (ATCC 29213). The clinical isolates are multiresistant *Acinetobacter baumannii* (64060/2), vancomycin-resitant *Enterococcus faecium*, extended-spectrum β-lactamase (ESBL)-producing *Klebsiella pneumoniae* (63735), multiresistant *Pseudomonas aeruginosa* (64658) and methicillin-resistant *Staphylococcus aureus* (64326). In further studies, the following strains were tested: MRSA, S. aureus, Staphylococcus epidermidis, B. subtilis, Moraxella catarrhalis, Str. pyogenes, Str. pneumoniae, Str. agalactiae, P. aeruginosa, E. coli, K. pneumoniae. The activities were not remarkable enough to require the determination of MIC values. The following species were active:

Plagiomnium cuspidatum (Hedw.) T.J.Kop.
Oxyrrhynchium hians (Hedw.) Loeske
Brachythecium rutabulum (Hedw.) Schimp.
Tortula muralis Hedw.
Paraleucobryum longifolium (Hedw.) Loeske
Plagiochila porelloides (Nees) Lindenb

4. DEREPLICATION STUDIES:

Literature data was collected for the analyzed species. A thorough literature search based on Web of Knowledge and Scifinder was carried out for all the 57 species. Species exerting antibacterial and antiproliferative activities were evaluated using LC-MS-DAD, in order to recognise already described compounds. By this method, several compounds were identified without the need of isolateing them.

5. MICROPROPAGATION STUDIES

In order to ensure sustainability of further research, *in vitro* micropropagation studies - of species regarded as the most promising - are being carried out. Optimization of surface sterilization (using 10% $CaCl_2O_2$ or 10% commercial Domestos) of *Brachythecium rutabulum*, *Oxyrrhynchium hians*, *Tortula muralis*, *Campylopus introflexus* and *Paraleucobryum longifolium* is completed. In the selected bryophytes selection of appropriate explants (shoot tips or lateral shoots), optimization of substrates (1/2 MS without sucrose) for *in vitro* cultivation were carried out. Various explants of bryophyte species, different surface sterilization methods, various culture media with different plant hormone combinations and without hormones (1 mg l^{-1} NAA/ 1 mg l^{-1} BA/ 1 mg l^{-1} 2, 4 D/ 1 mg l^{-1} BA+ 0.1 mg l^{-1} NAA/ 2 mg l^{-1} BA/ 1 mg l^{-1} NAA) were tested for optimal growth and morphogenesis. New moss shoots or protonema developments, depending on the hormonal supply, were observed. Growth response was recorded in every selected species during *in vitro* cultivation. In order to achieve intensive growth, hormone-free and 1 mg l^{-1} BA hormone supplied medium proved to be the most optimal. In addition, the *Campylopus introflexus* moss species was subjected to comparative ecophysiological studies. Based on the 4 examined ecophysiological characteristics (dry matter content per biomass unit, total soluble carbohydrate content, osmotic potential, photosynthetic pigment content), we found a difference between those derived from the natural habitat and those growing under *in vitro* conditions. A further aim is to increase the amount of plant biomass that can be produced by *in vitro* micropropagation, and to test and verify the presence of active substance production in *in vitro* conditions.

6. ISOLATION OF SECONDARY METABOLITES

From *Paraleucobryum longifolium*, Leucobryns A–E, axially chiral 9,10-phenanthrenequinone dimers, were isolated, together with diosmetin triglycoside. Leucobryns B and C were proved to be homodimeric atropodiastereomers containing both axial and central chirality elements, while leucobryns D and E were found to be heterodimeric atropodiastereomers containing central chirality in only one of the two monomeric units. Axial chirality of the compounds was determined by ECD measurements and sTDA ECD calculations, while the central chirality elements were assigned by TDDFT-SOR calculations. Leucobryns represent the first 9,10-phenanthrenequinone dimers, the monomers of which are linked through their C-8 atoms. Leucobryns B–E contain an uncommon C10 monoterpenoid side chain, in which isoprenoid units are joined by 3,4 linkages. Leucobryns A and B exhibited weak antiproliferative activity against several human cancer cell lines.

From *Pseudocleropodium purum*, 3 new benzonaphoxanthenone derivatives were isolated. and identified. Further 3 compounds heva een isolated, the structure elucidatioon of which is under progress.

7. SUMMARY

This project aimed at the investigation ogf Hungarian bryophyte species. As a result of our experiments, we identified new secondary metabolites of 2 species. New methods were developed for the micropropagation of bryophytes which may contribute to the sustainable raw material supply. This grant provided possibility to initiialize a new, still ongoing research topic and to establish scientific co-operations to achieve new results.