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

Ph.D. Thesis

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Supervisor:

Dezső Csupor DSc.

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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, Q1**

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, D1**

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

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Abbreviations

ABTS	2,2-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)
CB_1R	cannabinoid receptor type 1
CB_2R	cannabinoid receptor type 2
CC	Column chromatography
cis-PET	(−)-cis-perrottetinene
DMSO	Dimethyl sulfodixe
DNA	Deoxyribonucleic acid
DPPH	2,2-Diphenyl-1-picrylhydrazyl
ECD	Electronic circular dichroism
EtOAc	Ethyl acetate
FC	Flash chromatography
GFC	Gel fitration chromatography
HIV	Human immundeficiency virus
HPLC	High pressure liquid chromatography
HRESIMS	High resolution electrospray ionization mass spectrometry
HESI-II	Heated electrospray ionization
IPA	Isopropanol
LC-MS	Liquid chromatography coupled mass spectometry
MeCN	Acetonitrile
MeOH	Methanol
MIC	Minimal inhibition concentration
MS	Mass spectometry
MTPSL	microbial-terpene synthase-like
NMR	Nuclear magnetic resonance
PLC	Preparative layer chromatograpy
RT	Retention time
SR	Specific rotation
sTDA	Simplified Tamm-Dancoff Approximation
TDDFT	Time-dependent density-functional theory
Δ9-cis-THC	(−)-Δ9-cis-tetrahydrocannabinol
Δ9-trans-THC	(−)-Δ9-trans-tetrahydrocannabinol
UV	Ultra violet
VLC	Vacuum liquid chromatography

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 [1], intensive chemical and pharmacological studies have been performed only in the last few decades [2].

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 [1]. The Hungarian flora contains 659 species, with the predominance of mosses (2 hornworts, 146 liverworts, 511 mosses) [3].

From phytochemical and pharmacological point of view, bryophytes are poorly explored because of the difficulties of their collection and botanical identification. Still high numbers of new compounds were discovered from mosses, including more than 40 new carbon skeletons of terpenoids and phenolic compounds [4], [5]. Mono-, sesqui-, di-, and triterpenes, flavonoids, bibenzyls, acetogenins are the most common types of secondary metabolites of bryophytes. These compounds show interesing 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 [6]–[10].

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 [2].

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 [11], [12].

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* [13]. In 1959, an examination where 12 species of bryophytes were tested showed the remarkable antibacterial effect of *Anomodon rostratus, Orthotrichum rupestre* and *Mnium cuspidatum* [14]. 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 [15].

However, most of the Hungarian bryophytes have not been examined phytochemically and pharmacologically. These species might be considered as an udiscovered 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 Literature overview

3.1 Botany

There are two hypotheses which could explain the evolution of bryophytes. According to the first theory, bryophytes are originated from filamentous fresh water green algae or unicellular green algae and pteridophytes (ferns) are originated from these bryophytes. This theory is called progressive theory. The second, reductive theory states that vascular plants originated from green algae, and both bryophytes and pteridophytes evolved from the extinct Rhyniophytinia class [16]. The two theorys are shown in **Figure 1**.

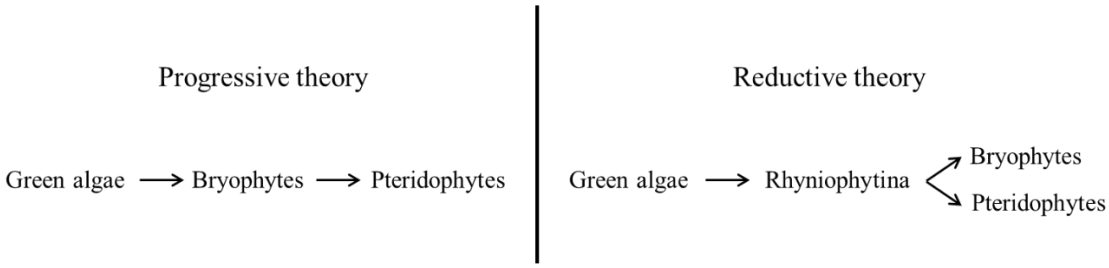


Figure 1. Theorys of bryophyte evolution.

In order to understand the evolution, the examination of the similarities of the chemical profiles is necessary. Certain compounds with very specific structures can be helpful in shedding light on questions about the evolution of certain taxa. Bis(bibenzyl)s and monomeric bibenzyls are the characteristic compound of the Hepaticae, which are also reported in some ferns. The first bis(bibenzyl) isolated from the pteridophytes is perrottetin H. This findings show clear relationship between ferns and liverworts, however the results are not trustable enough to decide which theory is acceptable [16].

3.1.1 Botany of *Paraleucobryum longifolium* and the *Paraleucobryum* genus

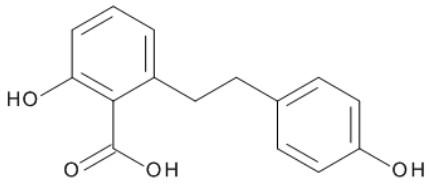
The *Paraleucobryum* genus belongs to Dicranaceae family, Dicranales order, Dicranideae subclass, Bryopsida class, Bryophyta division. *Paraleucobryum* genus is mainly native to the northern hemisphere and contains 7 species [17].

The genus was first introduced by Lindenberg [18]. The characteristics of the genus are the lanceolate and acuminate leaves with wide costa. The cross section of the leaves is made up of three layers of cells, ventral and dorsal hyalocysts which are hyaline cells, and a middle layer of chlorocysts. Alar cells are hyaline or brownish [17]–[19].

Paraleucobryum longifolium (Ehrh. ex Hedw.) Loeske, Hedwigia, 1908 is a silky, tufted plant, pale green when moist, whitish when dry. Plant are 40-60 mm long, leaves are 4-8 mm long, flexuose when dry, secund or falcate-secund when moist. The cross section of the costa is divided into 3 layers, ventral and dorsal layers of hyaline cells, and medium layer of chlorocysts. Dorsal layer contains some chlorocysts as well. Capsules are 1.5-4 mm long, simple, straight and smooth, operculum is 1-2 mm long [17]–[19]. *P. longifolium* is distributed in North America, Russia (Siberia), Europe, Iceland, Turkey, Caucasus, Madeira, Greenland [17], [20]. The plant grows on cliffs, tree trunks, stumps, and rotten logs, in moderate to high (400-2900 m) altitude [20].

3.2 Phytochemistry of bryophytes

Bryophytes produce oligo-, poly-, and trisaccharides that are different from higher plants and triglycerides, waxes, glycolipids and phospholipids. Unsaturated fatty acids like eicosapentaenoic acid and arachidonic acid are present [21]. Polyunsaturated fatty acids may play a role in freeze tolerant ability of bryophytes and that could be the reason why they can be found even in Antarctica [10], [22]. Liverworts produce lunularic acid (**1**), which is a dormancy factor and growth regulator [21].

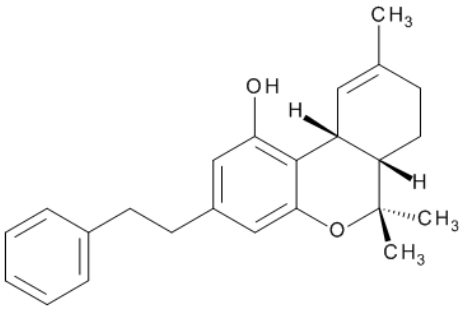


1

Characteristic secondary metabolites of bryophytes are mono-, sesqui-, di- and triterpenes, flavonoids, sterols, phenolic bibenzyls, bis (bibenzyls), dihydrostelbenes [2], [23], different lignans [24] and other aromatic compounds like benzoates, cinnamates, long-chain alkyl phenols, naphthalenes, phthalides, coumarins, and isocoumarins [25], [26]. These metabolites cause the specific fragrance, odour, pungency of the bryophytes. Interestingly, most of the sesqui- and diterpenoids reported from bryophytes are the enantiomers of compounds that can be found in higher ordered plants [2], [27], altough they may contain both enantiomers of the same compound [12], [16].

Nomerous cyclic and acyclic bis-bibenzyls have been reported from liwerworths especially from *Marchantiophyta* genus. These compounds are oligomers of polyhydroxylated dihydrostilbenes, formed mostly by cyclic dimerisation via biaryl (C─C) and/or diarylether (C─O)

linkage. Naturally occurring bis-bibenzyls are divided into three types based on the linkage between the macrocyclic rings: two biphenyl ether C─O bonds (type 1), a diphenyl ether C─O and a biaryl C─C bond (type 2), and two biphenyl C─C bonds (type 3). Macrocyclic bis-bibenzyls show nitric oxide production inhibitory, α-glucosidase inhibitory, apoptotic, antimicrobial, antifungal, cytotoxic, anticancer, and muscle relaxing activity [28]. Chlorinated bisbibenzyls were also reported, which are very rare in liverworts [29]. From the liverwort *Riccardia marginata* was the first time chlorinated bibenzyls were reported as a natural source [30]. A great investigation was carried out in 1994, when Asakawa and his co-workers isolated (−)-cis-perrottetinene (**2**), a bibenzyl cannabinoid from liverwort *Radula perrortetii* that structurally resembles to (−)-Δ9-trans-tetrahydrocannabinol (Δ9-trans-THC) (**3**) [31]. Later (−)-cis-perrottetinene (cis-PET) was reported from *Radula marginata* [32].



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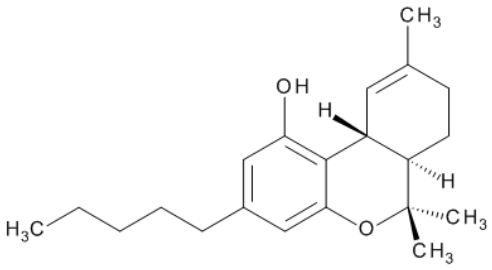


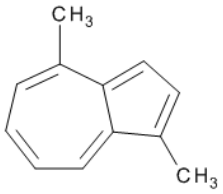
3

Because of the oil bodies, liverworts contains mono-, sesqui- and diterpenes with various type of skeleton, like gymnomitrane, pinguisane, 2,3-secoaromadendran, sphenelobane [27], however there are some mosses, like *Mnium, Plagiomnium, Homalia, Plagiothecium* and *Taxiphyllum* species, that contain mono-, sesqui- and diterpenes. Beside the typical type of terpene synthase genes, btyophytes have additional uniqe genes for terpene synthesis, which is called microbial-terpene synthase-like (MTPSL) genes. This is the reason why bryophytes, especially liverworts, are rich source of unique terpenoids. From liverworts more then 1600 terpenoids have been reported in the last 40 years [12].

Monoterpenes are characteristic compounds of the liweworths [16]. These compounds are responsible for the specific odor [12].

From liverworts about 900 sesquiterpenes have been isolated, divided in about 60 structural types. Eudesmane and aromadendrane-type are the most common, but cuparanes, pinguisanes, barbatanes [12], germacranolide, guaianolide, 2,3-secoaromadendrane, drimane-type compounds also occur frequently. Several sesquiterpene lactones which are responsible for allergic contact

dermatitis are reported [25]. It is noteworthy that africane-type sesquiterpenoids, which are very rare in nature, are reported from liverworts, and liverworts are the only natural source of pinguisane-type compounds [12]. From liverworts azulenes have been reported, 1,4-dimethylazulene (**4**) seems to be the main azulene, and oxygen substituted derivatives are also presented [33], [34].



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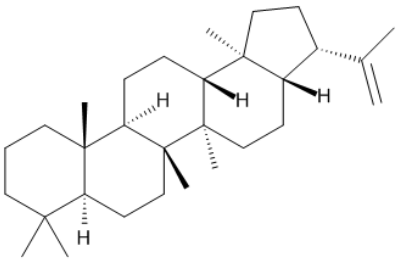
About 500 acyclic, di-, tri-, tetra-, and pentacyclic diterpenes have been isolated from liwerworths. The most abundant skeletons are labdane, clerodane and kaurane [12], but seco-clerodane, sacculatane, ent-kaurene, homoverrucosane and epi-neoverrucosane-type skeletons are also present [25], [29], [34]. Sacculatane, infuscane, abeo-labdane, spiroclerodane, 5,10-seco-clerodane, and 9,10-seco-clerodane-types are liverwort-specific diterpene skeletons [12].

The most common triterpenes in liverworts are hopane-type, such as diploptene (**5**), diplopterol (**6**), and α-zeorin (**7**) [12], [34], but lupane, oleanane, ursane, cycloartane, and serratane skeletons are also reported [12].



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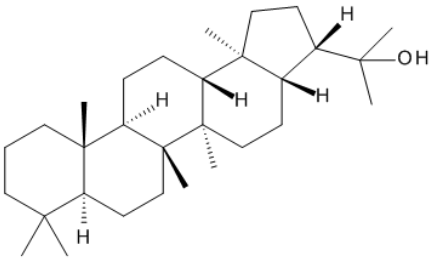

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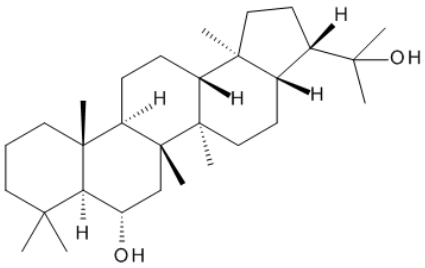

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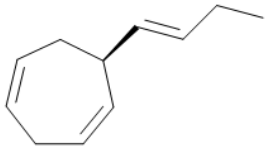
Acetogenins (*E*)-ectocarpene (**8**) and dictyopterene (**9**), which are brown-algae pheromones, are reported from the liverwort *Anastrophyllopsis involutifolia* [35].



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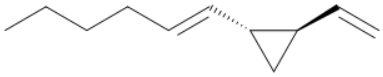

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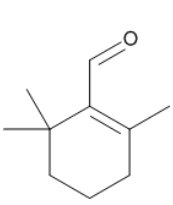
From mosses, the most abundant monoterpenes presented are β-cyclocitral (**10**), α- (**11**) and β-pinene (**12**), limonene (**13**), camphene (**14**), and bornyl acetate (**15**), and about 100 sesquiterpenoids and only a few diterpenoids are reported [12], [36]. Triterpenes are relatively rich in mosses, ursane-, fernane-, friedelane-, hopane-, lupane-, taraxane-, cycloartane-, obtusifolane-, dammarane-, polypodane-, and serratane-type triterpenoids are reported [12].

Hornworts are the less studied class among bryophytes, only a limited data is available. Common mono-, sesqui-, and diterpenes are reported. The most abundant monoterpenes are α- (**11**) and β-pinene (**12**), myrcene (**16**), terpinolene (**17**), and limonene (**13**). From sesquiterpenes aristolene (**18**), maaliol (**19**), veticadinoxide (**20**), diplophyllolide (**21**), anastreptene (**22**), β-bazzanene (**23**), β-barbatene (**24**), and δ-cuprenene (**25**) are present [12].



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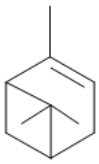

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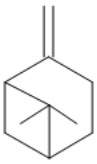

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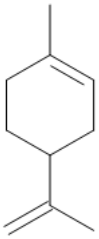

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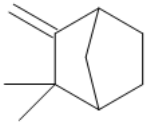

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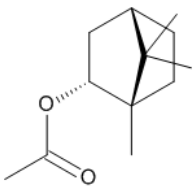

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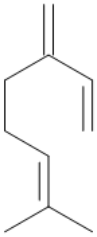

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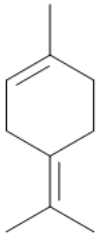

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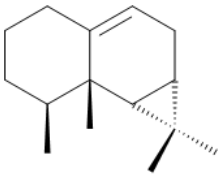

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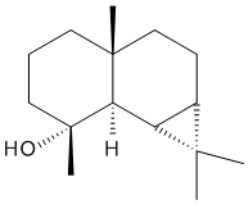

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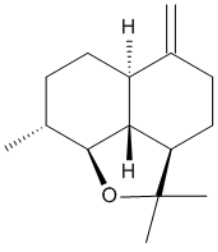

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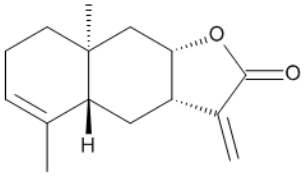

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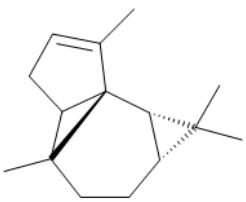

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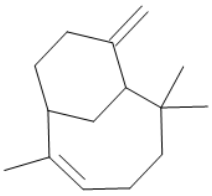

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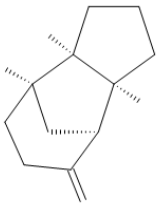

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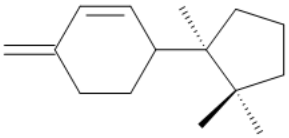

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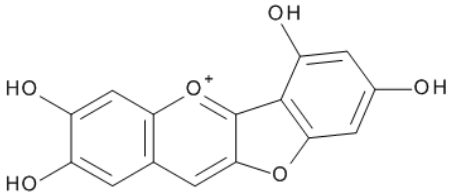
Anthocyanidins, as the pigments of bryophytes are reported, such as riccionidin A (**26**) and its dimer riccionidin B (**27**) [34], [37].



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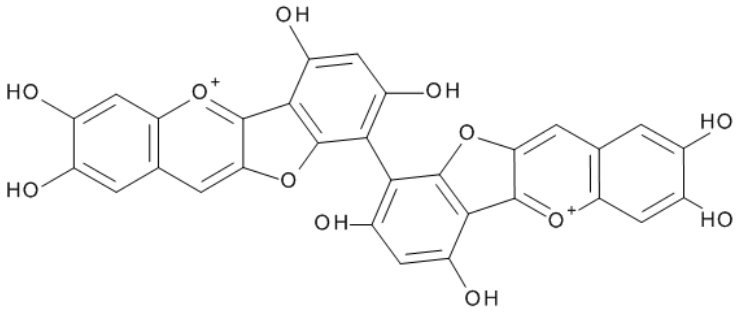

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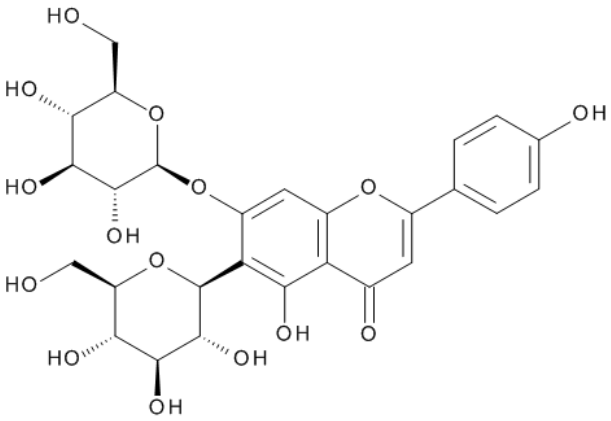
Flavonoids are common secondary metabolites of bryophytes, the first identified one was saponarin (isovitexin-7-*O*-glycuside) (**28**) from *Madotheka platypylla* [27]. Biflavonoids are the characteristic compounds of mosses among bryophytes, dimers have been reported from *Hypnum cupressiforme*, such as hypnogenol A (**29**) and hypnogenol B (**30**). The first identification of dihydroflavonols were reported from *Hypnum cupressiforme* [38], [39]. From mosses triflavonoids have also been reported [26].



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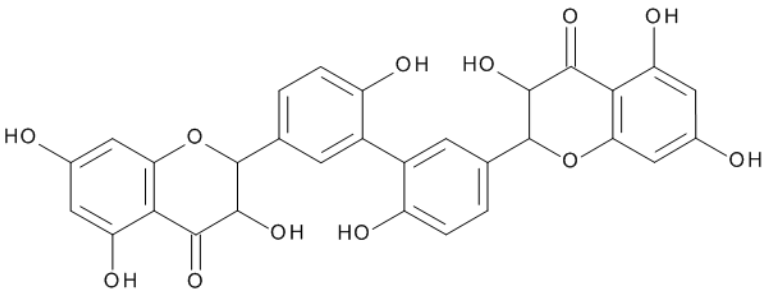

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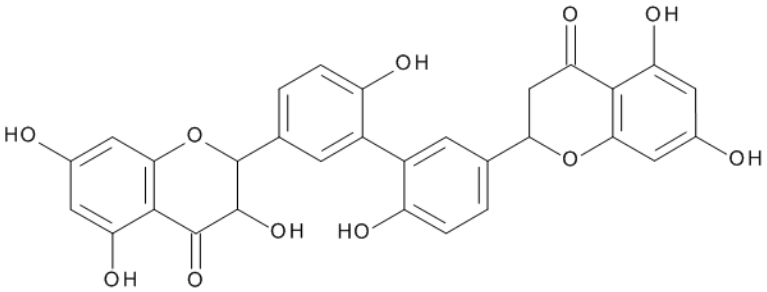
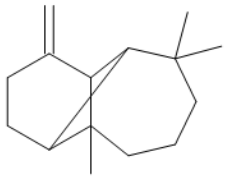


30

The oil of bryophytes contains high amount of mono- and sesquiterpenes, from them the most common compounds are α-pinene (**11**) and β-longipinene (**31**). Heptanal, tricyclene (**32**), α-thujene (**33**), camphene (**14**), thuja-2,4(10)-diene (**34**), β-pinene (**12**), 3-octanone, 2-amylfuran (**35**), octanal, α-phellandrene (**36**), α-terpinene (**37**), o-cymene (**38**), limonene (**13**), benzeneacetaldehyde, γ-terpinene (**39**), p-cymenene (**40**), nonanal, α-campholenal (**41**), *E*-pinocarveol (**42**), pinocarvone (**43**), terpinen-4-ol (**44**), myrtenal (**45**), decanal, 2E-decanal, 2E,4Z-decadienal, 2E,4E-decadienal, undecanal, α-cubebene (**46**), α- (**47**) and β-copaene (**58**), β-bourbonene (**49**), β-elemene (**50**), aromadendrene (**51**), aromadendrane-dehydro (**52**), α-humulene (**53**), α- (**54**) and γ-muurolene (**55**), β-selinene (**56**), viridiflorene (**57**), α-cadinene (**68**), γ-cadinene (**69**), δ-cadinene (**60**), *E*-cadina-1(2),4-diene (**61**), α-calacorene (**62**), caryophyllene oxide (**63**), α-selina-3,11-dien-6-ol (**64**), cubenol (**65**), *E*-calamenen-10-ol (**66**), cadalene (**67**), pentadecanol, hexahydrofarnesyl acetone, cyclohexadecanolide, eicosane, abietadiene (**68**), docosane, sandaracopimarinol (**69**), tricosane, tetracosane, pentacosane are presented in the oil in lower concentration [40]. Triterpenes have been identified, namely 21-hopene, 22(29)-hopene, ursolic acid, cyclolaudenol, 31-norcyclolaudenol. From sterols, campesterol, stigmasterol and β-sitosterol have been isolated. Normal alkanes from C_{23} to C_{32} and fatty acids from C_{12} to C_{24} also have been described [41].



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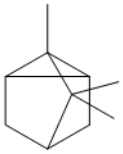

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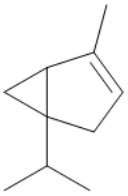

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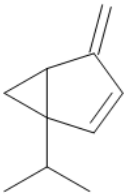

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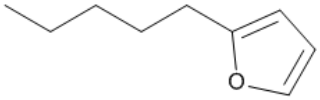

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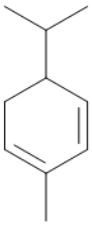

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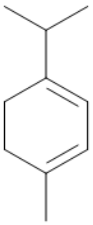

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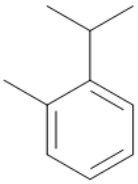

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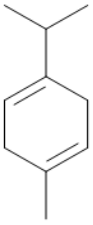

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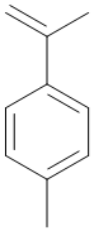

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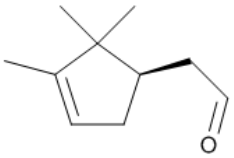

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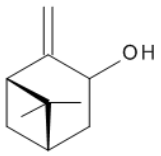

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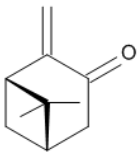

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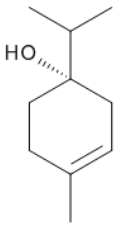

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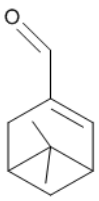

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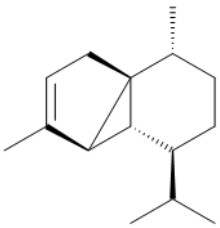

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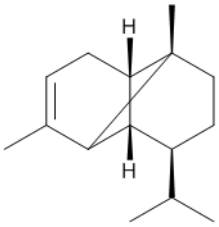

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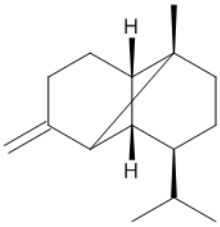

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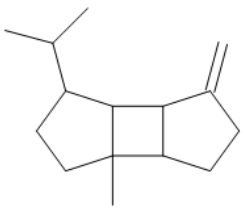

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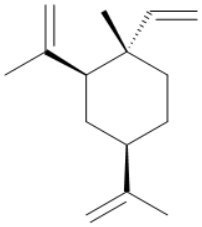

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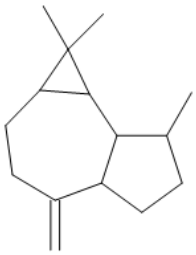

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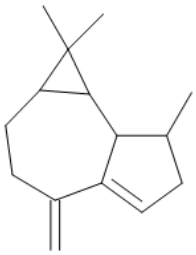

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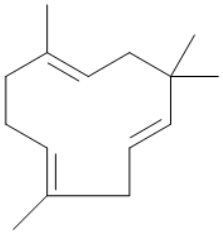

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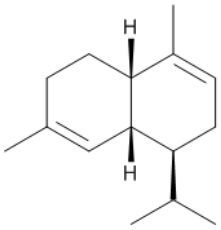

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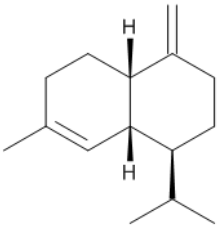

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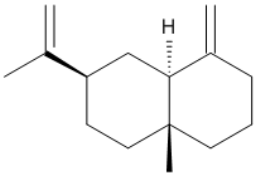

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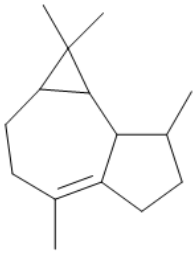

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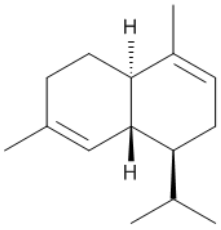

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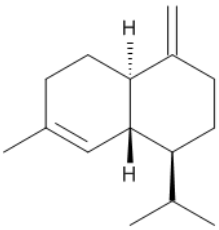

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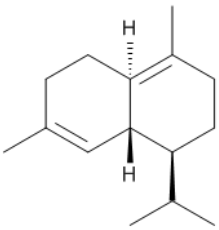

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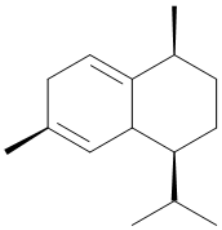

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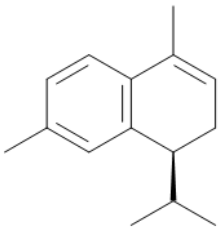

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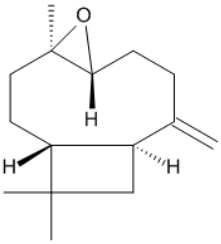

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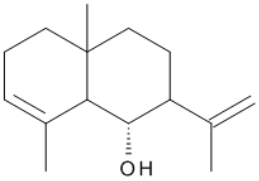

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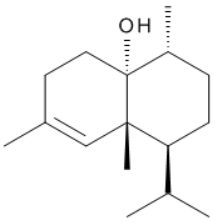

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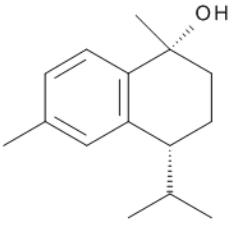

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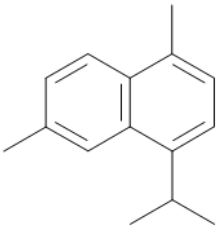

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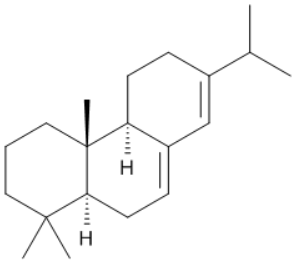

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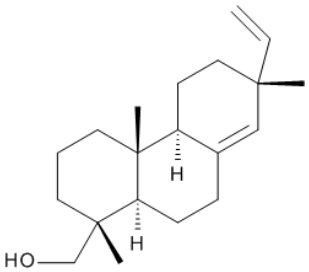

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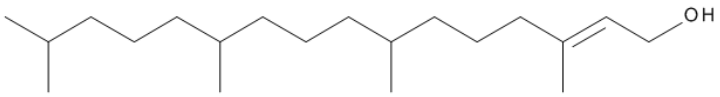
3.2.1 The chemical composition of *Paraleucobryum longifolium*

Paraleucobryum longifolium is an undiscovered species by phytochemical pont of view. In the first phytochemical studies, *P. longifolium* was investigated for lipid constituents; wax esters, hydrocarbons and steryl esters have been described from the plant [42]. Only fatty acids as palmitic acid , stearic acid, oleic acid, linoleic acid, linolenic acid [43] and wax esters as phytol (**70**) and phytenic acid (**71**) have been isolated [44]. No pharmacological studies have been reported.



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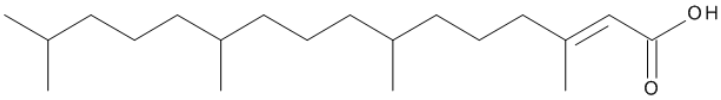

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3.2.2 Chemistry of phenanthrenes

Phenanthrenes are the secondary aromatic metabolites of higher plants, identified from Annonaceae, Aristolochiaceae, Cannabaceae, Combretaceae, Dioscoreaceae, Euphorbiaceae, Juncaceae, Lauraceae, Malpighiaceae, Orchidaceae, and Stemonaceae families [45], [46]. Phenanthrenes possess various biological activities such as antiproliferative, antimicrobial, anti-inflammatory, antioxidant, antiallergic, spasmolytic, and anxiolytic effects [45]–[47].

Phenanthrenes are formed from stilbenes by the oxidative coupling of the aromatic rings, and can be divided into three groups: mono-, di- and triphenanthrenes. Monophenanthrenes can be classified by the attached substituents. There are hydroxy- and/or methoxysubstituted, methyl-, hydroxymethyl-, formyl-, prenyl-, vinyl- and oxymethylsubstituted phenanthrenes and 9,10-dihydro- or dehydro derivatives and phenanthraquinones. Glycosides are relatively rare. Diphenanthrenes are classified by the type of connection of the phenanthrene monomers, which can be 1-1’, 1-3’, 1-8’, 3-3’, 7-7’, 8-3’, 8-8’ and 8-11’-linkages. The only isolated thriphenanthrene is monbarbatain D, which is formed by the coupling of three lusianthridin units [45]–[47].

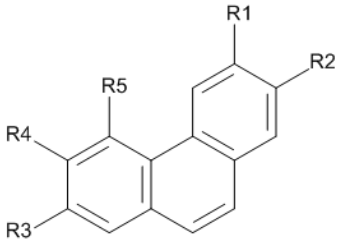
3.2.3 Phenanthrenes from bryophytes

In 1987, Asakawa isolated 2-hydroxy-3,7-dimethoxyphenanthrene (**72**) from *Marchantia polymorpha.* It was the first phenanthrene described from bryophytes [48]. From this species, two new phenanthrenes: 2,3-dimethoxy-7-hydroxyphenanthrene (**73**), 2,7-dihydroxy-3-methoxyphenanthrene (**74**) and three biphenanthrenes: 3,3'-dimethoxy-2,2',7,7'-tetrahydroxy-1,1'-biphenanthrene (**79**), 3-methoxy-2,2',3',7,7'-pentahydroxy-1,1'-biphenanthrene (**80**), 2,2',3,3',7,7'-hexahydroxy-1,1'-biphenanthrene (**81**) were described in 1994 [49]. In the *Marchantia* genus, *Marchantia paleacea* var. *diptera* produces 2-hydroxy-3,7-dimethoxyphenanthrene (**72**) [50] and 2-hydroxy-3,6-dimethoxyphenanthrene (**78**) [51] and *Marchantia tosana* produces 2,5-dimethoxy-3-hydroxyphenanthrene (**79**) [51], [52] and 2,7-dimethoxy-3-hydroxyphenanthrene (**80**) [52].

Plagiochila species contain various kinds of phenanthrenes. *Plagiochila spinulosa* contains two 9,10-dihydrophenanthrenes: 2-hydroxy-3,4,7-trimethoxy-9,10-dihydrophenanthrene (**82**), and 3,4,7-trimethoxy-9,10-dihydrophenanthrene (**83**) [51]. *Plagiochila oresitropha* contains one phenanthrene: 2-hydroxy-3,5-dimethoxy-phenanthrene (**78**), and eight 9,10-dihydrophenanthrenes: 2-hydroxy-3,5-dimethoxy-9,10-dihydrophenanthrene (**84**), 2,3-dimethoxy-5-hydroxy-9,10-dihydrophenanthrene (**85**), 2,3,5-trimethoxy-9,10-dihydrophenanthrene (**86**), 2-hydroxy-3,7-dimethoxy-9,10-dihydrophenanthrene (**87**), 2,3,7-trimethoxy-9,10-dihydrophenanthrene (**88**), 2,6-dimethoxy-5-hydroxy-9,10-dihydrophenanthrene (**89**), 3-methoxy-4,5-dihydroxy-9,10-dihydrophenanthrene (**90**), 2,3,5,7-tetramethoxy-9,10-dihydrophenanthrene (**91**) [52], [53].

Riccardia multifidi and *Riccardia jackii* are chemically similar, they both produce 3,4-dimethoxy-5-hydroxy-9,10-dihydrophenanthrene (**92**) [51], [54]. From the crude extract of the unidentified Indonesian and Tahitian *Frullania* species 2,3,5-trimethoxy-9,10-dihydrophenanthrene (**86**) was obtained [4].

Bis(bibenzyl)-phenanthrenes have also been investigated in bryophytes. From *Frullania convoluta* 2'-(11-hydroxy-1-bibenzyl-oxy)-1'-methoxy-6',10',11'-trihydroxy-7',8'-dihydro-phenanthrene (**93**), 2'-(10,11-dihydroxy-1-bibenzyl-oxy)-1'-methoxy-6',10',11'-trihydroxy-7',8'-dihydro-phenanthrene (**94**) [52], [55], from *Bazzania trilobata* bazzanin K (**95**) [52], [56], and from *Cavicularia densa* (+)-cavicularin (**96**) were isolated [52], [57].



72 R_1=R_3=OMe, R_2=OH, R_4=R_5=H

73 R_1=R_2=OMe, R_3=OH, R_4=R_5=H

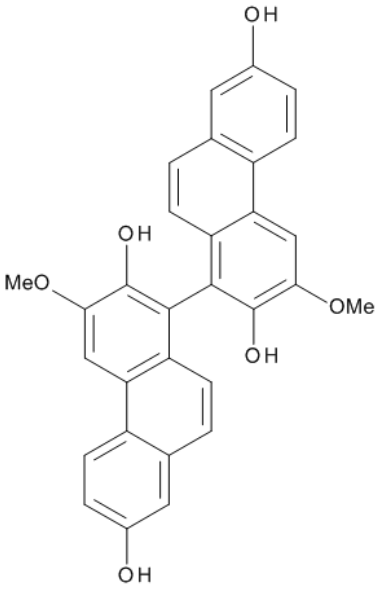
74 R_1=OMe, R_2=R_3=OH, R_4=R_5=H

75 R_1=R_4=OMe, R_2=OH, R_3=R_5=H

76 R_1=OH, R_2=R_5=OMe, R_3=R_4=H

77 R_1=OH, R_2=R_3=OMe, R_4=R_5=H

78 R_1=R_5=OMe, R_2=OH, R_3=R_4=H



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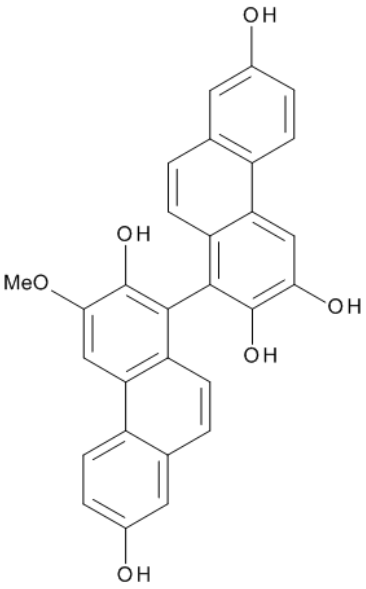

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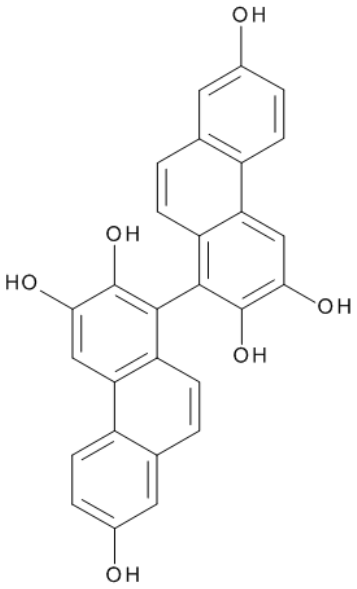

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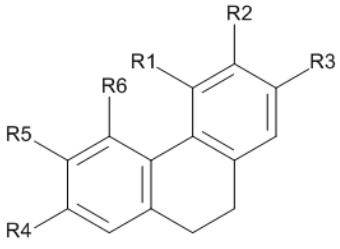


82 R_1=R_2=R_4=OMe, R_3=OH, R_5=R_6=H

83 R_1=R_2=R_4=OMe, R_3=R_5=R_6=H

84 R_1=R_4=R_5=H, R_2=R_6=OMe, R_3=OH

85 R_1=R_4=R_5=H, R_2=R_3=OMe, R_6=OH

86 R_1=R_4=R_5=H, R_2=R_3=R_6=OMe

87 R_1=R_5=R_6=H, R_2=R_4=OMe, R_3=OH

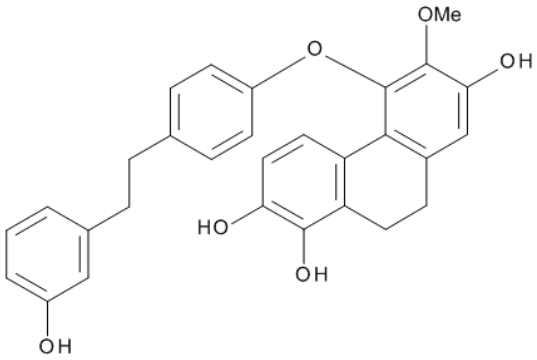
88 R_1=R_5=R_6=H, R_2=R_3=R_4=OMe

89 R_1=R_2=R_4=H, R_3=R_5=OMe, R_6=OH

90 R_1=R_6=OH, R_2=OMe, R_3=R_4=R_5=H

91 R_1=R_5=H, R_2=R_3=R_4=R_6=OMe

92 R_1=R_2=OMe, R_3=R_4=R_5=H, R_6=OH



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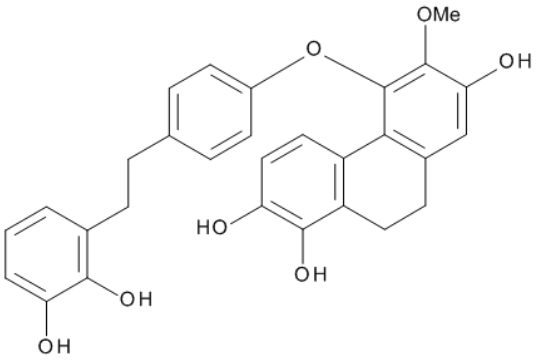

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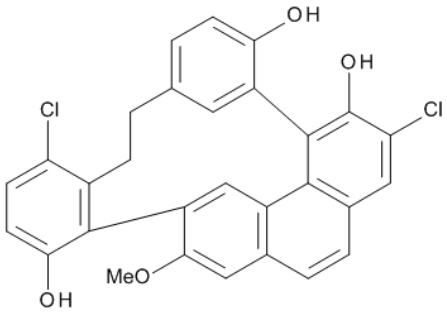

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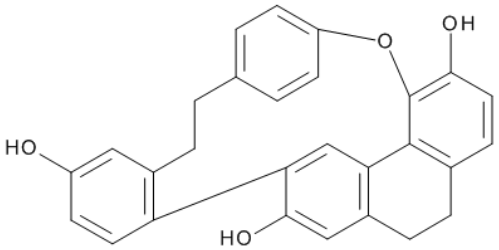

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3.3 Ethnomedicinal use of bryophytes

The folk medicinal use of bryophytes leads back to more than 1000 years. The Chinese medical text from the 11th century, the Jia You Ben Cao already mentioned the use of bryophytes [58].

Bryophytes are used in the folk medicine in China, India and among the American natives. There are still 63 species in medical use in China, 22 in India in the Himalayas. Native Americans used bryophytes not only as medicine, but as fiber and for chloting [6]. In China there are traditional markets in Guizhou (47 species in use), Yunnan (18 species in use) and Sichuan [59].

Polytrichum commune is a commoly occuring species, therefore it was widely used bryophyte in different cultures, especially in China. There are early notes about this species in the Jia You Ben Cao. The Compendium of Materia Medica (Ben Cao Gang Mu), which was written in the 16th century by Li Shinzen, mentions that species with the name “horse mane of the earth”. In China, *P. commune* was used as a diuretic and to treat fever, to stop bleeding and to reduce inflammation. In Europe, this species was mainly used as a fine fiber for clothing, however, the oil extract was used to strengthen the hair [58]. *Polytrichum alpinum* is used as anticatarrhal with the combination of *Sambucus nigra* and *Ramonda myconi* [60].

Amblystegium serpens was used to stop bleeding, and cure injuries [58]. *Barbula unguiculata* was used as an analgesic and to reduce fever, and *Bryum argenteum* was used as an antipyretic and as an antifungal agent in folk medicine [61].

The Doctrine of Signature is an acient method based on the concept that if the shape of a plant part is similar to a human organ, it can cure the disease of that body part. That is the reason why liverwors were considered to aid the liver [6], [61]. For example, in the folk medicine, *Marchantia polymorpha* is used to treat liver ailments, because its surface similar to the cross section of the liver. In China, it is still in use to treat jaundice of hepatitis and to clean the liver. In this genus, *Marchantia convoluta* and *Marchantia palacea* are also used with the same indications [6], [62]. In Europe *Marchantia polymorpha* was used to be a diuretic [5].

In China, *Rhobodryum* species are used to cure cardiovascular diseases and hypertension. There is a Chinese study, where the ether extract of *Rhodobryum giganteum* showed 30% increase in the flow rate in mice aorta, therefore the oxygen resistance was reduced [63]. Another *Rhobodryum* species, *Rhobodryum ontariense* may control hypertension. In an experiment, the bolus of *R. ontariense* normalized the arterial blood pressure in spontaneously hypertensive rats [64].

In Chinese tradicional medicine *Huperzia serrata* was used to improve blood circulation, redice fever and pain. The metabolite which can be responsible for the effect might be huperzine A, which is lycopodium alkaloid that reversible inhibits acetylcholinesterase *in vitro* and *in vivo* [65], [66]. In a double blind trial, huperzine A improved memory and behavior in patients with Alzheimer's disease and was more tolerated than donepezil and tacrine [65].

In the folk medicine, bryophytes were used to treat skin injuries, diseases and burns by promoting the healing of the skin. In the Himalayas, *Taxiphyllum taxirameum, Philonotis fontana, Plagiochasma appendiculatum, Bryum thomsonii, Marchantia polymorpha, Marchantia palmate* were widely used among the tribes. Also *Bryum*, *Mnium,* and *Philonotis* species and *Polytrichum juniperinum* were in used in China and in America in these indications [5], [6], [67]. In a Turkish study, the *in vivo* healing ability of the ethereal extracts of eight mosses were tested in two models. The ethereal extracts of *Reboulia hemisphaerica*, *Plagiochasma rupestre* and *Targionia hypophylla* showed potent wound healing capacity [67].

3.4 The use of bryophytes in the 21th century

The ability that mosses can take up nutrients and elements via their whole surface makes them perfect targets for biomonitoring air quality, especially heavy metal deposition. Nowadays, the

raw materials for biomonitoring are produced in bioreactors via an axenic *in vitro* cultivation process. With this development standardized and comparable raw material can be produced [68].

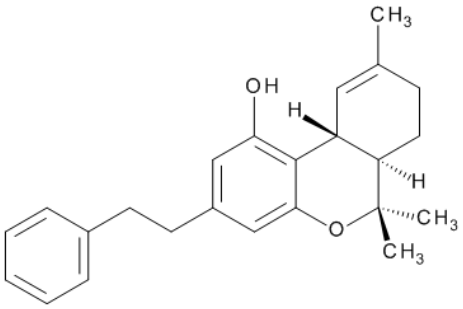
Moreover, *Physcomitrella patens* seems to be a perfect target for genetic engineering [69], [70]. In moss bioreactors various recombinant proteins are produced, such as recombinant human erythropoietin (rhEPO), which is neuro- and tissue-protective after stroke [70]. The production of recombinant glycoproteins plays an important role in the therapy of Fabry disease, a lysosomal storage disease where the patients suffer from deficient or defective α-galactosidase A [68]. Moss-aGal®, a recombinant human α-galactosidase, which is the innovation of Greenovation Biotech is in phase I clinical trial as the first moss produced drug candidate. By targeted knockout of a β1,2-xylo-syltransferase (XT) and an α1,3-fucosyltransferase (FT) genes, plant-specific sugar residue free glycoproteins can be produced. Unlike in the case of other plants, hyperglycosylated proteins, which are immunogenic in humans, are not produced and the adverse side effects are avoidable [68], [71]. Engineering seems to be usefeul in the production of therapeutical antibodies. Defucosylated moss-produced therapeutic immunoglubin G shows a higher antibody-dependent cellular cytotoxicity (ADCC) than the parenteral fucosylated IgG, as the defucosylated form is not affected by the blocking effect of endogenous IgG to the receptors [72].

In the cosmetic industry, Mibelle Biochemistry developed an anti-aging cosmetic ingredient named MossCellTec™ No. 1, based on the aquosus extract of *Physcomitrella patens* [68]. The extract may enhance the resilience the of the skin in stressful conditions by increasing the skin homogeneity and improving the hot-humid/cold-dry adaptation of reconstructed skin [73].

Sphagnum genus is used to produce materials for different aims. *Sphagnum* vegetation restoration is a process where *Sphagnum* mosses are re-established to areas in order to control erosion, absorb carbon-dioxide and for nature conservation. The re-established vegetation is not being harvested. Another aim is *Sphagnum* farming, which is a new type of peatland to produce moss biomass with a maximum yield, which provides raw material for horticultural growing media [74].

The liverwort *Radula marginata*, sold on the internet as a legal psychoactive drug, possesses cannabis-like effects. The compound responsible for this effect is the bibenzyl cannabinoid cis-PET (**2**). Despite the compound was first reported in 1994, no pharmacological studies were performed until 2018. Both cis-PET and its trans isomer have been tested for their pharmacological effects. For the *in vitro* and *in vivo* experiments on mice, trans-PET (**97**) was produced synthetically. This compound is an agonist of human CB_1R and human CB_2R and easily penetrates the brain. Through its effect on CB_1R, cis-PET induced analgesia, catalepsy,

hypolocomotion, and hypothermia. Similar to the diastereomers of THC, cis-PET is less potent CB_1R partial agonist than trans-PET. The only main difference between THC and PET is that PET diastereoisomers significantly reduced basal prostaglandin D2 and E2 levels in the brain in a CB_1R-dependent manner, limiting its adverse effects and reducing neuroinflammation. Cis-PET (**2**) is 10 times less potent on CB_1R than trans-THC (**3**), and the natural occurrence of cis-PET (**2**) is much lower than trans-THC (**3**), that is why the abuse potential of smoked *Radula marginata* is low [75]–[77].



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4 Materials and methods

4.1 Plant material

For phytochemical screening, 42 species of bryophytes were selected. Each of them 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. The plant was dried in room temperature, than was chopped in a Retch SM 100 cutting mill.

4.2 Purification and isolation of compounds from *P. longifolium*

Open column chromatography was carried out on plyamide (ICN Polyamide for column chromatography; **CC-P**: 600.0 g) and on normal phase SiO_2 (silica gel, 45-63 μm particle size, Molar Chemical Kft.; **CC-NP**: 30 g).

Mobile phases:

CC-P: MeOH-H_2O [2:8, 4:6, 6:4, 8:2, 1:0 (5 L, 5L, 12 L, 9 L, 13 L, respectively)].

CC-NP: toluene-$CHCl_3$-MeOH isocratic elution 2:6:1.5, 20 fractions collected, volume of collected fractions: fractions 1-4: 3 mL, fraction 5-8: 8 mL, fractions 9-18: 2 mL, fractions 19-20: 10 mL.

Vacuum liquid chromatography was carried out on normal phase SiO_2 (silica gel, 45-63 μm particle size, Molar Chemical Kft.; **VLC-NP I**: 101.16 g, **VLC-NP II**: 425.0 g) and on reversed phase SiO_2 (RP-18, **VLC-RP**: 4.5 g).

Mobile phases:

VLC-NP I: cyclohexane-$CHCl_3$-MeOH [90:9.9:0.1, 80:19.8:0.2, 70:29.7:0.3, 0:9:1, 0:8:2, 0:7:3, 0:6:4, 0:5:5, 0:4:6, 0:3:7, 0:2:8, 0:0:1 (1 L, 1.5 L, 1 L, 1 L, 4 L, 1 L, 3 L, 2 L, 2 L, 1 L, 2 L, 0.6 L)] volume of collected fractions: 100 mL.

VLC-NP II: $CHCl_3$-MeOH [10:0, 9.6:0.4, 9.3:0.7, 9:1, 8:2, 7:3, 0:1 (400 mL, 400 mL, 400 mL, 400 mL, 200 mL, 1200 mL, 600 mL)] volume of collected fractions: 200 mL.

VLC-RP: H_2O-MeOH [6:4, 5:5, 4:6, 3:7, 2:8, 0:1 (20 mL, 20 mL, 26 mL, 26 mL, 28 mL, 30 mL) volume of collected fractions: 2 mL.

Gel filtration chromatography was carried out on Sephadex® LH-20 (25-100 μm, Pharmacia Fine Chemicals; **GFC I**: 5.0 g, **GFC II**: 35.0 g, **GFC III**: 35.0 g, **GFC IV**: 35.0 g).

Mobile phases:

GFC I: MeOH; volume of collected fractions: fraction 1: 10 mL, fractions 2: 5 mL, fractions 3-35: 1 mL.

GFC II: MeOH, volume of collected fractions: fraction 1: 10 mL, fraction 2: 5 mL, fractions 3-20: 2 mL.

GFC III: MeOH, volume of collected fractions: fraction 1: 10 mL, fraction 2: 5 mL, fractions 3-32: 2 mL.

GFC IV: MeOH, volume of collected fractions: fraction 1: 10 mL, fraction 2: 5 mL, fractions 3-23: 2 mL.

High pressure liquid chromatography was performed on a Waters HPLC system (In-line Degasser AF, 600 Controller, 600 Pump, 2998 Photodiode Array Detector; Waters Corporation, Milford, Massachusetts, USA) with different reversed phase methods (HPLC-RP I, HPLC-RP II, HPLC-RP III, HPLC-RP IV).

Mobile phases:

HPLC-RP I: H_2O-IPA gradient system (0 min: H_2O-IPA 9:1, 1 min: 9:1, 10 min: 4:6, 11 min: 3:7, 12 min: 9:1, 18 min: 9:1), flow rate: 1 mL/min, column: Kinetex C-18 (100 A, 150×4.6 mm, 5 μm; Phenomenex, Torrance, USA), RT: 9.93 min. The detection was performed in the whole UV wavelength range and specifically at 254 nm.

HPLC-RP II: H_2O-MeCN gradient system (0 min: H_2O-MeCN 6:4, 1 min: 6:4, 10 min: 4:6, 11 min: 1:9, 12 min: 6:4, 17 min: 6:4). flow rate: 1.5 mL/min, column: Kinetex XB-18, 100 A, 250×4.6 mm, 5 μm (Phenomenex, Torrance, USA). The detection was performed in the whole UV wavelength range and specifically at 215 nm.

HPLC-RP III: H_2O-MeCN gradient system (0 min: H_2O-MeCN 25:75, 1 min: 25:75, 9 min: 15:85, 9.5 min: 0:1, 10.5 min: 0:1, 11 min: 25:75, 16 min: 25:75), flow rate: 1 mL/min, column: Kinetex XB-18, 100 A, 250×4.6 mm, 5 μm (Phenomenex, Torrance, USA). The detection was performed in the whole UV wavelength range and specifically at 215 nm.

HPLC-RP IV: H_2O-MeCN gradient system (0 min: H_2O-MeCN 65:35, 1 min: 65:35, 10 min: 62:38, 12 min: 62:38, 13 min: 0:1, 13.5 min: 65:35, 18 min: 65:35), flow rate: 1.5 mL/min, column: Kinetex XB-18, 100 A, 250×4.6 mm, 5 μm (Phenomenex, Torrance,

USA). The detection was performed in the whole UV wavelength range and specifically at 215 nm.

Flash chromatography was carried out on a Biotage Isolera^{TM} One system (Biotage, Uppsala, Sweden) with reversed phase method (RP-18, **FC-RP**: 60.0 g). Mobile phase:

FC-RP: H_2O-MeCN (from 5:5 to 0:1), flow rate: 36 mL/min.

Preparative layer chromatography was carried out on reversed phase SiO_2 (20×20 cm Silica gel 60 RP-18 $F_{254}S$, Merck; **PLC**). Detection was performed under UV light at 254 nm wavelength and on visible light. Mobile phase:

PLC: MeCN-H_2O 1:1.

4.3 Characterization and structure elucidation

NMR measurements played the main role in structure elucidation. The NMR spectra were recorded in methanol-d4 on a Bruker Avance DRX 500 spectrometer operating at 499.9 MHz for ^{1}H and 125.7 MHz for ^{13}C, and on a Bruker Avance NEO 700 spectrometer operating at 700.25 MHz (^{1}H) and 176.08 MHz (^{13}C), respectively. All measurements were performed at 298 K. The signals of the methanol-d_4 (1H: 3.35 ppm, 13C: 49.3 ppm) were taken as reference. 2D NMR data were acquired and processed with Bruker TopSpin 4.0.5 software. For 2D ^{1}H–^{1}H COSY, HSQC, HMBC, and EASY-ROESY experiments, Standard pulse programs were used.

The HRMS spectras were acquired on an FTHRMS-Orbitrap (Thermo-Finnigan) mass spectrometer equipped with ESI ion source in positive ionization mode.

ECD spectra were recorded on a JASCO J-810 spectropolarimeter. Solvents were distilled prior to use, and spectroscopic grade solvents were applied for spectroscopic measurements.

LC-MS was used to identify the possible compounds via the phytochemical screening from most potent extracts. The analysis of extracts was performed with a Thermo Q-Exactive Plus Orbitrap mass spectometer equipped with a HESI-II ion source coupled with an Agilent 1100 series model HPLC equipped with binary pump. For the separation the linear gradient of 0.1% formic acid in MS-grade water (eluent A) and 0.1% formic acid in MS-grade acetonitrile (eluent B) were used (from 5% to 95% B in 35 min) with a 0.3 mL/min flow rate. Columns used for the separation was Kinetex XB-C18 and Phenyl-Hexyl (Phenomenex; 2.1×100 mm, 2.6 um, 100 Å) columns. The mass accuracy was 0.25+/-0.01 ppm in the positive mode and 0.34+/-0.05 in the negative mode at the mass calibration just before the experiment. Acquisition was done in the data-dependent MS^2 scan mode by altering the charge state (positive/negative). The survey scan mass range was set between *m/z* 80–1000.

4.4 Pharmacological tests

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 (Universtity of Szeged, Albert Szent-Györgyi Medical School).

4.4.1 Antiproliferative assay

For phytochemical screening the antiproliferative activity was evaluated by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The extracts were assessed on human cervical (HeLa), ovarian (A2780), and breast (T47D) cancer cell lines, purchased from ECACC (European Collection of Authenticated Cell Cultures, Salisbury, U.K.) and were cultivated in minimal essential medium supplemented with 10% fetal bovine serum, 1% non-essential amino acids, and an antibiotic–antimycotic mixture. All media and supplements were obtained from Lonza Group Ltd. (Basel, Switzerland). The cells were maintained at 37 ºC in humidified atmosphere containing 5% CO_2. Near-confluent cancer cells were seeded onto a 96-well microplate (5000 per well) and attached to the bottom of the well overnight. 200 µL of new medium containing the tested substances (at 10 or 30 µg/mL) was added. After incubation for 72 h, the living cells were assayed by the addition of 20 µL of 5 mg/mL MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) solution. MTT was converted by intact mitochondrial reductase and precipitated as blue crystals during a 4 hour contact period. The medium was then removed, and the precipitated crystals were dissolved in 100 µL of DMSO during a 60 minutes period of shaking at 25 ºC. Finally, the reduced MTT was assayed at 545 nm using a microplate reader; wells with untreated cells were used as controls [78]. All experiments were carried out on two microplates with at least five parallel wells. Stock solutions of the tested substances (10 mg/mL) were prepared with DMSO. The highest DMSO content of the medium (0.3%) did not have any substantial effect on the cell proliferation. Cisplatin (Ebewe Pharma GmbH, Unterach, Austria) was used as a reference agent. The IC50 values of its antiproliferative action were 12.43, 1.30, and 9.78 µM against HeLa, A2780, and T47D cells, respectively.

The antiproliferative properties of the isolated compounds from *P. longifolium* were assayed by MTT method against cervical (HeLa and SiHa), breast (MDA-MB-231), and ovarian (A2780) cancer cells [78]. Cell lines were purchased from the European Collection of Cell Cultures (Salisbury, UK). The cells were grown in Minimum Essential Medium (MEM) supplemented with 10% fetal calf serum (FCS), 1% nonessential amino acids, and 1% penicillin-streptomycin. All media and supplements were obtained from Lonza Group Ltd. (Basel, Switzerland). Two

independent experiments were carried out with five wells for each condition. Clinically available drug cisplatin (Ebewe Pharma GmbH, Unterach, Austria) was used as a reference agent. All the calculations were performed using GraphPad Prism 5 software (GraphPad Software; San Diego, CA, United States).

4.4.2 Antimicrobial assay

The antimicrobial activity for the phytochemical screening was determined by the disc-diffusion method [79] on 11 standard strains. The standard Gram-positive strains were *Bacillus 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). Microbial cultures were grown on standard Mueller-Hinton agar plates or Columbia agar +5% sheep blood (COS) plates (bioMérieux, Marcy-l'Étoile, France) at 37 ºC under an aerobic or 5% CO_2 environment. The strains were stored in Cryobank vials (MAST Diagnostica, Rheinfeld, Germany) at -70 ºC and maintained at 4 ºC throughout the study to use as stock cultures. Bryophyte extracts were dissolved in DMSO (Sigma-Aldrich, St. Louis, MO, USA) or water at a concentration of 50 mg/mL. The sterile filter paper discs (6 mm in diameter) impregnated with the extracts (10 uL of redissolved extracts) were placed on the plate seeded with the respective bacterial suspensions (inoculums: 0.5 McFarland, 1–2 × 10^8 CFU/mL). DMSO served as the negative control, while ampicillin, erythromycin, imipenem, cefuroxime, and vancomycin antibiotic susceptibility discs were used as the positive control. The plates were incubated at 37 ºC for 24 h under aerobic or 5% CO_2 conditions. All measurements were carried out in triplicate. Positive activity was accepted above 6 mm of inhibition zone diameter.

5 Results

5.1 Phytochemical screening of Hungarian moss species

Air-dried, powdered plant materials were extracted using MeOH in an ultrasonic bath. After filtration and evaporation, the residues were dissolved in 50% aqueous MeOH and subjected to solvent–solvent partition with *n*-hexane that yielded aqueous layer I. and organic layer I, which after evaporation gave extract A. Aqueous layer I was further partitioned with $CHCl_3$ that yielded organic layer II and aqueous layer II. After evaporation, extraxt B and extract C was gained. The residual plant materials were dried and extracted with boiling H_2O. The filtered extracts were freeze-dried, affording extracts D (**Figure 2**).

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).

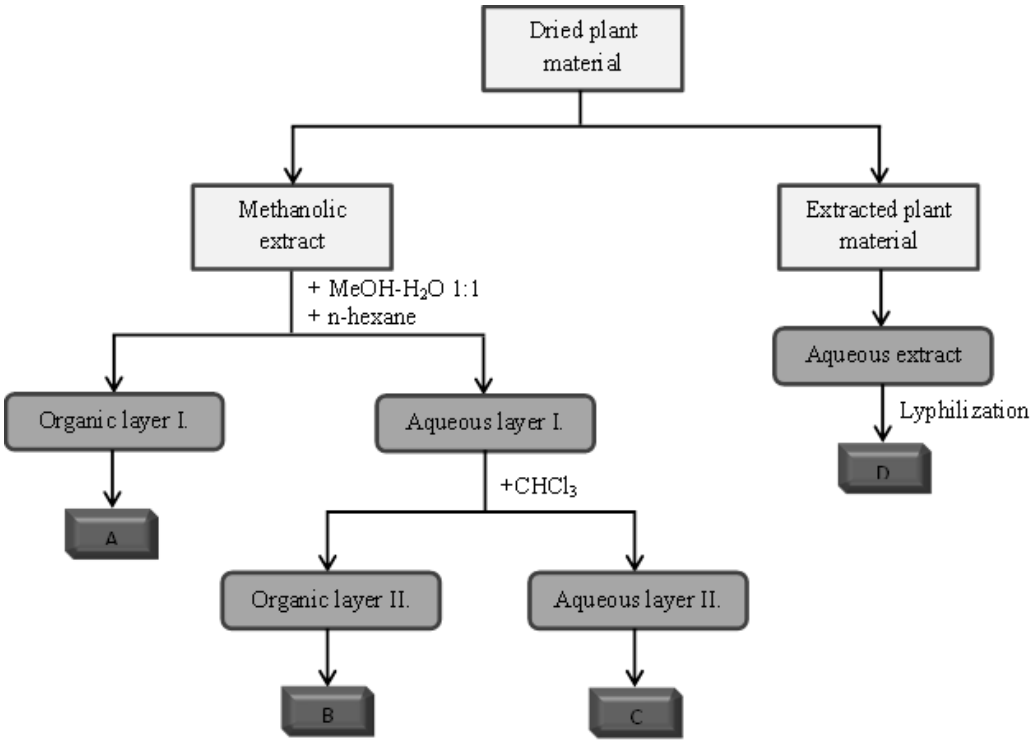


Figure 2. Extraction of bryophyte samples

5.2 Pharmacological activity of selected bryopytes via phytochemical screening

5.2.1 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. Results are presented in **Table 1**. 24/38/20/16 extracts were active from A/B/C/D fractions, respectively. From 17 species 25 extract showed more than 50% inhibition on

at least one of the cell lines at this concentration. This higher inhibition was most characteristic to B extracts (13), followed by A (7), C (4), and D (1). More than one extract was active in the case of six species, namely, *Brachythecium rutabulum*, *Climacium dendroides*, *Encalypta streptocarpa*, *Pleurozium schreberi* (A and B), *Neckera besseri*, and *Pseudoleskeella nervosa* (A, B, and C). At 30 g/mL, 36 samples of 26 species were inactive (25 of these were D extracts), hence further analysis of these extracts was unecessary. Apolar extracts (*n*-hexane and chloroform) were the most potent, polaric extracts showed weak or no activity. The B extract of *Paraleucobryum longifolium* showed the highest activity (78.54% inhibition on HeLa at 10 g/mL). Moreover, this extract was active on all the cell lines, and activities at 10 g/mL were not much lower than those at 30 g/mL (46.84–78.54% vs 56.87–83.93%). Interestingly, in the case of this species, only the $CHCl_3$ extract had remarkable activities. Concerning the sensitivity of the cell lines, the measure of inhibition was more pronounced in the cases of HeLa and T47D than A2780. On HeLa, 16 extracts; on T47D, 10 extracts; and on A2780, only 3 extracts exerted >50% inhibition at 10 g/mL. 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

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
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
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

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
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

5.2.2 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 ATCC 43300	*S. aureus* ATCC 29213	*S. epidermidis* ATCC 12228	*B. subtilis* ATCC 6633	*S. pyogenes* ATCC 19615	*S. pneumoniae* ATCC 49619	*S. agalactiae* ATCC 13813	*M. catarrhalis* 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**

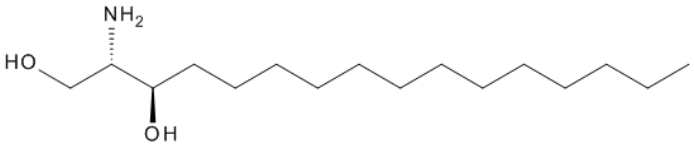
5.2.3 MS analysis

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 (Proteowizard), 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 (**Table 3**).

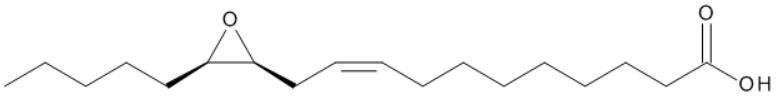
Table 3. Compounds identified by LC-MS from the most potent extracts

Species	Extract	Identified compounds
Atrichum undulatum	B	hexadecasphinganine (**98**), hydroxyoctadecadienoic acid, 18-oxooleate, vernolic acid (**99**)
Barbula unguiculata	B	hydroxyoctadecadienoic acid, 18-oxooleate, vernolic acid (**99**)

Species	Extract	Identified compounds
Bryum argenteum	A	hydroxyoctadecadienoic acid, lupenone (**100**), octadecanamide, 18-oxooleate, vernolic acid (**99**)
Encalypta streptocarpa	A	azelaic acid (**101**), hexadecasphinganine, hydroxyoctadecadienoic acid, 18-oxooleate, phytuberin (**102**), vernolic acid (**99**)
	B	abietic acid (**103**), dehydroabietic acid (**104**), neoabietic acid (**105**), alpha-amylcinnamaldehyde (**106**), artemorin (**107**), dehydrocostus lactone (**108**), isodehydrocostus lactone (**109**), eremanthin (**110**), dehydromyodesmone (**111**), glutinosone (**112**), hydroxyoctadecadienoic acid, ivalin (**113**), pseudoivalin (**114**), oblongolide (**115**), 18-oxooleate, santamarine (**116**), vernolic acid (**99**)
Neckera besseri	B	azelaic acid (**101**), hexadecasphinganine (**98**), hydroxyoctadecadienoic acid, 18-oxooleate, vernolic acid (**99**)
Paraleucobryum longifolium	B	buddledin A (**117**), hexadecasphinganine (**98**)
Pleurozium schreberi	A	hexadecasphinganine (**98**), glutinone (**118**), hydroxyoctadecadienoic acid, lupenone (**100**), mammeisin (**119**), 18-oxooleate, phytuberin (**102**), vernolic acid (**99**)
Porella platyphylla	A	azelaic acid (**101**), buddledin A (**117**), hexadecasphinganine (**98**), hydroxyoctadecadienoic acid, 18-oxooleate, vernolic acid (**99**)



98



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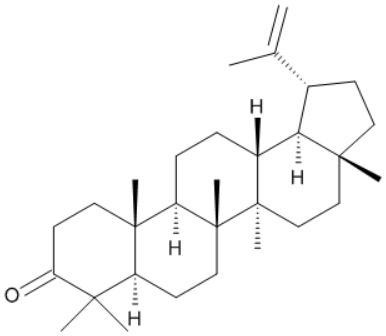

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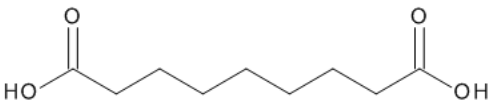

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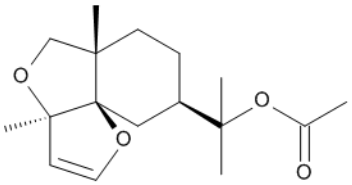

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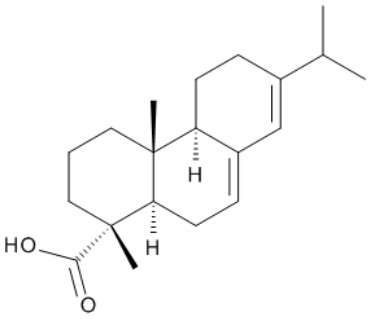

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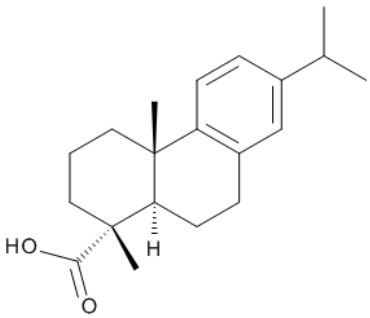

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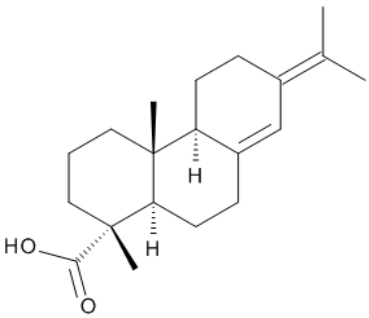

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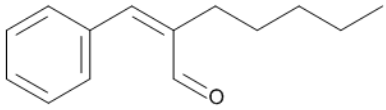

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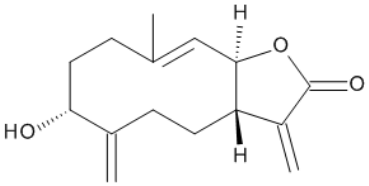

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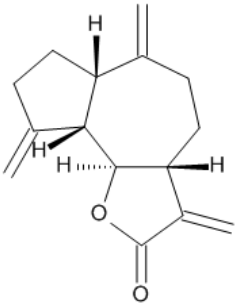

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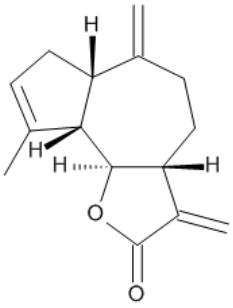

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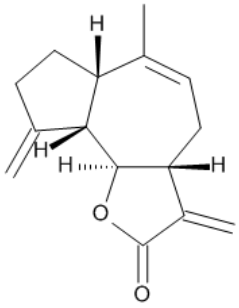

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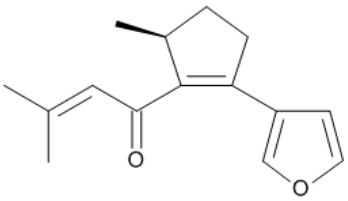

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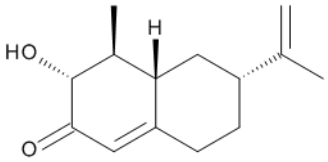

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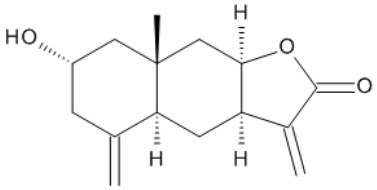

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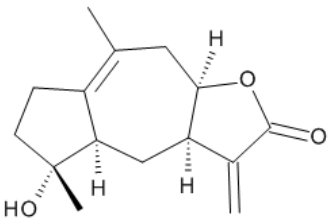

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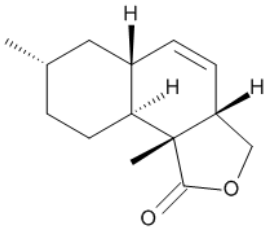

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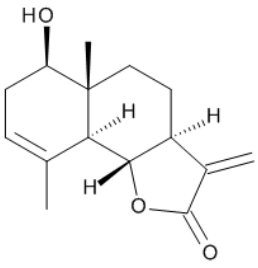

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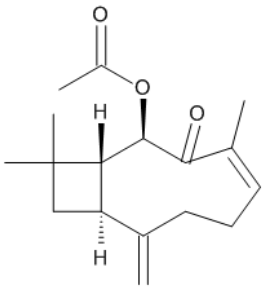

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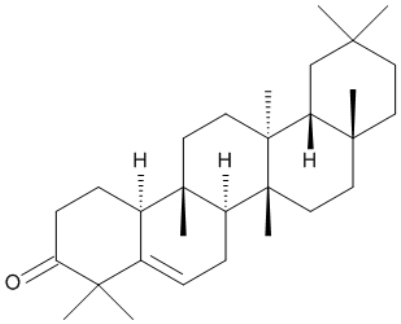

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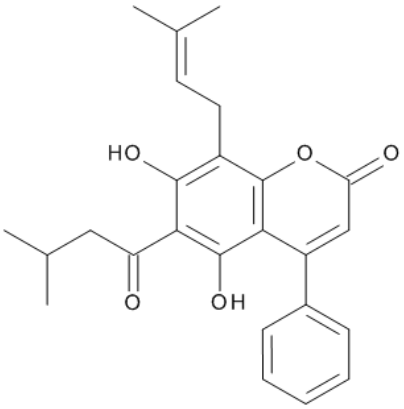

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5.3 Isolation of secondary metabolites

5.3.1 Isolation of secondary metabolites from *P. longifolium*

Since *P. longifolium* was active in both antiproliferative and antimicrobial assays and phytochemically less studied, hence it was selected for further extraction and purification (**Figure 3**). Plant material (4.0 kg) was extracted in a percolator with 75 L MeOH in room temperature. The extract was evaporated (331.1 g) and subjected to column chromatography on CC-P, eluted with different ratios of MeOH:water mixtures (20%, 40%, 60%, 80%, 100% MeOH). During the separation by 80% and 100% MeOH, the colour of elution changed, thus two separated subfractions (first and second part of elution: 80% a, 80% b and 100% a and 100% b) were collected from each of these two fractions. Fractions were evaporated, and the 60% MeOH fraction (CC-P60%) was chromatographed by VLC on normal phase SiO_2 using gradient system of cyclohexane-$CHCl_3$-MeOH with increasing polarity (**VLC-NP I**). Fraction with similar composition (VLC-NP I/134-149) were combined, evaporated and further separeted by VLC on reversed phase SiO_2 with gradient system of H_2O-MeOH (**VLC-RP**). Fractions eluted with H_2O-MeOH 4:6 (VLC-RP/2-8) were chromatographied by GFC eluted in MeOH (**GFC I**). Similar compositioned fractions (CGC I/16-20) were combined, evaporated and purified by HPLC using the gradient system of H_2O-IPA (**HPLC-RP I**). The purification yielded **Compound 6** (Rt=9.93 min).

The second part of the 80% metanol fraction (CC-P80% b) was separtated by VLC on normal phase SiO_2 using the gradient of $CHCl_3$-MeOH with increasing polarity (**VLC-NP II**). Fractions 11 (VLC-NP II/11) contained a chloroform soluble but metanol insolube black particle mixtures, and was choosen for further separation by CC on normal phase SiO_2 using the mixture

of toluene-$CHCl_3$-MeOH 2:6:1.5 with isocratic elution (**CC-NP**). The black particles were concentrated in fractions 9-16, fractions with similar compositon (CC-NP/9-11, CC-NP/12-13, CC-NP/14-16) were combined, and evaporated. The fraction CC-NP/9-11 was chromatographed with FC on reversed phase SiO_2 using the gradient of MeCN-H_2O with decreasing polarity (**FC-RP**). Fraction with similar composition were combined (FC-RP/6-7), evaporated, and chromatographed on GFC eluated in pure MeOH **(GFC II**). Fraction with similar composition were combined (CGC II/4-11), evaporated and purified by HPLC on reversed phase SiO_2 (**HPLC-RP II**). Elution was carried out with the gradient of H_2O-MeCN, that yielded enantiomers **Compound 2** (Rt=9.95 min) and **Compound 3** (Rt=10.27 min).

The fraction CC-NP/12-13 was chromatographed on GFC with MeOH (**GFC III**). Fractions containing black particles were combined (GFC III/12-16 and GFC III/17-21) and evaporated. Fraction GFC III/12-16 was chromatographed by HPLC on reversed phase with the gradient of H_2O-MeCN (**HPLC-RP III**). Fraction GFC III/17-21was purified by PLC on reversed phase SiO_2. However, none of these two separation yielded pure compound, but they contained the same compound in impure form, so the two fractions were combined and further separeted by HPLC method on reversed phase SiO_2 with the gradient of H_2O-MeCN (**HPLC-RP IV**). The purification yielded **Compound 4** (Rt= 14.42 min) and **Compound 5** (Rt= 14.96 min).

Fraction CC-NP/14-16 was chromatographed on GFC with MeOH (**GFC IV**), that gave **Compound 1**.

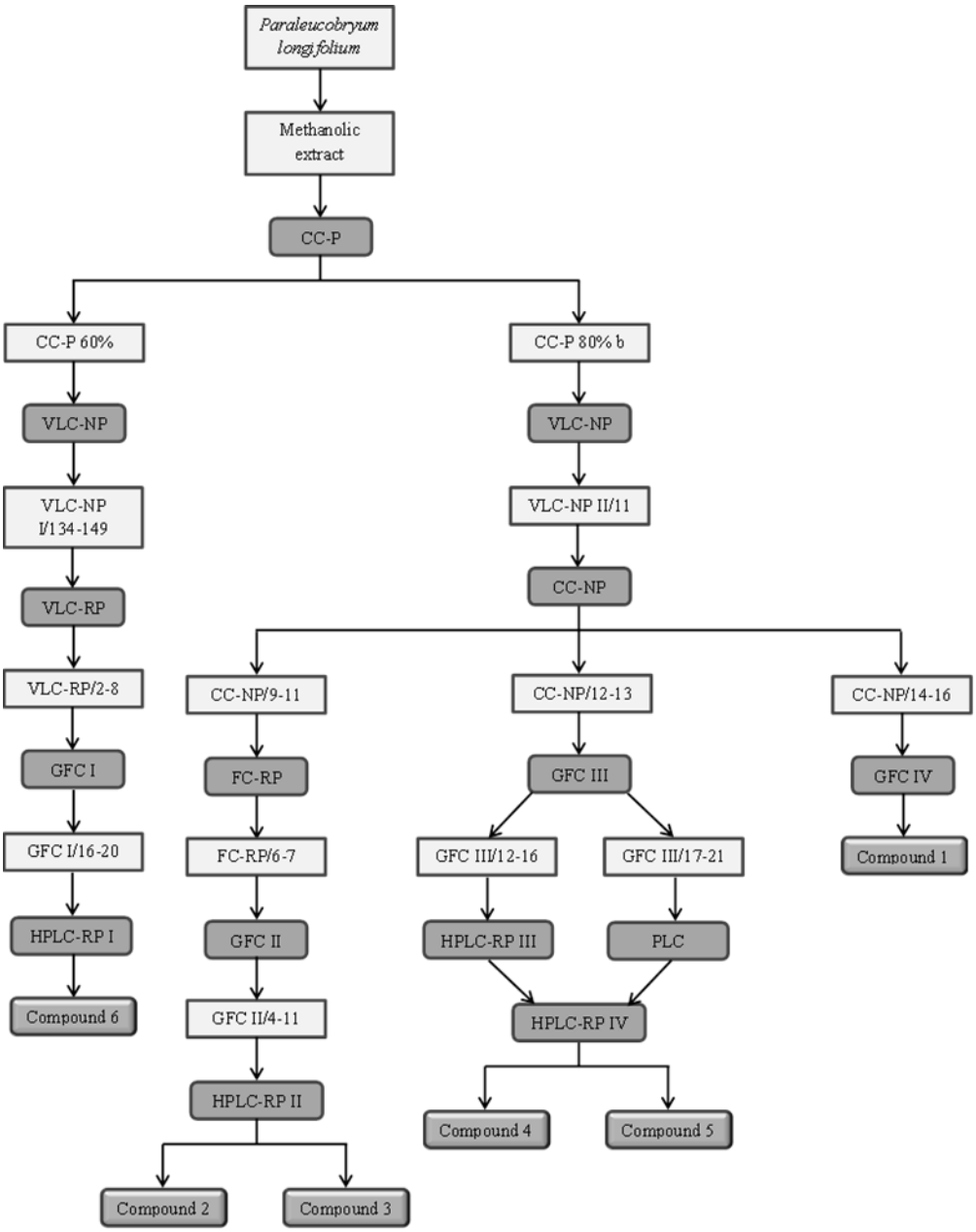


Figure 3. Isolation of compounds from *P. longifolium*

5.4 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 IC50 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 4**). 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 4. 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]

*: inhibition values less than 10% are considered negligible and not presented numerically.

5.5 Structure elucidation of isolated compounds

To determinate the structure of isolated compounds 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.

Compound **1** (leucobryn A) was obtained as a dark violet amorphous powder with UV maxima at 542.5, 370, 305, 272, and 213.5 nm. The molecular formula 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 (calcd for 733.2255 $C_{40}H_{38}O_{12}Na$). The 13C JMOD spectrum displayed 20 resonances, which indicated that compound **1** is a symmetric dimer.

The monomeric moiety of the molecule is constructed by 10 nonprotonated carbons (δ_C 71.98, 118.00, 121.19, 129.50, 131.07, 131.23, 133.91, 155.84, 156.81, and 157.65,), two carbonyl (δ_C 192.01, and 192.77), three methines (δ_C 107.65, 122.06, 129.97), two methylenes (δ_C 23.07,

44.21), a methoxy group (δ_C 56.29), and two methyls (δ_C 28.86, 28.99) (Table 1). The evaluation of 1D ^{1}H and 2D ^{1}H–^{1}H COSY spectroscopic data revealed the presence of two *ortho*-doublets (δ_H 8.44 d, 7.04 d), an isolated aromatic proton (δ_H 6.79 s), an ethylene group (δ_H, 3.00 td, 2.87 td, 1.74 td, 1.65 td), two methyls [δ_H 1.28 s (6H)] and a methoxy group [δ_H 3.65 s (3H)] (Table 2). HMBC correlations between H-6 and C-5, C-7, C-8, and C-5a, between H-3 and C-1, C-2, C-4, and C-4a, and between H-4 and C-2, C-1a, and C-10 disclosed that compound **1** contains a 9,10-phenanthrenedione parent system with *O*-functionalities at C-2 (δ_C 155.84), C-5 (δ_C 156.81), and C-7 (δ_C 157.65), and *C*-functionalities at C-1 (δ_C 133.91) and C-8 (δ_C 121.19) (**Figure 4**). The alternative anthraquinone structure was ruled out because the strong HMBC cross-peak observed between H-4 and C-5a suggested that they are separated by three rather than four bonds. The other three-bond correlations of H-4 with C-2 and C-1a had similar intensities. On the other hand, a weak four-bond heteronuclear interaction was detected between H-4 and the carbonyl carbon at $\delta_{C\text{-}10}$ 192.77. Furthermore, the proposed structure was confirmed by a weak cross-peak exhibited between H-6 and C 4a, which would not be expected in an anthraquinone, due to the long distance (5 bonds) between these atoms.

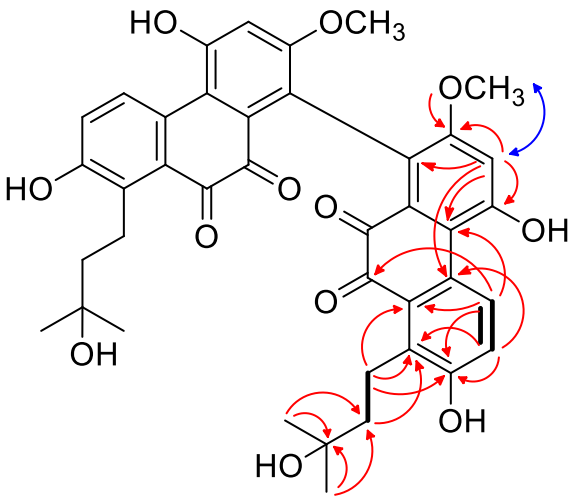


Figure 4. Key 2D NMR correlations in compound 1; – bold line: ^{1}H-^{1}H COSY, → red arrows: HMBC, ↔ blue arrow: NOESY

The presence of the methoxy group at C-7 was established by HMBC correlations between the OCH_3 protons and C-7 and C-6, and by a NOESY cross-peak between H-6 and the OCH_3 group. A 3-hydroxyisopentyl substituent was corroborated with the HMBC cross-peaks observed between the methyl protons at δ_H 1.28 (H_3-14, H_3-15) and C-13 (δ_C 71.98) and C-12 (δ_C 44.21); this group was placed at C-1 on the basis of the H-11/C-1, H-11/C-1a, H-11/C-2, and H-12/C-1

HMBC correlations. A hydroxy group, concluded from the HRESIMS data, is attached at C-7 as confirmed by its chemical shift value of δ_C 157.65. In accordance with all these data, 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 $[M+Na]^+$ (calcd for 869.3507 $C_{50}H_{54}O_{12}Na$). The ^1H NMR and ^{13}C JMOD spectra revealed a structure containing 25 carbon atoms only, suggesting 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**. Combined analysis of ^1H NMR, ^{13}C JMOD, and HSQC spectra proved that this C_{10} substituent consists of a nonprotonated, oxygenated carbon (δ_C 71.46), a disubstituted carbon-carbon double bond (δ_H 4.41 d, 4.57 dq; δ_C 112.61, 148.85), three sp^3 methylenes [δ_H 1.25 m, 1.36 m, 1.48 m (2H), 2.97 dd, 3.15 dd; δ_C 28.28, 31.26, 42.85], a methine (δ_H 2.31 m; δ_C 50.13), and three methyl groups (δ_H 1.11 s, 1.12 s, 1.64 br s; δ_C 18.56, 28.88, 29.30). Based on the ^1H–^1H COSY spectrum, two structural fragments could be unambiguously assigned for the C_{10} part: –CH_2-CHR-CH_2-CH_2– (A) and CH_2=(CH_3)C– (B) (**Figure 5**).

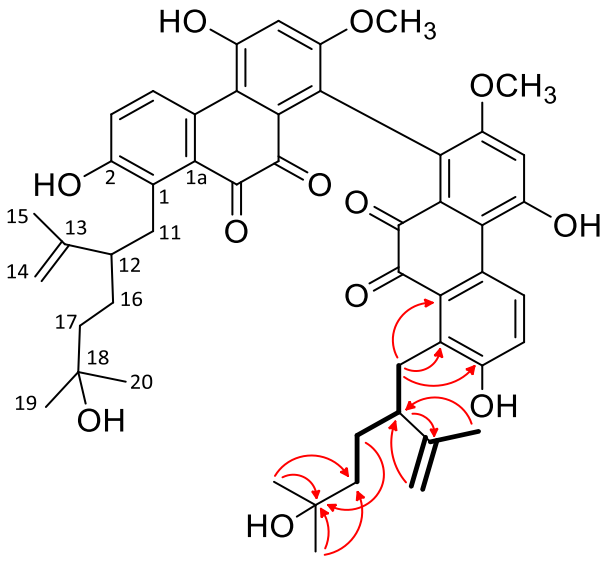


Figure 5. Key 2D NMR correlations in compound **2**; – bold line: ^1H-^1H COSY, → red arrows: HMBC.

Structural fragment A (C-11–C-12–C-16–C-17) was connected to the phenanthrenedione skeleton based on the long-range C–H correlations of H-11/C-1a, H-11/C-1 and H-11/C-2. In addition, a 2-

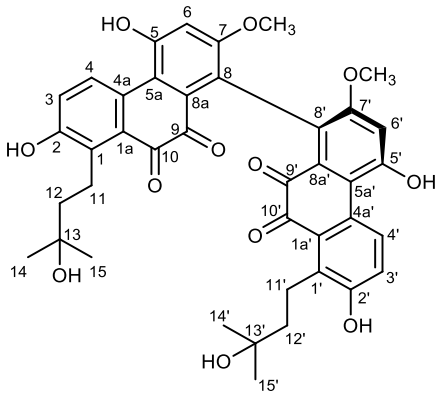
hydroxyisopropyl group, attached to C-17, was confirmed by the HMBC correlations between H_3-19/C-17, H_3-19/C-18, and H_3-20/C-17. HMBC cross peaks between H-14/C-12, H-15/C-12 and H-12/C-13 unambiguously established that unit B (C-13–C-14–C-15) is linked to C-12. The data above provided evidence for a C_{10} monoterpene side chain, in which, interestingly, the isoprenoid units are joined by 3,4 linkages, instead of the regular head-to-tail, tail-to-tail, or head-to-head connection. Collectively, the structure of compound **2** was established and named as leucobryn B.

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 minute differences observed in the chemical shifts of H-14, H-16, C-10, C-11, and C-14. These data suggest that the structural differences are explicable in terms of the stereochemistry of the compounds.

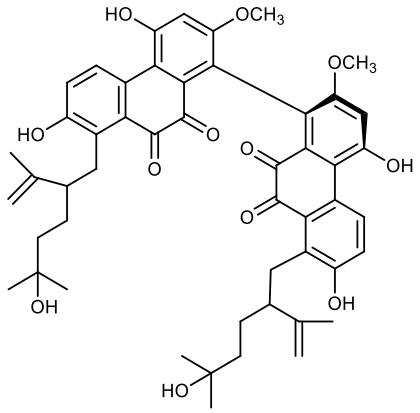
Compound **4** (leucobryn D) was isolated as a dark violet amorphous solid. HRESIMS data revealed a molecular formula of $C_{45}H_{46}O_{12}$ according to the sodium adduct ion at *m/z* 801.2876 $[M+Na]^+$ (calcd for $C_{45}H_{46}O_{12}Na$ 801.2881). ^1H NMR and ^{13}C JMOD data contained signals similar 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.

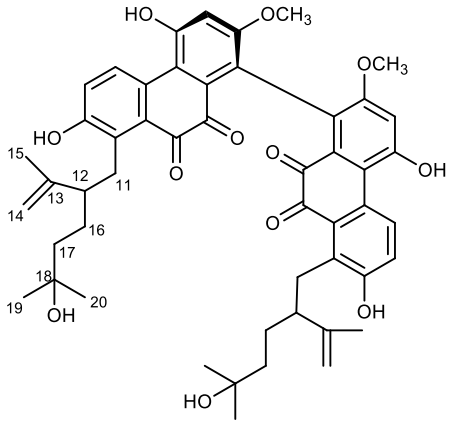
Compound **6** was identified as diosmetin 7-*O*-[2,4-di-*O*-(α-L-rhamnopyranosyl)]-β-D-glucopyranoside.



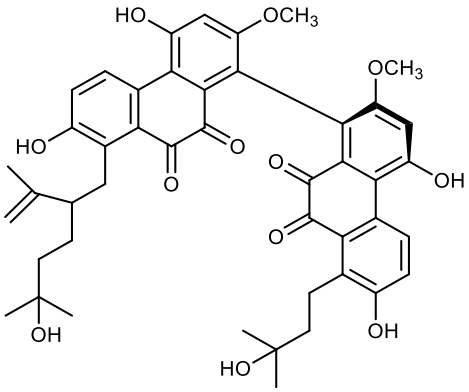
Leucobryn A



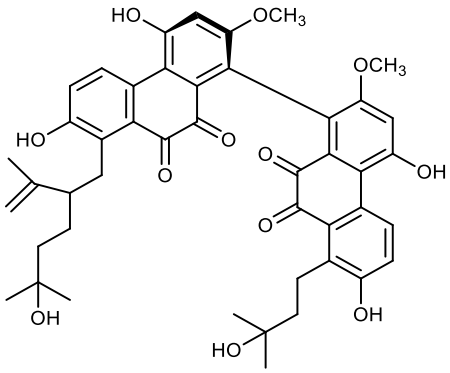
Leucobryn B



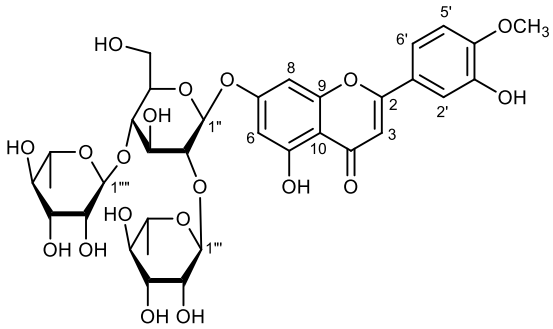
Leucobryn C



Leucobryn D



Leucobryn E



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

6 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*-(α-L-rhamnopyranosyl)]-β-D-glucopyranoside, which was previously described from *Dicranum scoparium* [80]) were isolated from this species. Although phentanthrenes are the specific pigments of bryophytes, leucobryns are the first natural occuring 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 [81], [82].

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

7 References

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