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Antibacterial Activity of Extracts from Some Bryophytes

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ABSTRACT

The antimicrobial activity of aqueous and ethanolic extracts of 11 *Bryophyta* species and 9 *Marchantiophyta* species collected in Latvia was tested against *Staphylococcus aureus*, *Escherichia coli* and *Bacillus cereus*. The extract of *Lophocolea heterophylla* inhibited the growth of *B. cereus*, but none of the tested extracts inhibited the growth of *E. coli*. 70% of bryophyte species demonstrated certain activity in relation to *S. aureus*. In general, 73% of ethanolic extracts and 39% of aqueous extracts exhibited antibacterial activity against *S. aureus*. The highest degree of antibacterial activity against *S. aureus* was shown by the ethanolic extract of *Dicranum scoparium* and aqueous extracts of *Atrichum undulatum* and *Rhytidiadelphus squarrosus*. The bactericidal action was not ascertained. For the first time antimicrobial activity has been proved for three moss species—*Eurhynchium angustirete*, *Rhytidiadelphus squarrosus* and *Rhodobryum roseum*, and for two liverwort species *Frullania dilatata* and *Lophocolea heterophylla*. Qualitative and quantitative differences of plant extracts were evaluated by FT-IR spectra.

Keywords: *Bryophyta*; *Marchantiophyta*; Antimicrobial Activity

1. Introduction

Bryophytes, including liverworts (*Marchantiophyta*), hornworts (*Anthocerotophyta*), and mosses (*Bryophyta*), are a diverse group of land plants that usually colonize habitats with moist or extremely variable conditions. Traditionally, because of their antimicrobial activity, mosses were used as a natural medicine in the Indian culture [1] and as natural diapers [2]. Today, mosses and liverworts are interesting for biotechnological use in medicine, agriculture, and pharmacology [1,3,4]. Liverworts have been proposed as ideal models for genetic studies and biotechnological applications [3].

The search for plants with antimicrobial activity has grown in importance in recent years, due to a growing concern about increase in the rate of infection caused by antibiotic-resistant microorganisms. Asakawa [5,6] has analyzed approximately 1000 bryophyte species from the world total of 27,000. However, few studies have been carried out about the antimicrobial properties of European bryophytes. In literature, reports have been found about antibacterial activity of 23 bryophyte species [7-15] that are common in Latvia [16] and other European countries [17-20].

In presented paper, the antimicrobial activity of 20 bryophyte species collected in Latvia was evaluated.

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2. Materials and Methods

2.1. Plant Material

Samples of all tested plants were collected from their native habitats in Salaspils, Kemeri, Iecava and Ropazi (Latvia) in August and September and the specimens were identified. Taxonomic references used were Abolina [16] and Smith [21-23] for liverworts and mosses, and Ignatov and Ignatova [24,25] for mosses. Once harvested, the plant material was maintained in the refrigerator (+4˚C) and processed in five days to obtain extracts.

2.2. Preparation of the Extracts

At first, plants were washed with sterile water to remove attached litter, dead material and fragments of epiphytic hosts. One gram of plant material per repetition was finely ground with a pestle and mortar, and then extract was made using 10 ml of sterile water or 50% ethanol. The suspensions were kept in refrigerator for 18 hours and then centrifuged (Eppendorf, 4000 rpm, 4˚C, 30 min). Autoclaved (121˚C, 15 min) aqueous extracts were used.

2.3. Antimicrobial Activity

Antimicrobial assays were performed on three species of microorganisms maintained in the Microbial Strain Collection of Latvia (MSCL). Following strains were used:

Bacillus cereus MSCL 330, *Escherichia coli* MSCL 332 and *Staphylococcus aureus* MSCL 334. Gentamicin (KRKA, Slovenia) 10 mg/ml was used as a positive control. For the evaluation of antimicrobial activity two methods were used: the agar-well diffusion method [26] and broth microdilution assay [27]. The tests were performed in triplicates for each microorganism evaluated. The final results were presented as the arithmetic averages.

2.3.1. Agar-Well Diffusion Method

Agar diffusion test was performed on Müller-Hinton Agar (Oxoid). Fresh inoculum approximately 10^6 CFU (colony-forming units)/ml of tested microorganisms was used. Aliquots of 70 µl of each test-sample solution and control (distilled water and 50% ethanol) were applied into 6.0 mm diameter wells. After incubation at 37˚C ± 1˚C for 18 hours the inhibition zone corresponding to the halo formed from well edge to the beginning of the zone of microbial growth was measured.

2.3.2. Broth Microdilution Assay

Müller-Hinton Broth (BD Difco™) was used. Test strains were suspended in broth to obtain a final density of approximately 10^6 CFU/ml. To confirm the initial bacterial counts, serially diluted bacterial cultures were plated on the Müller-Hinton Agar plates and enumerated. The test was performed using three concentrations of each extract (3%, 17%, and 33%, v/v) in test tubes, including growth (in water or ethanol dilutions) and sterility controls. Tubes were incubated at 37˚C ± 1˚C for 48 h. After incubation, the mixtures were subjected to successive 10-fold serial dilutions, mixed with a vortex shaker to ensure dispersion and quantitatively cultured in duplicate onto agar plates to determine the number of viable bacteria. Viable cell counts were expressed as CFU/ml and if applicable the minimal inhibitory concentration (MIC_{80}) according to Qaiyumi [28] was evaluated.

2.4. Identification of Chemical Constituents by FT-IR Spectroscopy

FT-IR absorption spectra of bryophyte extracts were registered on a microplate reader HTS-XT (Bruker, Germany). 50 - 330 µl of each sample were dried on a 96-place silicon plate at <50˚C. Spectra were collected over the wave-number range of 4000 - 600 cm^{-1}, 32 scans, resolution 4 cm^{-1}. Data were processed with OPUS 6.0 (Bruker, Germany) software. Spectra were Vector normalized and baseline corrected by the rubber-band method.

2.5. Statistics

Statistical analysis was done by analysis of variance and by Chi-square (χ^2) significance test. $P < 0.05$ was considered statistically significant.

3. Results

The agar-well diffusion method did not show any antibacterial effect of the tested extracts against the investigated microorganisms, and for that reason in subsequent experiments we used the method of broth microdilution assay. Using this method, in many cases significant influence of bryophytes on the growth of microorganisms was found (**Table 1**). None of the tested bryophyte species (*Lophocolea heterophylla*, *Nowellia curvifolia*, *Polytrichum commune*, *Rhodobryum roseum*) had a significant influence ($P > 0.05$) on the growth of *E. coli.* The growth of *Bacillus cereus* was inhibited by the aqueous extracts of *L. heterophylla* (MIC_{80} 27%), and *P. commune* (MIC_{80} was not achieved) but was not inhibited by the aqueous extracts of *N. curvifolia* and *R. roseum*. The most comprehensive researches have been made about the influence of bryophyte extracts on the growth of *Staphylococcus aureus*.

3.1. Antibacterial Activity of Mosses against *Staphylococcus aureus*

Of the 12 species, 7 species (58%) exhibited antimicrobial activity against *S. aureus* (**Figure 1**). Antibacterial activity against *S. aureus* was not established for *Fissidens taxifolius*, *Hypnum cupressiforme*, *Plagiomnium undulatum*, *Pleurozium schreberi* and *Sphagnum girgensohnii* aqueous extracts. On the contrary, *H. cupressiforme* and *S. girgensohnii* extracts stimulated the growth of *S. aureus* (**Figure 2**).

3.2. Antibacterial Activity of Liverworts against *Staphylococcus aureus*

Of the 8 species, 3 species (38%) exhibited antimicrobial activity against *S. aureus* (**Figure 3**). Antibacterial activity against *S. aureus* was not established for *Lepidozia reptans*, *Marchantia polymorpha*, *Nowellia curvifolia*, *Plagiochila asplenioides* and *Radula complanata* aqueous extracts. The extract of *N. curvifolia* stimulated the growth of *S. aureus* (**Figure 2**).

3.3. Main Constituents of Bryophytes Extracts

FT-IR spectra of extracts showed intensive band of C=C group at 1600 cm^{-1} and characteristic stretching bands of C=O in 1300 - 1720 cm^{-1} region indicating the carbonyl groups of phenolic esters [29], and C-O stretching bands of esters and phenols at 1240 and 1052 cm^{-1} [30]. The intensities of absorption bands in 1500 - 1720 cm^{-1} region varied in different samples thus indicating qualitative and quantitative differences of the biochemical com-

Table 1. Minimal inhibitory concentration (MIC_{80}) of plant extracts (in %) against *Staphylococcus aureus.*

Taxonomic position			Extract	
Division	Class	Species	Water	Ethanol
Bryophyta	*Bryopsida*	*Dicranum scoparium* Hedw.	30	3
		Eurhynchium angustirete (Broth.) T.J. Kop.	-	13 - 30[a]
		Fissidens taxifolius Hedw.	-	N.T.
		Hylocomium splendens (Hedw.) B., S. et G.	27	>33
		Hypnum cuppressiforme Hedw.	-	N.T.
		Plagiomnium undulatum (Hedw.) T. Kop.	-	-
		Pleurozium schreberi (Brid.) Mitt.	N.T.	-
		Rhodobryum roseum (Hedw.) Limpr.	24	13
		Rhytidiadelphus squarrosus (Hedw.) Warnst.	-	7
	Polytrichopsida	*Atrichum undulatum* (Hedw.) P. Beauv.	-	7
		Polytrichum commune Hedw.	>33	30
	Sphagnopsida	*Sphagnum girgensohnii* Russ.	-	N.T.
Machantiophyta	*Jungermannosida*	*Frullania dilatata* (L.) Dum.	13	33
		Lepidozia reptans (L.) Dum.	-	N.T.
		Lophocolea heterophylla (Schrad.) Dum.	30	10
		Nowellia curvifolia (Dicks.) Mitt.	-	N.T.
		Plagiochila asplenioides (L. emend. Tayl.)	-	N.T.
		Ptilidium pulcherrimum (G. Web.) Vainio	27	N.T.
		Radula complanata (L.) Dum.	-	N.T.
	Marchantiopsida	*Marchantia polymorpha* L.	-	N.T.

-Did not have MIC_{80}; [a]Only in the indicated interval of concentration; N.T. not tested.

position of bryophyte extracts depending on the extraction method and species (**Figure 4**). Thus the ethanolic extract of *Frullania dilatata* showed considerably higher concentration of phenolics (1600 cm^{-1}) while that of *Dicranum scoparium* much higher concentration of esters (1712 cm^{-1}). In aqueous extracts higher concentration of phenolics was found in extracts of *Marchantia polymorpha*, *Lophocolea heterophylla* and *Nowellia curvifolia.* In general, the concentration of phenolics was higher in ethanolic extracts.

In all extracts, the proportion of carbohydrates was higher than that of other determined compounds, except for four liverwort species (*Frullania dilatata*, *Lophocolea heterophylla*, *Marchantia polymorpha* and *Nowellia curvifolia*); in these species, the proportion of phenols was the highest. In other species, phenols have been proved to be the second largest group of substances; the only exception was *Plagiochila asplenioides*, for which the second largest group was made up by amides (**Table 2**). *Dicranum scoparium* and *Atrichum undulatum* extracts differed by significant ester content. In other extracts ester bands were weak and in many samples even missed.

4. Discussion

In Latvia, folk medicine and ethnopharmacological traditions of using bryophytes virtually do not exist; nevertheless more than 550 species of bryophytes have been found growing in this country [16]. In our study, extracts of 20 bryophytes collected in Latvia were screened for antibacterial activity. Microbiological tests indicated that different bryophytes possess different influence on the

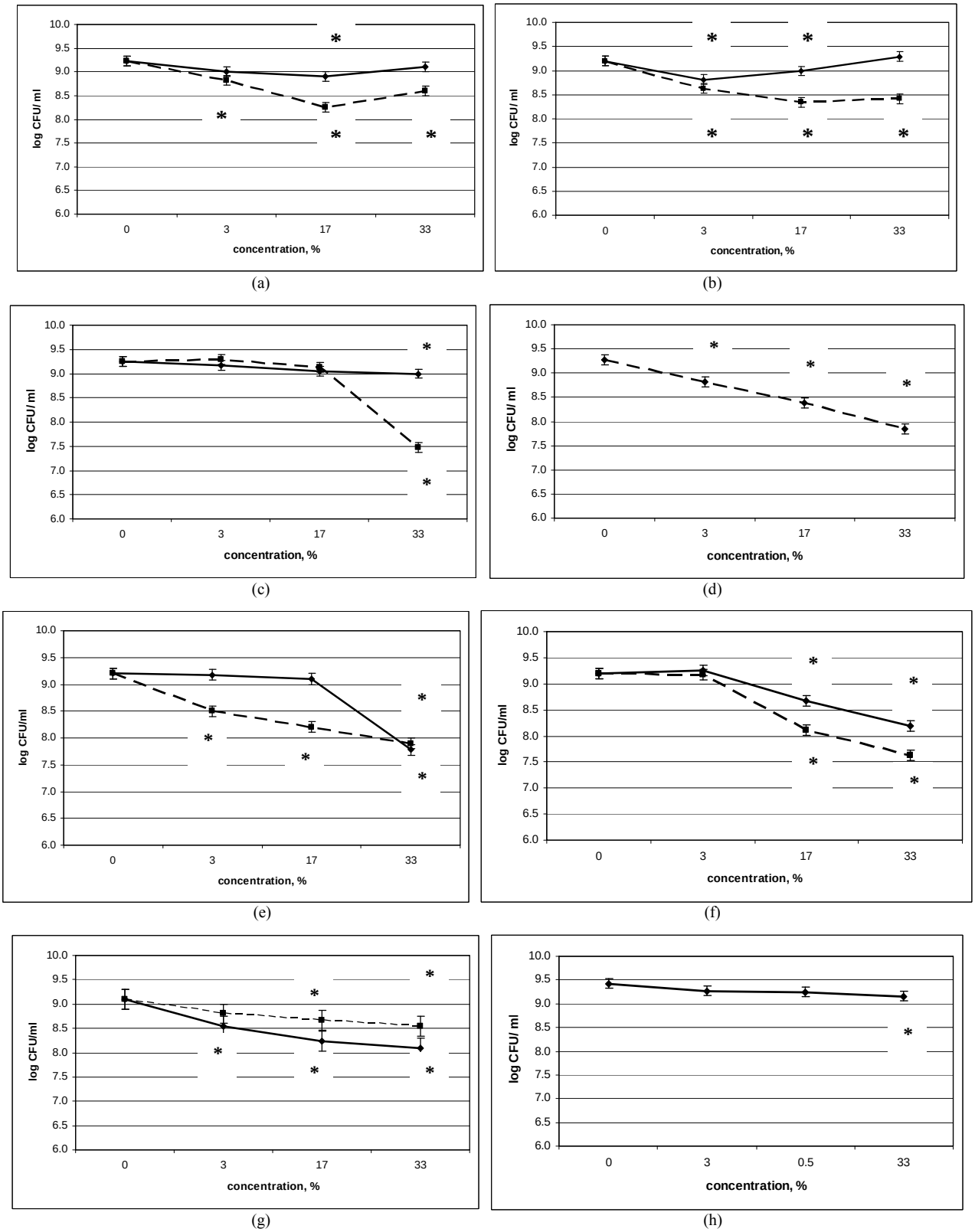


Figure 1. Inhibition of *Staphylococcus aureus* growth by mosses in dependence of the concentration of their aqueous or ethanolic extract. (a) *Eurhynchium angustirete*; (b) *Rhytidiadelphus squarrosus*; (c) *Polytrichum commune*; (d) *Atrichum undulatum*; (e) *Dicranum scoparium*; (f) *Rhodobryum roseum*; (g) *Hylocomium splendens*; (h) *Fissidens taxifolius*; ——— aqueous extract; – – – ethanolic extract; $^{*}P < 0.05$ in comparison with control, without extract.

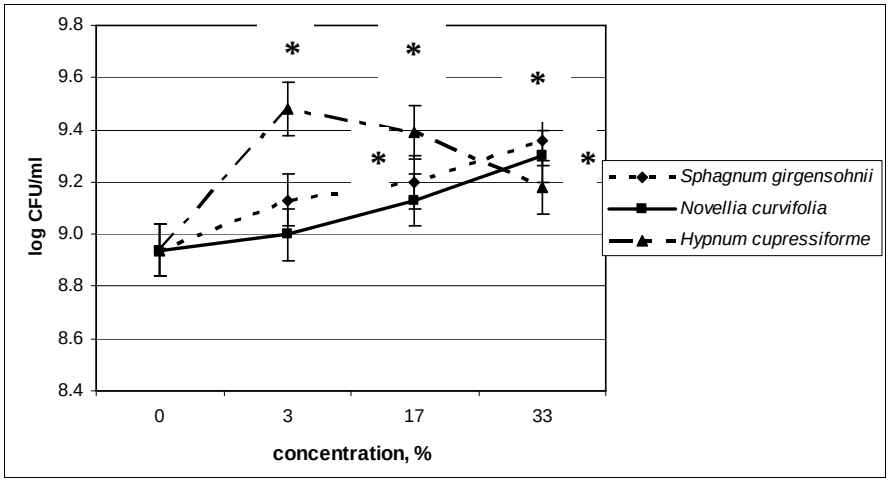


Figure 2. Stimulation of *Staphylococcus aureus* growth by three species of bryophytes in dependence of concentration of their aqueous extracts. $^{*}P < 0.05$ in comparison with control, without extract.

growth of microorganisms, from inhibition to stimulation.

70% of bryophyte species demonstrated certain activity in relation to *Staphylococcus aureus*. 55% of the species showed a more or less pronounced antibacterial activity. MIC_{80} was achieved and therefore could be estimated for 10 extracts (**Table 1**). Bactericidal effect has not been found in any case.

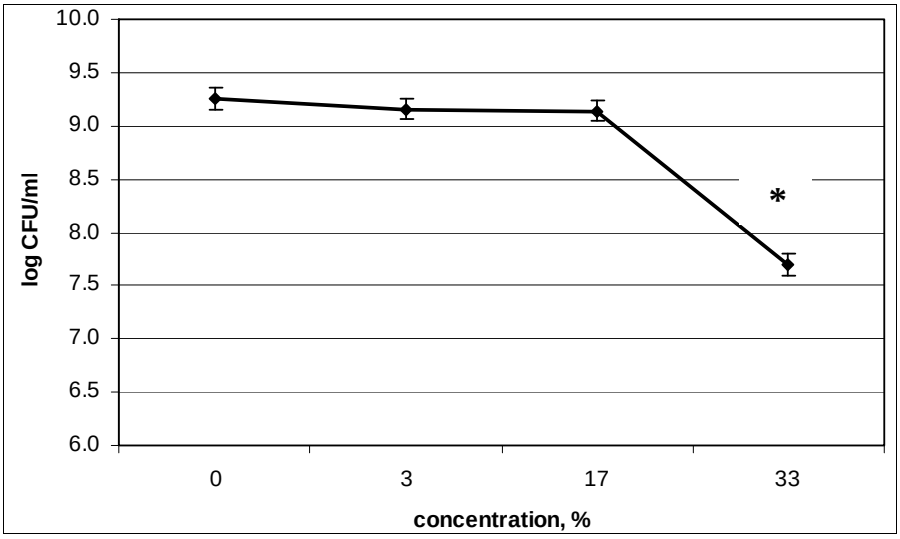
Chi-square test showed that significantly more moss species had antibacterial properties in comparison to liverwort species (**Table 2**, $P < 0.001$). Probably it was due to the fact that only for two liverwort species ethanolic extracts were studied instead of aqueous extracts. On the other hand, ethanolic extracts were studied for nine moss species.

In general, 73% of ethanolic extracts and 39% of aqueous extracts exhibited antibacterial activity against *S. aureus*. Ethanolic extracts exhibited also a higher degree of antimicrobial activity as compared with aqueous extracts with exception of *Frullania dilatata* and *Hylocomium splendens*, the aqueous extracts of these species showed higher antimicrobial activity than the ethanolic extracts (**Table 1**). In our experiments, the aqueous extract of *Marchantia polymorpha* did not influence the growth of *S. aureus*, although in literature data can be found about the antibacterial influence of this liverwort species on gram-positive bacteria among others [31]. This is also explainable by the type of extract. The ethanolic extracts of *M. palmata* have been described as having slightly higher antibacterial activity in comparison with the aqueous extracts [32]. It is known that one of characteristic features of *Marchantiophyta*, in difference from *Bryophyta* and *Anthocerotophyta*, is the presence of cellular oil bodies and production of a number of lipophilic terpenoids, aromatic compounds and acetogenins, several of which show biological activity including antibacterial and antifungal activities [4]. These oil bodies can be extracted with organic solvents.

The highest antimicrobial activity against *S. aureus* was shown by the ethanolic extract of *Dicranum scoparium* (MIC_{80} 3%) and aqueous extracts of *Atrichum undulatum* and *Rhytidiadelphus squarrosus* (MIC_{80} of 7% for both species) (**Table 1**). The antibiotically active substances of *Atrichum* and *Dicranum* spp. are considered to be polyphenolic compounds [33]. In particular, flavonoids, including phenolic acids, are the main group of phenols obtained from mosses [34]. It is important that the antibacterial activity of aqueous extracts in our experiments was heat stable. This fact can lighten the work in case if any of the extracts would be introduced in future practice.

The interconnection between the antimicrobial activity and content of phenolics, esters, amides and/or carbohydrates was not estimated.

To the best of our knowledge, the antimicrobial activity of five of above mentioned bryophyte extracts has not been previously reported. For the first time, antimicrobial activity has been found for three moss species, *Eurhynchium angustirete*, *Rhytidiadelphus squarrosus* and *Rhodobryum roseum*, and for two liverwort species, *Frullania dilatata* and *Lophocolea heterophylla*, although in the genus *Frullania*, antifungal activity had been previously reported for the species *F. muscicola* [35] and antibacterial activity—for species *F. nisquallensis* [36]. Previously, the chemical composition of *L. heterophylla* had been investigated. Asakawa [5] believes it is unique, because this liverwort species contains monoterpene 2-methylisoborneol together with calamenene-type sesquiterpenes and eudesmanolides. Further research is needed to obtain information about correlation between chemical composition and antimicrobial activity of bryophyte species.



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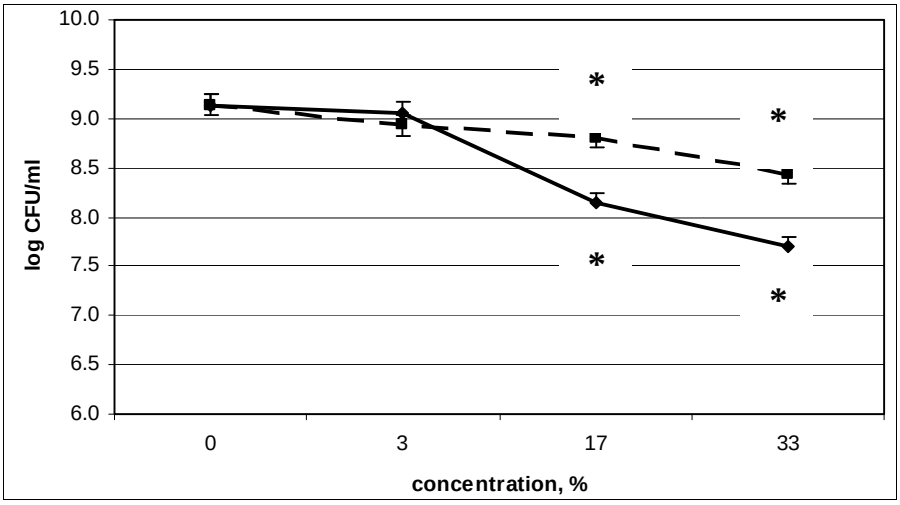

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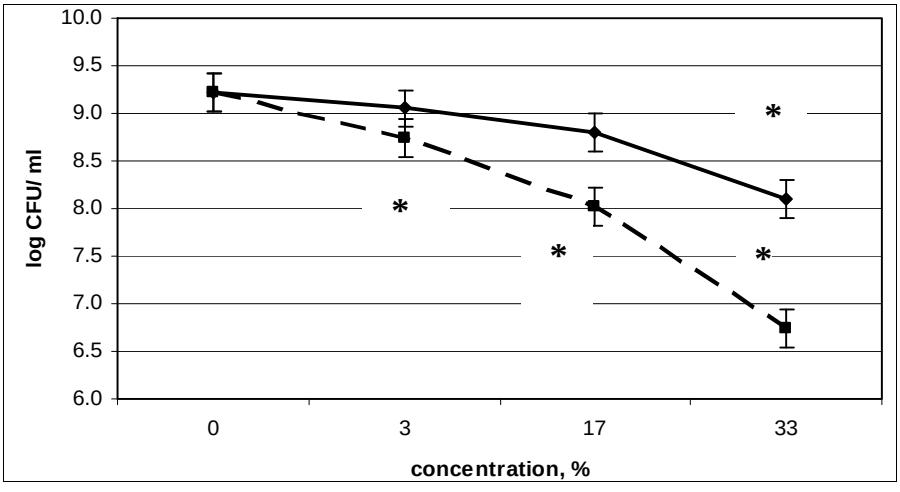

(c)

Figure 3. Inhibition of *Staphylococcus aureus* growth by liverworts in dependence of the concentration of their aqueous or ethanolic extract. (a) *Ptilidium pulcherrinum*; (b) *Frullania dilatata*; (c) *Lophocolea heterophylla*; —— aqueous extract; – – – ethanolic extract; $^{*}P < 0.05$ in comparison with control, without extract.

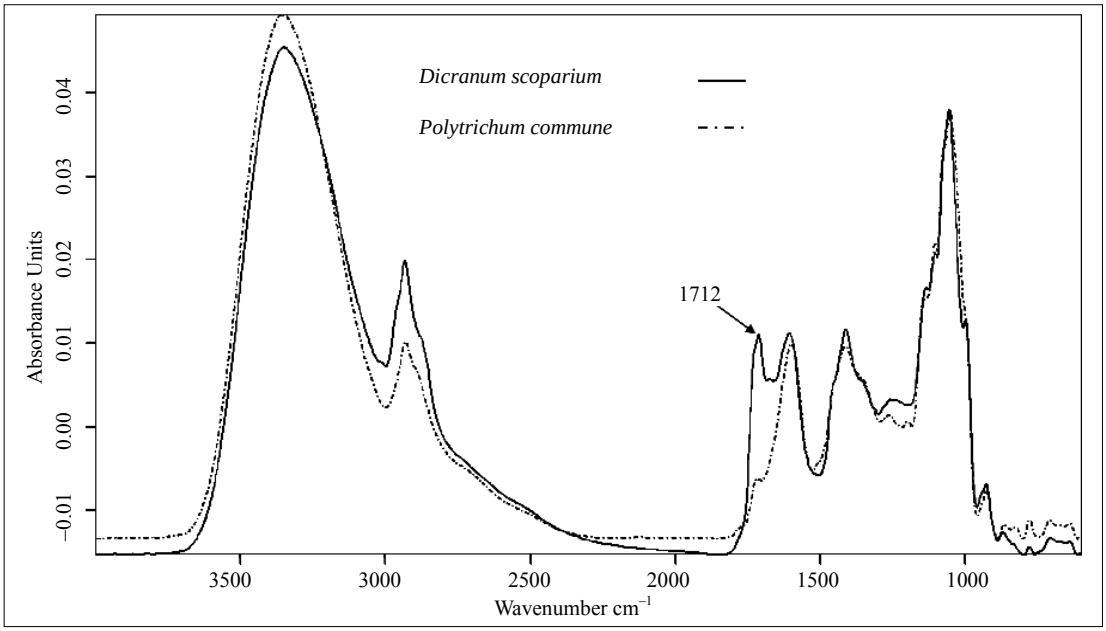

Figure 4. FT-IR absorption spectra of *Dicranum scoparium* and *Polytrichum commune* ethanolic extracts.

Table 2. Features of chemical composition of bryophytes extracts, expressed in relative units (FT-IR data).

Species	Amides		Esters		Phenolics		Carbohydrates	
	aqueous	ethanolic	aqueous	ethanolic	aqueous	ethanolic	aqueous	ethanolic
Bryophyta								
Atrichum undulatum	N.T.	0.024	N.T.	0.013	N.T.	0.042	N.T.	0.069
Dicranum scoparium	N.T.	0.022	N.T.	0.018	N.T.	0.030	N.T.	0.065
Eurhynchium angustirete	N.T.	0.027	N.T.	0.007	N.T.	0.040	N.T.	0.081
Fissidens taxifolius	0.016	N.T.	0.000	N.T.	0.024	N.T.	0.037	N.T.
Hylocomium splendens	0.016	N.T.	0.000	N.T.	0.024	N.T.	0.043	N.T.
Hypnum cupressiforme	0.015	N.T.	0.004	N.T.	0.028	N.T.	0.035	N.T.
Plagiomnium undulatum	N.T.	0.031	N.T.	0.000	N.T.	0.051	N.T.	0.069
Polytrichum commune	N.T.	0.025	N.T.	0.003	N.T.	0.038	N.T.	0.050
Rhodobryum roseum	0.014	0.030	0.000	0.000	0.015	0.026	0.045	0.045
Rhytidiadelphus squarrosus	N.T.	0.024	N.T.	0.005	N.T.	0.035	N.T.	0.066
Sphagnum girgensohnii	0.014	N.T.	0.000	N.T.	0.024	N.T.	0.041	N.T.
Marchantiophyta								
Frullania dilatata	N.T.	0.034	N.T.	0.000	N.T.	0.067	N.T.	0.036
Lepidozia reptans	0.015	N.T.	0.000	N.T.	0.023	N.T.	0.033	N.T.
Lophocolea heterophylla	0.023	0.040	0.000	0.000	0.041	0.068	0.031	0.057
Marchantia polymorpha	0.026	N.T.	0.000	N.T.	0.055	N.T.	0.028	N.T.
Nowellia curvifolia	0.017	N.T.	0.000	N.T.	0.032	N.T.	0.030	N.T.
Plagiochila asplenioides	0.014	N.T.	0.000	N.T.	0.013	N.T.	0.039	N.T.
Ptilidium pulcherrimum	0.017	N.T.	0.000	N.T.	0.026	N.T.	0.040	N.T.
Radula complanata	0.019	N.T.	0.000	N.T.	0.026	N.T.	0.040	N.T.

N.T.: not tested.

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