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OPTIMISATION OF CONDITIONS FOR EXTRACTION OF BIOLOGICALLY ACTIVE COMPOUNDS FROM COMMON BRYOPHYTES IN LATVIA

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Bryophytes are the second largest taxonomic group in the plant kingdom. They contain a high number of biologically active compounds. Studies of their composition are important for understanding evolutionary processes in the plant kingdom. The aim of this study was to assess bryophyte secondary metabolite extraction options and to increase the yields of polyphenols and substances determining the free radical scavenging activity of bryophyte extracts. Similar studies have been conducted using higher plants as model organisms, but not using bryophytes. Comparison of five extraction methods (conventional, Soxhlet extraction, treatment with microwaves, ultrasound, and supercritical CO_2 extraction) and several solvents with differing polarity showed microwave-assisted extraction as the most promising approach to obtain highest yields of extractives. The main factors that contributed to the efficiency of extraction were type of solvent, temperature, and the solvent to bryophyte mass ratio. The extracts obtained from bryophytes had remarkable antioxidant activity, the extent of which depended on the extraction conditions and bryophyte species. The extraction conditions can be optimised, and the total polyphenol content can be increased by up to 50% in comparison with the conventional approach.

Key words: *bryophyte chemistry, extraction optimisation, supercritical CO_2 extraction, microwave-assisted extraction, polyphenols, antiradical activity.*

INTRODUCTION

Bryophytes, which are the simplest land plants, are the second largest taxonomic group in the plant kingdom, with about 25 000 species (Asakawa *et al.*, 2013), including mosses (*Musci* ~18 000 species), liverworts (*Hepaticae* ~6000 species), and hornworts (*Anthocerotae* ~1000 species). Bryophytes can be found in all kinds of ecosystems (Glime, 2007). Yet, they have been neglected as a source of biologically active substances for a long time due to their small size and identification problems, and because they were considered as almost useless for humans, except in circumpolar regions as a common animal feed. Bryophytes are mainly composed of hemicelluloses and pectin (30 to 60% content, respectively, cellulose content from 15 to 25%). Bryophytes also contain proteins (content 5 to 10%) and lipids and phenolic compounds together with content 5 to 10% (Orlov *et al.*, 2005). The content of lignin has been found to be insignificant, although controversies in this respect exist (Balance *et al.*, 2008). The chemical composition of bryophytes differs depending on species, growth environment, and season (Glime, 2007;Goffinet and Shaw, 2008; Xie and Lou, 2009). Chemical composition studies can become an important dimension in the study of bryophyte biology and ecology (Goffinet and Shaw, 2008; Xie and Lou, 2009).

Bryophytes and their extracts have found application in ethnopharmacology, as medical plants for treatment of wounds and burns, and for curing tuberculosis, pneumonia, neurasthenia and other illnesses (Hotson, 1921; Spjut *et al.*, 1986; Sing *et al.*, 2006; Saboljevic *et al.*, 2010). The interest in bryophyte chemical composition has been growing ever since the presence of a high number of biologically active compounds in their composition was demonstrated (Basile *et al.*, 1999; Fu *et al.*, 2012; Asakawa *et al.*, 2013). Many compounds isolated from bryophytes have shown high biological activity (Krzaczkowski *et al.*, 2009; Üēüncü *et al.*, 2010). Likewise, extracts of bryophytes have shown multiple kinds of biological activity. For this reason, they are prospective in search of new pharmaceutically active compounds (Wang *et al.*, 2005; Asakawa, 2007). A number of bryophytes have demonstrated well-expressed antibacterial, antifungal and antiviral activities. Moreover, in several studies, their cytoxicity in respect to cancer cells has been demonstrated, as well as antioxidant, antiplatelet, antithrombin, insecticidal and neuroprotective activities, ability to inhibit a number of biochemically important enzymes, and other kinds of activities (Spjut *et al.*, 1986; Wang *et al.*, 2005; Sing *et al.*, 2006; Krzaczkowski *et al.*, 2009).

Another reason to study bryophyte composition is related to the need to understand their metabolism, which is quite dif-

ferent from that in higher plants (Goffinet and Shaw, 2008). Studies of secondary metabolites can help understand stress reactions (drought/wetness), oxidative stress, pollution stress (for example, heavy metal impact), UV radiation impact reaction in bryophytes, as well as functions of the main secondary metabolites in the overall metabolism (Tutschek, 1979; Goffinet and Shaw, 2008; Xie and Luo, 2009; Huang *et al.*, 2012).

Due to several reasons, studies of bryophyte biological activity have been concentrated on liverworts and relatively low-polar compounds amongst the secondary metabolites, considering the presence of oil bodies in the plants. Traditionally, low-polarity extrahents have been used in studying the presence of biologically active compounds in bryophytes (especially liverworts) (Singh *et al.*, 2006; Üçüncü *et al.*, 2010; Huang *et al.*, 2012), while the extraction process as such has not been specifically studied. However, to further advance the study of bryophyte secondary metabolites, it is important to study the possibilities of improving the extraction process by looking not only for some specific group of compounds but rather for opportunities to obtain a maximally high number of secondary metabolites.

The aim of this study was to assess the possibilities of improving the extraction efficiency of bryophyte secondary metabolites, especially regarding polyphenolic compounds and antioxidant activity, and to increase the yields of extracts.

MATERIALS AND METHODS

High diversity of bryophyte species is found in Northern Europe (Zinsmeister and Mues, 1990; Strazdiņa *et al.*, 2011; Glime, 2007). The bryophyte species used in this study are characteristic for this region. Samples were collected in moist coniferous forests near the Suda bog ($57^{o}15'99''$ N $25^{o}05'25''$ E), moist deciduous forests in the Kurzeme region near city Kabile ($56^{o}96'13''$ N $22^{o}28'07''$ E) and in the Cena bog ($56^{o}85'57''$ N $23^{o}80'99''$ E) in Latvia. Living parts of bryophytes were collected; then, the samples were washed with distilled water to clean them from microorganisms; after that, they were cleaned from dirt and other possible bryophyte species and needles. For further treatment, the top parts of plants (2–5 cm) were taken, and then the cleaned samples were stored at –20 °C. For reference, samples of each bryophyte species are stored at the Department of Environmental Science, Faculty of Geography and Earth Science, University of Latvia.

Extraction procedures. For extraction optimisation, two bryophyte species were used: *Rhytidiadelphus triquetrus* (RT) and *Sphagnum rubellum* (SR). The samples were dried at +40 °C in an oven until constant mass. All prepared extracts were filtered through paper filters and stored at 4 °C for up to 1 month until analysis. All used extrahents were analytical grade, company Fluka.

Conventional extraction of bryophytes. Dry samples were ground in a mill, and 0.3 g samples of bryophytes were weighed into 100 mL bottles with screw caps, adding 50 ml of solvent. Solvents such as ethanol (96%, 80%, 60%, 40%, 20%) diluted with demineralised (Millipore) water, acetone, dioxane, 10% hydrochloric acid, 10% formic acid, 5% hydrochloric acid, 5% formic acid, and DMSO (dimethyl sulfoxide) (100%, 40%, 20%) were used. The bottles were shaken in a shaker for 24 h at 140 rpm at room temperature.

Ultrasound-assisted extraction of bryophytes. Dry samples were ground in a mill, and 0.3 g samples of bryophytes were weighed into 100 ml bottles with screw caps, adding 50 ml of solvent. Solvents such as ethanol and methanol (96%, 80%, 60%, 40%, 20%) diluted with demineralised (Millipore) water, acetone, dioxane, and DMSO (100%, 40%, 20%) were used. Then, the samples were exposed to ultrasound (100 W) in an ultrasound bath (Cole Parmer) for 20 and 40 min. The temperature was kept constant at +40 ^{o}C by regularly adding cold water. The bottles were then shaken in a shaker for 24 h at 140 rpm at room temperature.

Extraction of bryophytes by microwave treatment. Dry samples were ground in a mill, and 0.3 g samples of bryophytes were weighed into Teflon extraction vessels, adding 50 ml of solvent (96%, 80%, 60%, 40%, 20% ethanol). The vessels were sealed using a Milestone Twister. Extraction was performed using a Milestone Ethos One microwave oven, at 120 °C and 150 °C temperatures, with 1500 W power. The extraction time was 40 min: 10 min to reach the chosen temperature, 20 minutes for steady extraction at the set temperature, and 10 minutes for the oven to cool down. After the extraction had completed, the samples in the extraction vessels were placed at room temperature and held for approximately 1 hour for the extract to cool down.

Supercritical CO_2 extraction. Dry bryophyte samples were ground in a mill, weighing 15 g of the sample in a metallic column. The column was inserted into a preheated (+ 60°C) oven, setting the CO_2 flow to 10 ml per minute. The extraction was done using a Separex CO_2 supercritical extractor. After the first trials, it was concluded that coupled extraction was required for best results; therefore, 96 % ethanol flow for 5 ml/min was used. The extraction experiments were conducted under 20 MPa pressure for 30 minutes and 60 minutes.

Soxhlet extraction of bryophytes. A 20 g dose of dry sample was weighed in a fabric bag, which was then sealed and inserted into an extraction tube. The extraction was done using a Soxhlet extractor and 96% ethanol as a solvent. The extraction process was done at 80 °C for 8 h and 24 h time periods.

Analytical methods. Before analysis, all bryophyte samples were kept at room temperature for ~1 hour. In all cases, three parallel measurements were carried out.

Determination of the total concentration of polyphenols in bryophyte extracts**.** Folin-Ciocalteu reagent was used for determination of the total amount of polyphenols (Singleton *et al.*, 1999). After maintaining extracts at room temperature for ~1 hour, 1 ml of bryophyte extract was put into a test tube, adding 5 ml of 10% Folin-Ciocalteu reagent (Aldrich). After 5 minutes, 4 ml of 7.5% sodium carbonate (Aldrich) was added. The test tube was shaken thoroughly and kept in a dark place at room temperature for 2 hours. Absorption

was then measured in a quartz cuvette (d=1 cm) on a spectrophotometer (Hach-Lange DR 2800) at 725 nm wavelength. Results were calculated using a standard curve, expressed as gallic acid/100g (GE mg/100 g) dry matter (Singleton *et al.*, 1999; Silverstein *et al.*, 2005).

Radical scavenging activity determination in bryophyte extracts using DPPH. After maintining extracts at room temperature for ~1 hour, 0.3 ml of bryophyte extract was put in a test tube and mixed with 3.6 ml of 4% 2-diphenyl-1-picrylhydrazyl solution in 96% ethanol (Aldrich). The mixture was incubated for 20 minutes in dark at room temperature. Absorption was measured in a quartz cuvette (d = 1 cm) with a spectrophotometer (Hach-Lange DR 2800) at 517 nm wavelength. Three parallel measurements were carried out. Radical scavenging activity was expressed as GE mg/100 g dry weight.

UPLC analysis of bryophyte extracts. Chromatography separation was performed with a Waters Acquity ultra-performance liquid chromatography (UPLC) system, equipped with a quaternary solvent manager, UV/Visible detector, thermo stated auto sampler and column heater. Data acquisition and analysis were performed using the Empower3 system. The chromatographic separations were carried out using the Acquity UHPLC BEH C8 (2.1 × 50 mm, 1.7 μm) column and solvent gradient elution programme. Two solvents — (A) water/acetonitrile (65:35 by volume) with 2% formic acid in water and (B) acetonitrile — were used as a mobile phase. Separation was made with the following time-gradient programme: initial (100% A), 10.0 min (78.0% A), 16.0 min (65.0% A), 21.0 min, (62.0% A), 30.0 min (50.0% A), 34.0 min (40.0%), 37.0 min (37.0% A), 39.0 min (30.0%), 40.0 min (23.0% A), 53.0 min (20.0%), and 54.0–60.0 min (10.0% A). The elution was performed at a flow rate of 0.25 mL/min, and the detector was set at 270 nm. The column was operated at 30 ^{o}C temperature. The sample injection volume was 5 μL, and the total run time was 60 min. The strong wash solvent was 70% acetonitrile in water, and the weak wash solvent — 10% acetonitrile in water.

RESULTS

Two well-characterised bryophyte species (Maksimova *et al.*, 2013) common in Northern Europe were used in the study to develop extraction procedures for bryophytes. The studied bryophytes showed relatively low variability in their elemental composition, and the basic element content ranges in the bryophyte species were: C 41 to 44%; O 49 to 52%; H 5.5 to 6%; N 0.4 to 2%; S 0%. The basic chemical characteristics of the studied bryophyte species were also given in a previous study (Maksimova *et al.*, 2013). Bryophyte extraction efficiency was compared using following criteria: yield of extracts (dry residue and D_{280}), total polyphenolic content, antiradical activity, and the number of individual compounds determined by means of UPLC analysis. Tested solvents for extraction were water, methanol, ethanol, acetone, dioxane, and dimethylsulphoxide. Several extraction methods were compared: a) conventional extraction (shaking at room temperature); b) Soxhlet extraction; c) ultrasound-assisted extraction; d) extraction using treatment with microwaves; and e) extraction with supercritical CO_2. Efficiency of different extraction conditions (time and temperature) were also compared for each selected method (Table 1).

Microwave extraction at 150 °C was the most efficient extraction method (Table 1), based on both total polyphenol content and radical scavenging activity. Conventional and Soxhlet extractions provided high yields, but, in comparison with intensive extraction methods, required much more time. Conventional extraction also consumed much more solvent than the other studied methods. Soxhlet extraction showed good results for radical scavenging activity; however, the total polyphenol levels were lower than, for example, obtained by ultrasound extraction. This indicates that polyphenolic compounds in bryophytes are not the only factor responsible for radical scavenging activity. Supercritical CO_2 extraction provided good yield of polyphenolics, while the overall yields and yields of radical scavenging substances were relatively low.

To better assess extraction efficiency using ultrasound for two bryophytes species, the effect of treatment time and ratio of ethanol/water mixtures were compared (Fig. 1). Ultrasound-assisted extraction allowed to decrease the extraction time and improve the extraction yield due to mechanical stress which induced cavitation with cellular breakdown and release of secondary metabolites.

As the chemical content of bryophytes varies from species to species (Asakawa, 2007), two widespread bryophyte species, one typical of forest and the other from mires, were chosen for study: *R. triquetrus* and *S. rubellum*. The increase of treatment time with ultrasound helped to significantly increase the yield of polyphenolics by up to 53% in the case of *R. triquetrus* and up to 70% for *S. rubellum* (Fig. 1). The difference in extraction efficiency between no treatment with ultrasound and 40 min treatment with ultrasound was approximately 20–50% in some cases, while the difference between the treatments for 20 or 40 min was less than 10% or none at all. In all extractions ultrasound treatment proved to be an efficient tool to improve extraction yields of total polyphenol. To achieve a higher optimal outcome for total polyphenol content, radical scavenging and dry matter outcome, various types of solvents were used, ensuring isolation of secondary metabolites and, especially, phenolic compounds from bryophytes (Table 2). Water, ethanol, methanol, acetone, dioxane, and DMSO were used as solvents. The selection of solvents was based on economic reasons, toxicity of solvents, and polarity of the substances of interest. Microwave extraction was found to be the most effective extraction technique and solvent optimisation was achieved by treating samples with ultrasound with subsequently shaking for 24 h.

Higher polyphenolic concentrations were found using solvents such as ethanol and DMSO. The use of acetone and methanol led to lower yields of polyphenolics. Antioxidant capacity (measured with the DPPH method) was also higher when using ethanol, but the differences were not as significant as in the case of total polyphenolics content and the total dry extract mass. The optimal yields of polyphenolics and radical scavenging substances and the total dry extract

Table 1

EXTRACTION EFFICIENCY FOR *R. triquetrus* USING DIFFERENT EXTRACTION TECHNIQUES (solvent: 60% ethanol)

Extraction method	Extraction conditions		Sample/extrahent ratio		Total polyphenol content, GE mg/ 100 g	Radical scavenging activity, GE mg / 100 g	Extraction yield (dry weight), mg/100 g
	Time, h	Temperature, °C	Volume of solvent, mL	Weight of moss sample, g			
Soxlet	8.0	80	300	11.2	165.5 ± 8.3	116.4 ± 5.8	205.2 ± 10.3
	24.0	80	300	11.2	239.6 ± 11.9	142.6 ± 7.1	231.5 ± 11.6
Microwave	0.5	120	30	0.3	111.2 ± 5.6	167.8 ± 8.4	195.6 ± 9.8
	0.7	150	30	0.3	486.9 ± 24.4	172.9 ± 8.6	150.2 ± 7.5
Ultrasound	0.5	50	40	0.5	243.7 ± 12.2	54.1 ± 2.7	195.5 ± 9.7
	0.7	70	40	0.5	254.6 ± 12.7	63.3 ± 3.2	150.6 ± 7.5
Supercritical CO_2	0.5	102	150	15.0	230.4 ± 11.5	162.4 ± 8.1	125.1 ± 6.3
	1.0	102	150	15.0	274.9 ± 13.5	143.5 ± 7.2	124.8 ± 6.2
Conventional	12.0	24	30	0.4	150.7 ± 7.6	25.8 ± 1.3	99.5 ± 4.9
	24.0	24	30	0.4	194.3 ± 9.7	36.9 ± 1.8	97.2 ± 4.9

Data are in three replicates. Mean ± SE values are given

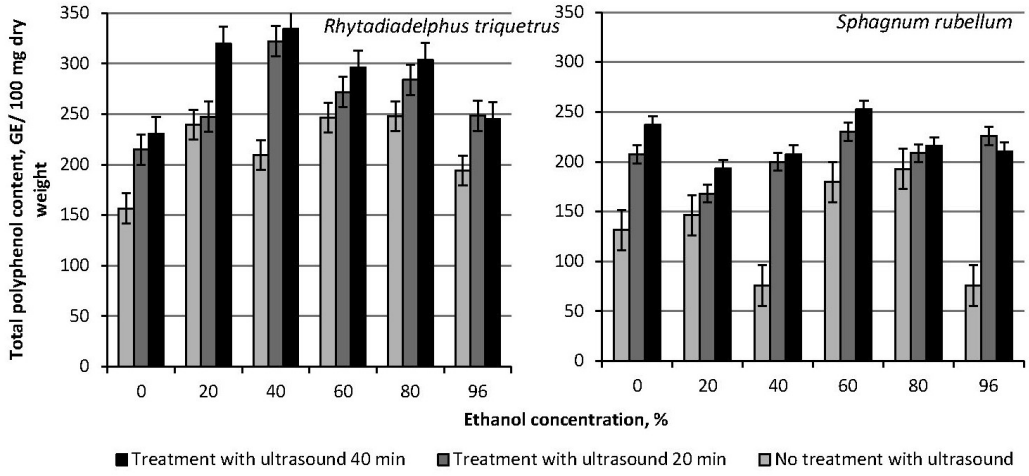

Fig. 1. Effect of the ethanol/water ratio and the extraction and ultrasound treatment time on the total polyphenol content in extracts from bryophytes *R. triquetrus* and *S. rubellum.*

mass were obtained from *R. triquetrus* and *S. rubellum* using aqueous methanol, ethanol and acetone in the concentration range from 60% to 80%. The optimal conditions in each specific case and each group of substances of concern are indicated in Table 2. The polar aprotic solvent DMSO in both pure solvent and aqueous mixture provided higher yields of extracts; however, the application possibilities of this solvent are limited by its cost and difficult removal after extraction.

The best results were obtained in microwave-assisted extraction, using ethanol as solvent Table 2. To better understand the factors controlling the extraction efficiency, an experiment was conducted to see how ethanol concentration affected the microwave-assisted extraction efficiency (Fig. 2). Higher polyphenol content was obtained with microwave treatment at 150 °C in comparison to 120 °C for both studied bryophyte species.

Microwave-assisted extraction, in comparison with ultrasound-assisted extraction, showed similar differences in efficiency among different optimal solvent concentrations and moss species used. The optimal ethanol concentration for *R. triquetrus* at 120 °C temperature was approximately 60%, while at 150 °C it was 20%; the optimal concentration of ethanol for *S. rubellum* at both tested temperatures was 60%. The optimal ethanol concentrations for *R. triquetrus* and *S. rubellum* in ultrasound-assisted extraction were 40% and 60%, respectively. Change of microwave temperature regime from 120 °C to 150 °C resulted in increase of total polyphenol content by 78% for *R. triquetrus* and by 44% for *S. rubellum.*

All of the analyses described provide some understanding about the biologically active substances in optimised extract; however, it is still unclear which substances or substance groups are responsible for the antioxidant activity. Therefore, analyses of some extracts were made using UPLC (Figs. 3, 4). The peaks of found substances were compared (Fig. 5).

According to Snyder's selectivity range, the extrahents used had well-expressed selectivity in respect to their ability to interact with substances in protonated forms. Addition of water to the extraction system increased its ability to interact with proton-accepting groups.

Table 2

EXTRACTION EFFICIENCY FROM *R. triquetrus* and *S. rubellum* USING DIFFERENT SOLVENTS AND SOLVENT MIXTURES (Extraction conditions: ultrasound treatment for 20 min with subsequent shaking for 24 h)

Extrahent		Total polyphenol content, GE/100 g		Radical scavenging activity, GE/100 g		Extraction yield (dry weight), mg/100 g	
		R. triquetrus	*S. rubellum*	*R. triquetrus*	*S. rubellum*	*R. triquetrus*	*S. rubellum*
Water		230.0 ± 11.5	237.2 ± 11.9	10.5 ± 0.5	11.9 ± 0.6	36.8 ± 1.8	51.8 ± 2.6
Methanol	100%	183.1 ± 9.2	132.9 ± 6.6	11.6 ± 0.6	45.9 ± 2.3	75 ± 3.8	91.6 ± 4.6
	80%	208.6 ± 10.4	132.9 ± 6.6	32.4 ± 1.6	48.7 ± 2.4	91.7 ± 4.6	91.4 ± 4.6
	60%	189.7 ± 9.5	135.3 ± 6.8	24.1 ± 1.2	42.6 ± 2.1	91.8 ± 4.6	75.1 ± 3.8
	40%	182.8 ± 9.1	120.4 ± 6.0	7.6 ± 0.4	31.2 ± 1.6	108.3 ± 5.4	50.2 ± 2.5
	20%	171.9 ± 8.6	116.2 ± 5.8	6.2 ± 0.3	27.6 ± 1.4	75.0 ± 3.8	50.8 ± 2.5
Ethanol	96%	254.0 ± 12.7	210.4 ± 10.5	11.5 ± 0.6	22.5 ± 1.1	652.5 ± 32.6	345.0 ± 17.3
	80%	304.0 ±15.2	215.7 ± 10.8	50.4 ± 2.5	26.4 ± 1.3	667.5 ± 33.4	287.5 ± 14.4
	60%	296.0 ± 14.8	252.6 ± 12.6	24.0 ± 1.2	33.5 ± 1.7	195.0 ± 9.8	97.5 ± 4.9
	40%	334.0 ± 16.7	207.5 ± 10.4	7.9 ± 0.4	11.5 ± 0.6	187.5 ± 9.4	179.5 ± 9.0
	20%	320.0 ± 16.0	193.2 ± 9.7	6.3 ± 0.3	1.6 ± 0.1	96.6 ± 4.8	78.0 ± 3.9
Acetone	100%	174.5 ± 8.7	127.9 ± 6.4	9.1 ± 0.5	15 ± 0 .8	36.8 ± 1.8	67.5 ± 3.4
	80%	195.7 ± 9.8	178.1 ± 8.9	47.2 ± 2.4	52.6 ± 2.6	91.5 ± 4.6	58.3 ± 2.9
	60%	238.1 ± 11.9	177.6 ± 8.9	46.2 ± 2.3	52.1 ± 2.6	116.3 ± 5.8	50.7 ± 2.5
	40%	214.2 ± 10.7	160.9 ± 8.0	31.2 ± 1.6	49.5 ± 2.5	125.6 ± 6.3	41.6 ± 2.1
	20%	191.1 ± 9.6	128.6 ± 6.4	12.5 ± 0.6	42.6 ± 2.1	50.4 ± 2.5	28.4 ± 1.4
Dioxane		151.2 ± 7.6	138.3 ± 6.9	13.5 ± 0.7	13.4 ± 0.7	15.0±0.8	147.0 ± 7.4
DMSO	100%	363.9 ± 18.2	75.7 ± 3.8	47.9 ± 1.6	31.5 ± 1.6	41.7 ± 2.1	97.5 ± 4.9
	40%	295.1 ± 14.8	75.7 ± 3.8	34.7 ± 2.2	43.6 ± 2.2	25.2 ± 1.3	108.0 ± 5.4
	20%	270.0 ± 13.5	75.7 ± 3.8	19.1 ± 1.0	16.6 ± 0.8	16.9 ± 0.8	68.3 ± 3.4

Data are in three replicates. Mean ±SE values are given.

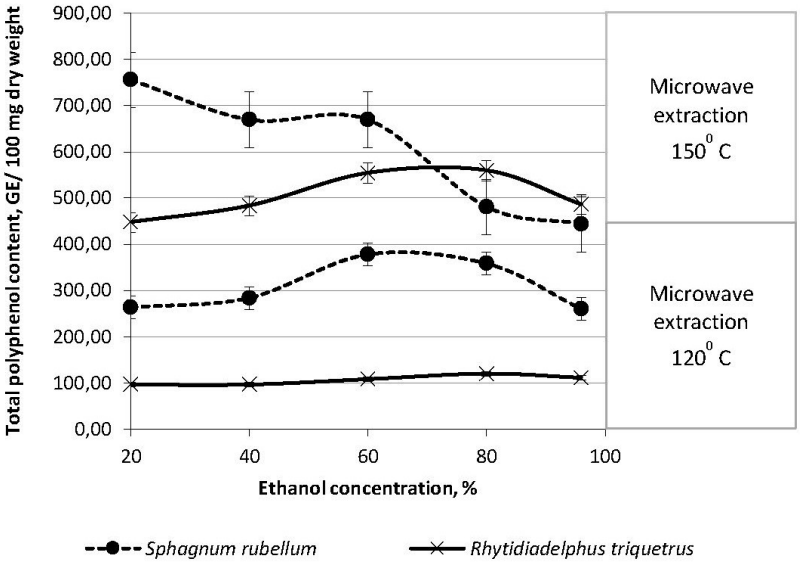

Fig. 2. Total polyphenol content after of microwave-assisted extraction from *R. triquetrus* and *S. rubellum* using aqueous ethanol (20–96%) as solvent.

UPLC analysis of bryophyte extracts (Figs. 4, 5) demonstrate presence of a high number of individual substances and the impact of the extraction conditions on the composition of extracts. The extraction efficiency under the elaborated extraction conditions was high, as indicated by the presence of individual components of bryophytes SR and RT in chromatograms, with the retention times 12.19; 18.50; 20.59; 26.57; 33.15; 37.24; 38.90; and 51.23 (Figs. 4, 5).

DISCUSSION

The bryophyte species for this study were selected considering their abundance in Northern Europe and results of previous studies (Maksimova *et al.*, 2013), the possible presence of biologically active substances in their composition, use in traditional medicine, and relevance in respect to understanding the secondary metabolite composition patterns in bryophytes. The following parameters were used as criteria

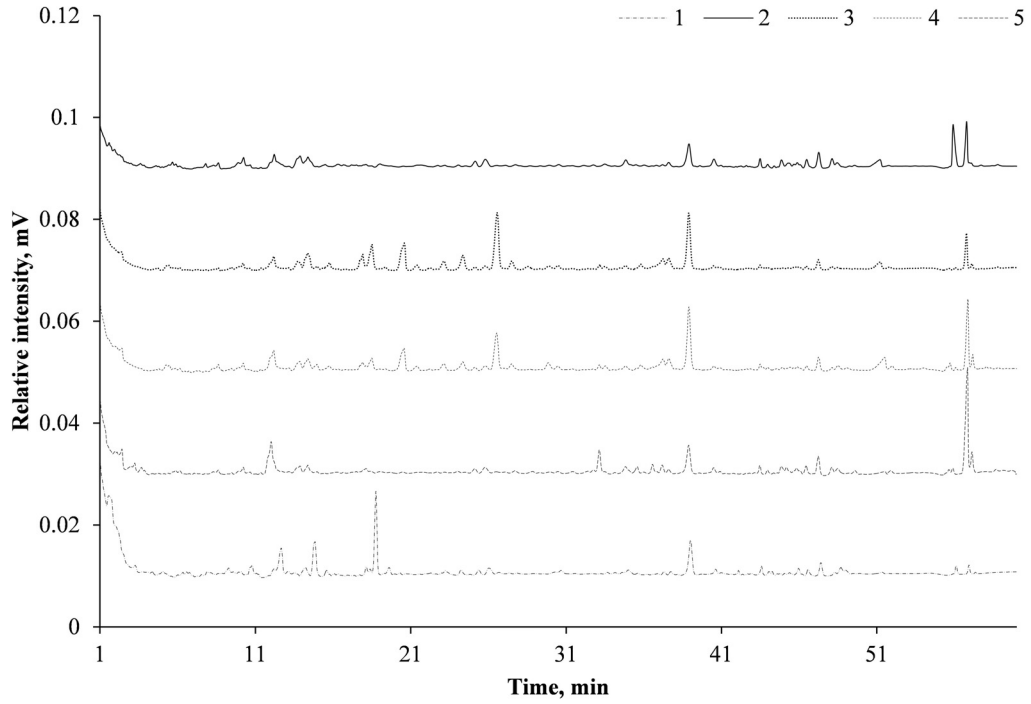

Fig. 3. UPLC chromatograms of optimised extracts of *S. rubellum* using the following solvents and 20 min ultrasound treatment*: 1. Dioxane; 2. 60% ethanol (24 h shaking* no ultrasound treatment); 3. 60% ethanol; 4. 100% methanol; 5. 80% acetone.

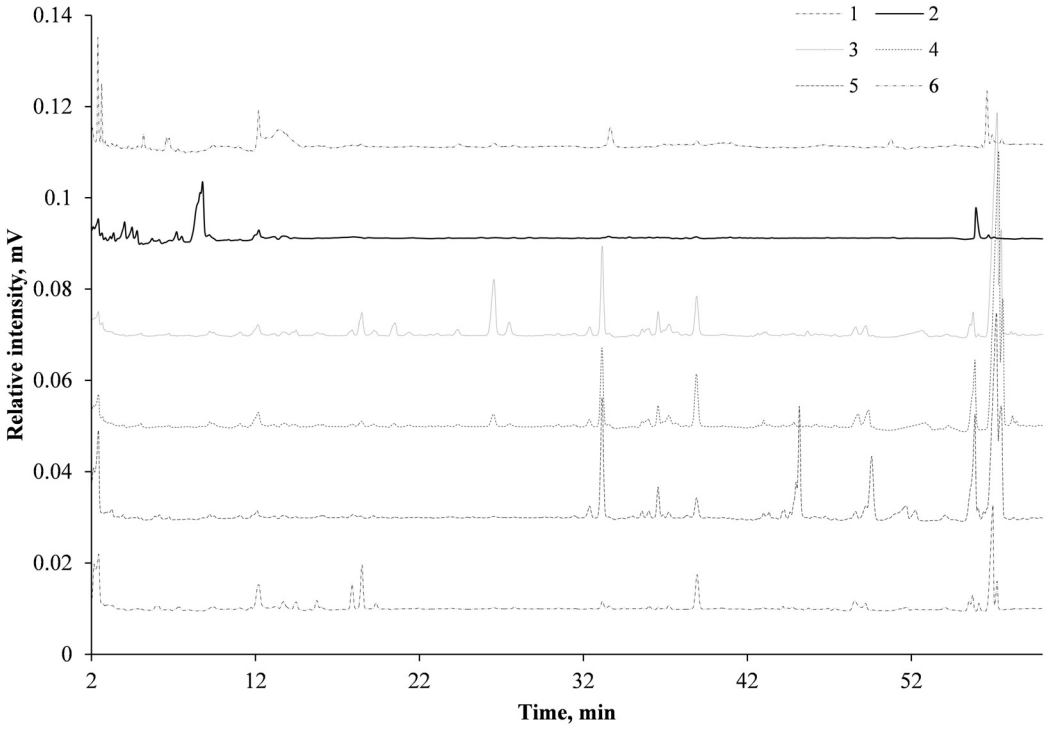

Fig. 4. UPLC chromatograms of optimised extracts of *R. triquetrus* using the following solvents and 20 min ultrasound treatment*: 1. dioxane; 2. 60% ethanol (24 h shaking* no ultrasound treatment); 3. 60% ethanol; 4. 100% methanol; 5. 80% acetone; 6. *Supercritical CO_2 extraction coupled with 96% ethanol treatment for 30 min.

for evaluating the extraction efficiency: yield of extracts (dry residue and D_{280}), total polyphenolic content, antiradical activity, and the number of individual compounds under UPLC chromatographic conditions. Amongst these criteria, major stress was placed on the yield of extracted substances and the antioxidant activities of the extracts, considering the recent interest in this kind of activity of natural compounds (Cheynier *et al.*, 2013). For this purpose, the DPPH radical scavenging activity analysis was used. The Folin-Ciocalteu assay has been used to estimate the total phenolic content in natural products (Cheynier *et al.*, 2013), although the basic mechanism of this assay is oxidation/reduction reaction, which can as such be considered as another method of antioxidant determination. Considering the interest in studies of biologically active compounds of bryophytes and, more broadly, in the composition of bryophyte secondary metabolites used for the extraction of low-cost, low-toxicity, volatile solvents with differing polarity, their mixtures were selected with regard to the ability of extracting substances with a possibly wider range of properties (water, methanol, ethanol, acetone, dioxane, dimethylsulphoxide). Five different extraction methods were used with the aim to ensure the highest extraction yield (Bucar *et al.*, 2013) prospective for obtaining prepara-

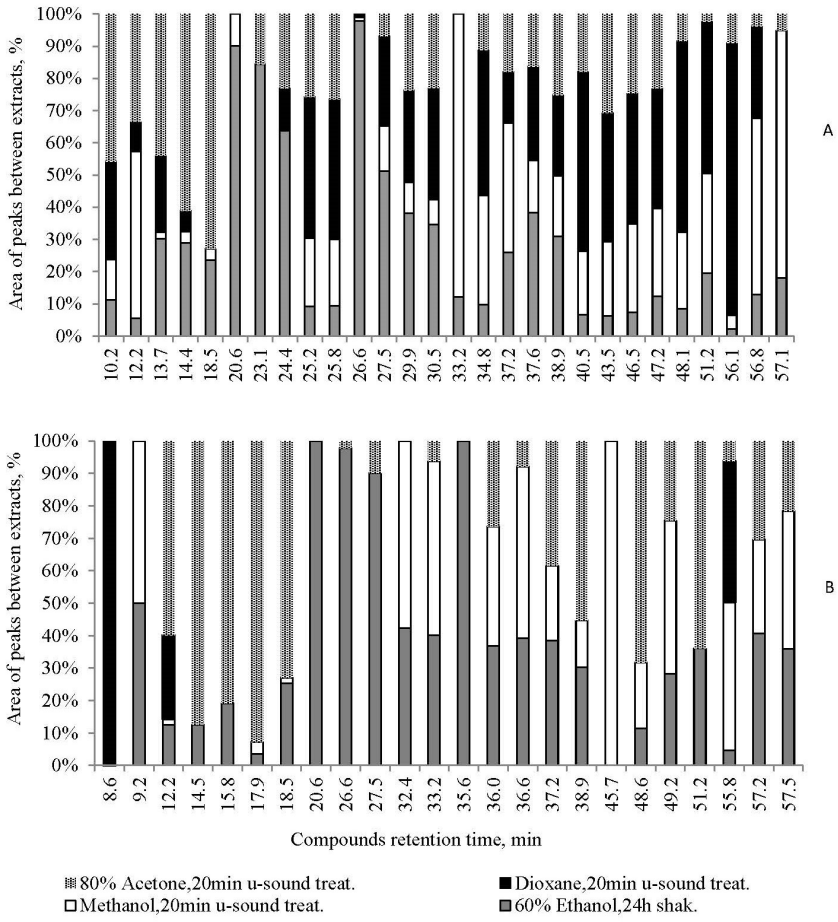

Fig. 5. Comparison of peak areas of substances detected using UPLC of bryophyte extracts (A-*Sphagnum rubellum;* B- *Rhytadiadelphus triquetrus*).

tive amounts of extracts: a) conventional extraction (shaking at room temperature); b) Soxhlet extraction; c) ultrasound-assisted extraction; d) extraction using treatment with microwaves; and e) extraction with supercritical CO_2. To study the impact of the extraction procedures, efficiency of extraction conditions (time and temperature) was also tested (Table 1).

Ultrasound extraction had high efficiency. Time period of sonification duration did not have major effect for times > 20 min. Optimal extraction conditions differed between the bryophyte species in respect to composition of the solvent mixture (ethanol and water). The highest yield of polyphenolics from *R. triquetrus* was obtained using 20–60% ethanol, and from *S. rubellum* using 20% ethanol. The differences found in the extraction yield of total polyphenolics indicated differences in the bryophyte composition and the need to estimate the optimal solvent concentration for each bryophyte species individually. 60% ethanol was found to be the best concentration for screening of bryophyte secondary metabolite composition, and was further used in this study.

For extracts from higher vegetation, a high correlation between total phenolic content and antioxidant activity has been observed (Tabart *et al.*, 2006; Ehala *et al.*, 2005). However, for extracts of bryophytes, the correlation was not high ($R < 0.5$), indicating that the antioxidative activity could be determined not only by phenolics but also by other groups of substances.

Increase of water content in the composition of extrahent resulted in higher yields for several compounds in the obtained UPLC chromatograms (Fig. 5), and thus potentially also for better better isolation of a larger number of secondary metabolites from bryophytes. The extraction method can be considered as an additional factor influencing the effect of water addition. Dioxane and acetone have medium selectivity in respect to their ability to interact with low-polarity compounds, and the use of dioxane does not ensure high yields of secondary metabolites (Fig. 5). Addition of water to the extraction mixtures increases the proton-donating ability of these solvents, as in the case of methanol and ethanol, although to a lesser extent. The composition of isolated compounds using an 80% acetone-water mixture did not differ much in qualitative composition from that obtained using 60% ethanol–water (Fig. 5). Considering that the chromatographic separation was achieved using an apolar column, the suggested extrahent systems helped to isolate a high number of compounds with relatively high polarity, despite differences in the qualitative composition of isolated compounds.

This study indicated that the extracts obtained from bryophytes had antioxidant activity, the extent of which depended on the extraction conditions. The principal factors that contribute to the efficiency of extraction are the type of solvent, temperature, the solvent to bryophyte mass ratio, etc. In this study, some of these parameters were tested for the extraction of polyphenolics and antioxidants. The main factors that contributed to the efficiency of extraction were

the type of solvent, temperature, and the solvent to bryophyte mass ratio. The extracts obtained from bryophytes had remarkable antioxidant activity, the extent of which depended on the extraction conditions and bryophyte species. The extraction conditions can be optimised, and the total polyphenol content can be increased by up to 50% in comparison with the conventional approach.

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LATVIJĀ AUGOŠU BRIOFĪTU EKSTRAKCIJAS APSTĀKĻU OPTIMIZĀCIJAS IETEKME UZ BIOLOĢISKI AKTĪVIEM SEKUNDĀRAJIEM METABOLĪTIEM

Briofītu sastāvā ir atrodams liels skaits bioloģiski aktīvu savienojumu, bet to ķīmiskā sastāva izpēte ir arī svarīga, lai izprastu augu valsts evolūcijas procesus. Šī pētījuma mērķis bija novērtēt briofītu sekundāro metabolītu ekstrakcijas iespējas un optimizēt polifenolu, un citu vielu ar antiradikālo aktivitāti iznākumu briofītu ekstraktos. Tika veikts piecu dažādu ekstrakcijas metožu salīdzinājums (tradicionālā ekstrakcija, Soksleta ekstrakcija, mikroviļņu ekstrakcija, ekstrakcija izmantojot ultraskaņu un ekstrakcija ar CO_2 superkritiskā stāvoklī). Tika veikts ekstrahentu salīdzinājums, izmantojot dažādas polaritātes šķīdinātājus. Galvenie faktori, kas nosaka ekstrakcijas efektivitāti, ir ekstrahenta veids, ekstrakcijas temperatūra un ekstrahenta/ briofītu parauga masas attiecība. Iegūtajiem ekstaktiem ir augsta spēja saistīt brīvos radikāļus, kas ir atkarīga no ekstrakcijas apstākļiem un briofītu sugas. Ekstrakcijas apstākļi var tikt optimizēti, un kopējais polifenolu daudzums var tikt palielināts par 50%, salīdzinot ar tradicionālajām ekstrakcijas metodēm. Par optimālu ekstrakcijas metodi uzskatāma ekstrakcija, izmantojot mikroviļņus, jo tā nodrošina augstāko ekstraktvielu iznākumu.