

SEASONAL VARIATION IN ANTIOXIDANT ACTIVITY OF SELECTED MOSSES
FROM POLANDADAM STEBEL¹, HELENA D. SMOLARZ², MAŁGORZATA JANKOWSKA-BŁASZCZUK³,
MACIEJ TRYŁOWSKI² AND ANNA BOGUĆKA-KOCKA²

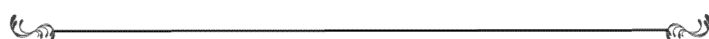
¹Department of Pharmaceutical Botany, School of Pharmacy with Division of Laboratory Medicine in Sosnowiec, Medical University of Silesia in Katowice, Ostrogórska Street 30, 41-200 Sosnowiec, Poland; e-mail: astebel@sum.edu.pl

²Chair and Department of Pharmaceutical Botany, Medical University of Lublin, Chodźki Street 1, 20-093 Lublin, Poland

³Department of Botany, Institute of Biology, Jan Kochanowski University, Świętokrzyska Street 15, 25-406 Kielce, Poland

ABSTRACT: The paper presents information about antioxidant activities and the seasonal variation of methanol extracts of eight moss species, namely *Atrichum undulatum* (Hedw.) P. Beauv., *Brachythecium rutabulum* (Hedw.) Schimp., *Callicladium haldanianum* (Grev.) H. A. Crum, *Hypnum cupressiforme* Hedw., *Kindbergia praelonga* (Hedw.) Ochyra, *Plagiothecium laetum* Schimp., *Polytrichastrum formosum* (Hedw.) G. L. Sm. and *Pohlia nutans* (Hedw.) Lindb. The antioxidant properties of the extracts from plants collected in spring, summer and autumn were evaluated using three methods - scavenging effect on DPPH radical, iron (II) chelating activity and inhibition of linoleic acid peroxidation. It was shown that the season when the sample was collected does not influence the antioxidant activity of the analyzed group of mosses. The systematic relations between the investigated species influence their antioxidant activity on the level of lower taxonomical units, i.e. families and the lower ones. The highest antioxidant activities show *Atrichum undulatum* and *Polytrichastrum formosum* thus these species could be considered as a potential source for antioxidants

KEY WORDS: *Bryophyta*, metal chelating activity, linoleic acid peroxidation, methanol extracts, mosses, scavenging effect on DPPH radical.



Introduction

Mosses Bryophyta are one of the three divisions of bryophytes and comprise about 13 000 species (Goffinet et al. 2009) occurring in almost all land and freshwater habitats of the Earth. Recent investigations have shown that mosses contain many biologically active compounds which can be used in medicine. Cytotoxic, anticancer, antitumor, antibacterial and antifungal properties were shown for many species, although mosses are less recognized in these fields than liverworts Marchantiophyta (Asakawa et al. 2013). Investigations on the antioxidant potential of mosses have been launched quite recently (for example Chobot et al. 2006; Bhattarai et al. 2008; Basile et al. 2011; Mukhopadhyay et al. 2013; Karim et al. 2014). Yet, the results are promising. A review of papers considering antioxidant potentials of bryophytes has recently been published by Dey and Nath De (2012). Since no information about antioxidant properties of Polish populations of mosses is available at the moment, we have decided to start the investigation. Another yet unsolved scientific problem are the seasonal variations and differences between haploid and diploid generations in antioxidant potential in mosses. The problem of seasonal variation of activity of biological compounds is fairly known in vascular plants (e.g. Ahmed et al. 2012; Olszewska 2011; Pliszka et al. 2009), but uninvestigated in bryophytes.

Goals of the work

The goals of the work are to determine: (1) the antioxidant activity of selected mosses using three methods: scavenging effect on DPPH radical, iron (II) chelating activity (CHEL) and inhibition of linoleic acid peroxidation (LPO), (2) the seasonal variation in antioxidant activity, (3) the comparison of results obtained from using the three methods and (4) the systematics relations between

species and their antioxidant activity. Four hypotheses were formulated: (1) the antioxidant activity of the chosen group of mosses expressed by the IP index differs significantly depending on the method applied, (2) the dates of sampling affected the antioxidant activity, (3) the IP index value was influenced by the concentration of the sample and (4) the systematic relations between the investigated species influence their antioxidant activity.

Materials and Methods

Collection of plant materials

Moss samples were collected from a plot localized in Katowice in southern Poland (GPS coordinates: lat. 50°15'30"N, long. 18°57'35"E; alt. 300 m). The plot comprises a patch of managed deciduous linden-oak-hornbeam forest *Tilio cordatae-Carpinetum betuli*. Mosses were gathered in the years 2012 and 2013 during three seasons – spring, summer and autumn. After collection, each sample was cleaned from admixtures of others plants and underwent careful selection. Only green parts of mosses, without necrosis and other harms were used for further investigations. All samples were divided into generations – gametophyte and, if present, sporophyte. Next, they were dried up at room temperature (about 20°C). Finally, for the investigations of their antioxidant activity, 9 gametophytes and 1 sporophyte which belong to following species were chosen: *Atrichum undulatum* (Hedw.) P. Beauv. (Polytrichaceae), *Brachythecium rutabulum* (Hedw.) Schimp. (Brachytheciaceae), *Callicladium haldanianum* (Grev.) H. A. Crum (Hypnaceae), *Hypnum cupressiforme* Hedw. (Hypnaceae), *Kindbergia praelonga* (Hedw.) Ochyra (Brachytheciaceae), *Plagiothecium laetum* Schimp. (Plagiotheciaceae), *Polytrichastrum formosum* (Hedw.) G. L. Sm. (Polytrichaceae), and

Pohlia nutans (Hedw.) Lindb. (Bryaceae). Representative specimen were deposited in the Herbarium of the Department of Pharmaceutical Botany of Medical University of Silesia in Katowice (SOSN).

Preparation of extracts

A 4g sample of air-dried and powdered plant material was soaked in 40 ml of 80% methanol for 1h and sonicated in ultrasonic bath at room temperature for 15 minutes. After triple extraction the supernatant was collected, evaporated under reduced pressure and dried. 200 mg of dry extract were dissolved in 10 ml of DMSO and 80% methanol mixture (1:1). The stock solution of the extract was diluted with 80% methanol to provide a series of five solutions of increasing concentration in the range of 1.25–20.00 mg/ml.

In vitro assay for evaluation of antioxidant activity

Antioxidant properties of the plant extracts collected in spring, summer and autumn were evaluated using three methods: scavenging effect on DPPH radical, iron (II) chelating activity and inhibition of linoleic acid peroxidation. Methanolic extracts from ten gametophytes and one sporophyte at concentrations of 20.00, 10.00, 5.00, 2.50 and 1.25 mg/ml were investigated. Ascorbic acid, EDTA and BHA were used as references. Every assay was performed three times.

Scavenging effect on DPPH radical (MET. 1 in statistical analysis)

The scavenging effect on DPPH radical was estimated according to the procedure described by Brand-Williams et al. (1995). 2.29 ml of freshly prepared DPPH in methanol (6×10^{-5}) was mixed with 0.06 ml of extract in methanol at different concentrations.

An equal amount of methanol was added to the control sample. Ascorbic acid was used as

reference. Thirty minutes later, the absorbance was recorded at 517 nm. The 30 minute scavenging time was based on preliminary experiment, where decrease in absorbance was tested with several extracts at concentration 10 mg/ml over a period of 30 min. Within the first minutes, the absorbance decreased rapidly and between 20 and 30 minutes, the decrease in absorbance did not exceed 5%. The antioxidant activity was expressed as a percentage of inhibition (IP) calculated according to the formula: $IP = [(AC(0) - AC(t)/A C(0)] \times 100$; where $A C(0)$ – absorbance of the control sample at time 0, $AA(t)$ – absorbance of the experimental sample measured every minute over 5 minutes. The IC_{50} (efficient concentration) values were calculated by linear regression of plots.

Metal chelating activity (CHEL) (MET. 2 in statistical analysis)

The chelation of iron (II) ions by the samples was carried out according to the method proposed by Guo et al. (2001), with an inconsiderable modification. Twenty μ l of 2 mM aqueous $FeCl_2$ were mixed with 1 ml of the extract of different concentrations and shaken. The control sample contained 1 ml of water instead of the extract. Next, 40 μ l of 5 mM ferrozine were added to each of the test tubes. After heating at room temperature for 10 minutes, the absorbance was measured at 562 nm. EDTA was used as the reference compound. Metal chelating activity effect was calculated using the following equation : % chelating activity = $[1 - As/Ac] \times 100$; where: As – absorbance of sample, Ac – absorbance of control reaction. The extract concentration causing 50% inhibition (EC_{50}) was calculated by interpolation from linear regression analysis.

Inhibition of linoleic acid peroxidation (LPO) (MET. 3 in in statistical anaysis)

The antioxidant activity of extracts in the linoelic acid peroxidation system was carried out according to the modified method described by Kuo et al. (1999). Ten microliters of extract were mixed with 0.37 ml of phosphate buffer (pH 7.0) containing 0.05% Tween 20 and 4 mM linoleic acid. The sample was preincubated at 37°C for 3 minutes. Linoleic acid peroxidation was initiated by the addition of 20 µl of 0.035% haemoglobin (or interchangeably 0.01 M FeCl₂) and incubated for 10 min at 37°C and terminated by the addition of 5 ml of 0.6% HCl. Next, 0.1 ml of 30% ammonium thiocyanate and 0.1 ml of 0.02 M ferrous chloride were added. After 5 minutes, the absorbance was measured at 480 nm and converted into the percentage of inhibition peroxidation using the equation; $IP = [1 - (As - A_0) / (A_{100} - A_0)] \times 100$. The reference absorbance (A_0) was obtained without the addition of haemoglobin to the reaction mixture. The control absorbance (A_{100}) was obtained with no sample addition to the aforementioned mixture.

Statistical analysis

The antioxidant properties of 9 groups of mosses were compared according to the obtained IP index. IP index values were analyzed depending on:

- 1) the method applied (IP_{MET1} , IP_{MET2} , IP_{MET3})
- 2) the date of collecting the plant material (IP_A – spring, IP_B – summer, IP_C – autumn)
- 3) the concentration of the extract (IP_{E1} – 1.25 mg/ml; IP_{E2} – 2.50 mg/ml; IP_{E3} – 5.00 mg/ml, IP_{E4} – 10.00 mg/ml)
- 4) the examined moss species (species 1–9: IP_1 , IP_2 , IP_3 , IP_4 , IP_5 , IP_6 , IP_7 , IP_8 , IP_9)

Because the condition of normality for the distribution of IP values was not met, the nonparametric Kruskal-Wallis analysis of ranks test for multiple comparisons with "METHOD"; "SEASON", "EXTRACT

CONCENTRATION" and "SPECIES" as the independent grouping variables was used.

The hypotheses that the following medians of IP values do not differ significantly was tested:

$$H_1^0: IP_{MET1} = IP_{MET2} = IP_{MET3}.$$

$$H_2^0: IP_A = IP_B = IP_C;$$

$$H_3^0: IP_{E1} = IP_{E2} = IP_{E3} = IP_{E4};$$

$$H_4^0: IP_1 = IP_2 = IP_3 = IP_4 = IP_5 = IP_6 = IP_7 = IP_8 = IP_9.$$

Moreover, the Spearman R correlation coefficient between the IP variables was computed:

obtained in MET.1, MET.2, MET.3: (R: IP_{MET1} - IP_{MET2} ; R: IP_{MET2} - IP_{MET3} ; R: IP_{MET1} - IP_{MET3});

The significance of the correlation coefficients was determined for $p < 0.05$. Statistica version 10 was used.

Results and discussion

The results of the antioxidant activity of moss methanol extracts over three seasons by means of three methods expressed as IC_{50} (mg/ml) are set in Tab. 1.

The research did not confirm the H_1^0 hypothesis as it showed that the antioxidant activity of the chosen group of mosses expressed by IP index significantly differs depending on the method applied. The comparison of the values of IP index obtained by every method showed a significantly higher IP value obtained by MET.3 method in comparison with MET.1 and MET.2 (Kruskal-Wallis test, $p < 0.05$; Fig.1). However, no significant differences between the IP values obtained by MET.1 and MET.2 were found (Fig. 1).

A significant positive correlation between IP values for every method was found. The coefficients of the Spearman correlation were (Tab. 2), respectively: $R = 0.78$ (for $IP_{MET.1}$ and $IP_{MET.2}$); $R = 0.60$ (for $IP_{MET.2}$ and $IP_{MET.3}$); $R = 0.47$ (for $IP_{MET.1}$ and $IP_{MET.3}$).

Table 1. Comparison of the antioxidant activity of moss methanol extracts over three seasons by means of three methods expressed as IC₅₀ (mg/ml). In the following assays, IC₅₀ values of ascorbic acid, EDTA and BHA were respectively 1.20, 19.22 and 12.45 (µg/ml).

Species	Spring			Summer			Autumn		
	DPPH	CHEL	LPO	DPPH	CHEL	LPO	DPPH	CHEL	LPO
<i>Atrichum undulatum</i> gametophyte	5.50	6.25	8.02	1.58	5.54	8.48	1.53	6.29	8.39
<i>Brachythecium rutabulum</i> gametophyte	82.61	56.78	11.55	24.15	28.02	10.78	40.09	19.97	7.11
<i>Callicladium haldanianum</i> gametophyte	36.62	92.31	16.63	46.66	50.13	14.74	4.30	92.31	7.38
<i>Hypnum cupressiforme</i> gametophyte	84.99	111.6	15.06	169.12	106.51	16.59	62.51	40.52	7.88
<i>Kindbergia praelonga</i> gametophyte	54.40	56.04	1.49	17.45	19.84	8.28	14.70	66.70	2.12
<i>Pohlia nutans</i> gametophyte	28.05	8.08	3.16	14.94	16.79	6.30	11.21	7.46	1.67
<i>Polytrichastrum formosum</i> gametophyte	12.82	19.20	5.61	15.32	19.20	5.61	-	-	-
<i>Plagiothecium laetum</i> gametophyte	9.44	7.19	9.08	5.12	7.63	1.46	5.20	7.21	1.67
<i>Atrichum undulatum</i> sporophyte	0.98	3.23	5.84	1.38	-	2.39	8.32	5.67	8.32

It was shown that the season when the material was collected did not influence the antioxidant activity of the particular groups of mosses – there are no significant differences between the median values of IP_{A-spring} IP_{B-summer} IP_{C-autumn} (Kruskal-Wallis test, $p > 0.05$; Fig. 2). Compared with the mosses, medical and nutritional value of the vascular plants depend on harvesting season (e.g. Ahmed, et al. 2012). The IP index value was influenced by the concentration of the sample. The higher the concentration, the higher the IP index. No significant differences for the index were found between the samples of the lowest

concentration: E1 – 1.25 mg/ml, E2 – 2.50 mg/ml and the highest concentration: E3 – 5.00 mg/ml and E4 – 10.00 ml/ml (Kruskal-Wallis test, $p < 0.05$; Fig. 3).

The obtained data shows that all species are characterized by the antioxidant effect. The comparative analysis of the IC₅₀ (Tab. 1) for the examined plant extracts showed significant differences in the antioxidant potential of particular species, especially for MET-1 and MET-2 methods. A less varied effect for the tested extracts was observed in the case of the inhibition of linoleic acid peroxidation (MET-3).

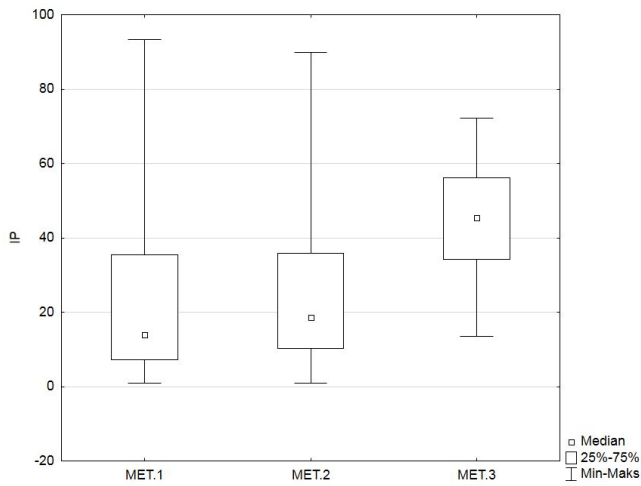


Fig. 1. The comparison of values of the IP index for a group of mosses evaluated by 3 methods (MET.1, MET.2, MET.3, explained in the paper). $[IP_{MET.1} = IP_{MET.2}] < IP_{MET.3}$; Kruskal-Wallis test, $p < 0.05$

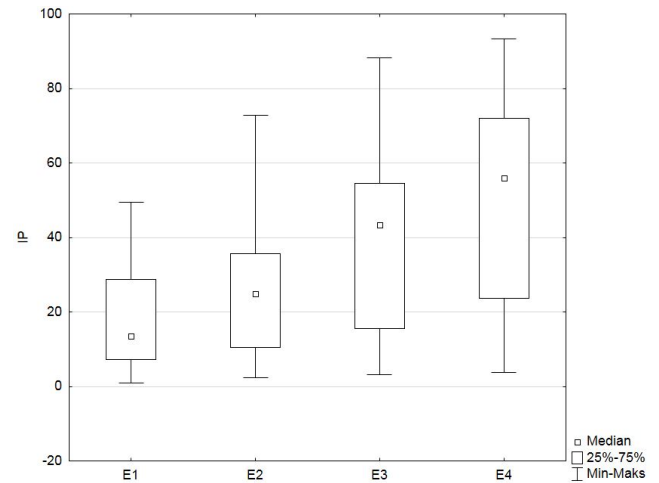


Fig. 3. The comparison of values of the IP index for a group of mosses using different extract concentrations: E1 – 1.25 mg/ml; E2 – 2.50 mg/ml; E3 – 5.00 mg/ml, E4 – 10.00 ml/ml. $[IP_{E1} = IP_{E2}] < [IP_{E3} = IP_{E4}]$. Kruskal-Wallis test, the "concentration" grouping variable, $p < 0.05$.

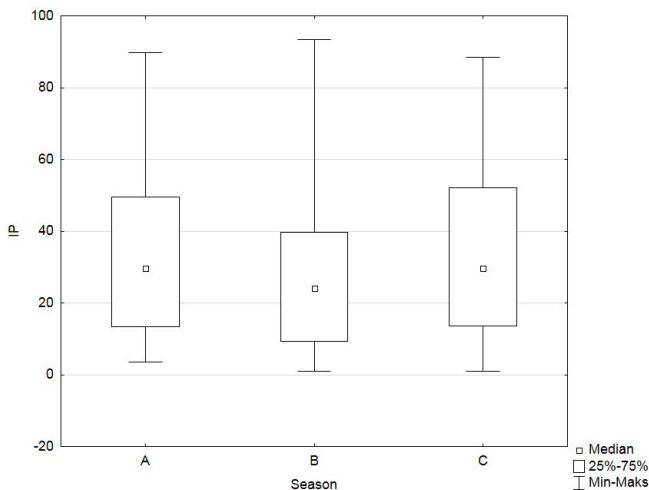


Fig. 2. The comparison of values of the IP index for a group of mosses collected during various seasons: A – spring, B – summer, C – autumn. $IP_A = IP_B = IP_C$; Kruskal-Wallis test, the "season" grouping variable, $p < 0.05$.

Table 2. Spearman's rang correlation coefficients for IP values which were obtained by three methods. Level of significance $p < 0.05$.

	$IP_{MET.1}$	$IP_{MET.2}$	$IP_{MET.3}$
$IP_{MET.1}$	x	0.78	0.47
$IP_{MET.2}$	0.78	x	0.78
$IP_{MET.3}$	0.47	0.60	x

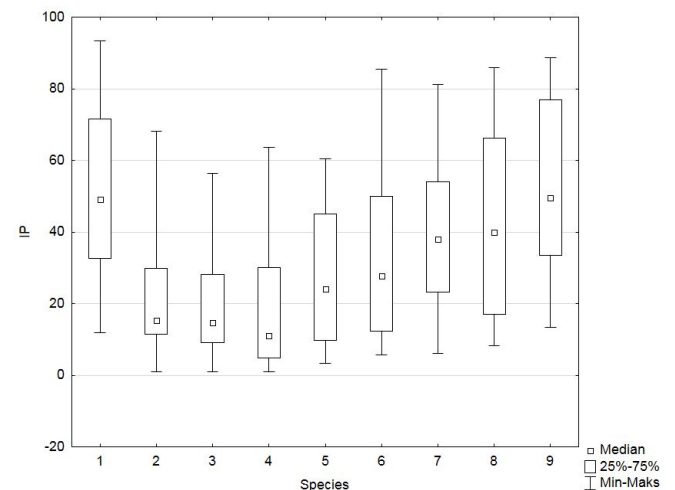


Fig. 4. The comparison of values of the IP index for 9 moss assays. 1 – *Atrichum undulatum* (gametophyte), 2 – *Brachythecium rutabulum* (gametophyte), 3 – *Callicladium haldanianum* (gametophyte), 4 – *Hypnum cupressiforme* (gametophyte), 5 – *Kindbergia praelonga* (gametophyte), 6 – *Pohlia nutans* (gametophyte), 7 – *Polytrichastrum formosum* (gametophyte), 8 – *Plagiothecium laetum* (gametophyte), 9 – *Atrichum undulatum* (sporophyte). Kruskal-Wallis test, for multiple comparisons, the "assay" grouping variable $p < 0.05$; $IP_{(1,7,8,9)} > IP_{(2,3,4,5)}$.

A statistically significant ability to scavenge DPPH free radicals is noted for the gametophyte and sporophyte of *Atrichum undulatum* and the gametophyte of *Plagiothecium laetum*. A slightly weaker antiradical effect was shown by the gametophyte of *Polytrichastrum formosum*. The extracts from the sporophyte and gametophyte of *A. undulatum*, also the extracts from gametophytes of *P. laetum* and *Pohlia nutans* are distinguished by their ability to chelate metals. *Hypnum cupressiforme* is characterized by the weakest activity of all the investigated species of mosses.

Statistical analysis of the IP index for all examined species confirmed significant differences in their antioxidant properties (Fig. 4). The assays of the highest antioxidant properties were: 1 (*Atrichum undulatum*, gametophyte), 9 (*Atrichum undulatum*, sporophyte), 7 (*Polytrichastrum formosum*, gametophyte) and 8 (*Plagiothecium laetum*, gametophyte), and of the lowest antioxidant properties: 2 (*Brachythecium rutabulum*, gametophyte), 3 (*Callicladium haldanianum*, gametophyte) and 4 (*Hypnum cupressiforme*, gametophyte) (Fig. 4; Kruskal-Wallis test, $p < 0.05$). The results confirmed earlier observations, that species of the family Polytrichaceae (here *Atrichum undulatum* and *Polytrichastrum formosum*) show the highest antioxidant activity (Chobot et al. 2008). Antioxidative potentiality of mosses probably depends on the presence of specific chemical groups and their relative concentrations (Mukhopadhyay et al. 2013).

The analysed mosses belong to 5 botanical families: Polytrichaceae (*Atrichum undulatum* and *Polytrichastrum formosum*), Bryaceae (*Pohlia nutans*), Plagiotheciaceae (*Plagiothecium laetum*), Brachytheciaceae (*Brachythecium rutabulum* and *Kindbergia praelonga*) and Hypnaceae (*Callicladium haldanianum* and *Hypnum cupressiforme*). In

Polytrichaceae the antioxidant activity is high, especially in sporophytes (diploid generation) of *Atrichum undulatum*. A fairly high antioxidant activity is also shown by *Plagiothecium laetum* and *Pohlia nutans*. The remaining four species show poor antioxidant activity (Tab. 1). The particular families belong to the taxonomic units of higher ranks – classes Polytrichopsida (Polytrichaceae) and Bryopsida (the remaining families). Among the Bryopsida class, the family Bryaceae is placed in the subclass Bryidae, whereas Brachytheciaceae, Hypnaceae and Plagiotheciaceae belong to the subclass Hypnidae, where they belong to the Hypnales order. On the basis of the preliminary observations, it is possible to state that the systematic relations are crucial in searching for antioxidant activity among mosses in lower taxonomical units, i.e. families and the lower ones.

Conclusions

This is the first report on seasonal variation in antioxidant activity of selected mosses. In conclusion we might say that (1) season during which a sample was collected does not influence the antioxidant activity of the analyzed group of mosses and (2) the systematic relations between the investigated species influence their antioxidant activity on the level of lower taxonomical units, i.e. families and lower ones.

The highest antioxidant activities show *Atrichum undulatum* (Fig. 5) and *Polytrichastrum formosum* thus these species need further investigation as a potential source for antioxidants



Fig. 5. *Atrichum undulatum*, moss with high antioxidant activity, commonly occurring in Poland (photo by A. Stebel).

Bibliography

Ahmed D., Baig H., Zara S. 2012. Seasonal variation of phenolocs, flavonoids, antioxidant and lipid peroxidation inhibitory activity of methanoloc extract of *Melilotus indicus* and its subfractions in different solvents. International Journal of Phytomedicine 4(3): 326–332.

Asakawa Y., Ludwiczuk A., Nagashima F. 2013. Chemical constituents of Bryophytes. Bio- and chemical diversity, biological activity, and chemosystematics. Progress in the chemistry of organic natural products 95: 1–796.

Basile A., Sorbo S., Conte B., Golia B., Montanari S., Cobianchi R. B., Esposito S. 2011. Antioxidant activity in extracts from *Leptodictyum riparium* (Bryophyta), stressed by heavy metals, heat shock and salinity. Plant Biosystems 145: 77–80

Bhattarai H. D., Paudel B., Lee H. S., Lee Y. K., Yim, J. H. 2008. Antioxidant activity of *Sanionia uncinata*, a polar moss species from King George Island, Antarctica. Phytotherapy Research 2(12): 1635–1639.

Brand-Williams W., Culiever M.E., Berset C. 1995. Use of a free radical method to evaluate antioxidant activity. LWT-Food Science and Technology 28: 25–30.

Chobot V., Kubicová L., Nabbout S., Jahodář L., Vytlačilová J. 2006. Antioxidant and free radical scavenging activities of five moss species. Fitoterapia 77: 598–600.

Chobot V., Kubicová L., Nabbout S., Jahodář L. Hadaceka F. 2008. Evaluation of antioxidant activity of some common mosses. Zeitschrift für Naturforschung 63c: 476–482.

Dey A. Nath De J. 2012. Antioxidant potential of bryophytes: stress tolerance and commercial perspectives: a review. Pharmacologia 3(6): 151–159.

Goffinet, B., Buck, W.R., Shaw A. J., 2009. Morphology, anatomy, and classification of the bryophyta, in: Goffinet, B., Shaw, A.J. (Eds), Bryophyte biology. Second edition: 55–138. Cambridge University Press, Cambridge.

Guo J. T., Lee H. L., Chiang S. H., Lin H. I. Chang C.Y. 2001. Antioxidant properties of the extracts from different parts of broccoli in Taiwan. Journal of Food and Drug Analysis 9: 96–101.

Karim F. A., Suleiman M., Rahmat A., Bakar M. F. A. 2014. Phytochemicals, antioxidant and antiproliferative properties of five moss species from Sabah, Malaysia. International Journal of Pharmacy and Pharmaceutical Sciences 6(10): 292–297.

Kuo J. M., Yeh D. B., Pan B. 1999. Rapid photometric assay evaluating antioxidative activity in edible plant material. Journal of Agricultural Food Chemistry 47: 3206–3209.

Mukhopadhyay S. T., Souvik M., Biswas A., Das N., Poddar-Sarkar M. 2013. Screening of Antimicrobial and Antioxidative Potential of Selected Eastern Himalayan Mosses. *European Journal of Medicinal Plants* 3(3): 422–428.

Olszewska M. A. 2011. Variation in the phenolic content and in vitro antioxidant activity of *Sorbus aucuparia* leaf extracts during vegetation. *Acta Poloniae Pharmaceutica* 68(6): 937–944.

Pliszka B., Waźbiańska J. Huszcza-Gałkowska G. 2009. Polyphenolic compounds and bioelements in fruits of Eastern Teaberry (*G. procumbens* L.) harvested in different fruit maturity phases. *Journal of Elementology*, 14(2): 341–348.