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Investigating Seasonal Variation in The Phytochemical and Antioxidant Capacities of Different *Sphagnum* Taxa

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Abstract

This study investigated the phytochemical content and antioxidant activities of four *Sphagnum* taxa (*S. centrale, S. palustre, S. teres* and, *S. auriculatum*) collected from the Black Sea region of Turkey during two different seasons. The phytochemical groups in the methanol extracts of Sphagnum taxa were identified using qualitative screening methods. The total phenol content of these taxa was quantitatively determined using Folin-ciocalteu reagent with gallic acid equivalents as the standard their antioxidant activities were evaluated by 1,2-diphenyl-1-picrylhydrazyl free radical scavenging ability (DPPH), the CUPRAC test, and phosphomolybdenum assay. Qualitative phytochemical screening of the methanol extracts showed that phenols, tannins, and saponins were present in the extracts, whereas alkaloids and flavonoids were absent. The highest DPPH free radical scavenging activity was observed in 100 µgmL^{-1} *S. teres* methanol extract (74.47±0.001% in the first season, September 2021 and 75.37±0.002% in the second season, May 2022). The highest total antioxidant capacity was found in 100 µgmL^{-1} *S. palustre* extract (81.00±0.027% in the first season and 84.87±0.002% in the second season). The results of our experiment showed that *Sphagnum* taxa collected in spring had higher antioxidant activity than those collected in autumn.

Keywords: Antioxidants, biological activity, bryophytes, seasonal changes, secondary metabolites

Farklı *Sphagnum* Türlerinin Fitokimyasal ve Antioksidan Kapasitelerindeki Mevsimsel Değişimin İncelenmesi

Öz

Bu çalışmada, Türkiye'nin Karadeniz bölgesinden iki farklı mevsimde toplanan dört *Sphagnum* taksonunun (*S. centrale, S. palustre, S. teres* ve *S. auriculatum*) fitokimyasal içeriği ve antioksidan aktiviteleri araştırılmıştır. *Sphagnum* taksonlarının metanol ekstraktlarındaki fitokimyasal gruplar kalitatif tarama yöntemleri kullanılarak tanımlanmıştır. Bu taksonların toplam fenol içeriği, standart olarak gallik asit eşdeğerleri ile Folin-ciocalteu reaktifi kullanılarak kantitatif olarak belirlenmiş, antioksidan aktiviteleri 1,2-difenil-1-pikrilhidrazil serbest radikal süpürme yeteneği (DPPH), CUPRAC testi ve fosfomolibden deneyi ile değerlendirilmiştir. Metanol ekstrelerinin kalitatif fitokimyasal taraması, ekstrelerde fenollerin, tanenlerin ve saponinlerin bulunduğunu, alkaloidlerin ve flavonoidlerin ise bulunmadığını göstermiştir. En yüksek DPPH serbest radikal süpürme aktivitesi 100 µgmL^{-1} *S. teres* metanol ekstraktında gözlenmiştir (ilk sezon olan Eylül 2021'de %74,47±0,001 ve ikinci sezon olan Mayıs 2022'de %75,37±0,002). En yüksek toplam antioksidan kapasite 100 µgmL^{-1} *S. palustre* ekstresinde tespit edilmiştir (ilk sezonda %81,00±0,027 ve ikinci sezonda %84,87±0,002). Deneyimizin sonuçları, ilkbaharda toplanan *Sphagnum* taksonlarının sonbaharda toplananlara göre daha yüksek antioksidan aktiviteye sahip olduğunu göstermiştir.

Anahtar kelimeler: Antioksidan, biyolojik aktivite, briyofit, mevsimsel değişimler, sekonder metabolitler

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1. Introduction

Since ancient times, plants have been used as remedies for a wide range of human ailments. Bioactive substances derived from plants are of profound interest in contemporary medicine due to their absence of adverse side effects and lower costs than synthetic drugs. Accordingly, traditional medicinal plants are currently the focus of medical research. Therefore, exploring the biological properties of plants and their metabolites that exhibit therapeutic effects is crucial. Previous studies have shown that bryophytes, having an almost global distribution represented by approximately 20,000-25,000 taxa worldwide, constitute the second largest group of plant biodiversity (Crum, 2001; Patiño and Vanderpoorten, 2018). According to a study conducted by Kürschner and Erdağ (2023), Turkish bryophytes are represented by ±1287 taxa (±1054 mosses, ±229 liverworts, and ±4 hornworts) (Kürschner and Erdağ, 2024; Ellis et al., 2024; Batan et al., 2024a, b). They, particularly those of the genus *Sphagnum*, play a crucial role in aquatic ecosystems, forming the main component of peat, commonly known as peat mosses. In Türkiye, marshes created by *Sphagnum* are scarce and located solely in the Northeastern region of the country (Northeastern Black Sea Region) (Kirmaci et al., 2019). There are 30 taxa belonging to the genus *Sphagnum*, which are naturally distributed in Türkiye. These taxa are classified into seven sections (*Sphagnum, Acutifolia, Squarrosa, Subsecunda, Cuspidata, Rigida and Hemitheca*) (Kirmaci et al., 2022; Özen-Öztürk et al., 2023).

Recently, there has been increasing interest in the chemical structure of bryophytes due to the identification of biologically active compounds that have significant biological potential (Krzaczkowski et al., 2009; Üçüncü et al., 2010; Fu et al., 2012; Cheng et al., 2012; Asakawa and Ludwiczuk, 2013). Extracts from bryophytes could constitute a significant source of new pharmaceutically active compounds (Asakawa, 2007; Klavina et al., 2018; Türker and Türkyılmaz Ünal, 2020). Mosses contain terpenoids as well as derivatives of benzoic, cinnamic, and phthalic acid coumarins and nitrogen-containing aromatic compounds that are structurally similar to those in vascular plants (Asakawa and Ludwiczuk, 2013). The various secondary metabolites in mosses play adaptive roles in their tissues. Investigating the structure of these compounds is crucial because it can offer insights into how mosses respond to environmental variations, including drought and humidity and it can provide protection from oxidative stress caused by pollution. Additionally, these studies reveal the functional roles of key substances involved in moss metabolism and help in understanding the mechanisms underlying secondary metabolite metabolism (Goffinet and Shaw, 2008; Xie and Lou, 2009). Several studies have explored the impact of seasonal variations in the phytochemical content, antioxidant activity and chemical element accumulation of select mosses belonging to the *Sphagnum* genus (Peters et al., 2018; Çelik et al., 2023).

Sphagnum taxa exhibit a variety of physical and structural properties that help them adapt to environmental stress. In addition, it is known to produce a wide range of biochemicals that could potentially protect cells from stress-induced damage. Bryophyta taxa have antibacterial, antifungal, antiviral, antioxidant, insecticidal, cytotoxic and antitumor effects (Cheng et al.., 2013; Onbasli and Yuvali, 2021; Yücel and Erata, 2021). However, the relationships between the anatomical, morphological, and biochemical characteristics of *Sphagnum* (i.e. metabolites, pigments, and antioxidant enzymes) and their responses to changes in the environment are not yet fully understood.

It is thought that there may be changes in the phytochemical content and antioxidant activities of mosses collected in different seasons. To confirm this hypothesis, this preliminary study aimed to investigate how change the phytochemical contents and antioxidant activities of different *Sphagnum* taxa collected from the Black Sea region of Türkiye in different seasons were collected in two seasons (September 2021 and late May 2022). To conduct this study, four different *Sphagnum* taxa (*Sphagnum centrale* C.E. O Jensen, *S. palustre* L., *S. auriculatum* Schimp., and *S. rubellum* Wilson) were collected in two seasons (Autumn, September 2021; Spring, Late May 2022). Methanolic extracts were obtained from the aforementioned taxa and the seasonal fluctuations in phytochemically active compounds were identified by screening methods. The antioxidant activity of the extracts was also evaluated by 1,1-diphenyl-2-picryl-hydrazyl radical (DPPH), CUPRAC, and phosphomolybdenum assays.

2. Materials and methods

2.1. Chemicals

1,1-diphenyl-2-picryl-hydrazyl radical (DPPH), ascorbic acid, and methanol were purchased from Sigma, Chem., Germany, and CUPRAC assay kit was purchased from BQC (Bioquochem) Redox Technologies, Asturias, Spain.

2.2. Collection Localities

The *Sphagnum* samples were collected from the following localities in two different seasons (Autumn, September 2021; Spring, Late May 2022) and identified by Prof. Dr. Mesut Kırmacı.

S. palustre: Trabzon, Sürmene, Çamburnu Üzeri, 280 m., N 40^0 53′ 58,2″ E 40^0 12′ 27,2″
S. auriculatum, S. rubellum: Rize, Kavrun Yaylası, 2050 m; N 40^0 53′ 49,8″ E 41^0 07′ 48,4″
S. centrale*:* Trabzon, Barma Yaylası, 1860 m; N 40^0 42′ 11,2″ E 40^0 08′ 57,7″

The fresh *Sphagnum* samples were collected from the above mentioned localities in autumn (September 2021) and spring (Late May 2022). The sphagnum samples were brought to the laboratory as soon as possible for phytochemical analysis and antioxidant activity determination, identified to taxa (using the relevant flora books and revisions) (Kirmaci et al., 2017), labelled, and stored in paper bags. Samples were deposited in the herbarium of Aydın Adnan Menderes University (AYDN).

2.3. Preparation of methanol extracts from Sphagnum taxa

15 g powdered dried mosses (*S. centrale*, *S. palustre*, *S. teres*, *S. auriculatum*, *S. rubellum*) were extracted with 500 mL methanol at room temperature for 24-48 h. After filtration, the extracts were evaporated. Once the solvent evaporated, the crude extract remained at the bottom of the jar. Thereafter, the percentage yields for the extracts were calculated using the formula below:

EY % = [Extract weight (g) / Dry sample weight (g)] x 100

The crude extracts were kept at +4°C until the experimental studies.

2.4. Phytochemical screening

We carried out phytochemical screening of extracts using standard qualitative protocols adapted from other studies. Sufficient amounts of dried extracts were dissolved in their respective solvents. We performed phytochemical analyses according to Ravishankara (2002) and Dominguez (1973). Details of the tests are described below (Ravishankara et al., 2002; Dominguez, 1973):

2.4.1. Detection of phenols

The prepared methanol extracts were dropped onto a filter paper. A drop of phosphomolybdic acid reagent was added to the drops and exposed to ammonia vapor. The blue color of the stains indicates the presence of phenols in the extract.

2.4.2. Detection of tannins

A 10% alcoholic solution of ferric chloride was added to 2-3 mL of the methanolic extract. Dark blue or greenish grey color of the solution indicates the presence of tannins in the extracts.

2.4.3. Detection of alkaloids

A small drop of precoate from the prepared methanol extract was dropped onto the TLC plate and then the plate was sputtered with Dragendorff's reagent. The orange color shows alkaloids.

2.4.4. Detection of flavonoids

A piece of magnesium strip and 1 mL of concentrated hydrochloric acid were added to a test tube containing 2-3 mL of methanol extract. A pink-red or red color of the solution indicates the presence of flavonoids in the extract.

2.4.5. Detection of saponins

In a test tube containing 10 mg of the extracts were mixed with 1 mL of distilled hot water was added. The test tube is then vigorously shaken for 30 s. The appearance of a froth layer indicates saponins (triterpene glycosides). The observed results are recorded as negative if no froth forms, and positive for froth.

2.5. Folin-Ciocalteu test for the determination of total phenolic content

The total phenolic content was spectrophotometrically analyzed using the Folin-Ciocalteu colorimetric method. (Singleton et al., 1999). 100 and 300 µL of properly diluted extract solutions were mixed with 1mL of FC reagent. The reagent was pre-diluted, 10 times, with distilled water. After standing for 3 minutes at room temperature, 3 mL of (2% w/v) sodium carbonate solution was added. The solutions were mixed and allowed to stand for 2h at room temperature. Then, the absorbance was measured at 760 nm, using a UV spectrophotometer (Shimadzu PharmaSpec UV1700, Japan). A calibration curve was prepared, using a standard solution of gallic acid (20, 40, 60, 80, and 100 mg mL^{-1}). It expressed the results as mg gallic acid per 100 gr dry mosses.

2.6. Determination of antioxidant activity

2.6.1. DPPH Free-Radical scavenging assay

The antioxidant activity of the extracts was measured by using 1,1- diphenyl-2-pycrylhydrazyl (DPPH) as a free radical model (Brand-Williams et al., 1995). This test is based on the change in purple color of the DPPH solution to yellow by antioxidant molecules, because of the scavenging of stable free DPPH radicals, which from purple to yellow measured at 517 nm (Blois, 1958). A

stronger yellow color shows a greater ability of the extract to scavenge free DPPH radicals and stronger antioxidant potential. One mL of 0.1 mM methanol solution of DPPH was added into 3 ml samples 5, 10, 20, 40, 60, 80, and 100 μgmL^{-1} concentrations of methanol extracts or standards. The solution was mixed vigorously and left to stand at room temperature for 30 minutes in the dark. The mixture was measured spectrophotometrically at 518 nm. Using a microplate reader (Elisa Reader, Biotek Co, USA) the absorbance of the mixture was measured spectrophotometrically at λ = 517 nm. 10 and 50 $\mu g\ mL^{-1}$ ascorbic acid was used as a positive control. We calculated the antioxidant activity as below:

DPPH Scavenging capacity (%) = $[(A_0 - A_1)/A_0] \times 100$

Where A_0 was the absorbance of the control and A_1 was the absorbance in the presence of the methanol extracts. We compared the actual decrease in absorption induced by the test with the positive controls. The analysis was performed in three replicates, and all data was reported as means ± standard deviations.

2.6.2. Phosphomolybdenum Assay for the Determination of Total Antioxidant Capacity

The total antioxidant capacity (TAC) assay of samples was carried out by the phosphomolybdenum assay (Ghafoor and Choi, 2009). A 0.1mL aliquot of the extract (10, 20, 40, 60, 80, and 100 μgmL^{-1}) solution was shaken with 1mL of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate, and 4mM ammonium molybdate). The test tubes were covered with aluminum foil and incubated in a water bath at 95 °C for 90 minutes. After the samples were cooled, the absorbances were measured at 765 nm. 10 and 50μgmL^{-1} ascorbic acid was used as standard. All experiments were performed in triplicate. The total antioxidant capacity (TAC) of the extracts was estimated using the following formula:

TAC (%) = [(Abs. of control - Abs. of sample) / (Abs. of control)] ×100

2.6.3. CUPRAC Spectrophotometric Assay for the Determination of Total Antioxidant Capacity

The antioxidant capacity of the *Sphagnum* methanol extracts was calculated by utilizing the reduction ability of the copper(II)-neocuproin complex (Cu(II)-Nc) formed by 2,9-dimethyl-1,10-phenanthroline (Neocuproin-Nc) with Cu(II) to the copper(I)-neocuproin [Cu(I)-Nc] chelate, which gives maximum absorbance at 450 nm (Apak et al., 2007). A total of 40 µL of the diluted extracts was added to 200 µL of the previously prepared working solution. Trolox was used as a standard in experiments. The mixture was incubated at room temperature for 30 min, and the absorbance was measured at 450 nm using a plate reader (Elisa Reader, Biotek Co, USA). The results were expressed as mM Trolox equivalents (TE mM).

2.7. Statistical Analysis

The results of the antioxidant screening were subjected to statistical analysis using SPSS 25 for Windows, IBM Corporation, New York, NY, USA. The results were expressed as mean ± SD and analyzed by One-Way ANOVA (Analysis of Variance) and a p-value was obtained. A significant difference was defined as $p \leq 0.05$.

3. Findings

3.1. Extraction amounts and extraction yield

The amounts of *Sphagnum* extract from the samples were measured in grams. The details of the extraction yield for each sample are given in Table 1. The extraction quantity obtained from *Sphagnum* samples collected in the first season varied between 0.4644 g and 1.0581 g and the extraction efficiency was calculated between 3.096% and 7.054%. The extraction quantity obtained from *Sphagnum* samples collected in the second season varied between 0.5013 g and 0.7640 g and the extraction efficiency was calculated between 3.342% and 5.093%. When both seasons were compared, the extraction quantities and extraction efficiency % obtained in the first season were found to be higher than the second season.

3.2. Phytochemical screening

Qualitative phytochemical screening results of methanol extracts indicated that phenols, tannins, and saponin components were present in the methanol extracts. During the first season, phenols were detected in only two of the four *Sphagnum* taxa (*S. auriculatum* and *S. rubellum*). In contrast, all samples collected in the second season were found to contain phenols (Table 2). In both seasons, tannins were found only in the methanol extract of *S. centrale*. *S. palustre* methanol extract contained tannins only in the first season, and tannins were not detected in the second season. Again, saponins were only found in the methanol extract of *S. centrale in* the first season, whereas saponins were not detected in the other three samples. The alkaloids and flavonoids were not detected in the methanol extracts of all Sphagnum samples used in the trials in both seasons (Table 2).

Table 1. Extraction yield of methanol extracts obtained from several taxa within the *Sphagnum* spp.

Taxa	Dry Sample Weight (g)	Extract Quantity (g)		Extraction Efficiency (%)	
		Season I	Season II	Season I	Season II
S. centrale	15	0.4644	0.7640*	3.096	5.093*
S. palustre	15	1.0581*	0.7279	7.054*	4.853
S. auriculatum	15	0.6996	0.5298	4.664	3.532
S. rubellum	15	0.8424*	0.5013	5.616*	3.342

*** p<0.05** Season I: September 2021; Season II: Late May 2022

Table 2. Phytochemical screening of different *Sphagnum* taxa methanol extracts

Taxa	Phenols		Tannins		Saponins		Alkaloids		Flavonoids	
	S I	S II	S I	S II	S I	S II	S I	S II	S I	S II
S. centrale	+	+	+	+	+	-	-	-	-	-
S. palustre	+	+	+	-	+	-	-	-	-	-
S. teres	+	+	-	-	+	+	-	-	-	-
S. auriculatum	-	+	-	-	-	-	-	-	-	-

Season: S; (+) present; (-) absent; Season I: September 2021; Season II: Late May 2022

Table 3. Total phenolic content in milligrams of gallic acid equivalents per gram of methanol extract of *Sphagnum* taxa

Taxa	mg GAE/g of extract ± SD	
	Season I	Season II
S. centrale	55,83 ± 0.72	69.17 ± 2.45*
S. palustre	51.25 ± 3.31	55.00 ± 1.25
S. auriculatum	55.42 ± 6.17	67.50 ± 7.60*
S. rubellum	57.50 ± 3.75	63.75 ± 4.48

*** p<0.05** Each value is the average of three analyses ± standard deviation Season I: September 2021; Season II: Late May 2022

3.3. Folin-Ciocalteu assay of total phenolic content

The quantification of total phenolic compounds (TPC) in methanol extracts was achieved using Folin-Ciocalteu reagent and expressed in gallic acid equivalents. Table 3 displays the TPC of the *Sphagnum* specimens

The mg GA/g extract of the *Sphagnum* specimens collected during the second season presented a greater TPC than did those collected during the first season. During the first season, the TPC in the collected samples ranged from 51.25 to 57.50 mg GAE/g. However, in the second season, the content ranged between 55.00 and 69.17 mg GAE/g. The second season samples of *S. centrale* (69.17 mg GAE/g) and *S. auriculatum* (67.50 mg GAE/g) had the highest total phenolic content.

3.4. Antioxidant activity

3.4.1. DPPH scavenging activity

The DPPH scavenging assay relies on the conversion of the DPPH solution from purple to yellow which is triggered by antioxidant molecules. These molecules effectively scavenged purple to yellow free radicals, which are stable DPPH radicals, as measured at 517 nm (Blois, 1958). A greater yellow color intensity signifies stronger scavenging of free DPPH radicals, indicating greater antioxidant potential in the extract. The DPPH scavenging activity of ascorbic acid ($10\mu gmL^{-1}$ and $50\mu gmL^{-1}$), used as the standard in the experiment, yielded a remarkable outcome (Table 4). Table 4 displays the antioxidant activity of methanol extracts prepared from *Sphagnum* taxa collected in two seasons at varying concentrations ($10\text{-}100\mu gmL^{-1}$). The results demonstrated that the methanol extract from selected *Sphagnum* taxa collected in both seasons exhibited a DPPH scavenging activity of more than 50% at all the tested concentrations. Additionally, there was an increase in DPPH scavenging ability with increasing extract concentration.

Table 4. DPPH radical scavenging activity results of four Sphagnum taxa methanol extract and ascorbic acid (%) with mean ± SD

Taxa	Concentrations	DPPH Scavenging Activity(%±SD) Season I (% ±SD) Season I	DPPH Scavenging Activity (%±SD) Season II	Total Antioxidant Capacity (%±SD) Season I	Total Antioxidant Capacity (%±SD) Season II
Ascorbic acid	10 μgmL^{-1}	81.56±0.001*	81.63±0.007*	36.16±0.013	36.10±0.003
	50 μgmL^{-1}	82.79±0.001*	82.83±0.002*	97.98±0.008*	98.02±0.018*
S. centrale	10 μgmL^{-1}	59.43±0.003*	59.56±0.003*	9.04±0.005	9.57±0.027
	20 μgmL^{-1}	62.70±0.002*	61.29±0.011*	11.86±0.016	12.13±0.024
	40 μgmL^{-1}	63.52±0.015*	64.20±0.002*	24.86±0.012	24.13±0.055
	60 μgmL^{-1}	63.93±0.001*	65.00±0.003*	30.51±0.014	30.19±0.004
	80 μgmL^{-1}	65.98±0.008*	66.38±0.007*	34.46±0.021	37.52±0.002
	100 μgmL^{-1}	67.62±0.006*	67.47±0.005*	36.71±0.008	42.55±0.006
S. palustre	10 μgmL^{-1}	66.80±0.009*	68.92±0.001*	33.89±0.011	34.64±0.003
	20 μgmL^{-1}	67.21±0.003*	70.82±0.002*	43.50±0.018	46.91±0.008
	40 μgmL^{-1}	68.03±0.002*	74.01±0.003*	54.01±0028*	55.66±0.003*
	60 μgmL^{-1}	68.95±0.006*	74.19±0.006*	65.31±0.032*	66.40±0.007*
	80 μgmL^{-1}	67.21±0.003*	74.83±0.004*	78.31±0.030*	78.47±0.002*
	100 μgmL^{-1}	65.98±0.005*	75.10±0.003*	81.00±0.027*	84.87±0.002*
S. auriculatum	10 μgmL^{-1}	63.82±0.003*	62.74±0.002*	12.75±0.004	13.05±0.006
	20 μgmL^{-1}	64.26±0.003*	64.83±0.005*	19.89±0.007	20.42±0.014
	40 μgmL^{-1}	65.11±0.001*	66.21±0.012*	38.93±0.021	40.00±0.015
	60 μgmL^{-1}	67.66±0.001*	67.93±0.002*	53.69±0.009*	55.53±0.005*
	80 μgmL^{-1}	68.94±0.002*	70.20±0.003*	59.73±0.010*	63.86±0.006*
	100 μgmL^{-1}	69.36±0.004*	72.48±0.015*	60.40±0.006*	65.26±0.009*
S. rubellum	10 μgmL^{-1}	58.55±0.006*	59.11±0.005*	17.82±0.007	19.53±0.012
	20 μgmL^{-1}	63.68±0.001*	63.38±0.003*	31.78±0.003	31.58±0.005
	40 μgmL^{-1}	64.10±0.004*	65.93±0.007*	31.78±0.003	32.42±0.008
	60 μgmL^{-1}	65.81±0.003*	66.84±0.004*	36.43±0.002	40.00±0.003
	80 μgmL^{-1}	68.80±0.005*	70.21±0.012*	46.51±0.005	50.53±0.013
	100 μgmL^{-1}	72.22±0.001*	74.39±0.005*	48.06±0.003	52.26±0.002*

*** p<0.05** Each value is the average of three analyses ± standard deviation, Season I: September 2021; Season II: Late May 2022

The DPPH radical scavenging activities of the extracts from the *Sphagnum* taxa collected during the second season were greater than those of the *Sphagnum* samples collected during the first season. Even though the antioxidant activities exceeded 50% at all concentrations tested, the methanol extracts of the *Sphagnum* samples collected in both seasons were somewhat lower than those of ascorbic acid. When the DPPH scavenging activities and total antioxidant capacities of the methanol extracts of mosses were compared, the highest activity was observed in the *S. palustre* methanol extract in both seasons (Table 4).

3.4.2. Phosphomolybdenum assay of total antioxidant capacity

Table 4 shows the total antioxidant capacity (TAC) results obtained by the phosphomolybdenum assay. The results indicated an increase in TAC with increasing concentration and the highest TAC value was found for the *S. palustre* methanol extract at 100µgmL^{-1} concentration. The TAC value exceeded 50% ($p<0.05$), with TAC values of 81.00% in the first season (September 2021) and 84.87% in the second season (late May 2022). The TAC of the samples collected during the second season was slightly higher than that in the first season. Ascorbic acid was utilized as the standard antioxidant for this study and the TAC of the samples in both seasons was measured at a concentration of 10 µgmL^{-1} 36.16% in the first season, which declined insignificantly to 36.10% in the later season. Nonetheless, the TAC value significantly increased to 97.98% in the first season and 98.02% in the second season upon the increase in the concentration to 50µgmL^{-1}.

3.4.3. CUPRAC spectrophotometric assay of total antioxidant capacity

The total antioxidant capacity of methanol extracts obtained from selected *Sphagnum* samples was determined by CUPRAC assay. The CUPRAC assay revealed lower antioxidant activity in methanol extracts obtained from *Sphagnum* samples collected in both seasons than in those obtained from the Trolox standard. The analysis outcomes were calculated and listed in Table 5 as mMTR/g. The second season *Sphagnum* samples showed a slight increase in antioxidant activity compared to the first season samples, but this increase was very slight compared to the Trolox standard.

Table 5. Total antioxidant capacity of methanol extracts of selected *Sphagnum* taxa

Taxa	CUPRAC value (mM TR/g)	
	Season I	Season II
Trolox (Standard)	1,000	1,000
S. centrale	0.0002	0.0036
S. palustre	0.0005	0.0023
S. airuculatum	0.0005	0.0016
S. rubellum	0.0006	0.0020

Season I: September 2021; Season II: Late May 2022

4. Results and Discussion

Mosses have been used in traditional medicine for centuries. Studies have shown that mosses extracts and phenolic compounds with antioxidant activity can be used in various fields to reduce oxidative damage. The genus *Sphagnum* is a source of many bioactive compounds. The content of the plant varies depending on the season, location of the research material, and plant taxa (Ramussen et al., 1995). It includes polysaccharides, amino acids, carotenoids, fatty acids, triterpenes, and sterols, as well as phenolic compounds like phenolic acids and flavonoids (Zych et al., 2023). *Sphagnum* taxa produce and can secrete different primary and secondary metabolites in their environment that play a role in their physiology, ecology, and stress tolerance (Fudyma et al., 2019; Hamard et al., 2019; Sytiuk et al., 2020). Secondary metabolites are typically produced from primary metabolites, which play a direct role in plant growth and metabolism, through various biosynthetic pathways. Secondary metabolites vary due to differences in living conditions, seasonal changes, exposure to water and moisture levels, and various substances derived from the environment. It is known that various primary and secondary metabolites, especially carbohydrates, carotenoids, phenols, proline, flavonoids, and tannins, play an important role in the growth of bryophytes, in increasing photosynthetic efficiency, and in gaining resistance to abiotic stresses, defend against pathogens and provide protection against microbial infections (Xie and Lou, 2009, Basile et al., 1999; Merkuria et al., 2005; Dey and De, 2012). Onbasli and Yuvalı (2021) reported the promising antioxidant, antimicrobial, antigenotoxic and anticancer activities of *Bryum capillare* ethanol extract. Although there are studies on the identification of *Sphagnum* metabolites (Klavina et al., 2018; Fudyma et al., 2019), there are not enough studies investigating the antioxidant activities of these mosses and seasonal differences in their antioxidant activities.

The contents of active compounds in medicinal plants are affected by seasonal changes and the constituents and active compounds of plants according to the seasons. As observed in the studies of Singh et al. (2008) and Jayanthy et al. (2013), secondary compounds are primarily obtained during the seasons when plants reach the highest maturity and concentration. The production of secondary metabolites is strongly influenced by both biotic and abiotic stresses as shown through genetic control (Naghdi et al., 2004). Various factors, such as climate, altitude, precipitation, sunlight duration, rainfall, outdoor temperature fluctuations, and other conditions, can affect the growth of a plant and the quality, physical properties, and chemical composition of its components in a particular species during different seasons. Stress factors often follow a seasonal pattern, causing plants to release secondary metabolites in varying amounts depending on environmental changes. These changes can also impact the therapeutic efficacy of the plant. While numerous studies have been conducted to investigate the harvest time and season of various medicinal plants and their parts (Özyigit, 2008; Soni et al., 2015), only a limited number of studies have explored the chemical and biochemical responses of bryophytes to seasonal changes (Klavina et al., 2018; Thakur and Kapila, 2017; Luni´c et al., 2022). For instance, a study on lipid composition in *Sphagnum* mosses demonstrated a distinct seasonal variation in the total lipid content of the moss (Karunen, 1982). Seasonal differences in the secondary metabolites contained in liverwort were found during an investigation of the chemical composition and concentration of specific metabolites in four different liverwort species (Klavina et al., 2018). Another study on different liverwort species showed seasonal differences in total phenolic and flavonoid content, as well as antioxidant and polyphenol oxidase enzymes (Thakur, and Kapila, 2017). Flavonoids constitute an important group of polyphenolic compounds (Chebil et al., 2007; Wang et al., 2017). The flavonoid pathway differs significantly between angiosperms and bryophytes and between each bryophyte group with regard to the classes of flavonoids produced (Kulshrestha et al., 2022). According to some researchers, flavonoid content varies according to seasonal changes. For example, Stefkov et al. (2009) reported that *Teucrium polium* had the highest total flavonoid content between late May and July. The results of our study were similar to previous studies and showed that the phytochemical content and antioxidant capacity of *Sphagnum* taxa collected in two different seasons varied seasonally (Tables 2, 3, 4 and 5). The secondary compounds increase in summer, probably enabling black moss to cope with abiotic stresses such as high temperatures and drought (Luni´c, et al., 2022). However, it has also been reported in the literature that among different bryophytes, most of the protective substances such as polyphenols are found in summer, while in others the concentration of phenolic substances is highest in spring and gradually decreases throughout the (Thakur and Kapila, 2017; Zhang et al., 2020; Perera-Castro et al., 2020). These data suggest that bryophytes respond species-specifically to different seasonal conditions. Furthermore, the assumptions of synergism and antagonism should be considered. According to the results obtained from *H. cupressiforme*, the season with the highest secondary metabolite content was summer (Luni´c et al., 2022). All moss samples used in our study were collected from the same location and from the same population. Therefore, variations in the secondary metabolite content in the methanol extract can be attributed to seasonal climatic characteristics such as temperature, precipitation, humidity and/or fluctuations in the duration and intensity of solar radiation.

Antioxidants are a diverse group of compounds that can be natural or synthetic. They play a crucial protective role against the harmful effects of oxidants, and negative effects of free radicals (Smolińska-Kondla et al., 2022). Antioxidant activity is widely regarded as a crucial aspect of bioactivity. The antioxidant defense systems protect the cell, cell membrane and organelles against oxidative damage under unfavorable conditions, thereby increasing its resistance to stress and prolonging the life span of the cell (Castro and Freeman, 2001). The study conducted by Duru et al., (2024) revealed the presence of several compounds with known biological activities that could contribute to antioxidant activity as a result of GC-MS analysis of *Calliergonella cuspidata.* The results of this study showed that the ethanol, methanol, and n-hexane extracts of *C. cuspidata* had similar DPPH radical scavenging ability as the positive control ascorbic acid. In another study in which phytochemical profile, antioxidant, fatty acid and mineral profile of *Polytrichum piliferum* Hedw. were evaluated, it was revealed that *P. piliferum* showed moderate antioxidant activity, total phenolic and flavonoid content (Çakır Sahilli and Alataş, 2024). The experimental findings of our study showed that the methanol extracts of mosses collected in two different seasons had DPPH scavenging activity above the 50% threshold at all concentrations tested (10-100µgmL^{-1}). The highest antioxidant activity was observed in moss during the last May.

The mosses tested showed the highest antioxidant activity in late May, which may be due to the need for increased antioxidant protection in mosses exposed to higher temperatures (Table 4). This is in accordance with the existing literature data, where it can be found that mosses exhibit higher antioxidant enzymatic activity during the summer (Kashyap et al., 2021).

Total antioxidant capacity (TAC) also increased with increasing concentration of methanol extract got from *Sphagnum* taxa (Table 4). It is well known that bryophytes possess strong antioxidative enzymatic machinery that helps them cope with extreme climates and other stresses (Day and De, 2012; Gahtori and Chaturvedi, 2020; Öztürk et al., 2021). The results of our study are similar to studies showing that bryophytes have strong antioxidative enzymatic mechanisms that help them cope with extreme climatic conditions, seasonal changes and other environmental stresses (Day and De, 2012; Gahtori and Chaturvedi, 2020; Öztürk et al., 2021). The higher free radical scavenging and total antioxidant capacity, especially in spring, the second collection season, compared to autumn, suggests that the bryophytes develop and grow in spring and develop protection mechanisms against the air warming. However, this estimate should be supported by comparative studies on different taxa living in different habitats and collected in different seasons.

In the first season, the antioxidant activity of the methanol extracts, analyzed using the CUPRAC assay, was weaker than that of the Trolox standard (Table 5). A slight increase in antioxidant activity was noted in the *Sphagnum* samples collected during the second season compared to those collected during the first season. Thakur and Kapila (2017) stated that, similar to other plants, the antioxidant activity of bryophytes is influenced by various factors, such as altitude, tissue type, and changing seasons. Additionally, the biochemical compounds responsible for antioxidant activity undergo changes in response to these factors, leading to differences in quality and quantity (Peters et al., 2018; Luni´c et al., 2022). According to current studies, bryophytes have a higher secondary metabolite content and antioxidant capacity in summer, which helps them to form biochemical adaptations in response to environmental changes (Luni´c et al., 2022). Due to variations in temperature, precipitation, environmental factors, sunshine length and intensity, and photoperiod, studies on *Sphagnum* taxa have revealed notable seasonal shifts in the phytochemical composition and antioxidant activity in extracts from these species. The results of our study reveal the antioxidant potential of *Sphagnum* mosses and the importance of seasonal fluctuations on secondary metabolite content and biological activities.

The results of this study showed that the amounts of secondary metabolites in eight different moss methanol extracts varied during different seasons. The highest antioxidant activity was observed in the second season (late May). The phytochemical content, free radical scavenging ability and high antioxidant activity of the methanol extract obtained from *Sphagnum* taxa, phytochemical content and antioxidant capacity were found to be higher in summer, so late May can be suggested as the most suitable collection season for medicinal purposes. The results of our study revealed that *Sphagnum* taxa that can be used as potential antioxidant sources, especially in the pharmaceutical and food industries, require further investigation. Seasonal variation in secondary metabolites and antioxidant activity is significant when using mosses for medicinal and cosmetic purposes.

Declarations

Authors' contributions

TAÇ, and ÖSA have designed the study and collected the data. MK collected moss samples from the field. TAÇ, ÖSA and GA have performed laboratory analysis and statistical analysis of the study. TAÇ has written manuscript; TAÇ, ÖSA and MK have reviewed and edited manuscript. All authors have read and approved the final manuscript.

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Conflict of interest

The authors have no competing interests to declare regarding the content of this article.

Ethics approval and consent to participate

This research did not involve human or animal subjects and therefore does not require ethical approval.

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