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Research Article

Determination of the chemical content of *Sphagnum molle* and its antiproliferative activity

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Abstract: For the first time, this study presents the phytochemical and anticancer activity studies on *Sphagnum molle* species. Chemical component analysis of *S. molle* species was carried out by HPLC/TOF-MS. Anticancer activity studies of its organic solvent extract against human prostate cancer (PC3) cells were performed. Chlorogenic acid, along with gentisic, 4-hydroxy-benzoic, protocatechic, vanillic, chicoric, ferulic, and cinnamic acids, 4-hydroxybenzaldehyde, and rutin, were identified as the main components in *S. molle* extract. In the antiproliferative activity studies of MeOH:$CHCl_3$ extract of *S. molle*, high dose was found to show the highest activity against human prostate cancer (PC3) cells with a 68% inhibition between the 10th and 15th hours.

1. INTRODUCTION

Bryophytes' extensive worldwide distribution contributes to their greater species richness in tropical and subtropical zones. Although mosses have a very primitive way of life, they create a lot of diversity in their lives. They can exist on almost every surface, from rocks to tree bark, from the soil surface to the surface of synthetic products (Horn *et al*., 2021). Bryophytes are divided into three classes: Anthocerotae, horned liverworts; Hepaticae, leafy and tallus liverworts; Musci, leafy liverworts (Schofield, 2001). They have been used as herbal medicine in China, Europe, and North America for over 400 years. There is data that bryophytes show antibiotic activity on fungal species and anticancer activity on different cancer cells (Asakawa & Ludwiczuk, 2018; Cianciullo *et al*., 2022; Commisso *et al*., 2021; Oztopcuvatan *et al*., 2012). Bryophytes have been shown to possess a wide range of medicinal properties, with applications in treating hepatic disorders, skin diseases, cardiovascular diseases, fever, and wounds. A range of novel pharmacologically active compounds can be made using the phytochemicals isolated from them (Bandyopadhyay & Dey, 2022). Bryophytes are utilized in different world regions for ecological and economic purposes. Due to the many active substances they contain, specific taxa of bryophytes have been investigated for their antimicrobial, antibiotic, antifungal, insecticidal, and repellent effects and their use as biomonitors in pollution monitoring (Commisso *et al*., 2021).

Many bryophytes find different uses in different geographies. In particular, China is known to

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utilize bryophytes more than other countries, followed by the United States and Canada. In general, most of the bryophyte utilization is reported from Asia, and it is stated that *Sphagnum*, *Marchantia*, and *Polytrichum* are the most commonly used bryophyte genera by people from different cultures around the world (Drobnik & Stebel, 2021; Motti *et al*., 2023). In a study conducted by Oztopcuvatan *et al*. (2012), the 48-hour cytotoxic effect of extracts of *Homalothecium sericeum* against C6 (mouse brain tumor) cells was investigated. It was found that extracts of *H. sericeum* had a dose-dependent cytotoxic effect on glioma cells (Oztopcuvatan *et al*., 2012). Some other studies determined that most bryophyte extracts showed antibacterial and antimicrobial activity (Kang *et al*., 2007; Sawant & Karadge, 2010).

Natural products from plants harbor diverse bioactive compounds with significant anticancer potential (Ouyang *et al*., 2014; Newman & Cragg, 2020). A more comprehensive and efficient assessment of the therapeutic potentials inherent in these natural products could substantially accelerate the identification of active pharmaceutical ingredients with anticancer efficacy (Dehelean *et al*., 2021). Their accessibility, low cytotoxicity, and potential for structural modification underscore their considerable promise in cancer treatment (Hashem *et al*., 2022). Secondary metabolites derived from plants represent a rich and valuable source for developing therapeutic agents. Their accessibility, low cytotoxicity, and potential for structural modification underscore their considerable promise in cancer treatment (Hashem *et al*., 2022).

Cancer is a general name given to diseases that constantly divide due to mutations in molecular mechanisms in different cells and tissues. It can have invasive-metastatic structures, be stable, or show continuous change. Cancer describes hundreds of diseases with different clinical courses and treatment responses (Brown *et al*., 2023). Cancer is an important public health problem as it is the second leading cause of death after cardiovascular diseases (Vincent *et al*., 2019). It is predicted that deaths from cancer would increase rapidly by a hundred percent until 2070 and, therefore, will rise to the first place (Soerjomataram & Bray, 2021). If the necessary measures against cancer are not taken and a continuous and effective control program is not implemented, it is predicted that new cancer cases will increase significantly in the 2030s, and treatment costs will reach unaffordable amounts (Iragorri *et al*., 2021).

Research on the *Sphagnum* genus is scarce, and as deep as we could reach into the scientific literature, there has been no study about the bioactivity/phytochemistry of *Sphagnum molle* species. This paper reports the findings of phytochemical analysis and anticancer activity studies on *Sphagnum molle* species as the first example in the literature.

2. MATERIALS and METHODS

2.1. Plant Material

Sphagnum molle, a moss species, is distributed in Turkey's Eastern Black Sea region. Plant material was collected from the Rize-Findikli zone (41°13′31.9″N, 41°17′26.9″E, altitude 377 m) in September 2012. The dried plant material was pulverized by grinding with liquid nitrogen. A methanol/chloroform mixture was used to prepare extracts: while chloroform disrupts cell wall, it extracts phenolics and other important molecules better with methanol. Approximately 100 g of *Sphagnum molle* dry plant material was macerated three times with MeOH:$CHCl_3$ (methanol:chloroform) (1/1) at room temperature for 24 hours. All extracts were combined, and the solvent was removed using a rotary evaporator (IKA RV10, USA).

2.2. Quantitative Analysis by HPLC-TOF/MS

The phenolic components of *Sphagnum molle* extract were analyzed by Agilent Technologies 1260 Infinity HPLC-TOF/MS. The analyses were performed using the same methods of Abay *et al*. (2015). Shortly, the ZORBAX SB-C18 (4.6 x 100 mm, 3.5 µm) analytical column was used during the analysis. The solvent method was as follows: Mobile phase A, water with 0.1% formic acid; mobile phase B, acetonitrile: 0-1 min. 10% B; 1-20 min. 50% B; 20-23 min. 80% B; 23-30 min. 10% B. Flow rate was 0.6 mL/min, and the injection volume was 10 µL. Mass

scanning was carried out in negative mode as the phenolics can structurally stabilize negative charge better (Kajdžanoska *et al*., 2010). For quantitative analysis, the extract was screened for 20 phenolic standards: Gallic acid, gentisic acid, chlorogenic acid, catechin, 4-hydroxybenzoic acid, protocatechuic acid, vanillic acid, caffeic acid, 4-hydroxybenzaldehyde, rutin, p-coumaric acid, chicoric acid, ferulic acid, apigenin-7-glucoside, rosmarinic acid, salicylic acid, quercetin, cinnamic acid, naringenin, and kaempferol.

2.3. Antiproliferative Activity Tests

Antiproliferative activity studies of the extracts were performed against the PC3 human prostate cancer cell line. Antiproliferative activity studies were performed in a sterile cabinet. 10 mg of *Sphagnum molle* extract was dissolved in sterile DMSO (20 mg/mL). The prepared sample was diluted 1:20 with DMEM in sterile microcentrifuge tubes and used in antiproliferative activity studies.

Antiproliferative activity studies were carried out using the previously reported method (Koldaş *et al*., 2015). E-plate 96 with 50 µL of medium in each well was placed in the sterile cabinet for 15 minutes and in the incubator for 15 minutes for thermal equilibration. The plate was placed on the RTCA device, and a background reading was performed. This reading took 1 minute, and the usability of Eplate 96 was evaluated. After the background reading, the plate was taken to the sterile cabinet. The desired number of cells in each well (25.000 cells/well) was calculated, and the appropriate cell suspension was prepared. Cedex HiRes Innovatis Analyzer cell counting device (Roche) was used for cell counting. 100 µL of cell suspension was added to all wells except three wells. 100 µL of medium (DMEM) was added to the wells without cells. The plate was kept in the sterile cabinet for half an hour to allow the cells to settle to the bottom and adapt to the medium. Then, it was placed back on the RTCA station in the carbon dioxide incubator, and the second stage was started, which lasted 80 minutes. During this time, the cells could attach to the bottom of the well and enter the growth process. At the end of this period, the E-plate was returned to the sterile cabinet, and a culture medium solution containing different concentrations of plant extract was added to the wells. The effect of three different concentrations of the plant extract was investigated: high (250 µg/mL), medium (100 µg/mL), and low dose (50 µg/mL). Each dose was studied in triplicate. After adding the samples, the plate was placed in the RTCA device for the last time. For 48 hours, the life/death status of the cells was recorded in real-time.

3. RESULTS and DISCUSSION

Component analysis of the plant extract was performed using an HPLC-TOF/MS device. The results showed that 10 out of 20 compounds were detected in MeOH:$CHCl_3$ extract of *Sphagnum molle*. Table 1 shows the result of quantitative phenolic analysis with retention times of the compounds in the extracts and their concentration. It was detected that chlorogenic acid was the most abundant compound in the extract. The diversity and levels of secondary metabolites of plants may be linked to geographical, physiological, and genetic factors (Özay & Pehlivan, 2024). The interplay of these factors represents significant dynamics that shape the biochemical profiles of plants.

In a study conducted on *Sphagnum palustre* by Eom *et al*. (2016), nine secondary metabolites from different classes, like phenolics, carboxylic acids, steroids, etc., were isolated. The only phenolic compound from their study is (-)-naringenin, which was not found in our study on a different species of *Sphagnum* genus. In another study on *Sphagnum palustre* by Nam *et al*. (2011), phytochemical constituents of ethanolic extract were investigated. They isolated seven phenolic compounds, including chlorogenic acid and rutin. Chlorogenic acid was found to be the most abundant compound in MeOH/$CHCl_3$ extract of *Sphagnum molle* in our study.

Çelik *et al*. (2024) compared the phytochemical compositions of four *Sphagnum* species (*S. centrale*, *S. palustre*, *S. teres,* and *S. auriculatum*) collected in spring and autumn. They showed that the phytochemical composition of methanol extracts of species varied depending on the

season. Only *S. auriculatum* and *S. rubellum* from the autumn season had phenolic content, while all four species from the spring season had phenolic content. However, none of the species showed alkaloid contents for both seasons. In a study by Kang *et al*. (2016), one new flavonoid, namely 6-methyl-(2R,3S)-alpinone, along with six known flavonoids, four known steroids, six known triterpenoids, and three known fatty acids, was isolated from the ethanol extract of *Sphagnum palustre*. They also showed that the ethanolic extract of *S. palustre* had a protective effect against anticancer drug-induced nephrotoxicity *in vitro*. Fudyma *et al*. (2019) investigated the metabolic profile of *Sphagnum fallax* by combining the spectral results of ^{1}H NMR, LC-MS-MS, LC-FTICR-MS/MS, and DI-FTICR-MS analyses. They identified 655 metabolites in total, consisting of lipids, acids, sugars, and their derivatives; 152 were lipids, and 329 were novel compounds. They asserted that because of these metabolites, especially acids, mosses play an important role in the peatlands' carbon cycle.

Table 1. HPLC-TOF/MS results of MeOH:$CHCl_3$ extract of *Sphagnum molle.*

Compound	RT (Retention Time)	Final Conc. (ng/mL)
Phenolic Acids		
Gentisic acid	4.321	30.6738
Chlorogenic acid	7.114	762.9612
4-hydroxybenzoic acid	6.790	270.5806
Protocatechic acid	6.939	50.7299
Vanillic acid	7.571	198.7690
Chicoric acid	9.310	5.8163
Ferulic acid	11.703	199.6831
Cinnamic acid	15.822	206.2353
Phenolic Aldehydes		
4-hydroxybenzaldehyde	9.042	156.8967
Flavonoids		
Rutin	9.668	285.2893

As a result of the analysis, it was determined that the plant was especially rich in phenolic acids (chlorogenic acid (8), 4-hydroxybenzoic acid (3), 4-hydroxybenzaldehyde (2), vanillic acid (5), ferulic acid (7) and cinnamic acid (6)) and flavonoids (rutin (9)). It was also found to contain small amounts of gentisic acid (1), protocatechuic acid (4), and chicoric acid (10). Their molecular structures can be seen in Figure 1. As a subgroup of plant phenolics, phenolic acids and flavonoids form an important part of the food chemicals that show activities and protection against several diseases, including cancer (Wani *et al*., 2023). Chlorogenic acid is one of the most studied phenolic acids, along with caffeic acid, ferulic acid, ellagic acid, gallic acid, etc., under in vivo and *in vitro* conditions. It shows anticancer activities against several cancer types, including breast (Naso *et al*., 2014), colon (Hou *et al*., 2017), leukemia (Bandyopadhyay *et al*., 2004), lung (Yamagata *et al*., 2018), and prostate (Lee *et al*., 2017). In their studies, Huang *et al*. (2019) found that chlorogenic acid shows its anticancer activity by preventing cell differentiation only in cancer cells, not normal cells. In an animal study by Zeng *et al*. (2021), chlorogenic acid showed its antitumor activity by impairing NF-kβ and stopping cells from metastasis.

During the experiment, it was observed that the medium dose (pink) and low dose (turquoise) of *S. molle* extract showed similar antiproliferative effects against PC3 cells. Although these different doses of the extract could not inhibit the proliferation of the cells in the wells to which they were added, they could still keep the CI values of these wells lower than the CI values of the control group. Low and medium doses of the extract could not completely stop the cells, but were able to suppress and slow down their proliferation significantly.

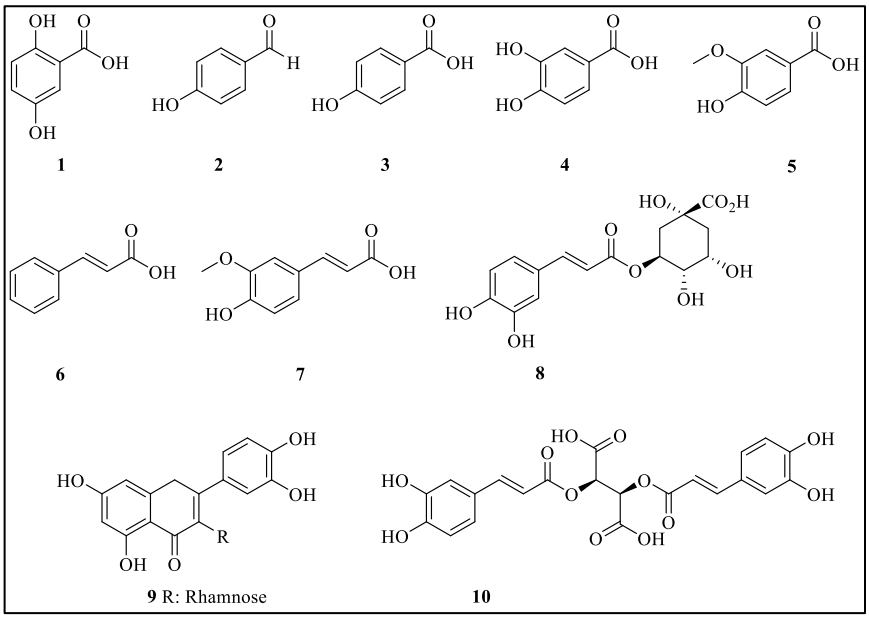


Figure 1. Molecular structures of phenolics determined by quantitative analysis of MeOH:$CHCl_3$ extract of *S. molle*.

The antiproliferative effect of MeOH:$CHCl_3$ extract of *Sphagnum molle* against human prostate cancer cells (PC3) is shown in Figure 2, and the changes in the CI value depending on the time intervals are given in Table 2. *S. molle*'s antiproliferative effect was examined at three different doses: high dose (250 µg/mL), medium dose (100 µg/mL), and low dose (50 µg/mL) (Figure 2). CI values obtained from wells containing only medium and PC3 cells (control group, red) increased throughout the experiment. The CI value obtained from the wells containing only medium (green, baseline) did not change and remained at zero until the end of the experiment. The cell index (CI) values obtained from the wells to which the plant extract was added were different from the CI values obtained from the control group. PC3 cells in the control group (red) did not encounter any antiproliferative effect and developed throughout the experiment, which increased the CI value to approximately 2.2. The CI value obtained from the wells to which the low-dose extract was added (turquoise) could only reach the values of the control group at 40 hours. This situation indicates that the molecules in the extract suppressed the proliferation of the cells.

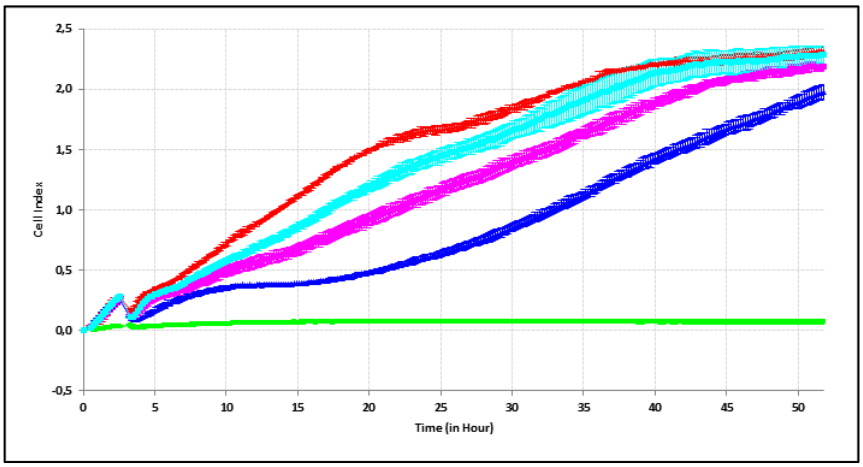


Figure 2. CI - Time plot showing the antiproliferative effect of MeOH:CHCl3 extract of *Sphagnum molle* against human prostate cancer cells (PC3) (Red: Control; Blue: 250 µg mL^{-1}; Pink: 100 µg mL^{-1}; Turquoise: 50 µg mL^{-1}; Green: Medium).

Table 2. Cell index (CI) values were obtained at 12, 24, 36, and 48 hours in the real-time antiproliferative activity analysis of the extract obtained from *Sphagnum molle* against PC3 cell lines at different concentrations.

	Cell Index		
	250 µg.mL^{-1}	100 µg.mL^{-1}	50 µg.mL^{-1}
12 h after treatment			
S. molle	0.390 ± 0.006	0.686 ± 0.040	0.871 ± 0.031
Control	1.133 ± 0.011	1.133 ± 0.011	1.133 ± 0.011
24 h after treatment			
S. molle	0.725 ± 0.023	1.261 ± 0.050	1.534 ± 0.055
Control	1.730 ± 0.031	1.730 ± 0.031	1.730 ± 0.031
36 h after treatment			
S. molle	1.390 ± 0.048	1.856 ± 0.047	2.106 ± 0.114
Control	2.192 ± 0.008	2.192 ± 0.008	2.192 ± 0.008
48 h after treatment			
S. molle	1.963 ± 0.055	2.181 ± 0.029	2.282 ± 0.070
Control	2.310 ± 0.036	2.310 ± 0.036	2.310 ± 0.036

Following the addition of the extracts to the wells, a delay was observed before their penetration into the PC3 cells. As depicted in the CI-Time graph, the sample addition occurred approximately 2.5 hours after the initiation of the experiment. After adding the samples, as the final phase commenced, the cells continued to proliferate, with the high dose showing significantly lower CI values compared to the other doses and the control group, starting from the 4th hour. At the 4th hour, the CI values were recorded as 0.112 for the high dose, 0.182 for the medium dose, and 0.218 for the low dose, whereas the control group exhibited a CI value of 0.278. While the cells maintained their metabolic activity, they became increasingly exposed to the antiproliferative effects of the extracts. Real-time measurements indicated that by the 10th hour, the PC3 cells in the control group had reached a CI value of 0.730 without any evident antiproliferative effects.

In contrast, the CI values at this time point were 0.586 for the low dose, 0.492 for the medium dose, and 0.356 for the high dose. The potent antiproliferative effect of the high dose persisted with comparable intensity until the 15th hour. At the 15th hour, the CI values were 1.125 for the control group, 0.866 for the low dose, 0.677 for the medium dose, and 0.388 for the high dose. Between the 5th and 10th hours, the CI value of the control group increased by approximately 50%, while the CI value of the high dose rose by only about 10%. In the subsequent hours, the strong effect of the high dose began to diminish, as evidenced by a gradual increase in the CI values obtained from the wells treated with the high dose, particularly after the 20th hour, when a more rapid rise in CI values was observed. Although the antiproliferative effects of the low and medium doses on PC3 cells were not as pronounced as the high dose, a clear separation from the control group was evident. The CI values of the *S. molle* extract at three different doses, along with those of the control group, at 12, 24, 36, and 48 hours after sample addition, are presented in Table 2. At the 12th hour, the CI value of the control group was approximately three times higher than that of the high dose. By the 24th hour, this difference had reduced to approximately 2.5 times; at the 36th hour, it was reduced to 1.5 times. By the 48th hour, the CI values of the high-dose group had approached those of the control group. A 50% difference in CI values was observed at the 12th hour when comparing the medium dose with the control group. The CI value of the low dose at the 12th hour was approximately 30% lower than that of the control group. The CI values in Table 2 further demonstrate that both the medium and low doses consistently separated from the control group throughout the experiment. At the 12th hour, the CI value of the low dose was more than twice that of the high dose, and the CI values of the

medium and high doses differed by approximately a factor of two. Throughout the experiment, the CI values for all doses of the extract remained lower than those of the control group.

Additionally, the dose-dependent effects were apparent in the CI values. While the low and medium doses slowed the proliferation of PC3 cells, the high dose notably suppressed PC3 cell proliferation between the 10^{th} and 15^{th} hours. In conclusion, it can be inferred that the MeOH:$CHCl_3$ extract of *S. molle* exhibits antiproliferative effects on PC3 cells.

4. CONCLUSION

Due to their bioactive properties, many molecules synthesized in plants, such as alkaloids, glycosides, phenolic compounds, and essential oils, are widely used in the food, cosmetics, and pharmaceutical sectors. Phenolic compounds (flavonoids, phenolic acids, anthocyanins, etc.) are highly valuable as natural products due to their strong biological effects, such as antioxidant, antiviral, and anticancer activity (Anwar *et al*., 2018; Farooq & Ngaini, 2019). Quantitative analysis of *Sphagnum molle* extract was performed for the first time, and the extract was scanned for 20 phenolic standards. Chlorogenic acid was determined as the main component. In addition, it was determined that it contains rutin, 4-hydroxybenzoic acid, cinnamic acid, ferulic acid, vanillic acid, 4-hydroxybenzaldehyde, protocatechic acid, and gentisic acid in order of quantity. Also, the antiproliferative effect against PC3 cells was investigated, and the extract was found to inhibit the proliferation of the cells for the first 20 hours.

Determination of the chemical constituents of *Sphagnum molle* and investigation of its antiproliferative activity against PC3 cells will provide important contributions to understanding the therapeutic use of secondary metabolites and the positive potential of natural products. In order to determine the biological activity of *Sphagnum molle* more clearly, it should be studied with different cancer cell lines and its purified components. Also, *in vivo* and *in silico* studies would shed light on investigating the potential benefits of *S. molle*.

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Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the authors.

Authorship Contribution Statement

Muhammed Altun: Investigation, Resources, Supervision, Formal Analysis, and Writing-original draft. **Serkan Koldas**: Methodology, Visualization, Software, and Validation.

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