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Determination of antimicrobial and antiproliferative activities of the aquatic moss *Fontinalis antipyretica* Hedw.

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Abstract: The purpose of the current study was to investigate the possible antimicrobial and anticancer properties of the aquatic moss *Fontinalis antipyretica* Hedw. (Fontinalaceae). We first obtained 8 different extracts (methanol; chloroform; acetone; ethyl acetate A, B, C, and D) by two different extraction processes. The antimicrobial activity of these extracts was then assessed by using the well diffusion method against 8 bacterial and 7 fungal strains. The results of antimicrobial studies showed that extracts of chloroform, acetone, and ethyl acetate A and C were active against almost all the species tested. The most effective extract was C, which was then screened through a preparative TLC (bioautography) for its active components. Spot 4 of extract C exhibited the highest antimicrobial activity against 5 bacterial strains (*B. cereus, B. subtilis, E. faecalis, E. aerogenes,* and *Y. enterocolitica*) and 3 fungal strains (*A. flavus, F. solani,* and *F. graminearum*), with MIC values of 93.8-375.0 µg/mL and 187.5-375.0 µg/mL, respectively. In addition, in vitro toxicity of the active component of extract C at the concentrations of 0.16, 1.6, 16, 80, and 160 µg/mL was tested. Depending on dose and time, only 80 and 160 µg/mL decreased rat glioma (C6) cell viability after 24 or 48 h. The present study suggests the possibility that *Fontinalis antipyretica* possesses antimicrobial and anticancer agent(s).

Key words: Activity, antibacterial, antifungal, Bryophyta, extract, toxicity

Sucul karayosunu *Fontinalis antipyretica* Hedw.' nin antimikrobiyal ve antikanser aktivitesinin belirlenmesi

Özet: Bu çalışmanın amacı sucul karayosunu *Fontinalis antipyretica* Hedw. (Fontinalaceae)' nin antimikrobiyal ve antikanser özelliklerinin incelenmesidir. İki farklı ekstraksiyon yöntemiyle 8 özüt (metanol, kloroform, aseton, etil asetat, A, B, C ve D) elde edilmiştir. Özütlerin antimikrobiyal aktivitesi 8 bakteri ve 7 küfe karşı kuyucuk difüzyon yöntemi ile belirlenmiştir. Kloroform, aseton, etil asetat, A ve C özütleri hemen hemen tüm test edilen türlere karşı etki göstermiştir. Ekstrakt C' nin aktif bileşenleri preperatif ince tabaka kromatografisi (Biyootografi) ile tarandı. Aktif bileşen (spot 4) test edilen bakteriler için 93,8-375,0 µg/mL aralığında; küfler için ise 187,5-375,0 µg/mL aralığında minimum inhibisyon konsantrasyonu göstermiştir. Ekstrakt C'nin bu bileşeni *B. cereus, B. subtilis, E. faecalis, E. aerogenes, Y. enterocolitica* olmak üzere 5 bakteri ve *A. flavus, F. solani, F. graminearum* olmak üzere 3 fungal straine karşı en yüksek antimikrobiyal aktivite göstermiştir. Ek olarak özüt C' nin aktif bileşeninin 0,16, 1,6, 16, 80 ve 160 µg/mL konsantrasyonlarının in vitro toksisitesi test edilmiştir. Sadece 80 ve 160 µg/mL 24 ve 48 saatte doza ve zamana bağlı olarak sıçan glioma (C6) hücre canlılığında azalmaya neden oldu. Bu çalışma *Fontinalis antipyretica*'nın yeni bir antimikrobiyal ve antikanser ajan olabilme olasılığını ortaya koymaktadır.

Anahtar sözcükler: Aktivite, antibakteriyal, antifungal, Bryophyta, özüt, toksisite

Introduction

Phylogenetically located between vascular plants and algae, bryophytes form a unique division in the plant kingdom (1). There are more than 22,000 members of the moss family (Bryophyta), which represents about 5.5% of plant species throughout the world (2). Although a few reviews concerning the biologically active chemical constituents of bryophytes have been published (2-4), the chemistry of bryophytes has been neglected for a long time for the following reasons: they are very small morphologically, difficult to collect in large amounts as pure samples, difficult to identify, and are considered nutritionally useless to humans. However, bryophytes have been used as medicinal plants for more than 400 years in China, Europe, and North America. Among other things, they have been used to cure cuts, burns, external wounds, bacteriosis, pulmonary tuberculosis, neurasthenia, fractures, convulsions, scalds, uropathy, and pneumonia (4-7). There is also evidence confirming the antibiotic activity of bryophytes against fungi and prokaryotic cells (7,8).

Of all the bryophyte extracts, flavonoids, biflavonoids, and isoflavonoids have been reported to be the most likely chemical barriers against microorganisms (9,10). Terpenoids and phenolic and volatile constituents have also been investigated in some species of mosses (2,11).

Cancer and microbes that have gained resistance to drug therapy are an increasing public health problem. Yet, there are few really effective antifungal preparations currently available for the treatment of fungal attack in agriculture, an occurrence which can be economically devastating. The logical progression of this knowledge would be to screen plants for any constituents that show activity against pathogenic fungi, bacteria, and cancer. Currently the most investigated taxa are from the angiosperm group. Very little data is available about other groups of plants, in particular bryophytes (5,6,10,12-16).

To the best of our knowledge, there is no published report concerning the antimicrobial and anticancer activity of *Fontinalis antipyretica* Hedw. (Fontinalaceae). Therefore, this study investigated, for the first time, the possible effects of *Fontinalis antipyretica* extracts against some bacterial and fungal species, and a rat glioma (C6) cell line.

Materials and methods

Plant material

Plant material was collected from the Yalımkaya Stream in the Sundiken Mountains (Eskişehir, Turkey) at a height of 1420 m in May 2006 and identified by Dr. F. Savaroglu, Eskişehir Osmangazi University. A voucher specimen (Savaroglu 764) was deposited at the herbarium of our department.

Preparation of the extracts

Fresh gametophytic samples of *Fontinalis antipyretica* were treated with 0.8% Tween 80 aqueous solution to remove the epiphytic hosts normally found on the surface, extensively washed in tap and distilled water, and dried on filter paper at room temperature. Extraction procedures were applied as described elsewhere (17,18). Extraction was carried out through two different processes.

First, 10 g of the sample in powder form was extracted with 250 mL of 80% methanol, chloroform, acetone, and ethyl acetate for 8 h using Soxhlet equipment. After filtering with Whatman filter paper (#1), all extracts were concentrated by rotary evaporation to dryness in vacuum (yield = 13.52%, 2.50%, 2.96%, and 1.95% respectively) and stored in desiccators for future use.

The second extraction process was completed in four steps. First, 30 g of gametophytic plant sample in powder form was extracted with 250 mL of petroleum ether for 8 h using Soxhlet equipment (extract A, yield 0.74%). In the second step, fat-free air-dried material (15 g) was extracted 4 times with methanol:water (70:30, v/v) at 40 °C for 30 min. The extract was then concentrated to dryness in vacuum (extract B, yield 9.23%). The third and fourth extracts were prepared as follows: fat-free air-dried material (15 g) was extracted 4 times with methanol:water (70:30 v/v) at 40 °C for 30 min, concentrated in vacuum, and the aqueous phase was extracted with ethyl acetate at room temperature. This was then concentrated to dryness in vacuum (extract C, yield 1.10%). The aqueous solution was separately concentrated by rotary evaporation to dryness in vacuum (extract D, yield 6.91%).

The yields from the different extracts were weighed, dissolved in dimethyl sulphoxide (DMSO) to a final concentration of 200 mg/mL, and then stored at 4 °C for further use.

Test microorganisms

The bacterial strains were recovered from long-term storage at –85 °C in the cryobank. The bacteria were refreshed in Nutrient Broth (Merck, Germany) at 35-37 °C and then inoculated on Nutrient Agar (Merck) plates to check the microbial purity. The molds were refreshed in Malt Extract Agar (Merck) at 27 °C. The strain numbers and sources of the acquired microorganisms are listed in Table 1.

Determination of antimicrobial activity

The moss extracts were studied for their antibacterial and antifungal activities through the well diffusion method, according to the National Committee for Clinical Laboratory Standards (NCCLS) (19,20). The bacterial test cultures were incubated in Mueller-Hinton broth (MHB) at 35-37 °C until they were visibly turbid. The density of these cultures was adjusted to a turbidity equivalent to that of the 0.5 McFarland standard used to standardize the inoculum's density (at 625 nm, 0.08-0.1 absorbance) with sterile saline. The bacterial cultures adjusted to this standard contained approximately 1×10^8 CFU/mL (19,20). In order to induce spore formation, the molds were grown on potato dextrose agar slants at 27 °C for 5 to 7 days. After being counted with a Thoma slide, the spore concentration was adjusted to 10^6 CFU/mL with sterile 0.1% Tween 80 for each mold. Mueller-Hinton agar (MHA) and Sabouraud dextrose agar, sterilized in a flask and cooled to 45-50 °C, were distributed among sterilized petri dishes (9 cm). The entire surface areas of the MHA plates and the Sabouraud 4% glucose medium (SGM) plates were inoculated with the bacteria and fungi by spreading with a sterile swab dipped into the adjusted suspensions (21). Six wells, each 6 mm in diameter, were cut out of the agar, and 20 µL of the extract solutions were placed into each well. The petri dishes were kept at 4 °C for 2 h, the plates inoculated with bacteria were incubated at 37 °C for 24 h and at 30 °C for 48 h for the fungal strains. The diameters of the inhibition zones were measured in millimeters. Penicillin and tetracycline (Bioanalyse) were used as positive controls for bacteria, Amphotericin B (Sigma) was used as a positive control for fungi, and DMSO was used as negative control. All assays were performed in duplicate.

Table 1. Bacterial and fungal strains used for antimicrobial activity test.

Bacterial strains	Source
Bacillus cereus NRRL B-3711	USDA, Agricultural Research Service, Peoria, IL, US
Bacillus subtilis NRRL B-209	USDA, Agricultural Research Service, Peoria, IL, US
Enterobacter aerogenes NRRL B-427	Department of Biology, Anadolu University, Turkey
Enterococcus faecalis ATCC 29212	Department of Microbiology, Eskişehir Osmangazi University, Turkey
Escherichia coli ATCC 25922	Department of Microbiology, Eskişehir Osmangazi University, Turkey
Salmonella typhimurium ATCC 14028	Department of Biology, Anadolu University, Turkey
Staphylococcus aureus ATCC 25923	Department of Microbiology, Eskişehir Osmangazi University, Turkey
Yersinia enterocolitica	Department of Biology, Anadolu University, Turkey
Fungal strains	
Aspergillus flavus ATCC 9807	Department of Biology, Anadolu University, Turkey
Aspergillus fumigatus NRRL 163	USDA, Agricultural Research Service, Peoria, IL, US
Aspergillus niger ATCC 10949	Department of Biology, Anadolu University, Turkey
Aspergillus parasiticus NRRL 465	USDA, Agricultural Research Service, Peoria, IL, US
Fusarium graminearum (wild type)	Department of Biology, Eskişehir Osmangazi University, Turkey
Fusarium solani (wild type)	Department of Biology, Eskişehir Osmangazi University, Turkey
Geotrichum candidum (wild type)	Department of Biology, Eskişehir Osmangazi University, Turkey

ATCC: American Type Culture Collection; NRRL: Northern Regional Research Laboratory

Analysis by TLC

Silica gel sheets (20 × 20 cm, 0.2 mm aluminum cards with a fluorescent indicator 254 nm, Fluka) were used for thin layer chromatography (TLC). Less than 1 mg crude extract material was dissolved in either petroleum ether or ethyl acetate and applied to form a single small spot about 1 cm from the bottom of the silica gel sheet. TLC plates were placed in a presaturated solvent chamber with n-buthanol: acetic acid: water (8:1:1), and the extract spots were allowed to develop on the plate. The TLC plates were then removed from the solvent chamber immediately before the solvent reached the top of the plates. These plates were allowed to air dry, and pigmented compounds were marked on the developed TLC plates with a pencil. Each plate was placed in an UV viewing cabinet (254 nm, Camag), and the locations of non-pigmented UV_{254}-fluorescing compounds were located directly on the plate with penciled-in circles (22,23).

Bioautographic method

To detect biological activity directly on the TLC plates, MHA or SGM-containing bacterial cells or fungal spores, respectively, were spread on silica gel plates in a thin layer. The bacterial inoculums and fungal spore suspensions, prepared as described above, were inoculated with the respective mediums (1:100) at 42 °C. A 0.5 mL aliquot of 1 mg/mL trifenil tetrazolium chloride solution (TTC, Merck) was added as a growth indicator, and the cultures were incubated as described above (22,23).

Minimum inhibitory concentration (MIC)

MIC was determined by the micro dilution method using a 96 well plate according to the NCCLS (20,24). First, 100 µL of MHB or Sabouraud dextrose broth (SDB) was placed in each well. The stock solutions of the extracts were diluted and transferred into the first well, and serial dilutions were performed so that concentrations in the range of 1.5-1500 µg/mL were obtained. The inoculums were adjusted to contain approximately 10^5 CFU/mL of bacteria and 10^4 CFU/mL of fungi, as described above. One hundred microliters of the inoculums were added to all the wells, and the plates were incubated at 37 °C for 24 h for bacteria and at 30 °C for 48 h for fungi. Antimicrobial activity was detected by adding 20 µL of 0.5% TTC aqueous solution. The MIC value taken was the lowest concentration of the extract that inhibited any visible bacterial or fungal growth, as indicated by TTC staining after incubation (19,24). Penicillin, tetracycline, and Amphotericin B were used again as the reference antibiotic controls.

Glioma cell culture and viability

For the rat glioma cell culture experiments all chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA), prepared immediately prior to use, and protected from light. The powder form of spot 4 from extract C, dissolved in DMSO, was diluted further in Dulbecco's Modified Eagle's Medium (DMEM) at a ratio of 1:10. The maximum concentration of DMSO was adjusted to 0.1% at the highest (160 µg/mL) concentration of spot 4.

The C6 cell cultures were maintained in 75 cm^2 flasks and incubated in DMEM with 10% fetal bovine serum and 1% penicillin-streptomycin solution at 37 °C in the humidified atmosphere of an incubator (Sanyo, Japan) containing 5% CO_2 and 95% air (25). When confluence was achieved, the glial cells were incubated with 3 mL of trypsin-EDTA (0.25%) solution for 5 min at 37 °C. After cell dispersion, trypsin activity was inhibited by adding growth medium. The cells were then centrifuged at 1000 rpm for 5 min at 4 °C and counted with a counter (Coulter, England). Cell viability was accessed by trypan blue dye exclusion and found to exceed 98%.

The cells were seeded in 2 × 10^5 cells/well (250 µL) in 96 well micro titer plates and incubated for 24 h. Each dose (0.16, 1.6, 16, 80, and 160 µg/mL) of extract C was then added to 8 wells, which contained the growth medium. The other 8 wells were reserved for the control and contained growth medium only. All plates were incubated at 37 °C for 24 or 48 h in a humidified atmosphere of 5% CO_2 and 95% air. Drug cytotoxicity screening was determined by using a 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay (26). A 25 µL MTT solution was added to each well and incubated at 37 °C for 4 h. The MTT solution was converted into blue formazan by the mitochondrial dehydrogenase activity of the viable cells. The amount of formazan produced is proportional to the number of living cells (27). After all the medium was removed from the wells 100 µL of DMSO was added to each well, and the crystal formazan particles produced in viable cells were dissolved for 5 min at room temperature with a shaker. The absorbance of the formazan dye was read at 550 nm using a microplate reader (Bio-Tek Instruments, USA), and cell survival percentages were calculated according to the following formula: absorbance of treated cells in each well × 100/mean absorbance of control cells. The dose response curves were calculated for extract C at the above-mentioned concentrations and expressed as the mean percent fraction of control ± SEM.

All statistical analyses were performed using one-way analysis of variance (ANOVA) and followed up with Tukey's multiple comparison tests. The results are the means of at least 3 independent assays, and a P value less than 0.05 was considered significant.

Results and discussion

The results of the antimicrobial activity of methanol, chloroform, acetone, ethyl acetate extracts, and the other 4 extracts (A, B, C, and D) from *Fontinalis antipyretica* are presented in Tables 2 and 3. Chloroform, acetone, ethyl acetate, and the A and C extracts showed significant antibacterial and antifungal activities against almost all the organisms. Among the tested bacteria *B. subtilis* was found to be the most sensitive, *E. aerogenes* came next, followed by *B. cereus*, *E. faecalis*, *Y. enterocolitica*, and *S. aureus*. Extract C exhibited high antimicrobial activity against *B. subtilis* (18 mm), *E. faecalis* (14 mm), *E. aerogenes* (12 mm), *Y. enterocolitica* (12 mm), and *S. aureus* (12 mm) and only modest activity against *B. cereus* (10 mm), *E. coli* (8 mm), and *S. typhimurium* (8 mm). Only extract C inhibited the growth of *S. typhimurium*. Furthermore, ethyl acetate and the A and C extracts exhibited some degree of activity against almost all of the fungi. The inhibition zone diameters against *F. solani, F. graminearum*, and *A. flavus* for extract C were bigger than those of the standard antibiotic, Amphotericin B (Table 3).

Extract C was examined by TLC on silica gel using the mobile phase described above. The bioautoghraphy method was applied to the crude extract C, which exhibited the highest antimicrobial activity. Four spots appeared on the TLC plates with Rf values of 0.34, 0.48, 0.54, and 0.67, respectively. Since only spot 4 had activity against *B. subtilis* and *F. graminearum*, it was scraped and used to determine its MIC values. Table 4 illustrates the MIC ranges of spot 4 against bacterial and fungal strains. Our results show that the fourth spot component of extract C produced favorable results against all the tested microorganisms with MIC values between 93.8 and 375.0 µg/mL for the bacterial strains and 187.5 and 375.0 µg/mL for the fungal strains. It was more effective against *B. cereus, B. subtilis, E. faecalis, E. aerogenes, Y. enterocolitica, A. flavus, F. solani,* and *F. graminearum* than the other strains tested.

The Figure demonstrates the effect of spot 4 of extract C on C6 cell survival. The highest concentration of DMSO was found to have no effect on cell viability when used alone. After 24 h the cell survival rates were 110%, 101%, 103%, 65%, and 49% with 0.16, 1.6, 16, 80, and 160 µg/mL spot 4 concentrations, respectively ($P < 0.001$ for 80 and 160 µg/mL). There

Table 2. Antibacterial activity of *Fontinalis antipyretica* extracts as inhibition zones (mm).

Tested bacterial strains	Methanol	Chloroform	Acetone	Ethyl acetate	Extract A	Extract B	Extract C	Extract D	Control 1*	Control 2**
B. cereus 3711	NS	7 ± 0.3	7 ± 0.3	9 ± 0.2	10 ± 0.2	8 ± 0.2	10 ± 0.2	NS	35 ± 0.2	30 ± 0.1
B. subtilis 209	NS	7 ± 0.3	7 ± 0.3	10 ± 0.2	10 ± 0.2	8 ± 0.2	18 ± 0.2	NS	13 ± 0.2	24 ± 0.1
E. aerogenes 427	NS	7 ± 0.3	7 ± 0.3	10 ± 0.2	10 ± 0.2	8 ± 0.2	12 ± 0.2	NS	R	10 ± 0.1
E. faecalis 29212	NS	7 ± 0.3	7 ± 0.3	8 ± 0.2	10 ± 0.2	NS	14 ± 0.2	NS	27 ± 0.2	15 ± 0.1
E. coli 25922	NS	NS	7 ± 0.3	NS	8 ± 0.2	NS	8 ± 0.2	NS	30 ± 0.2	25 ± 0.1
S. typhimurium 14028	NS	NS	NS	NS	NS	NS	8 ± 0.2	NS	21 ± 0.2	18 ± 0.1
S. aureus 25923	NS	8 ± 0.1	NS	12 ± 0.2	14 ± 0.2	NS	12 ± 0.2	NS	35 ± 0.2	27 ± 0.1
Y. enterocolitica	NS	7 ± 0.3	7 ± 0.3	10 ± 0.2	10 ± 0.2	NS	12 ± 0.2	NS	9 ± 0.2	29 ± 0.1

R: resistant; NS: not sensitive; *Penicillin (10 µg/disc); ** Tetracycline (30 µg/disc)

Table 3. Antifungal activity of *Fontinalis antipyretica* extracts as inhibition zones (mm).

Tested bacterial strains	Methanol	Chloroform	Acetone	Ethyl acetate	Extract A	Extract B	Extract C	Extract D	Control 1*
A. flavus 9807	NS	7 ± 0.2	7 ± 0.2	7 ± 0.2	8 ± 0.2	NS	9 ± 0.2	NS	7 ± 0.1
A. fumigatus 163	NS	NS	NS	10 ± 0.2	13 ± 0.2	NS	11 ± 0.2	NS	15 ± 0.1
A. niger 10949	NS	8 ± 0.3	8 ± 0.2	12 ± 0.2	9 ± 0.2	NS	10 ± 0.2	NS	13 ± 0.1
A. parasiticus 465	NS	NS	8 ± 0.2	9 ± 0.2	10 ± 0.2	NS	11 ± 0.2	NS	14 ± 0.1
F. graminearum	NS	NS	NS	23 ± 0.2	26 ± 0.2	NS	22 ± 0.2	NS	16 ± 0.1
F. solani	NS	7 ± 0.2	NS	15 ± 0.2	15 ± 0.2	NS	16 ± 0.2	NS	13 ± 0.1
G. candidum	NS	7 ± 0.2	NS	8 ± 0.2	11 ± 0.2	NS	10 ± 0.2	NS	11 ± 0.1

NS: not sensitive; * Amphotericin B (10 µg/disc)

Table 4. Minimum inhibitory concentrations (MIC, µg/mL) of the active component of extract C against bacterial and fungal strains.

Bacterial strains	MIC (µg/mL)	Penicillin (µg/mL)	Tetracycline (µg/mL)
B. cereus NRRL B-3711	93.8	1.5	1.5
B. subtilis NRRL B-209	93.8	187.5	1.5
E. aerogenes NRRL B-427	93.8	375.0	23.4
E . faecalis ATCC 29212	93.8	2.9	1.5
E. coli ATCC 25922	375.0	46.9	93.8
S. typhimurium	375.0	93.8	1.5
S. aureus ATCC 25923	187.5	1.5	1.5
Y. enterocolitica	93.8	187.5	5.9
Fungal strains	**MIC (µg/mL)**	**Amphotericin B (µg/mL)**	
A. flavus ATCC 9807	187.5	11.7	
A. fumigatus NRRL 163	375.0	5.9	
A. niger ATCC 10949	375.0	23.4	
A. parasiticus NRRL 465	375.0	46.9	
F. graminearum	187.5	11.7	
F. solani	187.5	11.7	
G. candidum	375.0	23.4	

is a clear dose-dependent decreasing action on cell survival. However, after 48 h the survival rates were 97%, 97%, 97%, 59%, and 16% with 0.16, 1.6, 16, 80, and 160 µg/mL of spot 4, respectively ($P < 0.001$ for 80 and 160 µg/mL). In the case of the 160 µg/mL concentration of spot 4 of extract C, there was a 33% more time-dependent reduction in cell survival when exposure time increased from 24 to 48 h.

Even though there are various methods for screening antimicrobial effects, the well diffusion method was preferred since it is a modification of the disc diffusion method and based on those described for standardized testing of antibiotics. This method can be used to simply determine whether or not antibacterial activity is present (28).

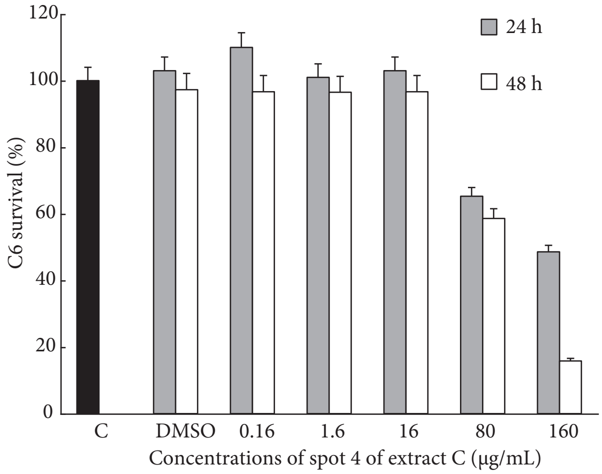

Figure. The effect of spot 4 of extract C on C6 cell survival (C: control, ***: $P < 0.001$).

The applied concentrations of methanol and B and D extracts of *Fontinalis antipyretica* did not demonstrate any inhibitory effect against any of the tested microorganisms. However, it is known that methanol and ethanol extracts of some mosses have shown antimicrobial activity against some strains of bacteria and fungi (7,12,13,15). Dulger et al. (12) investigated the antimicrobial activity of methanol extracts from the aerial parts of eight different moss species with the disc diffusion method and concluded that methanol extracts have a moderate activity against gram-positive and negative bacteria and a weak anti-yeast activity. İlhan et al. (13) studied the antimicrobial activity of methanol and acetone extracts from *Palustriella commutata* and indicated that methanol extract had a weak effect on some bacteria, but was inactive against yeast and mold strains. In another study, the antimicrobial activity of ethanol extracts from 15 Indian mosses was evaluated and only 7 of them were found to be active against all the organisms tested (7).

Our results indicated that chloroform, acetone, ethyl acetate, and A and C extracts demonstrated various antimicrobial activities against the tested organisms. The antifungal activity of extract C against *F. solani, F. graminearum*, and *A. flavus* was higher than that of the standard antibiotic, Amphotericin B. We were able to find only one report in support of our results, and this report also indicated that extracts from mosses displayed antifungal activity (29).

In the present study, generally gram-positive bacteria such as *B. subtilis, E. faecalis*, and *S. aureus* were more susceptible than gram-negative bacteria such as *E. coli* and *S. typhimurium*, with the exception of *Y. enterocolitica*. Similarly, Basile et al. (5) reported that the gram-positive bacterium *B. subtilis* showed a high sensitivity to extract of *Pleurochaete squarrosa*. The antibacterial activity of mosses against some gram-negative bacteria has been reported in other studies. In particular, *Leptodictyum riparium* extract was able to inhibit gram-negative more than gram-positive bacteria (29).

Since the complex chemical composition of plant extracts is generally a limiting obstacle to the isolation of antimicrobial compounds, we preferred to employ the bioautography method for the screening of active substances in crude extract and purification. The use of bioautography agar overlay bioassays allows the detection of active components in a crude plant extract (30). This method is a very convenient and simple way of testing plant extracts for their activity in the presence of both human and plant pathogenic microorganisms. There are various studies using this method for the same purpose (31,32). Through the bioautography method we ascertained that extract C was composed of 4 spots. Only spot 4 produced favorable results against all the tested bacterial and fungal strains with MIC values of 93.8-375.0 μg/mL and 187.5-375.0 μg/mL, respectively.

The growth-inhibitory effect of these extracts on the tested bacteria and fungi may be related to the presence of various lipid compounds. The presence of these lipids has also been reported in several mosses (33). Moreover, it is known that phenolic lipids, a large group of natural compounds, have different biological activities (34).

Since spot 4 of extract C of *Fontinalis antipyretica* was the most effective antimicrobial agent, we studied only this extract to determine its anticancer activity in the presence of C6 glioma cells. We found that spot 4 at high doses showed dose and time-dependent anticancer activity against glioma cells. Similarly, Yamada et al. (35) studied the cytotoxicity of Canadian Sphagnum peat on rat basophilic leukemia by MTT assay and found that low doses (0.001-10 μg/mL) did not show any decreasing effect after 48 h. In support of our data, Ivanova et al. (36) demonstrated that Sanionin A and B, from the moss *Sanionia georgico-uncinata*, had antiproliferative action after 72 h on human leukemia cells, mouse fibroblast cells, and human cervix carcinoma cells. A further study also indicated that 4 synthetic derivatives of lunularic acid, a bibenzyl found in mosses, had cytotoxicity on cell growth in a colon cancer cell line and estrogen (-) mammary tumor cells (37).

Microbes that have gained resistance to drug therapy are an increasing public health problem. While there are a few really effective antifungal preparations currently available for the treatment of systemic mycoses, the efficacy of existing drugs is rather limited. There is a need to screen plants for any constituents that show activity against pathogenic fungi and bacteria. The present study clearly indicates that bryophytes, a group which includes mosses, could be a promising new source of antimicrobial and anticancer agents. Our results offer the opportunity to work further on *Fontinalis antipyretica*, as well as on other bryophytes, for chemical and pharmacological validations. Further studies are warranted for the isolation and identification of individual phenolic compounds.

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