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The bioactive properties of a bryophyte collected from Bilecik (Turkey) Province

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ABSTRACT

Studies on the discovery of natural bioactive substances and the determination of their potential use in drug synthesis have gained importance due to the toxicity of existing chemical drugs and the increasing antibiotic resistance of pathogenic bacteria. In this context, bioactive secondary compounds synthesized by bryophyte samples collected from different geographic locations attract great interest. In this study, the antimicrobial, antioxidant, and anticancer activities of the extracts obtained from the bryophyte sample were investigated after the identification of the bryophyte sample collected from Bilecik Province. Agar Disk Diffusion (ADD) and Minimal inhibitory concentration (MIC) methods were used to determine the antimicrobial activity. DPPH removal activity and (3,4,5-dimethylthiazol-2-yl) -2−5-diphenyltetrazolium bromide (MTT) were carried out for the determination of the antioxidant capacity and anticancer activity, respectively. According to the results of the current study, the extracts of the bryophyte species, defined as *Leucodon sciuroides* (Hedw.) Schwägr, prepared with ethyl acetate and hexane, was found to have no significant antimicrobial and antioxidant activity, but methanol extract was found to have antifungal potential against *C. albicans* species. The ethyl acetate and methanol extracts from the bryophyte sample showed a higher antiproliferative effect against MCF-7 (ATCC number HTB-22^{TM}) (IC_{50}: 51.16 and 98.17) and MDA-MB-231 (ATCC number HTB-26^{TM}) (IC_{50}: 20.21 and 15.47). However, the same extract could not 50% inhibit the healthy cell line (3T3) up to the maximum concentration, >500 μg/mL. As a result, *L. sciuroides* has the potential to be used in the synthesis of new compounds with natural and selective toxicity.

1. Introduction

The bryophytes are a division of plants that includes all non-vascular land plants and can be split into three groups: Mosses, hornworts, and liverworts (Onele et al., 2018). There are between 18,000 to 25,000 species of bryophytes, which can be found in most ecosystems worldwide including mosses (11,000−13,000 species), liverworts (7000−9000 species), and hornworts (200−250 species) according to European Redlist (2022). On the other hand, these values are updated day by day as molecular studies on this subject always continue to reveal new species. Turkish mosses are composed of 726 species, subspecies, and varieties representing 164 genera and 42 families (Uyar and Çetin, 2004; Klavina et al., 2015).

Although bryophytes normally grow in humid habitats, they are relatively free from microbial attack. The scarcity of disease indicates that bryophytes can elaborate constitutive or inducible small-molecule antimicrobials (Xie and Lou, 2009). One of the features that help bryophytes survive and maintain their place in today's flora is their content of biologically active compounds, such as essential oils, flavonoids, terpenoids, fatty acids, and alcohols (Negi et al., 2020). These protect the otherwise delicate plants not only from fungi and other microorganisms but also from insects and slugs. In a way, these biochemical compounds make up for the lack of a thick cuticle and bark in bryophytes (Saxena and Harinder, 2004). Several bryophytes (in particular, mosses) have widely been used as medicinal plants in China, to cure burns, bruises, external wounds, etc. The mosses are said to possess certain biological activities and effects. Some bryophytes show characteristic fragrant odors and an intensely hot and bitter or saccharine-like taste. Generally, bryophytes are not damaged by insects, snails, slugs, and other small animals (Asakawa, 2007).

Bryophytes expressed interesting biological activities such as antibacterial, antifungal, antitumor, antiviral, antioxidant, etc. (Yayintas et al., 2019; Novakovic et al., 2021; Yıldırım Akatın et al., 2022). This study aims to investigate the bioactive properties of an identified bryophyte collected from the Bilecik (Turkey) province. There has been a rapid increase in studies carried out since the 2000s (Asakawa

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et al., 2013; Ertürk et al., 2015; Oztopcu-Vatan et al., 2017; Savaroglu et al., 2018). Important results have been reached regarding the bioactivities of bryophytes collected from different regions according to the sources in the recent literature (Cianciullo et al., 2022). However, there is limited information about the effects of extracts from *Leucodon sciuroides* (Leucodontaceae) species on antimicrobial or cytotoxic activities. To the best of our present knowledge, this is the first study that reported the antimicrobial and cytotoxic activities of *L. sciuroides* obtained from Bilecik, extracts using different solvents.

2. Materials and methods

2.1. Materials

2.1.1. Plant material

Plant material (Supplementary material 1) was collected from the Gökpinar Village (40.2147° N, 29.9241° E), Bilecik (Turkey), on rock, in June 2020. Plant samples were collected once in June, and a sufficient amount of samples were collected from the same region. While collecting the samples, care was taken not to mix the other plant species intertwined with the sample plants. The cluster of bryophyte samples was taken from the rock surface by hand and then put in paper bags (Rothero et al., 2005). The paper bag was labeled including the coordinate of the area where the samples were taken. The samples brought to the laboratory were purified from foreign materials during washing and dried at room temperature. The dried samples were immediately processed. The specimen was identified in the Department of Plant Biology at Eskisehir Osmangazi University. A voucher specimen (Savaroglu, 1494) was deposited at the Herbarium of the Department.

2.1.2. Test organisms

Four Gram-positive and four Gram-negative bacteria were tested for their susceptibility to the Bryophyte extracts. The chosen strains are representative of the most commonly involved pathogenic bacteria in human infections, including strains with high resistance to antibiotics or high environmental or antibiotic adaptability (Munita and Arias, 2016; Nowicki et al., 2019; Sionov and Doron, 2022). In addition, it was used to test the susceptibility of fungi to the extracts with 3 selected yeast strains representing eukaryotic cells. All test organisms were obtained from American Type Culture Collection (Table 1). The selected test organisms from the results of agar disk diffusion (ADD) tests were used in Minimum inhibition concentration (MIC) tests.

2.1.3. Cell cultures

NIH3T3 mouse healthy fibroblast cells (ATCC number CRL-1658™), MCF-7 (ATCC number HTB-22™), and MDA-MB-231 (ATCC number HTB-26™) were obtained from the American Type Culture Collection and used in MTT cell viability assays.

Table 1
Test organisms used in antimicrobial activity tests.

Species	Code	Type
Enterococcus faecalis	ATCC 2942	Gram + bacterium
Bacillus subtilis	ATCC 6633	Gram + bacterium
Staphylococcus aureus	ATCC 29213	Gram + bacterium
Staphylococcus epidermidis	ATCC 12228	Gram + bacterium
Escherichia coli	ATCC 05922	Gram - bacterium
Serratia marcescens	ATCC 8100	Gram - bacterium
Klebsiella pneumoniae	ATCC 13883	Gram - bacterium
Pseudomonas aeruginosa	ATCC 27853	Gram - bacterium
Candida albicans	ATCC 24433	Yeast
Candida krusei	ATCC 6258	Yeast
Candida parapsilosis	ATCC 22019	Yeast

2.2. Methods

Three different solvents (methanol, ethyl acetate, and hexane) were used to prepare bryophyte extracts. After the extraction step, all extracts are scanned to investigate the antimicrobial, antioxidant and antiproliferative properties. Agar Disc Diffusion (ADD) and Minimum Inhibition Concentration (MIC) methods were used to determine antimicrobial activity. DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) free radical method and MTT [(3,4,5-dimethylthiazol-2-yl)−2−5- diphenyltetrazolium bromide] assay were used for examining antioxidant and antiproliferative activity, respectively.

2.2.1. Preparation of bryophyte extracts

The defined bryophyte samples were washed 3 times with distilled water and dried at 50 °C for 24 h. The dried samples were pulverized with the help of a grinder. 10 gs of the prepared samples were weighed and extraction was carried out with 250 ml of methanol, ethyl acetate, and hexane solvents in the Soxhlet device for 24 h. The solvents used were evaporated in the Rotary evaporator device at 64 °C, 77 °C, and 69 °C for 30 min (until the solvent was fully removed), respectively (Mothana and Lindequist, 2005). The extracts obtained were stored at +4 °C to be used in studies.

2.3. Agar disc diffusion (ADD) method

Each of the bryophyte extracts was weighed 5 mg and dissolved in 1 ml of 20% dimethyl sulfoxide (DMSO) and stock lichen solutions with a concentration of 5 mg/ml (5000 ppm) were prepared. To test the antimicrobial activities of bryophyte extracts, the Agar Disc Diffusion (ADD) method (CLSI M02-A12, 2015) was applied first. Each microorganism used in the studies was activated for 18−24 h under optimum growth conditions in Mueller-Hinton Broth (MHB) and Sabouraud Dextrose broth (SDB) for bacteria and yeast cells, respectively. Absorbance values were measured in the spectrophotometer at 600 nm and the number of bacteria and yeast cells were adjusted to 10^6 and 10^5 CFU/ml for, respectively. The diluted bacteria and the yeasts (100 μl) were dropped into the Petri dishes containing Mueller-Hinton Agar (MHA), and Sabouraud Dextrose Agar (SDA) respectively, then inoculated with the smear sowing method using a swab.

Empty sterile discs were placed on the Petri plates at equal intervals and most 4 discs. 20 μl of stock bryophyte extract solutions were taken with a micropipette and absorbed into empty sterile discs with a diameter of 6 mm. As positive control discs Vancomycin (30 μg) for Gram (+) bacteria, Gentamicin (10 μg) for Gram (-) bacteria, and Fluconazole (5 μg) for yeast cells were used. Each microorganism was incubated for 18−24 h under its optimum growth conditions. At the end of the incubation, the zone diameters formed around the discs were measured with a millimeter ruler.

2.4. Minimum inhibition concentration (MIC) test

The Minimum Inhibition Concentration (MIC) test was performed by the microdilution method (CLSI M07-A9, 2012) using 96-well microplates. The wells were prepared to contain 100 μl volume in the final case. Using 5000 ppm stock bryophyte extract solutions, the bryophyte extract concentration in the first well was prepared to be 1250 μg/ml. Serial dilutions were made in a 1: 2 ratio and the concentrations of the next wells were adjusted to 625, 312.50, 156.25, 78.13, 39.06, 19.53, 9.77, and 4.88 μg/ml, respectively. The microbial suspension adjusted to a 0.5 McFarland standard was inoculated into wells. The same procedures were applied as the positive control, with Tetracycline for bacteria and Fluconazole for yeast cells. Each microorganism was incubated for 24 h following optimum growth conditions. Systems were measured at OD_{600} nm wavelength with a multi-well plate reader (Multiskan FC, Thermo Scientific, Massachusetts, USA). At the end of the incubation, the lowest extract

concentration at which growth was inhibited was determined as the MIC value. 96-well microplates were imaged using the G: BOX (Syngene, UK) imaging system with GeneSys software.

MHA and MHB for bacteria and SDA and SDB media were used for the growth of test microorganisms used in the antimicrobial experiments. All of the chemicals used in the experiments were purchased from Merck. All antimicrobial studies were carried out in three parallels with aseptic conditions, and their arithmetic averages were obtained.

2.5. Antioxidant activity tests

1,1-diphenyl-2-picryl hydrazyl (DPPH) free radical solution was used to determine the antioxidant activity properties of bryophyte extracts. In preparing the stock DPPH solution, 4 mg of DPPH was weighed and dissolved in 10 ml of ethanol (10−3 M) at 30 °C overnight in the dark with a magnetic stirrer. Butyl hydroxytoluene (BHT) was used as the standard antioxidant compound. Standard antioxidant compound and stock bryophyte extracts were finally prepared at concentrations of 0.10 mg/ml and their total volume was completed with ethanol to 3 ml. Finally, 1 ml of stock DPPH solution was added to each group. The systems were kept in a dark environment, at room temperature for 30 min, under 100 rpm shaking, and the values were measured at 517 nm wavelength in a spectrophotometer (Shahidi, 2015). As the antioxidant substances donate protons to DPPH, the absorbance at 517 nm decreases and the final absorbance value measured at this wavelength gives the amount of the remaining DPPH free radical (Blois, 1958). Using Eq. (1), the DPPH removal activity (%) of the samples was calculated.

$$\%\text{DPPHremoval} = [(A_C - A_S)/A_C]\text{x}100 \quad \text{(Eq. 1)}$$

In this equation; A_C is the absorbance value of control and A_S is the absorbance value of the sample.

In the experiments, the solution prepared by adding 1 ml of stock DPPH solution to 3 ml of ethanol was used as a control. Ethanol was used blindly while performing spectrophotometric measurements.

2.6. Cell culture and treatment

Cells were grown in RPMI 1640 medium supplemented with 2 mM L-glutamine, 10% fetal bovine serum, and 1% penicillin/streptomycin at 37 °C in a humidified incubator with a 5% CO_2 atmosphere. Compounds were dissolved in DMSO and diluted to working concentrations with a fresh medium. The control group (solvent control) was prepared with a medium containing 0.1% DMSO.

2.7. MTT cell viability assay

The viability of the cells was assessed by MTT [(3,4,5-dimethylthiazol-2-yl)−2−5- diphenyltetrazolium bromide] assay, which is based on the reduction of MTT by the mitochondrial dehydrogenase of intact cells to a purple formazan product. Yellow MTT is reduced to purple formazan in the mitochondria of living cells. This reduction takes place only when mitochondrial reductase enzymes are active, and therefore, the conversion is accepted as directly related to the number of viable (living) cells. In order to obtain IC_{50} concentrations of the samples, cell viability was determined by MTT assay. In short, cells were grown in 96-well plates at a density of 5×10^3 cells per well and subjected to different extract concentrations (500, 250, 125, 62.5, and 31.25 μg/mL). After 24 h incubation, MTT solution was added to wells to reach a final concentration of 0.5 mg/mL. The cells were incubated for another 4 h and then the current medium was removed and 100 μL of DMSO solution was added. The absorbance values were measured at 540 nm using a Cytation 3 Cell Imaging Multi-Mode Reader (Bio-Tek). Cell survival rates were expressed as the percentage of the DMSO (0.1%) solvent control and IC_{50} concentrations were calculated according to the analysis result.

2.8. Statistical analysis

The antimicrobial and antioxidant activity experiments were carried out in triplicate. SPSS 17 was applied to analyze the data and ANOVA (one-way) was used to compare average values. Correlation coefficients were calculated by using the Microsoft Excel package. The cytotoxicity results were also carried out in triplicate and statistical analysis was performed by SPSS v18.0. Graphics of MTT results were drawn using GraphPad Prism 6.0 software and statistically analyzed with one-way analysis of variance (ANOVA) and Tukey's post hoc test. Also using cell viability (%) data, IC50 values were calculated in Excel.

3. Results and discussion

3.1. ADD method assays

The results of the ADD test applied against Gram-positive bacteria species with bryophyte extract and Vancomycin (Bioanalyse VA 30) antibiotic disk impregnated in sterile empty discs (Bioanalyse BLK) are given in Fig. 1(a). According to the antibacterial activity results, it was observed that no inhibition zone was formed in the Petri dishes containing *E. faecalis, B. subtilis*, and *S. aureus* species, and therefore they were evaluated as having resistance to all used extracts (Fig. 1a). The zone diameters formed around the vancomycin (30 μg) disk used as positive control were measured as 20.33, 23.33, and 16.83 mm for the same bacterial species, respectively. On the other hand, there was no inhibition zone observed in the petri dish containing methanol extract against *S. epidermidis*. The inhibition zone formation was observed in the agar plates inoculating *S. epidermidis* with the extracts of ethyl acetate and hexane solvents (Table 2) and the zone diameter was measured as 7 mm around the Vancomycin (30 μg) disk used as the positive control.

The ADD assay results of bryophyte extracts prepared with all solvents against Gram (-) bacterial strains were given in Fig. 1(b). Gentamicin (10 μg) was used as the positive control discs in the assays. As a result of the ADD test applied against *C. albicans* and *C. krusei* species with fluconazole and 9 types of bryophyte extract-impregnated discs (Bioanalyse BLK), no zone diameter around the disk could be determined (Fig. 1c). As a result of the agar disk diffusion test applied against *C. parapsilosis* species in the same series of experiments, the zone diameter around the discs where bryophyte extracts were impregnated could not be determined.

The results of all the antibacterial assays were given in Tablo 2. As a result of the ADD test applied against *E. coli* with 3 types of bryophyte extract and Gentamicin (Bioanalyse CN 10), it was found that *E. coli* species were sensitive to all used bryophyte extracts. On the other hand, none of the extracts was as effective as the control disk. In the same series of experiments, it was observed that *S. marcescens* species were resistant to all used extracts and therefore no inhibition zone was formed. It had been determined that *K. pneumoniae* species were sensitive to all used extracts, and zone diameters of more than 7.5 mm have not been formed. Similarly, it was observed that *P. aeruginosa* species were sensitive to all extracts, while a maximum zone diameter of 6.5 mm was formed (Table 2). When Table 2 is examined, it is seen that Gram-positive bacteria tested are resistant to all bryophyte extracts except *S. epidermidis*. On the other hand, Gram-negative bacteria such as *E. coli, P. auroginosa*, and *K. pneumonia* seem to be sensitive to the extracts. Previously, Ilhan et al. (2006) showed that acetone extracts of a bryophyte species, *Palustriella commutata*, have more effective antibacterial activities on Gram-negative bacteria than the Gram-positive strains tested, using the ADD method in their study. The results of this study are similar to the results of the studies

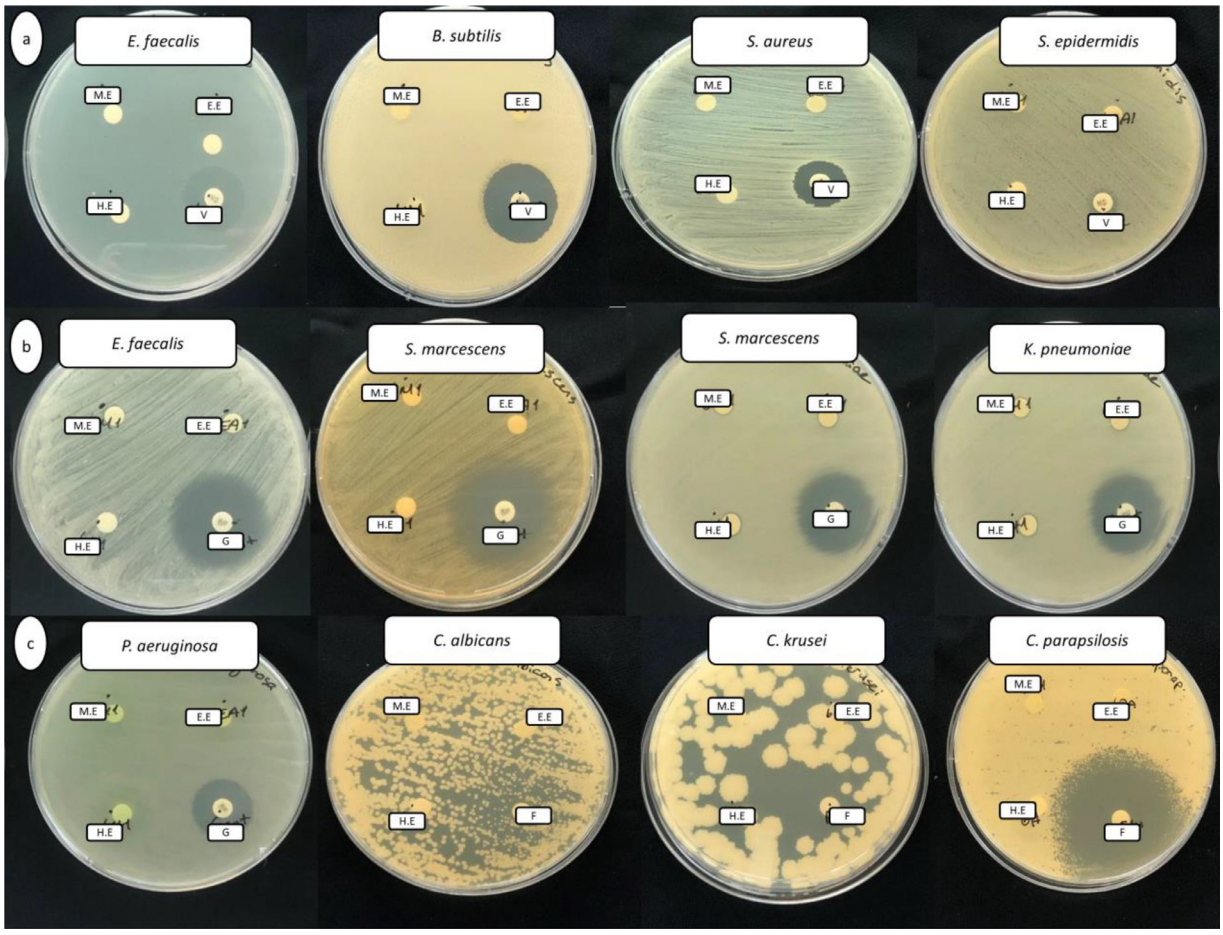

Fig. 1. **(a)** The images of ADD assay results; bryophyte extracts prepared with methanol (M.E), ethyl acetate (E.E), and hexane (H.E) solvents against Gram (+) bacterial strains and positive control discs 30 μg of Vancomycin (V); **(b)** The images of ADD assay results; bryophyte extracts prepared with methanol (M.E), ethyl acetate (E.E) and hexane (H.E) solvents against Gram (-) bacterial strains and positive control discs 10 μg of gentamicin (G); **(c)** The images of ADD assay results; bryophyte extracts prepared with methanol (M.E), ethyl acetate (E.E) and hexane (H.E) solvents against yeasts and positive control discs contained Fluconazole (F).

in the literature to determine the antimicrobial activities of different bryophyte species. As is known, traditional antibiotics are generally more active against Gram-positive bacteria than Gram-negative bacteria (Breijyeh et al., 2020). In this context, it is an interesting result that the bryophyte extract used in this study is more sensitive to Gram-negative bacteria. On the other hand, Savaroğlu et al. (2018) reported that a different bryophyte species, *Aulacomnium androgynum*, has antibacterial effects on both Gram-negative and Gram-positive bacteria. Similarly, in this study, ethanol and hexane extracts obtained from *Leucodon sciuroides* were found to have antibacterial effects on *S. epidermidis*, one of the Gram-positive bacteria tested. In fact, it is seen that this antibacterial effect is close to the effect of Gentamicin, which is used as a control antibiotic (Table 2). *Staphylococcus epidermidis*, one of the most important commensal microorganisms of human skin and mucosa, is also the cause of serious infections in immunocompromised patients, especially in association with the use of permanent medical devices that act as a scaffold for biofilm formation (Oliveira et al., 2021). A recent study reported that *S. epidermidis* is the most common cause of implant-related infections (Severn and Horswill, 2022). In addition, it has been reported that Staphylococcus epidermidis species show multidrug resistance properties (Aguila-Arcos et al., 2017). In this context, according to the results obtained from ADD tests, MIC experiments were carried out to examine in detail the effects of extracts against the *S. epidermis*.

Table 2
Zone Diameters (mm) Obtained by ADD Method (The bryophyte extracts using M.E: methanol; E.E: ethyl acetate; H.E: hexane; C*: Antibacterial agent used as positive control Vancomycin for Gr + and Gentamicin for Gr - bacteria).

	Microorganism	M.E	E.E	H.E	C*
		Zone diameter (mm)			
Gram (+)	*E. faecalis*	0.0	0.0	0.0	20.33
Gram (+)	*B. subtilis*	0.0	0.0	0.0	23.33
Gram (+)	*S. aureus*	0.0	0.0	0.0	16.83
Gram (+)	*S. epidermidis*	0.0	6.0	6.0	7.00
Gram (-)	*E. coli*	5.5	6.5	6.0	23.50
Gram (-)	*S. marcescens*	0.0	0.0	0.0	25.50
Gram (-)	*K. pneumoniae*	7.0	7.0	7.5	21.83
Gram (-)	*P. aeruginosa*	6.5	5.5	6.5	19.67

MIC experiments were continued using *S. aureus, S. epidermidis, E. coli*, and *K. pneumoniae* strains which were determined to be sensitive to bryophyte extracts as a result of ADD tests. Thus, the MIC values of the bryophyte extracts on the test organisms were determined. Due to the breeding morphology of species belonging to the genus *Candida* in solid media, the zone diameters could not be determined clearly (Fig. 1c). Therefore, MIC determination experiments have been applied for all *Candida* species.

3.3. Minimum inhibition concentration (MIC) test

The results of the MIC determination of bryophyte extracts (3 varieties) and tetracycline (positive control) against test bacterial species were given in Fig. 2. When the MIC values of the solutions

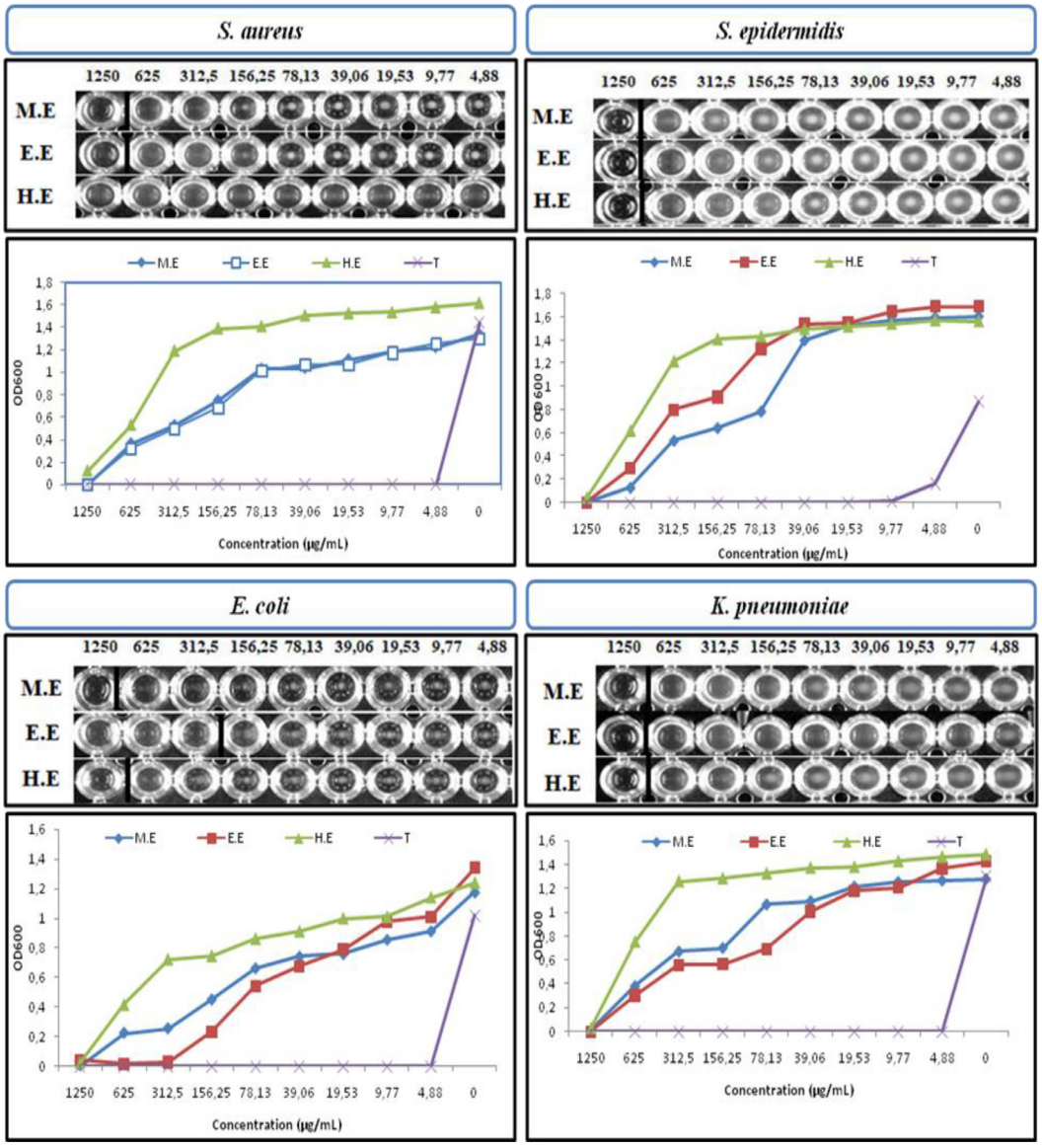

Fig. 2. Graphics of the antimicrobial activity of Bryophyte Extracts and Tetracycline (T) against test bacterial species by MIC assays and images of MIC tests (The bryophyte extracts using M.E: methanol; E.E: ethyl acetate; H.E: hexane).

were prepared by using extracts determined with methanol, ethyl acetate, and hexane solvents were examined; differently from the others, the MIC value of only ethyl acetate extract against *E. coli* was found 312.5 μg/ml (Fig. 2). The MIC values were 1250 μg/ml for other extracts against all test microorganisms (Fig. 2). It was determined that the MIC value of tetracycline used as a positive control in the experiments was less than 4.88 μg/ml. When the MIC results were evaluated, it was determined that the antimicrobial effect of bryophyte extracts against test bacterial organisms was low. Similarly, Cansu et al. (2013) examined the antibacterial effect of *Leucodon sciuroides* growing in Artvin, Turkey, and showed that the extract of the bryophyte dissolving in hexane had no antibacterial activity against *E. coli, P. aeruginosa, S. aureus, E. faecalis,* and *B. cereus.* Also, Vollár et al. (2018) reported that the n-hexane extract of *L. sciuroides* collected from Hungary showed no antibacterial effect against *S. aureus, S. epidermidis, B. subtilis, S. pneumonia,* and *S. pyogenes.* The results of antibacterial tests in the current study were compatible with the previous results in the literature.

The MIC test results of bryophyte extracts and yeast species belonging to the genus *Candida* are shown in Fig. 3. The MIC values of the methanol extract of bryophyte were 625 μg/ml for C. *albicans* and *C. parapsilosis* and 1250 μg/ml for *C. krusei*. The MIC values of the ethyl acetate extract of bryophyte were 625 μg/ml for *C. parapsilosis* and 1250 μg/ml for the other two species. The MIC values of the hexane extract for all test yeast species are 1250 μg/ml. As a positive control, the MIC values of fluconazole against *C. albicans, C. krusei,* and *C. parapsilosis* species were determined as 1250, 156.25, and 9.77 μg/ml, respectively. Similarly, Cansu et al. (2013), showed the antifungal activity of hexane extract of *L. sciuroides* against *C. albicans* depending on the presence of essential oils in the extract. Previously, Ertürk et al. (2015) reported that the ethanol extracts of *L. sciuroides* showed antifungal activity due to the attribution of active terpenoids presence in the extract. The MIC value of hexane extract of *L. sciuroides* obtained from Artvin, Turkey was found as 711 mg/ml, but in the current work the MIC value of methanol extract of *L. sciuroides* collected from Bilecik, Turkey was 625 mg/ml. The highest antifungal activity of the methanol extract of bryophyte for *C. albicans* indicates that this extract has the potential to be used in the synthesis of antifungal compounds for the treatment of *C. albicans* infections. When the antifungal activity results are examined, it is observed that the MIC method is more sensitive than the Agar Disk Diffusion method in determining the antifungal activity (Balouiri et al., 2016).

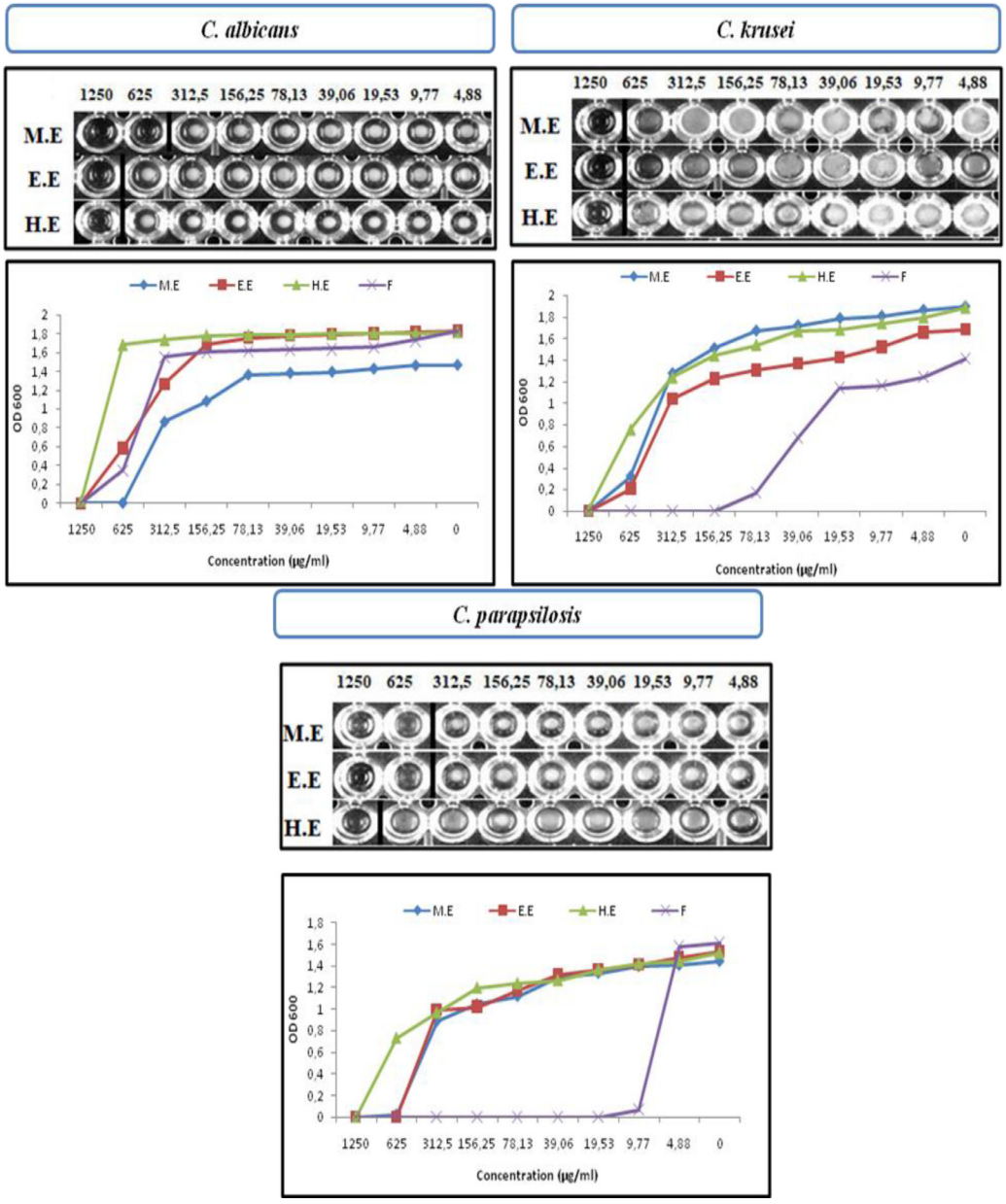

Fig. 3. Graphics of the antimicrobial activity of Bryophyte Extracts and Fluconazole (F) against test fungal species by MIC assays and images of MIC tests (The bryophyte extracts using M.E: methanol; E.E: ethyl acetate; H.E: hexane).

3.4. Antioxidant activity

DPPH removal activity (%) of all bryophyte extracts (0.10 mg/ml) was investigated in antioxidant activity tests. BHT was used as the standard antioxidant compound. The color changes in the experiments are quantitatively determined by spectrophotometric measurements and the calculations obtained from the results are given in Fig. 4.

When Fig. 4 was examined, it was determined that DPPH removal of methanol, ethyl acetate, and hexane extracts of the bryophyte were determined as 29.87%, 29.34%, and 28.27%, respectively.

3.5. Anticancer activity

Most of the recent studies focused on the cytotoxic effect of natural resources (Tan et al., 2017; Majolo et al., 2019). The results of cytotoxic effect experiments of extracts obtained from Bryophyte are given in Fig. 5. The results are expressed as mean ± standard deviation and $^{*}p < 0.5$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$ were significant compared to the negative control group.

IC_{50} values of Bryophyte ethyl acetate, methanol extracts, and doxorubicin (positive control) on MDA-MB-231, MCF-7 cancer, and 3T3 healthy cell lines were given in Table 3. The ethyl acetate and methanol extracts from the isolate of Bryophyte showed a higher antiproliferative effect against MCF-7 (IC_{50}: 51.16 and 98.17), MDA-MB-231 (IC_{50}: 20.21 and 15.47). However, the Bryophyte extracts could not 50% inhibit the healthy cell line (3T3) up to the maximum concentration, >500 μg/mL. Hexane extracts could not inhibit 3T3, MCF-7 and MDA-MB-231 cell lines.

Manoj et al. reported; methanolic and water extracts *Leucobryum bowringii Mitt.* inhibited cell proliferation at 10, 50, and 100 μg/mL concentrations on MCF-7 cell lines (Manoj et al., 2012). Vollar et al. studied the hexane and water extracts prepared from the *Leucodon sciuroides* species on T47D breast cancer cell lines. They used two concentrations, at 10 and 30 μg/mL. They showed a decrease in cell proliferation on the breast cancer cell line of 28.8% at 10 μg/mL and

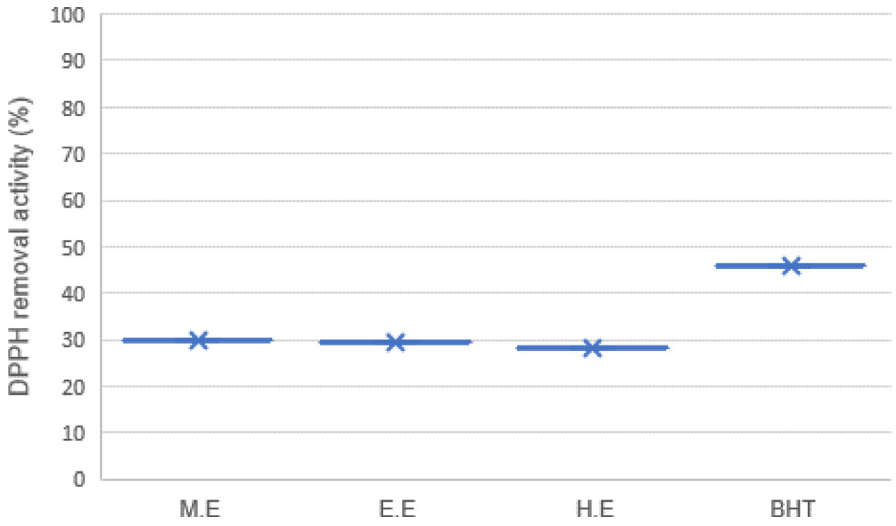

Fig. 4. DPPH removal activity (%) of bryophyte extracts(The bryophyte extracts using M.E: methanol; E.E: ethyl acetate; H.E: hexane).

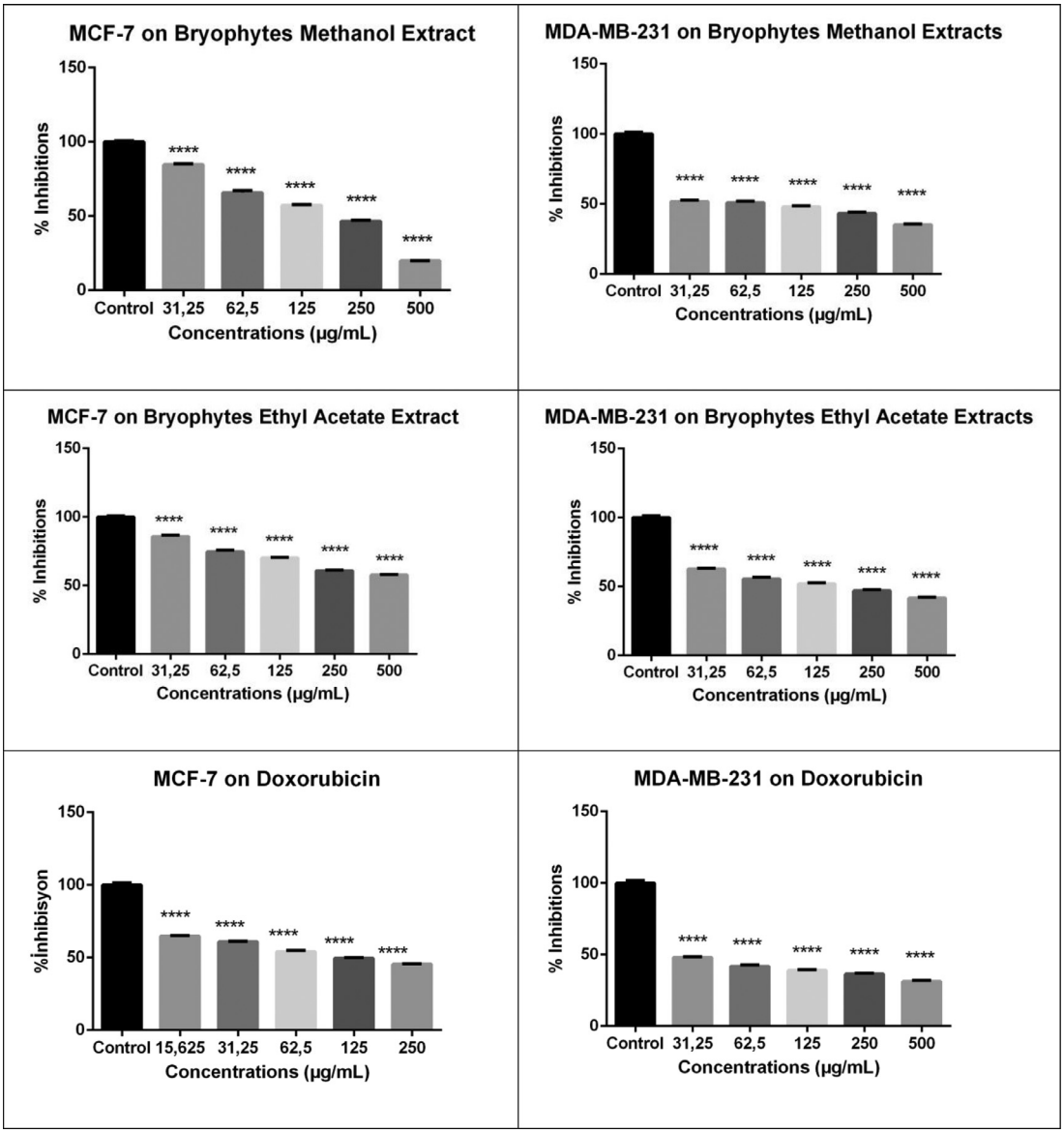

Fig. 5. Cytotoxic effects of the ethyl acetate, methanol extracts obtained from Bryophytes samples and doxorubicin (positive control) on MDA-MB-231, and MCF-7 cells at 24 h. (Control:% 0.1 DMSO containing medium; results are expressed as mean ± standard deviation; $n = 8$,; $p < 0.05^{*}$, $p < 0.01^{**}$, $p < 0.001^{***}$, $p < 0.0001^{****}$).

Table 3
IC50 values of different Bryophyte extracts including NIH3T3, MCF-7, and MDA-MB-231 cell lines.

IC50 value (μg/mL)	NIH3T3 (ATCC CRL-1658™)	MCF-7 (ATCC HTB-22™)	MDA-MB-231 (ATCC HTB-26™)
Bryophyte ethyl acetate	>500	51.16	20.21
Bryophyte methanol	>500	98.17	15.47
Bryophyte hexane	>500	>500	>500
Doxorubicin (positive control)	173.3	11.53	8.073

39.63% at 30 μg/mL. They observed that hexane extracts had no effect on cell proliferation in the same cell line. In our results, it was seen that hexane extracts did not cause a decrease in cell proliferation. There are very few studies in the literature about the Bryophyte species (*Leucodon sciuroides*) we have studied (Vallor et al., 2018).

4. Conclusion

It is known that bryophyte species have antimicrobial, antioxidant, and antiproliferative effects. However, few attempts have been made to test bryophytes against pathogenic strains. In addition, the bioactivity potentials of bryophytes vary from species to species. In this study, firstly the bryophyte species, which is abundantly observed and easily collected in Bilecik (Turkey), was defined. Since there is no information in the literature about the bioactive properties of the species, which was collected from this region and defined as *Leucodon sciuroides* (Leucodontaceae), the bioactive properties of this species were also examined in this study. For this purpose, extracts of bryophyte samples were obtained by using methanol, ethyl acetate, and hexane solvents. Then, ADD and MIC tests were performed to determine the antimicrobial activities of the extracts on Gram-positive and negative pathogen test organisms. DPPH free radical method and MTT assay were used for the determination of antioxidant and anticancer activities, respectively. As a result, the methanol extract of *L. sciuroides* showed high antifungal activity against *C. albicans*. Also, the ethyl acetate and methanol extracts of *L. sciuroides* had an antiproliferative effect on MCF-7 and MDA-MB-231 cells. The results of the current report indicated that the bryophyte species called *L. sciuroides* have antifungal and antiproliferative potential.

Supplementary material 1. The image of bryophyte sample: *Leucodon sciuroides*

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.sajb.2023.03.012.

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