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Investigation of Antimicrobial and Antioxidant Activities of some Lichens

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ABSTRACT

This study investigated the antimicrobial and antioxidant properties of methanol extracts from five lichen species: *Polycauliona candelaria* (Syn. *Xanthoria candelaria*), *Nephromopsis chlorophylla* (Syn. *Cetraria chlorophylla*), *Circinaria calcarea* (Syn. *Aspicilia calcarea*), *Bryoria capillaris* (Syn. *Alectoria cana*), and *Peltigera canina* (Syn. *Dermatodea canina*). Antimicrobial activities were determined using the agar disc diffusion method, while minimal inhibitory concentration (MIC) values were determined using the micro-well dilution method. Among the lichen species, methanol extract of *Circinaria calcarea* exhibited the highest antimicrobial activity, with a 15 mm zone of inhibition against *Escherichia coli* and *Bacillus cereus*. It also demonstrated the lowest MIC value (31.25 µg/mL) against *Bacillus cereus*, *Bacillus subtilis*, *Clostridium perfringens* and *Escherichia coli* were the most sensitive microorganisms to lichens. Various antioxidant determination methods were employed to assess the antioxidant activities of the lichens, including ferric ion (Fe^{+3}) reduction capacity, cupric ion (Cu^{+2}) reduction capacity, ferric ion reducing antioxidant power (FRAP), DPPH radical scavenging activity, DMPD radical scavenging activity and metal chelating activity using the bipyridyl reagent. All lichens exhibited excellent antioxidant activity, particularly in metal chelating activity using the bipyridyl reagent. *Peltigera canina* demonstrated the highest antioxidant activity among the studied lichen species across most of the applied method.

Keywords: Antimicrobial activity, Antioxidant activity, Lichen.

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Introduction

Lichens are symbiotic organisms of fungi and algae. These organisms that constitute the lichen form a new association with morphological and physiological features that are unlike themselves. These rootless, stemless, leafless organisms can live in the most difficult environmental conditions where algae and fungi cannot survive. They are common in almost every region of the world, from the Poles to the Equator, from the sea coast to the highest places in the mountains [1-5].

It is estimated that lichen symbiosis developed 400-600 million years ago [6]. It is stated that there are approximately 300 genera and 25,000 species of lichens in the world and more than 1800 of them are found in the flora of Turkey [4,7]. Various studies have stated that lichens were used for the treatment of diseases in ancient times [4,8]. Many types of lichens were believed to be effective against external burns, wounds, asthma, colds, tuberculosis, gastritis and other diseases [3,9]. Although lichens are occasionally used as food by humans, most lichen species are known to produce toxic substances. For this reason, it is recommended not to use lichens unconsciously [9].

Scientists studying lichen chemistry have found over 1050 compounds in lichens that are thought to be secondary metabolites. There are polyketide-derived aromatic compounds such as depsids, depsidons, dibenzofurans, xanthones and some other compounds such as pulvinic acid, esters, terpenes, steroids, among these compounds [2,3,5,10,11]. Lichens are evaluated as an important material in the fields of food, medicine and industry with these substances which they form as a result of their metabolic activities [3,5].

It has been documented in the literature that lichens and their metabolites have antibacterial, antifungal, antiviral, antiprotozoal, antitumor, anti-inflammatory, antipyretic, analgesic, antiallergic, antibiotic, cytotoxic effects [3,5,10,12,13].

Lichens contain phenolic compounds such as depsids, depsidones and dibenzofurans [5,10]. It has been stated in different studies that the antimicrobial activity of lichens comes from these compounds and their derivatives, lichen metabolites with acid character. As a matter of fact, it has been determined that lichen metabolites such as evernic acid (from the Depsid group), physodic acid, lobaric acid, fumarprotocetraric acid (from the Depsidon group), usnic acid (from the Dibenzofuran derivatives), protolicesterinic acid, pulvinic acid (from the Aliphatic acids) have high antimicrobial effects [3,9].

Numerous studies have highlighted the relationship between the antioxidant activities of lichens and the presence of phenolic compounds within their structures [12]. Literature has consistently reported that lichens harbor diverse secondary lichen substances known for their potent antioxidant properties, primarily attributed to their phenolic groups [2]. Specifically, certain depsides like atranorin (isolated from *Placopsis sp.*) and divaricatic acid (isolated from *Protousnea malacea*), as well as depsidones like pannarin (isolated from *Psoroma pallidum*) and 1'-chloropannarin (isolated from *Erioderma chilense*), have demonstrated notable antioxidant activity Furthermore, the extract of *Umbilicaria antarctica* has emerged as the most effective antioxidant in scavenging free radicals and superoxide anions, with lecanoric acid identified as the primary active compound [2]. These findings substantiate the notion that the remarkable antioxidant potential of lichens can be attributed to the presence of various phenolic compounds, which enable effective scavenging of harmful free radicals [3].

On the other hand, although synthetic antioxidants have been used instead of natural antioxidants in recent years, their use has been restricted or prohibited based on research results showing that they have been toxic and cancer-causing [2,3,8]. Reasons such as high side effects and multi-drug resistance of pathogenic microorganisms due to excessive use of antibiotics limit the use of synthetic drugs. This situation increases the interest in herbal-derived natural medicines with antimicrobial and antioxidant properties day by day. At the same time, researches in this direction on plants are constantly on the agenda. The use of plants for medicinal purposes is in demand all over the world as well as in our country. Among the reasons for this are that, unlike synthetic drugs, herbal medicines have much fewer side effects and are more easily obtainable and have several beneficial effects [5,8,14-16].

In this study, our aim to determine the in vitro antimicrobial and antioxidant activities of some lichens (*Polycauliona candelaria*, *Nephromopsis chlorophylla*, *Circinaria calcarea, Bryoria capillaris, Peltigera canina*) .

Materials and Methods

Collection and Identification of Lichens

In this study, we examined five lichen species. Lichen samples were collected by Prof. Dr. Ali ASLAN from the Oltu district of Erzurum province on 15 August 2011 and identified by using various flora books [17-20]. The lichen species investigated were as follows: Circinaria calcarea (L.) A. Nordin, Savić & Tibell (Syn. Aspicilia calcarea (L.) Körb), ATA-KKEF-772; Bryoria capillaris (Ach.) Brodo & D. Hawksw (Syn. Alectoria cana (Ach.) Leight), ATA-KKEF-771; Nephromopsis chlorophylla (Willd.) Divakar, A. Crespo & Lumbsch (Syn. Cetraria chlorophylla (Willd.) Poetsch;), ATA-KKEF-773; Peltigera canina (L.) Willd (Syn. Dermatodea canina (L.) A. St.-Hil.), ATA-KKEF-774; Polycauliona candelaria (L.) Frödén, Arup & Søchting (Syn. Xanthoria candelaria (L.) Th. Fr.), ATA-KKEF-775. Identified lichen samples were kept in herbarium of The Faculty of Pharmacy, Van Yüzüncü Yıl University.

Microorganisms

Microorganisms were obtained from Erzurum Provincial Health Directorate Public Health Laboratory and Atatürk University, Science Faculty, Biology Department. A total of 12 microorganisms (11 bacterial species and 1 fungus species) including, *Bacillus cereus* (ATCC 10876),*Clostridium perfringens* (Etlik Vet.5-10-7), *Escherichia coli* (ATCC 11229), *Enterococcus feacalis* (ATCC 29212),), *Klebsiella oxytoca* (ATCC 43086), *Listeria monocytogenes* (ATCC 7677), *Proteus mirabilis* (ATCC 15146), *Staphylococcus aureus* (ATCC 25923-12), *Streptococcus pyogenes* (ATCC 19615), *Bacillus subtilis* (ATCC 6633), *Salmonella enteritidis* (Clinical), *Candida albicans* (ATCC 60193)were used.

Preparation of Lichen Extracts

Lichens were dried and ground into powder. 10 g of each lichen was taken and extracted with 250 mL of methanol in a Soxhlet device (Soxhlet extractor (Isopad, Heidelberg, Germany)) for 72 hours. The mixture was filtered (with Whatman filter paper no: 1) and the solvent was removed in a rotary evaporator (Rotary evaporator, Buchi Labortechnic AG, Flawil, Switzerland) at 40°C. The obtained lichen extracts were stored at +4°C until use [12,21,22].

Antimicrobial Activity

Disc-diffusion method

The Agar disc diffusion method was applied to determine antimicrobial activity. 10 mg/mL solutions were prepared by dissolving 10 mg of each lichen extract in 1 mL of dimethyl sulfoxide (10% DMSO). Amounts of 30 µl (300µg/disc) taken from this solution were absorbed into each (6 mm diameter blank discs, Oxoid). The antibiotics [OFX: Ofloxacin (10 µg/disc), SCF: Sulbactam (30 µg) + Cefoperazona (75 µg) = (105µg/disc), NOV: Novobiocin (30 µg/disc) for bacteria, NYS: Nystatin (30µg/disc) for fungi] were used as a positive controls. Only discs impregnated with solvent (10% DMSO) were used as negative controls. Colonies taken with an inoculating loop from 18-24 hour pure cultures of microorganisms grown on the solid media plates were suspended in phosphate-buffered saline (PBS). The dilutions were prepared to be 10^8 CFU/mL according to McFarland turbidity standard (No.0.5). The samples taken from these dilutions using sterile cotton swab sticks were spread over the surface of proper agar plates (Nutrient Agar (NA,, Oxoid) for bacteria and Potato Dextrose Agar (PDA, Oxoid) for fungi) Then the absorbed discs were placed on the inoculated agar plates. Bacteria were incubated at 37°C for 24 -48 hours and fungi at 30°C for 48 hours. The antimicrobial activities of the lichens were evaluated according to the diameters of the inhibition zone that they were formed against the test microorganisms. The diameters of the inhibition zones

formed as a result of incubation were measured in a millimeter ruler. All the assays were performed in duplicate. [15,22,23]

Minimum Inhibitory Concentration (MIC)

MIC values of methanol extracts of 3 lichen species (*Nephromopsis chlorophylla*, *Circinaria calcarea*, *Bryoria capillaris*) which were found to be effective in terms of antimicrobial activity values were determined by modifying the microwell dilution method [30,31]. 100 µl of medium (Nutrient Broth, Oxoid) was poured into each well of 96-well ELISA microplates for this purpose.

On the other hand, 500 µg/mL dilutions of lichen extracts with dimethyl sulfoxide (10% DMSO) were prepared and 100 µl volume taken from here was transferred to the first well. Then, it was transferred volume of 100 µl from one well to the other. Thus it was prepared serial dilutions that the first well was 250 µg/mL and the other wells were at concentrations of 125 µg/mL, 62.5 µg/mL, 31.25 µg/mL, 15.625 µg/mL, 7.8125 µg/mL, respectively.

The same procedures were applied for Maxipine (µg/mL) antibiotic used as a positive control. DMSO solution was used as a negative control. Colonies taken with an inoculating loop from 18-24 hour pure cultures of microorganisms grown on the solid media plates were suspended in phosphate-buffered saline (PBS). The dilutions were prepared to be 10^8 CFU/mL according to McFarland turbidity standard (No.0.5). 5 µl was inoculated into each well with a micropipette from these dilutions. Then the plates were incubated for 24 hours at 37 °C in a shaker incubator. The lowest essential oil concentration without growth of test microorganisms was evaluated as MIC [15, 22-24]

Antioxidant Activity

Fe^{3+} reducing power activity

The Fe^{3+} reducing power activity of the lichen extracts was determined following the method described by Oyaizu [25]. Firstly, stock solutions of lichen extracts and standard antioxidant compounds (BHA, BHT, α-Tocopherol, and Trolox) were prepared at a concentration of 1 mg/mL. From these stock solutions, different concentrations (10, 20, and 30 µg/mL) were prepared by transferring the appropriate volume (1 mL) to test tubes and diluting with distilled water.

To each tube, 2.5 mL of phosphate buffer (0.2 M, pH 6.6) and 1% $K_3Fe(CN)_6$ were added sequentially, followed by incubation at 50°C for 20 minutes. Subsequently, 2.5 mL of 10% trichloroacetic acid (TCA) was added to the reaction mixture. From the upper phase of the solution, 2.5 mL was taken and mixed with 2.5 mL of distilled water and 0.5 mL of 0.1% $FeCl_3$. The absorbance of the resulting solution was measured at 700 nm against the blank sample. The blank sample consisted of distilled water.

In the control tube, no sample (lichen extracts or standard antioxidant compounds) was added, and the volume was made up with distilled water. Standard antioxidant compounds such as BHA, BHT, α-tocopherol, and trolox were used as positive controls. In this bioanalytical method commonly used in antioxidant studies, the yellow color of the test solution changes to various shades of green due to the reducing activities of the antioxidant substances present in the environment [25]. An increase in absorbance of the reaction mixture indicates an enhancement in the reduction capability.

Fe^{3+} reducing power activities of methanol extracts of the lichens used in the study was determined by measuring the absorbance of solutions of 10,20,30 µg/mL concentrations of the lichens at 700nm. Ferric ions (Fe^{3+}) reducing powers activities of the lichens and standard antioxidant compounds were compared with each other at this concentrations (10,20,30 µg/mL).

Cu^{2+} reducing power-CUPRAC assay

In this method, equal volumes (0.25 mL) of $CuCl_2$ solution (0.01 M), ethanolic neocuproine solution (7.5x10-3 M), and CH_3COONH_4 buffer solution (1 M) were added to test tubes containing lichen extracts at concentrations of 10, 20, and 30 µg/mL, respectively. The reaction mixtures were allowed to incubate for 30 minutes, after which the absorbance values were measured at 450 nm [26].

FRAP Assay

Solutions of lichen extracts and standard antioxidant compounds at concentrations of 10, 20 and 30 µg/ml were placed in test tubes. The volumes were made up to 0.5 mL with buffer solution. Afterward, the same volume (2250 µl) of $FeCl_3$ solution (20 mM) and FRAP reagent were added to the test tubes, respectively and the volume was completed to 5 mL. The mixture in the test tubes was homogenized using a vortex (tube mix). After about 10 minutes, absorbance values were measured at 593 nm wavelength [27].

Bipyridyl ferrous ions (Fe^{2+}) chelating activity

In this antioxidant activity determination method, firstly, $FeSO_4$ solution (0.25 mL, 2 mM) was placed in test tubes. 0.12 mL of lichen extracts or standard antioxidant compounds were added to the solution. Then, 1.5 mL of bipyridyl solution (0.2%) dissolved in 1 mL of Tris-HCl buffer (pH: 7.4) and HCl (0.2 M) were added, respectively. The absorbance of the solution was then measured spectrophotometrically at 562 nm [27].

DPPH· radical scavenging activity

In the method employed to determine antioxidant activity, a solution of 10^{-3}M 2,2-diphenyl-1-picrylhydrazyl (DPPH•) was used as the free radical. Test tubes were prepared with solutions of lichen extracts and standard antioxidant compounds at concentrations of 10, 20, and 30 µg/mL, with the total volume adjusted to 3 mL using ethanol. Subsequently, 1 mL of the stock DPPH• solution was added to each sample tube. The tubes were then incubated at room temperature, shielded from light, for 30 minutes. After the incubation period, the absorbance values were measured at a

wavelength of 517 nm. The decrease in absorbance indicates the amount of DPPH• solution remaining, thereby reflecting the scavenging activity of the samples against the free radical [28].

DMPD radical scavenging activity

In this antioxidant activity determination method, colored radical cation (DMPD•+) was obtained in the first step. 105 mg of DMPD was dissolved in 5 mL of distilled water to prepare 100 mM DMPD solution. Then, 1 mL of the prepared DMPD solution was added to 100 mL of phosphate buffer (0.1 M and pH 5.3). Finally, 0.2 mL of 0.05 M $FeCl_3$ was added. Measurements were made at 505 nm for 1 mL of this solution. Before using the DMPD radical solution, the absorbance value of the control solution was adjusted to 0.900±0.100 nm at 505 nm wavelength with phosphate buffer (0.1 M and pH 5.3). Then, solutions (10, 20, 30 µg/mL) prepared from lichen extracts and standard antioxidant compounds were placed in test tubes and the volume was made up to 0.5 ml with distilled water. Thereupon, DMPD (1ml) solution was added. After waiting for 50 minutes, absorbance values were measured at 505 nm wavelength [29].

Results

Antimicrobial Activities of Lichens

Disc- diffusion method

Metanol extracts of 5 lichen species used in our research were determined antimicrobial activities against 11 bacteria and 1 fungus species and the results are shown in Table 1. Methanol extracts of *C. calcarea*, *N. chlorophylla*, *B. capillaris* lichen species were determined that they showed antimicrobial activity against *B. cereus*, *B. subtilis, C. perfringens* ve *E. coli* with diameter of inhibition zone varying between 9-15 mm. it was determined that *C. calcarea* lichen species among these lichen species showed maximum antimicrobial with diameter of inhibition zone 15 mm against *E. coli* and *B. cereus* bacteria.

Table 1. Antimicrobial activities of methanol extracts of lichens against test microorganisms (Disc Diffusion Method)

Lichens	Diameter of inhibition zone (mm)											
	Proteus mirabilis (ATCC 15146)	*Staphylococcus aureus* (ATTC 25923-12)	*Escherichia coli* (ATCC 11229)	*Bacillus cereus* (ATCC 10876)	*Bacillus subtilis* ATCC 6633	*Clostridium perfringens* (Etlik Vet.5-10-7)	*Enterococcus fecalis* (ATCC 29212)	*Salmonella enteritidis* (Klinik)	*Streptococcus pyogenes* (ATCC 19615)	*Klebsiella oxytoca* (ATCC 43086)	*Listeria monocytogenes* (ATCC 7677)	*Candida albicans* (ATCC 60193)
Polycauliona candelaria	-	-	-	-	-	-	-	-	-	-	-	-
Nephromopsis chlorophylla	-	-	13	10	12	10	-	-	-	-	-	-
Circinaria calcarea	-	-	15	15	10	13	-	-	-	-	-	-
Bryoria capillaris	-	-	13	9	10	9	-	-	-	-	-	-
Peltigera canina	-	-	7	-	-	-	-	-	-	-	-	-
Negative control	-	-	-	-	-	-	-	-	-	-	-	-
Positive control (Antibiotics)	34 (OFX)	21 (SCF)	32 (OFX)	25 (NOV)	28 (OFX)	24 (OFX)	28 (NOV)	25 (OFX)	28 (OFX)	23 (OFX)	25 (SCF)	18 (NYS)

Antibiotics: OFX=Oflaxacin (10 µg/disc), SCF=Sulbactam (30 µg) + Cefoperazona (75 µg)= (105 µg/disc), NYS= Nystatin (30µg/disc) ve NOV= Novobiocin (30 µg/disc)
(-):No inhibition.

It was observed that the extract of the *P. canina* lichen species was effective only against *E. coli* bacteria with a 7 mm diameter of inhibition zone, while the extract obtained from the *P. candelaria* species did not show antimicrobial activity against any of the microorganisms studied.

E. coli, *B. subtilis*, *B. cereus*, *C. perfringens* was understood to be the most sensitive microorganism species against methanol extracts of lichen species in our study. It was determined that none of the methanol extracts of lichens showed antimicrobial activity against *E. feacalis*, *K. oxytoca*, *L. monocytogenes*, *S. pyogenes, S. enteritidis*, *P. mirabilis*, *S. aureus, C. albicans* microorganism species. Negative control discs prepared by impregnation with DMSO alone showed no antimicrobial effect on any of the test microorganisms.

Minimum Inhibitory Concentration (MIC)

MIC values of methanol extracts of *N. chlorophylla, C. calcarea, B. capillaris* lichen species which were effective in terms of antimicrobial activity among the 5 lichen species in our study were determined and the results were shown in Tables 2,3,4.

Among these 3 lichen species, methanol extract of *C. calcarea* lichen species showed the best (lowest) MIC value (31.25 µg/mL) against *E. coli* bacteria species.

Table 2. Antimicrobial activities and MIC values of *Nephromopsis chlorophylla* against test microorganisms

Microorganisms	*N. chlorophylla*		Antibiotics	
	[I]DD	[I]MIC	[a]DD	[a]MIC (Maxipime)
Enterococus fecalis	-	NT	28 (NOV)	125
Proteus mirabilis	-	NT	34(OFX)	125
Streptecoccus pyogenes	-	NT	28(OFX)	62.5
Klebsiella oxytoca	-	NT	23 (OFX)	125
Staphylococcus aureus	-	NT	21(SCF)	62.5
Escherichia coli	13	62.5	32(OFX)	31.25
Bacillus cereus	10	125	25(NOV)	125
Bacillus subtilis	12	250	28 (OFX)	125
Salmonella enteritidis	-	NT	25(OFX)	125
Clostridium perfringens	10	125	24(OFX)	125
Listeria monocytogenes	-	NT	25(SCF)	62.5
Candida albicans	-	NT	18 (NYS)	NT

[a]DD: Agar disc diffüsion method, OFX=Oflaxacin (10 µg/disc), SCF=Sulbactam (30 µg) + Cefoperazona (75 µg) = (105 µg/disc), NYS= Nystatin (30 µg/disc) ve NOV= Novobiocin (30 µg/disc) standard antibiotic discs were used (Oxoid).

[I]DD:, Disc diffusion method, diameters of inhibition zone (mm) formed against test microorganisms by methanol extracts of lichens (300g/disc)

[a]MIC: Minimum inhibitory concentration, standard antibiotic, Maxipime (µg/mL).

[I]MIC: Minimum inhibitory concentration formed against test microorganisms by methanol extracts of lichens (µg/mL)

(-): Inhibition zone not formed.

NT: Not Tested.

Table 3. Antimicrobial activities and MIC values of *Bryoria capillaris* against test microorganisms.

Microorganisms	*B. capillaris*		Antibiotics	
	[I]DD	[I]MIC	[a]DD	[a]MIC (Maxipime)
Enterococus fecalis	-	NT	28 (NOV)	125
Proteus mirabilis	-	NT	34(OFX)	125
Streptecoccus pyogenes	-	NT	28(OFX)	62.5
Klebsiella oxytoca	-	NT	23 (OFX)	125
Staphylococcus aureus	-	NT	21(SCF)	62.5
Escherichia coli	13	62.5	32(OFX)	31.25
Bacillus cereus	9	125	25(NOV)	125
Bacillus subtilis	10	125	28 (OFX)	125
Salmonella enteritidis	-	NT	25(OFX)	125
Clostridium perfringens	9	250	24(OFX)	125
Listeria monocytogenes	-	NT	25(SCF)	62.5
Candida albicans	-	NT	18 (NYS)	NT

[a]DD: Agar disc diffüsion method, OFX=Oflaxacin (10 µg/disc), SCF=Sulbactam (30 µg) + Cefoperazona (75 µg) = (105 µg/disc), NYS= Nystatin (30 µg/disc) ve NOV= Novobiocin (30 µg/disc) standard antibiotic discs were used (Oxoid).

[I]DD:, Disc diffusion method, diameters of inhibition zone (mm) formed against test microorganisms by methanol extracts of lichens (300g/disc)

[a]MIC: Minimum inhibitory concentration, standard antibiotic, Maxipime (µg/mL).

[I]MIC: Minimum inhibitory concentration formed against test microorganisms by methanol extracts of lichens (µg/mL)

(-): Inhibition zone not formed.

NT: Not Tested.

Table 4. Antimicrobial activities and MIC values of *Circinaria calcarea* against test microorganisms.

Microorganisms	*C.calcarea*		Antibiotics	
	[I]DD	[I]MIC	[a]DD	[a]MIC (Maxipime)
Enterococus fecalis	-	NT	28 (NOV)	125
Proteus mirabilis	-	NT	34 (OFX)	125
Streptecoccus pyogenes	-	NT	28(OFX)	62.5
Klebsiella oxytoca	-	NT	23 (OFX)	125
Staphylococcus aureus	-	NT	21(SCF)	62.5
Escherichia coli	15	31.25	32(OFX)	31.25
Bacillus cereus	15	62.5	25(NOV)	125
Bacillus subtilis	10	125	28 (OFX)	125
Salmonella enteritidis	-	NT	25(OFX)	125
Clostridium perfringens	13	250	24(OFX)	125
Listeria monocytogenes	-	NT	25(SCF)	62.5
Candida albicans	-	NT	18 (NYS)	NT

[a]DD: Agar disc diffüsion method, OFX=Oflaxacin (10 µg/disc), SCF=Sulbactam (30 µg) + Cefoperazona (75 µg) = (105 µg/disc), NYS= Nystatin (30 µg/disc) ve NOV= Novobiocin (30 µg/disc) standard antibiotic discs were used (Oxoid).

[I]DD:, Disc diffusion method, diameters of inhibition zone (mm) formed against test microorganisms by methanol extracts of lichens (300g/disc)

[a]MIC: Minimum inhibitory concentration, standard antibiotic, Maxipime (µg/mL).

[I]MIC: Minimum inhibitory concentration formed against test microorganisms by methanol extracts of lichens (µg/mL)

(-): Inhibition zone not formed.

NT: Not Tested.

Antioxidant activities of lichens

Fe^{3+} reducing power activity

As a result of the application of this antioxidant activity determination method, the yellow color of the test solution changes to green in different shades in the presence of antioxidant compounds. This is due to the reducing activities of antioxidant compounds. The reducing capacity of lichen extracts increases in direct proportion to the increasing concentration values. The reducing potential of lichen extracts was determined by measuring the absorbance of sample solutions at different concentrations (10, 20, 30 µg/mL) at 700 nm wavelength.

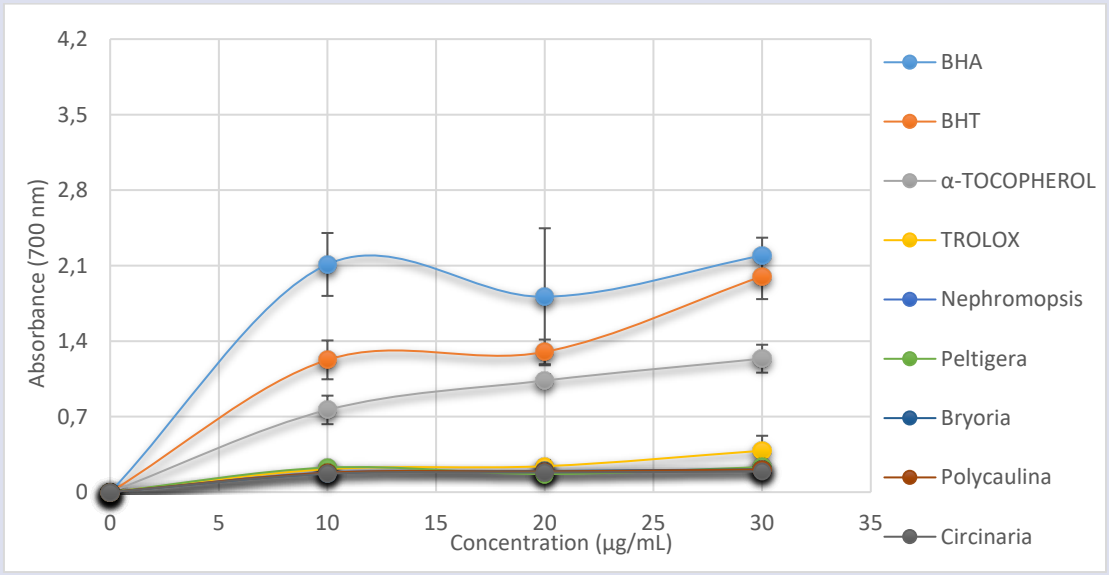

Figure 1. Fe^{3+} reducing activities of lichens (10-30 µg/mL)

The graph showing the reducing capacity of ferric ions (Fe^{3+}) to ferrous ions (Fe^{2+}) of lichen extracts is given in Figure 1. The absorbance values corresponding to 30 µg/mL for standard antioxidant compounds and lichen extracts were shown in Table 5. High absorbance values shown in Table 5 indicate high reducing capacity. For this antioxidant activity determination method, lichen extracts and standard antioxidants were compared with each other at a concentration of 30µg/mL.

As a result, lichens and the standard antioxidant compounds showed Fe^{3+} reducing power activity respectively to be BHA > BHT > -Tokoferol > Troloks > *P. canina*> *B. capillaris* > *P. candelaria* > *N. chlorophylla* > *C. calcarea.*

Cu^{2+} reducing power-CUPRAC assay

This assay is based on the measurement of the absorbance of a complex that results from the reaction of antioxidant with Cu^{2+}-neocuproine reagent. It was found that the reducing capacity of cupric ions (Cu^{2+}) to cuprous ions (Cu^{+}) of lichen extracts increased in direct proportion to the concentration. This reducing capacity of lichen extracts was determined by measuring the absorbance values of solutions of lichen extracts at different concentrations (10, 20, 30 µg/mL) at a wavelength of 450 nm. The graphs showing the cupric ions (Cu^{2+}) reduction results of lichen extracts and standard antioxidant compounds were shown in Figure 2, and the absorbance values corresponding to 30µg/ml for standard antioxidant compounds and lichen extracts were shown in Table 5.

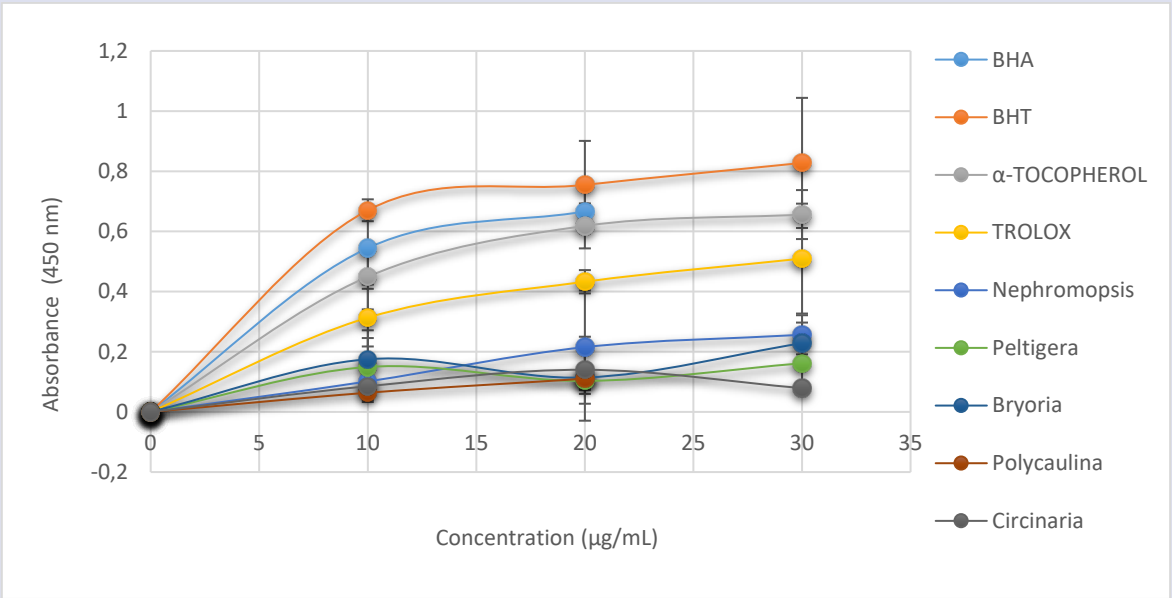

Figure 2. Cu^{2+} reducing activities of lichens (10-30 µg/mL)

The order of antioxidant activity of lichen extracts and standard antioxidants at a concentration of 30µg/ml as a result of this method was as follows; BHT> BHA> α-Tokoferol > Trolox > *N. chlorophylla* > *B. capillaris* > *P. candelaria* > *P. canina*> *C. calcarea*.

FRAP Assay

In the FRAP method, ferric ions (Fe^{3+}) are reduced to ferrous ions (Fe^{2+}). The ferrous (Fe^{2+}) ions formed form a blue complex with Tripyridyl triazine (TPTZ). This blue complex gives the maximum absorbance value at 593 nm wavelength. It was determined that the reducing capacity of lichen extracts, according to the FRAP method, increased in direct proportion to the concentration. The graphs of lichen extracts and standard antioxidant compounds showing the results of the FRAP method were shown in Figure 3 and the absorbance values corresponding to 30µg/mL for standard antioxidant compounds and lichen extracts were shown in Table 5. For this antioxidant activity determination method, when lichen extracts were compared with standard antioxidant compounds at a concentration of 30µg/mL; It was determined that there was a sequence as BHA>α-Tokoferol>BHT> *P. canina* > *B. capillaris* > *N. chlorophylla* > *P.candelaria* > *C.calcarea* > Troloks

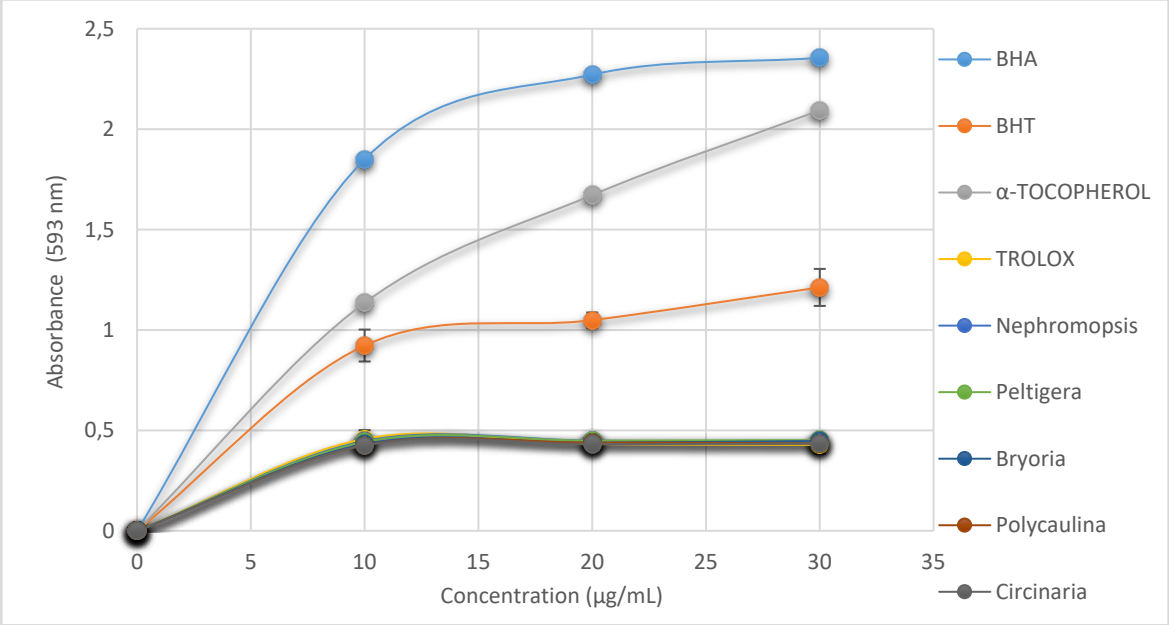

Figure 3. Ferric reducing activities (FRAP) of lichens (10-30 µg/mL)

Table 5. Absorbance values of lichens (30µg/mL) obtained from different antioxidant activity determination methods (Reducing capacity). Standard antioxidant compounds (BHA,BHT, α-Tocopherol, Trolox)

Antioxidants	Fe^{3+}reducing power activity (700nm)	Cu^{2+} reducing power (450nm)	FRAP Assay (593nm)
Control	0	0	0
BHA	2,194	0,691	2,355
BHT	2,001	0,828	1,212
α-Tokoferol	1,237	0,656	2,094
Troloks	0,384	0,510	0,428
Nephromopsis chlorophylla	0,210	0,256	0,440
Peltigera canina	0,232	0,162	0,452
Bryoria capillaris	0,212	0,228	0,447
Polycauliona candelaria	0,211	0,179	0,435
Circinaria calcarea	0,191	0,079	0,433

DPPH radical scavenging activity

Calculations related to the DPPH free radical scavenging activity were made according to the following equation

$$\text{DPPH}\cdot \text{ scavenging effect } (\%) = \left(\frac{\text{Acontrol} - \text{Asample}}{\text{Acontrol}}\right) \times 100$$

A sample is the absorbance value found after the addition of DPPH· solution to lichen extracts or standart antioxidants. A control is the absorbance value of the control value containing only DPPH solution.

The graphs showing the DPPH radical scavenging activity results of lichen extracts and standard antioxidant compounds were shown in Figure 4.

DPPH radical scavenging activities of lichen extracts increase in direct proportion to the concentration, as can be seen in Figure 4. Absorbance values of lichen extracts and standard antioxidant compounds at 30 µg/mL concentrations were shown in Table 6.

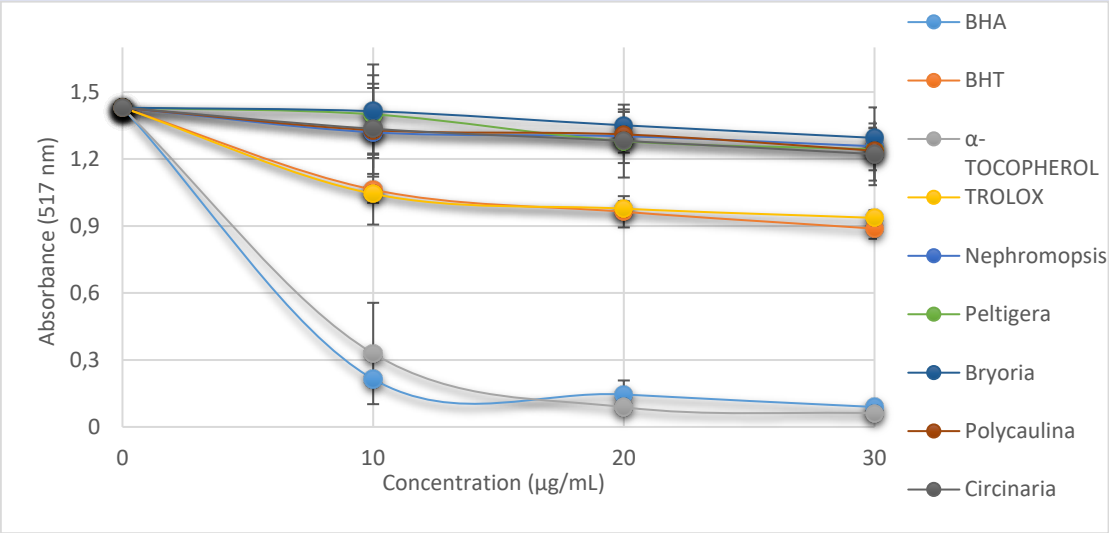

Figure 4. DPPH radical scavenging activities of lichens (10-30 µg/mL).

Lichen extracts and standard antioxidant compounds showed DPPH radical scavenging activity, respectively, as to be α-Tocopherol > BHA > BHT > Trolox > *C. calcarea* > *P. candelaria* > *P. canina* > *N.chlorophylla* > *B. capillaris* at 30µg/mL concentration.

DMPD Scavenging Activity

Calculations related to the DMPD free radical scavenging activity were made according to the following equation

$$\text{DPMD} \cdot \text{ scavenging effect } (\%) = \left(\frac{Acontrol - Asample}{Acontrol}\right) \times 100$$

A sample is the absorbance value found after the addition of DMPD solution to lichen extracts or standart antioxidant. A control is the absorbance value of the control value containing only DMPD solution.

Some standard antioxidant compounds (BHA and Trolox) were used as a positive control. As a matter of fact, BHT and α-Tocopherol, which are among the standard antioxidant compounds, do not show activity in DMPD radical scavenging activity. The graphs showing the results of this antioxidant activity determination method of lichen extracts and standard antioxidant compounds were shown in Figure 5, and the absorbance values of lichen extracts and standard antioxidant compounds at 30 µg/mL concentrations were shown in Table 6.

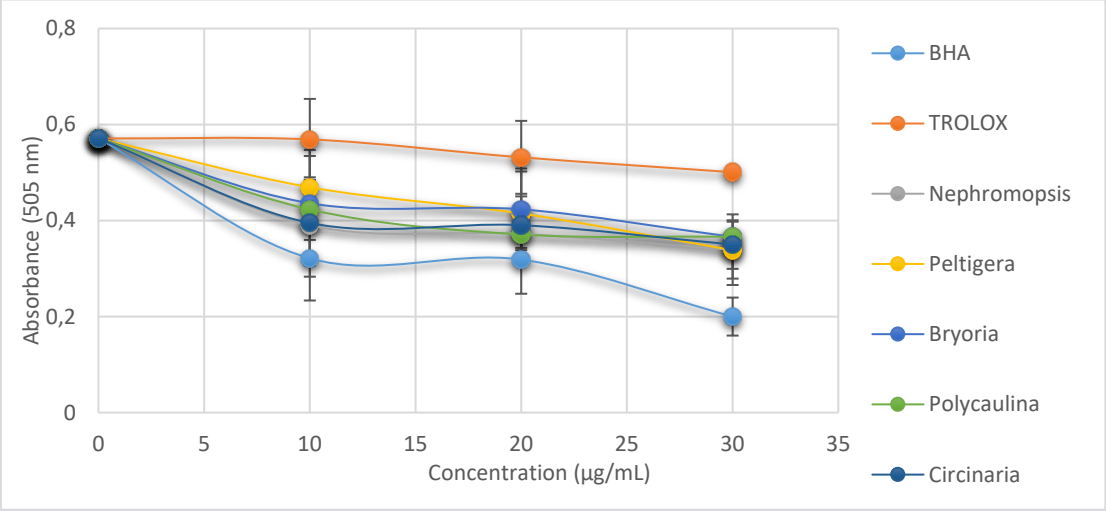

Figure 5. DMPD free radical scavenging activities of lichens (10-30 µg/mL).

Lichen extracts and standard antioxidant compounds showed DMPD radical scavenging activity, respectively, as to be; BHA> *P. canina* > *N. chlorophylla* > *C. calcarea* > *B. capillaris* =*P. candelaria* > Troloks at 30µg/mL concentration.

Bipyridyl Ferrous Ions (Fe^{2+}) Chelating Activity

Metal chelating activities of lichen extracts and standard antioxidant compounds were determined using bipyridyl. The graphs showing the results of this antioxidant activity determination method for lichen extracts and standard antioxidant compounds are given

in Figure 6, and the absorbance values of lichen extracts and standard antioxidant compounds at 30 µg/mL concentrations are given in Table 6. Lichen extracts and standard antioxidant compounds showed metal chelating activities,respectively, as to be Troloks> *P. candelaria* =*P. canina* > *C. calcarea* > *B. capillaris* > *N. chlorophylla* > BHT> BHA> α-Tokoferol at 30µg/mL concentration.

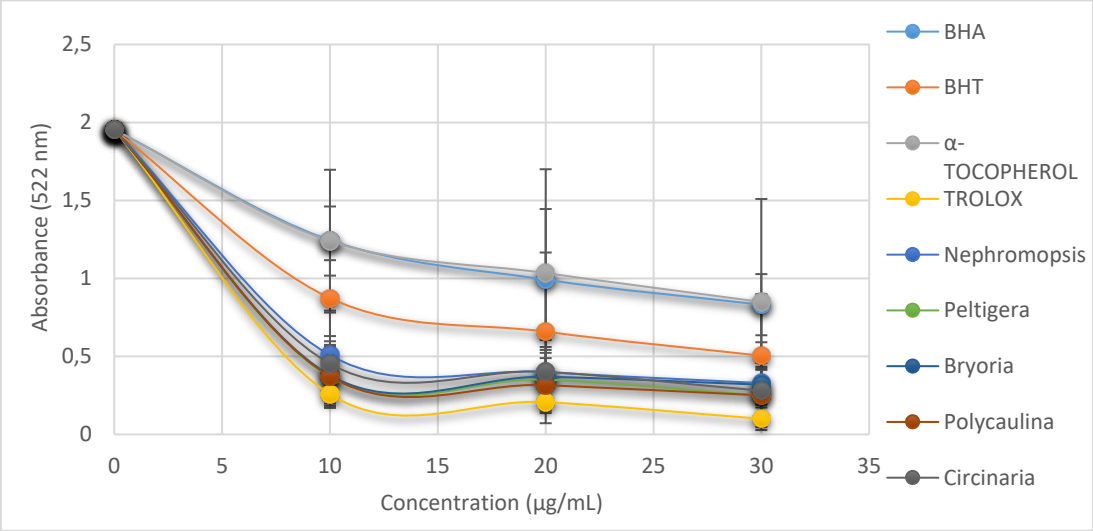

Figure 6. Bipyridyl metal chelating activities of lichens (10-30 µg/mL).

Table 6. Absorbance values of lichen extracts (30µg/mL) obtained from different antioxidant activity determination methods (radical removal). Standard antioxidant compounds (BHA, BHT, αTocopherol, Trolox).

Antioxidants	DPPH•scavenging activity (517 nm)	DMPD• scavenging activity (505 nm)	Bipyridyl metal chelating activity (522nm)
Kontrol	1,43	0,571	1,954
BHA	0,089	0,200	0,832
BHT	0,888	-	0,504
αTokoferol	0,061	-	0,849
Troloks	0,936	0,501	0,100
Nephromopsis chlorophylla	1,257	0,339	0,329
Peltigera canina	1,243	0,338	0,250
Bryoria capillaris	1,295	0,366	0,319
Polycauliona candelaria	1,237	0,366	0,250
Circinaria calcarea	1,222	0,350	0,280

Discussions

Preparation of Lichen Extracts

It is known that various solvents can be used for the preparation of lichen extracts, and each solvent enables the extraction of different lichen components. Therefore, the biological activities of the prepared lichen extracts can differ depending on the solvent used. In this case, it does not seem possible to determine exactly which solvent is more suitable for the preparation of a lichen extract with high biological activity. However, we preferred to use methanol as a solvent for the extraction processes of lichens evaluating some literature studies investigating the antimicrobial and antioxidant activities of lichens [10,12,13,30].

We suggest that extracts of lichens should be prepared using different solvents such as water, ethanol, acetone, as well as methanol, and the antimicrobial and antioxidant activities of these extracts should be determined.

Antimicrobial Activities of Lichens

Disc diffusion methods

The antimicrobial activity results of methanol extracts of lichens were shown in Table 1.

It was determined that a diameters of the inhibition zone forming against test microorganisms of methanol extracts of *N. chlorophylla, C. calcarea, B. capillaris* lichen species varied between 10-13mm;10-15mm;9-13mm, respectively. It was observed that methanol extract of *C. calcarea* among methanol extracts of lichens formed diameter of maximum inhibition zone (15 mm) against *E. coli* and *B. cereus* bacteria species.

It was understood that the most sensitive species to lichen extracts among test microorganisms were *B. cereus*, *B. subtilis*, *C. perfringens*, *E. coli. Escherichia coli* are Gram negative bacteria, *B. cereus, B. subtilis, C. perfringens* are Gram positive bacteria. When these results were examined, it was understood that the lichen extracts in our study were more effective on Gram-positive bacteria.

It was determined that lichen extracts showed a stronger antimicrobial activity against Gram positive bacteria compared to Gram negative bacteria in some

studies on the antimicrobial activities of lichens in the same as line with our results [11, 13, 15, 21,31].

Such a result is due to the differences in the cell wall structure of Gram-positive and Gram-negative bacteria according to various sources. Both Gram-positive and Gram-negative bacteria have a peptidoglycan layer in their cell wall structure. However, unlike Gram-positive bacteria, Gram-negative bacteria have an outer membrane over the peptidoglycan layer. This outer membrane has a lipopolysaccharide (LPS) layer. This layer is thought to slow down or prevent the entry of some compounds into the cell. Therefore, Gram-negative bacteria are less affected by antimicrobial compounds than Gram-positive bacteria [11, 22, 32].

The lichen species used in our study showed antimicrobial activity against test microorganisms with diameter of the inhibition zone varying between 7-15 mm. There have been many studies examining the antimicrobial activities of lichens 4,8,12,15,21,31]. When all these literature informations are evaluated, inhibition zone diameter sizes in our findings are comparable with other studies.

None of the lichen species in our study were effective on *Candida albicans*, which is the only fungus species among the test microorganisms. Some studies examining the antimicrobial activity of various lichen species have similar results to ours [12, 15, 34,35,]. According to a literature report due to differences in cell wall composition and permeability, bacteria are more sensitive to antimicrobial activity than fungi. In the same study, it has been stated that the cell wall of the fungus contains polysaccharides such as chitin and glucan and its permeability is weak [10]. In another a literature, it has been reported that the antibacterial properties of plants are more than their antifungal properties, and this is related to the structural differences between prokaryotic bacteria and eukaryotic fungal cells. As a matter of fact, it has been emphasized that while antimicrobial agents must bind to sterols in eukaryotic membranes to be effective, such binding is not necessary for bacterial cells [32]. When the antimicrobial activities of lichens in our study have been compared with other studies on this subject as considering the size of diameters of the inhibition zone and spectrum of antimicrobial activity formed against the test microorganisms, it has seen that the antimicrobial activities of methanol extracts of our lichens have been at an average level.

However, when all these research results are evaluated, it does not seem possible to determine exactly which lichen extract is stronger in terms of antimicrobial activity. As a matter of fact, there are many factors that affect the antimicrobial activities of lichens. Among these can be counted factors as differences in the methods of obtaining lichen extracts, the type of solvent used for lichen extraction, the contents of lichen species, the amount of lichen extracts absorbed into the discs, and which microorganism species are used [10,11,31,36,37].

MIC Values of Lichens

It was determined that the extracts of the lichens in our study formed MIC values ranging between 31.25-250 µg/mL against the test microorganisms. Among the lichens, the extract of *Circinaria calcarea* lichen species showed the best (lowest) MIC value (31.25 µg/mL) against *E. coli* bacteria.

When the MIC values obtained in some studies examining the antimicrobial properties of various lichen species are compared, it is understood that our results are at an average level.(8,23,38).

Antioxidant Activities of Lichens

Different antioxidant activity determination methods including (Fe^{3+}) reducing capacity, (Cu^{2+}) reducing capacity, Ferric reducing capacity (FRAP), DPPH radical scavenging activity, DMPD radical scavenging activity, Bipyridyl metal chelating activities were used to determine the antioxidant activities of *Polycauliona candelaria, Nephromopsis chlorophylla*, *Circinaria calcarea*, *Bryoria capillaris*, *Peltigera canina* lichen species in our study.

The absorbance values of methanol extracts of lichen species and standard antioxidant compounds (BHA, BHT, α-Tocopherol and Trolox) at 10, 20 and 30 µg/mL concentrations were measured for each antioxidant activity determination method applied to determine the antioxidant activities of lichens. Graphs showing the antioxidant activities of lichen extracts and standard antioxidant compounds were drawn considering these absorbance values. Graphs were shown in Figures 1-6. Absorbance values of lichen extracts and standard antioxidant compounds at concentrations of only 30 µg/mL were shown in Tables 5,6.

In order to determine the antioxidant properties of compounds or natural products, antioxidant activity determination methods such as reduction, radical scavenging and metal chelation must be applied. If positive results are obtained from at least three of these methods with separate principles, the studied compound can be evaluated as having potential antioxidant properties.

Bipyridyl metal chelation antioxidant activity determination method was used for the metal chelation test. It is necessary heavy metals such as Zn, Fe and Cu for the functioning of enzymes in metabolism. However, if the same metals are above the required level, they accumulate and can become toxic and cause harmful effects on biomolecules. In this case, it causes peroxidation of biological molecules such as lipids in the plasma membrane by inducing the formation of ROS and nitrogen species (RNS). If the substances or sources, whose antioxidant properties have been investigated, show the ability to chelate heavy metals, the formation of the above-mentioned radicals is prevented. Once metals are chelated with appropriate chelating agents, their damage in metabolism can be prevented and effectively removed from the body.

Antioxidant activity determination methods such as (Fe^{3+}) reducing, (Cu^{2+}) reducing Ferric reducing (FRAP) were used for the reduction test. Redox reactions in metabolism are the main reaction of biological oxidation. This is a chain of chemical reactions in which we use oxygen in the air to oxidize chemicals obtained from the breakdown of food to provide energy to the living system. If the metal ions present in metabolism are more than necessary, it negatively affects the metabolism as it increases the formation of free radicals. For example, Fe^{2+} is a reactive metal ion and its presence in free form is dangerous for metabolism. Therefore, its harmful potential is reduced by reducing it to Fe^{3+}. Otherwise, extremely dangerous free radicals and their precursors such as hydroxyl radicals are formed.

DPPH radical scavenging and DMPD radical scavenging antioxidant activity determination methods were used for the radical scavenging test. Determining the radical scavenging activities and antioxidant potentials of antioxidant compounds is important for biological systems, but also for the pharmaceutical industry. Free radicals may occur during normal biological processes in metabolism, and antioxidant systems and substances are needed to eliminate them. Otherwise, damage will occur in the biological system. In this respect, determining radical scavenging capacities is important to talk about antioxidant capacity (39,40).

As a result of the antioxidant activity determination methods that we applied, it was determined that the lichen species in our study showed an average antioxidant activity. *Peligera canina* lichen species among the lichens in our study showed the highest antioxidant activity in most of the antioxidant activity determination methods that we applied. It was determined that all lichens produced the best antioxidant activity in the bipyridyl metal chelating activity determination method.

Based on our antioxidant activity results, it does not seem possible to say which lichen species has the highest antioxidant activity among lichen species. As a matter of fact, there are many factors that affect the antioxidant properties of lichens. These include the climatic conditions in which lichens live, the contents of lichen species, the differences in the methods of obtaining lichen extracts, the type of solvent used in extraction processes and the determination of antioxidant activity applied [10,39].

On the other hand, it is known that the determination of lichen compounds that cause antimicrobial and antioxidant activity in lichens is also important in such studies. As a matter of fact, there have been many sources stating that the antimicrobial and antioxidant activities of lichens may be related to the phenolic compounds in their structures [12,40].

It is necessary also to purify the main components and then investigate their antagonistic or synergistic interactions with their each other and other components in order to fully understand the reason of biological activities of lichens [4,10,11].

Conclusions

Among the lichen species in our study, *Circinaria calcarea* showed the highest antimicrobial activity and *Peltigera canina* lichen species showed the highest antioxidant activity. These lichens can be evaluated for use in fields such as pharmacology, by conducting much more comprehensive studies on the antimicrobial and antioxidant properties.

Conflicts of interest

The authors declare that they have no conflict of interest.

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