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ORIGINAL ARTICLE

Antimicrobial and Antioxidant Activities of *Pseudevernia furfuracea* (L.) Zopf var. furfuracea and Evernia prunastri Lichens Collected from Black Sea Region

Bahar BİLGİN SÖKMEN[1♠], Kadir KINALIOĞLU[2], Sinem AYDIN[2]

[1]*Department of Chemistry, Faculty of Science and Arts, Giresun University, 28049 Giresun, TURKEY*
[2]*Department of Biology, Faculty of Science and Arts, Giresun University, 28049 Giresun, TURKEY*

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ABSTRACT

The antimicrobial and antioxidant activities of the acetone extracts of lichen Pseudevernia furfuracea var. furfuracea (PF) and Evernia prunastri (EP) were investigated. Different antioxidant tests were applied such as ABTS (2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) radical scavenging activity and reducing power. Antimicrobial activities of lichen extracts were determined by agar well diffusion method and micro-well dilution assay. All lichen extracts exhibited antimicrobial activity against test bacteria. Antioxidant activity of PF extract is higher than EP extract. Acetone extracts of PF and EP could be considered as a source of natural antioxidant and antimicrobial agent.

Key Words: *Antioxidant Activity, Antimicrobial Activity, Evernia prunastri, Pseudevernia furfuracea var. furfuracea.*

1. INTRODUCTION

Antioxidants play an important role in preventing or alleviating chronic diseases by reducing the oxidative damage to cellular components caused by reactive oxygen species [1]. There is growing interest in natural polyphenolic antioxidants, present in medicinal and dietary plants that help attenuate oxidative damage [2, 3].

Overuse of antibiotics has become the major factor for the emergence and dissemination of multi-drug resistant strains of several groups of microorganisms [4]. Thus, in the light of the evidence of rapid global spread of resistant clinical isolates, the need to find new antimicrobial agents is very important [5]. For this reason, plants are attracting much attention among researchers as significant new sources of bioactive substances.

Lichens is a plant which have been used for medicinal purposes by various ethnic groups from the dawn of the civilisations. PF was used as an old tonic for intestinal weakness [6]. PF was being imported to Egypt from Europe as foreign drugs [7]. PF is used in traditional medicine in Spain for respiratory ailments [8]. EP, along with PF and *Parmelia physodes* were the main ingredient in the "Lichen quercinus virdes", was used as a drug in Europe in the 15th century [9]. EP was an astringent and febribuge and recommended it for pulmonary affections [10].

In this study, we aimed to investigate antimicrobial and antioxidant activities of acetone extracts of EP (Parmeliaceae) and PF (Parmeliaceae) lichens collected from Black Sea Region.

2. MATERIALS AND METHODS

2.1. Chemicals

Butylated hydroxytoluen (BHT), ABTS, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) were purchased from Fluka Chemical Co. (Buchs, Switzerland). 2,2-diphenyl-1-picryl-hydrazyl (DPPH) and rutin were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Trichloroacetic acid (TCA) and ferric chloride were obtained from Merck. All other reagents were of analytical grade.

2.2. Preparation of the Lichen Extracts

Lichen specimens were collected from Dereli-Giresun which was located in the eastern part of the Black Sea region of Turkey between 40° 34' 23" N and 38° 24' 56" E and authenticated and identified by Dr. Kadir Kınalıoğlu. The vouchers; PF (herbarium no: 1820) and EP (herbarium no: 1819) were deposited in the herbarium of Giresun University, Faculty of Science and Arts, Department of Biology. Air dried and powdered lichens (20 g) were extracted with 200 mL of acetone by using a Soxhlet apparatus for 72 h at a temperature not exceeding the boiling point of the solvent, separately [11]. The extracts were filtered using Whatman filter paper (no.1) and then concentrated in vacuo at 40˚C using a rotary evaporator. Extracts were stored at -80˚C for further assays.

2.3. Test Microorganisms

Five bacteria strains were used to determine of antimicrobial activities of acetone extracts of PF and EP. Bacteria are used in the study as follows: *Staphylococcus calmii* (Lab isolate), *Bacillus pumilis* (Lab isolate), *Bacillus megaterium* (Lab isolate), *Acinetobacter baumannii* (Lab isolate), *Enterococcus faecium* (Lab isolate). Test microorganisms were supplied from Yeditepe University.

2.4. Antimicrobial Activity Assay

The dried lichen extracts were dissolved to obtain 30 mg/mL final concentration in dimethyl sulfoxide (DMSO) and extracts sterilized by filtration through 0.45 µm Millipore filters [12]. Antimicrobial tests were carried out by the agar well diffusion method. Inocula, corresponding to a value of 0.5 on the Mc Farland optical density scale, were prepared in Mhüller Hinton Broth and cultivated (100 µL) onto Müeller Hinton agar. Wells, 5 mm diameter, were punched in each agar plate. Wells were filled with 40 µL of acetone extract of EP, 40 µL of acetone extract of PF and DMSO (for negative control), separately [13]. All the plates were incubated at 37˚C for 24 h. The antimicrobial activities were evaluated by measuring the inhibiton zone diameter observed.

In minimal inhibitory concentration (MIC) assays, the inocula of microorganisms were prepared from overnight broth cultures and suspensions were adjusted to 0.5 McFarland standard turbidity. The 96 well plates were prepared by dispensing into each well 95 µL of Mhüller Hinton Broth and 5 µL of the inoculum. 100 µL each lichen extract initially prepared at the concentration of 1 mg/mL were added into first wells, separately. Then, 100 µL from their serial dilutions were transferred into ten consecutive wells. The last well containing 195 µL of nutrient broth without compound and inoculum on each strip was used as negative control. 96 well plates were incubated at 37°C overnight. The MIC was defined as the lowest concentration of the compounds to inhibit the growth of microorganisms [14].

2.5. Antioxidant Activity

2.5.1. DPPH radical scavenging activity

The DPPH radical scavenging activities of the lichen extracts were measured according to the procedure described by Brand-Williams et al. [15]. Appropriate dilution series (0.25-1.0 mg/mL) were prepared for acetone extracts in DMSO 0.1 mL of each dilution was added to 3.9 mL of a 6x 10^{-5} M methanolic solution of DPPH followed by vortexing. The mixture was shaken vigorously and allowed to stand in the dark at room temperature for 30 min. The decrease in absorbance of the resulting solution was then measured spectrophotometrically at 517 nm against methanol. The DPPH scavenging percentage was calculated as follows:

% the DPPH scavenging=[(absorbance of negative control-absorbance of sample)/absorbance of negative control)×100%].

BHT, Trolox and rutin (0.025-0.1mg/mL) were used as synthetic antioxidant reagents and all tests were carried out in triplicate.

2.5.2. Reducing power

The reducing powers of the lichen extracts and synthetic antioxidants were determined according to the method described by Oyaizu [16]. Different amounts of extracts (0.25–1.0 mg/mL) in 1 mL of DMSO were mixed with 2.5 mL of phosphate buffer (0.2 M, pH 6.6) and 2.5 mL potassium ferricyanide (1%), and then incubated at 50°C for 30 min. 2.5 mL of 10% trichloroacetic acid was added to the mixture to stop the reaction, and the mixture was centrifuged at 3000g for 10 min. The supernatant (2.5 mL) was mixed with 2.5 mL distilled water and 0.1% $FeCl_3$ (0.5 mL) and then the absorbance was measured at 700 nm. BHT, Trolox and rutin (0.025-0.1mg/mL) were used as standard antioxidants. The reducing powers of the tested samples increased with the absorbance values.

2.5.3. $ABTS^{\cdot+}$ radical scavenging activity

The $ABTS^{\cdot+}$ radical scavenging activities of the lichen extracts were measured according to the procedure described by Arnao et al. [17]. The stock solutions included 7.4 mM $ABTS^{\cdot+}$ solution and 2.6 mM potassium persulfate solution. The working solution was prepared by mixing the two stock solutions in equal quantities and allowing them to react for 12 h at room temperature in the dark. The solution was then diluted by mixing 1 mL $ABTS^{\cdot+}$ solution with 60 mL methanol to obtain an absorbance of 0.708±0.04 units at 734 nm using the spectrophotometer. Fresh $ABTS^{+}$ solution was prepared for each assay. 150 µL of lichen extracts (25-100 µg/mL) were allowed to react with 2850 µL of the $ABTS^{+}$ solution for 2 h in a dark condition. Then the absorbance was taken at 734 nm using the spectrophotometer. The $ABTS^{\cdot+}$ scavenging activity was calculated using the following equation:

ABTS radical scavenging activity (%)=[(absorbance of negative control−absorbance of sample)/absorbance of negative control) × 100%].

Extract concentration providing 50% inhibition (IC_{50}) was calculated form the graph plotted inhibition percentage against extract concentration. Synthetic antioxidant reagents (BHT, Trolox and rutin) (25-100 µg/mL) were used as positive control and all tests were carried out in triplicate.

2.5.4. Determination of total phenolic compounds

Total phenolic compounds were determined with Folin–Ciocalteu reagent, according to the method of Slinkard and Singleton [18] with some modifications. The dried lichen extracts were dissolved to obtain final concentration 1 mg/mL in DMSO. Aliquots (0.1 mL) of the extracts were transferred into test tubes and their volumes made up to 4.6 mL with distilled water. After addition of 0.1 mL Folin–Ciocalteu reagent (previously diluted 3-fold with distilled water) and 0.3 mL 2% Na_2CO_3 solution, tubes were vortexed and the absorbance of the mixture was recorded after 2 h at 760 nm against a blank containing 0.1 mL of extraction solvent. The amount of total phenolic compounds was calculated as mg gallic acid equivalents (GAE) from the calibration curve of gallic acid standard solution (covering the concentration range between 0.02 mg/mL and 0.1 mg/mL) and expressed as mg gallic acid per mg of extracts of the plant materials. The data were presented as the average of triplicate analyses.

3. RESULTS

3.1. Antimicrobial Activity

The antimicrobial activities of PF and EP extracts against microorganisms examined in the study were assessed by the presence or absence of inhibition zone diameter and MIC values. The results are given in Figure 1 and Figure 2.

The study showed that lichen extracts have antimicrobial effects against the tested bacteria at different rates. The maximum antimicrobial activity was observed by PF extract against *A. baumanii* and the minimum antimicrobial activity was observed by EP extract against *S. calmii* and *B. pumilis*.

The basic quantitative measurement of in vitro activity of antimicrobial agents with antimicrobial potential is the MIC. Demonstration of low MIC value is an indication that the phytoconstituents of the plant have the therapeutic properties [19]. MIC values of PF and EP lichen extracts are shown in Figure 2. Acetone extract of EP showed minimum inhibitory concentration (MIC) at 1000 µg/mL for *S. calmii, at 500* µg/mL for *B. pumilis, A. baumanii* and *B. megaterium*, acetone extract of EP also showed minimum inhibitory concentration (MIC) at 62.50 µg/mL for *E. faecium.* Acetone extract of PF showed minimum inhibitory concentration (MIC) at 500 µg/mL for *S. calmii, A. baumanii* and *B. megaterium* at *250* µg/mL for *E. faecium* and *B. pumilis.*

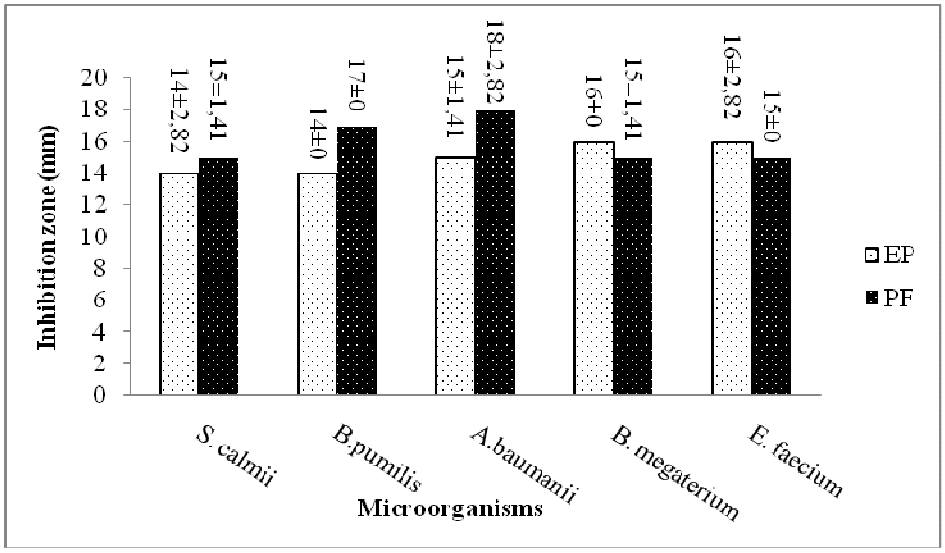

Figure 1. Inhibition zones which was created by lichen extracts against test microorganisms.

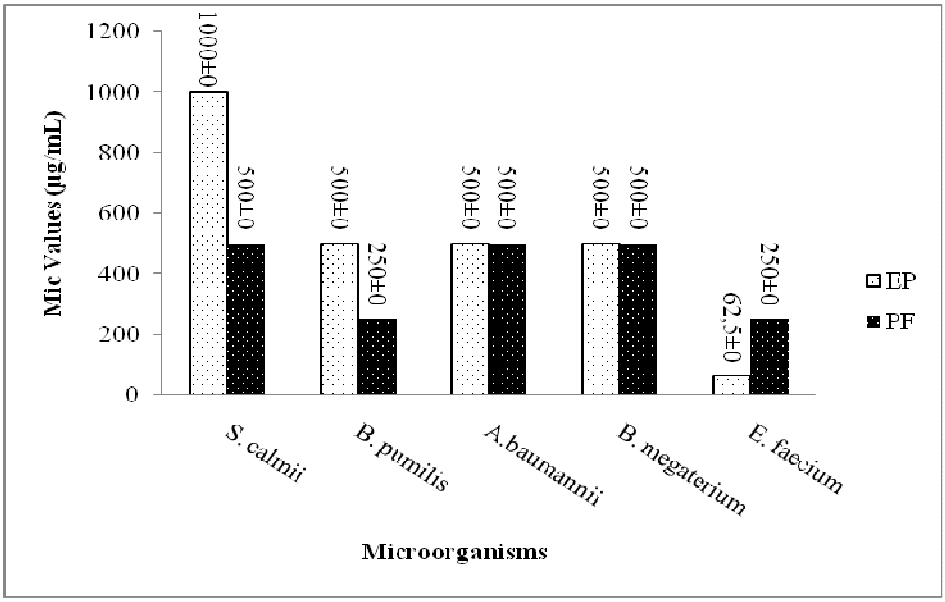

Figure 2. MIC values of lichen extracts.

3.2. Antioxidant Activities

3.2.1. Total phenolic compounds

Total phenolic compounds were estimated by the Folin-Ciocalteu method, based on the procedure of Slinkard and Singleton [18] using gallic acid as a standard phenolic compound. A linear calibration curve of gallic acid, in the range of 20-100 µg/mL with R^2 value of 0.9729, was constructed. In our study, it was determined that there were 187.27±0.020 and 146.36±0.004 µg/mL gallic acid equivalent of phenolic compounds in the 1 mg of acetone extracts of PF and EP, respectively.

3.2.2. *DPPH radical scavenging activity*

The DPPH radical scavenging effects of lichen extracts are presented in Figure 3. All the samples showed appreciable free radical scavenging activities. Radical scavenging activities of PF extract at 0.75 mg/mL and 1.0 mg/mL were higher when compared with rutin at 0.075 mg/mL. In addition, 0.75 mg/mL of PF extract had similar radical scavenging activity when compared with BHT at 0.075 mg/mL concentration. DPPH radical scavenging activity of PF extract at 1.0 mg/mL was higher than Trolox at 0.075 mg/ mL. Radical scavenging activities of EP extract of 1.0 mg/mL was higher when compared with rutin, Trolox and BHT at 0.025 mg/mL. From these results, it can be stated that PF extract have more ability to scavenge free radicals than EP extract.

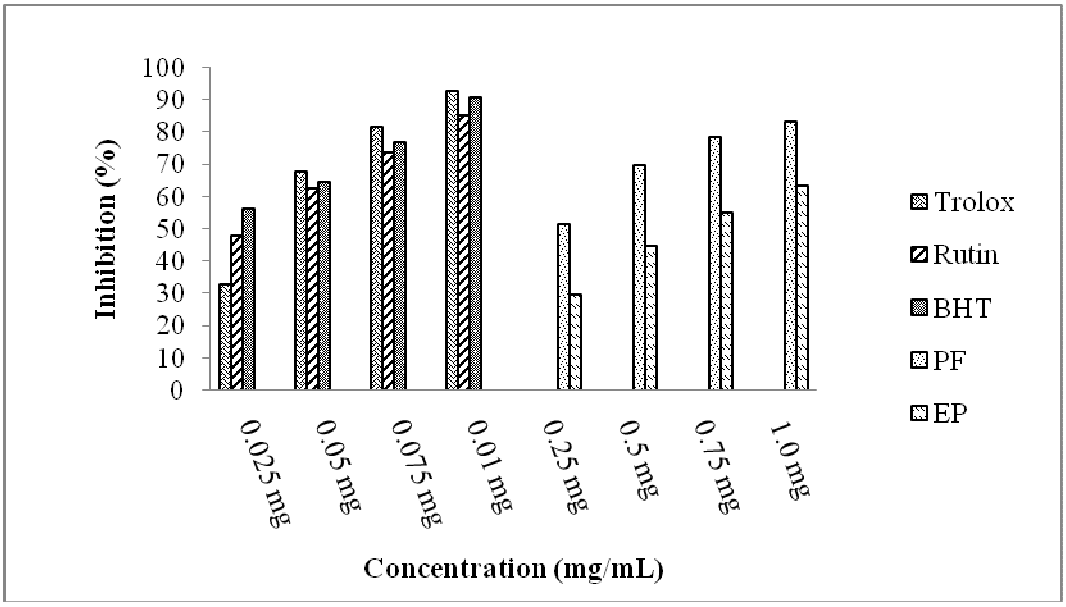

Figure 3. DPPH radical scavenging activities of lichens extracts and standarts.

Table 1. DPPH radical scavenging activities of lichens extracts and standarts.

Concentration (mg/mL)	Trolox	Rutin	BHT	EP extract	PF extract
0.025 mg	32,97±3,04	48±2,48	56,3±0,95	---	---
0,5 mg	68,07±3,37	62,77±2,51	64,6±1,1	---	---
0.075 mg	81,93±1,61	73,9±1,53	77±1,70	---	---
0.01 mg	92,83±1,95	85,33±1,15	90,73±1,96	---	---
0.25 mg	---	---	---	29,67±2,51	51,66±3,05
0.5 mg	---	---	---	45±2,64	70±2,64
0.75 mg	---	---	---	55,33±3,05	78,66±3,51
1.0 mg	---	---	---	63,66±3,21	83,33±3,05

3.2.3. Reducing power

Figure 4 shows the reducing powers of PF and EP acetone extracts. The reducing powers of extracts increased with increasing concentration. Reducing power of PF extract of 1.0 mg/mL was higher when compared with Trolox at 0.075 mg/mL. EP extract of 1.0 mg/mL had higher reducing power when compared with BHT, rutin and Trolox at 0.025 mg/mL. All concentrations of PF extract exhibited higher reducing power than all concentrations of EP extract.

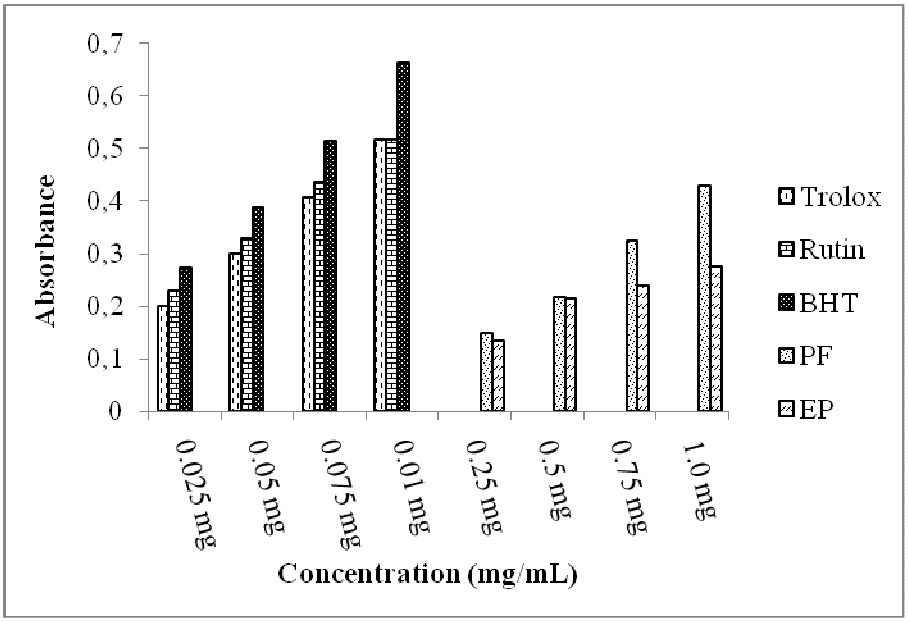

Figure 4. Reducing powers of lichen extracts and standarts.

Table 2. Reducing powers of lichen extracts and standarts.

Concentration (mg/mL)	Trolox	Rutin	BHT	EP extract	PF extract
0.025 mg	0,201±0,005	0,230±0,004	0,273±0,002	---	---
0,5 mg	0,301±0,003	0,33±0,005	0,338±0,003	---	---
0.075 mg	0,408±0,007	0,438±0,008	0,514±0,01	---	---
0.01 mg	0,516±0,004	0,517±0,01	0,663±0,002	---	---
0.25 mg	---	---	---	0,136±0,01	0,150±0,003
0.5 mg	---	---	---	0,216±0,02	0,218±0,04
0.75 mg	---	---	---	0,239±0,01	0,324±0,01
1.0 mg	---	---	---	0,277±0,008	0,430±0,02

3.2.4. $ABTS^{\cdot+}$ radical scavenging activity

The $ABTS^{\cdot+}$ scavenging activity of acetone extract of lichen compared to rutin and Trolox are shown in Figure 5. $ABTS^{\cdot+}$ scavenging activities increased with increasing concentration of the samples. $ABTS^{\cdot+}$ scavenging activities of PF, EP and standard compounds at 100 µg/mL exhibited the following order: rutin> EP>PF= Trolox. IC_{50} values for EP extract, PF extract, rutin and Trolox on $ABTS^{\cdot+}$ radical scavenging activity were found as 65.13 ± 3.33, 66.03 ± 3.98, 51.07 ± 0.92 and 66.03 ± 1.21 µg/mL, respectively.

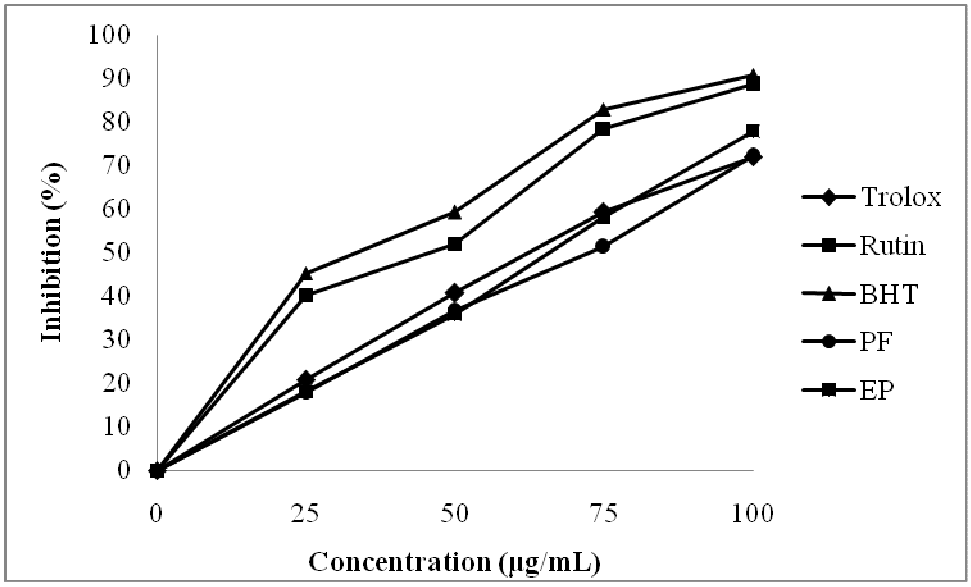

Figure 5. $ABTS^{+}$ radical scavenging activities of lichen extracts and standards.

Table 3. $ABTS^{+}$ radical scavenging activities of lichen extracts and standards.

Concentration (mg/mL)	Trolox	Rutin	BHT	EP extract	PF extract
0.025 mg	20,7±1,49	40,2±1,05	45,33±2,50	18,02±2,1	17,85±2,74
0,5 mg	40,73±0,92	52±1,50	59,26±2,50	35,87±2,58	36,57±2,21
0.075 mg	59,23±0,75	78,46±0,73	82,9±2,98	58,05±2,66	51,3±2,46
0.01 mg	72±1,13	88,66±0,41	90,86±1,32	77,97±3,21	72,17±2,55

4. DISCUSSION

The results obtained in this study indicate differences in antimicrobial activity between extracts depending on the species of lichen and as a function of the type of extracting solvent.

In investigation of the antimicrobial activity of the lichen extracts conducted by other authors, there are both similarities and differences in comparison with our results.

Aslan et al., [20] and Mitroviç et. al [21] studied antimicrobial activity of methanol extracts of *E. prunastri.* According these studies, methanol extracts of *E. prunastri* exhibited antimicrobial activity against some test bacteria and fungi. We also investigated whether antimicrobial activity of acetone extract of *E. prunastri* against tested bacteria and we found antimicrobial activity of acetone extract of *E. prunastri* against test bacteria. It can concluded, both methanol and acetone extracts of *E. prunastri* have antimicrobial effect against different test microorganisms.

Ilçim and Dığrak [22] studied antimicrobial activity of chloroform extract of *Parmelia furfuracea* (L.) Zopf which is synonym of *Pseudevernia furfuracea*. Chloroform extract of *P. furfuracea* exhibited antimicrobial activity against test bacteria but it couldn't exhibited antifungal activity. Osmanağaoğlu et al. [23] studied also antimicrobial activity of acetone and chloroform extracts of *P. furfuracea* against different test bacteria. Türkan et al. [24] investigated antimicrobial effects of raw skin treated with *Pseudevernia furfuracea* (L.) Zopf acetone extract. Acetone and chloroform extract of *P. furfuracea* exhibited antimicrobial activity against test bacteria. We also investigated antimicrobial activity of acetone extract of *P. furfuracea* against bacteria. According these results, it can concluded, acetone and chloroform extracts of *P. furfuracea* have antimicrobial activity against microorganisms at different ratio.

When antioxidant capacities of the extracts are compared with their phenolic constituents, it could be concluded that antioxidative nature of the extracts might depend on their phenolics [20]. As reported elsewhere, this activity is increased with presence of polyphenols in particular [25].

It has been reported that the antioxidant activity of phenols was mainly due to their redox properties, hydrogen donators, and single oxygen quenchers [2]. Another supportive report also emphasizes the correlation between antioxidative capacity and phenolic constituents, particularly methyl orsenillate, orsenillic acid, atranorin and lecanoric acid [26].

DPPH reduction was evaluated by decolorization of the radical solution (absorbance in the range of 515–528 nm) in the presence of the plant extract. Because the DPPH radical can accommodate many samples in a short period

of time and is sensitive enough to detect active molecules at low concentrations, it has been extensively used to screen the antiradical activities of vegetal extracts, juices and fruits [27].

Zovko et. al [28] studied antioxidant and antimicrobial activity of chloroform extract, ethanol extract and water extracts of lichen *Pseudevernia furfuracea* (L.) Zopf. In the assay, it concluded that chloroform and ethanol extract of *Pseudevernia furfuracea* demonstrated significant antioxidant activity and as it can be expected from the higher phenolic content ethanolic extract exhibited higher antioxidant activity than the chloroformic extract. According our study, acetone extract of *P. furfuracea* also exhibited antioxidant activity.

Aslan et al., [20] also investigated antioxidant activity of methanol extract of *Evernia divaricata*, *E. prunastri* , *Cladonia foliacea*, *Dermatocarpon miniatum* and *Neofuscella pulla* by scavenging of free radical DPPH and the inhibition of linoleic acid oxidation. Extracts of *C. foliacea*, *E. divaricata*, *E. prunastri* and *N. pulla* didn't exert any antioxidant activity. Mitrovic et. al. [21] also studied antioxidant activity of methanol extract of *E. prunastri* and it exhibited DPPH radical scavenging activity. According our study, acetone extract of *E. prunastri* also exhibit antioxidant activity by scavenging of free radical DPPH.

The $ABTS^{\cdot+}$ assay is based on the inhibition of the absorbance of the radical cation $ABTS^{\cdot+}$, which has a characteristic long-wavelength absorption spectrum showing absorption at 734 nm. Bleaching of a preformed solution of the blue-green radical cation $ABTS^{\cdot+}$ has been extensively used to evaluate the antioxidant capacity [29].

The reducing power has been used as one of the antioxidant capability indicators of plants [30]. In the reducing power assay, the presence of reductants (antioxidants) in the tested samples resulted in the reduction of the Fe^{+3}/ ferricyanide complex to the ferrous form (Fe^{+2}). Stojanavic et al., [31] studied reducing power and DPPH radical scavenging activity of *E. prunastri* and reported *E. prunastri* had reducing power and DPPH radical scavenging activity. In our study, we also investigated reducing power of extract of *E. prunastri* and we found it has reducing power. Our and literature results are consistent.

5. CONCLUSION

In conclusion, acetone extracts of *P furfuracea* and *E. prunastri* showed antimicrobial and antioxidant activity. Therefore, *P. furfuracea* and *E. prunastri* lichens as natural antioxidant sources appears to be an alternative to synthetic antioxidants. Purification and identification of the bioactive components which have antimicrobial and antioxidant activities are needed to examine the mechanism of these agents. In addition, the results suggest that the lichen extracts tested possess compounds with antimicrobial properties as well as antioxidant activity, which require further studies to determine antimicrobial agents for therapy of infectious diseases in human and plant diseases.

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