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Research Article

Comparison of Antioxidant Activity of *Roccella phycopsis* Ach. (Roccellaceae) and *Flavoparmelia caperata* L. Hale (Parmeliaceae) Lichens

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ABSTRACT

The antioxidant activities of *Roccella phycopsis* Ach. and *Flavoparmelia caperata* L. Hale lichens were determined by their ability to scavenge free radicals such as DPPH (2,2-diphenyl-1-picrylhydrazyl) and ABTS (2,2'-azinobis-(3-ethylbenzothiaziline-6-sulfonate). Total phenolic and flavonoid contents, metal chelating activity and reducing power of the extracs were also measured. Both lichens species displayed noticeable antioxidant activity. The total flavonoid contents of methanol and ethanol extracts of the lichens ranged from 110.06±0.004 µg/mL to 154.1±0.007 µg/mL and from 40.96±0.005 µg/mL to 65.23±0.007 µg/mL, respectively. *F. caperata* showed higher ferric reducing antioxidant power and DPPH radical quenching activity than *R. phycopsis*. This study reveals that ethanol and methanol extracts of *F. caperata* and *R. phycopsis* lichens might be utilized for a source of natural antioxidant agent.

Keywords: *Roccella phycopsis*, *Flavoparmelia caperata*, Free radical, Antioxidant activity

Roccella phycopsis Ach. (*Roccellaceae*) ve *Flavoparmelia caperata* L. Hale (*Parmeliaceae*) Likenlerinin Antioksidan Aktivitelerinin Karşılaştırılması

ÖZET

Roccella phycopsis Ach. ve *Flavoparmelia caperata* L. Hale likenlerinin antioksidan aktiviteleri DPPH (2,2-difenil-1-pikrilhidrazil) ve ABTS (2,2'-azinobis-(3-etilbenzotiyazilin-6-sulfonat) gibi serbest radikalleri süpürme yetenekleri kullanılarak belirlendi. Ekstraktların toplam fenol ve flavonoid içerikleri, metal şelatlama aktivitesi ve indirgeme gücü de belirlendi. Çalışılan her iki liken ekstraktı da dikkate değer bir antioksidan aktivite sergiledi. Likenlerin metanol ve etanol ekstraktlarının toplam flavonoid içerikleri sırasıyla 110.06±0.004 µg/mL-154.1±0.007 µg/mL ile 40.96±0.005 µg/mL-65.23±0.007 µg/mL arasında değişmektedir. *F. caperata*, *R. phycopsis*'den daha yüksek demir indirgeyici antioksidan gücü ve DPPH radikali süpürme aktivitesi gösterdi.Bu çalışma, *F. caperata* ve *R. phycopsis* likenlerinin etanol ve metanol ekstraktlarının doğal antioksidan kaynağı olarak kullanılabileceğini ortaya koymaktadır.

Anahtar Kelimeler: *Roccella phycopsis*, *Flavoparmelia caperata*, Serbest radikal, Antioksidan aktivite

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I. INTRODUCTION

Oxidative stress may be caused by reactive oxygen species (ROS) [1]. ROS have positive effect on phagocytosis, energy generation and formation of biologically effective compounds [2]. Nevertheless, excessive production of ROS can be toxic and may lead many diseases like arthritis, carcinogenesis, aging, diabetes mellitus, cancer, heart disease, neurodegenerative disease [3]. Antioxidants have great importance for preventing ROS induced oxidative damage, lipid peroxidation and DNA strand breaking [4].

Synthetic antioxidant compounds are generally used because they are influential and inexpensive when compared with natural ones [5]. However, synthetic antioxidants have some negations like toxicity and carcinogenicity. Therefore, remarkable interest has been dedicated to natural antioxidants [6].

Lichens have been used as folk medicine for centuries. Some lichen species have medicinal value. For example, *Ramalina bourgeana* was used as a diuretic, *Xanthoria parietina* was prescribed for kidney disorders and as an analgesic for pain and *Pseudevernia furfuraceae* was used to treat respiratory complaints. Many lichen species thought to have medicinal properties are enrolled in several pharmacopoeias [7].

Lichens possess depsides, depsidones, dibenzofurans and phenolic compounds. Most of them are unique to lichens. Antioxidant capacities of lichens have revealed by many scientists [8-10].

Roccella phycopsis is a fruticose lichen which belongs to Roccellaceae family. It has employed as dye and litmus source [11, 12]. *F. caperata* is known as greenshield lichen and used to heal the intestinal worms. Dried powder of the thallus can be used on skin burns. *F. caperata* was also used to dye wools in Man Island [13]. In China, *F. caperata* lichen has used as decoction to heat [14].

In view of the above, the main objective of the study is to assess antioxidant properties of ethanol and methanol extracts of *R. phycopsis* and *F. caperata* lichens which obtained from Eastern Black Sea Region.

II. MATERIAL AND METHOD

A. REAGENTS

2,2-diphenyl-1-picryl-hydrazyl ($DPPH^{\cdot}$), gallic acid, catechin, aluminium chlorid hydrat, butylated hydroxytoluen (BHT), trolox, ascorbic acid, rutin hydrate, 3–(2–pyridyl)–5,6–bis(4–phenyl–sulfonicasit)–1,2,4–triazine (ferrozine), ferrous chloride, ethanol, methanol, 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid (ABTS), ferric chloride, ethylene diamine tetraacetate (EDTA), potassium ferricyanide (III), potassium persulfate, sodium carbonate, sodium nitrite, sodium hydroxide were purchased from Sigma Chemical Co. Trichloroacetic acid (TCA), dimethyl sulfoxide (DMSO), Folin-Ciocalteu's phenol reagent were purchased from Merck.

B. LICHEN SPECIMENS

F. caperata and *R. phycopsis* species were collected from Giresun province between 25 September and 14 October 2011. Localities were presented in Table 1. Voucher specimens were preserved in the herbarium of the Faculty of Science and Arts, Giresun University, Giresun.

Table 1. Localities of collected lichen species

Species	**Locality Name**
R. phycopsis	Giresun Center, Gedikkaya Hill, 225 m
F. caperata	Giresun, Bulancak District, Ahmetli Village, 350 m

C. EXTRACTION PROCESS

Air-dried samples were grounded to powder with a blender. Powdered lichens (48 g) were subjected to Soxhlet extraction using 480 mL of ethanol or methanol, separetely. Each extract were filtered using Whatman filter paper (No.1) and solvents evaporated by rotary evaporator at 40°C. The residue were stored at -80°C for further use. The extraction process was done only once [15].

D. EXTRACTION YIELD (%)

The extracts were weighed and calculated as percentage of inhibition with the following eq.1;

$$Extraction\ Yield\ (\%) = \frac{\text{dry weight of extract} x 100}{\text{dry weight of original sample}} \quad (1)$$

E. ANTIOXIDANT ACTIVITY

The antioxidant activity of lichen samples was determined using ferric ions reducing antioxidant power (FRAP), DPPH radical quenching activity, ABTS radical quenching activity and iron chelating activity. Total phenolic and flavonoid contents of the lichen extracts also measured.

F. TOTAL PHENOLIC CONTENT

The total phenolic contents of extracts were defined by the Folin-Ciocalteu method [16]. This experiment is based upon the reduction of phosphor-wolfromate-phosphomolybdate complex by phenols; at the end of the reduction a blue reaction product is occurred [17]. 125 µL extract solutions which was prepared in 1000 µg/mL concentration, 4.5 mL distilled water and 0.1 mL Folin–Ciocalteu reagent (previously diluted 3-fold with distilled water) was mixed. After 3 min, 0.3 mL Na_2CO_3 (2%) was added to the mixture. After 2 h incubation at room temperature and dark, the absorbance of the mixture was read at 760 nm by using spectrophotometer (Shimadzu 1240 UV-Vis Spectrophotometer). Total phenolic content of the extracts was expressed as µg of gallic acid equivalents (GAE) by using the calibration curve (R^2: 0.9997).

G. TOTAL FLAVANOID CONTENT

Firstly, 0.25 mL lichen extract, 1.25 mL distilled water and 75 µL $NaNO_2$ (%5) were mixed and vortexed. After 6 min, 150 µL of $AlCl_3.6H_2O$ (%10) was added and the mixture was kept at room

temperature for 5 min. Then, 0.5 mL NaOH (1M) and 275 µL distilled water added to the mixture. Absorbance was measured at 510 nm. Catechin was used as standard and the results were expressed as µg catechin equivalent (QE) [18].

H. FERRIC IONS REDUCING ANTIOXIDANT POWER (FRAP)

Reducing power assay was performed by the method of Oyaizu [19]. 250-1000 µg/mL of extracts were prepared in DMSO. 2.5 mL of phosphate buffer (0.2 M, pH 6.6) and 2.5 mL potassium ferricyanide (1%) were added to extracts. This mixture was incubated at 50 °C in water bath for 20 min. After cooling, 2.5 mL of trichloroacetic acid (10%) was added to the mixture and centrifuged at 3000 rpm for 10 min. The upper layer of solution (2.5 mL) was mixed with distilled water (2.5 mL) and 0.5 mL $FeCl_3$ (0.1%). In this analysis, $[Fe(CN)_6]^{3-}$ complex was reduced to the $[Fe(CN)_6]^{-4}$ by antioxidant compounds. Therefore, the formation of colouring agent as $Fe_4[Fe(CN)_6]_3$ complex was read at 700 nm [20]. Ascorbic acid and BHT were used as standards.

I. *DPPH RADICAL QUENCHING ACTIVITY*

Blois's method was utilized to reveal DPPH radical quenching activity of test lichens. [21]. Appropriate dilution series (250-1000 µg/mL) were prepared for lichen extracts in DMSO. 0.75 mL of each solution was added to 1.5 mL of a $6x10^{-5}$ M methanolic solution of DPPH. The mixture was vortexed and left at room temperature for 30 min and the absorbance was measured at 517 nm. Synthetic antioxidant reagents were used such as BHT, trolox and ascorbic. When a hydrogen or a electron was transferred to DPPH radical, the absorbance diminished due to the later non-radical form [22]. The data were represented as SC_{50} (µg/mL) value.

J. *ABTS RADICAL QUENCHING ACTIVITY*

The ABTS test is attributed to the generation of a blue/green $ABTS^{\cdot+}$, which is practicable to hydrophilic and lipophilic antioxidant systems [22]. $ABTS^{\cdot+}$ solution was prepared by mixing 7.4 mM ABTS and 2.6 mM potassium persulfate and the mixture was kept at room temperature for 12 h in the dark to complete reaction. Then, $ABTS^{\cdot+}$ solution diluted with methanol to obtain an absorbance of 0.700±0.02 units at 734 nm [23]. Lichen extracts (150 µL) was allowed to react with 2850 µL of the $ABTS^{\cdot+}$ solution for 2 h in a dark condition and the absorbance was measured at 734 nm. SC_{50} (µg/mL) value was determined. BHT, rutin and ascorbic acid were used as reference standards.

K. METAL CHELATING ACTIVITY

The chelation of ferrous ions by lichen extracts in comparison with EDTA was investigated by the method of Dinis et al [24]. 5 mL of lichen extracts at different concentrations were added to a solution of 2 mM $FeCl_2$ (0.1 mL). The reaction was initiated by the addition of 5 mM ferrozine (0.2 mL). The mixture was waited at room temperature for 10 min and the absorbance was read at 562 nm. Ferrozine-Fe^{2+} complex formation was calculated with Eq. 2;

$$\text{İnhibition}\,(\%) = \left(\frac{AC - AS}{AC}\right) X100 \qquad (2)$$

AC: Absorbance of control; AS: Absorbance of the sample

III. RESULTS & DISCUSSION

The extraction yield is a measure of the solvent efficiency to extract spesific components from the prototype material. Table 2 demonstrates extraction yield percentages of the lichen extracts. The extraction yields varied from 12.34% to 19.38%. The highest and the lowest extraction yields were obtained from methanol extract of *R. phycopsis* and ethanol extracts of *F. caperata*, respectively.

Table 2. Extraction yields (%) of the lichen extracts

Lichen	Extraction Yield (%)
RPE	16.28
RPM	19.38
FCE	12.34
FCM	14.65

RPE: Ethanol extract of *R. phycopsis*, RPM: Methanol extract of *R. phycopsis*,
FCE: Ethanol extract of *F. caperata*, FCM: Methanol extract of *F. caperata*.

In terms of taste, aroma and healthy effects, phenolic compounds have nutritional and quality importance [25]. Furthermore, these molecules are significant for the protection of plants against pathogen, predators and UV radiation [26].

Phenolic compounds could give hydrogen to free radicals and they can terminate the chain reaction of the lipid oxidation at the early stage. Hydroxyl groups of phenolic compounds have capacity to scavenge radicals [27].

Total phenolic content of the extracts was determined by Folin-Ciocalteu method. Table 3 shows the phenolic content of extracts as μg of gallic acid equivalent. These lichen extracts had phenolic levels ranging from 62.44±0.004 and 109.35±0.005 μg of gallic acid equivalent. The RPE and FCE extracts were found to have maximum and minimum phenolic content, respectively.

Table 3. Total phenolic contents of extracts of *F. caperata* and *R. phycopsis*

Lichen	μg GAE/mg
RPE	109.35±0.005
RPM	106.55±0.007
FCE	62.44±0.004
FCM	63.50±0.002

Results are expressed as the mean ± standard deviation (S.D.) of each triplicate test.

Mitrovic et al. (2011) reported that FCM had 90.83±0.98 μg GAE/mg total phenolic content [28]. According to study was conducted by Stojanović et al. (2010), total phenolic content of FCM was 11.99±0.18 mmol of GAE/g of extract [29], but in our research total phenolic content was found as 63.5±0.002 μg GAE/mg. This different total phenolic contents in the same lichen species may be arisen from the geographical and climatic conditions in different regions [30].

Flavonoids are a class of secondary metabolites which have important functions such as free radical scavenging activity, anti-inflammatory activity and inhibition of hydrolytic and oxidative enzyme [31]. Flavonoids are one of important plant constituents which have antioxidant activities [32].

The total flavonoid content in the extracts of *F. caperata* and *R. phycopsis* are summarized in Table 3. Total flavonoid content of lichen extracts was expresses as µg of catechin equivalent in Table 4. Total flavonoid content in methanol extract varied from 110.06±0.004 µg QE/mg and 154.1±0.007 µg QE/mg, ethanol extract ranged from 40.96±0.005 µg QE/mg and 65.23±0.007 µg QE/mg. The maximum total flavonoid content was observed in the RPM and the minimum flavonoid content was observed in RPE.

Table 4. Total flavonoid content of lichen extracts

Lichen	µg QE/mg
RPE	40.96±0.005
RPM	154.10±0.007
FCE	65.23±0.007
FCM	110.06±0.004

Results are expressed as the mean ± standard deviation (S.D.) of each triplicate test.

Mitrovic et al. (2011) found that FCM had 33.55±0.93 mg Rutin/g of extract total flavonoid content [28] but in our current study total flavonoid content was determined as 110.06±0.004 µg cateshin/mg of extract.

Methanol extracts of both of the lichens have more total phenolic and flavonoid contents. Literature results are also supports this. Kamkar et al. (2014) stated that methanol extract of *Satureja hortensis* L. have higher total phenol, flavanoid contents, higher inhibition percentage of DPPH and β-carotene linoleic acid than ethanol extract of *S. hortensis* [33].

Compounds which have reducing power are electron donors and they can decrease the oxidized intermediates of lipid peroxidation processes, therefore they can play role as primary and secondary antioxidants. In FRAP experiment, the test solution which has yellow colour converts to green and blue according to the reducing power of extract. Existence of reducers induce the conversion of the Fe^{3+}/ferricyanide complex to the ferrous form. Measuring formation of Perl's Prussican Blue at 700 nm can reveal the Fe^{2+} concentration [34].

Table 5. Reducing power of extracts and standards

Lichen	250 µg/mL	500 µg/mL	750 µg/mL	1000 µg/mL
RPE	0.058±0.003	0.088±0.003	0.108±0.008	0.128±0.001
RPM	0.032±0.001	0.060±0.003	0.129±0.003	0.136±0.001
FCE	0.178±0.002	0.181±0.006	0.230±0.008	0.232±0.007
FCM	0.181±0.007	0.310±0.001	0.464±0.005	0.565±0.005
Ascorbic acid	0.811±0.004	0.829±0.005	0.892±0.005	1.0381±0.006

BHT	0.667±0.008	0.839±0.007	0.930±0.013	1.1912±0.003

Table 5 shows reducing power of lichen extracts and standards. The reducing power of lichens and standards at 750 and 1000 µg/mL exhibited in the following order: BHT>Ascorbic acid>FCM>FCE>RPM>RPE. Standards exhibited higher activity than extracts of *R. phycopsis* and *F. caperata*. Moreover, extracts of *F. caperata* lichen showed higher ferric ions reducing antioxidant power activity than extracts of *R. phycopsis*.

According a study which was carried out by Kosanic et al. (2011) acetone, methanol and aqueous extracts of *Parmelia caperata* which is synonym of *F. caperata* have reducing power [2].

As seen in Table 5, methanol extracts of lichens showed higher reducing activity than ethanol extracts. Smitha and Garampolli (2015) carried out a study about reducing power of methanol and ethanol extracts of *Ramalina pacifica* and *Roccella montagnei*. Methanol extracts of the lichens showed more reducing power when compared to the ethanol extract [35]

DPPH is used to free radical to investigate the radical scavenging effects of some natural products [36]. The DPPH radical includes an electron, which is responsible for the absorbance at 517 nm. When DPPH take an electron from an antioxidative compound, the decolourated DPPH can be measured from the changes in absorbance [37].

SC_{50} is the concentration of the antioxidant needed to scavenge 50% of DPPH present in the test solution. A lower SC_{50} indicates higher DPPH radical scavenging activity. The SC_{50} of the extracts and standards are presented in Table 6. The highest SC_{50} value was observed in RPE (1655.63±49.38 µg/mL) whereas the lowest SC_{50} value was observed FCE (526.87±10.55 µg/mL). In addition, extracts of *F. caperata* had lower SC_{50} than extracts of *R. phycopsis*.

Table 6. SC_{50} values of the lichen extracts and standards (µg/mL)

Lichen Extract	**SC_{50}**
RPE	1655.63±49.38
RPM	1533.74±75.46
FCE	723.59±12.57
FCM	526.87±10.55
BHT	672.04±8.13
Ascorbic Acid	494.56±3.42
Trolox	580.72±9.45

Results are expressed as the mean ± standard deviation (S.D.) of each triplicate test.

In a study was conducted by Mitrovic et al. (2011) it was found that SC_{50} FCM was 549.01±1.69 µg/mL [28]. According to a study was carried out by Stojanović et al. (2010) it was found that SC_{50} of FCM was 347.20±0.33 µg/mL [29] but in our study SC_{50} of FCM was found as 723.59±12.57 µg/mL.

Different SC_{50} might be associated with collecting lichens from different geography, different climates and using different extract concentration.

Kosanic et al. (2011) stated that acetone, methanol and aqueous extracts of *P. caperata* which is synonym of *F. caperata* have DPPH radical scavenging and superoxide anion radical scavenging activities [2]. Kosanic et al. (2012) also reported reducing power activity, DPPH radical scavenging activity and superoxide anion radical scavenging activity in acetone extract of *P. caperata* which is synonym of *F. caperata* lichen [38].

The $ABTS^{\cdot+}$ test is based on the inhibiton of the absorbance of radical cation $ABTS^{\cdot+}$ by antioxidants. This assay arise from the reaction between ABTS and potassium persulphate to produce the ABTS radical cation ($ABTS^{\cdot+}$). In the presence of antioxidant agent, the coloured radical turns to colourless $ABTS^{\cdot+}$ [39].

Table 7. SC_{50} values of the lichen extracts and standards (μg/mL)

Lichen Extract	SC_{50}
RPE	456.26±5.42
RPM	484.01±3.28
FCE	744.04±9.97
FCM	657.89±11.26
BHT	381.67±0.58
Ascorbic Acid	399.36±0.64
Rutin	393.35±0.62

Results are expressed as the mean ± standard deviation (S.D.) of each triplicate test.

The SC_{50} values of the extracts and standards were shown in table 7. A higher ABTS radical scavenging activity was associated with a lower SC_{50} value. RPE possessed the lowest $ABTS^{\cdot+}$ scavenging activity (456.26±5.42 μg/mL), while FCE showed the highest $ABTS^{\cdot+}$ radical scavenging activity (744.04±9.97 μg/mL) among the extracts.

Methanol extracts of the lichens exhibited better SC_{50} values. Oran et al. (2016) investigated antioxidant activities of ethanol and methanol extracts of *Usnea intermedia*, *Usnea filipendula* and *Usnea fulvoreagens*. Methanol extracts of all the tested lichens exhibited higher Trolox equivalent antioxidant capacity ($ABTS^{\cdot+}$ assay) than ethanol extracts of the lichens [40].

The method of Dinis et al. (1994) was used to determine the iron chelating activity of Fe^{2+} in the extracts. Ferrozine can generate complexes with Fe^{2+}but in the presence of chelating agents, the complex production is disrupted. As a result of this, the red colour of the complex is decreased. Estimation of colour reduction can reveal the chelating activity of the coexisting chelator [41].

Free iron ions plays significant role to constitute the free radicals. Also, high iron deposition in vital organs like liver and kidney etc. can cause the loss of function. Therefore, chelation of these free iron ions may hinder the production of free radicals and they can prevent the degeneration of vital organ function [42].

The chelating activity of extracts of *F. caperata* and *R. phycopsis* were shown in Table 8. Extracts of *R. phycopsis* exhibited higher metal chelating activity than extracts of *F. caperata*.

Table 8. Iron chelating activity (% inhibition) of the lichen extracts and standards

Lichen	250 µg/mL	500 µg/mL	750 µg/mL	1000 µg/mL
RPE	8.85±0.2	23.49±0.1	30.39±0.6	36.12±1.2
RPM	10.45±1.2	33.54±0.09	50.59±0.7	54.23±0.4
FCM	12.02±0.5	15.20±0.1	21.92±0.7	25.46±0.09
FCE	1,47±0.1	5.97±0.1	10.50±0.3	16.16±0.4
EDTA	49.96±1.4	72.28±1.2	89.05±0.7	98.75±0.2

Results are expressed as the mean ± standard deviation (S.D.) of each triplicate test.

Extracts of *R. phycopsis* exhibited higher metal chelating activity than extracts of *F. caperata*. Methanol extracts of both lichens showed higher activity than ethanol extracts of lichens. Moreover, the activity was dose dependent and increased with increasing concentration. The best activity was exhibited by EDTA.

Manojlovic et al. (2012) found that protocetraric and usnic acids which were detected as the major secondary metabolites in the acetone extract of *P. caperata* which is synonym of *F. caperata*. It had DPPH radical scavenging activity, superoxide anion scavenging activity and reducing power activity [43]. Caviglia et al. (2001) investigated *P. caperata* which is synonym of *F. caperata.* It was investigated the usnic acid amount in *P. caperata* lichen treated with Paraquat, a herbicide which transfer electrons from various transport systems to oxygen, producing $O_2^{\cdot-}$ superoxide radicals. Increasing usnic acid amount was measured in *P. caperata* thalli [44].

Until now, to the best of our knowledge, there is no such report available about ABTS radical scavenging, iron chelating activity of *F. caperata* and antioxidant activity of *R. phycopsis*.

Different extraction methods may influence the antioxidant activity of the extracts. Murugan and Parimelazhagan (2014) stated that soxhlet extraction method shows better for extracting polyphenolic compounds from plants (fractionation and maceration). Some of the heat sensitive compounds may decompose in the Soxhlet technique. However, thermolabile/thermostable compounds cannot be dehydrolyzed due to the stability of compounds. Therefore, thermostable compounds from the Soxhlet extraction method showed good antioxidant property compared to other techniques [45].

IV. CONCLUSION

It can be concluded that ethanol and methanol extracts of *R. phycopsi*s and *F. caperata* showed good antioxidant activity with rich total phenolic and flavonoid contents according to obtained results. Therefore, *R. phycopsi*s and *F. caperata* lichens as natural antioxidant sources appears to be an alternative to synthetic antioxidants. Purification and identification of the bioactive components which have antioxidant activities are needed to examine the mechanism of these agents.

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