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ANTIOXIDANT AND ANTIMICROBIAL PROPERTIES OF A LICHEN SPECIES, *CLADONIA RANGIFORMIS* GROWING IN TURKEY

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Abstract

The antioxidant activity (AA), reducing power (RP) and total phenolic compounds (TPC) of chloroform, methanol and water extracts of a lichen species, Cladonia rangiformis, was determined, in vitro. The chloroform extract of C. rangiformis showed significant antioxidant activity ($p<0.05$), although this activity was lower than that of trolox (positive control). Likewise, the chloroform extract of C. rangiformis contained the highest phenolic content as gallic acid equivalent ($\mu g/ml^{-1}$), its followed by the methanol and water extracts. However the reducing power assay of the methanol extract of C. rangiformis was higher than chloroform and water extracts. Antimicrobial activities of these extracts against 42 microorganisms consisting of 26 bacterial strains, 15 fungi and a yeast were also studied by using disk diffusion method. The extracts exhibited a weak antimicrobial activity against very limited number of microorganisms. However, extracts showed no antifungal activity.

***Key words**: Lichens; Cladonia rangiformis; antioxidant activity; antimicrobial activity*

Türkiyede Yetişen bir Liken Türü Olan *Cladonia rangiformis'*in Antioksidan ve Antimikrobiyal Özellikleri

Bir liken türü olan Cladonia rangiformis'in kloroform, metanol ve sulu ekstrelerinin in vitro antioksidan aktivitesi (AA), indirgeme güçleri (RP) ve toplam fenolik madde içerikleri (TPC) belirlenmiştir. C. rangiformis'in kloroformlu ekstresi troloks'tan (pozitif kontrol) daha düşük olmasına rağmen önemli bir antioksidan aktivite göstermiştir ($p<0.05$). Aynı şekilde C. rangiformis'in kloroformlu ekstresi gallik asit eşdeğeri ($\mu g/ml^{-1}$) olarak en yüksek fenolik içeriğine sahip olup bunu metanol ve su ekstreleri izlenmiştir. Diğer yandan C. rangiformis'in metanolül ekstresinin indirgeme gücü kloroform ve metanol ekstrelerinden daha yüksek bulunmuştur. Bu ekstrelerin antimikrobiyal aktiviteleride 26 bakteri suşu, 15 fungus ve bir maya'dan oluşan 42 mikroorganizmaya karşı disk-diffüzyon yöntemi kullanılarak çalışılmıştır. Ekstreler az sayıda mikroorganizmaya karşı zayıf bir antimikrobiyal aktivite gösterdi. Bununla beraber, ekstreler hiç antifungal etki göstermiştir.

***Anahtar Kelimeler**: Liken; Cladonia rangiformis; antioksidant aktivite; antimikrobiyal aktivite*

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INTRODUCTION

Antioxidants are important to prevent many human diseases. Antioxidant compounds have many functions such as free radical scavengers, complexers of pro-oxidant metals, reducing agents and quenchers of singlet oxygen formation (1). Antioxidants are also often used in oils and fatty foods to retard their autoxidation. The synthetic antioxidants such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA), previously widely used, are now doubted toxicologically (2). Therefore, many researchers have focused on natural antioxidants. The commercial development of plants as a source of antioxidants has led to their use for enhancing the properties of food for both nutritional purposes and preservation. Thus, in plant kingdoms, numerous crude extracts and pure natural compounds have been found to possess antioxidant properties. In general, typical natural antioxidants include tocopherols, flavonoids, quercetin, cinnamic acid, peptides and phenolic compounds as well as carotenoids (3,4).

Lichens are complex plants living in symbiotic relationship between fungi and algae, and the pertinent partners are defined as mycobiont and phycobiont, respectively. In all lichens, the fungus forms a thallus or lichenized stroma that may contain characteristic secondary metabolites (5). These secondary metabolites are unique with respect to those of higher plants. A number of these secondary metabolites have important biological activities as antimicrobial, antipyretic, cytotoxic, antitumor, analgesic, antiviral and allergenic compounds (6-13). Lichens have been used in folk medicine in many countries over a considerable period of time. Lichens were also rich species in terms of carbohydrate, crude fiber and minerals. Therefore, some lichen species have been used as food in many countries (14). However, antioxidant activities of lichens and their secondary metabolitis are poorly known, and only some recent works provide some useful information about this subject (4,14-18). On the other hand, the antimicrobial properties of various lichen species have been widely studied (14,19-24). There is also some studies which *C. rangiformis* has antiinflammatory effect (25) and cytotoxic activity on human cancer cell lines (26). However, no report on the relationship between antioxidant activity and phenolic content of *C. Rangiformis* has been available. Therefore, the aim of the present study was set to explore the relationship among phenolic contents, antioxidant activities and reducing powers of water, methanol and chloroform extracts of *C. rangiformis*. It was also of interest to find out whether the extracts possess antimicrobial activity. Therefore, the antimicrobial activities of water, methanol and chloroform extract of *C. rangiformis* against 42 microorganisms consisting of 26 bacterial strains, 15 fungi and 1 yeast were tested by using disc diffusion methods.

EXPERIMENTAL

Plant materials

Cladonia rangiformis Hoffm. (No.ATA1682) was collected from Trabzon region of Turkey in 2000. Lichen samples were identified by Dr. Aslan (27) and their voucher specimens has been deposited in the herbarium of Kazım Karabekir Education Faculty, Ataturk University, Erzurum (Turkey).

Extraction procedures

The lichen sample (100 g) was extracted separately with distilled water (60-80 °C, 200 ml x 4), methanol (40 °C, 200 ml x 4) and chloroform (40 °C, 200 ml x 4) for two days in a water bath with a shaking attachment. Then, water extracts were lyophilized under a 5 µm-Hg pressure. The methanol and chloroform extracts were concentrated under reduced temperature

and pressure using a rotary evaporator. Afterward, the residues of methanol extracts were dissolved in 50 ml of distilled water and re-extracted with *n*-hexane (3x50 ml) to remove lipophilic compounds. The water was then lyophilized and stored at -18 °C. The experiments were carried out using appropriate amount of lyophilized materials.

Determination of antioxidant activity

Antioxidant activities (AA) of the lichen extracts were determined using the thiocyanate method with minor modifications (28). Briefly, each sample (1mg) in 1 ml distilled water was mixed with 5 ml linoleic acid emulsion (0.02M, pH 7.0) and 5ml phosphate buffer (0.2M, pH 7.0). Linoleic acid emulsion was prepared by mixing 0.5608 g of linoleic acid with 0.5608 g of Tween 20 as emulsifier, and 100 ml phosphate buffer, and then the mixture was homogenized. The reaction mixture was incubated at 37°C. Aliquots of 0.1 ml were taken at different intervals during incubation. The degree of oxidation was measured according to the thiocyanate method by sequentially adding 4.7 ml of ethanol (75%), 0.1ml of ammonium thiocyanate (30%), 0.1ml of sample solution and 0.1ml of ferrous chloride (0.02M, in 3.5% HCl). The mixture stood for 3 minutes and then the peroxide value was determined by reading the absorbance at 500 nm using a UV-visible spectrophotometer (ThermoSpectronic-HEλIOS β). Test sample which contains the linoleic acid emulsion, but not extracts, was used as control. trolox and ascorbic acid solutions prepared in the conditions mentioned above were also used as positive control. Inhibition % was calculated by using the equation:

(1-absorbance of sample at 500 nm/absorbance of control at 500 nm) x 100

Determination of total phenolic contents

The amount of total phenolic compounds (TPC) in the lichen extracts was determined with the Folin-Coicalteu reagent according to the method previously published using gallic acid as standard (29). 500 µl of samples (three replicates) were introduced to test cuvettes, and then 2.5 ml of Folin-Colicalteu's reagent (diluted 1:10, v/v) and 2 ml of Na_2CO_3 (7.5%) were added. The absorbance was measured at 765 nm by using the ThermoSpectronic-HEλIOS β UV-visible spectrophotometer after incubating at 30˚C for 90 minutes. Results were expressed as milligrams of gallic acid equivalents (GAE) per ml of lyophylisates.

Reducing power assays

Reducing power (RP) was determined according to the method of Yen and Chen (30). Each lichen extracts (5 mg) was solved in 5 ml of distilled water and 0.5 ml of these solutions were mixed with 2.5 ml of phosphate buffer (0.2M, pH 6.6) and 2.5 ml potassium ferricyanide [$K_3Fe(CN)_6$] (1%). These mixtures were incubated at 50 ˚C for 30 minutes. Afterwards, 2.5 ml of trichloroacetic acid (10%) was added to these mixtures, which were then centrifuged at 3000 rpm for 10 minutes. Finally, 2.5 ml of the supernatant fractions were mixed with 2.5 ml of distilled water and 0.5 ml of $FeCl_3$ (0.1%), and the absorbances were measured at 700 nm. Increased absorbances of the reaction mixture indicated increased reducing power.

Antimicrobial activity assays

The lichen extracts were individually tested against microorganisms including total 42 microbial cultures consist of 26 bacteria strains (*Acinetobacter baumanii*A8; *Bacillus amyloliquefaciens*142; *Bacillus cereus*RK75; *Bacillus macerans*M58; *Bacillus megaterium*M3; *Bacillus subtilis*ATCC-6633; *Bacillus subtilis*A57; *Brucella abortus*A77; *Burkholdria cepacia*A225; *Clavibacter michiganense*A227; *Enterobacter cloacae*A135; *Enterococcus faecalis*-ATCC29122; *Escherichia coli*A1; *Klebsiella pneumoniae*A137; *Proteus vulgaris*A161; *Proteus vulgaris* Kukem1329; *Pseudomonas aeruginosa*-ATCC9027; *Pseudomonas aeruginosa*-ATCC27859; *Pseudomonas syringae* pv. *tomato*.A35; *Salmonella enteritidis*-ATCC13076; *Staphylococcus aureus*A215; *Staphylococcus aureus*-ATCC29213; *Staphylococcus epidermis*A233; *Streptococcus pyogenes*-ATCC176; *Streptococcus pyogenes*

Kukem676; *Xanthomonas campestris*A235), 15 fungi (*Alternaria alternate; Aspergillus flavus; Aspergillus variecolor; Fusarium acuminatum; Fusarium oxysporum; Fusarium solani; Fusarium tabacinum; Moniliania fructicola; Penicillium* spp.; *Rhizopus* spp.; *Rhizoctonia solani; Sclorotinia sclerotiorum; Sclorotinia minor; Trichophyton mentagrophytes; Trichophyton rubrum*) and 1 yeast species (*Candida albicans*A117). Microorganisms were provided from the Department of Clinical Microbiology, Faculty of Medicine and Plant Diagnostic Laboratory, Faculty of Agriculture at Atatürk University, Erzurum, Turkey. Identities of the microorganisms were confirmed by Microbial Identification System in Biotechnology Application and Research Center of Atatürk University.

For the disc-diffusion assay, the extracts were dissolved in water and chloroform to a final concentration of 30 mg/ml and sterilized by filtration by 0.45µm Millipore filters. Antimicrobial tests were then carried out by disc diffusion method (31,32) using 100µl of suspension containing 10^8 CFU/ml of bacteria, 10^6 CFU/ml of yeast and 10^4 spore/ml of fungi spread on nutrient agar (NA), sabouraund dextrose agar (SDA) and potato dextrose agar (PDA) medium, respectively. The discs (6 mm in diameter) were impregnated with the 30mg/ml extracts (300µg/disc) placed on the inoculated agar. Negative controls were prepared by using the same solvents employed to dissolve the plant extracts. Ofloxacin (10µg/disc) and sulbactam (30µg)+cefoperazona (75µg) (105µg/disc) were used as positive reference standards to determine the sensitivity of one strain/isolate in each microbial species tested. The inoculated plates were incubated at 37 ºC for 24 h for clinical bacterial strains, 48 h for yeast and 72 h for fungi isolates. Plant associated microorganisms were incubated at 27 ºC. Antimicrobial activity was evaluated by measuring the zone of inhibition against the test organisms. Each assay in this experiment was repeated twice.

Statistical analyses

Statistical analyses were calculated by using SPSS 9.0 software. To be able to determine the statistical significance of antioxidant activities, one-way variance analyzes (ANOVA) was applied, which showed that there was a statistically significant difference ($p<0.05$), then, multiple comparison was carried out by Scheffe's multiple comparison test. Pearson's bivariate correlation test was also carried out to calculate correlation coefficients (r) among antioxidant activity, reducing power and total phenolic content. All values are presented as mean ± S.D.

RESULTS

The antioxidant activities of methanol, chloroform and water extracts of *C. rangiformis,* on the peroxidation of linoleic acid were investigated. The results are represented in Figure 1. The results of antioxidant activity assays for methanol, chloroform and water extracts of *C. rangiformis* after 48 h incubation were also summarized as inhibition percentage in Table 1. trolox and ascorbic acid were used as positive controls for the hydrophilic antioxidants. Among the extracts tested, only chloroform extract of *C. rangiformis* showed antioxidant activity in comparison with control ($p<0.05$). It inhibited the peroxidation of linoleic acid by 48.3 %, but its inhibition effect was lower than that of the torolox. The water and methanol extracts did not show antioxidant activity ($p>0.05$).

Total phenolic contents (TPC) of methanol, chloroform and water extracts of *C. rangiformis* were given as gallic acid equivalents (GAE) in Table 1. As can be seen from this table, chloroform extract of *C. rangiformis* contains the highest phenolic content, its followed by methanol and water extracts.

The results of reducing power assay for the extracts of *C. rangiformis* were shown in Table 1. The highest reducing power was shown by methanol extract of chloroform extract of *C. rangiformis*, followed by chloroform and water extracts of *C. rangiformis*.

For the antimicrobial activity assays, methanol, chloroform and water extracts of *C. rangiformis* were tested against 42 microorganisms, consisting of 26 bacterial strains, 15 fungi and 1 yeast. Potency of the extracts was qualitatively and quantitatively assessed by the presence or absence of inhibition zones and zone diameter. Present results showed that the extracts tested exhibited weak antimicrobial activity against limited number of microorganisms, which are *Bacillus subtilis*-ATCC6633, *Bacillus amyloliquefaciens*142 and *Proteus vulgaris*A161. However, while all extracts of *C. rangiformis* were active against *Bacillus subtilis* ATCC6633 and *Bacillus amyloliquefaciens*142, only methanol extract of *C. rangiformis* was effective against *Proteus vulgaris*A161. The maximal inhibition zones for bacterial strains to which extracts of *C. rangiformis* are sensitive, were in the range of 7-9, 8-12 and 8-10 mm.

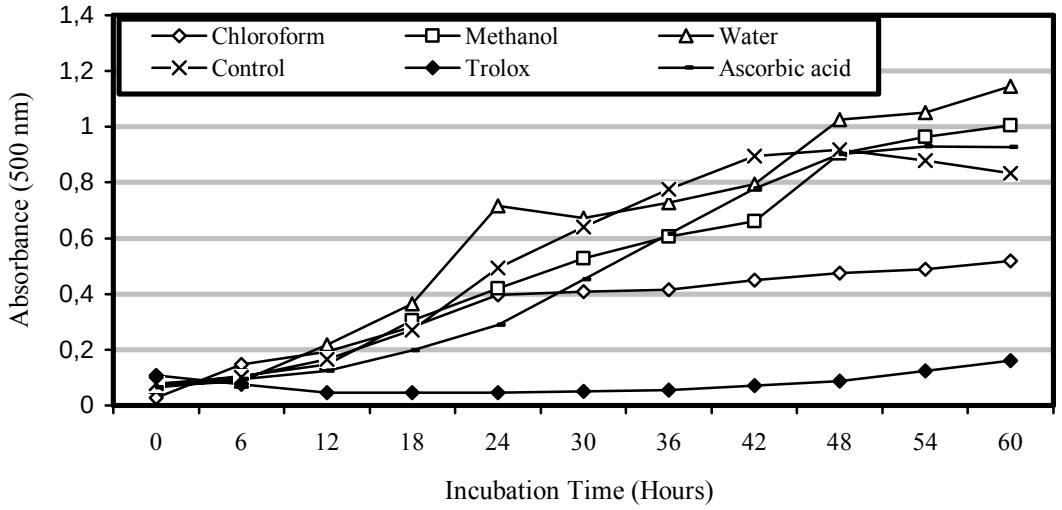

Figure 1. The antioxidant activities of chloroform, methanol and water extract of *C. rangiformis.* The indicated amounts of the extracts were present in 5 ml linoleic acid emulsion (0.02M, pH 7.0). The control was the linoleic acid emulsion without extracts. Results are means of three different extracts in each of which three measurements were made.

DISCUSSION

Previously, it has been reported that more natural compounds consisting of tocopherols, phenolics, flavonoids as well as carotenoids have antioxidant activity (3,4,33). Phenolic compounds are known as the high-level antioxidants because of their ability to scavenge free radicals such as singlet oxygen, superoxide and hydroxyl radicals (34). Therefore, the present study was aimed to explore the relationship between antioxidant potential and total phenolic content of *C. rangiformis*. For this purpose, antioxidant activity, reducing power and total phenolic content of the extracts of *C. rangiformis* were determined, *in vitro*. The results showed that there were not clear relationship between antioxidant activity and phenolic content. The highest antioxidant activity was shown by the chloroform extract of *C. rangiformis* (48.3 %) and it was also rich in terms of phenolic content (Table 1). However, reducing power of methanol extract of *C. rangiformis* was higher than chloroform extract, but not its antioxidant activity.

Table 1. The comparison of antioxidant activitiy, reducing power and total phenolic content of *C. rangiformis.*

	Antioxidant Activity		Total Phenolic Contents		RP Absorbasnce (700 nm)
	% inhibition[a]	Mean Abs. at 500 nm (48. h)	Absorbance (765 nm)	Gallic acid equivalent (μg/ml $^{-1}$)	
Cloroform	48.3	0.475±0.012*	0.528±0.007	0.048±0.001	0.083±0.001
Methanol	1.6	0.904±0.051	0.103±0.012	0.009±0.011	0.115±0.006
Water	-11.5	1.025±0.015	0.038±0.006	0.004±0.001	0.034±0.015
Control	-	0.919±0.011	-	-	-
Asc. acid	1.8	0.902±0.003	-	-	-
Trolox	90.5	0.087±0.001*	-	-	-

Values are mean ± SD of three sets of experiments, $p<0.05$ significance*, [a] The inhibition of linoleic acid oxidation

Reducing power is usually considered to be a good indicator of antioxidant capacity of extracts and pure compounds. Thus, the extracts, which had high reducing power, may be considered as an antioxidant source. The present results suggest that there is no correlation between antioxidant activity and reducing power. However, although chloroform extract of *C. rangiformis* showed antioxidant activity, the reducing power was high.

Many phenolic compounds incluiding depsides, depsidones, diphenyl ethers and monocyclic aromatics are biosynthezid by lichens (35,36). The antioxidant properties of some pure phenolic compounds isolated from lichen species have been also recently reported (4,15). It has been also found that orcinal-type lichen depsides and tridepsides have antioxidant activity by blocking toxic metal ions, which initiated free radical reactions (37). According to these reports, the antioxidant activity of chloroform extracts of *C. rangiformis* may be attributed to their high content of phenolic compounds. Nevertheless, it should be taken into consideration that there might be antagonastic or synergistic interactions between phenolic and non-phenolic compounds.

The antimicrobial properties of the crude extracts obtained from some lichen species against numerous microorganisms were previously screened (14,19-24). They exhibited a varying antimicrobial activities depending on microorganisms tested. On the other hand, many of crude extracts of various lichen samples usually showed an inhibition effects on the growth of *Bacillus* species (14,19,22,23). For *Bacillus* species, similar results were found in our study. For instance, all extracts of *C. rangiformis* showed antimicrobial activity against *B. subtilis* ATCC6633 and *B. amyloliquefaciens*142*,* but not against other *Bacillus* species tested in the present study.

In conclusion, the chloroform extract of *C. rangiformis* showed a potent antioxidant activity. The results of this study show that the chloroform extract of *C. rangiformis* may be used as possible pharmaceutical purposes. However, there was found a weakly relation between antioxidant and antimicrobial activities. The chloroform extract, which had potent antioxidant activity, showed weakly antimicrobial activity. The methanol extract of *C. rangiformis* was more effective in terms of antimicrobial activity in comparison to other extracts. This extract showed antimicrobial activity against limited number of microorganisms, which are only *B. subtilis* ATCC6633, *B. amyloliquefaciens*142 and *P. vulgaris*A161. However, it was not possible to find out the components of *C. rangiformis* responsible for antioxidant and antimicrobial activities in this study. Therefore, further work could be done on the isolation and chracterization of the individual compounds in the extracts, which are responsible for antioxidant and antimicrobial activity.

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