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Antioxidant and Antimicrobial Properties of the Lichens *Cladonia foliacea*, *Dermatocarpon miniatum*, *Everinia divaricata*, *Evernia prunastri*, and *Neofuscella pulla*

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Abstract

The aim of this study was to investigate *in vitro* antimicrobial and antioxidant activities of the methanol extracts of *Cladonia foliacea* Willd. Hudson, *Dermatocarpon miniatum* (L.) Mann., *Everinia divaricata* (L.) Ach., *Evernia prunastri* (L.) Ach., and *Neofuscella pulla* (Ach.) Essl. Antioxidant activity was evaluated by two separate methods: scavenging of free radical DPPH and the inhibition of linoleic acid oxidation. Extracts of *C. foliacea*, *E. divaricata*, *E. prunastri*, and *N. pulla* did not exert any activity in both assays, whereas those of *D. miniatum* provided 50% inhibition at 396.1 μg/ml concentration in the former and gave 49% inhibition in the latter. Total phenolic constituents of extracts from lichen species tested (*C. foliacea*, *D. miniatum*, *E. divaricata*, *E. prunastri*, and *N. pulla*) were 1.7% (w/w), 2.9% (w/w), 3.0% (w/w), 2.6% (w/w), and 1.5% (w/w), respectively (as gallic acid equivalent), implying that the observed activity could be related to the amount of polar phenolics. Extracts were also found to possess antimicrobial activity against some of the bacteria and fungi tested, but no activity was observed against the yeasts.

Keywords: Antimicrobial activity, antioxidant activity, *Cladonia foliace*, *Dermatocarpon miniatum*, *Everinia divaricata*, *Evernia prunastri*, *Neofuscella pulla.*

Introduction

Medicinal plants have been well-known natural sources for the treatment of various diseases since antiquity. According to a report issued by the WHO, there are about 20,000 plant species that are currently in use for medicinal purposes. Approximately 9000 plant species have been recorded from the flora of Turkey. However, chemical constituents and therapeutic importance of many plants have not been fully investigated yet (Ilcim et al., 1998).

Lichens, on the other hand, could be taken into account for this purpose, as they have very rich flora in this region. Concomitantly, this unique flora has attracted many researchers on the systematical basis (Aslan, 2000; Aslan et al., 2002a, 2002b). Nevertheless, the chemical or therapeutic utilization of these species has not been fully investigated. As far as our literature survey could ascertain, about 20,000 lichen species are available throughout the world, and 1000 of them have been reported from the Turkish flora (Aslan et al., 2002a). Biological activities and economic importance of some lichen species from Turkish flora as well as their chemical constituents have been reported in previous studies (Burkholder et al., 1944; Richardson & David, 1988; Huneck & Yoshimura, 1996; Dulger et al., 1998; Huneck, 1999; Perry et al., 1999; Gulcin et al., 2002).

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Therefore, the aim of this study was to evaluate *in vitro* antimicrobial and antioxidant activities of the methanol extracts obtained from five lichen species including *Cladonia foliacea*, *Dermatocarpon miniatum*, *Everinia divaricata*, *Evernia prunastri*, and *Neofuscella pulla*, which have not been studied yet.

Materials and Methods

Collection and identification of lichen samples

Lichen specimens were collected from Artvin province in year 2003. Samples were dried at room temperature for 48 h. Various flora books were used for identification of samples (Poelt & Vezda, 1981; Purvis et al., 1992; Wirth, 1995; Dobson, 2000). The lichen samples are stored in the herbarium of Kazım Karabekir Education Faculty, Atatürk University, Erzurum.

Preparation of the methanol extracts (MeOH)

Air-dried and powdered lichens (10 g) were extracted with 250 ml of methanol by using a Soxhlet extractor for 72 h at a temperature not exceeding the boiling point of the solvent (Lin et al., 1999). The methanol extracts were filtered using Whatman filter paper (no. 1) and then concentrated *in vacuo* at 40°C using a rotary evaporator. The residues obtained were stored in a freezer at −80°C until further tests.

DPPH assay

The hydrogen atom or electron donation ability of the corresponding extracts and some pure compounds was measured from the bleaching of purple-colored methanol solution of DPPH. This spectrophotometric assay uses stable radical diphenylpicrylhydrazyl (DPPH) as a reagent (Cuendet et al., 1997; Burits & Bucar, 2000). Fifty microliters of various concentrations of the extracts in methanol was added to 5 ml of a 0.004% methanol solution of DPPH. After a 30-min incubation period at room temperature, the absorbance was read against a blank at 517 nm. Inhibition free radical DPPH in percent (I%) was calculated in the following way:

$$I\% = (A_{blank} - A_{sample}/A_{blank}) \times 100$$

where A_{blank} is the absorbance of the control reaction (containing all reagents except the test compound), and A_{sample} is the absorbance of the test compound. Extract concentration providing 50% inhibition (IC_{50}) was calculated from a graph plotting inhibition percentage against extract concentration. Synthetic antioxidant reagent butylated hydroxytoluene (BHT) was used as positive control, and all tests were carried out in triplicate.

β-Carotene-linoleic acid assay

In this assay, antioxidant capacity is determined by measuring the inhibition of the volatile organic compounds and the conjugated diene hydroperoxides arising from linoleic acid oxidation (Dapkevicius et al., 1998). A stock solution of β-carotene–linoleic acid mixture was prepared as follows: 0.5 mg β-carotene was dissolved in 1 ml of chloroform (HPLC grade), and 25 µl linoleic acid and 200 mg Tween 40 was added. Chloroform was completely evaporated using a vacuum evaporator. Then 100 ml distilled water saturated with oxygen (30 min 100 ml/min) was added with vigorous shaking. A 2500-µl aliquot of this reaction mixture was dispersed to test tubes and 350 µl portions of the extracts prepared in ethanol at 2 g/l concentrations were added, and the emulsion system was incubated up to 48 h at room temperature. The same procedure was repeated with positive control BHT and a blank. After this incubation period, absorbance of the mixtures were measured at 490 nm. Antioxidative capacities of the extracts were compared with those of BHT at the same concentration and blank consisting only of 350 µl ethanol.

Assay for total phenols

The total phenolic content of the methanol extract of *C. foliacea*, *D. miniatum*, *E. divaricata*, *E. prunastri*, and *N. pulla* was determined employing literature methods (Slinkard & Singleton, 1977; Chandler & Dodds, 1983) involving Folin-Ciocalteu reagent and gallic acid as standard. An 0.1-ml aliquot of extract solution containing 1000 µg extract was taken in a volumetric flask, 46 ml distilled water and 1 ml Folin-Ciocalteu reagent were added, and the flask was shaken thoroughly. After 3 min, 3 ml of 2% Na_2CO_3 solution were added and the mixture was allowed to stand for 2 h with intermittent shaking. Absorbance was measured at 760 nm. The same procedure was repeated for all standard gallic acid solutions (0–1000 µg/0.1 ml), and standard curve was obtained with the equation given below:

$$\text{Absorbance: } 0.0012 \times \text{Gallic acid } (\mu g) + 0.0033.$$

Microbial strains

Methanol extracts were individually tested against a panel of microorganisms including a total of 54 microbial cultures belonging to 35 bacteria and 18 fungi and yeast species. The list of microorganisms used is given in Tables 1 and 2. Microorganisms were provided by the Department of Clinical Microbiology, Faculty of Medicine, and the Plant Diagnostic Laboratory, Faculty of Agriculture at Atatürk University, Erzurum, Turkey. Identity of the microorganisms used in this study was confirmed by the Microbial Identification System

Table 1. Antimicrobial activities of methanolic extracts of *Cladonia foliacea*, *Dermatocarpon miniatum*, *Evernia divaricata*, *Evernia prunastri*, and *Neofuscella pulla* against the bacterial strains tested based on disc-diffusion and micro-well dilution assay.

	Cladonia foliacea		*Dermatocarpon miniatum*		*Evernia divaricata*		*Evernia prunastri*		*Neofuscella pulla*		Antibiotics[a]	
Test microorganisms	DD[b]	MIC[c]	DD[b]	MIC[c]	DD[b]	MIC[c]	DD[b]	MIC[c]	DD[b]	MIC[c]	DD[a]	MIC[d] (max)
Acinetobacter baumanii A8	—	—	—	—	14	31.25	—	—	7	15.62	18 (OFX)	31.25
Acinetobacter lwoffi F1	—	—	—	—	—	—	—	—	—	—	24 (OFX)	62.50
Bacillus macerans A199	14	62.50	—	—	13	31.25	—	—	—	—	19 (OFX)	15.62
Bacillus megaterium A59	—	—	—	—	12	31.25	—	—	—	—	9 (SCF)	15.62
Bacillus subtilis ATCC-6633	11	31.25	16	62.50	18	62.50	—	—	12	31.25	28 (OFX)	62.50
Bacillus subtilis A57	7	15.62	—	—	9	31.25	—	—	9	15.62	28 (OFX)	125
Brucella abortus A77	—	—	—	—	12	31.25	—	—	—	—	12 (SCF)	62.50
Burkholdria cepacia A225	—	—	—	—	—	—	—	—	18	62.50	22 (SCF)	125
Clavibacter michiganense A227	16	62.50	18	62.50	18	62.50	9	15.62	14	31.25	25 (SCF)	16.62
Cedecea davisae F2	—	—	—	—	—	—	—	—	—	—	14 (OFX)	62.50
Enterobacter cloacae A135	—	—	—	—	16	62.50	—	—	—	—	20 (NET)	31.25
Enterococcus faecalis ATCC-29122	7	15.62	—	—	11	31.25	—	—	—	—	18 (SCF)	31.25
Escherichia coli A1	9	15.62	—	—	19	62.50	4	15.62	7	15.62	20 (OFX)	62.50
Klebsiella pneumoniae F3	—	—	—	—	—	—	—	—	—	—	12 (OFX)	125
Klebsiella pneumoniae A137	—	—	—	—	8	15.62	—	—	—	—	12 (OFX)	125
Morgonella morganii F4	—	—	—	—	—	—	—	—	—	—	14 (OFX)	125
Proteus vulgaris A161	—	—	—	—	7	15.62	—	—	—	—	13 (OFX)	125
Proteus vulgaris KUKEM1329	7	15.62	—	—	14	31.25	—	—	—	—	22 (NET)	31.25
Pseudomonas aeruginosa ATCC9027	—	—	—	—	9	15.62	—	—	—	—	22 (NET)	15.62
Pseudomonas aeruginosa ATCC27859	—	—	—	—	—	—	—	—	—	—	18 (NET)	125
Pseudomonas aeruginosa F5	—	—	—	—	—	—	—	—	—	—	18 (NET)	125
Pseudomonas pseudoalkaligenes F6	—	—	—	—	—	—	—	—	—	—	24 (OFX)	125
Pseudomonas syringae pv. *tomato* A35	13	31.25	—	—	14	31.25	9	31.25	9	31.25	14 (NET)	250
Salmonella cholerasuis arizonae F7	—	—	—	—	—	—	—	—	—	—	27 (SCF)	62.50
Salmonella enteritidis ATCC-13076	—	—	—	—	12	31.25	—	—	—	—	16 (NET)	125
Serratia plymuthica F8	—	—	—	—	—	—	—	—	—	—	24 (NET)	31.25
Shigella sonnei F9	—	—	—	—	—	—	—	—	—	—	22 (SCF)	31.25
Staphylococcus aureus A215	—	—	—	—	9	15.62	—	—	—	—	22 (SCF)	62.50
Staphylococcus aureus ATCC-29213	—	—	—	—	9	15.62	—	—	—	—	12 (SCF)	15.62
Staphylococcus epidermis A233	—	—	—	—	—	—	—	—	—	—	14 (SCF)	15.62
Staphylococcus hominis F10	—	—	—	—	—	—	—	—	—	—	10 (OFX)	62.50
Streptococcus pyogenes ATCC-176	14	31.25	—	—	15	31.25	7	15.62	10	31.25	13 (OFX)	31.25
Streptococcus pyogenes KUKEM-676	—	—	—	—	—	—	—	—	—	—	20 (SCF)	31.25
Xanthomonas campestris A235	19	62.50	—	—	17	62.50	14	31.25	—	—	16 (OFX)	62.50
Yersinia enterocolitica F11	—	—	—	—	—	—	—	—	—	—	—	—

[a]DD = Diameter of disk diffusion (mm); OFX = ofloxacin (10 μg/disk); SCF = sulbactam (30 μg) + cefoperazona (75 μg) (105 μg/disk); and NET = netilmicin (30μg/disk) were used as positive reference standards antibiotic discs (Oxoid).
[b]Inhibition zone in diameter (mm) around the disks impregnated with 300 μg/disk of methanol extract.
[c]Minimal inhibitory concentrations as (μg/ml).
[d]MIC = Maxipine (μg/ml) was used as reference antibiotic in micro-well dilution assay (Sigma).

at the Biotechnology Application and Research Center at Atatürk University.

Disk diffusion assay

The dried lichen extracts were dissolved in the same solvent (methanol) to a final concentration of 30 mg/ml and sterilized by filtration through 0.45-μm Millipore filters. Antimicrobial tests were then carried out by the disk diffusion method (Murray et al., 1995) using 100 μl of suspension containing 10^8 CFU/ml of bacteria, 10^6 CFU/ml of yeast, or 10^4 spore/ml of fungi spread on nutrient agar (NA), Sabouraud dextrose agar (SDA), and potato dextrose agar (PDA) medium, respectively. The disks (6 mm in diameter) were impregnated with 30 mg/ml extracts (300 μg/disk) and placed on the inoculated agar. Negative controls were prepared using the same solvents employed to dissolve the plant extracts. Ofloxacin (10 μg/disk), sulbactam (30 μg) + cefoperazona (75 μg) (105 μg/disk) and/or netilmicin (30 μg/disk)

Table 2. Anticandidal and antifungal activities of methanolic extracts of *Cladonia foliacea*, *Dermatocarpon miniatum*, *Evernia divaricata*, *Evernia prunastri*, and *Neofuscella pulla* against yeast and fungal isolates.

	Cladonia foliacea		*Dermatocarpon miniatum*		*Evernia divaricata*		*Evernia prunastri*		*Neofuscella pulla*		
Test yeast and fungi	DD[a]	MIC[b]	DD[a]	MIC[b]	DD[a]	MIC[b]	DD[b]	MIC[b]	DD[a]	MIC[b]	MIC[c] (AmpB)
Yeast											
Candida albicans A117	—	—	—	—	—	—	—	—	—	—	31.25
Fungi											
Alternaria solani	—	—	—	—	—	—	—	—	—	—	31.25
Aspergillus flavus	7	15.62	—	—	—	—	—	—	—	—	15.62
Aspergillus niger	—	—	—	—	—	—	31	62.50	—	—	15.62
Aspergillus variecolor	—	—	—	—	—	—	—	—	—	—	15.62
Fusarium acuminatum	—	—	—	—	30	62.50	—	—	—	—	62.50
Fusarium oxysporum	—	—	—	—	—	—	—	—	—	—	62.50
Fusarium solani	—	—	—	—	—	—	—	—	—	—	62.50
Fusarium tabacinum	—	—	—	—	—	—	—	—	—	—	62.50
Microsporum canis	—	—	—	—	16	31.25	—	—	—	—	62.50
Moniliania fructicola	—	—	—	—	—	—	—	—	—	—	15.62
Mortieraula alpina	—	—	—	—	—	—	—	—	—	—	62.50
Penicillum spp.	—	—	—	—	—	—	31	62.50	—	—	31.25
Rhizopus spp.	—	—	—	—	—	—	—	—	—	—	125
Rhizoctonia solani	—	—	—	—	19	31.25	—	—	—	—	31.25
Sclorotinia minor	24	125	—	—	—	—	—	—	—	—	125
Sclorotinia sclerotiorum	14	62.50	—	—	37	125	22	31.25	20	31.25	62.50
Trichophyton rubrum	—	—	—	—	—	—	—	—	24	62.50	31.25
Trichophyton mentagrophytes	—	—	—	—	—	—	—	—	—	—	15.62

[a]DD = Diameter of disk diffusion (mm). Inhibition zone in diameter (mm) around the disks impregnated with 300 µg/disk of methanol extract.
[b]Minimal inhibitory concentrations as (µg/ml).
[c]MIC = AmpB = amphotericin B (µg/ml) was used as refererence antibiotic in micro-well dilution (Sigma).

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°C for 24 h for clinical bacterial strains, 48 h for yeast, and 72 h for fungi isolates. Plant associated microorganisms were incubated at 27°C. Antimicrobial activity was evaluated by measuring the zone of inhibition against the test organisms. Each assay in this experiment was repeated twice.

Micro-well dilution assay

The minimal inhibition concentration (MIC) values were also studied for the microorganisms which were determined as sensitive to *C. foliacea*, *D. miniatum*, *E. divaricata*, *E. prunastri*, and *N. pulla* extracts in disk diffusion assays. The inocula of microorganisms were prepared from 12-h broth cultures, and suspensions were adjusted to 0.5 McFarland standard turbidity. The *C. foliacea*, *D. miniatum*, *E. divaricata*, *E. prunastri*, and *N. pulla* extracts dissolved in 10% dimethylsulfoxide (DMSO) were first diluted to the highest concentration (500 µg/ml) to be tested, and then serial two-fold dilutions were made in a concentration range from 7.8 to 500 µg/ml in 10-ml sterile test tubes containing nutrient broth. MIC values of *C. foliacea*, *D. miniatum*, *E. divaricata*, *E. prunastri*, and *N. pulla* extracts against bacterial strains and *Candida albicans* isolates were determined based on a micro-well dilution method (Sahin et al., 2002; Gulluce et al., 2004a, 2004b).

The 96-well plates were prepared by dispensing into each well 95 µl of nutrient broth and 5 µl of the inoculum. Amounts of 100 µl from *C. foliacea*, *D. miniatum*, *E. divaricata*, *E. prunastri*, and *N. pulla* extracts initially prepared at the concentration of 600 µg/ml were added into the first wells. Then, 100 µl from their serial dilutions was transferred into six consecutive wells. The last well containing 195 µl of nutrient broth without compound and 5 µl of the inoculum on each strip was used as negative control. The final volume in each well was 200 µl. Maxipime (Bristol-Myers Squibb, Fako Ilaclari As, Levent, Istanbul, Turkey) at the concentration range of 500–7.8 µg/ml was prepared in nutrient broth and used as standard drug for positive control. The plate was covered with a sterile plate sealer. Contents of each well were mixed on a plate shaker at 300 rpm for 20 s and then incubated at appropriate temperatures for 24 h. Microbial growth was determined by plating 5-µl samples from clear wells on nutrient agar medium. The extract tested in this study

was screened two times against each organism. The MIC was defined as the lowest concentration of the compounds to inhibit the growth of microorganisms.

Results and Discussion

Only a few reports concerning the antioxidative nature of lichens (Gulcin et al., 2002) are available in the literature. For example, *in vitro* antioxidant activity of aqueous extracts of *C. islandica* was reported earlier (Gulcin et al., 2002). The antioxidant activity of this species was found higher than that of α-tocopherol. Based on this information, free-radical scavenging and inhibition of linoleic acid oxidation of the methanol extracts of *C. foliacea*, *D. miniatum*, *E. divaricata*, *E. prunastri*, and *N. pulla* were investigated for the first time in this study.

Methanol extracts of all lichens tested did not exhibit notable free-radical scavenging activity and none of them was effective in the inhibition of linoleic acid oxidation (Table 3), except for that of *D. miniatum*. In the latter case, the extract provided 50% inhibition at 396 μg/ml concentration in DPPH assay and, moreover, exhibted significant activity on the inhibition of linoleic acid oxidation with 49% inhibition at 2 g/l concentration. Total phenolic constituents of these extracts and antioxidant activity results are also given in Table 3. When antioxidative 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. As reported elsewhere, this activity is increased with presence of polyphenols in particular (Behera et al., 2005). It has been reported that the antioxidant activity of phenols was mainly due to their redox properties, hydrogen donators, and single oxygen quenchers (Rice-Evans et al., 1995). Another supportive report also emphasizes the correlation between antioxidative capacity and phenolic constituents, particularly methyl orsenillate, orsenillic acid, atranorin and lecanoric acid (Jayaprakasha & Rao, 2000).

Table 3. Effect of the methanol extracts of *Cladonia foliacea*, *Dermatocarpon miniatum*, *Everinia divaricata*, *Evernia prunastri*, and *Neofuscella pulla* positive control on the *in vitro* free-radical scavenging and linoleic acid oxidation.

Sample	DPPH IC_{50} (μg/ml)	β-carotene/ linolieic acid (I%)	Total phenolic (%w/w)
Cladonia foliacea	NA	35 ± 1	1.7
Dermatocarpon miniatum	396 ± 0.5	49 ± 2	2.9
Everinia divaricata	NA	56 ± 1	3
Evernia prunastri	NA	46 ± 2	2.6
Neofuscella pulla	NA	32 ± 1	1.5
BHT (positive control)	19.8 ± 0.5	96 ± 1	—

NA, no activity observed.

The antimicrobial activities of *C. foliacea*, *D. miniatum*, *E. divaricata*, *E. prunastri*, and *N. pulla* extracts against microorganisms examined in the current study were qualitatively and quantitatively assessed by the presence or absence of inhibition zones and zone diameter and MIC values. The results are given in Tables 1 and 2.

The results showed that all of the extracts from five different lichen species have antibacterial activities against at least some of the bacterial species tested. The extract with the strongest antibacterial activity was obtained from *E. divaricata*, inhibiting 20 out of 35 bacteria tested. It was followed by *C. foliacea*, *N. pulla E. prunastri*, and *D. miniatum*, inhibiting 10, 8, 5, and 2 of the bacteria species tested, respectively.

The maximal inhibition zones and MIC values for bacterial strains, which were sensitive to the extracts of *E. divaricata*, *C. foliacea*, *N. pulla*, *E. prunastri*, and *D. miniatum*, were in the range of 9–19, 7–19, 9–18, 4–14, and 16–18 mm, 15.62–62.25, 15.62–62.25, 15.62–62.25, 15.62–31.25, and 62.25 μl/ml, respectively (Table 1). Except for *D. miniatum*, all other four lichen species were found to have antifungal activities against some of the fungal species (Table 2), but none of them have anticandidal activities.

The data in the current study confirmed the evidence in a previous study demonstrating the presence of antimicrobial substances in the extract of the some lichen species (Tay et al., 2004; Yilmaz et al., 2004). However this is the first study reporting antioxidant and antimicrobial activities of *Cladonia foliacea* Willd. Hudson *Dermatocarpon miniatum* (L.) Mann., *Everinia divaricata* (L.) Ach., and *Everinia prunastri* (L.) Ach., and *Neofuscella pulla* (Ach.) Essl. These findings may imply that the lichen species used differ from each other based on chemical constituents that were not identified in this study. Thus, further study is necessary to characterize the chemical constituents of the extracts from these lichen species. In addition, the data may also suggest that the extracts of lichen species tested possess compounds with antimicrobial properties as well as antioxidant activity, which requires further studies to determine antimicrobial agents in new drugs for therapy of infectious diseases in human and plant diseases.

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