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Evaluation of DNA Protective and Antimicrobial Properties of some Cladonia Species

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ABSTRACT

The present study evaluated the DNA protective properties and antimicrobial activities of the methanol extracts of nine *Cladonia* species, namely *C. pocillum, C. subulata, C. pyxidata, C. coniocraea, C. foliacea, C. firma, C. furcata, C. fimbriata* and *C. rangiformis* collected in Turkey. DNA protection properties efficiency of *Cladonia* extracts was evaluated using pBR322 plasmid DNA. In vitro antimicrobial activities of methanol extracts against two Gram-negative bacteria (*Escherichia coli* and *Proteus mirabilis*), three Gram-positive bacteria (*Staphylococcus aureus, Micrococcus luteus* and *Bacillus subtilis*) and two fungal strains (*Candida glabrata* and *Candida albicans*) were examined using the disc diffusion method and through the determination of minimal inhibitory concentrations (MIC). DNA protective studies, all *Cladonia* extracts protected pBR322 plasmid DNA against damage caused by the hydrogen peroxide (H_2O_2) with ultraviolet (UV). The results demonstrated that the inhibition zones in the disc diffusion method ranged from 6.5 to 19.0 mm. MIC results were ranged from 3.12 to 6.25 mg/mL. Cladonia extracts show a better antimicrobial effect against bacterial strains than fungal strains. The highest antimicrobial effect among lichen species was demonstrated by *Cladonia pocillum*. Our results demonstrated that tested *Cladonia* extracts had strong antibacterial and DNA protective effects. This is the first comprehensive study to evaluate the DNA protective properties activity of *Cladonia* extracts.

Keywords: Antimicrobial activity, *Cladonia*, DNA protective properties, Lichen

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Introduction

Lichens are natural resources used in the treatment of various diseases since ancient times. They are among the most fascinating organisms on earth and formed from the symbiotic relationship between the fungus ascomycetes and green algae or blue-green algae [1-3]. More than 1050 secondary metabolites have been isolated from lichens and have been found to have antibacterial, antiviral, anti-analgesic, antipyretic, and antiproliferative activities [4]. Lichens are of great interest to researchers as new important sources of bioactive substances due to the pronounced antimicrobial activity of secondary metabolites [5, 6]. Natural products are recommended as a therapeutic alternative to traditional antimicrobial therapy. Bacterial and fungal infection diseases remain the major causes of death worldwide [7]. Antibiotics are widely used to prevent and treat microbial infectious diseases, but new antibiotics and drugs are needed due to the emergence of antibiotic-resistant pathogens strains. The growing population of antibiotic-resistant micro-organisms motivated the investigation of lichens as an alternative antimicrobial drug [8-10]. Several studies have shown that some lichen species have antibacterial activity against micro-organisms [11-14].

The genus *Cladonia* is classified in the *Cladoniaceae* family (Ascomycota and Lecanorales) [15, 16]. The genus contains many secondary metabolites [17]. In previous studies, it has been determined that extracts of some *Cladonia* species have strong antioxidant, antimicrobial and anticancer activity in vitro [18-20].

This study aimed to determine the in vitro antimicrobial activities of extracts of *Cladonia* species (*C. pocillum* (Ach) O. J. Rich., *C. subulata* (L.) Weber ex F. H. Wigg., *C. pyxidata* (L.) Hoffm., *C. coniocraea* (Flörke) Spreng, *C. foliacea* (Huds.) Willd., *C. firma* (Nyl.) Nyl., *C. furcata* (Huds) Schrad, *C. fimbriata* (L.) Fr. and *C. rangiformis* Hoffm). All the species used in the study contain fumarprotocetraric acid. This acid has biological properties such as expectorant, antioxidant, antibacterial, antifungal, and anticancer [18, 21-24].

Oxidative stress-related DNA damage is associated with varied diseases. Numerous investigations have found that natural plant components have genotoxicity-protective action against oxidative stress and UV radiation [25]. As a result, the efficiency of *Cladonia* methanol extracts in protecting DNA from UV and oxidative stress was also investigated.

Materials and Methods

Lichen Samples

Lichen species were collected in field studies in different regions of Turkey. The morphological and anatomical features of the specimens were determined under the microscope, and their diagnosis was made using diagnostic keys [26, 27]. The species are preserved in Yozgat Bozok University, Boğazlıyan Vocational School Lichen Herbarium. The location information and herbarium numbers of the samples are given in Table 1.

Table 1. The Locality Information of Species and Herbarium Numbers

Species	Locality	Voucher
C. coniocraea→	Istanbul, Belgrad forests, Turkey, 28°55'683"E, 41°08'857"N, 20 m, 13 Eylül 2013	CLAD 77, 109
C. fimbriata→	Çankırı, Ilgaz, Turkey, 33°42'495"E, 41°00'848"N, 1200 m, 07 Temmuz 2014	CLAD 712, 713
C. firma→	Turkey, Çanakkale, Bayramiç, 26°45'634"E, 39°55'320"N, 220 m, 16 Eylül 2013	CLAD 52
C. foliacea→	Ankara, Güdül, Turkey, 32°09'54"E, 40°12'55"N, 750 m, 21 Temmuz 2014	CLAD 640
C. furcate→	Rize, Kackar Mountains National Park,Turkey, 41°08'801"E, 40°55'592"N, 1750 m, 16 Agustos 2014	CLAD 488
C. pocillum→	Mersin, Anamur, Turkey, 33°04'345"E, 36°05'592"N, 31 m, 19 Mayıs 2013 and Mersin, Çamlıyayla, 34°37'579"E 37°11'185"N, , 1350 m, 20 Mayıs 2013	CLAD 1, 55
C. pyxidata→	Çorum, Turkey, 34°49'277"E, 40°41'486"N, 1325 m, 25 Mayıs 2013	CLAD 135, 137
C. rangiformis→	Çorum, Turkey, 35°04'103"E, 40°31'855"N, 1186 m, 24 Mayıs 2013	CLAD 53
C. subulata→	Ordu, Çambaşı Plateau, Turkey, 37°56' 9"E, 40°44'06"N, 1560 m, 24 Eylül 2014	CLAD 998

Preparation of Lichen Extracts

Air-dried *C. pocillum* (5 g), *C. coniocraea* (4 g), *C. pxyidata* (3.5 g), *C. rangiformis* (30g), *C. foliacea* (10 g), *C. firma* (5 g), *C. furcata* (11 g), *C.subulata* (15 g), *C. fimbriata* (7g) thalli were pulverized and extracted three times in 70 percent methanol (MeOH) for 24 hours with periodic stirring. The extracts were removed from their solvents under vacuum (37 °C) after filtration. Before analysis, the extracts were lyophilized and kept at -18 °C.

Antimicrobial Assay

Antimicrobial activity of the *Cladonia* methanol extracts against bacterial strains (Gram-negative and Gram-positive), and fungal strains were examined by disc diffusion assay, MIC (Minimum inhibitory concentration), and MMC (Minimum microbicidal concentration). The test indicator bacteria included two Gram-negative bacteria (*Escherichia coli* ATCC25922*, Proteus mirabilis* ATCC25933), three Gram-positive bacteria (*Staphylococcus aureus* ATCT25923*, Micrococcus luteus* ATCC10240*,* and *Bacillus subtilis* ATCC6633*)* and two fungal strains (*Candida glabrata* ATCC90030, *Candida albicans* ATCC10231) were obtained from the culture collection of the Laboratory of Biotechnology, Faculty of Pharmacy, Erciyes University, Turkey. The bacterial strains were grown on Mueller Hinton Agar (MHA) medium and incubated at 37°C for 18-24h, and the fungal strains were grown on Sabouraud Dextrose Agar (SDA) medium and incubated 30°C for 36-48 h. A single colony was obtained from overnight strains using a sterile loop and inoculated into 5 mL of Mueller Hinton Broth (MHB) for tested bacterial strains and Sabouraud Dextrose Broth (SDB) for tested fungal strains. The inoculum strains were adjusted to the 0.5 McFarland standard turbidity. Then 100 µL micro-organisms (approximately 5 x10^5 CFU/mL) of the dilution was spread onto the agar plates containing MHA and SDA).

Sterile commercial paper discs (Oxoid; 6 mm) were placed on the above-inoculated media and impregnated with 20 µL of the methanol extract and incubated at 37 °C for 18-24 h (for bacteria) and 30°C for 36-48 h (for fungi). DMSO was used as a negative control, while Ampicillin: AMP and Nystatin: NS (10 µg/disc) were used as a positive control. All experiments were performed in duplicate, and the antibacterial/antifungal activities were assayed as the mean of a clear zone of inhibition diameter (mm) produced by the *Cladonia* extracts.

The Microdilution Method Determined the MIC values and minimum microbicidal concentration (MMC) (minimum bactericidal-fungicidal concentration (MBC-MFC)) values of the *Cladonia* methanol extracts in 96 multi-well microplates CLSI guidelines. The serial dilutions of *Cladonia* extracts were prepared with MHB for bacterial strains and SDB for fungal strains at a volume of 90 µL each well in microplates. A 100 µL of a stock solution (200 µg/mL) of all extract was added into the first well of the microplate. Then, serial dilutions were performed among the first and last wells. After, 10 µL of the diluted micro-organism suspension was added to all well to give a final concentration of 5 x 105 CFU/mL, making approximately 200 µL in each well. The obtained concentration range of the extracts was from 0.78 to 100 µg/mL. The added microplates were incubated at 37°C for 18-24 h for bacteria, 30°C for 36-48 h for fungi. Streptomycin and

ketoconazole were used as positive controls, and 10% DMSO solution was used as a negative control. The lowest concentration of the antimicrobial agent that did not produce visible growth (no turbidity observed) was defined as MIC. The MBC/MFC was determined on the agar medium by plating 10 µL of solution from each well where no visible growth was determined. After incubating at 37°C for 18-24h for bacteria and 30°C for 24-48h for bacteria. At the end of the incubation, the lowest concentration without growth was determined as MBC/MFC. All experiments were performed in duplicate.

DNA Cleavage Assay

The DNA cleavage activities of the *Cladonia* methanol extracts were shown by photolyzing hydrogen peroxide (H_2O_2) with ultraviolet (UV) in the presence of plasmid DNA (pBR322) and performing agarose gel electrophoresis. To prepare a 5% stock solution of extracts, 20 mg *Cladonia* extracts were weighed and dissolved in 400 µL dH2O. Up to 5 µL of the extract was added to each tube except for the control and 3 µL plasmid DNA (pBR322), and then 1 µL hydrogen peroxide (H_2O_2) in microcentrifuge tubes was added. All components with the *Cladonia* extracts, including tubes 2 and 4, were exposed to ultraviolet radiations for five mins. All tubes were incubated at 37°C for 1h. After irradiation, 5 µL of loading dye was added. They were loaded with Ethidium bromide (EtBr) staining 1% agarose gel at 90 V for 1.5-2 h in TAE (Tris base, acetic acid, and EDTA) buffer. The DNA fragments were visualized using ultraviolet illumination with a Bio-Rad Molecular Imager ChemiDoc XRS system (BioRad).

Statistical Analysis

The data are provided as mean values with a 95% confidence interval. ANOVA techniques were used for variance analysis. Tukey's pairwise comparison test was used to evaluate if there were significant differences between means at a threshold of $p<0.05$.

Results and Discussion

The antimicrobial activity of *Cladonia* methanol extracts was evaluated against microorganisms, and their potential effects were assessed qualitatively/quantitatively against the bacteria and fungi by the presence/ absence of inhibition zones MIC values. The *Cladonia* extracts inhibition zones obtained using disc diffusion assay are shown in Table 2. According to the results, the *Cladonia* extracts had great potential of antibacterial activity against all bacteria but did not have antifungal activity, except only the *C. pocillum* extract. The antimicrobial activity was checked with ampicillin and nystatin. These antibiotics had a more substantial effect than all extracts, as presented in Table 2. No inhibitory effect of DMSO, the negative control, was observed on the extracts. *Cladonia* extracts inhibited the bacterial strains produced a zone diameter of inhibition from 7.0 to 19 mm for Gram (-) and Gram (+) bacteria. In contrast, there was no antifungal activity for fungal strains of *Cladonia* extracts, except only *C. pocillum* extract. Antimicrobial activity analysis showed *C. pocillum* as the most potent extract and *C. pyxidata* as the weakest one. The methanol extract of *C. pocillum* was found to have the same significance ($p>0.05$) as the standard against Gram-negative *P. mirabilis*. It was also more effective against Gram-positive *B. subtilis* than the standard.

Table 2. Antimicrobial activity of extracts of Cladonia species(20 mg/mL) against tested bacteria and fungi using disc diffusion methods.

	Inhibition zone (mm)										
Micro-organisms	***Cladonia pocillum***	***Cladonia subulata***	***Cladonia pyxidata***	***Cladonia coniocraea***	***Cladonia foliacea***	***Cladonia firma***	***Cladonia furcata***	***Cladonia fimbriata***	***Cladonia rangiformis***	**Control (-)**	**Control (+)**
E. coli	14.96 ±0.60^{a}	NI*	NI	NI	6.93 ±0.45^{b}	NI	7.5 ±0.20^{b}	6.86 ±0.05^{b}	NI	NI	12.3 ±0.25^{c}
P. mirabilis	19.0 ±0.52^{a}	7.16 ±0.15^{b}	7.1 ±0.26^{b}	7.1 ±0.00^{b}	7.1 ±0.01^{b}	NI	NI	7.56 ±0.11^{b}	7.2 ±0.00^{b}	NI	20.02 ±0.2^{a}
S. aureus	14.13 ±0.06^{a}	7.53 ±0.4^{d}	NI	8.5 ±0.26^{b}	10.10 ±0.52^{c}	6.77 ±0.15^{d}	7.53 ±0.21^{d}	7.10 ±0.1^{d}	6.83 ±0.05^{d}	NI	21.03 ±0.25^{e}
B. subtilis	15.9 ±0.17^{a}	7.5 ±0.26^{b}	6.86 ±0.05^{b}	6.76 ±0.15^{b}	7.13 ±0.15^{b}	6.5 ±0.26^{b}	7.5 ±0.00^{b}	6.76 ±0.05b	8.0 ±0.17^{b}	NI	13.86 ±0.15^{c}
M. luteus	15.06 ±0.77^{a}	NI	NI	12.86 ±0.35^{b}	12.93 ±0.11^{b}	11.2 ±0.26 ^{c}	7.5 ±0.25 ^{d}	8.5 ±0.17^{e}	9.5 ±0.32^{e}	NI	22.06 ±0.31^{f}
C. albicans	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	15.16 ±0.15
C. glabrata	8.0 ±0.43^{a}	NI	NI	NI	NI	NI	NI	NI	NI	NI	14.86 ±0.21^{b}

Values with the same lower case letter (a–f) are not significantly ($p > 0.05$) different, n=3.

The MIC values of the tested *Cladonia* methanol extracts against the tested microorganisms ranged from 3.12 to 50 µg/mL (Table 3). The strongest antimicrobial activity was shown in the extract of the *C. pocillum*, which inhibited the tested bacteria and fungi species in a relatively low amount (3.12 to 25 µg/mL). The lowest activity was manifested by *C. pyxidata*, which inhibited the tested bacteria and fungi species at a concentration of 25 to 50 µg/mL. The most sensitive among the micro-organisms was *S. aureus*, and the highest resistance was shown in *B. subtilis* and fungal strains. The MIC and MBC/MFC that resulted in the same value for *Cladonia* extracts were observed against all micro-organisms. The strongest MBC and MFC were obtained with *C. pocillum* extract against *S. aureus* and *C. glabrata* (3.12 and 6.25 µg/mL), respectively.

Table 3. MIC and MMC of extracts of *Cladonia* species

Micro-organism	MIC(Minimum İnhibitory Concentration)($\mu g\ mL^{-1}$)/ *MBC/MFC(Minimum Bactericidal/Fumgucidal Concentration)($\mu g\ mL^{-1}$)								
	Cladonia pocillum	*Cladonia subulata*	*Cladonia pyxidata*	*Cladonia coniocraea*	*Cladonia foliacea*	*Cladonia firma*	*Cladonia furcata*	*Cladonia fimbriata*	*Cladonia rangiformis*
E. coli	3.12 *12.5	12.5 *25	25 *50	6.25 *25	6.25 *25	6.25 *25	12.5 *25	6.25 *25	12.5 *25
P. mirabilis	3.12 *12.5	25 *50	25 *50	25 *50	25 *50	25 *50	25 *50	25 *50	25 *50
S. aureus	3.12 *12.5	25 *25	25 *50	3.12 *6.25	3.12 *6.25	6.25 *12.5	12.5 *12.5	6.25 *6.25	6.25 *6.25
B. subtilis	3.12 *12.5	50 *50	50 *50	25 *50	25 *50	12.5 *50	25 *50	50 *50	50 *50
M. luteus	3.12 *12.5	12.5 *25	12.5 *25	3.12 *12.5	6.25 *12.5	12.5 *12.5	12.5 *25	6.25 *12.5	6.25 *12.5
C. albicans	25 *50	25 *50	50 *100	25 *50	25 *50	25 *50	25 *50	25 *50	25 *50
C. glabrata	6.25 *25	50 *100	50 *100	50 *100	100 *100	50 *100	50 *100	25 *50	25 *50

DNA cleavage assay has been investigated by inducing plasmid DNA damage by H_2O_2 and UV. The cleavage effect of *Cladonia* methanol extracts was assessed by the conversion of the plasmid DNA in the supercoiled (Form I) to its open circular (Form II) and the linear (Form III). The plasmid DNA (pBR322) damage results are shown in Fig. 1. The pBR322 ladder is clear in lane:1, while the pBR322 treated with UV and H_2O_2 revealed that plasmid DNA was damaged in lanes: 4. The H_2O_2 and UV together also induced pBR322 in lane:4. pBR322 treated with *Cladonia* extracts in the exposure of H_2O_2, and UV ırritation results are demonstrated in lanes: 5–13. In lane: 4, as a result of the interaction of the pBR322 with H_2O_2/UV, Form III is formed.

The antibacterial and antifungal properties of methanol extracts from *Cladonia* species (*C. pocillum, C. subulata, C. pyxidata, C. coniocraea, C. foliacea, C. firma, C. furcata, C. fimbriata,* and *C. rangiformis*) were investigated in this work. It is known that various *Cladonia* species exhibit different antimicrobial activities. The presence of diverse components is most likely responsible for the variances in antibacterial activity displayed by distinct *Cladonia* species [28]. In our experiments, *Cladonia* extracts demonstrated rather significant antibacterial activity but no antifungal activity. The severity of the antimicrobial effect depends on the lichen species, the concentration of their extracts, the contents of the extracts, and the tested microorganism. In our previous study, total phenol and flavonoid contents of *Cladonia* species were determined spectrophotometrically and fumarprotocetraric acid content was determined chromatographically (HPLC).

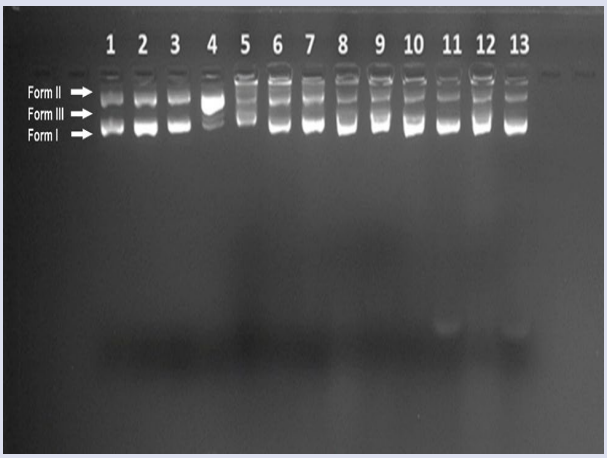

Figure 1. Line 1) PBR322 ladder, line 2) pBR322 / UV, line 3) pBR322 / H_2O_2, line 4) pBR322 / UV + H_2O_2, line 5) pBR322 and *C. pocillum* extract/ UV + H_2O_2, line 6) pBR322 and *C. subulata* extract/ UV + H_2O_2, line 7) pBR322 and *C. pyxidata* extract/ UV + H_2O_2, line 8) pBR322 and *C. coniocraea* extract/ UV + H_2O_2, line 9) pBR322 and *C. foliacea* extract/ UV + H_2O_2, line 10) pBR322 and *C. firma* extract/ UV + H_2O_2, line 11) pBR322 and *C. furcata* extract/ UV + H_2O_2, line 12) pBR322 and *C. fimbriata* extract/ UV + H_2O_2, line 13) pBR322 and *C. rangiformis* extract/ UV + H_2O_2

The amount of fumarprotocetraric acid was found to be between 1.89-23.82 mg g extract^{-1} by HPLC analysis. The highest total phenol content was found in *C. pocillum* with a value of 124 mg GAE g extract^{-1}. *C. fimbriata* has the highest fumarprotocetraric acid concentration (23.82±1.98 mg gextract^{-1}) [29] *C. pocillum* showed the strongest antibacterial effect among lichens studied at the same concentrations. This study demonstrated that the antibacterial action was mediated not just by fumarprotocetraric acid, but also by other phenolic compounds. Besides, these results proved that bacteria were more sensitive to antimicrobial agents than fungi in other studies. This difference is due to the cell wall structures of bacteria and fungi. The bacteria cell walls are composed of polysaccharide peptidoglycan, and fungal cell walls consist of chitin. It is thought that fungi are more resistant than bacteria because of the complex structure of the cell wall [19, 30]. *Cladonia* extracts were searched for antimicrobial effects in developing novel antimicrobial agents [31, 32]. Studzińska-Sroka et al. (2019) reported that an acetone extract of *C. uncialis* is active against *S. epidermidis and E. faecium* but did not display any activity against fungia. Kosanić et al. (2018) found that the five *Cladonia* lichens have strong antimicrobial activity against five bacteria and ten fungi strains. Mitrovic et al. (2015) investigated the antimicrobial activities of *C. foliacea* extract [13]. As a result of the study, C. *foliacea* extract had strong antimicrobial activity in all used tests. In addition, Açıkgöz et al. (2013) reported that acetone and chloroform extracts of two fruticose soil lichens, *C. rangiformis,* and *C. convoluta,* were active against two Gram-negative bacteria, two Gram-positive bacteria, and one fungal strain [33]. Our study of the antimicrobial effect of the extracts of *Cladonia* species showed a different degree of antimicrobial activity depending on the tested lichen species and the tested species of microorganisms. This is the first study of the antimicrobial activity of different *Cladonia* species originating from our region. In general, the tested lichen extracts showed good antibacterial activity.

Cladonia extracts were shown to have a significant protective effect, resulting in the formation of Form I and Form II fragments. These results demonstrated that UV/H_2O_2 induced plasmid DNA damage was protected. ROS (reactive oxygen species) may be produced by UV radiation and any oxygen factors. These destroy DNA or other cell components through oxidative stress [34]. It has been shown in some studies that free radicals induced DNA damage can be protected by using lichen extracts [35]. Recent research also supported the DNA damage protection efficacy of extracts of *Bryoria fuscescens*, *Umbilicaria decussata,* and *Parmelia tiliacea* lichens [36]. In another study, it has been shown that extracts of *Ganoderma lucidum* have significant radioprotective activity [37].This is the first study of the DNA damage protective effect of *Cladonia* species. Although it is not feasible to directly compare the results since no studies with *Cladonia* species have been undertaken, this study has demonstrated that the biological activity potential of lichens is substantial.

Conclusion

The antimicrobial activity of the nine lichen species from the famila *Cladoniaceae* (*C. pocillum, C. subulata, C. pyxidata, C. coniocraea, C. foliacea, C. firma, C. furcata, C. fimbriata,* and *C.rangiformis*) were demonstrated. *C. pocillum* methanol extract has the best antimicrobial activity against all bacterial strains. *Cladonia* methanol extracts also protected plasmid DNA (pBR322) from H_2O_2 and UV damage. It also demonstrated the potential of the extract to prevent DNA damage, which could be used in cancer research.

Further work will be done on the isolation and purification of components in the studied *Clodonia* species. Lichen compounds promise great potential for pharmaceutical applications as antimicrobial agents and in the development of novel formulations or molecules for the benefit of humanity.

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Conflicts of interest

The authors declare that they have no conflict of interest.

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