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Comparative Analyses of Antioxidant, Cytotoxic, and Anti-inflammatory Activities of Different *Cladonia* Species and Determination of Fumarprotocetraric Acid Amounts

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ABSTRACT
The study aimed to determine the biological activities of 70% methanol extracts of nine *Cladonia* species from Turkey. The chemical composition was analyzed via spectrophotometric and chromatographic (HPLC) techniques. DPPH• (1,1-diphenyl-2-picrylhydrazyl) and ABTS$^{+\bullet}$ (2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) radical scavenging activities, inhibition of ß-carotene/linoleic acid co-oxidation, and cytotoxicity on A549 and COLO205 cell lines were studied. Anti-inflammatory activity was assessed on the Raw 264.7 mouse macrophage cell line by determining nitric oxide (NO) levels. Among the studied *Cladonia* species *Cladonia* firma was found the most active extract in ABTS$^{+\bullet}$ test system (2.58±0.04 mmol/L/Trolox) and also in the cytotoxicity and anti-inflammatory experiments. *Cladonia pocillum*, *Cladonia rangiformis*, and *Cladonia foliaceae* were also found to be active. Fumarprotocetraric acid amounts are between 1.89-23.82 mg gextract^{-1} and fumarprotocetraric acid content did not show linearity with biological activities. It has been proved with this study that lichens traditionally used in treatment have as much biological activity as plants and can be evaluated in pharmaceuticals and cosmetics.

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Farklı *Cladonia* Türlerinin Antioksidan, Sitotoksik ve Antienflamatuvar Aktivitelerinin Karşılaştırmalı Analizleri ve Fumarprotosetrarik Asit Miktarlarının Belirlenmesi

ÖZET
Çalışmada, Türkiye'den 9 *Cladonia* türünün % 70 metanol ekstrelerinin biyolojik aktivitelerinin belirlemesi amaçlandı. Kimyasal bileşimi, spektrofotometrik ve kromatografik (HPLC) tekniklerle analiz edildi. Ekstrelerin DPPH• (1,1-difenil-2-pikrilhidrazil) ve ABTS$^{+\bullet}$ (2,2'-azino-bis (3-etilbenzotiyazolin-6-sülfonik asit) radikal süpürücü aktiviteleri, ß-karoten/linoleik asit birlikte oksidasyonunun inhibisyonu ve A549 ve COLO205 hücre hatlarında sitotoksisiteleri çalışıldı. Antienflamatuvar aktivite, Raw 264.7 fare makrofaj hücre hattında nitrik oksit (NO) seviyelerinin belirlenmesiyle değerlendirildi. Çalışılan *Cladonia* türleri arasında *Cladonia firma*, ABTS$^{+\bullet}$ test sisteminde (2.58 ± 0.04 mmol/L/Troloks) ve ayrıca sitotoksisite ve antienflamatuvar deneylerinde en aktif ekstre olarak belirlendi. *Cladonia pocillum*, *Cladonia rangiformis* ve *Cladonia foliaceae*'nin de aktif olduğu bulundu. Fumarprotosetrarik asit miktarları 1.89-23.82 mg gekstre^{-1} arasında olup fumarprotosetrarik asit içeriği biyolojik aktivitelerle doğrusallık göstermedi. Geleneksel olarak tedavide kullanılan likenlerin bitkiler kadar biyolojik aktiviteye sahip olduğu ve ilaç ve kozmetikte değerlendirilebileceği bu çalışma ile kanıtlanmıştır.

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INTRODUCTION

Lichens are declared as composite organisms, consist of the symbiotic relationship between the fungus and a green alga (and/or cyanobacteria) as a photosynthetic partner (Hodkinson and Lutzoni, 2009; Selbmann et al., 2010). Lichens have been used as additives to folk medicines for many years, as well as in food, spices, and cosmetics in countries such as Afghanistan, China, India, Ireland, Spain and New Zealand (Dayan and Romagni, 2001; Huneck, 1999; Malhotra et al., 2007; Shukla et al., 2014). Lichens have antibacterial, antiproliferative, antioxidant, anti-HIV, anti-cancer, immune regulation, and anti-protozoan effects (Caviglia et al., 2001; Behera et al., 2005; Bhattacharyya et al., 2016). A large number of secondary metabolites, including depsides, depsidones, dibenzofurans, xanthones and terpene derivatives are produced by lichens (Molnár and Farkas, 2010). The *Cladonia* genus is classified within the Cladoniaceae family (Lecanorales order and Ascomycota division) and the genus contains a large number of secondary metabolites including lichenic acids, phenolic compounds consisting mainly of depsides and depsidones (Huovinen and Ahti, 1982; Miadlikowska et al., 2006; Lumbsch and Huhndorf, 2010). *C. rangiferina*, known as reindeer lichens, is used as a medicinal tea for internal chest pains (Smith, 1973) as a medicine for diarrhea (Kari, 1987). Extracts of *Cladonia* species are used in folk medicine, therefore they have been evaluated in many studies for their biological activities (Koparal et al., 2006; Silva et al., 2010; Kosani et al., 2014; Coskun et al., 2015). Fumarprotocetraric acid, one of the depsidones of *Cladonia* species, has antioxidant, antitumor, antimicrobial, and also different biological activities (Ramos et al., 2014).

A common feature of the pathogenesis of most chronic diseases is the oxidative stress associated with the production of reactive oxygen species (ROS) (Antal, 2004). Due to its high reactivity and low stability, ROS enters lipids, proteins, and deoxyribonucleic acid (DNA) reactions. Oxidative damage caused by free radicals can initiate and promote the progression of certain chronic diseases, such as cancer, cardiovascular diseases, atherosclerosis, cataracts, and inflammation, in addition to neurodegenerative diseases (Parejo et al., 2002). The antioxidant mechanisms of phenolics include the removal of free radicals to terminate the radical chain reaction, the absorption of oxygen radicals (ROS), the chelation of the transition metals, the intervention of enzymes, the production and stimulation of ROS antioxidant enzyme activities. These activities of phenolics have been reported in many literatures to reduce the incidence of cancer. Phenolic antiproliferative agents have been involved in cell cycle arrest, induction of apoptosis, destruction of mitotic spindle formation, and inhibition of angiogenesis (Syiem et al., 2009; Hoshyar et al., 2015). Phenolics are also essential compounds among phytochemicals to suppress inflammation, and their anti-inflammatory capacity has also been demonstrated in studies (Shahidi and Yeo, 2018). A wide variety of chronic inflammatory conditions has been shown in many kinds of research that it predisposes sensitive cells to neoplastic transformation and that chronic inflammation is associated with cancer (Khansari et al., 2019).

Despite the traditional usage of lichens, there is a lack of information about their biological properties. Therefore, this study aims to evaluate the relationship between the *in vitro* antioxidant, cytotoxic and anti-inflammatory activities, and phenolic compound fumarprotocetraric acid amounts of nine traditionally used *Cladonia* species: *Cladonia coniocraea* (Flörke) Spreng., *Cladonia fimbriata* (L.) Fr., *Cladonia firma* (Nyl.) Nyl., *Cladonia foliacea* (Huds.) Willd., *Cladonia furcata* (Huds.) Schrad., *Cladonia pocillum* (Ach.) O.J. Rich., *Cladonia pyxidata* (L.) Hoffm., *Cladonia rangiformis* Hoffm., and *Cladonia subulata* (L.) Weber ex F.H. Wigg.

Herein, the radical scavenging activities of DPPH• and $ABTS^{+\bullet}$ and the inhibition of ß-carotene/linoleic acid co-oxidation were investigated. Besides, cytotoxic activities on the A549 (Human non-small adenocarcinoma alveolar basal epithelial cells) and COLO 205 (Human colon adenocarcinoma cell line) cell lines and anti-inflammatory activities in the Raw 264.7 (Mouse macrophage cell line) cell line were examined.

METHODS

Lichen Material and Reagents

The lichen samples were collected during 2013-2014 field studies. The collected specimens were diagnosed by using keys according to morphological and anatomical characteristics (Ahti and Hammer, 2002; Ahti et al., 2013). The samples were preserved in Bozok University, Bogazliyan Vocational School, Organic Agriculture Department. All chemical substances used in the experiments were of analytical quality and obtained from the Sigma Chemical Company (St. Louis, MO).

The locality information and herbarium numbers of *Cladonia species*

C. coniocraea: Turkey, Istanbul, Kemerburgaz on the way, Belgrad forests, *Pinus brutia* forests, 41°08'857"N, 28°55'683"E, alt. 20 m, 13/09/2013 (CLAD 77, 109); ***C. fimbriata***: Turkey, Çankırı, Ilgaz, East of Kastamonu-Ilgaz road, Başaraz village road, *Pinus nigra* and *Quercus* communities, 41°00'848"N, 33°42'495"E, alt. 1200 m, 07/07/2014 (CLAD 712, 713); ***C. firma***: Turkey, Çanakkale, Bayramiç, Northwest of

Hacıbekirler village, *Pinus brutia*, *Quercus* communities, siliceous rocks, 39°55'320"N, 26°45'634"E, alt. 220 m, 16/09/2013 (CLAD 52); ***C. foliacea***: Turkey, Ankara, Güdül, Between Güdül and Beypazarı, serpentine rocks, 40°12'55"N, 32°09'54"E, alt. 750 m, 21/07/2014 (CLAD 640); ***C. furcata***: Turkey, Rize, Çamlıhemşin, Kackar Mountains National Park, North of Ayder, Kavrun plateau road, *Picea* and *Fagus* mixed forest, 40°55'592"N, 41°08'801"E, alt. 1750 m, 16/08/2014 (CLAD 488); ***C. pocillum***: Turkey, Mersin, Anamur, Antalya-Mersin road, Northwest of the Bozyazı Peninsula, Puren neighborhood, *Pinus brutia* forest, maquis vegetation, serpentine, 36°05'592"N, 33°04'345"E, alt. 31 m, 19/05/2013 and Mersin, Çamlıyayla, the northern part of the cattle plateau, limestone bedrock *Juniperus* sp. communities, 37°11'185"N, 34°37'579"E, alt. 1350 m, 20/05/2013 (CLAD 1, 55); ***C. pyxidata***: Turkey, Çorum, North of Çatak Village, *Pinus nigra*-*Quercus* forest, serpentine bedrock, 40°41'486"N, 34°49'277"E, alt. 1325 m, 25/05/2013 (CLAD 135, 137); ***C. rangiformis***: Turkey, Çorum, Located east of Belen, step vegetation, *Rosa canina*, limestone bedrock, 40°31'855"N, 35°04'103"E, alt. 1186 m, 24/05/2013 (CLAD 53); ***C. subulata***: Turkey, Ordu, Çambaşı, Çambaşı Plateau, *Picea orientalis* and *Pinus nigra* forest, 40°44'06"N, 37°56' 9"E, alt. 1560 m, 24/09/2014 (CLAD 998).

Preparation of the extracts

Air-dried *C. pocillum* (4.9 g), *C. coniocraea* (3.9 g), *C. pxyidata* (3.3 g), *C. rangiformis* (31g), *C. foliacea* (12 g), *C. firma* (6 g), *C. furcata* (10 g), *C. subulata* (15.8 g), *C. fimbriata* (8 g) thalli materials were powdered with grinder (IKA MF10.1, China) and extracted 3 times for 24 h using 70 % methanol (MeOH) in a shaking water bath at room temperature. After the extracts were filtered, they were removed from their solvent under vacuum (37 °C). Following this procedure, the extracts were lyophilized and stored at -18 °C before analysis.

Total phenolic and flavonoid content

The total phenolic content of *Cladonia* extracts was measured by using the Folin-Ciocalteu assay and estimated as gallic acid equivalents (GAE). The total flavonoid content was determined by using the aluminum chloride assay and estimated as catechin (CA) equivalents (Zhishen et al., 1999). Extracts were prepared at a concentration of 2 mg mL^{-1} and dissolved in 70 % MeOH.

Determination of fumarprotocetraric acid amount with High Performance Liquid Chromatography (HPLC)

Dry *Cladonia* extracts were dissolved in 70 % MeOH and analyzed with HPLC (Shimadzu LC-20AT) instrument with a C18 column (Mediterranean-C18, 5µm; 4.6x250 mm) using PDA spectrophotometric detector between the wavelengths of 200 and 550 nm. Analysis time set to 20 minutes. Methanol-water-phosphoric acid (75:25:0.9, v/v/v) was used as a mobile solvent and the flow rate was set to 1 mL min^{-1}. The injection volume of the samples was applied as 10 µL. Fumarprotocetraric acid standard was dissolved in 70 % MeOH and diluted to five different concentrations to obtain calibration curve (Kosanic et al., 2018).

DPPH• radical scavenging activity

The method of Gyamfi et al. (1999) was used to evaluate the DPPH• radical removal effects of the extracts. Extracts were prepared at 10 different concentrations between 0.025-4 mg mL^{-1} concentrations ranges. Butylated Hydroxytoluene (BHT) was used as a standard and prepared at 10 different concentrations between 0.001-1 mg mL^{-1}. 50 µL sample, 450 µL Tris-HCl buffer (pH 7.4 50 mM), 1 mL DPPH• radical (0.1 mM in MeOH) were put into the test tubes, respectively. After incubating in darkness at room temperature for 30 min, their absorbance at 517 nm against the blind, which is composed of MeOH, was recorded. EC_{50} values of the extracts presented as the mean value of the triple analysis and the % inhibition calculated using Eq 1.

$$\%\ inhibition = [(Abscontrol - Abssample) / Abscontrol] \times 100 \quad \text{(Eq. 1)}$$

$ABTS^{+}$• radical scavenging activity

The method of Re et al. (1999) was used to evaluate $ABTS^{+\bullet}$ scavenging effects of the extracts. 2.45 mM $K_2S_2O_8$ and 7 mM $ABTS^{+\bullet}$ solutions were mixed in a ratio of 1:1 and incubated for 16 hours in the dark at room temperature. This prepared $ABTS^{+\bullet}$ radical solution was measured at 734 nm and diluted with ethyl alcohol until an absorbance of 0.700 (± 0.030) was reached. The reaction kinetics of the 990 µL radical solution, which was mixed with 10 µL sample solution for 30 minutes at 1-minute intervals, was measured at 734 nm. Extracts were studied at 1-4 mg mL^{-1} and standard BHT studied at 0.5-1 mg mL^{-1} concentrations. Percent inhibition of all samples was calculated as equivalent to Trolox (TEAC) and the mean value of the triple analysis was used.

Inhibition of β-carotene bleaching

In this assay, the antioxidant activity of lichen extracts was evaluated using the method of Velioglu et al. (1998). After mixing, 1.2 mL of *β*-carotene solution (1 mg mL^{-1} in chloroform), Tween 20, and linoleic acid, chloroform removed from media via rotary evaporator. The emulsion was prepared by adding distilled water to this mixture and mixing gently. For the blanks of the control and samples, the same procedure was performed without using β-carotene. BHT was used as a standard in the test system. After the extracts, standards, and controls were put into tubes together with the emulsion, these samples were kept in a water bath for 2 hours at 50 °C for autoxidation and the

fading level was measured at 470 nm every 15 minutes. Inhibition of ß-carotene bleaching was calculated using Eq. 2.

$AA\% = [1-(Abs0\ sample - Abs120\ sample)/(Abs0\ control - Abs120\ control)] \times 100$ (Eq. 2)

Cell culture

Mouse macrophage cell line (Raw 264.7), Human non-small adenocarcinoma alveolar basal epithelial cells (A549), and Human colon adenocarcinoma cell line (COLO 205) that we used in our experimental studies were purchased from the American Type Culture Collection (ATCC TIB-71, ATCC CCL-185, CCL-222, Manassas, VA, USA). The growing medium of cultures; DMEM, and RPMI (Invitrogen, Carlsbad, CA) with 1 % penicillin, streptomycin mixture solution (Invitrogen,) and 10 % FBS. The cultures were kept at 37 °C in 5 % CO_2 and 95 % air (Gaidhani et al., 2013).

Cytotoxicity on A549 and COLO 205 cells

Cell viability on A549 and COLO 205 cells, was assayed using a 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) test. Cultured cells were seeded (1×10^4 cell/well) in a 96-well microplate. Extracts prepared in the concentration range of 15.6-2000 µg mL^{-1} were then added to the plates kept at 37 °C for 24 hours for cell adhesion. After incubation 24 hours, the fluids in the wells were discharged and MTT solution in the fresh medium was added. Following 2 hours of incubation, dimethyl sulfoxide (DMSO) was added to each well to dissolve the purple MTT formazan crystals after removing the medium containing the MTT solution. In three repeated experiments, the absorbance of plates was read using Elisa (Biotek Synergy HT) at a wavelength of 570 nm and the results were given as mean ± SD.

Determination of NO in the Raw 264.7 cell line

The toxicity of the extracts in the Raw 264.7 cell line in the concentration range of 5-100 µg mL^{-1} was determined by the MTT method as mentioned above. After the non-toxic doses were determined, the cells were seeded at a number of $5x10^5$ to 6 well plates and incubated for 24 hours. After incubation, lipopolysaccharide (LPS) was added to other wells, except for the control well, at a concentration of 1 µg mL^{-1}. The extracts were added to wells at a concentration of 20 and 40 µg mL^{-1}, and indomethacin at a concentration of 25 µM after 3 hours. At the end of 24 hours, supernatants were collected, and centrifuged (10 min., 700 x g). Each 100 µL of culture supernatant was mixed with the same volume of Griess Reagent (Sigma-Aldrich, St. Louis, MO). After incubating 10 min. at room temperature absorbance was read at 540 nm using Elisa. Sodium nitrite was used as a standard to prepare the calibration curve.

Statistical analysis

Levene test was performed for variance homogeneity. One-way analysis of variance was used among multiple groups. Dunnett test and Tukey test were applied for multiple comparison test at $P<0.05$ level. EC 50 values were calculated using nonlinear regression curves (Sigma Plot 2001 version 7.0, SPSS Inc., Chicago IL).

RESULTS

Total phenolics and flavonoids

Total phenol and flavonoid amounts of the extracts were calculated using the spectrophotometric method and found that the *C. pocillum* and *C. coniocraea* contained a high amount of phenolic material (124.04±2.73; 103.53±1.59 $mg_{GAE}\ g_{extract}^{-1}$, respectively) (Table 1). The extract with the highest total flavonoid content was the *C. foliacea* extract (22.46±1.19 $mg_{CAE}\ g_{extract}^{-1}$).

Determination of fumarprotocetraric acid amount with HPLC

The chromatogram of *C. pocillum* extract and fumarprotocetraric acid amounts of the lichen extracts represented in Figure 1 and Table 1. The lowest fumarprotocetraric acid amount was identified in the *C. rangiformis* (18.89 mg $g_{extract}^{-1}$) and the highest amount was identified in *C. fimbriata* (232.82 mg $g_{extract}^{-1}$).

DPPH● radical scavenging

The capacity of extracts in physiological pH to remove the DPPH• radical, a stable and nitrogen-centered radical, has been investigated. Extracts were studied at 1, 2 and 4 mg mL^{-1} concentration. *C. pocillum* extract was found the most active extract in scavenging the DPPH• radical. *C. pocillum* extract showed higher inhibition and its EC_{50} value was found 0.24±0.05 mg mL^{-1}. The second-active ones were evaluated as *C. foliaceae* and *C. firma* showing similar inhibition percentages. EC_{50} value of the BHT was found to be 0.14±0.005 mg mL^{-1} (Table 2).

$ABTS^{+}$● radical scavenging

$ABTS^{+}$• radical scavenging capacity of extracts and BHT at concentrations of 0.50-4 mg mL^{-1} were evaluated in this experiment. All extracts exhibited the highest activity at 4 mg mL^{-1}, while none exceeded the activity of BHT used as a positive control at a concentration of 1 mg mL^{-1} Surprisingly *C. foliaceae* and *C. firma* showed more potent activity than *C. pocillum* in this test system (Table 2). $ABTS^{+}$• radical removal capacity at 4 mg mL^{-1} is evaluated and the ranking is as follows: *C. firma*> *C. foliaceae*> *C. fimbriata*> *C. rangiformis*> *C. coniocraea*> *C. pocillum*> *C. furcata*> *C.pxyidata* ~ *C. subulata*.

Table 1. Total phenol, flavonoid and fumarprotocetraric acid amount of *Cladonia* species
Çizelge 1. Cladonia türlerinin toplam fenol, flavonoit ve fumarprotosetrarik asit miktarı

Species (Türler)	Total phenols (Toplam fenoller) [mg_{GAE} $g_{extract}^{-1}$]	Total flavonoids (Toplam flavonoidler) [mg CAE $g_{extract}^{-1}$]	Fumarprotocetraric acid amount (Fumarprotosetrarik asit miktarı) (mg $g_{extract}^{-1}$)
C. pocillum	124.04±2.73	20.57±0.79	13.59±0.68
C. coniocraea	103.53±1.59	19.50±0.52	18.82±1.39
C. pxyidata	80.71±0.67	16.66±0.41	13.94±1.36
C. rangiformis	78.39±0.85	17.18±0.14	1.89 ±0.12
C. foliacea	91.20±2.85	22.46±1.19	18.58 ±0.68
C. firma	72.07±0.00	15.03±0.86	8.47±1.97
C. furcata	59.58±0.70	17.13±2.38	3.48±1.24
C. subulata	80.40±3.00	16.19±0.57	9.19±0.68
C. fimbriata	86.10±2.42	19.58±0.42	23.82±1.98

Data are expressed as mean ± standard error (n=3)

Figure 1. Chromatogram of *C. pocillum* lichen extract at 254 nm
Şekil 1. 254 nm'de C. pocillum liken ekstresinin kromatogramı

Table 2. DPPH and ABTS radical scavenging effects of *Cladonia* species
Çizelge 2. Cladonia türlerinin DPPH ve ABTS radikal süpürme etkileri

Species (Türler)	DPPH (% Inhibition, EC_{50}) *(% İnhibisyon, EC_{50})*			EC_{50} (µg mL^{-1})	ABTS (mmol/L/Trolox)			
	4 mg mL^{-1}	2 mg mL^{-1}	1 mg mL^{-1}		4 mg mL^{-1}	2 mg mL^{-1}	1 mg mL^{1}	0.5 mg mL^{1}
C. pocillum	89.74±0.12	86.30±0.69	85.17±0.05	0.24±0.05	2.41±0.07	2.01±0.08	1.29±0.05	
C. coniocraea	71.35±1.86	59.13±0.02	52.93±0.35		2.48±0.06	1.89±0.08	1.25±0.07	
C. pxyidata	72.46±0.94	68.32±0.04	57.27±0.07		1.67±0.08	1.30±0.05	1.26±0.05	
C. rangiformis	69.84±0.44	47.24±1.62	35.39±0.67		2.50±0.04	2.11±0.01	1.30±0.15	
C. foliacea	74.75±1.91	73.62±0.16	61.78±0.18		2.57±0.03	2.12±0.20	1.41±0.09	
C. firma	74.81±2.66	68.77±1.08	60.32±1.18		2.58±0.04	1.59±0.11	1.18±0.07	
C. furcata	56.62±0.15	32.91±2.42	24.46±0.81		2.34±0.10	1.88±0.15	1.49±0.13	
C. subulata	69.10±0.83	23.08±4.27	15.76±2.09		1.67±0.08	1.14±0.08	0.68±0.06	
C. fimbriata	73.97±0.83	52.82±0.55	38.73±0.43		2.52±0.05	1.94±0.11	1.23±0.03	
BHT				0.14±0.005			2.51±0.02	1.17±0.03

Data are expressed as mean ± standard error (n=3)

Inhibition of β-carotene bleaching

The oxidation inhibitory effect of the extracts was evaluated and the time-dependent change inhibition percentages were given in Figure 2. According to Figure 2, all extracts appear to inhibit oxidation but show less activity than positive control BHT. It was found that all extracts showed higher inhibition in the first 30 minutes, and at the end of 90 minutes, lipid peroxidation inhibitory effects decreased, while *C. rangiformis* was more active than other extracts.

Cytotoxicity on A549 and COLO 205 cells

The results obtained by the MTT cell viability method for the A549 cell line are given in Table 3. The results were given as percentage (%) and cell control group viability was accepted as 100 % and other groups were calculated. Significance of Levene statistics to test homogeneity of variance was found (P<0.05). Dunnet T3 test was applied to groups in which the assumption of normality was not provided for the variable. All the extracts at 15.6 µg mL^{-1} concentration were not found

statistically different (P>0.05) from the control group. At 2000 µg mL^{-1} concentration *C. pocillum, C. coniocraea, C. rangiformis, C. foliacea, C. firma, C. subulata* and *C. fimbriata* were found statistically different from the control group (P <0.001). *C. firma* extract was statistically more significant when inhibition values were examined in all concentrations.

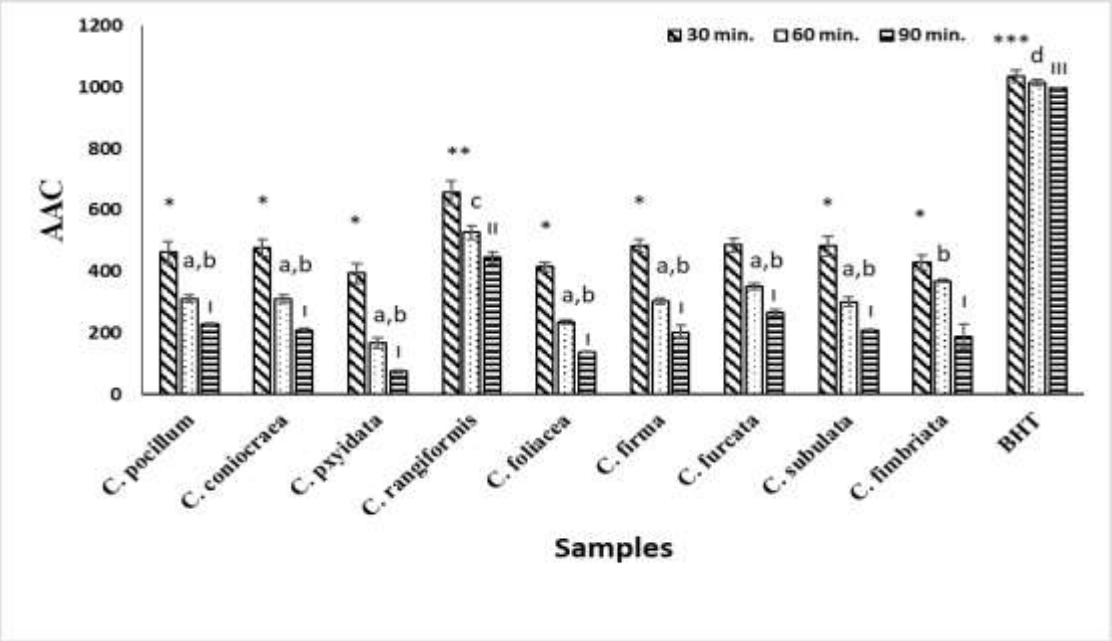

Figure 2. The effect of the *Cladonia* extracts and positive control on ß-carotene/linoleic acid co-oxidation. Bars with the same symbol, lower case letter and number (*,***), (a–d), (1–5) are not significantly (P>0.05) different (n=3)

Şekil 2. Cladonia ekstrelerinin ve pozitif kontolün ß-carotene/linoleik asit birlikte oksidasyonu üzerine etkileri. Aynı sembol, küçük harf ve sayıya (, ***), (a – d), (1-5) sahip değerler istatistiksel olarak farklı değildir(P> 0.05)*

The results obtained by the MTT cell viability method for COLO 205 cell line are given in Table 3. The results were given as percentage (%) and cell control group viability was accepted as 100 % and other groups were calculated. Significance of Levene statistics to test homogeneity of variance was found (P <0.05). Dunnet T3 test was applied to groups in which the assumption of normality was not provided for the variable. Among the all studied extracts only *C. foliacea* and *C. firma* were found statistically different from the control group (P<0.01) at 15.6 µg mL^{-1} concentration. At 1000 and 2000 µg mL^{-1} concentrations all the extracts showed potent inhibitory activity on COLO 205 cell line (P<0.001).

Determination of NO in the Raw 264.7 cell line

The results obtained by the MTT cell viability method for the Raw 264.7 cell line are given in Table 4. Considering the results of the MTT test, 20 and 40 µg mL^{-1} concentrations with the same significance compared to the control group were selected. When the control group and the LPS group were compared, the fact that the LPS group was significantly higher than the control group (P<0.001) showed that inflammation occurred (Table 5). Indomethacin administered at 25 µM doses brought the nitric oxide amount to the same level as the control group. None of the extracts were found to be statistically significant with indomethacin (P>0.05).

DISCUSSION

In this study, chemical composition and *in vitro* antioxidant, cytotoxic and anti-inflammatory effects of nine *Cladonia* species were investigated. The biological activity of most of the species investigated in this study was illuminated for the first time.

The total phenol, and total flavonoid amounts of the extracts which were prepared from the thalli part of lichens, were determined by spectrophotometric methods. Total phenol amounts were found between the range of 72.07±0.005 - 124.04±2.73 mg_{GAE} $g_{extract}^{-1}$ and total flavonoid amounts were found between the ranges of 16.19±0.57 - 22.46±1.19 mg_{CAE} $g_{extract}^{-1}$ (Table 1). Comparing the herein reported results about the total phenolic and flavonoid contents with Kosanic et al. (2018), our results seem higher than their results. Similar to us they have studied with *C. fimbriata, C. furcata, C. subulata* and *C. foliacea*. In a different study, the total phenolic amounts of the *C. subulata*, *C. furcata,* and *C. fimbriata* methanolic extracts were .

Table 3. Cytotoxic activity of *Cladonia* species on A549 and COLO 205 lines
Çizelge 3. Cladonia türlerinin A549 ve COLO 205 hatlarında sitotoksik aktivitesi

% Cell Viability (% Hücre Canlılığı) Concentrations (Konsantrasyonlar)									
Species (Türler)	**Cell Lines**	**15.6 µg mL^{-1}**	**31.25 µg mL^{-1}**	**62.5 µg mL^{-1}**	**125 µg mL^{-1}**	**250 µg mL^{-1}**	**500 µg mL^{-1}**	**1000 µg mL^{-1}**	**2000 µg mL^{-1}**
C. pocillum	A549	75.71±8.16	77.49±4.66	76.54±5.78*	70.52±5.48*	49.90±6.94**	51.23±6.33**	20.68±3.62***	11.50±0.21***
	COLO 205	71.81±6.35	72.47±8.19	62.44±10.26*	35.93±4.84***	29.39±5.09***	13.43±1.38***	5.74±0.12***	5.95±0.39***
C. coniocraea	A549	86.40±3.67	93.17±12.03	89.87±17.60	61.77±4.30**	55.17±4.96**	42.47±2.48**	17.82±2.93***	11.45±0.74***
	COLO 205	76.96±3.76	70.55±10.56	59.84±3.00*	56.50±4.95*	48.02±0.87**	45.65±5.73**	17.94±5.19***	5.80±0.32***
C. pxyidata	A549	88.96±7.30	81.69±7.22	94.61±15.56	70.65±3.74	69.65±9.00*	71.54±3.23	98.03±19.58	86.99±6.51
	COLO 205	84.68±3.70	85.18±2.06	74.61±4.87	65.13±7.91*	60.44±7.85**	57.79±6.18**	45.31±3.75**	27.99±6.23***
C. rangiformis	A549	94.38±14.48	84.20±18.22	67.87±11.35	54.21±9.53**	41.39±8.85**	42.49±5.43**	21.58±2.67***	15.76±0.84***
	COLO 205	71.27±3.35	65.32±4.80*	50.65±2.46**	36.27±2.71***	36.94±2.35***	7.00±0.67***	6.53±0.19***	6.09±1.36***
C. foliacea	A549	76.48±2.70	73.76±6.69	65.00±9.27*	61.97±3.57*	66.73±9.96*	49.13±5.16**	38.50±2.56***	11.07±1.25***
	COLO 205	54.83±1.33**	48.22±3.86**	44.32±4.81***	32.99±4.28***	24.35±2.18***	5.78±0.08***	6.45±0.26***	5.17±0.73***
C. firma	A549	97.30±5.28	65.41±11.53*	59.89±4.51**	57.56±4.40**	45.36±3.75**	42.66±2.65**	48.28±7.46**	40.83±2.62***
	COLO 205	66.69±4.73*	51.67±6.65**	52.54±4.93**	39.28±2.54***	41.56±4.80***	31.18±3.35***	14.39±3.55***	4.52±0.38***
C. furcata	A549	123.45±2.21	100,89±13.18	81.18±8.53	87.62±12.03	71.94±9.24	56.32±12.07*	64.63±7.97*	57.61±10.99*
	COLO 205	99.29±5.37	96.16±5.43	87.86±12.28	73.70±8.06*	59.87±8.49*	49.53±5.47**	5.48±0.43***	5.79±0.49***
C. subulata	A549	100.65±5.55	67.17±6.40*	64.45±4.09*	59.50±1.38*	48.57±4.90**	46.72±1.82**	46.70±1.50**	39.89±1.62***
	COLO 205	74.91±5.41	60.62±4.06**	58.61±3.56**	55.87±5.05**	47.23±2.80***	38.72±4.25***	25.77±5.48***	8.73±1.75***
C. fimbriata	A549	119.58±14.96	115.45±9.48	95.31±11.42	87.22±8.74	78.69±5.44	92.55±3.83	76.59±4.32*	13.81±0.15***
	COLO 205	97.44±8.64	97.57±5.85	102.67±7.69	82.18±5.79	84.62±13.98	34.36±5.87***	6.56±0.19***	5.25±0.87***

Data are expressed as mean ± standard error (n=3). Significant differences are indicated as * P<0.05, ** P<0.01,*** P<0.001

Table 4. Toxicity of Cladonia species on Raw 264.7 cell line
Çizelge 4. Cladonia türlerinin Raw 264.7 hücre hattı üzerinde toksisitesi

Species (Türler)	5 µg mL^{-1}	10 µg mL^{-1}	20 µg mL^{-1}	40 µg mL^{-1}	80 µg mL^{-1}	100 µg mL^{-1}
C. pocillum	99.37±1.71	100.23±1.77	98.93±2.00	91.08±1.36	80.31±0.84	56.78±0.42**
C. coniocraea	110.14±1.28	104.63±3.46	101.23±3.28	97.17±2.03	81.44±1.02	59.95±0.33**
C. pxyidata	111.38±9.69	105.41±1.65	107.56±4.94	91.21±1.55	96.38±3.14	62.44±2.61*
C. rangiformis	108.90±3.35	99.96±0.57	95.55±1.39	89.84±0.71	75.79±0.78	59.95±0.57**
C. foliacea	113.04±4.30	101.99±0.74	99.52±1.53	99.91±3.08	73.75±0.62	65.83±0.41*
C. firma	104.76±0.58	103.15±7.78	103.72±2.61	97.48±4.38	79.63±1.02	68.77±0.24*
C. furcata	107.93± 3.95	105.71±6.06	103.48±4.55	96.78±1.33	76.39±2.53	64.85±0.19*
C. subulata	111.02±3.24	103.01±2.61	98.54±3.02	98.03±2.61	78.76±3.73	60.13±0.56**
C. fimbriata	110.23±6.59	109.46±4.31	109.58±4.98	96.72±2.09	73.95±2.96	62.04±0.38*

In cytotoxicity experiment, data are expressed as mean ± standard error (n=3). Significant differences are indicated as * P <0.05, ** P<0.01.

Table 5. Effect of *Cladonia* species on NO amount
Çizelge 5. Cladonia türlerinin NO miktarına olan etkileri

Species (Türler)	Concentration (Konsantrasyon)	NO amount (µM) (NO miktarı)	Concentration (Konsantrasyon)	NO amount (µM) (NO miktarı)
C. pocillum	40 µg mL^{-1}	43.1±1.56^{a,b}	20 µg mL^{-1}	34.50±0.83^{c,d}
C. coniocraea	40 µg mL^{-1}	69.30±0.72^{k,l}	20 µg mL^{-1}	43.70±0.65^{a,b}
C. pxyidata	40 µg mL^{-1}	59.70±0.69^{j}	20 µg mL^{-1}	42.36±0.35 ^{a,b}
C. rangiformis	40 µg mL^{-1}	44.37±0.35^{b}	20 µg mL^{-1}	36.36±0.35^{c,d,e}
C. foliacea	40 µg mL^{-1}	40.03±0.78^{a,e,f}	20 µg mL^{-1}	30.23±0.70^{ı}
C. firma	40 µg mL^{-1}	40.90±0.83^{a,b,f}	20 µg mL^{-1}	31.00±2.21^{c,ı}
C. furcata	40 µg mL^{-1}	66.38±0.35^{b,k}	20 µg mL^{-1}	48.70±0.53^{g}
C. subulata	40 µg mL^{-1}	38.63±0.25^{e,f}	20 µg mL^{-1}	34.30±1.14^{c,d}
C. fimbriata	40 µg mL^{-1}	41.50±0.76^{a,b,f}	Control	8.44±0.18^{h}
Indomethacin	25 µM	8.30±0.35^{h}	LPS	72.37±0.67

In NO amount determination, data are expressed as mean ± standard error (n=3). The same letters (a-l) indicate the similarity between the groups (P>0.05) and the different letters represent the difference between the groups (P<0.05).

found as follows respectively, 0.211 ± 0.012, 0.494 ± 0.047 and 0.405 ± 0.008 mg mL^{-1} gallic acid. However, directly comparing this study and our results do not seem appropriate in terms of the units used. The fumarprotocetraric acid amounts of the lichen extracts are between the ranges of 18.89 – 232.82 mg $g_{extract}^{-1}$. Fumarprotocetraric acid has been found in many *Cladonia* species such as *C. foliaceae, C. verticillaris, C. furcata, C. rangiformis,* and *C. rangiferina.* (Laundon 1971; Yilmaz et al. 2004; De Barros et al. 2014; Narendra and Khurana, 2019). According to the Farkas et al. research, the concentration of fumarprotocetraric acid of *C. foliacea* acetone extract varied between 2.26 and 5.81 mg g^{-1} in winter collected samples. The higher content of *C. foliacea* in our study may be due to the samples obtained from different countries, the time of collection, and extraction method (Farkas et al., 2020).

DPPH• radical scavenging method is a rapid method for evaluating the free radical scavenging effect of extracts. We found that the scavenging effect of *Cladonia* species was not as high as BHT used as a positive control (Table 2). When comparing with the literature we set similar results found by Rankovic et al. (2011) and Kosanic et al. (2011; 2018). Hawry et al. demonstrated inhibition % of DPPH• radical for *C. subulata*, *C. furcata,* and *C. fimbriata*. According to the results, the highest activity was recorded for *C. fimbriata* as 21.57% inhibition. This result was considered to be lower than the percentage of inhibition at the lowest concentration used in our study. Besides, they found a 10% inhibition effect for *C. firma* extract but our data showed that at 1 mg mL^{-1} concentration *C. firma* extract scavenged the 60% of radical (Hawrył et al., 2020). In the antioxidant activity of lichens, environmental factors play a crucial role. Environmental factors like air pollution, high light, desiccation, high temperature, and rehydration act on decreasing the antioxidant activity and reducing the synthesis of antioxidant compounds in lichens (Bartak et al., 2004; Weissman et al., 2005, 2006).

ABTS^{+}• / TEAC method is one of the most preferred antioxidant activity measurement methods. The assay can be applied to both lipophilic and hydrophilic components. Water-soluble acid of Vitamin E also known as Trolox is used as the reference standard (Okan et al., 2013). ABTS^{+}• radical removal effect of extracts and standards were tested at four different

concentrations of 0.5, 1, 2, and 4 mg mL^{-1}. It has been determined that the scavenging effect of the extracts were not as high as BHT at the same concentration (Table 2). In this test system, *C. firma* and *C. foliacea* were found more active than *C. pocillum* which was active in DPPH• radical scavenging activity. Although ABTS radical scavenging activities of *Cladonia* species were not found in the literature, Paudel et al. reported the IC50 value of the methanol extract of *Cladonia* sp., whose species was not specified, as 51.6 µg mL^{-1} (Paudel et al., 2012). In a different study IC_{50} value of the fumarprotocetraric acid for ABTS radical scavenging was 277.8 ± 30.6 µg mL^{-1} and 417.5 ± 66.8 µg mL^{-1} for DPPH radical scavenging (Prokop′ev and Filippova, 2019). According to these results, it is understood that the antioxidant activity of fumarprotocetraric acid is not high, and it confirms that our fumarprotocetraric acid content and antioxidant activity are not correlated.

The *β*-carotene/linoleic acid method, which is one of the most widely used methods for this purpose, is based on the principle that the degradation products resulting from linoleic acid oxidation will turn the color of the *β*-carotene molecule. Extracts have been observed to inhibit lipid peroxidation due to time, but they were not as active as synthetic antioxidant BHT (Figure 2). There is only one study on the inhibition of ß-carotene/linoleic acid oxidation for *Cladonia* species. Aslan et al. (2006) reported that *C. foliacea* did not exhibit notable inhibition of linoleic acid oxidation.

The result of the cytotoxic activity of lichen extracts on the studied cell lines is detailed in Table 3. In A549 cell line *C. firma* extract was statistically more significant when inhibition values were examined in all concentrations. *C. firma* extract was also found to be more active in the $ABTS^{+\bullet}$ test system. However, other extracts except for *C. furcata* and *C. pxyidata* showed stronger inhibition than *C. firma* at a concentration of 2000 µg mL^{-1}. *C. pxyidata* showed no significant difference in all studied concentrations compared to the control group (P>0.05).

Against the COLO 205 cell line, all the extracts showed stronger inhibition than the A549 cell line. As in the A549 cell line, in this cell line, *C. firma* extract was significantly different compared to the control in all concentrations. *C. rangiformis* and *C. foliaceae* were also found more potent than other extracts. *C. fimbriata* extract in the COLO 205 cell line had a lower inhibition percentage than the other extracts and did not show a statistical significance below 250 µg mL^{-1} concentration. When the study of Kosanic et al. (2018) with *Cladonia* species is examined, the IC50 value of lichen extracts on the A549 cell line seems to below from our results. However, they incubated the cells for 72 hours with extracts and used a lower number of cells than we used in our study. In the study of Rankovic et al., evaluating the toxicity of *C. furcata*, they reported that the IC50 value of *C. furcata* on human colon cancer cell (LS174 cell line) was 40.22 µg mL^{-1}. In a study evaluating the toxic effects of *C. rangiformis* and *C. convoluta* on the MCF7 cell line, *C. rangiformis* was reported to inhibit the proliferation of cells in a dose-dependent manner (Coskun et al., 2015). Different inhibition percentages of the species in toxicity studies result from the differences in cell number, incubation time, and cell lines, and prevent us from making direct comparison with the data of our study. Also, no studies are evaluating the toxicity of most *Cladonia* species we studied in A549 and COLO cell lines.

Nitric oxide is synthesized from L-arginine by a family of enzymes called nitric oxide synthase. The interaction of nitric oxide with the superoxide radical results in a strong cytotoxic oxidant, peroxynitrite radical ($ONOO^{\bullet}$). Nitric oxide is produced in high amounts by cytokines and endotoxins in cases of infection and plays a role in killing parasites, but overproduction can cause reperfusion injury (Choudhari et al., 2013). The decrease in NO levels in all groups treated with *Cladonia* extracts except *C. coniocraea* (20 µg mL^{-1}) showed an anti-inflammatory effect. The most active species were found as *C. foliacea*, *C. firma,* and *C. subulata* (Table 4). Although there is no study evaluating the effects of *Cladonia* species on NO in Raw 264.7 cell line, the anti-inflammatory activities of some species have been evaluated by different methods in the literature. The anti-inflammatory activity of *C. clathrata* was studied using the paw edema model induced by 1% carrageenan. It has been reported that *C. clathrata* extract at a dose of 100-400 mg kg^{-1} reduced paw edema (Silva et al., 2010). In a formaldehyde-induced edema model, *C. rangiformis* exhibited 33.8%, 36.1%, 43.1% inhibition at 50, 100 and 200 mg kg^{-1} doses (Süleyman et al., 2002).

The results we obtained from our study, confirm the relationship between antioxidant activity and cytotoxic activity. At the same time, *C. firma* and *C. foliaceae*, which were active in cytotoxicity experiments, also exhibited anti-inflammatory activity. These results confirm the use of compounds with antioxidant properties in cancer and inflammation. Antioxidant, cytotoxic, and anti-inflammatory activities have not shown linearity with the fumarprotocetraric acid content of extracts. The results suggested us that presence of p-hydroxybenzoic acid derivatives; vanillic and protocatechuic acid derivatives and also dibenzofuran derivative usnic acid in *Cladonia* species can be related to antioxidant, cytotoxic, and anti-inflammatory activities (Zagoskina et al., 2013).

CONCLUSION

Antioxidant, cytotoxic, and anti-inflammatory

activities of 70% methanol extracts of the nine *Cladonia* species have been evaluated in this study. Although this study is superior to other studies conducted with *Cladonia* species in terms of the number of species studied, it also makes a significant contribution to the literature by comparing species activities to eachother. The *Cladonia* species which were active in the antioxidant test systems were also showed potent inhibition to the cancer cell lines than the other species, thus proven the correlation between the antioxidant and cytotoxic activity. The amount of fumarprotocetraric acid was not correlated with activity experiments. This study showed that lichens have biological activity and more detailed studies should be done on lichens for biologically active products, pharmaceuticals, or cosmetics.

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Author's Contributions

The contribution of the authors is equal.

Statement of Conflict of Interest

Authors have declared no conflict of interest.

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