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Original Research

In vitro evaluation of cytotoxic, anti-proliferative, anti-oxidant, apoptotic, and anti-microbial activities of *Cladonia pocillum*

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Abstract: The aim of this study was to investigate the anti-proliferative, apoptotic, cytotoxic, and anti-oxidant effects of extracts from the lichen *Cladonia pocillum*on human breast cancer cells (MCF-7), and to characterize the anti-microbial features. MCF-7 cells were treated with methanolic *C. pocillum* extract for 24h. The cytotoxicity of the extract was tested with MTT. Moreover, its anti-proliferative effects were examined with immunocytochemical method. Apoptosis and biochemical parameters were detected in MCF-7. The methanol and chloroform extracts of the lichen were tested for anti-microbial activity against *Staphylococcus aureus*, *Enterococcus faecalis*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Candida albicans* using the disc diffusion method and calculation of minimal inhibitory concentrations. Although BrdU incorporation was not observed in MCF-7 cells treated with methanol extract at a concentration above 0.2 mg/mL, a significant decrease was observed int he percentage of PCNA immunoreactive cells in groups treated with 0.2, 0.4, 06, and 0.8 mg/mL methanol extracts of *C.pocillum* (49±6.3, 44±5.2, 23±2.5, 0, respectively) compared to that of control (85±4.5). The percentage of apoptotic cells significantly increased in groups treated with 0.2, 0.4, 0.6, and 0.8 mg/mL extracts of the *C.pocillum* (54±3.5, 76±2.6, 77±1.8, 82±4.2, respectively) compared with that of control group (3.9±1.5). The half-maximal inhibitory concentration of the methanol extract against MCF-7 cells was 0.802 mg/mL .Although the chloroform extract showed more effective anti-microbial activity overall, the methanol extract showed higher anti-fungal activity. Collectively, the results of our study indicate that *C.pocillum* extracts have strong anti-microbial and apoptotic effects. This lichen therefore shows potential for development as a natural anti-microbial, anti-oxidant, and apoptotic agent.

Key words: Cell proliferation; Lichen; Microbial activity; MCF-7; TUNEL.

Introduction

Lichens are symbiotic organisms between fungi and algae. They are an integral part of many ecosystems. The lichens grow on rocks, trees and leaf surfaces. They can also colonize on rock surfaces or soil. They produce a wide variety of secondary metabolites which have a potential use as anti-microbial, anti-cancer, fungi toxic, herbicidal, anti-herbivory, anti-feedant, anti-oxidant and anti-inflammatory (1-4).

Despite the effort spent on research and funding, cancer is still a major public health problem and breast cancer is among the most common types of cancer worldwide. Breast cancer is the second leading causes of death among women (5). Oxidative stress results from an imbalance between production of free radicals and anti-oxidants.The overproduction of free radicals, such as reactive oxygen species (ROS) causes oxidative damage to proteins, lipids, and DNA. It is suggested that high levels of ROS have been detected in various cancer types. Moreover, tumor progression and aggressiveness may be promoted by production of ROS (6, 7). Hecht et al. (8) indicated that ROS has a role on breast cancer etiology and progression, and also more attention should be given to the development of ROS-generating and scavenging system for breast cancer therapy.

The efficacy of lichen metabolites in the treatment of cancers has been provenin the recent studies (9-12). The extracts from various lichens prepared in acetone, chloroform, and methanol etc. solvents were often used instead of defined compounds (13-15).The herbal medicine has been applied in the treatment of cancers for years. Several biological activities of various lichen species have been verified in the recent researches. A large number of studies have been performed regarding to lichen species acting as anti-cancer agent. However, generally those studies focus only on the cytotoxic activities of lichens. Therefore, we aimed in the present study to investigate the anti-proliferative, apoptotic, anti-microbial and anti-oxidative effects of *Cladonia pocillum* (Ach) O. J. Rich. Extract. Thus its effects on anti-proliferation were examined via bromide oxyuridine (BrdU) incorporation and expression of proliferating cell nuclear antigen (PCNA) and, on the cell death by inducing apoptosis and the changes of oxidative stress were determined in MCF-7 treated with methanol lichen extract.Moreover, anti-microbial activities of chloroform and methanol extracts from *C. pocillum* were tested as well.

Materials and Methods

Sample preparation

Samples of lichen material were collected in Kandira district of Kocaeli, a city in the East Marmara region of Turkey. The lichen samples were investigated under a stereomicroscope (Olympus SZ40; Olympus Medical Systems Corp., Tokyo, Japan) and the species were identified by G. Cobanoglu as *Cladonia pocillum* (Ach.) O. J. Rich (16). The air-dried samples were ground and the powdered lichen materials (4 g and 2 g) were extracted in a Soxhlet extractor using each 270 mL of methanol and chloroform as solvents, respectively. The extracts were filtered through Whatman No. 1 filter paper (Whatman, Maidstone, England). The solvents were then evaporated by a rotary evaporator under reduced pressure to yield 167 mg of methanol extracts and 10 mg of chloroform extracts of *C. pocillum*, respectively.

Cell culture

The human breast cancer (MCF-7) cell lines were obtained from the American Type Culture Collection (Manassas, VA, USA). MCF-7 cells were maintained in the recommended Dulbecco's modified Eagle's nutrient F-12 Ham (DMEM-F12) medium (Sigma-Aldrich, MO, USA) supplemented with 10 % fetal bovine serum (FBS) (Seromed, Istanbul, Turkey), L-glutamine, penicillin (50 units/mL) and streptomycin (0.05 mg/mL) (Biological Industries, Beit-Haemek, Israel). The cells were cultured in a humidified atmosphere with 5 % CO_2 at 37 °C.

Cytotoxicity assay

The effect of methanol lichen extract on cancer cell survival was determined using the thiazolyl blue tetrazolium bromide (MTT) cytotoxicity assay. MTT assay is taken up by viable cells and converted in the mitochondria to a soluble blue formazan product. MTT was performed according to the manufacturer's instructions of CellTiter 96® AQueous One Solution Cell proliferation Assay (Promega, USA).The cells were seeded at a density of 1 x 10^5 cells/well in 96-well plates. After 48 h, the cells were treated with different concentrations (0.01, 0.2, 0.4, 1, 2 mg / mL) of the lichen extract added for 24 h. After the treatment, media were carefully removed. MTT solution was added and the cells were incubated at 37 °C, 5 % CO_2 environment for 2 h. Absorbance was measured by ELISA reader (BioTek, Synergy H1 Hybrid Multi-Mode Microplate Reader, USA) at 490 nm. Each experimental condition was repeated 3 times. The percentage of relative cell viability was calculated using the following formula: % Cell viability = (OD_{490} treated cells / OD_{490} control) x 100.

Immunocytochemical detection of anti-proliferative activity

MCF-7 cells were seeded at a concentration of 1 x 10^5 cells/well in 24-well tissue culture plates and grown on coverslip. The MCF-7 cells were incubated with final concentrations (0.2, 0.4, 0.6, 0.8 mg/mL) of the methanol extracts from *C. pocillum* for 24 h. BrdU and PCNA antibodies were used in MCF-7 cells for immunocytochemical labeling by streptavidin-biotin-peroxidase technique.

In vitro labeling was carried out by applying 5-bromo-2′-deoxyuridine (1 mM) to 70 % confluent MCF-7 cells on coverslips for 1 h at 37 °C. After phosphatebuffered saline (PBS) washes, cells were fixed with methanol for 5 min at - 20 °C, and incubated with 2N HCL at 37 °C for 30 min. Following the incubation, boric acid (0.1 M, pH 8.5) was added on the cells for 10 min at room temperature. The cells were incubated for 1 h at 37 °C with a monoclonal anti-BrdU antibody (Neomarkers, USA) and then followed by detection using a streptavidin-biotin-peroxidase complex (Invitrogen, USA) and staining with 3-amino-9-ethylcarbazole (AEC) substrate solution (Invitrogen, USA).

The cells grown on the coverslips were incubated with the methanol extracts of *C. pocillum* as indicated. The cells on coverslips were washed with PBS and fixed with methanol for 5 min at – 20 °C. In order to avoid non-specific immunostaining, the cells were incubated with blocking solution for 20 min at room temperature and then, PCNA primary antibody (Neomarkers, USA; dilution 1:300, overnight at 4 °C) were used for labeling. After washing with PBS, biotinylated secondary antibodies and streptavidin, biotinylated horseradish peroxidase (Invitrogen, USA) were applied. Immunoreactivity was revealed by using AEC (Invitrogen, USA) and mounted in glycerin gelatin, preserved at 4 °C until microscopic examination. Images were captured using an Olympus BX - 50 bright-field microscope. The percentage of immunoreactive cells [(the number of immunoreactive cells /total cells) x 100] were expressed.

Analysis of apoptosis

For the determination of apoptosis, TUNEL assay was performed using the In situ Cell Death Detection Kit (Millipore, USA). The MCF-7 cells on coverslip were incubated with final concentrations (0.2, 0.4, 0.6, 0.8 mg/mL) of the methanol extracts from *C. pocillum* for 24 h. MCF-7 cells were fixed with methanol and then equilibration buffer was directly applied on the cells for 2 min at room temperature. Terminaldeoxynucleotidyltransferase (TdT) enzyme was mixed with reaction buffer and applied to the MCF-7 cells for 1 h at 37 °C. The anti-digoxigenin conjugate was added onto the coverslips and incubated at room temperature for 30 min. The reaction was observed with the diaminobenzidine (DAB) system. A minimum of 10 fields were randomly selected, and the all cells were counted in each field. The percentage of apoptotic cells [(the number of apoptotic cells / total cells) x 100] were expressed.

Biochemical assays

For biochemical analysis, MCF-7 cells were seeded at a concentration of 1 x 10^6 cells/well in 6-well tissue culture plates. The cells were incubated with final concentrations (0.2, 0.4, 0.6, 0.8 mg/mL) of the methanol extracts from *C. pocillum* for 24 h and then, the cells were sonicated for 10 min. Glutathione (GSH) levels were assayed in MCF-7 cells by the method of Beutler (17). Lipid peroxidation (LPO) levels in homogenates were determined by Ledwozyw's method (18). Catalase (CAT) activity was estimated by the method of Aebi (19). The protein content of the samples was determined according to Lowry's method (20).

Analysis of anti-microbial activity

The following microorganisms were used as test organisms in this study: *Pseudomonas aeruginosa*(ATCC 15442), *Escherichia coli* (ATCC 2592), *Enterococcus faecalis* (ATCC 29212), *Staphylococcus aureus* (ATCC 25923) and *Candida albicans* (ATCC 90028). The test microorganisms were maintained on nutrient broth medium (NB-No.3, for microbiology, 70149; Fluka, Munich, Germany) at 37 °C for 24 h, adjusted to 0.5 McFarland standard, approximately 10^8cfu/mL for bacteria and 10^6cfu/mL for *C. albicans*. The extracts were subjected to anti-microbial assay by measuring the diameter of zone of inhibition using disc diffusion method(21). For the disc diffusion assay, the dried extracts were dissolved and diluted in the respective solvents of methanol and chloroform. 20 µg chloroform extract and 134 µg methanol extract were impregnated to disc. Since all microbiological tests were made in laboratories with an International Quality Certification (ISO-15189), a large antibiotic control panel recommended by CLSI (Clinical Laboratory Standards Institute) was used. The zone of inhibition in each case was measured under the bacterial colony counter Colony Star (Funke-Gerber, Berlin, Germany) and data were recorded.

The minimal inhibitory concentration (MIC) of the extracts for anti-microbial testing was determined by agar dilution method. The minimum concentration of each extract with clear zone of inhibition was considered as MIC. The solvent without extracts served as negative control. A number of antibiotics were used as positive reference standards chloramphenicol, piperacillin/tazobactam for the bacteria, vancomycin and fluconazole for the yeast.

Statistical analysis

Experiments were carried out at least in triplicate and results were expressed as mean ± SD for microbial test and as mean ± SEM for proliferation, apoptosis and biochemical parameters. Statistical analysis of the differences in the measured properties of the groups were performed with one-way analysis of variance (ANOVA), followed by Tukey's *post-hoc* test. (SPSS 21.0software) . In all cases, $p<0.05$ was considered statistically significant.

Results

Effect of *C. Pocillum* on MCF-7 cell viability

The methanol extracts of *C. pocillum* at 0.01 to 2 mg/mL exhibited dose-dependent inhibitory effects on the proliferation of MCF-7 cells. Figure 1 shows the concentrations producing 50 % growth inhibition (IC_{50}) of the methanol extract on the MCF-7 cells for 24 h. The IC_{50} value of the methanol extract of *C. pocillum* on MCF-7 cells was 0.802 mg/mL.

Effects of the *C. Pocillum*on cell proliferation

It was detected that administration of methanol extract at above 0.2 mg/mL concentration of *C. pocillum* to MCF-7 cells was not shown BrdU incorporation (Figure 2A, B). Otherwise, the percentage of PCNA immunoreactive cells showed a significant decrease among groups ($p_{ANOVA}<0.001$) (Figure 2C, D). There was a significant decrease for the percentage of PCNA immunoreactive cells in concentrations of 0.2, 0.4 and 0.6 mg/mL of methanol *C. pocillum* compared to control cells respectively ($p<0.01$). Treatment with 0.6 mg/mL concentration of methanol *C. pocillum* extract exhibited a significant decrease as compared to 0.2 and 0.4 mg/mL concentrations respectively ($p<0.01$). Nevertheless, the PCNA immunoreactive cells were not observed in treatment with 0.8 mg/mL concentration of methanol *C. pocillum* extract (Figure 3A).

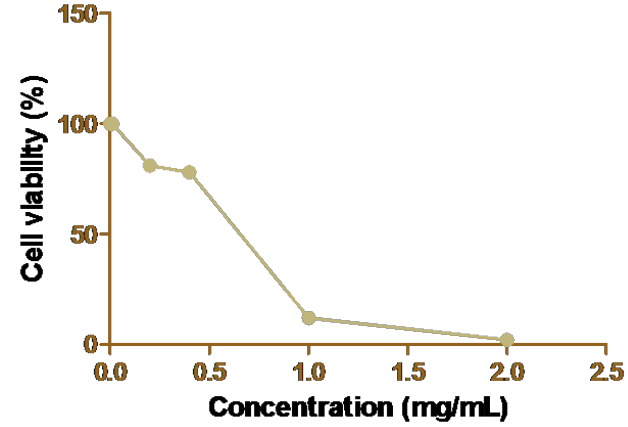

Figure 1. Cell viability in MCF-7 human breast cancer cells treated with methanol extract of *C. pocillum* for 24 h by MTT technique.

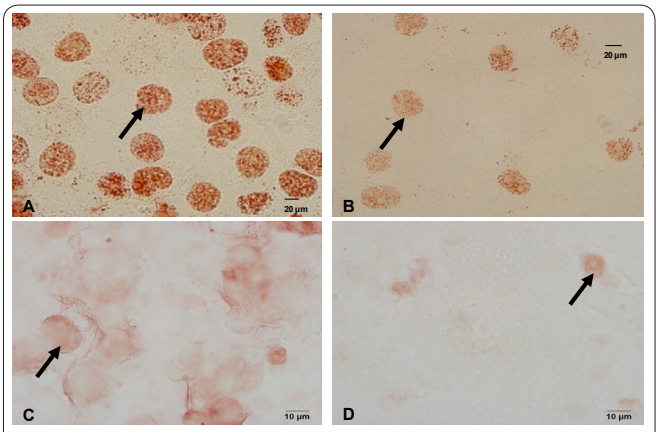

Figure 2. BrdU incorporation (A - B) and PCNAimmunreactivity (C - D) in MCF-7 cells treated with the methanol extract of *C. pocillum* (arrow). Control (A, C), treated with the methanol extracts of *C. pocillum* (0.2 mg / mL) (B, D). Streptavidin–biotin–peroxidase technique. Scale bar = 20 and 10 µm respectively.

Effects of the *C. Pocillum* on apoptosis

In contrast to the PCNA immunoreactivity, the percentage of apoptotic cells showed a significant increase with methanol *C. pocillum* extract treatment ($p_{ANOVA}<0.001$) (Figure 3B). All concentrations of methanol *C. pocillum* extract caused an increase in the percentage of apoptotic cells. Treatment with 0.2, 0.4, 0.6 and 0.8 mg/mL concentrations of methanol *C. pocillum* extract showed a significant increase in MCF-7 cells as compared to control cells respectively ($p<0.001$). Also, there was a significant increase in administration of 0.4, 0.6, 0.8 mg/mL concentrations of methanol *C. pocillum* extract as compared with 0.2 mg/mL group (Figure 4).

Biochemical results

GSH levels and CAT activity were the highest levels in 0.6 mg/mL concentration of methanol *C. pocillum* extract as compared to the other concentrations.

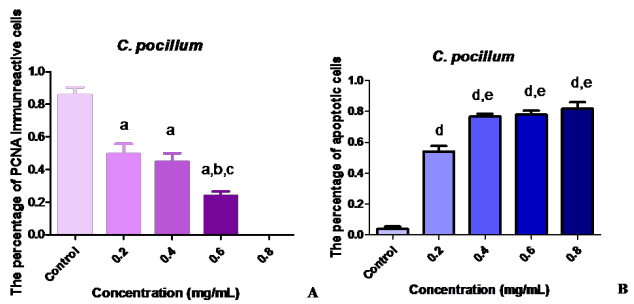

Figure 3. The percentage of PCNA immunreactive cells (A) and apoptotic cells (B) in MCF-7 cells treated with methanol extracts of *C. pocillum* for 24 h. Data values were expressed as mean ± SEM of triplicate determinations. [a]p< 0.01 versus control, [b]p< 0.01 versus concentration of 0.2 mg/mL, [c]p< 0.001 versus concentration of 0.4 mg/mL, [d]p< 0.001 versus control, [e]p< 0.001 versus 0.2 mg/mL.

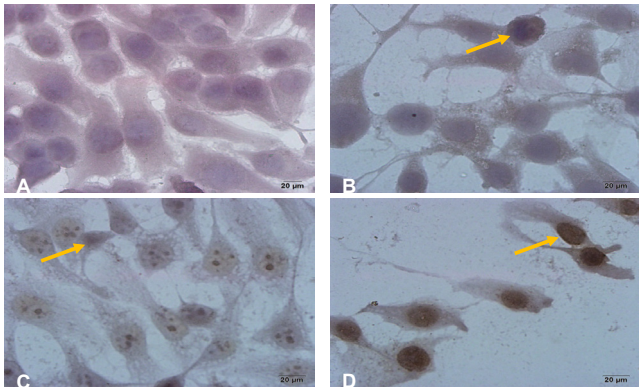

Figure 4. The programmed cell death induced *C. pocillum* in MCF-7 cells using TUNEL assay (arrows). TUNEL staining in control (A), treated with the methanol extracts of 0.2, 0.6 and 0.8 mg / mL *C. pocillum* (B - D), respectively. Scale bar = 20 µm.

However, these levels in GSH and CAT showed the reduction in 0.8 mg/mL concentrations of extract. A significant change in LPO levels was not observed among groups (Figure 5).

Effects of the methanol and chloroform extracts from *C. Pocillum* on anti-microbial susceptibility

The values for the diameters of the growth inhibition zones and the MIC are presented in Table 1 and 2. Although the chloroform extracts from *C. pocillum* were found to have anti-bacterial activity against *E. coli, P. aeruginosa* and *S. aureus* to some extent, they did not

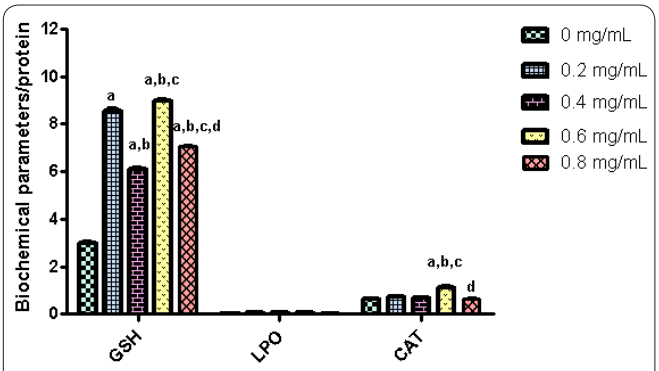

Figure 5. Glutathione (GSH, nmol/mg), lipid peroxidation (LPO, nmol/mg) levels and catalase (CAT, U/mg)) activity in all concentrations of the methanolic *Cladoniapocillum* extractin MCF-7 cells. Data values were expressed as mean ± SEM of triplicate determinations. [a]p< 0.001 versuscontrol, [b]p< 0.001 versus 0.2 mg/mL, [c]p< 0.001 versus 0.4 mg/mL, [d]p< 0.001 versus 0.6 mg/mL.

shown an effect against *E. faecalis* and an anti-fungal activity against *C. albicans*. The chloroform extracts were active against all tested microorganisms while the methanol extracts only showed an activity against *C. albicans*. Likewise *C. pocillum* extracts had higher anti-bacterial activity on Gram-negative bacteria then on Gram-positive bacteria. Especially the chloroform extract had maximum anti-microbial activity against *E. coli* with 32 mm inhibition zone, and was more effective than the tested antibiotics.

MIC determination indicated that the *C. pocillum* extracts inhibited the examined microorganisms. Both extracts showed anti-fungal activity, but it's obvious that the chloroform extract exhibited more anti-fungal activity according to the methanol extract. The negative control in this test did not indicate activity on any of the microorganisms.

Discussion

Plants have had an essential role in the traditional medicine and some plant-derived substances have anticancer and chemoprevention activity (22). Cytotoxic effects of the chloroform extracts from *Cladonia rangiformis* Hoffm. and *Cladonia convulata* (Lamkey) Cout. were observed and found that the two lichen species had activity on MCF-7 cells (23). Therefore, in the present study *C. pocillum* methanolic extract was examined as

Table 1. Anti-microbial activities of the extracts of *C. pocillum* in the disc diffusion assay.

Lichen species[a]		*E. coli*	*P. aeruginosa*	*E. faecealis*	*S. aureus*	*C. albicans*
C. pocillum						
	M	-	-	-	-	30 ± 0.50
	C	32 ± 0.58	31 ± 0.58	-	10 ± 1.00	23 ± 0.00
Antibiotics[b]						
	C				26 ± 0.58	
	FLU					25 ± 0.58
	TZP	26 ± 1.53	26 ± 1.52			
	Va			17 ± 1.15		

Values are mean inhibition zone (mm) ± SD of three replicates; "-" No inhibition observed

[a]C, chloroform extract (20 µg/disc); M, methanol extract (134 µg/disc).

[b]Antibiotics used as positive reference standards;-

C, chloramphenicol (30 µg/disc); FLU, fluconazole (25 µg/disc); TZP, piperacillin/tazobactam (110 µg/disc); Va, vancomycin (30 µg/disc).

Table 2. Minimum inhibitory concentration (MIC) of the extracts of *C. pocillum* against the test organisms.

		E. coli	*P. aeruginosa*	*E. faecealis*	*S. aureus*	*C. albicans*
C. pocillum	M	-	-	-	-	26,8 ± 0.00
	C	2 ± 0.58	4 ± 0.00	-	8 ± 1.00	6 ± 1.00

± SD of three replicates; "-" Non-affective on the bacteria
C, chloroform extract (µg/ml); M, methanol extract (µg/ml).

a new cytotoxic agent on MCF-7 cells by using MTT assays. The methanolic extract was able to inhibit the proliferation of the MCF-7 cell at 0.802 mg/mL. The decrease in viable cell percentage was evident as 24 hours of treatment with *C. pocillum* methanolic extract.

Similarly, Mitrović et al. (24) determined cytotoxicity of the methanol extracts from *Parmelia sulcate* Taylor, *Flavoparmelia caperata* (L.), *Evernia prunastri* (L.) Ach., *Hypogymnia physodes* (L.) and, *Cladonia foliacea*(syn: *Cladonia alcicornis* (Leightf.) Fr.) lichen species on the colon cancer adenocarcinoma (HCT - 116) cell line. It was suggested that *Hypogymnia physodes* and *Cladonia foliacea* had a better cytotoxic activity than the other lichens Ranković et al. (25) studied the anti-oxidant, anti-microbial and, anti-cancer activities of the lichens, such as *Cladonia furcated* (Hudson) Schrad, *Lecanoraatra* (Hudson) Ach., and *Lecanora muralis* (Schreber) Rabenh. The results revealed that the extract of *Cladonia furcata* had a slightly weaker cytotoxic activity than the other two. On the other hand, *Cladonia furcata* demonstrated the strongest anti-microbial activity in the same study. The recent literature supported that different species of *Cladonia* may cause inhibition of cancer cells. Also they have protective effects against various microorganisms.

In many studies on lichens, the inhibition of cell proliferation was referred as cytotoxic effects (9, 24,26). Anti-cancer activities of lichens were also depending on cytotoxic effects on cancer cells (25, 27-29). 5-Bromo-2′-deoxyuridine (BrdU), a thymidine analog, is commonly used to "birth-date" proliferative cells in vitro and in vivo (30). Like BrdU, PCNA is a potential anticancer target and has many cellular processes, such as DNA replication, cell-cycle progression and repair of DNA damage (31). We examined BrdU and PCNA antibodies using immunocytochemical labeling for antiproliferative effect of the methanol extract of *C. pocillum* in MCF-7 cell lines. BrdU incorporation was not determined in MCF-7 cells with the treated methanolic extractat above 0.2 mg/mL concentration. Increased concentration of *C. pocillum* methanolic extract reduced PCNA in the DNA synthesis phase of the cell cycle. The results of BrdU and PCNA immunoreactivity suggested that the methanol extract from *C. pocillum* could inhibit proliferation of MCF-7 cells as a human breast cancer cell line.

In other studies, cytotoxic effect of lichens is stated to result from lichen secondary metabolites. Atranorin, (+) (-) usnic acid and (+) (-) isousnic acid are some of these compounds found in *Cladonia* species. In particular, usnic acid was often tested for cytotoxic activity against cancer cell lines (32, 33). In the study of Bezivin et al. (13), lichen extracts from *Parmelia caperata, Cladonia convoluta*, *Cladonia rangiformis*, *Platisma glauca* and *Ramalina cuspidata* have shown cytotoxic activity against human cancer cell lines in vitro. Especially species of *Cladonia* were found more active than other lichen species. These results may be based on combination of varied metabolites in *Cladonia* species.

The researchers suggested that lichens secondary metabolites induce programmed cell death in human lung carcinoma A549, human ovarian carcinoma A2780 and human colon adenocarcinoma HT-29, thus lichens have a strong anti-cancer activity (14, 26). The methanol extract of *C. pocillum* was found to be inducing apoptosis in MCF-7 cells. The percentage of apoptotic cells was elevated depending on the concentration of the lichen extract. This state was an indication for some lichens species caused to programmed cell death in cancer cell lines.

Brisdelli et al. (34) observed that the lichen metabolites did not change the ROS level and prevent oxidative damage in HeLa cells. GSH is one of the most important scavengers of ROS.

In our study, *C. pocillum* increased GSH levels in MCF-7 cells and its optimal dose was determined to be 0.6 mg/mL. Yadav et al. (35) suggested that the lipid peroxidation produced the free radicals that cause to various human diseases including cancer and the inhibition of lipid peroxidation may be an indicator of therapeutic potential of plant extracts. According to the study of Fernández-Moriano et al. (36), lichen metabolites has the the ability to reduce the lipid peroxidation. The methanolic *C. pocillum* extract was unable change the lipid peroxidation level in MCF-7. An important enzyme in protecting the cells from oxidative damage is CAT enzyme. Its activity was increased in MCF-7 treated with *C. pocillum* extract, especially in 0.6 mg/mL concentration. 0.6 mg/mL concentration of *C. pocillum* extract may be optimal dose in terms ofbiochemical parameters.

Additionally, the strong anti-microbial effects of *C. pocillum* were demonstrated in the present study. To the contrary of many studies, the significant results against Gram-negative bacteria were recorded. Yilmaz et al. (37) examined the anti-microbial effects of (–)-usnic acid, atranorin, and fumar protocetraric isolated from *C. foliacea*, exhibiting high anti-microbial activity only against Gram-positive bacteria. Similarly, Ingolfsdottir (38) tested usnic acid against *E. coli*and *P. aeruginosa* but found no activity.On the other hand, according to some researches, several lichen species and its compounds were active against Gram-negative bacteria (25, 39). Two *Cladonia s*pecies; *C. convoluta* and *C. rangiformis* were tested against two Gram-negative bacteria; *E. coli* and *P. aeruginosa*. The chloroform extract of *C. pocillum* were more active than *C. convoluta* and *C. rangiformis* against *P. aeruginosa*. At the same time, the chloroform extract of *C. pocillum* had high antimicrobial activity against *E. coli* compared to *C. rangiformis* and the antibiotic used for control. Conversely, the methanol extract of *C. pocillum* showed lower activity

against *S. aureus* compared to *C. convoluta* and *C. rangiformis*. But, like *C. convoluta* and *C. rangiformis*, only a moderate effect was recorded for the both extracts of *C. pocillum* (23). Some *Cladonia* species showed anti-microbial effects against Gram-positive *S. aureus* (25, 40). The differences of anti-microbial effects are due to different metabolites in the *Cladonia* species. The results of this study indicate that the both extracts of *C. pocillum* exhibited anti-fungal activity. Particularly, the methanol extract was more active against *C. albicans*. If C. pocillum contains ursolic acid like as C. convoluta, the anti-fungal impact may be due to this substance. To reveal the possibility, the metabolites found in *C. pocillum* may be determined.

In conclusions, the results of the present study have indicated that the methanol extract from *C. pocillum* can inhibit cell proliferation while inducing programmed cell death in MCF-7 human breast cancer cells. The extract may prevent the oxidative damage to some extent. Also, the methanol extract of *C. pocillum* has anti-microbial activity, although not more than that of the chloroform extract. It is suggested that this lichen species, *C. pocillum* may be evaluated as a candidate for anti-microbial and anti-cancer treatments.

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Conflict of Interest
No conflict of interest was declared by the authors.

Author's contribution
The experimental design, the experiments of cell culture and article writing: Melike Ersoz and Zeynep Mine Coskun.
The collection of sample and identification: Gulsah Cobanoglu, Birkan Acikgoz and Cenk Sesal.
Microbial analysis: Iskender Karalti and Cenk Sesal.

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