Original Article

Anti-Proliferative and Apoptotic Effects of Methanolic Extracts from Different *Cladonia* Species on Human Breast Cancer Cells

(apoptosis / lichen / MCF-7 human breast cancer cells / proliferation)

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Abstract. This study tries to elucidate the anti-proliferative and apoptotic effects of methanolic lichen extracts from *Cladonia rangiformis* and *Cladonia convolute* in MCF-7 human breast cancer cells. Lichen extracts (0–2 mg/ml) were added to MCF-7 cells for 24 h. Cell viability was tested using 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) assay. Cell proliferation was observed using bromodeoxyuridine (BrdU) labelling and proliferating cell nuclear antigen (PCNA) by immunocytochemistry. The TUNEL method was used for cell death detection. The effective dose (ED_{50}) values of methanolic extracts from *C. rangiformis* and *C. convolute* were found to be 0.905 and 0.977 mg/ml, respectively. Treatment with *C. rangiformis* methanolic extract (0.2–0.8 mg/ml) dose-dependently inhibited proliferation of MCF-7 cells as detected by BrdU incorporation. The inhibition was started in 0.2 mg/ml concentration of *C. convoluta* methanolic extract. The percent of PCNA immunopositive cells showed a decrease in MCF-7 cells treated with two lichen extracts compared to control MCF-7. Both methanolic extracts showed a significant increase in percentage of apoptosis-positive cells. These results indicate that methanolic lichen extracts from *C. rangiformis* and *C. convolute* inhibited proliferation of MCF-7 cells and caused apoptosis in MCF-7 cells. The lichens may be novel natural agents for treating breast cancer disease.

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Abbreviations: AEC – 3-amino-9-ethyl-carbazole, ATCC – American Type Culture Collection, BrdU – bromodeoxyuridine, DAB – diaminobenzidine, DMEM-F12 – Dulbecco's Modified Eagle's nutrient F-12 Ham, ED_{50} – effective dose, FBS – foetal bovine serum, MCF-7 – human breast cancer cell line, MTT – 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide, OD – optical density, PBS – phosphate-buffered saline, PCNA – proliferating cell nuclear antigen, SEM – standard error of the mean, TdT – terminal deoxynucleotidyl transferase, TUNEL – terminal deoxynucleotidyl transferase dUTP nick end labelling, WHO – World Health Organization.

Introduction

Cancer is a major public health problem worldwide. According to the World Health Organization (WHO), 7.4 million people died of cancer in 2004 and 83.2 million more will have died by 2015 (WHO, 2008). Breast cancer is the second leading cause of cancer death among females (Siegel et al., 2013). It is expected that early detection and treatment with a positive result could decrease mortality (Vainio et al., 2002). Human breast cancer cell line MCF-7 is used as a prominent model system for the study of breast cancer (Arora et al., 2013; Wannous et al., 2013).

Lichens are symbiotic organisms composed of mycobiont and photobiont. They are a potential source in nutrition and in the production of dyes, perfumes and alcohol (Kosanić et al., 2012a). Lichens can be used as natural antioxidants, anti-microbial, anti-diabetics, anti-inflammatory and anti-cancer agents (Suleyman et al., 2002; Luo et al., 2006; Ranković et al., 2011; Zhang et al., 2012; Chauhan and Abraham, 2013). Recent studies suggest that lichen species may constitute a natural biosource for the risk reduction of cardiovascular diseases, diabetes, etc. (Behera et al., 2012; Bakir at al., 2013).

It is suggested that the extracts of *Cladonia* species, inclusive of Cladoniaceae family, have been used for

different remedies in folk medicine (Silva et al., 2010). In our previous study, both species (chloroform extracts from *C. rangiformis* and *C. convoluta*) have demonstrated biological actions such as anti-microbial and cytotoxic activities (Acikgoz et al., 2013). The aim of the present study is to evaluate the anti-proliferative and apoptotic activities of methanolic extracts from *C. rangiformis* and *C. convoluta* species in MCF-7 cells, respectively.

Material and Methods

Preparation of lichen samples

Lichen samples of *Cladonia rangiformis* Hoffm. and *Cladonia convoluta* (Lamkey) Cout. were collected from the Kandira district of Kocaeli province in the east Marmara region of Turkey, in April 2010. The lichen species were identified under a stereomicroscope (Olympus SZ40; Olympus Medical Systems Corp., Tokyo, Japan) and identified by G. Cobanoglu (Smith et al., 2009).

Extracts from 5 g and 3 g of air-dried aerial parts from *C. rangiformis* and *C. convoluta* were extracted, respectively, with the Soxhlet extractor using 270 ml of methanol solvent each. Extracted materials were filtered and the solvents volatilized under reduced pressure to yield 322 mg of *C. rangiformis* and 166 mg of *C. convoluta* methanolic extracts. The extracts were kept at -20 °C until assay.

Cell culture

MCF-7 human breast cancer cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA). Cell lines were cultured in Dulbecco's Modified Eagle's nutrient F-12 Ham (DMEM-F12) medium (Sigma-Aldrich, Hamburg, Germany) supplemented with 10 % foetal bovine serum (FBS) (Seromed, Istanbul, Turkey), penicillin (50 units/ml) (Biological Industries, Kibbutz Beit-Haemek, Israel), and streptomycin (0.05 mg/ml) (Biological Industries, Israel) in 75 cm^2 flasks at 37 °C, 5% CO_2 environment.

Cell viability by MTT

Cell viability studies were performed by measuring the amount of insoluble formazan formed in live cells based on the reduction of 3-(4,5-dimethylthiazol-2-yl) -2,5 diphenyltetrazolium bromide (MTT) salt (Mosmann, 1983). The MTT technique was performed according to the manufacturer's instructions of CellTiter 96® AQueous One Solution Cell proliferation Assay (Promega, Madison, WI). Briefly, 100 µl MCF-7 cell suspensions at 100,000 cell/ml were seeded in a 96-well microtitre plate. The methanolic lichen extracts in concentration range of 0.01, 0.2, 0.4, 0.6, 0.8, 1, 2 mg/ml were added to grown cells for 24 h. MTT reagent was added and 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, Winooski, VT) at 490 nm. Each experimental condition was repeated three times. The percentage of relative cell viability was calculated using the following formula: % cell viability = (OD_{490} treated cells / OD_{490} control) × 100.

Cell proliferation

MCF-7 cells were seeded at a concentration of 1×10^5 cells/well in 24-well tissue culture plates and grown on coverslips. The grown MCF-7 cells were incubated with various doses (0.2, 0.4, 0.6, 0.8 mg/ml) of the methanol extracts from *C. rangiformis and C. convoluta* for 24 h. Cell proliferation was analysed using bromodeoxyuridine (BrdU) labelling and by detecting the expression of the proliferating cell nuclear antigen (PCNA) using an immunocytochemical method.

In vitro labelling was carried out by administering 5-bromo-2′-deoxyuridine (1 mM) to 70% confluent MCF-7 cells on coverslips for 1 h at 37 °C. Following PBS washes, the cells on coverslips were fixed with methanol for 5 min, and DNA was denatured by incubating cells with 2 N HCl at 37 °C for 30 min. Following neutralization with 0.1 M boric acid (pH 8.5) for 10 min, cells were incubated for 1 h at 37 °C with a monoclonal anti-BrdU antibody (Neomarkers, Fremont, CA) followed by detection using a streptavidin-biotin-peroxidase complex (Invitrogen, Carlsbad, CA) and staining with AEC (3-amino-9-ethyl-carbazole) substrate solution (Invitrogen).

Cells grown on coverslips were incubated with the methanolic lichens as indicated. The cells on coverslips were washed with phosphate-buffered saline (PBS) and fixed with methanol for 5 min at -20 °C. In order to avoid non-specific immunostaining, cells were incubated with blocking solution for 20 min at room temperature. PCNA primary antibody (Neomarkers) (1 : 300) was applied to cells for 1 h at room temperature. After washing with PBS, biotinylated secondary antibodies and streptavidin, biotinylated horseradish peroxidase were applied. Cells were developed by using AEC as the substrate. Images were captured using an Olympus BX-50 bright-field microscope (Tokyo, Japan). The percentages of immunopositive cells [(the number of immunopositive cells / total cells) × 100] were expressed.

Detection of apoptotic cells

MCF-7 cells were then grown on coverslips. The cells were incubated with various doses (0.2, 0.4, 0.6, 0.8 mg/ml) of the methanol extracts from *C. rangiformis and C. convoluta* for 24 h. The transferase-mediated dUTP nick end-labelling (TUNEL) technique was performed to detect and quantitate apoptotic cell death using the *in situ* Cell Death Detection Kit (Millipore, Billerica, MA) according to the manufacturer's instructions. The grown cells on coverslips were fixed with methanol. Then, equilibration buffer was directly applied to the cells. Terminal deoxynucleotidyl transferase (TdT) enzyme was mixed with the reaction buffer and applied to the MCF-7 cells for 1 h at 37 °C. Anti-digoxigenin conju-

gate was added to the coverslips and incubated at room temperature for 30 min. The reaction was developed with the DAB (diaminobenzidine) system. A minimum of 10 fields were randomly selected, and the total cells were counted in each field. The percentages of apoptotic positive cells [(the number of apoptotic positive cells / total cells) × 100] were expressed.

Statistical analysis

The statistical analysis was performed using GraphPad Prism version 5.0 computer package software. Data were presented as means ± SEM. A comparison between two groups was performed using Mann-Whitney *U* nonparametric test and a comparison among multiple groups was performed using Kruskal-Wallis test. A probability of 0.05 or less was considered statistically significant.

Results

Determination of cell viability by MTT

To investigate the effects of methanolic extracts of *C. rangiformis* and *C. convolute* on MCF-7 cell cytotoxicity, cell viability was evaluated via the MTT assay. As shown in Fig. 1, the cells were exposed to 0.01, 0.2, 0.4, 0.6, 0.8, 1 and 2 mg/ml methanolic extracts of *C. rangiformis* and *C. convolute* for 24 h. The effective dose (ED_{50}) of methanolic extracts of *C. rangiformis* and *C. convolute* on the MCF-7 cells was recorded as 0.905 and 0.977 mg/ml, respectively.

Anti-proliferative effects of methanolic extracts of C. rangiformis Hoffm. and C. convolute (Lamkey) Cout. on MCF-7 cells

Cell proliferation was evaluated in MCF-7 cells treated with the methanolic extracts of *C. rangiformis* and *C. convolute*, respectively. Treatment with the methanolic extract of *C. rangiformis* (0.2–0.8 mg/ml) dose-dependently inhibited proliferation of MCF-7 cells, as detected by BrdU incorporation (data not shown). When the effects of methanolic lichen extracts were compared on MCF-7 cells, the effect of *C. convoluta* ($P < 0.001$) was poorer than that of *C. rangiformis* (Fig. 2). The inhibition was started in 0.2 mg/ml concentration of methanolic *C. convoluta* extract. We observed that all MCF-7 cells were thoroughly inhibited in 0.6 and 0.8 mg/ml concentrations. Both methanolic extracts inhibited the proliferation of MCF-7 cells (Fig. 2).

The respective changes in percentage of PCNA immunopositive cells in MCF-7 cells treated with the methanolic extracts of *C. rangiformis* and *C. convoluta* are shown in Fig. 3. The percent of PCNA immunopositive cells showed a decrease in MCF-7 cells treated with the methanolic extracts of *C. rangiformis* compared to control cells ($P < 0.001$). Similarly, PCNA immunopositive cells were significantly reduced in MCF-7 cells treated with the methanolic extracts of *C. convoluta* compared to control cells ($P < 0.001$) (Fig. 3). The percentage of PCNA immunpositive cells in *C. rangiformis* (0.4 mg/ml) significantly decreased as compared to 0.2 mg/ml concentration of *C. rangiformis* extract ($P < 0.05$). The cell inhibition was lower in 0.6 mg/ml concentration as compared with 0.4 mg/ml concentration of *C rangiformis* extract. Concentration of 0.8 mg/ml caused inhibition of all MCF-7 cells (Fig. 3). The inhibition of cells was significant in 0.2 mg/ml concentration of *C. convoluta* compared to the control ($P < 0.05$). We did not detect PCNA immunpositive cells in 0.6 mg/ml and above concentrations of *C. convoluta* extract (Fig. 3).

Detection of apoptotic cells

We confirmed apoptosis of MCF-7 cells with the two lichen species by the TUNEL assay. Both methanolic extracts from the two species (*C. rangiformis* and *C. convoluta*) showed a significant increase in percentage of apoptosis-positive cells ($P < 0.001$ and $P < 0.001$, respectively) that are shown in Fig. 4. Treatment with methanolic extracts of *C. rangiformis* (0.2 mg/ml) significantly decreased the cell counts and increased apoptosis in MCF-7 cells compared to the control ($P < 0.05$).

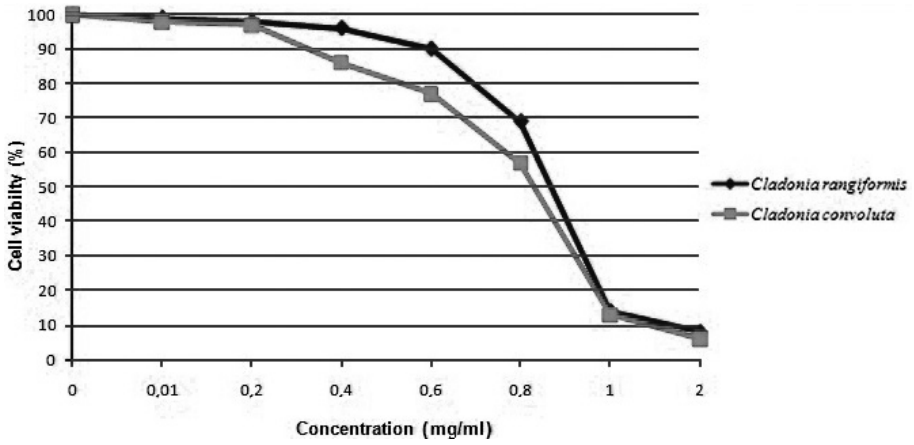

Fig. 1. Effects on cell viability following 24 h exposure to the methanolic extract of *C. convolute* and *C. rangiformis* in human breast cancer (MCF-7) cells as determined using MTT technique

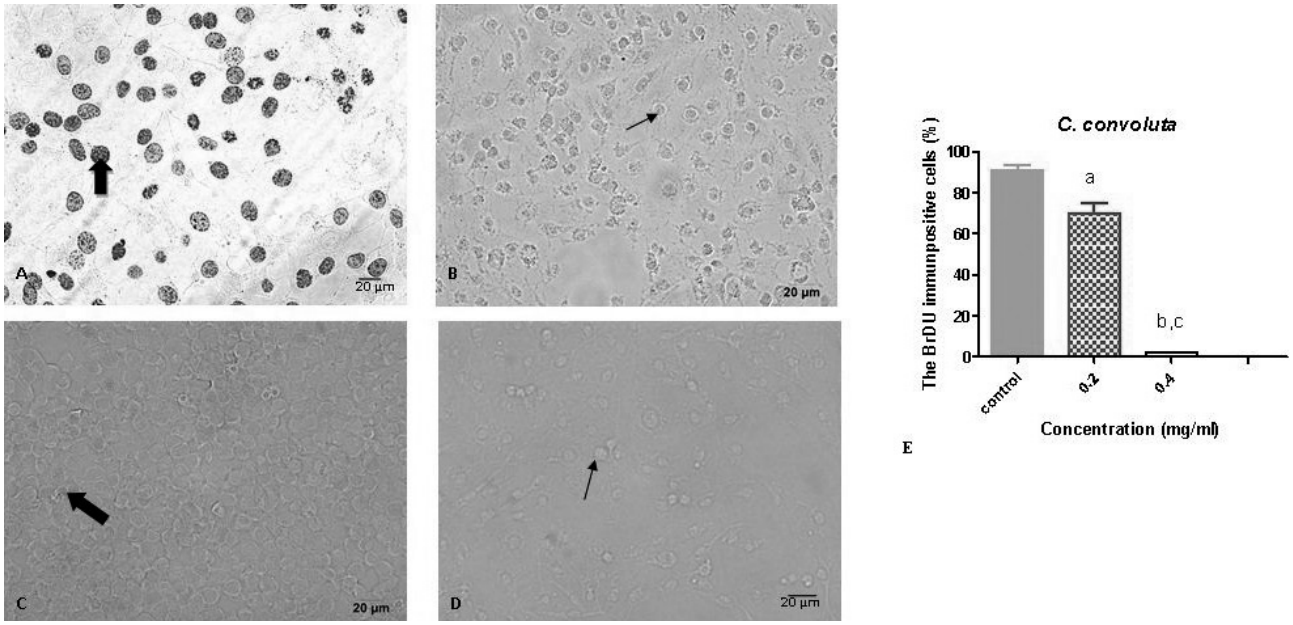

Fig. 2. Cell proliferation in human breast cancer (MCF-7) cells treated with the methanolic extract of *C. convoluta* for 24 h as determined using the BrdU immunohistochemistry method. BrdU immunopositive cells (thick arrow) and non-labelled cells (thin arrow) are seen. (**A**) Control, (**B**) treated with the methanolic extracts of 0.2 mg/ml *C. rangiformis*, (**C**) 0.4 mg/ml *C. convolute*, (**D**) 0.8 mg/ml *C. convolute*, streptavidin-biotin-peroxidase technique. Scale bar = 20 µm. (**E**) The percentage of BrdU immunopositive cells. $^{a}P < 0.01$ versus control, $^{b}P < 0.001$ versus control, $^{c}P < 0.001$ versus concentration of 0.2 mg/ml. Data values were expressed as mean ± SEM of triplicate determinations.

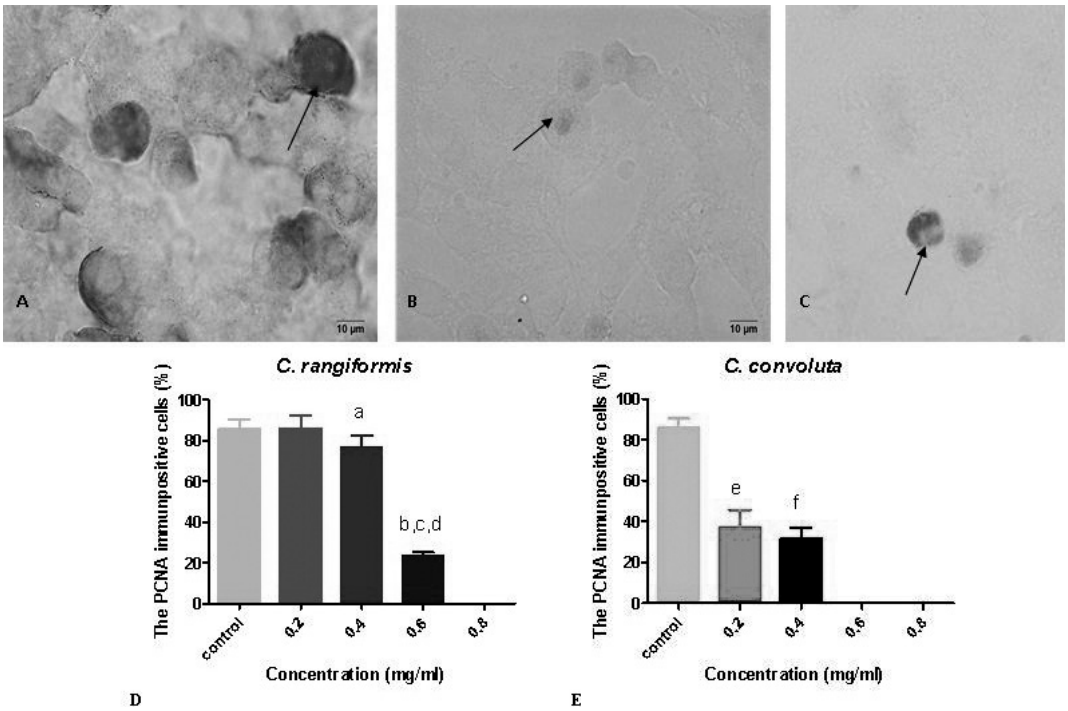

Fig. 3. Immunolocalization of PCNA (arrows) was observed in human breast cancer (MCF-7) cells treated with the methanolic extracts of *C. rangiformis* and *C. convoluta* for 24 h. Immunopositive cells labelled for PCNA in (**A**) control, (**B**) treated with the methanolic extracts of 0.2 mg/ml *C. rangiformis*, (**C**) 0.2 mg/ml *C. convolute,* streptavidin-biotin-peroxidase technique. Scale bar = 10 µm. The percentage of PCNA immunopositive cells (**D**) with *C. rangiformis*, (**E**) with *C. convolute*. Data values were expressed as mean ± SEM of triplicate determinations. $^{a}P < 0.05$ versus concentration of 0.2 mg/ml, $^{b}P < 0.01$ versus control, $^{c}P < 0.001$ versus concentration of 0.2 mg/ml, $^{d}P < 0.001$ versus concentration of 0.4 mg/ml, $^{e}P < 0.05$ versus control, $^{f}P < 0.01$ versus control.

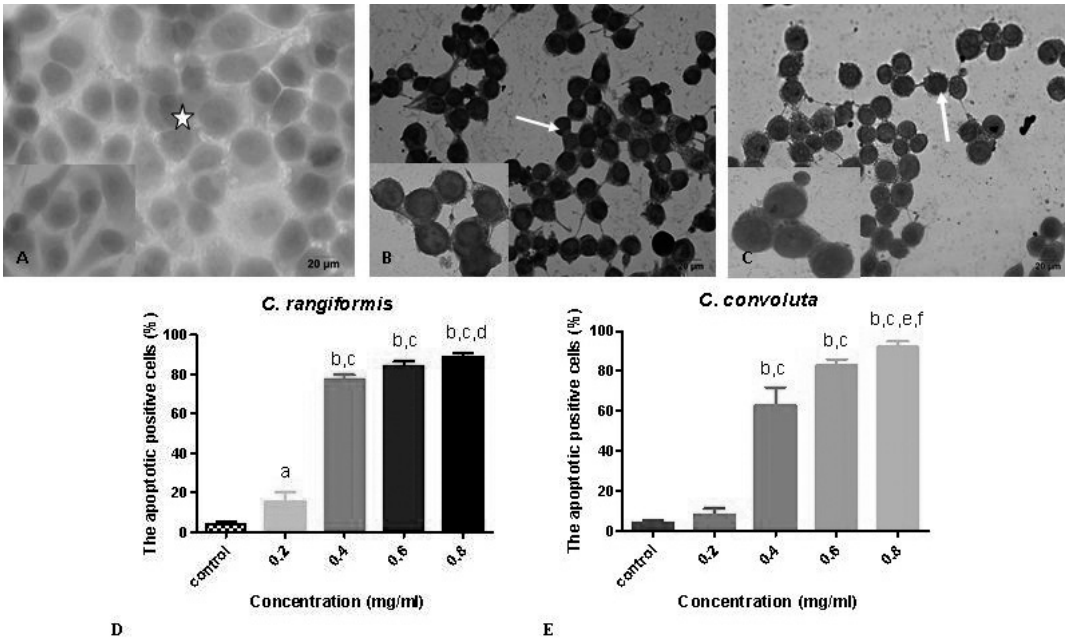

Fig. 4. Terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) staining of human breast cancer cells (MCF-7) (arrows) treated with the methanolic extracts of *C. rangiformis* and *C. convoluta* for 24 h. Non-labelled cells are shown by asterisk. TUNEL staining in (**A**) control, (**B**) cells treated with the methanolic extracts of 0.4 mg/ml *C. rangiformis*, (**C**) 0.4 mg/ml *C. convolute*. Scale bar = 20 µm. The percentage of apoptosis-positive cells (**D**) treated with *C. rangiformis*, (**E**) with *C. convolute*. Data values were expressed as mean ± SEM of triplicate determinations. $^{a}P < 0.05$ versus control, $^{b}P < 0.001$ versus control, $^{c}P < 0.001$ versus concentration 0.2 mg/ml, $^{d}P < 0.01$ versus concentration 0.4 mg/ml, $^{e}P < 0.001$ versus concentration 0.4 mg/ml, $^{f}P < 0.05$ versus concentration 0.6 mg/ml.

In 0.8 mg/ml of *C. rangiformis* extract, apoptosis was shown for almost all MCF-7 cells (Fig. 4). The percentage of apoptosis-positive cells was slightly increased in MCF-7 cells by treatment with *C. convoluta* (0.2 mg/ml) as compared to control cells. There were significant differences in changes in 0.4 mg/ml and above concentrations of *C. convoluta* extract in MCF-7 cells as compared to control cells and MCF-7 cells treated with 0.2 mg/ml concentration of *C. convoluta* extract. It was observed that almost all cells showed apoptosis in 0.8 mg/ml concentration of *C. convoluta* (Fig. 4).

Discussion

In our study, we have for the first time investigated the cytotoxic, anti-proliferative and apoptotic effects of methanolic extracts from *C. rangiformis* and *C. convoluta* species on MCF-7 cells.

Mitrović et al. (2011) evaluated anti-proliferative activities of the five lichens species *Parmelia sulcata, Flavoparmelia caperata, Evernia prunastri, Hypogymnia physodes* and *Cladonia foliacea* by MTT and reported that the lichens have anti-proliferative effects. The anti-cancer activities of *Umbilicaria* species (*U. crustulosa*, *U. cylindrica*, and *U. polyphylla*) were confirmed using only the MTT method by Kosanić et al. (2012b). The present study indicated that the methanolic extracts of *C. rangiformis* and *C. convolute* can show cytotoxic effects on MCF-7 cells. Furthermore, the methanolic lichen extracts show strong anti-proliferative activity against MCF-7 cells. In particular, BrdU incorporation is remarkably lower in the methanolic extract of *C. rangiformis* than in the extract of *C. convoluta* applied to MCF-7 breast cancer cell lines under *in vitro* conditions. It was observed that the PCNA immunopositive index exhibited a decrease in both *C. rangiformis* and *C. convoluta* methanolic extracts. The reduction of PCNA immunopositive index was more intensive depending on the concentration of the lichen extracts. Interestingly, the methanolic extract of *C. convolute* species was stronger than the methanolic extract of *C. rangiformis* in MCF-7 cells. It is known that DNA replication occurs at S phase of the cell cycle. It may be thought that *C. rangiformis* may reduce the number of cells in the S phase. Based on these results, it could be concluded that the methanolic lichen extracts of *C. rangiformis* and *C. convolute* might be anti-proliferative in several phases of DNA synthesis.

Ren et al. (2009) focused on the anti-proliferative and apoptotic mechanisms of lichen extracts from *Lethariella zahlbruckneri* in human colon cancer cells and observed that the extracts decreased the viability of cancer cells. The study of Bačkorová et al. (2012) showed that lichen metabolites activate programmed cell death in cancer cell lines. It has been suggested that the lichen extracts of *Parmelia* species may have strong antioxidant, antimicrobial and anti-cancer activities *in vitro*. Ranković et al. (2011) reported anti-cancer activity of the acetone extracts of *Cladonia furcata*, *Lecanora atra* and *Lecanora muralis* on the human melanoma FemX and hu-

man colon carcinoma LS174 cell lines. In addition, it has been suggested that *C. verticiallaris*, a species of Cladonia, produces fumarprotocetraric acid, which is an anti-oxidant for mice (de Barros Alves et al., 2014). According to the study of Kosanić et al. (2012a), lichens could also be used in the food and drug industry. In our previous study, we evaluated anti-microbial activities of *Cladonia* species. The results showed that the methanolic and chloroform extracts of *Cladonia* species had anti-microbial effects (Acikgoz et al., 2013).

In this study, the data clearly demonstrate that methanolic extracts of the studied lichens induce a significant apoptotic effect on the tested cancer cell lines. The apoptotic activity of *C. convoluta* species is relatively weaker than that of *C. rangiformis* species in MCF-7 cells. The lichens' apoptotic effects are probably the result of the apoptotic effects of individual lichen metabolites. Anti-cancer activity of different lichen metabolites such as usnic acid, lecanoric acid, gyrophoric acid, lobaric acid, evernic acid, vulpinic acid, and protolichesterinic acid is known (Bucar et al., 2004; Burlando et al., 2009). A lichen metabolite caused cell death via mitochondrial membrane depolarization and induction of apoptosis in human lung carcinoma cells (Singh et al., 2013).

In conclusion, the results of the present study for the first time clearly demonstrate that the methanolic extracts of two *Cladonia* lichen species display cytotoxic, anti-proliferative and apoptotic activities in MCF-7 cells under *in vitro* conditions. Thus, *C. rangiformis* and *C. convolute* species may have a great potential to be used as novel natural anti-cancer agents. Further studies aimed at the therapeutic effects of *Cladonia* lichen species' metabolites involving anti-proliferative and apoptotic pathways should provide new clues on the cell death mechanism in cancer diseases.

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References

Acikgoz, B., Karalti, I., Ersoz, M., Coşkun, Z. M., Cobanoğlu, G., Sesal, C. (2013) Screening of antimicrobial activity and cytotoxic effects of two *Cladonia* species. *Z. Naturforsch. C.* **68,** 191-197.

Arora, S., Aggarwal, A., Singla, P., Jyoti, S., Tandon, S. (2013) Anti-proliferative effects of homeopathic medicines on human kidney, colon and breast cancer cells. *Homeopathy* **102,** 274-282.

Bačkorová, M., Jendželovský, R., Kello, M., Bačkor, M., Mikeš, J., Fedoročko, P. (2012) Lichen secondary metabolites are responsible for induction of apoptosis in HT-29 and A2780 human cancer cell lines. *Toxicol. In Vitro* **26,** 462-468.

Bakir, T. O., Geyikoglu, F., Colak, S., Türkez, H., Aslan, A., Bakir, M. (2013) The effects of *Cetraria islandica* and *Pseudevernia furfuracea* extracts in normal and diabetic rats. *Toxicol. Ind. Health* DOI: 10.1177/0748233713475521

Behera, B. C., Mahadik, N., Morey, M. (2012) Antioxidative and cardiovascular-protective activities of metabolite usnic acid and psoromic acid produced by lichen species *Usnea complanata* under submerged fermentation. *Pharm. Biol.* **50,** 968-979.

Bucar, F., Schneider, I., Ogmundsdottir, H., Ingólfsdóttir, K. (2004) Antiproliferative lichen compounds with inhibitory activity on 12(S)-HETE production in human platelets. *Phytomedicine* **11,** 602-606.

Burlando, B., Ranzato, E., Volante, A., Appendino, G., Pollastro, F., Verotta, L. (2009) Antiproliferative effects on tumour cells and promotion of keratinocyte wound healing by different lichen compounds. *Planta Med.* **75,** 607-613.

Chauhan, R., Abraham, J. (2013) In vitro antimicrobial potential of the lichen *Parmotrema* sp. extracts against various pathogens. *Iran J. Basic Med. Sci.* **16,** 882-885.

de Barros Alves, G. M., de Sousa Maia, M. B., de Souza Franco, E., Galvão, A. M., da Silva, T. G., Gomes, R. M., Martins, M. B., da Silva Falcão, E. P., de Castro, C. M., da Silva, N. H. (2014) Expectorant and antioxidant activities of purified fumarprotocetraric acid from *Cladonia verticillaris* lichen in mice. *Pulm. Pharmacol. Ther.* **27,** 139-143.

Kosanić, M. M., Ranković, B. R., Stanojković, T. P. (2012a) Antioxidant, antimicrobial and anticancer activities of three Parmelia species. *J. Sci. Food Agric.* **92,** 1909-1916.

Kosanić, M., Ranković, B., Stanojković, T. (2012b) Antioxidant, antimicrobial and anticancer activity of 3 Umbilicaria species. *J. Food Sci.* **77,** T20-25.

Luo, H., Ren, M., Lim, K. M. (2006) Antioxidative activity of lichen Thamnolia vermicularis in vitro. *Mycobiology* **34,** 124-127.

Mitrović, T., Stamenković, S., Cvetković, V., Tošić, S., Stanković, M., Radojević, I., Stefanović, O., Comić, L., Dačić, D., Curčić, M., Marković, S. (2011) Antioxidant, antimicrobial and antiproliferative activities of five lichen species. *Int. J. Mol. Sci.* **12,** 5428-5448.

Mosmann, T. (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxic assay. *J. Immunol. Methods* **65,** 55-63.

Ranković, B. R., Kosanić, M. M., Stanojković, T. P. (2011) Antioxidant, antimicrobial and anticancer activity of the lichens Cladonia furcata, Lecanora atra and Lecanora muralis. *BMC Complement Altern. Med.* **11,** 97.

Ren, M. R., Hur, J. S., Kim, J. Y., Park, K. W., Park, S. C., Seong, C. N., Jeong, I. Y., Byun, M. W., Lee, M. K., Seo, K. I. (2009) Anti-proliferative effects of Lethariella zahlbruckneri extracts in human HT-29 human colon cancer cells. *Food Chem. Toxicol.* **47,** 2157-2162.

Siegel, R., Naishadham, D., Jemal, A. (2013) Cancer statistics, 2013. *CA Cancer J. Clin.* **63,** 11- 30.

Silva, J. A., Bomfim, R. R., Estevam, C., Antoniolli, A. R., Araújo, A. A., Thomazzi, S. M. (2010) Pharmacological properties of lichen Cladonia clathrata. *Pharm. Biol.* **48,** 745-752.

Smith, C. W., Aptroot, A., Coppins, B. J., Fletcher, A., Gilbert, O. L., James, P. W., Wolseley, P. A. (eds) (2009) *The li-*

chens of Great Britain and Ireland, British Lichen Society, London

Singh, N., Nambiar, D., Kale, R. K., Singh, R. P. (2013) Usnic acid inhibits growth and induces cell cycle arrest and apoptosis in human lung carcinoma A549 cells. *Nutr. Cancer* **65,** 36-43.

Suleyman, H., Yildirim, D., Aslan, A., Göçer, F., Gepdiremen, A., Güvenalp, Z. (2002) An investigation of the antiinflammatory effects of an extract from *Cladonia rangiformis* HOFFM. *Biol. Pharm. Bull.* **25,** 10-13.

Vainio, H., Bianchini, F. (eds) (2002) *IARC Handbooks of Cancer Prevention*, WHO, Lyon, France

Wannous, R., Bon, E., Mahéo, K., Goupille, C., Chamouton, J., Bougnoux, P., Roger, S., Besson, P., Chevalier, S. (2013) PPARβ mRNA expression, reduced by n-3 PUFA diet in mammary tumor, controls breast cancer cell growth. *Biochim. Biophys. Acta* **1831,** 1618-1625.

World Health Organization (2008) *WHO Health Statistics, 2008*. Geneva, Switzerland

Zhang, Y., Shi, J., Zhao, Y., Cui, H., Cao, C., Liu, S. (2012) An investigation of the anti-diabetic effects of an extract from Cladonia humilis. *Pak. J. Pharm. Sci*. **25,** 509-512.