

Drug and Chemical Toxicology

ISSN: (Print) (Online) Journal homepage: https://www.tandfonline.com/loi/idct20

Lichens exerts an anti-proliferative effect on human breast and lung cancer cells through induction of apoptosis

Sule Ozturk, Merve Erkisa, Seyhan Oran, Engin Ulukaya, Serap Celikler & Ferda Ari

To cite this article: Sule Ozturk, Merve Erkisa, Seyhan Oran, Engin Ulukaya, Serap Celikler & Ferda Ari (2021) Lichens exerts an anti-proliferative effect on human breast and lung cancer cells through induction of apoptosis, Drug and Chemical Toxicology, 44:3, 259-267, DOI: 10.1080/01480545.2019.1573825

To link to this article: https://doi.org/10.1080/01480545.2019.1573825

Published online: 05 Mar 2019.

Submit your article to this journal

Article views: 270

View related articles

View Crossmark data

Full Terms & Conditions of access and use can be found at
https://www.tandfonline.com/action/journalInformation?journalCode=idct20

DRUG AND CHEMICAL TOXICOLOGY
2021, VOL. 44, NO. 3, 259–267
https://doi.org/10.1080/01480545.2019.1573825

Taylor & Francis
Taylor & Francis Group

RESEARCH ARTICLE

Check for updates

Lichens exerts an anti-proliferative effect on human breast and lung cancer cells through induction of apoptosis

Sule Ozturk[a]*, Merve Erkisa[b]*, Seyhan Oran[a], Engin Ulukaya[b], Serap Celikler[a] and Ferda Ari[a]

[a]Department of Biology, Faculty of Science and Arts, Bursa Uludag University, Bursa, Turkey; [b]Department of Medical Biochemistry, School of Medicine, Istinye University, Istanbul, Turkey

ABSTRACT

Successful cancer treatment still requires new complexes or compounds from natural sources. Therefore, we investigated anti-growth/apoptotic effects of methanol extracts of the lichen species (*Xanthoparmelia somloensis* (Gleyn.) Hale, *Usnea intermedia* (A. Massal.) Jatta, *Bryoria capillaris* (Ach.) Brodo & D. Hawksw and *Lobaria pulmonaria* (L.) Hoffm.) on human lung (A549, H1299) and breast (MCF-7, MDA-MB-231) cancer cell lines. Anti-growth effects were monitored by the MTT and ATP viability assays. Cell death mode was evaluated by employing the fluorescence staining of nucleus, caspase-cleaved cytokeratin 18 detection, caspase 3/7 activity assay, Anneksin V cytofluorimetric assay and mitochondria membrane potential assay. Among the lichen extracts, *Usnea intermedia* exhibited strong anti-growth activity in a dose-dependent manner (1.56–100 μg/ml) compared to the others. *Usnea intermedia* was especially cytotoxic against MDA-MB-231 and H1299 cells (IC_{50} value for was found 3.0 and 10.2 μg/ml respectively). The cytotoxicity was resulted from apoptosis as proved by the presence of pyknotic nuclei, caspase 3/7 activity, phosphatidylserine translocation and loss of mitochondria membrane potential. In conclusion, *Usnea intermedia* warrants for further *in vivo* evaluation as a new alternative in cancer treatment.

ARTICLE HISTORY
Received 21 March 2018
Revised 10 January 2019
Accepted 16 January 2019

KEYWORDS
Apoptosis; cancer cell death; cytotoxicity; lichens; *Usnea intermedia*

Introduction

Cancer is known as a disease characterized by uncontrollable cell growth and proliferation. In recent years, there is an increase on new cancer cases, whereas the mortality of cancer patients decreases (Siegel *et al*. 2012). Cancer treatments by chemotherapy help to reduce the risk of cancer-related deaths. Unfortunately, the success rate still needs to be improved although new chemotherapeutics have been introduced into the market each year. A major problem is the resistance to chemotherapy or other therapies. For this reason, there is a need for more effective cancer treatments (Holohan *et al*. 2013).

Mutualistic life examples of lichens, algae and/or cyanobacteria and fungi have the ability to produce some substances that cannot be produced when they live independently (Ahmadjian 1993). Up to this date, hundreds of secondary metabolites for example depsides, depsidones, carotenes, terpens, xanthones, antraquinones and chomones have been detected in lichens (Boustie and Grube 2005, Huneck and Yoshimura 1996). Some of lichen species have established to be a reservoir of these metabolites for nutrients and medicinal purposes. Secondary metabolites of lichens have varied biological activities as antimicrobial, antiviral, antioxidant, antimutagenic, antiproliferative and cytotoxic effects *in vitro* and *in vivo* (Nguyen *et al*. 2014, Basile *et al*. 2015, Nithyanand *et al*. 2015, Ristić *et al*. 2016, Ebrahim *et al*. 2016). Several investigations were conducted related to cytotoxic activity and antiproliferative effects of lichens or secondary metabolites from lichen extracts on cancer cell lines (Bačkorová *et al*. 2012, Shrestha *et al*. 2015, Studzińska-Sroka *et al*. 2016). The extract of *Parmotrema reticulatum* showed anti-proliferative activity and inducing apoptosis on MCF-7 cell lines via induced cell cycle arrest (Ghate *et al*. 2013). In a different study, anti-proliferative and apoptotic activity was also reported for the methanol extracts of *Cladonia rangiformis* and *Cladonia convoluta* against MCF-7 cells (Coskun *et al*. 2015). In our previous study, the anti-proliferative activity of lichen species of *Parmelia sulcata* was investigated in breast cancer cell lines and genotoxic effects in human lymphocytes. This study showed that *Parmelia sulcata* extract inhibited anti-growth activity on breast cancer cell lines (Ari *et al*. 2015). In another study, we studied genotoxic, cytotoxic, and apoptotic effects of metanolic extract of *Hypogymnia physodes* on breast cancer cell lines (Ari *et al*. 2014a).

Bryoria capillaris and *Usnea intermedia* are fruticose form and belong to the family of Parmeliaceae. *Lobaria pulmonaria* and *Xanthoparmelia somloensis* are foliose form and belong to Lobariaceae and Parmeliaceae, respectively. Although the anticancer activity of many lichen species have been studied so far, the cytotoxic/apoptotic activities of this lichen species have been shown in this study. Our results suggest that these lichen species, especially *Usnea intermedia*, may provide

CONTACT Ferda Ari ferdaoz@uludag.edu.tr Department of Biology, Science and Arts Faculty, Uludag University, Bursa 16059, Turkey
*These authors contributed equally to this work

© 2019 Informa UK Limited, trading as Taylor & Francis Group

a novel approach for the treatment of breast and lung cancer.

Materials and methods

Lichen samples

Trunks of *Quercus* sp. (Uludag Mountain, Bursa, Turkey) were used to obtain lichen materials and identification was made through the aid of flora books (Purvis *et al.* 1994, Wirth 1995), followed by the deposition of it in the Herbarium of Uludag University (BULU), Bursa, Turkey. Lichen samples were air-dried and then cleansed of extraneous materials with caution and then it was finally ground into powder. 150 ml of solvent methanol (Merck, Darmstad, Germany) and water were added to 15 g of the ground material to extract it in a Soxhlet extractor. The process lasted for 24 h, followed by the concentration of the crude extracts using a rotary evaporator at 40 °C. Finally, lyophilization was taken placed and then it was stored at −20 °C by the time of the experimentation.

Chemicals and cell culture

Stock concentrations of lyophilized extracts of *Xanthoparmelia somloensis*, *Usnea intermedia, Bryoria capillaris*, and *Lobaria pulmonaria* were prepared in DMSO at a concentration of 0.05g/0.5 ml. The final concentrations were prepared by dilution with the culture medium. Varying doses (1.56–100 μg/ml) of lichen extract was applied to the cell cultures. RPMI 1640 supplemented with penicillin G (100 U/ml) and streptomycin (100 μg/ml) was used to culture the cells of human breast (MCF-7, MDA-MB-231) and lung (A549, H1299) cancers. L-glutamine and 10% fetal bovine serum (Invitrogen, Paisley, UK) were also added to the culture medium. The cells were kept at 37 °C with 5% CO_2.

Determination of cytotoxic activity

The MTT and ATP viability assays

Two hundred μl of culture medium was used to seed the cancer cells in triplicate at a density of 5000 cells/well of a 96-well plate. For control cells, they had 0.1% DMSO. For the tested cells, they had the lichen extracts. Both incubations were made for 72 h. The experiments were repeated at least two times in triplicate. Following treatments (72 h), each well got the MTT solution that contains 5 mg MTT powder in one ml PBS (pH 7.2). Following MTT application for 4 h, the cells were lysed with 100 μl of a solubilizing buffer that is freshly-prepared each time in 10% sodium dodecyl sulfate dissolved in 0.01 N HCl. And then, the absorbances were read by an ELISA plate reader at 570 nm following overnight incubation. Finally, percentage viabilities of treated cells were calculated in comparison with the untreated control cells. The formula following was used to calculations: % Viability = 100 x [(Sample Abs)/(Control Abs)].

For the confirmation of MTT results, the adenosine triphosphate (ATP) assay was performed. The reason for that was any possibility of interferences between the MTT compound and the lichen extracts. One or some of the unknown compounds of the extracts may show a reducing activity on the MTT compound, that is the reason for false positive results for viability. In addition, the ATP assay is a more sensitive assay than the MTT assay, thereby allowing to detect much lower cell numbers. The seeding densities and all the other things (e.g., treatments) in the ATP assay was similar to the MTT assay. ATP content of cells was measured following the recommendations of manufacturer (ATP Bioluminescence Assay, Sigma). In short, cells were lysed to extract ATP and then a luciferin-luciferase solution was added. A luminometer (Bio-Tek, USA) was used to read the luminescence. The results were presented in U/L.

Determination of cell death mode

Apoptosis detection by M30 antigen

Cytokeratin 18 (CK-18) is cleaved by specific caspases in apoptosis, followed by the occurrence of new CK-18 fragments (M30 antigens) whose concentrations are measured by an immunoassay kit (M30-Apoptosense ELISA kit, Peviva AB, Sweden). 10 000 cells were grown in a 96-well plate in 200 μl culture medium in triplicate and then treated for 72 h with 100 μg/ml of lichen extracts. As a positive control for cell death, paclitaxel (3.12 μM) was used because this agent is considered an appropriate apoptosis-inducer. Cells were exposed to 10% NP-40 for lysis for 10 min on a shaker at the end of the treatment. Wells were centrifuged at 2000 rpm for 10 s to remove the debris, followed by the placement of samples into wells that are coated with a mouse monoclonal antibody as a catcher. After cleansing the plate wells, M30 antibody that is horseradish peroxidase-conjugated was used for detection. An ELISA reader was used to read the absorbances at 450 nm (FLASH Scan S12, Eisfeld, Germany).

Annexin-V-FITC fluorescence imaging for apoptosis

The phosphoditylserine (PS) is found in the cytoplasmic site of the plasma membrane. PS translocation to outer membrane site occurs in early phase of apoptosis, thereby the detection of this translocation is used as a molecular marker for detection of apoptotic cells. Annexin-V-FITC is able to bind to PS, allowing the apoptotic cells to become visible. However, Annexin-V-FITC may also bind to necrotic cells. Therefore, propidium iodide (PI) is additionally used as a second dye to distinguish apoptotic and necrotic cells from each other. Necrotic cells are both Annexin-V-FITC and PI positive while apoptotic cells are only Annexin-V-FITC positive. Briefly, cancer cells were seeded in a 96-well plate at the density of 5×10^3 cells per well, and then the cells were treated for 24 h with *Usnea intermedia* at the 100 μg/ml dose. In addition, paclitaxel (3.12 μM) was used as a positive control for apoptosis. Following treatment, Annexin-V-Fluos kit (Roche, Germany), which contains Annexin-V-FITC and propidium iodide, was used to stain the cells, according to the protocol given in the booklet of the company. Moreover, a fluorescent dye (Hoechst dye 43332) which stains the nucleus

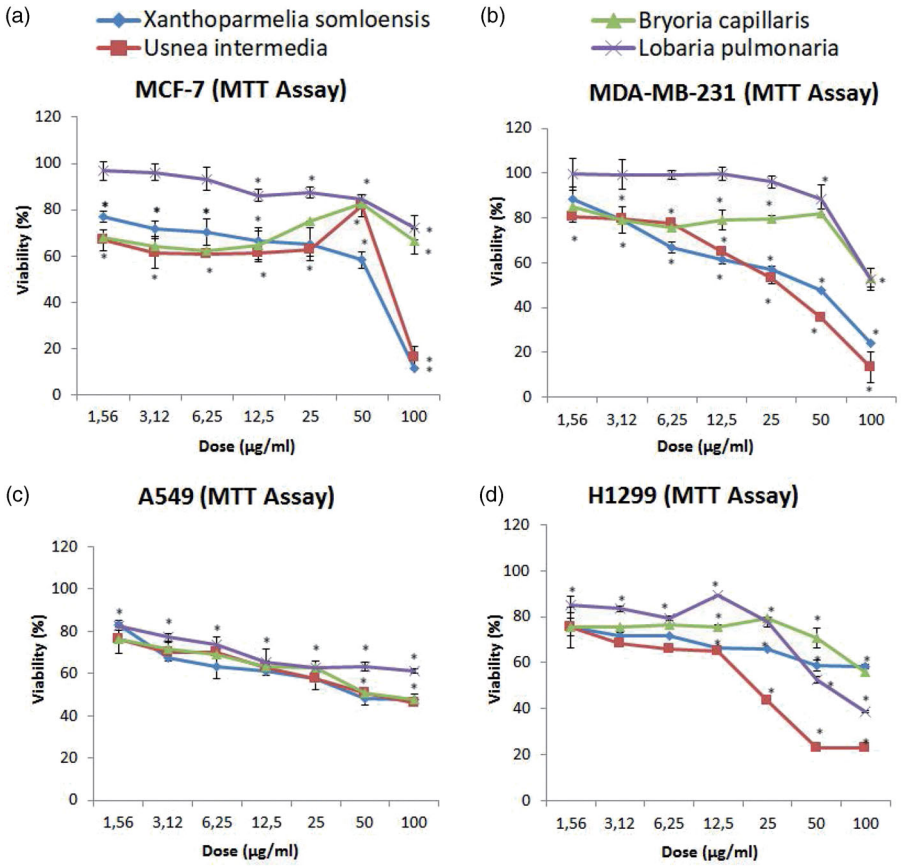


Figure 1. Anti-growth effect after the treatment with varying doses of *Xanthoparmelia somloensis*, *Usnea intermedia*, *Bryoria capillaris* and *Lobaria pulmonaria* for 72 h against MCF-7 (a), MDA-MB-231 (b) breast and A549 (c), H1299 (d) lung cancer cell lines by the MTT assay *Denotes statically significant differences in comparison with control ($p < 0.05$).

at the dose of 200 µg/ml was added to the staining solution to additionally detect apoptosis on the basis of nuclear morphology. Finally, a fluorescence microscope was used to examine the cells.

Caspase 3/7 and annexin V/dead cell assay

Treatment was made with *Usnea intermedia* (100 µg/ml) for 48 h. Human breast (MCF-7, MDA-MB-231) and lung (A549, H1299) cancer cell lines were flow-cytometrically analyzed to look at the early and late stage of apoptosis using Muse Annexin V/Dead Cell (cat #MCH100105, Merck Millipore, Billerica, MA, USA) and Muse Caspase 3/7 (cat #MCH100108, Merck Millipore) assays by Muse automated cell analyzer (Merck, Millipore), according to the protocol of the manufacturer. Non-apoptotic live cells appeared at the lower left panel (7-AAD negative, apoptosis negative) while apoptotic dead cells appeared at the upper right panel (7-AAD positive, apoptosis positive).

Mitochondria membrane potential assay

In apoptosis, mitochondrial events take place. For example, the membrane potential changes have been implicated in apoptotic cell death. Cells were treated for 48 h with 100 µg/ml of *Usnea intermedia* extract. For detection of mitochondrial membrane polarization, Muse Mitopotential assay kit (cat #MCH100110, Merck Millipore) was used according to the manufacturer's instructions. To show the cell membrane structural integrity, a dead cell marker (7-AAD) is also used. It is pumped out from live or healthy cells as well as early apoptotic cells. Dead cells thus show increased fluorescence in the viability axis.

In the cytometry-related diagrams, four distinctive populations of cells can be seen.

- (LL) Live cells with depolarized mitochondrial membrane: MitoPotential (−) and 7-AAD (−)
- (LR) Live cells with intact mitochondrial membrane: MitoPotential (+) and 7-AAD (−)
- (UR) Dead cells with depolarized mitochondrial membrane: MitoPotential (+) and 7-AAD (+)
- (UL) Dead cells with intact mitochondrial membrane: MitoPotential (−) and 7-AAD (+)

Statistical analysis

SPSS 20.0 statistical software package for Windows was used for all statistical analyses performed. Significance was calculated using one-way analysis of variance (ANOVA). For consideration of statistical significance, a value of $p < 0.05$ was accepted. The results were given as the mean ± SD (Standard Deviation).

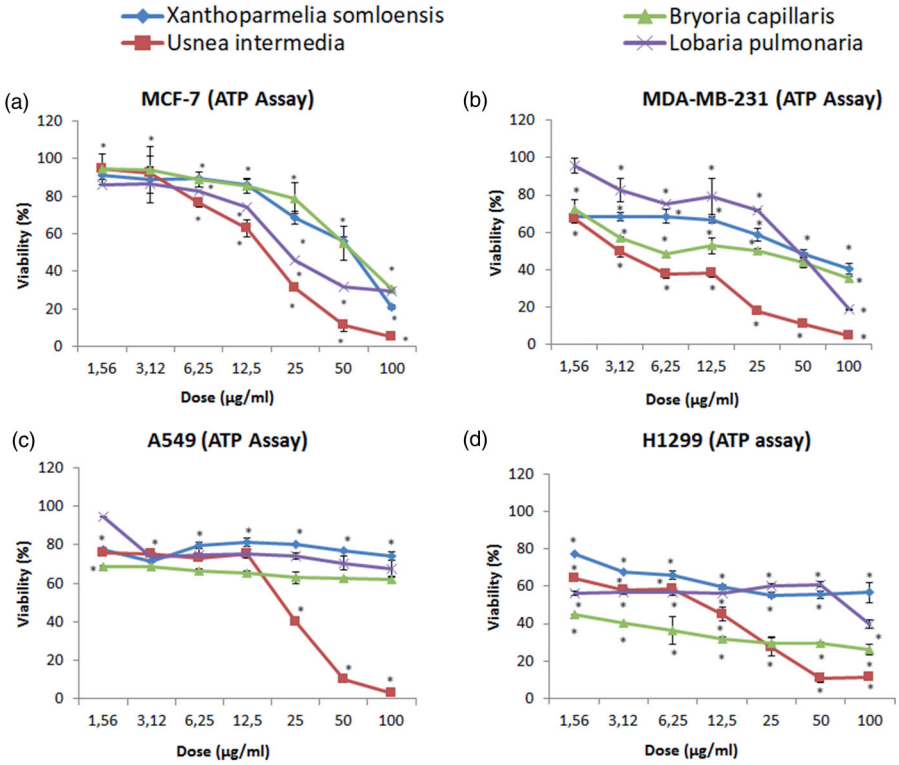


Figure 2. Anti-growth effect after the treatment with varying doses of *Xanthoparmelia somloensis*, *Usnea intermedia*, *Bryoria capillaris* and *Lobaria pulmonaria* for 72 h against MCF-7 (a), MDA-MB-231 (b) breast and A549 (c), H1299 (d) lung cancer cell lines by the ATP assay *Denotes statically significant differences in comparison with control ($p < 0.05$).

Table 1. Anti-growth parameters for the extracts *of Xanthoparmelia somloensis*, *Usnea intermedia*, *Bryoria capillaris* and *Lobaria pulmonaria* determined by the ATP viability assay following 72 h of treatment against cancer cell lines.

Cell Line	Lichen extracts	IC_{50} (µg/ml)
MCF-7	*Xanthoparmelia somloensis*	58.8 ± 0.23
	Usnea intermedia	17.5 ± 0.54
	Bryoria capillaris	59.8 ± 4.96
	Lobaria pulmonaria	23.1 ± 2.94
MDA-MB-231	*Xanthoparmelia somloensis*	45.8 ± 10.68
	Usnea intermedia	3.0 ± 0.23
	Bryoria capillaris	26.62 ± 2.82
	Lobaria pulmonaria	44.7 ± 1.67
A549	*Xanthoparmelia somloensis*	>100
	Usnea intermedia	21.4 ± 0.12
	Bryoria capillaris	>100
	Lobaria pulmonaria	>100
H1299	*Xanthoparmelia somloensis*	>100
	Usnea intermedia	10.2 ± 1.88
	Bryoria capillaris	<1.56
	Lobaria pulmonaria	75.7 ± 0.39

*IC_{50} is defined as the dose inhibiting 50% of viability.

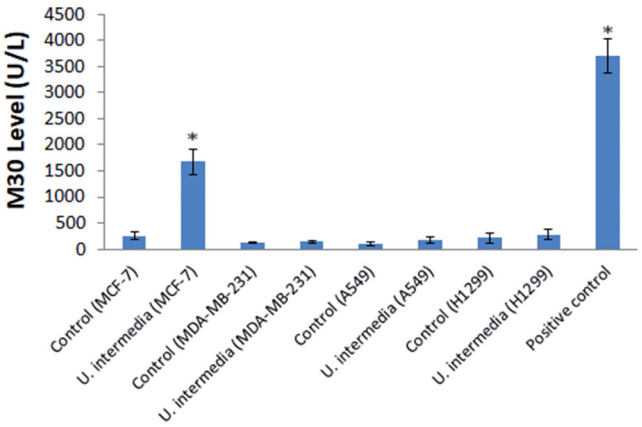


Figure 3. Measurement of caspase-cleaved cytokeratin 18 (M30) levels 48 h after the treatment with 100 µg/mL *Usnea intermedia*, in cancer cells. Paclitaxel (3.12 µM) was used as a positive control for M30. * denotes statistically significant change from control ($p < 0.05$).

Results

Anti-growth activity of lichen extracts by the MTT and ATP viability assays

The anti-growth effects of lichen extracts were investigated against human breast (MCF-7, MDA-MB-231) and lung (A549, H1299) cell lines by the MTT assay. Cancer cells were treated for 72 h with increasing doses of lichen extracts (1.56–100 µg/ml). It was found that lichen extracts exhibited anti-growth effects in a dose-dependent manner (Figure 1). According to the dose response curves of each cell line, breast cancer cells (MCF-7, MDA-MB-231) were particularly sensitive to lichen extracts.

Anti-growth effects can also be assessed by measuring the intracellular ATP levels in cells. Lichen extracts significantly reduced the cell viability in a dose-dependent manner ($p < 0.05$) (Figure 2). The cytotoxic effect of the lichen extracts was confirmed by the ATP viability assay. As shown in Figure 2, among lichen extracts, the *Usnea intermedia* significantly inhibited the growth of the cells at relatively lower doses ($p < 0.05$).

The IC_{50} values of lichen extracts were calculated on the basis of the ATP assay results (Table 1). The *Usnea intermedia* clearly resulted in higher cytotoxic activity in cancer cells compared to the others as shown in Table 1. IC_{50} values

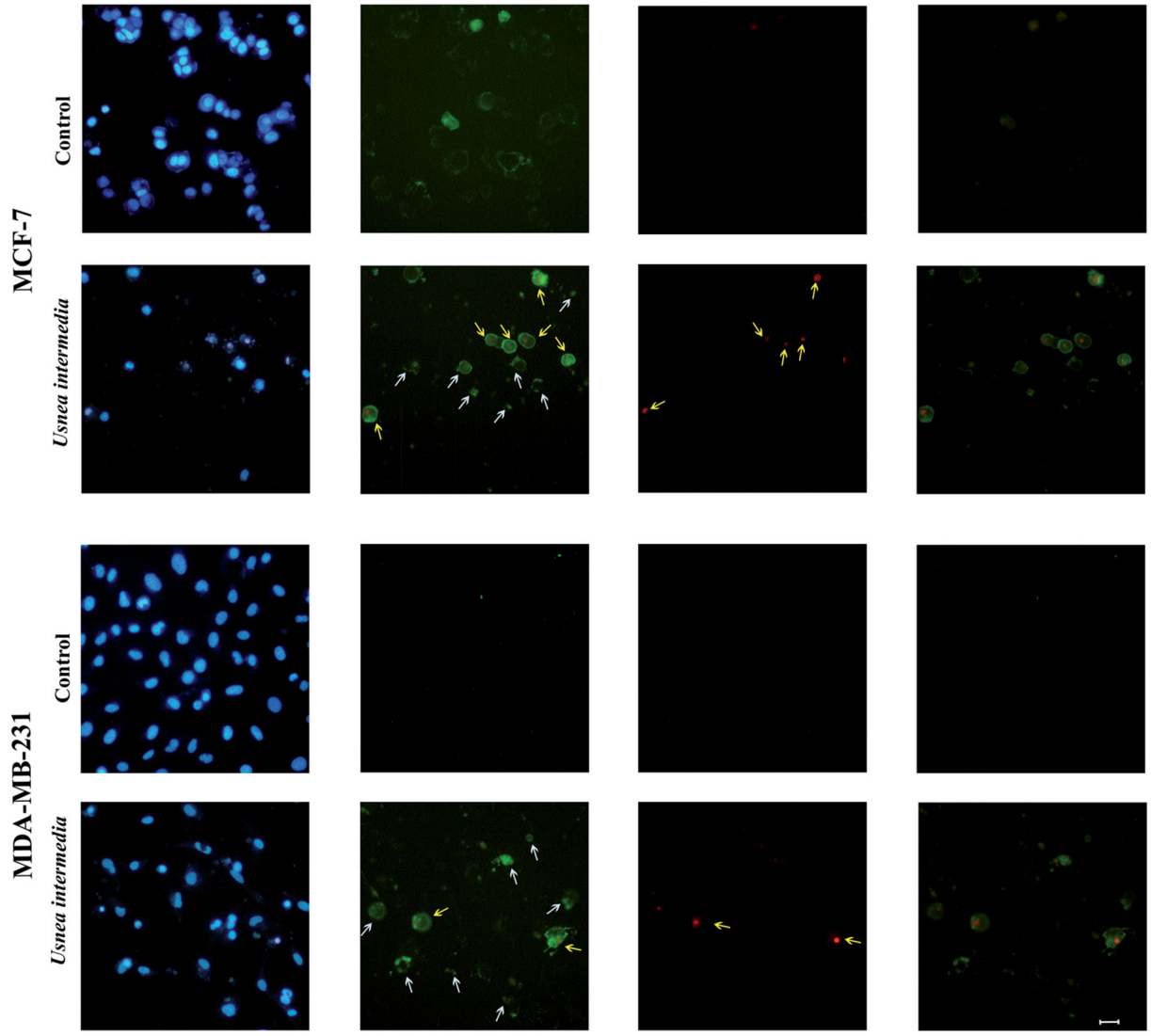


Figure 4. Flourescence imaging of MCF-7 and MDA-MB-231 breast cancer cells for determination of cell death mode. Cells were treated with 100 μg/mL *Usnea intermedia* for 24 h. Left panel shows Hoechst dye staining (blue); middle panels show Annexin-V-FITC (green) and PI (red) staining; right panel show merge of figures, respectively. White arrows show the presence of only Annexin-V-FITC staining for early apoptosis. Yellow arrows show the presence of both Annexin-V-FITC and PI staining for late apoptosis. A scale bar represents 20 μm.

were 17.5 ± 0.54 and 3.0 ± 0.23 μg/ml for MCF-7 and MDA-MB-231 cell lines, 21.4 ± 0.12 and 10.2 ± 1.88 μg/ml for A549 and H1299 cell lines, respectively.

Apoptosis-inducing effect of usnea intermedia on cancer cells

Because the *Usnea intermedia* was found to have the most promising cytotoxic activity, only this lichen was studied further for the elucidation of cell death mode. The apoptosis-inducing effect of *Usnea intermedia* was investigated by examining caspase-cleaved cytokeratin 18 (M30), which is a marker of apoptosis. In Figure 3, we found that M30 levels were significantly increased in MCF-7 breast cancer cells after the *Usnea intermedia* (100 μg/ml, 48 h) treatment compared to a positive control (paclitaxel, $p < 0.05$). M30 antigen levels were not changed in MDA-MB-231, A549 and H1299 cancer cells after the treatment with *Usnea intermedia*.

Annexin-V-FITC staining was performed in order to determine induction and progress of apoptosis and distinguish early and late apoptosis as well as necrosis. Hoechst dye 43332 was also used to observe all types of cells (alive and dead). Cells were treated with *Usnea intermedia* (100 μg/ml) for 24 h. We observed pyknotic nucleus (a marker of apoptosis) especially in breast cancer cell lines (MCF-7 and MDA-MB-231) by staining with Hoechst dye 43332 (blue). While primary necrotic or late apoptotic cells were positively stained with both Annexin-V-FITC (green) and PI (red), apoptotic cells were stained only Annexin-V-FITC positive (Figure 4). When considered together, our experiments suggest the apoptotic cell death in breast cancer cell lines (MCF-7 and MDA-MB-231). We observed that *Usnea intermedia* caused apoptotic cell death in A549 and H1299 lung cancer cell lines that was evident from the presence of Annexin-V-FITC staining. Some cells were observed to be positive for both Annexin-V-FITC (green) staining and PI (red) staining. However, these cells were still considered as apoptotic cells because the nucleus of those cells did not

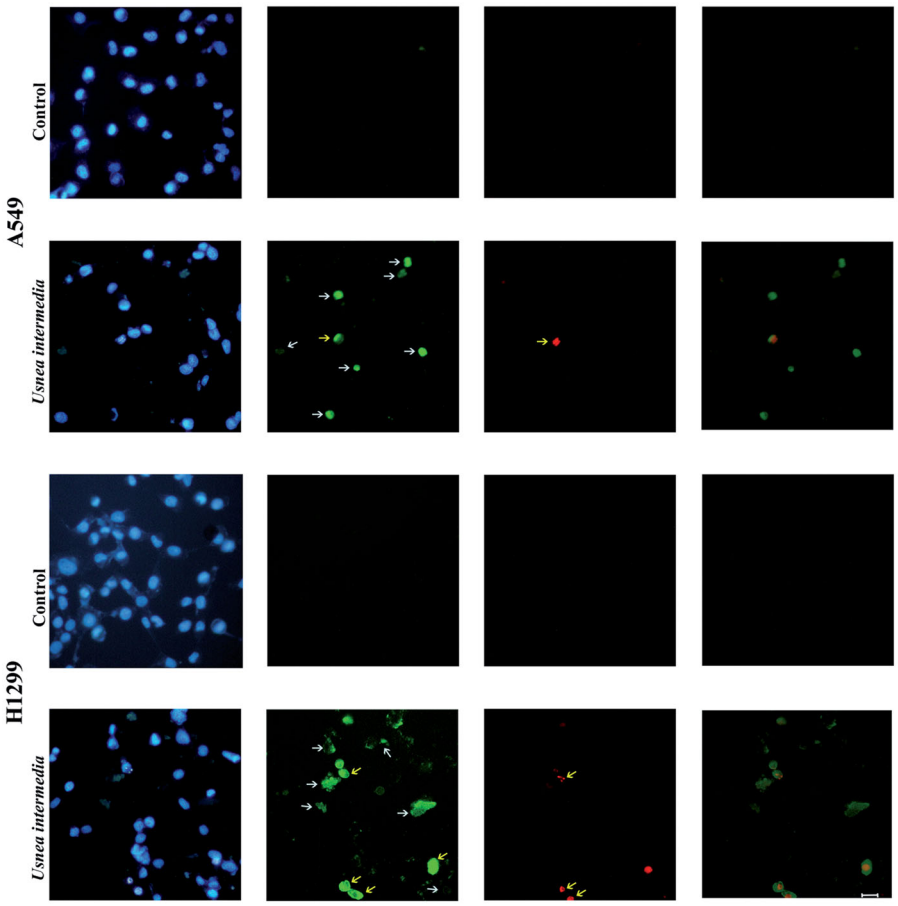


Figure 5. Flourescence imaging of A549 and H1299 human lung cancer cells for determination of cell death mode. The cells were treated with 100 μg/mL *Usnea intermedia* for 24 h. Left panel shows Hoechst dye staining (blue); middle panels show Annexin-V-FITC (green) and PI (red) staining; right panel show merge of figures, respectively. White arrows show the presence of only Annexin-V-FITC staining for early apoptosis. Yellow arrows show the presence of both Annexin-V-FITC and PI staining for late apoptosis. A scale bar represents 20 μm.

show any enlargement that is normally expected to occur in primary necrotic cells. We therefore considered those cells as being at the stage of late apoptosis (Figure 5).

The apoptosis inducing effects of the *Usnea intermedia* was also investigated by caspase 3/7, Annexin V and mitochondrial membrane potential by cytofluorimetric analysis. In Figure 6 shows that *Usnea intermedia* was increased in the caspase 3/7 activity (percentage of total apoptotic cells) compared with the controls at 48 h of treatment with 100 μg/ml dose. Data showed that the use of 100 μg/ml *Usnea intermedia* extract caused approximately 40–60% of the cells go to apoptotic stage. Our results suggest that *Usnea intermedia* induce caspase-dependent apoptosis not only on breast cancer cell lines but also on lung cancer cells.

The obtained results of Annexin V from flow cytometry, *Usnea intermedia* extract (100 μg/ml) stimulated an increase in the percentage of total apoptotic cells compared to the controls (Figure 7). Treatment with 100 μg/ml *Usnea intermedia* extract caused early stage of apoptosis approximately 50% of the cells (MDA-MB-231 and lung cancer cell lines, A549 and H1299). As for MCF-7 cell line, we found that about 12% of the MCF-7 cells were on early apoptotic stage and 20% were already in the late apoptotic stage.

During the cell death mitochondria plays important role and depolarization of transmembrane potential observed in the early stages of apoptosis. We found that mitochondrial membrane potential was depolarized when treated with (100 μg/ml) *Usnea intermedia* extract on breast (MCF-7, MDA-MB-231) and lung (A549, H1299) cell lines (Figure 8).

Discussion

Medicinal plants play a critical role in treatment of various diseases including cancer from far in the past. Lichens are valuable plant resources and are used in medicine, food, perfume and dye industry throughout the world (Shukla *et al.* 2010). Lichens and secondary metabolites have anticancer properties on cancer cells, therefore, in this study, the cytotoxic effects of the methanol extract of *Bryoria capillaris*, *Usnea intermedia*, *Lobaria pulmonaria* and *Xanthoparmelia somloensis* were investigated on human lung cancer (A549, H1299) and human breast cancer (MCF-7, MDA-MB-231) cells. We showed that lichen extracts exhibited significant anti-growth effects against cancer cell lines in a dose-dependent manner. Breast cancer cells (MCF-7, MDA-MB-231) were found to be more sensitive to lichen extracts for 72 h as seen by IC_{50} values (Table 1). In the literature, the lack of more studies using these lichen species on cancer cells prevented us from comparing our findings. In this aspect, this study is the first report showing increased cytotoxic activity of this lichen species on breast and lung cancer cells. As we have shown,

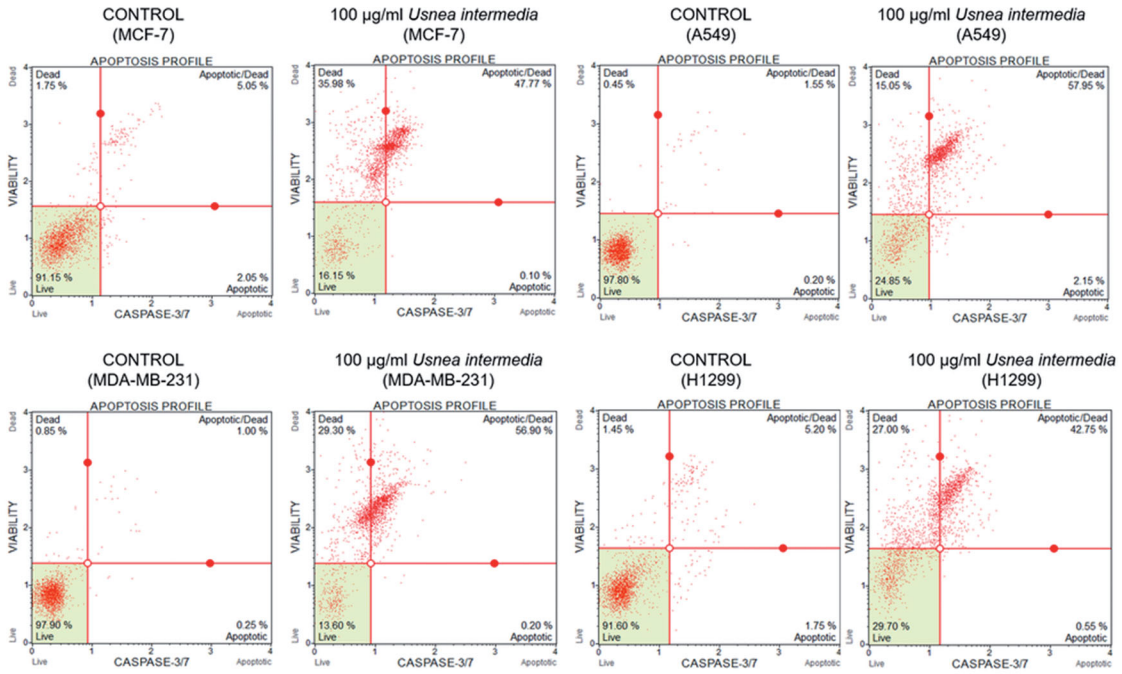


Figure 6. Measurement of caspase 3/7 activity, 48 h treatment with 100 µg/mL *Usnea intermedia,* in breast (MCF-7, MDA-MB-231) and lung (A549, H1299) cancer cell lines. Based on positivity for caspase 3/7 activity in apoptotic cells and simultaneous detection of dead cells (7-AAD dye positivity), assay enabled to differentiate four populations in each sample by cytofluorimetric separation on a Muse automated cell analyzer (Merck Millipore): non-apoptotic live (lower left (LL):7-AAD negative, apoptosis negative), non-apoptotic dead (upper left (UL):7-AAD positive, apoptosis negative), apoptotic live (lower right (LR):7-AAD negative, early apoptosis positive), and apoptotic dead (upper right (UR):7-AAD positive, late apoptosis positive) cells.

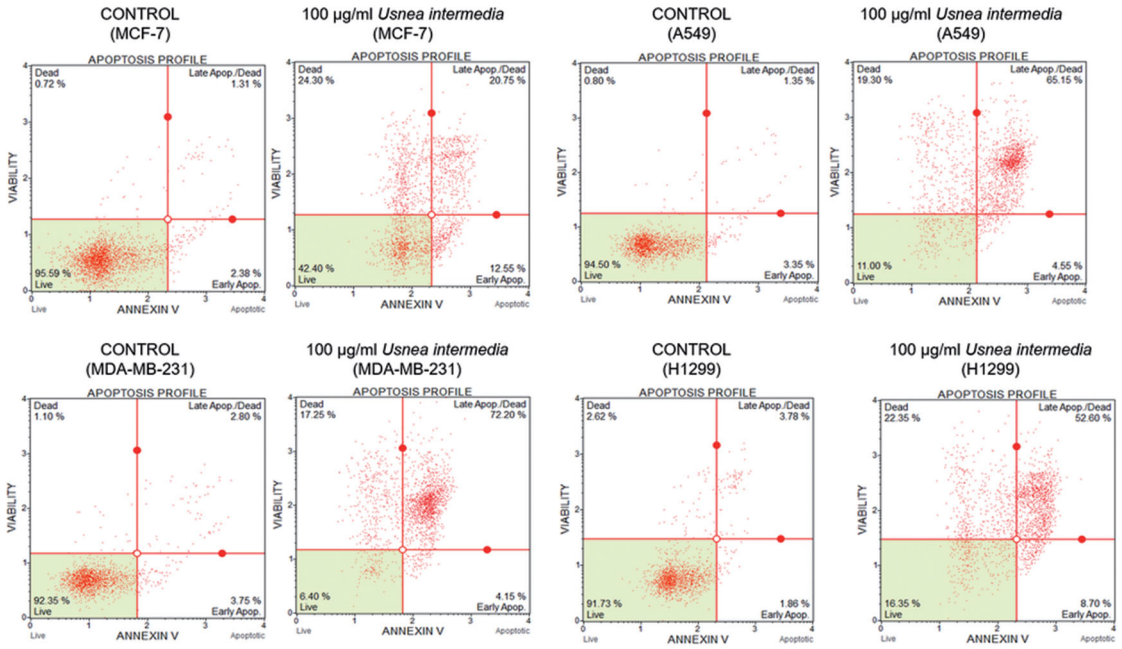


Figure 7. Measurement of phosphatidylserine exposure in apoptotic cells by Muse Annexin V/Dead Cell Assay. Cells treated at 48 h with 100 µg/mL *Usnea intermedia.* Based on positivity for phosphatidylserine exposure (positivity for annexin V) in apoptotic cells and simultaneous detection of dead cells (7-AAD dye positivity), assay enabled to differentiate four populations in each sample by cytofluorimetric separation on a Muse automated cell analyzer (Merck Millipore): non-apoptotic live (lower left (LL): 7-AAD negative, apoptosis negative), non-apoptotic dead (upper left (UL): 7-AAD positive, apoptosis negative), apoptotic live (lower right (LR): 7-AAD negative, early apoptosis positive), and apoptotic dead (upper right (UR): 7-AAD positive, late apoptosis positive) cells.

among lichen extracts, especially the *Usnea intermedia* significantly inhibited the growth of the cells at relatively lower doses. In our other study, we showed cytotoxic and apoptotic activity of *Usnea filipendula* extract in breast cancer cells (Celikler *et al.* 2014). The predominant phenolic compounds in *Usnea intermedia* extracts were stictic and usnic acids (Oran *et al.* 2016). Thus, it was shown that usnic acid was found to have strong anticancer activity toward several cell lines (Bačkorová *et al.* 2012, Singh *et al.* 2013, Koparal 2015).

We investigated the apoptosis-inducing effect of *Usnea intermedia* by examining caspase-cleaved cytokeratin 18, which is a marker of apoptosis. Interestingly, we found that M30 levels

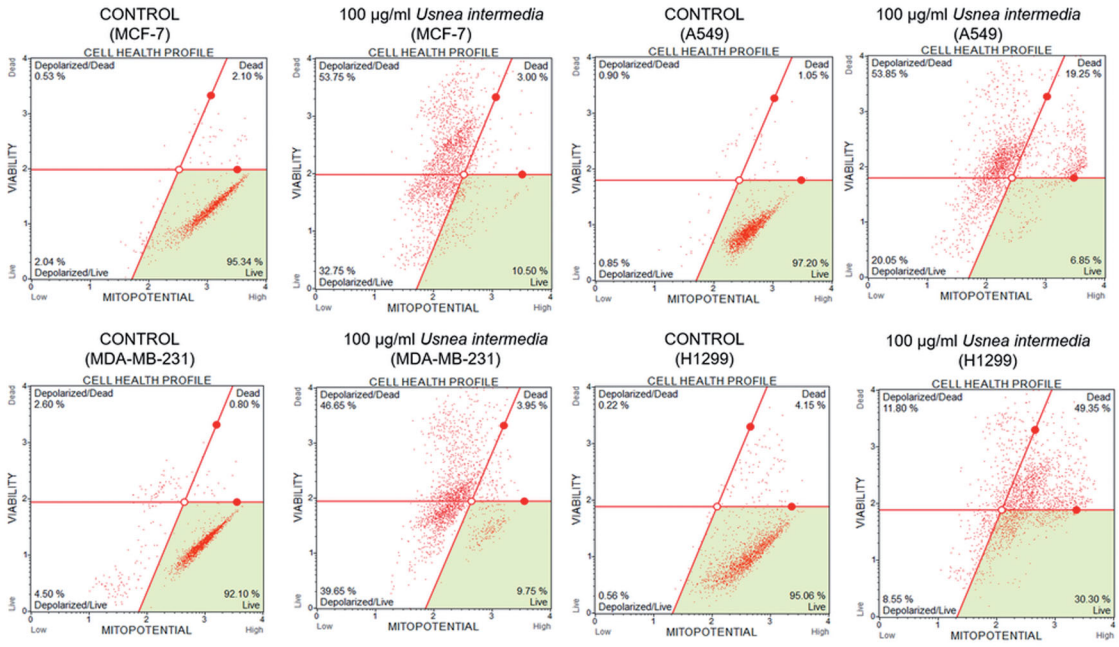


Figure 8. Determination of mitochondrial membrane potential (MMP) by Muse™ MitoPotential Assay. Cells were treated for 48 h with 100 µg/mL *Usnea intermedia*. The Muse™ MitoPotential assay enabled to differentiate four populations in each sample by cytofluorimetric separation on a Muse automated cell analyzer (Merck Millipore): (LL) Live cells with depolarized mitochondrial membrane: MitoPotential (−) and 7-AAD (−), (LR) Live cells with intact mitochondrial membrane: MitoPotential (+) and 7-AAD (−), (UR) Dead cells with depolarized mitochondrial membrane: MitoPotential (+) and 7-AAD (+), (UL) Dead cells with intact mitochondrial membrane: MitoPotential (−) and 7-AAD (+).

were significantly increased in MCF-7 breast cancer cells after the treatment with *Usnea intermedia* (100 µg/ml, 48 h treatment compared to a positive control). However M30 antigen levels were not changed in MDA-MB-231, A549 and H1299 cancer cells after the treatment with *Usnea intermedia* extract. In these cell lines, M30 levels did not increase compared to control that means these cells were either not dying via apoptosis or not expressing cytokeratin 18. Previously, we have showed that cytokeratin 18 expressions levels of H1299 (Cevatemre *et al.* 2015) and MDA-MB-231 (submitted for publication) cells were lower than A549 cells. Therefore, the results of the M30 assay should be interpreted cautiously. Morphological observation of cell death in cancer cells by fluorescence microscopy suggested there was an apoptotic cell death due to the presence of pyknotic nuclei and/or condensed chromatin by *Usnea intermedia*. Our data are consistent with the results of previous studies showing the methanol extracts of *Cladonia rangiformis* and *Cladonia convoluta* induced apoptosis in MCF-7 cells (Coskun *et al.* 2015). In another study, Ramalin, a compound of *Ramalina terebrata* caused apoptosis by activating the mitochondrial pathway in MCF-7 and MDA-MB-231 cells (Lee *et al.* 2016). In our previous study, we showed that extracts from *Parmelia sulcata* and *Usnea filipendula* exhibited apoptosis-like cell death on human lung cancer cells (A549, PC3) (Ari *et al.* 2014b). The fluorescence microscopy images of apoptosis were also confirmed by the cytofluorimetric analysis indicating how *Usnea intermedia* induce early (annexin V exposure at plasma membrane) as well as late apoptosis (caspase 3/7 activation) events. The *Usnea intermedia* induced alterations in mitochondrial membrane potential on cell lines by depolarization which has central role in apoptotic process. It was shown that usnic acid induced mitochondrial membrane depolarization in human lung cancer cells (Singh *et al.* 2013).

In conclusion, the lichen extracts from *Bryoria capillaris*, *Usnea intermedia*, *Lobaria pulmonaria* and *Xanthoparmelia somloensis* have anti-proliferative effect on breast and lung cancer cells in a dose-dependent manner. Particularly, *Usnea intermedia* induced apoptosis proved by the presence of pyknotic nuclei/Anneksin V staining and the increment of caspase 3/7 activity/loss of mitochondria membrane potential in breast and lung cancer cells. Our findings suggest that *Usnea intermedia* may deserve more attention regarding its potential as anticancer agent and warrants further *in vitro* and *in vivo* experiments.

Acknowledgement

The authors would like to thank O Akgün (Uludag University, Bursa, Turkey) for his assistance in editing of figures.

Disclosure statement

The authors report no conflicts of interest.

Funding

This study was financially supported by the Research Fund of Uludag University for the project that is numbered UAP(F)-2011/42.

References

Ahmadjian, V., 1993. *The lichen symbiosis*. New York, NY: John Wiley & Sons, Inc. pp 1–4.

Ari, F., *et al.*, 2014a. Genotoxic, cytotoxic and apoptotic effects of *Hypogymnia physodes* (L.) Nyl. on breast cancer cells. *Environmental Toxicology*, 29 (7), 804–813.

Ari, F., *et al.*, 2014b. *Parmelia sulcata* Taylor and *Usnea filipendula* Stirt induce apoptosis-like cell death and DNA damage in cancer cells. *Cell Proliferation*, 47 (5), 457–464.

Ari, F., *et al.*, 2015. Promising anticancer activity of a lichen, Parmelia sulcata Taylor, against breast cancer cell lines and genotoxic effect on human lymphocytes. *Cytotechnology*, 67 (3), 531–543.

Bačkorová, M., *et al.*, 2012. Lichen secondary metabolites are responsible for induction of apoptosis in HT-29 and A2780 human cancer cell lines. *Toxicology in Vitro*, 26 (3), 462–468.

Basile, A., *et al.*, 2015. Antiproliferative, antibacterial and antifungal activity of the lichen *Xanthoria parietina* and its secondary metabolite parietin. *International Journal of Molecular Sciences*, 16 (12), 7861–7875.

Boustie, J., and Grube, M., 2005. Lichens-a promising source of bioactive secondary metabolites. *Plant Genetic Resources: Characterization and Utilization*, 3 (02), 273–328.

Celikler, S., *et al.*, 2014. Genotoxic, cytotoxic, and apoptotic effects of crude extract of *Usnea filipendula* Stirt. in vitro. *Turkish Journal of Biology*, 38, 940–947.

Cevatemre, B., *et al.*, 2015. The M30 assay does not detect apoptosis in epithelial-derived cancer cells expressing low levels of cytokeratin 18. *Tumor Biology*, 36 (9), 6857–6865.

Coskun, Z.M., *et al.*, 2015. Anti-proliferative and apoptotic effects of methanolic extracts from different Cladonia species on human breast cancer cells. *Folia Biologica (Praha)*, 61, 97–103.

Ebrahim, H.Y., *et al.*, 2016. Norstictic acid inhibits breast cancer cell proliferation, migration, invasion, and in vivo invasive growth through targeting C-Met. *Phytotherapy Research*, 30 (4), 557–566.

Ghate, N.B., *et al.*, 2013. An antioxidant extract of tropical lichen, *Parmotrema reticulatum*, induces cell cycle arrest and apoptosisin breast carcinoma cell line MCF-7. *PLoS One*, 8 (12), e82293.

Holohan, C., *et al.*, 2013. Cancer drug resistance: an evolving paradigm. *Nature Reviews. Cancer*, 13 (10), 714–726.

Huneck, S., and Yoshimura, I., 1996. *Identification of lichen substances*. Berlin: Springer-Verlag.

Koparal, A.T., 2015. Anti-angiogenic and antiproliferative properties of the lichen substances (-)-usnic acid and vulpinic acid. *Zeitschrift Für Naturforschung C*, 70 (5-6), 159–164.

Lee, E., *et al.*, 2016. Ramalin-mediated apoptosis is enhanced by autophagy inhibition in human breast cancer cells. *Phytotherapy Research*, 30 (3), 426–438.

Nguyen, T.T., *et al.*, 2014. Lichen secondary metabolites in *Flavocetraria cucullata* exhibit anti-cancer effects on human cancercells through the induction of apoptosis and suppression of tumorigenic potentials. *PLoS One*, 9 (10), e111575.

Nithyanand, P., *et al.*, 2015. Usnic acid inhibits biofilm formation and virulent morphological traits of *Candida albicans*. *Microbiological Research*, 179, 20–28.

Oran, S., *et al.*, 2016. Antioxidant and antimicrobial potential, and hplc analysis of stictic and usnic acids of three Usnea species from Uludag Mountain (Bursa, Turkey). *Iran Journal Pharmaceutical Research*, 15, 527–535.

Purvis, O. W., *et al.*, 1994. *The lichen flora of Great Britain and Ireland*. 2nd ed. London, UK: Natural History Museum Publications in association with The British Lichen Society. pp. 710.

Ristić, S., *et al.*, 2016. Phytochemical study and antioxidant, antimicrobial and anticancer activities of *Melanelia subaurifera* and *Melanelia fuliginosa* lichens. *Journal of Food Science and Technology*, 53 (6), 2804–2816.

Shrestha, G., *et al.*, 2015. Anticancer activities of selected species of North American lichen extracts. *Phytotherapy Research*, 29 (1), 100–107.

Shukla, V., Joshi, G.P., and Rawat, M.S.M., 2010. Lichens as potential natural source of bioactive compounds: a review. *Phytochemistry Reviews*, 9 (2), 303–314.

Siegel, R., Naishadham, D., and Jemal, A., 2012. Cancer statistics, 2012. *CA: a Cancer Journal for Clinicians*, 62, 10–29.

Singh, N., *et al.*, 2013. Usnic acid inhibits growth and induces cell cycle arrest and apoptosis in human lung carcinoma A549 cells. *Nutrition and Cancer*, 65 (1), 36–43.

Studzińska-Sroka, E., *et al.*, 2016. Cytotoxic activity of physodic acid and acetone extract from *Hypogymnia physodes* against breast cancer cell lines. *Pharmaceutical Biology*, 54 (11), 2480–2485.

Wirth, W., 1995. *Die Flechten Baden-Württembergs. Teil 1–2*. Stuttgart: Verlag Eugen Ulmer, pp. 1006.