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Parmelia sulcata Taylor and *Usnea filipendula* Stirt induce apoptosis-like cell death and DNA damage in cancer cells

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Abstract

Objectives: Successful cancer treatments still require more compounds to be isolated from natural sources. Thus, we have investigated anti-proliferative/apoptotic effects of methanolic extracts of lichen species *Parmelia sulcata* Taylor and *Usnea filipendula* Stirt on human lung cancer (A549, PC3), liver cancer (Hep3B) and rat glioma (C6) cells.

Materials and methods: Anti-proliferative effects were monitored by MTT and adenosine triphosphate viability assays, while genotoxic activity was studied using the comet assay. Additionally, cell death mode and apoptosis assays (fluorescence staining, caspase-cleaved cytokeratin 18, caspase-3 activity and PARP cleavage) were performed.

Results: Extracts produced anti-population growth effects in a dose-dependent manner (1.56–100 μg/ml) by inducing apoptosis-like cell death. This resulted in the lines having the presence of pyknotic cell nuclei. In addition, significant increase in genetic damage in the cell lines was seen, indicating that DNA damage may have been responsible for apoptotic cell death.

Conclusion: In this study, methanolic extracts of *Parmelia sulcata* and *Usnea filipendula* induced apoptosis-like cell death by causing DNA damage, to cancer cells.

Introduction

Cancer remains one of the the leading causes of death globally. The International Agency for Research into Cancer (IARC) recently estimated that 7.6 million deaths were due to it, and 12.7 million new cases per year are reported worldwide (1). Thus, new anti-cancer active compounds from natural sources (plants and others) are required; *in silico* studies are leading the way in identifying pathways. Lichens are complex organisms living in a symbiotic relationship with fungi and algae (and/or cyanobacteria) (2). These associations lead to synthesis of a great number of secondary metabolites, the majority of which are unique to these groups. Through development of analytical techniques and experimental methods, approximately 1050 lichen compounds have been identified (3). Lichens have been used as medicines, food, fodder, perfumes, spices, dyes and for a wide variety of further purposes, throughout the world (4). Their metabolites have a number of possible biological activities, such as in antibiotics, in anti-tumour research, as antioxidants and anti-proliferative agents (2,3,5,6).

In the recent years, cytotoxic and genotoxic activities of lichen extracts have been investigated. Manojlović *et al.* evaluated cytotoxic potential of chloroform, ethyl acetate and methanolic extracts of *Thamnolia vermicularis* var. *subuliformis*. They observed that ethyl acetate and chloroform extracts had cytotoxic effects on HeLa cells 72 h after treatment (7). Anti-proliferative, apoptotic and genotoxic potential of *Hypogymnia physodes* and *Parmelia sulcata* extracts (PSE) has been studied by our group and we found that methanolic extracts of these lichen species, composed primarily of aliphatic compounds, had different effects (anti-proliferative or genotoxic) depending on doses used (8,9).

Parmelia sulcata and *Usnea filipendula* belong to the Parmeliaceae family and Kosanić *et al.* have shown that *P. sulcata* acetone extracts had anti-cancer activity on human melanoma and colon cancer cells (10). In a further study, Manojlović *et al.* found that salazinic acid (a major metabolite of *P. sulcata*), had high cytotoxic activity levels in human melanoma and colon cancer cell

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lines (11). However, there are limited data on cytotoxic and genotoxic activities of *U. filipendula* or *P. sulcata* in the literature.

In the present study, we have evaluated cytotoxic/apoptotic and genotoxic activities of methanolic extracts of *P. sulcata* and *U. filipendula* in human lung (A549, PC3), liver (Hep3B) and rat glioma (C6) cancer cell lines, by the comet assay, cell viability assays and apoptosis assays. We found that *P. sulcata* and *U. filipendula* had anti-population growth effects through induction of apoptosis-like cell death in human lung (A549, PC3) and liver (Hep3B) cancers and a rat glioma (C6), which warrants further *in vivo* experiments.

Materials and methods

Collection and identification of lichen samples

Lichen materials were collected from trunks of *Quercus* sp. (Uludag Mountain, Bursa, Turkey) in May 2010 and identified with the aid of flora academic literature (12,13). A voucher specimen was deposited in the Herbarium of Uludag University (BULU), Bursa, Turkey.

Extraction of lichen samples

Air-dried lichen samples were carefully cleansed of extraneous materials and ground into powder. Fifteen grams of ground material was extracted consecutively by adding 150 ml methanol solvent (Merck, Darmstad, Germany) and water, in a Soxhlet extractor, for 24 h. Crude extracts were concentrated using a rotary evaporator at 40 °C and thereafter, residues were lyophilized and stored at −20 °C until used in subsequent tests.

Chemicals and cell culture

Stock concentrations of lyophilized PSE and *Usnea filipendula* extract (UFE) samples were prepared in DMSO at 0.05 g/0.5 ml, while final concentrations were prepared in culture medium.

Lichen extract was used at different concentrations ranging from 1.56 to 100 μg/ml. Human lung (A549, PC3), liver (Hep3B) and rat glioma (C6) cell lines were cultured in RPMI 1640 supplemented with penicillin G (100 U/ml), streptomycin (100 μg/ml), L-glutamine, and 10% foetal calf serum (Invitrogen, Paisley, UK) at 37 °C in a humidified atmosphere containing 5% CO_2.

MTT viability assay

The various types of cancer cells were seeded in 200 μl culture medium in triplicate at 1×10^4 cells per well of 96-well plates. Cells were incubated either alone (controls with 0.1% DMSO) or in the presence of PSE or UFE for 72 h. Each experiment was conducted twice in triplicate. A 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) viability assay was performed as previously described (14). MTT was first prepared as a stock solution of 5 mg/ml in phosphate buffer (PBS, pH 7.2) and then was filtered. At the end of the treatment period (72 h), 20 μl of MTT solution (5 mg/ml PBS, pH 7.2) was added to each well. After incubation for 4 h at 37 °C, 100 μl of solubilizing buffer (10% sodium dodecyl sulphate dissolved in 0.01 N HCl) was added to each well. After overnight incubation, absorbance (Abs) was read using an ELISA plate reader at 570 nm, to determine cell viability, which was calculated in reference to untreated control cells using the formula:

$$\%\text{Viability} = 100\times\left(\frac{\text{Sample Abs}}{\text{Control Abs}}\right).$$

Adenosine triphosphate viability assay

Adenosine triphosphate (ATP) assay was performed to confirm results of the MTT assay due to possibility that the MTT assay itself could interfere with the lichen extracts. Moreover, the ATP assay is considered to be more sensitive than MTT (14). Seeding and treatment conditions, as well as viability calculation, were similar to those for the MTT assay (see above). At the end of the treatment period, ATP content was determined according to the manufacturer's recommendations (ATP Bioluminescence Assay; Sigma, St. Louis, MO, USA). Briefly, ATP was extracted from cells, and then luciferin-luciferase solution was added. Luminescence was determined in a luminometer (Bio-Tek, Winooski, VT, USA) and results were expressed in U/l. Cells were also morphologically observed by phase-contrast microscopy (Olympus CKX41, Tokyo, Japan) after exposure to 100 μg/ml of PSE and UFE for 72 h.

Fluorescence imaging for apoptosis

Detection of mode of cell death was performed on the basis of nuclear morphology, using fluorescence microscopy. Cells were stained with Hoechst 43332 dye to determine nuclear morphology. Hoechst dye stains all living or dead (primary necrotic or secondary necrotic) cells. Primary necrosis was decided on the basis of swelling and weak nuclear staining, as well as lack of fragmented or pyknotic nuclei. Secondary necrosis was decided by presence of pyknotic or fragmented nuclei with condensed (brighter) appearance. Briefly, cells were

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seeded in 6-well plates at 5×10^5 cells per well, then exposed to PSE and UFE at 100 μg/ml for 72 h. After treatment, cells were washed in PBS and incubated in Hoechst 43332 dye (5 μg/ml) solution for 15 min in the dark at 37 °C.

Caspase-cleaved cytokeratin 18 (M30) detection

Apoptosis was assayed by measuring levels of caspase-cleaved cytokeratin 18 (CCK18, M30) using a commercially available immunoassay kit (M30-Apoptosense ELISA kit; Peviva AB, Bromma, Sweden) according to the manufacturer's instructions. This method measures level of CK18-Asp396 neo-epitope (M30), a well-known marker of apoptosis. In 96-well plates, 1×10^4 cells were seeded per well in 200 μl culture medium in triplicate. Cells were treated for 72 h with 100 μg/ml PSE or UFE. Paclitaxel (3.12 μM) was used as positive control for apoptosis, as this agent is considered to be an appropriate apoptosis-inducer (15). At the end of the treatment period, cells were lysed in 10% NP-40 for 10 min on a shaker. Contents of identical wells were pooled and centrifuged at 295 g for 10 s to remove debris. All samples were placed in wells coated with mouse monoclonal antibody as catcher. After washing, horseradish peroxidase-conjugated antibody (M30-antibody) was used for detection, absorbance being determined using an ELISA reader at 450 nm (FLASH Scan S12, Eisfeld, Germany).

Measurement of active caspase-3 and cleaved PARP levels

Cancer cells (A549, PC3, Hep3B and C6) were seeded (1×10^6) in 25 cm^2 flasks and treated with 100 μg/ml of PSE or UFE for 72 h to detect active caspase-3 and cleaved PARP (poly(ADP-ribose) polymerase), markers of apoptosis. After treatment, cells were washed in ice-cold PBS and lysed in lysis buffer (Cell Signaling, MA, Danvers, USA) containing protease inhibitors (Sigma, St. Louis, MO, USA) and 1 mM phenylmethylsulphonyl fluoride. Cells were extracted at 4 °C for 5 min and centrifuged at 4 °C for 10 min at 14 000 ***g***. Cleaved PARP levels were estimated using PARP Cleaved [214/215] ELISA kit (Invitrogen, Camarillo, CA, USA) and human caspase-3(active) ELISA kit (Invitrogen Corporation) according to the protocols described in the manufacturers' instructions. For active caspase-3 determination, 100 μl cell lysates were incubated in microplate wells provided in the kit, at room temperature for 2 h. Samples were aspirated and washed 4 times in washing buffer and incubated in 100 μl detection antibody (anti-caspase-3) for 1 h at room temperature. After removal of antibody solution, wells were washed once more and incubated in 100 μl HRP anti-rabbit antibody for 30 min at room temperature. After aspirating the anti-rabbit antibody, blue colouration developed by adding 100 μl stabilized chromogen solution for 20 min at room temperature. The reaction was stopped by adding 100 μl stopping solution. For determination of cleaved PARP, 50 μl of each lysate was incubated with anti-cleaved PARP (detection antibody) for 3 h. After washing, each sample was incubated in a well with secondary IgG-HRP antibody solution for 30 min. Stabilized chromogen was added to each well for another 30 min, followed by addition of stop solution. Absorbance of each well was read at 450 nm using a microplate reader.

Comet assay

Alkaline version of the comet assay was used with modification (16). Briefly, cancer cells (A549, PC3, Hep3B and C6) were seeded (5×10^5 cells/well) in 6-well plates and incubated in 100 μg/ml of PSE or UFE, and 3.8 μg/ml cisplatin as positive control for 24 h. Cells were trypsinized and suspended in 60 μl PBS (phosphate buffered saline). Cell suspension containing 3×10^4 cells was mixed with 1% low melting point agarose (Sigma-Aldrich, Taufkirchen, German) and rapidly laid on slides coated with 1% normal melting point agarose(Sigma-Aldrich).

Cells were lysed and neutralized. Slides were stained with ethidium bromide (2 μg/ml) (Sigma-Aldrich) and visualized by fluorescence microscopy. Images were analysed using specialized software for COMET analysis (Kameram 21; Argenit, Istanbul, Turkey).

Comet length, tail length, tail DNA%, olive tail moment, head DNA%, genetic damage index (GDI) and percentage of damaged cell parameters were evaluated for each cell. GDI was calculated according to the following formula used by the COMET analysis program (16):

$$\mathrm{GDI} = \frac{(1 * \sum \mathrm{Type1}) + (2 * \sum \mathrm{Type2}) + (3 * \sum \mathrm{Type3}) + (4 * \sum \mathrm{Type4})}{\sum \mathrm{Type0} + \sum \mathrm{Type1} + \sum \mathrm{Type2} + \sum \mathrm{Type3} + \sum \mathrm{Type4}}$$

where ΣType0 is the total no damaged cells, ΣType1 is the total very low damaged cells, ΣType2 is the total low damaged cells, ΣType3 is the total high damaged cells, ΣType4 is the total very high damaged cells.

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Percentage of damaged cell (PDC) was calculated according to the following formula (16):

$$\%\text{Damaged cell} = \sum \text{Type2} + \sum \text{Type3} + \sum \text{Type4}$$

Statistical analysis

All statistical analyses were performed using the SPSS 20.0 statistical software package for Windows. Significance was calculated using one-way analysis of variance (ANOVA) and $P < 0.05$ was considered statistically significant. Results are expressed as mean ± SD.

Results

Anti-population growth activity of P. sulcata *and* U. filipendula *extracts by MTT and ATP viability assays*

The anti-population growth effects of PSE and UFE were investigated of different cancer cell lines by MTT assay. Cancer cells were treated for 72 h with increasing doses of PSE or UFE (1.56–100 μg/ml) and PSE and UFE exhibited anti-growth effects in a dose-dependent manner (Fig. 1). According to dose response curves of each cell line, Hep3B cancer cells were particularly sensitive to PSE and UFE.

Anti-growth effects can also be assessed by measuring intracellular ATP levels in cells; ATP level is sharply reduced after initiation of cell death. The extracts yielded clear dose–response curves for all cell lines used. Cell viability (considered as growth) significantly decreased after high doses of PSE and UFE (50 and 100 μg/ml, $P < 0.05$) (Fig. 2) treatment. However, it was found that PSE was slightly more cytotoxic to Hep3B and C6 cell lines than to A549 and PC3 cells (Fig. 2a). However, UFE had better cytotoxic effects on A549 and PC3s (Fig. 2b) compared to Hep3B and C6 cell lines.

IC_{50} values of PSE and UFE were calculated on the basis of ATP assay results. PSE clearly resulted in higher cytotoxic activity of Hep3B cells (47.3 μg/ml), while UFE was more cytotoxic to PC3 cells (32.9 μg/ml) compared to the others, as shown in Table 1.

Morphological evaluation by phase-contrast microscopy showed that PSE and UFE caused reduction in cell density, implying anti-population growth effects (Fig. 3). Cells were treated with PSE or UFE (100 μg/ml) for 72 h as shown in Fig. 3. PSE was more cytotoxic to

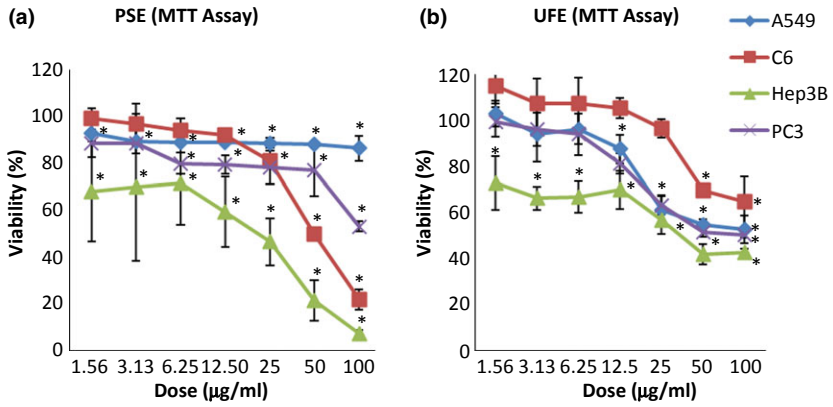

Figure 1. The anti-growth effect after the treatment with varying doses of *Parmelia sulcata* extract (PSE) (a) and *Usnea filipendula* extract (UFE) (b) for 72 h against cancer cell lines by the MTT assay as described in the Materials and methods section. *Denotes statically significant differences in comparison with control ($P < 0.05$).

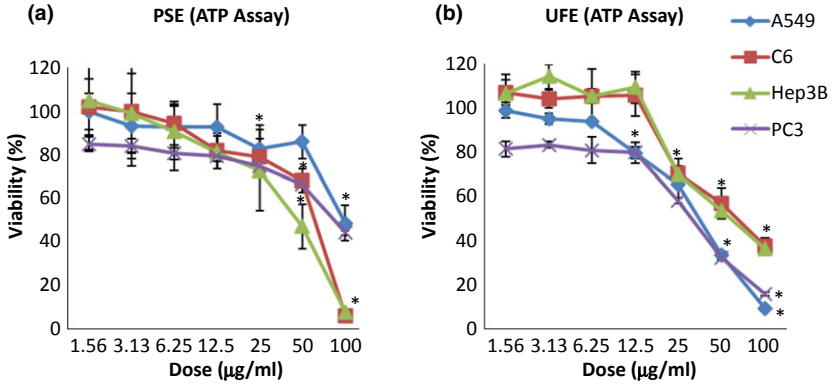

Figure 2. The anti-growth effect after the treatment with varying doses of *Parmelia sulcata* extract (PSE) (a) and *Usnea filipendula* extract (UFE) (b) for 72 h against cancer cell lines by the ATP assay as described in the Materials and methods section. *Denotes statically significant differences in comparison with control ($P < 0.05$).

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Table 1. Anti-growth parameters for *Parmelia sulcata* extract (PSE) and *Usnea filipendula* extract (UFE) determined by ATP assay following 72 h treatment to cancer cell lines

Cell line	Complex	IC_{50} (μg/ml)	IC_{90} (μg/ml)
A549	PSE	98.5	>100
	UFE	37.0	98.8
C6	PSE	64.7	96.8
	UFE	67.9	>100
Hep3B	PSE	47.3	97.7
	UFE	60.5	>100
PC3	PSE	87.3	>100
	UFE	32.9	>100

IC_{50} is defined as the dose inhibiting 50% of viability. IC_{90} is defined as the dose inhibiting 90% of viability.

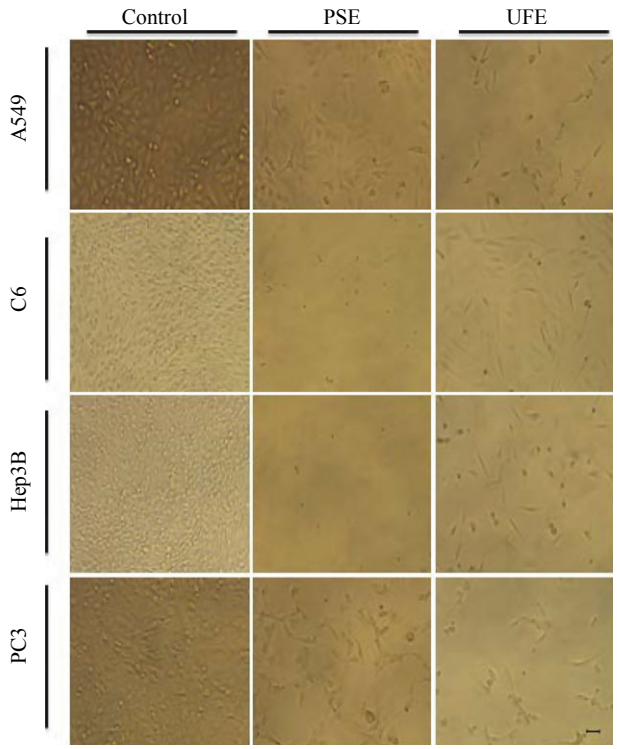

Figure 3. Images of phase contrast microscopy. Cells were treated with 100 μg/ml *Parmelia sulcata* extract (PSE) and *Usnea filipendula* extract (UFE) for 72 h. Controls included untreated cells. A scale bar represents 10 μm.

both C6 glioma and Hep3B liver cancer cells, while UFE yielded stronger cytotoxic effects against A549 and PC3 lung cancer cells. These observations were found to be in accordance with the MTT assay results.

Fluorescence staining for determination of cell death mode

According to the results of the viability assays and morphological evaluation, PSE and UFE displayed potent cytotoxic activity at 100 μg/ml against the chosen cancer cell lines. Thus, we further evaluated/confirmed the mode of cell death, by fluorescence imaging, on the basis of nuclear morphology. Cell nuclear morphology was examined after staining with a nucleus-staining fluorescent dye, Hoechst 43332. We observed that PSE and UFE treatments resulted in shrinkage (pyknosis) and brighter appearance (condensed chromatin) of nuclei, both of which are hallmarks of apoptosis (Fig. 4).

Apoptosis-inducing effects of P. sulcata *and* U. filipendula *extract on cancer cells*

Apoptosis-inducing effects of PSE and UFE were investigated by examining caspase-cleaved cytokeratin 18 (M30), a marker of apoptosis. As shown in Fig. 5, we found that M30 levels were significantly higher in A549 lung cancer cells after PSE and UFE (100 μg/ml, 72 h) treatments compared to positive controls (paclitaxel, $P < 0.05$). M30 antigen levels were not changed in PC3 and Hep3B cancer cells after treatment with PSE or UFE.

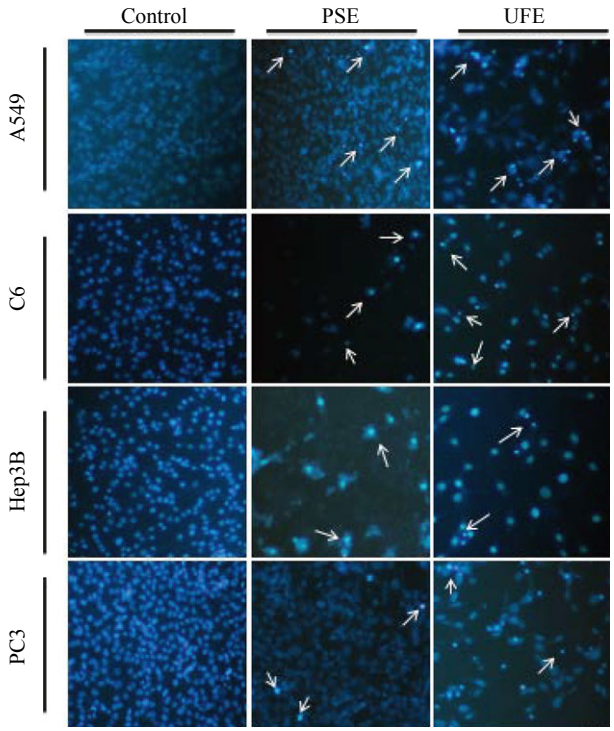

Figure 4. Flourescence imaging for determination of cell death mode. The cells were treated with 100 μg/ml *Parmelia sulcata* extract (PSE), *Usnea filipendula* extract (UFE) for 72 h and then stained as explained in the Materials and methods section, followed by the visualization by a fluorescence microscope. Arrows show pyknotic and brighter nuclei, that is a feature of apoptosis (magnification 100×).

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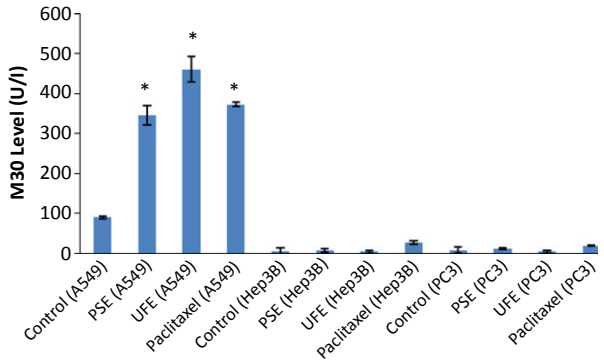

Figure 5. M30 (U/l) levels 72 h after the treatment with 100 μg/ml, *Parmelia sulcata* extract (PSE), *Usnea filipendula* extract (UFE) in cancer cells. Paclitaxel was used as a positive control for M30. *Denotes statistically significant change from control ($P < 0.05$).

Measurement of caspase-3 activation and PARP cleavage by ELISA

Effects of PSE and UFE on caspase-3 activation and PARP cleavage, determined by commercially available ELISA kits, are shown in Fig. 6. As seen there, no extracts resulted in increase either in active-caspase 3 or PARP cleavage, while positive control (paclitaxel) caused clear incremental increases.

Comet assay

Results of comet testing are summarized in Table 2. This shows effects of PSE and UFE (100 μg/ml) on comet frequency and genetic damage indices in human lung (A549, PC3), liver (Hep3B) and rat glioma (C6) cell lines, as well as positive (cisplatin, 3.8 μg/ml) and negative controls. PSE and UFE induced significant increases in genetic damage index and damage frequency compared to the controls in the cancer cell lines ($P < 0.0001$, Table 2).

Discussion

Natural products play an important role in cancer therapies, and as such, a substantial number of anti-cancer agents used in the clinic are either natural or derived from natural products from various sources (17). Recently, use of lichen and compounds extracted from lichens as alternative materials for natural therapeutic products has attracted considerable attention.

In the study presented here, possible cytotoxic and/or genotoxic activity of PSE and UFE were evaluated in human lung (A549, PC3), liver (Hep3B) and rat glioma (C6) cell lines. We showed that PSE and UFE exhibited significant anti-cell population growth activity against cancer cell lines in a dose-dependent manner. Lung cancer cells (A549, PC3) were found to be more sensitive to UFE than PSE for 72 h, as seen by IC_{50} values (Table 1). According to the literature, *U. filipendula* contains salazinic and usnic acids (12,18). Thus, it was shown that usnic acid has a potent cytotoxic effect through inducing apoptosis in A549 lung cancer cells (19).

We investigated the mode of cell death resulting from treatment with PSE and UFE, and morphological examination by fluorescence microscopy was first performed after staining the cells with fluorescent probes. It was clearly observed that PSE and UFE caused pyknosis, considered to be a hallmark of apoptosis. To confirm that it was indeed apoptosis, level of caspase-cleaved cytokeratin 18 (M30) was measured. Interestingly, we found that M30 level was significantly elevated only in A549 cells after treatment with PSE and UFE, but its level remained unchanged in PC3 and Hep3B cells. M30 assay, which detects only human-origin antigens, was not studied in C6 cells, as they are from rat, not human. However, we were curious as to why PC3 and Hep3B cells did not show any increase in M30 levels, although they underwent apoptosis, as revealed by their nuclear morphology. We found by western blotting that they expressed very low levels of cytokeratin 18 (data not shown). With regard to Hep3B cells, it has previously been reported that this line has high levels of vimentin expression (20). Thus, abundance of vimentin may cause cytokeratin lack in

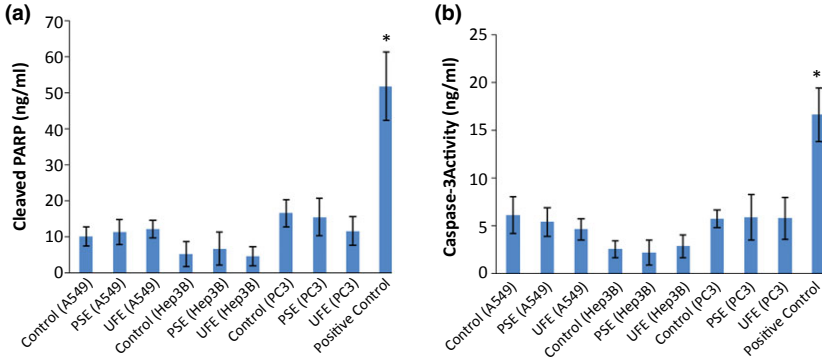

Figure 6. Effect of *Parmelia sulcata* extract (PSE) and *Usnea filipendula* extract (UFE) on PARP cleavage (a) and caspase-3 activity (b). The cells were treated with 100 μg/ml PSE, UFE and 3.12 μM Paclitaxel (as a positive control) for 72 h. *Denotes statistically significant change from control ($P < 0.05$).

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Table 2. Results of comet analysis after treatment with 100 μg/ml *Parmelia sulcata* extract (PSE), 100 μg/ml *Usnea filipendula* extract (UFE) positive (Cisplatin, 3.8 μg/ml) and negative controls (mean ± SD)

Cell type	Treatment[a]	Comet length[a]	Tail length[a]	Olive tail moment[a]	% Tail DNA[a]	% Head DNA[a]	Genetic damage index[a]	% Damaged cell[a]
A549	Negative Control	39.21 ± 24.77	10.63 ± 26.47	3.56 ± 13.78	9.01 ± 19.23	90.98 ± 19.23	0.54 ± 0.03	0.06 ± 0.01
	PSE	39.46 ± 18.20	13.47 ± 20.08	5.26 ± 10.83	17.65 ± 28.87	82.35 ± 28.87	0.94 ± 0.03***	0.20 ± 0.02***
	UFE	47.35 ± 27.86	18.76 ± 27.96	5.13 ± 9.81	14.29 ± 21.32	85.71 ± 21.32	0.72 ± 0.04***	0.4 ± 0.03***
	Cisplatin	46.27 ± 25.63	19.80 ± 26.98	6.90 ± 14.02	21.96 ± 22.64	78.04 ± 22.64	1.42 ± 0.03***	0.36 ± 0.02***
PC3	Negative Control	41.74 ± 19.70	11.64 ± 23.19	4.06 ± 12.23	9.84 ± 23.81	90.16 ± 23.81	0.46 ± 0.05	0.12 ± 0.02
	PSE	53.50 ± 39.94	23.22 ± 41.37	10.38 ± 21.91	20.76 ± 35.58	79.24 ± 35.58	0.86 ± 0.04***	0.25 ± 0.01***
	UFE	57.58 ± 39.85	27.85 ± 45.19	12.22 ± 23.96	23.32 ± 37.84	76.68 ± 37.84	0.98 ± 0.03***	0.29 ± 0.01***
	Cisplatin	50.33 ± 27.69	18.57 ± 27.95	5.48 ± 13.07	12.11 ± 22.51	87.89 ± 22.51	0.68 ± 0.03***	0.20 ± 0.02***
Hep3B	Negative Control	32.31 ± 14.83	8.34 ± 15.58	2.66 ± 8.10	11.06 ± 18.51	88.94 ± 18.51	0.7 ± 0.02	0.14 ± 0.03
	PSE	39.84 ± 20.89	13.86 ± 22.81	4.98 ± 11.88	15.98 ± 25.84	84.02 ± 25.84	0.98 ± 0.02***	0.19 ± 0.02***
	UFE	35.77 ± 13.99	9.20 ± 13.12	2.23 ± 5.18	10.70 ± 10.46	89.30 ± 10.46	1.06 ± 0.03***	0.19 ± 0.01***
	Cisplatin	33.62 ± 19.91	11.68 ± 21.50	3.75 ± 10.54	14.52 ± 17.84	85.48 ± 17.84	1.16 ± 0.03***	0.29 ± 0.02***
C6	Negative Control	30.24 ± 9.98	7.45 ± 9.78	1.94 ± 5.02	9.91 ± 16.75	90.09 ± 16.75	0.64 ± 0.02	0.09 ± 0.01
	PSE	40.12 ± 20.18	17.85 ± 22.05	6.55 ± 11.38	24.38 ± 27.32	75.62 ± 27.32	1.34 ± 0.02***	0.46 ± 0.04***
	UFE	38.41 ± 21.85	16.58 ± 23.67	6.02 ± 11.95	21.28 ± 26.28	78.72 ± 26.28	1.33 ± 0.02***	0.35 ± 0.01***
	Cisplatin	43.8 ± 34.24	20.67 ± 33.73	8.11 ± 16.10	22.03 ± 26.01	77.96 ± 26.01	1.46 ± 0.03***	0.50 ± 0.02***

PSE, *Parmelia sulcata* extract; UFE, *Usnea filipendula* extract; SD, standard deviation; Cisplatin (3.8 μg/ml); distilled water as negative control.
[a]Significance of PSE and UFE doses compared with DMSO solvent control.
***$P < 0.0001$.

the cytoskeleton of this particular cell line; Sommers *et al.* have reported that cell lines expressing vimentin have only low levels of cytokeratins (21). Due to this, results of M30 assay must be interpreted cautiously.

We used comet assay to determine the cell death mechanism. PSE and UFE induced significant increase in genetic damage index and damage frequency compared to controls at tested concentrations in all the cell lines ($P < 0.0001$); thus their genotoxic effects could be responsible for inducing apoptosis-like cell death through DNA damage.

We further investigated effects of PSE and UFE on caspase-3 activity and cleaved-PARP levels in our cancer cells. Caspase-3 (also known as CPP32, 32 kDa cysteine protease, or apopain), is a cysteine protease with aspartate specificity, and is a well-characterized effector of apoptosis. It is synthesized as an inactive proenzyme, whereupon, by cleavage at Asp175/Ser176, it is converted into the active enzyme. As caspase-3 activation followed by PARP cleavage is a key event in the apoptotic process, both are used as markers of apoptosis induction (22,23). Here, we sought to determine the role of PSE and UFE on caspase-3 activation and PARP cleavage. We observed no change in these compared to untreated control cells. To the best of our knowledge and from the literature, we found no information concerning mechanisms of action of PSE or UFE or how they cause cell death. Thus, it is tentatively suggested that they most likely induce apoptotic cell death through a caspase-independent pathway in these cells, or else caspases other than caspase-3 (perhaps caspases 6 or 7) may be involved in the process.

To our knowledge, there are no previously published data on cytotoxic/apoptotic and genotoxic activities of methanolic extracts of *P. sulcata* and *U. filipendula* on human lung (A549, PC3), liver (Hep3B) and rat glioma (C6) cell lines. Our results suggest that both cytotoxic and genotoxic properties of *P. sulcata* and *U. filipendula* induced apoptosis-like cell death by DNA damage in these cancer cells.

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