TÜBİTAK

Turkish Journal of Biology

http://journals.tubitak.gov.tr/biology/

Research Article

Turk J Biol
(2014) 38: 940-947
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doi:10.3906/biy-1405-23

Genotoxic, cytotoxic, and apoptotic effects of crude extract of *Usnea filipendula* Stirt. in vitro

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Received: 08.05.2014 • **Accepted:** 14.08.2014 • **Published Online:** 24.11.2014 • **Printed:** 22.12.2014

Abstract: Lichens are recently receiving great attention because they have potential anticancer activity. Therefore, in this study, genotoxic and antigrowth properties of lichen species *Usnea filipendula* Stirt. were tested against human breast cancer cell lines (MCF-7 and MDA-MB-231). Antigrowth effect was assayed by the MTT and ATP viability assays. Cell death modes (apoptosis/necrosis) were evaluated morphologically (fluorescence staining) and biochemically (caspase-cleaved cytokeratin 18, caspase-3 activity, and poly-(ADP-ribose) polymerase (PARP) cleavage). Genotoxic activity of *U. filipendula* was determined by using micronucleus, chromosomal aberration, and comet assays in human lymphocyte culture. *U. filipendula* inhibited growth in a dose-dependent manner and induced apoptosis by cleavage of PARP and induction of active caspase-3. It also showed genotoxic activity in doses (125 and 250 µg/mL) higher than that required for apoptosis. These results suggest that *U. filipendula* may induce apoptotic cell death at lower doses, while it may be genotoxic at higher doses.

Key words: *Usnea filipendula*, breast cancer, cell death, apoptosis, DNA damage

1. Introduction

Natural products play an important role in cancer therapy with substantial numbers of anticancer agents used in the clinic being either natural or derived from natural products from various sources (Mann, 2002). Recently, extracted lichen and lichen compounds as alternative materials of natural therapeutic products have attracted considerable attention from scientists. Lichens are complex organisms living in a symbiotic relationship with fungi and algae (and/or Cyanobacteria) (Müller, 2001). These associations give rise to a great number of secondary metabolites, the majority of which are unique to these organisms. Lichen substances have several possible biological activities: antibiotic, antiviral, antitumor, antimutagenic, antioxidant, antiinflammatory, antiproliferative, and cytotoxic (Müller, 2001; Oksanen, 2006; Molnar and Farkas, 2010; Mitrović et al., 2011).

Usnea filipendula belongs to the family Parmeliaceae and contains salazinic and usnic acid (Purvis et al., 1994; Brodo et al., 2001). Usnic acid, one of the most studied lichen compounds, was found to be effective against different types of cancer cell lines (Bačkorová et al., 2011). Salazinic acid, which is a major metabolite in *P. sulcata*, was found to possess high cytotoxic activity in human melanoma and colon cancer cell lines (Manojlović et al., 2012). In a recent study, methanol extracts of *Usnea articulata* and *Usnea filipendula* were reported to have strong antioxidative and antigenotoxic effects (Ceker at al., 2013). Anticancer activity of *Hypogymnia physodes* and *Parmelia sulcata* against breast cancer cell lines and genotoxic effect on human lymphocytes were previously investigated by our research group (Ari et al., 2014b, 2014c).

We think that further work needs to be done on the cytotoxic and genotoxic effects of lichens. Thus, in the present study, cytotoxic and genotoxic activities of the methanol extract of *U. filipendula* (UFE) were investigated in breast cancer cell lines and human lymphocytes, respectively.

2. Materials and methods

2.1. Lichen samples

U. filipendula was collected from the trunks of *Quercus* sp. (Mount Uludağ, Bursa, Turkey) in May 2010 and identified with the aid of flora books (Purvis et al., 1994; Wirth, 1995). A voucher specimen has been deposited in the Herbarium of Uludağ University (BULU), Bursa, Turkey.

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2.2. Extraction

An air-dried lichen sample was carefully cleansed of extraneous materials and ground into powder; 15 g of the ground material was extracted consecutively by adding 150 mL of solvent methanol (Merck) and water to a Soxhlet extractor for 24 h. The crude extracts were concentrated using a rotary evaporator at 40 °C; thereafter, the residues were lyophilized and stored at –20 °C until used in the tests.

2.3. Determination of cytotoxicity

2.3.1. Cell culture and chemicals

Breast cancer cell lines (MCF-7 and MDA-MB-231) were cultured in RPMI 1640 supplemented with penicillin G (100 U/mL), streptomycin (100 µg/mL), L-glutamine, and 5% fetal calf serum (Invitrogen, Paisley, UK) at 37 °C in a humidified atmosphere containing 5% CO_2. Lyophilized *U. filipendula* was dissolved in DMSO at a concentration of 0.05 g/0.5 mL, while the final concentrations were prepared in culture medium.

2.3.2. The MTT viability assay

MCF-7 or MDA-MB-231 cells were seeded in 200 µL of culture medium in triplicate at a density of 5×10^3 cells per well of a 96-well plate. Cells were incubated either alone (for control including 0.1% DMSO) or in the presence of UFE (1.56 to 100 µg/mL) 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 (Ulukaya et al., 2008). MTT was first prepared as a stock solution of 5 mg/mL in phosphate buffer (PBS, pH 7.2) and 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 sulfate dissolved in 0.01 N HCl) was added to each well. After overnight incubation, the absorbance (Abs) was read by an ELISA plate reader (FLASH Scan S12, Analytik Jena, Germany) at 570 nm to determine cell viability. Viability of treated cells was calculated in reference to the untreated control cells by using the following formula:

Cell viability (%) = [100 × (Sample Abs)/(Control Abs)].

2.3.3. The ATP viability assay

The seeding of cells and treatment conditions as well as the calculation of viability were similar to those of the MTT assay (see above). The ATP assay uses the highly sensitive reaction to determine the level of cellular ATP as a surrogate marker for the number of viable (ATP-producing) cells (Andreotti et al., 1995). ATP content was determined according to the manufacturer's recommendations (ATP Bioluminescence Assay, Sigma, St. Louis, MO, USA). Briefly, following the treatment, ATP was extracted from the cells, and then luciferin-luciferase solution was added. Luminescence was determined in a luminometer (Bio-Tek, USA).

2.4. Determination of apoptosis

2.4.1. Caspase-cleaved cytokeratin 18 (M30) detection

Apoptosis was assayed by measuring the level of caspase-cleaved keratin 18 (ccK18, M30) by a commercially available immunoassay kit (M30-Apoptosense ELISA kit, Peviva AB, Sweden) according to the manufacturer's instructions. This kit measures the levels of the CK18-Asp396 neo-epitope (M30), which is a well-known marker of apoptosis. Cells (1×10^4 per well of a 96-well plate) were seeded in 200 µL of culture medium in triplicate. MCF-7 or MDA-MB-231 cells were treated for 72 h with 100 µg/mL of UFE. Paclitaxel (3.12 µM) was used as a positive control for apoptosis as this agent is considered an appropriate apoptosis inducer (Park et al., 2004). At the end of the treatment period, the cells were lysed with 10% NP-40 for 10 min on a shaker. The contents of identical wells were pooled and centrifuged at 2000 rpm for 10 s to remove the debris. All samples were placed into wells coated with a mouse monoclonal antibody as a catcher. After washing, a horseradish peroxidase conjugated antibody (M30) was used for detection. The absorbance was determined with an ELISA reader at 450 nm (FLASH Scan S12, Eisfeld, Germany).

2.4.2. Fluorescence imaging for apoptosis

The seeding and treatment conditions as well as the detection of apoptosis were based on both the nuclear morphology of cells and their membrane integrity using a fluorescent microscope (Cevatemre et al., 2013). Therefore, the nuclear and membrane morphology of living (not fixed) cells was examined after staining them with fluorescent dyes, Hoechst 33342, calcein-AM, and propidium iodide (PI) (Cell Viability Imaging kit, Roche, Mannheim, Germany). Cells were analyzed with a fluorescent microscope at the end of the incubation.

2.4.3. Measurement of active caspase-3 and cleaved PARP levels

MCF-7 and MDA-MB-231 cells (1×10^6) were seeded in 25-cm^2 flasks and treated with 100 µg/mL of UFE for 72 h in order to detect active caspase-3 and cleaved poly(ADP-ribose) polymerase (PARP), which are the markers for apoptosis. After treatment, cells were washed in ice-cold PBS, and lysed in lysis buffer (Cell Signaling, MA, USA) containing protease inhibitors (Sigma, St. Louis, MO, USA) and 1 mM of phenylmethylsulfonyl fluoride (PMSF). Cells were extracted at 4 °C for 5 min, and centrifuged at 4 °C for 10 min at 14,000 × *g*. The level of cleaved PARP was estimated using the PARP Cleavage [214/215] human ELISA kit (Invitrogen, UK) and active caspase-3 level was determined with the Caspase-3 (Active) human ELISA

kit (Invitrogen, UK) according to the protocols described in the manufacturer's instructions. For determination of active caspase-3, 100 µL of cell lysates was incubated in the microplate wells provided in the kit at room temperature for 2 h. The samples were aspirated and washed 4 times with washing buffer and incubated with 100 µL of detection antibody (anti-active caspase-3) for 1 h at room temperature. After removal of the antibody solution, the wells were washed again and incubated with 100 µL of HRP anti-rabbit antibody for 30 min at room temperature. After the aspiration of the anti-rabbit antibody, a blue color was developed by adding 100 µL of stabilized chromogen solution for 20 min at room temperature. The reaction was stopped after the addition of 100 µL of stopping solution. For determination of cleaved PARP levels, 50 µL from each lysate was incubated with anti-cleaved PARP (detection antibody) for 3 h. After washing, each sample in a well was incubated with a secondary antibody, IgG-HRP solution, for 30 min. Stabilized chromogen was added to each well for another 30 min followed by addition of stopping solution. The absorbance of each well was read at 450 nm using a microplate reader.

The level of cleaved PARP was also determined by using Western blotting. MCF-7 cells were seeded in 75-cm^2 flasks and treated when the cells reached 70% confluence with 100 µg/mL of UFE for 72 h. Western blotting was performed using rabbit anti-PARP monoclonal antibody (1:1000 dilution; Cell Signaling, MA, USA) and rabbit anti-β-actin monoclonal antibody (1:1000 dilution; Cell Signaling, MA, USA). Bound antibodies were visualized on a Fusion FX-7 imaging device (Vilber Lourmat, Torcy, France).

2.5. Genotoxicity assays

Genotoxicity assays were performed as described in our previous study (Ari et al., 2014b). UFE was dissolved in 50% DMSO. A preliminary study was conducted to determine UFE doses (50, 100, 125, 250, 400, 500, and 750 µg/mL). The cells were then treated separately with a mutagenic agent, DMSO, as solvent control and diluted sterile UFE (125, 250, and 500 µg/mL) for 24 h prior to harvest. Metaphases were obtained by adding colcemid (0.2 µg/mL final concentration, Sigma) 2 h prior to harvest. For the micronucleus (MN) assay, cytochalasin B (6 µg/mL) was added at 44 h to block the cytokinesis process, and lymphocyte cultures were harvested after 72 h (Fenech, 2000). Chromosomes were prepared using standard procedures (Benn and Perle, 1992). Staining of the micronuclei was performed by immersing the air-dried slides in a 2% Giemsa solution.

2.5.1. Microscopic evaluation

The analysis of chromosomal aberrations (CAs) was performed in 50 metaphases for each culture. Total CA results were evaluated for both cases in which gaps and pulverisations were included and excluded. The CAs were classified according to the Environmental Health Criteria 51 for Short-term Tests for Mutagenic and Carcinogenic Chemicals (IPCS, 1985).

For the determination of MN, 2000 binucleated cells for each experimental point were examined following the scoring criteria adopted by the Human Micronucleus Project (Bonassi et al., 2001). We evaluated the binucleated micronucleated lymphocytes frequency as the number of binucleated lymphocytes containing 1 or more MN per 1000 binucleated cells.

2.5.2. Mitotic index (MI) and nuclear division index (NDI)

The MI was calculated from the number of metaphases in 2000 cells analyzed per culture for each dose group and donor in the CA assay. In the micronuclei assay, 500 lymphocytes were scored to evaluate the percentage of binucleated cells, and the NDI was calculated according to the following formula:

NDI= [MONO + 2BN + 3TRI + 4TETRA]/500,

where MONO, BN, TRI, and TETRA were mononuclear, binucleated, trinucleated, and tetranucleated lymphocytes, respectively.

2.5.3. Comet assay

The alkaline version of the comet assay was used (Singh et al., 1988) with some modifications. Venous blood samples from healthy donors (0.5 mL) were mixed with 5 mL of RPMI 1640 medium supplemented with 20% fetal calf serum, 1% penicillin/streptomycin (100 U/mL), and 2.5% phytohemagglutinin. The lymphocyte culture was incubated for 72 h at 37 °C. UFE (125, 250, and 500 µg/mL) and ethyl methanesulfonate (EMS, 1250 µg/mL, as positive control) were added 24 h prior to the end of incubation. The cells were lysed and electrophoresis was performed at 25 V for 30 min. The slides stained with ethidium bromide were visualized under a microscope with a fluorescence attachment. The images were analyzed by using specialized software for comet analysis (Kameram 21, Argenit, İstanbul, Turkey). Comet square, comet length, comet density, tail length, tail DNA percentage, tail moment length, olive moment length, 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 (Singh et al., 1988),

GDI = (1 × ΣType1) + (2 × ΣType2) + (3 × ΣType3) + (4 × ΣType4)/(ΣType0 + ΣType1 + ΣType2 + ΣType3 + ΣType4)

(ΣType 0: Total of undamaged cells; ΣType 1: Total of very low damaged cells; ΣType 2: Total of low damaged cells; ΣType 3: Total of high damaged cells; ΣType 4: Total of very high damaged cells).

2.6. Statistical analyses

All statistical analyses were performed using SPSS 20.0 for Windows. The significance was calculated using one-way analysis of variance and Tukey's honest significant difference tests with 95% confidence intervals. A value of $P < 0.05$ was considered statistically significant. Results were expressed as mean ± SD.

3. Results and discussion

3.1. Cytotoxic activities of UFE by the MTT and ATP assays

The antigrowth (cytotoxic) effect of UFE (1.56–100 µg/mL) was investigated on MCF-7 and MDA-MB-231 human breast cancer cell lines by the MTT (Figure 1A) and ATP assays (Figure 1B) for 72 h. It was found that UFE exhibited the antigrowth effect in a dose-dependent manner ($P < 0.05$). According to the dose response curves, UFE had a stronger growth-inhibitory effect on MCF-7 cells than on MDA-MB-231 cells. IC_{50} values (50% inhibitory concentration) of UFE were calculated on the basis of ATP assay results. UFE clearly resulted in cytotoxic activity on MDA-MB-231 cells (44.7 µg/mL), but it was more cytotoxic to MCF-7 cells (23.0 µg/mL).

In our previous study, we investigated the antiproliferative/apoptotic effects of methanol extracts of *U. filipendula* and *P. sulcata* on human lung cancer (A549, PC3), liver cancer (Hep3B), and rat glioma (C6) cells. We found that the lichen species induced apoptosis-like cell death by causing DNA damage to cancer cells (Ari et al., 2014a). The antigrowth effects of UFE may arise from its secondary metabolites. *U. filipendula* contains salazinic and usnic acids (Purvis et al., 1994; Brodo et al., 2001). Salazinic acid was found to possess high cytotoxic activity in human melanoma and colon cancer cell lines (Manojlović et al., 2012) and usnic acid had a potent cytotoxic effect through apoptosis in A549 lung cancer cells (Singh et al., 2013).

3.2. Apoptosis-inducing effect of UFE on MCF-7 and MDA-MB-231 breast cancer cells

The apoptosis inducing effects of UFE was investigated by M30 assay. Figure 2 shows that M30 levels (a marker of apoptosis) were not changed in MDA-MB-231 cells after 72 h of treatment with different concentrations (12.5–100 µg/mL) of UFE. In contrast, M30 levels were significantly increased in MCF-7 cells with 50 and 100 µg/mL UFE treatment ($P < 0.05$), suggesting an apoptosis-inducing effect of UFE in MCF-7 cells (Figure 2).

There are several studies reporting that lichen or lichen compounds resulted in apoptosis in different types of cancer cell lines (Mitrović et al., 2011; Russo et al., 2012; Singh et al., 2013). In our study, M30 levels were significantly increased in MCF-7 cells after the treatment

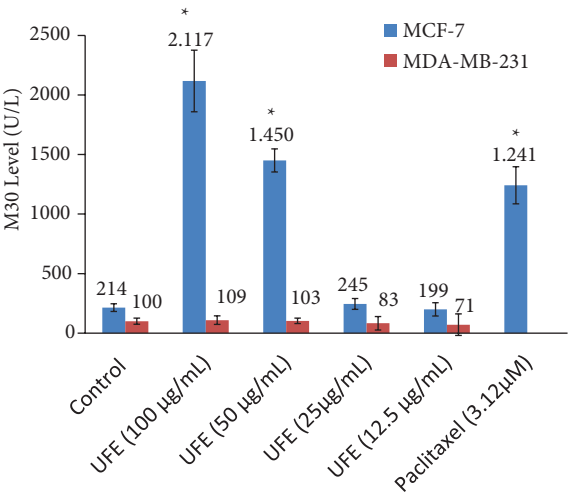


Figure 2. M30 (U/L) levels 72 h after treatment with *Usnea filipendula* extract (UFE) in MCF-7 and MDA-MB-231 breast cancer cells. Paclitaxel was used as positive control for M30 increase (apoptosis). * Denotes significant change from control ($P < 0.05$).

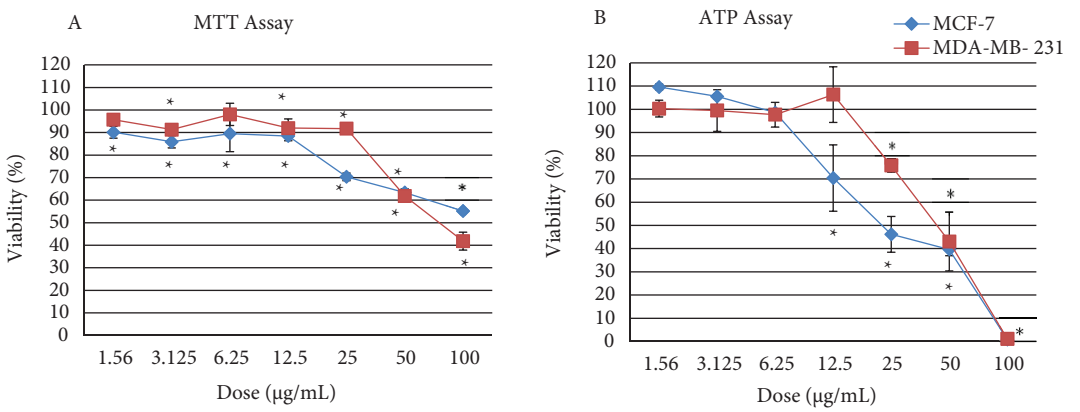


Figure 1. Assessment of viability of MCF-7 and MDA-MB-231 cell lines after 72 h of treatment with varying doses of *Usnea filipendula* extract (UFE) *Denotes significant differences in comparison with control ($P < 0.05$).

with UFE. However, MDA-MB-231 cells did not exhibit any increase in M30 levels, implying that the mode of cell death may be different from apoptosis or other reasons regarding the genomic status of the cells might play a role. For example, it was reported that cytokeratin levels may be low in breast cancer cell lines expressing high amounts of vimentin such as MDA-MB-231 (Sommers et al., 1989). Hence, the M30 assay may not be an ideal test to detect apoptosis in such cell lines. We further evaluated the mode of cell death induced by UFE by fluorescence imaging on the basis of nuclear morphology. According to the results, we observed that UFE resulted in cell/nuclear shrinkage in some cells, which is also a hallmark of apoptosis (Figures 3A and 3B). As a result, although we could not detect

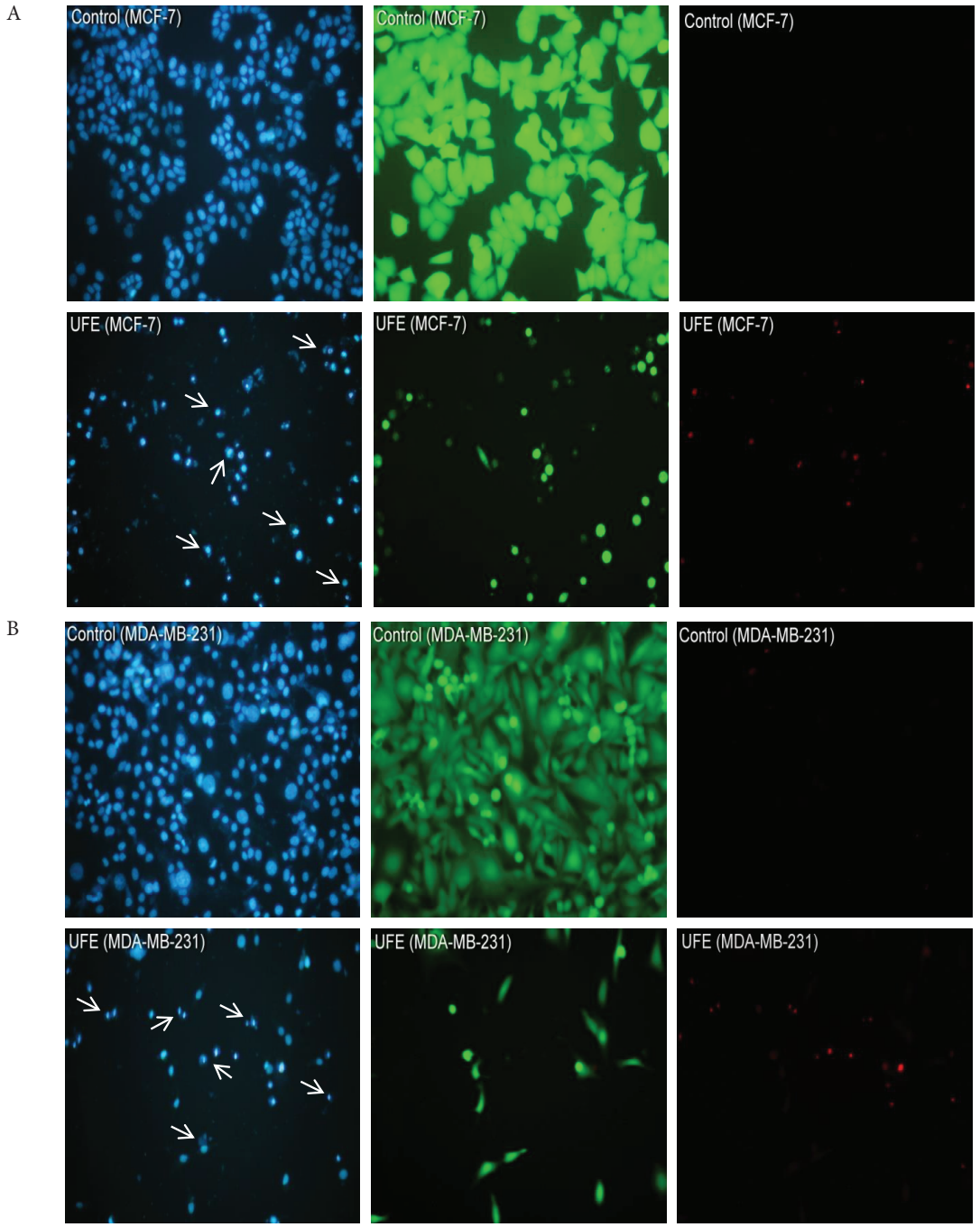


Figure 3. Fluorescence imaging for determination of cell death mode in MCF-7 (A) and MDA-MB-231 (B) cells. Cells were treated with 100 µg/mL UFE for 72 h. The left panel shows Hoechst 33342 staining, while the middle and right panels show calcein-AM and propidium iodide (PI) staining, respectively. Arrows show the pyknotic/fragmented nucleus of apoptotic cells.

apoptosis in the MDA-MB-231 cell line by measuring M30 levels, it was clearly evident from the Hoechst 33342/PI/calcein-AM triple staining experiment in which pyknotic and fragmented nuclei, a well-known feature of apoptosis, were present.

3.3. Effect of UFE on caspase-3 activation and cleaved PARP

To detect cleaved PARP, which is a marker of caspase-mediated apoptosis, we treated the MCF-7 and MDA-MB-231 cells for 72 h with 100 µg/mL of UFE. The cleaved-PARP levels were increased approximately 11-fold by UFE in MCF-7 cells with the ELISA assay (Figure 4A). ELISA assay results confirm the findings of Western blotting for PARP cleavage in MCF-7 cells (Figure 4B). On the other hand, in MDA-MB-231 cells, the cleaved-PARP levels were increased ~2-fold compared to untreated cells as a consequence of UFE treatment. The resulting PARP cleavage in both cell lines indicates apoptosis. In addition, caspase activation (cleavage of procaspase to active caspase) is regarded as a hallmark of apoptosis (Porter and Janicke, 1999) and caspase 3 is a central effector caspase in many types of cells and mediates the cleavage of itself, other downstream caspases, and other caspase substrates such as cytokeratin 18, and PARP (Gown and Willingham, 2002). In MDA-MB-231 cells, caspase-3 activation (~1.5 fold) and PARP cleavage (~2-fold) were induced by 100 µg/mL UFE (Figures 4A and 4C). Therefore, we suggest that UFE may induce apoptotic cell death through the caspase-dependent pathway in MCF-7 and MDA-MB-231 cell lines. In fact, in the literature, to the best of our knowledge, there is no information about the mechanism of action of *U. filipendula* that leads to cell death.

3.4. Genotoxicity assays

The results of genotoxicity tests are summarized in Tables 1 and 2. Table 1 shows the effect of UFE (125, 250, and 500 µg/mL), positive (EMS, 1250 µg/mL), solvent (DMSO 50%), and negative controls on the mitotic and nuclear division index, the number of CAs, the frequency of abnormal metaphases, and micronuclei frequency in human lymphocyte cell cultures.

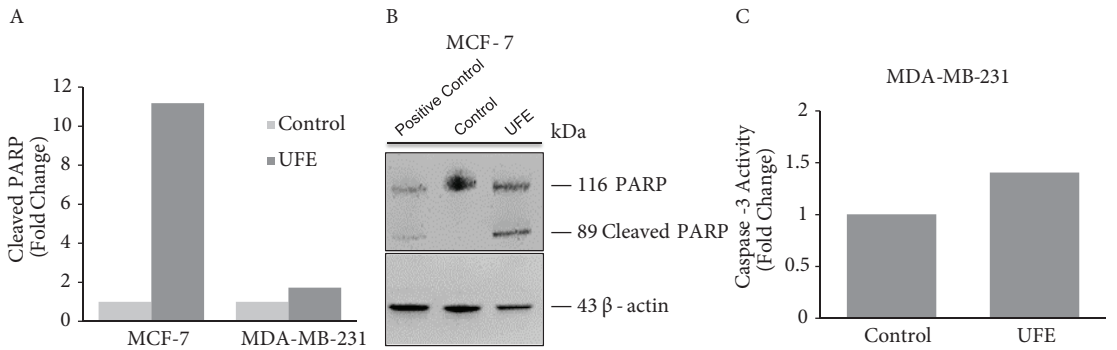


Figure 4. Cleaved PARP levels in MCF-7 and MDA-MB-231 cell lines after 72 h of *Usnea filipendula* extract (UFE) (100 µg/mL) treatment were examined by ELISA (A). Cleaved PARP levels in MCF-7 cell line examined by Western blotting (B). Active caspase-3 levels in MDA-MB-231 cell line examined by ELISA (C). *Denotes significant change from control ($P < 0.05$).

Table 1. Frequencies of chromosomal aberrations (CAs), micronuclei (MN), nuclear division index (NDI), and mitotic index (MI ‰) in cultured human lymphocytes treated with *Usnea filipendula* Stirt. extract (UFE), and positive and negative controls (mean ± SD).

Treatments	‰ MI ± SD[a]	Number of chromosome aberrations							TA(G+P) ± SD[a]	TA(G-P) ± SD[a]	MN ± SD[a]	NDI ± SD[a]
		CG	ICG	CB	ICB	EXC	SE	PLV				
Negative control	8.11 ± 2.54	0	0	0	0	0	0	0	0.00 ± 0.00	0.00 ± 0.00	12.00 ± 2.00	1.16 ± 0.04
Solvent control	8.50 ± 1.08	104	12	44	42	0	0	0	1.22 ± 0.31	0.63 ± 0.07	21.83 ± 5.10	1.38 ± 0.09
125 µg/mL UFE	7.30 ± 0.92	156	40	56	72	0	10	0	1.67 ± 0.63*	0.77 ± 0.24*	17.90 ± 3.48	1.36 ± 0.02
250 µg/mL UFE	6.70 ± 0.85	186	60	72	74	0	22	0	2.08 ± 1.08**	0.93 ± 0.51*	20.63 ± 5.12	1.21 ± 0.03**
500 µg/mL UFE	6.27 ± 0.15	108	42	34	72	0	14	0	1.40 ± 0.05	0.63 ± 0.22	24.63 ± 10.19	1.27 ± 0.04
EMS	3.34 ± 1.92	38	24	26	12	21	2	6	1.61 ± 0.37	0.66 ± 0.19	34.05 ± 2.12	1.09 ± 0.34

CG, chromatid gap; ICG, iso-chromatid gap; CB, chromatid break; ICB, iso-chromatid break, EXC, exchange figure; SE, spiralization error; PLV, pulverization; MI, mitotic index; TA(G+P), total chromosome aberration including gaps and pulverizations; TA(G-P), total chromosome aberration excluding gaps and pulverizations; MN, micronuclei frequency (‰); NDI, nuclear division index; UFE, *Usnea filipendula* methanolic extract; SD, standard deviation; EMS, methyl methane sulfonate as positive control (1250 µg/mL).

a, significance of UFE doses compared with DMSO solvent control; *, $P < 0.05$; **, $P < 0.005$

Table 2. Results of comet analysis in cultured human lymphocytes treated with *Usnea filipendula* Stirt. extract (UFE), and positive and negative controls (mean ± SD).

Treatments (µg/mL)	Comet ength[a]	Average comet density[a]	Tail length[a]	% Tail DNA[a]	Olive tail moment[a]	% Head DNA[a]	Genetic damage index (GDI)[a]	% Damaged cell[a]
Negative control	29.73 ± 3.02	0.14 ± 0.02	3.47 ± 1.29	2.43 ± 2.20	0.32 ± 0.30	97.57 ± 2.20	0.21 ± 0.03	0.01 ± 0.01
Solvent control	28.14 ± 3.07	0.1 ± 0.02	3.37 ± 1.00	2.76 ± 1.56	0.35 ± 0.20	97.24 ± 1.56	0.31 ± 0.05	0.00 ± 0.00
125	30.32 ± 6.02	0.12 ± 0.03	5.27 ± 5.82	5.33 ± 10.36	0.90 ± 2.08	94.67 ± 10.36	0.39 ± 0.09*	0.11 ± 0.03***
250	33.48 ± 10.52	0.12 ± 0.03	8.02 ± 10.91	7.91 ± 11.53	1.65 ± 4.14	92.09 ± 11.53	0.83 ± 0.08***	0.23 ± 0.01***
500	31.73 ± 6.32	0.12 ± 0.03	7.02 ± 6.41	9.83 ± 15.36	1.54 ± 2.74	90.17 ± 15.36	0.89 ± 0.03***	0.22 ± 0.02***
EMS	110.30 ± 17.22	94.31 ± 9.25	56.82 ± 10.54	5.92 ± 3.33	0.13 ± 0.03	3.69 ± 9.25	3.88 ± 0.04	0.97 ± 0.02

UFE, *Usnea filipendula* methanolic extract; SD, standard deviation; EMS, methyl methane sulfonate as positive control (1250 µg/mL).
a, significance of UFE doses compared with DMSO solvent control; *, $P < 0.05$; ***, $P < 0.001$

As presented in Table 1, 125 and 250 µg/mL UFE treatment significantly increased CAs in comparison with the solvent control when gap and pulverized metaphase were both included and excluded ($P < 0.05$ and $P < 0.005$, respectively). The 500 µg/mL dose did not show a genotoxic effect.

Chromatid gaps and breaks, iso-chromatid gaps and breaks, and other subchromatid aberrations were the most common chromosomal abnormalities. An insignificant decrease in the frequency of mitotic index was detected for the treatment with UFE ($P > 0.05$). There was a significant increase in the frequency of the CAs when gap and pulverized metaphase were both included (TGAP, $P < 0.05$–0.005) and excluded (TGEP, $P < 0.05$–0.005) compared with the control at 125 and 250 µg/mL concentrations.

The positive control group EMS at 1250 µg/mL concentration significantly increased the number of abnormal metaphases and the total structural CAs compared to the negative control ($P < 0.05$).

The results of the MN assay are also shown in Table 1. The effects of all doses of UFE were not statistically significant on MN frequency ($P > 0.05$). When we compared the nuclear division index (NDI), we also found significant differences in the 250 µg/mL dose of UFE ($P < 0.005$, Table 1). Table 2 shows the effect of UFE, positive, solvent, and negative controls on the comet frequency and genetic damage indexes in human lymphocyte cell cultures. UFE extract also induced a significant increase in GDI and damage frequency compared with the negative control at all tested concentrations ($P < 0.05$ and $P < 0.001$, Table 2).

In conclusion, the findings suggest that methanol extract of *U. filipendula* has anticancer activity against breast cancer cell lines by inducing apoptosis. In addition, *U. filipendula* had genotoxic activity on lymphocytes that was evident only at higher concentrations.

Acknowledgments
We thank the Research Fund of Uludağ University for project numbered HDP(F)-2013/27, providing us with the kits/chemicals. The authors would also like to thank S. Bozdemir for technical assistance.

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