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Evaluation of the anti-cancer potential of *Mahonia aquifolium* extracts via apoptosis and anti-angiogenesis

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Abstract

The cytotoxicity of *Mahonia aquifolium* ethanol and water extracts was examined using MTT test. The morphological changes were analyzed by fluorescence microscopy. Cell cycle distribution and possible activation of caspase-dependent pathway of cell death were assessed by flow cytometry. The effects of ethanol and water extracts on migration of endothelial EA.hy926 cells were analyzed by *in vitro* scratch assay and inhibition of angiogenesis was detected using tube formation assay. Both extracts demonstrated cytotoxic effects on cancer cell lines with very high selectivity. Morphological evaluation indicated apoptosis. These results were confirmed with cell cycle analysis, where there was accumulation of cancer cells in the subG1 phase. Ethanol and water extracts induced a caspase-dependent apoptosis in HeLa cells through activation of caspase-3 and caspase-8. Both extracts showed the ability to inbibit the migration of EA.hy926 cells and initial steps of angiogenesis. In addition, ethanol extract exerted significant anti-angiogenic effect.

Introduction

Mahonia is the second largest genus of the family Berberidaceae, which includes around 60 species and is distributed in Asia, America and Europe (Kost`alova et al., 2001). Plants of this genus are widely used in folk medicine as a cure for the psoriasis, dermatitis, fungal infections (Kost`alova et al., 2001) and in the Traditional Chinese Medicine for the treatment of tuberculosis, dysentery, pharyngolaryngitis, eczema, and wounds (He and Mu, 2015).

Many species of *Mahonia* were studied intensely and showed to possess antibacterial, antifungal, anti-inflammatory, anti-oxidant, anti-proliferative and pro-apoptotic effects (Kost`alova et al., 2001; He and Mu, 2015; Vollekova et al., 2003; Zeng et al., 2003; Hu et al., 2010; Wong et al., 2009).

Mahonia aquifolium (Pursh) Nutt. (*M. aquifolium*), an ornamental bush, is one of the most widespread plants of the genus *Mahonia* which contains many important alkaloids. It has been one of the most extensively studied plants of this genus. *M. aquifolium* has been used in the American Tradicional Medicine to treat different skin disorders (Kost`alova et al., 2001). Several studies indicate that *M. aquifolium* is especially effective in patients with mild to moderate psoriasis through activities on different components of inflammation process and on keratinocyte proliferation (Gulliver and Donsky, 2005; Augustin et al., 1999; Galle et al., 1994; Muller et al., 1995).

The major alkaloids of the genus *Mahonia* are iso-quinoline alkaloids. This group of alkaloids, especially berberine shows anti-inflammatory, antimicrobial, anti-proliferative, pro-apoptotic and antimutagenic effects

(Rackova et al., 2004). Berberine has a significant potential to be a new drug for the treatment of many diseases, including cancer. Many research studies demonstrate that berberine inhibits tumor progression, decreases metastatic potential of malignant tumor and in combination with other drugs or radiation shows more effectiveness than the single therapy (Tillhon et al., 2012).

Traditional use of *M. aquifolium* to treat inflammatory conditions suggests that this plant may have a potential to be used in the field of anti-cancer therapy. Based on the lack of data about anti-cancer potential of *M. aquifolium* and results that showed anti-proliferative and pro-apoptotic effects of different species of genus *Mahonia* (Hu et al., 2010; Wong et al., 2009) we decided to investigate the anti-cancer proprieties of this plant, especially their possible anti-angiogenic effects. Anti-angiogenic properties of *M. aquifolium* may be important for developing novel drugs with anti-metastatic activity. The objective of this investigation was to determine the effects of water and ethanol extracts of *M. aquifolium* on human malignant and normal cell lines. We investigated the cytotoxic effects of the extracts, cells death mode and distribution of cells at cell cycle phases after the treatment with extracts.

Materials and Methods

Plant extracts

The stem-bark of cultivated *M. aquifolium* was collected in October 2014 in the National Garden park in Pancevo, Serbia. Voucher specimen is deposited at the Herbarium of the Institute for Medicinal Plants Research, Dr. Josif Pancic, Belgrade (No. 046/14). Two types of the extracts were prepared. For the first extract, air dried and finely powdered *Mahonia* stem-bark was extracted with 70% ethanol at room temperature for 24 hours (1:5, w/v), and second one was prepared by ultrasound assisted extraction with water (1:10, w/v) for 30 min. Both extracts were filtered through a filter paper and concentrated under vacuum at 40°C on a rotary evaporator. The dried extracts were kept in refrigerator until further analysis.

LC/MS analysis

LC/MS analysis were performed on Agilent 1200 Series, Agilent Technologies with DAD detector, on a column Zorbax Eclipse XDB-C18 (RRHT, 50 × 4.6 mm i. d.; 1.8 µm) in combination with 6210 Time-of-Flight LC/MS system (Agilent Technologies). Drying gas (N_2) flow was 12 L/min; nebulizer pressure was 45 psi; drying gas temperature was 350°C. For ESI analysis, the parameters were: Capillary voltage, 4000 V; fragmentor, 70 V; positive mode. The mass range was from 100 to 1500 m/z. Processing of data was carried out with the software Mass Hunter Workstation. The examined water and ethanol extracts were dissolved in methanol and filtered through a 0.45 µm syringe filter. The operating temperature was 40°C, the injection volume was 0.1 µL and the detection was between 190-450 nm. Mobile phase was a mixture of two solvent compositions A (0.2% formic acid in water) and B (100% acetonitrile) with the following gradient elution: 0-0.5 min 5% B, 0.5-10 min 5-95% B, 10-15 min 95% B, 15-15, 5 min 95-5% B, 15.5-18.5 min 5% B, at a flow rate of 0.50 mL/min. The different concentrations of berberine standard (40 µg/mL, 0.2 mg/mL, 0.3 mg/mL, and 0.4 mg/mL) were analyzed, under the same conditions, for the construction of the calibration curve. Based on the equation of curve, the concentration of berberine was calculated. Berberin chloride was purchased from Sigma-Aldrich, purity ≥ 98%.

Cell lines

Human cervical adenocarcinoma (HeLa), colorectal adenocarcinoma (LS-174T) and normal, human embryonic lung fibroblast (MRC-5) cell lines were grown in RPMI-1640 medium. Somatic human umbilical vein endothelial (EA.hy926) cell line was grown in DMEM with high glucose (4.5 g/L). The conditions for cell culture growth were described previously (Matić et al., 2013a; Matić et al., 2013b). The cell lines used in the study were obtained from the American Type Culture Collection (USA). RPMI-1640, DMEM, FBS, L-glutamine, HEPES were products of Sigma-Aldrich (USA).

In vitro cytotoxicity

Treatment of cancer cell lines

Stock solutions of ethanol and water extracts (50 mg/mL) and berberine (10 mM) as a typical secondary metabolite of *M. aquifolium*, were dissolved in dimethyl sulfoxide (DMSO). HeLa, LS-174T, MRC-5 and EA.hy926 were seeded into 96-well microtiter plate (the seeding densities for each cell line were: 2000, 7000, 5000 and 5000 cells/well, respectively). 20 hours later five concentrations of extracts and berberine were added to the cells, except to the control cell samples, according to established procedure (Matić et al., 2013a; Matić et al., 2013b). Tested concentrations ranged from 12.5 to 200 µg/mL for extracts and from 2.1 to 67 µg/mL for berberine. The positive control was chemotherapy drug cisplatin. The final concentrations of DMSO to which the target cells were exposed were lower than 0.5% and non-toxic. Experiments were done in triplicates.

Determination of cell survival (Video Clip)

The effects of the extracts on survival of cells were determined by MTT cell survival test, according to the method of Mosmann (Mosmann, 1983), and modified and reported elsewhere by our research group (Matic et al., 2013a). After the 72 hours treatments with extracts and berberine (for HeLa, LS-174T and MRC-5

cell lines), and 24 and 48 hours treatments (for EA.hy926 cell line), 10 µL of MTT solution (3-(4,5-dimethylthiazol-2-yl)-2,5-dyphenyl tetrazolium bromide) (5 mg/mL) was added to each well. After 4 hours of incubation with MTT 100 µL of 10% SDS (100 g/L sodium dodecyl sulfate) was added to the wells. Absorbance was measured at 570 nm on the next day.

To determine the cell survival (%), absorbance (A) of a sample with cells grown in the presence of various concentrations of the investigated agents was divided by the absorbance of control, where cells were grown only in nutrient medium, and multiplied by 100 (Matić et al., 2013b). Absorbance of the blank was always subtracted from absorbance of the corresponding sample with target cells. IC_{50} was defined as the concentration of an agent that inhibited cell survival by 50% compared with a vehicle-treated control. The evaluation was conducted in accordance with the Protocol of the American Cancer Institute (NCI), which recommends that IC_{50} values ≤30 µg/mL should be considered significant for crude extracts of plant origin as well as IC_{50} values ≤4 µg/mL for pure substances (Geran et al., 1972). MTT and SDS were products of Sigma-Aldrich (USA).

Morphological evaluation of cell death

HeLa cells were seeded into plates on cover slips over night. The next day, the extracts and cisplatin, as a positive control, were added to cells at $2IC_{50}$ concentrations. After 24 hours of incubation, the cells were stained with a mixture of acridine orange and ethidium bromide dyes (3 µg/mL acridine orange and 10 µg/mL ethidium bromide in phosphate buffered saline (PBS), and visualized under a fluorescence microscope - Carl Zeiss PALM MicroBeam with Axio Observer.Z1 using AxioCam MRm (filters Alexa 488 and 568), as already described (Matić et al., 2013a; Živković et al., 2016).

Cell cycle analysis

HeLa cells were seeded into 6-well plate and after 24 hours they were treated with IC_{50} and $2IC_{50}$ concentrations of both investigated extracts for 24 and 48 hours. After incubation, the cells were collected by trypsinization, fixed in 70% ethanol on ice and stored at -20°C for one week (Matić et al., 2013a). Before staining, the cells were washed and treated with RNAse A (100 µg/mL) at 37°C for 30 min. Then cells were stained with propidium iodide (40 µg/mL). Cell cycle phase distribution was assessed using a FACSCalibur flow cytometer (BD Biosciences Franklin Lakes, NJ, USA) and analysis was performed using CELLQuest software (BD Biosciences), on a 10,000 cells per each sample (Matić et al., 2013a). The results were obtained from three independent experiments and presented with standard deviations.

Identification of caspases

HeLa cells were seeded into 6-well plate over night and pretreated for 2 hours with specific caspase inhibitors (40 µM) and then both extracts were added at $2IC_{50}$ concentrations. Each extract had a reference sample, which was not treated with an inhibitor. After 24 hours of incubation, the cell samples were collected, stored at -20°C for one week and analyzed according to established procedure (Matić et al., 2013a). The identification of target caspases, involved in the apoptotic pathway induced by the extracts, was performed by measuring the percentages of HeLa cells pretreated with caspase inhibitors in the subG1 phase. The caspase inhibitors were: Z-DEVD-FMK, a caspase-3 inhibitor, Z-IETD-FMK, acaspase-8 inhibitor and Z-LEHD-FMK, a caspase -9 inhibitor. Caspase inhibitors were purchased from R & D Systems (Minneapolis, USA).

In vitro scratch assay

EA.hy926 cells were seeded into 24-well plate in nutrient medium. After 24 hours cells created confluent monolayers, which were scarped with a p200 pipette tip to create a straight central line–scratch. The detached cells were washed and removed with nutrient medium. Cells in plate were then treated with sub-toxic concentrations (IC_{20}) of both extracts, 79.7 µg/mL for water extract and 48.3 µg/mL for ethanol extract, for 48 hours. Those concentrations were obtained for 48 hours treatment of EA.hy926 cells with investigated extracts. Photomicrographs were taken after making the wound 0, 24 and 48 hours later under the inverted phase-contrast microscope.

Tube formation assay

24-well plates were coated with 200 µL of Corning® Matrigel® basement membrane matrix. The assay was described elsewhere (Aranda and Owen, 2009; Matić et al., 2013a; Živković et al., 2016). Plates were incubated for 2 hours. After that, suspensions of EA.hy926 cells, which have the ability to develop tubules from elongated cell bodies when they grow on the surface of matrigel matrix, were added into plates. In control cell sample complete nutrient medium was added, while solutions of subtoxic concentrations 80 µg/mL (IC_{20}) of both extracts with nutrient medium were added to other samples. Those concentrations were obtained for 24 hours treatment of EA.hy926 cells with investigated extracts. After incubation, photomicrographs of cells were captured under the inverted phase-contrast microscope.

Results

Chemical analyses of plant extracts

In the examined extracts we detected that ethanol

Table I

Concentration of *M. aquifolium* extracts that induced 50% decrease in target cell survival, after 72 hours treatment

Extracts and compounds	IC_{50} (µg/mL)		
	HeLa	LS-174T	MRC-5
Water extract	42.9 ± 2.6	44.1 ± 2.9	163.7 ± 1.3
Ethanol extract	48.6 ± 3.5	39.0 ± 9.0	181.6 ± 7.8
Berberine	2.5 ± 0.2	7.8 ± 2.6	>67.3
Cisplatin	1.2 ± 0.1	5.3 ± 0.2	2.7 ± 0.3

IC_{50} values are presented as the mean ± standard deviation (SD) from three independent experiments

extract was more abundant in berberine (2.4%) compared to the water extract (1.3%).

In vitro cytotoxicity

Both ethanol and water extracts show moderate cytotoxic activity towards both tested malignant cell lines (Table I). In addition, the ethanol extract had slightly better cytotoxic activity against the LS-174T cells. However, berberine possessed cytotoxicity, very similar to conventionally used anti-cancer drug cisplatin. Both extracts exhibited a significantly weaker activity against normal, non-transformed MRC-5 cell line and therefore, good selectivity compared with cisplatin (data are summarized in Table II). The cytotoxicities of ethanol and water extracts were almost three to four times lower against the normal cells compared with the cancer cells (Table II).

Morphological evaluation of cell death mode

The human cervical adenocarcinoma HeLa cells treated with cisplatin showed typical signs of early apoptosis and late apoptosis. The same morphological characteristics, such as cell shrinkage and condensation of nucleus were detected in cells treated with extracts. For cells treated with extracts, no signs of necrosis had been observed. These results point out the pro-apoptotic effect of the investigated extracts as an important mechanism of their anti-cancer activity (Figure 1).

Cell cycle analysis

The analyses of the cell cycle changes indicate alterations in the percentage of cells; the increase of cells in sub G1 phase was concentration-dependent and accompanied with a significant increase of cells in S phase after 24 hours exposure (Figure 2). As expected, after 48 hours treatment both extracts induced the accumulation of significant number of cells in the sub G1 and S phase (Figure 2). It is known that the increase in the sub G1 cell population is related to the DNA fragmentation and apoptotic cell death. Likewise, we have seen that both extracts have the ability to cause the accumulation of HeLa cells in the S phase, which effectively blocks the cell division. This indicates that the ethanol and water exerts had anti-cancer effects on malignant cells through cell cycle arrest and apoptosis.

Table II

Selectivity index in the antitumor action of extracts and compounds

Extracts and compounds	IC_{50} MRC-5/IC_{50} (Cell line)	
	HeLa	LS-174T
Water extract	3.81	3.71
Ethanol extract	3.73	4.66
Berberine	>26.69	>8.58
Cisplatin	2.16	0.5

Determination of target caspases

The presence of caspase-3 and caspase-8 inhibitors notably decreased percentages of apoptotic cells in the sub G1 phase treated with water or ethanol extract (Figure 3).

Effects of extracts on endothelial cell migration

The examined ethanol and water extracts showed suppressive effects on migration of EA.hy926 cells (Figure 4). Ethanol extract demonstrated significant inhibitory effect on cell migration, while water extract showed slightly lower ability to suppress cell migration.

Anti-angiogenic effects

Ethanol extract showed significant anti-angiogenic potential. It inhibited the association of cells and formation of tubules and polygon structures (Figure 5). Water extract exerted weak anti-angiogenic effect. Cells samples treated with water extract had lower number of cell connections and tubular structures (Figure 5).

Discussion

Data from different studies suggest that plant species of genus *Mahonia* may have significant anti-cancer potential as well as isolated compounds from plants of

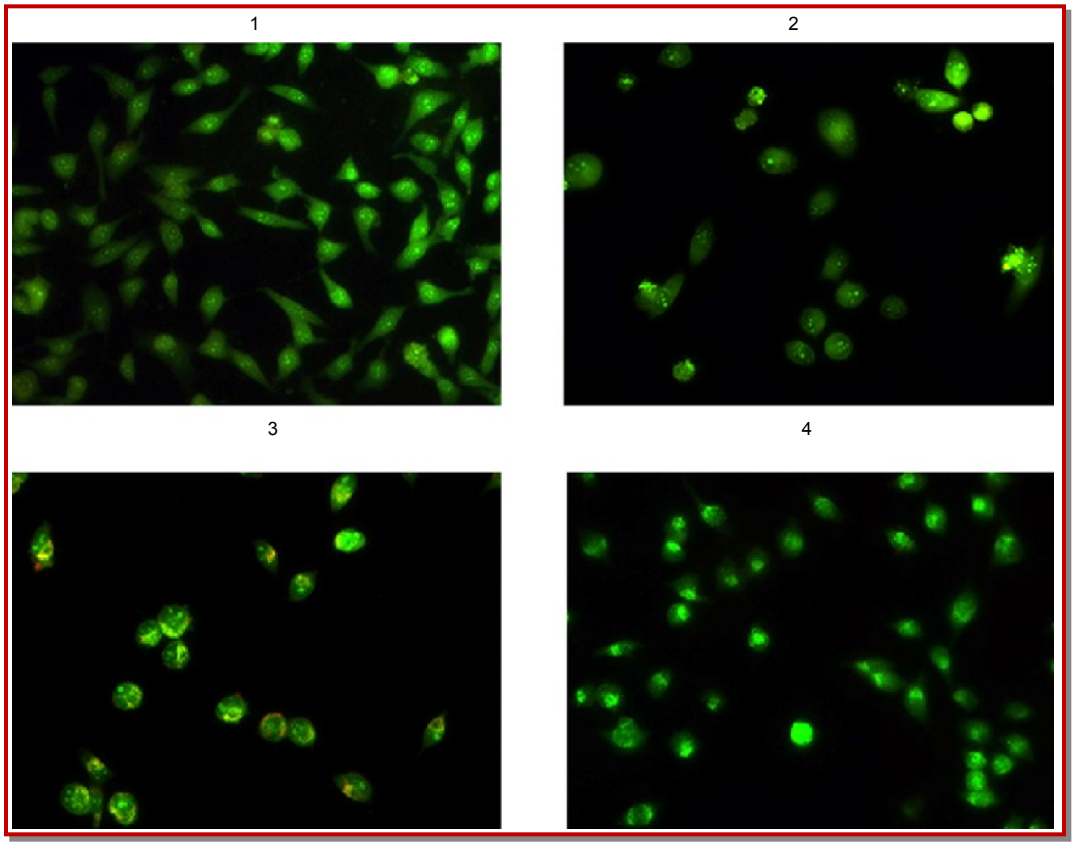

Figure 1: Photomicrographs of acridine orange/ethidium bromide stained HeLa cells (A): 1-control HeLa cells, 2- HeLa cells treated with cisplatin, 3- HeLa cells treated with water extract of M. *aquifolium*, 4- HeLa cells treated with ethanol extract of M. *aquifolium*

this genus.

The alkaloids are the major secondary metabolites of *Mahonia* species and it is assumed that they are responsible for most of the properties shown by the plants of this genus (He and Mu, 2015). Berberine and other alkaloids of this type are organic cations, which exhibit antimicrobial, anti-cancer, and antimutagenic activities. Berberine, palmatine, jatrorrhizine and magnoflorine are identified as the major alkaloids of *M. aquifolium*, while berbamine, oxyacanthine and columbamine are detected only in small amounts (Galle et al., 1994). Berberine significantly inhibits the growth of many different human cancer cell lines (Lin, 1996). While jatrorrhizine isolated from *M. balei* shows toxicity against mouse lymphoid macrophage (P388) cell line (Zhao et al., 1991). Berberine and jatrorrhizine demonstrate antimutagenic activity, while magnoflorine and jatrorrhizine isolated from *M. aqufolium* show better antiradical and anti-oxidative activities than berberine (Rackova et al., 2004).

It is reported that the ethanol extract of *M. oiwakensis* suppresses the growth of human lung cancer A549 cells *in vitro* and leads to apoptosis in human lung cancer cells *in vivo* (Wong et al., 2009). In addition, the water extract of *M. balei* inhibits the growth of human colon cancer HT-29 cells, while berberine, a chemical onstituent of mentioned water extract, shows antiproliferative activity on the same cells, suggesting that berberine may be responsible for the anti-cancer activity of extract (Hu et al., 2010).

For the first time, this study shows good to moderate cytotoxic activities of the examined water and ethanol extracts of *M. aquifolium* against cancer cell lines. IC_{50} values for both extracts for HeLa and LS-174T cell lines are nearly equal, regardless of the fact that content of the berberine is almost 2-times higher in the ethanol extract in comparison with water extract. This leads us to the conclusion that besides berberine as a bioactive component, the other compounds which exert cytotoxic activities are present in both extracts. To identify all active constituents of the extracts, further chemical characterization is needed.

The results demonstrate that extracts are several time less cytotoxic for normal, healthy cells than for cancer cells *in vitro*. Excellent selectivity in the anti-cancer action and good cytotoxic activity open the way for further investigations of the anti-cancer potential of these extracts and their active compounds.

Better understanding of molecular biology of cancer clarified that disturbances in apoptosis signalling pathways, induced by oncogenic mutations, lead to

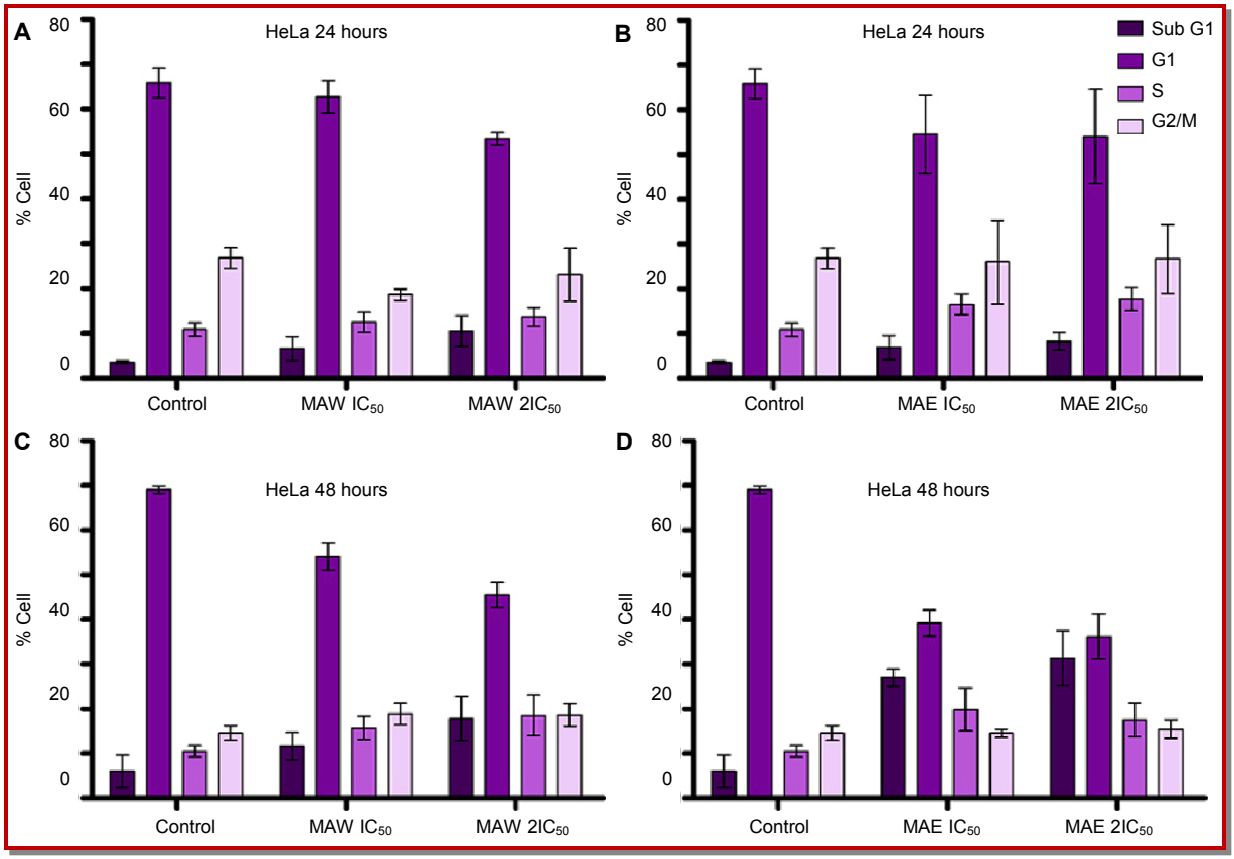

Figure 2: Changes in the cell cycle phase distribution of HeLa cells induced by *M. aquifolium* extracts after 24 (A, B) and 48 hours (C, D) of treatment

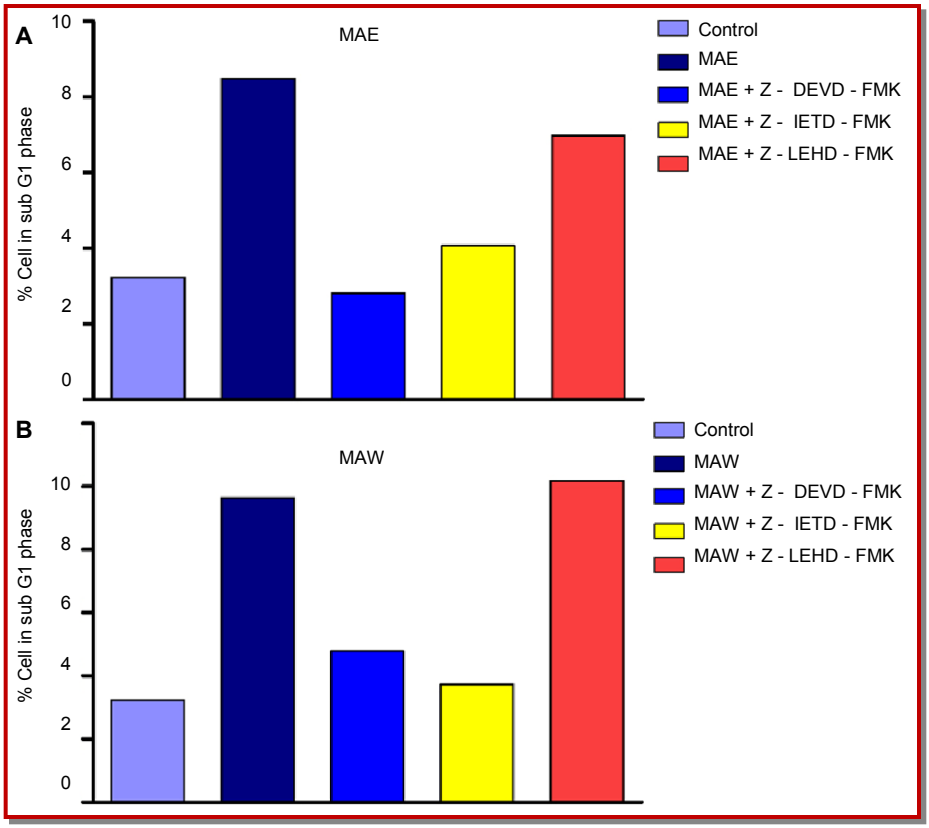

Figure 3: Effects of caspase inhibitors (caspase-3, -8 and -9 inhibitor) on the percentage of apoptotic HeLa cells after 24 hours treatment with ethanol extract (A) and with water extract (B)

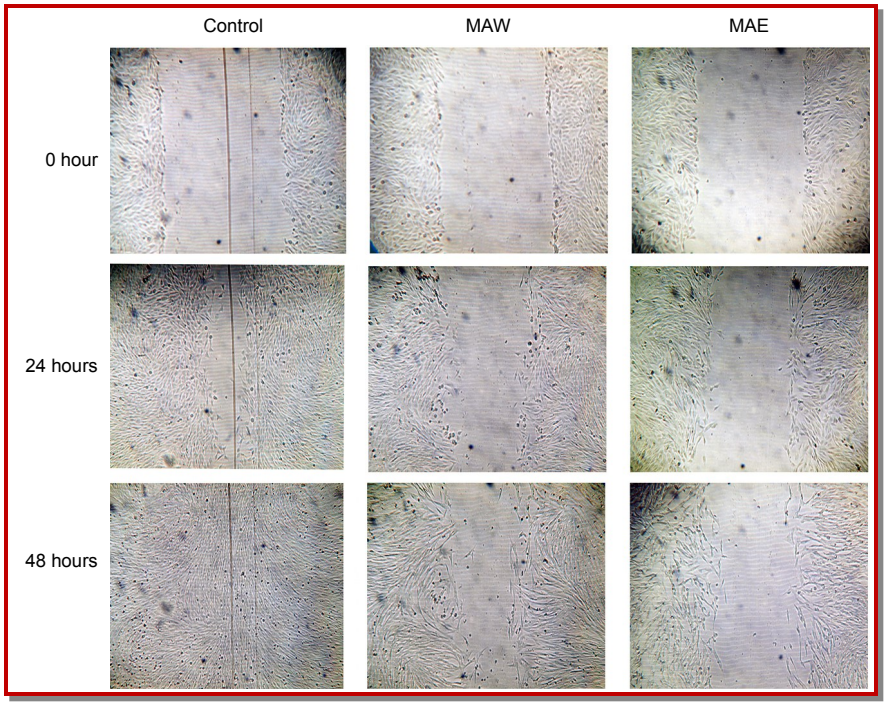

Figure 4: Effects of *Mahonia aquifolium* water (MAW) and ethanol (MAE) extracts on endothelial cell migration. Applied concentrations for MAW was 79.7 µg/mL for MAE was 48.26 µg/mL. Representative photomicrographs of one out of three independent experiments are shown

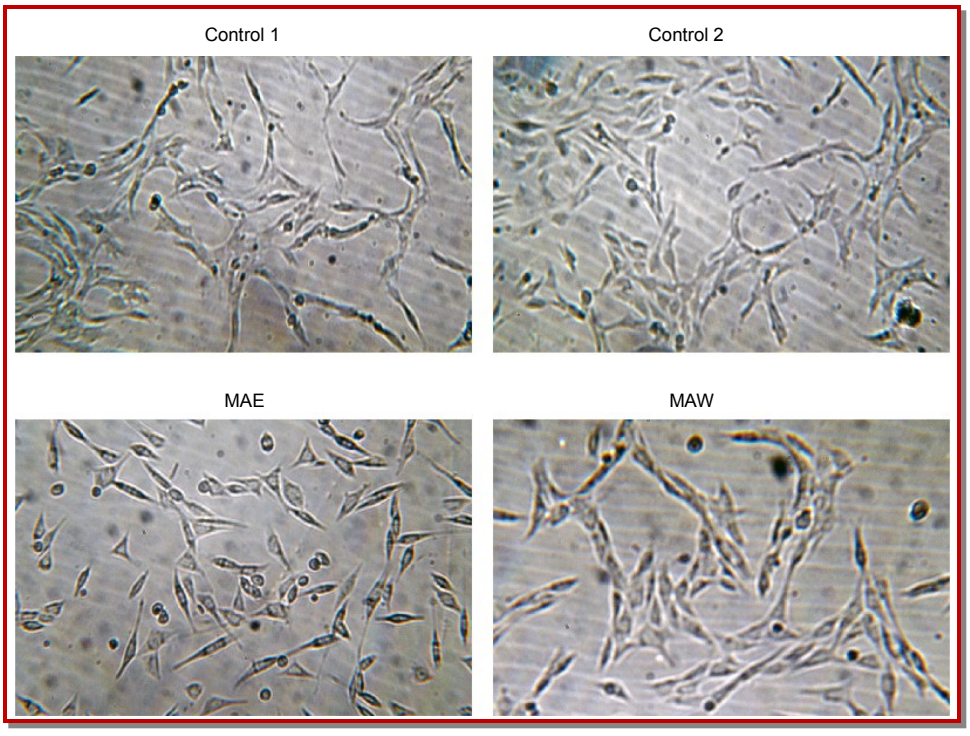

Figure 5: Effects of *Mahonia aquifolium* water (MAW) and ethanol (MAE) extracts on angiogenesis of endothelial cells. Applied concentrations of MAW and MAE were 80 µg/mL. Representative photomicrographs of one out of two independent experiments are shown

malignant transformation. It is well-known that majority of cytotoxic and anti-cancer drugs trigger apoptosis and that failures in cancer treatment may be due to defects in the apoptotic process (Lowe and Athena, 2000). The process of apoptosis is regulated by different mechanisms, which involve various signalling path-

ways. Our morphological examination of the cell death mode, cell cycle analysis and identification of involved caspases clearly demonstrate that water and ethanol extracts induce apoptosis in treated human cervical adenocarcinoma HeLa cells.

The studies showed that berberine may have an influence on many components of the apoptotic signalling pathways (Jabbarzadeh Kaboli et al., 2014). It has been shown that berberine significantly activates caspase-3, caspase-8 (Ho et al., 2009) and caspase-9 (Ho et al., 2009; Lin et al., 2006; Mantena et al., 2006). In this study, it is observed that the decreases of the percentages of the apoptotic sub G1 HeLa cells in the samples which are pretreated with caspase-3 and caspase-8 inhibitors and treated with extracts. Pretreatment with a caspase-9 inhibitor lead to insignificant decrease of cells in the sub G1 phase exposed to ethanol extract. The decrease in the presence of this inhibitor is absent when cells are treated with water extract. This result suggests that water and ethanol extracts induce a caspase-dependent apoptosis through activation of caspase-3 and caspase-8. The lack of caspase-9 activation might be explained by the presence of other active components in both extracts, which may predominantly trigger apoptosis through extrinsic signalling pathway.

Metastasis is strongly associated with ability of cells to migrate and to reduce capacity to attach to each other. It has been shown that berberine blocks Wnt/β-catenin signalling, inhibits cell migration (Park et al., 2012; Albring et al., 2013) and has the ability to regulate cell attachment through zonula occludens 1 protein (ZO-1) (Liu et al., 2009). Berberine can activate ZO-1, helps in the formation of tight junctions between cells and reduces cell mobility. Both tested extracts efficiently inhibited cell migration and mobility that are significant for suppression of the initial steps in invasion and metastasis and angiogenesis. The inhibitory effect of ethanol extract on cell migration is related to very good anti-angiogenic activity, suggesting the significant anti-angiogenic and anti-metastatic effects of this extract. The formation of new blood vessels is very important for the metastatic spread of cancer tissue and the new vascular network ensures supplying of cancer cells with oxygen and nutrients. *Pleurotus eous* polysaccharides suppress angiogenesis in breast cancer cells (Xu et al., 2015). Anti-cancer and anti-angiogenesis effects of the leaf extract of *Sargassum wightii* against osteosarcoma cancer cells have been reported (Yu et al., 2015).

The angiogenic inhibitors in therapy may contribute to reducing the mortality and morbidity in patients with cancer. The main characteristics of angiogenic antagonists, which are examined in clinical trials, are inhibition of proteases, endothelial cells migration and proliferation and angiogenic growth factors (Nishida et al., 2006). Both examined extracts show the ability to inhibit proliferation and migration of endothelial cells, while ethanol also inhibits association of endothelial cells and formation of tubules and polygon structures.

Conclusion

Both ethanol and water extracts of *M. aquifolium* exert cytotoxic activities, induce apoptosis in cancer cells and have the ability to inhibit cell migration and angiogenesis.

Acknowledgement

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Conflict of Interest

The authors declare that they have no conflict of interests.

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