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ORIGINAL ARTICLE

Antiproliferative and apoptotic effect of *Morus nigra* extract on human prostate cancer cells

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Abstract *Background: Morus nigra* L. belongs to the family *Moraceae* and is frequently used in traditional medicine. Numerous studies have investigated the antiproliferative effects of various extracts of different *Morus* species, but studies involving the *in vitro* cytotoxic effect of *M. nigra* extract are very limited. The purpose of this study was to evaluate the phenolic composition and antioxidant activity of dimethyl sulfoxide extract of *M. nigra* (DEM) and to investigate, for the first time, the probable cytotoxic effect in human prostate adenocarcinoma (PC-3) cells together with the mechanism involved. *Methods:* Total polyphenolic contents (TPC), ferric reducing antioxidant power (FRAP) and phenolic compounds of DEM were evaluated using spectrophotometric procedures and HPLC. The cytotoxic effect of DEM on PC-3 cells was revealed using the MTT assay. Mechanisms involved in the cytotoxic effect of DEM on PC-3 cells were then investigated in terms of apoptosis, mitochondrial membrane potential and cell cycle using flow cytometry, while caspase activity was investigated using luminometric analysis. *Results:* TPC and FRAP values were 20.7 ± 0.3 mg gallic acid equivalents and 48.8 ± 1.6 mg trolox equivalents per g sample, respectively. Ascorbic acid and chlorogenic acid were the major phenolic compounds detected at HPLC analysis. DEM arrested the cell cycle of PC-3 cells at the G_1 phase, induced apoptosis via increased caspase

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activity and reduced mitochondrial membrane potential. *Conclusions:* Our results indicate that *M. nigra* may be a novel candidate for the development of new natural product based therapeutic agents against prostate cancer.

1. Introduction

The genus *Morus*, commonly known as mulberries, contains 10–16 species and belongs to the family *Moraceae*. *Morus alba* L., *Morus rubra* L. and *Morus nigra* L. are the most extensive *Morus* species. *M. nigra* is native to western Asia and has been cultivated in Europe since Pre-Roman times (Ozgen et al., 2009; Kostic et al., 2013). The deep-colored *Morus* fruits are a rich source of phenolic compounds, including flavonoids, anthocyanins and carotenoids (Kostic et al., 2013). Due to their rich anthocyanin contents, the fruits of *M. nigra* in particular exhibit higher antioxidant activity than the other *Morus* species (Ozgen et al., 2009; Kostic et al., 2013). Recent studies have reported antimicrobial, antioxidant, antidiabetic, anti-HIV, anti-inflammatory, hypolipidemic, hepatoprotective, anticancer, antiobesity, and neuroprotective activities of different species of *Morus* and have attributed to those compounds (Sakagami et al., 2007; Khalid et al., 2011; Kostic et al., 2013; Ramesh et al., 2014; Grajek et al., 2015). *M. nigra* is used to treat urinary incontinence, dizziness, sore throat, depression, fever and cancer in traditional medicine (Khalid et al., 2011; Kostic et al., 2013).

Prostate cancer is the fifth most common cancer overall, the second most prevalent malignancy worldwide and the second greatest cause of cancer-related deaths among men after lung cancer (Shahneh et al., 2014; Huo et al., 2015; Kim et al., 2015). A combination of treatment options (radiation therapy, brachytherapy, cryosurgery, chemotherapy, hormonal therapy, and surgery) is often recommended for managing prostate cancer. Of the conventional modalities for prostate cancer treatment, chemotherapeutic drugs lead to various side effects. Natural anticancer drugs derived from medicinal plants that selectively induce apoptosis and/or growth arrest in cancer cells without causing detrimental effect in healthy cells are today available, and these natural products can serve as chemotherapeutic agents (Shahneh et al., 2014).

Numerous studies have investigated the antiproliferative effects of various extracts of different *Morus* species. Eo et al. reported that 80% methanol extract of root bark of *M. alba* L. exhibited a dose-dependent anticancer effect on human colorectal carcinoma cell line (SW480) via induced cell growth arrest and apoptosis (Eo et al., 2014). Fathy et al. demonstrated that *M. alba* extract had a cytotoxic effect on hepatocellular cell line (Fathy et al., 2013). However, studies involving the *in vitro* cytotoxic effect of *M. nigra* extract are very limited. Qadir et al. demonstrated that *M. nigra* leaf extract exhibited cytotoxic effect on human cervical cancer (HeLa) cell line (Qadir et al., 2014). However, to the best of our knowledge no previous study has investigated the cytotoxic effect of *M. nigra* extract on prostate cancer cells. The purpose of this study was to therefore to evaluate the phenolic composition and antioxidant properties of dimethyl sulfoxide extract of *M. nigra* to investigate, for the first time, the probable cytotoxic effect in human prostate adenocarcinoma cells together with the mechanism involved.

2. Materials and methods

2.1. Chemicals and reagents

All phenolic standards, methanol, folin phenol reagent, sodium carbonate, potassium ferricyanide, trichloroacetic acid, iron (III) chloride, gallic acid, trolox, acetonitrile, cisplatin, phosphate buffer saline (PBS) tablet, trypan blue solution, dimethyl sulfoxide (DMSO), and thiazolyl blue tetrazolium bromide (MTT dye) were purchased from Sigma (St. Louis, MO, USA). Kaighn's modification of Ham's F-12 (F-12K) and Eagle's minimal essential medium (EMEM) media were obtained from Lonza (Verviers, Belgium). Fetal bovine serum (FBS) was obtained from Biochrom (Berlin, Germany). Penicillin-streptomycin was purchased from Gibco (Paisley, England) and trypsin-EDTA solution from Biological Industries (Kibbutz Beit Haemek, Israel). All flow cytometry kits were obtained from BD Biosciences (San Diego, CA, USA).

2.2. Drug preparation and treatment

Cisplatin was used as a reference anticancer agent for cytotoxicity experiments due to its use in prostate cancer treatment (Dhar et al., 2011). It was dissolved in absolute DMSO to prepare a 1000 μg/mL stock solution.

External working concentrations of both extract and cisplatin were prepared by further dilution with DMSO. The final concentration of DMSO did not exceed 0.5% in culture media during any experiment, and this concentration did not affect cell morphology or viability.

2.3. Plant collection and extraction

Fully mature fruits of *M. nigra* were harvested from Kelkit town, Gumushane, Turkey. Samples were preserved in cool bags for transportation to the laboratory. The fruits were air-dried at room temperature for 20 days and converted into a fine powder using a blender and milling. The fruit powder (1 g) was extracted with 20 mL DMSO in a mechanical shaker (Shell Lab, Cornelius, OR, USA) in the dark for 24 h at 45 °C. The prepared 50 mg/mL stock DMSO extract of *M. nigra* (DEM) was filtered with Whatman No. 1 filter paper and a 0.2 μm filter and then stored at −20 °C until used in further experiments.

2.3.1. Estimation of total phenolic content (TPC)

Content of total phenolics of DEM was established by the spectrophotometric method (Slinkard and Singleton, 1977)

adapted to microscale using gallic acid as a standard. Briefly, 12.5 µL *M. nigra* extract was mixed with 62.5 µL freshly prepared Folin–Ciocalteu reagent and 125 µL 20% sodium carbonate. This was incubated at room temperature for 30 min, after which absorbance was measured at 760 nm. The results were calculated using a standard gallic acid chart and were expressed as milligrams of gallic acid equivalent per g sample.

2.3.2. Estimation of reducing power (FRAP)

The reducing power of DEM was established by FRAP assay (Oyaizu, 1986) adapted to microscale using trolox as a standard. Briefly, 40 µL *M. nigra* extract was mixed with 100 µL sodium phosphate buffer (pH = 6.6) and 100 µL 1% potassium ferricyanide. The mixture was incubated for 20 min at 50 °C and then cooled. Next, 100 µL 10% trichloroacetic acid was added. The mixture was then centrifuged at 3000*g* for 10 min, and 100 µL was taken from each of the upper phases. This was mixed with 100 µL pure water and 20 µL 0.1% of ferric chloride. The final mixture was incubated for 5 min in the dark and at room temperature. Absorbance was then measured at 700 nm. The results were calculated from a standard trolox chart and were expressed as milligrams of trolox equivalent per g sample.

2.3.3. HPLC analysis of phenolic compounds

Nine standards were used for HPLC analysis: ascorbic acid, gallic acid, 3,4-dihydroxy benzoic acid, protocatechuic acid, chlorogenic acid, caffeic acid, epigallocatechin gallate, *p*-coumaric acid and rutin hydrate.

HPLC analysis of phenolic compounds was performed using a reverse phase C_{18} column (150 × 4.6 mm i.d, 5 µm) (Macherey-Nagel, Duren, Germany), on a gradient program with a two solvent systems (A: 2% acetic acid in water and B: 0.5% acetic acid in acetonitrile:water (1:1)) at a constant solvent flow rate of 0.8 mL/min on a HPLC system (Agilent Infinity 1200, CA, USA) (De Villiers et al., 2004). Injection volume was 10 µL. Signals were detected at 240, 254, 260, 270, 275, 280, 300 and 324 nm by DAD. Column temperature was maintained at room temperature, 25 °C. Identification of compounds was performed by comparing retention times and spectral data with those of pure standards. Calibration curves of the standards were used for quantitation.

2.4. Cell culture

Human prostate adenocarcinoma (PC-3, ATCC-CRL-1435) cancer and human normal foreskin fibroblast cells (ATCC-CRL-2522) were supplied by the America Type Culture Collection (Manassas, VA, USA). PC-3 and fibroblast cells were cultured in F-12K and EMEM medium, respectively, supplemented with 2 mM L-Glutamine, 10% heat inactivated FBS, 1% penicillin and streptomycin with a 5% CO_2 supply at 37 °C.

2.4.1. Cytotoxicity assay

Cytotoxic effects of DEM and cisplatin on PC-3 and normal fibroblast cells were determined by MTT assay with 72 h treatment (Mosmann, 1983). MTT is a type of yellow tetrazolium salt and is converted to dark blue formazan crystals by metabolically active cells (Russo et al., 2004). Briefly, cells were seeded into a flat-bottomed 96-well cell culture plate at a density of 5×10^3 cells per well. After 24 h, the cells were treated with several concentrations of DEM (0–1000 µg/mL) and cisplatin (0–10 µg/mL) for 72 h. Next, 10 µL of MTT solution was added to each well, and the composed crystals were then dissolved in DMSO. Finally, absorbance was measured at 570 nm using a microplate reader (Versamax, Molecular Devices, California, USA). Optical densities were used to determine % cell viabilities using the formula (OD of treated group/OD of control group) × 100 in treated cells compared to control cells with no compound exposure (Deepa et al., 2012).

Log-concentrations versus % cell viabilities graphs were plotted. IC_{50} values were determined using this logarithmic graph. IC_{50} represents the concentration in "µg/mL" required for 50% inhibition of cell growth compared to negative control cells (Demir et al., 2016). Following the cytotoxicity experiments, the cytotoxic IC_{50} and IC_{90} concentrations of DEM and both IC_{50} (370 µg/mL) and IC_{90} (666 µg/mL) concentrations obtained were used for flow cytometry studies.

2.4.2. Cell cycle analysis

A total of 75×10^4 cells were cultured in T-25 flasks for 24 h. These were then treated with 370 and 666 µg/mL concentrations of DEM for 72 h and harvested. Cell suspensions were centrifuged for 5 min at 300*g* at room temperature. The supernatants were then aspirated, and cell pellets were washed twice with buffer solution. To the cell pellets was added 250 µL Solution A (trypsin buffer), and this was incubated at room temperature for 10 min. At the end of that time, 200 µL Solution B (trypsin inhibitor and RNase buffer) was added to the mixture, which was again incubated for 10 min at room temperature. Finally, the tubes were placed on ice and 200 µL cold Solution C (PI stain solution) was added. All procedures were carried out according to the manufacturer's recommendations (BD Biosciences, Cat No: 340242, San Diego, CA, USA). Data from 3×10^4 cells were collected and analyzed on a flow cytometer (FACSCalibur, Becton Dickinson, East Rutherford, NJ, USA). The results were compared with the negative (no treatment) control.

2.4.3. Annexin V analysis

A total of 75×10^4 cells were cultured in T-25 flasks for 24 h. These were then treated with 370 and 666 µg/mL concentrations of DEM for 72 h and harvested. Cell suspensions were centrifuged for 5 min at 300*g* at room temperature. Then, the supernatants were aspirated, and cell pellets were washed twice with cold PBS buffer. To the cell pellets was added 100 µL 1× Binding Buffer. Next, to the cell suspension were added 5 µL of FITC Annexin V and 5 µL PI. The mixture was then incubated for 15 min, in the dark at room temperature. All procedures were carried out according to the manufacturer's recommendations (BD Pharmingen, Cat No: 556547, San Diego, CA, USA). Data from 10^4 cells were collected and analyzed on a flow cytometer (FACSCalibur, Becton Dickinson, East Rutherford, NJ, USA). The results were compared with the negative (no treatment) control.

2.4.4. Determination of mitochondrial membrane potential

Mitochondrial membrane potential was determined using a JC-1 assay kit (BD Mitoscreen, Cat No: 551302, San Diego,

CA, USA). JC-1 remains as a monomer and emits green fluorescence at a low level of mitochondrial membrane potential, but the state of high mitochondrial membrane potential it forms aggregates and emits red fluorescence (Xuan et al., 2014).

A total of 75×10^4 cells were cultured in T-25 flasks for 24 h. These were then treated with 370 and 666 μg/mL concentrations of DEM for 72 h and harvested. Cell suspensions were centrifuged for 5 min at 300*g* at room temperature. The supernatant part was removed, and 500 μL JC-1 Working Solution was added to the cell pellets and incubated for 15 min at 37 °C. At the end of that time the mixture was centrifuged at 400*g* for 5 min. The supernatants were then aspirated, and cell pellets were washed twice with 1 × Assay Buffer. All procedures were carried out according to the manufacturer's recommendations. Data from 10^4 cells were collected and analyzed on a flow cytometer (FACSCalibur, Becton Dickinson, East Rutherford, NJ, USA). The results were obtained from the ratio of red to green fluorescence and were expressed as relative mitochondrial membrane potential compared to negative control samples (cells with no test compound).

2.4.5. Determination of caspase activity

Effector caspase activities were determined using a Caspase-Glo® 3/7 luminescent assay kit according to the manufacturer's recommendations (Promega, Cat No: G8090, Madison, WI, USA). Cells were seeded in a 96-well white-walled cell culture plate at a density of 5×10^3 cells/well. After 24 h, the cells were treated with several concentrations of DEM (250, 500, and 1000 μg/mL) for 18 h. After treatment, the media were discarded, and the cells were washed with PBS. Next, 100 μL of Caspase-Glo® 3/7 reagent was added to the wells, following by mixing on a plate shaker at 400 rpm for 30 s. The plate was incubated at room temperature for 30 min and the luminescence of each sample was read on a plate-reading luminometer (Molecular Devices SpectraMax Paradigm Multi-Mode, Sunnyvale, CA, USA). Results were given as relative caspase activity compared to negative control samples (cells with no test compound).

2.5. Statistical analysis

All experiments were carried out in triplicate, and results were expressed as mean ± standard deviation (mean ± S.D). Compatibility with normal distribution was determined using the Kolmogorov–Smirnov test. One-way ANOVA was used to compare differences among the groups. $p < 0.05$ was regarded as significant.

3. Results

TPC and FRAP values were 20.7 ± 0.3 mg gallic acid equivalents and 48.8 ± 1.6 mg trolox equivalents per g sample, respectively.

The chromatogram of phenolic standards is shown in Fig. 1. The phenolic compounds in *M. nigra* are shown in Table 1. The values were stated in μg/g sample. Ascorbic acid and chlorogenic acid were the most abundant compounds in *M. nigra* (Table 1). The small peak of ascorbic acid in Fig. 1 (at 280 nm) is due its maximum wavelength is 240 nm. 3,4-dihydroxy benzoic acid and protocatechuic acid had the same retention times and DAD spectra in our study. We therefore determined the quantities of these separately based on their calibration curves.

The PC-3 and fibroblast cells were treated with various concentrations of DEM and cisplatin. Their effects on cell proliferation were determined after 72 h based on cells' ability to metabolically reduce MTT to a formazan dye. The IC_{50} values for DEM and cisplatin are presented in Table 2. DEM exhibited moderate selective cytotoxicity against PC-3 cells compared to foreskin fibroblast cells.

The cell cycle analysis results are summarized in Fig. 2. Both concentrations of DEM significantly increased cell numbers at the G_0/G_1 phase ($p < 0.01$ and $p < 0.01$, respectively). Both concentrations of DEM also significantly reduced cell numbers at the S phase ($p < 0.01$ and $p < 0.05$, respectively).

The Annexin V analysis results are summarized in Fig. 3. Concentrations of 370 μg/mL of DEM increased the number of early apoptotic and dead cells, but the differences were not statistically significant ($p > 0.05$). However, concentrations of 666 μg/mL of DEM significantly reduced the number of viable cells and increased those of dead, necrotic/late apoptotic and early apoptotic cells ($p < 0.001$).

Mitochondrial membrane potential analysis results are presented in Fig. 4. Both concentrations of DEM significantly reduced mitochondrial membrane potential in PC-3 cells ($p < 0.001$). The percentage reductions in mitochondrial membrane potential by DEM were 26.4% and 62.5% for concentrations of 370 and 666 μg/mL DEM, respectively.

Caspase 3 and caspase 7 are key mediators of apoptosis because of their ability to cleave key cellular proteins. We measured the activity of caspase 3/7 using a luminometric kit for 18 h treatment. The percentage inductions of relative caspase 3/7 activity by DEM were 3%, 11% and 70% for concentrations of 250, 500 and 1000 μg/mL DEM, respectively, although the only significant increase ($p < 0.001$) was determined in the concentration of 1000 μg/mL DEM (Fig. 5).

4. Discussion

Cancer is a heterogeneous disease that can involve a genetic history due to multiple alterations of cell signaling pathways (Luo et al., 2009). Traditional chemotherapy is often associated with adverse effects on healthy cells and gradual drug resistance in cancer cells (Huang et al., 2011). Medicinal plants have been investigated as a potential new source of cancer therapy based on ability to produce apoptosis and/or growth arrest in cancer cells without causing cytotoxic effect in healthy cells (Shahneh et al., 2014).

Plants are excellent sources of food, chemicals and herbal medicines. Many important drugs have been derived from these (Khalid et al., 2011). *M. nigra* is one of the most important species of the genus *Morus*, the fruits containing substantial levels of phenolics, flavonoids, and ascorbic acid (Kostic et al., 2013). It has recently been reported to exhibit biological properties, such as antidiabetic, antioxidative, anti-inflammatory and antihyperlipidemic activities. These biological activities are due to the polyphenol components, including anthocyanins, present in some varieties (Kutlu et al., 2011; Kamiloglu et al., 2013). Various studies have investigated *in vitro* antiproliferative and proapoptotic characteristics of different *Morus* species in the recent years (Fathy et al.,

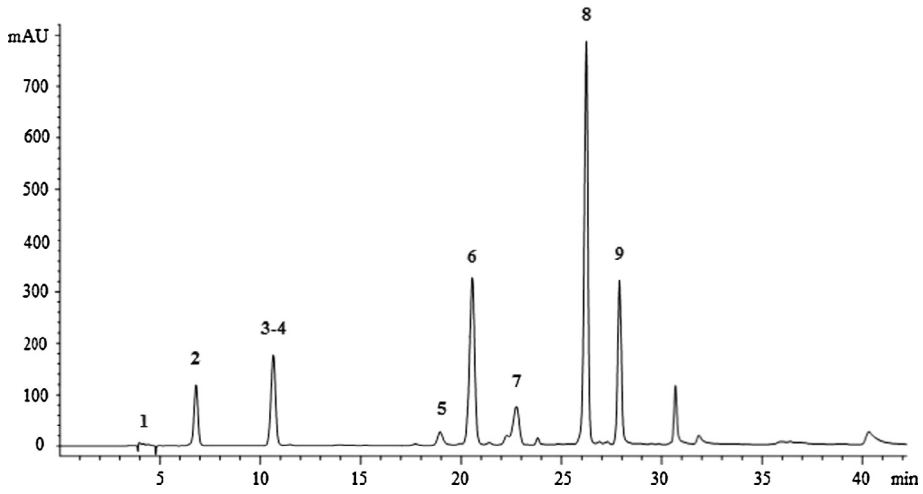

Figure 1 Chromatogram of standard phenolic acids (at 280 nm) in the optimum HPLC conditions in the experimental protocol. Peaks: ascorbic acid (1), gallic acid (2), 3,4-dihydroxy benzoic acid (3), protocatechuic acid (4), chlorogenic acid (5), caffeic acid (6), epigallocatechin gallate (7), p-Coumaric acid (8), rutin hydrate (9).

Table 1 Phenolic composition of the DMSO extract of *Morus nigra* L.

Phenolic compound assignment	Retention time (min)	Amount (μg/g sample)
Ascorbic acid	3.99	1995.7 ± 59.9
Gallic acid	6.80	7.5 ± 0.3
3,4-dihydroxy benzoic acid	10.65	88.6 ± 4.43
Protocatechuic acid	10.65	114 ± 5.13
Chlorogenic acid	18.95	309.6 ± 20.1
Caffeic acid	20.56	ND
Epigallocatechin gallate	22.28	ND
p-coumaric acid	26.23	134.7 ± 6.1
Rutinhydrate	27.88	71.6 ± 2.5

Results are expressed as mean ± SD of three determinations. ND, not detected.

Table 2 Cytotoxic activity (IC_{50}, μg/mL) of DEM and cisplatin ($n = 3$).

Test compound	PC-3 cells	Fibroblast cells
DEM	370.1 ± 5.8	424.9 ± 7.3
Cisplatin	0.608 ± 0.05	5.29 ± 0.24

2013; Eo et al., 2014), but studies involving the *in vitro* cytotoxic effect of *M. nigra* extract are very limited (Qadir et al., 2014). No previous studies have investigated the cytotoxic effect of *M. nigra* extract on prostate cancer. Sforcin and Bankova reported that *in vitro* methods are useful for preliminary investigation of the possible beneficial biological effects of a natural product. If positive results are obtained from *in vitro* studies, then *in vivo* or clinical trials are recommended (Sforcin and Bankova, 2011). The PC-3 cell line is an androgen receptor negative prostate cancer cell line with high metastatic potential frequently in cancer research for *in vitro* prostate cancer models (Shahneh et al., 2014; Huo et al., 2015). We therefore planned this study on the prostate cancer cell line (PC-3) under *in vitro* conditions.

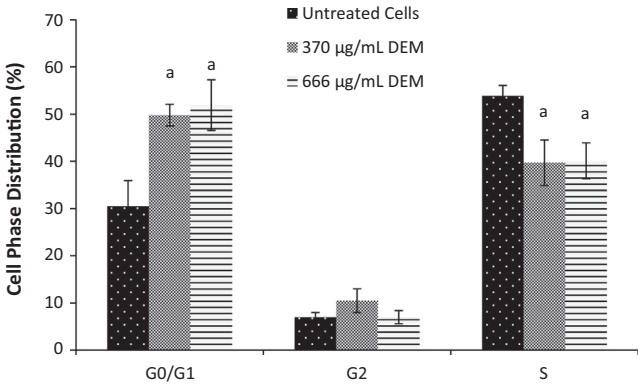

Figure 2 Cell cycle analysis of the effect of DEM extract on PC-3 cells after 72 h ($n = 3$). [a]Represents significant results ($p < 0.05$) compared with untreated PC-3 cells.

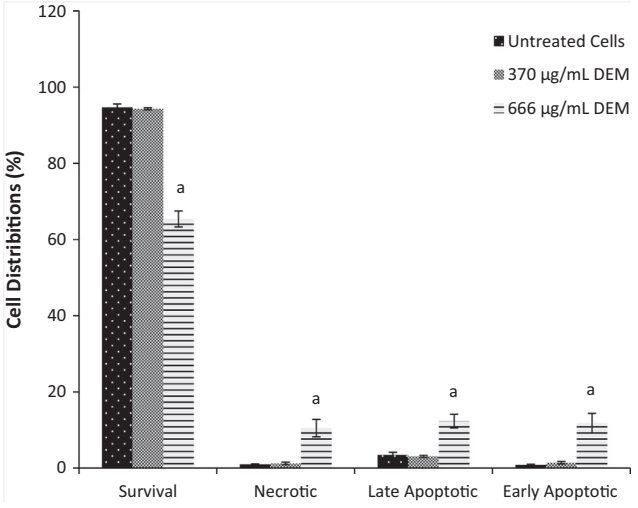

Figure 3 Annexin V-FITC analysis of DEM-treated PC-3 cells ($n = 3$). [a]Represents significant results ($p < 0.05$) compared with untreated PC-3 cells.

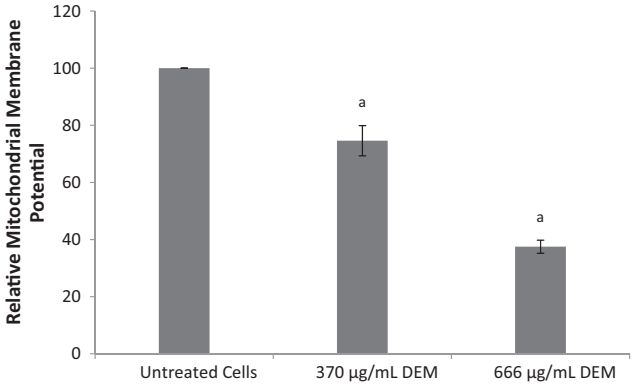

Figure 4 Effect of DEM on the integrity of the mitochondrial membrane of PC-3 cells after 72 h ($n = 3$). [a]Represents significant results ($p < 0.05$) compared with untreated PC-3 cells.

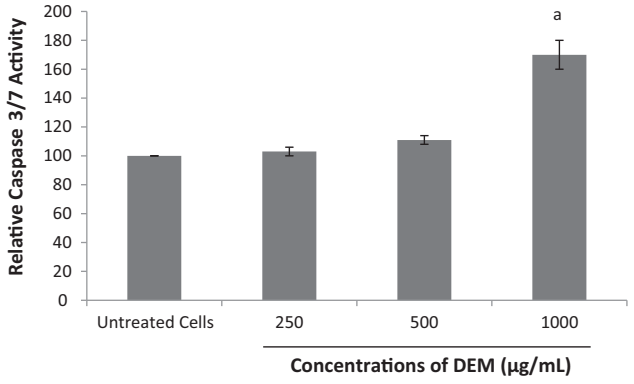

Figure 5 Detection of caspase-3/7 activity in PC-3 cells treated with DEM for 18 h ($n = 3$). [a]Represents significant results ($p < 0.05$) compared with untreated PC-3 cells.

Many methods are available for extraction of active components from plant materials. Maceration is one such technique. In this method, many solvents, such as water, ethanol, methanol, ethyl acetate, dimethyl sulfoxide, hexane or acetone, are used to extract the components from plant materials directly without compromising the structure (Dai and Mumper, 2010). The DMSO extract of *M. nigra* was prepared using the maceration technique in this study.

Various *in vitro* assays can be used to determine antioxidant activity of herbal extracts, and the use of at least two different methods is recommended (Nuutila et al., 2003). The TPC and FRAP methods were employed for the determination of antioxidant activity of DEM in this study. TPC and FRAP values of *M. nigra* extract were 20.7 ± 0.3 mg gallic acid equivalents and 48.8 ± 1.6 mg trolox equivalents per g sample, respectively. The TPC values of various extracts of *M. nigra* fruit from different regions range between 1.69 and 22.37 mg gallic acid equivalents/g sample (Kostic et al., 2013), while the FRAP value of 75% aqueous-methanol extract of *M. nigra* fruit from Turkey has been reported at 3.8 mg trolox equivalents/g sample (Kamiloglu et al., 2013). Our results were broadly similar to those of other studies. Any differences may have been due to the type of extraction method and solvent, environmental factors, soil, geographic region, harvest season, post-harvesting conditions and the maturity level of the fruits.

Several reports have described the use of HPLC with a diode array detector (DAD) for the characterization and quantification of phenolic composition (Boligon and Athayde, 2014). We used the HPLC-DAD spectra system for phytochemical analysis. Seven phenolic compounds (ascorbic acid, gallic acid, 3,4-dihydroxy benzoic acid, protocatechuic acid, chlorogenic acid, p-coumaric acid and rutin hydrate) were determined in the DEM. These results agree with previous research showing that the genus *Morus* is rich in polyphenolic compounds such as cyanidin-3-glucoside, kaempferol-3-O-rutinoside, rutin, quercetin, catechin, quercetin-3-O-glucoside, quercetin-3-O-rutinoside, taxifolin, chlorogenic acid, *p*-coumaric acid, vanillic acid, gallic acid, ferulic acid, caffeic acid, and syringic acid (Hassimotto et al., 2007; Gundogdu et al., 2011; Kostic et al., 2013).

An effective and acceptable chemopreventive or anticancer agent has to meet various criteria, including having no harmful effects on normal cells (Galati and O'Brien, 2004). We therefore performed cytotoxicity experiments in PC-3 cells coupled with human normal foreskin fibroblast cells. DEM exhibited reasonable selective toxicity against PC-3 cells compared to normal fibroblast cells via MTT assay, a nonradioactive, quick, and affordable method widely used in cytotoxicity studies (Russo et al., 2004). Qadir et al. demonstrated that *M. nigra* leaf extract exhibited cytotoxic effect on a human cervical cancer (HeLa) cell line (Qadir et al., 2014). Shi et al. isolate numerous flavonoids with isoprenoid groups from *Morus mongolica* and demonstrate selective cytotoxic activity in two human oral cancer cell lines (HSC-2 and HSG) against normal human gingival fibroblasts (Shi et al., 2001). In another study, Fathy et al. reported that *M. alba* extract exhibits cytotoxic effect on the hepatocellular cell line (Fathy et al., 2013), while Kofujita et al. demonstrated that prenylated flavanone separated from *M. alba* root shows cytotoxic effect against rat hepatoma cells (Kofujita et al., 2004). Additionally, Dat et al. isolated 11 flavonoids from the leaves of the white mulberry and evaluated their cytotoxic effect on three human cancer cell lines (HeLa, MCF-7 and Hep3B). Morusin was identified as the most potent cytotoxic compound against HeLa cells (Dat et al., 2010).

Many recent studies have concluded that cancer can be regarded as a disease of the cell cycle due to ineffective cell cycle checkpoint control. Cell cycle arrest of cancer cells is therefore regarded as one of the target mechanisms in cancer treatment (Diaz-Moralli et al., 2013). In this study, an IC_{90} concentration of DEM blocked the cell cycle of PC-3 cells at the G_0/G_1 phase, and both concentrations of DEM significantly reduced cell numbers at the S phase. This finding is significant because the cells did not achieve the next S phase involving intense protein and DNA synthesis. Both extracts of different *Morus* species and their constituents, such as lectin, gallic acid, ascorbic acid, and chlorogenic acid, can induce cell cycle arrest at different stages of the cell cycle, depending on the type of cancer cell by increasing the levels of cell cycle progression inhibitors or reducing levels of cyclins and CDKs (Thomas et al., 2005; Ou et al., 2010; Deepa et al., 2012; Liu et al., 2013; Eo et al., 2014).

Attenuate or extinct apoptosis capacity is reported in many cancer cells. Increasing apoptotic levels by various agents are therefore another target mechanisms in cancer cells (Reed,

1999). Caspases (cysteine-dependent aspartate-specific proteases) are activated during the early stages of apoptosis. These are synthesized as inactive zymogens but, once activated, can initiate a proteolytic cascade, resulting in the cleavage of key cellular components required for normal cellular function. Elevated caspase 3, 6 and/or 7 activity is regarded as an apoptosis marker. When cells are undergoing apoptosis, phosphatidylserine (PS), which makes up one part of the cell membrane, will translocate toward the extracellular side of the membrane. Annexin V, a 35–36 kDa phospholipid-binding protein, has a high affinity for PS. The translocation of PS to the outside or exposed side of the membrane is an early event in the apoptotic process. Annexin V staining is therefore regarded as a marker of early stage apoptosis (Deepa et al., 2012). Our results show that DEM potentially exhibited proapoptotic features in PC-3 cells both inducing caspase activity and reducing mitochondrial membrane potential. Huang et al. demonstrated that anthocyanins from mulberry exhibit activity against gastric cancer through the mechanism of induction of apoptosis via the activation of the p38/jun/Fas/FasL and p38/p53/Bax pathways that initiate cell death (Huang et al., 2011). Yang et al. showed that mulberry leaf polyphenol extract (especially rich in quercetin and rutin) induces apoptosis in p53-negative hepatocellular carcinoma cells via the p53-independent pathway (Yang et al., 2012). Kikuchi et al. showed that albanol A (isolated from the root bark extract of *M. alba*) exhibited cytotoxic activity on HL-60 leukemia cell line via inhibitory activity on topoisomerase II and inducing the caspase-dependent apoptosis pathway (Kikuchi et al., 2010). Recently, it has been reported that purified lectin from mulberry leaves induces apoptosis and cell cycle arrest in human breast (MCF-7) and colon (HCT-15) cancer cells in a caspase-dependent manner (Deepa et al., 2012).

It has been strongly suggested that the anticancer and proapoptotic properties of polyphenolic compounds derive from their pro-oxidant activities, rather than from antioxidant activity (Hadi et al., 2000). Although polyphenolic compounds are able to exhibit pro-oxidant effects under various conditions, they do not generally produce free radicals directly. Their pro-oxidant effect is widely associated with the redox status of the cell, and the basal redox levels of cancer cells are high compared to those of normal cells due to their greater levels of free heavy metal ions and increased metabolic rate (Lee et al., 2013). Jeong et al. reported that mulberry fruit extracts have cytotoxic effect on human glioma cells through ROS-dependent mitochondrial pathway and induction of apoptosis (Jeong et al., 2010). We therefore speculate that the apoptotic and antiproliferative effects of DEM in PC-3 cells may derive from its ROS formation capacity.

5. Conclusion

This study was the first to investigate the effect of proapoptotic and antiproliferative properties of *M. nigra* extract on prostate cancer cells. Further studies are required for the isolation and identification of individual phenolic compounds in the extracts. Also, the phytochemical studies together with *in vivo* biological activity investigations are essential for complete understanding of the medicinal applications.

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