Morus rubra Extract Induces G1 Cell Cycle Arrest and Apoptosis in Human Lung and Prostate Cancer Cells

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ABSTRACT

Objective: Cancer is one of the most deadly types of disease and evasion from apoptosis and unstoppable cell proliferation are accepted as its distinctive features. Many studies have evaluated the cytotoxic effect of different Morus species but, there is no study about cytotoxic effect of Morus rubra. In this study we aimed to evaluate phenolic composition, antioxidant properties and cytotoxic effect of acidified dimethyl sulfoxide extract of M. rubra (AMRE). Method: Antioxidant properties, phenolic composition and cytotoxic effect of AMRE were determined using spectrophotometric methods, HPLC, and MTT assay, respectively. Then, mechanisms of cytotoxic effect of AMRE on human prostate (PC-3) and lung (A549) cancer cells were examined in regard to cell cycle, apoptosis and mitochondrial membrane potential using flow cytometric methods. Results: Total phenolic content and reducing power values were 11.9 mg gallic acid equivalents and 42.9 mg trolox equivalents per g sample, respectively. Ascorbic and gallic acid were detected in AMRE as major antioxidant compounds. We determined that AMRE increased cell cycle arrest at G1 phase and exhibited apoptotic features via decreasing mitochondrial membrane potential in both prostate and lung cancer cells. Conclusion: These findings demonstrate that M. rubra extract can affect the behavior of human prostate and lung cancer cells in vitro conditions, and this effect now needs to be investigated in vivo.

Key words: Apoptosis, Cell Cycle, Cytotoxicity, Lung Neoplasms, Morus rubra, Prostate Neoplasms.

INTRODUCTION

Morus rubra, known as “red mulberry” belongs to the family Moraceae and the genus Morus. Mulberries are unique to temperate regions of Asia, Europe and North America.1,2 In folk medicines, fruits of Morus species are used to treat fever, hypertension, arthritis, anemia, oral and liver diseases. The dark-colored Morus fruits are rich source of phenolic compounds, containing anthocyanins and carotenoids. It has found that mulberry fruit extracts exhibit antioxidant, antimicrobial and anti-inflammatory properties as these activities are due to the above mentioned compounds.3 Cancer is one of the most deadly types of disease and evasion from apoptosis and unstoppable cell proliferation are accepted as its distinctive features.4 Prostate and lung cancer which are the most common cancer types and are the leading cause of cancer related death worldwide.4,5 Chemotherapy is frequently used against these cancers but, the major deficiencies of chemotherapeutic agents are systemic toxicity and drug resistance over time. To solve these
problems, scientific community enter new search for exploration of efficient chemotherapeutics and natural products are accepted as novel candidate for development new chemotherapeutics due their high anticancer activity and less toxicity in normal cells.\textsuperscript{6,7} There are many studies about cytotoxic effect of different berry species. Seeram et al reported that fresh fruit extracts of various berries have cytotoxic effects on human oral, breast, prostate and colon cancer cells,\textsuperscript{8} while Naowaratwattana et al demonstrated that \textit{M. alba} leaf extract inhibits the proliferation of HepG2 cells in a dose-dependent manner by inducing cell cycle arrest at G\textsubscript{2}/M phase, caspase activity and inhibiting topoisomerase II\textalpha{} activity.\textsuperscript{9} Recently, Deepa et al demonstrated that \textit{M. alba} extract induces apoptosis in HCT-15 and MCF-7 cells and downregulation of inducible nitric oxide synthase is suggested as a possible apoptotic mechanism.\textsuperscript{10} However, to the authors’ best knowledge there is no study reporting cytotoxic effect of \textit{M. rubra} in the literature. Therefore, the objective of the current study was to determine chemical composition and antioxidant properties of acidified dimethyl sulfoxide extract of \textit{M. rubra} (AMRE) and farther to search the probable cytotoxic effect in human prostate and lung adenocarcinoma cells coupled with mechanism of action for the first time.

\textbf{MATERIALS AND METHODS}

\textbf{Chemicals and Reagents}

Kaighn’s modification of Ham’s F-12 (F-12K) and Eagle’s minimal essential medium (EMEM) media were purchased from Lonza (Verviers, Belgium). Fetal bovine serum (FBS) was from Biochrom (Berlin, Germany). Penicillin-streptomycin and trypsin-EDTA solution from Biological Industries (Kibbutz Beit Haemek, Israel). All flow cytometric kits (DNA reagent, FITC Annexin V apoptosis detection and mitochondrial membrane potential detection kits) were purchased from BD Biosciences (San Diego, CA, USA). The other principal chemicals used were obtained from Sigma (St. Louis, MO, USA).

\textbf{Plant Collection and Extraction}

Fully mature fruits of \textit{M. rubra} were harvested from Gumushane, Turkey. The fruits were air-dried at room temperature and powdered using blender and milling into fine powder. The fruit powder (1 g) was extracted with 20 mL dimethyl sulfoxide (DMSO) plus 0.5\% hydrochloric acid in a mechanical shaker (Shell Lab, Cornelius, OR, USA) in the dark for 24 h at 45\textdegree{}C. The prepared 50 mg/mL stock AMRE was filtered with Whatman No. 1 filter paper and 0.2 \textmu{}m filter and then stored at \textdegree{}20 until used for further experiments.

\textbf{Drug Preparation and Treatment}

Cisplatin which was used as a reference chemotherapeutic drug for cytotoxicity studies due to its use in prostate and lung cancer treatments\textsuperscript{11,12} and was dissolved in DMSO. External working concentrations of both extract and cisplatin were prepared by further dilution with their solvents. The final concentration of solvents was never higher than 0.5\% in culture media. Under these conditions, solvents, were not toxic for studied cells.

\textbf{Estimation of Total Phenolic Content (TPC)}

Content of total polyphenols of AMRE was established with Folin-Ciocalteau procedure\textsuperscript{13} adapted to microscale using gallic acid as the standard. The results were calculated as mg of gallic acid equivalent (GAE) per g sample using a standard graph.

\textbf{Estimation of Reducing Power}

The reducing power of AMRE was established using the method described previously\textsuperscript{14} adapted to microscale using trolox as the standard. The results were calculated as mg of trolox equivalent (TE) per g sample using a standard graph.

\textbf{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, \textit{p}-coumaric acid, and rutin hydrate. The propylparaben was used as an internal standard.\textsuperscript{15} The analysis was performed by reversed phase HPLC on a Agilent Infinity 1200 (CA, USA) liquid chromatograph equipped with a diode array detector (DAD). The chromatographic separation was performed on a 5 \textmu{}m (150 × 4.6 mm i.d.) reversed-phase C\textsubscript{18} column (Macherey-Nagel, Duren, Germany). The solvent system used was a gradient of solvent A (2\% acetic acid in water), and solvent B (0.5\% acetic acid in acetonitrile:water (1:1)). Flow rate and injection volume were 0.8 mL/min and 10 \mu{}L, respectively.\textsuperscript{16} Signals were detected at 240, 254, 260, 270, 275, 280, 300 and 324 nm by DAD. Identification of compounds was performed comparing retention times and spectral data with those of pure standards. Calibration curves of the standards were used for quantitation.

\textbf{Cell Culture}

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Human prostate (PC-3, CRL-1435), lung (A549, CCL-185) cancer cells and human normal foreskin fibroblast (CRL-2522) cells were purchased from the American Type Culture Collection (Manassas, VA, USA). PC-3 and A549 cells were propagated in F-12K, fibroblast cells were propagated in EMEM, supplemented 10% FBS, 1% penicillin and streptomycin at 37°C with 5% CO₂.

Cytotoxicity Assay
The cytotoxicity of the extract and cisplatin were determined using the MTT assay, which is a tetrazolium salt and is reduced to purple formazan crystals by metabolically active cells. Briefly, the cells were seeded into a 96-well plate (5×10³ cells per well) and treated with several concentrations of AMRE (0-500 μg/mL) and cisplatin (0-10 μg/mL) for 72 h. After the incubation time, 10 μL of MTT dye (0.25 mg/mL) was added to the wells and the composed crystals were then dissolved in DMSO. The absorbance was detected at 570 nm using a microplate reader (Molecular Devices, CA, USA) and optical densities were used to determine % cell viabilities. The antiproliferative potential of AMRE and cisplatin were expressed as IC₅₀ values. Following the cytotoxicity experiments, calculated IC₅₀ and IC₉₀ concentrations of AMRE were used in flow cytometric studies.

Flow Cytometry Analysis for Cell Cycle Distribution
A549 and PC-3 cells in exponential phase of growth were treated with IC₅₀ and IC₉₀ concentrations of AMRE for 72 h, then harvested by trypsinization, and washed 2 times with buffer solution (contains sodium citrate, sucrose, and DMSO). 250 μL of solution A (trypsin buffer) was added to each tube and incubated for 10 min at room temperature. Then, 200 μL of solution B (trypsin inhibitor and RNase buffer) was added to each tube and incubated for 10 min at room temperature. Finally, 200 μL of cold solution C (PI stain solution) was added to each tube and incubated for 10 min in the dark on ice. Data from 30000 cells per sample were collected and analyzed on a flow cytometer (FACSCalibur, Becton Dickinson, East Rutherford, NJ, USA).

Measurement of Apoptosis by Flow Cytometry
A549 and PC-3 cells in exponential phase of growth were treated with IC₅₀ and IC₉₀ concentrations of AMRE for 72 h, then harvested by trypsinization, and washed 2 times with ice-cold phosphate buffered saline (PBS). Then, the cells were resuspended with 100 μL of the binding buffer. 5 μL of FITC Annexin V and 5 μL PI was added to each tube and incubated for 10 min at room temperature in the dark. Finally, 400 μL of the binding buffer was added to each tube and data from 10000 cells per sample were collected and analyzed on a flow cytometer within 1 h.

Measurement of Mitochondrial Transmembrane Potential
Mitochondrial transmembrane potential was evaluated by using JC-1 probe. Mitochondrial transmembrane potential depolarization is specifically indicated by a decrease in the red-to-green fluorescence intensity ratio. Cells were treated with IC₅₀ and IC₉₀ concentrations of AMRE for 72 h, then harvested by trypsinization, and washed 2 times with ice-cold PBS. 500 μL of the freshly prepared JC-1 working solution was added to each tube and incubated for 15 min at 37°C in a CO₂ incubator. Then, cells were washed 2 times with assay buffer and finally, each cell pellet was gently resuspended in 500 μL of assay buffer. Data from 10000 cells per sample were collected and analyzed on a flow cytometer. The results were stated as relative mitochondrial membrane potential compared to untreated cells.

Statistical Analysis
Data are from at least three independent experiments and expressed as means ± SD. Statistical evaluation of the data was performed using ANOVA, followed by post-hoc Tukey’s tests. A p value less than 0.05 was considered significant.

RESULTS
Total polyphenolic content and reducing power values of AMRE were 11.9±0.1 mg GAE and 42.9±1.6 mg TE per g sample, respectively. The chromatogram of standards is shown in Figure 1. The compounds in M. rubra are shown in Table 1 and the values were stated in µg/g sample. Ascorbic acid and gallic acid were the most abundant compounds in
M. rubra (Table 1). The small peak of ascorbic acid in Figure 1 (at 280 nm) is due to its maximum wavelength is 240 nm. 3,4-dihydroxy benzoic acid and protocatechuic acid had same retention time and DAD spectrum in our study. We therefore determined separately their quantities according to their calibration curves.

All cells were treated with various concentrations of AMRE and cisplatin for 72 h and their effects on cell growth were determined by MTT assay. The IC_{50} values for tested compounds are presented in Table 2. AMRE exhibited moderate selective cytotoxicity against both PC-3 and A549 cells compared to foreskin fibroblast cells.

The results of the cell cycle analysis of PC-3 cells are presented in Figure 2A. Both concentrations (360 and 648 µg/mL) of AMRE significantly increased the cell numbers at the G_0/G_1 phase (p=0.001 and p=0.0001, respectively) in PC-3 cells. Besides, both concentrations of AMRE significantly decreased cell numbers at the S phase (p=0.0001). The results of the cell cycle analysis of A549 cells are presented in Figure 2B. Both concentrations (290 and 520 µg/mL) of AMRE significantly increased the number of late apoptotic cells (p=0.001), while only the concentration of 520 µg/mL of AMRE extract significantly increased the number of early apoptotic cells (p=0.043).

Mitochondrial membrane potential analysis results of PC-3 and A549 cells are presented in Figure 4. Only the highest concentrations of AMRE significantly reduced mitochondrial membrane potential in PC-3 and A549 cells (p=0.0001). The percentage reductions in mitochondrial membrane potential by AMRE were 70.9% and 32.1% for concentrations of 648 and 520 µg/mL for PC-3 and A549 cells, respectively.

**DISCUSSION**

Cancer is one of the most important malignant disease and chemotherapy is often used its treatment, but it can cause some side effects, such as increased drug resistance in cancer cells and harmful effect in healthy cells over time. Thus, new approaches are needed to solve these problems, and natural products are accepted possible candidates for exploration of new anticancer agents due their high anticancer activity in cancer cells and less toxicity in normal cells. Plants are excellent source of foods, chemicals and herbal medicines. Many important drugs have been derived from them.

**M. rubra** is one of the most important species of the genus *Morus*, with fruits having substantial levels of

### Table 1: Phenolic compounds identified in acidified *M. rubra* extract by HPLC-DAD

<table>
<thead>
<tr>
<th>Phenolic compounds</th>
<th>Retention time (min)</th>
<th>Amount (µg/g sample)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ascorbic acid</td>
<td>3.99</td>
<td>177.2±7.1</td>
</tr>
<tr>
<td>Gallic acid</td>
<td>6.80</td>
<td>552.8±30.4</td>
</tr>
<tr>
<td>3,4-dihydroxy benzoic acid</td>
<td>10.65</td>
<td>37.1±1.6</td>
</tr>
<tr>
<td>Protocatechuic acid</td>
<td>10.65</td>
<td>52.2±2.5</td>
</tr>
<tr>
<td>Chlorogenic acid</td>
<td>18.95</td>
<td>74.1±3.3</td>
</tr>
<tr>
<td>Caffeic acid</td>
<td>20.56</td>
<td>ND</td>
</tr>
<tr>
<td>Epigallocatechin gallate</td>
<td>22.28</td>
<td>133.7±7.8</td>
</tr>
<tr>
<td>p-coumaric acid</td>
<td>29.00</td>
<td>ND</td>
</tr>
<tr>
<td>Rutin hydrate</td>
<td>27.88</td>
<td>9.0±0.3</td>
</tr>
</tbody>
</table>

**Notes:** Mean value of three determinations (three replicates) ± SD. ND, not detected.

### Table 2: Cytotoxic activity (IC_{50}, µg/mL)* of AMRE and cisplatin (n=4)

<table>
<thead>
<tr>
<th>Test Compounds</th>
<th>Cell Line</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A549</td>
</tr>
<tr>
<td>AMRE</td>
<td>288.5±4.7</td>
</tr>
<tr>
<td>Cisplatin</td>
<td>0.715±0.03</td>
</tr>
</tbody>
</table>

*IC_{50} is defined as the concentration inhibiting 50% of cell growth (viability) after the treatment with AMRE and cisplatin for 72 h, according to the MTT assay.
phenolics, anthocyanins, carotenoids and ascorbic acid. Many studies have investigated the cytotoxic effects of different berry species, however to the authors’ best knowledge there is no study reporting cytotoxic effect of *M. rubra* in the literature. It is advised that *in vitro* experiments are helpful and advisor for determining first biological effect of a natural or syntetic product. If favourable data are attained from these experiments, then clinical studies are advised. Prostate and lung cancer are the most common cancer types worldwide and the PC-3 and A549 cell lines are used frequently as *in vitro* prostate and lung cancer model. We therefore, planned this study on the A549 and PC-3 cancer cell lines under *in vitro* conditions.

Many *in vitro* assay are used for determination of antioxidant activity of herbal extracts and at least using two different methods are recommended. The TPC and FRAP values of AMRE were 11.9±0.1 mg GAE and 42.9±1.6 mg TE per g sample, respectively. The TPC values of different extracts of *M. rubra* fruit...
from Turkey range between 1.6 and 10.4 mg GAE/g sample (reviewed in Ref 27). Dimitrova et al reported that TPC and FRAP values of 70% ethanol extract of *M. rubra* from Bulgaria are 0.9 mg GAE and 1.8 mM TE per g sample, respectively. It is reported that *M. rubra* fruits are rich in phenolic compounds, such as quercetin, kaempferol, rutin, catechin, chlorogenic acid, *p*-coumaric acid, *o*-coumaric acid, vanillic acid, gallic acid, caffeic acid, and caffeoylquinic acid (reviewed in Ref 27). Gundogdu et al reported that *M. rubra* from Van (in Turkey) are rich in gallic acid, catechin, chlorogenic acid, caffeic acid, syringic acid, *p*-coumaric acid, ferulic acid, *o*-coumaric acid, phloridzin, protocatechuic acid, vanillic acid, rutin, and quercetin. Rutin and chlorogenic acid are most abundant phenolics in this study. We determined that seven compounds were in AMRE and gallic acid was the most abundant compound. There was not a fully overlapping between our phenolic composition results and literature data. This situation may have arisen from the plant species and number and kind of used standards. We think that the phenolic composition of *M. rubra* might reveal with further standard compounds in the future investigations. Some criteria, such as high effectiveness against multiple cancer cells and innocuous impacts on healthy cells, are determined for an effective chemopreventive agent. Thus, we carried out cytotoxicity studies in two cancer cells coupled with foreskin fibroblast cells as a normal cell line. We confirmed that only AMRE exhibited reasonable selective toxicity against A549 and PC-3 cells compared to normal fibroblast cells by MTT assay, which is a widely used method in cytotoxicity studies. There are many studies about cytotoxic affects of different berry species in the literature. It is reported that 80% acetone extracts of various raspberry (fresh heritage, kiwigold, goldie, and anne) have cytotoxic effect in human hepatocellular cancer (HepG2) cells, while Seeram et al demonstrated that fresh fruit extracts of various berries have cytotoxic effects on human oral, breast, prostate and colon cancer cells. Especially, black raspberry and strawberry extracts exhibit apoptotic features in colon cancer cells for 48 h treatment. Also, Skupien et al reported that *M. alba* leaf extract has cytotoxic effect both human leukaemic cell line (HL60) and its multidrug resistant sublines. Some researches now focus on the bioactive components of mulberry and concordantly Dat et al reported 11 flavonoids from the methanol extract of *M. alba* leaves, which were cytotoxic to HeLa, MCF-7, and Hep3B cancer cells. Interestingly, synergistic cytotoxic effect of a natural compound may not observed when its purified polyphenolic compounds were used. We therefore think that the mechanism of cytotoxic activity of AMRE may be explained with synergistic effects of all constituents of extract. Mammalian cell proliferation are occured via cell cycle progression, and defects in cell cycle are one of the most common features of cancer cells. Thus, cell cycle arrest is approved one of the target mechanisms for cancer therapy. Nguyen et al showed that bilberry extract inhibits human breast cancer cell proliferation in a dose-dependent manner in concert with induction of apoptotic cell death but no effect is observed on cell cycle in lowest concentrations. However, higher extract concentrations which cause an arrest of cells at the G2/M phase of the cell cycle and modify microtubule organization, while Naowaratwattana et al reported that *M. alba* leaf extract inhibits the proliferation of HepG2 cells in a dose-dependent manner by inducing cell cycle arrest at G2/M phase, caspase activity and inhibiting topoisomerase IIα activity. Recently, Kollar et al reported that prenylated flavonoids from *Morus alba* inhibit the growth of human monocytic leukemia (THP-1) cells through inducing G1 cell cycle arrest and apoptotic pathway. We determined that AMRE induced cell cycle arrest at G0/G1 phase in both A549 and PC-3 cells in a concentration dependent manner. Our results as soon as were similar with previous studies, the differences may have originated from the plant species, type of extraction method and solvent, cell type and cell line specificity. Suppression of apoptosis during carcinogenesis is known to play a role in the development of cancers and induction of apoptosis could be therefore an ideal target for the therapy of many cancer cells. When cells are undergoing apoptosis, phosphatidylserine (PS), which makes up a portion of the cell membrane, will translocate towards 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; therefore, Annexin V staining is considered a marker for early stage apoptosis. Mitochondria are essential organel for both vital (energy production) and lethal (apoptosis) functions in physiological and pathological scenarios and decreased mitochondrial membrane potential could caused to apoptotic cell death in cells. Lee et al demonstrated that morusin which is isolated from root bark of *Morus australis* and inhibits growth of human colon cancer cells via activation of caspases and inhibition of NF-κB pathway, while Faria et al reported that blueberry anthocyanin-pyruvic acid adduct extract exhibits cytotoxic effect on human breast cancer cells via caspase activation. In another study, abalonal A from the root bark induces apoptosis in leukemia cells, whereas
Deepa et al demonstrated that *M. alba* extract induces apoptosis in HCT-15 and MCF-7 cells and downregulation of inducible nitric oxide synthase is suggested as a possible apoptotic mechanism.\(^\text{10}\) Our results show that acidified *M. rubra* extract exhibited apoptotic features on both A549 and PC-3 cells by reducing mitochondrial membrane potential. Studies have suggested that the anticancer characteristics of phenolic compounds derive from pro-oxidant, rather than antioxidant activities. Such pro-oxidant activity is related to the cell's redox status. Higher basal redox levels have been determined in cancer cells than in normal cells as a result of their higher levels of free heavy metal ions and more elevated metabolic rate.\(^\text{10}\) Mounting evidence suggests that increasing oxidative stress by polyphenols in cancer cells may be an effective strategy for eliminating these cancer cells.\(^\text{43}\) Jeong et al reported that mulberry fruit extracts have cytotoxic effect on human glioma cells through reactive oxygen species (ROS) dependent mitochondrial pathway and induction of apoptosis.\(^\text{44}\) We therefore speculate that the antiproliferative effect of *M. rubra* extract in studied cancer cells may derive from its capacity to form ROS due to its polyphenolic components.

### CONCLUSION

This study is the first to investigate the effect of pro-apoptotic and antiproliferative properties of *M. rubra* extract on prostate and lung cancer cells. One limitation of this research is that *in vitro* studies cannot be extrapolated to possible activity *in vivo*. Further studies are now necessary to understand in more detail the exact interaction of the involved signaling pathways.

### ACKNOWLEDGMENTS

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### CONFLICTS OF INTEREST STATEMENT

None of the authors had any financial or personal relationships with other individuals or organizations that might inappropriately influence their work during the submission process.

### REFERENCES


Ibrahim et al.: Cytotoxic Effect of M. rubra on Prostate and Lung Cancer Cells


SUMMARY

- Morus rubra extract has had good antioxidant properties according to total phenolic content and reducing power analysis.
- Seven compounds have been determined in the extract and gallic acid is the most abundant compound.
- Extract has exhibited moderate selective cytotoxic effect against human lung and prostate cancer cells compared to human normal fibroblast cells.
- Extract has increased cell cycle arrest at G1 phase and exhibited apoptotic features via decreasing mitochondrial membrane potential in both prostate and lung cancer cells.

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