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Research Article
Cytotoxicity Effects of Flavonoid Extract of *Morus alba* Leaves in Hela Cell Line

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Abstract

Background and Objective: *Morus alba* leaves is a natural source of many compounds with different biological effects. It has been described to possess anti-inflammatory, antioxidant and cytotoxic activities. The aim of this study was to evaluate the cytotoxicity of total flavonoid extract from *Morus alba* (MTF) leaves and to determine its effect on viability of Hela cells. **Materials and Methods:** The MTT assay was used for quantitative and rapid measurement of Hela cancer cells viability. Apoptotic effect of MTF on Hela cells was also determined via DNA fragmentation analysis by diphenylamine assay and agarose gel electrophoresis. Also in order to determine if cytochrome c involves in apoptosis induced by flavonoid extract of *Morus alba* leaves, quantitative real-time PCR and western blotting were done. All statistical tests were performed using the SPSS statistical software and significant differences ($p<0.05$) were determined by one way ANOVA. **Results:** A strong and dose-dependent inhibition of cancer cell viability after treatment with MTF extract was achieved. DNA fragmentation was significantly observed at a concentration of 1.5 mg mL^{-1} of MTF when compared with controls. Cytochrome C release from mitochondria into cytosol was observed after treatment of Hela cells with MTF indicating apoptosis. **Conclusion:** These results demonstrated that MTF extract has cytotoxic and apoptotic activities on Hela cells and it can be used as an anticancer drug.

Key words: *Morus alba* leaves, MTT assay, DNA fragmentation, diphenylamine, cytochrome C

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

The genus *Morus* belongs to the Moraceae family which consists of 10-16 species that are distributed worldwide. Morus species are used in traditional Chinese medicine frequently[1]. Similar morphological characteristics are found in all species. *Morus alba* is a member of *Morus* genus which is commonly known as white mulberry[2]. It has many biological properties such as anti hyperlipidemic, anti hypertensive, anti hyperglycemic, anti microbial, anti allergic, anti inflammatory hepatoprotective, neuroprotective and also it is an immunomodulator and antivenom[2-5]. It has been known that flavonoids in different fruits and vegetables are responsible for many biological effects. They are polyphenolic compounds that are ubiquitous in nature and are categorized according to their chemical structure into different groups such as flavonols, flavones, flavanones, isoflavones, catechins, anthocyanidins and chalcones[2]. It has been reported that extracts of *Morus alba* leaves (LEM) have anti cancer properties for example they are inhibitor of neuroblastoma cells growth[6]. Likewise, root extract of *Morus* (REM) was also reported to induce programmed cell death in different cancer cell lines such as K562 and B380 human leukemia cells and B16 mouse melanoma cells[7]. The isolated albanol A from REM was also identified to induce the apoptosis in human leukemic HL-60 cells[8]. According to multiple lines of evidences, phenol derivatives of *Morus* spices are prescribed as chemotherapy drug for patients with cancer[9,10]. Fruit, leaf and branch of the white mulberry are rich of polyphenol and flavonoid derivatives. The leaves powder of white mulberry is used traditionally as food supplement in many countries such as China to treat many diseases including diabetes, romatoid and etc[2,11,12]. According to available data, flavonoids can affect different stages of cancer development by protecting DNA from oxidative damage, activating carcinogen metabolism and detoxification, preventing cellular proliferation and inducing cellular apoptosis but the exact mechanism of flavonoids action is poorly understood[2,10,13].

In apoptosis process cytochrome c is crucial for regulation of apoptosis via mitochondrial pathway. Today, the role of cytochrome c in cell death signaling and programmed cell death is fully identified[14]. This protein is located in the space between the inner and outer mitochondrial membranes. The release of cytochrome c from the mitochondria into cytosol where it binds to Apaf-1 in a 2:1 ratio (apoptosome) in the presence of dATP or ATP is triggered by apoptotic stimulus. Then caspase 3 will be activated following activation of caspase 9 via formation of cytochrome c/Apaf-1 complex[15]. This study showed that total flavonoids extracted from *Morus alba* leaves (MTF) induced programmed cell death in Hela cell line. It can be postulated that this natural compound might exert anticancer properties and might further be beneficial for cancer therapy however more studies are required to be done in order to identify its exact function.

MATERIALS AND METHODS

The study was done at Iran University of Medical Sciences in Iran in 2012.

Extraction and purification of flavonoids from *Morus alba* leaves: *Morus alba* leaves were collected from gardens in the North east of Tehran and confirmed by a herbalist and a herbarium code was considered for it. Dried *Morus alba* leaves were used for extraction of total flavonoids. A modified method of Chen[16] was used for extraction and determination of flavonoids. The absorbance of the extracted flavonoids solution was measured at 500 nm with a spectrophotometer (Bio-Varian Model, Cary Eclipse series). The MTF solution was diluted to 1, 1.5, 2 mg mL^{-1} and stored at 4°C before use.

Determination of flavonoids amount: The contents of flavonoids were expressed in mg per g of dry weight by comparing with standard curve. The yield of flavonoids was calculated using the following formula[17]:

$$Y = (6.404A+0.2806)\frac{BV}{W\ (mg\ g^{-1})} \quad (1)$$

Where:

A = Absorbance (500 nm)
B = Dilution factor
W = Dry weight precisely measured (mg g^{-1})
V = Volume of the extracting agent (mL)

Cell culture: The Hela cell line was obtained from National Center of Cell Sciences, Pasteur Institute of Iran (Iran-Tehran). The Hela cell line was cultured at 37°C with 5% CO_2 in RPMI medium containing 10% heat inactivated Fetal Bovine Serum (FBS) and 1% streptomycin. They were harvested and seeded into 96-well plates at a density of 15000 cells per well for further experiments. Cells were counted before and after being seeded in the 96-well plates. To do this, Trypsin-EDTA 0.25% was added to the plates and incubated at 37°C for 1-5 min. The cells were detached from the plates; 1 mL of pre-warmed culture medium was added and then the

cells were transferred to a 50 mL falcon tube and spun down at 1500 g. The cells were stained with trypan blue and counted using neobar lam[18].

Cell viability: MTT assay: Alive cells were quantified by MTT assay, which is based on the cleavage of the tetrazolium salt (MTT) by metabolically active cells to form a purple formazan dye that is water-insoluble. The MTT (3-(4,5 dimethylthiazol-2-yl) 2,5 diphenyl tetrazolium bromide) is cleaved by all living, metabolically active cells such as cancer cells (Hela cell in this study). The generated formazan amount is directly proportional to the homogenous cell population. More formazan was produced by active cells than resting cells. These properties are due to cleavage of MTT only by active mitochondria[18]. The MTT was dissolved in PBS at 5 mg mL^{-1} concentration and filtered to sterilize and remove the small insoluble residue present in some batches of MTT. Cells were seeded in 96-well culture plates (15000 cell per well in 100 µL culture medium, RPMI for Hela cell line) and incubated overnight at 37°C and 5% CO_2. Then cells morphology was observed using invert microscope (Optika-Italy) before exposure to flavonoid extract. After overnight growth, supernatants of culture plates were aspirated out and then 10 µL of flavonoid extract solutions were added in concentrations of 1, 1.5 and 2 mg mL^{-1} in each well of 96-well culture plates. Then 90 µL of culture medium was added to each well. Treated cells were incubated overnight at 37°C and 5% CO_2. Morphology of cells was observed by invert microscope following exposure to different concentrations of flavonoid extract. After overnight incubation, supernatants were aspirated out and 10 µL MTT solution (50 mg/10 mL PBS) was added to each well. Then 90 µL of culture medium (RPMI) was also added to each well and the plates were incubated for 4 h. Supernatants were replaced by 100 µL DMSO and plates were shaken at 37°C in shaker incubator for 15 min. Then absorbance at 570 nm wavelength was recorded using ELISA reader. All absorbance values were corrected against blank wells which contained growth media alone. The viability of the control and treated cells was calculated.

Apoptosis assay: DNA fragmentation: Cytoskeletal disruption, cell shrinkage, membrane blebbing, nuclear condensation and internucleosomal DNA fragmentation are phenotypical characters of apoptosis. Degradation of extracted nuclear DNA in an internucleosomal pattern is a critical pattern of apoptosis. DNA cleavage during apoptosis occurred at sites between nucleosomes at ≈180 bp intervals. Study of cellular responses to chemotherapy and anticancer agents could be possible by quantization of this process[19].

DNA fragmentation assay, diphenylamine method: Degraded DNA could be quantified by diphenylamine assay. Apoptosis evaluation was done by measurement of DNA fragmentation with the DPA colorimetric assay[19-21]. Optical density was read at 600 nm with a spectrophotometer reader (Bio-Varian Model, Cary Eclipse series), setting blank to 0. In this study, 3×10^5 cells were cultured in each well of the 96 well plates and treated with MTF extract in concentration of 1, 1.5 and 2 mg mL^{-1} for 48 h at 37°C.

DNA fragmentation assay, agarose gel electrophoresis: Hela cells were treated with 1, 1.5, 2 mg mL^{-1} of MTF for 72 h without any medium exchange. DNA was purified from treated and untreated Hela cells (control), using a DNA extraction kit (Primeprep Genomic DNA isolation kit, Fermentase Company) suitable for high molecular weight DNA. Then 4 µg of purified DNA of each sample was electrophoresed using a 2% agarose gel and stained with ethidium bromide.

Apoptosis assay: Cytochrome C assay: The K-ASSAY Ò cytochrome C (Cyt-c) releasing apoptosis assay kit (Cat. No. KT-147) provides an effective means for detecting cytochrome c translocation from mitochondria into cytosol during apoptosis. Cytochrome c releasing from mitochondria into cytosol is then determined by western blotting, using the cytochrome c antibody provided in the kit. According to kit producer the cytosolic and mitochondrial fractions of control and treated 5×10^7 HeLa cell line with MTF for 72 h are isolated as bellow.

5×10^7 cells were collected by centrifugation at 600×g for 5 min at 4°C. The cells were washed with 10 mL of ice-cold PBS and centrifuged at 600×g for 5 min at 4°C. The supernatant was removed. The cells were resuspended in 1.0 mL of 1X Cytosol Extraction Buffer Mix containing DTT and Protease Inhibitors and incubated on ice for 10 min. The cells were homogenized in an ice-cold Dounce tissue grinder for 30-50 passes.

The homogenate was transferred to a 1.5 mL microcentrifuge tube and centrifuged at 700 g for 10 min at 4°C. The supernatant was collected into a fresh 1.5 mL tube and centrifuged at 10,000×g for 30 min at 4°C. The supernatant was collected as cytosolic fraction. The pellet was resuspended in 0.1 mL mitochondrial extraction buffer mix containing DTT and protease inhibitors, vortex for 10 sec and saved as mitochondrial fraction. Protein concentrations were determined, using Bradford[22] method. About 10 µg of the cytosolic and mitochondrial fractions were subjected to 10% SDS polyacrylamide gel electrophoresis and then electro

transferred to polyvinylidene difluoride (PVDF) membrane for western blot analysis. For detection, first an anti-cytochrome c antibody, a mouse monoclonal antibody that reacts with denatured human, mouse and rat cytochrome C, was used and then horseradish peroxidase-labeled secondary antibody was added. After addition the enzyme substrate, the blots were developed in a chemiluminescence system (Pierce) and visualized by exposure to Kodak X-ray film. Densitometry was used in order to determine band density by using image J software.

RNA isolation, cDNA synthesis and quantitative real-time PCR: Total RNA of cytosolic and mitochondrial fractions of control and treated Hela cells was isolated according to the manufacturer's instruction of TRIZOL reagent (Invitrogen). Real-time PCR was performed using an ABI apparatus (Applied Biosystems, USA) according to the manufacturer's instructions of TRIZOL reagent (Invitrogen). The RNA concentrations were determined using a Nanodrop (ND-1000) spectrophotometer. cDNA was synthesized from 1 μg of total RNA using the QuantiTect Rev. Transcriptase kit (Qiagen) in a total volume of 20 μL and analyzed by QuntiFast SYBR Green PCR Kit (Qiagen). Each real-time PCR consisted of 1 μL cDNA, 10 μL SYBR Green PCR Master Mix and 1 μM forward and reverse primers (AnasPec). The specific primers were used as follows: cytochrome c, forward, 5'-TAAATATGAGGGTGTCGC-3' and reverse, 5'-AAGAATAGTTCCGTCCTG-3'; the normalizing control β-actin, forward, 5'-TGACAGGATGCAGAAGGAGA-3' and reverse, 5'-TAGAGCCACCAATCCACACA-3'). Reactions were carried out on a Rotor Gene 6000 System (Corbett, Australia) for 40 cycles (95°C for 20 sec, 62°C for 20 sec and 72°C for 20 sec) after initial 5 min incubation at 95°C. L19 (Forward 5'-GCGGAAGGGTACAGCCAAT-3' and reverse 5'-GCAGCCGGCGCAAA-3'), a non regulated ribosomal housekeeping gene, served as an internal control and was used to normalize for differences in input RNA. All measurements were performed in duplicate. The differences in the threshold cycle (CT) values of the target gene with the corresponding internal control L19 gene were calculated (ΔCT = CT gene - CT L19). The relative expression level of target gene to L19 was described using the equation 2-ΔCT[22].

Statistical analysis: All statistical tests were performed using the SPSS statistical software version 11.0 for Windows (SPSS Inc., Chicago, USA). The results are presented as means±standard deviation of the mean (SD). Significant differences were determined by one way ANOVA, followed by Bonferroni correction for multiple comparisons. p-value of less than 0.05 was considered as statistically significant[23].

RESULTS

The effect of flavonoids on cell growth: The flavonoids content was 246 mg g^{-1} in dried MTF powder after purification by NKA-9 macroporous resins. Cultivated cancer cell line (HeLa cell) was incubated with different concentrations (1, 1.5, 2 mg mL^{-1}) of the extract for 48 h and cell growth inhibition was determined using MTT assay. A strong and dose-dependent inhibition of cancer cell growth has been observed following treatment with MTF extract. This extract caused a significant decrease (p-value = 0.001) in viability or proliferation of HeLa cell line comparing to the untreated cells (IC_{50} = 2 mg mL^{-1}); cellular viability of the treated HeLa cell was reduced to 47.43% at a concentration of 2 mg mL^{-1} of MTF in comparison to the controls (Table 1).

Flavonoids induced DNA fragmentation in Hela cells: Diphenylamine method is a useful approach for investigating apoptosis by which DNA fragmentation to oligonucleosome segments could be detected. According to our results (Table 2) DNA fragmentation was observed after treatment of HeLa cell with MTF at concentrations of 1, 1.5 and 2 mg mL^{-1} for 48 h. The percentage of DNA fragmentation was elevated significantly by increasing the MTF concentration in comparison to the control (p-value = 0.001). It is worthy to point out that maximum DNA fragmentation percentage was significantly achieved at a concentration of 1.5 mg mL^{-1} of MTF when compared with controls. To confirm our results gel electrophoresis was also used for detection of DNA fragmentation. Agarose gel electrophoresis analysis of inter nucleosomal DNA fragmentation after treatment of Hela cells with different concentrations of MTF for 72 h without any cell culture medium exchange are shown in Fig. 1. The results

Table 1: Effect of different concentration of MTF on Hela cell viability compared to the control (untreated Hela cell)

Groups	Viability (%)	p-value
Control	100.00±0.000	0.001
1 (mg mL^{-1})	90.63±1.020	
1.5 (mg mL^{-1})	72.63±10.84	
2 (mg mL^{-1})	47.43±10.31	

Values are Means±SD

Table 2: Effect of different concentration of MTF on DNA fragmentation of Hela cell compared to the control (untreated Hela cell) using diphenylamine method

Groups	DNA fragmentation (%)	p-value
Control	25.40±1.80	0.001
1 (mg mL^{-1})	40.96±8.14	
1.5 (mg mL^{-1})	51.66±2.57	
2 (mg mL^{-1})	44.53±2.77	

Values are Means±SD

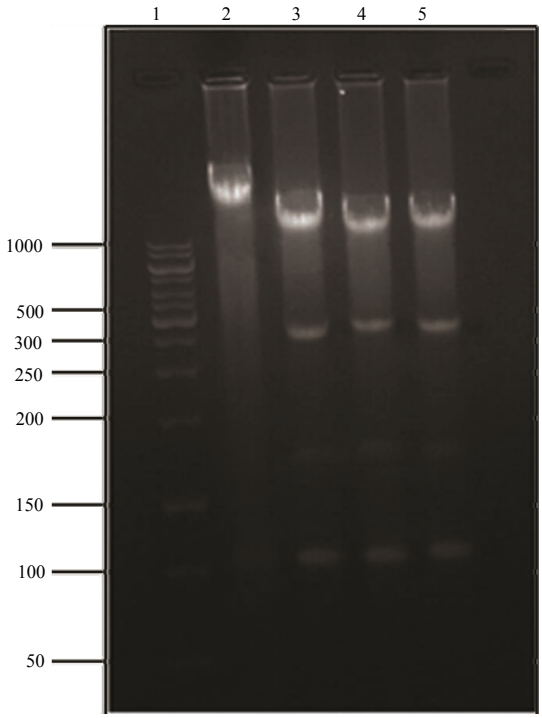

Fig. 1: Effect of different concentration of MTF on DNA fragmentation of Hela cell line using 2% agarose gel

Lanes 1 and 2: DNA ladder (shown in bp) and untreated Hela cells, respectively. Lanes 3-5: Treated Hela cells with concentrations of 2 mg mL^{-1} (Lane 3), 1.5 mg mL^{-1} (Lane 4) and 1 mg mL^{-1} (Lane 5) of MTF for 48 h

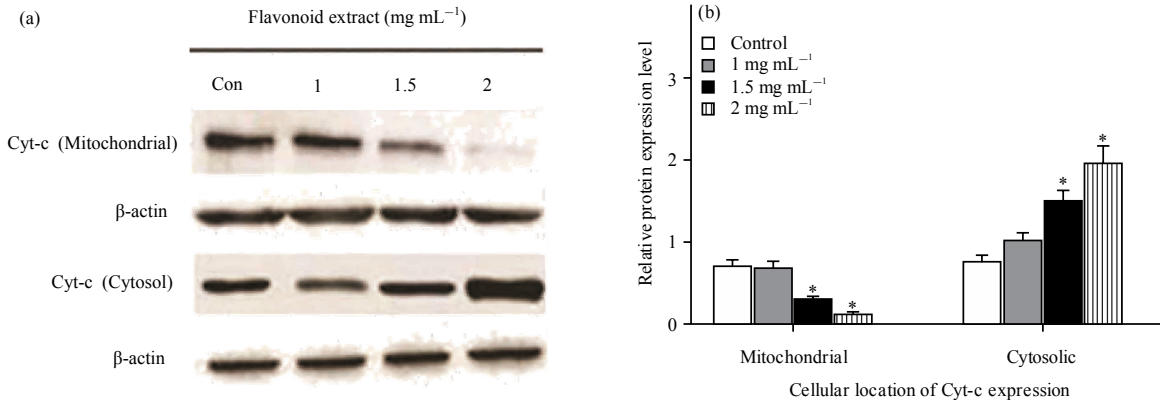

Fig. 2(a-b): Comparison between mitochondrial and cytosolic cyt-c levels of untreated and treated Hela cell with 1, 1.5 and 2 mg mL^{-1} of flavonoid extract ($p<0.05$), (a) Western blot analysis of Cyt-c level in mitochondrial and cytosolic fraction of Hela cells, (b) mRNA levels of Cyt-c in mitochondrial and cytosolic fraction of Hela cells

β-actin was used for normalization and loading as control. Values are means±SD. p-value less than 0.05 marked with asterisks, *Statistically significant

demonstrated the percentage of fragmented DNA of Hela cells following 72 h treatment with concentrations of 2 (lane 3), 1.5 (lane 4) and 1 (lane 5) mg mL^{-1} of MTF when compared to untreated cells (lane 2). As it is obvious, lane 2 shows the lake of DNA fragmentation in untreated cells but lanes 3-5 show DNA fragmentation caused by MTF at different concentrations.

Effect of flavonoids on protein and mRNA level of cytochrome C: Western blot data (Fig. 2a) revealed that the significant decrease in the level of Cyt-c in mitochondrial fractions and increase in the level of cytosolic fractions of the treated Hela cells with various concentration of MTF were observed ($p<0.05$). The mitochondrial and cytosolic fractions of cytochrome c at mRNA levels in treated Hela cells with

different concentrations of MTF and control cells were also investigated. In the presence of different concentrations of MTF, a significant reduction and elevation ($p<0.05$) was found for mitochondrial and cytosolic cytochrome C mRNA levels, respectively as shown in Fig. 2b.

DISCUSSION

Consumption of polyphenol compounds was suggested to correlate with a lower cancer risk. This study was designed to evaluate the cytotoxic effect of flavonoid extract, which was isolated from white mulberry leaves on the viability of Hela cells line. In addition, the goal of present study was to determine the apoptotic statues of Hela cells in response to administration of different concentrations of MTF comparing to untreated Hela cells. As it is obvious from our results, a strong and dose-dependent inhibition of Hela cell viability was found in response to MTF treatment. The MTF extract caused a significant decrease in viability of Hela cell line (IC_{50} = 2 mg mL^{-1}, p-value = 0.001). The most reduction in viability was found at the concentration of 2 mg mL^{-1} of MTF after 48 h in comparison to the control. Similarly, MTF may have effect on viabilities of different types of cancer cells. Consistently, the results of a study which was conducted by Choi *et al.*[10] showed that REM (Root extract from the mulberry) reduces the viability of doxorubicin-resistant MCF-7/Dox cells highly expressing MDR1 (multi drug resistance gene). They have suggested that REM might be useful for treatment of multidrug-resistant cancer cells. On the other hand, many reports indicated that flavonoids protect various cell types from oxidative stress[2].

The cytotoxic effect of three different flavonoids that were isolated from MA (kuwanon E, cudraflavone B and 4-O-methylkuwanon E) on proliferation of THP-1 cells and cell cycle progression of cancer cells were determined by Kollar *et al.*[24]. The cytotoxicity of many flavonoids including apigenin, eriodictyol, 3-hydroxyflavone, kaempferol, luteolin, naringenin, quercetin, rutin and taxifolin, toward cultured human normal cells, i.e., human lung embryonic fibroblasts (TIG-1) and Human Umbilical Vein Endothelial (HUVE) cells was also examined by Matsuo *et al.*[25]. Their results showed that flavonoids exert beneficial effects on human normal cells at relatively low concentrations, however toxic effects were observed at relatively higher concentrations. They suggested that flavonoids are incorporated into cells, increase intracellular ROS levels and then exert cytotoxicity. The structure–cytotoxicity relationship between flavonoids structure and cytotoxicity in normal human cells is unclear and their cytotoxic effects differ depending on the cell type. Flavonoids are recognized to act as prooxidants as well as antioxidants and their antioxidant or prooxidant properties may depend on the flavonoid concentrations, cell type and/or culture conditions. Also their cytotoxicity might be related to both their incorporation efficiency and intracellular ROS-generating ability[25]. Significant time and dose-dependant effects of flavonoids on cytotoxicity of cancer cell lines, such as HMECs (human mammary epithelial cells) were also reported[26]. Maximum cell death was reported at 72 h after initial treatment (p-value = 0.001). Flavonoids have been also shown to induce apoptosis in some cancer cell lines, while their molecular mechanism is not fully understood. Cytoskeletal disruption, cell shrinkage, membrane blabbing, nuclear condensation and internucleosomal DNA fragmentation are main phenotypical properties of apoptosis[14,19]. Based on the results of the present study, DNA degradation was observed for DNA of treated Hela cells, which is a hallmark of apoptosis. Apoptosis induction in Hela cells by different concentrations of MTF was detected by both diphenylamine and gel electrophoresis analysis. The apoptosis was suggested to occur for treated Hela cells with different concentration of MTF according to Fig. 1. As it is shown, DNA fragmentation pattern of the treated Hela cells did not change with increasing the MTF concentration. Consistently with our results, root extract of *Morus alba* (REM) caused apoptotic cell death in different types of cancer cells such as K562 and B380 human leukemia cells and B16 mouse melanoma cells which was reported by Nam *et al.*[7]. Degradation of nuclear DNA into nucleosomal units is indicated as one of the hallmarks of apoptotic cell death, which will take place in response to various apoptotic stimuli in various cell types[27]. Probable changes in gene and protein expression of cytosolic and mitochondrial fractions of cytochrome C in treated and control Hela cells were also analyzed in this study. As it was found in results, a significant difference ($p<0.05$) was identified for Cyt-c protein and gene expression levels between mitochondrial and cytosolic fractions of treated and control Hela cells. It is suggested that numerous physiological and molecular changes might be occurred during treatment of Hela cells with different concentration of MTF. The significant alteration ($p<0.05$) in Cyt-c protein and gene expression levels of cytosolic and mitochondrial fractions in treated Hela cells indicates that mitochondria might be involve in induction of apoptosis toward application of MTF. The study of dose related gene expression of Cyt-c is critical for a better understanding of its molecular and physiological changes during treatment. In previous study by Wang *et al.*[28], the mechanism of flavonoid-induced apoptosis in HL-60 leukaem was investigated. Consistently with our results, they have suggested that flavonoid-induced apoptosis is stimulated by the release of cytochrome c to the cytosol, by procaspase-9

processing and through a caspase-3-dependent mechanism. The induction of apoptosis by flavonoids was attributed to their cancer chemopreventive activity[27,28].

Wang *et al.*[28] showed that treatment of HL-60 leukaemic cells with flavonoids (such as apigenin, quercetin, myricetin and kaempferol) caused a rapid induction of caspase-3 activity and stimulated proteolytic cleavage of poly-(ADP-ribose) polymerase (PARP). Furthermore, they also showed that these flavonoids induced loss of mitochondrial transmembrane potential, elevation of Reactive Oxygen Species (ROS) production, release of mitochondrial cytochrome C into the cytosol and subsequent induction of procaspase-9 processing. Furthermore, the potency of flavonoids for inducing apoptosis was suggested to be dependent on the numbers of hydroxyl groups in the 2-phenyl group and on the absence of the3-hydroxyl group.

Taken together, the results of present study suggest that induction of apoptosis by MTF may provide a probable pivotal mechanism for their cancer preventive function. Although several molecules and pathways have been proposed as targets of flavonoids, the precise mechanism by which these compounds exert their cancer-protective effects are poorly understood and more studies are needed to clearly understand the mechanisms of action of flavonoids as modulators of cell apoptosis, which is crucial for the evaluation of their potential effects as anticancer agents.

CONCLUSION

The present data indicates that flavonoids extracted from *Morus alba* leaves have cytotoxicity effect in Hela cells. Also apoptosis occurred after treatment of the Hela cells with extracted flavonoids as DNA fragmentation confirmed it. Furthermore, apoptosis was stimulated by the release of cytochrome c from the mitochondria to the cytosol.

SIGNIFICANCE STATEMENT

This study discovers the cytotoxic and apoptotic activities of flavonoid extract of *Morus alba* leaves on Hela cells that can be beneficial for further use in chemotherapy. This study will help the researcher to uncover the critical area of cancer treatment with natural drugs.

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