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Anticancer activity of *Morus nigra* on human breast cancer cell line (MCF-7): the role of fresh and dry fruit extracts

Ahmed A.[1], Ali M. [1], El-Kholie E. [1], El-Garawani I.[2]* and Sherif N. [1]

[1]Department of Nutrition and Food Science, Faculty of Home Economics,Menoufia University, Menoufia, Egypt
[2*]Department of Zoology, Faculty of Science, Menoufia University, Menoufia, Egypt.

(Corresponding author-email: dr.garawani@yahoo.com)

Abstract

Black mulberry (*Morus nigra* L.) has a long history of medicinal use in folk medicine. In this study, in vitro anticancer properties of fresh and dry black mulberry (BM) ethanolic extracts on breast adenocarcinoma cell line (MCF-7) were investigated. Cytotoxic effect of both extracts was investigated by neutral red uptake method and the IC_{50} was assessed. The morphological changes, apoptosis and/or necrosis, were detected by Giemsa staining and acridine orange/ethidium bromide dual fluorescent staining methods, in addition to DNA fragmentation assay by gel electrophoresis and comet assay. Mitotic index was evaluated in MCF-7 groups to investigate the antiproliferative potential of fresh and dry BM extracts. Furthermore, micronucleus and other nuclear abnormalities were also investigated. BM extracts administration gave significant ($p < 0.05$) morphological

evidence of apoptosis and increased cell death after 48 hours more than that of 24 hours-treated MCF-7 cells among fresh and dry extract groups. DNA analysis after 24 h of BM extracts treated groups has no fragmentation while apoptosis is generated after 48h showing more fragmentation in fresh BM groups. The mitotic index records were significantly decreased in dose and time dependent manner showing the better antiproliferative effect with fresh extract treatments. DNA single strand breaks were also increased among the treatment groups at dose and time dependent manner with the best results with fresh extract. It can be concluded that black mulberry fruit is a considerable source of health dietary supplement and its integration for pharmaceutical industries has to be in concern.

Keywords: *Morus nigra*, MCF-7, Apoptosis, Micronucleus test, Comet assay and Mitotic index.

1. Introduction

Black mulberry (*Morus nigra* L.), belonging to the Moraceae family, native to southwestern Asia. It has a long history of Chinese folk medical use (Pawlowska et al., 2008 and Kutluet al., 2009). Black mulberry (BM) is one of the medicinal plants that have a great value in many treatments. Mulberry fruits as deep-colored fruits are good sources of phenolics, including anthocyanins and other flavonoids, and carotenoids (Kutluet al., 2009 and Song et al., 2009). Anthocyanin pigment molecules are a subclass of flavonoids. They are responsible for the red, purple, and blue colors observed in many plants (Nikkhah et al., 2010 and Kostic et al., 2013). Much progress has been made in identifying possible causes of cancer (Kirkegaard et al., 2010). Recently, an attempt to overcome these carcinogenic agents using natural materials has paid a great concern. Breast cancer is one of the most common malignancies all over the world. Human breast adenocarcinoma (MCF-7) cell line has become a prominent model system for the study of breast cancer as it relates to the susceptibility of the cells to apoptosis. Anthocyanins are thought to contribute to the nutritive value of fruits and berries due to their antioxidative, anti-carcinogenic, anti-inflammatory, and anti-angiogenic properties (Igarashi and Inagaki, 1991). Anthocyanins, the main active ingredient of mulberry,

could decrease the in vitro invasiveness of cancer cells (Chen et al., 2006). It could inhibit the growth and metastasis of B16-F1 cells (Huang et al., 2008), induced apoptotic death in gastric carcinoma cells (Huang et al., 2011). Anthocyanins and phenolic acids have antiproliferative effect on various cell lines including MCF7 cells (Heredia et al., 2015).The present work investigated , in vitro, anticancer properties of fresh and dry black mulberry (BM) ethanolic extracts on breast adenocarcinoma cell line (MCF-7).

2. Materials and Methods

2.1. Plant materials

Black mulberry fruits were obtained from local markets, Shanawan, Menuofia Governorate, Egypt. BM fruits were identified by a taxonomist, Faculty of Agriculture, Menufia University, Egypt.

2.2. Preparation of black mulberry fruits ethanolic extract

Both fresh and dried fruits (50g of each) were extracted with 10x (w/v) of 70% ethanol. The mixture was filtered, evaporated in rotary evaporator (Buchi, France) until the constant weight was reached. The obtained ethanolic extracts of mulberry fruits was kept at -20℃ until used.

2.3. In vitro anticancer study on MCF-7

2.3.1. Cells maintenance

Human breast cancer cell line (MCF-7) was purchased from (VACSRA), Giza, Egypt. Cells were maintained in complete growth medium [RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin (100 U/ml)/streptomycin (100 µg/ml)]. 5x 10^5 cells were grown in each T25 culture flask containing 7ml of complete growth medium to reach 70% confluency at 37°C, and then the treatments were added in triplicates. All culture reagents were obtained from (Lonza) supplier, Egypt.

2.3.2. Determination of tumor cells viability and counts

The visualization of MCF-7 cells viability was done according to trypan blue staining method (Berry et al., 1991). The concentration of cells per ml was determined using haemocytometer and they were calculated using the following calculations:

Cells / ml = 10^4 x (Average count per square) x (Dilution factor).

2.3.3. Study design

MCF-7 cultured flasks were divided into five groups as follow: **Group I**: untreated cells; **Group II**: Fresh black mulberry extract (FBME) (100 µg/ml); **Group III**: FBME (300 µg/ml); **Group IV**: Dried black mulberry extract (DBME) (100 µg/ml) & **Group V**: DBME (300 µg/ml).

2.3.4. Neutral red uptake assay

The assay was performed according to the protocol described by Siddiqui et al. (2010). Post treatment, cells were washed with (PBS; 0.01 M; pH 7.4), and 40 µg/ml of neutral red containing medium were added. After 3 h of incubation, the supernatant was removed, and the cells were washed with a solution of 0.5% formaldehyde and 1% calcium chloride. Subsequently, a solution of 1% acetic acid and 50% ethanol was added, and the dye was extracted for 10 min. The absorbance was then read at a wavelength of 540 nm. Cytotoxicity was calculated and IC_{50} was assessed. The experiment was conducted in triplicate.

2.3.5. Giemsa staining

Cells were smeared on a glass slide and air-dried. The smears were fixed in (3 parts methanol: 1 part glacial acetic acid) for 5 min. Cells were washed with PBS for 1 min then stained in Geimsa solution for 15 minutes and washed with PBS. Two hundred cells were examined (400 x) using light microscope (Olympus BX 41, Japan) and digitally photographed (Thippeswamy and Salimath, 2006).

2.3.6. Acridine orange/ ethidium bromide dual fluorescent staining

Cells from the control and treated groups were smeared on a glass slide and air-dried. Smears were fixed by freshly-prepared 3:1 (v/v) methanol : glacial acetic acid for 10 minutes then they were hydrated with PBS for 1 minute and stained with a mixture (1:1) of acridine orange (50 µg/ml)/ethidium bromide (5 µg/ml) solutions for 5 minutes. Cells were immediately washed with PBS and examination was done using fluorescent microscope (Olympus BX 41, Japan). Two hundred of cells per group were evaluated (400x) and the damaged (apoptotic and necrotic) cells were recorded according to the affinity and pattern of nuclear fluorescent staining, and then representative photos were digitally photographed (El-Garawani., 2015).

2.3.7. Micronucleus test

Cells were grown on cover slips and the treatments were applied after reaching 70% confluency. After the desired treatment period, cells were fixed in 95% methanol for 5 min then stained with 5% (Geimsa / May-Grünwald) for 10 min. Cells were washed twice with PBS then 300 cells were examined in each sample for micronucleus, binucleated and nuclear budding evidence according to the criteria of Tolbert et al. (1992) using bright field microscope (Olympus BX 41, Japan) and representative photos were digitally captured.

2.3.8. Total genomic DNA extraction and apoptosis detection

DNA extraction and detection of apoptosis (DNA fragmentation assay) were done according to "salting out extraction method" of Aljanabi and Martinez (1997) with some modifications by Hassab El-Nabi and Elhassaneen (2008). Cells were incubated in lysing buffer two hours at 37°C then; proteins were precipitated using 4M NaCl. The resultant supernatant was transferred to a new tube then DNA was precipitated using cold isopropanol. The pellets of nucleic acids were washed with 70% ethyl alcohol. The pellets were resuspended in TE buffer and were incubated with loading mix (0.1% RNase + loading buffer), and then loaded directly into 1.8% normal melting electrophoretic grade agarose in 1X Tris borate EDTA buffer. The apoptotic bands of DNA fragmentation appeared and located at 180 bp and its multiples 360, 540 and 720 bp against thirteen bands of DNA marker (100–3000 bp). The intensity of DNA apoptotic bands were measured by (ImageJ software) as a maximum optical density values.

2.3.9. Comet assay

Alkaline single-cell gel electrophoresis (comet assay) was performed according to Singh et al. (1988). Briefly, cells were embedded into low melting point agarose gels on microscope slides and immersed in lysis buffer (2.5 M NaCl, 100 mM EDTA and 10 mM Tris, pH 10.0) with freshly added 1% Triton X-100 (Sigma) and 10% DMSO for 1 h at 4 °C. Subsequently, the slides were incubated in freshly made alkaline buffer (300 mM NaOH and 1 mM EDTA, pH > 13) for 20 min at 4 °C. An electric current of 300 mA and 25 V was applied for 15 min. The slides were then neutralized (0.4 M Tris, pH 7.5), Then they were stained with ethidium bromide. Cells were visualized using fluorescence microscope (Olympus BX 41, Japan), and representative images were photographed. More than 100 randomly selected cells were examined for severity of DNA damage as comet fragmentation length. The results were divided as normal with no tail, damage with migrated tail not more than the diameter of the nucleus and strong damage with no distinct nucleus.

2.3.10. Chromosomal preparation and mitotic index assessment

For the analysis of MCF-7 chromosomes, colcemid (10µg/ml) was added to the cells and left 2 hours, then cells were harvested and resuspended in hypotonic solution (0.56% KCl) at 37 °C for 20 minutes, fixed with freshly-prepared 3:1 (v/v) methanol : glacial acetic acid. Fixed cells were resuspended and they were dropped onto glass slides and left to air-dry. The slides were stained with 3% (w/v) Giemsa in phosphate buffer (Evans, 1987). Cells were examined (400x) under light microscope (Olympus BX 41, Japan) then the representative photomicrographs were captured (100x) using digital camera. The frequency of mitotic index was estimated in 500 cells for each group according to the following formula:

Mitotic index (MI) = (Number of dividing cells/ Total examined cells) x100.

2.5. Statistical analysis

Data were presented as mean ± standard error (M ± SE). Comparisons were made between the untreated and treated groups were statistically analyzed using Duncan's Multiple Range Test, ($p < 0.05$) were considered positive.

3. Results

3.1. Evaluation of cytotoxicity using neutral red uptake assay

The inhibition of MCF-7 cells growth rates was observed after both extracts administration as more effectiveness of fresh extract than dry one in dose dependent manner with no effect of treatments exposure time. The recorded IC_{50} were 575±15 and 900±25µg/ml for fresh and dry extracts respectively after 24h and 48h.

3.2. Morphological changes after Giemsa staining method in MCF-7 cells treated with black mulberry extracts

The results of morphological changes revealed that fresh black mulberry extract was significantly ($p<0.05$) exerted antitumor activities on MCF-7 cells than that of dried black mulberry extract after 48 hours while a little antitumor effect was observed among two types of extracts after 24 hours. Cytological analysis of all treated groups of MCF-7 cells using Giemsa staining method indicated typical apoptotic morphology of membrane blebbing when compared to the untreated cells Figure (1) and Table (1).

Table (1): Morphological changes in MCF-7 cells after Giemsa staining for treated and untreated groups.

	24h		48h	
	%Dead	**%Apoptotic**	**%Dead**	**%Apoptotic**
Control	5±0.4	0.6±0.5	6.1±1.7	1.0±0.9
100 µg/ml(FBME)	32±3.0*	5.6±1.0*	15.0±2.0*	40.0±2.5*
300 µg/ml(FBME)	49±4.1*	6.3±1.0*	18.0±1.0*	37.6±1.2*
100 µg/ml(DBME)	19±4*	15.0±0.9*	16.3±2.1*	21.7±2.0*
300 µg/ml (DBME)	28±1.0*	13.0±1.7*	20.3±2.0*	29±1.0*

* Significant difference at ($p< 0.05$) compared with control group. **FBME**: fresh black mulberry extract and **DBME**: dry black mulberry extract.

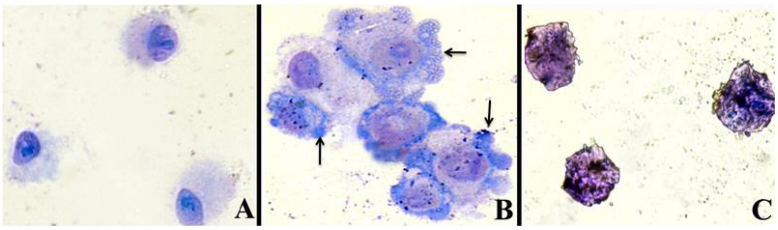

Figure (1): representative photomicrographs of Giemsa- stained MCF-7 cells showing morphological changes under light microscope (400 x). Where (A): normal control cells, (B): blebbing cytoplasm as a feature of apoptosis (arrows) and (C): dead cells.

3.3. Morphological changes after acridine orange/ ethidium bromide fluorescent staining

The formation of apoptotic bodies and nuclear condensation with bright orange color which are the characteristic features of apoptosis after acridine orange/ ethidium bromide staining was significantly (p<0.05) observed among all treated groups of MCF-7 cells with dose and time dependant values as shown in Figure (2) and Table (2). While the untreated control cells showed intact nuclear architecture.

Table (2): Morphological changes in MCF-7 cells after acridine orange/ ethidium bromide staining for treated and untreated groups.

	24h		48h	
	%Dead	**%Apoptotic**	**%Dead**	**%Apoptotic**
Control	4.0±1.0	0.3±0.1	5.3±1.5	2.0±1.0
100 µg/ml(FBME)	61.0±1.0*	0.2±0.3	46.0±2.0*	27.7±1.5*
300 µg/ml(FBME)	70.6±1.5*	0.5±0.3*	39.7±2.1*	32.3±2.5*
100 µg/ml(DBME)	32.4±2.1*	0.7±0.5*	31.6±1.5*	6.0±1.0*
300 µg/ml (DBME)	64.9±2.6*	0.3±0.2	55.6±3.1*	7.6±1.2*

* Significant difference at (p < 0.05) compared with control group. **FBME**: fresh black mulberry extract and **DBME**: dry black mulberry extract.

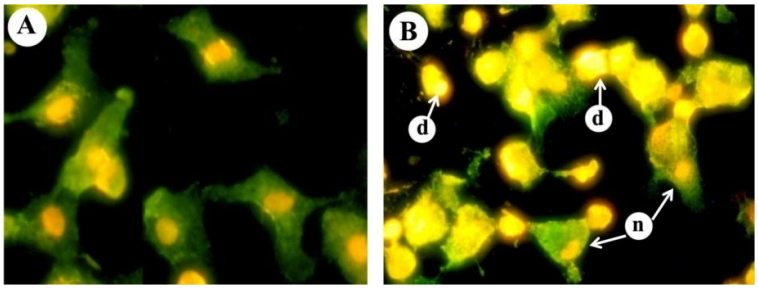

Figure (2): Representative photomicrograph for MCF-7 cells nuclear morphology after acridine orange / ethidium bromide fluorescent staining, (400x). Where (A): normal control cells, (B): showing dead cells with high intensity of fluorescence, where d: dead and n: normal.

3.4. Micronucleus and other nuclear abnormalities assay

The frequency of micronucleus, nuclear budding and binucleated cells induction in MCF-7 cells after BM extracts treatment was significantly (p<0.05) elevated among all treated groups at dose and time dependant manner (Table 3). Among the abnormalities observed budding nuclei were the most frequent after 24h. While after 48h of treatment exposure, binucleated cells were the most frequent (Figure 3).

Table (3): Micronucleus and other nuclear abnormalities in MCF-7 cells for treated and untreated groups.

	24h				48h			
	% Bud	**% MN**	**% Bn**	**%Total**	**% Bud**	**% MN**	**% Bn**	**% Total**
Control	3.2±0.47	0.33±0.47	4.1±1.2	7.2±1.6	1.9±0.77	0.6±0.94	3.8±1.3	7.2±1.3
100µg/ml (FBME)	9.1±1.4*	0.9±0.6	2.9±0.9	12.9±2.3*	5.8±1.1*	6.7±1.9*	6.1±1.8	18.6±1.1*
300µg/ml (FBME)	6.9±1.6*	-	2.4±0.2	9.9±1.5*	12.1±1.2*	4.0±0.6*	11.2±2.6	27.3±1.4*
100µg/ml (DBME)	3.2±0.47	1.2±0.42*	3.8±1.3	12.9±3.8	3.0±0.01*	-	3.6±1.3	12.7±1.8
300µg/ml (DBME)	3.6±1.2	2.2±1.1*	2.9±0.8*	9.9±0.8	4.3±2.3	1.2±0.4*	8.0±2.1*	9.9±0.8*

* Significant difference at (p < 0.05) compared with control group. **FBME**: fresh black mulberry extract and **DBME**: dry black mulberry extract.

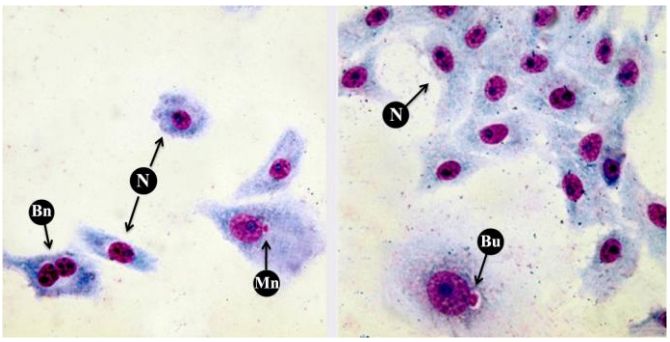

Figure (3): Representative photomicrograph for MCF-7 cells micronucleus, nuclear budding and binucleated occurrence after Geimsa / May-Grünwald staining, (400x). Where (N): normal cells, (Bn): binucleated cell, (Bu): nuclear budding and (Mn): micronucleus.

3.5. Total genomic DNA fragmentation in MCF-7 cells treated with black mulberry extracts

DNA from control MCF-7 cells was found to be intact (undamaged) as seen in (lane 1, Figure 4&5) with no release of DNA. The results of 24 hours treatment revealed no DNA damage was occurred (Figure 4), while the DNA from all treatments groups after 48 hours indicated the presence of DNA damage and marked by the migration of released fragmented DNA (Figure 5).

The administration of fresh black mulberry extract (lane 2&3) was significantly the best treatment which induced sever DNA damage which observed as a late stage of apoptosis with laddering pattern of DNA fragmentation after 48h of treatment when compared with control group. Dry black mulberry extract showed DNA damage less than that of fresh black mulberry extract as shown in (lane 4&5) Table (4) and Figures (4,5).

Table (4): Optical density of fragmented DNA in treated and untreated MCF-7 cells after 48h.

	180bp	360bp	540bp	720bp
Control	-	-	-	-
100 µg/ml(FBME)	32.8±8.9	44.3±8.4	95.6±13	127.5±7.5
300 µg/ml(FBME)	65.7±10.7	68.7±12.6	116.0±4.3	156.3±23.7
100 µg/ml(DBME)	25.6±7.6	26.9±2.6	35.0±4.4	52.2±12.8
300 µg/ml (DBME)	36.7±4.0	15.7±6.8	19.9±5.7	35.7±9.8

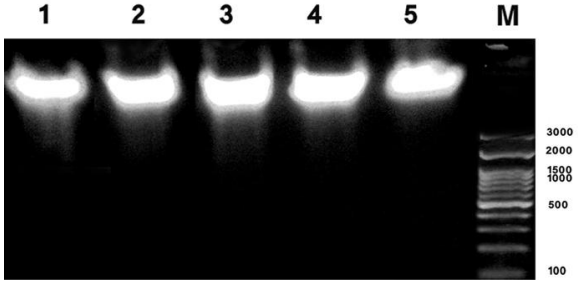

Figure (4): Representative digital photograph of total genomic DNA electrophoresis shows the effect of 24 hours treatments on MCF-7 cells. Where, lane: 1 resembles control MCF-7 cells group and lanes: 2-5 resemble fresh black mulberry treated cells 100µg/ml, fresh black mulberry treated cells 300µg/ml, dry black mulberry treated cells 100µg/ml and dry black mulberry treated cells 300µg/ml respectively. M: (100-3000 bp) ladder.

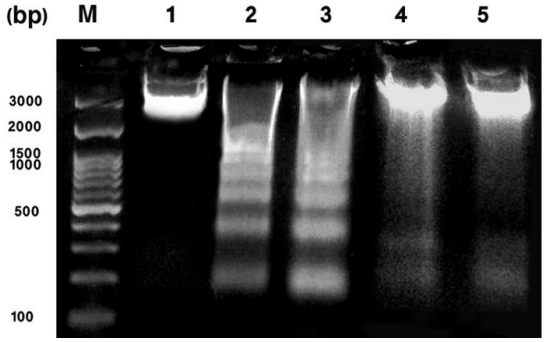

Figure (5): Representative digital photograph of total genomic DNA electrophoresis shows the effect of 48 hours of treatments on MCF-7 cells. Where, lane: 1 resembles control MCF-7 cells group and lanes: 2-5 resemble fresh black mulberry treated cells 100µg/ml, fresh black mulberry treated cells 300µg/ml, dry black mulberry treated cells 100µg/ml and dry black mulberry treated cells 300µg/ml respectively. M: (100-3000 bp) ladder.

3.6. DNA single strand breaks in MCF-7 cells treated with black mulberry extracts

The results of comet assay revealed that the presence of three forms of cells to be evaluated. They were normal (N), damaged (D) and strong damaged cells (SD) with fan-like tail. The DNA damage was increased, as well as, DNA tail migration length and intensity were increased when compared to intact undamaged normal cells (Figure 6). The recorded cells showed slightly increase in two forms of damaged cells percentage after 24 hours of both extracts administrations when compared to control group (Table 6). Moreover, after 48 hours of treatments, the results recorded significant ($p < 0.05$) elevation in the percentage of DNA-damaged cells especially with fresh extract treatments rather than that of dry one (Table 6).

Table (6): Comet assay in MCF-7 cells for treated and untreated groups.

	24h		48h	
	% damage	% strong damage	% damage	% strong damage
Control	8.5±1.5	-	10.5±1.5	4.0±1.1
100µg/ml (FBME)	15.1±5.2*	21.3±3.7*	15.1±4.5	32.6±3.7*
300µg/ml (FBME)	12.2±4.7*	22.4±5.7*	5.4±2.4	40.3±9.0*
100µg/ml (DBME)	5.2±0.35	26.1±5.5*	5.0±2.0	26.3±5.1*
300µg/ml (DBME)	4.7±2.5	25.3±3.3*	6.3±1.75	35.4±6.4*

* Significant difference at ($p < 0.05$) compared with control group. **FBME**: fresh black mulberry extract and **DBME**: dry black mulberry extract.

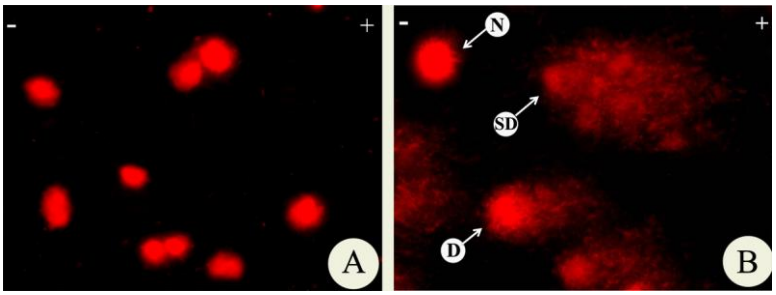

Figure (6): Representative photomicrograph for MCF-7 cells (400x) after comet assay technique. Where A: control untreated cells and B: treated cells showing grades of DNA damage (N: normal, D: damaged and SD: strong damaged nuclei).

3.7. Mitotic index frequency in MCF-7 cells treated with black mulberry extracts

The mitotic indexes were significantly ($p < 0.05$) inhibited in dose and time dependent manner when compared with control divided cells among all treatments illustrating the more effectiveness of antiproliferative properties of fresh extract than the dry one. They recorded the highest inhibition of mitotic frequency with (300 µg/ml) fresh black mulberry extract after 48 hours of treatment. While, (100 µg/ml) dry black mulberry extract gave the lowest inhibition index (Table 7).

Table (7): Mitotic index in MCF-7 cells for treated and untreated groups.

	% Mitotic index	
	24h	48h
Control	36.2±3.3	27.0±2.4
100 µg/ml (FBME)	20.4±2.7*	13.4±3.3*
300 µg/ml (FBME)	4.2±1.6*	1.3±3.1*
100 µg/ml (DBME)	21.6±2.3*	16.7±2.9*
300 µg/ml (DBME)	8.8±2.1*	3.6±2.1*

* Significant difference at ($p < 0.05$) compared with control group. **FBME**: fresh black mulberry extract and **DBME**: dry black mulberry extract.

4. Discussion

Despite the fact that many tumors initially respond to chemotherapy, breast cancer cells can survive and gain resistance to the chemotherapy (Campbell et al., 2001). Additionally, the non selectivity of chemical drugs has to be considered and trials for alternative medications have to be paid more concern. Accumulating evidences suggests that berry fruits may have potential for cancer prevention that may be due to their contents of macro- and micronutrients and the large number of bioactive phytochemicals (Ramos, 2008 and Huang et al., 2010). Moreover, due to their antioxidant properties, consumption of polyphenolic-rich foods play a role in the prevention of certain types of cancer and other diseases related to aging (Borbalanet al., 2003). These compounds are classified as flavonoids, tannins, phenolic acids and their analogues, and others depending on their chemical structures (Ramos, 2008; Huang et al., 2010). Flavonoid antioxidants have been demonstrated to exhibit anti-cancer effects against several epithelial cancers (Jiang et al., 2000). The relationship between natural polyphenols, apoptosis and cancer was identified by studding chemopreventive and/or chemotherapeutic ability of these compounds against cancer. One such important group of berry phytochemicals is anthocyanins. Anthocyanins are known to acts as anticarcinogenic agents against several cancers (Bishayee et al., 2011). Moreover, anthocyanin and other phenolic compounds of BM extracts may have potent effects, including anticancer with antioxidant, anti-inflammatory, and chemoprotective properties (Morse et al., 1993; Middleton et al., 2000, Li et al., 2013). Pure anthocyanins suppressed the growth of HCT116 colon and HS578T breast cell lines by G2/M cell cycle arrest and apoptosis (Chen et al., 2005; Yun et al., 2009). Apoptosis was identified as the major mode of anthocyanin-induced cell death (Chen et al., 2005; Seeram et al., 2006; Srivastava et al., 2007). The data of researches demonstrated proapoptotic effect of anthocyanins on numerous cancer cell lines (Katsube et al., 2003; Olsson et al., 2004; Chen et al., 2005; Seeram et al., 2006, Forester and Waterhouse, 2010). Also, anthocyanins induced apoptosis in MCF-7 cells as increased the amounts of oligonuceosomal-sized fragmentation (Devi et al., 2011).

In this study, the results of apoptotic anticancer properties were proved by evaluation of morphological changes and DNA fragmentation of MCF-7 treated with BM extracts. The results are in parallel to the pro-apoptotic properties of several pure anthocyanins and anthocyanin-rich extracts from berries on multiple cell types through both apoptotic pathways (Seeram et al., 2006 and Afaq et al., 2007).

Cytotoxicity test using neutral red method and mitotic index evaluated results of treated groups were in parallel with previous studies that reported high antiproliferative properties of mulberry juices (Seeram et al., 2006; Huang et al., 2008 and Zhang et al., 2011). These activities may be due to the contents of phenolic compounds that have been considered to be responsible for the various health benefits including cancer prevention (Huang et al., 2008).

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