



Chemotherapeutic effect of *Ulmus pumila* leaves methanolic extract against N-methyl-N-nitrosourea-induced mammary carcinoma in female rats: An *in vitro* and *in vivo* study

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ABSTRACT

Searching for a chemopreventive agent is an important approach for breast cancer management. The aim of the study was to evaluate the chemopreventive potential of *Ulmus pumila* (UP) leaves extract on breast tumorigenesis induced in experimental animals by N-methyl-N-nitrosourea. This target was undertaken through preparing several extracts from the fresh leaves of UP using different solvents against the breast adenocarcinoma cell line (MCF-7). Our *in vitro* results demonstrated that the methanolic extract of UP (UPME) showed the highest cytotoxic activity against the growth of MCF-7 cells. After determination of UPME safe dose (1/10) of a lethal dose, the *in vivo* results revealed that UPME treatment significantly decreased the activities of liver enzymes, kidney function, cancer antigen 15-3 (CA 15-3) level, urokinase plasminogen activator, heparanase, basic fibroblast growth factor, B-cell leukemia lymphoma 2, and cyclooxygenase-2. By contrast, total antioxidant capacity (TAC) was increased in therapeutic, protective, and prophylactic groups as compared to the tumor group. These improvements were supported with histopathological changes. These results indicated that the chemotherapeutic potential of UPME through stimulation of apoptosis and the suppression of angiogenesis, proliferation, and metastasis.

INTRODUCTION

Breast cancer (BC) is the most frequent malignant tumor in women in over 100 countries.

In 2018, about 2.1 million female BC cases were newly diagnosed worldwide with an estimated death number of 626,679 (Bray *et al.*, 2018). Egypt according to the National Cancer Registry Program was stratified into three geographical strata: lower, middle, and upper Egypt with the frequency of 33.8%, 26.8%, and 38.7%, respectively, and age-standardized incidence rate of 48.8/100,000 (Ibrahim *et al.*, 2014).

Cancer cells need to inhibit apoptosis and stimulate proliferation, angiogenesis, and metastasis for the growth and

enlargement of the tumor (Ali *et al.*, 2018). Angiogenesis is a process resulting in the formation of new blood vessels. In a wide variety of malignant solid tumors, basic fibroblast growth factor (bFGF) is known to have strong angiogenic activity (Ribatti *et al.*, 1999). It is activated by the action of the heparanase (HPA) enzyme mediating the formation of new blood vessels (Spaccapelo, 2016). Angiogenesis is a crucial process for metastasis where new blood vessels are formed and considered as the main way for cancer cells to leave their own primary place and grow as secondary tumors at a distant site (Kapoor *et al.*, 2015). Apoptosis is a critically important mechanism to control tissue growth, homeostasis, and for the removal of mutated or damaged cells through causing their suicide. Active caspases modulates the apoptotic process which occurs under internal or external stimuli as pathogenic infection or other irreparable cell damage (Kitazumi and Tsukahara, 2011). Inflammation is a natural defense mechanism in which immune cells orchestrate the release of several mediators such as prostaglandins, cytokines, and nitric oxide, which play a part in

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the defense process (Gomez-Cambronero *et al.*, 2002). Chronic inflammation may contribute to carcinogenesis through the up-regulation of angiogenesis, growth, and metastasis in a number of neoplasms (Sobolewski *et al.*, 2010). Oxidative stress was shown to cause DNA mutations and cell death and affect cell proliferation. The proliferation of genetically unstable cells might eventually progress toward carcinogenesis (Arya *et al.*, 2012).

New treatment regimens with minimum toxicity to normal cells need to be urgently developed as a conventional treatment of BC radiotherapy, chemotherapy, and local surgery, which suffer from various limitations including genetic mutation of normal cells, toxicity, and spreading of cancer cells to healthy tissues (Shakil *et al.*, 2018). BC aggressiveness, inhibition of cancer cell proliferation, and modulation of cancer-related pathways can be fought by natural products (Mitra and Dash, 2018).

Ulmus pumila (UP) is a natural herb that has traditionally been used for the treatment of infections. It belongs to the botanical classification of *Ulmaceae* (Jeong and Kim, 2012). Polysaccharides isolated from plants belonging to *Ulmus* genus are used as effective components for the treatment of glycosuria, cancer (Hwa *et al.*, 2001), acquired immunodeficiency syndrome (AIDS), as well as pathogenic virus diseases (Jung *et al.*, 2007) and possess anti-inflammatory and immune reinforcing ability (Hamed *et al.*, 2015).

The current study was targeted to prepare several extracts from the fresh leaves of UP using different solvents, which were screened for their cytotoxicity and anti-proliferative activities against breast adenocarcinoma cell line (MCF-7). The most effective extract was subsequently used to evaluate its anti-tumor effect against BC induced in rats.

MATERIALS AND METHODS

Chemicals

Dulbecco's modified eagle's medium was provided from Cambrex (Biowhittaker, Lonza, Basel, Switzerland). N-Methyl-N-nitrosourea, dimethyl sulfoxide (DMSO), penicillin, streptomycin, trypan blue, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and heat-inactivated fetal calf serum were obtained from Sigma Chemical Company (St. Louis, MO). Green and fresh leaves of UP were procured from El-Orman Garden (Giza, Egypt) and were identified by Prof. Khaled Mahmoud (Pharmacognosy Department, National Research Centre, Giza, Egypt). The taxonomical nomenclature was in accordance with Boulos (2002). The fresh leaves of UP were dried for 1 week in a solar oven at a temperature of 40°C and then powdered. The powdered leaves were kept at 4°C in a dark container for the phytochemical study and extraction.

Preparation of plant extract

A weight of 100 g dried powdered leaves was transferred to dark-colored flasks, soaked in 1 l of solvents with different polarities (water, methanol, methylene chloride, and hexane), and stored at room temperature with regular stirring. After 48 hours, the extracts were filtered through filter papers, the residues were re-extracted with equal volumes of the same solvent for 24 hours, and the filtration was repeated. Under reduced pressure, combined filtrates were evaporated at a temperature of 40°C using Büchi Rotary Evaporator R-114 (Switzerland), freeze-dried using a

Snijders Freeze Dryer (Tilburg, Holland) then the extraction yields were calculated. The obtained powder extracts were kept in sterile vials and stored at -20°C (Balasubramanian *et al.*, 2010). The freeze-dried plant extract was deposited at the Extract Bank of the *in vitro* Bioassay Laboratory in the National Research Centre (Giza, Egypt).

In vitro cytotoxicity study

The cytotoxic effect of different UP extracts (water, methanol, methylene chloride, and hexane) was evaluated against human BC adenocarcinoma MCF-7 cell line and normal retinal pigment epithelial RPE-1 cell line using a colorimetric MTT assay procedure according to Rashad *et al.* (2012).

Phytochemical screening of the crude UPME

The phytochemical screening of the UPME including the qualitative detection of alkaloids and terpenoids, saponins and flavonoids was done according to the method of Wadood *et al.* (2013), Yadav and Agarwala (2011), and Sofowora (1993), respectively. Phytochemical screening for phenols and tannins was fulfilled according to the method of Yadav and Agarwala (2011), whereas analysis for carbohydrates and steroids were estimated by the method of Yadav and Agarwala (2011) and Al-Daihan *et al.* (2013), respectively.

Animals

Inbred 5-weeks old female Wistar rats were housed under standard laboratory conditions (12 hour light dark cycles) in a room with controlled temperature (24°C ± 3°C) during the experimental period and provided with tap water and commercial pelleted diets. All animal experiments were approved by the Research Ethical Committee at the National Research Centre (Registration Number 15/097).

Determination of the median lethal dose (LD₅₀)

The median lethal dose (LD₅₀) of UPME was evaluated according to Wilbrandt (1952). Briefly, different UPME doses up to 2,000 mg/kg body weight in DMSO were orally administered to adult female Wistar rats (six rats per group) and a group of six rats was given orally the respective amount of DMSO and left as a control. The toxic symptoms and mortality rate in each group were recorded after 24 hours and continued for 1 month.

Experimental design

After an acclimatization period of 1 week, female rats were divided into six groups (Fig. 1) according to the following scheme; Control group (10 rats): normal intact female rats received a daily oral dose of DMSO 5 ml/kg body weight using an intragastric tube throughout the experimental period. UPME-treated group (Extract, 15 rats): rats were treated daily with 1/10 of the LD₅₀ of UPME (141.6 mg/kg body weight dissolved in DMSO) using an intragastric tube at the 7th week of age for four consecutive weeks. Tumor group (20 rats): female rats were intraperitoneally (i.p.) injected with two doses of freshly prepared N-methyl-N-nitrosourea (MNU) dissolved in sterile physiological saline at a dose of 50 mg/kg body weight at 7 and 16 weeks of age (Perše *et al.*, 2009). Prophylactic group (15 rats): rats were orally pretreated daily for four consecutive weeks with UPME before

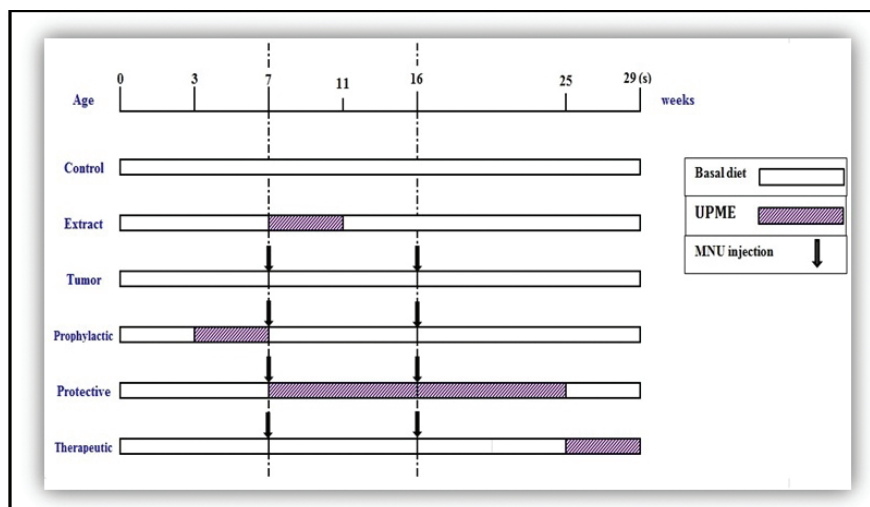


Figure 1. Design for the experimental protocol (s: sacrifice).

induction of BC. Protective group (15 rats): at the first injection of MNU, rats were treated daily with UPME and continued till the end of the experiment. Therapeutic group (15 rats): female rats were intraperitoneally injected with two doses of MNU followed by a daily oral administration of UPME starting from the age of 25 weeks for four consecutive weeks. At the end of the experiment, the animals were anesthetized, blood samples were collected and allowed to clot at room temperature. The blood was centrifuged at $6,000 \times g$ for 15 minutes at 4°C . Serum was separated and aliquoted for further biochemical analysis. At autopsy, intact mammary glands, as well as mammary tumors, were carefully excised and fixed in formalin for histopathological examination.

Biochemical analyses

Alanine aminotransferase (ALT), aspartate aminotransferase (AST), and total antioxidant capacity (TAC) were determined in serum using commercial assay kits (Biodiagnostic, Egypt). Also, serum alkaline phosphatase (ALP) activity, creatinine, and urea levels were analyzed using kits supplied by Reactivos GPL, Spain. Serum cancer antigen 15-3 (CA 15-3) as a marker of BC and urokinase plasminogen activator (uPA) level as a marker for tumor growth were assayed using rat-specific enzyme-linked immunosorbent assay kits provided by LifeSpan Biosciences, Inc. (Seattle, Washington). Serum HPA level as metastasis marker, bFGF concentration as a marker of angiogenesis, B-cell leukemia lymphoma 2 (Bcl-2) level as anti-apoptotic marker, as well as cyclooxygenase-2 (COX-2) concentrations as a marker of inflammation were assayed using rat-specific kits supplied by Glory Science Co. (TX).

Histopathological study

Fixed mammary glands were processed and cut with a microtome with $5 \mu\text{m}$ thick sections, stained with hematoxylin and eosin (H&E) and examined by high microscopy.

Statistical analysis

One-way analysis of variance (ANOVA) followed by post-hoc least significant difference test was performed in order to estimate significant differences among groups. Data are reported as

mean values \pm SE, and differences between groups were considered to be significant at $p < 0.05$. To determine the median inhibitory concentration (IC_{50}) of different UP leaf extracts, the viability percent was plotted against logarithmic concentrations and the resulting plot was fitted to a nonlinear regression curve using GraphPad Prism version 5.0 software (GraphPad Software Inc., San Diego).

RESULTS

In vitro cytotoxicity study

The median inhibitory concentration values (IC_{50}) of the four UP leaves extract (water, methanol, methylene chloride, and hexane) against the growth of the MCF-7 cell line were 51.79, 32.02, 95.14, and 102 $\mu\text{g}/\text{ml}$, respectively. The preliminary screening against different cell lines showed that the methanolic extract of UP leaves was the most active one to inhibit the growth of MCF-7 cells (Fig. 2). In addition, the methanolic extract showed no cytotoxicity effect against RPE-1 cells.

Phytochemical screening of UPME

As shown in Table 1, the results revealed the presence of high flavonoids concentration, moderate concentration of alkaloids, carbohydrates and steroids, whereas terpenoids, phenols, tannins, and saponins are present in low concentrations.

In vivo toxicity study

In Table 2, the median lethal dose (LD_{50}) of UPME was found to be 1,416.6 mg/kg body weight. The 1/10 of the LD_{50} of UPME was used in this study (141.66 mg/kg body weight).

Effect of different treatments on hepatic and renal function test

Data presented in (Fig. 3A) showed a significant increase in serum liver enzymes activity (ALT, AST, and ALP) in tumorized rats, compared to the control group. By contrast, a significant reduction was observed in serum liver enzymes activity in the prophylactic and the protective groups, compared to the tumor group. Oral administration of UPME after tumor induction

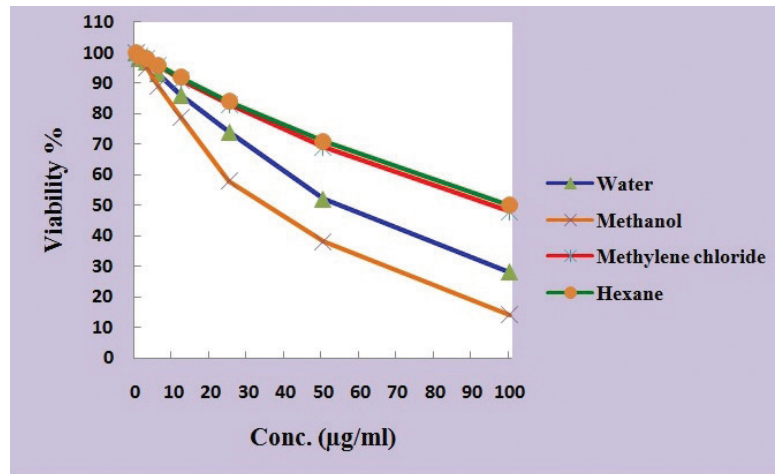


Figure 2. The viability percent of MCF-7 cells following treatment with different UP leaf extracts for 48 hours.

Table 1. Phytochemical screening of UPME.

Constituents	UPME
Flavonoids	+++
Alkaloids	++
Carbohydrates	++
Steroids	++
Terpenoids	+
Phenols & tannins	+
Saponins	+

+ Weak reaction, ++ Moderate reaction, +++ Strong reaction.

Table 2. Calculation of the half lethal dose (LD₅₀) in female rats.

Dose (mg/kg body weight)	Number of animals	Number of dead animals	Z	d	(Z) × (d)
100	6	0	0	100	0
250	6	0	0	150	0
500	6	0	0	250	0
1000	6	2	1	500	500
2000	6	4	3	1000	3000

$$LD_{50} = 2000 - (3500/6) = 1416.6 \text{ mg/kg bw.}$$

(therapeutic group) was able to normalize the aforementioned enzymes activity. Similarly, serum creatinine and urea levels were significantly increased in tumorized rats, compared to normal controls. On the other hand, a slight significant elevation in the urea level (10%) along with a significant reduction in serum creatinine level (37%) was observed in the prophylactic group, compared to the untreated tumor group. In addition, co-administration of UPME during tumor induction (protective group), as well as oral administration of UPME after tumor induction (therapeutic group) normalized serum creatinine and urea levels (Fig. 3B).

Effect of different treatments on serum CA 15-3 and uPA levels

Induction of BC in female rats with MNU produced a significant increase in serum CA 15-3 level by 337.5%, compared

to normal controls. On the other hand, pretreatment of female rats with UPME before tumor induction resulted in a significant reduction in serum CA 15-3 level by 66%, compared to the untreated tumor group. Similarly, co-administration of UPME during tumor induction significantly reduced the aforementioned tumor marker level by 70%, compared to the tumor group. Administration of UPME after tumor induction was able to normalize serum CA 15-3 level (Fig. 4A). Also, the induction of BC in female rats by MNU resulted in a significant elevation in serum uPA concentration (54.76%), compared to the normal control group. Meanwhile, pretreatment of female rats with UPME in the prophylactic group resulted in a significant reduction in serum uPA concentration by 15.38%, as compared to the untreated tumor group. By contrast, co-administration of UPME during tumor induction (protective group) and oral administration of UPME after tumor induction (therapeutic group) normalized serum uPA concentration (Fig. 4B).

Effect of different treatments on serum HPA and bFGF levels

Induction of BC in female rats resulted in a significant elevation in serum HPA concentration by 54.82%, compared to the normal control group. Pretreatment of female rats with UPME (prophylactic group) was unable to significantly lower serum HPA, compared to the tumor group. Co-administration of UPME during tumor induction (protective group) significantly decreased serum HPA by 21.56%, compared to the untreated tumor group. By contrast, oral administration of UPME after tumor induction (therapeutic group) normalized serum HPA concentration (Fig. 5A). In Figure 5B, induction of BC in female rats resulted in a significant elevation in serum bFGF concentration by 66.60%, compared to normal controls. Pretreatment of female rats with UPME (prophylactic group) resulted in a significant reduction in serum bFGF concentration by 24.97%, compared to the tumor group. Whereas co-administration of UPME during tumor induction (protective group) and oral administration of UPME after tumor induction (therapeutic group) normalized serum bFGF.

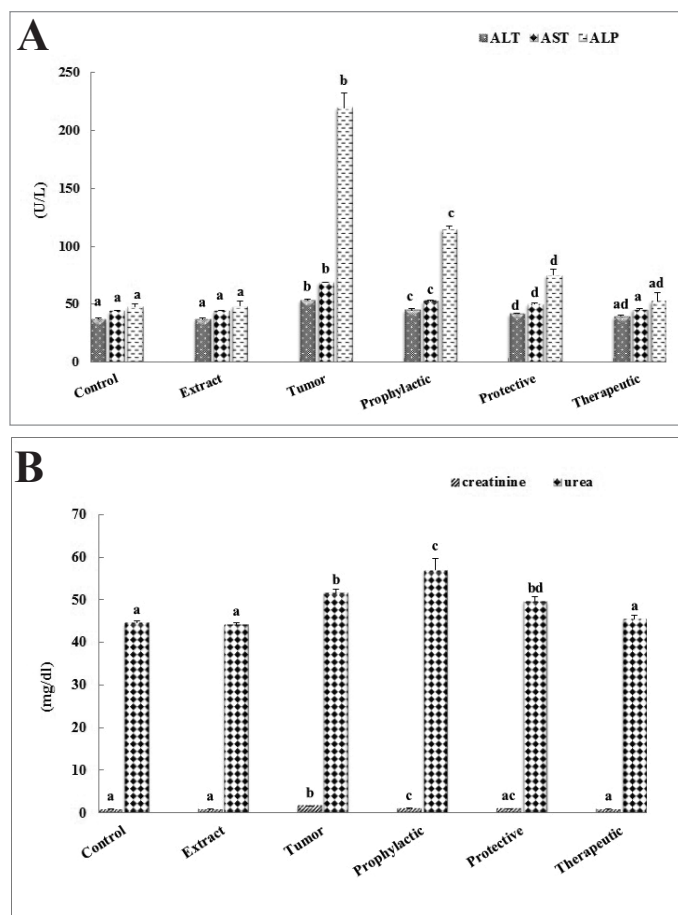


Figure 3. (A) Statistical significance of serum ALT, AST, and ALP activities in different groups. Values are expressed as mean \pm SE of 10–20 rats. Groups sharing the same letters for each parameter are not significantly different. (B). Statistical significance of serum creatinine and urea levels in different groups. Values are expressed as mean \pm SE of 10–20 rats. Groups sharing the same letters for each parameter are not significantly different.

Effect of different treatments on serum TAC, Bcl-2, and COX-2 levels

The result of TAC demonstrates that induction of BC in female rats resulted in a significant depletion in serum TAC level (33.83%), compared to normal controls. Pretreatment of female rats with UPME in the prophylactic group resulted in a significant enhancement of serum TAC level (15.79%), compared to the untreated tumor group. In addition, co-administration of UPME during tumor induction (protective group), as well as its administration after tumor induction (therapeutic group), normalized serum TAC level (Fig. 6A). Meanwhile, induction of BC in female rats resulted in a significant increase in serum Bcl-2 concentration by 55.65%, compared to normal controls. On the other hand, pretreatment of female rats with UPME before tumor induction (prophylactic group) resulted in a significant decrease in serum Bcl-2 concentration by 23.41%, compared to the untreated tumor group. Furthermore, co-administration of UPME during tumor induction (protective group), as well as its administration after tumor induction (therapeutic group), normalized the serum Bcl-2 concentration (Fig. 6B). Also, the induction of BC in female rats resulted in a significant increase in serum COX-2 concentration

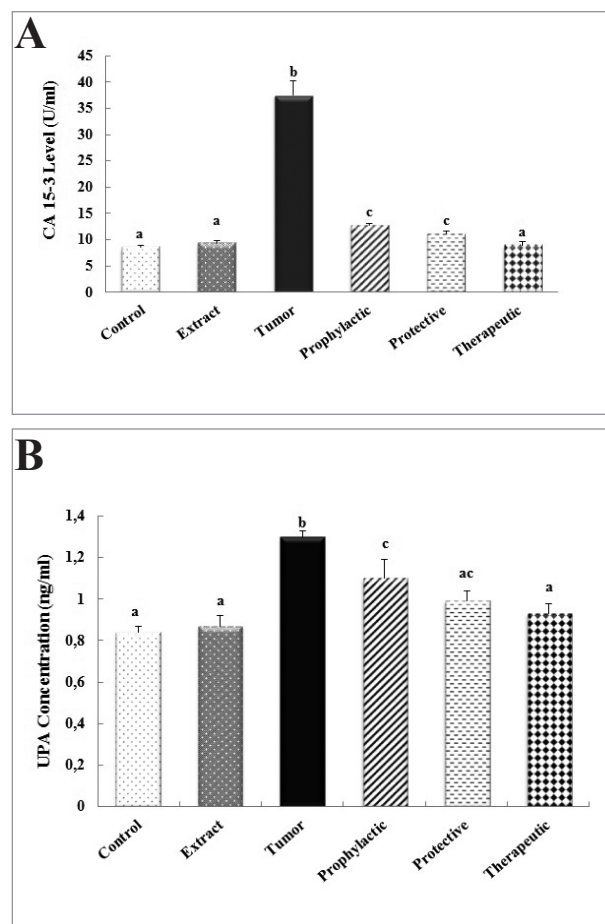


Figure 4. (A) Statistical significance of serum CA 15-3 level in different groups. Values are expressed as mean \pm SE of 10–20 rats. Groups sharing the same letters are not significantly different. (B) Statistical significance of serum uPA concentration in different groups. Values are expressed as mean \pm SE of 10–20 rats. Groups sharing the same letters are not significantly different.

by 42.12%, compared to normal controls. Pretreatment of female rats with UPME (prophylactic group) resulted in a significant reduction in serum COX-2 concentration by 13.36%, compared to the untreated tumor group. Moreover, co-administration of UPME during tumor induction (protective group), as well as its administration after tumor induction (therapeutic group), normalized serum COX-2 concentration (Fig. 6C).

Histopathological examination

Histological examinations of the mammary glands (Fig. 7A) of control and UPME treated rats (Fig. 7B) showed the normal histological structure of the acinus, ducts with periacinar and periductal fibrous tissue and all embedded in adipoblasts. On the other hand, the induction of BC in female rats by MNU-induced proliferation of anaplastic cancer cells with hyperchromasia, polarity, pleomorphism, and few mitosis replacing the acini and ducts in a focal manner in the mammary glands of rats from the tumor group (Fig. 7C). Mammary glands of female rats pretreated with UPME showed stratification in lining epithelium of some lactiferous ducts with anaplastic cancer cells and fibrous tissue, as well as restored of normal architecture (Fig. 7D). Mammary

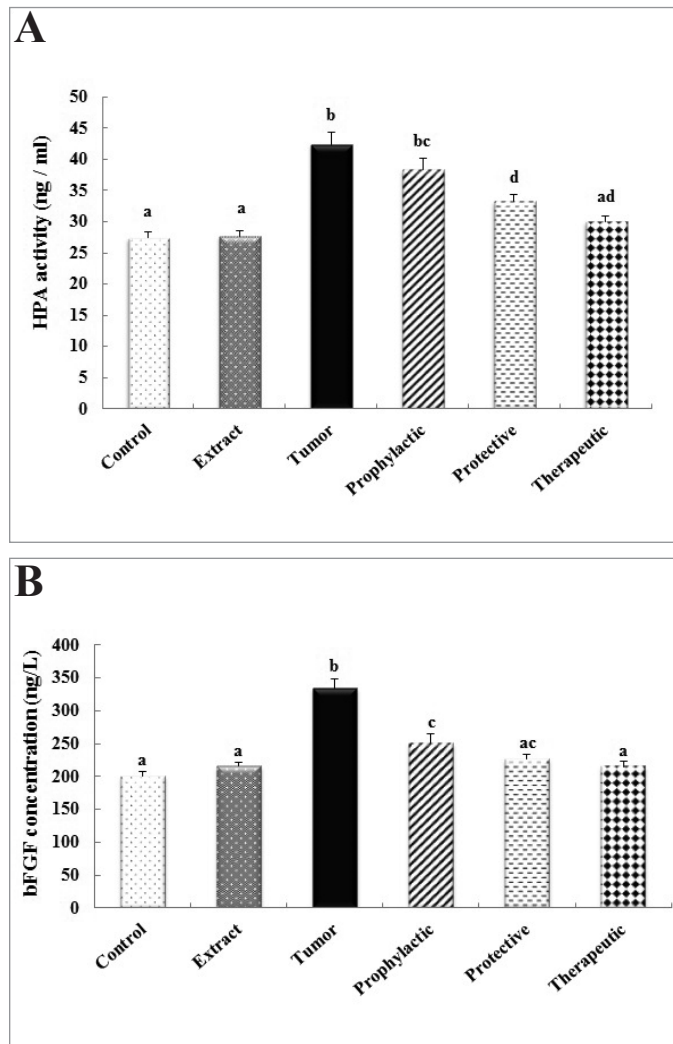


Figure 5. (A) Statistical significance of serum HPA concentration in different groups. Values are expressed as mean \pm SE of 10–20 rats. Groups sharing the same letters are not significantly different. (B) Statistical significance of serum bFGF concentration in different groups. Values are expressed as mean \pm SE of 10–20 rats. Groups sharing the same letters are not significantly different.

glands in the protective group showed no histopathological alteration with the normal histological structure of the ducts in the adipose tissue (Fig. 7E). Furthermore, mammary glands in the therapeutic group structure showed that the treatment of female rats with UPME after tumor induction improved the changes in the mammary gland with congestion in stromal blood vessels and mild periductal fibrosis (Fig. 7F).

DISCUSSION

There is a constant need for the development of novel and improved chemotherapeutic agents for the prevention and treatment of cancer due to the adverse effects of the conventional non-selective cytotoxic chemotherapies and the resistance developed for the existing anticancer drugs (Eldehna *et al.*, 2019). Natural products represent complementary and alternative medicines that have become popular due to its low toxicity and efficacy in patients with advanced cancer (Yue *et al.*, 2017).

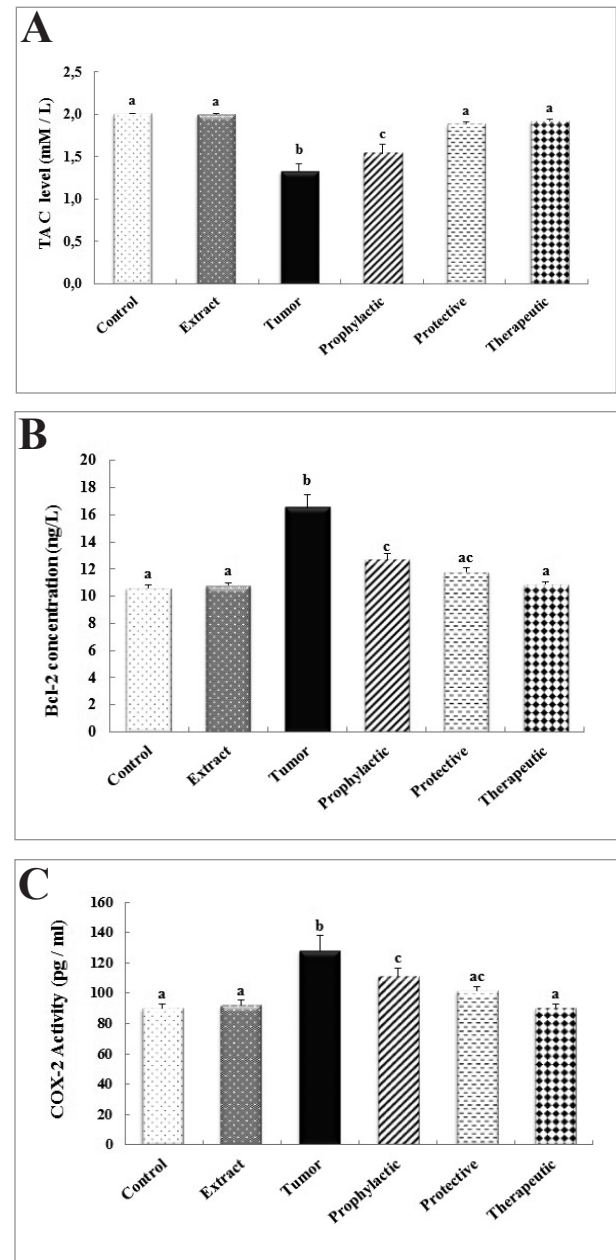


Figure 6. (A) Statistical significance of serum TAC level in different groups. Values are expressed as mean \pm SE of 10–20 rats. Groups sharing the same letters are not significantly different. (B) Statistical significance of serum Bcl-2 concentration in different groups. Values are expressed as mean \pm SE of 10–20 rats. Groups sharing the same letters are not significantly different. (C) Statistical significance of serum COX-2 concentration in different groups. Values are expressed as mean \pm SE of 10–20 rats. Groups sharing the same letters are not significantly different.

UP is a natural herb that has traditionally been used for the treatment of infections (Jeong and Kim, 2012). Polysaccharides isolated from plants belonging to *Ulmus* genus possess anti-inflammatory and immune reinforcing ability (Hamed *et al.*, 2015), are an effective component for the treatment of glycosuria, cancer (Hwa *et al.*, 2001), AIDS, as well as pathogenic virus diseases (Jung *et al.*, 2007). Furthermore, leucoanthocyanins and catechin, mucilage,

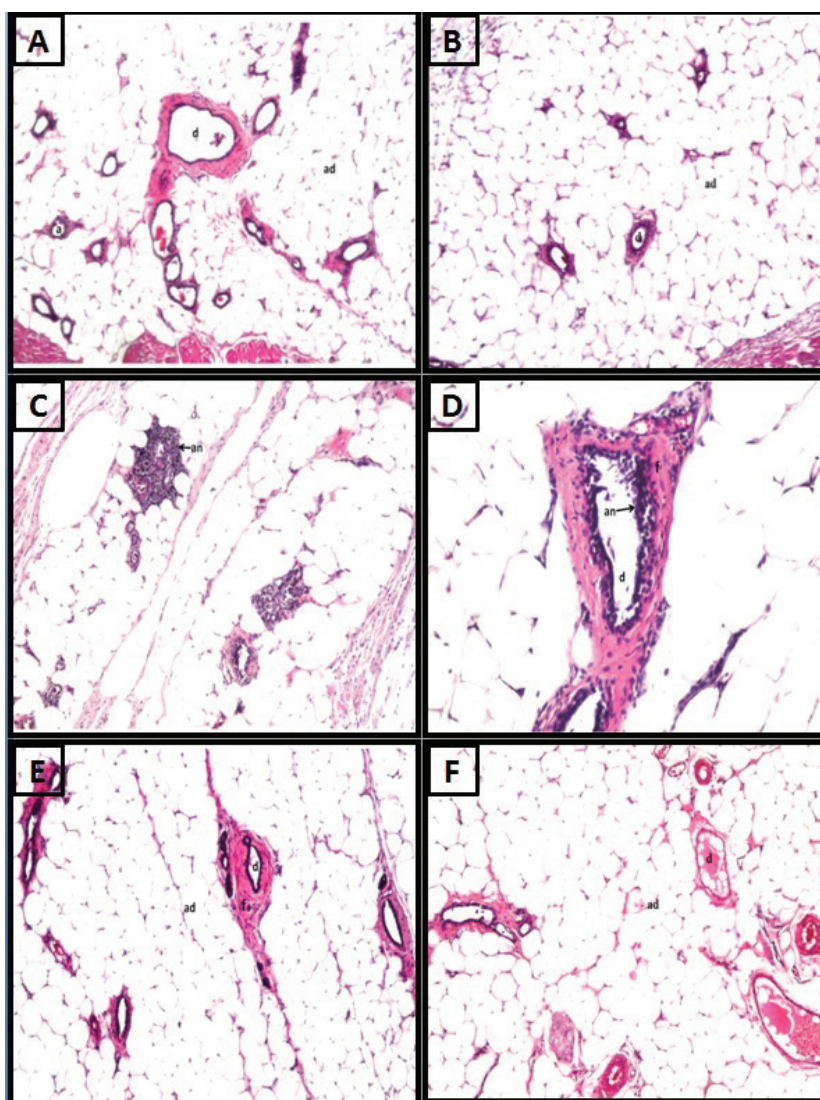


Figure 7. Photomicrograph of mammary glands in control (A) and UPME-treated rats (B) showing intact histological structure of the acinus (a), ducts (d) with periacinar and periductal fibrous tissue and all embedded in adipoblasts (ad). (C) A photomicrograph of the mammary gland of a rat from the tumor group showing proliferated anaplastic cancer cells (an) with hyperchromasia, polarity, pleomorphism, and few mitosis replacing the acini and ducts in a focal manner. (D) Mammary gland of a rat from the prophylactic group showing stratification in lining epithelium of some lactiferous ducts with anaplastic cancer cells and fibrous tissue (f). (E) The mammary gland of a rat from the protective group showing no histopathological alteration and the normal histological structure of the ducts in the adipose tissue. (F) Mammary gland of a rat from the therapeutic group showing that treatment with UPME after tumor induction improved the changes in the mammary gland with congestion in stromal blood vessels and mild periductal fibrosis.

polyphenols, flavonoids, tannins, lignans, proanthocyanidins, tannins, triterpenes, and sterols were previously isolated from *Ulmus* species (Jung *et al.*, 2007).

The aim of this study is to investigate the anti-carcinogenic activity of UPME through the study of its inhibitory effects on cancer cell proliferation, inflammation, angiogenesis, tumor growth, metastasis, as well as inducing apoptosis.

Our *in vitro* results demonstrated that the methanolic extract of UP (UPME) showed the highest anti-proliferative activity against the growth of MCF-7 cells compared to the other prepared extracts (water, methylene chloride, and hexane) and the phytochemical screening of this extract revealed that its anti-proliferative activity is due to the presence of high flavonoids concentration.

The results showed that UPME per se was safe as revealed by the non-significant change in liver or kidney functions, compared to normal controls.

Serum liver enzymes (ALT, AST, and ALP) activity was significantly increased in tumorized rats, which indicates the severity of hepatic tissue damage by MNU, this is in accordance with the previous findings of Ahn *et al.* (2004). These can be explained by the fact that, in cancer conditions, disturbance in the transport function of hepatocytes resulting in the leakage of enzymes due to altered permeability of plasma membrane and thus causing an increased level of these marker enzymes in serum and decreased level in the cells. In toxicity-induced animals, there is a cytoplasmic leakage of the enzyme into the bloodstream due to the destruction of the structural integrity of the cells

(Uma *et al.*, 2014). Also, Lakshmi and Subramanian (2014) reported that the rise in ALP activity in cancer-bearing animals may be due to the toxic effect of MNU in the liver, the disturbance in secretory activity, or due to altered gene expression in these conditions.

On the other hand, a significant reduction in the aforementioned enzyme activities compared to the untreated tumor group was observed in the prophylactic group and protective group. Oral administration of UPME after tumor induction was able to normalize serum ALT, AST, and ALP activities indicating the nontoxic nature of UPME. These results firmly establish the role of UPME in decreasing the severity of cancerous alteration in rats and suggest the possibility of the extract to stabilize the permeability of the plasma membrane (Hoshyar *et al.*, 2015).

Kidney function disorder results in the accumulation of toxic substances in the blood that should be removed from the body (Yener *et al.*, 2016). Our results demonstrated that serum creatinine and urea levels were significantly increased in tumorized rats. By contrast, a significant reduction in serum creatinine level was observed and a slight significant elevation in the urea level was recorded in the prophylactic group, compared to the untreated tumor group. Co-administration of UPME during tumor induction (protective group) resulted in a significant reduction in serum creatinine, accompanied by a slight significant decrease in serum urea level compared to the untreated tumor group. Similarly, Raj *et al.* (2015) documented an increase in serum creatinine levels in BC-induced rats as a consequence of the toxic effect of carcinogenic agents. On the other hand, oral administration of UPME after tumor induction normalized serum creatinine and urea levels indicating the non-toxic nature of UPME. These results trustworthy showed the role of UPME in decreasing the severity of cancerous alteration in rats (Hoshyar *et al.*, 2015). From foregoing results, it can be indicated that there is no overall adverse effects of the current extract on the liver and kidney function tests in the group received extract alone. Consequently, it can be concluded that the methanolic extract can be used safely.

Owing to the high sensitivity and specificity of tumor markers, they have become important markers for the early diagnosis of cancer as well as the evaluation of prognosis (Hou *et al.*, 2016). Induction of BC in female rats with MNU produced a significant increase in serum CA 15-3 level, compared to normal controls. Pretreatment of female rats with UPME before tumor induction (prophylactic group) resulted in a significant reduction in serum CA 15-3 level, compared to the untreated tumor group. Similarly, co-administration of UPME during tumor induction (protective group) significantly reduced the aforementioned tumor marker level, compared to the tumorized group. Whereas oral administration of UPME after tumor induction (therapeutic group) was able to normalize serum CA 15-3 level. These results agree with that of Santhalakshmi and Gayathri (2015) who reported that BC tumor marker CA 15-3 was significantly increased in 7,12-Dimethylbenz(a)anthracene-BC tumorized rats when compared to controls. Whereas rats treated with quercetin showed suppressed levels of tumor markers when compared to the induced group of rats. Authors attributed their results to quercetin which is one such plant-derived flavonoid that acts as an anticancer agent by suppressing the tumor markers, thereby preventing the initiation and progression of BC.

The uPA is one of the two enzymes responsible for the generation of the protease plasmin. In physiological and pathological processes in cell migration, tumor invasion and metastasis uPA is thought to be primarily involved in the generation of extracellular proteolytic activity (Duffy *et al.*, 2014; Omar *et al.*, 2012). Our results revealed a significant elevation in serum uPA level of tumorized rats, compared to the control group. Female rats pretreated with UPME before tumor induction resulted in a significant reduction in serum uPA compared to the tumor group. Co-administration of UPME during tumor induction as well as oral administration of UPME after tumor induction normalized serum uPA. Our results agree with Weinmann *et al.* (2002) who showed that elevated uPA activity in rat DS-sarcoma may be an indication for the uPA system functional role in tumor progression. The higher expression of uPA-induced proteolysis may be a result from oncogenic transformation. A wide variety of hormones, cytokines, and growth factors are known to regulate the expression of components of the uPA system and are up-regulated as a consequence of oncogenic transformation (Minisini *et al.*, 2007). The decrease in uPA concentration in our study may be due to the anti-tumor activity of flavonoids that are present in UPME and resulted in the suppression of invasion and migration. Due to its chemical structure similar to estrogen, flavonoids have unique advantages in the treatment and prevention of BC (Mahmoud and Ali, 2014). Also, Devipriya *et al.* (2006) investigated the effect of quercetin on the plasminogen activator system. They found that the administration of quercetin significantly reduced the tumor volume in rats induced for mammary carcinoma. Authors attributed their results to the quercetin role as a dietary bioflavonoid in mammary tumor treatment.

Metastasis is the main cause of death of cancer patients and poses the biggest problem to cancer treatment (van Zijl *et al.*, 2011). HPA cleaves the heparan sulfate (HS) chains of proteoglycans (HSPGs) and promotes tumor angiogenesis by releasing growth factors and angiogenic molecules that are sequestered in depot forms within HSPG making them available to bind and activate their tyrosine kinase receptors and to promote endothelial cell proliferation and neovascularization (Quiros *et al.*, 2006).

Our results showed that serum HPA level was significantly increased in tumorized rats, compared to normal controls. Prophylactic group serum HPA was insignificantly decreased compared to the untreated tumor group. Whereas co-administration of UPME during tumor induction (protective group) significantly decreased serum HPA compared to the untreated tumor group. Furthermore, oral administration of UPME after tumor induction (therapeutic group) normalized serum HPA activity. Similarly, Nobuhisa *et al.* (2005) reported that HPA expression correlates with the metastatic potential of the tumor, facilitating cell invasion through the extracellular matrix (ECM) barrier. Extensive studies have confirmed that over expression of HPA accelerates cell proliferation and elicits a marked pro-angiogenic response resulting in acceleration of tumor growth and degradation of the ECM (Cohen *et al.*, 2006). Yuan *et al.* (2012) reported that HPA over expression enhances the phosphorylation of molecules in the extracellular-signal-regulated kinase (ERK) and protein kinase B (AKT) pathways to stimulate the proliferation and migration of tumor cells. At the same time, HPA cleaves heparan sulfate and releases growth factors such as FGF and vascular endothelial growth factor (VEGF) from ECM

storage (Suzuki *et al.*, 2015). The decrease in HPA concentration following UPME treatment in tumorized rats in our study suggests an anti-metastatic ability for the extract that participates in repressing tumor invasion, angiogenesis, and metastasis.

Angiogenesis is a complex process regulated by a balance between pro-angiogenic and anti-angiogenic factors (Kazerounian and Lawler, 2018). Basic FGF (bFGF) has strong angiogenic activity (Westwood *et al.*, 2002). In this study, a significant elevation in serum bFGF concentration of tumorized rats, compared to the control group. By contrast, a significant reduction in serum bFGF concentration was observed in the prophylactic group. In agreement with our results, Siddiqi *et al.* (2018) found a significant increase in the serum level of bFGF in tumor-induced control rats, supporting the fact that angiogenesis is an important mechanism used to promote the growth of BC. Similarly, Kazerounian and Lawler (2018) found that the positive rate of bFGF expression in patients with BC with lymph node metastasis was significantly higher compared to patients without lymph node metastasis, which indicates that the high level of bFGF expression was associated with tumor invasion. On the other hand, co-administration of UPME during tumor induction (protective group) and oral administration of UPME after tumor induction (therapeutic group) normalized serum bFGF; this could be due to the presence of flavonoids which have prominent effects in preventing and treating BC. Their anti-tumor mechanisms mainly include inhibition of tumor angiogenesis.

Oxidative stress occurs in response to the oxidative damage caused when the body's anti-oxidative and scavenging activities cannot cope with the active oxidants produced by a harmful stimulant (Chikara *et al.*, 2018). The formation of reactive oxygen species (ROS) is involved in the occurrence and development of malignant tumors through the induction of DNA damage and genetic mutations, promotion of the proliferation, invasion, metastasis of malignant cells, and inhibition of apoptosis (Valko *et al.*, 2006). In the present study, there was a significant reduction in TAC in the serum of tumorized rats, compared with the control group. Previously, Giri *et al.* (1995) found that the toxic manifestation of MNU is associated with its oxidative metabolism, leading to the formation of reactive metabolites capable of generating free radicals. Similar report mentioned that breast carcinoma is associated with a decrease in antioxidant capacity in blood cells. Moreover, oxidative damage in cancer is associated with a great number of pathobiochemical pathways that lead to free radical DNA damage and cellular neoplastic transformation (Feng *et al.*, 2012). On the other hand, co-administration of UPME during tumor induction (protective group), as well as its administration after tumor induction (therapeutic group) normalized serum TAC level which showed that UPME contains a high level of flavonoids that play an important role as antioxidants. Martinez-Perez *et al.* (2014) found that the numerous hydroxy groups in the flavonoid structure, in combination with a highly conjugated π -electron system, allow them to act as free radical scavengers via hydrogen atom or electron-donating activities. Furthermore, they can block the formation of ROS, such as the hydroxyl radical, through chelation of redox-active transition metal ions. Thus, UPME has a positive effect on cancer treatment by the minimization of phenomena such as oxidative damage to DNA, indicating the non-toxic nature of UPME.

Apoptosis is a tightly regulated process of programmed cell death. Bcl-2 is an anti-apoptotic protein that is capable of blocking most of the pro-apoptotic stimuli (Suhaili *et al.*, 2017) and therefore promotes cell survival. Induction of BC in female rats by MNU resulted in a significant increase in serum Bcl-2 concentration compared to normal controls. In contrast, co-administration of UPME during tumor induction (protective group), as well as its administration after tumor induction (therapeutic group), normalized serum Bcl-2 level.

These results agree with that of Tsutsui *et al.* (2006) which revealed a significantly higher serum Bcl-2 level in BC patients than in normal healthy controls. The increase in the Bcl-2 level in cancer cells points to a potentially critical role of this anti-apoptotic protein in BC progression. Over expression of Bcl-2 protein may serve as a determinant of advantageous cell survival in breast tumor cells, ultimately leading to tumor progression and metastases. The ameliorative effect of UPME on Bcl-2 level in the therapeutic group and the protective group was due to the presence of flavonoids that induce apoptosis in BC through both main apoptotic pathways: intrinsic, caspase-9, and mitochondrial-driven apoptosis; and extrinsic, caspase-8, and death receptor-driven apoptosis (Abotaleb *et al.*, 2019). Also, flavonoids can modulate other important regulators of apoptosis, including anti-apoptotic protein families such as Bcl-2 (Martinez-Perez *et al.*, 2014).

Inflammation may contribute to carcinogenesis through the upregulation of growth, angiogenesis, and metastasis in a number of neoplasms. Over expression of COX-2 induce tumorigenesis, promote the growth and invasion of tumors (Galal *et al.*, 2014; Temirak *et al.*, 2014). COX-2 level was significantly increased in tumorized rats, compared to normal controls. By contrast, pretreatment of female rats with UPME (prophylactic group) resulted in a significant decrease in COX-2 concentrations, compared to the untreated tumor group. Whereas co-administration of UPME during tumor induction (protective group) and oral administration of UPME after tumor induction (therapeutic group) normalized COX-2 concentration.

These results agree with Dai *et al.* (2012) who mentioned that there was a high expression of COX-2 mRNA in BC cells. Also, Korbecki *et al.* (2013) showed that over production of ROS resulted in COX-2 mRNA and protein induction through enhancing promoter activity and ultimately prostaglandin (PG) synthesis stimulation. This increase results in rising PG levels which is implicated in different mechanisms in tumorigenesis as promoting tumor growth and angiogenesis, as well as down-regulating apoptosis (Rasmuson *et al.*, 2012). In our study, the decrease in COX-2 activity may be due to the presence of terpenoids and total saponin in our extract. These results agree with Xu *et al.* (2016) who found that terpenoids and total saponin inhibit COX-2 expression and decrease PGE2 production in cancer cell models, leading to inhibition of tumor development, metastasis, and survival and achieving more apoptotic effects. Moreover, the reduction in COX-2 activity may be due to the presence of flavonoids which play an important role in the cell-cycle arrest. Flavonoids arrest proliferation at the G2/M checkpoint in several different cancer types mainly through modulation of the expression level of different cyclins. They can also modulate proliferation, invasion, or inflammatory signals as they inhibit COXs

(cyclooxygenases) 1 and 2, involved in the production of prostaglandins that subsequently leads to cell proliferation, angiogenesis, and immunosuppression (Wei *et al.*, 2018).

The histological investigations supported the biochemical results of our study, as mammary gland of a rat from the control group showed the normal histological structure of the acinus, ducts with periacinar and periductal fibrous tissue and all embedded in adipoblasts. UPME treated group mammary gland showed the normal histological structure of ducts embedded in adipose tissue as the control group, indicating the non-toxic nature of methanolic extract of UP. Induction of BC in female rats by MNU-induced proliferation of anaplastic cancer cells (an) with hyperchromachia, polarity, pleomorphism, and few mitosis replacing the acini and ducts in a focal manner in the mammary gland of a rat from the tumor group. Treatment with UPME improved the toxic changes in prophylactic, protective and therapeutic groups. The ameliorative effect in the therapeutic group was more effective than that in protective and prophylactic groups. Moreover, the prophylactic group showed stratification in lining epithelium of some lactiferous ducts with anaplastic cancer cells and fibrous tissue, as well as restored normal architecture. Protective group mammary gland showed no histopathological alteration and the normal histological structure of the ducts in the adipose tissue. While therapeutic group showed that the treatment of female rats with UPME after tumor induction improved the changes in the mammary gland with congestion in stromal blood vessels and mild periductal fibrosis.

CONCLUSION

From the present study, it is obvious that UPME could be considered as a promising chemotherapeutic agent endowed with cytotoxic action against BC and with no toxic effect on normal cells. This cytotoxic action appeared via repressing the tumorigenesis incidence, restoring the biochemical parameters and improving the histological investigations. In addition, it had a therapeutic effect rather than a protective effect against MNU-induced BC in rats. It has an anti-angiogenic, anti-metastatic, and anti-proliferative role in cancer management. It also activates apoptosis which leads to cancer controlling, indicating that we almost achieved our goal. Future elucidation is required to interpret the detailed mechanism of action which may result in the identification of potent molecules from UPME.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest in the study.

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