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Antioxidant, cytotoxic and apoptotic activities of extracts from medicinal plant *Euphorbia platyphyllos* L.

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In the present study, potential antioxidant activities of crude extracts (diethyl ether, petroleum ether, ethyl acetate, methanol and water (infusion and decoction)) from *Euphorbia platyphyllos* L. (Euphorbiaceae) were evaluated for 1,1-diphenyl-2-picryl-hydrazyl (DPPH) scavenging, cytotoxic, DNA damaging and apoptotic activities of the extracts on MCF-7 breast cancer cells of extracts by DPPH assay, trypan blue exclusion assay, comet assay and Hoechst 33258/propidium iodide double staining, respectively. *E. platyphyllos* extracts showed significant DPPH scavenging activity, except diethyl ether and petroleum ether extracts. In addition to their antioxidant activity, the same extracts and also petroleum ether extract showed a significant cytotoxic effect on Michigan Cancer Foundation - 7 (MCF-7) human breast cancer cell line. Although all of the extracts induced significant DNA damage, diethyl ether extract induced apoptosis in the middle level and the others induced apoptosis in significant level. The results of this study suggested a dose-response relationship for all extract samples. Moreover, this study confirms that crude extracts of *E. platyphyllos* possess antioxidant properties and provoke DNA fragmentation, a sign of induction of apoptosis. These results suggest that this plant has potential for source of anticancer agents for breast cancer treatments. Further studies are required for the isolation and identification of individual phenolic compounds in the extracts.

Key words: Antioxidant activity, apoptosis, comet assay, cytotoxic activity, *Euphorbia platyphyllos* L., Michigan Cancer Foundation - 7 (MCF-7) cells.

INTRODUCTION

Numerous physiological and biochemical processes in the human body and environmental factors may produce oxygen-centered free radicals and other reactive oxygen species as byproducts. Overproduction of free radicals can cause oxidative damage to biomolecules in the body, such as lipids, proteins and DNA. Oxidative damage of biomolecules eventually leads to several chronic diseases, such as diabetes, atherosclerosis, aging, cancer and other degenerative diseases in humans (Halliwell and Gutteridge, 1998; Poulson et al., 1998; Cai et al., 2004). Medicinal plants have been used for pharmaceutical and dietary therapy for several millennia. Although medicinal plants have been used for such a long time, long time, their chemical contents and pharmacological effects are not well understood in most cases.

The most commonly found active constituents in plants (fruits, vegetables, medicinal herbs etc.) include a wide variety of free radical scavenging molecules, such as phenolic compounds (e.g. phenolic acids, flavonoids, quinines, coumarins, lignans, stilbenes, tannins), nitrogen compounds (alkaloids, amines, betalains), vitamins (vitamin C and E), terpenoids (including carotenoids, sesquiterpenes, diterpenes, triterpenes) and some other endogenous metabolites (curcuminoids, polysaccharides), some of which are rich in antioxidant activity (Velioğlu, 1998; Mosaddik, 2003; Panovska et al., 2005;

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Cai et al., 2004; Larson, 1988; Ho et al., 1994; Ng et al., 2000; Cai et al., 2004). Antioxidant activity is a fundamental and important phenomenon for human life. Epidemiological studies have shown that many of these antioxidant compounds possess anti-inflammatory, anti-atherosclerotic, antitumor, anti-mutagenic, anti-carcinogenic, antibacterial or antiviral activities (Chung et al., 1998; Gali-Muhtasib et al., 2001; Nagakawa and Yokozawa, 2002; Cai et al., 2004; Yang et al. 2001). The intake of natural antioxidants has been associated with reduced risk of cardiovascular disease, diabetes and other diseases associated with aging and especially cancer (Cai et al., 2004; Mclarity, 1997; Niki, 1997). Cancer is the second leading cause of death in the world and there is as yet no completely effective medicine to treat most cancers; furthermore, today many cancer treatments are very expensive. During the 1960s, the National Cancer Institute (USA) began to screen plant extracts with antitumor activity (Monks et al., 2002). As rich sources of novel anticancer drugs, natural compounds isolated from medicinal plants have received increasing interest ever since (Cai et al., 2004).

The genus *Euphorbia* is a member of the Euphorbiaceae family, which consists of about 2000 species ranging from annuals to trees (Shi and Jia, 1997). About 102 species are dispersed in Turkey, many of which have a series of applications in either traditional medicine or as folkloric herbs. Furthermore, rubber and raw material for stain are obtained from these plant species (Sanchez et al., 1998). The compounds isolated from *Euphorbia* genus include flavonoids, triterpenoids, alkanes, amino acids and alkaloids (Singla and Pathak, 1990). Flavonoids from *Euphorbiaceae* family are well documented for their various activities such as anti-tumor (Bomser et al., 1996), anti-inflammatory (Bani et al., 2000), antioxidant (Lin et al., 2002), anti-diuretic (Yoshida et al., 1998), anti-diarrheic (Agata et al., 1991) or anti-malaria (Tona et al., 1999). The genus *Euphorbia* has been subject to intense phyto-chemical examination because of its medicinal usage for the treatment of numerous diseases such as skin diseases, gonorrhea, migraine, intestinal parasites and wart cures (Baytop, 1999; Chaabi et al., 2007).

Euphorbia platyphyllos L. (broad-leaved spurge) is a glabrous or pubescent annual plant that is found mainly in southern parts of Europe. The plant produces white, milky latex which is characteristic of Euphorbiaceae species. The latex and other plant parts of *E. platyphyllos* have been used in traditional medicine for the treatment of warts, wens, and hangnails (Hartwell, 1969). Hohmann et al. (2003) suggested that *E. platyphyllos* accumulates highly functionalized jatrophane diterpenes. Some of these compounds are of considerable interest because of their antitumor, cytotoxic, and antiviral activities (Hohmann et al., 2003). In spite of these medicinal benefits, no data have been reported previously on the antioxidant, cytotoxic and apoptotic activities of the species. Therefore, the aim of our study was to evaluate the potential antioxidant activity, cytotoxic and apoptotic activities on Michigan Cancer Foundation - 7 (MCF-7) breast cancer cells of diethyl ether, petroleum ether, ethyl acetate, methanol and water (infusion and decoction) extracts from *E. platyphyllos* L.

MATERIALS AND METHODS

Chemicals

1,1-Diphenyl-2-picryl-hydrazyl radical (DPPH), rutin, dimethyl sulfoxide (DMSO), Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), trypan blue, Triton X-100, low melting point agarose, normal melting agarose and ethidium bromide were purchased from Sigma Aldrich Co (St. Louis, USA).

Collection of plant material

Aerial parts of *E. platyphyllos* L. were collected on the 19th of April, 2006, from the vicinities of Koçarlı, Aydin, Turkey, in the Eastern Mediterranean region. The botanical identification was made by Dr. Özkan EREN. A voucher specimen was deposited in the herbarium (AYDN 877) of the Faculty of Arts and Science, University of Adnan Menderes, Turkey.

Preparation of plant extracts

Dried, ground whole plants of *E. platyphyllos* L. (200 g) were extracted with a solvent series of increasing polarity (diethyl ether, petroleum ether, ethyl acetate, methanol and water). Two liters of solvent was added to 200 g plant material. After completing the first soxhlet extraction (at 40°C for approximately 12 h, until the solvent became colorless) with diethyl ether and filtration, the plant material was dried and subjected to the second extraction with petroleum ether, the third extraction with ethyl acetate, and the fourth extraction with methanol (Goffin et al., 2003; Lee et al., 2004; Miliauskas et al., 2004; Avcı et al., 2006). The extracts were evaporated and yielded 2.795, 0.987, 1.011 and 5.327 g dried mass, respectively. After the methanol extraction, plant material was dried and subjected to the water (infusion) extraction. For water (infusion) extraction, 2 L distilled water at 80°C was added to plant material for 10 min and extract was filtered. For the second water extraction (decoction), 2 L distilled water was added to 200 g dried plant material and boiled for 10 min and extract was filtered (Ljubuncic et al., 2005). Filtered extracts were lyophilized and yielded 6.173 and 5.930 g dried mass, respectively. Extracts were sealed in glass bottles and stored at -20°C until they were used.

Antioxidant activity

DPPH radical scavenging activity

Assay for DPPH free radical scavenging potential is based on the scavenging activity of stable 1,1-diphenyl-2-picryl-hydrazyl radical (DPPH). The free radical scavenging activity of diethyl ether, petroleum ether, ethyl acetate, methanol and water (infusion and decoction) extracts from *E. platyphyllos* L was tested for their ability to bleach the stable 1,1-diphenyl-2-picryl-hydrazyl radical (DPPH) (Brand-Williams et al., 1995). One milliliter of 0.1 mM DPPH methanol solution was added to 3 ml of various concentrations (10, 25, 50, 75,100, 150, 200 and 300 µg/ml) of extracts in methanol.

The mixture was shaken vigorously and left at room temperature. After 30 min, the absorbance of mixture was measured at λ=517 nm. Tests were carried out in triplicate. Rutin (50 and 100 µg/ml), a citrus flavonoid glycoside, was used as a standard, and the Shimadzu PharmaSpec UV–1700 UV-Visible spectrophotometer was used for measurement. Finally, the DPPH radical scavenging activity of extracts was calculated using the following equation:

Scavenging capacity % = 100 - [(Ab of sample-Ab of blank) × 100/Ab of control].

Extract concentration providing 50% inhibition (IC_{50}) was calculated from the plot of inhibition percentage against extract concentration.

Cytotoxicity assay

Cell line and culture

MCF-7 hormone dependent breast cancer cell line was used in this study. Cells were grown and maintained in humidified incubator at 37°C and in a 5% CO_2 atmosphere. DMEM supplemented with 10% FBS, 1 mM glutamine, 1% non essential amino-acids, 100 units/ml of penicillin and 100 mg/ml of streptomycin was used as the culture medium for MCF-7 cell culture. Prior to the assay, concentrated stock solutions of extracts were prepared in dimethyl sulfoxide (DMSO) and stock solutions were diluted to the required concentrations using DMEM media. The maximum percentage of DMSO present in the wells was 0.1% (v/v), a concentration that did not affect growth of cells. This datum was incorporated as a control element in all experiments. Cells were seeded at density of 5×10^4 cells/well into a sterile 24-well plate and allowed to adhere overnight. After 24 h incubation at 37°C under a humidified 5% CO_2 to allow cell attachment, the cells were treated with different concentrations (10, 25, 50, 75,100, 150, 200 and 300 µg/ml) of diethyl ether, petroleum ether, ethyl acetate, methanol and water (infusion and decoction) extracts from *E. platyphyllos* and incubated for 24 and 72 h under the same conditions. Furthermore, in order to avoid the effect of DMSO on cell proliferation and apoptosis, solvent controls were treated with similar concentrations of DMSO as used for sample preparation (in general 0.1% DMSO). A control of growth medium was also run in parallel for each time period in cell line.

Cell viability assay

For the determination of cytotoxic activity of extracts, viable cell numbers were determined using the trypan blue exclusion method (Son et al., 2003; Lee et al., 2005). Trypan blue dye exclusion was added to all cultures in a ratio of 1:1. Then, extract treatments and preparations were examined under the standard light microscope at 100X magnification. In principle, viable cells exclude an acid dye such as trypan blue; its uptake is indicative of irreversible membrane damage proceeding cell death. The ratio of live cells to dead cells (cell viability) was also determined. Standard curves were prepared and 50% cytotoxic concentrations of extracts (CC_{50}), which caused a 50% decrease in cell viability, were derived.

Determination of DNA damaging effects of the extracts

Alkaline comet assay

The comet assay is a versatile and sensitive method for measuring single-strand and double-strand breaks in DNA (Collins et al., 2008). Among the variety of methods developed for detecting DNA damage, comet assay or single cell gel electrophoresis (SSGE) assay is often used since it is fast, convenient and easy to apply. It is particularly attractive as a method to undertake *in vivo* and *in vitro* studies (Singh et al., 1988; Hartmann et al., 2003). The genotoxic effect of the extracts was determined by alkaline comet assay µg/ml (Singh et al., 1988). For the comet assay, 100, 150, 200 and 300 extract concentrations, which have high cytotoxic effect, were used. Cells were seeded at density of 5×10^4 cells/well in to a sterile 24-well plate and allowed to adhere overnight. After 24 h incubation at 37°C under a humidified 5% CO_2 to allow cell attachment, the cells were treated with 100, 150, 200 and 300 µg/ml concentrations of diethyl ether, petroleum ether, ethyl acetate, methanol and water (infusion and decoction) extracts from *E. platyphyllos* and incubated for 72 h under the same conditions. Subsequently, the cells were trypsinized and centrifuged.

After the centrifugation, cells were suspended in 150 µL of molten 0.5% low melting point agarose (LMPA) in phosphate buffered saline (PBS) without calcium and magnesium. Then 150 µL aliquots of the cell suspension were rapidly spread on three slides pre-coated with 85 µL of 1% normal melting agarose (NMA) and cover-slipped (24 × 50 mm). After the agarose was allowed to solidify for 5 min at 0°C, the coverslips were gently removed and a third layer of 75 µL LMPA was added. The slides were then placed in a tank filled with lysis solution (2.5 M NaCl, 0.1 M ethylenediaminetetraacetic acid (EDTA), pH 10, 10% DMSO and 1% Triton X-100 both freshly added) at 4°C. After 1 h, the slides were removed from the lysis solution and incubated in fresh electrophoresis buffer (0.3 M NaOH and 1 M EDTA, pH>13) for 20 min at room temperature for unwinding of DNA. Electrophoresis was then carried out at room temperature in the same electrophoresis buffer for 30 min at 0.7 V/cm and 300 mA. Finally, after electrophoresis, the slides were gently washed twice for 5 min in fresh neutralization buffer (0.4 M Tris-HCL, pH 7.59, followed by dehydration in absolute methanol. Then the slides were stained with 75 µL 1X ethidium bromide and cover slipped. For each treatment concentration, 100 randomly selected cells from each of three slides (300 cells per concentration) were evaluated for DNA damage visually using a 40X objective on a fluorescent microscope (Olympus BX 51).

The DNA damage was quantified by visual classification of cells into five categories of “comets” corresponding to the tail length (Anderson et al., 1994): (i) undamaged: Type 0, (ii) low-level damage: Type I, (iii) medium-level damage: Type II, (iv) high-level damage: Type III and (v) complete damage: Type IV. The extent of DNA damage was expressed as the mean percentage of cells with low, medium, high and complete damaged DNA, which was calculated as the sum of cells with damage from Types I, II, II and IV. From the arbitrary values assigned to the different categories (from Type 0=0 and Type 4=4) a genetic damage index (GDI) was calculated for each concentration level (Pitarque et al., 1999).

Apoptosis assay

Hoechst 33258 and propidium iodide (HOPI) double staining

Apoptotic effect of the extracts (at 100, 150, 200 and 300 µg/ml concentrations) was determined by Hoechst 33258, and propidium iodide staining, which allows for distinguishing between apoptosis and necrosis, was performed according to the method described by Grusch et al. (2002). MCF-7 cells (5×10^4 per ml) were seeded in 24-well plate and treated with 100, 150, 200 and 300 µg/ml concentrations of the extracts for 72 h. Hoechst 33258 (HO) and propidium iodide (PI) were added directly to the culture medium to final concentrations of 5 and 2 mg/ml, respectively. After an incubation period of 1 h at 37°C, the cells were examined under an Olympus BX 51 fluorescence microscope equipped with appropriate filters for Hoechst 33258 and PI. This method allows for a distinction between early apoptosis, late apoptosis and necrosis. Cells were counted under the microscope and the number of

Table 1. DPPH radical scavenging activity of the *E. platyphyllos* L. extracts.

Sample	IC_{50} value (µg/ml)±SD
Rutin	7.77 ± 0.03
Diethyl ether	-
Petroleum ether	-
Ethyl acetate	11.71 ± 0.02
Methanol	8.50 ± 0.03
Infusion (Water)	9.70 ± 0.01
Decoction (Water)	12.50 ± 0.01

*p<0.05.

apoptotic cells was given as a percentage.

Statistical analysis

All experiments were performed in triplicate and analyzed by one way ANOVA (SPSS 11.5 program). Statistically significant difference was considered at the level of p<0.05.

RESULTS

Antioxidant activity of *E. platyphyllos*

DPPH is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule (Soares et al., 1997). The reduction capability of DPPH radicals was determined by the decrease in its absorbance at 517 nm induced by antioxidants. Hence, DPPH is generally used as a substrate to evaluate anti-oxidative activity of antioxidants (Oyaizu, 1986). The concentration of an antioxidant needed to decrease the initial DPPH concentration by 50% (EC_{50}) was used to measure the antioxidant activity of crude extract and isolated compounds (Du Toit et al., 2001; Sanchez et al., 1998; Tshikalange et al., 2007). The EC_{50} values of crude EP extract and rutin are reported in Table 1. Results indicated that the tested crude extract and rutin showed a potent DPPH radical scavenging activity with EC50 values ranging from 11.7 ± 0.02 to 7.77 ± 0.03 µg/ml. The crude extract showed a concentration-dependent radical scavenging activity. The ethyl acetate, methanol and water (infusion and decoction) extracts showed a stronger DPPH scavenging activity, while diethyl ether and petroleum ether extracts showed no DPPH scavenging activity. Moreover, the studied extracts have very high anti-oxidative capacity at different concentrations. The lower the EC_{50} value, the higher is the antioxidant activity (Atoui et al., 2005; Banerjee et al., 2005; Chan et al., 2007; Loo et al., 2008). In our results, it is important to note that ethyl acetate, methanol, and water (infusion and decoction) extracts showed a high antioxidant activity and these values are comparable to those of rutin (7.77 ± 0.03).

Cytotoxic activity of the *E. platyphyllos* extracts

The cytotoxic effects (CC_{50}) of crude *E. platyphyllos* (EP) extracts on MCF-7 cells are reported in Table 2. When MCF-7 cells were treated with various concentrations of different extracts from EP for 24 and 72 h, it was found out that cell survival rates were decreased compared with the control. The CC_{50} for different extracts was calculated using the trypan blue assay (Table 2). Petroleum ether, ethyl acetate, methanol and water (infusion and decoction extracts) extracts exhibited significant inhibition of cell viability, while the diethyl ether extract did not show a consistent effect on viability. On the other hand, diethyl ether extract showed no significant influence on viability of MCF-7 cells in the ranges of applied concentrations and treatment times. Therefore, CC_{50} value of the diethyl ether extract was not determined.

On the other hand, the water (infusion and decoction) and ethyl acetate extracts showed even better cytotoxic effect against MCF-7 compared to other extracts in terms of the percentage survival of cancer cell lines for highest concentrations at exposure time 72 h. Cytotoxic effects were seen at highest test concentration (300 µg/ml) with cytotoxicity percentage of 92.33, 93.67 and 91.33%, respectively. Calculation of the CC_{50} values for the extracts confirmed that the water (infusion and decoction) and ethyl acetate extracts were more effective against MCF-7 (CC_{50}; 38.29 ± 0.57, 27.79 ± 0.58 and 46.24 ± 0.57, respectively). Ethyl acetate, methanol and petroleum ether extracts had low activity against MCF-7 cancer cells, which was inhibited at 24 h exposure time (CC_{50}; 296.10 ± 0.58, 200.00 ± 0.58 and 186.72 ± 0.57, respectively); however, at 72 h exposure, cytotoxic activity of these extracts increased significantly (CC_{50}; 46.24 ± 0.57; 97.16 ± 0.51 and 98.07 ± 0.58, respectively). These data indicate that the ethyl acetate and water extracts of *E. platyphyllos* are more active on especially breast cancer cells.

DNA damaging effects of *E. platyphyllos* extracts on MCF-7 cells

In these experiments, MCF-7 cells were treated with different extract doses of EP for 72 h. Table 3 shows the percentage of damaged nuclei, genetic damage index and arbitrary units as measured in the alkaline comet assay. Data obtained from the comet assay indicated that all *E. platyphyllos* plant extracts were found to induce significant DNA damage after 72 h of treatment compared to control groups (p<0.05) (Figure 1). Data generated from the comet assay also indicated a significant dose-dependent increase in DNA damage in MCF-7 cells associated with EP treatment. Genetic damage index and arbitrary unit values also increased in extract treatment

Table 2. Cytotoxic activity of the *E. platyphyllos* L. extracts on MCF-7 cells

Sample	CC_{50} value (µg/ml) ± SD at 24 h	CC_{50} value (µg/ml) ± SD at 72 h
Control	-	-
Solvent control (DMSO)	-	-
Diethyl ether	> 300	> 300
Petroleum ether	186.72 ± 0.57	98.07 ± 0.58
Ethyl acetate	296.10 ± 0.58	46.24 ± 0.57
Methanol	200.00 ± 0.58	97.16 ± 0.51
Infusion (Water)	141.66 ± 0.60	38.29 ± 0.57
Decoction (Water)	80.36 ± 0.57	27.79 ± 0.58

*p<0.05.

Table 3. Analysis of DNA damage as measured by comet assay in MCF-7 cells treated with *E. platyphyllos* L. extracts.

Samples	Concentrations (µg/ml)	Proportion of damaged nuclei (%)				Percent of damaged cells	Genetic damage index (GDI)	Arbitrary units (Au)
		Type I	Type II	Type III	Type IV			
Control	-	1.00	1.33	0.00	1.00	3.33	0.10	30
Solvent control (DMSO)	0.1%	1.33	0.33	1.67	2.00	5.33	0.15	46
Diethyl ether	100	0.67	2.00	7.00	22.00	32.33*	1.14*	341
	150	0.00	2.67	8.67	32.66	43.33*	1.62*	486
	200	0.00	11.00	5.33	34.67	51.00*	1.77*	530
	300	5.67	9.00	14.67	38.00	67.33*	2.20*	659
Petroleum ether	100	3.67	7.00	11.00	24.00	45.67*	1.47*	440
	150	6.33	9.00	10.00	28.33	53.66*	1.68*	503
	200	6.67	8.00	10.00	33.67	58.33*	1.87*	562
	300	4.00	4.00	7.67	60.33	76.00*	2.76*	829
Ethyl acetate	100	3.33	2.33	3.00	5.67	14.33	0.40	119
	150	3.33	2.67	3.33	7.00	16.33	0.47	140
	200	3.67	3.33	5.33	10.00	22.33*	0.66*	199
	300	9.33	6.67	11.00	13.67	40.66*	1.10*	331

Table 3. Contd.

Methanol	100	19.67	13.33	10.33	19.00	62.33*	1.53*	460
	150	24.67	17.33	15.67	26.33	84.00*	2.12*	635
	200	13.67	10.66	15.00	50.00	89.33*	2.80*	840
	300	13.33	11.67	13.66	57.00	95.66*	3.06*	917
Infusion (water)	100	15.67	13.33	15.66	28.00	72.66*	2.01*	604
	150	15.00	11.33	12.33	39.00	77.66*	2.31*	692
	200	13.67	14.33	13.00	53.00	93.67*	2.93*	880
	300	9.66	11.67	12.33	60.66	94.33*	3.13*	930
Decoction (water)	100	15.33	12.33	14.00	28.33	70.00*	2.08*	623
	150	15.00	14.00	12.67	37.67	79.33*	2.32*	695
	200	13.33	14.67	13.00	50.33	91.33*	2.83*	849
	300	10.33	11.00	12.00	58.00	92.00*	3.00*	901

*p<0.05. GDI: (Type 1+ 2.Type II+ 3.Type III+ 4.Type IV) / (Type 0+ Type I+ Type II+ Type III+ Type IV) (38).

groups depending on dose compared to control groups and reached a statistical significance, mainly by the increased percentage of type II, III and IV damages in the extract treatment groups ($p<0.05$). We observed a significant increase in percentages of DNA damage at all testing doses, which is an evidence of EP-induced DNA damage in MCF-7 cells. DNA damaging effect of extracts on MCF-7 cells after 72 h increased in the order of infusion > methanol > decoction > petroleum ether > diethyl ether > ethyl acetate. Taken together, our findings suggest that exposure significantly ($P < 0.05$) reduces cellular viability and induces DNA damage in MCF-7 cells.

Apoptotic effects of the *E. platyphyllos* extracts on MCF-7 cells

MCF-7 cells were treated with various doses (100, 150, 200 and 300 µg/ml) of *E. platyphyllos* extracts for 72 h. As indicated in Table 4, apoptotic induction was moderate (8.66 to 22.00%) after treating with diethyl ether extract, while the other extracts induced significantly high apoptotic induction after 72 h and this effect was dose-dependent (Table 4). Although diethyl ether induced moderate apoptosis, these values were found to be statistically significant. Apoptotic effect of extracts on MCF-7 cells after 72 h increased in the order of decoction (water) > infusion (water) > methanol > ethyl acetate > petroleum ether > diethyl ether. Furthermore, it was found that the extracts induced slight necrosis in MCF-7 cells. The change of nuclear morphology in the MCF-7 cells after treatment with 100, 150, 200 and 300 µg/ml extracts for 72 h was observed under fluorescence microscopy (Figure 2). Hoechst 33258 staining showed that there were significant morphological changes in the nuclear chromatin. In the untreated group, the cells were stained with a less bright blue color which was homogeneous. After treating with *E. platyphyllos* extracts for 72 h, the blue emission light in apoptotic cells was much brighter than the control cells and necrotic cells appeared in violet-red fluorescence without chromatin condensation and apoptotic bodies. As shown in Table 4, all of the cells treated by extract show typical apoptotic features in the treated cells, including cell shrinkage, nuclear fragmentation marginalization, chromatin condensation and production of membrane-bound apoptotic bodies. Apoptotic bodies, a specific and distinct feature of apoptotic cells, were found in the majority of treated cells. These results suggest that their inhibitory effect on cell growth might be through induction of apoptosis.

DISCUSSION

Historically, plants, herbs and spices are a folkloric source of medicinal agents. However, as

Table 4. Apoptotic effects of the *E. platyphyllos* L. extracts on MCF-7 cells.

Sample	Concentration (µg/ml)	Total cells	Apoptotic cells (% ± SD)	Necrotic cells (% ± SD)
Control	-	300	1.66 ± 0.00	-
Solvent control (DMSO)	0.1%	300	2.00 ± 0.01	-
Diethyl ether	100	300	8.66 ± 0.54*	1.00 ± 0.00
	150	300	11.00 ± 0.57*	2,33 ± 0.01
	200	300	14.66 ± 0.60*	2.66 ± 0.01
	300	300	22.00 ± 0.60*	3.66 ± 0.02
Petroleum ether	100	300	32.00 ± 1.00*	4.00 ± 0.01
	150	300	37.33 ± 0.60*	6.00 ± 0.03
	200	300	40.66 ± 0.58*	6.66 ± 0.05
	300	300	53.00 ± 1.00*	8.00 ± 0.06*
Ethyl acetate	100	300	28.00 ± 0.57*	4.66 ± 0.02
	150	300	37.33 ± 0.60*	6.00 ± 0.05
	200	300	41.66 ± 1.00*	7.66 ± 0.06*
	300	300	51.66 ± 1.00*	9.00 ± 0.06*
Methanol	100	300	29.00 ± 0.60*	7.00 ± 0.05
	150	300	38.33 ± 0.58*	9.33 ± 0.06*
	200	300	50.66 ± 1.00*	11.00 ± 0.09*
	300	300	57.33 ± 1.00*	13,66 ± 0.10*
Infusion (water)	100	300	37.66 ± 0.54*	6.00 ± 0.05
	150	300	45.00 ± 0.58*	8.66 ± 0.06*
	200	300	57.33 ± 0.60*	7.00 ± 0.05
	300	300	66.00 ± 1.00*	10.33 ± 0.09*
Decoction (water)	100	300	40.00 ± 0.50*	7.66 ± 0.06*
	150	300	47.33 ± 0.57*	9.00 ± 0.06*
	200	300	58.00 ± 0.60*	11.66 ± 0.09*
	300	300	71.00 ± 1.00*	14.00 ± 0.10*

*$p<0.05$.

modern medicine developed, several useful medicines were developed from lead compounds discovered in medicinal plants. This approach has provided leads against various pharmacological targets, including cancer, HIV, malaria and pain, and remains an important route to new pharmaceuticals (Akalere, 1993; Crag and Newman, 2005; Tan et al., 2006; Balunas and Kinghorn, 2005; Jones et al., 2006). Many chemo-preventive agents have been associated with anti-proliferative and apoptotic effects on cancer cells because of their high antioxidant activity, targeting signaling molecules, and preventing or protecting cells from further damage or transformation into cancer cells (Khan et al., 2007).

Free radical scavenging is generally the accepted mechanism for antioxidants to inhibit lipid oxidation. Antioxidants (inhibitors of lipid peroxidation) are important not only for preservation of food, but also for the defense of living cells against oxidative damage (Barbaste et al., 2002). The preferred method for evaluation of the scavenging free radicals activities is the 1,1-diphenyl-2 - picrylhydrazyl (DPPH) test (Brand- Williams et al., 1995; Blois, 1958). The screening of herbal extracts and their com-ponents by the DPPH scavenging assay has become a routine parameter for testing their antioxidant efficacy (Mothana et al., 2008; Jung et al., 2003). The inhibition of free radical DPPH is one of the oldest and most frequently used methods for total antioxidant potential/capacity of food and biological extracts. It is based on the ability of an antioxidant to give hydrogen radical to synthetic long-lived nitrogen radical compound DPPH, which is able to evaluate the scavenging free radical activities in a relatively short time compared to other

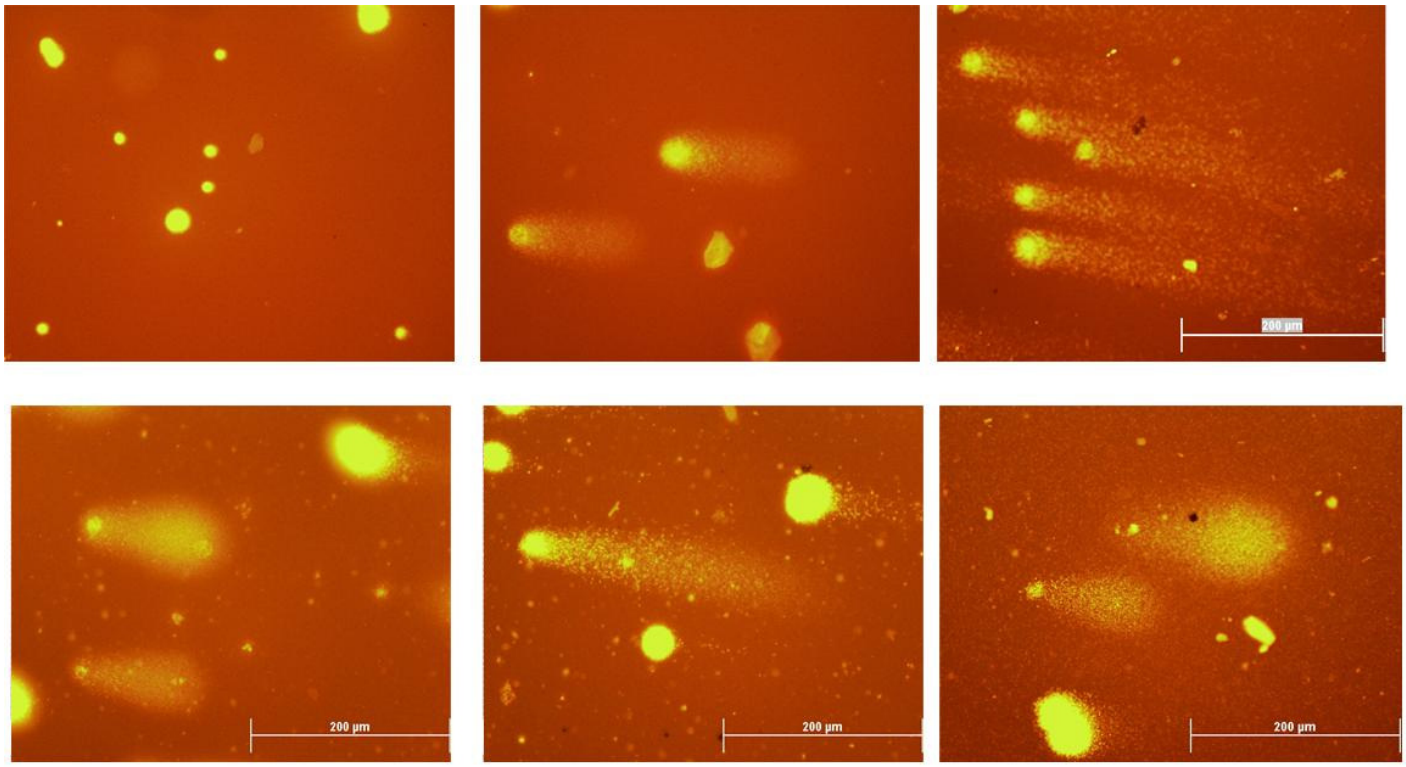

Figure 1. DNA damages after the *E. platyphyllos* extract treatments in the MCF-7 cells visualized with fluorescence microscopy. (A) control (undamaged); (B) 300 µg/ml diethyl ether extract treatment (Type III); (C) 300 µg/ml petroleum ether extract treatment (Type III); (D) 300 µg/ml methanol extract treatment (Type IV); (E) 300 µg/ml infusion extract treatment (Type I and Type IV); (F) 300 µg/ml decoction extract treatment (Type IV).

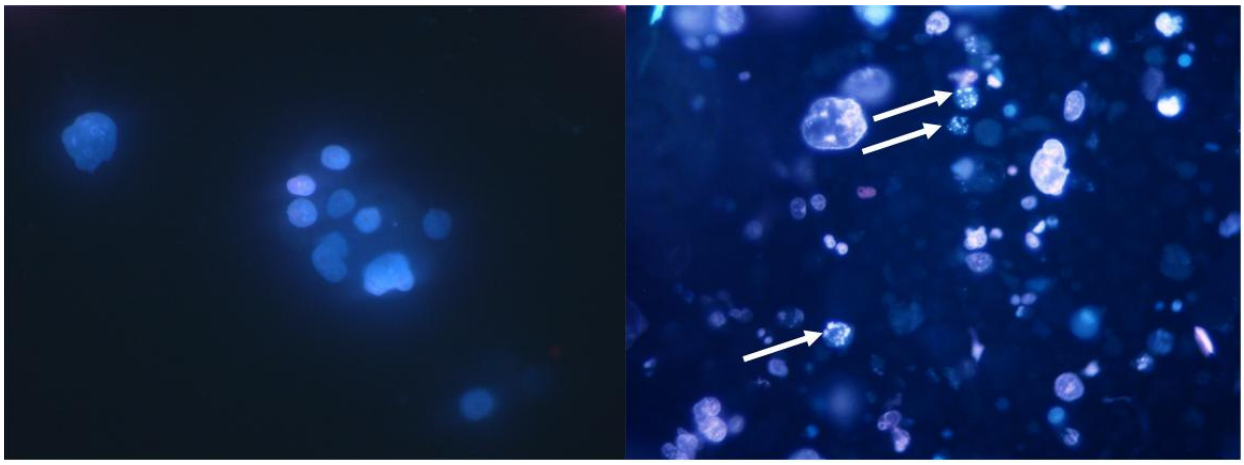

Figure 2. Apoptotic cells after the *E. platyphyllos* extract treatments in the MCF-7 cells visualized with fluorescence microscopy after HO/PI double staining. (A) control (normal) cells; (B) apoptotic cells indicated white arrow after treatment with 300 µg/ml decoction extract.

methods (Gil-Izquierdo et al., 2001).

The aim of this study was to evaluate the potential antioxidant activity and cytotoxicity and apoptotic effect of different crude extracts of E. *platyphyllos* on MCF-7 breast cell line. The different crude extracts [diethyl ether, petroleum ether, ethyl acetate, methanol and water (infusion and decoction)] of *E. platyphyllos* were assessed for their DPPH scavenging ability which was compared with the activity of well-known antioxidant, flavonoid rutin. The results of DPPH scavenging assay

suggested that extracts had radical scavenging activity. The ethyl acetate, methanol and water (infusion and decoction) extracts displayed highly significant DPPH radical scavenging activity. Additionally, antioxidant activity of rutin was determined in parallel. DPPH scavenging activities of the extracts were found to be concentration-dependent. However, the diethyl ether and petroleum ether extracts were considerably less effective radical scavengers compared to the others. It should be pointed out that diethyl ether and petroleum ether extracts during test were dissolved in methanol, and this procedure could have some effect on the measurements of radical scavenging activity as long as the extracts were not fully soluble in methanol (Miliauskas et al., 2004). Several plants have been studied for their antioxidant status. The difference in antioxidant capacity of different plant extracts may be attributed to differences in their chemical composition such as ascorbic acid, limonoids, carotenoids, terpenoids and flavonoids (Jayaprakasha et al., 2008). In the light of the proven medicinal properties of this plant, the DPPH scavenging ability of *E. platyphyllos* crude extracts implicate the strong medicinal properties of the plant.

The anti-oxidative activities of the extracts which could contribute to their cytotoxic properties were also studied. *E. platyphyllos* has also potent cytotoxicity activity. The strong cytotoxic properties of the *E. platyphyllos* extracts could be due to its high antioxidant activities. The effect of crude extracts on the viability of MCF-7 cell line was investigated by the trypan blue assay. Results of these assays suggest that all of the extracts of *E. platyphyllos,* with the exception of diethyl ether extract, were highly active against MCF-7 cell line. The water extract (decoction and infusion) and ethyl acetate extract exhibited the highest cytotoxic effect among the other extracts with CC_{50} values 27.79 ± 0.58, 38.29 ± 0.57 and 46.24 ± 0.57 against MCF-7 cell line, respectively. As for *E. platyphyllos,* this is the first study to report its cytotoxic activity against MCF-7 cell line. Different extracts of *E. platyphyllos* exhibited different activity on MCF-7 cell line. This different activity could be due to the fact that most of the active compounds in the whole plant parts may dissolve in the water instead of the organic solvent. Hohmann et al. (2003) suggested that *E. platyphyllos* accumulates highly functionalized jatrophane diterpenes. Some of these compounds are of considerable interest because of their antitumor, cytotoxic, and antiviral activities (Hohmann et al., 2003). Miglietta et al. (2003) tested jatrophanes from *Euphorbia semiperfoliata* Viv. for their interaction with purified bovine brain tubulin by an *in vitro* polymerization assay and electron microscopy. They also investigated the effects of jatrophanes on microtubular architecture, nuclear morphology, cell viability, cell cycle perturbations, as well as p53 and Raf-1/Bcl-2 involvement at a cellular level. They suggested that jatrophanes: (i) inhibited the growth of some human cancer cell lines (MCF-7 breast, HL-60 leukemia, Caco-2 colon and SK-OV-3 ovarian cells) without inducing cell cycle arrest in the G2/M phase and (ii) influenced p53 expression and Raf-1/Bcl-2 activation. Cytotoxic activity of the extracts from *E. platyphyllos* on MCF-7 cells in our study may be due to presence of jatrophane diterpenes in extracts.

The comet assay is based on the alkaline lysis of labile DNA at sites of damage, wherein cells are immobilized in a thin agarose matrix on slides, gently lysed and subjected to electrophoresis. The unwound, relaxed DNA emigrates from cells and after staining with a nucleic acid stain, the cells that have accumulated DNA damage appear as fluorescent comets with tails of DNA fragmentation or unwinding. In the present study, extract which treated MCF-7 cells with damaged DNA displayed increased migration of DNA fragments (comet tail) from the nucleus (comet head), which may also be a feature of DNA fragmentation associated with the necrotic/apoptotic death process (Fainbairn et al., 1996; Olive, 1999; Olive and Banath, 2006). Plants of *Euphorbia* genus are known for their rich content in secondary metabolites (Haba et al., 2007). This genus contains diterpenes (Lima et al., 2003; Shi et al., 2005), macrocyclic diterpenes (Rēdei et al., 2003), steroids (Tanoka et al., 1999 in Haba et al., 2007) flavonoids, triterpenoids, alkanes, amino acids, alkaloids (Singla and Pathak, 1990) and aromatic compounds (Öksüz et al., 2002). Flavonoids from Euphorbiaceae family are well documented for their various activities such as being antioxidant (Lin et al., 2002), anti-tumor (Bomser et al., 1996), anti-inflammatory (Bani et al., 2000), anti-diuretic (Yoshida et al., 1998), anti-diarrheic (Agata et al., 1991) or anti-malaria (Tona et al., 1999). Antitumor activity against sarcoma 180 ascites, leukemia in mice and cytotoxic activity against certain cancer cell lines have been reported in several species of the genus *Euphorbia* (Itokawa et al., 1989; Wu et al., 1991; Fatope et al., 1996; Betancur-Galvis et al., 2002).

DNA damage in MCF-7 cells was increased by the extract treatments at all concentrations (Table 3, Figure 1). The DNA damaging effect observed at all concentrations of the extracts might be induced by flavonoids. It has been suggested that flavonoids can intercalate with the DNA molecule (Havsteen, 1983) and it has been shown that some flavonoids are mutagenic with pro-oxidant effects (De Carvalho et al., 2003). Flavonoids might therefore cause cytotoxic effect by inducing DNA damage. Flavonoids and possibly also other components in the crude extracts can have dual actions (Demma et al., 2009). DNA fragmentation, which we observed in the MCF-7 cells treated with *E. platyphyllos* extracts, could be a result of apoptosis. Direct interaction between a DNA-reactive agent and DNA is one of several pathways that may lead to primary DNA damage, but major end-points measured in the comet assay such as DNA strand breaks and alkali-labile sites may also follow from other more indirect events such as cytotoxicity (Demma et al.,

2009).

Apoptosis is an important homeostatic mechanism that balances cell division and cell death, thus maintaining the appropriate cell number in the body. Disturbances of apoptosis in cancer cells have been studied in detail, and induction of apoptosis was one of the strategies for anticancer drug development (Martin and Green, 1995; Hu and Kavanagh, 2003). Emerging evidence has demonstrated that the anticancer activities of certain chemotherapeutic agents are involved in the induction of apoptosis and have no side effects on normal tissues, which are regarded as the preferred method of treating cancer (Xiao, 2007). Apoptosis is a form of programmed cell death that is characterized by a variety of morphological features, including changes in the plasma membrane such as loss of membrane asymmetry and attachment, cell shrinkage, chromatin condensation and chromosomal DNA fragmentation (Reed, 1997; 2000; Finkel, 1999; Sun, Hail, and Lotan, 2004). Findings of this study revealed that *E. platyphyllos* extracts have efficient apoptotic activity on MCF-7 cells and the number of apoptotic cells increased significantly after extract treatments. Data obtained from cell morphological assessment showed that *E. platyphyllos* extract could induce MCF-7 cell shrinkage as well as nuclear fragmentation marginalization, chromatin condensation and production of membrane-bound apoptotic bodies. These results indicated that *E. platyphyllos* extract may induce apoptosis in MCF-7 cells.

Conclusion

To the best of our knowledge, this is the first publication about antioxidant, cytotoxic and apoptotic effects of *E. platyphyllos* extracts. Results of this study suggest a dose-response relationship for all extract samples and indicate that this plant has potential as a source of anticancer agents for breast cancer treatments. Further studies involved the investigation about cytotoxic activity against other human cancer cell lines and molecular mechanism of isolated compound against breast cancer comparative with *E. platyphyllos* extract. Thus, the phytochemical studies together with pharmacological and toxicological investigations are essential for complete understanding of the medicinal application.

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