

RESEARCH ARTICLE

Antioxidant and Apoptotic Effects of an Aqueous Extract of *Urtica dioica* on the MCF-7 Human Breast Cancer Cell Line

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Abstract

Breast cancer is the most prevalent cancer and one of the leading causes of death among women in the world. Plants and herbs may play an important role in complementary or alternative treatment. The aim of this study was to evaluate the antioxidant and anti-proliferative potential of *Urtica dioica*. The anti oxidant activity of an aqueous extract of *Urtica dioica* leaf was measured by MTT assay and the FRAP method while its anti-proliferative activity on the human breast cancer cell line (MCF-7) and fibroblasts isolated from foreskin tissue was evaluated using MTT assay. Mechanisms leading to apoptosis were also investigated at the molecular level by measuring the amount of anti and pro-apoptotic proteins and at the cellular level by studying DNA fragmentation and annexin V staining by flow cytometry. The aqueous extract of *Urtica dioica* showed antioxidant effects with a correlation coefficient of $r^2=0.997$. Dose-dependent and anti-proliferative effects of the extract were observed only on MCF-7 cells after 72 hrs with an IC_{50} value of 2 mg/ml. This anti proliferative activity was associated with an increase of apoptosis as demonstrated by DNA fragmentation, the appearance of apoptotic cells in flow cytometry analysis and an increase of the amount of calpain 1, calpastatin, caspase 3, caspase 9, Bax and Bcl-2, all proteins involved in the apoptotic pathway. This is the first time such in vitro antiproliferative effect of aqueous extract of *Urtica dioica* leaf has been described for a breast cancer cell line. Our findings warrant further research on *Urtica dioica* as a potential chemotherapeutic agent for breast cancer.

Keywords: Breast cancer - *Urtica dioica* - MCF-7 - apoptosis - anti oxidant

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Introduction

Breast cancer is the most prevalent cancer and one of the leading causes of death among women in the world. New statistics showed that in the US one out of every eight women will suffer from breast cancer and annually more than 200,000 new cases of breast cancer are diagnosed (DeSantis et al., 2011). According to a recent assessment based on varying levels of human development it was predicted that the incidence of all-cancer cases including breast cancer as the most common cancer in women will almost double by 2030 (Bray et al., 2012). Therefore, providing effective treatment strategies will play an important role in promoting community health, particularly women's health. Surgery, chemotherapy and radiation therapy are currently used in the treatment of breast cancer, but have serious side effects. Plants and herbs due to some advantages such as being natural sources, having low side effect and low cost and also being easily accessible in comparison to common treatment methods, may play an important role in the treatment of the cancer. In recent years, many attempts have been

made to use herbs for cancer cure (Olaku and White, 2011; Huang et al., 2013; Suzuki et al., 2013). Due to the presence of flavonoids, aromatic compounds with antioxidant properties, plants can be effective in reducing the high level of oxidative stress present in cancerous cells. An association between consumption of fruits and vegetables with lower risk of cancer is widely accepted (Terry et al., 2001; Campbell et al., 2009). *Urtica dioica* (stinging nettle) is an herbaceous perennial flowering plant which grows wild in Iran. *Urtica dioica* has a variety of uses as a medicine in disease treatment such as diabetes, atherosclerosis, cardiovascular disease, prostate cancer (Konrad et al., 2000; Testai et al., 2002; El Haouari et al., 2006; Bnouham et al., 2010). In this study, we investigated the antioxidant and antiproliferative activity of the aqueous extract of *Urtica dioica* on breast cancer MCF-7 cell line with various cellular and molecular methods.

Materials and Methods

Plant extracts' preparation

Plant materials used in this research were leaves of

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Urtica dioica harvested in September, 2011 from Babol (Iran). The leaves were cleaned with water and then dried at room temperature away from direct sunlight and finely grounded with a grinder. Aqueous extraction procedures were performed by boiling 15 gr leaves in 300 ml of 5% ethanol by stirring for 15 min and then filtering through #1 filter paper (Whatman Inc., Hillsboro, OR, USA), followed by centrifugation at 5000 rpm for 15 min. The supernatant was evaporated at room temperature until dryness and the crude extracts were dissolved in deionized water and then filtered through a 0.2 μm filter and stored at -20°C for further analysis.

Antioxidant assay by MTT

Antioxidant activity was evaluated according to Muraina et al. (Muraina et al., 2009) with slight modification. Briefly, two-fold dilution of 12 mg/ml of extract was prepared with 100 ml of distilled water in a 96-well plate. Then, 10 μl of MTT (5 mg/ml) was added to every well and OD was measured at 590 nm after 0 and 8 hrs incubation at 37°C . Also, for extract stability and blank, different concentrations of extract without MTT were prepared. 250 μM Ascorbic acid was used as the positive control. All assays were repeated three times.

Ferric reducing antioxidant power assay

The ability to reduce ferric ions was measured using a modified version of the method described by Benzie and Strain (1996). An aliquot (50 μl) of the extract was added to 1.5 ml of FRAP reagent containing 10 parts of 0.3M sodium acetate buffer at pH 3.6, 1 part of 10 mM 2, 4, 6- tris (2-pyridyl)-1, 3, 5-triazine (TPTZ) solution and 1 part of 20 mM $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ solution and the reaction mixture was incubated at 37°C for 15 min. After which the absorbance was measured at 593 nm. A calibration curve was prepared, using an aqueous solution of ascorbic acid [62.5-1000 μM (serial dilution), $r^2=0.999$]. FRAP values were expressed on a fresh weight basis as micromoles of ascorbic acid equivalent per gram of sample.

Cell lines

The estrogen-receptor MCF-7 cell line was purchased from Pasteur institute, Tehran, Iran. The cells were cultured in RPMI-1640 medium (PAA, Austria) supplemented with 10% fetal bovine serum and 1% antibiotics (penicillin/streptomycin (Invitrogen) in a humidified atmosphere containing 5% CO_2 and 95% air, at 37°C . Primary human Fibroblasts were isolated from fresh foreskin isolated from children aged between 1.5-30 months who underwent routine circumcision as previously described (Pandamooz et al., 2012). Briefly 1-2 mm^3 pieces of foreskin were incubated at 37°C for 2 hrs in a 15 ml falcon tube in the presence of 0.5% dispase II (Sigma-Aldrich) then digested with 0.1% crude collagenase (Sigma-Aldrich, C2674). Cells were collected upon release every 20 minutes from the aqueous phase of the tube, passed through 70 μm cell strainer (BD Bioscience) and centrifuged before resuspending in high-glucose DMEM (PAA cat : E15-883), supplemented with 10% fetal bovine serum (FBS) (PAA, cat: A 15-15) and 1% penicillin/streptomycin (PAA cat: P11-010).

MTT assay

Anti proliferative activities of the *Urtica dioica* against MCF-7 breast cancer cell line and Fibroblasts was evaluated by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. For this purpose MCF-7 cells were plated at a density of 5×10^3 cells/well in a 96-well plate with 100 μl of culture medium in each well. Fibroblast cells were plated at a density 5×10^5 cells/well in a 24-well plate with 1 ml of culture medium in each well. After 24 hrs incubation at 37°C in 5% CO_2 , culture media were removed and replaced by new media containing different concentrations (0.375, 0.75, 1.5 and 3 mg/ml) of the extract and cells were incubated for 24, 48 and 72 hrs. After period time, the medium was discarded and the adherent cells were washed with phosphate buffer solution (PBS), then MTT stock solution (5 mg/ml) was added to each well and the plates were further incubated for 4 hrs at 37°C . Following which resulted crystals were dissolved in acidic isopropanol. After complete dissolving of formazan blue, absorbance of formazan dye at 570 nm was measured using a microplate reader (Rayto, China).

Apoptosis assay with ethidium bromide and acridine orange staining

To determine if the aqueous extract of *Urtica dioica* induced apoptosis in MCF-7 cells, cells were stained with acridine orange (AO) and ethidium bromide (EB) as fluorescent probes. The fluorescent cells were examined by fluorescence microscope. AO is a vital dye which stains cells with intact (normal cell) or fragmented nuclei (apoptotic) in green, while Ethidium bromide is taken up only by dead cells (necrotic cells) and stains them orange-red. Briefly, cells were treated with 2 mg/ml (IC_{50}) aqueous extract of *Urtica dioica* for 72 hrs in a 6-well plate. Floating and attached cells were then collected and washed with PBS, the pellet was dissolved in 250 μl PBS. Eight μl of a mixture of fluorescent dyes containing 100 pg/ml AO (Sigma Chemical, St. Louis, MO) and 100 pg/ml EB (Sigma, USA) was added to the 100 μl cells (1×10^6) and mixed gently at room temperature. Ten μl of the mixture was loaded on a neobar and examined under a fluorescence microscope.

Flow cytometry with annexin V-propidium iodide (PI) staining

Apoptotic cells were quantified by Annexin V-FITC and Propidium iodide (PI) double staining, using an Annexin V-FITC apoptosis detection kit (BioVision Research Products, Mountain View, CA, USA) according to manufacturer's protocols. MCF-7 Cells were incubated with 0, 0.375, 0.75, 1.5 and 3 mg/ml aqueous extract of *Urtica dioica* for 72 hrs, the floating and trypsinized-adherent cells were collected and washed twice with PBS, adjusted to 500 μl of the binding buffer (5×10^5 cells). Then, 5 μl of Annexin V-FITC and 5 μl of PI were added and cells were gently vortexed. Cells were then incubated for 15 min at room temperature (25°C) in the dark. Finally, cells were analyzed by a flow cytometer (Becton Dickinson).

DNA fragmentation assay

DNA preparation and agarose gel electrophoresis was

carried out according to a method previously reported by Herrmann et al. (Herrmann et al., 1994) with slight modification. Briefly, cells were cultured in 25 cm³ flask and treated with 2 mg/ml of extract for 72 hrs. After harvesting the cells samples were washed with phosphate buffer saline and pelleted by centrifugation. The cell pellets were suspended in 100 μ l lysis buffer (1% NP-40 in 20 mM EDTA, 50 mM Tris-HCl, and pH 7.5) and after incubation at room temperature for 15 min, treated for 10 min with SDS (final concentration 1%) and RNase A (final concentration 5 mg/ml) at 37°C followed by digestion with proteinase K (final concentration 1 mg/ml) for at least 5 hrs at 56°C. After addition of 1/4 vol. 10 M ammonium acetate the DNA was precipitated with 1 vol. ice-cold isopropanol, mixed thoroughly and kept for 1 h at -20°C after which the solution was centrifuged for 20 min at 12,000 rpm. The nucleic acids pellet was washed with 200 μ l 75% ice-cold ethanol and air-dried for 10 min at room temperature and finally dissolved in gel loading buffer for further separation by electrophoresis in 1.2% agarose gels.

Immunoassay (Enzyme-linked immunosorbent assay) ELISA

To evaluate the apoptotic pathway in MCF-7 cells after treatment with *Urtica dioica* extract, the amounts of calpain1, calpestatin, caspase 3, caspase 9, Bax and Bcl-2 were measured by ELISA kit (Glory Science Co., Ltd, USA). After treating cells with *Urtica dioica* extract for 72hrs, floated and adherent cells were pelleted and washed in cold PBS, and then cells were dissolved in PBS and lysed with one freeze/thaw cycle at -80°C. Lysed cells were centrifuged at 2000 rpm for 10 min to remove cell-debris. Protein concentration was determined according to the method proposed by Bradford (1976), using BSA as a standard protein. Equal amounts of protein were subjected to ELISA kit according to kit instructions.

Results

The antioxidant activity of *Urtica dioica* leave extract

The 15 gr of the powdered leave of *Urtica dioica* were observed to yield 2.32 \pm 0.288 gr of the aqueous extract corresponding to 15.46 \pm 1.92%. The antioxidant activity of the aqueous extract of *Urtica dioica* leave was evaluated by two methods, MTT and Ferric Reducing assays, Ascorbic acid, was considered as positive control. Due to the absorption of the aqueous extract at 590 nm, we measured the absorption of different concentrations of the extract (12-0.75 mg/ml) in the beginning and after eight hrs (Table1 and Figure 1A). As shown in Figure 1B, the aqueous extract of *Urtica dioica* have antioxidant effect with correlation $r^2=0.997$.

In Ferric reducing assay, because of extract absorption in 593 nm wavelength and rapid enzymatic reactions, we only made the measurement for 0.75 mg/ml of the extract which showed no absorption at 593 nm (Table2).

Effects of aqueous extracts on cell viability

Anti proliferative activities of different concentrations of the aqueous extract of *Urtica dioica* on the growth of

Table 1. Antioxidant Activities of the Aqueous Extract of *Urtica Dioica* and Ascorbic Acid Evaluated by MTT Assay

Concentration mg/ml	0 h OD Mean \pm SD	After 8 hrs OD Mean \pm SD
12	0.352667 \pm 0.023965*	0.793 \pm 0.077582*
6	0.241667 \pm 0.021032*	0.450667 \pm 0.02318*
3	0.199333 \pm 0.033081*	0.302667 \pm 0.032624*
1.5	0.173667 \pm 0.026083	0.212 \pm 0.032924
0.75	0.168 \pm 0.032078	0.192667 \pm 0.030989
0	0	0
250 μ M VC [†]	0.112 \pm 0.012583	0.246 \pm 0.020339

*The p value <0.05; [†]Vitamin C (Ascorbic Acid)

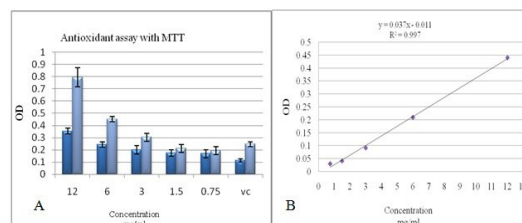


Figure 1. A) Antioxidant Activity of the Aqueous Extract of *Urtica Dioica*, 0 h (Dark Blue) and After 8 hrs (Light Blue) by MTT; and B) Regression Factor Squared for the Linear Regression, $r^2=0.997$

Table 2 Antioxidant Activity of *Urtica dioica* Extract by FRAP Assay

	Ascorbic acid	<i>Urtica dioica</i>
Concentration	250 μ M	0.75 mg/ml
OD Mean \pm SD	0.508 \pm 0.0132	0.113 \pm 0.0176

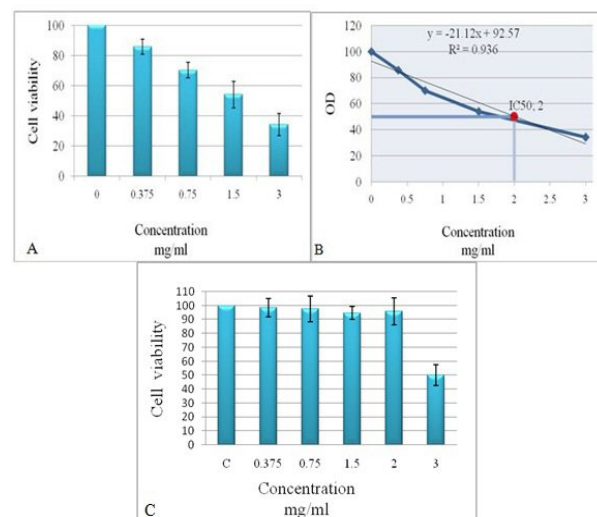


Figure 2. Dose-dependent Antiproliferative Effects of Aqueous Extracts of *Urtica dioica* Against MCF-7 cell line (A) and Fibroblast (C) after 72 hrs Incubation. B) IC₅₀ Value of Aqueous Extract of *Urtica dioica* towards MCF-7 Cell Line. Each value represents mean of three independent experiments \pm SD

the human breast cancer MCF-7 cell line and fibroblast are represented in Figure 2. Cell proliferation was analyzed after 24, 48 and 72 hrs. Data analysis showed that there was no regular difference in cell viability after 24 and

48 hrs (data not show) but after 72 hrs a dose-dependent manner anti proliferative activity was observed with correlation $r^2=0.936$ for MCF-7 but not fibroblast (Figure 2B and 2C). Statistical analysis showed that there was no significant difference in cell viability for MCF-7 cell line between 0.75 mg/ml and 1.5mg/ml and also between 1.5 and 3 mg/ml concentrations ($p>0.05$). But the difference between 0.375 mg/ml and 0.75 mg/ml was significant ($p<0.05$). Also fibroblasts treated with 0.375-2 mg/ml extract showed no significant difference in viability compared to non treated control, but in the presence of 3 mg/ml extract, a significant viability difference with non treated control was observed. Concentrations of the extracts which exhibited 50% growth inhibition (IC_{50} value) for MCF-7 cells were 2 mg/ml as shown in Figure 2B.

Urtica dioica induced apoptosis in MCF-7 cells.

Most anti-cancer drugs induce apoptosis, as a primary mechanism for inhibition of cell proliferation. To further investigate the cause of the decrease of cell viability by the

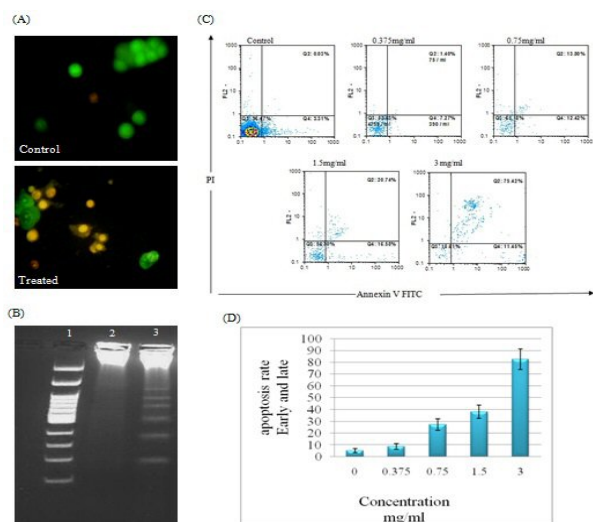


Figure 3. A) Morphological Observation with Acridine Orange and Ethidium Bromide (AO/EB) Stain. MCF-7 cells were treated without and with *Urtica dioica* aqueous extract, 2 mg/ml for 72 hrs. Magnification: 400x. **B) DNA Ladder Formation in MCF-7 Cells Treated with 2 mg/ml *Urtica dioica* for 72 hrs.** Lane 1, standard DNA marker (100bp ladder), lane 2, control; lane 3, 2mg/ml treated cells. **C) Flow Cytometric Analysis of Apoptosis/Necrotic Cell Death using Annexin-V-FITC/PI Staining of MCF-7 Cells Treated with *Urtica dioica*.** Quadrant 2 represents late apoptotic cells; Quadrant 3 represents live cells and Quadrant 4 represents early apoptotic cells. **D) Percentage of Apoptotic (Early and Late) MCF-7 Cells Induced by Different Concentrations of *Urtica dioica* after 72 hrs Treatment**

Table 3. Amount of Different Apoptotic Proteins After Treatment with *Urtica dioica* Extract

Concentration mg/ml	Total protein loaded μ g	Calpain 1 concentration IU/L	Calpestatin concentration IU/L	Caspase 3 ng/ml	Caspase 9 IU/L	Bax ng/ml	Bcl-2 ng/ml
Control	20	139.4	267.2	2.2	318.9	19.05	2.77
0.75	20	1273	3039	29.4	3331	181	24.76
1.5	20	385	850	11.41	1294	57.2	7.03
3	20	377.4	938	10.47	1019	59.3	8.3

aqueous extract of *Urtica dioica*, cells were stained with acridine orange and ethidium bromide fluorescence dyes. Annexin-V and PI were used to detect apoptotic cells. The morphological changes of the MCF-7 cells-treated with 2 mg/ml *Urtica dioica* extract for 72 hrs were compared to untreated control after acridine orange/ethidium bromide (AO/EB) fluorescence staining. As shown in Figure 3A, the apoptotic nuclei clearly had highly condensed chromatin that was uniformly fluorescent. In the control the cells nuclei were bigger than those of the cells treated with *Urtica dioica* extracts. Quantitative assessment of apoptosis was conducted using an Annexin-V assay kit. As shown in Figure 3C and 3D, treatment with different concentrations of *Urtica dioica* resulted in an increase in the percentage of early and late apoptosis (lower and upper right quadrant, respectively) in a concentration dependent manner. We also assessed the effects of *Urtica dioica* on induction of apoptosis in MCF-7 cells by DNA fragmentation assay. DNA extracted after 72 hrs treatment with 2 mg/ml of aqueous extract of *Urtica dioica* revealed a ladder corresponding to DNA fragmentation in agarose gel electrophoresis (Figure 3B) which gave an indication of apoptotic cell death induction by *Urtica dioica* extract. Furthermore, ELISA results emphasized that *Urtica dioica* extract caused cell death in MCF-7 cells via apoptosis proteins increase (Table 3). As shown in Table 4, the ratio of different apoptotic proteins is approximately equal to 1 for each concentration of aqueous extract of *Urtica dioica* analyzed and therefore the amounts of those proteins are similar with no significant difference between them.

Discussion

Recent analyses raise concerns about the increase of cancer prevalence in the near future (Bray et al., 2012). Therefore, looking for natural and low cost drugs that prevent cancer development is becoming an important purpose for researchers. In this study, we investigated antioxidant, antiproliferative and apoptotic effects of aqueous leaf extract of *Urtica dioica* on MCF-7 cell line. The antioxidant activity of the aqueous extract of *Urtica dioica* leave was evaluated by MTT and FRAP assay. Both methods confirmed that the aqueous extract of *Urtica*

Table 4. Increase Rate of Different Apoptotic Proteins After Treatment with *Urtica dioica* Extract

Different Concentration/control	Calpain 1	Caplestatin	Caspase		Bax	Bcl-2
			3	9		
0.75/Control	9.12	11.37	13.3	10.44	9.5	8.94
1.5/Control	2.76	3.18	5.1	4.05	3	2.76
3/Control	2.7	3.5	4.7	3.19	3.11	3.26

dioica contains antioxidant activity. Although while comparing to ascorbic acid, 15 gr powdered leave of *Urtica dioica* had an antioxidant activity approximately equivalent to 26.192 mg and 30.311 mg of pure ascorbic acid in MTT and FRAP assays respectively, but this difference in the amount of antioxidant activity may be due to different substrates used in these two methods. Moreover, Flavonoids such as Quercetin, Kaempferol (Apak et al., 2007) and so which may be present in *Urtica dioica* may have different abilities to convert substrate to product. As drugs used to treat cancer are cytotoxic and/or cytostatic, the potential cytotoxic effect of *Urtica dioica* was investigated by determining its effect on the viability of MCF-7 cell line and fibroblast cells with MTT assay. The results showed that *Urtica dioica* aqueous extract caused significant growth inhibition of MCF-7 cells in a dose-dependent manner with correlation $r^2=0.936$. In a related study, the cytotoxic activity of methanolic extract of *Urtica dioica* on prostate cancer cells determined by MTT assay showed a 30% maximum growth reduction at concentrations between 1-6 mg/ml after day 5 (Konrad et al., 2000). In another study, the percentage of cell survival of MCF-7 cells following 72 hrs exposure to 50 μ g/ml ethanol extracts fractions from *Urtica dioica* leaves and stems were demonstrated to be 93.12 ± 8.88 (Abu-Dahab and Afifi, 2007). Recently, the cytotoxicity effect of *Urtica dioica* on MCF-7 and MDA 231 cell lines was determined using the XTT assay (Guler, 2013). The survival differences observed could be due to differences in cell types investigated (as there are different MCF-7 cell line types) and the extraction procedures. The potent cytotoxicity activity of the extract of *Urtica dioica* could be due to its polyphenols which have a vast range of actions such as antioxidant, antibacterial, antimutagenic, and antiproliferative properties (Peterson and Dwyer, 1998; Nijveldt et al., 2001; Apak et al., 2007). Interestingly, Bosetti et al. reported an inverse association between flavanoids and breast cancer risk (Bosetti et al., 2005). The observed cytotoxic effect of 3 mg/ml *Urtica dioica* extract on foreskin fibroblasts might be due to the fact that foreskin fibroblasts are in some characters similar to foetal fibroblasts (Chen et al., 1989). In fact, previous studies indicated that tumor-derived fibroblasts, skin fibroblasts of breast cancer patients and foetal fibroblasts secrete a migration stimulating factor (MSF) which is not secreted by normal fibroblasts (Schor et al., 1988a; 1988b). Schor et al. (1987) showed that cancer development susceptibility in adults increased in the presence of foetal-like fibroblasts. Therefore, foreskin fibroblasts growth inhibition caused by the high concentration of *Urtica dioica* extract, can be interpreted as an anticancer effect. Since, most drugs used in cancer treatment also induce apoptosis, a type of programmed cell death characterized by specific morphological changes such as nuclear fragmentation, condensation of chromatin, blebbing of the plasma membrane, and the presence of apoptotic bodies (Janicke, 2009), to further investigate the cause of the decrease of cell viability by the aqueous extract of *Urtica dioica*, we used different methods to evaluate qualitatively and quantitatively apoptotic cells. Quality assessment with AO/EB staining showed that after treating cells with

Urtica dioica extract for 72 hrs, apoptotic nuclei clearly had highly condensed chromatin that was uniformly fluorescent in comparison with control. This suggests that the cell death observed was due to apoptosis, but not necrosis. For further analysis of apoptosis, quantitative assessment was conducted using flow cytometry with Annexin-V FITC kit. Our findings indicated that treatment with different concentrations of *Urtica dioica* extract resulted in an increase in the percentage of early and late apoptotic cells in a concentration dependent manner with $r^2=0.984$ similar to the antiproliferation assay. This data emphasizes that *Urtica dioica* affects cell proliferation by activating the apoptosis pathway. A family of cytosolic proteases, the caspases, plays an essential role in apoptosis. They can be divided into two major subgroups, initiator (caspases-2, -8, -9, and -10), which activate the effector caspases (caspases-3, -6 and -7). Initiator caspases are activated by apoptotic signals, resulting in the activation of the effector caspases (Devarajan et al., 2002; Mc Gea et al., 2002). One of the major pathways for activation of caspase-9 (initiator caspase) involves calcium overload of mitochondria to trigger cytochrome c release (Yu et al., 2007), leading to the activation of caspase-9, and the subsequent activation of caspase-3. Calpain1 and its endogenous specific inhibitor, calpastatin, which is also activated in some apoptotic systems, like caspase-3, is a cytosolic cysteine protease, but requires Ca^{2+} for its activity (Cui et al., 2007; Salehin et al., 2010). Caspase 3, the most commonly activated caspase that plays a critical role in apoptotic pathway by cleaving some proteins, exhibits some of the typical morphological and biochemical features such as membrane blebbing, induction of phagocytes migration to the site of apoptotic cells and DNA fragmentation (Jänicke, 2009). The Bcl-2 proteins family also have important role in apoptosis and are divided into anti-apoptotic (Bcl-2, Bcl-xL and Bcl-W) and pro-apoptotic proteins (Bax, Bak and Bid). By modifying mitochondrial membrane permeability, these proteins cause the release of cytochrome c and subsequently caspase cascade activation and consequently initiate apoptosis (Harris and Thompson, 2000). Bax/Bcl-2 ratio may be used to determine cell survival or death after treatment by an apoptotic drug (Oltvai et al., 1993). Our data indicate that following the addition of *Urtica dioica* aqueous extract, MCF-7 cells displayed significant increases in calpain 1, calpastatin, caspase 3, caspase 9, Bax and Bcl-2 activity in an inversely dose-dependent manner with the ratio of these different proteins remaining approximately equal to 1. The lower activity of apoptotic proteins observed with high concentrations of *Urtica dioica* extract, could be due to the fact that apoptosis takes place sooner with those concentrations compared to low concentrations (as confirmed by our flow cytometry analyses (Figure 3C, D), leading to subsequent proteins degradation. Although molecular studies concerning the mechanism of apoptosis induction in MCF-7 cell lines are contradictory, indicating for some caspase 9 activation (Cui et al., 2007; Liang et al., 2001), others demonstrating both caspase 9 and caspase 3 independent or independent mechanisms (Ofir et al., 2002; Shim et al., 2007) or caspase 3 dependent activation under some circumstances

(Jänicke et al., 1998; Nizamutdinova et al., 2007) we believe that these different results may be due to the usage of different MCF-7 cell lines, which for some have no active pro-caspase 3 protein. O'Donovan et al. (O'Donovan et al., 2003) showed that the levels of both precursor and active forms of caspase 3 were significantly higher in carcinomas compared to both fibroadenomas and normal breast tissues. Also Devarajan et al. (2002) suggested that the loss of caspases 3 expression may represent an important cell survival mechanism in breast cancer patients and reported that MCF-7 cells without active caspase 3 show chemoresistance to some drugs. Since the MCF-7 cell line used in this study has active caspase3, it is then a valuable *in vitro* model for studying the effect of plant extracts or synthetic drugs on breast cancer cells survival as most carcinomas possess active caspase 3. However, the use of *Urtica dioica* extract on MCF-7 variants without active caspase 3 can be considered for studying the efficacy and the possible caspase 3 independent mechanisms of action of naturally derived or synthetic drugs on breast cancer treatment.

Furthermore, in this study although the amount of both Bax and Bcl-2 increased, their ratio remained close to 1 after 72 hrs treatment with *Urtica dioica* aqueous extract. The increase of Bax as a pro-apoptotic protein leading to apoptosis increase was expected as confirmed by flow cytometry and MTT assay results. While the increase of Bcl-2 as an anti-apoptotic protein can be intriguing. However, Lam et al. (2009) reported an increase of Bcl-2 levels and breast carcinoma cells growth inhibition upon treatment with antiestrogens (Tamoxifen and ICI 182,780). Bcl-2 may induce apoptosis via interaction with nuclear orphan receptor Nur 77/TR3 (Lin et al., 2004) and convert to Bax-like death effector (Cheng et al., 1997). Tophkhane et al. (2007) also reported a relationship between the overexpression of Bcl-2 in breast cancer and diagnosis and hormonal therapy response improvement. They demonstrated that the interaction of genistein, an anti-tumor drug, with Bcl-2 convert Bcl-2 to a pro-apoptotic protein and subsequently increase cytochrome c, leading to apoptosis (Tophkhane et al., 2007).

Taken together, our findings warrant further researches on *Urtica dioica* as a potential chemotherapeutic agent for breast cancer including the evaluation of its antitumor activity in animal models.

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