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Original Research

Effects of Urtica dioica dichloromethane extract on cell apoptosis and related gene expression in human breast cancer cell line (MDA-MB-468)

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Abstract: Breast cancer is the most common cancer among women in worldwide, especially in developing countries. Therefore, a large number of anticancer agents with herbal origins have been reported against this deadly disease. This study is the first to examine the cytotoxic and apoptotic effects of Urtica dioica in MDA-MB-468, human breast adenocarcinoma cells. The 3-(4,5-dimethylethiazol-2 yl)-2,5- diphenyltetrazolium (MTT) reduction and trypan-blue exclusion assay were performed in MDA-MB-468 cells as well as control cell line L929 to analyze the cytotoxic activity of the dichloromethane extract. In addition, Apoptosis induction of Urtica dioica on the MDA-MB-468 cells was assessed using TUNEL (terminal deoxy transferase (TdT)-mediated dUTP nick- end labeling) assay and DNA fragmentation analysis and real-time polymerase chain reaction (PCR). The results showed that the extract significantly inhibited cell growth and viability without inducing damage to normal control cells. Nuclei Staining in TUNEL and DNA fragments in DNA fragmentation assay and increase in the mRNA expression levels of caspase-3, caspase-9, decrease in the bcl2 and no significant change in the caspase-8 mRNA expression level, showed that the induction of apoptosis was the main mechanism of cell death that induce by Urtica dioica extract. Our results suggest that urtica dioica dichloromethane extract may contain potential bioactive compound(s) for the treatment of breast adenocarcinoma.

Key words: Urtica dioica, breast cancer, MDA-MB-468 cell line, cytotoxicity, apoptosis.

Introduction

Breast cancer is second cause of cancer death among women following lung cancer. Thus, seeking a successful treatment for this disease is a priority (1). Using herbals for the treatment of malignancies are popular in many Asian cultures, because some herbals contain several anti-cancer compounds including, flavonoid, tannin and etc. In addition, beneficial compounds from these herbals are being used in the production of different modern anticancer drugs (2-4).

Urtica dioica is a family of plants containing 500 species and 40 genera that are widely distributed around the world, especially, in Europe, Asia, and northern Africa (5). Urtica dioica is frequently used herb in cancer therapy such as, prostate cancer (6). Both stem and leaves of this plant use. In fact, it is traditional herb used as an adjuvant therapeutic agent in several diseases including rheumatoid arthritis (7). Some evidence of immune-modulator properties of *Urtica dioica* has also appeared in the literature (8, 9). In breast cancer, phytopharmaceuticals are commonly prescribed (10).

In an animal study, it has been found that dichloromethane extract of Urtica diorica significantly inhibited experimentally induced prostate growth (11).

Apoptosis is a biological process that preserve homeostasis in living cells without causing inflammation (12, 13). Caspases play a notable role in main stages of administration phase of apoptosis (14). During the detection of Caspases, Caspase-3 typically is activated by numerous death signals (15, 16). Fundamentally, there are two key signaling pathways for cellular apoptosis: 1) the intrinsic or mitochondria pathway that response to intracellular stimulant and results in cytochrome c release from the mitochondria causing to the activation of Caspase-9; and 2) the extrinsic death receptor pathway begins by ligand binding to extracellular cell death receptors causing in caspase-8 activation. Both pathways cause activation of caspase-3 (17). Caspase activation effects particular substrates, resulting biochemical and morphological alteration in the cells such as cell condensation of chromatin, shrinkage, and cleavage of DNA (18-20). Consequently, the caspase activity can be a marker for apoptosis. Otherwise, Bcl-2 is an anti-apoptosis protein acting as an inhibitory role in apoptosis (21-23).

There have been no previous studies of the effects of *Urtica dioica* (*U. dioica*) on the breast adenocarcinoma cell line (MDA-MB-468). In the present study, we explored the effects of *Urtica dioica* extract on the expression levels of Caspase-3 and Bcl-2 genes, as the main markers of apoptosis. To specify whether the apoptosis was organized via intrinsic or extrinsic pathway, changes in the expression of Caspase-9 mRNA was examined by quantitative real-time PCR.

Materials and Methods

Preparation of the Plant extract

Plant materials used in this research were leaves of *Urtica dioica* harvested in May 2014 from East Azerbaijan (Iran). Plants' materials were dried at room tempe-

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rature and dark. Then, the plants samples were crushed by a mechanical grinder to optimum particle size. Then transfer herbs to10 litter extracting Reactor. 2.5 litter dichloromethane was added for each 200gr of *Urtica dioica* powder and mixed for 24 h. All extracts were filtered using Whatman no. 400 filter paper. The filtrate was subjected to evaporation under reduced pressure whereby a gummy mass was obtained which was stored at 4 ˚C for further use.

Cell line and culture

The MDA-MB-468 breast adenocarcinoma cell line and L929 fibroblast cells (Pasteur Institute, Tehran, Iran) were grown in RPMI-1640 medium (Sigma-Aldrich, St. Louis, MO, USA) containing 10% fetal bovine serum (FBS) (Sigma-Aldrich, St. Louis, MO, USA), 1% antibiotics (100 IU/ml penicillin, 100 µg/ml streptomycin) (Sigma-Aldrich, St. Louis, MO, USA), at 37˚C in a humidified atmosphere containing 5% CO2. The cells were sub-cultured 24-48 h later with an initial concentration of 4×10^4 cells/ml and used in the logarithmic phase in all experiments.

Cell proliferation assays

MTT assay for cytotoxicity

The effect of *Urtica dioica* on the breast cancer cells was measured by MTT assay. The cells (8×10^4 per ml) were plated into 96-well culture plates. After overnight incubation, the cells were treated with *Urtica dioica.* The experiment was subdivided into seven groups: treated cells with *Urtica dioica* 10, 20, 30, 40, 50, 60 µg/ml and non-treated cells (Control). After incubation for the 24 and 48h, 2 mg/mL of MTT (Sigma-Aldrich, St. Louis, MO, USA) was added to each well for an additional 4 h. The blue MTT formazan precipitate was then dissolved in 200 µL DMSO. The absorbance at 570 nm was measured on a multi-well plate reader. The concentration that produced 50% cytotoxicity (IC50) was determined using GraphPad Prism 6.01 software (GraphPad Software Inc., San Diego, CA, USA).

Cell viability assay

Cells were seeded on 24-well tissue culture plates at the density of 5×10^4 cell per well in RPMI supplemented with 10% FBS in the presence and absence (as controls) of *Urtica dioica* extract at different concentrations as indicated. The cultures were maintained at 37 ˚C in a tissue culture incubator containing 5% CO2 for 5 days. The cells were collected by trypsinization and stained with 0.4% trypan blue. Then it was allowed to stand at room temperature for 3 min. The dead cells and the total cells were counted. The dead cells and the total cells were counted. Viable cells (%) = [(total cells - dead cells) / total cells] × 100%.

Apoptotic effects of the dichloromethane extract of Urtica dioica

TUNEL

The Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) (Roche, Germany) assay is based on the activity of Terminal deoxynucleotidyl Transferase (TdT) enzyme that adds probed nucleotides to the ends of broken DNA strands. Probed nucleotides bind to the free 3'-hydroxyl end of double- or single-stranded DNA. The MDA-MB-468 breast adenocarcinoma cells were cultivated at a density of 15×10^3 cells/well in 96-well plates and then after overnight incubation, the cells were treated with IC50 dose of ***Urtica dioica*** extract. After 48 h of incubation, cells were fixed with 4% paraformaldehyde (Merck, Germany) solution in PBS for 1 h at room temperature, treated with 0.3% H2O2-methanol solution, and then permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate solution for 2 min on ice. The TUNEL assay was carried out following the manufacturer's instruction.

DNA fragmentation assay

DNA fragmentation patterns were examined by electrophoresis of extracted genomic DNA from the MDA-MB-468 cells following treatment with dichloromethane extract of *urtica dioica* (50 µg/ml) to detect oligonucleosomal DNA fragmentation, which is a hallmark of apoptosis. Cells were grown in T-25 culture flasks and incubated with the dichloromethane extract of *Urtica dioica* for 24 h. After incubation, the cells were harvested and washed with phosphate-buffered saline (PBS). Cells were re-suspended in 200 µl of PBS supplemented with 20 µl of proteinase K. The DNA was extracted using with phenol, chloroform, ethanol by following the Abcam DNA isolation kit manufacture protocol. The samples were subjected to electrophoresis at 80 V for 2 h in 1 % agarose gel containing 2µl of DNA green view. DNA fragmentation patterns were visualized using UVP image analyzer.

RT-PCR analysis

The total cellular RNA from control as well as cells treated with *Urtica dioica* extract was extracted by RNA-XPLUS following manufacturer's protocol. RNA was precipitated with isopropanol and the concentration was estimated by Nanodrop (Thermo scientific), 5µgr/µl of total RNA was used for each RT-PCR reaction. The primers used were as follows: β-actin-F: 5′TCCCTGGAGAAGAGCTACG 3′ R: 5′ GTAGTTTCGTGGATGCCACA 3′, Bcl2- F:5′ CCTGTGGATGACTGAGTACC 3′ R: 5′ GAGACAGCCAGGAGAAATCA 3′, Caspase 3-F: 5′ TGTCATCTCGCTCTGGTACG 3′, R: 5′AAATGACCCCTTCATCACCA 3′, Caspase 9-F: 5′ GCAGGCTCTGGATCTCGGC 3′, R: 5′GCTGCTTGCCTGTTAGTTCGC 3′. Caspase 8-F: 5′ TGAAAAGCAAACCTCGGGGA 3′, R: 5′ TGAAGCTCTTCAAAGGTCGTG 3′. PCRs was performed in a 20 µl reactions system containing 1 µl of cDNA template, 12 µl of SYBR green reagents, 0.2 pM of each of the primers and 6 µl of nuclease-free distilled water. The PCR conditions were 95 °C for 10 min followed by 45 cycles at 95 °C for 10 sec, 57 °C for 30 sec and 72 °C for 20 sec. Relative Bcl2, Caspase 3, Caspase 9 and Caspase 8 mRNA expression were calculated with the 2 ($2^{-\triangle\triangle CT}$) method, using β-actin as an internal control.

Results

***Urtica dioica* treatment decreased cell viability in breast cancer cells**

We first assessed the cell proliferation assay in MDA-MB-468 and L929 cells after exposure to 10, 20,

Table 1. (IC50) concentrations of *urtica dioica* for MDA-MB-568 and L929 cell lines after 24 and 36 incubations.

IC50 (µg/ml)		
Time	24h	48h
L929	47.21	58.24
MDA-MB-468	29.46	15.54

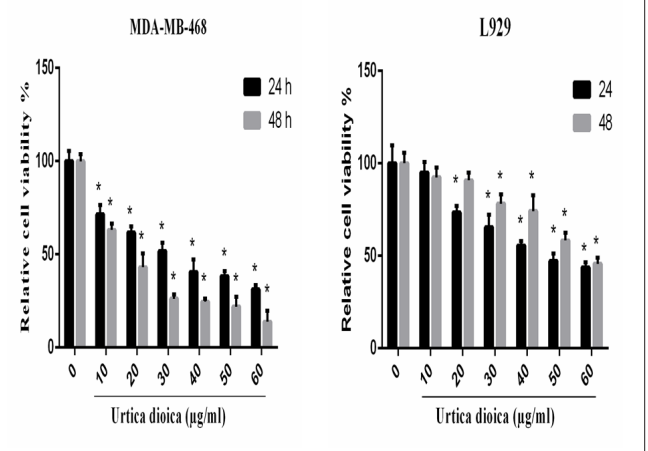

Figure 1. Dichloromethane extract of *Urtica dioica* induces dose and time-dependent cell death in Human Breast Cancer Cell Line. MDA-MB-468 and L929 cells were treated with 10, 20, 30, 40, 50, 60µg/ml *Urtica dioica* for 24 and 48 h and cell viability was determined by MTT assay. Data are means ± SD (n = 3) and are representative of three independent experiments carried out in triplicate. *p < 0.05 vs. untreated control.

30, 40, 50, 60 µg/ml of *Urtica dioica* for 24 and 48 h. MTT analyses showed that exposure to the *Urtica dioica* caused a marked decrease in cell viability in a concentration- and time-dependent manner in MDA-MB-468 cells and L929cells but cytotoxic effects of *Urtica dioica* extract on the MDA-MB-468 cells were significantly higher than on the L929 cells ($p<0.05$) (Figure 1). Table 1 shows the IC50 (50 % inhibitory concentrations) values of the *Urtica dioica* dichloromethane extract on the cells.

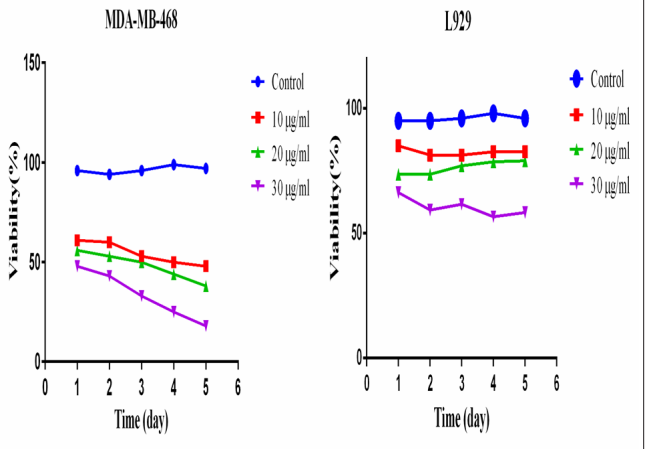

Figure 2. Cytotoxic effects of *Urtica dioica* on MDA-MB-468 and L929 cells. The cells were seeded on 24-well tissue culture plates and treated with *Urtica dioica* extract as indicated for 5 days. The cells were harvested by trypsinization, stained with trypan blue and the viable and dead cells were counted. Viable cells (%) = [(total cells-dead cells) / total cells] × 100.

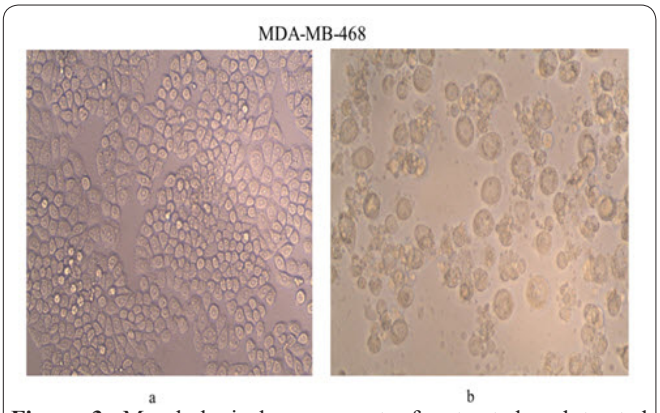

Figure 3. Morphological assessment of untreated and treated MDA-MB-468 cells with IC50 doses of *Urtica dioica* at 24h (magnification 20). (a): untreated MDA-MB-468 cells. (b): treated MDA-MB-468 cells with *Urtica dioica* extract.

We characterized the cytotoxic effects of *Urtica dioica* on MDA-MB-468 and L929 cells by conducting cell viability assay stained with trypan blue. Cultures of the MDA-MB-468 and L929 cells were treated with *Urtica dioica* extract at various concentrations for 5 day. As shown in Fig. 2, the results indicated that *Urtica dioica* extract had obvious cytotoxicity on MDA-MB-468 cells and L929 cells but cytotoxic effects of *Urtica dioica* extract on the MDA-MB-468 cells were significantly higher than on the L929 cells ($p<0.05$).

Morphological changes in MDA-MB-468 cells after exposure to IC50 dose of *Urtica dioica* for 24 h. As shown in Figure 3, the *Urtica dioica* treatment induced the majority of cells to shrink, float, and exhibit many cytoplasmic vacuoles, which is a typical apoptotic appearance.

Urtica dioica treatment caused apoptosis in MDA-MB-468 cells

A series of experiments was carried out to examine the cell death profile caused by the *Urtica dioica*. First, we assessed the apoptosis by using a TUNEL test. As shown in Figure 4, cell nuclei became brown after 24 hours of treatment with IC50 of the *Urtica dioica*, while this did not occur in control cells.

DNA fragmentation ladders, a characteristic of apoptosis, were also observed in cells treated with *Urtica dioica* for 24 h (Figure 5).

Bcl2, Caspase-3, Caspase-9 and Caspase-8 activities were measured, in order to investigate the role of bcl2 and caspases in *Urtica dioica* –induced apoptosis. As shown in Figure 6D, caspase-3 and caspase-9 activities were markedly enhanced but it has not seen any change in caspase-8 levels in a time-dependent manner following *Urtica dioica* treatment.

Discussion

Breast cancer is one of the most frequent types of cancer among women all over the world (24). Because of its high mortality and morbidity, there is a great deal of interest in finding an appropriate medication for the treatment of breast cancer. Among these treatments, medicinal plants are considered to be important sources of therapeutic substances and a variety of plants have been shown to contain bioactive and anti-cancer compounds (25-28).

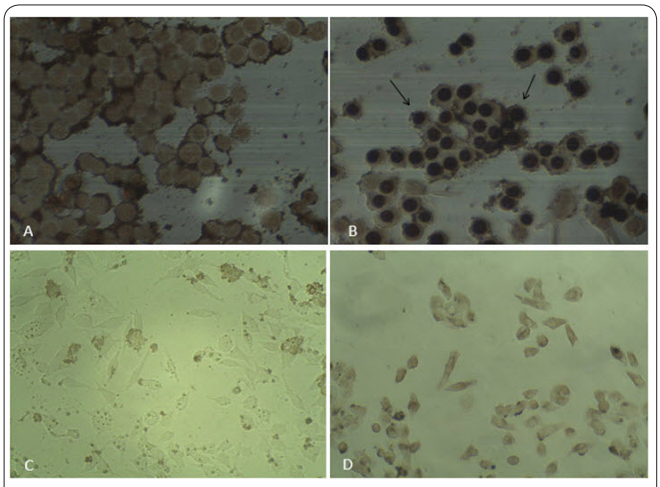

Figure 4. Apoptotic effects of *Urtica dioica* on MDA-MB-468 and L929 cells observed by TUNEL assay. (A) MDA-MB-468 control, (B) treated MDA-MB-468, (C) L929 control and (D) treated L929. Arrows indicate representative apoptotic cells. A-D: 20× magnification.

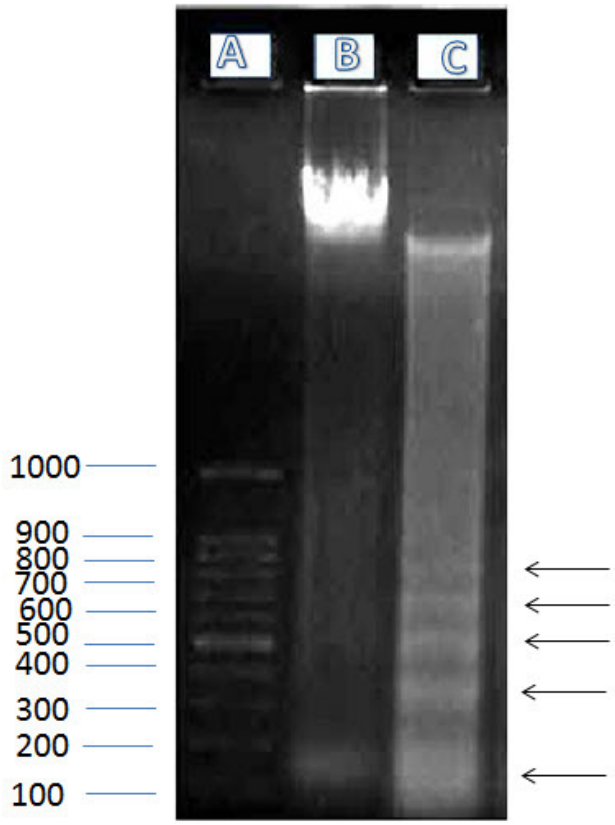

Figure 5. DNA fragmentation assay in MDA-MB-468 cells exposed to IC50 concentration of *urtica dioica* for 24 hr. (A) size marker (ladder), (B) Control cells (untreated cells) and (C): treated MDA-MB-468 cells with *Urtica dioica*. DNA fragments were separated in the 1.5% agarose gel electrophoresis.

A successful anti-cancer drug should kill cancer cells without causing excessive side effects to normal cells. This ideal situation is achievable by apoptosis induction in cancer cells (29). Increasing evidence demonstrates that plants are an important source of bioactive compounds that can induce apoptosis in human cancer cells (3, 30).

In the present study, as a first step to provide scientific evidence for anti-cancer property of *Urtica dioica*, the cytotoxic and apoptotic activity of dichloromethane extract on MDA-MB-468 human breast adenocarcinoma cells were evaluated.

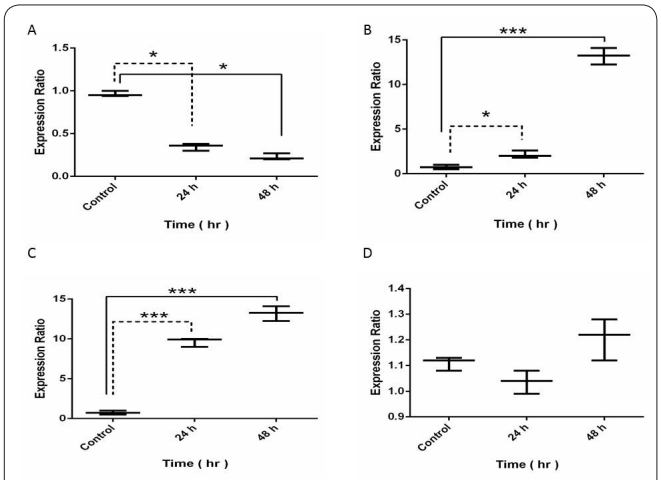

Figure 6. Effects of *urtica dioica* on (A) Bcl-2, (B) Caspase-3, (C) Caspase-9 and (D) caspase-8 mRNA expression in MDA-MB-468 cells at 24 h. Relative expression was acquired by qRT-PCR using $2^{(-\Delta\Delta Ct)}$ method. The results are presented as mean ± SD (n = 3); *p<0.05 versus control.

Our results showed that dichloromethane extracts could inhibit the growth of MDA-MB-468 cells through the induction of apoptosis. The IC50 values strongly indicated that the dichloromethane extract had a potent cytotoxic effect on MDA-MB-468 cells. Moreover, dichloromethane extract did not inhibit significantly noncancerous cells (L929) proliferation. The different sensitivity to dichloromethane extract between human breast carcinoma cells and normal cells suggested dichloromethane extract as a chemotherapeutic drug. There is evidence that naturally occurring compounds and many chemotherapeutic agents can trigger the apoptosis of cancer cells. In apoptosis, the earliest recognized morphological changes are chromatin condensation and nuclear fragmentation (31). Progression of the condensation is accompanied by convolution of the nuclear followed by breaking up of the nucleus into discrete fragments (32). Based on this, to confirm the induction of apoptotic process by dichloromethane extract, TUNEL assay and DNA fragmentation analysis were performed. TUNEL as a detection of DNA fragments in situ using the terminal deoxyribonucleotidyl transferase (TdT)-mediated biotin-16-dUTP nick-end labeling, revealed that dichloromethane extract of *urtica dioica* induced the apoptosis in a dose- and time dependent manner. In the current study, the pattern of TUNEL staining in treated MDA-MB-468 cells confirmed that DNA fragmentation is initiated at the nuclear periphery and progresses towards the center (Figure 5). To understand the potential anti-cancer mechanism of *Urtica dioica*, the mRNA expression levels of four apoptotic-related genes, caspase-3, bcl2, caspase-9 and caspase-8 were investigated. We found that the apoptosis elicited by the dichloromethane extract on MDA-MB-468 cells was mediated via bcl2, caspase-3 and caspase 9 but interestingly we have not seen any significantly change in caspase-8 levels. This suggests that the major pathway for the cell death through urtica dioica extract treatment is mitochondrial pathway (intrinsic pathway) activated by caspase 9. These findings were in agreement with many studies that described the role of these genes in inducing apoptosis (33). The family of caspases plays an

important role in apoptosis cell death processes. Among the caspases, caspases-3 is considered to be the most commonly executioner caspases during apoptosis. Caspases-9 and caspase-8 investigates as correspondence of intrinsic or extrinsic pathway of apoptosis (34).

Therefore, the results confirmed that the dichloromethane extract of *Urtica dioica* induced apoptosis in MDA-MB-468 cells through the activation of the caspase-3 pathways. In conclusion, the present study strongly suggests that the dichloromethane extract of *Urtica dioica* may contain bioactive compound that kill human breast adenocarcinoma cells, MDA-MB-468 cells without inducing substantial damage to noncancerous cell line L929, thus possibly suggesting a new potential chemotherapeutic agent for the treatment of breast cancer.

Author Contributions

Behzad Baradaran, Ali mohammadi and behzad mansoori planned the experiments, Ali Mohammadi, Behzad Mansoori, Samira Goldar, Leila Mohammadnejad and Elham Baghbani conducted the experiments, Vahid khaze and Dariush Shanehbandi analysed the data, and Ali mohammadi and Behzad Mansoori wrote the paper.

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