

Original Research

## **Urtica dioica dichloromethane extract induce apoptosis from intrinsic pathway on human prostate cancer cells (PC3)**

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**Abstract:** Prostate cancer is considered as the major cause of death among men around the world. There are a number of medicinal plants triggering apoptosis response in cancer cells, thus have a therapeutic potential. Therefore, further studies to characterize beneficial properties of these plants in order to introduce novel anti-cancer drugs are the interest of recent researches on the alternative medicine. On the other hand, due to traditional uses and availability of *Urtica dioica* extract, we decided to evaluate the efficacy of this medicinal herb on pc3 prostate cancer cell line. In the present study the cytotoxic effects of *Urtica dioica* extract were assessed by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay and trypan blue viability dye. Then, DNA fragmentation and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay were exploited to measure cell death and apoptosis stage. The expression levels of caspase 3, caspase 9 and Bcl-2 genes were quantified by Real-Time PCR. Finally, Cell cycle was analyzed by flow cytometry. MTT assay showed that dichloromethanolic extract of *Urtica dioica* significantly inhibited the cell growth. According to the DNA fragmentation and TUNEL assay results, the herbal extract was able to induce apoptosis in prostate cancer cells. Our findings also demonstrated that the plant extract substantially increases the caspase 3 and 9 mRNA expression, while decreases Bcl-2. Cell cycle arrest was occurred in G2 stage, due to the results of flow cytometry. These results indicate that dichloromethanolic extract of *Urtica dioica* can successfully induce apoptosis in PC3 cells. Therefore, it could be used as a novel therapeutic candidate for prostate tumor treatment.

**Key words:** Urtica dioica extract, Apoptosis, Cytotoxicity, Pc3.

### **Introduction**

Cancer is a process in which normal cells go through an uncontrolled and abnormal growth. It is a fatal condition with high mortality percentage followed with economic losses (1). However, prevention and treatment of these situations is a worldwide concern. Prostate cancer (PCA) is the second frequent occurring cancer and sixth leading cause of death in developing countries. It is well-documented that the incidence of PCA in western countries including United States and western EU is much higher than Eastern EU, Africa and Asian countries (3-8 per 100000 men annually) (2, 3). PCA solely constitutes 33 percent of all cancers (2, 4).

Several therapeutic approaches including conventional chemotherapy, radiation therapy and surgical removing were employed for treatment. However, the high rate of mortality in under treatment patients reveals the inefficiency of such strategies (5).

The development of an effective treatment requires more researches on underlying mechanisms involved in tumor formation. Inducing apoptosis is one of the significant characterizations of cytotoxic antitumor agents (6). It has been reported that a number of natural compounds such as plants can switch apoptotic pathways on which were already turned off in cancer cells. The capability for inducing apoptosis in cancer cells and preventing unwanted cell proliferation are crucial subjects in immunopharmacological studies. The environmental factors which cause DNA damages and consequently cancer malignancies are important in tumor pathogenesis (7).

Natural compounds, especially plants are potential candidates for production of pharmaceutical agents. Many of synthetic anticancer drugs including taxans, Vinca alkaloids, podophilotoxin and camptotocins are herbal derivative and are extensively used for treatment of both metastatic and non-metastatic tumors (8, 9).

*Urtica dioica* is an herbaceous plant with 40 species and 500 genera which most of them are native to North America, India, Malaysia and tropical areas. The species of this order are rarely found in Europe and Africa (11). The plant contains tannin, mucilage, wax-like substance, formic acid, phytoestrine, calcium and potassium nitrate, iron and a type of glucoside with irritating effects on skin. Orticine, a colored compound, are isolated from the top of the plant (12).

The progressive nature of PCA, increasing numbers of this malignancy in past two decades and high mortality rate in cancer patients(4) were the main reasons for evaluating the potency of *Urtica dioica* extract as an antitumor and antiproliferative candidate on pc3 prostate cancer cell line.

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## Materials and Methods

### Preparation of herbal extract

The leaves of *Urtica dioica* were collected from wild plants in mountains of Tabriz (Eastern Azerbaijan province, Iran) and were air dried in the shade at room temperature and 10-15% humidity. Then, the dried materials were ground using domestic mill and added to the 10 liter extraction reactor. The procedure performed using dichloromethane solvent up to 24 h. The extract was then filtered through whatman paper no.40 and the resultant filtrate was evaporated under negative pressure using a rotary vacuum evaporator.

### Cell culture

PC3 cells, a human prostate adenocarcinoma (NCBI code: C427), were purchased from National Cell Bank of Iran (Pasteur Institute, Iran-Tehran) and grown in RPMI-1640 supplemented with 10% fetal bovine serum (FBS) in the presence of 2 mM L-glutamine, penicillin 100 unit/ml and streptomycin 100 µg/ml in a humidified incubator with an atmosphere of 5% CO<sub>2</sub> at 37°C.

In order to determine the efficacy of plant extract, time and dose variables were considered. The cultured cells were subjected to different concentrations of herbal extract (5, 10, 20, 30, 40, 50, 60 µg/ml) for 24 and 48 h. Those cells that were not treated with plant extract served as vehicle. Moreover, all experiments carried out in triplicate.

### MTT assay

Cell cytotoxicity was analyzed by MTT (3-[4, 5-dimethyl-2-thiazolyl]-2, 5 diphenyl tetrazolium bromide), which is a yellow and water soluble compound and can be converted by mitochondrial reductase of live cells to blue formazan salt that readily detected by microplate reader at 570 nm. Briefly, 15×10<sup>3</sup> cells were seeded into each well of 96-well plate. After 12 h incubation, cells were treated to varying concentrations of *Urtica dioica* extract for different time periods (24 and 48 hours). Following incubation, supernatants of all wells were removed and washed with PBS. Then, MTT stock solution (2 mg/ml PBS) was added to each well. After 4 h incubation at 37°C, liquid phase of wells were discarded again and DMSO with 200 µl value was added to each well and shacked for 20 min. The absorbance of specimens was measured using ELISA reader at a wavelength of 570 nm.

### Trypan blue dye exclusion assay

The trypan blue exclusion test is a practical method for evaluating the integrity of cell membrane, which selectively stains dead cells, thus indirectly determines the number of viable cells. Briefly, 5×10<sup>4</sup> cells were distributed in 24-well plate and then treated with 10, 30 and 50 µg/ml of *Urtica dioica* extract. After incubation periods (1-5 days) the number of viable cells were counted by trypan blue dye.

### DNA fragmentation assay

Apoptosis or programmed cell death is a physiological process that distinctly characterized by morphological hallmarks such as membrane blebbing, DNA fragmentation and cytoplasm condensation. This method

was used to measure the extent of cell death. PC3 cells (5×10<sup>5</sup>) were seeded into 6-well plate and treated with plant extract. After 24 h, cell DNA was isolated By Apoptotic DNA Ladder Isolation kit (Abcam, USA, ab65627). Briefly, the cells were washed with PBS and then centrifuged at 500 g for 5 min. The supernatant was removed and then lysed by 50 µg of DNA Ladder extraction Buffer. Following 10 s incubation in room temperature, the cell supernatant was transferred to microcentrifuge tube. Afterwards, 5 µl of enzyme A was added and incubated for 10 min at 37°C. Then, enzyme B solution was added into each well and kept in 50°C for 30 min. After adding 5 µl of Ammonium acetate and 100 µl of isopropanol, samples were centrifuged at 16000 g and the supernatant was discarded. The DNA pellet was rinsed with 0.5 ml 70% ethanol and the precipitate was dissolved in 30 µl DNA suspension buffer. Finally, DNA samples were separated by electrophoresis in 1.5% agarose gels and results were visualized by trans-illumination with UV light.

### TUNEL assay for detection of DNA fragmentation

As the genomic DNA undergoes breaks during apoptosis process, the result is formation of two low molecular strands of DNA (mono and oligonucleosom) and also terminal strands which can be labeled under enzymatic reaction at the 3'-OH ends. PC3 cells (15×10<sup>3</sup>) were seeded into 96-well plate and subsequently treated with dichloromethane extract of *Urtica dioica* at desired concentration (IC<sub>50</sub>). After 24 h TUNEL assay was performed using Roche Molecular Biochemicals kit. Briefly, cells were fixed with 4% paraformaldehyde in PBS (pH 7.4). Following washing with PBS, the fixed cells were incubated with blocking solution (3% H<sub>2</sub>O<sub>2</sub> in methanol) at 25°C for 10 min. After removing the blocking solution and washing with PBS, cells were further incubated in permeabilisation solution (0.1% Triton X-100 in 0.1% sodium citrate) for 2 min on ice. The samples were resuspended in 50 µl of TUNEL reaction mixture (TdT enzyme and nucleotide) and incubated at 37°C for 1 h in the dark, covered with foil. Then, 50 µl of converter-POD streptavidin HRP solution was added and the cells were kept in humidified chamber, at 37°C for 30 min. After rinsing three times with PBS, 100 µl of DAB solution was added and incubated for 10 min in the dark. Eventually, stained cells were analyzed under a light microscope.

### RNA extraction

Total RNA from treated and untreated PC3 cells manually extracted according to the following steps. First, 5×10<sup>5</sup> cells were seeded into 6-well plate and after treatment were detached by Trypsin-EDTA. Then, 200 µl of TRIzol lizing buffer were added to cell precipitate and after repeated pipetting the mixture was transferred to 1.5 ml RNase-free microcentrifuge tube. After adding 200 µl of chloroform, the samples were vortexed vigorously for 1 min and incubated at room temperature for 5 min. The resultant mixture was centrifuged at 4°C at 12000 g for 15 min. Following centrifugation, the colorless upper aqueous phase which contains DNA was transferred to a fresh microtube. The cold isopropanol was added to precipitate RNA from the aqueous phase. The mixture was kept at -20 °C overnight. Afterwards,

**Table 1.** Primer sequences.

β-actin	F	5'-TCCCTGGAGAAGAGCTACG-3'
	R	5'-GTAGTTTCGTGGATGCCACA-3'
Caspase 3	F	5' TGTCATCTCGCTCTGGTACG 3'
	R	5' AAATGACCCCTTCATCACCA 3'
Caspase 9	F	5' GCAGGCTCTGGATCTCGGC 3'
	R	5'GCTGCTTGCCTGTTAGTTCGC 3'
Bcl2	F	5' CCTGTGGATGACTGAGTACC 3'
	R	5' GAGACAGCCAGGAGAAATCA 3'

the solution was centrifuged at 12000 g for 45 min at 4°C, the supernatant was discarded and the transparent precipitate was mixed with cold 70% ethanol and vortexed to detach the pellet. The resuspended solution was exposed to centrifugation for 15 min. The supernatant was then removed and the precipitate air-dried at room temperature. The RNA pellet was dissolved in DEPC-treated water and stored at -70 °C freezer for further use. To determine sample concentration, the absorbance of the solution was measured at 260 nm wavelengths and the purity of extracted RNA calculated through  $A_{260}/A_{280}$  proportion.

#### cDNA synthesis and quantitative Real-Time PCR (qRT-PCR)

Complementary DNA (cDNA) was constructed by using total RNA that already isolated from PC3 cells. The reaction mixture which contained 1 μl Random Hexamer primer, 2 μl 10X Buffer (50 mM Tris-HCl, 75 mM KCl, 3 mM MgCl and 10 mM DDT), 2 μl of 10 mM dNTP mix, and 1 μl Transcriptase enzyme was reached to the final volume of 25 μl. The reverse transcription was performed at 42°C for 60 min. The enzyme activity was stopped by setting the heat at 70°C and for 10 min.

Quantitative expression of caspase 3, caspase 9 and Bcl2 mRNA were analyzed using Quantifast SYBR Green Takara PCR kit and Real-Time PCR Thermocycler device, according to the manufacturer's instruction. The PCR primers were designed by primer 3 software and sequences have listed in Table 1. A PCR reaction mixture of 10 μl containing 3μl of each forward and reverse primer, 2 μl of cDNA, 5 μl of SYBR green reagent and 2.7 μl of DDW were employed. The PCR conditions for corresponding mRNAs were 94°C for 10 min followed 40 cycle in three steps include: 94°C for 10 sec, 59°C for 35 sec and 72°C for 20 sec, followed by one cycle at 72°C for 5 min. All qRT-PCR reactions were carried out in triplicated with aid of β-actin as an internal control. The relative expression levels were calculated using Ct ( $2^{-\Delta\Delta t}$ ) method. Ultimately, the difference between reference and target genes was subjected to Paired T statistical test via Graphpad prism 5 software.

#### Cell cycle assay

Treated cells were harvested by trypsin and fixed in ice-cold 70% ethanol. Then 1ml Propodium Iodid (PI) Master Mix solution (40 μl PI, 10 μl RNase, and 950 μl PBS) was added and incubated at 37°C for 30 min. The cell cycle arrest was analyzed by flow cytometry FACS Calibur (Beckman Coulter, Fullerton, CA, USA). The percentage of cell populations in G1, S and G2/M phases were determined by Cell Quest program.

#### Statistical analysis

All results were represented as mean±SD of three independent experiments. The IC50 of treated samples (concentration required for 50% inhibition of cancer cells) calculated via Graphpad Prism 6. The student's t test was exploited to analyze the effect of different concentrations of plant extract on PC3 cells.

#### Results

##### Cytotoxic effects of *Urtica dioica* dichloromethane extract on PC3 cells

To determine cytotoxic effects of plant extract on PC3 cancer cells, MTT assay was performed. Data analysis of PC3 cells treated with *Urtica dioica* extract at 0-60 μg/ml concentrations in 24 and 48 h have been illustrated in Figure 1. The IC50 concentration of *Urtica dioica* extract is shown in Table 2.

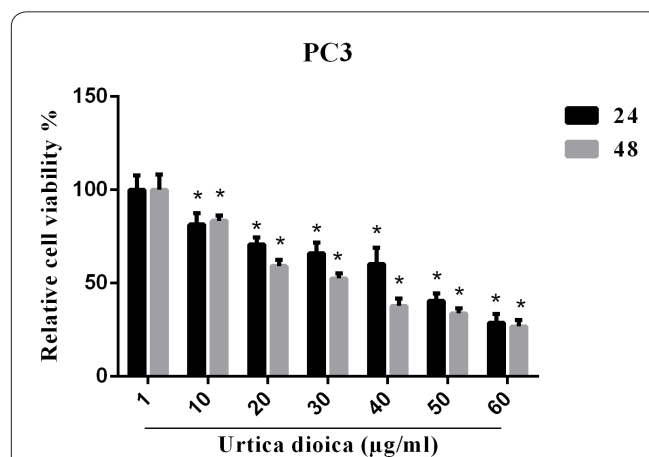
Direct counting for live and dead cells using trypan-blue exclusion dye revealed that *Urtica dioica* dichloromethane extract successfully decreases the viability of PC3 cells which was in dose and time dependent manner (Figure 2).

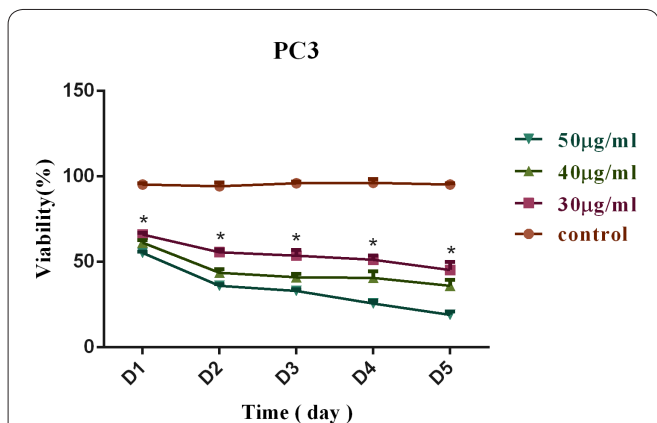
##### DNA fragmentation test

As displayed in Figure 3, findings of DNA fragmentation assay confirmed the presence of histone-com-

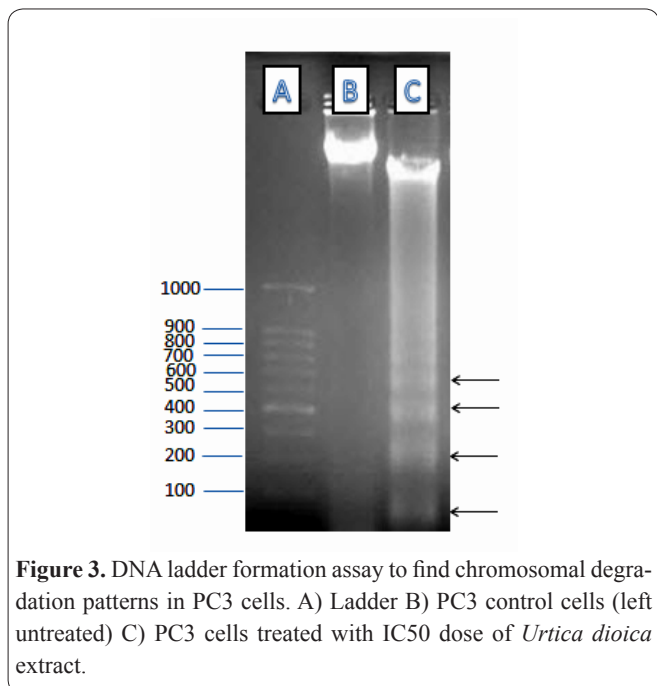
**Table 2.** Concentration is producing 50% inhibition (IC50) of *Urtica dioica* extract on PC3 cells in 24 and 48.

	IC50 (μg/ml)	
Time	24 h	48 h
PC3	29.46	15.54

**Figure 1.** Anti-proliferative effect of dichloromethane extract of *Urtica dioica* in 24 and 48 h. \*p<0.05 was considered as significant compared to untreated-control group (zero concentration of the extract) (N=3).



**Figure 2.** Viability of PC3 cells treated with various concentrations of dichloromethane extract of *Urtica dioica* (30, 40 and 50 µg/ml) for 1, 2, 3, 4 and 5 days. \* $p < 0.05$  was considered as significant compared to untreated-control group (N=3).



**Figure 3.** DNA ladder formation assay to find chromosomal degradation patterns in PC3 cells. A) Ladder B) PC3 control cells (left untreated) C) PC3 cells treated with IC50 dose of *Urtica dioica* extract.

plexed DNA fragments in cytoplasm of cells treated with *Urtica dioica* extract for 24 h.

**TUNEL assay**

After the treatment of PC3 cells with dichloromethane extract of *Urtica dioica* for 24 h, the observation of apoptotic bodies using TUNEL test was further supported the DNA fragmentation results. The fragmented nuclei of apoptotic cells colored light to dark brown in comparison with untreated cells. Representative images of TUNEL were illustrated in Figure 4.

**qRT-PCR**

To further reveal the exact mechanism involved in cell death induced by *Urtica dioica* extract, we examined the mRNA expression of active caspase 3 and caspase 9. These proteolytic caspases are pivotal mediator of cell death process that present in all apoptotic pathways. Findings of qRT-PCR technique showed that the expression levels of caspase 3 and 9 mRNA significantly increased following the treatment of PC3 cells with IC50 concentration of *Urtica dioica* extract for 24 and 48 h in contrast with vehicle control, indicating

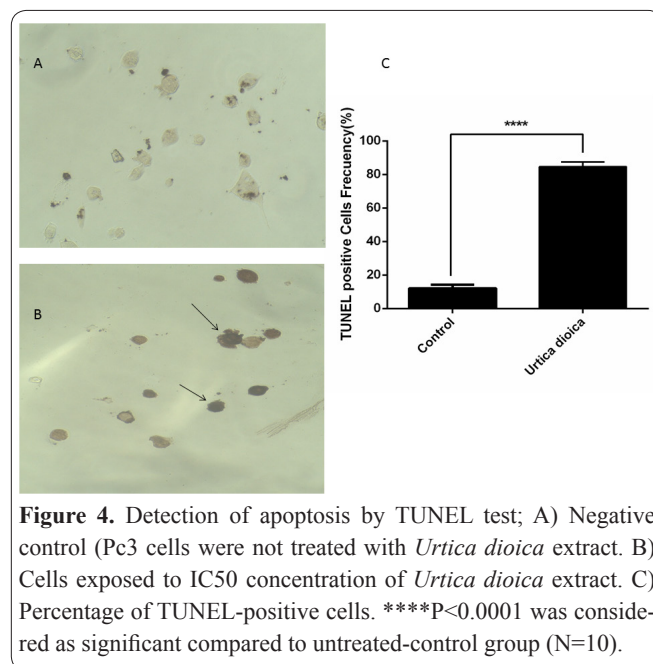
caspase-dependent apoptotic activity of *Urtica dioica* extract. Moreover, the expression levels of Bcl-2 anti-apoptotic mRNA were assessed by qRT-PCR and results showed a downregulation of Bcl-2 protein in cells treated with plant extract compared to control (Figure 5).

**Cell cycle arrest by Flow cytometry**

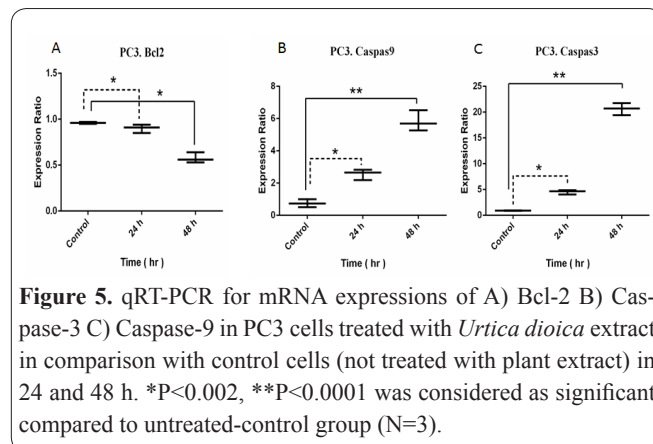
To investigate the effects of *Urtica dioica* extract on cell cycle distribution, PC3 cells incubated with 30 µg/ml of dichloromethane extract and after 48 h subjected to FACS analysis and PI staining for assessing cell cycle progress. After 48 h proportion of cells at G2/M phase was 37.23, respectively showing a significant increment as compared to vehicle control (25.73) which subsequently accompanied with a decrease in cell population at G1 and S phases. SubG0/G1 ratio was markedly increased to 22.54 in 48 h after treatment. These results obviously demonstrated that *Urtica dioica* dichloromethane extract can considerably arrest cell cycle at G2/M phase and inhibit progressive cell growth (Figure 6).

**Discussion**

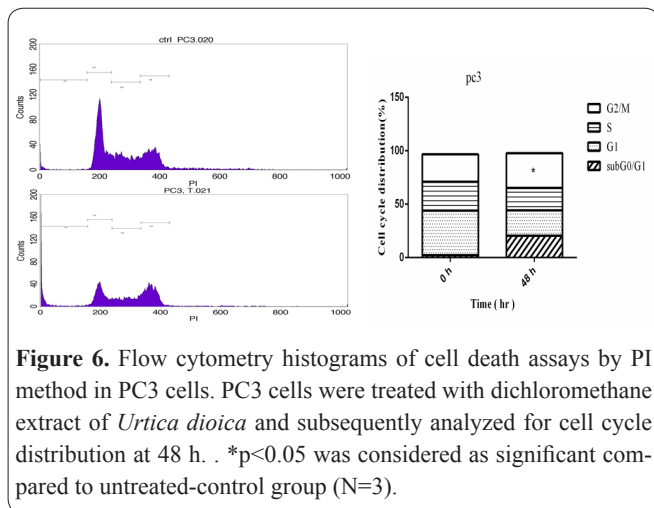
For many years, traditional medicine has been considered as a great source of effective drugs that introduced to the pharmaceutical market internationally and Iranian herbal medicine was one of the pioneers of such a the-



**Figure 4.** Detection of apoptosis by TUNEL test; A) Negative control (Pc3 cells were not treated with *Urtica dioica* extract. B) Cells exposed to IC50 concentration of *Urtica dioica* extract. C) Percentage of TUNEL-positive cells. \*\*\*\* $P < 0.0001$  was considered as significant compared to untreated-control group (N=10).



**Figure 5.** qRT-PCR for mRNA expressions of A) Bcl-2 B) Caspase-3 C) Caspase-9 in PC3 cells treated with *Urtica dioica* extract in comparison with control cells (not treated with plant extract) in 24 and 48 h. \* $P < 0.002$ , \*\* $P < 0.0001$  was considered as significant compared to untreated-control group (N=3).



**Figure 6.** Flow cytometry histograms of cell death assays by PI method in PC3 cells. PC3 cells were treated with dichloromethane extract of *Urtica dioica* and subsequently analyzed for cell cycle distribution at 48 h. \* $p < 0.05$  was considered as significant compared to untreated-control group (N=3).

rapeutic approach. At the moment, 75-80% of world people, especially in developing countries use herbal drugs for alleviating wide variety of diseases since they believe that besides the availability and reasonable cost, the side effects of these kinds of drugs are potentially lower than synthetic ones. A good anticancer drug should eliminate cancer cells without exerting any side effects on normal cells. In this case, induction of apoptosis in cancer cells would be an ideal plan. Many of common in clinical use drugs contain herbal derivatives. Moreover, a number of primary drugs such as Aspirin, digoxin and morphine synthesized from at least one herbal compartment (willow bark, fox glove and opium poppy, respectively) (10). Today, the world has been faced with increasing number of cancer patients and cancer has become the second cause of death after cardiac diseases. Therefore, understanding the underlying mechanisms of cancer pathogenesis will be the important part of generating novel therapeutic approaches (8). Some mutations in cells make them resistant to cell death self-stimulation, for that induction of apoptosis by chemotherapeutic agents could be one of the desired targets for the treatment of cancer (11). Medicinal plants have a pivotal role both in the prevention and treatment of cancer. The bioactive compounds of these plants act differently; however, induction of apoptosis is the ultimate point for each of these distinct pathways.

Several studies have shown the cytotoxic and anti-tumor effects of different species of *Urtica dioica*. It was found that *Urtica dioica* extract inhibits prostate  $\alpha 5$ -reductase and aromatase enzyme activity in a concentration dependent manner (12).

Results of present study showed that dichloromethane extract of *Urtica dioica* exert have a strong cytotoxic effect on PC3 cells compared to control group which was time and dose dependent. Our results were in consonance with previous findings of US harput which was performed using lymphocyte cell line (13).

Apoptosis process which begins with cell shrinkage and membrane blebbing subsequently followed by nuclei fragmentation and finally the cell breaks up into several apoptotic bodies. To investigate the induction of cell death by *Urtica dioica* extract, TUNEL assay was performed and results clearly show that the extract induces apoptosis in PC3 cells.

DNA fragmentation which is one of the important characteristics of apoptosis was assessed by gel electro-

phoresis. Results of this assay were in agreement with Zabit yener *et al.*, study which investigated the effect of *Urtica dioica* L. seed on aflatoxin-induced injury in rats (13).

Caspases are proteolytic proteins from cysteine proteases family which play a pivotal role in triggering effector phase of apoptosis. Once these enzymes are activated, they act on distinct substrates and cause various biochemical and morphological changes in apoptotic cells such as chromatin condensation, DNA fragmentation and etc. Thus, the caspases activity is considered as biochemical index of apoptosis.

Detection of caspase activity is a well-established procedure for distinguishing apoptosis from necrosis. Therefore, for further investigation the expression levels of caspase 3 and caspase 9 mRNAs were measured using qRT-PCT. Data analysis showed that caspase 3 and 9 apoptotic genes significantly overexpressed following treatment of PC3 cells with the *Urtica* extract as compared to negative control.

Bcl-2 protein is one the early-recognized antiapoptotic proteins which resides on the outer mitochondrial membrane (14). This protein efficiently suppresses apoptotic response through blocking permeability pore of mitochondria and inhibiting the release of cytochrome c and apoptosis-inducing factor (Aif). In addition, Bcl-2 inhibits cell death by regulating activation of caspases (15). Exposure of PC3 cells to IC50 dose of dichloromethane extract of *Urtica dioica* resulted in significant downregulation of Bcl-2 gene. In consistent with our findings, CL Kao *et al.*, showed that prostate cancer cell line that was treated with *Urtica dioica* extract expresses low levels of Bcl-2 in comparison with control group(16). We did not investigate the possibility that *urtica dioica*-induced apoptosis can occur dependently of cell cycle arrest. our results indicate that treatment with *urtica dioica* caused, in a dose-dependent manner, G2/M arrest in prostate cancer cells. G2/M arrest and apoptotic cell death in PC3 cells, as determined by flow cytometric analysis through PI assay. The critical elements of *Urtica dioica*-induced cell cycle arrest and apoptosis, including the checkpoint elements of the cell cycle, activation of caspases and mitochondrial function with associated release of apoptogenic factors. *Urtica dioica* extract initiated apoptosis might be induced of the cells existing in the G2/M-phase, which are committed to G2/M arrest (17, 18, 19).

Our results demonstrate that treatment of the PC3 cells with the dichloromethane extract of *Urtica dioica* considerably changes cell morphology and exerts antiproliferative effects with dose and time dependent manner. On the other hand, results of qRT-PCR were obviously showed that caspase 3 and caspase 9 downregulated in treated cells while the expression of Bcl-2 gene was decreased. In conclusion, dichloromethane extract of *Urtica dioica* could be a potential candidate for finding novel anticancer active compounds. In the future, studies focusing on cell signaling and the biological significance of *urtica dioica* induced apoptosis and cell cycle arrest would explain the mechanisms of the chemotherapeutic potency of *urtica dioica* in human cancer especially prostate cancer.

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