Cellular and Molecular Biology

Ghasemi *et al. Cell. Mol. Biol.*2016, 62 (9): 90-96
ISSN: 1165-158X
doi: 10.14715/cmb/2016.62.9.14

Original Research

Cytotoxic effects of *Urtica dioica* radix on human colon (HT29) and gastric (MKN45) cancer cells mediated through oxidative and apoptotic mechanisms

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Abstract: Defects in the apoptotic pathways are responsible for both the colorectal cancer pathogenesis and resistance to therapy. In this study, we examined the level of cellular oxidants, cytotoxicity and apoptosis induced by hydroalcoholic extract of *U. dioica* radix (0-2000 µg/mL) and oxaliplatin (0-1000 µg/mL, as positive control) in human gastric (MKN45) and colon (HT29) cancer, as well as normal human foreskin fibroblast (HFF) cells. Exposure to *U. dioica* or oxaliplatin showed a concentration dependent suppression in cell survival with IC_{50} values of 24.7, 249.9 and 857.5 µg/mL for HT29, MKN45 and HFF cells after 72 h treatment, respectively. ROS formation and lipid peroxidation were also concentration-dependently increased following treatment with *U. dioica*, similar to oxaliplatin. In addition, the number of apoptotic cells significantly increased concomitantly with concentration of *U. dioica* as compared with control cells, which is similar to oxaliplatin and serum-deprived cancer cells. In conclusion, the present study demonstrated that *U. dioica* inhibited proliferation of gastric and colorectal cancer cells while posing no significant toxic effect on normal cells. *U. dioica* not only increased levels of oxidants, but also induced concomitant increase of apoptosis. The precise signaling pathway by which *U. dioica* induce apoptosis needs further research.

Key words: *Urtica dioica*, Cytotoxicity, Apoptosis, Gastrointestinal cancer, MKN45 cells, HT29 cells.

Introduction

Recent analyses raise worries about the increase of cancer prevalence in the near future (1). In this way, looking for natural and low cost drugs that prevent cancer development is becoming an essential reason for scientists. Gastric cancer (GC) is a common malignancy and the second leading cause of cancer death worldwide (2). In the United States, there were approximately 22220 new cases and 10990 deaths in 2014 (3). Likewise, colorectal cancer (CRC) is the third most regularly analyzed cancer worldwide, with almost 1.4 million new cases in 2012 (4). Surgery, chemotherapy and radiation therapy are presently utilized as parts of the treatment of cancers, but have serious side effects.

The areas of cancer and infectious diseases have a leading position in utilization of medicinal plants as a source of drug discovery. Among FDA approved anticancer and anti-infectious drugs of natural origin have a share of 60% and 75% respectively (5). Recent studies on tumor inhibitory compounds of plant origin have yielded an impressive array of novel structures. It is worthy to mention the vivid current interest in discovery of natural drugs for cancer treatment and chemoprevention (6, 7). Huge number of plants species are screened and bio-assayed for this purpose in worldwide (8). There has been a growing interest in the use of naturally occurring compounds with chemo-preventive and chemotherapeutic properties for the treatment of cancers. Besides, epidemiological studies suggest that consumption of diets containing fruits and vegetables, major sources of phytochemicals, flavonoids, aromatic compounds with antioxidant properties and micronutrients, may reduce the risk of developing cancer by diminishing the abnormal state of oxidative stress present in carcinogenic (9).

Urtica dioica (stinging nettle) is a herbaceous perennial flowering plant which develops wild in Iran. *U. dioica* has a variety of uses as a medicine in diseases treatment such as diabetes, atherosclerosis, cardiovascular diseases and prostate cancer (10). Lignans, phenolic compounds, and sterols have been highlighted to be mainly involved in the antioxidant, anti-inflammatory, and cytoprotective effects of the plant (11).

In the present study, we examined the inhibitory effects of *U. dioica* on human colon (HT29) and gastric (MKN45) cancer cell lines.

Materials and Methods

Plant material

The roots of *U. dioica* were collected from Hosseinabad valley (2100 m height) in Pivejan, a village 65 Km South-West of Mashhad, Khorasan Razavi Province, Iran. The plant was identified by herbarium of Ferdowsi University of Mashhad, Iran (voucher specimen No. 527-8106-2). For preparation of hydroalcoholic extract of *U. dioica,* dry powdered roots (100 g) was extracted with ethanol (56%): H_2O (4 × 0.5 L), concentrated under reduced pressure to dryness and then kept at -20 ºC until use (yielded 35%).

Received June 28, 2016; **Accepted** August 24, 2016; **Published** October 15, 2016

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Cell lines and reagents

MKN45, HT29 and HFF cells were obtained from Pasteur Institute (Tehran, Iran). The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium (MTT), 2',7'-dichlorofluorescein diacetate (DCF-DA) were purchased from Sigma-Aldrich company (St. Louis, MO, USA). DMEM, fetal bovine serum, trypsin, penicillin, and streptomycin were obtained from Gibco BRL Life Technologies (Grand Island, NY, USA). Annexin V-FITC / propidium iodide (PI) kit was purchased from BioVision (Mountain View, CA, USA). Trichloroacetic acid and thiobarbituric acid (TBA) were obtained from Merck (Darmstadt, Germany).

Cell culture

MKN45, HT29 and HFF cells were maintained at 37° C in a humidified atmosphere (90%) containing 5% CO_2. Dulbecco's Modified Eagle's Medium (DMEM) with 10% (v/v) fetal bovine serum, 100 units/ml penicillin, and 100 µg/ml streptomycin. Cells were seeded overnight, and then incubated with various concentrations of *U. dioica* extract and oxaliplatin for 24, 48 and 72 h. For each concentration and time course study, there was a control sample which remained untreated and received the equal volume of medium. All different treatments were carried out in triplicate.

Cell viability

The cell viability was determined using a modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium (MTT) assay (12). Briefly, cells were seeded (5000/well) onto flat bottomed 96-well culture plates and allowed to growth for 24 h and then treated with *U. dioica* extract (0, 16, 31, 62, 125, 250, 500, 1000 and 2000 µg/ml) and oxaliplatin (0, 0.025, 0.25, 2.5, 25, 250, 500 and 1000 µM) for 24, 48 and 72 h. After removing the medium, cells were then labeled with MTT solution (5 mg/ml in PBS) for 2 h and the resulting formazan dye was solubilized with DMSO (100 µl). The absorption was measured at 570 nm (620 nm as a reference) in an ELISA plate reader.

Measurement of ROS generation

In brief, cells were seeded (10^5/well) in 12-well plates overnight, loaded with DCF-DA (20 µM) for 30 min in the dark and then treated with U. *dioica* extract (0, 16, 125 and 1000 µg/ml) or oxaliplatin (0, 25, 125 and 250 µM) for 1 or 3 h. After that, the medium was removed to a 75-mm Falcon polystyrene tube and the adherent cells were trypsinized and collected into the same tube. After washing twice with PBS, The DCF fluorescence intensity was detected using a FLUO-star galaxy fluorescence plate reader (Perkin Elmer 2030, multilabel reader, Finland) with excitation wavelength set at 485 nm and emission wavelength set at 530 nm (13).

Lipid peroxidation assay

The end product of lipid peroxidation is malondialdehyde (MDA), which reacts with TBA to form a pink-colored complex with a peak absorbance at 530 nm. To perform the assay, cells were seeded (10^5/well) in 12-well plates overnight with or without treatment with *U. dioica* extracts (0, 16, 125, and 1000 µg/ml) or oxaliplatin (1000 µM) for 24 and 48 h, then were scraped into trichloroacetic acid (2.5%, 1 mL) and centrifuged at 13000 × g at 4°C for 2 min. The lysate supernatant (500 µL) was removed and added to trichloroacetic acid (15%, 400 µL) and TBA 0.67%/butylated hydroxytoluene 0.01% (800 µL). This mixture was vortexed, boiled for 20 min and then the reaction was stopped by cooling in ice water bath. After centrifugation at 2500 rpm for 10 min at 4°C, the fluorescence intensity of supernatant was read in excitation/emission of 530/550 nm (ref).

Apoptosis assay

Apoptotic cells were detected using annexin V/PI staining of treated cells followed by flow cytometry. MKN45 and HT29 cells were cultured overnight and treated with *U. dioica* extracts or oxaliplatin for 48 h. Untreated and *U. dioica* or oxaliplatin-treated cells were then removed using trypsin-EDTA, extensively washed with PBS and adjusted to 1.5×10^6 cells/ml in calcium buffer and stained with annexin V-FITC and PI in accordance with the manufacture's instruction. Samples were then analyzed by flow cytometry. A total of 10,000 events per sample were obtained and the data was analyzed using WINMDI software. Viable cells were not stained; apoptotic cells excluded PI and expressed phosphatidyl serine (PS) stain by green color while necrotic cells were permeable to PI which associates with nuclear DNA, and were visible by red fluorescence. Three independent experiments were performed.

Statistical analysis

All results were expressed as mean ± SEM. The significance of difference was evaluated with two-way or one-way analysis of variance (ANOVA) followed by Tukey's post hoc test for multiple comparison. A probability level of $p < 0.05$ was considered statistically significant.

Results

U. dioica decreased cell viability in a concentration dependent manner

MKN45, HT29 and HFF cell lines were exposed to increasing concentrations of *U. dioica* or oxaliplatin for up to 72 h; after which cell viability was measured by MTT assay. Exposure to *U. dioica* or oxaliplatin showed a concentration dependent suppression in cell survival (Figs. 1A-C and 2A-C). As compared with the HFF cells, *U. dioica* and oxaliplatin-mediated toxicity were significantly higher in the MKN45 and HT29 cells. The results are graphically shown in Figures 1A-C and 2A-C and summarized in Table 1.

The IC_{50} (concentration of 50% inhibition) values of *U. dioica* in HT29 cells were 17.6, 36.1 and 24.7 µg/mL at 24, 48 and 72 h of treatment, respectively, as compared with 1189.0 and 857.5 µg/mL for normal HFF cells (at 48 and 72 h, respectively). On the other hand, After 24, 48 and 72 h treatment, the IC_{50} values of oxaliplatin were found to be 161.4, 29.8 and 29.8 µM in HT29 cells, respectively. These values for normal HFF cells were reported to be about 353.1 and 340.8 µM at 48 and 72 h exposure, respectively (Table 1). Treatment of MKN45 cells with 2000 µg/mL *U. dioica*, for 24 and 48

Table 1. IC_{50} (concentration of 50% inhibition) values of different treatments against human normal foreskin fibroblast (HFF), gastric (MKN45) and colon (HT29) cancer cells at 24-72 h.

Cell lines / Treatments	HFF		MKN45			HT29		
	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h
***U. dioica* (µg/ml)**	1189.0	857.5	1374.0[a]	488.0[a]	249.9	17.6	36.1	24.7
Oxaliplatin (µM)	353.1	340.8	308.0	243.0	213.8	161.4	29.8	29.8

[a] Predicted IC_{50} value.

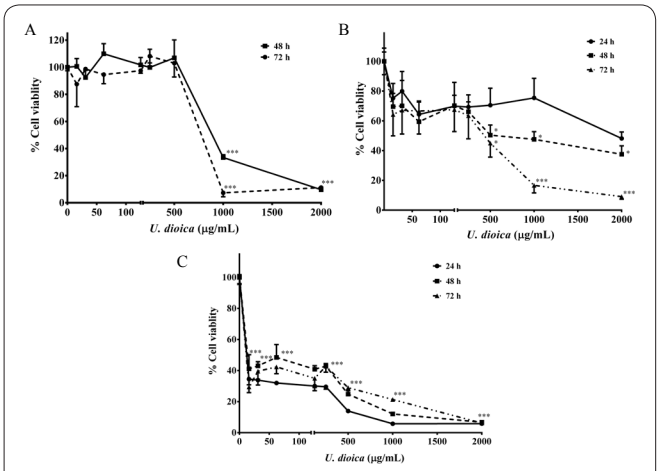

Figure 1. Anti-proliferative effects of hydroalcoholic extract of *U. dioica* radix on human normal foreskin fibroblast (HFF, A), gastric (MKN45, B) and colon (HT29, C) cancer cells. Cells were treated with increasing concentrations of *U. dioica* for 24-72 h. The percentage cell viability (quantitated by MTT assay) was normalized against the negative controls for each cell type. Mean and SEM of the three independent experiments were shown. $^{*}p < 0.05$, $^{***}p < 0.001$ as compared with coresponding control value.

h significantly reduced cell viability to 48 % and 37 %, respectively. While at 72 h, the IC_{50} value of *U. dioica* was 249.9 µg/mL (Fig. 1B, Table 1). The data also indicated that treatment of MKN45 cells with oxaliplatin significantly decreased cell viability with IC_{50} values of 308.0, 243.0 and 213.8 µM for 24, 48 and 72 h, respectively, as compared with control HFF cells (Fig. 2B, Table 1).

U. dioica concentration-dependently increased formation of ROS

Our findings showed a regular statistically time and concentration-dependent increase in ROS generation in MKN45 and HT29 cells, 1 and 3 h after treatment with *U. dioica* compared to control group (Fig. 3A-B). On comparing with control HT29 cells, 64 µg/mL *U. dioica* induced the formation of ROS (3.8 fold) during the 3 h of incubation (Fig. 3B, $p < 0.001$), similar to oxaliplatin (100 µM) (Fig. 4B). There is also a significant increase in ROS content in MKN45 cells 3 h after treatment with *U. dioica* compared to control group (Fig. 3A, $p < 0.001$). Also, treatment with 125 and 250 µM oxaliplatin significantly induced ROS generation in MKN45 cells (Fig. 4A).

U. dioica concentration-dependently increased lipid peroxidation

As shown in Figure 5A, exposure of MKN cells to *U. dioica* for 24 and 48 h concentration dependently increased MDA level, as compared to cells cultured in

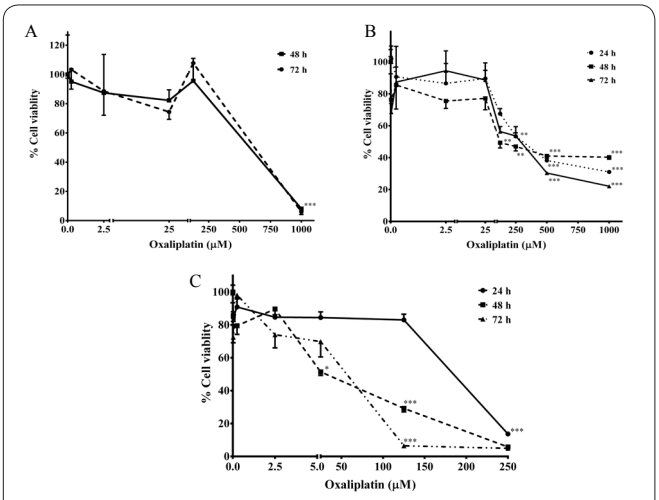

Figure 2. Anti-proliferative effects of oxaliplatine on human normal foreskin fibroblast (HFF, A), gastric (MKN45, B) and colon (HT29, C) cancer cells. Cells were treated with increasing concentrations of oxaliplatine for 24-72 h. The percentage cell viability (quantitated by MTT assay) was normalized against the negative controls for each cell type. Mean and SEM of the three independent experiments were shown. $^{**}p < 0.01$, $^{***}p < 0.001$ as compared with coresponding control value.

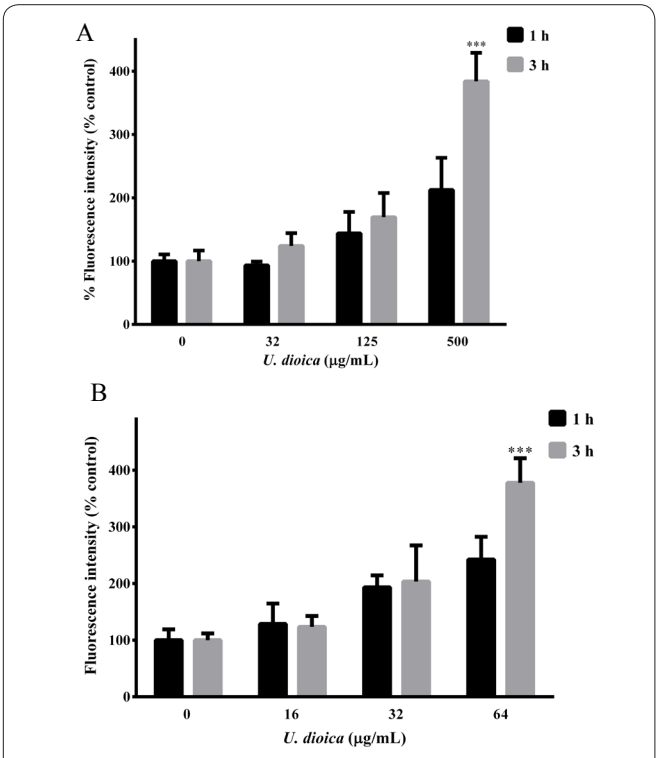

Figure 3. Effect of hydroalcoholic extract of *U. dioica* radix on intracellular reactive oxygen species (ROS) in human gastric (MKN45, A) and colon (HT29, B) cancer cells. Cells treated with increasing concentrations of *U. dioica* for 1 or 3 h. Data expressed as mean ± SEM (n=9). $^{***}p < 0.001$ as compared with coresponding control value.

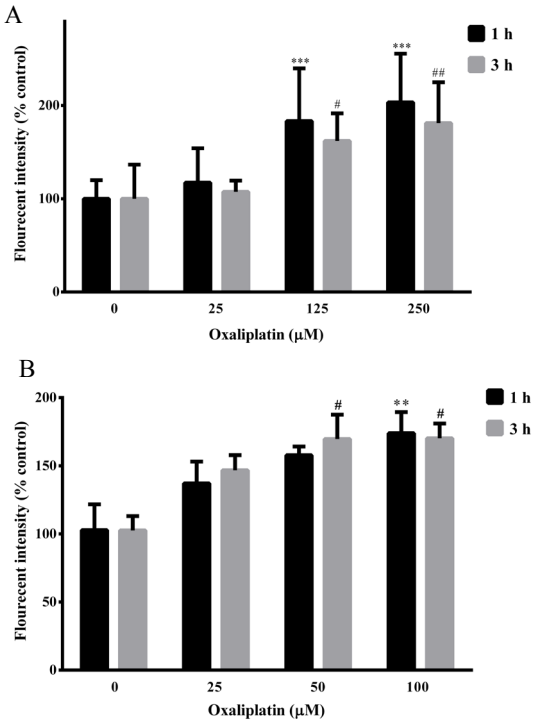

Figure 4. Effect of oxaliplatin on intracellular reactive oxygen species (ROS) in human gastric (MKN45, A) and colon (HT29, B) cancer cells. Cells treated with increasing concentrations of oxalipaltin for 1 or 3 h. Data expressed as mean ± SEM (n=9). $^{***}p < 0.001$, $^{**}p < 0.01$, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ as compared with coresponding control value.

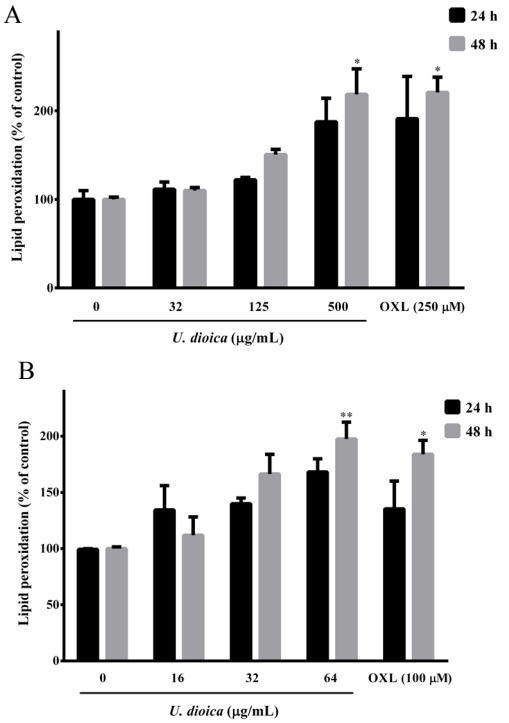

Figure 5. Effect of hydroalcoholic extract of *U. dioica* radix and oxaliplatin (OXL) on malondialdehyde content of human gastric (MKN45, A) and colon (HT29, B) cancer cells. Cells treated with increasing concentrations of *U. dioica* for 24 or 48 h. Data expressed as mean ± SEM (n=9). $^{*}p < 0.05$, $^{**}p < 0.01$ as compared with coresponding control value.

control condition. 500 μg/mL *U. dioica* increased MDA content about 2.2 fold, after 48 h treatment ($p < 0.05$), which is similar to that of oxaliplatin (250 μM)-treated cells. Likewise, the level of MDA in HT29 cells treated with 64 μg/ml was significantly ($p < 0.01$) higher than that of untreated cells (about 2 fold). Oxaliplatin (100 μM) also significantly increased the level of lipid peroxidation in HT29 cells after 48 h treatment (Fig. 5B).

U. dioica concentration-dependently induced apoptotic cell death

Figure 6A-G shows that *U. dioica* at high concentrations induces apoptosis in MKN45 cells, significantly. After 48 h treatment, only 3% of untreated cells undergone apoptosis, but 25% (32 μg/ml, $p > 0.05$), 36% (125 μg/ml, $p < 0.01$) and 44% (500 μg/ml, $p < 0.001$) of *U. dioica*-treated cells have entered apoptosis stages. Oxaliplatin-treated and serum deprived MKN45 cells also showed considerable apoptosis (36% and 33%, respectively) as compared to control untreated cells ($p < 0.01$ for both) (Fig. 6E-G).

The percentages of apoptosis, as determined with annexin V-FITC/PI flow cytometric analysis (Fig. 7A-G) were 30% ($p > 0.05$), 36% ($p < 0.05$) and 49% ($p < 0.001$) at 16, 32 and 64 μg/ml *U. dioica*-treated HT29 cells, while oxaliplatin and serum deprivation increased apoptotic cell death by 35% ($p < 0.05$) and 57% ($p < 0.001$), respectively. These results showed that the number of apoptotic cells increased concomitantly with concentration, as compared with control cells.

Discussion

Gastric and colorectal cancers are major worldwide health problem owing to its high prevalence and mortality rates (14, 15). It has been proposed that abnormalities in apoptotic inhibition contribute to both the pathogenesis of gastric and colorectal cancers and the resistance to chemotherapeutic drugs and radiotherapy (15, 16). Natural products have long been utilized to prevent and treat neoplasms and therefore searching for natural products directed at inducing apoptosis of cancer cells may be great strategy for gastroenterological cancer chemoprevention (17). Gözüm *et al*. reported more than 40% of patient with a diagnosis of cancer used medicinal herb as an alternative treatment and the most commonly used herb was stinging nettle leaf (*u. dioica*) or seed of nettle (18). This study is the first to investigate the antineoplastic and pro-apoptotic effects of *U. dioica* in gastric and colorectal cancer cells. We report here that *U. dioica* exhibits significant cytotoxicity in the cancer cells at selective concentrations. The cytotoxic effect of *U. dioica* was more pronounced against the neoplastic cells than human normal fibroblast cells. In addition, human colorectal HT29 cells were found to be more sensitive to *U. dioica* anti-proliferative effect, than human gastric MKN45 cancer cells. The MKN45 cell line came from poorly differentiated gastric adenocaricinoma with cancer stem cell properties and is usually resistant to chemotherapy (19). Tominaga *et al*. also reported that MAP kinases (in particular ERKs and p38 MAPK) followed by cyclooxygenase-2 (COX-2) is in part involved in HT29 cell proliferation, while this might not be associated with MKN45 cell proliferation (20). Several evidences show that endogenous COX-2 is involved in carcinogenesis and in tumor growth for colorectal cancer (21, 22). The role of MAP kinase signal transduction pathway in gastrointestinal cancers

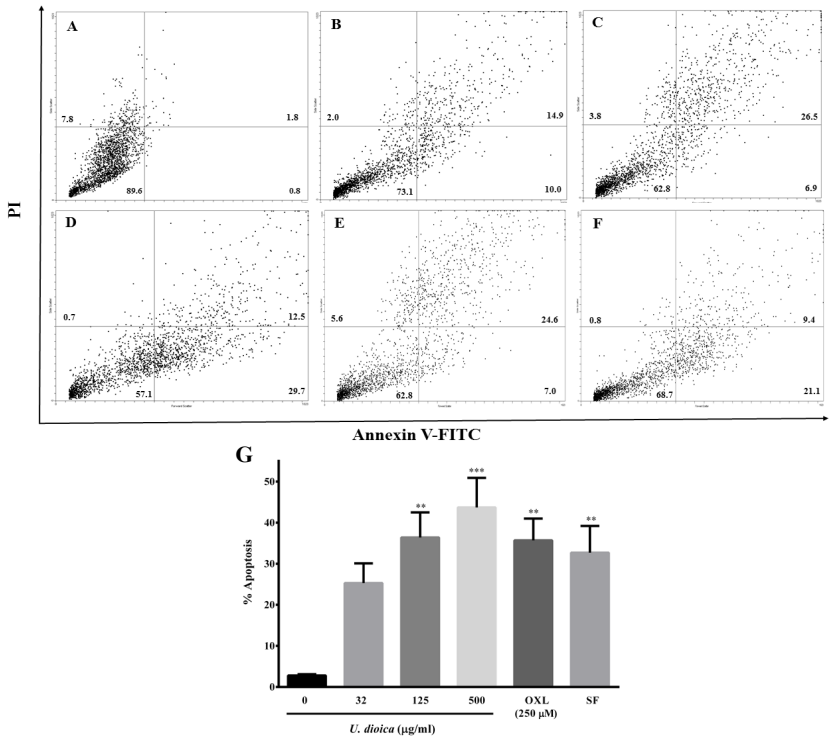

Figure 6. Apoptotic cell death induced by hydroalcoholic extract of *U. dioica* radix, oxalipaltin or serum free condition in human gastric (MKN45) cancer cells. Cells were incubated with increasing concentrations of *U. dioica* or oxalipaltin (OXL) for 48 h or serum free (SF) condition for 12 h. Apoptosis was assayed by annexin V-FITC staining kit and analysed by flow cytometry. (A) MKN45 control cells; (B) MKN45 cells treated with 32 µg/mL *U. dioica*; (C) MKN45 cells treated with 125 µg/mL *U. dioica*; (D) MKN45 cells treated with 500 µg/mL *U. dioica*; (E) MKN45 cells treated with 250 µM oxaliplatin (OXL); (F) MKN45 cells treated under serum free condition (SF); (G) Apoptosis rate shown by bar graph. The data shown are the means ± SEM from three independent experiments. $^{**}p < 0.01$, $^{***}p < 0.001$ as compared with control value.

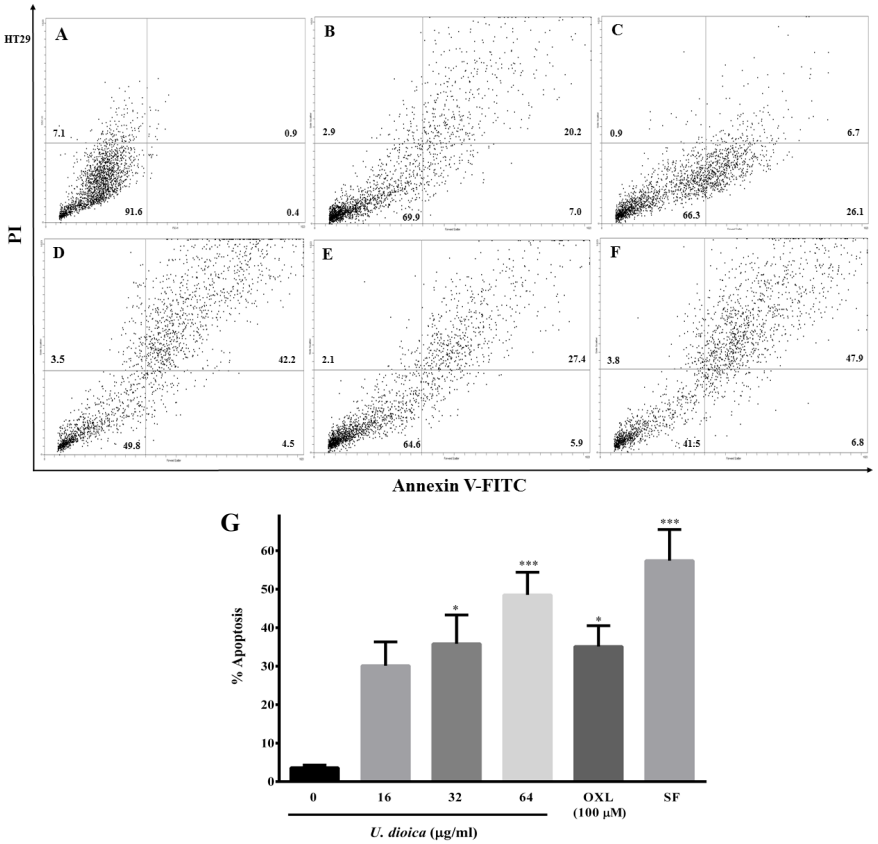

Figure 7. Apoptotic cell death induced by hydroalcoholic extract of *U. dioica* radix, oxalipaltin or serum free condition in human gastric (MKN45, A) and colon (HT29, B) cancer cells. Cells were incubated with increasing concentrations of *U. dioica*, oxalipaltin for 48 h or serum free condition for 12 h. Apoptosis was assayed by annexin V-FITC staining kit and analysed by flow cytometry. (A) HT29 control cells; (B) HT29 cells treated with 16 µg/mL *U. dioica*; (C) HT29 cells treated with 32 µg/mL *U. dioica*; (D) HT29 cells treated with 64 µg/mL *U. dioica*; (E) HT29 cells treated with 100 µM oxaliplatin (OXL); (F) HT29 cells treated under serum free condition (SF); (G) Apoptosis rate shown by bar graph. The data shown are the means ± SEM from three independent experiments. $^{*}p < 0.05$, $^{***}p < 0.001$ as compared with control value.

and its potential as an approach to cancer treatment was also fully elucidated (23). Our paper also provides evidence that *U. dioica* induces concentration-dependent apoptosis. In the same manner, HT29 cells exhibited increased sensitivity towards apoptotic effect of *U. dioica*, compared with MKN45 cells. It is noteworthy to mention that these effects are comparable with standard anti-neoplastic drug, oxaliplatin.

In this study, we also showed that cellular ROS content and lipid peroxidation were increased after treatment with *U. dioica* or oxaliplatin, in a time- and concentration-dependent manner, suggesting the possible role of oxidant stress in their potential cytotoxic and apoptogenic activities. Several studies demonstrated that increased ROS generation and resultant altered redox status may play an important role in the initiation and progression of cancer (24, 25). Indeed, such cellular oxidative stress and ROS-mediated mitogenic and anti-apoptotic signaling in cancer cells can be selectively targeted by direct- or indirect-acting redox modulators, representing a novel class of promising anticancer agents, called redox chemotherapeutics (25). Because of such intrinsic oxidative stress, cancer cells are more vulnerable to damage by further ROS insults induced by exogenous agents (26). Considerable evidences exist implicating oxidative stress as a mediator of apoptosis (27, 28). To this regard, apoptosis has considered as a mechanism to eliminate cells which large amounts of ROS (29). Several well-known chemotherapy agents including anthracycline antibiotics and platinum compounds induce high levels of ROS and apoptosis (27). The involvement of oxidative injury in lipid-mediated apoptotic signaling has been also reported in various cancer cells. In such view, the end products of lipid peroxidation (such as 4-hydroxynonenal and malondialdehyde), considered as second messengers of oxidative stress, can also induce apoptosis in cancer cells (26, 30, 31). On the other hand, the increased intracellular antioxidant capacity is a common phenomenon in tumor cells (including cancer stem cells) resistant to many anticancer agents. In these cells, pharmacologic agents with pro-oxidant and lipid peroxidation-inducing properties have shown to improve the effectiveness of chemotherapy by abrogation such adaptation mechanisms (26). The pro-oxidant capacity of *U. dioica* described here regarding its well-characterized antioxidant properties (32, 33), could suggest *U. dioica* as a potential redox chemotherapeutic.

A limited number of investigations have explored the anticancer effects of different parts of *U. dioica* on cancer cells. In agree with our findings, Levy *et al*. reported aqueous extract of *U. dioica* leaves produced a significant and dose dependent reduction in viability of human prostate carcinoma LNCaP cells with IC_{50} value of 42 µg/ml. The cytotoxic activity was mediated through oxidative stress and apoptosis (34). Wagner *et al*. showed a rare lectin (*U. dioica* agglutinine, UDA) isolated from *U. dioica* rhizomes, probably by blocking epidermal growth factor (EGF) receptor, possess in vitro antiproliferative properties on A431 epidermoid cancer cells (35). In a study by Konrad *et al*., treatment with 20% methanolic extract of *U. dioica* root led to significant concentration- and time-dependent inhibition of human prostatic epithelial (LNCaP) and not stromal cells proliferation (36). Di Sotto *et al.* indicated a protein fraction from the aerial part of *U. dioica* (namely UDHL30) possesses strong antimutagenic and radical scavenging effects suggesting its potential chemopreventive properties (37). Gul *et al.* also found significant correlation between concentration of *U. dioica* essential oil and chromosomal aberrations, micronuclei formation and cytotoxicity (38). In contrast, in a study conducted by Taghizadeh Kashani *et al.*, ethanolic extract from aerial parts of *U. dioica* showed poor cytotoxic activity against HT29 and Caco-2 (colorectal adenocarcinoma) cells with IC_{50} values > 1000 µg/ml (39). Trouillas *et al.* also demonstrated that the water-soluble fraction from aerial parts of *U. dioica* devoid significant antioxidant, anti-inflammatory and cytotoxic activities against B16 mouse melanoma cells (40).

Empirical studies use daily doses of 4-6 g of nettle root as an infusion or 300-756 mg for dried hydroalcoholic extracts. Phytosterols, lignans, polysaccharides, isolectins (mainly UDA), coumarins (e.g. scopoletin), phenols (e.g. p-hydroxy-benzaldehyde), triterpenoic acids and monoterpendiols are considered to be among the active principles of *U. dioicae* radix (10). In the present report the mechanism could not be studied because the effects observed could be due to several molecules and molecules which are not yet identified.

In conclusion, the present study demonstrated that the hydroalcoholic extract of *U. dioica* radix inhibited the growth and proliferation of gastric and colorectal cancer cells while posing no significant toxic effect on normal cells. The marked difference in cytotoxicity between cancer and normal cells suggests an exciting potential for *U. dioica* as novel alternatives to cancer therapy. The IC_{50} values reported in our study may be considerably lower if the pharmacological active compounds become pure. *U. dioica* not only increased levels of oxidants (ROS and lipid peroxidation), but also induced concomitant increase of apoptosis. The precise signaling pathway by which *U. dioica* induce apoptosis needs further research.

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