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Original Research

Urtica dioica inhibits cell growth and induces apoptosis by targeting *Ornithine decarboxylase* and *Adenosine deaminase* as key regulatory enzymes in adenosine and polyamines homeostasis in human breast cancer cell lines

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Abstract: Breast cancer is a heterogeneous and multifactorial disease with variable disease progression risk, and treatment response. *Urtica dioica* is a traditional herb used as an adjuvant therapeutic agent in cancer. In the present study, we have evaluated the effects of the aqueous extract of *Urtica dioica* on Adenosine deaminase *(ADA)* and Ornithine decarboxylase *(ODC1)* gene expression in MCF-7, MDA-MB-231, two breast cancer cell lines being estrogen receptor positive and estrogen receptor negative, respectively. Cell lines were cultured in suitable media. After 24 h, different concentrations of the extract were added and after 72 h, *ADA* and *ODC1* gene expression as well as *BCL2* and *BAX* apoptotic genes were assessed by Taqman real time PCR assay. Cells viability was assessed by MTT assay, and apoptosis was also evaluated at cellular level. The intra and extracellular levels of ODC1 and ADA enzymes were evaluated by ELISA. Results showed differential expression of *ADA* and *ODC1* genes in cancer cell lines. In MCF-7 cell line, the expression level of *ADA* was upregulated in a dose-dependent manner but its expression did not change in MDA-MB cell line. *ODC1* expression was increased in both examined cell lines. Also, increased level of the apoptotic *BAX/BCL-2* ratio was detected in MCF-7 cells. These results demonstrated that *Urtica dioica* induces apoptosis in breast cancer cells by influencing *ODC1* and *ADA* genes expression, and estrogen receptors. The different responses observed with these cell lines could be due to the interaction of *Urtica dioica* as a phytoestrogen with the estrogen receptor.

Key words: Breast cancer cell line; *Urtica dioica*; Adenosine deaminase (ADA); Ornithine decarboxylase (ODC1).

Introduction

Breast cancer is the most common cancer in the world and the second cause of cancer death among women after lung cancer (1-2). A variety of typical medical treatments such as chemotherapy, radiotherapy, hormone therapy, and surgery, as well as newer nanotechnology approaches are currently used for treatment of breast cancer, but they are associated with various side effects (3-4). A recent study showed that adjuvant therapy such as use of herbal medicine can help to prevent developing cancer and improve the survival rate of patients (5). There are many different types of herbal medicine (6). *Urtica dioica* belongs to the plant family of *Urticaceae* that contains different beneficial compounds such as anti-oxidants, formic acid, lectins, alkaloids, tannins, flavonoids, etc. (7-9). Our previous study has shown that the aqueous extract of *Urtica dioica* could induce the apoptotic pathway and inhibit proliferation of MCF-7 breast cancer cell line (10). Several studies pointed out the important effects of adenosine and polyamine-sin apoptosis (11-13). Polyamines and adenosine are essential molecules for normal cell processes (14-15). Ornithine decarboxylase (ODC) and adenosine deaminase (ADA) enzymes are involved in polyamines and adenosine metabolism, respectively (13, 16).

The aim of this study was to evaluate the effects of *Urtic adioica*, a natural herb widely distributed, on cell growth by evaluating *ADA* and *ODC1* genes expression in two breast cancer cell lines (MCF-7 and MDA-MB-231).

Materials and Methods

Plant materials

Aqueous extract preparation from *Urtica dioica* leaves was described previously (10). Briefly, 300 ml of 5% ethanol were added to 15 g of powdered plant leaves and the mixture was boiled for 15 min. After filtration, centrifugation and supernatant evaporation, the crude extract was stored at-20 °C for further use.

Cell culture

Two human breast cancer cell lines, MCF-7(estrogen receptor-positive) and MDA-MB-231 (estrogen receptor-negative) were obtained from Pasteur institute, Tehran, Iran. Primary fibroblasts were isolated from fresh foreskin, as previously described by Pandamooz et al. (17). Cell lines were cultured in suitable media (MDA-MB-231 and fibroblast cell lines in DMEM culture me-

dium; and MCF-7 in RPMI 1640), supplemented with 10% FBS, and 1% antibiotics (penicillin/ streptomycin (Invitrogen) in a humidified atmosphere containing 5%CO2 and 95% air, at 37°C.

Cell viability assay

Breast cancer cell lines (MCF-7 and MDA-MB-231) and foreskin fibroblast cells were seeded in 48 and 24-well plates at a density of 2×10^4 and 5×10^4 per well, respectively. Cells were treated with various concentrations (0, 0.75, 1.5 and 3 mg/ml) of *Urtica dioica* extract for 72 h. Next, cells were washed with PBS, and then MTT was added into each well for 4 h at 37 °C under 5% CO2, 95% air and complete humidity. Formazan crystals dissolved in DMSO and their absorbance was read at 570 nm using a microplate reader.

Dual acridine orange/ethidium bromide fluorescent staining for apoptosis detection

For cellular assessment of apoptosis using acridine orange/ethidium bromide (AO/EB) staining, cells were seeded in a 6-well plate. Cells were treated with *Urtica dioica* at a IC 50 value concentration for 72 h in the CO2 incubator, and then 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 cells. After staining, cells were examined under a fluorescence microscope.

Treatment with *Urtica dioica* for gene expression

Cells were seeded in a 25 cm^2 flask at a density of 4×10^5 cells. After 24 h, different concentrations (0.75, 1.5 and 3 mg/ml) of the extract were added and cells were cultured for 72 h. After period time, supernatants were collected and extracellular levels of ADA and ODC were measured using ELISA method. The adherent cells were detached with 0.025% trypsin-EDTA and cell suspensions were centrifuged and the pellets were divided into two aliquots after re-suspension in medium. Then, one aliquot was used for RNA extraction, and the other was used for intracellular ADA and ODC1 levels determination.

QPCR assay

Total RNA was extracted from treated and untreated cultures of MCF-7, MDA-MB-231, and fibroblast cells in the presence of Tripure isolation reagent (Roche, USA) according to the manufacturer's protocol. For gene expression analysis, complementary DNA (cDNA) was synthesized as previously described (18).Gene expression analysis was performed using a stem-loop Taqman assay in the presence of a universal Taqman probe and HotStarTaq Plus DNA Polymerase (Qiagen, Hilden, Germany), using StepOne real-time PCR apparatus (Applied Biosystems, USA). Amplification protocols for all studied genes were set at 5 min at 95°C, followed by 45 cycles of 15s at 95°C, and 1 min at 60°C. Primers and probes for *ADA*, *ODC1*, *BAX*, *BCL2* and *GAPDH* were designed within exon-exon regions using AlleleID 6.0 software according to a method described by Fattahi et al. (18-19). The sequences of primers are provided in Table 1. The sequence of the universal Taqman probe was FAM 5' TGGATGTGTCTGCGGCGTTTTATCAT 3' BHQ-1 and the sequence of the universal reverse primer was 5' GTATCCAGTGCTGCGACCGT 3'.

Enzyme-linked immune sorbent assay (ELISA)

Intracellular and extracellular concentrations of ADA and ODC1 were measured using human-specific ELISA kits (Bioassay technology laboratory, Shanghai, China) according to the manufacturer's protocols.

Table 1. Sequences of primers used in stem-loop Taqman assays.

Gene Name	Accession Number	Primers 5' 3'
		Specific forward primer:
		CATGGAGCTGCAGAGGATGATTGC
BAX	NM_001291429.1	RT-PCR primer
		GTCGTATCCAGTGCTGCGACCGTATGGATGTGTCTGCGGCGTTTTAT-CATGCACTGGATACGACCCAGTTGAAGTTGC
		Specific forward primer:
		GTGTGTGGAGAGCGTCAACC
BCL2	NM_000633.2	RT-PCR primer
		GTCGTATCCAGTGCTGCGACCGTATGGATGTGTCTGCGGCGTTTTAT-CATGCACTGGATACGAC CATCCCAGC CTC
		Specific forward primer:
		GACGCCCTACGAGTGGATG
ADA	NM_001804.2	RT-PCR primer
		GTCGTATCCAGTGCTGCGACCGTATGGATGTGTCTGCGGCGTTTTAT-CATGCACTGGATACGAC GAGTCTTACCGC
		Specific forward primer
		ACACTGTGATTGCCAGGAGAG
ODC1	NM_001291285.1	RT-PCR primer
		GTCGTATCCAGTGCTGCGACCGTATGGATGTGTCTGCGGCGTTTTAT-CATGCACTGGATACGAC CAAGAGTCCAGTC
		Specific forward primer
		TGGAGTCCACTGGCGTCTTCAC
GAPDH	NM_001289745	RT-PCR primer
		GTCGTATCCAGTGCTGCGACCGTATGGATGTGTCTGCGGCGTTTTAT-CATGCACTGGATACGAC AGGCATTGCTGA

Statistical analysis

Results are presented as mean ± SD of three independent experiments. Relative gene expression of target RNA level was determined using the $2^{-\Delta\Delta CT}$ method. The Pearson correlation coefficient was used to measure the strength of a linear association between RNA and protein levels. Comparisons of results between groups were performed by paired Student's *t* test. A value of $P< 0.05$ was considered as statistically significant.

Results

Cell viability

The cytotoxic activity of different concentrations of *Urtica dioica* extract on the growth of the human breast cancer cell lines, and fibroblasts are summarized in Figure 1. MCF-7 and MDA-MB-231 cell proliferation was inhibited in a dose-dependent manner after exposure to different extract concentrations whereas no significant changes were detected for human fibroblast cells. Data analysis also showed that there was no significant difference in cell viability between MCF-7 and MDA-MB-231 cells at 0.75-1.5 mg/ml ($P > 0.05$). But the difference in cell viability was significant at 3 mg/ml of the extract ($P < 0.05$). Approximately, the IC 50 value for both breast cancer cell lines was 2 mg/ml.

Apoptosis induction by *Urtica dioica* in breast cancer cell lines

AO/EB was used to confirm whether the reduced cell viability was due to apoptosis. As shown in Figure 2, the breast cancer cells showed obvious segmented chromatin after 72 h treatment with *Urtica dioica* extract, confirming their apoptotic state, while fibroblast cells were alive and showed bright green nucleus with uniform intensity.

Expression of *ADA* and *ODC1*

The expression levels of *ADA* and *ODC1* genes in MCF-7, MDA-MB-231and fibroblasts are shown in Figure 3a-3b. In MCF-7 cell line, *ADA* was up-regulated in a dose-dependent manner. In contrast, fibroblast cells exhibited *ADA* down-regulation following treatment with different concentrations of *Urtica dioica* aqueous extract. There was a significant difference of *ADA* gene expression between untreated and treated MCF-7 and fibroblast cells ($P< 0.05$) while no expression change was observed in MDA-MB-231 cell line before and after treatment with different plant extract concentrations. In all examined cell types, *ODC1* was down-regulated following treatment with 0.75 mg/ml of *Urtica dioica* extract, but its expression was increased in a dose dependent manner (Figure 3).

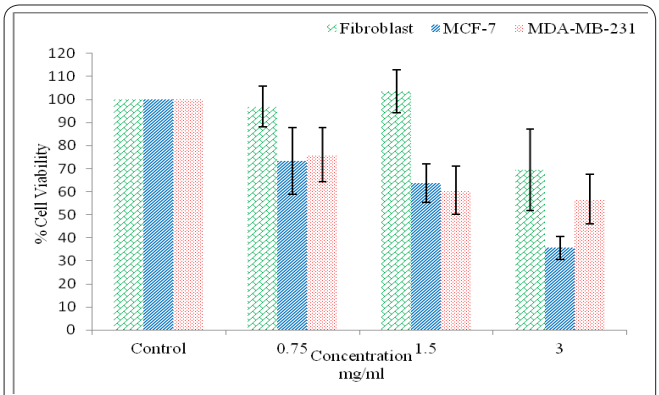

Figure 1. Dose-dependent cytotoxic activity effect of different concentrations of *Urtica dioica* extract on breast cancer cell lines and fibroblasts.

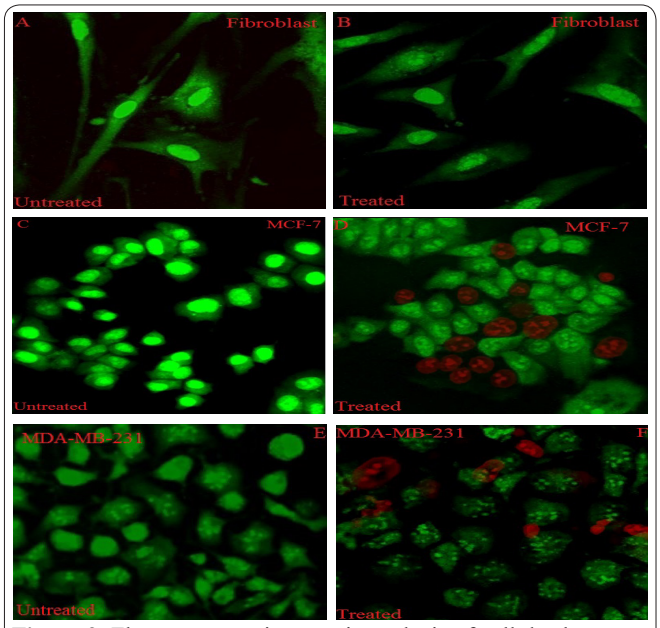

Figure 2. Fluorescence microscopic analysis of cell death pattern measured by acridine orange/ethidium bromide double staining assay. The cells are divided into four categories: Live cells with a green nucleus, Early apoptotic cells have green nuclei, but the chromatin is fragmented, Late apoptotic cells have orange to red nuclei with segmented chromatin. Necrotic cells have orange to red staining with a condensed nucleus (Magnification, ×400).

BAX/*BCL-2* ratio

The expression levels of anti and pro-apoptotic *BCL2*-related genes are shown in figure 4. A significant higher *BAX*/*BCL2*ratio for MCF-7, MDA-MB-231 and fibroblasts was found at all tested concentrations of *Urtica dioica* aqueous extract. Notably, the highest increase of *BAX*/*BCL2*ratio was observed in MCF-7 cells after 72h incubation with 1.5 mg/ml of plant extract (Figure 4d).

Intracellular and extracellular ADA and ODC1 levels

The extracellular and intracellular concentrations of ADA and ODC1 proteins were measured after treatment with different concentrations of *Urtica dioica*. As shown in Table 2, ADA intracellular concentration was in-

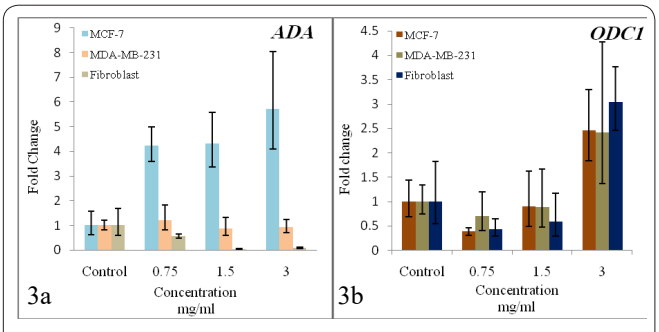

Figure 3. The gene expression levels of *ADA* (3a) and *ODC1* (3b) after 72h treatment with *Urtica dioica*. *GAPDH* gene expression was used for normalization. The results are expressed as fold changes. Data are expressed as the mean ± SD.

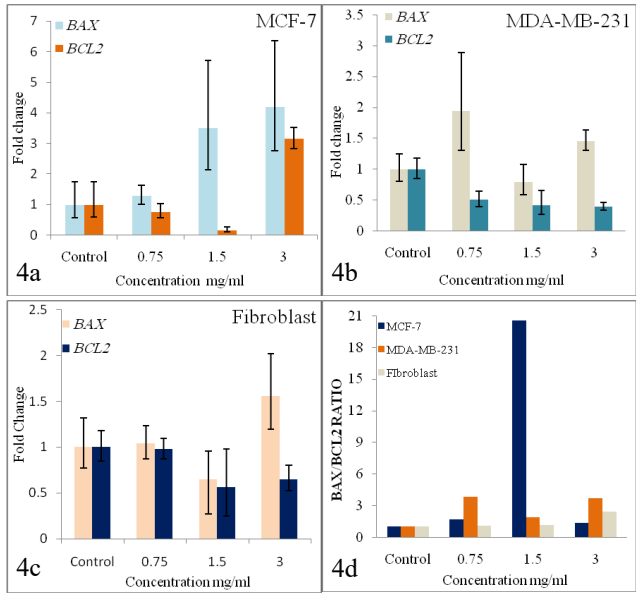

Figure 4. Effects of *Urtica dioica* aqueous extraction the levels of *BAX* and *BCL-2* mRNA expression by real-time PCR assay in MCF-7 (4a), MDA-MB-231(4b) and fibroblast (4c) cells. *BAX/BCL-2* ratio (4d). Data are expressed as the mean ± SD.

creased in a dose-dependent manner in MCF-7 cell line while its intracellular concentration was decreasedin a dose-dependent manner in fibroblasts. Moreover, ADA intracellular concentration was approximately constant following treatment with different concentrations of *Urtica dioica* in MDA-MB-231 cell line. All cell lines showed the highest positive correlation between mRNA level and intracellular ADA protein levels ($r > 0.9$). The lowest intracellular ODC1 level was found at 1.5 mg/ml of *Urticadioica*aqueous extract for fibroblasts and MDA-MB-231 cell lines, and at0.75 mg/ml for MCF-7 cell line. MCF-7 and fibroblasts showed the highest positive correlation between mRNA levels and intracellular ODC1 protein levels($r > 0.86$) while weak correlation between mRNA and intracellular ODC1 levels was found in MDA-MB-231 cells ($r=0.618$).

Discussion

Herbal medicine may be used in cancer treatment and help to prevent or reduce the symptoms of cancer without presenting the side effects of different traditional treatments (1). Several studies have shown anti proliferative and apoptotic effects of *Urtica dioica* on different cancer cell lines (20-22). The present study aimed to evaluate the effects of the aqueous extract of *Urtica dioica* on *ADA* and *ODC1* gene expression in breast cancer cell lines (MCF-7, MDA-MB-231) and fibroblasts as a control. Also, the apoptotic effect of *Urtica dioica* in breast cancer cell lines (MCF-7, MDA-MB-231) and fibroblasts was investigated at the molecular and cellular level. *ADA* is a key regulator of adenosine homeostasis which deaminates adenosine to inosine (23). Several studies showed that the level of *ADA* is increased in cancerous cells (12, 24). Also, *ADA* expression and activity was shown to be inhibited by plant extracts (25). Durak et al. showed that *Urtica dioica* extracts significantly inhibited *ADA* activity in prostate cancer tissues (26). *ADA* enzyme inhibition by *rosemary* extract has also been reported in gastric cancer tissues and their adjacent non tumoral tissues, but not in colon tissues (27). In the presen tstudy we observed an up regulation of *ADA* in MCF-7 cells but no expression change was noticed in MDA-MB cell line. These results suggest that *ADA* expression may vary in different types of tissues and cells due to the pleiotropic effects of this enzyme. Recent studies have shown that adenosine levels are increased in cancer. Adenosine interacts with four different types of receptors (A1R, A2AR, A2BR, and A3R). Adenosine increase can cause immune-suppressive effects through A2A and A2B receptors, depending on cell types (28-29). MCF-7 cells seem to be devoid of any significant amount of adenosine receptors, whereas MDA-MB-231 cells express very high levels of A2B receptors which show low affinity for adenosine (30), resulting therefore in reduced effects on immune system suppression.A2B receptors also inhibit ERK1/2 phosphorylation by stimulating adenylyl cyclase (AC) and phospholipase C (PLC) (31-32), although the exact mechanism is not completely understood yet. Many studies have shown that MAPK signaling pathway is involved in cell growth, proliferation and survival (33-34). Therefore, ERK inhibitors can be considered as candidate targets for cancer therapy. We suggest that the aqueous extract of *U. dioica* might stimulate A2B receptor activity in MDA-MB-231, and this effect may inhibit RAS-ERK signaling in this cancer cell line. ODC1 is the key enzyme in biosynthesis of polyamines, which can play a regulatory role in cellular processes, including proliferation, cell growth and apoptosis (35-36). Several studies have suggested that the inhibition of ODC1 expression and activity may be an approach

Table 2. Influence of *Urtica dioica* extract on extracellular and intracellular concentrations of ADA and ODC1 proteins. Correlation (Spearman test) was evaluated between mRNA expression (ADA and ODC1) analyzed by real-time quantitative PCR, and protein production evaluated by the Enzyme-linked immune sorbent assay (ELISA). Fib: fibroblasts.

Sample		ADA (ng/ml)			ODC1 (ng/ml)		
		MCF-7	*MDA-MB231*	Fib	MCF-7	*MDA-MB231*	Fib
Control	Extracellular	325.4	253.5	229	460.9	365.6	393
	Intercellular	469.9	633.9	539.5	761.9	924.1	727.7
0.75 mg/ml	Extracellular	227.7	265.3	257.8	341.1	195.4	424.6
	Intercellular	717.5	629.9	473.1	750.5	1031.7	737.4
1.5 mg/ml	Extracellular	268.6	208.4	220.9	336.7	289.1	376.8
	Intercellular	700.2	605.8	141	1753.6	966.7	650.1
3 mg/ml	Extracellular	186.7	169.9	261.4	316.6	275.1	408.9
	Intercellular	828.7	659.7	123.9	1014.9	1027.1	1251
Correlation with mRNA	Extracellular	0.3631	0.9731	0.407	0.5	0.6048	0.488
	Intracellular	0.9253	0.9594	0.9474	0.9385	0.6603	0.8607

for inhibiting cancer development (13, 37). Neto et al. reported that *Cranberry* extracts inhibited ODC activity which impacted cell proliferation in epidermal ME 308 cells (38). Furthermore, polyamines interact with histone acetyltransferase (HAT) and histone deacetylases (HDAC) enzymes that effect on histone acetylation status (39). Over expression of ODC in murine skin tumors was shown to lead to high level of acetylated p53, which can induce downstream target genes (40). In the present study, we found that treatment of breast cancer cell lines with *Urtica dioica* induced *ODC1*expression correlated with apoptosis promotion. Therefore, *ODC1* overexpression may lead to transcriptional repression of genes controlling cellular proliferation and growth through histone acetylation alteration. As *Utrica diocia* is a phytoestrogen (41), it may also influence cell growth and apoptosis via interaction with the estrogen receptor (42). MCF7 cells possess estrogen and progesterone receptors, and are HER2 negative, whileMDA-MB-231 cells are devoid of these three receptors. This difference regarding hormone receptors could be responsible for different responses observed with these two cell lines. Another possibility explaining differences in gene expression pattern in these cell lines may be the difference in P53 activity. MDA-MB-231 harbors *P53* mutations and MCF-7 have wild-type *P53* (43). P53 can control the transcription of *BCL-2* family of genes particularly *BCL-2* (anti-apoptotic) and *BAX* (pro-apoptotic). Also, P53 can induce the expression of P21cyclin-dependent kinase inhibitor, which triggers cell cycle arrest in response to various stress stimuli by interacting with factors that influence cell cycle progression (44-45). So, it is possible that *Urtica diocia* induces apoptosis via a P53-independent pathway in MDA-MB-231 breast cancer cells. Also, we observed a marked increase of apoptosis with 1.5 mg/ml of *Urtica dioica* extract, which decreased with higher extract dose. This could be due to increased synthesis of heat shock proteins in response to a variety of stresses able to prevent apoptosis. In conclusion, our results demonstrated that *Urtica dioica* extract effects on *ODC1* and *ADA* genes expression and inhibits growth and induces apoptosis of breast cancer cells. This is a first study showing that cytotoxicity of *Urtica dioica* can induce *ODC1* and *ADA* expression in breast cancer cell lines. Further studies are necessary to achieve more definitive results and determine the diagnostic value of *ADA* and *ODC1* genes in cancer development and also the mechanism of action of *Urtica dioica* on cell growth and apoptosis in different types of cancer cells.

Conflict of Interest: The authors declare that they have no conflict of interest.

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