Evaluation of p53 and Bcl-2 genes and proteins expression in human breast cancer T47D cells treated with extracts of *Astrodaucus persicus* (Boiss.) Drude in comparison to Tamoxifen

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ABSTRACT

Background and purpose of the study: Screening of different plant components for new anticancer drugs is one of the main research activities throughout the world. In this study, the anticancer effects of *Astrodaucus persicus*, an Iranian species of family of Umbelliferae, in human breast cancer T47D cells was investigated. Also since tumorigenesis is thought to result from a series of progressive gene alterations, including activation of oncogenes and inactivation of tumor suppressor genes, expression of two such genes, p53 and Bcl-2 that are believed to play a crucial role in tumorigenesis and cell death were determined.

Materials and Methods: The p53 and Bcl-2 genes and proteins expression alterations in T47D cells at RNA synthesis level was studied by using RT-PCR analysis and protein synthesis using immunocytochemistry technique.

Results: p53 gene expression increased significantly in the presence of both plant extracts but Bcl-2 expression increased significantly in the presence of aerial and decreased significantly in the presence of root extract. In addition, treatment of T47D cells with plant extracts decreased the nuclear staining of p53 and cytoplasmic staining of Bcl-2 proteins.

Conclusion: These results suggest that the methanolic extracts of *Astrodaucus persicus* especially its root extract may contains bioactive compounds, probably coumarins that prevents proliferation of T47D breast carcinoma cells by mechanisms such as apoptosis. These data are the first report on the possible molecular mechanisms of action of *Astrodaucus persicus* extracts in breast cancer cell proliferation.

Keywords: *Astrodaucus persicus*, T47D cells, p53 and Bcl-2 gens, RT-PCR, Immunocytochemistry

INTRODUCTION

Breast cancer is one of the most common cancers in women and is the second leading cause of cancer death after lung cancer among women worldwide (1). Out of one million of new cancer cases in the world, breast cancer comprises 18% of all female cancers (2). The molecular aspects of breast cancer biology and pathogenesis have been greatly increased during the last decade. Development of breast cancer involves activation and deactivation of several types of genes to promote malignancy (3). Wild type p53 is an important regulatory protein in induction of apoptosis following DNA damage induced by anti-cancer drugs (4, 5). This tumor suppressor protein leads to either arrest of growth of viable cells in G1 phase or apoptosis (6, 7).

The Bcl-2 is the founding member of family of genes that either prevents or promotes cellular apoptosis (8, 9). The Bcl-2 itself is an anti-apoptotic gene that prevents initiation steps of apoptosis and programmed cell death. Expression of the Bcl-2 gene has been shown to be regulated by estrogens in normal and cancerous mammary epithelial cells that are estrogen receptor (ER) positive. Expression of the Bcl-2 protein can be determined by immunohistochemical methods in ~80% of breast tumor samples with either node positive or negative (10, 11).

Natural products have played an important role throughout the world in treatment and preventing

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different human diseases including cancers (12, 13). Vinca alkaloids and cytotoxic podophyllotoxins were discovered in the 1950s as first anti-cancer agents from plants (14, 15). In addition to several plants extracted compounds such as camptothecin, vincristine, vinblastine, taxol and podophyllotoxin, many of these natural compounds were structurally modified to yield stronger anti-cancer analogues with less adverse effects (16, 17).

In this concept, the *Astrodaucus persicus*, a medicinal plant which has been used as a remedy in cancer-related diseases was evaluated for its properties (18, 19). In Iran, the genus *Astrodaucus* of the family Umbelliferae is represented by two species, *Astrodaucus persicus* (Boiss.) Drude and *Astrodaucus orientalis* (L.) Drude which grow wild in different regions of Iran and nearby countries such as central and southern Russia, northern and north-western Caucasus, the western desert of Syria, inner Anatolia and central Asia. In Iran, *Astrodaucus persicus* is mainly distributed in Mazandaran, Tehran, Semnan, Ghazvin and Golestan provinces. The composition of the essential oils of the root, leaves and flowers/fruits of *Astrodaucus persicus* have been reported previously (20). The cytotoxicity and alterations of the pattern of cell cycle by this plant extracts has also been investigated (21). The main aim of this study was to evaluate the anti-cancer effects of *Astrodaucus persicus* extracts in human breast cancer T47D cells in comparison to tamoxifen. The expression of p53 and Bcl-2 genes and proteins was also determined to study the molecular mechanisms of anticancer effects of plant extracts on human breast cancer T47D cells.

MATERIALS AND METHODS

Chemicals and reagents

RPMI 1640, pen-strep were obtained from biosera. FBS was purchased from Gibco Co. Tamoxifen was purchased from a commercial source. Tamoxifen was solubilized in 96% ethanol and PBS (1:1 v/v) at 10^{-3} M concentration as stock solution, were stored at 4°C and diluted with culture medium and added to the plate at the defined concentrations. Mouse anti-human p53 and Bcl-2 were obtained from Dako. Tir Pure isolation reagent, protector RNase inhibitor, Taq DNA plymerase, PCR Nucleotide Mix (dNTP), $MgCl_2$ stock solution, 5x buffer for M-MuLV RT, DNA molecular weight marker, M-MuLV reverse transcriptase (RT) were purchased from Roche. DTT (Dithiothreitol) was obtained from Boehringer Mannheim (Germany). p53 and Bcl-2 primers were purchased from MWG (Germany) and β-actin primers were obtained from MOLBIOL (TIB).

Preparation of A. persicus extracts

Astrodaucus persicus plant (Voucher No. 6642-TEH) was collected from the Taleghan road. The plant was identified by Dr. Amin GR. The aerial part and root were isolated, dried and chopped finely using a blender. Plant powders were kept in closed container at -20°C. One hundred grams of the dried material (aerial part and root) were exhaustively extracted with methanol by soxhlet extraction. The methanolic extracts were filtered and evaporated to dryness under reduced pressure by a rotatory evaporator (Rotavapor). The resulting residues of each part of plants were stored at -20°C. The recovery weights of dried material of aerial part and root was about 14% and 5%, respectively. The extract was dissolved in DMSO (Sigma), sterilized by 0.22 µm Syringe filter and subsequently diluted to appropriate working concentrations.

Cell lines and culture medium conditions

The human breast cancer T47D cell line (ATCC HTB-133, USA) was obtained from the Cell Bank of Pasteur Institute in Tehran (Iran). This hormone-sensitive breast cancer cell line was grown routinely as monolayer culture in RPMI-1640 culture medium supplemented with 100U/mL of penicillin, 100µg/ml of streptomycin and 10% heat-inactivated FBS at 37°C in 5% CO2 incubator.

RT-PCR

The mRNA expression levels of p53 and Bcl-2 carried out using semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR). Briefly, the T47D cells were cultured in T-25 flasks and maintained in RPMI medium for 48 hrs. The RPMI medium was supplemented with FBS and pen-strep. To the flask was added the required concentration of aerial part and root of *A. persicus* methanolic extracts to inhibit 50% growth and incubated for 48 hrs. Total cellular RNA was isolated from the untreated (control RPMI flask) and treated cells using Tri Reagent according to manufacturer's protocol. cDNA was synthesized from 2 µg of total isolated RNA by incubation for 1hr at 42°C with M-MLV reverse transcriptase (Fermentas) and $oligo(dT)_{18}$ primer according to the manufacturer's instruction. Then 2.5µl of the reaction mixture was subjected to PCR for amplification of p53 and Bcl-2 cDNAs using specifically designed primers by BLAST analysis of Genebank sequences available for these genes (p53 Forward: 5′-C T G A G G T T G G C T C T G A C T G T A C C A C C A T C C-3′; p53 Reverse: 5′-C T C A T T C A G C T C T C G G A A C A T C T C G A A G C G-3′ and Bcl-2 Forward: 5′-G T T C G G T G G G G T C A T G T G T G T G G A G A-3′; Bcl-2 Reverse: 5′-G C T G A T T C G A C G T T T T G C C T G A A G A C-3′). As an internal control, the house keeping gene β-Actin (β-Actin Forward: 5′-A C G G G G T C A C C C A C A C T G T G C-3′; β-Actin Reverse: 5′-C T A G A A G C A T T T G C G G T G G A C G A T G-3′) was co-amplified in each reaction. The

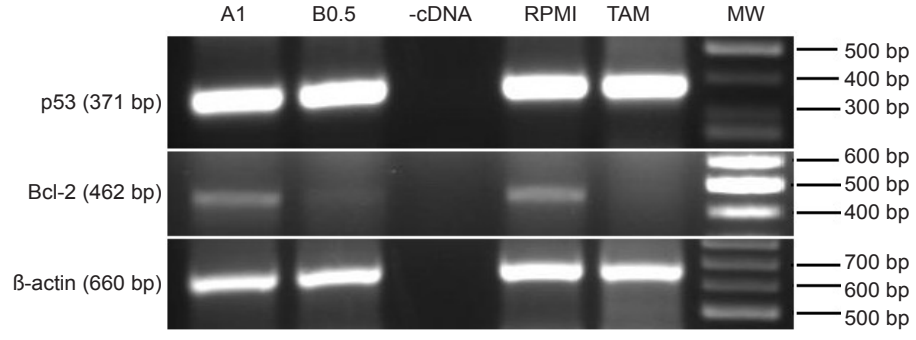

Figure 1. Effects of IC_{50} concentration of methanolic extracts of aerial and root of *A. persicus* in comparison to RPMI and 10^{-5}M TAM on the mRNA levels of p53 and Bcl-2 in T47D cells. After 48 hrs incubation with IC_{50} concentrations of extracts, total RNA was isolated from treated and control samples and alteration in the expression of genes were analyzed using RT-PCR.

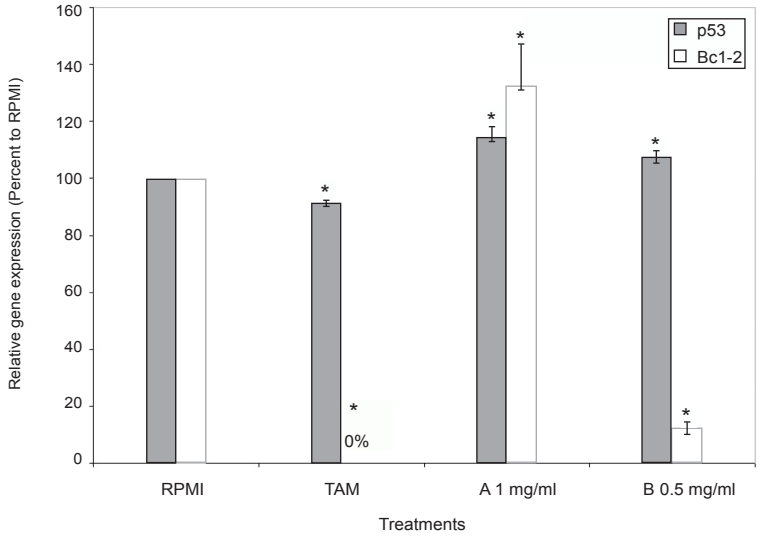

Figure 2. Effects of IC_{50} concentration of methanolic extracts of aerial and root of *A. persicus* in comparison to RPMI and 10^{-5}M TAM on the mRNA levels of p53 and Bcl-2 in T47D cells. After 48 hrs incubation with IC_{50} concentrations of extracts, total RNA was isolated from treated and control samples and expression alterations of genes were analyzed using RT-PCR. Quantification of each band was performed by densitometry analysis software and results expressed as the ratio of p53/β-actin or Bcl-2/β-actin in percent to RPMI (* $p < 0.05$).

PCR reactions was carried out in a final volume of 50 µl containing 1x PCR buffer and 5 U/µl Taq-polymerase (Fermentas), 1.5 mM $MgCl_2$, 0.2mM of each dNTP and 0.4 µM of each primer. The template was denatured for 5min at 94°C, followed by amplification cycles at 94°C for 1min, 69°C (for p53, 64°C for Bcl-2 and 57°C for β-actin) for 1min and 72°C for 1.20 min, and terminated with an additional extension step for 8 min at 72°C. The PCR products were visualized using 1.2% agarose gel electrophoresis with ethidium bromide staining. In negative control, template cDNA was replaced by DEPC water.

Immunocytochemical assay of p53 and Bcl-2 proteins

The T47D cells were seeded (5×10^4 cells/well) in Lab-Tek chamber slides (8 wells glass slide, Nunc, USA). After 48 hrs fresh culture medium was added to a pair of wells. The extract of aerial part and root of *A. persicus* was added to another pair of wells. After 48 hrs incubation at 37°C these wells were suctioned and cells were twice rinsed with PBS. Cells were fixed in ethanol:acetone (9:1) for 30 min at -20°C and then rinsed again with cold PBS at room temperature. For immunocytochemical assay the cells were incubated overnight with primary monoclonal antibody against Bcl-2 and against p53 at dilution of 1:75 at 4°C. Cells were then washed with Tris buffer and incubated with biotinylated immunoglobulin secondary antibody. Then the results were visualized by addition of streptavidine conjugated horseradish peroxidase, rinsing with Tris buffer and finally 3-amino-9-ethylcarbazole (AEC) as chromogen substrate. The slides were then mounted and examined under light microscope.

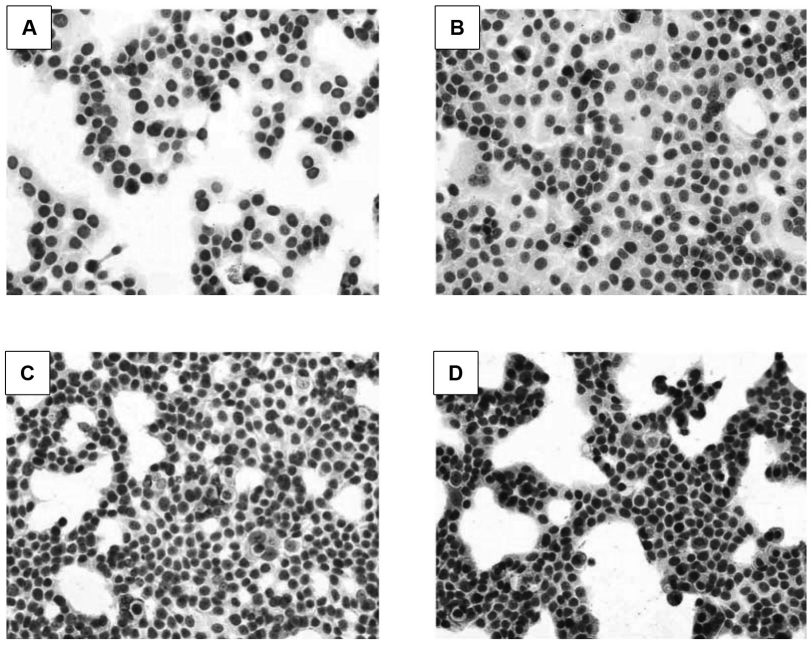

Figure 3. Immunostaining of p53 in T47D cells. Cells were immunostained with primary antibody for p53 and HRP detection system using AEC chromogen. Then slides were mounted and studied under light microscope. (Magnification 400X). A (RPMI), B (TAM 10^{-5}M), C (A 1mg/ml), D (B 0.5 mg/ml).

Statistical analysis

Experiments were performed in triplicate and results are reported as means ± SE, and comparisons were analyzed by one-way analysis of variance (ANOVA) with a Tukey post hoc test to identify between-group differences ($p < 0.05$) using SPSS software (version 11.5).

RESULTS

Levels of expression p53 and Bcl-2 genes in T47D cells treated with methanolic extract of A. persicus

Results of the experiments are represented in figures 1 and 2. As it is obvious in these figures p53 increased significantly in the presence of the extract of both aerial and root of the plant. Bcl-2 expression increased significantly in the presence of aerial extract of the plant and decreased significantly in the presence of root extract.

Immunostaining pattern of p53 and Bcl-2 in T47D cells treated with methanolic extracts of A. persicus

The T47D cells were immunostained with primary antibodies for p53 and Bcl-2 as described in the section of methods. In the presence of plant extracts nuclear staining of p53 and cytoplasmic staining of Bcl-2 decreased in T47D cells in comparison to RPMI control as shown in figures 3 and 4.

DISCUSSION

In the early 1990s, the first introductory data were presented about novel treatments for breast cancer including the taxanes (22, 23). The expression of mutant p53 protein in breast cancers seems to be related to poor prognosis associated with a high histological grade, epidermal growth factor receptor (EGFR) positivity, and Bcl-2 and estrogen receptor (ER) negativity (24, 25). The Bcl-2 oncoprotein suppresses or delays the induction of apoptosis in prostate, skin, lymphoid tissues, and mammary gland (26, 27).

Natural products are still important sources to discover new anticancer drugs. These products are mainly secondary metabolites, produced by organisms in response to external stimuli such as nutritional changes, infection and competition. Natural Products which are produced by plants, fungi, bacteria, insects and animals have been isolated as biologically active pharmacophores. The National Cancer Institute have collected about 35,000 plant samples from 20 countries and has screened around 114,000 extracts for anticancer activity (13).

In this study, the effects of methanolic extracts of aerial and root parts of *Astrodaucus persicus,* an endemic species of Umbelliferae in Iran was investigated for changes in p53 and Bcl-2 gene and protein expression in T47D cell line in comparison to tamoxifen. In the previous studies, the IC_{50} of aerial and root extracts on T47D cells determined to be 1 and 0.5 mg/ml, respectively which means both extracts were cytotoxic (21). In this study, the mRNA expression levels of two cancer-related genes, p53 and Bcl-2 in T47D cells treated with aerial and root extracts of the plant were investigated. While the

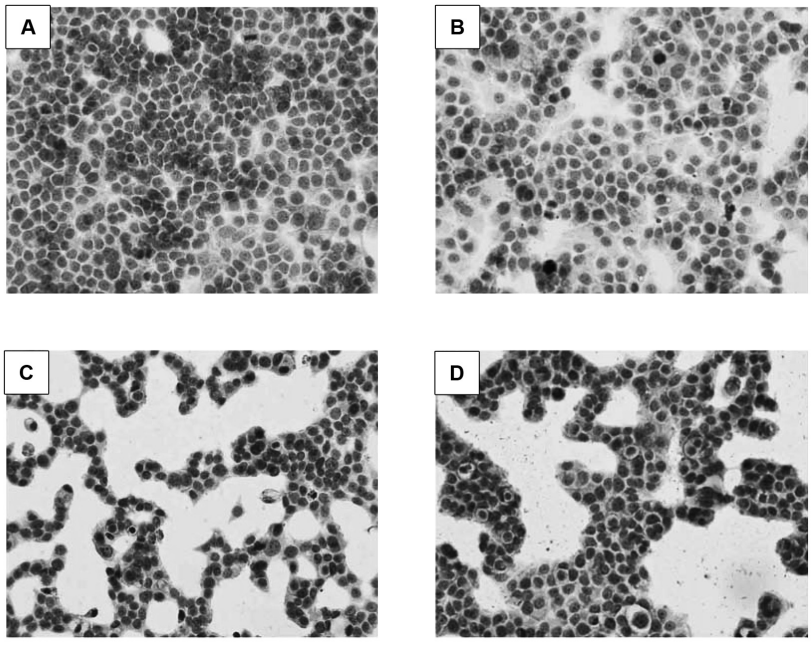

Figure 4. Immunostaining of Bcl-2 in T47D cells. Cells were immunostained with primary antibody for Bcl-2 and HRP detection system using AEC chromogen. Then slides were mounted and studied under light microscope. (Magnification 400X). A (RPMI), B (TAM 10^{-5}M), C (A 1mg/ml), D (B 0.5 mg/ml).

p53 gene expression increased in the presence of aerial and root extracts, the Bcl-2 gene expression increased significantly in the presence of aerial extract and decreased in the presence of root extract. Interestingly both the aerial and root extracts decreased the p53 and Bcl-2 protein expression. The activities of this plant may be due to the presence of coumarin compounds that are present in family of Umbelliferae. Coumarins comprise a very large class of compounds which are found throughout the plant kingdom. Their biological effects include anti-bacterial, anti-thrombotic and vasodilatory, anti-mutagenic, lipoxygenas and cycloxygenas inhibition scavenging of reactive oxygen species, and anti-tumourigenic (28, 29). In conclusion, results of the present study suggest that the methanolic extracts of *Astrodaucus persicus* and particularly the root extract may contain bioactive compounds, probably coumarins that prevents proliferation of T47D breast carcinoma cells by mechanisms such as induction of apoptosis. These data are the first report on the possible molecular mechanisms of action of *Astrodaucus persicus* extracts on cancer cell proliferation. Additional studies are required to characterize the bioactive compounds responsible for the observed activities of *Astrodaucus* plants as a novel resource for new anticancer drugs.

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