Anticancer effects and cell cycle analysis on human breast cancer T47D cells treated with extracts of *Astrodaucus persicus* (Boiss.) Drude in comparison to doxorubicin

[1,5]Abdolmohammadi M.H., [1,2]Fouladdel Sh., [3]Shafiee A., [4]Amin Gh., [5]Ghaffari S.M, [*1,2]Azizi E.

[1]Molecular Research Lab., Department of Pharmacology and Toxicology, [2]National Center of Excellence of Toxicology, [3]Department of Medicinal Chemistry, and [4]Department of Pharmacognosy, Faculty of Pharmacy, Medical Sciences/University of Tehran (MS/UT), and [5]Institute of Biochemistry and Biophysics, University of Tehran, Tehran, Iran

Received 12 Nov 2007; Revised 22 Jan 2008; Accepted 25 Jan 2008

ABSTRACT

Background and purpose of the study: There are considerable efforts to identify naturally occurring substances as new drugs in cancer therapy. Many components from dietary or medicinal plants have been identified that possess substantial chemopreventive properties. Iran has unique plant varieties yet to be studied for anticancer components. Therefore, anticancer effects and cell cycle alterations caused by *Astrodaucus persicus* (Boiss.) Drude, an Iranian species of family of Umbelliferae, in human breast cancer T47D cells was investigated.

Material and Methods: The T47D cells were seeded in 96-well culture plates in the presence and absence of different concentrations of either aerial or root extracts of *A. persicus* to determine their anticancer effects in comparison to doxorubicin using MTT assay. The changes in the cell cycle pattern of T47D cells using DAPI reagent in flow cytometric analysis was also studied.

Results: Both extracts of *A. persicus* showed strong antiproliferative effects on T47D cells when compared to RPMI control and doxorubicin. The cytotoxicity of the root extract was greater than aerial extract of *A. persicus*. Both extracts showed pattern of cell cycle relatively similar to RPMI and significantly different from doxorubicin.

Conclusion: These data are first report on potential anticancer activity of *A. persicus* extracts and its possible mechanism of action on cancer cell proliferation.

Keywords: Breast cancer, T47D cells, MTT assay, Flow cytometry, *Astrodaucus persicus*

INTRODUCTION

Breast cancer is one of the most common malignancies which affects women worldwide especially in western countries (1-2). It is both genetically and histopathologically heterogeneous, and the mechanism(s) underlying breast cancer development remains largely unknown (3). The development of breast cancer involves several types of genes that need to be activated or inactivated in order to promote malignancy (4).

A major problem with present cancer chemotherapy is the serious deficiency of active drugs for the curative therapy of tumors (5-6). For thousands of years, natural products have played an important role throughout the world in treatment and prevention of human diseases (7). Over 60% of the currently used anticancer agents are derived in one way or another from natural sources (8-10). The search for anti-cancer agents from plant sources started in the 1950s by discovery and development of the vinca alkaloids, vincristine, and the isolation of the cytotoxic podophyllotoxins (11-14).

The chemotherapeutic drugs including etoposide, camptothecin, vincristine, *cis*-platinum, cyclophosphamide, paclitaxel (Taxol), 5-fluorouracil and doxorubicin have been observed to induce apoptosis in cancer cells (15-17). Among them, the agents that alter the cell cycle have been of particular interest, since cell cycle regulation is the basic mechanism underlying cell fate, i.e., proliferation, differentiation or acquire death (18). Thus uncontrolled cell proliferation is one of the main hallmarks of cancer, and tumor cells damages in genes that are directly involved in regulation of the cell cycle (19-21). Most, if not all, human cancers show a deregulated control of G1 phase progression, a

Correspondence: aziziebr@tums.ac.ir

period when cells decide whether to start proliferation or to stay quiescent (22-23). Doxorubicin (DOX) is an anthracycline antineoplastic antibiotic with several different mechanisms including: intercalation into DNA, free radicals formation, and inhibition of topoisomerase II that leads to G2/M arrest in cell cycle and eventually induces apoptotic cell death (24-25).

One of the approaches used in drug discovery, is the ethnomedical data approach, in which the selection of a plant is based on the prior information on the use of the plant in the folk medicine. It is generally known that ethnomedical data provides substantially increased chance of finding active plants relative to random approach (26-27). Thus, *Astrodaucus persicus* (Boiss.) Drude, a medicinal plant of the family of Umbelliferae (Apiaceae) used as a remedy in cancer-related diseases have been evaluated for its properties. In Iran, the genus *Astrodaucus* is represented by two species, *Astrodaucus orientalis* (L.) Drude and *Astrodaucus persicus* (Boiss.) Drude, which grow wild in different regions of Iran and nearby countries such as central and southern Russia, the western desert of Syria, inner Anatolia, Transcaspia 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 *A. persicus* have been investigated but cytotoxicity and anticancer activity of this plant have not been reported previously (28-32). Therefore, the main aim of this study was to evaluate the cytotoxic properties and anticancer effects of *Astrodaucus persicus* extract and to determine the possible mechanisms of cell cycle alterations elicited by the extract on breast carcinoma cancer cells.

MATERIAL AND METHODS

Chemicals and reagents

RPMI 1640, pen-strep and FBS were purchased from Gibco (UK). Doxorubicin (Adriablastina, Italy), MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide), dimethyl sulfoxide (DMSO) were purchased from Sigma (Germany). DAPI and Nonidet P40 were purchased from Roche (Germany).

Preparation of A. persicus extracts

A. persicus plant (Voucher No. 6642-THE, Herbarium of faculty of Pharmacy) was collected from the Taleghan road and was identified by Dr. Amin GR. The aerial part and root were isolated, dried and chopped finely using a blender. Hundred grams of the dried material of aerial part or root were extracted with methanol by soxhlet extraction. The hydroalcoholic extracts were filtered and evaporated to dryness under reduced pressure by a rotatory evaporator. The resulting residues for each part of plants were stored at -20^{o}C. The recovery weight of aerial part was about 14% and for root it was 5% of the dried material. The extract was dissolved in DMSO (Sigma), sterilized by filtration and subsequently diluted to appropriate working concentrations with RPMI culture medium.

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 100 U/mL of penicillin, 100 ng/mL of streptomycin and 10% heat-inactivated FBS at 37 °C in 5% CO2 incubator (15, 25).

In vitro cytotoxicity assay

Cells were used in cytotoxicity studies when 90% confluence was reached in T25 flasks. Cells were harvested with trypsin/EDTA, washed with PBS and counted using trypan blue dye exclusion method. T47D cells were seeded into 96-well plates at a density of 10^4 cells/well and left to attach to the plates for 48 hr. Then, cells were incubated for 2, 4 and 6 days with various concentrations of extracts. After the exposure time, the cells were incubated with 25 microliter of MTT (4 mg/ml) at 37°C for 3 hr. After dissolving the formazan crystals in DMSO, plates were read in a microplate reader (SUNRISE TECAN, Austria) at 540 nm against 620 nM (15, 33). This experiment was performed in triplicates and repeated 3 times. For each concentration Mean values ± SE was determined.

Analysis of the cell cycle

Cell cycle phase distribution was determined by analytical DNA flow cytometry. T47D cells were incubated for 48 hours with IC_{50} concentration of aerial and root part extracts of *A. persicus*. Extracts, doxorubicin and control RPMI treated T47D cells were harvested and adjusted to $5x10^5$ cells/ml and stained with DAPI reagent at 4^{o}C for 30 min in dark. The PARTEC flow cytometer with FloMax software was used to analyse DNA content using UV light at FL4. The percentage of cells in the various phases was determined and statistical analysis of data from flow cytomtric experiments was carried out (18).

Statistical analyses

Statistical analyses were performed by the SPSS 11.5 software. Statistical differences among treated and untreated cells were determined by one-way ANOVA (Analysis of Variance). To compare several groups, Tukey post-hoc test was applied and mean differences with $p< 0.05$ were considered statistically significant.

RESULTS

Cytotoxicity of the extracts of A. persicus on T47D cells

The antiproliferative effects of plant extracts in comparison to 250nM of doxorubicin on T47D cells was determined by MTT method. In vitro screening of the extracts of aerial part and root of *A. persicus* on breast carcinoma T47D cell line produced a time and dose-dependent inhibition of the cell growth (Figures 1-3) (15,33).

Cell cycle distribution of T47D Cells treated with extracts of aerial part and root of A. persicus

In order to determine the cell cycle pattern of T47D cells, the DNA content of RPMI, doxorubicin and extracts treated cells were analyzed following DAPI staining and flow cytometric method. The cell cycle phase distribution was quantitated from 3 independent sets of measurements that showed relatively similar pattern to RPMI which was completely different than Doxorubicin (Figures 4 and 5) (18).

DISCUSSION

Cancer chemotherapeutic agents can often provide temporary relief from symptoms, prolongation of life and occasionally complete remission. A successful anticancer drug should kill or incapacitate cancer cells without causing excessive damages to normal cells. This ideal situation is achievable by inducing apoptosis in cancer cells. Chemopreventive agents comprise diverse groups of compounds with different mechanisms of action with ultimate ability to induce apoptosis. Understanding the modes of action of these compounds should provide useful information for their possible applications in cancer prevention and perhaps in cancer therapy (34-35).

Cell cycle modulation by various natural and synthetic agents is gaining widespread attention in recent years. Given that disruption of cell cycle plays a crucial role in cancer progression, its modulation by phytochemicals seems to be a logical approach in control of carcinogenesis (36). There are a number of herbs that have shown the ability to induce cell cycle arrest and to play an important role in cancer prevention and therapy. Genistein, daidzein and isoflavonoids in soybean are thought to play an important role in breast cancer prevention (37-38). Two other examples of natural compounds with anticancer properties are quercetin and apigenin. Quercetin is one of the major flavonoids found in the human diet which exerts a dose-dependent inhibitory effect on cell proliferation with cell cycle arrest in G2/M phase. Quercetin has also been shown to inhibit cell proliferation in colon carcinoma (HCT-116 and HT-29) and mammary adenocarcinoma (MCF-7) cell lines after 24 h of exposure (39-40). Apigenin a flavone found in celery has antiproliferative effect with cell cycle arrest in G2/M in MCF-7 cells and induces caspase activities in HL-60 cells (41-42).

In the present study, the extracts of *Astrodaucus persicus,* an endemic species in family of Umbelliferae (Apiaceae) in Iran were evaluated for antiproliferative effects on T47D breast cancer cell line and cell cycle alterations in the presence of plant extracts in comparison to doxorubicin.

The Umbelliferae family is rich in species containing active components such as coumarins and furanocoumarins. It has been reported that these compounds show antibacterial, antifungal, antitumoral, and spasmolytic activities. Some furanocoumarins, such as psoralen and bergapten are used clinically in the treatment of skin diseases including vitiligo and psoriasis. *Petroselinum sativum* is used to treat hypoglycemia and bronchitis. *Lepidium sativum* has been used to treat hemorrhoids (43). *Coriandrum sativum* fruits are know as a sedative, abortifacient and a remedy for pimples. *Eryngium billardieri* roots have been used for maturation of abscess (43). The chloroform extract of the root of *Angelica japonica* show high inhibitory activity against human gastric adenocarcinoma (MK-1) cell growth. From this extract, a new furanocoumarin named japoangelone and four furanocoumarin ethers of falcarindiol, named japoangelols A-D have been isolated (44). Falcarindiol and panaxynol from the root of *Heracleum moellendorfii* are constituents which inhibit the growth of nude mouse-transplantable human gastric adenocarcinoma (MK-1), human uterus carcinoma (HeLa), and murine melanoma (B16F10) cells (44).

In this study, after 2, 4 and 6 days of exposure, extract treated cells showed a decreased viability of T47D cells in comparison to RPMI control by MTT assay. Both the aerial and root extracts inhibited cell proliferation at various concentrations with time and in dose dependent pattern. As a result, the IC_{50} of aerial extract was found to be 1 mg/ml and for the root extract it was 0.5 mg/ml.

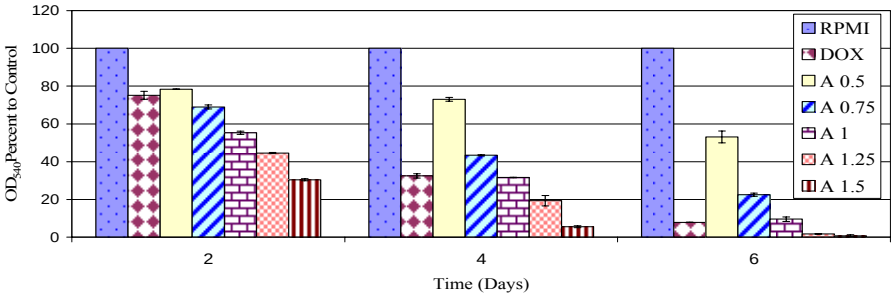

Figure 1. Cytotoxicity of different concentrations of aerial part extract of *A. persicus*. Cells were seeded into 96-well plates (10^4 cells/well). Two days later, the cells were incubated with RPMI as negative control, Doxorubicin 250 nM as positive control and different concentrations of extract of *A. persicus* aerial part (A) for 2, 4 and 6 days. Cell proliferation was determined by MTT assay. The data represent the mean±SE of 3 independent experiments each in triplicate format.

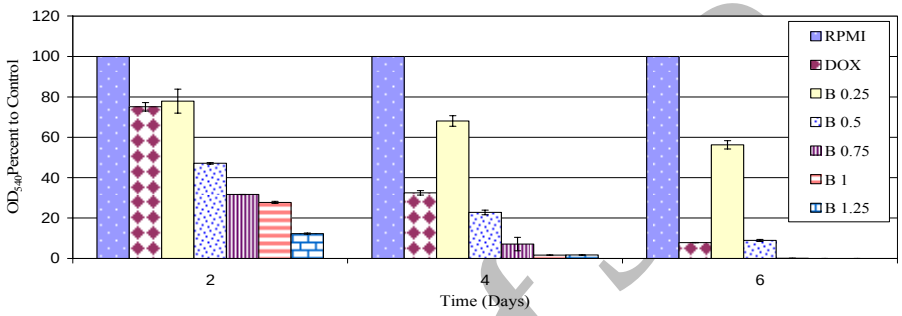

Figure 2. Cytotoxicity of different concentrations of root extract of *A. persicus*. Cells were seeded into 96-well plates (10^4 cells/well). Two days later, the cells were incubated with RPMI as negative control, Doxorubicin 250 nM as positive control and different concentrations of extract of *A. persicus* root (B) for 2, 4 and 6 days. Cell proliferation was determined by MTT assay. The data represent the mean±SE of 3 independent experiments each in triplicate format.

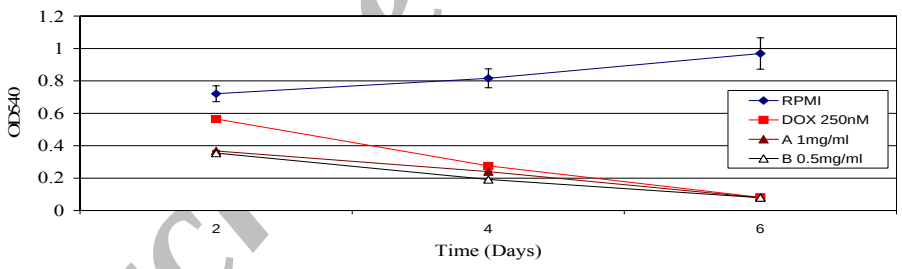

Figure 3. Effect of IC_{50} Concentrations of aerial and root extract of *A. persicus*. Cells were seeded into 96-well plates (10^4 cells/well). Two days later, the cells were incubated with RPMI as negative control, Doxorubicin 250 nM as positive control and IC_{50} concentrations of extract of *A. persicus* aerial part (A) and root (B) for 2, 4 and 6 days. Cell proliferation was determined by MTT assay. The data represent the mean±SE of 3 independent experiments each in triplicate format.

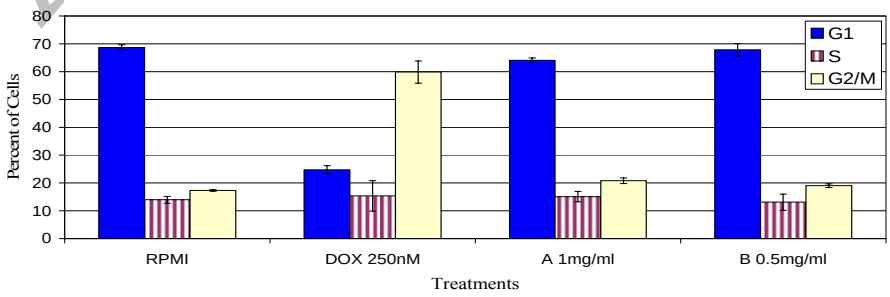

Figure 4. Cell cycle phase distribution of T47D cells. T47D cells (5×10^5 cells/ml) treated with Aerial (A 1mg/ml) and root (B 0.5mg/ml) as well as RPMI or Doxorubicin 250nM were prepared for cell cycle analysis after 2 days exposure. Cells stained with DAPI reagent were analysed with PARTEC flow cytometer equipped with UV light at FL4. The data represent the mean±SE of 3 independent experiments.

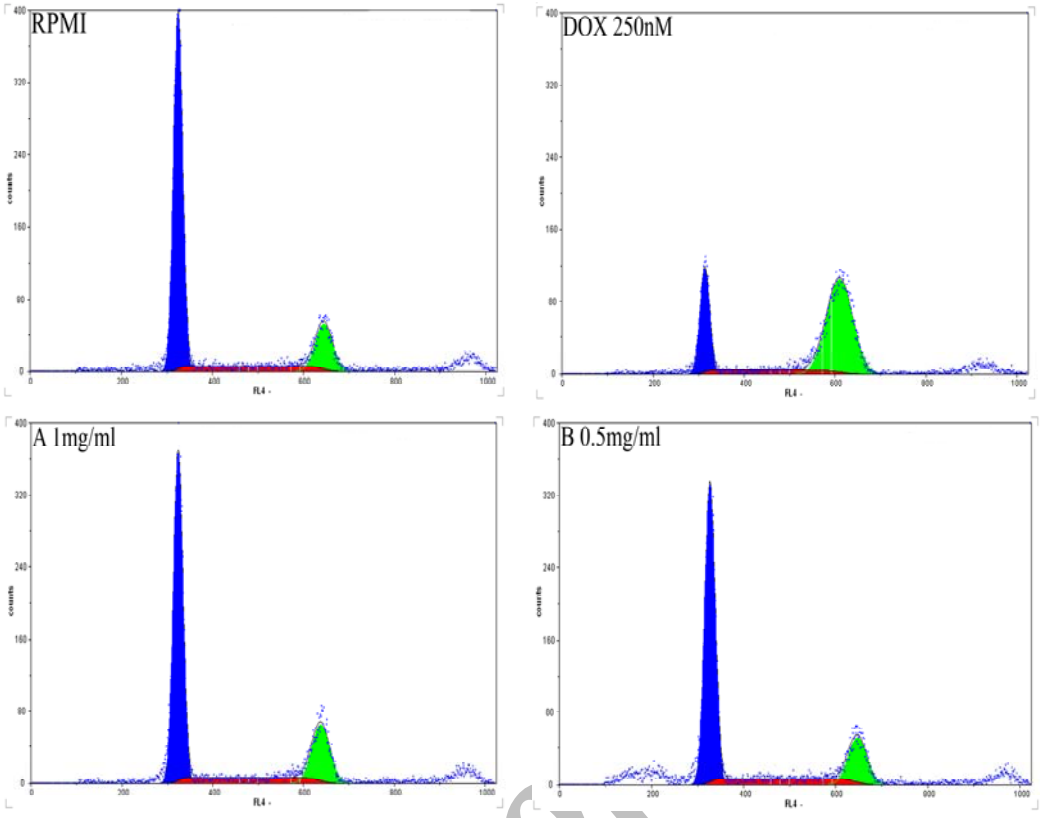

Figure 5. Cell cycle patterns of treated T47D cells. Flow cytograms of T47D cells treated with RPMI, Doxorubicin 250nM, aerial (A 1mg/ml) and root (B 0.5 mg/ml) extracts stained with DAPI reagent show G1, S, G2/M phases of cell cycle under different assay conditions.

In addition, to study the mechanism behind the anticancer activity of *A. persicus*, the effects of IC_{50} concentration of extracts on the cell cycle phases of T47D cells were investigated. Our results showed that the pattern of distribution of phases of cell cycle of T47D cells following exposure to both extracts of *A. persicus* was relatively similar to control RPMI. However unlike doxorubicin, the inhibitory effect of extracts on proliferation of T47D cells was not related to cell cycle arrest in G2/M.

In conclusion, extracts of *A. persicus* have concentration and time-dependent anticancer activities without significant alteration on cell cycle pattern in comparison to control RPMI. Extract of root in comparison to the extract of aerial part shows higher anticancer activity. Further molecular studies are undergoing to elucidate the mechanism(s) of action of these extracts on cancer cells.

ACKNOWLEDGMENTS

The authors would like to thank the financial support of National Center of Excellence of Toxicology (NCET) and also kind assistance of office of vice-chancellor for research of medical sciences university/university of Tehran.

REFERENCES

1. Coleman WB, Tsongalis GJ. The Molecular Basis of Human Cancer. Human Press Inc., Totowa, N J. 2002; 347-363.
2. McPherson K, Steel CM, Dixon JM. Breast cancer-epidemiology, risk factors, and genetics. BMJ 2000; 321(9): 624-628.
3. Hedenfalk IA, Ringner M, Trent JM, Borg A. Gene Expression in Inherited Breast Cancer, Advances in Cancer Reaearch. Academic Press 2002; 1-34.
4. Ingrasson S. Breast Cancer: Introduction, Seminar in Cancer Biology. 2001; 11: 323-326.
5. Valeriote F, Grieshaber CK, Media J, Pietraszkewics H, Hoffmann J, Pan M, McLaughlin S. Discovery and development of anticancer agents from plants. Journal of Experimental Therapeutics and Oncology 2002; 2: 228-236.

6. Kinghorn AD, Farnsworth NR, Soejarto DD, Cordell GA, Swanson SM, Pezzuto JM, Wani MC, Wall ME, Oberlies NH, David J, Keoll DJ, Kramer RA, Rose WC, Vite GD, Fairchild CR, Peterson RW, Wild R. Novel Strategies for the Discovery of Plant-Derived Anticancer Agents. Pharmaceutical Biology 2003; 41: 53-67.
7. Chin YW, Balunas MJ, Chai HB, Kinghorn AD. Drug Discovery from Natural Sources. The AAPS Journal 2006; 8(2): E239-E253.
8. Cragg GM, Newman DJ. Plants as a source of anti-cancer and anti-HIV agents. Ann. Appl. Biol. 2003; 143: 127-133.
9. Balunas MJ, Kinghorn AD. Drug discovery from medicinal plants. Life Sciences 2005; 78: 431-441.
10. Cragg GM, David JN. Plant as source of anti-cancer agents. Journal of Ethnopharmacology 2005; 100: 72-79.
11. Reddy L, Odhav B, Bhoola KD. Natural products for cancer prevention: a global perspective. Pharmacology & Therapeutics 2003; 99: 1-13.
12. Tsuda H, Ohshima Y, Nomota H et al. Cancer Prevention by Natural Compounds. Drug Metab. Pharmacokin. 2004; 19(4): 245-263.
13. Srivastava V, Negi AS, Kumar JK, Gupta MM, Khanuja SPS. Plant-based anticancer molecules: A chemical and biological profile of some important leads. Bioorganic & Medicinal Chemistry 2005; 13: 5892-5908.
14. Pezzuto JM. Plant-Derived Anticancer Agents. Biochemical Pharmacology 1997; 53: 121-133.
15. Tan ML, Sulaiman SF, Najimuddin N, Samian MR, Muhammad TST. Methanolic extract of Pereskia bleo (Kunth) DC. (Cactaceae) induces apoptosis in breast carcinoma, T47-D cell line. Journal of Ethnopharmacology 2005; 96: 287-294.
16. Kaufmann SH, Earnshaw WC. Induction of Apoptosis by Cancer Chemotherapy. Experimental Cell Research 2000; 256: 42-49.
17. Johnstone RW, Ruefli AA, Lowe SW. Apoptosis: A Link between Cancer Genetics and Chemotherapy. Cell 2002; 108(25): 153-164.
18. Dobashi Y, Takehana T, Ooi A. Perspectives on cancer therapy: cell cycle blockers and perturbators. Curr Med Chem. 2003; 10(23): 2549-58.
19. Garrett MD. Cell cycle control and cancer. Current Science 2001; 81(5): 515-522.
20. Tachibana KK, Gonzalez MA, Coleman N. Cell-cycle-dependent regulation of DNA replication and its relevance to cancer pathology. Journal of Pathology 2005; 205: 123-129.
21. Sandal T. Molecular Aspects of the Mammalian Cell Cycle and Cancer. The Oncologist 2002; 7: 73-81.
22. Golias CH, Charalabopoulos A, Charalabopoulos K. Cell proliferation and cell cycle control: a mini review. Int J Clin Pract. 2004; 58(12): 1134-1141.
23. Hung DT, Jamison TF, Schreiber SL. Understanding and controlling the cell cycle with natural products. Chemistry & Biology 1996; 3: 623-639.
24. Mizutani H, Tada-Oikawa ST, Hiraku Y, Kojima M, Kawanishi S. Mechanism of apoptosis induced by doxorubicin through the generation of hydrogen peroxide. Life Sciences 2005; 76: 1439-1453.
25. Gruber BM, Anuszewska EL, Skierski JS. Activation of programmed cell death (apoptosis) by adriamycin in human neoplastic cells. Mutation Research 2001; 484: 87-93.
26. Lee KH. Novel Antitumor Agents from Higher Plants. Joun Wiley & Sons, Inc. Med Res Rev. 1999; 19(6): 569-596.
27. Montbriand MJ. Herbs or Natural Products That Decrease Cancer Growth. Oncology Nursing Forum 2004; 31(4): E75-E90.
28. Bazargani YT, Almasirad A, Amin G, Shafiee A. Chemical composition of the essential oils of Astrodaucus persicus (Boiss.) Drude root, stem/leaves and flowers/fruits, Flavour Fragr. J. 2006; 21: 294-296.
29. Mojab F, Kamalinejad M, Ghaderi N, Vahidipour HR. Phytochemical Screening of Some Species of Iranian Plants. Iranian Journal of Phrmaceutical Research: 2003; 77-82.
30. Rechinger KH. Flora Iranica No. 162: 112-115.
31. Mozaffarian V. A Dictionary of Iranian Plant Names. Farhang Moaser, 1988; 64.
32. Bigdeli M, Rustaiyan M, Nazak N, Masoudi S. Essential oil of Astrodaucus persicus (Boiss.) Drude. from Iran. J. Essent. Oil Res. 2004; 16(5): 420-421.
33. Ostad S. N., Motahhary P., Beshkar M., Ghahremani M.H. 17β-estradiol and progesterone upregulate cyclooxygenase-2 expression in the human gingival fibroblasts. DARU 2006; 14(4):190-196.
34. Taraphdar AK, Roy M, Bhattacharya RK. Natural products as inducers of apoptosis: Implication for cancer therapy and prevention. Current Science 2001; 80(11): 1387-1396.

35. David H. Vegetables, fruits and phytoestrogens in the prevention of diseases, Journal of Postgraduate Medicine 2004; 50(2): 145-149.
36. Singh RP, Dhanalakshmi S, Agarwal R. Phytochemicals as Cell Cycle Modulators. Cell Cycle 2002; 1(3): 156-161.
37. Wang HZ, Zhang Y, Xie LP, Yu XY, Zhang RQ. Effects of genistein and daidzein on the cell growth, cell cycle, and differentiation of human and murine melanoma cells. J Nutr Biochem. 2002; 13: 421-426.
38. Chen WF, Huang MH, Tzang CH, Yang M, Wong MS. Inhibitory actions of genistein in human breast cancer (MCF-7) cells. Biochim Biophys Acta 2003; 1638: 187-196.
39. Ramos S. Effects of dietary flavonoids on apoptotic pathways related to cancer chemoprevention. Journal of Nutritional Biochemistry 2007; 18: 427-442.
40. Ong C, Tran E, Nguyen T, Lee S, Lee J, et al. Quercetin-induced growth inhibition and cell death in nasopharyngeal carcinoma cells are associated with increase in Bad and hypophosphorylated retinoblastoma expressions. Oncol Rep. 2004; 11: 727-733.
41. Yin F, Giuliano A, Law R, Van Herle A. Apigenin inhibits growth and induces G2/M arrest by modulating cyclin-CDK regulators and ERK MAP kinase activation in breast carcinoma cells. Anticancer Res. 2001; 21: 413-420.
42. Wang I, Lin-Shiau S, Lin J. Induction of apoptosis by apigenin and related flavonoids through cytochrome c release and activation of caspase-9 and caspase-3 in leukaemia HL-60 cells. Eur J Cancer 1999; 35: 1517-1525.
43. Ozcelik B, Kusmenoglu S, Turkoz S, Abbasoglu U. Antimicrbial Activities of Plants from the Apiaceae. Pharmaceutical Biology 2004; 42(7): 526-528.
44. Fujika T, Furumi K, FujiiH, Okabe H, Mihashi K, Nakano Y, Matsunaga H, Katano M, Mori M. Antiproliferative Constituents from Umbelliferae Plants. A New Furanocoumarin and Falcarindiol Furanocoumarin Ethers from the Root of *Angelica japonica.* 1999; Chem. Pharm. Bull. 47(1) 96-100.