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Full Length Research Paper

Effects of Geven root extract on proliferation of HeLa cells and bcl-2 gene expressions

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Geven (*Astragalus* L.) root extract is used for asthma, diarrhea, and cancer therapy in Chinese Medicine. Liquid *Astragalus* root extracts are traditionally used in Anatolia for leukemia and wound healing. 439 species of this plant, which has 3000 species in the world, grow in Turkey and 204 of these are endemic. In this study, molecular mechanism of apoptotic and cytotoxic effects of root ethyl acetate extract of *Astragalus chrysochlorus* on HeLa cells was investigated. For this purpose, 1, 0.5, 0.15 and 0.1 mg/ml concentrations of *Astragalus* root extract were applied to HeLa cell cultures for 24, 48 and 72 h. Cell kinetic parameters as proliferation rate (MTT assay), Mitotic Index and apoptotic index were used for experimental cytotoxicity assay of root extract. Furthermore, effects of root extract on gene expression rates which take place in apoptosis mechanism as *Bcl-2, Bax, Bak, Bcl-x, Bik, Mcl-1, Bfl-1* were determined by reverse transcription-polymerase chain reaction (RT-PCR) technique. As a result of investigated parameters, root extract of *Astragalus chrysochlorus* is found to have cytotoxic effect on HeLa cell cultures. This cytotoxic effect which appeared in our experiments achieved statistically significant decrease on proliferation rate of HeLa cells depending on application time and concentration. Also mitotic index and apoptotic index presented statistically significant increase on particular concentrations and hours ($p < 0.05$). Likewise, it is determined on molecular basis that root extract causes significant changes on especially antiapoptotic genes.

Key words: *Astragalus chrysochlorus*, HeLa cells, Cytotoxicity, Apoptosis, bcl-2 gene family.

INTRODUCTION

Cancer is a disease which occurs when somatic cells proliferate uncontrolled and occupy nearby tissue or spread from the tissue from which it originated to another tissue by blood-lymph path. Cancer research studies have passed to a new era by developing genomics and proteomics technologies in recent years. In this new era, cancer is accepted as a genetic disease. This new view of point requires new diagnosis and treatment approaches.

It is generally known that tumor cell killing mechanism of cancer drugs is inducing apoptosis. For this reason, it is very important to determine apoptosis to investigate responses of tumor cells to anti-cancer drugs (Arican, 2008; Ilgar and Arican, 2009).

Apoptosis takes place in various developmental and physiological processes, and also in controlling cell amount by killing tumor cells induced by chemotherapy. Genetically regulated biological processes are driven by the rates of proapoptotic and antiapoptotic proteins. Recently, apoptosis inducers are used in cancer therapy. Apoptosis breakdown is seen in many human tumors, it is assumed that, this breakdown contributes to transformation of normal cell to tumor cell. Apoptosis is a genetically regulated biological process. Changes on apoptotic cells occur after series of events which regu-

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lated by caspases and various cell signals that regulate proapoptotic and antiapoptotic proteins. Receptor-induced apoptotic pathway is induced by ligands and receptors such as FAS, TNF, TRAIL, and also by molecules such as caspases and bcl-2 family members (Arican et al., 2008; Bhalla, 2003; Chang and Yang, 2000; Evan and Vousden, 2001).

Extrinsic and intrinsic pathways can be activated separately but most caspases' activation is common in apoptotic pathway. It has been tried to interfere different parts of apoptotic pathway to induce apoptosis, and for this purpose TRAIL receptor, *bcl-2* family proteins, caspases and IAP's' are targeted. Most of these treatments are in pre-clinic experimental stage because their efficiency is low and they are drug resistance trended. It is known that *bcl-2* blocks programmed cell death, increases metastasis potential and increases resistance to anti-cancer therapy. High-level expression of *bcl-2* proto-oncogene is found in various solid tumors. Antisense approaches are applied by various levels in preclinical studies to decrease expression levels of various proapoptotic genes including bcl-2 (Bhalla, 2003; Chang et al., 2000; Corazza et al., 2009; Werner et al., 2002).

For many years medicinal plants played important role in treatment of various diseases. Studies including natural plant products are very important for finding new and more efficient anticancer agents. 62% of cancer drugs, which are used in America, are made of natural products or natural product derivatives.(Bhalla, 2003; Chang and Yang, 2000; Corazza et al., 2009; Frenzel et al., 2009).

Astragalus L. is the largest genus of flowering plants, containing up to 3000 species (Akan, 2009). Due to the rich content of saponines, flavonoids, polysaccharides (El-Sedakhy, 1994; Evan and Vousden., 2001; Frenzel et al., 2009) and biological activities related to their secondary metabolites, *Astragalus* roots from various species have been used in the traditional Chinese medicine, as immunostimulants, hepatoprotectors, antiperspirants, diuretics, etc., and for the treatment of nephritis, diabetes, leukemia, and cancer. It has been reported that crude extracts of some *Astragalus* species have anti-viral and cytotoxic activity (Karagoz et al., 2007; El-Sedaky et al., 1994). *Astragalus* species also have great economical values for the production of tragacanth gum which is used as an emulsifier, stabilizer and thickening agent in pharmaceuticals and foodstuffs. Turkey and Iran produce almost all of the world's tragacanth gum supply (He et al., 1991).In Turkey the genus *Astragalus* (Leguminoseae) is represented by approximately 439 species and 204 of them are endemic (Akan, 2009). *Astragalus chrysochlorus* (2n=16) is one of the endemic species in Turkey, growing in 32°-36° meridian of southhern Anatolia (Aytac, 1997). In Anatolia, an aqueous extract of *Astragalus* roots are traditionally used for treatment of leukemia and wound-healing (Dogan et al., 1985).

In our previous study, antioxidant, cytotoxic and phagocytic effects of *A. chrysochlorus* was determined. Tissue culture conditions for producing phenolic substances was established and also selenium accumulation capacity of *A. chrysochlorus* was determined (Hasancebi 2003; Karagoz et al., 2007; Cakir & Ari, 2009; Ari et al., 2010). Ethylacetate and chloroform extracts of *Astragalus chrysochlorus* have cytotoxic activity against Vero cells and inhibited cell viability up to 70% (Karagoz et al., 2007).

The aim of this study was to apply *A. chrysochlorus* root extracts to HeLa cell lines and determine changes on cytotoxicity and viability. For this aim, kinetic parameters as proliferation rate, apoptotic index(AI) and mitotic index(MI) have been examined. Also some apoptosis-related genes have been examined by reverse transcription-polymerase chain reaction (RT-PCR) method.

MATERIALS AND METHODS

Cell culture

HeLa (cervix adenocarcinoma) cells were obtained from laboratory of Tokiyo Technology Institute and were grown routinely by doing passage twice a week.

Cells were grown in Minimum Essential Medium (Sigma, MEM) containing %10 FBS (Gibco Lab.), penicillin (100 unit/ml) and streptomycin (50 mg/ml) and placed in 25 cm^2 cell culture flasks at 37°C under a humidified atmospheric condition of 5% CO_2 and 95% air. Cells were washed with Hank's Balanced Salt Solution (HBSS) and harvested using 6 ml trypsin (Gibco Lab.) for 3 min. After that cells were centrifuged at 1500 rpm for 3 min. Then supernatant was discarded and pellet was diluted with MEM. Cells were seeded at a concentration of 30000 cells/well in to 96 wells.

Collection and identification of *A. chrysochlorus*

The plants were collected in June 2004 from Sertavul, Karaman/TURKEY and were identified by Prof. Dr. Tuna Ekim (İstanbul University, Faculty of Science, Department of Botany). A voucher specimen was deposited in the herbarium ISTF no: 40006 (Istanbul University, Faculty of Science Herbarium).

Preparation of extracts

Crude extracts of roots were prepared by decoction of 10 g of pulverized material in 100 ml of ethyl acetate for two days. Then samples were extracted at room temperature using a warring blender. Plant residues were removed by centrifugation (12000 x g, 30 min, 10°C) and the supernatant was filtered and evaporated to dryness under reduced pressure. In this way, crude ethyl acetate root extracts were obtained.

Experimental design

40 mg *A. chrysochlorus* root extracts were dissolved in MEM as a 4 mg/ml stock solution. Then final concentrations (D1, D2, D3 and D4, respectively, 1, 0.5, 0.15 and 0.1 mg/ml) were obtained by diluting aliquots of the stock solution in MEM supplemented with 10 % FBS. Prepared doses were treated to HeLa cells in the time period of 24, 48 and 72 h.

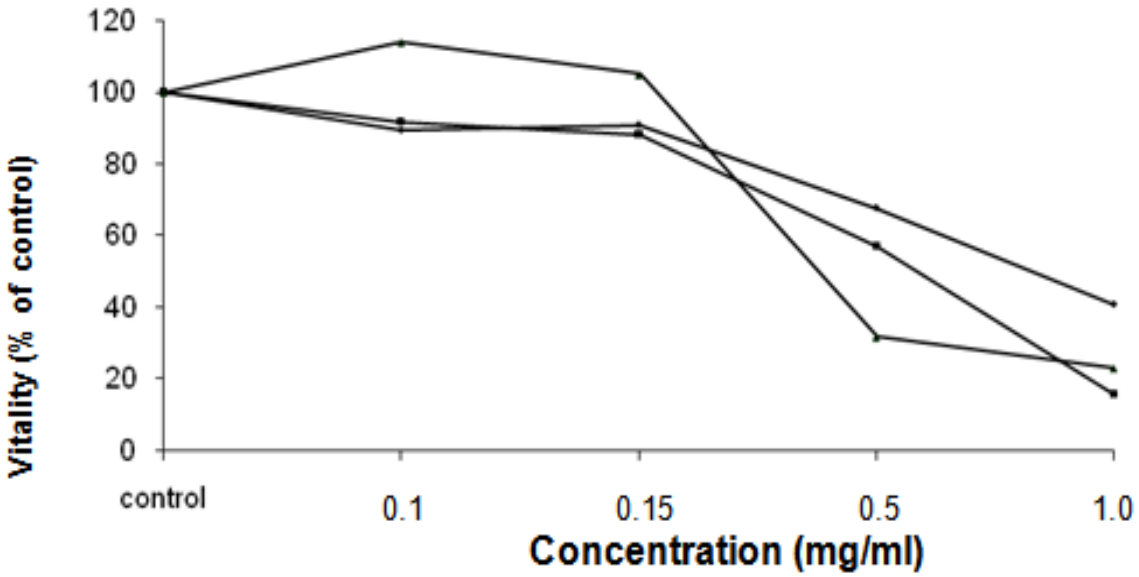

Figure 1. Effect of *Astragalus chrysochlorus* root extracts treatment on vitality in HeLa cells at different concentrations for 24 h, ♦; 48 h, ■; 72 h, ▲.

Cytotoxicity assays

Antiproliferative effects were measured *in vitro* on HeLa cell line by using the 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT; Sigma) assay. Cells were seeded onto a 96 well micro plate attached to the bottom of the well overnight. Second day, medium was removed and 40 µl/well MTT solution was added. After treatment with MTT, plates were incubated at 37°C and Formosan crystals were precipitated during 4 h. The crystals were dissolved in 200 µl dimethyl sulfoxide (DMSO) during 1 h period in shaker incubator at 37°C. Finally the micro plate was read on Elisa reader at 540 and 570 nm reference of 690 nm (Kurashige et al., 1999). The same procedure was used for the other plates at 48 and 72 h, too.

Mitotic index analysis

Feulgen method was used to determine at mitotic index. Cells were treated with Feulgen that prepared with 1N HCl at room temperature for 1 min. and then hydrolyzed with 1N HCl for 10.5 min. at 60 ˚C. After slides were treated with Feulgen, they were rinsed for 5 min in distilled water and stained with 10% Giemsa stain solution pH 6.8 for 3 min and washed phosphate buffer for two times. After staining, the slides were rinsed in distilled water, then air dried. Mitotic index was calculated by counting metaphases, anaphases and telophases for each tested plant extract concentration and control. As a result, 1000 cells were counted from each slides for mitotic index.

Apoptotic index analysis

For the determination of the apoptotic index, cells were fixed with methanol and stained with 4',6-diamidino-2-phenylindole (DAPI). After slides were washed in phosphate buffered saline (PBS), they were scored in double-blind under the fluorescence microscope. The apoptotic index represents the percentage of fragmented nuclei and was determined on a microscopic field of at least 30 areas/each slides (Mossman, 1983).

RNA extraction and reverse transcriptase (RT)-PCR

Total RNA was isolated from $6x10^6$ cells for each samples (control, 1mg/ml and 0,1 mg/ml concentrations, 72 hours) and 50 µl total RNA was used to performed one-step reverse transcriptase polymerase chain reaction (Invitrogen PureLink Micro-to-Midi Total RNA Isolation Kit, Cat. No: 12183-180). Primers were used for *bcl-2, bax-α, bak, bcl-x, bik, mcl-1, bfl-1* and *β-actin* (Takara Bcl-2 family, Cat no. 6623). The PCR cycles consisted of initial incubation at 55 ˚C for 30 min. The reaction mixture was then held at 94°C for 2 min to denature the RNA/cDNA hybrid. The double-stranded cDNA was produced and amplified in following PCR conditions: Denaturation at 94 ˚C for 15 s.; annealing at 60 ˚C for 30 s.; and extension at 68 ˚C for 1 min., for 40 cycles; and final extension at 68 ˚C for 5 min. for 1 cycle. RT-PCR products were electrophoresed on 1.8% agarose gels and visualized under UV light after ethidium bromide staining.

Statistical analysis

The data (mitotic index and apoptotic index) were analyzed by one-way ANOVA, followed by Dunnett's test for separate comparisons with the control group. Differences were considered significant at $p < 0.05$.

RESULTS

Proliferation rates, which were determined by MTT assay that was conducted on HeLa cells for which we applied five different concentrations of Astragalus root extracts for 24, 48 and 72 h to show rate of cytotoxicity effect are presented on Figure 1. Determined %vitality values are

Table 1. Mitotic index values of various treatment groups in HeLa cells growing *in vitro*, statistical significance, given in ±SD.

Treatment group (mg/ml)	Mitotic index ± SE		
	24 (h)	48 (h)	72 (h)
Control	2.469 ± 0.294	3.352 ± 0.445	2.100 ± 0.456
0.1	2.290 ±0.342	1.603 ± 0.289[1]	2.208 ± 0.532
0.15	1.868 ± 0.300	1.968 ± 0.320	2.681 ± 0.381
0.5	3.046 ± 0.412	3.392 ± 0.447	2.433 ± 0.299
1	3.887 ± 0.451 [1]	4.250 ± 0.641	3.066 ± 0.449

[1]$p < 0.05$

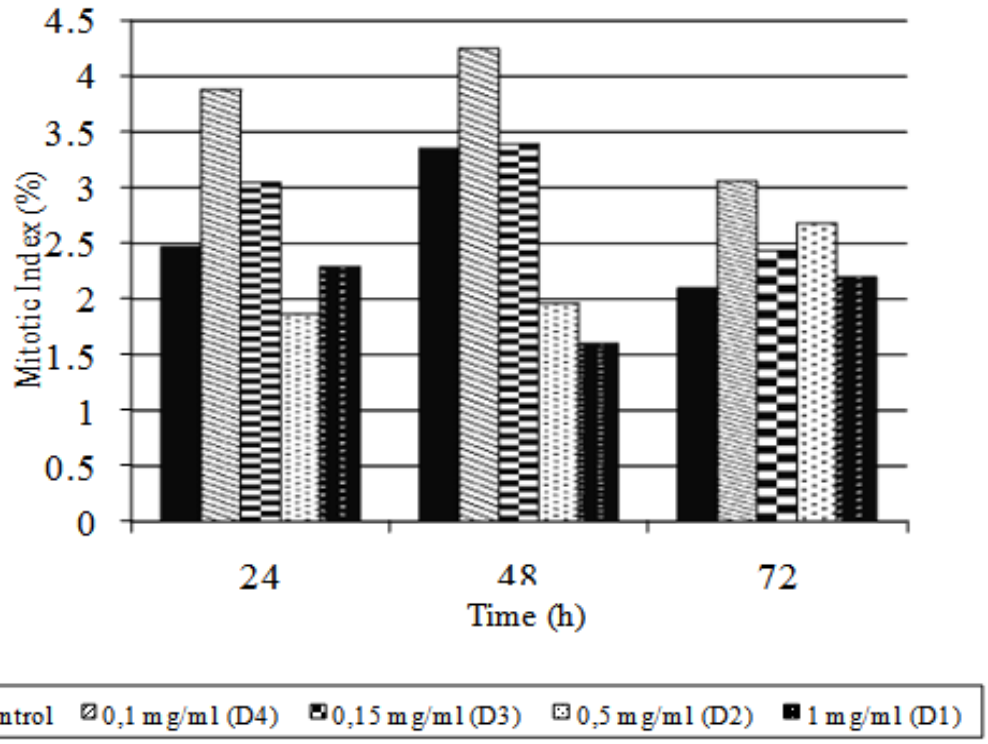

Figure 2. Mitotic index of HeLa cell lines treated with *Astragalus chrysochlorus* root extracts for 24, 48 and 72 h.

calculated by assuming control group as 100%. As it can be seen on (Figure 1) cytotoxic effect of root extract was appeared in first 24 h of application time and increased depending on application time and dose. According to the statistical analysis, differences between groups were statistically significant compared to the control group ($p < 0.05$).

Same experimental groups were carried out in parallel for mitotic index and apoptotic index parameters to explain which cell cycle phase was effected by the extract to cause this appeared cytotoxic effect. MI values of experimental groups and their significance compared to the control are presented on Table 1. Expected decrease or increase was not determined on MI values depending on applied dose and hours (Figure 2). Apoptotic cell percentage of extract applied on HeLa cells presenting AI values are shown in Table 2. According to the statistical analysis, statistically significant increase was determined on AI values between control group and extract applied on experimental groups on particular doses and hours. As it can be seen in Figure 3, AI value of D1 and D2 doses which were applied for 24 h presented significant increase. For 48 h of application group, only D4 dose presented significant AI increase. On 72 h group, D1 and D2 doses applied on HeLa cells presented AI increase. When we compare all extract applied on experimental groups to each other, it can be seen that statistically, the lowest AI percentage was on 72 h applied D4 dose and the highest AI percentage was on 24 h applied D1 dose.

Furthermore, we examined effects of the highest and

Table 2. Apoptotic index values of various treatment groups in HeLa cells growing *in vitro*, statistical significance.

Treatment group (mg/ml)	Apoptotic index ± SE		
	24(h)	48 (h)	72 (h)
Control	8.738± 1.098	8.686±0.886	9.151±0.800
0.1	11.494±1.232	13.708±1.910 [2]	4.880±0.684 [1]
0.15	13.490±1.068 [1]	8.599±0.604	6.441±0.920
0.5	12.087±1.352	8.114±0.473	13.981±1.309 [2]
1	16.776±1.337 [2]	7.320±0.869	10.408±1.254

[1]p<0, 05 [2] p<0.01

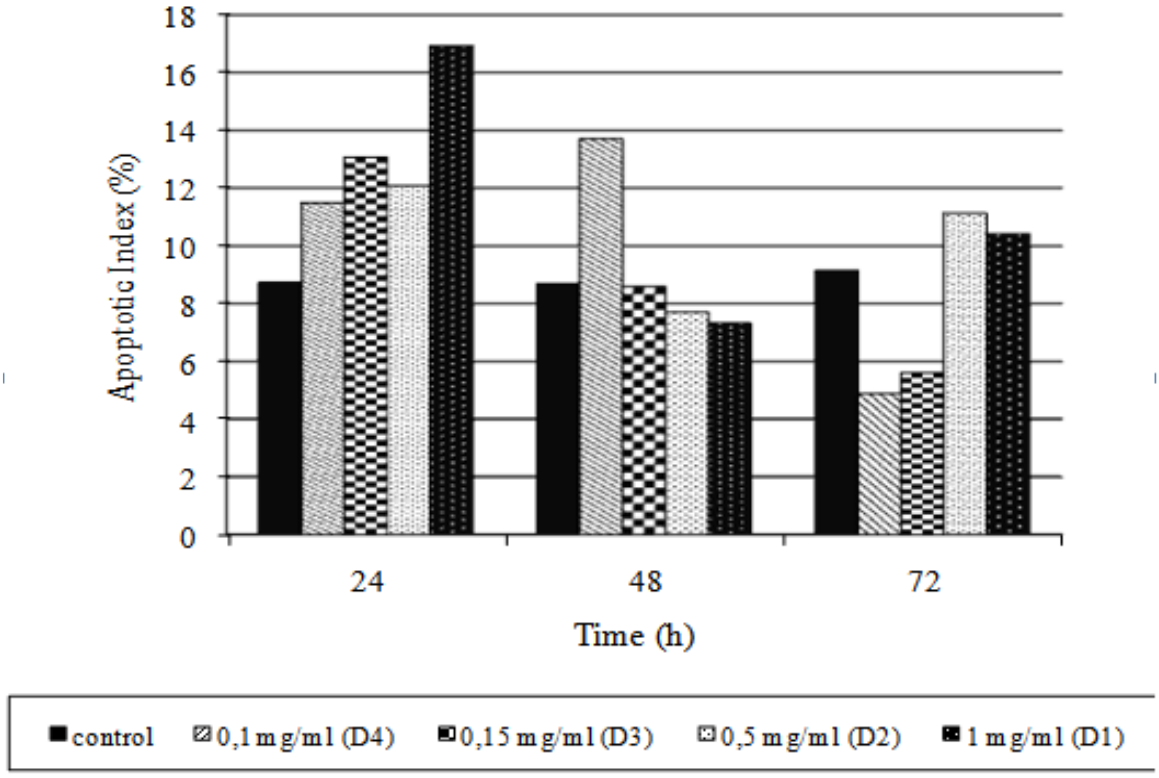

Figure 3. Apoptotic index of HeLa cell lines treated with *Astragalus chrysochlorus* root extracts for 24, 48 and 72 h.

the lowest doses, which cause apoptosis on HeLa cells, on expression rates of *bcl-2* gene family to illuminate molecular mechanism of extract stimulated apoptosis. For 72 h application group, expression rates of proapoptotic (*Bax*), antiapoptotic (*bcl-2*) genes of *bcl-2* family and b-actin as internal standard are presented on (Figure 4). On 72 h experimental group, we determined that proapoptotic bax gene was not expressed on the highest D1 dose, bak gene was expressed on both (D1 and D2) doses, proapoptotic bik gene was expressed on D1 dose and expression rate of bik was increased on D4 dose. Likewise, expression rates of antiapoptotic genes belonging to the bcl-2 gene family are presented on Figure 4 for Hela cells which were applied to the highest and the lowest doses for 72 h.

Expression rates of antiapoptotic bcl-x and mcl-1 presented significant decrease compared to the control on D1 dose which is the highest. Likewise, bcl-2 gene of antiapoptotic members was not expressed on the same D1 dose when it is applied for 72 h.

In this study, expression rates of proapoptotic *bax* and *bik* genes, antiapoptotic *bcl-2* gene were investigated on D1 and D4 doses applied on experimental groups for 24 and 48 h in addition to the 72 h (Figures 5 and 6,

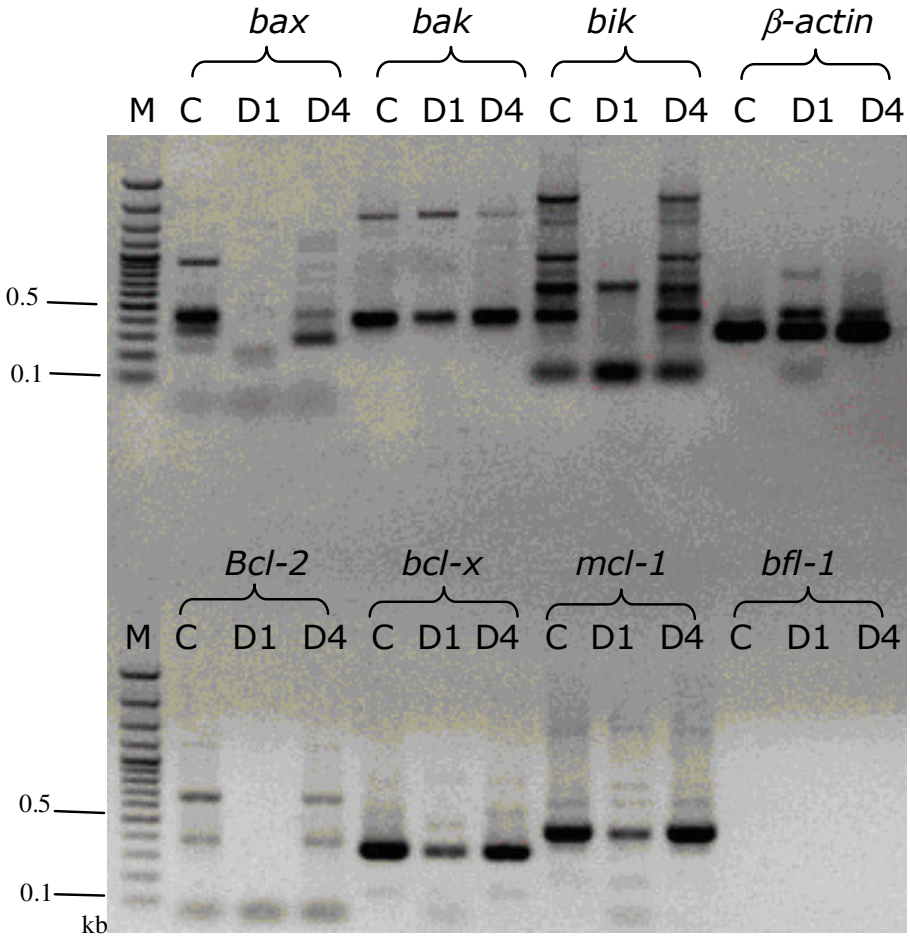

Figure 4. Gel electrophoresis of RT-PCR reaction for *HeLa cells.* M, marker; C, Control; D1, 1 mg/ml; D4, 0.1 mg/ml for 72 h.

respectively). Expression rates of proapoptotic bax and bik genes were not presented with significant increase or decrease compared to the control, on 24 and 48 h applied groups. Antiapoptotic bcl-2 gene presented expression on 24 h applied D1 dose, but that expression disappeared when application time of D1 dose extended to 48 h.

DISCUSSION

We can describe cancer as a disease of genome which occurs by dynamic changes of DNA in cells' life cycle. Even though imbalance between genetic and environmental factors may start cancer, genomic changes which can occur in any cells genome and affect various cellular pathways are always primary cause of cancer.

Apoptosis occurs by two main pathways. These are; extrinsic pathway which is triggered by death ligand pathway and intrinsic pathway which occurs by mitochondrial pathway. In mitochondrial pathway, cytochrome c, which is a member of mitochondrial transport system, has to be released for activation of caspase cascade. Release of cytochrome c from mitochondria to cytosol in first stage of apoptosis is regulated positively or negatively by members of *bcl-2* family. *Bcl-2*, *bcl-xL* and *mcl-1* of *bcl-2* family member block apoptosis and *bax, bak, bid, bik*, *bim*, and *bad* triggers apoptosis (Periman et al., 1999; Rozalski et al., 2005; Corazza et al., 2009; Werner et al., 2002).

Control and regulation of mitochondrial permeability are managed by proteins of bcl-2 family. Proapoptotic and antiapoptotic members create heterodimers. Antiapoptotic *bcl-2* gene family members have all four bcl-2 homology areas (BH 1-4). Proapoptotic proteins have only three of these areas (BH1, BH2 ve BH3) or have only the BH3 area. These members present pro- or antiapoptotic effects by different mechanisms depending on these homology areas (Bhalla , 2003; Hanada et al., 1995; Sitorus et al., 2009; Tang et al., 1992).

Kim et al. (2009) studied with genistein which is a product of soybean and has anti-cancerogenic effect on multiple tumor cells. They applied 5 to 60 µmol/L dose of

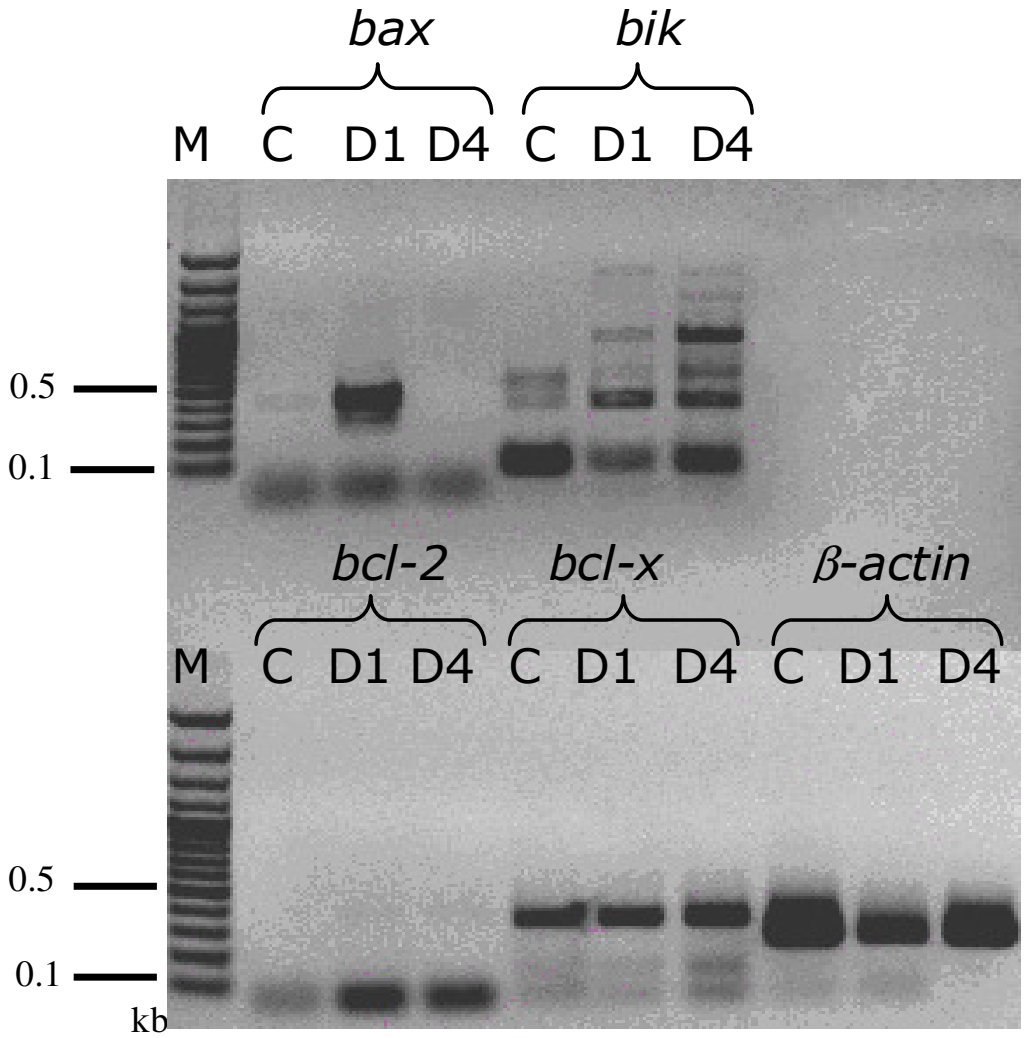

Figure 5. Gel electrophoresis of RT-PCR reaction for *HeLa cells.* M, marker; C, Control; D1, 1 mg/ml; D4, 0.1 mg/ml for 24 h.

genistein on HeLa, CaSki ve C33A cells (cervical cancer cells) for 48 h to investigate apoptosis pathway of this product. They presented that genistein is effective on both intrinsic and extrinsic pathways. Furthermore, they found that gene expressions of t-bid and bax which play a role on cytochrome c released from mitochondria are increased; *bcl-2* gene expression is not effected by genistein and therefore, bax/bcl-2 rate increases.

Sitorus et al. (2009) investigated gene expressions which play role on different pathways of apoptosis on retinoblastoma. They studied on total 19 incident including 10 internal eye tumors and nine external eye tumors. In various tumors, AI and *bcl-2* gene expression are inversely proportional but they could not find statistical significance between these two groups. Over-expression of *bcl-2* gene is found for all tumors but apoptotic cell amount increased. Also they reported that *bcl-2* gene did not express in 11 tumors. Four of these did not present obvious apoptosis and they concluded that over-expression of *bcl-2* gene does not always block apoptosis.

Determination of AI parameter, that indicates rate and intensity of apoptosis, is an assistant technique to estimate tumor type, stage, course of disease, results, resistance and the future of the patient (Arican et al., 2008; Yin et al., 2003).

Gottardi et al. (1996) investigated apoptosis mecha-nism of malignant CD5+ B cells by presenting expression of *bcl-2* gene family. As a result of RT-PCR, theydetermined that expression level of *bax* was high and *bcl-xS* gene expression was slightly lower in some samples.

The aim of this study is applying *Astragalus chrysochlorus* root extracts to HeLa cell lines and determine changes on cytotoxicy and viability. As a

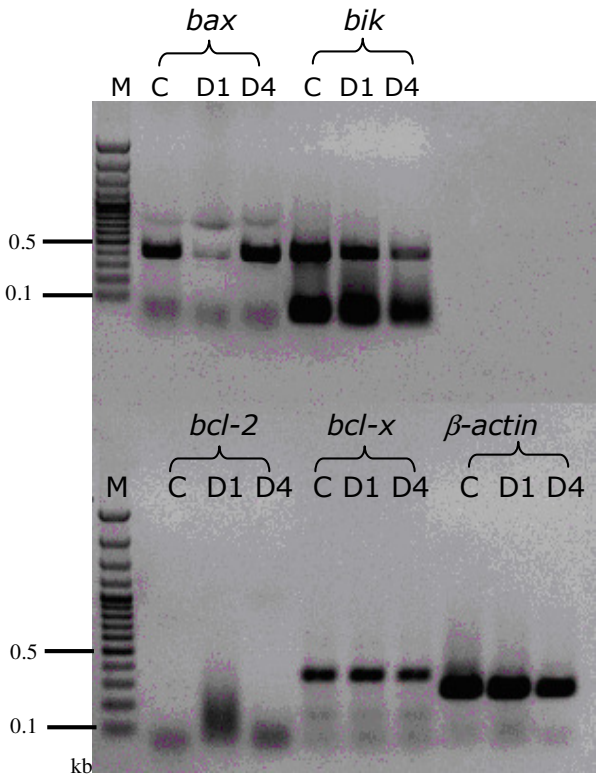

Figure 6. Gel electrophoresis of RT-PCR reaction for *HeLa cells.* M, marker; C, Control; D1, 1 mg/ml; D4, 0.1 mg/ml for 48 h.

result, kinetic parameters as proliferation rate, apoptotic index, mitotic index and also some apoptosis-related genes have been examined. Time dependant cytotoxic effect of 5 different concentrations of *Astragalus* plant extract was evaluated on HeLa cell cultures. For this aim colorimetric MTT assay was used and % vitality values were determined. As a result of these, it's been determined that extract which was applied to HeLa cells caused cytotoxic effect depending on dose and application time beginning from 24 to 48 hrs and 72 hrs (Figure 1). Apoptotic index values of 5 different applied concentrations of this extract were compared to understand if that determined cytotoxicity, which was found *Astragalus* extract applied HeLa cells, was related to apoptosis. As a result of this, the highest apoptotic index values were determined for D1 dose which was applied for 24 hrs. We assume that decrease on apoptotic index values of 72 hrs applied group was because of the high toxicity on this duration. When cytotoxicity and apoptotic index values of all experimental groups were considered, the most effective concentration of *Astragalus* plant extract on HeLa cells was determined as DIdose (1 mg/ml). In our study, gene expressions of *bcl-2* family were investigated by RT-PCR technique on D1 and D4 concentrations applied HeLa cells for whole apoptosis process which was appeared in experimental time periods. As a result it was determined that expressions of *bcl-x*, *mcl-1* and *bcl-2* genes belong to *bcl-2* family decreased significantly on D1 dose when they were compared to the control and D4 dose for 72 hrs. Furthermore, expression of proapoptotic *bax* gene presented significant increase on D1 dose compare to control and D4 dose for 24 hrs. Natural products are generally used to create new anti-carcinogenic agents and producing its derivatives. Furthermore, it's crucial to understand mechanism of these anti-carcinogenic agents that they take place. Results of this study are very important on basis of investigating molecular mechanism by determining anti-carcinogenic and also apoptotic effects of applied endemic plant extract on HeLa cells, investigating apoptotic index values and presenting gene expressions of *bcl-2* family which have anti-apoptotic and pro-apoptotic properties. This study has an importance to be the first one on this area. Result that obtained from this study is thought to be encouraging for the forthcoming studies that would plan to use plant extracts. Also, this results will contribute to present cellular and molecular based effects of natural products which are used on cancer therapy and improving their efficiency.

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