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Original Research Article

Investigation of grape and raisin extracts in induction of apoptosis and necrosis and their genetic expression on cancer stem cells (SH-SY5Y)

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ABSTRACT

Grapes and raisins with and without seeds were extracted using ethyl acetate/H_2O solvent. The antioxidant activity of each extract was confirmed by the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging method and free radical scavenging assay. The results confirmed the antioxidant property of the extract in such a way that the most antioxidant activity of the extract was on radical activity. Free radicals were observed in extracts of seedless raisins and seeded grapes. Their cytotoxicity was investigated on cancer stem cells (SHSY5Y) using the MTT (p-value = <0.0001) method. The results obtained from the MTT test were confirmed using the cell counting method and Trypan blue staining. Cells (SHSY5Y-1) were identified with IC50=66.87 µg/mL in seeded grapes extract and IC50=12.63 µg/mL in raisins without seeds extract. The FITC-V Annexin$^+$ and PI$^+$ cell populations were considered as secondary apoptosis, and FITC-V Annexib$^-$ and PI$^+$ cell populations as necrosis. Data analysis was done using device software. Flow cytometry analysis showed that 62.2 % of cells underwent cell apoptosis after treatment with seedless raisin extract and 78% of cells after treatment with seeded grapes. Real time PCR method was performed for the molecular analysis of treated cells. The change was evaluated in the expression of apoptotic genes (BAX, BCL2, P53, and GAPDH). Expression of apoptotic genes for BAX and BCL2 is high under treatment with seeded grape extract. However, the expression of apoptotic genes for BCL2 and P53 is high under treatment with raisin without grape extract.

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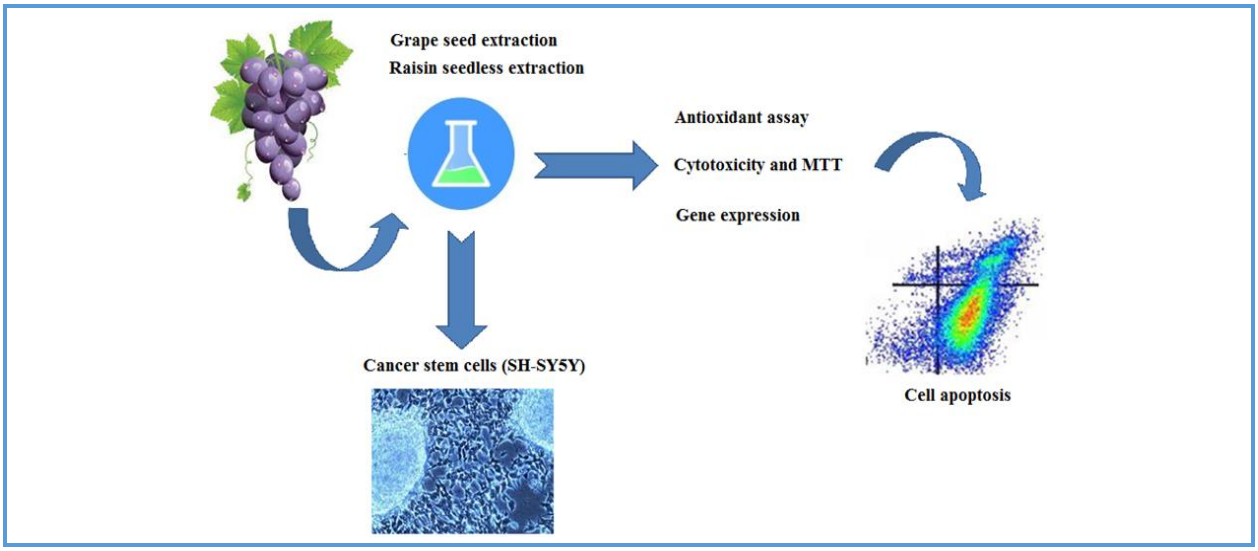
Graphical Abstract



Introduction

Anticancer properties of grapes and raisins

The grape fruit is divided into two types, seeded and seedless, and each type can be seen in different colors, red, black, yellow, and almost green [1]. Grape seed is a waste of grape juice industry. Depending on its type, these seeds contain protein, lipid, carbohydrate, and polyphenol. Polyphenols in grape seeds are mainly flavonoids. Grape seed extract is known as a powerful antioxidant that supports the body against aging, disease, and decay [2]. Extensive research shows that grape seed extract is useful in all areas of health, including youthful skin, cellular health, elasticity and flexibility due to its antioxidant effect to bond with collagen [3-6]. Other studies have shown that proanthocyanidins help support the body from cancer and power blood circulation by reinforcement capillaries, arteries, and veins [7]. Cancer occurs when the body's normal cells undergo genetic and biochemical changes and become uncontrollable in growth and reproduction [3].

Glioma stem cells (GSCs) and SH-SY5Y cells

Cancer stem cells are a small group of cancer cells that, like normal stem cells, have maintained the properties of self-renewal, proliferation, and differentiation and at the same time have tumorigenic properties [8]. These cells remain in the G0 phase which is cellular state outside the repetitious cell cycle and causes spontaneous proliferation away from the center of cell growth regulation, which leads to the spread of cancerous masses. This phenomenon is the main reason for the expansion and increase in the volume of cancer stem cell tumors in the bone marrow cancer cells [9]. These tumor-initiating cells are responsible for the emergence of various types of cancer cells with a very high growth rate in the mass and tumor due to their high rate of renewal and division. They are a factor for the maintenance and survival of the cancer cell population and high resistance to treatment by maintaining cell metabolism and ultimately lead to the death of the patient [10-18].

Glioma stem cells (GSCs) introduce a subpopulation of cells within glioblastoma that

has been specified by growing resistance to chemotherapy and radiotherapy. Therefore, GSCs may cause cancer treatment failure. Hence, GSCs are a suitable target for the glioblastoma treatment and GSCs removal is very important in the treatment of glioblastoma (brain tumors). Strategies to target the GSCs mainly involve direct effects and toxicity on GSCs, which are focused on targeting cell surface markers and particular pathways required to maintain GSCs structure [8-10].

Cell lines are often used for toxicity or viability experiments aimed at finding disease mechanisms at the cellular level [19-24]. The SH-SY5Y cell line is a triple cloned sub-line of SK-N-SH cells established in the early 1970s from the bone marrow mass of a patient with neuroblastoma of sympathetic adrenergic ganglionic origin [11]. This cell line exhibits neuronal marker enzyme activation (tyrosine and dopamine beta-hydroxylases), special perception of norepinephrine (NA), and expresses some neurofilament proteins. These cells also express opioid, muscarinic and nerve growth factor receptors. In addition, SH-SY5Y cells have the ability to proliferate in culture for long periods, which is a prerequisite for the development of a contamination-free in vitro cell model [12].

Literature

It was investigated the effect of methanolic extracts of leaves, peels, cores, and juice of five varieties of grapes cultivated and was found the most lethality in grape juice [13-15]. Shantaram S. Joshi *et al.* (2000) found interesting results related to the lethal effect of grape seed on liver cancer cells [15]. They also investigated the lethal effect of grape seed extract on breast cancer cells. The result showed that grape seed extract reduced the growth of cancer cells in a period of 24, 48, and 72 hours [16]. Manjinder *et al.* examined the metabolite of Gallic acid in grape seed on mouse animal tumors. They noticed apoptosis and its anticancer effects [17]. Amir Ala Iqbali investigated the effects of grape seed extract on oral cancer and umbilical cord stem cells. They found that grape seed extract could significantly reduce the survival of oral cancer cells, but had little effect on the lethality of human umbilical cord natural stem cells [18]. Numerous studies have been done on grape and raisin extract and gene expression related to cancer, but there was a little research that specifically compared grapes and raisins [12-18].

Experimental

Chemical materials and instruments

The chemical materials of ethyl acetate solvent, 2,2-diphenyl-1-picrylhydrazyl (DPPH), dimethyl thiazyl diphenyl tetrazolium bromide (MTT), Dimethyl sulfoxide (DMSO) with analytical grade were used. MTT test was performed with Microplate reader Model Elisa microplate reader. The flow cytometry test was done with Flow Cytometers Model BD FACSlyric. Gene expression was done with real time PCR Cycler with Rotor-Gene Model Q MDx. Antioxidant test was under DPPH method. UV-Vis spectroscopy data was performed with spectrophotometer Model LEKI SS2110.

Planting and preparation of extract

To prepare grape fruit extract, Shahroudi cultivar was harvested from the garden of Tehran University of Agriculture and Natural Resources Research Center. Samples include five groups: grapes with seeds, grapes without seeds, raisins with seeds, raisins without seeds

and grape seeds alone. They were washed and completely dried in an oven at 45 °C. After grinding, the samples were extracted with ethyl acetate solvent and distilled water in a ratio of 1:9 for 24 hours on a shaker with a speed of 180 rpm and a temperature of 25 °C. The extracts were concentrated after passing through filter paper using a rotary device under vacuum conditions and at 40 °C, and then dried using the low pressure of the device, and then the extracts were filtered using a 0.22 micron filter under sterile conditions.

Cell culture

SH-SY5Y cell line (glioma cancer stem cell) was purchased from Pasteur Institute cell bank (Tehran-Iran) and placed in 25 mL flasks in DMEM-F12 culture medium containing 10% FBS (fetal bovine serum) and 1% antibody [11]. Penicillin-streptomycin biotic was cultured at 37 °C in a humid environment containing CO_2 (5%). Every 24 hours, the cells were checked with an inverted microscope and the culture medium was changed if necessary. The day after the initial culture, cell density was checked under an inverted microscope. If the cell growth reached 80%, cells were passaged.

Antioxidant assay

DPPH free radical inhibition measurement was used to determine the antioxidant capacity of grape extract. Accordingly, a similar volume of DPPH with a concentration of 100 µM was added to a fixed number of samples. The stirred solution was kept in dark for 20 min at room temperature and absorbance was measured at 515 nm against a methanol control (without DPPH). The inhibition percentage was calculated from equation 1. Based on the inhibition percentage, only two samples (grapes with seeds and raisins without seeds) were selected for further investigation.

$$\%\text{Inhibition} = \left[\frac{A_{control} - A_{sample}}{A_{sample}}\right] \times 100 \quad (1)$$

Cytotoxicity and MTT method

The effect of cytotoxicity of seedless raisin and seeded grape extract on SH-SY5Y was investigated by colorimetric method (MTT) with micro-culture tetrazolium test compared to the control. This is based on the activity of succinate dehydrogenase in the mitochondria of living cells and converts the yellow MTT solution into purple insoluble formazan crystals, which can be detected after dissolving in DMSO with an ELISA plate reader. Flow cytometry text, real time PCR was performed in Pasteur Institute cell bank, and university of Tehran specialized laboratory.

Result and discussion

Effect of cytotoxicity by MTT method

After passing the cells several times and reaching the quorum, the cells were transferred to a 96-well plate, so that 15,000 cells were placed in DEMEM_F12 culture medium containing 10% FBS in each well. The cells were placed in the vicinity of different concentrations of extracts (0.5, 10, 30, 50, 150, 250, 300, and 500 µg). The micro plates containing the extract and cells were incubated for 24 hr under the same conditions. On the day of reading, 200 µL of DMSO was replaced with the medium incubated with MTT dye and it was gently pipetted to dissolve the purple formazan crystals. Light absorption was measured at 570 nm. The effect of each extract concentration was evaluated with 3 repetitions. The percentage of cytotoxicity in the negative control group was 100. The percentage of cytotoxicity is the average of the

negative control minus the average of each dose divided by the average of the negative control and multiplied by a hundred. A concentration of the tested compounds that reduces the percentage of cell life by half was considered as Inhabitation Concentration 50 (IC50). This value was determined by the graph obtained from the interpretation with the software (Graph pad prism).

Figure 1 displays survival percentages of SH-SY5Y after treatment with extracts. In this diagram, the SHSY5Y-1 is related to treatment with seeded grapes and the SHSY5Y-2 is related to raisins without seeds. With increase in the concentration of the extracts, the survival percentage or viability decreases significantly in both selected samples. A concentration of the tested compounds that reduces the percentage of cell life by half was considered as Inhabitation Concentration 50 (IC50).

Measuring the antioxidant activity of each extract using DPPH radical scavenging method and free radical scavenging assay, confirmed the antioxidant properties of the two extracts. The results obtained from the MTT test were confirmed using the cell counting method and Trypan blue staining. Cells (SHSY5Y-1) were identified with IC50=66.87 µg/mL in seeded grapes extract and IC50=12.63 µg/mL in raisins without seeds extract.

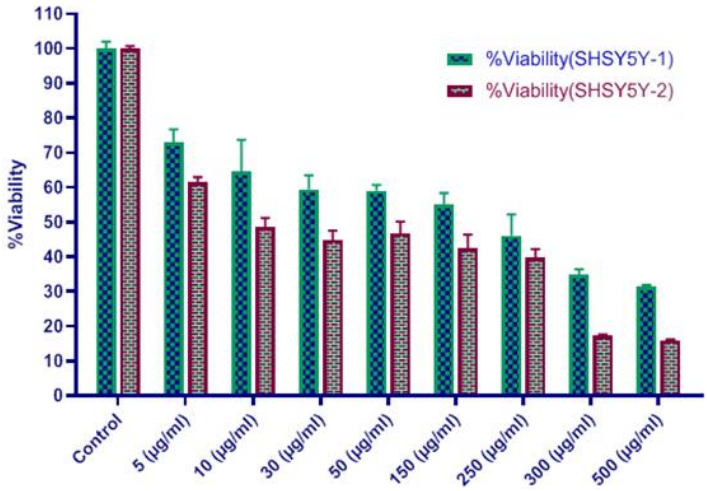


Figure 1. Survival percentages in (SHSY5Y-1) and (SHSY5Y-2).

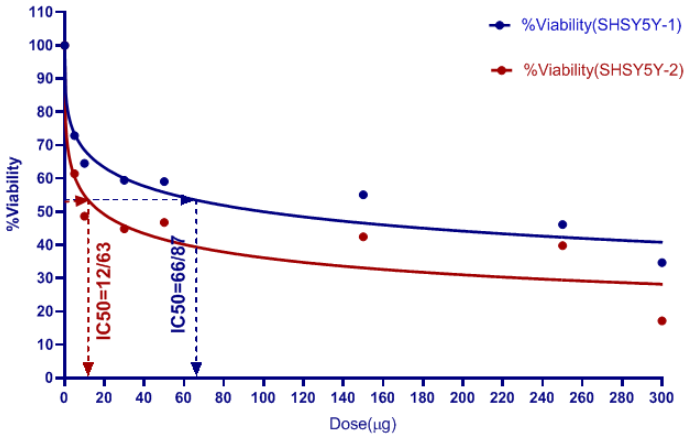


Figure 2. Inhabitation concentrations (IC50) in (SHSY5Y-1) and (SHSY5Y-2).

Investigating of apoptosis and necrosis with flow cytometry

A number of $5x10^5$ of SH-SY5Y were placed in each well of a 6-well plate and treated with concentrations of 12.63 and 66.87 µg/mL extract. After 24 hr incubation, the cells were incubated with FITC-V Annexin and propidium iodide (PI) and analyzed by flow cytometry. The FITC-V $Annexin^+$ and PI^+ cell populations were considered as secondary apoptosis, and FITC-V $Annexib^-$ and PI^+ cell populations were considered as necrosis [11,12]. In this test, culture medium without drugs and dyes is considered as negative control. In the following, double staining (PI/ FITC-V Annexin) was used to diagnose necrosis and apoptosis to provide a general description of the selective toxicity of a chemical substance. Apoptosis is a type of death in the organism's cells in which the cells are determined by receiving generation signals. As soon as these cells receive a signal (from inside or outside the cell), they witness a series of cascade events of the reaction of molecules, and as a result, they undergo cell death.

Data analysis was done using device software and dividing the points recorded in the two-dimensional curve into four regions. Double staining of cells with Annexin V-FITC and PI provides conditions to divide cells into four regions. Region Q1 shows necrotic cells, Q2 late apoptotic cells, Q3 live cells and Q4 early apoptotic cells [16]. Flow cytometry analysis showed that 62.2 % of cells underwent cell apoptosis after treatment with seedless raisin extract and 78% of cells after treatment with seeded grapes (Figure 3).

Although a very small percentage of cells suffered necrosis, but this amount was very small compared to the percentage of apoptotic cells. The findings indicate that both substances in high concentration can advance healthy cells towards apoptosis and prevent them from multiplying.

The changes in the expression of apoptosis and necrosis genes

First, one million SH-SY5Y were cultured in 6 house plates. Total RNA (Total Ribo nucleic acid) was extracted for reverse polymerase chain reaction (RT-PCR) test, after 24 hr of exposure to extracts with concentrations of 12.63 and 66.87 µg/mL. Extraction was done using Trizol kit and RNA extraction according to the manufacturer's own protocol with amounts of RNAs extracted using the cDNA production kit. The cDNA synthesis was converted to single-stranded DNA. The obtained cDNA combination was used to check the expression of BAX, BCL2, and GABDH genes using real time PCR technique [14-16]. Primers for any genes are listed in Table 1.

Afterwards, real time PCR test was used to validate the effects of apoptosis by the extracts on SH-SY5Y. Real time PCR is a type of polymerase chain reaction that during the amplification of cDNA values in the real time machine, at the same time [10-12]. The results of the multiplication of strings are displayed on the device software at the same moment so that SH-SY5Y after treatment with extracts, RNA extraction, cDNA synthesis, and performing real time PCR on apoptotic genes caused more expression and overexpression of genes. This is if the expression of genes is higher than the internal control gene GAPDH, which proves the effect of these extracts on the expression of apoptotic genes. According to Table 2 and Figure 5, expression level of apoptotic genes for BAX and BCL2 is high under treatment with seeded grape extract.

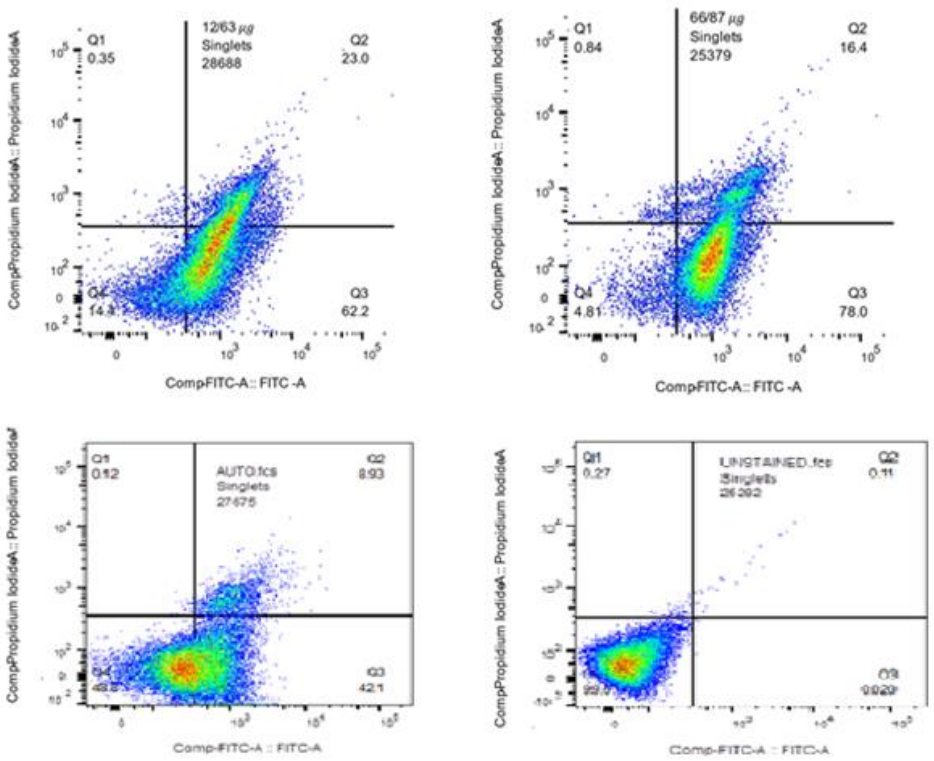


Figure 3. Flow cytometry test and the percentage of apoptotic and necrotic cells.

Table 1. Primers for gene expression and cDNA synthesis

Gene	Primers
GABDH	5'-CGT GGA AGG ACT CAT GAC CA-3', 5'-TCC AGG GGT CTT ACT CCT TG-3'
Bcl-2	5'-CGA CGA CTT CTC CCG CTA CCG C-3', 5'-CCG CAT GCT GGG GCC GTA CAG TTC C-3'
Bax	5'-TCC ACC AAG AAG CTG AGC GAG-3', 5'-GTC CAG CCC ATG ATG GTT CT-3'
GAPDH	5'-GTCTCCTCTGACTTCAACAGCG-3', 5'-ACCACCCTGTTGCTGTAGCCAA-3'

Table 2. Expression level of apoptotic genes in the seeded grape extract sample

Gene	Type	Reaction Efficiency	Expression	Std. Error	95% C.I.	P(H1)	Result
GAPDH (control)	REF	0.8225	1.000				
P53	TRG	0.8675	2.666	1.669 - 4.901	1.142 - 5.296	0.000	UP
BAX	TRG	0.8513	2.843	2.041 - 4.173	1.545 - 4.663	0.000	UP
BCL2	TRG	0.8488	2.190	0.912 - 4.750	0.877 - 6.255	0.141	

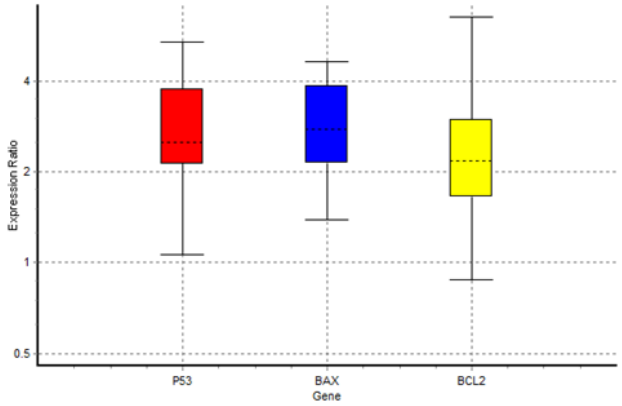


Figure 4. Expression levels of apoptotic genes in the extract of seeded grape sample.

Table 3. Expression level of apoptotic genes in the seeded grape extract sample

Gene	Type	Reaction Efficiency	Expression	Std. Error	95% C.I.	P(H1)	Result
GAPDH (control)	REF	0.8538	1.000				
BAX	TRG	0.8713	1.525	0.792 - 2.952	0.593 - 4.069	0.286	
BCL2	TRG	0.8775	1.871	1.127 - 2.803	0.919 - 3.863	0.036	UP
P53	TRG	0.8725	1.761	1.185 - 2.470	0.834 - 2.966	0.023	UP

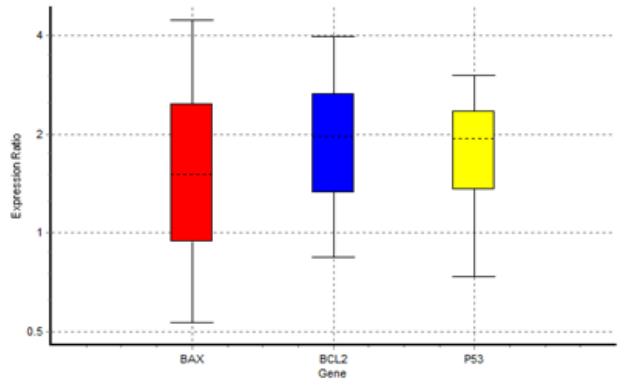


Figure 5. The expression level of apoptotic genes in the extract of seedless raisin sample.

According to Table 3 and Figure 5, expression level of apoptotic genes for BCL2 and P53 is high under treatment with raisin without grape extract. The present study showed that the extracts of seedless raisins and seeded grapes are effective on the SH-SY5Y lethality. More experiments are needed in the field of the effect of the mentioned extracts on various induced cancer tumors in animal models and its side effects in the local use of this drug.

Conclusion

The effect of cytotoxicity of seedless raisin and seeded grape extract on SH-SY5Y was investigated by colorimetric method (MTT), flow cytometry text, and real time PCR. The results obtained from the MTT test were confirmed using the cell counting method and Trypan blue staining. Cells (SHSY5Y-1) were identified with IC50=66.87 µg/mL in seeded grapes extract and IC50=12.63 µg/mL in raisins without seeds extract. A number of $5x10^5$ of SH-SY5Y in a 6-well plate were treated with concentrations of 12.63 and 66.87 µg/mL extract. After 24 hr incubation, the cells were incubated with FITC-V Annexin and propidium iodide (PI) and analyzed by flow cytometry. Flow cytometry analysis showed that 62.2 % of cells underwent cell apoptosis after treatment with seedless raisin extract and 78% of cells after treatment with seeded grapes. The obtained cDNA combination was used to check the expression of BAX, BCL2 and GABDH genes using real time PCR technique. Expression level of apoptotic genes for BAX and BCL2 is high under treatment with seeded grape extract. Expression level of apoptotic genes for BCL2 and P53 is high under treatment with raisin without grape extract.

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Molla Sadra Student Research Center located in the national hub of stem cells and regenerative medicine, Karaj Biotechnology Research Institute, Tehran University Agriculture and Natural Resources Campus Center Garden, Tehran University Pasteur Institute cell bank, University of Tehran specialized laboratory.

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