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Effect of Garlic Extract on the Expression of BAX and Bcl-Xl Genes in Rat Lymphocytes

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Abstract

Introduction: In addition to their nutritional value, medicinal herbs have received great attention, since ancient times, for their potential in controlling and curing diseases. Such effects are originated from various compounds, including antioxidants, available in their structure. Amongst others, garlic has been used by both traditional medicine and modern medicine to treat various diseases. Thanks to various antioxidant combinations and sulfur-contained substances, including allysine, phytoalexin, diallyl sulfide disulfide and tri-sulfide, garlic plays a vital role in curing diseases including cancer. In this study, we examined the effect of garlic extract on the expression of BAX and BCL-XL genes.Materials and Methods: A total number of 30 male Wistar rats (weight=250 ± 20 gr) were selected from the experimental animal breeding center of Jundishapur University of Ahwaz in random. The rats were grouped into three groups namely: healthy rats, 3-week old rats and 8-week old rats. The latter two groups were treated by garlic extract, extracted by percolation method, at two concentrations i.e. 100 and 200 mg /ml. All rats were evaluated using Real Time PCR method.Results: The expression of BAX showed a significant increase at the concentrations of 100mg/ml and 200 mg/ml of garlic extract in weeks 3 and 8 in all groups while the expression of Bcl-Xl showed a significant decrease at both concentrations in both weeks.Discussion: It can be argued that garlic extract has a positive effect on the opposite expression of Bax and Bcl-Xl genes contributing to the apoptosis of cancer cells.

Keywords: Apoptosis, Bax, Bcl-Xl, Garlic Extract

Introduction

Immunology is the science of body defense against infections. Immune system cells possess specific properties. Despite slight differences in their morphology, all of them are responsible for suppressing foreign pathogens. They are categorized into two categories in terms of the number of nucleus: multinucleated phagocytes such as neutrophil, basophil and eosinophil and single-nucleated phagocytes including monocytes, macrophages, lymphocytes and natural killer cells (NK cells). Lymphocytes are small circular cells with a diameter of 7-15 microns with a big spherical nucleus that induce the specialty of immune response. They are categorized into two categories: A) B-lymphocytes: in mammals, B-lymphocytes are produced in bone marrow while and in birds, they are produced in bursa of fabricious. It is activated by the reaction between gene and the antibody of B cell where it converts to operational cell secreting antibody (Janeway,2017). B T-lymphocytes: it is produced in bone marrow and goes to thymus to be matured there and this is why it is called T cell. T-lymphocytes kills those cells that generate foreign antigens and are protected from antibodies like virus-infected cells or inter-cell microbes. T-lymphocytes, themselves, are categorized into two functional categories.

Cancer is the first cause of mortality preceded only by cardiovascular diseases. It is the uncontrolled growth of cells. Cellular manipulation results in the formation of a mass that is called neoplasm or tumor. They may migrate to far areas and lead to metastasis. The mechanism for the formation of all neoplasms is the lack of suitable responses to the control of normal growth and lack of cellular cycle control (Carpino, 2012).

Bcl-2 family proteins play a vital role in the permeability of the outer membrane of mitochondrion and adjusting mitochondrion dynamics during apoptosis. BAX is in the form of ring-shaped holes inside mitochondrion that is responsible for releasing cytochrome c while Bcl-Xl controls the formation of the holes (Bleicken et al., 2016). Bcl-Xl is located in zone 21 and the molecular weight of its protein is 25 Kdalton with a length of 239 amino acids. This protein adjusts the activity of caspase enzymes. This protein releases cytochrome c from mitochondrion. This, in turn, activates caspase 9 and caspase 3 and, finally, results in cellular suicide. Bcl-Xl protein plays a role in the activation and inhibition of apoptosis (Nguyen et al., 2013).

Today, diseases are recommended to be treated by medicinal herbs, instead of chemical drugs, with different biological properties originated from their lower side effects and costs. Garlic is an example of such medicinal herbs. Garlic, which has been used as an herb from old times, contains two main

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antioxidants including flavonoids and sulfur compounds that result in anti-tumor, hypolipidemia, hypocholestrolemia and anti-atherosclerosis effects. With the scientific name of sativum allium, garlic is an umbelliferae plant and an important indigenous plant of Iran with diverse amino acids, minerals, vitamins, flavonoids, and volatile and non-volatile compounds with considerable medicinal and therapeutic value (Hussein et al., 2013). Allysine, phosphorus compounds, and alliinase, peroxidase, myrosinase enzymes and ajoene, citral and geraniol are the most important compounds of garlic. Studies show that garlic increases cytokines and activates macrophages, lymphocytes and neutrophils and, in turn, improves and stimulates immune system (Khodadadi et al., 2013). Garlic extract affects the regulation of immune response through increasing the production of T-cells and increasing the number of NK cells (Shirzad et al., 2012). Garlic possesses various anti-microbial, anti-cancer and anti-fungal effects and improves nutritional indicators, stimulates growth and immune system and is an anti-stress compound with antioxidant effects balancing blood pressure (Fazlolahzadeh et al., 2011). Garlic improves the activity of immune system which directly fights with tumors. Therefore, anyone who is at cancer risk or had involved in cancer should be benefited from the effects of garlic by adding it to his/her food regime. One of three people will be involved in cancer in his/her life. This means that all us are at cancer risk. Nevertheless, the apoptotic effects of garlic on tumor-affected tissues have not been well identified. This study, therefore, aims to evaluate the effect of garlic extract on the expression of Bax and Bcl-Xl genes in rat lymphocytes.

Method of Study

This study selected 30 male Wistar rats (weight=250 ± 20 gr) from the experimental animal breeding center of Jundishapur University of Ahwaz in random. Prior to filling garlics into the percolator, for extracting garlic extract, they were separated, crushed and wetted by 30% of ethanol 96%. The wetted garlics, then, were passed through special sieves and filled into percolator in a homogenous manner. The remainder of the solvent was added to the garlic mass. When the solvent is added to the garlic mass, the percolator valve should be kept in open position in order to completely drain trapped air. As soon as the first drop of garlic extract exited the percolator, the valve was closed and percolation process was continued for 24 to 48 hours while the solvent was completely covered the surface of garlic mass inside the percolator. During this period, swelling is completed and interstitial maceration occurs. After this period, garlic extract was removed from percolator drop by drop while the solvent was continually added from the top of the percolator. This study required two different concentrations. Therefore, the produced extract was diluted and separated at 100mg/ml and 200 mg/ml concentrations. During mice treatment, garlic extract was fed by gavage. This study used duration and concentration parameters to measure gene expression. The blood of all mice groups was mixed in order to dilute and purify RNA. Then, RNA was extracted from blood using the same stages defined for the used Kit. This study uses Yekta Tajhiz RNA extraction kit. In addition, the special CDNA synthesis kits of Yekta Tajhiz Company were used to convert RNA to CDNA. This kit can be used for 50 reactions. According to relevant protocol, CDNA synthesis should be carried out in a cold environment. To conduct Real time PCR reaction and to extract RNA from the cells separated from culture, for 5 to 10 million cells, one ml trizole was added to cell-carrying micro-tubes. The cells, then, were lysed by a sampler at room temperature (25^{oc}) for 5 minutes. In order to completely separate nucleoprotein complex, the obtained homogenized solution was incubated at room temperature for 15 minutes. For one ml trizole, used in the beginning of extraction, 200 ml chloroform was added to the sample and the solution was shaken severely for 15 seconds. The solution, then, was incubated at room temperature for 15 minutes and centrifuged at 12000 g at 4^{oc} for 15 minutes. Then, the samples were gently removed from the centrifuge. A total number of three layers were observed in each micro-tube. The colorless layer above the tube contained RMA. This layer was transferred to a 1.5 ml sterilized micro-tube. To sediment RNA, 500 µl isopropanol was added to the collected solution and the solution was positioned upside down for several times and was kept at freezer temperature for 10 minutes. Then, the samples were centrifuged at 12000g at 4^{oc} for 10 minutes. When centrifuging process was completed, the surface layer of solution was accurately and completely removed and one ml ethanol (70%) was added to the solution in order to wash RNA sediment. The remained solution was then positioned upside down for 5 minutes and was kept at freezer temperature for 10 minutes and was centrifuged at 7500 g at 4^{oc} for 10 minutes. After completion of centrifuge process, the surface layer was accurately and completely removed and the remained ethanol in the micro-tube was removed by air flow (care was taken that RNA not to be excessively dried in order to be easily solved again). Finally, RNA sediment was solved in 40ml water treated by diethyl-pyrocarbonate (DEPC) and was incubated at 56^{oc} for 10 minutes. The purity and amount of extracted RNA were determined using colorimetry.

The primers used in this study were designed by Allel ID. The sequence of the studied mRNA genes was obtained from National Center of Biological Information (NCBI) and the accurate connection of primers to relevant sequence was evaluated. Then, the connection of primers to other sequences was evaluated using Blast in NCBI. Table 1 shows the sequence of the studied primers.

Table 1: the sequence of primers used in this study

1	BAX F	CCAGGACGCATCCACCAAGAAGC
1	BAX R	TGCCACACGGAAGACCTCTCG
2	BCL-XL F	TATTGGTGAGTCGGATTGCAA
2	BCL-XL R	AACGCTCCTGGCCTTTCC

Real time PCR reaction was carried out in The U.S. made ABI thermocycler using ΔΔCt method. Table 2 shows PCR data.

Table 2: PCR data

	Pre denaturation	Denaturation	Annealing	Extension	Post extension
Temperature	94	94	60	72	72
Time	10 min	30 second	15 second	20 second	10 min
		40 cycle			

Eventually, after necessary calculations, required materials were filled in Real time CR micro-tubes at the following volumes:

Table 3: required materials for tests to be done in Real time PCR micro-tubes

material	Volume (µ.l)
Master mix	8
PF	1
PR	1
DNA	2
D.W	8

Finally, 20ml PCR solution was obtained. The micro-tubes were placed in PCR devices in accordance with cycles shown in table 4:

Table 4: PCR cycles

cDNA synthesis	Inactive RT	Denature	Anneal/extend
42	95 c	95 c	60 c
5 minutes	3 minutes	3 seconds	20 seconds
		40 cycles	

Mean CTs, derived from readings associated with samples introduced as triplicate to ABI Stepone, was analyzed using REST-RG V.3 and SPSS. The obtained graphs should be statistically analyzed by specialists who are skilled in statistical analysis software in order to enable the evaluation of test results. Real time PCR graphs were analyzed by t-test and analysis of variance was carried out by SPSS.

Results

This study was conducted on 30 male Wistar rats (weight=250±20gr). They were selected from the experimental animal breeding center of Jundishapur University of Ahwaz in random. The rats were grouped in five groups as follows: two healthy rats groups with 5 rats in each group and four case groups, each with 5 rats, for studying the considered two genes. In order to evaluate the quality of extracted RNA by agarose gel, 10 ml of each sample was loaded on agarose gel (1%) and 18s and 28s bonds were studied. Fig. 1 shows the electrophoresis of several RNA samples.

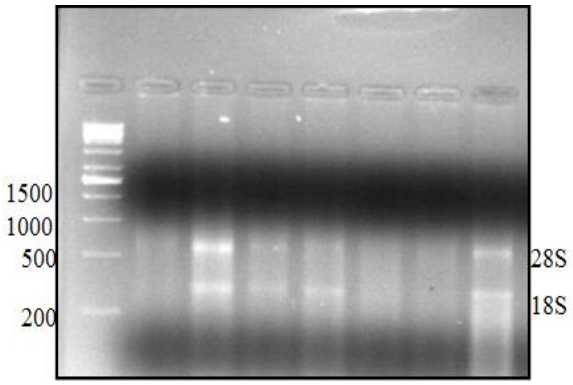
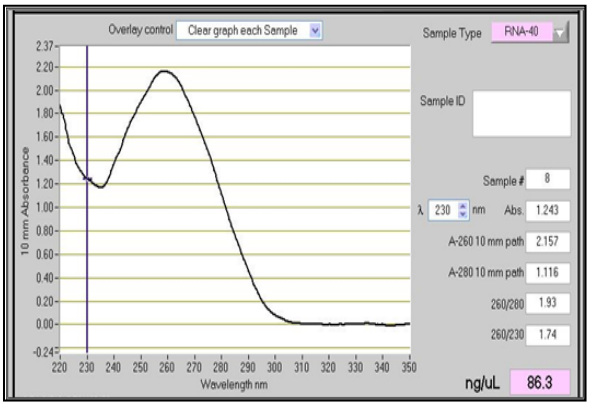


Fig. 1: 18s and 28s bonds derived from electrophoresis gel of extracted RNA samples

To evaluate the quantity of extracted RNA, 1ml of each sample was located inside Nano-drop device. Then, the concentration and light absorbance of them were read in required wavelengths. Graph 1 shows the Nano-drop graph of a sample.



Graph 1: determining the quantity and purity of by spectrophotomet

Mean CTs, derived from readings associated with samples introduced as triplicate to ABI Stepone, was analyzed using REST-RG V.3 and SPSS. The relative expression of BAX and Bcl-Xl was obtained for each group of mice in considered weeks. Table 5 and graph 2 show the expression of BAX and Bcl-Xl. The comparison of P-value between BAX and Bcl-Xl shows a significant difference ($p<0.05$) indicating that the expression of both gens experienced significant changes. This agrees with the considered hypothesis.

Table 5: expression of BAX and BCL-Xl in different groups in considered weeks

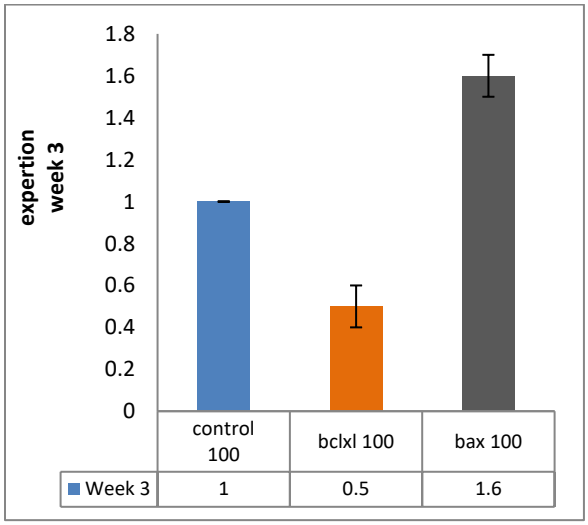
Group	BAX concentration 100	BAX concentration 200	BCL-Xl concentration 100	BCL-Xl concentration 200
Expression in week 3	1.68	2.76	0.5	0.53
Expression in week 8	1.96	3.67	0.25	0.11

According to statistical results, time affects the trend of the expression of both gens ($p<0.05$) and this effect is significant. In addition, concentration has a positive effect on the expression of both gens ($p<0.05$).

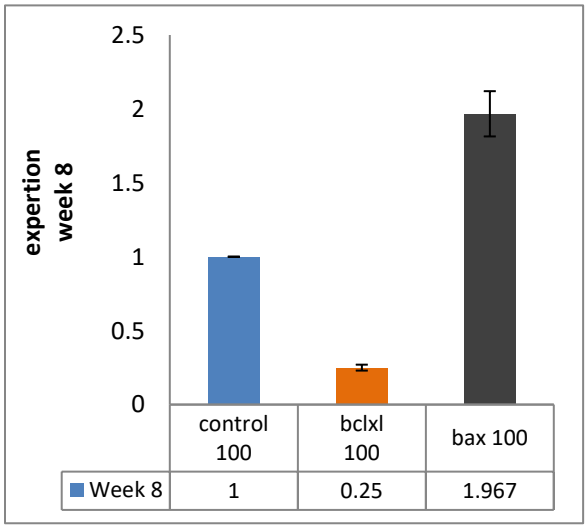
Table 6: expression of BAX and Bcl-Xl in different groups compared to control group in considered week

Gen	Week 3		Week 8	
	100mg	200mg	100mg	200mg
BAX	+1.68↑	+2.76↑	+1.96↑	+3.76↑
Bcl-Xl	-2↓	-2↓	-4↓	-9↓

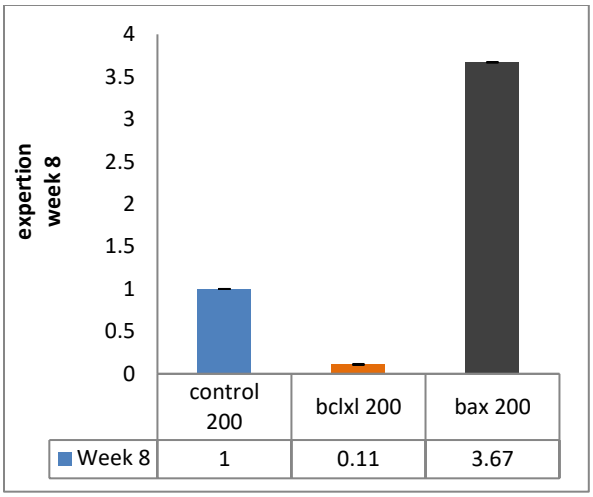
The expression of BAX in both concentrations showed an increase compared to control group in weeks 3 and 8. However, the expression of Bcl-Xl showed a fall compared to control group. This changes are significant ($p<0.05$) (graphs 2 to 5).



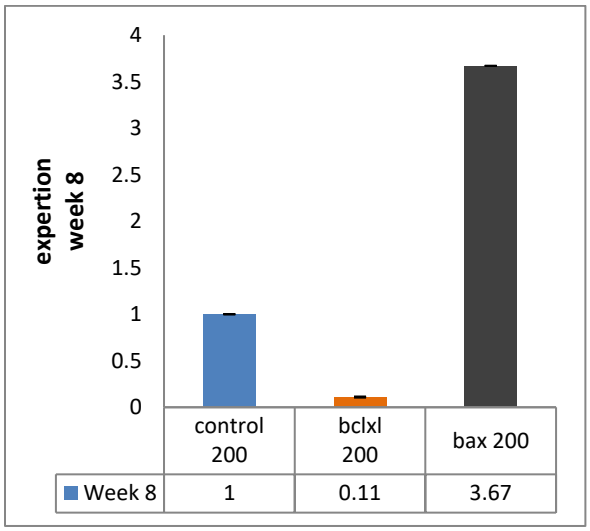
Graph 2: expression of BAX and Bcl-Xl in week 3 (concentration=100mg/ml)



Graph 3: expression of BAX and Bcl-Xl in week 3 (concentration=200mg/ml)

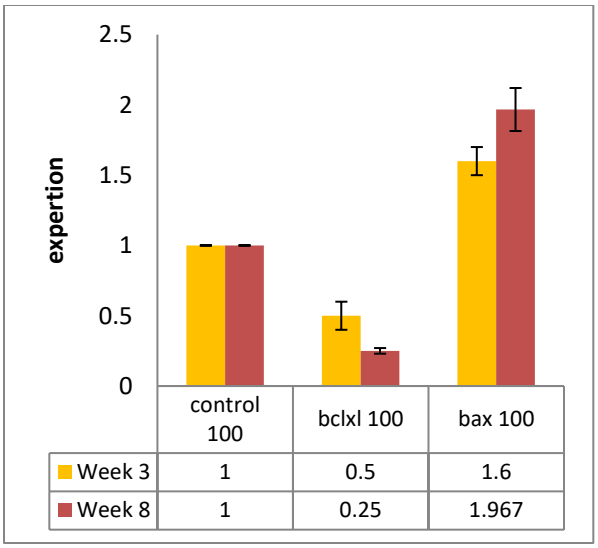


Graph 4: expression of BAX and Bcl-Xl in week 8 (concentration=100mg/ml)

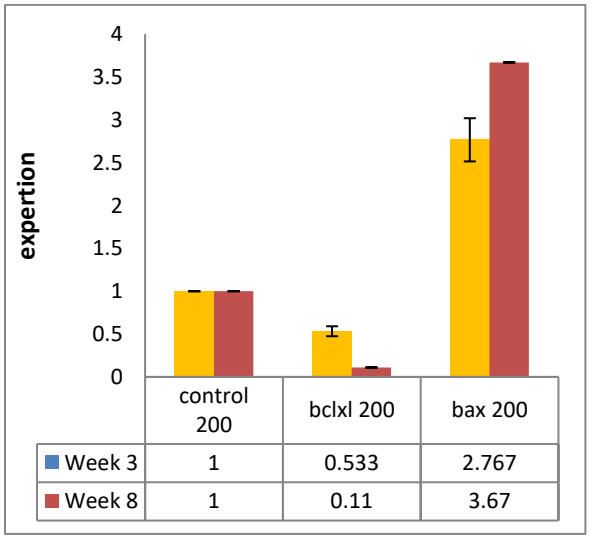


Graph 2: expression of BAX and Bcl-Xl in week 8 (concentration=200mg/ml)

In both weeks, the expression of BAX was higher for the concentration of 200 mg/ml than the concentration of 100 mg/ml while the expression of BCL-Xl was lower for the concentration of 200 mg/ml than the concentration of 100 mg/ml and the differences were significant ($p<0.050$ (graphs 6 and 7). According to results, the maximum expression of BAX and the minimum expression of Bcl-Xl were observed in week 8 and at the concentration of 200 mg/ml.



Graph 6: expression of BAX and BCL-Xl in weeks 3 and 8 (concentration=100 mg/ml)



Graph 7: expression of BAX and BCL-Xl in weeks 3 and 8 (concentration=200 mg/ml)

Discussion

Environmental impacts, including air pollution, and stress, life style and food diet are among factors associated with the rising state of the prevalence of cancer. It has been proved that consuming foods with antioxidant effects plays an effective role in preventing and decreasing cancer (Nguyen et al., 2013; Bayan et al., 2014). On the other hand, despite many therapy strategies including surgery, chemotherapy and radiotherapy, cancer-related mortality has still a high rate indicating the inefficiency of these strategies (Capasso, 2001). Considering these facts, people are interested, in recent years, in using natural products and nutritional supplements with anti-cancer effects. Garlic, which has been considered as a medicinal herb from old times, contains two main antioxidants: flavonoids and sulfur compounds. These antioxidants result in anti-tumor, hypolipidimia, hypocholestrolemia and anti-atherosclerosis effects (Suliman et al., 2001). Garlic strengthens immune system and it is beneficial for some types of cancer. The sulfur compounds of garlic are produced from an amino acid called alliin. During crushing the garlic, this amino acid produces allysine by allinase enzyme (Carpino, 2012). Garlic extract regulates the response of immune system by increasing and reproducing T-cells and increasing the number and activity of NK cells (Hengarther, 2000). The anti-reproduction effects of the s allele of sulfides, derived from garlic, are associated with the conversion of sulfate sulfur in tumor cells, or with the control of anti-proliferation activity of enzymes and signaling factors during the formation of cystine, or with the transformation of di-sulfides available in regulated redox proteins (Soberane et al., 2006). The immune system strengthening effects of garlic and the effects of its different sub-products or metabolites on some types of cancer have motivated us to concentrate on the evaluation of its effects on lymphocytes. Stenimetz's study showed the reduced risk of colon cancer between Americans who consume garlic (Steinmetz et al., 1994). The study of Lau and Morioka showed that garlic regulates immune system response (Backer & Abel, 1995; Moriokaet al., 1993). Different studies have confirmed the effect of garlic extract on regulating immune system response through increasing the reproduction of T-cells and NK cells (Steller, 1995). The study of Shirzad showed that the phenolic compounds and the antioxidant capacity of fresh garlic are moderately higher. The anti-cancer effect of fresh garlic is almost proportional to its phenolic compounds or antioxidant capacity. Therefore, a part of garlic effects may be rooted in these factors (Shirzad et al., 2012). S-aliel sulfides, molecules that are available in garlic, may play a vital role in preventing the groeth of cancerous cells. The molecular mechanism involving in the application of the anti-cancer effect of garlic works in a manner that, s-alliel sulfides can raise the endogenous levels of glutathione peroxides (Cartier et al., 2003; Castillo & Kowalik, 2002). However, since DNA is damaged prior to cancer, it seems that s-alliel sulfides can prevent DNA damage. Apoptosis is a type of planned cell death. It is necessary for many physiological functions such as the preservation of tissue homeostasis and removal of dangerous and damaged cells (Melino et al., 2011). Increased tubular apoptosis has been reported in old rats, compared to younger rats in physiological condition (Ryu & Kang, 2017). Similar to Bcl-2 (anti-apoptotic protein) and Bcl-associated protein x (Bax pro-apoptotic protein), the members of Bcl-2 (B-cell lymphoma 2) play a strong role in regulating the transferring holes of mitochondrion permeability (Milner, 2001). Bax pro-apoptotic protein increases by the reverse regulation of Bcl-2 anti-apoptotic protein. This increases cellular sensitivity to apoptosis.

This study well differentiated the positive effect of garlic extract on the opposite expression of the studied two genes after 3 weeks. The higher expression of BAX and the lower expression of Bcl-Xl show that garlic extract has an effective function in their expression. In week 8, increased expression of both gens was not well differentiated at both 100 and 200 mg/ml concentrations. According to above discussions, the increased expression of BAX

and decreased expression of Bcl-Xl result in the permeability of mitochondrion membrane and, in turn, result in apoptosis. Different studies show that both water-soluble and fat-soluble sulfur compounds have the anti-cancer advantage of garlic (Conti et al., 2003). In 2002, Lin et al reported that the allysine compound of garlic can prevent the endometrial cells of breast and colon from cancer (Lin et al., 2002). In 2003, Finlyie et al suggested that garlic can reduce the risk of some types of cancer (Finley, 2003). In 2009, Lee et al reported that cancer is the main cause of mortality across the world so that it accounts for 13% of mortalities on an annual basis (Lee, 2009). Mellino et al (2011) and Lee et al (2009) introduced garlic as an effective medicinal herb for strengthening immune system. There are different epidemiologic evidences indicating the beneficial effects of the different bioactive organosulfur compounds of garlic, including allile derivatives, against different types of cancer (Melino et al., 2011; Gayathri et al., 2009). Gayathri et al (2009) conducted a study on the effect of garlic extract on apoptosis and reported the increased expression of BAX and the decreased expression of Bcl-Xl for the concentration of 40mg/kg (Gayathri et al., 2009). Kweon et al reported in 2013 that garlic extract shows a preventive effect against cancer. They argued that garlic shows a combination of anti-tumor, controlled growth of cancerous cells and chemo-preventive effects (Kweon et al., 2003). The anti-cancer mechanisms of garlic include controlling the growth of cancerous cells, strengthening anti-toxicity, preventing DNA against caner-induced factors, controlling free radicals, antioxidant effects, regulating cell proliferation and regulating apoptosis and immune system (Finley, 2003). By clocking cellular circle in G2/M phase, garlic compounds decrease the growth of tumor cells (Su et al., 2006). Regarding the expression of BAX and Bcl-Xl, in the best state of this test the expression of Bcl-Xl reduced by 4 folds while that of BAX increased by 32 folds so that if tumor cells subject to such a gene expression, they will experience strong apoptosis. Garlic allicyne inhibits the proliferation of the cancerous endometrial cells of breast and colon and induces apoptosis in the cancerous cells of colon through their cytotoxic effects (Su et al., 2006; Sanz et al., Gayathri et al., 2009). Studies show that consuming selenium-rich plants including garlic decreases the risk of some malignancies and prevents the growth of liver, prostate, cyst, gullet, lung, skin and abdomen tumors (Melino et al., 2011, Gayathri et al., 2009).Diallyl tri-sulfide (DATS) is an organosulfur separated from garlic and shows anti-cancer activity (Jo et al., 2008).

Garlic strengthens macrophages and cytotoxic T-lymphocytes in tumors. It increases the production of lymphokine such as tumor necrosis factor, resulting in tumor destruction, through increasing the activity of NK cells and via interleukin 5 and interferon gama (Austyn & Wood, 1993) The increased rate of these factors, intervening in improved immune system, is associated with the increased apoptosis of tumor cells so that these factors increase by the increase of apoptosis.

In 2013, Capasso reported that garlic-treated cancerous cells show limited cell division in G2 phase so that the growth rate of cancerous cells decreased (Capasso, 2001). In 2014, Gu et al stated that Bcl-2-induced JNK activation generates mediatory mitochondrion signaling paths, transfers a considerable amount of BAX and circulates c-cytochrome. As a result, the activation of JNK and the biochemical mitochondrion transfer contribute to the allysine-induced apoptosis in SKOV3 cells (Xu et al., 2014). The selenium content of garlic plays a strong role in its effects on immune system. The proportion of selenium in garlic depends on the proportion of it is soil. A study on a mice sample showed that garlic activates anti-tumor effect and increases delayed response sensitivity that is an indicator of cell immune. Studies on liver cancerous cells show that garlic has anti-oxidant effect and its components prevent DNA damages (BayIr et al., 2008). BAX and Bcl-Xl have opposite role in controlling apoptosis. This means that as BAX expression increases and Bcl-Xl expression decreases, the apoptosis of cancerous cells increases in practice. According to our observations, the best expression of both genes was observed in week 8 and at the concentration of 200 mg/ml. The results of this study indicate that garlic extract can significantly affect the opposite expression of BAX and Bcl-Xl in practice at determined concentrations. The set of above processes as well as other unknown mechanisms may affect the inhibition of the growth of cancerous cell. This demands further studies. Considering above results, it appears that the high antioxidant capacity of garlic may inhibit the generation and growth of tumors. In addition, fresh garlic shows more efficiency than stale one. This may be associated with the phenolic compounds, antioxidant capacity and existence of sulfur compounds in fresh garlics.

In addition, it seems that higher concentrations of fresh garlic extract may have more effect on the expression of BAX and Bcl-Xl compared to lower concentrations. This study evaluated the effect of garlic extract on apoptosis process in lymphocytes for different durations and concentrations and obtained satisfactory results. According to results, duration and concentration are two main factors for the expression of both genes. Higher durations are not necessarily effective. On the other hand, higher concentrations may more affect the opposite expression of the genes. If garlic is consumed at a balanced level, it will have no harmful effects. Therefore, more accurate experiments are required to determine the accurate consumption concentration. Both duration and concentration, which were evaluated in this study for treating mice, showed significant effect in the expression of BAX and Bcl-Xl in the immune system of the studied mice.

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