Effects of Aged Garlic Extracts on Caspase-3 Activity in Myeloid Cancer Cell Lines

Yıllanmış Sarımsak Ekstraktlarının Miyeloid Kanser Hücre Serisi Üzerine Apoptotik Etkileri

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ABSTRACT

Garlic (Allium sativum) is a spice used for centuries for both condiment and medicinal purposes. Studies have shown that it has antibacterial, antiviral, anti-inflammatory, antifungal, antimutagenic, antioxidant, antiallergic, anti-aging, antitumoral and immunostimulatory activities. Activation of caspase-3 initiates the caspase activation chain, leading to apoptosis. In this study aimed to determine the apoptotic effect of DMSO-containing aged garlic extracts (AGE) on Myoid Cancer Cell lines (HL-60). Flow cytometry method and spectrophotometric caspase-3 activity analyzes were used using human lymphocyte cells as a control group. The highest apoptotic effect on HL-60 cell lines was observed in aged garlic extracts at a concentration of 12.5 mg/mL (10.9%). It was determined that caspase-3 activity in HL-60 cell lines increased by 1.28 to 3.02 times compared to lymphocyte cells. It was concluded that aged garlic extracts triggered apoptosis by increasing the activity of caspase-3 in HL-60 cell lines. It is thought that the aging of garlic has an anticancer effect and additional studies on this subject may bring new perspectives to cancer treatment.

Keywords: Apoptosis, Myeloid HL-60 cell line, Aged garlic extract (AGE), Caspase-3 activity

ÖZ

Sarımsak *(Allium sativum*) yüzyıllar boyunca gerek çeşni ve gerekse medikal amacıyla kullanılan bir baharattır. Yapılan çalışmalarla antibakteriyel, antiviral, antiinflamatuar, antifungal, antimutajenik, antioksidan, antialerjik, yaşlanmayı azaltıcı, antitümoral ve immünositimülatör aktiviteleri olduğu belirlenmiştir. Kaspaz-3'ün aktifleşmesi kaspaz aktifleşme zincirini başlatarak apoptozu gerçekleştirir. Bu çalışmada DMSO'lu yıllanmış sarımsak ekstraktlarının (AGE) Miyoid Kanser Hücre hatları üzerine (HL-60) apoptotik etkisi belirlenmesi amaçlandı. İnsan lenfosit hücreleri kontrol grup olarak kullanılarak akış sitometrisi yöntemi ve spektrofotometrik kaspaz-3 aktivitesi analizleri kullanıldı. HL-60 hücre hatlarına üzerine en yüksek apoptotik etki 12,5 mg /mL konsantrasyondaki yıllanmış sarımsak ekstraktlarınında gözlendi (%10,9). Lenfosit hücrelerine göre HL-60 hücre hatlarındaki kaspaz-3 aktivitesi ise 1,28 ile 3,02 kat oranında arttığı tespit edildi. Yıllanmış sarımsak ekstraktlarının HL-60 hücre hatlarında kaspaz-3'ün aktivitesini arttırarak apoptozu tetiklediği sonucuna ulaşıldı. Sarımsağın yaşlandırılmasının antikanser etkisi olduğu ve bu konuda ilave çalışmaların kanser tedavisine yeni bakış açıları kazandırabileceği düşünülmektedir.

Anahtar Kelimeler: Apoptoz, Miyoloid HL-60 hücre hattı, Yıllanmış sarımsak ekstraktı (AGE), Kaspaz-3 aktivitesi

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INTRODUCTION

From its earliest history, human beings have used various plants not only as food but as alternative medicines. Garlic (*Allium sativum*), which is used raw or cooked, is one of these plants.[1] It is understood from the historical sources that garlic was given to workers in the fight against diseases such as cholera and plague in Central Asia and during the construction of the pyramids in ancient Egypt.[2]

Dry tooth garlic; in addition to 65% water, it contains carbohydrates (especially fructose), sulfur compounds, amino acids, fiber, vitamin B-complex vitamins, protein, magnesium, sodium, iron, many organosulfur compounds and more.[3,4] It has been suggested that these components are responsible for the strong biological activities of garlic. It has been reported in various investigations that garlic has antioxidant, antiviral, antimicrobial, antifungal, antibacterial and antiplatelet activity.[5] Several studies have observed an inverse relationship between increased garlic intake and the risk of developing cancer.[6] Garlic has been reported to reduce the formation of colon, respiratory tract, muscle, liver, breast, prostate, stomach, bladder and kidney tumors.[7-10] It has been reported in several studies that the number of sulfur and allyl groups in the components of garlic also affects anticancer activity.[11]

Apoptosis is a necrosis-distinct form of cell death that occurs in both the physiological and pathological milieu, beginning with embryonic development and continuing to the death of the living being.[12] It performs apoptosis by initiating caspase waterfall by activation of passive caspases which are important mediators of programmed cell death (apoptosis) in the cell receiving the death signal. Among them, as a death protease, caspase-3 catalyzes the specific cleavage of many essential cellular proteins.[13]

In *in vivo* studies, it has been determined that with the increase in the aging time of the food, the food increases anti-cancer and antimetastatic.[14]

Although there are many studies on the anticancer activity of raw or cooked garlic, there are no studies on the anticancer activity of aged garlic extracts. In addition, since there is no study on the effect of garlic on HL-60 lines, it was aimed to determine the effect of aged garlic extracts on HL-60. In this study we investigated the effect of AGE supplements on caspase-3 to determine anticancer effect in HL-60 myeloid cancer cell line.

MATERIALS AND METHODS

Chemicals

Fetal Bovine Serum (FBS), L-Glutamine and RPMI 1640 were supplied from GIBCO (Paisley, England). Ethidium Bromide, Penicillin and streptomycin and Acridine Orange were products of Sigma (St. Louis, MO, USA). Trypsin/EDTA solution and Phosphate-buffered saline (PBS-Dulbecco) were supplied by Biochrom AG (Berlin, Germany). Ficoll (Lymphoprep) was supplied by Nycomed Pharma (Oslo, Norway).

Preparation of Aged Garlic Extracts

Aged garlic extracts were obtained from ready SARMEX AGE. These extracts were prepared with one kilo of garlic is peeled and crushed thoroughly. 1 liter of water and 5 pieces of lime are mixed into the crushed garlic put into a jar and 1 liter of vinegar is added into this mixture. After mixing these, the jar is tightly capped and kept in a dark and cool place for 6 to 10 months. Ready-made garlic extracts were sterilized by filtration through membrane filters with 0.2 pore mesh (Sch Schleicher & Schuell FB 030/3 0.2 m /7 bar max). AGE extract was prepared in 1+9 dilution with DMSO.

HL-60 Myeloid Cancer and Lymphocytes Cell Lines

Control cell cultures were performed by lymphocytes isolated from the blood of healthy individuals.[15] HL-60 Myeloid Cancer Series Cell Cultures were obtained from KTU Faculty of Medicine Hematology Laboratory.

Cell Culture and Incubation of AGE Extracts

Aged garlic extracts (AGE) at concentrations of 0, 12.5, 25 and 50 mg /mL were incubated in isolated lymphocytes and HL-60 myeloid cancer cells in RPMI 1640 containing 10% fetal bovine serum, 1% penicillin and streptomycin for 72 hours at 37^{o}C under 5% CO_2 pressure. Flow cytometric analyzes and caspase-3 activity assay procedures performed in these cultures with all extract preparation and cell culture addition were performed in duplicate for each concentration.

Cell Viability

Cell pellets after washing twice with PBS; fluorescent dye mixture containing acridine orange and ethidium bromide (AO/EB) was added to the cells. Cells were visualized under a fluorescent microscope at 510 nm by placing a drop of the mixture on a microscope slide.[16] All experiments were done in three times.

Determination of Apoptosis by Flow-Cytometric Fluorescence Analysis

For the DNA content of the stained nuclei; flow cytometry (Coulter Epics Elite ESP) was used. The distribution of the DNA content was determined using the G1, S and G2/M phases. Cells with DNA content less than G1 were distributed into pre-G1 (hypodiploid cells) and expressed as the apoptotic phase.[17] All experiments were performed in three times.

In Vitro Caspase-3 Assay

Using Biosource International USA, Catalog # CPP32-KHZ 0022 colorimetric protease assay kit, the CPP32/caspase-3 activity of the extracts was determined. All experiments were done in three times.[18]

Statistical Analysis

The values obtained were expressed as standard deviation and arithmetic mean. Compliance with normal distribution was measured with the Kolmogorov-Smirnov test, and differences between groups were measured with Student's t-test and One-way ANOVA.

Aspect of the Research Ethics

Since the research study titled “Apoptotic Effects of Aged Garlic Extracts on Myeloid Cancer Cell Series” was conducted before 2015, ethics committee approval was not obtained. The study was performed *in vitro* in cell culture medium, and no live animals or human subjects were used.

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RESULTS AND DISCUSSION

Apoptosis by Flow Cytometric Cell Cycle and DNA Analysis Determination

Aged garlic extracts (50, 25, 12.5 and 0 mg /mL, respectively) were added empirically to the lymphocytes cells as a control grup in decreasing concentrations. According to the flow-cytometric cell cycle and DNA analysis histograms at the end of the cell culture study made to investigate the effect of garlic extracts on lymphocytes (Table 1).

Table 1. Cell Cycle and Apoptosis Effects of Different Concentrations of AGE Extracts in Lymphocytes (N=3)

Parameter	Extract Concentration mg/mL			
Extract No.(mg/mL)	50	25	12.5	0
G_0/G_1(%)*	98.7±0.09	98.8±0.02	99.5±0.01	96.0±0.04
S (%)	1.3±0.01	1.2±0.00	0.5±0.00	3.8±0.01
G_2M (%)	0.0±0.00	0.0±0.00	0.0±0.00	0.2±0.00
Apoptosis (%)	Non observed	Non observed	Non observed	Non observed
Viability (%)	100	100	100	100

**G0=Gap0/Resting phase, G1= Gap1, S= Synthesis and G2=Gap2 are interphase, M= Mitosis period of cell cycle); p < 0.001*

At the highest dose (50 mg /mL), G_0/G_1 ratio was 98.7%, G_2M 0%, and S phase 1.3%. While it was determined that mitosis was blocked, apoptosis could not observed.

In the second highest dose (25 mg /mL), it was determined that G_0/G_1 ratio was 98.8%, G_2M was 0%, and S phase was 1.2%. In the third highest dose (12.5 mg /mL), G_0/G_1 ratio was 99.5%, G_2M 0%, and S phase was 0.5%. While it was determined that mitosis was blocked in both concentrations, no apoptosis was detected. In the 4th dose (0 mg /mL); G_0/G_1 ratio was 96.0%, G_2M 0.2%, S phase was 3.8% and apoptosis was not detected. According to these results, AGE suppressed normal cell cultures. It was observed that garlic extracts inhibited normal lymphocytes at the G_2M stage.

Table 2. Cell Cycle and Apoptosis Effects of Different Concentrations of AGE Extracts in HL-60 Cell Lines (N=3)

Parameter	Extract Concentration mg/mL			
Extract No.(mg/mL)	50	25	12.5	0
G_0/G_1 (%)*	20.5±0.01	36.6±0.03	12.6±0.01	52.0±0.03
S (%)	55.5±0.03	50.5±0.01	68.8±0.02	3.8±0.01
G_2M (%)	24.0±0.00	13.0±0.00	18.5±0.00	10.0±0.00
Apoptosis (%)	4.8±0.00	4.5±0.00	10.9±0.00	Non observed
Viability (%)	84.3±0.00	83.4±0.01	10.9±0.03	100

**G0=Gap0/Resting phase, G1= Gap1, S= Synthesis and G2=Gap2 are interphase , M= Mitosis period of cell cycle); p < 0.001*

According to the data in Table 2, garlic extracts (50, 25, 12.5 and 0 mg /mL, respectively) were added empirically to HL-60 cell lines in decreasing concentrations.

According to the flow-cytometric cell cycle and DNA analysis histograms performed as a result of the cell culture study conducted to investigate the effect of garlic extracts on HL-60 cell lines; the 1st dose is the highest dose with a concentration of 50 mg /mL. In this concentration, G_0/G_1 ratio was 20.5%, G_2M 24% and S phase 55.5%. It was observed that garlic extracts put 4.8% of the HL-60 tumor cell line into apoptosis. The DNA index (DI) of the apoptotic peak was determined to be 0.843. In the second dose (25 mg /mL), G_0/G_1 ratio was 36.6%, G_2M 13%, and S phase was 50.5%. At this dose, AGE extracts induced 4.5% apoptosis of HL-60 tumor cells. The DNA index (DI) of the apoptotic peak was measured as 0.834.

In the 3rd dose (12.5 mg /mL), G_0/G_1 ratio was 12.6%, G_2M 18.5%, and S phase 68.9%. It was observed that AGE extracts at this dose dragged HL-60 tumor cells to apoptosis at the highest rate (10.9%).

In the 4th dose (0 mg /mL), 52% of G0/G1, 10% of G2M, and 38% of S phase were detected. No apoptosis could be observed on HL-60 tumor cells of AGE extracts at this dose.

According to the results of these data; AGE extracts appear to have an apoptotic effect on the HL-60 tumor cell line. The highest apoptotic effect was observed at the 3rd dose (12.5 mg /mL).

Caspase Activity Results

Caspase-3 activity results obtained by incubation of control cells (lymphocyte series) and HL-60 myeloid series cells with AGE extracts with DMSO at various concentrations are given in Table 3.

Table 3. Effects of Different Concentrations of AGE Extracts on Caspase-3 Activity Fold Increase in Lymphocytes and HL-60 Cell Lines (N=3)

Parameter	Extract Concentration mg/mL				
	100	50	25	12.5	0
Increase in Caspase-3 Activity In Lymphocytes*	1.45±0.01	1.18±0.01	1.65±0.01	1.11±0.01	1.06±0.02
Increase in Caspase-3 Activity In HL-60*	4.38±0.00	2.14±0.03	1.21±0.02	1.43±0.01	0.86±0.03
Fold Increase of Caspase-3 Activity**	3.02±0.02	1.81±0.01	1.34±0.01	1.29±0.01	0.84±0.01

* $p < 0.001$ ** fold increase

According to the results after 72 hours of cell culture incubation of HL-60 cancer cell extracts with Control (lymphocyte) cells incubated with AGE extracts. there was an increase in activity (1.29-3.02-fold) observed in myeloid HL-60 series with garlic extracts (Table 3).

The health effects of allium vegetables come from sulfur-containing components and very rich content such as flavonoids. selenium. oligosaccharides. arginine.[19]

Allium vegetables are known to have many potential medical capacities. especially cancer. apart from being consumed in the diet worldwide. Epidemiological studies have also stated that allium vegetable consumption has the potential to have a protective effect against cancer. Increased garlic intake significantly reduced the risk of developing cancer.[20. 21]

Garlic and its sulfur compounds have been reported to suppress tumors against many cancers such as lung. colon. breast. skin. esophagus cancers in experimental studies. [22-24]

Although there is no certainty about the anticancer effect mechanism of garlic some mechanisms have been suggested. Among these mechanisms are suppression of bioactivation of various carcinogens. blockage of N-nitroso compound (NOC) formation. reduced cell proliferation with enhanced DNA repair. triggering apoptosis in cancer cells.[25]

Fukushima et al. (2001) attributed the anticancer effect of garlic to the orgonosulfur compounds in its content. In their study on *TA100 Salmonella Typhimurium*. they determined that the fat-soluble diallyl disulfide (DADS) and water-soluble S-allylcysteine (SAC) contained in the garlic have anticancer effects by delaying mutagenesis.[26]

Greenblatt et al. (2006) determined that garlic has an anticarcinogenic effect on hormone-sensitive cancers. especially breast and prostate cancer. in their in vitro and in vivo studies. They suggested that garlic achieves this effect by blocking cytochrome P450 enzymes. which trigger the activation of carcinogens and/or by increasing cytochrome P450s. which catabolize carcinogens to less reactive intermediates.[27]

Another anticancer mechanism is related to garlic's ability to trigger apoptosis. This theory has been supported by some studies that ajoene. DADS. dialylsulfide (DAS). allicin. and S-allylmercaptocysteine SAMC compounds found in garlic have apoptotic effects on cancer cells.[28-30]

Lamm et al. (2001) determined that garlic suppresses the growth of 180 types of tumor cells in vivo and in vitro. and that the most common carcinogen. UV radiation. is inhibited by garlic.[31]

De Greef et al. (2021) emphasized that these sulfur-containing compounds such as allicin. diallyl sulfide. diallyl disulfide. diallyl trisulfide. alliin. S-allylcysteine and S-allylmercaptocysteine in the structure of garlic affect various stages of carcinogenesis through some different mechanisms. They suggested that the possible anticancer action mechanisms of these derived phytochemicals are to alter mitochondrial permeability. inhibit angiogenesis. potentiate antioxidative and proapoptotic properties. and regulate cell proliferation.[32]

Caspase-3 is an effector caspase that forms the junction point in the pathway of apoptosis in various pathways (Fas L death ligand or any apoptotic stimulus via bcl-2).[13]

Oommen et al. (2004) reported that allicin triggered the formation of apoptotic bodies by triggering cleavage of poly (ADP-ribose) polymerase and activation of caspase 3.8.9. and inhibited the growth of cancer cells of murine and human origin.[33]

In their study Gore et al. (2021) determined that due to allin and allicin in the structure of garlic. it showed breast. prostate. colon. hepatic and cervical cancer cell line exhibits anticancer and anticancer stem cell activity. Therefore. they stated that garlic can be used as a natural supplement. especially in patients receiving chemotherapy.[34]

Su and colleagues (2006) treated with garlic extracts in a study colo 205 cell series; garlic was observed to reduce percentange of viable cells by triggering apoptosis. increasing Bax cytochrome c and caspase-3 and decreasing the level of Bcl-2. In this study. they also determined that raw garlic extracts reduce mitochondrial membrane potential and increase the activity of gene expression and caspase-3.[35]

Iciek et al. (2011) determined that the diallyl trisulfide (DATS) have the highest biological activity in HepG2 cells; which increases H_2O_2 formation. decreases thiol level. inhibits cell proliferation. and provides the greatest induction of caspase 3 activity in HepG2 cells.[36]

Knowles et al. (2001) showed that the anticancer activities of the garlic organosulfur compounds were; they vary in the number of allyl and sulfur groups present in the solution. depending on their concentration. cell density. the stability of the composition. solubility in water and oil. According to them the alil group is required for maximum development inhibition. In one study. they found that although 100 pmol/L of DADS decreased tumor cell proliferation by 90%. dipropyldisulfide (DPDS) in the same molarity did not delay development.[37]

According to Srivastava and his friends (1997); anticancer effects of garlic depend on the number of allyl and sulfur groups in the antitumoral effects of the organosulfur compounds of garlic. Organosulfur compounds such as DAS, DADS, DATS containing allyl have important properties when propyl-containing organosulfur compounds in the lungs; both in the lung and in the lungs. increase the activity of GST and lack anticancer capacities. The number of sulfur groups affects the organ selectivity and chemical protection of allyl-sulfur compounds. For example. DATS inhibits Benzo (a) pyrene-induced gastric neoplasm 2.9 times more potent than DAS.[38]

Claudia et al. (2011) reported that the process leading to apoptotic cell death may occur following the cessation of the mitotic effect occurring at early sites in cancer cells without damaging the healthy cells of the organosulfur compounds found in garlic.[39]

In some *in vivo* studies they determined that the aging of the food changes its bioactivity due to a change in the content of the food.[14]

In a study by Khosravi and Razani (2021). they determined that the amount and type of polyphenols contained in soybean products. which are fermented and aged. are highly altered in their bioactivities such as anti-cancer. anti-diabetes. antiobesity. which are on health. since both the amount and type are altered by the glucosidase enzyme of the microorganisms.[40]

There are very limited studies on the health of aged garlic extracts. In addition. since there is no study on the effect of garlic on HL-60. In this study. we studied the apoptotic effect of aged garlic in cell cultures without isolating any component as a target. Since ethanol has a cytotoxic effect on the cell. DMSO was used as a solvent in less than 0.1 % of our study.

In our study. garlic extracts are shown in two ways that apoptosis occurs. The first is flow cytometric cell cycle and DNA analysis. The second is the determination of caspase-3 activity in cytosol fractions obtained from cell cultures.

In the first study. aged garlic extracts showed an apoptotic effect on the HL-60 tumor cell line with the method of flow-cytometric analysis. The highest apoptotic effect was observed in the 12.5 mg /mL of AGE. Therefore. this dose is the result of the optimal dose in cell culture studies. It has been observed that garlic extracts inhibit normal lymphocytes at the G_2M stage. The results of caspase activity were also found to support flow-cytometric findings. In the myeloid HL-60 cell series. an increasing caspase-3 activity (1.33-3.02 fold) was observed with aged garlic extracts.

CONCLUSION AND RECOMMENDATIONS

The results of this study show that aged garlic extracts increase apoptosis of HL-60 cell lines. Further investigation into which active substances or substances (organosulfur compounds. polyphenol substances. flavonoids. selenium. tellurium etc.) in the garlic induce apoptosis can be examined. On other possible anticancer mechanisms of garlic extracts can be investigated in other cancer cell cultures.

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