

Comparison of Fresh and Aged Garlic Extracts in Terms of Antioxidative Power and Allicin Content

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Abstract

The different nutrients in garlic provide numerous health benefits. As the most active and important bioactive compound found in garlic, allicin offers antimicrobial, anticancer, antioxidant, cholesterol-lowering and cardiovascular-preventive effects. In this study, fresh and aged (dried) garlic extracts were compared in terms of the active ingredient allicin, constituents, and antioxidative properties. Ultrasonic apparatus was used for extraction because of its higher speed and shorter extraction time, improved quality of the extract with respect to antioxidative properties, and the possibility of extracting heat-sensitive compounds. The results showed that the highest allicin content (0.27%) was observed in the fresh garlic extract. Also the largest amounts of phenolic compounds (0.311 mg gallic acid equivalent) were observed in the fresh garlic extract. The highest inhibition rate (50% at 1500 ppm) was that of the fresh garlic extract. The results showed that the allicin content and antioxidative properties of fresh and dried garlic extracts were significantly different. Therefore, fresh garlic extract can be a good alternative to use in functional food.

Keywords: Garlic, Ultrasonic, Antioxidant, Allicin

Introduction

According to recent studies, diets containing fruits, vegetables, and fibres (of plant origin) can prevent life-threatening diseases (heart diseases, obesity, and diabetes) or reduce their risks. Research on the relationship between food of plant origin and human health indicates that herbal bioactive compounds have beneficial properties [1]. Cancer and cardiac diseases are the main causes of mortality in Iran and the world [2]. It has been estimated that one-third of cancer deaths can be prevented in industrialized countries through developing appropriate formulations. Making changes in diets and lifestyles are practical measures to considerably reduce the incidence of cancer [3]. Epidemiological studies have established a link between food intake of plant origin and a range of health benefits. The most important plant bioactive compounds present in the diet with health benefits are glucosinolates (sulphur-containing compounds found in plants of the garlic family), terpenoids (carotenoids, monoterpenes, and phytosterols) and various groups of polyphenols (such as anthocyanins, flavones, isoflavones, and ellagic acid) [4,5].

Cooked or raw, garlic (*Allium sativum* L.) can be mixed with different foods and offers countless therapeutic benefits, especially in traditional medicine. Also known as *A. sativum*, garlic belongs to the Alliaceae family and is considered a gramineous herb with an onion compound containing several small bulbs [6].

In addition to vitamins A, B, and C, garlic contains effective drugs such as volatile oils, mucilages, mineral salts, alliin, allicin, alliinase enzyme, and inulin. Garlic has antioxidant properties and exhibits therapeutic effects on some cancers [7]. In addition, garlic has antimicrobial, antibacterial, antiviral, antifungal effects on cardiovascular and immune systems [8,9].

Most medicinal and health properties of garlic are attributed to allicin-containing the thiosulfinate functional group. This compound is not naturally produced in the plant, but is a secondary product derived from the destruction of a kind of cysteine sulfide called alliin, is as the most important enzyme in this plant family, alliinase reacts with alliin after the destruction of garlic plant tissues to produce allicin, a highly unstable compound, and pyruvic acid. Given that the amount of this composition is dependent on environmental factors and processing conditions, these parameters can be tailored to

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prevent the reduction of this valuable compound [7]. Allicin and thiosulfates trap free radicals and inhibit lipid peroxidation and platelet aggregation, stimulate fibrinolysis, and reduce the amount of blood lipids [6].

Khanam *et al.* (2004) reported that allicin alone had no inhibitory effect on cancer cell growth, and the inhibitory effects of garlic pertain to the breakdown of alliin into allicin. These effects had a direct relationship with intracellular glutathione consumption. In other words, the lower the level of blood glutathione is (the more intracellular glutathione is consumed), the greater the inhibitory effect of allicin on cancer cell growth will be. Allicin exhibits its protective power against cancer through stimulating various mechanisms such as blocking the formation of nitrosamine (one of the most important carcinogens), preventing cancer cells growth and spread, and boosting the immune system. Organosulfur compounds prevent liver cancer through blocking the formation of non-nitrosamine carcinogens such as aflatoxin B₁. Moreover, diallyl disulfide is an effective inhibitor of colon cancer cell growth and regular consumption of garlic reduces colon cancer development [10].

A study on garlic showed its antioxidative properties in preventing age-related and cardiovascular diseases. Raw garlic extract was effective in improving oxidative stress and decreasing blood lipids in rats, which was attributed to the antioxidative properties of garlic. Antioxidative properties of garlic oil protect the stomach against ethanol. The free radical scavenging activity and high phenolic content in aqueous extracts of garlic result from the presence of allicin as an active ingredient. Researchers have shown that the therapeutic effects of garlic on some cancers are due to its antioxidative properties. Gorinstein *et al.* reported that heat had substantial effects on antioxidative properties of garlic [11].

According to Indu *et al.*, garlic oil had important antimicrobial properties in addition to allicin, and other authors showed that garlic oil had excellent antimicrobial activity at different concentrations on various bacterial strains such as *Escherichia coli* [12]. Fratianni *et al.* reported that garlic oil had excellent antimicrobial against *Listeria monocytogenes*, *Salmonella enteritidis*, *E. coli*, and *Staphylococcus aureus* [13]. Ankri and Mirelman (1999) stated that allicin (thio-2-propene-1-sulfinic acid-S-allyl ester) was the most important active ingredient in fresh, chopped garlic and had various antimicrobial properties against a wide range of Gram-positive and Gram-negative bacteria (*Escherichia*, *Salmonella*, *Staphylococcus*, *Streptococcus*, and *Klebsiella*). Allicin influences the metabolic activity of cysteine protease through reacting with the thiol group of different enzymes such as alcohol dehydrogenase and RNA polymerase [14].

Ultrasonic extraction is a simple technique that can be a good alternative to traditional extraction methods. Increased the extraction speed and efficiency is one of the main advantages of the ultrasound method. Moreover, the ultrasound technique can reduce the operating temperature and allow extraction of heat-sensitive compounds. In this study, fresh and aged garlic (6 months) extracts were compared in terms of allicin content, other constituents, and antioxidative properties. Ultrasonic apparatus was used for extraction because of its higher speed and shorter extraction time, improved quality of the extract with respect to antioxidative properties, and possibility to extract heat-sensitive compounds.

Material and Methods

Materials

Garlic cubes were purchased from the local market of Rasht (Iran). The garlic was washed and peeled. Before extraction, the garlic was ground in a blender and the resulting particles were immediately used for extraction.

Chemicals, Reagents, and Instruments

2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical, Folin-Ciocalteu reagent, and the standard substances including gallic acid were purchased from Sigma (Sigma Aldrich GmbH, Sternheim, Germany), and methanol was prepared from Merck KGaA (Darmstadt, Germany). An ultrasonic bath process (purchased from Elma, Germany) was used to perform the ultrasonic-assisted extraction.

Methods

Extraction

Extracts of fresh garlic and aged garlic were performed according to the Loghmanifar *et al.* (2020) method. Cloves taken from fresh and dried garlic were separately peeled, washed, and turned into uniform and small pieces using a blender. They were then mixed with distilled water (ratio 1:2) and placed in an ultrasound water bath model (Elma, Germany) (40 Watt power and 37 Hz) at 30 °C for 10 min. The obtained solution was filtered with Whatman paper No. 1 and then after freezing at -80 °C, powdered in a freeze drier [15].

Determination of Total Phenolic Content

The content of total phenolic compounds in the garlic extracts was determined using the Folin-Ciocalteu reagent [16]. Absorbance was measured at 765 nm using a UV-VIS spectrophotometer (Shimadzu, Japan). The content of phenolic compounds was expressed as gallic acid equivalents (GAE) per dry weight of extract using the gallic acid calibration curve. All experiments were performed in three replicates.

DPPH Test

The free-radical scavenging capacity of garlic extracts was determined as described by Fenfang *et al.* (2017). Different amounts of the extract were mixed with methanol (95%) and DPPH in order to obtain different final concentrations of the extract. After 60 min at room temperature and in darkness, the absorbance was measured at 517 nm using a UV-VIS spectrophotometer (Shimadzu, Japan). All experiments were performed in three replicates. Radical scavenging capacity (%RSC) was calculated from the following equation [17]:

$$\%RSC = \frac{A_{\text{blank}} - A_{\text{sample}}}{A_{\text{blank}}} \times 100 \quad (\text{Equation 1})$$

IC₅₀ is a parameter commonly used to compare the free radical scavenging activity of different extracts. IC₅₀ refers to the half-maximal inhibitory concentration of the extract against the free radical in the reaction medium [18]. The amount of IC₅₀ Obtained using Graph Pad Prism8 software.

The FRAP Assay

The reducing capacity of an extract is an important indicator of its anti-oxidative activity [19]. A volume of 0.02 mL of the extract (1 mg/mL) was mixed with 1 mL of a FRAP working solution (including 25 mL sodium acetate, 2.5 mL 2,4,6-Tris (2-pyridyl)-s-triazine, and 2.5 mL iron chloride). The mixture was kept for 5 min at room temperature and its absorbance was read at 595 nm. The reducing activity of the extract was calculated using a standard curve (in µmol iron against mg dry weight of the extract) [20-21].

Quantitative Determination of Allicin

The quantity of Allicin in the optimum extract was measured by High-Performance Liquid Chromatography (HPLC) based on the method described by British Pharmacopoeia (2015) [22]. The internal standard was used of butyl parahydroxy benzoate and column size was 0.25 m and 4 mm diameter. The mobile phase consisted of mixing 60% methanol and 40% anhydrous formic acid solution (v/v) at a flow rate of 0.7 ml/min and detection done with Spectrophotometer at 254 nm.

$$\%allicin = \frac{S1 \times C2 \times (Vis/Vt) \times 8.65}{S2 \times C1 \times (Vs/Vt)} \times 100$$

(Equation 2)

S1: area of the peak corresponding to allicin

S2: area of the peak corresponding to butylparahydroxybenzoate

C1: sample concentration

C2: internal standard concentration

Vis: internal standard volume

Vs: sample volume

Vt: total volume

Identification of the compounds in the optimum garlic extract by using gas chromatography-mass spectrometry (GC-MS).

This was done based on the criteria in chromatography (retention time) and spectrometry (mass spectral interpretation, comparison with library information and standard compounds). Chromatographic and spectroscopic data were collected using a gas chromatograph (Thermo Scientific Trace OQ301) attached to a DSQ mass spectrometer (electron impact ionization, eV 70; Thermo Scientific) [23].

Results and Discussion

The results of total phenolic compounds in mg gallic acid per gram dry weight of the extract are presented in Table 1. The amount of phenolic compounds was higher in fresh garlic than in aged garlic, and a significant difference were observed between them in this respect ($p < 0.05$).

The results regarding the inhibition rate of DPPH free radical of the fresh and aged garlic extracts are presented in Figure 2. There was a significant difference between the two extracts in this respect ($p < 0.05$). The inhibition rate of the fresh and aged garlic extracts reached 50% at 1500 ppm and about 5000 ppm, respectively ($p < 0.05$).

The high inhibition rate of DPPH free radical in this method is due to the higher contents of phenolic and tocopherol compounds. Inhibitory power of various extracts largely depends on the number and position of their hydroxyl groups and on the molecular weights of their phenolic compounds [24]. As in other studies, the results of this research showed that treatments with higher phenolic compounds had greater free radical scavenging activity [25].

The HPLC chromatogram of allicin for fresh and aged garlic extracts are shown in Figures 4.

The percentages of allicin calculated based on the areas under the peaks are listed in Table 3.

Comparison of the results showed that the allicin content was 0.27% in the fresh garlic extract and 0.086% in the aged garlic extract ($p < 0.05$).

The present study indicated that the fresh garlic extract had higher antioxidative power than the aged garlic extract, which can be partly contributed to its sulphur-containing constituents. Milner (2001) showed that the antioxidative activity of some *Allium* species depended on their sulphur-containing constituents and their precursors [26]. *In vitro* studies have introduced garlic and its constituents as anti-carcinogenic materials that also reduce blood lipid levels [27]. The strong garlic smell causes gastrointestinal disorders and anemia [28-7]. These events are caused by allicin and other water-soluble sulphur-containing components produced from it through chemical reactions [29-7]. Yoshida *et al.* (1984)

showed that the aqueous ethanol extract of aged garlic lacked the pungent garlic smell, did not lead to these events, and could be used with complete certainty of its safety in medical experiments [30]. Aged garlic extract has the potential to boost the immune system and antioxidative properties, improve peripheral blood flow, increase production of natural killer cells, prevent reduced activity of the immune response in patients with advanced cancer, activate the transcription factor, and protect DNA against free radicals [31]. The mechanism of antioxidative action of aged garlic extracts is accompanied by phagocytic action of reactive oxygen species and increased levels of cellular antioxidant enzymes, superoxide dismutase, catalase, glutathione peroxidase, and glutathione in cells [32]. Moreover, the dried garlic used in the present experiment had been stored for several months. It seems that its active ingredients and even its effective enzymes that give it the antioxidative properties, in addition to its pungent smell, had decreased compared to fresh garlic. These effects probably resulted from the lower contents of allicin and other water soluble sulphur-containing compounds in the dried garlic.

The results related to the reducing power of the extracts on trivalent iron in micromole iron per gram dry weight of the fresh and aged garlic extracts are presented in Figure 6.

The results of ANOVA showed that the effect of concentration on the reducing power of the extract and hence on its antioxidative activity was significant ($P < 0.05$). At all concentrations tested, the reducing power of ascorbic acid was significantly ($P < 0.05$) higher than that of garlic extracts.

The reducing power of the fresh garlic extract was much closer to that of the synthetic antioxidant Ascorbic acid. Zou *et al.* (2008) studied the antioxidative properties of polysaccharide obtained from *Pteridium aquilinum* and showed that its reducing power increased from about 200 to 600 μM when the concentration of the extract was raised from 25 to 400 $\mu\text{g/mL}$ [33]. Tan *et al.* (2015) attributed the remarkable reducing power of the polysaccharide derived from *Dipsacus asperoides* root to the presence of hydroxyl groups in the polysaccharide structure that can participate in the reduction of Fe^{3+} to Fe^{2+} [34].

Table 4 lists the quantities of the compounds identified in the fresh garlic extract (because of its more antioxidant properties) using a GC-MS machine.

The two main compounds in this garlic extract were diallyl disulfide (34.87%) and dipropyl trisulfide (25.88%). Pyrogallol (13.38%) and methyl propyl trisulfide (11.36%) were among the other compounds found in the ultrasonic aqueous extract (Table 5, Figure 5). The amounts of volatile compounds in garlic extract depend on various factors including genetic factors (type and species of garlic) and agricultural factors (planting,

caring for the crop and harvesting). For example, the diallyl disulfide contents of Cameroonian, Chinese, Moroccan, French and Mexican garlic are 37.3, 35, 23.2, 21.8 and 17.2%, respectively [35-36].

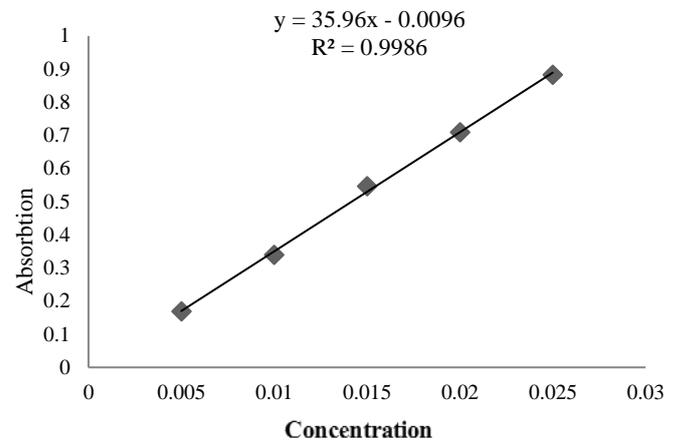


Fig. 1 Calibration curve for absorbance of gallic acid concentrations

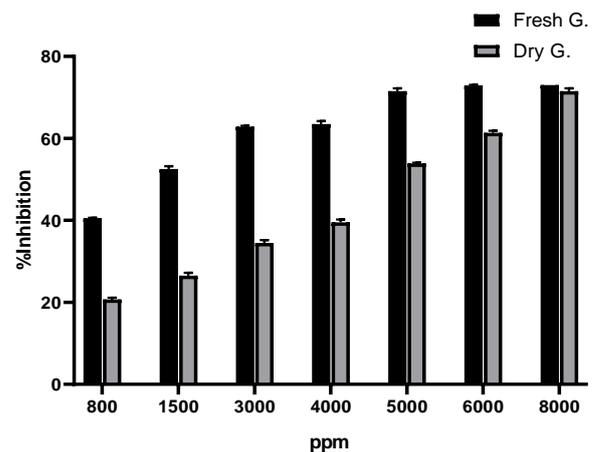


Fig. 2 Comparison of inhibition rate of DPPH free radical of fresh and aged garlic extracts

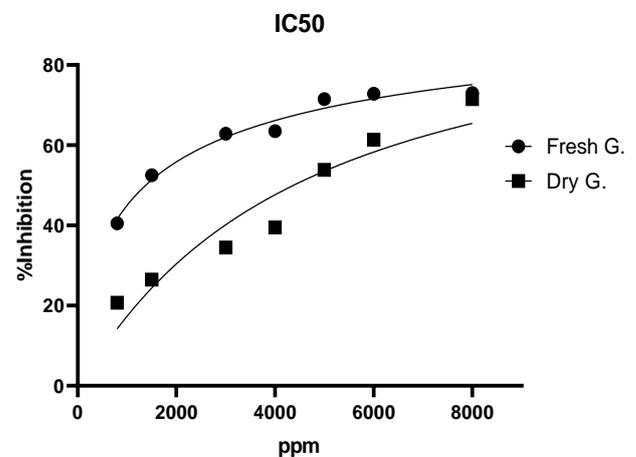


Fig. 3 IC_{50} values of fresh and aged garlic extracts

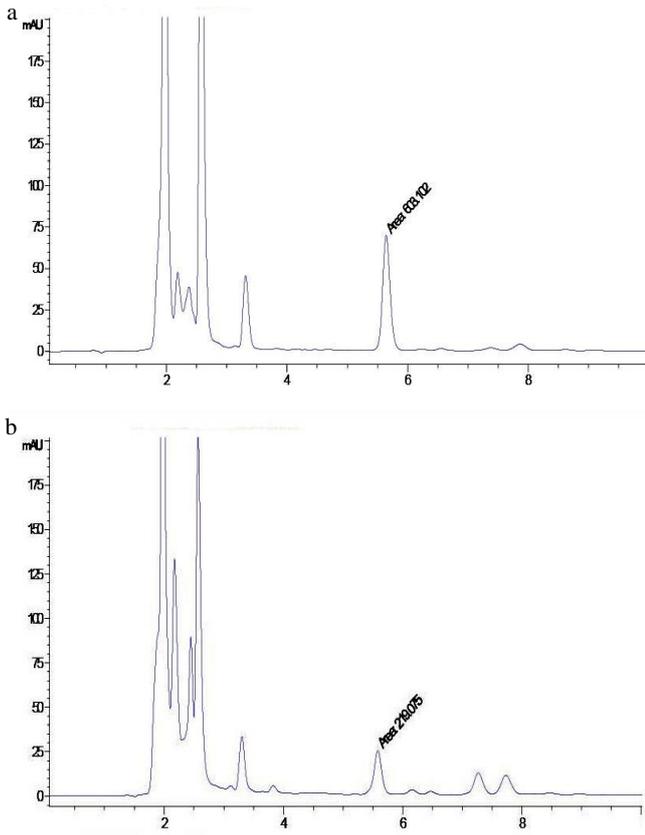


Fig. 4 HPLC chromatogram regarding allicin content in the (a) fresh garlic extract (b) aged garlic extract

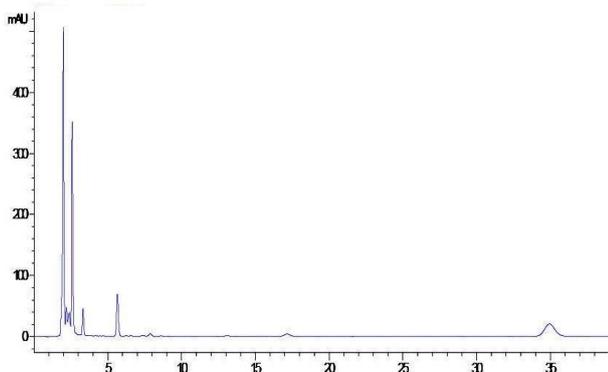


Fig. 5 HPLC chromatogram of the internal standard of butyl parahydroxy benzoate

Table 1 Comparison of total phenolic content (in mg gallic acid in gram dry weight of the extracts obtained from fresh and aged garlic)

Sample	Aged garlic extract	Fresh garlic extract
Total phenolic content	0.311 b±0.05	0.42 a±0.08

*Different small letters in each row represents a significant difference in the level of 5%

Table 2 IC₅₀ values of fresh and aged garlic extracts

Sample	Aged garlic extract	Fresh garlic extract
IC ₅₀	4376 a	1378 b
R squared	0.91	0.97

*Different small letters in each row represents a significant difference in the level of 5%

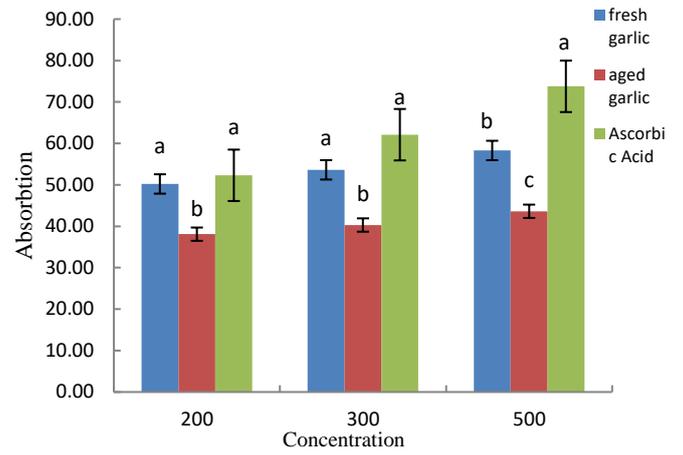


Fig. 6 Comparison of different concentrations of fresh and aged garlic extract and Ascorbic acid in trivalent iron reduction

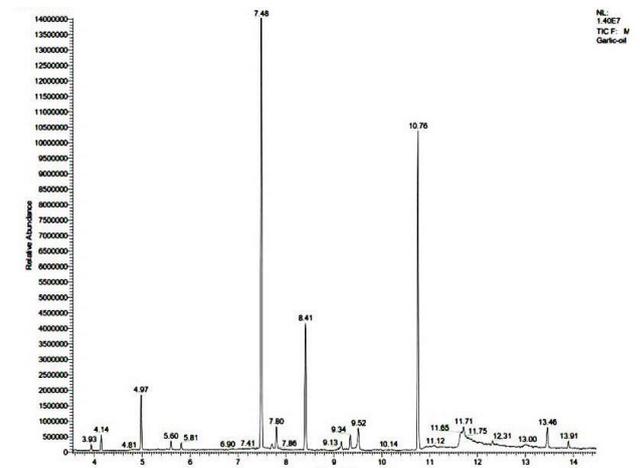


Fig. 7 GC-MS chromatogram of fresh garlic extract

Conclusion

Functional and nutritious foods are popular worldwide for their health benefits and therapeutic properties. Traditional herbs are used to treat many diseases and physiological problems.

Table 3 Allicin content in fresh and aged garlic extracts

Sample	Peak area (sample)	Peak area (internal standard)	Percentage of Allicin
Fresh garlic extract	603.10	1007	0.27 a
Aged garlic extract	219.08	1158	0.09 b

*Different small letters in each row represents a significant difference in the level of 5%

Table 4 The amount of compounds in fresh and aged garlic extract

Num.	Title	RT (min)	Result (%)
1	1,3-Dithiane	4.97	4.37
2	Dimethyl trisulfide	5.81	0.56
3	Diallyl disulfide	7.48	34.78
4	Diallyl disulfide (isomer)	7.8	2.71
5	Trisulfide, methyl 2-propenyl	8.41	11.36
6	3-Vinyl-1,2-dithiocyclohex-4-ene	9.16	0.85
7	N-Amyl sec-butyl disulfide	9.34	1.23
8	3-Vinyl-1,2-dithiocyclohex-5-ene	9.52	2.31
9	Trisulfide, di-2-propenyl	10.76	25.88
10	Pyrogallol	11.71	13.38
11	Hexathiane	13.46	1.99
12	Diallyl tetrasulfide	13.91	0.59

Among functional food materials, use of garlic is ever-expanding due to its potential health benefits. The health benefits of garlic are due to its sulfur-containing compounds such as allicin and S-methyl-1-cysteine sulfoxide whereas its pharmacological effects result from its organosulfur compounds such as cysteine sulfoxide and thiosulfinate contents. Fresh garlic has more antioxidative properties because of its higher sulfur-containing compounds and higher allicin contents compared to aged garlic. Therefore, it is recommended to use fresh garlic extract in therapeutic and research projects.

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