

A Comparison between the Antioxidant Strength of the Fresh and Stale *Allium sativum* (Garlic) Extracts

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Abstract

Background: Fruits and vegetables are considered as the source of antioxidant and the factor of destroying reactive oxygen species. The effect of this antioxidant might decrease in time. This study was aimed at examining and comparing the antioxidant effect and the level of phenolic and flavonoid compounds as well as allicin level, between fresh and three-month stale *Allium sativum* (garlic).

Materials and Methods: In an experimental study, the ethanolic extract of bushes of fresh and three-month-old *Allium sativum* was prepared and their antioxidant activities were assessed in linoleic acid and α -carotene linoleate system. The amount of phenolic compounds was measured by Folin-Ciocalteum method, based on gallic acid; the amount of flavonol and flavonoid compounds by aluminum chloride base on rutin base; and the amount of allicin by spectrophotometry method. SPSS-15 Software and *t*-statistic tests were used to analyze the mean difference between the results of two groups and $p < 0.05$ was considered as significant.

Results: The efficiency of fresh garlic (35.36) in inhibiting the oxidation was higher ($p < 0.05$) comparing to three-month dated *Allium sativum* (10.2). Phenolic compounds of the fresh garlic (12.61 mg/g) were more than the three-month dated *Allium sativum* (2.89 mg/g). The amount of allicin was respectively 15 $\mu\text{g/ml}$ and 8 $\mu\text{g/ml}$ in the fresh and three-month dated *Allium sativum* ($p < 0.05$).

Conclusion: The fresh garlic contains more useful substances and it is recommended to be used in its fresh estate.

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Introduction

Reactive oxygen species are instable and very reactive molecules capable of harming cell, DNA and having mutagenicity [1]. There is an abundance of antioxidants in fruits and vegetables having the potential to neutralize reactive oxygen species and transform them into harmless molecules [2]. Antioxidants lead to oxidation process delay, prevention from polymerization of the cycle started by the reactive oxygen species and other oxidation reactions [3]. Consuming flavonoid-rich foods protect human against diseases related to oxidative stress such as heart diseases and cancer [4]. The protective effect induced by fruits and vegetables against cancer; cardiovascular and cerebrovascular diseases are attributed to their antioxidant substances [5]. In the meantime, *Allium sativum* known as garlic belonging to Liliaceae family, is a herbaceous plant with small bulbs [6] and effective medicinal substances such as allein, allicin, allinase enzyme, inulin,

vitamins A, B and C [7]. *Allium sativum* is a traditional plant used not only as a spice, but also for various biological qualities like anticancer, anti-arthrosclerosis, antithrombotic, antimicrobial, anti-inflammatory and antioxidant for ages [8].

The study carried out on *Allium sativum* has shown the role of its antioxidant qualities in preventing from the infection to age-relevant and cardio-vascular diseases [9]. Rawgarlic extract is effective in improving oxidative stress and rats' blood lipids decrease and the effect is ascribed to the antioxidant activity of *Allium sativum* [10]. *Allium sativum* oil protects stomach against ethanol damage for its antioxidant qualities [11]. The activity of destroying reactive oxygen species and high phenolic content of *Allium sativum* aquatic extract depends, in part, on the presence of allicin as an active substance [10]. Also, *Allium sativum*' healing effect on some cancers is proved to be due to its antioxidant activity [12]. In the

study of Gazzani et al the significant effect of temperature on the antioxidant activity of *Allium sativum* was shown [13]. In fact, a great deal of therapeutic effect of *Allium sativum* is assigned to its antioxidant compounds which might be related to its phenolic compounds and flavonoid substances. There is a need to study the antioxidant activity and different amounts of phenolic, flavonoid and allicin contents in various forms of *Allium sativum* such as fresh and stale garlic. Accordingly, this study was carried out aiming at the assessment and comparison between the antioxidant effect and measurement of the amount of phenolic, flavonoid, and allicin in fresh and three-month dated *Allium sativum*.

Materials and Methods

Upon preparing *Allium sativum* hydroalcoholic extract, antioxidant strength, the amount of phenolic, flavonoid and flavonol compounds as well as the allicin content of fresh and three-month dated *Allium sativum* extract were determined in this experimental study. Fresh *Allium sativum* of Fereidoon-shahr in Isfahan, was cleaned and smashed after being collected, left in room temperature for half an hour and extracted using maceration method. Also, an amount of the same garlic was extracted after three months and under ordinary humidity.

Preparing *Allium sativum* methanolic extract: first, the smashed garlic (50g) was poured in a 1l balloon and ethanolic alcohol (96%) (400 g) was added and placed on a shaker for 24h. Then, the resulting extract was filtered using filter paper and Buchner funnel and the ethanol (70%) was poured on the residue. After 24h, it was again filtered and added to the first extract. Then, the extract was condensed in vacuum (at 50 and 70 rpm) till the residual volume reached one fifth of the basic volume. The residue was poured in container with certain weight and dried in the oven under 50 °C. Upon getting completely dried; the resulting extract was weighed. The amount of the resulting dried extract was 3g kept under -70 °C till being used [14].

To assess the antioxidant activity in β -carotene linoleate model; chloroform (0.5ml), 5ml β -carotene (0.2 mg), 20ml linoleic acid (20mg) and 0.2ml tween 40 (polyoxyethylene sorbitan monopalmitate, 200 mg) were poured in a test tube, and incubated for 10min under 50 °C. The chloroform evaporated. Resulting solution was diluted with water and 4ml aliquots of it were added to the samples (control and test). The control sample contained ethanol (0.2 ml) and test sample contained ethanol (0.2ml) plus *Allium sativum* extract (0.05 ml). As with the extract and standard sample, optical absorption of the control sample was recorded at 0min and a half an hour later at 90min on the wavelength 470 nm.

The distilled water and β -carotene solvent blank used in the test was chloroform. The samples were incubated in Ben Marry under 50 °C. The antioxidant activity was examined based on the capability of the samples in inhibiting β -carotene activity [15]. To determine antioxidant power in linoleic acid model, the extract solvent (2ml, 200 mg/l), linoleic acid 2.51% in ethanol (2

ml), phosphate buffer (4ml, 0.05M with pH=7) and distilled water (2 ml) were mixed in a capped tube and transferred into the oven (40 °C). After 6 and 12h, the sample absorption was measured using thiocyanate assay and repeated each 12h. To read the samples absorption, 0.1ml of the emulsion prepared by ethanol 75% (9.7 ml) and Ferro-chloride (0.1 ml, 0.02 M) in chloridric acid (10%) were mixed. After 3min, ammonium thiocyanate (0.1 ml, 30%) was added to the mixture and then the solution absorption was read on wavelength 500nm. The method is based on the oxidation of FeII by peroxides. Resulting Fe III creates a red complex by ammonium thiocyanate with maximum absorption on wavelength 500nm and is considered as an index of the amount of the existing peroxide [16].

The amount of phenolic compounds was measured using Folin-Ciocalteu and based on gallic acid [17]. First, standard solutions (with concentrations 12.5, 25, 62.5, 100 and 125 units in millions of gallic acid in methanol [60%] solution) were prepared. Then, 0.1 ml of each one was transferred into the test tube and Folin-Ciocalteu reagent solution (0.5ml, 10%) was added. 3 to 8min later, sodium carbonate solution (0.4 ml, 7.5%) was added. The tubes were kept under lab temperature for 30min and then the amount of absorption was measured in triplet on the wavelength 765nm using spectrophotometer. To determine the phenolic compounds of all extracts, 0.01 to 0.02g of them were solved in methanol (60%) and reached to the volume of 10ml and the phenolic content was determined using Folin-Ciocalteu reagent, but here 0.1ml of the extract solution was added (instead of the standard solution). Then, total amount of phenolic content was calculated (mg of gallic acid in one gram of the extract powder, based on the value of the absorption read).

Aluminum chloride technique was used for measuring total flavonoid amount [18]. First, the standard solutions (with concentrations 25, 50, 100, 250 and 500 part per million (PPM) of the rutin solution in methanol (60%) were prepared, then 1ml of each solution was transferred into a test tube and 1ml aluminum chloride (2%) was added to it. Then, potassium acetate solution (6 ml, 5%) was added to it and the absorption was read on 415nm wavelength, after 40min.

Each of the standard concentrations was measured in triplet and to determine total flavonoid contents of the extracts, about 0.01 to 0.02g of the extracts powder was solved in methanol (60%) and reached to %10ml volume. Then, total flavonoid level was determined using aluminum chloride technique, but here 1ml of the extract solution was added (instead of the standard solution). Then, total amount of flavonoid was determined using aluminum chloride technique and based on the rutin assay, but here the absorption value was read after 2.5h on the wavelength 440nm [19].

The amount of allicin (as the effective substance in the particular qualities of *Allium sativum*) was measured using spectrophotometry technique. 2-Nitro-5-benzoic acid was required for measuring allicin. Accordingly, first, tris solution (50ml, 0.5 M) was prepared, then 2-

mercaptoethanol solution (5ml) and nitrobenzoic acid solution (21g or 21ml) was added and, immediately after 5min, the medium was acidified up to pH 1.5ml using hydrochloric acid and kept for a night under 4 . In the meantime the orange crystals formed were washed with diluted chloridric acid and dried under vacuum [20]. We prepared a mixture (7.2pH) by combining 2-nitro-5-banzoic acid (0.2×10⁻⁴M) and sodium phosphate (50mM) and EDTA (1Mm), and added some of the prepared extracts (with its allicin no more than 10µg) and after 30min, we measured the absorption amount on the wavelength 412nm and placed it in equation below to determine the allicin amount:

$$C_{allicin} (ng/ml) = \frac{A_{412} \times 12}{280} = \frac{(A_2 - A_1)_{412} \times 12}{280}$$

A2: the absorption amount of 2-nitro-5-banzoic acid before the extract addition, A1: the absorption amount of 2-nitro-5-banzoic acid and the extract after 30min. SPSS-15 Software and t-statistic tests were used to analyze the mean difference between the results of two groups. $p < 0.05$ was considered as significantly different [10].

Results

Results showed that - based on -carotene linoleate model for assessing the antioxidant power – the amount of antioxidant activity was more in the fresh *Allium sativum* extract comparing to the three-month dated *Allium sativum* extract ($p < 0.05$). The absorption amount in -carotene model (on wavelength 470nm) was 35.36 in the fresh *Allium sativum* extract and 10.2 the three-month dated *Allium sativum* extract. Also, based on linoleic acid model, the amount of antioxidant activity was more in the fresh *Allium sativum* extract comparing to the three-month dated *Allium sativum* ($p < 0.05$). The absorption amount index on the wavelength 500nm after 6 and 12h (repeated each 12h) is represented in figure 1. There was a statistically significant difference ($p < 0.05$) in the amount of absorption (on the wavelength 500nm) observed between the fresh *Allium sativum* extract and the three-month dated *Allium sativum* extract. Additionally, the total phenol and flavonoid contents in the fresh *Allium sativum* extract were statistically more than the amounts in the three-month dated *Allium sativum* extract ($p < 0.05$).

A comparison of the amount of the existing flavonol showed that there was no statistically significant difference between the two *Allium sativum* extract groups ($p > 0.05$) (Fig.2). A comparison between the antioxidant power of the two extracts using -carotene method showed a significant difference between the fresh *Allium sativum* extract comparing to the three-month dated *Allium sativum* extract regarding the absorption amount on wavelength 470nm ($p < 0.05$).

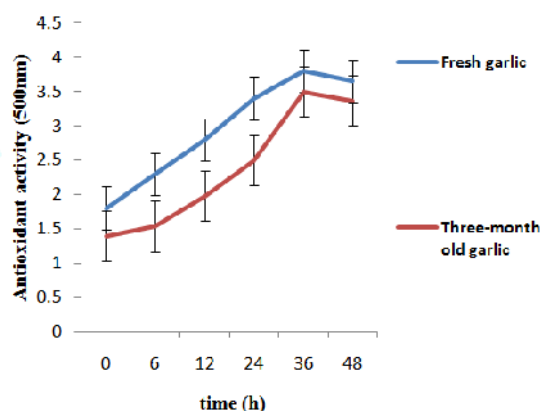


Figure 1. The antioxidant activity of the two types of *Allium sativum* extracts based on linoleic acid model

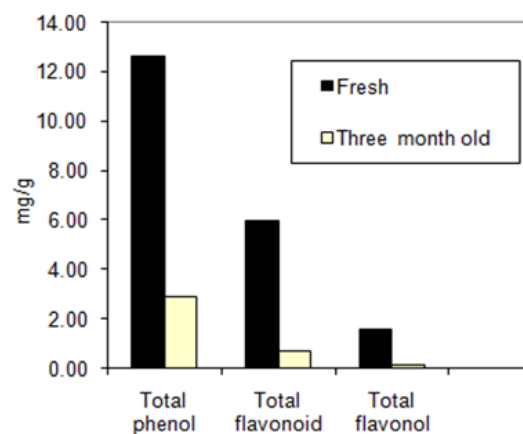


Figure 2. Total phenolic, flavonoid and flavonol contents of the two types of *Allium sativum* extracts

A significant difference was observed between the two types of *Allium sativum* extracts regarding the amounts of phenolic and flavonoid compounds ($p < 0.05$). The comparison between flavonol amount of the fresh and three-month dated *Allium sativum* did not show a significant difference. Also, the comparison between the two types of *Allium sativum* extracts demonstrated that the amount of allicin was respectively 15µg/ml and 8µg/ml in the fresh and three-month dated *Allium sativum* ($p < 0.05$).

Discussion

This study, it was determined that the fresh *Allium sativum* extract had higher antioxidant activity comparing to the three-month dated *Allium sativum* extract. On the other hand, phenolic, flavonoid compounds and allicin existing in the fresh *Allium sativum* were more than the three-month dated *Allium sativum*. It seems that the higher antioxidant activity level of the fresh *Allium sativum* is due to the presence of sulfur substances.

A study demonstrated that the antioxidant activity of some allium species is related to the presence of sulfur

substances and their pre-structures [21]. A study showed that a great deal of the antioxidant capacity of plants depends not only on the presence of vitamins C, E and -carotene, but also on other substances including polyphenols with strong antioxidant activity [22]. It was shown in a study that a process like baking will soften cell wall and facilitate carotenoids extraction [23] and lead to their extraction into water and reduction of their levels in the tissue. Also, during the baking, vitamins existing in vegetables will decrease using temperature degree [24]. So, it seems that in our study fresh *Allium sativum* (away from the processes like baking) has phenolic, flavonoid and flavonol compounds as well as allicin. Accordingly, it seems totally logical that the fresh *Allium sativum* comparing to the three-month dated *Allium sativum* maintain their compounds such as carotenoids and vitamins (including A, B, C). It seems that likely the high antioxidant activity of the fresh *Allium sativum* in this study is to some extent related to the presence of vitamin C and its high phenolic level (phenol, flavonoid and flavonol).

Studies have showed that *Allium sativum* is among foods resulting in the improvement of the quality of the individuals' lives. Experimental studies have introduced *Allium sativum* and its substances as the inhibitors of cancerogenesis and blood cholesterol level decrease [25] so that – in addition to its strong odor – it results in a wide range of effects like gastro-enteric disorders and anemia [26]. These events are induced by allicin and other sulfur substances soluble in water which produced by waterfall chemical reactions from allicin [27].

It was demonstrated in a study that *Allium sativum* extract created by long term extraction of *Allium sativum* in aquatic ethanol without any burning odor has not lead to the events, in the clinical experiments [28]. Also, the potential effects of immunity, antioxidant [29], improving the peripheral blood flow, increasing natural killing cells and prevention from immunity system function decrease in the patients with advanced cancer, activating transcription factor and maintaining DNA against reactive oxygen species by the slate of *Allium sativum* extract represented [30]. The *Allium sativum* actionis accompanied with reactive oxygen species (ROS) activity, increase in cellular antioxidant enzymes, superoxide dismutase, catalase, glutathione peroxidase and glutathione in cells [31]. On the other hand, it seems that, by three months after keeping the fresh *Allium*

sativum in this study, it is likely that the amount of its effective substances or even effective enzymes in enacting the antioxidant property and also burning odor have decreased comparing to the fresh *Allium sativum* which finally results in the decrease of the antioxidant activity and phenolic content of the three-month dated *Allium sativum* extract which these effects are probably ascribed to the reduction of allicin and other sulfur substances solved in water in the three-month dated *Allium sativum* extract. In a study regarding the effect of raw *Allium sativum* extract on the inhibition of copper ion inclined to LDL oxidation and liver antioxidant enzymes reduction, it was showed that this effect of *Allium sativum* is attributed to its antioxidant quality [10]. There is a positive relationship between removing reactive oxygen species and the phenolic amount of all different parts of *Allium sativum* extract [10]. Maybe, the total phenol amount in various sections of the plants change based on the processed they carry out [32]. Results of the study correlate with the results from the previous reports regarding the direct relationship between the phenolic substances and antioxidant activity [33].

Further studies are suggested regarding the application of more types of the extracts to gain correlation coefficient and draw the desirable diagram for better representation of results, as well as the detection and assessment of the amount of each one of the phenols having different effects based on their various amounts in various parts of the plant and probably responsible for a wide range of antioxidant activity. The fresh *Allium sativum* extract has higher antioxidant capacity, phenolic, flavonoid and flavonol compounds comparing to three-month dated *Allium sativum* extract. As a result, studying the exact cellular mechanism of the effects, separating other effective compounds existing in the extracts and also determining the effect mechanism of the separated purified effective compounds, and comparing between the compounds existing in various parts of the plant are recommended. Furthermore, studying how the preparation assays or processes make their effects seems necessary.

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