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Determination of Antioxidant Activity in Garlic (*Allium sativum*) Extracts Subjected to Boiling Process *in vitro*

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Abstract Garlic is a vegetable known to be a good antioxidant food resource around the world. Several studies on the antioxidant activity of garlic, mainly conducted using one or two methods, have been reported. However, comparison of the antioxidant activity of garlic before and after cooking is rarely reported. In this study, we compared the antioxidant activity of garlic aqueous and methanol extracts processed before and after boiling to mimic the cooking process. By testing the antioxidant activities of the extracts in different chemical mimic systems *in vitro*, namely, ABTS [2, 2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid)] and DPPH (2, 2-diphenyl-1-picrylhydrazyl) radical scavenging activities, reducing power, and metal chelating ability, we found the following data: (1) no significant difference was observed on the ABTS radical scavenging activities of garlic aqueous and methanol extracts before and after boiling process; (2) the reducing power of garlic aqueous and methanol extracts decreased by 25.9% and 14.1%, respectively, whereas the metal chelating activity of boiled garlic aqueous extracts increased by 54.7%; and (3) DPPH radical scavenging test may not be suitable to examine the garlic extracts. In addition, the ABTS radical scavenging activities of garlic extracts were very stable at pH ranges similar in human bodies, and both sulfhydryl and phenolic compounds were probably responsible for the antioxidant ability of garlic. The boiling process destroyed only a small part of garlic bio-ingredients related to antioxidant activity properties.

Keywords: *aqueous, methanol extract, radical scavenge, reducing power, metal chelating*

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1. Introduction

Reactive oxygen species (ROS) in human bodies include peroxyl radical, superoxide anion radical ($^{\cdot}O_2^-$), hydrogen peroxide, hydroxyl radical, hypochlorous acid, singlet oxygen, and nitric oxide radical [1]. Formation of ROS, such as $^{\cdot}O_2^-$ and hydroxyl, is an unavoidable consequence of aerobic metabolism during respiration. Evidence suggests that mitochondria converts 1% to 2% of the oxygen consumed in $^{\cdot}O_2^-$ under normal physiological conditions and turns them into a series of very destructive ROS, such as hydroxyl [2]. Another endogenous resource of ROS is inflammatory reaction, such as release of ROS by polymorpho-nuclear leukocytes and macrophage peroxisomes. Exogenous sources of ROS include smoke, air pollution, and pesticides [3,4]. ROS has important roles in the body, including its involvement in the signal transduction of inflammation to resist pathogen infection [5]. However, overproduction of ROS may easily cause damage to cells or tissues by lipid peroxidation and denaturation of proteins or nucleic acid. This damage has severe consequences on overall metabolism [6], which may then cause series of clinical symptoms, such as arteriosclerosis, arthritis, obesity, heart disease, and cancer. Radical damage theory of aging is one of the most acceptable theories in the world [4,7,8].

In the long history of evolution, surviving organisms have developed considerable strategies to eliminate these dangerous radicals, which could be summarized into two systems. One group includes gene-coded antioxidant enzymes, such as superoxide dismutase, catalase, peroxidase, thioredoxin systems, and glutathione systems; another group includes non-gene coded metabolic products, such as uric acid, ascorbic acid, α-tocopherol, carotenes, glutathione, lipoic acid, and ubiquinol [9,10,11,12].

As an important part of food, vegetables not only provide carbohydrates and minerals but also supply antioxidants, such as vitamins, carotenoids, and polyphenols. Garlic is a vegetable known to be a good antioxidant food resource worldwide. Considerable studies on the antioxidant activity of garlic, mainly conducted using one or two methods, have been reported. However, comparison of the antioxidant activity of garlic before and after cooking is rarely reported. To investigate the effects of cooking on the antioxidant activity of garlic, we extracted garlic by using water and methanol. Garlic aqueous and methanolic extracts were prepared, subjected to boiling to mimic the cooking procedure, and examined for their antioxidant activity by different assays *in vitro*.

2. Materials and Methods

2.1. Garlic Samples and Extracts Preparation

Fresh garlic was cleaned by distilled water, and 1 g of edible parts was cut and completely grinded with 5 mL of distilled water or methanol (Xilonghuagong Co., Ltd., Chengdu, China). Samples were centrifuged at 13 500 g for 10 min. Subsequently, 4 mL of supernatants were collected and separated into two equal parts, one of which is boiled for 30 min and centrifuged at 13 500 g for 10 min. The supernatants were collected for antioxidant tests.

2.2. ABTS Radical Scavenging Test

ABTS (Amresco) radicals were obtained based on the report made by Mathew [13]. ABTS radical solutions were diluted to make the absorbance at 734 nm to be 0.8 ± 0.030. Approximately 2 µL of samples was added into 48 µL of solution, and absorbance at 734 nm was detected by Ultrospec 2100 pro UV/Visible Spectrophotometer (Amersham Bioscience). ABTS radical scavenging percentage was calculated as follows: Inhibition (I) % = [(AB − AA)/AB] × 100, where AA stands for absorbance at 734 nm after sample incubation, and AB stands for absorbance at 734 nm before sample addition. Afterward, 1.2 mM of α-tocopherol (sigma) and 1.2 mM of butylated hydroxytoluene (BHT, Alfa Aesar, China) were used as positive controls. α-Tocopherol and BHT have similar concentration in the following tests.

2.3. DPPH Radical Scavenging Test

DPPH radicals (Sigma-aldrich) were obtained based on the report made by Mathew [14]. Briefly, 2 µL of samples was added into 48 µL of 60 µM DPPH dissolved in methanol. After incubation in darkness at room temperature, absorbance at 517 nm was detected by Ultrospec 2100 pro UV/Visible Spectrophotometer (Amersham Bioscience). DPPH radical scavenge rates were calculated as follows: I% = [(AB−AA) / AB] × 100, where AA stands for absorbance at 517 nm after sample incubation, and AB stands for absorbance at 517 nm before sample addition.

2.4. Determining Reducing Power

Using BHT and α-tocopherol dissolved in methanol as positive controls, the reductive potential was determined based on the method described by Oyaizu [15]. All the following chemicals and buffers used in this study are analytical grade products from Tianjin Fengchuan Chemical Reagent Technologies Co., Ltd., Tianjin, China. Briefly, 20 µL of samples was mixed with phosphate buffer (50 µL, 0.2 M, pH 6.6) and potassium ferricyanide (50 µL, 1% w/v). The mixture was incubated at 50 °C for 20 min. Approximately 50 µL of trichloroacetic acid (10%, w/v) was added to the mixture. The mixture was then centrifuged at 1 200 g for 10 min. The upper layer of solution (50 µL) was mixed with 50 µL of distilled water and ferric chloride (0.1% w/v). The absorbance was monitored at 700 nm by Ultrospec 2100 pro UV/Visible Spectrophotometer (Amersham Bioscience).

2.5. Chelating Effects on Ferrous Ions

All the chemicals and buffers used in this study are analytical grade products from Tianjin Fengchuan Chemical Reagent Technologies Co., Ltd., Tianjin, China. The chelating effects on metals were determined based on the method of Denis et al. with slight modifications [16]. Briefly, 20 µL of the samples or solvents of the same volume was added to a solution of 2 mM of $FeCl_2$ (10 µL). The reaction was initiated by the addition of 5 mM of ferrozine (40 µL). The mixture was vigorously shaken, diluted with 230 µL of water, and left standing at room temperature for 10 min. Absorbance of the solution was then measured at 562 nm by Ultrospec 2100 pro UV/Visible Spectrophotometer (Amersham Bioscience). The percentage of inhibition of ferrozine-Fe^{2+} complex formation was calculated using the following formula. I% = [(A0 − A1) / A0] × 100, where A0 is the absorbance of the control, and A1 is the absorbance in the presence of the samples. Moreover, 1.2 mM of EDTA was used as control.

2.6. pH Effects on ABTS Radical Scavenge

All the chemicals and buffers except for ABTSs used in this study are analytical grade products from Tianjin Fengchuan Chemical Reagent Technologies Co., Ltd., Tianjin, China. pH studies were conducted by dissolving sample into 20 mM of sodium acetate, pH 5.0; 20 mM of sodium phosphate, pH 6.0, 7.0, or 8.0; or 20 mM of glycine-OH, pH 9.0. The exact concentrations of ABTS radical were determined by $\varepsilon 415 = 3.6 \times 10^4\ M^{-1}cm^{-1}$ [17]. Reaction rates during the first minute were obtained by analyzing the absorbance values detected by Ultrospec 2100 pro UV/Visible Spectrophotometer (Amersham Bioscience) using the software Swift II Reaction Kinetics.

2.7. Alkylation of Thiol Groups

All the buffers used in this study are analytical grade products from Tianjin Fengchuan Chemical Reagent Technologies Co., Ltd., Tianjin, China. Thiol groups were alkylated by incubating sample with 22 mM of indoacetamide (IAA, sigma) in phosphate-buffered saline (10 mM of sodium phosphate, pH 7.4, 120 mM of NaCl, and 3 mM of KCl) for 1 h in darkness at room temperature. The same volume of water and IAA were used as controls. Initial rates were calculated as described in pH effects on ABTS radical scavenge.

2.8. Absorbance Spectrum Test for Phenols

Samples were added to standard $ABTS^+$ solutions, and the absorbance spectrum was read at 0 s, 2 s, 30 s, 2 min, 5 min, 10 min, and 20 min. Finally, end point scanning of the reaction products was immediately accomplished after adding NaN3 (Sigma-aldrich) to a final concentration of 60 mM to reduce remaining ABTS radicals [18]. The absorbance values at 550 nm were corrected using the following formula. A550 = 0.5 × (A550 (observed) − 0.403 × A735) + 0.5 × (A550 (observed) − 0.163 × A415) [19].

2.9. Statistical Analysis

Data were expressed as means ± SD of three replicates and then analyzed by SPSS V13. One-way analysis of

variance and Duncan's new multiple-range test were used to determine the differences among the means. P values < 0.05 were regarded as significant.

3. Results and Discussion

3.1. ABTS Radical Scavenging Activity

Data in Figure 1 demonstrated that no significant difference was observed on the ABTS radical scavenging activities of garlic aqueous and methanolic extracts before and after boiling ($p > 0.05$). These results also showed that the ABTS radical scavenging test is very stable in different types of solvents. We then took this method to test pH effects on the antioxidant activity of garlic and ingredients that are responsible for its antioxidant activity.

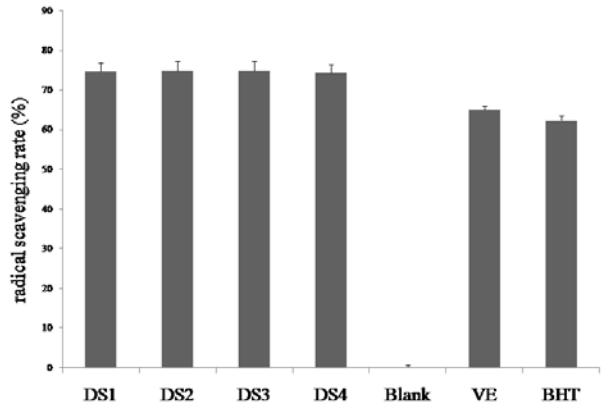

Figure 1. ABTS radical scavenging activity of garlic extracts. DS1, aqueous extracts; DS2, boiled aqueous extracts; DS3, methanol extracts; and DS4, boiled methanol extracts

3.2. DPPH Radical Scavenging Activity

Quenching of DPPH radical is mainly achieved by electron transportation, which is hard to be achieved in hydrophobic systems. The results are usually lower than the real antioxidant activity. The results are easily influenced by solvents and pH [20,21]. Stasko et al. demonstrated that 50% (v/v) of methanol in water is most widely suitable for all kinds of samples, whereas 60% of methanol in water can cause precipitation of DPPH in this system [22].

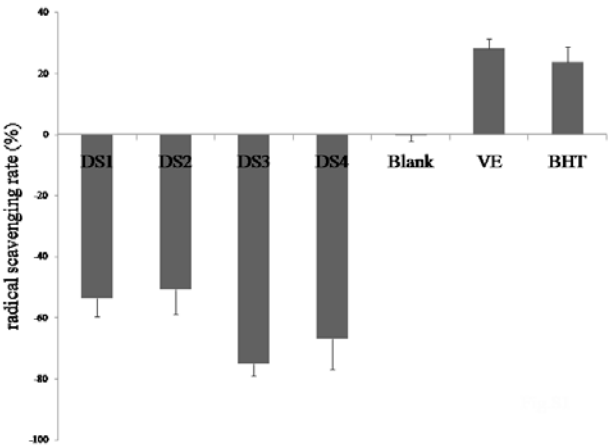

Figure 2. DPPH radical scavenging activity of garlic aqueous extracts. DS1, aqueous extracts; DS2, boiled aqueous extracts; DS3, methanol extracts; and DS4, boiled methanol extracts

We adopted the 100% methanol system in DPPH radical scavenging test and found that BHT and α-tocopherol have effects on DPPH radical scavenge, but garlic extracts resulted in a negative result via the calculation formula (Figure 2). As a powerful alkalic food, garlic may vigorously change the pH of the reaction system, which may then cause unexpected results in the system. Purple color precipitates after addition of garlic extracts into the system. These precipitations may block the optical path and cause negative DPPH radical scavenging results calculated by the formula.

3.3. Reducing Power

Data from Figure 3 indicated that the reducing power of both garlic aqueous and methanol extracts declined with $p < 0.05$ after boiling. The results showed that the boiling procedure, despite of the water solvents or organic solvents, could destroy the reducing power of garlic extracts. Although the statistical analysis value is significant, the reducing power of garlic aqueous and methanol extracts are reserved as 74.1% and 85.9%, respectively.

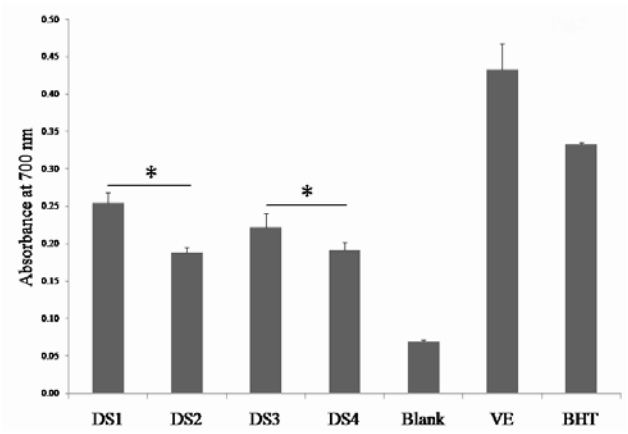

Figure 3. Reducing power of garlic aqueous extracts. DS1, aqueous extracts; DS2, boiled aqueous extracts; DS3, methanol extracts; and DS4, boiled methanol extracts. Absorbance at 700 nm represents the reducing power. * means $p < 0.05$

3.4. Chelating Effects on Ferrous Ions

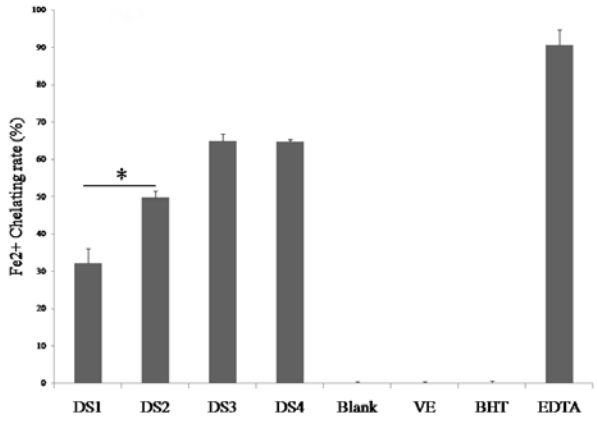

Figure 4. Metal chelating of garlic aqueous extracts. DS1, aqueous extracts; DS2, boiled aqueous extracts; DS3, methanol extracts; and DS4, boiled methanol extracts. * means $p < 0.05$

Data from Figure 4 indicated that α-tocopherol and BHT showed no ferrous ion chelating activities despite of

their powerful ABTS and DPPH radical scavenging activities. Garlic methanol extracts showed excellent metal chelating activity, which is about 71.5% of that of 1.2 mM EDTA. Notably, ferrous ion chelating activities of garlic aqueous extracts increased about 54.7% after boiling procedure. This result may be contributed to the complexes formed during the boiling process.

3.5. pH effects on ABTS Radical Scavenge

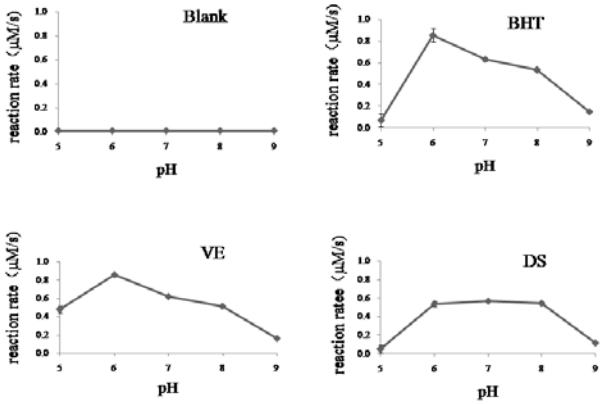

Figure 5. pH effects on the ABTS radical scavenging activity of garlic aqueous extracts (DS)

Data from Figure 5 showed that garlic aqueous extracts demonstrated a powerful ABTS radical scavenging activity compared with α-tocopherol and BHT under pH = 7 solvents. Although the result is not the most powerful ABTS radical scavenger under all of the pH environments tested, garlic aqueous extracts showed faster radical scavenging rate under pH 6 to 8, which is the most widespread pH conditions of the human bodies except for several special organs, such as the stomach. These results showed that garlic not only has powerful antioxidant ability *in vitro* but also has important radical scavenger roles in the body.

3.6. Sulfhydryl Compounds Responsible for ABTS Radical Scavenging Activity

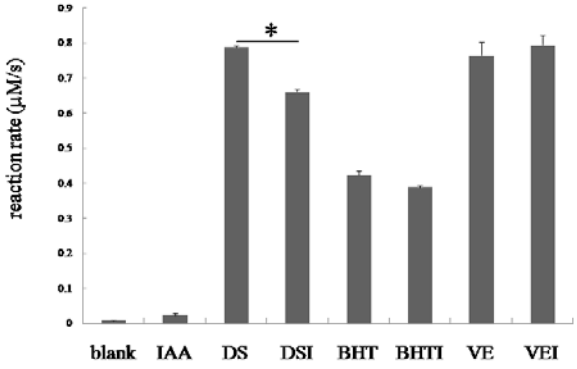

Figure 6. Alkylation of thiol groups by IAA inhibited part of the ABTS radical scavenging activity of garlic aqueous extracts (DS). * means $p < 0.05$

Data in Figure 6 presented that only garlic aqueous extracts showed a decline on ABTS radical scavenging activity after IAA inhibition ($p < 0.05$). IAAs have no effects on the radical scavenge activity of α-tocopherol and BHT. These results showed that sulfhydryl compounds containing thiol groups in garlic, maybe diallyl trisulfide, are responsible for part of the ABTS radical scavenging activity of garlic [23].

3.7. Phenolic Compounds Responsible for ABTS Radical Scavenging Activity

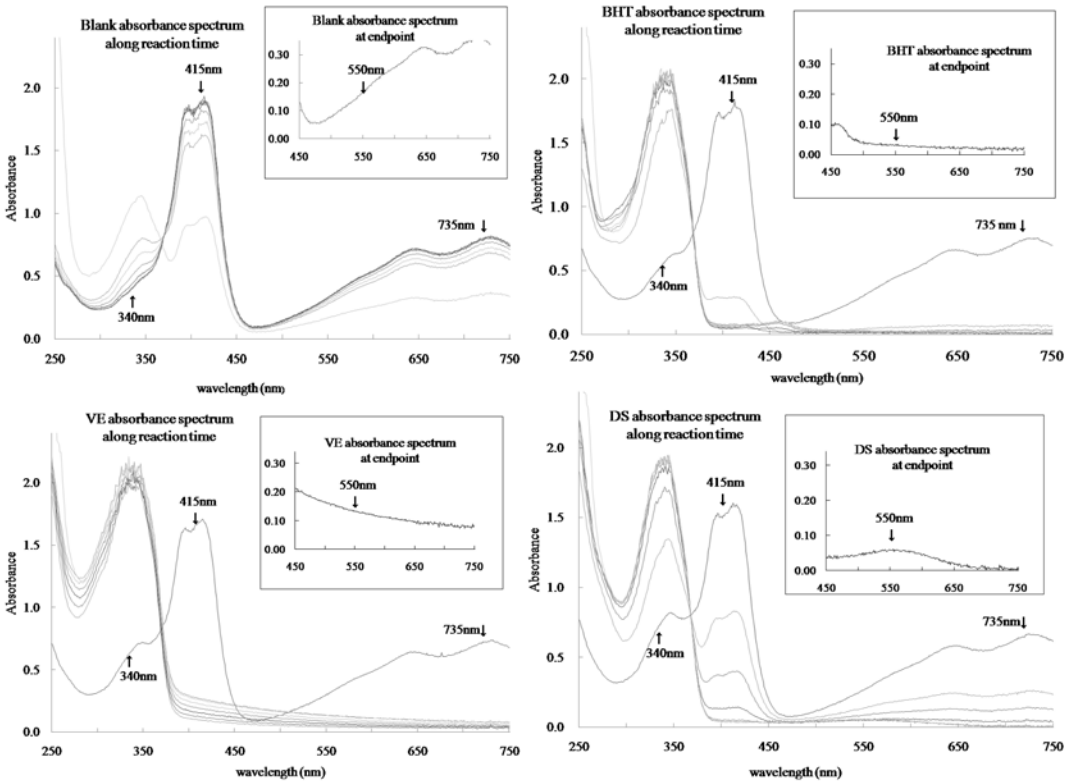

Figure 7. Wave scan of ABTS radical scavenging process by garlic aqueous extracts (DS) and controls. Samples were added to standard $ABTS^{+}$ solutions, and the absorbance spectrum was read at 0 s, 2 s, 30 s, 2 min, 5 min, 10 min, and 20 min. Finally, end point scanning of the reaction products was immediately done after adding NaN3 to a final concentration of 60 mM to reduce remaining ABTS radicals

During the ABTS radical scavenging process, radicals with a specified absorbance at 415 nm are turned into ABTS molecules with a specified absorbance at 340 nm. α-Tocopherol and BHT have faster radical scavenging rates in the initial 30 s, whereas garlic aqueous extracts similarly nearly cleaned all of the radicals after 2 min (Figure 7). Notably, no specified compounds were detected at the corrected absorbance at 550 nm in ABTS radical scavenging process by α-tocopherol and BHT. However, specified compounds were detected after the end point scan of garlic aqueous extracts. Phenolic compounds are reported to form purple products with ABTS molecules after their scavenging of ABTS radicals [24]. Formation of compounds that have specified absorbance at the corrected absorbance at 550 nm in ABTS radical scavenging process demonstrated that phenolic compounds are responsible for ABTS radical scavenging activity of garlic aqueous extracts.

4. Conclusion

In this study, we compared the antioxidant activity of garlic aqueous and methanol extracts processed before and after boiling to mimic the cooking procedure. No significant difference was observed on the ABTS radical scavenging activities of garlic aqueous and methanol extracts before and after boiling. The reducing power of boiled garlic aqueous and methanol extracts decreased by 25.9% and 14.1%, whereas the metal chelating activity of boiled garlic aqueous extracts increased by 54.7%. Furthermore, the ABTS radical scavenging activity of garlic extracts was very stable at pH ranges similar in human bodies, and both sulfhydryl and phenolic compounds were probably responsible for the antioxidant ability of garlic. The boiling process destroyed only a small part of garlic antioxidant activity.

Statement of Competing Interests

The authors have no competing interests.

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