Food Sci Biotechnol (2018) 27(1):219–225
https://doi.org/10.1007/s10068-017-0246-4

Antioxidant and antimicrobial activities of fresh garlic and aged garlic by-products extracted with different solvents

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Received: 3 August 2017 / Revised: 26 September 2017 / Accepted: 6 October 2017 / Published online: 12 December 2017

Abstract This study aims to investigate antioxidative and antibacterial properties of fresh garlic (non-aged, NG) and aged garlic (AG) by-products extracted with distilled water, ethanol, or chloroform. To determine their antioxidative and antibacterial capacities, 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay, 2,2′-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) radical scavenging assay, and H_2O_2 radical scavenging activity, Fe^{2+} chelating activity, total ferric reducing antioxidant power (FRAP), and disc diffusion tests were performed. Total phenol and flavonoid contents from distilled water extract of AG were significantly higher than those of NG. DPPH, ABTS, FRAP, and H_2O_2 scavenging activities of distilled water extract of AG were higher than those of NG. However, Fe^{2+} chelating activities of ethanol and chloroform extracts were higher than those of distilled water extracts for both NG and AG. Antibacterial effects of AG were higher than those of NG. In conclusion, aged garlic showed more potent antioxidant and antibacterial effects than fresh garlic.

Keywords Garlic · Aged garlic · Antioxidant · Antibacterial · Extract

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Introduction

Garlic (*Allium sativum* L.) is originated from western Asia and Mediterranean coast. It has been used as spice and natural medicine. Garlic is known to contain natural antioxidants that can remove reactive oxygen species (ROS) and reduce lipid peroxides and low-density lipoprotein (LDL) oxidation [1, 2]. Because garlic contains allicin, diallyl sulfides, and other sulfur compounds, it shows many physiological effects and activities in various metabolic pathways [3].

However, the strong and tangy flavor of garlic is big problem when developing products using garlic compounds. This is related to alliin and its precursor, *S*-allyl-(L)-cysteine. When cell structure of garlic is broken, alliin is transformed to allicin by alliinase. Allicin is responsible for the strong flavor of garlic. Because allicin is unstable, it is decomposed to lipophilic organic sulfur compounds such as diallyl sulfide, diallyl disulfide, and diallyl trisulfide [4]. The most common and traditional method to reduce the strong flavor of garlic is by steaming or roasting. When garlic is heat treated, alliinase activity is greatly reduced and the flavor is softened [5]. Aged garlic or black garlic is attracting attention of customers these days. It is a type of processed garlic produced by aging of whole garlic under constant temperature and humidity [6]. During the aging process, volatile components of garlic are decreased due to high temperature. On the other hand, water soluble flavonoids and phenolic compounds are increased [7].

There have been studies about aged garlic, aged garlic juice, and products such as bread (flour dough) and *Yakju* (Korean rice-wine) that contain aged garlic [8, 9]. However, research about the by-product after the extraction process of garlic juice is insufficient. Therefore, the purpose of this study was to determine the antioxidative

activity of garlic and aged garlic by-products after extracting them with different solvents (distilled water, ethanol, and chloroform).

Materials and methods

Samples

Samples of non-aged garlic (NG) and aged-garlic (AG) were obtained from OZL DNF Inc. (Dam Yang, Korea). Experiments were carried out using powdered garlic by-product samples. Briefly, after pressing out juice from whole garlic without removing the husk, the remaining was dried at 60 °C for 6 h (powdering). Then 50 g of NG or AG powder was mixed with 250 mL of solvent (distilled water, ethanol, or chloroform) and incubated at 25 °C in a shaking (250 rpm) incubator for 24 h. The extract was then filtered with Whatman filter paper (No. 2).

Chemicals

Folin–Ciocalteu's phenol reagent, Na_2CO_3, MeOH, $AlCl_3$, 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), potassium persulfate ($K_2S_2O_8$), potassium ferricyanide, trichloroacetic acid, ferric chloride, $FeCl_2$, ferrozine, Peroxidase from horseradish, hydrogen peroxide (H_2O_2) were obtained from sigma (Sigma-Aldrich GmbH, Sternheim, Germany).

Antioxidant activities

Determination of total phenol contents

Total phenol contents were determined using Folin–Ciocalteu's phenol according to published method [10] with slight modification. Briefly, tenfold diluted sample (0.5 mL) and Folin–Ciocalteu's phenol reagent (2.5 mL) were mixed and reacted at room temperature for 5 min. After that, 75 g/L Na_2CO_3 (2 mL) was added to the mixture and reacted at room temperature for 2 h. Absorbance value was then measured at 760 nm on a spectrophotometer. MeOH was used as blank. Garlic acid equivalents were used to construct standard curve.

Determination of total flavonoid contents

Total flavonoid contents were determined according to Dowd method [11] with slight modifications. Briefly, 2% $AlCl_3$ (1.5 mL) and tenfold diluted sample (0.5 mL) were mixed and reacted at room temperature for 10 min. Absorbance of the mixture was then measured at 415 nm on a spectrophotometer. Quercetin was used to obtain standard curve.

DPPH free radical scavenging activity

Free radical scavenging activity of each sample was measured using the method of Blois [12], with some modifications. Briefly, 5 mL of 0.1 mM DPPH in 95% ethanol was added to 1 mL of each tenfold diluted sample. The mixture was shaken vigorously and incubated at room temperature for 30 min in the dark. After that, the mixture was filtered through a nylon syringe filter (0.45 μm). Its absorbance was then measured at 517 nm. DPPH radical scavenging activity was then calculated using the following equation:

$$\text{DPPH radical scavenging effect}\,(\%) = \{1 - (\text{absorbance}_{\text{sample}}/\text{absorbance}_{\text{control}})\} \times 100$$

ABTS radical cation scavenging activity

ABTS radical cation scavenging activity was determined using the method of Re et al. [13]. Briefly, 2 mM ABTS was dissolved in distilled water (DW) containing 2.45 mM potassium persulfate ($K_2S_2O_8$) and kept at room temperature for 12 h in the dark. The $ABTS^{\cdot+}$ solution was adjusted with sodium phosphate buffer (0.1 M, pH 7.4) to an initial absorbance of about 0.75 ± 0.005 at 734 nm. Then 0.1 mL of each tenfold diluted sample was mixed 3 mL of $ABTS^{\cdot+}$ solution. After incubating at room temperature for 10 min, the absorbance was measured at 734 nm. $ABTS^{\cdot+}$ radical scavenging activity was then calculated using the following equation:

$$ABTS^{\cdot+}\ \text{radical scavenging effect}\,(\%) = \{1 - (\text{absorbance}_{\text{sample}}/\text{absorbance}_{\text{control}})\} \times 100$$

Ferric reducing antioxidant power (FRAP)

FRAP assay was performed following the modified Oyaizu method [14]. Briefly, 1 mL of each tenfold diluted sample was mixed with 2.5 mL of sodium phosphate buffer (0.2 M, pH 6.6) and 2.5 mL of 1% potassium ferricyanide (potassium hexacyanoferrate). The mixture was then incubated at 50 °C for 20 min. After adding 2.5 mL of 10% trichloroacetic acid, the mixture was then centrifuged at 4000 rpm for 10 min. The upper layer (2.5 mL) was mixed with 2.5 mL of distilled water and 0.5 mL of 0.1% $FeCl_3$ (ferric chloride). After 5 min of reaction at room temperature, the absorbance was measured at 700 nm.

Table 1 Total phenol and flavonoid contents in fresh garlic and aged garlic by-products extracted with different solvents

	NG			AG		
	DW	Ethanol	Chloroform	DW	Ethanol	Chloroform
Total phenol	5.68 ± 0.51^{c}	2.86 ± 0.14^{c}	0.27 ± 0.06^{c}	147.58 ± 5.27^{a}	43.01 ± 2.85^{b}	0.69 ± 0.11^{c}
Total flavonoid	70.82 ± 0.22^{c}	35.07 ± 0.78^{d}	25.44 ± 1.08^{e}	338.04 ± 1.60^{a}	124.08 ± 3.22^{b}	28.07 ± 1.97^{de}

Total phenolic contents: mg GAE/g, total flavonoid content: μg QE/g

NG non-aged garlic powder, *AG* aged garlic powder, *DW* distilled water

Values with different superscripts are significantly different among samples ($p < 0.05$)

Ferrous ions (Fe^{2+}) chelating activity

Fe^{2+} chelating activity of the extract was determined using published method [15]. Briefly, 0.5 mL of each tenfold diluted sample was mixed with 2 mL of 1 mM $FeCl_2$ in 95% ethanol. The reaction was initiated by adding 2.5 mL of 2 mM ferrozine in 95% ethanol. The mixture was then vortexed and incubated at room temperature for 10 min. It was then filtered through a nylon syringe filter (0.45 μm). The absorbance of the filtrate was then measured at 562 nm. Ferrous ions chelating activity was then calculated using the following equation:

$$\text{Ferrous ions chelating activity}\,(\%) = \{1 - (\text{absorbance}_{\text{sample}}/\text{absorbance}_{\text{control}})\} \times 100$$

Hydrogen peroxide (H_2O_2) scavenging activity

Hydrogen peroxide scavenging activity was determined using previously described method [16]. Briefly, each extract sample (0.1 mL) was mixed with H_2O_2 (0.01 mL, 50 mM), peroxidase from horseradish (0.6 mL, 10 U/mL), ABTS (0.6 mL, 0.1%), and 1.8 mL of 0.1 M phosphate buffer (pH 6.0). The solution was then incubated at 37 °C for 15 min. Absorbance of the resulting solution was then measured spectrophotometrically at 414 nm. Garlic acid equivalents were used to construct standard curve.

Antimicrobial activity based on disc diffusion test

Antimicrobial activity of each extract (NG or AG) was tested against *Salmonella enteritidis*, *Staphylococcus aureus*, *Escherichia coli*, *Bacillus cereus*, and *Listeria monocytogenes* using agar diffusion technique [17]. Bacterial colonies were suspended in normal saline to obtain approximate concentrations (10^6–10^7 CFU/mL). Filter paper discs (Whatman, 6 mm in diameter) were dipped in each sample (50 μL) and dried for 3 h. All plates were incubated at 37 °C for 24 h. The diameter of the resulting zone of inhibition was measured in mm. Penicillin was used as positive control while distilled water was used as negative control.

Statistical analysis

All experiments were carried out in triplicates. Results are presented as mean ± standard error of means. Data were analyzed using IBM SPSS statistics 24.0. One-way analysis of variance (ANOVA) was performed. Significant differences between different solvents and treatments (NG and AG) were analyzed using Tukey test. A p value of less than 0.05 was considered statistically significant.

Results and discussion

Determination of total phenol and flavonoid contents

Results of total phenol and flavonoid contents of NG and AG extracts using different solvents are shown in Table 1. Distilled water extract from AG had significantly ($p < 0.001$) higher total phenol content (147.58 mg GAE/g) than other extracts. Mean values of total phenol contents were in the following order: AG-E (43.01 mg GAE/g) > NG-D (5.68 mg GAE/g) > NG-E (2.86 mg GAE/g) > NG-C = AG-C (0.27 and 0.69 mg GAE/g, respectively). In general, antioxidant activities of plant extracts are mostly interactive with phenolic-type compounds [18]. Regardless of solvents, flavonoid contents of AG groups were significantly higher compared to those of NG groups. In particular, flavonoid content of water extract from AG was approximately 4.5-fold higher than that of NG (AG-W, 338.04 mg GAE/g; NG-W, 70.82 mg QE/g). Consequently, water extract from AG showed the highest total phenols and flavonoid contents. The significant increase in total phenol and flavonoids in AG might be due to the conversion of some components in garlic into these highly hydrophilic compounds. According to Bozin et al. [19], 80% methanol extract from aged garlic had higher total phenol and flavonoids contents (0.98 mg GAE/g and 6.99 μg QE/g, respectivley) than that from fresh garlic

Table 2 DPPH and ABTS radical scavenging activity (%) of fresh garlic and aged garlic by-products extracted with different solvents

	NG			AG		
	DW	Ethanol	Chloroform	DW	Ethanol	Chloroform
DPPH radical scavenging activity	20.86 ± 0.29^{c}	11.22 ± 0.14^{e}	5.28 ± 0.33^{f}	79.21 ± 0.74^{a}	51.16 ± 0.13^{b}	13.44 ± 5.14^{d}
ABTS radical scavenging activity	35.92 ± 0.43^{c}	19.78 ± 0.15^{d}	17.43 ± 0.09^{e}	99.56 ± 0.06^{a}	83.49 ± 0.28^{b}	19.23 ± 0.20^{d}

NG non-aged garlic powder, *AG* aged garlic powder, *DW* distilled water

Values with different superscripts are significantly different among samples ($p < 0.05$)

(0.05 mg GAE/g and 4.16 μg QE/g, respectively). Nencini et al. [20] have found that aged garlic extract with 15% ethanol contains 0.73 mg GAE/g (bulbs) and 1.23 mg GAE/g (leaves) of total phenol. Flavonoids and phenolic compounds have antioxidant abilities such as regeneration of α-tocopherol, free radical scavengers, and metal chelation [13].

DPPH and ABTS radical scavenging activity

Results of DPPH radical scavenging activity showed a decreasing tendency according to the extraction solvent (DW > ethanol > chloroform) as shown in Table 2. DPPH radical scavenging activities of the AG group extracted with DW, ethanol, and chloroform were significantly ($p < 0.05$) higher than those of the NG group: AG-D (79.21%) > AG-E (51.16%) > NG-D (20.86%) > AG-C (13.44%) > NG-E (11.22%) > NG-C (5.28%). ABTS radical cation scavenging activity results were similar to results of DPPH radial scavenging test. That is, AG-D had the highest activity and AG group had higher activity than the NG group. Radical cation scavenging activities of extracts were in the following order: AG-D (99.56%) > AG-E (83.49%) > NG-D (35.92%) > AG-C (19.23%) = NG-E (19.78%) > NG-C (17.43%). Accordingly, AG group showed greater radical scavenging activities for DPPH and ABTS than NG group, with AG-D showing the greatest activity. Previous studies have suggested that aged garlic contains plentiful phenol, flavonoid, and various sulfur compounds such as *S*-ally-(L)-cysteine (SAC, hydrophilic) and disulfide (hydrophobic) compared to fresh garlic. In addition, SAC has high radical scavenging activities [21, 22]. The number of phenolic compounds and flavonoids has positive correlation with DPPH and ABTS radical scavenging activities due to hydrogen and electron donation from hydroxyl groups of these compounds [23, 24]. In particular, ABTS method is regarded as potentially more efficient than DPPH method since ABTS can measure both hydrophilic and hydrophobic substances [25].

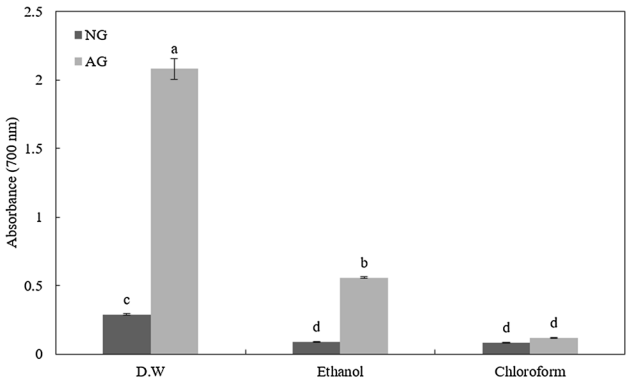

Fig. 1 Ferric reducing antioxidation power (FRAP) of non-aged garlic powder (NG) and aged garlic powder (AG) by-products extracted with distilled water (DW), ethanol, and chloroform. Values with different superscripts are significantly different among samples ($p < 0.05$)

Ferric reducing antioxidation power (FRAP)

FRAP is a method of verifying antioxidant activity through electron donating ability. In FRAP, ferric tripyridyltriazine (Fe^{3+}-TPTZ) complex is reduced to ferrous tripyridyltriazine (Fe^{2+}-TPTZ) by the sample. The higher the absorbance, the higher the antioxidant activity. Results of total ferric reducing power showed that the AG group had a decreasing tendency of reducing power according to extraction solvent (DW > ethanol > chloroform) based on absorbance at 700 nm (Fig. 1). Among these sample extracts, AG-D had significantly ($p < 0.001$) higher reducing power than other groups. The reducing power of these samples was in the following order: AG-E > NG-D > NG-E = NG-C = AG-C. This result might be due to high phenol and flavonoid contents in distilled water and ethanol extracts of aged garlic compared to those of non-aged garlic extracts. Positive correlation between polyphenol content of *Allium* species and FRAP value ($r^2 = 0.669$) has been observed previously [20].

Ferrous ions (Fe^{2+}) chelating activity

Ferrozine reacts with Fe^{2+} to form a complex and become purple. The chelating activity of a sample can inhibit the formation of Fe^{2+}-ferrozine complex, resulting in degradation of color. Results of ferrous ion chelating activity of aged and fresh garlic extracts using different solvents are shown in Fig. 2. Ferrous ions chelating activity was detected in all groups. Especially, extracts using ethanol and chloroform (organic solvent) had significantly ($p < 0.05$) higher ferrous ion chelating activity than extracts using DW. Distilled water extract from aged garlic showed significant ($p < 0.001$) higher ferrous ion chelating activity than all groups. However, ethanol and chloroform extracts did not show significant difference in ferrous ion chelating activity for aged or non-aged garlic. Similarly, it has been reported that aqueous extract from garlic has higher reducing power and lower metal chelating ability than methanol extracts [26]. The inhibition effect of diallyl sulfide and diallyl disulfide derived from garlic on Fe^{2+} lipid oxidation has been proved previously [27]. The high metal chelating effect of ethanol and chloroform extracts could be due to thiol groups such as hydrophobic disulfides with metal chelating ability [28].

Hydrogen peroxide (H_2O_2) scavenging activity

Results of hydrogen peroxide scavenging activities of various extracts are show in Fig. 3. AG-D extract had the highest hydrogen peroxide scavenging activity (235.02 mg GAE/g), followed by AG-E (196.11 mg GAE/g), NG-D (86.48 mg GAE/g), NG-E (29.68 mg GAE/g), AG-C (19.00 mg GAE/g), and NG-C (5.32 mg GAE/g). NG and AG groups showed a decreasing tendency in hydrogen peroxide scavenging activity according to extraction solvent (DW > ethanol > chloroform). Hydrogen peroxide is

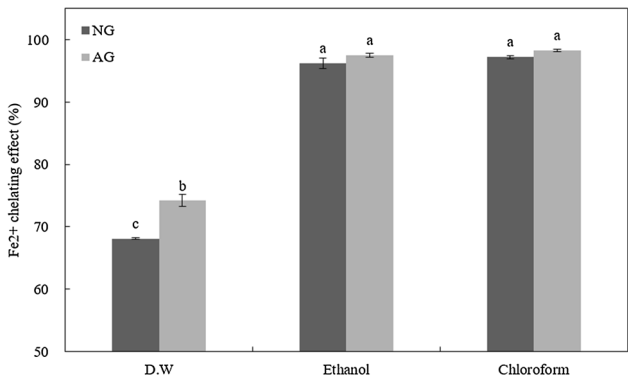

Fig. 2 Fe^{2+} chelating activity (%) of non-aged garlic powder (NG) and aged garlic powder (AG) by-products extracted with distilled water (DW), ethanol, and chloroform. Values with different superscripts are significantly different among samples ($p < 0.05$)

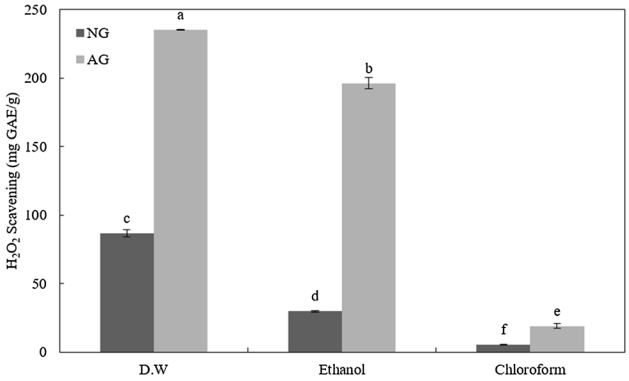

Fig. 3 Hydrogen peroxide (H_2O_2) scavenging activity of non-aged garlic powder (NG) and aged garlic powder (AG) by-products extracted with distilled water (DW), ethanol, and chloroform. Values with different superscripts are significantly different among samples ($p < 0.05$)

considered a non-free radical species. It has high toxicity in presence of some metal ions such as Fe^{2+} and Cu^{2+}. Lipid oxidation generated by Fenton reaction (Fe^{2+} and hydrogen peroxide) can be inhibited by garlic extracts [19]. Ide et al. [29] have reported that sulfur compounds such as SAC and alliin of aged garlic can scavenge hydrogen peroxide. Furthermore, fructosyl arginine generated from Maillard reaction during aging of garlic has shown hydrogen peroxide scavenging ability [30]. Therefore, the high hydrogen peroxide scavenging activity of aged garlic extract using distilled water might be useful for protection against oxidative toxicity.

Antimicrobial activity

Results of antimicrobial activities of aged and non-aged garlic extracts using different solvents are shown in Table 3. Non-aged garlic extracts except chloroform extract did not show significant antimicrobial activity against *S. aureus*, *S. enteritidis*, *E. coli*, *B. cereus*, or *L. monocytogens*. Similar results have been reported by Dziri et al. [31] showing that antibacterial activity of rosy garlic (*Allium roseum var. odoratissimum*) against pathogenic bacteria was mostly not detected. In contrast, Benkeblia [32] has demonstrated that essential oil extract from garlic has high antimicrobial activity against or *S. aureus* and *S. Enteritidis*. However, chloroform extracts of both non-aged and aged garlic inhibited ($p < 0.05$) the growth of *B. cereus*. Yin and Cheng [27] have also observed that the addition of lipophilic sulfur compounds such as diallyl sulfide and diallyl disulfide into meat products can reduce the growth of pathogenic bacteria whereas hydrophilic sulfur compounds such as *s*-ethyl cysteine and *n*-acetyl cysteine have low antibacterial activities. Inhibition of *B. cereus* growth is sensitively affected by solvent used to

Table 3 Disc diffusion test results of fresh garlic and aged garlic by-products extracted with different solvents

unit: mm	NG			AG			SEM
Extract solvent	DW	Ethanol	Chloroform	DW	Ethanol	Chloroform	
S. aureus	nd	nd	nd	9.67[a]	nd	nd	0.11
S. enteritidis	nd	nd	nd	nd	nd	nd	–
E. coli	nd	nd	nd	10.67[a]	10.00[a]	nd	0.34
B. cereus	nd	nd	9.67[b]	nd	nd	13.3[a]	0.29
L. monocytogenes	nd	nd	nd	nd	nd	nd	–

NG non-aged garlic powder, *AG* aged garlic powder, *DW* distilled water, *nd* not detected, *SEM* standard error of means

Values with different superscripts are significantly different among samples ($p < 0.05$)

extract plants [33]. The growth of *E. coli* was significantly ($p < 0.05$) reduced by distilled water and ethanol extracts of aged garlic. This result might be due to the fact that hydrophilic sulfur compounds and phenol/flavonoids contents were increased in aged-garlic.

Acknowledgements This work was supported by the Brain Korea 21 Plus Project of Department of Food Science and Biotechnology of Animal Resources, Konkuk University in 2017.

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