

Antioxidant Activities, Phenolic Contents and Electronic Nose Analysis of Black Garlic

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Abstract: Black garlic is a processed garlic product with a moisture-controlled high temperature heat treatment for a long time. In order to determine the secondary metabolites of black garlies treated in the study, firstly, in vitro antioxidant activities of black garlies purchased from Edovital company, Kastamonu, Turkey were determined, followed by qualitative and quantitative measurement of the phenolic compound content by HPLC and finally the electronic nose analysis of the content of nebulizer vapors in wood vinegar extract of black garlies were done successfully. Chlorogenic acid, vanillic acid, benzoic acid, gallic acid contents in detected 13 phenolic acids were quite high. All quantitative results were expressed as mg gallic acid equivalent (GAE) per g dry matter of black garlic sample. ABTS and DPPH antioxidant activities were very low according to BHT standart and 2-Methylene-4-pentalen (18%) and Furfural (25%) were detected in high amount with electronic nose in nebulvapor contents of black garlic wood vinegar extract.

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1. INTRODUCTION

Black garlic is a processed state of fresh garlic under high temperature and humidity by the Maillard reaction. D,L-lactic acid, 5-hydroxymethyl-2-furfural, adenosine, uridine, (1S,3S)-1-methyl-1,2,3,4-tetrahydro- β -carboline-3-carboxylic acid, (1R,3S)-1-methyl-1,2,3,4-tetrahydro- β -carboline-3-carboxylic acid, and 2-acetylpyrrole were detected in black garlic ethyl acetate extracts with HPLC coupled with diode array detection analysis and NMR spectrometry [1]. Black garlic has many health-promoting properties: effective in the treatment of colon cancer, effective in the treatment of diabetes, having anti-allergic, anti-inflammatory, antioxidant, anticancer and cardiac protective effects [1-5]. Processed black garlic components are polysaccharides, reducing sugars, proteins, phenolic compounds, organic sulfur compounds and melanoidins [6]. The antioxidant capacity of black garlic is related to polyphenols [7]. Black garlic can prevent the development of atherosclerosis by clearing cholesterol [8]. In black garlic, the content of allicin, which gives a harsh aroma taste, is reduced and allisin in the browning process is transformed into antioxidant compounds such as bioactive alkaloid and

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flavonoid compounds [9]. Black garlic have been consumed in extracted form as well as being used widely as an food ingredient including beverages, candy due to sour taste of it [10]. Black garlic whose main volatiling components are organosulfur compounds such as thiosulfates and sulfur [11]. High antioxidant activity of black garlic have been observed with S-allyl cystein (watersoluble compound) increases by fermentation [12]. In one study, as the concentration of black garlic increased, the DPPH radical clearing ability increased due to increased polyphenol content of treated black garlic [13]. In other study, ABTS scavenging activity was the highest in aged black garlic extract [14]. The aim of this study is finding out of phenolic and antioxidant profile of black garlic. In addition to this, with different method from literature, wood vinegar extract of black garlic converted into vapor phase by nebulizer and these vapors were analyzed to determine of content with electronic nose.

2. MATERIAL AND METHODS

2.1. Procurement of Plant Material and Extraction Process

Fermentable black Garlics (200 g, stock code: HBV000002R8CH, from Taskopru) were purchased Edovital Company, Kastamonu, Turkey in May, 2018. For extraction of dry black garlic sample powders (30 g, powdered via a blender (Model SHB 3062; Sinbo, Istanbul, Turkey)) were used methanol (300 mL) solvent with shaker for 24 h at room temperature. The methanolic extracts of the black garlic were filtered and collected into flasks, before they were dried with rotary evaporator stored at +4 °C in refrigerator (Bosch, Germany) until use [15].

2.2. DPPH (1,1-diphenyl-2-picrylhydrazyl) Radical Scavenging Activity

DPPH radical scavenging ability was determined with slight modifications of method of Zhang et al. (2011) at 517 nm by using a spectrophotometer [16]. 1 mL of 0.1mM DPPH radical (CAS Number 1898-66-4, 1 g, Sigma-Aldrich) solution prepared in methanol was mixed with 1 mL of the test sample (2 mg / mL, methanolic black garlic extract at 2 h 6000 rpm (Nuve laboratory technology)) dissolved in 100 mM acetate buffer (pH 7.2). The mixture was shaken and left to stand for 20 min in the dark until spectrophotometric measurement (with double beam UV-Vis spectrophotometer, Shimadzu (190 nm-1100 nm)) and butylated hydroxy toluene (BHT) ($\geq 99\%$, Sigma-Aldrich) was used as a positive control.

The half inhibitory concentration (IC_{50}) (represents the concentration that caused a 50% inhibition of radical formation) value was used to express the results [16]. IC_{50} value (Table 1) was obtained by using DPPH inhibition graph.

2.3. FRAP (Ferric Reducing Antioxidant Power) Activity

FRAP assay (is based on electron transfer) was performed by following the method described by Benzie and Straine (1996) [17], with soft modifications (FRAP reagent consist on mixing acetate buffer at pH 3.6, 10 mM 2,4,6-tripyridyl-S-triazine (TPTZ) ($\geq 99.0\%$, HPLC grade, Merck) acidic solution and 20 mM $FeCl_3 \cdot 6H_2O$ (97%, 5 g, Sigma-aldrich, CAS Number: 10025-77-1)). The reaction mixture (FRAP reagent and black garlic methanolic extract (2 mg / mL) (3:1)) was then incubated at 37 °C for 4 min (Nuve laboratory technology, BM 30 water bath). Absorbance was determined at 595 nm against a blank prepared using distilled water. The result was given as the average Trolox equivalent (TEAC) of 3 repeat measurements [17] (Table 1).

2.4. CUPRAC (Cupric Ion Reducing Antioxidant Capacity) Assay

The chromogenic redox reagent, bis (neocuproine) copper (II) chelate for the CUPRAC assay produced a stable and colorful Cu (I)- neocuproine chelate as a result of the redox reaction with the polyphenols at pH 7. The absorbance of the color was measured at 450 nm. Antioxidant activity was calculated as the average Trolox equivalent (TEAC) of 3 repeat measurements.

Black garlic methanolic extract (2 mg/mL) and CUPRAC reagent (1:1) were mixed and measured in 1 min [18] (Table 1).

2.5. ABTS (2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) Radical Scavenging Activity

The determination of ABTS radical activity was based on the mechanism of Re et al. (1999) [19]. The stock solution of ABTS was mixed in dark bottle by dissolving 0.250 g of ABTS salt and 0.045 g $K_2S_2O_8$ in 100 mL of ultra pure water, then it was left in the fridge. The prepared ABTS solution was diluted with ultra pure water by using Milli-Q® IQ 7003/7005 Ultrapure Lab Water System (Merck) and before use it was kept at 25°C in darkness for 24 h. To measure the radical scavenging ability 0.05 mL of the black garlic methanolic extract (2 mg / mL) was added to 2.5 mL of a diluted ABTS solution. Absorbance was determined after 5 min incubation at 734 nm against water as blank. Butylated hydroxy toluene (BHT) ($\geq 99\%$, Sigma-Aldrich) was used as a positive control. The rate of 50% inhibition (IC_{50}) was calculated [19] (Table 1).

Table 1. Antioxidant activity comparison of black garlic methanolic extract according to four different methods.

DPPH (IC_{50}) (mg / mL)	ABTS (IC_{50}) (mg / mL)	FRAP (TEAC) (μ M / methanolic extract (mg))	CUPRAC (TEAC) (μ M / methanolic extract (mg))
0.18 \pm 0.02	0.28 \pm 0.03	42.16 \pm 0.04	56.18 \pm 0.02

\pm SD: Average Standart Deviation, 95 % confidence interval, critical ratio: $p < 0.05$

2.6. Phenolic Analysis with HPLC

RP-HPLC-DAD analysis system (Agilent 1100 Technologies, Waldbronn, Germany) of phenolic compounds was combined by using a purospher star reverse phase column (4.6 \times 250 mm, 5 μ m) (Merck, Germany), on a isocratic program with a solvent system (A: 2% formic acid in methanol:water [1:1]) at a constant solvent flow rate of 0.7 mL.min⁻¹. Injection volume was 50 μ L and analysis time was 40 min. Signals were detected at 235, 240, 253, 265, 280, 295, 312, 345 nm by DAD and at 280 nm (For the phenolic compounds, maximum absorption) by UV detection. Column temperature has been set at room temperature, 25°C. 13 phenolic components were identified by comparing to standards in black garlic methanolic extract (2 mg / mL), qualitatively and quantitatively [20] (Figure 1) (Table 2).

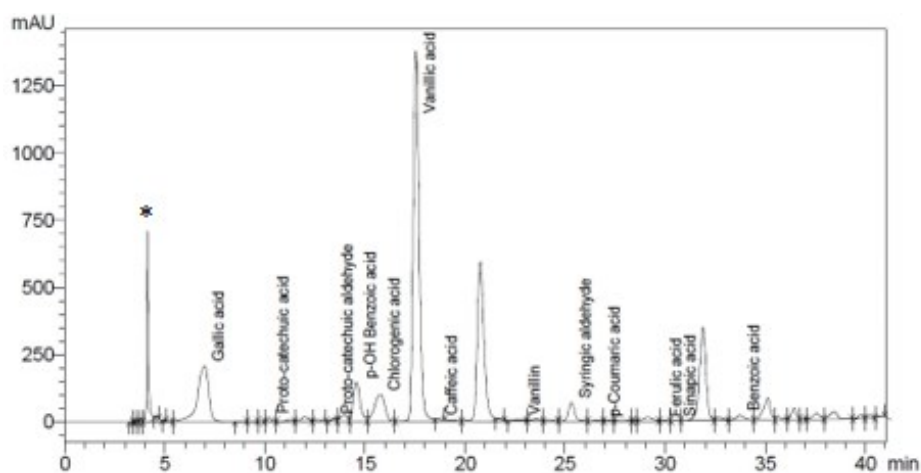


Figure 1. HPLC peak chromatogram of black garlic methanolic extract at 280 nm

Table 2. Detected qualitative and quantitative phenolic components of black garlic methanolic extract with HPLC at 280 nm (\pm SD: Average Standard Deviation, 95% confidence interval, critical ratio: $p < 0.05$)

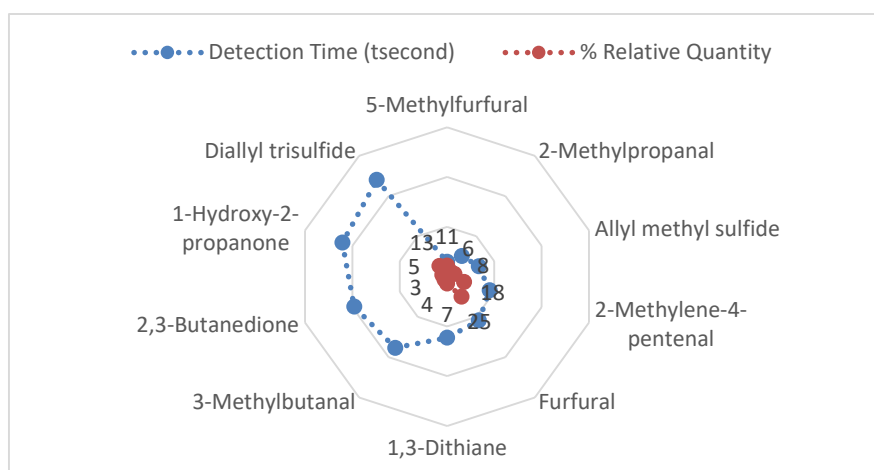
Number	Phenolic Component Name	Retention Time	% Area	Concentration (mg / L)
*	Unknown	4.15	0.01	0.1 \pm 0.01
1	Gallic acid	6.98	18.32	172.14 \pm 0.03
2	Proto-catechuic acid	10.22	0.49	4.89 \pm 0.02
3	Proto-catechuic aldehyde	13.41	0.20	1.11 \pm 0.04
4	<i>p</i> -OH Benzoic acid	14.57	7.48	94.62 \pm 0.02
5	Chlorogenic acid	15.77	7.69	183.08 \pm 0.03
6	Vanillic acid	17.54	60.97	750.95 \pm 0.01
7	Caffeic acid	18.63	0.07	0.92 \pm 0.03
8	Vanillin	22.77	0.36	2.88 \pm 0.01
9	Syringic aldehyde	25.31	3.10	95.27 \pm 0.03
10	<i>p</i> -Coumaric acid	26.76	0.16	1.50 \pm 0.01
11	Ferulic acid	29.84	0.06	1.24 \pm 0.04
12	Sinapic acid	30.53	0.01	0.09 \pm 0.01
13	Benzoic acid	33.73	1.10	223.72 \pm 0.03

2.7. Electronic Nose Analysis of Nebulizer Vapors of Black Garlic Wood Vinegar Extraction Oils

Wood vinegar (250 mL), which is a kind of dark brown liquid produced by slow pyrolysis of plant biomass, was purchased from Tu Hong Biotech Co., Ltd, Hebei, China (Mainland) and was used for clevenger distilled extraction (Sesim Kimya Laboratuvar, Ankara, Turkey) method of black garlic (30 g) powdered via a blender (Model SHB 3062; Sinbo, Istanbul, Turkey). Collected extraction oils of black garlic were placed in the chamber of nebulizer device (particle size $< 4 \mu\text{m}$, Bayer company, Germany). Components analysis of obtained nebulous vapors were realized with electronic nose (PERES foodsniffer (Swiss Technology)) including volatile organic compound sensors combined smartphone (Samsung Galaxy S5 (Seoul, South Korea)) network library in a short time (120 second) [21, 22] (Figure 2).

2.8. Statistical analysis

All qualitative and quantitative statistical analysis were reported significantly ($p < 0.05$) with average standard deviation of 3 repeated measurements. Statistical analysis was carried out by using SPSS Version 21.0 software program and Microsoft Excel (Microsoft Office Corporation, 2010, Redmond, Washington).

**Figure 2.** Volatile organic compounds detected with electronic nose

3. RESULTS and DISCUSSION

In one study, DPPH radical clearance of black garlic ethyl acetate extract was 30 % and was equivalent to 5 mg / mL gallic acid [1]. In another study, black garlic contained many organic acids occurring in nature. Lactic acid was the main organic acid in the black garlic detected in liquid analysis [23]. Therefore, lactic acid may be responsible for unique taste. Total phenolic content in black garlic was increased by about four to 10 times compared to white garlic. Hydroxycinnamic acid derivatives were found to be the major phenolic acids of black garlic at different processing stages [24].

In the other study, malondialdehyde content in black garlic groups at 20 and 40 mg / kg animal study doses significantly was reduced ($p < 0.05$). In addition to this, Serum superoxide dismutase activities which are showing the effectiveness of antioxidant system in black garlic groups at 20 and 40 mg / kg animal study doses were significantly higher ($p > 0.05$) [25]. Table 1 antioxidant activity data in this study support this. On the other hand, in volatile components analysis of black garlic, the main sulfur volatiles in the black garlic exhibited an inverse behavior throughout the heating. While the concentration on plant volatiles decreased throughout the warming, the volatile and roasted aroma volatile concentrations increased [26]. In the HPLC profile analysis of free, soluble esters and glycosylated phenolic acids, trans-hydroxycinnamic acids (caffeic, *p*-coumaric, ferulic and sinapic) in garlic were shown to be twice as high as onions [27]. In this fence, because of the fact that contents of phenolic acids and trans-hydroxycinnamic acids of black garlic were valuable for responsible about antioxidant activity, HPLC phenolic peak profiles were examined in this study.

Electronic nose method is a new trend for volatile content analysis as an alternative to GC-MS. For showing heat process effect on garlicks, in a study, electronic nose was used [28]. The electronic nose's (have six sensors) spider radar graph analysis values showed that raw and heat-treated garlic's odour component characteristics were different [28].

In this study, ABTS and DPPH with CUPRAC and FRAP antioxidant activity results of black garlic methanolic extract supported each other in addition to this, DPPH activity was more effective to show the radical scavenging capacity of high antioxidative components than ABTS with increasing percentage of negative slope and CUPRAC activity was also more effective than FRAP about black garlic methanolic extract. Because copper ions were more effective linked to phenolic content from iron ions according to their reduction potentials about antioxidant capacity with positive slope in proportion to the increasing spectrophotometric color intensity (Table 1).

In a study the FRAP value in Taşköprü black garlic ($640.76 \pm 86.98 \mu\text{mol.g}^{-1}$) slightly decreased from FRAP value of black garlic in Chinese type ($678.20 \pm 77.56 \mu\text{mol.g}^{-1}$) [29] but in this study, FRAP value of black garlic ($42.16 \pm 0.04 \mu\text{M/mg}$) was found and this value was quite high per gram (Table 1). In the other study, The IC_{50} value of polyphenol extracted from black garlic for DPPH· radical inhibition was $140.79 \mu\text{g/mL}$ [25] but in this study, the IC_{50} value of black garlic methanolic extract for DPPH· radical inhibition was found as $180 \mu\text{g/mL}$ (Table 1). The reason for this is that the total content of black garlic including flavonoid and sugar groups decreases the antioxidant activity. In an another study, n-hexane, dichloromethane, ethyl acetate, n-butanol and water extracts of black garlic in different polarities were used to isolate the active ingredients (total polyphenol content) [30]. In the DPPH· radical scavenging and iron reducing power antioxidant activity tests, the aqueous extract of black garlic was more effective [30]. Therefore, the methanolic extract form closest to the polarity of the water was preferred in this study. In the same study, DPPH· radical scavenging activity of the aquatical extract of black garlic was high at a concentration of 0.6 mg / mL and concentrations below 0.2 mg/mL were not studied [30]. The DPPH· radical scavenging activity of black garlic was measured as 0.18 ± 0.02 in this study (Table 1), with the

idea that different solvent extracts (such as methanol) may exhibit better activity if these concentrations are studied. ABTS radical scavenging activity was higher in hexane, chloroform and ethyl acetate fractions of black garlic and reducing power was also significantly lower in butanol and water fraction (its polarity is close to methanol) [31] as opposite that ABTS radical scavenging activity was high in methanolic extract of black garlic in this study (Table 1).

While in a study, in phenolic analysis with HPLC, coumaric acid was the main ingredient in black garlic residue [32], in this study, in phenolic analysis with HPLC, from detected 13 components at 280 nm, vanillic acid (60,97 % peak area) was in the foreground as main flavor component of black garlic. Gallic acid (18,32 % peak area), chlorogenic acid (7,69 % peak area) and *p*-OH Benzoic acid (7,48 % peak area) with vanillic acid (60,97 % peak area) formed the major components of total phenolic content (Table 2) (Figure 1).

In the HPLC analysis of methanolic extract of black garlic, it is estimated by considering retention time and uv spectrum that the unknown component with asterisk is vitamin C (Figure 1) (Table 2). 2-Methylene-4-pentenal (18 %), furfural (25 %), diallyl trisulfide (13 %), 5-methylfurfural (11 %) were detected as volatile major organic compounds from 10 components with electronic nose (Figure 2). In other studies, for fermented black garlic according to purple garlic, there was no difference in the amount of ferulic acid, fermented black garlic had high amounts of coumaric acid and caffeic acid and chlorogenic acid decreased significantly [33]. In this study, as opposed to other studies [33], chlorogenic acid was quite high (Table 2). In other studies, in black garlic, volatile compounds of allyl alcohol and S-alk(en)-yl-L-cysteine derivatives were strongly high and furfural rate was approximately 4 % of total area of volatile compounds [33] but in this study, furfural compound and its derivatives were strongly high (Figure 2).

4. CONCLUSION

Black garlic can be a strong source of antioxidants and phenolic acids and can be used as an alternative fermented food product against oxidative stress. It can also be an aromatic food source as a component of flavor and odor. Most susceptible vitamins especially like ascorbic acid responsible for antioxidant activity can be lost during the heating process for fermented black garlic [33]. In the study, it was found that black garlic had high phenolic acid content especially like vanillic acid (60,97 % peak area) which have been responsible for sweet aroma from detected 13 components with HPLC-DAD-UV analysis and great antioxidant properties of black garlic were supported by 4 different methods (CUPRAC, FRAP, DPPH, ABTS). The results were statistically significant ($p < 0.05$) (Table 1 and Table 2). Electronic Nose Analysis of Nebulizer Vapors of Black Garlic Wood Vinegar Extraction Oils was applied firstly, in literature. Wood vinegar extraction increased the formation and involvement of furfural derivative compounds (furfural (25 %), 5-methylfurfural (11 %)) in oil phase (Figure 2). Thus, compounds which have low molecular weight and can easily fly were better detected with electronic nose combined nebulizer.

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