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The Effects of Processing Time on the Total Phenolic, Flavonoid Content, and Antioxidant Activity of Multi Bulb and Single Bulb Black Garlic

[Pengaruh Waktu Pengolahan Bawang Hitam dan Bawang Tunggal Hitam terhadap Kadar Fenol Total, Flavonoid dan Aktivitas Antioksidan]

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ABSTRACT

Black garlic is produced by processing multi-bulb garlic (*Allium sativum*) or single-bulb garlic in high temperature and high humidity for several days. Black garlic has many health benefits, such as an antioxidant activity resulting from its compound, including groups of flavonoid and phenolic compounds. This study aimed to analyze the effect of aging time on multi-bulb and single-bulb black garlic on the content of total phenolic, flavonoid, and antioxidant activity. Black garlic was processed at a 60-70°C heating temperature and 70-80% relative humidity for 25 days. Determination of total phenol and flavonoid contents was conducted using spectrophotometric methods with gallic acid as a standard of total phenolic and quercetin as a standard of flavonoid, while the antioxidant activity was determined by DPPH radical reduction. The results showed that total phenolic contents (% w/w GAE), flavonoids contents (% w/w QE), and EC_{50} values at 0 until day 25 increased on a particular day in multi-bulb and single-bulb black garlic. The optimal total phenolic content of both black garlic was obtained by heating for 20 days, flavonoid content of multi-bulb garlic for 10 days, and single-bulb black garlic for 15 days. Highest antioxidant activity was obtained on days 20 and 25 for single-bulb black garlic and multi-bulb black garlic, respectively. The aging time of black garlic affects total phenolic, flavonoid content, and antioxidant activity. In general, longer processing time caused an increase in the total phenolic content, flavonoid content, and antioxidant activity of both black garlics. Multi-bulb black garlic showed higher phenolic or flavonoid content and antioxidant activity than single-bulb black garlic.

Keywords: aging, antioxidant, black garlic, flavonoid, phenolic

ABSTRAK

Bawang hitam merupakan hasil dari pengolahan bawang putih (Allium sativum) atau bawang lanang (tunggal) menggunakan suhu pemanasan dan kelembapan tinggi selama beberapa hari. Bawang hitam mempunyai banyak khasiat bagi kesehatan salah satunya sebagai antioksidan yang dihasilkan dari kandungannya antara lain kelompok senyawa flavonoid dan fenol. Penelitian ini bertujuan menganalisis pengaruh waktu pengolahan bawang hitam dan bawang lanang hitam terhadap kadar fenol total, flavonoid dan aktivitas antioksidan. Metode pengolahan bawang hitam menggunakan pemanasan pada suhu 60-70°C dan kelembapan 70-80% yang dilakukan selama 25 hari. Penetapan kadar fenol total dan flavonoid menggunakan metode spektrofotometri dengan standar asam galat dan kuersetin, sedangkan penetapan aktivitas antioksidan menggunakan peredaman radikal DPPH dengan parameter nilai EC_{50}*. Kadar fenol total (% b/b GAE), flavonoid (% b/b QE) dan nilai* EC_{50} *pada pemanasan hari ke-0 sampai hari ke-25 mengalami peningkatan pada hari tertentu baik pada bawang hitam maupun bawang lanang hitam. Terdapat pengaruh waktu pengolahan terhadap kadar fenol, flavonoid dan aktivitas antioksidan. Secara umum semakin lama waktu pengolahan, semakin meningkat kandungan dan aktivitas dari kedua jenis bawang hitam. Bawang hitam memiliki kandungan fenol, flavonoid dan aktivitas antioksidan yang lebih tinggi dibanding bawang lanang hitam.*

Kata kunci: *antioksidan, bawang hitam, fenol, flavonoid, waktu pengolahan*

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INTRODUCTION

Garlic (*Allium sativum*) is one of the herbal plants used to treat diseases and has health benefits. Furthermore, it is used by Indonesians as a food plants and spice in their daily diet (BPOM, 2016). The major component of garlic is allicin, an organosulfur compound with several pharmacological activities (Zhang *et al.,* 2016). Some of the other garlic compounds are alliin, ajones, phenols, and alkaloids (Batiha *et al.,* 2020). Several recent studies have found that garlic has antioxidant, antimicrobial, anticancer, anti-inflammatory, anticancer, antihypertensive, and hepatoprotective properties (Batiha *et al.,* 2020; Choi *et al.,* 2014). Subsequently, garlic can be classified into two varieties based on the number of cloves, *i.e*. single-bulb and multi-bulb garlics. The single-bulb garlic, also known as single garlic or *bawang lanang*, is used by Indonesians to maintain stamina and prevent diseases caused by hyperlipidemia (Lestari and Rifai, 2019). Moreover, its taste and smell are stronger than multi-bulb garlic, and it has higher efficacy because it contains the level of volatile oil higher and accumulated in a single-bulb (Phadar *et al.,* 2014; Rahmawati and Reny, 2012).

Recently, garlic has been processed into different forms, such as black garlic, which is obtained from fermentation at high temperatures and humidity for a set period until it turns black, has a sweet taste, and softer consistency (Kimura *et al.,* 2017). Several factors can influence the processing of black garlic, that are the heating temperature, humidity, and heating time, which are 40-90°C heating temperature, 50-70% humidity, and 15-45 days, respectively (Kim *et al.,* 2013; Bae *et al.,* 2014; Zhang *et al.,* 2016; Agustina *et al.,* 2020).

Processing procedures at high temperatures and humidity over a long period can cause changes in the chemical content and activity of compounds in garlic, such as the total phenols, flavonoids, allicin compounds, *S*-allyl cysteine (SAC), and antioxidant activity (Kim *et al.,* 2013; Bae *et al.,* 2014; Zhang *et al.,* 2016; Xiong *et al.,* 2018; Najman *et al.,* 2020). Additionally, it is important to note that black garlic has a SAC compound that is higher than raw (white) garlic, but the allicin compound of black garlic is lower due to the conversion of allicin to several other compounds, such as SAC during heating (Choi *et al.,* 2014).

Previous research on the effect of processing on antioxidant activity and chemical content has been carried out on multi-bulb black garlic, but not on single-bulb black garlic. Therefore, it is necessary to examine the effect of the processing time of multi-bulb compared with single-bulb black garlic on the contents of total phenolic, flavonoid, and antioxidant activity. The quality of black garlic can be measured through the compound content and activity. The differences in physicochemical parameters in multi-bulb and single-bulb black garlic include water and methanol solubility of extract, total ash value, acid insoluble ash, and loss of drying that can affect the phenolic content and antioxidant activity have been measured (Phadar *et al.,* 2014).

MATERIALS AND METHODS

Materials

The plant materials used in this study were multi-bulb and single-bulb garlic (*Allium sativum*) from traditional market in Malang, East Java. Meanwhile, the chemicals used were 70% ethanol (Onemed, Indonesia), p.a ethanol (Merck, Germany), $AlCl_3$ (Merck, Germany), 5% acetic acid, concentrated HCl (Merck, Germany), 5% $FeCl_3$ (Merck), gallic acid (Sigma Aldrich, USA), Folin-Ciocalteu (Merck, Germany), DPPH (Sigma Aldrich, USA), and distilled water.

Additionally, the apparatus used included the OHAUS MB35 Moisture Analyzer, analytical balance (Sartorius B1 210 S, Germany), gram balance (Ohaus cen-O-Balance, US), UV-Vis spectrophotometer (Shimadzu UV-1800, Japan), rice cooker, oven, blender, mesh sieve 30, maceration apparatus, rotary evaporator, water bath, ultrasonic bath, micropipette, and laboratory glassware.

Processing black garlic

Black garlic was made by incorporating garlic wrapped in aluminum foil into a 7.0 L ripening chamber without peeling the skin at temperature and humidity levels of 60-70°C and 70-80%, respectively, and leaving it to stand for 5, 10, 15, 20, and 25 days (Choi *et al.,* 2014; Zhang *et al.,* 2016). Five kilograms of garlic were inserted at the beginning and were taken one kilograms every five days. Temperature and humidity were controlled using alumunium foil and the addition of water, both of which were monitored using a thermohygrometer. Subsequently, the skin of the black garlic is removed, thinly sliced, put in the oven at 50°C until dry before being mashed with a blender and sieved at a mesh size of 30. The moisture content (MC) determination of the fine and homogeneous powder was conducted using the moisture analyzer tool (OHAUS MB35).

Extraction of black garlic

About 100 g of black garlic powder was weighed and macerated in 500 mL of 70% ethanol for 24 hours at room temperature. Subsequently, the residue was re-extracted two times with the same method, while all the filtrate was concentrated using a rotary evaporator and a water bath in order to obtain a thick extract of black garlic (Azizah *et al.,* 2014).

Measurement of total phenolic content

The total phenolic content of black garlic extract was determined by spectrophotometric method using gallic acid standards by dissolving 15.0 mg of thick extract in 5.0 mL of distilled water. 300 μL of the mixture was pipetted and 1.5 mL of Folin-Ciocalteu and 1.2 mL of 7.5% solution of Na_2CO_3 were added. Subsequently, the admixture was homogenized and allowed to stand for 20 minutes at room temperature before the absorbance of the admixture was measured at a wavelength of 765 nm. Furthermore, the total phenol content expressed as % w/w GAE (gallic acid equivalent) (Khoddami *et al.,* 2013).

Measurement of flavonoid content

The flavonoid content of black garlic extract was determined using the spectrophotometric methods using quercetin standard. The procedure involved the dissolution of 50.0 mg of thick black garlic extract in 5.0 mL of ethanol. The admixture was pipetted 1.0 mL and was added 1.0 mL of 10% $AlCl_3$ and 8.0 mL of 5% acetic acid. Subsequently, the homogenized mixture was allowed to stand for 15 minutes at room temperature before the absorbance was measured at a wavelength of 354.6 nm. The total flavonoid content expressed as % w/w QE (Quercetin Equivalents) (Kumar *et al.,* 2017).

Determination of antioxidant properties

The antioxidant activity of black garlic extract was determined through DPPH attenuation using a UV-Vis spectrophotometer. The procedure involved the dissolution of the garlic extract in 70% ethanol, which was further diluted to prepare five different test solution concentrations. Subsequently, the mixture of 2.0 mL test solution and 4.0 mL DPPH was allowed to stand for 15 minutes at room temperature and avoid light before the absorbance was measured at a wavelength of 518 nm. The antioxidant activity of black garlic extract was expressed as the EC_{50} of the attenuation activity (Xie and Schaich, 2014).

Data analysis

The data in this study were analyzed with the SPSS 16 program using one-way ANOVA (Analysis of Variance). Meanwhile, the significant differences were concluded at *p*-value <0.05 using the LSD test as a post hoc test. All measurements were used three times for replication (Rahma *et al.,* 2014).

RESULTS AND DISCUSSION

Moisture content of black garlic powder

The results showed that the moisture content (MC) or water content of the black garlic powder was less than 10% (in wet basis) each time it was dried in an oven. Multi-bulb and single-bulb black garlic produced an MC ranging from 2.7 to 9.8% between day 0 to 25, as shown in Table 1. According to the quality specifications of traditional medicine in Perka BPOM RI No. 12/2014, traditional medicine preparations in the form of simplicia powder should have a water content less than or equal to 10% (BPOM RI, 2014). The greater the moisture content of simplicia, become greater the risk of microorganism contamination during storage, which can reduce the stability of the simplicia (Saifuddin *et al.,* 2011).

Table 1. The moisture content (MC in wet basis) of black garlic powder

Test Material	Processing Time (days)	MC %
Multi-bulb black garlic	0	7.3±1.0
	5	3.3±0.1
	10	3.3±0.0
	15	6.8±1.2
	20	8.9±1.1
	25	7.5±0.2
Single-bulb black garlic	0	8.3±0.2
	5	6.5±0.1
	10	2.7±0.1
	15	6.7±1.0
	20	5.7±1.3
	25	2.8±0.4

Black garlic extract

The black garlic powder with the suitable MC was extracted using 70% ethanol as the solvent through the maceration method. This solvent can extract active compounds such as phenols and flavonoids from a plant more effectively than other solvents such as water, methanol, acetone, and some ethanol with different concentrations (Suhendra *et al.,* 2019; Padmawati *et al.,* 2020). Maceration is the simplest extraction method, which involves soaking without the use of heat. Therefore, it protects the active compounds in simplicia, such as flavonoid compounds from being degraded by heat (Sa'adah *et al.,* 2017).

Total phenolic content

The principle of the total phenolic measurement of black garlic extract is based on the conversion of phenolic compounds that is expressed as GAE (gallic acid equivalents), which is a related response of all the major phenolic compounds found in plants (Kim *et al.,* 2013). The results showed that the total phenolic content of multi-bulb black garlic on days 0, 5, 10, 15, 20, 25 were 0.95, 5.72, 5.62, 6.02, 8.28, and 4.45% w/w GAE, respectively. Meanwhile, the total phenolic of the single-bulb black garlic were 0.85, 4.95, 4.19, 5.50, 5.84, and 4.32% w/w GAE, as shown in Figure 1. The graph of total phenol content showed that the processing time had an effect on the total phenol content of multi-bulb and single-bulb black garlic.

The highest total phenol content in both multi-bulb and single-bulb black garlic were produced on the 20th day of processing and decreased on the 25th day. Additionally, multi-bulb black garlic had the highest total phenol content with a processing time of 20 days, which was 8.28% w/w GAE, while single-bulb black garlic produced 5.84% w/w GAE. It's caused by the phytochemical compounds of multi-bulb black garlic extract being more water soluble than single-bulb black garlic (Phadar *et al.,* 2014). The increase in total phenol content can be attributed to the heating process due to the breakage of the bonds of complex phenolic compounds into free forms through the glycosylation process (Kim *et al.,* 2013), whereas the decrease in the total phenol contents can be attributed to compound degradation when exposed to overheating for over 25 days (Kim *et al.,* 2013; Zhang *et al.,* 2016; Mardiah *et al.,* 2017).

Flavonoid content

Flavonoids have many derivative compounds, including flavonols, isoflavonoids, and flavones that have antioxidant activity and widely used as a new source of bioactive compound in the development of drug or functional foods (Kim *et al.,* 2013). The flavonoid contents of black garlic extract were determined using quercetin as the standard because it has a group of flavonol with a hydroxyl group on the C-3 and C-5 atoms and a keto group on the C-4 atom (Azizah *et al.,* 2014). According to Figure 2, the results showed that the flavonoid content of the black garlic during processing time 0, 5, 10, 15, 20, and 25 days were 3.56, 24.83, 25.12, 16.23, 18.98, and 16.01% w/w QE, respectively. Meanwhile, the flavonoid content of the single-bulb black garlic was 2.36, 8.32, 17.25, 17.98, 16.20, and 14.10% w/w QE. The data on the flavonoid content were statisti-cally analyzed as same as phenolic content and exhibited a significant difference in flavonoid content of multi-bulb and single-bulb black garlic.

According to the graph of the flavonoid contents in the processing of multi-bulb and single-bulb black garlic, it can be inferred that the processing time has an effect on flavonoid contents, as well as the total phenol contents. The highest flavonoid content in multi-bulb black garlic was observed during 10 days of processing, which decreased drastically on the 15th day and slowly increased until the 25th day. The processing of single-bulb black garlic on the 15th day produced the highest flavonoid contents, which decreased until the 25th day but not significantly. The multi-bulb black garlic produced the highest flavonoid content of 25.12% w/w QE after ten days of processing, while the single-bulb black garlic was 17.25% w/w QE. Meanwhile, the highest flavonoid content in the single-bulb black garlic was produced after 15 days of processing at 17.98% w/w QE, while the multi-bulb black garlic was 6.23% w/w QE.

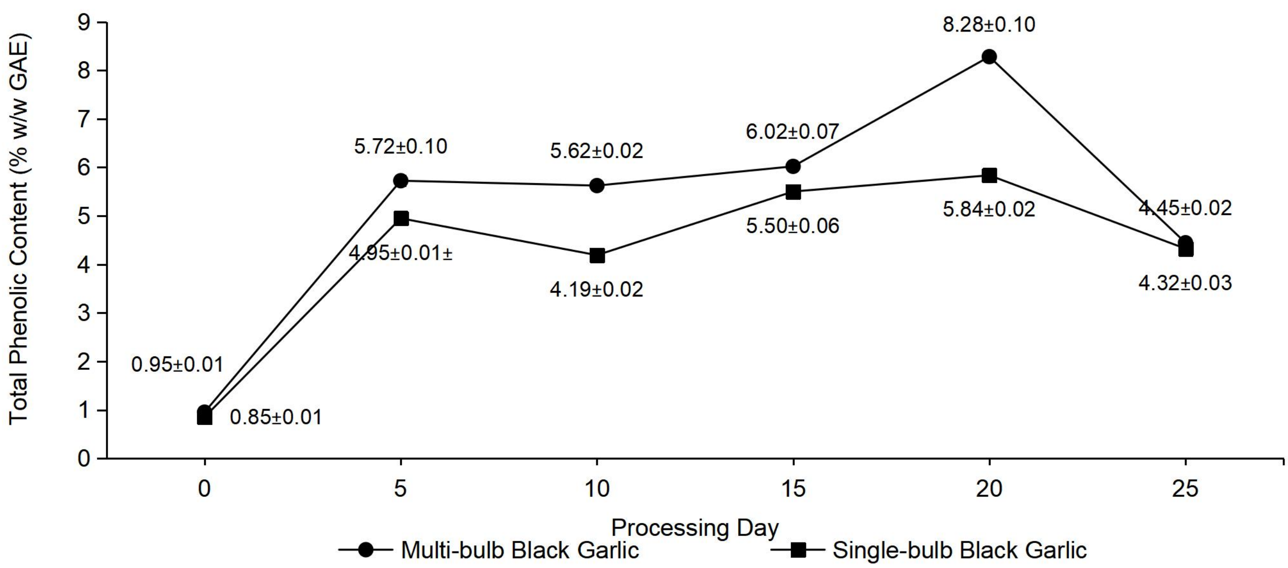

Figure 1. The difference of the total phenol between multi-bulb and single-bulb black garlic on processing day 0 to day 25

The increased contents of flavonoid compounds in black garlic can be caused by the processing method, which uses variable heating and humidity parameters. Additionally, this is due to the fact that the process can degrade the structure of cellulose in plant cells and generate bioactive compounds, such as flavonoids (Kim *et al.,* 2013). The flavonoid content of multi-bulb black garlic is also higher than single-bulb black garlic as the same as total phenolic content, because flavonoids and phenols are components with high solubility in polar solvents such as water, ethanol, and methanol (Phadar *et al.,* 2014).

Antioxidant properties

The presence of phenols and flavonoids content in black garlic indicates antioxidant activity. The non-enzymatic browning reaction that occurs during the heating process of garlic, such as the Maillard reaction and chemical oxidation of phenols, can give the dark brown colour of garlic and lead to the formation of some compounds that have antioxidant activity (Zhang *et al.,* 2016). In addition, the flavonoid content of garlic has functional hydroxyl groups that have an antioxidant effect by chelating metal ions or scavenging free radicals (Kumar and Pandey, 2013).

The antioxidant activity of black garlic extract was determined using a DPPH (*1,1-diphenyl-2-picrylhydrazyl*) free radical scavenger, with the purple color of the DPPH solution at an absorbance of 518 nm. DPPH is a radical since it has an unpaired atom, but it can be converted to a non-radical (*1,1-diphenyl-2-picrylhydrazyl*) when the unpaired atom receives a hydrogen atom donor from an antioxidant compound. Subsequently, the purple color fades when the DPPH solution is mixed with antioxidant compounds, which indicates changes in non-radical compounds (Agustina *et al.,* 2020; Setiawan *et al.,* 2018).

The EC_{50} value is used to determine the antioxidant activity of multi-bulb and single-bulb black garlic extracts in reducing DPPH radicals. The EC_{50} value in determining antioxidant activity is defined as the effective concentration that can reduce free radicals by up to 50% (Chen *et al.,* 2013). The results showed that the EC_{50} values of multi-bulb black garlic extract were 2042.61, 219.99, 737.58, and 24.38 ppm during the 0, 15, 20, and 25 days, respectively. Meanwhile, the EC_{50} of the single-bulb black garlic extract was 4721.95, 37.42, 27.49, and 144.42 (Figure 3). The EC_{50} value had obtained a *p*-value < 0.05 when statistically analyzed using the One Way ANOVA test. Analysis continued with post hoc using the LSD test and exhibited that there was a significant difference in all the data for each test sample, which can be used to compare the parameters in this study.

According to the graph showing the antioxidant activity of multi-bulb and single-bulb black garlic, it can be inferred that there is a decrease in the EC_{50} value when black garlic is processed at longer times since that EC_{50} with a lower value indicates a higher antioxidant activity. Multi-bulb black garlic processed for 25 days had the highest antioxidant activity with an EC_{50} value of 24.39 ppm, while single-bulb black garlic had an EC_{50} value of 144.42 ppm. Meanwhile, single-bulb black garlic had the highest antioxidant activity processed for 20 days with an EC_{50} value of 27.49 ppm, while multi-bulb black garlic had an EC_{50} value of 737.59 ppm.

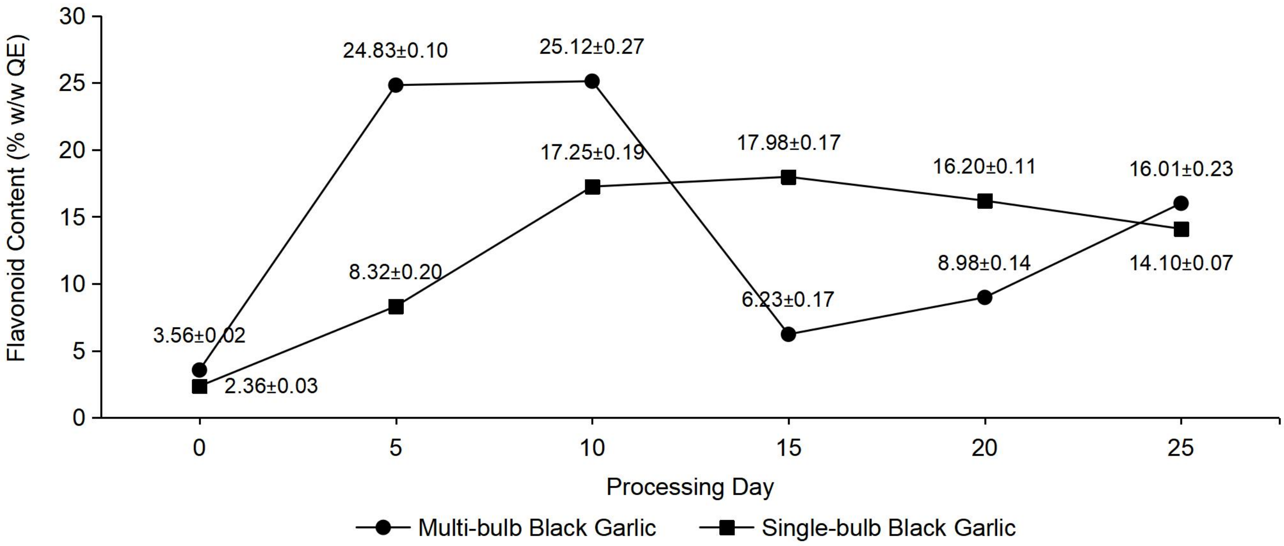

Figure 2. Flavonoid content of multi-bulb and single-bulb black garlic on processing day 0 to day 25

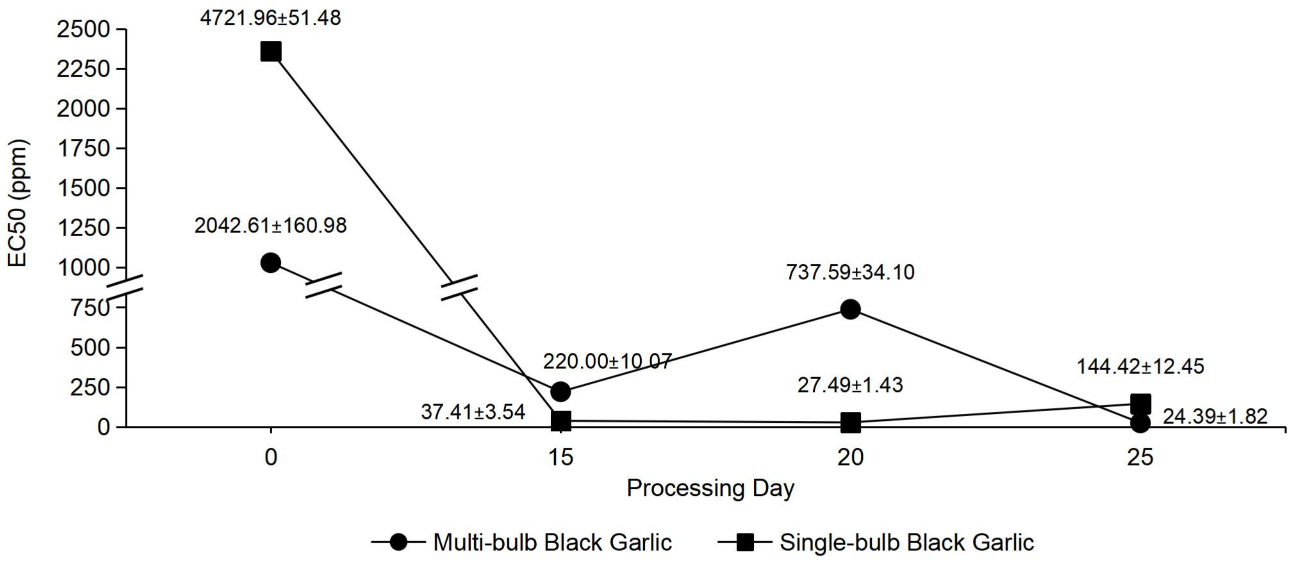

Figure 3. The EC_{50} value of multi-bulb and single-bulb black garlic on processing day 0 to day 25

A previous study by Choi *et al.* (2014) revealed that the highest antioxidant activity of black garlic was obtained on day 21 using a temperature of 70°C and 90% humidity (Choi *et al.,* 2014). The antioxidant activity may be due to the influence of the SAC (*S-allyl cysteine*) compound formed from allicin during the heating process, which may be the reason black garlic has a higher SAC content than white garlic (Bae *et al.,* 2014; Colín-González *et al.,* 2012). *S-allyl cysteine* compound in black garlic has activities as antidiabetic (Kim *et al.,* 2016) and cardioprotector (García-Villalón *et al.,* 2016).

CONCLUSIONS

The processing time for multi-bulb and single-bulb black garlic over 0-25 days at temperature and humidity levels of 60-70°C and 70-80%, respectively, had an effect on the content of total phenolic and flavonoid, as well as the antioxidant activity. The longer processing time caused an increase in the total phenolic content, flavonoid content, and a decrease in the value of EC_{50} at a particular time. Conversely, content and activity were decreased if the processing time was too long. The highest content of total phenolic in multi-bulb and single-bulb black garlic was observed on the 20th day of processing, and multi-bulb black garlic was greater than single-bulb black garlic. Meanwhile, the highest flavonoid content of both black garlic was observed on different days. Multi-bulb black garlic was observed on the 10th day of processing, which was greater than that of single-bulb black garlic during the 15th day of processing. Lastly, the highest antioxidant activity of multi-bulb black garlic was observed during the 25th day of processing, which was higher than that of single-bulb black garlic on the 20th day. Therefore, the optimal process is obtained by heating for 20th days because the content of phenol, flavonoid, and antioxidant activity was relatively high. More efforts should be made to measure single active compounds such as allicin and SAC to determine the quality of each black garlic through its processing time.

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