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Antioxidant Activity of the Ethyl Acetate Fraction, N- Hexane Fraction from the Ethanol Extract of Black Garlic (Allium Sativum L.) using the 2,2- Diphenyl 1-Picrylhydrazyl (DPPH)

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A B S T R A C T

Garlic (Allium sativum L.) is a plant of the Amaryllydaceae family which is known to have potential as an antioxidant. Black garlic is a fermented product of heating garlic at 70-80°C with 70-80% humidity for 21 days. This study aims to test the antioxidant activity of the ethyl acetate fraction, n-hexane fraction and ethanol extract of black garlic. Black garlic was extracted using maceration method with 70% ethanol solvent and also using ethyl acetate and n-hexane solvents for fractinations. The results of the phytochemical test showed that the ethyl acetate fraction contained alkaloids, flavonoids, phenols and tannins; The N- hexane fraction contains steroid and terpenoid compounds; The ethanol extract contains alkaloids, flavonoids, phenols, tannins, saponins, steroids and terpenoids. The antioxidant activity with the DPPH method obtained IC50 the ethyl acetate fraction, n-hexane fraction and ethanol extract respectively of 31.6818 µg/mL, 194.5385 µg/mL and 6.6000 µg/mL. The results showed that the ethanol extract and ethyl acetate fraction had very strong antioxidant activity, while the n-hexane fraction had weak antioxidant activity.

Keywords: Antioxidant, Black Garlic, DPPH

INTRODUCTION

Garlic (Allium sativum. L) type of plant that has been widely used both in the food and health sectors. Garlic has potential as a raw material for medicines to cure various diseases (Samadi, 2000). Aside from being a cooking spice, traditionally garlic bulbs are used to treat high blood pressure, respiratory problems, headaches, hemorrhoids, constipation, bruises or cuts, intestinal worms, cholesterol, flu, urinary tract disorders, and others (Thomas, 2000). ;Rukmana, 1995). Garlic contains about 63% water, 28% carbohydrates (fructans), 2.3% organosulfur component acids, 2% protein (alliinase), 1.2% free amino acids (arginine), and 1.5% fiber (Kimura et al., 2017).

Black garlic is the result of processing garlic through a heating process with a temperature of 70°C and 70-80% humidity for 30-40 days without the addition of other substances or any additional treatment (Wang et al., 2012). Black garlic has a strong antioxidant content (Kimura et al, 2017). Antioxidants are compounds that can inhibit oxidation reactions by binding to free radicals and highly reactive molecules (Winarsi, 2007). Several studies have shown that black garlic has antioxidant activity which is very useful for preventing several diseases, including inhibiting the growth of cancer cells and anti-allergy (Kimura et al., 2017), preventing premature aging (Kim et al., 2013), anti-obesity (Wei et al., 2017), lowers blood lipid levels (Ha et al., 2015) and lowers blood sugar levels (Rosita, 2016).

Based on research conducted by Choi et al., (2014) black garlic extracted with deionized water was prepared for 7, 14, 21, 28 and 35 days under controlled conditions of 70°C and 90% relative humidity. The results of this study showed an increase in antioxidant activity in black garlic occurred at a maximum when heated for 21 days, the results of these tests on fresh garlic and black garlic had antioxidant activity of 4.65% and 74.48%.

As for other studies that have been conducted by Amin (2015) garlic bulbs extracted with ethanol solvent, and fractionated respectively with n-hexane, ethyl acetate, and water solvents, obtained ethanol extract and ethyl acetate fraction had antioxidant activity that was strong with the IC50 ethanol extract was 13.85 ppm and the ethyl acetate fraction was 7.27 ppm.

In research of Yani (2019) the antioxidant activity test of black garlic ethanol extract with a heating time of 12 days, the black garlic ethanol extract obtained an IC50 value of 637.7955 µg/mL (very weak antioxidant). Based on the description above, the researchers were interested in testing the antioxidant activity of the ethyl acetate fraction, n-hexane fraction of ethanol extract of black garlic using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) method.

METHOD

Tools

The tools used in this study was a UV visible spectrophotometer (UV-1700 PharaSpec), rotary evaporator (Heidolph), separatory funnel (Iwaki), analytical balance (Biobase), rice cooker (Polytron), blender (Philip®), filter paper, spatula, aluminum foil, maceration container (dark bottle), beaker glass (Iwaki®), volumetric flask (Iwaki®), evaporating cup, porcelain crucible, dropping pipette, measuring pipette (Iwaki®), test tube (Iwaki®), funnel (Pyrex®), and test tube rack.

Material

The ingredients used are 2 kg of fresh garlic (Allium sativum L.). The chemicals used were Ethanol (C2H5OH) 70% (PT.Novalindo), Hydrochloric Acid (HCl) (Merck), Iron (III) Chloride (FeCl3) (Merck), Potassium Iodide (KI) (Merck), Acetic Acid Anhydrous (CH3COOH) (Merck), Mercury (II) Chloride, Mg powder, aquadest (PT.Novalindo), Methanol (CH3OH) pa (PT. Novalindo), Ethyl Acetate (CH3CH2OC(O)CH3) (PT. Novalindo), N Hexane (PT.Novalindo), 2,2-dyphenyl-1-picrylhydrazyl (DPPH) (Sigma), Gallic acid (C7H6O5) (PT.Nitra Kimia), Sulfuric Acid (H2SO4) (PT Bratachem).

RESEARCH PROCEDURE

Sample

Sample used in this study was garlic (Allium sativum. L) which was purchased as much as 2 kg at Nanggalo Market, Padang City, West Sumatra.

Identification of Plant

Identification of garlic conducted at the Herbarium Laboratory of Andalas University (ANDA), Department of Biology, Faculty of Mathematics and Natural Sciences (FMIPA) Andalas University (UNAND) Padang, West Sumatra.

Making Black Garlic

Garlic as much as 2 kg was chosen which is large, not rotten, and still intact with other cloves, not broken ones. Garlic is left unpeeled and left dry and not moist. Garlic is put into the rice cooker with a temperature of 70-80º C and arranged not overlapping each other to prevent damage to the shape of the black garlic. The rice cooker is closed and set in keep warm mode and left for 21 days.

After 21 days, garlic that has been black is removed and garlic are selected which have non-scorched clove skin, garlic which is black and wrinkled inside so that black garlic is obtained (Aini&Shovitri, 2018). The black garlic that has been obtained is then refined using a blender.

Preparation Ethanol Extract of Black Garlic

Simplicia was weighed as much as 200 g was put into a maceration bottle and then 2000 mL of 70% ethanol solvent was added (ratio 1:10 b/v). For the first 6 hours it is soaked while occasionally stirring, then left to stand for 18 hours. The macerate is separated by filtering, the maceration process is repeated 2 times, using the same type and amount of solvent. All macerate was collected, then evaporated with a rotary evaporator at 50℃ to obtain a thick extract. The yield obtained was weighed and recorded (Ministry of Health of the Republic of Indonesia, 2010). The yield percentage is the weight of the thick extract divided by the weight of the dry simplicia multiplied by 100% (Ministry of Health of the Republic of Indonesia, 2010).

Fractionation of Ethanol Extract

Viscous black garlic of ethanol extract obtained was successively fractionated using n-hexane, ethyl acetate and water as solvents. The ethanol extract of black garlic was weighed 10 mg and then diluted first using 100 mL of heated aquadest in a separating funnel, added 100 mL of n-hexane solvent, shaken and then allowed to stand until a boundary was seen between the two solvents, after which the fractions were separated. n-hexane was removed from the separatory funnel. The remaining water fractions were added with 100 mL ethyl acetate, then shaken and allowed to stand until a separation boundary was seen between the two solvents and then removed from the separatory funnel.

The extraction of each fraction was repeated three times using 100 mL of solvent for each extraction. The first, second and third extraction are collected. The fractionated extract was thickened using a rotary evaporator vacuum evaporator (Kurniasih et al, 2015). Phytochemical Screening Ethanol Extract, Ethyl Acetate Fraction and N-Hexane Fraction of Black Garlic

1. Flavonoid Test

A total of 0.5 g ethanol extract, ethyl acetate fraction and n-hexane fraction of garlic (Allium sativum L.). Each was dissolved with 3 mL of 70% ethanol, then 0.1 g of Mg powder and 2 drops of concentrated HCl were added. Positive results are indicated by the appearance of a yellow-orange color to dark red (Ministry of Health of the Republic of Indonesia, 1995).

2. Alkaloid Test

Weight as much as 0.5 g of ethanol extract, ethyl acetate fraction, n-hexane fraction of black garlic (Allium sativum L.) was added 1 mL of 2 N hydrochloric acid and 9 mL of distilled water, heated over a water bath for 2 minutes, cooled and filtered. The filtrate obtained was used to test alkaloids. Into 2 test tubes, put 0.5 ml of filtrate. In each test tube: 1. Add 2 drops of Bouchardat LP to form a brown to black precipitate. 2. Add 2 drops of Mayer LP reagent to form a white or yellow precipitate. Alkaloids are positive if there is sediment or turbidity in the reagent above (Ministry of Health of the Republic of Indonesia, 1995).

3. Saponin Test

Weight as much as 0.5 g of ethanol extract, ethyl acetate fraction, and n-hexane fraction of black garlic. Each put into a test tube, then add 10 ml of distilled water. Then shake vigorously for 10 seconds. If foam is formed for not less than 10 minutes, as high as 1 cm – 10 cm and on the addition of 1 drop of 2 N HCl, the foam does not disappear (Ministry of Health of the Republic of Indonesia, 1995).

4. Tannin Test

Weight as much as 0.5 g of ethanol extract, ethyl acetate fraction and n-hexane fraction of black garlic. Each of them was added 3 drops of 1% FeCl3 reagent to produce a greenish or blue-black color indicating the presence of tannins (Ministry of Health of the Republic of Indonesia, 1995).

5. Terpenoids and Steroids Test

Weight as much as 0.5 g of ethanol extract, ethyl acetate fraction and n-hexane fraction of black garlic. Each was added 3 ml of chloroform or 3 ml of

70% ethanol and added 2 ml of concentrated sulfuric acid (H2SO4) and 2 ml of anhydrous acetic acid. A change in color from purple to blue or green indicates the presence of steroid compounds and the formation of a brownish color between the surfaces indicates the presence of terpenoid compounds (Ministry of Health of the Republic of Indonesia, 1979).

6. Phenol Test

Weight as much as 0.5 g of ethanol extract, ethyl acetate fraction and n-hexane fraction of black garlic. Drop of hydrochloric acid P, the part containing phenol derivatives was intense red (Department of Health of the Republic of Indonesia, 1995)

Antioxidant Activity Test with DPPH Method

a) Preparation of 30 µg/mL DPPH Solution

Weigh approximately 10 mg of DPPH (BM 394.33). Then it was dissolved with methanol up to 100 mL, then placed in a volumetric flask lined with aluminum foil. Enough of the solvent up to the mark then shake until homogeneous and a DPPH solution with a concentration of 100 µg/mL is obtained. Then it was diluted by pipetting 15 mL of DPPH solution with a concentration of 100 µg/mL, put it in a 50 mL volumetric flask, enough solvent up to the mark, then shaken until homogeneous and a DPPH solution with a concentration of 30 µg/mL was obtained (Molyneux, 2004).

b) Preparation of Blanko Solution and Optimization of Maximum Wavelength of DPPH

Pipette 3.8 mL of DPPH solution (30 µg/mL) into a vial. Then 0.2 mL of methanol pa was added, closed the vial, then the mixture was homogenized and incubated in a dark room for 30 minutes. Determine the absorption spectrum using a UV-Visible spectrophotometer at a wavelength of 400-800 nm and determine the maximum wavelength.

c) Preparation Standard Solution of Gallic Acid

Weigh 10 mg of pure gallic acid, put it in a volumetric flask and then add methanol pa to 100 mL (100 µg/mL). Then a concentration series of 1 µg/mL, 2 µg/mL, 3 µg/mL, 4 µg/mL, 5 µg/mL was made, by pipetting 0.1 mL, 0.2 mL of main liquor (100 µg/mL). 0.3 mL, 0.4 mL and 0.5 mL then added with methanol pa to the mark of the 10 mL volumetric flask. Then pipette 0.2 mL of each concentration into the vial and add 3.8 mL of DPPH solution (30 µg/mL) then the vial is closed with aluminum foil. Incubated in a dark room for 30 minutes. The absorbance of various concentrations was measured with a UV-Visible spectrophotometer at the maximum wavelength of the DPPH (Andayani et al., 2008).

d) Antioxidant Activity Test of Black Garlic a. Ethanol Extract

Ethanol extract was weighed as much as 25 mg, then dissolved with methanol pa in a 25 mL volumetric flask, sufficient to mark the mark to obtain a concentration of 1000 µg/mL. Perform dilution from a concentration of 1000 µg/mL to a concentration of 100 µg/mL by pipetting 2.5 mL of a solution

of a concentration of 1000 µg/mL, put it in a 25 µg/mL volumetric flask, fill the volume with methanol up to the mark (Main solution 100 µg/mL) . Then a series of concentrations of 1 µg/mL, 2 µg/mL, 3 µg/mL, 4 µg/mL and 5 µg/mL were made. Make dilutions by pipetting 0.1 mL, 0.2 mL, 0.3 mL, 0.4 mL, and 0.5 mL, put in a 10 mL volumetric flask and bring the volume up to the mark. Then pipette 0.2 mL of each concentration into the vial and add 3.8 mL of DPPH solution (30 µg/mL) then the vial is closed with aluminum foil. Incubated in a dark room for 30 minutes. The absorbance of various concentrations was measured with a UV-Visible spectrophotometer at the maximum wavelength of the DPPH.

The calculation results of the antioxidant activity are entered into the equation line y = a + bx with the concentration (µg/mL) as the abscissa (x axis) and the % antioxidant activity value of 50% will be obtained from the line equation (Andayani et al., 2008). Do the same procedure for ethyl acetate fraction and N-Hexane fraction.

Data Analysis

Antioxidant activity

Antioxidant activity is determined based on the percentage reduction in DPPH free radical uptake by calculating the percentage inhibition of DPPH uptake by using the formula

Notes:

$$\% \text{ Inhibition} = \frac{\text{Controlabsorbancel-sample absorbance}}{\text{Control absorbance}} x\ 100$$

a) Absorbance control: absorption 30 ppm DPPH radical absorption at the maximum DPPH wavelength

b) Absorbance sample: sample absorption in 30 ppm DPPH radicals at the maximum DPPH wavelength.

Then it was compared with gallic acid based on the calibration curve and linear regression equation of gallic acid on the percent inhibition of DPPH solution by gallic acid.

c). IC50

From the calculation of % inhibition, a regression equation y = bx + a will be obtained so that the IC50. IC50 is the concentration of the sample solution that provides 50% (y = 50%) inhibition of DPPH radicals.

RESULTS AND DISCUSSION

After conducting research on the antioxidant activity test of the ethyl acetate fraction, N-hexane fraction from the ethanol extract of garlic (Allium sativum L.), the following results were obtained: that the sample used was garlic (Allium sativum L.) with the Amaryllidaceae family.

The yield of black garlic (Allium sativum L.) ethanol extract. The yield of black garlic ethanol extract was 34.5382%. Results of phytochemical screening performed on ethanol extract, ethyl acetate fraction and n-hexane fraction. The ethanol extract showed positive results for the presence of alkaloids, flavonoids, phenols, tannins, saponins, steroids and terpenoids. The ethyl acetate fraction showed positive results, containing alkaloids, flavonoids, phenols and tannins. The n-hexane fraction showed positive results for the presence of steroid and terpenoid compounds.

Fresh garlic bulbs are processed into black garlic by placing them in a rice cooker with a temperature of 70-80ºC and arranging them not to overlap each other to prevent damage to the shape of the black garlic. The rice cooker is closed and set in keep warm mode and left for 21 days. During the heating process, garlic changes color, texture, taste and aroma until it becomes black garlic. The color change that occurs is from white to yellow, then light brown, dark brown until finally it becomes black. The texture of garlic changes from firm, then soft and runny to dry and becomes black garlic. The taste of garlic changes from spicy to sweet when it becomes black garlic. Changes in the aroma of garlic from pungent to not overpowering when it becomes black garlic. During the heating process, the unstable components of garlic including allicin turn into stable components such as S-Allyl Cysteine (SAC) (Lee et al., 2009). The increase in S-Allyl Cysteine (SAC) occurs due to the enzymatic hydrolysis of γ-glutamyl-S-allyl cysteine by γ-glutamyl transpeptidase to become S-Allyl Cysteine (SAC). S-Allyl Cysteine (SAC) is a component that functions as an antioxidant.

SAC is an important water-soluble organosulfur component (Jones et al., 2007). The amount of SAC increases significantly during the heating process (Bae et al., 2014). In addition, the content of total polyphenols and total flavonoids also increased (Choi et al., 2014). Goristein et al., (2008) showed that processing garlic into black garlic causes change bioactive compounds, such as polyphenols, flavonoids during the heating process. In the research conducted by Choi et al., (2014). During the heating process, the increase in total polyphenolic compounds and total flavonoids occurred in optimal heating for 21 days.

The maximum absorption wavelength measurement results of 2,2-diphenyl-1-picrylhydrazyl (DPPH) solution with a concentration of 30 µg/mL in methanol pa produce a maximum absorption at a wavelength of 515.50 with absorbance 0.646. The longer it wards off free radicals in DPPH and the greater the percentage of inhibition (Bahriul et al., 2014). The IC50 or free radical scavenging activity of 50% obtained from standard gallic acid was 4.7182 µg/mL (very strong antioxidant <50 µg/mL). Gallic acid is used as a comparison because gallic acid is a type of phenolic compound that has strong antioxidant activity and is a cheap, pure compound with high stability. The 515.50 wave obtained is included in the visible light wavelength range of 400-800 nm, and is included in the DPPH wavelength range of 515-520 nm (Molyneux, 2004). (Fig.1)

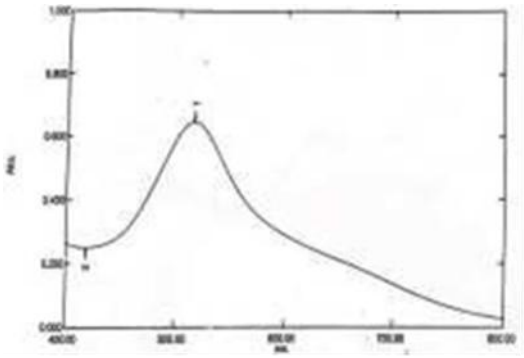

Fig 1. The maximum absorption wavelength of DPPH 30 µg/mL

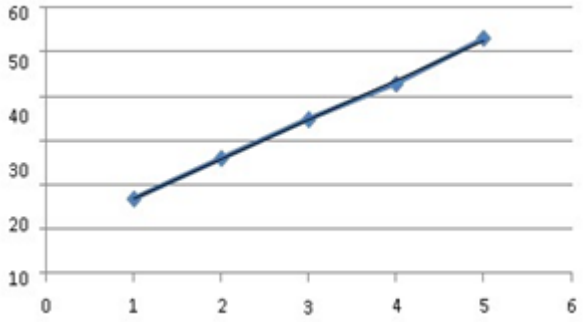

Fig 2. Gallic Acid Calibration Curve

Testing the antioxidant activity of gallic acid as a comparison, the absorbance of the solution was obtained 0.537; 0.478; 0.421; 0.369; 0.304 and percent radical scavenging activity 16.8730%; 26.0061 %; 34.8297%; 42.8792%; 52.9411% (Fig.2). Judging from the absorbance results, it can be seen that the greater the concentration of the sample, the smaller the absorbance value obtained, this is because the higher the antioxidant compound that is able to reduce free radical.

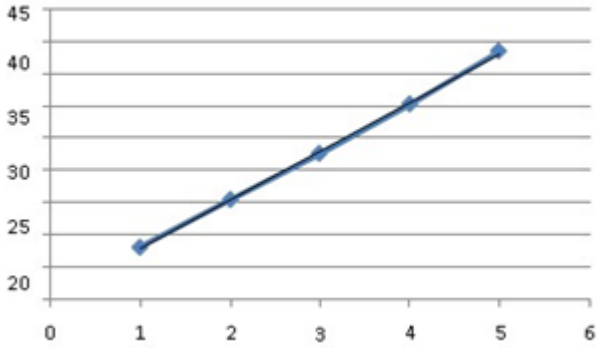

Fig 3. Calibration Curve of Ethanol Extract

In testing the antioxidant activity of black garlic ethanol extract, the absorbance was obtained solution 0.593; 0.546; 0.500; 0.451; 0.398 and percent radical scavenging activity 8.2043%; 15.4798%; 22.6006%; 30.1857%; 38.3900% (Fig.3). Judging from the absorbance results it can be seen that the greater the concentration of the sample, the lower the absorbance value obtained, this is because the higher the antioxidant compounds are able to reduce or counteract free radicals in DPPH and the greater the percentage of inhibition (Bahriul et al., 2014). The IC50 or 50% free radical scavenging activity obtained from black garlic ethanol extract was 6.6000 µg/mL (a very strong antioxidant <50 µg/mL).

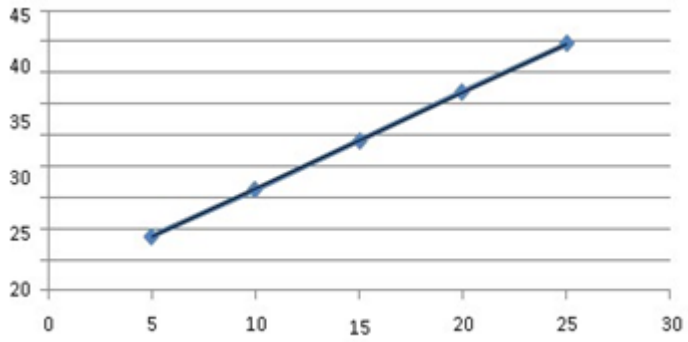

Fig 4. Ethyl Acetate Fraction Calibration Curve

In testing the antioxidant activity of the ethyl acetate fraction from blackened garlic ethanol extract, the absorbance of the solution was 0.589; 0.541; 0.490; 0.439; 0.390 and percent radical scavenging activity 8.8235%; 16.2537%; 24.1486%; 32.0433%; 39.6284% (Fig.4). Judging from the absorbance results, it can be seen that the greater the concentration of the sample, the lower the absorbance value obtained, this is due to the higher antioxidant compounds that are able to reduce or counteract free radicals in DPPH and the greater the percentage of inhibition (Bahriul et al., 2014) . The IC50 or free radical scavenging activity of 50% obtained from the ethyl acetate fraction was 31.6818 µg/mL (a very strong antioxidant <50 µg/mL).

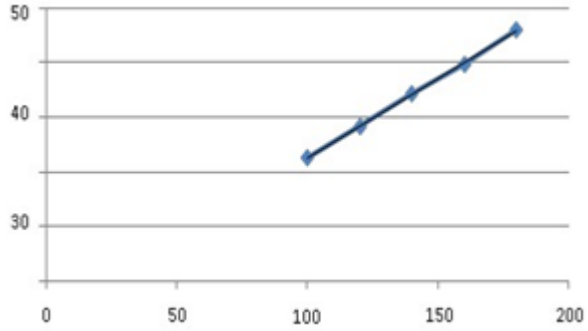

Fig 5. N-Hexane Fraction Calibration Curve

In testing the antioxidant activity of the n-hexane fraction from blackened garlic ethanol extract, the absorbance of the solution was 0.500; 0.463; 0.424; 0.390; 0.349 and percent radical scavenging activity 22.6006%; 28.3281%; 34.3653%; 39.6284%; 45.9752% (Fig.5). Judging from the absorbance results, it can be seen that the greater the concentration of the sample, the lower the absorbance value obtained, this is due to the higher antioxidant compounds that are able to reduce or counteract free radicals in DPPH and the greater the percentage of inhibition (Bahriul et al., 2014) . The IC50 or free radical scavenging activity of 50% obtained from the n-hexane fraction was 194.5385 µg/mL (weak antioxidant >100 µg/mL).

The difference in the IC50 ethanol extract and each fraction is caused by the distribution of secondary metabolite compounds that act as antioxidants (flavonoids, phenols and tannins) based on the polarity of the solvent used. The results showed that the ethanol extract was polar and had stronger antioxidant properties with 6.600 µg /mL when compared to the ethyl acetate and n-hexane fractions. This is presumably due to the content of compounds that can reduce free radicals more, because they can attract polar and semipolar compounds which generally have OH groups found in phenolics and flavonoids. Polyphenols in ethanol extract produce strong antioxidant activity in capturing free radicals.

In the previous research, namely Yani, (2019) tested the antioxidant activity of garlic ethanol extract and black garlic ethanol extract by heating the garlic to black for 12 days, the garlic extract obtained an IC50 at a concentration of 670 .0333 µg/mL ± 1.8609 (very weak antioxidant 200-1000 µg/mL), for black garlic the IC50 value was at a concentration of 637.7955 µg/mL ± 1.7879 (very weak antioxidant 200-1000 µg/mL). Based on this, it can be concluded that the heating time for black garlic can affect the antioxidant activity obtained.

CONCLUSION

Based on research conducted on antioxidant activity tests of the ethyl acetate fraction, n-hexane from black ethanol extract of garlic (Allium sativum L.), it can be concluded that:

1. The content of secondary metabolites in the ethyl acetate fraction contains alkaloid compounds, flavonoids, phenols and tannins. The N-hexane fraction contains steroid and terpenoid compounds. The ethanol extract contains alkaloids, flavonoids, phenols, saponins, tannins, steroids and terpenoids. The conclusion is that the ethanol solvent attracts the most chemical compounds.

2. The ethyl acetate fraction, N-hexane fraction and ethanol extract have antioxidant activity. Antioxidant activity of the ethyl acetate fraction IC50 31.6818 µg/mL (very strong <50 µg/mL), the n-hexane fraction IC50 194.5385 µg/mL (weak >100 µg/mL), and ethanol extract IC50 6.6000 µg/mL (very strong <50 µg/mL). The antioxidant activity of the ethanol extract was higher than the ethyl acetate and n-hexane fractions.

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