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Characterization and Biological Activity Test of Garlic and Its Fermentation as Antioxidant, Analgesic, and Anticancer

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ABSTRACT
The purpose of this study was to characterize and biological activity test of garlic and its fermented products (black garlic) as antioxidants, analgesics, and anticancer properties. The subject of this study was garlic and its fermented products (black garlic). Characterization includes analysis of total phenol content, flavonoid content, and identification of isolated components by FT-IR and GC-MS. The analysis of total phenolic and flavonoid content was carried out by spectroscopic methods. The antioxidant activity test used the DPPH (2,2-diphenylpicrylhydrazyl) method. The analgesic test used the mouse stretching method induced by acetic acid. Anticancer tests are carried out through cytotoxicity tests on several cancer cell lines. The cytotoxicity test method was carried out with the MTT Cell Proliferation Kit using a colorimetric method. The results showed that the garlic and black garlic extracts had high phenolic and flavonoid content. Chromatographic separation of ethyl acetate extract from black garlic obtained compound A with a purity of 67.69%. The results of the identification of compound A using IR and GC-MS showed that the isolated compound had similarities with 5-hydroxymethylfurfural. The antioxidant activity test of each extract and fraction of garlic and black garlic was classified as low, except for the ethanol extract of black garlic which showed high antioxidant activity with IC_{50} 38.609 ± 0.11µg/mL. The ethanol extracts of garlic and black garlic showed analgesic activity but showed low toxicity in several types of cancer cells T47D, 4T1, MCF7 / Her2, HeLa, and WiDr.

Keywords: *Allium sativum, antioxidant, analgesic, anticancer, Garlic, fermented garlic, black garlic, phenolic.*

1. INTRODUCTION

The garlic plant has been cultivated since ancient Egypt, used as a spice and flavoring, and has medicinal potential, so it is widely used in the cultures of various nations. The health benefits of the chemical component garlic in treating various disorders have been studied in both animals and humans [1]. Several studies have shown that garlic and its fermented (black garlic) have antioxidants [2; 3; 4; 5; 6], anti-genotoxic [5], anti-inflammatory [6], anti-leukemic [7], and anti-allergic [8]. Single garlic or with the local name "bawang lanang" is included in the Alliaceae family of the genus Allium, with the species *Allium sativum* L. Single garlic or with the local name "bawang lanang" is included in the Alliaceae family of the genus Allium, with the species *Allium sativum* L. Today the use of garlic for health has developed in various product forms.

The black garlic produced from fresh garlic which is fermented for some time at a controlled temperature (60-90) °C under high humidity (80-90) % [9]. When compared to fresh garlic, black garlic does not give off a strong taste because of the reduced allicin content, and unlike regular garlic, this black garlic has a soft, chewy, savory texture and a slightly sweet taste. Figure 1 shows the image of garlic and black garlic. The increased bioactivity of black garlic compared to fresh garlic was associated with changes in physicochemical properties. Several studies showed differences in the composition and activity properties of fermented garlic (black garlic) and garlic [10]. This study aims to characterize and test the biological activity of garlic and its fermented products (black garlic) as antioxidants, analgesics, and anticancer.

Figure 1. Garlic (" bawang lanang") and its fermented (black garlic)

2. EXPERIMENTAL SECTION

2.1. Apparatus and reagent

In this study used various equipment including evaporator Buchi Rotavapor R-215, FTIR Shimadzu Prestige 21, GCMS QP 2010S Shimadzu, 722N Visible Spectrophotometer, vortex mixer, column chromatography, TLC chamber, tissue culture flask, 96 microtiter plates well, and glassware. Materials used include organic solvents such as ethanol, methanol, *n*-hexane, ethyl acetate, folin-ciocalteu reagents, sodium carbonate, gallic acid (Sigma), rutine (Sigma), aluminum nitrate, potassium acetate, white garlic, black garlic, 2,2-diphenyl-1-picrilhidrazil (DPPH, Aldrich), ascorbic acid (Aldrich), aquadest, T47D, 4T1, MCF7 / Her2; HeLa; and WiDr cell lines, some reagents such as washing solutions (99% RPMI (Gibco), 1% repellent, (Gibco); storage media -70 ºC (20% FBS (Gibco), 70% RPMI requiring 1% repellent and 1% fungizon, 10% DMSO); culture media (89% RPMI, 1% sprayer, 10% FBS); nitrocellulose filter paper 0.22 µm (Whatman)); the blue dye tripan (Sigma); MTT solution (3- (4,5-dimethyltiazol-2-il) -2,5-diphenyltetrazolium bromide dissolved in PBS at a concentration of 5 mg/ml; formazan solvent (10% SDS in 0.01 N hydrochloric acid), acetic acid, acetosal, carboxyl methyl celulose (CMC).

2.2. Procedures for extracting, isolating, and identifying components from garlic

Each garlic and black garlic are mashed to form a powder. A total of 1 kg of each garlic and black garlic was then macerated with ethanol for 24 hours at room temperature while stirring occasionally. The filtrate was separated and the residue was macerated again 3 times. The filtrate obtained was collected and concentrated using a vacuum evaporator, so that a thick ethanol extract was obtained. The ethanol extract obtained was then fractionated with n-hexane, chloroform, and ethyl acetate. Each extract from the fractionation results is then concentrated by vacuum, and used for further tests. Data from the extraction and partition results of each type of garlic are listed in Table 1. This study also extracted water from each type of garlic. Water extract is made by adding 500 ml of distilled water to 100 g of garlic sample then heated for 30 minutes. The mixture is then separated by filtering. The filtrate obtained is then used for research. Chromatographic separation of components was only carried out on the ethyl acetate fraction of black garlic that showed component A having a purity of 67.89 %. Component identification was carried out by IR and GC-MS.

2.3. Determination of total phenolic and flavanoid content

The determination of total phenolic content was carried out using the Folin-Ciocalteau reagent [11]. A total of 1 ml of 15% Na2CO3 solution and 2 ml of distilled water were added to the test tube containing the 0.5 ml (1000 µg/ml) sample solution. The solution was incubated for 10 minutes at 500C. The solution was then homogenized using a vortex mixer and let stand for 1 hour. The absorption of each solution was measured using a Visible Spectrophotometer at a wavelength of 760 nm. Gallic acid is used as a standard made at various concentrations. Total phenolic content is defined as milligrams of gallic acid per gram of extract. Each sample was repeated three times.

The determination of total flavonoid levels in each extract and fraction of white garlic and black garlic was carried out by the spectrophotometric method by adding $AlNO_3$ and CH_3COOK solutions [12]. A total of 0.5 ml of 10% $AlNO_3$ solution, 0.1 ml of CH_3COOK 1 M solution, and 4.3 ml of distilled water were added to the test tube containing 0.5 ml of sample solution (10.000 µg/ml). Each solution was repeated three times, then homogenized and allowed to stand for 40 minutes at room temperature. The absorbance of each sample solution was measured using a Visible Spectrophotometer at a wavelength of 416 nm. As a standard solution, rutine compounds are used at various concentrations. Total flavonoid levels were interpreted as rutine equivalent milligrams per gram of extract mass.

2.4. Antioxidant activity test

The antioxidant activity test was carried out by the DPPH method, namely using 2,2-diphenyl-1-picrylhydracil (DPPH) as a source of radicals [13]. The sample was dissolved in ethanol at various concentrations. The sample solution was reacted with DPPH solution (with a ratio of 1: 1). The solution was then placed in a dark room at room temperature for 30 minutes. The absorption of each solution was measured using visible spectroscopy at a wavelength of 516 nm and compared with the absorbance of the control solution. The antioxidant activity of each sample is calculated using the following formula:

$$\% \text{ Antioxidant activity} = \frac{[\text{A Control} - \text{A sample}]}{[\text{A Control}]} \times 100\%$$

The IC_{50} value of each sample was calculated using a regression equation of the percentage of antioxidant activity at various concentrations. As a positive control, ascorbic acid was used.

2.5. Analgesic test

The analgesic test used the writhing method in mice [14]. Induction in male mice was done intraperitoneally by injecting acetic acid. Pain in male mice is indicated by stretching namely, the stomach (abdomen) touches the base where the male mice are placed and the male mice's legs are fully pulled back. Mice were divided into eight groups, each using 5 male mice. In group 1 (positive control), each mice was given acetosal orally and then induced with acetic acid. In group 2 (negative control), each mice was given CMC 0.5% orally then induced with acetic acid. In groups 3,4 and 5 each mice was given white garlic ethanol extract sequentially at a dose of 100; 200; 400 mg/kg BW, then induced with acetic acid. In groups 6,7,8, each mouse was given ethanol extract of black garlic sequentially, each with a dose of 100; 200; 400 mg/kg BW respectively. After 5 minutes of treatment, the number of stretches of each mice was counted every five minutes continuously for one hour. The percentage of protection against acetic acid induction with the formula:

$$\%\text{Protection} = \frac{\text{AWC-AWE}}{\text{AWC}} \times 100\%$$

AWC =The average number of writhing mice in negative control group
AWE = The average number of writhing mice in experiment group

2.6. Cytotoxicity activity test against cancer cells

The in vitro cytotoxicity test was investigated using 96 wells plate [15]. The sample is dissolved in a suitable phosphate buffer or solvent. Each well was put in 100 µl of RPMI media containing 4% penstrep, 100 µl of samples at various concentrations of 25 µg to 0.05 µg, then added 100 µl of cancer cells (Cancer cell lines used in this research: T47D, 4T1; MCF7 / Her2; HeLa; and WiDr) in culture media with the number of cells 5 x 104 each well. As a blank, 100 µl of potassium phosphate buffer pH 7.2 was used as a series dilution, and two replications were performed for each sample. At the end of the incubation, 10 µl of MTT (50 µg / ml) was added to each well, then incubated again for 4 hours. Living cells will react with MTT to form formazan which gives a purple color. The reaction was stopped by adding 100 µl SDS 10% in HCl 0.01 N, then incubated at room temperature overnight then read with a Benchmark microplate reader at a wavelength of 595 nm. Cell viability was calculated by the formula:

% of living cells = (abs P - abs M) / (abs K - abs M) x 100%
abs P = absorbance of cells treated; abs M = absorbance of the media
abs K = absorbance of control cells
The IC_{50} value is determined by probit analysis in the SPSS 11.5 program

3. RESULTS AND DISCUSSION

The garlic used in this study is local garlic obtained from farmers in the Wonosobo area of Central Java. Partially fermented garlic at 70ºC using magic com for two weeks to form black garlic. The sample in this study used 1 kg of garlic and 1 kg of black garlic. The two samples of each garlic are then peeled and blended until smooth. Each sample was then macerated using ethanol as a solvent. The maceration method is carried out by immersing the sample in ethanol solvent for 24 hours while stirring occasionally. Ethanol solvent was chosen because it can extract the desired substance, ethanol also has a low boiling point and can dissolve almost all secondary metabolites. Soaking is done three times. Each ethanol extract obtained was then concentrated using a Buchi R-215 rotary evaporator to obtain concentrated ethanol extract. Each extract was then partitioned successively using *n*-hexane, chloroform, and ethyl acetate, to obtain *n*-hexane fraction, chloroform fraction, ethyl acetate fraction, and ethanol-soluble residual fraction. Each fraction is then concentrated to obtain solids. The extract and fraction obtained are then used for further research. Data from the extraction and partition of each sample can be seen in Table 1. In this study, we also made water extracts from each type of garlic. The water extract was prepared by adding 500 ml of distilled water to 100 g of each sample of garlic and then heating it for 30 minutes. The mixture is then separated by filtering. The filtrate obtained was then used for research.

The determination of the total phenolic content was carried out by the method of adding Folin-Ciocalteau reagent [11]. The absorption of each mixture was measured at a wavelength of 760 nm. As a standard phenolic compound gallic acid was used at various concentrations (50-200 µg / ml) and the absorbance was measured at the same wavelength. The measurement results obtained by the calibration curve with the linear regression equation $y = 0.0043x + 0.0339$ ($R^2 = 0.993$). Total phenolic content is expressed as mg of Gallic Acid Equivalents (GAE) per g of plant extract. The determination of total flavonoid levels in each extract and fraction of garlic and black onions was carried out by the spectrophotometric method [12]. In this study, to determine the total flavonoid levels, Rutine standard compounds were used at various concentrations (50-200 µg / ml) and the absorbance was measured at a wavelength of 415 nm. The measurement results obtained by the regression equation as a calibration curve with a linear regression equation $y = 0.0032x + 0.0999$ ($R^2 = 0.9631$). The total flavonoid content is expressed as mg of Rutine Equivalents (RE) per g of plant extract. The results of the analysis of phenolic and flavonoid levels of each extract and fraction of garlic and black garlic are shown in Table 1.

Table 1. Extract and fraction result of white garlic and black garlic, total phenolic and flavonoid content

Sample		weight (g)	Phenolic content (mg galic acid/g sampel	Flavonoid content (mg rutin/g sampel)
A	Ethanol extract garlic	300	71.54±11.31	2.32 ± 0.22
	n-hexana fraction of garlic	80	79.59±6.64	6.55±0.75
	Chloroform fraction of garlic	120	77.77±11.57	2.11±0.87
	Ethyl acetate fraction of garlic	0	Not detected	Not detected
	Residu of ethanol fraction of garlic	95	68.56±5.13	0.23±0.09
B	Ethanol extract of black garlic	354	306.09±32.23	9.25±0.55
	n-hexana fraction of black garlic	75	36.86±37.16	1.11±0.23
	Chloroform fraction of black garlic	1.5	409.81±40.12	Not detected
	Ethyl acetate fraction of black garlic	3.5	293.76±28.61	10.66±0.58
	Residu of ethanol fraction of black garlic	210	411.209±17.75	9.81±0.43
C	Water extract of garlic (100 g/200 mL water)	150 ml extract water (not evaporated)	165.16±13.81	Not detected
	Water extract of black garlic (100 g/200 mL water)	150 ml extract water (not evaporated)	296.32±30.25	Not detected

The separation of components in the ethyl acetate fraction of black garlic was carried out using gravity column chromatography. From this separation, one component is obtained indicating high purity. Identification of these components using IR and GC-MS. The IR data of the isolated compounds are shown in Figure 1. The functional groups present in the isolated compound can be identified through an IR spectroscopic analysis. The results of the analysis using IR indicate that the compound contains a hydroxyl group, C = O; and C = C.

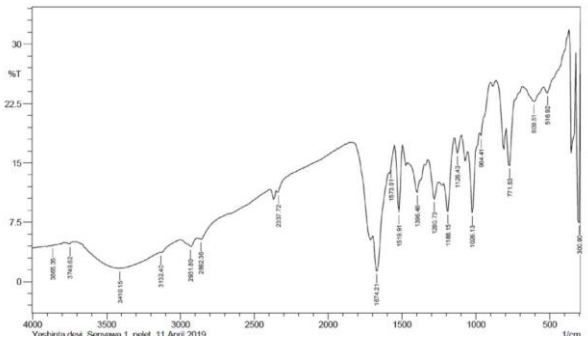


Figure 1. IR Spectrum of the compound isolated from the ethyl acetate fraction of black garlic

GC-MS analysis was used to identify the relative mass (m/z) and fragmentation of the isolated compound from the ethyl acetate fraction shown in figure 2. Based on the GC-MS spectrum, the isolated compound was still mixed with several other compounds.

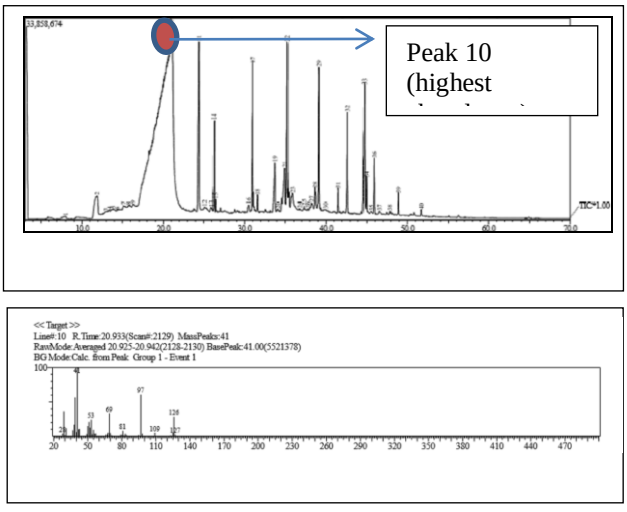


Figure 2. Spektrum GC-MS isolated compound

Table 2. The antioxidant activity test of each extract and fraction from garlic and black garlic

Sample		Antioxidant activity (IC_{50} (µg/mL)
A	Total ethanol extract garlic	938.897± 0.12
	n-Hexane fraction garlic	713.134± 1.26
	Chloroform fraction of garlic	1316.671± 2.12
	Residu of ethanol fraction of garlic	345.58± 0.19
B	Total ethanol extract black garlic	38.609± 0.11
	n-Hexane fraction of black garlic	363.69± 1.12
	Ethyl acetate fraction of black garlic	287.21± 0.34
	Residu of ethanol fraction of black garlic	311.09± 1.07
C	Water extract of garlic	913.083± 2.32
	Water extract of black garlic	220.71± 1.14

This is because the spectrum obtained has 40 peaks, but the highest abundance is at the 10th peak with an abundance of 67.89%. After conducting a literature study, the compound in the GC-MS data library, the fragmentation pattern that most closely matches the peak spectrum fragmentation pattern of 10th is the spectrum of 2-furancarboxyldehyde compound or often called 5-hydroxymethylfurfural. The compound has the molecular formula $C_6H_6O_3$, molecular weight m/z 126, and a similarity index of 83.

The mass spectrum at peak 10th has a molecular weight of 126 m/z which is thought to be the peak of molecular ion [M +]. The ion peak shown at m/z 125 is the release of the hydrogen molecule. Meanwhile, the peak that appears at m/z 97 is the termination of the side chain (-CO) attached to carbon atom number 2 followed by a ring change from pentagonal to hexagonal shape. According to According to the Metabolomics Innovation Center, the 5-hydroxymethyl-2-furancarboxaldehyde compound or commonly known as 5-hydroxymethylfurfural (5-HMF) can be found in black garlic [16]. This compound is obtained from various carbohydrate compounds. 5-HMF compound is a compound found in many plant species, including in tomatoes, tobacco oil, and others. This compound is used as an index of heating treatment and damage to foods such as tomato paste, honey, and fruit juices. A 5-HMF compound is a furan group, which is a compound containing a furan ring indicated by a pentagonal aromatic ring with one oxygen atom and four carbon atoms formed from amino acids with reducing sugars. These reactions usually require heat and occur during the preparation or manufacture of various types of food or beverages by roasting. This reaction causes changes in color, taste, and nutritional content[17]. 5-HMF was only obtained in the ethyl acetate fraction of black garlic, whereas in garlic it did not produce ethyl acetate fraction. This shows that the fermentation process has changed the carbohydrates or sugars in garlic to 5-HMF.

The antioxidant activity test of each extract and fraction from garlic and black garlic was carried out using the DPPH method. The test results of each extract and fraction are shown in Table 2. The results of this study indicate that the levels of phenolic compounds from the extract and fraction of black garlic are higher than those in white garlic. This is because in the fermentation process there is a hydrolysis reaction to break down phenolics in the form of glucosides. The presence of high phenolic compounds in the extracts and fractions affects their antioxidant activity. The results of this study indicate that the total ethanol extract of black garlic has the highest antioxidant activity with IC_{50} 38.609 ± 0.11µg / mL. Phenolic compounds are very good chemical compounds as oxygen radical scavengers. Phenolic compounds can act as reactive oxygen radical scavenger intermediates without triggering further oxidation reactions so that phenolic compounds are known to have antioxidant and antiradical activity[18].

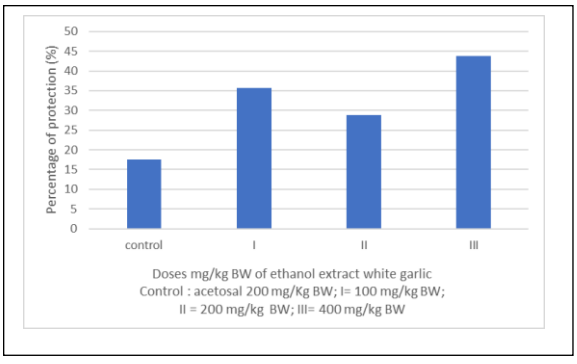


Figure 3. The in vivo analgesic activity test of the ethanol extract of garlic

The in vivo analgesic activity test of the ethanol extract of garlic and black garlic is shown in Figure 3 and 4. The data shows that the analgesic activity test carried out in vivo shows that the ethanol extract of black garlic has a higher analgesic activity. From these data, it

can also be seen that the ethanol extracts of garlic and black garlic have higher analgesic activity compared to the positive control group using acetosal. This is by the results of a previous study reported by Dange [19]. The main phytoconstituents such as thioacremonone, allicin, ajoene, and quercetin in garlic can inhibit cyclooxygenase peripherally and act on opioid receptors which centrally lead to analgesics.

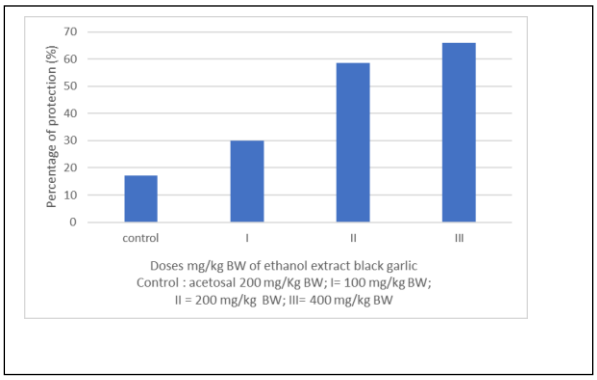
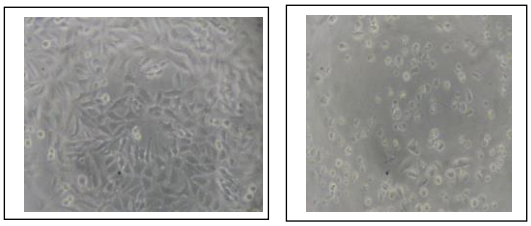
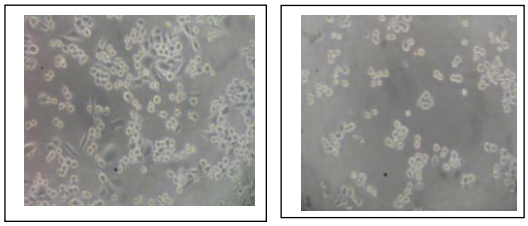


Figure 4. The in vivo analgesic activity test of the ethanol extract of black garlic



A B

Figure 5. The results of observations of T47D breast cancer cell before treatment (A) and breast cancer T47D cell after treatment (B) with 700 µg/mL chloroform fraction of black garlic



A B

Figure 6. The results of observations of Hela S3 cell before treatment (A) and Hela S3 cell after reatment (B) with 300 µg/mL chloroform fraction of black garlic

Anticancer activity tests were carried out in vitro using cancer cells T47D, 4T1; MCF7 / Her2; HeLa; and WiDr. The activities expressed in the IC_{50} chloroform fraction of black garlic against cancer cells T47D, 4T1; MCF7 / Her2; HeLa; and WiDr are 447.455; 227.562; 200.806; 510.466; and 429.001 µg / mL.Whereas other extracts and fractions of garlic and black garlic showed IC_{50} values greater than 1000 µg/mL which indicated very lower cytotoxic activity. The results of observations of T47D and HeLa cancer cells in treatment with chloroform extract of black garlic are shown in Figure 5 and 6.

4. CONCLUSION

The total phenolic and flavonoid levels of garlic and black garlic extracts showed a difference. The total phenolic and flavonoid levels of the ethanol extract of garlic were 71.54 ± 11.31 mg GAE / g sample and 2.32 ± 0.22 mg RE/g sample, respectively, whereas in the ethanol extract of black garlic it was 306.09 ± 32.23 mg GAE/g sample and 9.25 ± 0.55 mg RE/g sample. The results of the isolation and identification of the ethyl acetate fraction of black garlic showed the presence of 5-hydroxymethylfurfural compounds with a percentage of 67.89 %. The antioxidant activity of black garlic extract showed high activity with IC_{50} 38.609 ± 0.11 µg/mL, while the ethanol extract of garlic showed lower activity. The ethanol extract of garlic and black garlic has higher analgesic activity than acetosal, while its activity against some cancer cells shows low activity.

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