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Antibacterial Potential of Single Black Garlic Extract Organosulfur Compounds on *Staphylococcus aureus* Bacteria

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Abstract: Single black garlic (SBG) is a product of processing garlic (*Allium sativum* L.) by heating it for several days. SBG has antibacterial activity due to the presence of organosulfur, phenolic and flavonoid compounds. This research aims to determine the antibacterial potential of SBG extract on Staphylococcus aureus and determine its chemical compound content based on Liquid Chromatography-Mass Spectrometer (LC-MS) analysis. The extract was obtained from the maceration method combined with a magnetic stirrer using 0, 30, 60 and 90% ethanol solvent. The antibacterial test used the disc diffusion method with a positive control of ciprofloxacin and a negative control of distilled water. LC-MS analysis of SBG extract with the best antibacterial activity. The results of the antibacterial activity test showed that the SBG extract had antibacterial activity based on the presence of an inhibition zone. The largest zone of inhibition in the 90% ethanol solvent SBG extract was 27.71 mm, and the positive control was 22.58 mm. The SBG extract with the most optimal antibacterial activity was analyzed using LC-MS to produce 109 compounds which were divided into 15 different compound groups, some of which were allicin (5.900%) which was the compound with the most composition and innulotetraose (0.065%) which was the compound with the smallest composition in the SBG extract. Data from the antibacterial test results were processed using the IBM statistical program SPSS 25, a One-way ANOVA test, which aims to determine the effect of antibacterial activity on Staphylococcus aureus bacteria, and data on the chemical compound content of the SBG extract were analyzed descriptively. SBG extract has the potential as an antibacterial which is classified as very strong in-vitro, so further in-vivo research is needed to apply the extract as an economical product that can treat skin infections and there are several compounds that dominate SBG extract with the highest antibacterial activity, such as organosulfur compounds, amino acids, flavonoids and phenolics based on LC-MS analysis.

Keywords: Antibacterial; Inhibition Zone; Organosulfur Compounds; Single Black Garlic (SBG).

Introduction

Black garlic can be produced by heating garlic at a temperature of 60℃ for 10 days [1]. Garlic is divided into two types based on the number of cloves, namely compound garlic (multi-bulb garlic) and single garlic (single-bulb garlic) [2]. Black garlic contains many amino acids, polysaccharides, reducing sugars, proteins, melanoidins, and several bioactive compounds, including organosulfur compounds, phenolics, alkaloids and flavonoids [3]. The bioactive compounds in black garlic are higher than in garlic due to the heating process. The bioactive compounds of black garlic are dominated by organosulfur compounds. The content of organosulfur compounds is 8,432 nmol/g in garlic and 66,452 nmol/g in black garlic [4]. One of the organosulfur compounds in black garlic is *allicin*. *Allicin* has unstable properties, so it can change into other organosulfur compounds with higher stability, such as *S-allyl-L-cysteine* (SAC) and organosulfur sulfide groups [5]. The organosulfur compounds contained in black garlic can be obtained by extraction [6].

Extraction is a technique of withdrawing certain chemical compounds from a sample using a solvent [7]. The effectiveness of extraction depends on the conditions during extraction such as the extraction method and the type of solvent used [8]. Solvents that can be used are water and ethanol because they have lower toxicity compared to other solvents so they are safer for all extraction methods and for extracts that will be used as medicine [9]. The extraction process can be carried out using several methods, including maceration, sonication and distillation methods. The most common black garlic extraction method used during the extraction process is the maceration method because it uses simple equipment and procedures [10]. However, this method requires a long time in the extraction process [11]. Magnetic stirrer maceration (M-MS) is a maceration method that combines a magnetic stirrer to it can speed up the extraction process because it provides constant stirring, which can increase contact between the solvent and the sample. Contact between the solvent and the sample results in the rupture of the sample's cell walls and membranes due to the pressure difference between inside and outside the cell, so that the solvent enters the cytoplasm and dissolves the sample's bioactive compounds out of the cell [12]. The result of the black onion extraction process is black onion extract containing chemical compounds which can be used as antioxidants, anti-inflammatory, anti-obesity, anti-cancer, anti-diabetic and anti-bacterial [1].

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The high content of organosulfur compounds in black onions and the presence of secondary metabolite compounds cause black onion extract to have the potential to be antibacterial [13]. Bacteria can cause several infections in the body. The most frequently encountered infections are skin infections because the skin often comes into direct contact with the surrounding environment [14]. Most skin infections are caused by *Staphylococcus aureus* bacteria. *Staphylococcus aureus* can infect the skin due to scratches on the skin, so that when the body's immunity is weakened, body tissue is more easily damaged, which can cause infection [15]. *Staphylococcus aureus* infection of the skin can cause acne, boils, impetigo, and can even cause dermatosis with several characteristic signs such as inflammation and abscess formation [16]. Black onion extract is able to act as an antibacterial on the skin, so it can be used as an additional ingredient in ointments and gels [17]. The presence of chemical compounds that act as antibacterials, such as organosulfur compounds and secondary metabolite compounds, in SBG extracts can be identified using the Liquid Chromatography-Mass Spectrometer (LC-MS) analysis instrument.

This research examines the antibacterial ability of *Staphylococcus aureus* bacteria from a single black onion extract obtained by extraction using the maceration method combined with a magnetic stirrer, which can speed up the extraction process using several variations in the concentration of ethanol-water solvent based on the diameter of the bacterial inhibition zone using the disc diffusion method. Apart from that, this research also examines the chemical compound content in SBG extract at the most effective concentration of ethanol solvent in inhibiting the activity of *Staphylococcus aureus* bacteria based on LC-MS analysis.

Research Methods

Single Black Garlic Extraction

10 g of ground SBG was weighed, then put into a 250 mL beaker, and 100 mL of solvent (1:10) was added, covered with aluminium foil and extracted at room temperature for 3 hours using a magnetic stirrer at a speed of 1000 rpm. After that, the results obtained in the form of extracts were centrifuged at a speed of 1500 rpm for 1 hour and filtered using filter paper to obtain SBG extract [6]. The extract obtained was evaporated using a *rotary evaporator* at a temperature of 40-45°C with a speed of 100 rpm to obtain a thick SBG extract with a concentration of 100 mg/mL.

$$\text{Yield (\%)}: \frac{\text{mass of raw material}}{\text{mass of extract}}\ x\ 100\%$$

Testing of Antibacterial Activity of Single Black Garlic Extract

Tool Sterilization

Sterilization of tools is carried out on tools that have been washed, dried and put in heat-resistant plastic. Sterilize the equipment using an autoclave set at a pressure of 1 atm and a temperature of 121°C for approximately 20 minutes.

Creation of Mueller Hinton Agar (MHA) media

A total of 3.8 g of Mueller Hinton Agar (MHA) media was dissolved little by little in sterile distilled water, then diluted to 100 mL, heated and stirred using a magnetic stirrer hot plate until completely dissolved. The media was sterilized in an autoclave at 121°C for 15 minutes, then the media was poured into sterile Petri dishes and cooled until it solidified.

Preparation of 0.5 McFarland standard solution

9.95 mL of 1% H_2SO_4 was put into a sterile test tube, and 0.05 mL of 1% BaCl2 solution was added, then shaken until cloudy.

Preparation of physiological NaCl solution

0.9 g of NaCl was dissolved in 100 mL of distilled water, then 5 mL was taken into a test tube and covered with cotton wool. After that, it was sterilized in an autoclave at a temperature of 121°C for 15 minutes.

Bacterial inoculation

Staphylococcus aureus bacteria were taken in 1 dose and streaked onto the finished MHA medium, then incubated at 37°C for 24 hours.

Preparation of solution suspension

Bacterial culture colonies on MHA media were placed in a test tube containing 5 mL of NaCl and then incubated at 37°C for 24 hours.

Antibacterial activity test

The antibacterial activity test was carried out by taking 1 dose of the bacterial suspension and inoculating it on MHA media, then spreading it with sterile cotton buds and leaving it to dry (±5 minutes). To test antibacterial activity, paper discs were prepared, each of which had been soaked in distilled water, DMSO, SBG extract from varying concentrations of ethanol solvent, at several variations in extract concentration and ciprofloxacin solution on the test medium, then incubated at 37ºC for 24 hours [18]. Antibacterial testing of SBG extract was carried out in triplicate using the same method and controls on different bacterial suspensions.

Analysis of Chemical Compounds of Single Black Garlic Extract Using LC-MS

Single black onion extract was analyzed using LC-MS to produce retention time and composition (%), which would then be analyzed for the chemical compound content in single black onion extract using LC-MS spectral database guidelines.

Data Analysis Techniques

Data from the antibacterial test results were processed using the IBM statistical program SPSS 25, One-way

analysis of variance (ANOVA) test, which aims to determine the effect of antibacterial activity on *Staphylococcus aureus* bacteria. Data obtained from the results of a single black garlic extraction using ethanol solvent, based on LC-MS analysis, were analyzed descriptively.

Results and Discussion

Single Black Garlic Extraction

Single black garlic (SBG) has a sweet and sour taste, a black color, a weak smell, and a soft texture. SBG extraction uses a maceration method combined with a magnetic stirrer to speed up the reaction by increasing contact between the solvent and the sample through a constant stirring process. Contact between the solvent and the sample results in the breaking of the sample cell walls and membranes due to the pressure difference between inside and outside the cell, so that the solvent enters the cytoplasm and dissolves the sample's bioactive compounds out of the cell [12]. SBG extraction begins by refining the SBG with the aim of expanding the SBG area and facilitating contact between the SBG and the solvent so as to produce a more optimal extract [19]. The SBG extraction process uses ethanol solvent with varying concentrations of 0, 30, 60 and 90%. The use of ethanol as a solvent is because ethanol is a universal solvent that is able to attract polar and non-polar compounds, thereby allowing more metabolite compounds to be extracted; besides that, ethanol has low toxicity [9]. The SBG extract before the evaporation process showed that the extract in 0, 30 and 60% concentration of ethanol solvent produced a brownish color, and the extract in 90% concentration of ethanol solvent produced a yellowish color. The higher the concentration of ethanol solvent, the fewer tannin compounds are extracted. Tannin compounds are compounds that can give a darker color to the extract [20]. The SBG extract obtained was evaporated to produce a thick extract, so that the yield values were obtained as in Table 1.

The yield value of SBG extract increased along with the increase in solvent concentration up to 60% ethanol solvent, and decreased with 90% ethanol solvent. The highest yield was in the SBG extract using 60% ethanol solvent. This shows that the chemical compounds in SBG tend to dissolve easily in ethanol, which has a lower water content [21]. There are several factors that influence the yield value, namely sample size, type of solvent, level of solvent polarity and length of extraction time [22]. The difference in the yield value of SBG extract is caused by the influence of the solvent's strength in attracting polar and non-polar compounds.

Table 1. Yield Value of Single Black Onion Extract

Sample	Extract Mass Sample (g)	SBG Extract Yield After Evaporation (%)
SBG extract in 0% ethanol solvent	1.938	19.380
SBG extract in 30% ethanol solvent	5.184	51.840
SBG extract in 60% ethanol solvent	5.946	59.460
SBG extract in 90% ethanol solvent	4.663	46.630

The SBG extract contains several polar and non-polar compounds such as amino acids, polysaccharides, reducing sugars, proteins, organosulfur compounds, phenolics, alkaloids, and flavonoids [23]. The most prominent compound in black garlic is an organosulfur compound in the form of allicin. This compound is produced when garlic is heated to become black garlic, allowing the hydrolysis reaction of the alliin compound to produce allicin via the alliinase enzyme, as illustrated in Figure 1 [8]. Because this compound has low stability, it can decompose into more stable organosulfur compounds such as diallyl disulfide (DADS), diallyl trisulfide (DATS), diallyl sulfide (DAS), and S-allyl-L-cysteine (SAC) [1][10]. In the SBG extract, allicin is one of the most abundant organosulfur compounds, and it is believed to have antibacterial potential [13].

Antibacterial Activity Test of SBG Extract

The SBG extract obtained was tested for antibacterial activity using the disc diffusion method on *Staphylococcus aureus* bacteria. The disc diffusion method is a testing method used to see the activity of a compound in inhibiting microbes, using disc paper as a medium to absorb antimicrobial compounds in the extract. The presence of a clear zone around the disc indicates that there is an obstacle to microbial activity. The diameter of the inhibition zone of SBG extract was measured using a calliper, and the results obtained were subtracted from the diameter of the paper disc [24].

Table 2. Antibacterial Activity Test Results of Single Black Garlic Extract

Inhibition Zone Diameter (mm)	SBG extract in 0% ethanol solvent	SBG extract in 30% ethanol solvent	SBG extract in 60% ethanol solvent	SBG extract in 90% ethanol solvent	Positive Control	Negatve Control
1	27.50	28.23	28.56	28.94	23.21	-
2	24.65	25.84	26.06	26.33	21.85	-
3	25.98	26.22	26.39	27.87	22.67	-
Average	26.04[a]	26.76[a]	27.00[a]	27.71[a]	22.58[b]	-

Note: Values followed by different letters indicate significant differences ($p<0.05$)

The results of the research show that the diameter of the inhibitory zone for *Staphylococcus aureus* bacteria from SBG extract with a variety of ethanol solvents of 0, 30, 60 and 90% has an inhibition zone in the range of 26.04 to 27.71 mm, where the highest inhibition zone is shown at the 90% ethanol concentration of 27.71 mm which can be seen in Figure 1. The SBG extract at this concentration has an average inhibition zone of ≥20 mm, so that its antibacterial properties are classified as very strong. The higher the extract concentration, the more optimal the ability of the

extract to inhibit the growth of *Staphylococcus aureus* because the more active compounds it contains so that the inhibitory power against bacterial growth is greater [25]. This inhibition zone showed higher results than in research from [26], which is 8.28 mm, and in the research of [27], which is 9.67 to 24 mm, with different methods and bacteria. Another study used the dilution method on black garlic extract, which was related to observing the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). Black garlic extract inhibits Bacillus cereus bacteria with an MIC value of 0.023, the MIC obtained has an absorbance value close to 0. Meanwhile, when observing bacteria using the MBC test for black garlic extract, it shows that there is still growth of *Bacillus cereus* bacteria. This means that black garlic extract is not bactericidal or kills Bacillus cereus, but it can inhibit the growth of Bacillus cereus [28].

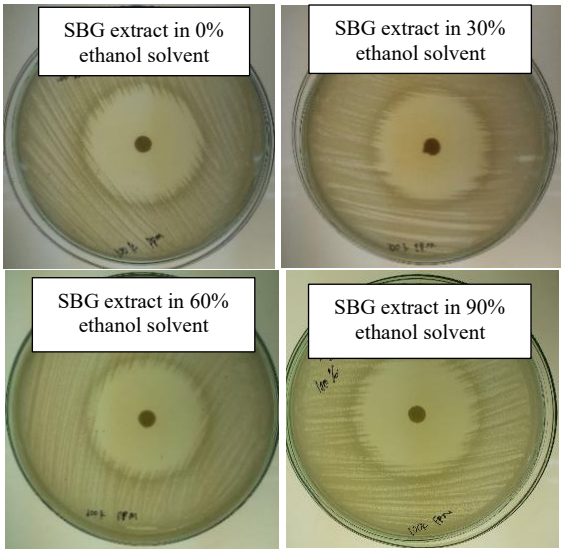

Figure 1. Antibacterial Test Results of SBG Extract

The antibacterial activity of SBG extract is thought to be due to the content of *allicin* and its derivative organosulfur compounds, as well as the content of secondary metabolite compounds [29]. Organosulfur compounds can inhibit bacterial activity through the reaction between the sulfur group in organosulfur compounds and the sulfhydryl (SH) group in bacterial enzymes, which can form mixed disulfide bonds which can damage the cytoplasmic membrane of the bacterial wall, causing cell lysis and bacterial death [29]. The presence of secondary metabolite compounds in SBG such as flavonoids and phenolics can also have an effect on inhibiting bacterial growth. Flavonoids act as antibacterials by inhibiting nucleic acid synthesis, cytoplasmic membrane function and energy metabolism so that the exchange of nutrients and energy supply in bacterial cells is hampered [13]. Phenolic compounds inhibit bacterial activity by denaturing bacterial proteins, inhibiting cell wall synthesis and damaging bacterial cell membranes [30].

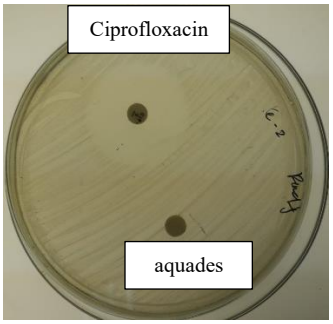

Figure 2. Antibacterial Test Results of Positive and Negative Controls

Analysis of Chemical Compounds of SBG Extract Using LC-MS

The SBG extract with the highest antibacterial activity was analyzed for its chemical compound composition using *Liquid Chromatography-Mass Spectrometer* (LC-MS). This research used SBG extract in 90% ethanol solvent. Based on LC-MS analysis of the SBG extract with the highest antibacterial activity, 109 different chemical compounds were obtained, which were classified into fifteen compound groups as in Table 3. The results obtained were in the form of compound chromatograms as in Figure 3.

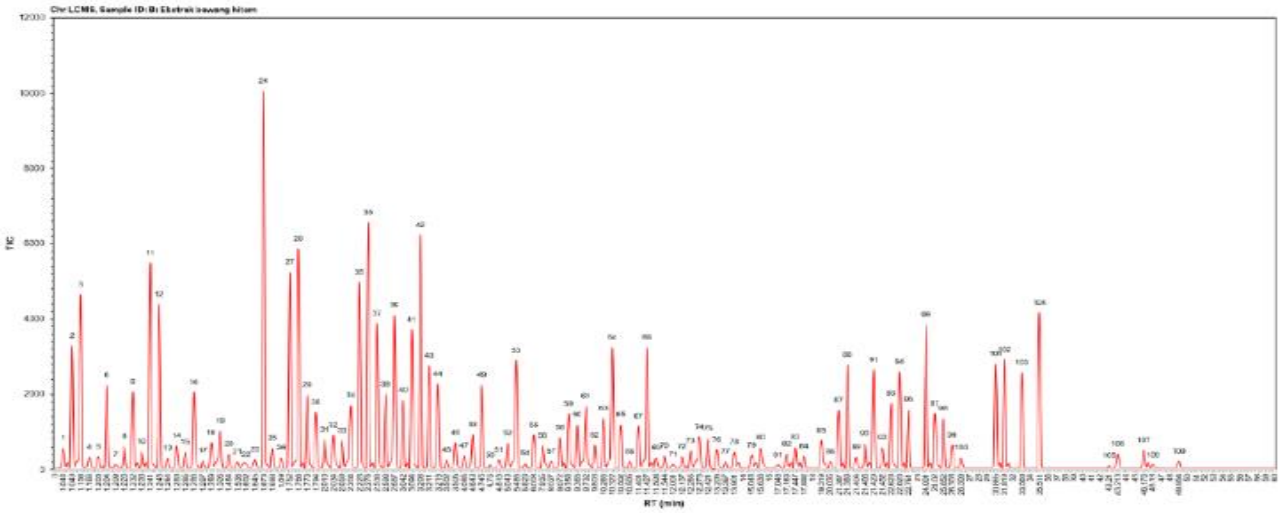

Figure 3. Chromatogram of LC-MS Analysis Results of Single Black Garlic Extract

The peaks in the resulting chromatogram mean the amount and percentage composition of the chemical compound in the extract. The highest peak is the compound with the highest percentage, such as peak number 24, which is the organosulfur allicin compound. The lowest peak is shown at number 105, which is the fructooligosaccharide

compound inulinotetraose. The chemical compounds of SBG extract are dominated by several compounds with a composition of ≥2%, namely organosulfur compounds, amino acid compounds and flavonoid compounds.

Table 3. Results of LC-MS Analysis of Single Black Garlic Extract Chemical Compounds

No	Compound Type	Number of Compounds	Total Composition (%)
1.	Organosulfur	22	16.58
2.	Flavonoid	21	27.11
3.	Asam Amino	20	48.64
4.	Fenolik	10	5.08
5.	Steroid	5	1.15
6.	Terpenoid	2	0.59
7.	Organoselenium	2	0.23
8.	Lakton	3	1.22
9.	Sterol	3	0.82
10.	Furan	1	0.37
11.	Alkaloid	2	0.82
12.	Keton	6	2.31
13.	Sakarida	9	1.69
14.	Thiol	2	0.58
15.	Aldehid	1	0.28

Conclusion

Single black garlic extract (SBG) from several variations in ethanol solvent concentration has effectiveness as an antibacterial against *Staphylococcus aureus* bacteria, as indicated by the formation of an inhibitory zone diameter, which increases with increasing ethanol solvent concentration. SBG extract has the potential as an antibacterial, which is classified as very strong in vitro, so further in vivo research is needed to apply the extract as an economical product as an addition to ointments and gel that can treat skin infections. Chemical compound analysis using LC-MS on the SBG extract with the highest inhibition zone as an antibacterial produced 109 compounds, which were divided into fifteen groups of compounds with different compositions. There are several groups of compounds with dominant compositions, such as organosulfur compounds, amino acid groups and flavonoid groups.

Author's Contribution

Rindy Tya Destiana: Collecting research data and preparing articles; Nuniek Herdyastuti: Responsible person and article compiler; Wahyu Setyarini: Antibacterial test facility provider; and Radita Yuniar Arizandy: Antibacterial test facility provider.

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