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EFFECTS OF ETHANOL AND ETHYL ACETATE EXTRACTS OF GARLIC (*ALLIUM SATIVUM*) ON THE GROWTH OF EXTENDED SPECTRUM B-LACTAMASE *ESCHERICHIA COLI*

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ABSTRACT

Background: The prevalence of Extended Spectrum β-Lactamase (ESβL) *Escherichia coli* has increased by 0.91 – 2.31% per year and causes β-lactam antibiotics to be useless. Natural medicines such as garlic can be used to treat antibiotic resistance. It has been reported that garlic ethanol extract can inhibit the growth of Metallo-β-lactamase *E. coli*, but there have been no reports of garlic ethyl acetate extract activity against resistant bacteria. **Objective:** This study aims to prove the effect of ethanol and ethyl acetate extracts of garlic in inhibiting the growth of ESβL *E. coli*. **Methods:** The ethanol and ethyl acetate extracts of garlic were prepared in concentrations of 25, 50, 75 and 100% w/v, and were tested on ESβL *E. coli* using the disc diffusion assay. This study used a post-test-only control group design with meropenem as the positive control. The effectiveness of both garlic extracts was assessed from the inhibition zones formed around the discs. Phytochemical tests were carried out to see the compound content of garlic extract. **Results:** All concentrations of garlic ethanol extract were not significantly different in inhibiting ESβL *E. coli* growth (with inhibition zone diameter 2.82 - 3.30 mm). However, for the ethyl acetate extract, the higher the concentration the higher the activity of the extract in inhibiting ESβL *E. coli* (p-value <0.05). The best inhibition zone of ethyl acetate extract was 4.18 mm at a concentration of 100%. Meropenem as a positive control produced a 17 mm inhibition zone. The ethanol and ethyl acetate extracts of garlic had no difference in the active compound content, both contain tannins, saponins, and essential oils. **Conclusion:** The ethanol and ethyl acetate extract of garlic had weak potential to inhibit ESβL *E. coli* growth when compared to meropenem as a control drug.

Keywords: *antibacterial agent, β-lactamases, garlic, meropenem, phytochemicals*

INTRODUCTION

Microbial infection is a health problem in developing countries, such as Indonesia, and is the main cause of high morbidity and mortality.[1] Bacterial infections can be treated with antibiotics. However, the excessive and inappropriate use of antibiotics has led to an increase in the incidence of antibiotic resistance.[2] One of the resistant bacteria that has increased significantly over the last few decades is Extended Spectrum β-Lactamase (ESβL) producing bacteria, including *Escherichia coli*. Based on the CDC report in 2017, there were at least 197,400 cases of hospitalized patients and 9,100 deaths due to resistance of ESβL-producing bacteria to antibiotics.[3] The prevalence of ESβL *Escherichia coli* has increased by 0.91% per year in community infections. A higher increase occurred in healthcare-associated infections of 2.31% per year.[4] *Escherichia coli* is a bacilli-negative bacterium from the Enterobacteriaceae family that can produce beta-lactamase enzymes that break the amide ring in beta-lactam antibiotics.[5]

ESβL *E. coli* can be treated with carbapenems or non-beta lactam antibiotics such as ciprofloxacin and gentamicin. However, the risk of resistance to non-beta lactam antibiotics is greater due to the mediated mutation of the ESβL gene.[6] The inability of antibiotics to kill ESβL *E. coli* has led to the search for new drugs from traditional ingredients. Traditional medicine is a treatment method used by the community for a long time, using materials or substances derived from plants, animals and minerals.[7,8] One of the plant ingredients that can be used as herbal or traditional medicine is garlic (*Allium sativum*), a tuber that is often found in Indonesia.[9-10]

Garlic is known to have many benefits such as anti-microbial, anti-cancer, anti-inflammatory, anti-diabetic, anti-hypertensive, hepatoprotective, and anti-alzheimer.[11] Garlic is known to have several components of active compounds that have

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antibacterial properties such as alkaloids, flavonoids, saponins, tannins, essential oils, organosulfur and allicin. However, the extraction solvent may affect the types of compounds that can be extracted from garlic and cause differences in antimicrobial activity.[12] For example, garlic aqueous extract is known to inhibit *Streptococcus mutans*[13], but cannot inhibit the growth of *E. coli*.[14] Meanwhile, garlic ethanol extract can inhibit the growth of *Pseudomonas aeruginosa* [15], Metallo-β-lactamase *E. coli* [16], and kill Methicillin-Resistant *Staphylococcus aureus* (MRSA).[17] Ethyl acetate can also be used for garlic extraction and the results showed that ethyl acetate extract was better at inhibiting the growth of *E. coli* compared to ethanol extract.[18]

Not many studies have assessed the effectiveness of garlic extract on ESβL *E. coli*. Therefore, research is needed to examine its effectiveness as an antibacterial. This study used two types of solvents (ethanol and ethyl acetate) to extract secondary metabolites from garlic. The effectiveness of both extracts in inhibiting the growth of ESβL *E. coli* was evaluated. In addition, the secondary metabolites content in both types of extracts was analysed using phytochemical tests.

METHODS

The garlic used in this research was purchased from the Bandungan Traditional Market. The ESβL *E. coli* used in this study were clinical isolates from the collection of the Microbiology Laboratory, Faculty of Nursing and Health Sciences, Universitas Muhammadiyah Semarang. This study was a post-test-only control group design and was conducted at the Microbiology Laboratory, Faculty of Biology, Universitas Negeri Semarang.

Garlic Extracts Preparation

As much as 1 kg of washed garlic is cut into small pieces and mashed using a blender. Then the garlic was soaked in a 96% ethanol solution with a ratio of 1:10. The solution was stirred for 30 minutes and placed in a closed and dark container protected from light for 24 hours. After 24 hours the solution was filtered, and the garlic precipitate was soaked again for 24 hours and repeated 3 times. The filtered liquid is collected and then evaporated with the help of a vacuum rotary evaporator (Ika RV10) and a water bath (Memmert) at 96 °C.[19] The thick extract is redissolved with 96% ethanol (ratio of 1:3) in a separatory funnel, and then add three parts of ethyl acetate and shaken gently until the separation of the ethyl acetate and ethanol solutions occurs. The ethyl acetate and ethanol fractions were collected and dried using a rotary evaporator and water bath.[20]

Phytochemical Analysis of Garlic Extracts

The ethanol and ethyl acetate extracts of garlic were tested for chemical compounds using phytochemical test procedures. Qualitative analysis aimed to determine the content of flavonoids, tannins, saponins, alkaloids, and essential oils. A flavonoid test was carried out by putting 1 ml of each extract into a test tube and adding 2 drops of concentrated hydrochloric acid. After vigorous shaking, the sample tested positive for flavonoids if there was a change in colour to yellow, red or orange.[21]

For the tannin test, as much as 1 ml of each extract was added 2 drops of 1% $FeCl_3$. The sample is tested positive for tannins when there is a change in colour to blackish blue. The saponin test was carried out by adding 10 mL of warm water to it 2-3 ml of each extract. After shaking it for 10 seconds with the addition of 2N HCl. The sample is positive for tannins if a stable foam forms for not less than 10 minutes. For the alkaloid test, 2 ml of each extract was added with 2 ml of 2N HCL, then Mayer's reagent was added. The sample is tested positive for alkaloids when there is a white precipitate. The qualitative test of essential oils was done by evaporating 1 ml until only the residue remained. The sample is declared positive if there is a characteristic odour of the compound in the residue.[22]

Antibacterial Activity Test of Garlic Extracts

ESβL *E. coli* were grown using Mueller Hinton Broth (MHB) media according to Mc Farland turbidity of 0.5. The bacterial suspension was implanted into the Mueller Hinton Agar (MHA) plate with a thickness of 4 mm using the spread method.[23]

In this study, the antibacterial activity test was carried out using the disc diffusion method. Garlic ethanol and ethyl acetate extract were prepared in four concentrations, namely 25, 50, 75 and 100% w/v. Water was used as a negative control and meropenem was set as a positive control. As much as 10 µl of each concentration of the garlic extract solution was dripped onto empty discs and then

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placed on MHA media that had been inoculated with ESβL *E. coli*. The amount of each garlic extract on the tested discs became 10, 7.5, 5, and 2.5 mg. The media was incubated at 37 ºC for 18-24 hours, and then the inhibition zone diameter was measured using a ruler.[24] The criterion for the strength of zone inhibition is categorized as weak, medium, strong and very strong.[25] Differences in the inhibition zone of each concentration in both garlic extracts were analysed using a parametric One-Way ANOVA test.

RESULTS

A phytochemical test for the content of garlic compounds was carried out on ethanol extract and ethyl acetate extract. In this study, the two extracts contained the same compounds, namely tannins, saponins, and essential oils (Table 1).

Table 1. Phytochemical result analysis of garlic (*Allium sativum*) extract.

Phytochemical Test	Ethanol 96% Extract	Ethyl Acetate Extract
Flavonoid	-	-
Tannin	+	+
Saponin	+	+
Alkaloid	-	-
Essential oil	+	+

Table 2. Mean ± standard deviation as a result of antibacterial activity of garlic (*Allium sativum*) extract against ESβL *E. coli* using disk diffusion method.

Extract/Control	T 10 mg	T 7.5 mg	T 5 mg	T 2.5 mg	NC mm	PC mm
Ethanol 96%	3.18 ± 1.48	3.30 ± 0.70	2.82 ± 0.59	3.00 ± 1.00	17.00 ± 1.00[a]	0.00 ± 0.00
Ethyl Acetate	4.18 ± 0.98	3.17 ± 0.76	2.42 ± 0.52[b]	1.43 ± 1.25[b,c]		
Inhibition category	Weak	Weak	Weak	Weak	Strong	No Inhibition

Note: T = Treatment (mg); NC = Negative Control (Sterile Water); PC Positive Control (Meropenem 10 µg)
[a]significant differences with all extract
[b]significant different with T (10)
[c]significant different with T (10) and T (7.5)

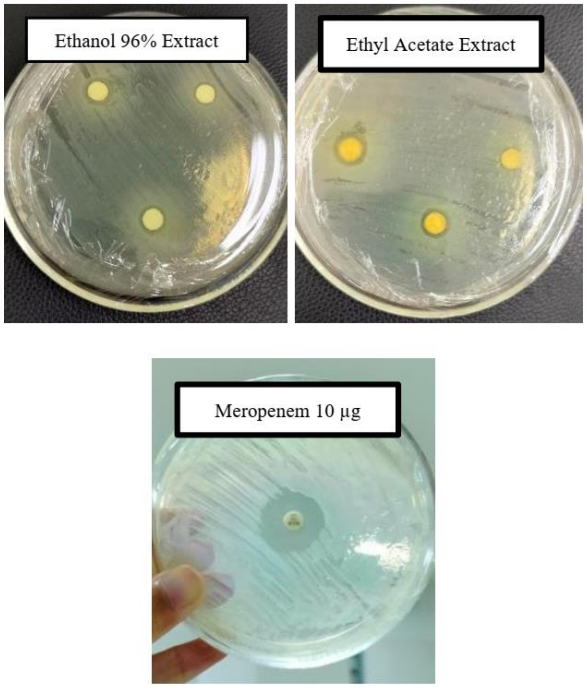

Figure 1. Image of the ESβL *E. coli* inhibition zone formed caused by 10 mg ethanol garlic extract, 10 mg ethyl acetate garlic extract, and 10 µg meropenem.

The inhibitory activity of the two garlic extracts from the highest concentration, namely 10 mg, was categorized as weak inhibition. The growth inhibition zone of ESβL *E. coli* formed from 10 mg of ethanol extract from garlic was 3.18 ± 1.48 mm and that of 10 mg of ethyl acetate extract from garlic was 4.18 ± 0.98 mm, both of which were significantly different from the inhibition produced by meropenem 10 µg of 17.00 ± 1.00 mm (Table 2 and Figure 1).

DISCUSSION

This study conducted an antibacterial activity test to determine the effectiveness of the ethanol extract and ethyl acetate extract of garlic (*Allium sativum*) in inhibiting the growth of ESβL *E. coli* bacteria. The activity test method used is disk diffusion, to determine the bacterial inhibition zone by measuring the diameter of the clear zone around the disc which has been incubated for 24 hours.[26]

This study produced data that the ethanol and ethyl acetate extracts of garlic were able to inhibit the growth of ESβL *E. coli*, even though they were included in the category of weak inhibition. At the

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highest concentration of the extract, which was 10 mg, the inhibition of the two extracts was significantly different from the positive control of 10 µg meropenem. The amount of garlic extract used in the test was 1000 times greater than that of meropenem.

Research related to the effect of garlic extract in inhibiting the growth of *E. coli* has been reported by Prihandani et al. (2015), which stated that garlic powder dissolved in distilled water had antibacterial activity against *E. coli* with an inhibition zone diameter of 9 mm.[27] The inhibition zone was bigger than the results of this study, but the *E. coli* used in that research was wild-type. Meanwhile, the activity of ethanol and ethyl acetate extracts of garlic on *E. coli* ESβL has not been reported. Garlic extract that has been tested on *E. coli* ESβL is water extract. The extract has minimum inhibition and minimum bactericidal concentration of 125-250 mg/ml and 250-500 mg/ml against *E. coli* ESβL bacteria. This shows that garlic has antibacterial activity but requires very high concentrations to inhibit and kill the *E. coli* ESβL.[28]

Aprillia (2023) conducted a phytochemical analysis related to the compound content of garlic bulbs, which proved that garlic bulbs contain secondary metabolites such as flavonoids, saponins, and tannins.[29] In another study conducted by Nadia et al. (2022) explained that organosulfur allicin, saponins, flavonoids, tannins and essential oils are compounds contained in garlic extract.[10] Meanwhile from other research, alkaloid content was reported to be found in garlic ethyl acetate extract.[30] In this study, both the ethanol and ethyl acetate extracts of garlic did not contain flavonoids and alkaloids. Ethanol and ethyl acetate extracts of garlic in this study only contained tannin, saponin, and essential oil. These results are in line with research which states that garlic flavonoids can be extracted only using water and methanol as solvents.[31]

Differences in the content of compounds in garlic can be caused by high heating factors during the extract evaporation process. Other factors that can affect the stability of garlic's bioactive components are storage time, pH, temperature, and processing methods.[32] According to research conducted by Wayan et al. (2017), temperature and extraction time have a significant effect on total flavonoid levels and antioxidant activity.[33] The type of solvent also affects the chemical compounds contained in the extract. In this study, it was found that the ethyl acetate extract of garlic contained polar compounds like saponins and tannins. This is caused by the presence of electrons that resonate in the benzene ring which results in the reduction of the compound polarity, and more attracted to ethyl acetate which is semi-polar in nature. Ethyl acetate solvent is also a type of semi-polar solvent that has a methoxy group that can form hydrogen bonds which can attract polar compounds in the extract.[34]

Garlic has antibacterial activity presumably because it contains antibacterial compounds from the tannins, essential oils, and saponins.[35] Tannins are known to inhibit bacterial growth through changes in the stability of protein molecules, changes in protein structure and damage to the cell cytoplasmic membrane, causing damage to the bacterial cell wall.[29] Essential oils work as an antibacterial by interfering with membranes or cell walls so that they are not formed or formed imperfectly.[36] Meanwhile, saponins as antibacterial work by increasing the permeability of the bacterial cell wall so that it can cause lysis and death of bacteria.[37] Saponins have hydrophilic and lipophilic molecules that can dissolve fat and reduce cell surface tension destroying bacterial cells. Saponins diffuse through the outer membrane and the vulnerable cell wall, binding to the cytoplasmic membrane and resulting in disruption and reduced stability. This causes the cytoplasm to leak out of the cell and results in bacterial cell death.[38]

CONCLUSION

The ethanol and ethyl acetate extracts of garlic contain tannins, saponins and essential oils. An amount of 10 mg of garlic extract on discs can inhibit the growth of ESβL *E. coli* in the weak inhibition category. This growth inhibition is significantly different from meropenem as a control antibiotic which can inhibit the growth of ESβL *E. coli* in the category of strong inhibition. Therefore, the development of garlic extract as an antibiotic needs to be evaluated further.

ETHICAL APPROVAL

This study has received research ethics approval from the Medical Health and Research Ethics Commission (KEPK), Medical Faculty of

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CONFLICTS OF INTEREST

None.

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AUTHOR CONTRIBUTIONS

Author contributions are explained as follows "conceptualization, methodology, analysis MDR, RU, and MPA; investigation and resources, RU; writing-original draft preparation, RU; writing-review and editing, MDR; visualization, MDR; supervision, MDR and MPA; project administration, MDR, RU, and MPA".

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