Journal of Nutraceuticals and Herbal Medicine

Journal Homepage: http://journals.ums.ac.id/index.php/jnhm

Antibacterial Activity of Combination of Ethanol Extract of Pepermine Leaves (*Mentha piperita* L.) and Amikacin Against *Klebsiella pneumonia, Staphylococcus aureus*, and *Escherichia coli*

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Abstract

Peppermint leaves have been known to have antibacterial and antifungal activity. Amikacin is a semisynthetic derivative of kanamycin which is active against both Gram-positive and Gram-negative enteric bacteria. The combination of plant extracts with antibiotics is one way or alternative to overcome bacterial resistance to antibiotics. The purpose of this study was to determine the combined effect of the ethanolic extract of peppermint (*Mentha piperita* L.) and amikacin against *Klebsiella pneumonia*, *Staphylococcus aureus*, and *Escherichia coli* as well as the compounds contained in the ethanolic extract of peppermint (*Mentha piperita* L.) leaves. Antibacterial activity was tested using the disk diffusion method (Kirby Bauer) and the phytochemical screening test using the tube test method. The concentration of peppermint leaf ethanol extract for the combination test was 200 mg/mL and 400 mg/mL, for the concentration of amikacin used was 5 mg/mL with three comparisons made, namely 25:75, 50:50, and 75:25. The results showed a synergistic effect with the largest inhibition zone diameter at a ratio of 25:75 at a concentration of 200 mg/mL, which was 36.25±2.5 mm on *Klebsiella pneumonia* bacteria. While at a concentration of 400 mg/mL the diameter of the largest inhibition zone was 40±1.63 mm in *Klebsiella pneumoniae* bacteria with a ratio of 75:25. The results of statistical tests using the t-test showed a significance value of 0.000 <0.05 so that there was a significant difference in the administration of each concentration to the resulting inhibition zone. The results of the phytochemical screening test contained alkaloids, phenolic compounds, flavonoids, tannins.

Keywords: *Mentha piperita L.*, *Klebsiella pneumonia, Staphylococcus aureus, Escherichia coli*, disk diffusion, amikacin.

INTRODUCTION

Infectious diseases are still at the top of the list of causes of illness and death. One of the causes of infectious diseases is bacteria, symptoms that arise due to infection can be fever, chills, hypotension, and toxicity. Treatment for infectious diseases usually uses antibiotics, one of which is amikacin (Grayson et al., 2017). Amikacin is a semisynthetic derivative of kanamycin which is active on Gram-positive and Gram-negative enteric

bacteria, including strains of Proteus, Enterobacter, Serratia, *Mycobacterium tuberculosis* (Katzung and Bertram, 2004), *Staphylococcus aureus, Pseudomonas aeruginosa,* and *Escherichia coli* (Mohamed, 2013). The difference between Gram-negative and Gram-positive bacteria lies in the crystalline dye at the time of gram staining. Negative bacteria will not retain the crystal violet dye so it will turn red. Whereas in Gram-positive bacteria it is purple and the cell wall is thicker (Nurhidayati et al., 2015). Amikacin belongs to the aminoglycoside group whose mechanism of action is to inhibit protein biosynthesis by irreversibly binding aminoglycosides to the 30S subunit of the bacterial ribosome (Katzung and Bertram, 2004).

The increase in bacterial resistance to antibiotics is not balanced with the discovery of new antibiotics (Ventola, 2015). There are many plants that have the potential to treat infectious diseases, for example, peppermint leaves. Peppermint leaves (*Mentha piperita* L.) have antifungal activity on *Candida albicans, Candida tropicalis, Candida glabrata, Candida parapsilosis,* and *Candida krusei* (Furlan et al., 2010). In addition, peppermint leaves also have antibacterial activity, the diameter of the inhibition zone of distilled water extract of peppermint leaves using the disk diffusion method against the bacteria *Streptococcus mutans,* and *Aggregatibacter actinomycetemcomitans*, respectively 20.16 ± 0.36 mm, 18.34 ± 1.09 mm (Raghavan et al., 2018).

One way or alternative to overcome resistance is to combine antibiotics with plant extracts. Related to plant extracts which are traditional medicines added with synthetic isolation materials for research purposes can be developed further (BPOM, 2018). Study Stefanovi and Comic, (2012) explained that a plant when combined with antibiotics can cause a synergistic effect and have antibacterial activity, for example, research conducted on an ethanol extract of lemon balm leaves with a combination of streptomycin antibiotics showed synergistic results within the diameter of the streptomycin inhibition zone in *Bacillus subtilis* and *Enterobacter cloacae* bacteria previously 27.5 ± 0.71 mm, 1.41 ± 1.09 mm after being combined with ethanol extract of lemon balm leaves to 29.5 ± 0.71 mm, 32.5 ± 0.71 mm.

Peppermint leaves (*Mentha piperita* L.) are still in the same family as lemon balm leaves, namely from the Lamiaceae family, while streptomycin and amikacin antibiotics belong to the aminoglycoside group. Based on this research, a combination of ethanol extract of peppermint leaf (*Mentha piperita* L.) and amikacin was conducted to test the antibacterial activity against *Klebsiella pneumoniae, Staphylococcus aureus,* and *Escherichia coli bacteria*.

METHODS

1. Tools and materials

This study uses equipment, namely glassware, Laminar Air Flow (LAF), shaker incubator (New Brunswick), weighing equipment (Ohaus), oven (Memmert), incubator (Memmert), micropipette (Socorex), autoclave (Hirayama), filter paper, water bath (Memmert), rotary evaporator (Laborota). While the materials used for this study were *Klebsiella pneumoniae, Staphylococcus aureus, Escherichia coli*, 96% ethanol, peppermint leaves (*Metha piperita* L.) taken from Tawangmangu, Karanganyar, Central Java, amikacin, ampicillin, chloramphenicol, tetracycline, erythromycin, distilled water, blank disk, MH (Mueller Hinton) medium, BHI (Brain Heart Infusion) liquid medium, 100% Dimethyl Sulfoxide (DMSO), 0.9% sterile NaCl, glacial CH3COOH, H2SO4, sodium chloride

20 mg/mL, gelatin 10 mg/mL, HCl 1 N, Mg powder, concentrated HCl, $FeCl_3$ 10 mg/mL, Mayer's reagent,

2. Research Place

The research was conducted at the Pharmacy Microbiology Laboratory, Faculty of Pharmacy, Universitas Muhammadiyah Surakarta.

3. Plant Determination

The determination of peppermint leaf samples was carried out at the Plant Systematics Laboratory of the Biology Study Program, Faculty of Mathematics and Natural Sciences, Sebelas Maret University.

4. Material Preparation

Fresh peppermint leaves are cleaned of dirt attached to the leaves with clean water then the leaves are dried and powdered using a blender. Before being extracted, the powder was sieved first and then extracted.

5. Extraction

Peppermint leaf extract was made using the maceration method. The dried Simplicia powder was weighed with a weight of 300 g, soaked in 3000 ml of 96% ethanol using a maceration vessel, then the marinade was allowed to stand for 3 days protected from sunlight while stirring several times. After 3 days the ethanol filtrate was filtered using a Buchner funnel. Then the ethanol extract of the peppermint leaves is separated from the chlorophyll by adding hot water with a ratio of 1:1 between the hot water and the ethanolic extract of the peppermint leaves, then wait for it to cool and settle. After that, it was filtered and then evaporated using a rotary evaporator at a temperature of 45ºC then thickened above the water bath.

6. Sterilization of Tools and Materials

Utensils such as glassware are washed and then dried and wrapped in paper. Heat-resistant equipment such as Petri dishes, test tubes, glass beakers, and other glassware was sterilized using dry heating in an oven for 1 hour at 170ºC. Meanwhile, tools and materials that are not heat resistant such as media, tips (blue, yellow, white), dropper pipettes, and other tools are sterilized by wet heating using an autoclave at 121ºC for 20 minutes. Ose needles and spreader glass are sterilized by burning.

7. Preparation of Media

The media used for this research are MH (Mueller Hinton) and BHI (Brain Heart Infusion) media. Preparation of MH (Mueller Hinton) Media was carried out by dissolving 1.14 grams in 30 mL of distilled water for each one petri dish. Meanwhile, for the manufacture of BHI (Brain Heart Infusion) media, it is done by dissolving 185 mg in 5 mL of distilled water for each test tube. Then the media solution made was sterilized using an autoclave at 121ºC for 20 minutes and then poured into a petri dish and allowed to stand at room temperature until solid. The principle of autoclaving in killing bacteria by using steam at high temperature and pressure is 121ºC, the high temperature can kill microorganisms or bacteria.

8. Bacterial culture

Each of the bacteria *K. pneumoniae, S. aureus*, and *E. coli* was taken one end of the round loop from the parent bacteria then streak plated on MH media (Mueller Hinton)

then incubated for 18-24 hours at 37ºC, after 18-24 hours. hours and inoculated colonies growing in Petri dishes were stored in a refrigerator at 4ºC.

9. Bacterial Suspension Preparation

K. pneumoniae, S. aureus, and *E. coli* bacteria from overnight culture on MH (Mueller Hinton) plates were taken 3-5 colonies each, then a bacterial suspension was made with 5 ml of Brain Heart Infusion (BHI) media and incubated using a shaker incubator at 200 rpm at 37ºC for 2 hours. The concentration of the bacterial suspension was equalized using the Mc Farland standard of 10^8 CFU/mL by diluting it using a sterile 0.9% NaCl solution. The bacterial suspension was taken 200 L each time it was used for the antibacterial test.

10. Antibiotic Sensitivity Test

The bacterial suspension which was equivalent to the standard Mc Farland 108 CFU/mL was taken 200 L and then inoculated on solid MH (Mueller Hinton) media then leveled with a spreader glass and waited for a while to dry. The antibiotic discs used were ampicillin 10 g, chloramphenicol 30 g, tetracycline 30 g, erythromycin 15 g, Amikacin 30 g were placed on MH media that had been inoculated with bacterial suspension and then incubated at 37ºC for 18-24 hours. The diameter of the inhibition zone formed was measured and compared with the standard of bacterial resistance to antibiotics.

11. Production of Stock and Concentration Series of Peppermint Leaf Ethanol Extract

The stock concentration of the peppermint leaf ethanol extract made was 800 mg/mL, the peppermint leaf ethanol extract was weighed 8 g and then dissolved to 10 mL using 100% DMSO. The concentration series made were 200 mg/mL, 400 mg/mL and 800 mg/mL with 125 L stock-taking, 250 L dissolved in 100% DMSO to 500 L and for a concentration of 800 mg/mL 500 L was taken directly from the stock solution.

12. Amikacin Concentration Series and Stock Preparation

Amikacin stock with 50 mg/mL concentration was prepared by dissolving 500 mg amikacin with aqua pro injection until the volume was 10 mL. The concentration series that were made were 5 mg/mL, 10 mg/mL, and 20 mg/mL from the stock solution taken as much as 100 L, 200 L, 400 L and dissolved with the aqua pro injection to 1 mL.

13. Comparison Series of Peppermint Leaf Ethanol Extract and Amikacin

Comparison of the concentration series of peppermint leaf ethanol extract with amikacin made 3 comparisons, namely 25:75, 50:50, 75:25. The concentration of peppermint leaf ethanol extract used was 200 mg/mL and 400 mg/mL, while the amikacin concentration series used was 5 mg/mL, with the total volume of the combination in disk 20 L. The intake for the combination test of each comparison is 5 L:15 L, 10 L:10 L, 15 L:5 L.

14. Antibacterial Activity Test of Peppermint Leaf Ethanol Extract and Amikacin with Disk Diffusion Method

Each suspension of *K. pneumoniae, S. aureus*, and *E. coli* bacteria was taken 200 L then inoculated on MH media and then flattened using a spreader glass, and waited for a while to dry. The DMSO control 100% and the concentration series of peppermint leaf ethanol extract were each taken 20 L and then dripped onto a blank disk that had been placed on MH media that had been inoculated with bacterial suspension. After that, it

was incubated for 18-24 hours at 37ºC and then observed and measured the diameter of the inhibition zone formed. Pretreatment of the amikacin concentration series was carried out in the same way as the peppermint leaf ethanol extract, but the negative control used was aqua pro injection. This single test was replicated 4 times.

15. Antibacterial Activity Test Combination of Peppermint Leaf Ethanol Extract and Amikacin

Bacterial suspensions of *K. pneumoniae, S. aureus,* and *E. coli* which were equivalent to the standard Mc Farland 10^8 CFU/mL were taken 200 L then inoculated on MH media then leveled with a spreader glass, and waited for it to dry. The ethanol extract of peppermint leaves used were concentrations of 200 mg/mL and 400 mg/mL then the volume of the combination of ethanolic extract of peppermint leaves with amikacin was in the ratio (25:75, 50:50, 75:25) with positive control of 5 mg/mL, The negative control used was 100% DMSO and aqua pro injection, each of which was dripped on a blank disk of 20 L which had been placed on MH media with bacterial suspension inoculated. Then it was incubated for 18-24 hours at 37ºC and then observed and measured the diameter of the inhibition zone formed.

16. Phytochemical Screening

a. Alkaloid Test

The extract was weighed as much as 40 mg then added with 2 mL of chloroform and ammonia and then filtered. The mixture is then added 3-5 drops of concentrated H_2SO_4 and then shaken until two layers are formed. The acid portion (bottom) was taken and added with 4-5 drops of Mayer and Dragendorff reagents each. The sample contains alkaloids if a precipitate is formed, using Mayer's reagent the precipitate is white, and Dragendorff's reagent the precipitate formed is yellow-red.

b. Phenolic Test

The ethanol extract of peppermint leaves was weighed as much as 40 mg and then added with 10 mg/mL $FeCl_3$ solution as much as 10 drops. The presence of phenolic in the sample if the solution is green, blue, purple, dark black, or red.

c. Flavonoid Test

Peppermint leaf ethanol extract was weighed 40 mg then added 100 mL of hot water and then boiled for 5 minutes and filtered. Then 5 mL was taken, added with 50 mg of Mg powder and 1 mL of concentrated HCl, and then shaken vigorously. Observe the color changes that occur, the presence of flavonoids if the solution is yellow or orange and red.

d. Saponin Test

Extract 40 mg added with 10 mL of water then shaken for 1 minute, then added with HCl 1 N 2 drops. Observe the foam formed for 7 minutes, if the foam formed is stable in the extract containing saponins.

e. Tannin Test

Peppermint leaf ethanol extract was taken sufficiently then added 1 mL of 20 mg/mL sodium chloride solution and then added 5 mL of 10 mg/mL gelatin. The formation of a precipitate indicates the presence of tannins in the sample.

f. Steroid or Titerpenoid Test

Forty mg of extract was weighed and then 10 drops of glacial CH_3COOH and 2 drops of H_2SO_4 were added and then shaken gently. Extracts containing steroids are indicated by green or blue color in the solution, extracts indicate the presence of triterpenoids if the solution formed is red or purple.

17. Data analysis

a. Antibacterial Activity Data Analysis

Data analysis was performed by measuring the diameter of the inhibition zone on the ethanol extract of peppermint leaves, amikacin, DMSO, and the combination of the ethanolic extract of peppermint leaves with amikacin. The results showed synergism if the diameter of the combined inhibition zone was greater than that without the combination.

b. Phytochemical Screening Data Analysis

Data analysis to determine the compounds contained in the extract was carried out by looking at the test reaction results and color changes in the test tube.

RESULTS AND DISCUSSION

1. Plant Determination

Plant determination was carried out at the Plant Systematics Laboratory of the Biology Study Program, Faculty of Mathematics and Natural Sciences, Sebelas Maret University. The purpose of plant determination is to determine the correct identity of a plant used in research and to avoid errors in collecting samples for research (Diniatic, 2015). The key to the determination of these plants is as follows:

Identification key:

1b-2b-3b-4b-12b-13b-14b-17b-18b-19b-20b-21b-22b-23b-24b-25b-26b-27a-28b-29b-30b-31b-403b-404b-405a- 406b-409a-410b-411b ——————————————————

190.Lamiaceae 1b-2b-3a-4c-5b-7b-8c-11a-12a-13b-15c-20b-21b-23b-24b

22.Ocimum 1b-2b-4b-5b-6a-7b-8a ——————————

7.Mentha x piperitaL. ——————————————————

The results of the identification key indication that the plant used for this research is *Mentha piperita* L.

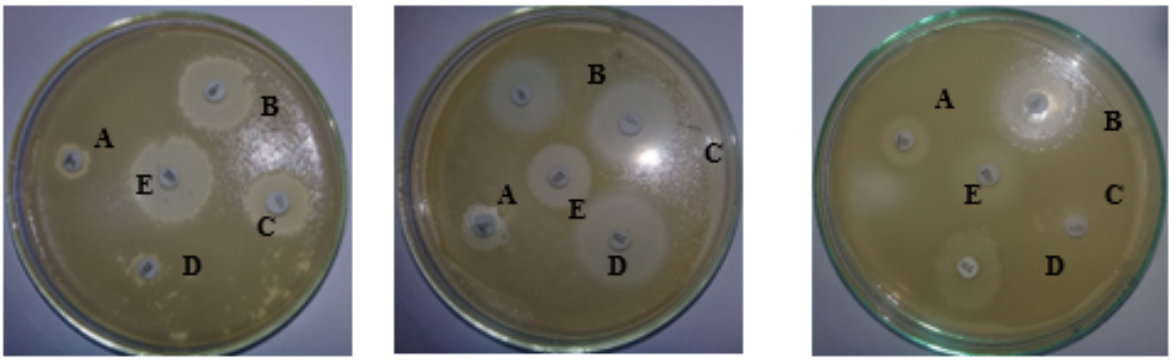
2. Extraction

Extracts were made by the maceration method using 96% ethanol as solvent. Ethanol is a mixed hydroalcoholic solvent that can dissolve substances that are soluble in alcohol and water so that it can extract the active compounds contained in plant simplicia (Ansel, 1989). The plant parts used in this study were leaves that contained chlorophyll compounds. Chlorophyll is an unstable compound so it is necessary to separate chlorophyll (Ernaeni et al., 2012). The yield of the extract obtained was 9.333% from 300 grams of dried simplicia peppermint leaves obtained 28 grams of thick extract.

3. Bacterial Sensitivity Test To Antibiotics

The sensitivity test is used to determine the sensitivity and resistance of bacteria to antibiotics (Cahyono and Indrayudha, 2013), the bacteria used were *K. pneumoniae, S. aureus,* and *E. coli* using the disk diffusion method. The sensitivity test used several antibiotic discs, namely Ampicillin, Chloramphenicol, Tetracycline, Erythromycin, and Amikacin. The diameter of the inhibition zone from the results of the bacterial sensitivity test to antibiotics can be seen in Figure 1.

The results of the bacterial sensitivity test to antibiotics (Table 1) on *K. Pneumoniae* bacteria showed intermediates on ampicillin and tetracycline antibiotic disks while showing sensitivity on chloramphenicol, erythromycin, and amikacin antibiotic disks*. S. aureus* bacteria showed sensitivity to all antibiotic disks, while *E. coli* bacteria showed intermediates to ampicillin antibiotic disks, sensitivity to chloramphenicol, tetracycline, amikacin antibiotic disks, and resistance to erythromycin antibiotic disks, bacterial resistance to erythromycin due to the methylation process on rRNA receptors resulting in loss of receptors on ribosomes (Jawetz et al., 2005).



I II III

Picture 1. Bacterial sensitivity test results to antibiotics Ampicillin (A), Chloramphenicol (B), Erythromycin (C), Tetracycline (D), Amikacin (E)
Description: *Klebsiella pneumoniae* (I), *Staphylococcus aureus* (II), *Escherichia coli* (III)

Table 1. Bacterial Sensitivity Test Results Klebsiella pneumoniae, Staphylococcus aureus, and Escherichia coli

Bacteria	Antibiotic Disc	Bacterial inhibition zone standard (mm)			Inhibition zone diameter (mm)	Information
		Resis-tance	Inter-mediate	Sensi-tive		
Klebsiella Pneumoniae	Ampicillin 10 g	13	14 - 16	17	16	Intermediates
	Chloramphenicol 30 g	12	13 - 17	18	21	sensitive
	Tetracycline 30 g	11	12 - 14	15	12	Intermediates
	Erythromycin 15 g	15	16 -20	21	21	sensitive
	Amikacin 30 g	14	15 -16	17	27	sensitive
Staphylococcus aureus	Ampicillin 10 g	13	14 - 16	17	19	sensitive
	Chloramphenicol 30 g	12	13 - 17	18	22	sensitive
	Tetracycline 30 g	11	12 - 14	15	27.5	sensitive
	Erythromycin 15 g	15	16 -20	21	25.5	sensitive
	Amikacin 30 g	14	15 -16	17	20	sensitive

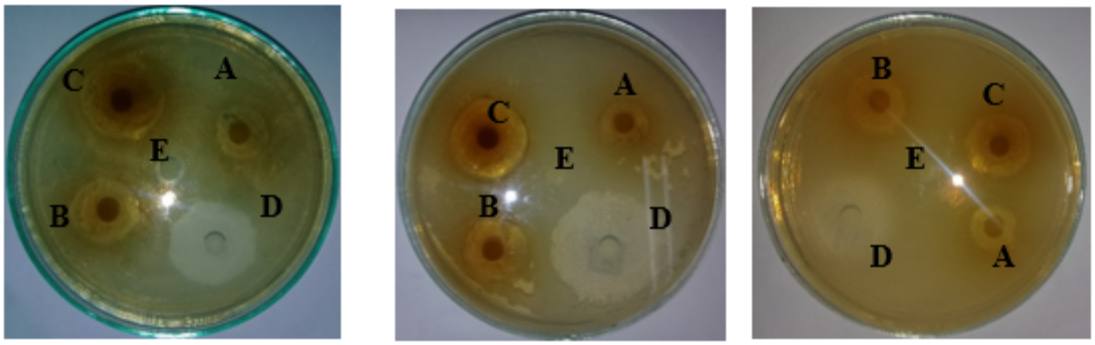
Bacteria	Antibiotic Disc	Bacterial inhibition zone standard (mm)			Inhibition zone	Information
Escherichia coli	Ampicillin 10 g	13	14 - 16	17	15	Intermediates
	Chloramphenicol 30 g	12	13 - 17	18	27.5	sensitive
	Tetracycline 30 g	11	12 - 14	15	28	sensitive
	Erythromycin 15 g	15	16 -20	21	6	resistance
	Amikacin 30 g	14	15 -16	17	20	sensitive

Note: Inhibition zone diameter including disk diameter (6mm)

4. Antibacterial Activity of Peppermint Leaf Ethanol Extract and Amikacin

The antibacterial test of single peppermint leaf ethanol extract and amikacin single test was carried out to determine the antibacterial activity of the peppermint leaf ethanol extract and the sensitivity of the amikacin antibiotic used against bacteria (Hanik et al., 2012). The bacteria used were *K. pneumoniae, S. aureus,* and *E. coli*. The test was carried out using the disk diffusion method, this method was also used by AL-sum and Al-arfaj, (2013) and also Raghavan et al., (2018).

The concentration of peppermint leaf ethanol extract used was 200 mg/mL, 400 mg/ml, and 800 mg/mL with each disc concentration of 4mg/disk, 8 mg/disk and 16 mg/disk. The positive control used amikacin 20 mg/mL with a concentration of 0.4 mg/disk per disk, and the negative control used 100% DMSO. The concentrations of amikacin used were 5 mg/mL, 10 mg/mL, 20 mg/mL, with the concentration of each disk being 0.1 mg/disk, 0.2 mg/disk and 0.4 mg/disk, the negative control used was used aqua pro injection. The results of the diameter of the inhibition zone were influenced by the ethanol extract of peppermint leaves on the growth of K. pneumoniae, S. aureus, and E. coli bacteria.



(I) (II) (III)

Picture 2. The results of the antibacterial activity test of peppermint leaf ethanol extract with a concentration of 4 mg/disk (A), concentration 8 mg/disk (B), concentration 16 mg/disk (C), amikacin 0.4 mg/disk (D), DMSO (E)

Description: *Klebsiella pneumoniae* (I), *Staphylococcus aureus* (II), *Escherichia coli* (III)

Table 2. The results of the antibacterial activity of peppermint leaf ethanol extract

Test Material mg/disk	*K. pneumoniae* inhibition zone diameter (mm)	*S. aureus* inhibition zone diameter (mm)	*E. coli* inhibition zone diameter (mm)
Extract 4	18±1.63	18.5±1	16.25±0.5
Extract 8	20.5±0.58	22.25±0.5	19.75±0.5
Extract 16	25.5±0.58	25.25±0.5	25.25±0.5
Amikacin 0.4	36.25±2.5	26.5±2.38	23±0

Test Material	*K. pneumoniae* inhibition	*S. aureus* inhibition	*E. coli* inhibition zone
DMSO	6	6	6

Note: Inhibition zone diameter including disk diameter (6mm), is the average of 4 times of replication

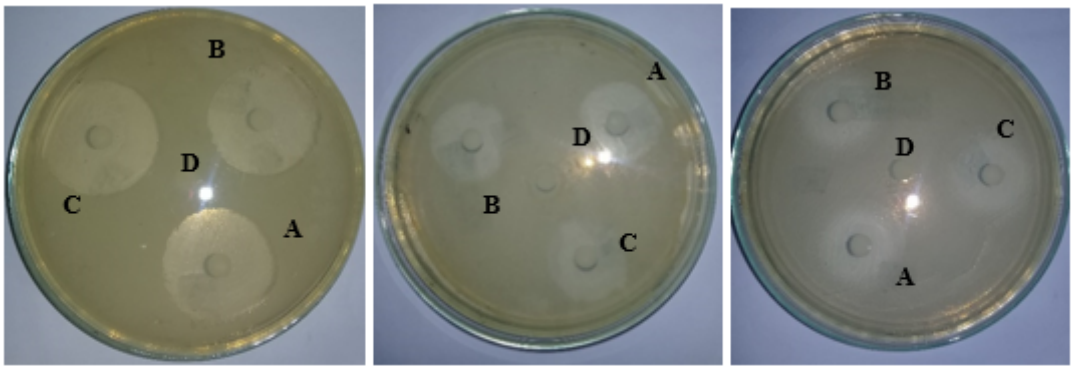
The single test results of antibacterial activity of peppermint leaf ethanol extract against *K. pneumoniae, S.aureus,* and *E.coli* bacteria (Table 2) at the tested concentration formed a radical inhibition zone (Figure 2), meaning that the peppermint leaf ethanol extract has antibacterial activity. The diameter of the inhibition zone of peppermint leaf ethanol extract had the greatest activity at a concentration of 800 mg/mL with a concentration of 16 mg/disk per disk, which was equal to25.5±0.58 mm in *K. pneumoniae* bacteria, 25.25±0.5 mm in *S. aureus*, and *E.coli*. The greater the concentration tested, the greater the diameter of the inhibition zone, this is influenced by the large number of compounds contained in the disk so that the peppermint leaf ethanol extract is able to inhibit the growth of K. pneumoniae, S. aureus, and E. coli bacteria as well.

The results of the diameter of the inhibition zone on the ethanol extract of peppermint leave formed (Figure 2) were compared with the positive control used, namely amikacin 20 mg/mL with a concentration of 0.4 mg/disk each with an inhibition zone of 36.25±2.5 mm at *K. pneumoniae*, 26.5 ± 2.38 mm in *S. aureus*, and 23 ± 0 mm in *E. coli*. While the negative control used was 100% DMSO as a solvent for peppermint leaf ethanol extract that did not show the diameter of the inhibition zone so that the resulting inhibition zone was not influenced by the solvent (Handoko et al., 2013). The diameter of the inhibition zone in the ethanol extract of peppermint leaves was smaller than the positive control, but the ethanolic extract of peppermint leaves still had antibacterial activity because it could inhibit the growth of *K. pneumoniae, S. aureus,* and *E. coli bacteria.*

Research conducted by Al-sum and Al-arfaj, (2013) Peppermint leaf aqueous extract with a concentration of 200 mg/ml can inhibit the growth of *K. pneumoniae, S. aureus*, and *E. coli* bacteria, with inhibition zone diameters of 8 mm, 18 mm, and 14 mm, respectively. Results of research conducted Al-sum and Al-arfaj, (2013)The diameter of the resulting inhibition zone is smaller than the test results on the ethanol extract of peppermint leaves in this study. This can be caused by differences in the solvents used, in this study 96% ethanol solvent was used, and leaves were obtained from the Indonesian region, while in this study Al-sum and Al-arfaj, (2013) The solvent used was water, and the leaves used were obtained from the Riyadh garden area in Saudi Arabia.

The results of a single test of amikacin on bacteria *K. pneumoniae, S. aureus*, and *E. coli* (Table 3) with a concentration of 5 mg/mL, 10 mg/mL, 20 mg/mL with a concentration of 0.1 mg/disk per disk, 0 ,2 mg/disk, 0.4 mg/disk indicated the zone of inhibition respectively 31.5±2.38 mm, 33.75±1.5 mm, 38.75±2.98 in bacteria *K. pneumoniae*, 23.25±1.25 mm, 26.25±1.25mm, 28±0.81 mm in bacteria*S. aureus* and 25±0 mm, 26.75±1.5 mm, 30±0 mm in bacteria *E. coli*. The negative control used was Aqua pro injection as the solvent did not show an inhibition zone, so the resulting inhibition zone was not affected by the solvent. Based on the diameter of the resulting inhibition zone (Figure 3), a concentration of 5 mg/mL (0.1 mg/disk) was chosen for the combination test, a concentration of 5 mg/mL (0.1 mg/disk) was chosen because the diameter of the

resulting inhibition zone was large when the largest concentration was used, which was 20 mg/mL with a concentration of 0.4 mg/disk per disk when combined the diameter of the resulting inhibition zone would be larger.



(I) (II) (III)

Picture 3. Preliminary test results of amikacin 0.1 mg/disk (A), 0.2 mg/disk (B), 0.4 mg/disk (C), aqua pro injection (D)

Description: Klebsiella pneumoniae (I), Staphylococcus aureus (II), Escherichia coli (III)

Table 3. Amikacin preliminary test results

Test Material mg/disk	*K. pneumoniae* inhibition zone diameter (mm)	*S. aureus* inhibition zone diameter (mm)	*E. coli* inhibition zone diameter (mm)
Amikacin 0.1	31.5±2.38	23.25±1.25	25±0
Amikacin 0.2	33.75±1.5	26.25±1.25	26.75±1.5
Amikacin 0.4	38.75±2.98	28±0.81	30±0
Aqua pro injection	6	6	6

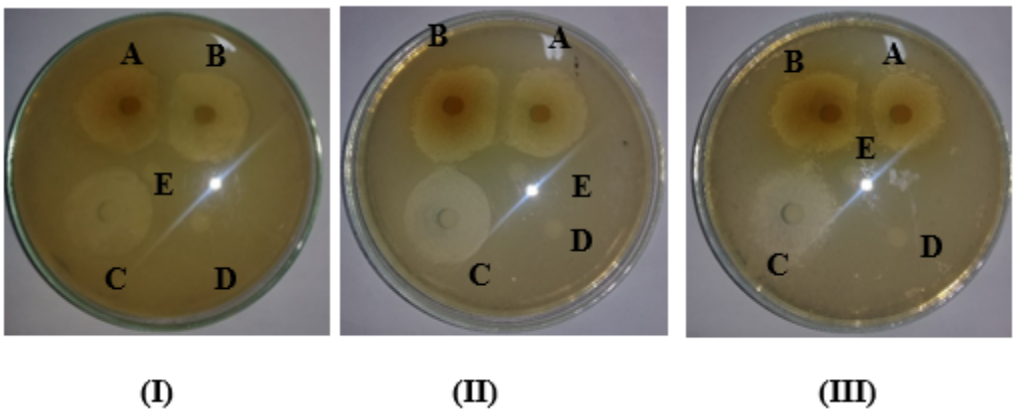
Note: Inhibition zone diameter including disk diameter (6mm), is the average of 4 times of replication

5. Antibacterial Activity Combination of Peppermint Leaf Ethanol Extract and Amikacin

The purpose of the antibacterial activity test of the combination of peppermint leaf ethanol extract and amikacin was carried out to determine the effect of antibacterial activity after being combined rather than given alone. This test was carried out using the disk diffusion method with a concentration of peppermint leaf ethanol extract of 200 mg/mL and 400 mg/mL with a concentration of each disk of 4 mg/disk and 8 mg/disk combined with amikacin at a concentration of 5 mg/mL with a concentration of 0, each disk. 1 mg/disk. The comparison between the ethanol extract of peppermint leaves and amikacin was 25:75, 50:50, 75:25 with a total volume of 20 L, the positive control used was amikacin 5 mg/mL with a concentration of 0.1 mg/disk each, while the negative control was used. used DMSO and aqua pro injection.

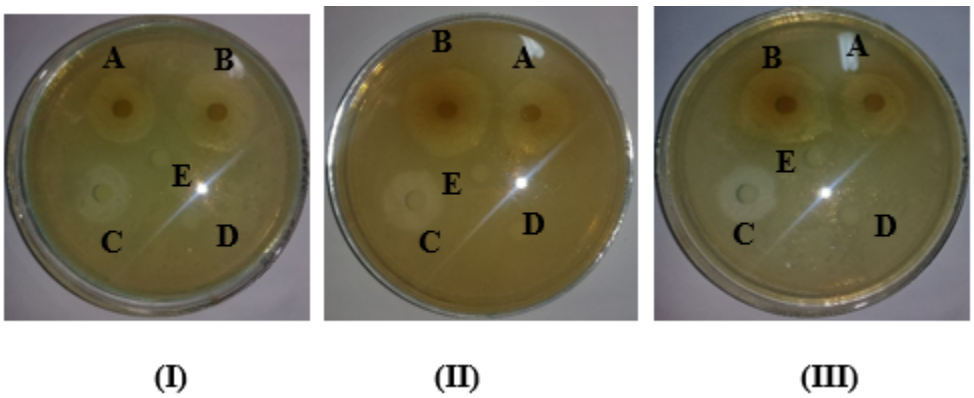
The results of the combination antibacterial activity test on *K. pneumoniae, S. aureus,* and *E. coli* bacteria (Tables 4 and 5) showed that the diameter of the inhibition zone was larger than that of the ethanol extract of peppermint leaves alone. Inhibition zone diameter of peppermint leaf ethanol extract concentration of 200 mg/mL (4 mg/disk) in combination with amikacin 5 mg/mL (0.1 mg/disk) at a ratio of 25:75, 50:50, 75:25 in bacteria K. pneumoniae in a row, namely36.25±2.5 mm; 32±1.5mm; 34±1.71 mm.

Whereas in bacteria*S. aureus* that is 27.25±1.71 mm; 27.25±1.89 mm; 26±1.41 mm and in bacteria*E. Coli* that is 26.75±1.5 mm; 28±1.16mm; 26.75±2.62 mm. Diameter of single inhibition zone of amikacin concentration 5 mg/mL in bacteria *K. pneumoniae*, *S. aureus*, and *E. coli*, respectively, namely 30±0; 20±0; 20±0. At concentrations of 200 mg/mL (4 mg/disk) and 400 mg/mL (8 mg/disk) with amikacin 5 mg/mL (0.1 mg/disk) resulted in a larger diameter than given without the combination. These data indicate that the combination of peppermint leaf ethanol extract with amikacin antibiotics shows a synergistic effect.



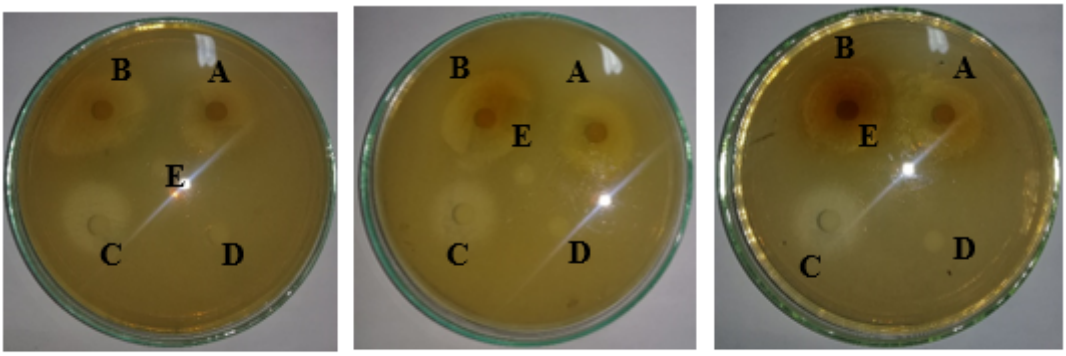
Picture 3. The results of the antibacterial activity of the combination of peppermint leaf ethanol extract and amikacin against bacteria K. pneumoniae 4 mg/disk (A), 8 mg/disk (B), Amikacin 0.1 mg/disk (C), Aqua pro injection (D), DMSO (E)

Note: Comparison 25:75 (I), Comparison 50:50 (II), Comparison 75:25 (III)



Picture 4. The results of the antibacterial activity of the combination of peppermint leaf ethanol extract and amikacin against S. aureus bacteria 4 mg/disk (A), 8 mg/disk (B), Amikacin 0.1 mg/disk (C), Aqua pro injection (D), DMSO (E)

Note: Comparison 25:75 (I), Comparison 50:50 (II), Comparison 75:25 (III)



(I) (II) (III)

Picture 5. The results of the antibacterial activity of the combination of peppermint leaf ethanol extract and amikacin against E. coli bacteria 4 mg/disk (A), 8 mg/disk (B), Amikacin 0.1 mg/disk (C), Aqua pro injection (D), DMSO (E)

Note: Comparison 25:75 (I), Comparison 50:50 (II), Comparison 75:25 (III)

Table 4. The results of the antibacterial activity of the combination of peppermint leaf ethanol extract 4 mg/disk and amikacin 0.1 mg/disk

Test Material	*K. pneumoniae* inhibition zone diameter (mm)	*S. aureus* inhibition zone diameter (mm)	*E. coli* inhibition zone diameter (mm)
Extract 4 mg/disk + Amikacin 0.1 mg/disk (25:75)	36.25±2.5	27.25±1.71	26.75±1.5
Extract 4 mg/disk + Amikacin 0.1mg/disk (50:50)	32±1.5	27.25±1.89	28±1.16
Extract 4 mg/disk + Amikacin 0.1mg/disk (75:25)	34±1.71	26±1.41	26.75±2.62
Amikacin 0.1mg/disk	30±0	20±0	20±0
DMSO	6	6	6
Aqua pro injection	6	6	6

Note: Inhibition zone diameter including disk diameter (6mm), is the average of 4 times of replication

Table 5. The results of the antibacterial activity test of a combination of peppermint leaf ethanol extract 8 mg/disk and amikacin 0.1 mg/disk

Test Material	*K. pneumoniae* inhibition zone diameter (mm)	*S. aureus* inhibition zone diameter (mm)	*E. coli* inhibition zone diameter (mm)
Extract 8 mg/disk + Amikacin 0.1mg/disk (25:75)	39.75±2.36	31.5±1.73	31.5±1.73
Extract 8 mg/disk + Amikacin 0.1mg/disk (50:50)	375±2.45	32.75±1.5	31±0.82
Extract 8 mg/disk + Amikacin 0.1mg/disk (75:25)	40±1.63	29.5±0.58	29±1.15
Amikacin 0.1mg/disk	30±0	20±0	20±0
DMSO	6	6	6
Aqua pro injection	6	6	6

Note: Inhibition zone diameter including disk diameter (6mm), is the average of 4 times of replication

The synergistic effect of the combination of peppermint leaf ethanol extract and amikacin in inhibiting bacterial growth showed a mutually supportive effect between the two. Research conducted by Pramila et al., 2012 showed Peppermint leaf extract compounds that can inhibit bacterial growth is flavonoid compounds and tannins. The combination of antibiotics and flavonoid compounds can provide a synergistic effect in inhibiting bacterial growth because flavonoid compounds are the largest group of phenolic compounds so that when combined with antibiotics can increase the diameter of the inhibition zone in inhibiting bacterial growth. (Amin et al., 2016). The mechanism of action of amikacin, which is an aminoglycoside group, is to inhibit protein biosynthesis by irreversibly binding aminoglycosides to the 30S subunit of the bacterial ribosome (Katzung and Bertram, 2004), so that it can inhibit the function of the 30S subunit of the bacterial ribosome and will cause the bacteria to die. The addition of extract to the use of amikacin can increase activity as an antibacterial by producing a larger diameter of the inhibition zone without the combination.

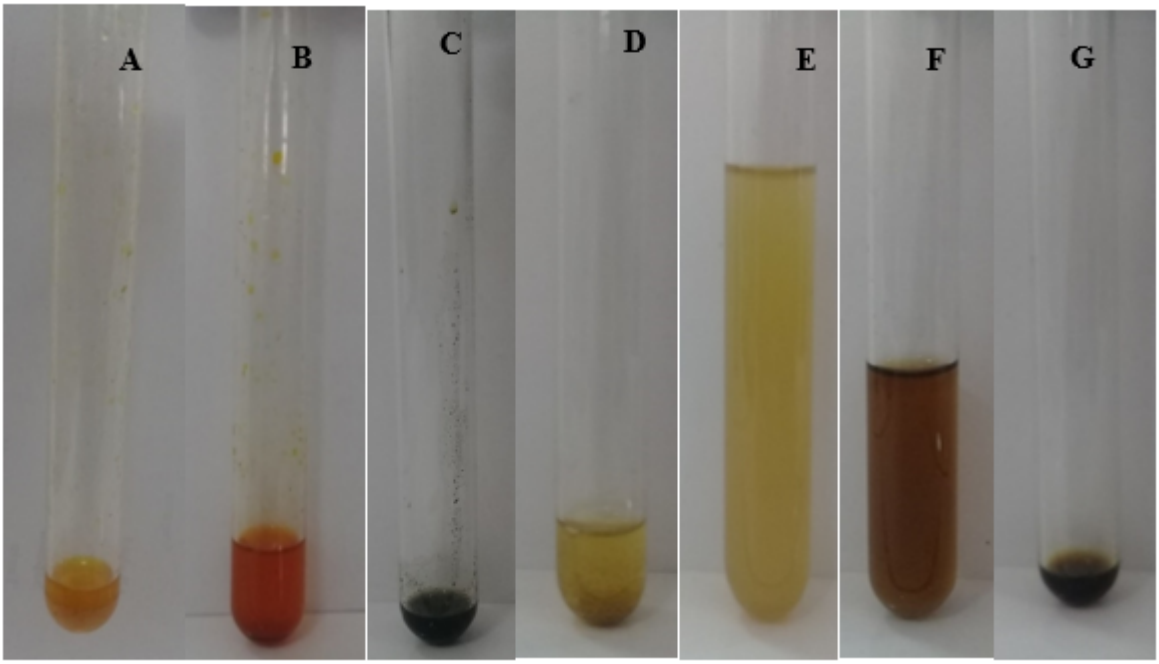
The data calculated using the t-test showed that the significance value obtained was 0.000 <0.05. It could be concluded that there was a significant difference in the administration of each concentration to the resulting inhibition zone. The results showed a synergistic effect with the largest diameter of the inhibitory zone at a ratio of 25:75 with a concentration of 200 mg/mL, which was 36.25±2.5 mm on *Klebsiella pneumoniae* bacteria, which before combined the diameter of the inhibition zone was 18±1.63 mm. Meanwhile, at a concentration of 400 mg/mL, the diameter of the largest inhibition zone was 40±1.63 mm in *Klebsiella pneumoniae* bacteria with a ratio of 75:25 which before combined the diameter of the resulting inhibition zone was 20.5±0.58 mm.

6. Phytochemical Screening Results

The purpose of the phytochemical screening test was to determine the content of compounds contained in the ethanol extract of peppermint leaves. This phytochemical screening test was carried out using the tube method, namely by using several color reagents (Simaremartye, 2014). The results of phytochemical screening of peppermint leaf ethanol extract showed negative results on saponins and steroids, this was because the glycosyl groups on saponins which act as polar groups were not active on the surface so that when shaken with water the saponins would not show foam, while on steroids the color changed to green. caused by oxidation but the results did not show a color change so that there was no color change due to the absence of oxidation of steroid compounds through the formation of conjugated double bonds (Risky and Suyatno, 2014).

Positive results were shown on alkaloids, phenolic compounds, flavonoids, tannins, and triterpenoids. In the alkaloid tested using Meyer's reagent a white precipitate will form and with Dragendorff's reagent a red color will be formed, this is due to the presence of an alkaloid complex with potassium so that a precipitate will form (Marliana and Suryanti, 2015). The test results on phenolic showed positive in the presence of black color in the reaction tube, the black color formed was due to the reaction of $FeCl_3$ with the aromatic –OH group. In the flavonoid test, the formation of yellow color was due to the reduction by concentrated HCl with Mg powder (Minarno, 2015). Test on tannins using sodium chloride and gelatin, the formation of a precipitate due to the reaction between tannins and gelatin will form a copolymer that is insoluble with water so that it will precipitate protein from gelatin (Datin et al., 2014). Positive results on triterpenoids

are indicated by a red-brown color due to the addition of H_2SO_4 so that a brownish ring is formed in the test solution (Qurrota and Laily, 2011).



Picture 6. Phytochemical screening test results of ethanol extract of peppermint leaf alkaloids (A, B), phenolics (C), flavonoids (D), saponins (E), tannins (F), steroids (G)

Table 6. Phytochemical screening results of peppermint leaf ethanol extract

Compound	References (Pulipati et al., 2016)	Phytochemical Screening Results	Derivative Compound
Alkaloids	**+**	**+** (With Meyer's reagent a white precipitate is formed, with Dragendorff's reagent a yellow-red precipitate is formed)	Hydrocotyl alkaloids
Phenolic	+	+ (Dark Black)	Phenolic acid, chlorogenic, rosmarinic
Flavonoids	+	+ (Yellow)	Quercetin, mentoside,
Saponins	_	_ (No foam formed)	isoroifolin, vitamin K,
Tannins	+	+ (Chocolate precipitate)	eugenol, thymol, rutin
Steroids / Triterpenoids	+/+	-/+ (Red-brown)	Squalene, urosolic acid, a-amyrin

Description: + (positive), - (negative)

The results of the research conducted by Pulipati et al., (2016) showed Peppermint leaf ethanol extract contains alkaloids, phenolic compounds, flavonoids, tannins, steroids, and triterpenoids, while the results are negative for saponins. Research conducted by Pulipati et al., (2016) compared to the test results in this study, the ethanol extract of peppermint leaves was almost the same but in this study, there were no steroid compounds, this was due to differences in the reagents used.

The mechanism of action of alkaloids as an antibacterial is by interfering with the constituent components of peptidoglycan in bacterial cells so that the cell wall layer is

not fully formed and can cause cell death (Darsana et al., 2012). The mechanism of action of phenolics in killing microorganisms is by denaturing cell proteins. The hydrogen bonds formed between phenol and protein cause the protein structure to be damaged. The hydrogen bonds will affect the permeability of the cell wall and cytoplasmic membrane. Permeability of cell walls and cytoplasmic membranes that are disrupted can cause an imbalance of macromolecules and ions in the cell so that the cell becomes lysed (Poongothai and Rajan, 2013).

The mechanism of action of flavonoids in inhibiting bacterial growth is divided into three, namely inhibiting nucleic acid synthesis, inhibiting cell membrane function, and inhibiting energy metabolism. The antibacterial mechanism of flavonoid compounds inhibiting nucleic acid synthesis is the location of the hydroxyl group at the 2',4' or 2',6' dihydroxylation position on rings B and 5,7 dihydroxylation on ring A plays an important role in antibacterial activity. Flavonoids cause damage to the permeability of bacterial cell walls, microsomes, and lysosomes as a result of interactions between flavonoids and bacterial DNA (Hendra et al., 2011).

The mechanism of action of flavonoids in inhibiting cell membrane function is to form complex compounds with extracellular proteins so that they can damage bacterial cell membranes and are followed by the release of intracellular compounds (Nuria et al., 2009). Flavonoids can inhibit energy metabolism by inhibiting the use of oxygen by bacteria. Flavonoids will inhibit cytochrome C reductase so that the formation of metabolism is inhibited, energy is needed by bacteria for macromolecular biosynthesis (Rao, 2013).

The mechanism of action of tannins as an antibacterial is by inhibiting the enzyme reverse transcriptase and DNA topoisomerase so that bacterial cells cannot be formed (Concerned et al., 2018). The mechanism of triterpenoids as an antibacterial is related to lipid membranes and sensitivity to triterpenoid components that cause leakage in lysosomes. Triterpenoids can interact with cell phospholipid membranes which are permeable to lipophilic compounds, causing decreased membrane integrity and cell membrane morphology to change which causes cell brittleness and lysis (Puspita, 2011).

CONCLUSION

Based on the results of the study, it can be concluded that the peppermint leaf ethanol extract has antibacterial activity against *Klebsiella pneumoniae, Staphylococcus aureus*, and *Escherichia coli* bacteria, the combination of peppermint leaf ethanol extract and amikacin has a synergistic effect or a unidirectional effect indicated by the diameter of the combined inhibition zone is greater than which is without combination. Peppermint leaf ethanol extract contains alkaloids, phenolic compounds, flavonoids, tannins, and triterpenoids.

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