The Effectiveness of Karamunting Leaf’s Fraction (Rhodomyrtus tomentosa (Aiton) Hassk) as Antimicrobials in Carbapenemase Resistant Klebsiella pneumonia

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Abstract

In the world, the incidence of nosocomial infections in hospitals has increased. There had been an increase in the incidence of infections caused by Enterobacteriaceae, one of them is Klebsiella pneumonia, which resistant to carbapenem in the worldwide. The consequences of increased rates of resistance to many drugs pose a high need for the discovery of new types of antibiotic drugs. Rhodomyrtus tomentosa (aiton) hassk has an antibacterial effect that has long been used by Indonesians as a traditional drug. This study aims to find out the effectiveness of karamunting leaf fraction as an antimicrobial in carbapenemase resistant K. pneumonia bacteria and to find out the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the active compound of karamunting leaves as antimicrobial in Carbapenemase resistant Klebsiella pneumonia (CRKP) bacterial isolates. This research was an experimental laboratory research in vitro that exploratory analytical. The results of the study obtained the value of MIC ethyl acetate fraction started at a concentration of 125 μg/ml and n-hexan fraction at a concentration of 4000 μg/ml. MBC value was 125 μg/ml for ethyl acetate fraction and n-hexan fraction of karamunting leaves was 8000 μg/ml. From the bacterial activity test obtained at a concentration 32000 μg/ml of n-hexan fraction, the average diameter of the inhibitory zone 7.80 ± 1.30 mm and ethyl acetate fraction at a concentration of 4000 μg/ml can inhibit the growth of CRKP bacteria with an average of inhibition zone diameter was 9.40 ± 1.67 mm. From the results of the analysis using the Independent T Test and mann whitney test obtained a probability value between all groups with positive control was <0.05. It can be concluded that the ethyl fraction of acetate and n-hexan leaves of karamunting leaves contains active compounds that can interfere with the integrity of CRKP bacterial cell walls or membranes so that the bacteria can die.

Keywords: Fractions, Karamunting Leaves, Klebsiella pneumonia Resistant Carbapenemase

1. Introduction

The incidence of nosocomial infections in the world’s hospitals has increased. From WHO’s data, the incidence of nosocomial infections shows a figure of 8.7% from 55 hospitals from 14 countries where from Southeast Asia, Europe, the Middle East and the Pacific.1 Klebsiella pneumonia (K.pneumonia) is the second most bacterial after Eschericia coli which causes nosocomial infection.2

The drug of choice used for K.pneumonia infection is cephalosporins. However, due to increased resistance to the drug then another treatment option used as a therapeutic option is carbapenem.3 Carbapenem is an antibiotic that has the widest spectrum activity and the greatest potential action to kill gram positive bacteria as well as Gram negatif bacteria.4

In recent decades there has been an increase in the incidence of infections caused by Enterobacteriaceae, one of which is K. pneumonia resistant to carbapenem in the worldwide.5 Enterobacteriaceae, that is resistant to carbapenem, has been reported worldwide as a consequence of most acquisitions of the carbapenemase gene.6 Carbapenemase production is one of the main
mechanisms for resistance of \textit{k.pneumonia resistant} carbapenemase strains. Loss of outer membrane protein (OMP) from bacteria is also the cause of resistance.\textsuperscript{7,8}

The consequences of increased rates of resistance to many drugs pose a high need for the discovery of new types of antibiotic drugs. Karamunting Leaf (\textit{Rhodomyrtus tomentosa (aiton) hassk}) has an antibacterial effect that has long been used by Indonesians as a folk remedy.\textsuperscript{9} The fraction of karamunting leaves has been widely studied to have an antibacterial effect on Gram positive bacteria and Gram negative bacteria. This is due to the very high content of flavanoids and tannins in karamunting leaves.\textsuperscript{10}

Previous research has looked for the antibacterial effects of karamunting leaves on \textit{K.pneumonia} bacteria, but few have examined the antibacterial effectiveness of karamunting leaves against \textit{Carbapenemase resistant Klebsiella pneumonia} bacteria. Therefore, this study aims to find out the effectiveness of karamunting leaf fraction as an antimicrobial in \textit{carbapenemase resistant K.pneumonia} bacteria and to know the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the active compound of karamunting leaves (\textit{Rhodomyrtus tomentosa (aiton) hassk}) as antimicrobial in carbapenemase resistant \textit{Klebsiella pneumonia} (CRKP) bacterial isolates.

2. Method

This research was an in vitro laboratory experimental study with analytical exploratory character to test the antibacterial activity of caramunting leaf fraction (\textit{Rhodomyrtus tomentosa (aiton) hassk}) against carbapenemase resistant \textit{Klebsiella pneumoniae}.

The samples of this study were carbapenemase resistant \textit{Klebsiella pneumoniae} bacteria which were taken from \textit{K. pneumoniae} isolates in infected patients at Dr. Moh. Hoesin Palembang which has been detected as a \textit{Carbapenemase Resistant K. pneumoniae} by the VITEK-2 compact system automatic tool. This bacterial isolate was then cultured on Mc Conkey medium which was then incubated in an incubator at 37.5\degree C for 24 hours.

This research was conducted at the Biomedical Laboratory of FK UNSRI and the Microbiology Laboratory of FK UNSRI in January-April 2020. This research has received ethical approval from the Ethical Committee for Health Research at the Central General Hospital Mohammad Hoesin and FK UNSRI with Certificate of Ethics No.018/ kepkrsmhfkunsri/2020.

The test solution (karamunting leaf fraction) with a concentration of 125 µg/ml, 250 µg/ml and 500 µg/ml was dissolved with 10 ml of nutrient broth. Then added 0.1 bacterial suspension. Incubate for 24 hours at 37\degree C. Then look at the turbidity. MIC is the lowest concentration of bacterial suspension that does not occur growth.

From the clear MIC results, planting was carried out on nutrient agar by scratching it on the media. Then incubate for 24 hours at 37\degree C. Look at the growth of bacteria, if there is no growth, it means that at this concentration is MBC.

Determine which fraction the active compound was in. Performed with the Kirby-Bauer diffusion method, as follows on a petri dish containing Muller Hinton agar smeared with 1 ose of test bacteria. Place a 6 mm disc paper that has previously been added with ethyl-acetate, n-hexane and water ethanol fractions with concentrations of 125 µg/ml, 250 µg/ml and 500 µg/ml, respectively. Also place the disc paper containing the control solution (DMSO) and the tigecyclin antibiotic disc on the agar medium. Incubated 24 hours at 37\degree C. After being stored for 24 hours at
37°C, the resistance zone formed was measured. The antibacterial activity test is said to be positive if there is a clear zone around the disc paper that is free from bacterial growth.

3. Results

3.1 Results of Determination Minimum Inhibitory Concentration Value (MIC) of Karamunting Leaf Fraction (Rhodomyrtus tomentosa (aiton) hassk)

Based on the research of Salni (2018) which reports that karamunting leaf extract (Rhodomyrtus tomentosa (aiton) hassk) with a minimum concentration of 250 µg/ml has an inhibition zone of 8 mm against Enterobacteriaceae bacteria, so we use a concentration of 250 µg/ml as the reference concentration for determining Minimum inhibitory concentrations (MIC). MIC determination was carried out by dilution method, looking at the turbidity in the test tube. The ethyl acetate fraction was divided into five concentrations, namely 250, 125, 62.5, 31.25, 15.625 µg/ml with the addition of nutrient broth. In the n-hexane fraction, the concentration increased and divided into five concentrations, namely 8000, 4000, 2000, 1000, 500 µg/ml. This is because at the previous concentration the MIC value could not be determined. The results of determining the minimum inhibitory concentration (MIC) by the dilution method can be seen in the following table.

| Type of fraction | Concentration (µg/ml) | Turbidity |
|------------------|-----------------------|-----------|
| Ethyl Acetate    | 250 -                 |           |
|                  | 125 +                 |           |
|                  | 62.5 ++               |           |
|                  | 31.25 ++              |           |
|                  | 15.625 ++             |           |
| N-heksan         | 8000 -                |           |
|                  | 4000 +                |           |
|                  | 2000 ++               |           |
|                  | 1000 ++               |           |
|                  | 500 ++                |           |

Note:
++: turbid
+: little turbid
-: clear

3.2 Results of Determination Minimum Bactericidal Concentration (MBC) of Karamunting Leaf Fraction (Rhodomyrtus tomentosa (aiton) hassk)

The results of determining the minimum bactericidal concentration (MBC) of the karamunting leaf ethyl acetate fraction on CRKP bacteria was 125 µg/ml and the MBC of the karamunting leaf n-hexane fraction was 8000 µg/ml.

3.3 Antibacterial Activity Test of Karamunting Leaf Fraction (Rhodomyrtus tomentosa (aiton) hassk) on CRKP bacteria

The results of the karamunting leaf fraction antibacterial activity test showed that n-hexane and ethyl acetate fractions had antibacterial activity against Carbapenemase Resistant Klebsiella pneumonia (CRKP). This was indicated by the formation of an inhibition zone which is then averaged from...
five repetitions and can be classified as a compound that has antibacterial activity. At a concentration of 32000 µg/ml, n-hexane fraction of karamunting leaves could inhibit the growth of CRKP bacteria with an average inhibition zone diameter of 7.80 ± 1.30 mm. Ethyl acetate fraction at a concentration of 4000 µg/ml can inhibit the growth of CRKP bacteria with an average inhibition zone diameter of 9.40 ± 1.67 mm.

**Tabel 2. Results of the Antibacterial Activity Test of Caramunting Leaf Fraction (Rhodomyrtus tomentosa (aiton) hassk) on CRKP bacteria**

| Fraction     | Concentration (µg/ml) | Number of repetitions | Average inhibition zone diameter (mm) |
|--------------|-----------------------|-----------------------|--------------------------------------|
| N-hexan      | 32000                 | 5                     | 7.80 ± 1.30                          |
| Ethyl Acetate| 4000                  | 5                     | 9.40 ± 1.67                          |
| Control(-)   | 5                     | 6.20 ± 0.40           |
| DMSO         | 5                     | 0.40                  |
| Control(+)   | 5                     | 16.00 ± 1.22          |
| Tigecyclin   |                       |                       |

From table 2, it is known that the ethyl acetate fraction produces a larger inhibition zone in inhibiting the growth of CRKP bacteria than the n-hexane fraction.

### 3.4 Normality and Homogenity Test of Research Sample

In this study, the normality test of the minimum inhibitory concentration was carried out against *Carbapenemase Resistant Klebsiella pneumonia* (CRKP). From the Kolmogorov Smirnov test, the minimum inhibitory concentration of *Klebsiella pneumonia* was obtained from the EDK 4000, HDK 32000 group and positive control> 0.05, which means that the distribution of EDK 4000, HDK 32000 data and normal positive controls.

**Tabel 3. The result of Normality and Homogenity Test**

| Concentration | Treatment group (n = 5 per group) | Mean ± SD | p* |
|---------------|-----------------------------------|-----------|----|
| Klebsiella pneumonia | Negative control | 0.62 ± 0.045 | 0.001 |
|               | EDK 4000                          | 0.94 ± 0.167 | 0.200 |
|               | EDK 2000                          | 0.86 ± 0.089 | 0.046 |
|               | EDK 1000                          | 0.88 ± 0.109 | 0.026 |
|               | EDK 500                           | 0.70 ± 0.000 | 0.079 |
|               | EDK 250                           | 0.70 ± 0.000 | 0.001 |
|               | EDK 125                           | 0.70 ± 0.000 | 0.001 |
|               | HDK 32000                         | 0.78 ± 0.130 | 0.161 |
|               | HDK 16000                         | 0.70 ± 0.000 |      |
|               | HDK 8000                          |            |    |
|               | HDK 4000                          | 0.68 ± 0.045 |      |
|               | Positive control                  | 0.68 ± 0.045 |      |
|               | control                           | 1.60 ± 0.123 |      |

Kolmogorov Smirnov, p = 0.05

**3.5 Effectiveness of Karamunting Leaf Fraction against Carbapenemase Resistant Klebsiella pneumonia (CRKP)**

The minimum inhibitory concentrations of all groups against *Carbapenemase Resistant Klebsiella pneumonia (CRKP)* were measured then averaged and tabulated, then compared each group. With the Kolmogorov Smirnov normality test (table 3), the probability value of the EDK 4000, HDK 32000 group and positive control group was> 0.05, which means that the data distribution of the three groups was normal. The comparison between the two groups with normal distribution used the Independent T Test, while the comparison between the two groups involving groups with abnormal data used the Mann Whitney test. The results of the analysis can be seen in table 4.

From the analysis, it was found that the probability value between all groups with positive control was <0.05, so it could be concluded that there was a significant difference in the mean minimum inhibitory concentration of all treatment groups with positive control on CRKP. The minimum
inhibitory concentration of positive control was greater than all treatment groups.

Table 4 Comparison of the Minimum Inhibitory Concentration of Karamunting Leaf Fraction to CRKP

| Group         | Mean ± SD | Group | Mean ± SD | p value |
|---------------|-----------|-------|-----------|---------|
| Negative      | 0.62 ± 0.045 | EDK 4000 | 0.94 ± 0.167 | 0.009* |
| group         |           | EDK 2000 | 0.86 ± 0.089  | 0.006* |
|               |           | EDK 1000 | 0.88 ± 0.109  | 0.005* |
|               |           | EDK 500  | 0.70 ± 0.014* | 0.000  |
|               |           | EDK 250  | 0.70 ± 0.014* | 0.000  |
|               |           | EDK 125  | 0.70 ± 0.014* | 0.000  |
|               |           | HDK 32000| 0.78 ± 0.130  | 0.000  |
|               |           | HDK 16000| 0.70 ± 0.014* | 0.000  |
|               |           | HDK 8000 | 0.68 ± 0.072* | 0.045  |
|               |           | HDK 4000 | 0.68 ± 0.072* | 0.045  |
|               |           | Positive | 1.60 ± 0.123  | 0.007* |
|               |           | control  | 1.60 ± 0.123  | 0.007* |
| EDK 4000      | 0.94 ± 0.167 | EDK 2000 | 0.86 ± 0.089  | 0.334* |
|               |           | EDK 1000 | 0.88 ± 0.109  | 0.455* |
|               |           | EDK 500  | 0.70 ± 0.018* | 0.000  |
|               |           | EDK 250  | 0.70 ± 0.018* | 0.000  |
|               |           | EDK 125  | 0.70 ± 0.018* | 0.000  |
|               |           | HDK 32000| 0.78 ± 0.129* | 0.000  |
|               |           | HDK 16000| 0.70 ± 0.018* | 0.000  |
|               |           | HDK 8000 | 0.68 ± 0.045  | 0.017* |
|               |           | HDK 4000 | 0.68 ± 0.045  | 0.017* |
|               |           | Positive | 1.60 ± 0.123  | 0.000* |
|               |           | control  | 1.60 ± 0.123  | 0.000* |
| EDK 2000      | 0.86 ± 0.089 | EDK 1000 | 0.88 ± 0.109  | 0.811* |
the results obtained were different from the KBM results of the ethanol extract in MRSA bacteria, namely between 3.91-62.5 µg/ml. This can be caused by differences in the group of bacteria, namely between gram positive and gram negative.\textsuperscript{12}

The results of the antibacterial activity test showed that the n-hexane and ethyl acetate fractions had antibacterial activity which was indicated by the formation of an inhibition zone on the media. Between the two test samples, the ethyl acetate fraction had a large enough inhibition zone diameter compared to the n-hexane fraction, which was 9.40 ± 1.67 mm in CRKP bacteria. Ethyl acetate and n-hexane fractions contain flavonoids and from the Salni, 2019, namely at a concentration of 250 µg/ml. Based on the MIC level, the strength of the active content of the ethyl acetate fraction was categorized as quite strong because it ranged from 100-500 µg/ml. The strength of the antibacterial content can be seen in the MIC value. The MIC value of antibacterial active compounds is divided into several classes, namely active compounds that have a MIC value of less than 100 µg/ml have very strong antibacterial active compounds, if the MIC value between 100-500 µg/ml is strong enough, the KHM value is 500-1000 µg/ml is weak and if the MIC value is more than 1000 µg/ml it means that the compound does not have antibacterial activity.\textsuperscript{11}

Based on the results of determining the value of the Minimum Bactericidal Concentration (MBC), the MBC for the ethyl acetate fraction in CRKP bacteria was 125 µg/ml. The MBC value indicates that at the fraction concentration there are no bacteria that can grow. The results of the MBC value of the ethyl acetate fraction were the same as the research conducted by Limsuwan et al., 2009, where ethanol extracts of methicillin-resistant \textit{S. aureus} (MRSA) bacteria were 30-1000 µg/ml. Whereas in the n-hexane fraction the results obtained were different from the KBM results of the ethanol extract in MRSA bacteria, namely between 3.91-62.5 µg/ml. This can be caused by differences in the group of bacteria, namely between gram positive and gram negative.\textsuperscript{12}

In addition, the results show that there is a significant difference in the mean minimum inhibitory concentration of all treatment groups with negative control on CRKP. The minimum inhibitory concentration of all groups was greater than the negative control group.

4. Discussion

The MIC results of CRKP bacteria were different from the MIC results of the n-hexane fraction of \textit{S. dysenteriae} and \textit{S. typhi} bacteria and \textit{K. pneumoniae} bacteria. The \textit{S. typhi} bacteria had a large enough inhibition zone compared to the \textit{K. pneumoniae} bacteria, which had a smaller inhibition zone. The MIC results from this study showed that the ethyl acetate fraction had a large enough inhibition zone compared to the n-hexane fraction. This can be caused by differences in the group of bacteria, namely between gram positive and gram negative.\textsuperscript{12}

The results of the antibacterial activity test showed that the n-hexane and ethyl acetate fractions had antibacterial activity which was indicated by the formation of an inhibition zone on the media. Between the two test samples, the ethyl acetate fraction had a large enough inhibition zone diameter compared to the n-hexane fraction, which was 9.40 ± 1.67 mm in CRKP bacteria. Ethyl acetate and n-hexane fractions contain flavonoids and

|       | HDK 8000 | HDK 4000 | Positive control |
|-------|----------|----------|------------------|
| EDK 125 | 0.70 ± 0.000 | 0.78 ± 0.130 | 1.60 ± 0.123 |
|       | 0.68 ± 0.045 | 0.317\textsuperscript{a} | 0.005\textsuperscript{a} |
| HDK 32000 | 0.78 ± 0.130 | 0.70 ± 0.136\textsuperscript{a} | 1.60 ± 0.123 |
|       | 0.68 ± 0.045 | 0.095\textsuperscript{a} | 0.008\textsuperscript{a} |
| HDK 16000 | 0.70 ± 0.000 | 0.70 ± 0.136\textsuperscript{a} | 1.60 ± 0.123 |
|       | 0.68 ± 0.045 | 0.317\textsuperscript{a} | 0.005\textsuperscript{a} |
| HDK 8000 | 0.68 ± 0.045 | 0.317\textsuperscript{a} | 1.60 ± 0.123 |
|       | 0.68 ± 0.045 | 1.000\textsuperscript{a} | 0.007\textsuperscript{a} |
| HDK 4000 | 0.68 ± 0.045 | 0.005\textsuperscript{a} | 0.123 |

\textsuperscript{a} Independent \textit{T} Test, \textit{p} = 0.05 \textsuperscript{-} Uji Mann Whitney, \textit{p} = 0.05
phenols. The ethyl acetate fraction provides a larger zone of inhibition because it contains semi-polar solvents, which can attract flavonoids and phenols and can also attract polar and non-polar compounds.\textsuperscript{11}

The mechanism of action of flavonoids, especially catechins, as antimicrobials in Gram positive and Gram-negative bacteria involves the interaction of flavonoids with a double lipid layer. This occurs through two mechanisms, the first is associated with the partitioning of more non-polar compounds in the hydrophobic interior of the membrane, while the second involves the formation of hydrogen bonds between the polar head groups of lipids and flavonoids which are more hydrophilic at the membrane interface.\textsuperscript{13}

Phenolic compounds contained in the ethyl acetate fraction work by several mechanisms, namely modification of cell membrane permeability, changes in various intracellular functions caused by hydrogen bonding from phenolic compounds to enzymes or by modification of cell wall stiffness with loss of integrity due to different interactions with cell membranes.\textsuperscript{14}

From the comparison of positive control (tigecyclin) with ethyl acetate fraction and karamunting leaf n-hexane fraction to CRKP bacteria, the results were significant differences in the ten concentrations with positive control. This is because the ethyl acetate fraction compound and the n-hexane fraction are not pure compounds that can work directly on the target bacterial organ as antibacterial.

Pure Karamunting leaf compounds, for example Rhodomyrton, will provide better antibacterial effects than compounds in the ethyl acetate fraction and the n-hexane fraction of Karamunting leaves. Rhodomyrton interferes with the functions of glycolysis, gluconeogenesis and amino acid metabolism in bacteria.\textsuperscript{15} Rhodomyrton also has a mechanism of action by suppressing acid production and reducing biofilm biomass formed by \textit{Streptococcus mutans} bacteria.\textsuperscript{16}

Proteomic studies on rhodomyrton-treated methicillin \textit{Staphylococcus aures} (MRSA) resistant cellular proteins have suggested changes in proteins associated with cell wall biosynthesis and cell division, protein degradation, stress response and oxidative stress, cell surface antigens and virulence factors, and various metabolic pathways.\textsuperscript{7} It is suspected that this mechanism of action supports the results of rhodomyrton's ability to overcome resistance to \textit{carbapenemase}.

5. Conclusion

From the research results, it can be seen that there is a significant difference between the average MIC of the ethyl acetate fraction and the n-hexane fraction of CRKP bacteria. The fraction of karamunting leaf extract had antibacterial activity of Carbapenemase Resistant \textit{Klebsiella pneumonia} (CRKP) at a concentration of 125 \textmu g/ml of ethyl acetate fraction and 4000 \textmu g/ml of n-hexane fraction. And ethyl acetate and n-hexane fractions contain compounds that can interfere with the integrity of the bacterial cell wall/membrane so that the bacteria can die.

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