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Activity of Karamunting Leaf Extract (Rhodomyrtus tomentosa (Aiton) Hassk) as an Antibiofilm on *Klebsiella pneumoniae*

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ABSTRACT

Klebsiella pneumoniae can cause serious infections. The use of antibiotics is often an option in treating infections, but bacteria can adapt by forming biofilms. Therefore, the search for alternative antibiofilms from natural sources is important. One potential plant is karamunting leaves (*Rhodomyrtus tomentosa* (aiton) hassk). This study aims to describe the potential of the ethanol extract of karamunting leaf as an antibiofilm against *Klebsiella pneumoniae*. This study is an in vitro descriptive laboratory test. A thick ethanol extract of karamunting leaves was then prepared in three concentration variations: 62.5µg/mL, 125µg/mL, and 250µg/mL. The positive control used was meropenem, and the negative control used was DMSO. The parameter measured for the inhibition and eradication test was biofilm thickness, which was read using a 590nm Microplate reader. In this study, the percentage of inhibition in the three concentrations was 90.65% at a concentration of 250 µg/ml, 86.69% at a concentration of 125µg/ml, and 87.22% at a concentration of 62.5µg/ml. During the eradication test, the percentages obtained were -10.67% at a concentration of 250µg/ml, 3.58% at a concentration of 125µg/ml, and 68.23% at a concentration of 62.5µg/ml. The ethanolic extract of karamunting leaves demonstrates good biofilm inhibitory activity against *Klebsiella pneumonia* at all tested concentrations. At a concentration of 62.5µg/ml, the extract exhibits good biofilm eradication activity, while at 125µg/ml and in the positive control, it shows poor activity. Interestingly, at a concentration of 250µg/ml, the extract acts as a biofilm promoter, enhancing biofilm formation.

INTRODUCTION

Infectious diseases continue to be a significant global health issue, affecting both developed and developing countries (Ebeledike et al., 2021). Among these, pneumonia is a leading cause of morbidity and mortality, often associated with the opportunistic pathogen *Klebsiella pneumoniae* (Abbas et al., 2024; Biscevic-Tokic et al., 2013). This Gram-negative bacterium can cause severe infections in various organ systems, including the urinary tract, soft tissues, central nervous system, and digestive system. In hospitals, *Klebsiella pneumoniae* is a major contributor to hospital-acquired infections (HAIs) due to its ability to spread rapidly through medical equipment and personnel (Ebeledike et al., 2021; Gorrie et al., 2022). Its virulence factors, including capsular polysaccharides, fimbriae, and biofilm formation, play a critical role in its ability to evade the immune system and establish persistent infections (Guerra et al., 2022; Riwu et al., 2022).

Treatment of *Klebsiella pneumoniae* infections often involves antibiotics such as carbapenems and cephalosporins. (Cui et al., 2025; Prince et al., 1997). However, the emergence of antibiotic resistance due to irrational use has significantly reduced the effectiveness of these treatments. Bacterial biofilms, which are estimated to be involved in 65–80% of all infections, further complicate therapy by providing a protective barrier against antimicrobial agents. Biofilm formation enables bacteria to survive in hostile environments by reducing metabolic activity and slowing cell division, thereby increasing their resistance to conventional antibiotics

(Guerra et al., 2022; Vestby et al., 2020). These conventional strategies face major challenges due to the paradoxical promotion of biofilms triggered by hormesis. Several studies have demonstrated that exposure to antibiotics or other stressors at sub-inhibitory concentrations can enhance biofilm formation by activating stress-response pathways and regulating quorum sensing (e.g., the SOS response, RpoS, and EPS production). The use of antimicrobials at inadequate doses may actually strengthen, rather than weaken, bacterial communities, thereby complicating the development of resistance (Shenkutie et al., 2020). To overcome these challenges, novel biofilm-control strategies are being explored, including quorum-sensing inhibitors such as Tanreqing in *K. pneumoniae* (Zhang et al., 2024), EPS-degrading enzymes, and compounds targeting QS and efflux pumps that suppress bacterial communication without triggering adaptive responses (Brackman & Coenye, 2015; Hetta et al., 2024). These non-cidal strategies are expected to complement conventional bactericidal therapies by suppressing biofilm formation while preventing the activation of hormetic mechanisms. This challenge underscores the urgent need for alternative therapies to combat biofilm-related resistance (Lukito, 2023).

Natural compounds derived from medicinal plants offer promising alternatives for addressing antibiotic resistance. Karamunting (*Rhodomyrtus tomentosa*), a traditional herbal remedy in Indonesia, is known for its high content of flavonoids and tannins, which exhibit antibacterial and antibiofilm properties. Studies have shown that karamunting extract effectively inhibits bacterial growth and disrupts biofilms in carbapenem-resistant *Klebsiella pneumoniae* and other pathogens (Sabrina et al., 2021). Moreover, the active compound rhodomyrtone has demonstrated significant activity against biofilm formation and eradication in previous research (Saising et al., 2011).

Despite these findings, research on the antibiofilm activity of karamunting extract against *Klebsiella pneumoniae* remains limited. Most studies focus on its antibacterial effects rather than its role in inhibiting and eradicating biofilms. This study aims to describe the activity of karamunting leaf extract against *Klebsiella pneumonia*. This research aims to provide a foundation for alternative therapies that address the growing threat of antibiotic resistance in *Klebsiella pneumoniae*.

METHOD

This study is an in vitro descriptive laboratory test to investigate the potential of an ethanol extract of karamunting leaf as an antibiofilm agent against *Klebsiella pneumoniae*. The independent variable in this study is exposure to karamunting leaf extract (*Rhodomyrtus tomentosa* Aiton (Hassk)) in ethanol extract, with concentrations of 62.5μg/ml, 125μg/ml, and 250 μg/ml, and a duration of exposure of 24 hours (Sabrina et al., 2021). The dependent variable in this study was the activity of inhibition and eradication of biofilm formed by *Klebsiella pneumoniae* bacteria. The population in this study consisted of all Enterobacteriaceae bacteria, meeting the following inclusion criteria: *Klebsiella pneumoniae* bacterial isolates from the Microbiology Laboratory at the Faculty of Medicine, Sriwijaya University's collection, and bacterial colonies capable of forming biofilms in the biofilm growth test. The exclusion criteria for this study were unverified *Klebsiella pneumoniae* bacteria.

Preparation simplicia

The initial step in this study was to prepare karamunting leaf extract using the maceration method. Simplicia was blended until smooth, then soaked using 96% ethanol, and then filtered using filter paper. This method was repeated three times for 2 x 24 hours.

Rejuvenation and identification *of Klebsiella pneumonia*e producing biofilm

Klebsiella bacteria were inoculated into McConkey medium and incubated in an incubator at 37.5°C for 24 hours. Prior to the test, *Klebsiella pneumonia*e were revived by inoculating onto Nutrient Agar media and incubating at 37°C in room air for 24 hours on two occasions. A sterile microplate with 180μL of TSB medium was set up, and then 20μL of the test bacteria were added to each well. Incubation was performed at 37°C in room air for a duration of 24 hours. Following the incubation, the media from the tube was eliminated and washed with distilled water. The tube was dried by exposing it to air at room temperature. Once dry, the tube was immersed in a 0.1% crystal violet solution for 30 minutes. The test results were analyzed using a microplate reader and subsequently reported as optical density. After that, the sample was stained using crystal violet, and the test results showed positive results if film lines formed, which were visible at the bottom and walls of the test tube.

Microtiter plate biofilm eradication assay

The determination of antibiofilm formation activity is divided into two categories: the assessment of biofilm inhibition activity and the evaluation of biofilm eradication activity. Five treatment groups were used, with each group having five repetitions. In the biofilm inhibition activity experiment, the thick extract of karamunting leaves was first diluted with Dimethyl Sulfoxide (DMSO) to concentrations of 62.5 µg/ml, 125 µg/ml, and 250 µg/ml, and then added to wells containing TSB media, DMSO, and bacterial suspensions. The positive control used meropenem antibiotic, TSB media, and bacteria, while the negative control contained TSB media, bacteria, and DMSO. After incubation at 37°C for 48 hours, the biofilm was washed with distilled water, stained with 0.1% crystal violet for 30 minutes, and analyzed using a microplate reader at 590 nm to measure optical density (OD). The percentage of biofilm inhibition was calculated using the formula:

$$\%\ \text{inhibition} = \frac{Mean\ control\ OD - Mean\ treatment\ OD}{Mean\ OD\ control} \times 100\%$$

Antibiofilm activity was classified as good (>50%), poor (0-50%), or biofilm promoter (<0%).

The biofilm eradication activity experiment was conducted by adding 10µL of bacterial suspension and 110µL of TSB medium into each well, and then incubating at 37°C for 48 hours. After the biofilm was formed, treatment was carried out by adding 40µL DMSO to the negative control, meropenem antibiotic to the positive control, and 40µL DMSO with extracts at various concentrations to the treatment wells. After incubation for 48 hours, the biofilm was washed with distilled water, stained with 0.1% crystal violet for 30 minutes, and read using a microplate reader at 590 nm to measure optical density (OD). The formula calculated the percentage of biofilm eradication:

$$\%\ \text{eradication} = \frac{Mean\ control\ OD - Mean\ treatment\ OD}{Mean\ OD\ control} \times 100\%$$

Antibiofilm activity was classified as good (>50%), poor (0-50%), or biofilm promoter (<0%).

This research has received a certificate of ethical approval from the Medical and Health Research Ethics Committee, Faculty of Medicine, Universitas Sriwijaya, with protocol number 227-2024.

RESULTS

A total of 200 grams of dried karamunting leaf powder macerated in 6 liters of 96% ethanol, 30.704 grams of thick extract were obtained, yielding an extraction efficiency of 15.35%. Minimum Inhibitory Concentration (MIC) testing is carried out to determine the lowest concentration of antimicrobial agents that can effectively inhibit the growth of microorganisms. The MIC test was carried out at various concentrations of 62.5µg/mL, 125µg/mL, and 250µg/mL using the dilution method.

The results obtained showed that the concentration of 125µg/ml is half the MIC of the negative control tube, where no microorganism growth occurred, thus illustrating the ability of karamunting leaf extract at a concentration of 125µg/ml to inhibit growth at a specific concentration. This data serves as the basis for assessing the effectiveness of karamunting leaves tested in this study.

The identification of Klebsiella pneumoniae biofilm production was conducted using the test tube method. The test tube method produced positive results, as visible film lines were observed on the bottom and walls of the test tubes.

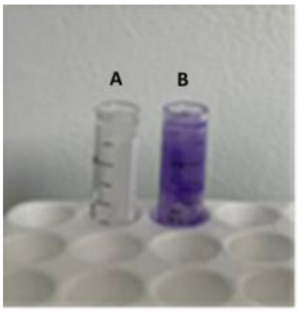

Figure 1. Results of the identification of *Klebsiella pneumoniae* bacterial biofilm A. control (-) Empty tube; B. Biofilm formation is indicated by purple color in the tube

Figure 1 shows that a purple color appears throughout the entire tube with 0.1% gentian violet dye, indicating the formation of a biofilm in the test tube on the right. In the biofilm growth inhibition test carried out by diluting the thick extract of karamunting leaves using Dimethyl Sulfoxide (DMSO) into concentrations of 62.5μg/ml, 125μg/ml, and 250μg/ml.

Table 1. Microplate reader inhibition results

Treatment	1	2	3	4	5	Mean ± SD
Control (-)	0.839	1.041	0.809	1.047	0.835	0.914 ± 0.119
Control (+)	0.074	0.077	0.110	0.080	0.084	0.085 ± 0.014
250μg	0.105	0.090	0.088	0.072	0.072	0.085 ± 0.014
125μg	0.076	0.107	0.268	0.072	0.085	0.122 ± 0.083
62.5μg	0.082	0.104	0.09	0.17	0.138	0.117 ± 0.037

Table 2. Inhibition test results

Treatment	Mean	Inhibition (%)
Control (-)	0.9142	
Control (+)	0,08	90.70
250μg/ml	0.085	90.65
125μg/ml	0.121	86.69
62,5μg/ml	0.116	87.22

Among all treatment groups, the 250μg/ml concentration of karamunting leaf extract exhibited the highest inhibition value, with 90.65% inhibition of biofilm formation compared to the negative control. The inhibition results are also close to the inhibition rate in the treatment using antibiotics, differing by only about 0.04% compared to the karamunting leaf extract at a concentration of 250μg/ml. At a concentration of 125μg/ml, the inhibition percentage was 86.69% and at a concentration of 62.5μg/ml, it was 87.22%. All concentrations have an inhibition percentage above 50% which represents good anti-biofilm activity.

Table 3. Microplate reader eradication results

Treatment	1	2	3	4	5	Mean ± SD
Control (-)	1.305	0.133	0.298	0.839	0.441	0.603 ± 0.471
Control (+)	0.549	0.243	0.284	1.020	0.257	0.471 ± 0.332
250μg	0.618	0.997	0.880	0.368	0.475	0.668 ± 0.266
125μg	0.220	0.792	0.394	0.974	0.528	0.582 ± 0.303
62.5μg	0.174	0.167	0.188	0.213	0.216	0.192 ± 0.022

Furthermore, the percentage of biofilm eradication was calculated, yielding the following results.

Table 4. Eradication test results

Treatment	Mean	Eradication(%)
Control (-)	0.60	
Control (+)	0.47	21.98
250µg/ml	0.66	-10.67
125µg/ml	0.58	3.58
62.5µg/ml	0.19	68.23

Of all treatment groups, karamunting leaf extract with a concentration of 62.5µg/ml had the highest eradication value compared to the negative control at 68.2%. This value is higher compared to the positive control, which has a percentage of only 21.98%, and the negative control. However, the results obtained decreased as the concentration of karamuting leaf extract used increased. At a concentration of 125µg/ml, the eradication percentage was 3.58% and at a concentration of 250µg/ml, it was -10.67%. The concentration of 62.5µg/ml has a percentage above 50%, so it can be considered to have good anti-biofilm activity (good activity). At a concentration of 125µg/ml, the positive control has a percentage between 0% and 50%, indicating poor anti-biofilm activity. At a concentration of 250µg/ml, it has a percentage below 0%, indicating no anti-biofilm activity (biofilm promoter).

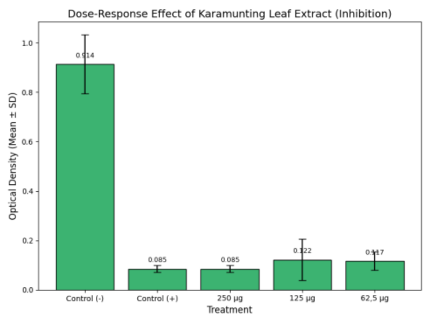

Figure 2. Dose-response effect of karamunting leaf extract (inhibition)

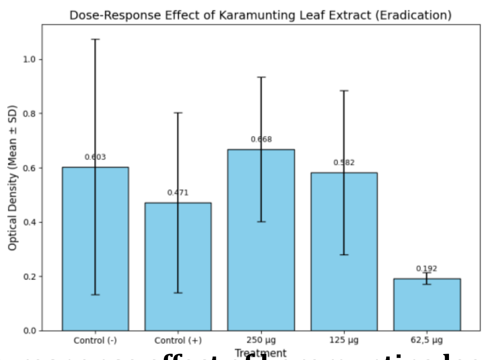

Figure 2. Dose-response effect of karamunting leaf extract (inhibition)

DISCUSSION

Solvents are needed when performing an extraction to attract active compounds. The solvent used during extraction will affect the results of the active compounds produced later. Solvent selection is considered based on the amount and type (Sasadara & Wiranata, 2022). Solvents commonly used in the maceration process include butanol, hexane, ethyl acetate, methanol, and ethanol. Polar compounds tend to dissolve in polar solvents, while nonpolar compounds dissolve in nonpolar solvents. In this study, ethanol was chosen as the extraction solvent for karamunting due to its polar nature, which is not only able to dissolve polar compounds but also nonpolar compounds. Ethanol has a superior extractive ability for most low-molecular-weight compounds, such as saponins and flavonoids.

Additionally, ethanol can penetrate cell walls, enabling the extraction process of active compounds to occur more quickly (Karepu et al., 2020; Yulianti et al., 2020). The ethanol concentrations commonly used as solvents in maceration are 96% and 70%. 70% ethanol has more polar properties than 96% ethanol, but its disadvantage is that it is less effective in extracting nonpolar compounds. In this study, 96% ethanol was used as a solvent due to its ability to extract polar, semipolar, and nonpolar active compounds, resulting in extracts with a more optimal active compound content compared to those obtained with 70% ethanol.

In this study, DMSO (dimethyl sulfoxide) was used as a diluent due to its ability to dissolve a wide range of compounds, including both polar and nonpolar substances. Additionally, DMSO was chosen because it does not affect bacterial growth, thereby not interfering with the results of antibacterial activity testing conducted by the disc diffusion method. DMSO also served as a negative control, because it was proven not to affect the observations in the extract test against bacterial growth (Aruperes et al., 2021; Rahmi & Putri, 2020).

The extraction results in this study have a slightly higher yield percentage than those of Sari et al. (2017), which produced a yield of 14.222% using the same maceration method. In the study, the researchers used 500 grams of karamunting leaf powder and a 96% ethanol solvent. The filtrate obtained was concentrated using a rotary vacuum evaporator and obtained 71.1133 g of extract (Sari et al., 2017).

The biofilm activity test is very dependent on the isolate strain used. The results of the biofilm formed will vary from one strain to another. In addition, in some cases, the amount of biofilm formed will be higher on minimal media than on those that are richer in nutrients. The biofilm yield will also be higher in LB (Luria Broth) media, but not in minimal media without casamino acids and glucose or glycerol as an energy source. The use of different protocols will also significantly affect the yield of biofilm formed.

In summary, the process of biofilm formation in *Klebsiella pneumoniae* bacteria depends on the nature of the strain and is highly modulated by culture conditions, environmental factors, and methodology (Naves et al., 2008). In a study by Surgers et al. (2019), a total of 82 isolates of *Klebsiella pneumoniae* bacteria obtained from patients at Saint-Antoine Hospital were examined. The study found that 8 isolates did not produce biofilm at all; meanwhile, in another study by Shadkam et al. In 2021, a total of 100 isolates obtained from hospitals in Iran were analyzed, revealing that 31 isolates produced weak biofilms, 19 produced medium biofilms, 25 produced strong biofilms, and 25 isolates did not produce biofilms at all (Shadkam et al., 2021; Surgers et al., 2019).

In the results of biofilm inhibition experiments, the obtained inhibition of biofilm formation was consistent across all treatments, regardless of the karamunting extract concentration. The highest concentration of inhibiting biofilm, compared to the negative control, was 250 μg/ml, and the inhibitory activity decreased as the concentration decreased. The results in this study have a higher value of inhibition results when compared to Abidah's research. In 2020, a study used a 96% ethanol extract of black mulberry leaves on *Escherichia coli* bacteria. In this study, a concentration of 160μg/ml yielded a biofilm inhibition percentage of 65.83%, while a concentration of 80μg/ml resulted in an inhibition percentage of 58.15%. This difference can be attributed to the varying contents between black mulberry leaf extract and karamunting leaves. In the experiment, the phytochemical content of black mulberry was found to contain no flavonoids, saponins, or alkaloids (Abidah, 2020). The content of rhodomirton, which is a derivative of flavonoids in karamunting leaves, is proven to have significant activity as an antibiofilm (Saising et al., 2011).

Additionally, a study by Khotimah. Using 96% ethanol extract of black mulberry leaves on *Klebsiella pneumoniae* bacteria, the inhibition activity at a concentration of 160 μg/ml was 8.10% and at a concentration of 80 μg/ml, it was 15.70%. In the phytochemical test of this study, flavonoids, saponins, and alkaloids were not found (Khotimah, 2020).

In the results of biofilm eradication experiments, biofilm degradation was observed at two concentrations of karamunting extract. The highest biofilm eradication result compared to the negative control was at a concentration of 62.5μg/ml. At a concentration of 125μg/ml, biofilm eradication activity was also observed, with a percentage that was not significantly higher than that of the negative control. At concentrations higher than 125μg/ml, no eradication activity was found. This phenomenon can be attributed to the fact that high concentrations of extracts can stimulate biofilm formation, as bacteria respond to environmental changes resulting from antibiotic exposure and attempt to adapt by producing large amounts of biofilm (Taufiq & Darah, 2020). This finding aligns with the results of Walraven et al. (2014), which revealed that the use of high antifungal drugs can facilitate biofilm formation in *Candida albicans* isolates resistant to echinocandins (Walraven et al., 2013). However, the discussion does not fully address why lower concentrations were more effective in eradication. A possible explanation is the hormesis effect, where sub-inhibitory or lower concentrations of antimicrobial agents can trigger stress responses that increase susceptibility and destabilize biofilms. In comparison, higher concentrations may paradoxically promote protective biofilm formation (Calabrese & Mattson, 2017).

The results in this study yield a higher eradication rate value compared to Alif's research. In 2020, a study used a 96% ethanol extract of black mulberry leaves on *Klebsiella pneumoniae* bacteria. In this study, a concentration of 160µg/ml yielded a 25.70% eradication percentage, and 18.48% at a concentration of 80µg/ml (Khotimah, 2020). Meanwhile, in Abidah's research from 2020, which used a 96% ethanol extract of black mulberry leaves on Escherichia coli bacteria. In this study, a concentration of 160µg/ml yielded a 52.07% eradication percentage, while 36.88% was achieved at a concentration of 80µg/ml (Abidah, 2020).

The eradication results in this study tended to decrease at higher concentrations. In contrast, previous studies on the ethanol extract of *Rhodomyrtus tomentosa* against *Staphylococcus aureus* bacteria reported that biofilm destruction activity is dose-dependent, with effectiveness increasing with increasing concentration (Saising et al., 2011). This can be caused by several factors, including changes in pH, temperature fluctuations, variations in oxygen levels, or errors in measurement and protocol implementation techniques (Mathlouthi et al., 2018).

In addition, the results of this study are strongly influenced by the phytochemical content present in the secondary metabolites of karamunting leaves. Therefore, phytochemical tests were conducted to identify the compounds present in the material. It is known that karamunting leaf extract contains various compounds, including alkaloids, flavonoids, steroids, saponins, tannins, and quinones. All of these compounds fall into the category of polar compounds, which dissolve in polar solvents (Hasibuan & others, 2017).

Flavonoids have a mechanism that inhibits quorum sensing in bacteria, resulting in the disruption of signaling between bacteria and thereby inhibiting biofilm formation. Tannins can interfere with the growth and reproduction of bacteria by making them iron-deficient. Alkaloids function similarly to flavonoids, which inhibit biofilm attachment by inhibiting sortase. In addition, saponins also play a role in binding lipids, which can cause cell leakage in bacteria (Boakye et al., 2016; Salim et al., 2021; Serrano et al., 2009).

This study has certain limitations that should be acknowledged. First, the findings are based on a single bacterial strain, which limits the generalizability of the results to diverse clinical isolates of Klebsiella pneumoniae or other biofilm-forming pathogens. Second, the study did not include molecular-level investigations, such as the assessment of quorum-sensing gene expression or other biofilm-associated regulatory pathways, which could have provided deeper mechanistic insights into the antibiofilm effects of Karamunting leaf extract. To strengthen the evidence, future research should involve fractionation of the extract to isolate and characterize the active bioactive compounds responsible for the observed effects. Furthermore, in vivo studies are necessary to validate the antibiofilm efficacy under physiological conditions and to explore the therapeutic potential of Karamunting leaf as a candidate for novel antimicrobial strategies.

CONCLUSION

The ethanol extract of karamunting leaves (*Rhodomyrtus tomentosa*) has a Minimum Inhibitory Concentration (MIC) of 125µg/mL. The extract demonstrated good biofilm inhibition at all tested concentrations and effective biofilm eradication at 62.5µg/mL, while promoting biofilm formation at 250µg/mL. The findings suggest that karamunting extract may serve as a promising source of antibiofilm agents at lower concentrations. However, its potential to promote biofilm at higher doses highlights the importance of dose optimization.

AUTHOR'S DECLARATION

Authors' contributions and responsibilities

TS: contributed to writing original draft, visualization, funding acquisition, conceptualization; **RS**: Writing original draft (supporting); **ER**: Supervision (main), validation (equal), review and editing; **EZ**: Formal analysis, Conceptualization; **EL**: validation (equal), visualization (equal).

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Availability of data and materials

All data in this research are available from the authors.

Competing interests

This research has no competing interests with others.

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