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Comparative Antibacterial Activity of *Citrus hystrix* DC. Leaf Essential Oil Against ESBL *Klebsiella pneumoniae* and Multidrug-Resistant *Serratia marcescens*

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Abstract: The increasing antimicrobial resistance has driven the development of natural antibacterial sources, one of which is essential oils. This study aimed to analyse the antibacterial activity of lime leaf essential oil (*Citrus hystrix* DC.) against Extended Spectrum β-Lactamase (ESBL)-producing *Klebsiella pneumoniae* and multidrug-resistant *Serratia marcescens*. The study used a true experimental design with a posttest-only control group design. The essential oils were tested at concentrations of 50%, 60%, 70%, 80%, 90%, and 100% using the well diffusion method with four replications. Ciprofloxacin served as a positive control and dimethyl sulfoxide as a negative control. Data were analysed using the Shapiro–Wilk test, Levene's test, one-way ANOVA, or Welch ANOVA, followed by post hoc tests according to data characteristics at a significance level of $p<0.05$. The results showed that lime leaf essential oil had antibacterial activity against both bacteria at all concentrations tested. For *Klebsiella pneumoniae* ESBL, the inhibition zone diameter increased from 6.83 mm at 50% concentration to 9.30 mm at 90% concentration, then decreased to 8.08 mm at 100%. For multidrug-resistant *Serratia marcescens*, the inhibition zone diameter increased from 7.80 mm to 14.00 mm at 90% concentration, then decreased to 11.20 mm at 100%. Statistical analysis showed a significant difference between the concentrations for both bacteria ($p<0.001$). In general, *Serratia marcescens* showed higher sensitivity than *Klebsiella pneumoniae* ESBL. Although its inhibitory power was still lower than ciprofloxacin, lime leaf essential oil has the potential to be developed as a natural antibacterial source against resistant Gram-negative bacteria.

Keywords: Antibacterial activity; antimicrobial resistance; *Citrus hystrix*; essential oil; *Klebsiella pneumoniae*; *Serratia marcescens*.

INTRODUCTION

Infectious diseases remain a leading cause of morbidity and mortality worldwide. Infections occur when pathogenic microorganisms, such as bacteria, viruses, fungi, and parasites, invade and multiply in the body, causing tissue damage and impaired organ function[1]. In healthcare settings, nosocomial infections remain a serious challenge, increasing length of stay, treatment costs, and patient mortality. Globally, the mortality rate from nosocomial infections is reported to be around 2.8%[2]. In Indonesia, various pathogenic bacteria are still frequently found as causes of nosocomial infections, one of

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which is *Serratia marcescens*, which was reported to be found in 7.4% of isolates causing nosocomial infections at Prof. Dr R. D. Kandou General Hospital in Manado[3]. Furthermore, research by Nugroho et al. (2024) showed that *Klebsiella pneumoniae* is one of the Gram-negative bacteria successfully isolated from wound swabs of patients with diabetes mellitus[4].

Klebsiella pneumoniae and *Serratia marcescens* are Gram-negative bacteria from the Enterobacteriaceae family that are opportunistic and frequently cause infections in patients with compromised immunity. *Klebsiella pneumoniae* is known to cause pneumonia, urinary tract infections, bloodstream infections, wound infections, and various nosocomial infections[5]. Meanwhile, *Serratia marcescens* is a significant cause of surgical site infections, urinary tract infections, pneumonia, and healthcare-associated bacteremia[3]. The increasing irrational use of antibiotics has led to the emergence of bacterial strains resistant to various antibiotic classes, complicating infection management.

One resistance mechanism commonly found in *Klebsiella pneumoniae* is the production of Extended Spectrum β-Lactamase (ESBL) enzymes, which can hydrolyse most β-lactam antibiotics, thus decreasing therapeutic effectiveness[6]. In Indonesia, the prevalence of ESBL-producing *Klebsiella pneumoniae* is reported to range between 50–82% based on a national antimicrobial resistance survey[7]. On the other hand, *Serratia marcescens* has been reported to exhibit resistance to various antibiotic classes, including penicillin, cephalosporin, macrolide, tetracycline, nitrofurantoin, colistin, aminoglycosides, and fluoroquinolones[8,9]. Research by Tavares et al. (2023) also showed that *Serratia marcescens* isolates exhibited resistance to three or more antibiotic classes (multidrug resistance/MDR)[10]. This condition makes these two bacteria priority pathogens requiring the development of new antibacterial agents to address increasing antimicrobial resistance[11,12].

Utilising natural products as antibacterial sources is one approach that continues to be developed to address the problem of antibiotic resistance. Indonesia boasts rich biodiversity, including a variety of plants that produce essential oils with antibacterial activity. One such plant is the kuit lime (*Citrus hystrix* DC.), a plant native to South Kalimantan that bears a resemblance to the kaffir lime, but differs in the morphology of its fruit and leaves[13,14]. Kuit lime populations are abundant in the Astambul and Aranio Districts of Banjar Regency[15]. Its leaves produce essential oils containing various bioactive compounds, such as γ-terpinene, prehnitene, (+)-4-causene, β-cis-osimene, and β-pinene, which are known to have antibacterial activity through cell membrane damage and disruption of bacterial metabolism[14,16,17].

Several studies have demonstrated that essential oils from various plants have activity against antibiotic-resistant Gram-negative bacteria. Zataria multiflora essential oil was able to inhibit the growth of *Klebsiella pneumoniae* ESBL with an MIC value of 0.05% and an inhibition zone diameter of 29.7 mm at a concentration of 100%[18]. Camellia seed essential oil produced an inhibition zone diameter of 16 mm against *Klebsiella pneumoniae* ESBL[19], while lemongrass essential oil (Cymbopogon citratus) produced an inhibition zone diameter of 9.9 mm[20]. In *Serratia marcescens* bacteria, essential oil of kaffir lime leaves (*Citrus hystrix* DC.) showed an inhibition zone diameter of 9.04–12.07 mm at a concentration of 100%[21], while parijoto fruit extract (*Medinilla speciosa Blume*) produced an inhibition zone diameter of 17.42 mm against *Serratia marcescens* at a concentration of 50%[22]. However, research on the activity of lime leaf essential oil against these two priority bacteria, particularly comparing its effectiveness against ESBL-producing *Klebsiella pneumoniae* and MDR-resistant *Serratia marcescens* using the same method and concentration range, is still very limited.

This study aimed to analyse the antibacterial efficacy of lime leaf essential oil (*Citrus hystrix* DC.) against the growth of ESBL-producing *Klebsiella pneumoniae* and MDR-resistant *Serratia marcescens* at various concentrations using the well diffusion method.

MATERIALS AND METHODS

Research Design

This was a true experimental study with a posttest-only control group design. The aim was to evaluate the antibacterial efficacy of lime leaf essential oil (*Citrus hystrix* DC.) against the growth of Extended Spectrum β-Lactamase (ESBL)-producing *Klebsiella pneumoniae* and multidrug-resistant *Serratia marcescens* (MDR *S. marcescens*). The treatment groups consisted of essential oils at concentrations of 50%, 60%, 70%, 80%, 90%, and 100%, while the control groups consisted of a positive control (ciprofloxacin), a negative control (DMSO), and a media control. The number of replicates was determined using the Federer formula, i.e., $(t-1)(r-1) \geq 15$, resulting in four replicates for each treatment.

Four independent experimental replicates were performed for each treatment group. For each replicate, a new bacterial suspension was prepared and adjusted to a 0.5 McFarland standard, followed by independent inoculation onto Mueller–Hinton agar and antibacterial testing. Thus, each replicate represented an independent experimental run rather than repeated measurements from the same culture plate.

Research Ethics

This research has obtained ethical approval from the Health Research Ethics Committee of the Banjarmasin Ministry of Health Polytechnic under license number 733/KEPK-PKB/2025 for the study on MDR *Serratia marcescens* and number 735/KEPK-PKB/2025 for the study on ESBL *Klebsiella pneumoniae*.

Research Location and Time

The research was conducted from October 2025 to January 2026. The essential oil distillation process was conducted in the Chemistry Laboratory, Faculty of Mathematics and Natural Sciences, Lambung Mangkurat University, while antibacterial activity testing was conducted in the Bacteriology Laboratory, Department of Medical Laboratory Technology, Poltekkes Kemenkes Banjarmasin.

Research Samples

The plant material used in this study was limau kuit, a locally *recognised citrus* plant obtained from Astambul District, Banjar Regency, South Kalimantan, at an altitude of approximately 10–15 m above sea level. Botanical identification classified the plant specimen as *Citrus hystrix* DC. Although *C. hystrix* is also commonly associated with kaffir lime (jeruk purut), the term limau kuit is retained throughout this manuscript to specifically refer to the local plant material investigated in this study. The botanical identification was conducted to confirm the taxonomic identity of the plant material prior to essential oil extraction. The leaves were selected based on the following criteria: fresh, free from pest infestation, and free from visible holes or physical damage.

The test organisms consisted of one clinical isolate of ESBL-producing *Klebsiella pneumoniae* obtained from a wound swab and one clinical isolate of *Serratia marcescens* obtained from a wound specimen at Ulin Regional Hospital, Banjarmasin. Bacterial identification and antimicrobial susceptibility testing were performed using the VITEK system. The *K. pneumoniae* isolate was identified with 90% probability and was reported as ESBL phenotype positive by the VITEK system. The *S. marcescens* isolate was identified with 99% probability, and its antimicrobial susceptibility profile demonstrated resistance to multiple antimicrobial classes. Based on the predefined MDR criterion, the isolate was classified as multidrug-resistant (MDR).

Essential Oil Preparation

1,500 g of lime leaves were cleaned, wilted for one week at room temperature, and then cut into approximately 0.5 cm lengths. The essential oil was obtained using steam-water distillation for six hours. The distillate was separated using a separating funnel and then purified by adding anhydrous sodium sulfate (1–3 g/L) to obtain a water-free essential oil. The oil yield was calculated based on the ratio of the volume of oil obtained to the weight of the starting material.

Preparation of Test Solutions

Essential oil formulations were prepared at concentrations of 50%, 60%, 70%, 80%, 90%, and 100% (v/v). For the 50–90% formulations, pure dimethyl sulfoxide (DMSO) was used as the diluent to obtain a final volume of 500 µL. The 50%, 60%, 70%, 80%, and 90% formulations contained 250, 300, 350, 400, and 450 µL of essential oil, respectively, supplemented with 250, 200, 150, 100, and 50 µL of pure DMSO. The 100% formulation consisted of 500 µL of essential oil without DMSO. Each formulation was homogenised using a vortex mixer before use.

Bacterial preparation

Klebsiella pneumoniae ESBL isolates were rejuvenated on Eosin Methylene Blue (EMB) agar and then transferred to chocolate agar, while MDR *Serratia marcescens* isolates were rejuvenated on MacConkey agar and then transferred to chocolate agar. Bacterial colonies were suspended in 0.9% NaCl to a McFarland standard of 0.5.

Antibacterial activity test

Antibacterial activity was tested using the well diffusion method on Mueller–Hinton agar. For each replicate, a new bacterial suspension was independently prepared and standardised to a 0.5 McFarland turbidity standard. A total of 180 µL of the standardised bacterial suspension was evenly inoculated onto the surface of the agar using a sterile cotton swab. Eight wells, each 6 mm in diameter, were prepared on each plate, consisting of six treatment wells, one positive control, and one negative control. Each treatment well was filled with 40 µL of the corresponding essential oil formulation, while the positive control was filled with ciprofloxacin solution equivalent to 5 µg/well and the negative control was filled with pure DMSO. Each concentration was tested in four independent biological replicates, with a newly prepared bacterial suspension and a separate agar plate used for each replicate. After a 10-minute diffusion period, the plates were incubated at 37°C for 18 hours. The diameter of the inhibition zone was measured using a vernier calliper and expressed in millimetres.

Data analysis

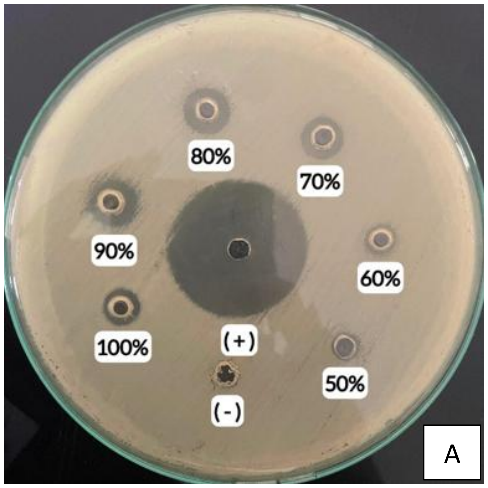
The diameter of the inhibition zone is presented as the mean ± standard deviation. Data distribution was tested using the Shapiro–Wilk test, while homogeneity of variance was tested using the Levene test. The selection of comparative tests was based on the results of both tests: one-way ANOVA if parametric assumptions were met, Welch ANOVA if the data were normally distributed but variances were not homogeneous, or Kruskal–Wallis if the data were not normally distributed. Analysis continued with appropriate post hoc tests to determine differences between groups, with a significance level of $p < 0.05$.

RESULTS AND DISCUSSION

The test results showed that the essential oil of lime leaves (*Citrus hystrix* DC.) has antibacterial activity against ESBL *Klebsiella pneumoniae* and MDR *Serratia marcescens* at all concentrations tested. For ESBL *Klebsiella pneumoniae*, the average inhibition zone diameter increased with increasing concentration from 6.83 mm at 50% concentration to 9.30 mm at 90% concentration, then decreased to 8.08 mm at 100%. Meanwhile, for MDR *Serratia marcescens*, the average inhibition zone diameter increased from 7.80 mm at 50% concentration to a peak of 14.00 mm at 90% concentration, then decreased to 11.20 mm at 100%. The positive control in the form of ciprofloxacin produced an average inhibition zone diameter of 27.23 mm against ESBL *Klebsiella pneumoniae* and 30.10 mm against MDR *Serratia marcescens*, while the negative control (DMSO) did not produce an inhibition zone in either bacterium. In general, the pattern of results showed that the antibacterial activity of lime leaf essential oil increased up to a concentration of 90%, then decreased at a concentration of 100% in both test bacteria. In addition, the diameter of the inhibition zone formed in MDR *Serratia marcescens* tended to be larger than that of ESBL *Klebsiella pneumoniae* at the same concentration, which indicated that lime leaf essential oil produced larger inhibition zones against MDR *Serratia marcescens* than against ESBL *Klebsiella pneumoniae*.

Table 1. Inhibition Zone Diameters of *Citrus hystrix* DC. Leaf Essential Oil Against ESBL *Klebsiella pneumoniae* and MDR *Serratia marcescens*

Bacteria	Concentration (%)	Replicate I	Replicate II	Replicate III	Replicate IV	Mean (mm)
ESBL *Klebsiella pneumoniae*	50	6.3	6.8	6.9	7.3	6.83
	60	6.9	7.2	7.6	7.8	7.38
	70	8.5	8.2	8.3	8.5	8.38
	80	9.2	8.6	8.7	8.7	8.80
	90	9.9	9.0	9.2	9.1	9.30
	100	8.1	7.9	8.1	8.2	8.08
	Pos control	26.8	27.4	27.8	26.9	27.23
	Neg control	0	0	0	0	0.00
MDR *Serratia marcescens*	50	7.9	7.7	7.4	8.1	7.80
	60	9.2	8.7	8.3	11.0	9.30
	70	11.2	9.5	10.0	12.5	10.80
	80	12.8	10.4	10.7	13.7	11.90
	90	15.6	12.9	11.5	15.3	14.00
	100	11.4	10.7	10.4	12.3	11.20
	Pos control	29.8	30.0	30.3	30.3	30.10
	Neg control	0	0	0	0	0.00



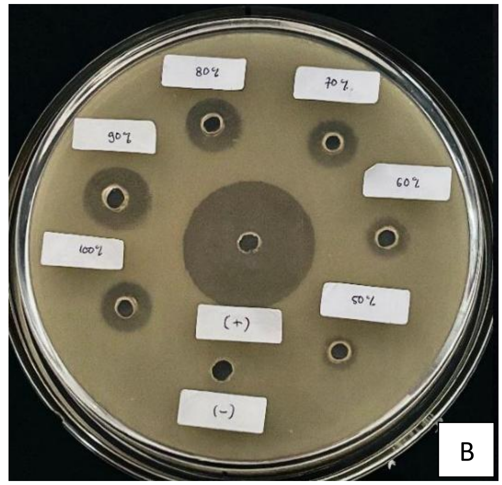


Figure 1. Inhibition Zones Produced by *Citrus hystrix* DC. Leaf Essential Oil Against ESBL *Klebsiella pneumoniae* (A) and MDR *Serratia marcescens* (B) Using the Well Diffusion Method.

Table 2. Statistical Analysis of the Inhibition Zone Diameters of *Citrus hystrix* DC. Leaf Essential Oil Against ESBL *Klebsiella pneumoniae* and MDR *Serratia marcescens*

Bacterium	Normality Test (Shapiro–Wilk), *p*	Homogeneity Test (Levene), *p*	Comparative Test	*p*-value	Interpretation
ESBL *Klebsiella pneumoniae*	0.859	0.275	One-way ANOVA	<0.001	Significant difference among concentrations
MDR *Serratia marcescens*	0.272–0.850	0.017	Welch ANOVA	<0.001	Significant difference among concentrations

Statistical test results showed that the inhibition zone diameter data for ESBL *Klebsiella pneumoniae* were normally distributed (Shapiro–Wilk, p=0.859) and homogeneous (Levene, p=0.275), so the analysis continued with a one-way ANOVA. The results showed a significant difference in inhibition zone diameter among lime leaf essential oil concentrations (p<0.001). Tukey's further test showed that most concentration pairs differed significantly, except for the 50%–60%, 60%–100%, 70%–80%, 70%–100%, and 80%–90% concentrations (p>0.05). The highest inhibition zone diameter was obtained at 90%, while 100% showed decreased activity compared with 90%.

For MDR *Serratia marcescens*, the Shapiro–Wilk test showed a normal distribution (p=0.272–0.850), but the homogeneity test revealed heterogeneity of variance (p=0.017). Therefore, the analysis was continued using the Welch ANOVA test. The analysis revealed a significant difference in inhibition zone diameter between essential oil concentrations (p<0.001). Further Games–Howell tests showed significant differences primarily between the 50% concentration and all other concentrations, and between the

60% and 90% concentrations. Most concentration pairs at 70%, 80%, 90%, and 100% showed no significant differences ($p>0.05$). These findings indicate that increasing the essential oil concentration increases antibacterial activity up to 90%, after which activity tends to plateau at 100%.

Table 3. Post Hoc Analysis of Inhibition Zone Diameters of *Citrus hystrix* DC. Leaf Essential Oil Against ESBL *Klebsiella pneumoniae*

Comparison	*p*-value	Significant
50% vs 60%	0.195	No
50% vs 70%	<0.001	Yes
50% vs 80%	<0.001	Yes
50% vs 90%	<0.001	Yes
50% vs 100%	<0.001	Yes
60% vs 70%	0.004	Yes
60% vs 80%	<0.001	Yes
60% vs 90%	<0.001	Yes
60% vs 100%	0.058	No
70% vs 80%	0.442	No
70% vs 90%	0.007	Yes
70% vs 100%	0.765	No
80% vs 90%	0.277	No
80% vs 100%	0.046	Yes
90% vs 100%	<0.001	Yes

Tukey's HSD test; significance level $p < 0.05$.

Table 4. Post Hoc Analysis of Inhibition Zone Diameters of *Citrus hystrix* DC. Leaf Essential Oil Against MDR *Serratia marcescens*

Comparison	*p*-value	Significant
50% vs 60%	0.010	Yes
50% vs 70%	0.014	Yes
50% vs 80%	0.045	Yes
50% vs 90%	0.013	Yes
50% vs 100%	0.008	Yes
60% vs 70%	0.307	No
60% vs 80%	0.250	No
60% vs 90%	0.047	Yes
60% vs 100%	0.053	No
70% vs 80%	0.826	No
70% vs 90%	0.200	No
70% vs 100%	0.542	No
80% vs 90%	0.858	No
80% vs 100%	1.000	No
90% vs 100%	0.776	No

Games–Howell post hoc test; significance level $p < 0.05$.

Overall, the two bacteria responded differently to increasing concentrations of lime leaf essential oil. ESBL *Klebsiella pneumoniae* showed more significant differences between concentrations than MDR *Serratia marcescens*, while the inhibition zone diameters produced by MDR *Serratia marcescens* were generally larger at the same concentration.

This study aimed to determine the antibacterial efficacy of lime leaf essential oil (*Citrus hystrix* DC.) against resistant Gram-negative bacteria, namely ESBL *Klebsiella pneumoniae* and MDR *Serratia marcescens*, using the well diffusion method. Before testing, the plant underwent a determination test to confirm that it was a suitable *Citrus hystrix* DC. strain for use as a research material. The essential oil was obtained through steam-water distillation with a yield of 0.6% (v/w). This value is slightly lower than the study by Afifah (2025), which obtained a yield of 0.73% (v/w)[23]. These yield differences may be influenced by environmental factors such as geographic conditions, rainfall, soil organic carbon content, soil pH, and macronutrient content in plants[24].

The bacterial isolates used were clinical isolates whose identities had been confirmed through colony morphology, Gram staining, and VITEK identification results from the Microbiology Laboratory of Ulin Banjarmasin Regional Hospital. The *Klebsiella pneumoniae* isolate possesses an extended-spectrum β-lactamase (ESBL) mechanism. At the same time, *Serratia marcescens* is classified as Multidrug-Resistant (MDR), making both bacteria highly resistant and challenging to treat[25,26].

The results showed that lime leaf essential oil had antibacterial activity against both test bacteria, indicated by the formation of inhibition zones at all concentrations. This activity indicates that lime leaf essential oil has potential as a natural antibacterial source against resistant Gram-negative bacteria. Based on GC-MS analysis, the main components of essential oils are γ-terpinene, β-cymene, 3-carene, α-thujene, and o-cymene, which belong to the monoterpene hydrocarbon group. These compounds damage bacterial cell membranes, increase membrane permeability, cause leakage of intracellular components, and disrupt cell metabolism, thus inhibiting bacterial growth[14,27].

The same pattern was observed for both bacteria: the inhibition zone diameter increased with increasing essential oil concentration from 50% to 90%. The 90% concentration produced the largest inhibition zone diameter, at 9.30 mm against ESBL *Klebsiella pneumoniae* and 14.00 mm against MDR *Serratia marcescens*. These results indicate that increasing concentration increases the amount of active compounds that diffuse into the medium, thus increasing the number of antibacterial components that interact with bacterial cells. The higher the concentration of an antimicrobial compound, the greater the ability to inhibit bacterial growth until it reaches the optimum concentration[28,29].

Interestingly, for both bacteria, the inhibition zone diameter decreased at 100% concentration, to 8.08 mm for ESBL *Klebsiella pneumoniae* and 11.20 mm for MDR *Serratia marcescens*. This phenomenon indicates that increasing essential oil concentration does not always increase antibacterial activity. One cause is the increased viscosity of essential oils at high concentrations, which reduces the diffusion capacity of active compounds into the agar medium. Furthermore, the hydrophobic nature of essential oils results in suboptimal distribution in hydrophilic agar media, reducing the zone of inhibition[30,31,32]. In addition to diffusion, interactions between essential oil components at high concentrations can also be antagonistic, thus inhibiting proportional antibacterial effectiveness[33].

Although both bacteria are Gram-negative and resistant, essential oils demonstrated greater activity against MDR *Serratia marcescens* than against ESBL *Klebsiella pneumoniae*. The maximum zone of inhibition against *Serratia marcescens* reached 14.00 mm, while against *Klebsiella pneumoniae* it was only 9.30 mm. The biological characteristics of each bacterium likely influence this difference. *Klebsiella pneumoniae* has a thick polysaccharide capsule and an outer membrane rich in lipopolysaccharide (LPS), along with resistance mechanisms such as ESBL enzyme production, decreased porin expression, and increased efflux pump activity. This combination of mechanisms makes antibacterial compound penetration more difficult, resulting in relatively lower essential oil activity[25,34].

Meanwhile, *Serratia marcescens* also possesses an LPS outer membrane and MDR mechanisms such as efflux pumps, antibiotic target modification, protective enzyme production, and the ability to form biofilms. However, the results of this study indicate that essential oils can still produce larger inhibition zones than ESBL *Klebsiella pneumoniae*. This suggests that the multi-target mechanism of action of essential oils through cell membrane damage can still affect MDR bacteria. However, their effectiveness is lower than that of standard antibiotics[26,35].

The inhibition zones produced by essential oils on both bacteria were significantly smaller than those of the positive control, ciprofloxacin. The average diameter of the ciprofloxacin inhibition zone reached 27.23 mm on ESBL *Klebsiella pneumoniae* and 30.10 mm on MDR *Serratia marcescens*. These findings align with various studies showing that standard antibiotics still have higher antibacterial activity than essential oils. However, some essential oils still show promising activity against resistant Gram-negative bacteria[26,36,37]. The chemical composition of the essential oils also influences this difference in effectiveness. Lime leaf essential oil is dominated by monoterpene hydrocarbons, which are known to have lower antibacterial activity than essential oils rich in phenolic compounds such as thymol and carvacrol[17,18].

The statistical analysis confirmed the biological results of the study. In ESBL *Klebsiella pneumoniae*, the data were normally distributed and homogeneous, so they were analysed using One-Way ANOVA, which showed a significant difference between concentrations ($p<0.05$), followed by the Tukey test to determine which pairs of groups differed significantly. In contrast, in MDR *Serratia marcescens*, the data were normally distributed but not homogeneous, so the Welch ANOVA test was used, which also showed a significant difference between concentrations ($p<0.05$), followed by the Games-Howell test. Both analyses showed that increasing the concentration of essential oils significantly affected the diameter of the inhibition zone; however, after reaching the optimum concentration, further increases did not significantly increase antibacterial activity[38].

Overall, the results indicate that lime leaf essential oil has antibacterial activity against ESBL *Klebsiella pneumoniae* and MDR *Serratia marcescens*. A concentration of 90% demonstrated the best activity against both bacteria, while increasing the concentration to 100% did not improve antibacterial effectiveness. Although the inhibitory effect was still below that of standard antibiotics, these findings suggest that lime leaf essential oil has the potential to be developed as a natural antibacterial or as an adjunct to antibiotic therapy to address the increasing cases of Gram-negative bacterial resistance. Therefore, further research on MIC/MBC determination, formulation, molecular mechanism of action, and combination with antibiotics is warranted.

This study has several limitations. First, antibacterial activity was evaluated only using the well diffusion method, so the results are significantly influenced by the diffusion capacity of the essential oil in the agar medium, particularly because of the hydrophobic nature of essential oils. Second, the study did not determine the minimum inhibitory concentration or minimum bactericidal concentration, so antibacterial potential could not be assessed quantitatively. Third, the test used only one clinical isolate for each bacterium, so it could not describe variation in response between strains. Fourth, the study did not evaluate possible synergistic effects of essential oils with antibiotics or the molecular mechanisms underlying their antibacterial activity. Furthermore, the main active components identified by chromatography have not been tested separately, so the contribution of each compound to antibacterial activity cannot be confirmed.

CONCLUSION

The essential oil of lime leaves (*Citrus hystrix* DC.) has antibacterial activity against ESBL *Klebsiella pneumoniae* and multidrug-resistant *Serratia marcescens*, with optimum activity at 90% and a tendency to decrease at 100%. The antibacterial activity against *S. marcescens* is higher than against ESBL *K. pneumoniae*, although its inhibitory power is still lower than that of ciprofloxacin. These findings suggest that lime leaf essential oil has potential as a natural antibacterial against resistant Gram-negative bacteria. Further research is needed to evaluate the minimum inhibitory concentration and minimum bactericidal concentration, the mechanism of action, synergistic effects with antibiotics, formulations that enhance effectiveness, and toxicity and in vivo testing to support its development as a candidate for complementary therapy.

CONFLICT OF INTEREST

In this study there is no conflict of interest

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