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Synergistic antibacterial activity of kersen (Muntingia calabura L.) and beluntas (Pluchea indica L.) leaf extracts against Staphylococcus aureus ATCC 25923

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Abstract: Bacterial infections remain a major public health problem in Indonesia, with increasing concern related to antimicrobial resistance. The use of antibacterial combinations has been proposed as a strategy to enhance antibacterial efficacy. This study aimed to evaluate the antibacterial interaction between kersen leaf extract (*Muntingia calabura L.*) and beluntas leaf extract (*Pluchea indica L.)* against *Staphylococcus aureus* ATCC 25923. This study employed an experimental laboratory design using a broth microdilution method with a checkerboard assay. Antibacterial activity was assessed by determining the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC), and the interaction between extracts was evaluated using the fractional inhibitory concentration index (FICI). The MIC values of kersen and beluntas leaf extracts tested individually were 10 mg/mL and 2 mg/mL, respectively. When combined, bacterial growth inhibition was achieved at reduced concentrations of 2.5 mg/mL kersen leaf extract and 0.5 mg/mL beluntas leaf extract. The MBC values of kersen and beluntas leaf extracts alone were 10 mg/mL and 4 mg/mL, respectively, while the combination showed bactericidal activity at 5 mg/mL kersen leaf extract and 0.5 mg/mL beluntas leaf extract. The calculated FICI value was 0.5, indicating a synergistic interaction between the two extracts. In conclusion, the combination of kersen and beluntas leaf extracts exhibited synergistic antibacterial activity against *Staphylococcus aureus* in vitro, as demonstrated by reduced MIC and MBC values. These findings suggest that this plant extract combination has potential for further development as an alternative antibacterial strategy, pending additional in-depth studies.

Keywords: Muntingia calabura L.; Pluchea indica L.; antibacterial combination; Staphylococcus aureus; fractional inhibitory concentration index.

INTRODUCTION

Staphylococcus aureus is a major human pathogen responsible for a wide range of clinical infections, ranging from mild skin infections to severe systemic diseases[1]. This bacterium is recognized as one of the leading causes of healthcare-associated and community-acquired infections worldwide[1]. A major concern associated with *S. aureus* infections is its ability to develop resistance to various antibiotics, particularly β-lactam antibiotics such as penicillin, which significantly complicates treatment strategies[2].

The increasing prevalence of antibiotic resistance has encouraged the exploration of alternative antibacterial agents derived from natural products. Medicinal plants are considered promising sources of bioactive compounds with antibacterial potential. One such plant is kersen (*Muntingia calabura L.*), whose leaves have been reported to contain various bioactive metabolites, including

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alkaloids, flavonoids, tannins, saponins, and terpenoids/steroids [3,4]. Flavonoids are considered the major active constituents in kersen leaves and play a crucial role in their antibacterial activity. The antibacterial mechanisms of flavonoids include inhibition of nucleic acid synthesis, disruption of bacterial energy metabolism, and impairment of cell membrane function, ultimately leading to the inhibition of bacterial growth [5].

Another medicinal plant with antibacterial potential is beluntas (*Pluchea indica L.)*. Phytochemical analyses have shown that beluntas leaves contain several secondary metabolites, including alkaloids, flavonoids, phenolic compounds, steroids, and terpenes, with phenolic compounds being the dominant constituents[6,7]. Phenolic compounds exert antibacterial effects primarily by disrupting bacterial cell membranes, deactivating essential enzymes, and inducing protein denaturation, which leads to increased membrane permeability and subsequent bacterial cell damage[8].

The antibacterial combination method is a method that can be used to increase the efficacy of an antibacterial and reduce the potential side effects of the antibacterial [9]. The antibacterial combination method can produce several effects, including synergistic, additive, indifferent, and antagonistic[9,10,11]. The synergistic effect indicates that the antibacterial combination has a better effect than the use of antibacterial in the individual compounds [10].

Combination therapy is a strategy commonly employed to enhance antibacterial efficacy while minimizing potential adverse effects and the development of resistance. The interaction between combined antibacterial agents may result in synergistic, additive, indifferent, or antagonistic effects. A synergistic effect indicates that the combined antibacterial activity is greater than the sum of the effects of each agent used individually, whereas an additive effect reflects comparable efficacy between the combination and individual agents. In contrast, indifferent and antagonistic interactions indicate no improvement or reduced antibacterial effectiveness, respectively.

Synergistic or additive effects are more likely to occur when antibacterial agents possess different mechanisms of action, thereby targeting multiple bacterial pathways simultaneously. Although the antibacterial activities of Muntingia calabura L. and Pluchea indica L. leaf extracts have been reported individually, studies evaluating their combined antibacterial effects against Staphylococcus aureus remain limited.

Therefore, this study aims to investigate the antibacterial activity of the combination of kersen (*Muntingia calabura L.*) and beluntas (*Pluchea indica L.)* leaf extracts against *Staphylococcus aureus*, with particular emphasis on determining the nature of their interaction.

MATERIAL AND METHOD

Research design

This study employed an experimental laboratory design using the broth microdilution method combined with a checkerboard assay to evaluate the antibacterial activity and interaction between kersen leaf extract (*Muntingia calabura L.*) and beluntas leaf extract (*Pluchea indica L.*) against *Staphylococcus aureus.*

Plant Materials and Extract Preparation

Fresh kersen (*Muntingia calabura L.*) and beluntas (*Pluchea indica L.*) leaves were collected from Kotagede and Umbulharjo, Yogyakarta, Indonesia, respectively. Plant identification was conducted at an accredited laboratory based on morphological characteristics and taxonomic references. The leaves were washed, air-dried, milled into powder, and extracted using 96% ethanol at a ratio of 1:10 (w/v). A total of 100 g of powdered sample was macerated in 1 L of solvent for 24 hours with intermittent shaking. The filtrates were concentrated under

reduced pressure using a rotary evaporator at 50°C, followed by further evaporation on a water bath to obtain viscous extracts.

Extract Standardization and Phytochemical Screening

Extract standardization included organoleptic evaluation (color, odor, and consistency) and determination of moisture content using a halogen moisture analyzer. Phytochemical screening was conducted qualitatively using tube tests and thin-layer chromatography (TLC) to identify the presence of alkaloids, flavonoids, phenolics, terpenoids/steroids, and saponins following standard procedures[15,16,17].

Bacterial Strain and Inoculum Preparation

Staphylococcus aureus ATCC 25923 was used as the test organism. The bacterial strain was rejuvenated on Brain Heart Infusion Broth (BHIB) and incubated at 37°C for 24 hours. The bacterial suspension was adjusted to match the 0.5 McFarland standard (approximately 1×10^8 CFU/mL) and further diluted in BHIB to obtain a final inoculum concentration of 1×10^6 CFU/mL.

Determination of MBC

Minimum bactericidal concentration (MBC) was determined by subculturing aliquots from wells showing no visible growth onto Mueller-Hinton Agar (MHA) plates, followed by incubation at 37°C for 18–24 hours [20,21]. The lowest concentration showing no bacterial growth on agar was defined as the MBC.

Data Analysis

The interaction between KLE and BLE was evaluated by calculating the Fractional Inhibitory Concentration Index (FICI) using the following formula:
FICI = (MIC_A combination / MIC_A alone) + (MIC_B combination / MIC_B alone)
FICI values were interpreted as follows: ≤0.5 (synergistic), >0.5–1.0 (additive), >1.0–4.0 (indifferent), and >4.0 (antagonistic)[12].

RESULTS AND DISCUSSION

The determination test sample of kersen and beluntas were conducted at the Biology Laboratory of the Faculty of Science and Technology, Universitas Ahmad Dahlan, Yogyakarta. The test was carried out using fresh plants with plant parts intact, starting from the roots, stems to leaves in the Laboratory and comparing of morphology of the plants with taxonomy on the literature. The plant determination test results showed that the plants used as samples were kersen (Mutingia calabura L.) and beluntas (Pluchea indica L.) plants.

Table 1. Organoleptic characteristics of kersen and beluntas leaf extracts

Parameter	Kersen leaf extract (KLE)	Beluntas leaf extract (BLE)
Color	Dark brown to black	Dark green
Odor	Characteristic extract odor	Characteristic extract odor

The organoleptic evaluation (table 1) showed that the kersen leaf extract (KLE) exhibited a dark brown to black color, while the beluntas leaf extract (BLE) appeared dark green. Both extracts presented a characteristic extract odor. These observations indicate that the extraction process produced concentrated plant extracts with distinct visual characteristics.

The dark brown to black coloration observed in the kersen leaf extract may be attributed to the presence of high levels of polyphenolic compounds, particularly flavonoids and tannins, which are known to undergo oxidation during the extraction and concentration processes. In contrast, the dark green color of the beluntas leaf extract is likely associated with chlorophyll derivatives and phenolic compounds commonly found in leafy plant materials.

The characteristic odor detected in both extracts suggests the presence of volatile secondary metabolites, which is consistent with previous reports on ethanolic extracts of medicinal plants. Overall, the organoleptic properties observed in this study indicate acceptable extract quality and support the suitability of the extracts for subsequent antibacterial activity testing. Moisture content of the

extracts was determined using a halogen moisture analyzer to assess extract quality and stability. Low moisture content indicates improved stability and reduced risk of degradation during storage.

Table 2. Moisture content of kersen and beluntas leaf extracts

No	Material	Moisture content (%)
1	Kersen leaf extract	4.05
2	Beluntas leaf extract	7.18

The moisture content analysis showed (table 2) that the kersen leaf extract had a moisture content of 4.05%, while the beluntas leaf extract exhibited a moisture content of 7.18%. The moisture content values obtained for both extracts were below 10%, indicating acceptable extract quality and stability. Low moisture content is an important parameter in extract standardization, as excessive residual water may promote degradation of secondary metabolites and reduce shelf stability. The lower moisture content observed in the kersen leaf extract compared to the beluntas leaf extract suggests a potentially higher stability during storage. Overall, the moisture content results support the suitability of both extracts for further antibacterial activity testing.

The phytochemical screening test is a test used to determine the content and component of secondary metabolite compounds in samples that have the potential to be antibacterial[14].

Table 3. Phytochemical screening results of kersen and beluntas leaf extracts (tube tests)

Phytochemical test	Kersen leaf extract (KLE)	Beluntas leaf extract (BLE)
Alkaloids	+ (orange-red precipitate)	+ (orange-red precipitate)
Flavonoids	+ (red precipitate formed)	+ (red precipitate formed)
Phenolics	+ (blackish-green color)	+ (blackish-green color)
Terpenoids	− (no precipitate/color change)	− (no precipitate/color change)
Steroids	− (no precipitate/color change)	− (no precipitate/color change)
Saponins	+ (stable foam formation)	+ (stable foam formation)

Phytochemical screening using tube tests (table 3) revealed that both kersen and beluntas leaf extracts contained alkaloids, flavonoids, phenolic compounds, and saponins, as indicated by positive reactions[15]. In contrast, terpenoids and steroids were not detected in either extract based on the absence of color change or precipitate formation. The presence of alkaloids, flavonoids, phenolic compounds, and saponins in both extracts supports their potential antibacterial activity, as these classes of secondary metabolites are widely reported to exhibit antimicrobial properties through various mechanisms. The absence of detectable terpenoids and steroids may be influenced by extraction conditions, solvent polarity, or low compound concentrations below the detection limit of qualitative assays[17]. Overall, the phytochemical profiles of both extracts indicate a similar composition of major bioactive compounds, which may contribute to their antibacterial effects either individually or in combination.

TLC tests are the separation method between chemical components based on the solubility level so that is obtained that to more specific compounds respond test reagents.

Table 4. Thin-layer chromatography (TLC) screening results of kersen and beluntas leaf extracts

Phytochemical class	Kersen leaf extract (KLE)	Beluntas leaf extract (BLE)
Alkaloids	+	+
Phenolics	+	+
Flavonoids	+	+
Terpenoids	−	−
Steroids	+	+

Note: (+) presence of compound indicated by positive reaction; (−) compound not detected.

Thin-layer chromatography analysis demonstrated (table 4) the presence of alkaloids, phenolic compounds, flavonoids, and steroids in both kersen and beluntas leaf extracts, as indicated by positive reactions with specific detection reagents. Terpenoids were not detected in either extract under the experimental conditions applied. The TLC results confirmed the phytochemical profiles observed in the tube tests, indicating consistency between qualitative screening methods. The presence of alkaloids, flavonoids, phenolic compounds, and steroids suggests that these secondary metabolites may contribute to the antibacterial activity of both extracts, either individually or synergistically. The absence of terpenoids may be attributed to solvent polarity, low compound concentration, or limitations of the detection method. Overall, the similar TLC profiles of kersen and beluntas leaf extracts support their potential compatibility for use in combination antibacterial assays.

Table 6. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of kersen leaf extract (KLE), beluntas leaf extract (BLE), and their combinations against *Staphylococcus aureus*

Treatment	MIC (mg/mL)	MBC (mg/mL)
KLE alone	10	10
BLE alone	2	4
KLE : BLE (combination)	2.5 : 0.5	5 : 0.5

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of kersen leaf extract (KLE), beluntas leaf extract (BLE), and their combination against Staphylococcus aureus are presented in Table 6. The MIC and MBC values for KLE alone were both 10 mg/mL, whereas BLE exhibited a MIC of 2 mg/mL and an MBC of 4 mg/mL. Notably, the combination of KLE and BLE reduced the effective concentrations required for both inhibition and bactericidal activity, with MIC values of 2.5 mg/mL and 0.5 mg/mL, and MBC values of 5 mg/mL and 0.5 mg/mL, respectively. The higher MBC values compared to MIC values observed for both single extracts indicate that higher concentrations were required to achieve bactericidal effects than to inhibit bacterial growth. The combined use of KLE and BLE resulted in a marked reduction in both MIC and MBC values, suggesting enhanced antibacterial efficacy when the extracts were applied together. This improvement may be attributed to complementary or synergistic interactions between bioactive compounds present in both extracts, leading to more effective disruption of bacterial cellular processes. These findings further support the use of combination therapy and justify subsequent evaluation using the fractional inhibitory concentration index (FICI).

Analysis by the FICI method is an index that can show inhibitory activity in a bacterium from a combination of antibiotics[12].

$$\text{FICI} = \frac{\text{MIC A combination}}{\text{MIC A alone}} + \frac{\text{MIC B combination}}{\text{MIC B alone}}$$

$$\text{FICI} = \frac{2.5\ \text{mg/ml}}{10\ \text{mg/ml}} + \frac{0.5\ \text{mg/ml}}{2\ \text{mg/ml}}$$

$$\text{FICI} = \frac{1}{4} + \frac{1}{4} = \frac{2}{4} = \frac{1}{2}$$

FICI = 0.5

The interpretation of FICI values according to can be seen in the following table 7.

Table 7. Interpretation criteria of the fractional inhibitory concentration index (FICI)

FICI value	Interpretation
≤ 0.5	Synergistic
> 0.5 – ≤ 1.0	Additive
> 1.0 – ≤ 4.0	Indifferent
> 4.0	Antagonistic

The interaction between kersen leaf extract (KLE) and beluntas leaf extract (BLE) was evaluated using the fractional inhibitory concentration index (FICI). The FICI values were interpreted as follows: ≤0.5 indicated synergistic interaction, >0.5–1.0 additive interaction, >1.0–4.0 indifferent interaction, and >4.0 antagonistic interaction.

The FICI value of 0.5 obtained in this study indicates a synergistic interaction between kersen leaf extract (KLE) and beluntas leaf extract (BLE) against *Staphylococcus aureus.* This finding demonstrates that the combined antibacterial effect of both extracts is greater than their individual effects, as evidenced by the reduced MIC and MBC values observed in the combination treatment.

Synergistic interactions are commonly observed when antibacterial agents possess different or complementary mechanisms of action[9,11,18,19]. The synergistic effect observed is consistent with the principle that multi-target antibacterial strategies can improve efficacy and reduce the required dosage of individual agents[18]. This reduction in effective concentration may contribute to minimizing potential toxicity and delaying the development of bacterial resistance. Moreover, the comparable phytochemical profiles confirmed by tube tests and TLC analysis support the compatibility of both extracts when used in combination.

Despite these promising findings, this study is limited to in vitro evaluation against a single bacterial strain. Further studies involving additional pathogenic strains, quantitative phytochemical analysis, and in vivo models are required to validate the antibacterial potential and safety of the extract combination. Nonetheless, the results of this study provide a scientific basis for the development of plant-based antibacterial combinations as alternative or complementary therapeutic agents.

CONCLUSION

The combination of kersen leaf extract (KLE) and beluntas leaf extract (BLE) exhibited a synergistic antibacterial effect against Staphylococcus aureus, as indicated by a fractional inhibitory concentration index (FICI) value of 0.5. The combined treatment reduced the minimum inhibitory and bactericidal concentrations compared to the individual extracts, demonstrating enhanced antibacterial efficacy. These findings suggest that the combination of KLE and BLE has potential as a plant-based antibacterial formulation and may serve as a basis for further investigation into alternative antibacterial strategies against Staphylococcus aureus. However, additional studies, including quantitative phytochemical analysis, evaluation against multiple bacterial strains, and in vivo assessment, are required to confirm its therapeutic applicability.

AUTHORS' CONTRIBUTIONS

All authors have equal responsibility in the research and preparation of the manuscript. ABS has conducted the experiment and data analysis. NS has supervised the experiment and data analysis. NS and ABS has written the manuscript.

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DATA AVAILABILITY STATEMENT

The data supporting the findings of this study are available from the corresponding author upon reasonable request for academic and research purposes.

DISCLOSURE STATEMENT

The views and opinions expressed in this article are solely those of the authors and do not necessarily reflect the official policies or positions of their affiliated institutions. The data presented in this study are original, were generated by the authors, and have not been previously published or submitted for publication elsewhere.

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