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Effectiveness of the Ethanol Extract Combination of Binahong and Patikan Kebo Against *Staphylococcus aureus* Causing Cellulitis

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Abstract
Cellulitis is a skin infection caused by *Staphylococcus aureus*. The use of antibiotics in treatment often has the potential to increase bacterial resistance. The secondary metabolites present in binahong and patikan kebo leaves have the potential to act as antibacterial agents. This study aimed to determine whether the combination of binahong and patikan kebo leaf extracts is more effective in inhibiting the growth of *S. aureus* compared to their single extracts. The antibacterial activity was tested using the disc diffusion method with negative control (DMSO 10%) and positive control (amoxicillin 25 mcg). The concentrations of single extracts used were 40%, 50%, and 60%, while the combination extract test ratios were 1:3, 2:2, and 3:1. The data were analyzed using the Kolmogorov-Smirnov test and one-way ANOVA, followed by the Duncan test. The results of the antibacterial activity test showed that the combination of ethanol extracts of patikan kebo and binahong leaves had better effectiveness as an antibacterial against *S. aureus* compared to the single extract. The optimal combination of ethanol extracts of patikan kebo and binahong leaves in inhibiting the growth of *S. aureus* was in the ratio of 1:3, as it produced the largest inhibition zone with an average diameter of 1.11 ± 0.10 cm. Therefore, ethanol extracts combination of patikan kebo and binahong leaves can be used as the main ingredients for skin infection drugs.

Keywords: infection; secondary metabolites; antibacterial; inhibition zone.

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INTRODUCTION

In 2020, the Indonesian Ministry of Health reported a prevalence of 0.49 (49%) cases per 10,000 population and 4.2 new cases of skin disease per 100,000 population. Cellulitis is one of the common skin diseases caused by bacterial infection, which ranks 18th in the Global DALYs Disease Burden Ranking and is the fourth leading cause of universal disability (Xue et al., 2022; Njim et al., 2017). Cellulitis has a high incidence phase, with approximately 24.6% of cases per 1,000 patients annually. The incidence rate of cellulitis is increasing year by year (Rositawati and Sawitri, 2016). The primary cause of cellulitis is gram-positive bacterial infections such as *Streptococcus* spp. or *Staphylococcus aureus* (Bennett et al., 2019).

Skin infections are often treated using antibiotics (Hidayah et al., 2016). However, there is a negative impact that occurs, namely causing an increase in bacterial resistance if antibiotics are used uncontrollably (Angelica, 2014). This can occur if the administration of antibiotics is less precise regarding the dose, duration, and type used (Negara, 2016).

This situation requires further research for the treatment of infections by microorganisms that are resistant to antibiotics, developing new antibiotics at affordable costs that can eliminate or prevent the growth of resistant bacteria is very important. An alternative to these problems is to explore the active ingredients contained in medicinal plants (Hendriani et al., 2016).

Based on the research of Tshikalange et al. (2005), it is suggested that binahong leaves are believed to cure various infectious diseases caused by bacteria. According to Ekaviantiwi et al. (2013), the presence of active compounds that have antibacterial power from the phytochemical test results of binahong leaves, namely alkaloids, steroids/triterpenoids, tannins, tannin gallate, and saponins, was

shown. Garmana et al. (2014) have also found active compounds in the form of steroids/triterpenoids, saponins, and flavonoids by conducting phytochemical screening on binahong leaves.

In addition, another plant that can be utilized as herbal medicine is patikan kebo (Euphorbia hirta L). This plant contains active compounds namely tannins, quercitrin and myricitrin (flavonoids), terpenoids, alkaloids, polyphenols, and triterpenoids, which contribute to antiseptic, anti-inflammatory, antifungal, and antibacterial effects (Iskandar et al., 2022; Karim et al., 2015).

Plants known as binahong and patikan kebo are widespread throughout Indonesia. The leaf extracts of patikan kebo (*E. hirta* L.) and binahong (*Andredera cordifolia*) both contain secondary metabolite compounds that have antibacterial qualities. However, there has been no research comparing the combined extracts and single extracts to determine their effectiveness in inhibiting bacterial growth. Combining the two extracts may result in different effectiveness compared to using a single extract as an antibacterial agent. This calls for further research to compare the effectiveness of single and combined extracts in blocking the growth of infection-causing bacteria. Then, this research aims to determine whether the combination of binahong and patikan kebo leaf extracts is more effective than the single extract in inhibiting *S. aureus*.

MATERIALS AND METHODS

This research was conducted from November 19, 2023 to May 4, 2024, at the Genetics and Biomolecular Lab, Undergraduate Program of Biology, Faculty of Mathematics and Natural Sciences, State University of Surabaya. Binahong and patikan kebo leaf samples were collected from Kediri and Jombang, East Java. The samples were dried for approximately one week. After drying, the leaves were pulverized using a blender, and as much as 300 g of each leaf was obtained. The leaf symplisia was then macerated using 96% ethanol solvent three times at a ratio of 1:3, 1:2, and 1:2 for 24 hours in each treatment. The maceration process produces a filtrate. Furthermore, the filtrate was evaporated with a rotary evaporator to separate the binahong leaf and basil leaf extracts from the solvent. The concentration of the single extract tested for each leaf was 40%, 50%, and 60%. To obtain some of these concentrations, the extract was diluted by weighing as much as 4 g, 5 g, and 6 g and then dissolved using 10% DMSO solvent in a volume of 10 mL.

Subculture of bacteria was performed using NA and NB media. NA media was made by dissolving 0.92 g of NA in 40 mL of distilled water. NB media was made by dissolving 0.4 g of NB in 50 mL of distilled water. For MHA media, 38 grams of MHA were dissolved in 1,000 mL of distilled water. Then, it was homogenized and heated using a hot plate, after which it was sterilized using an autoclave at 121^{o}C and 2 atm pressure for 15 minutes. The bacterial culture was rejuvenated by taking one ose of *S. aureus* and inoculating it into the slanted NA media aseptically by the streak method. Next, it was incubated in an incubator for 24 hours at 37^{o}C. After 24 hours, one ose of bacterial culture was taken from the slanted NA, inoculated on sterile NB media, and then incubated in the incubator.

The bacterial suspension on NB media aged 24 hours was taken in 1 mL and put into a test tube containing 9 mL of sterile 0.9% NaCl to calculate the bacteria using the spectrophotometric method. The solution was vortexed, and the calculation of bacterial density was carried out with a spectrophotometer with a wavelength of 625 nm until it obtained the same absorbance value as the McFarland 0.5 standard solution of 0.08-1; the value is comparable to a bacterial concentration of 1.5x10^{8} CFU/mL (Rosmania and Yanti, 2020; Rijal and Asri, 2024).

Disc diffusion was used to test for antibacterial properties. Antibacterial activity testing refers to and modifies the research of Handayani et al. (2017), namely by pouring 1 mL of test bacterial suspension into a Petri dish, then 15 mL of MHA media was added and homogenized until it solidifies. The paper disc was soaked in 40%, 50%, and 60% concentration extract and 10% DMSO negative control solution for 30 minutes. Afterward, a Petri dish filled with media and bacteria was connected to the paper disc and incubated for 24 hours at 37 °C.

Furthermore, the combined extract of binahong and patikan kebo leaves was tested with the same testing procedure as the single extract, using a ratio of 1:3, 2:2, and 3:1. A similar method was used in other comparisons to make a combination of extracts, namely combining the ethanol extract of binahong leaves as much as 1 ml with 3 ml of the ethanol extract of patikan kebo leaves to obtain a ratio of 1:3 with each extract concentration of 60%.

The data were examined using the Kolmogorov-Smirnov normalcy test to determine whether or not they were expected. Then, using the IBM SPSS Statistic Version 22 program, an ANOVA analysis of variance and the Duncan test was used to determine the effect of concentration on the antibacterial activity of *S. aureus*.

RESULTS

Binahong and patikan kebo leaf ethanol extracts, as well as the combination of the two extracts, were shown to have antibacterial activity through the formation of inhibitory zones, tests on both extracts alone and in combination revealed that the combined extract's antibacterial activity was superior to that of the single extract, as evidenced by the fact that the diameter of the inhibition zone generated from the combination extract was longer than that of the inhibition zone generated from the single extract (Figure 1).

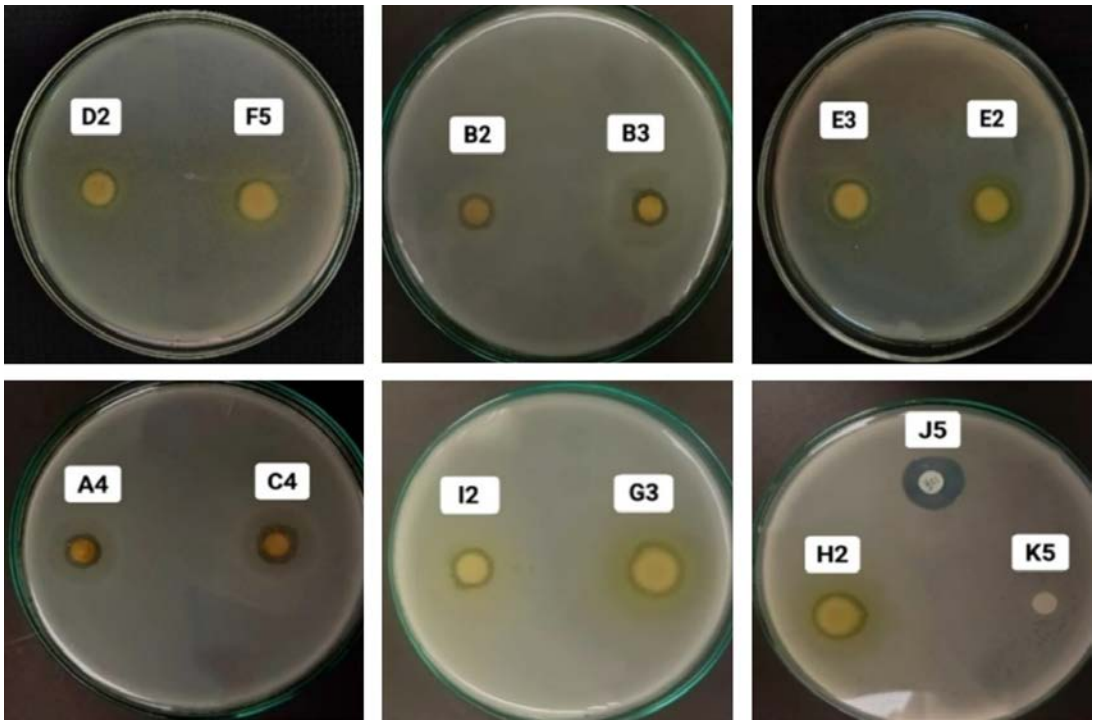

Figure 1. Results of antibacterial activity testing of extract treatments (A) Binahong Leaf 40%, (B) Binahong Leaf 50%, (C) Binahong Leaf 60%, (D) Patikan Kebo Leaf 40%, (E) Binahong Leaf 50%, (F) Binahong Leaf 60%, (G) Combination 1^{B}:3^{PK}, (H) Combination 2^{B}:2^{PK}, (I) Combination 3^{B}:1^{PK}, (J) Positive Control, and (K) Negative Control. Numbers 2, 3, 4, and 5 replicates each test.

The Kolmogorov-Smirnov test was used to determine whether the obtained data had a normal distribution. The results showed a significance value of 0.200 > 0.050, indicating a normal distribution. Subsequently, a one-way ANOVA test was conducted, yielding a significance value of 0.000 < 0.050 ($p < 0.050$). This suggests a significant difference in the impact of the treatment on the growth of *S. aureus*. To further analyze the differences between treatments, a Duncan test was performed at a 5% confidence level ($\alpha = 0.050$), revealing significant variations among the treatments (Table 1).

Table 1. Antibacterial activity of ethanol extracts of patikan kebo leaves and binahong leaves and their combination against *S. aureus*

Test	Mean Inhibition Zone Diameter (cm) + SD
Binahong 40%	$0.33 + 0.08^{b}$
Binahong 50%	$0.40 + 0.05^{b}$
Binahong 60%	$0.62 + 0.05^{c}$
Patikan Kebo 40%	$0.70 + 0.13^{c}$
Patikan Kebo 50%	$0.91 + 0.15^{de}$
Patikan Kebo 60%	$0.99 + 0.06^{e}$
Combination (1^{B}:3^{PK})	$1.11 + 0.10^{f}$
Combination (2^{B}:2^{PK})	$0.84 + 0.09^{d}$
Combination (3^{B}:1^{PK})	$0.65 + 0.03^{c}$
Positive control (Amoxicillin 25 mcg)	$1.14 + 0.06^{f}$
Negative control (DMSO 10%)	$0.00 + 0.00^{a}$

Notes: Notations a, b, c, d, e, and f indicate significant differences based on Duncan's test analysis ($\alpha = 0.05$). While code B in the treatment indicates the administration of binahong extract, PK suggests the administration of patikan kebo extract.

In Table 1, it is observed that the therapy outcomes using a 40% concentration of binahong leaf extract were not significantly different from the outcomes using a 50% concentration of binahong leaf extract, but they were significantly different from the outcomes using a 60% concentration of binahong leaf extract. The 60% concentration of binahong leaf extract was not significantly different from the treatment using a 40% concentration of patikan kebo extract and the combination (3:1). The treatment using a 50% concentration of patikan kebo leaf extract was not significantly different from the treatment using a 60% concentration of patikan kebo leaf extract and the combination (2:2). Additionally, the combination treatment (1:3) was not significantly different from the positive control treatment. The combination therapy (1:3) and the amoxicillin positive control treatment, however, both generated the biggest average diameter of the inhibition zone in comparison to the others, measuring 1.11 ± 0.10 for the former and 1.14 ± 0.06 for the latter.

DISCUSSION

This study was conducted to see the antibacterial effectiveness of ethanol extracts of patikan kebo and binahong leaves combined or singly against S. aureus, using positive control amoxicillin 25 mcg. Based on the testing of amoxicillin 25 mcg that has been done, the average diameter of the inhibition zone of 1.14 ± 0.06 cm is classified as weak according to the classification of Davis and Stout (1971). The results of this test are in line with the research of Al-Shammari et al. (2021), showing the results of 25 mcg amoxicillin testing using the disc diffusion method, which has an average inhibition zone diameter of 1.10 cm.

In the study, the role of 10% DMSO was as a negative control as well as an extract diluent. The use of 10% DMSO as a negative control is in line with Suryani et al. (2019), explaining that DMSO does not have any effect on the formation of the inhibition zone so that the test will not have an impact (Nonci et al., 2016). These findings align with the studies by Rahmi and Putri (2020), explaining that DMSO has no antibacterial effect on *Escherichia coli, Candida albicans*, or *S. aureus*.

Based on the test results of ethanol extracts of patikan kebo leaves and binahong leaves, it shows that both of them have the ability to inhibit and kill *S. aureus* bacteria. From the statistical results, it explains that there are significant differences in each treatment. It can be seen from the average diameter produced that the length of the diameter will increase along with the higher concentration of extract given. The antibacterial ability of patikan kebo leaf extract is supported by the research of Muiz et al. (2022), explaining that patikan kebo leaf extract can inhibit the growth of *S. aureus* bacteria. Meanwhile, the antibacterial ability of binahong leaf extract is supported by the research of Sulistyarsi and Pribadi (2018), explaining that binahong leaf extract can inhibit the growth of *Pseudomonas aeruginosa* and *S. aureus* bacteria.

The test results showed that the ethanol extract of patikan kebo leaves produced a better antibacterial effect than the single ethanol extract of binahong leaves. Meanwhile, the combination of extracts from both with a concentration ratio of 1^{B}:3^{PK}, where the content of patikan kebo extract is higher, showed better results, and these results were significantly different from the ratio of 2^{B}:2^{PK} and 3^{B}:1^{PK}. This is due to differences in the content of active compounds in plants, which are influenced by internal and external factors that affect the composition of these compounds (Katuuk et al., 2019). Supported by the statement of Kinho et al. (2011) about the antibacterial content in the form of euphorbol, tirukalol, eufosterol, taraxerol, friedlin, β-amyrin, β-eufol, and hentriacontane owned by patikan kebo plants until now there has been no research that mentions that binahong plants also have it.

The test results of ethanol extracts of patikan kebo and binahong leaves show that the chemical compounds contained have pharmacological effects as antibacterials. The results of antibacterial tests produce inhibition zone diameters that vary greatly. This is due to several factors, such as the availability of extract concentrations and the antibacterial components' content (Rahman et al., 2017).

Active ingredients found in the patikan kebo leaves' ethanol extract include phenolics, tannins, quercitrin and myricitrin (flavonoids), alkaloids, triterpenoids, taraxerol, friedlin, β-amyrin, β-eufol, euforbol, tirukalol, eufosterol, and seta hentriacontane (Iskandar et al., 2022; Karim et al., 2015; Kinho et al., 2011). According to Ekaviantiwi et al. (2013), the phytochemical screening of binahong leaf extract revealed the presence of active chemicals, including triterpenoids, flavonoids, alkaloids, tannins, steroids, and saponins. The two plants have quite different amounts of compounds. The mechanism by which certain compounds inhibit or kill bacteria in multiple ways is as follows.

Cell wall breakdown is the first step in the formation of secondary metabolite chemicals that prevent bacterial growth and development. By acting as antibacterials, flavonoids stop bacteria from

forming cell walls (Na'im, 2014). Additionally, by breaking down the components of peptidoglycan, alkaloids harm the bacterial cell wall layer (Taufiq et al., 2015).

Secondary metabolite chemicals penetrate deeper and disrupt bacterial cell membrane when its cell wall is destroyed. The stability of the cell is lowered by saponins' capacity to attach to the cytoplasmic membrane. Cell death results from this cytoplasmic leakage (Robinson, 1995; Karlina et al., 2013). Furthermore, the mechanism of membrane permeability can be interfered with by flavonoids (Voutquenne-Nazabadioko, 2018). In addition, because phenolic compounds form hydrogen bonds with bacterial cell proteins, they help to denaturize cells proteins, resulting in damages of protein structure, leading in cell damage and death (Carolia and Noventi, 2016; Hafsari et al., 2015).

Chemicals that are secondary metabolites have the ability to damage bacterial cells' nuclei. Proteins can be precipitated, coagulated, and denatured by tannin compounds, which can also deactivate enzymes and damage genetic material, making bacteria dormant. Additionally, they block bacterial DNA from synthesizing by inhibiting the enzymes reverse transcriptase and DNA topoisomerase in DNA synthesis (Dewi et al., 2014; Rosidah and Wila, 2012; Nuri et al., 2013). Bacteria lyse and destroy the cell membrane as a result of tannins penetrating the bacterial cell and targeting its core (Agustina, 2018).

Combining the patikan kebo and binahong leaf ethanol extracts results in more antibacterial activity than using them alone. The diameter of the inhibition zone created lends credence to this. With an average diameter of 1.11 ± 0.10 cm, the ratio of 1:3 between the ethanol extracts from patikan kebo and binahong leaves is the most efficient for preventing the growth of *S. aureus*.

CONCLUSION

Based on the research, combination of ethanol extracts of patikan kebo and binahong leaves shows stronger antibacterial effects against *S. aureus* compared to using just one kind of leaves extract. The best ratio for preventing *S. aureus* growth is 1:3, where the average inhibition zone reaches 1.11 ± 0.10 cm in diameter. These findings suggest that patikan kebo and binahong leaf extracts have great potential as key ingredients for developing natural remedies to treat skin infections.

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CONFLICT OF INTEREST

There is no conflict of interest.

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