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Research Article

Antibacterial and Antibiofilm Activity of the Extract and Fractions of Kelakai (*Stenochlaena palustris* (Burm.F) Bedd) against Methicillin-resistant *Staphylococcus aureus*

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Abstract

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a major concern in healthcare due to its resistance to antibiotics and ability to form biofilms. This study investigated the antibacterial and antibiofilm activity of the extract and fractions of kelakai (*Stenochlaena palustris*) leaves against MRSA. Phytochemical screening revealed the presence of alkaloids, flavonoids, steroids, tannins, and triterpenoids in *S. palustris* leaves. The ethanolic extract of *S. palustris* leaves exhibited dose-dependent antibacterial activity against MRSA at concentrations up to 500 ppm. Among the fractions, the *n*-hexane fraction exhibited antibacterial activity at 500 and 1000 ppm, while the methanolic fraction showed inhibition only at 1000 ppm. The ethyl acetate fraction did not show any inhibition. All fractions and extract demonstrated antibiofilm activity, with the *n*-hexane fraction exhibiting the strongest activity (91.33 ± 1.52%) at 125 ppm. The crude extract of *S. palustris* leaves showed the weakest antibiofilm activity (32.66 ± 8.14%). These findings suggest that *S. palustris* leaves contain compounds with antibacterial and antibiofilm properties against MRSA, with the *n*-hexane fraction being the most promising. Further studies are needed to isolate and characterize the active compounds responsible for these activities.

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INTRODUCTION

Infectious diseases continue to represent a significant global public health crisis, a challenge significantly exacerbated by the escalating emergence of bacterial species resistant to conventional antibiotics[1]. Since the seminal discovery of antibiotics in 1928, these agents have been pivotal in saving millions of lives; however, over the past few decades, antibiotic resistance has evolved into a critical health threat. A primary catalyst for this resistance is the inappropriate use of existing antibiotics[2]. The resulting impact on healthcare systems is substantial, encompassing inflated treatment costs, significant productivity loss, and increased patient mortality[3]. The issue of Antimicrobial Resistance (AMR) constitutes one of the most urgent global health challenges, estimated to be responsible for 1.27 million deaths worldwide in 2019, with over 2.8 million infections and more than 35,000 deaths occurring annually in the United States alone. Current surveillance indicates a worrying rise in hospital-onset AMR infections, underscoring how events like the COVID-19 pandemic have intensified the burden of antimicrobial resistance globally[4]. Recognizing this severity, the World Health Organization (WHO) released a priority list of bacterial pathogens in 2017 to guide the urgent discovery of new antimicrobial agents[5].

A key mechanism contributing to the high prevalence of antibiotic resistance is the capacity of many bacterial species to form biofilms[6]. A biofilm serves as a robust bacterial defense mechanism against external threats, particularly antibiotic

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molecules, and acts as a significant virulence factor[7]. Methicillin-resistant *Staphylococcus aureus* (MRSA), a globally recognized multidrug-resistant pathogen, frequently utilizes biofilm formation as a resistance strategy, physically blocking the penetration of antibiotics into the bacterial cells[8]. The complex structure of the biofilm, comprising an extracellular polymeric substance (EPS) matrix that includes polysaccharides, proteins, and extracellular DNA (eDNA), physically slows or blocks the diffusion of antibiotics. Furthermore, the EPS matrix can inactivate antibiotics through chelating cationic molecules or via deactivating enzymes, while the heterogeneous metabolic states within the biofilm contribute to increased antibiotic tolerance and resistance[4].

Given the urgent need for novel antimicrobial compounds, Indonesia's vast biodiversity, which ranks second globally, represents a crucial reservoir for potential medicinal plant species, many of which remain unexplored. Specifically, Borneo, the country's largest island, is home to numerous indigenous plants with potential as raw materials for new antibiotics. One such native Bornean plant is kelakai (*Stenochlaena palustris* (Burm.F) Bedd). While traditionally consumed as a food source by local communities, recent research reports its antibacterial activity against *Cutibacterium acne*[9]. However, comprehensive studies investigating its antibacterial and, critically, its antibiofilm activity against multi-resistant strains, such as MRSA, are currently lacking. Therefore, this study investigates the antibacterial and antibiofilm activity of the extract and fractions derived from *S. palustris* leaves against MRSA.

MATERIALS AND METHODS

Materials

Fresh leaves of *S. palustris* (approximately 5 kg) were harvested from Banjar Regency, South Kalimantan, Indonesia. Plant identification was formally authenticated by the Laboratory of Banua Botanical Garden, Banjarbaru, South Kalimantan (Certificate No. 070/11100/LIT/KRB/2024). The preparation of the crude drug adhered to standard protocols: the leaves were initially washed to remove external debris and impurities, then coarsely chopped to expedite the drying process. Drying was performed in a drying cabinet to mitigate potential microbial contamination and prevent the degradation of thermolabile metabolites or chemical reactions induced by UV light (as would occur with sun drying), thus maintaining product safety and quality. The dried crude drug was subsequently ground into a fine powder to increase the surface area, maximizing the potential extraction yield. The physical quality of the resulting crude drug powder was verified in accordance with the standards outlined in the Farmakope Herbal Indonesia, 2nd Edition[10]. The resulting powder was stored in a sealed container under cool, dry conditions until the extraction phase.

Methods

Extraction and fractionation

Extraction was executed using the Ultrasound-Assisted Extraction (UAE) method[11]. Approximately 500 g of the crude drug powder was placed in an Erlenmeyer flask, and 70% ethanol (five times the weight of the powder, or sufficient to submerge the material) was added. The mixture was sonicated using a Biobase sonicator set at a frequency of 40 kHz and a temperature of 30°C for 30 minutes. The resulting extract was separated from the residual crude drug powder by filtration through flannel cloth. The filtrate was then concentrated using a Biobase rotary evaporator. The concentrated extract was hermetically sealed and stored at a low temperature (<8°C) until subsequent testing. Fractionation of the ethanolic extract followed a modified established procedure, yielding three fractions: *n*-hexane, ethyl acetate, and methanol[12]. Specifically, 1.5 g of the concentrated ethanolic extract was applied to the top of a glass column chromatography tube (40 cm length, 2 cm diameter) that was wet-packed with 5 g of Silica gel 60 (Merck) using *n*-hexane as the solvent. Sequential elution was performed by pouring 5 mL of each solvent through the column, resulting in three polarity-dependent fractions (*n*-hexane, ethyl acetate, and methanolic fractions). All fractions were sealed and stored at a low temperature (<8°C) awaiting evaluation.

Phytochemical screening

The secondary metabolite profile of *S. palustris* leaves was investigated following the protocol published by Farnsworth[13]. Briefly, 10 g of *S. palustris* dry leaf powder was macerated, and the resulting extract was used to qualitatively identify the presence of six major secondary metabolite groups: alkaloids, flavonoids, phenolics, steroids, tannins, and triterpenoids. All

phytochemical screening experiments were executed in triplicate. The identification was achieved through specific spot tests using the following characteristic reagents:

Alkaloids: The plates were sprayed with Dragendorff reagent; the appearance of orange-brown spots indicated a positive result[14].

Flavonoids: Plates were exposed to ammonia vapors and subsequently sprayed with 5% aluminum chloride ($AlCl_3$). The visualization of yellow fluorescent spots under UV 366 nm confirmed the presence of flavonoids[15].

Phenolics and tannins: The application of a 1% $FeCl_3$ solution was used, with greenish-black or blue spots signifying the presence of these compounds[16].

Terpenoids and steroids: Plates were sprayed with the Liebermann-Burchard reagent; the development of violet to purple spots indicated terpenoids, whereas bluish-green spots confirmed the presence of steroids[17].

Preparation of bacterial suspension

A stock culture of MRSA ATCC 43300 bacteria, retrieved from glycerol storage, was initially inoculated onto Nutrient Agar (NA) medium (Merck) using an inoculation loop and incubated at 37°C for 24 hours. A single, isolated colony of MRSA was then transferred into Nutrient Broth (NB) medium (Merck) and incubated at 37°C for 24 hours to achieve an overnight culture. This overnight bacterial suspension was subsequently diluted with fresh NB medium until its turbidity matched that of McFarland Standard No. 0.5 for use in the assays.

Antibacterial activity

The antibacterial efficacy of *S. palustris* extract and fractions was assessed using the well diffusion method[18]. The extract and all fractions were diluted in 1% Dimethyl Sulfoxide (DMSO) to obtain three test concentrations: 1000 ppm, 500 ppm, and 250 ppm. Streptomycin (10 µg/mL) was employed as the positive control, while 1% DMSO served as the negative control. Two hundred µL of the standardized bacterial suspension was uniformly spread across the surface of a Petri dish containing Mueller-Hinton Agar (MHA) medium (Merck) using a sterile L-rod. Wells were created in the MHA medium using a sterile cork borer. One hundred µL of each test solution (extract, fractions, and controls) was introduced into the respective wells. The plates were then incubated at 37°C for 24 hours. All antibacterial experiments were performed in triplicate.

Antibiofilm activity

The antibiofilm potential of *S. palustris* extract and fractions was determined via a modified microplate assay protocol[7]. The extract and fractions were prepared at a final test concentration of 125 ppm (50 µL in 1% DMSO) in a 96-well plate. The overnight MRSA suspension was diluted in NB medium (Merck) to achieve an optical density (OD_{600}) of 1.6. Fifty µL of this bacterial suspension was added to each well containing the extract or fractions; the final well, lacking any test compound, was reserved as the growth control. The 96-well plates, containing the mixture of bacteria and test substances, were incubated at 37°C for 18 hours to allow for biofilm formation. Following incubation, the non-adherent bacterial cells were gently discarded by washing the wells three times with 120 µL of sterile phosphate-buffered saline (PBS). The plates were then oven-dried by heating at 60°C for 1 hour. Biofilm staining was performed by adding 120 µL of a 0.1% crystal violet solution to each well, followed by incubation at room temperature for 15 minutes. Excess crystal violet was removed using tap water, and the plates were air-dried. To quantify the biofilm mass, 150 µL of a 30% acetic acid solution was added to each well to release the bound crystal violet-biofilm complex. The released crystal violet solution from each well was diluted 10-fold, and the absorbance was measured at a wavelength of 585 nm using a Jenway visible spectrophotometer. The antibiofilm activity for each extract and fraction was calculated based on the percentage of biofilm production inhibition relative to the control. All experiments were conducted in triplicate.

Data analysis

The results from all experiments, including phytochemical screening, antibacterial activity, and antibiofilm activity, were collected from triplicate measurements. The diameter of the zone of inhibition for antibacterial testing and the absorbance values for antibiofilm activity were used for comparative analysis. For the antibiofilm assay, the percentage inhibition was calculated using **Equation 1**. The data obtained were used to determine the efficacy of *S. palustris* extract and its fractions, as well as to identify the optimal concentration or fraction exhibiting the highest inhibitory effect against the target bacteria. All

quantitative results were presented as mean ± standard deviation (SD), and statistical significance was determined using a one-way ANOVA test, with a p-value ≤0.05 considered statistically significant. Figures were generated using GraphPad Prism software, V.10.3.1.

$$\text{Inhibition (\%)} = \left(1 - \frac{\text{Sample absorbance}}{\text{Control absorbance}}\right) \times 100\% \qquad [1]$$

RESULTS AND DISCUSSION

The phytochemical screening results, presented in **Table I**, revealed the presence of alkaloids, flavonoids, steroids, tannins, and triterpenoids in *S. palustris* leaves, with the notable exception of saponins. This finding aligns with previous reports on the plant's secondary metabolite profile[9]. The absence of saponins, which are often associated with bitterness or foaming properties, may contribute to the historical use of *S. palustris* leaves as an edible vegetable. It is essential to acknowledge that this initial screening was qualitative; thus, future research focusing on a more thorough, quantitative analysis of these metabolite groups and their specific biological roles is warranted.

Table I. Phytochemical screening of *S. palustris*.

Secondary metabolite class	Result
Alkaloid	+
Flavonoid	+
Saponin	-
Steroid	+
Tannin	+
Triterpenoid	+

The ethanolic extract of *S. palustris* leaves (**Figure 1**) demonstrated notable antibacterial activity against MRSA, supporting the plant's traditional use in managing infectious and inflammatory conditions. As shown in **Table II**, the diameter of the inhibition zone increased with concentration; however, a critical observation was the plateauing of activity between the 500 ppm (12.00 ± 1.00 mm) and 1000 ppm (11.33 ± 1.53 mm) concentrations. This non-linear, dose-dependent behavior is somewhat unusual, as the activity of pure compounds typically increases proportionally until saturation. The plateau observed here may indicate that the active components within the crude extract reach a maximal effective concentration at 500 ppm, or it might reflect antagonistic interactions among the complex mixture of phytochemicals, such as the flavonoids, tannins, and phenolics identified in the screening, that limit further enhancement of activity at higher doses[19-21]. Given the use of a crude ethanolic extract, which contains both bioactive and inactive constituents, fractionation was performed to elucidate the location of the active compounds further.

Figure 1. (left) Herbal and (right) extract of *S. palustris* leaves.

Table II. Zone of inhibition of ethanolic extract of *S. palustris* leaf.

Treatment	Concentration	Zone of inhibition (mm) ± SD
Positive control (streptomycin)	10 µg/mL	19.33 ± 0.58
Negative control (DMSO)	1%	-
Stenochlaena palustris extract	1000 ppm	11.33 ± 1.53
Stenochlaena palustris extract	500 ppm	12.00 ± 1.00
Stenochlaena palustris extract	250 ppm	7.67 ± 0.58

Analysis of the fractions provided clearer insight into the distribution of antibacterial compounds, as detailed in **Table III**. At the lowest concentration (250 ppm), no fraction exhibited inhibition. The *n*-hexane fraction showed activity at both 500 ppm and 1000 ppm, while the methanolic fraction only demonstrated inhibition at the highest concentration (1000 ppm). Interestingly, the ethyl acetate fraction exhibited no antibacterial activity at any of the tested concentrations. This outcome suggests that the primary antibacterial agents are not semipolar compounds soluble in ethyl acetate. Conversely, the superior performance of the *n*-hexane fraction implies that the most active antibacterial constituents are primarily nonpolar secondary metabolites, which partition readily into the *n*-hexane solvent.

Table III. Zone of inhibition of fraction of *S. palustris* extract.

Treatment	Concentration	Zone of inhibition (mm) ± SD
n-hexane fraction	250 ppm	-
	500 ppm	9.43 ± 0.67
	1000 ppm	9.23 ± 0.97
Ethyl acetate fraction	250 ppm	-
	500 ppm	-
	1000 ppm	-
Methanolic fraction	250 ppm	-
	500 ppm	-
	1000 ppm	8.71 ± 0.79
Positive control (streptomycin)	10 µg/mL	19.33 ± 0.47
Negative control (DMSO)	1%	-

The evaluation of antibiofilm activity against MRSA, quantified by the reduction in crystal violet staining absorbance (**Table IV**), revealed that all fractions and the crude extract exhibited inhibitory effects at a concentration of 125 ppm. The results, summarized in **Figure 2**, clearly demonstrate that the *n*-hexane fraction exhibited the strongest antibiofilm activity (91.33 ± 1.52%), while the crude ethanolic extract showed the weakest effect (32.66 ± 8.14%). This marked difference suggests that the compounds responsible for inhibiting biofilm formation are predominantly nonpolar secondary metabolites, which are preferentially concentrated in the *n*-hexane fraction.

Table IV. Absorbance values indicating antibiofilm activity of *S. palustris* extract and fractions (125 ppm concentration).

Replication	Absorbance (Å)				
	Negative control (DMSO 1%)	*n*-hexane fraction	Ethyl acetate fraction	Methanol fraction	Ethanol extract
1	0.417	0.042	0.195	0.263	0.239
2	0.419	0.038	0.192	0.274	0.295
3	0.410	0.031	0.195	0.252	0.302
Mean	0.415	0.037	0.194	0.263	0.279
SD	0.005	0.006	0.002	0.011	0.033

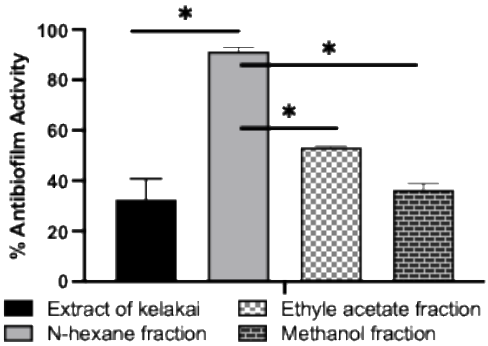

Figure 2. Antibiofilm activity of the extract and fractions of *S. palustris* leaves. The *n*-hexane fraction shows the strongest antibiofilm activity. (*) indicates a significant difference between two related groups (p-value = 0.05).

The strong antibiofilm effect in the nonpolar *n*-hexane fraction aligns with the phytochemical profile of *S. palustris*, which includes terpenoids and steroids. These nonpolar compounds are well-documented for their ability to disrupt bacterial membranes, interfere with quorum sensing, and inhibit the synthesis of EPS, all critical steps in biofilm development and maintenance[22]. Conversely, the lower activity of the crude ethanolic extract is likely due to the dilution effect of the active nonpolar compounds by a large proportion of polar, inactive, or even antagonistic constituents, such as highly polar flavonoids and tannins. This observed variation in potency among the fractions is consistent with differences in solvent polarity, emphasizing that fractionation is an effective strategy for concentrating the key nonpolar antibiofilm agents present in *S. palustris*.

The ability of natural products to inhibit bacterial biofilm formation is of paramount importance in the context of AMR, as the EPS matrix provides significant protection to MRSA against antibiotics. Numerous medicinal plants, including *Curcuma longa*, *Camellia sinensis*, and *Allium sativum*, are known to possess potent antibiofilm properties, often acting through multi-targeted mechanisms such as disrupting bacterial adhesion, interfering with quorum sensing, and degrading EPS[23-30]. In this context, the strong antibiofilm activity identified in the *n*-hexane fraction of *S. palustris* marks it as a promising, yet underexplored, natural source for combating MRSA-associated infections.

In summary, the ethanolic extract and its subsequent fractions of *S. palustris* exhibit significant antibacterial and antibiofilm activities against MRSA. The nonpolar *n*-hexane fraction consistently demonstrated the highest potency for both activities, indicating that the primary bioactive agents are likely nonpolar secondary metabolites, such as terpenoids or steroids. However, a significant limitation of this study is the use of crude and semi-purified extracts, which prevents the identification of the specific active molecules. Future work is therefore essential to isolate and structurally characterize these nonpolar compounds, as well as to elucidate their molecular mechanisms of action against bacterial targets and biofilm components.

CONCLUSION

The study conclusively demonstrates that *S. palustris* leaf extract, along with its *n*-hexane and methanolic fractions, exhibits significant inhibitory activity against MRSA when tested at the highest concentration of 1000 ppm. Furthermore, all tested extracts and fractions displayed notable antibiofilm activity. Crucially, the *n*-hexane fraction proved to be the most potent, achieving the strongest inhibition of biofilm formation at a minimum inhibitory concentration of 125 ppm (91.33 ± 1.52%). These findings strongly suggest that the *n*-hexane fraction contains the most promising active compounds. Consequently, future research should prioritize conducting a follow-up study focused on the isolation and detailed structural characterization of the specific antibacterial and/or antibiofilm compound(s) responsible for this pronounced activity within the *n*-hexane fraction of the *S. palustris* leaf.

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DATA AVAILABILITY

None.

CONFLICT OF INTEREST

The authors declared no conflict of interest related to this research.

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