PHARMACON
Jurnal Farmasi Indonesia
Pharmaceutical Journal of Indonesia

Pharmacon: Jurnal Farmasi Indonesia
Vol. 23, No. 1 (2026), pp. 86-91
DOI:10.23917/pharmakon.v23i1.7592
p-ISSN: **1411-4283**, e-ISSN: **2685-5062**

Unveiling the Antibacterial Activity and Phytochemical Screening of Extract and Fractions from Berebat (*Spatholobus ferrugineus*) Stem

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ARTICLE HISTORY:
Submitted : 2024-12-12
Accepted : 2026-06-25
Published : 2026-06-30

KEYWORDS:
Antibacterial; Berebat; Fractionation; *Spatholobus ferrugineus*

Citation:
Setyaningsih, E.P., Bahri, S., Aryati,R. (2026). Unveiling The Antibacterial Activity And Phytochemical Screening Of Extract And Fractions From Berebat (*Spatholobus ferrugineus*) Stem. Pharmacon: Jurnal Farmasi Indonesia, 23(1), 86-91. https://doi.org/10.23917/pharmakon.v23i1.7592

ABSTRACT

Berebat (*Spatholobus ferrugineus*) is a plant that grows in East Belitung, and has been used empirically by local people to treat sprue mouth, but scientific evidence supporting its antibacterial activity is still limited. This study aimed to evaluate the antibacterial activity of Berebat stem extract and fractions and to identify the most active solvent fraction. The dried stem powder was macerated with 96% ethanol, and the concentrated extract was then fractionated using n-hexane and ethyl acetate . Phytochemical screening was performed to identify secondary metabolites in the extract and fractions. The antibacterial activity was tested against Streptococcus sanguinis using the disc diffusion method at concentrations of 5%, 15%, 25%, 35%, and 45%, with 0.2% chlorhexidine gluconate as a positive control and 10% DMSO as a negative control. Phytochemical screening showed that flavonoids and tannins were present in the crude extract and both fractions, while saponins were absent in the n-hexane fraction and triterpenoids were absent in the ethyl acetate fraction. Berebat stem extract and fractions have antibacterial activity against *Streptococcus sanguinis* at 5%, 15%, 25%, 35%, and 45%. The ethyl acetate fraction showed the highest antibacterial activity, with an inhibition zone of 16.53 mm at 45% concentration, close to the positive control of 16.64 mm. Statistical analysis using the Kruskal–Wallis test revealed a significant difference in antibacterial activity among the ethanol extract, n-hexane fraction, and ethyl acetate fraction ($p < 0.001$).

INTRODUCTION

Berebat (*Spatholobus ferrugineus*) is a climbing shrub that grows by wrapping its stems around larger plants and is found in wet tropical biomes (Saputri et al., 2023). In East Belitung, Berebat stems are used empirically by the community to treat various diseases, one of which is sprue mouth. People use boiled Berebat stems to drink and also as a mouthwash (Abdussalam et al., 2021).

Berebat is a plant in the Fabaceae family. The Fabaceae family is a group that produces secondary metabolites from the flavonoid and isoflavonoid groups (Cahyana & Adiyanti, 2021). Preliminary research was conducted by Marliana (2007) regarding the antioxidant activity of the active compounds contained in Berebat. Based on the results of phytochemical screening, It is known that Berebat stems contain alkaloids, flavonoids, polyphenols, and terpenoids/steroids. Saputri et al. (2023) have successfully isolated pterocarpan derivative compounds from the bark of Berebat, namely the compounds variabilin and medicarpin. In another study conducted by Aliviyanti et al (2021), 96% ethanol reflux extract was proven to increase the LC50 of Berebat and contains anti-breast cancer substances.

Other research previously carried out on members of the Spatholobus genus included *Spatholobus littoralis* Hassk extract against *Pseudomonas aeruginosa* bacteria. *Spatholobus littoralis* Hassk has antibacterial effectiveness at a concentration of 100% and has a clear zone of 22.4 mm, which can be expressed as a strong

inhibitory response (above> 20) against the growth of *Staphylococcus aureus* with 96% ethanol solvent. It has inhibitory power at concentrations of 10%, 20%, and 30% of 7.63 mm, 8.26 mm, and 8.83 mm. *Spatholobus littoralis* Hassk contains phenolic compounds, flavonoids, alkaloids, terpenoids, and tannins (Latu & Wahid, 2023; Weldy et al., 2022).

The use of Berebat stems, which has been empirically used but lacks scientific evidence of its efficacy, prompted this study to evaluate its antibacterial activity against *Streptococcus sanguinis*, a common oral commensal that has been implicated in recurrent aphthous stomatitis and other oral microbial imbalances (Okahashi et al., 2022; Seoudi et al., 2015). Extraction using maceration and fractionation was chosen to prove the solvent variation on the resulting compound's characteristics and maximize its antibacterial activity.

METHODS

Chemical Material

Streptococcus sanguinis bacteria were obtained from the University of Indonesia. Ethanol 96%, ethyl acetate, n-hexane, and nutrient agar base were purchased from Merck. Chlorhexidine Gluconate 0,2% was used as a positive control from PT Minorock Mandiri. Dimethyl Sulfoxide (DMSO) 10% was used as a negative control from Merck. NaCl 0,9% was obtained from Otsuka, and Nutrient Agar (NA) was purchased from Merck.

Plant Materials

Stem of *Spatholobus ferrugineus* was collected from Lenggang village, East Belitung, Indonesia. this plant was determined in the Herbarium Depokensis (UIDEP) University of Indonesia.

Extraction Process

Extract preparation was carried out using maceration and fractionation methods. 500 g of Berebat powder was soaked using 96% ethanol solvent until completely submerged for 3x24 hours with occasional stirring; remaceration was carried out twice. The extract was concentrated using a rotary evaporator at a temperature of about 50°C, and the evaporation process was continued over a bath until a thick extract was obtained. For liquid-liquid partitioning, the thick extract was first suspended in distilled water and then partitioned successively with n-hexane and ethyl acetate in a separating funnel using a solvent ratio of 1:10 w/v. Each partition was repeated until the organic layer became clear. The obtained fractions were then concentrated to obtain the n-hexane fraction and ethyl acetate fraction (Astuti et al., 2014; Wijianto et al., 2024).

Phytochemical Screening

Phytochemical screening was performed on Berebat powder, extract, and fractions to identify alkaloids, saponins, tannins, flavonoids, triterpenoids, and steroids. Alkaloids were tested by heating 0.5 g of sample with 1 mL of 2 N HCl and 9 mL distilled water for 2 min, cooling, filtering, and reacting the filtrate with Mayer, Dragendorff, and Bouchardat reagents. Saponins were identified by shaking 0.5 g of sample with 10 mL distilled water for 1 min and observing stable foam after 10 min. Tannins were tested by adding 2 drops of 10% FeCl3 to 0.5 g of sample extracted with 10 mL hot water. Flavonoids were detected by adding concentrated HCl and magnesium powder to the heated sample. Triterpenoids and steroids were identified by reacting the sample in chloroform with acetic anhydride and concentrated H2SO4, where red or purple indicated triterpenoids and blue-green indicated steroids (Shaikh et al., 2020).

Preparation of Test Solution

The ethanol extract solution and Berebat fractions were divided into five concentration groups (w/v): 5%, 15%, 25%, 35%, and 45%. Each is made by weighing 100 mg, 300 mg, 500 mg, 700 mg, and 900 mg, then adding 10% DMSO to 2 mL.

Antibacterial Assay

The antibacterial activity test of the Berebat stem extract and fractions was carried out using the disk diffusion method; the paper disks used had a diameter of 6 mm. Approximately 15 mL of sterile Nutrient Agar (NA) medium is poured into a sterile petri dish until it solidifies. 0.1 mL/100 µl of bacterial suspension standardized to McFarland No. 0.3 was added to each petri dish and spread using a spreader over the surface of the medium until it dried, then dripped the paper disc with 20 µl of 5%, 15%, 25%, and 35% test solutions, place them regularly on agar medium containing the test bacteria and then labeled. The positive control was the antiseptic Chlorhexidine gluconate

0.2%, and the negative control was DMSO 10%. Incubate for 24 hours at 37°C, then observe the growth of the test bacteria and measure the inhibitory diameter (Hossain, 2024).

Data Analysis

The experiments were performed in triplicate. The inhibition zone diameter was measured for each replicate, and the results were expressed as mean ± standard deviation (SD). Antibacterial activity data were presented in tabular form and analyzed descriptively and inferentially. The normality of data distribution was assessed using the Shapiro–Wilk test. Since the data were not normally distributed, differences in antibacterial activity among the ethanol extract, n-hexane fraction, and ethyl acetate fraction of *Spatholobus ferrugineus* stem against *Streptococcus sanguinis* were evaluated using the Kruskal–Wallis test. Statistical significance was established at $p < 0.05$.

RESULT

The maceration of 500 g of Berebat stem powder yielded 82.3 g of ethanol extract, corresponding to an extraction yield of 16.46% relative to the initial plant material. The fractionation was performed using 20 g of the crude ethanol extract as the starting material. Based on this crude extract, the n-hexane fraction yield was 14.84%, and the ethyl acetate fraction yield was 35.1%. Extract yields are fundamental to calculating plant-to-extract ratios (Monagas et al., 2022). The extraction solvent plays a crucial role in influencing the properties of extracts. Various extraction solvents may have various effects on the solubility, the amount of extractable material in the starting plant, extraction yield, and pharmacological activity of the plant (Azabou et al., 2020; Monagas et al., 2022). Thus, a suitable solvent should be selected for the extraction of compounds targeted.

The phytochemical profiles of all samples (Berebat powder, extract, and fractions) were screened using various reagents to confirm the presence of the significant phytochemicals group, as depicted in Table 1.

A clear zone around the disc paper indicates that the extract can inhibit bacterial growth, as depicted in Table 2. Pan et al. (2009) also classify antibacterial activity based on the inhibition zone diameter, as depicted in Table 3.

Table 1. Phytochemical screening of powder, extract, and Berebat fraction

Secondary metabolite	Results			
	Powder	ethanol extract	ethyl acetate fraction	n-hexane fraction
Flavonoid	+	+	+	+
Tanin	+	+	+	+
Saponin	+	+	+	-
Alkaloid	+	+	+	+
Steroid	-	-	-	-
Triterpenoid	+	+	-	+

Note : + = presence of secondary metabolite; - = absence of secondary metabolite

Table 2. Test result of inhibition zone diameter Berebat extract and fraction against *Streptococcus sanguinis* bacteria

Samples	Concentration of test solution	Average of inhibition zone diameter (±SD)	Category
Ethanol extract	5%	9.32±0.63	Moderate
	15%	11.15±0.90	Moderate
	25%	11.53±0.92	Moderate
	35%	13.95±0.44	Strong
	45%	14.35±0.38	Strong
Ethyl acetate fraction	5%	10.63±0.99	Moderate
	15%	13.77±2.13	Strong
	25%	14.29±1.45	Strong
	35%	15.83±1.69	Strong
	45%	16.53±2.18	Strong
N-hexane fraction	5%	7.87±0.54	Weak
	15%	8.57±0.71	Weak
	25%	8.76±0.53	Weak
	35%	9.04±0.14	Moderate
	45%	9.21±0.36	Moderate
Positive control	0.2%	16.47±1.07	Strong
Negative control	10%	0	-

So, it was concluded that the ethyl acetate fraction had the best antibacterial activity against *Streptococcus sanguinis* bacteria. As a semi-polar solvent, ethyl acetate has more complex chemical compounds than polar and nonpolar solvents (Esati et al., 2024). The Kruskal–Wallis test revealed a statistically significant difference in the inhibition zone diameters among the ethanol extract, n-hexane fraction, and ethyl acetate fraction of *Spatholobus ferrugineus* stem against *Streptococcus sanguinis* ($H = 28.883$, $df = 2$, $p < 0.001$).

Table 3. Classification of antibacterial activity based on inhibition zone diameter

No.	Diameter	Category
1	> 12 mm	Strong
2	9<x≤12 mm	Moderate
3	7<x≤9 mm	Weak
4	= 6 mm	No inhibitory activity

Based on the mean rank values, the ethyl acetate fraction exhibited the highest antibacterial activity (mean rank = 34.17), followed by the ethanol extract (mean rank = 25.93), while the n-hexane fraction showed the lowest antibacterial activity (mean rank = 8.90). These findings indicate that fractionation with ethyl acetate enriched antibacterial constituents and showed the highest antibacterial activity among the tested samples.

DISCUSSION

The fractionation method and semi-polar solvents yielded better extractions, which may tell us that the Berebat stem is enriched with semi-polar secondary metabolites. Fractionation is a process of separation of plant extracts into various fractions. The fractionation method can maximize the extraction process, extracting compounds based on their polar properties (Abubakar & Haque, 2020). The ethyl acetate solvent is semi-polar, causing the extraction of compounds to be larger, whereas non-polar and polar compounds will also be attracted to the ethyl acetate fraction (Herdiana & Aji, 2020).

Ethanol extract can dissolve flavonoids, tannins, saponins, alkaloids, and triterpenoids in ethyl acetate fraction, flavonoids, tannins, saponins, and alkaloids. Meanwhile, flavonoids, tannins, alkaloids, and triterpenoids were also found in the n-hexane fraction. Flavonoids and alkaloids are semi-polar and are found in all three solvents. They can dissolve in all types of solvent polarity (Setyaningsih et al., 2024). Flavonoids have sugar groups (simple carbohydrates) because they can dissolve in water, which causes them to dissolve quickly in polar or semi-polar solvents. Alkaloids contain nitrogen in their cyclic part and bond with various groups, including amine, amide, phenol, and methoxy groups, which causes alkaloids to be semi-polar (Putri et al., 2023).

Saponin is a surface-active compound that is easily detected through its ability to form foam. The glycosidic bond component contained in saponin causes this compound to tend to be polar. Therefore, saponin is not found in the non-polar fraction (Suleman et al., 2022). The ethyl acetate fraction showed the strongest antibacterial activity, even though it was negative for triterpenoids in the phytochemical screening. This suggests that the inhibitory effect was more likely due to the combined or synergistic action of flavonoids, tannins, and alkaloids detected in the extract and fractions. These compounds are known to contribute to antibacterial activity through multiple mechanisms, including membrane disruption, protein precipitation, enzyme inhibition, and interference with bacterial genetic material. (Setyaningsih et al., 2024). More compounds were present in simplicia powder, which had not undergone an extraction process, and in ethanol extract. Secondary metabolites could be reduced during the extraction process. The extraction process can affect the concentration and therapeutic effect of simplicia due to the stability and nature of secondary metabolites in simplicia (Issusilaningtyas et al., 2023).

Ethyl acetate can attract more flavonoids. Flavonoids are metabolite compounds often contained in the Fabaceae family (Cahyana & Adiyanti, 2021). Berebat is included in this family. This was proven based on the extraction results, which showed that the yield of the ethyl acetate fraction was greater than that of the ethanol extract and n-hexane fraction. Flavonoid compounds cause damage to cell membranes and the release of intracellular bacterial compounds (Samputri et al., 2020). Besides flavonoids, tannins may also contribute to the antibacterial activity of the ethyl acetate fraction by complexing with bacterial proteins, disrupting cell wall integrity, and interfering with microbial adhesion, thereby inhibiting the growth of *Streptococcus sanguinis*. The increase in extract content is directly proportional to the increase in inhibitory diameter. The higher the concentration of a substance, the greater the number of microorganisms that can be inhibited (Fatriadi et al., 2018; Setyaningsih et al., 2024).

CONCLUSIONS

Antibacterial activity test on ethanol extract, ethyl acetate fraction, and n-hexane fraction using disc diffusion showed that the ethyl acetate fraction of Berebat had the best antibacterial

activity. Among the tested samples, the ethyl acetate fraction exhibited the strongest antibacterial activity against Streptococcus sanguinis and showed significantly higher overall inhibitory effects than the other fractions based on Kruskal–Wallis analysis.

ACKNOWLEDGMENT

The author would like to thank the Faculty of Pharmacy, National Institute of Science and Technology (ISTN) for supporting research facilities.

AUTHORS' CONTRIBUTIONS

EPS and RA conducted the experiment, SB and RA conducted an antibacterial activity test, and EPS and SB wrote and revised the manuscript. All authors agreed on the final version of this manuscript.

CONFLICT OF INTERESTS

The authors state that they have no conflict of interest with respect to the work described in this manuscript.

ETHICAL CONSIDERATION

The authors state that they have no ethical issues.

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