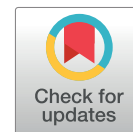


Antibacterial activity of betel (*Piper betle* L.) fruit against *Pseudomonas aeruginosa*



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ABSTRACT

Background: Betel (*Piper betle* L.) is a well-known medicinal plant for its numerous health benefits. Saponins, flavonoids, polyphenols, and essential oils are among the chemical constituents of betel plants. Flavonoids are one of the most common groups of secondary metabolites found in plant tissues, including in betel plants.

Objective: The purpose of this research is to isolate flavonoids from betel fruit and to determine the antibacterial activity of betel fruit extract against *Pseudomonas aeruginosa*.

Methods: Thin layer chromatography (TLC) with the eluent chloroform: methanol: water was used to isolate flavonoids. UV-Vis spectrophotometry was used to determine the presence of flavonoids in betel fruit. The antibacterial activity of extract and TLC-isolates of betel fruit was tested by using the disc method.

Results: TLC analysis resulted in the formation of a brown stain. The UV-Vis spectrophotometry results revealed two absorption bands at 366 nm and 268 nm, indicating that flavonoids are present in betel fruit. Antibacterial activity test against *Pseudomonas aeruginosa* bacteria showed that the concentration of 30% and 60% of betel fruit extract had strong antibacterial activity.

Conclusion: The results revealed that the betel fruit contains flavonoid compounds, and the extract has medium to strong antibacterial activity.

Keywords: antibacterial, flavonoid, betel fruit, piper betle, pseudomonas aeruginosa

Introduction

Antibiotic-resistant bacteria are becoming more common, necessitating the development of new natural compounds to kill bacteria. *Pseudomonas aeruginosa* is one of the bacteria that has developed resistance to antibiotics. This bacterium is commonly found in burns, ear infections, and surgical wounds [1].

One of the medicinal plants in Indonesia that can be used as traditional medicine is betel (*Piper betle* L) [2]. Traditionally, betel has been used to treat thrush, sore throats, coughs, eyewash, vaginal discharge, stop nose bleeding, speed wound healing, and eliminate bad breath. The chemical content of the betel plant is saponins, flavonoids, polyphenols, and essential oils. Flavonoid

compounds are thought to have a mechanism of action by denaturing bacterial cell proteins and damaging cell membranes. Flavonoids are one of the secondary metabolites found in plants. This compound can be used as antimicrobial, wound infection medicine, antifungal, antiviral, anticancer, and antitumor.

Betel plants have been reported as antibacterial activity, including their activity against *P. aeruginosa* [3,4]. So far, many studies focus on the leaves of betel, although the fruit part has been widely used by people in several areas of North Sulawesi, Indonesia. Therefore, this study aimed to analyze the flavonoid content in betel fruit, as well as to test the antibacterial activity of the betel fruit extract against *P. aeruginosa*.

Methods

Sample preparation

Betel fruits were taken from Airmadidi Atas, North Minahasa Regency, North Sulawesi, Indonesia, with an altitude of 331 meters above sea level with a north latitude of 1°25'46" and a south latitude of 124°58'56. The sample was determined as *Piper betle* L. at the Biology Laboratory of Sam Ratulangi University. Betel fruits were washed, drained, sorted and weighed. Then, they were allowed to dry at room temperature before being mashed into powder and sieved.

Sample extraction

A total of 420 grams of powder obtained from 1000 grams of fresh fruit was put into an extraction container. The extraction was carried out by immersing the sample with 1260 mL of ethanol 96% for 3×24 hours at room temperature while stirring occasionally. The extraction results were then filtered and the residue was separated from the filtrate. The residue was extracted again with ethanol 96% for 3×24 hours. Then, the sample was filtered to obtain a second filtrate. The filtrates obtained were combined and then evaporated at 42°C in incubator to obtain a thick extract.

Flavonoid compound isolation

Isolation of flavonoid compounds was carried out using thin layer chromatography. Sample extract and quercetin as a control were spotted on a previously activated silica gel F₂₅₄ plate. Elution was carried out with the mobile phase chloroform: methanol: water (65:25:4) [5], then the plate was observed at a wavelength of 365 nm. After that, the stains on the plate were scraped off and analyzed by UV-Vis spectrophotometer. The isolate was centrifuged, then read on a UV-Vis spectrophotometer using methanol as a standard solvent. The spectrum is observed at a wavelength of 200-400 nm [6].

Antibacterial activity test of betel fruit extract

Antibacterial activity test uses the disk diffusion method [7]. Ethanol extract of betel fruit was

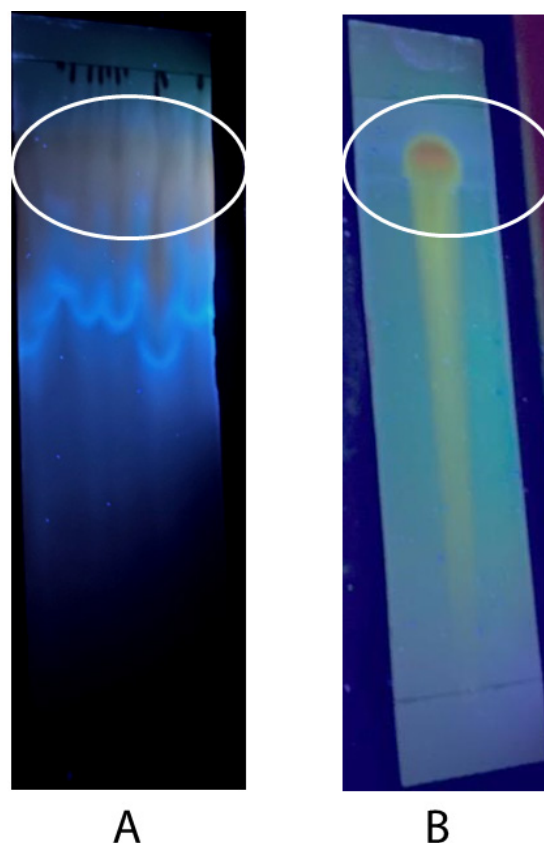


Figure 1. TLC results with chloroform: methanol: water (65:25:4). (A) betel fruit thick extract; (B) quercetin

weighed as much as 1.5 g; 3 g; and 6 g, then each was added with 10 ml of distilled water to obtain a concentration of 15%, 30% and 60%. For the negative control solution, sterile distilled water was used. Additionally, as a positive control, paper discs containing the antibiotic chloramphenicol at a concentration of 30 µg/disk was used. *Pseudomonas aeruginosa* ATCC 27833 from pure cultures were taken as much as 1 ose and then grown or inoculated by scratching on slant nutrient agar. Bacterial culture in each agar slant was incubated at 37°C for 24 hours. The rejuvenation culture was taken 1 ose in nutrient agar medium then suspended in 0.9% sodium chloride and shaken.

A total of 100 mL of liquid nutrient agar was mixed with 3 mL of bacterial suspension and then put into a petri dish of 30 mL each and allowed to solidify. Then, using sterile tweezers, each paper disc that has been dripped with the test sample was aseptically placed on the surface of solid nutrient agar. Then the petri dish was

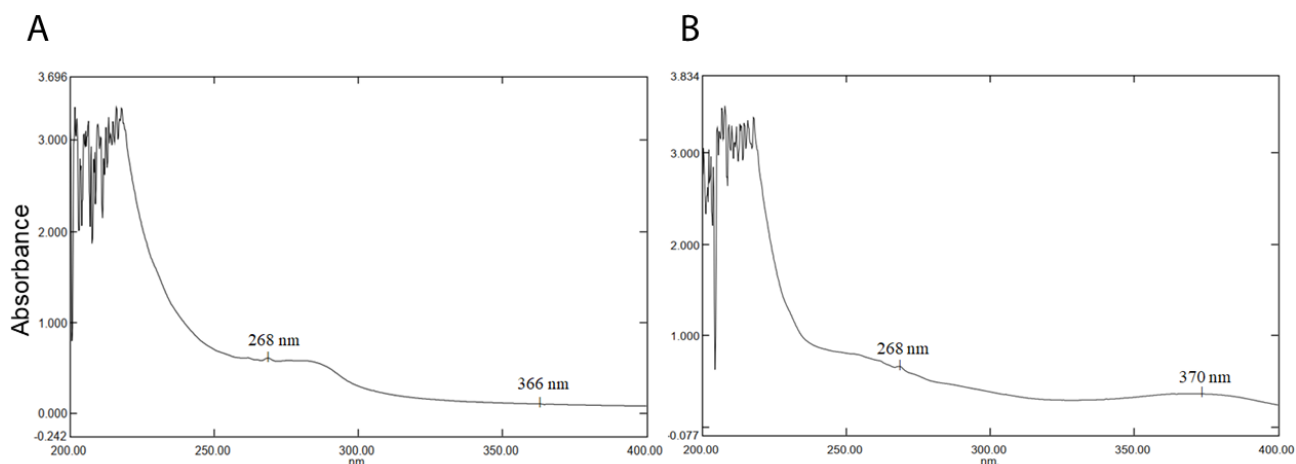


Figure 2. UV-Vis spectrophotometry analysis. (A) betel fruit flavonoid isolation; (B) quercetin

incubated at 37°C for 1x24 hours. The clear zone formed was observed and the diameter of the inhibition zone was measured using a caliper.

Antibacterial activity test of flavonoid compounds

A total of 100 mL Mueller Hinton agar (MHA) was sterilized and mixed with 3 mL bacterial suspension before being poured into 30 mL petri dishes and allowed to solidify. Each paper disc was dripped with flavonoid samples with concentrations of 15%, 30% and 60% aseptically using sterile tweezers, then placed on the solid medium. The petri dish was then incubated for 1x24 hours at 37°C. The clear zone formed was observed, and the inhibition zone's diameter was determined using a caliper.

Data analysis

The graphs were generated by using GrapPad Prism 8. The obtained data were subjected to the One-Way ANOVA statistical test and Holm-Sidak multiple comparisons test. $p < 0.05$ was considered significant.

Results

Isolation of flavonoid compounds from betel fruit extract by thin layer chromatography

A thick extract of 69.27 g (yield 16.49%) was obtained from the extraction process using the

maceration method. Figure 1 showed that betel fruit extract exhibits a brownish-colored stain when exposed to UV 366 light, whereas quercetin exhibits the same color, indicating the presence of flavonoids. The Rf values obtained were 0.86 and 0.875 for betel fruit extract and quercetin, respectively.

Analysis of isolates with UV-Vis spectrophotometer

UV-Vis spectrophotometer analysis in visible spectrum, two bands were presented at a wavelength of 366 nm and at a wavelength of 268 nm (Figure 2A). The results obtained have the same absorption range as quercetin (Figure 2B). The first band appeared in the range 350-385 nm, which is 370 nm, and the second band appeared in the range 250-280 nm, which is 268 nm. This further establishes the presence of flavonoids in betel fruit extract. The difference in the wavelength of the band in betel fruit extract and quercetin can be influenced by several factors, such as type of solvent, the pH of the solution, the concentration of the solution, the thickness of the solution, and the width of the light gap.

Antibacterial activity of betel fruit extract and TLC-isolates

As shown in Figure 3A, each concentration of betel fruit extract was able to inhibit the growth of *P. aeruginosa*. Concentration of 15% resulted

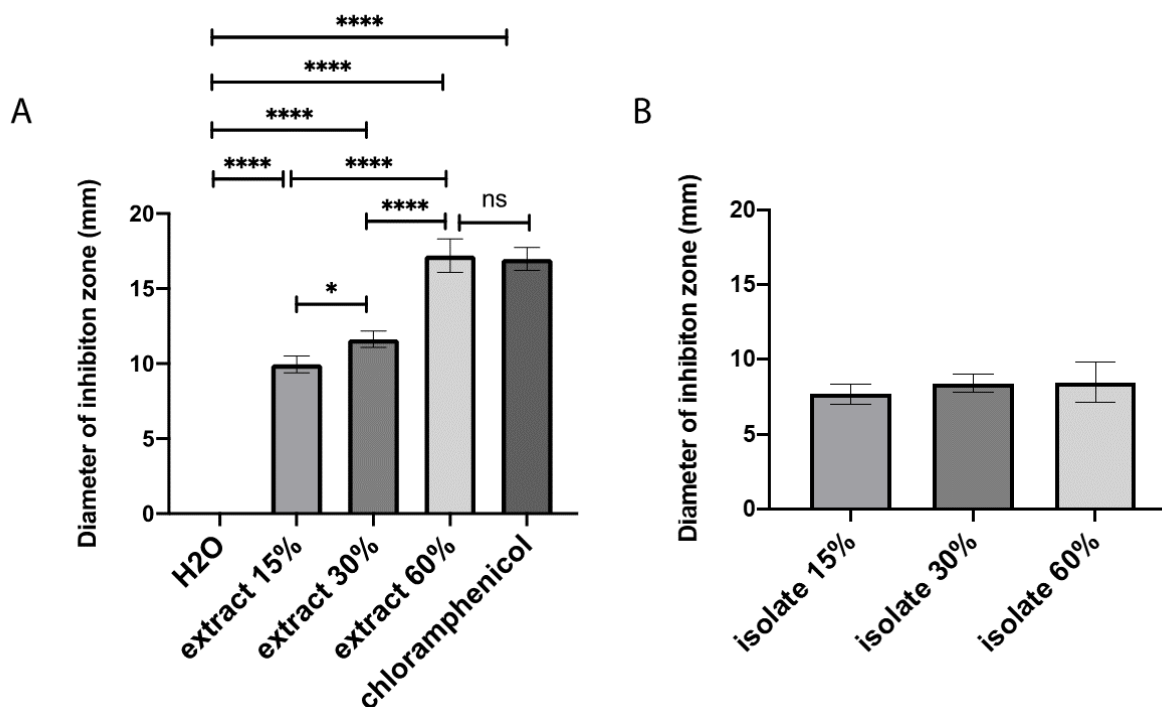


Figure 3. Diameter of inhibition zones as a result of antibacterial activity. (A) betel fruit extract; (B) flavonoid isolated from betel fruit extract

in an inhibition zone diameter of 9.95 ± 0.550 mm (a moderate antibacterial activity), while a concentration of 30% and 60% resulted in an inhibition zone with a diameter of 11.63 ± 0.551 mm and 17.20 ± 1.100 mm, respectively (both have a strong category of antibacterial activity). The negative control did not have an inhibitory zone, while the chloramphenicol (a positive control) had an inhibition zone with diameter 16.97 ± 0.777 mm (a strong antibacterial activity). Interestingly, the extract concentration of 60% equal to chloramphenicol in inhibiting bacterial growth.

The results of antibacterial testing using thin layer chromatography with brown stains (flavonoids from betel fruit extract) are shown in Figure 3B. The results of the isolation from thin layer chromatography were divided into three concentrations, namely 15%, 30% and 60%, and produced an inhibitory zone of 7.68 mm; 8.4 mm, and 8.47 mm (a medium antibacterial activity). According to the results, this activity is below the extract activity, suggesting not only flavonoid that have contribution to antibacterial activity.

Discussion

The results of UV-Vis spectrophotometry analysis of isolate showed absorption at wavelengths of 366 nm and 268 nm, suggesting that the isolate was positive for flavonoids. The absorption spectrum of flavonols has a wavelength of 350-385 nm in the first band and the second band at a wavelength of 250-280 nm [8].

The results of testing the antibacterial activity of betel fruit extract showed that the higher the concentration the stronger the inhibitory activity, this is in line with betel leaf extract research results [7, 11]. The bacterial inhibitory is categorized as very strong (>20 mm), strong (10-20 mm), moderate (5-10 mm), weak (<5 mm) [9]. Based on this category, the betel fruit extract concentration of 15% which means it has a moderate category of antibacterial activity. While extracts with concentrations of 30% and 60% which means that it has a strong category of antibacterial activity. Meanwhile the positive control chloramphenicol has strong antibacterial activity.

The formation of the inhibition zone was caused by the presence of flavonoid secondary metabolite

compounds contained in the isolate which acted as antibacterial. The difference in the diameter of the inhibition zone formed in the antibacterial activity test between flavonoid isolates and betel fruit extract could be attributed to betel fruit containing several other antibacterial compounds. The number of antibacterial active substances contained in the isolate can cause a difference in the inhibition zone; the more antibacterial compounds in the isolate, the better the isolate works in inhibiting bacterial growth.

In addition to flavonoids, it is possible that other chemical compounds as saponins, polyphenols and essential oils contained in betel extract can contribute to inhibiting the growth of bacteria. Flavonoids can interfere with the permeability of bacterial cells, by forming protein complexes that disrupt the integrity of the bacterial cell membrane [10]. Saponins can increase the permeability of cell membranes so that they can cause lysis or rupture of bacterial cell membranes [11]. Polyphenols are able to damage cell membranes, denature proteins, and inactivate enzymes so that bacterial cell walls will experience a decrease in permeability which can interfere with the exchange of substances needed for metabolism [12]. The antibacterial activity of green betel leaf essential oil is caused by the presence of phenolic compounds and their derivatives that can denature bacterial cell proteins. Based on this study, it was found that the ethanol extract of betel fruits has antibacterial activity.

Conclusion

The betel fruit extract showed moderate to strong antibacterial activity against *Pseudomonas aeruginosa*.

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Author contribution

Conceptualization, A.H.; Methodology, A.H.; Investigation, O.S.D; Writing – Original Draft, A.H.; Writing – Review & Editing, T.E.L; Supervision, F. All authors have read and agreed to this manuscript.

Declaration of interest

The authors declare no conflict of interest.

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