Antimicrobial Activity of *Piper ribesoides* Root Extract *Against Staphylococcus aureus*

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Abstract

The methanol extract of *Piper ribesoides* Wall, was evaluated for potential antimicrobial activity against *Staphylococcus aureus* (Rosenbach). The methanol extract of *P. ribesoides* root was effective on *S. aureus*. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values of the methanol extract of *P. ribesoides* were 3.125 mg/ml and 6.250 mg/ml, respectively. Apart from the antibacterial effects, imaging using scanning electron microscopy (SEM) was done to determine the major alterations in the microstructure of the *S. aureus*. The main abnormalities noted via SEM studies were alterations in morphology and complete collapse of the bacterial cells after 36 h of exposure to the extract. The effect of the extract on the growth profile of the bacteria was also examined. The extract changed the normal growth profile of *S. aureus*, thus confirming the bactericidal effect of the extract on *S. aureus*.

Key words: Antibacterial activities, *P. ribesoides* , SEM, *Staphylococcus aureus*

INTRODUCTION

The Piperaceae (pepper) family contains approximately 2,000 species, which are widely grown and commonly used in tropical regions as medicines, spice, and condiments in regional cuisine [1-2]. Malaysia is endowed naturally with a very rich plant life. *Piper ribesoides* Wall (Piperaceae) widely grown throughout Malaysia has been locally known as Sirih rimba. These *Piper* species have been traditionally used in Malaysia for food and medicinal purposes. *P. ribesoides* is reported as good remedy used for treatment of diabetes and poultice to reduce swellings [3]. *P. ribesoides* also commonly used in folk medicine as a carminative [4].

Staphylococcus aureus (Rosenbach) is one of the most important pathogens that cause suppuration, abscess formation, a variety of pyogenic infection, and even fatal septicemia in human beings. *S. aureus*, which can induce bacteremia (associated with 80% mortality in the preantibiotic era), proved to be susceptible to the earliest antimicrobial substance; however, as antibiotic use increased, staphylococcal resistance rapidly developed [5-6]. Hence, new prototype antimicrobial agents are needed to address this situation. The antimicrobial compound from plants may inhibit microbial growth by different mechanism than those presently used antimicrobial agents and may have significant clinical value in treatment of resistant microbe [7]. Because the medicinal plant extract does not exhibit the harsh side effects and high cost of pharmaceuticals, it is becoming the alternative health choice for the general public.

This encouraged us to evaluate *P. ribesoides* as a source of antimicrobial agent against *S. aureus,* based on their ethnomedical use. The current study was carried out to determine the antimicrobial activity of the crude extract of *P. ribesoides* root against *S. aureus*.

MATERIALS AND METHODS

Plant material

P. ribesoides root was collected from various areas in Peninsular Malaysia, in December 2003 and authenticated by the botanist of the School of Biological Sciences at Universiti Sains Malaysia

Microorganism

S. aureus (ATCC 25923) was used as the test organism and was obtained from the laboratory stock culture. The bacterium was cultured on nutrient agar slants at 37 ^{O}C for 18 h. The stock culture was maintained on nutrient agar slants at 4 ^{O}C.

Antimicrobial activity

The antimicrobial activity of the crude extract was determined following the method described by NCCLS with slight modifications [8].

Disk diffusion technique

The test microbes were removed aseptically with an inoculating loop and transferred to a test tube containing 5.0mL of sterile distilled water. Sufficient inoculum's was added until the turbidity equaled 0.5 McFarland (10^{8}CFU/mL) standards (bioMerieux, Marcy Petoile, France). One milliliter of the test tube suspension was added to the 15–20mL of nutrient agar before setting aside the seeded agar plate (9 cm in diameter) to solidify for 15 min. Three Whatman filter paper no. 1 disks of 6-mm diameter were used to screen the antimicrobial activity. Each sterile disk was impregnated with 20 µL of the extract

(corresponding with 10 mg/mL of crude extract), Streptomycin (10µg/mL), as positive control), and methanol (as negative control), before it was placed on the surface of the seeded plates. The plates were incubated at 37 ^{O}C overnight and examined for zones of growth inhibition.

Determination of minimum inhibitory concentrations (MIC)

A 16-h culture was diluted with a sterile physiologic saline solution [PS; 0.85% (w/v) sodium chloride] with reference to the 0.5 McFarland standards to achieve inoculums of approximately 10^6 colony forming units (CFU) per milliliter. A serial dilution was carried out to give final concentrations between 1.56 and 200.00 µg crude extract per milliliter. The tubes were inoculated with 20 µL of the bacterial suspension per milliliter nutrient broth, homogenized, and incubated at 37 ^{O}C. The minimum inhibition concentration (MIC) value was determined as the lowest concentration of the crude extract in the broth medium that inhibited the visible growth of the test microorganism.

Determination of minimum bactericidal concentrations (MBC)

MBC was determined by sub culturing the test dilution on to a fresh drug-free solid nutrient agar medium and incubating further for 18-24 h. The highest dilution that yielded no single bacterial colony on a solid medium was taken as MBC.

Scanning electron microscope observations

Scanning electron microscope observations were carried out on *S. aureus* cells. One milliliter of the *S. aureus* cells suspension at the concentration of $1x10^6$ cells per milliliter was inoculated on a nutrient agar plate and then incubated at 37 ^{O}C for 12 h. The extract (2 mL), at the concentration of 3.125 mg per mL, was then dropped onto the inoculated agar and was further incubated for another 36 h at the same incubation temperature. A 10% DMSO-treated culture was used as a control. A small block of bacteria containing agar was withdrawn from the inoculated plate at 0 and 36 h and fixed for scanning [9]. The small agar pieces were fixed in 3% glutaraldehyde buffered with 0.1M sodium phosphate buffer (pH 7.2) for an hour at room temperature and then washed four times in sodium phosphate buffer, and post-fixed in 1% osmium tetroxide in the same buffer for an hour then washed four times in the same buffer. They were then dehydrated in a graded alcohol series. The last stages of dehydration were performed with propylene oxide (CH_3CHCH_2O). The specimens were then dried in the incubator at 30^{o}C overnight. The dried specimens were mounted onto stubs by double-sided carbon tape. The specimens were coated with a thin layer of gold by a Polaron SC 502 sputter coater, and were examined in the Scanning Electron Microscope.

Growth profile of *S. aereus* in the presence of the *P. ribesoides* root extract

In order to assess the bactericidal effect of the crude extract with MIC and MBC concentration over time, a growth profile curve was plotted. A 16-h culture was harvested by centrifugation, washed twice with PS, and resuspended in PS. The suspension was adjusted using the McFarland standard and was then further diluted in PS to achieve approximately 10^7 CFU/mL. The crude extract was added to aliquots of 25mL Mueller-Hinton broth (MHB) in 50mL Erlenmeyer flasks in a shaking water bath at 37 ^{O}C in an amount that would achieve a concentration of 0 (control) and 3.125 mg/mL (MIC concentration) after the addition of the inoculums. Later, a solution of 1mL inoculums was added to all Erlenmeyer flasks. Immediately after the addition of the inoculums, a 1mL portion was removed from each Erlenmeyer flask and the growth of *S. aereus* was monitored by measuring the optical density (OD) with a UV-spectrophotometer (UV-9100, Ruili Co., China) at 540 nm. The growth of *S. aereus* was measured every 4 h for 48 h using the above method.

RESULTS

Disk diffusion technique

The results of antimicrobial activity of the crude extract tested by the disk diffusion method against *S. aereus* are given in Table 1. The extract exhibited a favorable activity against the bacteria tested. The zone of clearance produced by the commercial antibiotic (Streptomycin) disk was larger than those produced by the extract disk (Figure 1). The blind control (methanol) did not inhibit the microorganisms tested, *S. aereus*.

Determination of MIC and MBC

The antimicrobial activity of the extract and their potency were quantitatively assessed by determining the MIC and MBC, respectively, as given in Table 2. The MIC and MBC value of *P. ribesoides* was 3.125 mg/ml and 6.25 mg/ml, respectively. The MIC and MBC value of Streptomycin against *S. aereus* were 0.065 and 0.104 µg/ml, respectively.

Table 1. Antimicrobial activity (Zone of inhibition) of *Piper ribesoides* root extract

Microorganism	Zone of inhibition (mm)[a]		
	Crude extract	Streptomycin	Methanol
Staphylococcus aureus	10.21	20.72	0.00

[a] The values (average of triplicate) are diameter of zone of inhibition at 10 mg/mL crude extract and 10µg/mL Streptomycin.

Table 2. Antimicrobial activity (MIC[a] and MBC[b]) of *Piper ribesoides* root extract

Microorganism	Methanol extract (mg/mL)		Streptomycin (µg/mL)	
	MIC	MBC	MIC	MBC
Staphylococcus aureus	3.125	6.25	0.065	0.104

[a] MIC, minimum inhibitory concentration

[a] MBC, minimum bactericidal concentration.

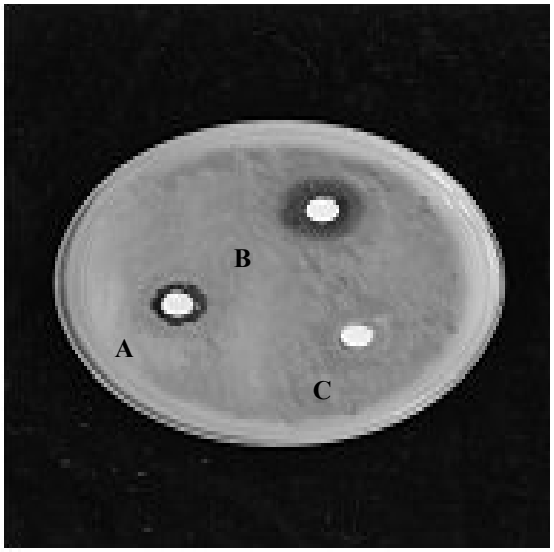


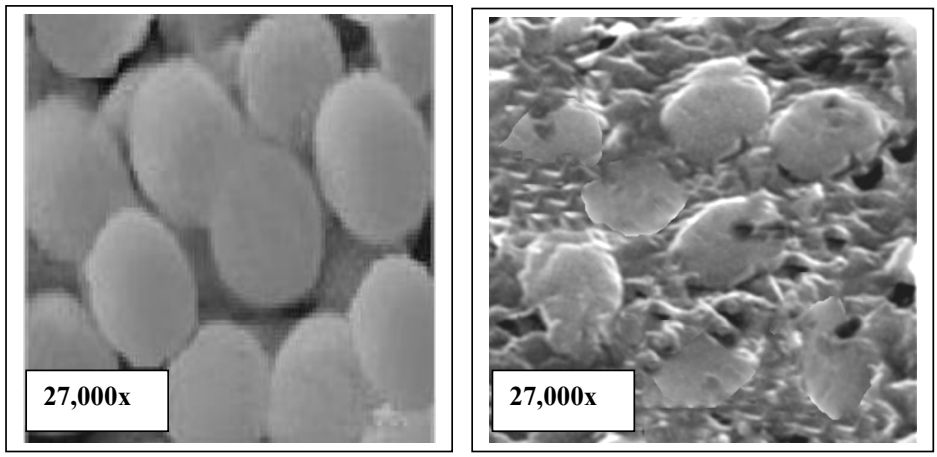
Figure 1. Agar plate showing inhibitory zones: (A) crude extract (10mg/mL), (B) Streptomycin (10µg/mL) and (C) methanol.

Scanning electron microscope observation

Cells treated with MIC (3.125mg/ml) concentration of the crude extract underwent considerable morphologic alterations in comparison with the control when observed by SEM (Figure 2). Figure 2 shows the SEM photomicrographs of the untreated and extract treated cells of *S. aereus* at 36-h time of exposure. Untreated cells (Figure 2A) appear as coccus and smooth cells. After 36 h of exposure (Figure 2B), complete collapsed cells were seen. It is believed that at this stage, the cells had lost their metabolic functions completely.

Growth profile of *S. aereus* in the presence of the *P. ribesoides* root extract

The growth profile of *S. aereus* in MHB at MIC and 0 (control) concentrations is shown in Figure 3. The MIC (3.125mg/ml) concentration altered the normal growth profile for *S. aereus* compared with the control (0 concentrations). This finding confirmed the bactericidal effect of the extract on *S. aereus* at the MIC concentration.



(A) **(B)**

Figure 2. Scanning electron microscope photomicrograph of the untreated (A) and extract-treated (B) cells of *Staphylococcus aureus.*

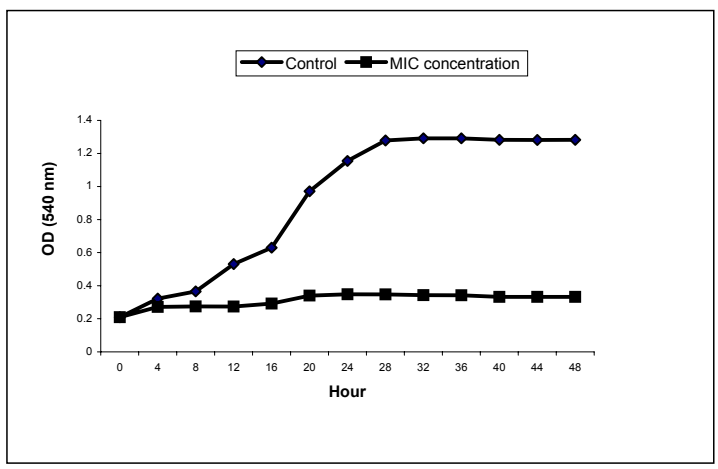


Figure 3. Growth profile for *Staphylococcus aureus* in Mueller-Hinton broth with 0 (Control) and 3.125mg/mL (MIC concentration) of the crude methanol extract of the *Piper ribesoides* root.

DISCUSSION

The use of higher plants and preparations made from them to treat infections is a longstanding practice in a large part of the world population, especially in developing countries, where there is dependence on traditional medicine for a variety of ailments [10]. Interest in plants with antimicrobial properties has revived as a consequence of current problems associated with the use of antibiotics [11-12]. The present studies aimed at the investigation of a Malaysian medicinal plant with antimicrobial activity against *S. aereus*.

P. ribesoides is used as medicinal plant in various localities of Malaysia. The purpose of this study was to assess the antimicrobial potential of root extract of *P. ribesoides* against *S. aereus*. The results obtained from the diffusion method, showed that the extract exhibit a favorable antimicrobial activity against *S. aereus*. The MIC and MBC values obtained for the extracts against the *S. aereus* also support the findings of the diffusion method. The area of concern is that MIC values of the plant extracts obtained in this study were lower than the MBC values, suggesting that the plant extracts were bacteriostatic at lower concentration and bactericidal at higher concentration. This was further confirmed by the alteration of the normal growth profile of *S. aereus* by the extract. Furthermore, the SEM study showed that the extract could completely collapse the bacterial cells and inhibit the growth of the *S. aereus*, which can cause infection in human being.

Hence, *S. aereus* infection could be treated by the extract, as the MIC for this bacterium was found to be only 3.125 mg/ml. In addition, it may be used as an antibacterial agent in known dosages, especially in rural communities where conventional drugs are unaffordable or unavailable and the health facilities are inaccessible. The results presented here indicate that the natural products analyzed seem to be a good choice for the development of new strategies to treat staphylococcal infections, including those caused by methicillin-resistant *S. aureus*.

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