Antibacterial Spectrum of Ethanol Extract of Indonesian Red Piper Betel Leaf (*Piper crocatum* Ruiz & Pav) Against Staphylococcus species

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Abstract

Purpose: The present study was performed to evaluate the antibacterial spectrum of red piper betel leaf ethanol extract from Indonesia against Staphylococcus species as follows: *Staphylococcus aureus* ATCC 11784, *S. xylosus* ATCC 3342, *S. warneri* ATCC 3340, *S. haemolyticus* ATCC 3741, dan *S. epidermidis* ATCC 13228.

Methods: The phytochemical screening of the ethanol extract of the red piper betel leaf was performed using standard procedures. The antibacterial activity test was assessed using the agar diffusion method. The extracts were tested for determining minimum inhibitory concentration value (MIC) using macrodilution method.

Results: Phytochemical screening results indicated that ethanol extracts of the red piper betel leaves contain flavonoids, tannins, polyphenols, saponins and steroid. Based on its inhibitory diameter, the extract has a broad-spectrum antibacterial of the entire species of Staphylococcus. The strongest antibacterial effect of the extract was against *S. aureus* and the weakest against *S. xylosus*. In a comparative analysis of the extracts with neomycin sulfate, indicated that neomycin sulfate demonstrated greater antibacterial activity than the extract at the same concentration. The results of the MIC value determination as follows: *S. xylosus* ATCC 3342 (2.25-3.06% w/v); *S. warneri* ATCC 3340 (0,56-1.00% w/v); *S. haemolyticus* ATCC 3741 (0.25-0,56% w/v), *S. epidermidis* ATCC 13228 (0.25-0,56% w/v); and *S. aureus* ATCC 11784 (0.25-0,56% w/v).

Conclusions: It can be concluded that ethanol extract of red piper betel leaves produced a broad spectrum antibacterial against Staphylococcus species.

Keywords: red, piper betel, staphylococcus, antibacterial, MIC**.**

Introduction

Staphylococci can cause many forms of infection. S. *aureus* colonize the nasal passage and axillae. *S epidermidis* is a common human skin commensal. Other species of staphylococci are infrequent human commensals. While *S. saprophiticus* causes urinary tract infections, especially in girls. Other species of Staphylococci such as: *S lugdunensis, S haemolyticus, S warneri, S schleiferi, S intermedius*) are infrequent pathogens. Infectious disease caused by Staphylococcus contributes to high rates of infectious diseases and transmitted annually. *Staphylococcus aureus* causes superficial skin lesions (boils, styes) and localized abscesses in other sites [1]. In addition, *Staphylococcus aureus* is a major cause of bacteremia, and *S. aureus* bacteremia is associated with higher morbidity and mortality, compared with bacteremia caused by other pathogens [2]. *S. aureus* causes deep-seated infections, such as osteomyelitis and endocarditis and more serious skin infections (furunculosis). *S aureus* can causes food poisoning by releasing enterotoxins into food. *S. aureus* causes toxic shock syndrome by the release of superantigens into the blood stream [1]. The problem of Staphylococcal infection is particularly serious in many hospitals and its complicated by the fact that a large proportion of hospital strains are antibiotic resistant [3].

Infections acquired outside hospitals can usually be treated with penicillinase-resistant β-lactams. Hospital acquired infection is often caused by antibiotic resistant strains and can only be treated with vancomycin. Multiple antibiotic resistance is increasingly common in *S aureus* and *S epidermidis*. Methicillin resistance is indicative of multiple resistance. Methicillin-resistant *S aureus* (MRSA) causes outbreaks in hospitals and can be epidemic [1]. The resistance patterns of S. aureus isolates from patients in Baghdad, were Levofloxacin (20 %) , Norfloxacin 16 %) , Ofloxacin (18 %), Ciprofloxacin (16 %), Lomofloxacin (14 %) and Nalidixic acid (50 %) [4]. Strains of *S. aureus* have developed resistance to antibiotics, including penicillin, cephalosporins, methicillin, vancomycin, and linezolid [2]. The problem of bacterial Antibiotic resistance has achieved a global dimension, being one of the leading unresolved problems in public health. The relentless evolution of resistance, in the face of a decrease in the development of new antimicrobial agents active against resistant pathogens, has led to an increasing number of cases in which the pathogen is resistant to most, or even all, drugs available for

clinical use [4]. This changing epidemiology of *S. aureus* bacteremia, in combination with the inherent virulence of the pathogen, is driving an urgent need for improved strategies and better antibiotics to prevent and treat *S. aureus* bacteremia and its complications. The burden of *S. aureus* bacteremia, particularly methicillin-resistant *S. aureus* bacteremia, in terms of cost and resource use is high [2].

The discovery of antibiotics from natural can be used as an opportunity to overcome Staphylococcus resistance against antibiotic synthesis. One of the plants that are empirically can cure various kinds of diseases and have antibacterial efficacy is the red piper betel (*Piper crocatum R*uiz & Pav.) [5]. The red piper betel leaves have silver-colored, and when it was ripped then it would be slimy. Its aroma is more fragrant than the betel leaf green and this is one of the advantages that can be utilized as a natural deodorizer. In addition, the red piper betel leaves contain chemicals as follows: polifenolat, alkaloids, saponins, tannins, flavonoids, essential oils (karvakrol, eugenol, hidroksikavikol, kavikol, kavibetol, alilpirokatekol, p-simen, signal, kariofilen, kadimen, estragol terpinen , and phenyl propane) [6]. Those chemical contents can be used as antibacterials [6-7]. Until now, other studies have cited their antibacterial activity of ethanol extract of red betel leaf green and against *S. aureus* either pure strains or clinical isolates [8-11). But its antibacterial against Staphylococcus species has not been studied yet. In this study, the antibacterial spectrum of ethanol extract of red piper betel leaf against several Staphylococcus species has been investigated.

Materials and methods

Materials

Red betel (*Piper crocatum* Ruiz & Pav) leaves were obtained from the experiment garden of Manoko, Lembang, Indonesia. The determination is carried out in the Department of biology, Faculty of mathematics and natural sciences, the University of Padjadjaran, Jatinangor. The tested bacteria used in this study were *Staphylococcus aureus* ATCC 11784, *S. epidermidis* ATCC 13228, *S. haemolyticus* ATCC 3741, *S. warneri* ATCC 3340 and S. *xylosus* ATCC 3342, obtained from Microbiology Laboratory, Padjadjaran University, Bandung, Indonesia. The culture media that were used are *Mueller-Hinton Agar* (MHA-Oxoid) and *Mueller-Hinton Broth* (MHB-Oxoid). The chemicals used are ethanol 70% (Brataco), distilled water, chloroform, ether, anhydrous acetic acid, concentrated sulfuric acid, potassium iodide (Merck), mercury (II) chloride (Merck), bismuth subnitrat (Merck), amyl alcohol, 1% gelatin solution, iron (III) chloride (Merck) , magnesium metal powder (Merck), hydrochloric acid 2 N (Merck) , aqueous ammonia, potassium hydroxide (Merck), vanillin, dimethyl sulfoksida (DMSO-Merck) and neomisin sulfate (PT. cendo).

Preparation of samples

Leaves were washed in tap water and dried, dried leaves were grounded in grinder and powdered leaves were used for preparation of extract [8]. The extraction was done using the maceration method. This method was chosen to prevent damage to the compounds contained in the Red betel leaves by high temperatures. This process was done by soaking the powder of red piper betel simplisia in 70% ethanol during 3x24 hours with the replacement of solvent every 24 h. . The extracts were evaporated using a rotary evaporator at 40-50 ^{O}C, then continued to evaporate on a water bath until dried extract with constant weight was obtained. The extract was stored in a refrigerator at 4 ºC until time of use. The percentage yields (w/w) of the extracts were calculated using the formula below [12] :

(Weight of extract ÷ weight of starting plant material) × 100%

Phytochemical screening

Phytochemical analysis of red Piper betel leaves was carried out for ethanolic extracts to evaluate the presence of secondary metabolites like alkaloids, flavonoids, Quinones, polyphenols, tannins, saponins, steroids, monoterpenoid, triterpenoid, and seskuiterpenoid by using various standard methods [13].

Preparing bacterial suspension

The bacterial suspension was prepared by transferring a loopful of inoculum from slant agar into normal saline (0.9%) under aseptic conditions. The turbidity was adjusted to McFarland turbidity standard tube No. 0.5 (10^8cfu/ml) by adding sterile normal saline [14-15].

Antibacterial Activity Test

Antibacterial activity test of the red piper betel extract was conducted by agar diffusion-well method. The volume of 20 µl standardized bacterial inoculum was uniformly spread using a sterile cotton swab on a sterile MH agar. Four serial extracts dilutions yielded concentrations of 80, 60, 40, and 20%w/v using dimethyl sulfoxide as the solvent. 50µL of each concentration was added to each of the 4 wells (9 mm diameter holes cut in the agar gel). The plates were incubated for 24 h at 37ºC, under aerobic conditions. After incubation, confluent bacterial growth was observed. Inhibition of the bacterial growth was measured in mm. Tests were performed in duplicate [16].

Determination of Minimum Inhibitory Concentration (MIC)

Determination of minimum inhibitory concentration of red *Piper betel* ethanol extract was done using macrodillution method. The bacterial suspension was prepared by transferring a loopful of inoculum into normal saline (0.9%) from the stock culture. The density of each microbial suspension was adjusted to equal that of 10^6cfu/ml (standardized by 0.5 McFarland standard) [17]. Minimum inhibitory concentration (MIC) of red *Piper betel* against Staphylococcus sp. was assessed by serial dilution method. The ethanol extracts of red *Piper betel* leaf were dissolved in DMSO and then diluted with MHB medium until achieved concentration as follows: 0,06%; 0,25%; 0,56%; 1%; 1,56%; 2,25% and 3,06%w/v. The volume of 10 µL standardized cell bacterial suspensions was put into each tested concentration. The tubes then were incubated for 20 h with temperature at 37^{o}C. MIC was determined from the smallest concentration which did not show any turbidity. MHA media in Petri dishes were sub-cultured from tubes without growth and incubated at 37°C for 20 h. The petri dishes were observed macroscopically. The highest dilution that yielded no bacterial colony on a solid medium was taken as MIC [18].

Results and discussion

Yield of the extract

Maceration is a widely used technique in medicinal plants research. Maceration involved soaking plant materials (coarse or powdered) in a stoppered container with a solvent and allowed to stand at room temperature for a period of minimum 3 days with frequent agitation. The processed intended to soften and break the plant's cell wall to release the soluble phytochemicals [19]. From the results of maceration as much as 158.43 grams of crude drugs, obtained ethanol extract condensed as much as 21.35 grams. Therefore, the ethanol leaf extract of *Piper betel* yielded 13,47 %w/w. The characteristic of the extract was a thick, sticky solid, distinctive-smelling, and brownish Green.

Result of phytochemical screening

The results of phytohemical tests of red piper betel leaves extracts were presented in Table 1. In the phytochemical screening, ethanolic extract yielded flavonoids, tannins, polyphenols, saponins and steroid.

Table 1: phytochemical of red piper betel extract

Phytochemical	Result
Alkaloids	+
Flavonoids	+
Tannins	+
Polyphenols	+
Saponins	+
Monoterpenoids	-
Triterpenoids	-
Steroids	+
Quinones	-

Notes: (+) indicates presence; (-) indicates absence

In another study stated that the ethanolic extract of red piper betel obtained from Simpang Balik village (Aceh, Indonesia) contained the same secondary metabolites with our extract and reported that the ethanol extract gave antibacterial effect againt Methicillin Resistant Staphylococcus aureus (MRSA) [20].

Antibacterial Test Results

The results of the antibacterial activity test of red piper betel leaf ethanol extract can be seen in Fig 1. and The average diameter of inhibitory extracts to test bacteria, can be seen in Figure 2.

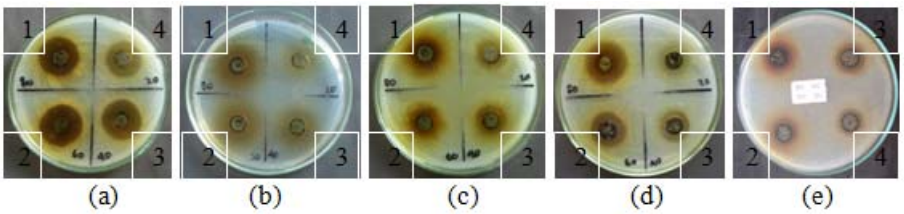

Fig. 1: Antimicrobial activity of ethanolic extract of red piper betel leaves with various concentrations against tested bacteria

Notes: (a)*S. aureus* ATCC 11784; (b) *S. haemolyticus* ATCC 3741; (c) *S. warneri* ATCC 3340; (d) *S. epidermidis* ATCC 13228; (e) S. *xylosus* ATCC 3342; 1= 80%w/v; 2= 60%w/v; 3=40%w/v; 4=20%w/v

Table 2: The everage of inhibition zone diameters

Extract concentration (%w/v)	Inhibitory diameter (mm)±SD				
	S. aureus	*S. haemolyticus*	*S. warneri*	*S. epidermidis*	S. *xylosus*
20	20.35±0.141	16.15±0.212	13.00±0.282	14.95±0.070	13.15±0.070
40	21.60±0.283	17.15±0.212	14.95±0.070	17.40±0.141	14.00±0.000
60	22.35±0.495	18.90±0.141	15.75±0.212	17.95±0.212	14.35±0.070
80	24.85±1.060	19.35±0.070	17.95±0.141	20.50±0.141	15.10±0.141

Based on the data in the table above, the ethanol extract of red betel leaf can inhibit the growth of all species of Staphylococcus. It can be concluded that the extract has a broad-spectrum against staphylococcus species used. The extracts provides the greatest activity against the *S. aureus* ATCC 11784 and the smallest activity against bacteria *S. xylosus* ATCC 3342. Based on the diameter of inhibition zones, all tested concentrations of the extract were categorized as a very active antibacterial (above 11 mm) [21].

Minimum Inhibitory Concentration Determination Result

Minimum inhibitory concentrations (MIC) refer to the lowest concentration of an antimicrobial that will inhibit the visible growth of a microorganism. The result of MIC determination can be seen in Table 3.

Table 3: Minimum inhibitory concentration value

Extract concentration (%w/v)	**Tested bacteria**				
	S. aureus	***S. epidermidis***	***S. haemolyticus***	**S. *xylosus***	***S. warneri***
3.06	-	-	-	-	-
2.25	-	-	-	+	-
1.56	-	-	-	+	-
1.00	+	-	-	+	-
0.56	+	+	+	+	+
0.25	+	+	+	+	+
0.06	+	+	+	+	+

Note : (+) = no growth inhibition; (-) = minimal growth inhibition

The minimum inhibitory concentration of the extract was determined by visual observations and it was observed that the MIC for the bacterial strains was in the various range, as follows: *S. xylosus* ATCC 3342 (2.25-3.06% w/v); *S. warneri* ATCC 3340 (0,56-1.00% w/v); *S. haemolyticus* ATCC 3741 (0.25-0,56% w/v), *S. epidermidis* ATCC 13228 (0.25-0,56% w/v); and *S. aureus* ATCC 11784 (0.25-0,56% w/v). These MIC value indicating that the extracts are bacteriostatics at lower concentrations than MIC value.

Conclusion

It can be concluded that ethanol extract of red piper betel leaves produced a broad-spectrum antibacterial against Staphylococcus species.

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Conflicts of interest

The authors confirm that this article content has no conflicts of interest.

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