Antibacterial activity of red betel (*Piper crocatum*) leaf methanolic extracts aginst methicillin resistant *Staphylococcus aureus*

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Abstract. Red betel (Piper crocatum) is one of traditional medicine that has antibacterial activity. This research was conducted to determine the antibacterial activity of the methanol extract of red betle leaves against growth of Methicillin Resistant *Staphylococcus aureus* (MRSA). This research used Completely Randomized Design (CRD) method with 5 treatment groups consist of red betle leaves extract with concentration 150 mg/mL, 300 mg/mL, 450 mg/mL, 600 mg/mL, and negative control (sterile water) with 4 times repetition. The methanol extract of red betle leaves was obtained by maceration technique using methanol 96% as solvent. Antibacterial activity test conducted with Kirby Bauer disc diffusion method. Data of this research were analyzed by ANOVA followed by Duncan Test. The results showed that red betel leaf extracts in concentration 150 mg/mL, 300 mg/mL, 450 mg/mL, 600 mg/mL provide average inhibition zone respectively 9,0 mm, 11,2 mm, 13,6 mm and 15,7 mm. The result showed that red betel leaf extracts in tested concentration showed antibacterial activity against MRSA. The higher concentration of extract, the greater inhibition area was formed.
Keywords: Red betel methanolic extract, MRSA, antibacterial activity

Introduction

MRSA bacterium was first found in the United States in 1968. It is included in *emerging infectious pathogen*, and can spread through contacts between the infected medical workers and patients in hospitals. In the last few decades, the incidents of MRSA infection have kept increasing in many parts of the world (Arnita, 2007). A research conducted in 9 countries in 1974 showed that 2% of the overall *S. aureus* infection cases were MRSA. This figure went up to 22% in 1995 and 85% in 2008, and has kept rocketing eversince (Sheen, 2010). A research conducted by Maulana (2011) proved that *S. aureus* can be isolated from the sputum of chronic coughing patients in RSUDZA Banda Aceh (9 isolates out of 100 speciments (9%). From those 9 isolates, 5 isolates (5%) were identified as MRSA. MRSA mortality rate reached 50% in the period of 1999-2005 in all American hospitals which far exceeded the increase of mortality rate of *Methicillin-Susceptible Staphylococcus aureus* (MSSA) which was only 18% in the same period (Klein *et al*., 2007).

An increased bacterial resistance to the existing antibiotics should be balanced with the discovery of new medication. An alternative product which is more potent and low-cost, with fewer side effects and continuous large number availability needs to be discovered to overcome antibiotic resistance. One way of doing this is by using a antibacterial active substance contained in the medicinal plants. One of medicinal plants commonly used by people is betel plant. Betel plant has been known as an antiseptic since 600 BC. The types of betel generally used as medicine in Indonesia are green betel (*Piper betel*) and black betel. However, there is another type gaining popularity i.e. red betel. Red betel is the type of betel often used as ornamental plant in the 1990s, but now it has shifted to medicinal function since its introduction by Sudewo (2010), a medicinal plants producer in Blunyahrejo.

Antibacterial capacity of red betel leaves has proven effective in combating pathogenic bacteria such as *Staphylococcus aureus* and *Escherichia coli (*Juliantina *et al*., 2009) Ethanolic extract of red betel leaves also shows antibacterial activities against *Pseudomonas aeruginosa* and *Shigella dysenteriae* (Noor, 2011)

This research is conducted to test the antibacterial activity of the red betel leave extract against the growth of MRSA. Red betel leave extract is used with methanolic dilution in this research. Methanol is a polar dilution so it can attract all polar active metabolic substances such as tannin and flavonoid, thus increasing the antibacterial capacity of the extract (Pradipta, 2007; Wresdiyati (2003).

Material and Methods

This reaserach is a laboratory experiment using Completely Randomized Design pattern with 4 repetitions. The design of this research consists of 5 treatment groups namely extract concentration of 150 mg/mL, 300 mg/mL, 450 mg/mL, 600 mg/mL and negative control (sterile water).

Preparation of Red Betel Leaves

The red betel leaves were obtained from Simpang Balik village, Wih Pesam sub-district, Bener Meriah. Geographically, Bener Meriah is located in a tropical region with average humidity of 75%, at the elevation of 100-1200 m above sea level. The average annual precipitation ranges from 1000 to 2000 mm. The soil texture and drainage system are good and the average temperature ranges from 26^0C to 27^0C. The leaves used were 500 gr fresh red betel leaves. The leaves were picked in the morning to allow a proper drying process. Using clean cutting tools, the picking started from the third leaf below the buds to the ninth leaf in every sprig. This method was applied due to the leaves' high content of active substance with strong scent and bitter flavor(Sudewo, 2010).

Red Betel Leaf Herbarium Test

Herbarium test was performed to identify and ensure the family and species of red betel plant to be examined based on its morphological characteristics. The result showed that the red betel used belonged to Piperaceae family and of *Piper crocatum* species.

The Formulation of Red Betel Leave Methanolic extract

500 gram of fresh red betel leaves was soaked in water for 30 minutes and then washed, clean, and drained. The leaves were then airdried for 7×24 hours in room temperature with no direct sunlight, after which 173 gram of dried red betel leaves was obtained. The dried leaves were ground to a powdery consistency. The powder was soaked in 2 litres of 96% methanol dilution for 3×24 hours, changing the dilution every 24 hours, and then strained using filter paper. The filtrate resulted from the straining was vaporized using *rotary vacum evaporator* at 40^0C to get a concentrated extract of red betel leaves (Safithri and Fahma, 2005). This extract was then divided into several testing concentrations.

Phytochemical Screening

Flavonoid Testing. 0.05 gram of red betel extract and 96% methanol were heated for 5 minutes. H_2SO_4 was then added to the filtrate. Flavonoid compound was indicated by the red color caused by the addition of H_2SO_4. *Alkaloid Testing*. 5 ml chloroform and ammoniac was added to 0.05 gram of red betel extract.The chloroform fraction was then separated and acidified using 1 drop of H_2SO_4 2M. The acid fraction was divided into three cylinders, each of which was added Dragendorf, Meyer and Wagner reagent. The presence of alkaloid was indicated by the forming of white sediment in the Meyer reagent, red sediment in Dragendorf reagent, and brown sediment in Wagner reagent. *Tannin Testing*. 0.05 gram of red betel extract was added 12.5 mL of sterile water, and then brought to a boil for 5 menit. The solution was filtered and 5 drops of $FeCl_3$ 1% (b/v) was added to the filtrate. The formed color of dark blue or greenish black showed the presence of tannin. *Steroid and Triterpenoid Testing*. 0.05 gram of red betel extract was added 12.5 mL of ethanol 30% and heated for 5 minutes and strained. Its filtrate was vaporized and ether was added. Lieberman Buchard reagent (3 drops of acetate anhydride and 1drop of concentrated H_2SO_4). The formed red or purple color indicated the presence of triterpenoid and green showed steroid. *Saponin Testing*. 0.05 gram of red betel extract was added 5 mL of sterile water and heated for 5 minutes. It was then shaken for 5 minutes. Saponin test showed a positive result if foam of at least 1 cm high was formed and stayed stable after being set aside for 10 minutes.

Reidentification of Tested Bacterium

The MRSA isolate was obtained from the *stock culture* isolated from the pus of diabetic ulcer patients at the Zainal Abidin Hospital's Microbiology Laboratory. Next, reidentification of the tested bacterium was performed through macroscopic and microscopic (Gram Staining) examination, catalase test, coagulation test, as well as test of resistance to *Methicillin* (Brown, 2005).

Macroscopic Examination. The bacterium grown in the inoculation medium was macroscopically identified. The examination comprised shape, surface, border and color of the

colony growing in the *Nutrient Agar* medium. *Microscopic Examination*. This examination was performed through Gram staining.

Catalase Test. Bacterium culture was smeared on the glass object evenly and then given 1 or 2 drops hydrogen peroxide solution (H_2O_2) 3%. The catalase activities on the microbes can be identified by examining whether or not the gas bubbles were formed. *Coagulation Test*. Bacterium culture was obtained using sterilized ose and then inoculated into a cylinder containing 0.5 ml of human plasma. Positive coagulation test was indicated by the forming of coagulated plasma in the cylinder afte being incuated for 24 hours at 37^0C. *Test of Resistance to Methicillin*. Inocula was gradually suspended into sterile NaCl 0,9% on the wall of the test tube to produce a fine bacterial suspension. The bacterium was inoculated to a MHA medium by dipping a sterilized cotton bud into the inocula. The cotton bud was applied onto the surrounding of the agar surface. The inocula was left to dry for a few minutes at room temperature. Resistance test was performed by using oxacillin 5 µg antibiotic disc, which was incubated at 35^0C. After that, the diameter of the formed inhibition zone was measured using a vernier caliper and the result was stated in milimeter (mm). Should the inhibition be ≤ 10 mm, it is indicated that the *S. aureus* strain is resistant to methicillin/oxacillin.

Antibacterial Activity Test of Red Betel Leaf Methanolic extract against MRSA

This test applied the Kirby-Bauer disc diffusion method using Mueller Hinton Agar (MHA) medium, in accordance with the procedures of *European Committee on Antimicrobial Susceptibility Testing*/EUCAST (2009). Inocula was suspended gradually into sterile NaCl 0.9% on the wall of the test tube to produce a fine bacterial suspension. Next, bacterial turbidity was measured using spectrophotometer at the wavelength of 625 nm and absorbency of 0.08-0.1. The bacterium was inoculated to a MHA medium by dipping a sterilized cotton bud into the inocula. This testing of red betel leaf methanolic extract used an empty disc of 6 mm in diameter. The empty disc was soaked in the extract solution of different concentrations for 30 minutes. It was then set on an inoculated petri dish using sterilized tweezers. The negative control used an empty disc soaked in sterile water. Each treatment was repeated 4 times. The next step was incubation for 24 hours at 37^0C.

Results and Discussion

Identification and Extraction of Red Betel Leaves

The identification result of red betel leaves based on the herbarium test performed at the Herbarium Laboratory of Biologi Department, Math and Science Faculty, Syiah Kuala University, showed that the fresh red betel leaves collected from Wih Pesam sub-district, Bener Meriah, were from red betel plant (*Piper crocatum*). 500 gram of fresh leaves was airdried to produce 173 gram of dried red betel leaves. After being macerated in 2 litres of 96% methanolic dilution for 3×24 hours, it produced 26.1 gram red betel leaf methanolic extract of blackish green color and thick consistency. The extract was then divided into concentrations of 150 mg/mL, 300 mg/mL, 450 mg/mL, 600 mg/mL.

Phytochemical Screening

The result of the red betel leaf methanolic extracts phytochemical screening showed the presence of alkaloid, flavonoid, saponin, triterpenoid, and tannin compounds (Table 1).

Table 1. Result of phytochemical screening of red betel leaf methanolic extracts

No	Compound		Result*
1	Alkaloid	Mayer Reagent	-
		Dragendorf Reagent	+
		Wagner Reagent	-
2	Flavonoid		+
3	Saponin		+
4	Triterpenoid		+
5	Steroid		-
6	Tannin		+

*(+) = containing tested compound; (-) = not containing tested compound.

Flavonoid contained in red betel leaves comprises *flavonol, flavanon, isoflavon*, *auron*, *cathechin, antocyanidines dan chalcones (*Arishandy, 2010; Lenny, 2006). The total flavonoid concentrations of the extract of fresh red betel leaf boiling water, the extract of commercial red betel leaf boiling water, and the methanolic extract of fresh red betel leaves are 0.57% b/b, 0.54% b/b dan 1.89% b/b (Wirdani, 2008). Therefore, the methanolic dilution produced higher flavonoid compared to the extract of red betel leaf boiled water which constitutes the most common use of the leaves by Indonesian communities. Flavonoid content was also found in the red betel leaf ethanolic extract. This is possible because flavonoid is a polar compound and only dilutes in polar dilutions such as ethanol/methanol (Hasbi 2011). A number of other active substances such as tannin, saponin and alkaloid were also found in the extract used in the research, but not in the red betel leaf ethanolic extracts (Hasbi, 2011). The difference can be caused by a lot of factors such as organ differences, cells, age of plants, seasons, and geographical location of the plants (Bohm, 2009; Shahidi and Ho, 2000). In addition, the difference was also the result of the characteristics of a number of active substances against temperatures or a very low content, thus unable to be detected in the qualitative phytochemical screening.

Reidentification Result of Methicillin-Resistant *Staphylococcus aureus*

The result of macroscopic observation on the *Nutrient Agar* (NA) medium showed that *S. aureus* formed a colony of 0.5 mm in size, of white color, round shape with fine border and even surface. Gram Staining result showed a bacterium in coccus shape, purple color, and forming a small group resembling grapes which indicated Gram positive bacterium. The coagulation test using plasma in tubes produces a positive result namely the forming of plasma coagulation. From the catalase test, a positive result was obtained which was indicated by the forming of gas bubbles. The result of resistance test of *S. aureus* to *methicillin* showed that *S. aureus* isolates are resistant to antibiotic *methicillin*. All the above mentioned results show that the tested bacterium was MRSA.

Result of Actibacterial Activity Test of Methanolic Extract of Red Betel Leaf Against MRSA

The antibacterial activity test of red betel leaf methanolic extract against the growth of MRSA at the 15%, 30%, 45% and 60% concentrations resulted in inhibition zones of average diameter of 9.0 mm, 11.2 mm, 13.6 mm and 15.7 mm each. The average diameter of the inhibition zone formed in sterile water was 0 mm. The data obtained was then analyzed using *Analisis of Variance* (ANOVA) to examine the impact of each treatment. The result of ANOVA revealed that there was a significant difference in the antibacterial activity indicated by each concentration of red betel leaf methanolic extracts. Statistical test was then continued with Duncan Test at $p<0,05$.

Table 2. Average diameter of inhibition zone ± deviation standard of red betel leaf methanolic extract against the growth of MRSA. The average value of inhibition zone was followed by different superscripts showing significant differences (P<0,05)

Treatment	Diameter of inhibition zone in each repetition (mm)				Average diameter of inhibition zone (mm) ± SD
	I	II	III	IV	
Negative control	0	0	0	0	0 ± 0,00[a]
150 mg/ml extract concentration	10	9	8	9	9,0 ± 0,81[b]
300 mg/ml extract concentration	12	12	11	10	11,2 ± 0,95[c]
450 mg/ml extract concentration	15	14	13	12,5	13,6 ± 1,10[d]
600 mg/ml extract concentration	17	16	15	15	15,7 ± 0,95[e]

.The red betel leaf methanolic extracts of 450 g/ml and 600 mg/ml concentrations could still be used as antibacterial material against MRSA. This is based on the general standard stipulated by *National Committe for Clinical Laboratory Standards*/NCCLS (2002) in Tambekar and Dahikar (2010), that a bacterium is considered susceptible to antibacterial materials from plants when the inhibition zone diameter is more than 12 mm. The red betel leaf methanolic extract of 15% and 30% concentrations could not be used as antibacterial material against MRSA as its inhibition zone diameter is less than 12 mm.

The capability of red betel leaf methanolic extracts to combat the growth of MRSA owes to the active compounds contained in the plant such as flavonoid, alkaloid, tannin, saponin and triterpenoid. This is in line with the statement of Tsuchiya, Sato and Miyazaki (1996) that 2', 4' or 2', 6'-dihydroxylation of B ring and 5,7- dihydroxylation of A ring within the flavanon structure is important to anti-MRSA activity. Alcaraz *et al*., (2000) also reports the importance of hydroxyl group at 5 position from flavon and in the activity against MRSA, which supports the finding of Tsuchiya, Sato and Miyazaki (1996).

A research by Sakagami, Mimura and Kajimura (1998) asserts that sophoraflavanon G has an intensive antibacterial power against MRSA (Tsuchiya dan Linuma, 2000). The effect of sophoraflavanon G on the fluidity of membranes was studied using liposomal model of membranes. At the concentration compatible with minimum inhibitory concentration, sophoraflavanon G was proven to significantly increase the fluorescence polarization of liposome. The increase showed that sophoraflavanon G reduced the fluidity of the outer and inner layers of bacterial cell membranes.

According to Aniszewski's research (2007, alkaloid has antibacterial activities despite of its unclear mechanisms. However, it is connected to the chemical elements of carbon rings, aromatic substitution and oxidation nature of alkaloid which can be antibacterial. Alkaloid can cause bacterial cells lysis and contains toxin which can inhibit bacterial growth.

Tannin is a chelating agent which can cause the cell membrans to shrink hence distorting the cell's permeability. Lim *et al*., (2006) expose that hydrolyzed tannin can slow down the growth of cells by restricting the synthesis of cell membranes development of the cell's wall. Abnormality in the cell membranes then causes a change in the cell's permeability and death of the bacteria. Besides through the reaction to the cell's membranes, the effect of tannin antimicrobes can also be observed from the enzyme inactivity and destruction or the inactivity of genetic material function. Tannin also has antibacterial activities related to its ability to set adhesin and enzyme inactive and join the cell's wall. Tannin can cause the membranes to burst (Cowan, 1999).

Saponin can function as antimicrobes. Saponin has molecules that attract water or hidrophillic and molecules that can dilute lipid or lipophillic, thus decreasing the pressure of cell surface which eventually causes the destruction of the bacterium. Triterpenoid can actively fight against bacteria, but its antibacterial mechanism is still really unclear. The antibacterial activity of triterpenoid is assumed to involve bursting of membranes by the lipophillic componenets (Cowan, 1999). Triterpenoid compounds can also result in the decreased semipermeability of cell's membranes causing the nutrients and enzymes to abandon cytoplasm. Bacterial metabolism will be hindered resulting in the reduced production of ATP. This causes inhibition of bacterial growth and reproduction.

References

Fuda, C and Hesek, D. 2006. *Mechanistic Basic For the Action of New Cephalosporin Antibiotics Effective against Methicillin and Vancomicyn resistant staphylococcus aureus (MRSA)*. The Journal of biological chemistry. 281: 10035-41.

Modric, J 2008. *Virulence Factors of Staphylococcus aureus*. Available at http://www.healthhipe.com

Noviana, H. 2004. Evaluation of MRSA Resistance to Quinolone in Atma Jaya Hospital. Atma Jaya Medical Magazine. 3(2) pp. 113

Prakash, M, Rajasekar, K and Karmegam, N. 2007. *Prevalence of Methicillin Resistant Staphylococcus aureus in Clinical Samples Collected from Kanchipuram Town, Tamil Nadu, South India*, INSInet Publication. 3(12) pp 1705-1709

Arnita. 2007. *4th Symposium of Indonesia Antimicrobial Resistance Watch (IARW)*, MRSA update : Diagnose anf Treatment. Symposia Magazine. August 2007. Vol 7(1):64.

Sheen, B. 2010. *Diseases & Disorders: MRSA*, Cengage Learning, Gale.

Klein, E, Smith, DL and Laxminarayan, R. 2007. *Hospitalizations and Deaths Caused by Methicillin-Resistant Staphylococcus aureus,* United States 1999-2005, Emerging Infectious Diseases.13:1840-46.

Maulana, RD. 2011. Anti bacterial Activity of *Sechium edule (jacq) Swart) Ethanolic Extract Against MRSA Isolated from Sputum. Thesis.* Banda Aceh: Faculty of Medicine, Syiah Kuala University.

Sudewo, B. 2010. *Red Betel*. Jakarta. PT Agromedia Pustaka. pp 37-47

Noor, HH. 2011. Antibacterial Activity of Red Betel Leaf Ethanolic Extract Against *Pseudomonas aeruginosa and Shigella dysenteriae*. *Thesis*. Surakarta : Faculty of Pharmacy Muhammadiyah University of Surakarta.

Pradipta, IV, Nikodemus, TW and Susilawati, Y. 2007. Isolation and Identification of Xanthon from *isolasi dan identifikasi Senyawa Golongan Xanton Garcinia Magostana L.,* Pharmaceutical Science Journal. 4:65-77.

Safithri, M and Fahma. 2005. Red Betel Leaf Water Extract as antihyperglycemia. Research Report. Bogor: LPPM IPB

Brown, DFJ, Edwards, DI, Hawkey, PM, Marison, D, Ridgway, GL, Towner, KJ dan Wren, MWD. 2005. *Guidelines for Laboratory Diagnosis and Susceptibility Testing of Methicillin-Resistant Staphylococcus aureus (MRSA)*. Journal of Antimicrobial Chemotherapy. 56:1000-1018

European Committee on Antimicrobial Susceptibility Testing 2009, *Antimicrobial Susceptibility Testing EUCAST Disk Diffusion Method*. Available at http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Disk_test_documents/Disk_method_description_v1.0.pdf

Arishandy. 2010. Isolation and Identification of Flavonoid on Piper Betel Leaf Extracts. *Thesis*. Malang. Technology and Science Institute of Malang.

Lenny, S. 2006. Flavonoid, Fenil Ptopanoid and Alkaloid. Faculty of Scince and Mathematics. Sumatra Utara University. Available at http://repository.usu.ac.id/bitstream/ 123456789/1842/1/06003489.pdf

Bohm, BA .2009. *The Geography of Phytochemical Races*. Springer Science Business Media BV.Canada

Shahidi, F dan Ho, C. 2000. *Phytochemicals and Phytopharmaceuticals*. AOCS Press. USA

Tambekar, DH dan Dahikar, SB .2010. *Exploring Antibacterial potential of Some ayurvedic preparations to Control Bacterial Enteric Infections*, Journal of Chemical and Pharmaceutical Research, 2(5):494-501

Tsuchiya, H, Sato, M dan Miyazaki, T *et al.* 1996. *Comparative Study on the Antibacterial Activity of Phytochemical flavanones against Methicillin-Resistant Staphylococcus aureus,* J Ethnopharmacol. 50:27-34

Tsuchiya, H and Linuma, M. 2000. *Reduction of Membrane Fluidity by Antibacterial Sophoraflavanone G Isolated from Sophora exigua*, Phytomedicine, 7:161-5

Alcaraz, LE, Blanco, SE, Puig, ON, Tomas, F dan Ferretti, FH. 2000. *Antibacterial Activity of Flavonoids against Methicillin-Resistant Staphylococcus aureus Strains*, J Theor Biol. 205:231-40.

Ikigai, H, Nakae, T, Hara, Y and Shimamura, T. 1993. *Bactericidal Catechins Damage the Lipid Bilayer*. Biochim Biophys Acta. 1147:132-6

Lim, SH, Darah, I dan Jain, K. 2006. *Antimicrobial of Tannins Extracted from Rhizophora apiculata Barks*, J. Tropical Forest Science 18(1):59-65

Cowan, MM. 1999. *Plant Product as Antimicrobial agent*, Clinical Microbiology Reviews, 12(4):564-82.