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Research article

Betel Nut (Areca Catechu) Extract Against Vancomycin- Resistant Enterococcus

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Abstract.

Vancomycin-resistant *Enterococcus* (VRE) is a bacterium that is resistant to various antibiotics, especially vancomycin. The VRE resistance mechanism is caused by a change of amino acid residue in the terminal subunit of peptide NAM/NAG, D-alanyl-D-alanine, which is the vancomycin site. D-alanyl-D-alanine and D-alanyl-D-serine variations only provide one site for vancomycin which is used for four spot hydrogen interactions. These changes cause the affinity to decrease by 1000 times so antibiotics cannot perform their functions. Betel nut is known for its action as a natural remedy. Betel nuts have antimicrobial properties against gram-positive and gram-negative bacteria. It is thought that this is through their phenolic compounds. The aim of this research was to determine the antimicrobial activity of betel nuts against VRE. The research was conducted in a microbiology laboratory in June-July 2021. The antimicrobial action of the betel nut was assessed using the microdilution method and scanning electron microscopy. Betel nut extract was prepared using water, n-hexane, and ethyl acetate. The control used was tetracycline. It was found that the concentrations that were able to inhibit VRE were 256 µg/mL of ethyl acetate extract, > 1024 µg/mL of water extract, 1024 µg/mL of n- hexane extract, and 128 µg/mL of tetracycline. The results showed a clear spot in the thin layer chromatography that was in contact with agar with VRE, and the clear spot belonged to the phenolic substances of the betel nut. The microscopy results showed that the VRE cells were destroyed when they came in contact with the betel nut extract. Therefore, we can conclude that the betel nut extract was able to inhibit VRE growth. These findings can be used to support research on alternative new drug compounds.

Keywords: antimicrobial, betel nut, microdilution, SEM, VRE

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1. Introduction

Vancomycin resistant enterococci (VRE) is a bacterium that is resistant to various antibiotics especially vancomycin[1]. VRE grows in the skin, gastrointestinal tract, genitourinary (GU) tract, and in the oral cavity[2]. Resistance to bacteria causes serious infections and can be spread from an infected person to others through casual contact or through contamination[3] VRE can also be spread directly to people after they touch surfaces that are contaminated with VRE. VRE is not spread through the air by coughing or sneezing[4]. The main mechanism of resistance to vancomycin or other glycopeptides is caused by changes in the peptidoglycan synthesis pathway in enterococci, particularly

the substitution of D-Alanine-D-Alanine (D-Ala-D-Ala), to D-Alanine-D-Lactate (D-Ala-Ala). D-Lac) or D-Alanine-D-Serine (D-Ala-D-Ser)[5]. The mechanism of resistance is due to the replacement of d-Ala-d-Ala with d-Ala-d-lac as the terminal amino acid in Lipid II, resulting in a 1000-fold decrease in binding between vancomycin and peptidoglycan due to the loss of one hydrogen bond[6], [7]. Vancomycin widely used for Gram positive bacteria, but not effective for Gram- negative bacteria because antibiotics cannot across the outer membrane of bacteria [8].

Vancomycin widely used for Gram positive bacteria, but not effective for Gram-negative bacteria because antibiotics cannot across the outer membrane of bacteria. There are some various vancomycin-resistant phenotype, i.e VanA , vanB , VANC , VanD, and vanE. VanA manifest as the highest level resistance against vancomycin and teicoplanin induced. Phenotype VanB resistant against vancomycin but sensitive againts teicoplanin. Strain VanC intermediate until moderate resistance against vancomycin. VanD phenotype has moderate resistance against vancomycin, susceptible and low resistance againts teicoplanin[9], [10]. VanE phenotype resisntant against vancomycin and sensitive to teicoplanin[11]. Infection epidemiology of resistant bacteria is changing rapidly. In past 10 years infection caused by resistant organism improving become community infection. Resistant improved therefore the alternative drug is needed[12]–[14]. The drugs against infection caused by antibiotic resistant bacteria are come from plant. The drug agents usually have different structure and action with standard antibiotic [15]. by antibiotic resistant bacteria are come from plant. the drug agents usually have different structure and action with standard antibiotic [8].

Betel nut is widely known in Indonesia as traditional medicine. Betel nut has wide spectrum antimicrobial actitivity[16], [17]. Research was done by Karphorm et all (2009) showed the component of betel nut is soluble in water. The extract is able to inhibit Gram positive and Gram negative. Water and acetone fraction showed biggest inhibition againts bacteria. MIC of betel nut extract againts againts *Bacillus cereus* ATCC 1729, *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, and *Salmonella typhimurium* ATCC 13811 is 0,78 mg/mL[18]. Betel nut is also able to inhibit the growth of resistant bacteria such as MRSA and MRCNS [19], [20]. Betel nut contain 0,3-0,6% alkaloid, such as Arekolin ($C_8H_{13}NO_2$), arekolidine, arekain, guvakolin, guvasine and isoguvasine, red tanin 15%, fat 14% (palmitic acid, oleic acid, stearic acid, caprilic acid, lauric acid, myristitate acid), starch and resin [21]. Previous studies have only tested the antimicrobial activity of areca nut against pathogenic bacteria. Previously, no one had researched the mechanism of action of areca nut against bacteria. Areca nut can

inhibit several resistant bacteria such as MRSA and MRCNS. This study performed an unprecedented test on VRE bacteria.

2. Methods

2.1. Extract preparation, maceration, and fractionation with different polarity solvent.

Betel nut macerated by ethanol for 3x24 hours then evaporated. Extract then fractionated by water, ethyl acetate, and n-hexane. Each fraction evaporated. The extracts and it fractions were determined phytochemicals. Extract and fractions are separated by thin layer chromatograph TLC).

2.2. microdilution method to determined minimum inhibition concentration

Minimum inhibitory concentration (MIC) determined with micro dilution method. This method used micro plate and brain heart infusion (BHI) broth (OXIOD CM1135). To the first until twelfth column of micro plate added 100 µL BHI broth. Five microns VRE added to 10 mL BHI broth to prepare the test bacteria and homogenized by vortex stirrer.

To all column of micro plate added 100µL BHI broth. Then to Second until twelfth column added 100µL VRE, which prepared before. To the twelfth column added 100µL extract solution of *Betel nut,* then homogenized. From twelfth column, took 100µL solution the added to eleventh column, homogenized. From eleventh column took 100µL solution, homogenized. The dissolution performed until third column. Microplate is incubated in 37°C for 24 hours then observed the clear section of plate. The smallest concentration showed clear section stated as minimum inhibitory concentration (MIC). 5µL aliquot from the clear section was streak to nutrient agar and incubated in 37°C four 24 hours. If there are not microbial growth on nutrient agar is stated as minimum killing concentration (MKC). Positive control obtained by added erythromycin, tetracycline, and penicillin G instead of the betel nut extract solution.

2.3. Bioautography method

Bioautography was done by contact and agar overlay method. Contact method was done by touch TLC plate on media agar which before was streak by VRE. Then incubated

in 37˚C for 24 hours. After incubated, check the clear area in around TLC's spot Agar-overlay method is the mix method between contact and direct method. Antimicrobial agent in agar poured into TLC plate, then TLC plate which containing microbe incubated for 24 hours. After 24 hours, check the clear area in TLC spot [22]

2.4. Betel Nut mechanism

The Betel nut mechanism againts VRE is determined by scanning electron microscope (SEM) method[23]

3. Results

Phytochemical properties of Betel nut showed in Table 1.

TABLE 1: Phytochemical Profile of *Betel Nut* Extract and Fraction.

Substances	Betel Nut			
	Ethanol Extract	n-hexane	Ethyl acetate	water
Alkaloid	+	+	-	-
Flavonoid	+	-	+	+
Saponin	+	+	+	+
Quinone	+	+	+	+
Tannin	+	-	+	+
Terpenoid	+	+	-	-
Steroid	-	-	-	-

Notes:
+ = Extract/fraction is containing the substances
- = Extract/fraction is not containing the substances

The next step is thin layer chromatography (TLC) to separating the substances in extract and fractions.

Antimicrobial activity against VRE is determined by broth microdilution method. The result showed Betel Nut ethyl acetate fraction is able to inhibit the growth of VRE. Ethyl acetate fraction is able to inhibit VRE with concentration 128 µg/ (table 2).

Antimicrobial activity of *Betel Nut* showed that all fractions are able to inhibit VRE growth. It showed by the clear area in the plate. Autobiography showed the result that spot with RF 0.6 is able to inhibit the VRE growth show with clear zone in the spot area.(fig. 2)

To determine the VRE bacteria morphology after exposed by *Betel Nut* extract and fraction is using scanning electron microscopy (Figure 3).

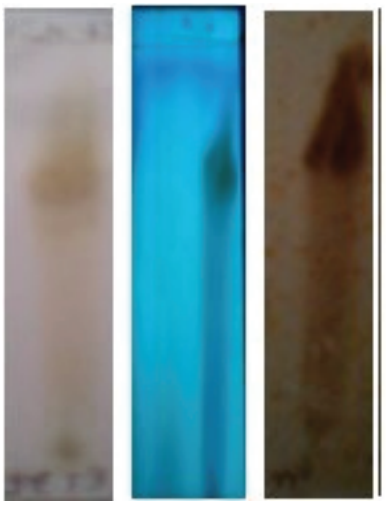


Figure 1: Chromatogram betel nut fraction used silica GF254, (1) betel nut extract, (2) λ 254 nm, (3) sprayed by FeCl3.

Table 2: Minimum Inhibition Concentration and Minimum Killing Concentration *Betel Nut*.

Microbe	F. Water		F. Ethyl acetate		F. n-Hexana		Tetracyline	
	(µg/mL)		(µg/mL)		(µg/mL)		(µg/mL)	
	MIC	MKC	MIC	MKC	MIC	MKC	MIC	MKC
VRE	>1024	>1024	256	>1024	1024	>1024	64	512

Notes:
MIC = Minimum Inhibition Concentration MKC = Minimum Killing Concentration

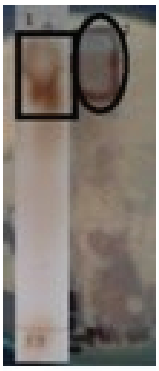
1. 

Figure 2: Bioautography spot in TLC against VRE (1) spot in TLC that used for autobiography (2). Clear zone of bacteria after media contained bacteria were contacting with spot in TLC no 1.

4. Discussion

The test results of areca nut extract against VRE showed that the best fraction was the ethyl acetate fraction. Phenol is thought to have an important role and is responsible as an antibacterial against VRE. This study is in line with previous studies that areca nut extract can inhibit other resistant bacteria which are MRSA and MRCNS[19], [24].

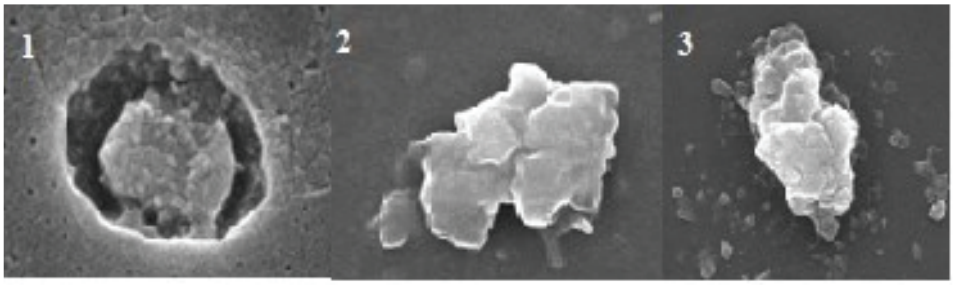


Figure 3: Scanning Electron Microscopy of VRE (1) VRE exposed by ethyl acetate fraction (2) VRE exposed by erythromycin, (3) MRNCS exposed by tetracycline.

Betel nut is widely known has an antimicrobial activity against bacteria. Betel nut is able to inhibit Gram positive and Gram negative[25]. Chewing of betel nut after every meal reduces the population of oral micro flora which may be responsible for dental carries and bad breath[26]. It was also seen that enteric pathogens like *Staphylococcus aureus, Salmonella typhi, Escherichia coli* and *Shigella flexneri* could be inhibited by ingredients of betel nut[27]. By looking at the research results, it can be seen the scientific implication that areca nut extract has good effectiveness against bacteria. This is in line with research that conducted antimicrobial tests on bacteria[28].

The result showed phenolic substances is the antimicrobial properties that can inhibit VRE, due to it reaction to $FeCl_3$. Phenolic substances showed dark purple to black when it reacted with $FeCl_3$. Phenolic substances widely spread in plant and it is secondary metabolite in metabolism pathway of plant[29]. Phenolic compounds with less complex structures, such as catechol and coumarin, have also shown to exhibit bactericidal and fungicidal activities. Increased accumulation of phenolic phytoalexins in plants can promote host defense against pathogens. One outstanding example is the resistance of grape phenolic stilbenes to fungal colonization[30]. The results of this study are in line with research that proves that polyphenols are a promising source of effective, safe, and inexpensive antibacterial compounds. Although polyphenols with a higher minimum inhibitory concentration than antibiotics cannot be used in antimicrobial monotherapy due to insufficient therapeutic effect, the application of combination therapy with antibiotics can improve their pharmacokinetic and pharmacodynamic properties[31].

Polyphenols have a significant impact on human health. Polyphenols can pass through the digestive system without absorption, so they can affect the gut microbiota. This can cause polyphenols to become their active forms and polyphenols to change the composition of the gut microbiota thereby inhibiting pathogenic and beneficial bacteria[32]. Phenolic substances have antimicrobial activity through inhibition of oxidative substances which reacted with sulfhydryl or through nonspecific interaction

with protein or membrane cell bacteria[33]. Simple phenols and phenolic acids have simple phenol ring structures with single substitutions such as cinnamic and caffeic acids so that they have many substitutions and hydroxylation. The site and degree of hydroxylation affect the degree of toxicity. Metabolites appear to be more strongly inhibited the more easily their structures are oxidized[34].

The SEM result showed VRE contacted to betel nut extract is destroyed. Some antibacterial compounds inhibit bacterial growth by damaging cell walls. The results of this study showed that exposure to phenol from the extract was able to damage the cell wall structure. This is in line with previous studies of phenolic compounds having a good ability to destroy bacterial cell walls. Phenol compounds show results in terms of induce greater ion leakages and higher proton influx than hydroxybenzoic acids[35]. Antibacterial targets the peptidoglycan layer at the time of bacterial cell wall assembly. Antibacterial can bind to the peptide substrate on the peptidoglycan layer thereby preventing the reaction with the enzyme. It acts by reducing peptidoglycan cross-linking and consequently weakening the cell wall [36], [37].

The limitation of this research is that it only looks at the exposure to the extract and its fraction but does not see the exposure to secondary metabolites from the isolates. For further research, it is necessary to isolate secondary metabolites from areca nut, to determine the damage to the bacterial structure. In addition, it is necessary to do an acute toxicity test for extracts to determine the safety of extract consumption.

5. Conclusions

Betel nut can inhibit vancomycin resistant enterococcus (VRE). This finding could be an alternative for the discovery of new drug compounds. This research needs to be continued by testing the mechanism on other targets using isolates of secondary metabolites from areca nut.

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