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RESEARCH ARTICLE

MIC and MBC of red fruit extract (*Pandanus conoideus* Lam) against periodontal pathogens bacteria

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ABSTRACT

There are only few studies on the antibacterial activity of red fruit extract (*Pandanus conoideus* Lam) against oral pathogenic bacteria. Thus, this study aims to determine the effectiveness of red fruit extracts **by looking at** the Minimum inhibitory Concentrations (MIC) and Minimal Bactericidal Concentrations (MBC) against periodontal pathogenic bacteria. The subjects of this study were *Streptococcus mutans* (ATCC 25175), *Fusobacterium nucleatum* (ATCC 25586), and *Porphyromonas gingivalis* (ATCC 33277). The antibacterial effectiveness of red fruit extract was tested by the liquid dilution method (microdilution). The data were analyzed using the one-way ANOVA test followed by a double comparison test with the Post Hoc Least Significance Different (LSD) test method. The red fruit extract effectively inhibited and eliminated test bacteria ($p < 0.05$). Our study showed that the red fruit extracts at a concentration of 20% could inhibit the growth of Streptococcus mutans and Porphyromonas gingivalis, which was determined as the MIC strength of 80% as MBC of both bacteria tested. Furthermore, red fruit extract at the concentration of 10% showed an inhibitory effect on the growth of *Fusobacterium nucleatum*, which was determined as MIC of *Fusobacterium nucleatum* and the strength of 40% as MBC of *Fusobacterium nucleatum*. The red fruit extracts were significantly effective against the growth of *Streptococcus mutans, Fusobacterium nucleatum*, and *Porphyromonas gingivalis* provide essential information for further *in vivo* clinical studies to determine the exact dosage and clinical effectiveness of periodontal disease.

Keywords: antibacterial; periodontal pathogens; red fruit (*Pandanus conoideus* Lam)

INTRODUCTION

The severity of the periodontal disease has been correlated with an increase in pathogenic potential and the number of bacteria that form plaque mass.[1] The earliest microbiota in the development process of gingivitis is dominated by *gram-positive rods, gram-positive cocci*, and *gram-negative bacteria; periodontitis* is dominated by *gram-negative* bacteria obligate anaerobes.[1-3] Several studies have shown that *Streptococcus mutans*, *Fusobacterium nucleatum*, and *Porphyromonas gingivalis* have pathogenicity and synergism in causing and aggravating the periodontal disease.[4-6] *Streptococcus mutans* is the initial colonization in plaque mass formation and ecological changes.[4] Ecological changes in plaque mass lead to changes in mass plaque colonization.[1] *Fusobacterium nucleatum* is a secondary colony in plaque mass that plays an essential role in the development and maturation of plaque.[5]

Herbal medicine is currently an alternative therapy that is increasingly popular because some plants are proven to be nutritious and safe for health care, treatment, and disease prevention and are cheaper and easier to obtain.[7,8] One of the endemic plants that are pretty interesting is the red fruit. *(Pandanus conoideus* Lam).[9] Red fruit is generally processed into food for consumption for the Papuan since it is known to provide several health benefits such as having antioxidant, anti-inflammatory activity, enhancing immunity, and antimicrobial activity.[10]

Ida Indrawati's research, which examined the sensitivity of pathogenic bacteria to red fruit, found that the red fruit extract could inhibit the growth of pathogenic bacteria, *Salmonella typhi, Bacillus cererus, Staphylococcus aureus, Klebsiella*

pneumonia, and *Escherichia coli*.[11] Damayanti et al. also showed that the red fruit extract had antibacterial activity against *Enterococcus faecalis*, *Streptococcus mutans*, and *Streptococcus sanguinis*.[12] Based on previous findings, the red fruit showed potential antibacterial activity against several oral pathogenic bacteria. Still, there was no research publications on the effectiveness of the red fruit extract (*Pandanus conoideus* Lam) against the growth of periodontal pathogenic bacteria, which urged the researchers to conduct this study.

MATERIALS AND METHODS

The red fruit extract was made by maceration using 70% ethanol for 72 hours. 900 g of red fruit was cleaned with water, mashed with a mechanical grinder, and macerated with 70% ethanol solvent for 72 hours at room temperature. The red fruit extract was filtered to produce a filtrate. Then the filtrate was evaporated using a rotary evaporator at a temperature not more than 40 °C that generated a thick extract. Furthermore, the thick red fruit extract was sterilized using UV light and stored in a refrigerator. This research procedure has been reviewed and approved by the Research Ethics Commission of Faculty of Medicine of Universitas Sumatera Utara based on the certificate number: 72/TGL/KEPK FK-RSUP HAM/2020.

The thick red fruit extract was diluted into five concentrations of 80%, 40%, 20%, 10% and 5% with the ratio of the red fruit extract in DMSO (Dimethyl Sulfoxide) solvent, respectively at 4 : 1 v / v, 2 : 3 v / v, 1 : 4 v / v, 1: 9 v / v, and 1 : 19 v / v. Afterwards, it was stored in a sterile vial container and in a refrigerator before use. Before testing the bacteria, the red fruit extract had phytochemical screening to identify the chemical compounds contained in the red fruit extract.

This study used three types of bacteria, namely *Streptococcus mutans* (ATCC 25175), *Fusobacterium nucleatum* (ATCC 25586), and *Porphyromonas gingivalis* (ATCC 33277). They were utilized to test the antibacterial activity of red fruit extract against *Fusobacterium nucleatum* (ATCC 25586) and *Porphyromonas gingivalis* (ATCC 33277) using solid media Brucella Agar Sheep Blood 5% and broth media Brain Heart Infusion Broth (BHI-B) incubated for 48-72 hours at 37 °C in the anaerobic atmosphere. Meanwhile, Streptococcus mutans (ATCC 25175) used Blood Agar solid media and Nutrient Broth (NB) broth media incubated for 24 hours at 37 °C aerobic atmosphere.

Streptococcus mutans bacterial suspensions were prepared with turbidity levels equal to 0.5 McFarland and Porphyromonas gingivalis and Fusobacterium nucletum with bacterial turbidity similar to 3 McFarland. The procedure started from the preparation of three test tubes, each of which was filled with 15 ml of sterile 0.45% NaCl solution. Furthermore, 4-10 colonies of one type of tested bacteria were inserted into one of these tubes using a sterile loop needle and then homogenized using a vortex. Afterwards, the turbidity level was tested or equalized using a nephelometer (density check) according to the turbidity level of each type of tested bacteria.

The antibacterial effect of red fruit extract against Streptococcus mutans (ATCC 25175), Fusobacterium nucleatum (ATCC 25586), and Porphyromonas gingivalis (ATCC 33277) was observed using the liquid dilution method (macro dilution). The test was started by preparing a test tube containing 8 ml of broth, as much as the number of repetitions and treatments. Afterwards, 1 ml of the bacterial test suspension was inserted into each tube and1 ml of extract was added according to each concentration, then vortexed and incubated. After the incubation period, the MIC value was determined by considering each tube's clarity level. To determine the value of MBC, subcultures of all dilution tubes were resulted from incubation on solid media with the whole streak and incubation according to each test bacterium's growth conditions. Subsequently, the bacterial colony count was carried out using the TPC (Total Plate Count) method.

However, because the pigment of the extract was concentrated, it is necessary to sub-culture the entire diluent tube at each concentration on a

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solid medium to determine whether the turbidity is due to bacterial growth and to determine the value of Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). All dilution tubes were continued until the subculture stage on solid medium. The MIC was measured as the lowest concentration with an optical density below or equal to that group that is not given extract. MBC was determined by subculturing the MIC dilutions and higher concentrations onto sterile Mueller Hinton agar plates incubated at 37 °C for 24 h. In addition, each Petri dish was counted for bacterial colonies using the Total Plates Count (TPC) method. Calculations were performed on plates with a bacterial colony count of 30 to 300 by visual inspection and counting. The principle of colony counting is that a live bacterial cell will grow into a colony when cultured on a solid medium. The calculation is conducted when the shape of the colony expands, and the point is considered a colony. If the shapes of the two colonies intersect, they are considered as two colonies.[13,14]

This study used the One-Way ANOVA test to see the differences between research groups. The multiple comparability test used the Post Hoc Least Significance Different (LSD) method to determine the average difference between the treatment groups.

RESULTS

Phytochemical screening of red fruit was carried out to identify secondary metabolite compounds in the red fruit extract used in this study. Table 1 on the results of the phytochemical screening of red fruit delineates that the metabolites contain alkaloids, flavonoids, glycosides, and triterpenoids/steroids.

The minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) of the red fruit extract against the growth of periodontal pathogenic bacteria are are in Table 2. Table 2 shows the highest average number of colonies in *Streptococcus mutans Porphyromonas gingivalis* and *Fusobacterium nucleatum* from dilution tube culture at 5%. In contrast, at a

Table 1. Phytochemical screening of Red Fruit Extract (*Pandanus conoideus* Lam)

Secondary Metabolite	Reactor	Results
Alkaloid	Dragendroff	+
	Bouchard	+
	Meyer	+
Flavonoid	Mg Powder + Amil Alcohol + HCl_p	+
Glycoside	Molish + H_2SO_4	+
Saponin	Hot Water/Whipped	-
Tanin	$FeCl_3$	-
Triterpenoid/ Steroid	Lieberman- Bourchat	+

Table 2. The average number of colonies of *Streptococcus mutans, Porphyromonas gingivalis,* and *Fusobacterium nucleatum* from dilution tube culture

Red Fruit Extract Concentration	The Average Number of Colonies of Bacteria (CFU/ml)		
	Streptococcus mutans	*Porphyromonas gingivalis*	*Fusobacterium nucleatum*
80%	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
40%	56.00 ± 13.78	189.33 ± 14.30	0.00 ± 0.00
20%	185.00 ± 7.51	286.00 ± 9.85	38.00 ± 9.54
10%	380.67 ± 3.06	457.00 ± 3.61	277.67 ± 18.18
5%	387.00 ± 2.00	474.00 ± 6.08	387.00 ± 11.00

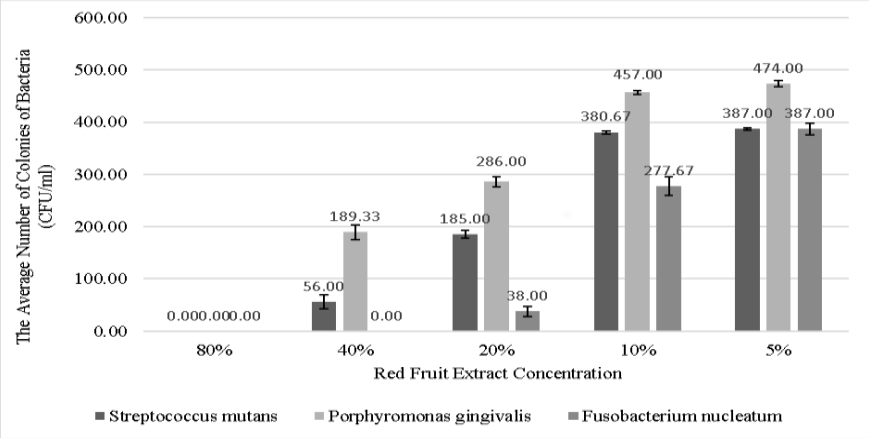

Figure 1. The average number of bacteria colonies of different red fruit extracts concentrations

Table 3. The effectiveness test of the red fruit extract results in the growth of *Streptococcus mutans, Porphyromonas gingivalis,* and *Fusobacterium nucleatum* bacteria

Bacteria	Red Fruit Extract Concentration	P-value				
		80%	40%	20%	10%	5%
Streptococcus mutans	80%	-	0.000*	0.000*	0.000*	0.000*
	40%	-	-	0.000*	0.000*	0.000*
	20%	-	-	-	0.000*	0.000*
	10%	-	-	-	-	0.240
Porphyromonas gingivalis	80%	-	0.00*	0.00*	0.00*	0.00*
	40%	-	-	0.00*	0.00*	0.00*
	20%	-	-	-	0.00*	0.00*
	10%	-	-	-	-	0.057
Fusobacterium nucleatum	80%	-	1.000	0.001*	0.000*	0.000*
	40%	-	-	0.001*	0.000*	0.000*
	20%	-	-	-	0.000*	0.000*
	10%	-	-	-	-	0.000*

LSD test
*p sig <0.05

concentration of 80%, there was no growth of bacterial colony.

As shown in Figure 1, the MIC and MBC values of red fruit extract on the growth of *Streptococcus mutans* bacteria are the same as the *Porphyromonas gingivalis* bacteria, namely at concentrations of 40% and 80%. In comparison, the MIC and MBC values of red fruit extract were on the growth of *Fusobacterium nucleatum* bacteria at concentrations of 10% and 40%. The low MIC and MBC values of the extract indicated that the extract had strong antibacterial activity.

Table 3 shows that the effectiveness of the red fruit extract on the growth of *Streptococcus mutans* bacteria is the same as the *Porphyromonas gingivalis*, namely, at a concentration of 5% with 10%, which indicated no significant difference ($p > 0.05$). At a concentration of 20%, 40%, and 80 % there was a significant difference ($p < 0.05$). meanwhile, the effectiveness of the red fruit extract on the growth of *Fusobacterium nucleatum* bacteria showed that at a concentration of 80% with 40%, there was no significant difference ($p > 0.05$), and at a

concentration of 5%, 10%, and 40% there was a significant difference ($p < 0.05$).

DISCUSSION

The MIC value in this study was determined by a tube indicator that turned out to be visually apparent. However, the observations show that the tube does not look clear, possibly due to the red fruit grain pigment colouring. Red fruit (*Pandanus conoideus* Lam) contains carotenoids that produce an orange-red pigment.[15] Therefore, to see MIC and MBC in this study more clearly, the process continued by culturing all the tested bacteria onto solid media by counting the number of tested bacterial colonies.

This study showed that the red fruit extract effectively inhibited and killed *Streptococcus mutans, Fusobacterium nucleatum*, and *Porphyromonas gingivalis* bacteria. The red fruit extract had different MIC and MBC values against the three tested bacteria. The results showed that giving red fruit extract with a concentration of 80% resulted in no growth of *Streptococcus mutans* and *Porphyromonas gingivalis* colonies, so it was determined as the value of MBC for the two tested bacteria. The MIC value of red fruit extract against *Streptococcus mutans* and *Porphyromonas gingivalis* was set at a concentration of 20% because at a concentration of 20%, there was a significant reduction in the number of bacteria. The addition of red fruit extract with a concentration of 40% caused no growth of *Fusobacterium nucleatum* bacteria colonies. It was determined as the value of MBC against *Fusobacterium nucleatum* bacteria. The MIC value of red fruit extract against *Fusobacterium nucleatum* was set at a concentration of 10% because at a concentration of 10%, there was a significant reduction in the number of bacteria.

Previous research findings by Ida Indrawati supported the results of this study in that the ethanol extract of red fruit at a concentration of 5%, 10%, 20%, 40%, and 80% could inhibit the growth of pathogenic bacteria such as *S. typhi, E. coli, K. pneumonia, B. cereus, S. aureus, and S. pyogenes.* The results of this study showed that the average diameter size of the inhibition zone of red fruit extract against each tested pathogenic bacterium was categorized as moderate to very strong.[12] The research of Damayanti et al. also showed that the methanol extract of red fruit that had been partitioned using ethyl acetate solvent and had MIC and MBC values against *Streptococcus mutans*, respectively at 0.312% and 0.625%.[12]

The factors that influence the differences in MIC and MBC values were the test organism, the size of the inoculum, the composition of the culture media, the incubation time, the extract's antibacterial ability, and the incubation conditions. Incubation conditions affect the temperature, aeration, and pH. The difference in the value of MIC and KBM in this study was thought to be attributed to each test bacterium which had a different susceptibility to an antibacterial compound.[16] The activity of antibacterial compounds is also influenced by the composition and structure of the bacterial cell wall, because it determines the penetration and bonding of active compounds against bacteria.[17] *Fusobacterium nucleatum* and *Porphyromonas gingivalis* are obligate anaerobic gram-negative bacteria, while Streptococcus is gram-positive bacteria.[18-20] Gram-negative bacteria have cell wall layers that are more complex than bacteria gram-positive both structurally and chemically.[18-19] The structure of the gram-negative cell wall is formed from a thin peptidoglycan layer adjacent to the cytoplasmic membrane, and the outer membrane is composed of phospholipids and lipopolysaccharides. However, some compounds currently can disrupt the outer membrane of bacterial cell walls through the release of lipopolysaccharides.[19-21]

The active compounds cause the antibacterial effect caused by the red fruit against the three tested bacteria. The phytochemical tests conducted by Ratna Djamil and colleagues revealed that red fruit contains many antibacterial compounds such as flavonoids, tannins, coumarins, emodols, essential oils, glycoside steroids, flavonoids, anthocyanins, and saponins.[22] The phytochemical screening of

red fruit shows that the metabolites are alkaloids, flavonoids, glycosides, and triterpenoids/steroids. This may be related to the origin of the red fruit, according to Murtiningrum et al., which state that the chemical composition and characteristics of the red fruit vary according to the ecogeographic conditions in which the fruit grows.[23] This is the same as most other plant extracts. The phytochemical screening of the ethanol extract of soursop leaves (*Annona muricata L*.) indicates a womb's secondary metabolite compounds in saponins, terpenoids, steroids, flavonoids, and tannins alkaloids.[24]

The alkaloid content in red fruit as antibacterial can inhibit bacterial growth by disrupting peptidoglycan components in the bacterial cell wall, which can prevent the entire formation of the bacterial cell wall and cause cell death.[19,25] Flavonoids are polyphenolic compounds with three main mechanisms for inhibiting bacterial growth: nucleic acid synthesis, cytoplasmic membrane function, and bacterial energy metabolism.[26,27] The mechanism of action of steroids as antibacterials is by damaging the lipid membrane so that liposomes leak.[28] Steroids are also known to interact with phospholipid membranes because their properties' permeability to lipophilic compounds decreases the integrity of the membrane, and disturbs the morphology of the cell membrane, resulting in the cell being lysed and brittle.[28,29]

The solvent and the extraction technique strongly influence the extracted active compound. The red fruit extraction technique in this study is the maceration technique. Maceration is a conventional extraction method by immersing plant parts intact or coarsely ground in a solvent in a closed vessel at a room temperature for at least two days with repeated stirring until all soluble plant material dissolves. The maceration method can prevent the destruction of thermolabile compounds. This study used 70% ethanol as a solvent in extracting fruit because it can dissolve polar and non-polar active compounds. This solvent was chosen based on the principle of a suitable solvent, that the polarity value is the same as the compound to be extracted, has a high distribution coefficient and selectivity for active ingredients, is stable to heat and chemicals, is not dangerous, is readily available in large quantities and is compatible.[30]

CONCLUSION

This study has proven that the ethanol extract of red fruit (*Pandanus conoideus* Lam) effectively inhibits and kills the growth of periodontal pathogenic bacteria. The antibacterial activity of the ethanol extract of the red fruit is broad-spectrum because it can inhibit and kill the growth of gram-positive and gram-negative bacteria. The MIC and MBC values of red fruit extracts against *Fusobacterium nucleatum* were lower than those of *Streptococcus mutans* and *Porphyromonas gingivalis*. This condition shows that Fusobacterium nucleatum is more susceptible to antibacterial activity from the red fruit extract than *Streptococcus mutans* and *Porphyromonas gingivalis* bacteria.

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