Antibacterial Activity of Buah Merah (Pandanus conoideus Lam.) Against Bacterial Oral Pathogen of Streptococcus sanguinis ATCC10556, Streptococcus mutans ATCC 25175, and Enterococcus faecalis ATCC 29212: An in Vitro Study

Lisda Damayanti¹, Ida Ayu Evaangelina¹, Avi Laviana¹, Yetty Herdiyati¹ and Dikdik Kurnia²,*

¹Department of Pediatric Dentistry, Faculty of Dentistry, University of Padjadjaran, Bandung, Indonesia. 
²Department of Chemistry, Faculty of Mathematics and Natural Science, University of Padjadjaran, Bandung, Indonesia

Abstract:
Background:
Caries and periodontitis are dental diseases caused by bacteria of S. sanguinis, S. mutans, and E. faecalis with three main etiological factors of the host, substrate, and time.

Objective:
This study proposed to investigate the antibacterial effects of Buah Merah (Pandanus conoideus Lam.) against oral bacteria of E. faecalis, S. mutans, and S. sanguinis.

Materials and Methods:
The Buah Merah was extracted with different solvents to yield n-hexane, ethyl acetate, methanol, and H₂O extracts. The concentrations of single and mixture extracts were adjusted for antibacterial assay against bacteria of E. faecalis, S. mutans, and S. sanguinis strains through agar well diffusion assay with chlorhexidine, fosfomycin, and quercetin used as positive controls.

Results:
The ethyl acetate extract showed highest antibacterial activity against three oral bacterial of E. faecalis, S. mutans, and S. sanguinis with inhibition zones values of 9.3, 12.3, and 17.9 mm at 40%, respectively, together with their MIC and MBC values of 1250 & 2500, 0.312 & 0.625, and 0.312 & 0.625 ppm, respectively. For the formulation of extracts, combinations samples test gave various effects to different bacteria, with the best activity showed by methanol-ethyl acetate (M-Ea) extracts against S. mutans with an inhibition zone of 16.25 mm at 40 ppm. The strong and synergistic effect of methanol extract against S. mutans was supported by inhibition zones of the formulation of methanol extract-fosfomycin which showed an inhibition zone of 25.9 mm at 10 ppm.

Conclusion:
The extracts of Buah Merah demonstrated antibacterial activity against oral bacteria of E. faecalis, S. mutans, and S. sanguinis and gave important information for further in vivo clinical studies to determine the exact dosages and its effectiveness in practical application. These results prove the antimicrobial effects of Buah Merah extracts as alternative natural drugs with synergistic effects of active constituents.

Keywords: Buah Merah, Pandanus conoideus Lam., antibacterial compound, S. sanguinis, S. mutans, E. faecalis.

1. INTRODUCTION
Caries is a disease of teeth’s hard tissue, which are enamel, dentin, and cementum. There are three main etiological factors of caries: host, substrate, and time. Streptococcus mutans is included as cariogenic bacteria because it is able to produce acid and carbohydrate quickly. The pathogenesis process of teeth caries is marked by the ability to grow in an acid surrounding and quick sugar metabolism characteristic for organic acid including lactic acid [1, 2].

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Caries and periodontitis are types of dental diseases caused by bacteria. Some bacteria are responsible for dental disease including S. sanguinis, S. mutans, and E. faecalis [3, 4]. All the three are interconnected to each other to cause infection in teeth. Two of them, S. mutans and S. sanguinis, contribute to form dental plaque which develops caries [5]. If it is not treated immediately, it will lead to further infection such as root canal infection. However, E. faecalis is one of the bacteria which exists in the root canal [6]. Systemic diseases will occur if infection in that step is not treated wisely [7].

S. mutans is able to metabolize all kind of sugar and glycosides, such as glucose, fructose, sucrose, lactose, and others. Because of the presence of extracellular glucose and sucrose, S. mutans is able to synthesize Intracellular Polysaccharide Glycogen (IPGs) and produce mutation (bacteriocin) which is considered as an important factor of teeth biofilm’s colonization and production [1].

One of the methods to maintain teeth with big and infected caries on the pulp surface is by doing a root canal or endodontic treatment. Approximately 24-77% of endodontic treatment failures are caused by E. faecalis bacteria. This microbe is able to produce biofilm, go through the dentin tubule, survive in low pH levels, and resist many intracanal medicines [8 - 10].

Intracanal medicament for every visit is recommended to reduce whole bacteria inside the root canal. Root canal medicament must have antibacterial characteristics, able to reduce the rest of microbial biofilm, not irritate periapical tissue, help the regeneration of periapical tissue, and also easy to clean and apply [10, 11].

Teeth surface is covered with biofilm layer: a layer containing millions of bacteria, polymer saliva, and food leftovers and not every teeth covered with biofilm shows the signs of caries [10 - 12]. The prevention of caries and periodontal diseases are based on the control of bacteria and plaques and the production of plaques begins with the formation of pellicle on the teeth surface.

The first bacteria contacted by the pellicle is S. sanguinis which is able to facilitate nutrition and surrounding for another new bacteria inside the oral cavity. The ongoing accumulation of plaques on the upper area of gingival margin, interproximal, pit, and fissure is able to cause caries. The plaque production can be inhibited through adhesion, proliferation, and bacterial aggregation [12].

As there is the bacteria resistance, the discovery of antibacterial agents is still ongoing. Medicinal plants and other natural products are a source of new antibacterial agents that have to be explored [13, 14]. Recently, many researchers have given more concern for herbal medicine because of the variety of safety and synergic effects [15]. A large number of plants that can be used as herbal medicine are available in nature. One of them is Buah Merah.

Buah Merah, known as Pandanus conoideus Lam., is an indigenous plant from Papua, Indonesia. Local people usually consume it directly as food or use its oil for treatment to cure diseases including stroke, HIV, and cancer [17]. In the previous studies, Buah Merah showed that it has antibacterial activity against C. albicans, S. aureus, and M. gypseum [16]. Moreover, Oil of Buah Merah has been used as a major content in hand soap formulation [18]. Based on the studied data that Buah Merah showed potential antibacterial activity, it was suggested to contain antibacterial agents for dental disease.

The acceptance of Herbal medicines as an alternative to modern medicine has led researchers to investigate antibacterial agents of medical plants [19]. Selection of Buah Merah as the source for an antibacterial agent was done with an assumption that the process for drug development would be simpler because the toxicity of Buah Merah is negligible since it is consumed on a daily basis.

2. MATERIALS AND METHODS

2.1. Materials

Fresh Buah Merah (Pandanus conoideus Lam.) was collected in June 2017 from West Papua, Indonesia. Sample extracts were prepared by an extraction method with organic solvents of methanol, n-hexane, and ethyl acetate.

2.2. Instruments

Laminar airflow, incubator (Memmert, IN55), anaerobic jar (Oxoid, AG0025A), autoclave, microplate reader (Biochrom EZ read 400, 80-4001-40), micropipette (Eppendorf, 3120000062 and 3120000054), colony counter (Schuett-Biotec, 3081502).

2.3. Preparation of the Buah Merah Extracts

The fresh fruit of Buah Merah was extracted with methanol (1:3 m/v) for 72 hours, and filtered and evaporated in vacuo at 40°C yielded crude extract. The methanol extract was subsequently partitioned between n-hexane-water and ethyl acetate-water resulting in n-hexane, ethyl acetate, and water extracts, respectively.

2.4. Preparation of the Combination Extracts and References Compound

Each extract and fraction of M. pendans were made in concentrations 40, 20, 10, and 5% (all samples made in 1 ml). The antibacterial activity of the sample was divided into three categories. First, to evaluate the antibacterial activity of a single fraction, concentrations used are 40, 20, and 10%. Second, extract at concentration 5% was used to evaluate the antibacterial activity of the combined fraction. Samples were combined following Table 1, 10 µl of each fraction were combined to another fraction to provide six mixtures. Finally, all samples were combined with positive control of Quercetin, Fosfomycin, and Chlorhexidine to give twelve combinations. Each sample in each category (20 µl) was added into the paper disc.
2.5. Preliminary Phytochemical Screening

Screening for alkaloids, terpenoids, and flavonoids secondary metabolites was performed to methanol, n-hexane, ethyl acetate, and water extracts previously reported [20 - 22].

2.6. Microorganism Assay

The bacteria of E. faecalis ATCC 29212, S. mutans ATCC 25175, S. sanguinis ATCC 10566 were used for antibacterial test on Muller Hinton broth and Muller Hinton agar as a medium, chlorhexidine (purchased from Merck Co. Ltd. and Sigma Aldrich) as a positive control, Brain Heart Infusion broth (Oxoid, CM1135), Muller Hinton agar (Oxoid, CM0337), paper disc 6 mm (Sigma-Aldrich, Z741310), aquabest (Ikapharmindo Putramas), microplate 96 well (Iwaki, 3820 024), filter tips (Biologix, code 22-0010, 22-0200, and 22-1000), parafilm (Sigma-Aldrich P7688-IEA).

2.7. Antibacterial Activity Assay

Antibacterial effects of Buah Merah extracts against E. faecalisATCC 29212, S. mutans ATCC 25175, S. sanguinis ATCC 10566 were observed using Kirby-Bauer disk diffusion. This determination of the sensitivity or resistance of E. faecalis, S. mutans, and S. sanguinis to compounds was based on CLSI protocols (CLSI, 2012) [23, 24]. All samples were diluted with methanol except chlorhexidine (control) with water. The concentration used for all samples and control was 40, 20, and 10%. Paper discs (6 mm) were impregnated with 20 μL of each sample and then placed on the surface of the agar. Tests were performed in duplicate.

The MIC and MBC activities of compounds 1 and references of antibiotics against E. faecalis ATCC 29212, S. mutans ATCC 25175, S. sanguinis ATCC 10566 were determined by the micro-dilution method in 96-well microplates (CLSI, 2012). The bacterial cells were precultured in Muller Hinton broth at 37°C under aerobic conditions and incubated in the presence of compounds with the concentrations obtained by serial two-fold dilution at 37°C, without shaking in the same broth for 24h on the micro. Their MICs were estimated as the lowest concentrations, where the bacterial cells were not observed visually as reported previously and were given based on triplicate experiments. Water or methanol used for dissolving compounds had no effect on the bacterium. The positive control, chlorhexidine and fosfomycin, were dissolved in water and the tests were performed in duplicates.

3. RESULTS

3.1. Buah Merah Extracts

The fresh fruit of Buah Merah (3 kg) was extracted with 15 L of methanol (1:3 v/v) for 72 hours, and filtered and evaporated in vacuo at 40°C yielded 820 g of crude extract. The methanol extract was subsequent partitioned solvent between n-hexane-water (1:1, v/v) and ethyl acetate-water (1:1, v/v) resulting in n-hexane (525 g), ethyl acetate (22 g) and water (18 g) extracts, respectively.

3.2. Phytochemicals Screening of the Buah Merah Extracts

Data of secondary metabolite constituents samples by phytochemical analysis as shown in Table 1, indicated that phenolic and flavonoid compounds were found in all of the fractions; n-hexane fraction contains steroid and triterpenoid; ethyl acetate and H2O fraction contain phenolic, flavonoid, steroid, triterpenoid, alkaloid, and tannin. The H2O fraction contains all secondary metabolites of phenolic, flavonoid, steroid, triterpenoid, saponin, tannin, and alkaloid, while in n-hexane fraction only steroid and triterpenoid. In the ethyl acetate and methanol fractions, similar major secondary metabolites of flavonoids were identified [25].

The analysis data for secondary metabolites constituents of Buah Merah (P. conocidus Lam.) as shown in Table 1, showed that all factions contained different components. Research data gave support that a previous study indicated flavonoids as an important antimicrobial compound [26].

Table 1. Data of phytochemical analysis of the extracts of Buah Merah.

| No. | Secondary metabolites | Reagent | Samples fraction |
|-----|-----------------------|---------|-----------------|
| 1   | Phenolic              | FeCl3 5%| - H2O n-Hexane  |
| 2   | Flavonoid             | a. HCl (g.a) + Mg | - H2O EtOAc |
|     |                       | b. H2SO4 2N     | - H2O MeOH   |
|     |                       | c. NaOH 10%     | -             |
| 3   | Steroid               | Lieberman Burchard | + + + +      |
| 4   | Triterpenoid          | Lieberman-Burchard | + + + +      |
| 5   | Saponin               | HCl + H2O      | + - - -      |
| 6   | Tanin                 | FeCl3 1%       | + - - -      |
| 7   | Alkaloid              | a. Dragendorff  | + - + +      |
|     |                       | b. Wagner       | - - - -      |

3.3. Antibacterial Activity

3.3.1. Antibacterial Activity of the Extracts

To determine the potential of Buah Merah as natural sources of antibacterial agents, the extracts to inhibit bacterial growth were tested against E. faecalis, S. mutans, and S. sanguinis. Susceptibility of Buah Merah extracts against bacteria can be evaluated from their inhibition zone of sample on bacteria growth by Kirby-Bauer method, and the sample was conducted at concentrations of 40, 20, and 10%, with chlorhexidine as positive control and methanol & H2O as negative controls. Based on the assay data in Table 2, this study found that only ethyl acetate extract was active against E. faecalis, S. mutans, and S. sanguinis with different inhibition zone value for each different bacteria, while the most active against S. sanguinis with inhibition zone values of 14.5, 16.1, and 17.9 mm at a concentration of 10, 20, and 40%, respectively. Further analysis for their MIC values was conducted for ethyl acetate extract against E. faecalis, S. mutans, and S. sanguinis, and the assay data showed that MIC and MBC were the same values against S. mutans and S. sanguinis, while less active for E. faecalis.
Table 2. Antibacterial activity of the extracts of Buah Merah against pathogenic oral bacteria E. faecalis ATCC 29212, S. mutans ATCC 25175, and S. sanguinis ATCC 10556.

| No. | Extracts | Inhibition Zones (mm) at concentration (%) | MIC (ppm) | MBC (ppm) |
|-----|----------|------------------------------------------|-----------|-----------|
|     |          | 40 | 20 | 10 |            |            |
| 1   | MeOH     | 0  | 0  | 0  | -          | -          |
| 2   | n-Hexane | 0  | 0  | 0  | -          | -          |
| 3   | EtOAc    | 12.3 | 10.4 | 0.312 | 0.625       |
| 4   | CHX 2%   | 16.6 | 15.7 | 0.003 | 0.006       |

Note: CHX: Chlorhexidine

E. faecalis

3.3.3. Antibacterial Activity of the Combinations Extracts and References Compounds

Further analysis determines activity effects of active constituents in the single and references compounds of chlorhexidine, fosfomycin, and quercetin, the formulated mixtures extracts were made and their antibacterial activity was re-evaluated against bacteria of E. faecalis ATCC 29212, S. mutans ATCC 25175, and S. sanguinis ATCC 10556, respectively.

The effects of additional reference compounds on the antibacterial activity of extracts are shown in Table 4. All reference compounds give different effects depend on each bacteria. The assay data contained some different inactive combination extracts against all bacteria. On the other hand, the activities represented by inhibition values were in the range of 6.85 to 25.9 mm.

Table 4. Data of antibacterial activity of the combination extracts of Buah Merah (P. conoideus Lam.) and reference compounds at a concentration of 10% against pathogenic oral bacteria E. faecalis ATCC 29212, S. mutans ATCC 25175, and S. sanguinis ATCC 10556.

| No. | Samples | Inhibition zones at concentrations of 10% | E. faecalis | S. mutans | S. sanguinis |
|-----|---------|-------------------------------------------|------------|-----------|-------------|
| 1   | M+Chx   | 10.15                                    | 0          | 9.75      |
| 2   | M+F     | 21.6                                    | 25.9      | 6.9       |
| 3   | M+Q     | 0                                        | 7.05      | 10.15     |
| 4   | Hex+Chx | 0                                        | 13.25     |
| 5   | Hex+F   | 20.7                                    | 23.9      | 0         |
| 6   | Hex+Q   | 6.85                                    | 12.7      |
| 7   | Ea+Chx  | 7.7                                       | 12.8      | 11.85     |
| 8   | Ea+F    | 19.65                                    | 21.9      | 11.3      |
| 9   | Ea+Q    | 7.7                                       | 6.85      | 11.85     |
| 10  | H2O+Chx | 12.15                                    | 18.7      | 11.15     |
| 11  | H2O+F   | 22.1                                    | 22.1      | 0         |
| 12  | H2O+K   | 0                                        | 0         | 0         |

Note: M: methanol, Hex: n-Hexane, Ea: Ethyl acetate, Chx: Chlorhexidine, Q: Quercetin, F: Fosfomycin

4. DISCUSSION

Recently, potential challenges for the sustainability of modern new drug discovery for antimicrobial drug resistance have increased markedly over the last decades. Current treatment for carries disease caused by infection of some pathogenic oral bacteria of E. faecalis, S. mutans, and S. sanguinis mainly used 2% chlorhexidine as the gold standard [27, 28], but it may cause discoloration of the teeth and drug resistance. Previous papers reported some alternatives for the prevention of dental plaque related diseases and the improvement of dental health by complement or substitute active antibacterial agents i.e. probiotics, xylitol and sea salt [29 - 35]. Therefore, to solve this problem, there is a need to find and develop new antibacterial compounds that are more selective, effective, and efficient with no or a very limited negative side effect.

Natural products are potential sources that synthesize...
The role of single and combinations extracts contributing to antibacterial activity was then evaluated against all bacteria. By this assay, sample formulation could be determined the study of the synergistic effects of the extracts. As shown in Table 3, the antibacterial activity of combinations extracts described all combination extracts were active against two oral bacteria of S. mutans ATCC 25175 and S. sanguinis ATCC 10556 with different inhibition zone values, while against bacteria of E. faecalis ATCC 29212, two combination extracts of n-Hex+Ea and n-Hex+H₂O were inactive. This finding predicted that the addition of ethyl acetate extract with other extracts caused synergistic effect their antibacterial activity against S. mutans ATCC 25175 and S. sanguinis ATCC which identified by increasing of their inhibition zones values. On the other hand, it was conversely observed that antagonistic effect resulted from combination extracts between n-Hex+Ea and n-Hex+H₂O against E. faecalis ATCC 29212.

According to the main target of this study for discovering new antibacterial agents as complemented or substituted antibacterial drugs, the effect of combinations between extracts and reference compounds against their antibacterial activity was also evaluated. In this study, chlorhexidine, fosfomycin, and quercetin were used as reference compounds. Chlorhexidine has substantivity for a period of 10-12 hours. It has long-lasting antibacterial activity with a broad spectrum of action [27]. Fosfomycin is bactericidal with putative activity against several bacteria, including multidrug-resistant Gram-negative bacteria, by irreversibly inhibiting an early stage in cell wall synthesis [28]. Quercetin is a polyphenolic flavonoid with potential chemoprotective properties. The present studies show the effectiveness of quercetin as an antibacterial agent on selected organisms [29].

The effects of extracts addition into reference compounds on their antibacterial activity as shown in Table 4, showed that different combinations resulted in increasing antibacterial activity against each bacteria. The highest antibacterial activity against E. faecalis, S. mutans, and S. sanguinis was combination of H₂O+F, M+F, and Hex+Chx with vales of 22.1, 25.9, and 13.25, respectively. This fact suggested that the bioactive constituents in methanol and H₂O extract together with fosfomycin were synergically combined to increase their activity to inhibit bacterial growth of E. faecalis and S. mutans. Since fosfomycin was known as inhibitor enzyme murA, the H₂O+F, M+F extracts were active as antibacterial agents by inhibiting the formation of the bacterial cell wall of E. faecalis and S. mutans [30]. Then, the increasing antibacterial activity caused by the addition of Hex+Chx extracts presented the possible mechanism that bacterial growth of S. sanguinis was inhibited via damage to the membrane of bacteria [38]. Though there has not been any thorough research into the mechanisms underlying the antibacterial activity for extracts, previous research suggested that different compounds would target components of bacterial cells differently. Based on the data in this study, the results showed that the edible plant of Buah Merah (Pandanus conoideus Lam.) has potential antibacterial agents. The different activity values of single and combination extracts together with the added effect of reference antibacterial drugs were given important biomarkers as antibacterial agents according to oral bacteria species. The structure and identity of antibacterial lead compounds in active extracts could be determined in single or pure compounds by guided combination phytochemical screening and antibacterial activity data.

**CONCLUSION**

The edible plant of Buah Merah (Pandanus conoideus Lam.) demonstrated in vitro antibacterial activity against pathogenic oral bacteria of E. faecalis ATCC 29212, S. mutans..
ATCC 25175, and S. sanguinis ATCC 10556. This finding is an important database for further in vivo clinical studies to determine the exact dosages and its effectiveness in practical application. Since the plant was used as daily consumption, toxicity studies should be conducted to determine the safety aspect. Further work is required to isolate and determine the lead antibacterial compounds that play the role in inhibiting or eliminating bacteria. Discovery of new antibacterial agents from promising of the edible plant may open the possibilities of finding new clinically effective antibacterial compounds against dental caries and other bacterial oral pathogens.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE
Not applicable.

HUMAN AND ANIMAL RIGHTS
No animals/humans were used for studies that are the basis of this research.

CONSENT FOR PUBLICATION
Not applicable.

AVAILABILITY OF DATA AND MATERIALS:
Not applicable.

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CONFLICT OF INTEREST
The authors declare no conflict of interest, financial or otherwise.

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