

RESEARCH ARTICLE

Combination of *Piper betel* Leaves and *Areca catechu* Nuts
Ethanollic Extract Effects on *In Vitro* Antimicrobial Test against
Aerobic Mouth Microbiota

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ABSTRACT

Introduction: Oral health awareness in Indonesia is still low with more and more oral and/or dental infection becomes more prevalent such as gingivitis, endodontitis, and pericoronitis. These infections could be caused by mouth microflora such as lactobacilli, *Streptococcus mutans*, *Streptococcus mitis*, *Streptococcus oralis*, and *Streptococcus sobrinus*. As time goes by, these bacteria could also become more problematic as antibacterial resistance emerges. Betel nut and betel leaf are well known traditional herbs that is often used in combination for "nyirih"; an Indonesian tradition to chew both plants often after meal. It is believed that these plants treat bad breath and also increases oral health as it could inhibit bacterial growth. **Method:** Betel nut and betel leaf were dried, powdered and macerated using 96% ethanol. The extract was tested on mouth microflora of volunteers grown in BHI using agar using disk diffusion test and modified E-test strip. **Results:** The betel leaves extract showed antibacterial activity in most of concentration used (1.25%, 2.5%, 5%, 10%) while betel nuts only gave antimicrobial effect in 10% concentration. The E-test results also showed the synergistic action between *Piper betel* leaves and *Areca catechu* nuts with some giving significant difference compared to the single treatment ($P < 0.05$). **Conclusion:** *Piper betel* leaves gives antimicrobial effect with MIC value of 1.25% while *Areca catechu* have MIC value of 10%. Furthermore, combination of both resulted in synergistic effect as indicated with larger diameter of inhibition compared to the single treatments.

Keywords: *Piper betle*; *Areca catechu*; Antimicrobial activity; Synergistic effect; Ethanollic Extract

INTRODUCTION

Traditional uses of both betel leaf and betel nut are very well known especially in Indonesia to increase the oral health of the consumer. Aromatic traditional plants have been shown to have inhibition against bacteria, fungi, and yeasts (Shwetha, Chaitanya, Babu & Prakruthi, 2017). Betel leaf is widely used as a post-meal mouth freshener and is extensively grown in Southeast Asian countries, including in Indonesia. Betel leaves can be used with other types of plants or condiments for chewing purpose where in Indonesia it is called *nyirih*. Betel leaves have also shown its antimicrobial and antileishmanian properties in several studies (Shwetha, Chaitanya, Babu & Prakruthi, 2017). Betel nut is also another plant that is traditionally used for treatment of oral diseases such as gingivitis by chewing the plant itself (Shwetha, Chaitanya, Babu & Prakruthi, 2017). Alkaloids and tannins are bioactive compounds of betel nut and these classes of compounds have been shown to have inhibitory effects on several microorganisms (Shwetha, Chaitanya Babu & Prakruthi, 2017). Dried form of the betel nut have shown to strengthen the gums, sweeten the breath, remove bad taste, and also a direct antimicrobial effect against oral bacteria including *Streptococcus mutans*, *Streptococcus salivarius*, *Candida albicans*, and *Fusiform nucleatum* (Shwetha, Chaitanya, Babu & Prakruthi, 2017).

In Indonesia, the awareness of oral health is still low. Pathogen in the mouth is mostly responsible for the oral and dental infections that occur which may include periodontal disease, gingivitis, pericoronitis, endodontitis, and peri-implantitis (Salam, Khokon, Baidya & Mussa, 2014). The most common bacteria that causes dental cavities are *Streptococcus mutans*, *Streptococcus sobrinus*, *Streptococcus oralis*, *Streptococcus mitis* and *lactobacilli* (Shwetha, Chaitanya, Babu &

Prakruthi, 2017). Increase in antibacterial resistance and some adverse effects of antibacterial have been a problem for synthetic antibacterial drugs. Therefore, alternative antibacterial therapy that are non toxic and effective are needed and antibacterial agents from plants may hold the answer.

This study aims to evaluate the antimicrobial combination of betel leaf and betel nut ethanolic extract using *in vitro* testing against mouth microbiota. The results may give an insight into consideration of using the combination of both extracts to formulate a new toothpaste product to combat oral health problems especially in Indonesia.

MATERIAL AND METHODS

Extraction of betel leaves

Betel leaves were washed and wiped to remove excess water. The leaves were dried in an oven with a temperature of 40 °C (Hoque *et al.*, 2012). Leaves were then pulverized and macerated using 96% ethanol with a ration of 1:4 (leaves : ethanol) for 1 day with agitation. Solvent were collected and filtered using vacuum filter via Whatman paper no. 1. Filtered extract were then collected and the solvent was evaporated using rotary evaporator. Concentrated extract then diluted using DW type III into a concentration of 10%, 5%, 2.5%, 1.25%, 0.625%, and 0.3% (w/v) (Datta, Ghoshdastidar & Singh, 2019). Diluted extract then kept in refrigerator 5 °C for further use.

Extraction of betel nut

Ripe fruit of *Areca catechu* were separated from the seeds and the seeds were washed and wiped until dry. Seed then were dried using oven at a temperature of 60 °C. Dried seeds were pulverized and macerated with 96% ethanol with a ratio of 1:4 (seeds : ethanol) for 5 days with agitation. Solvent then collected filtered using vacuum filter via

Whatman paper no. 1. Solvent then collected and evaporated using a rotary evaporator. Concentrated extract was diluted using DW Type III into concentration of 10%, 5%, 2.5%, 1.25%, 0.625%, and 0.3% (w/v). Diluted extract then kept in the refrigerator 5°C until further use (Sutrisno, Wahdaningsih & Handini, 2019).

Microbial preparation

Oral microbiota were obtained by swabbing oral cavity of volunteer and swabs were dipped into brain heart infusion broth. The microbes were incubated in a 37 °C incubator for 24 hours. Growth of bacteria then observed and kept in refrigerator 5 °C until further use. The microbes were prepared into 0.5 McFarland Standard using brain heart infusion broth to achieve cell density of 10^8 CFU/mL as working solution.

Preparation of antimicrobial control positive

Positive control used in this experiment was an antibacterial toothpaste. The solutions were made by diluting toothpaste with DW type III to achieve 2000 ppm concentration.

Antimicrobial assay with disk diffusion method

Agar plates were inoculated with 100 µL bacteria with concentration of 10^5 CFU/mL to every plate. Sterile disc papers that had been previously stored in diluted extracts then added according to the mapping shown in figure 1. The agar plates were then incubated for 24 hours in a 37 °C incubator. After incubation, diameter of inhibition zones were measured.

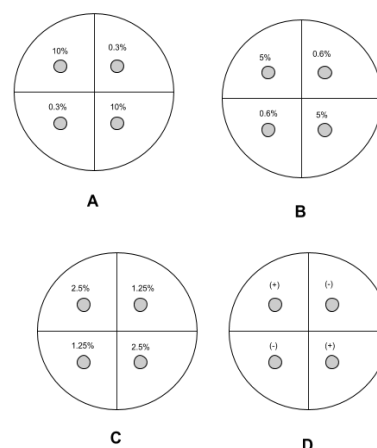


Figure 1. Mapping for disk diffusion test.

Combination antimicrobial assay with modified E-test using conventional antibiotic strip

Agar plates were inoculated with 100 µL bacteria with concentration of 10^5 CFU/mL. Sterile paper strip that had been previously stored in diluted extracts then added according to mapping below. After incubation for 24 hours in a 37 °C incubator, the diameter of inhibition zones were measured (Orhan, *et al.* 2005).

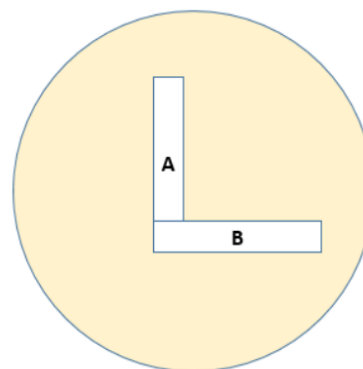


Figure 2. Diagram for E-test strip.

Table 1. Mapping for E-test extract combination assay.

Dish Number	Concentration of Betel Leaves Extract (BLE) (A)	Concentration of Betel Nuts Extract (BNE) (B)
1	10%	2.5%
2	10%	1.25%
3	5%	2.5%
4	5%	1.25%

Statistical analysis

The data obtained were statistically analyzed using two tail student t-test. The P values of 0.05 was used to determine the significance level.

RESULTS AND DISCUSSIONS

Antimicrobial strip test

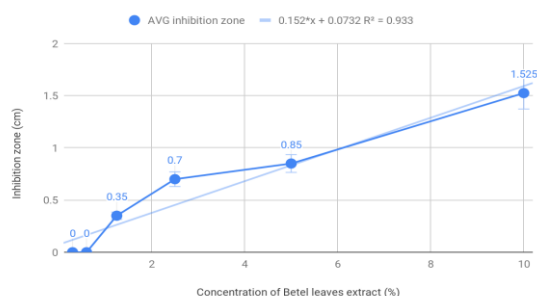


Figure 3. Concentration vs Inhibition zone of Betel Leaves extract towards mouth bacteria.

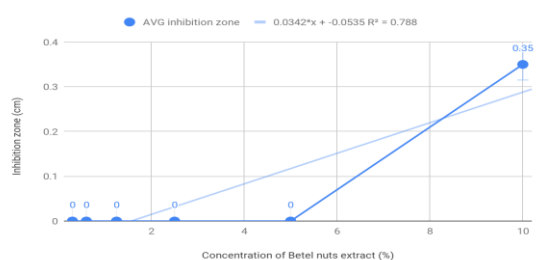


Figure 4. Concentration vs Inhibition zone of Betel Nuts extract towards mouth bacteria.

From the graph above, both of the extracts have antimicrobial effect starting from the concentration of 1.25% and 10% for BLE and BNE respectively. BLE had more potent antimicrobial activity towards aerobic mouth microflora compared to BNE as BLE shown to have antimicrobial activity starting at

concentration 1.25%. BNE on the other hand shown to have antimicrobial activity at significantly higher concentrations of 10%. MIC of both extracts as can be seen from figure 1 and 2 were 1.25% and 10% for BLE and BNE respectively. Both formulas shown in figure 1 and 2 also can not be used as the linearity (r^2) is below than 0.99.

Table 2. E-Test combination test of BLE and BNE at various concentration.

BNE		BLE		Combination
Concentration (%)	Average Inhibition zone (cm)	Concentration (%)	Average Inhibition zone (cm)	Average Inhibition zone (cm)
10	0	2.5	1	2.95*
10	0	1.25	0.95	2.45*
5	0	2.5	1.05	1.8
5	0	1.25	0.9	1.3

*Indicated the significant difference between combination treatment compared to both BLE and BNE ($P < 0.05$)

Table 2 showed that the combination of BNE and BLE resulted in an increase of antimicrobial potency of the extracts. This trend was clearly evident in all of the concentration combination. Although the 10% and 5% concentration of BNE did not show any inhibition zone, the combination of both BNE and BLE shown a bigger inhibition zone compared to the inhibition zone on BLE alone. Furthermore, table 2 demonstrated that combinations of 10% BNE with 2.5% and 1.25% BLE, respectively, gave a significant difference compared to the single treatment ($P < 0.05$).

Based on the experiment, MIC of betel leaf extract is 1.25% while betel nut extract is 10%. Previous study showed that MIC of BLE extract against food borne bacteria were around 0.625-0.75% while betel nut ethanolic extract were around 0.188-0.377 mg/ml (Hoque *et al.*, 2012; Rahman *et al.*, 2016). On the E-test experiment, all combination concentration shows that there is a synergistic effect of both betel leaf and betel nut extract. All of the combination diameter

of inhibition is higher than diameter of inhibition of each extract. Synergistic effect is mostly evident at 5% of betel nut extract, lower than BNE MIC, when combined with 1.25% of betel leaf extract because the diameter of inhibition increases by 2 fold or more.

The BLE contains phytochemicals such as; alkaloids, flavonoids, polyphenols, tannins, monoterpenoids and sesquiterpenoids (Rusminah, Susanto, 2017). The alkaloids causes the incomplete production of peptidoglycan of the bacterial cell wall causing cell death. The flavonoids in the BLE forms a complex compound towards the extracellular proteins which affects the bacterial cell membrane (Rusminah, Susanto, 2017). The phenols contained in BLE are responsible to bind with the bacterial cell wall causing it to denature and increase the permeability. It will also cause changes to the protein molecules and causes protein coagulation. Due to denaturation and coagulation of the proteins, it loses their function and physiological properties. This results in the increase of permeability, cell damage and inhibit cell growth. Meanwhile, tannin has two antibacterial mechanism, the first mechanism is by causing the cell wall to shrink and the second mechanism affects the cell membrane of the bacteria, which increases permeability causing inhibition of bacterial growth (Rusminah, Susanto, 2017). The areca nut extract also contains tannins. It was found that tannin was effective towards gram-positive bacteria such as *Streptococcus mutans* (Endang *et al.*, 2017). The tannin in the areca nut binds with the peptidoglycan unit, disturbing the cell wall and cause cell leakage. The formation of peptidoglycan is influenced by three types of enzyme such as; transpeptidase enzyme, carboxypeptidase and transglycosylase. Tannin also inhibits the transpeptidase enzyme function, which is to combine one peptidoglycan unit to the other

in cell wall formation (Endang *et al.*, 2017). Although, as shown in the results above, BNE only capable to exhibit its antimicrobial activity in concentration as high as 10%. This can be due to the low tannins level contained in the extract itself.

There are many types of microorganisms in the mouth but some of them may be pathogenic and can further causes certain types of dental diseases (Daniluk *et al.*, 2006). Some types of pathogenic oral microorganisms including bacteria and fungi that are inhibited by betel leaf and betel nut are *Streptococcus mutans*, *Streptococcus salivarius*, *Candida albicans*, and *Fusiform nucleatum* (Daniluk *et al.*, 2006). However there are still many types of aerobic bacteria including *Staphylococcus spp*, *Streptococcus mitis*, *Streptococcus sanguis*, *Neisseria mucosa*, and etc (Daniluk *et al.*, 2006). The mouth microflora can be a mixture of many types of microorganisms including gram positive and negative bacteria or even fungi. Although, in this study the type of microorganism was not standardized as microflora were obtained from volunteer's mouth.

Further studies could be done to standardize the type of bacteria used as the study subject. Mechanism of this synergistic effect also could be studied to provide more information about the effect of both extracts toward mouth microflora.

CONCLUSION

Only BLE yields a noticeable antimicrobial effect towards mouth microbiota. Interestingly, when BLE was used in combination with BNE, the combination resulted in a significant synergistic effect. Although this is the case, the mechanism of synergism between both extracts is not well understood. It is recommended to further standardize the method and therefore will

give a clear result for the antimicrobial effect towards mouth microbiota. Nevertheless, this result could provide an insight of how to utilize this herbal product to improve oral health.

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