# THE EFFECT OF ETHANOLIC EXTRACTS FROM VARIOUS TYPES OF BETEL LEAVES ON THE GROWTH OF *PORPHYROMONAS GINGIVALIS*

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***Abstract***

People in Southeast Asia have widely used betel leaves for traditional daily medicine. Several types of betel leaves have been empirically proven to treat various dental and oral diseases. Four of them are black betel (*Piper betle var. Nigra*), green betel (*Piper betle* L.), earth betel (*Peperomia pellucida*) and red betel (*Piper crocatum*). This research aimed to compare the effect of the four types of betel leaf extract inhibiting the growth of *Porphyromonas gingivalis* (*P. gingivalis*), the periodontal pathogenic bacteria. The design of this study was a post-test control group design using *in vitro* disc diffusion method. There were 4 test groups, namely the black betel, green betel, earth betel, and red betel groups, each consisting of a concentration of 25%, 50%, 75%, and 100%. Sterile distilled water was used as the negative control, while the positive control is Metronidazole gel 25%. Sixteen repetitions were performed for each group, and then the average diameter of the inhibition zone formed in each group was calculated using a caliper. The results showed that all groups were effective in inhibiting the growth of *P. gingivalis* bacteria, with the highest effectiveness obtained from 100% concentration, sequentially 19.65(±0.64) mm for black betel, 19.85(±0.61) mm for green betel, 21.70(±0.53) mm for earth betel, and 25.16(±1.00) mm for red betel. Even so, these results are still below the effectiveness of metronidazole, producing an inhibition zone of 27.50(±0.09) mm. There is a significant difference in the comparison between concentrations for each type of betel leaf (P-value < 0.05). Likewise, comparing each concentration between each kind of betel leaf, the result is also a significant difference (P-value: 0.000). These results prove that betel leaf has efficacy as an antibacterial agent, which might be due to the presents of flavonoids, alkaloids, tannins, and essential oils.

***Keywords***: Antibacterial Agent, Betel Leaf Extract, *Porphyromonas gingivalis*

## *Introduction*

Periodontal disease, including periodontitis, is a chronic inflammation that occurs in the periodontium, and its severity can be assessed from the loss of the periodontal ligament and damage to the alveolar bone around the periodontal tissue (1). The results of the 2018 Indonesian Basic Health Research show that the prevalence of periodontitis in Indonesia is 74.1% (2). Meanwhile, a survey conducted by the Global Burden of Disease Study (2019) shows that the majority of periodontitis burden was observed among those aged 55–59 years, the incidence of periodontitis has shown an increasing trend among younger individuals (3). Periodontal disease is one of the leading causes of missing teeth, which results in a decrease in the function of the teeth as mastication, aesthetics, and a reduction in the level of self-confidence and quality of life (4).

A microbial biofilm, a supragingival and subgingival dental plaque, is the leading cause of periodontitis. Supragingival plaque is dominated by aerobic bacteria such as *Streptococcus sanguinis* and *Actinomyces viscosus*, while subgingival plaque is dominated by anaerobic bacterium such as *Porphyromonas gingivalis* (*P. gingivalis*) (5, 6). *P. gingivalis* is a Gram-negative bacterium, rod-shaped anaerobes, non-motile, non-spore-forming, and forms black color colonies on blood agar. They use the fermentation process for metabolism. This bacterium is found in subgingival plaque in approximately 87.75% of chronic periodontitis patients (7). The pathogenicity of *P. gingivalis* depends on its virulence factors. It's structural components such as lipopolysaccharide (LPS), which activate inflammatory mediators (cytokines, prostaglandins, IL-1, and TNF), fimbriae which induce T cell responses and produce antibodies, as well as proteins,

and secretory components. Active virulence factors rapidly and significantly destroy the structure of the periodontal tissue, resorption of alveolar bone, and induction of host responses by the production of inflammatory mediators so that they can inhibit the body's protective mechanisms (6–9).

The periodontitis treatment procedure begins with eliminating plaque bacteria and controlling factors that can facilitate the formation and retention of plaque. Scaling and root planing are two mechanical treatments necessary for treating periodontitis. To maximize the effectiveness of eliminating plaque bacteria, adding antibacterial agents under specific conditions may occasionally be essential (10). Antibacterial drug administration can be administered locally or systemically. Systemic antibiotics for periodontal therapy are usually given in the form of amoxicillin, metronidazole, tetracycline, clindamycin, and ciprofloxacin. Even though these drugs are effective against bacteria, it is common to find side effects from their use. Symptoms such as rashes, changes in blood pressure, chills, arrhythmias, and even difficulty breathing can arise as a side effect of using chemical drugs (11, 12). Given this, other options for antibacterial therapy—natural and made from herbal plants that Indonesians are familiar with—have been brought up. This herbal medication is considered safer overall and has no side effects consequences. Betel leaf is one of the numerous medicinal herbs utilized for years; it mainly contains antibacterial characteristics (13, 14).

Many types of betel grow in Indonesia, some of which are black betel (*Piper betle var nigra*), green betel (*Piper betle* L), earth betel (*Peperomida pellucida*), and red betel (*Piper crocatum*). Flavonoids, saponins, tannins, alkaloids, terpenoids, and phenols are all present in betel leaf. It is well known that these active ingredients have antibacterial and anti-inflammatory properties (15, 16). Although, in general, despite having the same composition, betel leaves might vary in antibacterial efficacy based on the concentration of their active compounds (17, 18). In light of this, we are looking for studies that compare how well the four different betel leaf extracts work to stop *P. gingivalis* from growing at concentrations of 25%, 50%, 75%, and 100%.

## Materials and Methods

The samples used in this study were black, green, earth, and red betel leaves taken from the Manoko plantation in Lembang, which is a highland area in West Jawa, Indonesia, and determined by the Indonesian Institute of Sciences in Bogor, also in West Jawa, Indonesia. *P. gingivalis* ATCC 33277 was used as an indicator strain and identified microscopically at the Microbiology Laboratory, Faculty of Dentistry, Airlangga University, Surabaya, East Jawa, Indonesia.

This research is a post-test control group design experimental laboratory, observing the control and experimental groups at a predetermined time after being given treatment. The disc diffusion method was used in the study to ascertain how much the four betel leaf extracts inhibited *P. gingivalis* growth.

### Extract manufacture

To lower the water content, up to 1 kg of each leaf was dried for two days at room temperature (avoid direct sunshine). Using the maceration procedure, 250 grams of cleansed and water-rinsed betel leaves were crushed and dried in an oven to produce a 70% ethanol extract. The betel leaves are dried, ground into a powder, and combined with 70% ethanol solvent. After letting it sit for three days (seventy-two hours), strain it through filtering paper, then evaporate it with a rotating vacuum evaporator. Using 2 milliliters of pure water, the concentrations employed in the inhibition test were 25, 50, 75, and 100%. The positive control in this experiment was metronidazole gel 25%, while the negative control was distilled water (19–21).

### Preparation of P. gingivalis suspension

50 µl of hemin and vitamin K were put into an Enlenmeyer tube containing 4.7 grams of BHI agar and 100 mL of Aquadest. After covering the medium with aluminum foil, it was autoclaved for 15 minutes at 121°Celsius to sterilize. After that, the media is placed into a 10 cm diameter by 4 mm thick sterile petri dish and left there to solidify. One loop of *P. gingivalis* was taken in a sterile loop and dissolved in 2 milliliters of BHIB liquid medium in a test tube to create the bacterial suspension, which was generated by a turbidity standard of 0.5 Mc Farland ($1.5 \times 10^8$ CFU/ml). After being incubated for 24 hours at 37°C in an anaerobic environment in a desiccator, the reaction tube was diluted with distilled water and homogenized in a centrifuge. A spectrophotometer was used to measure the absorbance and match it to a standard 0.5 McFarland turbidity at a wavelength of 560 nm and an absorbance of 0.05 (20, 22).

### Antibacterial test

The disc diffusion method was used for the *P. gingivalis* inhibition test procedure. To create a final 100 mg/mL concentration, 0.1 g of extract was dissolved in 100 mL of distilled water to make a stock solution. The extract was then diluted to contain 25, 50, 75, and 100 mg/mL of the stock solution. 6 mm blank discs were sterilized and impregnated with 20 µl of each dilution. To ensure exact impregnation, 5 µl of the extract was spotted alternately on both sides of the discs and then allowed to dry before spotting the next 5 µL. Metronidazole 25% was used as the positive control, and sterile distilled water discs were used as the negative control. Before being applied to the bacterial lawn, each disc was completely dried.

Antibacterial activity was assessed by measuring the diameter of the inhibition zone (IZ) surrounding the discs. There were sixteen iterations of the experiment. The leaf extract's mean zone of inhibition diameters (mm) was used to express the antibacterial activity (19).

### *Measurement of the bacterial inhibition zone*

The clear zone of inhibition formed around the disc was measured using a caliper. Measurements were made from the paper disc's outer site to the inhibition zone's outer limit. If the inhibition zone is circular, the diameter of the inhibition zone can be measured from opposite edge to opposite edge and through the center of the disc (19).

Data analysis

Data analysis was performed using the SPSS calculation application. The data results will be tested for normality using the Shapiro-Wilk test and the homogeneity test using the Levene test if the results show a p-value > 0.05, meaning they are normally distributed. A One-Way ANOVA test is run if the data's variance is homogeneous and the distribution is normal. A non-parametric Kruskal-Wallis statistical test is run if the data is homogenous and regularly distributed. The T-independent test was used if the data were homogeneous, and the Mann-Whitney test, if they weren't, to assess whether or not there was a significant difference in each group.

## *Results*

### *Mean of inhibition zone*

Sixteen repetitions of the test were carried out in each group. The mean results of the bacterial inhibition zone formed can be seen in Table 1 below.

**Table 1:** Mean of inhibition zone

| Group | | N | Mean (mm) | *P value* | Anova | Antibacterial Power Chategory |
|---|---|---|---|---|---|---|
| | Black betel | 16 | 08.53 (±0.31) | | | Medium |
| | Green betel | 16 | 10.27 (±0.91) | | | Strong |
| 25% | Earth betel | 16 | 14.40 (±0.69) | 0.000* | 377.977 | Strong |
| | Red betel | 16 | 17.62 (±0.18) | | | Strong |
| | Metronidazole | 16 | 27.50 (±0.90) | | | Very strong |
| | Black betel | 16 | 13.13 (±0.94) | | | Strong |
| | Green betel | 16 | 14.93 (±1.17) | | | Strong |
| 50% | Earth betel | 16 | 16.54 (±0.53) | 0.000* | 122.905 | Strong |
| | Red betel | 16 | 20.92 (±0.81) | | | Strong |
| | Metronidazole | 16 | 27.50 (±0.90) | | | Very strong |
| | Black betel | 16 | 17.07 (±0,42) | | | Strong |
| | Green betel | 16 | 17.77 (±0,62) | | | Strong |
| 75% | Earth betel | 16 | 19.07 (±0,18) | 0.000* | 95.163 | Strong |
| | Red betel | 16 | 23.53 (±1,29) | | | Very strong |
| | Metronidazole | 16 | 27.50 (±0,90) | | | Very strong |
| | Black betel | 16 | 19.65 (±0,64) | | | Strong |
| | Green betel | 16 | 19.85 (±0,61) | | | Strong |
| 100% | Earth betel | 16 | 21.70 (±0,53) | 0.000* | 61.843 | Very strong |
| | Red betel | 16 | 25.16 (±1,00) | | | Very strong |
| | Metronidazole | 16 | 27.50 (±0,09) | | | Very strong |

* anti-bacterial power category based on the area of the inhibition zone formed according to Davis and Stout (1971): <5 mm (weak); 5-10 mm (medium); 10-20 mm (strong); >20 mm (very strong).

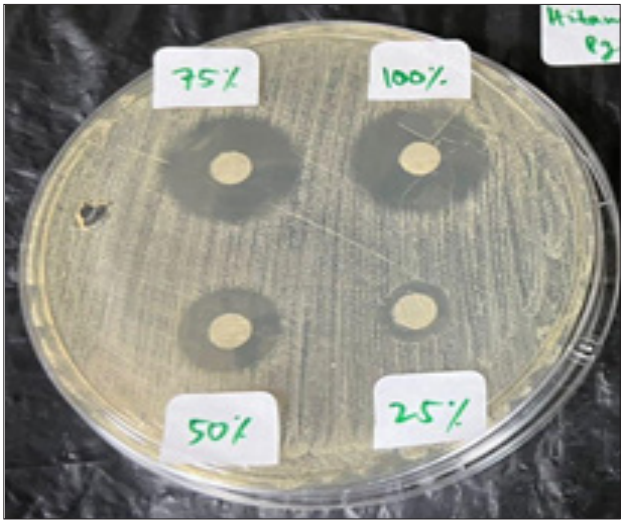


**Figure 1:** Inhibitory zone of black betel

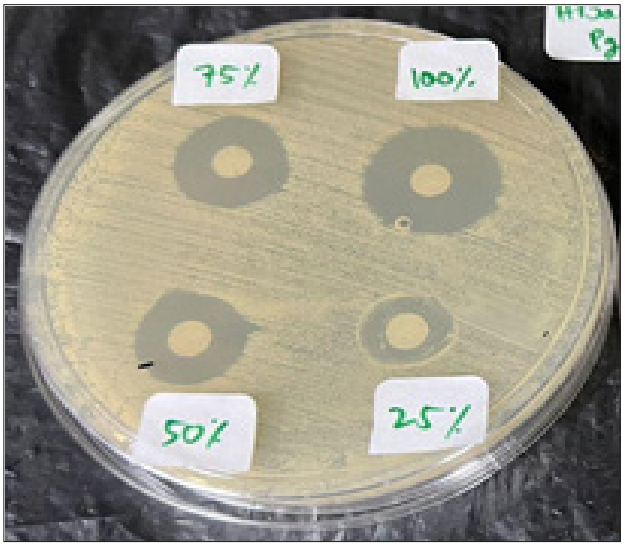


**Figure 2**: Inhibitory zone of green betel

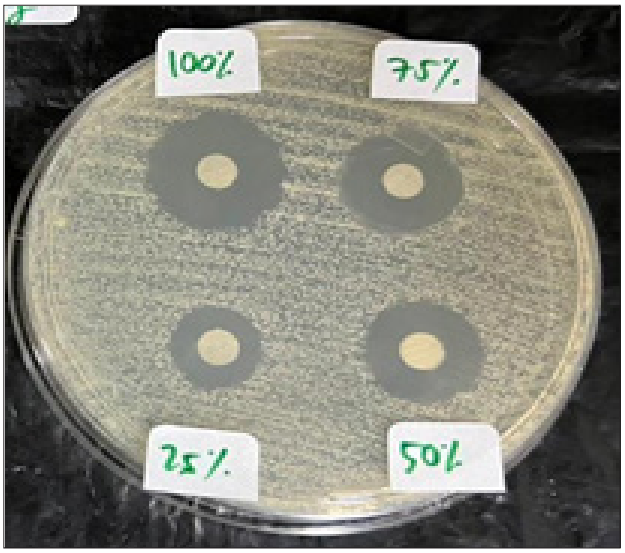


**Figure 3:** Inhibitory zone of earth betel

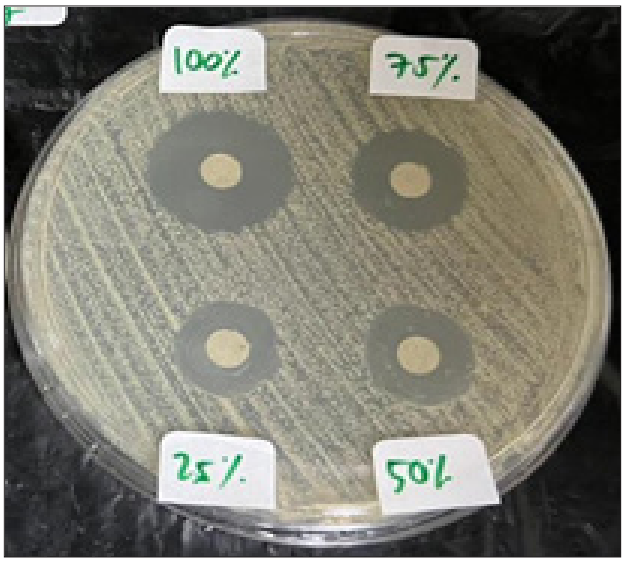


**Figure 4**: Inhibitory zone of red betel

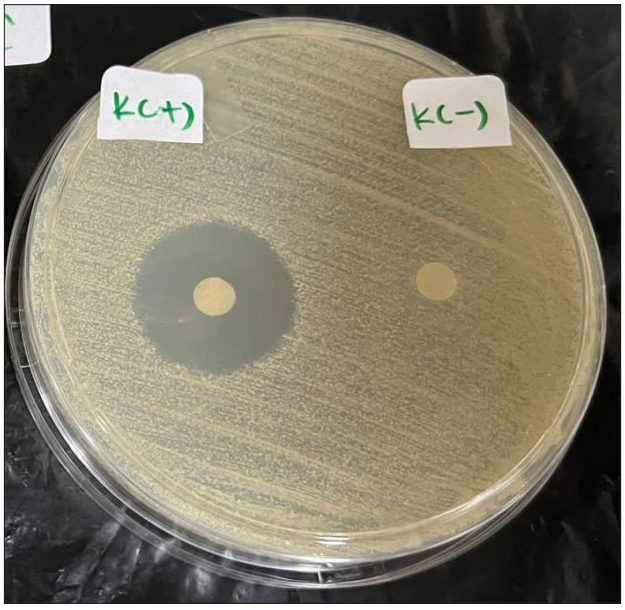


**Figure 5:** Inhibitory zone of control groups

The results in Table 1 show that the 100% concentration produced the highest inhibition zone on all types of betel leaves. Among all, red betel has the highest average inhibition zone compared to the other three types of betel. However, the inhibition zone in all betel leaf groups was still lower than the inhibition zone formed in the metronidazole group.

### *Comparison effectiveness between all concentration*

Each concentration on each type of betel leaf is then compared to one another. The results can be seen in Tables 2 to 5 below.

**Table 2:** Comparison of effectiveness between concentrations of black betel

| Test Group | 25% | 50% | 75% | 100% | (+) |
|---|---|---|---|---|---|
| 25% | | 0.001* | 0.000* | 0.000* | 0.000* |
| 50% | 0.001* | | 0.002* | 0.001* | 0.000* |
| 75% | 0.000* | 0.002* | | 0.022* | 0.000* |
| 100% | 0.000* | 0.001* | 0.022* | | 0.000* |
| (+) | 0.000* | 0.000* | 0.000* | 0.000* | |

Table 2 compares concentrations in black betel leaf, Table 3 for green betel leaf, Table 4 for earth betel leaf, and Table 5 for red betel leaf. They all show a significant difference, where the p-value is ≤0.05.

**Table 3:** Comparison of effectiveness between concentrations of green betel

| Test Group | 25% | 50% | 75% | 100% | (+) |
|---|---|---|---|---|---|
| 25% | | 0.005* | 0.000* | 0.000* | 0.000* |
| 50% | 0.005* | | 0.041* | 0.003* | 0.000* |
| 75% | 0.000* | 0.041* | | 0.003* | 0.000* |
| 100% | 0.000* | 0.003* | 0.003* | | 0.000* |
| (+) | 0.000* | 0.000* | 0.000* | 0.000* | |

**Table 4:** Comparison of effectiveness between concentrations of earth betel

| Test Group | 25% | 50% | 75% | 100% | (+) |
|---|---|---|---|---|---|
| 25% | | 0.013* | 0.005* | 0.000* | 0.000* |
| 50% | 0.013* | | 0.001* | 0.000* | 0.000* |
| 75% | 0.005* | 0.001* | | 0.001* | 0.000* |
| 100% | 0.000* | 0.000* | 0.001* | | 0.001* |
| (+) | 0.000* | 0.000* | 0.000* | 0.001* | |

**Table 5:** Comparison of effectiveness between concentrations of red betel

| Test Group | 25% | 50% | 75% | 100% | (+) |
|---|---|---|---|---|---|
| 25% | | 0.002* | 0.001* | 0.000* | 0.000* |
| 50% | 0.002* | | 0.042* | 0.005* | 0.000* |
| 75% | 0.002* | 0.042* | | 0.005* | 0.001* |
| 100% | 0.000* | 0.005* | 0.005* | | 0.040* |
| (+) | 0.000* | 0.000* | 0.000* | 0.040* | |

## *Discussion*

The findings demonstrated that the growth of *P. gingivalis* was successfully reduced by the extracts of all varieties of betel leaves examined at concentrations of 25%, 50%, 75%, and 100%. The disc paper's surrounding clear zone is probably the result of metabolites that have antibacterial properties. Alkaloids, flavonoids, polyphenols, saponins, and quinones were detected in all varieties of betel leaf extracts after a phytochemical test screening. The content of active substances in betel leaves is also thought to cause the growth of *P. gingivalis* to be disrupted.

Alkaloids can impede the growth of bacteria by causing damage to the peptidoglycan's constituent parts, which prevents bacteria from growing normally. Because they can avoid transpeptidase peptidoglycan from being formed, flavonoids are also referred to as antibacterial substances because this prevents the bacterial cell wall from withstanding internal osmotic pressure. Bacterial growth will be slow because flavonoid compounds disrupt metabolic processes (22, 23).

Polyphenol-active substances contained in betel leaves can also be antibacterial agents. The ability of these polyphenols can cause damage to bacterial cells, cell leakage, protein denaturation, and interfere with enzymes in bacteria. Polyphenols are toxic to bacteria because they can precipitate proteins in bacterial cells, cause damage to the cells and penetrate the cell walls. Saponin is also a betel leaf metabolite compound that acts as an antibacterial. Saponins can cause hemolysis of bacterial cells. This active substance can make the cell membrane unstable by increasing the permeability of the cell membrane (22, 23).

Quinones can inhibit bacterial growth because they can interfere with bacterial cells. Quinone generates substances such as transmembrane proteins' nucleophilic amino acid residues, enzymes on the surface of cell membranes, and cell wall polypeptides (23). Additionally, essential oils found in betel leaves also have antimicrobial properties. Essential oils prevent the formation of germs by interfering with the formation of membranes or cell walls (22).

In the negative (-) control group, there was no evidence of any inhibition of *P. gingivalis* by distilled water. Distilled sterile water is an inert substance., so it will not have the ability to inhibit bacterial growth. This reason makes the writer choose sterile distilled water as a negative control (24). In contrast to the control (+), metronidazole effectively inhibited the growth of *P. gingivalis*. An efficient antibiotic for anaerobic bacteria is metronidazole. This medication functions by obstructing the synthesis of DNA. Metronidazole opens the DNA superhelix during deoxyribonucleic acid (DNA granules) formation, preventing DNA replication (22).

The diameter of the inhibitory zone that betel leaf extract created against *P. gingivalis* increased proportionally to

the concentration increase. The variation in the inhibition zones seen at each concentration can be attributed to the concentration-dependent variation in the amount of active antibacterial component in the betel leaf extract. When betel leaf extract is extracted at 100% concentration, it is highly pure because no sterile distilled water is used as a diluent, unlike when extracts are prepared at 25%, 50%, and 75% concentrations. Conversely, more diluent is required to lower the extract concentration. As a result, these components' active ingredients likewise diminish (25).

## Conclusion

Extracts of the four types of betel leaf at all concentrations effectively inhibit the growth of *P. gingivalis*.

Comparison of the effectiveness of the four types of betel leaf extract between each concentration was significantly different. The higher the concentration, the higher the effectiveness in inhibiting the growth of *P. gingivalis*.

Red betel leaf extract has significantly better effects than other types of betel leaf extract in inhibiting the growth of *P. gingivalis*.

## Acknowledgement

The authors would like to thank the support from the Laboratory of Microbiology Research Center, Faculty of Dentistry, Universitas Airlangga, Surabaya, East Jawa, Indonesia, for assistance to the author in completing the research.

## Competing interests

The authors declare that they have no competing interests.

## Ethical Clearance

We obtained approval from the Research Ethics Committee (MREC) Universitas Padjadjaran, Bandung, West Jawa, Indonesia, registered under number 1331/UN6.KEP/EC/2022.

## Financial support

The autors did not receive support from any organization for the submitted work.

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