Antibacterial effect of binahong (*Anredera cordifolia* (Ten.) Steenis) leaf infusion against black pigmented bacteria

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Abstract. Periodontitis is a highly prevalent periodontal disease in Indonesia. Black pigmented bacteria, such as *Porphyromonas gingivalis* and *Prevotella intermedia*, are the major pathogens associated with chronic periodontitis. Binahong has been scientifically proven to have an antimicrobial effect. This study investigated the effectiveness of a binahong leave infusion as an antibacterial agent against black pigmented bacteria in vitro. The concentrations of the infusion used in the study were 50%, 65%, 80%, 95%, and 100%. The blank disc diffusion method was performed to measure the zone of inhibition in brucella agar. From the diffusion test on brucella agar, the inhibitory zones were 0.42 mm (50%), 1.21 mm (65%), 1.18 mm (80%), 1.19 mm (95%), and 1.36 (100%). In conclusion, a binahong leaf infusion inhibits the growth of black pigmented bacteria.

1. Introduction

Periodontal disease is one of the most common infectious oral diseases in Indonesia. Based on SKRT 2004, the prevalence of periodontal disease in Indonesia is 96.58% [1]. Chronic periodontitis is the most common disease among all periodontal diseases.

The major periodontopathogen in chronic periodontitis is a group of bacteria living in the subgingival region. In the initial stage of chronic periodontitis, bacteria produce toxins that trigger an immuno-inflammatory response, leading to direct tissue destruction [2]. Chronic periodontitis is a polymicrobial infection including various bacterial species. The major pathogens in chronic periodontitis are the black pigmented bacteria *Porphyromonas gingivalis* and *Prevotella intermedia* [2].

Binahong is a plant that is easily found in Indonesia and is known for its medicinal use in curing various diseases such as diabetes mellitus and vomiting blood. It contains high levels of secondary metabolic compounds including saponins, flavanoids, and phenol [3], which have antibacterial and antioxidant effects. These compounds are found in all parts of the plant, but leaves are the easiest to use [4]. Leaves contain flavanoids, aporins, tannins, alkaloids, and polyphenols [5]. Flavanoids and saponins are antibacterial molecules that bring about bacteriolysis by interfering with bacterial cell wall permeability [3,4]. Alkaloids are DNA-intercalating agents that interfere with DNA transcription. Phenol denatures proteins on the bacterial cell wall [6]. In vitro assays have shown that 15% of a binahong leaf infusion can kill *Streptococcus mutans* serotype c [7].

Herbal medicine or botanical medicine can be processed in the form of a decoction, an infusion, an extract, a fraction, or an isolate. However, processing these medicines as extracts, fractions, and isolates require a laboratory and pharmaceutical skills [8]. Infusions are made by extracting materials in 90°C water for 15 min [9]. Processing botanical medicine using this infusion technique is better than processing using exposure or boiling because of its lower temperature, which keeps materials well preserved. Infusion is an easy and affordable process that does not require any special treatment.
or equipment [9]. Binahong leaf has an antibacterial effect on S. mutans. However, the antibacterial effect of binahong against black pigmented bacteria is still unknown.

2. Materials and Methods

2.1. Sampling
Patients were placed upright in a dental unit, and their lips were fixed with a fixture. The working area in the mouth was isolated with a cotton roll and was dried with an air jet. Supragingival plaque was removed using a cotton swab, and subgingival plaque was taken into the periodontal pocket using an excavator. The subgingival plaque sample was placed in an Eppendorf tube containing 1 ml of PBS and was then immediately placed on ice.

2.2. Specimen processing
Specimens were centrifuged by gradient centrifugation at 700 rpm, 800 rpm, and 900 rpm, each for 1 min to separate supernatants from pellets. The supernatant was removed using an Eppendorf tip, and the pellets were added to 1 ml of PBS. After final centrifugation, the pellet was dissolved with 1 ml of NaCl and then homogenized using an Eppendorf tip.

2.3. Preparation of bacterial culture on brucella agar
Homogenization products were scraped onto brucella agar using sticks that were sterilized and then cooled by placing the tip in empty agar. The bacteria on brucella agar were then placed in an airtight container and then filled with N₂, H₂ and CO₂ for 1 min. The container was incubated for 72 h at 37°C.

2.4. Purification of bacteria
After incubation for 72 h, bacterial colonies on brucella agar were selected and Gram staining was performed. Black pigmented bacteria were collected and then put in brucella broth. Brucella broth was placed in an airtight container and then filled with N₂H₂ for 1 min and incubated for 6 days.

2.5. Producing the binahong leaf infusion
Fifty grams of fresh binahong leaves was cleaned and cut into small pieces and then placed on a glass tray filled with 500 ml of aqua bidest. The leaves were steamed in a pan of boiling water for 15 min and occasionally stirred. After steaming was finished, leaves were filtered and then heated using a water bath until 100% concentration was reached. The infusion was inserted into an airtight glass container that was wrapped with aluminum foil. Then, tyndallization was conducted for 3 consecutive days by steaming at 65°C for 30 min [10,11].

To obtain 50%, 65%, 80%, and 95% binahong leaf infusion solutions, the original infusion sample was mixed with the appropriate amounts of double distilled water (aqua bidest) in Eppendorf tubes. Then, 1 ml of chlorhexidine was used as the positive control, and 1 ml of water was used as the negative control.

2.6. Blank disc diffusion method
Petri dishes containing brucella agar were prepared, and blank discs were labeled based on the concentration of the binahong leaf infusion. Cultures of black pigmented bacteria (result of serial dilution of ½ McFarland standard containing 15 × 10⁷ CFU bacteria) were placed on petri dishes and were then incubated at 37°C for 15 min. Approximately 0.2 ml of the binahong leaf infusion was dripped onto a blank disc and then placed on the agar surface. Two experiments were performed for each concentration of the binahong leaf infusion (50%, 65%, 80%, and 95%). The petri dishes were incubated in an aerobic jar for 24 h at 37°C. The diameters of inhibitory zones were measured after 24 h [12]. The inhibitory zone diameter was measured in millimeters (mm) using calipers by calculating the diameter of clear zones, then subtracting the diameter of the blank disc, and then dividing by two [2].

3. Results
Wild strains of black pigmented bacteria were cultured on brucella agar (Figure 1). Figure 2 demonstrates gram staining on brucella agar and liquid brucella.
The average inhibitory zone of bacterial colonies was 0.954 mm on the first medium and 1.188 mm on the second medium (Table 1 and Figure 3). Therefore, the average inhibitory zone of black pigmented bacterial colonies was 1.071 mm.

Table 1. Measurement of inhibitory zones of binahong leaf infusion against black pigmented bacteria.

| Solutions                 | Black pigmented bacterial colonies on brucella agar 1 (mm) | Black pigmented bacterial colonies on brucella agar 2 (mm) |
|---------------------------|-------------------------------------------------------------|-------------------------------------------------------------|
| 50% binahong leaf infusion| 0.59                                                        | 0.25                                                         |
| 65% binahong leaf infusion| 1.21                                                        | 1.21                                                         |
| 80% binahong leaf infusion| 0.62                                                        | 1.74                                                         |
| 95% binahong leaf infusion| 0.99                                                        | 1.38                                                         |
| 100% binahong leaf infusion| 1.36                                                        | 1.36                                                         |
| 0.2% chlorhexidine         | 4.49                                                        | 2.91                                                         |
| Aqua bidest               | 0                                                           | 0                                                            |
Inhibitory Zones

**Figure 3.** Histogram of inhibitory zones of binahong leaf infusion against black pigmented bacteria.

### 4. Discussion

In the transfer of bacterial culture from the brucella solid medium to the liquid medium, there was contamination showing cocci and brown bacteria, which are characteristic of gram-positive bacteria. Contamination may be caused by the origin of specimens, cross-sectional contamination (contaminated culture and presence of viruses and fungi), and direct contamination (contamination of laboratory equipment, reagents, and medium). Contamination may also be caused by operator errors such as careless removal techniques and lack of disinfection [13].

Infusion had a weak antibacterial effect because its active materials were mixed with other substances; therefore, it could not work optimally. A binahong leaf infusion cannot be used to determine the KHM in the dilution method because of its dark colour.

The bacterial sensitivity test was performed using the blank disc diffusion test to determine the inhibitory zone of the binahong leaf infusion against black pigmented bacteria. The formation of the clear zone indicated the inhibition of growth of black pigmented bacteria due to secondary metabolite compounds in the binahong leaf infusion [14].

Binahong leaves contain antimicrobials such as flavanoids, saponins, alkaloids, terpenoids, and phenols. Flavanoids and saponins damage the permeability of bacterial cell walls, leading to bacteriolysis [5]. Saponins can also form complex compounds with cell membranes through hydrogen bonds [14], which can damage the permeability of the cell wall and cause cell death [15]. Alkaloids react with nitrogen and amino acids and can damage bacterial cell walls. This reaction leads to changes in the structure of amino acids and DNA, promoting bacterial cell lysis. Phenols acts by inhibiting bacterial growth by inactivating enzymes in the cell membrane, leading to bacterial cell lysis [16,17].

In the present study, the inhibitory zone test for black pigmented bacteria was conducted in duplicate. The inhibitory zone is a clear zone formed because of the antibacterial effects of an infusion. If there was no inhibitory zone on the plate, the bacteria were resistant to the infusion. If there were inhibitory zones on the plate, the bacteria were sensitive to the infusion. The largest inhibitory zone was 1.36 mm at the 100% concentration, and when compared with 0.2% chlorhexidine, the inhibitory zone was 1.36/3.7.

The inhibitory zone showed a tendency to increase in diameter as the concentration of the infusion increased. The inhibitory zone diameter increased from an average of 0.42 mm at 50% concentration to that of 1.21 mm at 65% concentration. The inhibitory zone diameter increased also from 80%, 95%, to 100% concentration. In general, the effectiveness of the material is related to the concentration of the materials [18].

In the present study, the inhibitory zone diameter decreased from 1.21 mm at 65% concentration to 1.18 mm at 80% concentration. Several factors, such as the viscosity of the medium, the speed of the
infusion, the concentration of the infusion on filter discs that did not distribute properly, the 
sensitivity of the organism to the infusion, the interaction of the infusion with the medium, and the 
size of the inoculum that was not distributed properly, may have caused this decrease in the inhibitory 
zone diameter. The binahong leaf infusion used in this study still contained other non-antibacterial 
compounds that could reduce the antibacterial effects of flavanoids, saponins, phenols, alkaloids, and 
terpenoids. Thus, antibacterial effects may be impaired due to the attachment of active antibacterial 
compounds to other compounds [19].

Black pigmented bacteria are gram-negative bacteria, which have cell walls consisting of three 
components. The outermost membrane contains protein molecules called porins, lipopolysaccharides, 
and lipids, and it also has a thin peptidoglycan layer. Porin in the outermost cell membrane is 
hydrophilic, which may make it difficult for certain molecules to enter the bacterial cell [20]. 
Approximately 20% of the outer membrane of bacteria contains lipids, which is why these secondary 
metabolites find it difficult in entering the bacterial cell wall [21]. In addition, the infusion contains 
united polar and non-polar compounds, making bioactive materials less optimal. This factor may 
cause the low sensitivity of the inhibitory zone in the binahong leaf infusion against black pigmented 
bacteria. The inhibitory zone diameter of the binahong leaf infusion against Salmonella typhi was 
12.58 mm [12]. The use of binahong leaves in an extract has been proven to have more antibacterial 
effects than in an infusion. In conclusion, the binahong leaf infusion has antibacterial effects and has 
the ability to inhibit the growth of black pigmented bacteria.

5. Conclusion 
This study showed black pigmented bacteria were sensitive to the binahong leaf infusion. This 
infusion could inhibit bacterial growth at concentrations of >50%. The effectiveness of the active 
substances in the binahong leaf infusion generally increased along with the increasing concentrations 
of the infusion.

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