Explication of the mixture of *Piper betle* and *Eugenia polyantha* on oral bacteria

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Abstract

The aim of this study was to analyze the effectiveness of the mixtures of *Eugenia polyantha* and *Piper betle* leaves against oral bacteria. Four compositions of EP and PB were made and observed on tryptic soy agar, blood agar, MacConkey Agar (MCA), and Mannitol Salt Agar (MSA). Inhibition analysis was conducted at concentrations of 20%, 30%, 40%, 50%, and 60%. Our study results indicate that *Staphylococcus aureus* exhibited growth on BA, bacterial colonies were identified on MSA, and serological tests confirmed coagulation. A biochemical test conducted on a MCA sample revealed several species within the *Enterobacteriaceae* family, including *Escherichia coli*, *Pseudomonas*, *Klebsiella*, and *Proteus*. The highest level of inhibition was observed with the sixth toothbrush (K4). Significant differences in inhibition were noted among the various groups (K1, K2, K3, K4, and controls), with the diameter of inhibition for each combination yielding $p<0.05$. However, the differences in inhibition between the combinations themselves were not statistically significant, with $p>0.05$. *Streptococcus sp, Klebsiella sp, Enterobacteriaceae sp (Coliform sp), Pseudomonas,* and *Staphylococcus aureus* are the contaminant bacteria on used toothbrushes. The highest inhibition level and bactericidal properties were achieved by mixing 70% bay leaf (*Eugenia polyantha*) and 30% betel leaf infusion.

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Introduction

The teeth, gums, tongue, mucous membranes, throat, and buccal mucosa provide various surfaces for microbial colonization. In total, there are more than 700 species of microorganisms present in the oral cavity.[1,2] The organisms present as commensal and pathogenic microorganisms. Over time, pathogenic microorganisms can lead to tooth decay and periodontal diseases. Some of these microorganisms adhere to solid surfaces, forming dental biofilm, while others remain suspended in aqueous environments. Under adverse circumstances, bacteria can lead to bacteremia and spread to vital organs, such as the heart valves, forming a persistent plaque.

Therefore, biofilm management or plaque control is needed to prevent oral diseases. Many studies have proven the effectiveness of mechanical plaque controls, such as toothbrushing, to manage dental biofilm.[3] Nevertheless, toothbrushes may become infected with microorganisms from the buccal cavity, environment, hand aerosol, and storage locations.[4,5]

The microbiomes found on used toothbrushes are associated with the microbiome from various sites in the oral cavity.[4,5] The microbiota can lead to cross-contamination with microorganisms in the oral cavity, such as *S. mutans, Staphylococcus aureus, Streptococcus pyogenes* and *Candida albicans*,[6] *Pseudomonas, Coliforme Corynebacterium., Bacillus sp, Gram (-) Neisseria sp*.[4,7] Moreover, antimicrobial-resistant bacteria has been found in used contaminated toothbrushes.[8] Glass observed that the defect of oral tissue was exacerbated through a contaminated toothbrush instead of a sterile toothbrush. Some studies found that toothbrush handle material and the tightness of brush fibers have an impact on the retention of bacteria.[9] Therefore, it is necessary to disinfect the toothbrush after use or before putting it in storage.[10,11]

Several methods are recommended for the decontamination of toothbrushes, including the immersion of toothbrushes into alcohol and spraying toothbrush fibers with antimicrobial solutions such as chlorhexidine, sodium hypochlorite, hydrogen

peroxide, essential oil vinegar, and warm salt water.[5,12-19] However, the disadvantage of manufactured chemical products is that they are costly and can adversely affect long-term use. Considering the aforementioned factors, natural products that are standardized and safety-oriented are becoming more accepted globally. Some previous studies investigated some herbs as toothbrush disinfectants.[10] Furthermore, mixing multiple herbs into one formula enhances antimicrobial potential.[20,21] *Piper Betle* is a popular antibacterial, mouthwash, and wound-healing component in Southeast Asian and Ayurvedic traditional medicine, and *Eugenia polyantha* is also used to treat gastrointestinal problems.

Eugenia Polyantha is a tropical tree belonging to the kingdom *Plantae*, division *Spermatophyta*, subdivision *Pinophyta,* class *Coniferopsida*, order *Myricales*, family *Myrtaceae,* genus *Eugenia*, and species *Polyanthum (Wight)*. *Eugenia polyantha* leaf has long been known as a spice with traditional healing use, antimicrobial, and antioxidant,[22,23] which is easy to find in the market. The leaves are rich in phenolic compounds, flavonoids, phenol, tannins, saponins, steroids, terpenoids, and essential oils.[24,25] As an antioxidant, *Eugenia polyantha* is stable in frying crude coconut oil.[25] The solutions have been shown to inhibit the growth of oral bacteria and candida.[7,26,27]

Green betel (*Piper betle (L)*) is an herbaceous plant that grows or climbs on other tree trunks or wood. It is classified as follows: kingdom: *Plantae*; subkingdom: *Tracheobionta*; division: *Spermatophyta*; subdivision: *Angiospermae*; class: *Dicotyledonae*; order: *Piperales*, family: *Piperaceae*; genus: *Piper*; species: *Piper betle (L)*. Research conducted by Nurjannah *et al.* showed that boiled *Piper betle* has an inhibitory effect on bacterial colonies carried by toothbrushes.[7] Besides essential oils, other compounds are flavonoid acid, organic acids, amino acids, steroids, sugars, tannins, proteins, terpenoids, saponin, fats, starch, and carbohydrates.[28] *Piper betle* has been recognized for its antibacterial and antifungal properties and is considered safe for oral use.[29-31] Traditionally, it is used for skin diseases, hemorrhoids, smelly sweat, cleaning the eyes, reducing blood vessels, and as a cough medicine. In dentistry, *Piper betel* leaf infusion can be used to eliminate bad breath and to stop gum bleeding and toothache. The general purpose of this study was to analyze the effectiveness of the infusion containing the mixtures of *Eugenia polyantha* leaves and *Piper betle* leaves against bacterial growth on used toothbrushes. The analysis includes the determination of oral microorganisms on used toothbrushes, inhibition, and contact time.

Materials and Methods

Infusion preparation

Fresh *Eugenia polyantha* leaves and *Piper betle* leaves were washed, drained, and sliced. We weighed 600 g of EP leaves and added 1 L of distilled water, and 350 g slices of PB leaves and added 1 L of distilled water. Both were heated in a water bath at a temperature of ±90ºC for 20 minutes, then cooled and filtered. The four compositions of the solutions between *Eugenia polyantha* and *Piper betle* were made as follows: K1 (40%:60%), K2 (50%:50%), K3 (60%:40%), K4 (70%:30%), with sterile water as a negative control, and ciprofloxacin as a positive control. The K1, K2, and K3 combination in that composition has been balanced between the two herbs for comparison. Meanwhile, K4 was made based on the observations conducted by Putri *et al*.[32]

Toothbrushing implementation

Eight primary school students were chosen randomly as participants. Each participant was given a toothbrush with the same size and type of brush. Recommended tooth brushing techniques were demonstrated by the dentist prior to tooth brushing implementation. Participants brushed their teeth systematically for 2 minutes, consisting of 20 seconds for each region, starting from the upper right to the upper front, upper left, lower left, lower front, and lower right regions. After tooth brushing, without rinsing, the toothbrush was put into a sterile test tube containing NaCl. The sterile test tube was firmly sealed and transported to the lab for testing. After the successful breeding of microorganisms, the used toothbrushes were placed in the medical waste bin and discarded.

Specimen breeding and microorganism identification

50 µL of bacterial suspension from the participant's used toothbrush was inoculated on Tryptic Soy Broth (TSB), incubated at 37ºC for 24 hours, then observed for turbidity. TSB serves as a base medium for a variety of specialized growing conditions. It is high in nutrients and is widely used to cultivate microorganisms for diagnostic and sensitivity testing. To identify the type of contaminant bacteria on the toothbrush, the gram stain was performed in TSB agar. It was planted for a four-way streak on the following selective medium to determine microorganisms in mixed cultures.

Colony characteristics were observed on TSA. TSA is commonly used in clinical microbiology to culture and isolate non-fastidious microorganisms for further testing. It is an enriched, non-selective medium that supports the growth of both gram-positive and gram-negative bacteria.

Coccus gram-positive and gram-negative were isolated on Blood Agar (BA). Blood agar is an enhanced medium containing general nutrients and animal blood. It is used to cultivate bacteria and detect pathogens by hemolytic activity.

Gram-negative bacteria were identified using MacConkey Agar (MCA), which inhibits the growth of gram-positive bacteria due to the presence of bile salts and crystal violet.

Agar is a selective and differential medium that isolates and distinguishes gram-negative enteric bacteria based on their capacity to ferment lactose. Lactose fermenters (such as *Escherichia coli*) produce acid, which causes them to appear pink/red on the medium. Non-lactose fermenters (such as Salmonella and Shigella) stay colorless or pale.

Mannitol salt agar (MSA) is a selective and differential medium used to isolate *Staphylococcus* species, particularly *Staphylococcus aureus*, using mannitol fermentation differentiation.

Inhibition analysis

Using a sterile cotton swab, 100 µL of bacterial suspension from each TSB was evenly spread over the surface of Mueller-Hinton Agar (MHA). Six mm wells were made using a sterile tube. Poured 50µL of each mixture (K1, K2, K3, and K4) infusion at concentrations of 20%, 30%, 40%, 50%, 60%, sterile distilled water as a negative control, and 5 µg ciprofloxacin as a positive control into the well. The media was wrapped in brown paper, labeled, and incubated at 37˚C for 24 hours. The clear area was analyzed as an inhibition zone on each well. The inhibition zone, or clear area, was coded as 1 and 0 for no clear area.

Contact time analysis

The micropipetting solution from each tube containing microorganisms from the used toothbrush was added to the first

tube containing 9 mL of 0.85% NaCl and mixed until homogeneous. The solution was pipetted from the first tube to the second tube, and this process was repeated until the fifth tube. Then, 1 mL of solution from each NaCl tube was transferred into a labeled petri dish. Next, approximately 15 mL of Plate Count Agar (PCA) media was added at a temperature of 40-45ºC. The treatment was conducted for eight used toothbrushes. The number of bacterial colonies in each PCA medium using a colony counter was counted. Points 1 and 2 were carried out for contact times of 10, 20, 30, 40, 50, and 60 minutes, and each treatment was repeated 4 times.

Statistical analysis

All data were statistically analyzed by using SPSS software package 25 for Windows. The normality test was used prior to the differential test. The Levene test based on the mean (α=0.05) was used to analyze the homogeneity. The Kruskal-Wallis test was used to see the effectiveness differences of the infusions (K1, K2, K3, K4, control +, and control –) on inhibition of bacterial growth. The Mann-Whitney test was carried out to see the inhibition efficacy differences between each combination on bacterial growth.

Results

After incubating, the solution containing the used toothbrush in TSB became cloudy, as shown in Figure 1. To study the presence of bacteria contained in toothbrushes and to determine gram-positive and gram-negative colonies, the bacterial suspension of the TSB media was inoculated in the blood agar. The result shows that the shape, elevation, color, and characteristics of bacterial colonies were round, convex, and cloudy for β-hemolysis, while round, convex, and murky or slimy color for non-hemolysis.

The suspected *Staphylococcus aureus* on Mannitol Salt Agar (MSA) is based on a round shape, 1-2 mm in size, convex elevation, gold color, and hemolysis. The serological test detected the presence of bacteria coagulating from all used toothbrushes.

Inoculation on MCA

Table 1 shows the observed results for the shape and size, elevation, color, and characteristics of bacterial colonies on MC agar. The bacterial characteristics were lactose fermenter and non-lac-

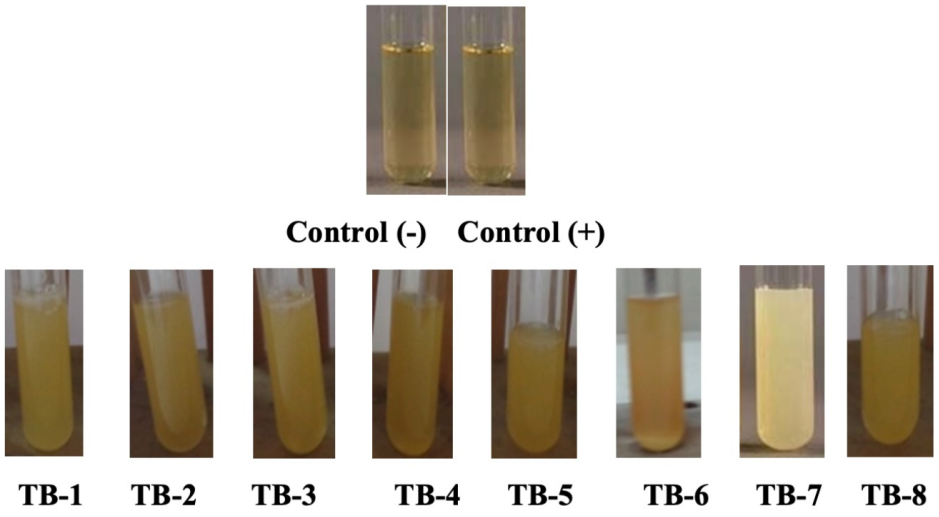

Figure 1. The bacterial turbidity of solution containing used toothbrushes in tryptic soy broth (TSB).

Table 1. Results of observations of bacterial colonies on MacConkey agar.

Sample code	Colony	Shape and Size	Elevation	Color	Characteristic
TB 1	1	Round, 2-3 mm	Convex	Pink	Lactose fermenter
	2	Round, 1-1.5 mm	Convex	Yellow	Non lactose fermenter
TB 2	1	Round, 3-4 mm	Convex	Pink, slippery	Lactose fermenter
TB 3	1	Round, 2mm	Convex	Pink, slippery	Lactose fermenter
	2	Round, 3mm	Convex	Pink, rough	Lactose fermenter
TB 4	1	Round, 3-4 mm	Convex	Pink, slippery,slimy	Lactose fermenter
TB 5	1	Round, 2mm	Convex	Pink, slippery	Lactose fermenter
	2	Round, 3mm	Convex	Pink, rough	Lactose fermenter
TB 6	1	Round, 3mm	Convex	Pink, mucoid	Lactose fermenter
	2	Round, 2-3 mm	Convex	Yellow	Non lactose fermenter
TB 7	1	Round, 2-3 mm	Convex	Pink	Lactose fermenter
	2	Round, 4mm	Convex	Yellow	Non lactose fermenter
	3	Round, 3mm	Convex	Yellow, mucoid	Non lactose fermenter
TB 8	1	Round, 3-4 mm	Convex	Pink, slippery	Lactose fermenter

TB, toothbrush.

tose fermenter. To determine the growth characteristics of the bacteria from the *Enterobacteriaceae* group, a biochemical test was carried out on each colony on MC agar. The bacteria were *Escherichia coli, Enterobacter cloacae, Pseudomonas sp, Klebsiellaozaena,* and *Proteus sp.* The results of biochemical tests are shown in Table 2.

Inhibition test of bay leaf and betel leaf infusion on bacterial growth on toothbrushes

The mean inhibitory levels of the four combinations K1, K2, K3, and K4 compared to the positive control (ciprofloxacin 5 mg) are presented. The diameter of the inhibitory zone on the negative control on all toothbrushes was 0, while the positive control on all toothbrushes was 38, 30, 28, 25, 19, 27, 24, and 28, respectively. The four combinations of the mixtures were below positive control. The inhibition zone of the combinations was K1: 4, 13, 16, 17, 14, 17, 16, and 0, K2: 15, 16, 13, 14, 15, 17, 14, and 0, K3: 10, 14, 13, 13, 14, 17, 12, and 12, K4: 17, 17, 13, 13, 0, 5, 19, 12, and 0.

Table 3 displays the average number of bacteria at different time intervals (initial, 10, 20, and 30 minutes) after immersing the toothbrushes in a mixture of *Eugenia polyantha* and *Piper betle* infusion (K4).

Statistical analysis

Based on the Kruskal-Wallis test, there was a significant difference between the diameter of the inhibition of betel leaf infusion in the six data groups (K1 K2 K3 K4 cipro 50 µg/ 50 µL, and sterile aqua dest as a negative control). The Whitney test showed the diameter of inhibition for each combination of K1, K2, K3, K4, ciprofloxacin, and aqua dest was $p<0.05$. However, the diameter of the inhibition of betel leaves among combinations was not significantly different ($p>0.05$).

Discussion

In this study, all incubated solutions containing samples from used toothbrushes were cloudy. This indicates that oral bacteria from the used toothbrushes successfully grew in TSB. In healthy individuals, toothbrush contamination occurs immediately after use and increases with repeated use.[3,6] Moreover, the used toothbrush will likely be contaminated by pathogenic opportunists in a sick condition.[4] A recent study found that used toothbrushes har-

Table 2. Biochemical test results from MacConkey agar media.

Sample code	Colony	Observation result				Suspect
		Indole	MR	VP	SC	
TB 1	LF	+	+	–	–	*Escherichia coli*
	LF	–	–	+	+	*Enterobacter cloacae*
	NLF	–	–	–	–	*Pseudomonas sp*
TB 2	LF	+	+	–	–	*Escherichia coli*
	LF	–	–	+	–	*Klebsiellaozaena*
TB 3	LF	+	+	–	–	*Escherichia coli*
	LF	–	–	+	–	*Klebsiellaozaena*
	NLF	–	–	–	–	*Pseudomonas sp*
	NLF	+	+	–/+	+/–	*Proteus sp*
TB 4	LF	+	+	–	–	*Escherichia coli*
	LF	–	–	+	–	*Klebsiellaozaena*
TB 5	LF	+	+	–	–	*Escherichia coli*
	LF	–	–	+	–	*Klebsiellaozaena*
TB 6	LF	+	+	–	–	*Escherichia coli*
	NLF	–	–	–	–	*Pseudomonas sp*
TB 7	LF	+	+	–	–	*Escherichia coli*
	LF	–	–	+	–	*Klebsiellaozaena*
	NLF	–	–	–	–	*Pseudomonas sp*
	NLF	+	+	–/+	+/–	*Proteus sp*
TB 8	LF	+	+	–	–	*Escherichia coli*
	LF	–	–	+	–	*Klebsiellaozaena*

TB, toothbrush; LF, lactose fermenter; NLF, non-lactose fermenter; MR, methyl red; VPs, Voges-Proskauer; SC, Simmons Citrate. TB, toothbrush.

Table 3. Average number of bacteria on a toothbrush after soaking with a mixture of *Eugenia polyantha and Piper betle* Infusion (K4).

Dilution of the infusion mixture	Average number of bacteria (CFU/mL) at contact time (minutes)			
	0	10	20	30
1: 4	81,667	62,967	23,689	32,900
1: 9	>111,000	>111,000	>111,000	>111,000

bored antimicrobial-resistant bacteria such as *Staphylococcus aureus*, *E. coli,* and *Pseudomonas*.[8] Therefore, based on the findings, to reduce the risk of contamination, the used toothbrush should be reconsidered to disinfect immediately after use.

The commensal oral flora was identified by cultivating samples on various types of media The findings of this study were consistent with previous research that identified *Streptococcus* growth on blood agar, *Klebsiella sp, Enterobacteriaceae sp (Coliform sp), Pseudomonas* growth on McConkey agar, and *Staphylococcus aureus* on mannitol salt agar.[33-35] In addition, as a normal flora in dental plaque, *Streptococcus mutans* is also higher in teeth with cavities. The selected participants had more than three cavities due to caries. As a result, the findings indicated that the growth of microorganism colonies on blood agar, Mannitol Salt Agar (MSA), and MacConkey Agar (MC) consisted of oral microorganisms that were transferred by toothbrushes.

All compositions of the mixtures can inhibit the growth of bacterial colonies, which was evidenced by the formation of an inhibition zone around the bacterial colonies grown on Mueller-Hinton Agar (MHA). The inhibition zone of the mixture was varied for each toothbrush, ranging from 12 mm to 19 mm; the lowest inhibition zone was found on toothbrush number 8 with an inhibition zone of 12 mm (K3), and the highest inhibition zone was on toothbrush number 6, which is 19 mm (K4). Therefore, the infusion composition of the combination of 70% *Eugenia polyantha* + 30% *Piper betle* has the greatest inhibitory power against the growth of decontaminant bacteria compared to the other compositions. However, the average diameter of the K4 inhibition zone (70% *Eugenia polyantha* + 30% *Piper betle*) is 13.47 mm, which is relatively slightly smaller when compared to the average diameter of the inhibition zone for bay leaf infusion in the previous study.[36] This is probably due to a reduction in the concentration of each part at the combined concentration.

This study concluded that both formulations of *Eugenia polyantha* and *Piper betle* have potential effects on gingivitis prevention. They had an effectiveness level almost similar to chlorhexidine gluconate 2%.

The F-II formula demonstrated superior physical indicator values, as these were closer to the standard values. For its antibacterial properties and improved physical indicators, which align better with herbal standard values, the F-II formula is recommended as a mouthwash for gingivitis.

The absence of bacterial growth on the PCA medium indicated that a 70% *Eugenia polyantha* and 30% *Piper betle* (K4) combination exhibited bactericidal properties. In addition, the K4 mixture was diluted at 1:4 and 1:9. The results revealed that diluting K4 in a 1:4 ratio was better than 1:9. However, bacterial colonies in K4 dilutions at ratio 1:4 with contact times of 0, 10, 20, and 30 minutes were >300. O'Toole stated that if the number of colonies reaches 300, the bacterial count will not meet the required standards.[37] Therefore, further examination with a different dilution ratio is necessary. Furthermore, the extract mixture was tested against four isolated pathogenic bacteria: *Streptococcus sp, Klebsiella sp, Enterobacteriaceae sp (Coliform sp), Pseudomonas and Staphylococcus*. The aqueous extract exhibited antibacterial activity against gram-positive and gram-negative bacteria. Several studies have found that these plants effectively inhibit bacterial growth. *Piper betle* is widely used in Ayurvedic and Southeast Asian traditional medicine for antimicrobial, mouthwash, and wound healing. *Eugenia polyantha* is also used in traditional medicine to treat gastrointestinal problems and as an antibiotic. Although *Piper betle* is often used as a mouthwash, this study discovered that a higher concentration of *Eugenia polyantha* produced better results.

The widespread use of *Eugenia polyantha* for gastrointestinal disorders, along with the similarity of microbiome strains in oral and intestinal populations, highlights its potential effectiveness and merits further investigation.

Conclusions

Streptococcus sp., Klebsiella sp., Enterobacteriaceae sp. (Coliform sp.), Pseudomonas and *Staphylococcus aureus* are the contaminant bacterial colonies that remain on used toothbrushes after washing with water. The optimal bactericidal properties and highest inhibition levels can be achieved by combining a 70% infusion of *Eugenia polyantha* leaves with a 30% infusion of *Piper betle* leaves.

Further studies are necessary to comply with pharmaceutical regulations to prevent side effects regarding quality testing, safety, efficacy, pricing, and marketing approval procedures.

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