Protective effects of mouthwash formulations of *Syzygium polyantha* (L.) and *Piper betel* (L.) on oral microbiota-induced gingivitis

Megananda Hiranya Putri,[1] Neneng Nurjanah,[1] Dewi Sodja Laela,[1] Susi Sukmasari[2]

[1]Dental Health Department, Politeknik Kesehatan Kemenkes Bandung, Bandung, Indonesia; [2]Paediatric Dentistry and Dental Public Health Department, Kulliyyah of Dentistry, International Islamic University Malaysia, Malaysia

Abstract

Using a combination of natural ingredients as a mouthwash was expected to have a synergistic effect in preventing gingivitis, a common oral disease. The objective of this study was to elucidate the anti-inflammatory effect of different proportions of mouthwash infusions: F1 (75% Syzygium polyantha and 25% Piper betle) and F2 (25% Syzygium polyantha and 75% Piper betle) on oral microbiota causing gingivitis. Twenty-four Rattus norvegicus were divided into four groups, and bacteria were injected into the periodontal sulcus. The anti-inflammatory effect was assessed by calculating the reduced number of polymorphonuclear (PMN) leukocytes. A cytotoxicity test was carried out on the normal fibroblast cell line 3T3-L1. There were no significant differences in the decreased number of PMN leukocytes ($p=0.079>0.05$). Both F1 and F2 showed results of cell viability approaching 100% of living cells at concentrations of 0.29 ppm and 0.04 ppm, equivalent to 0.058% and 0.029%, respectively. This study concluded that both formulations of Syzygium polyantha and Piper betle have potential effects on gingivitis prevention. They had an effectiveness level almost similar to Chlorhexidine gluconate 2%. The toxicity value of formulation F1 is superior to that of formulation F2. Further studies concerning the toxicity of the mixtures and their effect on oral biofilm are needed.

Correspondence: Susi Sukmasari, Paediatric Dentistry and Dental Public Health Department, Kulliyyah of Dentistry, International Islamic University Malaysia, Malaysia. E-mail: sukmasari@iium.edu.my

Key words: mouthwash formulations, *Syzigium polyanta* leaves, *Piper betle* leaves, anti-inflammation, gingivitis.

Contributions: MHP, conceptualization, data curation, formal analysis, methodology, validation, visualization, original draft writing. NN, conceptualization, investigation, methodology, validation, and writing – original draft writing. DSL, supervision, and original draft writing. SS, writing, visualization, review and editing.

Conflict of interest: the authors declare no conflict of interest.

Funding: this research was supported by a research grant from Director of Bandung Polytechnique of Health: No LB.02.01/3.1/ 3388.C /2021.

Ethics approval and consent to participate: the research has received ethical approval from the Health Research Ethics Commission, Bandung Polytechnique of Health No 28 KEPK/EC/VII/2022.

Patient consent for publication: not available.

Availability of data and materials: all data generated or analyzed during this study are included in this published article.

Acknowledgement: we are fully appreciative of all the researchers who helped in this study. We are grateful to have received grants from the Indonesian Ministry of Health (RISBINAKES).

Received: 11 September 2023.
Accepted: 6 November 2023.
Early access: 19 December 2023.

Healthcare in Low-resource Settings 2024; 12:11768
doi:10.4081/hls.2023.11768

Introduction

The quality of life can be affected by periodontal diseases. Gingivitis is the early sign of periodontal diseases, with a prevalence of about 20-50% in the global population, including both developed and developing countries. Gingivitis is gum inflammation caused by certain oral microbiota harbored on dental biofilm.[1] The clinical symptoms include swelling at the edges of the gums, redness, and bleeding when brushing teeth. The inflammation occurs when dental plaque is dominated by anaerobic Gram-negative bacteria.[2] The bacteria that cause gingivitis are Gram-negative bacteria, such as *Porphyromonas gingivalis*, *Tannerella forsythia*, *Treponema denticola*, *Actinomyces viscosus*, *Selenomonas anaerobius*, *Aggregatibacter actinomycetemcomitans*, and Gram-positive bacteria like *Streptococcus sanguinis*, *Streptococcus mutans*, and *A. viscosus*.[3,4] Globally, the disease affects more than 90% of the population and found in Hyderabad that 70.4% children ages 5-15-year-old.[5]

Untreated gingivitis can develop into periodontitis.[6] Referring to the Indonesian Basic Health Survey in 2018, the prevalence of periodontitis among individuals aged 15 to 65 years old was more than 67.8%.[7] Gingivitis in the early stages is characterized by increasing levels of PMN leukocytes. Lipopolysaccharide (LPS) is a major component of the cell wall of Gram-negative bacteria that activates neutrophils. Activated neutrophils then release large amounts of pro-inflammatory substances. These substances are chemoattractants, causing the migration of neutrophils from blood vessels to injured tissues and enabling them to kill microbes.[8] The continued activation of neutrophils and the production of hydrolytic enzymes by other Gram-negative bacteria in the plaque cause damage to the intercellular components of the gingival epithelium and the underlying connective tissue. It is evident that the number of hydrolytic enzymes in the gingiva and saliva of patients with gingivitis is increased.[9] The increasing number of

PMN leukocytes in the tissue represents the inflammation process. Besides tooth brushing as a mechanical practice to control plaque, safe preventive substances are needed to prevent gingivitis or to stop the process from progressing into a severe condition. Mouthwash is one of the delivery methods that can incorporate certain active ingredients to prevent gingivitis.[10]

The content of herbal plants contains metabolite compounds that have the potential to act as antibacterial, antifungal, anti-inflammatory, and palliative or pain-relieving agents.[1,10,11] Bay leaves (Syzygium polyantha Wight) have been known for a long time as a plant that can be used for treatment and has many benefits. The metabolites found in Syzygium polyantha plants include saponins, triterpenoids, flavonoids, polyphenols, alkaloids, tannins, and essential oils consisting of sesquiterpenes, lactones, and phenols. In dentistry, bay leaf extract can be used for root canal treatment, as an active ingredient in toothpaste, mouthwash solutions, and toothbrush disinfectants, and it appears that research on this subject will continue to grow.[12-14]

Green betel leaf (*Piper betle* L.) is one of the medicinal plants that is widely used in traditional medicine in several countries for strengthening teeth, healing minor mouth wounds, eliminating body odor, stopping bleeding gums, and serving as a mouthwash. The antimicrobial properties of green betel leaf (*P. betle*) are highly valuable when used to treat infections caused by pathogenic microorganisms in the human body, such as inhibiting the growth of *C. albicans*.[15] The content of phenol (carvacrol) and phenylpropane (eugenol and kavikol) in the essential oil of green betel leaf (*P. betle*) functions as a potent antimicrobial (bactericide) and fungicide.[16,17] The primary reason behind the antibacterial effect of betel leaf is that it contains 4.2% essential oil, with its main component consisting of betel phenol and its derivatives, which possess antibacterial properties.[18,19]

Research conducted by Sung Ho Lee (2021) demonstrated that the combined effects of natural ingredient extracts, when compared with the effects of single extracts and the control group (chemical-based mouthwash), showed a more beneficial effect.[20] This research suggests that similar outcomes may occur with other combinations of natural products.

This study aims to evaluate the anti-inflammatory effect of a mouthwash based on a combination of infusions (aqueous extracts) in two different proportions of Syzygium polyantha and Piper betle as active ingredients. This evaluation will be conducted by observing the histopathological description of polymorphonuclear (PMN) leukocytes in the gingiva of white rats (Rattus norvegicus) of the Wistar strain induced with gingivitis. Additionally, the study aims to determine the safe concentrations that allow for 100% cell viability after exposure for 24 hours to both types of mouthwash. It is anticipated that the mixtures will determine the best mouthwash formula out of two different proportion of *Syzygium polyantha* and *Piper betle.*

Materials and Methods

The mouthwash formula and PMN leukocyte measurements were conducted at the Chemistry and Pharmacology Laboratory at the Faculty of Medicine. The gingival histopathology of rats was examined at the Histopathology Laboratory.

Mouthwash production

Fresh leaves were purchased from the local market and transported to the laboratory on the same day. The research was conducted at the Faculty of Medicine, Padjadjaran University, Bandung, Indonesia. The leaves were identified by an expert from the Integrated Laboratory at Bandung Polytechnic of Health. It cleaned and cut into smaller pieces. One kg of freshly ground Syzygium polyantha leaves and one kilogram of freshly ground Piper betle leaves were boiled in separate glass beakers, each containing 1 liter of distilled water, at 90°C for 20 minutes. After cooling, a mouthwash formulation 1 (F1) was created by mixing 75% Syzygium polyantha leaves with 25% Piper betle leaves, and a mouthwash formulation 2 (F2) was made by mixing 25% Syzygium polyantha leaves with 75% Piper betle leaves. he following additives, Tween 80 (10%), Peppermint oil (1%), Sodium benzoate (0.4%), Sodium saccharin (6%), and food coloring (0.2%), were added to both formulations in the same proportion. In the formulation, Tween was used as a solvent and suspending agent to homogenize the solution; Sodium benzoate served as a preservative because it can inhibit the growth of bacteria and fungi in acidic conditions; Saccharin was used as a sweetener; Peppermint oil was added to provide a distinctive aroma, and food coloring was included as an additive to enhance attractiveness, maintain uniformity, stabilize the color, and prevent discoloration of the solution. The resulting solution was homogeneous, clear, light brown in color, with a mint aroma and a sweet taste. The pH, specific gravity, and viscosity of F1 were 4.59, 1.0126 g/cm^3, and 2.0784 cSt, respectively, while the pH, specific gravity, and viscosity of F2 were 4.49, 1.0155 g/cm^3, and 2.1607 cSt, respectively.

Anti-inflammation test on wistar rats

Animal research was conducted with ethical approval from the Health Research Ethics Commission, Bandung Polytechnic of Health, under the reference number 28 KEPK/EC/VII/2022. A total of 24 male white rats (Rattus norvegicus) of the Wistar strain, aged 2-3 months and weighing 180-200 grams, were acclimatized for 5 days to ensure their healthy condition. The rats were then grouped into 4 groups, with each group consisting of 6 rats according to the intervention they were to receive.

All rats were induced with gingivitis by injecting 0.02 ml of Porphyromonas gingivalis and Streptococcus sanguinis bacterial suspension (10 McFarland turbidity ratio 1:1) into the gingival pocket of the left and right mandibular first incisors on the labial surface. The injections were performed using a 30 mg insulin needle. Prior to the induction, 0.3 ml of Ketamine was injected into the upper thigh muscles of the rats as a sedative. This gingivitis induction was repeated for 5 consecutive days, once a day.

On the 5th day after induction, gingivitis reached a peak condition. Which was marked by the clinical appearance of redness and swollen gums.

Two rats from each group had their gum tissue collected, which was then stored in 8 containers containing Formalin solution and sent to the histopathology lab for the preparation of smear samples and the examination of the number of PMN leukocytes (neutrophils). The remaining four rats in each group were subjected to the following treatment plan: Group I: The negative control group received mouthwash without active ingredients. Group II: The positive control group received a comparison mouthwash containing Chlorhexidine Gluconate 0.2%. Group III: Mouthwash formula F1, which consisted of 75% Syzygium polyantha and 25% Piper betle as active ingredients, was administered. Group IV: Mouthwash formula F2, with an active ingredient combination of 25% Syzygium polyantha and 75% Piper betle, was used. The solutions were applied to the labial gingiva using a dropper, and the rats were held in position for 1 minute, simulating the principle of gargling with a mouthwash solution with a normal exposure

time of 1 minute. This process was repeated for 2 consecutive days, with a frequency of 2 times a day. On the 2nd day (D+2) and 5th day (D+5) after induction, 2 rats were selected from each group. Their gum tissue was cut and stored in 8 containers containing a 10% formalin solution, and then sent to the histopathology laboratory for the preparation of smear samples and neutrophil counts. The analysis of the difference in the average decrease in the number of PMNs from the peak of gingivitis until the 5th day after the administration of the 4 groups of mouthwashes was conducted using the Kruskal-Wallis and Mann Whitney tests.

Cytotoxicity assays (Cytotoxicity assays) with the MTT Assay method

The cells tested were the normal fibroblast cell line 3T3-L1, cultured in complete Roswell Park Memorial Institute (RPMI) culture media containing 10% Fetal Bovine Serum (FBS) and 1% Penicillin-streptomycin antibiotics. The cells used were at 80% confluence. The media was removed from the flask, and the cells were rinsed twice with 10 mL of PBS. Next, 3 mL of Trypsin-EDTA solution was added, and the cells were incubated for 5 minutes until the cell layer detached. Afterward, the cells were centrifuged at 1500 rpm for 5 minutes, and the cell pellet was reconstituted with a new complete medium.

The cell culture was seeded into 96-well plates and incubated for 24 hours. After incubation, the media from each well was removed. Each well was then refilled with various concentrations of formulas F1 and F2. Triplicates of each dilution dose were prepared, and the treated cells were re-incubated for 24 hours. The MTT Assay kit reagent was applied, and the absorbance was measured using the Elisa reader Multiskan EX with a wavelength of 550 nm.

Results

In this study, the potential anti-inflammatory effects of the mouthwash were observed by assessing its ability to reduce the number of PMN leukocytes in the gum tissue of Wistar rats induced with gingivitis on the 2nd and 5th days after exposure to the four groups of test solutions. The initial measurements of the decreasing PMNs in the negative control, positive control, formula 1, and formula 2 were 31.9, 43.8, 31.8, and 93.5, respectively. The second measurements were 62.1, 28.6, 11.7, and 7.5, respectively. The final measurements were 7.7, 27.2, 5.2, and 11.5, respectively (Figure 1).

The Kruskal-Wallis test was employed to compare the mean decrease in the number of PMNs after mouthwash instillation among the four groups following three observations. A p-value of 0.079 was obtained. The comparison of the average decrease in the number of PMNs after the instillation of the four mouthwash groups, between the two treatments and another group's solutions, was conducted using the Mann-Whitney U test. The results showed that when comparing Formula 1 to the negative control and positive control, the values were -1.091 and -1.091, respectively. For Formula 2, the comparison to the negative control resulted in 0.000, and to the positive control, it was -0.655. When comparing Formula 1 to Formula 2, the value was -0.218.

The results of the MTT assay show the average number of living cells for mouthwash Formula 1 (75% Eugenia polyanta: 25% Piper betle) at various concentrations (ppm): 0, 0.07, 0.15, 0.29, 0.59, 1.17, 2.34, 4.69, 9.37, 18.75, 37.5, 75, and 150. The values were 100, 102.46, 137.22, 119.80, 69.69, 15.30, 4.37, 1.38, 2.42, 4.58, 7.25, -1, 34, and -0.95, respectively. Meanwhile, the average living cells at these concentrations were 0, -2.46, -37.22, -19.80, 30.31, 84.70, 95.63, 98.62, 97.58, 95.42, 92.48, 101.34, and 100.95, respectively.

The results of the MTT assay also show the average number of living cells for mouthwash Formula 2 (25% Eugenia polyanta: 75% Piper betle) at various concentrations (ppm): 0, 0.07, 0.15, 0.29, 0.59, 1.17, 2.34, 4.69, 9.37, 18.75, 37.5, 75, and 150. The values were 100, 96.84, 108.30, 112.36, 76.70, 40.51, 2.032, 1.69, 1.64, 5.53, 8.26, 0.09, and 1.60, respectively. Meanwhile, the average living cells at these concentrations were 50, 48.43, 54.17, 56.23, 38.45, 20.45, 1.41, 1.62, 2.38, 5.89, 10.38, 12.54, and 25.80, respectively.

Discussion

Periodontal microbiota contained in dental biofilm induce an inflammatory reaction of gingival tissue.[21] If the inflammation is not treated, it can develop into periodontitis, which may result in damage to the alveolar bone, tooth loss, and potentially pose risks to systemic health. In this study, Streptococcus sanguinis is considered an early colonizer that facilitates the invasion of gingival cells by periodontopathic pathogens in dental biofilm, such as *Porphyromonas gingivalis*.[22] Both of them represent oral microbiota that can cause gingivitis.[23-25] This study has proven that gingivitis was successfully developed in rats both clinically and histologically following the injection of both *Porphyromonas gingivalis* and *Streptococcus sanguinis* in all groups. The gingiva under the two lower incisor teeth swelled after the injection, supported by an increasing number of PMNs.

The exposure of rat gingiva to *P. gingivalis* continued to induce neutrophils and other inflammatory cells to produce cytokines and proinflammatory enzymes, which led to more neutrophils migrating from the vascular stream to inflamed tissues. This is evidenced by the high number of neutrophils on the second day. Cytokines produced in the inflammatory process can either increase or inhibit the inflammation process. As inflammation develops, neutrophils

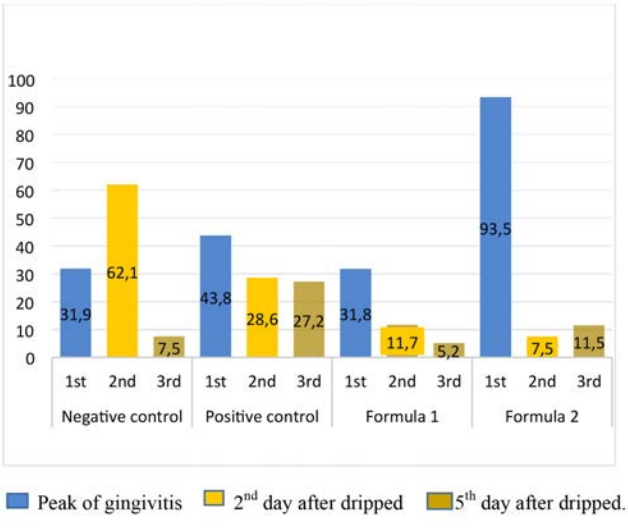

Figure 1. The average number of PMNs at the peak of gingivitis and after dripping on 2nd day and 5th day.

undergo an apoptotic mechanism through the production of TNF-α, leading to the death of the neutrophils themselves. Therefore, the number naturally decreased by the 5th day.[13,26]

In addition to mechanical actions for controlling dental biofilms, such as toothbrushing and flossing, therapeutic topical mouthwashes with active ingredients that are followed by rinsing and gargling can help control dental biofilm, reduce gingivitis, bad breath, and tooth decay.[12,27] The practice of mouth rinsing by humans has been documented for over two millennia, beginning as traditional Chinese medicine.[28] Here is a wide variety of mouthwashes available in the market and prescribed as adjuvants for managing complex oral conditions, including oral mucositis and even xerostomia. Furthermore, due to the Covid-19 outbreak, some mouthwashes have been suggested for pre-treatment rinsing prior to oral or dental procedures.[29,30] However, it's essential to consider the risks of antimicrobial drug resistance, adverse effects, or misuse.[13,31]

Chlorhexidine 2%, as a second-generation mouthwash, has been proven to reduce plaque by 70–90% and remains effective for 18 hours or more. The electrostatic and hydrophobic interactions of Chlorhexidine directly damage bacteria. It is effective against both Gram-positive and Gram-negative bacteria in the oral cavity, and it has been demonstrated to be one of the antiseptic mouthwash ingredients that can reduce and alleviate various oral cavity complaints.[12,20,28,30] Chemical-based mouthwashes, such as Chlorhexidine, have drawbacks, including the potential for teeth staining and frequent complaints of burning or stinging sensations in the user's oral mucosa. Prolonged use of Chlorhexidine can also disrupt the normal flora balance in the oral cavity.[15,20] In this study, Chlorhexidine gluconate 2% was used as a positive control, demonstrating anti-inflammatory activity. This was indicated by a decrease in the average number of PMN leukocytes from 43.8 at the peak of gingivitis to 28.6 on the second day, with a slight further decrease on the 5th day after exposure.[30,32,33]

There is evidence from many clinical and experimental studies emphasizing the role of several herbs in reducing inflammation. Some compounds contained in these herbs have been verified to have a potential effect against microorganisms.[20,34] Tannins and flavonoids are the main polyphenolic compounds found in the combination of bay leaf and betel leaf, which exhibit anti-inflammatory effects. The infusion of the combination of bay-betel leaves also contains essential oils and phenolic compounds that possess stronger antibacterial properties.[35]

Flavonoids, which are widely distributed and relatively low in toxicity, can be safely consumed in the diet and show potential anti-inflammatory and antioxidant effects. Flavonoid interventions with low cost are widely used in the clinical treatment of various diseases.[36] The anti-inflammatory mechanisms of flavonoids include the inhibition of the formation of proinflammatory enzymes, such as Cyclooxygenase-2, Lipoxygenases, and NO-inducing enzymes. They also inhibit NF-κB and activate protein-1 (AP-1), as well as phase II activation of antioxidant detoxifying enzymes, protein kinase C, and erythroid factor-2.[37,38] The superiority of mouthwash based on a combination of herbal extracts has been proven in several previous studies.[20,39,40]

Considering the intended purpose of formulating mouthwash, the extraction method used was infusion. Water-based extraction is a highly polar solvent, which is inexpensive, nontoxic, nonflammable, and contains polar substances.[44] Bay leaves (Syzygium polyanta) and Piper betel are traditionally and widely used as herbs and medicine throughout India, Asia, and the Western world. Both herbs have been established to contain various compounds, including flavonoids and tannins. Tannins and flavonoids are the main polyphenolic compounds found in the combination of Syzygium polyanta leaves and Piper betel leaves.[12-14,19,35]

F1 is a mouthwash formula with an active ingredient in combination with an infusion of Syzygium polyanta leaves and Piper betle (75%: 25%). It demonstrates anti-inflammatory properties, as evidenced by the decrease in the average number of PMN leukocytes from the peak of gingivitis to the second and fifth days after application. It is observed that when gargling is performed during the peak condition of gingivitis, the effect of reducing PMN leukocytes continues until the fifth day. However, something different occurs with the F2 mouthwash, which contains the active ingredient combination of an infusion of Syzygium polyanta leaves and Piper betle leaves (25%: 75%). The PMN numbers showed a sharp decrease between the first application and the second day but increased again on the fifth day. This suggests that the condition can be influenced by various factors related to the rats or the interaction between active compounds in different concentrations. However, this issue requires further investigation. This study successfully demonstrates an anti-inflammatory effect in the positive controls, formulas F1, and F2. The analysis indicates that there are no significant differences among the three solutions. It can be assumed that F1 and F2 have almost the same effect as Chlorhexidine in reducing the inflammatory reaction in gingivitis.

The cytotoxicity test, using the MTT Assay method, determined the levels of active ingredients in sample formula F1 at a tested concentration of 30%, equivalent to 150 ppm. The results indicated that the IC50 value for F1 was 0.7032 ppm, which is equivalent to a concentration ranging between 0.117% and 0.234%. The safe concentration for F1 is defined as the highest concentration that results in cell viability approaching 100%. According to the absorbance table (Table 5), this concentration value is 0.29 ppm, equivalent to a concentration of 0.058%.

For sample formula F2, tested at a concentration of 30% or equivalent to 50 ppm, the results showed that the IC50 value for F2 was 0.3206 ppm, equivalent to a concentration ranging from 0.117% to 0.234%. The safe concentration for F2 is also defined as the highest concentration leading to cell viability approaching 100%. From the Absorbance Table (Table 6), the concentration value was found to be 0.04 ppm, which is equivalent to a concentration of 0.029%.

The results of the toxicity test for both F1 and F2 formulas showed values of 0.058% and 0.029%, respectively. These values indicate that the mouthwash formulas are non-toxic when used at concentrations lower than these figures. However, when considering the IC50 value, F1, with a value of 0.7032 ppm, is higher than F2, which has a value of 0.3206 ppm. This suggests that the toxicity of F2 is greater than that of F1 because, at a lower concentration, F1 can cause the death of approximately 50% of living cells compared to F2. It's important to note that the toxicity values of the F1 and F2 mouthwash formulas in this study were much lower than the toxicity value of essential oil (Myrrh oil) on fibroblast cells and human epithelial cells, as investigated by Tipton *et al.* (2003) using the same method type.

In addition to developing the ideal requirements of a mouthwash to achieve the best antimicrobial activity, there is an emphasis on the capability to maintain beneficial commensal species in the dental biofilm or saliva, which is an essential component of human health. This aspect holds promise for further study. Understanding the interactions among potential herbs at the molecular level may hold the key to rationalizing herb combinations for future drug discovery.

Conclusions

This study concludes that both formulations of Syzygium polyantha and Piper betle have the potential to prevent gingivitis, with an effectiveness level nearly similar to Chlorhexidine gluconate 2%. The toxicity value of formulation F1 is superior to that of formulation F2.

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