pH And Antibiofilm Analysis Of Elephant Ginger (*Zingiber Officinale Var. Officinale)* Mouthwash Formulations Against *Streptococcus Mutans* – An In Vitro Study

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ABSTRACT

Background: *Caries is the most common oral disease. Streptococcus mutans is the main microorganism in caries etiology due to its ability to form biofilm. Biofilm can be eliminated using mouthwash. Elephant ginger (Zingiber officinale var. officinale) can be developed as a herbal mouthwash because it is able to inhibit S. mutans. This study aimed to analyze pH and antibiofilm effect in vitro of elephant ginger mouthwash formulations on S. mutans.*
Method: *Elephant ginger was macerated with 96% ethanol, then formulated into 2.5%, 5%, 10%, and 15% mouthwash formulations, underwent pH measurement for 28 days, and their antibiofilm effect on S. mutans were measured using microplate reader for 1h (therapeutic assay) and 24h (preventive assay).*
Result: *All formulations showed pH values ranging from 6.42-6.87, which remained stable for 21 days and decreased significantly on day 28. All formulations were able to reduce S. mutans biofilm adherence for 1h better than commercialized herbal mouthwash and similar to 0.1% CHX. Furthermore, 5% and 10% mouthwash formulations showed similar effectivity to 0.1% CHX and commercialized herbal mouthwash in inhibiting S. mutans biofilm formation for 24h, while 15% mouthwash formulation was more effective than 0.1% CHX and similar to commercialized herbal mouthwash, and 2.5% mouthwash formulation was less effective than 0.1% CHX and commercialized herbal mouthwash.*
Conclusion: *Elephant ginger mouthwash formulations showed pH values that were stable for 21 days and had the potential as an herbal mouthwash due to its antibiofilm effect on S. mutans.*

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INTRODUCTION

Many measures have been taken to overcome dental caries.[1] Even so, caries still affects most of world's populations compared to other oral diseases according to 2017 *Global Burden of Disease Study*.[2]

Dental caries is the demineralization of teeth hard tissues brought about by acid which is the end-product of oral acidogenic and aciduric microorganisms' metabolism.[3] Dental plaque (biofilm), an accumulation of oral microbiota that has complex structure and abundant extracellular polymeric substances (EPS), possesses a significant role in caries pathogenesis.[4] It has been a common understanding that *Streptococcus mutans* acts as the main microorganism involved in caries etiology.[5] *Streptococcus mutans* is a highly cariogenic pathogen, owing to its ability to form biofilm by synthesizing glucan from sucrose which promotes biofilm matrix formation, metabolize carbohydrates into organic acid, and thrive in acidic environments.[6] Extracellular polymers are insoluble and act as a resistant matrix structure or barrier to the diffusion of salivary metabolites and buffers into plaque.[5,7]

Dental plaque can be removed through mechanical approach (toothbrush, dental floss, toothpick, and interdental brush) and chemical approach (mouthwash). Despite being the most widely used method to improve oral hygiene, most people are unable to brush their teeth effectively.[8] Mouthwash provides a simpler alternative to reduce dental plaque for patients with a lack of dexterity and motivation to brush their teeth.[9] Mouthwash can also deliver itself into areas that are not accessible to toothbrush.[10]

Chlorhexidine gluconate (CHX) is the most common mouthwash used in dentistry. However, its usage can inflict some unfavourable side effects, such as brown-discoloration of teeth, restoration material, and tongue, taste disturbance, burning sensation and erosion on oral mucosa, parotid gland swelling, and increasing supragingival plaque formation.[8,9,11]

The search for another mouthwash alternatives has shifted its focus to herbal ingredients as a way to cope with CHX's side effects. Many herbal mouthwashes have proved its efficacy in controlling plaque, as well as exerted anti-inflammatory and antioxidant effect.[12] Herbal mouthwash also has some advantages as it doesn't cause any side effects and doesn't contain alcohol which can induce halitosis.[13]

Ginger (*Zingiber officinale*) is one of the most broadly used spices all around the world. Aside from its extensive use in foods and beverages, ginger also has anti-inflammatory, analgesic, antipyretic, and antibacterial effects.[14] Ginger has good potential to be developed in Indonesia.[15] Indonesia's climate, soil condition, and geographical location are suitable for ginger cultivation.[16] Ginger extract has also acquired GRAS (*generally recognized as safe*) status from *Food and Drug Administration* in 2017.[17]

Ginger comes in three varieties according to its morphology: elephant ginger (*Z. officinale* var*. officinale*), red ginger (*Z. officinale* var*. rubrum*), and emprit ginger (*Z. officinale* var*. amarum)*.[18] The chemical constituents in ginger is classified into volatile oil (essential oil), nonvolatile oil (oleoresin), and starch. Essential oil has antimicrobial effect due to its ability to impair the formation of bacterial cell wall. Meanwhile, oleoresin is able to damage the outer membrane as well as the cytoplasmic membrane of the bacterial cell wall. Red ginger has the highest concentration of essential oil and oleoresin, thus its effect is also the highest among those varieties.[19] Unfortunately, the spicy taste of red ginger is quite intense.[18,20] Therefore, with its less spicy taste than red ginger, elephant ginger can be considered as a more acceptable mouthwash active ingredient. Elephant ginger also demonstrated a better antibacterial effect than emprit ginger.[18]

Previous studies noted that elephant ginger extract with a concentration of 50% was able to inhibit the growth of *S. mutans*.[21] Meanwhile, no research has been conducted on the effect of elephant ginger in mouthwash formulation on the growth of *S. mutans*. Therefore, further research is needed on the development of elephant ginger extract as a mouthwash to inhibit the growth of *S. mutans* biofilm *in vitro*.

Before a mouthwash undergoes clinical trials, it is necessary to evaluate the stability of the mouthwash for a month, which includes organoleptic tests (color, odor, consistency, taste), pH, viscosity, density, and sedimentation.[22,23] In this study, researchers will conduct pH test on several formulations of elephant ginger mouthwash (*Z. officinale* var. *officinale*).

RESEARCH METHOD

This research is experimental (in vitro) with a posttest-only control group design. The preparation of 96% ethanol extract of elephant ginger was carried out at Balai Penelitian Tanaman Rempah dan Obat (BALITTRO), Bogor, Indonesia. The preparation of mouthwash formulation and antibacterial effect testing was done at the Microbiology Center of Research and Education (MiCORE) Laboratory, Faculty of Dentistry, Trisakti University, Jakarta, Indonesia. The pH test was carried out at the Pharmacy Physics Laboratory, Faculty of Pharmacy, University of Indonesia, Depok, Indonesia.

1. Preparation of 96% Ethanol Extract of Elephant Ginger (*Z. officinale* var. *officinale*) by Maceration Method

The 10-month-old elephant ginger was harvested, then the 10 kg ginger rhizome was washed, aerated, and sliced with a thickness of 5 mm. The ginger rhizome was dried in an oven (40-50^{0}C) for 3 days, then mashed using a grinder and filtered to produce 1 kg of powder. The elephant ginger powder was soaked in 6 to 10 L 96% ethanol solvent, then stirred for 3 h. The mixture was allowed to stand for 3 days, then filtered to obtain the filtrate. The filtrate was evaporated with a rotary vacuum evaporator (Buchi, Switzerland) for 6 h at a 40-50^{0}C to become a thick extract of $\pm$100 g and stored at 4^{0}C.

2. Preparation of Mouthwash Formulation of Elephant Ginger (*Z. officinale* var. *officinale*)

Elephant ginger extract was dissolved with propylene glycol, distilled water, and glycerine (M1). Menthol was ground and dissolved in 70% ethanol (M2). Xylitol and sodium benzoate were dissolved in 10 mL of distilled water (M3). M1, M2, and M3 were mixed and then distilled water was added to a volume of 100 mL (Table 1). The mouthwash was centrifuged at 6,000 rpm (4^{0}C, 10 min), then filtered with gauze and Whatman filter paper no. 1. After that, the mouthwash was stored in a tight container at 4^{0}C (Figure 1).

Table 1. Mouthwash formulation of elephant ginger (*Z. officinale* var. *officinale*)

Ingredients	Number of ingredients per formulation			
	F1	F2	F3	F4
Elephant ginger extract (g)	2.5	5	10	15
Glycerine (mL)	15	15	15	15

Propylene glycol (mL)	10	10	10	10
Xylitol (g)	0.1	0.1	0.1	0.1
Menthol (g)	0.25	0.25	0.25	0.25
70% ethanol (mL)	0.1	0.1	0.1	0.1
Sodium benzoate (g)	0.1	0.1	0.1	0.1
Distilled water (mL)	ad 100	ad 100	ad 100	ad 100

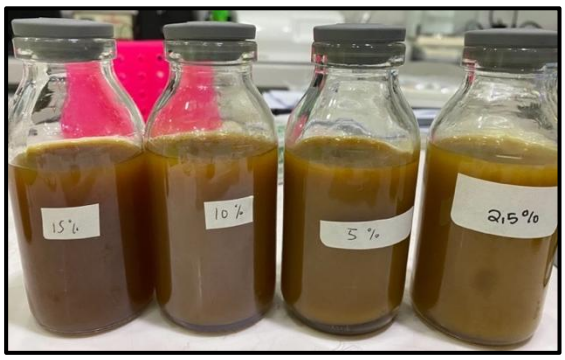


Figure 1. The mouthwash formulation of elephant ginger (*Z. officinale* var. *officinale*) 2.5%, 5%, 10%, and 15%.

3. pH Test of Elephant Ginger (*Z. officinale* var. *officinale)* Mouthwash Formulation

The pH measurement begun with calibrating the pH meter (Jenway, United Kingdom) with a liquid with pH 4 and 7. After calibration, the electrode was rinsed with distilled water, inserted into the sample, and the number that appears was then recorded. pH measurements were carried out three times on day 1, 7, 14, 21, and 28.

4. Preparation of *S. mutans* culture in BHI

BHI (Brain Heart Infusion) broth medium was prepared by mixing 37 g of BHI powder (Sigma-Aldrich, United States) with 100 mL of distilled water, then sterilized in an autoclave (Tomy, Japan) 121^0C for 15 min. A 10 µL culture of *S. mutans* ATCC 25175 from MiCORE Laboratory was cultured in 10 mL of BHI broth and incubated in an incubator (Jisico, Seoul) at 37°C for 24 h. Turbidity was measured using a microplate reader (SAFAS Monaco, Monaco) with a wavelength of 595 nm. The bacterial culture was diluted to a concentration of 108 CFU/mL (OD_{595}=0.132) and homogenized with a vortex mixer (DLAB, United States).

5. Test of Antibacterial Effects of Elephant Ginger (*Z. officinale* var*. officinale*) Mouthwash against *S. mutans* Biofilm Using Microtiter Plate Assay

In the preventive assay*,* 100 µL *S. mutans* culture was transferred to a 96-well plate (Nest Biotechnology, Jiangsu) using a micropipette (Corning, New York), then 100 µL of each mouthwash formulations, Enkasari mouthwash and 0.1% CHX and positive controls, and aquadest as negative control, were added to each culture and incubated at 37°C for 24 h. In the therapeutic assay, 200 µL S. mutans culture were transferred into each well of a 96-well plate and incubated at 37°C for 24 h, allowing it to form a biofilm. After that, all seven treatments were added to the bacterial culture and incubated at 37°C for 1 h. Each treatment had five replications.

After incubation, the liquid in the 96-well plate was removed using a micropipette and the biofilm attached to the 96-well plate was fixed with fire. Next, 0.05% crystal violet (Merck Millipore, Darmstadt) was added to each well, allowed to stand for 15 minutes, and then rinsed with water. Finally, 200 µL of 99% ethanol (Merck Millipore, Darmstadt) was added to the culture, then the mass of the biofilm was measured with a microplate reader at 490 nm.

6. Data Analysis

Analysis of pH data was performed using Repeated Measures ANOVA test and post-hoc pairwise comparisons test ($p<0.05$). The antibacterial effect data were analyzed by One-way Analysis of Variance (ANOVA) test and continued with post-hocTukey test ($p<0.05$).

RESULTS

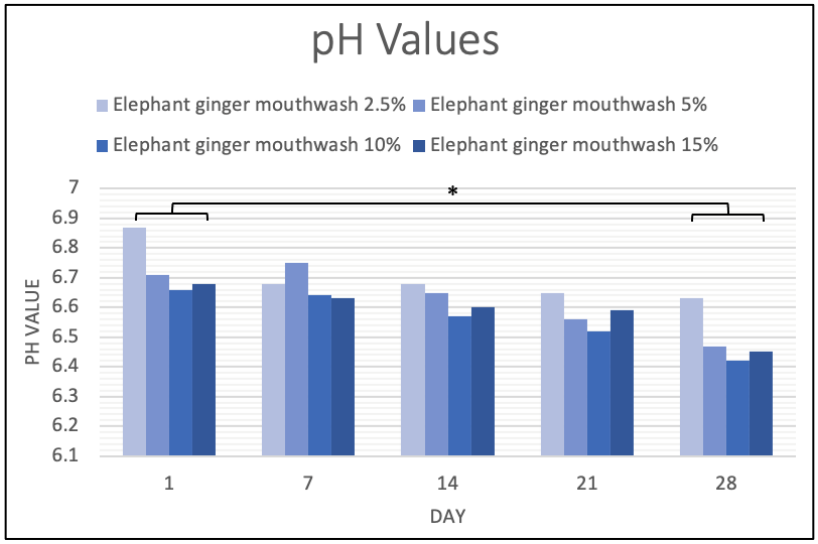
a. pH Value

The pH values of the four mouthwash formulations ranged from 6.42 to 6.87 (Table 2). The data were normally distributed ($p>0.05$) and from the Repeated Measures ANOVA test, p value <0.05 was obtained, which means that there were significant differences in pH values in the four mouthwash formulations during the 28-day measurement. The results of pairwise comparisons test can be seen in Figure 2, which showed that pH values of four formulations on day 1 differed significantly ($p<0.05$) from day 28.

Tabel 2. pH values of *Z. officinale* var. *officinale* mouthwash formulations for 28 days.

Formulation (n=3)	pH value at day					p value
	1	7	14	21	28	
2.5%	6.87	6.68	6.68	6.65	6.63	0.001*
5%	6.71	6.75	6.65	6.56	6.47	0.001*
10%	6.66	6.64	6.57	6.52	6.42	0.001*
15%	6.68	6.63	6.60	6.59	6.45	0.001*

**Repeated Measures* ANOVA ($p<0.05$)



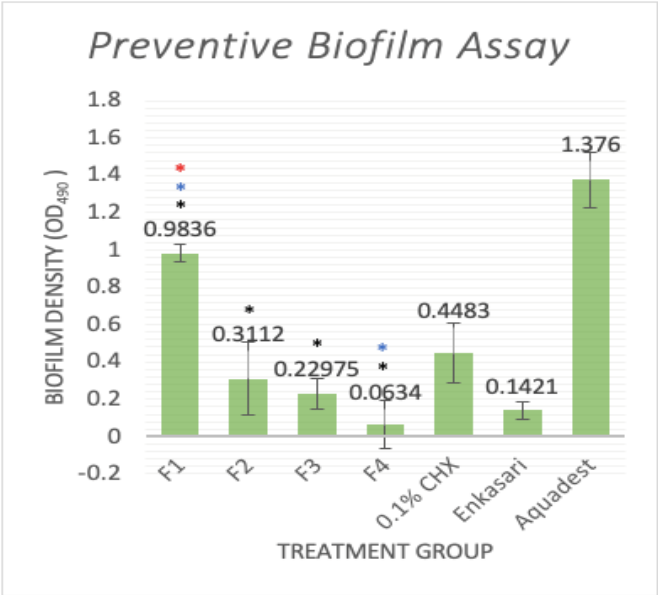
*Pairwise comparisons test (p<0.05)

Figure 2. pH value of a mouthwash formulation containing *Z. officinale* var. *officinale* for 28 days.

b. Antibacterial Test Results against *S. mutans* Biofilms

In the preventive assay, the density of the *S. mutans* (OD_{490}) biofilm formed after interacting for 24 h with a mouthwash containing *Z. officinale* var. *officinale* 2.5% is 0.98±0.05. At a concentration of 5% of 0.31±0.19; 10% concentration of 0.23±0.08, and at a 15% concentration of 0.06±0.13. Interaction with 0.1% CHX and Enkasari resulted in *S. mutans* biofilm density of 0.45±0.16 and 0.14±0.05, respectively. Exposure with distilled water resulted in a *S. mutans* biofilm density of 1.38±0.15.

One-way ANOVA test showed that there was a significant difference in *S. mutans* biofilm density in the seven treatments (p<0.05). The results of the Tukey test are shown in Figure 3.

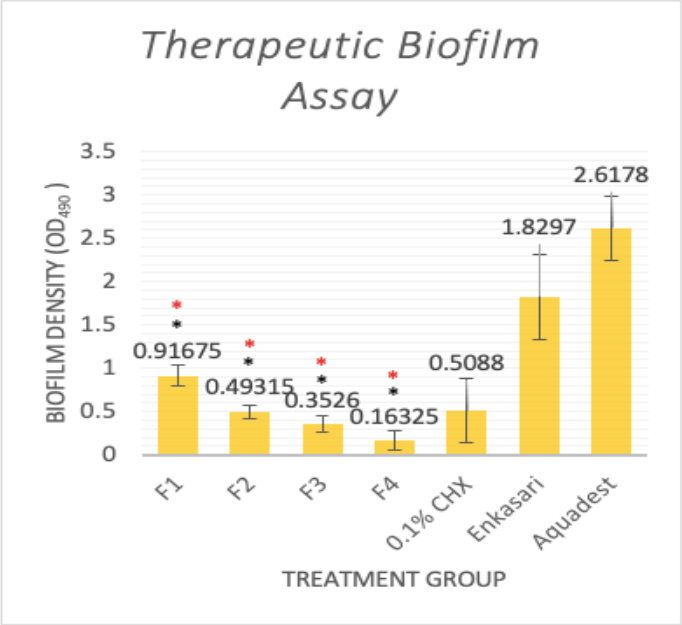


*: significantly different from aquadest (p<0.05)
*: significantly different from 0.1 % CHX (p<0.05)
*: significantly different from Enkasari (p<0.05)

Figure 3. Mass density of *S. mutans* (OD_{490}) biofilm formed after exposure to the seven treatments for 24 h in a preventive assay (Tukey's post-hoc test (p<0.05)).

In the therapeutic assay, the density of the *S. mutans* biofilm that remained attached after interacting for 1 h with a mouthwash containing *Z. officinale* var. *officinale* 2.5% is 0.92±0.11. At a concentration of 5% it is 0.49±0.08; 10% concentration of 0.35±0.1, and 15% concentration of 0.16±0.11. Interaction with 0.1% CHX, Enkasari, and distilled water resulted in a mass density of *S. mutans* biofilms of 0.51±0.37; 1.83±0.49; and 2.62±0.37.

One-way ANOVA test showed that there was a significant difference in *S. mutans* biofilm density in the seven treatments ($p<0.05$). Tukey test results can be seen in Figure 4.



*: significantly different from aquadest ($p<0.05$)
*: significantly different from Enkasari ($p<0.05$)

Figure 4. Mass density of *S. mutans* biofilm (OD_{490}) that remained attached after exposure to the seven treatments for 1 h in a therapeutic assay (Tukey's post-hoc test ($p<0.05$)).

DISCUSSION

Although not being the main method in maintaining oral hygiene, use of mouthwash can serve as an alternative method for some people under certain conditions, or adjunction to tooth brushing to increase its efficacy in removing plaque.[24] This study compared four mouthwash formulations containing *Z. officinale* var. *officinale* with different concentrations. In the acute (24 h) and subacute (28 days) toxicity tests conducted by five studies, administration of 5000 mg/kg *Z. officinale* extract or powder did not cause signs of toxicity and mortality in experimental animals. *Z. officinale* extract also does not have embryotoxic or teratogenic effects. Meanwhile, in clinical trials, the side effects caused by *Z. officinale* occurred in low frequency and intensity.[25] Thus, *Z. officinale* extract is safe to be formulated into mouthwash.

When making the mouthwash, the first step was *Z. officinale* var. *officinale* that had been dried, crushed into powder, then extracted using the maceration method and 96% ethanol solvent. The maceration method using ethanol as a solvent has been shown to produce *Z. officinale* extract with a higher content of polyphenols and 6-gingerol compared to the reflux method and other solvents, namely acetone and methanol.[26] Another study

also revealed that extraction of *Z. officinale* using dry samples with 95% ethanol resulted in the highest gingerol content.[27]

The measurement of pH value for 28 days showed that the four mouthwash formulations had pH values that were within the pH range of the oral cavity (6-7), so they possibly wouldn't cause tooth erosion if used for a long time.[28,29] The four mouthwash formulations showed pH values that remained stable with no significant difference for 21 days, then decreased significantly ($p<0.05$) on day 28. Mouthwash pH stability can be further maintained by adding a buffer, such as benzoic acid or citric acid.

The positive controls used in this study consisted of two types of mouthwash, namely 0.1% CHX and Enkasari containing extracts of green tea (*Camellia sinensis*) and Java turmeric (*Curcuma xanthorrhiza*). As the gold standard for anti-plaque agents, CHX has been shown to reduce the amount of S. mutans in saliva in several clinical studies.[30] Green tea contains epigallocatechin-3-gallate, which is able to inhibit the growth of *S. mutans* in planktonic and biofilm conditions, and causes a decrease in acidogenicity and stress tolerance of *S. mutans*.[31] Java turmeric contains xanthorrizol compounds which can damage the peptidoglycan layer of *S. mutans*, and several in vitro studies have also proven its antibacterial and anti-biofilm effects against *S. mutans*.[32]

Based on the preventive assay results, the four mouthwash formulations of *Z. officinale* var*. officinale* was capable of significantly reducing *S. mutans* biofilm formation for 24 h compared to distilled water ($p<0.05$). The effect produced by the mouthwash formulation of *Z. officinale* var. *officinale* 2.5% was significantly lower than CHX 0.1% and Enkasari ($p<0.05$), whereas the mouthwash formulation *Z. officinale* var. *officinale* 5% and 10% had an effect equivalent to CHX 0.1% and Enkasari ($p>0.05$), and the mouthwash formulation *Z. officinale* var. *officinale* 15% had a better effect than CHX 0.1% ($p<0.05$) and was comparable to Enkasari ($p>0.05$). The therapeutic assay results proved that the four mouthwash formulations of *Z. officinale* var. *officinale* had a significantly better effect on reducing *S. mutans* biofilm adhesion for 1 hour than distilled water and Enkasari ($p<0.05$), and not significantly different from CHX 0.1% ($p>0.05$). The four mouthwash formulations containing *Z. officinale* var. *officinale* has an antibacterial effect on *S. mutans* biofilm. This effect also increased with increasing concentration of *Z. officinale* var. *officinale* in mouthwash.

The density of the *S. mutans* biofilm resulting from the interaction with the negative control in the preventive assay was lower than that in the therapeutic assay. This is due to the shorter incubation time of the preventive assay compared to the therapeutic assay. In the preventive assay, after *S. mutans* was incubated with the treatment for 24 h, the biofilm that formed was immediately measured, whereas in the therapeutic assay, after *S. mutans* was incubated for 24 h and a biofilm formed, the biofilm was again incubated with the treatment for 1 hour before measured.

The antibacterial effect demonstrated by the mouthwash formulation of *Z. officinale* var. *officinale* is produced by secondary metabolites contained in the extract of *Z. officinale* var. *officinale* and other excipients in the mouthwash formulation. The results of the phytochemical test of *Z. officinale* var. *officinale* conducted by previous studies stated that elephant ginger extract contains terpenoids, flavonoids, alkaloids, tannins, and steroids.[21] Terpenoid compounds extracted from ant nests (*Myrmecodia pendans*) as much as 50 ppm were shown to be able to inhibit *S. mutans* biofilms and cause 40% eradication of *S. mutans* biofilms.[33] Flavonoids contained in cranberries (*Vaccinium subg. Oxycoccus*), can cause a reduction of EPS by more than 80%, reduce the accumulation of *S. mutans* in biofilms, create an environment with a higher pH, and cause damage

to the biofilm structure so as to facilitate biofilm detachment.[34] A group of alkaloid compounds synthesized from marine plants (marine alkaloids), namely macavolamine, has been shown to have bactericidal and anti-biofilm effects against *S. mutans*.[35] Tannins have astringent properties that can inhibit the glucosyltransferase (gtf) enzyme so that they can inhibit bacterial attachment.[36] One study also noted that the tannin fraction of neem (*Azadirachta indica*) extract had antibacterial and anti-biofilm effects against *S. mutans*.[37] The steroids contained in *Withania somnifera*, along with other compounds, are thought to contribute to inhibiting acid production, acid tolerance, attachment through a sucrose-dependent mechanism, and EPS formation in *S. mutans* biofilms.[39]

The crude extract and methanol fraction of *Z. officinale* have been shown to be able to reduce *S. mutans* biofilm formation, inhibit glucan synthesis and *S. mutans* attachment, cause damage to biofilm structure, reduce expression of genes that play an important role in *S. mutans* virulence in vitro, and reduce development of caries in vivo.[38] A clinical study that examined the use of mouthwash containing 0.5% essential oil of *Z. officinale* in orthodontic device users, noted that the mouthwash reduced the amount of biofilm and *S. mutans* for 1 minute and 15 min after use.[39]

In previous studies, it was known that the minimum inhibitory concentration (MIC) of *Z. officinale* var. *officinale* against *S. mutans* is 50%,[21] but the concentration used in the mouthwash formulation in this study was sub-MIC (2.5-15%). The mouthwash has been shown to inhibit the formation and adhesion of *S. mutans* biofilms. Other research also found that a natural substance with sub-MIC concentrations was able to reduce *S. mutans* biofilm formation by reducing *S. mutans* virulence factors, but did not affect its viability.[40,41]

Several other ingredients contained in this mouthwash also have antibacterial and anti-biofilm effects against *S. mutans*. 50% propylene glycol and 100% glycerin were able to kill planktonic *S. mutans* in vitro.[42] 0.1% sodium benzoate was noted to be able to significantly reduce the viability of *S. mutans* in biofilms.[43] Mentha piperita essential oil, which has a main component of menthol, is able to reduce the expression of gtfB, C, and D genes which play an important role in the synthesis of glucan by *S. mutans*.[44] Xylitol has been shown to inhibit the formation of S. mutans biofilms by reducing the viability and production of *S. mutans* polysaccharides.[45]

The shortcoming of this study is the mouthwash formulation produced is still too thick so it has a cloudy color. Further research is needed to make mouthwash formulations with lower extract and glycerin concentrations, and other ingredients can be added to improve its pH stability and clarity.

CONCLUSION

Mouthwash formulations containing *Z. officinale* var. *officinale* 2.5%, 5%, 10%, and 15% showed stable pH values for 21 days, then decreased significantly on day 28. The pH values of the four mouthwash formulations ranged from 6.42-6.87.

Formulation of elephant ginger mouthwash (*Z. officinale* var. *officinale*) 5% and 10% is as effective as 0.1% CHX and Enkasari in inhibiting *S. mutans* biofilm formation for 24 h, while the mouthwash formulation is 15% more effective than 0.1% CHX and equivalent to Enkasari. The 2.5% mouthwash formulation was less effective than the 0.1% CHX and Enkasari. The mouthwash formulation of *Z. officinale* var. *officinale* 2.5%, 5%, 10%, and 15% were able to reduce *S. mutans* biofilm adhesion for 1 h better than Enkasari and equivalent to 0.1% CHX. The mouthwash formulation of *Z. officinale* var. *officinale* has the potential to be further developed into a herbal mouthwash.

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CONFLICT OF INTEREST

None.

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