Formulation of Anti-Oral Mouthwash Nanoemulsion Biofilm Based on Propolis Extract *Heterotrigona itama, Tetragonula sapiens*, and *Tetragonula clypearis*

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ABSTRACT

The use of mouthwash is one of the actions against biofilms that are often used. However, commercial mouthwashes have a fairly high alcohol content, which is around 26.9% of the total volume, which is considered to have a prolonged impact where high alcohol content in direct contact with the oral mucosa can cause lesions or abnormalities, resulting in a shift in the medical paradigm towards eco-friendly widely considered as a solution. Propolis with antibacterial ability was formulated using the nanoemulsion steps, which were initiated by separating pure propolis through drying, and then there were variations in the formulation of 3 types of bee propolis: H*eterotrigona itama, Tetragonula sapiens*, and *Tetragonula clypearis* along with the addition of Tween 80, propylene glycol, glycerin and then the effect on microbial growth of *S. mutans* was compared with antimicrobial agents in Brazilian propolis with the identification and comparison of the antibacterial activity stability of the organoleptic formula. Where the active ingredient content of propolis is the highest in *H. itama* propolis with a total flavonoid content of 38.94 mgQE/L sample and *T. clypearis* propolis has the lowest total flavonoid content of 14.23 mgQE/L sample with its function as an anti-oral biofilm agent by inhibiting the glucosyltransferase was proven with a minimum percentage of 49% inhibition of *S. mutans* and degradation of 18% with the use of a combined surfactant proved to be able to increase the stability of the preparation shown at 2:1 (v/v).

1. Introduction

There were thirty percent of the Indonesian population who had dental and oral problems with an increase of 70% from the same suffering due to the formation of oral biofilms (Soulissa 2020). The emergence of this problem was reinforced by the lack of preventive measures from plaque growth from the lack of mechanical cleaning. Actions that are considered good to do as one of the preventive measures to deal with this are to improve oral hygiene with anti-microbial agents as an anti-oral biofilm function. Seeing the side effects of preventive measures against oral biofilm removal in the form

of commercial mouthwashes and the existence of multi-drug resistance bacteria strains due to overuse of antibiotics requires a shift in the medical paradigm to using green perceptions in its application, where the use of propolis as an antimicrobial agent as an anti-oral biofilm agent can be a solution (Shahani and Reddy 2011).

Supported by the flavonoid content of propolis which can inhibit the activity of the glucosyltransferase enzyme which triggers carbohydrate metabolism to become acid so that a biofilm layer is formed (Dai and Mumper 2010). The active ingredient of propolis is in the form of polyphenols which act as active ingredients for antimicrobial agents which at this writing function to inhibit the growth of *Streptococcus mutans* as a gram-positive bacterium from the Staphylococcaeae family in the form of

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rods that do not form spores and are non-motile with fluctuating anaerobic properties in oral biofilm growth (Harris *et al.* 2002).

Numerous studies have shown multiple biological activities of the propolis produced by *Apis mellifera* honeybees and stingless bees, including antimicrobial, antioxidant, anti-inflammatory, cardioprotective, antiproliferative, and dental medicine (Abdullah *et al.* 2020; Akmar *et al.* 2022; Popova *et al.* 2022; Zullkiflee *et al.* 2022). Therefore, it has great potency of propolis to be used as an alternative complementary medicine for the potential treatment of dentistry, oral health, and medicine (Zulhendri *et al.* 2021).

The aim of this research is to obtain the formulation of mouthwash preparations from 3 sources of stingless bee propolis (*Heterotrigona itama, Tetragonula sapiens, Tetragonula clypearis*) all in from Indonesia based in mostly Sulawesi to produce antibacterial activity and stability that are effective and better than commercial mouthwashes.

2. Materials and Methods

2.1. Materials

Crude propolis samples were collected from stated stingless bees from South Sulawesi Province. Ethanol 96%, Tween-80, propylene glycol, distilled water, and phosphate buffered saline (PBS) were purchased from Brataco Company (Brataco, Indonesia). Brain Heart Infusion (BHI) broth and agar was purchased from Sigma Aldrich (Sigma-Aldrich, St Louis, USA), Streptococcus mutans ATCC 25175 collected from Faculty of Dentistry Universitas Indonesia.

2.2. Propolis Extract Production

The production process of propolis extract was prepared for the propolis extraction process with 96% food grade ethanol. Propolis raw in each batch was weighed as much as 250 g. Propolis raw was mixed with 1,250 ml 96% with 1:5 (g/ml) ratio, and it was agitated using agitator (IKA RW 20) at 500 rpm for 8 hours. Then the ethanol propolis solution was allowed to stand for 8 hours to separate between the clear extract solution and the resin. The filtrate (96% extract ethanol propolis (EEP) and the resin were filtered using Whatman paper no.1 (GE Healthcare). The supernatant was then mixed, and the concentration was reduced to 70% ethanol-water solution. The previous solution was cleaned of wax and impurities before stirring and centrifuged 1,000x g for 30 minutes then the pulp was separated from the propolis extract by filtering the supernatant using filter paper. EEP was evaporated with rotavapor to separate the propolis extract from ethanol at 60°C until no ethanol had evaporated anymore.

2.3. Measurement of Flavonoid Content

The extracted propolis was then identified the levels of the flavonoid active ingredients using spectrophotometer measurements by first making a matrix or placebo, making simulation or standard samples, and measuring the levels of both standard samples with targets which resulted in a standard quercetin curve.

At first, calibration data was needed in the form of 5 standard solutions with a predetermined concentration level with a difference of 2 mg/L in the range of 3-11 mg/L and the absorbance value was calculated. The target sample was measured for its quercetin content, previously diluted 10x with distilled water as a diluent factor. After obtaining a standard curve for quercetin, the total flavonoid value of the sample can be obtained.

2.4. Preparation of Nanoemulsion

Mouthwash was made according to the formulation in Table 1 in accordance with the provisions where the determination of the composition of this preparation pays attention to the nanoemulsion component (surfactant) as a concentration variation for optimizing the mouthwash preparation. First, glycerine was mixed with propylene glycol solution in a container and then Tween 80 (aqueous phase) was added.

The propolis extract was then mixed with distilled water and added to the aqueous phase. This aqueous phase was mixed with the extract (non-polar) which has previously been mixed with peppermint oil, acting as the oil phase (dispersed phase), followed by a homogenization process using homogenizer a high-pressure magnetic stirrer at a temperature of 30-40°C for 15 min and ultra-sonification at 53 Hz for 10 min. As for the safety of the formulation, the concentration of each raw material has been adjusted according to the concentration recommended by the Food and Drug Administration in the Inactive Ingredients Database, 2013 (Zhang *et al.* 2022).

Table 1. Nanoemulsion formulation anti-oral biofilm mouthwash

Material	Function	Formulation (%v/v)						
		A	B	C	D	E	(-)	(+)
Propolis *T. clypearis*	Pharmaceutical agent	7.00	2.00	12.00	7.00	7.00	0.00	0.00
Propolis *H. Itama*	Pharmaceutical agent	7.00	12.00	7.00	0.00	12.00	0.00	0.00
Propolis *T. sapiens*	Pharmaceutical agent	7.00	7.00	2.00	14.00	2.00	0.00	0.00
Propolis *A. mellifera*	Pharmaceutical agent	0.00	0.00	0.00	0.00	0.00	0.00	21.00
Tween 80	Surfactant	3.75	5.00	2.50	0.00	7.50	5.00	5.00
Propylene glycol	Solubilizer	1.25	1.25	1.25	1.25	1.25	1.25	1.25
Peppermint oil	Nanoemulsion Oil Dispersant	0.25	0.25	0.25	0.25	0.25	0.25	0.25
Glyceryn	Humectant	3.75	2.50	5.00	7.50	0.00	2.50	2.50
Aquadest	Solvent	70.00	70.00	70.00	70.0	70.00	91.00	70.00

2.5. Physical Evaluation of Nanoemulsion Mouthwash

The evaluation was conducted to all formula, in term of physical characterization for 3 weeks per week 0, and also particle size and pH identification. The physical characterization was done with organoleptic analysis texture, appearance, taste, odor, and homogeneity. The pH measurement was done with Eutech pH-meter (Eutech Instruments Pte Ltd, Singapore). Particle size measurement was done with Particle Size Analyzer (Horiba SZ-100). In the evaluation of mouthwash preparations, the following equation used to calculate HLB of formulation in order to conduct organoleptic as a stability test. Where:

$$HLB_{max} = f_A \times HLB_A + (1 - f_A) \times HLB_B \qquad \text{Eq. (1)}$$

HLB_{max} = HLB mixed surfactant A and B
f_A = surfactant weight fraction A
HLB_A = HLB surfactant A
HLB_B = HLB surfactant B

2.6. Stability Assay of Nanoemulsion Mouthwash

Within the taking after physical evaluation, stability assay was moreover conducte to all formulation. The measure comprises of storage testing through a shelf-life stability test carried out for 3 weeks at room temperature (28±2°C) which was carried out in parallel with physical evaluation of the preparation with organoleptic observations and weekly pH measurements. Another measure that was too conducted was centrifuge testing (5,000 rpm, 30 mins), where the observational data was in the form of comparison of physical condition after centrifugation with the physical condition of the preparation before centrifugation. Also four cycles of freeze-thaw cycling test which carried out by constant transfer to extreme temperatures in the mouthwash preparation. The preparation was stored at 4±2°C for 24 hours which was then transferred directly to storage in an oven at 40±2°C for 24 hours, and the treatment was counted for one cycle.

2.7. Anti-Oral Biofilm Activity *In vitro* Assay of Nanoemulsion Mouthwash

This *in vitro* test was carried out to see the ability of the preparations to the growth of oral biofilms due to the growth of *Streptococcus mutans* bacteria by paying attention to the effect of the concentration of the active substance (dose dependent) and the concentration of *S. mutans* (concentration dependent). This test involves a formulation that has passed the physical test and stability test that has been carried out and compared with the effectiveness of the control preparation. The natural bacteria found in the mouth used as the test bacteria was *Streptococcus mutans*. To create *in vitro* conditions, a pellicle surface calculation was carried out to represent the condition of the mouth by means of which the test bacteria will be cultured on an artificial pellicle layer.

3. Results

3.1. Extract Purification and Flavonoid Test

Propolis extract was shown in Figure 1. The darkest precipitate belongs to the extraction of *Heterotrigona itama* proplis and the extraction results with the least precipitate owned by *Tetragonula clypearis* shows that the amount of residual wax in the solution, to
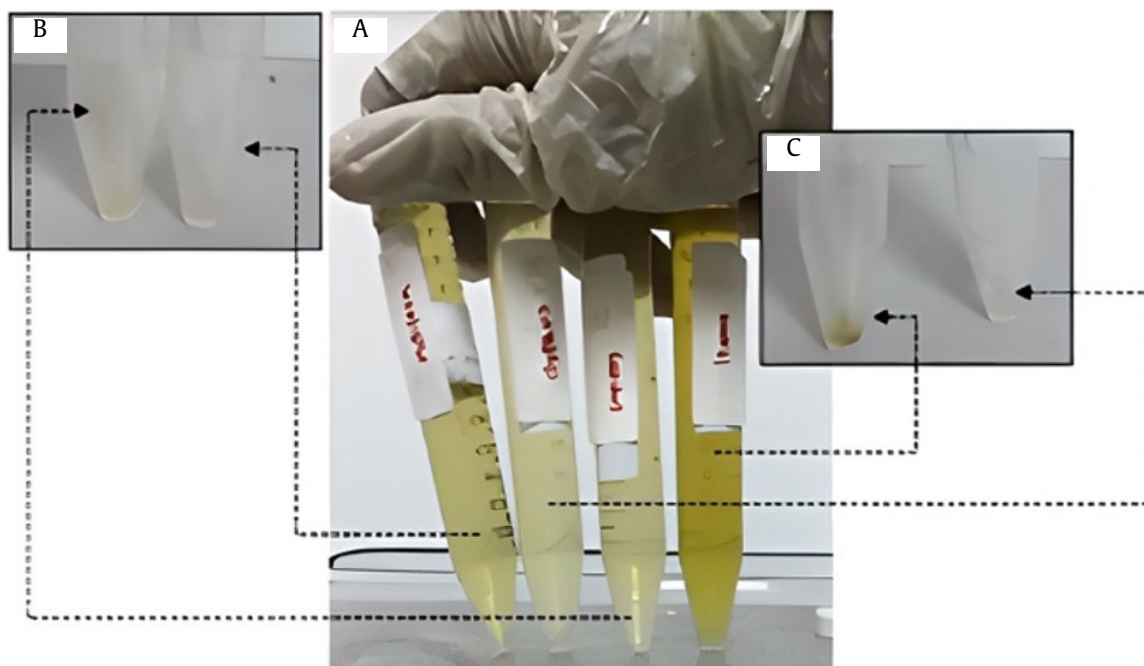
Figure 1. (A) Propolis extract before centrifugation, (B) precentrifuged wax precipitated by *T. sapens* and *A. melifera* propolis, (C) wax precipitated from *H. itama* and *T. clypeqaris* propolis centrifugation

obtain pure propolis extract, then the supernatant was filtered using filter paper to obtain the most optimal separation results. Furthermore, the propolis extract will go through a test for the content of the active flavonoid material which was carried out by measuring the spectrophotometer as the result shown in Table 2 below.

It can be concluded that the higher the absorbance value, the higher the total flavonoid content, so that based on the measurement results H*eterotrigona itama* propolis has the highest total flavonoid content, which is 38.94 mgQE/L sample and *Tetragonula clypearis* propolis has the lowest total flavonoid content, which is equal to 14.23 mgQE/L sample.

3.2. Evaluation of Nanomemulsion Formulation

Preparation of propolis mouthwash nanoemulsion was shown in Figure 2. Figure above showed that the nanoemulsion results with various physical appearances, this may be due to the composition of the nanoemulsion-forming materials as important factor in the formation of nanoemulsion formulations. The formulation with the most different physical form is in formula D with high mixed turbidity, this is due to the formulation without the content of Tween 80 and 7.5% (v/v) glycerin in the composition.

Table 2. Total flavonoid per sample

Sampel	Abs	Total flavonoid (mg quarcetin/L sample)
Heterotrigona itama	0.3118	38.94
Tetragonula clypearis	0.1183	14.23
Apis melifera	0.1492	18.17
Tetragonula sapiens	0.1198	14.42

3.3. Physical Evaluation

The value of the Hydrophilic-Lipophilic Balance (HLB) was shown in Table 3. From the result, the HLB value was based on the ratio of surfactants of each formulation. Most of the preparations have good HLB values as O/W emulsions except for formulations D and E. Formulation E shows a large HLB value, It has very weak dispersibility properties as evidenced by the physical appearance that there was no homogeneity between phases making the preparation look cloudy. This was thought to be due to the dispersed phase having larger molecules so that the aqueous phase was unable to hold and the dispersed substance will agglomerate to form a coarse emulsion system. As for Formula E, although it has the best physical appearance, the HLB value of the preparation is considered less precise with the application it should be.
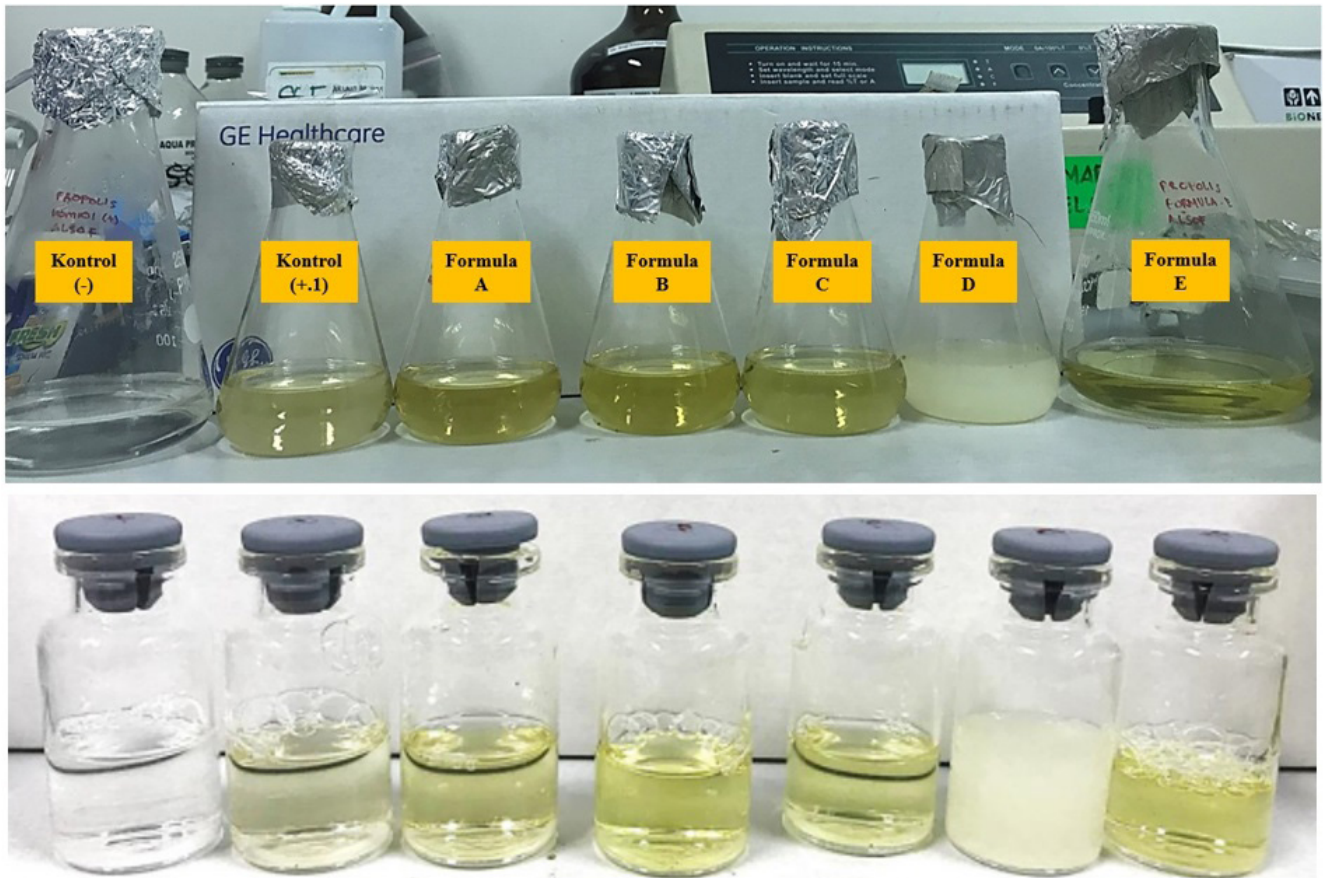
Figure 2. Preparation of propolis mouthwash nanoemulsion on week 0; (from left to right) control (-), control (+), formula A, formula B, formula C, formula D, formula E

Table 3. Hydrophilic-lipophilic balance of formulation

Formula	Tween 80: glyceryn (v/v)	Surfactant A weight fraction	HLB_{max}	Visual	Application
(-) control	2:1	0.67	11.67	Translucent	O/W Emulsion
(+) control	2:1	0.67	11.67	Translucent	O/W Emulsion
A	1:1	0.50	10.00	Translucent	O/W Emulsion
B	2:1	0.67	11.67	Translucent	O/W Emulsion
C	1:2	0.33	8.33	Milk like dispertion after mixing	O/W Emulsion
D	0:1	0.00	5.00	Weak dispertion	W/O Emulsion
E	1:0	1.00	15.00	clear	Detergent

The pH of propolis nanoemulsion was shown in Table 4. Based on the table of pH measurement result, it can be seen that formula D has the lowest pH value among other formulations.

The results of particle size evaluation (Figure 3), the majority of formulations are still classified as nanoemulsions which are characterized by an average globule size in the range of 1-100 nm, except for formulations D and E.

Table 4. pH measurement result per sample

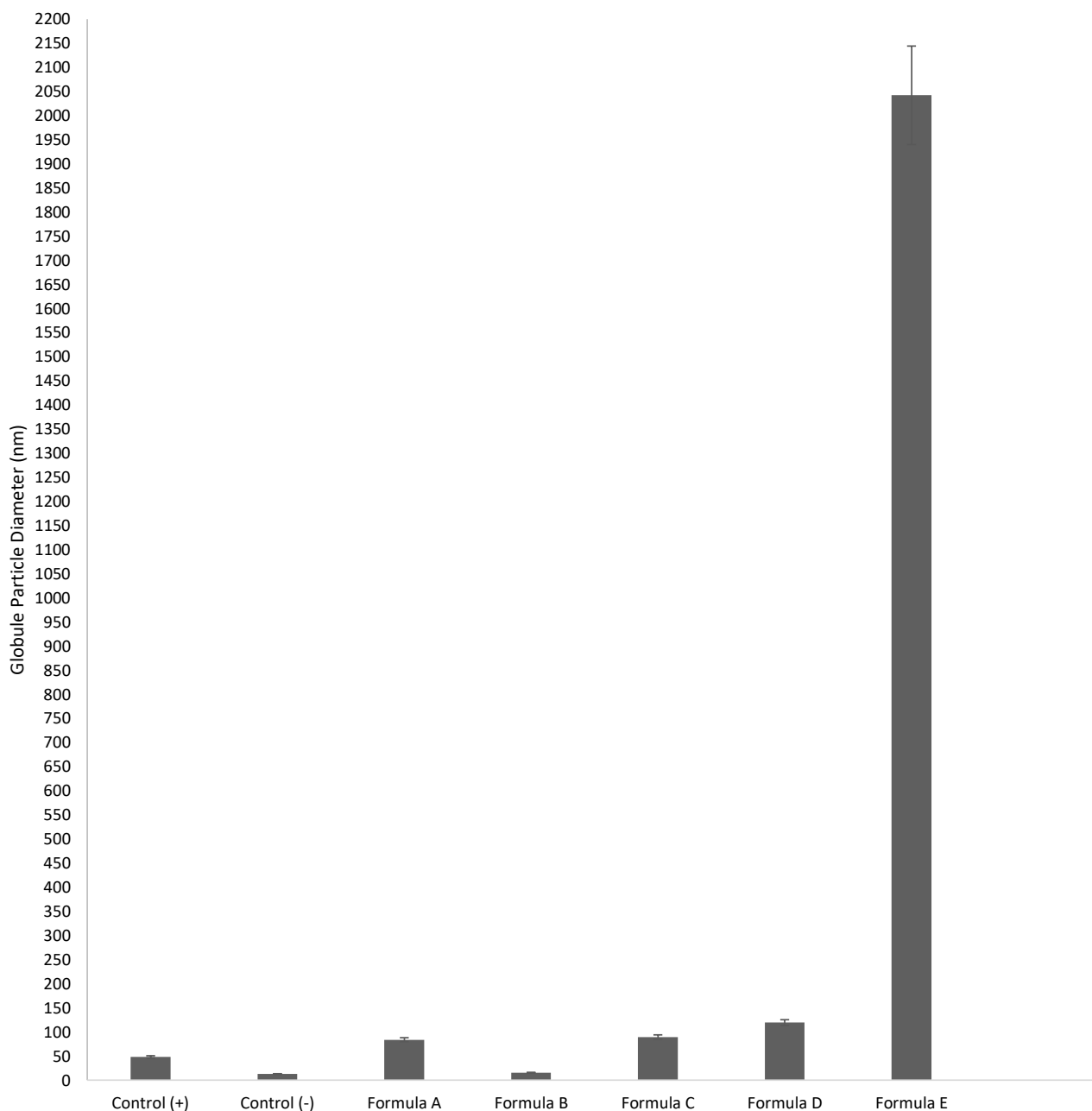| pH	Week-2	Week-3
(-) control	6.14	7.91
(+) control	6.39	7.29
A	5.87	6.93
B	5.94	7.04
C	5.87	6.92
D	5.78	6.79
E	5.95	7.03

Figure 3. Graph of avarage globule particle diameter in week-3

3.4. Evaluation of Stability Assay

Shelf-life stability characteristic was shown in Figure 4. Based on the graph of the effect of temperature and shelf life on pH values causing a linear increase.

Centrifuge testing result was shown in Table 5. Visually, all preparations including controls did not experience phase separation except for formulation D, there was a difference in color in the solution where the bottom part looked much more yellow while the upper part of the mixture indicated a separation of the oil phase attached to the container and the water phase so that it can be said to be unstable against one year's storage gravity.

The results of the cycling test which can be seen below are assessed in the last cycle where the control (-) looks clear, the control (+.1) looks transparent with a slight yellowish tint, formulas A and C look transparent with a little white mist forming on the bottom. preparations with a
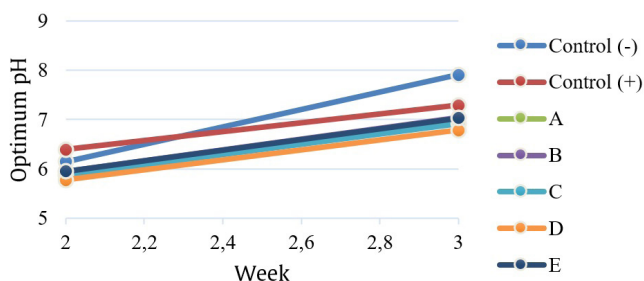
Figure 4. Graph of correlation between shelf-life and pH

Table 5. Tabulation of centrifugation test results per sample

Formulation	Result
(-) control	Stable
(+) control	Stable
A	Stable
B	Stable
C	Stable
D	Phase separation occurs
E	Stable

yellowish color, formulas B and E look clear with a yellowish color, while formula D looks cloudy, and the preparations were foggy almost like milk and there was a separation of the water and oil phases as has happened since the beginning of the preparation of the preparation in week 0.

3.5. Formulation Passed the Stability Test

After carrying out all stages of physical testing and stability of the preparation for 3 weeks, the results of the propolis-based mouthwash formulation with the best stability fell on formulation B with a surfactant ratio of 2:1 (v/v) with an average pH of 6.49 and an average size globule particle size was 14.67. The results of storage, organoleptic, HLB calculations, centrifugation tests and cycling tests showed results for stable nanoemulsions with yellowish transparent physical properties.

Even so, Control (-) as a preparation without active ingredients was proven to act as a control (-) in terms of its ineffective ability to stabilize during the evaluation which was evident from the physical appearance which showed growth in the preparation in the form of whitish mucus which clearly indicated the instability of the preparation. In addition, additional control (+.2) was given in the form of a commercial propolis-based mouthwash with the trademark propolinse as a comparison at the anti-oral biofilm test stage.

3.6. Results of the Oral Antibiofilm Activity Test Results for Mouthwash Preparations

Evaluation of the oral antibiofilm activity was carried out using the Total Plate Count (TPC) method after an incubation period of 24 hours at 37°C. Before the Total Plate Count was carried out, *in vitro* conditions were prepared for testing to mimic the natural condition of the mouth using a 96-well plate. Each well was filled with a pellicle layer sample containing a ratio of 1:1 (v/v) saliva with mouthwash which then went through an incubation process to produce a pellicle coating which was marked by turbidity at the bottom of the well as shown in Figure 5.

Prior to the growth of cultures for both *S. mutans* and planktonic bacteria, serial dilution was carried out to facilitate the colony calculation process. Cultures on agar media can then be performed using the spread plate method.

Based on the table, the three-mouthwash formulation were reactive to the control of saliva, as seen from the decrease in the number of bacterial colonies, the order of decreasing the number of bacteria was most effectively carried out by commercial control (+.2), formula B, and control (+.1).

The Figure 6 above was consistent with the colony calculation results in Table 6 and shown the relationship between the active ingredient content of the preparation and the antibiofilm activity that can be assessed from the growth of *S. mutans*. The hypothesis that can be drawn from the graph was that from the three preparations there was no significant difference based on the exposure of calculated data to the antibiofilm activity in Table 6, especially in commercial control (+.2) preparations and formula B. It can be concluded that the decrease in the number of bacterial colonies in the biofilm layer was comparable. With an increase in the percentage of the active substance in each preparation. P3 or Formula B contains propolis extract with the highest active substance content owned by *Heterotrigona itama* propolis of 38.94 mgQE/L sample and the addition of quercetin flavonoid content of 14.23 mgQE/L sample of *Tetragonula clypearis* and 14.42 mgQE/L sample of *Tetragonula sapiens*, the sample ws considered more effective as an atibiofilm agent compared to P_1 as a control (+.1) with the active ingredient content
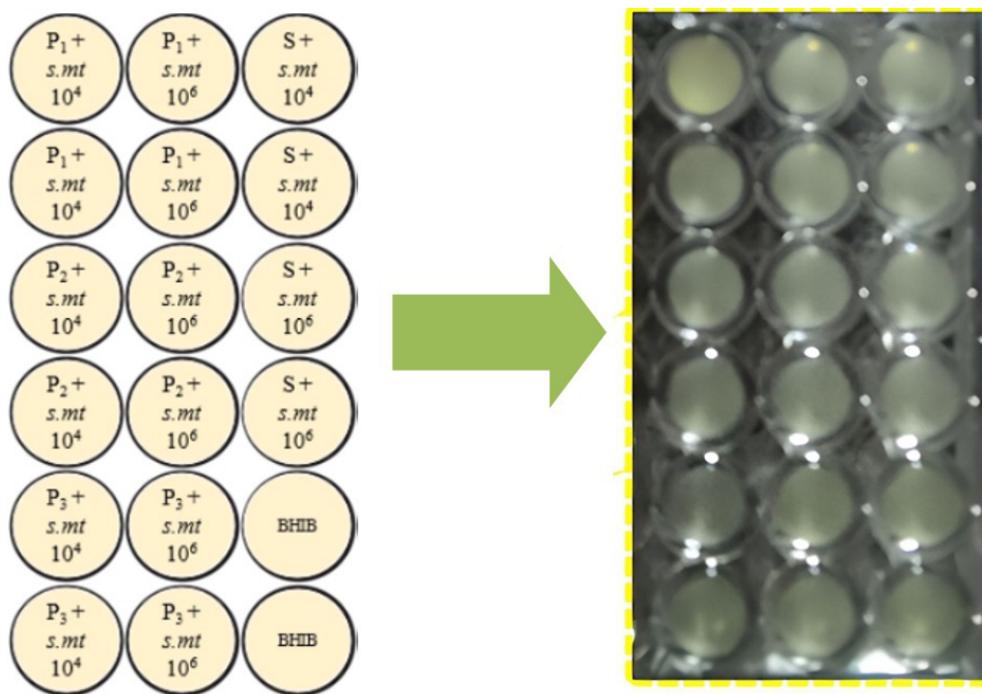
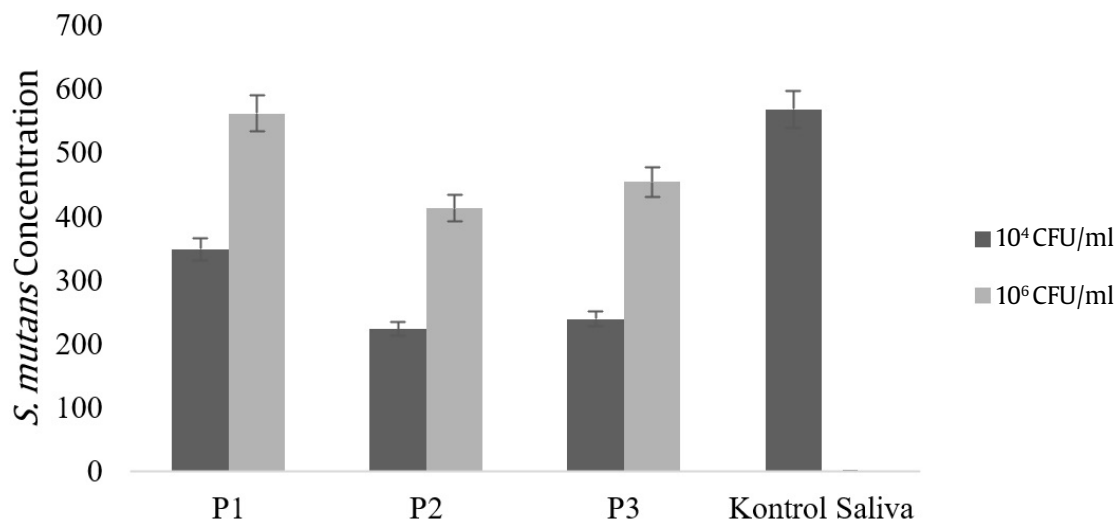Figure 5. Pellicle coating using 96-well plate

Figure 6. Relationship of active ingredients content on growth of *S. mutans*. P1: saliva + control (+.1); P2: saliva + control commercial (+.2); P3: saliva + formula B

Table 6. Number of colonies (TPC) of microorganisms after 24 hours culture

S. mutans	Mouthwash	24H incubation	
	Formulation	S (x10^5)	P (x10^5)
	P1	349	578
10^4 CFU/ml	P2	223	480
	P3	239	534
	S	568	630
	Formulation	S (x10^6)	P (x10^6)
	P1	562	621
10^6 CFU/ml	P2	413	536
	P3	454	572
	S	764	724

S = number of *S. mutans* colonies on the biofilm layer; P = number of colonies of planktonic bacteria (supernatant); P1: saliva + control (+.1); P2: saliva + commercial control (+.2); P3: saliva + formula B

represented by *Apis melifera* propolis with a quercetin content of 18.17 mgQE/L the sample is considered the least effective in inhibiting *S. mutans*.

The percentage inhibition of the formulation against *S. mutans* bacteria is shown in the Figure 7.

P_2 has the average inhibition of 53% and the degradation was 25% so then considered the highest, this was analyzed because the P_2 formulation had additional content of *Camellias sinensis*.

Concentration dependent assay of nanoemulsion was shown in Table 7. Based on the table above, it can be seen that formulation B mouthwash with the predominant content of *Heterotrigona itama* propolis works effectively to inhibit growth and degrade biofilms because it is considered to be able to compete with commercial propolis mouthwashes

which are commercially available that contains green tea as an antibacterial agent other than propolis.

4. Discussion

4.1. Extract Purification and Flavonoid Test

Extraction was carried out by maceration using a technical solvent of 96% ethanol against honeycomb (raw propolis) with a certain ratio without any heating process to eliminate the possibility of denaturation of the active ingredient content 96% ethanol solvent is semi polar so that active compounds with different polarity can separate themselves by diffusion. The propolis extract obtained was related to the amount of solvent used where the more ethanol used, the more cavities in the solvent that can be filled by
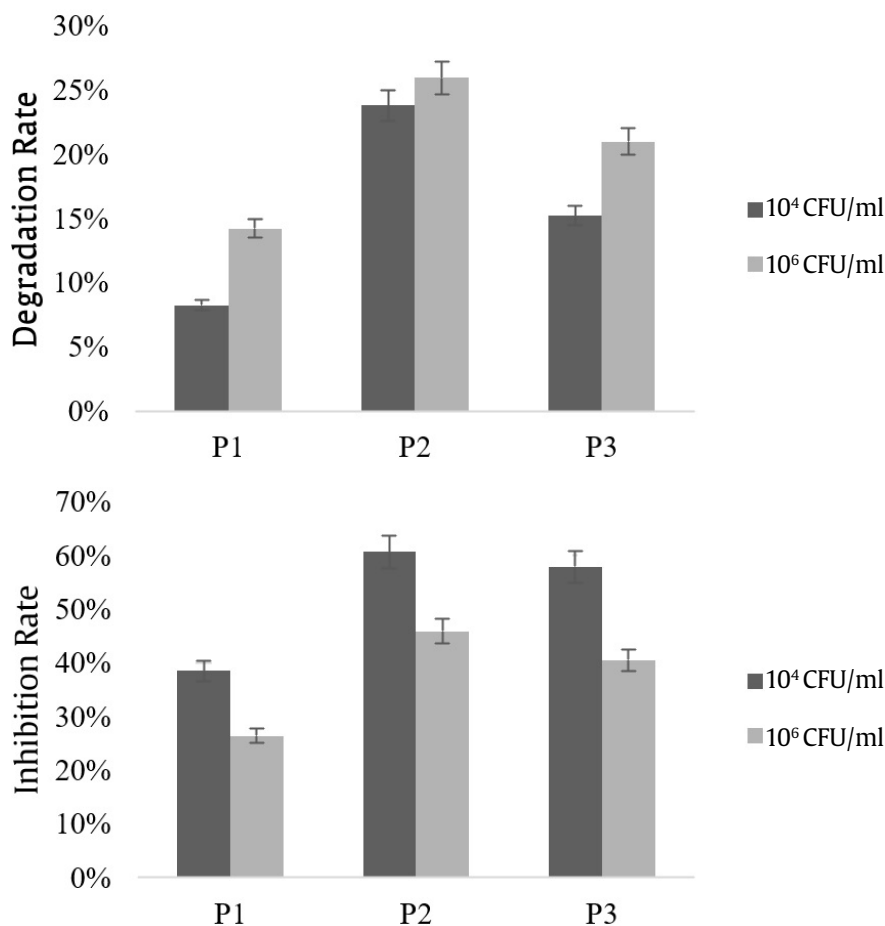

Figure 7. Graph of biofilm inhibition and degradation percentage. P1: saliva + control (+.1); P2: saliva + control comersil (+.2); P3: saliva + formula B

Table 7. Number of colonies (TPC) of microorganisms after 24 hours culture

Treatment (24 H)	*S. mutans* concentration	
	10⁴ CFU/ml	10⁴ CFU/ml
	Biofilm inhibition rate	
P1	39%	26%
P2	61%	46%
P3	58%	41%
Avg	45%	
	Biofilm degradation rate	
P1	39%	26%
P2	61%	46%
P3	58%	41%
Avg	18%	

the extracted honeycomb component (Oroian *et al.* 2020).

4.2. Evaluation of Nanomemulsion Formulation

The nanoemulsion results with various physical appearances, this may be due to the composition of the nanoemulsion-forming materials as important factor in the formation of nanoemulsion formulations. The formulation with the most different physical form was in formula D with high mixed turbidity, this was due to the formulation without the content of Tween 80 and 7.5% (v/v) glycerin in the composition. Meanwhile, Tween 80 acts as a surfactant that acts as an oil phase and is a compound with lipophilic and hydrophilic groups that function as a lowering of surface tension and produces energy to change the size of the globules into smaller ones where when the surfactant is in water the hydrophilic molecules will gather on the surface and then enter the water. liquid while the hydrophobic part will be outside the wall (container) where the presence of this oil phase affects the lipophilic group (Kothekar *et al.* 2007).

Meanwhile, glycerin acts as a cosurfactant that is useful in reducing surface tension by its presence as a co-emulsifier which also reduces the hydrophilicity of non-polar solvents (Wu *et al.* 2021) so that the excess content of glycerin without being accompanied by the content of tween 80 makes the composition more hydrophobic and does not mix well. As for the composition of other nanoemulsions in the form of humectants which influence the formulation, in this study propylene glycol as a humectant functions to keep the active substance from evaporating with the side function of increasing the activity of natural preservatives owned by propolis. Peppermint oil, apart from acting as an aromatic agent, also acts as

a cosolvent in increasing the solubility of its volatile constituents.

4.3. Evaluation of Physical Characteristic

In the manufacture of nanoemulsions using various concentrations and types of excipients, it is necessary to pay attention to the relationship between polarity that contributes to the value of the Hydrophilic-Lipophilic Balance (HLB) of the preparation to determine an emulsion type based on the results of the relationship to hydrophilic and lipophilic properties where the molecular value is nonionic is in the 0-20 HLB range (Pavoni *et al.* 2020). Although propolis is known as a non-polar part of honey, structurally it has been proven to have semi-polar properties due to its phenolic acid content which contains phenolic acid with polar properties (Cetin-Karaca 2011). Propolis, which turns out to be semi-polar, is an important ingredient for producing a cloudy/blurred appearance of the product, the increasing appearance of a hazy and cloudy appearance which can be determined mathematically from the mixed HLB calculation.

Even so, by verification the pH of the mouthwash ranges from Ph 5-7 (Loke *et al.* 2016). Where, the pH value was considered to affect the type of bacteria that can grow in preparations where most optimum bacteria grow at a pH of 6.5-7.5 (Mirhashemi *et al.* 2021), therefore the standard pH of the formulation must be in the range of the optimum pH value for bacterial growth because the desired nature of the formulation is antibacterial, besides that it is also necessary to pay attention to the optimum pH of the oral cavity considering the research was carried out in vitro where an acidic atmosphere will cause the demineralization process to be faster which causes

more tooth minerals to melt (Willis and Gabaldón 2020) so based on the research it looks like that all preparations are still at a suitable pH range for the manufacture of mouthwash but there is an increase in pH so that further analysis needs to be carried out regarding its relationship with shelf life at room temperature.

Based on the results of particle size evaluation, the majority of formulations are still classified as nanoemulsions which are characterized by an average globule size in the range of 1-100 nm (Zhang *et al.* 2017). The formulations D and E have globule sizes that are much larger than the nanoemulsion requirements, this could be due to the much higher concentration of glycerin surfactant in formulation D, with high levels of glycerin indicating that the emulsion is lipophilic. Theoretically, a solution with lipophilic properties indicates more oil phase in the preparation, with an oil density lower than water density, the mixture will form an aggregate of oil droplets where the greater the aggregation will be directly proportional to the size of the globule of the preparation and potentially on its stability (Barradas and de Holanda e Silva 2021). The drastic decrease in the control (-) formulation and Formula B with the equations owned in the ratio of the amount of surfactant. It is assessed that combined surfactants can increase stability by the formation of a flexible interfacial film layer to prevent globule incorporation this can be because the combined surfactant can reduce the surface tension of the globule emulsion using a single or dominant surfactant.

4.4. Evaluation of Stability Assay

Based on the result of shelf-life stability characteristic, the effect of temperature and shelf life on pH values causing a linear increase, while based on research conducted by Sahlan et al analyzed that mouthwash should not be too acidic because it can cause erosion of tooth enamel (Sahlan *et al.* 2018). As explained in the previous section, the pH of the formulated preparations tends to be neutral to acidic which shows a slight increase during the storage period, indicating that the pH of the preparation is less stable during the storage period which is characterized by an increase in pH which is considered to be influenced by the presence of phenolic acid in the preparation where the concentration of the ingredients active and the ratio of surfactants affect changes in pH during storage.

4.6. Results of the Oral Antibiofilm Activity Test Results for Mouthwash Preparations

Prior to the growth of cultures for both *S. mutans* and planktonic bacteria, serial dilution was carried out to facilitate the colony calculation process. Cultures on agar media can then be performed using the spread plate method. Commercial propolis mouthwash with the trademark propolisse was considered the most effective in inhibiting the growth of *S. mutans*, this analysis was influenced by the type of propolis used in this trademark in the form of Brazilian propolis with its high flavonoid content of 55 mg/L (Lisbona-González *et al.* 2021) which was much higher than the levels of flavonoids in formulations P_1 and P_2 with the addition of other active ingredients from excipients in the form of *Camellia sinensis* as a reinforcement of bacterial inhibition. Based on previous research, it was found that the characteristics of the best *Camellia sinensis* extract at an initial brewing temperature of 95°C for 15 minutes produced a high total flavonoid (Wang *et al.* 2022). *Camellia sinensis* extract could be natural ingredients considered to be the most effective as an antibacterial agent formulation in dentistry (Goenka *et al.* 2013).

The selection of variations in bacterial concentration refers to a study by Nakas, 2007 where the minimum concentration of *S. mutans* bacteria to form colonies on the surface of teeth and gums is 10^4 CFU/ml, while the concentration of 10^6 CFU/ml indicates an excessive level of *S. mutans* potential to cause caries (Nakaš and Zukanović 2007).

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