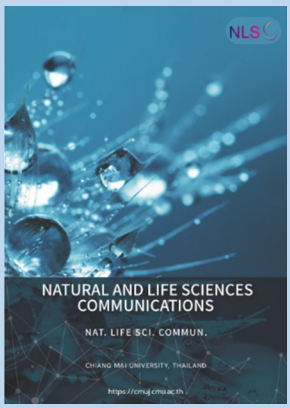
Research article



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Development of Toothpaste Containing *Morus alba* Stem Extract and Its Antimicrobial Activity Against Oral Pathogens

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ABSTRACT

Oxyresveratrol has been reported to combat the microorganisms that lead to gingival inflammation and tooth decay. The previous study found that *Morus alba* stems contained a high amount of oxyresveratrol compared to other parts of the tree. Therefore, the objectives of this research were to develop a toothpaste containing *M. alba* stem extract (MSE) and to evaluate its antimicrobial activity against oral pathogens. The ethanolic extract was first prepared, and its oxyresveratrol content was analyzed using high performance liquid chromatography (HPLC). Various toothpastes containing MSE were developed and characterized for the required characteristics. The toothpastes that satisfied the requirements were then chosen and subjected to a stability test. Lastly, the effectiveness of MSE and the most stable toothpaste in suppressing oral pathogens was assessed. Analysis of the MSE showed that oxyresveratrol was present at 8.74% by weight. The toothpaste containing 0.25% MSE was successfully developed. It showed good physical and chemical characteristics: the desired color, odor, and taste, with the right quantity of foam, a pH in the range of 5.5–10.5, and did not exhibit phase separation. Stability testing showed that it retained the required characteristics. MSE inhibited *Porphyromonas gingivalis* more effectively than *Streptococcus mutans*. The minimum inhibitory concentration (MIC) values of MSE against *P. gingivalis* and *S. mutans* were 62.50 µg/mL and 4 mg/mL, whereas the minimum bactericidal concentration (MBC) values were 125 µg/mL and 4 mg/mL. The toothpaste containing MSE effectively inhibited the growth of *P. gingivalis* and *S. mutans*, the causes of periodontitis and tooth decay.

Keywords: *Morus alba* stem extract, Toothpaste, Antimicrobial activity, Oral pathogens, Periodontitis

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INTRODUCTION

Toothpaste is a cosmetic that is essential to oral health. It reduces dental issues and fights oral pathogens. Oral pathogens play roles in the etiology of several oral diseases, including dental caries, gingivitis, periodontitis, and endodontic infection. Among these pathogens, *Streptococcus mutans* and *Porphyromonas gingivalis* are major microorganisms that cause dental caries and periodontitis (Capasso and Supuran, 2016). *S. mutans*, a gram-positive anaerobic bacterium, is the main pathogen responsible for dental caries and dental plaque (biofilm) formation. *S. mutans* digests sucrose into glucose and fructose. These monosaccharide sugars enter the glycolytic metabolic pathway and fermentation processes, where they are converted to pyruvate and then to lactic and other acids. These acids can dissolve enamel and dentin and facilitate the formation of cavities. In addition, it produces three glucosyltransferases (Gtfs) enzymes (GtfB, GtfC, and GtfD) (Zhang et al., 2021). GtfB produces mainly insoluble glucans containing α-1,3-linked glucose; GtfC produces a mixture of insoluble and soluble glucans (α-1,6-linked glucose); and GtfD synthesizes mostly soluble glucans (Paes Leme et al., 2006). The water-insoluble glucans contribute to the adhesion of bacteria to dental surfaces and facilitate bacterial colonization (Zhang et al., 2021). Conversely, the water-soluble glucans serve as backup energy when exogeneous fermentable carbohydrates are depleted, producing acids as by-products as mentioned above. These acids lower the pH of the dental plaque, leading to the demineralization of tooth enamel and subsequently the formation of cavities (Zhang et al., 2021). In addition, *S. mutans* can also cause serious oral health issues, such as mouth pain, tooth abscesses, poor nutritional status (Zhang et al., 2022), and inflamed pulp linked to severe dental caries (Nomura et al., 2016). *P. gingivalis* is a gram-negative anaerobic bacterium and plays a critical role in the progression of periodontal disease. Periodontal disease can range in severity from gingivitis (i.e., a moderate and reversible inflammation of the gingiva) to chronic deterioration of connective tissues, which forms periodontal pockets and eventually leads to tooth loss (How et al., 2016; Gerits et al., 2017; Supandi et al., 2023). Although more than 500 different bacterial species have been found to be present in human subgingival plaque, extensive studies have revealed that *P. gingivalis* is the main cause of chronic periodontitis (How et al., 2016).

Oxyresveratrol, or trans-2, 3', 4, 5'-tetrahydroxystilbene, is a natural compound found in various plants, such as grapes, *Artocarpus lakoocha*, and *Morus alba*, or white mulberry. Oxyresveratrol has been reported to inhibit the growth of *S. mutans* and the production of water-insoluble glucans, thereby reducing the formation of biofilm (Wu et al., 2020). It has also been found to suppress the production of acids by *S. mutans* during carbohydrate fermentation, which is crucial for preventing the demineralization of tooth enamel and the development of cavities. Wu et al. (2022) reported that oxyresveratrol, in conjunction with *Lactobacillus casei*, has been demonstrated to reduce the ability of *S. mutans* to adhere to tooth surfaces and inhibit the growth of biofilm formation of *S. mutans*. Oxyresveratrol has also shown promising effects against *P. gingivalis* by inhibiting its growth (Phoolcharoen et al., 2013) and attenuating the inflammatory response (Phoolcharoen et al., 2013; Sekiya et al., 2018), which is crucial for preventing tissue destruction and the progression of periodontal disease.

Apart from antimicrobial effects, oxyresveratrol also possesses anti-inflammatory effects. It has been shown to suppress LPS-induced inflammatory responses in the RAW264.7 murine macrophage cell line by downregulating the activity of various inflammatory signalling pathways, including NF-κB and MAPK pathways, which play crucial roles in orchestrating inflammatory responses (Lee et al., 2015). In the human periodontal ligament (hPDL) cell model, it was proven to suppress the mRNA and protein expression of the pro-inflammatory cytokines IL-6, IL-8, and IL-1ß (Phoolcharoen et al., 2013; Yiemwattana et al., 2018).

Our previous study determined quantities of oxyresveratrol in various parts of the mulberry tree (Thongsuk, 2007). The results found that the stems have the maximum amount of oxyresveratrol compared to that obtained from the twigs, with the least quantity in the leaves. We also confirmed the anti-inflammatory activities of *M. alba* stem extract (MSE) using LPS-stimulated RAW 264.7 cells (Soonthornsit et al., 2017). It attenuates the inflammation through the inhibition of nitric oxide production by suppressing mRNA and protein expression of the inducible nitric oxide synthase (iNOS). It has also been shown to inhibit cyclooxygenase (COX)-2 mRNA expression. Overall, the extract prepared from *M. alba* stems, which contains a high amount of oxyresveratrol, holds promise as a potential therapeutic agent for preventing and treating oral diseases, including dental caries and periodontal disease. Therefore, the objectives of this research were to further develop a toothpaste containing MSE and to evaluate its antimicrobial activity against oral pathogens.

MATERIAL AND METHODS

Materials

Brain Heart Infusion (BHI) Agar, and L-cysteine hydrochloride were obtained from HiMedia Laboratories Pvt. Ltd., Maharashtra, India. Tryptic Soy Broth (TSB) Agar and yeast extract were bought from Becton, Dickinson and Company, Maryland, USA. Vitamin K1, and hemin were purchased from Sigma Chemical Co., St. Louis, Missouri, USA. Horse blood was received from Oxoid Ltd., Cheshire, UK. Oxyresveratrol (purity 98%) was supplied by Chanjao Longevity Co., Ltd., Bangkok, Thailand. Sodium fluoride was received from Ajax Finechem, Australia. Carboxymethylcellulose (CMC FVH6, viscosity 2400–2600 mPa.s at 2% solution), was purchased from Changshu Wealthy Sciences & Technology Co., Ltd., China. Hydrated silicas (Zeodent® 113 and Zeodent®165) (Evonik Operations GmbH, Essen, Germany) were received as gifts from Jebsen & Jessen Ingredients (Thailand) Ltd. (Bangkok, Thailand). Cocamidopropyl betaine (CAPB) was bought from Galaxy Surfactants Ltd. (Maharashtra, India). PEG-40 hydrogenated castor oil (PEG-40) was obtained from BASF SE (Ludwigshafen, Germany). Tocopheryl acetate was obtained from Sigma-Aldrich, Massachusetts, USA. Butylated hydroxytoluene (BHT) was bought from Namsiang Co., Ltd., Bangkok, Thailand.

Preparation of *Morus alba* stem extract (MSE)

The stems of *M. alba* (var. Buriram 60) were supplied by the Queen Sirikit Sericulture Center, Phrae Province, Thailand. The sample stems were chopped and dried in a hot air oven at a controlled temperature of 50°C. The extraction protocol followed the method previously described by Soonthornsit et al. (2017). The chopped and dried stems (500 g) were macerated in 3,500 mL of 80% ethanol. The extraction process was repeated twice. The filtrates were collected, pooled, and dried using a rotary evaporator (Buchi, Flawil, Switzerland), followed by drying in a hot air oven (Memmert GmbH, Schwabach, Germany) at 50°C. The yield of the extraction was calculated based on the dry weight of the plant materials.

HPLC analysis of oxyresveratrol in MSE

Oxyresveratrol content in the MSE was quantified by HPLC analysis using the method described by Yhirayha et al. (2021) with some modifications. An HPLC system, the Nexera LC-40 (Shimadzu, Kyoto, Japan), was equipped with a photodiode array detector (PDA). The detection wavelength was 320 nm. An analysis was performed on a C18(2) LC column (Phenomenex Luna, 5 µm, 150 × 4.6 mm, Torrance, USA) at a controlled temperature of 30°C using the isocratic mode. Acetonitrile mixed with 0.05M phosphate buffer pH 3 (ratio 1:3) was used as the mobile phase at a flow rate of 1 mL/min. A standard oxyresveratrol was dissolved in

methanol to obtain concentrations ranging from 5 to 50 µg/mL. The stock solution of MSE was prepared by dissolving the extract in methanol at a concentration of 0.25 mg/mL.

Formulation of toothpastes containing MSE

The preparation process for the toothpaste containing MSE started with preparing the liquid base by dissolving water-soluble ingredients, including sodium fluoride and sodium benzoate, in water. This solution was added to a rheology modifier that had been pre-wetted with humectants like glycerin or sorbitol. After allowing the mixture to swell for 30–60 min, MSE and an antioxidant (tocopheryl acetate or BHT), which were dissolved in PEG-40 hydrogenated castor oil, were combined. Subsequently, each powder ingredient was added, one at a time, and mixed well. Flavoring and coloring agents were added and mixed thoroughly. Surfactants were added last and mixed gently to avoid foaming.

Evaluation of toothpaste

The physical and chemical characteristics of each of the various toothpaste samples were determined as follows.

Organoleptic evaluation

The organoleptic properties of the formulated toothpaste, including appearance, color, odor, and phase separation, were evaluated based on the criteria outlined by Thai Industrial Standard (TIS) S41-2019 (TISI, 2019), which state that an herbal toothpaste shall be homogeneously semi-solid, show no separation, and be free of foreign matter. It should have a good natural color from the ingredients used and no other undesirable odors such as musty, rancid, or spoiled. Although the taste of a toothpaste did not specify this requirement, it was noted in this study.

Spreadability

A 1 g sample of each toothpaste was placed on a glass plate 10 x 10 cm and 0.5 cm thick. Another identical-sized glass plate was used to cover the toothpaste, and a 1,000-gram weight was placed on top for 30 sec. The diameter of the spread on the plate was measured. A commercial herbal toothpaste was used as the control sample to benchmark the spreadability.

pH measurement

One gram of each toothpaste was weighed into a test tube. Three grams of water were then added and thoroughly mixed. The pH of the suspension was measured at 25 ± 1 °C within 10 min using a pH meter. The pH must be in the range of 5.5–10.5 as outlined by (TIS) S41-2019 (TISI, 2019).

Foam-forming test

The sample preparation was the same as for the pH test. However, the shaking was performed by placing the tube on a vortex mixer (Genie-2, Scientific Industries, Inc., USA) using touch-mode operation for 1 min. The level of foam produced was immediately measured and again after the tube was allowed to stand for 1, 3, and 5 min. The foam height at t = 0 min indicates foamability, while the foam height at t = 1, 3, and 5 min indicates foam stability.

The formulated toothpastes that exhibited favorable physical and chemical characteristics were chosen and subjected to a stability test.

Determination of stability

According to Thai Industrial Standard recommendations, heating-cooling cycling tests were used to determine the stability of each toothpaste (TIS S41-2019). The toothpaste samples, filled in flexible plastic tubes, were kept at 4 ± 2 °C for 24 hours and then stored at 45 ± 2 °C for 24 hours. This process was performed for four

cycles. Subsequently, the samples were allowed to cool to room temperature. Their organoleptic properties, pH, and foaming properties were then examined using the procedure mentioned previously, compared with their initial characteristics.

In addition, "How hard or easy is the toothpaste to squeeze from the tube?" was determined using the TA.XTplus texture analyzer (Stable Micro Systems, Surrey, UK) equipped with a 30 kg load cell. A cylinder aluminium probe, 20 mm in diameter (P/20 probe), was used. The parameters for the testing included a pre-test speed of 1 mm/sec, a test speed of 2 mm/sec, and a post-test speed of 10 mm/sec. The trigger force of 5 g was set, and the compression distance of 15 mm was targeted. The experiments were performed at 25°C. Peak forces of the force-time curves were recorded via Texture Exponent software (version 2.0.7.0 Stable Microsystems).

Following the stability tests which were carried out on the toothpastes containing MSE that had passed the characteristics tests, the toothpaste that remained unchanged was chosen and further tested for its antimicrobial activities.

Antimicrobial activity testing

The experiment protocols for the antimicrobial activities against *Porphyromonas gingivalis* (ATCC 33277) and *Streptococcus mutans* (ATCC 25175) were reviewed and approved by the Naresuan University Institutional Biosafety Committee (NUIBC MI 66-04-10).

The determination of the minimum inhibitory concentration (MIC) of *P. gingivalis* and *S. mutans* was performed by the broth macrodilution method in accordance with the previously described method (Yiemwattana et al., 2018) and CLSI standards (CLSI, 2020), with some modifications. A stock solution of MSE was prepared by dissolving the extract in a 2% DMSO solution. The MSE toothpaste was prepared by dispersing it in a culture medium. Two-fold dilutions were performed in 1 mL of supplemented TSB for *P. gingivalis* and 1 mL of BHI for *S. mutans*. The ingredients of the supplemented TSB were 30 mg/mL of tryptic soy broth, 5 mg/mL of yeast extract, 0.5 mg/mL of L-cysteine hydrochloride, 1 µg/mL of vitamin K1, 5 µg/mL of hemin, and 50 mg/mL of horse blood. After that, each bacterial inoculum with a 0.5 McFarland scale density was added to each tube to give a final concentration of 2.5×10^6 CFU/mL for *P. gingivalis* and 1×10^6 CFU/mL for *S. mutans*. The tubes were incubated under anaerobic conditions at 37°C for 18–24 hours for *P. gingivalis* and 48–72 hours for *S. mutans*. The growth of bacteria is indicated by the turbidity or the formation of a pellet of bacteria at the bottom of the tubes. The lowest concentration of the tested samples that inhibited the visible growth of the bacteria was recorded as the MIC.

Following the MIC determination, the MBC was determined by taking aliquots from the tubes that showed no visible growth of bacteria and inoculating them on supplemented TSB blood agar plates for *P. gingivalis* and on BHI agar plates for *S. mutans* using the drop plate method. Nutrient agar shared the same composition as nutrient broth, except 15% of agar powder was added. The plates were incubated and observed for bacteria forming colonies for 3-5 days. The minimum concentration of the test sample that kills 99.9% or more of the test microorganisms in the original inoculum was defined as the MBC (Rahmanian et al., 2015).

An uninoculated medium was used as a negative control, and a chlorhexidine solution (0.2%) was used as a positive control. Inoculated medium was employed as a bacterial growth control. Additionally, the 2% DMSO vehicle for MSE was utilized as a vehicle control.

Statistical analysis

The statistical analysis for before and after stability tests was determined by the paired t-test. A *P*-value < 0.05 was considered a statistically significant difference.

RESULTS

Extraction yield and oxyresveratrol content

The extract prepared from the stems of *M. alba* was dark brown in color, with a percentage yield of 5.5% of the dried plant material. The amount of oxyresveratrol in the extract was 8.74%.

Formulation of toothpaste

Toothpastes generally consist of abrasive, humectant, rheology modifier, surfactant, therapeutic agent, preservative, flavoring agent, sweetener, coloring agent, and water. In this study, they were prepared using Zeodent® 113 as an abrasive, glycerin or sorbitol as a humectant (to prevent toothpaste from drying out), CMC or Zeodent®165 as a rheology modifier, sodium lauroyl sarcosinate or CAPB as a surfactant, sodium fluoride as an anti-cavity agent, sodium benzoate as a preservative, spearmint as a flavoring agent, maltitol as a sweetener, brilliant blue as a coloring agent, and water as a solvent. MSE was the therapeutic agent used for its potential for preventing and treating dental caries and periodontal disease. The formulations, coded variously as Toothpastes A to H, are shown in Table 1.

Table 1. Ingredients of toothpastes containing *M. alba* stem extract formulated.

	Quantity (%w/w)							
	A	**B**	**C**	**D**	**E**	**F**	**G**	**H**
Glycerin	54.83	39.50	30.27	23.14	25.14	-	-	-
Sorbitol	-	-	-	-	-	36.48	29.61	29.61
Maltitol	2.52	12.57	17.64	17.64	17.64	-	8.82	8.82
CMC	0.45	0.45	0.45	0.21	0.21	0.58	0.58	0.58
MSE	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
PEG-40	7.00	7.00	7.00	7.00	5.00	0.83	1.67	1.67
Tocopheryl acetate (Vitamin E)	-	-	-	-	-	0.50	0.50	-
BHT	-	-	-	-	-	-	-	0.50
Sodium fluoride	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10
Sodium benzoate	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10
Zeodent 113®	20.00	20.00	20.00	20.00	20.00	20.00	20.00	20.00
Zeodent 165®	3.50	5.00	4.00	4.00	4.00	4.00	1.67	1.67
Titanium dioxide	0.50	1.00	0.50	0.50	0.50	0.50	0.50	0.50
Sodium lauroyl sarcosinate	1.65	1.65	2.40	4.50	4.50	4.50	3.00	3.00
CAPB	-	-	-	-	-	-	1.50	1.50
Flavoring agent	1.10	1.10	1.16	1.16	1.16	1.20	1.20	1.20
Brilliant blue	-	-	-	-	-	-	0.37	0.37
Water qs to	100	100	100	100	100	100	100	100

Glycerin and sorbitol are the most frequently used humectants in toothpaste. The glycerin-based toothpastes (i.e., Toothpastes A–E) were formulated. They showed a homogeneous and creamy texture without phase separation and were yellowish-brown in color (Table 2). The desirable level of sweetness was achieved using maltitol, which is a sugar alcohol that is not metabolized by oral bacteria

(Godswill, 2017). Toothpastes A–E had spreadability ranging from 3.9 to 5.9 cm. Their pH was about 7, which was in the acceptable range specified by TIS S41-2019, i.e., 5.5–10.5. The foaming ability of Toothpastes A–D was low and was enhanced when the content of PEG-40 hydrogenated castor oil was decreased, as obtained in Toothpaste E (Table 2). As seen in Table 2, the spreadability of Toothpaste E was nearly identical to that of the commercial toothpaste used as a benchmark. Also, it demonstrated the highest foamability (i.e., foam height at t = 0 min). Toothpaste E was therefore selected and subjected to the stability test.

Table 2. Organoleptic properties, pH, spreadability and foam formation of toothpaste developed compared to the commercial toothpaste (n = 2).

Parameter	Commercial	A	B	C	D	E	F	G	H
Appearance	-	homogeneous, creamy	homogeneous , creamy	homogeneous, creamy	homogeneous, creamy	homogeneous, creamy	homogeneous, creamy	homogeneous, creamy	homogeneous , creamy
Color	-	yellowish brown	yellowish brown	yellowish brown	yellowish brown	yellowish brown	yellowish brown	mint green	mint green
Odor	-	light mint smell	light mint smell	pleasant smell and feel fresh	pleasant smell and feel fresh	pleasant smell and feel fresh	pleasant smell and feel fresh	pleasant smell and feel fresh	pleasant smell and feel fresh
Taste	-	unpalatable, light sweet	sweetness increase	palatable, fresh, and cool	palatable, fresh, and cool	palatable, fresh, and cool	bitter, fresh, and cool	palatable, fresh, and cool	palatable, fresh, and cool
Spreadability (cm)	5.10	5.90	4.20	4.60	3.90	5.00	4.90	4.90	5.10
pH	-	7.24	7.07	7.12	7.05	7.11	7.20	7.00	7.32
Foam height (cm)									
t = 0 min	2.60	0.20	0.10	0.50	0.50	0.90	1.10	2.70	2.40
t = 1 min	2.50	0.20	0.10	0.50	0.50	0.80	1.1	2.60	1.90
t = 3 min	2.40	0.20	0.10	0.40	0.50	0.60	1.00	2.40	1.80
t = 5 min	2.30	0.20	0.10	0.40	0.50	0.50	1.00	2.30	1.50

It was noted that the appearance, color, odor, taste, and pH of Toothpaste E remained unchanged throughout the stability test (Figure 1, Table 3). At this point, a TA.XTplus texture analyzer fitted with a cylinder aluminum probe (20 mm in diameter) was used to measure the force required to extrude toothpaste from a tube. Following the stability test, the extrusion force required to extrude Toothpaste E increased significantly (10,748.10 ± 887.42 g) from the initial value (8,444.50 ± 574.76 g).

Next, Toothpastes (F–H) were further developed using sorbitol as the humectant. To enhance foam formation, the content of PEG-40 hydrogenated castor oil was further decreased, and the water component was increased by taking out maltitol from the formulation. Although the foamability of Toothpaste F only slightly increased, foam stability (i.e., foam height at t = 1, 3, and 5 min) greatly improved over that of Toothpaste E (Table 2). However, a bitter taste developed in Toothpaste F and became dominant. This is because glycerin and sorbitol have similar sweetness levels but are lower than those of maltitol. The sweetness value relative to the sucrose of glycerin is 0.6, sorbitol is 0.6, and maltitol is 0.9 (Godswill, 2017). Also, the reduction in PEG-40 hydrogenated castor oil content caused difficulty in the solubilization of MSE so the PEG-40 hydrogenated castor oil content was increased. Maltitol was re-added to the formulation to improve its palatableness. An antioxidant, tocopheryl acetate was added to Toothpaste F and G, while BHT was added to Toothpaste H to prevent unpleasant smell and taste due to chemical alteration in a product in the long term. Cocamidopropyl betain (CAPB), an amphoteric surfactant, was added to both Toothpaste G and H. CAPB does not have as strong foamability as an anionic surfactant such as sodium lauroyl sarcosinate but is commonly used as a foam booster to improve the foamability and stability of various products. CAPB was

combined with sodium lauroyl sarcosinate to enhance the foaming capability of both Toothpaste G and H. It was observed that both Toothpaste G and H were also found to have acceptable organoleptic characteristics, and the spreadability of Toothpaste G was 4.9 and Toothpaste H was 5.1 cm (Table 2). The pH of both formulations was in the acceptable range. The foamability and stability of both formulations were greatly enhanced (Table 2).

Following the stability test, Toothpaste G did not show phase separation (Figure 1). Its color and taste remained unchanged, but it smelled rancid (Table 3). On the other hand, Toothpaste H remained stable throughout the stability test (Figure 1, Table 3), so it was chosen for further investigation on its antimicrobial properties.

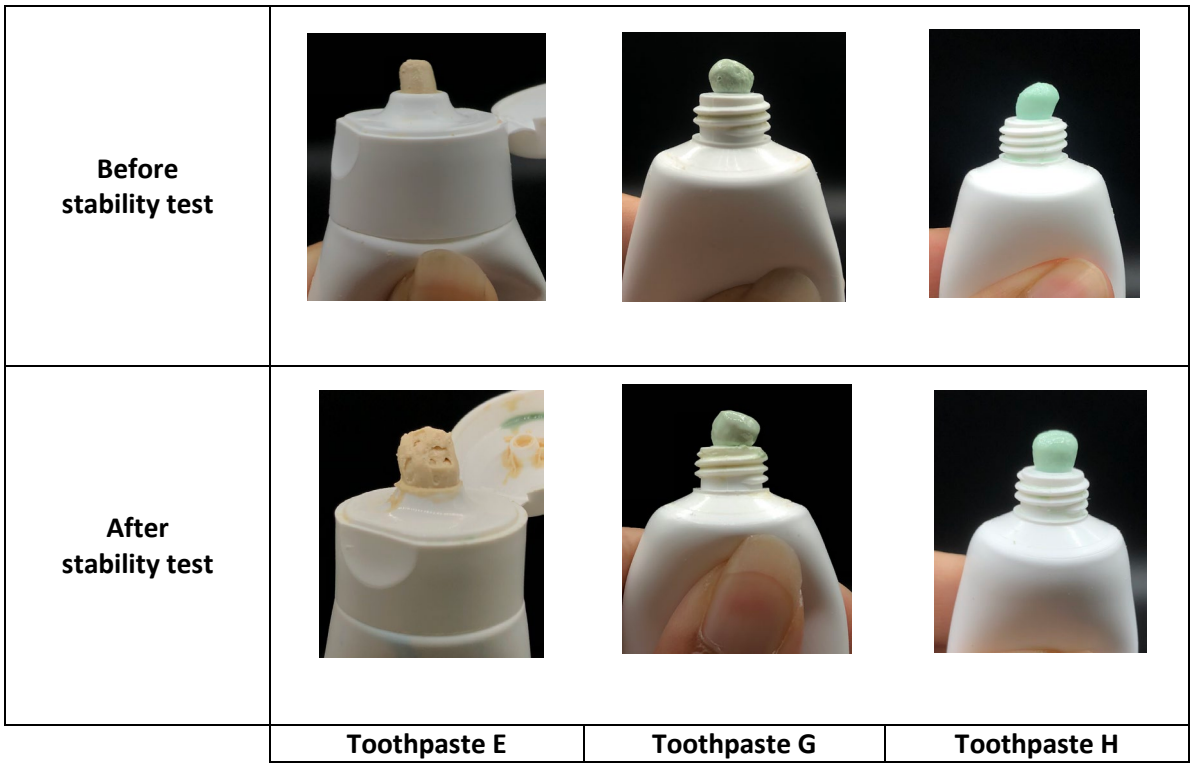

Figure 1. Appearance and color of Toothpastes E, G, and H before and after stability test.

Table 3. Characteristics of toothpaste containing *M. alba* stem extract (Toothpaste E, G, and H) before and after stability study (n = 3).

Parameters	Before stability test			After stability test		
	Toothpaste E	Toothpaste G	Toothpaste H	Toothpaste E	Toothpaste G	Toothpaste H
Organoleptic property						
Appearance	homogeneous, creamy	homogeneous, creamy	homogeneous, creamy	not change	not change	not change
Color	yellowish brown	mint green	mint green	not change	not change	not change
Odor	pleasant smell & fresh	pleasant smell & fresh	pleasant smell & fresh	not change	Rancid smell	not change
Taste	palatable, fresh, and cool	palatable, fresh, and cool	palatable, fresh, and cool	not change	not change	not change
pH	7.11 ± 0.05	6.99 ± 0.01	7.31 ± 0.01	7.19 ± 0.02	7.15 ± 0.00	7.36 ± 0.01
Foam height (cm)						
t = 0 min	0.87 ± 0.21	2.70 ± 0.29	2.15 ± 0.23	1.43 ± 0.33	4.05 ± 0.35	3.03 ± 0.57
t = 1 min	0.80 ± 0.16	2.60 ± 0.29	2.10 ± 0.12	1.33 ± 0.37	3.92 ± 0.35	2.89 ± 0.74
t = 3 min	0.57 ± 0.05	2.43 ± 0.25	2.03 ± 0.21	1.20 ± 0.36	3.70 ± 0.29	2.60 ± 0.80
t = 5 min	0.50 ± 0.00	2.30 ± 0.22	1.92 ± 0.16	1.17 ± 0.39	3.60 ± 0.29	2.28 ± 0.76
Extrudability (g)	8,444.50 ± 574.76	2,454.53 ± 108.76	2,012.30 ± 44.98	10,748.10 ± 887.42*	2,743.57 ± 161.85	1,587.23 ± 165.59

Note: * $P < 0.05$

Antimicrobial activity

As the negative control, the uninoculated medium did not show any colonies. The positive control, a 0.2% chlorhexidine solution, demonstrated bactericidal action against *P. gingivalis* and *S. mutans*. Conversely, bacterial growth was observed in the inoculated medium without MSE (i.e., the bacterial growth control). Furthermore, the MSE vehicle (i.e., 2% DMSO) exhibited no antimicrobial activity against any of the two oral pathogens.

MSE showed antimicrobial activity against the tested oral pathogens. However, it showed stronger inhibitory activity against *P. gingivalis* than *S. mutans* (Table 4). The MIC and MBC of MSE against *P. gingivalis* were 0.0625 and 0.1250 mg/mL, respectively. Its MIC and MBC values against *S. mutans*, however, were 4 mg/mL. MSE 0.0625 mg, 0.125 mg, and 4 mg are equivalent to MSE toothpaste 25 mg, 50 mg, and 1,600 mg, respectively. Nevertheless, Toothpaste H demonstrated total inhibition against *P. gingivalis* and *S. mutans* even after being diluted to 7.8125 mg/mL (or MSE 0.02 mg/mL) and 62.5 mg/mL (or MSE 0.16 mg/mL).

Table 4. Antimicrobial activity of *M. alba* stem extract (MSE) and Toothpaste H containing MSE at 0.25% w/w (n = 3).

Sample	*Porphyromonas gingivalis*		*Streptococcus mutans*	
	MIC	MBC	MIC	MBC
	(mg/mL)		(mg/mL)	
M. alba stem extract (MSE)	0.0625	0.1250	4.0000	4.0000
Toothpaste H	<7.8125	<7.8125	<62.5000	<62.5000

Note: Controls for the antimicrobial tests were uninoculated medium, inoculated medium, medium with 2% DMSO without MSE, and 0.2% chlorhexidine solution.

DISCUSSION

In this study, toothpastes containing MSE at 0.25% w/w with the desired characteristics were formulated. The content of MSE in the toothpaste was set at 0.25% w/w. This is because the recommended amount of toothpaste is pea-sized, or 0.25 g (EFSA, 2005), but 0.7 g is typically used (Ponikvar, 2008). Therefore, the dosage of the MSE toothpaste in this study was aimed at between 0.25 and 0.5 g, containing 0.625 and 1.25 mg of MSE extract per usage. This range permits dilution during brushing because it is five to ten times greater than the MBC value (i.e., 0.125 mg/mL).

The desired characteristics of a toothpaste are a homogeneous and creamy texture, a pleasant smell, being fresh and cool, as well as being palatable. Apart from these, a toothpaste's viscosity is a crucial feature that has an impact on customer satisfaction. The toothpaste should extrude out of the tube into a ribbon that can maintain its ribbon shape on a toothbrush's bristles without collapsing.

The spreadability and extrudability were used as indicators for toothpastes' viscosity. It was observed that the viscosity of the toothpastes was dependent mainly on the ratio of liquid components in the mixture, especially humectant, maltitol, and water. Humectant is one of the essential ingredients in toothpaste, which can prevent water loss and provide creamy structure. A large amount of water loss can cause the toothpaste to dry out, leading to hardening paste in the tube. Glycerin was used as the humectant in Toothpaste A–E, while sorbitol was used as the humectant in Toothpaste F–H. Both glycerin and sorbitol also act as thickening and sweetening agents. Maltitol, a sugar alcohol, was used as the sweetener. It provides a cooling sensation because, upon dissolving in water, an endothermic (heat-absorbing)

reaction occurs. Like glycerin, due to its viscous nature, maltitol also acts as a rheology modifier. The sugar alcohols, including glycerin, sorbitol, and maltitol, were used in this because they were not metabolized by oral bacteria. Therefore, they do not cause tooth decay (Bradshaw and Marsh, 1994; Godswill, 2017). Water was used as the solvent for water-soluble components and to adjust the final weight of the formulation. The viscosity properties of Toothpaste H were similar to those of the commercial toothpaste, as evidenced by its spreadability (5.1 cm) and extrudability (2,012.30 ± 44.98 g). The commercial toothpaste has a spreadability of 5.1 cm and an extrudability of 1919.2 ± 128.79 g. Following the stability test, the extrudability of Toothpaste H remained unchanged, suggesting that Toothpaste H possesses an appropriate viscosity.

The pH must be in the range of 5.5–10.5. This is because enamel is the outermost layer of the tooth. The main component of tooth enamel is calcium hydroxyapatite ($Ca_5(PO_4)_3(OH)$ or $Ca_{10}(PO_4)_6(OH)_2$), which naturally demineralizes, especially in environments with a pH value lower than 5.5 (called critical pH) (Amaechi and Loveren, 2013). All toothpastes developed in this study showed a pH of around 7, suggesting a low risk of enamel demineralization.

Many customers prefer toothpaste with high foaming ability since it gives a feeling of deep cleaning. Foam height determined at t = 0 min indicated foam ability, while that determined at the following time points indicated foam stability. Surfactant is known to facilitate the formation of foam by reducing the surface tension of a liquid. It can also enhance the stability of foam by inhibiting the coalescence of bubbles. In addition, lowering the surface tension property of the surfactant helps to remove debris from the surface of teeth. Thus, it functions as a cleansing agent. It also helps to disperse the toothpaste in the mouth. Sodium lauroyl sarcosinate, an anionic surfactant, was used as the surfactant in this study. Anionic surfactants are widely used as primary surfactants because they typically produce larger amounts of foam than other types of surfactants. However, some of them are known to be irritating, including sodium lauryl sulfate. Sodium lauroyl sarcosinate is mild and biodegradable. From the results, an increasing amount of sodium lauroyl sarcosinate could increase foam height to some extent. However, in combination with CAPB, foam forming ability was enhanced. The synergistic effect of sodium lauroyl sarcosinate (an anionic surfactant) and CAPB (a zwitterionic surfactant) has been reported by Wei et al. (2020).

The antimicrobial activities of MSE against *P. gingivalis* and *S. mutans* were clearly shown, indicating that it has therapeutic potential for preventing and treating periodontal disease and dental caries. Toothpaste H containing MSE at 0.25% w/w possessed strong bactericidal activities against *P. gingivalis* and *S. mutans* even after being diluted and stronger than the extract itself. This may be attributed to the toothpaste base. As mentioned above, the toothpaste base contains preservatives and surfactants, which may exert antimicrobial effects (Lindstedt et al., 1990; Yapar et al., 2017).

CONCLUSION

Toothpaste containing MSE at 0.25% w/w with the desired characteristics was successfully developed. It effectively inhibited the growth of *P. gingivalis* and *S. mutans*. Therefore, it has potential to be used for preventing and treating periodontal disease and dental caries. Nevertheless, further investigations into the cytotoxicity of MSE toothpaste in 3D oral tissue models or in volunteers are recommended.

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AUTHOR CONTRIBUTIONS

Angkanee Chatthong and Anyaphat Meepian designed and conducted the experiments and data analysis. Tasana Pitaksuteepong designed the experiments, supervised the project, and wrote the manuscript. Jantipa Jobsri was responsible for antimicrobial activity against *Porphyromonas gingivalis*. All authors have read and approved the final manuscript.

CONFLICT OF INTEREST

The authors declare that they hold no competing interests.

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