Original Article

pISSN, eISSN 0125-5614
M Dent J 2021; 41 (1) : 83-90

Efficacy of alcohol-free mouthwash containing essential oil from the fruits of *Zanthoxylum limonella* Alston on dental biofilm, gingivitis, and *Streptococcus mutans* controls

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Objective: To evaluate the efficacy of mouthwash containing essential oil from the fruits of Ma-Khwaen (*Zanthoxylum limonella* Alston) on dental biofilm, gingival inflammation, and *Streptococcus mutans* controls.
Materials and Methods: The crossover clinical trial was conducted in twenty-four volunteers who were allocated into three groups according to the types of mouthwash including 0.12% chlorhexidine digluconate (CHX), 0.5% Ma-Khwaen mouthwash (Ma-Kh), and distilled water (DW). After receiving professional prophylaxis and 2 -weeks run-in period, the volunteers started to rinse with their allocated mouthwashes, immediately after brushing in the morning and at night for 14 days. Clinical parameters, plaque index (PI), gingival index (GI), as well as *S.mutans* colony forming unit (CFU) count from collected supragingival plaque of four molars were conducted at baseline, Day 7, and Day 14. At the end of each experimental phase, the volunteers underwent 2-weeks washout period before starting the second and third allocations.
Results: Both CHX and Ma-Kh groups demonstrated the reduction on PI and GI statistically significant difference from baseline to Day 7 and Day 14. DW had no effects on PI and GI reduction. There was statistically significant difference when compared the efficacy in PI and GI reduction between CHX and DW groups as well as Ma-Kh and DW groups, but no significant difference between CHX and Ma-Kh groups. *S.mutans* CFU count significantly decreased in CHX group from baseline to Day 7 and Day 14, and in Ma-Kh group from baseline to Day 7. DW had no effect on *S.mutans* CFU reduction.
Conclusion: The alcohol-free mouthwash formulation containing essential oil from Ma-Khwaen fruits demonstrates the clinical efficacy on dental biofilm and gingival inflammation reduction but has little effect on *S.mutans* control. Ma-Khwaen mouthwash seems to be possible alternative to CHX mouthwash as part of the daily oral hygiene of patients with gingivitis.

Key words: dental biofilm, essential oil, gingivitis, mouthwash, *Zanthoxylum limonella* Alston

How to cite: Vachirarojpisan T, Karnchanarungroj K, Posiri C, Hongsamsipjet P, Charoensup R, Worapamorn W. Efficacy of alcohol-free mouthwash containing essential oil from the fruits of *Zanthoxylum limonella* Alston on dental biofilm, gingivitis, and *Streptococcus mutans* controls. M Dent J 2021; 41: 83-90.

Introduction

Dental biofilm is well recognized as a major role in the etiology of the two most common human oral diseases, including dental caries and gingivitis [1]. Mechanical plaque control is highly effective in controlling dental biofilm, however, this measure is difficult to be done evenly and thoroughly on a regular basis. The use of chemical plaque control is therefore considered as a useful adjunct to daily self-performed oral hygiene [2]. To date, mouthwashes containing various active agents including chlorhexidine (CHX), essential oils (EOs), cetylpyridinium chloride, zinc compounds, stannous fluoride, and herbs have been reported to be effective in reducing dental biofilm formation and gingivitis [3, 4]. CHX mouthwash, a broad-spectrum antiseptic, is nowadays accepted to be the gold standard for dental biofilm control. However, there

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Received: 24 November 2020 Revised: 20 January 2021 Accepted: 22 January 2021

are some undesirable side effects such as staining of teeth and tongue, altered taste sensation, and desquamation of oral mucosa, which limit patients' acceptability and its long-term use [5]. While the synthetic chemicals have a main action to kill or reduce microorganisms, the mechanism of mixture of herb- or plant-derived substances natural compounds in promoting oral health is related to anti-inflammatory and antioxidant properties, apart from antimicrobial activity [6]. Previous studies have reported for the potentiality on reducing dental biofilm and gingival inflammation of the mouthwashes containing herbal extracts, such as *Camellia sinensis* (green tea) and *Azadirachta indica* (neem) [4, 6]. EOs which obtained from the plant metabolism as byproducts, are hydrophobic liquids containing a mixture of volatile compounds such as alkaloids, phenols and terpenes. EOs have been widely used in the traditional medicine as antimicrobial, anti-inflammatory, antioxidant, expectorant, digestive, and diuretic agents [7, 8]. The EO mouthwash containing a complex mix of phenolic compounds has been found to be as effective as CHX in controlling gingival inflammation after 6 months of use [9].

Ma-Khwaen (Thai word of *Zanthoxylum limonella* Alston) is a local plant widely found in the Northern part of Thailand. Many parts of Ma-Khwaen tree have been used in Thai and Chinese traditional medicine, for examples, its bark and root have been used for relieving stomachache, indigestion and vomiting. Ma-Khwaen fruit is non-toxic and usually used as food flavor for northern Thai dishes due to its lemon scent and spice. There have been evidences suggesting that Ma-Khwaen fruits may be helpful in medicine because the essential oil extracted from Ma-Khwaen fruits contains three major constituents, including sabinene ($C_{10}H_{16}$); terpinen-4-ol; and limonene, which have potentiality of anti-inflammation and antimicrobes [10-15]. As Chiang Rai is one of the four provinces where the government promotes herbal city development to enhance the competitiveness of Thai herbal industry, therefore, it is of interest to study the usefulness of Ma-Khwaen EO in oral health medicine. It should take into consideration that the ethanol which is used to dissolve and stabilize the numerous substances present in the EO mouthwash may relate to the burning sensation and effect on the surfaces of composite restorations as well as its possible role in the development of oropharyngeal cancer [16, 17]. Therefore, the purpose of this study was to evaluate the efficacy of alcohol-free mouthwash formulation containing essential oil from the fruits of Ma-Khwaen on dental biofilm, gingival inflammation, and *Streptococcus mutans* controls.

Materials and methods

Essential oil Extraction Process

Ma-Khwaen fruits were collected during winter season in Chiang Rai province. Extraction of essential oil from Ma-Khwaen fruits was performed according to the previously described method [11]. Briefly, the 100 grams of clean and air - dried fruits were powdered and hydrodistilled in a Clevenger - type apparatus for 6 hours. The oil obtained on the top of the aqueous distillate was separated and dried over anhydrous sodium sulfate. The essential oil was stored in a hermetically sealed glass bottle at 4 °C until use.

Mouthwash Preparation

Three mouthwashes were used in the study; 0.12% CHX as a positive control, Ma-Khwaen (Ma-Kh) as the experimental mouthwash, and distilled water (DW) as a negative control. The mouthwash containing essential oil from Ma-Khwaen was prepared by thoroughly mixing 0.5 ml essential oil with 1 ml polysorbate 20 (TWEEN® 20), then gently added distilled water with constant stirring to provide 100 ml mixture before adding coloring agent (blue color; Food color, Winner®). The prepared product was thoroughly mixed until the mixture appeared optically transparent. A negative control was prepared with distilled water, coloring agent and flavoring agent (Pure lemon extract, Mc Cormick®).

Preparation of Mitis Salivarius Agar (MSA)

Mitis Salivarius Agar culture media (Mitis Salivarius Agar Medium; Difco Laboratorios, Detroit MI, USA) supplemented with 20% sucrose

and 0.2 U/ml bacitracin were formulated follow the standard protocol [18]. The poured agar plates were sealed and stored at 4ºC.

Dental plaque sampling

Supragingival dental plaque on buccal and lingual surfaces of four molars was collected with a sterile Gracey curette. Pooled plaque sampling was transferred into 1.0 ml of 0.05 M phosphate buffer solution pH 7.4 in microcentrifuge tube, then mixed well with Vortex mixer for 1 minute. 100 µl of the sampling was transferred into sterile tubes which contain 0.9 ml phosphate buffer solution pH 7.4 for serial 10x dilution, then mixed and diluted until the concentration of the solution was $1:10^6$ of the initial concentration. The diluted solution was spread on the prepared MSA plates, incubated in anaerobic condition (5% CO_2, 37 ºC) for 48 hours. The bacterial colony was observed and its morphological appearance was recorded. The number of CFUs of *S.mutans* was counted twice by a single rater who had been standardized intra-rater reliability.

Study protocol

Twenty-four healthy volunteers who had a minimum of 20 sound natural teeth without probing depth > 4 mm, a mean gingival index (GI; Loe & Silness 1963) [19] between 0.1 - 2.0 and a plaque index (PI; O'Leary 1972) [20] between 60 - 100%, participated in this double-blind, randomized, crossover clinical trial. The volunteers were randomly distributed into 3 groups (n = 8) which subsequently underwent a rotation of rinsing 0.12% CHX, Ma-Kh, and DW. Each volunteer was identified by a code. All the mouthwashes were prepared in identical looking plastic bottles which were coded as 1, 2, and 3. They were provided with measuring cups with 20 ml marking in order to use the correct volume of mouthwash. All studied mouthwashes were fresh prepared and gave to the volunteers on the day of appointment.

All volunteers received scaling and root planing before entering each allocation of the trial. After a 2-weeks run-in period, the volunteers were instructed to rinse twice daily with 20 ml of their allocated mouthwashes for 1 min, immediately after brushing in the morning and at night for 14 days. Subsequent rinsing with water was not allowed. To check for compliance, the volunteers were asked to make a mark on the provided time schedule when they used mouthwashes. At each follow-up appointment, they were asked to bring back the bottles to assess the volume of mouthwashes. The clinical parameters, GI and PI, were recorded and dental plaque sampling was collected at baseline, Day 7, and Day 14. After completing each allocation, the volunteers were asked to rate a 5-point scale questionnaire regarding the satisfaction of the mouthwashes they used, in terms of smell, taste, color, duration of rinsing and overall satisfaction. The volunteers underwent 2-weeks washout period before starting the second and third allocations.

The study was approved by Mae Fah Luang University Research and Ethical Committee (REH-62072).

Statistical analysis

Statistical analyses were performed with the RStudio program version 1.2.5033. The difference of dental biofilm and gingival inflammation reduction, and the antibacterial against *S.mutans* of the studied mouthwashes were compared within group, at each studied time to the baseline, by using Wilcoxon signed-ranks test. Mann-Whitney U test was used to compare the difference of these parameters between groups at each studied time. Statistically significant difference was considered if p value was < 0.05.

Results

The effect of the studied mouthwashes on dental biofilm formation

After twenty-four volunteers finished the three allocations of using their assigned mouthwashes, the results showed that there was statistically significant reduction on PI from baseline to Day 7 and Day 14, both in CHX group and in Ma-Kh group ($p < 0.01$). DW group did not demonstrate this plaque reduction effect. When compared the effect on dental biofilm reduction among the studied mouthwashes at each time, the results demonstrated that there was statistically significant difference from baseline to

Day 7 between CHX and DW ($p < 0.05$), and between Ma-Kh and DW ($p < 0.01$). The statistically significant difference on dental biofilm reduction was also demonstrated from baseline to Day 14 between CHX and DW ($p < 0.01$), and between Ma-Kh and DW ($p < 0.01$). No statistically significant difference was found between CHX and Ma-Kh groups, both at Day 7 and Day 14. (Figure 1, Table 1)

The effect of the studied mouthwashes on gingival inflammation

The effect on gingival inflammation reduction was significantly demonstrated from baseline to Day 7, both in CHX group and in Ma-Kh group ($p < 0.05$). The reduction constantly continued to Day 14, which showed statistically significant difference from baseline in CHX group and in Ma-Kh group ($p < 0.01$). DW group did not demonstrate this effect, both from baseline to Day 7 and baseline to Day 14. When compared the effect on gingival inflammation reduction among the studied mouthwashes at each time, the results demonstrated that there was statistically significant difference from baseline to Day 7 between CHX and DW ($p < 0.05$), and between Ma-Kh and DW ($p < 0.01$). The statistically significant difference on gingival inflammation reduction was also demonstrated from baseline to Day 14 between CHX and DW ($p < 0.05$), and between Ma-Kh and DW ($p < 0.01$). No statistically significant difference was found between CHX and Ma-Kh groups, both at Day 7 and Day 14. (Figure 2, Table 1)

The effect of the studied mouthwashes on Streptococcus mutans counts

Streptococcus mutans were distinguished among others by their morphology which described as raised, convex, undulate, opaque, pale blue colonies, granular frosted glass appearance on MSA. The antibacterial effect against *S.mutans* was significantly demonstrated from baseline to Day 7 and Day 14 ($p < 0.01$) in CHX group. Ma-Kh group was significantly demonstrated the effect only from baseline to Day 7 ($p < 0.05$) but not at Day 14. DW group did not demonstrate this effect from baseline to Day 7 and Day 14. When compared the antibacterial effect against *S.mutans* among the studied mouthwashes at each time, there was no statistically significant difference except the effect between Ma-Kh and DW groups at Day 7 ($p < 0.05$) (Table 1)

Satisfaction of the studied mouthwashes

The results of the satisfaction questionnaire demonstrated that the volunteers rated the highest satisfaction for DW in all aspects conducted including smell, taste, duration of rinsing and overall satisfaction except color. The satisfaction of Ma-Kh mouthwash regarding smell, taste, duration of rinsing, and overall satisfaction was better than CHX.

Discussion

It is widely accepted that the control of dental biofilm formation, both mechanical and chemical means, is the cornerstone for the prevention of dental caries, gingivitis as well as periodontitis [1, 2]. The present clinical study is the first report to provide the evidence that mouthwash containing the essential oil from Ma-Khwaen (*Zanthoxylum limonella* Alston) fruits may be of value in the control of dental biofilm and gingival inflammation.

The antiplaque effect is the capacity of agents that can prevent the formation of bacterial aggregate on tooth surfaces by their antimicrobial properties and/or directed against specific adherence mechanisms i.e. ionic charge, surfactants, and wettability properties. During a 14 - day period of twice daily rinsing of Ma-Kh mouthwash in the present study demonstrated the constantly and significantly reduced plaque formation in the similar pattern to CHX mouthwash. This effect may due to the major components in Ma-Kh EO, sabinene and terpinen-4-ol, which have been known for their antimicrobial properties [11, 21]. The mechanism of this antiplaque effect, however, may attribute to microemulsion of the mouthwash which may provide better penetration throughout the biofilm or direct against specific adherence of bacterial aggregation on the tooth surface. This speculation should be explored with further investigations.

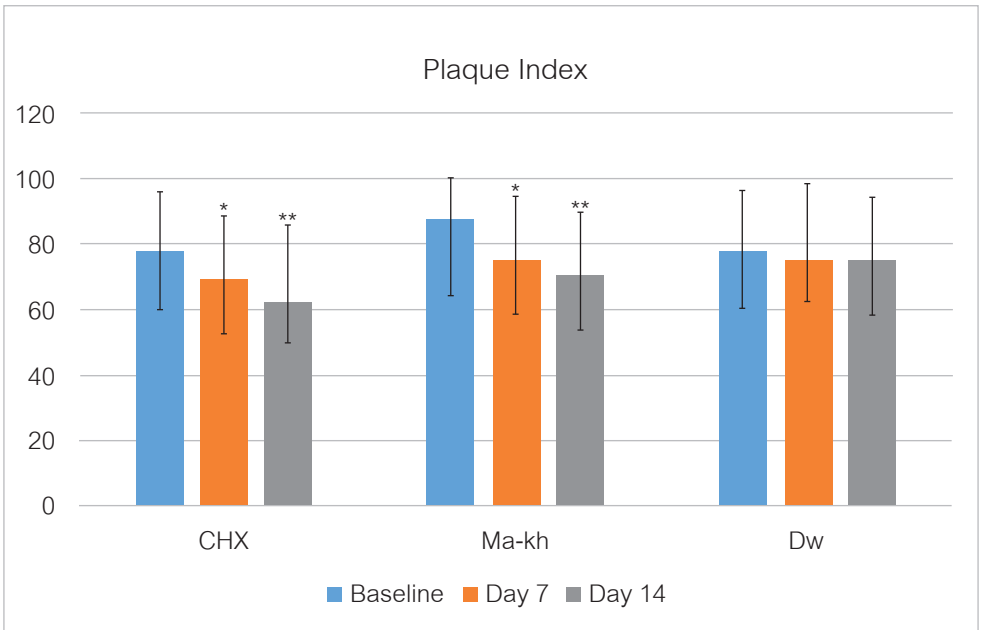

Figure 1 The effect of the three mouthwashes on dental biofilm reduction represented by Plaque Index (PI) at baseline, Day 7 and Day14.
CHX: 0.12% chlorhexidine digluconate, Ma-Kh: 0.5% Ma-Khwaen mouthwash, DW: Distilled water
*$p < 0.01$ statistically significant difference when compared Day 7 to baseline
**$p < 0.01$ statistically significant difference when compared Day 14 to baseline (Wilcoxon signed-ranks test)

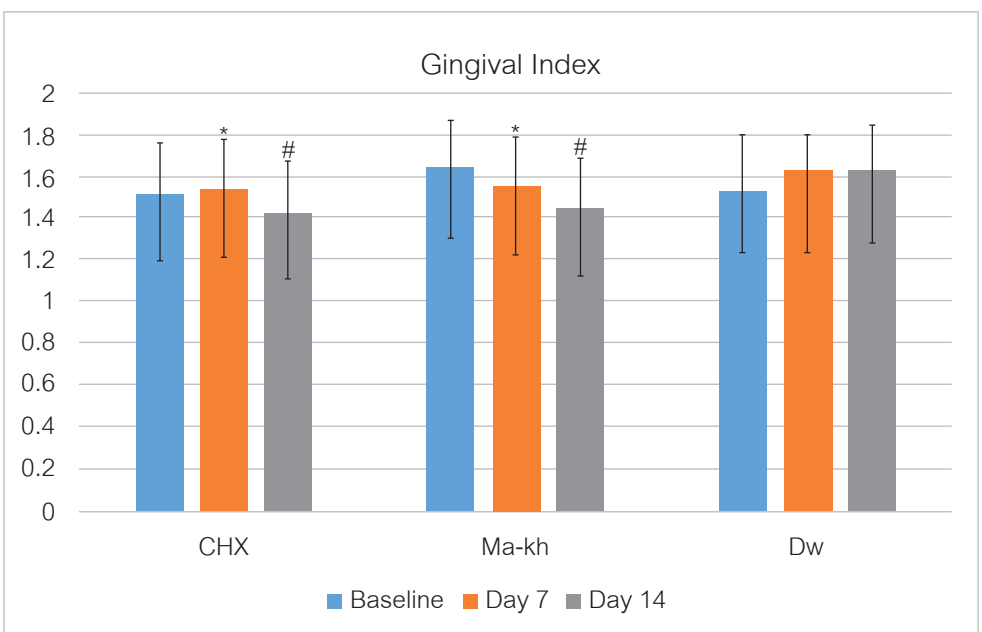

Figure 2 The effect of the three mouthwashes on gingival inflammation reduction represented by Gingival Index (GI) at baseline, Day 7 and Day14.
CHX: 0.12% chlorhexidine digluconate, Ma-Kh: 0.5% Ma-Khwaen mouthwash, DW: Distilled water
*$p < 0.05$ statistically significant difference when compared Day 7 to baseline
#$p < 0.01$ statistically significant difference when compared Day 14 to baseline (Wilcoxon signed-ranks test)

Table 1 Comparison the effect on dental biofilm formation (Plaque Index), gingival inflammation (Gingival Index) and *Streptococcus mutans* colony forming unit (CFU) counts at baseline to Day 7 and baseline to Day 14 among the three mouthwashes

	CHX	Ma-Kh	DW	*p*-value[c,d,e]
Plaque Index (%)				
Baseline	77.59 ± 18.38	87.98 ± 12.78	77.93 ± 18.91	
Day 7	69.16 ± 19.49	75.1 ± 20.33	75.58 ± 22.61	0.04[c*], 0.00[d#], 0.67[e]
p-value[a]	0.00[#]	0.00[#]	0.77	
Day 14	62.82 ± 23.13	70.89 ± 18.45	75.91 ± 18.01	0.00[c#], 0.01[d#], 0.33[e]
p-value[b]	0.00[#]	0.00[#]	0.43	
Gingival Index (0-3)				
Baseline	1.52 ± 0.24	1.65 ± 0.22	1.54 ± 0.26	
Day 7	1.55 ± 0.23	1.57 ± 0.23	1.61 ± 0.2	0.03[c*], 0.00[d#], 0.14[e]
p-value[a]	0.02*	0.03*	0.14	
Day 14	1.43 ± 0.25	1.45 ± 0.24	1.53 ± 0.26	0.05[c*], 0.01[d#], 0.40[e]
p-value[b]	0.01[#]	0.00[#]	1.0	
Streptococcus mutans **(*10^6 CFU/mL)**				
Baseline	0.87 ± 0.80	5.41 ± 4.03	8.03 ± 9.83	
Day 7	0.54 ± 0.71	5.17 ± 4.78	11.31 ± 12.06	0.25[c], 0.05[d*], 0.07[e]
p-value[a]	0.00[#]	0.03*	0.51	
Day 14	0.48 ± 0.45	6.73 ± 5.36	9.45 ± 8.42	0.61[c], 0.53[d], 0.69[e]
p-value[b]	0.00[#]	0.48	0.89	

CHX: 0.12% chlorhexidine digluconate, Ma-Kh: 0.5% Ma-Khwaen mouthwash, DW: Distilled water

[a] Comparison with the baseline (before rinsing) and after 7 days (Willcoxson signed-rank test)

[b] Comparison with the baseline (before rinsing) and after 14 days (Willcoxson signed-rank test)

[c] Comparison with the DW group and CHX group (Mann-Whitney *U*-test)

[d] Comparison with the DW group and Ma-Kh group (Mann-Whitney *U*-test)

[e] Comparison with the Ma-Kh group and CHX group (Mann-Whitney *U*-test)

*$p < 0.05$, #$p < 0.01$

The anti-inflammatory effect on gingival inflammation of Ma-Kh mouthwash was demonstrated by decreasing gingival bleeding which showed no statistically significant difference when compared to CHX mouthwash but significantly better than DW. This finding supported previous studies regarding anti-inflammatory property of the components in EOs, sabinene and terpinen-4-ol. Sabinene has been reported for the strong anti-inflammatory activity through nitric oxide production inhibition in lipopolysaccharide plus interferon gamma (IFN-γ)-triggered macrophages [14]. It was also reported for anti-inflammatory activity against carrageenan-induced paw edema in mice [15]. Meanwhile, terpinen-4-ol, has been reported to suppress the production of TNF-alpha, IL-1 beta, IL-8, IL-10 and PGE_2 by LPS-activated monocytes [22, 23].

Interestingly, the recent study has been reported the inhibitory effects of sabinene on the growth, acid production, biofilm formation, and adherence of *S. mutans* [24]. The study demonstrated that sabinene inhibited the growth of *S. mutans* in a dose-dependent manner and showed a tendency to decrease the expression of the spaP gene, which may relate to the inhibition of *S. mutans* adhesion. It was found in this present study that Ma-Kh mouthwash had the effect on *S.mutans* reduction only in a short period of time. Whether this finding related to the concentration of EO in the mouthwash still requires further studies.

Traditionally, most of the formulations of mouthwashes including EO and herbal mouthwashes have contained alcohol as a solvent for other substances. The presence of alcohol in mouthwashes was a controversial issue in many of the previous studies regarding the risk of oral cancer, oropharynx or other head and neck cancers. Recent systematic review concluded that the use of alcohol containing mouthwashes has not represented an independent risk factor for the development of head and neck cancer. However, the risk does increase when it occurs in association with other carcinogenic risk factors [25]. In the present study, polysorbate 20 (TWEEN® 20) was used as an emulsifying agent for stable oil-in-water emulsions of Ma-Kh EO in the mouthwash. Polysorbate 20, a polyoxyethylene sorbitol ester, is well known as a nonionic surfactant with its hydrophilic–lipophilic balance (HLB) 16.7 which is suitable for EOs dispersed in aqueous phase and works as an effective solubilizer for EO [8]. Non-alcoholic formulation should be in consideration because it showed a favorable response and in order to avoid the risk of alcohol side effects. People who experience dry mouth due to medicinal side effects, radiation therapies or some systemic diseases such as Sjogren's syndrome or diabetes, can all benefit from using alcohol free mouthwashes [16].

The preferable of selecting and complying with mouthwashes are closely related to taste, color, smell and the pleasant sensation that follows use. The combination of lemon-liked smell from limonene and the woody and spicy scent from sabinene in Ma-Kh EO made the specific taste and unique smell of Ma-Kh mouthwash. This clinical trial demonstrated that the participants were more satisfied with Ma-Kh mouthwash than CHX mouthwash by its odor and taste.

Conclusion

The alcohol-free mouthwash formulation containing essential oil from Ma-Khwaen fruits demonstrates the clinical efficacy on dental biofilm and gingival inflammation reduction but had little effect on *S.mutans* control. Ma-Khwaen mouthwash seems to be possible alternative to CHX mouthwash as part of the daily oral hygiene of patients with gingivitis.

Acknowledgement

We would like to thank Assoc.Prof.Dr. Rawiwan Charoensup for her generous providing Ma-Khwaen (Zanthoxylum limonella Alston) essential oil and Mr.Dhiti Osatakul for his advice in statistical analysis.

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