Original Article

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In Vitro Anti-Cariogenic Plaque Effects of Essential Oils Extracted from Culinary Herbs

Microbiology Section

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ABSTRACT

Introduction: Cariogenic bacteria including mutans streptococci and lactobacilli are partly but significantly involved in dental caries development. An effective prevention strategy against dental caries is to decrease the accumulation of this microbiota either in planktonic or in biofilm form.

Aim: To examine the antimicrobial and anti-plaque effects of some culinary herbs (spices), so the herbs are plausibly used as alternative and effective herbal plaque control supplements to promote good oral health.

Materials and Methods: Essential oils extracted from sweet basil *(Ocimum basilicum)*, cinnamon bark *(Cinnamomum zeylanicum)*, sweet fennel *(Foeniculum vulgare)*, kaffir lime *(Citrus hystrix)*, black pepper *(Piper nigrum)*, peppermint *(Mentha piperita)*, and spearmint *(Mentha spicata)* were primarily examined for their antimicrobial activities against the cariogenic bacteria *(Streptococcus mutans* KPSK2 and *Lactobacillus casei)* using the agar disk diffusion and broth microdilution methods, respectively. These essential oils were then analysed for anti-plaque effects (retardation of *S. mutans* biofilm formation and reduction of the in vitro established biofilm). This experimental study was performed at the Department of Oral Microbiology, Faculty of Dentistry, Mahidol University during June 2015 till August 2016.

Results: All selected essential oils showed different degrees of antimicrobial activity against the planktonic form of both cariogenic bacteria. Cinnamon bark essential oil expressed the strongest inhibitory effect against *S. mutans* {MIC of 0.08% (v/v)} and *L. casei* {MIC of 0.16% (v/v)}, whereas the weakest effect was found in kaffir lime essential oil {MIC values of 2.5% and 5.0% (v/v) for *S. mutans* and *L. casei*, respectively}. Up to 80% of *S. mutans* biofilm was retarded to form on the substratum primed with these spice essential oils, especially cinnamon oil. The preventive effect of these oils was in dose- and exposure time-dependent manners. For reductive effect against the 24-hour pre-established *S. mutans* biofilm, at least 50% of the biofilm mass was reduced when the biofilm was treated with each essential oil at the MIC for an hour. The reductive effect against the in vitro established *S. mutans* biofilm of these culinary herb essential oils only depended on the exposure time.

Conclusion: Cinnamon and sweet basil essential oils with impressive in vitro anti-cariogenic bacteria and anti-plaque effects may be proposed as alternative and effective supplements to promote oral health status.

Keywords: Antimicrobial activity, Cariogenic bacteria, Anti-plaque effects

INTRODUCTION

Dental caries affects directly every age group worldwide, particularly in developing countries. As a multifactorial oral disease, both intrinsic (host-susceptibility: age, immunity, and behavior) and extrinsic (time, diet, and microbiota) factors significantly influence caries development [1]. Mutans streptococci and lactobacilli are oral bacteria predominantly isolated from initial and advanced caries, respectively [2-5]. There are several predisposing factors leading to a heavy accumulation of oral microflora as well as the cariogenic bacteria and the ones causing dental caries. These factors include high consumption of sugar and glutinous diet (snacks) of children, poor oral hygiene due to impairment of physical potential and xerostomia of the elderly and cancer patient undergone chemical- or radio-therapy. Acidic products derived from fermentable carbohydrates by the catabolism process of oral microflora, especially cariogenic bacteria enhance demineralization of the teeth leading to dental caries development [6]. Therefore, one of effective strategies in oral health promotion is to control microbial accumulation (biofilm/plaque) on hard and soft surfaces of the mouth by natural cleaning (mastication, etc.,), mechanical cleaning (brushing and flossing), and chemical plaque control (fissure sealant, fluoride supplement, sugar substitution and antimicrobial supplements) procedures [7,8]. However, several adverse effects including staining on the teeth, unpleasant taste, and induction of antimicrobial resistant strain after long-term application of some antimicrobial agents have been reported [9]. Culinary herbs, especially the ones with medicinal properties (anti-inflammatory, antimicrobial) have become choices of interest even though their safety, bioavailability, and pharmacokinetic interactions have yet to be extensively investigated [10].

Seven culinary herbs used as spices in Thai cuisine were the focus of this study. The spices included black pepper (*Piper nigrum*), cinnamon (*Cinnamomum zeylanicum*), kaffir lime (*Citrus hystrix*), peppermint (*Mentha piperita*), spearmint (*Mentha spicata*), sweet basil (*Ocimum basilicum*), and sweet fennel (*Foeniculum vulgare*). Several reports clearly demonstrate that these culinary herbs contain antioxidant, anti-carcinogenic and antimicrobial properties, especially against medically important microorganisms [11-16]. However, there are relatively few studies regarding their antimicrobial property against oral microflora related to dental caries development [17]. An investigation of the inhibitory effect of these culinary herb essential oils against planktonic (floating) and biofilm forms of the caries-related microorganisms (mutans streptococci and lactobacilli) is required before the herbs may be proposed as naturally derived plaque control agents to promote oral health status. Therefore, the selected culinary herbs were examined for the in vitro antimicrobial and anti-plaque effects against the cariogenic bacteria in this study. It may provide informative guidance for further investigations and development of natural anti-caries supplements beneficial for dental caries management.

MATERIALS AND METHODS

Essential oils extracted from the bark of cinnamon; leaves of sweet basil, peppermint, spearmint, kaffir lime, and sweet fennel; peel of kaffir lime; seeds of black pepper were commercially acquired from Tropicalife Company, Ltd. (Bangkok, Thailand). Brain Heart Infusion (BHI) agar and broth were purchased from BBL™ whereas Rogosa SL and Mueller-Hinton agar were from Difco™ Becton, Dickinson and Company (Bangkok, Thailand). Artificial saliva was purchased from Ramathibody Hospital (Bangkok, Thailand). *Streptococcus mutans* KPSK2 and clinical strain *Lactobacillus casei* were provided by the Department of Oral Microbiology, Faculty of Dentistry, Mahidol University, Bangkok, Thailand. Chlorhexidine mouthwash (0.2% and 0.5%) was prepared by the Faculty of Dentistry, Mahidol University, Bangkok. This experimental in vitro study was carried out during June 2015 to August 2016.

Antimicrobial Effect against Planktonic Cariogenic Bacteria

All selected herbal essential oils were screened for their anti-microbial activity by agar disk diffusion method [18]. Briefly, *S. mutans* KPSK2 and *L. casei* clinical strains were freshly sub-cultured on BHI agar and incubated at 37ºC with 5% CO_2 for 48 hours. Few colonies of each bacterium were collected, cultured in BHI broth and incubated under the condition mentioned for 24 hours. All culinary herb essential oils (200 µL) were primarily diluted with 10% Dimethyl Sulfoxide (DMSO) (10 mL DMSO in 90 mL BHI broth) (800 µL) to be 20% (v/v). A 20 µL of diluted herbal essential oil was added into each sterile 6 mm-diameter paper disk and air dried. The disks were placed on the Mueller Hinton agar that was previously inoculated evenly with bacterial suspension (0.5 McFarland standards, 1-2x10^8 CFU/mL). The plates were incubated at 37ºC with 5% CO_2 for 24 hours. Expression of antimicrobial activity of each herb was analysed from inhibition zone appearance, measured and recorded. The 0.2% chlorhexidine and 10% DMSO were used as positive and negative controls, respectively. The experiment was done in triplicate each with three repeats.

Each herbal essential oil was further examined for its Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) against both cariogenic bacteria by broth microdilution method with some modification [19]. In brief, each herbal essential oil was 2-fold serially diluted with 10% DMSO in Phosphate-Buffered Saline (PBS) to obtain concentrations ranging from 20% to 0.04% (v/v). The cariogenic bacteria were prepared as mentioned previously. Each well of a 96-flat bottom well polystyrene plate (substratum) was filled with 15µL of bacterial suspension in BHI (5x10^6 CFU/mL). Then, a 135µL of each concentration of herbal essential oil (columns 1-10), 0.2% chlorhexidine in BHI (column 11) and 10% DMSO (column 12) was added and designated as tests, positive and negative controls, respectively. The plate was incubated at 37ºC with 5% CO_2 for 24 hours. MIC was the lowest concentration of a certain herb required to impede the growth of cariogenic bacteria (visually clear well). Minimum bactericidal concentration (MBC), defined as the lowest concentration of herb capable of eliminating the tested organisms, was evaluated by transferring 25µL of the solution in each clear well to BHI agar and incubated in the environment described above for 24 hours. The experiment was done in triplicate each with three repeats.

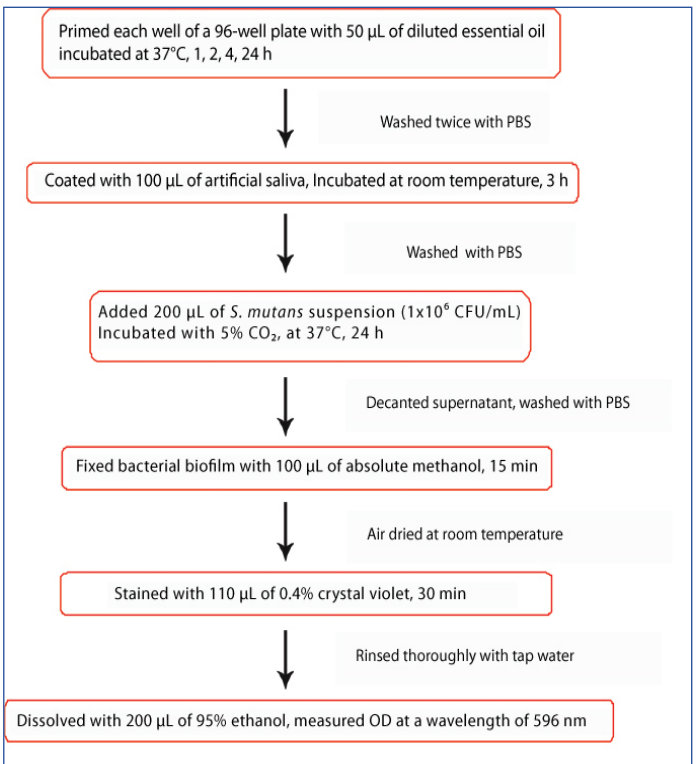
The culinary herb essential oils with potent antimicrobial property, especially against the planktonic *S. mutans* KPSK2 were finally examined for their anti-plaque effects against *S. mutans* biofilm which include preventive effect on the formation of bacterial biofilm in vitro and reductive effect on the pre-established biofilm, with minor modification [20].

Anti-plaque effects against *S. mutans* biofilm

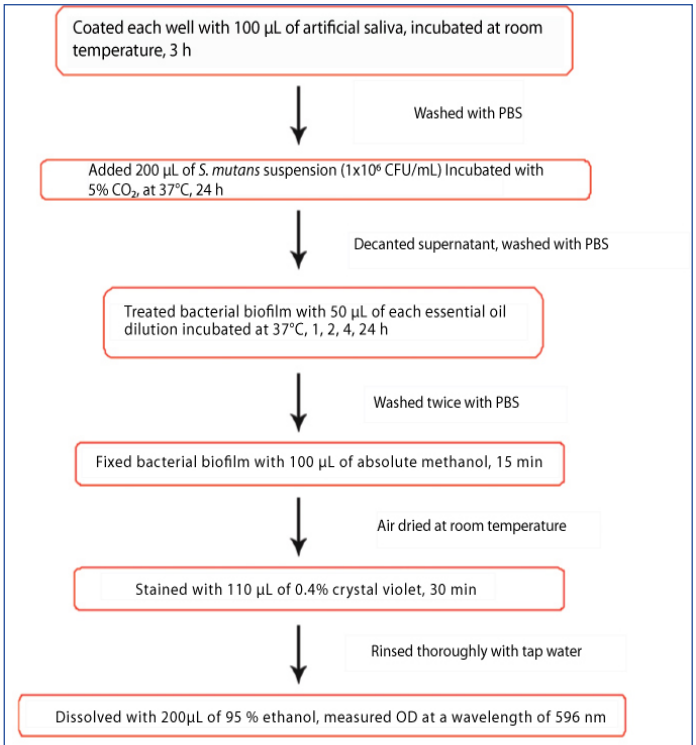
Preventive effect on the formation of *S. mutans* biofilm in vitro [20]: A 96-well plate was firstly coated with 50µL of varied concentrations of herbal essential oils ranging from MIC, 2xMIC to 4xMIC, 0.2% chlorhexidine as well as BHI with 3% sucrose (BHI-S) and was designated as tests, positive and negative (untreated substratum) controls, respectively then the plate was incubated at 37ºC in 5% CO_2 incubator for different time-intervals (1, 2, 4, and 24 hours). Each well was emptied and washed twice with PBS, pH 7.2. The plate was then applied with 100 µL artificial saliva, incubated at room temperature for three hours and washed with PBS before applied with 200µL of *S. mutans* suspension containing 1x10^6 CFU/mL or BHI-S (blank control). This was then incubated aerobically with 5% CO_2 at 37ºC for 24 hours. The supernatant was gently decanted and washed with 200µL PBS. The bacterial biofilm was fixed with 100µL absolute methanol for 15 minute then air dried at room temperature. The biofilm was stained with 110 µL of 0.4% crystal violet solution for 30 min before rinsed thoroughly with tap water until the well of blank control appeared colorless. The biofilm mass was quantitated by the addition of 200µL of 95% ethanol, shaken for 30 minute, then 100µL of solution was transferred to a new 96-well plate and absorbance (optical density, OD) was measured at a wavelength of 596 nm [Table/Fig-1]. Preventive effect of the spice essential oils tested was interpolated to be the percentage of inhibition on the formation of bacterial biofilm by using the equation shown below. The experiment was done in triplicate each with three repeats.

$$\{1 - (OD_{596} \text{ of the test}/OD_{596} \text{ of negative control})\} \times 100$$

Reductive effect against the *S. mutans* biofilm established in vitro [20]: A 96-well plate was first coated with 100µL of artificial saliva and incubated at room temperature for three hours. The plate was washed twice with PBS before 20 µL of *S. mutans* inoculum (1x10^6 CFU/mL) was applied into each well containing BHI-S that was 200µL in total volume. The bacterial biofilm was allowed to form in 37ºC incubator with 5% CO_2 for 24 hours. As mentioned in the section of preventive effect, various



[Table/Fig-1]: Methodology to determine preventive effect of essential oil on the formation of *S. mutans* biofilm in vitro.



[Table/Fig-2]: Methodology to determine reductive effect of essential oil against the *S. mutans* biofilm established in vitro.

concentrations of herbal essential oils, chlorhexidine and BHI-S were added to the pre-established biofilm for different time-interval ranging from 1, 2, 4 to 24 hours and designated as tests, positive, and negative controls, respectively. After being washed, the biofilm mass was finally determined by crystal violet staining as described above [Table/Fig-2]. Reductive effect of the tested herbal essential oils against the pre-established *S. mutans* biofilm was interpolated to be the percentage of bacterial biofilm being disrupted by using the equation described below. The experiment was performed in triplicate each with three repeats.

$$\{1 - (OD_{596} \text{ of the test}/OD_{596} \text{ of negative control})\} \times 100$$

STATISTICAL ANALYSIS

Anti-cariogenic plaque efficacy (preventive and reductive effects) was described as the percentage of retardation of biofilm formation and reduction of the pre-established biofilm, respectively. Influence of the culinary herb essential oil concentration and exposure time on the anti-plaque efficacy was further analysed by two way analysis of variances and Tukey's multiple comparison tests at significance level of 0.05 using SPSS program version 18.0.

RESULTS

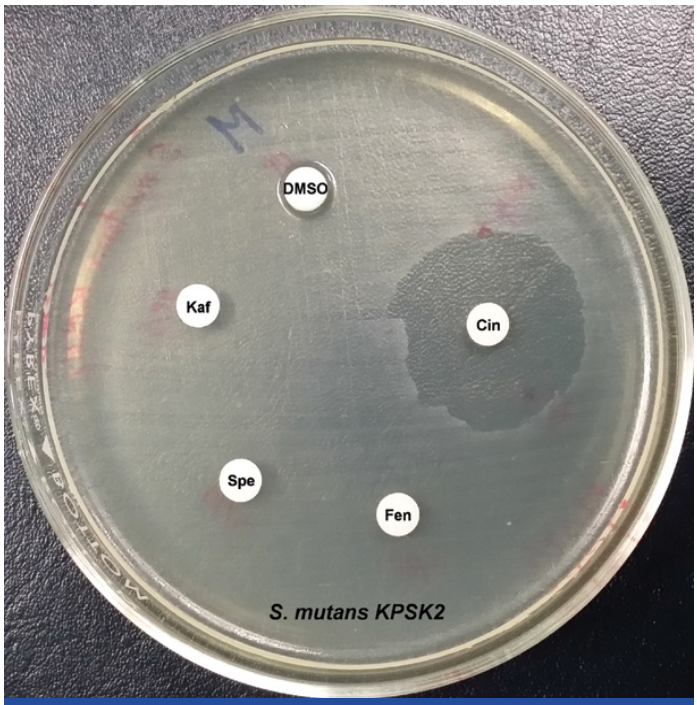
Antimicrobial Effect against Planktonic Cariogenic Bacteria

All tested herbal essential oils at concentration of 20% (v/v), except black pepper displayed different degrees of inhibitory activity against both *S. mutans* KPSK2 and *L. casei* through the agar disk diffusion method. No inhibitory activity against *L. casei* was observed from black pepper essential oil even though the concentration of the oil was increased to 50% (v/v). Cinnamon bark essential oil expressed the most potent antimicrobial activity against both cariogenic bacteria whereas, 10% DMSO used as a diluent did not show such activity. Inhibitory efficacy of all tested essential oils against the cariogenic microorganisms was summarized in [Table/Fig-3,4].

The essential oils with antimicrobial activity against both tested microorganisms were further examined for their MIC and MBC values by broth microdilution method. The growth of planktonic *S. mutans* KPSK2 and *L. casei* was strongly inhibited by the essential oil extracted from the cinnamon bark with MIC and MBC values of

Tested agents	Inhibition zone {mean of diameter (mm) ± SD}	
	S. mutans KPSK2	*L. casei*
Cinnamon (bark)	32.17±1.32	17.33±1.03
Sweet basil (leaf)	14.67±0.82	11.83±0.75
Peppermint (leaf)	11.33±1.03	9.00±0.89
Spearmint (leaf)	9.50±1.04	8.33±0.52
Black pepper (seed)	14.00±0.63	-
Sweet fennel (seed)	9.17±0.75	8.67±0.52
Kaffir lime (peel)	8.67±0.52	7.33±0.52
Kaffir lime (leaf)	8.50±0.55	7.16±0.41
10% DMSO	-	-
0.25% Chlorhexidine	29.83±0.75	27.50±0.55

[Table/Fig-3]: Antimicrobial activity against *S. mutans* KPSK2 and *L. casei* of all tested herbal essential oils with concentration of 20% (v/v) determined by agar disk diffusion method and expressed in mean value of inhibition zone diameter (mm) with Standard Deviation (SD).
(-): no inhibition zone



[Table/Fig-4]: Inhibitory effect of 20% (v/v) of culinary herb essential oils including cinnamon (Cin), sweet fennel (Fen), spearmint (Spe), kaffir lime (Kaf) and 10% (v/v) dimethyl sulfoxide (DMSO) (negative control) against *S. mutans* KPSK2, determined by agar disk diffusion method.

0.08% and 0.16% (v/v), respectively. Essential oils extracted from either peel or leaf of kaffir lime expressed the weakest antimicrobial activity with MICs and MBCs of 2.5% and 5.0% (v/v) against *S. mutans* KPSK2 and *L. casei*, respectively. The MIC and MBC values of the selected herbal essential oils were illustrated in [Table/Fig-5].

Anti-plaque effects against *S. mutans* biofilm

Preventive effect on the formation of *S. mutans* biofilm in vitro: After the substratum was primed with 0.25% chlorhexidine mouthwash, the formation of *S. mutans* biofilm was affected differently ranging from >60% inhibition (24-h priming) down to >30% inhibition (1-h priming). Inhibition of *S. mutans* biofilm development was also observed on the substratum being primed with each tested herbal essential oil. Similar to antimicrobial effect against the planktonic culture, the formation of *S. mutans* biofilm was retarded by >80% on the substratum that was primed with cinnamon bark essential oil at its MIC for 4 or 24 hours, compared to the negative control (untreated substratum) [Table/Fig-6]. In contrast, the formation of bacterial biofilm was inhibited by approximately 60% on the substratum primed with this herb for only one or two hour. Additionally, high concentration {4xMIC;

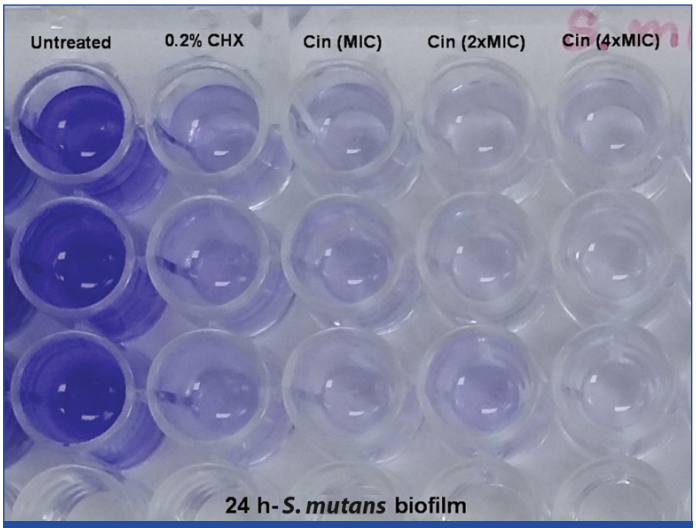
Tested agents	*S. mutans* KPSK2		*L. casei*	
	MIC [%(v/v)] (µL/mL)	MBC [%(v/v)] (µL/mL)	MIC [%(v/v)] (µL/mL)	MBC [%(v/v)] (µL/mL)
Cinnamon (bark)	0.08 (0.8)	0.08 (0.8)	0.16 (1.6)	0.16 (1.6)
Sweet basil (leaf)	0.31 (3.10)	0.31 (3.10)	1.25 (12.5)	1.25 (12.5)
Peppermint (leaf)	1.25 (12.5)	1.25 (12.5)	2.50 (25.0)	2.50 (25.0)
Spearmint (leaf)	1.25 (12.5)	1.25 (12.5)	2.50 (25.0)	2.50 (25.0)
Black pepper (seed)	1.25 (12.5)	2.50 (25.0)	NT	NT
Sweet fennel (seed)	1.25 (12.5)	2.50 (25.0)	5.00 (50.0)	5.00 (50.0)
Kaffir lime (peel)	2.50 (25.0)	2.50 (25.0)	5.00 (50.0)	5.00 (50.0)
Kaffir lime (leaf)	2.50 (25.0)	2.50 (25.0)	5.00 (50.0)	5.00 (50.0)

[Table/Fig-5]: Antimicrobial activity of all tested herbal essential oils against *S. mutans* KPSK2 and *L. casei* determined by broth microdilution method and expressed in MIC and MBC values.
NT: Not tested.

Tested agents	Inhibitory effect against formation of *S. mutans* biofilm (%)± SD			
	Priming time of herb on substratum (h)			
	1	2	4	24
Cinnamon (bark)				
MIC	67.40±5.0*	77.53±5.0*	80.50±4.0	85.42±6.0
2xMIC	73.36±6.0*	79.40±5.0*	85.48±3.0	87.01±6.0
4xMIC	80.45±2.0*,†	84.07±5.0*,†	87.63±5.0†	88.22±2.0
Sweet basil (leaf)				
MIC	58.10±5.0*	64.98±4.0*	69.35±6.0	70.20±5.0
2xMIC	70.60±4.0*,†	71.44±5.0*,†	79.89±6.0†	80.09±4.0†
4xMIC	78.47±7.0*,†	78.81±5.0*,†	81.66±4.0†	86.77±3.0†
Peppermint (leaf)				
MIC	55.52±7.0*	64.02±8.0*	66.55±6.0	66.60±6.0
2xMIC	66.53±8.0*,†	74.88±7.0†	77.11±4.0†	78.93±5.0†
4xMIC	73.93±5.0*,†	77.17±6.0†	82.41±2.0†	83.42±5.0†
Spearmint (leaf)				
MIC	54.18±7.0*	64.72±5.0*	64.96±4.0	65.77±5.0
2xMIC	72.33±5.0*,†	74.54±6.0*,†	76.42±5.0†	77.01±5.0†
4xMIC	77.91±4.0*,†	78.21±7.0*,†	82.30±1.0†	82.75±5.0†
Black pepper (seed)				
MIC	53.64±5.0*	61.78±4.0*	64.79±4.0	65.77±5.0
2xMIC	70.36±5.0*,†	71.04±3.0*,†	73.80±4.0†	78.07±3.0†
4xMIC	74.37±5.0*,†	75.51±4.0*,†	77.74±4.0†	82.74±3.0†
Sweet fennel (seed)				
MIC	53.44±4.0*	60.14±6.0*	64.33±4.0	65.53±5.0
2xMIC	68.60±5.0*,†	68.90±5.0*,†	78.21±4.0†	78.81±3.0†
4xMIC	76.64±7.0*,†	78.51±5.0*,†	81.09±4.0†	84.39±3.0†
Kaffir lime (peel)				
MIC	52.26±3.0*	59.64±7.0*	63.81±5.0	64.05±5.0
2xMIC	62.48±6.0*,†	68.85±5.0*,†	72.85±4.0†	76.10±3.0†
4xMIC	72.62±7.0*,†	73.47±4.0*,†	76.33±5.0†	77.98±3.0†
Kaffir lime (leaf)				
MIC	52.15±4.0*	59.98±6.0*	63.81±7.0	64.72±4.0
2xMIC	62.66±6.0*,†	68.40±5.0*,†	73.31±4.0†	73.61±3.0†
4xMIC	72.42±2.0*,†	73.39±4.0*,†	77.65±4.0†	81.51±3.0†
0.25% chlorhexidine	34.42±2.0*	44.71±4.0*	52.70±2.0*	62.75±2.0

[Table/Fig-6]: Preventive effect of the tested herbal essential oils individually primed on substratum for different time periods against the in vitro formation of *S. mutans* biofilm.
*Significantly different from the substratum primed with the agent for 24 h ($p<0.05$)
†Significantly different from the substratum primed with agent at its MIC ($p<0.05$)

0.32% (v/v)} of the cinnamon bark oil with 1-h priming on the substratum inhibited the formation of *S. mutans* biofilm by >80%. Sweet basil essential oil was the second best among the tested herbs in restraining the formation of such bacterial biofilm (>70% inhibition). Other essential oils illustrated lower degree of preventive effect (not statistically significant, $p>0.05$) against the formation of *S. mutans* biofilm, compared to those of cinnamon bark and sweet basil oils. Preventive effect of all tested herbal essential oils against the formation of *S. mutans* biofilm in vitro was summarized in [Table/Fig-6,7].



[Table/Fig-7]: In vitro formation of *S. mutans* biofilm on the untreated substratum and those substrata being primed with various concentrations (at MIC, 2xMIC and 4xMIC) of cinnamon bark essential oil (Cin) or 0.2% chlorhexidine (CHX) for 24 h.

Reductive effect against the in vitro established *S. mutans* biofilm: A 24 hours in vitro-established *S. mutans* biofilm mass was reduced by ≤35% and ≤19% after the bacterial biofilm was treated with 0.25% chlorhexidine for 24 hours and one hour, respectively. Cinnamon bark essential oil exhibited the strongest reductive effect but not statistically significant ($p>0.05$) against the 24 hours pre-established *S. mutans* biofilm among the tested herbal essential oils. Approximately, 80% of biofilm mass was reduced after the pre-established bacterial biofilm was exposed to the cinnamon essential oil at its MIC, 2xMIC {0.16% (v/v)}, and 4xMIC {0.32% (v/v)} for 24 hours. Within the shortest exposure time (1 h), the oil was able to reduce the biofilm mass by 60% approximately. The reductive effect of all tested essential oils against the 24 hours pre-established *S. mutans* biofilm was demonstrated in [Table/Fig-8].

DISCUSSION

Culinary herbs or spices used in Thai cuisine have been well-known not only as food flavoring but also as herbal medicine in the traditional remedy. These edible herbs contain many medicinal properties including antimicrobial (especially against medically important pathogens) and anti-inflammatory activities [11-16]. Presently, the safety and side effects of the medicinal herbs used worldwide have not been extensively analysed yet. However, these criteria are required before the herbs will be used as pharmaceutical drugs but not as dietary supplements [21]. Dental plaque control by lowering the levels of dental caries related microorganisms (mutans streptococci, lactobacilli, etc.,) and restraining their biofilm formation has been recommended as an effective strategy for oral health promotion [22]. The study clearly illustrates that the essential oils extracted from cinnamon, sweet basil, peppermint, spearmint, sweet fennel, black pepper, and kaffir lime not only were antimicrobials against cariogenic bacteria including *S. mutans* and *L. casei*, but also were anti-plaque agents to retard the formation of *S. mutans* biofilm in vitro.

Different degrees of antimicrobial activity of those culinary herb essential oils against the growth of both cariogenic bacteria were observed and categorized into 3 groups according to the MIC values: strong {MIC ≤ 0.1% (v/v)}, moderate {MIC ≤ 1.0% (v/v)}, and weak [MIC > 1.0% (v/v)] activities, respectively. Cinnamon bark essential oil demonstrated the most potent inhibitory activity against the growth of planktonic *S. mutans* KPSK2 and clinical strain *L.*

Tested agents	Reduction of the established *S. mutans* biofilm mass (%) ± SD			
	Time of treatment (h)			
	1	2	4	24
Cinnamon bark				
MIC	59.16±0.03*	64.62±0.03*	67.47±0.01*	77.33±0.02
2xMIC	59.24±0.02*	65.42±0.03*	67.59±0.02*	77.45±0.03
4xMIC	63.08±0.03*	67.75±0.03*	69.03±0.02*	81.21±0.02
Sweet basil leaf				
MIC	58.91±0.01*	65.50±0.02*	67.90±0.02	70.25±0.03
2xMIC	61.63±0.03*	66.91±0.03*	68.13±0.02	72.13±0.03
4xMIC	62.36±0.03*	67.76±0.03*	68.33±0.02	73.28±0.02
Peppermint leaf				
MIC	55.06±0.02*	61.66±0.02	63.32±0.02	64.67±0.03
2xMIC	56.83±0.03*	63.69±0.02	66.99±0.02	69.59±0.02
4xMIC	65.45±0.03*,†	66.11±0.02†	68.18±0.02	71.01±0.02†
Spearmint leaf				
MIC	55.70±0.02*	62.22±0.02	63.78±0.02	64.16±0.02
2xMIC	57.54±0.02*	64.48±0.02	64.92±0.03	65.52±0.02
4xMIC	60.46±0.02*	65.38±0.02	66.49±0.02	71.52±0.02
Black pepper seed				
MIC	55.31±0.03*	61.36±0.02	62.96±0.02	64.59±0.02
2xMIC	57.23±0.03*	62.94±0.02	64.91±0.03	65.81±0.03
4xMIC	59.45±0.02*	66.34±0.03	66.77±0.05	67.81±0.02
Sweet fennel seed				
MIC	55.44±0.05*	61.43±0.02	63.19±0.03	64.97±0.02
2xMIC	56.58±0.03*	62.89±0.03	64.37±0.05	65.82±0.03
4xMIC	58.75±0.03*	65.58±0.03	68.11±0.03	69.66±0.05
Kaffir lime peel				
MIC	54.83±0.06*	61.24±0.04	61.99±0.06	63.66±0.06
2xMIC	59.48±0.05*	64.28±0.05	64.39±0.04	64.87±0.06
4xMIC	60.18±0.06*	66.32±0.04	65.86±0.06	66.79±0.06
Kaffir lime leaf				
MIC	55.12±0.05*	60.77±0.05	62.14±0.06	62.96±0.06
2xMIC	58.33±0.05*	62.28±0.06	62.87±0.06	63.27±0.03
4xMIC	58.87±0.06*	63.02±0.05	64.95±0.06	66.94±0.06
0.25% chlorhexidine	18.90±0.02*	20.20±0.04*	25.66±0.02*	35.16±0.02

[Table/Fig-8]: Reductive effect of all tested herbal essential oils against the in vitro established 24-h *S. mutans* biofilm.
*Significantly different from the treatment of established biofilm with agent for 24 h ($p<0.05$)
†Significantly different from the treatment of established biofilm with agent at MIC ($p<0.05$)

casei (strong activity group). This finding was associated with the previous study by Zainal-Abidin et al., but it was significantly different from those studied by Fani and Freires [23-25]. Sweet basil leaf essential oil moderately inhibited the growth of such cariogenic *Streptococcus* (moderate activity group) then followed by peppermint leaf, spearmint leaf, black pepper seed, sweet fennel seed, kaffir lime peel and leaf essential oils (weak activity group). Antimicrobial potential of sweet basil examined in this study was slightly weaker than those reported previously [26]. Dissimilar to the report by Wongsariya et al., a much stronger antimicrobial activity of kaffir lime essential oil against *S. mutans* KPSK2 was observed [27]. Intriguingly, both peppermint and spearmint oils did express inhibitory activity against the tested streptococci with moderate degree. This was completely different from the previous study showing that antimicrobial activity against such oral *Streptococcus* was observed from neither peppermint nor spearmint essential oils [11]. These discrepancies may be the consequences of active component variation in herb origin due to geographic location, seasons, harvesting time, extraction procedure, etc., strains of the tested microorganisms as well as testing methods [28].

Biofilm form, a favored mode of growth, of oral streptococci is found to be a major cause of dental caries development and hard to control apart from its planktonic counterpart. So, it is necessary to assess the anti-plaque effects of the selected spice essential oils in terms of preventing bacterial biofilm formation and reducing or disrupting the pre-established biofilm. For preventive effect, the formation of *S. mutans* biofilm was inhibited by 80% at least on substratum being primed with cinnamon bark essential oil as low as its MIC or four times the MIC for four or one hour, respectively. The present finding has restated the previous report that this oil contained preventive effect against the formation of *S. mutans* biofilm [29]. Relatively weaker degree of preventive effect against the formation of *S. mutans* biofilm was found in other essential oils extracted from sweet basil, peppermint, spearmint, sweet fennel, black pepper and kaffir lime, compared to the one of cinnamon bark oil. Only 25-40% of *S. mutans* biofilm was allowed to form on the substratum primed with these essential oils at the MIC for 24 hours or at four times the MIC for one to four hours. Interestingly, the study clearly shows that the preventive effect of all selected culinary herb essential oils against the formation of *S. mutans* biofilm was in dose- and exposure time-dependent manners. Different degrees of preventive effect among these culinary herb essential oils observed may result from a variety of phytochemicals or bioactive components with distinct properties, especially the adhesion inhibition property, and quantities comprised in individual plant. Previous report has demonstrated that cinnamon extract contained glucosyltransferase adherence-inhibition effect on *S. mutans* [30].

All tested essential oils demonstrated reductive effect on the *S. mutans* biofilm pre-established in vitro, apart from their preventive effect against the formation of such cariogenic biofilm. Cinnamon bark essential oil also showed the strongest efficacy in disrupting the mass of pre-established bacterial biofilm. Approximately 80% of biofilm mass was reduced after the pre-established biofilm exposed to the cinnamon bark oil with the concentration at 4 times the MIC for 24 hours, whereas other oils were able to reduce the biofilm mass by at least 50%. This finding was associated with the previous study [31]. The reductive effect of each essential oil against the pre-established *S. mutans* biofilm clearly depended on the exposure times rather than the doses. This experiment has reiterated that the complex infrastructure of bacterial biofilm acts as a shelter to protect the embedded microorganisms from any antimicrobial agents. In other words, the biofilm form is more tolerant to any harmful substances, especially antimicrobials than its planktonic counterpart because several mechanisms have been proposed to contribute to phenotypic resistance of biofilm. These mechanisms include decrease in antimicrobial penetration, different growth rates and nutrient ingredients within the biofilm, persister phenomenon, induction of resistance mechanisms, and mutational resistance [32].

Additionally, all tested essential oils, except black pepper seed oil, demonstrated different degrees of antimicrobial activity against another cariogenic bacterium, *L. casei* (clinical strain). Cinnamon bark essential oil was still the best culinary herbal oil that strongly inhibited the growth of planktonic *L. casei*, as shown against *S. mutans* KPSK2. This study is probably the first report demonstrating MIC values of cinnamon bark oil against both cariogenic microorganisms. Interestingly, floating *L. casei* seems to be less susceptible to all tested spice essential oils because high concentrations of the oils were required to restrain the growth of the bacterium. Dissimilar to previous study, sweet basil leaf essential oil illustrated potent inhibitory effect on the tested *Lactobacillus* [25]. The peppermint leaf oil inhibited the growth of *L. casei* whereas the black pepper seed oil did not affect adversely on such bacterium as reported previously [33,34].

The primary bacterial colonizers such as streptococci and Gram-positive rods (*Actinomyces* species) interact with receptor molecules in the pellicle through specific stereo-chemical molecular interactions, alter microenvironment on the tooth surfaces and allow the

secondary and tertiary colonizers to adhere and to form biofilm at last [35,36]. The impediment in primary colonization of oral streptococci should directly impact on the dental biofilm development. Therefore, anti-*L casei* plaque effect of the selected herbal essential oils was not studied.

LIMITATION

S. mutans biofilm was developed as single-species plaque under the static environment. This in vitro pre-established bacterial biofilm may not represent the actual properties of multi-species dental plaque with structural complexity and dynamic condition developed on hard and soft tissues of oral cavity. Different responses of dental plaque to those culinary herb essential oils may be observed which could not be done in the present study.

CONCLUSION

This study has indicated that cinnamon, sweet basil, peppermint, spearmint, sweet fennel, kaffir lime and black pepper are excellent not only as culinary but also as anti-caries herbs. The essential oils extracted from these herbs, especially cinnamon bark illustrated potent medicinal properties including antimicrobial (against planktonic cariogenic bacteria) and anti-plaque (retardation of *S. mutans* biofilm formation and reduction of the established biofilm) effects. Further investigations for other pharmacological properties, toxicity, etc., of these herbal essential oils are required prior to being qualified and applied as clinically effective, inexpensive and safe plaque control supplements to promote oral health condition.

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