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Research article

Anti-oral streptococci and anti-biofilm properties of *Etlingera pavieana* essential oil and its bioactive compounds proposed for an alternative herbal mouthwash

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ABSTRACT

Oral streptococci are the major group of bacteria in the oral cavity. Some of their species cause oral diseases that may lead to tooth loss and quality-of-life reduction, such as dental caries. One of prevention techniques to promote oral health is rinsing mouthwash after toothbrushing. This study aimed to determine the potential uses of local food, also remedy, plant in Thailand called Reaw-Horm or *Etlingera pavieana* for alternative herbal mouthwash. The essential oil from *E. pavieana* rhizome (Eo) is used for anti-streptococci including *Streptococcus mutans* and *Streptococcus sobrinus* and anti-biofilm activities. The main components of Eo are methyl chavicol (MC) and *trans*-anethole (TA). The disk diffusion method showed the inhibition zone of Eo in a dose-dependent manner. The minimum inhibitory concentration (MIC) of Eo and TA was >1.6 % v/v, and 0.4 % v/v of MC. Regarding anti-biofilm activities, MC showed nearly equal anti-biofilm formation of *S. mutans* and *S. sobrinus*, whereas Eo and TA acted toward *S. sobrinus* more than *S. mutans* biofilm. Sub-MIC killing effects on cells under biofilm were observed in Eo and MC. Therefore, MC was recommended as an active compound for anti-streptococci activities. Biocompatibility of Eo and MC were shown to be safe for epidermal cell lines. Herbal mouthwashes containing Eo were developed and had antioxidant and antimicrobial actions with established for 3 months. This study provides *in vitro* support on the use of herbal mouthwash with antioxidant and antimicrobial activities for dental caries prevention and well-being of individuals.

1. Introduction

The aromatic plants of genus *Etlingera* belong to the Zingiberaceae family are regarded as vegetables and herbal medicines indigenous to Southeast Asia. The rhizome of *Etlingera pavieana* (Pierre ex Gagnep.) R.M. Sm. (Thai name: Reaw-Horm) is used as food ingredient for noodle soup. In folk medicine, *E. pavieana* rhizome has been used as anti-pyretic, anti-flatulence, anti-emetic, and diuretics in southeastern Thailand and Cambodia. Previous studies showed the pharmacological activity of *E. pavieana* rhizome including

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antibacterial, anticancer, antioxidant, anti-inflammation, and anti-atherosclerosis [1–5]. Chemotaxonomic studies revealed that *E. pavieana* was grouped to species containing mainly phenylpropanoids. Analysis determined that the oil of *E. pavieana* rhizome consisted of *trans*-anethole, methyl chavicol, *p*-anisaldehyde and δ-cadinene [6]. Isolated compounds such as anethole and methyl chavicol were used as flavoring agents in food products [7,8]. *In vivo* studies of these constituents were highlighted for promising alternative medicine candidates, including for anti-inflammation [9,10]. In accordance with the traditional uses, *E. pavieana* could be feasibly developed as herbal healthcare products for treatment and promoting health among population, however, additional scientific evidence is required.

Oral health is a continuing global problem challenge. Dental caries is one of the most prevalent situations, affecting more than two billion individuals of all ages worldwide [11]. Dental caries is an infectious biofilm-facilitated condition. Oral streptococci including *Streptococcus mutans* and *Streptococcus sobrinus* play critical role in carious lesions and produce dental plaque or biofilm which are the primary etiologic factor of dental caries. Microbial biofilms are composed of extracellular polymeric substance (EPS), microbial communities, and other virulent factors. The acidic microenvironment and physical barrier of biofilm causes increased resident pathogen bacteria, resulting in the aggressive progression of dental caries [12]. Moreover, bacterial cells in biofilm are less susceptible to antimicrobial agents than planktonic cells [13]. Antimicrobial agents are used in oral-care products for the prevention of dental caries, with the aim to restore health and prevent progression and recurrence [14–16].

Mouthwashes or mouthrinses are widely used for deodorizing, refreshing or antiseptic effect in holistic medicine [17]. Herbal extracts or natural substances are considered as an attractive choice to conventional antibiotics and chemical agents because of their lesser undesirable effect, and likewise for the effort to overcome the antimicrobial resistance crisis [18]. Anti-plaque biofilm agents with properties other than bactericidal or bacteriostatic activities may be used for the control or primary prevention of oral diseases. An anti-plaque effect of minimum concentration may be reasonable and desirable for preventing recolonization of pathogenic microorganisms and enhancing mechanical plaque control [14].

In this study, the essential oil from *E. pavieana* rhizome and its bioactive compounds including methyl chavicol and *trans*-anethole were determined for anti-streptococci and anti-biofilm activities. The plant was identified and extracted for essential oil, which was then analyzed for its composition. The effect of *E. pavieana* essential oil on two cariogenic species of oral streptococci, namely *S. mutans* and *S. sobrinus* were assessed in both planktonic and biofilm stages. Anti-biofilm formation was determined and reported as minimum biofilm formation inhibitory concentration (MBIC). Additionally, the sub-minimum inhibitory concentration (sub-MIC) of substances on biofilm formation was also determined. Biocompatibility of *E. pavieana* essential oil was evaluated. Then the essential oil was used to formulate the mouthwashes with antioxidant and antimicrobial actions. Considering this information, *E. pavieana* rhizome may be potential for the application in oral health care products.

2. Materials and methods

2.1. Extraction and composition analysis of E. pavieana rhizome essential oil

E. pavieana rhizome was purchased from local farmer in Chantaburi province. The plant was further identified by the botanists and the specimen was collected at the Herbarium of Pharmaceutical Botany Mahidol University (voucher specimen number PBM005650). The fresh rhizome was washed, cut, and air dried. The dried sample was extracted by hydrodistillation to obtain the volatile oil using clevenger apparatus. The sample was kept in refrigerator until analysis.

The composition analysis of essential oil was obtained by gas chromatography mass spectrometry (GC-MS) (Model GC-MSD-PAL3, Agilent Technology, USA) using HP-5MS UI column (30 m × 0.25 mm, film thickness 0.25 μM. The oven temperature was settled at 50–250 °C and the injector temperature was settled at. 250 °C. *E. pavieana* essential oil was prepared at the concentration 10 % v/v (in hexane). 1 μl of sample was injected at the split ratio of 100:1 using helium as carrier gas and the rate of 2 mL/min. The mass spectra were scanned at the range between 30.0 u and 550.0 amu. The compounds were identified in the Wiley10n14.l library. Standard alkane solution, methyl chavicol (MC), and *trans*-anethole (TA) (Sigma-Aldrich, USA) was injected at the same condition.

2.2. Bacteria and culture condition

S. mutans ATCC 25175 and *S. sobrinus* ATCC 33402 were cultivated in brain heart infusion (BHI) medium in microaerobic condition (5 % CO_2 in an extinction candle jar). A few colonies from well-isolated bacteria on BHI agar were inoculated in BHI broth overnight at 37 °C. The overnight culture was then sub-cultured in BHI broth for 4 h to obtain the mid-exponential phase and optical density at 600 nm (A_{600}) were adjusted to 0.2 which correspond to 10^8 colony forming units (CFU)/mL.

2.3. Anti-oral streptococci activity

Disc diffusion method was carried on BHI agar with swabbed 10^8 CFU/mL microbial culture. The sample was prepared in DMSO (Dimethyl sulfoxide, >99.0 %, Emplura®, Merck, Germany) at various concentration with negative control, then pipetted to the 6 mm paper disc for 20 μl [19]. The discs were put onto agar and incubated for 18–24 h under microaerobic condition. The halo region on the plate was recorded as inhibition zone.

Broth microdilution method was performed to determine the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). The sample, methyl chavicol (MC), and *trans*-anethole (TA) were prepared in 5 % v/v Tween-80 and 5 % v/v absolute ethanol in BHI broth and then started for two-fold dilution. Chlorhexidine (CHX, Sigma-Aldrich, USA) was used as positive

control. The bacterial suspension was added to the well for the final amount of 10^5 CFU. The microplates were incubated for 24 h at 37 °C. The MIC was then determined visually, and the MBC was assessed by taking the solution from the no-growth wells and plated on BHI agar, subsequently incubated for 48 h. The lowest concentration plate with no growth was noted as MBC [20].

2.4. Anti-biofilm formation

The log-phase culture ($A_{600} = 0.2$) was diluted to the final amount at 10^5 CFU in a well. The final volume of each well was 100 μl BHI broth containing 2 % w/v sucrose and various concentration of sample from two-fold dilution prepared as above. The microplate was incubated for 24 h at 37 °C. Then, the planktonic cells were removed carefully, and the remaining biofilm was washed with sterile phosphate-buffered saline (PBS). Biofilm biomass was determined using crystal violet (CV) assay by staining the biofilm with 0.01 % w/v CV and eluted with 100 μl 95 % ethanol. Absorbance at 550 nm was measured to determine the biofilm biomass [20]. Percentage of biofilm inhibition was calculated by (NT-T)/NT*100, where NT was biofilm biomass of normal condition and T was biofilm biomass when incubated in sample. The minimum concentration that can decrease the biofilm biomass for 90 % was defined as minimum biofilm formation inhibitory concentration (MBIC).

2.5. Cell death under biofilm conditions

The effect of substances on cells under biofilm was evaluated. Briefly, biofilm was established by inoculating the log-phase culture in BHI containing 2 % w/v sucrose for 24 h. Then, the media and planktonic cells were removed carefully, and the preformed biofilm was incubated with test substances for an hour [21]. The viable cells under biofilm were determined using MTT (3-[4,5-diethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide) colorimetric assay. The untreated biofilm was performed every step parallelly using sterile water instead of samples. Percentage of biofilm killing was calculated as 1-(T/NT)*100, where T was viability cells of treated biofilm and NT was viability cells of untreated biofilm.

2.6. Biocompatibility

Essential oil was tested against human epithelial (KB) and keratinocyte (HaCat) and L929 mouse fibroblast. Cells were cultivated in Dulbecco's Modified Eagle's medium (DMEM) supplement with 8 % fetal bovine serum (FBS) and 100 μg/mL penicillin G-streptomycin at 37 °C in 5 % CO_2 incubator. After cell confluence, 1×10^5 cells in 200 μl in 96-well tissue culture plate and incubated with the presence of various concentration of tested sample for 24 h [21]. Cell viability was determined using MTT colorimetric assay after the exposure of sample at 24 h. The percentage of cytotoxicity was calculated as ((T-NT)/T)*100 where T was the absorbance of treated cells and NT was the absorbance of untreated cells. Half-maximal inhibitory concentration (IC_{50}) was reported based on % cytotoxicity.

2.7. Herbal mouthwash formulation

The prototype of herbal mouthwash was formulated. The surfactant including tween 80 and 95 % ethanol were used to solubilize the essential oil at the minimum volumes. Erythritol, a sugar alcohol, was used as sweeteners. Sodium benzoate was added to the formulation as preservatives. Distilled water, propylene glycol and glycerin were used as solvents and co-solvents. Base formulation was prepared without essential oil. Formulated mouthwashes were inspected based on the specification of Thai Industrial Standard (TIS) no. 2342–2550. Stability of products were determined at accelerated condition at 40 ± 2 °C for 3 months. Prototype and base mouthwashes were determined for antioxidant and anti-*S. mutans* activities.

Total phenolic content (TPC) was determined using Folin-Ciocalteu reagent (FCR) and demonstrated as mg gallic acid equivalence. Briefly, 20 μl mouthwash was mixed with 50 μl FCR for 3 min. Then, 80 μl 7.5 % w/v sodium carbonate was added and incubated the reaction for 2 h. Absorbance at 765 nm was determined and inferred to the standard curve of gallic acid [22].

Antioxidant activities were evaluated by DPPH assay. 100 μl mouthwash was mixed with 100 μl DPPH reagent (152 μM 1,1-diphenyl-2-picrylhydrazyl in methanol) and incubated for 30 min. Absorbance at 517 nm was determined and calculated as % inhibition which equal to [(Absorbance of control - Absorbance of sample)/Absorbance of control] x 100 [23].

For evaluation of anti-*S. mutans* activity, 0.5 ml mouthwashes were added to the pellet of 0.5×10^8 CFU of *S. mutans* and mixed for 30 s. Then the mixture was centrifuged, removed, and washed with phosphate buffer saline. The final number of *S. mutans* were resuspended and quantified by plating on BHI agar [24].

2.8. Statistical analysis

The data were expressed as the mean ± standard deviation (SD) of at least three independent experiments. For mouthwash formulation, the *t*-test of Microsoft excel was used to calculate *p* value and the level of significance was $p < 0.05$.

3. Results

3.1. Chemical composition

The GC-MS profile of the *E. pavieana* rhizome essential oil (Eo) is shown in Fig. 1. The major components were methyl-chavicol

(MC) and *trans*-anethole (TA) (Table 1).

3.2. Anti-oral streptococci activities

According to disc diffusion assay, the *E. pavieana* rhizome essential oil (Eo) showed moderate inhibition depending on concentration (Fig. 2(A-B)). MIC and MBC of Eo and its dominant compounds, which are methyl chavicol (MC) and *trans*-anethole (TA), were determined using broth microdilution and the results are shown in Table 2.

3.3. Anti-biofilm formation

Fig. 3 presents the percentage of inhibition for anti-biofilm. MBIC of Eo was 1.6 % v/v for *S. sobrinus* which exhibited 97.02 ± 0.64 % inhibition of biofilm formation. Moreover, the maximum concentration of Eo that can be prepared for the test was 1.6 % v/v, whereas Eo has shown the anti-*S. mutans* biofilm formation at 39.48 ± 3.05 %. The major compounds of *E. pavieana* essential oil, methyl-chavicol (MC) and *trans*-anethole (TA), were evaluated for their antibiofilm formation as well. MC had an MBIC at 0.1 % v/v and 0.4 % v/v and showed inhibition at 92.92 % and 95.59 % for *S. mutans* and *S. sobrinus*, respectively. TA had an MBIC at >1.6 % v/v and 1.6 % v/v and showed inhibition at 43.55 % and 95.55 % for *S. mutans* and *S. sobrinus*, respectively.

The effects of sub-minimum inhibitory concentrations (sub-MIC) of substances on biofilm formation are shown in Fig. 4. For *S. mutans* biofilm, MC demonstrated >90 % inhibition at the range of 1/4XMIC–1XMIC, whereas Eo and TA had inhibitory activities against *S. mutans* biofilm formation at a concentration (designated as 1XMIC) of approximately 40 % and at half of maximum concentration (designated as 1/2XMIC) at approximately 20 %. Almost 100 % of *S. sobrinus* biofilm formation was inhibited by 1XMIC of MC, TA, and Eo, whereas the 1/2XMIC concentrations of MC, Eo, and TA declined % inhibition to 38 %, 29 %, and 8 %, respectively.

3.4. Cell death under biofilm conditions

Biofilm cell death was assessed for potential antiplaque effects. After biofilm was formed for 24 h, essential oil and its major compounds at various concentrations was incubated with biofilm for 1 h. The viability of cells under biofilm was calculated and shown as % biofilm killing (Fig. 5). Eo can inactivate *S. mutans* biofilm cells at 2XMIC (MBC),1XMIC and 0.5XMIC (sub-MIC), with the percentage of killing at 64.79 ± 1.48, 59.43 ± 2.99, 52.89 ± 1.73 respectively. For *S. sobrinus* biofilm cells, 2XMIC (MBC), 1XMIC and 1/2XMIC (sub-MIC) were shown percentage of killing at 81.51 ± 0.71, 74.27 ± 0.39, 69.78 ± 0.78, respectively. Furthermore, MC showed the sub-MIC killing effects on both *S. mutans* and *S. sobrinus* biofilms.

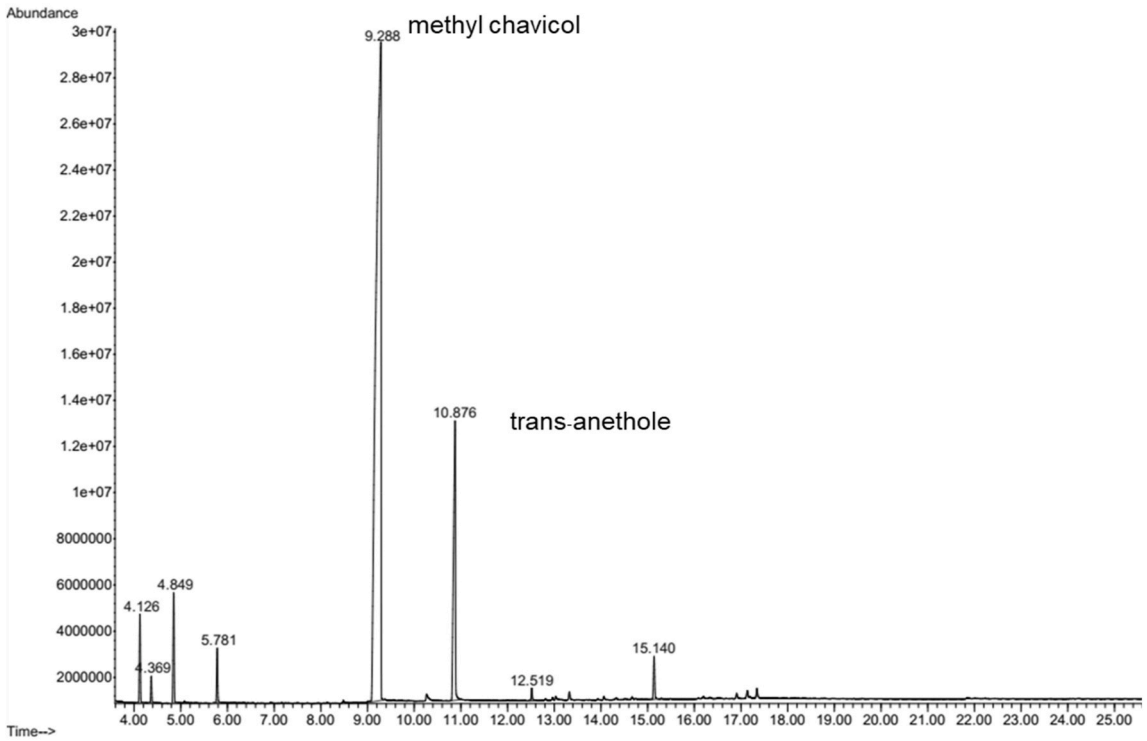

Fig. 1. A typical chromatogram of *E. pavieana* oil (Eo).

Table 1
GC-MS Analysis of *E. pavieana* oil (Eo).

Linear retention index (LRI)	Retention time (RT)	Compound	Molecular Formula	% Area
952	4.13	2-Pinene	$C_{10}H_{16}$	2.05
964	4.37	2,2-dimethyl-3-methylene-bicyclo [2.2.1]heptane	$C_{10}H_{16}$	0.56
990	4.85	2(10)-Pinene	$C_{10}H_{16}$	2.68
1040	5.78	D-Limonene	$C_{10}H_{16}$	1.34
1227	9.29	Anisole, p-allyl-	$C_{10}H_{12}O$	79.34
1312	10.88	trans-Anethole	$C_{10}H_{12}O$	12.41
1387	12.52	.alfa.-Copaene	$C_{15}H_{24}$	0.37
1544	15.14	.delta.-Cadinene	$C_{15}H_{24}$	1.26
			Total	**100**

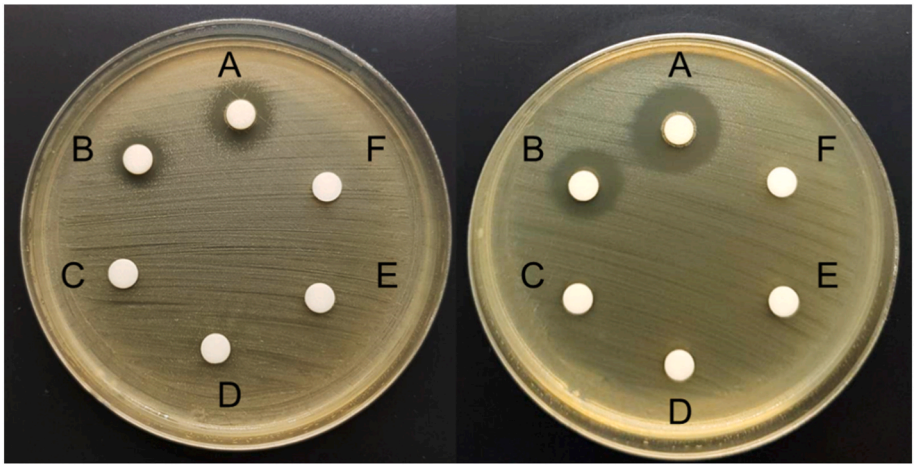

Fig. 2. The inhibition zones of *E. pavieana* essential oil against *S. mutans* (Left) and *S. sobrinus* (Right) with concentration dependent manner (A. non-dilute (100 %), B. 1:2 (50 %), C. 1:4 (25 %), D. 1:8 (12.5 %), E. 1:16 (6.25 %), F. DMSO (100 %) as negative control).

Table 2
Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of *E. pavieana* essential oil (Eo) and its major compounds: methyl-chavicol (MC) and *trans*-anethole (TA). Chlorhexidine (CHX) was used as control.

Microorganisms	*S. mutans*		*S. sobrinus*	
Concentration	MIC	MBC	MIC	MBC
Eo (% v/v)	>1.6	>1.6	1.6	>1.6
MC (% v/v)	0.4	0.8	0.4	0.8
TA (% v/v)	>1.6	>1.6	1.6	>1.6
CHX (% w/v)	0.000625	0.00125	0.000625	0.00125

3.5. Biocompatibility

Eo was nontoxic against fibroblast cells, oral epithelium, and epidermal keratinocytes cells (Table 3). Cell density and morphology after 24-h incubation with Eo and MC is shown in Fig. 6(A-C). Its active compound, MC, was evaluated for cytotoxicity and showed a higher IC_{50} value than Eo indicating less toxicity except for HaCaT cell. The cytotoxicity of 0.2 % v/v Eo were 80.71 ± 0.50 %, 74.88 ± 0.14 %, and 42.35 ± 0.14 % against L929 fibroblast cells, KB oral epithelial cell, and HaCaT epidermal keratinocytes cells, respectively.

3.6. Mouthwash formulation

Table 4 shows the ingredients and concentration of developed herbal mouthwashes. Erythritol was selected as sweetener to obscure the spice flavor from *E. pavieana* rhizome. Sodium benzoate was added to the formulation as preservative at a concentration of 0.1 % w/v. The base formula without *E. pavieana* essential oil was prepared. Table 5 presents the TPC (mg per gallic acid equivalence (GAE)), DPPH (% inhibition), and killing assay.

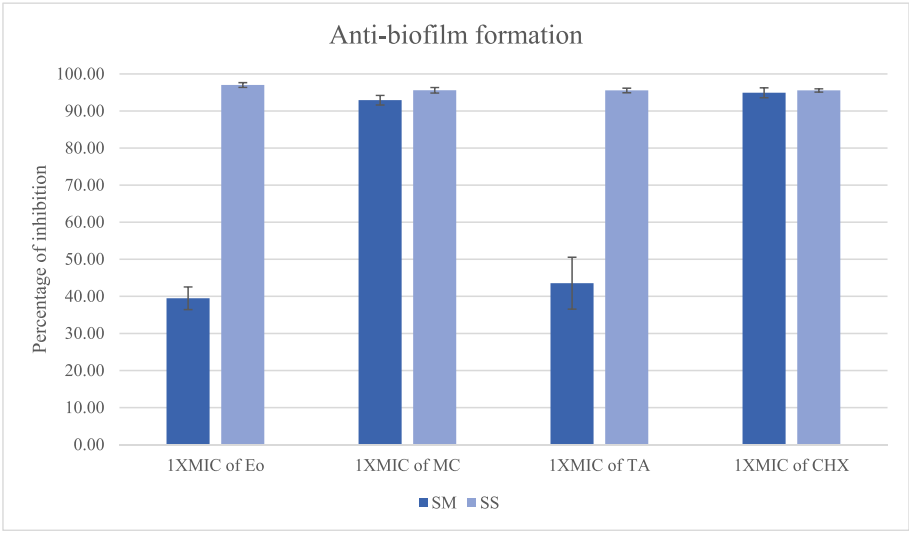

Fig. 3. Anti-biofilm formation (% inhibition) of *E. pavieana* essential oil (Eo) and its major compounds: methyl-chavicol (MC) and *trans*-anethole (TA) against *S. mutans* (SM) and *S. sobrinus* (SS). 0.000625 % w/v Chlorhexidine (1XMIC CHX) was used as control.

4. Discussion

The main components of *E. pavieana* essential oil (Eo) were found to be methyl chavicol (MC) and *trans*-anethole (TA). Antimicrobial activities of *E. pavieana* and its main constituents were investigated. A previous report found that *E. pavieana* essential oil affects various pathogens including *Bacillus cereus*, *Staphylococcus aureus* and *Listeria monocytogenes* [3]. In this study, the maximum concentration of oil that could be dissolved in Tween and ethanol was 1.6 % v/v or 16 μl/ml. At this maximum concentration, Eo and TA demonstrated the MIC and MBC at >1.6 % v/v for *S. mutans*, whereas MC had an MIC and MBC for both *S. mutans* and *S. sobrinus* at 0.4 and 0.8 % v/v, respectively. The isolated compounds of essential oil including menthol, eugenol, thymol, and cinnamaldehyde were intensively studied considering a prospective applicability in oral-care products [25]. Methyl chavicol (synonym as estragole, *p*-allylanisole) are aromatic compounds that are well-known major bioactive compound of *Ocimum basilicum* and *Artemisia dracunculus* [26]. The compound was methoxy derivatives of phenylpropane. Previous studies reported antioxidant, antilipase, insecticidal, anti-inflammatory, and antimicrobial activities as responsibility of MC [27]. In the present study, MC was found to possess an anti-streptococci activities against *S. mutans* and *S. sobrinus*, which are famous species of oral streptococci known to cause human caries. These two cariogenic bacteria can rapidly generate and tolerate acids and are able to colonize on tooth surfaces [28,29]. Current data showed that MC could be induce favorable effects against these oral pathogens.

Dental plaque is the biofilm deposited on tooth surfaces which causes oral diseases. In this study, anti-biofilm formation of Eo and its major compounds against two streptococcal species was assessed. As a result, *S. sobrinus* biofilm was susceptible to Eo, MC, and TA than *S. mutans* biofilm with >95 % inhibition at MIC level. However, *S. mutans* biofilm was inhibited less than 50 % for Eo and TA at >1.6 % v/v, and MC at >90 % at sub-MIC level (lowest as 1/4XMIC or equal to 0.1 % v/v, 1 μl/ml). The anti-biofilm activities of Eo and TA except for MC on *S. mutans* biofilm was observed at a dose-dependent manner. Several studies have reported the effect of compounds on biofilm but not planktonic cells. The suppression of biofilm formation at sub-MICs level with less impact on the planktonic cell growth was noted in lemon essential oil and tea extract [30–32]. Since TA possessed little effect on anti-streptococci and anti-biofilm formation, this suggested that MC in Eo may play a key role in anti-plaque activity. Further experiments should focus on which mechanisms of MC obstruct biofilm formation including adherence, suppression of key enzymes, reduction of extracellular polymeric substance (EPS) production, etc.

Moreover, biofilm appeared as a shelter for microorganisms which promote the survival of pathogens. Antimicrobial agents were assessed for their feasibility including potency, rate of bacteria killing, and efficacy against biofilm including bacterial death or inhibiting biofilm formation [13]. Cell death under biofilm conditions was critical for possible antiplaque properties. Several essential oils extracted from culinary herbs have been demonstrated anti-cariogenic plaque effects including cinnamon bark oil of which 1XMIC for 1 h showed a biofilm mass reduction by CV assay of 59 % [33]. In our studies, MTT assay was used to determine the viability of cells under the biofilm. Eo and MC can kill almost 80 % of viable biofilm cells. Moreover, the result showed that they can inactivate *S. mutans* and *S. sobrinus* cells under biofilm at MBC, MIC and sub-MIC in a dose-dependent manner. Abovementioned, the activity at sub-MIC level showed that Eo and MC as promising candidate for formulating products for preventive treatment.

Mouthwashes is a popular oral product for routine uses. In the present study, the *E. pavieana* essential oil and its potent compounds, methyl-chavicol (MC) were assessed for biocompatibility using cytotoxicity tests against various cell types. While MC is permitted for use in synthetic flavoring substances, its safety in oral cells was considered [34]. Nontoxicity of essential oil in cell-based assay were considered at more than 50 μg/ml or 0.005 % w/v [21,35]. In this study, Eo was tested against the cell line including epithelium, fibroblast and keratinocytes and demonstrated a 50 % inhibition at the concentration of 0.05–0.2 % v/v. The result showed that Eo and

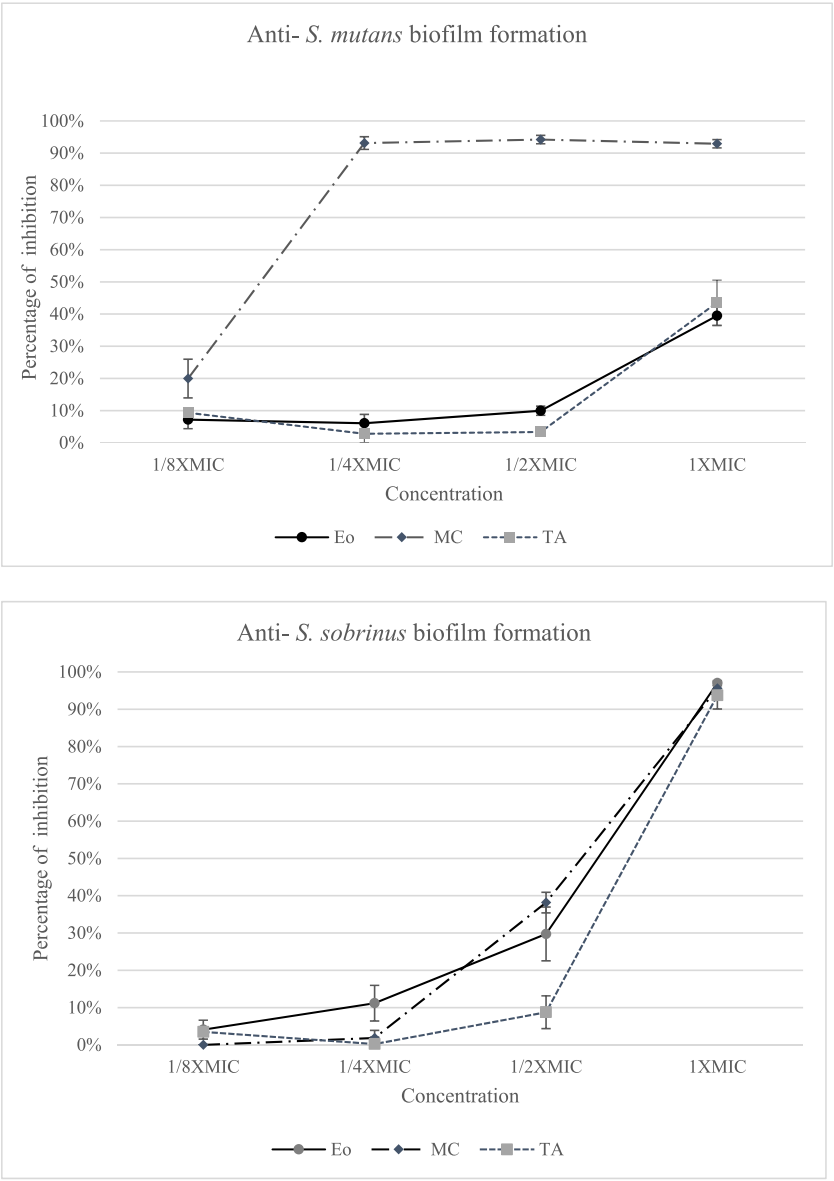

Fig. 4. Effects of sub-minimum inhibitory concentrations (sub-MIC) of substances (Eo, MC, and TA) on biofilm formation. The upper panel is anti-*S. mutans* biofilm formation. The lower panel is anti- *S. sobrinus* biofilm formation.

MC were not toxic at the concentrations 0.2 % v/v or approximately 2 mg/ml on HaCaT or keratinized epithelium cell. Essential oils could have antiproliferative effects and are selectively used against tumor cell lines but not normal cell lines [35]. Additionally, the application of mouthwashes was short time exposure and should be considered as less toxic.

It has been previously revealed that essential oils used in over-the-counter mouthwashes showed antimicrobial, anti-inflammatory and antioxidant effects [36,37]. In the present study the herbal mouthwash containing 1 % v/v or about 10 mg/ml E. *pavieana* essential oil as active ingredient exhibited antioxidant and antibacterial activities. The formula was alcohol-free and had a transparent appearance and good dispersion in water with a pleasant aroma of Reaw-Horm. This herbal formulation contains a total phenolic content of 13.79 ± 1.14 mg GAE/ml and has an antioxidant activity by DPPH inhibition of 27.32 ± 0.53 %. However, this outcome was not high as those reported from previous studies of such novel mouthwash as Ulvan-silver nanoparticles at 50 μl/ml or 5 % v/v which exhibited great antioxidative activity. It should be considered that higher formulation concentrations could demonstrate higher activities [38]. In addition, the present mouthwashes demonstrated anti-*S. mutans* reduction at 1.7 log or decrease for 50-folded after 30 s of exposure. A previous study has reported that commercial mouthwashes could induce a 1.23–7.51 log-reduction of *S. mutans* [24]. Accordingly, the herbal mouthwash formulation was a prototype product and carried out for human study in the future.

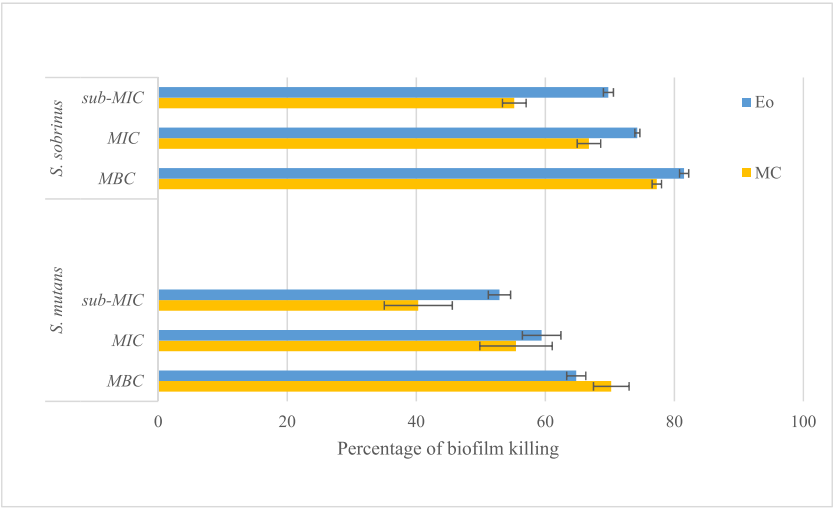

Fig. 5. Percentage of cell death under biofilm conditions or % biofilm killing at sub-MIC, MIC, and MBC of *E. pavieana* essential oil (Eo) and methyl-chavicol (MC).

Table 3
Biocompatibility of essential oil from *E. pavieana* rhizome (Eo) and its active compounds, methyl chavicol (MC) on various types of cells.

Cell line	Cell derivation	Cell type	IC_{50} of Eo	IC_{50} of MC
L929	Mouse subcutaneous connective tissue	Fibroblast	0.05 ± 0.01	0.17
KB	Human oral epithelial carcinoma cell line	Epithelial cell	0.07 ± 0.00	0.17
HaCaT	Human skin	Epidermal keratinocytes	>0.2	0.16

5. Conclusion

Dental caries is an infectious disease. Its prevention includes mechanical removal of biofilm by toothbrushing in combination with the use of additional oral-care products such as mouthwash. In this study, *E. pavieana* which is commonly used as food and traditional medicine was evaluated for its potential use for herbal mouthwash formulation. The major components of Eo were found to be MC and TA. Two species of oral streptococci, namely, *S. mutans* and *S. sobrinus* were investigated in the form of planktonic and under biofilm cells. MC was shown to be responsible for anti-streptococci activities. Moreover, Eo and its constituents can inhibit and kill biofilm cells at sub-MIC in a dose-dependent manner. For mouthwash formulation, Eo was biocompatible with the normal cell line. Herbal mouthwash containing Eo showed the antioxidant and anti-*S. mutans* effects and could be used as complementary to conventional approach to prevent dental caries and maintain good oral health.

Ethical statement

Not applicable.

Data availability

All the research data included in the manuscript. No new data associated with the manuscript.

CRediT authorship contribution statement

Karn Wongsariya: Writing – original draft, Supervision, Methodology, Investigation, Formal analysis, Conceptualization. **Jinthana Lapirattanakul:** Writing – review & editing. **Savita Chewchinda:** Writing – review & editing, Writing – original draft, Supervision. **Pimpikar Kanchanadumkerng:** Writing – review & editing, Writing – original draft, Validation, Supervision, Resources, Methodology, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

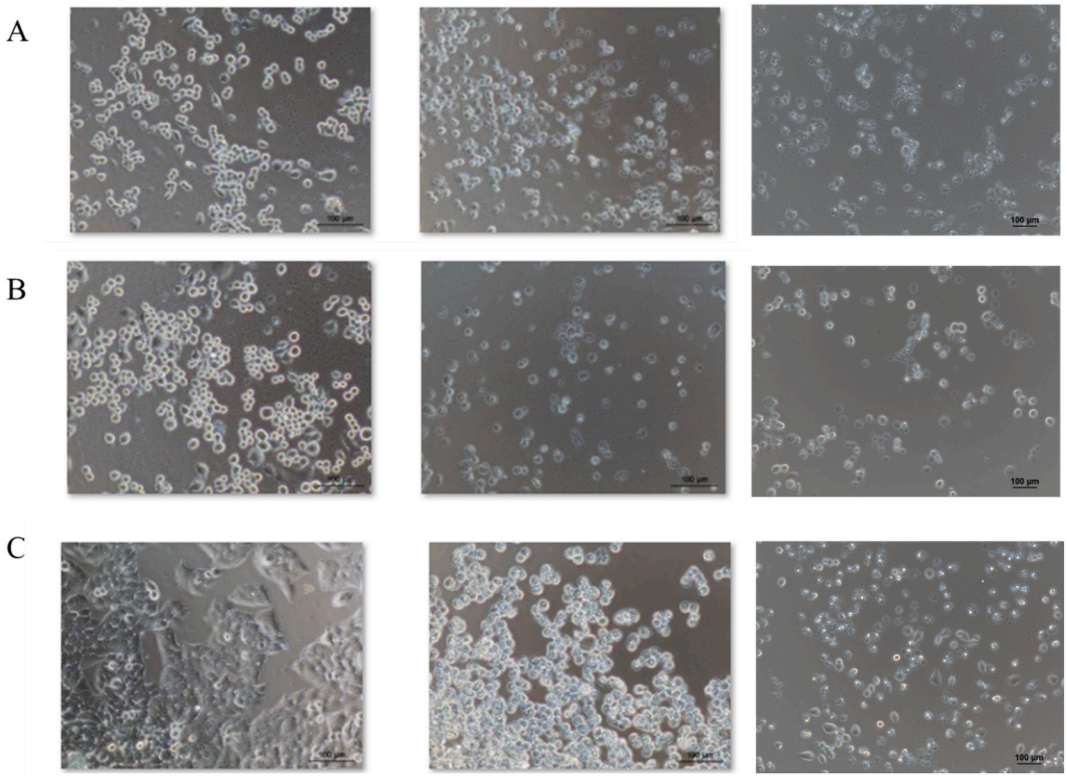

Fig. 6. Morphology view of the L929 fibroblast cells (A), KB oral epithelial cell (B), and HaCaT epidermal keratinocytes cells (C). The middle and right panel demonstrated the cells after 24-h treatment of Eo and MC, respectively, while the left panel was a control. The images of cells were observed under an inverted microscope (Nikon Eclipse Ti) with 200X magnificence.

Table 4
Developed mouthwash and their ingredients.

Ingredient	Function	% w/w
E. pavieana essential oil	Active ingredient	1
Propylene glycol	Co-solvent/humectant	10
Glycerine	Co-solvent/humectant	5
Tween-20	Surfactant	20
Erythritol	Sweetener	2
Sodium benzoate	Preservative	0.10
Water qs to	Solvent	100

Table 5
The biological activities of herbal mouthwash formulation (F1) and base formula without *E. pavieana* essential oil (F4).

Mouthwash formulation	Initial		At 3 months		*p*-value
TPC (mg per GAE)					
F1	13.794	$\pm 1.137^{A}$	21.359	$\pm 1.164^{A}$	0.002
F4	10.179	$\pm 1.345^{B}$	17.871	$\pm 2.856^{A}$	0.014
DPPH (% Inhibition)					
F1	27.32	$\pm 0.53^{A}$	49.90	$\pm 0.20^{A}$	0.0002
F4	28.64	$\pm 0.33^{B}$	32.06	$\pm 0.55^{B}$	0.0192
Killing assay (Number of cells in log CFU after treatment)					
F1	7.06	$\pm 0.15^{A}$	6.99	$\pm 0.08^{A}$	0.571
F4	7.56	$\pm 0.04^{B}$	7.61	$\pm 0.28^{B}$	0.796
Number of initial cells	8.70	±0.55	9.17	±0.15	–

Different superscripts indicate a statistical difference between F1 and F4 in each activity test.

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