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Original Article

Effectiveness of Maprang "*Bouea macrophylla* Griffith" Seed Extract against Endodontopathogenic Microorganisms in a Multispecies Bacterial-fungal Biofilm

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Abstract

Objective: This study aimed to assess the antimicrobial efficacy of seed extract derived from Bouea macrophylla Griffith (Maprang wan) grown in northern Thailand against *Entercoccus faecalis, Streptococcus gordonii* and *Candida albicans* in planktonic and multispecies biofilm.

Methods: Agar diffusion and broth microdilution methods were performed to investigate the antimicrobial activities of ethyl acetate extract of Maprang wan seed against planktonic *E. faecalis, S. gordonii* and *C. albicans*. Growth curves of *E. faecalis, S. gordonii* and *C. albicans* in 1:1:1 and 1:1:0.1 ratio, respectively) were performed to measure the appropriate ratio of the species within biofilm. Multispecies of *E. faecalis, S. gordonii* and *C. albicans* biofilms were grown in RPMI for 48 hours at 37°C. Following this, the biofilms were exposed to 25 mg/ml of Maprang seed extracts. 0.02 mg/ml chlorhexidine was used as the positive control and RPMI as the negative control. After exposure, time-kill assay was conducted to evaluate time-point of the extract which showed bactericidal and fungicidal effects. The colony forming unit (CFU) data were analyzed with one-way ANOVA and Dunnett's test ($p<0.05$).

Results: Maprang seed extract demonstrated the antimicrobial activity against planktonic and multispecies *E. faecalis, S. gordonii* and *C. albicans*. The time kill assay showed a time dependent action of Maprang seed extract, it demonstrated significant reduction of viable organisms at the time periods.

Conclusions: Maprang seed extract had antibiofilm property against multispecies *E. faecalis, S. gordonii* and *C. albicans* biofilms. The antimicrobial activity of the extract was comparable to chlorhexidine. Maprang seed extract has potential as a novel antimicrobial agent and may be an alternative to treat superficial infections or as a root canal medicament.

Keywords: *Bouea macrophylla* Griffith (Maprang wan) seed extract, multispecies biofilm, root canal medicament

Introduction

Root canal infections are polymicrobial biofilm-mediated diseases. This together with the complexity and variability of the root canal anatomy make disinfection of this system extremely challenging. Moreover, the most important factor for failure of root canal treatment is microbial persistence and resistance.[1] In endodontic treatment, biofilm removal is accomplished by a chemo-mechanical preparation. The specific instruments and disinfecting agents in the form of irrigants and/or intra-canal inter-appointment medicaments are used.

E. faecalis is a Gram-positive facultative anaerobic coccus which is frequently isolated from sites of oral infection including carious lesions, chronic periodontitis, and persistent apical periodontitis.[2] *E. faecalis* can penetrate deep into dentinal tubules and adapt itself to the harsh environmental conditions.[2] It is occasionally detected in primary endodontic infections and it is a common finding in persistent endodontic biofilm infections.[2] *E. faecalis* resist to chemo-mechanical treatment and it also survives in nutrient-deficient conditions.[2]

S. gordonii are Gram-positive facultative anaerobes, alpha-hemolytic, appeared in spherical and clustered pairs or short chains. It is commonly found as a commensal microbial of the skin, intestine, and oral cavity.[3] In oral cavity, *S. gordonii* are the most commonly streptococcus found in root canal and shown significantly associated with post-treatment apical periodontitis due to its clinical isolated incident from infected root canal.[4] *S. gordonii* also possess ability of invading dentinal tubule via collagen type I binding, which aids intratubular invasion and growth.[4]

C. albicans is an opportunistic pathogen which colonizes the gastrointestinal tract, upper respiratory tract and oral and vaginal mucosa in both of healthy and medically compromised individuals.[5] In the oral cavity, *C. albicans* is mainly found at sites where endogenous oral commensal bacteria are also present. It only causes infection if its amount exceeds the tolerance of the underlying mucosae, or the host immune responses are locally or systemically impaired.[6] One of the major virulence factors of *C. albicans* is its ability to form biofilms.[6]

Mixed bacterial–fungal biofilms are always present in the oral environment including infected root canals. *E. faecalis, S. gordonii* and *C. albicans* are the three most commonly recovered species in root canals undergoing retreatment due to failure of the primary endodontic treatment and with persistent infection.[7] The complex structure of the biofilm allows some degree of interspecies metabolic interactions and commensal relationships to develop between the two populations.[8] *C. albicans* is the most studied fungal pathogen in bacterial–fungal biofilms.[8] The study on dual species biofilm of *C. albicans* and *S. gordonii* indicated that bacteria enhance fungi' filament by physical and chemical influence. In addition, biomass volume of the multispecies biofilm was higher compared to single species condition.[9] Likewise, *C. albicans* and *E. faecalis* had synergism effect in forming biofilm, which also resulted in increase biofilm volume.[10]

Chemical disinfectants have been used as intra-canal medication between appointments. An ideal intra-canal medication kills remaining microorganisms; moreover, it should minimize ingress of infection in case of a leaking restoration.[11] Chlorhexidine in 0.02 mg/ml concentration has been used in endodontics in both paste and solution form, it is used as a root canal medicament since it has broad-spectrum antibacterial activities against microorganisms found in infected root canals.[12] In addition, chlorhexidine acquires the antimicrobial substantivity in the dentin medicated, the slowly released positively charged ions are adsorb into dentin and prevent microbial colonization on the dentin surface up to four weeks.[13] Nowadays, there are attempts to find an alternative agent from natural sources which possess both antimicrobial activity and exert minimal tissue-irritating effect.

Bouea macrophylla (Maprang) is a tropical fruit tree which widely cultivated in Southeast Asia. In Thailand, Maprang wan (sweet Maprang) are the most widely consumed cultivar in Thailand.[14] The phytochemical compositions of Maprang extraction showed numerous bioactive compounds including alkaloid, flavonoids, anthraquinones, saponin, total phenol, tannin, sterol, triterpenes, and vitamin.[14] Maprang seed extract distributed resourceful of polyphenolic substance in 80 -700 mg GAE/g.[15] The extract has been reported to contain potential medicinal properties that can be used as antioxidant, anti-inflammatory, anti-cancer, or antimicrobial agents.[14] Maprang seed extract showed antimicrobial activity against wide range of microorganisms including gram-positive bacteria *(Staphylococcus aureus, Listeria monocytogenes, E. faecalis* and *Bacillus cereus)*, gram-negative bacteria *(Escherichia coli, Shigella flex-*

neri, Vibrio cholera and *Pseudomonas aeruginosa)* and fungus *(C. albicans)*.[14,16] However, there are no studies on the anti-biofilm activities of Maprang seed extract on bacterial, fungal, or multispecies biofilms. The hypothesis is that seed extract of Maprang Wan could be potentially novel agents for difficult-to-treat root canal infections. The aim of the present study was to determine the activity of the seed extract of Maprang Wan on planktonic and preformed multispecies biofilms of *E. faecalis, S. gordonii* and *C. albicans in vitro.*

Materials and Methods

This study was approved by the Human Experimentation Committee of the institution where the study was conducted (Certificate of ethical clearance No. 85/2020).

Microorganisms and growth conditions

E. faecalis ATCC 29212, *S. gordonii* ATCC 10558, and *C. albicans* ATCC 10231 were maintained in frozen stocks at -80°C. *E. faecalis* and *S. gordonii* were subcultured overnight on Brain Heart Infusion (BHI) (BD, Franklin Lakes, NJ, USA), Todd Hewitt Broth (BD, Franklin Lakes, NJ, USA) with 0.3% yeast extract (THBYE 0.3%) at 37°C for 24 hours.[17] One colony was then inoculated into 20 ml LB broth (Lennox, Massachusetts, USA) and grown overnight at 37°C in a shaking incubator (250 rpm maximum). The cells were harvested by centrifugation (4,500 rpm for 5 minutes), washed twice with sterile phosphate-buffered saline (PBS) (Sigma-Aldrich, Missouri, USA) and then re-suspended in 1xRPMI-1640. The cell density was measured by using a spectrophotometer and adjusted to 0.1 OD at 600 nm, which was equivalent to $1.0x10^8$ cells/ml.

C. albicans was cultured on Sabouraud Dextrose (SAB) agar (Oxoid, Basingstoke, UK)[18] and incubated at 37°C for 48 hours before being used to check viability (by plating) and purity (by microscope). One colony was then inoculated into 20 ml yeast peptone dextrose (YPD) broth (Melford, Ipswich, UK) and grown overnight at 37°C in the shaking incubator (250 rpm maximum). The cells were harvested by centrifugation (3,000 rpm for 5 minutes), washed twice with sterile PBS and then resuspended in 1xRPMI-1640. To produce the standardized biofilm inoculum, the cell density was measured by using a Neubauer haemocytometer and adjusted to $1.0x10^6$ cells/ml. The cell counts were confirmed by plating technique.

Media

RPMI-1640 (R0883, Sigma-Aldrich, Missouri, USA) with 2.0 g/l D-glucose was prepared with morpholinepropanesulfonic acid (MOPS) (Oxoid, Basingstoke, UK) and buffered to pH 7.4. The medium was filtered through a 0.22 μm membrane filter (Sigma-Aldrich, Missouri, USA) and stored at 4°C. The final concentrations of RPMI-1640 used to prepare multispecies biofilms was triple strength (3x).

Plant materials and extract preparation

Maprang Wan (sweet Maprang) fruits were derived from Nakhon Nayok Province, Thailand during the period of March to April 2020. Seeds were removed from the fruits, weighed, and minced. The samples of tested seeds were then dried at a temperature of 45°C for 24 hours using a hot-air oven. Extraction was carried out following the method of Dechsupa *et al.*[14] with some modifications. Briefly, minced seeds were triple macerated in ethyl acetate in 1:10 ratio for 48 hours with daily shaking. The extraction solutions, which were pooled together, were filtered through Whatman filter paper No. 1 and the filtrate were then dried by rotary evaporation. The crude ethyl acetate extract of the maprang seeds was collected and stored at room temperature until usage.

Susceptibility testing

Agar diffusion assay was used to determine the antimicrobial activities of the extracts followed method recommended by the clinical and Laboratory Standards Institute (CLSI).[19,20] Briefly, *E. faecalis, S. gordonii,* and *C. albicans* suspensions were diluted by BHI broth to a final concentration of approximately $1x10^6$ CFU/ml (OD_{600}=0.1). The diluted bacterial suspension (200 ml) was streaked onto Mueller-Hinton agar. The diluted fungal suspension (200 ml) was streaked onto Mueller-Hinton Agar + 2% Glucose and 0.5 μg/ml Methylene Blue Dye (GMB). The agar was excavated and each sample, dissolved in 25%v/v ethanol in culture broth at a concentration of 50 mg/ml (50 μl), was added into each well (6 mm diameter). The inoculated plates of *E. faecalis, S. gordonii,* and *C. albicans* were incubated at 37°C, under anaerobic condition for 24 hours. The diameter of the inhibition zone was measured in millimeters using digital vernier caliper (Insize Co., Ltd., USA). Chlorhexidine solution in 0.02 mg/ml concentration was used as positive controls.

Normal saline solution served as a negative control. The experiments were performed in triplicates.

Broth microdilution methods were used to determine the susceptibility of the planktonic *E. faecalis, S. gordonii,* and *C. albicans* to the extracts. Maprang seed extract was diluted from a stock 50 mg/ml aqueous solution into RPMI broth. Chlorhexidine (CHX) digluconate in 0.02 mg/ml, Corsodyl® (Glaxo Smith Kline, Middlesex, UK), was used as positive controls; RPMI medium was used as a negative control to determine the maximum microbial growth. The minimum inhibitory concentration (MIC) was read spectrophotometrically after 24 hours of incubation. Microbial suspensions (20 μl) were added to broth (180 μl) in 96-well cell-culture microtiter plates (F96 MicroWell Plates, NUNC, ThermoFisher Scientific, Leicestershire, UK). Microbial growth was determined by a spectrophotometer Multiscan RC analyser (ThermoFisher Scientific) at 490 nm. The inhibitory concentration of Maprang seed extract that prevented 50% of the bacterial or candidal growth was determined by changes in optical density. In addition, the microbiocidal activity of Maprang seed extract was tested after MICs were determined by streaking 20 μl of the test suspensions from representative wells onto agar and incubating for 48 hours; the results were shown as minimum bactericidal and fungicidal concentration (MBC/MFC). All samples were analyzed in triplicates within separated experiments.

Multispecies biofilm development

The culture suspensions of the three microorganisms were mixed in 1:1:1 and 1:1:0.1 of *E. faecalis: S. gordonii: C. albicans* proportions to create the multispecies biofilms inoculum. 100 μl of prepared dilutions were pipetted into the wells of a flat-bottom tissue-culture treated 24-well microtiter plate (Corning® Incorporated, Corning, NY, USA). The plates were incubated statically for 48 hours at 37°C and 5% CO_2. After incubation, the growth curves were established to understand the growth pattern of each isolate. Cells were harvested after 0, 6, 12, 24, 36, and 48 hours without addition of new nutrients. Ten-fold serial dilutions were made into PBS, and then 10 μl of each dilution was spread on BHI agar plates. Cell plating were used to determine cell counts at each time point. These procedures were done in triplicate.

In vitro time-kill assay

The suspension of 1: 1: 1 *E. faecalis: S. gordonii: C. albicans* was incubated in an anaerobic incubator (5% CO_2) at 37°C to develop into biofilms which were then grown for 48 hours before exposure to reagents. The Maprang seed extract at 10xMBC/MFC concentration and a control (sterile water) were incubated with pre-formed multispecies biofilm in 3x RPMI media in 24-well plates. Cells were harvested at 0, 24, 48, 36 and 168 hours. Ten-fold serial dilutions were prepared and 10 μl of each dilution was spread on BHI agar plates. After overnight incubation at 37°C, the number of colony forming units (CFUs) was counted. The experiments were performed in triplicates within separated experiments. The time-kill curve of Maprang seed extract was calculated and expressed as time v.s. - $\log_{10}$ survivors curves.

Statistical analysis

The statistical analysis was performed with IBM SPSS Statistics Version 20 (SPSS Inc, Chicago, IL, USA). One-way Analysis of Variance (ANOVA) with a Dunnett's test was used to investigate significant differences between independent groups of data which followed normal Gaussian distribution. Statistical tests were interpreted at the 5% significance level.

Results

Planktonic susceptibility testing

The ethyl acetate extract derived from Maprang seed presented a strong inhibitory effect against *E. faecalis, S. gordonii,* and *C. albicans* grown planktonically according to their broad inhibition zone (Table 1). However, the antimicrobial activities against *S. gordonii* and *C. albicans* of the extract were lower than those of chlorhexidine.

Table 1: Mean and standard deviation of the diameter of the growth inhibition zones (mm) provided by different testing agents against *E. faecalis, S. gordonii* and *C. albicans* isolates.

Solution	Zone of inhibition (mm ± SD)		
	E. faecalis	*S. gordonii*	*C. albicans*
Maprang seed extract	19.00±0.45	24.00±0.15	13.60±1.11
CHX (positive control)	18.70±2.11	34.00±2.36	15.80±1.30
NSS (negative control)	0	0	0

CHX: Chlorhexidine, NSS: Normal Saline Solution

The effects of Maprang seed extract on *E. faecalis, S. gordonii,* and *C. albicans* grown planktonically are summarized in Table 2. The extract shown to have antibacterial and antifungal activities: the MIC for *E. faecalis, S. gordonii* was 0.39 mg/ml and the MIC for *C. albicans* was 0.25 mg/ml. Maprang seed extract was bactericidal (MBC) and candicidal (MFC) at 2.50 mg/ml.

Table 2: The activity of Maprang seed extract against planktonic *E. faecalis, S. gordonii* and *C. albicans* isolates.

Organism and phenotype	Destination	Maprang seed extract	
		MIC (mg/ml)	MBC/MFC (mg/ml)
E. faecalis	ATCC 29212	0.39	2.50
S. gordonii	ATCC 10558	0.39	2.50
C. albicans	ATCC 10231	0.25	2.50

MBC, minimum bactericidal concentration; MFC, minimum fungicidal concentration; MIC, concentration that inhibits 50% of the growth.

Growth curve

From the multispecies analysis, the number of colony forming units per ml for each microorganism after 24 hours of growth, are shown in Figure 1. The total microbial count based on the colonies formed on BHI agar, which supported all microorganisms in the multispecies biofilms, confirmed the media types that best supported the biofilm viability for the multispecies biofilms. 1:1:1 *E. faecalis: S. gordonii: C. albicans* biofilm showed that the growth of neither was affected by the presence of the other (Figure 1a). However, 1:1:0.1 *E. faecalis: S. gordonii: C. albicans* biofilm showed that *E. faecalis* inhibited the growth of *C. albicans* at 36 hours (Figure 1b).

The 1:1:1 ratio was selected for the biofilm stage as this reflected better the situation in root canal infections. The growth of *E. faecalis, S. gordonii* and *C. albicans* followed a similar pattern (Figure 1a); the amount of all living cells increased exponentially during the first 12 hours of incubation (exponential growth phase). Between 12 and 24 hours the amount of living cells remained stable (stationary phase), and the cells were exponentially growth between 24 and 36 hours. The amount of living cells then decreased after 36 hours (starvation/death phase).

Lethal effect against multispecies of *E. faecalis, S. gordonii* and *C. albicans* biofilms of Maprang seed extract by time-kill assays

Maprang seed extract in concentration of 25 mg/ml (10x MBC/MFC) was determined for its lethal effects (Figure 2). The viable count of *E. faecalis, S. gordonii* and *C. albicans* treated with Maprang seed extract was decreased over time ($p<0.05$). Generally, 3-log10 reduction in the number of CFU/ml is considered as a bactericidal/fungicidal effect.[21] Maprang seed extract presented bactericidal and fungicidal effect within 3 days, and the effect was significantly better in 7 days ($p<0.05$). However, chlorhexidine was a fast-killng agent as it inhibited completely the growth of organisms within 24 hours.

Discussion

In vitro models have traditionally been used to screen potential agent and determine the mechanism of action

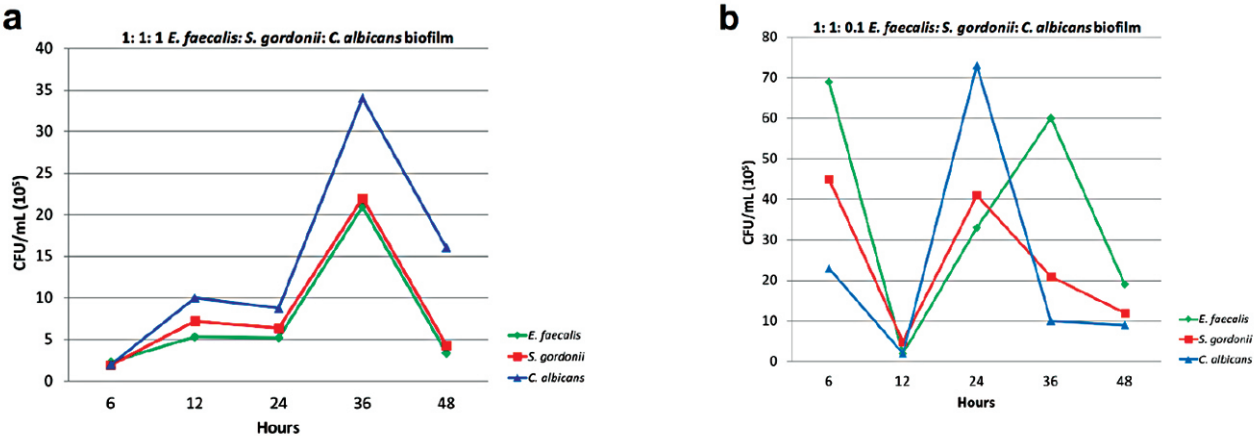

Figure 1: The growth of *E. faecalis, S. gordonii* and *C. albicans* in the presence of each other enumerated by CFU counting. The different ratios of multispecies biofilm were presented as 1: 1: 1 *E. faecalis: S. gordonii: C. albicans* (a) and 1: 1: 0.1 *E. faecalis: S. gordonii: C. albicans* (b).

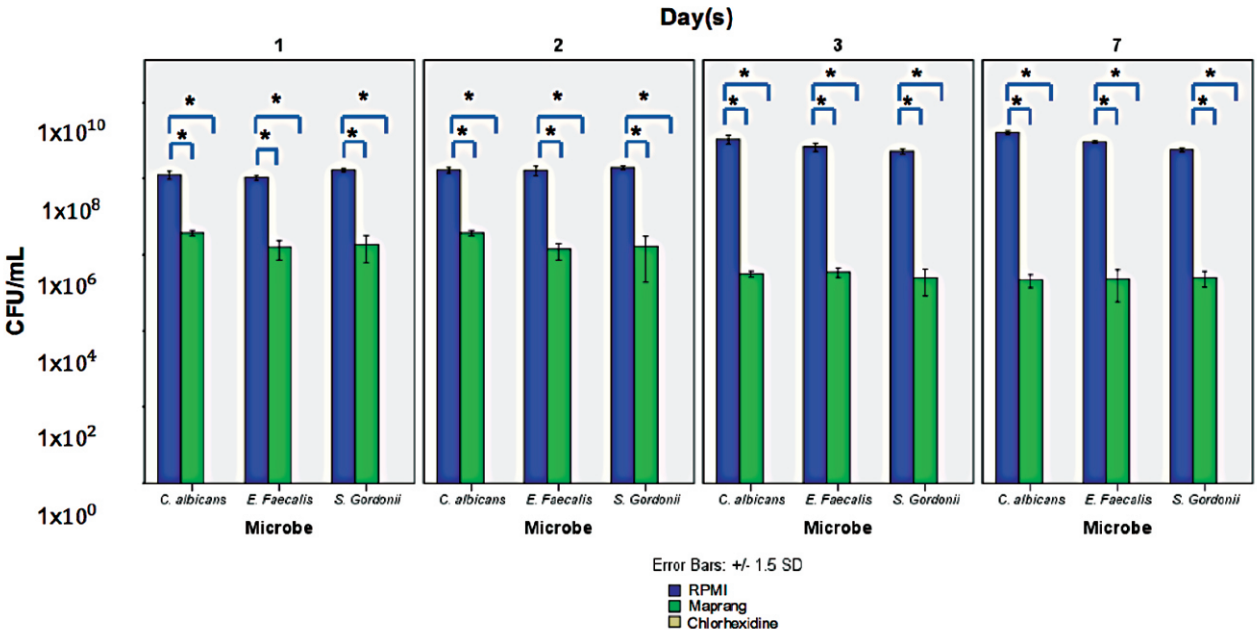

Figure 2: 7 days growth of multispecies 1: 1: 1 *E. faecalis: S. gordonii: C. albicans* biofilm in quantitative *in vitro* assays to measure the antimicrobial activity of Maprang seed extract. Viability was determined at the indicated time points by serial dilution plating. The time-kill curves presented here are time points in days. Results of two independent experiments done in triplicate are presented (*p<0.05: one way- ANOVA and Dunnett's test).

using precisely controlled experimental conditions. This study reported the effectiveness of Maprang seed extract against planktonic and multispecies *E. faecalis, S. gordonii* and *C. albicans.* The selected microorganisms in this study were representative bacterial and fungal in persistent root canal infection, and have been reported a high prevalence in failed endodontically treated teeth.[7] The disc diffusion susceptibility method was used for screening preliminary screening of pure natural substances. The broth dilution method was further used in preliminary screening of potential new antimicrobial agents, this method was also used to identify the concentration of the extract that inhibit and kill microorganisms.[22] In the present study, Maprang seed extract at 25 mg/ml showed bactericidal and fungicidal effect, this concentration was in line with the other *in vitro* study tested on *E. faecalis* and *C. albicans.*[14] In addition, time-kill assay was used to assess the dynamic interactions between antimicrobial agents and microbial strains and time required for bactericidal or fungicidal effect. This study performed the time-kill assay until 168 hours in order to replicate the period of intra-canal medicament in clinical scenario.[23] Maprang seed extract represented bactericidal and fungicidal effect within 3 days.

The composition of the growth medium has a major effect on the *in vitro* biofilm architecture, protein expression profiles and the antifungal susceptibility.[24] In this study, RPMI medium was used to mimic a physiological environment. The D-glucose concentration in RPMI medium was 2,000 mg/l. As biofilm growth and maturation is enhanced by the presence of glucose, hyperglycemic media are commonly used in biofilm studies[25], it is important that the *in vitro* model used mimics the conditions *in vivo* at the site of infection as far as possible. We used RPMI medium without additional D-glucose throughout the biofilm experiments to achieve this.

Following the selection of the RPMI 1640 as the microbiological media, we tested various inoculum concentrations on the growth of the multispecies biofilms. In 1:1:1 *E. faecalis: S. gordonii: C. albicans* biofilm, the growth of each species has similar pattern. We see an overall balance in the total microbial counts for the multispecies biofilm, which supports the overall synergistic and balancing effect these microorganisms have *in vitro*. This supported the previously reported commensal relationships between *E. faecalis* and *S. gordonii.*[26] Previous studies have also shown that synergistic interactions exist between *S. gordonii* and *C. albicans,* and that these interactions enhance *C. albicans'* hyphal develop-

ment and therefore pathogenicity *in vitro*.[9] Moreover, previous study reported that *E. faecalis* and *C. albicans* promote a mutually beneficial association with the host, in effect choosing a commensal lifestyle over a pathogenic one.[10] Therefore, 1:1:1 *E. faecalis: S. gordonii: C. albicans* biofilm was suitable to be used to assess the antimicrobial properties of Maprang seed extract.

The time kill assay showed a time dependent action of antimicrobial agents against multispecies biofilm. In this study, chlorhexidine was able to thoroughly eliminate the biofilm within 1 day. This finding was in line with other study which demonstrated that 2% CHX-containing medications were able to eliminate the 1- and 3-day biofilms of *E. faecalis*.[27] Maprang seed extract demonstrated significant reduction of viable organisms at 3 days, it was comparable to chlorhexidine (p=0.126). However, antibiofilm activity of the extract was significantly improved within 7 day. Therefore, Maprang seed extract has potential to be used as a root canal medicament, the higher concentration of the extract or the longer period of medication was required in order to completely eliminate the multispecies biofilm within the root canal.

On the other hand, there are some limitations of this study. Firstly, only three isolates of microorganisms were included in the biofilm model although oral infections typically involve multi-species biofilms.[28] However, persisting root canal infections often have only few dominant species.[29] Nevertheless, in the future, the antimicrobial activity of Maprang seed extract should be studied against more diverse biofilms. Secondly, the possible interactions between Maprang seed extract and root canal substances (such as dentin and hydroxyapatite) have not been tested. This topic should be addressed in further studies in order to evaluate the potential of the agent as an inter-appointment intra-canal medicament in endodontics. Finally, this study is performed *in vitro*. To mimic the environments in clinical situations, the antimicrobial activity of Maprang seed extract against multispecies biofilm in an *ex vivo* tooth model is required.

Conclusions

Within the limits of the present study, it is concluded that the ethanolic extract of Maprang seed had antibacterial and antifungal effect against planktonic *E. faecalis, S. gordonii* and *C. albicans*. Moreover, the extract had potential to eliminate multispecies *E. faecalis, S. gordonii* and *C. albicans* biofilms within 7 days. The antimicrobial activity of Maprang seed extract was comparable that of chlorhexidine. Further *ex vivo* studies using infected root canal models and clinical trials are required before the clinical use of Maprang seed extract as the inter-appointment intra-canal medicaments.

Conflicts of interest

The authors declare no conflict of interest.

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