

## Potential of *Piper betle* extracts on inhibition of oral pathogens

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### Summary

In the present study, antimicrobial activity of *Piper betle* crude ethanol extract against 4 strains of oral pathogens; *Candida albicans* DMST 8684, *C. albicans* DMST 5815, *Streptococcus gordonii* DMST 38731 and *Streptococcus mutans* DMST 18777 was compared with other medicinal plants. *P. betle* showed the strongest antimicrobial activity against all tested strains. Fractionated extracts of *P. betle* using hexane, ethyl acetate, and ethanol, respectively, were subjected to antimicrobial assay. The result revealed that the fractionated extract from ethyl acetate (F-EtOAc) possessed the strongest antimicrobial activity against all tested strains. Its inhibition zones against those pathogens were  $23.00 \pm 0.00$ ,  $24.33 \pm 0.58$ ,  $12.50 \pm 0.70$  and  $11.00 \pm 0.00$  mm, respectively and its minimum inhibitory concentrations were 0.50, 1.00, 0.50 and 1.00 mg/mL, respectively. Interestingly, the minimum concentration to completely kill those pathogens was the same for all strains and found to be 2.00 mg/mL. Killing kinetic study revealed that the activity of F-EtOAc was dose dependent. HPLC chromatograms of *P. betle* extracts were compared with its antimicrobial activity. An obvious peak at a retention time of 4.11 min was found to be a major component of F-EtOAc whereas it was a minor compound in the other extracts. This peak was considered to be an active compound of *P. betle* as it was consistent with the antimicrobial activity of F-EtOAc, the most potential extract against the tested pathogens. It is suggested that F-EtOAc is a promising extract of *P. betle* for inhibition of oral pathogens. Separation and structure elucidation of the active compound of this extract will be further investigated.

**Keywords:** *Piper betle*, oral pathogen, extract, antibacterial activity, antifungal activity

### 1. Introduction

Infectious disease occur in oral cavity is one of the serious dental problems. It causes impairment and functional limits of the infected organs and disability of the associated organs in oral cavity. According to the lesion and type of pathogens, oral infectious disease can be divided into many types such as oral candidiasis, gingivitis, periodontal disease, and dental caries. *Candida albicans* is a major cause of oral candidiasis (1). The oral streptococci bacteria such as *Streptococcus sanguinis*,

*Streptococcus gordonii*, *Streptococcus intermedius*, and *Streptococcus oralis* enriching in oral biofilm are the major cause of gingivitis (2,3). Whereas many studies confirm that *Streptococcus mutans* is the most severe bacteria in oral cavity and is a major cause of dental caries (4,5).

Oral candidiasis, gingivitis, and dental caries can be treated and prevented by using denture cleanser and mouthwash, respectively. Previous studies presented that chemical agents such as alkaline peroxide, alkaline hypochlorite, chlorhexidine, and disinfectant in denture cleanser and mouthwash can reduce dental plaque and the number of oral pathogens, significantly (6,7). However, long use of these agents can cause some defects in oral cavity such as certain adverse effects (8), increasing of tooth staining, and calculus formation

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(9). Moreover, changing oral taste can be easily found including bleaching of acrylic resin which is a denture base material (8,9).

Research in activity of plant extracts have been increasing interested for utilizing as alternatives and replacing the use of those chemical compounds for treatment or prevention of infection of oral cavity (10). Many plant extracts show high potential on antibacterial activity for these purposes. For example, the ethanol crude extract from aerial part of *Andrographis paniculata* shows antimicrobial activity against *Escherichia coli*, *Staphylococcus aureus*, *Vibrio alginolyticus*, *Shigella sonnei*, *Salmonella typhimurium*, *Vibrio cholera*, *Shigella boydii*, and *Vibrio alginolyteus* (11). The ethyl acetate extracts of *Momordica charantia* leaves and *Sesbania grandiflora* bark have the inhibitory activity against *S. aureus*, *E. coli*, and *Bacillus cereus* (12,13) whereas the extract of *Phyllanthus emblica* that can inhibit *Bacillus subtilis*, *Bacillus cereus*, *Salmonella typhi*, *Salmonella paratyphi* and *S. aureus* (14). It is noted that most plant extracts possess the activity against both Gram positive and Gram negative bacteria. However, some plant extracts exhibit the inhibitory activity against only Gram positive such as the extracts of *Psidium guajava* leaves that has the inhibitory activity against *S. aureus* and *B. cereus* but cannot inhibit the growth of *E. coli* and *Salmonella enteritidis* (15).

*Piper betle* is a medicinal plant in family Piperaceae. Many studies have explored its biological properties such as antimicrobial, radioprotective, antioxidant, anti-inflammatory and immunomodulatory activities (16-19). For the antimicrobial activity, *P. betle* have been reported to inhibit the growth of various kinds of bacteria such as Gram positive *S. aureus* and *Enterococcus faecalis* as well as Gram negative *Pseudomonas aeruginosa*, *Proteus vulgaris*, and *Klebsiella pneumoniae* (18,20). However, the research on activity of *P. betle* extracts against oral pathogens including both fungi and bacteria which are the major cause of oral candidiasis, gingivitis, and dental caries is still less. It is therefore interesting to investigate the activity of this plant against these oral pathogens to find an alternative option for the treatment of oral infectious disease or using as an active extract in oral hygiene products.

## 2. Materials and Methods

### 2.1. Chemicals

Ethanol, ethyl acetate, and hexane were from RCI Labscan (Bangkok, Thailand). Dimethyl sulphoxide (DMSO) was from Merck (Darmstadt, Germany). Brain heart infusion broth (BHI), brain, heart infusion agar (BHA), sabouraud dextrose broth (SDB) and sabouraud dextrose agar (SDA) were purchased from Difco (Maryland, USA). Human blood was supported by Maharaj Nakorn Chiang Mai Hospital (Chiang Mai,

Thailand). Tystatin (Nystatin) was from T.O. Pharma Co., LTD (Bangkok, Thailand). Other chemicals and solvents are of the highest grade available.

### 2.2. Plant Materials

*P. betle* and other five medicinal plants including *A. paniculata*, *M. charantia*, *P. emblica*, *S. grandiflora* and *P. guajava* were collected from the northern area of Thailand. The voucher specimens of these plants were deposited in the Herbarium of the Faculty of Pharmacy, Chiang Mai University, Thailand. The reference number and part used of these plants were presented in Table 1. The fresh medicinal plants were dried in a hot air oven at 50°C for 24-48 h. The dried medicinal plants were ground into fine powder.

### 2.3. Preparation of plant extracts

The dried powder samples of all plants were macerated in ethanol for 24 h. Then, the mixture was filtered through Whatman No. 1 filter paper. The dried macerated plant residue was re-macerated in ethanol and filtered again. The obtained filtrates were gathered and subjected to a rotary vacuum evaporator in order to remove the solvent. The crude ethanol extract (CE) obtained was kept in the refrigerator until used.

The fractionated extracts of *P. betle* were prepared using organic solvents with different polarity. Hexane was the first solvent used to macerate the plant material. Then, the dried macerated plant residue was further extracted with ethyl acetate, and ethanol, respectively. The hexane fractionated extract (F-Hexane), ethyl acetate fractionated extract (F-EtOAc) and ethanol fractionated extract (F-EtOH) obtained after solvent evaporation were kept in the refrigerator until used.

### 2.4. Oral pathogenic strains and growth conditions

The reference strains of oral pathogens used in this study were *C. albicans* DMST 8684, *C. albicans* DMST 5815, *S. gordonii* DMST 38731 and *S. mutants* DMST 18777. *C. albicans* DMST 8684 and *C. albicans* DMST 5815 were cultured in SDB at 37°C under aerobic condition for 24-48 h whereas *S. gordonii* DMST 38731 and *S. mutants* DMST 18777 were cultured in BHI at 37°C under anaerobic condition (5% H<sub>2</sub>, 5% CO<sub>2</sub> and 90% N<sub>2</sub>) for 24-48 h. The suspension of these pathogenic strains was prepared and the concentration was adjusted using a McFarland densitometer (DEN-1 Biosan, Riga, Latvia) to the turbidity of 0.5 McFarland standard.

### 2.5. Comparing antimicrobial activity of *P. betle* CE with other plants

Stock solutions of CE of each plant were prepared by

dissolving in DMSO to a concentration of 100 mg/mL. The antimicrobial test was performed using the disk diffusion method. An exact amount of 20  $\mu$ L stock solution of CE was added on a paper disk (CE-disk). The suspension of pathogenic strains after adjusting to 0.5 McFarland standard was swabbed over the entire surface of SDA for *Candida spp.* and 5% human blood in BHA (bBHA) for bacterial strains. The CE-disks were placed on the surface of each cultured medium. The plates were inverted and incubated in an aerobic condition for *Candida* strains and in anaerobic condition for bacterial strains at 37°C for 16-18 h. After incubation, the diameter of the clear zone indicating complete inhibition was measured. Nystatin suspension at a concentration of 100,000 unit/mL (20 mg/mL) and 1.2 mg/mL chlorhexidine solution (CHX) were used as positive controls for antifungal and antibacterial determinations, respectively. DMSO was used as a negative control.

#### 2.6. Antimicrobial activity of *P. betle* fractionated extracts in comparison with its CE

This experiment was performed using broth dilution method. Stock solutions of *P. betle* extracts including CE and the fractionated extracts; F-Hexane, F-EtOAc, and F-EtOH were prepared by dissolving in DMSO to have a final concentration of 32 mg/mL. Two fold serial dilutions of the stock solution were prepared until the lowest concentration obtained was 0.03 mg/mL were prepared in a 96-well plate using SDB or BHI media as a diluent for *Candida* strains or bacterial strains, respectively. The suspension of each pathogen was adjusted to have final microorganism concentrations of  $1 \times 10^4$  and  $1 \times 10^6$  cfu/mL for *Candida* strains and bacteria strains, respectively. The plates were inverted and incubated in aerobic condition for *Candida* strains and in anaerobic condition for bacterial strains at 37°C for 16-18 h. Minimum inhibitory concentration (MIC) of the extracts that inhibited the visual growth of the microorganism in this step was recorded. All dilutions were subsequently streaked on the entire surface of SDA for *Candida* strains and bBHA for bacterial strains and further incubated in the same conditions as in the determination of MIC. After incubation, the minimum concentrations of the extracts that showed complete inhibition of *Candida* strains and bacterial strains were determined as minimum fungicidal concentration (MFC) and minimum bactericidal concentration (MBC), respectively.

#### 2.7. Killing kinetic study

The study was performed according to the method previously described by Okonogi *et al.* (19). F-EtOAc of *P. betle* was used in this experiment as it showed the highest antifungal and antibacterial activities against all tested pathogens. F-EtOAc was firstly dissolved in

DMSO to have a concentration of 100 mg/mL. Then the extract solution was further diluted with SDB or BHI, a diluent for *Candida* strains or bacterial strains, respectively to obtain concentrations of 1-fold, 2-fold, and 4-fold MFC and MBC for *Candida* strains and bacterial strains, respectively. Suspensions of the pathogenic strains having microorganism concentration of  $10^4$ - $10^6$  cfu/mL were added to the extract solution in the 96-well plates. The cultures were then incubated in the same conditions as in the determination of MIC for 24 h. Viable counts were determined at the time intervals of 0, 1, 2, 4, 6, 12 and 24 h by plating 20  $\mu$ L of known dilutions of the culture samples on the entire surface of SDA and bBHA for *Candida* strains and bacterial strains, respectively. The SDA and bBHA plates were subsequently incubated for up to 24-48 h in the suitable condition of each strain. The plates with 30 to 300 colonies were used for cfu counts. Log cfu/mL was plotted against time for construction of the killing kinetic curves. Nystatin and CHX were used as a positive control for kinetic study of antifungal and antibacterial activities, respectively. All assays were analyzed in triplicate.

#### 2.8. High performance liquid chromatography (HPLC) analysis

HPLC analysis of *P. betle* extracts including CE, F-Hexane, F-EtOAc, and F-EtOH was performed using a Hypersil ODS column (4.6 i.d.  $\times$  250 mm) with an Agilent 1100 HPLC system (Massachusetts, USA). The exact weight of 1 mg extracts were dissolved in 1 mL ethanol (HPLC grade) and filtered through a 0.22  $\mu$ m filter membrane before injection with an injection volume of 20  $\mu$ L. The HPLC mobile phase consisting of methanol (A) and water (B) at a volume ratio of 7:3 was isocratic pumped at a flow rate of 0.70 mL/min for 20 min and detected by the UV/VIS detector at a wavelength of 280 nm.

#### 2.8. Statistical analysis

All experiments were done in triplicate and the results are expressed as mean  $\pm$  SD. Statistical analysis was done by using one-way ANOVA and *p*-value at a level of 95% confidence limit.

### 3. Results

#### 3.1. Preparation of plant extracts

The outer appearance of *P. betle* CE was dark green fluidized mass with specific intense odor whereas that of *P. emblica*, *S. grandiflora*, and *P. guajava* were solid mass and easily to be ground into powder. The CE of *A. paniculata* and *M. charantia* appeared as viscous mass. It was noted that the color of CE extracted from the leaves was dark green whereas that extracted from

the barks was rusty brown. The yield of CE obtained from each plant was presented in Table 1. It was found that *P. emblica* gave the highest yield followed by *A. paniculata* and *P. betle*. Fractionated extraction of *P. betle* resulted the fractionated extracts with different yield. The yield of F-EtOAc was the highest of 8.8% w/w whereas that of F-Hexane and F-EtOH were 8.2 and 4.2% w/w, respectively.

### 3.2. Comparing antimicrobial activity of *P. betle* CE with other plants

The results as shown in Table 2 demonstrated that CE of *P. betle* possessed the strongest antimicrobial activity against both antifungal and antibacterial activity against the tested pathogens with the inhibition zones of  $22.30 \pm 2.10$  and  $17.30 \pm 0.67$  mm for *C. albicans* DMST 8684 and *C. albicans* DMST 5815, respectively and

$7.80 \pm 0.30$  and  $7.10 \pm 0.00$  mm for *S. gordonii* DMST 38731 and *S. mutans* DMST 18777, respectively. The CE of *M. charantia* and *P. guajava* showed inhibition to *C. albicans* DMST 5815 and *S. gordonii* DMST 38731 whereas the CE of *P. emblica* and *A. paniculata* showed only antifungal activity. In addition, it was found that the CE of *S. grandiflora* showed no activity against the tested oral pathogens. In comparison with the control, Nystatin showed the inhibitory effect to both *C. albicans* DMST 8684 and *C. albicans* DMST 5815 and CHX showed the strong effect to both *S. gordonii* DMST 38731 and *S. mutans* DMST 18777. From these results, *P. betle* was considered to be suitable for further study.

### 3.3. Antimicrobial activity of *P. betle* fractionated extracts in comparison with its CE

The results as shown in Table 3 demonstrated that different fractionated extracts of *P. betle* possessed antimicrobial activity in different levels. F-Hexane possessed the inhibition zone of  $21.00 \pm 1.40$  and  $20.67 \pm 0.58$  mm, against both *C. albicans* DMST 8684 and *C. albicans* DMST 5815 whereas only *S. gordonii* DMST 38731 could be inhibited with the inhibition zone of  $8.00 \pm 0.00$  mm. F-EtOAc possessed obviously clear inhibition zone of  $23.00 \pm 0.00$  and  $24.33 \pm 0.58$  mm against *C. albicans* DMST 8684 and *C. albicans* DMST

**Table 1. Detail of plants used and the yield of CE**

Plant samples	Voucher specimen	Used part	Yield (%)
<i>A. paniculata</i>	004046	Leaf	24.6
<i>M. charantia</i>	023225	Leaf	6.6
<i>P. emblica</i>	008895	Leaf	40.1
<i>S. grandiflora</i>	023176	Bark	8.9
<i>P. guajava</i>	008610	Leaf	19.5
<i>P. betle</i>	008612	Leaf	19.2

**Table 2. Inhibition zone of CE against oral pathogens**

CE of plant samples and controls	Inhibition zone (mm)			
	<i>C. albicans</i> DMST 8684	<i>C. albicans</i> DMST 5815	<i>S. gordonii</i> DMST 38731	<i>S. mutans</i> DMST 18777
<i>A. paniculata</i>	NZ	$10.00 \pm 0.00$	NZ	NZ
<i>M. charantia</i>	NZ	$10.33 \pm 0.58$	$7.7 \pm 0.70$	NZ
<i>P. emblica</i>	$10.70 \pm 0.80$	NZ	NZ	NZ
<i>S. grandiflora</i>	NZ	NZ	NZ	NZ
<i>P. guajava</i>	NZ	$12.00 \pm 0.00$	$8.10 \pm 0.0$	NZ
<i>P. betle</i>	$22.30 \pm 2.10$	$17.30 \pm 0.67$	$7.80 \pm 0.30$	$7.10 \pm 0.00$
Nystatin	$19.67 \pm 0.58$	$17.00 \pm 0.00$	-	-
CHX	-	-	$8.00 \pm 0.00$	$15.00 \pm 0.00$
DMSO	NZ	NZ	NZ	NZ

NZ: no inhibition zone. Data were represented as mean  $\pm$  SD.

**Table 3. Inhibition zone of *P. betle* extracts against oral pathogens**

<i>P. betle</i> extracts and controls	Inhibition zone (mm)			
	<i>C. albicans</i> DMST 8684	<i>C. albicans</i> DMST 5815	<i>S. gordonii</i> DMST 38731	<i>S. mutans</i> DMST 18777
F-Hexane	$21.00 \pm 1.40$	$20.67 \pm 0.58$	$8.00 \pm 0.00$	NZ
F-EtOAc	$23.00 \pm 0.00$	$24.33 \pm 0.58$	$12.50 \pm 0.70$	$11.00 \pm 0.00$
F-EtOH	NZ	NZ	NZ	NZ
CE	$21.00 \pm 0.71$	$20.00 \pm 0.00$	$11.30 \pm 0.40$	$10.67 \pm 0.58$
Nystatin	$19.67 \pm 0.58$	$17.00 \pm 0.00$	-	-
CHX	-	-	$8.00 \pm 0.00$	$15.00 \pm 0.00$
DMSO	NZ	NZ	NZ	NZ

NZ: no inhibition zone. Data were represented as mean  $\pm$  SD.

**Table 4. MIC, MFC and MBC (mg/mL) of *P. betle* extracts**

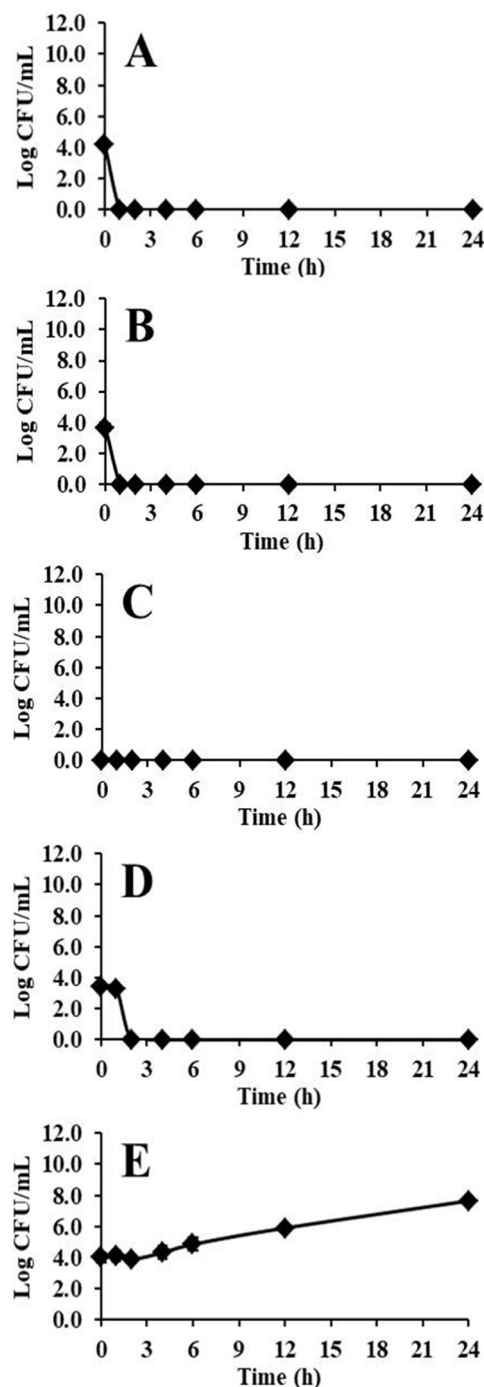
<i>P. betle</i> extracts and controls	<i>C. albicans</i> DMST 8684		<i>C. albicans</i> DMST 5815		<i>S. gordonii</i> DMST 38731		<i>S. mutans</i> DMST 18777	
	MIC	MFC	MIC	MFC	MIC	MBC	MIC	MBC
F-Hexane	1.00	2.00	1.00	4.00	1.00	2.00	2.00	2.00
F-EtOAc	0.50	2.00	1.00	2.00	0.50	2.00	1.00	2.00
CE	1.00	2.00	1.00	2.00	1.00	4.00	2.00	8.00
Nystatin	$6 \times 10^{-4}$	$2.4 \times 10^{-3}$	$3.1 \times 10^{-3}$	$9.8 \times 10^{-3}$	-	-	-	-
CHX	-	-	-	-	$< 3 \times 10^{-4}$	$< 3 \times 10^{-4}$	$< 3 \times 10^{-4}$	$6 \times 10^{-4}$

5815 and of  $12.50 \pm 0.70$  and  $11.00 \pm 0.00$  mm against both *S. gordonii* DMST 38731 and *S. mutans* DMST 18777, respectively. F-EtOH showed no inhibitory effect to all tested pathogens. In comparison with CE, it was found that CE also possessed the inhibitory activity against both tested fungi and bacteria. However, the inhibition zones of CE against all tested oral pathogens were significantly less than F-EtOAc.

Determination of MIC, MFC and MBC of *P. betle* extracts was performed in order to confirm the results of inhibition zone. F-EtOH was not subjected to this experiment as it showed no inhibition zone. The results were shown in Table 4. F-EtOAc showed the highest inhibitory activity against all tested oral pathogens. Antifungal activity of CE was almost similar to F-EtOAc, however, antibacterial activity was significantly lower. It was found that the MBC values of F-EtOAc against *S. gordonii* DMST 38731 and *S. mutans* DMST 18777 were 2 times and 4 times, respectively, less than CE indicating that F-EtOAc was significantly higher effective than CE. F-Hexane showed minor antimicrobial activity against the oral pathogens. Nystatin, a positive control for antifungal activity, showed an activity against *C. albicans* DMST 8684 and *C. albicans* DMST 5815 with MIC values of  $6.1 \times 10^{-4}$  and  $3.1 \times 10^{-4}$  mg/mL and MFC values of  $2.4 \times 10^{-3}$  and  $9.8 \times 10^{-3}$  mg/mL, respectively. CHX, a positive control for antibacterial activity, showed an activity against *S. gordonii* DMST 38731 and *S. mutans* DMST 18777 with the same MIC value of  $< 3 \times 10^{-4}$  mg/mL and MBC values of  $< 3 \times 10^{-4}$  and  $6 \times 10^{-4}$  mg/mL, respectively.

### 3.5. Killing kinetic study

As F-EtOAc showed the highest inhibitory activity against all tested oral pathogenic strains, it was selected for this experiment. The killing kinetic of F-EtOAc against *C. albicans* DMST 8684 was dose dependent as shown in Figure 1. It was found that at concentration of 1-fold MFC (2 mg/mL) and 2-fold MFC (4 mg/mL), the efficiency of F-EtOAc to completely kill the microorganism could be done within 1 h. However, strong killing efficiency was obviously seen when the concentration of F-EtOAc was increased to 4-fold MFC (8 mg/mL). At this concentration, F-EtOAc could



**Figure 1. Killing kinetics of F-EtOAc at the concentration of 1-fold MFC (A), 2-fold MFC (B), 4-fold MFC (C) in comparison with nystatin (D) and DMSO (E) ( $n = 3$ ) against *C. albicans* DMST 8684.**

completely kill the microorganism suddenly once after the microorganism exposed to the extract. Nystatin could completely kill the pathogen within 2 h. It was obviously seen that the killing rate of F-EtOAc against *C. albicans* DMST 8684 was significantly faster than nystatin.

The killing kinetic of F-EtOAc against *S. gordonii* DMST 38731 was dose dependent as shown in Figure 2. At concentration of 1-fold MBC (2 mg/mL), F-EtOAc could completely kill the pathogenic bacteria within 4 h. However, increasing concentration of the extract to 2-fold MBC (4 mg/mL) and 4-fold MBC (8 mg/mL), higher killing efficiency was obviously seen. The

extract at these concentrations could completely kill the bacteria within 2 and 1 h, respectively. CHX, a positive control for antibacterial activity, could completely kill this pathogenic bacterial strain within 4 h. It was obviously seen that the killing rate of F-EtOAc against *S. gordonii* DMST 38731 was significantly faster than CHX.

The killing kinetic of F-EtOAc against *S. mutans* DMST 18777 was also dose dependent as shown in Figure 3. It was found that at a low concentration of 1-fold MBC (2 mg/mL), F-EtOAc could completely kill all pathogenic bacteria. However, after comparison with the bacterial growth curve in the negative

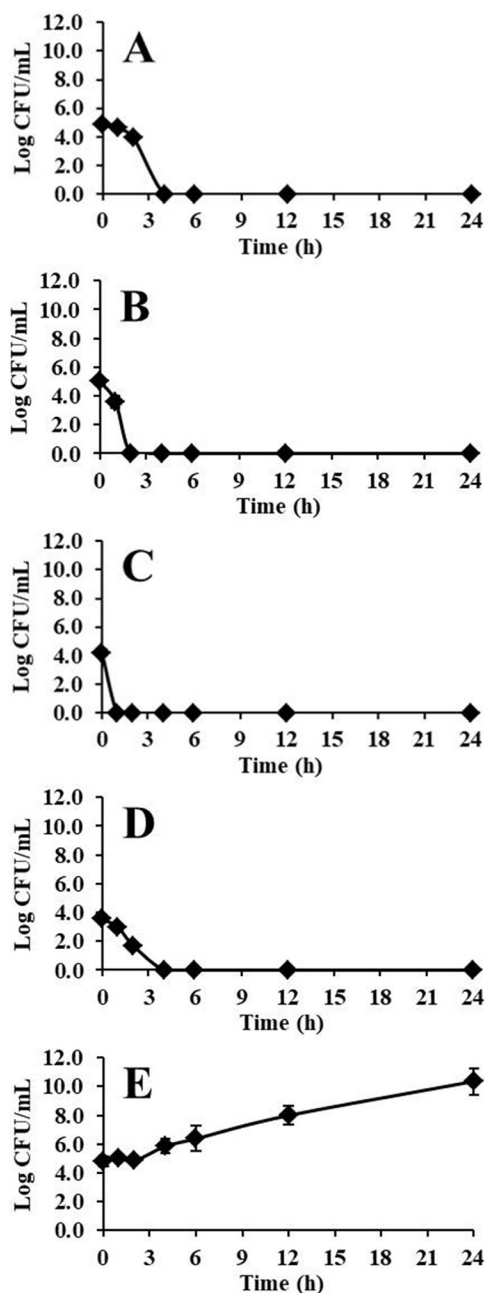


Figure 2. Killing kinetics of F-EtOAc at the concentration of 1-fold MBC (A), 2-fold MBC (B), 4-fold MBC (C) in comparison with nystatin (D) and DMSO (E) ( $n = 3$ ) against *S. gordonii* DMST 38731.

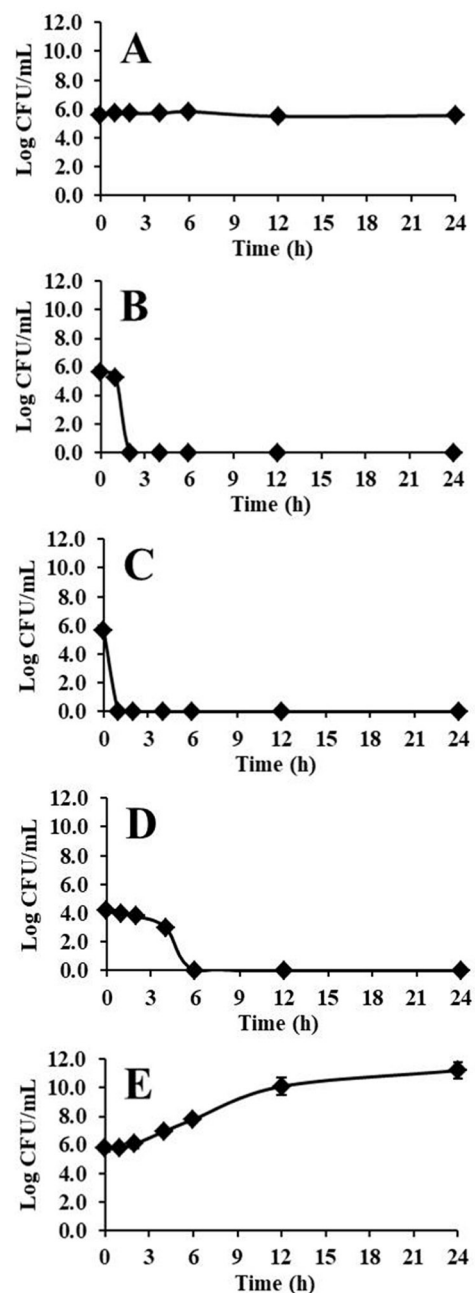


Figure 3. Killing kinetics of F-EtOAc at the concentration of 1-fold MBC (A), 2-fold MBC (B), 4-fold MBC (C) in comparison with nystatin (D) and DMSO (E) ( $n = 3$ ) against *S. mutans* DMST 18777.

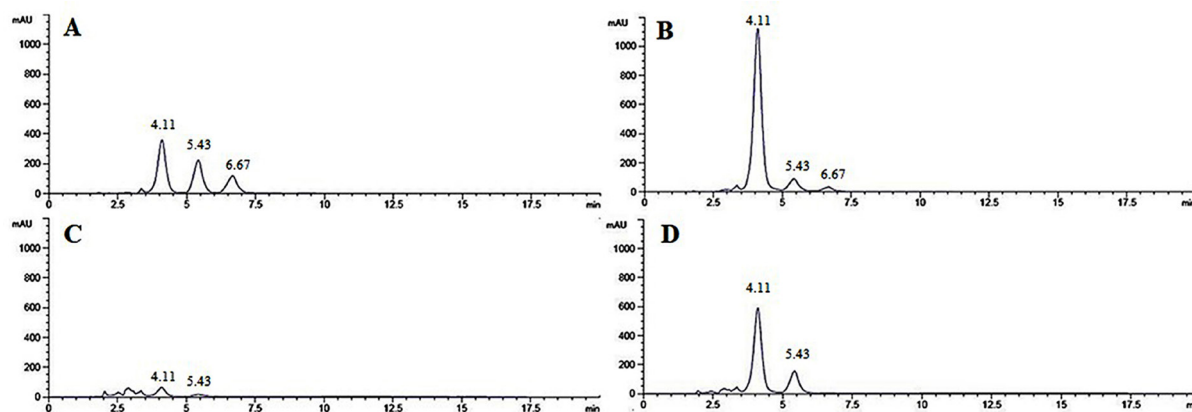


Figure 4. HPLC chromatograms of *P. betle* extracts; F-Hexane (A), F-EtOAc (B), F-EtOH (C) and CE (D).

control, it could be clearly seen that the extract exactly showed some inhibition to the growth of the pathogen. Increasing concentration of the extract to 2-fold MBC (4 mg/mL) and 4-fold MBC (8 mg/mL), higher killing efficiency was obviously seen. The extract at 4 mg/mL could completely kill the bacteria within 2 h whereas at 8 mg/mL of F-EtOAc, the pathogen was completely killed within 1 h. CHX, a positive control for antibacterial activity, could completely kill this pathogenic bacterial strain within 6 h. It was obviously seen that the killing rate of F-EtOAc against *S. mutans* DMST 18777 was significantly faster than CHX.

### 3.6. HPLC analysis of *P. betle* extracts

HPLC chromatograms of *P. betle* extracts including F-Hexane, F-EtOAc, F-EtOH and CE were demonstrated in Figure 4. F-Hexane and F-EtOAc contained 3 major compounds at the same retention time of 4.11, 5.43 and 6.67 min but different quantity. The compound of F-EtOAc at a retention time of 4.11 min was obviously seen as a major ingredient. Whereas, F-EtOH and CE contained 2 compounds that were presented in the same retention time of 4.11 and 5.43 min.

## 4. Discussion

It has been reported worldwide of the side effects of chemical antiseptic compounds used in oral hygiene products (8). Meanwhile, researches have explored many biological activities of plants extracts for treatment of various diseases (13,16,22). For inhibition of oral pathogens, many plant extracts have been reported to have the potential on this purpose, for example, ethyl acetate extract of *Camellia sinensis* leaves was reported to decrease the incidence of dental caries (23) whereas water extract of *Vaccinium macrocarpon* fruits showed an inhibitory effect against protease enzyme of *Porphyromonas gingivalis* (24). Moreover, the extract from *Salvadora persica* stem can inhibit many oral pathogenic bacteria, such as *S.*

*mutans*, *Lactobacillus acidophilus*, *Aggregatibacter actinomycetemcomitans*, and *P. gingivalis* (25). Present study was done on *P. betle*, a plant widely grown in Southeast Asian countries. The leaves of this plant have been widely consumed by local people as a mouth freshener. There are some reports on its antibacterial activity against many types of bacteria (18,20) but none of them are oral pathogens. The current study reports on inhibitory effects of *P. betle* extracts against four strains of oral pathogens including two strains of oral pathogenic fungi; *C. albicans* DMST 8684 and *C. albicans* DMST 5815 and two strains of oral pathogenic bacteria; *S. gordonii* DMST 38731 and *S. mutans* DMST 18777. These pathogenic microorganisms are the major cause of oral candidiasis, gingivitis and dental caries, respectively (2,26,27). The antibacterial and antifungal activities were investigated by two methods which are disk diffusion method and broth dilution method following the standard protocol of the National Committee for Clinical Laboratory Standards (NCCLS) (28,29) The antimicrobial activity of CE of *P. betle* against these pathogens was firstly compared with the CE of other five plants which have been reported to have antimicrobial activity. The results indicate that even the tested plant extracts showed antibacterial activity, but not all of them can inhibit the oral pathogens. CE of *P. betle* was found to be the most effective against all tested strains with inhibition zones of  $22.30 \pm 2.10$  and  $17.30 \pm 0.67$  mm for *C. albicans* DMST 8684 and *C. albicans* DMST 5815, respectively and  $7.80 \pm 0.30$  and  $7.10 \pm 0.00$  mm for *S. gordonii* DMST 38731 and *S. mutans* DMST 18777, respectively. However, the activity against these oral pathogens of the CE is significantly lower than the fractionated extract from ethyl acetate (F-EtOAc). F-EtOAc showed the highest effective with the inhibition zones of  $23.00 \pm 0.00$  and  $24.33 \pm 0.58$  mm against *C. albicans* DMST 8684 and *C. albicans* DMST 5815 and of  $12.50 \pm 0.70$  and  $11.00 \pm 0.00$  mm against *S. gordonii* DMST 38731 and *S. mutans* DMST 18777, respectively.

The inhibition zone can roughly indicate the

inhibitory effects of the tested extracts but MIC, MBC, or MFC values present deeper and more proper information particularly for comparative effects of the extracts. The results confirm that F-EtOAc is the most effective extract against the oral pathogenic bacteria and fungi. The MBC values of F-EtOAc against *S. gordonii* and *S. mutans* show that F-EtOAc is 2 times and 4 times, respectively, higher effective than CE. In addition, antimicrobials are usually regarded as bactericidal or fungicidal if the MBC/MIC or MFC/MIC ratio is  $\leq 4$  and bacteriostatic or fungistatic if  $> 4$  (30). The ratios obtained for all the test organisms were above 4 which indicated that F-EtOAc was bactericidal and fungicidal actions against the tested oral microorganisms.

The bactericidal and fungicidal actions of F-EtOAc were confirmed by the killing kinetic study. Bactericidal and fungicidal agents can completely inhibit the growth or multiplication of pathogenic microorganisms (31). From the killing kinetic patterns of F-EtOAc, it is indicated that F-EtOAc exhibits bactericidal and fungicidal actions. The pharmacological actions of some antimicrobial agents are dose dependent and some are time dependent (21,32). The effective antimicrobial activity of F-EtOAc from the killing kinetic patterns is considered to be dose and time dependent.

Many previous reports have shown that *P. betle* contains extensive biologically active compounds such as eugenol, allylpyrocatechol, chavibetol, chavibetol acetate, caryophyllene, hydroxychavicol, which are related to its activities (33-35). The HPLC condition used in the current study was modified from the previous studies of Ferreres *et al.* that the major chemical compounds in *P. betle* were reported (33). Considering the HPLC patterns of *P. betle* extracts, it is considered that the antimicrobial activity of the extracts against the tested oral pathogens are likely according to the compound at a retention time of 4.11 min. This compound was found to be the most abundant of F-EtOAc and extremely higher amount than other extracts. This compound therefore is considered to be an active compound of *P. betle* for antimicrobial actions against the oral pathogens.

In conclusion, *P. betle* is a medicinal plant that possessed the strong potential action against oral pathogens causing candidiasis, gingivitis and dental caries. The evaluation of antimicrobial activity of fractionated extracts of *P. betle* confirms that the major bioactive compound of *P. betle* extract is a moderate polar compound similar to ethyl acetate. *P. betle* extract is the promising natural source of antimicrobial compounds against oral pathogens. Purification and structure elucidation of the active compounds as well as clinical trials are challenges for further studies.

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