

Effects of *Piper betle* fractionated extracts on inhibition of *Streptococcus mutans* and *Streptococcus intermedius*

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Summary

The overgrowth of certain strains of normal flora in oral cavity can cause many kinds of oral infections or diseases such as caries, periodontitis, and gingivitis. Prevention and treatment of these diseases are usually achieved by chemical antiseptics. However, these chemicals are found as negative impacts of human health hazards and accession of microbial resistance. The present study explores the potential of *Piper betle* extracts on inhibition of two oral pathogenic bacteria; *Streptococcus mutans* DMST 41283 and *Streptococcus intermedius* DMST 42700. *P. betle* demonstrated significantly higher inhibitory activity against both pathogenic strains than *Acacia catechu*, *Camellia sinensis*, *Coccinia grandis*, *Solanum indicum*, and *Streblus asper*. Among fractionated extracts of *P. betle* from several solvents, the extract from ethyl acetate (Pb-EtOAc) possessed the widest inhibition zone of 11.0 ± 0.1 and 11.3 ± 0.4 mm against both bacterial strains, respectively. Pb-EtOAc showed the same minimum inhibitory concentration of 0.5 mg/mL against both strains, whereas its minimum bactericidal concentrations were 2.0 and 0.5 mg/mL against *S. mutans* and *S. intermedius*, respectively. HPLC analysis demonstrated that the major active compound of Pb-EtOAc was 4-allylpyrocatechol. It was found that the killing kinetics of Pb-EtOAc against both test strains were time and dose dependent. Scanning electron microscopy micrographs showed the morphological changes and depletion of the tested pathogens indicating cell destruction after exposure to Pb-EtOAc. It is confirmed that Pb-EtOAc is potentially effective against both oral pathogens and might be used as natural alternative agents in prevention and treatment of oral infections caused by oral pathogenic bacteria.

Keywords: 4-allylpyrocatechol, killing kinetics, cell morphology, oral infection, plant extract

1. Introduction

Oral health is categorized as the important issue to overall health and wellness. World health organization (WHO) concerns this situation and promotes many policies to boost a good oral health and prevent oral diseases (1). Poor oral health is mainly due to diseases

or infections in oral cavity caused by overgrowth of some oral bacteria. *Streptococcus* is the major group of bacteria causing oral infectious diseases. *Streptococcus mutans* causes pathogenesis of dental caries whereas *Streptococcus intermedius* locates in subgingival tissue and causes gingivitis. The violence of these oral diseases leads to loose of teeth and periodontitis which is a chronic severe oral infection (2,3). Prevention and treatment of oral infection can be achieved by oral hygiene practices and use of some antiseptics. Various chemical antimicrobial agents were used as effective medicine and contain in oral health products. However, prolong use of these chemicals presents negative impacts

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of human health hazards and acquisition of microbial resistance. The use of natural products such as plant extracts are of increased interest due to safer than those chemicals for human and more environmental friendly. Therefore, several medicinal plants have been investigated for antibacterial activities in order to be an alternative choice for prevention and treatment of infectious diseases including oral infections (4). Many plant extracts including plant essential oils have been reported to have potential inhibitory effects against many strains of Gram-negative and Gram-positive pathogenic bacteria (5-7). The *in vitro* inhibition of some oral bacteria by the extracts of *Antrodia camphorate* (8) and *Prunus mume* (9) has been reported. However, there are still few reports of plant extracts on the inhibitory effects against oral pathogens. Therefore, it is essential to search for potential plants that have high inhibitory activity against the oral pathogens.

Piper betle is an edible plant in Family Piperaceae. This plant has been used as a main active composition in many Asian folk medicinal remedies for a long time. The extracts of *P. betle* have been reported to have several pharmacological effects such as anti-inflammatory, anti-allergic, wound healing, antiplatelet, antioxidant including antibacterial and antifungal activities (10). The bioactive compounds found in *P. betle* are several types such as allylpyrocatechol, eugenol, chavibetol, caryophyllene, and hydroxychavicol. The concentration of these compounds distributed in the plant is different depended on the different parts of the plant as well as source and season of harvesting (11,12). Among extensive reports on its biological activities, the report on inhibitory effects against the pathogenic microorganisms causing oral infectious diseases is still less. We previously reported the antimicrobial activity of *P. betle* against *Candida albicans* DMST 8684, *C. albicans* DMST 5815, *Streptococcus gordonii* DMST 38731 and *S. mutans* DMST 18777 and demonstrated that *P. betle* had significantly higher activities against these oral pathogens than *Andrographis paniculata*, *Momordica charantia*, *Phyllanthus emblica*, *Sesbania grandiflora* and *Psidium guajava* (13). In the present study, we further investigated the inhibition potential of *P. betle* against another strains of oral pathogenic bacteria; *S. mutans* DMST 41283 and *S. intermedius* DMST 42700, and compared its activity with five different plants having antibacterial activity against other microorganisms. Moreover, this study explores the bioactive compound of the most effective fractionated extract of *P. betle*. The work was to explore the inhibition potential of *P. betle* on oral pathogenic bacteria and that it can be used as an active agent for prevention and treatment of the oral infections caused by those pathogens.

2. Materials and Methods

2.1. Culture media and chemicals

Brain heart infusion broth (BHI) and brain heart infusion agar (BHA) were purchased from Difco (Maryland, USA). Human blood was supported by Maharaj Nakorn Chiang Mai Hospital (Chiang Mai, Thailand). Ethanol, ethyl acetate, and hexane were from RCI Labscan (Bangkok, Thailand). Dimethyl sulphoxide (DMSO) and glutaraldehyde were from Merck (Darmstadt, Germany). 4-Allylpyrocatechol (APC) was purchased from Fluka (Missouri, USA). Other chemicals and solvents are of the highest grade available.

2.2. Plant materials and extraction

P. betle and other five Thai medicinal plants, including *Acacia catechu*, *Camellia sinensis*, *Coccinia grandis*, *Solanum indicum*, and *Streblus asper*, were collected from the northern part of Thailand. The plants were identified and their voucher specimens were kept at the Herbarium of the Faculty of Pharmacy, Chiang Mai University, Thailand. The details of these plants are shown in Table 1. Fresh medicinal plants were dried in hot air oven at 50°C for 24-48 h before pulverizing into fine powder. To prepare their crude ethanol extract (CE), the plant powders were macerated in ethanol for 24 h. Subsequently, the mixture was filtered through a Whatman No.1 filter paper. The filtrate was collected and the plant residues were re-macerated in ethanol and filtered again. The obtained filtrates were gathered and subjected to a rotary vacuum evaporator (EYELA N-100, Tokyo, Japan) for removing the solvent. The obtained CE of each plant was kept in 4°C for further experiment.

The fractionated extracts of *P. betle* were prepared using hexane, ethyl acetate, and ethanol. The extraction method was according to the previous reported (13). Briefly, hexane was used as the first macerating solvent. The plant residue from hexane extraction was further macerated with ethyl acetate, and ethanol, respectively. The filtrates from each solvent were subjected to the rotary evaporator. After the solvents were completely removed, the fractionated extracts from hexane (Pb-Hexane), ethyl acetate (Pb-EtOAc) and ethanol (Pb-EtOH) were obtained. All extracts were kept at 4°C until use.

2.3. Pathogenic strains and inoculum preparation

Two strains of oral pathogenic bacteria; *S. mutans* DMST 41283 and *S. intermedius* DMST 42700 were used as the test microorganisms. *S. mutans* is a facultative anaerobic bacteria, but the optimal atmospheric condition for cultures should be anaerobic or contain only a low percentage of oxygen with 5-10% carbon dioxide whereas *S. intermedius* is an aerotolerant anaerobic commensal bacteria. The inoculum of these strains was prepared by culturing the oral pathogens in

broth media. Both strains were cultured in BHI at 37°C under anaerobic condition (5% H₂, 5% CO₂ and 90% N₂) for 24-48 h. The stock inoculum was adjusted to the turbidity of 0.5 McFarland standard using a McFarland densitometer (DEN-1 Biosan, Riga, Latvia).

2.4. Antibacterial activity investigation

The antibacterial activities of the extracts were investigated by means of two standard methods, the disk diffusion and the broth dilution methods.

The disk diffusion method was used to investigate antimicrobial activity of the CE of *P. betle* (Pb-CE) and the CE of other five plants (*A. catechu*, *C. grandis*, *C. sinensis*, *S. indicum*, and *S. asper*) as well as the fractionated extracts of *P. betle* (Pb-Hexane, Pb-EtOAc, and Pb-EtOH). The stock solutions of the test extracts were prepared by dissolving the extracts in DMSO. An exact amount of 20 µL of the extract solution was added onto the 6-mm diameter disk papers. The entire surface of BHA containing 5% human blood (bbHA) was swabbed with the stock inoculum of the test bacteria after adjusting to 0.5 McFarland standard. The extract loaded disks were placed on the surface of this medium. The plates were then incubated in an anaerobic condition at 37°C for 24 h. The diameter of inhibition zone was measured. Chlorhexidine 1.2 mg/mL solution (CHX) and DMSO were used as a positive and negative control, respectively.

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of *P. betle* extracts were evaluated by a broth dilution method. Stock solutions of Pb-Hexane, Pb-EtOAc, and Pb-CE were prepared by dissolving the extracts in DMSO. These stock solutions were diluted with BHI media to obtain the two-fold serial dilutions in a 96-well plate. The stock inoculum of each pathogen was added to the plates to have a final concentration of 1×10⁶ cfu/mL. The plates were incubated in anaerobic condition at 37°C for 24 h. The lowest concentration of the extract that inhibited microbial growth after 24 h incubation was recorded as MIC. All dilutions were subsequently streaked on the entire surface of bbHA and further incubated in the same conditions as in the determination of MIC. After 24-h incubation, a minimum concentration of the extracts that showed complete inhibition of the bacteria was observed and recorded as MBC.

2.5. HPLC analysis

Pb-EtOAc was subjected to HPLC using a Hypersil ODS column (4.6 i.d. × 250 mm) with an Agilent 1100 HPLC system and UV detector set at 280 nm. An isocratic mobile system composed of methanol and water at a volume ratio of 7:3 was used to elute the samples at a flow rate of 0.70 mL/min for 20 min. The extract was dissolved in ethanol and filtrated through a

0.22 mm Millipore filter, type GV (Millipore, Bedford, Massachusetts, USA) prior to HPLC injection. The sample volume of 20 µL was injected and the HPLC was run in an ambient temperature. The samples were analyzed in triplicate. Standard APC was used as a biomarker and run separately under the same HPLC condition. The HPLC peak of the extract which appeared at the same retention time of APC was detected and expectedly identified as APC, a bioactive compound of the extract.

2.6. Killing kinetic study

Pb-EtOAc was used in this experiment. The extract solution was prepared by dissolving Pb-EtOAc in DMSO. The obtained solution was further diluted with BHI until the extract concentrations were 1-fold, 2-fold and 4-fold MBC. The inoculum of the pathogenic strains having microorganism concentrations of 1 × 10⁶ cfu/mL was added to the extract solution in the 96-well plates. The plates were incubated in an anaerobic condition at 37°C for 24 h. Viable bacteria were determined at the time intervals of 0, 1, 2, 4, 6, 12, and 24 h by plating 20 µL of the known dilutions of the culture samples on the entire surface of bbHA. The plates were further incubated for 24-48 h. The plates with 30 to 300 colonies were used for colony forming unit (cfu) counts. The killing kinetic curves showing relationship between log cfu/mL and time were plotted. CHX and DMSO were used as positive and negative controls, respectively.

2.7. Microbial morphology study

Assessment of a possible mechanism of the active compound against oral pathogens, a morphological analysis of the test bacteria was carried out by treating the pathogens with Pb-EtOAc. Morphology of the pathogens before and after treatment was observed using scanning electron microscopy (SEM) (JEOL JSM-6610LV, Tokyo, Japan). The pathogenic strains having the concentration of 1 × 10⁶ cfu/mL were added to the 0.5 mg/mL Pb-EtOAc solution in the 96-well plates. It was noted that this concentration was the MIC of Pb-EtOAc against all test pathogens. The cultures were then incubated in the same conditions as in the determination of MIC for 24 h. In the preparation of sample for SEM analysis, the suspension in each well was filtered through nylon membrane. Bacterial cells were fixed using 2% glutaraldehyde in phosphate buffer solution (pH 7.4) for 2 h-1 week. The cells were washed to remove glutaraldehyde and other suspended materials in the same solution for 10 min, this step was repeated 3 times. Then, the cells were dehydrated in increasing concentration of ethanol as follows; 50% for 5 min, 70% for 15 min, 85% for 15 min, 95 % for 15 min, and 100% for 15 min. This step was repeated 2 times. The chips of nylon membranes that covered with

Table 1. Plants and the percentage yield of their CE

Plant samples	Family	Local name	Voucher specimen	Used part	Yield (%)
<i>A. catechu</i>	Fabaceae	Seesiad	009208	Wood	2.7
<i>C. grandis</i>	Cucurbitaceae	Tamleung	007620	Leaf	18.1
<i>C. sinensis</i>	Theaceae	Miang	005584	Leaf	16.3
<i>P. betle</i>	Piperaceae	Plu	008612	Leaf	19.2
<i>S. indicum</i>	Solanaceae	Mawangton	023226	Fruit	22.5
<i>S. asper</i>	Moraceae	Khoi	005531	Bark	6.6

bacteria cells after dehydration were dried by critical point drying technique. Afterwards, the chips were mounted on aluminum stub and coated with gold in a sputter coater (JEOL JFC-1100E, Tokyo, Japan). The cells on the chip were observed under the SEM at 15 kV accelerating voltage with magnification of 15,000X. CHX was used as a positive control.

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 followed by Tukey's HSD test using SPSS software version 17.0 and *p*-value at a level of 95% confidence limit.

3. Results

3.1. Preparation of CE

The CE of the studied plants showed different appearances and yields. All plant extracts showed different specific intense odor. Pb-CE was dark green fluidized mass whereas that of *C. grandis*, *C. sinensis*, *S. indicum*, and *S. asper* were a viscous mass. The CE of *A. catechu* appeared as dried powder. It was noted that the color of the extracts from the wood, bark, and fruit was rusty brown to light yellow, while that from the leaf was dark green. The yield of CE obtained from each plant was different as presented in Table 1.

3.2. Comparing the inhibitory efficiency of *P. betle* with other plants

The results as shown in Table 2 demonstrated that Pb-CE had the highest antimicrobial activity against both pathogenic bacteria. The inhibition zones of *S. intermedius* was slightly wider than that of *S. mutans*, indicating that *S. intermedius* was slightly more sensitive to Pb-CE than *S. mutans*. The CE of *S. asper* showed inhibition against *S. intermedius*, almost same as *P. betle*, but showed no activity against *S. mutans*. The CE of *A. catechu* showed inhibition against *S. intermedius* but significantly less than that of *P. betle* and no activity against *S. mutans*. The inhibition zones of the positive control, CHX, against *S. mutans* and *S. intermedius* were 14.4 ± 0.6 and 14.6 ± 0.6 mm, respectively, while the

Table 2. Inhibition zone of plant CE against the test oral pathogens

Plant samples	Inhibition zone (mm)	
	<i>S. mutans</i>	<i>S. intermedius</i>
<i>A. catechu</i>	6.2 \pm 0.1c	6.7 \pm 0.3b
<i>C. grandis</i>	6.1 \pm 0.1c	6.2 \pm 0.1c
<i>C. sinensis</i>	6.1 \pm 0.2c	6.3 \pm 0.1c
<i>P. betle</i>	8.5 \pm 0.7a	8.9 \pm 0.3a
<i>S. indicum</i>	6.0 \pm 0.1c	6.1 \pm 0.1d
<i>S. asper</i>	6.4 \pm 0.1b	7.8 \pm 0.3a

Values are mean \pm SD followed by different lowercase letters imply the significant differences (*p* < 0.05) between values in the same column.

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

<i>P. betle</i> fractionated extracts	Inhibition zone (mm)	
	<i>S. mutans</i>	<i>S. intermedius</i>
Pb-Hexane	6.2 \pm 0.1b	8.0 \pm 0.0b
Pb-EtOAc	11.0 \pm 0.0a	11.3 \pm 0.4a
Pb-EtOH	6.1 \pm 0.2b	6.1 \pm 0.1c

Values are mean \pm SD followed by different lowercase letters imply the significant differences (*p* < 0.05) between values in the same column.

Table 4. MIC and MBC (mg/mL) of *P. betle* extracts against the test oral pathogens

<i>P. betle</i> extracts	<i>S. mutans</i>		<i>S. intermedius</i>	
	MIC	MBC	MIC	MBC
Pb-Hexane	2.0	2.0	1.0	1.0
Pb-EtOAc	0.5	2.0	0.5	0.5
Pb-CE	2.0	8.0	1.0	2.0

negative control, DMSO, showed no inhibition zone for both strains.

3.3. Comparative antimicrobial activity of *P. betle* fractionated extracts

Among the fractionated extracts of *P. betle*, Pb-EtOAc possessed obviously the highest antimicrobial activity with the inhibition zones of 11.0 ± 0.1 and 11.3 ± 0.4 mm against *S. mutans* and *S. intermedius*, respectively as shown in Table 3. Pb-Hexane showed the activity against only *S. intermedius* but significantly less potent than Pb-EtOAc. Moreover, Pb-Hexane showed no inhibitory activity against *S. mutans*. Pb-EtOH showed no inhibitory effect to both test pathogens.

3.4. Determination of MIC and MBC of *P. betle* extracts

The results obtained from broth dilution method could obtain MIC and MBC of the extracts. The results as shown in Table 4 confirmed that among *P. betle* extracts, Pb-EtOAc was the most effective extract of *P. betle* against both oral pathogenic bacteria. It was found that the MIC of Pb-EtOAc against both pathogens was the same value of 0.5 mg/mL. However, the MBC values against both strains were significant different, 2.0 mg/mL for *S. mutans* and 0.5 mg/mL for *S. intermedius*. Pb-Hexane and Pb-CE demonstrated similar level of inhibitory activity. Both extracts showed antimicrobial activity with the same value of MIC against both pathogens. Their MIC values were higher concentration than that of Pb-EtOAc, indicating less potential on pathogenic inhibition than Pb-EtOAc. CHX, a positive control for antibacterial activity, showed an inhibitory activity against *S. mutans* and *S. intermedius* with the same MIC and MBC values of $< 3 \times 10^{-4}$ mg/mL.

3.5. HPLC analysis

HPLC chromatograms of Pb-EtOAc in comparison with APC standard compound are shown in Figure 1. The results showed that Pb-EtOAc contained three major compounds at the retention time of 4.11, 5.43 and 6.67 min. APC showed a peak at a retention time of 4.10 min which is resemblance with the HPLC peak that found at 4.11 min of Pb-EtOAc.

3.6. Killing kinetic study

The killing kinetics of Pb-EtOAc against the two pathogens were investigated and the results indicated that the killing activity of Pb-EtOAc was dose and time dependent. At 1-fold MBC, Pb-EtOAc completely killed *S. mutans* within 6 h as shown in Figure 2. Increasing concentration of Pb-EtOAc, higher killing efficiency was obviously seen. The bacteria were completely killed within 2 h and 1 h by the concentrations of 2-fold

and 4-fold MBC, respectively. Whereas CHX (0.006 mg/mL) could completely kill this pathogenic strain within 4 h. The killing kinetic of Pb-EtOAc against *S. intermedius* was shown in Figure 3. At 1-fold MBC, Pb-EtOAc completely killed the bacteria within 6 h. However, increasing concentration of the extract to 2-fold and 4-fold MBC, higher killing efficiency was obtained. The extract at these concentrations

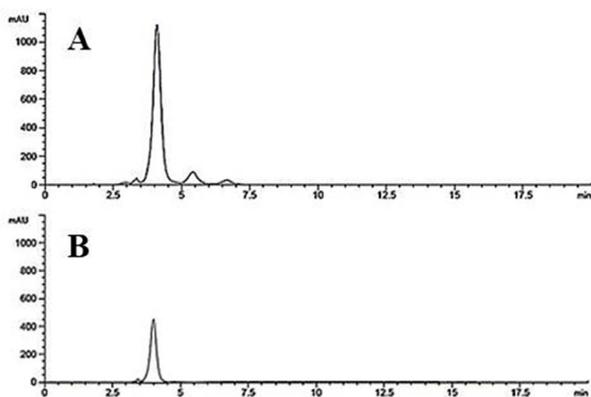


Figure 1. HPLC chromatograms of Pb-EtOAc (A) and 4-Allylpyrocatechol (B).

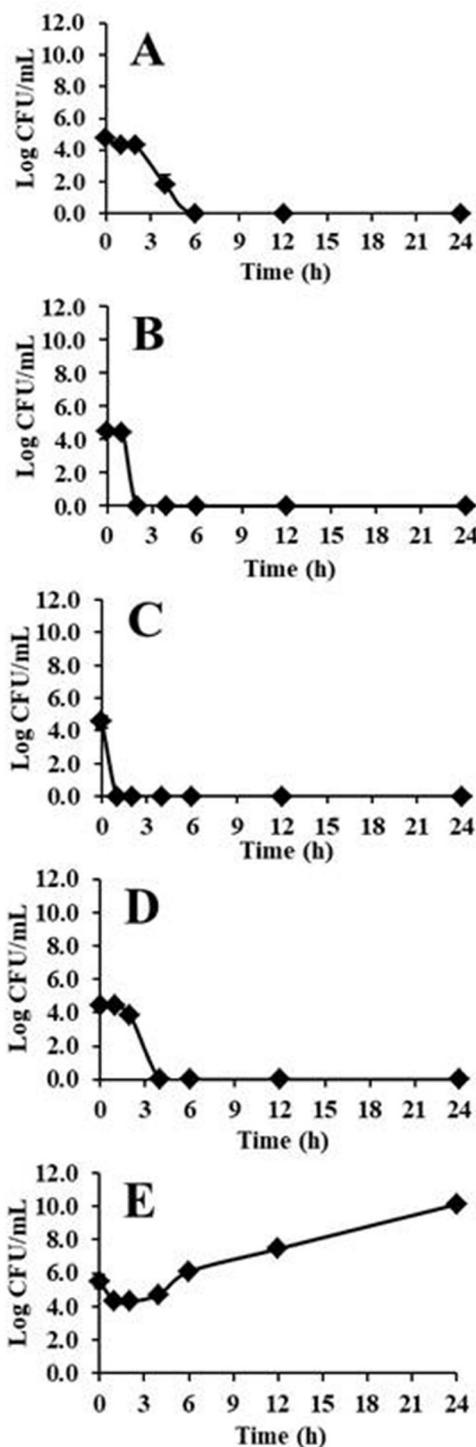


Figure 2. Killing kinetics of Pb-EtOAc at the concentrations of 1-fold MBC (A), 2-fold MBC (B), 4-fold MBC (C) in comparison with CHX (D) and DMSO (E) against *S. mutans* ($n = 3$).

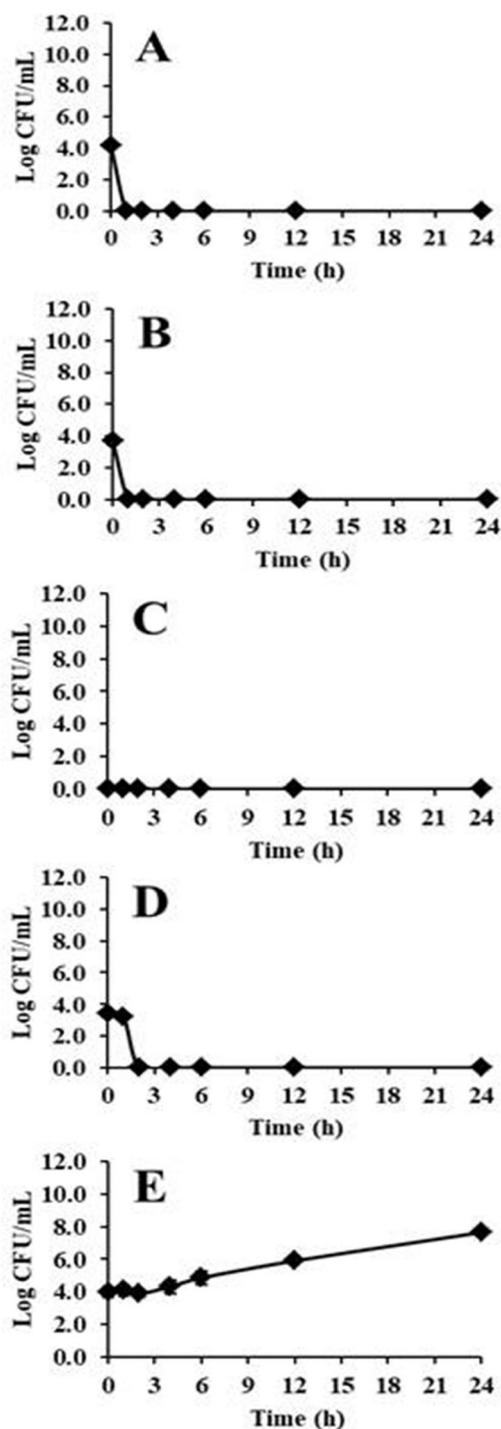


Figure 3. Killing kinetics of Pb-EtOAc at the concentrations of 1-fold MBC (A), 2-fold MBC (B), 4-fold MBC (C) in comparison with CHX (D) and DMSO (E) against *S. intermedius* ($n = 3$).

could completely kill the bacteria within 4 and 1 h, respectively. CHX at 0.006 mg/mL could completely kill this pathogen within 2 h.

3.7. Microbial morphology study

The morphology of the test oral pathogens were observed using SEM. The morphology of the untreated

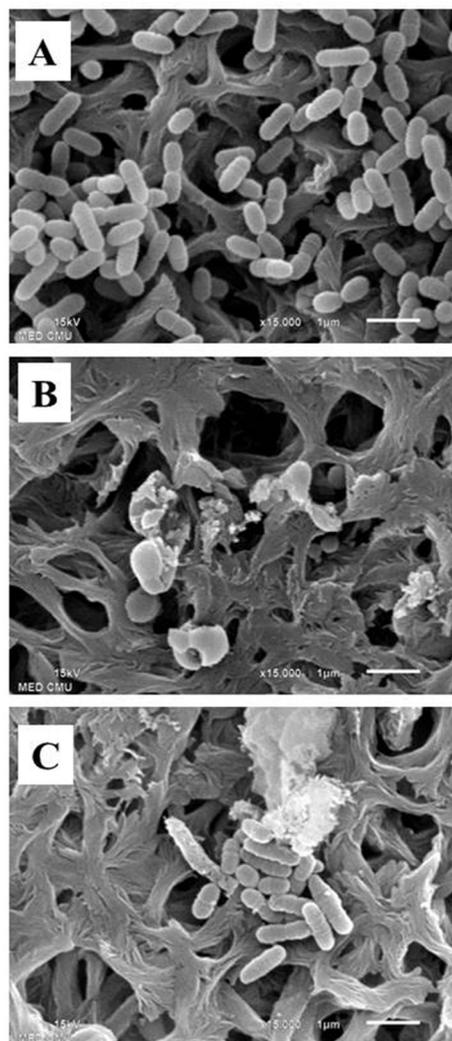


Figure 4. SEM micrographs of untreated and treated oral pathogens on nylon membrane; untreated *S. mutans* (A), treated *S. mutans* with Pb-EtOAc (B), and treated *S. mutans* with CHX (C).

S. mutans in BHI medium as control cells was elongated ellipse shape and smooth surface as shown in Figure 4A. After incubation with 0.5 mg/mL of Pb-EtOAc, the damage of *S. mutans* cells could be seen as presented in Figure 4B. Furthermore, morphology of the cells was changed. The size of the cells were swollen and disruption. The cell population was significantly reduced when compared to the control group, same as the positive control (Figure 4C). The control cells of *S. intermedius* in BHI media without being treated with the extract were complete round and smooth surface as shown in Figure 5A. After incubation with 0.5 mg/mL of Pb-EtOAc, the bacterial cells were damaged and the cell size was slightly enlarged with roughly surface as seen in Figure 5B. Moreover, the population of the cells was significantly decreased in comparison with a control sample. Treating with 0.006 mg/mL CHX, morphology and size of *S. intermedius* were slightly changed and the cell population was significantly decreased as presented in Figure 5C.

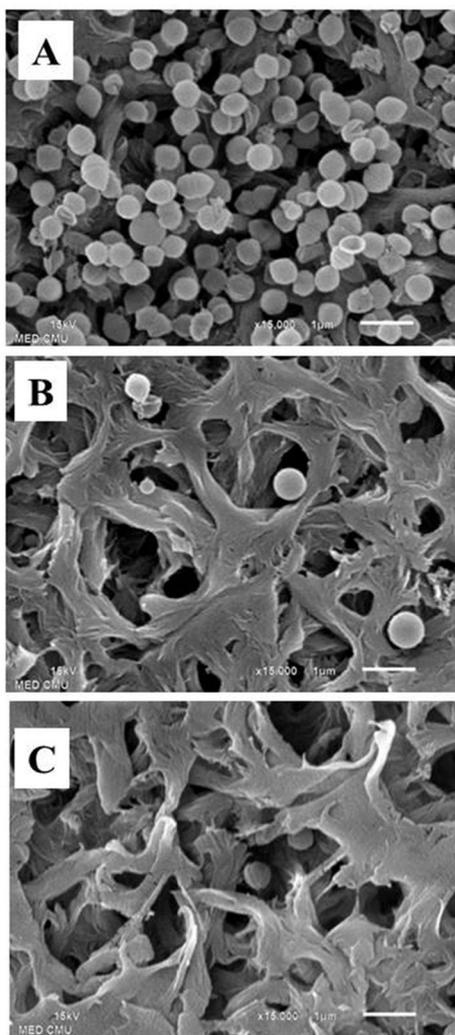


Figure 5. SEM micrographs of untreated and treated oral pathogens on nylon membrane; untreated *S. intermedius* (A), treated *S. intermedius* with Pb-EtOAc (B), and treated *S. intermedius* with CHX (C).

4. Discussion

Oral diseases infected by oral pathogenic bacteria do not affect only the oral cavity, but also influence the others parts in the body via blood circulation (14,15). For example, periodontitis caused by oral pathogenic bacteria has been reported to be a significant risk of developing cardiovascular diseases; atherosclerosis, myocardial infarction and stroke (16). Utilizing chemical aseptic or antimicrobial compounds to promote oral hygiene and inhibit oral pathogens causes several side effects (17,18). Moreover, microbial resistance has always been reported after long term using of these chemicals (19). The use of antimicrobial agents from plants instead of these chemicals, therefore, is our interest.

In our works on searching for potential plant extracts having high activity on inhibition of oral bacteria, *P. betle* is one of many interesting plants. In the current experiment, we compared the activity of *P.*

betle with five different potential plants (*A. catechu*, *C. grandis*, *C. sinensis*, *S. indicum*, and *S. asper*) that have been previously reported to have antimicrobial activities (20-24). The yield of Pb-CE was 20.1%, slightly higher than that previously reported, which might be due to the different time and area of plant collection (25). Among the fractionated extracts of *P. betle*, the yield of Pb-EtOAc was the highest, followed by that of Pb-Hexane and Pb-EtOH. The difference of the yield among the different fractionated extracts is due to the different extracting solvents used and the solubility of the compounds existing in *P. betle* (26). These results confirm that not only the source of plants but also the extracting solvent and the method of extraction that affect the yield of the extracts. The antimicrobial activity of *P. betle* extract has been reported by other groups (10). However, they did not report its activity against the oral pathogens. We are the first group who demonstrate the activity of *P. betle* against oral pathogenic microorganisms. We previously reported its activity against the oral pathogenic fungi and certain bacterial strains (13). However, there are several bacteria existing in oral cavity that can be predominated and cause oral diseases, particular *S. mutans* and *S. intermedius*. The results of this study provide extensive data from the previous report and can be the scientific evidence to confirm the potential of *P. betle* on inhibition of various pathogenic bacterial strains in oral cavity as well as a supported scientific data of a historical use of *P. betle* by local people in the South and Southeast Asian countries as a mouth freshener (27).

In antibacterial activity investigation, the inhibition zone can approximately indicate the inhibitory effects of the test extracts, but MIC and MBC demonstrate deeper and more proper data for particularly comparative efficacy of the extracts. From the results of inhibition zone, MIC and MBC, it is confirmed that Pb-EtOAc was the highest potential extract on inhibitory activity against both oral pathogenic strains; *S. mutans* and *S. intermedius*.

Antimicrobial activity is usually regarded as bactericidal if the MBC/MIC ratio is ≤ 4 and bacteriostatic if > 4 (28). For Pb-EtOAc, the ratios of MBC/MIC calculated for *S. mutans* and *S. intermedius* were 4 and 1, respectively. From these results, the ratios of MBC/MIC are ≤ 4 , indicating that Pb-EtOAc possesses bactericidal actions against both oral pathogens.

The pharmacological actions of some antimicrobial agents are dose dependent and some can be dose or time dependent (29,30). In our study on killing kinetics, the results indicate that the pharmacological action of antibacterial activity of Pb-EtOAc is dose and time dependent.

The morphological characteristic of bacterial cells is essential for understanding the possible mechanism

of action of antimicrobial agent against the pathogens. Generally, SEM is one of the potential tools used to analyze the morphology of the cells. It can provide the information on number, shape and size of cells (31,32). Using SEM, the morphology of *S. mutans* and *S. intermedius* was obviously observed in the current study. After treating with Pb-EtOAc, the morphology and size changes as well as population depletion of both strains were well seen. These effects caused by Pb-EtOAc are similar to the effects caused by CHX, the positive control. CHX was previously reported that it can destroy pathogen cells by disruption of cell membrane with its positive charge (33). The results from this study support that the antibacterial activity of Pb-EtOAc against *S. mutans* and *S. intermedius* is disruption of cell membrane of those pathogens leading to the leakage of essential cell compositions and cell dead.

The active compounds in plant extracts can be possibly identified by HPLC analysis using known active compound as a marker (34). Many compounds including APC have been reported from *P. betle*. APC has been reported to have many biological actions, for example induce apoptosis of cancer cells (35) and inhibit platelet aggregation (36). For antimicrobial activity, this compound has been reported to have an inhibitory activity against *Staphylococcus aureus* and *Escherichia coli* (37). In the present study, HPLC chromatogram of Pb-EtOAc demonstrates many peaks indicating many compounds existing in the extract. However, the major peak of Pb-EtOAc appears at the same retention time as the peak of APC. Therefore, the active compound of *P. betle* for antibacterial activity against the oral pathogens is considered to be APC. The inhibitory activity against anaerobic bacteria of APC has never been reported elsewhere. This is the first report demonstrates this activity of the compound, particularly against anaerobic strains of oral pathogens.

In conclusion, the findings in the present study confirm that *P. betle* is a potential medicinal plant for inhibition of *S. mutans* and *S. intermedius*, the oral pathogenic bacteria and the major cause of dental caries and gingivitis. Pb-EtOAc is the most effective extract of *P. betle*. The major active compound of Pb-EtOAc is APC. The antibacterial activity of Pb-EtOAc bactericidal action and it is time and dose dependent. The mechanism of action of Pb-EtOAc is the ability to destroy the pathogen cells by causing disruption of cell membrane causing the leakage of essential components of the cells and cell dead.

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