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Original article

Screening for antibacterial and antibiofilm activity in Thai medicinal plant extracts against oral microorganisms

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ABSTRACT

To evaluate the antibacterial activity of 12 ethanol extracts of Thai traditional herb against oral pathogens. The antibacterial activities were assessed by agar well diffusion, broth microdilution, and time-kill methods. Antibiofilm activity was investigated using a 3-[4,5-dimethyl-2-thiazolyl]-2,5-diphenyl-2H-tetrazolium-bromide (MTT) assay. High performance liquid chromatography (HPLC), thin layer chromatography (TLC) fingerprinting, and TLC-bioautography were used to determine the active antibacterial compounds. *Piper betle* showed the best antibacterial activities against all tested strains in the minimal inhibitory concentration and minimal bactericidal concentration, ranged from 1.04–5.21 mg/mL and 2.08–8.33 mg/mL, respectively. Killing ability depended on time and concentrations of the extract. *P. betle* extract acts as a potent antibiofilm agent with dual actions, preventing and eradicating the biofilm. The major constituent of *P. betle* extract was 4-chromanol, which responded for antibacteria and antibiofilm against oral pathogens. It suggests that the ethanol *P. betle* leaves extract may be used for preventing oral diseases.

1. Introduction

Herbal medicines are valuable and available resources of primary health care for thousands year in the traditional medicine including the Thai folk medicine. Generally, the bioactive compounds in herbal medicines are secondary metabolites which act as various pharmacological properties.[1–3] They are used as the substances of modern drugs e.g. morphine from *Papaver somniferum* for pain treatment,[4,5] colchicine from *Colchicum autumnale* for treatment in pericardial disease.[6] Some studies have reported antibacterial activity from various herbs[7–9]; however, sufficient evidence to support their active compounds and their effectiveness for antibacteria and antibiofilm, particularly on oral pathogens is limited.

An imbalance of bacteria in the dental biofilm can cause dental caries and periodontitis which are major oral infections.[10] The high doses of antimicrobial agents are need for removing the biofilm in clinical approach.[11,12] Moreover, in long term use of chlorhexine (CHX) mouth-rinse can cause side effects; disturbance in taste sensation, brown discoloration at dorsum of tongue and desquamative lesions of oral mucosa.[13,14]

Our previous study has presented anticandidal and antibiofilm of some Thai herbs in Thailand.[15] All herbs tested were commonly found in tropical areas of Southeast Asia. Herbal medicines may be an alternative way for being antibacterial agents to prevent oral infectious diseases. This extended study was to evaluate the antibacterial and antibiofilm activities of 12 ethanol extracts of Thai traditional herb included *Alpinia galanga*, *Curcuma longa*, *Curcuma zedoaria*, *Piper betle*, *Piper chaba*, *Piper nigrum*, *Piper sarmentosum*, *Mentha cordifolia*, *Ocimum africanum*, *Ocimum basilicum*, *Ocimum sanctum* and *Zingiber officinale*, and to investigate the active compounds of the most effective extract.

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2. Materials and methods

2.1. Preparation of medicinal herb extracts

Twelve herbs were purchased from the local market, and the details were shown in Table 1. The herbs were identified by an expert in the Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Prince of Songkla University, Songkhla, Thailand, where voucher specimens (Table 1) were deposited in the herbarium. Dried plants were macerated with ethanol for 3 days, and then filtrated through Whatman No. 4 filter paper. Each filtrate was dried using a rotary evaporator at 40 °C and kept at −20 °C. For testing, 0.1 mg of each dried extract was initially dissolved with 100 μL of dimethyl sulfoxide (DMSO) and then adjusted to 1 mL by adding sterile distilled water, giving a final concentration of 10% (w/v) extract in 10% (v/v) dimethyl sulfoxide (DMSO).

2.2. Bacterial strains and growth conditions

A total of 7 oral microorganisms were employed in the study including 5 Gram positive cariogenic bacteria, *Enterobacter faecalis* ATCC 19433 (*Ef*), *Lactobacillus fermentum* ATCC 14931 (*Lf*), *Lactobacillus salivarius* ATCC 11741 (*Ls*), *Streptococcus sobrinus* ATCC 33478 (*Ss*) and *Streptococcus mutans* ATCC 25175 (*Sm*), and 2 Gram negative periodontopathogenic bacteria, *Aggregatibacter actinomycetemcomitans* ATCC 33384 (*Aa*) and *Fusobacterium nucleatum* ATCC 25586 (*Fn*). Microorganisms were maintained on either brain heart infusion agar (BHA) with 5% (v/v) blood for facultative bacteria, and supplemented with 0.5% (w/v) yeast extract, hemin and vitamin K for anaerobic bacteria. The strains were grown under aerobic or anaerobic (10% H_2, 10% CO_2 and 80% N_2) conditions as appropriate.

2.3. Antibacterial assay

2.3.1. Agar well diffusion method

One hundred microliters of inoculum, equivalent to 10^7 CFU/mL, was mixed with 20 mL of warm melted BHA. The mixture was then poured into the plate with a 6 mm diameter metal cup. After solidifying of BHA, the metal cups were removed and the well was added with 100 μL of each plant extract. The plate was incubated at 37 °C for 24 h. A 10% DMSO were taken as a negative control. The antimicrobial activity of each plant extract was determined by measuring the diameter of the zone of the inhibition in millimeters. Duplicates were maintained and the experiment was repeated thrice.

2.3.2. Broth microdilution method

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were carried out as recommended instruction of the Clinical and Laboratory Standards Institute (CLSI). Briefly, 10% stock solution of each extract was diluted in the brain heart infusion broth (BHI) in two-fold serial dilutions to obtain concentrations from 0.02 to 25 mg/mL at a total volume of 100 μL per well in 96-well microtiter plates. Each tested strain (100 μL) at a final concentration of 1×10^6 CFU/mL was added to each well and incubated at 37 °C in appropriate conditions. The medium, 0.1% (w/v) CHX and 10% DMSO were used as the non-treated, positive and negative controls, respectively.

MIC was defined as the lowest concentration of the extract that completely inhibited growth in comparison with the non-treated control. MBC was defined as the lowest concentration of wells that did not allow visible growth when 10 μL of the well contents was plated on agar and grown at 37 °C in appropriate conditions. All experiments were repeated thrice in duplicate.

2.3.3. Time-kill assay

As the results of the screening of herb extracts revealed that *P. betle* leaves extract gave the strongest antibacterial activity. Thus, *P. betle* extract was used for the time-kill assay. The growing cultures (10^6 CFU/mL) of representative strains, *S. mutans* ATCC 25175 and *A. actinomycetemcomitans* ATCC 33384, were incubated in BHI broth supplemented with the extract at concentrations equivalent to 1 ×, 2 ×, and 4 × MIC at 37 °C. Surviving bacteria were observed at 0, 0.5, 1, 2, 4, 6, 8, 10, 12 and 24 h by culturing on agar plates. The procedure was repeated in triplicate, and the $\log_{10}$ CFU/mL was plotted against time. A 0.1% CHX and extract free medium were used as positive and non-treated controls, respectively.

2.4. Antibiofilm assay

2.4.1. Inhibition of biofilm formation

The effect of *P. betle* extract on biofilm formation of each representative strain, *S. mutans* ATCC 25175 and *A. actinomycetemcomitans* ATCC 33384, was examined by using the modified microdilution method.[16] Briefly, two-fold serial dilutions of *P. betle* extract were prepared, with final concentration ranged from 0.02 to 25 mg/mL. A cell suspension of the tested strains was prepared as described in the MIC assay, and 100 μL (1×10^6 CFU/mL) were inoculated in each of a 96-well plate. A 0.1% CHX, phosphate buffered saline (PBS) and extract

Table 1
Plants and susceptibility of oral microorganisms.

Scientific name	Voucher specimen no.	Used part	Antibacterial activity of microorganisms						
			Ef	*Lf*	*Ls*	*Ss*	*Sm*	*Aa*	*Fn*
Mentha cordifolia Opiz.	SKP 095130301	Leaf	++	+	+	−	−	+	+
Ocimum africanum L.	SKP 095150201	Aerial	+	+	+	−	−	.	+
Ocimum basilicum Lour.	SKP 095150101	Aerial	+	+	+	−	−	−	−
Ocimum sanctum L.	SKP 095151901	Aerial	+	+	+	−	−	−	−
Piper betle L.	SKP 146160201	Leaf	++	++	++	+++	+++	+++	+++
Piper chaba Hunter	SKP 146160301	Fruit	+	−	−	−	−	−	+
Piper nigrum L.	SKP 146161401	Fruit	+	−	−	−	−	−	+
Piper sarmentosum Roxb.	SKP 146161906	Leaf	+	−	−	−	−	−	++
Alpinia galanga (L.) Willd.	SKP 206010701	Rhizome	−	−	−	−	+	++	++
Curcuma longa L.	SKP 206031201	Rhizome	+	−	−	−	+	−	++
Curcuma zedoaria Roscoe	SKP 206032601	Rhizome	−	−	−	−	+	−	++
Zingiber officinale Roscoe	SKP 206261501	Rhizome	++	−	−	+	−	−	++

+, 0 < zone < 10 mm; ++, 10 ≤ zone < 20 mm; +++, 20 ≤ zone; −, absence of inhibition zone.