

Original Research Article

Screening for Anticandidal and Antibiofilm Activity of Some Herbs in Thailand

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Abstract

Purpose: To evaluate the anticandidal activity of the ethanol extracts of 12 herbs from Thailand.

Methods: The herbs studied were *Alpinia galanga*, *Curcuma longa*, *Curcuma zedoaria*, *Mentha cordifolia*, *Ocimum africanum*, *Ocimum basilicum*, *Ocimum sanctum*, *Piper betle*, *Piper chaba*, *Piper nigrum*, *Piper sarmentosum* and *Zingiber officinale*. Various *Candida* spp. were examined for minimal inhibitory concentration (MIC) and minimal fungicidal concentration (MFC) using microdilution method; time-kill assay was also used to assess the plants. Antibiofilm activity was investigated using a 3-[4, 5-dimethyl-2-thiazolyl]-2, 5-diphenyl-2H-tetrazolium-bromide (MTT assay). Gas chromatography mass spectrometry (GC-MS) analysis, thin layer chromatography (TLC) fingerprinting and TLC-bioautography were used to determine the active anticandidal compounds.

Results: All tested herbs, except extracts of *P. nigrum* and *Limiaceae* family, showed varying zones of inhibition against *Candida albicans* ATCC 90028. *P. betle* revealed the strongest anticandidal activity against all tested strains with MIC ranging from 1.56 to 3.13 mg/ml, and MFC from 3.13 to 8.33 mg/ml. Killing activity depended on time and concentrations of the extract. The concentration of *P. betle* extract required to inhibit $\geq 90\%$ biofilm formation of *C. albicans* ATCC 90028 was 3.13 ± 0.15 mg/ml, while that to remove $\geq 90\%$ biofilm growth was 12.50 ± 0.69 mg/ml. The result of GC-MS analysis showed the major compound of *P. betle* extract responsible for anticandidal activity as 4-chromanol.

Conclusion: *P. betle* extract contains 4-chromanol which is a good potential anticandidal agent for the treatment of oral infectious diseases caused by certain *Candida* spp.

Keywords: *Piper betle*, 4-Chromanol, Anticandida, Biofilm, Candidiasis, Herbs

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INTRODUCTION

The genus *Candida* contains more than 150 species, however, only a limited number of these species regularly cause diseases in humans [1]. *Candida* species belong to the normal microbiota of human mucosal surfaces including the oral cavity. There has been a significant increase in incidence of oral candidiasis due to higher numbers of immunocompromised patients, the

use of dental devices and the use of broad spectrum antifungal drugs. *Candida albicans* is the main cause of oral candidiasis, however, non-*C. albicans* (NCAC) species such as *Candida glabrata*, *Candida krusei* and *Candida tropicalis* are now frequently reported as human pathogens [2].

The pathogenesis of oral candidiasis is facilitated by a number of factors including the ability to

adhere to medical devices and/or host cells, and to form biofilm. Biofilm formation is an important virulence factor for a number of *Candida* species as it confers significant resistance to antifungal therapy by limiting their penetration through the matrix and protecting cells from host immune responses [3]. It has been demonstrated that biofilms formed by *C. albicans* and/or NCAC strains have been associated with high morbidity and mortality rates [4].

Chlorhexidine (CHX) has been used as an adjunctive therapeutic option for topical use owing to its broad-spectrum antimicrobial efficacy in oral candidiasis. However, it has been known to have side effects with prolonged use [5,6].

Natural products have increasingly received attentions due to their antimicrobial activity against a wide range of human pathogens. Few studies have reported anticandidal activity from various herbs [7,8]. In addition, the information on their active compounds is scarce. The aim of this study was to evaluate of the ethanolic extracts of 12 herbs, which are traditionally used in Thai folk medicine against various *Candida* spp. The phytochemical profile of the most effective extract was also investigated.

EXPERIMENTAL

Preparation of medicinal herb extracts

Twelve herbs included in this study were purchased from the local market, and their details are shown in Table 1. The herbs were identified by Dr. Supreeya Yuenyongsawad; 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. The dried plants were macerated with ethanol for 3 days and then filtered through Whatman no. 4 filter paper. The filtrate, each case, was concentrated using a rotary evaporator at 40 °C and kept at -20 °C. A 10 % (w/v) stock solution in 10 % dimethyl sulfoxide (DMSO) of each extract was used in this study.

Candida strains and growth conditions

A total of eleven *Candida* spp. were tested, and the details of strains used shown in Table 2. In this study, all tested strains were cultured on Sabouraud Dextrose Agar (SDA; Difco Laboratories, Detroit, MI, USA) or Sabouraud Dextrose broth (SDB; Difco Laboratories, Detroit, MI, USA) at 37 °C for 24 h.

Antifungal assay

Agar diffusion test

A 100 µl of inoculum (10^7 cfu/ml) was mixed with 20 ml of warm SDA, and poured into the plate with a 6 mm diameter metal cup. After solidifying the SDA, the metal cups were removed and the well filled with 100 µl of each extract. A 10 % DMSO was included as control. Inhibitory activity was determined by measuring the diameter of the inhibited zone. All tests were performed in triplicates.

Broth microdilution test

Minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) were determined using the broth microdilution method of the Clinical and Laboratory Standards Institute [9]. Each extract was subjected to a two-fold serial dilutions in the SDB to obtain concentrations from 0.02 mg/ml to 25 mg/ml at a total volume of 100 µl per well in 96-well plate, and 100 µl of each tested strain (1×10^3 cfu/ml) was added. The medium as well as 0.1 % CHX and 10 % DMSO were used as blank, positive control and non-treated control, respectively. MIC was defined as the lowest concentration of the extract that showed no detectable growth. MFC was defined as the lowest concentration of wells that did not grow when 10 µl of the well contents was plated on agar. All experiments were repeated thrice in duplicates.

Time-kill assay

As the screening of extracts revealed that *P. betle* extract gave the strongest anticandidal activity, it was used for the time-kill assay [10]. Growing culture (10^6 cfu/ml) of representative *C. albicans* ATCC 90028 was added to SDB and exposed to 1, 2 and 4 x the MIC of extract, and extract free medium was used as growth control. Samples were removed for colony counts at 0, 0.5, 1, 2, 4, 6, 8, and 24 h. The viable counts were determined using the serial dilution method after incubation. The procedure was repeated in triplicate, and the \log_{10} cfu/ml was plotted against time. A 0.1 % CHX was used as positive control. All assays were carried out in triplicates.

Antibiofilm assay

Inhibition of biofilm formation

The effect of *P. betle* extract on biofilm formation of representative *C. albicans* ATCC 90028 was examined using the modified microdilution method [11]. Two-fold serial dilutions of extract

and a cell suspension of the tested strain were prepared as described in the MIC assay. A 0.1 % CHX was used as the positive control. The phosphate buffered saline (PBS) at pH 7.0 and the medium alone were used as the non-treated and blank controls, respectively. After overnight incubation, supernatants were discarded and washed 3 times with PBS. Biofilm formation was quantified using a 3-[4, 5-dimethyl-2-thiazolyl]-2, 5-diphenyl-2H-tetrazolium-bromide (MTT; Sigma-Aldrich, St Louis, MO, USA) assay. The numbers of surviving *Candida* were determined by measuring their ability to reduce the yellow tetrazolium salt to a purple formazan product at OD 570 nm. Percentage inhibition was calculated by equation 1.

$$I\% = [1 - (A_{570} \text{ of the test} / A_{570} \text{ of non-treated control}) \times 100] \dots \dots \dots (1)$$

The biofilm inhibition concentration (MBIC₅₀ and MBIC₉₀) was defined as the concentrations that showed 50 % and 90 % inhibition of biofilm formation. All experiments were repeated in triplicate [11].

Eradication of biofilm formation

The eradication of biofilm was also examined using minimum biofilm eradication concentration (MBEC) assay. A 200 µl (10³ cfu/ml) aliquot of *C. albicans* ATCC 90028 was inoculated into 96-well plates to form biofilm overnight. The biofilm was washed 3 times with PBS. The biofilm was exposed to a 200 µl of various concentrations of extract ranged from 0.02 to 25 mg/ml. The number of surviving *Candida* were determined using MTT assay at OD 570 nm. Percentage eradication was calculated by equation 2.

$$E\% = [1 - (A_{570} \text{ of the test} / A_{570} \text{ of non-treated control}) \times 100] \dots \dots \dots (2)$$

MBEC value was defined as the concentrations that showed 50 and 90 % eradication of biofilm formation. The medium alone, 0.1 % CHX and PBS were used as the blank control, positive and non-treated, respectively. All experiments were repeated thrice in duplicate [11].

GC-MS analysis of phytoconstituents in *P. betle* extract

One microliter of *P. betle* extract was investigated for its phytoconstituents using gas chromatography-mass spectrometer (GC-MS, Trace GC Ultra, USA), the TR-5MS capillary column (0.25 mm x 30 m x 0.25 µm film thickness) was used for the separation of various phytoconstituents. The GC conditions were:

injection temperature, 250 °C; column temperature, isothermal at 70 °C for 2 min, then programmed to 305 °C at 10 °C/min and held at this temperature for 5 min. Helium gas with a flow rate of 1.0 ml/min was used as a carrier gas. The effluent of the GC column was introduced directly into the source of MS and spectra obtained in the electron ionization mode with 70 eV ionization energy, the sector mass analyzer was set to scan between 35-500 amu for 2 s. The identification of individual unknown compound is based on comparing its retention time relative to those of the known components by matching spectral peaks available with Wiley 9 Mass Spectral Library [12].

Thin layer chromatography (TLC) fingerprinting of *P. betle* and TLC-bioautography-assisted separation of anticandidal

For TLC-fingerprinting study, *P. betle* extract (100 mg) and pure eugenol (5 µl) were prepared in 1 ml of 95 % ethanol. A 10 µl of each sample was applied onto the alumina silica gel GF₂₅₄ TLC-plate, and was run through a series of solvent systems using a mixture of 90 volumes of toluene and 10 volume of ethyl acetate as the mobile phase. The plates were dried in the air, and were stained with a 20 % (w/v) of phosphomolybdic acid in ethanol [13].

TLC-bioautography was performed using agar overlay method. TLC-plates were placed on plate contained 6 ml of SDA, and 10 ml of top agar with 100 µl of inoculum (10⁷ cfu/ml) was covered on TLC plate. After overnight incubation, the inhibited zone was observed as clear zone. The relative front values (Rf) was calculated as: Rf = distance travelled by solute / distance travelled by solvent [14].

Statistical analysis

The data are expressed as mean ± standard deviation (SD) by using Microsoft Excel 2007 12.0.6611.1000 (Microsoft Corporation, Redmond, WA, USA) from the three experiments with duplicate independent experiments. The graph of time-kill analysis is plotted using SigmaPlot Version 11.0 (Systat Software, Inc., San Jose, CA, USA).

RESULTS

Anticandidal activity of 12 herbs against *C. albicans* was shown in Table 1. These extracts showed different inhibited zone against *C. albicans* ATCC 90028. The diameter of inhibited zone for all the extracts ranged from 1 to 20 mm,

except *P. nigrum* and extracts of Lamiaceae did not show any inhibitory zone. Two herbs *P. betle* and *C. zedoaria*, revealed the good anticandidal activity, and were further examined for their MIC and MFC against 11 *Candida* strains. *P. betle* exhibited a better anticandidal activity than *C. zedoaria* against all tested strains with the MICs ranged from 1.56 to 3.13 mg/ml and MFCs ranged from 3.13 to 8.33 mg/ml, respectively (Table 2).

Time kill assay of *P. betle* extract was performed for *C. albicans* ATCC 90028, it was depended on time and concentrations of extract. At 1 × MIC, 2 × MIC and 4 × MIC, the strain was killed after 8, 2 and 1 h, respectively. The killing of 0.1 % CHX was observed within 30 min (Figure 1). The concentrations of *P. betle* extract required to inhibit ≥ 50 % and ≥ 90 % biofilm formation of *C. albicans* ATCC 90028 were 0.78 ± 0.31 mg/ml

and 3.13 ± 0.15 mg/ml, respectively. The concentrations of *P. betle* extract to remove of ≥ 50 % and ≥ 90 % biofilm formation were 1.56 ± 0.44 mg/ml and 12.5 ± 0.69 mg/ml, respectively. CHX could completely inhibit or remove biofilm formation (Figure 2). The GC-MS analysis of *P. betle* extract showed 2 major chemical components of 4-chromanol (62.33 %) and eugenol (17.10 %), which their retention times were 10.46 and 9.11, respectively (Figure 3A). It was demonstrated that there were several bands of components appeared on the TLC-plate (Figure 3B), and one major band with R_f at 0.38 gave the large inhibited clear zone against tested *Candida* in TLC-bioautography assay (Figure 3C). Another minor band with R_f at 0.74 matched to the standard eugenol did not show any inhibited zone.

Table 1: Plants and susceptibility of *Candida albicans* ATCC 90028

Family	Scientific name	Voucher specimen no.	Used part	Anticandidal activity
Lamiaceae	<i>Mentha cordifolia</i> Opiz.	SKP 095130301	Leaf	-
	<i>Ocimum basilicum</i> L.	SKP 095150201	Aerial parts	-
	<i>Ocimum africanum</i> Lour.	SKP 095150101	Aerial parts	-
	<i>Ocimum sanctum</i> L.	SKP 095151901	Aerial parts	-
Piperaceae	<i>Piper betle</i> L.	SKP 146160201	Leaf	+++
	<i>Piper chaba</i> Hunter	SKP 146160301	Fruit	+
	<i>Piper nigrum</i> L.	SKP 146161401	Fruit	-
	<i>Piper sarmentosum</i> Roxb.	SKP 146161906	Leaf	+
Zingiberaceae	<i>Alpinia galanga</i> (L.) Willd.	SKP 206010701	Rhizome	+
	<i>Curcuma longa</i> L.	SKP 206031201	Rhizome	+
	<i>Curcuma zedoaria</i> Roscoe	SKP 206032601	Rhizome	++
	<i>Zingiber officinale</i> Roscoe	SKP 206261501	Rhizomes	+

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

Table 2: MIC and MFC of tested extracts against *Candida* spp

Strain	Concentration of extracts (mg/ml)			
	<i>Curcuma zedoaria</i>		<i>Piper betle</i>	
	MIC	MFC	MIC	MFC
<i>Candida albicans</i> ATCC 10231	2.6±0.9	>25	3.13±0	4.17±1.8
<i>Candida albicans</i> ATCC 90028	2.6±0.9	>25	3.13±0	3.13±0
<i>Candida dubliniensis</i> MYA-577	2.6±0.9	25±0	2.60±0.9	4.17±1.8
<i>Candida dubliniensis</i> MYA-646	2.6±0.9	25±0	2.08±0.9	3.13±0
<i>Candida glabrata</i> ATCC 66032	6.25±0	>25	3.13±0	6.25±0
<i>Candida glabrata</i> ATCC 90030	6.25±0	>25	2.60±0.9	5.21±1.8
<i>Candida krusei</i> ATCC 6258	8.33±3.6	>25	3.13±0	8.33±3.6
<i>Candida krusei</i> ATCC 34135	8.33±3.6	>25	3.13±0	6.25±0
<i>Candida tropicalis</i> ATCC 750	2.60±0.9	>25	2.60±0.9	4.17±1.8
<i>Candida tropicalis</i> ATCC 13803	2.60±0.9	>25	2.08±0.9	3.13±0
<i>Candida tropicalis</i> ATCC 66029	1.56±0	25±0	1.56±0	4.17±1.8

MIC – the minimum inhibitory concentration; MFC – the minimum fungicidal concentration; the values represent data obtained from three independent assays and where the appropriate mean ± S.D. is given

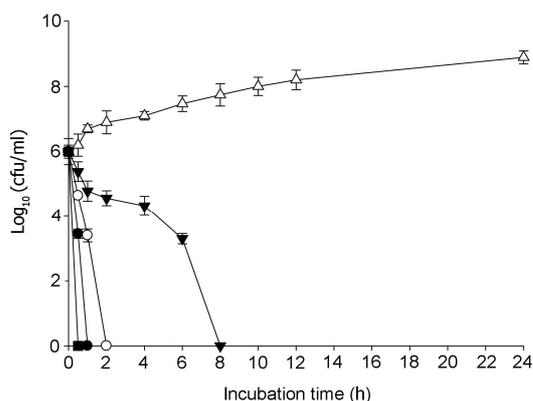


Figure 1: Time-kill curves of *C. albicans* ATCC 90028 at different concentrations of *P. betle* extract: 0 × MIC (Δ), 1 × MIC (\blacktriangledown), 2 × MIC (\circ) and 4 × MIC (\bullet); 0.1 % CHX (\blacksquare). Each symbol indicated the mean \pm SD

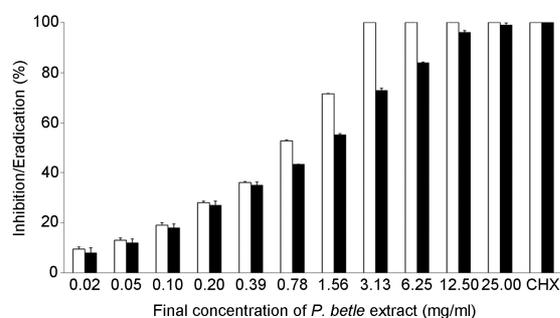


Figure 2: Inhibition (\square) and eradication (\blacksquare) of biofilm of *C. albicans* ATCC 90028 by *P. betle* extract at various concentrations; 0.1 % (w/v) CHX was used as control. Error bars indicate standard deviations; n = 6

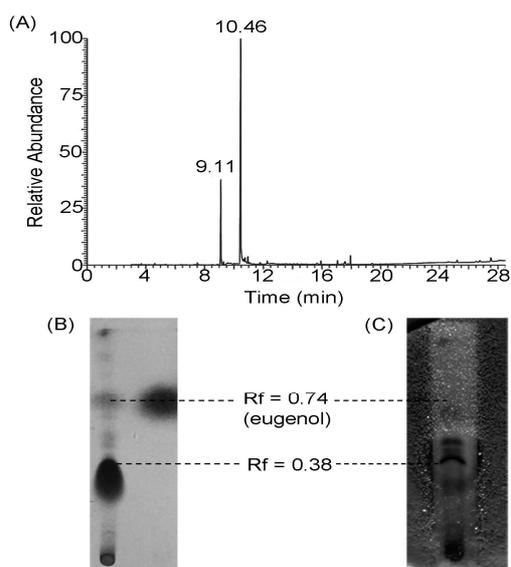


Figure 3: GC-MS chromatogram (A), TLC fingerprinting (B) and TLC-bioautography against *C. albicans* ATCC 90028 (C) of *P. betle* leaves extract

DISCUSSION

Candida species are the most common fungal pathogens of humans and are the causative agents at various locations in the body, giving rise to severe morbidity in millions of individuals worldwide [15]. In oral cavity, *C. albicans* is the organism most frequently implicated in a range of oral candidiasis like oropharyngeal candidiasis, oral thrush and denture stomatitis [15], and NCAC have been found dramatically in oral candidal infection. A topical use of CHX mouthwash is the most common antimicrobial substance to control oral candidiasis. However, CHX has been reported as having many unwanted effects including taste disturbance, desquamative lesions, and brown staining of the teeth and oral mucosa [5,6] and having limited use in the pregnant and the young children.

Medicinal herbs are widely employed in folk medicine, mainly in communities with low hygiene and poor sanitation. Plants products are considered to have low side effects compared with synthetic ones. Some studies have demonstrated that herbs possess the anticandidal activity against various *Candida* strains [7,8]. In this study, 7 of 12 Thai herbs, *A. galanga*, *C. longa*, *C. zedoaria*, *P. betle*, *P. chaba*, *P. sarmentosum* and *Z. officinale* showed potential anticandidal activity against tested *C. albicans*. However, *P. nigrum* and plant extracts in family of Lamiaceae did not any activity possible because their major potent compounds are volatile oil which could have evaporated during preparation of extracts by a rotary evaporator. Among those plants tested, *P. betle* extract exhibited the strongest anticandidal activity against tested *Candida* species.

In addition, *P. betle* extract has shown an antibiofilm activity. Biofilm of *C. albicans* and NCAC are associated with high incidence of hospital morbidity and mortality [4] due to the increased use of medical devices such as lens, implants and dentures. Such biomaterials facilitate *Candida* strains to colonize and form biofilm leading to the development of fungal resistance to antifungal agents such as amphotericin B, ketoconazole and fluconazole [16]. Experiments comparing biofilm of *Candida* in this study have demonstrated that higher concentrations of MBEC₉₀ of *P. betle* extract (4 folds) than of MBIC₉₀ are required to significantly remove existing biofilm cells. This is an expected result since *Candida* in the biofilm is strongly protected and less susceptible to antifungal

agents than in planktonic form [17]. It was shown that *P. betle* extract was able to remove *Candida* in the dose and time dependent manner. Our results indicated that *P. betle* extract acts as a potent antibiofilm agent that has dual actions of preventing biofilm formation and removing existing biofilm.

P. betle has been extensively used in traditional medicines in many countries. It was reported to have various pharmacological activities such as antifungal, antimicrobial, immunomodulatory and anti-inflammatory [18].

The antimicrobial activity has been previously shown to be a broad spectrum of both bacteriostatic and fungistatic effects against *C. albicans*, *Pseudomonas aeruginosa*, *Aspergillus flavus* and *Escherichia coli* [19,20]. Some studies reported an anticandidal effect of *P. betle* on a wide range of *Candida* spp. [7,20]. Our results are in concordance with the study of Himratul-Aznita *et al* [20] who demonstrated the anticandidal activity of aqueous crude extract of *P. betle* leaves against 7 oral *Candida* species. However, there was no information of antibiofilm or active compounds mentioned in that study. The ethanol extract in this study revealed the lower MICs and MFCs of anticandidal activity compared to the aqueous extract [20]. This was also found in a study of the ethanol extract of *P. betle* leaves with stronger antibacterial activity compared with aqueous extract [21]. It indicates that the ethanol may be the appropriate solvent for *P. betle* leaves extraction.

The results of the GC-MS analysis, TLC fingerprinting and TLC-bioautography in the present study indicated that 4-chromanol could be the active compound responsible for the anticandidal activity. There were only 2 major peaks found in the GC-MS analysis, and the one with the lower peak matched to the eugenol by the TLC fingerprinting. Eugenol did not exhibit any anticandidal activity in TLC-bioautography assay. Therefore, the major band found on the TLC fingerprinting with Rf of 0.38 showing a large inhibited zone against *Candida* strain should be the highest peak of 4-chromanol found in GC-MS analysis.

A number of phytochemical constituents of *P. betle* extract have been documented in some studies [18,22]; however, 4-chromanol has not been reported. The finding of the phytochemical profile of *P. betle* extract in this study is in agreement with Deshpande and Kadam [21], in which the extract revealed the presence of 4-

chromanol. However, the presence of 4-chromanol was a major component in this study (62.33 %), which was higher than earlier reported that was higher than their report. The finding of chemotypic variations in this study and others may be due to ecological and geographical conditions, age of herb and time of harvesting. The apparent variation in the chemical profile may influence their anticandidal activity.

CONCLUSION

The current study supports the traditional uses of the studied plant and suggests that the *P. betle* leaves extract is a potential source of natural anticandidal agent. It possesses 4-chromanol with good anticandidal properties that may be used for oral infectious diseases caused by certain *Candida* spp. Further work should be performed to determine its mode of action as well as *in vivo* activity.

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