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Original article

Antifungal activities of extracts from Thai medicinal plants against opportunistic fungal pathogens associated with AIDS patients

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Summary

In this study, 36 extracts derived from 10 plant species were selected to screen for their antifungal activity against clinical isolates of *Candida albicans*, *Cryptococcus neoformans* and *Microsporum gypseum*. Selection was based on their use by traditional Thai healers or their reported antimicrobial activities in an attempt to find bioactive medicines for use in the treatment of opportunistic fungal infections in AIDS patients. The disc diffusion and hyphal extension–inhibition assays were primarily used to test for inhibition of growth. Minimum inhibitory concentration was determined by dilution methods. The chloroform extracts of *Alpinia galanga* and *Boesenbergia pandurata* had pronounced antifungal activity against *C. neoformans* and *M. gypseum*, but exhibited weak activity against *C. albicans*. *Alpinia galanga* and *B. pandurata* are excellent candidates for the development of a remedy for opportunistic fungal infections in AIDS patients.

Key words: AIDS, *Alpinia galanga*, antifungal, *Boesenbergia pandurata*, *Candida albicans*, *Cryptococcus neoformans*, *Microsporum gypseum*.

Introduction

Opportunistic fungal infections are increasing as a consequence of the unprecedented increase in numbers of immunocompromised patients from various areas of the health care system. The situation has become even more alarming with the current pandemic of AIDS. The commonly encountered fungal infections in HIV patients are candidiasis, cryptococcosis and histoplasmosis. In Thailand, cryptococcosis and candidiasis are among the top five opportunistic infections in AIDS patients.[1] *Microsporum gypseum* is a geophilic fungus with worldwide distribution. It generally causes infections of the skin and scalp.[2] Although it is not common, it can cause disseminated infections in AIDS patients.[3–5]

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Approximately 46.6% of AIDS cases in Thailand have been labourers with low income.[1] Thus, Thai HIV/AIDS patients tend to seek remedies to relieve their AIDS symptoms and opportunistic infections using traditional and cheaper medicines. Therefore, we investigated the antifungal activities of plant extracts used for self-medication by AIDS patients in southern Thailand. Selection of plants was based on their use by traditional healers and/or on previously reported antimicrobial activities.[6–16]

Materials and methods

Plant materials and extract preparation

Plants used in this study with their reported biological activities are shown in Table 1. Plant materials were collected in Songkhla Province, Thailand and deposited at the Herbarium of the Faculty of Pharmaceutical Sciences, Prince of Songkla University. The extraction procedures had been described previously by Tewtrakul *et al.* [17]. Briefly, dried plants were successively extracted with chloroform, methanol and boiling water.

Table 1 Plants used in this study.

Botanical name (voucher number)	Family	Parts used	References
Antifungal			
Alpinia galanga (L.) Willd. (SN4412030)	Zingiberaceae	Rhizome	6–9
Boesenbergia pandurata (Roxb.) Schltr. (SN4412015)	Zingiberaceae	Rhizome	7, 10
Eclipta prostrata (L.) L. (SN4412025)	Compositae	Whole plant	11
Murraya paniculata (L.) Jack (SN4412040)	Rutaceae	Leaf	12
Piper betle L. (SN4412035)	Piperaceae	Leaf	7, 13
Antibacterial			
Piper chaba Hunter (SN4412020)	Piperaceae	Fruit	7
Spilanthes acmella (L.) Murray (SN4412045)	Compositae	Whole plant	14
Zingiber zerumbet (L.) Roscoe ex Sm. (SN4412010)	Zingiberaceae	Rhizome	7
Treatment of skin diseases/infected wound			
Acanthus ebracteatus Vahl.(SN4501010)	Acanthaceae	Leaf, stem	15
Coccinia grandis (L.) Voigt (SN4412050)	Cucurbitaceae	Leaf	16

The solvents were removed under reduced pressure. Each dried extract was dissolved at a concentration of 100 mg ml^{-1} in dimethyl sulphoxide (DMSO; Merck, Darmstadt, Germany) and kept at −20 °C before assay.

Fungal strains and inoculum preparation

Two clinical isolates of *Candida albicans* were from the Dental Hospital, Faculty of Dentistry, Prince of Songkla University (Songkhla, Thailand). Clinical isolates of *Cryptococcus neoformans* and *Microsporum gypseum* were obtained from Siriraj Hospital, Faculty of Medicine, Mahidol University (Bangkok, Thailand). *Candida albicans* NCPF3153 was used as a control strain. Fungi were cultured on Sabouraud dextrose agar (Becton Dickinson and Company, Sparks, MD, USA) at 35 °C (*C. albicans* and *C. neoformans*) and 25 °C (*M. gypseum*) and are maintained at Prince of Songkla University. All transfers of fungal strains were carried out under biological safety cabinet (Microflow, North Summerset, UK). Inocula of yeast were prepared in Sabouraud dextrose broth (Becton Dickinson and Company) and adjusted to 0.5 McFarland turbidity. Inoculum of *M. gypseum* was prepared in Sabouraud dextrose broth and adjusted to 4×10^3 conidia ml^{-1}.

Assay for antifungal activity

Yeast inoculum was spread onto Sabouraud dextrose agar plate with a sterile cotton swab. Three sterile filter paper discs, 6 mm in diameter (Schleicher and Schuell, Dassel, Germany), were impregnated with 10 μl (1 mg) of extract solution, allowed to air dry and placed on the inoculated agar surface. A negative control consisting of 10 μl DMSO and a positive control consisting of 10 μg amphotericin B (E. R. Squibb & Sons, Princeton, NJ, USA) were also prepared. Plates were incubated at 35 °C for 24 h (*C. albicans*) and 48 h (*C. neoformans*) at which time the diameter of the inhibition zone was recorded. An extract was classified as having antifungal activity when the diameter of the inhibition zone was ≥6.5 mm, 0.5 mm larger than the diameter of the paper disc.

For the antifungal activity against *M. gypseum*, the hyphal extension–inhibition assay was used.[18] Briefly, discs impregnated with 10 μl (1 mg) of extract solution were placed on the Sabouraud dextrose agar surface in front of the advancing fungal colony. The plates were incubated at 25 °C for 3–7 days. Dimethyl sulphoxide 10 μl and miconazole 30 μg (Sigma, St Louis, MO, USA) were used as negative and positive controls respectively. The antifungal activity was indicated by a crescent-shaped zone of growth inhibition around the disc.

Determination of minimum inhibitory concentrations

A modified agar microdilution method was used to determine the minimum inhibitory concentrations (MIC) of the plant extracts that produced inhibition zones against *C. albicans* and *C. neoformans*.[19] Extract solutions were mixed with melted Sabouraud dextrose agar in the ratio of 1 : 10 to give the final concentrations of 0.5–512 μg ml^{-1}. One hundred microlitres of each concentration was dropped into each microtitre well (Nunc, France). One microlitre of yeast suspension containing approximately 10^4 CFU was then dropped onto the surface of the agar. DMSO was used as negative control and amphotericin B was used as the standard antifungal drug. Plates were incubated at 35 °C for 24 h (*C. albicans*) and 48 h (*C. neoformans*). MICs were recorded by reading the lowest plant extract concentration that showed no visible growth.

A modification of the NCCLS M38-A broth microdilution test was performed against *M. gypseum*.[20] Equal volumes of a suspension of conidia (approximately 4×10^3 conidia ml^{-1}) were added to each test dilution to make a final concentrations of 1–512 $\mu g\ ml^{-1}$ in triplicate. Plates were incubated at 25 °C for 72 h. DMSO was used as negative control and miconazole was used as a standard antifungal agent. The MICs were recorded for the lowest concentration that resulted in a reduction approximately 50% of the fungal growth.

Results

The results of the antifungal screening of chloroform, methanol and aqueous extracts of 10 plant species are given in Table 2. Chloroform extracts from *Alpinia galanga*, *Boesenbergia pandurata* and *Piper betle* containing 1 mg of extract had antifungal activity against all the fungi tested. Chloroform extracts from *A. galanga*, *B. pandurata*, *P. betle* and *Zingiber zerumbet* and methanol extract from *B. pandurata* produced inhibition zones against *C. albicans* that ranged from 8.1 to 10.4 mm compared with zones of 15.6–16.6 mm with 10 μg amphotericin B. *Cryptococcus neoformans* was more

Table 2 Antifungal activity of chloroform, methanol and aqueous extracts of medicinal plant species (concentration 1 mg $disc^{-1}$).

Botanical name	Extraction solvent	Mean diameter[1] (mm) of inhibition zone of fungal strains[2]				Inhibition
		Ca3153	Ca43	Ca48	Cn	Mg
Acanthus ebracteatus	Chloroform					
	Methanol					
	Water					
Alpinia galanga	Chloroform	10.4	10.3	9.6	27.5	+
	Methanol					
	Water					
Boesenbergia pandurata	Chloroform	8.4	8.2	8.3	9.3	+
	Methanol	8.1	8.6	8.2	9.5	ND[3]
	Water					
Coccinia grandis	Chloroform					
	Methanol					
	Water					
Eclipta prostrata	Chloroform				9.8	+
	Methanol					
	Water					
Murraya paniculata	Chloroform				19.7	+
	Methanol					
	Water					+
Piper betle	Chloroform	10.3	8.7	9.2	17.4	+
	Methanol					
	Water					
Piper chaba	Chloroform				8.3	+
	Methanol					
	Water					
Spilanthes acmella	Chloroform				7.5	+
	Methanol					
	Water					
Zingiber zerumbet	Chloroform	9.8	9.5		8.6	
	Methanol					
	Water					
Amphotericin B (10 μg $disc^{-1}$)		16.5	16.6	15.6	21.6	
Miconazole (30 μg $disc^{-1}$)						+

[1] $n = 3$, minimum diameter possible = 6 mm. Standard error was <15% of mean in all cases.

[2] Ca, *Candida albicans*; Cn, *Cryptococcus neoformans*; Mg, *Microsporum gypseum*.

[3] ND, not done.

Table 3 Minimum inhibitory concentration (MIC) of crude medicinal plant extracts against *Candida albicans* (Ca), *Cryptococcus neoformans* (Cn) and *Microsporum gypseum* (Mg).

Medicinal plants	Extraction solvent	MIC (μg ml^{-1})				
		Ca3153	Ca43	Ca48	Cn	Mg
Alpinia galanga	Chloroform	>512	>512	>512	128	16
Boesenbergia pandurata	Chloroform	>512	>512	>512	64	64
Boesenbergia pandurata	Methanol	>512	>512	>512	128	ND
Eclipta prostrata	Chloroform	ND	ND	ND	128	256
Murraya paniculata	Chloroform	ND	ND	ND	256	512
Murraya paniculata	Water	ND	ND	ND	ND	>512
Piper betle	Chloroform	>512	>512	>512	128	128
Piper chaba	Chloroform	ND	ND	ND	128	512
Spilanthes acmella	Chloroform	ND	ND	ND	128	256
Zingiber zerumbet	Chloroform	>512	>512	>512	128	ND
Amphotericin B		0.06	0.06	0.06	0.06	
Miconazole						4

ND, not done because there was no activity by disc diffusion method.

susceptible to the plant extracts than was *C. albicans. Cryptococcus neoformans* was inhibited by chloroform extracts of *A. galanga*, *B. pandurata*, *Eclipta prostrata*, *Murraya paniculata*, *P. betle*, *Piper chaba*, *Spilanthes acmella* and *Z. zerumbet* and methanol extract of *B. pandurata* with zones of inhibition that ranged from 7.5 to 27.5 mm and the maximum zone was obtained with the chloroform extract of *A. galanga*. Chloroform extracts of *A. galanga*, *B. pandurata*, *E. prostrata*, *M. paniculata*, *P. betle*, *P. chaba* and *S. acmella* exhibited antifungal activity against *M. gypseum*.

The MIC values of extracts with significant effects against *C. albicans*, *C. neoformans* and *M. gypseum* are presented in Table 3. Although chloroform extracts of *A. galanga*, *B. pandurata*, *P. betle* and *Z. zerumbet* and methanol extract of *B. pandurata* exhibited inhibition zones against *C. albicans* using the disc diffusion assay, in the MIC assays none of the extracts inhibited the growth of this fungus at the highest concentration tested (512 μg ml^{-1}). The MICs of the extracts against *C. neoformans* ranged from 64 to 256 μg ml^{-1}. A chloroform extract of *B. pandurata* was the most active with an MIC value of 64 μg ml^{-1}. The MICs of the extracts against *C. neoformans* did not correlate to the inhibition zones and this might have a consequence on the solubility of the active components present in the extracts. Among the medicinal plants tested, a chloroform extract of *A. galanga* showed very strong activity against *M. gypseum* with an MIC value of 16 μg ml^{-1} which was only four times higher than that of miconazole at 4 μg ml^{-1}.

Discussion

Due to the increasing development of drug resistance in human pathogens as well as the appearance of undesirable effect of certain antifungal agents, there is a need to search for new agents. In this study, we evaluated the antifungal activity, of extracts from selected medicinal plants, used in a primary health care project by AIDS patients in southern Thailand. Their traditional uses suggest that these plants are favoured by low overall toxicity to humans. From 10 plant species, extracts from three species inhibited growth of all fungal strains, four species inhibited only *C. neoformans* and *M. gypseum* and one species inhibited *C. albicans* and *C. neoformans*. It is noted that most of the chloroform extracts showed significant antifungal activity. This finding indicates that the active antifungal compounds in these plants are most likely to be non-polar. In traditional Thai medicine, liquid extracts are most often made by boiling or infusion or alcohol maceration.[15] These preparations are more suitable for polar compounds but in those cases the plant materials used have not been previously extracted with chloroform. However in order to get the most benefit from these plants, methods of preparation that involve purification from chloroform extracts should be devised.

Among these active extracts, only chloroform extracts from *A. galanga* and *B. pandurata* rhizomes were shown to have pronounced antifungal activities towards *C. neoformans* and *M. gypseum* with MIC values of 16–128 μg ml^{-1}. *Alpinia galanga* and *B. pandurata* are from the same Zingiberaceae family and are commonly used in traditional medicine. Previous work on antimicrobial activities of these two plants has been reported.[6–10] It is well documented that 1′-acetoxychavicol acetate isolated from *A. galanga* has antifungal and antimycobacterial activities.[6, 21] Jantan *et al.* [10] reported that the oil of *B. pandurata* rhizomes was effective against dermatophytes, filamentous fungi and yeast-like fungi including *C. albicans* and *C. neoformans*. The essential oil of *B. pandurata* contained high levels of camphor, geraniol, 1,8-cineole, methyl cinnamate and camphene. Tuchinda *et al.* [22] isolated panduratin A, sakuranetin, pinostrobin, pinocembrin and dihydro-5,6-dehydrokawain from chloroform extract of *B. pandurata* and found that these compounds are responsible for the anti-inflammatory effect. Pinocembrin has also been found in the bee product propolis. It has been shown to have very strong antimicrobial activity.[23] Lopéz *et al.* [24] reported that pinocembrin chalcone from *Piper lanceaefolium* inhibited the growth of *C. albicans* with an MIC value of 100 $\mu g\ ml^{-1}$. However, Metzner & Schneidewind [25] tested the effect of pinocembrin on mice infected with *Candida* and reported that it did not protect the infected mice. Thus, the antifungal bioactive components present in the chloroform extract from *B. pandurata* need to be further characterised. In addition, the chloroform and methanol extracts of *B. pandurata* and the methanol extract of *A. galanga* are reported to have HIV-1 protease inhibitory activity.[17] Our findings confirm the antifungal properties of *A. galanga* and *B. pandurata* against *C. neoformans*, and *M. gypseum*. *Alpinia galanga* and *B. pandurata* are therefore potential candidates for treatment of opportunistic fungal infections in AIDS patients.

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