ภาคผนวก 9 : ผลงานวิจัยตีพิมพ์ เรื่อง The anti-amoebic activity of some medicinal plants used by AIDS patients in southern Thailand

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ORIGINAL PAPER

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The anti-amoebic activity of some medicinal plants used by AIDS patients in southern Thailand

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Abstract The anti-amoebic activities of chloroform, methanol and water extracts from 12 Thai medicinal plants (39 extracts) commonly used by AIDS patients in southern Thailand were screened, at a concentration of 1,000 μg/ml, against *Entamoeba histolytica* strain HTH-56:MUTM and strain HM1:IMSS growing in vitro. The extracts were incubated with 2×10^5 *E. histolytica* trophozoites/ml of medium at 37°C under anaerobic conditions for 24 h. The cultures were examined with an inverted microscope and scored (1–4) according to the appearance and numbers of the trophozoites. The extracts that caused inhibition were selected and retested using the same conditions but with concentrations that ranged from 31.25 to 1,000 μg/ml using *E. histolytica* strain HM1:IMSS, and the IC_{50} values for each extract were calculated. The chloroform extracts from *Alpinia galanga* (IC_{50} 55.2 μg/ml), *Barleria lupulina* (IC_{50} 78.5 μg/ml), *Boesenbergia pandurata* (IC_{50} 45.8 μg/ml), *Piper betle* (IC_{50} 91.1 μg/ml) and *Piper chaba* (IC_{50} 71.4 μg/ml) and the methanol extract from *B. pandurata* (IC_{50} 57.6 μg/ml) were all classified as "active", i.e. with an IC_{50} of less than 100 μg/ml, whereas those from *Murraya paniculata* (IC_{50} 116.5 μg/ml) and *Zingiber zerumbet* (IC_{50} 196.9 μg/ml) were classified as being "moderately active". The IC_{50} of a standard drug, metronidazole, was 1.1 μg/ml.

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Introduction

Amoebiasis is an increasingly important parasitic disease among patients with HIV infection regardless of whether they have AIDS. Although HIV/AIDS patients are not especially prone to infection with *Entamoeba histolytica*, it has been suggested that they are more susceptible to an invasive form of the disease than are normal patients (Fätkenheuer et al. 1997; Hung et al. 1999; Liu et al. 2001). Infection with *E. histolytica* has also been reported to be an important cause of acute and chronic diarrhoea in HIV patients (Waywa et al. 2001; Joshi et al. 2002; Arenas-Pinto et al. 2003).

The most commonly prescribed drug for treating intestinal protozoan infections, including the invasive form of *E. histolytica*, is metronidazole. However, metronidazole can have undesirable side-effects, and failures in treatment have been reported (Llibre et al. 1989; Johnson 1993; Voolmann and Boreham 1993; Tracy and Webster 1996; Lemee et al. 2000). These problems lead our team to search for an alternative drug that could be suitable for use in preventing and treating *E. histolytica* infections in HIV-positive patients.

The use of medicinal plants by people in developing countries is popular because these products are safe, widely available at low cost and easy to access. We therefore evaluated the in vitro activity of selected medicinal plants, used in a primary health care project by AIDS patients in southern Thailand, against *E. histolytica*. Furthermore, in previous studies, we found that some of these plants are active against growth of *Giardia intestinalis* in vitro (Sawangjaroen et al. 2005). Although the in vitro assays may not be related to direct in vivo activities in some studies (Ghoshal et al. 1996), it still an important approach to activity screening, which may provide a firm basis for improving basic community health care to the population.

Materials and methods

Test organisms

E. histolytica strain HTH-56:MUTM, originally described by Thammapalerd et al. (1993), and strain HM1:IMSS used in this experiment were gifts from Assoc. Prof. Nittaya Thammapalerd and Asst. Prof. Chutathip Siriphant, Mahidol University, Bangkok, respectively. They were cultured axenically in screw-capped tubes at 37°C, under anaerobic conditions, on YI medium (Diamond et al. 1995) supplemented with 10% heat-inactivated calf bovine donor serum. Subculture was performed every 48 h. For the assays, cells were harvested by chilling the tube on ice for 15 min to detach the monolayer and then centrifuged at 300×*g* for 5 min. The supernatant was decanted, and cells were resuspended in fresh medium. The numbers of viable cells were calculated using a haemocytometer and 0.4% (w/v) trypan blue. The criteria for viability were motility and dye exclusion.

Preparation of plant extracts

The preparations of plant materials used in this experiment were described by Tewtrakul et al. (2003). Briefly, the plants were collected in the area of Songkhla Province, Thailand, with voucher specimens deposited at the herbarium of the Faculty of Pharmaceutical Sciences, Prince of Songkla University, Thailand. Dried plants were successively extracted with chloroform, methanol and boiling water. The solvents were removed under reduced pressure. Each dried extract was dissolved, at a concentration of 100 mg/ml, in dimethyl sulphoxide (DMSO) before assay. The extracts were further diluted with culture medium to a concentration of 2 mg/ml. The maximum concentration of DMSO in the test did not exceed 1%, at which level no inhibition of amoeba growth occurred.

Anti-amoebic activity of medicinal plants

For screening, *E. histolytica* strain HTH-56:MUTM and strain HM1:IMSS were used. *E. histolytica* trophozoites, 2×10^5 cells/ml, in triplicate, were incubated with each plant extract, at a concentration of 1,000 μg/ml, in 96-well tissue culture plates (200 μl/well). Metronidazole and complete medium with added DMSO were used as negative and positive controls, respectively. After 24 h of incubation at 37°C under anaerobic conditions, the trophozoites from each well were examined and counted with an inverted microscope. The appearance and numbers of trophozoites were scored and presented as score values from 1 to 4, with 1 showing the most inhibition of growth and 4 showing no inhibition, according to Upcroft and Upcroft (2001).

Plant extracts that were active against both strains of *E. histolytica* (more than 90% of the trophozoites rounded up), at a concentration of 1,000 μg/ml, were selected and retested against *E. histolytica* strain HM1:IMSS for determination of their IC_{50}. Briefly, *E. histolytica* trophozoites, 2×10^5 cells/ml, were incubated with the same conditions as used for screening but in the presence of serial twofold dilutions of plant extracts that ranged from 31.25 to 1,000 μg/ml. After 24-h incubation, the plates were chilled for 15 min to detach the trophozoites. The numbers of viable cells from every well were counted twice, using trypan blue and a haemocytometer. The results were calculated as the percentage of growth inhibition when compared with the controls grown without plant extracts. The plot of the probit value against log of the plant extract concentration was

Table 1 Scores for *Entamoeba histolytica* (strains HTH-56:MUTM and HM1:IMSS) growing in vitro after incubation with plant extracts (1,000 μg/ml) for 24 h

Botanical name	Family	Part used	Score[a]		
			$CHCl_3$	MeOH	H_2O
Acanthus ebracteatus Vahl	Acanthaceae	Leaf, stem	4	4	4
Alpinia galanga (L.) Willd.	Zingiberaceae	Rhizome	1[b]	4	4
Barleria lupulina Lindl.	Acanthaceae	Leaf	4	3	4
		Stem	1[b]	4	3
Boesenbergia pandurata (Roxb.) Schltr.	Zingiberaceae	Rhizome	1[b]	1[b]	4
Coccinia grandis (L.) Voigt	Cucurbitaceae	Leaf	4	4	4
Eclipta prostrata (L.) L.	Asteraceae	Whole plant	3	4	4
Gynura pseudochina (L.) DC.	Asteraceae	Leaf	4	4	4
Murraya paniculata (L.) Jack	Rutaceae	Leaf	1[b]	3	4
Piper betle L.	Piperaceae	Leaf	1[b]	4	4
Piper chaba Hunter	Piperaceae	Fruit	1[b]	3	4
Spilanthes acmella (L.) Murray	Asteraceae	Whole plant	3	3	4
Zingiber zerumbet (L.) Roscoe ex Sm.	Zingiberaceae	Rhizome	1[b]	4	4

[a]Scores were graded using the following criteria (Upcroft and Upcroft 2001): 1=dead or significantly fewer cells than the control well (not more than 20% coverage of well surface) or more than 90% of the trophozoites rounded up; 2=20–50% coverage of the well surface and some parasite motility; 3=an almost confluent well, much motility; 4=a confluent well

[b]The extract was considered active at a concentration of 1,000 μg/ml when all three test wells graded 1

Table 2 Anti-amoebic activity (IC_{50}) of plant extracts on *E. histolytica* (HM1:IMSS) growing in vitro after 24-h incubation

Plants	Extraction solvent	Anti-amoebic activities (IC_{50}, μg/ml)	
		Average[a]	Standard deviation
Alpinia galanga	Chloroform	55.2[b]	0.8
Barleria lupulina	Chloroform	78.5[b]	0.8
Boesenbergia pandurata	Chloroform	45.8[b]	1.4
	Methanol	57.6[b]	1.2
Murraya paniculata	Chloroform	116.5[c]	3.5
Piper betle	Chloroform	91.1[b]	12.0
Piper chaba	Chloroform	71.4[b]	6.1
Zingiber zerumbet	Chloroform	196.9[c]	37.0
Metronidazole		1.1[d]	0.1

[a]Mean values obtained from duplicate samples obtained on at least two separate occasions
[b]$20<IC_{50}\leq100$ μg/ml=active
[c]$100<IC_{50}\leq250$ μg/ml=moderately active
[d]$IC_{50}<20$ μg/ml=highly active

made. The best straight line was determined by regression analysis, and the concentrations that caused 50% inhibition (IC_{50}) were calculated. Each concentration was tested in duplicate, and at least two experiments were performed on separate occasions.

Results

The results of the testing of plant extracts against *E. histolytica* trophozoites, including botanical names and parts of the plants used, are presented in Table 1. Chloroform extracts from *Alpinia galanga*, *Barleria lupulina*, *Boesenbergia pandurata*, *Murraya paniculata*, *Piper betle*, *Piper chaba* and *Zingiber zerumbet* and methanol extracts from *B. pandurata* at a concentration of 1,000 μg/ml produced good inhibition; the remaining extracts showed no activity.

The calculated IC_{50} values of the eight extracts, which proved to be active against the in vitro growth of *E. histolytica*, are shown in Table 2. As the criteria used for determining the degree of the anti-amoebic effects seem to vary between different research groups, we used the slightly modified criteria from Tona et al. (1998), as follows:

- $IC_{50}<20$ μg/ml=highly active
- $20<IC_{50}\leq100$ μg/ml=active
- $100<IC_{50}\leq250$ μg/ml=moderately active
- $250<IC_{50}\leq500$ μg/ml=weekly active
- $IC_{50}\geq500$ μg/ml=inactive

Discussion

In our search for natural compounds aimed for use as anti-amoebic agents for HIV/AIDS patients, we tested 12 medicinal plants (39 extracts) commonly used by AIDS patients in southern Thailand. We found that at least seven plants exhibited good anti-amoebic activity with amoeba growing in vitro. Among the seven active plant extracts, three of them (*B. pandurata*, *P. chaba* and *Z. zerumbet*) are prescribed in cases of mucous bloody stools and dysentery in Thai traditional medicine (Farnsworth and Bunyapraphatsara 1992). This condition may be due to infection with *E. histolytica*. Our finding confirms the traditional therapeutic claims for these herbs. Little is known about the nature of the compounds with anti-amoebic activity from the plants investigated. Furthermore, these plant extracts were also active against another intestinal protozoan parasite pathogenic to humans, *G. intestinalis* growing in vitro (Sawangjaroen et al. 2005). To obtain information on the type of compounds that could be responsible for the anti-amoebic activities, we reviewed the articles on active plants for the relevance of this finding.

The leaves of *B. lupulina* are normally used to prevent inflammation in Thai traditional medicine. Yoosook et al. (1999) demonstrated an effect of a methanol extract against herpes simplex virus, growing in vitro. However, in this experiment, only the stem of *B. lupulina* shows anti-amoebic activity.

The fresh rhizome of *B. pandurata*, currently known as *B. rotunda* (Larsen 1996) or "Kra-chai" in Thai, is an edible rhizome that is commonly used to treat colic disorders and used against inflammation. Panduratin A, sakuranetin, pinostrobin, pinocembrin and dihydro-5,6-dehydrokawain, isolated from chloroform extracts of this rhizome, are responsible for the anti-inflammatory effect, according to Tuchinda et al. (2002). The antimutagenic effect (Trakoontivakorn et al. 2001) as well as the hepatocarcinogenic effect (Tiwawech et al. 2000) of several agents extracted from this rhizome was also reported. In addition, its chloroform extract was shown to have potent HIV-1 protease inhibitory activity (Tewtrakul et al. 2003). Whether or not the anti-amoebic activities stem from these compounds still needs to be researched.

A. galanga or *Languas galanga* ("Khaa" in Thai) is commonly used as a flavouring agent in various Thai dishes. It may contain agents that augment the hepatocarcinogenicity effect (Tiwawech et al. 2000). *A. galanga* and *Z. zerumbet* are in the same Zingiberaceae family as *B. pandurata*. It is possible that the essential oils, responsible for the characteristic odour as well as for their reported use in (folk) medicine, are responsible for their anti-amoebic activity.

P. betle is a tropical plant and its leaves are often chewed in Thailand and many other Southeast Asian countries to prevent malodour. It was found that hydroxychavicol, a major phenolic compound in *P. betle* leaves, is related to the incidence of oral submucous fibrosis (Jeng et al. 2004). The anti-adherence effect on early plaque settlers (Razak and Rahim 2003) and the hepatoprotective and antioxidant effect of aqueous extracts of *P. betle* were also reported (Saravanan et al. 2002, 2003). Allylpyrocatechol from *P.*

betle leaves, which shows activities against obligate oral anaerobes that cause halitosis (Ramji et al. 2002), could be responsible for its anti-amoebic effect.

The anti-amoebic activity of *P. longum*, a closely related species to *P. chaba*, has been evaluated by several researchers. Ghoshal et al. (1996) reported the potential role of an ethanol extract of *P. longum* fruit against *E. histolytica*, both in vitro and in vivo. The activity of extract from *P. longum* fruit against amoebiasis in mice (Sawangjaroen et al. 2004) and against *Blastocystis hominis* in vitro (Sawangjaroen and Sawangjaroen 2005) was also reported. In addition, its aqueous and ethanol extracts inhibited growth of *Giardia lamblia*, both in vitro and in vivo (Tripathi et al. 1999), and it has been successfully used in part of a drug formulation to treat giardiasis in patients in India (Agarwal et al. 1997). It is not surprising that the results of the experiments presented in this paper correlate well with those of other researchers. However, it is still necessary to carefully identify the active components in each extract.

In conclusion, several plants which are being used for their reputed medicinal properties by the AIDS patients of this region are good candidates to be further studied for their potential in the systemic therapy and/or prophylaxis of *E. histolytica* infections. This finding would be an advantage in initiating therapy to reduce the morbidity and mortality among such patients due to these pathogens.

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