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In vitro Efficacy of the Antifungal Activity of Some Thai Medicinal-Plants on the Pathogenic Fungus, *Saprolegnia parasitica* H2, from Fish

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ABSTRACT

Ethanol crude extracts of galanga (*Alpinia galanga*) (rhizome), betel vine (*Piper betel* Linn.) (leaves), *Rhinacanthus nasutus* Linn. (leaves), *Kaempferia galanga* Linn. (leaves and roots) were studied for fungistatic and fungicidal activity against water mold *Saprolignea parasitica* H2. Minimal inhibitory concentration (MIC) of crude extracts of betel vine leaves and *K. galanga* Linn. roots against *S. parasitica* H2 were 500 and 125 μg/ml, respectively. The crude of *R. nasutus* Linn. leaves at 500 μg/ml could inhibit fungal growth for 46%. The crude extracts of galanga rhizome and *K. galanga* leaves at 500 μg/ml had minimal fungistatic activity against *S. parasitica* H2. The fungicidal concentrations of crude extracts of betel vine leaves, *R. nasutus* Linn. leaves and *K. galanga* Linn. leaves against *S. parasitica* H2 were 500 μg/ml after 24 hr-immersion, 5,000 μg/ml after 24 hr-immersion and 50 μg/ml after 120 min-immersion, respectively. Crude extracts of galanga rhizome and *K. galanga* Linn. leaves at 5,000 μg/ml had no fungicidal effects in this study. In addition, the fungicidal activity varied between concentration of plant extract and exposure time. This study presented that the crude extracts from betel vine leaves and *K. galanga* Linn. roots had high antifungal activity against *S. parasitica* H2, while *R. nasutus* Linn. leaves had moderate activity.

Key words: crude extracts, galanga rhizome (*Alpinia galanga*), betel vine (*Piper betel* Linn.), *Rhinacanthus nasutus* Linn., *Kaempferia galanga* Linn., *Saprolignea parasitica* H2, antifungal activity

INTRODUCTION

Infectious disease is one of the most important diseases in fish and causes an economic loss in many aspects. It is caused by bacteria, fungus, virus and parasites (Plumb, 1999). These pathogens are generally found on fish skin, gill, water and environment surrounding fish. Fish fungus is commonly known as water mold or oomycete which is found in soil, freshwater and estuarine water. Water mold is in the family: Saprolegniaceae, and there are 3 important genera, Saprolegnia, Aphanomyces and Achlya, causing fungal infection in fish. Any kind and age of fish could be infected with water mold, including freshwater and estuarine fish (Noga, 1993). Chemical or drugs used to treat infected fish are limited; for example, malachite green, potassium

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permanganate, copper sulphate and formalin (Treves-Brown, 2000). In addition, some chemical have harmful effects such as malachite green which is shown as a carcinogen and mutagen (Meyer and Jorgenson, 1983).

Recently, natural plant products have been known for their medicinal and antimicrobial properties. Garlic, galanga rhizome, betel vine, *Sapindus* sp., *Rhinacanthus nasutus* Linn. and *K. galanga* Linn. had an antifungal activity against dermatophytes in human and veterinary medicine (George and Pandalai, 1949; Wuthi-udomlert *et al*., 2000; Trakranrungsie *et al*., 2004;). In addition, herbal products such as D-limonene, neem seed extract, tea tree oil, eugenol, hinokitiol, citral and allyl-isothiocyanate have an antifungal activity against fish water mold, e.g. Saprolegnia, Aphanomyces and Achlya (Hussein *et al*., 2000; Campbell *et al*., 2001; Mori *et al*., 2002). For this reason, an attention has been diverted to an alternative, safe and cheap method for the management of fish water mold. Thus, the fungistatic and fungicidal activities of crude extract of galanga rhizome, betel vine leaves, *Rhinacanthus nasutus* Linn. leaves, *Kaempferia galanga* Linn. leaves and roots against water mold, *S. parasitica* H2, were studied.

MATERIALS AND METHODS

Plant and extraction

Galanga rhizome (*Alpinia galanga*), betel vine leaves (*Piper betel* Linn.), *Rhinacanthus nasutus* Linn. leaves, *Kaempferia galanga* Linn. leaves and roots were air-dried and grounded. Plant crude extracts were prepared with soxhlet extraction using ethanol as solvent.

Fungal isolates

Saprolegnea parasitica H2 (ATCC 90213) was cultured on glucose yeast extract (GY) agar (1 g glucose, 0.25 g yeast extract, 1.5 g agar and 100 ml distilled water) (Yuasa and Hatai, 1979). Fungal isolate were incubated at 20°C. Agar blocks 4 × 4 mm, taken from the advancing edge of 2-3 days old colonies, were used as a fungal inoculum in all experiments.

Fungistatic activity

Plant crude extracts were diluted with sterile water at 20 times the desired final concentrations following Hussein *et al*. (2000). One ml of crude suspension was placed in sterile petri dish and 19.0 ml of melted GY agar was added with gentle mixing to distribute crude suspension in the medium evenly. GY agar was used as control medium. The fungal blocks were inoculated centrally on prepared GY plates and kept at 20°C. Three replicates were prepared for each concentration. The mycelial growth of fungus was measured after 24, 48 and 72 hr comparing with the control and then compared a percentage of the fungal colony diameter on GY media containing the crude extracts with the control GY media.

Fungicidal activity

The fungal blocks were immersed in a single concentration (as prepared in fungistatic test) of each plant crude extract for 5, 10, 30, 60 and 120 min and 24 hours. The agar blocks were washed with sterilized distilled water before being inoculated onto GY agar plates (without crude extracts). Control block agars were immersed in sterilized distilled water and then inoculated onto the GY medium. All culture plates were incubated at 20°C. The mycelial growth was measured after 24, 48 and 72 hr. The fungicidal activities of crude extracts were shown as no fungal growth on GY agar within 48 hour.

RESULTS

Fungistatic effect of plant crude extracts

Plant ethanol crude extracts used in our study had antifungal activity against *S. parasitica*

Table 1 Antifungal activity of various concentrations of crude extracts on the growth of *Saprolignea parasitica* H2.

Concentration of Crude Extract (μg/ml)	Percentage of fungal growth *				
	Rhizome of galanga (*Alpinia galanga*)	Leaf of Betel vine (*Piper betel* Linn.)	Leaf of *Rhinacanthus nasutus* Linn.	Leaf of *Kaempferia galanga* Linn.	Root of *Kaempferia galanga* Linn.
0	100.00	100.00	100.00	100.00	100.00
5	103.87	104.97	96.59	96.59	98.86
50	104.60	94.29	93.18	97.73	59.09[a]
125	97.97	62.62	90.91	88.64	0.00[a]
250	89.87	25.78[a]	78.41[a]	97.73	0.00[a]
500	83.61	0.00[a]	54.55[a]	90.91	0.00[a]

* Percentage of the fungal colony diameter on GY media containing the crude extracts compared with the control GY media.

[a] significantly inhibited fungal growth compared to the control without crude extract using t-test, $p < 0.05$.

H2 at various concentrations (Table 1). Crude extracts were dissolved completely in water at prepared concentrations (5,000 μg/ml), but high concentrations (> 5,000 μg/ml) of crude extracts from galangal rhizomes and *R. nasutus* Linn. leaves were not completely suspended. The study of fungistatic activity showed that crude extracts of betel vine leaves and *K. galanga* roots had high activity and crude extract of *R. nasutus* Linn. leaves had moderate activity. Crude extracts from galangal rhizomes and *K. galanga* leaves showed minimal fungistatic activity.

Minimal inhibition concentrations (MIC) of betel vine leaf and *K. galanga* Linn. root crude extract were 500 and 125 μg/ml (ppm), respectively in which caused completely inhibition of fungal growth. In addition, the crude extracts of *R. nasutus* Linn. leaves, galangal rhizomes and *K. galanga* Linn. leaves at 500 μg/ml inhibited the fungal colony growth approximately 45.45, 16.39 and 9.09 %, respectively.

Fungicidal effect of plant crude extracts

The fungicidal activities of plant ethanol crude extracts against *S. parasitica* H2 were shown in Table 2. The vegetative form of *S. parasitica* H2 were killed after immersed in the 500 μg/ml of betel vine leaf extract, 5,000 μg/ml of *R. nasutus* Linn leaf extract and 50 μg/ml of *K. galanga* root crude extract. In addition, the fungicidal activity varied between concentration of plant extract and exposure time. Crude extract of betel vine leaves at 500, 1,250, 2,500 and 5,000 μg/ml killed *S. parasitica* H2 after 24 hr, 30 min, 5 min and 5 min immersion, respectively. *Kaempferia galanga* root extract at 50, 500, 1,250, 2,500 and 5,000 μg/ml killed the fungus after 120 min, 120 min, 60 min, 30 min and 5 min immersion, respectively, and *R. nasutus* leaf extract at 5,000 μg/ml could killed the fungus after 24 hr immersion. Nevertheless, crude extracts of *A.galanga* rhizomes and *K. galanga* leaves at 5,000 μg/ml (high concentration in this study) had no fungicidal effects.

DISCUSSION

In our study, it was presented that crude extracts from betel vine and *R. nasutus* Linn. leaves, and *Kaempferia galanga* Linn. roots had antifungal activity against fish water mold, *S. parasitica* H2. The root extract of *K. galanga* Linn. had greatest antifungal activity, fungistatic andfungicidal activity, compared with extracts of betel vine and *R. nasutus* Linn. leaves, which *K. galanga* Linn. root extract showed lowest

Table 2 Fungicidal effects of various concentrations of herbal crude extracts and immersion times (5 min until 24 hr) on vegetative growth of *Saprolignea parasitica* H2.

Crude Extract	Concentration of crude extract (µg/ml)	Exposure time of fungal isolate in crude extract					
		5 min	10 min	30 min	60 min	120 min	24 hour
Rhizome of galanga *(Alpinia galangal)*	0 (Control)	+	+	+	+	+	+
	50	+	+	+	+	+	+
	500	+	+	+	+	+	+
	1,250	+	+	+	+	+	+
	2,500	+	+	+	+	+	+
	5,000	+	+	+	+	+	+
Leaf of betel vine (*Piper betel* Linn.)	0 (Control)	+	+	+	+	+	+
	50	+	+	+	+	+	+
	500	+	+	+	+	+	-
	1,250	+	+	-	-	-	-
	2,500	-	-	-	-	-	-
	5,000	-	-	-	-	-	-
Leaf of *Rhinacanthus nasutus* Linn.	0 (Control)	+	+	+	+	+	+
	50	+	+	+	+	+	+
	500	+	+	+	+	+	+
	1,250	+	+	+	+	+	+
	2,500	+	+	+	+	+	+
	5,000	+	+	+	+	+	-
Leaf of *Kaempferia galanga* Linn.	0 (Control)	+	+	+	+	+	+
	50	+	+	+	+	+	+
	500	+	+	+	+	+	+
	1,250	+	+	+	+	+	+
	2,500	+	+	+	+	+	+
	5,000	+	+	+	+	+	+
Root of *Kaempferia galanga* Linn.	0 (Control)	+	+	+	+	+	+
	50	+	+	+	+	-	-
	500	+	+	+	+	-	-
	1,250	+	+	+	-	-	-
	2,500	+	+	-	-	-	-
	5,000	-	-	-	-	-	-

\+ Vegetative growth appeared within 48 hour.

\- No vegetative growth detected within 48 hour

concentration against water mold.

Dilokkunanant *et al.* (2000) found that extractions of dried betel vine which extracted with 6 different kinds of solvent (hexane, chloroform, diethyl ether, acetone, ethanol and methanol) could inhibit the growth of 4 fungi spp.: *Aspergillus niger, A. flavus, A. japonica* and *Penicillium monoticillate,* which isolated from bark of Nontri plant (*Peltophorum dasyrachis* Miq.) at concentration of 100,000-250,000 ppm. They also found that these betel vine extracts at 100,000-200,000 ppm could inhibit 5 fungi (*A. flavus, Penicillium sp., Furarium sp., Cladosporium spp* and *Curvalaria lunata*) which were isolated from

a bathroom and a highly-humidity room.

The efficacy of antimicrobial activities from plant crude extracts were affected by diluents such as DMSO, ethanol, hexane, propylene glycol and Tween 80 (Hussein *et al*., 2000, 2002; Dilokkunanant *et al*., 2002). In our study, water was used to dissolve plant crude extracts in which differ from other studies. All test extracts could dissolve completely at 5,000 μg/ml and showed some antifungal activities against *S. parasitica* H2.

CONCLUSION

Our results showed that crude ethanol extracts of betal vine leaves, *R. nasutus* Linn. leaves, and *K. galanga* Linn. roots had antifungal, fungistatic and fungicidal activity against fish water mold, *S. parasitica* H2. MIC of betal vine leaves and *K. galanga* Linn. roots against *S. parasitica* H2 were 500 and 125 μg/ml, respectively. The fungicidal concentrations of betel vine and *R. nasutus* Linn. leaves, and *K. galanga* Linn. roots crude extracts against *S. parasitica* H2 were 500 μg/ml after 24 hr immersion, 5,000 μg/ml after 24 hr immersion and 50 μg/ml after 120 min, respectively. Therefore, these 3 plant crude ethanol extracts may be alternative antifungal products against fish water mold and it is needed to further investigation in fish toxicity tests.

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