International Journal of Phytomedicine 6 (2014) 237-242
http://www.arjournals.org/index.php/ijpm/index

Original Research Article

ISSN: 0975-0185

In vitro antifungal activity of *Cassia fistula* L. against selected pathogenic water molds

Manassanan Borisutpeth[1]*, Pithai Kanbutra[1], Sompoth Weerakhun[1], Shinpei Wada[2], Kishio Hatai[2]

*Corresponding author:

Manassanan Borisutpeth

[1]Faculty of Veterinary Medicine, Khon Kaen University, Khon Kaen 40002, Thailand
[2]Laboratory of Aquatic Medicine, Nippon Veterinary and Life Science University, Musashino, Tokyo

Abstract

Water mold infections in both cultured and wild aquatic animals, caused by members of the genera Saprolegnia, Achlya, and Aphanomyces, have widely occurred worldwide. Outbreaks in aquatic creatures, especially in aquacultural facilities and fish hatcheries are common problem. *Cassia fistula* L. has been used as medicinal plant for broad purposes including for fungal infectious diseases remedy, but efficacy against water molds is still not apparently known. The present study was aimed to investigate *in vitro* antifungal activity of *C. fistula* stem-bark alcoholic extracts against the selected pathogenic water molds isolated from fish. The results showed that the *C. fistula* extract was capable to kill hyphae and zoospores of *Saprolegnia paracitica* NJM 8604 and *S. diclina* NJM 0005 at levels ranging from 1,000-4,000 μg/mL evidently within 24 h-exposure, while, a level of 500 μg/mL was sufficient to kill the both stages of *Aphanomyces invadans* NJM 9701. In addition, the antifungal activity of *C. fistula* absolute alcoholic extract comparing to 50% aqueous alcoholic extract were seemingly not different. Conclusively, the *C. fistula* stem-bark extracts, both absolute and 50% aqueous alcoholic extractions, were effectively able to inhibit growth and kill the 3 strains of pathogenic water molds by affect throughout the 2 important, zoospore and hyphal, stages.

Keywords: Antifungal activity, Aphanomyces spp, Aquatic animals, Cassia fistula L., Saprolegnia sp.,Water molds

Introduction

Outbreaks of waterborne fungal infections in aquatic creatures including fish, amphibians, and reptiles are common problem, especially in aquacultural facilities and fish hatcheries. Such infection can develop at all stages of the life cycle of these creatures. Water mold infections caused by members of the family Saprolegniaceae, particularly, the genera *Saprolegnia*, *Achlya* and *Aphanomyces*, have widely reported in freshwater fish [1, 2, 3, 4, 5, 6]. Although malachite green is mostly effective antifungal agent against pathogenic water molds which can inhibit zoospore germination and hyphal growth, its teratogenic, carcinogenic properties and residual properties have been reported [7]. Consequently, as the use of malachite green in the fish culturing industry is prohibited, alternative effective agents against water molds are necessary for fish farmer, but no effectual agents have been reported [8, 9, 10, 11, 12, 13, 14, 15]. Recently, many researchers have paid attention on application of herbal products as antifungal agents because of their safety originating from natural resource.

Cassia fistula L., a semi-wild and widespread throughout the tropics, is always planted as an ornamental and commonly used as medicinal plant. Various plant parts have been used for indigenous remedy in both ethnomedicine and ethnoveterinary and recommended in traditional pharmacopoeia, especially in south and south-east Asia. *C. fistula* has also been reported to exhibit antimicrobial activities including antidermatophytic activities in both human being and animals [16, 17, 18]. Extracts of various parts such as leaves, stem-bark, root-bark and fruit pulp have been explored for medicinal applications. In addition, this plant has been pointed as a probable source of drugs that could improve the treatment of fungal infections [18].

This study was conducted to determine the antifungal activities of *C. fistula* stem-bark alcoholic extract against three common pathogenic water molds.

Materials and methods

Plant and extraction

Fresh *C. fistula* stem-bark was collected from Khon Kaen province, Thailand. Crude absolute and 50% aqueous methanolic extracts were prepared. Firstly, cleaned fresh stem-bark was minced and subsequently ground using electric grinder. One part of the ground bark was soaked in 3-5 parts of each absolute and 50% aqueous methanol at room temperature for two days. Liquid extract was collected using gauze filtration and centrifugation. After methanol evaporation, the extract was subjected to freeze-drying using a freeze-dryer (Telstar® LyoAlfa 6-50, Terrassa, Spain) and powdered. The obtained extract powder was weighed and stored at –20 C. Just prior to use, the extract powder was primarily

reconstituted with either absolute or 50% aqueous ethanol at a concentration of 100,000 μg/mL and filtered through 0.2 μm-pore membrane. Because of lesser toxicity, ethanol was used for alcoholic reconstituting instead of methanol.

Fungal strains

Three selected pathogenic water molds causing fungal infection were isolated from diseased fish in Japan and used in this study (Table 1). The water molds were grown and maintained on glucose-yeast extract (GY) agar [19] at 20 C by monthly sub-culturing on fresh GY agar.

Table 1 The water mold strains used in this study

Isolate	Host	Site	Location	Year
Saprolegnia paracitica NJM 8604	Coho salmon, *Oncorhynchus kitsutch*	Body surface	Miyagi Prefecture, Japan	1986
Saprolegnia diclina NJM 0005	Coho salmon, *O. kitsutch*	Body surface	Miyagi Prefecture, Japan	1986
Aphanomyces invadans NJM 9701	Ayu, *Plecoglossus altivelis*	Muscle	Shiga Prefecture, Japan	1997

Fungistatic effect on hyphae

The primarily reconstituted *C. fistula* extract solutions were diluted with sterile distilled water (SDW) adjusting to concentrations of 40,000, 20,000, 10,000, 5,000 and 2,500 μg/mL. The fungistatic effect were tested by incorporating 2.0 mL of each solution directly into 18.0 mL of melting GY agar in sterile Petri-dishes (90×20 mm) with gentle thoroughly shaking. The final concentrations of the test dishes were 4,000, 2,000, 1,000, 500 and 250 μg/mL, respectively. Control plates containing GY agar and 2.0 mL SDW instead of *C. fistula* extracts were also prepared. A fungal agar block of each strain, which taken from advancing edge of pre-growing vegetative colony using No.2 cork borer, was placed at the center of each plate and then incubated at 20 C. The growth of hyphae was measured using a vernier caliper along two radii at right angle to each other at day 3 and day 7 after inoculation for *Saprolegnia sp.* and *Aphanomyces sp.*, respectively and the mean colonial radius was calculated. The fungistatic activity was shown by percentage of hyphal growth inhibitory which was calculated using the formula:

$$\left[\frac{(\text{control colonial radius}) - (\text{test colonial radius})}{(\text{control colonial radius})}\right] 100$$

Fungicidal effect on hyphae

Firstly, *C. fistula* extract solution was diluted to test concentrations of 4,000, 2,000, 1,000, 500 and 250 μg/mL using SDW. The fungal agar blocks, taken from vegetative colony as described above, were submersed in Petri-dishes containing 20 mL of the test solutions. After 10, 30 min, 1, 2 and 24 h, the fungal blocks were dipped in SDW to rinse all the test solution out of the blocks. Then, the fungal blocks were inoculated onto GY agar plates and incubated at 20 C for 3 and 7 days for *Saprolegnia sp.* and *Aphanomyces sp.*, respectively. As negative control, the fungal agar blocks were submersed in SDW without *C. fistula* extract. The fungal growth was daily monitored comparing to each corresponding control. No fungal growth appearance indicated the fungicidal effect of *C. fistula* extract against the hyphae.

Fungicidal effect on zoospores

Firstly, two fungal agar blocks of each strain were incubated in 20 mL of GY broth at 20 C for 2 days. The growing mycelia were harvested, washed in sterile tap water (STW), and transferred into Petri-dish contained 25 mL of STW to incubate at 20 C for 24 h allowing zoospore production. The zoospores were collected by aseptically filtering through sterile gauze, enumerated under microscope using a Neubauer chamber (Erma, Tokyo) and adjusted to gain 1,000 spores/mL zoospore suspensions. One ml of each test concentration (40,000, 20,000, 10,000, 5,000 and 2,500 μg/mL) of *C. fistula* extract was added into 9 mL of the zoospore suspension. The mixture was incubated at 20 C for 10, 30 min, 1, 2 and 24 h. As control, the zoospore suspension was incubated in SDW without *C. fistula* extract. At each incubation time, 0.1 mL of each mixture was drawn to inoculate into 30 mL of GY broth and incubated at 20 C. The fungicidal effect of *C. fistula* extract against zoospores was determined by observing the presence of fungal growth after 3 days post inoculation.

Results

Fungistatic effect on hyphae

Growth of *S. paracitica* NJM 8604, *S. diclina* NJM 0005 and *A. invadans* NJM 9701 were completely inhibited when exposed to absolute ethanol reconstituted *C. fistula* extracts at doses of 2,000, 2,000 and 500 μg/mL, respectively. While, 50% aqueous ethanol reconstituted *C. fistula* extracts showed completely inhibition on the hyphal growth at doses of 4,000, 4,000 and 500 μg/mL, respectively (Table 2).

Table 2 Fungistatic effect of *Cassia fistula* extracts on hyphae

	Extracts Concentration (μg/mL)	*Saprolegnia paracitica* NJM 8604	*Saprolegnia diclina* NJM 0005	*Aphanomyces invadans* NJM 9701
Control: without *C. fistula* extract	0	37.5 (0)	37.5 (0)	26.5 [a] (0) [b]
Cassia fistula: absolute alcoholic extract	250	-	-	2.33 (91.2)
	500	3.75 (90.0)	-	NG (100)
	1000	2.09 (94.4)	13.66 (63.6)	NG (100)
	2000	NG (100)	NG (100)	-
	4000	NG (100)	NG (100)	-
Cassia fistula: 50% aqueous alcoholic extract	250	-	-	2.35 (91.1)
	500	8.49 (77.4)	-	NG (100)
	1000	3.42 (90.9)	21.57 (42.5)	NG (100)
	2000	0.52 (98.6)	8.79 (76.6)	NG (100)
	4000	NG (100)	NG (100)	NG (100)

[a], mean radius of hyphal colony (mm);
[b], percentage of hyphal growth inhibitory compared to the control without *C. fistula* extract;
-, Not tested;
NG, No hyphal growth

Fungicidal effect on hyphae

When the water molds exposed to extracts for 24 h, *C. fistula* absolute alcoholic extracts were able to kill hyphae of *S. paracitica* NJM 8604, *S. diclina* NJM 0005 and *A. invadans* NJM 9701 at the concentrations of 1,000, 2,000 and 250 μg/mL, respectively (Table 3). After exposure for 24 h, *C. fistula* 50% aqueous alcoholic extracts showed hyphal killing ability against *S. diclina* NJM 0005 and *A. invadans* NJM 9701 at the concentrations of 2,000 and 250 μg/mL, respectively, while, *S. paracitica* NJM 8604 was killed at 2,000 μg/mL when exposure time was either 2 or 24 h (Table 3).

Table 3 Fungicidal effect of *Cassia fistula* extracts on hyphae

Fungal strains	Exposure time	Control	*Cassia fistula* concentration (μg/mL)				
			250	500	1,000	2,000	4,000
S. paracitica NJM 8604	10 min	+	+/+ [a]	+/+	+/+	+/+	+/+
	30 min	+	+/+	+/+	+/+	+/+	+/+
	1 h	+	+/+	+/+	+/+	+/+	/
	2 h	+	+/+	+/+	+/+	+/	/
	24 h	+	+/+	+/+	/+	/	/
S. diclina NJM 0005	10 min	+	+/+	+/+	+/+	+/+	+/+
	30 min	+	+/+	+/+	+/+	+/+	/+
	1 h	+	+/+	+/+	+/+	+/+	/+
	2 h	+	+/+	+/+	+/+	+/+	/
	24 h	+	+/+	+/+	+/+	/	/
A. invadans NJM 9701	10 min	+	+/+	+/+	+/+	/	/
	30 min	+	+/+	+/+	+/+	/	/
	1 h	+	+/+	+/+	+/+	/	/
	2 h	+	+/+	+/+	/	/	/
	24 h	+	/	/	/	/	/

+, Growth; , No growth; [a], absolute alcoholic extract/50% aqueous alcoholic extract

Fungicidal effects on zoospores

C. fistula absolute and 50% aqueous alcoholic extracts were able to kill the zoospores of *S. paracitica* NJM 8604 and *S. diclina* NJM

0005 at doses of 1,000 and 2,000 μg/mL for even 2 and 24 h-exposure, respectively. While, the zoospores of *A.invadans* NJM 9701 was killed by the both alcoholic extracts at a concentration of 500 μg/mL for 24 h-exposure. (Table 4)

Table 4 Fungicidal effect of *Cassia fistula* extracts on zoospores

Fungal strains	Exposure time	Control	*Cassia fistula* concentration (μg/mL)				
			250	500	1,000	2,000	4,000
S. paracitica NJM 8604	10 min	+	+/+ [a]	+/+	+/+	/+	/+
	30 min	+	+/+	+/+	+/+	/+	/
	1 h	+	+/+	+/+	+/+	/+	/
	2 h	+	+/+	+/+	/	/	/
	24 h	+	+/+	+/+	/	/	/
S. diclina NJM 0005	10 min	+	+/+	+/+	+/+	+/+	/
	30 min	+	+/+	+/+	+/+	+/+	/
	1 h	+	+/+	+/+	+/+	+/+	/
	2 h	+	+/+	+/+	+/+	/	/
	24 h	+	+/+	+/+	+/+	/	/
A. invadans NJM 9701	10 min	+	+/+	+/+	+/+	/+	/
	30 min	+	+/+	+/+	+/+	/+	/
	1 h	+	+/+	+/+	/+	/+	/
	2 h	+	+/+	+/+	/+	/	/
	24 h	+	+/+	/	/	/	/

+, Growth; , No growth; [a], absolute alcoholic extract/50% aqueous alcoholic extrac

Discussion

C. fistula has been used as alternative medicine for infectious diseases with scientific approves appearing its antimicrobial activities including antifungal activity [20], however, efficacy against water molds has not been revealed. The present study showed that *C. fistula* stem-bark alcoholic extracts were able to inhibit the fungal growth and to kill both hyphae and zoospores of *S. paracitica* NJM 8604 and *S. diclina* NJM 0005. The effective doses against the 2 molds were ranged from 1,000-2,000 μg/mL evidently within 24 h-exposure, while the lower concentrations (250-500 μg/mL) were sufficient to kill the both fungal stages of *A. invadans* NJM 9701. Among the strains, this may point that *A. invadans* NJM 9701 is more sensitive to the *C. fistula* extracts than the 2 *Saprolegnia* strains. Similarly, Hussein et al. [21] and Mori et al. [22] described that *A. piscicida* (syn. *A. invadans*) strains were more sensitive against some chemicals/phytochemicals including eugenol, hinokitiol, citral and allyl isothiocyanate than *Saprolegnia* strains. Likewise, the aroma components from *Alpinia galangal* were more efficient against *A. piscicida* than *Saprolegnia* sp. and *Achlya* sp. [23]. In addition, concurrent evidences were stated that Thai medicinal plants including: guava leaves, betel pepper leaves, tamarind raw fruits, salapeepa flowers and red rose flowers were potent antifungal agents given their effects against hyphal growth of *A. invadans* more effective than *S. diclina* and *Achlya* sp. [14]. These results also demonstrated that the efficacy of phytochemicals or chemicals against fungi were dependent on the fungal species, as well as, the prolong exposure to the phytochemicals or chemicals gave more effective. The fungistatic and fungicidal effectiveness of the plant extracts may depend upon the toxicity of phytochemical constituents to the molds. Within stages sensitivity, the zoospores of *S. paracitica* NJM 8604 and *S. diclina* NJM 0005 seemingly showed more sensitivity to *C. fistula* extracts than its hyphal stage when kept shorter exposure time at the even dose. Zoospores are single cells, and therefore, they are more vulnerable to chemicals including the plant extracts [24]. On the other hand, the zoospores of *A. invadans* NJM 9701 showed slight tolerance against the *C. fistula* extracts in comparison with its hyphal stage. Because of the zoospores play an important role in the development of water mold infection in aquatic animals, this figure that the prophylaxis use of the *C. fistula* extracts may has

been effective. Thus, administration with 1,000-2,000 μg/mL of the *C. fistula* alcoholic extracts for 2-24 h-exposure, which may be effective to control infection by zoospore stages of all causative fungi, was suggested as a practical procedure. The present results showed that the antifungal activities were not different between the *C. fistula* absolute and 50% aqueous alcoholic extracts, however, the absolute alcoholic extracts trended giving more efficacious. This may point that the active antifungal phytochemicals are alcoholic soluble. Medicinal and antimicrobial properties of the natural plant products have been nowadays known and used, however, phytochemical and biological investigations of the effective medicinal plant are the urgent need to characterize antimycotic principles and to assess toxicity in order to develop new drugs.

Conclusion

In conclusion, *C. fistula* stem-bark alcoholic extracts were effectively able to inhibit hyphal growth and kill both of the zoospores and hyphae of the three pathogenic water molds.

Authors' contributions

Manassanan Borisutpeth conceived of the study and participated in study design, carried out the plant extraction and antifungal activity tests, and participated in the sequence alignment and drafted the manuscript. Pithai Kanbutra carried out the plant collection and extraction, involved in analysis of data, and participated in drafting the manuscript. Sompoth Weerakhun carried out the antifungal activity tests. Shinpei Wada carried out the antifungal activity tests and helped to draft the manuscript. Kishio Hatai conceived of the study, participated in its design and coordination, revised the manuscript in it critically for important intellectual content, and given approval of the manuscipt. All authors read and approved the final manuscript.

Acknowledgment

We would like to express grateful appreciation to members of Laboratory of Aquatic Medicine, Nippon Veterinary and Life Science University, Tokyo, Japan for supporting facilities available throughout the experiments.

References

[1]. Carballo M, Muñoz MJ. Effect of sublethal concentrations of four chemicals on susceptibility of juvenile rainbow trout (*Oncorrhynchus mykiss*) to Saprolegniasis. Appl Environ Microbiol 1991; 57: 1813-1816.

[2]. Hatai K, Hoshiai G. Saprolegniasis in cultured coho salmon. Fish Pathol 1992a; 27: 233-234.

[3]. Hatai K, Hoshiai G. Mass mortality in cultured coho salmon, *Oncorhynchus kisutch*, due to *Saprolegnia parasitica* Coker. J Wildl Dis 1992b; 28: 532-536.

[4]. Blaustein AR, DG Hokit, RK O'Hara, RA Holt. Pathogenic fungus contributes to amphibian losses in the Pacific-Northwest. Biol Cons 1994; 67: 251-254.

[5]. Kitancharoen N, Yuasa K, Hatai K. Morphological aspects of *Saprolegnia diclina* type I isolated from pejerrey, *Odonthetes bonariensis*. Mycoscience 1995; 36: 365-368.

[6]. Hussein MMA, Hatai K. *Saprolegnia salmonis* sp. nov. isolated from sockeye salmon, *Oncorhynchus nerka*. Mycoscience 1999; 40: 385-389.

[7]. Meyer FP, Jorgenson TA. Teratological and other effects of malachite green on the development of rainbow trout and rabbits. Trans Am Fish Soc 1983; 112: 818-824.

[8]. Srivastava RC. Fungal parasites of certain freshwater fishes of India. Aquaculture 1980; 21: 387-392.

[9]. Prabhuji SK, Srivastava GC, Rizvi SJH, Mathur SN. 1,3,7-Trimethylxanthine (caffeine); a new natural fish fungicide. Cell Mol Life Sci 1983; 39: 177-179.

[10]. Perrucci S, Cecchini S, Pretti C, Cognetti AMV, Macchioni G. *In vitro* antimycotic activity of some natural products against *Saprolegnia ferax*. Phytother Res 1995; 9: 147-149.

[11]. Kitanchroen N, Yamamoto A, Hatai K. Fungicidal effect of hydrogen peroxide on fungal infection of rainbow trout eggs. Mycoscience 1997a; 38: 375-378.

[12]. Kitanchroen N, Yamamoto A, Hatai, K. The fungistatic effect of NaCl on rainbow trout egg saprolegniasis. Fish Pathol 1997b; 32: 159-162.

[13]. Barnes ME, Ewing DE, Cordes RJ, Young GL. Observation on hydrogen peroxide control of *Saprolegnia* spp. during rainbow trout egg incubation. Prog Fish-Cult 1998; 60: 67-70.

[14]. Borisutpeth P, P Kanbutra, C Hanjavanit, K Chukanhom, D Funaki, K Hatai. Effects of Thai herbs on the control of fungal infection in Tilapia eggs and toxicity to the eggs. Aquaculture Sci 2009; 57(3): 475-482.

[15]. Borisutpeth P, P Kanbutra, C Hanjavanit, E Horiuchi, S Wada, K Hatai. The *in vitro* antifungal effects of chlorine dioxide on water molds. Aquaculture Sci 2010; 58(2): 219-224.

[16]. Rajan S, DS Baburaj, M Sethuraman, S Parimala. Stem and stem bark used medicinally by the Tribals Irulas and Paniyas of Nilgiri District, Tamil Nadu. J Nat Remedies 2001; 1(1): 49-54.

[17]. Prashanth KV, Chauhan NS, Padh H, Rajani M. Search for antibacterial antifungal agents from selected Indian medicinal plants. J Ethnopharmacol 2006; 107: 182-188.

[18]. Duraipandiyan V, Ignacimuthu S. Antibacterial and antifungal activity of *Cassia fistula* L.: An ethnomedicinal

plant. J Ethnopharmacol 2007; 112: 590-594.

[19]. Hatai K, Egusa S. Studies on pathogenic fungus of mycotic granulomatosis III development of the medium for egg-fungus. Fish Pathol 1979; 13: 147-152.

[20]. Ali MA, Sayeed MA, Bhuiyan MSA, Sohel FI, Yeasmin MS. Antimicrobial screening of *Cassia fistula* and *Mesua ferrea*. J Med Sci 2004; 4(1): 24-29.

[21]. Hussein MMA, Wada S, Hatai K, Yamamoto A. Antimycotic activity of eugenol against selected water molds. J Aquat Anim Health 2000; 12: 224-229.

[22]. Mori T, Hirose H, Hanjavanit C, Hatai K. Antifungal activities of plant extracts against some aquatic fungi. Biocontrol Sci 2002; 7: 187-191.

[23]. Chukanhom K, Borisutpeth P, Hatai K. Antifungal activities of aroma components from *Alpinia galanga* against water molds. Biocontrol Sci 2005; 10: 105-109.

[24]. Willoughby LG, Pickering AD. Viable Saprolegniaceae spores on the epidermis of the salmonid fish *Salmo trutta* and *Salvelnus alpinus*. Trans Brit Mycol Soc 1977; 68: 91-95.