Note

Antifungal Activities of Plant Extracts against Some Aquatic Fungi

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Hinokitiol, citral and allylisothiocyanate, which are chemicals of plant extracts, were investigated for their effectiveness as antifungal agents against the genera *Saprolegnia*, *Aphanomyces* and *Achlya*. The minimum inhibitory concentrations (MICs) of these chemicals were 12.5 µg/ml of hinokitiol against all fungal isolates, and 100.0-200.0 µg/ml of citral and 50.0-100.0 µg/ml of allylisothiocyanate were appeared to inhibit the growth of *Saprolegnia* and *Aphanomyces*, but not *Achlya*. Among these chemicals, 100.0 µg/ml of hinokitiol was the most effective agent to inhibit the hyphal growth of *S. parasitica* ATCC 90213 and *Aphanomyces* spp. but not of *S. diclina* ATCC 90215 and *Achlya*. Hinokitiol also inhibited at 100.0 µg/ml the zoospore germination of *Saprolegnia* spp., whereas 12.5-25.0 µg/ml of hinokitiol inhibited the zoospore germination of *Aphanomyces* spp. (but not *Achlya*). Treatment with a higher concentration of citral (800.0 µg/ml) had an effectiveness of inhibiting the hyphal growth of all fungal isolates but the zoospores of all isolated strains showed higher sensitivity to citral (200.0-400.0 µg/ml) than their vegetative stages did. The treatment with 100.0-400.0 µg/ml of allylisothiocyanate was found to inhibit the vegetative growth of all fungal isolates except *Achlya*. On the other hand, the zoospores of all isolates showed higher sensitivity to allylisothiocyanate (200.0-400.0 µg/ml) than their vegetative stages did.

Key words : Antifungal activity/Aquatic fungi/Allylisothiocyanate/Citral/Hinokitiol/Plant extracts.

Mycotic disease in freshwater fishes is usually caused by fungi in the family Saprolegniaceae, the genera *Saprolegnia*, *Aphanomyces* and *Achlya* (Hatai, 1980). The minimum inhibitory concentrations (MIC) of three chemicals, malachite green, formalin and sodium chloride (NaCl), against those three genera were investigated (Yuasa and Hatai, 1995). The most effective chemical was malachite green, which inhibited zoospore germination and hyphal growth at

a low concentration. However, malachite green has teratogenic and carcinogenic properties (Merer and Jorgenson, 1983), so its use as a fungicide in fish culture is prohibited in the U.S. by the Food and Drug Administration (FDA) (Marking et al., 1994; Fitzpatrick et al., 1995). NaCl has been frequently used as a fungal control agent, but it is effective at only a high concentration, and its fungicidal activity is poor in comparison with malachite green (Hatai et al., 1977).

Recently, the use of extracts from plants for antibacterial purpose has spread because of their safety

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(Kobori and Tanabe, 1993, 1994a and b). Plant extracts, such as hinokitiol, citral and allylisothiocyanate, are widely used for the preservation of food and antibacterial treatment of household articles (Yokota et al., 1994). An objective of this study was to investigate the effectiveness of treatment with these plant extracts on the vegetative and zoosporic stages of three generic fungi, in comparison with those of treatments with malachite green, formalin and NaCl.

Malachite green (including malachite green oxalate 99% solution: Wako Pure Chemical Industries, Ltd.), formalin (Wako Pure Chemical Industries, Ltd.), NaCl (Manaku, Ltd.), hinokitiol (Kanto Chemical Ltd.), citral (*cis+trans*: Wako Pure Chemical Industries, Ltd.) and allylisothiocyanate (SOH-Ei, Ltd.) were used as antifungal agents.

Details of fungal isolates used in the present study are presented in Table 1. The fungi, *Saprolegnia*, *Aphanomyces* and *Achlya*, were cultured in Petri dishes of glucose yeast (GY) media with 1.5% w/v agar (Difco) at 20°C to obtain vegetative hyphae, and edges of colonies were cut and put into GY broth before being transferred to sterilized tap water to obtain zoospores, as described by Hatai and Egusa (1979).

The antifungal agents used in this experiment were malachite green, formalin, NaCl, hinokitiol, citral and allylisothiocyanate. The fungi, *Saprolegnia*, *Aphanomyces* and *Achlya* were cultured on 20 ml of GY agar at 20°C. Culturing was done for 2-3 d for *Saprolegnia* spp. and 5-6 d for *Aphanomyces* spp. and *Achlya diffusa* NJM 9228. Each chemical was mixed with 20ml of GY agar. The actively growing mycelia were cut off with a No. 2 Cork borer (5.5mm in dia.) and centrally placed onto the media. The effectiveness of the antifungal agents was determined by measuring the diameter of the colony every 2 days for 6 days. Colony diameters of the fungi were measured at different times depending on the genus: diameter of *Aphanomyces* spp. were determined at day 6, that of *Achlya diffusa* NJM 9228 was determined at day 4

and those of *Saprolegnia* spp. at day 2. A control group for each fungus on GY agar without any antifungal agent was also observed.

The plant extracts used in this experiment were hinokitiol, citral and allylisothiocyanate. The chemicals were tested for their fungicidal activities against the vegetative and zoosporic stages. The activity against the vegetative stage was carried out as follows: the fungal isolates were prepared according to the culture conditions mentioned above. An actively growing part of each isolate was excised and placed into the various concentrations of the plant extracts for 10min, 30min, 1 h, 2 h and 24h, respectively. Then it was washed twice with 40ml of sterilized distilled water and placed onto 20ml of GY agar without plant extracts. The effectiveness of the three plant extracts against the vegetative stages was determined by comparing colony diameters of the treated ones with those of the control group which had been immersed in sterilized distilled water. In the case that no growth of fungi appeared after 24h, the fungi were kept for 3d to observe their viability.

The investigation of the activity of plant extracts against fungi in the zoosporic stage was conducted by preparing the 10-time-concentrated solution of the plant extract in sterilized distilled water. Zoospore production was carried out in sterilized tap water, after which they were harvested by aseptic filtration through gauze. The number of zoospores was evaluated and adjusted to 100-200 spores/ml. Then 1 ml of the concentrated solution prepared was added to 9 ml of zoospore suspension and 500 µl of the mixture was inoculated into 40 ml of GY broth after 10min, 30 min, 1 h, 2 h and 24h. This dilution ratio had been determined by a preliminary test to show that there was no effect on the germination of viable zoospores. The viability of zoospores was determined by the appearance of germinating thalli during a 3-4 d period under the microscope. Control groups without the plant extract treatment were also observed. All of the experiments were performed at 20°C. The minimal

TABLE 1. The fungal isolates used in the present study.

Species	Isolate no.	Host/habitat ^a	Origin	Host location	Year isolated
<i>Saprolegnia parasitica</i>	ATCC 90213 (NJM 8604)	Coho salmon	Fish surface	Miyagi	1986
<i>Saprolegnia diclina</i>	ATCC 90215 (NJM 8605)	Coho salmon	Fish surface	Miyagi	1986
<i>Aphanomyces piscicida</i>	ATCC 32712 (NJM 8997)	Ayu	Internal viscera	Tochigi	1989
<i>Aphanomyces</i> sp.	NAV 9201	Nihon ishigame	Skin surface	Tokyo	1992
<i>Achlya diffusa</i>	NJM 9228	Pond water	—	Tokyo	1992

^aCoho salmon: *Oncorhynchus kisutch* Walbaum; ayu: *Plecoglossus altivelis* Temminct et Schligel; nihon ishigame: *Mauremys japonica*.

concentration of each plant extract at which the growth of each fungus was inhibited, was expressed as MIC in these experiments.

As seen in Tables 2-3, the MICs of malachite green were 2.0 μ g/ml against *S. parasitica* ATCC 90213 and 1.0 μ g/ml against both *S. diclina* ATCC 90215

TABLE 2. Antifungal effects of malachite green, formalin and NaCl on the growth of three fungal genera.

Concentration	Isolate				
	<i>S. parasitica</i> ATCC 90213	<i>S. diclina</i> ATCC 90215	<i>A. piscicida</i> NJM 8997	<i>Aphanomyces</i> sp. NAV 9201	<i>Achlya diffusa</i> NJM 9228
Control					
0 μ g/ml	100 ^a	100	100	100	100
Malachite green					
0.5 μ g/ml	15.5	26.9	4.1	0.3	22.9
1.0	5.2	0	0	0	17.5
1.5	4.6	0	0	0	9.0
2.0	0	0	0	0	10.0
2.5	0	0	0	0	8.8
3.0	0	0	0	0	7.9
Formalin					
12.5 μ g/ml	90.0	95.2	98.8	76.0	112.1
25.0	85.5	87.0	60.7	0	79.8
50.0	59.0	57.6	0	0	44.7
100.0	0	0	0	0	0
NaCl					
0.5%	105.3	86.0	64.4	78.2	78.6
1.0	69.6	58.5	7.4	48.1	28.6
1.5	42.2	43.0	0	4.7	0
2.0	19.3	34.0	0	0	0
2.5	0	13.7	0	0	0
3.0	0	0	0	0	0

^aPercentage of the colony diameter on media containing different concentrations of malachite green, formalin and NaCl compared with the control group of each isolate.

TABLE 3. Antifungal effects of hinokitiol, citral and allylisothiocyanate on the growth of three fungal genera.

Concentration	Isolate				
	<i>S. parasitica</i> ATCC 90213	<i>S. diclina</i> ATCC 90215	<i>A. piscicida</i> NJM 8997	<i>Aphanomyces</i> sp. NAV 9201	<i>Achlya diffusa</i> NJM 9228
Control					
0 μ g/ml	100 ^a	100	100	100	100
Hinokitiol					
3.2 μ g/ml	88.2	102.6	98.0	99.3	128.0
6.3	80.6	94.9	81.1	98.0	102.6
12.5	0	0	0	0	0
25.0	0	0	0	0	0
Citral					
12.5 μ g/ml	96.1	73.0	66.3	98.3	96.7
25.0	85.0	71.8	45.7	97.3	113.1
50.0	67.6	46.3	23.8	95.1	102.0
100.0	53.0	6.5	0	69.9	43.9
200.0	0	0	0	0	2.8
Allylisothiocyanate					
12.5 μ g/ml	93.1	97.2	94.3	109.2	122.2
25.0	85.5	79.1	54.9	94.0	120.3
50.0	83.2	90.4	0	85.2	116.7
100.0	0	0	0	0	27.9

^aPercentage of the colony diameter on media containing different concentrations of hinokitiol, citral and allylisothiocyanate compared with the control group of each isolate.

TABLE 4. Effect of the dosage of hinokitiol, citral, and allylisothiocyanate on the vegetative growth and zoospore germination of tested fungi exposed for 60 min.

Plant extracts	Isolates	MIC	
		Vegetative growth	Zoospore germination
Hinokitiol	<i>Saprolegnia parasitica</i> ATCC90213	100 ^a	100
	<i>Saprolegnia diclina</i> ATCC90219	>100	100
	<i>Aphanomyces piscicida</i> NJM8997	100	<12.5
	<i>Aphanomyces</i> sp. NAV9201	100	25
	<i>Achlya diffusa</i> NJM 9228	>100	>100
Citral	<i>Saprolegnia parasitica</i> ATCC90213	800	400
	<i>Saprolegnia diclina</i> ATCC90219	800	400
	<i>Aphanomyces piscicida</i> NJM8997	800	200
	<i>Aphanomyces</i> sp. NAV9201	800	400
	<i>Achlya diffusa</i> NJM 9228	800	400
Allylisothiocyanate	<i>Saprolegnia parasitica</i> ATCC90213	400	200
	<i>Saprolegnia diclina</i> ATCC90219	400	400
	<i>Aphanomyces piscicida</i> NJM8997	400	100
	<i>Aphanomyces</i> sp. NAV9201	200	200
	<i>Achlya diffusa</i> NJM 9228	>800	400

^a $\mu\text{g/ml}$.

and *Aphanomyces* spp., whereas no concentrations of malachite green had any effect on the fungal growth of *Achlya diffusa* NJM 9228. The MICs of formalin were 100.0 $\mu\text{g/ml}$ against both *Saprolegnia* spp. and *Achlya diffusa* NJM 9228; 50.0 $\mu\text{g/ml}$ against *A. piscicida* NJM 8997 and 25.0 $\mu\text{g/ml}$ against *Aphanomyces* sp. NAV 9201. The MICs of NaCl were 2.5% against *S. parasitica* ATCC 90213; 3.0% against *S. diclina* ATCC 90215; 1.5% against both *A. piscicida* NJM 8997 and *Achlya diffusa* NJM 9228, whereas it was 2.0% against *Aphanomyces* sp. NAV 9201. The MIC of hinokitiol was 12.5 $\mu\text{g/ml}$ against all fungal isolates used. The MICs of citral were 200.0 $\mu\text{g/ml}$ against both *Saprolegnia* spp. and *Aphanomyces* sp. NAV 9201; 100.0 $\mu\text{g/ml}$ against *A. piscicida* NJM 8997, whereas no concentrations of citral had any effect on *Achlya diffusa* NJM 9228. The MICs of allylisothiocyanate were 100.0 $\mu\text{g/ml}$ against both *Saprolegnia* spp. and *Aphanomyces* sp. NAV 9201; 50.0 $\mu\text{g/ml}$ against *A. piscicida* NJM 8997 but there was no effect on *Achlya diffusa* NJM 9228.

As shown in Table 4, the treatment with 100 $\mu\text{g/ml}$ of hinokitiol was effective in killing both the hyphae and zoospores of *S. parasitica* ATCC 90213, but it only killed the zoospores of *S. diclina* ATCC 90215 and hyphae of both *A. piscicida* NJM 8997 and *Aphanomyces* sp. NAV 9201. The zoospores of *A. piscicida* NJM 8997 and *Aphanomyces* sp. NAV 9201 were more sensitive to hinokitiol (12.5 $\mu\text{g/ml}$ and 25.0 $\mu\text{g/ml}$, respectively) than their hyphae, whereas both stages of *Achlya diffusa* NJM 9228 had a high

tolerance to hinokitiol. The treatment with citral at the highest concentration of 800.0 $\mu\text{g/ml}$ was effective in killing the vegetative stages of all fungal strains used (Table 4). On the other hand, 400.0 $\mu\text{g/ml}$ of citral could kill the zoospores of almost all fungal strains tested, and *A. piscicida* NJM 8997 showed an even a higher sensitivity to citral (200.0 $\mu\text{g/ml}$) (Table 4). The treatment of 400.0 $\mu\text{g/ml}$ of allylisothiocyanate was effective in killing the vegetative stage of *S. parasitica* ATCC 90213 but its zoospore stage was more sensitive than its vegetative stage. The concentrations of 400.0 $\mu\text{g/ml}$ and 200.0 $\mu\text{g/ml}$ of allylisothiocyanate were also effective in killing both stages of *S. diclina* ATCC 90215 and *Aphanomyces* sp. NAV 9201, respectively. The hyphae of *A. piscicida* NJM 8997 showed higher sensitivity to allylisothiocyanate than its zoospores. Various concentrations of allylisothiocyanate were not effective in killing the vegetative stages of *A. diffusa* NJM 9228, whereas the concentration of 200.0 $\mu\text{g/ml}$ was able to kill its zoospores (Table 4).

Malachite green, formalin and NaCl have been reported as antifungal agents in the culturing of fish and fish eggs; namely 2.0 $\mu\text{g/ml}$ of malachite green, 125.0 $\mu\text{g/ml}$ of formalin and 2.5% of NaCl were effective in controlling fungal infection (Kitancharoen and Hatai, 1998; Yuasa and Hatai, 1995). The result of the present study is similar to those of Yuasa and Hatai (1995) and Kitancharoen and Hatai (1998). It demonstrated that the range of concentrations of chemicals that had an effectiveness in inhibiting the growth

of the genera *Saprolegnia*, *Aphanomyces* and *Achlya* was as follows: 1.0-2.0 $\mu\text{g/ml}$ of malachite green (except against *Achlya*); 25.0-100.0 $\mu\text{g/ml}$ of formalin and 1.5-3.0% of NaCl.

Kobori and Tanabe (1993, 1994a and b) reported that the chemical extracts from plants could be used as antibacterial and bactericidal agents. According to Yokota et al. (1994), plant extracts, e.g. hinokitiol, generally were used in hair tonic and cosmetics; citral was used as an aromatic agent in medicine and had a potential bactericidal action against pathogenic bacteria; allylisothiocyanate is used to preserve the freshness of food. Matsumoto et al. (1994) reported that a concentration of 50.0-200.0 $\mu\text{g/ml}$ of hinokitiol was the MIC against bacteria. The present results showed that a lower concentration of hinokitiol, 12.5 $\mu\text{g/ml}$, was effective in inhibiting the growth of hyphae of the genera *Saprolegnia*, *Aphanomyces* and *Achlya*. Therefore, fungi in the family Saprolegniaceae were more sensitive to hinokitiol than bacteria were. Other plant extracts, i.e. citral and allylisothiocyanate, were also used as antifungal agents in this study. In a comparison study, it was shown that a concentration of 50.0 $\mu\text{g/ml}$ of allylisothiocyanate appeared to be more effective in inhibiting growth of *A. piscicida* NJM 8977 than 100.0 $\mu\text{g/ml}$ of citral. Also 100.0 $\mu\text{g/ml}$ of allylisothiocyanate had a greater effectiveness in inhibiting the growth of both *Saprolegnia* spp. and *Aphanomyces* sp. NAV 9201 than 200.0 $\mu\text{g/ml}$ of citral. However, the high concentrations of 100.0 $\mu\text{g/ml}$ of citral and 200.0 $\mu\text{g/ml}$ of allylisothiocyanate had no effect in inhibiting the hyphal growth of *Achlya diffusa* NJM 9228. The effectiveness of plant extracts in inhibiting hyphal growth may be dependent on the toxicity of plant extracts. Furthermore, the zoospores of these genera were more sensitive to the plant extracts than the hyphae were. The zoospore is a single cell, which is easily to vulnerable to plant extracts, and the zoospore stage is important in the development of saprolegniasis in fish (Willoughby and Pickering, 1977). This is the first report to demonstrate the sensitivities of the plant extracts to control the hyphal growth and zoospore germination of fungi, family Saprolegniaceae *in vitro*. However, it is necessary to investigate the efficacy of these plant extracts in controlling naturally occurring fungal infections in fish and fish eggs.

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