Antifungal activity of lichen extracts and usnic acid for controlling the saprolegniasis

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Abstract**—***Aquatic oomycetous fungi often cause serious damage to fresh water fishes. Antifungal activity of acetone extracts of three fruticose lichens namely,* Cladonia amaurocraea, Cladonia rangiferina *and* Usnea longissima *were investigated against three pathogenic oomycete fungi which can cause serious saprolegniasis:* Saprolegnia parasitica, Achlya bisexualis *and* Pythium *sp.. Usnic acid was also examined for antifungal activity against the pathogenic fungi. The minimum inhibitory concentration of usnic acid and lichen extracts for the tested fungi* Saprolegnia parasitica *and* Achlya bisexualis *were 2 mg* L^{-1}*, 200 mg* L^{-1} *respectively. The higher concentration was needed to inhibit mycelial growth of* Pythium *sp.. The result suggests that the potential of using usnic acid and lichen extracts for controlling the saprolegniasis.*

Keywords—antifungal activity, lichen, Oomycota, saprolegniasis, usnic acid.

I. INTRODUCTION

Saprolegniasis is a major disease problem and of great concern for the aquaculture industry. It plays a serious threat to fish populations in natural habitats. It is usually recognized as a fungal disease caused by any of several species of water molds of the family Saprolegniaceae, Oomycota, however, "oomycetous fungi" belong to the lineage of biflagellate "heterokont" organisms, commonly referred to as "stramenopiles", within the kingdom Chromista [1,2]. They do not belong to the kingdom Fungi, although they are usually studied by mycologists. *Saprolegnia parasitica* and *Achyla bisexalis* are the two most important oomycete fish pathogens. Some species in *Pythium* can also cause the saprolegniasis. Natural recovery by infected fish is almost impossible [1,3]. Malachite green was quite an effective antifungal agent but it is mutagenic, teratogenic and carcinogenic [4,5,6]. It has been banned since 1991 in many countries [7]. Formalin is the drug currently permitted by FDA for controlling fungus on fish diseases in the United States. Hydrogen peroxide, sodium chloride and bronopol are also worth mentioning in preventing and treating saprolegniasis [8]. Recently, nikkomycin Z was employed to inhibit the growth of the mycelium of *S. parasitica* [9]. Due to the lack of efficient methods to control pathogenic Saprolegniaceae, there is a dramatic reemergence of saprolegniasis in aquaculture. Thus, there is an important need for developing efficient and sustainable methods to stop the spread of these pathogens.

Lichens are symbiotic organisms of fungi and algae or cyanobacteria that can produce unique secondary metabolites and have been used in folk medicine since ancient times [10]. Studies in the last three decades proved the antimicrobial (antibacterial and/or antifungal), antiviral, antiprotozoal, antipyretic, antitumour, antiproliferative, anti-inflammatory, photoprotective, analgesic, as well as growth and enzyme inhibitory activities of some of the lichen extracts and compounds [10,11]. Various studies have demonstrated that many of lichen species contain usnic acid could play various biological roles and appear to function as allelopathic agents in nature. Usnic acid was proved to have the inhibitory effect on the growth of mold, bacterium, and yeast [12]. It was also reported that the acetone, methanol and light petroleum extracts of *Usnea* were effective against *Bacillus licheniformis, B. megatarium* and *Staphylococcus aureus* [13]. The results of study on the antibacterial activities of different solvent extracts of *Usnea florida* showed that the extracts had certain inhibition effect on *Staphylococcus aureus, Escherich coli, Pseudomonas aeruginosa, Micrococcus luteus,* and *Aspergillus flavus* [14].

The efficacies of usnic acid and the acetone extracts of three lichens (*Cladonia amaurocraea, Cladonia rangiferina* and *Usnea longissima*) were evaluated for controlling the growth of *Saprolegnia parasitica, Achyla bisexalis* and *Pythium* sp. in the present study. To the best of our knowledge, no information about the activities of lichen extracts and lichen acids against aquatic oomycetous fungi is available at present.

II. MATERIAL AND METHOD

Three species of lichen were used: *Cladonia amaurocraea*, *Cladonia rangiferina*, *Usnea longissima*. Three oomycetous fungal species included: *Saprolegnia parasitica, Achlya bisexualis* and *Pythium* sp.. *S. parasitica* was purchased from CBS (540.67). *A. bisexualis* (GHL 2012.2) and *Pythium* sp. (GHL 2012.3) were isolated from diseased fishes by using castor seeds. All three strains can infect tilapia and cause saprolegniasis. The strains were cultured on potato dextrose agar (PDA) slants and were stored at 4 ^{o}C until use. To obtain long-term growth cultures, the fungal strains were subcultured on 9-cm diameter PDA plates at 25 ^{o}C.

The lichen thalli were removed dust particles. The extracts were prepared by the extraction of 2 g of ground lichen in 30 ml acetone for five hours followed by filtration, at last get 20 ml lichen extracts. The stock solution of usnic acid was prepared by 20 mg usnic acid was fully dissolved in 20 ml acetone. The lichen extracts and usnic acid were stored at 4 ^{o}C for further bioassay. Final concentrations of stock solution for usnic acid and lichen extracts were 1 and 100 mg mL^{-1} respectively.

The medium of the following composition was prepared: dextrose 20 g, agar 20 g, potato 200 g, distilled water 1000 ml. The usnic acid and lichen extracts were mixed in 100 ml standardized medium, to give concentration of usnic acid were 32, 16, 8, 4, 2 mg L^{-1}, the lichen extracts were 3.2, 1.6, 0.8, 0.4, 0.2 g L^{-1}. In controls, 800 µl acetone was used in place of usnic acid and lichen extracts.

The pure culture of fungal species, growth and maintained on PDA medium at 25 ^{o}C was used for the experiments. Each treatment was duplicated three times. These treated Petri plates were incubated at 20 ^{o}C Radial growth for each test was recorded by taking the mean diameter of colonies from three plates. The measurements of colony diameter were made after different incubation periods depending on the hyphal growth rate of each species: 2 days for *Saprolegnia parasitica*; 2, 6 and 8 days for *Achlya bisexualis*; 2, 7, 8 and 10 days for *Pythium* sp.. Inhibitory effects of lichen compounds were determined by comparing treatment plates with control plates. The activity of each test compound was expressed as percent inhibition and, where appropriate, in both control and treated Petri dishes, was measured diametrically and the percentage inhibition of growth (% I) was calculated using the following formula.

$$I\ (\%) = (C\text{-}T)/C \times 100$$

Where C = Diameters of fungal colony in control plate, T = Diameters of fungal colony in treatment plate.

Data were subjected to one-way analysis of variance (ANOVA) using the Statistical program SPSS16.0, and means separation was made using Least Significant Difference (LSD). Difference on statistical analysis of data was considered statistically significant at $p < 0.05$.

III. RESULTS AND DISCUSSION

3.1 Activity of usnic acid

The activity of usnic acid concentration at 2, 4, 8, 16, 32 mg L^{-1} inhibited the growth of three pathogenic fungi is shown in Table 1. Usnic acid absolutely inhibited the mycelial growth of *Saprolegnia parasitica* at 32 mg L^{-1}. Compared with the activity of nikkomycin Z (about 100 mg L^{-1}, for totally inhibition), usnic acid has better effect to inhibit the growth of the mycelium of *S. parasitica* [9]. Mycelial growth of *Achlya bisexualis* and *Pythium* sp. are totally inhibited by usnic acid at 16 mg L^{-1}. Among all three oomycetous fungi, usnic acid showed good antifungal activity on *S. parasitica* at the concentration more than 4 mg L^{-1} (with percentage control >50%). The inhibitory potency of usnic acid against *S. parasitica, A. bisexualis* and *Pythium* sp. demonstrated that the Minimal Inhibition Concentration (MIC) to be 2 mg L^{-1}, 2 mg L^{-1}, 8 mg L^{-1}, respectively. The MIC was defined as the lowest concentration of significant inhibition of the visible mycelial growth. Recently, 30 fungicidal chemicals in agriculture were screened for antifungal activity against *Saprolegnia*, among them, kresoximmethyl and azoxystrobin showed very good effect in vitro with minimum inhibitory concentration (MIC) values of 1.0 and 0.5 mg L^{-1}, respectively [15]. However, the MIC of azoxystrobin was close to its safe concentration (SC 0.553 mg L^{-1}) and kresoximmethyl showed higher toxicity to goldfish (*Carassius auratus*) with MIC>SC (0.131 mg L^{-1}), thus, they may need chemical modifications to enhance their inhibitive effects or could be used in combination with other drugs [15]. In our study, That acute toxicity to goldfish of usnic acid performed as described in literatures [15,16] with SC (4.152 mg L^{-1}) showed it can be used to control the saprolegniasis caused by *S. parasitica* and *A. bisexualis*. Among the three species of oomycetous fungi tested, *S. parasitica* showed high susceptibility to the usnic acid. At concentration of 2, 4 mg L^{-1}, usnic

acid showed growth promoting activities for *Pythium* sp. (Table 1). *S. parasitica* grows very quickly and the colony could cover the whole Petri dishes for about 2 days, while *Pythium* sp. grows slowest among the three oomycetous fungi.

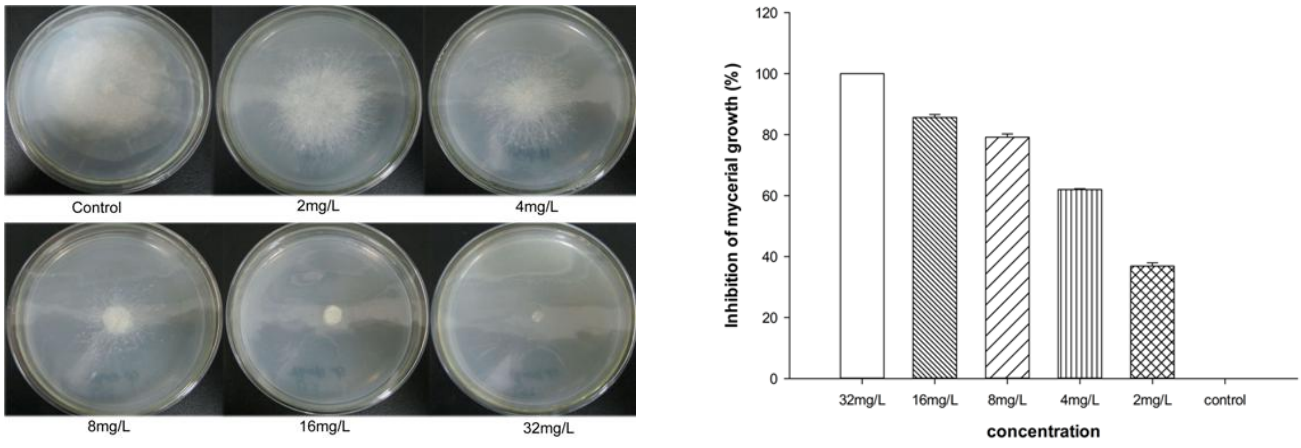
3.2 Activity of lichen extracts

Acetone extracts of *Usnea longissima*, *Cladonia rangiferina* and *Cladonia amaurocraea* at different concentrations (200, 400, 800, 1600, 3200 mg L^{-1} and with 800 µl acetone as control) for antifungal activity against *Saprolegnia parasitica*, *Achlya bisexualis* and *Pythium* sp. with their percentage inhibition, which is an average of three replicates and its standard error are given in Table 2. As an example, Mycelial growth of *S. parasitica* in different concentrations of *Cladonia rangiferina* extracts was also shown in Fig. 1 a & b. Effects of lichen extracts vary at different concentrations tested. In all cases, the fungal growth was completely inhibited with each extract at the concentration 3200 mg L^{-1}. No mycelial growth was observed from all discs treated with extracts of *U. longissima* and *C. rangiferina* on *Pythium* sp.; the same results were observed on *Achlya bisexualis.*

In the present study, the three lichens extracts significantly reduced the mycelial growth of the oomycetous fungi. Acetone extracts of the three lichens were found to show excellent activity against all the test fungi except *Pythium* sp. when compared with other two fungi. At a low concentration, extracts of lichens showed growth promoting activities for *Pythium* sp.

Among three lichen species, the acetone extract of *C. amaurocraea* showed minimum inhibition against *Saprolegnia parasitica* of 36 % at the concentration of 200 mg L^{-1}. Different concentrations of extracts exhibited significantly different antifungal activity. In the case of the fungal growth was completely inhibited with each extract with the concentration 3200 mg L^{-1}. Minimal inhibitory concentration (MIC) of acetone extract of tested lichen was determined and shown in TABLE 3. The minimal inhibitory concentration of the extracts against *Saprolegnia parasitica* and *Achlya bisexualis* is being found to be 200 mg L^{-1}, in contrast against *Pythium* sp. was 800 mg L^{-1} (TABLE 3).

The acetone extract effects of *U. longissima*, *C. rangiferina* and *C. amaurocraea* at different concentrations on the mycelial growth of *Achlya bisexualis* and *Pythium* sp. are also shown in TABLE 2. All extracts gave almost the same minimum inhibitory concentrations against *S. parasitica* and *A. bisexualis* (TABLE 3). The results indicate that mycelial growth of *A. bisexualis* is totally inhibited by extracts of the three lichens at 1600 mg L^{-1} and 3200 mg L^{-1}. At the concentration of 200 mg L^{-1} and 400 mg L^{-1} extracts of three lichens showed growth promoting activities for *Pythium* sp.. While extract of *C. amaurocraea* also showed growth promoting activities for *Pythium* sp. at the concentration of 800 mg L^{-1}. Acetone extracts of *U. longissima* was the better growth inhibitor when compared with acetone extracts of *C. rangiferina* and *C. amaurocraea* (TABLE 2). In the case of the extracts of *U. longissima* and *C. amaurocraea*, usnic acid should be effective compound, while for *C. rangiferina*, atranorin and fumarprotocetraric acid or their combination may be the main agents.



(A) **(B)**

FIGURE 1: (A): MYCELIAL GROWTH OF *SAPROLEGNIA PARASITICA* IN DIFFERENT CONCENTRATIONS (100×) OF *CLADONIA RANGIFERINA* EXTRACTS IN POTATO DEXTROSE AGAR MEDIUM.
(B) EFFECT OF DIFFERENT CONCENTRATIONS (100×) OF *CLADONIA RANGIFERINA* EXTRACTS ON THE MYCELIAL GROWTH INHIBITION OF SAPROLEGNIASIS PATHOGEN *SAPROLEGNIA PARASITICA*. ERROR BARS REPRESENT THE STANDARD ERROR OF THE MEAN.

TABLE 1
EFFECT OF USNIC ACID ON THE MYCELIAL GROWTH AND PERCENTAGE CONTROL OF *SAPROLEGNIA PARASITICA*, *ACHLYA BISEXUALIS* AND *PYTHIUM* SP.

Fungal strain	Percentage control at various concentrations of usnic acid at 20 °C					
	0 mg/L	2 mg/L	4 mg/L	8 mg/L	16 mg/L	32 mg/L
Saprolegnia parasitica CBS 540.67	0	36.8±1.10	62.0±0.29	79.1±1.05	85.6±0.94	100±0
Achlya bisexualis GHL 2012.2	0	2.55±1.93	13.2±1.25	49.6±1.66	100±0	100±0
Pythium sp. GHL 2012.3	0	-19.5±3.05	-5.6±3.78	23.5±1.68	100±0	100±0

TABLE 2
ANTIFUNGAL ACTIVITY OF ACETONE EXTRACTS OF *USNEA LONGISSIMA*, *CLADONIA RANGIFERINA* AND *CLADONIA AMAUROCRAEA*

Lichen	Concentration	Percentage inhibition at various concentrations at 20 °C(%)		
		Saprolegnia parasitica	*Achlya bisexualis*	*Pythium* sp.
Usnea longissima	0 mg/L	0	0	0
	200 mg/L	66.82±0.31 b	33.08±1.13 a	–49.45±5.00 a
	400 mg/L	70.73±0.64 c	60.34±0.30 b	–24.75±4.57 b
	800 mg/L	58.07±1.44 a	100±0.00 c	33.30±4.95 c
	1600 mg/L	79±1.52 d	100±0.00 c	100±0.00 d
	3200 mg/L	100±0.00 e	100±0.00 c	100±0.00 d
Cladonia rangiferina	0 mg/L	0	0	0
	200 mg/L	41.76±1.15 a	12.00±1.85 a	–1.44±16.8 a
	400 mg/L	62.57±1.54 b	32.59±1.61 b	–1.11±8.50 a
	800 mg/L	79.39±0.87 c	67.59±1.45 c	–9.47±6.31 b
	1600 mg/L	89.40±0.42 d	100±0.00 d	100±0.00 c
	3200 mg/L	100±0.00 e	100±0.00 d	100±0.00 c
Cladonia amaurocraea	0 mg/L	0	0	0
	200 mg/L	36.13±1.46 a	12.60±0.69 a	–23.85±2.64 a
	400 mg/L	59.71±1.47 b	28.35±1.17 b	–2.00±1.39 b
	800 mg/L	77.86±1.18 c	64.84±1.50 c	24.78±2.58 c
	1600 mg/L	87.31±0.37 d	100±0.00 d	80.42±0.63 d
	3200 mg/L	100±0.00 e	100±0.00 d	100±0.00 e

a-e: Different small letters indicate significant differences among 5-level concentration within the same lichen extract (One-way ANOVA and Least Significant Difference (LSD), P< 0.05). Data are mean ± SE (standard error) based on n = 3.

TABLE 3
MINIMAL INHIBITORY CONCENTRATION (MIC) OF USNIC ACID AND LICHEN EXTRACTS AGAINST PATHOGENIC OOMYCETOUS FUNGI

Fungal strain	The MIC of Usnic acid (mg L^{-1})	The MIC of lichens tested (mg L^{-1})		
		Usnea longissima	*Cladonia rangiferina*	*Cladonia amaurocraea*
Saprolegnia parasitica CBS 540.67	2	200	200	200
Achlya bisexualis GHL 2012.2	2	200	200	200
Pythium sp. GHL 2012.3	8	800	1600	800

IV. CONCLUSION

In conclusion among all the lichen extracts and usnic acid tested for antifungal activity on the oomycetous fungi, good efficacy was demonstrated, except at a low concentration, the growth promoting activities for *Pythium* sp. were observed. Therefore, lichen secondary metabolites could be used as an effective agent for the treatment against the infection of some pathogenic oomycetous fungi. The lichens have the potential for use in developing a novel therapy to control saprolegniasis in aquaculture.

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