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RESEARCH ARTICLE

IN VITRO SCREENING OF ANTIFUNGAL ACTIVITY OF AQUEOUS EXTRACTS OF SOME MEDICINAL PLANTS AGAINST PATHOGENIC FUNGI *SAPROLEGNIA PARASITICA*

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Abstract

The objective of the study was to evaluate the antifungal activity of aqueous extracts of *Brassica nigra, Andrographis paniculata, Targetes erectus* and *Pinus roxberghaii* against *Saprolegnia parasitica*. Pathogenic fungus *Saprolegnia parasitica* was isolated from fungal infected specimen of cold water fish *Tor putitora.* SDA (Sabouroud's Dextrose agar) was used to obtain bacteria free pure culture of the test fungus. Aqueous extracts of leaves of all the test plants prepared at three different concentrations (5%, 8%, 10% w/v) were tested against the mycelial mass of the *Saprolegnia parasitica* for three different time periods (20 min., 40 min., and 60 min.). Results revealed that all the tested extracts possess high degree of antifungal activities against the test pathogen. Extract of *Brassica nigra* was found most effective with MIC (minimum inhibitory concentration) of 5% w/v concentration for 20 min. exposure time.

Introduction

Fisheries and aquaculture contribute significantly to foods and nutrition security. The last few decades have witnessed the great advances in the productivity of the fisheries sector in India. But unfortunately in the quest of higher economic returns, increasing production by adopting high yielding techniques, and maximum utilization of all the niches of aquaculture system, we usually forget about the health of the fish. Disease is the single largest cause of economic losses in aquaculture (Meyer, 1991). Fungal infections are second only to bacterial diseases in economic importance. In general, the diseases caused by fungi are known as "MYCOSIS" and those in fishes are called as "FISH MYCOSIS". Mainly genus *Saprolegnia* has been accused of causing mycosis in fishes (Srivastava, 1987). *Saprolegnia parasitica* (Saprolegniaceae) is very common pathogenic fungus infecting cold water fish and their eggs. Most of the chemical which are used for the treatment of fungal disease have restricted use due to their toxicity and persistence in the environment (Gieseker *et al.,* 2006; Meyer and Jorgenson, 1983). Malachite green is very effective fungicide but it is carcinogenic, mutagenic and teratogenic (Meyer and Jorgenson, 1983; Meinertz *et al.,* 1995). Hence present age of green and organic aquaculture demands the use of some ecologically safe, effective and economically feasible herbal products for the treatment of fungal infections. Plants have been known for their medicinal and antimicrobial properties since ancient times. Rohani *et al.* (2006) announced that *Zataria multiflora* is an appropriate alternative of malachite green. Antifungal activities of Lemon grass, Lantana, Nerium, Basil and Olive leaves have also been demonstrated *in vitro* by Meyer & Jorgenson (1983). There are numerous reports which show that certain plant extracts are highly effective in suppressing the growth of fungal infections in agricultural crops but use of plant extract to control fungal growth in freshwater fish is scarce in the literature (Rai *et al.,* 2002). Therefore, the present investigation was aimed at *in vitro* screening of antifungal activity of aqueous extracts of *Brassica nigra, Andrographis paniculata, Targetes erectus* and *Pinus roxberghaii* against pathogenic fungi *S. parasitica*.

MATERIALS AND METHODS

Experiment was carried out at Directorate of Coldwater Fisheries Research (DCFR), Bhimtal (Latitude $29^0$21´ N, Longitude $79^0$34´ E, altitude 1370 m MSL) Uttarakhand, India.

Isolation of test fungus: Fungal infected samples of cold water fish *T. putitora* were collected from the experimental cemented fish tanks of DCFR, Bhimtal (Fig.-1). Infected tissues were dissected out to isolate the pathogenic fungi. Tissue samples were homogenized with sterilized double distilled water in a pestle and mortar. The resultant solution was incubated in petri plate having the hemp seeds as bait (Butty *et al.,* 1989). After 2-3 days white cottony fungal colonies appeared on the hemp seeds (Fig.-2). These fungal colonies were then washed with sterile double distilled water for 3-4 times and transferred to new petri plates with some new hemp seeds and incubated at 14-16^0C for 48-72 hours. A small piece of fungus was cut out from it, rinsed with sterile distilled water and transferred to SDA (Sabouroud's Dextrose agar) plate under aseptic condition. Again a small piece of fungus was cut out from SDA plate, rinsed with sterile distilled water and transferred to a new SDA plate. This process was repeated for 4 to 5 times to obtain a bacteria free, pure fungal culture. Fungus was identified as *Saprolegnia parasitica* as per the methods described by Coker and Matthews (1937), Beakes *et al.* (1994) and Khulbe (2001) (Fig.-3&4).

Procurement of test plants and preparation of aqueous extracts: *B. nigra, T. erectus, P. roxberghaii* and *A. paniculata* were selected for the present study on the basis of their antimicrobial properties. Leaves of *A. paniculata* were obtained from Research Center, Central Institute of Medicinal and Aromatic Plants (CIMAP), Pantnagar while Leaves of *P. roxberghaii* were collected from the forest area adjacent to DCFR, Bhimtal. Plants of *B. nigra, T. erectus* were obtained from DCFR premises. For aqueous extract preparation, leaves of all the test plants were washed with clean tap water several times, rinsed with sterilized distilled water and left for air drying. Air dried leaves were grind well in grinder to obtain fine powder. This fine powder was then soaked in sterilized double distilled water for 48 hours at room temperature at three different concentrations (5%, 8%, 10% w/v) and sieved through Whatman filter paper. Finally the extracts were filtered through the 0.22 µm syringe filter to obtain bacteria free extracts and preserved into refrigerator at low temperature of 4°C until used.

Mycelial growth inhibitory test: The agar plugs of 6 mm diameter were cut from the active growing fungal colony and immersed into aqueous extracts of different concentrations for three different time periods (20 min., 40 min., and 60 min.). Such treated agar plugs were put over SDA plates under aseptic condition and allowed to incubate at 18-20^0C for 48 hours (Fig.-6-9). Agar plugs immersed into sterile double distilled water for different time periods (20 min., 40 min., 60 min.) were serving as control (Fig.-5). Experiment was performed in triplicates. Results obtained were subjected to two-way ANOVA at 5% level of significance.

RESULTS

Mycelial growth after treatment with aqueous extracts at the different concentrations and exposure times is given in table-1. Minimum inhibitory concentrations (MIC) of aqueous extracts of test plants are presented in table-2.

DISCUSSION

Similar inhibitory properties of essential oils of five plants from asteraceae family were reported by Rai *et al.* (2002) against the fungus, *Saprolegnia ferax.* Zaidan *et al. (*2005) screened out *in vitro* antibacterial activity of *A. paniculata* using disc diffusion. Bobbarala (2009) investigated the antimicrobial activities of *A. paniculata* against phytopathogenic bacteria and fungi. Khomavilai *et al.* (2006) announced that horse radish extract is the effective antifungal against Saprolegniosis and can stop the *S. parasitica* growth with concentration of 68 mg/l for 60 minutes exposure time. Ilondu *et al.* (2009) used the aqueous extracts of *Vernonia amygdalina* for the control of saprolegniosis in *Clarias gariepinus*. Bokhari (2009) investigated the antifungal activities of *Cymbopogon. citrates, Lantana camara*, *Nerium oleander, Ocimum basilicum* and *Olea europaea* with methanolic extract (*in vitro)* against different pathogenic fungi including *Microsporum canis, M. gypseum,* and *Trichophyton mentagrophytes* and observed that the extract of *C. citrates* was most effective followed by *Lantana*. Similar to our findings Azizi *et al.* (2012) investigated the effect of aquatic and alcoholic extracts of *Citrullus colocynthis* on growth of the *S. parasitica.*

The results not only reflect the antifungal activity of aqueous extracts of all the tested plants against *S. parasitica* but also hold great prospect especially for the peasant local fish farmers who cannot afford the high cost of chemicals and synthetic fungicides. Further *in vivo* studies are suggested, which could reduce the severe losses due to saprolegniosis in eggs and larvae of cold water fish during breeding and rearing.

Table-1: Colony diameter (mm) of *S. parasitica* after treatment of plant aqueous extracts at various concentrations and exposure times.

	Andrographis paniculata			*Pinus roxberghaii*			*Targetes erectus*			*Brassica nigra*		
Extract conc. (%w/v)	Exposure time (min.)			Exposure time (min.)			Exposure time (min.)			Exposure time (min.)		
	20	40	60	20	40	60	20	40	60	20	40	60
Control	38±2	38±1	38±2	38±2	38±1	38±2	38±2	38±1	38±2	38±2	38±1	38±2
5	23±4*****	15±2*****	7±1******	14±2*****	12±2******	11±2******	14±2*****	13±2*****	13±1*****	0±0******	0±0******	0±0******
8	18±1*****	7±1******	0±0******	9±1******	8±1******	0±0******	0±0******	0±0******	0±0******	0±0******	0±0******	0±0******
10	9±1******	0±0******	0±0******	0±0******	0±0******	0±0******	0±0******	0±0******	0±0******	0±0******	0±0******	0±0******

*****significant difference with control ($p<0.05$) ****** highly significant difference with control ($p<0.05$)

Table 2: MIC for the different plant extracts

Plant Extracts	**Minimum Inhibitory Concentration (MIC)**
Andrographis paniculata	8% w/v conc. with 60 min. exposure time
Targetes erectus	8% w/v conc. with 20 min. exposure time
Pinus roxberghaii	8% w/v conc. with 60 min. exposure time
Brassica nigra	5% w/v conc. with 20 min. exposure time

Figure-1. Fungal infected *Tor putitota*

Figure-2. Isolation of *S. parasitica* using hemp seeds

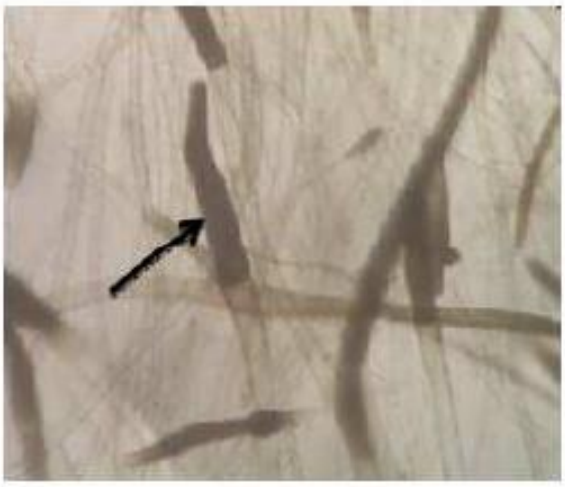


Figure-3. Mycelial mass of *S. parasitica* with Zoosporangia (20X)

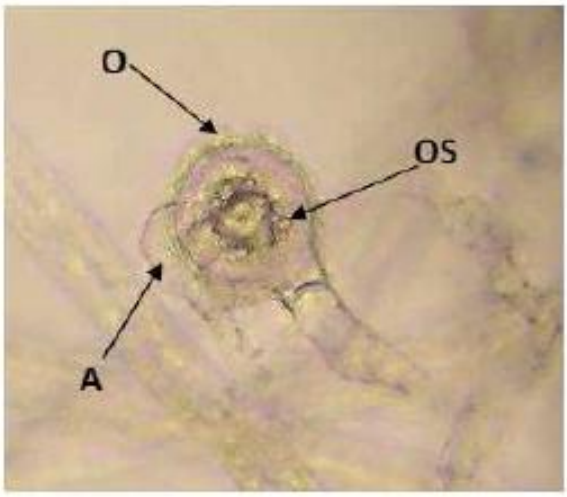


Figure-4. Fertilized oogonia of *S. Parasitica* (20X) (OS- Oosphere, A- Antheridium, O- Oogonium)

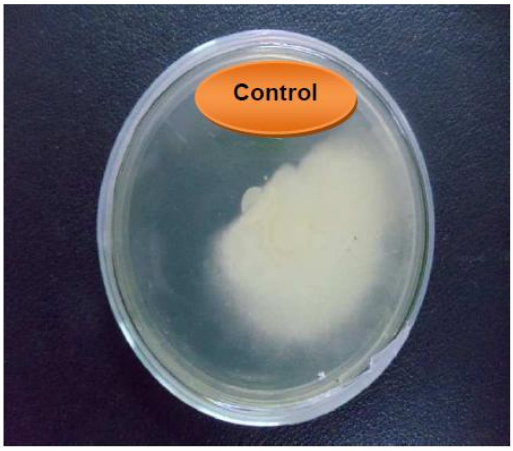
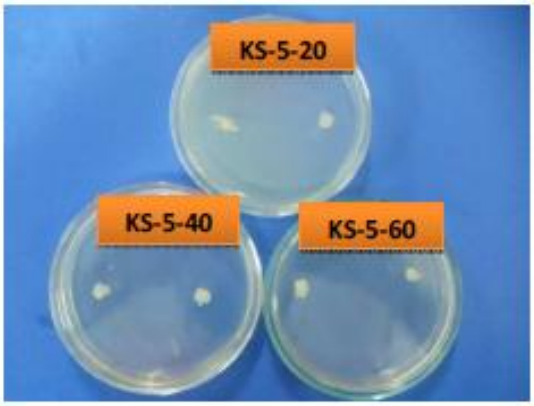
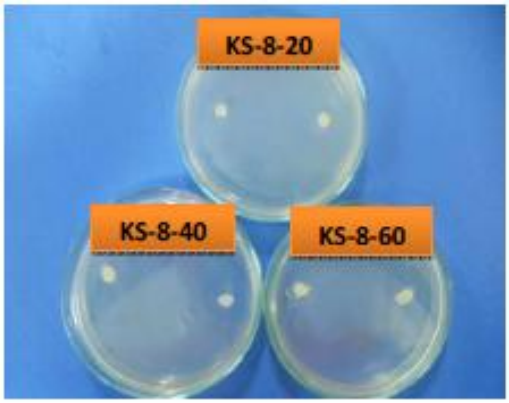


Figure-5. Control: Colony diameter of *S. parasitica* without any treatment of plant extract





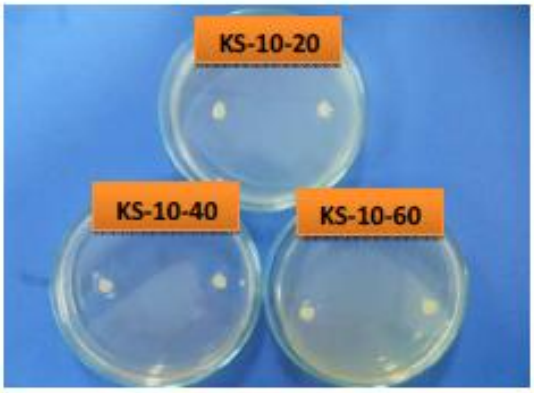
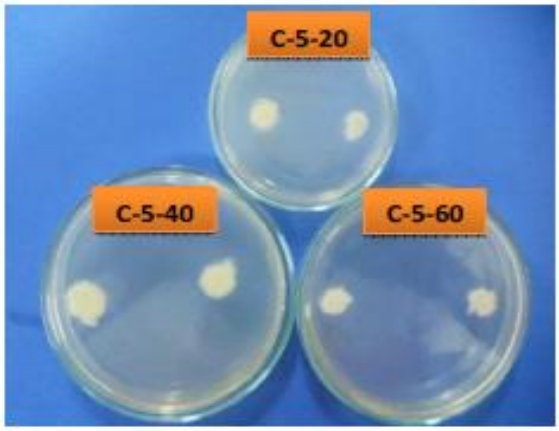
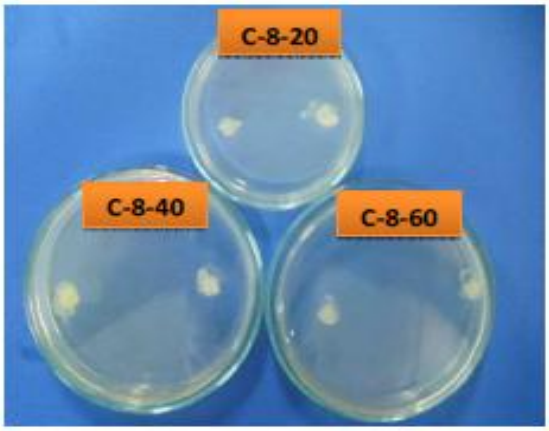


Figure-6. Mycelial growth after treatment with *Brassica nigra* leaf extract

KEY: KS-5-20: *B. nigra* leaf Extract of 5% conc. with 20 min. exposure time (Similarly other codes can be interpret)





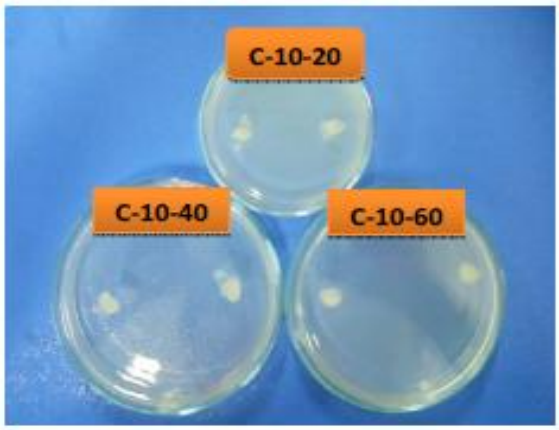
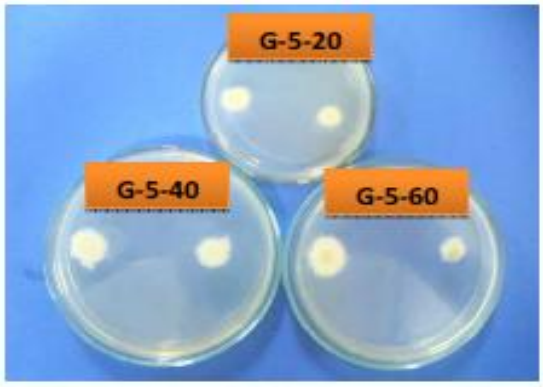
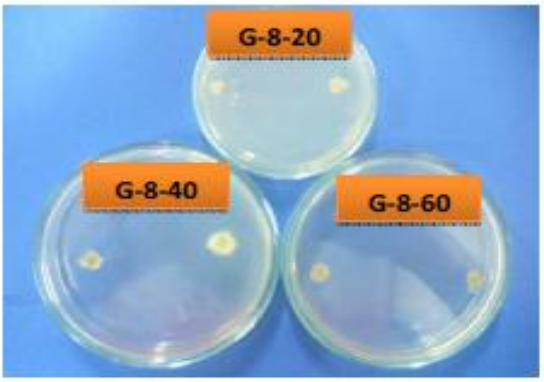


Figure-7. Mycelial growth after treatment with *Pinus roxburghaii* leaf Extract

KEY: C-5-20: *P. roxburghaii* leaf Extract of 5% conc. with 20 min. exposure time (Similarly other codes can be interpret)





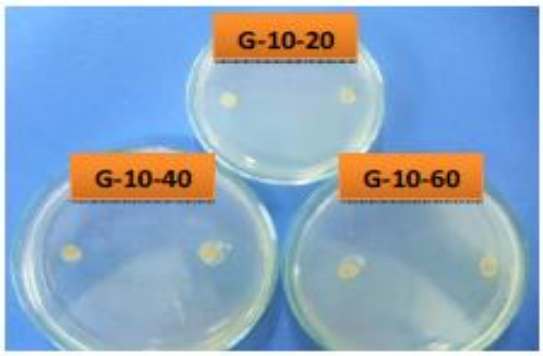


Figure-8. Mycelial growth after treatment with *Targetes erectes* leaf Extract

KEY: G-5-20: *T. erectes* leaf Extract of 5% conc. with 20 min. exposure time (Similarly other codes can be interpret)

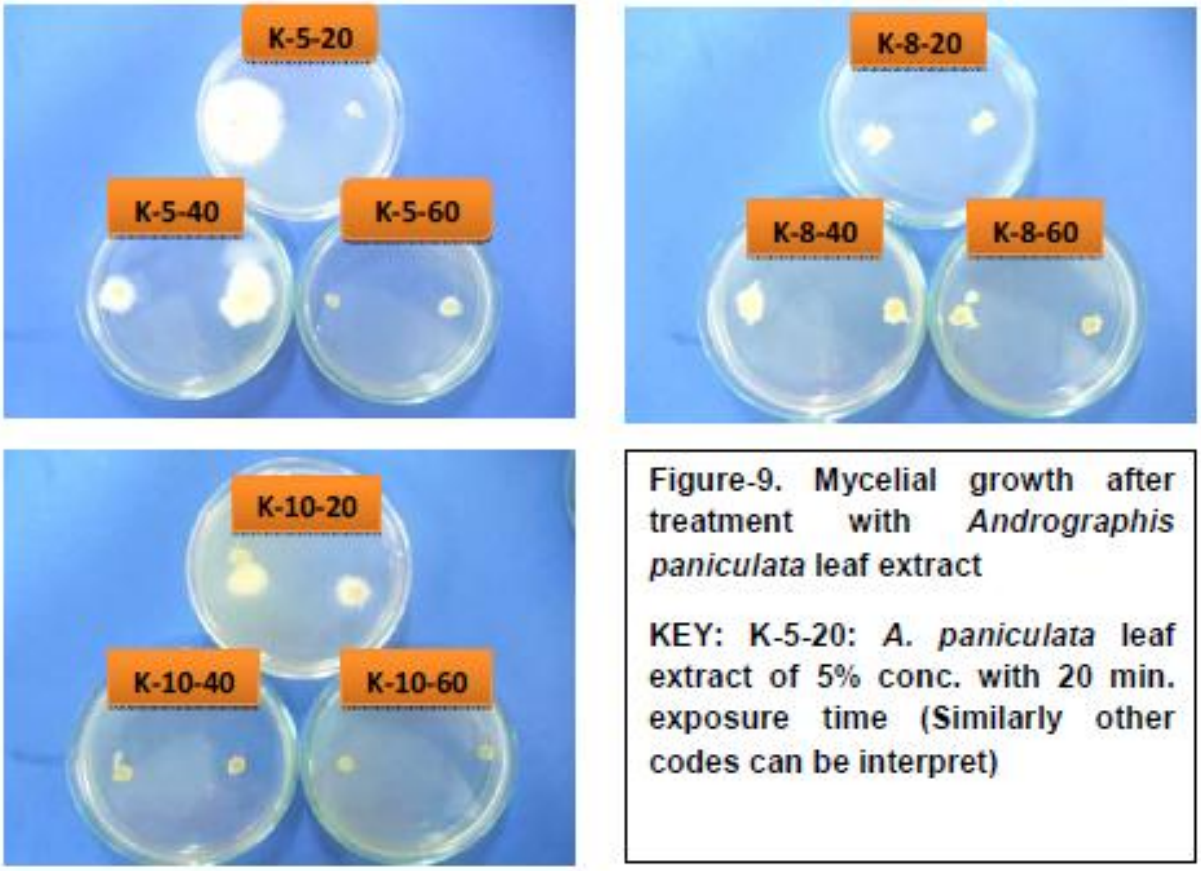


Figure-9. Mycelial growth after treatment with *Andrographis paniculata* leaf extract

KEY: K-5-20: *A. paniculata* leaf extract of 5% conc. with 20 min. exposure time (Similarly other codes can be interpret)

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