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A STUDY ON THE EFFECT OF CHEMOTHERAPY AND MEDICINAL PLANT EXTRACTS ON FUNGAL FISH PATHOGENS

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ABSTRACT

Pancreatic alpha amylase (PAA) inhibitory activity of aqueous extracts of medicinal plants was evaluated in vitro to search new anti-diabetic agents as alternatives to synthetic medicines. Rhizome and leaves of C. longa and leaves of Moringa oleifera L., Azadirachta indica L., Psidium guajava L. and Murraya koenigii L were extracted with hot water and six extracts were tested for presence of PAA inhibitory activity quantitatively and their modes of inhibition were determined. Presence of alpha amylase inhibitors were identified in all extracts in quantitative assay. Aqueous extract of leaves and rhizome of C. longa showed highest anti-amylase potential with IC_{50} values of 0.53±0.10 and 0.96±0.29 mg/ml respectively. IC_{50} values of other extracts ranged between 1.24±0.49 to 4.50±0.38 mg/ml. Three highest inhibition potential showing extracts of C. longa leaves and rhizome and aqueous extract of Moringa oleifera displayed non-competitive, mixed and non-competitive mode of inhibition respectively as determined in terms of changes in Vmax and km values. In conclusion, active constituents of these three extracts possess anti-diabetic properties and can be used in management of diabetes mediated complications.

KEY WORDS

Alpha amylase, hyperglycemia, inhibition potential, medicinal plants, turmeric

1.INTRODUCTION

Fish disease caused by fungal infection is common in both farmed and wild fishes throughout the world. Fungal disease in water bodies and fish culture systems in Tamil Nadu has also been recognized as one of the emerging disease problems in fish production (Habib *et al.,* 2007). Fungi are primarily regarded as a secondary invader and known to attack the host when it gets injured either mechanically or as a result of infection (Scott and O' Bier, 1962). The family Saprolegniaceae contains the majority of fungi that have been associated with diseases in fish and shellfish such as *Aphanomyces, Saprolegnia, Leptolegnia, Achlya* and *Dictyuchus* (Srivastava, 1980).

Chemotherapeutics is mainly used in aquaculture for the treatment and prophylaxis of disease problems. An advantage of chemical application is that it achieves quick result (Tonguthai, 1996). Successful chemotherapy against fungal diseases have been reported using different treatments. The fungicides used are 'protectant' rather than 'systemic' and are applied to the water in which they are held rather than to the fish themselves (Willoughby and Roberts, 1994). In laboratory treatment trial, Scott and O' Warren (1964) recommended 2 ppm concentrations of malachite green for complete destruction of fungal hyphae within 24-72 h. treatment. Sati and Khulbe (1985) found 0.05% formalin treatment for 20 minutes to be effective against some water molds while Roberts *et al.* (1992) reported that only very low concentrations (0.25 ppm) of malachite green are required to kill *Saprolegnia parasitica* zoospores and cysts and Das and

Das (1993) suggested lime is suitable to control EUS through improving or disinfecting the water quality.

Medicinal plants are part and parcel of human society to combat diseases from the dawn of civilization (Biswas *et al.,* 2002). Different types of medicinal plants are now used against fish disease in Asia but its application and research on the herbal therapy is still under experimental stages. Kraus (1995) and Khan (2001) found that extracts of neem fruit, seeds, seed kernel, twigs, stem bark and root have fungicidal and bactericidal properties. The snake-head fish farmers used the bark of cork wood tree (*Sesbania grandiflora*) for the treatment of haemorrhage lesions (Direkbusarakom, 2000) to counter EVS in Thailand and Talukder (2005) reported that extracts of *Azadirachta indica*, *Curcuma zedoaria* and *C. longa* were inhibitory against the fungal growth.

Considering the importance, the present study was attempted to develop a suitable control measure using low-cost chemotherapeutics and locally available medicinal plants.

2.MATERIALS AND METHODS

2.1 Selection of Pathogens

Due to low virulent characters of laboratory stock pathogens, fresh isolates were collected from diseased organisms in this study. Characterization, identification and pathogenicity of the fungal isolates were determined according to the techniques followed by Zahura (2001), Habib (2002) and Muniruzzaman (2004). Newly isolated *Aphanomyces invadans*, *Saprolegnia* sp. and *Achlya* sp. were detected as strong to moderate virulents based on their pathogenic performance and selected for further studies.

2.2 Selection of Chemotherapeutics

Based on preliminary investigations, availability in markets and reports of the previous workers, four different chemotherapeutics – lime + salt, brilliant green (BDH Chemicals), methylene blue (Loba Chemie) and malachite green (Merck Ltd.) were selected for this study in various doses.

2.3 Selection of medicinal plants and extract preparation

Medicinal plants were primarily selected based on their recognized medicinal properties (Dastur, 1977; Anawer, 2001; Muniruzzaman and Chowdhury, 2004; Talukdar, 2005). Turmeric (*Curcuma longa)*, Zedoary holud (*C. zedoaria),* and mixed extracts of neem + turmeric and neem + Zedoary were selected for the present study.

The desired parts of plants were washed with clean water, rinsed with sterilized distilled water and cut into small pieces. Before making the crude extract, each medicinal plant was weighed and then the paste was prepared using a homogenizer. Fibrous particulates from the extracts were screened out by pressing through a fine meshed cloth and finally filtered through Whatman 541 filter paper to get fine extracts. Crude extracts were then collected in conical flasks and preserved in refrigerator at low temperature for future use.

2.4 *In vitro* efficacy test

In vitro efficacy test of the fungicidal chemotherapeutic and medicinal plants against the fish pathogenic fungi were carried out in separate vials, containing 10 ml GP broth. Subculture technique of fungal isolates was performed following the method described by Muniruzzaman (2004) and Talukdar (2005). The fungi were sub-cultured aseptically on GP agar plates and incubated at 22 °C for 18 h and the pattern of fungal growth was observed daily up to 10 days. For *in vitro* test of chemotherapeutics, agar blocks containing fungal hyphae were cut by sterile Cork Borer and kept into individual vials containing 10 ml GP broth and 1 ml of each chemotherapeutic agent. Four different doses (Table-1) of fungicidal agents were used and the minimum inhibitory dose (MID) was detected through the observation of inhibitory growth performances of fungus. Effective dose was determined when growth of fungal mycelium was fully stopped. The inactive fungal mycelium block was further placed on GP-agar plate to assess the efficacy of chemotherapeutic. No fungal growth indicated the appropriate dose for each case.

For *in vitro* test of medicinal plants, extracts of different doses (Table-2) were taken into vials containing 10 ml GP broth and a fungal block and incubated at 22°C. No extract was mixed in the case of control. Efficacy of mixed plant extracts was also tested by mixing the extract of *A. indica* together with *C. zedoaria* and *C. longa* at a ratio of 1:1. Treated hyphal blocks were then re-cultured to ensure their inhibition of growth. The MID of the effective plants tested was found to vary with different fungal species.

Table-1 Selected chemotherapeutics with their varied doses applied on fungal isolates

Sl. No.	Name of fungicidal chemicals	Doses
1.	Salt + lime	0.5% + 10 ppm 1.0% + 15 ppm 1.5% + 20 ppm 2.0% + 25 ppm
2.	Brilliant blue	1.1 ppm 1.2 ppm 1.3 ppm 0.4 ppm
3.	Malachite green	1.4 ppm 0.6 ppm 0.8 ppm 1.0 ppm
4.	Methylene blue	2 ppm 4 ppm 6 ppm 8 ppm

Table-2 Various doses of selected medicinal plants applied on pathogenic fungal isolates

Sl. No.	Name of medicinal plants	Doses
1.	Tumeric (bulb)	20 mg/ml 15 mg/ml 10 mg/ml 5 mg/ml
2.	Zedoary (bulb)	20 mg/ml 15 mg/ml 10 mg/ml 5 mg/ml
3.	Neem (leaf) + Zedoary (bulb)	20 mg/ml 15 mg/ml 10 mg/ml 5 mg/ml
4.	Neem (leaf) + Turmeric (bulb)	20 mg/ml 15 mg/ml 10 mg/ml 5 mg/ml

Table 3 Effect of chemicals on experimentally infected fish with fungal pathogens

Treatment	Dose (mg/l)	Recovery (%)
T_1: Salt + lime	2.0% + 25 ppm	80.00 ±2.89
T_2: Brilliant green	0.3 ppm	80.00 ±2.31
T_3: Methylene Blue	6 ppm	70.00 ± 1.73
T_4: Malachite Green	0.8 ppm	80.00 ±1.15
T_5: Control	No dose	0 g

Table 4 The effect of medicinal plants on experimentally infected fish with fungal pathogens

Treatment	Dose (mg/l)	Recovery (%)
T1: Turmeric (*C. longa)*	20	80.00 ± 1.15
T2: Zedoary *(C. zedoaria)*	20	60.00 ±5.77
T3: Neem + Zedoary (*A. indica* + *C. zedoaria)*	15	70.00 ± 3.33
T4: Neem + Turmeric *(A. indica* + *C. longa)*	15	80.00±2.89
T5: Control	No extract	0

2.5 Investigation on the therapeutic effects (*in vivo*)

Preparation of zoospore suspension and abrasion of fish: Zoospore suspension of the pathogenic fungal isolates was prepared as described by Willoughby and Roberts (1994) and Lilley (1997). These zoospores were used for experimental infection. Disease free young weighing 15 to 20 gm were used. Each group of fish (10 fish per 15 litre of water) was exposed to zoospore suspension of (30 ml autoclave pond water (APW) containing zoospore/1 litre tap water.) *A. invadans*, *Saprolegnia* sp. and *Achlya* sp. separately under aerated condition in a glass aquarium. Controlled fishes were released into the aquarium having only APW and no zoospore. After few days, the fungal infection was observed and clinical signs of infection, moribundness, death of fish and recovery of disease were recorded for each aquarium.

Chemotherapeutic effects on the infectivity of fungi: After releasing motile zoospores in each aquarium, the infection was observed within few days. The exposed fish were transferred to other aquaria for chemotherapy. The treatments selected for this purpose were T1 (salt + lime), T2 (brilliant green), T3 (methylene blue), T4 (malachite green) and T5 (control). Short bath treatment was applied separately in individual chemotherapeutics for about 30 minutes twice per day. Chemotherapeutic trials was continued up to 10 days with the same dosage.

Therapeutic effects of medicinal plants: Extracts of turmeric, Zedoary, and two mixed extracts of neem + Zedoary and neem + turmeric were used for treatment trials (T1, T2, T3 and T4 respectively) whereas control (T5) received no plant extract. Dip bath treatment was applied to observe their therapeutic effects. The whole process was performed according to studies conducted by Muniruzzaman (2004) and Talukdar (2005). Clinical signs were observed and recorded. After 10 days of experimental period, fish muscle from the abraded location was aseptically removed, placed on GP agar and incubated at 25ºC to check fungal growth.

3. RESULTS AND DISCUSSION

The present study was designed to examine the medicinal efficacies of some selected chemotherapeutics and available medicinal plants against fungal fish pathogens causing diseases in cultured fishes. Growth of fungus was found to be affected by different doses of various chemotherapeutics under *in vitro* condition. Brilliant green (MID 0.3 ppm) was effective against all the three fungal isolates. Salt + lime and malachite green (MID 2.0% + 25 ppm and 0.8 ppm, respectively) showed better performances against fungal isolates than that of methylene blue (MID 6 ppm).

Literature reveals that Chowdhury *et al.* (2003) reported a combination of salt and lime to suppress the effect on the growth of Aphanomyces in GP broth under laboratory conditions while Scott and O' Warren (1964) used 2 ppm of malachite green for complete destruction of fungal hyphae within 24-72 h treatment and Roberts *et al.* (1992) found that 0.25 ppm of malachite green was required to kill *S. parasitica*. Nevertheless, Borghetti *et al.* (1994) found no significant differences among mortality rates after evaluating the efficiency of different treatments of malachite green and methylene blue.

In the present study, the use of chemotherapeutic treatments which contained a suspension of salt + lime together (T1) was found to be significantly ($p < 0.05$) effective (80 ⍰ 2.88% recovery rate) in reducing *A. invadans*, *Saprolegnia* and *Achlya* infection in fish. This is similar to the findings of Alam (2004). The use of Brilliant green (T2) also appeared to be a good fungicide with 80.00 ⍰ 2.31% recovery rates. However, the application of malachite green (T4) eventhough was effective (80.00 ⍰ 1.15% recovery rate) in reducing the *Saprolegnia* and *Achlya* infection, it was very weak

against *A. invadans* infection. This result is similar to the observations made by Muniruzzaman (2004) and Alam (2004). The present study also reveals that the use of methylene blue (T3) had a medium effect with fewer (70.00 ± 1.73%) recovery rate. The therapeutic effects of the tested chemotherapeutic are shown in Table-3.

In the case of medicinal plants, the extracts of turmeric showed best inhibitory response against all the fungi tested whereas the extracts of Zedoary exhibited medium effects at the same dose. In the case of mixed plants extracts, inhibitory effect increased when neem was applied equally by mixing it with Zedoary and turmeric. The MID in this respect were found to vary with the plant species and fungal pathogens tested. The highest inhibitory effect of turmeric and Zedoary was recorded when MID was 20 mg/ml. In mixed cases, (neem + Zedoary and neem + turmeric) the dose was effective at 10 mg/ml but fungal growth fully stopped at MID 15 mg/ml against the plants tested.

Among the four different treatments, a successful therapeutic effect against pathogenic fungal infection was found in T1, where extract of turmeric recovered 80.00% ± 1.15 of challenged fish (Table-4). Similar fungicidal performance was also observed when neem was mixed with either turmeric (T3) or Zedoary (T4) with good recovery rates of 80.00% ± 2.88 and 70.00% ± 2.88 respectively. This finding is similar to the earlier work done by Muniruzzaman (2004) and Talukdar (2005). The Zedoary (T2) extract also had a medium effect in reducing fungal infection with the rate of recovery being 60%.

Literature reveals that the essential oil fractions from *C. longa* rhizomes of various habitats exhibited fungicidal activity particularly against *Aspergillus niger* and *Physalospora tucumanesis*, *Ceratocystis paradora*, *Sclerotium solfsii*, *Curvularis lunata*, *Helminthosporium sacchari*, *Fusarium moniliforms* and *Cephalosporium sacchari* (Khanna, 1999). Campbell *et al.* (2001) in an *in vitro* trial, found that neem extracts as well as malachite green, ash and potassium permanganate had a strong antifungal property against *A. invadans* while Anon (1994) suggested that a paste prepared from ground neem leaves and tumeric were effective against EUS by inhibiting the spread of infection as well as influencing the growth and survival of recovered fish. Thus, the present study clearly suggests that medicinal plants can be used as an effective control measure along with chemotherapeutics against fungal diseases of fish.

Growth of fungus was found to be affected by different doses of various chemotherapeutics under *in vitro* condition. Brilliant green (MID 0.3 ppm) was effective against all the three fungal isolates. Salt + lime and malachite green (MID 2.0% + 25 ppm and 0.8 ppm, respectively) showed better performances against fungal isolates than that of methylene blue (MID 6 ppm). In the case of medicinal plants, the extracts of turmeric showed best inhibitory response against all the fungi tested whereas the extracts of Zedoary exhibited medium effects at the same dose. In the case of mixed plants extracts, inhibitory effect increased when neem was applied equally by mixing it with Zedoary and turmeric.

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