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Efficacy of medicinal plants on microbial fish pathogens

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Abstract

Studies were conducted to examine the efficacy of fifteen medicinal plants against common microbial (bacterial and fungal) fish pathogens. Crude extracts were prepared from these medicinal plants and applied to the fresh culture of pre-isolated bacterial (*Aeromonas hydrophila*, *Pseudomonas fluorescens* and *Edwardsiella tarda*) and fungal pathogens (*Aphanomyces invadans, Saprolegnia* sp. and *Achlya* sp.) under *in-vitro* condition. A high dose was initially used to detect antimicrobial effects of these plants and garlic, turmeric, neem, shoti, akand etc. were screened out strong and moderate effective on the pathogens. In the case of bacterial pathogens, strong inhibitory response was exhibited by garlic and mixed extract of akand + neem; followed by turmeric and akand. Four different doses of plants extract were exposed under the *in-vitro* condition to determine minimum inhibitory dose (MID). However, garlic offered the best result with 93.33 ± 3.33% recovery of challenged fish (*Barbonymus gonionotus*) in aquarium trial. Akand + neem, turmeric and akand showed moderate to weak recovery rate with the same dose. In the case of fungi, *in-vitro* test on the individual culture was done at four pre-selected doses. Treatment trial was performed using the minimum inhibitory dose (MID) of the highly effective plants (*viz.,* turmeric, shoti, neem + turmeric and neem + shoti) on experimental infected of fish. Turmeric (80.00 ± 1.15% recovery rate) and neem + turmeric (80.00 ± 2.88% recovery rate) were found to be the most effective plants. These could be used as an effective, low-cost and alternative disease control measure in fish health management.

Keywords: Efficacy, Medicinal plants, Microbial fish pathogens

Introduction

Infectious microbial diseases of fish is one of the most significant threats to successful aquaculture of Bangladesh. Every year both culture and wild fish production are hampered by disease incidence. As a result, fish production declines affecting aquatic protein supply and national economy of the country may face problems (Muniruzzaman, 2004). Control of fish disease is currently based almost entirely on chemotherapy and it will retain a role in the management of fish culture systems (Roberts, 1995). Many aquaculture chemicals are, by their very nature, biocidal, and may be released to the surrounding environment at toxic concentrations either through misuse, or in some cases, even by following generally accepted procedures for use (Weston, 1996). Habitual use of these strong chemotherapeutics can lead to problems with microbial resistance and unacceptable residues in aquaculture products and environment. The resistant bacterial strains could have a negative impact on the therapy of fish diseases or human diseases and environment of fish farms (Smith *et al.,* 1994). Now a days, different chemicals such as, malachite green, methylene blue, copper sulphate, formalin are sometimes applied as therapeutics against fungal diseases which have some carcinogenic properties. Besides, rigidity should also be maintained to dose and treatment schedule which may be hard to continue for small-scale fish farmers (Rahman, 2007)

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 being used against fish disease in Asia. Bacterial and fungal diseases in Chinese fish farms were prevented and treated applying seven medicinal plants (Liping, 1994). In Thailand, during the outbreak of epizootic ulcerative syndrome (EUS) the snake-head fish farmers in Uthaitanee, used the bark of cork wood tree (*Sesbania grandiflora*) for the treatment (Direkbusarakom, 2000).

Research on the introduction of medicinal plants in the Bangladesh aquaculture is still under experimental stages. A scientific study to investigate the antibacterial activity of the medicinal plant guava (*Psidium guajava*) against bacteria pathogenic for shrimp was initiated by Direkbusarakom and Aekpanithanpong, 1992. Khan (2001) recommended Neem as a preventive treatment for EUS in small ponds and also mentioned that neem treatment is less hazardous compared to other fungicidal treatments. Since many species of herbs are used as human food/medicine, it is probable that their use would be safe for aquatic animals and for human consumers of aquatic animals' products (Muniruzzaman and Chowdhury 2006). Considering the importance, the present works seem will contribute to develop a low-cost, alternative and eco-friendly therapeutic measure in favour of proper fish health management with locally available medicinal plants.

Materials and Methods

Isolates were collected from diseased wild and farmed fishes for using in this study. High virulent bacterial species *Aeromonas hydrophila* (TL-2), *Pseudomonas fluorescens* (AK-2) and *Edwardsiella tarda* (PL-1) were identified upto species level based on their biochemical characters and compared with old laboratory stocks. Besides, newly isolated *Aphanomyces invadans* (TK-1), *Saprolegnia* sp. (Sap-4) and *Achlya* sp. (Ach-2) were detected as strong to moderate virulent based on their high pathogenic performance and selected for further studies.

Fifteen medicinal plants, effective against common microbial fish pathogens were selected as recognized by Dastur, 1977; Anawer, 2001; and previous studies conducted by Muniruzzaman and Chowdhury, 2004; Rahman, 2005 and Talukdar, 2005. These were *Allium sativum, A. cepa, Terminalia arjuna, Curcuma longa, C. zedoaria, Ocimum sanctum, Polygonum hydropipper, Amaranthus sanguinalis, Callotropis gigentia, Tamarindus indica, Colocasia esculenta, Psidum guajava, Azadirachta indica, Tagetes erecta, Datura metal.* Most of these plants were collected from the adjacent area of Bangladesh Agricultural University, Mymensingh.

Parts of medicinal plants were washed, rinsed and cut into small pieces. Each plant was weighed and paste was prepared using stone made homogenizer. Fibrous particulates from the extracts were screened out with fine meshed markin cloth and then filtered through Whatman 541 filter paper to get fine extracts. Crude extracts were then collected in conical flasks and preserved into refrigerator at low temperature of 10°C.

Growth of fish pathogens by the action of collected plant extracts was determined through *in-vitro* efficacy test. Extracts of different plants were applied on the fresh culture of three selected bacterial and fungal isolates at pre-fixed doses. The microbial growth status was graded by comparing with the control ones and inhibitory performance was detected.

Suspension of freshly cultured bacteria, *A. hydrophila* (TL-2), *P. fluorescens* (AK-2) and *E. tarda* (PL-1) were prepared at the concentration of 2.5×10^7 CFU/ml by following agar plate dilution method. 0.1 ml of each bacterial suspension was spread over Tryptone Soya Agar (TSA, Oxoid) plates using a sterilized glass rod. 50 µl of crude plant extract at pre-fixed doses (8, 6, 4 and 2 mg/ml) were inoculated separately on the sterile disc of blotting paper (3 mm diameter), dispensed earlier on the culture plates. A clear inhibitory zone was observed surrounding the herbal extracts applied and extracts were categorized as strong, moderate and poor inhibitory.

Efficacy test of the selected plants against three fish pathogenic fungi *viz.*, *A. invadans* (TK-1), *Saprolegnia* sp. (Sap-4) and *Achlya* sp. (Ach-2) were carried out in separate vials containing 10 ml GP broth under *in-vitro* condition. The fungi were sub-cultured aseptically on GP agar plates and incubated at 22ºC for 18 hrs. Circular agar blocks (4 mm in diameter), containing fungal hyphae were cut by sterile Cork Borer and kept into individual vial. Plant extracts of different doses (20, 15, 10 and 5 mg/ ml) were then mixed with vials containing 10 ml GP broth and a fungal block and incubated at 22°C. No extract was mixed in the case of control. Antifungal effects of the plants were detected following Muniruzzaman and chowdhury (2006). Efficacy of mixed plant extracts was also tested by mixing the extract of *A. indica* with *C. zedoaria* and *C. longa* individually at a ratio of 1:1. Pattern of fungal growth was observed daily up to 10 days of experimental periods. Treated hyphal blocks were then recultured to ensure their inhibition of growth. The minimum inhibitory dose of the effective plants tested was found to vary with different fungal species.

Selected bacterial isolates (TL-2, AK-1 and PL-2) were cultured on TSA, inoculation was done and incubated at 25°C for 18-24 hours. Around 25 mg of bacterial colonies were weighed to make homogenous solution with 10 ml of 0.85% sterile physiological saline in a vial using auto vortex machine. To obtain the expected concentration ($3X10^7$ CFU/ml) of bacterial suspension, decimal dilution technique of the stock solution was applied and made it ready for injection.

Healthy young Thai silver barb (*Barbonymus gonionotus)* weighing 15 to 20 gm, were injected smoothly and carefully with 1.0 ml disposable syringes at a dose of 0.1 ml/fish comprising $3X10^7$ CFU/ml with three different bacterial (*A. hydrophila, P. fluorescens* and *E. tarda*) suspensions. The experimental infection of the injected fish was expressed as lesion on fins, skin, head or body surface.

Immediately after injection, fishes were exposed into four plants extract *viz.*, garlic, turmeric, akand and extracts of neem + akand, mixed earlier with five litres of settled tap water in four small aquaria for dip bath treatment at the selected dose of 8 mg/ml. Dip treatment was performed for twice a day up to their tolerance level. The whole process was continued up to 10 days. Fish were observed for pathological change, moribund condition and subsequent healing process owing to herbal treatment. Three replications were taken for each test.

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 Thai silver barb, *B. gonionotus* (weight 15-20 g) were abraded on 3-4 mm^2 area by removing patches of scales from each side of the body below the dorsal fin. Each group of fish was exposed to zoospore suspension of (30 ml APW containing zoospore/1 litre tap water.) *A. invadans*, *Saprolegnia* sp. and *Achlya* sp. separately under aerated condition (10 fish per 15 litre of water) in a 30-litre capacity glass aquarium. Controlled fishes were released into the aquarium having only APW and no zoospore. After few days, the fungal infection was observed in the form of reddening and raised ulcerated form on the abraded part (base of scales) of the experimental fish.

Extracts of turmeric, shoti, and two combined extracts of neem + shoti and neem + turmeric were selected for dip treatment to observe their therapeutic effects. Whole process was performed according to studies conducted by Muniruzzaman (2004) and Talukdar (2005). Clinical signs was observed and recorded daily. 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. Three replications were taken for each test.

The analysis of data was done following one way analysis of variance (ANOVA) using MSTAT programme. The mean differences among the treatments were adjudged with Duncan's Multiple Range Test (Gomez and Gomez, 1984).

Results and Discussion

Out of fifteen medicinal plants tested, ten (66.67%), eleven (73.33%) and eight (53.33%) species showed antibacterial effect against *A. hydrophila*, *P. fluorescens* and *E. tarda*, respectively (Table 1) under *in-vitro* condition. Best growth inhibition was observed when garlic (*A. sativum*) was used. Akand (*C. gigantean*) was better inhibitory against *A. hydrophila* followed by *P. fluorescens* and *E. tarda*. Lower inhibitory response of turmeric (*C. longa*) was observed on the bacterial isolate, *P. fluorescens*, but medium growth inhibition was detected against *A. hydrophila* and *E. tarda*. On the other hand, combination of akand + neem (*A. indica*) was found to strongly inhibit the growth of *A. hydrophila* followed by *E. tarda*, but weak inhibitory response was detected against the growth of *P. fluorescens*. Minimum inhibitory dose (MID) of garlic was determined as 8 mg/ml against all the bacterial isolates. MID for the mixed plants extract of akand + neem was also found as 8 mg/ml against *A. hydrophila* and *E. tarda*. Turmeric showed lower inhibitory response at the MID of 6 mg/ml on *A. hydrophila*, but growth inhibition was detected against *P. fluorescens* and *E. tarda* when MID was elevated to 8 mg/ml. Moreover, same MID was also detected in the case of akand.

Table 1. Inhibitory response of medicinal plants against microbial fish pathogens

Sl. No.	Medicinal plants (Used parts)	Inhibitory response of plants extracts on bacterial and fungal growth					
		A. hydrophila	*P. fluorescense*	*E. tarda*	*A. invadans*	*Saprolegnia* sp.	*Achlya* sp.
1.	Garlic (B)	+++	+++	+++	-	-	-
2.	Onion (B)	++	+	+	++	+	+
3.	Arjuna (Bk)	+	+	+	+	+	+
4.	Turmeric (B)	++	+	++	+++	+++	+++
5.	Shoti (B)	++	++	++	+++	++	+++
6.	Basil (L)	+	-	-	+	-	-
7.	Water piper (L)	-	+	-	+	-	-
8.	Lalpata (L)	-	-	-	-	-	-
9.	Akand (L)	+++	++	++	++	++	++
10.	Tamarind (L)	+	-	-	+	-	-
11.	Kachu (L)	-	-	-	-	-	-
12.	Guava (L)	+	+	-	+	-	-
13.	Neem (L)	++	++	++	++	+++	++
14.	Marigold (L)	-	-	-	-	-	+
15.	Datura (L)	-	+	-	-	+	-
*	Akand (L) + Neem (L)	+++	+++	+++	Nt	Nt	Nt
*	Neem (L) + Shoti (B)	Nt	Nt	Nt	+++	+++	+++
*	Neem (L) + Turmeric (B)	Nt	Nt	Nt	+++	+++	+++

L: Leaf B: Bulb Bk: Bark
+++: Highly effective, ++: Medium effective, +: Low effective -: Not effective against the pathogens tested Nt: Not tested, *: Application of mixed plants extracts

In case of fungal pathogens, ten (66.67%), seven (46.67%) and seven (46.67%) species of medicinal plants showed antifungal effect against *A. invadans*, *Saprolegnia* sp. and *Achlya* sp. respectively (Table 1) under laboratory condition. Four pre-selected doses of shoti (*C. zedoaria*) turmeric, mixed extracts of neem + shoti and neem + turmeric were selected for *in-vitro* study. Bulb extracts of turmeric showed best inhibitory response against all the fungi tested whereas bulb extracts of shoti exhibited as medium effective at the same dose. In the case of mixed plants extracts, inhibitory effect increased when neem was applied equally mixing with shoti and turmeric. The minimum inhibitory doses (MID) in this respect were found to vary with the plant species and fungal pathogens tested. Higher inhibitory effect of turmeric and shoti was recorded when MID was 20 mg/ml (Table 2). In mixed cases, both neem (L) + shoti (B) and neem (L) + turmeric (B) were found to be low effective at 10 mg/ml but fungal growth were fully stopped at MID 15 mg/ml against the plants tested.

Table 2. Selected minimum inhibitory dose (MID) of various medicinal plants against bacterial and fungal fish pathogens

Fish pathogens	Name of medicinal plants applied	Determined MID after *In-vitro* study	Selected dose for *In-vivo* study
Bacteria	Garlic	6 mg/ml	8 mg/ml
	Turmeric	8 mg/ml	8 mg/ml
	Akand	6 mg/ml	8 mg/ml
	Akand + Neem	6 mg/ml	8 mg/ml
Fungi	Turmeric	15 mg/ml	20 mg/ml
	Shoti	20 mg/ml	20 mg/ml
	Neem + Turmeric	15 mg/ml	15 mg/ml
	Neem + Shoti	15 mg/ml	15 mg/ml

Recovery rate of experimentally infected fish varied with the concentration of extracts and species of the medicinal plants. Best result was obtained in the case of garlic (T_1) where recovery rate was significantly ($p<0.05$) higher (93.33% ± 3.33) (Table 3) than all other treatments, performed against the bacterial infection of fish at the concentration of 8 mg/ml within the 10 days of experimental period. The extract obtained from turmeric (T_2) was medium effective (63.33% ± 3.33 recovery rate) but lower effect was found in the case of T_3, where 56.66% of the infected fish were recovered by the extract of akand. Moreover, mixture of akand + neem showed the prominent effect with 86.67% ± 3.33 recovery rate against the infectivity of bacterial pathogens which was ultimately suited with the previous studies conducted by Rahman (2005). Graphical presentation of therapeutic effects was shown in Fig. 1. The study also revealed that the pathological changes have an inverse relation with the efficacy of the treatments.

Table 3. The effect of medicinal plants on experimentally infected fish with bacterial pathogens

Treatment	Dose (mg/ml)	Recovery (%)
T_1: Garlic *(A. sativum)*	8	$93.33^{a} \pm 3.33$
T_2: Turmeric (*C. longa)*	8	$66.67^{ab} \pm 3.33$
T_3: Akand *(C. gigentia)*	8	$53.33^{b} \pm 3.33$
T_4: Akand + Neem *(C. gigentia + A. indica)*	8	$86.67^{a} \pm 3.33$
T_5: Control	No extract	0^{e}
Level of significant		**

Values bearing different superscripts (a, b, ab, c, d, e) in the same column are significantly different but with the same superscripts are not significantly different ($p<0.05$)

** : Significant ($p<0.05$), Recovery (%): Mean ± S.E.

The bulbs of *A. sativum* are used both for medicinal and culinary purposes (Villachira, 1998). The bulbs contain an acrid volatile oil (0.25%), starch, mucilage, albumin and sugar. The major component of volatile oil is propyl disulphide which is a powerful germicide (Anawer, 2001). Garlic or onion has been mixed to the shrimp pellet and fed every day to protect the bacterial infection (Direkbusarakom, 2000). The extract of akand contains several proteinases as well as calotropin and other cardiac glycosides. A powder of dried leaves of akand is an efficacious local application for ulcer, eczema and other skin diseases (Anawer, 2001). Crude extract of different parts of neem have been used as traditional medicine for treatment of various diseases (Biswas *et al.*, 2002).

Among four different treatments, a successful therapeutic effect against pathogenic fungal infection was found from T_1, where extract of turmeric recovered 80.00% ± 1.15 of challenged fish. Similar fungicidal performance was also observed when neem was mixed with either turmeric (T_3) or shoti (T_4) with good recovery rates of 80.00% ± 2.88 and 70.00% ± 2.88 respectively. Such findings were similar to the previous studies (Muniruzzaman, 2004 and Talukdar, 2005). Moreover, shoti (T_2) also performed as medium effective plant in reducing fungal infection and the rate of recovery was recorded upto 60%. Therapeutic effects of different medicinal plants on the experimental infection of fish with the challenged pathogens are shown in Table 4 and its graphical presentation was illustrated in Fig. 2.

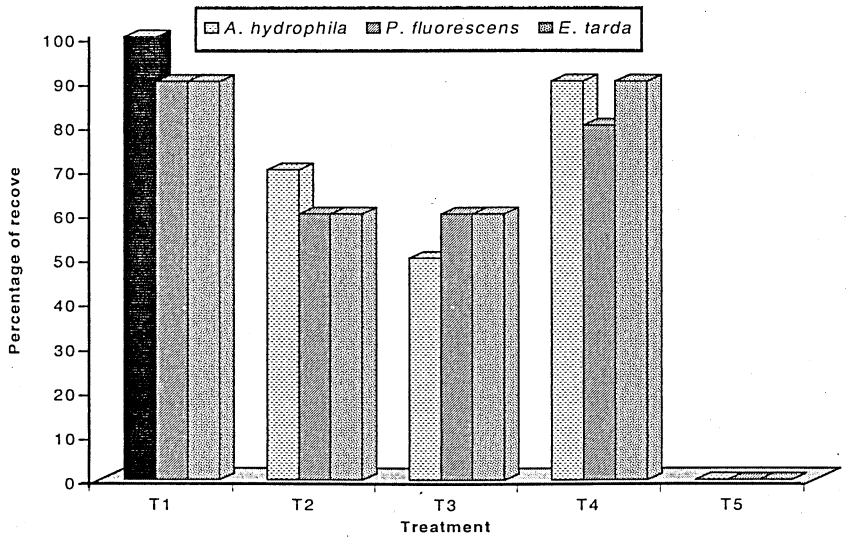
Table 4. The effect of medicinal plants on experimentally infected fish with fungal pathogens

Treatment	Dose (mg/ml)	Recovery (%)
T_1: Turmeric (*C. longa*)	20	$80.00^{a} \pm 1.15$
T_2: Shoti (*C. zedoaria*)	20	$60.00^{c} \pm 5.77$
T_3: Neem + Shoti (*A. indica* + *C. zedoaria*)	15	$73.00^{b} \pm 3.33$
T_4: Neem + Turmeric (*A. indica* + *C. longa*)	15	$80.00^{a} \pm 2.88$
T_5: Control	No extract	0^{h}
Level of significant		**

Values bearing different superscripts (a, b, c, d, e, f, g, h) in the same column are significantly different but with the same superscripts are not significantly different ($p<0.05$)
** : Significant ($p<0.05$)
Recovery (%): Mean ± S.E.

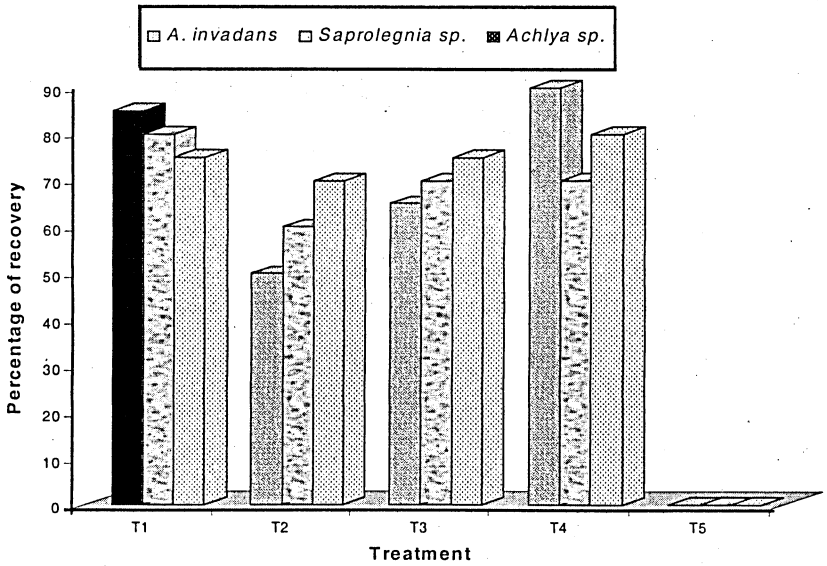
The essential oil fractions from *C. longa* rhizomes of various habitats exhibited fungistatic activity particularly against *Aspergillus niger* and *Physalospora tucumanesis, Ceratocystis paradora, Sclerotium solfsii, Curvularis lunata, Helminthosporium sacchari, Fusarium moniliforms* and *Cephalosporium sacchari* (Khanna, 1999). In the *in-vitro* trial, Campbell *et al.* (2001) found that neem extracts as well as malachite green, ash, potassium permanganate had a strong antifungal property against *A. invadans*. Anon, 1994 suggested that a paste prepared from ground neem (*A. indica*) leaves and turmeric were effective against EUS by inhibiting the spread of infection as well as influencing the growth and survival of recovered fish.

The investigation indicated that native medicinal plants could be used as low-cost alternative therapeutic measure against the fish diseases caused by pathogens tested. Further detailed studies regarding the application of medicinal plants at field level are necessary to establish the facts.



T_1: Garlic T_2: Turmeric T_3: Akand T_4: Akand + Neem T_5: Control

Fig. 1. Therapeutic effects of different medicinal plants on the experimental infection of fish with bacterial pathogens



T_1: Turmeric T_2: Shoti T_3: Neem + Shoti T_4: Neem + Turmeric T_5: Control

Fig. 2. Therapeutic effects of different medicinal plants on the experimental infection of fish with fungal pathogens

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