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Evaluation of the efficacies of selected antibiotics and medicinal plants on common bacterial fish pathogens

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Abstract

An experiment was conducted to compare the efficacies of some selected antibiotics and medicinal plants against common bacterial fish pathogens *viz., Aeromonas hydrophila*, *Pseudomonas fluorescens* and *Edwardsiella tarda.* Four different antibiotics *viz.,* CFCIN (ciprofloxacin), Renamycin (oxytetracycline), DT-10 (doxycicline) and Sulfatrim (sulphadiazine + trimethoprim) were exposed in different doses (100, 75, 50 and 25 ppm) to the culture of freshly isolated bacteria under the *in vitro* condition for sensitivity test and minimum inhibitory dose (MID) was determined. Based on *in vitro* results, antibiotics were applied to the experimental infection of Thai silver barb, *Barbonymus gonionotus*. CFCIN showed the best result with 100% recoveries of challenged fish in prolonged bath treatment. Medicinal plants were selected on the basis of previous studies. Crude extracts were prepared from various parts (leaves and bulb) of garlic, turmeric, akand and neem and four different doses were applied to the fresh culture of pathogenic isolates under the *in-vitro* condition to determine minimum inhibitory dose (MID). However, garlic offered the best result with 90.00 ± 2.89% recoveries of challenged fish in aquarium trial. Akand + neem, turmeric and akand showed moderate to weak recovery rates with the same dose. The present study thus showed that medicinal plants would be an effective control measure along with antibiotics against bacterial fish diseases.

Keywords: Efficacy, Medicinal plants, Bacterial fish pathogens

Introduction

Bacteria, the major group of pathogens, pose one of the most significant threats to successful fish production throughout the world. Bacterial diseases are responsible for heavy mortalities in both culture and wild fishes throughout the world and most of the causative microorganisms are naturally occurring opportunist pathogens which invade the tissue of a fish host rendered susceptible to infection (Roberts, 1989). Among all other bacteria, Aeromonad, Pseudomonad and *Edwardsiella tarda* are the major bacterial fish pathogens which are widely distributed in aquatic organisms in nature (Banu, 1996 and Islam, 1996). In coastal regions, fish have been suffered from vibriosis, a bacterial disease causing losses in the fish production (Rahman, 2005).

Control of fish disease is currently based almost entirely on chemotherapy and it will entirely retain a role in the management of fish culture systems (Roberts, 1995). Anti-bacterial chemotherapy has been applied in aquaculture for over 50 years (Inglis, 1996). Antibiotics are also used prophylactically in carp culture at times of year when haemorrhagic septicaemia is most likely to occur (Inglis *et al*., 1994). But habitual use of anti-bacterials can lead to problems with bacterial 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). This situation actually brings human to new medical dilemma (Muniruzzaman and Chowdhury, 2004). Medicinal plants possess therapeutic properties; exert beneficial pharmacological effects on the animal body, widely available in nature and eco-friendly. A scientific study to investigate the antibacterial activity of the medicinal plants, guava (*Psidium guajava*) against bacteria pathogenic for shrimp was initiated by Direkbusarakom and Aekpanithanpong (1992). Kraus (1995) found that extract of neem fruit, seeds, seed kernel, twigs, stem bark and root have fungicidal and bactericidal properties. Externally garlic (*Allium sativum*) is used as disinfectant and it is applied to indolent tumours, ulcerated surface and wounds (Dastur, 1977). Chowdhury *et al*. (1991) reported that extract obtained from garlic was also highly effective against two tested bacteria, *A*. *hydrophila* and *P*. *fluorescens* (MIC 0.6 mg/ml). Garlic, turmeric, akand and neem could be used as an alternative therapeutic measure against bacterial infection of fish (Rahman, 2005). But sporadic findings from the previous studies could not satisfy a suitable prevention and control measures.

Considering the above aspects, the present study was conducted to suggest a package in favour of proper health management in the aquaculture of Bangladesh by comparing the efficacies of selected antibiotics and medicinal plants on common bacterial fish pathogens.

Materials and Methods

Selection of Pathogens

Suspected pathogens were isolated from diseased wild and farmed fishes for using in present studies. Methods of bacterial isolation, their characterization and pathogenicity test were completed according to the method described by Barrow and Feltham (1993) and Chowdhury and Muniruzzaman (2002). Finally, high virulent species *viz., 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 and selected for further studies.

Selection of antibiotics

In the aquaculture of Bangladesh, locally available veterinary grade antibiotics are used for the prophylaxis and treatment of fish disease problems. Based on preliminary investigation, availability in markets and reports of the previous workers, four available antibiotics *viz.,* CFCIN (Ciprofloxacin 10%; FnF) Renamycin (Oxytetracyclin, USP 200 mg; Renata Ltd.), DT-10 (Doxycicline 10% + Trimethoprime 10%; FnF) and Sulfatrim (Sulphadiazine BP 40% + Trimethoprim BP 8%; Techno Drugs) were selected for this study.

Selection of Medicinal Plants and Extracts Preparation

Primarily, fifteen medicinal plants were selected based on their recognized medicinal properties described by Dastur (1977) and Anawer (2001) and the previous studies conducted by Muniruzzaman and Chowdhury (2004) and Rahman (2005). Among these, most effective garlic *(A. sativum),* turmeric (*Curcuma longa)*, Akand *(C. gigentia)* and mixed extracts of Akand + Neem *(C. gigentia* + *A. indica)* were selected for the present studies. Most of the medicinal plants were collected from the adjacent area of Bangladesh Agricultural University, Mymensingh.

Desired parts of plants were washed with clean water, rinsed with sterilized distilled water and cut into small pieces. Before making crude extract, each medicinal plant was weighed and then paste was prepared using stone made homogenizer. Fibrous particulates from the extracts were screened out by pressing through fine meshed cotton cloth and finally 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 to use in future under the laboratory condition.

In-vitro Efficacy Test

Suspensions of freshly cultured bacterial isolates were prepared at the concentration of $2.5x10^7$ 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. Fifty µl of testing agents were inoculated separately at pre-fixed doses on the sterile disc of blotting paper (3 mm diameter), dispensed earlier on the culture plates.

a. Antibiotic sensitivity test

Effects of selected antibiotics were determined by antibiotic sensitivity test using drug disc (paper disc) method against the most virulent bacterial isolates. Due to unavailability of different antimicrobiotic discs, selected antibiotics were diluted in four different concentrations *viz.,* 25, 50, 75, 100 ppm and 50 µl was dropped on each blotting paper (3 mm in diameter) disc and incubated for 5 days at 20^oC. Sensitivity was recognized with clear zone surrounding the disc. The diameters of the restricted halos around the paper disc were measured time to time for determining the minimum inhibitory dose (MID). Selected antibiotics with their varied doses are illustrated in Table 1.

Table 1. Selected antibiotics and their varied doses applied on bacterial isolates

Sl. No.	Name of antibiotics		Doses
	Trade name	Type of antibiotics	
1.	CFCIN	Ciprofloxacin	25 ppm
			50 ppm
			75 ppm
			100 ppm
2.	Renamycin	Oxytetracyclin	25 ppm
			50 ppm
			75 ppm
			100 ppm
3.	DT-10	Doxycicline	25 ppm
			50 ppm
			75 ppm
			100 ppm
4.	Sulfatrim	Sulphadiazine + Trimethoprim	25 ppm
			50 ppm
			75 ppm
			100 ppm

b. Efficacy test with medicinal plants

Based on our previous study (Muniruzzaman and Chowdhury, 2004), extracts of different plants were applied on the fresh culture of the three bacterial isolates (*Aeromonas hydrophila*, *Pseudomonas fluorescens* and *Edwardsiella tarda* sp.) for *in-vitro* efficacy test at pre-fixed doses *viz., 2*, 4, 6 and 8 mg/ml (Table 2). The microbial growth inhibition status was graded as strong, moderate, poor and not effective by comparing with the control ones to different doses and inhibitory performance was detected.

Table 2. Various doses of selected medicinal plants applied on pathogenic bacterial isolates

Sl. No.	Name of medicinal plants	Doses
1.	Garlic (Bulb)	2 mg/ml
		4 mg/ml
		6 mg/ml
		8 mg/ml
2.	Turmeric (Bulb)	2 mg/ml
		4 mg/ml
		6 mg/ml
		8 mg/ml
3.	Akand (Leaf)	2 mg/ml
		4 mg/ml
		6 mg/ml
		8 mg/ml
4.	Akand (Leaf) + Neem (Leaf)	2 mg/ml
		4 mg/ml
		6 mg/ml
		8 mg/ml

Investigation on the Therapeutic Effects (*In-vivo*)

Preparation of bacterial suspension and injection: Selected bacterial isolates (TL-2, AK-1 and PL-2) were cultured on TSA, inoculation was done carefully and incubated at 22°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 the suspensions of three pre-selected pathogenic bacterial isolates. The experimental infection of the injected fish was expressed as lesion on fins, skin, head or body surface. Three replications were used for each test.

a. Effects of antibiotics on bacterial infection

After experimental infection with virulent isolates, selected antibiotics were applied individually to a group of ten strong and disease free fish to observe their effects. The treatments selected for this purpose were T_1 with CFCIN, T_2 with Renamycin, T_3 with DT-10, T_4 with Sulfatrim and T_5 as control. Prolonged bath treatment was applied separately for about 3-4 hours and twice per day. Temperature was maintained by room temperature and dissolved oxygen was ensured by regular aeration.

b. Therapeutic effects of medicinal plants

Immediately after injection, fishes were exposed for treatment trial into four plant extracts *viz*., garlic (T_1), turmeric (T_2), akand (T_3) and extracts of neem + akand (T_4), mixed earlier with five litres of settled tap water in four small aquaria. Another aquarium was set as control (T_5) without mixing any plants extract. Dip bath treatment was performed up to the tolerance level of fish at the selected dose of 8 mg/ml for twice a day. 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.

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

Effect of different antibiotics on the fish pathogenic bacteria under laboratory condition provided useful information for treatment of bacterial fish diseases. Antibiotic sensitivity test of each pathogenic species was performed under *in-vitro* condition to determine minimum inhibitory dose (MID). All tested isolates (100%) were sensitive to CFCIN followed by Renamycin (86.67%) at 75 ppm. DT-10 was not so effective in lower concentration but sensitive for 80% isolates at 100 ppm. Moreover, only 53.33% pathogens were sensitive in case of salfatrim at 100 ppm. After investigation on 106 (out of 132) mesophilic aeromonads Yucel, *et al.,* (2005) found that all strains (*A. hydrophila, A. veroni bv. sobria, A. caviae*) were susceptible to ciprofloxacin. Wolska *et al.,* (1999) also reported that 99% of *Pseudomonas aeruginosa* strains were susceptible to ciprofloxacin. Sarker *et al.* (2000) performed drug sensitivity test and found that 50% of the *Aeromonas sobria* isolates were highly sensitive to oxytetracycline, oxolinic acid and chloramphonical and resistant to erythromycin and sulphamethoxazole.

Therapeutic effects of the antibiotics tested were examined through experimental infection. Best result was obtained with 100% recovery (Table 3) of infected fish when the antibiotic, CFCIN was used for prolonged bath treatment in laboratory condition. Renamycin was also found to be effective in healing bacterial infection (90.00 ± 2.89%) followed by DT-10 (80.00 ± 5.78%). Sulfatrim was detected as less effective with 60.00 ± 4.62% recoveries of challenged fish. Kou *et al.* (1988) and Liao *et al.* (1996) used oxytetracycline in aquaculture as bactericide. Lio-Po and Sanvictores (1987) found positive effect of oxytetracycline in controlling *Pseudomonas* sp. in Tilapia fry. According to Shariff *et al.,* (1996) oxytetracycline (about 20 ppm) in a dip or bath solution is used against bacterial disease in Malaysia and Singapore. Chowdhury *et al.* (2003) found positive effect of Renamycin (oxytetracycline) against bacterial infection.

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) at the concentration of 8 mg/ml where recovery rate was significantly ($p<0.05$) higher (90.00% ± 2.89) (Table 4) than all other treatments performed within the 10 days of experimental period. The extract obtained from turmeric (T_2) was medium effective (60.00% ± 4.62 recovery rate) but lower effect was found in the case of T_3, where 50.00% ± 2.89 of the infected fish were recovered by the extract of akand. Moreover, mixture of

akand + neem showed the prominent effect with 80.00% ± 5.78 recovery rate against the infectivity of bacterial pathogens which was ultimately suited with the previous studies conducted by Rahman (2005). The study also revealed that the pathological changes have an inverse relation with the efficacy of the treatments.

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

Treatment	Dose (mg/l)	Recovery (%)
$T_{1:}$ CFCIN	75	100.00^{a} ± 0.00
$T_{2:}$ Renamycin	75	90.00^{b} ± 2.89
$T_{3:}$ DT-10	100	80.00^{c} ± 5.78
$T_{4:}$ Sulfatrim	100	60.00^{e} ± 4.62
$T_{5:}$ Control	No dose	0^{h}
Level of significance	-	**

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.

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

Treatment	Dose (mg/ml)	Recovery (%)
$T_{1:}$ Garlic *(A. sativum)*	8	90.00^{a} ± 2.89
$T_{2:}$ Turmeric (*C. longa)*	8	60.00^{c} ± 4.62
$T_{3:}$ Akand *(C. gigentia)*	8	50.00^{d} ± 2.89
$T_{4:}$ Akand + Neem *(C. gigentia + A. indica)*	8	80.00^{b} ± 5.78
$T_{5:}$ Control	No extract	0^{e}
Level of significance	-	**

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). Akand is used as a traditional medicinal plant with unique properties (Oudhia and Tripathi, 1998). 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).

In aquarium trial, use of antibiotics took quickest possible of time (seven days only) rather than medicinal plants to recover the experimental infection. However, findings of the present studies provided useful information (regarding the application of antibiotics medicinal plants) for making a package in favour of proper fish health management. Detailed studies on field trial are necessary to establish the facts.

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