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SENSITIVITY OF FISH PATHOGENIC BACTERIA TO VARIOUS MEDICINAL HERBS

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ABSTRACT

The sensitivity of certain local medicinal herbs was evaluated against pathogenic *Aeromonas hydrophila*, *Pseudomonas fluorescens* and *Edwardsiella tarda* bacteria of fish during the period from April 2001 to March 2002. A suspension of freshly cultured experimental bacteria (2 x 10^7 CFU / ml) was prepared and 0.1 ml of this suspension was spread over the tryptic soya agar (TSA) plates. Then crude extract (0.1ml / plate) of each herb was inoculated in the middle of the cultured plate to detect the response. The herbal extract was categorized as high, medium and low inhibitory responded by observing the clear zone of inhibition. Fine extract of the high inhibitory responded herbs was applied under *in vitro* conditions against the bacteria tested. Twenty-one (80.76%), twenty-four (92.30%) and twelve (46.15%) species of the herbs tested showed antibacterial effect against *A. hydrophila*, *P. fluorescens* and *E. tarda*, respectively. Among them, high inhibitory responded herbs were 38.09% for inhibition of *A. hydrophila* and *P. fluorescens* and 38.33% for *E. tarda*. However, the extract collected from bulb of *Allium sativum* for inhibition of *A. hydrophila* and *P. fluorescens* and the decoction obtained from leaves of *Calotropis gigantea* for *E. tarda* were detected to be the most promising herbs considering effectiveness with minimal inhibitory concentration (MIC) among all of the herbs tested.

Key words : Sensitivity, pathogenic bacteria, herbal extract, minimal inhibitory concentration

INTRODUCTION

Bacteria constitute the most economically significant group of pathogenic agents. 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). Bacterial infection of fish and fish products may influence human health either directly by inducing disease or indirectly through the effect on the human beings by residues of antimicrobial agents used to treat such infection in fishes (Inglis *et al.*, 1993). Weston (1996) stated that 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. Thus, there is a potential for mortality of non-target organisms. Habitual use of antibacterials can lead to problems with bacterial resistance and with 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 the fish farms (Smith *et al.*, 1994). The predominant public concerns on microbial resistance due to the use of antibiotics are the possible impacts on human health resulting from the emergence of drug-resistant bacteria in animals caused by the prolonged use of low level antibiotics in animal feed (Sorum *et al.*, 1992) and that antibiotic residues may persist in sediments for a long time. These situations actually bring human to new medical dilemma. A scientific study to investigate the antibacterial activity of the herb, guava (*Psidium guajava*) against bacteria pathogenic for shrimp was initiated by Direkbusarakom and Aekpanithanpong (1992). Now, many kinds of herbs have been widely used in veterinary and human medicine. In part because herbs are the natural products, that seen as safe for consumers but also they are readily available in Asia. Herbs or herbal products also have a role in aquaculture at present time (Direkbusarakom, 2000). In Vietnam, Institute of Ecology and Bioresources have undertaken applied research on some medicinal herbs for prophylaxis and treatment of fish and shrimp diseases, e. g., white mouth, white head, red skin, and red spot in fish and luminescent and brown spot disease in shrimp (Dung, 1990). Wild satavari (*Asparagus racemous*) is widely used in India as ayurvedic medicine for promoting growth in humans and was used in *Labeo rohita* fry and found to produce similar results (Kavita and Sharma, 1996). In Bangladesh, different kinds of medicinal herbs are available which grow in roadside, small jungles or fellow lands and most of them are cultivatable with very low cost. Many species of these herbs are used as directly human food or as medicine such as, *Andrographis paniculata*, *Azadirachta indica*, *Basella alba*, *Allium cepa*, *Allium sativum*, *Calotropis gigantea*, *Momordica charantia*. Externally garlic (*Allium sativum*) is used as disinfectant and it is applied to indolent tumours, ulcerated surfaces and wounds (Dastur, 1977). As an emollient crushed onion (*Allium cepa*) or its juice is applied over skin diseases and insect bites. The leaf juice of giant milk weed (*Calotropis gigantea*) is applied to aphthous sores in the mouth of children and skin diseases. Therefore, in all likelihood, their use would be safe for aquatic animals, environment and also for human. That is why, it was of

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keen interest to observe whether herbs might be used as an alternative treatment for bacterial diseases of fish. Many scientists of Asia have been used herbs to control fish and shrimp diseases and have obtained positive and fruitful results but research regarding use of herbs in aquaculture to control bacterial diseases not yet done in Bangladesh, although the Government of Bangladesh has given emphasis on herbal treatment not only for human but also for other animals including fish. Considering the above, in the first attempt, the objective of the study was to examine the sensitivity of medicinal herbs of Bangladesh against the three important fish pathogenic bacteria viz., *Aeromonas hydrophila*, *Pseudomonas fluorescens* and *Edwardsiella tarda* usually cause disease in Bangladesh aquaculture.

MATERIALS AND METHODS

The sensitivity of certain local medicinal herbs against bacteria pathogenic for fish was studied during the period from April 2001 to March 2002. Fifteen isolates of three pathogenic bacteria (viz., *Aeromonas hydrophila*, *Pseudomonas fluorescens* and *Edwardsiella tarda*) each containing of five isolates, maintained in the laboratory of the Department of Aquaculture, Bangladesh Agricultural University, Mymensingh were used for this study.

Preparation of crude herbal extracts

The herbs selected for this study were *Acacia arabica, Allium cepa, Allium sativum, Andrographis paniculata, Azadirachta indica, Basella alba, Calotropis gigantea, Cephalandra indica, Curcuma zedoaria, Curcuma longa, Cuscuta reflexa, Cynodon dactylon, Datura metel, Enhydra fluctuence, Heliotropium indicum, Hydrocotyle asiatica, Mimosa pudica, Momordica charantia, Ocimum sanctum, Piper betle, Polygonum hydropiper, Psidium guajava, Sesbania grandiflora, Sesbania sesban, Tagetes erecta* and *Tamarindus indica.* The fresh leaves of each of the selected herb were washed by sterilized distilled water and prepared their paste using stone made homogenizer. The herb extracts was collected from the paste and filtered through Whatman 541 filter paper and then centrifuged by BR 401 Bench Refrigerated Centrifuge. Finally extract was collected and preserved at 4^0C for further studies. It may be mentioned that in some cases, the extract was collected from bulb / bark of the herb.

Preparation of fine herbal extracts

According to method of Direkbusarakom *et al.* (1998), the leaves of the high inhibitory responded herbs were dried in the sunlight and extracted by ethanol using a soxlet apparatus. This extract was further prepared as granules with polyvinylpyrolidone (PVP) to promote easy dissolution of the herb extracts in antibacterial tests.

Sensitivity tests

A suspension of freshly cultured experimental bacteria (2×10^7 CFU / ml) was prepared and 0.1ml of this suspension was spread over the tryptic soya agar (TSA) plates. Then crude extract (0.1 ml / plate) of each herb was inoculated in the middle of the cultured plate to detect the response. Inhibitory response of herbal extract was recorded according to normal growth response of bacteria after incubation at 30° C for 18 to 24 h and the extracts were categorized as high, medium and low inhibitory response to observe the sensitive zone. Normal growth was recognized as resistant to extracts and clear zone was recognized as a sensitive one. Only high inhibitory responded herbs were used for further sensitivity tests.

Sensitivity tests for the fine extracts of high inhibitory responded herbs were conducted by an agar plate dilution method according to Tragen (1983). Serial two-fold dilutions of each extract were prepared to furnish concentration higher to lower. One milliliter of each dilution was mixed with 9 ml of Muller Hinton Agar and dispensed into agar plates. For the control, polyvinylpyrolidone was used at a dose of 10 mg / ml instead of herbal extract. In order to prepare the inoculum for the test plates, bacterial strains were precultured at 30° C for 18 h in tryptic soya broth. The precultured broth (0.1 ml) was then mixed with 1 ml of the same broth before overlaying on the test plates. Antibacterial effect was determined by observation of the growth of bacteria after incubation at 30° C for 24 h.

RESULTS AND DISCUSSION

Twenty-one (80.76%), twenty-four (92.30%) and twelve (46.15%) species of the 26 herbs (In some cases, different parts of a same herb used) tested showed antibacterial effect against *A. hydrophila*, *P. fluorescens* and *E. tarda*, respectively (Table 1) and it was observed that 53.84% herbs did not showed any effect on *E. tarda* whereas, in the case of *A. hydrophila* and *P. fluorescens* 19.23% and 7.69% herbs did not showed inhibitory effect, respectively. It indicates that *E. tarda* is less sensitive to herbs. Among the herbs tested, eight species (38.09%) viz., *A. arabica, A. cepa, A. sativum, C. gigantea, M. charantia, P. hydropiper, P. guajava* and *T. indica* were high inhibitory responded herbs for inhibition of *A. hydrophila* and *P. fluorescens* and seven (58.33%) were for *E. tarda*. The high inhibitory responded herbs were created clear inhibitory zone when herb extracts inoculated on the middle of the cultured plates. In case of *A. hydrophila*, the medium inhibitory responded herbs were *A. indica* (Ld), *B. alba* (L).

C. zedoaria (B), *C. longa* (B), *C. indica* (L), *H. indicum* (R), *H. asiatica* (L) and *P. betle* but *A. indica* (Ld), *B. alba* (L), *C. zedoaria* (B), *C. longa* (B), and *H. asiatica* (L) were for *P. fluorescens* and only *A. paniculata* (L) for *E. tarda*. On the other hand, the low inhibitory responded herbs were *A. paniculata* (L), *M. pudica* (Ld), *S. grandiflora* (Bark extract), *S. sesban* (L) and *T. erecta* (Inf.) for inhibition of *A. hydrophila* whereas *C. indica* (L), *C. longa* (L), *C. reflexa* (Whole), *C. dactylon* (L), *D. metel* (L), *E. fluctuence* (L), *H. indicum* (R), *P. betle* (L), *S. grandiflora* (Bark extract), *S. sesban* (L) and *T. erecta* (Inf.) were for *P. fluorescens* and *C. zedoaria* (B), *C. longa* (B), *P. betle* (L) and *T. erecta* (Inf.) were for *E. tarda*.

Table 1. Effect of medicinal herbs on the three bacterial fish pathogens

Herbs species (used part)	Inhibitory response of herbal extracts on bacterial growth		
	Aeromonas hydriphila	*Pseuomonas fluorescens*	*Edwardsiella tarda*
1. *Acacia arabica* (Bark inf.)	+++	+++	+++
2. *Allium cepa* (B)	+++	+++	-
3. *Allium sativum* (B)	+++	+++	+++
4. *Andrographis paniculata* (L)	+	-	++
5. *Azadirachta indica* (L)	-	-	-
6. *Azadirachta indica* (Ld)	++	++	-
7. *Basela alba* (L)	++	++	-
8. *Calotropis gigantea* (Ld)	+++	+++	+++
9. *Cephalandra indica* (L)	++	+	-
10. *Curcuma zedoaria* (L)	-	-	-
11. *Curcuma zedoaria* (B)	++	++	+
12. *Curcuma longa* (L)	-	+	-
13. *Curcuma longa* (B)	++	++	+
14. *Cuscuta reflexa* (Whole)	-	+	-
15. *Cynodon dactylon* (L)	-	+	-
16. *Datura metel* (L)	-	+	-
17. *Enhydra fluctuence* (L)	-	+	-
18. *Heliotropium indicum* (L)	-	-	-
19. *Heliotropium indicum* (R)	++	+	-
20. *Hydrocotyle asiatica* (L)	++	++	-
21. *Mimosa pudica* (Ld)	+	-	-
22. *Momordica charantia* (L)	+++	+++	+++
23. *Ocimum sanctum* (L)	-	-	-
24. *Piper betle* (L)	++	+	+
25. *Polygonum hydropiper* (Ld)	+++	+++	+++
26. *Psidum guajava* (L)	+++	+++	+++
27. *Sesbania grandiflora* (Bark extract)	+	+	-
28. *Sesbania sesban* (L)	+	+	-
29. *Tagetes erecta* (L)	-	-	-
30. *Tagetes erecta* (Inf.)	+	+	+
31. *Tamarindus indica* (L)	+++	+++	+++

B = Bulb, L = Leaf, Ld = Decoction of leaves, Inf. = Infusion, R = Root, +++ = High, ++ = Medium, + = Low, - = No response.

A. paniculata is one of most famous medicinal herbs for treatment of bacterial infections among Thai and Chinese people. It is also used China for the treatment of enteritis in freshwater fish (Rajandra, 1990; Rath, 1990). In addition, ethanol extracts of this herb have been reported to be effected against α-*Streptococcus* group (Laopaksa *et al.*, 1988). However, in this study, the extracts of *A. paniculata* showed medium effect on the tested bacterial strains of *E. tarda* and low effect on *A. hydrophila* but did not show any effect on *P. fluorescens*. The essential oil of holly basil (*O. sanctum*) had been shown to have antibacterial activities (Dey and Choudhuri, 1981). In the present study, extracts from the leaves of *O. sanctum* (white variety) had no inhibitory effect on any of the bacteria tested, which support the findings of Direkbusarakom *et al.* (1998). The extracts from leaves of the marigold (*T. erecta*) did not show any antibacterial effect but infusion of the leaves of this herb showed low antibacterial activity against the bacteria tested. The extracts of root of the herb, *H. indicum* was low to medium effective against A. *hydrophila* and

P. fluorescens but the extracts of leaves did not show any antibacterial activity. It indicates that antibacterial property of this herb does not reside in the leaves. This type of result was observed in the case of *C. zedoaria* where the extracts of bulb showed effect against the bacteria tested but the extracts of leaves had no antibacterial effect. In our experiments, it was observed that the extracts from the leaves of *C. longa*, *C. reflexa*, *C. dactylon*, *D. metel* and *E. fluctuence* had no antibacterial effect on *A. hydrophila* and *E. tarda* but showed low effect on *P. fluorescens*. In Thailand, during the outbreak of epizootic ulcerative syndrome (EUS) in 1983 farmers who cultured snake head fish in Uthaitanee province used the bark of cork wood tree (*Sesbania grandiflora*) for the treatment of the haemorrhage lesions. They found that most of the fishes recovered after treatment (Direkbusarakom, 2000). In the present study, the extracts collected from the bark of *S. grandiflora* showed low effect on the tested bacteria, *A. hydrophila* and *P. fluorescens* . These two types of bacteria were associated with epizootic ulcerative syndrome of fish in Asia (Boonyaratpalin, 1987). The inhibitory effect of onion oil against the growth of various isolates of bacteria representing Gram-positive (4 isolates) and Gram-negative (4 isolates) species were studied by Zohri *et al.* (1995). Results showed that onion oil was highly active against all Gram-positive bacteria tested and only one isolate (*Klebsiella pneumoniae*) of Gram-negative bacteria. The findings of the present study showed that the extracts obtained from bulb of onion (*A. cepa*) had inhibitory effect on *P. fluorescens* followed by *A. hydrophila*.

Among the eight species of high inhibitory responded herbs, the extract from bulb of *A. sativum* showed the highest effect against *A. hydrophila* and *P. fluorescens* and minimum inhibitory concentration (MIC) was 0.6 mg / ml (Table 2). The leaves extracts of *M. charantia* and P. *guajava* and the decoction of leaves of *P. hydropiper* had the second highest activity at MIC 1.2 mg / ml. *A. cepa* showed effect only on *P. fluorescens* at MIC 1.2 mg / ml. On the contrary, the decoction of the leaves of *C. gigantea* showed the best effect to inhibit the bacteria, *E. tarda* at 0.6 mg / ml but MIC for other two bacteria was 5 mg / ml. The MIC of *T. indica* was 10 mg / ml for all of the tested bacteria (Table 2).

In the case of prevalence of inhibitory effect of the eight high inhibitory responded herbs, the bulb extracts of *A. sativum* was the most effective since it inhibited 20% of the tested strains of *A. hydrophila* at 0.6 mg / ml and 80 and 100% were inhibited at 1.2 and 2.5 mg / ml, respectively (Fig. 1) whereas in case of *P. fluorescens*, 15%, 70% and 100% were inhibited at 0.6, 1.2 and 2.5 mg / ml, respectively (Fig. 2). The second most effective herb was *M. charantia* in that 70% of the tested bacterial strains of *A. hydrophila* were inhibited at 1.2 mg / ml and 100% at 2.5 mg / ml. *P. guajava* also showed more or less similar results. The decoction of leaves of *P. hydrophyla* was inhibited all of the bacteria tested at 1.2 mg / ml. On the other hand, The decoction of leaves of *C. gigantea* was the most effective herb against the tested bacterial strains of *E. tarda* where it inhibited 25% of the tested strains at 0.6 mg / ml and 75% and 100% were inhibited at 1.2 and 2.5 mg / ml, respectively (Fig. 3).

M. charantia and *P. guajava* were found to be the most effective against fish and shrimp pathogenic bacteria (Direkbusarakom *et al.*, 1998). *M. charantia* extract inhibited 70% and 100% of the tested bacteria at 1.25 and 2.5 mg / ml, respectively (Table 2). *P. guajava* inhibited 50% of the tested bacterial strains. Sindermsuk *et al.* (1989) found that *P. guajava* could inhibit *V. cholerae* and *V. parahaemolyticus*, that causative agents of diarrhea in humans. However, in this study, *M. charantia* followed by *P. guajava* were found to be the second highest effective herbs to inhibit *A. hydrophila* and *P. fluorescens* at MIC 1.2 mg / ml. *P. hydropiper* showed inhibitory effect against *A. hydrophila* to control bacterial disease of pangasius fish (Dung, 1990). The decoction of leaves of this herb showed good inhibitory effect at MIC 1.2 mg / ml on *A. hydrophila* and *P. fluorescens* in the present study.

Many kinds of herbs have been introduced to shrimp farms suffering from infectious diseases in Thailand, since 1990. For example garlic or onion has been mixed to the shrimp pellet and fed every day to protect the bacterial infection (Direkbusarakom, 2000). Garlic contains acroline, crotonic, aldehyde and allyl sulphide, which act as a powerful germicide. It is a natural antiseptic agent (Anawer, 2001). Spices have been shown to possess medicinal value, in particular, antimicrobial activity (Arora and Kaur, 1999). Of the different spices tested only garlic and clove were found to possess antimicrobial activity. The bactericidal effect of garlic extract was apparent within 1 h of incubation and 93% killing of *Staphylococcus epidermidis* and *Salmonella typhi* was achieved within 3 h. Some bacteria showing resistance to certain antibiotics were sensitive to extracts of both garlic and clove. Anti-candidal activity was shown by garlic than by nystatin. However, spices might have a great potential to be used as antimicrobial agents. Chowdhury *et al.* (1991) found that the aqueous extract of garlic (*Allium sativum*) and allicin, a naturally occurring antibiotic from garlic both showed significant *in vitro* antibacterial activity against isolates of multiple drug-resistant *Shigella dysenteriae* 1, *Sh. flexneri* Y, *Sh. sonnei* and enterotoxigenic *Escherichia coli*. The minimum inhibitory concentrations of the aqueous extract and allicin against *Sh. flexneri* Y were 5 and 0.4 μl / ml, respectively. In our experiments, the extracts obtained from bulb of garlic (*A. sativum*) was highly effective against the tested two bacteria, *A. hydrophila* and *P. fluorescens* and the minimum inhibitory concentration of the extracts was at 0.6 mg / ml.

Table 2. Minimum inhibitory concentrations (MIC) of the eight selected herbs against three fish pathogenic bacteria

S / L	Herbs species (used part)	Concentration of herb (mg / ml)	Pathogenic bacteria of fish		
			Aeromonas hydrophila	*Pseuomonas fluorescens*	*Edwardsiella tarda*
1.	*Acacia arabica* (Bark inf.)	10	+	+	+
		5	+	+	+
		2.5	+	+	+
		1.2	-	-	-
		0.6	-	-	-
		0.3	-	-	-
2.	*Allium cepa* (B)	10	+	+	-
		5	+	+	-
		2.5	+	+	-
		1.2	-	-	-
		0.6	-	-	-
		0.3	-	-	-
3.	*Allium sativum* (B)	10	+	+	+
		5	+	+	+
		2.5	+	+	+
		1.2	+	+	-
		0.6	+	+	-
		0.3	-	-	-
4.	*Calotropis gigantea* (Ld)	10	+	+	+
		5	-	-	+
		2.5	-	-	+
		1.2	-	-	+
		0.6	-	-	+
		0.3	-	-	-
5.	*Momordica charantia* (L)	10	+	+	+
		5	+	+	+
		2.5	+	+	+
		1.2	+	+	-
		0.6	-	-	-
		0.3	-	-	-
6.	*Polygonum hydropiper* (Ld)	10	+	+	+
		5	+	+	+
		2.5	+	+	+
		1.2	+	+	-
		0.6	-	-	-
		0.3	-	-	-
7.	*Psidum guajava* (L)	10	+	+	+
		5	+	+	+
		2.5	+	+	-
		1.2	+	+	-
		0.6	-	-	-
		0.3	-	-	-
8.	*Tamarindus indica* (L)	10	+	+	+
		5	+	+	+
		2.5	-	-	-
		1.2	-	-	-
		0.6	-	-	-
		0.3	-	-	-

B = Bulb, L = Leaf, Ld = Decoction of leaves, Inf. = Infusion, + = Positive response, - = No response.

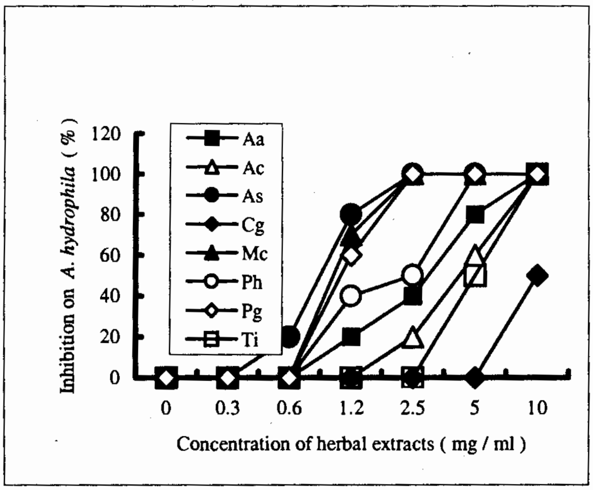

Fig. 1. Inhibitory effect of eight herbal extracts on the survival of *A. hydrophila*

Aa : *Acacia arabica* Ac : *Allium cepa* As : *Allium sativum* Cg : *Calotropis gigantea*
Mc : *Momordica charantia* Ph : *Polygonum hydropiper* Pg : *Psidium guajava* Ti : *Tamarindus indica*

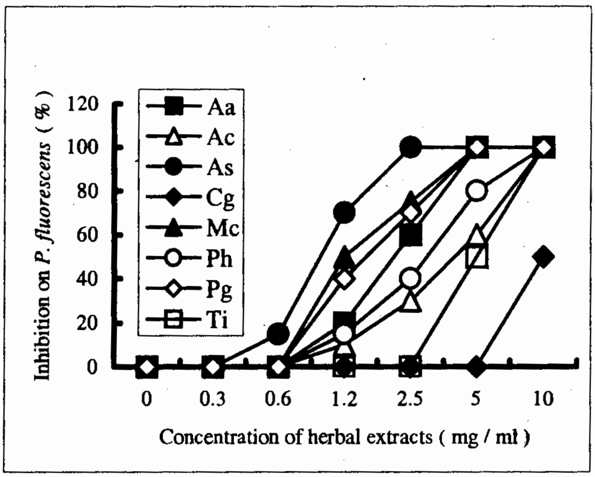

Fig. 2. Inhibitory effect of eight herbal extracts on the survival of *P. fluorescens*

Aa : *Acacia arabica* Ac : *Allium cepa* As : *Allium sativum* Cg : *Calotropis gigantea*
Mc : *Momordica charantia* Ph : *Polygonum hydropiper* Pg: *Psidium guajava* Ti: *Tamarindus indica*

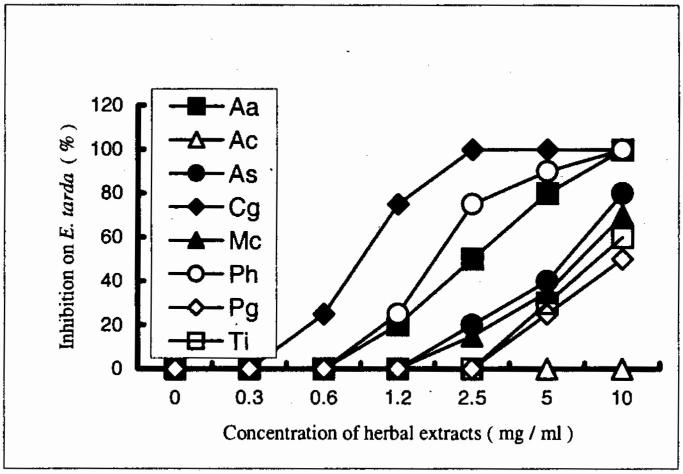

Fig. 3. Inhibitory effect of eight herbal extracts on the survival of *E. tarda*

Aa : *Acacia arabica* Ac : *Allium cepa* As : *Allium sativum* Cg : *Calotropis gigantea*
Mc : *Momordica charantia* Ph : *Polygonum hydropiper* Pg : *Psidium guajava* Ti : *Tamarindus indica*

A powder of dried leaves of *C. gigantea* is an efficacious local application for ulcer, eczema and other skin diseases (Anawer, 2001). A decoction of leaves is also a useful washing and rapidly healing agent for ulcer disease in human. In the present study, the decoction of leaves of this herb was found to be the most effective against *E. tarda*. In contrast, the extracts collected from the bulb of *A. cepa*, *C. zedoaria* and *C. longa* had no effect on this bacterium but showed low effect on other two bacteria. It indicates that probably no antibacterial property for inhibition of *E. tarda* is present in the bulb extract of *A. cepa*, *C. zedoaria* and *C. longa*. However, the present study provides useful information for the first time in Bangladesh regarding the efficacy of medicinal herbs against common fish pathogenic bacteria in aquaculture of Bangladesh. *A. sativum* for inhibition of *A. hydrophila* and *P. fluorescens* and *C. gigantea for E. tarda* were detected to be the most promising herbs among all of the herbs tested. On the basis of the results achieved, studies are necessary for its application in field condition.

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