SYNOPSIS
**Introduction**

Aquaculture, beyond doubt, is the fastest growing food-producing sector in the world and fish is one of the most widely consumed low-cost protein source in many parts of the world (FAO, 2005). Increased worldwide demand for fish and shellfish has resulted in intensification of aquaculture. Intensive aquaculture has led to a higher incidence of disease-outbreaks with an increasing range of pathogens. So far, conventional approaches such as the use of disinfectants and antimicrobial drugs have had limited success in the prevention or cure of aquatic diseases. Massive use of antimicrobials for disease control and growth promotion in animals increases the selective pressure exerted on the microbial world and hence resulting in natural emergence of antibiotic resistance. Though application of vaccines in fish farming to prevent bacterial diseases is effective (Siwicki et al., 1998) but, protection is species (pathogen) specific and expensive (Robertson, 1999). Problem with present vaccines, antibiotics, drugs and chemical treatments to prevent diseases in fish has set the stage for a new concept in disease prevention, the use of immunostimulants. Immunostimulants are natural or synthetic substances able to activate the non-specific immune mechanisms and specific immune responses if the treatment is followed by infection or vaccination (Anderson, 1992). The use of immunostimulants for the prevention of fish diseases progresses, several preparations and regimes have become more promising (Anderson, 1992; Raa et al., 1992; Jeney and Jeney; 2002). The actual number of synthetic substances or drugs used in fish farming is low and more emphasis is now given to natural replacements (Cuesta et al., 2002).

Herbal drugs are known to possess immunomodulatory properties and generally act by stimulating both specific and non-specific immunity. Many plants used in Indian traditional medicine are reported to have immunomodulating properties
in mammals (Singh and Shukla, 1998; Colic and Savic, 2000; Ali et al., 2000; Thakur et al., 2007). Recently, some of these plants have also been reported to exhibit immunomodulatory activity in fish (Sahu et al., 2007; Divyagnaneswari et al., 2007; Christybapita et al., 2007; Chakrabarti and Rao, 2006; Sudhakaran et al., 2006; Yin et al., 2006).

_Nyctanthes arbor-tristis_ L. commonly called as night jasmine or coral jasmine, widely used in the traditional medicinal systems of India, has hepatoprotective, antileishmanial, antiviral and antifungal activities (Puri et al., 1994). Earlier workers have reported the presence of polysaccharides, nyctanthoside, nyctanthic acid, β-sitosterol, 6β-hydroxyloganin and arbortristoside - A and B (Purushothaman et al., 1985; Rathor et al., 1989; Tuntiwahwuttikul et al., 2003). Arbortristoside - A and B have been reported to possess leishmanicidal (Tandon et al., 1991), antiplasmodial (Tuntiwahwuttikul et al., 2003), antispermatic (Gupta et al., 2006) and antiallergic activities (Gupta et al., 1995) in mice. Strong stimulation of antigen specific and non-specific immunity, evidenced by increased humoral and delayed type hypersensitivity (DTH) responses to sheep red blood cells (SRBC) and in macrophage migration index (MMI), has been demonstrated in mice fed with 50% ethanolic extract of seeds, flowers and leaves of NAT in mice (Puri et al., 1994). As, there has not been any immunological investigation on the effect of _Nyctanthes arbor-tristis_ L. (NAT) seeds in fish species, the NAT seeds have been chosen for detailed investigation on the immunity of _Oreochromis mossambicus_. In this context, the present study aimed to investigate the immunomodulatory property of intraperitoneally and orally administered NAT seed extracts on humoral and cellular non-specific immune responses, specific immune response and disease resistance in _O. mossambicus_ against _Aeromonas hydrophila_.

Objective

The objective of the present study was to study the effect of chloroform (CE), methanol (ME) and aqueous (AE) extracts of NAT seed on the immune responses of *Oreochromis mossambicus* with special reference to,

1. Non-specific immune mechanisms with reference to
   a. Humoral parameters
      - total and differential count of blood leucocytes
      - serum lysozyme activity
      - serum myeloperoxidase activity
      - serum alternative complement activity
      - serum antiprotease activity
      - serum bactericidal activity and
   b. Cellular parameters
      - intracellular reactive oxygen species (ROS) production
      - intracellular reactive nitrogen intermediates (RNI) production
      - intracellular myeloperoxidase activity

2. The specific immune response as assessed in terms of antibody response to heat killed *Aeromonas hydrophila* tested by bacterial agglutination assay and ELISA

3. Disease resistance of *Oreochromis mossambicus* as tested against live virulent *Aeromonas hydrophila*.

In addition, the antibacterial properties of *Nyctanthes arbor-tristis* L. seed extracts have been tested against four common bacterial fish pathogens.
Methods

NAT seeds were successively extracted with (solvents with increasing polarity) petroleum ether, chloroform, ethyl acetate, methanol and finally with aromatic water (CHCl₃ 0.25% v/v) and filtered. The successive extraction was done by cold maceration process for seven days (Cooper and Gunn, 2005; Singh et al., 2007; Ghule et al., 2006). Chloroform (CE), methanol (ME) or aqueous (AE) extraction yielded sufficient amount of extract concentrate and so these extracts were chosen for detailed investigation. The desired doses of CE were prepared using pure coconut oil (to dissolve the non-polar compounds) and ME and AE in sterile distilled water (to dissolve polar compounds). Fish were administered intraperitoneally with NAT seed extracts at doses of 2, 20 or 200mg Kg⁻¹ body weight. Appropriate control groups were maintained. To investigate the non-specific immune parameters, fish were bled on 2, 4, 6, 8 and 10 days after the intraperitoneal administration of seed extracts and various immune parameters were assayed.

In another set of experiments, fish were fed with balanced fish diet supplemented with extracts of NAT seeds at levels of 0.01%, 0.1% or 1% w/w of the feed for selected feeding schedules and the control groups for these schedules were fed with normal diet. The different feeding schedules include Group I: one week feeding; Group II: two weeks feeding; Group III: three weeks feeding. Fish were tested at the end of every week of feeding for various immunological parameters and disease resistance.

Results

The results of the present study indicate immunostimulatory and disease protective properties of NAT seed extracts administered either intraperitoneally or orally as feed supplement in *O. mossambicus*. Following section deals with the major
findings of the investigation involving intraperitoneal administration of NAT seed extracts.

1. **Immunomodulatory effect of NAT seed extracts administered intraperitoneally in *O. mossambicus***

- In general, NAT seed extracts caused an increase in total peripheral blood leucocyte count, which was due to a corresponding increase in the number of circulating granulocytes and lymphocytes.

- Serum lysozyme activity was increased in all the treated groups and particularly those treated with CE showing increased lysozyme activity throughout the test days irrespective of the dose used.

- In general, all the three extracts of the seed significantly enhanced serum MPO activity on most of the days tested.

- The serum \( \text{ACH}_{50} \) activity was enhanced by CE or ME treated groups on different days.

- In general, all the three extracts enhanced the serum antiprotease activity on most of the days tested.

- By and large, the seed extracts were effective in increasing serum bactericidal activity on most of the days tested.

- NAT seed extracts increased the ROS production in one or two days post treatment.

- The RNI production in the peripheral blood leucocytes was significantly increased by all the seed extracts on certain post treatment days.

- The NAT seed extracts significantly enhanced myeloperoxidase activity of peripheral blood leucocytes on most of the post treatment days.
With reference to specific immune response in terms of antibody response to heat-killed *A. hydrophila* (assessed by bacterial agglutination assay and ELISA), in general, only selected doses increased the primary antibody response on certain days whereas most of the doses of NAT seed extracts significantly enhanced the secondary antibody response on most of the days tested.

Although the seed extracts administered as single or double dose caused considerable increase in protection against live virulent *A. hydrophila*, the single dose treatment was found to be comparatively more effective than the double dose. The maximum RPS value of 43.48 was observed in the fish administered with single dose of 200 mg Kg\(^{-1}\) of AE.

2. **Immunomodulatory effect of NAT seed extracts orally administered as feed supplement in *O. mossambicus*  
   - Significant increase in total circulating leucocytes was observed in all the NAT seed extract fed groups only after two weeks. This enhancement was evidently due to increased numbers of circulating granulocytes and lymphocytes to certain extent.
   - Serum lysozyme and myeloperoxidase activities were significantly increased in the fish fed with NAT seed extracts supplemented diet.
   - Feed supplemented with CE of NAT seed increased serum alternate complement activity irrespective of the dose and duration of treatment and only certain doses and duration of ME or AE enhanced alternate complement activity.
   - In general, serum antiprotease activity was significantly increased on administration of AE in most of the treatment regimens whereas only certain doses and durations of treatment with CE and ME caused an increase in antiprotease activity.
• Generally, the serum bactericidal activity was significantly increased after feeding with diet containing CE, ME or AE of NAT seed.

• While ROS production by peripheral blood leucocytes was significantly enhanced in the fish administered with NAT seed extracts irrespective of the dose and duration of treatment, the RNI production was generally enhanced only after the treatment for a week or two.

• The MPO activity in peripheral blood leucocytes was increased by most of the regimens of treatment with all the three NAT seed extracts.

• In general, specific immune response in terms of antibody response assessed by bacterial agglutination assay and ELISA was enhanced by all NAT seed extracts with highest dose normally causing maximal enhancement.

• When fish fed with NAT seed extract - supplemented feed were challenged with virulent A. hydrophila, compared to the untreated control, the percent mortalities in general were significantly reduced reflecting certain levels of protection. Among the treated groups, the fish fed with CE-supplemented diet for 3 weeks exhibited maximum protection with the RPS value of 59.10 followed by AE and ME.

3. **Antibacterial property of NAT seed extracts against fish pathogens**

• In agar-well diffusion test, NAT seed extracts did not develop inhibition zone against any of the fish pathogens tested. However, in minimum inhibitory concentration (MIC) test, ME of NAT seed alone inhibited the growth of A. hydrophila and *Flavobacterium columnare* with MIC values of 50 and 6.25 mg/ml respectively.

Qualitative tests were performed to analyze the NAT seed extracts for their chemical constituents (Table 5). The results indicated the presence of coumarins in all
the NAT seed extracts. Alkaloids and phytosterols were exclusively present in CE and ME. Carbohydrates were present in ME and AE. Saponins and tannins were present in AE.

**Conclusion**

The results of the present study indicate the absence of effective antibacterial activity of NAT seed extracts against the four fish pathogens tested. Hence, whatever disease resistance or protection observed in this study presumably, was due to the immunostimulatory action of certain compounds present in the NAT seed extracts. Thus, the study indicates the immunostimulatory effect of NAT seed extracts especially by chloroform extract (CE) and recommends the use of NAT seed or its extract as dietary supplement to augment fish health in finfish aquaculture. However, before applying them in fish culture there is a need to understand the mechanism behind this immunostimualtion at cellular and molecular levels and to rule out any possible adverse side effects of using these plant products in culture situation. Appropriate field trials are also necessary before using the *Nyctanthes arbor-tristis* seed extracts prophylactically to prevent infectious diseases in finfish aquaculture systems.