MATERIALS AND METHODS
1) FISH - BIOLOGY AND MAINTENANCE

1.1. Biology of Oreochromis mossambicus (Peters)

*Oreochromis mossambicus* is a freshwater teleost, native to Africa. In Greek, *oreos* means ‘of the mountains’; *chrom* means ‘color’ (Boschung and Mayden, 2004) and mossambica describes the geographic area, Mozambique, to which the species is native (Jubb, 1967; Moyle, 1976). It is placed taxonomically as follows:

<table>
<thead>
<tr>
<th>Kingdom</th>
<th>Animalia</th>
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<tbody>
<tr>
<td>Phylum</td>
<td>Chordata</td>
</tr>
<tr>
<td>Subphylum</td>
<td>Vertebrata</td>
</tr>
<tr>
<td>Super class</td>
<td>Osteichthyes</td>
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<tr>
<td>Class</td>
<td>Actinopterygii</td>
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<tr>
<td>Order</td>
<td>Perciformes</td>
</tr>
<tr>
<td>Family</td>
<td>Cichlidae</td>
</tr>
<tr>
<td>Genus</td>
<td><em>Oreochromis</em></td>
</tr>
<tr>
<td>Species</td>
<td><em>mossambicus</em></td>
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In Asia, it was the first introduced tilapia species. In the year 1930, *O. mossambicus* was introduced to Indonesia from South Eastern Africa (Guerrero, 2001). Later in 1952 it was introduced into India from Bangkok (Panikkar and Thambi, 1954). Tilapia is third only to carps and salmon as the most widely farmed freshwater fish in the world.

1.2. Habitat

*O. mossambicus* has a broad salinity tolerance (Trewevas, 1983) and it can survive at high range of salinity of upto 40 ppt (Knaggs, 1977; Dial and Wainright, 1983). It can also endure high water temperature, low dissolved oxygen and high
ammonia concentration. It breeds in all types of waters such as saline, brackish, fresh or foul and also in waters at an altitude of 7000 feet above sea level (Sterba, 1962).

1.3. Sexual Maturity and Dimorphism

Tilapia is known for their ability to sexually mature at a small size, females mature at the size of 150-160 mm and males mature at 170-180 mm (Hodgkiss and Man, 1978; Arthington and Milton, 1986). Adult fish are known to live six to eight years, but some of them with longevity of eleven to twelve years have been reported (Boschung and Mayden, 2004; Fryer and Illies, 1972). Sexes are separate and there is a marked sexual dimorphism. Males are larger and darker than females, have longer anal, dorsal fins and more robust jaws (Oliveira and Almada, 1995). In males the genital opening is circular at the end of the papilla, whereas in the females it is a transverse slit (Pandian and Muthukrishnan, 1988). During the breeding season, the male develops a reddish colour along the fringes of the dorsal, caudal and anal fins. The shape of the dorsal and anal fin in females is round and in males they are pointed. The relative length of pelvic fin in females is not reaching the anus, but in males it is reaching beyond the anus (Oliveira and Almada, 1995).

1.4. Reproduction and Fertilization

Males construct nests in sparse to moderately vegetated bottoms where fertilization of the eggs takes place (Bruton and Bolt, 1975). Several females lay eggs in the same nest. Single female can lay 50-1780 eggs, based on individuals' size and environmental conditions (Trewevas, 1983). Once fertilized, the female take the eggs into her buccal cavity (hence they are called African mouth breeders) and brood them until hatching. Hatching occurs in approximately 3-5 days. Once hatched, the females continue to mouth-brood the fry until they are approximately 14-21 days old.
1.5. Feeding habit

*O. mossambicus* is placed in the opportunistic omnivore category by Boschung and Mayden (2004). This species is largely herbivorous, consuming planktonic algae, diatoms, aquatic plants and small fish (Moyle 1976; Chacko and Krishnamoorthi, 1954; Boschung and Mayden 2004). Juveniles are carnivorous; adults tend to be herbivorous (Panikkar and Thambi, 1954). They exhibit considerable plasticity in their feeding habits. It readily accepts artificial feed and worms in aquarium and ponds.

1.6. Fish maintenance

Male fish alone were used for this study to maintain the homogeneity of the sample avoiding the data being distorted by possible immune modulation by hormones in mature females. Further, tilapia is grown worldwide as male monosex culture because males grow faster and are more uniform in size (Chapman *et al.*, 1992). Females use considerable energy in egg production and do not eat when they incubate eggs. Male fish weighing 30±5 g were used for all the assays except for non-specific cellular assays (which require large number of peripheral blood leucocytes), fish weighing 50±5 g were used. Fish were procured from a local fish farmer and acclimated for two weeks in fibre reinforced plastic (FRP) tanks (vol. 400 L) at a density of 4gL⁻¹. Canister water filters (Eheim, Germany) were used to maintain the water quality during this study. Temperature was not maintained in the fish tanks since earlier studies in this laboratory indicated only minor daily fluctuation in water temperature i.e. 28±2°C. Other water quality parameters were monitored and maintained (pH 7.3±0.3 and dissolved oxygen content 5.2±0.1 mg L⁻¹). Fish were fed *ad libitum* with balanced fish diet prepared in this laboratory (Prabakaran *et al.*, 2006).
2) NYCTANTHES ARBOR-TRISTIS L. AND EXTRACT PREPARATION

2.1. Nyctanthes arbor-tristis L.

The systematic position of *Nyctanthes arbor-tristis* L. (NAT) according to Bentham and Hooker (1965) is as follows:

- **Kingdom**: Plantae
- **Division**: Magnoliophyta
- **Class**: Magnoliopsida
- **Order**: Lamiales
- **Family**: Oleaceae
- **Genus**: *Nyctanthes*
- **Species**: *arbor-tristis* L.

2.2. Preparation of extracts

*N. arbor-tristis* L. seeds were collected from a nearby residential premise during the month of November 2005. The plant was authenticated as *Nyctanthes arbor-tristis* L. and the voucher specimen (CFI 03-102) was deposited in the herbarium of Department of Botany, Lady Doak College, Madurai (Plate 1). The fully ripened fresh seeds were washed 3-4 times in sterile distilled water, dried in shade, coarsely powdered and stored at -20ºC until used. One thousand grams of coarsely powdered seed was successively extracted with petroleum ether, chloroform, ethyl acetate, methanol and finally with aromatic water (CHCl$_3$ 0.25% v/v). Each extraction was done by cold maceration process for seven days with agitation twice daily (Cooper and Gunn 2005; Singh *et al.*, 2007; Ghule *et al.*, 2006) (Flow chart - 1) and filtered through sterile muslin cloth and filter paper. The extracts were concentrated in rotary vacuum evaporator separately (BUCHI, Switzerland) and stored at -20ºC until use. Sufficient amount of extract concentrate was yielded from chloroform (CE),
PLATE 1. *NYCTANTHES ARBOR-TRISTIS* L.

- **PLANT**
- **FLOWER**
- **FRUITS**
- **SEEDS**
FLOW CHART - 1

PREPARATION OF NYCTANTHES ARBOR-TRISTIS L. SEED EXTRACT

(Cooper and Gunn, 2005; Singh et al., 2007; Ghule et al., 2006)

NAT seeds were soaked in petroleum ether for 7 days at 15ºC

- Filtered and Dried ➔ Petroleum ether extract

- Marc ➔ Soaked in chloroform for 7 days at 15ºC
  - Filtered and Dried ➔ Chloroform extract

- Marc ➔ Soaked in Ethyl acetate for 7 days at 15ºC
  - Filtered and Dried ➔ Ethyl acetate extract

- Marc ➔ Soaked in Methanol for 7 days at 15ºC
  - Filtered and Dried ➔ Methanol extract

- Marc ➔ Soaked in aromatic water (CHCl₃ 0.25% v/v) for 7 days at 15ºC
  - Filtered and Dried ➔ Aqueous extract

- Marc Discarded
methanol (ME) or aqueous (AE) extraction. The desired dose of CE was prepared using pure coconut oil (in order to dissolve the non-polar compounds) and ME/AE in sterile distilled water. These NAT seed extracts were tested for immunomodulatory activity in *O. mossambicus*.

3) ROUTE OF ADMINISTRATION

Injection is the most effective method of administration of immunostimulants to fish (Sakai, 1999). But for practical application in the field, administration of immunostimulants through feed would be much easier, non-stressful, less labour-intensive and also it allows large scale administration. In the present study, administration through intraperitoneal route was done to understand the perfect dose-response relationship in immunomodulation by the NAT seed extracts. Equivalent experiments involving administration of NAT seed extracts as feed supplements were also performed to test the possible efficacy of the seed extract for application in the field.

3.1. Intraperitoneal route

The experimental fish were administered intraperitoneally with 0.2 ml of CE/ME or AE of *N. arbor-tristis* L. seeds at 10 fold increasing doses of 2, 20 or 200 mg Kg\(^{-1}\) body weight (bw). The control groups received 0.2 ml of sterile water or purified coconut oil (Flow chart 2). The group administered with sterile water was maintained as control for groups treated with ME or AE and coconut oil administered group was the control for the group treated with CE. Intraperitoneal administration of the extracts was carried out using one ml tuberculin syringe with 24-gauge needle. After the administration of the extracts, various immunological parameters were assayed.
FLOW CHART - 2

Experimental design to study the effect of *N. arbor-tristis* L. seed extracts administered intraperitoneally in *O. mossambicus*

<table>
<thead>
<tr>
<th>Non-specific Immune Mechanisms (3 sets x 66 = 198 fishes)</th>
<th>Specific Immune Mechanism (Antibody Response) (66 fishes)</th>
<th>Over all Functional Immunity (Disease Resistance) (2 sets x 330 = 660 fishes)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Haematological Parameters</td>
<td>Bacterial agglutination assay</td>
<td>Single dose of seed extracts</td>
</tr>
<tr>
<td>Serum bactericidal activity</td>
<td>ELISA</td>
<td>Double dose of seed extracts</td>
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<tr>
<td>Serum Lysozyme activity</td>
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<td>Serum MPO activity</td>
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<td>Serum ACH$_{50}$ activity</td>
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<td>Serum antiprotease activity</td>
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<tr>
<td>ROS production by PB L</td>
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<td></td>
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<tr>
<td>RNI production by PBL</td>
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<td>MPO activity by PBL</td>
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Total no. of fishes used for IP experiments = **924**
3.2. Oral route

Fish were fed with diet supplemented with NAT seed extracts at concentrations of 0.01%, 0.1% or 1% (of the feed) whereas the control groups were fed with normal balanced diet (appendix 1). The fish were fed at rate of 2% body weight per day on different feeding schedules. The different feeding schedules were, Set I: one week feeding; Set II: two weeks feeding; Set III: three weeks feeding (Flow chart 3). At the end of every week of feeding, various immunological parameters were assayed.

4) NON-SPECIFIC IMMUNE MECHANISMS

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 experiments concerning administration of plant extracts along with feed, fish (n=6 per group) were bled at the end of each week of feeding. The fish were bled serially using 1 ml tuberculin syringe fitted with 24 gauge needle from the common cardinal vein situated just below the gills (Michael et al., 1994). The total time for bleeding a fish is around 30 seconds and interval between two successive bleeding is two days or one week. A control group was also bled in a similar schedule. The cortisol secreted if any due to handling stress in *O. mossambicus* will enter blood stream only after 4 minutes and it reaches back to normal level well within 24 hours (Foo and Lam, 1993; Binuramesh *et al*, 2006). Hence, the serial bleeding would not have caused any stress to influence the assay results. The blood was collected in serological tubes (70x10 mm) and allowed to clot overnight at 4°C and was then centrifuged at 400 g for 10 min for the serum to be separated. The serum was collected and stored in sterile micro centrifuge tubes at
Experimental design to study the effect of *N. arbor-tristis* L. seed extracts supplemented with feed in *O. mossambicus*

**FLOW CHART - 3**

6 sets of 10 groups each (n=6 fishes/group; 360 fishes)  
3 sets of 10 groups each in triplicates (n=10 fishes/group; 900 fishes)

1 control + (3 treatments x 3 doses)  
(CE, ME, AE) (0.01, 0.1 or 1%)

**Non-specific Immune Mechanisms**  
(3 sets x 60 = 180 fishes)

- Haematological Parameters
- Serum bactericidal activity
- Serum Lysozyme activity
- Serum MPO activity
- Serum ACH$_{50}$ activity
- Serum antiprotease activity
- ROS production by PBL
- RNI production by PBL
- MPO activity by PBL

**Specific Immune Mechanism**  
(Antibody Response - BA & ELISA)  
(3 sets x 60 = 180 fishes)

- Set I
  - One week feeding & Immunization
- Set IV
  - One week feeding & Immunization
- Set V
  - Two week feeding & Immunization
- Set VI
  - Three week feeding & Immunization

**Over all Functional Immunity**  
(Disease Resistance)

- Set I  
  - One week feeding & Challenge
- Set II  
  - Two weeks feeding & Challenge
- Set III  
  - Three weeks feeding & Challenge

Total no. of fishes used for feed experiments = 1260
-20°C until used for assays. The leucocytes were separated from peripheral blood leucocytes to study the non-specific cellular immune mechanisms.

**4.1. Haematological parameters**

**4.1.1. Total count of leucocytes**

Fish were bled from common cardinal vein to collect 0.2ml of blood in microcentrifuge tubes previously coated with 5% EDTA (Ethylene diamine tetraacetic acid). For total count, 100µl of collected blood was diluted with dilute acetic acid (1:20 dilution) (appendix 2) in Sahli’s pipette. The cells were counted in the Neubauer chamber (haemocytometer) under 400X magnification.

**4.1.2. Differential counting of leucocytes**

From the undiluted blood collected for total count, 0.1ml was put on a clean glass slide to make thin and even smears. The smears were then air dried and fixed in 100% methanol for 2-3 minutes. The fixed slides were then stained with Leishman’s stain by immersing the slide in it for 5 minutes and rinsed thoroughly. The cells were counted under 400 X magnification.

**4.2. Serum lysozyme activity**

Serum lysozyme activity was analysed by using a turbidimetric assay described by Parry *et al.* (1965) with the microplate adaptation of Hutchinson and Manning (1996). A suspension of 0.3mg ml$^{-1}$ *Micrococcus lysodeikticus* in 0.05M sodium phosphate buffer (appendix 3) was used as the substrate. Ten microlitres of serum (in duplicate wells) was added to 250µl of the bacterial suspension in 96-well microtitre plate and the reduction in absorbance at 490nm was determined after 0.5 and 4.5min of incubation at 28°C in a microplate reader (Biorad, USA). One unit of lysozyme activity was defined as a reduction in absorbance of 0.001 min$^{-1}$ (Ellis 1990).
4.3. Serum Myeloperoxidase activity

Serum Myeloperoxidase activity was measured by the method described by Quade and Roth (1997) with partial modification (Sahoo et al., 2005). To 10μl of serum, 90μl of phenol red-free Hank’s balanced salt solution (HBSS) (appendix 4) containing Mg\(^{2+}\) and EGTA (Ethylene glycol tetraacetic acid) was added (in duplicate wells) in a 96-well microtitre plate. To this mixture, 50μl of TMB (3, 3’, 5, 5’- tetramethylbenzidine hydrochloride) - H\(_2\)O\(_2\) (Genei, India) was added and incubated for 2 minutes at room temperature. To stop the reaction, 50μl of 2M H\(_2\)SO\(_4\) was added. Optical density was read in a microplate reader at 450nm against 100μl of HBSS as blank.

4.4. Alternate complement activity (ACH\(_{50}\))

The alternate complement activity was studied using sheep red blood cells (SRBC) as targets (Ortuno et al., 1998). The SRBC stored at 10°C in Alsever’s solution (pH 6.1) (appendix 5) were washed and resuspended at 3% (v/v) in phenol red-free HBSS containing 10mM Mg\(^{2+}\) and 10mM EGTA (ethylene glycol tetraacetic acid). One hundred microliters of serum was serially diluted in HBSS and was mixed with an equal volume of SRBC (in duplicate wells) in a 96-well microtitre plate. After incubation for 1h at 22°C, the samples were centrifuged at 400g for 5 min at 4°C to remove unlysed erythrocytes. The haemoglobin content of the supernatants was assessed by measuring the absorbance at 450nm in microplate reader (Biorad, USA). The values of maximum and minimum haemolysis were obtained by adding 100μl of distilled water or HBSS to 100μl of SRBC, respectively. Lysis curves were generated by plotting percent haemolysis against the volume of test serum added (ml) on \(\log_{10}\) - \(\log_{10}\) scaled paper. The volume yielding 50% haemolysis (ACH\(_{50}\) units ml\(^{-1}\)) was determined for each group.
4.5. Serum antiprotease activity

This experiment is a modification of a method described by Bowden et al. (1997). The assay uses an aniline-arginine dye ester as a substrate for trypsin, which hydrolyses the aniline dye resulting in a colour change that can be measured spectrophotometrically. Two millimolar BAPNA (sodium-benzoyl-DL-arginine-p-nitroanilide HCl, Himedia, India) was used as the substrate. Ten microlitres of serum was incubated with trypsin solution (0.1% trypsin bovine pancreas, in 0.01M Tris HCl, pH 8.2, Himedia, India), in duplicate, then 500μl substrate was added and the volume was made up to 1ml with 0.1M Tris HCL (pH 8.2). It was incubated at 22°C for 25 min. The reaction was stopped with 30% acetic acid and optical density was read at 415 nm in a plate reader against a blank. The inhibitory capacity of antiprotease was expressed in terms of percentage trypsin inhibition as described by Zuo and Woo (1997). Trypsin inhibition (%) = (A1-A2/A1) x 100; where A1 = control trypsin activity (without serum); A2 = activity of trypsin remained after addition of serum.

4.6. Serum bactericidal activity

Bactericidal activity was determined by the method of Welker et al. (2007). Twenty microliters of sample serum was added to duplicate wells of a 96-well microtiter plate and incubated for 150 min with 20μl of a 24h culture of A. hydrophila. Hank’s Balanced Salt Solution was used as positive control. To each well, 25μl of 3- (4, 5 dimethyl thiazolyl-2) -2, 5- diphenyl tetrazolium bromide (MTT; 2.5 mg/ml) (Hi-Media, Mumbai) was added and incubated for 10 min to allow the formation of formazan. Plates were centrifuged at 2000 g for 10 min, the supernatant was discarded and the precipitate was dissolved in 200 μl of dimethyl sulfoxide (DMSO). The absorbance of the dissolved formazan was read at 490 nm. Bactericidal
activity was calculated as the decrease in the number of viable *A. hydrophila* cells by subtracting the absorbance of samples from that of HBSS control and reported as absorbance units.

4.7. Separation of leukocytes from peripheral blood

Peripheral blood leucocytes were separated following the method of Miller and Mc Kinney (1994) with minor modifications. The fish were bled using 5 ml syringe filled with 2 ml of blood collecting medium (RPMI-1640 supplemented with 50,000 IU l\(^{-1}\) sodium heparin, 1,00,000 IU l\(^{-1}\) penicillin, and 100 mg l\(^{-1}\) streptomycin). The diluted blood was carefully overlaid onto an equal volume of Lymphosep-lymphocyte separation medium with a density of 1.077 (Eurobio, France) and the cells were spun down at 800g for 20 min. The leucocytes in the interface were collected and washed twice with wash medium (RPMI-1640 supplemented with 10,000 IU l\(^{-1}\) sodium heparin, 1,00,000 IU l\(^{-1}\) penicillin and 100 mg l\(^{-1}\) streptomycin) and resuspended in culture medium (RPMI-1640 supplemented with 3% (v/v) of pooled tilapia serum, 1,00,000 IU l\(^{-1}\) penicillin, 100 mg l\(^{-1}\) streptomycin and 4 mM L-glutamine, Biochrom AG, Germany). Numbers of viable cells were enumerated by trypan blue exclusion method and adjusted to 4x10\(^7\) ml\(^{-1}\) using culture medium.

4.8. Production of reactive oxygen species (ROS)

The intracellular respiratory burst activity was measured by the method of Secombes (1990) with minor modifications. Peripheral blood leucocytes (1 x 10\(^6\) cells per well, in triplicate) were incubated with 25μl of nitroblue tetrazolium (NBT, 1 g l\(^{-1}\)) in 175μl culture medium for 2 h at 28°C. The supernatant was removed and the cells were fixed in 100% (v/v) methanol for 5 min. Each well was washed twice with 125μl of 70% (v/v) methanol. The fixed cells were allowed to air-dry overnight. The reduced NBT (in the form of formazan) was dissolved using 125 μl of 2N potassium
hydroxide (KOH) and 150 µl of dimethyl sulfoxide (DMSO) per well. Optical density was measured spectrophotometrically at 650 nm.

4.9. Production of reactive nitrogen species (RNI)

Nitric oxide (NO) released by peripheral blood leucocytes in the medium was measured using Griess reagent (Green et al., 1982). The leucocytes produce nitric oxide that is rapidly converted into more stable nitrite in the medium. The nitrite present in the culture supernatant can be measured colorimetrically by converting nitrite into a pink dye by adding Griess reagent. Peripheral blood leucocytes (1 x 10^6 cells per well, in triplicate) were cultured in 175 µl culture medium for 96h in a moist chamber, moistened with 1% copper sulphate solution at 28ºC. Fifty microlitres of culture supernatant was collected and transferred to a separate microtitre plate. To each well containing the culture supernatant, 50 µl of Griess reagent (appendix 6) was added. After 10 min of incubation, the optical density was measured spectrophotometrically at 570 nm. Molar concentration of NO_2^- in culture medium was read from a standard curve generated from a graded series of NaNO_2 concentration.

4.10. Myeloperoxidase activity in peripheral blood leucocytes

Total myeloperoxidase content in peripheral blood leucocytes was measured according to Palic et al. (2005) with minor modifications. The release of myeloperoxidase mostly by the azurophilic granules of neutrophils during respiratory burst activity was measured through the peroxidase content. Twenty five microlitres of cell suspension containing 1 x 10^6 cells per well (in triplicate) were incubated for 20 min with 125 µl of 0.02% cetyltrimethylammonium bromide (CTAB, Himedia, India) in flat-bottom 96-well plates. Fifty microlitres of TMB (3, 3’, 5, 5’, -tetramethylbenzidine hydrochloride) - H_2O_2 (Genei, Bangalore) was added. Exactly
after 2 min, 50 μl of 2 M sulphuric acid was added to stop the reaction. Plates were centrifuged at 600 g for 15 min and 200 μl of supernatant was transferred to fresh 96 well plates. The optical density was measured at 450 nm.

5) SPECIFIC IMMUNE RESPONSE

5.1. Antigen preparation

5.1.1. Preparation of heat-killed whole cell vaccine

The virulent strain, *Aeromonas hydrophila* AHO21 used in this assay was kindly provided by the Department of Animal Sciences, Bharathidasan University, Tiruchirappalli, India. Pure *A. hydrophila* colony was isolated with Aeromonas isolation medium (Hi media, India) and then enriched in tryptone soy broth (Hi media, India) for experimental purpose. Single cell colony of *A. hydrophila* from tryptone soy agar plate was inoculated in 30 ml of tryptone soy broth. After overnight incubation at 37°C, the culture was exposed to 60°C for 1h in water bath for heat-killing the organisms (Karunasager *et al.*, 1997). The cultures were then centrifuged at 800g for 15 min. The packed cells were washed thrice with PBS and the required dose was prepared by adjusting with phosphate buffered saline (Appendix 7).

5.2. Immunization, serial bleeding and serum separation

To study the specific antibody responses, experimental fish (n=6/group) were injected with different doses of the seed extracts. Two days after the seed extract administration, fish were immunized intraperitoneally with heat killed *A. hydrophila* whole organism vaccine (10⁹ cells/fish). To study the secondary antibody response, fish were administered with the same dose of antigen on day 56-post primary immunization (i.e. after the complete decline of primary antibody response). For the groups treated through feed, fish were immunized at the end of each feeding schedule (i.e. after 1, 2 or 3 weeks).
Treated fish were bled at regular intervals of 7 days and the blood was collected in serological tubes. Blood was allowed to clot overnight at 4°C and was then centrifuged at 400 g for 10 min for the serum to be separated. The serum was collected and stored in sterile microcentrifuge tubes at -20°C until used. For bacterial agglutination assay, the serum was kept at 47°C in a water bath for 30 min to inactivate complement (classical pathway) before storing at -20°C (Sakai, 1981). Primary and secondary antibody responses were measured by both bacterial agglutination assay and ELISA (Enzyme Linked Immunosorbent Assay).

5.3. Bacterial Agglutination Assay

Antibacterial antisera were titrated using bacterial agglutination assay following the method of Roberson (1990). It was performed in 96 well “V” bottom microtitre plates (Laxbro, Pune, India). The wells in the rows received 25 µl of phosphate buffered saline. In the first well of a row, 25µl of a sample antiserum was added and two fold serial dilutions were made in that row upto 11th well, leaving the 12th well as negative control and the same was repeated in other rows with samples of other antisera. A volume of 25µl of heat killed *A.hydrophila* suspension (prestained with 0.02% crystal violet) having a cell concentration of 10^9 cells/ml was added to each well. The microtitre plate was hand-shaken for effective mixing and incubated overnight at 37°C. The highest dilution of serum giving detectable macroscopic agglutination was expressed as log2 antibody titre of the antiserum.

5.4. Enzyme linked immunosorbent assay (ELISA)

Serum antibody levels to *A. hydrophila* was measured using an indirect ELISA method described by Delamare and associates (2002) with minor modifications (Binuramesh *et al.*, 2006). The bacteria were harvested from overnight liquid culture and washed thrice with 0.15M NaCl solution and adjusted to 5x10^7 cells
ml$^{-1}$ in carbonate bicarbonate buffer pH 9.6 (appendix 8). Microtitre plates were coated with (100µl/well) whole bacterial cells at a concentration of 5x10$^7$ml$^{-1}$. The plates were incubated overnight at 4°C and then washed thrice with PBS containing 0.05% Tween 20 (PBS-T). Non-specific binding sites were blocked with 100 µl/well of 1% [w/v] BSA in PBS. The plates were incubated at 28°C for 1 h and washed thrice with PBS-T. One hundred microlitres of test serum samples in triplicate were added to wells and incubated for 1 h at 28°C. After incubation, the plates were washed thrice with PBS-T and 100µl of Rabbit anti-tilapia Ig polyclonal antibody (raised in this laboratory) diluted to 1:10 in PBS was added to each well. The plates were allowed to stand for 1h at 37°C followed by washing thrice with PBS-T. Then 100µl of Goat anti-rabbit IgG antibody conjugated with horse-radish peroxidase (Sigma, USA) diluted to 1:2000 in PBS was added to each well and incubated for 1h at 37°C. The plates were again washed thrice with PBS-T. The coloring reaction was developed by adding 100µl of 3, 3', 5, 5'- tetramethyl benzidine - H$_2$O$_2$ (Genei, India). The reaction was stopped with 25µl of 1M Sulphuric acid and read at 450nm using ELISA plate reader. The mean absorbance value for triplicate wells was used to express serum antibody level.

6) DISEASE RESISTANCE

Groups of 10 fish each, in triplicates were administered intraperitoneally with 0.2 ml of extract having 2, 20 or 200 mg kg$^{-1}$ body weight of seed extract of NAT either as single dose (Day 1) or as double dose (Day 1 and 4). Distilled water and oil controls were also maintained. Seven days after the last administration of seed extracts, fish were challenged intraperitoneally with LD$_{80}$ dose (0.2 ml PBS containing 1 x 10$^8$ cells) of live virulent A. hydrophila bacteria (Courtesy: Dr.P.K.Sahoo, Central Institute of fresh water Aquaculture, Bhubaneshwar,
Orissa, India). Earlier, the LD\(_{80}\) dose was determined by administering graded doses to untreated fish. Cumulative mortality was recorded for 15 days.

In experiments involving administration of plant extracts along with feed, groups of 10 fish each, in triplicate were fed with 0.01, 0.1 or 1\% chloroform extract, methanol extract or aqueous extract supplemented feed. The control group, in triplicate received normal feed. After 1, 2 and 3 weeks of feeding, fish were challenged (injected) with virulent \textit{A. hydrophila} (LD\(_{80}\) dose, 0.2 ml PBS containing 1 x 10\(^8\) cells) and the cumulative mortality was recorded for 15 days.

The mortality symptoms observed include, haemorrhagic septicemia, distended abdomen and lesions on the ventral surface of the body. The cause of death was confirmed by re-isolating the organisms from liver of 10\% of dead fish using Aeromonas isolation medium (Hi media, Mumbai, India). Relative percent survival (RPS) was calculated by the following formula, RPS = 1 – (percent mortality in treated group/percent mortality in control group) x 100 (Ellis, 1988).

7) QUALITATIVE CHEMICAL EVALUATION

The extracts obtained were subjected to qualitative tests for the identification of various plant constituents.

7.1. Detection of carbohydrates (Kokate et al., 1999)

Small amount of extract was dissolved in 5ml of distilled water and filtered. The filtrate was subjected to Molish’s test and Benedict’s test to detect the presence of carbohydrates.

7.1.1. Molish’s test

Filtrate was treated with 2-3 drops of 1\% alcoholic O-naphthol and 2ml of concentrated sulphuric acid was added along the sides of the test tube. Most
carbohydrates (mono, oligo and polysaccharides) are converted into furfural (furan-2-aldehyde) by dehydration with concentrated sulphuric acid. The furfural formed gives a violet colour with an alcoholic solution of O-naphthol.

**7.1.2. Benedict’s test**

Filtrate was treated with few drops of Benedict’s reagent (appendix 9) and boiled. The cupric ion (Cu++) is reduced to metallic copper by reducing sugars. Formation of characteristic coloured precipitate ranging from green to brick red indicates the presence of reducing sugar.

**7.2. Detection of glycosides (Evans, 1989)**

Small quantity of extract was stirred with 10 ml of boiling distilled water. This was filtered through Whatman No.1 filter paper and 2 ml of the filtrate was hydrolyzed with a few drops of concentrated HCl and the solution rendered alkaline with a few drops of ammonia solution. Five drops of this solution was added to 2 ml of Benedict’s qualitative reagent and boiled. A reddish brown precipitate showed the presence of glycosides.

**7.3. Detection of proteins and free amino acids (Kokate et al., 1999)**

A small quantity of the extract was dissolved in 10ml of distilled water and filtered through Whatman No.1 filter paper. The filtrate was subjected to Millon’s, Biuret and Ninhydrin tests to detect the presence of proteins and free amino acids.

**7.3.1. Millon’s Test**

The extract was treated with Millon’s reagent (that contains mercuric sulphate and sulphuric acid) (appendix 10) followed by heating gives a red precipitate or colour. The red coloration is produced by the interaction of an aromatic phenol and Millon’s reagent.
7.3.2. Biuret Test

To a portion of the extract, equal volume of 5% sodium hydroxide and 1% copper sulphate were added. A characteristic reddish violet colour indicates the presence of peptide linkage. The colour is due to the formation of a copper complex with peptide linkage. All proteins, peptones and peptides answer the test.

7.3.3. Ninhydrin Test

The extract was treated with ninhydrin reagent (appendix 11). Amino acids give a characteristic colour ranging from blue to purple due to the formation of triketohydrindene hydrate.

7.4. Detection of alkaloids (Mukherjee, 2002)

Small quantity of the extract was treated with a few drops of dilute hydrochloric acid and filtered. The filtrate was treated with various reagents such as Wagner’s reagent, Dragendorff’s reagent and Mayer’s reagent. On treatment with dilute hydrochloric acid basic alkaloids are made soluble in water by forming salts. The alkaloids in acid solution form insoluble precipitate when treated with different reagents containing metal ions.

7.4.1. Wagner’s Test

To 1ml of the filtrate, few drops of Wagner’s reagent that has iodine and potassium iodide (appendix 12) were added. Formation of reddish brown precipitate indicates the presence of alkaloids.

7.4.2. Dragendorff’s Test

To 1ml of the filtrate, few drops of Dragendorff’s reagent (solution of sodium bismuth iodide) (appendix 13) was added. Formation of orange brown precipitate indicates the presence of alkaloids.
7.4.3. Mayer’s Test

To 1 ml of the filtrate, few drops of Mayer’s reagent (solution of potassiomercuric iodide solution) (appendix 14) was added. Formation of cream coloured precipitate indicates the presence of alkaloids.

7.5. Detection of phytosterols (Klyne, 1965)

Small quantity of extract was dissolved in 5 ml of chloroform. Then this chloroform solution was subjected to Salkowski and Liebermann Burchard test for the detection of phytosterols. Sterols undergo dehydration with acetic anhydride solution and concentrated sulphuric acid.

7.5.1. Salkowski Test

To 1ml of the extract, few drops of concentrated sulphuric acid were added along the sides of the tubes. The formation of red or pink ring is due to dehydration with concentrated sulphuric acid confirms the presence of phytosterols.

7.5.2. Liebermann Burchard Test

The above mentioned chloroform solution was treated with 1ml of acetic anhydride solution followed by few drops of concentrated sulphuric acid. Formation of colour ranging from blue to green is due to dehydration with acetic anhydride or concentrated sulphuric acid indicates the presence of phytosterols.

7.6. Detection of saponins ((Kokate et al., 1999)

The extract was diluted with 20ml of distilled water and it was agitated in a graduated cylinder for 15 minutes. Steroidal glycosides (saponins) are surface active hence reduces surface tension like soap solution. The formation of foam indicates the presence of saponins.
7.7. Detection of coumarins (Dean, 1963)

To 1ml of the extract, 1ml of 10% NaOH was added. On treatment with bases like ammonia or sodium hydroxide, coumarin gives yellow colour. The formation of yellow colour is due to the cleaved products of coumarin containing phenolic groups, which form salt with sodium hydroxide.

8) STUDIES ON ANTIBACTERIAL ACTIVITY

8.1. Inhibition zone assay (Perez et al., 1990)

Test organisms Aeromonas hydrophila, Edwardsiella tarda, Flavobacterium columnare and Pseudomonas aeruginosa (Courtesy: Dr. B.K. Das, Central Institute of Freshwater Aquaculture, Bhubaneshwar, Orissa, India) were selected based on their pathogenic nature in fish. NAT seed extracts (200 mg/ml) were selected to determine the antibacterial efficacy. The antibiotic, Oxytetracycline (OTC) was used as positive control. This ensured that the tested organisms were susceptible to a common antibiotic and were not resistant strains. Further OTC control is used to assess the efficacy of the NAT seed extract compared to that of OTC. The solvent used for extraction were used as negative control because it is necessary to prove that the solvent has no inhibitory action of its own.

Antibacterial activity of plant extracts was tested by well-in agar method. Three hour old culture of A. hydrophila, E. tarda, F. columnare or P. aeruginosa was spread uniformly over the agar plate using sterile cotton swab to get uniform distribution of bacteria. Wells of 8 mm diameter were made in the inoculated plate using a sterile borer. To test the antibacterial effect of CE, it is dissolved in 2% DMSO. The ME or AE were dissolved in sterile distilled water. The NAT seed extracts were filtered through 0.45µm millipore filter and 0.2 ml of each extract was filled into the wells aseptically. A positive control (OTC) and the negative control
(solvent used to dissolve the extract) have also been maintained. The plates were incubated for 24 h at 37°C. The results were recorded by measuring the diameter of inhibition zone.

8.2. Determination of minimum inhibitory concentration

The antibacterial activity was also studied in terms of minimum inhibitory concentration (MIC) for CE, ME and AE of NAT seed. The extracts of this medicinal plant were filtered through 0.45µm millipore filter and the MIC values were determined for *A. hydrophila, E. tarda, F. columnare* and *P. aeruginosa* by following the procedure of Eloff (1998). Briefly, fifty microliters of plant extract was serially diluted with sterile distilled water with a initial concentration of 100mg/ml in a 96 well microtitre plate and 50 µl of 3 hr old culture was added to each well separately. The microplate was covered and incubated at 37°C for 18h. Then, 40 µl of 0.2mg/ml of p-iodonitrotetrazolium (INT) was added to each well and incubated for 10min. The appearance of red colour in the wells indicates the growth of bacterial culture. The lowest concentration of extract that completely inhibited the bacterial growth was considered as the MIC values.

9) STATISTICAL ANALYSIS

The data were expressed as arithmetic mean ± standard error (SE). Statistical analysis of data involved one-way analysis of variance (ANOVA) followed by Tukey’s posthoc multiple comparison test. The levels of significance were expressed as *P*-value less or greater than 0.05. All statistical calculations were performed using the software, Sigma stat 2.0 (Jandel Corporation, USA). A sample one way ANOVA followed by Tukey’s test is given in appendix 15.