Immunostimulatory effect of *Tinospora cordifolia* Miers leaf extract in *Oreochromis mossambicus*

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Immunostimulatory effect of leaf extract of *T. cordifolia* on (i) specific immunity (antibody response), (ii) non-specific immunity (neutrophil activity) and (iii) disease resistance against *Aeromonas hydrophila* was investigated in *O. mossambicus*. Ethanol and petroleum ether extracts of the leaves were used. Both ethanol and petroleum ether extracts administered at doses of 0.8, 8 or 80 mg/kg body weight, prolonged the peak primary antibody titres upto one to three weeks. Ethanol extract at the dose of 8 mg/kg and petroleum ether extract at the doses of 0.8 or 8 mg/kg enhanced the secondary antibody response. All the doses of ethanol extract significantly enhanced neutrophil activity. Fish injected with petroleum ether or ethanol extract at a dose of 8 mg/kg were protected against experimental infection with virulent *A. hydrophila*. The results indicates the potential of *T. cordifolia* leaf extracts for use as an immunoprophylactic to prevent diseases in finfish aquaculture.

Keywords: Immunostimulants, *Oreochromis mossambicus*, Tilapia, *Tinospora cordifolia*

Fish culture is an age-old practice in India, which is the second largest culture fish producer in the world¹. One of the major bacterial fish pathogens in India is *Aeromonas hydrophila* which causes a variety of diseases such as haemorrhagic septicaemia, infectious dropsy, tropical ulcerative disease and finrot leading to heavy mortality in aquaculture farms²-⁴. Fish diseases can be controlled with the help of antibiotics and other drugs. However, the emergence of antibiotic resistant microorganisms is an important obstacle to their extensive use. Recently the use of immunostimulants was introduced as a prophylactic measure⁵,⁶. An immunostimulant is a substance that elevates non-specific defense mechanisms and specific immune response if the treatment is followed by vaccination or infection⁵.

So far, a number of immunostimulants that include a very heterogeneous group of substances like levamisole, lipopolysaccharides, glucans, peptidoglycans, and muramyl dipeptide have been tested in a variety of fish species⁵,⁷. Though many synthetic and natural substances have been tested for their immunostimulating abilities, traditional medicinal herbs seem to have the potential to be a rich source of active substances for immunomodulation⁸,⁹. Indian medicinal plants are a rich source of substances that are claimed to induce paraimmunity, the nonspecific immunomodulation of granulocytes, macrophages, natural killer cells and complement functions in mammalian models. Indian medicinal plants have immunomodulatory properties have been reviewed in mammalian models¹⁰. Indian medicinal plants with antioxidant and immunomodulatory activities have been identified and their antioxidant and immunomodulatory effects in mammalian species reviewed¹¹.

Traditional use of medicinal herbs in India and China to control fish diseases has been reviewed¹²,¹³. Immunostimulatory properties of the leaf extracts of *Ocimum sanctum*¹⁴,¹⁵ and Azadirachta, a seed kernel compound in *Azadirachta indica*¹⁶,¹⁷ have been reported in the tilapia, *Oreochromis mossambicus*. The immunomodulatory effects of plant products in mammals¹⁸ and fish¹⁹-²⁵ have also been extensively reviewed.

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Tinospora cordifolia Miers belongs to the family Menispermaceae. It is commonly called 'amrita', 'gur-chala' or 'jetwatika' in Sanskrit. It is mentioned in Ayurvedic literature as a constituent of several preparations used for treating general debility, dyspepsia, fever and urinary diseases. T. cordifolia is also claimed to be useful for treatment of jaundice, skin diseases, diabetes, anaemia, emaciation and infection.

The objective of the present study is to investigate the effect of ethanol and petroleum ether extracts of T. cordifolia leaves on the immune response and disease resistance in O. mossambicus.

Materials and Methods

Fish and maintenance—Oreochromis mossambicus (Mossambique tilapia), a common freshwater and brackish water cichlid fish was used. Male fish weighing 25-30 g were used. All experiments were carried out in circular plastic tanks (vol. 70 l) except disease resistance tests, which were carried out in circular cement tanks (vol. 160 l). Water was changed on alternate days. Water temperature was not maintained in the fish tanks since earlier studies in this laboratory indicated only minor daily fluctuations in temperature i.e. 28±1.5°C. The fish were fed once a day ad libitum with a balanced fish diet prepared in the laboratory.

Plant material—Tinospora cordifolia leaves were collected from the botanical garden of the American College, Madurai in November 2002. The plant species was identified and confirmed by Dr. D. Stephen, P.G. Department of Botany, The American College, Madurai, India. The voucher specimen (Specimen No: CFI03-101) was deposited in the Herbarium of Department of Botany, Lady Doak College, Madurai.

Preparation of extracts—Dried powdered leaves of T. cordifolia were defatted by extraction with petroleum ether and then extracted sequentially with ethanol according to the method described by Corrinne et al. with minor modifications.

Petroleum ether extract—The freshly collected leaves were shade dried. The dried leaves were crushed and stirred in petroleum ether for 30 min. The extract was separated from debris by filtration using a sterile muslin cloth. The procedure was repeated twice and the filtrate obtained was centrifuged. The supernatant was pooled and then using a rotary vacuum evaporator the petroleum ether was evaporated. The residue was then dissolved in purified coconut oil (as nonpolar substitute for the toxic petroleum ether) to get the desired doses.

Ethanol extract—The filtered debris obtained during petroleum ether extract preparation was used for ethanol extraction. The procedure for ethanol extraction is similar to that of petroleum ether and after rotary vacuum evaporation, from the residue, different doses were prepared in distilled water (as the polar substitute for ethanol).

Preparation of heat killed whole cell vaccine—Overnight cultures of A. hydrophila were subjected to 60°C for 1 hr in a water bath. The cultures were centrifuged at 1500 g for 15 min. The packed cells were washed and the required dose was prepared in phosphate buffered saline.

Primary and secondary antibody responses—Experimental fish (n=7/group) were administered, ip, with 0.2 ml ethanol or petroleum ether extracts of T. cordifolia leaves at doses of 0.8, 8 or 80 mg/kg body weight whereas the corresponding control fish received 0.2 ml water or purified coconut oil. Two days after the leaf extract administration, the fish were vaccinated, ip, with heat killed A. hydrophila vaccine (10⁹ cells/fish). Both extract administration and vaccination was done using a tuberculin syringe with a 24-gauge needle. To study the secondary antibody response, fish were administered with the same dose of antigen on day 42 post primary immunization. Fish were bled serially from the common cardinal vein at regular interval of 7 days after immunization. The serum was separated and the complement was inactivated at 47°C in a water bath for 30 min and stored at -20°C until further use. Serially double diluted antibacterial antisera were titrated using bacterial agglutination assay. The highest dilution of serum giving detectable macroscopic agglutination was expressed as log₂ antibody titre (to represent log₂ of the inverse of titre) of the serum.

Neutrophil activity—Fish (n=7/group) were injected with ethanol or petroleum ether extracts of T. cordifolia leaves at doses of 0.8, 8 or 80 mg/kg whereas the corresponding control fish received 0.2 ml water or purified coconut oil. Two days after the extract administration the fish were vaccinated with heat killed A. hydrophila (10⁹ cells/fish). The nitroblue tetrazolium (NBT) assay was performed for every two days post immunization. For NBT assay, method of Secombes was followed with slight modification. From common cardinal vein, 200 μl blood was drawn into a syringe with 20 μl heparinized saline. The heparinized blood was collected in silicon coated eppendorf tubes. Accurately 200 μl hepa
rinsed blood was overlaid onto 300 μl Histopaque 1077 (Sigma, USA) and centrifuged at 1500 g for 15 min. The cells in the interface were collected and washed twice in RPMI 1640 medium (Biochrom AG, Germany). The pellet was resuspended in 50 μl of RPMI-1640, which was placed in a 96 well flat bottom plate. To this cell suspension, 50 μl NBT (0.4%) was added and incubated for 90 min at 28°C. The NBT solution was pipetted out and the cells were fixed with 100% methanol and washed twice with 70% methanol. The formazan produced inside the cells were solubilised in 60 μl of 2 N KOH and 70 μl dimethylsulfoxide. The developed turquoise blue coloured solution was then read in a microplate reader (Model 550, Biorad, USA) at 655 nm.

**Disease resistance test**—Fish (n=30/group) were injected, ip, with ethanol or petroleum ether extract at doses of 0.8, 8, or 80 mg/kg on day one and four. The control fish received 0.2 ml water or oil on the same days. The groups were challenged on the seventh day after leaf extract administration by an ip injection of virulent *A. hydrophila*. Earlier the challenge dose was standardized to give 75-80% mortality in the untreated groups. Ninety-six hour mortality was recorded.

**Statistical analysis**—The data were expressed as arithmetic mean ± SE. Analysis of variance (two way) and two-tailed Student’s t-test were employed for statistical analysis.

**Results**

**Effect of ethanol extract of *T. cordifolia* leaves on the antibody response**—All the doses tested enhanced (*P*<0.05) the antibody titres on day 21 post immunization, during the primary response (Fig. 1). The enhanced antibody titres caused by the doses 0.8 and 80 mg/kg persisted for another week (till day 28). During the secondary response, the dose of 0.8 mg/kg significantly (*P*<0.001) increased the antibody titre on the peak day (day 56).

**Effect of petroleum ether extract of *T. cordifolia* leaves on the antibody response**—The ether extract though did not cause significant increase in the peak antibody titre during primary response, the peak titres persisted till day 35 (Fig. 2). After the booster administration of the antigen on day 42 post primary immunization, the secondary antibody response was significantly (*P*<0.05) enhanced on days 49 and 56 (7th and 14th day after secondary immunization) in the groups treated with petroleum ether extract at doses of 0.8
and 8 mg/kg. The peak day was found to be on day 7 for the treated and control groups.

Effect of T. cordifolia extracts on the neutrophil activity—All the doses of the ethanol extract tested significantly ($P<0.001$) enhanced the neutrophil activity (Fig. 3) on day 8. On the other hand, the immunostimulation by petroleum ether extract was not impressive and only the dose of 0.8 mg/kg caused higher ($P<0.01$) neutrophil activity (Fig. 4) on day 2 and 8.

Disease protective effect of T. cordifolia extracts—Administration of ethanol extract to O. mossambicus enhanced the protection against A. hydrophila (Fig. 5). All the three doses reduced the percent mortality when compared with that of the control group. In all the petroleum ether treated groups (Fig. 6), the percent mortality observed for 96 hrs was reduced in a dose dependent manner when compared with that of the control group.

Discussion

The present study focuses on the immunostimulant activity of ethanol and petroleum ether extracts of T. cordifolia leaves. In general both the extracts were found to stimulate (i) specific (ii) nonspecific and (iii) protective immunity.

Both the leaf extracts of T. cordifolia prolonged the primary antibody response. The secondary immune response was enhanced in the groups treated either with ethanol or petroleum ether extracts at a dosage of 8 or 0.8 mg/kg. There are no published reports on the immunostimulatory effect of T. cordifolia in fish though immunostimulatory properties of this plant have been shown in some mammalian models. The

Fig. 3—Effect of ethanol extract of T. cordifolia leaves on the neutrophil activity in heat killed A. hydrophila immunized group. Each point represents the arithmetic mean (n=7/group) ± SE. (* $P<0.001$)

Fig. 4—Effect of petroleum ether extract of T. cordifolia on neutrophil activity in heat killed A. hydrophila immunized group. Each point represents the arithmetic mean (n=7/group) ± SE. (* $P<0.01$).

Fig. 5—Effect of ethanol extract of T. cordifolia leaves on the disease resistance to a challenge with live virulent A. hydrophila in O. mossambicus expressed as 96 hr percent mortality (n=30/group).
crude extract formulation of *T. cordifolia* has been shown to enhance the humoral immunity in golden hamster and oral administration of crude extract of *T. cordifolia* stem to mice for 15 days enhanced humoral response to sheep red blood corpuscles.

The nitroblue tetrazolium (NBT) reduction assay estimates the ability of neutrophils, and macrophages to produce oxygen radicals (O₂⁻, OH, O₃, H₂O₂). The ability of macrophages to kill the pathogenic microbes is probably one of the most important mechanisms of protection against diseases in fish. Increased respiratory burst activity i.e. production of oxygen radicals can be correlated with increased killing activity. From the results (Figs 3 and 4) obtained, it is evident that fishes injected with ethanol extract at doses of 0.8, 8 and 80 mg/kg significantly enhanced the neutrophil activity on day 8 whereas the petroleum ether extract was not very effective in enhancing the neutrophil activity. However, 0.8 mg, the lowest dose of petroleum ether extract tested increased the level of neutrophil activity on day 2 and day 8. Earlier, ethanolic extract of *T. cordifolia* has been shown to improve phagocytic function in mice. In an in vitro study, *T. cordifolia* extract has been shown to increase the phagocytic and killing capacity of macrophages of rat in a dose dependent manner. The active principles of *T. cordifolia*, cordioside (Tc2), cordifolioside A (Tc5) and cordiol (Tc7) have been reported to cause macrophage activation in mice. The enhanced neutrophil activity observed in the present study may have been due to increased number of activated neutrophils as reported in mice orally fed with extract of *T. cordifolia* preparation or enhanced ROS production due to phagocyte activation by immunostimulatory compounds present in the extracts as shown in mice treated with *T. cordifolia* preparation. The enhanced antibody response and neutrophil activity in the present study were logically reflected in the disease resistance test where both the extracts were found to be effective in inducing disease resistance against an experimental challenge with a virulent pathogen. Maximum protection was conferred by 80 mg/kg dose of both the extracts. Pretreatment with the whole aqueous standardized extracts of *T. cordifolia* has been shown to reduce the mortality in mice injected with *E. coli* intraperitoneally. However it is not clearly known how at cellular and molecular level the extract works to stimulate the specific and nonspecific defense mechanism.

The stimulation of specific and nonspecific immunity and the protection against fish pathogen *A. hydrophila* by ethanol and petroleum ether extracts of *T. cordifolia* observed in the present study indicate the presence of active principles in both the ethanol and petroleum ether extracts of *T. cordifolia*. The active principle responsible for the stimulatory effect of the extract is yet to be identified. After confirming the immunostimulatory properties of *T. cordifolia* in studies involving other immunological parameters particularly those of nonspecific immunity and sufficient field trials, the plant extract can be used as an immunoprophylactic to prevent infectious diseases in finfish aquaculture systems.

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