DISCUSSION
The present investigation indicates that leaf extract and fractions of *Tinospora cordifolia* administered intraperitoneally or orally as feed supplement can stimulate immune response and increase the disease resistance to *Aeromonas hydrophila* infection in *Oreochromis mossambicus*.

**EFFECT OF T. CORDIFOLIA LEAF EXTRACT AND FRACTIONS ADMINISTERED INTRAPERITONEALLY OR ORALLY ON NONSPECIFIC IMMUNE RESPONSE IN O. MOSSAMBICUS**

**Total and differential count of peripheral blood leucocytes**

In the present study, most of the doses of extract and fractions of *T. cordifolia* leaves when administered intraperitoneally had significant stimulatory effect on the total and differential count of peripheral blood leucocytes. The aqueous extract and hexane fraction when supplemented with diet increased the total peripheral blood leucocyte count after 2 weeks of feeding and the water soluble fraction after 3 weeks of feeding. Similar results have been reported in various animal models when the same plant extract was used. Aqueous extract of *T. cordifolia* stem has shown to produce leucocytosis with predominant neutrophilia and also stimulated macrophage function in rats (Thatte and Dahanukar, 1988). Intraperitoneal administration of *T. cordifolia* stem methanolic extract to mice, for 5 days increased the total white blood cell count significantly (Mathew and Kuttan, 1999). Oral treatment of mice with water and ethanol extracts of *T. cordifolia* stem, there was significant increase in the total count of leucocytes (Manjrekar et al., 2000). Experiments with *T. cordifolia* alcoholic and water extracts resulted in an increase in total leucocyte count in mice (Grover et al., 2000). Badar et al. (2005) have reported an increase in WBC count in the patients treated with Tinofend® tablets containing water extract of stem of *T. cordifolia*. A formulation of total extracts of *T. cordifolia* resulted in increased white cell count in mice (Diwanay et al., 2004). Preparation from
T.cordifolia administered intraperitoneally to mice before whole body γ-irradiation restored total leucocyte count (Goel et al., 2004). An herbal formulation with five plants including T.cordifolia resulted in increased haemocyte count in shrimp (Citarasu et al., 2006). Similar enhancement in total leucocyte count was observed when mice were treated with abrin, a lectin derived from the seeds of Abrus precatorius (Ramnath et al., 2002) and an extract from the roots of Withania somnifera (Davis and Kuttan, 2000). Dip treatment of Cyprinus carpio with aqueous leaf extract of Azadirachta indica significantly increased the total WBC count (Harikrishnan et al., 2003; 2005). Sahu et al. (2007) have reported contradictory result of decreased total count of leucocytes when Labeo rohita fingerlings were fed with garlic, Allium sativum incorporated diet. As one of the first lines of body defence, the number of leucocytes is known to increase sharply when infections occur. While no work has been reported earlier on the effect of T.cordifolia on total and differential leucocyte count on any fish model, the present study clearly indicates that the extract and fractions of T.cordifolia leaf could stimulate the leucopoietic system.

**Serum globulin level**

The serum globulin level was significantly increased on intraperitoneal or oral administration of T. cordifolia preparations. Many workers have reported similar findings on herbal immunostimulants. Diets supplemented with methanolic extracts of Ocimum sanctum leaf and Withania somnifera root significantly increased serum immunoglobulin level in grouper, Epinephelus tauvina against Vibrio harveyi infections (Sivaram et al., 2004). Labeo rohita fed with 0.5% of Achyranthes aspera root extract (Rao et al., 2004), 0.5% of Achyranthes aspera seed powder (Rao et al., 2006) and 5 or 10 g Kg⁻¹ Allium sativum powder (Sahu et al., 2007) incorporated diet were shown to have elevated serum globulin level. Catla catla, fed with a diet containing seed extract of Achyranthes aspera
(0.5%) for four weeks exhibited increased serum globulin levels (Rao and Chakrabarti, 2005). Increase in serum globulin level is thought to be associated with a stronger innate immune response of fish (Wiegertjes et al., 1996). Increase in total immunoglobulins is good indicator of health status (Rao et al., 2006). These results indicate that T.cordifolia leaf preparations, improve the health status of O.mossambicus, by increasing the serum globulin levels.

**Lysozyme activity**

Lysozyme, known to be an important nonspecific immune mediator against bacterial infections has been found in the present study to increase its activity on treatment with T.cordifolia leaf preparation. All the doses of extract and fractions of T.cordifolia leaf, administered intraperitoneally enhanced serum lysozyme activity on all the post treatment days tested. Similarly, when fed with diet, all the doses enhanced serum lysozyme activity irrespective of duration of feeding. Recently, it was reported by this laboratory, that the lysozyme activity was enhanced on intraperitoneal administration of water or hexane soluble fractions of Solanum trilobatum leaf in O.mossambicus (Divyagnaneswari et al., 2007). Similarly, Nyctanthes arbor-tristis leaf extract and fractions also had a positive effect on serum lysozyme activity in O.mossambicus (Devasree, 2006). In another recent study in this laboratory all the doses of aqueous extract of Eclipta alba incorporated diet significantly enhanced lysozyme activity after 1, 2 and 3 weeks (Christybapita et al., 2007). A recent study elsewhere had also shown similar enhancement of lysozyme activity when tilapia was fed with feed containing 0.1% and 0.5% of Astragalus radix root for 1 week (Yin et al., 2006). When the effectiveness of traditional Chinese medicine (TCM) formulation from Astragalus root (Radix astragali seu Hedysari) and Chinese Angelica root (R.Angelicae sinensis) was evaluated on nonspecific immunity of the freshwater fish, Jian carp, a significant increase in
lysozyme activity of the experimental fish fed with TCM (Jian and Wu, 2004) was observed. The activity of serum lysozyme also increased significantly in *Oncorhynchus mykiss* fed with a marine alga, *Dunaliella salina* (Amar, 2004). *Labeo rohita* fed with 0.5% of *Achyranthes aspera* seed (Rao *et al.*, 2006) and *Allium sativum* incorporated diet were shown to have enhanced lysozyme activity (Sahu *et al.*, 2007). Immunostimulants can increase serum lysozyme activity, due to either an increase in the number of phagocytes secreting lysozyme, or to an increase in the amount of lysozyme synthesized per cell (Engstad *et al.*, 1992). Changes in lysozyme activity are greatly influenced by the potency and type of immunostimulants to which fish are exposed (Lapatra *et al.*, 1998).

**Serum natural haemolytic complement activity**

The alternative complement pathway is important in recognition and clearance of pathogens in the absence of antibodies (Stahl *et al.*, 2003). Bactericidal activity of complement has been well recognized as one of the key killing mechanisms of clearing bacteria in teleosts (Ellis, 1999; 2001). The ACH$_{50}$ activity in the present study was elevated by all the doses of aqueous extract and water soluble fraction and by middle and highest doses of hexane soluble fraction when administered intraperitoneally. On the other hand there was no modulation in the serum alternate complement activity in the group fed with aqueous extract of *T. cordifolia* leaf irrespective of the feeding schedule. However, fish fed for 2 or 3 weeks with certain doses of water or hexane soluble fraction incorporated diet showed enhanced ACH$_{50}$ activity. This enhanced activity is in agreement with the recent finding in *O.mossambicus* fed with aqueous extract of *Eclipta alba* leaf as feed supplement (Christybapita *et al.*, 2007). Large yellow croaker, fed with traditional Chinese medicine (TCM), a formulation from Astragalus root and Chinese Angelica root at 1.0% and 1.5% rates (Jian and Wu, 2004) exhibited elevated complement haemolytic activity. An α-D-glucan isolated and characterized from *T. cordifolia* also
activates the alternate pathway of complement (Nair et al., 2004). This activity of α-D-glucan is comparable to the activity of fungal β-glucan. β-glucan enhancing serum alternate complement activity has long been reported in various fish models (Engstad et al., 1992; Matsuyama et al., 1992; Jeney and Anderson 1993). The alternative pathway of complement activation is known to be one of the powerful nonspecific defence mechanisms helping to protect fish from a wide range of potentially invading organisms, such as bacteria, fungi, viruses or parasites (Li and Lovell, 1985). Haemolytic complement activity seems to vary widely in fish as a consequence of the administration of immunostimulants. The activation of the above mentioned nonspecific immunological function is associated with increased protection against pathogens and infectious diseases.

**Serum haemolysin activity**

In general, the haemolysin activity was not much influenced by the extract and fractions of *T.cordifolia* leaf. When administered intraperitoneally, though the middle and highest doses of aqueous extract and lowest dose of hexane soluble fraction caused significant enhancement in serum haemolysin activity on certain days, none of the water soluble fraction caused any modulation. Further, when the leaf preparations were administered orally there was no significant modulation in the haemolysin activity in any of the groups fed with *T. cordifolia* diet supplemented with any of the preparations at any period of measurement. While there is no report on modulation of heamolysis by plant derived immunostimulant available in literature, a few studies with other immunostimulants had dealt with this immune parameter. Feeding β-glucan to rohu, *Labeo rohita* though raised the mean serum haemolytic titre, the increase was not significant (Sahoo and Mukherjee, 2001). The results of the present investigation is in agreement with the finding of Kumari et al. (2003) where the dietary intake of lactoferrin
had no significant effect on the serum haemolytic activity of *Clarias batrachas* at any time during the experiment.

**Serum antiprotease activity**

In general, the serum antiprotease activity in terms of trypsin inhibition was significantly increased after intraperitoneal or oral administration of *T. cordifolia* leaf preparations. Fish plasma contains a number of protease inhibitors, principally α1-antiprotease, α2-antiplasmin and α2- macroglobulin that may play a role in restricting the ability of bacteria to invade and grow *in vivo* (Ellis, 1987; 2001). Protease inhibitors could selectively arrest replication of microbial pathogen without untoward toxicity to the host (McKerrow *et al.*, 1999). *In vitro* study with 50% alcohol extract of *T. cordifolia* showed a moderate inhibition of trypsin induced hydrolysis of bovine serum albumin, confirming the antiproteolytic activity of the extract (Gacche and Dhole, 2006). This enhanced antiprotease activity observed in the present study is also in agreement with the finding in *O. mossambicus* fed with *Eclipta alba* leaf aqueous extract incorporated diet (ChristyBapita *et al.*, 2007); in *Labeo rohita* fed with aqueous extract of *Achyranthes aspera* root (Rao and Chakrabarti, 2004) and in *Catla catla* fed with water extract of *Achyranthes aspera* seed (Rao and Chakrabarti, 2005). Further, rainbow trout fed with a natural carotenoid, astaxanthin supplemented diet exhibited enhanced serum antiprotease activity (Thompson *et al.*, 1995b). The results of the present study indicates that extract and fractions of *T. cordifolia* enhance the activity of natural antiproteases in the serum, which may provide effective defence against invading bacterial pathogens.

**ROS and RNI production**

The activities of phagocytic cells can be detected by phagocytosis, killing and chemotaxis. Stimulation of phagocytic cell membrane during phagocytosis triggers the production of microbicidal reactive oxygen species (ROS), such as super oxide anion,
singlet oxygen and hydrogen peroxide during the phenomenon termed as the respiratory burst (Chung and Secombes, 1988; Secombes, 1990). Production of these substances is a crucial effector mechanism for limiting the growth of fish pathogens (Olivier et al., 1995). The importance of reactive nitrogen species (RNS) in macrophage killing has also been indicated (Nathan and Hibbs, 1991). The reactive oxygen and nitrogen species are considered to be toxic for fish bacterial pathogens (Miyazaki, 1998). Enhancement of pathogen killing is the most important aspect in the macrophages of fish treated with immunostimulants (Sakai, 1999). The measurement of O$_2^-$ concentration has been accepted as an accurate parameter to quantify the intensity of a respiratory burst. In the present investigation, the extract and fractions of *T.cordifolia* leaf enhanced the ROS production and RNI production in *O.mossambicus* when administered intraperitoneally or orally. Earlier study in this laboratory on the same fish using ethanol extract of *T.cordifolia* leaf indicated increase in neutrophil activity (Sudhakaran et al., 2006). Further, methanolic extracts from five herbal medicinal plants including *T.cordifolia* mixed with the basal diet significantly enhanced the production of intracellular superoxide anion in shrimps (Citarasu et al., 2006).

Singh et al. (2006) have reported that an alcoholic extract of *T. cordifolia* enhanced the production of another reactive species, NO in tumor bearing mice. Similarly there was an increase in neutrophil count in rats treated with *T.cordifolia* extracts (Thatte and Dahanukar, 1989). Extract of this herb has been demonstrated to augment the activity of murine peritoneal macrophages, T lymphocytes and B cells (Dahanukar et al., 2000). The active principles of *T. cordifolia*, cordioside, cordifolioside A and cordial have been identified as glycosides, reported to cause macrophage activation in mice (Kapil and Sharma, 1997). An α-D-glucan exhibiting unique immune stimulating properties such as increase in nitric oxide production was isolated and characterized from
this plant (Nair et al., 2004). Intraperitoneal administration of methanolic extract of 
*T. cordifolia* stem to mice caused an enhancement in macrophage activation (Mathew and 
Kuttan, 1999).

Many other plant preparations have also been shown to modulate the ROS 
production by phagocytes. Enhanced superoxide anion production by peripheral blood 
leucocytes was also reported in *O. mossambicus* on intraperitoneal administration of water 
or hexane soluble fraction of *S. trilobatum* leaves (Divyagnaneswari et al., 2007) or when 
fed with *Eclipta alba* aqueous extract incorporated diet (Christybapita et al., 2007). *Labeo 
rohita* fed with 0.5% of *Achyranthes aspera* seed (Rao et al., 2006) and *Allium sativum* 
incorporated diets (Sahu et al., 2007) were shown to have enhanced superoxide anion 
production by peripheral blood leucocytes. Enhanced production of reactive oxygen upon 
in vitro treatment with glycyrrhizine isolated from the herb, *Glycyrrhiza glabra* in 
*Oncorhynchus mykiss* was reported by Jang (1995). The rainbow trout fed with ginger 
(*Zingiber officinale*) extract showed significant increase in the extracellular activity of 
phagocytic cells in blood, but it had not enhanced the intracellular respiratory burst 
activity (Dugenci et al., 2003).

Chintalwar et al. (1999) have shown that the aqueous extract of *T. cordifolia* 
contains polyclonal B-cell activator, which may be attributed to an acidic arabinogalactan 
polysaccharide. Similar immunomodulatory polysaccharides have been reported with 
other species of medicinal plants in mammalian models (Wagner and Stuppner, 1988; 
Wagner and Jordan, 1988). The squeezed sap of flowers of *Echinacea purpurea* 
(*Echinacin®*) containing arabinogalactan, a polysaccharide is found to increase the 
oxidative burst in mouse macrophages (Parnham, 1996). The primary target of the 
immunomodulatory compounds is believed to be the macrophages, which play a key role 
in the generation of an immune response (Devasagayam and Sainis, 2002). It is well
known that activated macrophages display increased phagocytosis and intracellular killing of pathogens that are important defence mechanisms against pathogenic bacteria. In the present study, superoxide anion production by the fish treated with *T. cordifolia* leaf preparations indicates that this herbal preparation stimulates phagocytes to produce higher amounts of ROS indicating that more phagocytes were present in the blood of treated fish or that their activity was increased.

**Humoral and cellular Myeloperoxidase activity**

Neutrophils are an important component of host defence against many bacterial, viral and fungal infections and the evaluation of neutrophil function is valuable for assessment of the health status of human and animal populations (Densen and Mandell, 1990). Fish neutrophils have phagocytic, chemotactic and bactericidal functions, an intense respiratory burst and a peroxidase (myeloperoxidase, MPO) activity (Rodrigues *et al.*, 2003; Palic *et al.*, 2005). The process of degranulation is essential for the release of MPO and activation of the halide production pathway, as well as release of a diverse mixture of antimicrobial enzymes. Myeloperoxidase activity was significantly increased by all the *T. cordifolia* leaf preparations measured as a humoral or cellular immune parameter. Similar result has been reported in *O. mossambicus* fed with *Eclipta alba* aqueous extract incorporated diet (Christybapita *et al.*, 2007). No study has been reported earlier on the effect of *T. cordifolia* on myeloperoxidase activity in any animal model including fish. As mentioned earlier, Nair *et al.* (2004) have isolated and characterized a α-D-glucan from *T. cordifolia* with immune stimulating properties. Thatte and Dahanukar (1988) reported the potent immunostimulation by *T. cordifolia*, with effects comparable to lithium and glucan. Glucans are complex polysaccharide components of cell walls found in a large variety of organisms such as bacteria, fungi and plants. Stimulatory effects of glucans on neutrophils, as well as other components of the
immune system, have long been recognized (Engstad et al., 2002; Hong et al., 2004). Glucan-specific receptors are present on phagocytic cell membranes of several species, including fish neutrophils (Ainsworth, 1994) and potent activation of neutrophil function, including an increase in phagocytosis and killing, has been described in vitro (Chen and Ainsworth, 1992; Couso et al., 2001). Palic et al. (2006) have demonstrated in vitro treatment of fathead minnows, *Pimephales promelas* neutrophils with yeast β-glucan resulted in increased oxidative burst and total MPO activity. Phytochemical analysis of *T. cordifolia* leaf extract and fractions in this study also have shown the presence of carbohydrates and glycosides. One or more of these compounds might be the reason for the activation of the neutrophils and subsequent increase in myeloperoxidase activity observed in the present study. Moreover, there are many reports on the antioxidant property of *T. cordifolia* (Prince and Menon, 1999; Desai et al., 2002; Singh et al., 2004; Rawal et al., 2004). Thus, the neutrophil activity is strongly influenced by *T. cordifolia*.

**EFFECT OF TINOSPORA CORDIFOLIA LEAF EXTRACT AND FRACTIONS ADMINISTERED INTRAPERITONEALLY OR ORALLY ON SPECIFIC IMMUNE RESPONSE TO A. HYDROPHILA**

The extract and fractions of *T. cordifolia* leaf when administered intraperitoneally at different dose levels significantly enhanced both primary and secondary antibody responses to *A. hydrophila*. Although all the doses of extract and fractions enhanced antibody response, no clear dose dependency in immunostimulation was observed. This finding confirms an observation made in this laboratory on the enhancement of antibody response in *O. mossambicus* by petroleum ether and ethanolic extracts of *T. cordifolia* leaf (Sudhakaran et al., 2006). Similar reports on the effect of *T. cordifolia* on the specific immune response of mammalian models have been reported. Humoral immunity in
golden hamster was enhanced as evidenced by the haemagglutination titre by the crude extract formulation of *T. cordifolia* (Sohni *et al*., 1996). The active principles, syringin and cordiol obtained from *T. cordifolia* caused significant increase in IgG antibodies in mice serum (Kapil and Sharma, 1997). Sainis *et al.* (1999) have reported that oral administration of crude extract of *T. cordifolia* stem to mice for 15 days increased humoral response to sheep red blood corpuscles. Intraperitoneal administration of *T. cordifolia* stem methanolic extract to mice for 5 days increased the number of plaque forming cells in the spleen and circulating antibody titre (Mathew and Kuttan, 1999). A formulation of total extracts of *Tinospora cordifolia* resulted in protection towards Cyclophosphamide induced immunosuppression showing significant increase in haemagglutinating and haemolytic antibody titres in sarcoma bearing mice (Diwanay *et al*., 2004).

Earlier studies conducted in this laboratory also revealed similar enhancement of both primary and secondary antibody responses to sheep erythrocytes by various plant extracts. Intraperitoneal administration of leaf extract of *Acalypha indica*, *Phyllanthus niruri* and seed kernel of *Azadirachta indica* have enhanced the antibody response in *O.mossambicus* (Hemapriya *et al*., 1997). Similarly enhancement of both primary and secondary immune responses by aqueous leaf extract of *Ocimum sanctum* (Logambal *et al*., 2000; Venkatalakshmi and Michael, 2001) in *O.mossambicus* was reported. *Azadiractin*, a triterpenoid extracted from neem seed kernel of *Azadirachta indica* administered intraperitoneally in *O.mossambicus* enhanced primary and secondary antibody responses to sheep erythrocytes (Logambal and Michael, 2001). The present finding is in agreement with the recent reports in *Catla catla* (Rao and Chakrabarti, 2005; Chakrabarti and Rao, 2006) fed with diet containing *Achyranthes aspera* seed elicited significant increase in haemagglutination antibody titres against chicken red blood cells.
and bovine serum albumin respectively. *Labeo rohita* (Rao et al., 2004) fed with *Achyranthes aspera* root supplemented diet caused significant increase in antibody titres against chicken red blood cells.

Enhancement of specific immune response by various plant extracts in different mammalian models has been also reported. Ethanol extract of *Andrographis paniculata* (Puri et al., 1993), crude extract of *Panax ginseng* (Singh et al., 1984), ethanolic extract of *Picrorhiza kurroa* (Atal et al., 1986), root extract of *Withania somnifera* (Davis and Kuttan, 2000) and alcoholic extract of stem bark of *Mangifera indica* (Makare et al., 2000) have been shown to induce significant stimulation of antibody response to SRBC in mice. A herbal immunomodulator preparation, consisting of aqueous ethanolic extracts of *Thujae summitates*, *Baptisiae tinctoriae radix*, *Echinaceae purpurae radix* and *Echinaceae pallidae radix* when administered orally caused significant increase in the numbers of splenic plaque forming cells and the titres of specific antibodies in sera of treated mice (Bodinet et al., 2002). Administration of *Ocimum sanctum* seed oil produced a significant increase in anti SRBC antibody titre in rats (Mediratta et al., 2002). The extract of *Achyranthes aspera* was found to enhance the induction of ovalbumin (OVA) specific humoral antibody response in mice, on intraperitoneal injection of extract along with ovalbumin (Vasudeva et al., 2002).

The significant enhancement of specific immune response by extract and fractions might be due to the presence of phytoconstituents in *T. cordifolia* leaves which include alkaloids, diterpenoid lactones, glycosides, steroids, sesquiterpenoid, phenolics, aliphatic compounds and polysaccharides (Singh et al., 2003). Qualitative analysis of the extract and fractions in the present study also confirmed the presence of carbohydrates, alkaloids, glycosides, phytosterols, saponins and coumarin, which have been shown to have immunomodulatory effect in other animal models. Chintalwar et al., (1999) have
identified an acidic arabinogalactan polysaccharide from the aqueous extract of *T. cordifolia*, which is proved to have polyclonal mitogenic activity. Similarly, a polysaccharide isolated from leaf gel of *Aloe vera* (t’Hart et al., 1989), the glycosides from rhizomes of *Curculigo orchioides* Gaerten (Lakshmi et al., 2003), the alkaloidal fraction of *Boerhaavia diffusa* (Mungantiwar et al., 1999), oral administration of Picroliv, an irridoid glycoside derived from *Picrorhiza kurroa* (Puri et al., 1992), methanolic extracts of whole plant of *Eclipta alba* containing coumarin compounds (Jayathirtha and Mishra, 2004) and triterpenoid compounds such as oleanolic acid (Raphael and Kuttan, 2003) caused an increase in humoral antibody titres to SRBC in mice. The enhancement exhibited by these immunostimulatory compounds might have also reflected in the present study as evident by the presence of these compounds in the leaves of *T. cordifolia*.

**EFFECT OF TINOSPORA CORDIFOLIA LEAF EXTRACT AND FRACTIONS ADMINISTERED INTRAPERITONEALLY OR ORALLY ON DISEASE RESISTANCE AGAINST *A. HYDROPHILA***

The overall consequence of an immunostimulant administration is reflected in the host’s ability to resist infection. In the present study analysis of percent mortality following challenge with live *A. hydrophila* showed significant protection in treated *O.mossambicus*. Results of the present investigation on disease resistance revealed that single or double dose of AE, WSF or HSF when administered intraperitoneally enhanced the protection. The double dose treatment was found to be more effective than single dose treatment. Similarly, the feed supplemented with extract or fraction when given continuously on selected feeding schedules, enhanced the protection against challenge with live *A. hydrophila*. The protection was better in the groups fed with supplemented diet for 2 and 3 weeks. The present finding unequivocally confirms the earlier finding in
this laboratory on the same animal using ethanol and petroleum ether extracts of *T.cordifolia* leaf indicating increased protection against *A. hydrophila* (Sudhakaran et al., 2006). Methanolic extracts from five herbal medicinal plants including *T.cordifolia* mixed with the basal diet significantly enhanced the survival and reduced the viral load in shrimps (Citarasu et al., 2006). *T. cordifolia* extract have shown to protect mice against mortality due to induced *E.coli* sepsis (Thatte and Dahanukar, 1988).

Extracts of other plants investigated earlier in this laboratory also revealed the protective properties in them. The intraperitoneal injection of *O. sanctum* leaf extract (Logambal et al., 2000), water or hexane soluble fractions of *Solanum trilobatum* leaf (Divyagnaneswari et al., 2007), aqueous extract, water or hexane soluble fractions of *Nyctanthes arbor-tristis* (Devasree, 2006) and the aqueous extract of *Eclipta alba* (Christybapita et al., 2007) enhanced disease resistance against *A. hydrophila* in *O. mossambicus*. Recently, decreased mortality on challenge with *A. hydrophila* was reported in *Labeo rohita* fed with 0.5% *Achyranthes* seed incorporated diet (Rao et al., 2006). *Labeo rohita* fingerlings fed with garlic, *Allium sativum* incorporated diet increased the rate of survival against *A. hydrophila* (Sahu et al., 2007). Similarly, the report of Abutbul et al. (2004) on tilapia fed with a diet containing ethyl acetate extract of *Rosmarinus officinalis* leaf powder and Fujiki et al., (1994) on carp administered with the fraction of *Undaria pinnatifida* showed an increased protection.

The methanolic herbal extract (*Solanum trilobatum, Andrographis paniculata* and *Psoralea corylifolia*) increased the survival and growth and reduced the bacterial load in the shrimp, *Penaeus monodon* post larvae (Citarasu et al., 2003). Reduced mortalities against pathogenic challenges at lower dosages of herbal principles were also reported by Kim *et al.* (2001) and by Jain and Wu (2003). Methanolic extract of *Ocimum sanctum* leaf and *Withania somnifera* root incorporated diet significantly decreased the rate of
mortality in greasy grouper, *Epinephelus tauvina* against *Vibrio harveyi* infections (Sivaram *et al.*, 2004). Dip treatment of *C. carpio* with aqueous leaf extract of *Azadirachta indica* significantly protected the fish from *A. hydrophila* infection (Harikrishnan *et al.*, 2005). Seaweed polysaccharides like sodium alginate and carrageenan increased the resistance from *V. alginolyticus* infection in *Epinephelus coicoides* (Cheng *et al.*, 2007). Citarasu *et al.* (2002) developed an artemia enriched herbal diet for *Penaeus monodon* with a combination of five herbs, which significantly increased the growth and survival during stress conditions. The increased protection in *T. cordifolia* preparations treated groups indicates that various humoral/cellular factors involved in innate and/or adaptive immunities are elevated to protect the host effectively from infection. In general, immunostimulants were found to stimulate antibody response, lysozyme, phagocytosis and other immunological functions in fish (Sakai, 1999).

In the present study, oral administration of *T. cordifolia* fractions and extracts conferred lesser degree of disease protection when compared to that of intraperitoneal administration. The lowered level of protection observed in groups fed with *T. cordifolia* (extract and fractions) supplemented feed could be due to many reasons such as, that all the fish might not have ingested the optimal dosage as there will be unequal competition for food due to hierarchy among the individuals of the group. According to Winberg and Lepage (1998), socially subordinate fishes have scarce and unreliable access to food and other resources. They also experience general lack of control and predictability as well as a constant threat of aggressive action from dominant animals. Further, the digestive enzymes in the digestive tract of fish might have denatured the immunostimulatory compounds present in the supplemented feed resulting in reduced absorption of immunostimulatory compounds into the system ultimately resulting in decreased
immunostimulation and disease protection. Hence it has to be protected to escape digestion in order to be efficiently delivered to immunocompetent cells in the gut or lymphoid organs (Companjen et al., 2006).

Numerous questions remain to be investigated about the dosage, duration of feeding with supplemented diet and persistence of stimulatory effect of the preparation in *O.mossambicus* and other species of fish. Dosage and duration cannot be separated because the duration of feeding affects not only the efficacy but also the possible accumulation and therefore, the persistence in the fish (Shoemaker et al., 2002). Moreover, some of the immune parameters measured in the present study exhibited peak activity at different times with different doses of *T.cordifolia* leaf extract and fractions. Such differential effects on various immune parameters are common in this kind of study concerning the modulation of fish nonspecific host defences (Fletcher, 1986). In addition, innate defences result from a combination of several cellular and humoral factors and that may present differential specificity to a given immunomodulatory principle. Due to these peculiar characteristics, each activity showed its peak that disappeared with time and dose, and that might not coincide exactly in magnitude or time with the peaks of other activities as observed by earlier workers (Ortuno et al., 1999). So considering *T.cordifolia* leaf preparations as potent immunostimulant in finfish aquaculture optimal doses, administration times and the duration necessary to elicit an enhanced immune response have to be established in an attempt to provide a useful approach for protecting cultured fish against infectious diseases.

**MECHANISM OF IMMUNOSTIMULATION BY HERBAL PREPARATIONS**

The immunostimulation observed in the present study might be due to phytoconstituents of *T.cordifolia*, which include protein, alkaloids, diterpenoid lactones, glycosides, steroids, sesquiterpenoid, phenolics, aliphatic compounds and polysaccharides
Phytochemical analysis of *T.cordifolia* leaf extract and fractions in this experiment also confirms the presence of carbohydrates, glycosides, protein, alkaloids, phytosterols, saponins and coumarins. It was suggested that plant constituents might directly activate innate defence mechanisms by acting on receptors and trigger gene activation, resulting in the production of anti-microbial molecules (Bricknell and Dalmo, 2005). Therefore it seems very likely that at least a part of the stimulatory capacities of *T.cordifolia* extract and fractions might be associated with one or more components present in it.

Several studies have revealed the mechanism of action of *T.cordifolia* in mammalian models. Singh *et al.* (2006) have reported that an alcoholic extract of *T. cordifolia* enhanced the differentiation of tumor associated macrophages to dendritic cells, in response to granulocyte/macrophage colony stimulating factor, interleukin-4 and tumor necrosis factor. Leyon and Kuttan (2004) investigated the antiangiogenic activity of *T. cordifolia* in mice and analyzed the serum cytokine profile which showed a drastic increase of proinflammatory cytokines such as IL-1β, IL-6, TNF-α and granulocyte monocyte-colony stimulating factor (GM-CSF). Cytokines play crucial roles in regulating various aspects of immune responses. Among cytokines, interleukin (IL)-12 plays a central role in co-ordinating innate and cell mediated adaptive immunity (Watford *et al.*, 2003).

A α-D glucan (RR1) composed of (1-4) linked back bone and (1-6) linked branches with a molecular mass of >550 kDa and exhibiting unique immune stimulating properties was isolated and characterized from *T.cordifolia*. It activated different subsets of the lymphocytes such as natural killer (NK) cells, T cells and B cells. Immune activation by α-D glucan (RR1) in normal lymphocytes elicited the synthesis of interleukin (IL)-1β, IL-6, IL-12, IL-18, IFN- γ, tumor necrosis factor (TNF)-α and
monocyte chemoattractant protein. The similarity of α-D glucan’s structure to the conserved molecular pattern of the cell wall components of fungal β-glucans may be the reason for the activation of immune system (Nair et al., 2004). Immunostimulation by induction of cytokines and synthesis of NO, activation of macrophages, induction of phagocytic, cytotoxic and antitumor activities has been reported recently in polysaccharide or polysaccharide containing fractions of *Morinda citrifolia*, *Panax ginseng* and *Echinacea* (Hirazumi and Furusawa, 1999; Shin et al., 2002; Goel et al., 2002). Many polysaccharides are known immunostimulants of which β-glucans have recently received considerable attention (Bohn and BeMiller, 1995). β-glucans activate the immune system by binding to specific receptors (pattern recognition receptors) of the innate immune system and stimulate phagocytic, cytotoxic, and antimicrobial activities by the synthesis and release of cytokines, chemokines and reactive oxygen and nitrogen intermediates (Brown and Gordon, 2003). β-glucans also induce proinflammatory responses such as tumor necrosis factor (TNF)-γ and IL-12 synthesis, required for IFN-γ production (Rosenberg, 2001).

The mechanism by which glucan enhance immunity of fish is still not completely elucidated till date. In mammals, the existence of glucan receptors on macrophages and neutrophils has been revealed, and the first step for interaction between glucan and phagocytes involves the binding of glucan to the receptor (Williams, 1997; Williams et al., 1996). Biochemical studies have identified specific receptors for β-glucan on fish macrophages and neutrophils (Engstad and Robertsen, 1993, 1994; Ainsworth, 1994). Administering β-glucan begins a chain reaction of events, which heightens cellular immune response. It stimulates the production of white blood cells, such as macrophages, neutrophils and monocytes for combating the invading pathogens. Cellular mobilization is increased, helping the cells of the immune system to recognize antigens and to move
where they are needed most. The immune system’s capacity to engulf non-self cells is augmented and the production of antimicrobial agents is increased. Triggering of innate immune activation paves the way for adaptive immune response by antigen-specific T and B-lymphocytes. The above said immunostimulating mechanism of β-glucan may be applicable to the immunostimulating mechanism of α-glucan that has been isolated from *T.cordifolia*.

The present results suggest that the extract and fractions of *T.cordifolia* leaf would improve the nonspecific immunity of fish and prevent bacterial infections in culture systems. Further purification of the active compounds and their evaluation may substantially improve quality as well as their usage in the culture system. However, there is a need to understand the mechanisms behind these immunostimulatory effects, to rule out any possible adverse side effects in using these plant products and to confirm the efficacy in field trials before applying them as potent therapeutic agents in finfish culture.