

Immunomodulatory Activity of *Solanum trilobatum* Leaf Extracts in *Oreochromis mossambicus*

M. DIVYAGNANESWARI, D. CHRISTYBAPITA
and R. DINAKARAN MICHAEL

*Centre for Fish Immunology, PG Department of Zoology and Biotechnology
Lady Doak College, Madurai- 625002, India*

ABSTRACT

The application of plant-derived immunostimulants in aquaculture for the prevention of diseases is a promising new development. The objective is to study the effect of crude extract, water and hexane soluble fractions of *Solanum trilobatum* leaves administered intraperitoneally on the specific immune response, non specific immunity and disease resistance in *Oreochromis mossambicus*. The specific immune response was assessed in terms of primary and secondary antibody responses to *Aeromonas hydrophila* and serum lysozyme activity was measured to study the non-specific immune mechanism. The functional immunity in terms of percentage mortality and Relative Percent Survival (RPS) was assessed by challenging the fish with live *A. hydrophila*. All the doses of three fractions except 32 mg kg⁻¹ of crude extract and 800 mg kg⁻¹ of water soluble fraction significantly enhanced the primary and secondary antibody responses on most of the days tested. Almost all the doses of both water and hexane soluble fractions enhanced the serum lysozyme activity. In disease resistance test, the percentage mortality was significantly reduced in the treated groups which are reflected by RPS values ranging from 45 to 70. This preliminary study indicates the potential of using *S. trilobatum* for controlling infectious diseases in aquaculture.

Divyagnaneswari, M., Christybapita, D. and Dinakaran, R.M. 2008. Immunomodulatory activity of *Solanum trilobatum* leaf extracts in *Oreochromis niloticus*, pp. 221-234. In Bondad-Reantaso, M.G., Mohan, C.V., Crumlish, M. and Subasinghe, R.P. (eds.). Diseases in Asian Aquaculture VI. Fish Health Section, Asian Fisheries Society, Manila, Philippines. 505 pp.

Corresponding author: R. Dinakaran Michael, rdmichael2000@yahoo.co.in

INTRODUCTION

Intensive fish farming creates a stressful environment for the animal resulting in suppression of their immune response (Kajita *et al.*, 1990), which leads to disease and disease outbreaks in aquaculture. There are basically three methods for controlling fish diseases: vaccination, chemotherapeutics and immunostimulants. Treatment with synthetic chemicals and antibiotics to prevent the above problem in fish has had limited success and the emergence of antibiotic resistant microbes is an important consideration for their continued use. The higher dependence of fish on the nonspecific immune mechanisms than on the specific immunity and problems associated with the development of efficacious vaccines such as cost, antigenic heterogeneity of microbial strains and viral antigenic drift have forced fish health scientists to look for effective immunostimulants from different sources including plants for prophylactic and therapeutic purposes. The use of immunostimulants enhances specific and non-specific immunity of farmed fish and offers a promising alternative to antibiotics and vaccines (Anderson, 1992).

The immunostimulants which have been tested for application to aquaculture include peptides like FK-565 (Kitao and Yoshita, 1986; Kitao *et al.*, 1987), glucan (Chen and Ainsworth, 1992), extracts from a tunicate (McCumber *et al.*, 1981), or an abalone (Sakai *et al.*, 1991), lactoferin (Sakai, 1999), levamisole (Anderson and Jeney, 1992), chitosan (Siwicki *et al.*, 1994), GH (Sakai *et al.*, 1996), Vitamin C (Qin *et al.*, 2000; Sahoo *et al.*, 1999), extra cellular products of *Mycobacterium* spp. and oligonucleotides (Sakai *et al.*, 2001). These immunostimulants have been reported to increase various aspects of innate immunity such as the number of phagocytes, lysozyme and complement activities as well as serum Ig levels. Some immunostimulants have been shown to protect rainbow trout (Wahli *et al.*, 1998) against furunculosis and to decrease unspecific mortality in rockfish (Kim *et al.*, 1999).

Solanum trilobatum Linn (Family: Solanaceae) is one of the common Indian medicinal plants and it has been used in traditional medicine for many centuries (Mohan *et al.*, 1998). This plant is commonly used to treat asthma, cough, dyspnoea, chronic febrile infections and difficult parturition. Its pivotal action is cardiac, tonic and carminative. The constituents of this plant include, sobatum, β solamarine, solaine, solasodine, glycoalkaloid, diosogenin and tomatidine. This plant possesses a broad spectrum of antibiotic, antibacterial, and anticancer activity (Mohan and Devi, 1996). This plant is being widely used in human medicine in India but there is no report on its application in preventing fish diseases.

The objective was to study the effect of crude extract, water and hexane soluble fractions of *Solanum trilobatum* leaves administered intraperitoneally on the specific, nonspecific immune responses and the disease resistance in *Oreochromis mossambicus*.

MATERIALS AND METHODS

Fish and their maintenance

Oreochromis mossambicus (Mossambique tilapia), a common fresh and brackish water cichlid fish was used in this study. Male fish weighing 25 ± 5 g were used. All experiments

were carried out in Fiber Reinforced Plastic tanks (vol. 150 l). They were kept at an ambient, uncontrolled temperature of $28\pm 2^{\circ}\text{C}$ under natural photoperiod. Water was changed on alternate days. Fish were fed *ad libitum* with a balanced fish diet prepared in the laboratory.

PLANT EXTRACT PREPARATION

The plant *Solanum trilobatum* was procured from the market and the plant species was identified and confirmed by Dr. D. Stephen, P.G. Department of Botany, The American College, Madurai, Tamil Nadu, India. The voucher specimen (Specimen No: CFIS01) was deposited in the Herbarium of Department of Botany, Lady Doak College, Madurai. The leaves were collected and washed in sterile distilled water. They were shade-dried, powdered and stored at -20°C until further use. The extraction was done by following the methods of Lee *et al.* (2000) and Xu *et al.* (2000).

Preparation of crude extract

Ten grams of leaf powder was exhaustively extracted with sterile distilled water and it was filtered through sterile muslin cloth and the filtrate was allowed to stand for 30 min at room temperature. The filtrate was collected and the solvent was removed using rotary vacuum evaporator (Buchi SMP, Switzerland). The residue obtained after evaporation was dissolved and the desired doses were prepared in sterile distilled water.

Preparation of water and hexane soluble fractions (WSF/HSF)

Ten grams of leaf powder were exhaustively extracted with methanol and it was filtered through sterile muslin cloth and the filtrate was allowed to stand for 30 min at room temperature. The filtrate was collected and the solvent was removed using rotary vacuum evaporator (Buchi SMP, Switzerland). The residue obtained after evaporation was mixed with sterile distilled water. The extract was taken in a separating funnel and equal volume of hexane was added and mixed carefully by intermittent releasing of the pressure inside the separating funnel. The content was allowed to stand without any disturbance until two distinct layers (lower water soluble and upper hexane soluble fraction) were seen. The fractions were collected separately. This process was repeated until the colourless hexane fraction was obtained which indicates the completion of hexane fractionation. The water and hexane soluble fractions were concentrated in rotary vacuum evaporator. The desired doses of water and hexane soluble fractions were prepared in distilled water and pure coconut oil (to dissolve the non-polar compounds), respectively and stored at -20°C until used for experimentation.

Bleeding and serum collection

Fish were bled from common cardinal vein using 1 ml tuberculin syringe fitted with 24-gauge needle (Michael *et al.*, 1994). For serum separation, 200 μl of blood was drawn and the whole bleeding procedure was completed within 1 min to minimize the stress to

fish. The blood was collected in serological tubes and the clot was stored in a refrigerator overnight. The clot was then spun down at 400g for 10 min. The serum collected was stored in sterile Eppendorf tubes at -20°C until used for assays.

Bacterial agglutination assay

Preparation of heat killed whole cell vaccine

Overnight culture of *A. hydrophila* was subjected to 60°C for 1 hr in a water bath (Karunasagar et al., 1997). The culture was centrifuged at 800g for 15 min. The packed cells were washed and the required dose was prepared in phosphate buffered saline.

Primary and secondary antibody responses

After acclimation for a period of 2 weeks, fish (n=6) were intraperitoneally injected with 6.4, 32, 160 or 800 mg kg⁻¹ body weight (0.2 ml/fish) of crude extract, water or hexane soluble fractions of *S. trilobatum* whereas the corresponding control fish received 0.2 ml of water or purified coconut oil (control for HSF group). Two days after the leaf extract administration, the fish were vaccinated intraperitoneally 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 59-post primary immunization. The blood was collected at the interval of seven days and the bacterial agglutination assay was performed. The serum was separated and the complement was inactivated at 47°C (Sakai, 1981) in a water bath for 30 min and stored at -20°C until used for assay. Serially double diluted antibacterial antisera were titrated by bacterial agglutination assay (Roberson, 1990). 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.

Lysozyme activity

After acclimation, fish (n=6) were intraperitoneally injected with (0.2ml/fish) 0, 4, 40 or 400 mg kg⁻¹ body weight of crude extract, water or hexane soluble fraction whereas the corresponding control fish received 0.2 ml of distilled water or purified coconut oil respectively. The fish were bled 2 days prior to and 2, 4, 6, 8 and 10 days after treatment. Lysozyme activity was measured by the method of Parry et al. (1965) in combination with the microplate adaptation of Hutchinson and Manning (1996). In this turbidimetric assay, 0.03% lyophilized *Micrococcus lysodeikticus* in 0.05mM sodium phosphate buffer (pH 6.2) was used as substrate. Ten microlitres of fish serum was added to 250 µl of bacterial suspension in a “U” bottom microtitre plate and the reduction in absorbance at 490nm was determined after 0.5 and 4.5min incubation at 22°C using a microplate reader (Biorad, USA). One unit of lysozyme activity was defined as a reduction in absorbance of 0.001 per min.

Disease resistance

Group of 10 fish in triplicates were administered with 0.2ml of 0, 4, 40 or 400 mg kg⁻¹ body weight of crude extract, water or hexane soluble fraction of *S. trilobatum* on day 1.

The control fish received 0.2 ml of water or oil. After the administration of plant extracts fish were challenged (injected) with virulent *A. hydrophila* (1×10^8 cells/fish) on day 7. Earlier, the challenge dose was adjusted to give 80% mortality in the untreated groups. An untreated and a phosphate buffered saline injected control groups were also maintained. Mortality was recorded for 15 days and RPS was calculated by the following formula (Ellis, 1988) below:

$$\text{RPS} = 1 - \left\{ \frac{\text{Percent mortality in treated group}}{\text{Percent mortality in control group}} \right\} \times 100$$

Statistical analysis

Data were expressed as arithmetic mean \pm standard error (SE). Statistical analysis of data involved one-way analysis of variance (ANOVA) followed by Tukey's pairwise comparison test. The levels of significance were expressed as P-value less or greater than 0.05.

RESULTS

Bacterial agglutination assay

The highest dose of 800 mg kg^{-1} of crude extract significantly enhanced the primary and secondary antibody responses on most of the days tested ($P < 0.05$). The enhancement in the secondary antibody response was found to be on day 21 in all other groups (Figure 1). The highest dose of water soluble fraction (800 mg kg^{-1}) was found to be lethal to fish. The other doses have advanced the peak antibody response by a week and significantly enhanced the primary and secondary antibody responses on most of the days tested ($P < 0.05$, Figure 2). All the doses of hexane soluble fraction except 800 mg kg^{-1} , advanced the peak day by 1 week ($P < 0.05$), whereas 800 mg kg^{-1} treated groups showed the peak response on day 14. Further, all the concentrations enhanced the antibody response on most of the days tested ($P < 0.05$). The enhanced secondary antibody response for the group administered with 160 mg kg^{-1} of hexane fraction was found to be on 7, 14 and 21 ($P < 0.05$). For 800 mg kg^{-1} treated groups, it was only on 14 ($P < 0.05$, Figure 3).

Lysozyme activity

The lysozyme activity was enhanced by the administration of 40 mg kg^{-1} of crude extract (Figure 4) or WSF (Figure 5) on day 4, 6 and 8 ($P < 0.05$). The 400 mg kg^{-1} of crude extract or 4 mg kg^{-1} of WSF enhanced the activity on day 2 and 4 ($P < 0.05$). It is also enhanced in the group treated with 4 mg kg^{-1} or 400 mg kg^{-1} of HSF on most of the days tested ($P < 0.05$) (Figure 6).

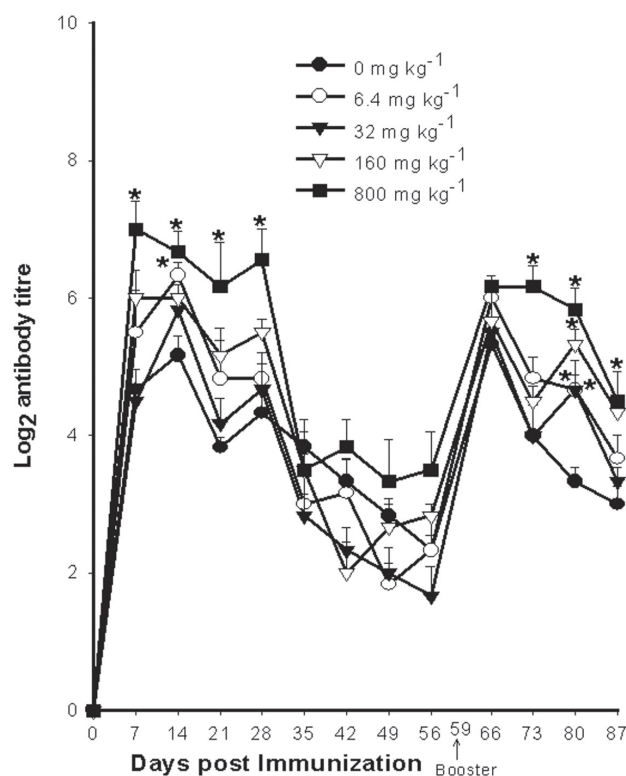
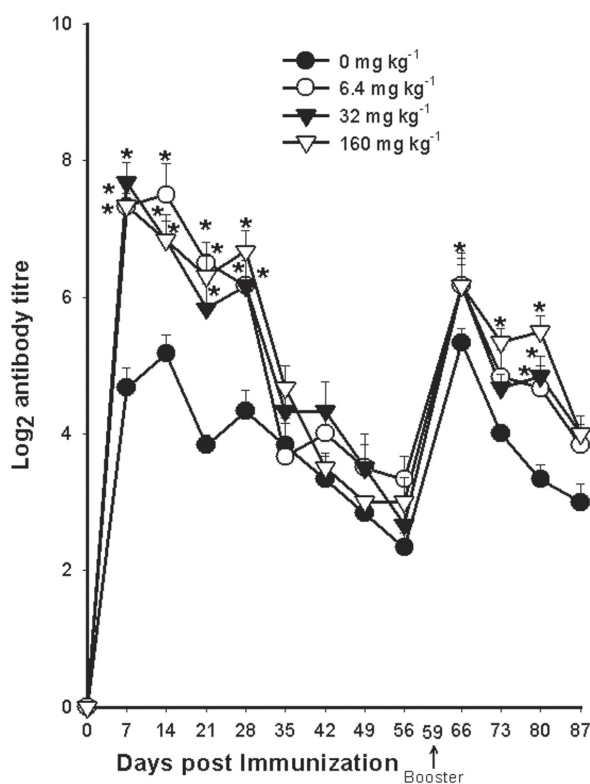


Figure 1. Effect of *S. trilobatum* crude leaf extract on the antibody responses against heat killed *A. hydrophila* in *O. mossambicus*. Each point represents the arithmetic mean value of 6 fish \pm standard error (* $P < 0.05$).

Figure 2. Effect of *S. trilobatum* water soluble fraction (WSF) on the antibody responses against heat-killed *A. hydrophila* in *O. mossambicus*. Each point represents the arithmetic mean value of 6 fish \pm standard error (* $P < 0.05$).



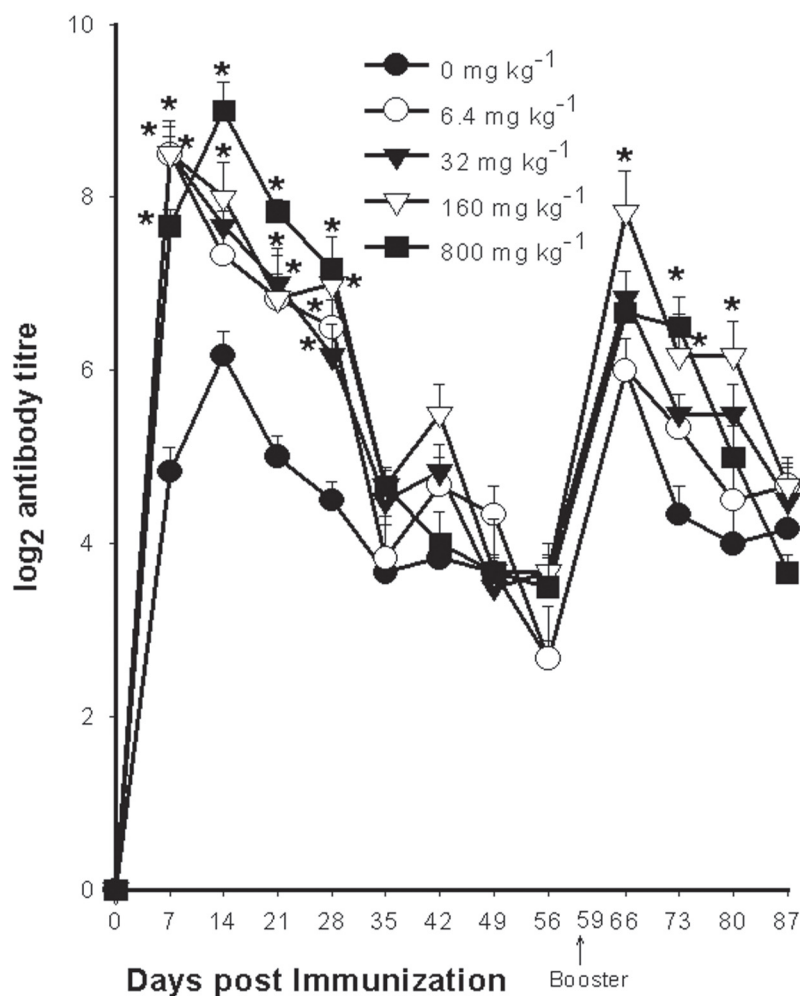


Figure 3. Effect of *S. trilobatum* hexan soluble fraction (HSF) on the antibody responses against heat killed *A. hydrophila* in *O. mossambicus*. Each point represents the arithmetic mean value of 6 fish \pm standard error (* $P < 0.05$).

Disease resistance

The percentage mortality was decreased (Table 1) in fish challenged with live *A. hydrophila* by extract administration. Among the groups administered with plant extracts, the lowest percentage mortality of 23.33% was observed in the group treated with highest dose of 400 mg kg⁻¹ of WSF or 4 mg kg⁻¹ of HSF with the RPS value of 70.84, and 65.01, respectively (Table 1).

Figure 4

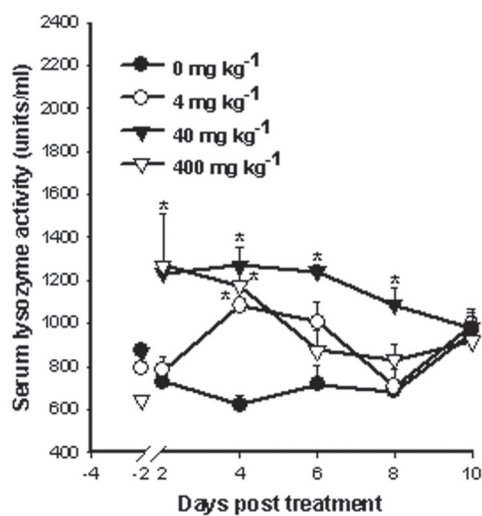


Figure 5

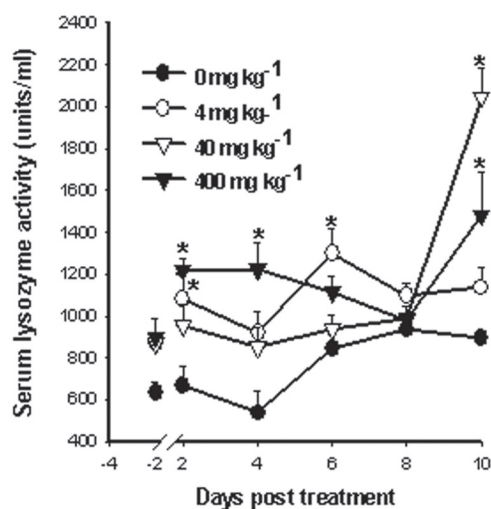
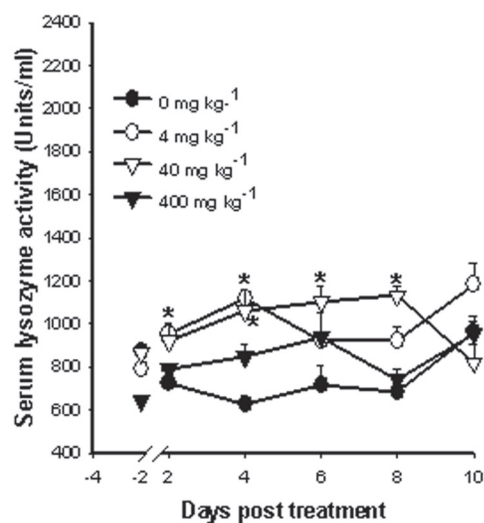


Figure 6

Figures 4-6. Effect of *S. trilobatum* crude leaf extract, water and hexane soluble fractions (WSF/HSF) on the serum lysozyme activity in *O. mossambicus*. Each point represents the arithmetic mean value of 6 fish \pm standard error (* $P < 0.05$).

Table 1. Effect of *S. trilobatum* leaf extracts on the percentage mortality and Relative Percent Survival (RPS) in *O. mossambicus* challenged with live *A. hydrophila*. The percentage mortality values are mean \pm SE, n=10 fish per group in triplicate; a posteriori Tukey comparison of control and treated groups shown with different alphabets representing significant difference ($P<0.05$).

No.	Type of sample	Percentage mortality (%)	Relative percent survival (RPS)
1	Distilled Water (Control)	80.00 \pm 0.00 ^a	-
2	Crude – 4 mg kg ⁻¹	40.00 \pm 5.77 ^{bc}	50.00
3	Crude - 40 mg kg ⁻¹	30.00 \pm 0.00 ^c	62.50
4	Crude - 400 mg kg ⁻¹	46.67 \pm 3.33 ^b	41.66
5	Distilled Water (Control)	80.00 \pm 0.00 ^a	-
6	WSF – 4 mg kg ⁻¹	30.00 \pm 5.77 ^b	62.50
7	WSF - 40 mg kg ⁻¹	33.33 \pm 3.33 ^b	58.34
8	WSF - 400 mg kg ⁻¹	23.33 \pm 3.33 ^b	70.84
9	Oil (Control)	66.67 \pm 6.67 ^a	-
10	HSF – 4 mg kg ⁻¹	23.33 \pm 6.67 ^b	65.01
11	HSF - 40 mg kg ⁻¹	30.00 \pm 5.77 ^b	55.00
12	HSF - 400 mg kg ⁻¹	30.00 \pm 5.77 ^b	55.00

DISCUSSION

In the present study, crude extract, water and hexane soluble fractions of *S. trilobatum* leaves were found to have significant stimulatory effect on the specific, nonspecific immunity tested and the disease resistance in *O. mossambicus*. Most of the doses significantly enhanced the primary and secondary antibody responses to *A. hydrophila*. The magnitude of the antibody response is very high in the groups treated with water or hexane soluble fractions. This finding is in agreement with an earlier finding where the leaf extract of *Ocimum sanctum* produced a significant stimulatory effect on both primary and secondary responses to *A. hydrophila* (Logambal *et al.*, 2000) and SRBC (Venkatalakshmi and Michael, 2001) in the same species of fish. This treatment not only enhanced the magnitude of the response but also shortened the lag period of both primary and secondary responses with corresponding advancement of the peak day of antibody response as seen in the present study. The primary and secondary response to sheep erythrocyte was enhanced by the treatment of Azadiractin, a triterpenoid extracted from neem seed kernel of *Azadirachta indica* (Logambal and Michael, 2001) in *O. mossambicus*. Similarly, feeding of 0.5% root extract of *Achyranthes aspera* for 4 weeks significantly enhanced the haemagglutination titre in *Labeo rohita* (Rao *et al.*, 2004) and anti BSA antibody level in *Catla catla* (Chakrabarti and Rao, 2006).

Lysozyme is an important component in the immune system of fish. It is bactericidal by hydrolyzing β (1 \rightarrow 4) linkages of bacterial cell wall peptidoglycans resulting in bacteriolysis. It is also known to act as opsonin and activate the complement system and phagocytes (Magnadottir, 2006). In the present study, it was observed that the lysozyme activity was substantially enhanced on treatment with crude extract, water or hexane soluble fractions of *S. trilobatum*. Similar results of elevated lysozyme activity was observed on 20, 25 and 30 days after feeding Jian carp (Jian and Wu, 2004) and large yellow croaker, *Pseudosciaena crocea* (Jian and Wu, 2003) with traditional Chinese medicine (TCM) formulated from Astragalus root (*Radix astragalini seu heydsari*) and chinese Angelica root (*Radix angelicae sinensis*) at a ratio 5:1 (w/w). *Oreochromis niloticus* fed with 0.1 and 0.5% *Astragalus radix* for 1 week (Yin et al., 2006) and *Labeo rohita* fed with 0.5% of *Achyranthes aspera* seed extracts for 4 weeks were shown to enhance lysozyme activity (Rao et al., 2006).

For testing efficacy of an immunostimulant, it is very essential to estimate the increased protection in treated fish. (Sakai et al., 2001). The enhancement of nonspecific immune parameters by *S. trilobatum* leaf preparation is possibly an important factor in reducing the percentage mortality and thereby protecting the fish against live *A. hydrophila* challenge. Earlier studies in this laboratory also revealed that dietary supplementation of *Ocimum sanctum* (Logambal et al., 2000) and *Nyctanthes arbortristis* (L. D. Devasree, Centre for Fish Immunology, Lady Doak College, unpublished data) leaves and intraperitoneal injection of water and hexane soluble fraction of *Eclipta alba* (D. Christyapita, Centre for Fish Immunology, Lady Doak College, unpublished data) leaves enhanced the disease resistance against *A. hydrophila* in *O. mossambicus*. The present finding is in agreement with the results of Abutbul et al. (2004) in tilapia fed with a diet containing ethyl acetate extract of *Rosmarinus officinalis* leaf powder and Rao et al. (2006) where the disease resistance against *A. hydrophila* was enhanced in *L. rohita* fed with 0.5% of *A. aspera*. The methanolic herbal extracts (*S. trilobatum*, *Andrographis paniculata* and *Psoralea corylifolia*) helped to increase the survival and growth and reduced the bacterial load even in the shrimp, *Penaeus monodon* post larvae (Citarasu et al., 2003).

Even though all the fractions enhanced the specific and nonspecific immunity, the water and hexane soluble fractions were more protective than the crude extract was. The immunostimulatory effect of water soluble fraction might be due to alkaloids and carbohydrates. The low and high molecular weight compounds of several medicinal plants have been shown to have immunostimulating potential *in vivo* and *in vitro* (Wagner, 1990). The enhancement in nonspecific immune responses and the protection by hexane soluble fraction might be due to the presence of the compound, sobatum. Sobatum, a partially purified component of the plant *S. trilobatum* was obtained from the petroleum ether/ethyl acetate (75:25) extractable portion, which was identified as β -sitosterol by comparison with an authentic sample and proved to be an anticancer agent by *in vitro* and *in vivo* experiments (Govindan et al., 2004). This phytosterol seems to enhance the activity of lymphocytes and the production of cytokines in mammalian models (Bouic et al., 1996).

CONCLUSION

The present study reveals that the administration of crude extract, water or hexane soluble fraction enhanced the specific, nonspecific immune parameters and disease resistance against *A. hydrophila* in tilapia. The water and hexane soluble fractions seem to be a better immunostimulant, which can have a promising role in aquaculture to prevent diseases and disease outbreaks. Also further investigation on the immunostimulatory and disease protective effects of this plant preparations when administered along with feed (which is the preferred route of administration in the culture situation) for disease prevention in aquaculture is warranted.

ACKNOWLEDGMENTS

The authors are grateful to the Fish Health Section of Asian Fisheries Society for providing travel assistance to present this paper in the Sixth Symposium on Diseases in Asian Aquaculture at Colombo, Sri Lanka. The financial support from Department of Biotechnology, Government of India, New Delhi for the study and the technical guidance for the plant extract preparation by Dr. S. Premsingh, Department of Chemistry, The American College, Madurai are acknowledged.

REFERENCES

- Abutbul, S., Golan-Goldhirsh, A., Barazani, O. and Zilberg, D. 2004. Use of *Rosmarinus officinalis* as a treatment against *Streptococcus iniae* in tilapia (*Oreochromis* sp.). *Aquaculture* 238: 97-105.
- Anderson, D. P. 1992. Immunostimulants, adjuvants and vaccine carriers in fish: application to aquaculture. *Ann. Rev. Fish Dis.* 2: 281-307.
- Anderson, D. P and Jeney, G. 1992. Immunostimulants added to injected *Aeromonas salmonicida* bacterin enhance the defense mechanisms and protection in rainbow trout (*Oncorhynchus mykiss*). *Vet. Immunol. Immunopathol.* 34:379-389.
- Bouic, P. J. D., Etsebeth, S., Liebenberg, R.W., Albrecht, C.F., Pegel, K. and Van Jaarsveld, P.P. 1996. Beta-sitosterol and Beta-sitosterol glucoside stimulate human peripheral blood lymphocyte proliferation: Implications for their use as an immunomodulatory vitamin combination. *Int. J. Immunopharmacol.* 18: 693-700.
- Chakrabarti, R and Rao, Y.V. 2006. *Achyranthes aspera* stimulates the immunity and enhances the antigen clearance in *Catla catla*. *Int. J. Immunopharmacol.* 6: 782-790.
- Chen, D and Ainsworth, A.J. 1992. Glucan administration potentiates immune defense mechanisms of channel catfish, *Ictalurus punctatus* Rafinesque. *J. Fish Dis.* 15: 295-304.

- Citarasu, T., Venkatramalingam, K., Babu, M.M., Sekar, R.R.J. and Petermarian, M. 2003. Influence of the antibacterial herbs, *Solanum trilobatum*, *Andrographis paniculata* and *Psoralea corylifolia* on the survival, growth and bacterial load of *Penaeus monodon* post larvae. *Aquaculture International* 11: 583-595.
- Ellis, A. E. 1988. Fish Vaccination. Academic Press, London.
- Govindan, S., Viswanathan, S., Vijayasekaran, V. and Alagappan, R. 2004. Further studies on the clinical efficacy of *Solanum xanthocarpum* and *Solanum trilobatum* in bronchial asthma. *Phytotherapeutic Research* 18:805-809.
- Hutchinson, T. H and Manning, M.J. 1996. Seasonal trends in serum lysozyme activity and total protein concentration in dab (*Limanda limanda* L.) sampled from Lyme Bay, U.K. *Fish and Shellfish Immunol.* 6:473-482.
- Jian, J. and Wu, Z. 2003. Effects of traditional Chinese medicine on nonspecific immunity and disease resistance of large yellow croaker, *Pseudosciaena crocea* (Richardson). *Aquaculture* 218:1-9.
- Jian, J. and Wu, Z. 2004. Influence of traditional Chinese medicine on non specific immunity of Jian Carp (*Cyprinus carpio* var. Jian). *Fish Shellfish Immunol.* 16: 185-191.
- Kajita, Y., Sakai, M., Atsuta, S. and Kobayashi, M. 1990. The immunomodulatory effects of levamisole on rainbow trout, *Oncorhynchus mykiss*. *Fish Pathol.* 25:93-98.
- Karunasagar, I., Ali, A., Otta, S.K. and Karunasagar, I. 1997. Immunisation with bacterial antigens: infection with motile *Aeromonas*. *Dev. Biol. Stand.* 90:135-141.
- Kim, K. H., Hwang, Y.J. and Bai, S.C. 1999. Resistance to *Vibrio alginolyticus* in juvenile rockfish (*Sebastes schlegeli*) fed diets containing different doses of aloe. *Aquaculture* 180:13-21.
- Kitao, T. and Yoshida, Y. 1986. Effect of an immunopotentiator on *Aeromonas salmonicida* infection in rainbow trout (*Salmo gairdneri*). *Vet. Immunol. Immunopathol.* 12:287-296.
- Kitao, T., Yoshida, T., Anderson, D.P., Dixon, O.W. and Blanch, A. 1987. Immunostimulation of antibody producing cells and humoral antibody to fish bacterins by a biological response modifier. *J. Fish Biol.* 31:87-91.
- Lee, T. H., Qiu, F., Waller, G.R. and Chou, C.H. 2000. Three new flavanol Galloylglycosides from leaves of *Acacia confusa*. *J. Nat. Prod.* 63:710-712.
- Logambal, S. M., Venkatalakshmi, S. and Michael, R.D. 2000. Immunostimulatory effect of leaf extract of *Ocimum sanctum* Linn. in *Oreochromis mossambicus* (Peters). *Hydrobiologia* 430:113-120.
- Logambal, S. M. and Michael, R.D. 2001. Azadirachtin - an immunostimulant for *Oreochromis mossambicus* (Peters). *J. Aquac. Trop.* 16:339-347.
- Magnadottir, B. 2006. Innate immunity of fish (Overview). *Fish and Shellfish Immunology.* 20:137-151.

- McCumber, L.J., Trauger, R. and Sigel, M.M. 1981. Modification of the immune system of the American eel, *Anguilla rostrata*, by ETE. *Dev. Biol. Stand.* 49:289-294.
- Michael, R.D., Srinivas, S.D., Sailendri, D. and Muthukkaruppan, V.R. 1994. A rapid method for repetitive bleeding in fish. *Indian. J. Exp. Biol.* 32:838-839.
- Mohan, P.V. and Devi, K.S. 1996. Cytotoxic potential of the preparations from *Solanum trilobatum* and the effect of *Sobatum* on tumor reduction in mice. *Cancer Lett.* 110:71-76.
- Mohan, P.V., Madhusudana Rao, J., Sumathykutty, M.A. and Devi, K.S. 1998. Cytotoxicity of extracts of *Solanum trilobatum* and anticarcinogenic activity of *Sobatum*. *Biomedicine* 18:106-111.
- Parry, R.M., Chandan, R.C. and Shahani, R.M. 1965. A rapid sensitive assay of muramidase. *Proc. Soc. Exp. Biol. Med.* 119:384-386.
- Qin, Q.W., Wu, Z.H., Zhou, Y.C. and Pan, J.P. 2000. Non-specific immunomodulatory effects of dietary vitamin C on Grouper, *Epinephelus awoara*. *Tropic Oceanol.* 19:58-63.
- Rao, Y.V., Romesh, M., Singh, A. and Chakrabarti, R. 2004. Potentiation of antibody production in Indian major carp *Labeo rohita*, rohu, by *Achyranthes aspera* as a herbal feed ingredient. *Aquaculture* 238:67-73.
- Rao, Y.V., Das, B.K., Jyotirmayee, P. and Chakrabarti, R. 2006. Effect of *Achyranthes aspera* on the immunity and survival of *Labeo rohita* infected with *Aeromonas hydrophila*. *Fish Shellfish Immunol.* 20:263-273.
- Roberson, B. S. 1990. Techniques in Fish Immunology. SOS Publications, Fair Haven, USA. 81p.
- Sahoo, P.K., Mohanty, J. and Mukherjee, S.C. 1999. The effect of three immunomodulators on haematological parameters and immunity level in rohu (*Labeo rohita*) fingerlings. *J. Aquac. Trop.* 14:127-135.
- Sakai, D.K. 1981. Heat inactivation of complements and immune hemolysis reactions in rainbow trout, masu salmon, coho salmon, goldfish and tilapia. *Bull. Jap. Soc. Sci. Fish.* 47:565-571.
- Sakai, M., Kamiya, H., Atsuta, S. and Kobayashi, M. 1991. Immunomodulatory effects on rainbow trout, *Oncorhynchus mykiss*, injected with the extract of abalone, *Haliotis discus hannai*. *J. Appl. Ichthyol.* 7:54-59.
- Sakai, M., Kobayashi, M. and Kawauchi, H. 1996. *In vitro* activation of fish phagocytic cells by GH, prolactin and somatolactin. *J. Endocrinol.* 151:113-118.
- Sakai, M. 1999. Current research status of fish immunostimulants. *Aquaculture* 172:63-92.
- Sakai, M., Taniguchi, K., Mamoto, K., Ogawa, H. and Tabata, M. 2001. Immunostimulant effects of nucleotide isolated from yeast RNA on carp, *Cyprinus carpio* L. *J. Fish Dis.* 24:433-438.

- Siwicki, A. K., D. P. Anderson and G. L. Rumsey. (1994). Dietary intake of immunostimulants by rainbow trout affects non-specific immunity and protection against furunculosis. *Vet. Immunol. Immunopathol.* 41: 125-139.
- Venkatalakshmi, S. and Michael, R.D. 2001. Immunostimulation by leaf extract of *Ocimum sanctum* Linn. in *Oreochromis mossambicus* (Peters). *J. Aquac. Trop.* 16: 1-10.
- Wagner, H. 1990. Search for plant derived natural products with immunostimulatory activity (recent advances). *Pure Appl. Chem.* 62:1217-1222.
- Wahli, T., Verlhac, V., Gabaudan, J., Schuep, W. and Meier, W. 1998. Influence of combined vitamins C and E on non-specific immunity and disease resistance of rainbow trout, *Oncorhynchus mykiss* (Walbaum). *J. Fish Dis.* 21:127-137.
- Xu, Y. J., Wu, X.H., Tan, B.K.H., Lai, Y.H., Vittal, J., Imiyabir, Z., Madani, L., Khozirah, K.S. and Goh, S.H. 2000. Flavanol-Cinnamate Cycloadducts and Diamide derivatives from *Aglaia laxiflora*. *J. Nat. Prod.* 63:473-476.
- Yin, G., Jeney, G., Racz, R., Xu, P., Jun, X. and Jeney, Z. 2006. Effect of two Chinese herbs (*Astragalus radix* and *Scutellaria radix*) on non specific immune response of tilapia, *Oreochromis niloticus*. *Aquaculture* 253:39-47.