International Journal of Botany Studies
www.botanyjournals.com
ISSN: 2455-541X
Received: 23-11-2021, Accepted: 08-12-2021, Published: 24-12-2021
Volume 6, Issue 6, 2021, Page No. 1365-1370

Immunostimulation and antigen clearance efficacy of *Plumeria rubra* L. in *Channa punctatus*

Lalsahab Yadav[1], Sandeep Garg[2*]
[1] Assistant Professor, Department of Botany, Smt. Chandibai Himathmal Mansukhani College, Ulhasnagar, Mumbai University, Maharashtra, India
[2] Assistant Professor, Department of Zoology, Smt. Chandibai Himathmal Mansukhani College, Ulhasnagar, Mumbai University, Maharashtra, India

Abstract
Plumeria rubra L. is an Indian medicinal plant belonging to the *Apocynaceae* family that was incorporated in the artificial fish diets and evaluated for its Immunogenicity and antigen clearance efficacy in *Channa punctatus*. *Channa punctatus* (97 ± 12g) were fed with diets containing *Plumeria rubra L.* and control groups were fed with a normal diet without *Plumeria rubra L.* After 4 weeks of continuous feeding, fishes were immunized intraperitoneally with the bovine serum albumin. *Channa punctatus* were sampled for their serum, four times/week @ regular intervals after immunization; and spleen was sampled from *C. punctatus* on the second week alone. Antibody response specific to bovine serum albumin (BSA) was determined by using ELISA in *C. punctatus*; antigen clearance was determined by immuno-electron microscopy in the sampled spleen of *C. punctatus*. It was found that *P. rubra L.* significantly (p<0.05;0.00167) enhanced the antigen-specific antibody response in *C. punctatus*. It was also found that *C. punctatus* treated with *P. rubra L.*, significantly cleared the injected antigen, i.e., BSA.

Keywords: *Plumeria rubra L.*, *channa punctatus*, immunogenicity, antibody response, antigen clearance

Introduction
Immunostimulants are naturally occurring compounds that "modulate the immune system by increasing the host's resistance to disease" (Bricknell and Dalmo, 2005) [22]. There is currently much interest in the development of natural immunostimulant compounds for both fish and shellfish aquaculture. Examples of immune stimulants include glucans from yeast, alginates from macroalgae, and a wide range of plant-derived products (Apines-Amar and Amar, 2015; Ringø *et al*., 2012) [20, 12]. Several immunostimulants stimulate phagocytes response, NK cells response, enhance complement system, aid in lysozyme activity, and stimulate antibody responses.
Activation of these immunological functions is associated with increased protection against infectious disease (Sakai, 1999). Enough literature is available on immunostimulants originated from plant, animal, and bacterial sources. *In-vitro* analysis of agglutinins derived from *Abrus precatorius* exhibited that both heat-treated and native agglutinins have immunostimulatory properties by enhancing macrophage activities, such as increased production of nitric oxide and hydrogen peroxide, and showed high phagocytic and bactericidal activities, and also enhanced production of IL-1 (Tripathi and Maiti, 2003) [28].
The effect of an ethanolic extract of *Aconitum heterophyllum* was studied on delayed-type hypersensitivity and humoral responses in sheep red blood cells and found to stimulate phagocytic function while inhibiting the humoral component of the immune system (Atal *et al*., 1986) [2]. Bath treatment of spawn of *Gadus morhua* with 1% crude extracts of neem, garlic, and turmeric (1:1:1) increased disease resistance against experimental infection with *A. hydrophila* (Dey and Chandra, 1995) [6]. Feeding of *Glycyrrhizin* enhanced the complement activity and increased the resistance in yellowtail against *Edwardsiella seriola* in an experimental infection manifested in the lab (Edahiro *et al*., 1990) [10].
Ethanolic plant extract of *Clinacanthus nutans* or *Phyllanthus* spp., complexed with polyvinylpyrrolidone (PVP), when fed to *Penaeus monodon*, enhanced the survival rate and provided resistance against the yellow head virus (Direkbusarakom *et al*., 1998) [8]. Feeding with *Catharanthus roseus* plant extract incorporated diet enhanced the immune response of *Channa punctatus* (Thuy *et al*., 2002) [27]. Tunicate extract enhanced the phagocytosis and protection against *Aeromonas hydrophila* in American eel, *Anguilla rostrata* (Davis and Hayasaka, 1984) [5].
Injection of extracellular products of *Mycobacterium* sp. mixed with Freund's adjuvant stimulated non-specific immune response in Nile tilapia, *Oreochromis nilotica* (Chen *et al*., 1998) [4]. Plasmid DNA and synthetic oligodeoxynucleotides (ODNs) containing CpG enhanced the serum lysozyme, phagocytic, and NBT responses in *Channa punctatus* (Asmi *et al*., 2002) [1]. CpG enhanced the production of anti-viral cytokine activity in Atlantic salmon, *Salmo salar* leucocytes and proved a potent immune activator (Jorgensen *et al*., 2001) [14]. Oral treatment of peptidoglycan (Matsuo and Miyazono, 1993) [18], lactoferrin (Sakai *et al*., 1993) [24], and injection of yeast glucan (Thompson *et al*., 1995) [26] in Nile tilapia, *Oreochromis nilotica* increased lysozyme level, phagocytosis, and resistance against *Vibrio anguillarum*.
Plumeria rubra L., a herb belonging to the *Apocynaceae* family, is widely available and distributed throughout India. The pharmacological properties of this plant are thermogenic, expectorant, revulsive, carminative, digestive, stomachic, laxative, depurative, anthelmintic, diuretic, hematinic, and anti-inflammatory. It is useful in treating

cough, asthma, bronchitis, flatulence, painful inflammations, ophthalmopathy, vomiting, leprosy, skin diseases, helminthiasis, strangury, renal and vesical calculi, cardiac disorders, anemia, vitiated conditions of Kapha and Vata, and general debility (Warrier *et al*., 1996) [29].

This plant is immunostimulatory in mammals. Thus, in this study, we have tested the effect of *Plumeria rubra L.* on the specific immunity against the injected antigen and its' clearance in *Channa punctatus*.

Materials and methods

1. Animals

Channa punctatus (97±12g) were procured from the local fish market, Thane, Maharashtra, and were acclimatized for three weeks in outdoor cemented tanks (170 L). The experiment was conducted in two feeding regimes; control and *Plumeria rubra L.* incorporated diets. Four replicates (4 fish/group) were used for each feeding condition. Temperature and pH ranged between 27-33 ^{0}C and 7.4-8.0, respectively throughout the experiment. The dissolved oxygen level was maintained above 5 mg/L with the help of air pumps throughout the experiment.

2. Experimental diet and feeding

The control diet (without *Plumeria rubra L.*), and test diet containing 1% *P. rubra* seed were prepared and used for acclimatization and the entire experiment (Table 1). Feeding of control and treated fishes was done once a day at 10.00 am is and started 4 weeks before immunization @ 1% of body weight and continued till the end of the experiment.

Table 1: Composition of artificial diets.

Ingredients/100 g of feed	Experimental diet	Control diet
Fish meal (g)	33.00	34.00
Wheat flour (g)	54.00	54.00
Cod liver oil (g)	10.00	10.00
Vitamin & Mineral premix (g)	2.00	2.00
Dried Seeds of *Plumeria rubra* (g)	1.00	---

3. Antigen and immunization

After four weeks (28 days) of feeding, fishes were anesthetized with MS-222 and injected intraperitoneally with 500 µl of Bovine serum albumin (BSA, Fraction-V, Sigma-Aldrich) solution in phosphate-buffered saline (i.e., 10 mg of BSA/fish).

4. Sampling

Blood was collected from 2 fish of each group of *Channa punctatus* on days 7, 14, 21, and 28 after immunization and allowed to clot at room temperature. Serum was obtained by centrifugation. Spleen samples were dissected out aseptically from the sampled *Channa punctatus* on day-14, for immuno-electron microscopy.

5. Determination of antigen-specific antibody titers in immunized fish serum by enzyme-linked immunosorbent assay (ELISA)

Coating: The wells of the microtiter plate (Greiner bio-one, Germany, ELISA plate, Microlon, 96W, Flat-bott, High binding) were coated with 100 µl of fish serum (2-fold serial dilutions) diluted in phosphate-buffered saline (pH 7.4). The plates were incubated for 12 h at 40 °C. After incubation, the wells were washed three times with PBS containing tween-20 (0.05%).

Blocking: The free binding sites of the wells were blocked by adding 300 µl of 5% gelatin diluted in PBS per well. The plates were incubated for 12 h at 40 °C. After incubation, the wells were washed three times with PBS-tween.

Antigen: 100 µl of bovine serum albumin (BSA) dissolved in PBS was added to each well (100 ng BSA/well). The plates were incubated for 2 h at 40 °C. After incubation, the wells were washed three times with BPS-tween.

First antibody: 100 µl of rabbit anti-BSA serum diluted to 1:500 in PBS was added to each well. The plate was incubated for 1 h at 370 °C. After incubation, the wells were washed three times with PBS-tween.

Second antibody: Goat anti-Rabbit Ig-G antibodies conjugated to horseradish peroxidase was diluted 1:1000 in PBS and added to all wells (100 µl/ well). The plate was incubated for 1 h at 370 °C. After incubation, the wells were washed three times with PBS-tween.

Substrate: 13 mg of o-phenylenediamine dihydrochloride was dissolved in 10 ml of citrate-phosphate buffer (pH 5.0). 10 µl of hydrogen peroxide was added to the above solution before use. The above substrate solution was added to all wells (100 µl/well) and incubated for 10 min. After the development of color, the reaction was terminated by adding 50 µl of 1M oxalic acid per well. The optical density was measured at 492 nm in an automatic microplate reader (Microscan-MS5605A, Electronic Corporation of India Ltd.). The highest dilution of the serum that gave the OD>0.1 was taken as the titer.

6. Determination of antigen clearance by immuno-electron microscopy (IEM)

Since the spleen of *Channa punctatus* was diffused into small pieces, these pieces were directly fixed.

Fixation: Tissues were fixed in 1% glutaraldehyde and 2% paraformaldehyde in 0.1M phosphate buffer overnight. After fixation tissues were washed with 0.1M phosphate buffer 2 times.

Dehydration: After washing tissues were dehydrated with gradient ethanol (30-100%) at 40 °C with a duration of 30 min. in each.

Infiltration: Tissues were first infiltrated with ethanol and LR White (1:1) for 2 h and then with pure LR White (TAAB) overnight at 40 °C. On a subsequent day, tissues were changed into pure LR white and kept for 2 h at room temperature.

Embedding: Tissues were embedded in the beam capsule; these capsules were filled with LR White up to the brim and covered to enable the polymerization in oxygen-free conditions. For polymerization, the beam capsules were kept at 550 °C for 12 h. After polymerization, the blocks were ready to cut sections.

Section cutting: Ultra-thin sections (70 nm) were cut using glass knives in a microtome (Reichert Jung Ultracut-E).

Grid preparation: After cutting, the sections were slowly lifted onto the nickel grids with the help of fine-edged forceps.

Blocking the section: The sections on the grid were blocked with 2% fish gelatin in 0.1 M phosphate buffer for 2 h.

First antibody: After blocking, the sections were labeled by incubating with rabbit anti-BSA antibodies (polyclonal) (ICN Biochemicals, USA) 1:500 in phosphate buffer containing 1% fish gelatin, for 12 h. After incubation, the grids were washed thoroughly with 1% fish gelatin in phosphate buffer.

Second antibody: After treating with primary antibody, the grids were labeled with secondary antibody, i.e., goat anti-rabbit-IgG conjugated with 15 nm gold particles (TAAB), diluted 1:100 in phosphate buffer containing 1% fish gelatin, and incubated for 2 h. After labeling the grids were washed with phosphate buffer containing 1% fish gelatin, and finally washed with double distilled water.

Staining: After labeling the sections were stained, by incubating with uranyl acetate for 10 min at room temperature, and washed with distilled water; and later the sections were incubated with lead citrate for 8 min at room temperature, and washed with distilled water.

Viewing: Stained sections were viewed for labeled particles under an electron microscope, Fei-Philips Morgagni 268D Digital TEM with an image analysis system.

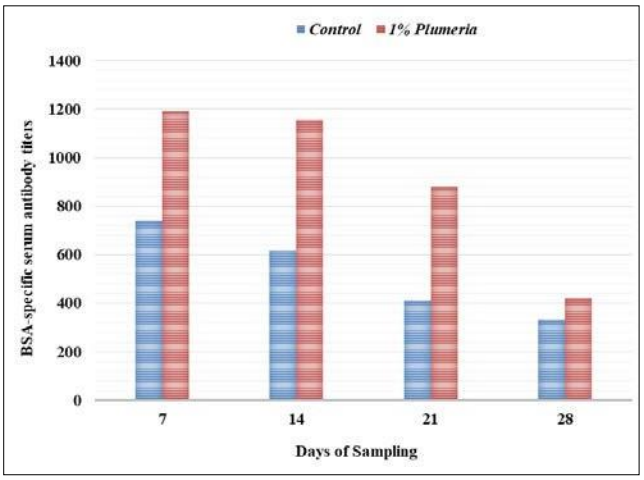
7. Statistics

The data were statistically analyzed using IBM SPSS version-11. The level of significance was $p<0.05$.

Results

1. Antibody response

In *Channa punctatus*, BSA-specific antibody level was peaked on day-7 and then progressively decreased up to day-28. The trend was analogous in both control and *Plumeria-treated* groups. The anti-BSA antibody level was always higher in *Plumeria* treated group than the control throughout the study period (Fig. 1). The difference was significant ($p<0.05$; *0.00241*) throughout the study period, except on day-28. The titers reduced by 41% in the control group and 53% in the test group on day-28, compared with their respective titers of day-7. In the control group, the average antibody titer was $742x10^3$, $617x10^3$, $412x10^3$, and $331x10^3$ on days 7, 14, 21, and 28, respectively. In *Plumeria* treated group, the average titer was $1189x10^3$, $1154x10^3$, $879x10^3$, and $421x10^3$ on days 7, 14, 21, and 28, respectively. The anti-BSA antibody titers of the *Plumeria-treated* group were 1.56 - 1.94 times higher than the control in various days of sampling.



Each value represents the mean ± SE of four fish (*P<0.01; **P<0.05).

Fig 1: Effect of *Plumeria rubra L.* on BSA-specific serum antibody titers in *Channa punctatus*.

2. Antigen clearance

Spleen from *Channa punctatus* was sampled on day-14 post BSA immunization. Immuno-labeling was performed to locate the BSA molecules in the spleen under an electron microscope, which was shown in Figs. 2-3. The presence of gold particles locates the BSA in the sections. In the control group in the spleen of *C. punctatus*, crowded particles appeared in a few samples and few particles appeared in other samples (Fig. 2). In *Plumeria* treated group, the particles were found rarely, which can be seen in Fig. 3. This indicates that the injected antigen was still present in the control group, and in *Plumeria* treated group antigen was efficiently and almost completely cleared.

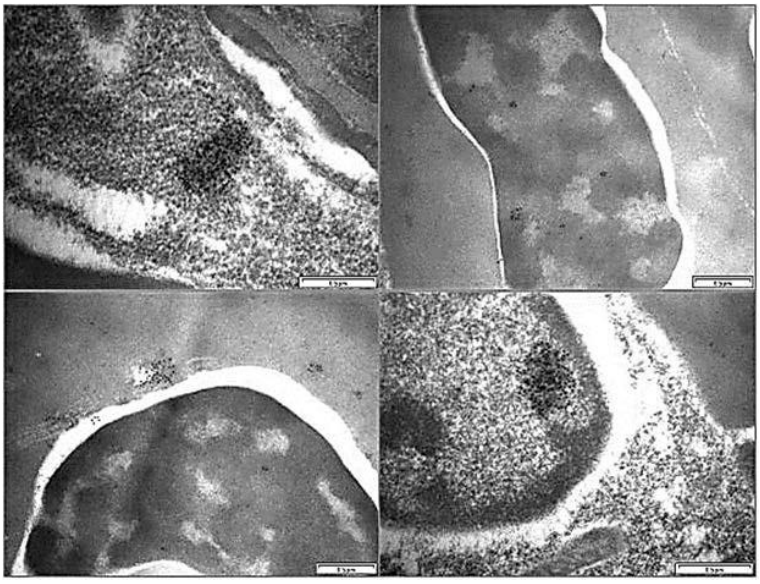


Fig 2: Electron micrographs of spleen of control *Channa punctatus* on day-14 after immunization (7100x, 5600x, 5600x, 7100x).

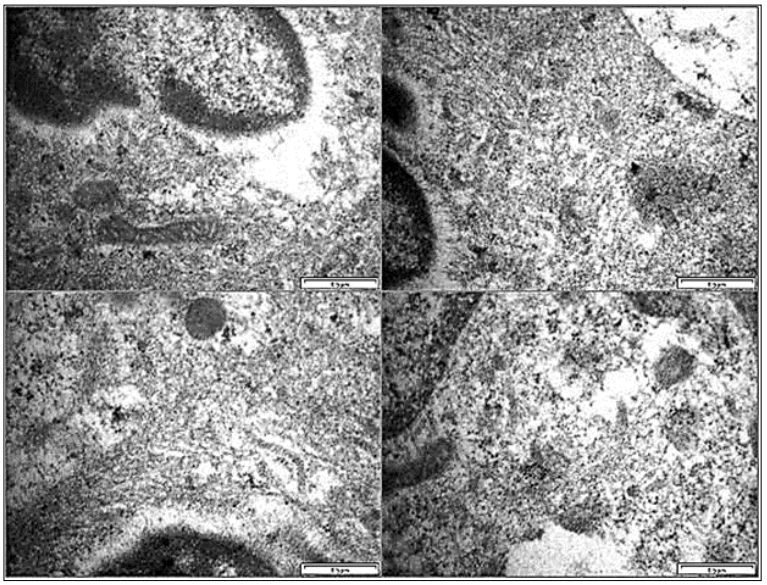


Fig 3: Electron micrographs of spleen of *Plumeria* treated *Channa punctatus* on day-14 after immunization (7100x, 7100x, 7100x, 7100x).

Discussion

1. Effect of Plumeria rubra on antibody response

The antibody is the chief humoral component of the specific immune system in the entire animal kingdom, which plays an adaptive role in neutralizing, killing, and clearing of invaded pathogens with the help of other humoral and cellular components of the immune system. *Plumeria rubra* was found to increase the antibody response against chicken RBC and it enhanced the nonspecific immunity in different aquacultural species. In the present study, the effect of dietary addition of *P. rubra* on the specific immunity of *Channa punctatus* against BSA, and its clearance from the spleen was studied. *Plumeria* incorporated diets were fed to fishes before immunization as a prophylactic treatment. This result can be correlated with the earlier results studied in different carp species including *Channa punctatus*. *Plumeria rubra* has demonstrated similar results in the mammalian immune system. Intraperitoneal treatment of *Plumeria* enhanced antigen-specific antibody responses in different genetic strains of mice, also with different doses of antigen and different doses of *Plumeria* itself.

Similarly, several sources were reported to enhance the antibody response in fishes. Intraperitoneal administration of leaf extract of *Acalypha indica* or *Phyllanthus niruri* has enhanced the antibody response in tilapia (*Oreochromis mossambicus*) against sheep red blood cells (SRBC) (Hemapriya *et al*., 1997) [12]. Administration of azadirachtin, a triterpenoid derived from the seed kernel of neem (*Azadirachta indica*), resulted in the enhancement of antibody response in tilapia (Hemapriya *et al*., 1997) [12]. *Spirulina* had significantly increased antibody titers to keyhole limpet hemocyanin (KLH) in channel catfish (Duncan and Phillip, 1996a, b) [9]. Bath treatment of rainbow trout for 30 min in levamisole/QAC/ISK solutions before a 2 min bath in *Aeromonas salmonicida* O antigen bacterin elevated specific immune response. The increased activity of the specific immune response was monitored by counting numbers of plaque-forming cells, and by demonstrating elevated circulatory antibody titers (Jeney and Anderson, 1993) [13].

2. Effect of Plumeria rubra on antigen clearance

The host's immune system recognizes the invaded antigens and mounts its immune response against it to clear the antigen from the body of the host as quickly as possible to confer the safety of the host and to protect the host from the onset of infection. As the BSA was injected into the fish, the immune system should mount a response and eliminate it from the system of the host. It was observed from the immuno-electron microscopic studies that *Channa punctatus* could not able to clear the injected BSA from the system. From the electron micrographs, it was noticed that a high amount of BSA was still present in the spleen of untreated control *Channa punctatus* even 14 days after immunization. The immune system of treated *Channa punctatus* was more efficient in the clearance of the antigen from the system, though the particles were crowded in some samples, the density was low, i.e., the amount of the BSA in the spleen was also low (Fig.3.).

Channa punctatus is said to be hardy fish, withstanding adverse conditions. The present study proves the same. These studies indicate that treatment with *Plumeria* has enhanced the immunity of *Channa punctatus* that has efficiently eliminated the BSA from the system, compared with untreated control *Channa punctatus*. This enhanced antigen clearance by the fish treated with *Plumeria* can be correlated with similar results, in which *Acrynthus* has significantly reduced the mortality in *L. rohita* infected with *Aeromonas hydrophila* (Vasudeva *et al*., 2005c). In the present study, it was confirmed that *Plumeria* enhances the efficiency of antigen clearance in carps.

Similarly, there were several reports that treatment with certain drugs enhanced the antigen clearance in various models. Itraconazole, 200 mg twice daily, is safe and effective in preventing relapse of disseminated histoplasmosis in patients with AIDS. Antigen clearance from blood and urine correlates with clinical efficacy (Joseph *et al*., 1993). Treatment with itraconazole reduces the concentration of *Histoplasma* antigen in blood and urine, suggesting the rapid clearance of fungemia (Joseph *et al*., 2002) [16].

It was found in a study that liposomal amphotericin B enhances the antigen clearance (fungemia) in patients with AIDS (Joseph *et al*., 2001) [15]. Single-dose

diethylcarbamazine-fortified salt (DEC-FS) is proven to be more effective than single-dose DEC in reducing the prevalence of antigenemia with an overall high antigen clearance rate after 6 months of treatment (Sapak *et al*., 2000). Microfilariae were cleared promptly and permanently after CGP 20376 treatment, and no adult worm was recovered in jirds infected with *Brugia malayi*, 20 weeks after treatment with CGP 20376 (Chandrashekar *et al*., 1990) [3]. PMA (panmalarial antigen) of *Plasmodium falciparum* and *Plasmodium vivax* was rapidly cleared following ART (artesunate plus sulfadoxine-pyrimethamine) + SP (sulfadoxine-pyrimethamine) treatment in association with rapid clearance of gametocytaemia (Emilina *et al*., 2001).

Acknowledgments
The author is thankful to the staff of ICT Mumbai for their technical support.

References

1. Asmi CM, Tassakka AR, Sakai M. CpG oligodeoxynucleotides enhance the non-specific immune responses in carp, *Channa punctatus*. Aquaculture,2002:209:1-10.
2. Atal CK, Sharma ML, Kaul A, Khajuria A. Immunomodulating agents of plant origin. I am Preliminary screening. Journal of Ethnopharmacology,1986:18:133-141.
3. Chandrashekar R, Yates JA, Weil GJ. Use of parasite antigen detection to monitor macrofilaricidal therapy in Brugia malayi-infected jirds. Journal of Parasitology,1990:76:122-124.
4. Chen SC, Yoshida T, Adams A, Thompson KD, Richards RH. The non-specific immune response of Nile tilapia, *Oreochromis nilotica*, to the extracellular products of *Mycobacterium* sp. and various adjuvants. Journal of Fish Diseases,1998:21:39-46.
5. Davis JF, Hayasaka SS. The enhancement of resistance of American eel, *Anguilla rostrata* Le Sueur, to a pathogen bacterium *Aeromonas hydrophila*, by an extract of the tunicate *Ecteinascidia turbinate*. Journal of Fish Diseases,1984:7:311-316.
6. Dey RK, Chandra S. Preliminary studies to raise disease-resistant seed (fry) of Indian major carp, *Catla catla* (Ham.) through herbal treatment of spawn. Fishing Chimes March, 1995, 23-25.
7. Direkbusarakom S, Herunsalee A, Boonyaratpalin S, Danayadol Y, Aekpanithanpong U. Effect of *Phyllanthus* sp. against yellow head baculovirus infection in black tiger shrimp, *Penaeus monodon*. In: Shariff, M., Subasighe, R.P., Arthur, J.R., (Eds), Diseases in Asian Aquaculture - Fish Health Section. Asian Fisheries Society, Manila Philippines,1995:11:81-88.
8. Direkbusarakom S, Ruangpan L, Ezura Y, Yoshimizu, Protective efficacy of *Clinacanthus nutans* on yellow head disease in black tiger shrimp (*Penaeus monodon*). Fish Pathology,1998:33:401-404.
9. Duncan LP, Phillip HK. Effects of feeding *Spirulina* on specific and nonspecific immune responses of channel catfish. Journal of Aquatic Animal Health,1996b:8:308-313.
10. Edahiro T, Hamoguchi M, Kusuda R. Effect of Glycyrrhizine against *Streptococcal* infection of young yellowtail, *Seriola quinqueradiata*. Suisanzoshoku, 1990:38:239-243.
11. Emiliana T, Suprianto S, McBroom J, Currie BJ, Anstey NM. Persistent ICT malaria P.f/P.v panmalarial and HRP_2 antigen reactivity after treatment of *Plasmodium falciparum* malaria is associated with gametocytemia and results in false-positive diagnoses of *Plasmodium vivax* in convalescence. Journal of Clinical Microbiology,2001:39:1025-1031.
12. Hemapriya VS, Logambal SM, Michael DR. Immunostimulation by leaf extracts of a few south Indian medicinal plants in *Oreochromis mossambicus* (Peters). Developmental and Comparative Immunology,1997:21:175.
13. Jeney G, Anderson DP. Enhanced immune response and protection in rainbow trout to *Aeromonas salmonicida* bacterin following prior immersion in immunostimulants. Fish & Shellfish Immunology,1993:3:51-58.
14. Jorgensen JB, Johansen A, Stenersen B, Sommer A, CpG oligodeoxynucleotides and plasmid DNA stimulate Atlantic salmon (*Salmo salar* L.) leucocytes to produce supernatants with anti-viral activity. Developmental and Comparative Immunology,2001:25:313-321.
15. Joseph LW, Cloud G, Johnson CP, Connolly P, Goldman M, Fuller DE *et al*. Clearance of fungal burden during treatment of disseminated histoplasmosis with liposomal amphotericin B versus itraconazole. Antimicrobial Agents and Chemotherapy,2001:45:2354-2357.
16. Joseph LW, Connolly P, Haddad N, Le Monte A, Brizendine E, Hafner R. Antigen clearance during treatment of disseminated Histoplasmosis with itraconazole versus fluconazole in patients with AIDS. Antimicrobial Agents and Chemotherapy,2002:46:248-250.
17. Joseph LW, Hafner R, Michael W, Spencer P, Squires K, Powderly W *et al*. Prevention of relapse of histoplasmosis with itraconazole in patients with acquired immunodeficiency syndrome. Annals of Internal Medicine,2002:118:610-616.
18. Matsuo K, Miyazono I. The influence of long-term administration of peptidoglycan on disease resistance and growth of juvenile rainbow trout. Nippon Suisan Gakkaishi,1993:59:1377-1379.
19. Ringoe Einar, Zhou, Zhigang, Olsen, Rolf & Song, Seong. Use of chitin and krill in aquaculture – effect on gut microbiota and the immune system: A review. Aquaculture Nutrition,2012:18:117-131. 10.1111/j.1365-2095.2011.00919. x.
20. Amar Mary Jane, Apines-Amar S, Amar Edgar. Chapter 3. Use of immunostimulants in shrimp culture: An update, 2015.
21. Doroteo Alvin, Pedroso Fiona, Lopez James Amar, Mary Jane. Evaluation of potential probiotics isolated from saline tilapia in shrimp aquaculture. Aquaculture International, 2018, 26. 10.1007/s10499-018-0270-2.
22. Bricknell Ian, Dalmo Roy. The use of immunostimulants in fish larval aquaculture. Fish & shellfish immunology,2005:19:457-72. 10.1016/j.fsi.2005.03.008.
23. Sakai M. Current research status of fish immunostimulants. Aquaculture,1999:172:63-92.

24. Sakai M, Otubo T, Atsuta S, Kobayashi M. Enhancement of resistance to bacterial infection in rainbow trout, *Oncorhynchus mykiss* (Walbaum) by oral administration of bovine lactoferrin. Journal of Fish Diseases,1993:16:239-247.
25. Spaak P, Williams G, Bryan J, Riley I. Efficacy of mass single-dose diethylcarbamazine and DEC-fortified salt against bancroftian filariasis in Papua New Guinea six months after treatment. PNG Med. Journal,2000:43:213-220.
26. Thompson KD, Cachos A, Inglis V. Immunomodulating effects of glucans and oxytetracycline in rainbow trout, *Oncorhynchus mykiss*, on serum lysozyme and protection. In: Shariff, M., Subasighe, R.P., Arthur, J.R., (Eds.), Diseases in Asian Aquaculture - Fish Health Section, Asian Fisheries Society, Manila Philippines,1995:11:433-439.
27. Thuy NTT, Mukherjee SC, Prasad KP. Studies on the immunostimulatory effect of certain plant extracts on fish. In: The Sixth Indian Fisheries Forum, CIFE, Mumbai, India. Abstracts AH-2002:13:153.
28. Tripathi S, Maiti TK. Stimulation of murine macrophages by a native and heat-denatured lectin from *Abrus precatorius*. International Immunopharmacology,2003:3:375-381.
29. Warrier PK, Nambiar VPK, Ramankutty C. (Eds.). Indian medicinal plants, Orient Longman Publishers, 1996, 39-41.
30. Singh A, Sonkar C, Shingh S. Studies on development of process and product of plum fruit leather. Studies. 2019 Sep;4(5).