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The effect of Tulsi leaf extract on innate immunity by quantifying the serum immunoglobulin level in Tilapia

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Abstract

The impact of Tulsi (*Ocimum sanctum* Linn.) plant methanolic leaf extract on the innate immunity of serum immunoglobulin levels in Tilapia *Oreochromis mossambicus* (Peters) are elaborately discussed here. Fishes were administrated orally 0.01, 0.1 and 1% and intraperitoneally injected with 0.32, 1.6 and 8 mg kg^{-1} body weight, of Tulsi leaf extract respectively. The non-specific humoral assay specifically serum immunoglobulin level was monitored. The serum immunoglobulin assay was performed at the end of every week by following three weeks feed supplement and intraperitoneal administration of Tulsi leaf extract. First week post administration of extract showed significant increase in serum immunoglobulin level on days 7, 21 and 28 at 0.1 and 0.01% ($P<0.01$) of the treated groups. Second weeks treatment groups showed a significant increase in immunoglobulin level on all days tested at 0.1 and 0.01% ($P<0.01$). Similarly, three week treatment groups of 0.1 and 1% ($P < 0.01$) showed a moderate increase in the serum immunoglobulin level on all treated days tested. In the intraperitoneal treatment groups were tested significantly increased the level of immunoglobulin on day 14 and 21 at 1.6 and 0.32 mg kg^{-1} ($P<0.01$). The results strongly revealed that the immunstimulatory properties of *Ocimum sanctum* leaf extract and it can be used as an immunostimulant in local fish farm regions.

Keywords : *Ocimum sanctum*, *Oreochromis mossambicus*, methanolic leaf extract, serum immunoglobulin, Immunostimulant and Innate immunity.

Introduction

Aquaculture is one among the most prevalent resource of protein production sector in the world. India produced the aquaculture product over 4.2 million tonnes and also it holds second position among world production Chakravartty (2015). World per capita fish supply has been increased year by year from an average of 9.9 kg and 18.6 kg in 1960 and 2010 respectively Mehana *et.al.* (2015). According to Fisheries Statistics Division reported that the disposition of Fish Catch in Indian Sub-continent was 121.79 Lakhs Tonnes. The first five states were occupied for top fish consumption, such as Tripura 29.29 Kg, Kerala 19.41 Kg, Manipur 14.Kg, Odisha 13.79 Kg, Assam 11.72 Kg Per Capita/ Per Year, respectively in the year of 2019-2020. India exported fish and fish products were during the year of

2019-2020 and its value Rs. 46,662.85 Crores Handbook on Fisheries Statistics (2020). In this sector, which is severely affected by various fish pathogens and leads to decline fish production and reducing the economic status of the fish farmers as well as their nation. We can be eradicating the fish diseases from infection through increasing the level of immunity and administration of bioimmunostimulants in fishes4. People in India have been utilized over the 500 medicinal plants since ancient times for curing various ailments of humans and animals Vaidyaratnam (2020). Medicinal plants have multipotential bioactive secondary metabolites present, which are against various disease causing pathogens due to compounds like alkaloids, flavanoids, pigments, phenolics, terpenoids, steroids and essential oils Citarasu *et.al.* (1998); Citarasu *et. al.* (1999); Citarasu *et al.* (2001); Citarasu *et.al.* (2002); Sivaram *et.al.* (2004); Anjana *et.al.* (2009); Bairwa *et. al.* (2012). Commonly in fresh water fishes, primarily affected by gram negative bacterial pathogen namely *Aeromonas hydrophila* which can produce diseases like haemorrhagic septicaemia, infectious abdominal dropsy, and fin and tail rot and epizootic ulcerative syndrome Roberts (1992); Austin and Austin (1993) These diseases are found in all kind environmental conditions such as warm, cool and fresh water around the world Pandey *et.al.* (2012). Herbal plant extract acts as a immunostimulant which may leads to enhance the level of various components of innate immunity such as complement, phagocytic activity, serum lysozyme, antiprotease, myeloperoxidase, bactericidal activity, and disease resistance as well as serum immuno-globulin level in *Tilapia* Jeney *et. al.* (1997); Chung *et.al.* (2010); Catherine *et.al.* (2010); Sahoo and Mukherjee (2001); Arulraj *et.al.* (2016). Nowadays, we turn on immunoprophylactic method to control microbial infection in fishes by using plant based immunostimulants, which are effective biomedicines alternative to vaccines and antibiotics Anderson (1992); Secombes (1994); Loghothetis & Austin (1996); Devasree *et.al.* (2012). The usage of immunostimulants in fishes can increase the strength of defense mechanisms against pathogens Gibson (1997); Harikrishnan (2003); Harikrishnan *et.al.* (2010); Sharifuzzaman and Austin (2009). The liver dysfunction, kidney disorders and other immune disorders related illness which are major diseases due to lack of action in the immune system; those diseases should be monitored by Total protein, albumin and globulin tests Nafisi Bahabadi *et.al.* (2014). In this study, the serum immunoglobulin production following natural infection and humoral antibody production in Tilapia are measured, through non-specific immune mechanism by feed supplement and intraperitoneal administration of *Methanolic Tulsi* leaf (*Ocimum sanctum* Linn.) extract.

Materials and Methods

Oreochromis mossambicus were used as an experimental animal for non-specific immune mechanism study. Tilapia weighing around 45 ± 5 g were collected from Devi fish farm Kalligudi village, samayanallur, Madurai and acclimated for two weeks in 70 L fiber reinforced plastic (FRP) tanks Gabriel et al., (2011). Fish diet was prepared in the laboratory Christybapita (2007). Fishes were kept in laboratory condition at a room temperature of 28 ± 2°C. Water was changed 2 times per week. The excess food and faecal matter were removed on alternate days Muralitharan and Dinakaran Michael (1990).

Phytoimmunostimulant Preparation and Administration

A protease producing bacterial strain was isolated from abattoir waste sample collected from Wadala local market, Mumbai, India. The isolate was identified as *Bacillus subtilis* by a 16S rRNA method. Media used for growth and fermentative production of proteaseare mentioned earlier (Badhe *et.al.*, 2016).

After 24 h the broth contents were centrifuged at 5000 rpm at 4ºC for 20 min and the cell-free supernatant obtained was used as the crude enzyme sample.

To analyse the non-specific immune mechanisms, Fishes were fed with diet supplemented with Tulsi leaf extract at concentrations of 0.01%, 0.1%, 1%, whereas the control groups were fed with normal balanced diet. Fishes were intraperitoneally injected with 0.2 ml of methanolic extract of Tulsi leaves at the dosage of 0 (control), 0.32, 1.6 and 8 mg kg-1 body weight using 1 ml tuberculin syringe with 24-gauge needle on day 0. The control fish received 0.2 ml of sterile distilled water. At the end of every week, Three weeks feed supplement and intraperitoneal treatment groups and control group fishes were carried out for serum immuno globulin level assay. The fishes were bled at regular intervals of 7 days for four weeks after 0 day post extract administration of Tulsi leaf extract in Tilapia.

Blood Collection and Serum Separation

A maximum of 200 µl of blood was bled from common cardinal vein situated just below the gills Michael *et.al.* (1994). using 1 ml tuberculin syringe fitted with 24-gauge needle and collected in serology tubes. The blood was allowed to clot overnight at 4°C and was then centrifuged 3000 rpm for 10 min for the serum to be separated. The serum was collected and stored in sterile micro centrifuge tubes at -20°C until used for assays. The separated serum was used to study the non-specific immune mechanisms.

NON-IMMUNE MECHANISM

Serum Immunoglobulin Level

The total protein content in serum was measured following the method of Lowry Lowry *et.al.* (1951). and albumin was measured by the Bromo Cresol Green (BCG) method which utilizes the dye-binding properties of albumin Doumas et.al. (1971). The globulin fraction was calculated by subtracting the albumin value from the total protein value.

For the estimation of total protein, 10µl of serum was diluted with 30µl of saline in 96-well microtitre plates. To this, 200µl of reagent A that contains sodium tartrate, copper sulphate, sodium carbonate and sodium hydroxide were added and incubated for 10 minutes at room temperature. Cu^{++} in alkaline solution forms a tartrate complex that reacts with nitrogen atom of the peptide bonds. This mixture is reduced by 20µl of Folin Phenol reagent when incubated for 30 minutes at room temperature and a blue colour is developed. The optical density was read at 490nm in a plate reader. Total protein was estimated from a standard graph prepared using Bovine Serum Albumin (BSA).

To measure albumin, 10µl of serum was diluted with 40µl of succinate buffer and 150µl of working dye solution [BCG stock solution, succinate buffer and brij] was added and this mixture was incubated for 2 minutes. When the anionic BCG reacts with albumin by means of electrostactic forces, tertiary Vander Wall's forces and hydrogen bonding, the absorbance of the solution increases in direct proportion to the albumin concentration. The optical density was read at 630nm in a plate reader. A standard graph was prepared using bovine serum albumin as a standard.

Statistical Analysis

All the data were expressed as mean ± standard error (SE). Statistical analysis of data involved one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. The levels of significance were expressed as P-value less or greater than 0.05 and 0.01. All statistical calculations were performed using the software, SigmaStat 3.5 (Systat Software, Inc, USA).

RESULTS

Quantification of Serum Immunoglobulin – One week feed supplement

The quantification of serum immunoglobulin in Tilapia ranged from 500 to 2500 µg/ml among the desired different groups including the control for one week feed supplement with methanolic Tulsi leaf extract Fig.-1. The maximum serum immunoglobulin level increased significantly in the dose 0.1% on day 7 treated groups (1692.2 µg/ml P<0.01) and on day 21 in the dose 0.01% treated groups (1904.2 µg/ml P< 0.01).

Quantification of Serum Immunoglobulin – Two weeks feed supplement

During two weeks of dietary intake of Methanolic Tulsi leaf extract in Tilapia for serum immunoglobulin ranged from 500 to 3000 µg/ml Fig.-2. The serum immunoglobulin increased significantly in the doses 0.1%, on day14 (1026.6 µg/ml P<0.01). Similarly, in the dose 0.1% significant increase was noticed (2080.1 µg/ml P < 0.01) on day 28.

Quantification of Serum Immunoglobulin – Three weeks feed supplement

Fish fed for 3 weeks with desired doses of *Methanolic Tulsi* leaf extract for quantifying the serum immunoglobulin level in Tilapia Fig.(3). The serum immunoglobulin level increased significantly on day 7 in the doses 1%, 0.1% and 0.01% treated groups (1536 µg/ml, 1508.8 µg/ml and 717.4 µg/ml P<0.01). and further, in the doses 0.1% and 1% exhibited moderately increase of serum immunoglobulin level on day 21 (796 µg/ml and 541.9 µg/ml P<0.01) and in the doses 0.1% and 1% increased significantly on day 28 respectively (772.9 µg/ml and 720.7 µg/ml P < 0.01). However, significant 1% (P < 0.01) difference was found in the serum globulin level after 3 weeks of treatment.

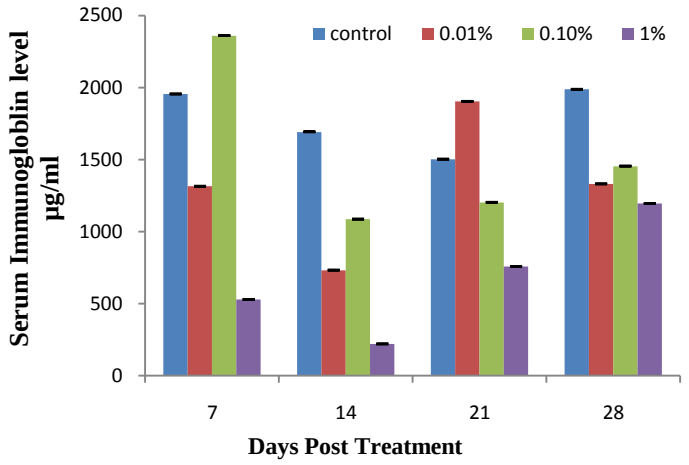

Fig.-1. Serum Immuno globulin level of *O. mossambicus* fed with *O. sanctum* methanolic leaf extract (One week) supplemented diet. Data are represented as mean ± SE (n = 6 wells of serum, in duplicate in the microtitre plate). Statistical differences ($P < 0.05$) among the groups of a feeding schedule are indicated by different letters. No significant differences appear among the groups marked with the same letter.

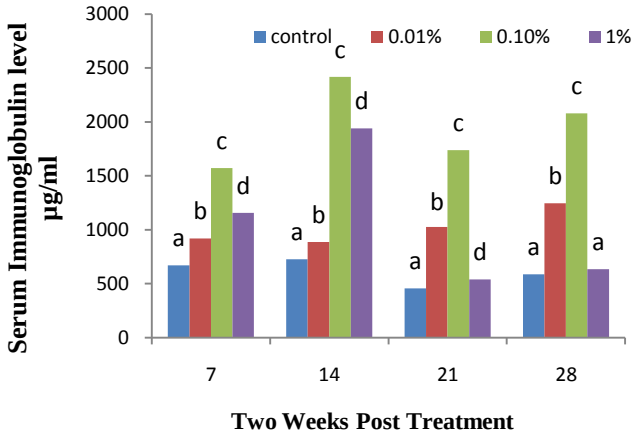

Fig. -2. Serum Immuno globulin level of *O. mossambicus* fed with *O.sanctum* methanolic leaf extract (Two weeks) supplemented diet. Data are represented as mean ± SE (n = 6 wells of serum, in duplicate in the microtitre plate). Statistical differences ($P < 0.05$) among the groups of a feeding schedule are indicated by different letters. No significant differences appear among the groups marked with the same letter.

Quantification of Serum Immunoglobulin – Intra peritoneal

Effect of methanolic Tulsi leaf extract on serum immunoglobulin ranged from 200 to 1600 µg/ml in Tilapia in Fig.-4. The administration of extract at different desired doses such as 0.32, 1.6 and 8 mgKg^{-1} with control, the difference was highly significant (P< 0.01) throughout the period of this experiment. The highest serum immunoglobulin level was observed in the doses 0.32, and 1.6 mgKg^{-1} treated groups on day 21 (P<00.1) and on day 14 in the 1.6 mgKg^{-1} treated group than the control (µg/ml P<0.01).

DISCUSSION

The serum immunoglobulin level was elevated significantly on intraperitoneal or diet supplement of Ocimum sanctum leaf extract. Scientifically there were many such reports on plant derived immunostimulants. In the current investigation, stated that First week post administration of extract showed significantly elevated serum immuoglobulin level on days 7, 21 and 28 at 0.1 and 0.01% (P<0.01) of treated groups. Second weeks treatment groups showed significantly elevated immunoglobulin level on all days tested at 0.1 and 0.01% (P<0.01). Similarly three weeks treatment groups of 0.1 and 1% (P<0.01) were moderately elevated the serum immunoglobulin level on all treated days tested. In the intraperitoneal treatment groups were tested significantly increased the level of immunoglobulin on day 14 and 21 at 1.6 and 0.32 mg kg^{-1} (P<0.01). Oral administration of methanolic extracts of *Ocimum sanctum* leaf and *Withania somnifera* root significantly enhanced serum immunoglobulin (level in grouper,) *Epinephelus tauvina* against *Vibrio harveyi* infections. *Labeo rohita* fed with 0.5% of *Achyranthes aspera* root extract Rao and Chakrabarti (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.*

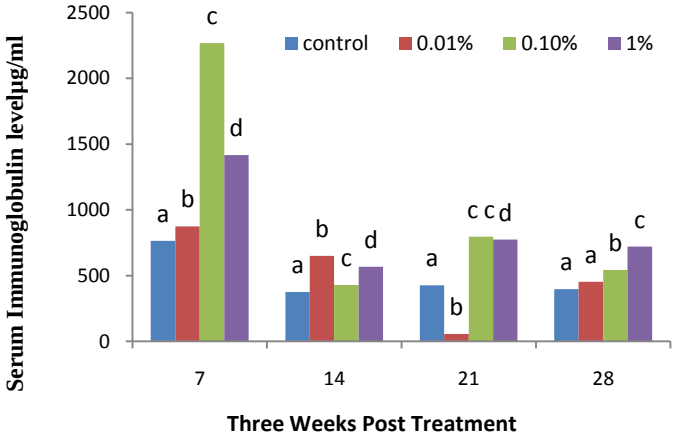

Fig.(3). Serum Immuno globulin level of *O. mossambicus* fed with *O. sanctum* methanolic leaf extract (Three weeks) supplemented diet. Data are represented as mean ± SE (n = 6 wells of serum, in duplicate in the microtitre plate). Statistical differences ($P < 0.05$) among the groups of a feeding schedule are indicated by different letters. No significant differences appear among the groups marked with the same letter.

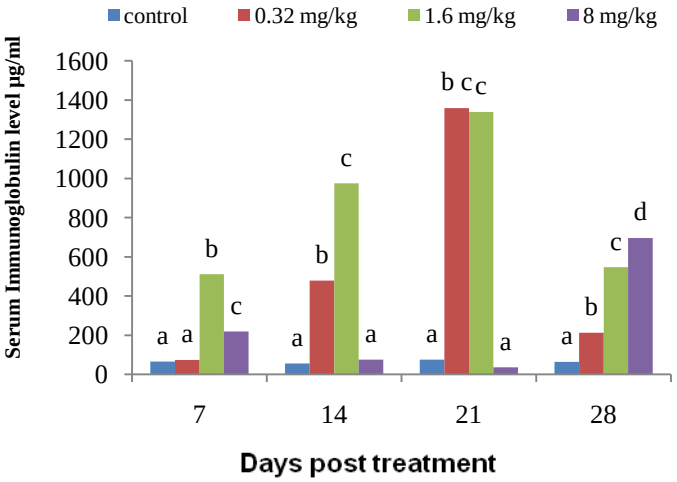

Fig.-4. Effect of methanolic fraction (ME) of *O. sanctum* on the serum Immuno globulin level in *O. mossambicus* (i.p). Each point represents mean ± SE of 6 fish (serum samples assayed in duplicate in the microtitre plate); a posterior Tukey comparision of control and treated groups on weeks shown with different alphabets represents significant difference (P < 0.05). No significant differences appear among the groups marked with the same letter.

(2007) supplemented diet were observed to have enhanced serum globulin level.

Feed supplement of 0.5% of *Achyranthes aspera* seed extract was increased serum globulin level for four weeks Rao and Chakrabarti (2005). It reveals that serum immunoglobulin level which was very important part of innate immunity of fishes Wiegertjes *et.al.* (1996). The highest level of serum immunoglobulin level which strongly associated with good health condition in fishes Rao *et.al.* (2006). Aqueous extract, Water soluble fractions and Hexane soluble fractions of *Tinospora cordifolia* leaves strongly exhibited the increasing the level of serum immunoglobulin on day 4 post extract treatment in the doses of 6, 60, 600 mg Kg^{-1} than the control, likewise that 0.1% feed supplement of *Tinospora cordifolia* leaves exhibited serum globulin level increased in Tilapia Catherine and Dinakaran Michael (2007). Similar study reported from *Tinospora cordifolia*, syringin and cordiol which were showed significant enhanced the level of IgG antibodies and cell-mediated immunity in mice Maurya *et.al.* (1996). The effect of 0.50%, 1% and 2% of lupin, mango and stinging nettle in rainbow trout fed for 14 days and 1% of all desired doses showed highest value for globulin level in humoral immune response Elham (2010).

The effect of *Solanum trilobatum* leaves extracts administered either intraperitoneally and orally in *Oreochromis mossambicus*, there was significantly increased the serum immunoglobulin level, especially hexane soluble fraction doses such as a 4, 40, 400 mg Kg^{-1} administrated by intraperitoneally similarly that feed supplement of *Solanum trilobatum* leaf extract exhibited the highest globulin level at 1% hexane soluble fraction Divyagnaneswari *et.al.* (2007). Both extracts of Echinacea or Ginseng fed groups showed a significant enhancement of total globulin than that fed groups of oxytetracycline Sabry *et.al.* (2014). Plasma immunoglobulin level increased by treated with epin and glucan in Siberian sturgeon (*Acipenser baeri*). The treatment of chitosan and finnstim in Russian sturgeon and led to decrease the level of the immunoglobulin Magnadóttir (2006); Kolman (2001). Similarly the aerial parts of 2.5% and 5% feed supplementation of crude extract of *Ocimum sanctum* elevate the level of serum globulin level in *Clarias batrachus* Nahak and Sahu (2014). The diet administration of 0.1 and 1 % yarrow extract (*Achillea millefolium* L.) significantly enhanced the level of globulin in plasma of rainbow trout (*Oncorhynchus mykiss*), on day 30 Nafisi Bahabadi *et.al.* (2014).

The water soluble compounds phenolic groups, and other ingredients such as eugenol, methyl eugenol, and caryophyllene which were derived from *Ocimum sanctum* (Tulsi) leaf extract and they acted as a potential immunostimulant Chopra et.al. (1956). Likewise that, the antibody level and neutrophils content increased by the effect of *Ocimum sanctum* leaf extract in Tilapia (*Oreochromis mossambicus*) Venkatalakshmi and Michael (2001). According to statement of Arulraj et.al. (2014), Eugenol, Caryophyllene, Germacrene, Hexade-canoic acid methyl ester, 9,12, 15-Octade- catrienoic acid methyl ester (z,z,z), Phenol, 2-methoxy-4-(1-propenyl), Farnesol, a'cubebene which were present from methanolic *Ocimum sanctum* (Tulsi) leaf extract by GC-MS report. Those major compounds act against the human as well as fish bacterial pathogens and also which have antimicrobial and immunostimulatory properties and proved by in scientific report. It was clearly known that *Ocimum sanctum* Methanolic leaf extract developed the health status in *Oreochromis mossambicus* by increasing the level of serum immunoglobulin against fish pathogen.

Since the change in blood serum immunological parameter is very useful strategic technique for

identifying, predict and monitor the health condition of fishes. This parameter is very useful for plant based drug development against various disease causing pathogens. In this current investigation on the immuno-logical parameter of *Oreochromis mossambicus* revealed that this Holy basil Tulsi herbal plant would be important in the curing of various ailments of marine animals.

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