Available online at www.ijpab.com

DOI: http://dx.doi.org/10.18782/2320-7051.5120

ISSN: 2320 – 7051

Int. J. Pure App. Biosci. **5 (5):** 765-775 (2017)

Research Article

International Journal of Pure & Applied Bioscience

OPEN ACCESS

Tinospora Cordifolia Extract Induced Effects on Cellular Immune Reactions of *Labeo rohita* (Hamilton) Challenged Against *Aeromonas hydrophila*

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Received: 3.07.2017 | Revised: 13.08.2017 | Accepted: 21.08.2017

ABSTRACT

The present study was conducted to evaluate the effect of methanolic Tinospora cordifolia stem extract on cellular immune reactions of Labeo rohita fingerlings against Aeromonas hydrophila infection. Fish were fed with pelleted diet without Tinospora cordifolia stem extract (control), 100 mg (TC_1), 200 mg (TC_2) and 300 mg (TC_3) methanolic stem extract of T. cordifolia kg^{-1} diet for 60 days. Immune reactions viz. NBT level, phagocytic activity, total immunoglobulin, lysozyme activity, antiprotease activity and myeloperoxidase activity of fish were examined at 0, 15, 30, 45 and 60 days of feeding. Fish were challenged with A. hydrophila after 60 days post feeding and relative percentage survible (%), immune reactions and agglutination antibody titre were recorded over 14 days post infection. The results demonstrated that fish fed with T. cordifolia stem extract showed enhanced NBT level, phagocytic activity, total immunoglobulin level, lysozyme activity, antiprotease activity and myeloperoxidase activity ($p<0.05$) compared with the control. The survival rate was higher in experimental diets than the control group. Dietary T. cordifolia stem methanolic extract at the level of 200 mg kg^{-1} showed significantly ($p<0.05$) higher protection (RPS 44.36±0.65%) and agglutination antibody titre (81.36±0.65) against A. hydrophila infection than control. The results suggest that the T. cordifolia stem methanolic extract have increases specific and non-specific immunity and disease resistance in L. rohita fingerlings against A. hydrophila infection.

Key words: *Tinospora cordifolia. Aeromonas hydrophila. Non-specific immunity. Labeo rohita. Disease resistance*

Cite this article: Sharma A., Chadha, N.K., Das, S.K., Sen, A., Roy, S.D., Chanu, T.I., Sawant, P.B., Prakash, C., *Tinospora Cordifolia* Extract Induced Effects on Cellular Immune Reactions of *Labeo rohita* (Hamilton) Challenged Against *Aeromonas hydrophila*, *Int. J. Pure App. Biosci.* **5(5):** 765-775 (2017). doi: http://dx.doi.org/10.18782/2320-7051.5120

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INTRODUCTION

To date, disease control strategies for bacterial pathogens have been centred on the use of antibiotics and chemotherapeutics which has been widely criticized for their negative impacts[10]. Furthermore, pathogen like the bacterium *Aeromonas hydrophila* no effective vaccines available due to the complex antigenic structure[4]. Therefore, attention has focused on immunostimulant of plant origin which have been reported to possess various activities like immunomodulatory, growth promoters, antimicrobial and Antistress[6].

The herb *Tinospora cordifolia* is extensively used in the Indian System of Medicine; the extract of different parts of the herb has found wide use in variety of diseases. The alcoholic and aqueous extracts of *T. cordifolia* have been tested successfully for immuno-modulatory activity in rat and mice[28,8]. Both ethanol and petroleum ether extract of *T. cordifolia* (Miers) administered intraperitoneally injected separately in *Oreochromis mossambicus* provided protection against *A. hydrophila* infection[27]. Similarly in *Oreochromis mossambicus* intraperitoneally injected with water-soluble fraction of *T. cordifolia* (Miers) enhanced non-specific immunity and disease resistance against *A. hydrophila* infection[2]. In this backdrop, it was planned to systematically evaluate the effect of dietary administration of the methanolic stem extract of *T. cordifolia* on cellular immune reactions of *Labeo rohita* fingerlings against *A. hydrophila* infection.

MATERIALS AND METHODS

Experimental animal and management

600 nos. of clinically healthy fish (*L. rohita)* average weight of 16.5 ± 0.14 g and average length of 8.27±0.75 cm were collected from the private fish farm of Assam and brought to Fishery Division wet laboratory, ICAR Research Complex for NEH region, Meghalaya. Fishes were acclimatized in the laboratory condition in 500L cemented tanks for 15 days at 26-28^{0}C before the commencement of the experiment. Fish were provided with adequate aeration and fed with control diet at the rate 3% of the body weight twice a day.

Collection of plant and preparation of herbal extract

Tinospora cordifolia (Guduchi) plants were collected from the reliable source and scientifically identified and authenticated by Dr. Rajendra D. Shinde, Associate professor, Department of Botany, St. Xavier's College, Mumbai (India). A specimen of the plant was deposited in the form of herbarium for authentication of the plant (accession no. 01194). The fresh stems were air dried under the shade and then powdered. The dried stems were pulverized by an electrical blender and passed through 20 µm mesh sieve. The sieved powder was then soaked in methanol (1:1 ratio) for 48 hr[7]. The slurry was then filtered, washed to remove non-soluble fractions and filtrate was centrifuged (20,000 X g for 30 min) for clarification. The clarified extracts were condensed at 35^{0}C until the solvent residue had evaporated. The yield was 15% dried extract in terms of dried starting material.

Preparation of herbal diets

The experimental diet was prepared with the locally available ingredients containing 0.01%, 0.02%, 0.03% of *T. cordifolia* stem extract (Table 1). Initially all ingredients except vitamin mineral mixture and extracts were weighed upto desired quantity, blended properly with water to make dough. The dough was steam cooked for 20 min in a pressure cooker at 15 psi. The herbal extract prepared was added at different doses along with Vitamin-mineral pre-mix to the steam ingredients mixture so as to prepare experimental feed and then made into pellets by using a hand pelletizer[33] and then dried at 40^{0}C for 12 hr. The dried pellets were stored in an air sealed container and stored in cool dry place for further use.

Experimental design and feeding diet

The experiment was performed in 500L cemented tanks in the Fishery Division wet laboratory. Fish were divided into four groups (Control, TC_1, TC_2 and TC_3) and each group was maintained in triplicate set containing 50

nos. of fish. The control group diet was devoid of stem extract. The remaining groups TC_1, TC_2 and TC_3 were fed with feed containing 0.01%, 0.02% and 0.03% of *T. cordifolia* stem extract. Fish were provided with adequate aeration and fed at the rate of 3% of body weight twice a day in the 7:00 a.m. and 7:00p.m. The experiment was conducted for 60 days and the sampling for various immunological parameters was carried out on 0^{th} day, 15^{th} day, 30^{th} day, 45^{th} day and 60^{th} day of feeding trials. For each sampling 8 fish were selected randomly from each tank and analyzed for various parameters.

Collection of blood from the fish and separation of serum

Blood from the fish were drawn with the help of a sterilized 2 ml hypodermal syringe and 24 gauge needles directly from the caudal vein containing EDTA as an anticoagulant. Before drawing blood, fishes were anaesthetized with CIFECALM (50µl l^{-1}) [31]. For serum separation the blood was collected without anticoagulant in serological tubes and stored in a refrigerator overnight. The clot was then spun down at 3000 x g for 10 min. The serum collected was stored in sterile serum tubes at -20^0C until used for assays. All the procedures were carried out in the sterilized condition. After drawing blood fishes were given 1% $KMnO_4$ dip treatment and released in to the tank.

Culture of pathogens

Pathogenic strain of *Aeromonas hydrophila* was procured from (*ATCC* 35654), Himedia, India. *A. hydrophila* was grown on nutrient broth (HiMedia Ltd., India) for 24 hr at 37^0C.The culture broth was centrifuged at 3000 x g for 10 min. The supernatant was discarded and the pellet was resuspended in sterile phosphate buffer saline (PBS, p^H 7.2) and the OD of the solution was adjusted to 0.5 at 456 nm, which corresponded to $1x10^7$ cells ml^{-1}.

Determination of immune reaction

Nitroblue tetrazolium (NBT) assay**,** phagocytic activity assay, total immunoglobulin assay and lysozyme activity assay were performed followed by Sharma *et al*.[25]. Serum antiprotease activity a modification of the method described by Ellis [9]. Briefly, 10 µl of serum were incubated with the same volume of standard trypsin solution for 10 min at 22^0C. To this, 100 µl of 0·1 M phosphate buffer, pH 7·0 and 125 µl 2% azocasein were added and incubated for 1 hour at 22^0C. Then 250 µl of 10% trichloro acetic acid (TCA) was added and incubated for 30 min at 22^0C. The mixture was centrifuged at 6000*g* for 5 min. 100 µl of the supernatant was transferred to a 96 well non-absorbent microtray (Nunc) containing 100 µl/well of 1 N NaOH. The O.D. was read at 430 nm. The blank was phosphate buffer in place of serum and trypsin and the reference sample was phosphate buffer in place of serum. The percentage inhibition of trypsin activity compared to the reference sample was expressed for each serum sample as described by Zuo and Woo[34]. Trypsin inhibition (%) = (Trypsin blank OD- sample OD/Trypsin blank OD) x100. Myeloperoxidase content was measured according to Quade and Roth[17] with slight modification. About 10 µl of serum was diluted with 90 µl of Hank's Balanced Salt Solution (HBSS) without Ca^{2+} or Mg^{2+} in 96-well plates. Then 25 µl of 10 mM 3,3´, 5,5´-tetramethylbenzidine hydrochloride (TMB) (Himedia, India) and 25 µl 5 mM H_2O_2 (both substrates of MPO and prepared on same day) were added. The colour change reaction was stopped after 2 min by adding 50 µl of 4 M sulphuric acid (H_2SO_4). The OD was read at 450 nm in a microplate reader (Biorad, USA). Agglutination antibody titre assay were determined according to Plumb and Areechon[16] with slight modification. Briefly, Sera samples collected as earlier at the end of 14^{th} day of challenge were serially diluted in two fold steps with PBS using 25µl of samples and PBS in Microtitre plates. An equal volume (25µl) of formalin killed *A. hydrophila* (10^7cells/ml) was added to each well depending on the source of fish sera challenge with *A. hydrophila*. Then the microtitre plate was incubated overnight at room temperature. The antibody titre was determined at the greatest serum dilution where no agglutination occurred as indicated by presence of a button in the well and values were expressed as reciprocal of that dilution.

Challenge study and relative percentage survivals (RPS)

After feeding trial of different herbal extracts through feed over 60 days the fish in various experimental groups, 10 fish from each experimental tank were injected intraperitoneally with 100µl of bacterial suspension and the mortality was observed for 7 days. Sampling of the survivors was carried out on the 14th day of *A. hydrophila* infection. The confirmation of the infection was accomplished after re-isolating and the bacteria from, kidney, liver and muscle of the dead fish. The reconfirmation was done after performing all the biochemical and the confirmatory tests. Relative percentage survivals (RPS) = (Number of surviving fishes after challenge/Number of fishes injected with bacteria) x100[14].

Statistical analysis

All the data were expressed as arithmetic mean ± SE. Statistical analysis of data involved one way analysis of variance (ANOVA) followed by the comparison of means following Duncan's Multiple Range Test available with SPSS windows 16.0 software. The levels of significance were expressed as P-value at 0.05 level.

RESULTS

Immune reactions

NBT level in all the all the experimental groups were significant ($p< 0.05$) on 15th, 30th, 45th and 60th days of sampling compare to control. The highest NBT level were observed in TC_2 group on 15th, 30th and 60th days of sampling and TC_3 group on 45th days of sampling. Moreover, after the challenge also the NBT level was significantly ($p< 0.05$) higher in all the experimental groups compare to control (Fig. 1). Phagocytic activity in all the experimental groups were significant ($p< 0.05$) on 15th, 30th, 45th and 60th days of sampling compare to control. The highest phagocytic activity was observed in TC_2 group on all the days of sampling followed by TC_3 and TC_1 group. Moreover, after the challenge also the phagocytic activity was significantly ($p< 0.05$) higher in all the experimental groups compare to control (Fig. 2). The level of lysozyme activity in all the experimental groups TC_1, TC_2 and TC_3 was significantly ($p< 0.05$) higher on 30th, 45th, and 60th days of sampling in comparison with the control. However, the highest lysozyme activity was observed after the challenge in TC_2 group (Fig. 3). Total immunoglobulin level in all the experimental groups of fishes fed with diet containing *T. cordifolia* stem extract showed increasing trend from 15th to 60th days of sampling. The highest immunoglobulin level was observed after the challenge in TC_2 group (Fig. 4). Serum antiprotease activity in all the experimental groups TC_1, TC_2 and TC_3 was significantly ($p< 0.05$) higher on 15th, 30th, 45th, and 60th days of sampling in comparison with the control. However, the highest serum antiprotease activity was observed after the challenge in TC_2 group (Fig. 5). Myeloperoxidase activity in all the experimental groups TC_1, TC_2 and TC_3 was significantly ($p< 0.05$) higher on 15th, 30th, 45th, and 60th days of sampling in comparison with the control. The highest myeloperoxidase activity was observed in TC_2 group. However, after the challenge the serum myeloperoxidase activity was observed to be significantly ($p< 0.05$) higher in all the experimental groups compare to control (Fig. 6).

Relative percentage survival (RPS) and agglutination antibody titre. After injection with *A. hydrophila*, the first mortality was recorded after 12 h. The highest survival was recorded in the TC_2 (44.36 ±0.65%) group and lowest percentage survival was recorded in the control (4.42 ± 0.02%) group (Fig. 7). The highest antibody titre was recorded in the TC_2 (81.36 ± 0.65) group and lowest antibody titre was recorded in the control (16.42 ± 0.02) group (Table 2).

DISCUSSION

Immune reaction

The present study on *Labeo rohita* revealed increase in NBT level in all the experimental groups supports the findings of Vikhe *et al*[32]., in *Candida albicans* administered with alcoholic stem extract of *T. cordifolia*, in *L.*

rohita fed with varying *percentages* of garlic Sahu *et al*[21]. Respiratory burst activity is one of the most important bactericidal mechanisms in fish (Secombes and Fletcher [24]). Moreover, in the present study after the challenge also the NBT level was significantly ($p< 0.05$) higher indicates the production of reactive oxygen species (ROS) such as superoxide anion and hydrogen peroxide after challenge which played a role in the clearance of pathogens[30]. In the present study phagocytic activity in all the experimental groups of fishes fed with diet containing *T. cordifolia* stem extract showed increasing trend are in line with the report of Vikhe *et al*[32]., in *C. albicans* administered with alcoholic stem extract of *T. cordifolia*, in *L. rohita* fed with mango kernel[22]. The increase in phagocytic activity in the present study signifies the role of *T. cordifolia* in enhancing the nonspecific immune response after the challenge also the phagocytic activity was significantly ($p< 0.05$) higher indicates the continuous protection from invading pathogen through the intra cellular killing mechanism. The lysozyme activity in the present study showed a significantly increasing trend which can be compared with the findings in *Oreochromis mossambicus* treated with water-soluble fraction of *T. cordifolia*[2], in *L. rohita* fed with the diet containing herb *Achyranthes aspera*[19]. Increase of lysozyme activity in the present study revealed the higher non-specific defence system of the fish as lysozyme activity is an important index of innate immunity of fish[23]. The total immunoglobulin level in all the experimental groups of fishes fed with diet containing *T. cordifolia* stem extract showed increasing trend are in line with the report in rats orally administration of *T. cordifolia* alcoholic extract[1], in *L. rohita* with *W. somnifera* root powder [25]. Serum antiprotease activity in all the experimental groups of fishes fed with diet containing *T. cordifolia* stem extract showed increasing trend indicates its restricting ability of bacteria to invade and to grow *in vivo*[18], are in agreement with the findings in *Oreochromis mossambicus* treated with water-soluble fraction of *T. cordifolia*[2] and aqueous extract of *E. alba*[5].

The phagocytic enzyme myeloperoxidase (MPO), hemoprotein present in neutrophils and monocytes, has an essential role in immune surveillance and host defence mechanisms by the formation of reactive species involved in microbial killing kill (bacteria and other pathogens) by generating hypochlorous acid (HOCl) from H_2O_2[11]. In the present study myeloperoxidase activity in all the experimental groups of fishes fed with diet containing *T. cordifolia* stem extract showed increasing trend this finding is supported by Alexander *et al*[2]., in *O. mossambicus* treated with water-soluble fraction of *T. cordifolia* and *E. alba* leaf extract[5]. However, after the challenge the serum myeloperoxidase activity was observed to be significantly ($p< 0.05$) higher indicates hypochlorous acid production and providing protection by host defence reaction.

Challenge study and agglutination antibody titre

In the present study, even after the challenge the immunoresponse was observed to be significantly higher in all the experimental groups which can be substantiated by the fact that, in normal condition i.e. before challenge *T. cordifolia* has enhanced the basal levels of immune response and in immune-suppressed conditions i.e. post challenge, significantly recovered the immune profile, as because in healthy conditions, immune-stimulants are expected to serve as prophylactic or promotive agent and in individuals with suppressed immune system as therapeutic agent[20].

The agglutination antibody titre was highest (81.36 ± 0.65) in the TC_2 group followed by TC_1 (38.26 ± 0.45) group and TC_3 (30.32 ± 0.14) group. The lowest antibody titre was recorded in the control (16.42 ± 0.02) group. Present findings are in agreement with broiler birds feeding with *T. cordifolia*[13], in *L. rohita* administered with *A. aspera*[29]. From our results, specific immunity measured showed higher number of antibodies produced by the experimental diet feeding groups than the control. It proved that the *T. cordifolia* stem methanol extract could develop or induce the

specific antibody in fish against the antigen. The challenge test with *A. hydrophila* showed highest percentage survival rate in the treatment group TC_2 followed by TC_1 (17.26%) and 14.32% survival in the group TC_3 when compared with the control. The decreased in mortality rate with dietary *T. cordifolia* after injection of bacteria, *A hydrophila* is in agreement in *O. mossambicus* administered with water-soluble fraction of *T. cordifolia*[3], in *L. rohita* fed with the diet containing herb *Achyranthes aspera*[29]. Challenge experiments have shown that *T. cordifolia* increased protection in *L. rohita* challenge with *A. hydrophila* may be by the boosting of nonspecific immunity as evident by the production of reactive oxygen species (ROS), hypochlorous acid and enhanced engulfment, intracellular killing mechanisms, lysozyme antibiotic activity, serum antiprotease bactericidal activity coupled with specific immunity evident by increased in total immunoglobulin level, agglutination antibody titre.

The activation of non-specific immune response observed in the present study might be substantiated by the fact that *T. cordifolia* containing promising bioactive phytoconstituents particularly, glycosides principles of *T. cordifolia*, cordioside (TC-2) and cordiofolioside A (TC-5) through macrophage activation[12], magnoflorine (alkaloides), tinocordiside (glycosides) through enhancement in phagocytic activity and increase in nitric oxide and reactive oxygen species generation[26] and through novel 1,4-alpha-D-glucan activation of macrophages[15]. The stimulation of specific defence may be by the active glycosides principles of *T. cordifolia* Syringin (TC-4) compounds through the rise in antibodies in serum[12].

Table 1: Composition of control and experimental diets (in 100g of feed)

Ingredients	Control	TC_1	TC_2	TC_3
Ground nut oil cake (g)	40	40	40	40
Fish meal (g)	25	25	25	25
Rice bran (g)	20	19.99	19.98	19.97
Soyabean meal (g)	12	12	12	12
Vitamin-mineral mix[a] (g)	2	2	2	2
Starch (g)	1	1	1	1
Herbal extract (mg)	0	0.01	0.02	0.03

[a]Vitamin–mineral mix (EmixTM plus) (quantity/2.5 kg): Vitamin A-55,00,000 IU; Vitamin D3-11,00,000 IU; Vitamin B2-2,000 mg; Vitamin E-750 mg; Vitamin K-1,000 mg; Vitamin B6-1,000 mg; Vitamin B12-6 mg; Calcium panthothenate-2,500 mg; Niacinamide-10 gm; Choline chloride-150 gm; Mn-27,000 mg; Iodine-1,000 mg; Fe-7,500 mg; Cu-2,000; Zn-5,000 mg; Co-450 mg; Ca-500 g; P-300 g; Se-50 ppm; L-Lysine-10 g; DL-methionine-10 g.

Table 2: Agglutination antibody titre of *Labeo rohita* after challenged with *A. hydrophila*

Group	Agglutination antibody titre
Control	16.42 ± 0.02^{a}
TC_1	38.26 ± 0.45^{b}
TC_2	81.36 ± 0.65^{c}
TC_3	$30.32 \pm 0.14^{b,d}$

All the data are represented as mean ± SE. Mean values with different superscript with in a column for a parameter is significantly different ($p < 0.05$).

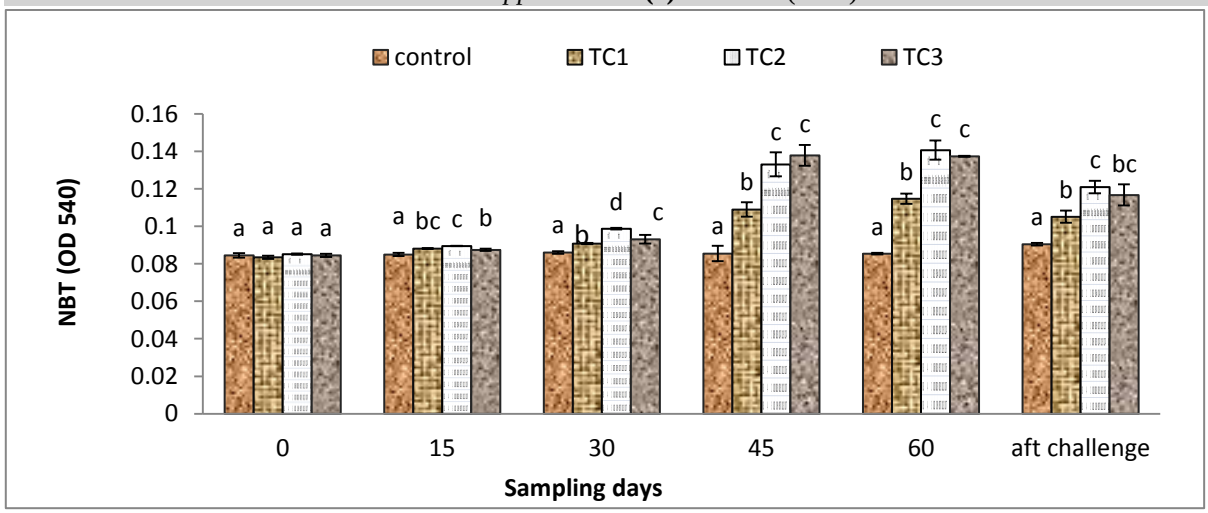

Fig. 1: NBT level on various sampling days of different experimental groups fed with *T. cordifolia* stem methanolic extract (mean ± S.E.). Mean values with different superscript with in a column for a parameter are significantly different, (p < 0.05)

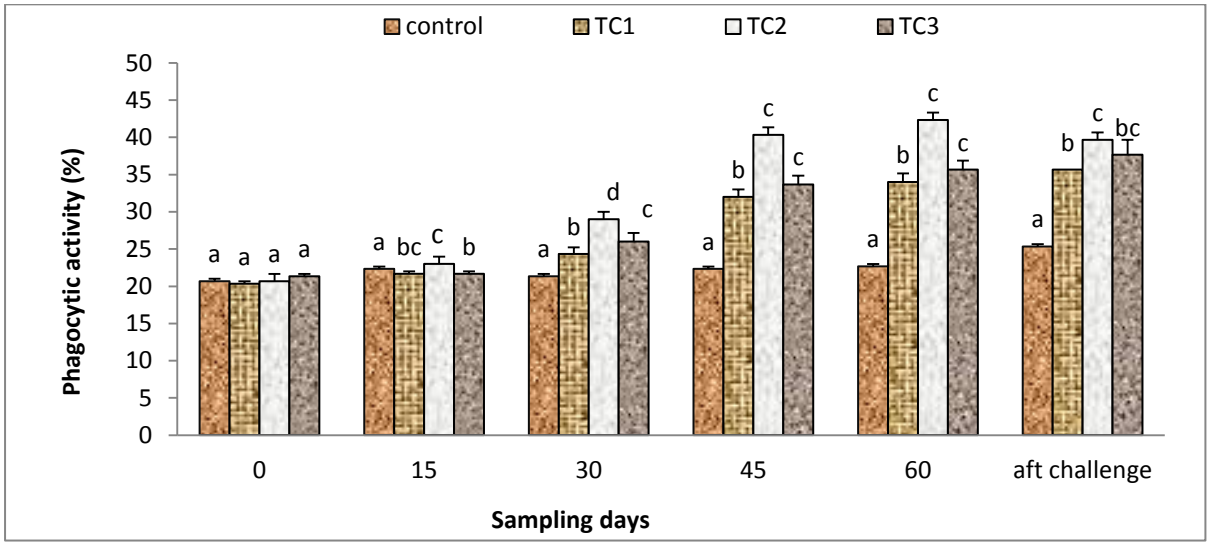

Fig. 2: Phagocytic activity (%) on various sampling days of different experimental groups fed with *T. cordifolia* stems methanolic extract (mean ± S.E.). Mean values with different superscript with in a column for a parameter are significantly different, (p < 0.05)

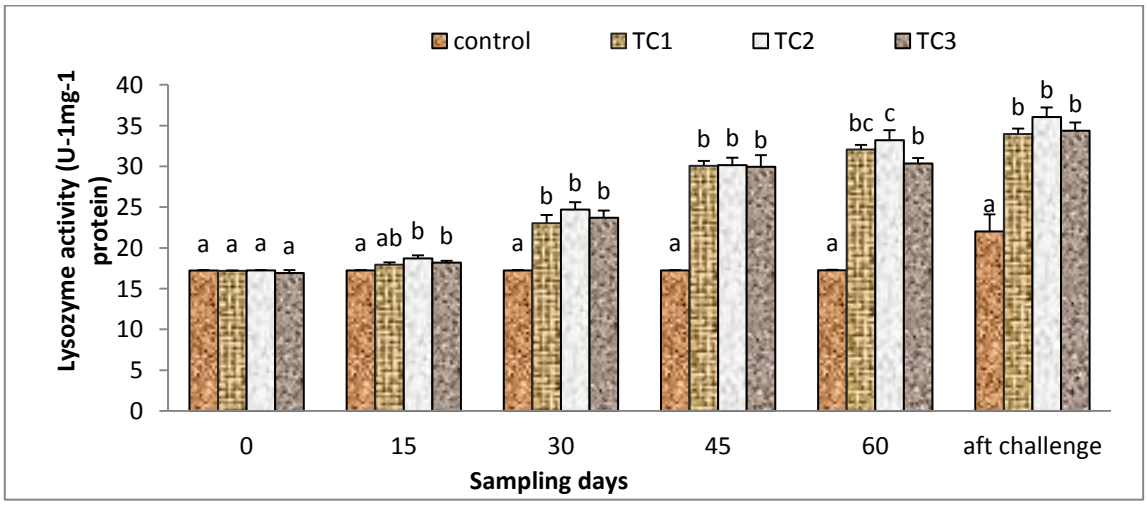

Fig. 3: Lysozyme activity ($U^{-1}mg^{-1}$protein) on various sampling days of different experimental groups fed with *T. cordifolia* stems methanolic extract (mean ± S.E.). Mean values with different superscript with in a column for a parameter are significantly different, (p < 0.05)

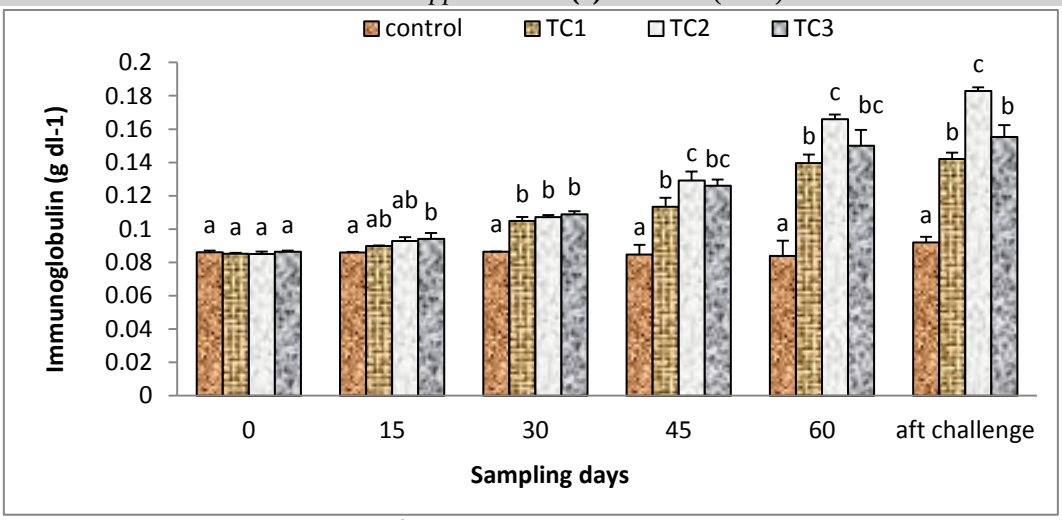

Fig. 4: Total Immunoglobulin level (g-dl^{-1}) on various sampling days of different experimental groups fed with *T. cordifolia* stems methanolic extract (mean ± S.E.).Mean values with different superscript with in a column for a parameter are significantly different, (p < 0.05)

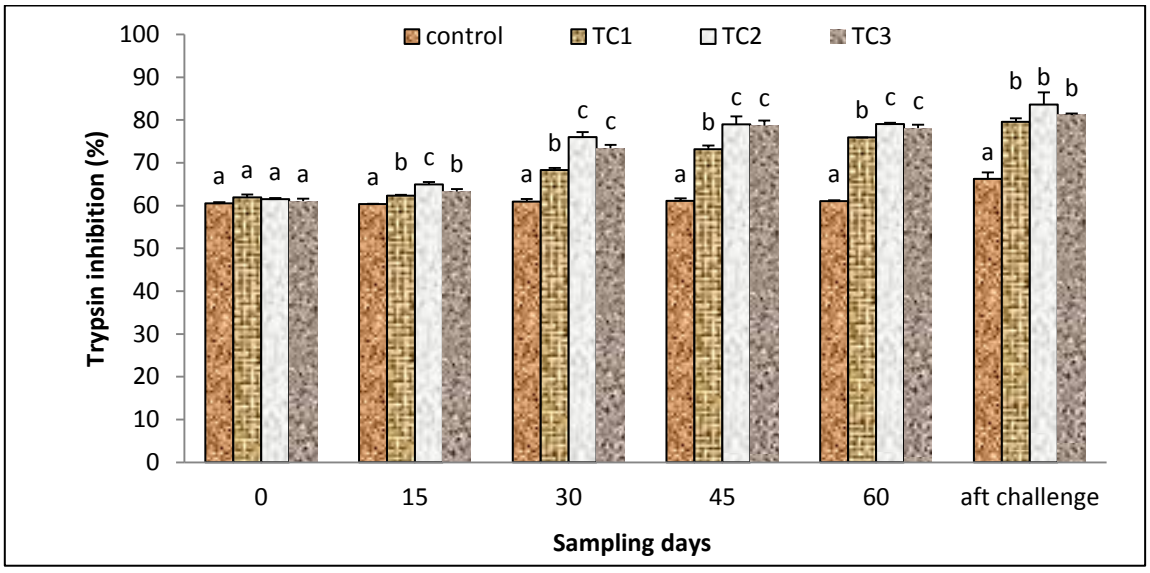

Fig. 5: Antiprotease activity (%) on various sampling days of different experimental groups fed with *T. cordifolia* stems methanolic extract (mean ± S.E.) Mean values with different superscript with in a column for a parameter are significantly different, (p < 0.05)

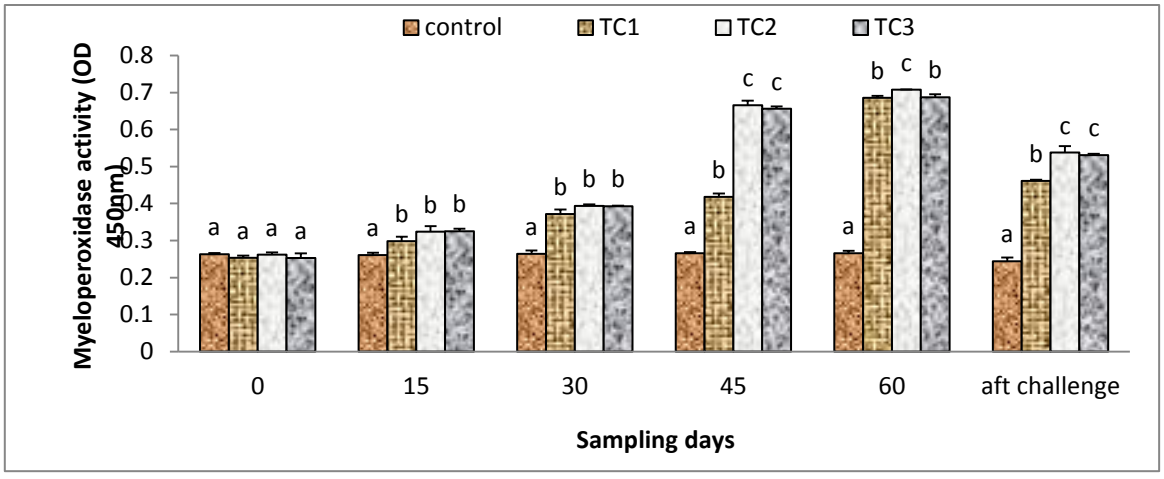

Fig. 6: Myeloperoxidase activity on various sampling days of different experimental groups fed with *T. cordifolia* stems methanolic extract (mean ± S.E.) Mean values with different superscript with in a column for a parameter are significantly different, (p < 0.05)

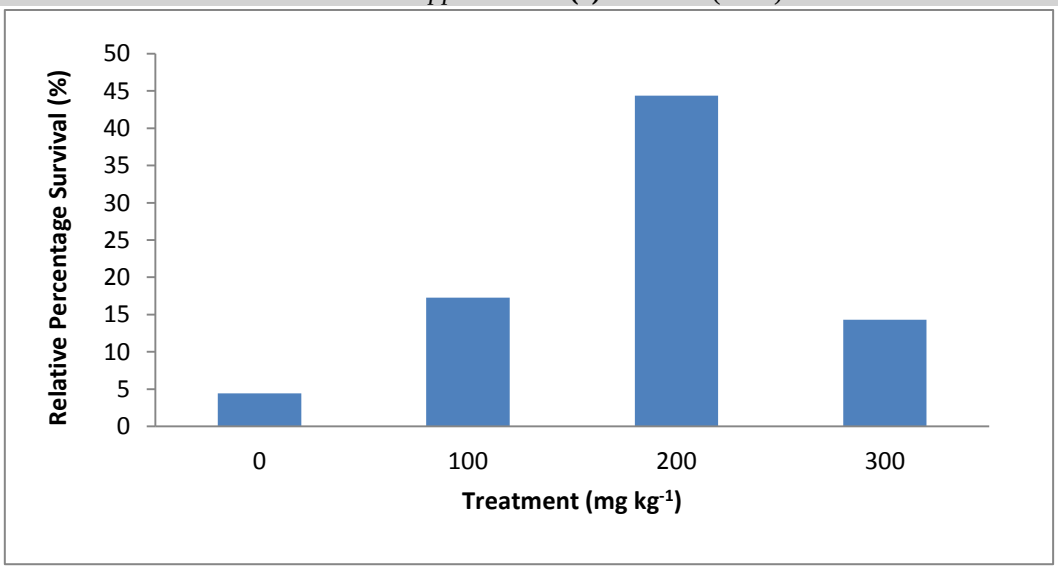

Fig. 7: Relative percentage survival (RPS) of different experimental groups fed with *T. cordifolia* stem methanolic extract (values are mean ± S.E.) in *Labeo rohita* challenge with *A. hydrophila*

CONCLUSION

Thus, from the present study it can be deduced that feed containing 200 mg *T. cordifolia* stem extract/kg diet is a potent immune booster and might be the most appropriate dose which activated the receptors and the corresponding genes responsible for the secretion of immune defence factors also might be found useful in enhancing the immunity in immune compromised individuals.

However, present study opens up new avenues for future study on most effective dose under pond conditions, as well as the effect of *T. cordifolia* through different modes of administration should be further investigated in order to ascertain its molecular mechanism, administrative regime for different age group of fish and time of application to ensure enhanced growth and production in culture ponds.

Acknowledgements

The authors would like to express their gratitude to Dr. S.V. Ngachan, Director, ICAR (RC) for NEH region, Meghalaya, India for providing all the infrastructure facilities needed for the conduct of the work. Authors also thankful to Dr. W. S. Lakra, Ex-Director, Central Institute of Fisheries Education, Mumbai, India for allowing to carrying out the present work in collaboration with ICAR (RC) for NEH region, Meghalaya, India.

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