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Stress mitigation of fucoidan-based nutraceuticals in *Labeo rohita* fingerlings challenged with *Aeromonas hydrophila*

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Abstract
Fucoidan was extracted productively from *Sargassum wightii* and its antioxidant potential was investigated. The isolated fucoidan content of 5.6% of dried seaweed powder was recorded. The *in vitro* antioxidant activity by 1, 1-diphenyl-2-picrylhydrazyl (DPPH) scavenging, total phenolic contents and reducing power assays of fucoidan rich extract (FRE) were evaluated. The extract showed enhanced antioxidant properties in a concentration dependent manner. Later, feeding trial of 60 days was conducted to observe any synergistic effect of methionine supplementation along with FRE on mitigation of stress due to *Aeromonas hydrophila* challenge, in which 420 *Labeo rohita* fingerlings were randomly distributed into seven groups in triplicates fed with different experimental diets by following a completely randomised design. There was no significant ($P>0.05$) effect on stress related enzymes like superoxide dismutase, catalase, aspartate transaminase and alanine transaminase before the bacterial challenge, indicating stress free condition. But, the activity of enzymes in the group containing 2% FRE along with low dose methionine was significantly reduced among all the treatments during the post *A. hydrophila* challenge period. These results proved that the stress resulted from the bacterial challenge was mitigated synergistically by 2% FRE along with low dose methionine in the diets of *L. rohita* fingerlings.

Keywords: Antioxidant; *Sargassum wightii*; Fucoidan; methionine; *Aeromonas hydrophila*

1. Introduction
The freshwater fish species, especially the carps dominate the global aquaculture production. In the Indian aquaculture sector, Indian major carps (IMCs) viz. *Labeo rohita (*Rohu), *Catla catla*, and *Cirrhinus mrigala*, contribute the major aquaculture production [1]. Among the three species, *L. rohita* comprises the major part of the culture due to its high consumer preference and market demand, which is one of the reasons for its intensive farming. But the high stocking culture of this species leads to stress and enhances its susceptibility to many infections and immune suppression [2]. During stress production of free radicals increases, which are unstable molecules resulting in extensive damage and oxidative stress. Oxidative stress takes place when there are the structural and functional modifications of biomembranes as a result of chemical alterations of biomolecules or there may be a low antioxidant defence [3]. Free radicals result in the generation of reactive oxygen species (ROS) which are a group of highly reactive molecules due to presence of unpaired electron. These include hydroxyl, hydrogen peroxide radicals and superoxide anions generated due to oxidative damage to lipids, proteins and DNA molecules [4].

Hence, several therapeutic agents have been proposed as an ideal prophylactic method to mitigate the stress level and protect the cultured fish from oxidative damages. The use of chemotherapeutic drugs and antibiotics has been limited in fish culture due to certain issues like development of antibiotic resistant bacteria, environmental pollution and the accumulation of antibiotic residues in animals including fish [5]. Recent studies in fish have reported that immunostimulants can reverse the deleterious effects mediated by stress and can promote various defensive functions [6]. To mitigate stress and safe guard the fish from the diseases, feeding of nutraceuticals appears to be an ideal strategy to improve their non-specific defence mechanism.

Several nutraceuticals have been used as modulators of the immune system in aquafeeds. In this context, Fucoidan, a polysaccharide containing substantial percentages of L-fucose and sulphate ester groups is one such bioactive molecule which has anti-oxidative functions [7] and

has the potential to mitigate the stress in fish. Brown seaweeds and some marine invertebrates are the important sources of such sulphated polysaccharides. Fucoidan also display a variety of pharmaceutically relevant biological activities, including antitumor [8], anticoagulant and antithrombotic [9], anti-inflammatory [10] and antiviral [11] activities. Fucoidan has been repeatedly proved to be immunostimulant in the diets of fishes [12, 13].

Several additives in the aquafeeds have been reported to modulate the immune system. Amino acids are the additives which act as the precursors of endogenous synthesis of specific protein meant for other metabolic functions [14]. Particularly methionine is usually the first limiting and important essential amino acid required by fishes for their normal growth and several other functions in the body [15]. Many studies revealed that methionine exhibits the immunostimulating activity and can mitigate the stress level [16].

The aim of the present study was first to check the *in vitro* antioxidant activity of fucoidan rich extract (FRE) and then its stress mitigating effect in *L. rohita* fed with formulated diet containing the FRE along with methionine. Dietary intervention of such nutraceutical may be an ideal approach to mitigate oxidative stress and promote immunity of fish. Therefore, it was hypothesised that the supplementation of methionine will enhance the stress mitigation response of FRE synergistically, as it has been reported that the over-sulphation of fucoidan enhances its antioxidant activity [17].

2. Material and methods

2.1 Collection of seaweeds

Brown seaweed, *Sargassum wightii*, was collected from Mandapam, Tamil Nadu, India. Fresh and healthy brown seaweed samples were collected from the sea and thoroughly washed with freshwater and then with distilled water and dried in shade for about three days. Next, the dried seaweeds were pulverized, sieved and the powder was packed in an airtight container.

2.2 Extraction of fucoidan

The extraction of fucoidan was carried out as described by [18] with slight modifications. The fine seaweed powder (100g) was dissolved in 1000 ml of 85% ethanol and kept stirring overnight at room temperature. The next day, the alcohol was decanted and the residual seaweed was washed with acetone followed by centrifugation at 5000 r/min for 15 min. Then, the supernatant was decanted and the residual seaweed obtained was dried at room temperature for overnight to remove the residual acetone. After this, seaweed was mixed with one litre of distilled water and stirred for 7 hrs at 70 °C. The hot mixture obtained was subjected to filtration through a muslin cloth and the residual seaweed was again mixed with 500 ml of water followed by stirring for 7 hrs at 70 °C. Once again the water was filtered out with the help of muslin cloth. Both the extracts were pooled together and centrifuged at 5000 r/min g for 15 min. The collected supernatant was mixed with calcium chloride pellets to make the concentration to 1% of the extract and the solution was kept overnight at 4◦C for precipitation of alginic acid. The dark orange brown aliquot was crude fucoidan rich extract.

2.3 Purification and quantification of fucoidan

The crude fucoidan was purified by using dialysis membrane (Himedia, Mumbai, India). The membrane was activated before use by boiling it in 1mM EDTA solution for 10 min. Then, the crude fucoidan was put inside the dialysis tube whose both ends were closed by a thread. The tubing was then kept in a two-litre beaker with distilled water and the water was stirred using magnetic stirrer at room temperature. The water was replaced regularly as per the saturated level of impurities exuding from the tubing. This was done for 24 hrs until there was no visible impurity. The product which was purified fucoidan was used for fucoidan yield quantification.

The dialyzed fucoidan rich extract sample was quantified and the L-fucose content was analyzed according to the method of [19]. The absorbance was measured at 480 nm with a characteristic orange yellow solution. Then, from the L-fucose content, the fucoidan yield was calculated empirically that 1 µg of fucoidan is equivalent to 1.75 × fucose (µg) [20, 21].

2.4 *In vitro* antioxidant activity

2.4.1 DPPH (Diphenyl Picrylhydrozyl) scavenging assay

DPPH scavenging activity of fucoidan rich extract expressed as % inhibition of DPPH radical was measured by slightly modified method of [22]. The DPPH radical has a deep purple colour which is reduced by antioxidant compound to the corresponding pale-yellow hydrazine. The fucoidan rich extract sample with different concentrations was taken to which 2 ml of 0.06M methanolic DPPH was added. Then it was mixed well and kept in dark for 30 min. Optical density was measured at 517 nm against the reagent blank. The control containing no fucoidan extract was also run along with the samples.

2.4.2 Estmation of total phenolic contents

Total phenolic contents in the FRE were determined according to the method described by [23]. Graded levels of FRE concentrations were prepared. Thirty microlitre of FRE was taken in a test tube and the volume was made up to 3 ml with distilled water, after which 0.5 ml of Folin-Ciocalteau reagent (1N) was added followed by 2 ml of 20% sodium carbonate after 3 min. The tubes were then placed in boiling water for 1 min, cooled and later, the absorbance was taken at 650 nm against the reagent blank. Gallic acid was used as the standard and the standard curve of absorbance against different concentrations was prepared. The total phenolic content was expressed in mg phenols/100g dry weight of sample.

2.4.3 Ferric reducing antioxidant power (FRAP) assay

The FRAP assay was carried out according to the method of [24] with some modifications. The FRAP reagent was freshly prepared and warmed to 37 °C in water bath prior to use. Twenty microlitre of sample was added to 1.8ml of the FRAP reagent and the absorbance of the reaction mixture was then recorded at 593 nm after 4min. The standard curve was made using iron (II) sulphate solution (100-2000 µM) and the results were expressed as µmol Fe (II)/g dry weight of plant extract.

2.5 Experimental diets

After the *in vitro* antioxidant activity of FRE, diets were formulated and prepared with the purified ingredients as indicated in Table 1. L-methionine supplementation was done to study any synergistic effect on antioxidant activity (stress related enzymes) of fucoidan extract and methionine in *L. rohita*. The dietary methionine requirement for Indian major carps was reported to be 1.2% of the diet at constant 1%

dietary cysteine level [25]. L-methionine was supplemented above the requirement of fish in two different levels at constant 1% cysteine level, while taking the sulphur containing amino acid profile of casein and gelatin (0.9% methionine and 0.14% cysteine) into consideration. Methionine content of gelatin and casein together was 0.9%, so in order to fulfil the basic requirement (1.2%) of the particular amino acid, a 0.3% extra L-methionine was added in all the treatments along with control. Therefore, low dose and high dose methionine were added at 1.5 times (1.8%) and 2 times (2.4%) of the fish's methionine requirement. Seven diets with same composition except the levels of methionine, FRSE and cellulose to keep the crude protein and lipid level at 35% and 8%, respectively, were prepared in the Fish Nutrition Laboratory of ICAR-Central Institute of Fisheries Education. All the selected ingredients were properly mixed with a requisite amount of water in order to make the dough except the vitamin-mineral mix, oil and BHT. Then, the dough was steam cooked for 15 min in an autoclave. After cooling, vitamin-mineral mix, oil and BHT were mixed thoroughly to get the even distribution of vitamin-mineral premix in the dough. Then, pellets were prepared with the extruder of 2 mm diameter followed by drying in the moisture-free environment at 60 °C in a hot air oven and finally packed.

2.6 Fish and Experimental Design

L. rohita fingerlings used for the present study were procured from Hans Aquaculture, Raigarh, Mumbai, India. Fish were acclimatised for 15 days in the Wet Laboratory of CIFE. Fish were fed two times daily with control diet to satiation under a natural photoperiod and continuous aeration.

The experiment was conducted for a time period of 60 days in 21 plastic tanks (150 L capacity) covered with plastic lids. Prior to experimentation fish were fasted for 24 h and weighed. Twenty fish per tank of initial weight 8 to 8.5 g were randomly distributed in the seven distinct treatment groups in triplicates following CRD (Table 2). The fishes were fed to satiation twice daily at (08:00 and 18:00 h) for 60 days.

2.7 Analytical methods

The water quality was analysed for parameters like temperature, pH, dissolved oxygen, free carbon dioxide, total hardness, ammonia, nitrite and nitrate levels following 'APHA' procedures [26].

2.8 Tissue homogenate preparation

The muscle, liver and gill of the fish were removed carefully and were weighed. Then, tissues were homogenized with chilled sucrose solution (0.25M) in a glass tube using tissue homogenizer. The homogenate was centrifuged at 5000 rpm for 10 min at 4 °C. The supernatant was stored at -20 °C until use. A 5% homogenate was prepared for muscle, liver and gill. Total protein of each tissue sample for enzyme assays was estimated by Bradford method [27].

2.9 Enzymes of Oxidative Stress

2.9.1 Superoxide dismutase (SOD)

Superoxide dismutase was assayed according to the method described by [28] based on the oxidation of epinepherine–adrenochrome transition by the enzyme. Fifty microlitre of the sample was taken in the cuvette and 1.5 ml 0.1M carbonate–bicarbonate buffer containing 57mg/dl EDTA (pH 10.2) and 0.5 ml epinephrine (3 mM) was added and mixed well. Change in optical density at 480 nm was read immediately for 3 min in a Shimadzu–UV spectrophotometer. One unit of SOD activity was the amount of protein required to give 50% inhibition of epinephrine auto-oxidation.

2.9.2 Catalase (CAT)

Catalase activity was carried as per the method of [29]. To a reaction mixture of 2.45 ml phosphate buffer (50 mM, pH 7.0), enzyme source was added and the reaction was started by the addition of 1.0 ml of hydrogen peroxide (H_2O_2) solution. The decrease in absorbance was measured at 240 nm at 15 sec intervals for 3 min. The enzyme blank was run simultaneously with 1.0ml distilled water instead of H_2O_2 solution. CAT activity was expressed as nano moles hydrogen peroxide decomposed/min /mg protein.

2.9.3 Aspartate and alanine aminotransferase (AST and ALT)

The AST and ALT activity were assayed in different tissue homogenates as described by [30]. The substrate for AST comprised of 0.2 M D, L-aspartic acid and 2mM α-ketoglutarate in 0.05M phosphate buffer (pH 7.4). The AST activity was expressed as nmoles of pyruvate released/mg protein/min at 37 °C. Similarly, for ALT the substrate comprised of 0.2 M L- alanine and 2 mM α-ketoglutarate in 0.05M phosphate buffer (pH 7.4) and such activity was expressed as nmoles of oxaloacetate released/min/mg protein at 37 °C. In the experimental and control tubes, 0.5ml of substrate was added. The reaction was started by adding 0.1 ml of tissue homogenate. The assay mixture was incubated at 37 °C for 60 min. The reaction was terminated by adding 0.5ml of 1mM 2,4dinitrophenyl hydrazine (DNPH). In the control tubes the enzyme source was added after DNPH solution. The tubes were held at room temperature for 20 min with occasional shaking. Then 5ml of 0.4ml NaOH solution was added, the contents were thoroughly mixed. After 10 min, the OD was recorded at 540nm against the blank.

2.10 Challenge study

After 60 days of feeding, 10 fish from each replicate with 30 fish from treatment were intraperitoneally injected with virulent *A. hydrophila*, which was obtained from the Aquatic Animal Health and Management Division CIFE Mumbai. The bacteria were grown on nutrient broth (Himedia, Mumbai, India) for 24 h at 28 °C in a BOD incubator. Then, broth culture was centrifuged at 3000 g for 10 min at 4 °C. The supernatant was discarded and the pellet was washed three times with sterile phosphate buffer saline (pH 7.4) and finally suspended in PBS. The OD value of the solution was maintained to 0.5-0.6 at 456 nm by serial dilution to obtain the final bacterial cell concentration of 1.8×10^7 CFU mL^{-1} which at 0.2 ml dose was used for challenge study [31]. Mortality was recorded for all groups for 10 days. Post-challenge sampling of the fish survived was carried out on the 10^{th} day for enzyme analysis. *A. hydrophila* was confirmed by re-isolating it from the dead fish.

2.11 Statistical Analysis

The data analysis was carried out by using statistical software package SPSS (ver. 22, USA) through one way ANOVA and Duncan's multiple range tests, to determine the significant differences between the means. Paired t-test was also used to find out the significant difference between the mean of pre-

and post-challenge condition. Comparisons were made at the 5% probability level.

3. Results

3.1 Quantification of Fucoidan

The fucoidan was quantified by estimating the L-fucose content. Fucoidan yield was obtained by multiplying a factor of 1.75 to L-fucose content. The L-fucose content of the concentrated and dialysed extract was 32 mg/g dried seaweed powder. Hence, the fucoidan yield was 56mg/g or 5.6g/100g dried seaweed powder.

3.2 *In vitro* antioxidant properties of FRE

3.2.1 DPPH (2, 2-Diphenyl-1-picrylhydrazyl) radical scavenging method

The free radical scavenging capacity of the FRE with different concentrations was evaluated by using the DPPH (stable free radical). Each concentration of FRE taken was observed to scavenge DPPH radical, represented as percentage inhibition (Table 3). But it has been seen that ability to scavenge free radicals enhances as the concentration of fucoidan extract was increased. All the concentrations of FRE varied significantly ($P<0.05$), except that there was no significant different in the scavenging capacity of 8 mg/ml and 10 mg/ml concentrations.

3.2.2 Total phenolic contents (TPC) assay

The total phenolic contents of all the concentrations of fucoidan extract showed a similar trend as that of DPPH assay. Phenolic contents in all the concentrations were significantly ($P<0.05$) different, but highest TPC were observed at 12 mg/ml followed by 10mg/mlof FRE (Table 3).

3.2.3 Ferric reducing antioxidant power (FRAP) assay

The values of this assay were found to be significantly different, except in the 6mg/ml and 8mg/ml concentrations (Table 3). It was observed that as the concentration of extract increased the FRAP values were also elevated.

3.3 Physicochemical Parameters of Water

The different physicochemical parameters of water were maintained within the normal ranges, such as temperature (28-32^{0}C), pH (7.2-8.4), dissolved oxygen (5.2-7 mgml^{-1}), ammonia (0.06-0.1 mgml^{-1}), Nitrite-N (0.06-0.1 mgL^{-1}) and Nitrate-N (0.05-1 mgL^{-1}).

3.4 Enzymes of Oxidative Stress

3.4.1 Superoxide Dismutase (SOD) activity

The SOD activity of all the experimental groups was determined both in the pre and Post-challenge period. In the pre-challenge period there was no significant difference in SOD activity ($P>0.05$) among the different experimental groups both in liver and gill (Fig. 1). When the fish were challenged with *A. hydrophila*, high enzyme activity was observed in the control group. A significant difference ($P<0.05$) was found in different experimental groups in the post-challenged period with least activity in T_6 group both in liver and gill tissues.

3.4.2 Catalase activity

The catalase activity of liver and gill was analysed both in pre- and post-challenge periods (Fig. 2). In the pre-challenge period no significant difference was observed in catalase activity ($P>0.05$) among the experimental groups in liver and gill. Lowest activity was observed in T_6 group both in the liver and gill tissues during post-challenge, which was significantly ($P<0.05$) different from the other groups. Highest catalase activity was found in the control group indicating a high level of stress in that group.

3.4.3 Aspartate transaminase (AST) and Alanine transaminase (ALT) activities

The activity of enzyme in liver and muscle did not differ significantly ($P>0.05$) during the pre-challenge period (Fig. 3). But, when fish were challenged with bacteria high activity of enzyme was observed in control and low activity was seen in T_6 group which was significantly ($P<0.05$) different from the other groups.

The ALT activity in liver and muscle was not significantly different ($P>0.05$) before the fish were challenged with bacteria (Fig. 4). In the liver as well as in muscle, highest activity was recorded in control and lowest was observed in T_6 group which was significantly ($P<0.05$) different from rest of treatments.

4. Discussion

Stress is a general but non-specific response to any factor disturbing homeostasis. Stress is defined as the response of the cell or organism, to any demand placed on it such that it leads to an extension of a physiological state beyond the normal resting state [32]. Stress is the result of low antioxidant defence and/or excessive free radical production and this causes the structural and functional modifications due to chemical alterations of biomolecules [3]. Although there are reports about the antioxidant activity of fucoidan but, the synergistic effect on the stress mitigation due to methionine supplementation along with fucoidan extract is the first study as per our knowledge. In the present study, *in vitro* antioxidant activity of fucoidan rich extract was carried out. Then, fucoidan extract and L-methionine based diets were formulated and prepared to check whether there was enhanced effect on the mitigation of stress due to bacterial challenge.

Fucoidan is a term used for a class of fucose rich sulfated polysaccharides found in the fibrillar cell walls and intercellular spaces of brown seaweeds of the class Phaeophyceae and some invertebrate species, including the sea cucumber and sea urchin [33]. The current study found that the yield of fucoidan was 5.6%, which is in agreement with the previous report [12], who reported a yield of 5.5% from *S. wightii*.

The antioxidant activity of the fucoidan extract was determined by DPPH radical scavenging mechanism. DPPH is a free radical compound that has been usually employed to assess the free radical scavenging ability of different samples possessing antioxidant activities [34]. The free radical scavenging activity of the FRE was expressed as inhibition %. Fucoidan extract exhibited free radical scavenging activity in a concentration-dependent manner. The results have shown that as the concentration of the extract increased, the free radical scavenging activity was also enhanced. These results were found in accordance with the earlier study [35] who reported that antioxidant properties of most of the plant extracts was concentration dependent.

The total phenolic contents (TPC) of the plant extract is a good indicator of the total antioxidant power of the extract. The TPC of all the concentrations FRE were significantly ($P<0.05$) different. An increase in the TPC was noted with

the increasing concentration of FRE. The result of the present study is also supported by report of others, who suggested that phenolic contents are responsible for their antioxidant activity in plant extracts [36, 37].

FRAP assay is one of the very useful and most rapid test for routine analysis of antioxidant activity. The presence of the reductants in the seaweed extract causes the reduction of the Fe^{3+}/ferricyanide complex to the ferrous form. The reducing power was consistently elevated with the increasing concentration of FRE. Previous reports suggested that the reducing properties of polyphenols have been shown to exert antioxidant action as determined by the FRAP assay [38]. Similarly, previous study [39] revealed that many selected medicinal plants exhibited antioxidant activity as estimated by reducing power assay.

Feeding trial was conducted to study the stress mitigation through the antioxidant fucoidan rich extract along with the methionine supplementation at different levels in the diets. All the physiochemical parameters of water were maintained within the normal ranges of requirements for the fish, which is very essential for controlling the stress effect from environmental side.

SOD and catalase are antioxidant enzymes present in the body which scavenge free radicals (reactive oxygen species) thereby protecting lipids, membranes and other compounds being oxidized or destroyed [40]. Antioxidants also act in conjunction with these enzymes in detoxifying effect of ROS. In the present study, the SOD and catalase activity was diminished significantly with the increasing level of dietary FRE along with low dose methionine. This indicates that the free radicals are effectively scavenged by the added methionine. It may be due to the presence of phenolic compounds in the fucoidan extract which synergistically acted with supplemented methionine as a powerful antioxidant.

The increase in the activity of AST and ALT in the control for both the tissues is the indicator of aspartate and alanine mobilization via gluconeogenesis for glucose production to tackle the stress during the post-challenge period. The higher activity of AST and ALT is mainly the manifestation of metabolic breakdown of protein for energy provision in order to mitigate stress [41]. In the present study, both AST and ALT were significantly reduced in the treatment group supplemented with 2% fucoidan along with low dose methionine in comparison to other groups. Our results corroborate with the earlier study [42], who reported that the transaminase activity was reduced in the treatment groups supplemented with the nutraceutical like probiotics after challenged with bacteria.

Table 1: Composition of the Experimental Diets (% DM basis)

Ingredients	Control	T1	T2	T3	T4	T5	T6
Casein	33	32	31	33	33	31	32
Gelatin	7.25	7.25	7.25	7.25	7.25	7.25	7.25
Dextrin	16.75	16.75	16.75	16.75	16.75	16.75	16.75
Starch	19.5	19.5	19.5	19.5	19.5	19.5	19.5
Cellulose	9.84	10.24	10.64	8.84	7.84	9.64	8.24
CMC	1.5	1.5	1.5	1.5	1.5	1.5	1.5
Cod liver oil	4	4	4	4	4	4	4
Sunflower oil	4	4	4	4	4	4	4
Vitamin-mineral mix	2	2	2	2	2	2	2
BHT	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Choline Chloride	0.9	0.9	0.9	0.9	0.9	0.9	0.9
L-cysteine	0.86	0.86	0.86	0.86	0.86	0.86	0.86
L-methionine	0.3	0.9	1.5	0.3	0.3	1.5	0.9
Fucoidan	0	0	0	1	2	1	2
	100	100	100	100	100	100	100

Table. 2: Experimental Design

Treatments	Condition
Control (C)	Basal diet (No fucoidan+0.3% methionine)
Treatment (T_1)	Basal diet + methionine (Low dose- 0.9%)
Treatment (T_2)	Basal diet + methionine (High dose- 1.5%)
Treatment (T_3)	Basal diet + 1% fucoidan+0.3% methionine
Treatment (T_4)	Basal diet + 2% fucoidan+0.3% methionine
Treatment (T_5)	Basal diet + 1% fucoidan+ methionine (High dose- 1.5%)
Treatment (T_6)	Basal diet + 2% fucoidan+ methionine (Low dose- 0.9%)

Table 3: *In vitro* antioxidant activity of fucoidan rich extract

Concentrations(mg/ml)	DPPH (inhibition %)	Phenolic contents (mg/100g)	FRAP µmol Fe (II)/100g
0.25	11.46^{h}±0.25	1.45^{i}±0.05	9.81^{i}±0.09
0.50	21.75^{g}±0.05	1.84^{h}±0.02	12.25^{h}±0.07
1.00	26.27^{f}±0.14	4.66^{g}±0.09	17.35^{g}±0.11
2.00	33.10^{e}±0.06	5.07^{f}±0.05	28.53^{f}±0.06
4.00	38.13^{d}±0.09	8.95^{e}±0.08	51.88^{e}±0.49
6.00	59.20^{c}±0.13	16.52^{d}±0.06	78.30^{d}±0.50
8.00	60.56^{b}±0.29	20.95^{c}±0.15	97.09^{c}±0.72
10.0	61.39^{b}±0.77	28.42^{b}±0.04	113.75^{b}±0.65
12.0	64.99^{a}±0.10	35.50^{a}±0.06	131.03^{a}±0.16

Mean values in the same column with different superscript differ significantly ($P<0.05$). Data expressed as Mean±SE, n=3.

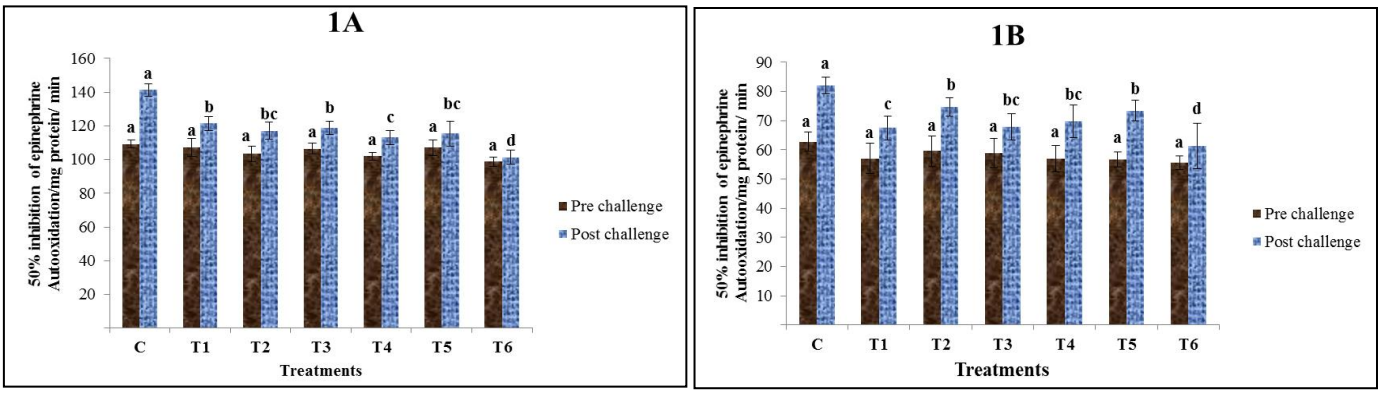

Fig 1: Pre- and post-challenge SOD in liver (1A) and gill (1B) of *Labeo rohita* fingerlings fed with different experimental diets. Different superscripts (a, b, c, d) in each series under each panel signify statistical differences (ANOVA, Duncun's test, $P<0.05$). Values are expressed as mean ± SE (n = 3).

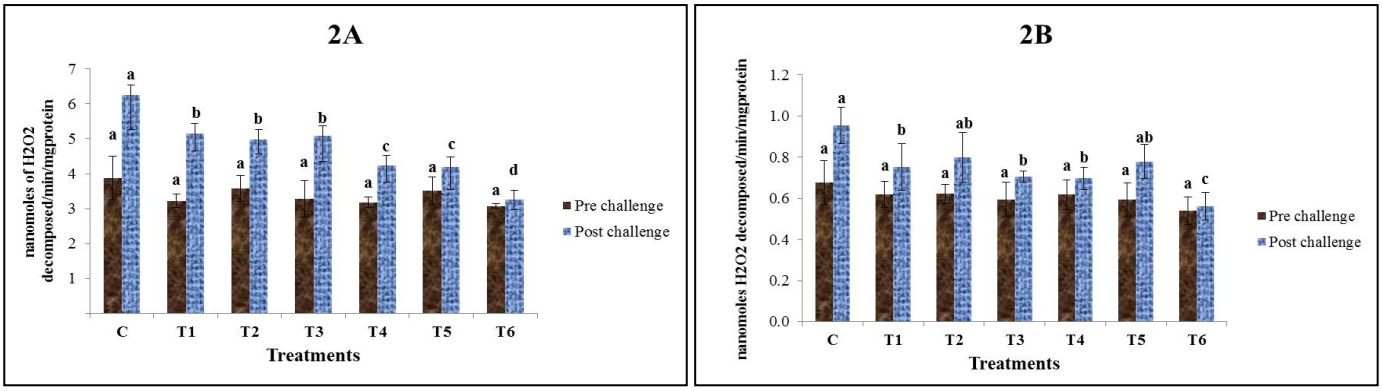

Fig 2: Pre- and post-challenge Catalase activity in the liver (2A) and gill (2B) of *Labeo rohita* fingerlings fed with different experimental diets. Different superscripts (a, b, c, d) in each series under each panel signify statistical differences (ANOVA, Duncun's test, $P<0.05$). Values are expressed as mean ± SE (n = 3).

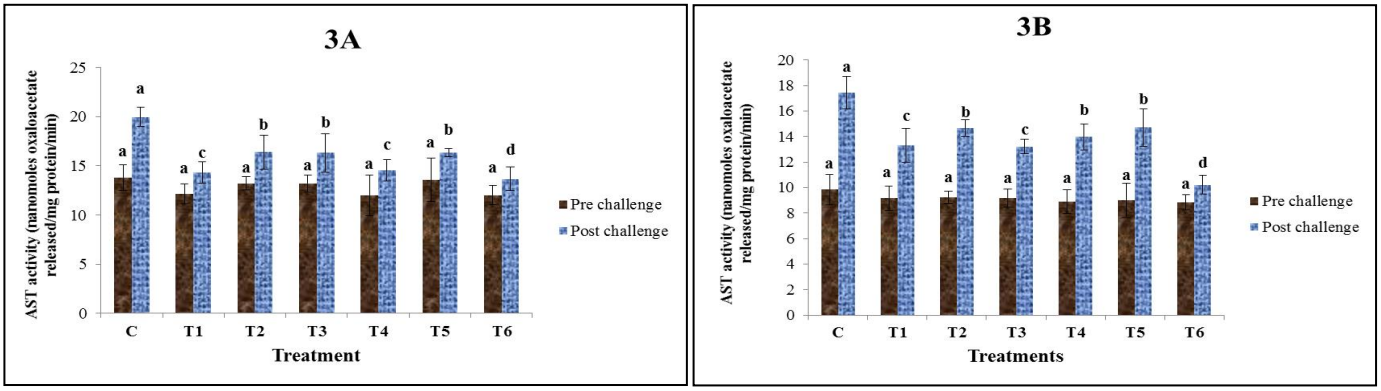

Fig 3: Pre- and post-challenge AST activity in the liver (3A) and muscle (3B) of *Labeo rohita* fingerlings fed with different experimental diets. Different superscripts (a, b, c, d) in each series under each panel signify statistical differences (ANOVA, Duncun's test, $P<0.05$). Values are expressed as mean ± SE (n = 3).

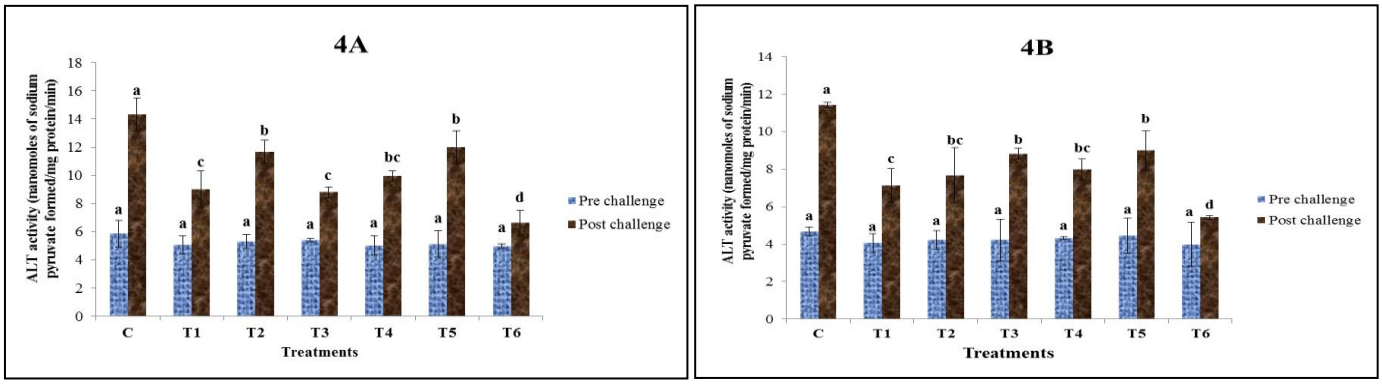

Fig 4: Pre- and post-challenge ALT activity in the liver (4A) and muscle (4B) of *Labeo rohita* fingerlings fed with different experimental diets. Different superscripts (a, b, c, d) in each series under each panel signify statistical differences (ANOVA, Duncun's test, $P<0.05$). Values are expressed as mean ± SE (n = 3).

5. Conclusion

In conclusion from the above investigation, using several *in vitro* assays, fucoidan rich extract was found to scavenge free radicals and bestows it the bioactive potential of antioxidant activity. However, the stress level of fish challenged with *A. hydrophila* was mitigated in a synergistic manner when the FRE was supplemented along with the low dose methionine. It can thus be suggested that 2% fucoidan rich extract along with low dose methionine supplementation can synergistically increase the potential of stress mitigation in *L. rohita*. Further research might be undertaken to study the stress related enzymes at molecular level.

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