



Effects of nutraceutical conglomerate on growth and antioxidant status of *Labeo rohita* fingerlings

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ABSTRACT

Four month feeding trial was conducted to evaluate the effect of a vitamin-mineral based nutraceutical conglomerate on *Labeo rohita* fingerlings (Hamilton, 1822) exposed to different stressors like crowding (normal stocking density NSD, 10 number of fish in 75 L water or high stocking density, HSD, 20 number of fish in 75 L water) and feed restriction (satiation feeding, SF or restricted feeding, RF, at 1% of body weight). One thousand and eighty (1080) fingerlings of *Labeo rohita* with an average size of $8.5 \text{ g} \pm 0.5$ were randomly distributed in 6 distinct experimental groups, viz, THR0 (HSD, RF and 0% nutraceutical), THR1 (HSD; RF; 0.1 % nutraceutical), THR5 (HSD, RF, 0.5 % nutraceutical), TNS0 (NSD, SF and 0% nutraceutical), TNS1 (NSD; SF; 0.1 % nutraceutical) and TNS5 (NSD; SF; 0.5 % nutraceutical). Feeding of nutraceuticals improved the overall growth of the fish both in stress exposed and non-exposed groups. The hepatic antioxidant genes (superoxide dismutase-1, SOD-1; glutathione peroxidase-1, GPx-1) and enzymes (SOD and GPx) showed significantly higher values in stress exposed group (THR0) during the experimental trial in all the four months while catalase gene and enzyme showed a higher activity from 2nd month onwards. Reduced glutathione was found lower at the end of 3- and 4-months trial. Serum total antioxidant status (TAS) was significantly reduced due to stress at the end of each trial period, whereas feeding of 0.1 % nutraceutical increased the TAS in stress exposed groups. Hence, it can be concluded that feeding of 0.1 % nutraceutical conglomerate containing zinc, selenium, vitamin E and methionine could mitigate the crowding and feed restriction-mediated oxidative stress in *Labeo rohita* fingerlings at the end of 3- and 4-months period. The result of this study will be highly applicable for the production of stunted carp fingerlings for short duration crop in seasonal ponds or water bodies.

1. Introduction

The world population is growing at a faster rate and expected to reach 9 billion by 2050 and therefore, the daunting challenges for the present-day researchers are to meet the food requirement for this projected population (World Bank Report, 2013). Even though it is minimally recognized, but promising fact that aquaculture can share a major protein requirement of the middle-income families along with a sizeable livelihood security to the poor. By 2050, the global aquaculture must produce an additional 47.5 million tonnes of fish to meet the expected consumption demand (FAO, 2016). This higher production from aquaculture sector could only be possible

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through either horizontal or vertical expansion of the aquaculture farms. The horizontal expansion of aquaculture farms is difficult probably due to increasing urbanization and industrialization globally. Therefore, the vertical expansion of aquaculture is the only option left for augmenting enhanced production to meet the expected demand. Vertical expansion of aquaculture is mostly intending to increase in stocking density of the fish, which has the impact on growth, survival, and yield variation for different species (Zhu et al., 2011; Khatune-Jannat et al., 2012; M'balaka et al., 2012). Nevertheless, it may result in increased competition for food, aggression and reduced reproductive performance (Tave, 1986) as high stocking density not only cause crowding stress but also results in feed restriction, hypoxia etc. Moreover, it is very well reported that crowding and feed deprivations are considered as chronic stressors in aquaculture (Wedemeyer et al., 1990).

Higher stocking density or over-crowding causes a decrease in the availability of sufficient food to all fish in the group leading to reduced feed intake, feed efficiency and undergrowth. Under such conditions, body reserves will be channelized to satisfy energy requirements resulting in growth retardation or stunting in fish (Trenzado et al., 2006; Shamna et al., 2020). Most of the times growth retardation or stunting of any organism is a phenotypic reversible or irreversible negative change (Bhujel et al., 2007; Sardar et al., 2006). This is mainly because of the oxidative stress generated, in other words, the uncontrolled production of reactive oxygen species (ROS) in response to the stressors. The unpaired electron in these reactive oxygen species can oxidize steroids, nucleic acids, carbohydrates, proteins, and interact with membrane lipids resulting in peroxidation. This can affect cell division, differentiation, immune status, and metabolism (Di Giulio et al., 1989). Body antioxidant defense system including enzymatic antioxidants like superoxide dismutase (SOD), catalase (CAT), thioredoxins and glutathione peroxidase (GPx) etc. along with non-enzymatic antioxidants like vitamin A, vitamin E, vitamin C, copper (Cu), manganese (Mn), zinc (Zn), selenium (Se), glutathione, carotenoids etc. continually either neutralize or inhibit the production of ROS/free radicals to prevent cellular damage. Though there is such an efficient antioxidant defence system present in fish, the spontaneous production of ROS produced due to stress factors like crowding, feed restriction etc. can reduce the stress mitigation efficiency of the cells leading to increased concentration of ROS, which is detrimental to fish health. Dietary intervention by providing additional non-enzymatic antioxidants or nutraceuticals could be a potential strategy to stimulate the entire antioxidant defence system of the fish for mitigating the oxidative stress effects.

Among the vitamins, tocopherol (vitamin E) plays a key role in protecting the quality of aquaculture products through its antioxidant property (NRC, 2011). However, our previous works in this line indicated that feeding of vitamin E was found to be effective as an antioxidant during nitrite toxicity in *Labeo rohita* fingerlings (Ciji et al., 2012) and vitamin C reduced endosulfan stress in *Channa punctatus* (Sharma et al., 2009). Tejpal et al. (2009) had reported that dietary inclusion of L-tryptophan mitigates crowding stress in mrigal fingerlings. Similarly, synergistic effect of citric acid and dietary microbial phytase was observed in *Labeo rohita* fingerlings when fed with sub-optimum protein (Baruah et al., 2007). Hence, the present research was designed to study the synergistic effect of zinc, selenium, vitamin E and methionine-containing dietary nutraceutical conglomerate on growth, enzymatic antioxidants and their gene expression along with total antioxidant status of *Labeo rohita* exposed to crowding and feed restriction for different duration.

2. Materials and methods

2.1. Experimental animal

Fingerlings of *Labeo rohita* (Hamilton, 1822) with an average weight of 8.5 ± 0.5 g were procured from Prem fish farm, Ankaleswar, Gujarat and were transported in polyethylene bags to the wet laboratory of the ICAR-Central Institute of Fisheries Education (ICAR-CIFE). During the acclimatization period of 7 days, fishes were kept at a well aerated circular tank of 1000 L capacity and fed with a diet containing 35 % protein twice daily.

2.2. Formulation and preparation of experimental diets

Purified ingredients like gelatin and casein (protein sources), starch and dextrin (carbohydrate sources), carboxy methyl cellulose (binder), vitamin and mineral mixture, and BHT (synthetic antioxidant) procured from Hi-Media and vegetable oil and fish oil (lipid sources) procured from local market were used to formulate and prepare the three experimental diets viz. control (C), diet A and diet B with supplementation of nutraceutical conglomerate at 0, 0.1 and 0.5 % level, respectively (Supplementary data; Table 1a). The nutraceutical mixture contained methionine (360 mg), vitamin E (21 g) zinc (1000 mg) and selenium (50 mg) in 1Kg in which cellulose was used as filler. Hence, when given in diet at 0.1 and 0.5 % levels these nutrients were above the requirement levels of the fish (Supplementary data; Table 1a & 1b).

2.3. Experimental design and maintenance of fish

The feeding trial was conducted in plastic rectangular tubs ($57 \times 36 \times 47$ cm, 75 L capacity) covered with perforated lids and continuous aeration. The water quality parameters were checked at regular intervals which were within the optimum range for freshwater fish culture viz; temperature $25-29^\circ\text{C}$, pH 7.5–8.1, DO 6.3–7.5 and ammonia $0.05-0.09\text{ mg L}^{-1}$. One thousand and eighty fingerlings of acclimatized *Labeo rohita* (Hamilton, 1822) with an average size of 8.5 ± 0.5 g were randomly distributed in 6 distinct experimental groups exposed to different stressors like crowding (normal stocking density NSD, 10 number of fish per 75 L water or high stocking density, HSD, 20 number of fish per 75 L water) and feed restriction (satiation feeding, SF or restricted feeding, RF, at 1% of body weight). Hence, the groups were THR0 (HSD, RF and 0% nutraceutical) THR1 (HSD; RF; 0.1 % nutraceutical), THR5 (HSD, RF, 0.5 % nutraceutical), TNS0 (NSD; SF and 0% nutraceutical), TNS1 (NSD; SF; 0.1 % nutraceutical) and TNS5 (NSD; SF; 0.5 %

nutraceutical) in triplicates with four similar experimental set for the duration of 1, 2, 3 or 4 months (each month are independent), respectively (Fig. 1a).

2.4. Sample collection

Sampling was done from all groups at the end of feeding trial of 1, 2, 3 or 4 months, respectively for assessing enzyme activities, gene expression, glutathione levels and total antioxidant status. The liver tissues used for gene expression study were stored in cryovials containing 1.5 mL RNA Later solution (Ambion Biosciences). Partial characterization of all the three genes were done at Molecular laboratory of MBT division, CMFRI, India, under the DBT sponsored training on Marine Biotechnology. Blood was collected using 1 mL syringe and allowed to clot in 1.5 mL tubes; serum was collected in a new tube after centrifugation at 4000xg for 10 min. Serum samples were used for studying the total antioxidant status of the fish. For studies of antioxidant enzyme activities and reduce glutathione level, 5 % tissue homogenate (liver) was prepared in 0.25 M sucrose solution and stored in -80°C till further analysis.

2.5. Expression of enzymatic antioxidant genes

Expression studies were conducted for the three enzymatic antioxidant genes viz., SOD-1, GPx-1 and CAT as follows:

2.5.1. Total RNA extraction

Total RNA was isolated from liver tissue of rohu using TRIzol™ Reagent (Invitrogen, USA) by acid guanidinium thiocyanate-phenol-chloroform extraction method (Chomczynski, 1993). The mRNA was quantified using BioPhotometer® plus with Hellma Tray Cell (Eppendorf, Hamburg, Germany) at 260 nm. The integrity of RNA was checked in 1.2 % agarose gel.

2.5.2. cDNA synthesis

cDNA synthesis of extracted RNA was done using First Strand cDNA Kit (Fermentas, Germany) using anchored oligodT primer following the instructions of the manufacturer.

2.5.3. qPCR analysis

The real time PCR Primer was also designed using Gene runner V.3.0 software based on the partial sequences obtained for SOD, catalase and GPX transcripts. Primers were synthesized in Xcelris Lab Limited, Gujarat, India. List of primers used in this study is given in Table 1d (Supplementary data).

The qRT-PCR analysis of SOD, CAT and GPx transcripts was done in the treatment groups of at the end of feeding trial of 1, 2, 3 and 4 months, respectively. β -actin was used as a reference and the qRT-PCR was performed in Roche LC480 Light Cycler using Maxima SYBER green /ROX qPCR master mix (2x) (Fermentas, USA). The T_m value for all primers were 59°C . In order to confirm that only one PCR product was amplified and detected, the amplified products were subjected to melting curve analysis at the end of each PCR reaction. The relative expression of mRNA was estimated using comparative CT method and by subtracting CT value of internal control from target gene, the ΔCT was calculated. The mean ΔCT was obtained from normalized ΔCT value and $\Delta\Delta CT$ was calculated by subtracting mean ΔCT of control treatment from mean ΔCT of target gene. Fold change at various concentrations of SOD-1, CAT, and GPx-1 were calculated by $2^{-\Delta\Delta CT}$. Internal control for normalization was β -actin gene.

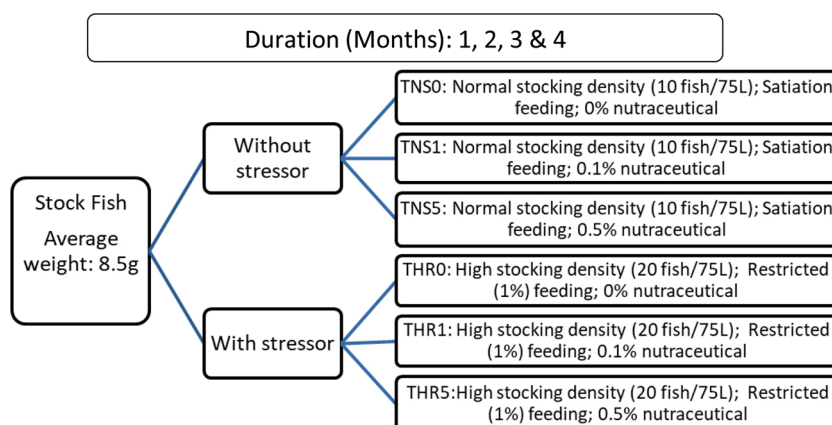


Fig. 1. a) Experimental design with experimental set (each month indicate 1 set) showing various treatments.

2.6. Analysis of antioxidant enzyme activities

2.6.1. Superoxide dismutase (SOD)

Hepatic SOD activity was assayed according to the method described by [Mishra and Fridovich \(1972\)](#) based on the oxidation of epinephrine–adrenochrome transition by the enzyme with little modification. Tissue homogenate (50 μ L) was taken in the cuvette and 1.5 mL 0.1 M carbonate – bicarbonate buffer containing 57 mg dL^{-1} EDTA (pH 10.2) and 0.5 mL epinephrine (3 mM) was added and mixed well. Change in optical density was read at 480 nm for 3 min in a UV spectrophotometer (Shimadzu, Japan). SOD activity was expressed as unit activity (amount of protein required to give 50 % inhibition of epinephrine auto oxidation).

2.6.2. Catalase (CAT)

Catalase activity of liver tissue was assayed according to the method described by [Takahara et al. \(1960\)](#). To a reaction mixture of 2.45 mL phosphate buffer (50 mM, pH 7.0) and tissue homogenate (50 μ L), H_2O_2 solution (1.0 mL) was added and the decrease in absorbance was measured at 240 nm at 15 s intervals for 3 min. The enzyme blank was run simultaneously with 1.0 mL distilled water instead of H_2O_2 solution. Enzyme activity was expressed as nanomoles H_2O_2 decomposed / min / mg protein.

2.6.3. Glutathione peroxidase (GPx)

Hepatic GPx activity was determined according to the method of [Hafeman et al. \(1974\)](#). Tissue homogenate (100 μ L) was treated with 5 mM glutathione (100 μ L), 1.2 mM H_2O_2 (100 μ L), 25 mM NaN_3 (100 μ L) and 2.1 mL of 1 M phosphate buffer (pH 7.0) at 37 °C for 6 min. The reaction was stopped by adding 1.65 % phosphoric acid (2.0 mL) and the mixture was centrifuged at 3000 g for 10 min. The supernatant (2.0 mL) was mixed with 0.4 M Na_2HPO_4 (2.0 mL) and 1 mM DTNB prepared in buffer (1 mL). The yellow colour was measured at 412 nm after 10 min incubation at room temperature. GPx activity was expressed as nano moles H_2O_2 decomposed / min / mg protein.

2.6.4. Glutathione -s - transferase (GST)

GST activity of liver tissue was determined by the method of [Habig et al. \(1974\)](#), in which the formation of the conjugate 1-chloro-2, 4- dinitrobenzene (CDNB) using as substrate in the presence of reduced glutathione (GSH), which is measured as increase in absorbance (for 3 min) at 340 nm. The reaction mixture was a cocktail of 1.5 mL sodium phosphate buffer 0.1 M (pH 6.5), 0.2 mL GSH 9.2 mM, 0.02 mL CDNB 0.1 M and 0.1 mL of the sample. The activity of enzyme was expressed as units/ mg protein/ min.

2.7. Determination of reduced glutathione (GSH)

Reduced glutathione of liver tissue was estimated according to the method of [Moron et al. \(1979\)](#). To a 500 μ L tissue homogenate, 125 μ L of 25 % TCA was added and kept in ice for 5 min. To the cooled mixture 500 μ L of 5 % TCA was added and subjected to centrifugation (3000 g, 5 min). From the supernatant collected 150 μ L was mixed with 350 μ L of 0.2 M phosphate buffer (pH 8.0) and 0.6 mM DTNB prepared in buffer (1 mL). The yellow colours formed were measured against TCA containing blank at 412 nm. The reduced glutathione was measured using standard graph and expressed as nmol/g protein.

2.8. Serum total antioxidant status

Serum total antioxidant status was measured using total antioxidant assay kit (Cayman Chemical, Ann Arbor, USA) and expressed in mM.

2.9. Statistical analysis

Data were statistically analyzed using one way and two - way ANOVA at 5 % level of significance in SPSS software version 22 after checking the normality and homogeneity of the variance. The results of one-way ANOVA are expressed in the form of mean $\pm$ SE, while two-way data as mean $\pm$ SEM. DMRT was used for post-hoc analysis to compare the means. PCA was used to express the effect of the treatments on growth and total antioxidant status of the fish using XLSTAT software.

3. Results

3.1. Growth response

Dietary nutraceutical did not elicit any significant changes ($p > 0.05$) in the fortnightly growth performance of *L. rohita* for the first 60 days of the trial, but the group fed 0.1 % nutraceutical showed a significantly higher weight after 75 days of feeding compared to other groups ($p < 0.05$). The fish exposed to stress showed lower weight compared to the non-stress exposed counterpart, however, the effect of nutraceutical was only seen after 90 days of feeding with the group fed 0.1 % nutraceutical exhibiting a significantly higher value compared to the non-supplemented (THR0) and 0.5 % (THR5) fed groups ($p < 0.05$). Stress caused a significant effect on the growth performance of the fish throughout the feeding trial ($p < 0.05$). Dietary nutraceutical had no effect on the fish for the first 60 days of the trial ($p < 0.05$), however, from day 90–120, 0.1 % (THR1) nutraceutical significantly improves the weight of the fish compared to the non-supplemented and 0.5 % fed groups ($p < 0.05$). There was a significant interaction effect of stress and

nutraceutical on the fortnightly increase in weight of the fish from day 75–120 ($p < 0.05$) (Supplementary data, Table 2a).

3.2. Principal component analysis of the weight gain and total antioxidant status

A positive correlation was observed between the monthly increase in weight and total antioxidant status of *L. rohita* fingerlings exposed to stress and fed different levels of nutraceutical (Supplementary data, Table 2b). The PCA analysis showed that the two components (PC1 and PC2) represented 93 % of the total variance in the fed *L. rohita* fingerlings. The TAS 60, WG, TNS1 and TNS5 were found grouped together and are in close proximity to each other, and these accounted for 79 % of the total variance (PC1). However, THR1 and THR5, and THR0 were found separated from each other and other parameters and accounted for 13 % (PC2) of the total variance (Supplementary data, Fig. 1b).

3.3. Enzymatic antioxidants

3.3.1. Hepatic SOD-1 gene expression and SOD enzyme activity

The SOD-1 gene expression was significantly increased ($P < 0.05$) in liver of fish due to stress at the end 1, 2, 3 and 4 months of trial period with the highest increment in the 2 months trial (Fig. 2, Table 1). Compared to THR0 fed group, feeding of nutraceutical, especially 0.1 % (THR1) level significantly reduced ($P < 0.05$) the SOD-1 gene expression at the end of 2, 3 and 4 months of experiment. The interaction between stress and dietary nutraceutical had significant effect on the hepatic SOD-1 gene expression in rohu at the end of each trial period. One-way ANOVA analysis revealed that dietary nutraceutical did not affect hepatic SOD-1 gene expression in without-stress exposed fish, whereas both 0.1 and 0.5 % dietary nutraceutical significantly reduced ($P < 0.05$) the hepatic SOD-1 gene expression in the stress exposed fish (THR1 and THR5) at the end of 2, 3 and 4 months of feeding trial compared to THR0 group (Fig. 2).

The hepatic SOD enzyme activity in fish did not vary significantly due to stress exposure and feeding nutraceutical at the end of 1-month trial, while stress significantly increased ($P < 0.05$) hepatic SOD activity in fish at the end of 2, 3- and 4-months trial. Conversely, feeding of dietary nutraceutical (both 0.1 % and 0.5 %) significantly decreased ($P < 0.05$) the hepatic SOD activity at the end of 2, 3- and 4-months trial (Table 1, Fig. 2). Fish fed with 0.1 % nutraceutical supplemented diet exhibited significantly lower hepatic SOD activity at the end of 3 months trial. However, the significant effect on liver SOD activity in fish due to interaction between stress and nutraceutical were observed at the end of each trial period. One-way ANOVA showed that hepatic SOD enzyme activity was significantly higher ($P < 0.05$) in THR0 throughout the experimental trial (Fig. 2). Dietary nutraceutical though could not change hepatic SOD activity significantly in stress exposed fish at the end of 1 month trial, significantly lower ($P < 0.05$) enzyme activity was found in THR1 (0.1 % nutraceutical) and THR5 (0.5 % nutraceutical) groups at the end of 2-, 3- and 4-months trial (Fig. 2).

3.3.2. Hepatic catalase gene expression and catalase enzyme activity

Catalase gene expression in the liver of fish (Fig. 3, Table 2) was significantly increased ($P < 0.05$) due to stress at the end of 1, 2, 3- and 4-months trial while catalase enzyme activity was significantly increased in 1, 2 and 4 months. Feeding of 0.5 % nutraceutical significantly reduced ($P < 0.05$) the hepatic catalase gene expression at the end of each trial period, however, 0.1 % dietary nutraceutical significantly reduced ($P < 0.05$) the gene expression at the end of 2, 3- and 4-months trial period. Feeding of 0.5 % nutraceutical significantly reduced the hepatic catalase enzyme activity in 1, 2 months, whereas 0.1 % nutraceutical could reduce the enzyme activity in all the 4-months.

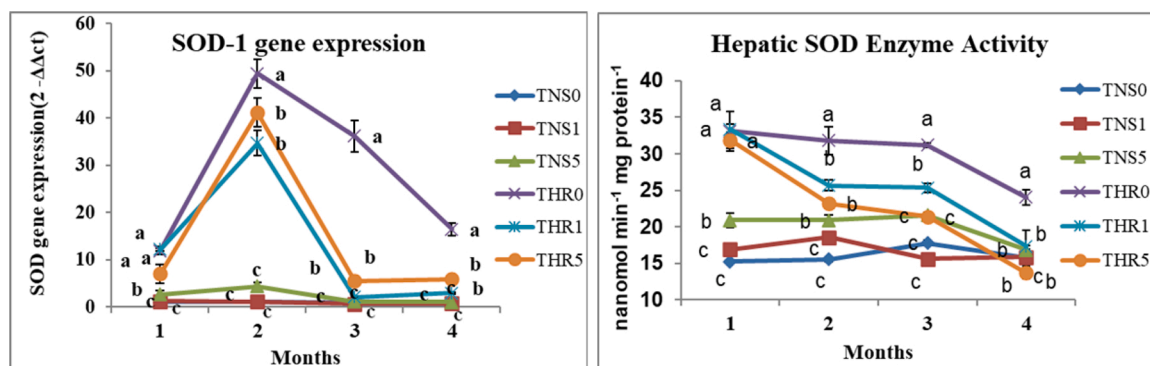


Fig. 2. Effect of stress and nutraceutical on hepatic SOD-1 gene expression and SOD enzyme activity in rohu fingerlings at the end of 1, 2, 3- and 4-months experimental periods.

Data are expressed as mean $\pm$ SE ($n=3$) and the lines with different superscripts differ significantly ($p < 0.05$).

THR0 (high stocking density, HSD, 20 nos 75 L⁻¹ water; restricted feeding, RF, at 1% of body weight; 0% nutraceutical containing diet) THR1 (HSD; RF; 0.1 % nutraceutical containing diet), THR5 (HSD, RF, 0.5 % nutraceutical containing diet), TNS0 (normal stocking density, NSD, 10 nos 75 L⁻¹ water; satiation feeding, SF, 3 % of body weight; 0% nutraceutical containing diet), TNS1 (NSD; SF; 0.1 % nutraceutical containing diet) and TNS5 (NSD; SF; 0.5 % nutraceutical containing diet).

Table 1

Effect of stress (high stocking density & feed restriction), level of nutraceutical and the interaction between both on hepatic SOD gene expression and enzyme activity in rohu, *Labeo rohita* fingerlings.

Two-way ANOVA	SOD-1 gene expression (L) ($2^{-\Delta\Delta CT}$)				SOD ¹ enzyme (L)			
Effect of stress	Month 1	Month 2	Month 3	Month 4	Month 1	Month 2	Month 3	Month 4
Without stress	1.70 ^b	2.24 ^b	0.96 ^b	0.94 ^b	17.68	18.33 ^b	18.33 ^b	16.18 ^b
With stress	10.42 ^a	41.78 ^a	14.63 ^a	8.46 ^a	26.89	26.89 ^a	25.97 ^a	18.36 ^a
SEM	0.353	1.863	1.497	0.655	1.584	0.524	0.218	0.707
p value	0.002	0.001	0.001	0.003	0.159	<0.001	0.002	0.034
Effect of nutraceutical level								
0.0	6.52 ^a	25.26 ^a	18.62 ^a	8.74 ^a	24.18	23.68 ^a	24.49 ^a	19.93 ^a
0.1	6.76 ^a	17.96 ^c	1.44 ^b	1.85 ^c	25.15	22.11 ^b	20.47 ^c	16.60 ^b
0.5	4.91 ^b	22.81 ^b	3.34 ^c	3.52 ^b	26.38	22.05 ^b	21.50 ^b	15.29 ^b
SEM	0.432	2.281	1.833	0.802	1.940	0.641	0.267	0.866
p value	0.021	0.001	<0.001	0.002	0.913	0.027	<0.001	0.023
Effect of stress*nutraceutical								
p value	<0.001	0.001	<0.001	0.002	0.049	0.001	<0.001	0.007

Data expressed as Mean & SEM, n=3 (two-way ANOVA); Mean values in the same column with different superscripts differ significantly. Significance of mean value was observed at 5% probability level ($P < 0.05$); L-liver.

¹ SOD activity is expressed as nanomoles/min/mg protein.

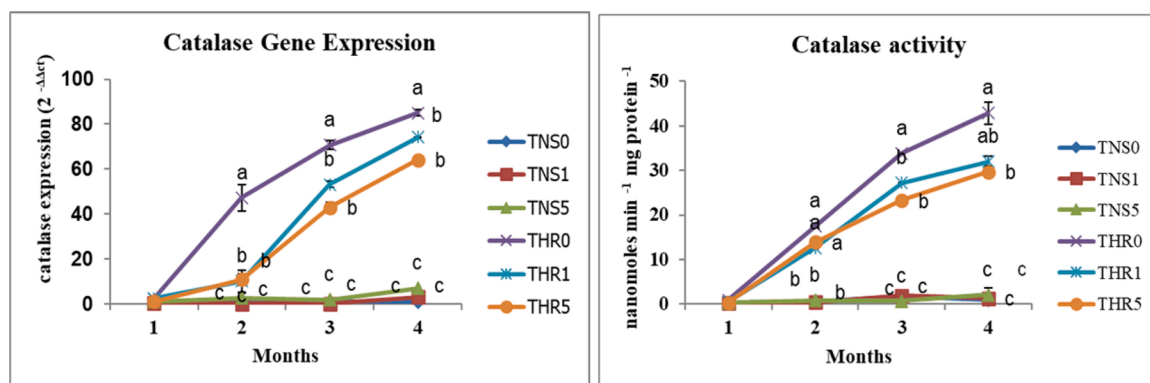


Fig. 3. Effect of stress and nutraceutical on hepatic catalase gene expression and enzyme activity in rohu fingerlings at the end of 1, 2, 3- and 4-months experimental period.

Data are expressed as mean $\pm$ SE (n=3) and the lines with different superscripts differ significantly ($p < 0.05$).

THR0(HSD, 20 nos 75 L⁻¹ water; RF, at 1% of body weight; 0% nutraceutical containing diet) THR1 (HSD; RF; 0.1 % nutraceutical containing diet), THR5(HSD, RF, 0.5 % nutraceutical containing diet), TNS0 (normal stocking density, NSD, 10 nos75L⁻¹ water; satiation feeding, SF, 3% of body weight; 0% nutraceutical containing diet), TNS1 (NSD; SF; 0.1 % nutraceutical containing diet) and TNS5 (NSD; SF; 0.5 % nutraceutical containing diet).

The interaction between stress and dietary nutraceutical had significant effect ($P < 0.05$) on hepatic catalase gene expression and enzyme activity in fish at the end of each trial period. Though stress significantly increased ($P < 0.05$) the hepatic catalase enzyme activity at the end of 1, 2- and 4-months trial, it was significantly reduced at the end of 3 months trial. On the other hand, in comparison to without nutraceutical fed group, 0.1 % dietary nutraceutical significantly reduced ($P < 0.05$) the hepatic catalase enzyme activity in fish at the end of each trial period, while 0.5 % dietary nutraceutical though significantly reduced ($P < 0.05$) the enzyme activity at the end of 1- and 2-months trial, failed to do it at the end of 3- and 4-months trial period. The one-way ANOVA indicated that the hepatic catalase gene expression and enzyme activity did not change significantly in the normal fish (without stress exposure) due to feeding of nutraceutical, whereas in stress exposed fish, dietary nutraceutical both at 0.1 and 0.5 % level (THR1 and THR5) significantly reduced ($P < 0.05$) the gene expression at the end of 2-, 3- and 4-months trial but not at the end of 1 month trial (Fig. 3). However, feeding of 0.1 and 0.5 % dietary nutraceutical could only reduce ($P < 0.05$) the enzyme activity at the end of 3- and 4-months trial in comparison to without-nutraceutical (THR0) fed group (Fig. 3).

3.3.3. Hepatic GPx gene expression and GPx enzyme activity

Stress exposure resulted in significant increase ($P < 0.05$) in hepatic GPx-1 gene and the GPx enzyme activity at the end of each period of experiment (Fig. 4, Table 3). In comparison to without-nutraceutical fed group, dietary nutraceutical significantly reduced ($P < 0.05$) the hepatic GPx-1 gene expression. Feeding of 0.1 % nutraceutical reduced the GPx-1 expression in 1, 3- and 4-month stress

Table 2

Effect of stress (high stocking density & feed restriction), level of nutraceutical and the interaction between both on catalase gene and enzyme activity in rohu, *Labeo rohita* fingerlings.

Two-way ANOVA	Catalase gene expression (L) ($2^{-\Delta\Delta CT}$)				¹ Catalase enzyme (L)			
Effect of stress	Month 1	Month 2	Month 3	Month 4	Month 1	Month 2	Month 3	Month 4
Without stress	0.89 ^b	1.32 ^b	1.05 ^b	3.72 ^b	0.28 ^b	0.65 ^b	2.36 ^a	14.59 ^b
With stress	2.13 ^a	22.79 ^a	55.66 ^a	74.74 ^a	0.59 ^a	1.27 ^a	1.74 ^b	28.88 ^a
SEM	0.13	2.23	0.77	0.38	0.02	0.03	0.11	0.88
<i>p</i> value	0.002	<0.001	0.004	<0.001	0.001	<0.001	0.004	0.002
Effect of nutraceutical level								
0.0	1.76 ^a	24.11 ^a	35.90 ^a	43.03 ^a	0.60 ^a	1.19 ^a	2.47 ^a	26.23 ^a
0.1	1.56 ^{ab}	5.22 ^b	26.67 ^b	38.59 ^b	0.37 ^b	0.86 ^b	1.64 ^b	17.70 ^b
0.5	1.18 ^b	6.82 ^b	22.50 ^c	35.65 ^c	0.33 ^b	0.82 ^b	2.03 ^a	21.28 ^{ab}
SEM	0.16	2.74	0.94	0.50	0.03	0.04	0.14	1.08
<i>p</i> value	<0.001	0.001	0.002	0.001	<0.001	0.001	0.013	<0.001
Effect stress*nutraceutical								
<i>p</i> value	0.056	0.012	0.002	<0.001	0.254	<0.001	0.015	0.008

Data expressed as Mean & SEM, n = 3.

Mean values in the same column with different superscripts differ significantly. Significance of mean value was observed at 5% probability level ($P < 0.05$); L-liver.

¹ Catalase activity is expressed as nanomoles/min/mg protein.

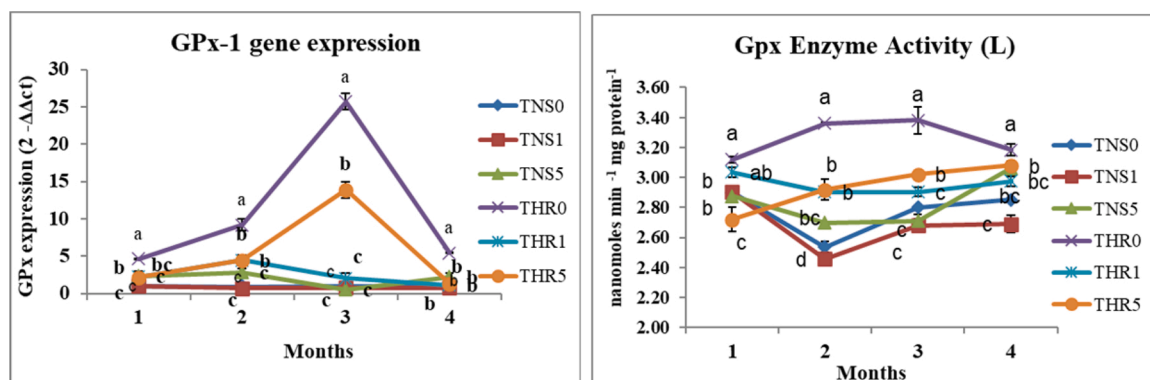


Fig. 4. Effect of stress and nutraceutical on hepatic GPx-1 gene expression and GPx enzyme activity in rohu fingerlings at the end of 1, 2, 3- and 4-months experimental period.

Data are expressed as mean $\pm$ SE (n=3) and the lines with different superscripts differ significantly ($p < 0.05$).

THR0(HSD, 20 nos 75 L⁻¹ water; RF, at 1 % of body weight; 0% nutraceutical containing diet) THR1 (HSD; RF; 0.1 % nutraceutical containing diet), THR5 (HSD, RF, 0.5 % nutraceutical containing diet), TNS0 (normal stocking density, NSD, 10 nos75L⁻¹ water; satiation feeding, SF, 3% of body weight; 0% nutraceutical containing diet), TNS1 (NSD; SF; 0.1 % nutraceutical containing diet) and TNS5 (NSD; SF; 0.5 % nutraceutical containing diet).

exposed groups. The effect of nutraceutical level revealed that at the end of 3 months trial both 0.1 and 0.5 % dietary nutraceutical and at the end of 4 months trial only 0.1 % dietary nutraceutical significantly reduced ($P < 0.05$) the hepatic GPx enzyme activity in comparison to without-nutraceutical fed group (Table 3).

The interaction between stress and dietary nutraceutical had a significant effect ($P < 0.05$) on hepatic GPx-1 gene expression in fish at the end of each trial period, whereas it had significant effect ($P < 0.05$) on GPx enzyme activity only at the end of 2-, 3- and 4-months trial. One-way ANOVA revealed that 0.5 % dietary nutraceutical significantly enhanced hepatic GPx-1 gene expression in normal fish (without exposure to stress) at the end of 1 and 2 months trial, whereas in comparison to without –nutraceutical fed group (THR0), stress exposed fish fed either 0.1 or 0.5 % nutraceutical exhibited significantly reduced ($P < 0.05$) hepatic GPx gene expression at the end of each trial period, where 0.5 % dietary nutraceutical resulted in highest reduction ($P < 0.05$) at the end of 1 and 2 months trial (Fig. 4). Feeding 0.5 % dietary nutraceutical significantly enhanced ($P < 0.05$) the GPx enzyme activity in normal fish at the end of 2 and 4 months trial, while 0.1 % dietary nutraceutical at the end of 1 month trial and both 0.1 and 0.5 % dietary nutraceutical at the end of 2, 3 and 4 months trial significantly reduced ($P < 0.05$) the hepatic GPx enzyme activity in stress exposed fish in comparison to without-nutraceutical fed group (THR0).

Table 3

Effect of stress (high stocking density & feed restriction), level of nutraceutical and the interaction between both on GPx gene and enzyme activity in rohu, *Labeo rohita* fingerlings.

Two-way ANOVA	GPx-1 gene expression (L) ($2^{-\Delta\Delta CT}$)				¹ GPx enzyme (L)			
Effect of stress	Month 1	Month 2	Month 3	Month 4	Month 1	Month 2	Month 3	Month 4
Without stress	1.46 ^b	1.51 ^b	0.76 ^b	1.33 ^b	2.89 ^b	2.56 ^b	2.73 ^b	2.93 ^b
With stress	3.01 ^a	6.02 ^a	13.86 ^a	2.65 ^a	3.03 ^a	2.87 ^a	3.09 ^a	3.08 ^a
SEM	0.09	0.27	1.42	0.12	0.03	0.02	0.03	0.02
p value	0.004	0.001	0.001	<0.001	0.003	0.001	0.002	<0.001
Effect of nutraceutical level								
0.0	2.83 ^a	5.09 ^a	13.34 ^a	3.23 ^a	3.01	2.76	3.08 ^a	3.11 ^a
0.1	1.63 ^c	2.58 ^b	1.38 ^c	0.96 ^c	2.97	2.68	2.79 ^b	2.83 ^b
0.5	2.27 ^b	3.62 ^{ab}	7.20 ^b	1.78 ^b	2.90	2.71	2.87 ^b	3.07 ^a
SEM	0.11	0.33	1.73	0.15	0.03	0.03	0.03	0.02
p value	<0.001	0.001	0.002	<0.001	0.085	0.123	<0.001	0.001
Effect of stress*nutraceutical								
p value	<0.001	0.001	0.003	<0.001	0.201	<0.001	0.009	0.005

Data expressed as Mean & SEM, n = 3.

Mean values in the same column with different superscripts differ significantly. Significance of mean value was observed at 5% probability level ($P < 0.05$).

¹ GPx (Glutathione peroxidase) activity expressed as nanomoles/min/mg protein; L-liver.

3.3.4. Hepatic glutathione-s-transferase (GST) activity

Stress caused a significant increase ($P < 0.05$) in Glutathione-s-transferase (GST) activity at the end of 1- and 4-months trial, while it resulted in a significant decrease in the GST activity at the end of 2- and 3-months trial (Table 4). Dietary nutraceutical significantly increased the GST activity level in fish only at 3rd month of experiment with a higher value in 0.5 % nutraceutical fed group. The significant interaction effect of stress and dietary nutraceutical on GST activity in fish was observed at 2nd, 3rd and 4th month of experiment. One-way ANOVA indicated that at 3rd month of experiment, the THRO group showed a lower GST activity, whereas at 4th month of experiment the fish of all the three stress- exposed groups showed a significantly higher activity of GST.

Table 4

Effect of stress (high stocking density & feed restriction) and level of nutraceutical on hepatic GST activity in rohu, *Labeo rohita* fingerlings.

One-way ANOVA	GST (Units/ mg Protein/ min)			
Treatments	Month 1	Month 2	Month 3	Month 4
TNS0	10.51 ± 1.47	4.70 ^a ± 0.17	7.16 ^b ± 0.06	4.93 ^c ± 0.23
TNS1	6.25 ± 0.68	4.71 ^a ± 0.16	9.81 ^a ± 0.60	10.49 ^b ± 1.31
TNS5	10.87 ± 0.82	5.34 ^a ± 0.27	10.0 ^a ± 1.20	13.71 ^b ± 0.81
THRO	11.85 ± 0.44	1.23 ^b ± 0.03	1.79 ^c ± 0.09	24.75 ^a ± 3.20
THR1	12.33 ± 1.17	2.75 ^b ± 0.63	8.67 ^a ± 0.20	16.88 ^a ± 3.79
THR5	13.11 ± 1.88	2.26 ^b ± 0.03	9.09 ^a ± 0.22	15.65 ^{ab} ± 1.2
P value	0.19	0.001	<0.001	0.001
Effect of stress (Two-way ANOVA)				
Without stress	9.21 ^b	4.91 ^a	8.99 ^a	9.71 ^b
With stress	12.43 ^a	2.08 ^b	6.52 ^b	19.09 ^a
SEM	0.68	0.17	0.33	1.26
p value	0.006	<0.001	0.001	0.003
Effect of nutraceutical level				
0.0	11.18	2.97	4.48 ^c	14.84
0.1	9.29	3.73	5.24 ^b	13.68
0.5	11.99	3.80	9.54 ^a	14.68
SEM	0.84	0.21	0.40	1.54
p value	0.105	0.193	<0.001	0.850
Effect of Stress*nutraceutical				
p value	0.148	0.011	<0.001	0.004

Data expressed as Mean ± SE, n=3 (one-way ANOVA) and Mean & SEM, n=3 (two-way ANOVA); Mean values in the same column with different superscripts differ significantly. Significance of mean value was observed at 5% probability level ($P < 0.05$).

3.4. Serum total antioxidant status (TAS)

Serum TAS was significantly higher ($P < 0.05$) in the stress exposed fish than the unstressed one at the end of each trial period (Table 5). But compared to without-nutraceutical fed group, feeding of 0.1 and 0.5 % nutraceutical at the end of 1- and 2-months trial and 0.1 % nutraceutical at the end of 4 months trial significantly increased ($P < 0.05$) the serum TAS value. However, dietary nutraceutical significantly reduced ($P < 0.05$) the serum TAS at the end of 3 months trial period. Serum TAS was significantly affected ($P < 0.05$) by the interaction between stress and dietary nutraceutical. The one-way ANOVA analysis revealed that among the unstressed fish, 0.5 % dietary nutraceutical fed group (TNS5) at the end of 1 and 4 months and both 0.1 and 5% dietary nutraceutical groups (TNS1 and TNS5) at the end of 3 months trial period exhibited significantly higher ($P < 0.05$) serum TAS. Significantly higher ($P < 0.05$) serum TAS value was recorded in stress exposed fish fed with dietary nutraceutical than their without-nutraceutical fed counterpart at the end of each trial period.

3.5. Hepatic reduced glutathione (GSH) level

Stress caused a significant increase ($P < 0.05$) in hepatic reduced glutathione (GSH) at the end of 1- and 2-months trial, while it resulted a significant decrease in the GSH level at the end of 3- and 4-months trial like hepatic GPx enzyme activity (Table 5). Feeding of nutraceutical at 0.1 % level significantly ($P < 0.05$) increased the GSH levels at the end of 2, 3- and 4-months trial. Interaction between stress and dietary nutraceutical had significant effect ($P < 0.05$) on hepatic GSH at the end of each trial period. One way ANOVA revealed that unstressed fish fed with 0.5 % nutraceutical exhibited significantly decreased GSH at the end of 2- and 3-months trial in comparison to TNS0 and TNS1 groups, however, at the end of 4 months trial, all the without stress exposed group showed a significantly higher ($P < 0.05$) GSH value. In stress exposed fish, compared to without-nutraceutical fed group, 0.1 % dietary nutraceutical significantly increased ($P < 0.05$) GSH level at the end of 2nd, 3rd and 4th months. However, feeding of 0.5 % nutraceutical significantly decreased the GSH level in stress exposed fish compared to 0.1 % nutraceutical counterpart at the end of 3- and 4-months trial.

4. Discussion

4.1. Growth

Crowding and/or diet restriction are a known stressor and have been noted to cause growth depression in fish (Sumpter et al., 1991; Tejpal et al., 2009; Das et al., 2015; Shamna et al., 2020). In the present study, crowding, and diet restriction was observed to cause a significant growth retardation in *L. rohita*, however, feeding of nutraceutical positively improve the performance of the fish after 60 days of feeding. Similarly, lower body weight gain was observed in hybrid tilapia subjected to 2- and 4-week feed restriction compared to the control (Wang et al., 2004). Nevertheless, long-term feeding of nutraceutical at 0.1 % mitigated this effect and improve the fish performance. Kumar et al. (2012) reported that the supplementation of methyl donors such as methionine, choline, and betaine enhanced growth in stress-exposed *L. rohita*. The higher dose of nutraceutical (0.5 %) was noticed to elicit growth reduction compared to the 0.1 % counterpart, and this could be due to higher level of some compounds such as selenium which at higher level was reported to cause toxicity in channel catfish (Gatlin and Wilson, 1984). Hence, the current study showed that 0.1 % of the fed nutraceutical is the optimal supplemental dose for mitigating multiple stressors like crowding and diet restriction in *L. rohita* fingerlings.

4.2. Hepatic enzymatic antioxidants

Most organisms adapt to oxidative stress by increasing the activity of enzymatic antioxidants (Hermes-Lima, 2004). The oxidative damage mediated by starvation and crowding stress can be reduced by the proper functioning of enzymatic antioxidant system of many vertebrates (Moody and Hassan, 1984; Valavanidis et al., 2006; Antonopoulou et al., 2013). Superoxide dismutase (SOD) is well-known to deactivate the reactive oxygen species through conversion to hydrogen peroxide, which is further detoxified either by catalase and/or glutathione peroxidase according to its concentrations (Yu, 1995). The data obtained in this study showed that both the SOD-1 gene expression and SOD enzyme activity were significantly higher in the liver of stress exposed *Labeo rohita* (crowding and feed restriction) than their without-stress counterpart at the end of 2, 3 and 4 months trial period, which indicates that these enzymes play a major role in oxidative stress mediated by crowding and feed restriction from 2nd months onwards in *L. rohita*. During feed restriction higher level of acetyl CoA is generated through the active beta-oxidation process for meeting the energy requirement of fish (Lanza, 1998). This process generates a large number of NADH and FADH₂ to be oxidized in the electron transport chain with the production of higher level of ROS in intra-cellular condition (Lanza, 1998). This condition induces the action of antioxidant enzymes especially in the liver. Similar trend was observed in GPx enzyme activity and gene expression as that of SOD. The catalase gene expression and enzyme activities were significantly higher in stress exposed without-nutraceutical fed group (THR0), which indicates that stress (crowding and feed restriction) had a significant effect in the activation of catalase gene and enzyme activity in *L. rohita* in all 4 months of experimental trial. Similar to our results, diet restriction resulted in a significantly higher activity of SOD, catalase and glutathione peroxidase in liver of *Sparus aurata* (Pascual et al., 2003). Likewise, 42 and 22 % increased activity of SOD and catalase, respectively, was observed due to starvation in *Dentex dentex* (Morales et al., 2004). According to Das et al. (2004), multiple stressors in *L. rohita* resulted in increased activity of SOD, CAT and GST. SOD, GPx, and CAT activities in the serum of three cichlid fish (*Oreochromis niloticus*, *Tilapia rendalli*, and *Geophagus brasiliensis*) taken from polluted water in different seasons were found to be higher

Table 5

Effect of stress (high stocking density & feed restriction), level of nutraceutical and the interaction between both on hepatic reduced glutathione levels and total antioxidant status in rohu, *Labeo rohita* fingerlings.

One-way ANOVA Treatments	Reduced glutathione, GSH (micromoles/ g tissue)				Serum total antioxidant status (TAS) (mM)			
	Month 1	Month2	Month3	Month4	Month1	Month2	Month3	Month4
TNS0	7.79 ^c ± 0.51	6.33 ^b ± 0.10	6.36 ^a ± 0.08	5.61 ^a ± 0.02	0.59 ^a ± 0.001	0.84 ^a ± 0.005	0.79 ^a ± 0.002	0.83 ^a ± 0.007
TNS1	7.02 ^c ± 0.41	6.29 ^b ± 0.11	6.01 ^a ± 0.21	5.89 ^a ± 0.09	0.58 ^a ± 0.004	0.88 ^a ± 0.006	0.44 ^b ± 0.003	0.81 ^a ± 0.003
TNS5	8.31 ^c ± 0.32	4.91 ^c ± 0.06	4.07 ^b ± 0.08	5.06 ^a ± 0.06	0.57 ^b ± 0.002	0.87 ^a ± 0.003	0.47 ^b ± 0.003	0.53 ^b ± 0.007
THR0	19.26 ^a ± 0.14	3.13 ^c ± 0.89	2.25 ^c ± 0.40	2.11 ^c ± 0.65	0.44 ^c ± 0.001	0.43 ^d ± 0.020	0.24 ^d ± 0.004	0.19 ^c ± 0.001
THR1	15.07 ^a ± 0.04	8.03 ^a ± 0.14	5.90 ^a ± 0.07	4.31 ^b ± 0.20	0.56 ^b ± 0.001	0.62 ^b ± 0.003	0.42 ^b ± 0.003	0.80 ^a ± 0.004
THR5	12.38 ^b ± 0.54	8.10 ^a ± 0.19	2.85 ^c ± 0.06	2.92 ^c ± 0.06	0.57 ^b ± 0.002	0.59 ^c ± 0.002	0.36 ^c ± 0.015	0.48 ^b ± 0.002
P value	<0.001	0.014	0.005	0.038	<0.001	0.003	0.005	<0.001
Effect of stress (Two-way ANOVA)								
Without stress	7.71 ^b	5.84 ^b	5.48 ^a	5.52 ^a	0.58 ^a	0.86 ^a	0.57 ^a	0.73 ^a
With stress	15.57 ^a	6.42 ^a	3.67 ^b	3.11 ^b	0.52 ^b	0.55 ^b	0.34 ^b	0.49 ^b
SEM	0.24	0.22	0.11	0.16	0.001	0.005	0.004	0.003
p value	0.001	<0.001	<0.001	0.001	<0.001	<0.001	0.001	0.002
Effect of nutraceutical level								
0.0	13.53 ^a	4.73 ^b	4.31 ^b	3.86 ^b	0.52 ^b	0.64 ^b	0.52 ^a	0.51 ^b
0.1	11.05 ^{bc}	7.16 ^a	5.96 ^a	5.10 ^a	0.57 ^a	0.75 ^a	0.43 ^b	0.81 ^a
0.5	10.35 ^c	6.50 ^{ab}	3.46 ^b	3.99 ^b	0.57 ^a	0.73 ^a	0.41 ^c	0.51 ^b
SEM	0.30	0.27	0.14	0.19	0.001	0.006	0.005	0.003
p value	0.001	<0.001	<0.001	<0.001	<0.001	0.002	<0.001	0.001
Effect of stress*nutraceutical								
p value	<0.001	<0.001	0.001	<0.001	<0.001	0.001	0.002	<0.001

Data expressed as Mean & SEM, n = 3 (two-way ANOVA); Significance of mean value was observed at 5% probability level (P < 0.05). Mean values in the same column with different superscripts differ significantly.

(Bonafé et al., 2008).

Reactive oxygen species produced in fish can be neutralized by enzymatic and non-enzymatic antioxidants (Seyle, 1950). For removal of ROS, non-enzymatic antioxidants like selenium, zinc etc. act in combination with enzymatic antioxidants while other non-enzymatic antioxidants like vitamin E, glutathione etc. play their role individually as well as in combination with enzymatic antioxidants (Martinez-Alvarez et al., 2005). In the present study, there was a significant reduction of SOD, catalase and GPx gene and enzyme activities in 0.1 % nutraceutical fed group at the end of each trial period of 1, 2, 3 and 4 months. On the other hand, the 0.5 % nutraceutical fed group showed a reduced activity of SOD at the end of each trial period but a higher catalase and GPx activities were observed in stress exposed fish at the end of 3rd and 4th months trial. The decreased enzymatic antioxidant activities in 0.1 % nutraceutical fed group is due to the effective scavenging of the reactive oxygen species by the extra supplemented vitamin E as suggested by McCoy and King (1980). Methionine act as an antioxidant as its sulphur can be oxidized to sulfoxide; furthermore, via repair of methionine sulfoxide through methionine sulfoxide reductase (Luo and Levine, 2009). Dietary supplemented extra methionine could have neutralized ROS resulting in the reduction of SOD, CAT and GPx activities in fish of 0.1 % nutraceutical fed group (Sears, 2013). It is well documented that the dietary supplementation of nutraceutical like vitamin E, selenium and methionine reduces the SOD and catalase activity in several fish species exposed to stress (Dandapat et al., 2000; Lin and Shiau, 2007; Ciji et al., 2012; Kumar et al., 2012). Suarez et al. (1999) reported that there was a decrease in catalase activity in liver tissues of rat subjected to stress and subsequent feeding of vitamin E supplemented diet. However, supplementation of higher level of selenium can cause stress with enhanced catalase and GPx activity as reported by Ighodaro and Akinloye (2018). Probably due to this reason 0.5 % nutraceutical supplementation (25 % extra Se than the requirement of the species) in the diet of stress exposed groups could increase activities of these antioxidant enzymes at the end of 3- and 4-months trial (Messaudi et al., 2009).

4.3. Reduced glutathione (GSH) and glutathione -s- transferase (GST) activity

The glutathione plays an important role in mitigating oxidative stress (Hidalgo et al., 2002) and can be considered as an important biomarker of stress (Caipang, 2012). GSH is a cellular protecting non-enzymatic agent, which reduce the reactive oxygen species by its sulfhydryl group (Zhang et al., 2004). In the present study, the glutathione levels were significantly higher in the stress exposed groups in 1st and 2nd month. However, GSH level was lower in 3rd and 4th month in stress exposed groups. The GSH level in the cell can be varied either by the synthesis of GSH or by the reduction of GSSG (oxidized glutathione) catalyzed by glutathione reductase to GSH (Wu et al., 2004). The reduction in GSH is mainly due to increased utilization of GSH or the depression of its regeneration (Upadhyay and Panda, 2010). Under normal physiological state the balance of GSH in the body is maintained through its cellular synthesis and it is interrupted due to its utilization during stress (Li et al., 2010). GSH is synthesized from cysteine, glycine and glutamate, but cysteine is the major limiting amino acid for its synthesis (Wu et al., 2004). However, cysteine synthesized from dietary methionine (via methionine transsulfuration) can support in vivo synthesis of GSH (Kaplowitz et al., 1985), which protect the cell from oxidative stress

(Finkelstein, 2000). Therefore, the increased level of GSH in stress exposed fish at the end of 2- and 3-months trial of the present study due to feeding of 0.1 % nutraceutical is the indication of its mitigating action against stress-induced ROS probably mediated through enhanced level of cysteine synthesized from 25 % extra (0.1 % nutraceutical) supplemental level of methionine in the diet. However, supplementation of 46.0 % extra methionine (0.5 % nutraceutical) in the diet than the requirement of the species did not give any extra benefit to fish in relation to stress mitigation.

Glutathione γ -transferase (GST) is a detoxification enzyme which work parallel to the glutathione. Though the slight stress exposure enhances its activity, while severe stress exposure suppresses its activity (Yildirim and Yaman, 2019). The present results in stress exposed groups showed a similar trend of reduced GSH level. From this result, it can be concluded that the activation of GST occurs when there is an exhaustion of GSH. This result is supported by Li et al. (2010) and Zhang et al. (2004), who also observed a similar trend of GST activity in fish due to effect of xenobiotic. In the present study, a higher GPx level in all 4-months with decreasing GSH level from 1, 2 and 3 months in THRO group and a lower GPx activity with higher GSH levels in 1, 2 and 3 months in THR1 (0.1 % nutraceutical fed and stress exposed) group indicate a relationship between GSH levels and GPx activity. The non-enzymatic antioxidants like vitamin E and glutathione are considered as first line of defence during oxidative stress (Martinez-Alvarez et al., 2005) and is widely accepted that the enzymatic antioxidant activities are influenced by glutathione (Halliwell and Gutteridge, 2000). Moreover, an increase in glutathione (GSH) levels, indicate a higher productivity of the glutathione peroxidase (GPx) through GSSG $\rightarrow$ GSH cycle and vice versa. Hence, it can be concluded that feeding of 0.1 % nutraceuticals positively influenced the GPx gene expression, GPx activity, GSH levels and GST activity.

4.4. Total antioxidant status

During oxidative stress in the body, the stress mitigation action is first performed by non-enzymatic antioxidants and then by enzymatic antioxidants (Martinez-Alvarez et al., 2005). This process reduces the level of antioxidants in the system. In the present study, significantly lowered total antioxidant status (TAS) in stress exposed fish at the end of each trial period of 1, 2, 3 and 4 months might be due to the same mechanism. But, dietary supplementation of 0.1 % nutraceutical could compensate with higher level of TAS in stress exposed fish at the end of 1, 2- and 4-months trial. Similar result was found at the end of 1- and 2-months trial due to feeding of 0.5 % nutraceutical but failed to do so in fish at the end of 3- and 4months trial, probably due to lower level of GSH as confirmed in our study. In agreement with our result, Blomhoff (2005) and Gutteridge and Halliwell (2000) reported that dietary supplementation of nutraceutical (like vitamin E, ascorbic acid etc) improved the total antioxidant and reduced the oxidative stress in rat exposed to restricted feeding.

5. Conclusion

Exposure of *Labeo rohita* fingerlings to crowding (high stocking density) and feed restriction for 1, 2, 3- and 4-months period increased expression of SOD-1, catalase and GPx-1 genes and activity of catalase and GPx enzymes. There was a change in expression of antioxidant enzymes gene and their activities in 3- and 4- months in stress exposed groups. However, the SOD enzyme activity was higher only in 2, 3, and 4 months. The improved total antioxidant status and reduced glutathione levels along with the activity of antioxidant enzymes revealed that though feeding 0.5 % nutraceutical conglomerate showed positive effect in some parameters, however, feeding of nutraceutical at 0.1 % (16.66, 2.5, 2.5 and 5% extra vitamin E, methionine, zinc and selenium, respectively) level supported the overall stress mitigation in *L. rohita* after 3 months of stress exposure (i.e. high stocking density and restricted feeding). Hence it can be concluded that 0.1 % nutraceutical can reduce oxidative stress due to crowding and feed restriction in aquafarms where fishes are exposed to stress for more than 3 months for the production of stunted carp fingerlings. This strategy will help to harvest the short duration crop in the seasonal pond or waterbodies.

Declaration of Competing Interest

The authors declare that they do not have any personal or financial interest with any one or any institution which can influence the present work.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.anifeedsci.2021.115045>.

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