

Additional feeding of vitamin–mineral-based nutraceutical to stress-exposed rohu, *Labeo rohita*, enhances the IGF-1 gene expression and growth

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Abstract

One thousand and eighty rohu fingerlings with an average size of 8.5 ± 0.5 g were randomly distributed in 6 distinct experimental groups in triplicates, where the first three groups were exposed to high stocking density (HSD; 20 nos/ 75 L water) with restricted feeding (RF), viz. THR0 (HSD, RF, 0% nutraceutical), THR1 (HSD, RF, 0.1% nutraceutical) and THR5 (HSD, RF, 0.5% nutraceutical), and second three groups were exposed to normal stocking density (NSD; 10 nos /75 L water) with satiation feeding (SF) and designated as TNS0 (NSD, SF, 0% nutraceutical), TNS1 (NSD, SF, 0.1% nutraceutical) and TNS5 (NSD, SF, 0.5% nutraceutical). The weight gain (WG) %, specific growth rate (SGR), protein efficiency ratio (PER) and IGF-1 expression were significantly lower, whereas feed conversion ratio (FCR), serum superoxide dismutase (SOD) and catalase activities were significantly higher in fish exposed to HSD with RF at monthly sampling. Feeding of 0.1% dietary nutraceutical resulted in a significantly higher WG%, SGR, PER and IGF-1 expression and lower FCR, SOD and catalase activities at the end of the third and fourth month of feeding in HSD with RF groups. The present study concluded that 3-month feeding of 0.1% nutraceutical could improve growth and reduce stress in fish.

KEYWORDS

body composition, feed restriction, high stocking density, IGF-1, stress, weight gain

1 | INTRODUCTION

The global aquaculture must produce an additional 47.5 million tonnes of fish to meet the expected consumption demand (FAO, 2016) by 2050. This higher production could only be possible from the aquaculture sector through either horizontal or vertical expansion. Considering the constraints of natural resources like land and water, the vertical expansion of aquaculture is the only option left for augmenting the production to meet the expected demand. This is possible through innovative strategies and intervention of advanced technologies to make the aquaculture production sustainable and eco-friendly with enhanced productivity. Vertical expansion of

aquaculture is mostly intended to increase in stocking density of the fish with the aim of enhanced productivity, which has the impact on growth, survival and yield variation for different species (Khatune-Jannat, Rahman, Bashar, Hasan, & Ahamed, 2012; M'balaka, Kassam, & Rusuwa, 2012; Zhu et al., 2005). Nevertheless, it may result in increased competition for food, aggression and reduced reproductive performance (Tave, 1986). Higher stocking density or overcrowding causes a decrease in the availability of sufficient food to all fish in the group leading to reduced feed intake, feed efficiency and undergrowth. This can result in changes in normal fish physiology and immune functions (Ellis, Hastings, & Munro, 1981). Under such conditions, body reserves will be channelized to satisfy energy

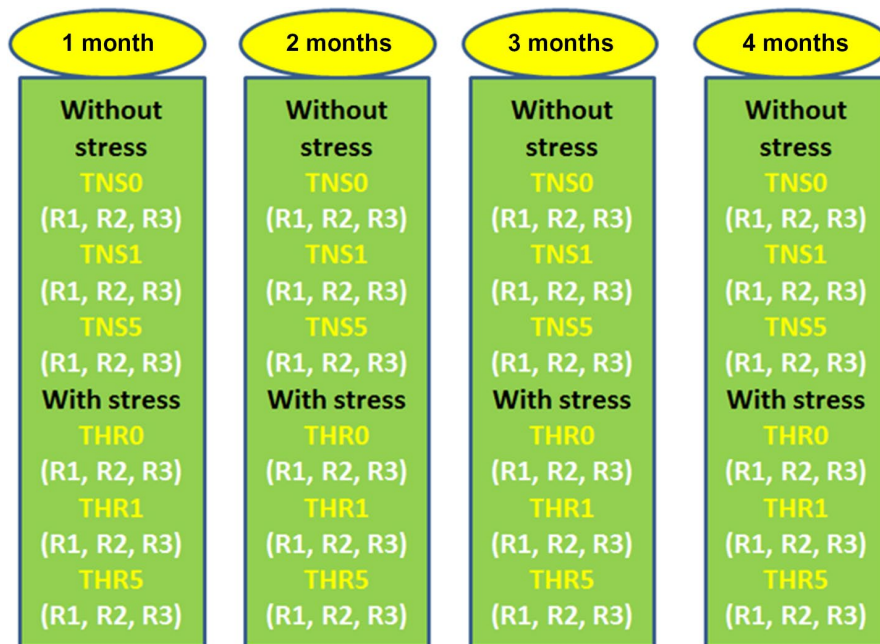


FIGURE 1 Experimental design showing various treatments of experiment. TNS0, normal stocking density, satiation feeding, 0% nutraceutical; TNS1, normal stocking density, satiation feeding, 0.1% nutraceutical; TNS5, normal stocking density, satiation feeding, 0.5% nutraceutical; THR0, high stocking density, restricted feeding, 0% nutraceutical; THR1, high stocking density, restricted feeding, 0.1% nutraceutical; and THR5, high stocking density, restricted feeding, 0.5% nutraceutical [Colour figure can be viewed at wileyonlinelibrary.com]

requirements resulting in growth retardation or stunted growth in fish (Trenzado, Morales, & de la Higuera, 2006).

Crowding along with feed restriction can cause further stress resulting in an increased reactive oxygen species (ROS) production at tissue levels, which can directly affect the normal functioning of the antioxidant and immune system of the fish, thus leading to an outbreak of disease and mortality. Therefore, crowding and feed deprivations are considered as chronic stressors in aquaculture (Wedemeyer, 1997). In order to produce a stunted population and ensure fish availability throughout the year, farmers expose fish to high stocking density and feed restriction. These stress exposures activate the general adaptive syndrome (GAS) in fish and result in the release of flight hormones. Increase of catecholamine levels in the body further alters various metabolic and physiological pathways and hence results in an increased energy demand. There are various biochemical responses in fish to mitigate the stress effect by adjusting the metabolic pathways to meet the increasing energy demand (Mommsen, Vijayan, & Moon, 1999). This may not only affect the growth performance but also affect the health of the fish. The increased production of catecholamines and further changes in the body ultimately weaken the immune system of the fish, and this can be the reason for disease outbreak often noticed in higher stocking density ponds (Tejpal et al., 2009).

Growth and health status of fish can be improved by dietary supplementation of micronutrients (Cho & Blaser, 2012). There are several studies which indicated that dietary supplementation of vitamins, minerals, amino acids, fatty acids, herbal extract and prebiotics could successfully mitigate the severity of stress effects in fish (Chung et al., 2005; Ciji, Sahu, Pal, Dasgupta, & Akhtar, 2012; Fawole, Sahu, Pal, & Kumar, 2016; Manush, Pal, Das, & Mukherjee, 2005). In sturgeon, the stress due to hypoxia could be mitigated by dietary supplementation of omega-3 fatty acids and vitamin E (Agradi et al., 1993), whereas, zinc (Zn), selenium (Se), manganese (Mn) and copper

are very well-studied antioxidants in fish (Maret, 2000; Powell, 2000). Decrease in the selenium content in body results in decreased activity of glutathione peroxidase in erythrocyte, plasma, liver and other tissues of fish (Bell, Cowey, Adron, & Shanks, 1985; Lall, 2002), and the deficiency of selenium results in growth reduction in carp, catfish and rainbow trout (Baidya & Murthy, 2015). However, the effect of deficiency or supplementation over and above the requirement level of these nutrients during stress in fish is not studied yet. Similarly, from the literature survey, it is well understood that there is a lack of study on the synergistic effect of the combination of various micronutrients over and above their optimum requirement on growth and stress mitigation in fish. Some studies like the feeding of organic acids and yeast together to tackle stress are found to be effective (Jha, Pal, Sahu, Kumar, & Mukherjee, 2007). Hence, in the present experiment, we studied the synergistic effect of zinc, selenium, vitamin E and methionine-containing dietary nutraceutical on growth, serum superoxide dismutase (SOD) and catalase enzymes activity, and IGF-1 expression of *Labeo rohita*, a highly cultured fish in the South and South-East Asia, subjected to multiple stressors.

2 | MATERIALS AND METHODS

2.1 | Experimental animals

The fingerlings of *Labeo rohita* (Hamilton, 1822) (8–9 g) were procured from Prem fish farm, Ankaleshwar, Gujarat, India. Polythene bags with oxygenated water were used to transport the fish to the wet laboratory of ICAR-Central Institute of Fisheries Education (ICAR-CIFE) and carefully transferred to a circular tank (1,000 L). The fish were left undisturbed the whole night. The stock was acclimatized under aerated conditions for a period of 12 days with a feed containing 30% crude protein.

TABLE 1 Ingredients and proximate composition of the experimental diet

Ingredient (g/kg)	Control (C)	0.1% nutraceutical	0.5% nutraceutical
Casein (vit.free) ^a	300	300	300
Gelatin ^a	90	90	90
Dextrin ^a	100	100	100
Starch soluble ^a	300	300	300
Cellulose powder ^a	108.9	107.9	103.9
Vegetable oil ^b	30	30	30
Cod liver oil ^b	30	30	30
Carboxymethyl cellulose ^a	20	20	20
Vit-min premix ^c	20	20	20
Butylated hydroxyl toluene ^a	0.1	0.1	0.1
Choline chloride ^a	1	1	1
Nutraceutical ^d	0	1	5
Proximate composition (g/kg on dry matter basis)			
Moisture	84.90	71.50	71.60
Dry matter	915.1	928.5	928.4
Crude protein	354.5	357.8	359.8
Ether Extract	61.20	61.30	61.10
Total Ash	52.00	48.10	47.4
Crude fibre	106.3	102.4	106.8
Nitrogen-free extract	425.9	430.3	423.7
Calculated Digestible energy ^e (MJ/kg)	15.37	15.50	15.41

^aHimedia Laboratories, India.^bProcured from local market.^ccomposition (quantity 2.5/kg): vitamin A, 55,00,000 IU; vitamin D3, 11,00,000 IU; vitamin B2, 2,000 mg; vitamin E, 6,750 mg; vitamin K, 1,000 mg; vitamin B6, 1,000 mg; vitamin B12, 6 mcg; calcium pantothenate, 2,500 mg; nicotinamide, 10 g; choline chloride, 150 g; Mn, 27,000 mg; I, 1,000 mg; Fe, 7,500 mg; Zn, 5,000 mg; Cu, 2,000 mg; Co, 450; L-lysine, 10 g; DL-methionine, 10 g; selenium, 125mg; and vitamin C, 2,500 mg.^dComposition of nutraceuticals (quantity per kg): methionine, 360 mg; vitamin E, 21g; zinc, 1,000 mg; and selenium, 50 mg; (patent number—201,821,027,496). 0.1% nutraceutical contained 17.00, 25, 2.5 and 5% extra supplementation of vitamin E, methionine, zinc and selenium, respectively, whereas 0.5% dietary supplementation contained 80.0, 46.0, 12.5 and 25% extra supplementation of vitamin E, methionine, zinc and selenium respectively.^eCalculated energy = $[16.74 \times \text{CP (g/kg)} + 37.7 \times \text{EE (g/kg)} + 16.74 \times \text{NFE (g/kg)}] / 1,000$ (Halver, 2002).

2.2 | Experimental setup

One thousand and eighty fingerlings with an average size of 8.5 ± 0.5 g were randomly distributed in 6 distinct experimental groups in triplicates, where first three groups were exposed to high stocking density (HSD; 20 nos/ 75 L water) with restricted feeding (RF; 1% feed restriction) and second three groups were exposed to normal stocking density (NSD; 10 nos /75 L water) with satiation feeding (SF) (Figure 1). Nutraceutical conglomerates were fed to both the groups at 0, 0.1 or 0.5%. Hence, the six experimental groups were designated as THRO (HSD, RF, 0% nutraceutical), THR1 (HSD, RF, 0.1% nutraceutical), THR5 (HSD, RF, 0.5% nutraceutical), TNSO (NSD, SF, 0% nutraceutical), TNS1 (NSD, SF, 0.1% nutraceutical) and

TNS5 (NSD, SF, 0.5% nutraceutical). The nutraceutical mix contained methionine, 360 mg/kg; vitamin E, 21g/kg; zinc, 1,000 mg/kg; and selenium, 50 mg/kg. The experiment was conducted for 120 days. No ethical approval was required for conducting this study.

2.3 | Experimental diets

Three purified diets (Table 1), viz a control diet, 0.1% and 0.5% nutraceutical-based diets, were used in the present experiment. All the ingredients were thoroughly mixed, and the dough was prepared after adding the required quantity of water. The dough was subjected to steam cooking for 15 min in a pressure cooker. Vitamin–mineral

mix and nutraceutical were added to the cooled dough. The feed was prepared using a hand pelletizer, and the air-dried pellets were stored at 4°C until use.

2.4 | Proximate composition of fish and feed

The proximate composition of the whole fish and feed (Table 1) was done following the standard protocol of AOAC (1995). Moisture (%) was estimated after drying the samples at $100 \pm 2^\circ\text{C}$ in a hot air oven until attaining a constant weight. Crude protein was analysed in a semi-automatic nitrogen analyzer (Kjeltec-2200, Foss Tecator, Hoganas, Sweden). Crude lipid was estimated in an ether extraction unit (Socsplus-SCS-08-AS, Pelican Equipment, Chennai, India). The crude fibre of the feed was determined in FibroTRON (Tulin Equipments, India) through acid (1.25% HCl) and alkali (1.25% NaOH) digestion of sample followed by ashing of fibre. The total ash estimation was performed through ignition of the sample in a muffle furnace (550°C for 4 hr). Nitrogen-free extract (NFE) and total carbohydrate (TC) were calculated based on the following formulae.

$$\text{NFE (\%)} = 100 - [\text{CP (\%)} + \text{EE (\%)} + \text{TA (\%)} + \text{CF (\%)}]$$

$$\text{TC (\%)} = 100 - [\text{CP (\%)} + \text{EE (\%)} + \text{TA (\%)}]$$

2.5 | Digestible energy value

The digestible energy (DE) was calculated using the formula described by Halver (1976) as follows.

$$\text{Digestible energy (Kcal/100g)} = \text{CP (\%)} \times 4 + \text{EE (\%)} \times 9 + \text{NFE (\%)} \times 4$$

2.6 | Growth parameters

Body weight of the fish was determined at 15-day interval. Fishes were starved overnight before taking the weight. The growth parameters were calculated using the following formulas:

$$\begin{aligned} \text{Weight gain (\%)} &= (\text{Final live weight} - \text{Initial live weight}) / \text{Initial live weight} \times 100; \\ \text{SGR} &= (\ln \text{ final live weight} - \ln \text{ initial live weight}) / \text{Number of experimental days} \times 100; \\ \text{FCR} &= \text{Total dry feed intake} / \text{Body weight gain (wet weight)}; \\ \text{PER} &= \text{Body weight gain (wet weight)} / \text{Protein intake (dry weight)}. \end{aligned}$$

2.7 | Hepatosomatic index and intestinal somatic index

At the end of each month of feeding trial, three fish were taken from each experimental tank and anaesthetized using clove oil (50 mg/L). Immediately, liver and intestine tissues were dissected out and pooled together and weight was taken for calculation of Hepatosomatic index (HSI) and intestinal somatic index (ISI) as follows:

$$\text{HSI (\%)} = (\text{Wet weight of liver (g)}) / (\text{Wet weight of fish (g)}) \times 100$$

$$\text{ISI (\%)} = (\text{Wet weight of intestine (g)}) / (\text{Wet weight of fish (g)}) \times 100$$

2.8 | Sample collection for gene expression

Liver tissues were used for the gene expression study, and the tissues were collected aseptically with utmost care to avoid any contamination. The samples were collected in cryovials containing 1.5 ml RNA later solution (Ambion Biosciences) and stored according to the manufacture's instruction.

2.9 | Total RNA extraction

The 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 HellmaTrayCell (Eppendorf, Hamburg, Germany) at 260 nm. The integrity of RNA was checked in 1.2% agarose gel.

2.10 | Synthesis of the first-strand cDNA

After the synthesis, the RNA was checked for qualitative and quantitative integrity, and further, the RNA samples were subjected to DNase treatment (Fermentas, USA). The first-strand cDNA synthesis was done with reverse transcriptase kit (Fermentas, USA) using 2 µg RNA according to the manufacture's protocol. The reaction mix was subjected to the given temperature profile: 70°C for 5 min, then 25°C for 5 min, 42°C for 60 min and 70°C for 10 min in an S1000 PCR machine (Bio Rad, Hercules, CA, USA).

2.11 | mRNA Expression of IGF-1 in liver tissue by real-time PCR

The quantification of IGF-1 mRNA in the liver tissue of *Labeo rohita* was done in Light Cycler® 480 detection system (Roche, Switzerland) with β -actin as the reference gene (Acc No.—EU184877). The total reaction volume used in the study was 10 µl containing 1 µl (5 pmol) of each gene-specific primer (IGF-1) (Acc No.—KX455870), 5 µl of 2X Maxima™ SYBR Green qPCR Master Mix (Thermo Scientific, USA) and 1 µl of cDNA. Real times PCR was performed after standardizing the primer with different picomol (2.5 pm, 5 pm, 7.5 pm and 10 pm) of RNA and have used SYBR Green qPCR Master Mix without ROX (Fermentas, USA). After analysing the melting curve after every cycle, the comparative CT method was used to determine the relative expression of IGF-1 mRNA. The values were normalized by $2^{-\Delta\Delta\text{CT}}$ method (Livak

TABLE 2 Effects of stress (high stocking density and feed restriction), level of nutraceutical and interaction between stress and nutraceutical on weight gain % and specific growth rate of *Labeo rohita* fingerlings

One-way ANOVA		Weight gain (WG, %)				Specific growth rate			
Treatments*		1st Month	2nd Month	3rd Month	4th Month	1st Month	2nd Month	3rd Month	4th Month
TNS0		51.21 ^a ± 0.93	76.05 ^a ± 4.47	135.01 ^b ± 2.86	156.30 ^b ± 2.86	1.54 ^a ± 0.014	1.53 ^a ± 0.012	1.20 ^a ± 0.014	1.20 ^a ± 0.006
TNS1		51.66 ^a ± 0.29	76.84 ^a ± 0.31	147.16 ^a ± 1.28	175.35 ^a ± 1.28	1.56 ^a ± 0.010	1.57 ^a ± 0.004	1.18 ^a ± 0.010	1.17 ^a ± 0.001
TNS5		50.23 ^a ± 1.28	75.80 ^a ± 2.23	120.95 ^b ± 1.43	150.00 ^b ± 1.43	1.58 ^a ± 0.032	1.51 ^a ± 0.029	1.06 ^b ± 0.003	1.08 ^b ± 0.004
THR0		1.89 ^c ± 0.34	6.60 ^c ± 1.79	12.95 ^d ± 0.33	14.54 ^d ± 0.33	0.18 ^e ± 0.006	0.13 ^c ± 0.012	0.14 ^c ± 0.006	0.11 ^d ± 0.004
THR1		5.44 ^b ± 0.09	12.08 ^b ± 1.51	14.97 ^c ± 0.83	28.13 ^c ± 0.83	0.53 ^d ± 0.014	0.11 ^c ± 0.023	0.12 ^c ± 0.019	0.13 ^c ± 0.017
THR5		7.75 ^b ± 1.42	8.48 ^{bc} ± 0.80	13.82 ^{cd} ± 1.72	21.90 ^c ± 1.72	0.74 ^c ± 0.065	0.15 ^c ± 0.012	0.14 ^c ± 0.065	0.20 ^c ± 0.056
p value		.005	.004	<.001	<.001	<.001	.012	.001	.028
Effect of stress (Two-way ANOVA)									
Without stress		51.04 ^a	76.23 ^a	134.33 ^a	160.55 ^a	1.56 ^a	1.50 ^a	1.14 ^a	1.15 ^a
With stress		5.02 ^b	9.05 ^b	13.91 ^b	18.19 ^b	0.49 ^b	0.14 ^b	0.13 ^b	0.14 ^b
SEM		0.40	0.55	0.85	0.81	0.02	0.004	0.03	0.02
p value		.001	<.001	<.001	.001	<.001	<.001	.007	.001
Effect of nutraceutical level									
0.0		26.55 ^b	41.32 ^b	73.98 ^b	85.42 ^b	0.47 ^a	0.58	0.69 ^b	0.51 ^c
0.1		28.55 ^a	42.46 ^{ab}	81.07 ^a	101.74 ^a	0.48 ^a	0.59	1.37 ^a	1.41 ^a
0.5		28.99 ^a	44.14 ^a	67.39 ^b	85.95 ^b	0.39 ^b	0.57	1.16 ^{ab}	1.02 ^b
SEM		0.49	0.67	1.05	0.99	0.03	0.03	0.17	0.24
p value		.003	.016	.001	.006	.003	.078	.003	<.001
Effect of stress × nutraceutical									
p value		.003	.019	<.001	.014	<.001	.04	.002	.002

Note: Data expressed as mean ± SE, n = 6 (one-way ANOVA) and mean & SEM (two-way ANOVA); mean values in the same column with different superscripts differ significantly. Significance of mean value was observed at 5% probability level ($p < .05$)

*TNS0, normal stocking density, satiation feeding, 0% nutraceutical; TNS1, normal stocking density, satiation feeding, 0.1% nutraceutical; TNS5, normal stocking density, satiation feeding, 0.5% nutraceutical; THR0, high stocking density, restricted feeding, 0% nutraceutical; THR1, high stocking density, restricted feeding, 0.1% nutraceutical; and THR5, high stocking density, restricted feeding, 0.5% nutraceutical.

& Schmittgen, 2001) with the reference gene. The calculations were done using the formulae below:

Δct = target gene CT value – reference gene CT value

$\Delta\Delta ct$ value = Δct value of treatment group – Δct value of control group

2.12 | Serum Superoxide dismutase and catalase

Serum was collected as per the standard procedure, and SOD and catalase (CAT) activities were measured from serum using Cayman's SOD assay kit and catalase assay kit respectively (Cayman chemical, Ann Arbor, USA). The activity of SOD enzyme was calculated based on the regression equation obtained from the standard graph, and the calculation was done according to the following formula:

SOD activity (U/ml) = ((Sample OD – Y intercept) $\times$ 0.23 ml $\times$ sample dilution) / (Slope $\times$ 0.01 ml) $\times$ 100

The catalase activity in serum was analysed based on the peroxidation function of catalase. Reading was taken at 540 nm, and values were calculated from the following formula and expressed in nmol min⁻¹ ml⁻¹ (1 unit = amount of enzyme that causes the formation of 1nm of formaldehyde/min at 25°C).

CAT activity (U/ml) = (μ M of sample/20 min) $\times$ sample dilution

2.13 | Statistical analysis

All the data were statistically analysed after checking the normality and homogeneity of the variance using one-way and two-way ANOVA in SPSS software version 22. The one-way results were mentioned in the form of mean $\pm$ SE, while two-way data expressed as mean $\pm$ SEM. The significance level considered in the present study was at 5% level ($p < .05$).

3 | RESULTS

3.1 | Weight gain percentage (WG %)

The WG % was significantly lower ($p < .05$) in stress-exposed groups than their non-exposed counterpart during 1, 2, 3 and 4 months of the experimental period (Table 2). Feeding of 0.1% nutraceutical improved the WG% during each of the four-month feeding trial. In comparison with non-supplemented (control) and 0.5% nutraceutical-supplemented groups, feeding of 0.1% nutraceutical significantly ($p < .05$) increased the WG % in fish at the end of 3rd and 4th month of the experiment. There was a significant interaction effect of stress and nutraceutical on weight gain of the fish.

According to one-way ANOVA analysis, dietary nutraceutical did not cause any significant variation of WG% in non-stress-exposed fish at the end of 1 and 2 months of the experiment. However, among the stress-exposed groups, the fish fed with 0.1% nutraceutical

exhibited significantly higher ($p < .05$) WG% than its non-supplemented counterpart at the end of each experimental period of 1, 2, 3 and 4 months. On the other hand, the stress-exposed fish of 0.5% nutraceutical-fed group (THR5) showed similar WG% to THR1 within the same period (1–4 months) of the experiment. Overall, 0.1% dietary nutraceutical exhibit significantly higher ($p < .05$) WG% in fish than the non-supplemented group at the end of 1, 2, 3 and 4 months of experiment.

3.2 | Specific Growth Rate

Stress exposure significantly ($p < .05$) reduced the specific growth rate (SGR) of fish at the end 1, 2, 3 and 4 months of experimental period. Dietary nutraceutical had a significant effect on SGR in 1st, 3rd and 4th month of the experiment with a significantly higher value recorded in 0.1% nutraceutical-fed groups (Table 2). There was a significant interaction effect of stressors and nutraceutical on SGR of the fish. One-way ANOVA revealed that SGR was significantly lower in THR0 group in 1st and 4th month, while both 0.1 and 0.5% nutraceutical-fed groups showed similar values in 2nd, 3rd and 4th month of stress exposure. Among the non-stress-exposed groups, 0.5% nutraceutical-fed group (TNS5) showed a significantly lower SGR in 3rd and 4th month compared to TNS0 and TNS1 groups.

3.3 | Feed conversion ratio

Exposure to stress significantly increased ($p < .05$) the feed conversion ratio (FCR) of fish at the end of 1, 2, 3 and 4 months of feeding (Table 3). Dietary nutraceutical did not cause any significant change in FCR of control group fish (non-stressed), but stress-exposed fish fed with 0.1% dietary nutraceutical exhibited significantly lower FCR than the stress-exposed fish of non-supplemented and 0.5% nutraceutical-supplemented groups at the of each experimental period of 1, 2, 3 and 4 months. On the other hand, though the non-stress-exposed fish of 0.5% dietary nutraceutical group (TNS5) showed significantly lower ($p < .05$) FCR at the end 1, 2, 3 and 4 months of experiment, the stress-exposed fish of same supplemented group (THR5) showed significantly higher FCR than its non-supplemented counterpart (THR0) at the end of 3rd month of the experiment. Overall, dietary nutraceutical did not produce any significant change in FCR in fish at the end of 3 and 4 months of feeding but a significant interaction effect between stress and nutraceutical was seen in each of the trial period of 1, 2, 3 and 4 months.

3.4 | Protein efficiency ratio

Stress-exposed fish exhibited significantly lower ($p < .05$) protein efficiency ratio (PER) than their non-exposed counterpart in all the four months of the experiment. Dietary nutraceutical had a

TABLE 3 Effects of stress (high stocking density and feed restriction), level of nutraceuticals and interaction between stress and nutraceutical on protein efficiency ratio and feed conversion ratio of *Labeo rohita* fingerlings

One-way ANOVA	Protein Efficiency Ratio (PER)				Feed Conversion Ratio (FCR)			
Treatments*	1st Month	2nd Month	3rd Month	4th Month	1st Month	2nd Month	3rd Month	4th Month
TNS0	1.11 ^a ± 0.03	1.36 ^a ± 0.02	1.37 ^b ± 0.02	1.47 ^a ± 0.01	1.89 ^d ± 0.04	1.98 ^d ± 0.03	2.11 ^d ± 0.03	2.60 ^d ± 0.03
TNS1	1.13 ^a ± 0.02	1.38 ^a ± 0.05	1.52 ^a ± 0.05	1.48 ^a ± 0.03	1.86 ^d ± 0.03	1.96 ^d ± 0.01	1.91 ^d ± 0.05	2.56 ^d ± 0.01
TNS5	1.10 ^a ± 0.01	1.39 ^a ± 0.01	1.32 ^b ± 0.01	1.46 ^a ± 0.01	1.84 ^d ± 0.01	1.98 ^d ± 0.01	2.19 ^d ± 0.03	2.64 ^d ± 0.02
THR0	0.16 ^d ± 0.03	0.42 ^d ± 0.05	0.51 ^c ± 0.05	0.42 ^c ± 0.02	6.12 ^a ± 0.59	7.08 ^a ± 0.95	5.64 ^b ± 0.16	6.93 ^a ± 0.29
THR1	0.20 ^c ± 0.01	0.51 ^d ± 0.07	0.43 ^c ± 0.07	0.52 ^b ± 0.07	3.91 ^c ± 0.10	3.74 ^c ± 0.71	4.49 ^c ± 0.45	4.77 ^c ± 0.89
THR5	0.25 ^b ± 0.04	0.78 ^c ± 0.05	0.53 ^c ± 0.05	0.61 ^b ± 0.02	4.51 ^b ± 0.21	6.08 ^b ± 0.22	6.82 ^a ± 0.25	5.80 ^b ± 0.19
<i>p</i> value	<.001	.018	<.001	.007	.027	.011	.021	.042
Effect of stress (Two-way ANOVA)								
Without stress	1.12 ^a	1.37 ^a	1.41 ^a	1.47 ^a	1.86 ^b	1.97 ^b	2.07 ^b	2.60 ^b
With stress	0.16 ^b	0.57 ^b	0.49 ^b	0.52 ^b	4.84 ^a	4.97 ^a	5.12 ^a	5.63 ^a
SEM	0.01	0.03	0.01	0.02	0.17	0.34	0.13	0.22
<i>p</i> value	<.001	.002	.001	<.001	<.001	.001	<.001	.001
Effect of nutraceutical level								
0.0	0.59 ^b	0.89 ^b	0.94 ^b	0.94 ^b	4.01 ^a	4.03 ^a	3.58	4.52
0.1	0.65 ^a	0.94 ^a	0.98 ^a	1.00 ^a	2.89 ^c	2.85 ^{ab}	3.20	3.67
0.5	0.67 ^a	1.09 ^a	0.93 ^b	1.04 ^a	3.18 ^b	3.53 ^b	4.01	4.17
SEM	0.01	0.04	0.02	0.03	0.20	0.42	0.16	0.28
<i>p</i> value	<.001	.007	.046	.040	<.001	.041	.065	.055
Effect of stress × nutraceutical								
<i>p</i> value	<.001	.017	<.001	.035	<.001	.004	.007	.047

Note: 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* < .05)

*NS0, normal stocking density, satiation feeding, 0% nutraceutical; TNS1, normal stocking density, satiation feeding, 0.1% nutraceutical; TNS5, normal stocking density, satiation feeding, 0.5% nutraceutical; THR0, high stocking density, restricted feeding, 0% nutraceutical; THR1, high stocking density, restricted feeding, 0.1% nutraceutical; and THR5, high stocking density, restricted feeding, 0.5% nutraceutical.

TABLE 4 Effect of stress (high stocking density and feed restriction), level of nutraceuticals and interaction between stress and nutraceuticals on IGF-1 expression in *Labeo rohita* fingerlings

Two-way ANOVA	Insulin-like growth factor-1 (IGF-1) gene expression (liver) (2-ΔΔct)			
	1st Month	2nd Month	3rd Month	4th Month
Effect of stress				
Without stress	0.80 ^a	1.09 ^a	1.35 ^a	3.69 ^a
With stress	0.09 ^b	0.19 ^b	0.46 ^b	0.49 ^b
SEM	0.04	0.03	0.14	0.20
<i>p</i> value	<.001	<.001	.007	.001
Effect of nutraceutical level				
0.0	0.47 ^a	0.58	0.69 ^b	0.51 ^c
0.1	0.48 ^a	0.59	1.37 ^a	1.41 ^a
0.5	0.39 ^b	0.57	1.16 ^{ab}	1.02 ^b
SEM	0.03	0.03	0.17	0.24
<i>p</i> value	.003	.078	.003	<.001
Effect stress × nutraceuticals				
<i>p</i> -value	.001	<.001	<.001	.003

Note: 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* < .05).

significant effect on PER in 1, 2, 3 and 4 months of the experiment. Though, both the levels (0.5 and 0.1%) of nutraceutical showed higher PER, but 0.1% nutraceutical-fed group showed significantly higher PER values throughout the experimental period. An interaction effect of stress and nutraceutical on PER was observed, and one-way ANOVA revealed that PER was significantly lower in THRO group in 4th month of stress exposure. Overall, both 0.1 and 0.5% dietary nutraceutical caused significantly higher PER in fish than their non-supplemented counterparts at the end of 1, 2 and 4 months of experiment, whereas, at the end of 3 months of the experiment, the fish fed with 0.1% nutraceutical resulted in significantly higher ($p < .05$) PER than the non-supplemented and 0.5% nutraceutical-supplemented group.

3.5 | Insulin-like growth factor-1 (IGF-1) expression

Stress exposure significantly reduced the IGF-1 expression in all the four months of experiment, and feeding of 0.1% nutraceutical increased the IGF-1 expression in 3rd and 4th month (Table 4). There was a significant interaction effect of stress and level of nutraceutical on IGF-1 expression. One-way ANOVA showed that feeding of both 0.1 and 0.5% nutraceutical significantly increased ($p < .05$) the IGF-1 expression in stress-exposed fish in comparison with control group at the end of 1, 2, 3 and 4 months (Figure 2 and supplementary data). Among the stress-unexposed groups, 0.1% nutraceutical-fed group exhibited significantly higher ($p < .05$) IGF-1 expression than the non-supplemented and 0.5% nutraceutical-fed groups at the end of 3 and 4 months of experiment, but at the end of 1 and 2 months of experimental period, no significant variation was noticed among the groups (i.e. TNS groups). Overall, 0.1% dietary nutraceutical resulted

in a significantly higher ($p < .05$) IGF-1 expression in fish fed nutraceutical than its non-supplemented counterpart at the end of 3 and 4 months of experiment (Table 4).

3.6 | Hepatosomatic and intestinal somatic index

Stress significantly reduced ($p < .05$) the HSI in fish at the end of 4 months and ISI in fish at the end of 3 and 4 months of experiment in comparison with non-stress-exposed fish, but at the end of other experimental period there was no significant variation in HSI and ISI between the stress exposed and non-exposed groups. Dietary nutraceutical did not cause any significant variation in HSI and ISI in non-stress-exposed fish in all the four months. Among the stress-exposed groups, 0.1% nutraceutical group exhibited significantly higher HSI than the fish of non-supplemented and 0.5% nutraceutical-supplemented groups at the end of 3 and 4 months of experiment, but at the end of 1 and 2 months of experiment there was no significant variation of HSI among the different groups of stress-exposed fish. Moreover, different groups of stress-exposed fish did not show any significant variation of ISI among themselves at the end of each experimental period of 1, 2, 3 and 4 months. Overall, the dietary nutraceutical did not result in any significant changes in HSI and ISI value compared to the non-supplemented group at the end of each experimental period. There was no significant effect of nutraceutical and stress on HSI and ISI values ($p > .05$) (Table 5).

3.7 | Whole-body proximate composition

The stress-exposed groups showed a significantly higher ($p < .05$) moisture level compared to the non-exposed groups at the end of 1, 2, 3 and 4 month of experiment. Compared to non-supplemented groups with or without stress exposure, the fish of nutraceutical-supplemented groups did not show any significant variation of moisture content at the end of 1, 2, 3 and 4 months of experiment. Furthermore, level of dietary nutraceutical also did not cause any significant variation of moisture in any group irrespective of duration of feeding. Stress exposure significantly reduced the CP content in fish at the end of 3 and 4 months of experiment. Dietary nutraceutical did not produce any significant variation in the CP content in fish of non-stress-exposed groups, whereas 0.1 and 0.5% nutraceutical-fed groups at the end of 3 month and 0.5% dietary nutraceutical at the end of 4 month of experiment resulted in significantly higher ($p < .05$) body protein content than their non-supplemented counterpart. The whole-body ether extract (EE) content also varied significantly among the different treatments ($p < .05$). Compared to non-stress-exposed fish, stress significantly reduced ($p < .05$) the whole-body EE content in fish at the end of 1, 2, 3 and 4 months of the experiment with an inverse relationship between EE and moisture (Tables 6 and 7) content. Though dietary nutraceutical did not produce any significant variation in EE content of whole body in stress-exposed fish at the end of 1 and 2 months of the experiment,

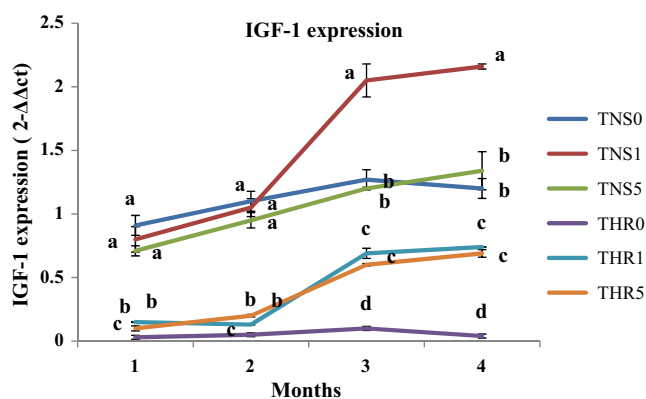


FIGURE 2 IGF-1 expression shown by *Labeo rohita* fingerlings of different treatments during different culture period with or without stress exposure; TNS0, normal stocking density, satiation feeding, 0% nutraceutical; TNS1, normal stocking density, satiation feeding, 0.1% nutraceutical; TNS5, normal stocking density, satiation feeding, 0.5% nutraceutical; THRO, high stocking density, restricted feeding, 0% nutraceutical; THR1, high stocking density, restricted feeding, 0.1% nutraceutical; and THR5, high stocking density, restricted feeding, 0.5% nutraceutical [Colour figure can be viewed at wileyonlinelibrary.com]

TABLE 5 Effects of stress (high stocking density and feed restriction), level of nutraceuticals and interaction between stress and nutraceuticals on hepatosomatic index (HSI) and intestinal somatic index (ISI) of *Labeo rohita* fingerlings

One-way ANOVA	Hepatosomatic index (HSI)				Intestinal somatic Index (ISI)			
Treatments*	1st Month	2nd Month	3rd Month	4th Month	1st Month	2nd Month	3rd Month	4th Month
TNS0	1.51 ± 0.04	1.80 ± 0.23	1.58 ^a ± 0.24	1.93 ^a ± 0.15	3.49 ± 0.21	3.50 ± 0.32	3.56 ± 0.09	3.65 ± 0.21
TNS1	1.92 ± 0.13	1.33 ± 0.10	1.69 ^a ± 0.11	1.92 ^a ± 0.03	3.42 ± 0.19	3.33 ± 0.13	3.52 ± 0.49	3.49 ± 0.13
TNS5	1.56 ± 0.12	1.44 ± 0.14	1.69 ^a ± 0.08	1.89 ^a ± 0.20	2.85 ± 0.16	2.92 ± 0.27	3.15 ± 0.17	3.07 ± 0.05
THR0	1.48 ± 0.11	1.34 ± 0.13	1.07 ^c ± 0.30	1.05 ^c ± 0.19	2.81 ± 0.10	2.96 ± 0.33	2.51 ± 0.08	2.29 ± 0.25
THR1	1.24 ± 0.16	1.35 ± 0.16	1.47 ^a ± 0.11	1.21 ^b ± 0.02	2.62 ± 0.29	2.88 ± 0.08	2.33 ± 0.18	2.20 ± 0.10
THR5	1.27 ± 0.61	1.18 ± 0.39	1.20 ^{bc} ± 0.34	0.83 ^c ± 0.12	2.69 ± 0.16	3.29 ± 0.38	2.59 ± 0.19	2.63 ± 0.63
P value	.085	.126	.020	.001	.446	.073	.058	.052
Effect of stress (Two-way ANOVA)								
Without stress	1.66	1.53	2.48	1.92 ^a	3.25	3.25	3.41 ^a	3.40 ^a
With stress	1.33	1.29	1.24	1.03 ^b	2.71	3.05	2.48 ^b	2.37 ^b
SEM	0.09	0.10	0.11	0.11	0.16	0.15	0.13	0.16
p value	.230	.125	.617	<.001	.310	.348	<.001	.001
Effect of nutraceutical level								
0.0	1.50	1.57	1.47	1.49	3.15	3.23	3.03	2.97
0.1	1.58	1.34	1.19	1.57	3.02	3.11	2.93	2.84
0.5	1.41	1.31	1.16	1.36	2.77	3.11	2.87	2.85
SEM	0.11	0.13	0.13	0.12	0.19	0.18	0.16	0.19
p value	.586	.313	.215	.629	.405	.863	.762	.871
Effect of stress × nutraceutical level								
p value	.153	.421	.913	.546	.485	.186	.357	.198

Note: 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 ($p < .05$).

*TNS0, normal stocking density, satiation feeding, 0% nutraceutical; TNS1, normal stocking density, satiation feeding, 0.1% nutraceutical; TNS5, normal stocking density, satiation feeding, 0.5% nutraceutical; THR0, high stocking density, restricted feeding, 0% nutraceutical; THR1, high stocking density, restricted feeding, 0.1% nutraceutical; and THR5, high stocking density, restricted feeding, 0.5% nutraceutical.

the stress-exposed fish of 0.1% nutraceutical-supplemented group exhibited significantly higher ($p < .05$) whole-body EE content than their non-supplemented and 0.5% nutraceutical-supplemented counterparts at the end of 3 and 4 months of experiment.

The stress-exposed fish exhibited significantly higher ($p < .05$) whole-body total ash (TA) content than its non-stress counterpart at the end of 4 months of experiment (Table 8). Whole-body total carbohydrate (TC) level of fish was significantly lowered ($p < .05$) due to the effect of stress for all of 1, 2, 3 and 4 months (Table 8). The stress-exposed fish of 0.1% nutraceutical group exhibited significantly higher ($p < .05$) whole-body TC level than its non-supplemented and 0.5% nutraceutical-supplemented counterparts at the end of 3 and 4 months of experiment. The stress-unexposed fish of 0.5% nutraceutical (TNS5)-fed group showed significantly lower ($p < .05$) whole-body TC content than its non-supplemented and 0.1% nutraceutical-supplemented counterparts at the end of 4 months of experiment. Overall, the fish of 0.1% nutraceutical group exhibited significantly higher ($p < .05$) whole-body TC content than its non-supplemented and 0.5% nutraceutical-supplemented counterparts at the end of 4 months of experiment.

3.8 | Serum superoxide dismutase and catalase

The serum SOD activity significantly increased in the fish of stress-exposed groups during each of all the four months of experiment (Table 9). But, the effect of nutraceutical on this enzyme in fish was seen only after 2nd month of the experiment with significantly lower ($p < .05$) value in the fish fed 0.1% nutraceutical-supplemented diet at 2nd, 3rd and 4th month. One-way ANOVA indicated that fish of THR0 and THR5 groups showed significantly higher serum SOD activity during all the four months of the experiment. The highest activity of the enzyme was noted in 2nd month in stress-exposed groups (Figure 3 and supplementary data). TNS5 groups showed a significantly higher activity ($p < .05$) compared to TNS0 and TNS1 in 1st and 2nd month of the experimental period.

Serum catalase activity in fish was significantly increased in stress-exposed groups in 2, 3 and 4 months, and feeding of nutraceuticals at 0.1% reduced the activity at 2nd, 3rd and 4th month of experiment (Table 9). One-way ANOVA analysis revealed that catalase activity was significantly higher in THR0 group in all the four months, whereas non-stress-exposed groups and 0.1% nutraceutical-fed

TABLE 6 Effects of stress (high stocking density and feed restriction), level of dietary nutraceutical and interaction between stress and nutraceutical on moisture content of *Labeo rohita* fingerlings

One-way ANOVA		Dry matter (DM, %)				Moisture (%)			
Treatments*		1st Month	2nd Month	3rd Month	4th Month	1st Month	2nd Month	3rd Month	4th Month
TNS0		26.63 ^a ± 0.71	27.41 ^a ± 0.72	28.51 ^a ± 0.55	27.08 ^a ± 0.19	73.37 ^b ± 0.70	72.59 ^b ± 0.72	71.49 ^b ± 0.55	72.92 ^b ± 0.19
TNS1		26.68 ^a ± 1.85	27.20 ^a ± 0.11	27.30 ^a ± 1.08	26.59 ^a ± 0.18	73.32 ^b ± 0.85	72.80 ^b ± 0.11	72.70 ^b ± 0.09	73.41 ^b ± 0.18
TNS5		25.33 ^a ± 0.27	29.33 ^a ± 1.86	28.03 ^a ± 0.23	26.19 ^a ± 0.69	74.67 ^b ± 0.27	70.67 ^b ± 2.17	71.97 ^b ± 0.23	73.81 ^b ± 0.69
THR0		20.67 ^b ± 0.54	23.18 ^b ± 0.91	20.71 ^b ± 0.38	19.70 ^b ± 0.47	79.33 ^a ± 0.54	76.82 ^a ± 0.91	79.29 ^a ± 0.38	80.30 ^a ± 0.47
THR1		20.14 ^b ± 0.62	21.98 ^b ± 1.55	21.26 ^b ± 0.20	21.07 ^b ± 0.19	79.86 ^a ± 0.62	78.02 ^a ± 1.45	78.74 ^a ± 0.20	78.93 ^a ± 0.19
THR5		24.52 ^{ab} ± 0.65	23.29 ^b ± 1.33	21.76 ^b ± 1.04	20.18 ^b ± 1.33	75.48 ^{ab} ± 0.65	76.71 ^a ± 1.34	78.24 ^a ± 0.05	79.82 ^a ± 0.33
p value		.042	.022	.030	.004	.042	.022	.030	.004
Effect of stress (Two-way ANOVA)									
Without stress		26.22 ^a	27.98 ^a	27.95 ^a	26.62 ^a	73.78 ^b	72.02 ^b	72.05 ^b	73.38 ^b
With stress		21.78 ^b	22.82 ^b	21.24 ^b	20.31 ^b	78.23 ^a	77.18 ^a	78.76 ^a	79.69 ^a
SEM		0.36	0.81	0.18	0.23	0.36	0.81	0.18	0.23
p value		.000	.001	.001	.001	.000	.001	.001	.000
Effect of nutraceutical levels									
0.0		23.65	25.29	24.61	23.39	76.35	74.71	75.39	76.61
0.1		23.41	24.59	24.28	23.83	76.59	75.41	75.72	76.17
0.5		24.92	26.31	24.90	23.18	75.08	73.69	75.10	76.82
SEM		0.45	0.99	0.22	0.28	0.45	0.99	0.22	0.28
p value		.071	.488	.170	.282	.071	.488	.170	.282
Effect of stress × nutraceutical levels									
p value		.001	.815	.027	.088	.001	.815	.027	.088

Note: 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 ($p < .05$).

*TNS0, normal stocking density, satiation feeding, 0% nutraceutical; TNS1, normal stocking density, satiation feeding, 0.1% nutraceutical; TNS5, normal stocking density, satiation feeding, 0.5% nutraceutical; THR0, high stocking density, restricted feeding, 0% nutraceutical; THR1, high stocking density, restricted feeding, 0.1% nutraceutical; and THR5, high stocking density, restricted feeding, 0.5% nutraceutical.

TABLE 7 Effects of stress (high stocking density and feed restriction), level of nutraceutical and interaction between stress and nutraceutical on crude protein and total lipid content of *Labeo rohita* fingerlings

One-way ANOVA		Crude Protein (CP, %)				Total Lipid (TL, %)			
Treatments*		1st Month	2nd Month	3rd Month	4th Month	1st Month	2nd Month	3rd Month	4th Month
TNS0		16.45 ± 0.33	16.46 ± 0.34	17.15 ^a ± 0.54	16.87 ^a ± 0.40	4.26 ^a ± 0.15	4.20 ^a ± 0.03	4.14 ^a ± 0.23	4.58 ^a ± 0.09
TNS1		15.53 ± 0.51	16.27 ± 0.58	17.34 ^a ± 0.35	17.06 ^a ± 0.32	4.09 ^a ± 0.18	4.07 ^a ± 0.25	3.22 ^a ± 0.28	4.40 ^a ± 0.15
TNS5		16.46 ± 0.40	17.07 ± 0.43	17.46 ^a ± 0.16	17.68 ^a ± 0.08	4.20 ^a ± 0.04	4.11 ^a ± 0.08	3.95 ^a ± 0.10	4.22 ^a ± 0.06
THR0		14.92 ± 0.50	14.40 ± 0.21	13.27 ^c ± 0.27	11.33 ^c ± 0.51	2.44 ^b ± 0.05	2.02 ^b ± 0.14	1.43 ^c ± 0.11	1.10 ^c ± 0.14
THR1		14.67 ± 0.58	14.53 ± 0.53	14.39 ^b ± 0.14	11.91 ^{bc} ± 0.48	2.71 ^b ± 0.06	2.48 ^b ± 0.06	1.56 ^b ± 0.26	1.38 ^b ± 0.07
THR5		15.82 ± 0.09	14.81 ± 0.52	15.15 ^b ± 0.53	12.83 ^b ± 0.28	3.06 ^{ab} ± 0.04	2.49 ^b ± 0.08	1.37 ^c ± 0.08	1.06 ^c ± 0.13
p value		.087	.076	.011	.041	.013	.009	.032	.010
Effect of stress (Two-way ANOVA)									
Without stress		16.14	16.49	17.31 ^a	17.20 ^a	4.18 ^a	4.13 ^a	3.77 ^a	4.40 ^a
With stress		15.14	14.27	14.26 ^b	12.02 ^b	2.74 ^b	2.33 ^b	1.46 ^b	1.18 ^b
SEM		0.25	0.28	0.21	0.22	0.06	0.07	0.11	0.07
p value		.100	.051	<.001	.002	.001	<.001	<.001	.001
Effect of nutraceutical level									
0.0		15.69	15.74	15.21 ^b	14.10 ^b	3.35 ^b	3.11	2.78	2.84
0.1		15.56	15.28	15.86 ^a	14.48 ^a	3.48 ^a	3.28	2.39	2.89
0.5		15.68	15.12	16.30 ^a	15.25 ^a	3.63 ^a	3.30	2.66	2.64
SEM		0.31	0.35	0.26	0.27	0.07	0.09	0.14	0.08
p value		.948	.457	.042	.029	.014	.276	.158	.112
Effect of stress × nutraceutical									
p value		.067	.615	.049	.044	.066	.063	.037	.154

Note: 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 < .05).

*TNS0, normal stocking density, satiation feeding, 0% nutraceutical; TNS1, normal stocking density, satiation feeding, 0.1% nutraceutical; TNS5, normal stocking density, satiation feeding, 0.5% nutraceutical; THR0, high stocking density, restricted feeding, 0% nutraceutical; THR1, high stocking density, restricted feeding, 0.1% nutraceutical; and THR5, high stocking density, restricted feeding, 0.5% nutraceutical.

TABLE 8 Effects of stress (high stocking density and feed restriction), level of nutraceutical and interaction between stress and nutraceutical on total ash and total carbohydrate level of *Labeo rohita* fingerlings

One-way ANOVA	Total ash (TA, %)				Total carbohydrate (TC, %)			
Treatments*	1st Month	2nd Month	3rd Month	4th Month	1st Month	2nd Month	3rd Month	4th Month
TNS0	2.15 ± 0.02	2.09 ± 0.01	2.10 ± 0.06	2.26 ± 0.05	4.84 ± 0.77	3.90 ± 0.01	5.01 ^a ± 0.24	3.71 ^a ± 0.43
TNS1	2.17 ± 0.06	2.29 ± 0.10	2.07 ± 0.01	2.17 ± 0.03	4.80 ± 0.44	4.82 ± 0.1	5.62 ^a ± 0.11	3.72 ^a ± 0.38
TNS5	2.26 ± 0.08	2.25 ± 0.70	2.13 ± 0.05	2.12 ± 0.07	4.56 ± 1.59	5.67 ± 0.67	4.17 ^a ± 0.27	3.23 ^b ± 0.65
THR0	2.42 ± 0.08	2.19 ± 0.05	2.14 ± 0.02	2.07 ± 0.02	3.91 ± 1.09	4.09 ± 0.46	2.73 ^b ± 0.22	1.89 ^c ± 0.79
THR1	2.38 ± 0.09	2.28 ± 0.09	2.18 ± 0.01	2.11 ± 0.01	4.84 ± 0.77	2.68 ± 0.09	3.26 ^a ± 0.13	3.37 ^b ± 0.42
THR5	2.21 ± 0.02	2.24 ± 0.09	2.07 ± 0.02	2.09 ± 0.03	3.24 ± 1.39	3.07 ± 0.09	2.81 ^b ± 0.22	1.86 ^c ± 0.29
p value	.532	.442	.411	.312	.069	.066	.011	.001
Effect of stress (Two-way ANOVA)								
Without stress	2.34	2.23	2.13	2.09 ^b	4.73 ^a	5.13 ^a	4.93 ^a	3.55 ^a
With stress	2.19	2.21	2.10	2.18 ^a	3.99 ^b	3.28 ^b	2.93 ^b	2.37 ^b
SEM	0.04	0.04	0.02	0.02	0.30	0.60	0.24	0.12
p value	.230	.700	.189	.016	.001	.049	.002	<.001
Effect of nutraceutical level								
0.0	2.18	2.16	2.09	2.18	3.87	4.00	3.87	2.30 ^b
0.1	2.30	2.24	2.10	2.12	4.44	3.75	4.02	3.30 ^a
0.5	2.32	2.27	2.15	2.11	3.49	4.87	3.84	2.55 ^b
SEM	0.05	0.05	0.02	0.03	0.37	0.74	0.29	0.15
p value	.107	.372	.082	.241	.542	.542	.437	.002
Effect of stress × nutraceutical								
p value	.350	.239	.217	.195	.583	.224	.726	<.001

Note: 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 ($p < .05$).

*TNS0, normal stocking density, satiation feeding, 0% nutraceutical; TNS1, normal stocking density, satiation feeding, 0.1% nutraceutical; TNS5, normal stocking density, satiation feeding, 0.5% nutraceutical; THR0, high stocking density, restricted feeding, 0% nutraceutical; THR1, high stocking density, restricted feeding, 0.1% nutraceutical; and THR5, high stocking density, restricted feeding, 0.5% nutraceutical.

stress-exposed groups showed a significantly lower ($p < .05$) activity in 1st, 2nd and 3rd month of experiment (Figure 4). Among the stress-exposed groups, THR1 showed a significantly lower catalase activity in 4th month of the experiment.

4 | DISCUSSION

4.1 | Growth, nutrient utilization and IGF-1 expression

Fishes reared under high stocking density or feed restriction alone or in combination for a definite period, resulting in stunting or growth suppression (Das, Mishra, Mishra, & Jayasankar, 2015; Wedemeyer, 1997). Similarly, in the present study, the stressors like high stocking density and feed restriction significantly reduced the WG%, SGR, IGF-1 expression and PER with increased FCR in fish at the end of different experimental period. According to Tejpal et al. (2009), the growth and nutrient utilization were significantly reduced in mrigal, when subjected to crowding stress, while feed restriction or starvation resulted in cessation

of growth and higher FCR in rainbow trout (Sumpter, Le Bail, Pickering, Pottinger, & Carragher, 1991). The poor growth, lower IGF-1 expression and high FCR in the stress-exposed groups fed diet without nutraceutical supplementation (THR0) compared to stress-unexposed fish (TNS0) might be due to severity of stress mediated through overcrowding and feed restriction (Multiple stressor). According to Very, Kittilson, Norbeck, and Sheridan (2005), growth is mediated by hepatic derived IGF-1 gene expression and the interaction between growth hormone and its receptors. Changes during stress, in growth hormone–insulin-like growth factor-1 (GH-IGF-1) axis, resulted in variation in feed intake and growth, which in turn causes reduced growth and lipolysis (MacKenzie, VanPutte, & Leiner, 1998). In the present study, overstocking mediated stress reduced the growth of rohu probably by the same mechanism, which was aggravated further by feed restriction. In gilthead seabream, similar condition was observed when fish fed diet with low ration size and energy content (Company, Calduch-Giner, Kaushik, & Perez-Sanchez, 1999). Salmons raised under high stocking density and feed restriction was reported to have lower IGF-1 mRNA and proteins levels in circulation (Reinecke et al., 2005; Wong, Zhou, Jiang, & Ko, 2006)

TABLE 9 Effect of stress (high stocking density and feed restriction), level of nutraceutical and the interaction between both on serum SOD and catalase enzyme activity in *Labeo rohita* fingerlings

Two-way ANOVA	Serum SOD				Serum Catalase			
	1st Month	2nd Month	3rd Month	4th Month	1st Month	2nd Month	3rd Month	4th Month
Effect of stress								
Without stress	7.56 ^b	9.03 ^b	9.51 ^b	10.76 ^b	1.53	1.64 ^b	2.28 ^b	1.63 ^b
With stress	10.81 ^a	22.36 ^a	14.08 ^a	12.67 ^a	1.67	1.85 ^a	2.98 ^a	4.06 ^a
SEM	0.753	0.919	1.676	0.416	0.003	0.002	0.026	0.031
P value	.010	.002	.012	.02	.400	.002	.001	.013
Effect of nutraceutical level								
0.0	9.35	17.80 ^a	13.13 ^a	12.67 ^a	1.60	1.89 ^a	2.79 ^a	3.19 ^a
0.1	8.66	12.17 ^b	10.07 ^b	11.52 ^{ab}	1.61	1.69 ^b	2.50 ^b	2.58 ^b
0.5	9.54	17.12 ^a	12.18 ^{ab}	10.95 ^b	1.59	1.66 ^b	2.66 ^a	2.77 ^{ab}
SEM	0.92	1.126	2.053	0.51	0.004	0.002	0.031	0.038
p value	.780	.023	.019	.015	.201	.001	.023	.031
Effect stress × nutraceuticals								
p value	.476	.530	.095	.131	.001	.002	.001	.827

Note: 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 ($p < .05$). Serum SOD and catalase are expressed as nanomoles/min/mg protein.

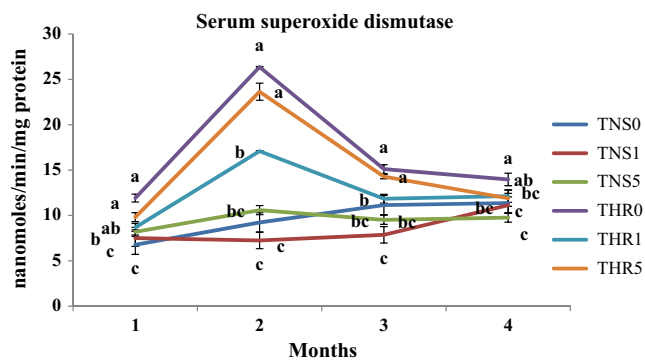


FIGURE 3 Serum SOD activity shown by *Labeo rohita* fingerlings of different treatments during different culture period with or without stress exposure; TNS0, normal stocking density, satiation feeding, 0% nutraceutical; TNS1, normal stocking density, satiation feeding, 0.1% nutraceutical; TNS5, normal stocking density, satiation feeding, 0.5% nutraceutical; THR0, high stocking density, restricted feeding, 0% nutraceutical; THR1, high stocking density, restricted feeding, 0.1% nutraceutical; and THR5, high stocking density, restricted feeding, 0.5% nutraceutical [Colour figure can be viewed at wileyonlinelibrary.com]

and the liver IGF-1 gene expression reduced during fasting of fish (Fox, Riley, Hirano, & Grau, 2006). This reduction might be owing to the result of dampening the IGF-1 response to GH probably due to increased levels of plasma growth hormone and reduced growth hormone receptor expression (Saera-Vila, Caldach-Giner, & Pe'rezSa'Nchez, 2005). Accordingly, in our study, the feed restriction and overcrowding resulted in a growth suppression (stunting) and lower IGF-1 expression in rohu fingerlings at the end of each experimental period of 1, 2, 3 and 4 months. In agreement with

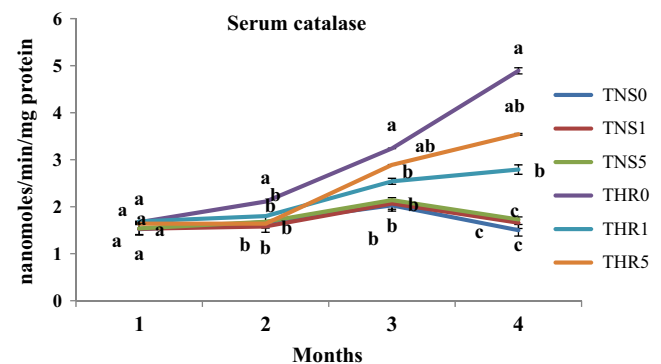


FIGURE 4 Serum catalase enzyme activity shown by *Labeo rohita* fingerlings of different treatments during different culture period with or without stress exposure; TNS0, normal stocking density, satiation feeding, 0% nutraceutical; TNS1, normal stocking density, satiation feeding, 0.1% nutraceutical; TNS5, normal stocking density, satiation feeding, 0.5% nutraceutical; THR0, high stocking density, restricted feeding, 0% nutraceutical; THR1, high stocking density, restricted feeding, 0.1% nutraceutical; and THR5, high stocking density, restricted feeding, 0.5% nutraceutical [Colour figure can be viewed at wileyonlinelibrary.com]

our findings, Wang, Yang, & Cai (2004) reported that hybrid tilapia when subjected to 2- and 4-week feed restriction showed significantly lower body weight gain than the control fish. Similarly, Fox et al. (2010) observed significantly lower levels of IGF-1 in plasma of tilapia subjected to three-week feed restriction.

Higher weight gain % and IGF-1 expression in stress-unexposed fish of 0.1% nutraceutical-supplemented group at the end of 3 and 4 months of experiment indicated growth-promoting effect mediated through long-term feeding of nutraceutical containing zinc

(2.5%), which probably played major role in protein synthesis and cell division (Frassinetti, Bronzetti, Caltavuturo, Cini, & Della Croce, 2006); selenium (5%) which played a major role in thyroid functions (Kohle, Jakob, Contempe, & Dumont, 2005) and antioxidant action; vitamin E, which could reduce the metabolic energy requirement (Randall et al., 1992); and methionine as methyl donor for methylation of different biomolecules for optimum performance (Kumar et al., 2012). Similarly, increased weight gain and feed efficiency were observed in rohu (Sau, Paul, Mohanta, & Mohanty, 2004), seabream (Tocher et al., 2002) and mrigal (Paul, Sarkar, & Mohanty, 2004) due to long-term feeding of vitamin E supplemented diet. Additionally, long-term feeding trial confirmed the growth-promoting effect of dietary zinc in common carp (Ogino & Yang, 1979) through the mechanism of zinc-gene interaction and protein synthesis as suggested by Chesters (1991). Moreover, Shan et al. (2017) had reported that dietary supplementation of encapsulated methionine improved the overall growth and IGF-1 expression in Jian carp.

Furthermore, the growth depressing effects (lower WG% and IGF-1 gene expression) of stress (overcrowding and feed restriction) exposed rohu fingerlings were mitigated by feeding 0.1% nutraceutical (17.0, 25, 2.5 and 5% extra supplementation of vitamin E, methionine, zinc and selenium respectively) at the end of 1, 2, 3 and 4 months of experiment, and this could probably be due to the combined actions of vitamin E, methionine, zinc and selenium present in the nutraceutical premix. In agreement with our finding, Kumar et al. (2012) demonstrated that the supplementation of methyl donors such as methionine, choline, lecithin and betaine improved growth in stress-exposed rohu. In the present study, growth was significantly higher in stress-exposed fish of 0.1% nutraceutical-fed group than their non-supplemented counterpart at the end of each experimental period, whereas stress-exposed fish of 0.5% nutraceutical group could exhibit significantly higher growth than their non-supplemented counterparts only at the end of 1st and 4th month of experiment. This finding indicated better stress mitigation and growth-promoting effects of 0.1% dietary nutraceutical, which might contribute the optimum dose of nutraceutical to support growth. Higher doses of some nutraceutical may induce stress rather than its mitigation action. For example, higher selenium levels such as 13 and 15 mg/kg dry feed resulted in selenium toxicity in rainbow trout and catfish respectively (Gatlin & Wilson, 1984; Hilton, Hodson, & Slinger, 1980).

4.2 | Hepatosomatic index and intestinal somatic index

In the present study, the significantly reduced HSI value recorded in rohu exposed to overstocking and feed restriction stress at the end of 4 months of experiment might be due to the mobilization of stored glycogen and lipid from the liver for energy production during stress exposure. In agreement with our finding, Hanna (1962) reported that glycogen could be mobilized from various tissues such as liver, spleen and muscles of stomach, thus leading to decreased weight of these organs in *Clarias lazera* at the end of 2 and 7 months

of feed restriction. In this study, the significantly lower intestinal somatic index (ISI) observed in stress-exposed rohu fingerlings at the end of 3 and 4 months of experiment might be due to less functional intestine as a result of insufficient availability of nutrients for digestion and absorption, hence leading to reduced growth. Similarly, reduction in ISI was observed in rohu subjected to diet restriction (Yengkokpam et al., 2014), feeding with phorbol ester containing feed (Shamna et al., 2017) and overcrowding (Wang et al., 2019). The stress-exposed rohu of 0.1% nutraceutical group exhibited significantly higher HSI than the fish of non-supplemented and 0.5% nutraceutical-supplemented groups. This finding again confirmed that 0.1% nutraceutical appears to be effective dose for mitigating stress effect caused due to overstocking and restricted feeding in rohu.

4.3 | Whole-body proximate composition

In the present study, stress (overstocking and feed restriction) significantly increased the whole-body moisture content whereas the whole-body CP, EE and TC contents were found to decrease and this effect could be due to hydration of tissue and mobilization of lipid, glycogenolysis and breakdown of body **protein for fulfilling the stress-induced enhanced energy need**. Inverse relationship between whole-body moisture and EE levels of rohu was maintained at the end of each experimental period of 1, 2, 3 and 4 months. In accordance with the result, Weatherley and Gill (1987) and Quinton and Blake (1990) also reported that starvation or feed restriction leads to an increase in moisture content and a decrease in lipid content in fish tissues. Moreover, Yengkokpam et al. (2014) found the considerable variation of the body lipids, proteins and moisture levels in rohu with differential mobilization of both protein and lipid due to food deprivation. In agreement with the current finding, starvation and restricted feeding resulted in proportionate variation of lipid level in *Anguilla anguilla* (Larson & Lewander, 1973), rainbow trout (Quinton & Blake, 1990) and *Sparus macrocephalus* (Zhang, Wu, Cai, & Zhu, 2007).

Stress exposure (overcrowding and feed restriction) significantly reduced the whole-body EE and TC level in rohu at the end of 1, 2, 3 and 4 months of experiment, whereas significant reduction of CP content in fish was recorded at the end of 3 and 4 months of experiment. This indicated that fish utilize lipid and carbohydrate, whereas body protein break down and mobilization took place later for fulfilling the stress-induced enhanced energy needs. Many authors have also reported that the lipid mobilizes first and acts as immediate energy source during starvation in plaice (Jobling, 1980) and rainbow trout (Reinitz, 1983). Fish have the capacity of converting amino acid to glucose (Bever & Dunn, 1981) through gluconeogenesis during stress to fulfil the additional energy requirement (Kumar et al., 2012). Stress significantly reduces the TA content in rohu only at the end of 4 months of experiment indicating that long-term stress exposure only could affect the mineral contents in fish as supported by the observation of Srijila, Rani, Babu, and Tiwari (2014). Similarly, there was no significant difference in ash content in juvenile tilapia starved for 3 weeks (Hsieh & Shiau, 2000).

Dietary supplementation of nutraceutical containing methionine, vitamin E, Zn and Se did not produce any significant effect on whole-body proximate composition of non-exposed rohu. Similarly, diets containing graded levels of vitamin E did not show any significant variation in the proximate composition of *Labeo rohita* (Ciji et al., 2012), Atlantic salmon (Hamre, Berge, & Lie, 1998), seabass (Pirini, Gatta, Testi, Trigari, & Monetti, 2000), rainbow trout (Mustafa, 2004) and beluga (Amlashi, Falahatkar, Sattari, & Gilani, 2011). Moreover, Yang, Liu, Tian, Liang, and Lin (2010) reported that dietary supplementation of methionine at 0.432% (10.25% extra level over the requirement) lowered the body lipid composition in grass carp. On the contrary, it has been reported that under normal condition, excess consumption of amino acids undergoes catabolism rather than taking part in body protein synthesis leading to enhanced tissue fat deposition (Chatzifotis, Takeuchi, & Seikai, 1996). Though whole-body moisture and TA contents of stress-exposed fish were not significantly affected by dietary nutraceutical, dietary supplementation of 0.1% nutraceutical containing 17.0, 25.0, 2.5 and 5.0% extra levels of vitamin E, methionine, zinc and selenium, respectively, over their requirement significantly increased whole-body EE and TC content in stress-exposed rohu in comparison with the non-supplemented and 0.5% dietary nutraceutical (containing 80.0, 46.0, 12.5 and 25.0% extra levels of vitamin E, methionine, zinc and selenium, respectively, over requirement) groups at the end of 3 and 4 months of experiment. However, both levels of nutraceutical and only 0.5% dietary nutraceutical significantly increased the whole-body CP content in stress-exposed rohu in 3 and 4 months of experiment respectively. But the whole-body CP content did not vary significantly between the stress-exposed fish of 0.1 and 0.5% nutraceutical groups at the end of 4 months of experiment. This finding indicated that long-term feeding 0.1% nutraceutical is the more appropriate dose over 0.5% for mitigating overcrowding and restricted feeding induced stress in rohu fingerlings. In accordance with the results, rohu fed with selenium supplemented diet for 8 weeks did not show any significant difference in body moisture and dry matter content, while the lipid level was significantly enhanced in selenium fed groups in comparison with the control (Baidya & Murthy, 2015).

4.4 | Serum Superoxide dismutase and catalase

There are reports saying that there will be parallel induction of SOD and CAT during stress (Dimitrova, Tishinova, & Velcheva, 1994). In the present study, the serum SOD activity significantly increased in the fish of stress-exposed groups during each of all the four months of experiment while CAT activity was significantly increased only after 2nd month. The non-significant activity of catalase in 1st month stress exposure might be due to the action of glutathione peroxidase instead of CAT in the first month. Stressors such as feed restriction and crowding can result in generation of ROS in the system which is converted to hydrogen peroxide (H_2O_2) by superoxide dismutase (SOD) and further neutralized by the catalase (CAT) or glutathione peroxidase (GPx) (Robinson et al., 1997; Tejpal et al.,

2009). The higher activity of SOD enzyme noted on 2nd month in stress-exposed groups indicates that there can be higher level of stress response intermediated by enzymatic antioxidants during this time period. However, the higher value observed in non-stress exposed group fed 0.5% nutraceutical and stress exposed group (TNS5 and THR5 groups) indicate that there was some toxic effect of higher level (25% extra) of selenium. This result is in corroboration with Hamilton et al. (2002), who reported that selenium is one of the important trace elements; however, it causes toxicity when administered in excess. The activity of SOD and catalase enzymes in 0.1% nutraceutical fed, stress exposed (THR1) and non exposed (TNS1) groups indicate that at 0.1% level, selenium, methionine, zinc and vitamin D had a synergistic and positive effect to bring down the oxidative stress in fish.

5 | CONCLUSION

Feed restriction and overcrowding are common stressors in aquaculture systems that can affect the growth of the cultured species. Overcrowding along with restricted feeding for 1, 2, 3 and 4 months period considerably decreased the WG%, SGR, IGF-1 expression, PER and whole-body EE and TC and increased FCR and whole-body moisture content in rohu, *Labeo rohita* fingerlings, whereas long-term stress exposure (3–4 months) only severely decreased HSI, ISI and whole-body CP contents and increased TA level in fish. Feeding of 0.1% nutraceutical (containing 17.0, 25.0, 2.5 and 5.0% extra levels of vitamin E, methionine, zinc and selenium, respectively, over requirement) for 3- to 4-month period significantly enhanced WG% and IGF-1 expression in rohu in comparison with the non-supplemented and 0.5% nutraceutical groups. Thus, dietary supplementation of 0.1% nutraceutical was found to be more superior to 0.5% level for mitigation of the stress-induced (overstocking and restricted feeding) effects in fish with significantly higher WG%, SGR, IGF-1 expression, PER, HSI and whole-body CP, EE and TC and, lower FCR, SOD and CAT activities than the non-supplemented group.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

AUTHOR CONTRIBUTION

Shamna N. designed the study, conducted the feeding trial, analysed the primary data, and wrote the first manuscript draft. Sahu, N.P. conceptualized, supervised, and corrected the draft and approved the final version of the manuscript. Femi Fawole and Sarvendra Kumar conducted the laboratory analysis. Sardar P. contributed to reviewing the literature and statistical analysis.

DATA AVAILABILITY STATEMENT

The data that support the findings of this paper are available from the corresponding author upon reasonable request.

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