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Dietary supplementation of Moringa leaf meal for Nile tilapia *Oreochromis niloticus*: Effect on growth and stress indices

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ABSTRACT

The current study investigated the effects of Moringa *(Moringa oleifera)* leaf meal (MLM) on growth and stress indices of Nile tilapia (*O. niloticus*). Experimental fish (2.0 ± 0.5 g average body weight) were divided into two groups; one was fed with a diet not supplemented with MLM (control diet), and the other group was fed with a diet supplemented with 1.5% MLM (diet 2) for three-month period. The results showed that MLM incorporation in the diet improved growth indices (body mass gain, specific growth rate, length gain rate and feed conversion ratio) and somatic indices (spleen somatic index, hepatosomatic index and intestine somatic index). Moreover, all hematological indices were also significantly increased. Additionally, stress indices were significantly improved, including antioxidants; superoxide dismutase, glutathione peroxidase, catalase and lipid peroxidase; and biochemical indices including enzymes activities of Aspartate Aminotransferase and Alanine Transaminase, as well as glucose and cortisol concentrations. Also, markedly up-regulated the expression of related genes; Insulin-Like Growth Factor-1 (IGF-1), Transforming Growth Factor-β (TGF-β) and superoxide dismutase (SOD) genes; in fish group fed diet supplemented with MFM as compared with the control group. These results show that MLM effectively alleviated starvation stress through retaining the enhanced growth and stress indices. In conclusion, Moringa supplementation to Nile tilapia has a positive effect as growth promoter and natural anti-stress phyto-therapy.

Introduction

Oreochromis niloticus is one of the ideal species for aquaculture as it is fast growing and highly tolerant of different environmental conditions (Nandlal and Pickering, 2004). Physiological stress response can be caused by several managemental procedures as high densities, handling, starvation and transportation stressors (Halliwell, 1994; Bowden, 2008; Wang and Nixon, 2001). Starvation is one of those stressors that can cause oxidative stress conditions through enhancing reactive oxygen species generation (Robinson et al., 1997).

Phyto-therapies can possess an antioxidative activity and thus can be used in aquaculture to overcome the oxidative conditions. Moringa is a fast growing, widely distributed plant in tropical and subtropical countries, which is characterized with several important industrial and medicinal uses. Its leaf extracts have hypo-cholesterolaemic and antioxidant activities (Chumark et al., 2008). It contains antioxidants, which can inactivate harmful free radicals produced during normal cellular activities and from different stressful conditions (Rapatsa and Moyo, 2014). The leaves, in particular, contain phenolics and flavonoids, which have various biological activities, including antioxidant, anticarcinogenic, immunomodulatory and hepatoprotective properties (Sherif et al., 2014).

Moringa has also become a potential alternative plant protein source in aquaculture. Crude protein content (CP) in Moringa leaf is estimated to be about 260 g/kg of leaf. Furthermore, essential amino acids content in leaves includes mainly methionine, cysteine, tryptophan and lysine (Abdulkarim et al., 2005; Sherif et al., 2014). Moringa contains low concentrations of harmful factors in its seeds, and its protein contents are higher than proteins of soybean seeds and other legumes (Ferreira et al., 2008).

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The current work aimed to evaluate the effects of dietary supplementation of *Moringa oleifera* on the growth and stress indices of Nile tilapia challenged with one week starvation.

Materials and methods

Fish

Oreochromis niloticus of average weight 2.0 ± 0.5 g were acquired from a private fish farm in Kafr El Sheikh City, Egypt (31°24′28.8″N 30°57′35.6″E). Fish were transported to the Wet Lab of Aquatic Animals Diseases and Management, Benha University, Egypt, and were kept in 750 L fiberglass tank. Later on, Fish were randomly divided among six fiberglass tanks and allowed to acclimatize for additional 10 days. This work was done according to the protocol of the Research Ethics Board, Faculty of Veterinary Medicine, Benha University (BUFVTM 02-08).

Preparation of experimental diets

Fresh *Moringa oleifera* was obtained from Faculty of Agriculture, Benha University, air dried for 24 h, powdered, packed in air tight bags and stored at 4 °C. Two diets were prepared. Basal diet in powder form (Table 1) was allocated into two parts, the first one was free control diet and the second part was incorporated with 15 g *M. oleifera* /kg feed and processed to obtain concentration of 1.5% (based on unpublished work done in our laboratory). After drying, diets were packed in clean dry plastic jars and stored at 4 °C in the refrigerator until use, according to (Elabd et al., 2016a; El-Asely et al., 2014)

Experimental design

Hundred and eighty fish were distributed into two groups in 750 L fiberglass tanks, each with three replicates. The control group was fed a free basal diet and the second group received 1.5% *M. oleifera*. Fish were fed to satiation twice a day for a period of three months. Water temperature was adjusted at 25.0 ± 0.8 °C and dissolved oxygen (DO) at 6.0 ± 0.9 mg/L. Mortality and fish health status were also checked daily throughout the experiment.

Table 1
Ingredients and proximate composition of the basal diet.

Ingredients	g/1000 g total diet
Fish meal[a]	300
Yellow corn	150
Soya bean	350
Wheat bran	25
Vegetable oil	25
Gluten	25
Bi-calcium phosphate	10
NaCl	2
Mineral and vitamin permixes[b]	3
Composition	Proximate analysis (%)
Crude protein (CP)	38.29
Ether extract (EE)	6.36
Crude fiber (CF)	8.33
Ash	9.12
Digestible energy (Kcal/kg)	3302

[a] Danish Fishmeal (INTRACO ltd., Belgium).
[b] Egavet premix: each 3 kg contain: vitamin A, 12 million IU; vitamin D, 2.5 million IU; vitamin E, 10,000 mg; vitamin K3, 1000 mg; vitamin B1, 1000 mg; vitamin B2, 5000 mg; vitamin B6, 1500 mg; niacin, 30,000 mg; biotin, 50 mg; folic acid, 1000 mg; pantothenic acid, 10,000 mg; Mn, 60,000 mg; Zn, 50,000 mg; Fe, 30,000 mg; Cu, 5000 mg; Se, 100 mg; Co, 100 mg; Mn, 250,000 mg; CaCo3.

The Starvation Challenge. Incorporated diet was stopped after three months feeding trial and then fish from each replicate were exposed to one week starvation stress.

Estimation of growth indices

Fish weight was estimated at the beginning of the study, and after starvation to calculate growth indices (after 3 months). All measurements were carried out on 30 fish in each group (10 samples from each replicate) according to methods previously mentioned in our previous studies (Elabd et al., 2016a; El-Asely et al., 2014) as following: Body Mass Gain (BMG) (%) = 100 × [final body mass (g) − initial body mass (g)/initial body mass (g)]; Specific Growth Rate (SGR) (% day^{-1}) = [(ln final body mass in g) − ln initial body mass in g)/number of trial days] × 100.; Feed Conversion Ratio (FCR) = $F/(Wf - Wi)$; Length Gain Rate (LGR) (%) = 100 × [Average terminal body length (cm) − Average initial body length (cm)/Average initial body length (cm)]; Spleen Somatic Index (SSI) = (weight of spleen (g)/total body weight (g)) × 100; Hepatosomatic Index (HSI) = (weight of liver (g)/total body weight (g)) × 100; Intestine Somatic Index (ISI) = (weight of intestine (g)/total body weight (g)) × 100.

Sampling

Blood and serum collection

This experiment included two sampling points; first point was after three months feeding on tested diet and before exposure to any stress condition; and the second one was after one week from exposure to starvation stress. Three fish were sampled from each replicate (9 fish per group). Blood was collected using 1 ml sterile syringe with EDTA anticoagulant for measurement of Red Blood Cell (RBCs) and White Blood Cell (WBCs) counts, Packed Cell Volume (PCV), Hemoglobin (Hb) and Corpuscular hemoglobin concentrations; Mean cell (or corpuscular) volume (MCV), Mean cell hemoglobin (MCH) and Mean cell hemoglobin concentration (MCHC); according to methods previously described by El-Asely et al. (2014). To obtain serum, other blood samples were collected without anticoagulant and allowed to clot at room temperature in a decline situation, then centrifuged at 3000*g* for 15 min and stored at −20 °C for measuring glucose and cortisol concentrations, plus the enzymes aspartate transaminase (AST) and aka alanine aminotransferase (ALT) activities following methods previously mentioned by Elabd et al. (2016a,b) and Elabd et al. (2017).

Tissue sampling

After blood collection, liver samples were isolated and weighted, then distributed into two portions. The first one was used for antioxidant assays and the second was stored at –80 °C in RNAlater solution for gene expression analysis.

Hematological assays

RBCs, WBCs counts; and PCV were counted according to Stoskopf (1993). Mean Hemoglobin (Hb) and Corpuscular hemoglobin concentrations (MCV, MCH and MCHC) were calculated according to the formula mentioned by Dacie and lewis (1975).

Biochemical measurements

Cortisol and glucose concentrations

Glucose concentration in serum was assayed spectrophotometerically in triplicates at 546 nm using BioMed, Egypt's commercial kit according to the following formulas: Glucose (mg/dL) = [(^{A} sample /^{A} Standard)/(^{A} Standard)] × [100 × (standard value)]. Where, Standard 100 mg/dL = 5.56 mmol/L. Cortisol con-

centration was assayed at 450 nm using the commercial kit LSBio, USA.

AST and ALT activities

AST and ALT activities were assayed using the commercial kits BioMed, Egypt, at 340 nm according to the following formulas: AST Activity U/L = 1746 × ΔA 340 nm/min and ALT activity (U/L) = ΔE/min Sample × 1746.

Antioxidants

Antioxidant enzymes activities; superoxide dismutase SOD; catalase CAT; glutathione peroxidase GPx; and Lipid peroxidation LPx; were measured spectro-photometerically at 560 nm (SOD), 510 nm (CAT), 340 nm (GPx) and 534 nm (LPx) using the colorimetric kits BIODIAGNOSTIC, Egypt, according to the following formulas:

SOD (U/gm tissue) = [(% inhibition) × (3.75) × (1/gm tissue)] × sample dilution. o CAT activity (U/g) = [(^{A} sample − ^{A} Standard)/(^{A} Standard)] × [1/gm tissue used per test]. o GPx (U/g) = [(^{A} sample − ^{A} Standard)/(^{A} Standard)] × [1/gm tissue used per test]. o LPx activity (nmol/gm tissue) = [(^{A} sample)/(^{A} Standard)] × [10/gm tissue used].

Gene expression

RNA was extracted using the RNeasy Mini kit (Qiagen, Germany) and other procedures including homogenization of samples, purification of total RNA and column DNase digestion to remove residual DNA, All these procedures were performed according to the manufacturer protocol. RNA quantity was checked using Nano-Drop spectrophotometry Thermo Scientific, USA, by absorption ratio 1.80: 2.00 and OD 260/OD280 nm. Primers in this study were obtained from Metabion, Germany (Table 2). *Rt-PCR* reaction was done in a Stratagene MX3005P, using 200 U/μL Reverse Transcriptase (Thermo Fisher, USA), 8.25 μl of water, and 3 μl of RNA template. The CT of each sample was compared with that of the positive according to the "ΔΔCt" method (Yuan et al., 2006) using the following ($2^{-\Delta\Delta ct}$) equation: Where ΔΔCt = ΔCt reference − ΔCt target, *ΔCt target = Ct control − Ct treatment* and *ΔCt reference = Ct control − Ct treatment*.

Statistical analysis

The data were analyzed by One-Sample T-Test to determine significant difference between groups and a value of $P < 0.05$ was considered significant using the (SPSS, version 16.0) software.

Results

Growth indices

Table 3 shows that incorporating *O. niloticus* diet with 1.5% Moringa for three months significantly enhanced the growth indices (BMG, LGR, SGR and FCR) and somatic indices (LSI, SSI and ISI) compared to the control group. Additionally, it efficiently mitigated the starvation stress through retaining the enhanced growth indices (BMG, SGR and FCR) after the starvation challenge compared to the control group (Fig. 1A, B, C and D).

Hematological assays

1.5% Moringa incorporated diet induced a significant increase in all hematological indices (RBCs, WBCs counts; and PCV, Hb, MCV, MCH and MCHC) compared to control group (Table 4). Moreover, Moringa incorporation positively mitigated the decrease in hematological parameters caused by starvation stress and increased all values over the control group (Table 4).

Antioxidants assays

The group with 1.5% Moringa supplementation didn't show any significant difference in antioxidants activities than the control group. However, starvation caused a significant decrease ($P < 0.05$) in SOD, GPx and CAT activities, while Moringa incorporation significantly increased those levels (Fig. 2A, B and C). Furthermore, Moringa incorporation enhanced LPx activity as it decreased its elevated level, which was caused by exposure to one-week starvation (Fig. 2D).

Biochemical assays

Adding 1.5% Moringa to Nile tilapia diet markedly decreased glucose and cortisol levels compared to control. Moreover, Moringa

Table 2
Primers sequences, target genes, amplicon sizes and cycling conditions for SYBR green rt-PCR.

Target gene	Primers sequences	Reverse transcription	Primary Denaturation	Amplification (40 cycles)			Dissociation curve (1 cycle)			Reference
				Secondary denaturation	Annealing	Extension	Secondary denaturation	Annealing	Final denaturation	
EF-1α	CCTTCAACGCTCAGGTCATC	50 °C	94 °C	94 °C	62 °C	72 °C	94 °C	62 °C	94 °C	Gröner et al. (2015)
	TGTGGGCAGTGTGGCAATC	30 min	15 min	15 s	30 s	30 s	1 min	1 min	1 min	
IGF1	TTCTCCAAAAACGAGCCTGCG									Vera cruz et al. (2006)
	TCTGCTACTAACCTTGGGTGC									
TGFB	GTTTGAACTTCGGCGGTACTG				60 °C			60 °C		Standen et al. (2016)
	TCCTGCTCATAGTCCCAGAGA				30 s			1 min		
SOD	GGTGCCCTGGAGCCCTA									Afifi et al. (2016)
	ATGCGAAGTCTTCCACTGTC									

Table 3
Growth indices of *O. niloticus* after three months feeding Moringa incorporated diet.

Moringa%/kg feed	Initial Wt (g)	Final Wt (g)	BMG (%)	SGR (%)	LGR (%)	FCR	LSI	SSI	ISI
Control	2.0 ± 0.5	12.1 ± 0.5	459.4 ± 0.0	2.6 ± 0.0	383.6 ± 0.0	0.08 ± 0.0	1.8 ± 0.0	3.4 ± 0.0	1.1 ± 0.07
1.5	2.0 ± 0.5	20.0 ± 0.7*	898.3 ± 0.0*	3.4 ± 0.0*	443.7 ± 0.0*	0.04 ± 0.0*	3.0 ± 0.0*	9.6 ± 0.0*	2.2 ± 0.07*

Values are mean (n = 30) ± SEM. Mean values with asterisk (*) are different significantly ($P < 0.05$). BMG = Body Mass Gain, SGR = Specific growth rate, LGR = Length Gain Rate, FCR = Feed Conversion Ratio, LSI = Hepatosomatic index, SSI = Spleen somatic index and ISI = Intestine somatic index.

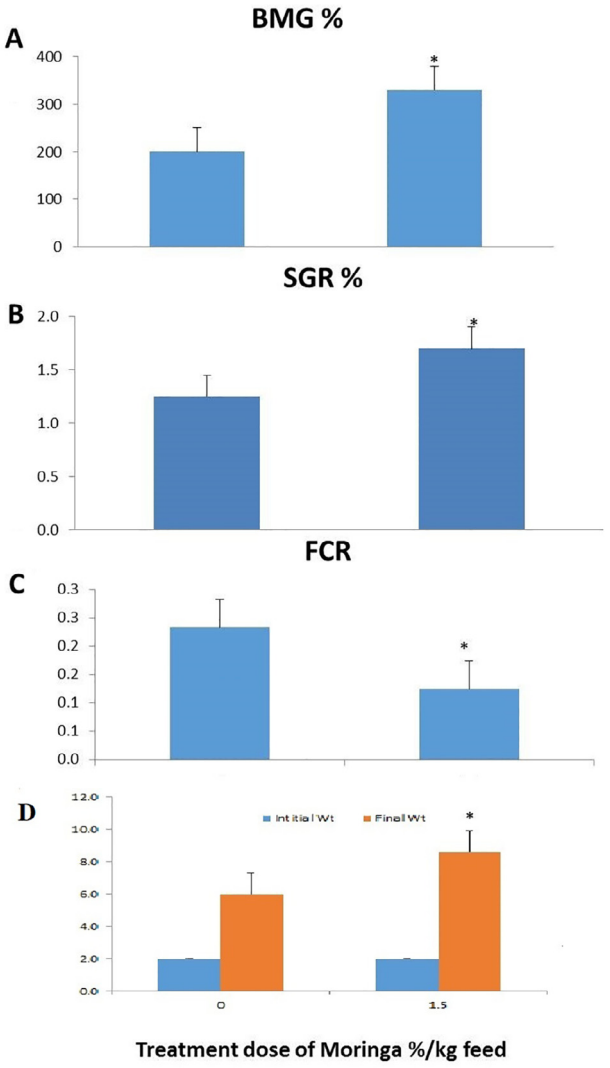

Fig. 1. BMG (%) (A), SGR (%) (B), FCR (C) and Weights (D) of Moringa fed *O. niloticus* at post-challenge. Values are mean (n = 30) ± SEM. Mean values with asterisk (*) are different significantly (P < 0.05).

incorporated diet was able to alleviate starvation stress and retained the decrease in glucose and cortisol levels (Fig. 3A and B). Moringa dietary incorporation also showed a significant decrease in both ALT and AST activities prior to and after the starvation challenge (Fig. 4A and B).

Expression of targeted genes

Group fed with 1.5% Moringa incorporated diet showed significant high (P < 0.05) expression of Insulin-Like Growth Factor-1 (IGF-1), Transforming Growth Factor β (TGF-β) and SOD genes than the control group before the starvation challenge (Fig. 5A and C). After the starvation stress, the group fed with diet supplemented with 1.5% Moringa was able to alleviate the starvation stress and continued showing significant up-regulation (P < 0.05) in the expression of IGF-1, TGF-β and SOD genes (Fig. 6A–C).

Discussion

In Aquaculture, starvation is one of the conditions that lead to oxidative stress through stimulating reactive oxygen species generation (Kumari and Sahoo, 2005; Robinson et al., 1997). Many herbal plants have the ability to inhibit the generation of oxygen anions and scavenge free radicals, thus act as natural antioxidants (Chakraborty and Hancz, 2011). In the current study we focused on *Moringa oleifera*, which is well known by its high antioxidant activity (Chumark et al., 2008; Iqbal and Bhanger, 2007; Nascimento et al., 2016).

The results obtained from this study highlight the benefits of *M. oleifera* incorporation in Nile tilapia feed. It markedly increased growth indices of *O. niloticus* before exposure to starvation and efficiently mitigated that stress through retaining the enhanced growth indices (BMG, SGR and FCR) after the starvation challenge. Similarly, Elabd et al. (2016b) reported that dietary supplementation of yellow perch with liquorice and astragalus significantly enhanced different growth parameters.

Previous studies revealed the ability of Moringa to significantly increase growth parameters and feed utilization of fish (Ozovehe, 2012; Sherif et al., 2014). Those positive results may be attributed to the fact that Moringa is a good source of fats, proteins and crude fibers (Francis et al., 2001), which consequently increases growth indices. In addition to its antioxidant activities, this enabled the supplemented group to retain enhanced growth indices even after exposure to starvation stress (Tejas et al., 2012).

The most important antioxidant enzymes are SOD, GPx, CAT and LPx (Madeira et al., 2013; Somogyi et al., 2007). In the current study, despite of the negative effect of starvation exposure on antioxidants activities, Moringa diet significantly alleviated this effect and returned them to control level. Parallel results were acquired by Elabd et al. (2016b) who reported that the incorporation of yellow perch's diet with liquorice and astragalus obviously decreased SOD and CAT activities, as well as GPx and LPx activities were elevated following starvation. This may be attributed to Moringa active components, which have been reported to possess antioxidative activities in fish (Chumark et al., 2008; Nascimento et al., 2016), as Moringa leaves possess a combination of antioxidants that are proven to be more powerful than a single antioxidant, due to synergistic mechanisms and enhanced antioxidant cascades (Mishra et al., 2011; Tejas et al., 2012).

Hematological indices values are known to reflect and indicate the health status of fish (Řehulka, 2000). The current study revealed that Moringa incorporation significantly increased all hematological parameters at pre and post starvation exposure. Likewise, Sherif et al. (2014) reported that haemogram (RBC, Hb,

Table 4
Hematological indices of Moringa fed *O. niloticus* at pre and post-exposure to starvation.

Moringa%/kg feed	RBCs count × 10^6/L	Hbg/dl	PCV %	MCV fl	MCH pg	MCHC g/dl	Tot. Leuck. Count × 10^3/L
Control	1.0 ± 0.0	6.6 ± 0.0	19.3 ± 0.0	173.8 ± 0.0	72.8 ± 0.0	37.8 ± 0.0	110.3 ± 0.0
1.5	1.3 ± 0.0	7.9 ± 0.0	23.4 ± 0.0	204.1 ± 0.0	56.1 ± 0.0	30.4 ± 0.0	171.6 ± 0.0
Control	1.0 ± 0.01	3.8 ± 0.0	10.3 ± 0.0	123.2 ± 0.0	42.8 ± 0.0	35.8 ± 0.0	43.5 ± 0.0
1.5	3.7 ± 0.01	4.9 ± 0.0	12.5 ± 0.0	115.5 ± 0.0	46.5 ± 0.0	40.4 ± 0.0	83.9 ± 0.0

Values are mean (n = 9) ± SEM. Mean values with asterisk (*) are different significantly (P < 0.05).
Packed Cell Volume = PCV, Mean Corpuscular Volume Hemoglobin = MCV, Mean Corpuscular hemoglobin concentrations = MCH and Mean Corpuscular hemoglobin concentrations = MCHC.

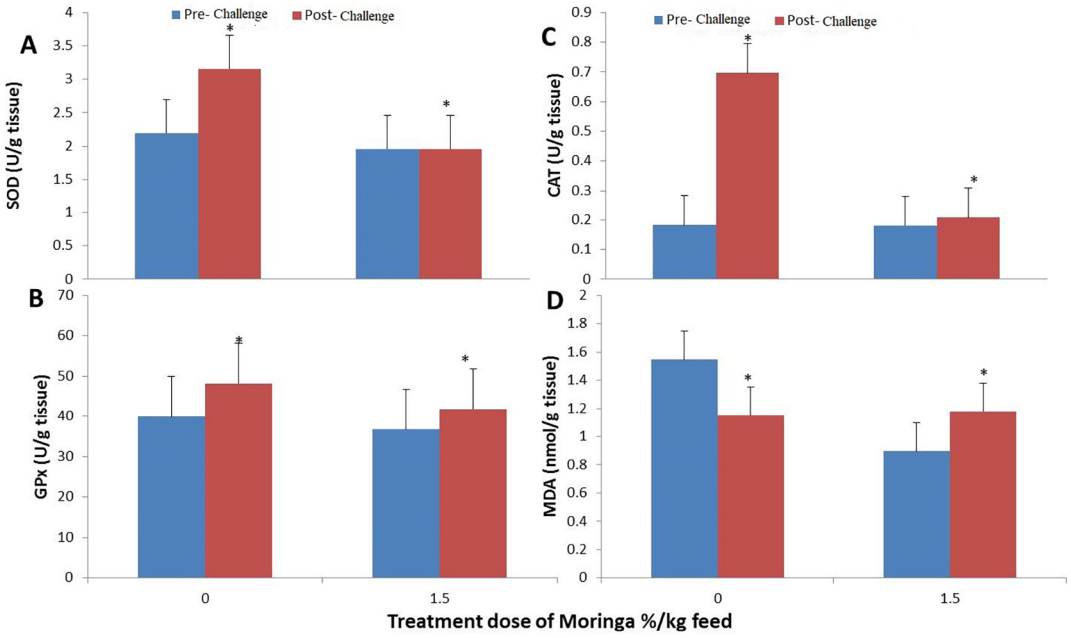

Fig. 2. SOD (A), GPx (B), CAT(C) and MDA (D) activities in liver of Nile tilapia (*O. niloticus*) after feeding with Moringa incorporated diet pre and post-challenge. Values are mean (n = 9) ± SEM. Mean values with asterisk (*) are different significantly (P < 0.05).

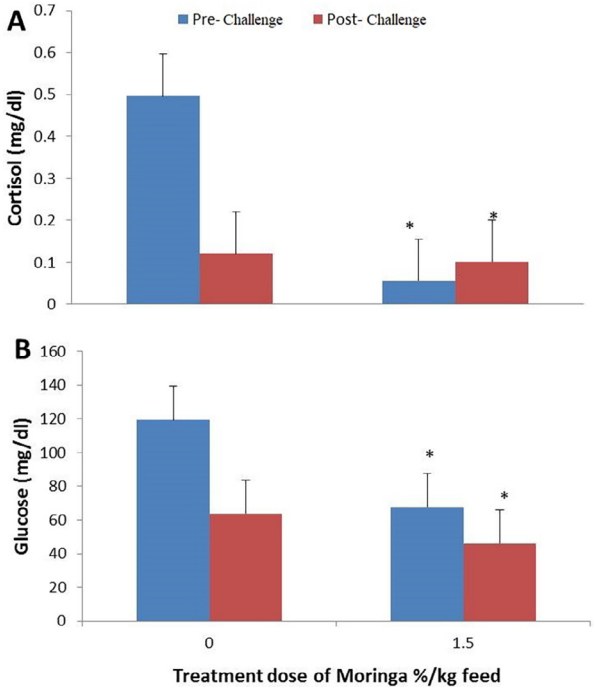

Fig. 3. Effect of Moringa incorporated diets on Cortisol (A) and Glucose (B) in liver of Nile tilapia (*O. niloticus*) at pre and post-challenge. Values are mean (n = 9) ± SEM. Mean values with asterisk (*) are different significantly (P < 0.05).

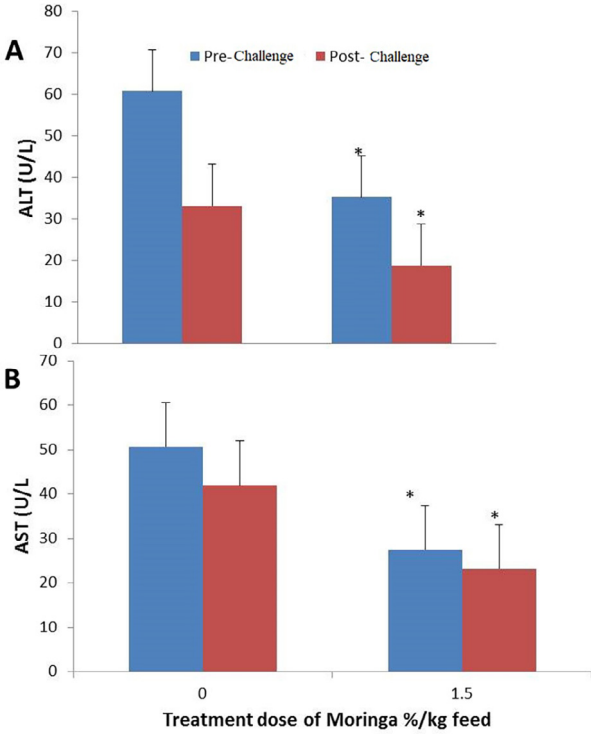

Fig. 4. Effect of Moringa incorporated diets on ALT (A) and AST (B) in liver of Nile tilapia (*O. niloticus*) at pre and post-challenge. Values are mean (n = 9) ± SEM. Mean values with asterisk (*) are different significantly (P < 0.05).

PCV, MCV, MCH and MCHC) of *O. niloticus* supplemented Moringa with different concentrations was significantly enhanced indicating that Moringa incorporation caused enhanced health status (Nascimento et al., 2016).

Serum glucose and cortisol levels are usually used as indicators for environmental stress in fish (Barton and Iwama, 1991). Glucose and cortisol levels gave significant decrease in the Moringa supplemented group throughout the experiment. This might be caused by the antioxidant properties of Moringa (Chumark et al., 2008) and that it might have encouraged the insulin activity and decreased glucose level as recorded in previous studies (Abasali and Mohamad, 2010; Elabd et al., 2017, 2016a,b; Talpur, 2014).

The current study revealed that Moringa effectively decreased ALT and AST activities. This can be due to the hepatoprotective

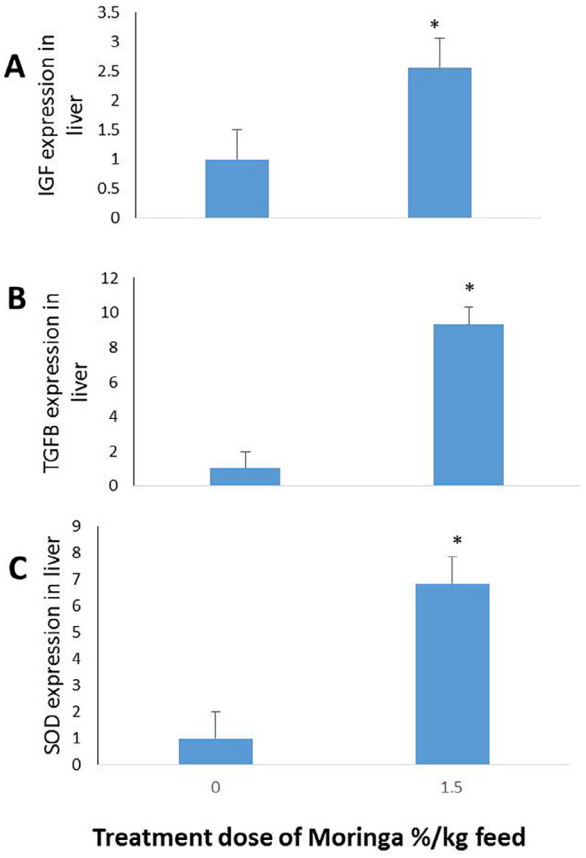

Fig. 5. IGF(A), TGF (B) and SOD (C) genes expression in Nile tilapia (*O. niloticus*) pre-challenge, Values are mean (n = 9) ± standard error. Mean values with asterisk (*) are different significantly (P < 0.05).

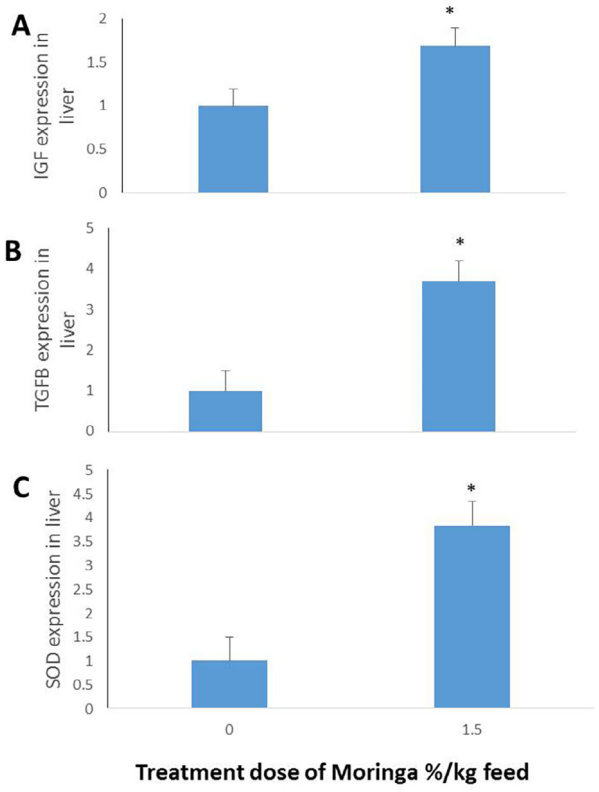

Fig. 6. IGF(A), TGF (B) and SOD (C) genes expression in Nile tilapia (*O. niloticus*) post-challenge. Values are mean (n = 9) ± standard error. Mean values with asterisk (*) are different significantly (P < 0.05).

effects of Moringa (Nascimento et al., 2016) and the presence of quercetin (Anwar et al., 2007), which have substantial effects on the levels of AST and ALT. Previous results agree with the current findings. Elabd et al. (2016a,b) reported that Liquorice and Astragalus effectively decreased ALT and AST activities at pre-exposure and post-exposure to starvation stress.

IGF-I is related to growth regulation (Rui et al., 2010). IGF-I expression clearly reflected growth indices data and showed significant up-regulation in Moringa supplemented group than the control. These results came in agreement with Elabd et al. (2016b). Also, immuneregulatory TGF-β expression was enhanced by Moringa incorporation throughout the entire experiment, indicating its beneficial role. In parallel, the expression ratio of target genes (IL-8, IL-1β and TGF-β) of the head kidney of rainbow trout *Oncorhynchus mykiss* fed with lupin, mango and stinging nettle showed markedly up-regulation compared to the control (Awad and Austin. 2010). This can be attributed to the capability of Moringa leaves to stimulate both cellular and humoral immune responses (Sudha et al., 2010), in addition to their antioxidant properties (Nascimento et al., 2016). In conclusion, the current study revealed that *Moringa oleifera* dietary supplementation enhanced growth indices and antioxidative stress response in Nile tilapia exposed to one-week starvation challenge and thus can be used as natural anti-stress agent.

Declaration of Competing Interest

Authors declare no conflict of interest.

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