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Effect of *Silybum marianum* seeds as a feed additive on growth performance, serum biochemical indices, antioxidant status, and gene expression of Nile tilapia, *Oreochromis niloticus* (L.) fingerlings

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ABSTRACT

A growth trial was conducted over 10 weeks to measure the effects of dietary *Silybum marianum* seeds on "growth performance, feed utilization, blood parameters, antioxidant enzymes activities and gene expression of Nile tilapia *Oreochromis niloticus* fingerlings". Five isonitrogenous and isoenergetic experimental diets were formulated to contain five levels of *S. marianum* seeds on the dry weight basis at 0 (control), 2.5, 5, 7.5 and 10 g kg^{-1} diet. The results showed that the highest weight gain (WG), specific growth rate (SGR), protein efficiency ratio (PER) and apparent protein utilization (APU) were obtained by fish fed diet supplemented with 7.5 and 10 g kg^{-1} *S. marianum*. In addition, the total serum protein concentration as well as albumin and globulin protein fraction contents were obviously higher in fish fed either 7.5 or 10 g *S. marianum* seeds kg^{-1} diet than other diets. The lowest level of aspartate and alanine aminotransferase was recorded in fish fed diet supplemented with 10 g kg^{-1} diet *S. marianum*. Results also showed that, the highest total antioxidant enzyme activity of superoxide dismutase (SOD) and catalase activity (CAT) was obtained by fish fed diet supplemented with 10 g kg^{-1} *S. marianum*. The highest transcripts accumulation of growth hormone was detected in pituitary of fish fed diet supplemented with 10 g kg^{-1} diet *S. marianum*, but the highest expression of immunoglobulin M-2 (IGM-2) was detected in liver of fish fed diet supplemented with 7.5 g kg^{-1} diet *S. marianum*. The highest up-regulated expression of SOD and CAT were detected in fish fed diet supplemented with 10 g kg^{-1} diet *S. marianum* seeds. These results suggested that the best dietary *S. marianum* level in the diet of Nile tilapia, *O. niloticus* fingerlings was 7.5 g or 10 g kg^{-1} diet (92.25 and 123 mg kg^{-1} silymarin) as a feed additive to promote growth, enhance the immune responses, increase antioxidant activity and gene expression.

1. Introduction

Aqua-feeds industry are looking forward to conveying dual benefits of improved performance and innate humoral immunity response to the cultured fish species (Daudpota et al., 2016; Hassaan et al., 2018; Hassaan et al., 2019), thus the nutritional status might be considered as one of the major aspects that stimulates the immune responses of cultured fish species. Recently, using of herbal medicine such *Silybum marianum* as an alternative way for health improvement in aquatic organisms has gain interest, due to increasing concerns on the detrimental effects of antibiotics and chemicals in cultured species and the environment (Van Hai, 2015). Furthermore, they may be readily available, inexpensive, and have no deleterious effects because of low side effects on fish and surrounding (Han et al., 2016; Na-Phatthalung et al., 2018). Active components of herbal medicine could enhance nutrient digestibility, absorption and assimilation capacity by enhancing the digestive enzymes secretion and maintaining healthy intestinal microflora as well as improving immune status (Mohiseni et al., 2017). Moreover, high content of natural antioxidant in herbal medicine as flavonoids, tocopherols, cinnamic acid and folic acid carotenoids can obstruct reactive oxygen species (ROS) generation and scavenge free radicals from tissues (Citarasu, 2010; Astuya et al., 2017).

Milk thistle, *Silybum S. marianum* fruits contains an isomeric of flavonolignans known as silymarin (Jia et al., 2013). The principal components of silymarin are taxifolin, silybin A, silybin B, isosilybin A, isosilybin B, silychristin A and silycrhristin B, and the major content of

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silymarin is silybin which is around 50–70% (El-Garhy et al., 2016). The hepatoprotective and anti-inflammatory properties of silymarin are not understood well in fish, however, some studies have reported that silymarin and silibinin achieve to reduce fat retention in the guts of mice by inhibiting lipogenic gene expression and/or improve lipolysis and plasma lipoprotein content by regulating plasma lipoproteins (Chou et al., 2012; Yao et al., 2013). Silymarin acts as an antioxidant due to its anti-inflammatory effects and has been used in the treatment of various liver damage (Surai, 2015; Yao et al., 2011). Few reports on the application of silymarin in fish diet are available. The available investigations have revealed that addition of silymarin in diets enhanced the growth performance of *Carassius auratus* gibelio (Yi et al., 2012), decreased plasma content of cholesterol of rainbow trout (Banaee et al., 2011), induced hepatoprotective effects of common carp exposed by CCl4 (Jia et al., 2013), improved the immune system in rainbow trout and improved the antioxidative stress capability of Atlantic salmon (Ahmadi et al., 2012; Sanchez et al., 2016). To the best of our knowledge, no reports are known about the effects of *S. marianum* seeds as a feed additive on growth hormone and immunoglobulin gene expression of fish. Therefore, the purpose of the present study was to evaluate the effects of *S. marianum* seeds as feed additive on the growth performance, serum biochemical indices and antioxidant status of Nile tilapia. Specifically, the study examined the genes expression of growth hormone (GH), immunoglobulin M-2 (IGM-2), superoxide dismutase (SOD) and catalase (CAT) of Nile tilapia.

2. Materials and methods

2.1. Fish and experimental management

Fish were obtained from stocks held at Fish Research Station, National Institute of Oceanography and Fisheries (NIOF), El-Kanater El-Khayria, Kalubiya Governorate, Egypt. Before the experiment, fish were acclimatized to the experimental conditions for 2 weeks with continuous aeration in fish nutrition lab of NIOF. The fish were treated in accordance with the guidelines of Banha Committee of animal research and ethics (BCARE). At the beginning of the experiment, the fish were weighed individually after acclimation period and total of 150 Nile tilapia (mean weight 2.32 ± 0.01 g) were randomly distributed into 15 cylindrical tanks (60 L), three replicate tanks were randomly distributed to each treatment at a rate of 10 fish per tank. The tanks were supplied with dechlorinated tap water and were continuously supplied with compressed air for oxygen requirement via air stone. Approximately, one-third of the water volume in each tank was daily changed after cleaning and eliminating the accumulated excreta. Fish were hand fed close to apparent satiation fourth daily (at 9:00 am, 11.00 am, 13.00 pm and 15.00 pm) for 70 days. During the study period, fish weighed biweekly to adjust the total amount of feeds consumed by the fish in each tank. Water temperature was recorded daily with a mercury thermometer suspended at 15-cm depth. pH was determined by using a pH meter (Orion pH meter, Abilene, Texas, USA). While dissolved oxygen (mg/L) was measured using YSI model 56 oxygen meter (YSI Company, Yellow Springs Instrument, Yellow Springs, Ohio, USA). Total ammonia was measured using DREL/2 HACH kits (HACH Co., Loveland, Co. USA). During the feeding trial, the water quality parameters averaged (± standard deviation): Water temperature 26.8 ± 0.3 °C; dissolved oxygen 5.98 ± 0.22; pH values 8.17 ± 0.35; total ammonia 0.16 ± 0.01 mg/L, all tested water quality criteria (temperature, dissolved oxygen, pH value and total ammonia) were within the acceptable limits for rearing Nile tilapia (Boyed, 1990).

2.2. Diets preparation

Five isonitrogenous (285.70 g kg^{-1} of crude protein) and isocaloric (18.65 MJ kg^{-1} gross energy) diets were formulated from practical ingredients (Table 1). The first diet was the control diet. *Silybum marianum* seeds were added to the other four diets at levels 2.5, 5, 7.5 and 10 g kg^{-1} diet. Fish meal and soybean meal were used as the main sources of protein and fish oil was incorporated as a source of lipid in the basal diet, *S. marianum* seeds was kindly provided by genetic engineering department, biotechnology lab, faculty of Agriculture, Benha University, Egypt, and prepared by Fish nutrition lab, Aquaculture division, National Institute of Oceanography and fisheries (NIOF), Cairo, Egypt. Total silymarin content in all experimental diets were 0.0, 30.75, 61.5, 92.25 and 123 mg kg^{-1} diet, respectively (Table 1). According to high-performance liquid chromatography (HPLC) analysis, *S. marianum* seeds contained taxifolin, silibinin A and B, isosilibinin A and B, silidianin, and silicristin (Fig. 1). Furthermore, the flavonolignans contents of *S. marianum* seeds in experimental diets according to high-performance liquid chromatography (HPLC) analysis was showed in Table 2. The ingredients were blended in a feed mixer using a homogenous mixture grinder. Powdered *S. marianum* seeds were mixed with wheat bran to achieve uniformity and were then added to the blend, followed by homogenization. Next, fish oil was added, followed by homogenization. Finally, distilled water was added to make dough. The dough was passed through pelleting hand-noodle maker with a 2 mm diameter. Pellets were air-dried at 24 ° C and then stored at −20 ° C until used.

Table 1
Formulation and proximate composition of the experimental diets (g kg^{-1} diet).

Ingredient	Control	2.5 g kg^{-1}	5 g kg^{-1}	7.5 g kg^{-1}	10 g kg^{-1}
Fish meal 65%	100.00	100.00	100.00	100.00	100.00
Soybean meal 44%	350.00	350.00	350.00	350.00	350.00
Corn gluten meal 62%	30.00	30.00	30.00	30.00	30.00
Yellow corn 8.5%	250.00	250.00	250.00	250.00	250.00
Wheat bran 14%	100.00	97.50	95.00	92.50	90.00
Rice polishing 13%	100.00	100.00	100.00	100.00	100.00
Fish oil	40.00	40.00	40.00	40.00	40.00
Premix[1]	25.00	25.00	25.00	25.00	25.00
Vitamin C	5.00	5.00	5.00	5.00	5.00
Silybum marianum seed	0.00	2.50	5.00	7.50	10.00
Total Silymarin mg kg^{-1}	0.00	30.75	61.50	92.25	123.00
Chemical analysis					
Protein	286.35	286.00	285.65	285.30	284.95
Lipid	70.30	70.43	70.55	70.68	69.90
Ash	56.69	56.64	56.60	56.55	56.05
Fiber	54.00	53.96	53.92	53.88	53.00
Nitrogen free extract[2]	532.70	533.00	533.30	533.60	536.10
Gross energy[3]	18.64	18.65	18.65	18.65	18.65

[1] Vitamin and mineral mixture kg^{-1} of mixture contains: 4800 I.U. Vit A, 2400 IU cholecalciferol (vit. D), 40 g Vit E, 8 g Vit K, 4.0 g Vit B_{12}, 4.0 g Vit B2, 6 g Vit B6, 4.0 g, Pantothenic acid, 8.0 g Nicotinic acid, 400 mg Folic acid, 20 mg Biotin, 200 g Choline, 4 g Copper, 0.4 g Iodine, 12 g Iron, 22 g Manganese, 22 g Zinc, 0.04 g Selenium. folic acid, 1.2 mg; niacin, 12 mg; d-calcium pantothenate, 26 mg; pyridoxine. HCl, 6 mg; riboflavin, 7.2 mg; thiamin. HCl, 1.2 mg; sodium chloride (NaCl, 39% Na, 61% Cl), 3077 mg; ferrous sulfate ($FeSO_4.7H_2O$, 20% Fe), 65 mg; manganese sulfate ($MnSO_4$, 36% Mn), 89 mg; zinc sulfate ($ZnSO_4.7H_2O$, 40% Zn), 150 mg; copper sulfate ($CuSO_4.5H_2O$, 25% Cu), 28 mg; potassium iodide (KI, 24% K, 76% I).

[2] NFE (Nitrogen free extract) =100-(crude protein + lipid + ash +fiber content).

[3] Gross energy calculated using gross calorific values of 23.63, 39.52 and 17.15 kj/g for protein, fat and carbohydrate, respectively according to Brett (1973).

2.3. Proximate composition of whole-body fish

Five individual fish were sampled from each tank and then oven-dried at 70 °C until constant weight and calculated weight loss, and the samples were also stored at −20 °C for subsequent analysis. All chemical analysis was conducted according to AOAC (1995). Chemical composition was analyzed on experimental diets and fish samples at the

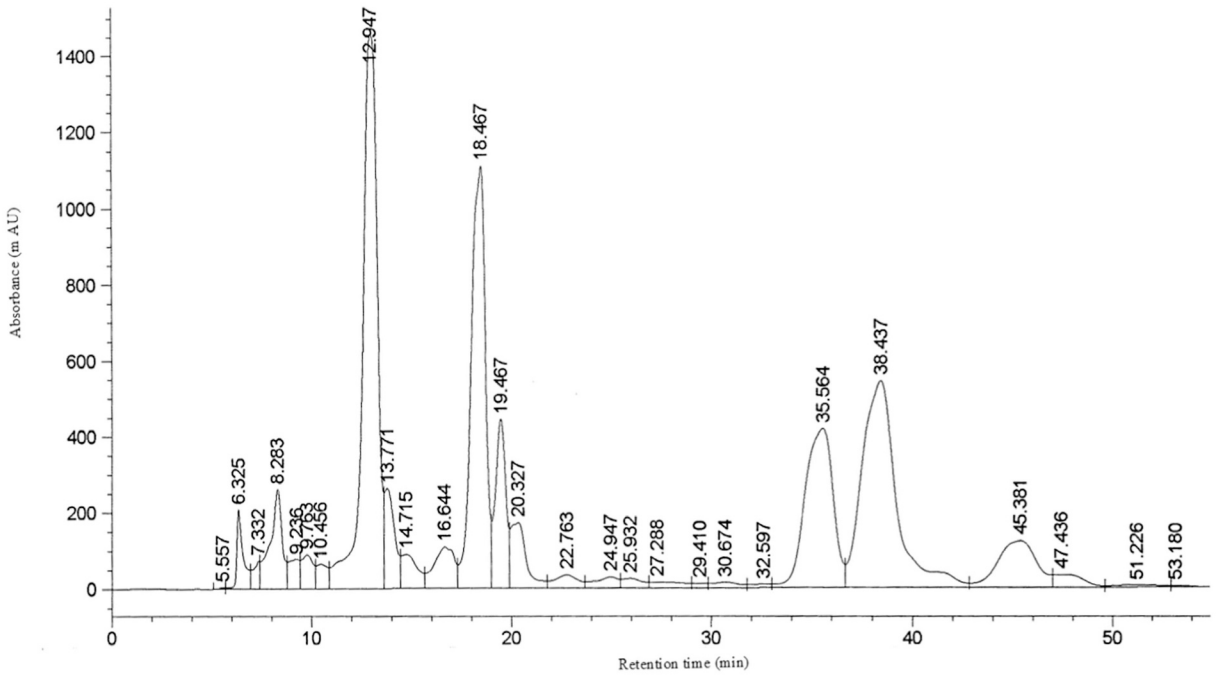

Fig. 1. Flavonolignans mixture consisted of Taxifolin, Silychristin, Silydianin, Silybin A, Silybin B, and iso Silybin A + B with different concentration determined by high performance liquid chromatography (HPLC).

Table 2
Flavonolignans of *Silybum marianum* seeds in experimental diets according to high-performance liquid chromatography (HPLC) analysis.

Diets	Flavonolignans (mg kg^{-1} dry weight)					
	Taxifolin	Silychristin	Silydianin	Silybin A	Silybin B	Iso Sb A + B
2.5 g kg^{-1}	7.25	6.25	2.50	4.25	6.50	2.10
5 g kg^{-1}	14.50	12.50	5.00	8.50	13.00	4.20
7.5 g kg^{-1}	21.75	18.75	7.50	12.75	19.50	6.30
10 g kg^{-1}	29.00	25.00	10.00	17.00	26.00	8.40

end of the experiment. Crude protein was estimated by micro-Kjeldahl method, N × 6.25 (using Kjeltec auto analyzer, Model 1030, Tecator, Höganäs, Sweden). Lipid content was determined by Soxhlet extraction with diethyl ether (40–60 °C). Ash was determined by incineration at 550 °C for 12 h. Fiber content of the experimental diets was determined using the method described by Van Soest et al. (1991). Nitrogen-free extract was computed by taking the sum of values for crude protein, crude lipid, crude fiber and ash and by subtracting this sum from 100.

2.4. Measurement of serum biochemical parameters

At the end of the experiment, three fish of each replicate were anesthetized 3-aminobenzoic acid ethyl ester (MS 222, 100 mg/L, Sigma, St. Louis, MO) and blood samples were collected using clean syringes from the caudal vein of fish. The blood samples were centrifuged at 1500*g* for 15 min at 4 °C. The blood serum was used for the determination the liver enzymes activities of serum transaminases; aspartate aminotransferase (AST) and alanine aminotransferase (ALT) (Reitman and Frankel, 1957). Total serum protein and albumin were estimated according to Henry (1964) and Wotton and Freeman (1982), respectively. Total serum protein and albumin were determined according to Doumas et al. (1981). However, the total serum globulin was calculated by subtracting the total serum albumin from the total serum protein according to Coles (1974).

2.5. Measurement of antioxidant enzymes activities in liver

The liver of three fish in each replicate were weighed, rinsed and grinded in glass homogenizer tubes with ice-cold saline (to 0.1 g of liver was added 0.9 mL saline, pH 7.0), and centrifuged at 3000*g* for 10 min. The supernatant was collected for assays of superoxide dismutase (SOD). Superoxidase dismutase activity was measured according to the method of Peskin and Winterbourn (2000), using water-soluble tetrazolium salt as a superoxide detector and expressed as units per milligram protein. Beers and Sizer (1952) method was modified for catalase (CAT) activity assay. A mixture of 2.5 ml of phosphate buffer (pH 7.0), 2 ml of H_2O_2 solution and 0.5 ml of sample was added to each tube. The hydrogen peroxide (H_2O_2 30 mM) was used as a substrate and the decrease in H_2O_2 concentration at 22 °C was measured spectrophotometrically at 240 nm for 1 min and expressed as specific activities (U/g protein).

Total antioxidant capacity (T-AOC) was estimated according to the method of Benzie and Strain (1996) and the activity was expressed as units per milligram protein.

2.6. Genes expression

2.6.1. Total RNA extraction and complementary deoxyribonucleic acid (cDNA) synthesis

On 70 days of the feeding trial, the livers and pituitary were excised from five fish randomly obtained from each treatment, then immediately were grinned by Tissue Lyser LT apparatus (QIAGEN GmbH, QIAGEN Strasse 1, Hilden, Nordrhein-Westfalen-40724, and Germany) followed by total RNA extraction from the suspension of cells using SV Total RNA Isolation System (Promega cat.no. #Z3100) following the manufacturer's protocol.

For effective elimination of genomic DNA contamination from starting RNA samples, Residual genomic DNA was eliminated by treating RNA with gDNA Wipeout Buffer that included in the QuantiTect® Reverse Transcription Kit, according to the manufacturer's recommendations. Reverse transcription (RT) of the RNA was carried out using QuantiTect® Reverse Transcription Kit (Qiagen, Cat. No. 205311). The total RNA and cDNA samples were stored at −80 °C until

Table 3
Oligonucleotide name and sequence of qRT-PCR primers.

Gene	Forward 5- > 3	Reverse 5- > 3	Acc
18 s rRNA	GGTTGCAAAGCTGAAACTTAAAGG	TTCCCGTGTTGAGTCAAATTAAGC	AF497908.1
GH	TCGACAAACACGAGACGCA	CCCAGGACTCAACCAGTCCA	KT387598.1
IgM-2	CCACTTCAACTGCACCCACT	TGGTCCACGAGAAAGTCACC	KC677037.1
SOD	CATGCCTTCGGAGACAACAC	ACCTTCTCGTGGATCACCAT	AY491056.1
Catalase	AGCTCTTCATCCAGAAACGC	GACGTCAGGCGTCACATCTT	JF801726.1

Table 4
Growth performance and feed utilization of Nile tilapia fed diet with different levels of *Silybum marianum* seeds for 70 days.

	Experimental treatments					*P* value
	Control	2.5 g kg^{-1}	5 g kg^{-1}	7.5 g kg^{-1}	10 g kg^{-1}	
Initial body weight (g fish^{-1})	2.30 ± 0.22	2.26 ± 0.31	2.36 ± 0.33	2.27 ± 0.14	2.40 ± 0.18	0.986
Final body weight (g fish^{-1})	20.70 ± 1.00^{c}	23.61 ± 0.99^{b}	23.81 ± 0.69^{b}	26.58 ± 0.96^{a}	26.25 ± 0.85^{a}	0.001
Weight gain (g fish^{-1})	18.40 ± 0.56^{c}	21.34 ± 0.87^{b}	21.44 ± 0.92^{b}	23.58 ± 0.88^{a}	24.32 ± 0.69^{a}	0.012
Specific growth rate (% day^{-1})	3.14 ± 0.04^{c}	3.35 ± 0.02^{b}	3.29 ± 0.01^{b}	3.52 ± 0.05^{a}	3.42 ± 0.02^{a}	0.001
Feed intake (g fish^{-1})	28.44 ± 2.36	29.03 ± 2.31	30.49 ± 2.09	30.26 ± 2.16	29.57 ± 2.07	0.002
Feed conversion ratio	1.55 ± 0.21^{a}	1.36 ± 0.15^{b}	1.42 ± 0.12^{b}	1.24 ± 0.18^{c}	1.23 ± 0.19^{c}	0.045
Apparent protein utilization %	57.48 ± 3.65^{b}	66.00 ± 2.21^{a}	63.07 ± 2.69^{a}	67.66 ± 3.12^{a}	66.46 ± 2.44^{a}	0.002
Protein efficiency ratio	2.33 ± 0.01^{c}	2.54 ± 0.02^{b}	2.42 ± 0.02^{b}	2.77 ± 0.01^{a}	2.78 ± 0.02^{a}	0.001
Survival rate%	92.33 ± 2.1^{b}	96.00 ± 1.5^{a}	97.00 ± 2.3^{a}	98.00 ± 1.6^{a}	97.65 ± 2.2^{a}	0.012

Means followed by different letters in the same row are significantly different ($P < .05$).

Table 5
Proximate composition (g kg^{-1}) of Nile tilapia fed diet with different levels of *Silybum marianum* seeds for 70 days.

	Experimental treatments					P value
	Control	2.5 g kg^{-1}	5 g kg^{-1}	7.5 g kg^{-1}	10 g kg^{-1}	
Dry matter	230.60 ± 0.78	225. 7 ± 0.82	245. 4 ± 0.96	216. 1 ± 0.88	220.4 ± 0.96	0.086
Protein content	568.80 ± 1.12^{c}	568.82 ± 1.85^{c}	569.60 ± 1.75^{c}	578.70 ± 1.11^{b}	588.82 ± 0.96^{a}	0.012
Ash content	150.00 ± 1.17^{c}	169.52 ± 1.85^{b}	172.50 ± 1.57^{b}	180.00 ± 2.01^{a}	177.50 ± 1.19^{a}	0.0001
Lipid content	206.40 ± 1.12^{a}	162.60 ± 1.11^{b}	168.00 ± 0.98^{b}	151.80 ± 0.97^{c}	133.50 ± 2.01^{d}	0.0001

Means followed by different letters in the same row are significantly different ($P < .05$).

use.

2.6.2. Differential expression analysis of genes by quantitative real time PCR (qRT-PCR)

The relative transcripts amount of growth hormone (GH), catalase (CAT), superoxide dismutase (SOD) and immunoglobulin M-2 (IGM-2) genes using the Step One Plus real time PCR system (Applied Biosystems). The mRNA level was measured using specific primers designed for these five genes (Table 3). Triplicate PCR reactions were carried out for each analyzed sample in addition to non-template control (NTC) and cDNA template negative. Each PCR reaction consisted of, 2.5 µl of cDNA (except for NTC), 12.5 µl SYBR Green PCR Master Mix (QuantiTect SYBR Green PCR Kit, Qiagen Cat. no. 204143), 0.3 µM of each forward and reverse primer, 1 µl RNase inhibitor and RNase-Free water to a final volume of 25 µl. Reactions were then evaluated on an Applied Biosystem 7500 Real time PCR Detection system under the following conditions: 95 °C for 15 min and 40 cycles of 95 °C for 30 s followed by 60 °C for 1 min. The fluorescence monitoring occurred at the end of each cycle and finally 95 °C for 15 min for melting temperature analysis. *18 s rRNA* gene was used as reference gene for qPCR data normalization. All experimentally induced changes in the expression of the studied genes are presented as n-fold changes relative to the corresponding controls. Relative gene expression ratios (RQ) between treated and control groups were calculated using the formula: $RQ = 2^{-\Delta\Delta CT}$ (Livak and Schmittgen, 2001).

2.7. Calculations and statistical analysis

Initial body weight (g) (IBW) and final body weight (g) (FBW) of individual fish were recorded for all fish from each aquarium at the initiation and the termination of the experiment. Weight gain (WG) = final weight (g) – initial weight (g).

Specific growth rate (SGR) = Ln W_2 – Ln W_1/t, Where, Ln = the natural log; W_1 = initial fish weight, W_2 = final fish weight in grams and t = period of study.

Feed conversion ratio (FCR) = Feed intake (g) / weight gain (g).

Protein efficiency ratio (PER) = Weight gain (g) / protein ingested (g).

Survival rate percentage (SR) = 100 x (total number of fish at the end of the experiment / total number of fish at the start of the experiment).

Apparent protein utilization (APU) = 100× (protein gain/ protein in diet).

All the collected data were subjected to one-way analysis of variance, ANOVA (SAS, version 6.03, Soft Inc., Tusla, OK, USA, SAS, 1993). Duncan's multiple range test was used to compare differences among treatment means when significant F values were obtained (Duncan, 1955) at a level of significance of $P < .05$. The values are expressed as means ± standard error.

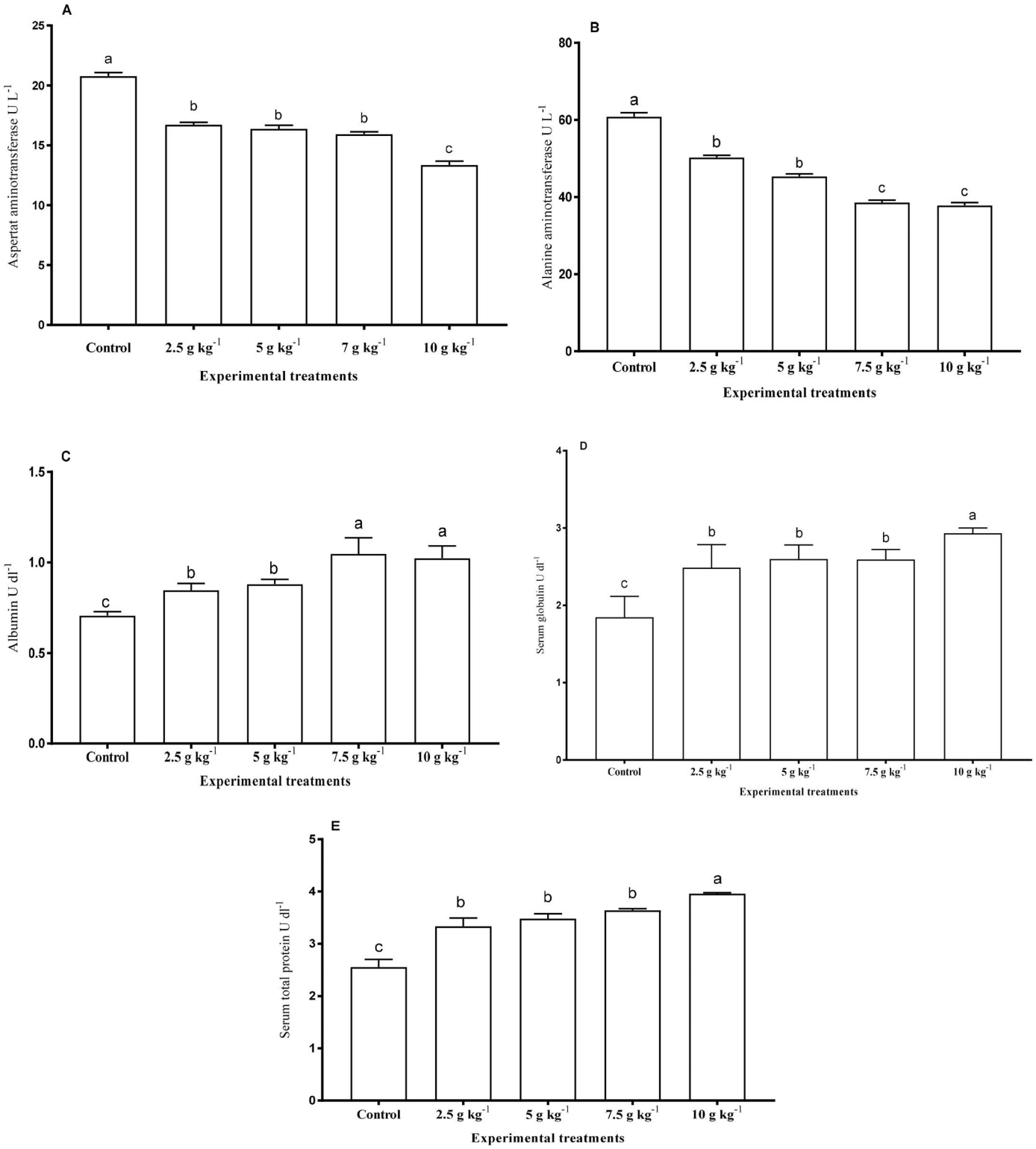

Fig. 2. (A, B, C, D, E): Serum biochemical analysis of different treatments; A: Aspartate amino transferase (ALT) U L^{-1}; B: Alanine amino transferase (ALT) U L^{-1}; C: Albumin U L^{-1}; D: Globulin U L^{-1} and E: Total protein U L^{-1}. Different letters in columns indicate significant differences among treatments ($P < .05$).

3. Results

3.1. Growth performance and feed utilization

Survival rate of fish fed diets supplemented with different levels of silymarin was significantly ($P < .05$) higher than control diet (Table 4). Application of dietary silybium improved growth performance and feed utilization of Nile tilapia (Table 4). Fish fed diet supplemented with 7.5 and 10 g kg^{-1} diet *S. marianum* recorded the highest FBW, WG, SGR, PER and APU compared to the control diets. The results showed that feed intake did not differ significantly ($P > .05$) among all treated groups, while, fish fed 7.5 and 10 g kg^{-1} diet *S. marianum* significantly ($P < .05$) recorded the best FCR values compared to the control diet.

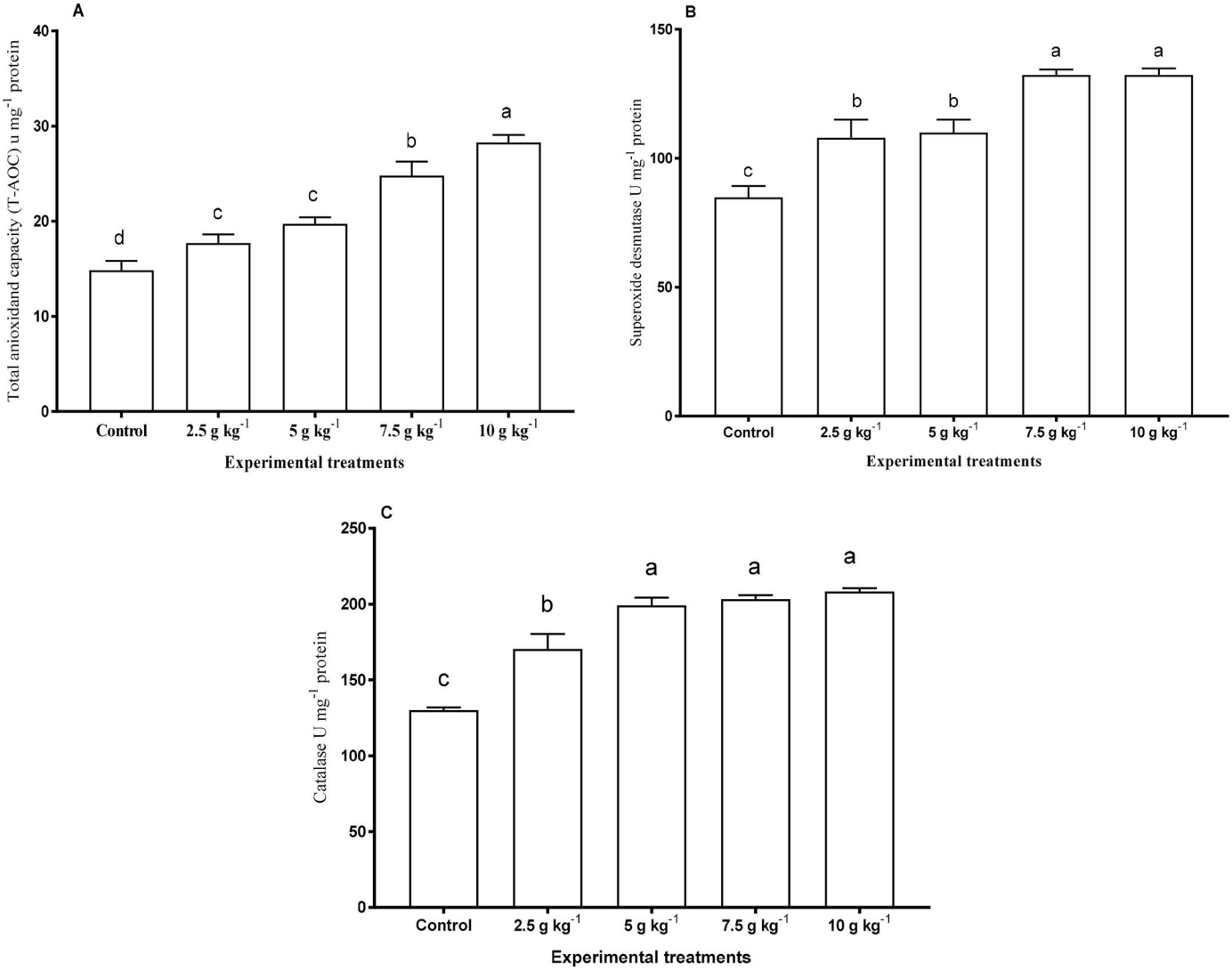

Fig. 3. (A, B and C): Antioxidant enzymes activities of Nile tilapia liver tissue fed control and different groups of *Silybum marianum* seeds diets; A: Total antioxidant capacity (T-AOC) u mg^{-1} protein; B: superoxide dismutase (SOD) u mg^{-1} protein; C: Catalase (CAT) u mg^{-1} protein. Different letters in columns indicate significant differences among treatments ($P < .05$).

3.2. Proximate compositions of whole-body fish

The chemical compositions of whole *O. niloticus* are presented in Table 5. No significant difference ($P > .05$) was found in dry matter content of the whole fish body. Protein and ash content were increased significantly ($P < .05$) with increasing level of *S. marianum* in fish diets. The highest protein and ash contents were observed in fish fed diets containing high level of silymarin (7.5 and 10 g kg^{-1} *S. marianum*). Lipid content was decreased significantly ($P < .05$) with increasing level of *S. marianum* in fish diets.

3.3. Serum biochemical parameters

Serum alanine aminotransferase (ALT) and aspartate amino transferase (AST) activities were decreasing with the increasing levels of *S. marianum* supplementation. Lowest level of AST and ALT was recorded in fish fed diet supplemented with10 g kg^{-1} diet *S. marianum* (Fig. 2 A and Fig. 2 B). The serum albumin, globulin and total protein contents of fish were affected by dietary *S. marianum* seeds supplementation (Fig. 2 C, D and E). The serum total protein and globulin content was markedly ($P < .05$) higher in fish fed either 7.5 or 10 g *S. marianum* seeds kg^{-1} diet than other experiment diets. However, the highest serum albumin was recorded in fish fed diets supplemented with 7.5 g and 10 g *S. marianum* seeds kg^{-1} diet with insignificant deference (Fig. 2 C).

3.4. Activities of antioxidant enzymes

Total antioxidant capacity (T-AOC) was significantly ($P < .05$) elevated in all treated diets that contained *S. marianum* compared to the control diet (Fig. 3 A). The highest T-AOC activity was obtained by fish fed diet supplemented with 10 g kg^{-1} diet *S. marianum*. Furthermore, there was no significant ($P > .05$) difference in T-AOC activity between diets supplemented with 2.5 and 5 g kg^{-1} diet. Fish fed either 7.5 or 10 g kg^{-1} *S. marianum* diet showed significantly increased activity of superoxide dismutase (SOD) than other diets (Fig. 3 B). No significant ($P > .05$) difference was found in SOD activity for fish fed diets supplemented with 2.5 or 5 g kg^{-1} diet *S. marianum*. There was an increasing tendency of catalase (CAT) activity of fish with increasing dietary *S. marianum* seeds (Fig. 3 C). The highest CAT activity was observed in fish fed diet supplemented with 5, 7.5 and 10 g kg^{-1} diet *S. marianum* which did not differ significantly ($P > .05$).

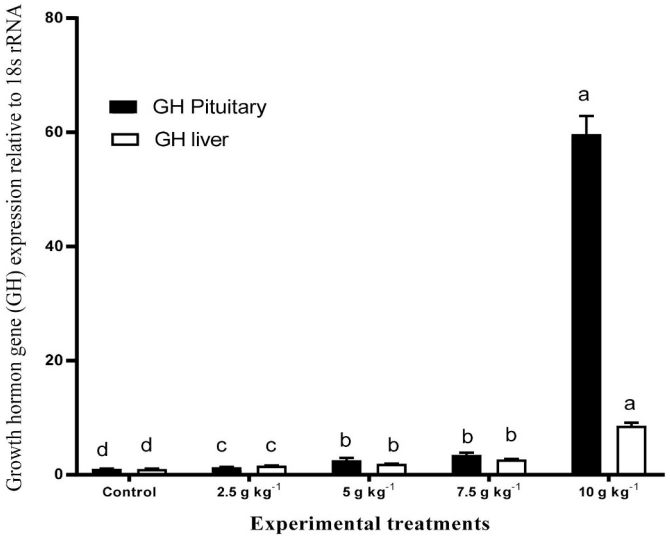

Fig. 4. The growth hormone (GH) of Nile tilapia fed control and different groups of *Silybum marianum* seeds using qRT-PCR. Different letters in columns indicate significant differences among treatments ($P < .05$).

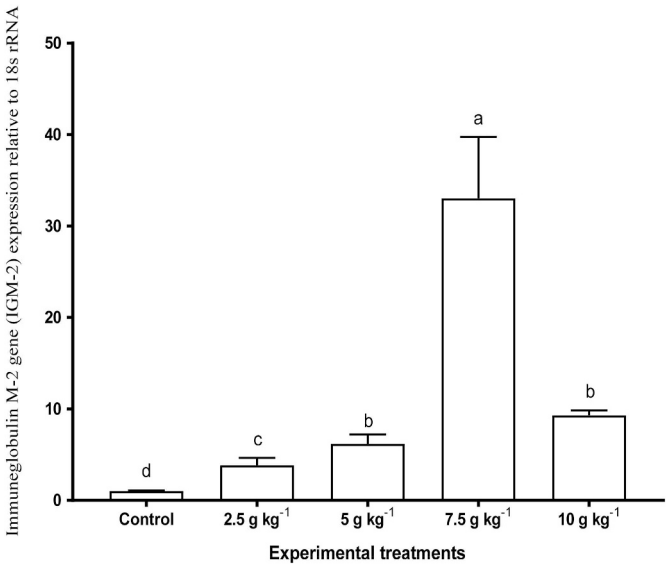

Fig. 5. The expression profiling genes of immunoglobulin heavy chain-2 gene (IGM-2) of Nile tilapia fed control and different groups of *Silybum marianum* seeds using qRT-PCR. Different letters in columns indicate significant differences among treatments ($P < .05$).

3.5. Genes expression by quantitative real time PCR (qRT-PCR)

Relative growth hormone (GH) gene expression was significantly ($P < .05$) up-regulated in liver and pituitary of fish fed different levels of *S. marianum* seeds as compared with fish fed control diet after 10 weeks (Fig. 4). Furthermore, the highest transcripts accumulation of GH was detected in pituitary of fish fed diet supplemented with 10 g kg^{-1} diet *S. marianum*. Correlation analysis showed that GH gene expression in liver and pituitary were positively correlated to weight gain ($r = 0.892$, $P < .024$; $r = 0.759$, $P < .046$), respectively.

Relative immunoglobulin M-2 (IGM-2) gene expression was highest in liver of fish fed diet supplemented with 7.5 g kg^{-1} diet *S. marianum* (Fig. 5). No significant difference was found in the relative expression of the IGM-2 gene between 5 and 10 g kg^{-1} diet *S. marianum* seeds. The superoxide dismutase (SOD) gene was significantly ($P < .05$) higher up-regulated in both 7.5 and 10 g kg^{-1} diet *S. marianum* than other groups (Fig. 6 A). No significant difference was showed in the relative expression of the SOD gene between 5 and 7.5 g kg^{-1} diet *S. marianum*. Expression of the catalase (CAT) gene was significantly ($P < .05$) increased with increasing levels of *S. marianum* seeds supplementation, and the highest up-regulated expression was detected in fish fed diet supplemented with 10 g kg^{-1} diet *S. marianum* seeds (Fig. 6 B).

4. Discussion

Using natural herbs or their extracts as feed additive is becoming useful for fish feeding, act as antioxidant, growth promotion, appetite stimulation and immunostimulants of fish due to the bioactive components, such as flavonoids, phenolics or essential oils, and pigments (Citarasu, 2010; Chakraborty et al., 2014; Ahmadi et al., 2012; Cao et al., 2016; Hassaan and Soltan, 2016). In the present study, dietary *S. marianum* seeds addition resulted in higher FBW, WG and SGR, indicating that *S. marianum* seeds improved growth performance. A significant growth promoting effect of 7.5 or 10 g *S. marianum* seeds kg^{-1} diet may be attributed to the high level of flavonolignans (total silymarin) as antioxidant (92.25 and 123 mg kg^{-1} dry weight, respectively) that stimulated protein synthesis via enzymatic system (Banaee et al., 2011). Furthermore, the lowest FCR and highest PER were detected for fish fed diet supplemented with *S. marianum* seeds compared to other diets. These results herein may be attributed to the presence of bioactive components in *S. marianum* that not only promotes feed efficiency, but also influences protein retention. In this context, Citarasu (2010) reported that active components of plant extract could improve the digestibility and the availability of nutrients resulting in an increase in feed utilization and leading to higher protein synthesis.

Following the same pattern, Xiao et al. (2017) and Jia et al. (2013) found beneficial effect of silymarin extract on growth performance and feed utilization efficiency in grass carp, *Ctenopharyngodon idellus* and common carp, respectively. On the other hand, supplementation of silymarin extract or flavomycin did not have any effects on growth performance or feed utilization of *Carassius auratus gibelio* (Yi et al., 2012). These discrepancies in results may be related to different fish species and culture conditions or different ingredients of diets. In addition, there was closed correlation between GH gene expression and growth performance in this study. The highest growth performance value was recorded by fish fed diet supplemented with *S. marianum*, also the relative expression of GH gene mRNA (Fig. 4) exhibited the same trend. This finding indicated that, *S. marianum* can exert a positive modulating effect on the transcription of GH gene which in turn might be involved in hyperplastic and hypertrophic muscular growth, Our results are in agree with Sruthi et al. (2018) and Midhun et al. (2016) who noted that the relative expression of GH gene was significantly up-regulated in tilapia fed diet supplemented with curcumin. Furthermore, previous study showed that several fish species have been made transgenic for GH recorded a significantly enhanced somatic growth via muscle hypertrophy and hyperplasia (Fuentes et al., 2013). Increasing of protein synthesis in muscle of rainbow trout is positively correlated with the increasing of GH which is a process absolutely required for muscle hypertrophy (Fauconneau et al., 1996). It has been reported that the whole-body composition could reflect fish quality and is affected by several factors, such as feed composition and feeding mode (Fauconneau et al., 1993; De Francesco et al., 2004). Our results revealed that dietary *S. marianum* in fish diets significantly increased the protein content of whole body of *O. niloticus*. However, lipid content was significantly decreased with increasing dietary level of *S. marianum*. It seems that *S. marianum* seeds which contained high level of silymarin play important roles in lipid accumulation in cell. In this context, Xiao et al. (2017) reported that dietary silymarin could decrease lipid accumulation during adipocyte differentiation in grass carp. Further studies are needed to provide information of the mode of action of dietary *S. marianum* to decrease lipid accumulation in tissues.

Normally, AST exists in hepatocyte mitochondria, while ALT is

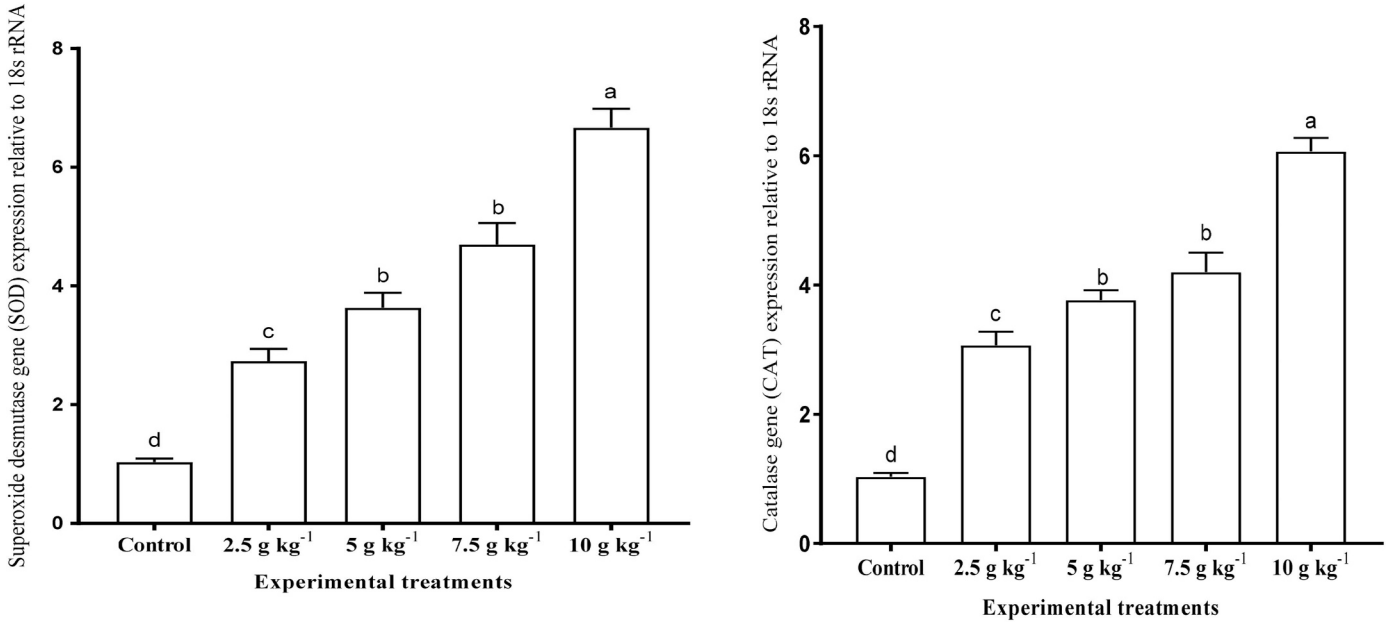

Fig. 6. The expression profiling genes of SOD and catalase (CAT) of Nile tilapia fed control and different groups of *Silybum marianum* seeds using qRT-PCR. Different letters in columns indicate significant differences among treatments ($P < .05$).

spread around the hepatic cells and bile duct. The increasing activities of serum AST and ALT in fish may reveal the leakage of enzymes across damaged plasma membranes and/or rising synthesis of enzymes by the liver tissue (Yang and Chen, 2003). Thus, the activity of serum AST and ALT are used as important indicators to reflect the health of liver and their functions of the fish (Zhai et al., 2014) as well as it can be used to assess the health status and as stress indicators in fish (Satheeshkumar et al., 2010). In the present study, serum ALT and AST activities were decreased with increasing dietary level of *S. marianum* seeds indicated that a better trend was occurred in the liver cells of tilapia. Also, the lowest level of AST and ALT may be related to the strong antioxidant of silymarin activity and their hepatoprotective properties which led to prevent lipid peroxidation of cell membranes and finally suppress the release of liver damage enzymes such as AST and ALT into plasma as stated previously by Kvasnicka et al. (2003) and Akrami et al. (2015). In this context, natural antioxidant flavonoids such as catechin and silybin improved the liver function and enhanced its ability against oxidative stress and tissue damage (Davila et al., 1989). Also, addition of extract silymarin from *S. marianum* in diets regulated ALT and AST activities in plasma of rainbow trout (Banaee et al., 2011).

Generally, increases in the levels of serum total protein, albumin and globulin in fish are thought to be related with a stronger innate immune response (Bernet et al., 2001). The results herein detected the potential enhancement of "total protein, albumin and globulin" in fish fed all diets with *S. marianum*, indicating that the increase in serum total protein level probably reflects the increase in the protein synthesis in liver tissue with close relationship. This enhancement may be related to the enhancing effect of silymarin flavonolignans on the formation of ribosomes and stimulation of DNA and protein synthesis in liver tissues, which subsequently increase the content of serum albumin which indicates that fish are immunologically strong (El-Kamary et al., 2009; Akrami et al., 2015). In the same context, serum albumin level of juvenile rainbow trout, *Oncorhynchus mykiss* was higher in fish fed diet supplemented with 0.1 g kg^{-1} of silymarin extract than control group (Ahmadi et al., 2012). Silymarin has an antioxidant activity and radical scavenging role which support its function as cytoprotection activities (Karimi et al., 2011). The possible known mechanisms of action of silymarin protection are blockade and adjustment of cell transporters, p-glycoprotein, estrogenic and nuclear receptors. Using yellow leader and Japanese honeysuckle (Ardo et al., 2008), ginger, mistletoe, stinging nettle (Dügenci et al., 2003) and fenugreek seed meal (Roohi et al., 2017) increase the serum level of total protein. The expression levels of IGM-2 in hepatic cells (Fig. 5) were up-regulated in the presence of *S. marianum* up to 7.5 g kg^{-1} diet. The elevated IGM-2 is indicator for the enhancement of the immunologically state in fish fed *S. marianum*, which may aid in innate immune system and resisting pathogenic injuries. There is limited information on the effects of *S. marianum* seeds or their extract on the gene expression of IGM-2, thus we need further studies in this aspect. The antioxidant defense systems include a series of antioxidative enzymes that is susceptible to attack by reactive oxidative stress (ROS) (Lewis-McCrea and Lall, 2007). Antioxidant defense systems including antioxidant enzymes represent protection against oxidative damage in tissue (Halliwell and Gutteridge, 1990; Sharawy et al., 2017). It known that antioxidant enzymes in fish are usually affected by nutritional factors. Cellular total hepatic antioxidant capacity (T-AOC) is critical to ensure physiological functions and protection against free radicals. Superoxide dismutase (SOD) production and catalase (CAT) activity are widely used as non-specific immune indices in fish as well as their activities are key indicators of the antioxidant capability of cells (Shiau et al., 2015). The present study showed evaluation in the activities of T-AOC, SOD and catalase (CAT) as well as gene expression of main enzymatic antioxidants such as superoxide dismutase and catalase (Fig. 6). In the present study, T-AOC, SOD and CAT were gradually increased with increasing *S. marianum* level in fish diets. Moreover, mRNA expression examination suggested that up-regulated level of SOD and CAT in fish fed diet supplemented with *S. marianum* led to increase the activities of oxidative enzymes. This finding herein was attributed to the presence of flavonoids and vitamin E in the active silymarin compound in *S. marianum* seeds which have highly efficient to scavenge free radical within tissues (Doehmer et al., 2008). Silymarin has medical property as an "antioxidant, anti-inflammatory, anti-carcinogenic and anti-fibrotic properties" as well as Silymarin affect on the cellular-reduced glutathione (GSH) content, cellular protein biosynthesis potency and increased stability of the cellular membrane in liver (Banaee et al., 2011). Moreover, Chand et al. (2011) revealed that the active silymarin in *S. marianum* seeds significantly elevated the antioxidant status of liver and it has direct effects on immune cells. In addition, Astuya et al. (2017) noted that the extracted phenolic compounds from *Pinus radiata bark* significantly reduced the relative expression of the pro-inflammatory enzymes of

salmonid cell lines. Results of similar studies indicated that other phenolic compounds have positive effects on SOD activities in oxidative stress conditions (Subash and Jayanthi, 2010). Our findings herein are concurred with Wang et al. (2018) who demonstrated that the antioxidant ability of Japanese seabass, *Lateolabrax japonicus* improved by Chinese herbal medicines mixture supplementation expressed by the higher CAT, SOD and T-AOC activities (Chen et al., 2013; Elabd et al., 2016). Nonetheless, Banaee et al. (2015) demonstrated a significant effect of silymarin extract treatment as a hepatoprotective agent in oxidative stress of fish. Our results are consistent with Subash and Jayanthi (2010) who indicated that phenolic compounds have positive effects on the CAT enzyme activity in oxidative stress conditions. Similarly, Nazeri et al. (2017) observed the highest CAT enzyme activity of *Oncorhynchus mykiss* when fed diet containing rutin. Previous studies have been investigated on the effects of *S. marianum* seeds or their extract as dietary supplementation on the antioxidant status of fish but there is no study showed their effects on antioxidant enzymes genes such as SOD and CAT. On the other hand, hepatic gene expressions of 3-Hydroxy-3-methylglutaryl-CoA reductase was detected for grass carp, *Ctenopharyngodon idellus* fed diet contained silymarin compared to control diet (Xiao et al., 2017). Dietary administration of inulin evaluated the CAT antioxidant genes expression of common carp, *Cyprinus carpio* (Hoseinifar et al., 2017a). Antioxidant gene expression such as SOD and CAT in common carp fed diet supplemented with palm fruit extracts was higher than control diet (Hoseinifar et al., 2017b).

In conclusion, our results indicated that supplementation of diets with *S. marianum* is beneficial for tilapia as growth promoting, modulating the immune response and antioxidant enzymes capacity due to the high active silymarin flavonolignans contents in *S. marianum*. Further studies are needed for gene expression investigation to detect the mode of action of silymarin on the health statues of fish.

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