Animal Feed Science and Technology 290 (2022) 115369

Contents lists available at ScienceDirect

Animal Feed Science and Technology

journal homepage: www.elsevier.com/locate/anifeedsci

Can dietary phytogenic mixture improve performance for growth, digestive enzyme activity, blood parameters, and antioxidant and related gene expressions of Nile tilapia, *Oreochromis niloticus*?

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ARTICLE INFO

Keywords:
Growth
Phytogenics
Bioactive compounds
Oxidative stress
Gene expression
Nile tilapia

ABSTRACT

The form of dietary phytogenic inclusion and its physiological causal mechanisms for growth promotion and immune stimulation in fish remain unknown. The study examined the effects of dietary phytogenic mixture extracted from lemon (*Citrus limon*), onion (Allium cepa), and garlic (*Allium sativum*) (LOG) on Nile tilapia performance, digestive enzymes, haemato-biochemical indices, oxidative stress, and associated gene expression for 70 days. In this experiment, diets were supplemented with 0, 10, 20, and 30 ml LOG kg^{-1} in isonitrogenous and isoenergetic diets and fed to tilapia with an average initial body weight (4.23 ± 0.09 g). Compared to the control diet, the dietary LOG at 20 ml kg^{-1} elicited the highest final body weight (FBW, 35.50 g $fish^{-1}$), weight gain (WG, 31.2 g $fish^{-1}$), specific growth rate (SGR, 3.02%, day $fish^{-1}$), and survival rate (99.33%). Significant quadratic differences in chymotrypsin, trypsin, amylase, and lipase were shown with increasing LOG supplementation. There was a quadratic response in hematology parameters of fish with increasing LOG levels. Significant linear decreases in ALT, AST, cholesterol, and triglyceride were shown with the increased LOG inclusion in the diets. A polynomial correlation in total protein, albumin, and globulin was found under different inclusion levels of LOG while significant quadratic increases in SOD, CAT, and Gpx and significant quadratic decrease in MDA was found with increasing LOG supplementation. The IGM-2, SOD, and CAT gene expressions were quadratically improved; the highest relative expression was obtained by fish received 20 ml LOG kg^{-1} diet. Growth hormone gene expression was quadratically modulated in the liver and pituitary of fish fed diverse doses of dietary LOG compared with the control. The phytogenic of LOG at 20 ml kg^{-1} elicited the best tilapia performance and hematological

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https://doi.org/10.1016/j.anifeedsci.2022.115369
Received 6 October 2021; Received in revised form 11 June 2022; Accepted 19 June 2022
Available online 22 June 2022

indices, increased antioxidant and digestive enzyme activities, and gene expressions of growth, immunoglobulin and superoxide dismutase.

1. Introduction

Aquaculture has been considered the fastest animal protein producing sector, however, the intensification of fish culture is urgently needed to maintain and increase aquaculture production to fulfill human needs (FAO, 2018). A parallel trend of intensification of aquaculture is the decline in growth performance, depression of immune system, and high susceptibility to infectious or inflammatory diseases (Hassaan et al., 2014; FAO, 2016; Omitoyin et al., 2019). Hence, improving fish performance and controlling diseases is the most important target in intensive aquaculture (Hassaan et al., 2015, 2018, 2019a; Baba et al., 2016, 2019b).

Over the last few years great attention was being given on the effects of inclusion of phytogenics as growth promoters and immune stimulants in aquaculture (Baba et al., 2016; Aanyu et al., 2018; Hassaan et al., 2021a). Preceding trials have been executed to evaluate the effects of phytogenics on the growth response of fish fed diets supplemented with limonene extracts from lemongrass and geranium or *Citrus limon* peels essential oil, carvacrol or thymol, essential oil (EO) extract from lemon fruit peels (Ngugi et al., 2017) for enhancing growth performance and immune status of fish (Giannenas et al., 2012; Baba et al., 2016; Aanyu et al., 2018; Al-Sagheer et al., 2018). Previous results stated that not all phytogenic compounds have optimistic impacts on fish performance and immune response of fish. For example, Shalaby et al. (2006) and Kesbiç et al. (2020) stated an enhancement of weight gain of tilapia fed diets contained garlic powder or bergamot (*Citrus bergamia*) peel oil. Moreover, the inclusion of dietary dried lemon (*Citrus lemon*) pomace into common carp (*Cyprinus carpio*) diets have improved fish growth indices (Laein et al., 2021). On the contrary, Mesalhy et al. (2008)

Table 1
Bioactive compounds identified from the LOG mixture by gas chromatography/mass spectrometry.

No	RT (Time)	Name	Area sum %
1	7.31	1- pyrroline,2-pheyle	31.08
2	9.64	Thiazolidine, 2-amino-N-aminoformyl-	0.44
3	9.67	D-Limonine	0.50
4	9.8	2- Methylthioiane	0.56
5	10.28	D-isoascorbic acid	0.52
6	10.41	Dianhydromannitol	0.71
7	10.84	2-Dodecanol	0.63
8	11.28	Gardnin	2.39
9	11.64	2- Hexadecanol	0.37
10	11.83	2′- Hydroxy-3,4,4′,6′-tetramethoxychacore	0.41
11	12.06	Luteoline	0.67
12	12.30	Geranyl isovalerate	0.53
13	12.24	β Carotene	0.32
14	12.46	Corymbolone	0.46
15	12.63	Dihydro- β- agarofuran	0.34
16	12.70	22- Tricosenoic acid	0.57
17	12.93	Caryophyllene oxide	5.3
18	13.66	Retinol	0.41
19	13.81	Phytanic acid	2.72
20	14.08	Hexa- hydro- farnesol	2.12
21	14.44	Phytol	1.04
22	14.63	Heptacosane	1.59
23	14.78	5 β,7 βH,10 α-Eudesm-11-en-1 α -ol	2.71
24	14.93	Apigenin 8-C-glucoside	2.69
25	15.35	Cis-9-Hexadecenoic acid	3.08
26	15.66	(s)-(-)-Citronellic acid	0.52
27	15.82	Squalene	2.34
28	16.31	Vitamin E	0.45
29	16.40	Palustrol	0.83
30	16.66	5,7- Dimethoxyflavone	4.5
31	16.96	Longiborneol	3.55
32	17.40	Ascaridole	0.53
33	17.49	Citronelly tglate	4.7
34	18.12	Shyobunone	0.50
35	18.27	Patchoulialcohol	1.7
36	18.59	(+)-Valeranone	1.2
37	19.79	Farnesane	0.46
37	19.82	4,5-dithia-1,7-octadiene	1.3
38	19.43	Thunbergol	1.4
39	19.91	Valeranone, (+)-	3.35
40	21.35	Elaidic acid	10.03
41	23.12	Octacosanol	0.48

found no enhancement of growth with different doses of garlic. These contradictions are mainly attributed to the chemical composition of bioactive compounds and the dose in the plant medicinal products (Chakraborty et al., 2014). Rainer and Lea (2015) found that inclusion a mixture of natural growth promoter compounds shows higher growth-boosting power than a single phytogenic compound because of the symbiotic effects of the interlinkage between bioactive molecules in the different phytogenic products (Costa et al., 2013). In general, several factors such as the type of phytogenic compounds, quantity, length of the growth trial, and species of fish elicit different performance responses in fish. More research is needed to identify the optimum dose of phytogenic compounds that can be functional in diverse species to improve growth and immune response. In Nile tilapia, the form of phytogenic inclusion in the diet (i.e., in the form of single phytogenic compound or in combination/mixture with other phytogenics) and the phytogenics' physiological causal mechanisms for growth promotion and immune stimulation remain unknown. Hence, the main objective of the study was to identify the optimum dose of mixture phytogenic compounds (i.e., lemon, onion, and garlic) that promote growth of Nile tilapia and immune system stimulation (i.e., hematology and molecular indices responses).

2. Materials and methods

2.1. Preparation of mixture of lemon, onion, and garlic extract

Fresh lemon (*Citrus limon*), onion (Allium cepa), and garlic (*Allium sativum*) (LOG) were purchased from a local market in Cairo, Egypt. The peels were carefully collected and dried for ten days at 36 °C, then they were kept in a room at constant temperature (25ºC) and humidity (60%). Dried components of LOG were ground using an electric blender to allow a better extraction; then the bioactive compounds were obtained from fresh LOG using hydro-distillation method using a modified Clevenger type apparatus according to the method of Baba et al. (2016). Around 100, 100, and 12.5 g of lemon, onion, and garlic, respectively, were placed in a flask containing 150 ml cold 0.9% NaCl for extraction. The hydro-distillation system was heated by a Genlab heating device (China, 98-IC model 1000 ml), which was placed in a flask containing LOG and solvent. The vapor was collected using a condenser attached to the top of the flask, and the mixture was then filtered and centrifuged at 3000 g for 15 min to separate the bioactive chemicals, with the clear supernatant discarded. The aqueous bioactive extract of LOG was kept in a dark bottle at − 20 °C until used. The bioactive compounds for the LOG mixture were estimated by gas chromatography/mass spectrometry (Shimadzu, Kyoto, Japan) (Table 1). Also, the total phenolic

Table 2
Formulation and proximate composition of the basal diets (g kg diet$^{-1,}$ dry matter).

Ingredient	g kg diet^{-1}
Fish meal 65 %	100
Soybean meal 44 %	370
Corn gluten meal 62 %	60
Yellow corn 8.5 %	250
Wheat bran 14 %	120
Fish oil	40
Starch	30
Vitamin and minerals[a]	30
Chemical analysis	
Protein	303. 05
Lipid	66.2
Ash	48.71
Fiber	45.15
Neutral detergent fiber (NDF)	159
Acid detergent fiber (ADF)	97
Nitrogen free extract (NFE)[2]	536.89
Gross energy[3] MJ kg^{-1}	18.978

[a] 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 gm 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 ($FeSO_4$.7 H_2O, 20% Fe), 65 mg; manganese sulfate ($MnSO_4$, 36% Mn), 89 mg; zinc sulfate ($ZnSO_4$.7 H_2O, 40% Zn), 150 mg; copper sulfate ($CuSO_4$.5 H_2O, 25% Cu), 28 mg; potassium iodide (KI, 24% K, 76% I), [2]NFE (Nitrogen free extract) = 100-(crude protein + lipid + ash +fibre content). [3]Gross energy calculated using gross calorific values of 23.63, 39.52 and 17.15 kjg^{-1} for protein, fat and carbohydrate, respectively according to Brett (1973).

compounds (190.2 mg gallic acid equivalent L^{-1}) and total antioxidant capacity (340 mg ascorbic acid equivalent L^{-1}) were measured according to Singleton et al. (1999) and Aguilar Urbano et al. (2013), respectively.

2.2. Diets

Four experimental diets (30% crude protein, 6.62% lipid, 4.871% ash, and 18.97 MJ gross energy, estimated according to AOAC (1995)) were formulated (Table 2). The diets were supplemented with 0 (control), 10, 20, and 30 LOG ml kg^{-1} diet. The dry ingredients were ground into fine powder and combined with soybean oil, then the LOG mixture was added and thoroughly mixed. Pellets (2 mm) were prepared using a laboratory pellet mill (California Pellet Mill, San Francisco, CA, USA), and sun-dried (35 °C) for 48 h and stored at 4 °C before use.

2.3. Fish and rearing conditions

The Committee of Animal Research and Ethics guidelines and the Animal Ethics Research Committee policies of the National Institute of Oceanography and Fisheries (NIOF, Cairo, Egypt) were carefully considered and followed. The growth trial was conducted at NIOF for 70 days.

Healthy Nile tilapia, *Oreochromis niloticus* (4.22 ± 0.09 g) were collected from the NIOF fish farm. The fish were stored in two 1-m^3 tanks for 15 d for acclimation. During this period, fish were fed a commercial diet (30 % crude protein). After the 15-d acclimation period, fish were randomly allocated in sixteen 220-L^3 fiberglass tanks, with 20 fish per tank. Water volume was changed partially (20 % replacement daily). Fish were fed until apparent satiation four times daily (09:00, 11.00, 13.00, and 15.00). Water quality was monitored every week throughout the feeding trial at 15 cm depth from each tank to evaluate the water quality parameters. Dissolved oxygen (DO) and temperature (°C) were measured in-site using a portable oxygen meter (Jenway, London, UK). The pH was measured using a pH meter (Digital Mini-pH Meter, USA). Unionized ammonia (NH_3) was measured using special kits (HACH Co., Loveland, USA). The ranges of the above-mentioned parameters were 27.5–28.7 °C, 6.7–6.8 mg L^{-1}, 7.2–7.6, and 0.131–0.222 mg L^{-1} for water temperature, DO, pH, and NH_3, respectively. The water quality was maintained and kept within permissible range for Nile tilapia *O. niloticus* fingerlings according to Boyd (1990). Light regime was maintained at 12-h light; 12-h dark (08:00–20:00) by using fluorescent bulb.

2.4. Growth performance

At the end of the experiment growth performance was measured using final body weight (FBW), weigh gain (WG), specific growth rate (SGR), feed conversion ratio (FCR), protein efficiency ratio (FCR), and survival rate as mentioned by Ibrahim et al. (2021); Hassaan b) et al. (2021) and El-Badawy et al. (2021).

2.5. Measurement of digestive enzymes activity

The middle intestines were collected (n = 5 per treatment) to measure digestive enzyme activities following the methods of Hassaan et al. (2019a), (2019b). Chymotrypsin and trypsin were estimated as described by Hummel (1959) and lipase activity was measured according to Zamani et al. (2009). Amylase activity was estimated according to Bernfeld (1951).

2.6. Hematological and biochemical blood indices

Fish were anesthetized using MS-222 to collect blood samples from the caudal vein from four fish per tank to measure hematological parameters; using 10% EDTA, blood was allowed to clot at 4 °C and then centrifuged (3000 g, 5 min). First blood sample was collected using heparinized 3 ml syringes from the caudal vein of fish to determine hematological parameters. Hematocrit (Htc) estimated according to the methods of Reitman and Frankel (1957), whereas, hemoglobin (Hb), was estimated using hemoglobin kits according to the standard methods of cyanmethemoglobin. Red blood cells (RBCs) was counted using light microscope. The second blood samples were collected from the caudal vein using non-heparinized 3-ml syringes and was allowed to clot for 30 min at 4 °C then centrifuge at 4000 g for 10 min to obtain serum for estimating biochemical analysis. Liver functions such as aspartate transaminase (AST), alanine transaminase (ALT) and alkaline phosphatase (ALP) were estimated according to Reitman and Frankel (1957). The total protein and albumin were measured according to Wotton and Freeman (1974), globulin content was calculated according to the method of Coles (1974), and alkaline phosphatase, cholesterol, and triglyceride were measured using Crest Biosystems® kits.

2.7. Measurement of hepatic antioxidant enzyme activities

Liver tissues from five fish per treatment were ground in ice cold saline according to Hassaan et al. (2019a), (2019b) then centrifuged at 3000 g for 5 min. The supernatants were collected to estimate the antioxidant enzymes. Water soluble tetrazolium was used to estimate superoxidase dismutase (SOD) activity according to the method of Peskin and Winterbourn (2000). The catalase (CAT), glutathione peroxidase (GPx), and malondialdehyde (MDA) activities were measured as described by Dogru et al. (2008).

2.8. Gene expressions

2.8.1. Total RNA extraction and cDNA synthesis

At the end of the feeding trial, the livers and pituitary were excised from five fish randomly obtained from each treatment, then immediately ground 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 included in the QuantiTect® Reverse Transcription Kit, according to the manufacturer's recommendations. Reverse transcription 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 used.

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

The relative transcripts amount of growth hormone (GH), CAT, SOD, and immunoglobulin M-2 (IGM-2) genes were analyzed using the Step One Plus real time PCR system (Applied Biosystems). The mRNA level was measured using specific primers designed for the 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. The 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).

2.9. Statistical analysis

Polynomial regression contrasts were used to measure the regression between various levels of LOG on the response parameters. The SAS package software was used for statistical analysis at significant levels of $P \leq 0.05$.

3. Results

3.1. Growth performance

As indicated in Table 4, dietary LOG supplementation quadratically improved FBW ($P = 0.0006$), WG ($P = 0.0006$), SGR ($P = 0.0007$), and survival rate ($P = 0.0006$). Compared to the control diet, the dietary LOG at 20 ml kg^{-1} elicited the highest growth response, including, final body weight (FBW, 35.50 g $fish^{-1}$), weight gain (WG, 31.2 g $fish^{-1}$), specific growth rate (SGR, 3.02%, day $fish^{-1}$), and survival rate (99.33%).

3.2. Activities of endogenous enzymes

Fig. 1(a, b, c and d) shows the quadratic ($P < 0.05$) regression of chymotrypsin, trypsin, amylase, and lipase activities ($P = 0.026$, $P = 0.023$, $P = 0.013$, and $P = 0.045$, respectively) with different doses of LOG. The optimum LOG levels to achieve the optimum activities of chymotrypsin (5.5 mg protein), trypsin (1.7 mg protein), amylase (730 mg protein), and lipase (1000 mg protein) was at 20 ml kg^{-1} diet.

3.3. Hematological and serum biochemical indices

Hematological and serum biochemical parameters are shown in Table 5. There was a quadratic response in hematocrit (Htc), hemoglobin (Hb), and red blood cells count (RBC) as the level of LOG increased in the diet ($P = 0.042$, $P = 0.049$, $P = 0.041$, and $P = 0.015$, respectively). In comparison to the control treatment, the group fed dietary LOG at level 20 ml kg^{-1} had the highest Htc

Table 3
Oligonucleotide names and sequences 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 diets with different levels of LOG.

Parameters	Control	Phytogenic			*P-Values*	
		10 ml LOG kg^{-1}	20 ml LOG kg^{-1}	30 ml LOG kg^{-1}	Linear	Quadratic
IBW[a] (g $fish^{-1}$)	4.20	4.14	4.30	4.26	0.09	0.5778
FBW[a] (g $fish^{-1}$)	28.83	32.97	35.50	29.17	0.545	0.002
WG[b] (g $fish^{-1}$)	24.63	28.83	31.2	24.91	0.689	0.006
SGR[c] (%, day $fish^{-1}$)	2.75	2.96	3.02	2.75	0.091	0.001
FCR[d]	1.62	1.35	1.28	1.67	0.254	0.025
PER[e]	2.06	2.47	2.60	2.00	0.251	0.042
Survival rate[f] (%)	97.00	99.00	99.33	97.33	0.630	0.016

[a] FBW = Final body weight (y) = $-3.79x^2 + 19.048x + 10.75$; $R^2 = 0.8978$
[b] WG = Weight gain (y) = $-3.815x^2 + 19.127x + 3.48$; $R^2 = 0.9065$
[c] SGR = Specific growth rate (y) = $-0.2175x^2 + 1.0765x + 1.2875$; $R^2 = 0.945$
[d] FCR = Feed conversion ratio (y) = $0.0017x^2$ - $0.0487x + 1.633$; $R^2 = 0.97$
[e] PER = Protein efficiency ratio (y) = $-0.0025x^2 + 0.0752x + 2.0375$; $R^2 = 0.9618$
[f] Survival rate, y = $-1.75x^2 + 9.782x + 86.085$; $R^2 = 0.9847$

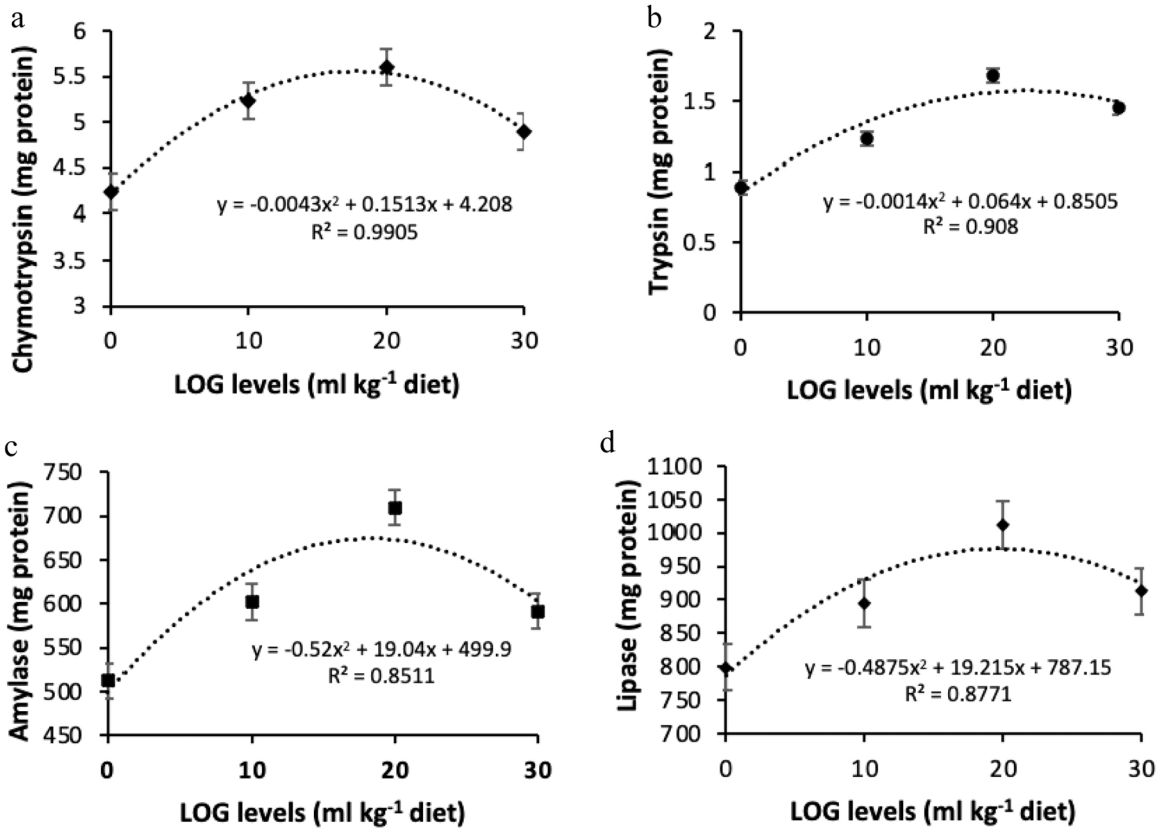

Fig. 1. Digestive enzymes: chymotrypsin, trypsin, amylase, and lipase of Nile tilapia fed diets with different levels of LOG.

(24.15%), Hb (13.74 g dl^{-1}), and RBC (2.93 $\times 10^6$ cmm^{-1}). Significant linear decreases in ALT ($P = 0.039$), AST ($P = 0.025$), cholesterol ($P = 0.031$), and triglyceride ($P = 0.335$) were shown with the increased supplementation of LOG. There were quadratic responses in total protein ($P = 0.001$), albumin ($P = 0.040$), and globulin ($P = 0.005$) under different addition levels of LOG. However, adding dietary LOG at a level of 20 ml kg^{-1} to the diet resulted in the highest total protein (3.82 UL^{-1}), albumin (1.97 UL^{-1}), and globulin (1.85 UL^{-1}).

3.4. Activities of antioxidant response

Estimated SOD, CAT, Gpx, and MDA values are shown in Fig. 2(a, b, c and d). Significant quadratic increases in SOD, CAT, and Gpx but a significant quadratic decrease in MDA were found with increasing supplementation levels of LOG in the diet.

Table 5
Hematological and serum biochemical of Nile tilapia fed diets with different levels of LOG.

Parameters	Control	Phytogenic			*P-Values*	
		10 ml LOG kg^{-1}	20 ml LOG kg^{-1}	30 ml LOG kg^{-1}	Linear	Quadratic
Hematocrit (%)	20.13	22.33	24.15	22.83	0.532	0.042
Hemoglobin (g dl^{-1})	10.05	11.11	13.74	11.05	0.524	0.049
Red blood cells ($\times 10^6$ cmm^{-1})	2.33	2.42	2.93	2.35	0.521	0.015
AST[a](UL^{-1})	15.40	13.2	11.02	10.80	0.025	0.223
ALT[b] (UL^{-1})	31.17	26.73	23.83	21.02	0.039	0.402
ALP[c] (UL^{-1})	41.07	26.50	24.99	24.27	0.042	0.402
Total protein (UL^{-1})	2.85	3.58	3.82	3.06	0.091	0.001
Albumin (UL^{-1})	1.52	1.90	1.97	1.69	0.505	0.040
Globulin (UL^{-1})	1.33	1.68	1.85	1.37	0.062	0.005
Triglyceride (mmol L^{-1})	5.47	4.40	3.57	2.43	0.022	0.403
Cholesterol (mmol L^{-1})	6.57	4.83	3.97	2.20	0.031	0.335

Htc, $y = -0.13\times 2 + 0.542x + 21.73$; $R^2 = 0.9776$
Hb, $y = -0.1875x^2 + 1.0005x + 9.1425$; $R^2 = 0.4733$
RBCs, $y = -0.0425x^2 + 0.2195x + 2.1525$; $R^2 = 0.9993$
Albumin, $y = -0.165x^2 + 0.883x + 0.8$; $R^2 = 0.9994$
Globulin, $y = -0.2075x^2 + 1.0665x + 0.4475$; $R^2 = 0.9411$
Total protein, $y = -0.3725x^2 + 1.9495x + 1.2475$; $R^2 = 0.9785$
Cholesterol, $y = -0.1397x + 6.488$; $R^2 = 0.9838$
Triglyceride, $y = -0.0995x + 5.46$; $R^2 = 0.9967$
[a] ALT = alanine aminotransferase, $y = -0.159x + 14.97$; $R^2 = 0.9129$
[b] ALT = aspartate aminotransferase, $y = -0.3335x + 30.69$; $R^2 = 0.9864$
[c] ALP = alkaline phosphatase, $y = -0.6233x + 38.922$; $R^2 = 0.9229$

3.5. Gene expressions

A significant quadratic improvement in IGM-2, SOD, and CAT gene expressions were noted with the highest relative expression obtained by fish fed 20 ml LOG kg^{-1} diet (Figs. 3, 4 and 5). Relative GH gene expression was significantly ($P < 0.05$) up-regulated in the liver and pituitary of fish fed different levels of dietary LOG compared with the control (Fig. 6). The transcripts expression of the GH gene was also noted in the diet supplemented with 20 ml LOG kg^{-1}.

4. Discussion

The use of antibiotics in aquaculture poses several risks and issues such as toxicity, the organism's sensitivity to antibiotic residues, and the potential to environmental contamination, among others. These factors direct aquaculturists and nutritionists to find safe and sustainable alternatives to antibiotics such as phytogenics (Heuer et al., 2009; Baba et al., 2016). Recently, few studies evaluated phytogenic components such as limonene, thyme, lemon peel, and bitter lemon as an antioxidant and growth promoters of different fish spices (Aanyu et al., 2018; Al-Sagheer et al., 2018). To date, no information is available on the effects of combinations of the phytogenic compounds lemon, onion, and garlic on growth, digestive and antioxidant enzyme activities, and associated gene expressions in Nile tilapia. The present study showed improvement of growth response of fish fed dissimilar amounts of dietary LOG, with the best performance exhibited in fish fed 20 ml kg^{-1}. These findings are similar with Zaki et al. (2012) and Maniat et al. (2014), which showed growth enhancement in fish fed diets enriched with phytogenics, including, fenugreek meal (*Trigonella foenum-graecum*), hot pepper meal, *Thymus vulgaris*, chamomile flowers meal (*Matricaria recutita*), and garlic (*Allium sativum*). In addition, rainbow trout fed diets containing a mixture of thymol and carvacrol showed improved ($P < 0.05$) feed efficiency and growth (Giannenas et al., 2012). On the contrary, other studies showed no impacts of phytogenics on growth and feed efficiency (Kim et al., 2013; Takaoka et al., 2016). The contradicting results have been linked to the amount of active compounds, length or duration of the experiment, and fish species used, causing dissimilar growth responses (Yang et al., 2015). Consequently, it is necessary to identify the optimum doses of phytogenic compounds for different fish species and the related physiological mechanisms behind the growth promotion and immune system stimulation of phytogenic compounds. This information could aid in formulating functional foods and feeds containing phytogenic compounds and direct decision makers to develop policies for using phytogenic compounds in fish species.

The enhancement of growth response of the Nile tilapia could be attributed to the biological properties of bioactive compounds present in LOG ingredients that stimulate different physiological responses, including: increased feed intake, digestive enzyme secretion, and antioxidant status (Fox et al., 2010); improved somatotropic axis as growth, insulin, and somatomedin C hormones serve as central factors in regulating and controlling fish growth and many other physiological process (Qiang et al., 2012); effect on taste, aroma, and flavour of the diets that also affect the palatability and feed intake (Syahidah et al., 2015); effect on Neuropeptide Y hormone secretion that plays a positive function in enhancing the appetite of fish (Kiris et al., 2007); bioactive components in the phytogenics could increase growth of desirable microflora in the fish intestine and digestive enzyme activities (Ngugi et al., 2017) which boost the growth response of fish; and positive correlation between phytogenics and growth and antioxidant hormones IGM-2, SOD, and CAT gene expressions.

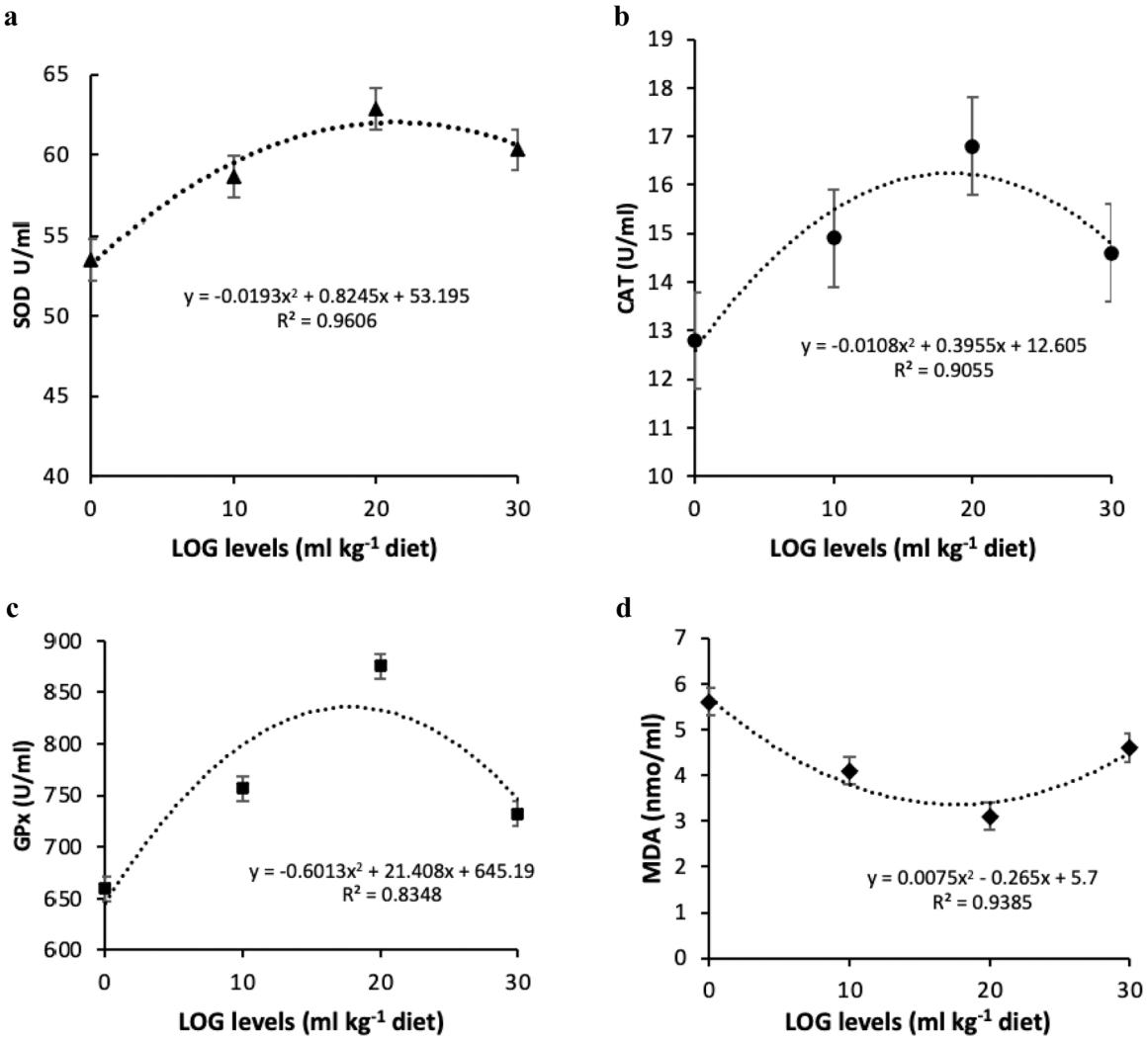

Fig. 2. Activities of antioxidant response: superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (Gpx), and malondialdehyde (MDA) of Nile tilapia fed diets with different levels of LOG.

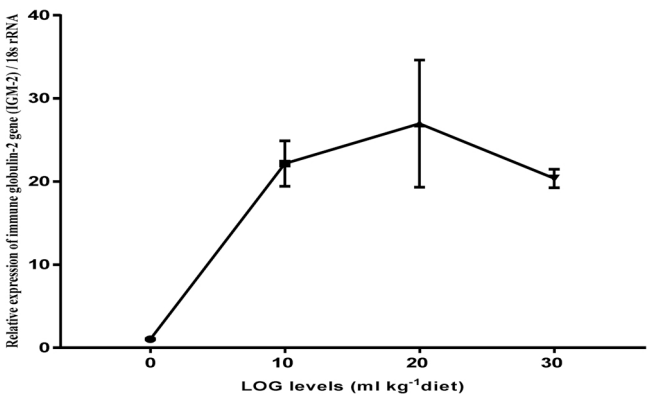

Fig. 3. Relative expression of immune globulin-2 gene (IGM-2) / 18 s rRNA of Nile tilapia, after feeding diets with different levels of LOG.

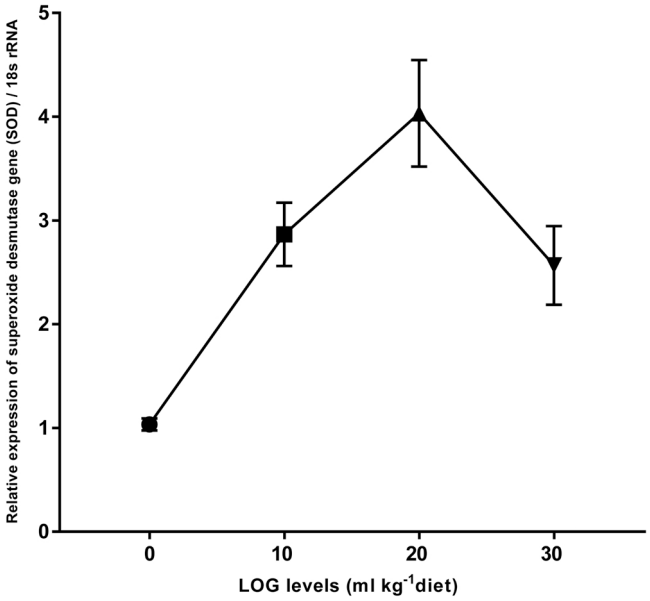

Fig. 4. Relative expression of superoxide dismutase (SOD) / 18 s rRNA of Nile tilapia, after feeding diets with different levels of LOG.

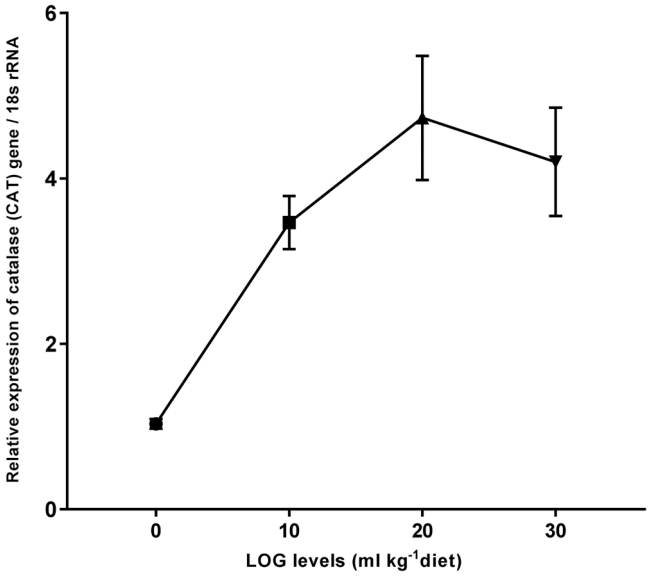

Fig. 5. Relative expression of catalase (CAT) / 18 s rRNA of Nile tilapia, after feeding diets with different levels of LOG.

Digestive enzymes play a vital role in the breakdown of nutrients to increase assimilation in the gastrointestinal tract (Hassaan et al., 2019a, 2019b). The present study showed that supplementation of 20 ml kg^{-1} LOG increased the activity of chymotrypsin, trypsin, amylase, and lipase. This might be due to the presence of short chain of fatty acids (SCFA), such as acetic, propionic, and butyric acids in phytogenic mixture (Table 2). These SCFA improve the secretion of digestive enzymes by decreasing the pH in the gastrointestinal tract which optimize the activity of digestive enzymes (Castillo et al., 2014). The essential oils in LOG could also contribute to increased digestive enzyme activities (Jang et al., 2007). Similarly, 2% lemon peel in mullet feeds improved the activities of lipase and amylase (Toutou et al., 2018). In the present study, a high level of LOG (30 ml kg^{-1}) showed an adverse effect on endogenous enzymes, partly due to the increase in the pH of gastric tract, which reduced secretion of digestive enzymes.

Monitoring of hematological parameters have been used as good indicators of fish health (Hassaan et al., 2019a, 2019b). The present research showed that all diets supplemented with LOG improved Htc, Hb and RBC. Similarly, hematological parameters were improved by supplementation of bioactive compounds (Harikrishnan et al., 2010; Acar et al., 2015; Hassaan and Soltan, 2016). Nonetheless, the dietary essential oil derived from citrus such as limon and fruit peels decreased Htc, Hb, and RBC in fish (Ngugi et al.,

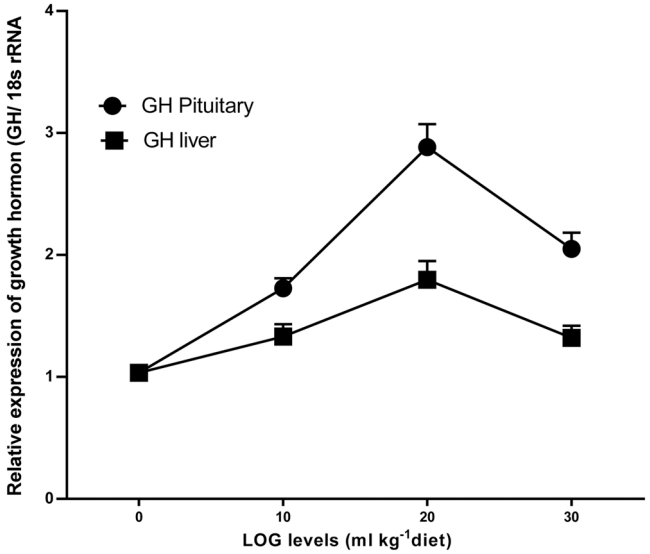

Fig. 6. Relative expression of growth hormone (GH) / 18 s rRNA of Nile tilapia, after feeding diets with different levels of LOG.

2017). The major variables of non-specific immune are serum total protein and albumin (Acar et al., 2015; Hassaan and Soltan, 2016; Hassaan et al., 2019a, 2019b).

In the present study, fish fed 20 ml LOG kg^{-1} improved the non-specific immune parameters compared with other diets. This is consistent with Baba et al. (2016) and Ngugi et al. (2017), in which fish fed different phytogenic components such as sativa oil, thyme, fennel oils, Citrus limon peels, and bitter lemon fruit peels exhibited increased values for the different hematological parameters.

The liver function parameters ALT and AST were enhanced in fish fed diet supplemented with LOG. Further research is warranted for the effect of dietary photogenics on ALT, AST and alkaline phosphatase. Triglyceride and cholesterol levels decreased with increasing LOG levels, which could be due to the presence of flavones and flavonoids compounds (Youssef et al., 2014) or short chain fatty acids (Zhao et al., 2017). Similarly, Acar et al. (2015) stated that Nile tilapia fed sweet orange peel (*Citrus sinensis*) exhibited lowered triglyceride and cholesterol.

To the best of our knowledge, there is very scarce research regarding the effect of mixture supplementation of phytogenics especially on the antioxidants defense enzymes as indicators of free radicles superoxide and hydrogen peroxide (Hassaan and Soltan, 2016). In the present study, supplementation with 20 ml LOG kg^{-1} achieved enhanced activities of the antioxidant enzymes. LOG compounds could easily lyse lysis free radicals by ROS and catching free radicals before arriving their target cell (Liu et al., 2008). Furthermore, the various bioactive compounds present in the lemon and garlic work synergistically and enhance antioxidative properties that create an effective defense system against free radicals (Nivitabishekam et al., 2009). Lemon and garlic also contain organo-sulphur compounds (Rahman et al., 2012). Lemon and garlic have immuno-modulatory properties on the intestinal microflora of the gut that help the organism to compete with pathogenic infections and stress factors like free radicals (Parham et al., 2020). Cell damage mediated by free radicals could have been prevented through the synergistic interactions between active compounds in LOG, such as flavonoids, organo-sulphur compounds, and fructo-oligosaccharides (Lee et al., 2012; Hayat et al., 2016).

The highest gene expression of GH, IGM-2, SOD, and CAT were recorded in fish fed 20 ml LOG ml kg^{-1} diet. The improvement of gene expression of growth and antioxidant hormones could have also contributed to enhanced tilapia performance. Aanyu et al. (2018) observed that the addition of limonene improved the insulin growth factor I gene expression while Li et al. (2016) reported that garlic could induce and boost key genes involved in antioxidant genes. Furthermore, in a clinical trial, the treatment of raw garlic showed a positive role in inhibiting bacterial infection caused by *Helicobacter pylori* in the stomach of human patients (Zardast et al., 2016).

5. Conclusions

The present study showed an enhancement of growth and immune response of fish fed different levels of dietary LOG. However, dietary LOG at level 20 ml kg^{-1} elicited the best growth and immune system response in tilapia.

CRediT authorship contribution statement

All authors contributed to this study conception and design. Material preparation, data collection and analysis were performed by [Mohamed S., Hassaan], [Eman Y., Mohammady], [Ayman Farrag], [Mohamed R., Soaudy] and [Bassuony]. The first draft of the manuscript was written by [Mohamed S., Hassaan], and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Funding

This manuscript was funding by the coauthors, and no funding agancy.

Availability of data and material

(data transparency).

Code availability

(software application).

Conflict of interest statement

The authors declare that they have no conflict of interest.

Acknowledgements

The authors acknowledge the National Institute of Oceanography and Fisheries, Cairo, Egypt for supporting during this research.

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