Fish and Shellfish Immunology 93 (2019) 1–7

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

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

Full length article

Optimal dietary alcoholic extract of lotus leaf improved growth performance and health status of grass carp (*Ctenopharyngodon idellus*)

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ARTICLE INFO

Keywords:
Alcoholic extract of lotus leaf
Grass carp
Growth performance
Body composition
Anti-oxidation
Immunity

ABSTRACT

Grass carp (*Ctenopharyngodon idellus*) is one of the most important aquaculture fish in China. This study tried to explore the effects of dietary alcoholic extract of lotus leaf (AELL) addition on the growth performance and health status of grass carp by feeding juvenile fish (average weight: 34 ± 1 g) with four different experimental diets: control, AELL7, AELL14 and AELL21 for 8 weeks. At the end of the growth trial, the highest values of final body weight (FBW), weight gain rate (WGR), specific growth rate (SGR) and feed intake (FI) all occurred in group AELL14 ($P < 0.05$). Compared to control, the crude lipid content of whole-body and the serum malondialdehyde (MDA) in the three experimental groups decreased, while the serum superoxide dismutase (SOD), glutathione peroxidase (GSH) and total antioxidant capacity (T-AOC) values almost all increased in the three experimental groups. The highest serum immunoglobulin M (IgM) concentration occurred in AELL14 group ($P < 0.05$). In AELL14 and AELL21 groups, both the serum complement 3 (C3) concentration and lysozyme (LYS) activity were significantly higher, whereas the final cumulative mortality in challenge test was significantly lower, when compared to those in control group ($P < 0.05$). The AELL exerted dose-dependent beneficial effects on grass carp health through up-regulating related gene expressions and enzyme activity. In conclusion, the optimal dietary AELL level is 0.14% for juvenile grass carp.

1. Introduction

Modern intensive aquaculture concerns the high output of fish to meet the rising global demand for aquatic protein [1], and exposes fish to various stress and pathogenic bacterium, resulting in growth retardation, depression of resistance to disease and tolerance to environmental stress, and meat quality degrading [2]. In addition, antibiotics were used as feed additives to promote fish growth and protect fish health, which successfully increased the fish supply in the past two decades. However, due to the bacterial resistance to antibiotics and the food safety caused by antibiotics residue, antibiotics have been prohibited to be used in fish diet [3]. Recently, plant extracts have been tried to be used as feed additives for the replacement of antibiotics to improve the growth, health and meat quality of fish due to the active components of these extracts, such as flavonoids, phenolics, and pigments [4,5].

Grass carp (*Ctenopharyngodon idellus*) is one of the most important aquaculture species in China, with a total production of more than 5.9 million tons in 2016 [6]. Some studies regarding new feed additives have been reported in grass carp: dietary 8–12% yeast culture supplementation improved fish growth and antioxidant capacity [7], dietary 100–200 mg/kg silymarin promoted growth and protein efficiency, suppressed lipid accumulation, and improved health status in grass carp fed with high-lipid diets [5], dietary geniposide supplement could increase muscle collagen content and decrease muscle lipid content and muscle fiber diameter, but did not affect fish growth [8]. As described above, plant extracts from different sources showed different effects on grass carp, which also might be at high cost. In the present study, a

Abbreviations: AELL, alcoholic extract of lotus leaf; FBW, final mean body weight; WG, weight gain rate; SGR, special growth ratio; FI, feed intake; FCR, feed conversion ratio; HSI, hepatosomatic index; VSI, viscerosomatic index; MDA, malondialdehyde; T-AOC, total antioxidant capacity; SOD, superoxide dismutase; GSH, glutathione peroxidase; LYS, lysozyme; IgM, immunoglobulin M; C3, complement 3; Ah-1, *Aeromonas Hydrophila 1*; qRT-PCR, Quantitative real-time polymerase chain reaction; MS-222, Tricaine methanesulfonate; *nrf2*, NF-E2-related factor 2; *mtor*, target of rapamycin; *s6k1*, ribosomal S6 kinase 1; *keap1*, kelch-like-ECH-associated protein 1; *ef1a*, elongation factor 1-alpha; ROS, reactive oxygen species

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https://doi.org/10.1016/j.fsi.2019.07.039
Received 21 May 2019; Received in revised form 8 July 2019; Accepted 13 July 2019
Available online 14 July 2019

traditional Chinese medicine, lotus leaf extract, was chosen to explore whether it can promote the growth and health of grass carp.

Lotus (*Nelumbo nucifera* Gaertn), a perennial aquatic herb widely distributed throughout Eastern Asia, has come into wide use due to the various beneficial effects. The extracts of rhizomes, seeds, flowers and leaves have been reported to have varied therapeutic potential, among which the leaves are widely used in traditional medicine [9]. The mature leaves are rich in flavonoids with a content of 84.21 mg/g, alkaloids with a content of 2.132 mg/g, and some other compounds [9–11]. The flavonoids are a group of polyphenolic compounds, which exhibit several biological functions, such as anti-obesity, anti-inflammatory, anti-allergic, and antioxidant potential [10,12]. The alkaloids have also been reported to be major ingredients for lowering hyperlipemia, resisting karyokinesis [13], anti-microbial, anti-fungal, and anti-obesity [14]. That the extract of lotus leaf could have potential biological effects has been proven in animals, including humans [15,16], rats [17,18] and fish [19]. Up to date, most relevant studies have been focused on its pharmacological functions in mammals as a traditional Chinese medicine [9]. Limited knowledge is available at present on the effects of dietary lotus leaf extract supplement on aquatic animals except the growth promoting effect [19].

In the present study, a growth trial with four groups of juvenile grass carps fed with different level of dietary alcoholic extract of lotus leaf (AELL) was conducted to explore the effects of dietary AELL supplement on the growth performance and health status of grass carp.

2. Materials and methods

2.1. Preparation of lotus leaf extract

Samples of lotus leaves were collected from Dazhaishan Farm (Wuhan, China). AELL were prepared as described previously [20] with slight modifications. In brief, the dried lotus leaf was ground into a fine powder through a 420-μm mesh. 0.5 g powder was extracted with 17.5 mL of 60% ethanol solution under ultrasonication for 30 min at 50 °C, 175 W in a SB-5200DT ultrasonic cleaner (Ningbo Scientz Biotechnology Co. Ltd, Ningbo, China), and then filtered. The filtrate was condensed by evaporating the alcohol through a Heizbad Hei-VAP rotary evaporator (Heidolph Instruments GmbH & Co. KG, Schwabach, Germany) to remove the solvent. Then the extract was stored at 4 °C.

A certain of the extract was dissolved in 50 mL of distilled water, and then the main component, lotus leaf flavonoids (LLF), content of the extract was calculated using the following linear equation based on the calibration curve that was prepared according to the colorimetric method [21] using rutin as the standard through a UV-2800 ultraviolet and visible spectrophotometer (Unico Instruments Co. Ltd, Shanghai, China) at 510 nm:

$$Y = 0.1012\ X - 0.0012,\ r = 0.9964$$

Where X is the absorbance; Y is the LLF content in mg/mL. A total of 400 g lotus leaves was extracted and 110.4 g AELL was obtained containing 47.6 g LLF (the AELL gain rate from the dried leaves was 27.5%). The dried extract was kept in airtight bottles at 4 °C in a refrigerator until use. All the chemicals used in this study were of analytical grade.

2.2. Experimental diets

Four isonitrogenous experimental diets, namely, control, AELL7, AELL14 and AELL21, were formulated to contain AELL level at 0, 0.07%, 0.14% and 0.21%, respectively. Fish meal, soybean meal, cottonseed meal and rapeseed meal were used as main protein sources, wheat flour and soybean oil were used as main carbohydrate source and lipid source, respectively. Ingredients and proximate composition of the experimental diets were given in Table 1. The raw materials were all crushed (through a 60-mesh sieve), thoroughly mixed, and then pelleted into cylinder (diameter: 2.0 mm, length: 6 mm) by a laboratory pellet presser. The pellets were then air-dried and stored at −20 °C for later use.

Table 1
Diet formulation and proximate composition of the experimental diets (% as fed basis).

Ingredient	Dietary lotus leaf extract levels			
	Control	AELL7	AELL14	AELL21
Fish meal	1.50	1.50	1.50	1.50
Soybean meal	30.00	30.00	30.00	30.00
Cottonseed meal	6.00	6.00	6.00	6.00
Rapeseed meal	30.00	30.00	30.00	30.00
Wheat flour	20.50	20.50	20.50	20.50
Soybean oil	4.00	4.00	4.00	4.00
Choline chloride (50%)	0.15	0.15	0.15	0.15
Ethoxyquin (30%)	0.05	0.05	0.05	0.05
Monocalcium phosphate	1.60	1.60	1.60	1.60
Microcrystalline cellulose	2.20	2.13	2.06	1.99
Salt	0.40	0.40	0.40	0.40
Compound premix[a]	1.00	1.00	1.00	1.00
AELL	0.00	0.07	0.14	0.21
Bentonite	2.50	2.50	2.50	2.50
Mould inhibitor	0.10	0.10	0.10	0.10
Nutrient content (%)				
Crude protein	30.12	29.87	29.76	29.74
Crude lipid	7.98	7.85	7.81	7.82
Ash	4.71	4.66	4.58	4.61
Moisture	8.91	8.87	8.81	9.13
Flavonoids (g/kg)	0.00	0.26	0.57	0.97

[a] Per kilogram of compound premix containing: vitamin A, 300,000 IU; vitamin D_3, 100,000 IU; vitamin E, 1.2 g; vitamin k_3, 0.5 g; vitamin B_1, 0.5 g; vitamin B_2, 0.7 g; vitamin B_6, 0.6 g; vitamin B_{12}, 0.0015 g . vitamin B_5, 2.5 g; vitamin C, 10 g; folic acid 0.15 g, D-calcium pantothenate 1.8 g, biotin 0.003 g, inositol 8 g, magnesium 10 g, manganese 2 g, iron 12 g, zinc 5 g, copper 0.4 g, iodine 0.1 g, cobalt 0.03 g, selenium 0.01 g.

2.3. Experimental fish and feeding management

Juvenile fish were obtained from Honghu Fisheries Co. Ltd, and were acclimated to experimental conditions for two weeks in an indoor recirculating aquaculture system. After the acclimation, 360 fish (average weight, 34 g; male and female mixed) were randomly distributed into twelve 300-L circular tanks at a density of 30 fish per tank. Triplicate groups of fish were fed twice a day (8:30 a.m. and 16:00 p.m.) for 8 weeks with four experimental diets containing different levels of AELL. During the feeding period, the feed intake was recorded daily and the fish were reared under the following conditions: water temperature: 24-29 °C; dissolved oxygen: > 5 mg/L; pH: 7.7; photoperiod: 12: 12 h (light: dark).

All procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of Huazhong Agricultural University (Wuhan, China) for laboratory animal use.

2.4. Sample collection

All fish were rapidly anesthetized with tricaine methanesulfonate (MS-222, Sigma, USA) (75 mg/L) at the end of the growth trial after 24 h of fasting, and then counted and collectively weighed to calculate the weight gain rate (WGR), specific growth rate (SGR) and feed conversion ratio (FCR). 5 fish were randomly selected from each tank, pooled as a sample and frozen at −80 °C for subsequent whole-body composition analysis. Another 5 fish were randomly selected from each tank, weighed individually, and then blood samples were collected from each fish using a 5-mL heparinized syringe from the caudal vein. Serum was obtained from the blood sample by centrifugation (3000 × *g*,

10 min, 4 °C), equal volume of serum from each of the five fish were mixed as a sample and frozen at −80 °C until subsequent analyses. Then the 5 fish from each tank were dissected on ice immediately, the visceral mass and the hepatopancreas weight were weighed to calculate the viscerosomatic index (VSI) and hepatosomatic index (HSI), and the muscle was sampled, fast-frozen in liquid nitrogen, and stored at −80 °C for gene expression analysis.

2.5. Determination of chemical composition of experimental diets and fish body, as well as serum biochemical indices

Analysis of moisture (105 °C, 24 h), crude protein (Kjeldahl nitrogen × 6.25), crude lipid (ether extraction by Soxhlet method) and ash (550 °C, 18 h) in diets and whole-body samples were performed following standard laboratory procedures [22].

The serum malondialdehyde (MDA) content, total antioxidant capacity (T-AOC), activities of superoxide dismutase (SOD) and glutathione peroxidase (GSH) were detected by kits purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). The serum concentrations of lysozyme (LYS), complement 3 (C3) and immunoglobulin M (IgM) were detected by kits purchased from Shanghai Enzyme-linked Biotechnology Co., Ltd (Shanghai, China). The amount of enzyme required to convert 1 μmol of substrate per minute at 37 °C was considered as a unit of enzyme activity (U).

2.6. Real-time quantitative PCR

Total RNA was isolated with RNAiso Plus Kit (Takara, Dalian, China) according to the manufacturer's instructions. The extracted RNA was resuspended in 50 μL RNase-free water. RNA quality and quantity were assessed by electrophoresis on 1% agarose gel and by NanoDrop® ND-1000 UV–vis spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) at 260 and 280 nm. Subsequently, 1 μg total RNA was used for reverse transcription with PrimeScriptTM RT reagent Kit (Takara, Dalian, China) according to the manufacturer's recommendation. Quantitative real-time polymerase chain reaction (qRT-PCR) was performed using a quantitative thermal cycler (Light Cycler 480 II, Roche) with the SYBR® Green Realtime PCR Master Mix (Toyobo, Osaka, Japan) in a total volume of 20 μL reaction mixture containing reverse transcriptase. For each target gene, specific primer pairs (Table 2) were designed through Primer Premier 6.0 software using known sequences in NCBI database. The PCR parameters were forty cycles at 95 °C for 5 s, annealing temperature (corresponding specific primer pairs) for 10 s and 72 °C for 15 s, with an additional initial 30 s pre-denaturation step at 95 °C. Melting curves were systematically monitored (temperature gradient at 0.5 °C/s from 55 to 94 °C) to confirm the only one fragment was amplified. The expression of the genes were quantified relative to means of *β-actin* and elongation factor 1-alpha (*ef1a*) using the $2^{-\Delta\Delta Ct}$ value method as described by Pfaffl [23].

Table 2
Primer sequences used for real-time PCR.

Gene Name	Description	Sequences of primers(5′-3′)	Accession number
nrf2	Forward	TGGACGAGGAGACTGGAGAG	KF733814
	Reserve	TGGTAGGTGGAACGGAAACAT	
mtor	Forward	ACACCTCCACCTTCTCCA	JX854449
	Reserve	TCCCACTTTCCACCAACT	
s6k1	Forward	ACATAAAGCAGCCTGACG	EF373673.1
	Reserve	TGGAGGAGGTAATGGACG	
keap1	Forward	GCTTCCAGAGAGTCCAGAGAG	KF811013.1
	Reserve	CTTCAGCCAGATGTTCTTCCTC	
ef1a	Forward	TGACTGTGCCGTGCTGAT	GQ266394.1
	Reserve	CGCTGACTTCCTTGGTGATT	
β-actin	Forward	AGACCACCTTCAACTCCATCAT	M25013.1
	Reserve	CTCCTGCTTGCTAATCCACATC	

Note: *nrf2*: NF-E2-related factor 2; *mtor*: target of rapamycin; *s6k1*: ribosomal S6 kinase 1; *keap1*: kelch-like-ECH-associated protein 1; *ef1a*: elongation factor 1-alpha.

2.7. Challenge test

The wild-type strain of *Aeromonas hydrophila 1* (Ah-1), provided from laboratory of Prof. Xiaoxuan Chen (College of Fisheries, Huazhong Agricultural University, China), was activated by streaking inoculation in the medium, and the single colony was selected for species amplification. Ah-1 was cultivated for 16 h at 28 °C, and then the bacterial solution was serially diluted and counted. The bacterial solution was resuspended with sterilized normal saline for fish, and the final concentration was 1.42×10^7 CFU/mL.

After the feeding experiment, 240 grass carp randomly selected from 12 tanks (20 fish from each tank) were intraperitoneally injected with bacterial solution at a dose of 0.1 mL per fish. The challenge dose was determined according to the preliminary test. The number of dead grass carp was recorded every two hours after injection.

2.8. Statistical analysis

Data were expressed as mean ± SE (standard error). All data were checked for normality by the Kolmogorov–Smirnov test and for homogeneity in variances by Bartlett's test. Statistical differences between treatments were firstly determined by one-way ANOVA and then by Tukey's post-hoc test. All statistical analyses were performed using SPSS computer program, version 19 (IBM, Armonk, NY, USA). Difference was considered significant at $P < 0.05$.

3. Results

3.1. Growth performance, feed intake and morphology parameters

The growth performance and morphological parameters are shown in Table 3. After 8 weeks of feeding trial, the final body weight (FBW) of fish was about 4 times of the initial body weight. The FBW in AELL14 group was significantly higher than that in other groups ($P < 0.05$), and the FBW in AELL21 group was significantly higher than that in the control group ($P < 0.05$), but was not significantly different from AELL7 group. Graded levels of dietary AELL exhibited no significant effects on the HSI and VSI of juvenile grass carp. Both weight gain rate (WGR) and specific growth rate (SGR) in AELL14 group were significantly higher than those in other groups ($P < 0.05$). The feed intake (FI) in AELL14 group was significantly higher than that in the control group and AELL21 group ($P < 0.05$). As the dietary AELL content increased gradually, the FCR showed a decreasing tendency, which was significantly lower in AELL21 group than that in the control and AELL7 group ($P < 0.05$).

3.2. Body proximate composition of fish fed with different levels of AELL

As shown in Table 4, the crude protein content of whole-body in control group was significantly lower than that in AELL7 and AELL21 group ($P < 0.05$), but was not significantly different from that in AELL14 group. As the dietary AELL content increased gradually, the crude lipid content showed a gradually decreasing tendency, and it was significantly higher in the AELL21 group than that in control group ($P < 0.05$). The ash and moisture content of whole-body showed no significant difference among groups.

3.3. Serum antioxidant capacity of fish fed with different levels of AELL

As shown in Fig. 1, the serum SOD value was significantly lower in control group compared with those in other groups ($P < 0.05$), but showed no significant difference among the other three groups. As the dietary AELL content increased gradually, both GSH and T-AOC values

Table 3
Effects of graded levels of dietary AELL on growth performance, feed intake and morphology parameters of juvenile grass carp.

Ingredients	Dietary lotus leaf extract levels			
	Control	AELL7	AELL14	AELL21
IBW (g/fish)	34.02 ± 0.26	34.72 ± 0.33	33.81 ± 0.37	34.43 ± 0.64
FBW (g/fish)	122.47 ± 1.86^{a}	125.66 ± 1.08^{ab}	132.38 ± 0.98^{c}	127.82 ± 1.34^{b}
WGR (%) [a]	260.06 ± 6.89^{a}	261.98 ± 4.39^{a}	291.62 ± 2.87^{b}	271.43 ± 6.66^{a}
SGR (%/d) [b]	2.13 ± 0.03^{a}	2.14 ± 0.02^{a}	2.28 ± 0.01^{b}	2.19 ± 0.03^{a}
FI (g/fish) [c]	163.82 ± 3.85^{a}	168.41 ± 2.45^{ab}	174.83 ± 1.56^{b}	161.77 ± 3.34^{a}
FCR[d]	1.85 ± 0.02^{b}	1.85 ± 0.01^{b}	1.77 ± 0.00^{ab}	1.73 ± 0.04^{a}
VSI (%) [e]	9.22 ± 0.41	8.29 ± 0.62	8.10 ± 0.58	8.44 ± 0.22
HSI (%) [f]	1.49 ± 0.13	1.47 ± 0.0.05	1.82 ± 0.34	1.27 ± 0.33

All data were expressed as mean ± SE (n = 3, except that n = 15 for VSI and HSI). Mean values not sharing a common superscript in the same row are significantly different ($P < 0.05$), while mean values in the same row without any superscript are not different.

[a] WGR (weight gain rate, %) = [final body weight (g) − initial body weight (g)] × 100/initial body weight (g).

[b] SGR (specific growth rate, %/d) = 100 × [ln (final body weight) − ln (initial body weight)]/56.

[c] FCR (feed conversion ratio) = feed intake (g)/[final body weight (g) − initial body weight (g)].

[d] FI (feed intake, g/fish) = total dry matter intake in each tank (g)/the number of fish.

[e] VSI (viscerosomatic index, %) = 100 × [final visceral weight (g)/final body weight (g)].

[f] HSI (hepatosomatic index, %) = 100 × [final hepatopancreasr weight (g)/final body weight (g)].

of the serum showed a first increasing and then decreasing tendency, and reached a peak value in AELL14 group ($P < 0.05$). The serum MDA value in control group was significantly higher than that in AELL14 and AELL21 group ($P < 0.05$), but was not significantly different from AELL7 group.

All data were expressed as mean ± SE of 3 parallel tanks and equal volume of serum from the 5 fish in each tank were mixed and analyzed (n = 3). Mean values not sharing a common superscript in the same row are significantly different ($P < 0.05$).

3.4. Serum immune parameters and survival after challenge test of fish fed with different levels of AELL

As shown in Fig. 2, both C3 concentration and LYS activity in the serum increased gradually with the dietary AELL level, and they were significantly higher in the AELL14 and AELL21 group than that in the control group ($P < 0.05$). The serum IgM concentration showed a first increasing and then decreasing tendency with increasing AELL content, reached a peak value in AELL14 group ($P < 0.05$).

During challenge test, dead fish began to appear at 8 h after the Ah-1 injection (Fig. 3). Generally speaking, the cumulative mortalities in the three experimental groups were t lower than that in the control group during 8–32 h after the Ah-1 injection. The final cumulative mortality of control, AELL7, AELL14 and AELL21 group was 81.7%, 75.0%, 66.7%, 60.0%, respectively. The final cumulative mortality in AELL14 group was only significantly lower than that in the control group ($P < 0.05$), while this parameter in AELL21 group was significantly lower than that in the control and AELL7 group ($P < 0.05$).

Values are means ± SE of 3 parallel tanks and the equal volume of serum from the 5 fish in each tank were mixed together and analyzed. Different letters above bars indicate statistically significant differences among treatments ($P < 0.05$).

The figure showed a dynamic change of fish survival during 8–32 h after challenged with Ah-1. Values are means ± SE (n = 3). Different letters at the end of the line indicate statistically significant differences among final cumulative mortalities in different groups ($P < 0.05$).

3.5. Relative mRNA expression of genes in muscle

Fig. 4 showed that the *mtor* expression was increased by the AELL supplement, which was the highest in the AELL14 and AELL21 group, followed by in the AELL7 group, and the lowest in the control ($P < 0.05$). The *s6k1* expression was significantly higher in the AELL7 and AELL21 group than that in the control group ($P < 0.05$), and it was also significantly higher in the AELL21 group than that in the AELL14 group ($P < 0.05$). The *nrf2* expression was significantly higher in the AELL14 group than that in other three groups ($P < 0.05$). As the dietary AELL content increased gradually, the *keap1* expression decreased significantly, which was lowest in AELL14 and AELL21 groups ($P < 0.05$).

Values are means ± SE of 3 parallel tanks and equal amount of cDNA from 5 fish in each tank were mixed and analyzed. Different letters above bars indicate statistically significant differences among treatments ($P < 0.05$).

4. Discussion

In present study, the highest value of FBW, WG and SGR all occurred in AELL14 group, suggesting that optimal AELL supplement improved the growth of grass carp. In addition, AELL14 group showed higher feed intake than the control and AELL21 group, and somewhat lower FCR value than the control although significantly lower FCR was only achieved by the 0.21% supplement of AELL, which indicated that the growth promotion was a synergistic effect of the enhanced feed

Table 4
Effect of dietary AELL levels on body composition of juvenile grass carp.

Body Composition	Dietary Lotus leaf extract levels			
	Control	AELL7	AELL14	AELL21
Crude Protein (%)	15.67 ± 0.11^{a}	16.56 ± 0.12^{b}	16.06 ± 0.04^{ab}	16.42 ± 0.22^{b}
Crude Lipid (%)	7.84 ± 0.29^{b}	7.77 ± 0.29^{ab}	7.20 ± 0.13^{ab}	7.07 ± 0.07^{a}
Ash (%)	2.91 ± 0.04	3.15 ± 0.07	3.06 ± 0.10	3.14 ± 0.07
Moisture (%)	73.87 ± 0.46	72.98 ± 0.30	73.10 ± 0.32	73.29 ± 0.48

All data were expressed as mean ± SE of 3 parallel tanks and 5 fish in each tank were pooled as one sample for analysis (n = 3). Mean values not sharing a common superscript in the same row are significantly different ($P < 0.05$), while mean values in the same row without any superscript are not different.

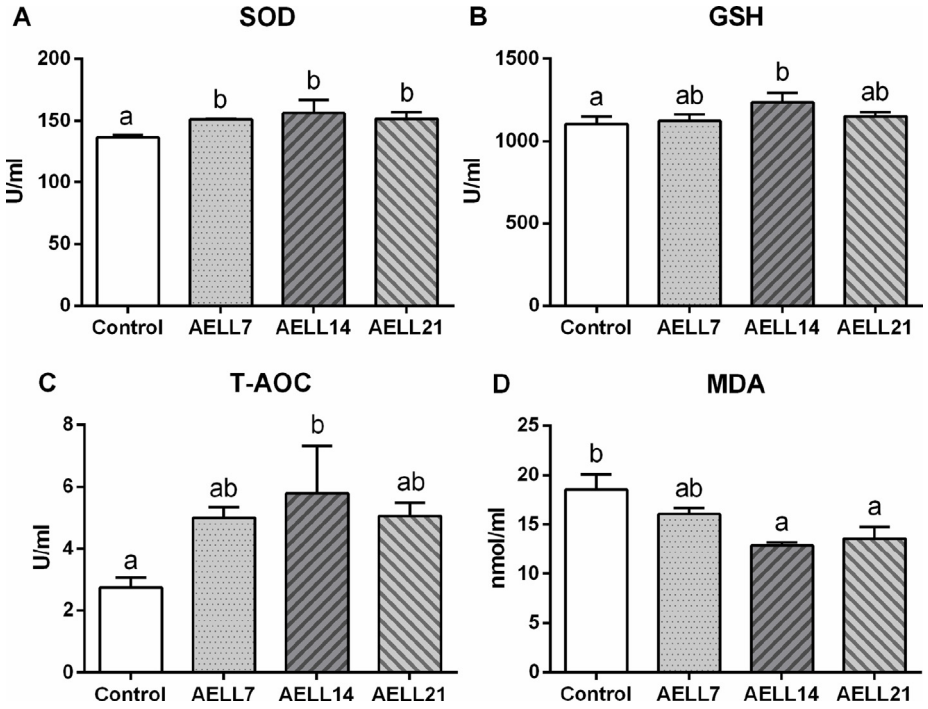

Fig. 1. Effects of graded levels of dietary lotus leaf extract on serum antioxidant parameters of juvenile grass carp.

intake and feed utilization at optimal AELL supplement level. The effect of lotus leaf extract on growth performance may be species-dependent and dose-dependent. A previous study indicated that dietary containing lotus leaf extract could improve growth performance of Nile tilapia at an optimal dietary inclusion level of 1% [19]. Another study on tilapia showed that the dietary containing different content of lotus peduncle extracts (0, 0.05, 0.1 and 1%) increased the growth performance of tilapia dose-dependently [24]. Similarly, catfish (*Clarias gariepinus*) fed the diets containing lotus stamen extract at different levels (0.1, 0.5 and 1%) also showed the promotion in growth and feed utilization in a positive dose-effect manner [25].

The specific mechanism of AELL on growth and feed utilization promotion in fish is still little known. It was reported that the villus height, villus width and goblet cell number both in the anterior and posterior parts of intestine increased in the catfish fed with different level of lotus stamen extracts, which all showed a positively dose-effect relationship [25]. The improvement of intestinal physiology might be a reasonable explanation for the promotion on fish growth by optimal AELL addition in the present study. However, in mammals, it was reported that lotus leaf extracts at IC50 value of 0.46 mg/mL could inhibit the activities of α-amylase and lipase in vitro [26]. Whether the higher dietary inclusion of AELL (AELL21) could decrease the digestive activities of grass carp to some extent thus to show no improvement on fish growth in this study deserves further investigation. Previous study

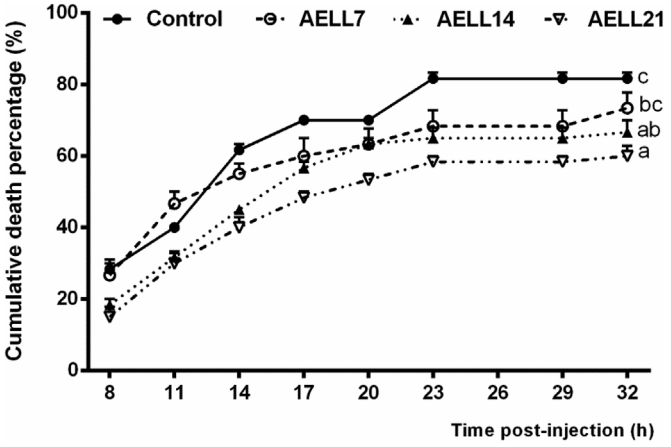

Fig. 3. Cumulative mortalities (%) of juvenile grass carp fed with graded levels of dietary AELL.

showed that the catechin in lotus leaf methanolic extract could dose-dependently enhanced insulin secretion [27], and the gene expressions of *mtor* and *s6k1* in muscle of grass carp increased dose-dependently with dietary AELL inclusion in this study, which might suggest that the promoting effect of the AELL on growth and feed utilization could be

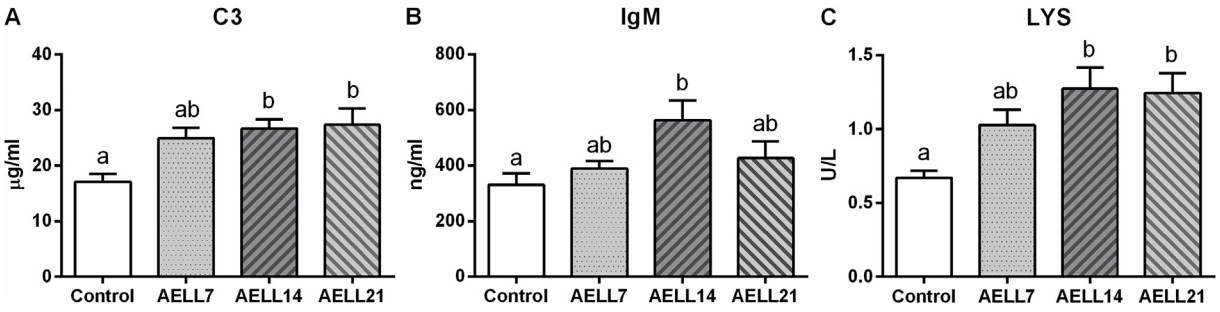

Fig. 2. Effects of graded levels of dietary AELL on serum immune parameters of juvenile grass carp.

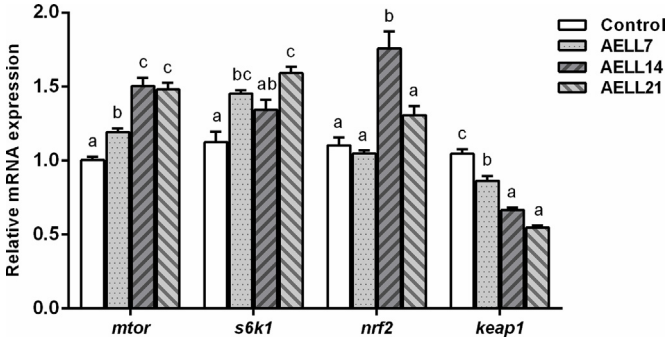

Fig. 4. Relative mRNA levels of mtor, s6k1, nrf2 and keap1 genes in the muscle of juvenile grass carp (*Ctenopharyngodon idellus*) fed with diets containing different lotus leaf extract levels for 8 weeks.

achieved through the activation of insulin-PI3K-mTOR signaling pathway to promote protein synthesis.

The gradually decreased crude lipid content of whole-body as dietary AELL increased indicated the hypolipidemic effect of AELL in this study. Similar hypolipidemic effects of AELL were discussed in alloxan-induced diabetic mice [20], diabetic or dyslipidaemic rats [28], female university students [15], and oleic acid-induced HepG2 cells [16]. The hypolipidemic mechanism of lotus leaf extract may be that diverse constituents of lotus leaves, including benzoisoquinoline alkaloids, metastigmanes and flavonoids with their specific action each, exert synergistic actions on inhibiting fat absorption and fat accumulation [29]. Moreover, the gradually increased crude protein content of whole-body revealed the potential effect of AELL on promoting protein synthesis. Combined the higher protein content with the higher muscle expression of *mtor* and *s6k1* of fish in the three experimental groups of this study, the increased growth was further proven to be due to the promotion on protein synthesis by AELL through up-regulation of muscle gene expressions.

In recent years, the systemic anti-oxidative effect of diverse compounds of the AELL was suggested to be protective and therapeutic in mammals [30–32]. Moreover, It has been proven that the antioxidant components of lotus leaves mainly are flavonoids, including catechin, quercetin, and some glucoside compounds of quercetin [21]. In the present study, the AELL14 group showed significantly higher serum SOD, GSH and T-AOC values than the control group, and other AELL supplemented groups also showed somewhat higher enzymatic values compared to the control, while the serum MDA showed the opposite trend to SOD, which indicated the antioxidant-promoting effect of AELL on fish when optimal AELL inclusion level was applied. Similar results were discussed in mice with exhaustive swimming exercise-induced oxidant stress [30], and in vitro studies on hepatocytes with H_2O_2-induced hepatic damage [31], human umbilical vein endothelial cells [32], and oleic acid-induced HepG2 cells [16].

Nrf2 signaling pathway can regulate the gene expression of antioxidant enzyme [33]. Under basal conditions, the Cullin3-Keap1 complex sequesters Nrf2 in the cytosol by binding its two motifs. The motif of Nrf2 is loosened from the Cullin3-Keap1 complex when cells are exposed to reactive oxygen species (ROS) and then Nrf2 translocates into the nucleus to initiate the gene transcriptions of antioxidant enzyme [33]. In present study, the higher expression of *nrf2* in AELL14 group and lower expression of *keap1* in three experimental groups indicated the anti-oxidative mechanism of AELL may be through the Nrf2 signaling pathway by up-regulation of *nrf2* expression and down-regulation of *keap1* expression. Moreover, *mtor* was suggested to modulate the *nrf2* expression in human hepatic carcinoma cells [34] and in human brain endothelial cells [35]. The higher expression of *mtor* and *s6k1* was consistent with the *nrf2* expression in the current study, which further indicated the possible role of *mtor* in the regulation of AELL on anti-oxidative capacity of grass carp.

Teleost fish have complex immune system, including innate immunity, such as lysozyme, complement system, and adaptive immunity, such as antibodies and lymphocytes [36]. Lysozyme is an important defense molecule of fish innate immune system to prevent fish from the infection of exogenetic pathogen [37]. The gradual increase in LYS activity with dietary AELL level suggested the enhanced innate immunity of fish by AELL, similar result was reported in Nile tilapia (*Oreochromis niloticus* L.) [38]. Complement acts as a rapid and efficient immune surveillance system by eliminating cellular debris and infectious microbes, orchestrating immune responses and sending 'danger' signals, contributing substantially to homeostasis [39], in which C3 is a central molecule whose activation is essential for all the important functions performed [40]. To our knowledge, this is the first report regarding the effect of lotus leaf or its extract on C3 activity of fish. The consistent increase of C3 with dietary AELL level suggested the strong promoting effect of AELL on complement system to increase immunity. IgM, one of the three major immunoglobulin isotypes reported in teleost fish, was thought to be the potent immunoglobulin responding to pathogens both in systemic and mucosal compartments [41]. The pentameric nature of IgM renders it very efficient in the process of opsonizing (coating) antigen for destruction and fixing complement [42]. In the present study, the higher IgM concentration in AELL14 group than the control also indicated that IgM could also be a target molecule of AELL in enhancing fish immunity. Furthermore, the cumulative mortalities of fish in challenge test, which is consistent with the results in Nile tilapia [38], might suggest a synergistic promoting-effect of AELL on innate immune and adaptive immune. Similarly, the quercetin extracted from lotus leaves was concluded to be a potential antibacterial agent [43]. In general, the AELL14 diet exhibited beneficial immune effects when compared to the control, while the more supplement of AELL (AELL21 diet) did not show any more benefits on the basis of AELL14 diet.

5. Conclusion

In conclusion, the supplementation of AELL in this study exhibited dose-dependent beneficial effects on grass carp. In combination with the fish growth performance, whole-body composition, antioxidant capacity, and immunocompetence, the 0.14% concentration of AELL is an appropriate dosage to be supplemented in grass carp diet.

Declaration of interest

The authors declare no financial and non-financial competing interests.

Funding sources and Acknowledgments

This work was supported by Fundamental Research Funds for the Central Universities of Huazhong Agricultural University (2662016PY105). Thanks to everyone who contributed to this experiment.

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