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Effects of dietary *Gelsemium elegans* alkaloids on growth performance, immune responses and disease resistance of *Megalobrama amblycephala*

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ABSTRACT

The present study aim to investigate the effects of dietary *Gelsemium elegans* alkaloids supplementation in *Megalobrama amblycephala*. A basal diet supplemented with 0, 5, 10, 20 and 40 mg/kg G. *elegans* alkaloids were fed to *M. amblycephala* for 12 weeks. The study indicated that dietary 20 mg/kg and 40 mg/kg G. *elegans* alkaloids supplementation could significantly improve final body weight (FBW), weight gain rate (WGR), specific growth rate (SGR), feed conversion ratio (FCR) and protein efficiency ratio (PER) ($P < 0.05$). The 20 mg/kg and 40 mg/kg G. *elegans* alkaloids groups showed significantly higher whole body and muscle crude protein and crude lipid contents compared to the control group ($P < 0.05$). The amino acid contents in muscle were also significantly increased in 20 mg/kg and 40 mg/kg groups ($P < 0.05$). Dietary 40 mg/kg G. *elegans* alkaloids had a significant effect on the contents of LDH, AST, ALT, ALP, TG, TC, LDL-C, HDL-C, ALB and TP in *M. amblycephala* ($P < 0.05$). Fish fed 20 mg/kg and 40 mg/kg dietary *G. elegans* alkaloids showed significant increase in complement 3, complement 4 and immunoglobulin M contents. The liver antioxidant enzymes (SOD, CAT and T-AOC) and MDA content significantly increased at 20 mg/kg and 40 mg/kg G. *elegans* alkaloids supplement ($P < 0.05$). The mRNA levels of immune-related genes *IL-1β*, *IL8*, *TNF-α* and *IFN-α* were significantly up-regulated, whereas *TGF-β* and *IL10* genes were significantly down-regulated in the liver, spleen and head kidney of fish fed dietary supplementation with 20 mg/kg and 40 mg/kg G. *elegans* alkaloids. After challenge with *Aeromonas hydrophila*, significant higher survival rate was observed at 20 mg/kg and 40 mg/kg G. *elegans* alkaloids supplement ($P < 0.05$). Therefore, these results indicated that *M. amblycephala* fed a diet supplemented with 20 mg/kg and 40 mg/kg G. *elegans* alkaloids could significantly promote its growth performance, lipids and amino acids deposition, immune ability and resistance to *Aeromonas hydrophila*.

1. Introduction

Megalobrama amblycephala (*M. amblycephala),* also called blunt snout bream, is an economically important freshwater fish species in the aquaculture industry in China and its production has increased rapidly in recent years [1,2]. In recent years, the intensive aquaculture and polluted aquatic environment produce poor physiological environment and frequent outbreak of diseases, which resulted into high mortality and high economic loss, especially in summer [3]. To avoid the outbreak of diseases in aquaculture, many antibiotics and chemical medicine have been used to prevent the diseases [4]. However, the abuse of antibiotics has caused many other problems, such as the generation of drug-resistant pathogens and environmental pollution [5]. Therefore, to reduce the use of antibiotics and find other ways to protect cultured fish are urgently needed.

Nowadays, some natural plant extracts, called immunostimulants, which contain several active compounds, have been shown to be effective alternatives to traditional antibiotics [6]. The role of immunostimulant on health management through enhance the immunity of organisms. These immune-enhancing plant extracts had turned into a new research area of top priority with aims to reduce disease outbreak and related economic losses [6]. Thus these plant extracts have a potential application as an immunostimulant in aquaculture. Furthermore, they are inexpensive, locally available and environment friendly

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[7]. Many research findings provide solid evidence that various plant extract have been developed to improve immunity and prevent pathogenic microorganism infection in aquaculture, such as gynura bicolor extract [8], hyacinth extracts [9], angelica sinensis extracts [10] and guava leaf extract [11]. To our knowledge, there are no reports in literature regarding the effects of dietary *Gelsemium elegans* (*G. elegans*) extracts on growth performance and immune responses in fish. *G. elegans,* a small genus of the family Loganiaceae, which widely distributed in China, is a kind of well-known Chinese herbal medicine for treatment of neuropathic pain [12], inflammatory [12], rheumatic arthritis and anxiety [12]. *G. elegans* Benth contains a variety of active compounds such as alkaloids, flavonoids, and triterpenes etc., and its main ingredients included koumine, gelsemine and gelsenicine etc [14,15]. Some compounds in plants including polysaccharide, alkaloids and flavonoids could positively induce effects on growth performance and general health of fish [16–18].

The wide use of *G. elegans* in traditional medicines, and its numerous beneficial effects on mammals had motivated us to explore its potential biological activities in fish. However, there is little information available on the effects of *G. elegans* alkaloids in aquaculture. Therefore, this study was carried out to investigate the impacts of dietary various levels *G. elegans* alkaloids on growth performance, immune responses, expressions of immune-related genes and disease resistance in *M. amblycephala*, which would encourage its use as a potential immunostimulant in aquaculture.

2. Materials and methods

2.1. Experimental diet

The diets of *G. elegans* alkaloids were formulated in Table 1 and the basal diet composition is based on previous studies [19]. The basal diet was used as control diet (0 mg/kg), and 5, 10, 20 and 40 mg/kg G. *elegans* alkaloids were supplemented to formulate as experimental diets. The *G. elegans* alkaloids were provided by Manst Biotechnology Co., Ltd (Chendu, China). The main component contents of *G. elegans* alkaloids including koumine, gelsemine and gelsenicine were provided in Fig. S1. All the ingredients were thoroughly mixed with fish oil, *G. elegans* alkaloids and water and then were made sinking pellet feed through the pelletizer. Lastly, the pellets were air-dried to below 10% moisture. After drying, all diets were sealed in bags and stored at −20 °C for further use.

2.2. Experimental fish and feeding trial

M. amblycephala were obtained from Lihong Fishery Company (Guangzhou, China). The experiment was performed in a recirculating aquaculture system in the laboratory. Prior to the feeding trial, the fish were fed with basal diet for 2 weeks to acclimate to the experimental diet and conditions. After acclimation period, the similar size *M. amblycephala* (3.73 ± 0.03 g) were randomly distributed into 15 tanks for 30 fishes per tank. Fish were fed twice daily at 8:00 and 17:00 until apparent satiation, and daily feed consumption was recorded. We put the feed in a special sealed bottle and weigh the unused feed at the end of each feeding to calculate the feed consumption. During the feeding trial, fish were reared under the following conditions: water temperature ranged from 25.0 to 28 °C, pH from 7.2 to 7.8 and dissolved oxygen concentration from 5.5 to 6.0 mg/L. The fish were reared and fed under 12-h light 12-h dark photoperiod. Each diet treatment was tested in triplicate, and the trial lasted 12 weeks.

2.3. Samples collection

After the feeding trial, fish were starved 24 h prior to sampling. The number and mean body weight of fish in each tank were measured. The fish were randomly selected from each tank and were euthanized using 100 mg/L tricaine methanesulfonate (MS-222, Sigma, USA), then were sampled and stored at −20 °C. The blood samples from the caudal vessel were collected into centrifuge tubes with 20 μL EDTA (15 g/L) inside using 1 mL syringes. After collection, the blood samples were centrifuged at 4 °C 3500 rpm for 15 min, then the supernatant was taken and stored at −80 °C for plasma biochemical indices measurement. Samples of the liver, spleen and head-kidney were also removed and stored at −80 °C for gene expression analysis. Additionally, the liver samples for the histology observations were fixed in 4% paraformaldehyde. Muscle samples from the dorsal sides were sampled and stored at −80 °C for further analysis of amino acid composition. Each diet treatment was tested in triplicate.

Table 1
Feedstuff and nutrient levels of experimental diets in this study.

Feedstuff (g/kg)	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5
Fish meal	50	50	50	50	50
Soybean meal	400	400	400	400	400
Rapeseed meal	150	150	150	150	150
Flour	272	272	272	272	272
Rice bran	40	40	40	40	40
Corn protein powder	5	5	5	5	5
Fish oil	15	15	15	15	15
Methionine	2	2	2	2	2
Calcium dihydrogen phosphate	25	25	25	25	25
Salt	1	1	1	1	1
Vitamin and mineral mixture	10	10	10	10	10
Choline chloride	10	10	10	10	10
G. elegans extracts	0	0.005	0.01	0.02	0.04
Carboxymethyl cellulose	20	20	20	20	20
Nutritional levels (%)					
Moisture	7.46 ± 0.03[a]	7.49 ± 0.04[a]	7.5 ± 0.04[a]	7.44 ± 0.01[a]	7.44 ± 0.06[a]
Crude protein	29.69 ± 0.02[a]	29.72 ± 0.01[a]	29.72 ± 0.01[a]	29.71 ± 0.02[a]	29.71 ± 0.02[a]
Crude lipid	3.66 ± 0.02[a]	3.68 ± 0.03[a]	3.69 ± 0.02[a]	3.7 ± 0.02[a]	3.69 ± 0.02[a]
Crude ash	7.36 ± 0.06[a]	7.36 ± 0.05[a]	7.38 ± 0.04[a]	7.35 ± 0.06[a]	7.37 ± 0.06[a]

Vitamin and mineral mixture provided by Guangzhou Fishtech Co., Ltd., China (Premix feed, 2111). Vitamin and mineral mixture provides the following: vitamin A (300000 IU/kg); vitamin D3 (80000 IU/kg); vitamin E (2500 mg/kg); vitamin K3 (400 mg/kg); vitamin B1 (600 mg/kg); vitamin B2 (850 mg/kg); vitamin B6 (600 mg/kg); inositol (4000 mg/kg); vitamin C (8500 mg/kg); nicotinamide (3000 mg/kg); calcium pantothenate (1500 mg/kg); biotin (12 mg/kg); Fe (6000 mg/kg); Mn (750 mg/kg); iodine (120 mg/kg); Cu (500 mg/kg); Zn (7000 mg/kg); Se (35 mg/kg); Co (100 mg/kg).

2.4. Proximate composition and amino acid profile analysis

The moisture, crude protein, crude lipid and crude ash in diets, muscles and whole body were determined by the AOAC standard method [20,21]. The moisture content of the samples was determined by air oven method at 105 °C. The crude protein was determined using Kjeldahl method (N × 6.25). The content of crude lipid was determined by Soxhlet extraction method. For crude ash content determine, samples were placed in a muffle furnace at 550 °C for 6 h. The amino acid profile was analysed using an Automatic Amino Acid Analyzer (Hitachi, Japan) based on previous studies [22]. The samples were performed in triplicate.

2.5. Liver histological processing

The fixed liver samples were washed twice in phosphate-buffered saline (PBS), dehydrated in a graded series (10, 20, 30%) of sucrose, and then embedded in opti-mum cutting temperature compound (OCT, Tissue-Tek 4583). The frozen liver samples were cut into section at 10 μm on cryomicrotome and then mounted in glass slides with 3-aminopropyl-triethoxysilane. The frozen sections were air-dried at room temperature and then stored at −20 °C. The next day, the frozen sections were rinsed with 50% ethanol, and then stained with a filtered 0.5% Oil red O for 30 min. After staining, used 50% ethanol and distilled water rinsed again for 1 min and stained with hematoxylin for 45 s, then covered with mixture of glycerol and gelatin. Frozen sections stored at −20 °C waiting for the next analysis [23]. Frozen sections were imaged on a microscope (Olympus, BX51).

2.6. Plasma biochemical and immunity parameters

Plasma biochemical indexes including lactate dehydrogenase (LDH), aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), triacylglycerol (TG), total cholesterol (TC), low density lipoprotein cholesterol (LDL-C), high density lipoprotein cholesterol (HDL-C), albumin (ALB) and total protein (TP) were analyzed using the automatic biochemical analyzer (Roche, Switzerland) by the colorimetric method previously studies [24]. Plasma complement 3 (C3), complement 4 (C4) and immunoglobulin M (IgM) were detected by ELISA method using commercial kits (Shanghai Enzyme-linked Biotechnology Co., Ltd, China).

2.7. Plasma and liver antioxidant indices assay

The liver samples were placed in a centrifuge tube with cold phosphate buffer (PBS, pH 7.4), then were homogenized in an ice bath. The liver samples were harvested by centrifugation of 3500 rpm for 15 min and washed twice with cold phosphate buffer solution (PBS, pH 7.4) at room temperature, and then suspended in PBS. Plasma and the supernatants were used to detected the SOD, CAT, T-AOC activities and MDA content using commercial kits (Nanjing Jiancheng Bioengineering Institute, China) in line with the manufacturer's protocol.

2.8. Gene expression analysis

Briefly, total RNA of the liver, spleen and head-kidney were extracted using a TransZol Up Plus RNA Kit (TransGen Biotech, China). The OD260/OD280 ratio was measured using UV spectroscopy and RNA integrity was evaluated by 2% agarose gel electrophoresis, and then reverse transcribed using Takara reverse transcription kit (Takara, Dalian, China) according to the manufacturer's instructions. The cDNA was used as templates for gene expression analysis using RT-qPCR (Reverse transcription quantitative real-time PCR). The mRNA levels of interleukin 1β (*IL-1β*), interleukin 8 (*IL8*), interleukin 10 (*IL10*), tumor necrosis factor α (*TNF-α*), interferon α (*IFN-α*), transforming growth factor β (*TGF-β*), Cu/Zn superoxide dismutase (*Cu/Zn-SOD*) and Mn superoxide dismutase (*Mn-SOD*) were performed using a SYBR Green Supermix (BIO-RAD, USA) on a BIO-RAD CFX96 Real-Time PCR Detection System (BIO-RAD, USA). Thermal cycling conditions were 95 °C for 2 min, followed by 39 cycles of 95 °C for 15 s, 60 °C for 30 s, 72 °C for 30 s and a final cycle of 72 °C for 7 min. Melting curves were performed from 60 to 90 °C. Specific primers of these genes were obtained from previous studies (Table S1). Relative gene expression levels were normalized against the expression level of *β-actin* [25].

2.9. Aeromonas hydrophila challenge experiment

After sampling, 15 fish obtained from each tank were moved to new tank to acclimate for one week. The *Aeromonas hydrophila* obtained from Dr. Chen's research team in Foshan University (Guangzhou, China). The *A. hydrophila* was cultured according to the method described by previous studies [26]. Briefly, The *A. hydrophila* was inoculated in Luria broth (LB) and incubated for 24 h at 160 rpm. The *A. hydrophila* were harvested by centrifugation at 3500 rpm for 5 min and washed twice in PBS. The colony forming unit (CFU) per mL (CFU/mL) was determined by smearing 10 μL tenfold serial dilutions onto LB agar plates. Fish were challenged by intraperitoneal injection with 50 μl bacterial suspension (3.2×10^8 CFU/mL). Fish are fed with *G. elegans* alkaloids normally after the injection. Fish were observed twice a day and the mortality was recorded during the challenge period (one week).

2.10. Detection of G. elegans alkaloids residues in blood and muscle

Determination of *G. elegans* alkaloids residues by gas chromatography, and the highest concentration group (40 mg/mL) was selected for residue detection. Briefly, the first step is to acidify blood and muscle samples. After acidification, the blood and muscle samples was soaked in 95% ethanol solution to extract *elegans* alkaloids. Then the acidified solutions were filtered and steamed to near drying and redissolve with absolute ethanol (muscle) and absolute ether (blood). Repeated above treatment until no obvious insoluble substance was found in absolute ethanol and absolute ether solution. Last, evaporate anhydrous ethanol and absolute ether to dry using rotary evaporator, and resolve the residue with methanol. The resolved methanol solutions were determined by gas chromatography [27]. Koumine standard was used as residual indicator, which has the highest content in *G. elegans* alkaloids. The retention time was used for qualitative analysis and peak area was used for quantitative analysis.

2.11. Statistical analysis

Experiments were carried out at least in triplicate. All data from this study were analyzed using SPSS statistical package version 19.0 (SPSS Inc., USA). Real-time PCR data were analyzed using the $2^{-\Delta\Delta Ct}$ method. Differences were calculated by analysis of variance (ANOVA). A value of $p < 0.05$ was considered statistically significant. All values were expressed as the mean ± standard deviation (SD).

3. Results

3.1. Effect of dietary G. elegans alkaloids on growth performance

Growth performance results were presented in Table 2, showing that final body weight (FBW), weight gain rate (WGR), specific growth rate (SGR) in the high concentration diet group (20 mg/kg and 40 mg/kg) were significantly higher than the control group ($P < 0.05$). Feed conversion ratio (FCR) was significantly lower in 20 mg/kg and 40 mg/kg treatment groups than control group ($P < 0.05$). Moreover, dietary supplementation with high concentration diets groups (20 mg/kg and 40 mg/kg) significant increased the protein efficiency ratio (PER) index compared to the control group ($P < 0.05$). However, There were no significant differences in condition factor (CF), viscerosomatic index

Table 2
Effects of *G. elegans* alkaloids on growth performance, feed utilization and body indices in *M. amblycephala*.

Item	Diet 1 (0 mg/kg)	Diet 2 (5 mg/kg)	Diet 3 (10 mg/kg)	Diet 4 (20 mg/kg)	Diet 5 (40 mg/kg)
IBW (g)	3.71 ± 0.11^{a}	3.73 ± 0.08^{a}	3.71 ± 0.12^{a}	3.73 ± 0.11^{a}	3.79 ± 0.13^{a}
FBW (g)	24.82 ± 1.53^{a}	24.41 ± 1.17^{a}	25.03 ± 0.69^{ab}	26.98 ± 0.22^{bc}	28.89 ± 1.55^{c}
WGR (%)	569.37 ± 56.22^{ab}	553.87 ± 29.91^{a}	574.8 ± 11.92^{ab}	623.59 ± 19.33^{bc}	661.97 ± 23.07^{c}
SGR (%/d)	2.26 ± 0.1^{ab}	2.23 ± 0.05^{a}	2.27 ± 0.02^{ab}	2.36 ± 0.03^{bc}	2.42 ± 0.03^{c}
FCR (%)	2.29 ± 0.11^{a}	2.35 ± 0.09^{a}	2.29 ± 0.08^{a}	2.11 ± 0.02^{b}	2.01 ± 0.09^{b}
PER(%)	147.01 ± 7.29^{a}	143.24 ± 5.69^{a}	147.31 ± 4.9^{a}	159.18 ± 1.77^{b}	167.42 ± 7.18^{b}
CF (g/cm^{3})	2.21 ± 0.07^{a}	2.17 ± 0.04^{a}	2.14 ± 0.07^{a}	2.27 ± 0.11^{a}	2.26 ± 0.18^{a}
VSI (%)	12.28 ± 0.32^{a}	12.22 ± 0.14^{a}	12.62 ± 0.25^{a}	12.5 ± 0.16^{a}	12.4 ± 0.33^{a}
HSI (%)	1.75 ± 0.1^{a}	1.72 ± 0.07^{a}	1.75 ± 0.04^{a}	1.74 ± 0.06^{a}	1.82 ± 0.14^{a}

Data are summarized as means ± SD. IBW = initial body weight; FBW = final body weight; Weight gain rate (WGR, %) = 100 × (final body weight-initial body weight)/initial body weight; Specific growth rate (SGR, %/d) = 100 × (Ln final body weight-Ln initial body weight)/number of days; Feed conversion ratio = (total diet fed, g)/(total wet weight gain, g); Protein efficiency ratio (PER) = wet weight gain/protein intake; Condition factor (CF, g/cm^{3}) = 100 × (body weight, g)/(body length, cm)^{3}; Hepatosomatic index (HSI, %) = 100 × (liver weight, g)/(whole body weight, g); Viscerosomatic index (VSI, %) = 100 × (viscera weight, g)/(whole body weight, g). Values within a row with no common superscript differ significantly ($P < 0.05$) or differ extremely significantly ($P < 0.01$).

(VSI) and hepatosomatic index (HSI) among all treatments ($P > 0.05$).

3.2. Effect of dietary G. elegans alkaloids on proximate composition and amino acid profile

The whole body and muscle proximate compositions were shown in Table 3. Compared to control group, the 20 mg/kg and 40 mg/kg groups shown significantly higher whole body and muscle crude protein content ($P < 0.05$). The 20 mg/kg and 40 mg/kg groups significantly improved crude lipid contents of fish whole body ($P < 0.05$), meanwhile the 40 mg/kg group had the higher crude lipid contents of muscle ($P < 0.05$). Whole body crude ash content only increased significantly at 40 mg/kg levels ($P < 0.05$), the muscle crude ash showed no statistical difference in all treatment groups. However, Whole body and muscle moisture content was not significantly affected by the graded dietary *G. elegans* alkaloids levels ($P > 0.05$).

Table 4 showed the amino acid composition of fish muscle. Essential and non-essential amino acids content increased significantly as *G. elegans* alkaloids increased ($P < 0.05$), especially in 20 and 40 mg/kg groups. Meanwhile, the total amount of amino acids increased significantly with the increase of *G. elegans* alkaloids ($P < 0.05$). Among the 16 common amino acids, all the amino acids increased in varying degrees with the increase of *G. elegans* alkaloids, except for the content of serine showed no statistical difference ($P > 0.05$).

3.3. Effect of dietary G. elegans alkaloids on the lipid deposition in liver

To understand the lipid deposition in the liver, we carried out the histological study. Thus Oil red O was used to stain lipid on the liver frozen section slides. As shown in Fig. 1, with the increase of *G. elegans* alkaloids concentration, the liver lipid deposition became more serious, especially in 20 mg/kg and 40 mg/kg groups (Fig. 1d and e). Specifically, lipid droplets extended more widely in the liver cells. However, lipid deposition did not change significantly in 5 mg/kg and 10 mg/kg treatment groups (Fig. 1b and c).

3.4. Effect of dietary G. elegans alkaloids on plasma biochemical and immunity parameters

Plasma biochemical indexes were provided in Fig. 2. The results showed dietary *G. elegans* alkaloids had a significant effect on the contents of LDH, AST, ALT, ALP, TG, TC, LDL-C, HDL-C, ALB and TP ($P < 0.05$). The contents of LDH, AST, HDL-C and TP increased significantly with the increase of *G. elegans* alkaloids ($P < 0.05$) (Fig. 2a, b and c). The ALT, ALP and ALB contents were significantly increase only at the highest levels (40 mg/kg) compared to the control group ($P < 0.05$) (Fig. 2a and c). The TG and TC decreased significantly at 5 mg/kg and 10 mg/kg levels ($P < 0.05$), but TG content increased significantly with the further levels (40 mg/kg) ($P < 0.05$) (Fig. 2b). Moreover, the HDL contents of all treatment groups were significantly lower than control group ($P < 0.05$) (Fig. 2b). In addition, we detected some immune indices including complement 3 (C3), complement 4 (C4) and immunoglobin M (lgM) in plasma. We found that the contents of C3, C4 and lgM increased significantly at different levels compared to the control group ($P < 0.05$), except for C3, it decreased significantly at 10 mg/kg concentration ($P < 0.05$) (Fig. 3).

3.5. Effect of dietary G. elegans alkaloids on liver and plasma antioxidant indices

The effect of *G. elegans* alkaloids on the liver and plasma oxidative status was shown in Fig. 4. These antioxidant indexes include superoxide dismutase (SOD) catalase (CAT), total anti-oxidizing capability (T-AOC) and Malondialdehyde (MDA). With the increase of *G. elegans*

Table 3
Effects of *G. elegans* alkaloids on whole body and muscle proximate composition of *M. amblycephala*.

Proximate composition	Diet 1 (0 mg/kg)	Diet 2 (5 mg/kg)	Diet 3 (10 mg/kg)	Diet 4 (20 mg/kg)	Diet 5 (40 mg/kg)
Whole body (%)					
Moisture	68.65 ± 0.69^{a}	68.03 ± 0.65^{a}	68.52 ± 0.13^{a}	68.51 ± 0.71^{a}	68.15 ± 0.71^{a}
Crude protein	49.72 ± 1.66^{a}	50.26 ± 1.33^{ab}	50.7 ± 1.81^{ab}	52.4 ± 0.76^{b}	52.35 ± 0.92^{b}
Crude lipid	36.65 ± 0.5^{a}	36.01 ± 1.17^{a}	36.92 ± 0.99^{ab}	39.41 ± 2.63^{bc}	39.77 ± 1.34^{c}
Crude ash	9.2 ± 0.56^{a}	9.22 ± 0.26^{a}	9.23 ± 0.14^{a}	9.45 ± 0.3^{ab}	9.97 ± 0.22^{b}
Muscle (%)					
Moisture	74.18 ± 0.71^{a}	74.5 ± 0.78^{a}	74.25 ± 0.41^{a}	74.4 ± 0.94^{a}	74.54 ± 0.67^{a}
Crude protein	70.36 ± 3.15a	71.57 ± 0.78a	72.66 ± 1.99^{ac}	75.63 ± 2.99^{bc}	77.05 ± 1.95^{b}
Crude lipid	21.03 ± 1.61^{a}	21.66 ± 0.53^{a}	22.2 ± 0.72^{a}	21.76 ± 0.84^{a}	23.99 ± 0.32^{b}
Crude ash	6.01 ± 0.24^{a}	6.21 ± 0.28^{a}	6.15 ± 0.23^{a}	6.28 ± 0.35^{a}	6.29 ± 0.26^{a}

Values are means ± SD of three replications. Values within a row with no common superscript differ significantly ($P < 0.05$) or differ extremely significantly ($P < 0.01$).

Table 4
Effects of *G. elegans* alkaloids on muscle amino acid composition of *M. amblycephala*.

Amino acids (%)	Diet 1 (0 mg/kg)	Diet 2 (5 mg/kg)	Diet 3 (10 mg/kg)	Diet 4 (20 mg/kg)	Diet 5 (40 mg/kg)
Threonine (Thr)	0.81 ± 0.02^{a}	0.85 ± 0.02^{bc}	0.82 ± 0.03^{bc}	0.82 ± 0.03^{b}	0.86 ± 0.02^{c}
Valine (Val)	0.81 ± 0.01^{a}	0.85 ± 0.02^{b}	0.87 ± 0.02^{b}	0.88 ± 0.01^{b}	0.91 ± 0.03^{c}
Methionine (Met)	0.55 ± 0.02^{a}	0.55 ± 0.01^{ab}	0.55 ± 0.01^{a}	0.56 ± 0.01^{ab}	0.57 ± 0.02^{b}
Isoleucine (Ile)	0.77 ± 0.02^{a}	0.8 ± 0.01^{b}	0.8 ± 0.02^{b}	0.81 ± 0.01^{bc}	0.83 ± 0.01^{c}
Leucine (Leu)	1.46 ± 0.02^{a}	1.52 ± 0.01^{b}	1.5 ± 0.01^{b}	1.51 ± 0.02^{b}	1.56 ± 0.02^{c}
Phenylalanine (Phe)	0.76 ± 0.01^{a}	0.8 ± 0.01^{b}	0.78 ± 0.01^{c}	0.81 ± 0.01^{bd}	0.82 ± 0.02^{d}
Lysine (Lys)	1.81 ± 0.02^{a}	1.86 ± 0.02^{b}	1.84 ± 0.03^{b}	1.85 ± 0.01^{b}	1.91 ± 0.01^{c}
Histidine (His)	0.62 ± 0.01^{a}	0.65 ± 0.01^{b}	0.63 ± 0.01^{a}	0.65 ± 0.00^{b}	0.67 ± 0.02^{c}
Aspartic acid (Asp)	1.84 ± 0.03^{a}	1.87 ± 0.04^{a}	1.86 ± 0.04^{a}	1.9 ± 0.04^{a}	2.02 ± 0.09^{b}
Serine (Ser)	0.7 ± 0.02^{a}	0.72 ± 0.01^{a}	0.71 ± 0.02^{a}	0.7 ± 0.03^{a}	0.71 ± 0.02^{a}
Glutamic acid (Glu)	2.69 ± 0.03^{a}	2.8 ± 0.03^{b}	2.7 ± 0.02^{a}	2.73 ± 0.03^{a}	2.84 ± 0.02^{b}
Glycine (Gly)	0.84 ± 0.03^{a}	0.89 ± 0.03^{b}	0.88 ± 0.03^{ab}	0.88 ± 0.02^{ab}	0.89 ± 0.03^{b}
Alanine (Ala)	1.1 ± 0.03^{ab}	1.13 ± 0.02^{b}	1.09 ± 0.03^{a}	1.11 ± 0.02^{ab}	1.14 ± 0.01^{bc}
Tyrosine (Tyr)	0.6 ± 0.02^{a}	0.63 ± 0.02^{ab}	0.61 ± 0.02^{ac}	0.63 ± 0.01^{bc}	0.64 ± 0.01^{b}
Arginine (Arg)	1.08 ± 0.01^{a}	1.13 ± 0.02^{bc}	1.1 ± 0.02^{ab}	1.11 ± 0.01^{ab}	1.15 ± 0.03^{c}
Proline (Pro)	0.69 ± 0.01^{b}	0.71 ± 0.01^{b}	0.65 ± 0.01^{a}	0.74 ± 0.01^{c}	0.69 ± 0.02^{b}
EAAs	7.58 ± 0.05^{a}	7.88 ± 0.04^{b}	7.8 ± 0.05^{b}	7.88 ± 0.03^{b}	8.13 ± 0.06^{c}
NEAAs	9.55 ± 0.04^{a}	9.88 ± 0.04^{b}	9.6 ± 0.02^{a}	9.8 ± 0.09^{b}	10.07 ± 0.14^{c}
TAAs	17.13 ± 0.07^{a}	17.76 ± 0.08^{b}	17.4 ± 0.06^{c}	17.69 ± 0.08^{b}	18.2 ± 0.20^{d}

EAAs = Essential amino acids; NEAAs = Non-essential amino acids; TAAs = Total amino acids. Values are means ± SD of three replications. Values within a row with no common superscript differ significantly ($P < 0.05$) or differ extremely significantly ($P < 0.01$).

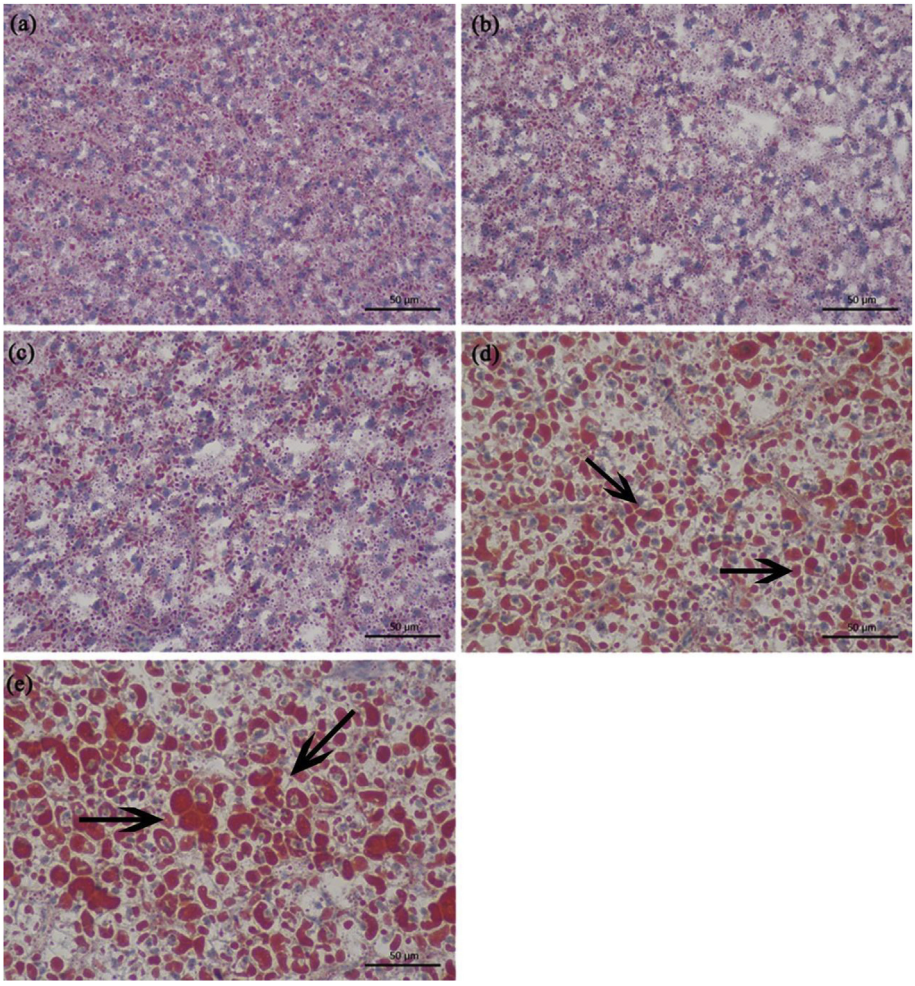

Fig. 1. Histological analysis of *M. amblycephala* liver after feeding with *G. elegans* alkaloids. The liver frozen sections stained with oil red O. (a) Negative control (NC), 0 mg/kg and *G. elegans* alkaloids added at (b) 5 mg/kg; (c) 10 mg/kg; (d) 20 mg/kg; (e) 40 mg/kg. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

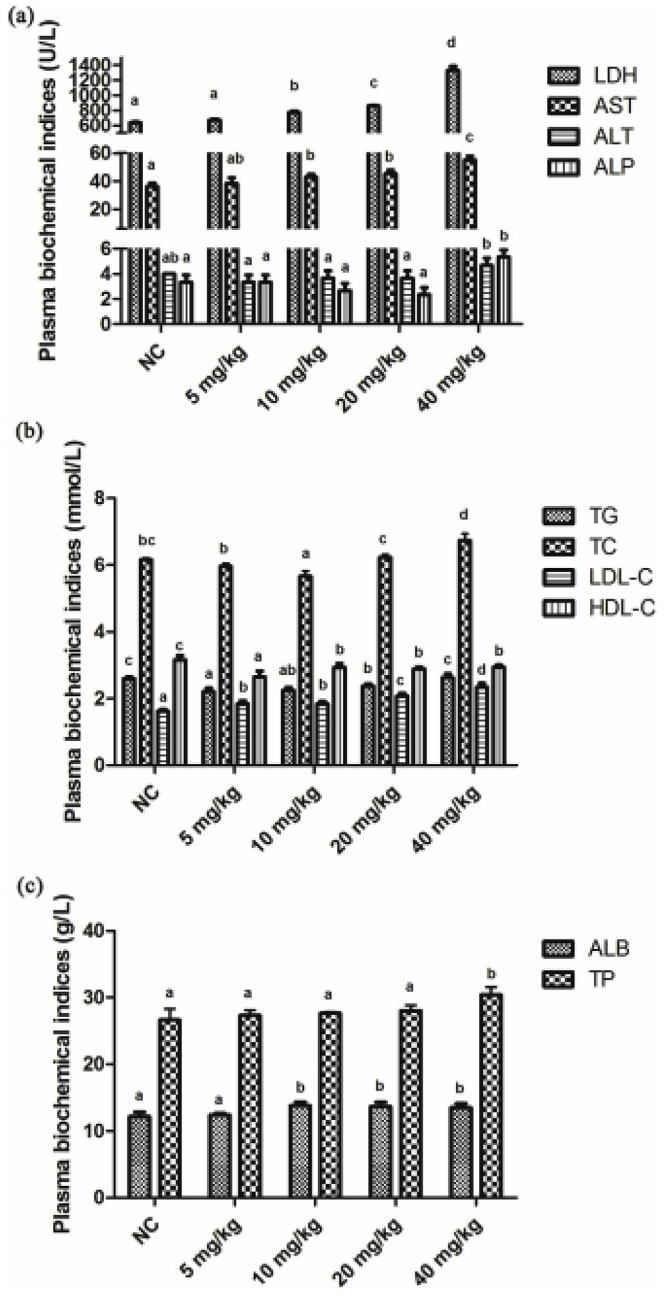

Fig. 2. ***Plasma biochemical parameters for M. amblycephala*** **fed with** ***G. elegans*** **alkaloids.** (a) Lactate dehydrogenase (LDH), aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP) (U/L); (b) Triacylglycerol (TG), total cholesterol (TC), low density lipoprotein cholesterol (LDL-C) and high density lipoprotein cholesterol (HDL-C) (mmol/L); (c) Albumin (ALB) and total protein (TP) (g/L). NC= (Negative control, 0 mg/kg). Results were expressed as mean ± SD (n = 3). Values with no common superscript differ significantly ($P < 0.05$) or differ extremely significantly ($P < 0.01$).

alkaloids, the liver SOD, CAT, T-AOC, MDA and plasma CAT, T-AOC, MDA levels decreased significantly at first and then increased significantly ($P < 0.05$) (Fig. 4a, b, c and d). The results showed the liver SOD, CAT, T-AOC, MDA and plasma CAT, T-AOC, MDA levels were the lowest in 10 mg/kg group and the highest in 40 mg/kg group. However, plasma SOD only increased significantly at the highest level (40 mg/kg) compared with the control group ($P < 0.05$) (Fig. 4c), while other concentrations did not change significantly.

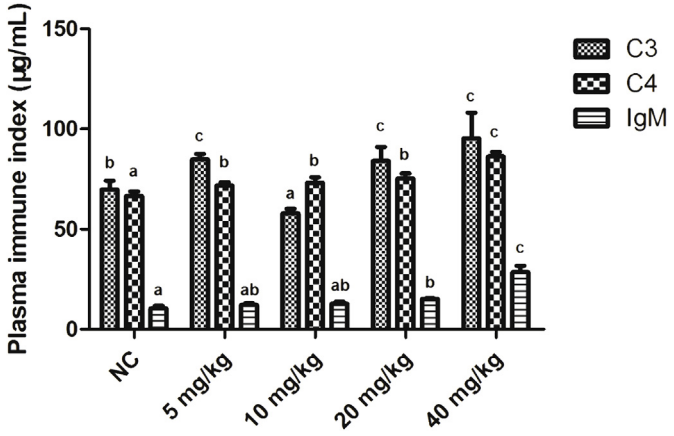

Fig. 3. ***Plasma immune parameters for M. amblycephala*** **after feeding with** ***G. elegans*** **alkaloids.** NC= (Negative control, 0 mg/kg). Data are expressed as mean ± SD for n = 3. Values with no common superscript differ significantly ($P < 0.05$) or differ extremely significantly ($P < 0.01$).

3.6. Immune-related genes expression

Fig. 5 showed the expressions of *IL-1β*, *IL8*, *IL10*, *TNF-α*, *IFN-α*, *TGF-β*, *Cu/Zn-SOD*, *Mn-SOD* in the liver, spleen and head kidney of *M. amblycephala* fed with *G. elegans* alkaloids for 12 weeks. The interleukin family genes (*IL-1β* and *IL8)* mRNA level in the liver, spleen and head kidney increased significantly in 20 mg/kg and 40 mg/kg treatment groups compared with the control group ($P < 0.05$) (Fig. 5 a and b). Especially in the liver, the expression of *IL-1β* and *IL8* were the lowest in control group compared with other tissues at low concentrations, but these genes expressed significantly increase at high *G. elegans* alkaloids levels ($P < 0.05$). However, the expression level of *IL10* reduced after feeding with *G. elegans* alkaloids (Fig. 5 c). Moreover, cytokine-related genes (*TNF-α* and *IFN-α*) mRNA levels in the liver, spleen and head kidney also had significant increase at 20 mg/kg and 40 mg/kg G. *elegans* alkaloids ($P < 0.05$), and these increases are dose-dependent (Fig. 5 d, e and f). However, *TGF-β* gene showed a trend of decreasing first and then increasing in the liver and spleen, and minimum expression was obtained in 5 mg/kg and 20 mg/kg groups ($P < 0.05$), respectively. In addition, *Cu/Zn-SOD* mRNA level in the liver, spleen and head kidney increase with increasing *G. elegans* alkaloids. *Cu/Zn-SOD* mRNA in the liver, spleen and head kidney significantly increased in 40 mg/kg group ($P < 0.05$) (Fig. 5 g). *Mn-SOD* mRNA level in the liver was a dose-dependent increase, and the relative mRNA expression level of *Mn-SOD* significantly increased in 20 mg/kg and 40 mg/kg groups, but no significant difference was found in the spleen and head kidney among the groups ($P > 0.05$) (Fig. 5 h).

3.7. The survival rate after A. hydrophila challenge and the residues of G. elegans alkaloids in blood and muscle

In order to verify the immune enhancement of the *G. elegans* alkaloids to *M. amblycephala*, we carried out challenging test using *A. hydrophila*. The challenge test result showed that the highest survival rate was observed in 20 mg/kg and 40 mg/kg G. *elegans* alkaloids groups ($P < 0.05$), and showed no difference between 20 mg/kg and 40 mg/kg groups ($P > 0.05$) (Fig. 6). Dietary supplementation with *G. elegans* alkaloids could significantly increase survival rate compared to the control group ($P < 0.05$). Due to the toxicity of *G. elegans* alkaloids, the *G. elegans* alkaloids residues in muscle and blood must be detected. Gas chromatographic results show that had no *G. elegans* alkaloids residue in blood and muscle at 40 mg/mL group (Fig. 7).

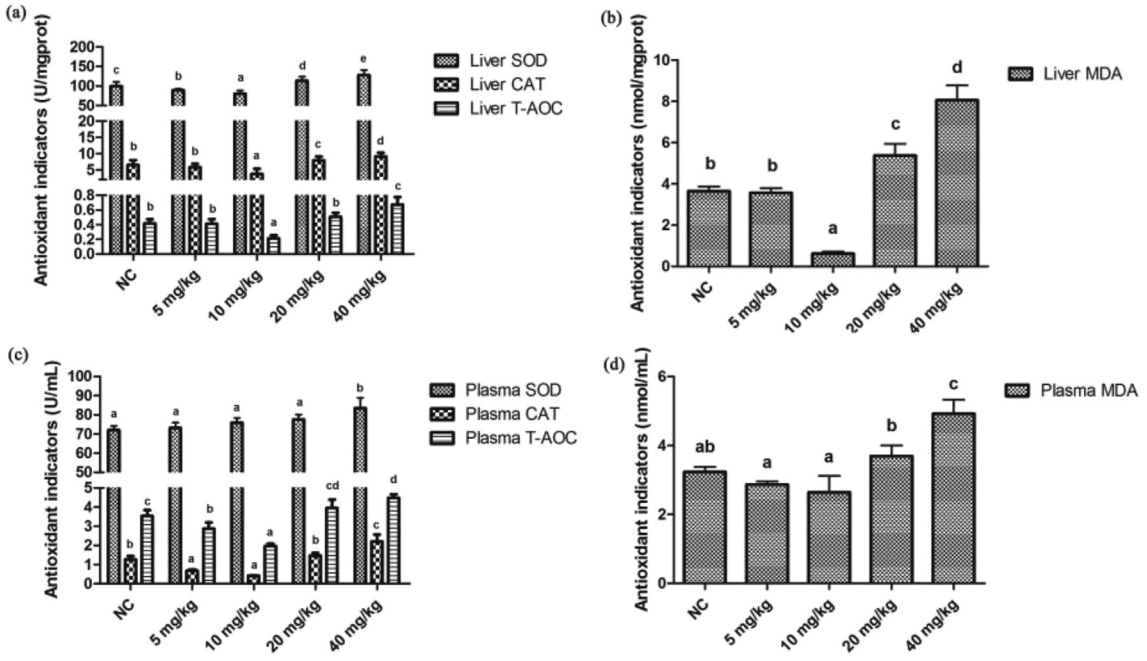

Fig. 4. ***Antioxidant parameters for M. amblycephala*** **fed with** ***G. elegans*** **alkaloids**. SOD: superoxide dismutase; CAT: catalase; MDA: malondialdehyde; T-AOC: total antioxidant capacity. (a) Liver SOD, CAT and T-AOC (U/mgprot); (b) Liver MDA (nmol/mgprot); (c) Plasma SOD, CAT and T-AOC (U/mL); (d) Plasma MDA (nmol/mL). NC= (Negative control, 0 mg/kg). Data are expressed as mean ± SD (n = 3). Values with no common superscript differ significantly ($P < 0.05$) or differ extremely significantly ($P < 0.01$).

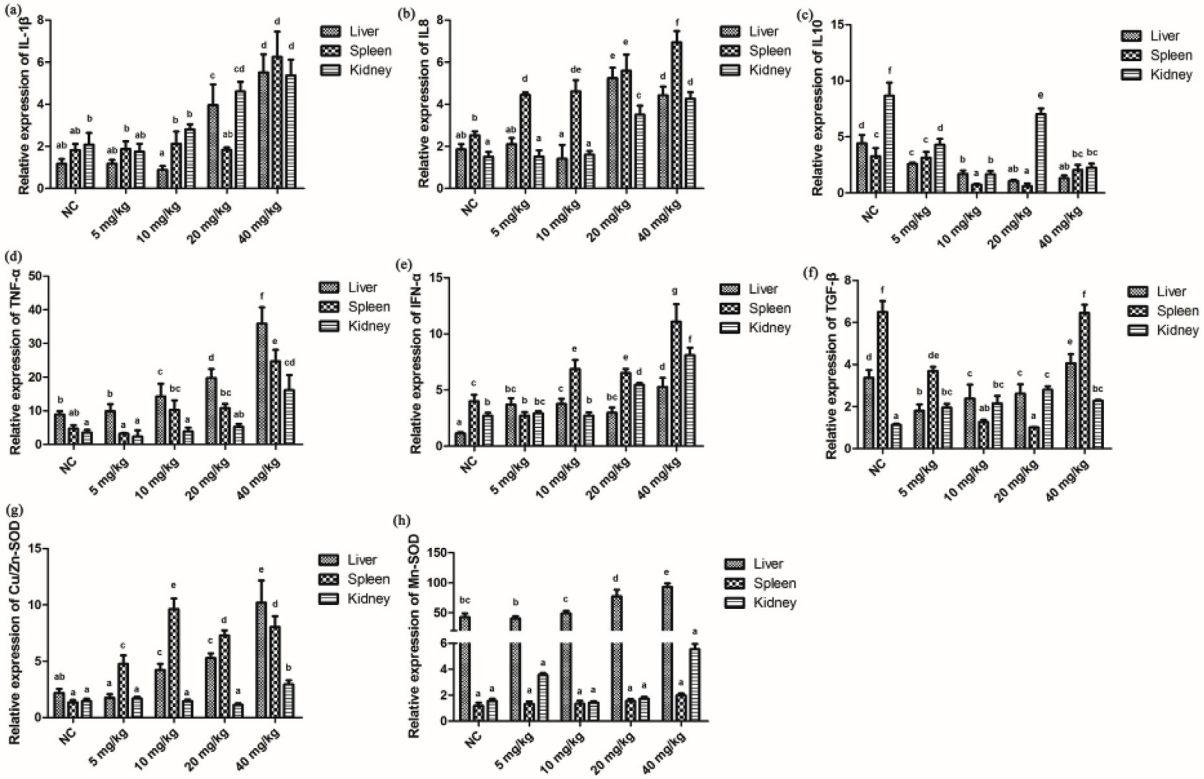

Fig. 5. Expression of immune related genes in ***M. amblycephala*** **after feeding with** ***G. elegans*** **alkaloids**. The detected tissues included liver, spleen and head kidney. (a) Interleukin 1β (*IL-1β*); (b) Interleukin 8 (*IL8*); (c) Interleukin 10 (*IL10*); (d) Tumor necrosis factor α (*TNF-α*), (e) interferon α (*IFN-α*); (f) Transforming growth factor β (*TGF-β*); (g) Cu/Zn superoxide dismutase (*Cu/Zn-SOD*); (h) Mn superoxide dismutase (*Mn-SOD*). NC= (Negative control, 0 mg/kg). Results were normalized against *β-actin.* Data were expressed as mean ± SD (n = 3). Values with no common superscript differ significantly ($P < 0.05$) or differ extremely significantly ($P < 0.01$).

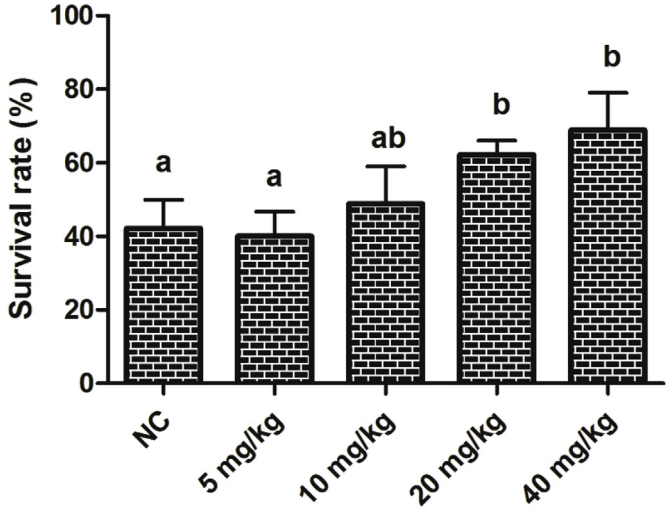

Fig. 6. Survival rate of *M. amblycephala* after *A. hydrophila* Challenge. NC= (Negative control, 0 mg/kg). Data were expressed as mean ± SD for n = 3. Values with no common superscript differ significantly ($P < 0.05$) or differ extremely significantly ($P < 0.01$).

4. Discussion

In recent years, with the rapid development of aquaculture and its high-density farming, the aquaculture environment is deteriorating and the diseases of aquaculture organisms occur frequently. Immunostimulants had been widely used in aquaculture as an effective substance to control fish diseases. At present, the most commonly immunostimulants are synthetic chemicals, microbial derivatives, plant extracts, vitamins and hormones. Chemical drugs have been widely used in aquaculture, meanwhile chemical drugs bring multiple negative impacts on environment and human health including drug resistance and drug residues [29], so it is necessary to find more effective and environment friendly immunostimulants. Plant extracts have attracted widespread attention due to inexpensive, locally available, easy to decompose and environmentally friendly characters [28]. Plant extracts are known to promote growth, stimulate appetite and immunostimulation, and anti-pathogenic properties in fish [29]. Plant extracts also has been reported to enhance growth performance and immunity in fishes [30,31]. *G. elegans* widely distributed in China is a kind of well-known Chinese herbal medicine, which has many biological functions including neuropathic pain [12], inflammatory [12], rheumatic arthritis and anxiety [13]. *G. elegans* has not been studied in aquaculture and its biological function is not clear in aquatic organisms. In the present study, We used *G. elegans* alkaloids as dietary supplementation in a 12 week feeding trial to study its effects in *M. amblycephala*. For instance, whether the *G. elegans* alkaloids had potential beneficial effects on fish growth performance and immunity. In the results, we observed a significant increase of WGR, SGR, PER and a significant decrease of FCR at 20 mg/kg and 40 mg/kg G. *elegans* alkaloids in the feeding trial. FCR and PER are important indicators of fish diet quality to evaluate protein utilization [32], the WGR and SGR are the growth index that we concerned [33]. Previous studies have shown that dietary supplementation of the alkaloids (betaine) can improve the growth parameters of *M. amblycephala* [34]. Our results showed the same trend that the growth performance and feed efficiency of fish could be improved by adding *G. elegans* alkaloids as dietary supplementation. Studies have shown that alkaloids can also improve growth performance of other species, such as chickens and pigs [35,36]. Deposition of related amino acids and lipids has an important effect on nutritive value and meat flavor [37,38]. The dietary supplementation of *G. elegans* alkaloids increased crude protein and lipid content in the whole body and muscle, and the essential and non-essential amino acid contents also increased significantly in 20 mg/kg and 40 mg/kg groups. In addition, histological analysis also showed that *G. elegan* alkaloids were associated with lipid deposition in 20 mg/kg and 40 mg/kg groups.

The plasma parameters are generally considered health condition indicators of fish and those parameters are useful for determining the fish in response to dietary supplements [39]. As far as we know, there are few studies available on the effects of dietary *G. elegans* alkaloids on

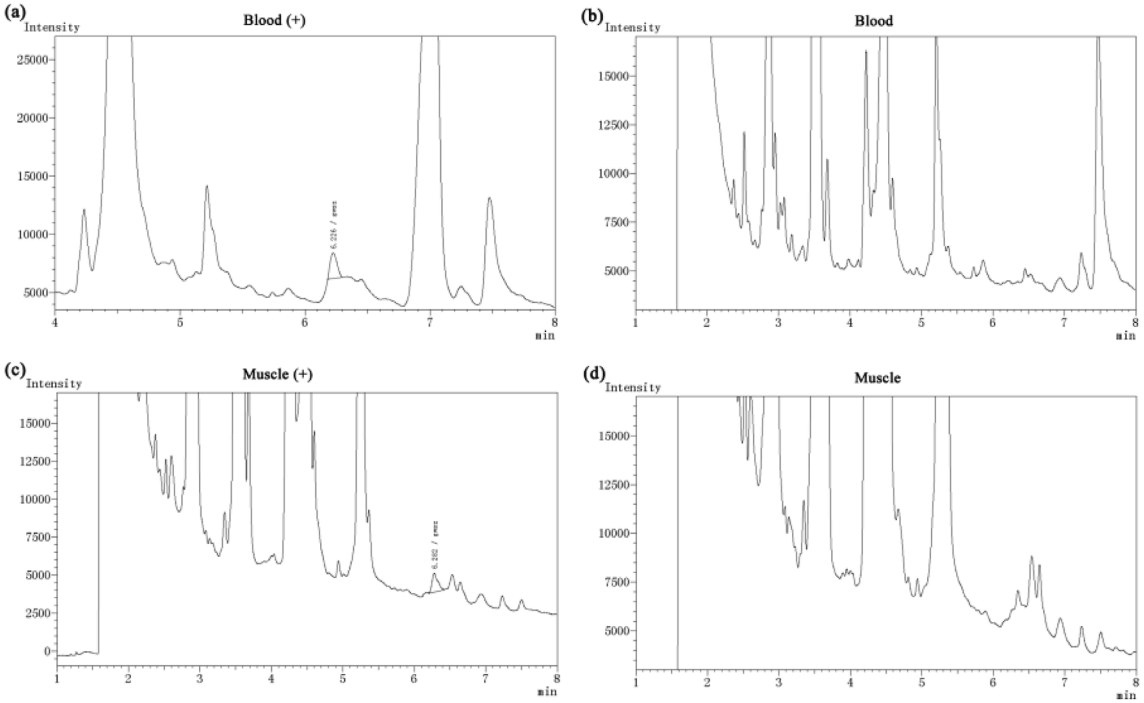

Fig. 7. *G. elegans* alkaloids residues in liver and blood were detected by gas chromatography. (a) Blood (+), Blood with koumine added as positive control; (b) Blood. (c) Muscle (+), Muscle with koumine added as positive control; (d) Muscle.

plasma parameters of fish. AST, ALT, ALP and LDH are important enzymes present in the liver was reported to be a marker for liver injury [40,41]. Meanwhile, AST and ALT are crucial amino transferases in fish and related to amino acid metabolism [42]. In our study, increments of AST, ALT, ALP and LDH in 20 mg/kg and 40 mg/kg groups indicates damage of liver that was caused by *G. elegans* alkaloids. This results may also be associated with abnormal metabolism of amino acids. Previous studies have shown that the plant extracts, such as dandelion and Moringa oleifera seed extracts, can increase the serum AST, ALT and ALP levels in fish [33,43], and the results are similar to our study. The TG, TC, LDL-C and HDL-C are important indexes of lipid metabolism in plasma [44,45]. The increase of TG, TC, LDL-C and HDL-C in plasma may show lipids metabolic disorders and liver damage [46,47]. Previous studies showed that the dandelion extracts can increase serum HDL-C content and decreased serum TG, TC and LDL-C contents in rabbits and mice [48,49]. In our study we found the opposite trend that the serum TG, TC and LDL-C levels were significantly increased with dietary *G. elegans* alkaloids addition. On the contrary, the HDL-C level decreased significantly as *G. elegans* alkaloids supplementation increased, which indicated that *G. elegans* alkaloids diets resulted in remarkable changes in the plasma lipid profile. This improved plasma component might be related to its body and muscle composition (lipid deposition) by increasing TG and TC synthesis, and improving LDL content and equally enhancing liver functioning enzyme activities such as AST, ALT, ALP and LDH of fish. In addition, the increase of plasma TP and ALB content in fish is thought to be associated with a stronger innate immunity response [50,51]. In this study, a significantly enhancement of TP and ALB contents were observed in fish fed with 40 mg/kg G. *elegans* alkaloids, which indicated that the *G. elegans* alkaloids also had some effect on the immune response of fish.

Complement and immunoglobulins play an important role in non-specific and specific immunity of fish [52]]. Complement is a plasma protein that exists in organisms blood and tissue fluids, which can mediate immune response and inflammatory reaction, and the content of C3 and C4 were higher than those of other complement molecules [53]. Fish lgM was the main immune mediator in fish specific humoral immune response, which was present in the blood and immune fluids [54]. Previous studies have shown that some plant immunostimulants may increase C3, C4 and IgM content in fish species [17,39]. Our study results also showed that the plasma C3, C4 and IgM content were significantly increased in *M. amblycephala* after feeding with *G. elegans* alkaloids. These results indicated that *G. elegans* alkaloids could improve the non-specific immunity of fish.

Antioxidant system is an important part of non-specific immune system [55]. Generally speaking, antioxidant enzymes including SOD, CAT and T-AOC and the MDA content are the most commonly used antioxidant indicators in fish [56,57]. SOD and CAT are natural scavenger of oxygen free radicals in organisms. SOD can convert harmful superoxide free radicals into hydrogen peroxide, then the CAT immediately decomposed it into completely harmless compounds, thus they work together to reducing the toxic effect [58]. The T-AOC directly reflected antioxidant capacity of fish and the MDA content indirectly reflected the severity of free radicals attacked in fish [57]. Previous studies have shown that plant extracts, such as palm fruits and *Origanum vulgare* leaf extracts can increase the antioxidant stress ability of fish [59,60]. *G. elegans* alkaloids monomers have been shown to exhibit antioxidant effects in *Tetrahymena rhermophila* cells [61,62]. Consistent with these studies, we found the treatment groups supplemented with *G. elegans* alkaloids had significantly increased contents of the plasma and liver SOD, CAT and T-AOC and MDA compared to the control group. The results further illustrate that *G. elegans* alkaloids may activate antioxidant system in fish.

The proliferation, differentiation and function of immune system cells are regulated by a series of cytokines, including interleukin, interferon, transforming growth factor and tumor necrosis factor family etc [63,64]. Cytokines can be divided into two categories: pro-inflammatory (IL-1β, IL8, TNF-α and IFN-α ect.) and anti-inflammatory (IL10 and TGF-β etc.), which are considered as important initiators and mediators of inflammation [65,66]. Many studies reported that plant immunostimulants up-regulated the pro-inflammatory cytokines and down-regulated the anti-inflammatory cytokines in fish fed with *Spirulina platensis* and guava leaves extracts [67,68]. In the present study, the mRNA levels of *IL-1β*, *IL8*, *TNF-α* and *IFN-α* genes were up-regulated, whereas *TGF-β* and *IL10* genes were down-regulated in the immune organ (liver, spleen and head kidney) of fish fed dietary supplementation with *G. elegans* alkaloids. The results indicated that *G. elegans* alkaloids trigger the immune response by promoting the expression of pro-inflammatory factors. However, understanding the underlying mechanism requires further study. In addition, superoxide dismutase (SOD) is classified into three forms based on the metal cofactors active site, including copper/zinc-SOD, iron-SOD and manganese-SOD [69]. In fish, two kinds of SODs have been commonly studied: Cu/Zn-SOD and Mn-SOD [70]. In our study, Cu/Zn-SOD and Mn-SOD mRNA levels were up-regulated in the liver, spleen and head kidney by *G. elegans* alkaloids, which showed a similar expression pattern with the plasma and liver SOD. This result once again confirms that the extract can activate the antioxidant system of fish.

The bacterial challenge trial provides an opportunity to assess the effectiveness of dietary therapy in preventing pathogens [71]. *A. hydrophila* is known to cause serious diseases in freshwater fish, especially epidemic septicaemia [72]. Previous studies have shown that plant extracts can enhance resistance of fish to several bacterial pathogens [30,73]. In our study, dietary *G. elegans* alkaloids showed a significantly increased survival rate in *M. amblycephala* after challenge with *A. hydrophila*. This result indicated that *G. elegans* alkaloids enhanced the immunity of *M. amblycephala* and showed positive effects against *A. hydrophila* infection. *G. elegans* alkaloids were improving growth parameter, amino acids and lipid deposition, organism immunity and resistance to disease, which is good news for aquaculture, but it had some kind of toxicity [74], so the residues in blood and muscle are concerned by people. We use koumine as the detection index to detect the residues, which was the highest component of *G. elegans* alkaloids. Gas chromatographic results showed no *G. elegans* alkaloids residue in blood and muscle. The results indicated *G. elegans* alkaloids can be used as daily immunostimulant additives in aquaculture.

5. Conclusions

Sum up, this is the first report showing that *G. elegans* alkaloids can be used as an additive in aquaculture. In our study, we found dietary *G. elegans* alkaloids promoted the growth performance, amino acid and lipid deposition of *M. amblycephala*, as well as improved immune ability and survival rate against *A. hydrophila* challenge, especially at 20 mg/kg and 40 mg/kg. Meanwhile, no *G. elegans* alkaloids residues were detected in the blood and muscle. Therefore, *G. elegans* alkaloids can be used as an efficient and environment friendly immunostimulant to improve fish immune ability and yield of aquaculture. This study preliminarily explored the application of *G. elegans* alkaloids as an immunostimulant in aquaculture, and its specific mechanism needs further study.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2019.05.026.

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