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Evaluation of Liver Damage and Herbal Detoxification of Coral Reef Fish Caused by Ammonia Stress

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ABSTRACT

Ammonia nitrogen can cause serious toxicological damage to fish and cause fish death. This study focused on liver damage caused by ammonia nitrogen stress in coral reef fish and used herbal extracts to improve ammonia poisoning and prevent liver tissue damage. Effective prevention of ammonia poisoning is critical in the large-scale aquaculture of coral reef fish. In this study, *Amphiprion ocellaris*, a large-scale coral reef fish aquaculture species, was selected as the experimental fish species. Natural botanicals were added to the feed to evaluate their ability to mitigate oxidative stress in the liver and *A. ocellaris* stress resistance. The natural botanicals used in this study included *Silybum marianum* (SM), *Lycium chinense* (LC), *Vigna radiate* (VR), *Vigna angularis* (VA) and *Colix lacrymaiobi* (CL), respectively. The concentrations added were 1000 (I) and 2000 (II) mg kg^{-1}, respectively. Twelve days of feeding were performed. After the end of the day, the fish were challenged with unionized ammonia-N 0.2 and 0.6 mg L^{-1}. Alanine aminotransferase (ALT), aspartate aminotransferase (AST), superoxide dismutase (SOD), catalase (CAT), growth and survival were detected to evaluate resistance to ammonia and oxidative stress in the liver. Histopathological studies were conducted on the liver tissues of each treatment group. The results demonstrated that SM(I) and SM(II) can improve *A. ocellaris* resistance to ammonia stress and increase its growth and survival rates. These results can be applied to the large-scale breeding of marine fish to prevent and control ammonia stress effectively.

1 | Introduction

Toxic substances can cause significant physiological damage to aquatic organisms. Poison fishing, feed toxins and poisons dissolved in the water can cause serious damage to the fish and coral reefs (Thornhill 2012). According to surveys, up to 80% of marine ornamental fish will die before or shortly after being caught (Hanawa et al. 1998). This may be caused by poisoning or stress-induced injuries. The liver is a vital detoxification organ in the fishes. If the survival rate of wild-caught marine ornamental fish can be improved by liver detoxification, it will be of significant value to the ornamental fish trade and large-scale circulating aquaculture. In addition to poisoning, ammonia is a toxin that causes fish death. The major sources in water are nitrogen-containing metabolic end products produced by the metabolism of proteins in aquatic organisms discharged into the water (Wood 1991; Wilkie 1997). Transamination through the liver is produced by the aerobic or anaerobic action of bacteria on aquatic organism faeces and residual food (Ballantyne 2001; Ip et al. 2001). Ammonia in water predominantly comprises ionic NH_4^+ and non-ionic NH_3. The former is a hydrophilic molecule that cannot penetrate the fish body from the outside world. The latter is a lipophilic molecule with smaller molecules that can easily penetrate lipids. The qualitative cell membrane enters the body cells of the fish, causing ammonia poisoning (Wang et al. 2006). Previous studies have demonstrated that an ammonia

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concentration of 0.006–0.34 mg L^{-1} in water causes pathological changes in fish organs and tissues. If the ammonia concentration is below the semi-lethal concentration (LC_{50}), it affects the health of the fish, thereby affecting their growth and survival (Ip et al. 2001). Generally, ammonia content in water between 0.6 and 2.0 mg L^{-1} will cause fish poisoning. The pH of seawater is high, and highly toxic NH_3 often remains in the water, posing a serious threat to the seawater fish (Ip et al. 2001). Under general intensive breeding conditions, the breeding density is high, and the amount of feed is large. This can easily cause the faeces to be unable to decompose, or there will be too much residual feed, resulting in excessive ammonia. This directly affects the growth, immune defence ability, or survival of cultured organisms. Therefore, if the ability to resist ammonia stress can be improved through feeding methods, it will be of significant value for large-scale aquaculture.

Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) are found in fish livers. Both are non-specific enzymes in the plasma and are relevant to cell damage in specific tissues. Transaminase activity in fish plasma can be used to diagnose liver function impairment (Hernandez et al. 2007; Suzumura et al. 2000). High ammonia stress also causes changes in the antioxidant systems of the fish. Previous studies have demonstrated that high ammonia stress affects ALT and AST activities in the fish livers (Lemarié et al. 2004; Siscar et al. 2014). AST and ALT levels are indirectly related to oxidative metabolism; therefore, we also measured changes in superoxide dismutase (SOD) and catalase (CAT) levels to evaluate the ability of natural botanicals to resist stress. In aerobic fish cells, reactive oxygen species, such as superoxide anions and hydroxyl radicals, are produced during normal metabolism, especially due to mitochondrial membrane oxidative metabolism. These compounds may harm cells and cause oxidative stress (Meng et al. 2014). Aerobic organisms have evolved defence systems to protect themselves from the toxic effects of increased ROS production by activating antioxidant systems, such as glutathione and Phase II detoxification enzymes. Specific antioxidant enzymes, the first line of defence against oxidative stress, include SOD and CAT. When fish are stressed by poisons, ROS are induced to produce SOD and CAT to resist oxidative stress (Zhang et al. 2004).

Herein, five natural botanicals were fed to evaluate their protective ability on fish livers, including *Silybum marianum* (SM), *Lycium chinense* (LC), *Vigna radiate* (VR), *Vigna angularis* (VA), and *Coix lacrymaiobi* (CL). SM possesses antioxidant properties, removes free radicals and inhibits lipid peroxidation. In recent years, it has often been used as a natural medicine to protect liver function and treat chronic liver diseases (Flora et al. 1998; Torres et al. 2013). LC contains trace elements, such as potassium, calcium, sodium, zinc, iron, copper, manganese, selenium, chromium, strontium, lead, nickel, cadmium, arsenic, cobalt and magnesium. It can improve immunity and have pharmacological effects in terms of health, including anti-ageing and liver protection (Chang and So 2008; Kim et al. 1999). VR is effective in treating burns and mumps and acts as a detoxifying agent and an antipyretic (Kim et al. 1998). After extraction with methanol, mung bean testa demonstrated good antioxidant and antioxidant inhibitory effects on the liposome model system and lipid peroxidation (Duh et al. 1997). VA is rich in anthocyanins and has antioxidant capabilities (Madhujith et al. 2004; Ranilla et al. 2007). CL has anti-inflammatory, antioxidant, immune-promoting, blood pressure regulatory, anticancer, antimutagenic, antiallergic and hypolipidemic effects (Kuo et al. 2001).

This study was conducted using clownfish (*Amphiprion ocellaris*), the most representative seawater ornamental fish. Clownfish are loved by aquariums, making the seawater ornamental trade of clownfish frequent globally. Ammonia stress caused by poisonous fishing or large-scale aquaculture in the wild affects fish physiology and survival. However, *A. ocellaris* is monogamous and can be reproduced quantitatively in tanks (Allen et al. 2008). These marine fishes are suitable for research purposes. This study aimed to evaluate the hepatoprotective and antioxidant effects of natural botanicals in clownfish.

2 | Materials and Methods

2.1 | Fish Sample

To ensure that the *A. ocellaris* used in the experiment came from the same batch and to ensure the accuracy of the experiment, self-cultivated F1 progeny were selected. The clownfish were moved to an experimental tank ($60 \times 60 \times 40$ cm^3) and isolated into groups with box nets ($20 \times 15 \times 15$ cm^3). Circulating water filtration was used to maintain stable water quality. Five fish were included in each treatment group, with three replicates each ($n = 15$ fish). Their weight was 20.00 ± 0.17 g. The daily feeding amount was 5% of the fish's body weight. The fish were fed twice (9:00 and 17:00) for a total of 12 weeks. A siphon method was used to drain the bottom 1 h after daily feeding to remove excrement and residual bait at the bottom.

2.2 | Feed

The natural botanicals selected for this study included SM, LC, VR, VA and CL, respectively. They were added to the prepared feed at two concentrations of 1000 (I) and 2000 (II) mg kg^{-1}, respectively, as depicted in Table 1. Refer to the extraction method for natural botanicals by Ding et al. (2023). The plant material was first ground and then soaked in 70% (v/v) ethanol for 48 h, followed by concentration using a rotary evaporator. After concentration, it is placed at −20°C and frozen to achieve freeze-drying. The freeze-dried powder can be used as a feed additive. These were denoted as SM I, LC I, VR I, VA I, CL I, SM II, LC II, VR II, VA II and CL II, respectively. The formulated diet without natural botanicals was used as the control group (C), with three replicates each. During the experiment, the survival rate and growth were recorded. The experiment was conducted for 12 weeks.

2.3 | Ammonia Stress Test

Diverse concentrations of the natural botanical compounds were used. After 12 weeks, NH_4Cl was added to the breeding tank for dilution. An ammonia stress test was conducted. Post-preparation, it was expressed as unionized ammonia-N (UIAN). A 96-h UIAN tolerance test was conducted on clownfish before the start of the experiment, with an LC_{50} concentration of

TABLE 1 | Clownfish natural botanicals additive feed formula.

Ingredients (g)	Treatment		
	Control	1000 (I)	2000 (II)
Fishmeal	42	42	42
Soy flour	20	20	20
Squid powder	5	5	5
Cellulose	6.1	6	5.6
Starch	16.6	16.6	16.6
Fish oil	3.3	3.3	3.3
Shrimp powder	3	3	3
Minerals	2	2	2
Vitamins	2	2	2
Natural botanicals	0	0.1	0.2
Feed nutrition(% dry weight)			
Crude protein	45	45	45
Crude fat	3.0	3.0	3.0
Crude fibre	1.2	1.3	1.3

TABLE 2 | Clownfish is culture water quality conditions.

Water quality conditions	
Temperature (mg L^{-1})	26.5 ± 0.5
Salinity (mg L^{-1})	32.5± 0.5
pH	8.0 ± 0.5
Nitrous acid (mg L^{-1})	0.01 ± 0.05
Nitric acid (mg L^{-1})	0.05 ± 0.05
Phosphate (mg L^{-1})	0.01 ± 0.01

0.65 mg L^{-1} UIAN. Therefore, this experiment used 0.6 mg L^{-1} UIAN as the high-concentration group for the ammonia nitrogen challenge and 0.2 mg L^{-1} UIAN as the low-concentration group. The water quality parameters used in the experiment are listed in Table 2. Survival rate was recorded every 12 h for 72 h. After the experiment, blood was collected from the caudal peduncle for AST and ALT activity detection to evaluate the liver-protective effects of the diverse natural botanical treatment groups on clownfish.

2.4 | Liver Function Enzyme Activity Measurement

A 1-mL plastic syringe was used to wet the inner wall of the syringe with 0.1 M EDTA. The needle was then inserted into the blood vessel below the lateral line of the caudal peduncle to draw approximately 0.8 mL of blood. After standing for 24 h, centrifuge and use the serum for analysis. The drawn blood was injected into a microcentrifuge tube and was centrifuged (Hitachi 18 PR-52, Japan) at 1000 × *g*, 4°C, for 15 min to obtain the crude enzyme extract.

2.5 | AST and ALT Detection

ALT enzyme activity was measured using the method of Reitman and Frankel (1957), and AST activity was measured using the method of Adeyemi et al. (2015). ALT and AST kits provided by France Randox company were used. Absorbance was measured at OD 340 nm. The absorbance of the samples was compared with that of the blank samples, and ALT and AST enzyme activities were calculated. The analytical methods were referenced from Shabanzadeh et al. (2016) and Al-Saadi and Al-Khshali (2018).

Calculation formula:

$$U/l = 1746\times \triangle A340 \text{ nm min}^{-1}$$

2.6 | Hemolytic Protein

The protein assay kit (Bio-Rad, No. 500-00060) 4×, 0.80 mL) was used as a diluent, and 0.2 mL was added to the appropriate multiple of the diluent. The absorbance value was measured on a spectrophotometer at 595 nm wavelength. Bovine serum albumin (BSA, 66 kDa, Sigma) was used as a protein standard to determine the protein concentration in the blood cell lysate.

2.7 | Antioxidant Enzyme Assay

The detection method of SOD refers to (Cheng et al. 2021; Ding et al. 2022), which is measured using a spectrophotometer. CAT was measured using a commercially available reagent set (Sigma). The experimental operation refers to the CAT measurement method for detection (Chen et al. 2022).

Sample preparation: Clownfish blood was extracted, and 10 mL of 0.1 mole PBS buffer was added and mixed in a 15-mL test tube. It was centrifuged at 1500 × *g* for 30 min, and the supernatant was collected as a sample. We added 5 μL of sample into the test tube and added 70 μL of 1 × Assay Buffer to it. For the blank, we added 75 μL of 1 × Assay Buffer. We added 25.00 μL colourimetric assay substrate solution to the sample and blank, respectively. We mixed it evenly, started the reaction, and let it stand for 1–5 min. We added 900 μL of stop solution to the sample and blank and mixed it by inverting. We took 10 μL of the reaction mixture into another test tube and added 1 mL of colour reagent. We waited for 15 min at room temperature and measured the absorbance value at OD 520 nm. All operations are performed at a constant temperature of 25°C.

2.8 | Liver Histology Studies

Clownfish liver tissue was first soaked in 4% formalin for 48 h for tissue fixation. Use ethanol for dehydration (50%–95%), and finally perform paraffin embedding and tissue sectioning. Tissue section thickness is 5.00 μm. Routinely processed for histology, sectioned and stained with haematoxylin and eosin (H&E).

2.9 | Feed Nutrition Analysis

2.9.1 | Crude Protein

Total nitrogen (crude protein) was determined by the micro Kjeldahl method (AOAC 1984). Weigh a known weight (0.50 g) of feed sample and place it in a digestive tube. Add 12 mL of digestion solution (9 mL nitric acid + 3 mL hydrochloric acid). Digest using a digester for 1 h until the solution becomes clear. After the solution cooled, it was brought to a final volume of 50 mL with deionized water. Total nitrogen was determined by colorimetry, and protein content was obtained from standard curves using protein standards of different concentrations.

2.9.2 | Crude Fat

The Soxhlet extraction method described by AOAC (2002) is used to determine crude fat. Place 2.00 g of anhydrous sample into a fat-free extraction tube, plug it with cotton and then place it into the extraction tube. Add 250 mL of petroleum ether to a clean, dry flask and extract for 6 h. The extractive solution was concentrated in a rotary evaporator using a vacuum pressure of 600 mmHg at a maximum temperature of 60°C. The flask was then placed in a fume hood and dried at 25°C for 45 min to remove residual solvent from the extract oil and to weigh (*W*2). Calculate crude fat using the following formula:

$$\text{Fat } (\%) = \frac{W1 - W2}{W1 \text{ of sample}} \times 100$$

where *W*1 is the weight of the empty flask; *W*2 is the weight of the flask and the fat deposits.

2.9.3 | Crude Fibre

Crude fibre was determined by acid–base digestion method (AOAC 2002). Place the residue obtained after lipid extraction of 2.00 g of sample into a 1-L beaker and add 200 mL of 2.5 M sulphuric acid. After heating for 30 min, the mixture was cooled, filtered through a Buchner funnel and then the residue was washed three times with 50 mL of boiling water. Return the washed residue to the beaker and add 200 mL of 2.5 M sodium hydroxide solution for 30 min. The resulting solution was filtered, washed three times with 50 mL of boiling water and then washed with 25 mL of ethanol. The washed residue was dried to constant weight in an oven at 130°C and then cooled in a desiccator. The residue was scraped into a pre-weighed crucible and weighed (*W*1). After ashing at 550°C for 2 h and cooling in a desiccator, it was weighed again (*W*2). The weight was calculated using the following formula:

$$\text{Crude fiber } (\%) = \frac{100 - [W1 - W2]}{W}$$

2.10 | Statistical Analysis

The data obtained from the experiment were analysed using the Statistical Package for Social Science (SPSS) software, single-factor analysis of variation and Duncan's Multiple Range Test (DMRT). They were used to determine the effects of two different concentrations of natural botanicals and ammonia stress. We evaluated the differences in the growth, survival and liver function index of *A. ocellaris*. The significance level was set at $p < 0.05$.

3 | Results

3.1 | Growth and Survival

The growth and survival after 12 weeks of feeding are depicted in Figure 1. The weight of C was 23.21 ± 0.22 g. The average weight of other treatment groups was 29.51 ± 0.29 g, with SM II 39.12 ± 0.19 g being the best ($p < 0.05$). The results demonstrate that the SM treatment group added to the feed had better growth performance than other groups and was significantly different from other treatment groups and C ($p < 0.05$). SM II resulted in a higher growth rate than SM I ($p < 0.05$). CL II had better growth than CL I. Compared with VR I, VR II, VA I and VA II, the CL II group was better ($p < 0.05$). There were no significant differences ($p > 0.05$) among the LC I, VR I, VR II, VA I and VA II groups. The survival rate results demonstrated that the survival rate of the diet supplemented with SM I and SM II groups was 100%, which was significantly better than the other groups ($p < 0.05$). When comparing the LC I, LC II and CL II groups, no significant differences were observed ($p > 0.05$). The C had the lowest survival rate, with a survival rate of only 20%.

3.2 | AST and ALT

Activity of dietary herbs on AST in *A. ocellaris* adult for 12 weeks. The results (Figure 2A) depict that SM I 2.25 ± 0.23 and SM II

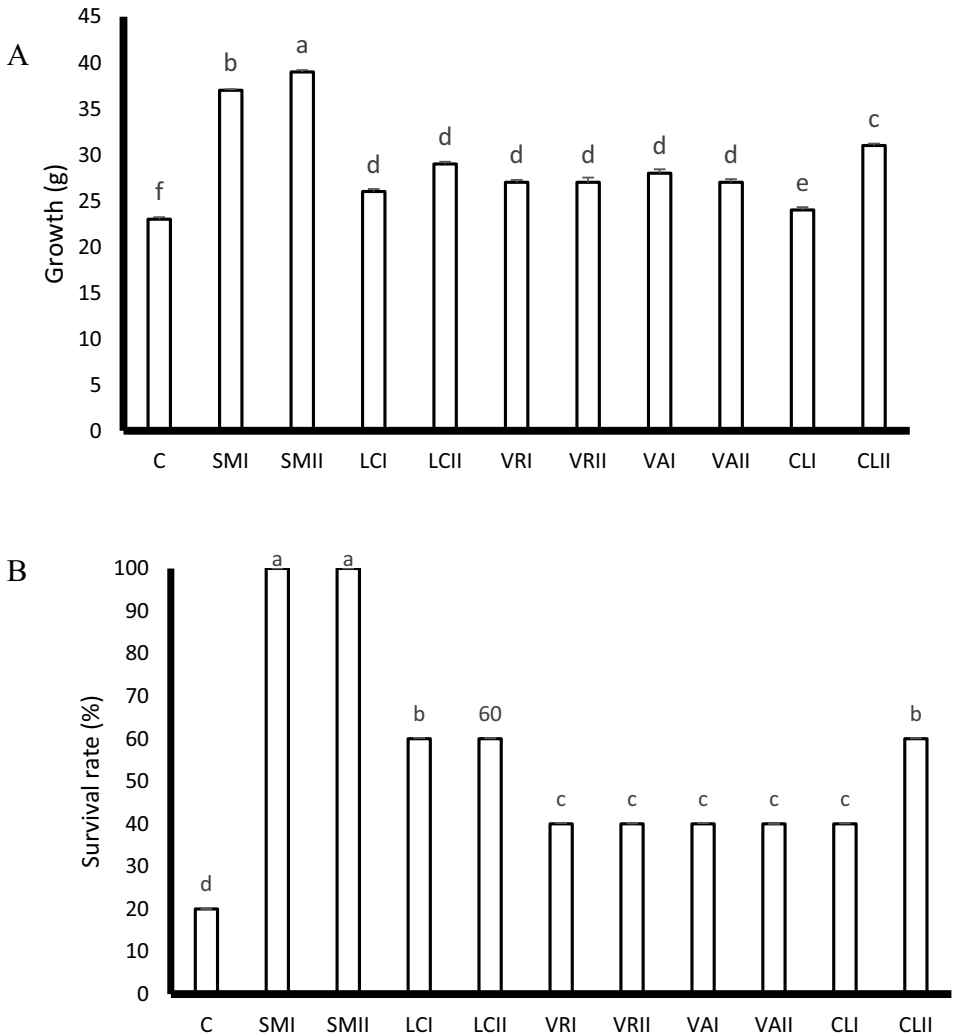


FIGURE 1 | Effects of dietary natural botanicals on growth and survival post 12 weeks of feeding *Amphiprion ocellaris*. Bars represents ±SD ($n = 15$ fish). The superscript English letters represent significant differences ($p < 0.05$). I: 1000, II: 2000 mg kg^{-1}. C, control group; CL, *Colix lacrymaiobi*; LC, *Lycium chinense*; SM, *Silybum marianum*; VA, *Vigna angularis*; VR, *Vigna radiate*. (A) Represents growth. (B) Represents survival.

2.03 ± 0.32 U mg $protein^{-1}$ had the lowest activity. Compared to the other treatment and C, they were significantly lower ($p < 0.05$). The group with the highest activity of 5.06 ± 0.28 U mg $protein^{-1}$ was the C. It had lower AST activity than other groups ($p < 0.05$). When comparing the two concentrations of SM, LC, VR, VA and CL treatment groups, there was no significant difference in AST activity regardless of the added concentration ($p > 0.05$). The results demonstrated that adding S to the feed could significantly reduce AST activity.

The ALT activity performance is depicted in Figure 2B. The ALT activity of SM I and SM II was significantly lower than that of the other treatment groups and the C ($p < 0.05$). The ALT activity of the C was the highest. There was a significant difference compared to the other treatment groups ($p < 0.05$). There were no significant concentrations in LC, VR, VA and CL groups ($p > 0.05$). According to these results, feeding *A. ocellaris* with SM effectively reduced ALT activity.

3.3 | Toxicity Test

After feeding *A. ocellaris* with natural botanicals for 12 weeks, the results of a toxicity test with 0.21 mg L^{-1} UIAN demonstrated that the AST activity was lowest with SM(I) 4.12 ± 1.10 and SM(II) 3.51 ± 0.78 U mg $protein^{-1}$ ($p < 0.05$). Compared to the C, it was reduced by 9.57 times (Figure 3A). The AST activity in each treatment group was lower than that in the control group. Except for the VR group, all other treatment groups demonstrated inhibited AST activity as the concentration of natural botanicals increased. The ALT activity is depicted in Figure 3B. The SM I, SM II, VA II and CL II groups had significantly lower ALT activities than the other groups ($p < 0.05$).

Among the treatment groups, the VR I and VR II groups had the highest activity, with an average of 10.43 ± 0.77 U mg $protein^{-1}$, second only to C. Hepatic metabolism has been demonstrated to be potentially compromised.

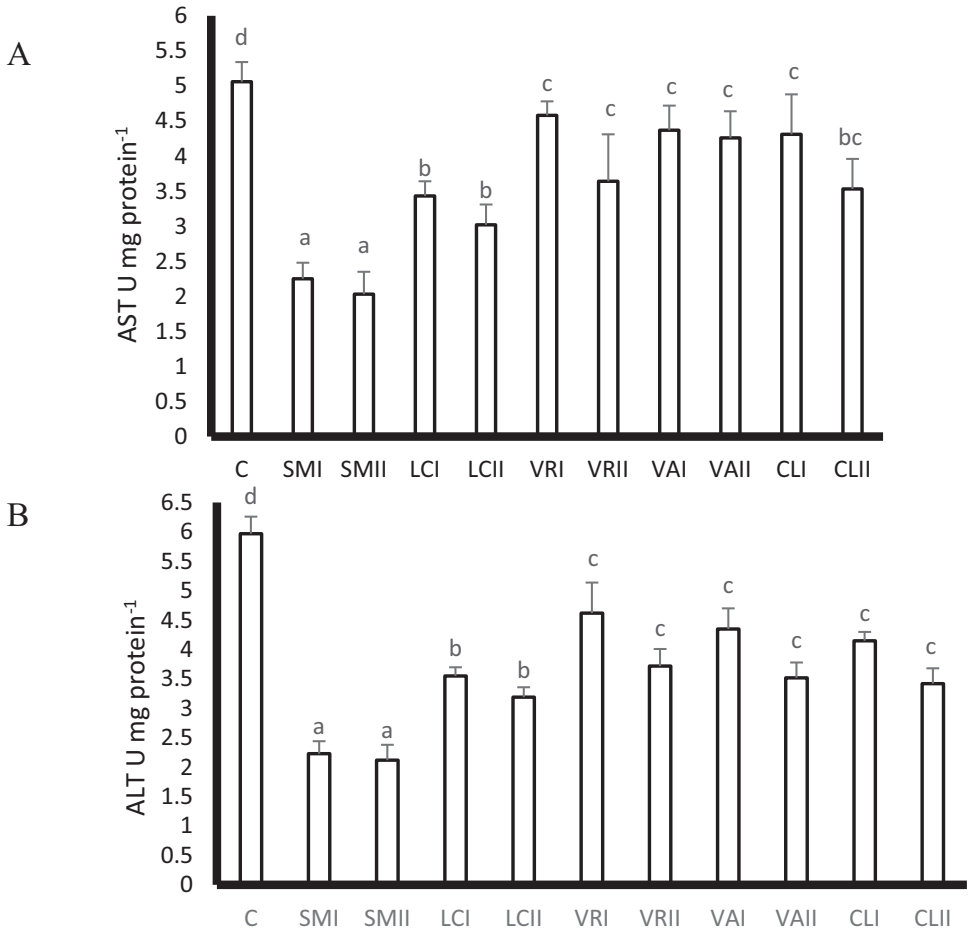


FIGURE 2 | Effects of dietary natural botanicals on AST and ALT post 12 weeks of feeding *Amphiprion ocellaris*. Bars represents ±SD ($n = 15$ fish). The superscript English letters represent significant differences ($p < 0.05$). I: 1000, II: 2000 mg kg^{-1}. ALT, alanine aminotransferase; AST, aspartate aminotransferase; C, control group; CL, *Colix lacrymaiobi*; LC, *Lycium chinense*; SM, *Silybum marianum*; VA, *Vigna angularis*; VR, *Vigna radiate*. (A) Represents AST. (B) Represents ALT.

3.4 | Ammonia Toxicity Test

The results of the toxicity test at the high UIAN concentration of 0.6 mg L^{-1} (Figure 4A) demonstrate that the lowest average AST of SM I and SM II was 3.01 ± 0.70 U mg $protein^{-1}$. This is significantly different from other groups ($p < 0.05$). LC(II) (5.47 ± 0.96 U mg $protein^{-1}$) and LC I (6.27 ± 0.79 U mg $protein^{-1}$) were significantly lower than VR I, VR II, VA I, VA II, CL I, CL II and the control group ($p < 0.05$). ALT activity was the lowest in the SM I and SM II groups (Figure 4B. At low concentrations of ammonia nitrogen, the ALT activity of the other treatment groups was lower than that of the control group. Among the treatment groups, the VR group had higher ATL activity, which was significantly different from the other treatment groups ($p > 0.05$).

A. ocellaris After being challenged with high and low concentrations of UIAN, AST and ALT activities in the blood were the lowest in the SM-supplemented group. LC II activity increased as the ammonia nitrogen concentration increased. No significant differences were observed between the other treatment groups. AST and ALT activities in each treatment group were lower than those in the control group, which indicates that these natural botanicals can reduce liver damage caused by UIAN in *A. ocellaris*.

3.5 | Treat Stress Responses

After being challenged with a high concentration of 0.62 mg L^{-1} UIAN, feeding SM I and SM II to *A. ocellaris* significantly reduced the SOD and CAT activities in the body. This was 2.5 and 6.5 times lower than that of the control group ($p < 0.05$) (Figure 5). Compared with the control group, SOD and CAT activities in the other treatment groups were significantly reduced ($p < 0.05$) and indicated that other natural botanicals may have antioxidant effects, but these effects may not be as significant as those of SM.

3.6 | Histopathological Examination

Excessive ammonia-N can cause ammonia poisoning in clownfish, resulting in increased ammonia levels in the blood. The liver absorbs potentially harmful substances from the blood circulation, leading to liver cell necrosis and tissue fibrosis. We can observe the production of vacuolated tissue in liver tissue

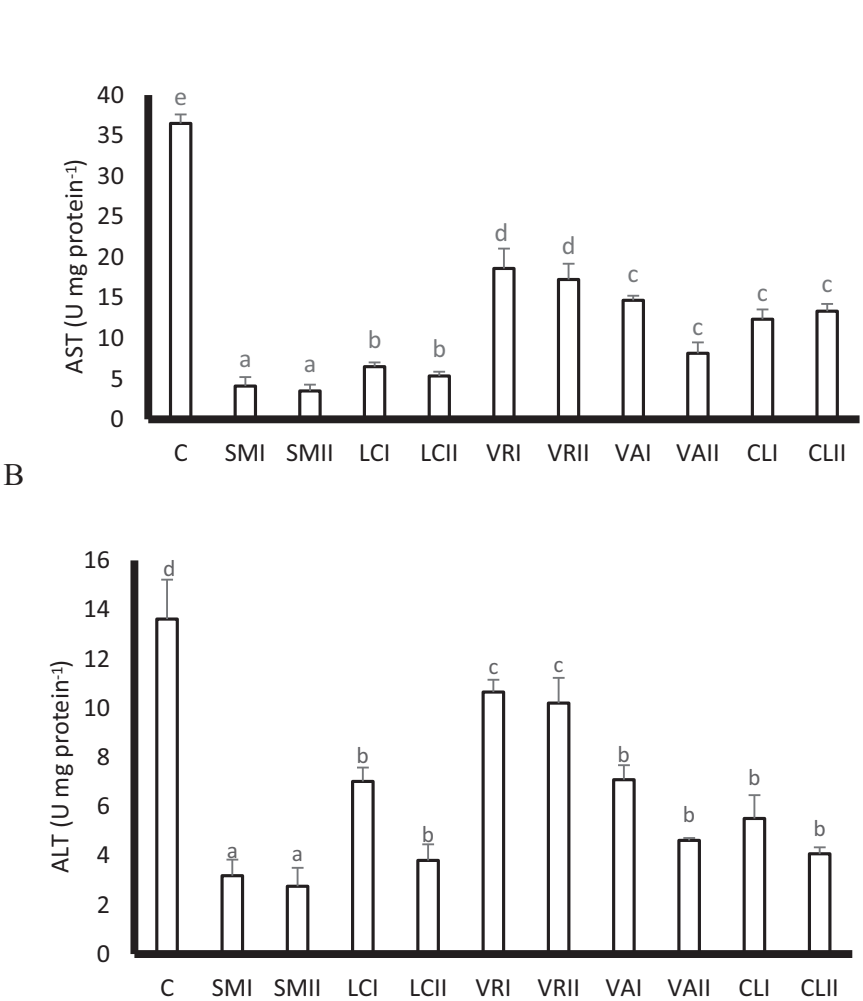


FIGURE 3 | Ammonia toxicity test (0.2 mg L^{-1}) for AST and ALT activity performance in fish serum post 72 h. Bars represent ±SD ($n = 15$ fish). The superscript English letters represent significant differences ($p < 0.05$). I: 1000, II: 2000 mg kg^{-1}. ALT, alanine aminotransferase; AST, aspartate aminotransferase; C, control group; CL, *Colix lacrymaiobi*; LC, *Lycium chinense*; SM, *Silybum marianum*; VA, *Vigna angularis*; VR, *Vigna radiate*. (A) Represents AST. (B) Represents ALT.

in C, VA and CL. And the distribution of Kupffer cells, a type of macrophage induced by liver inflammation and damage, was observed in CL (Figure 6). Fatty degeneration cells can be observed in both LC and LA. This means that tissue degeneration begins to occur in liver cells after being poisoned by ammonia, causing cells to begin to be damaged, inflamed and so forth. In the SM group, intact liver cells were observed, and no vacuolated tissue or fatty degeneration cells were found. According to histopathological examination, it was found that SM can effectively improve the metabolism of clownfish liver against ammonia toxicity. When the ammonia-N concentration in the water is within the range of 0.6 mg L^{-1}, it can reduce liver cell damage.

4 | Discussion

4.1 | Effects of Natural Botanicals on Growth and Survival

This study found that feeding *A. ocellaris* with natural botanicals can improve its growth and survival. The addition of SM to the feed resulted in better growth and survival rates of *A. ocellaris* than in the other groups. Post-statistical analysis, SM I and SM II growth were significantly different from that of the other treatment groups and the C ($p < 0.05$). The survival rates in the SM I and SM II groups were significantly higher than those in the other groups ($p < 0.05$). These results indicate that SM is a medicinal plant that has long been used as a hepatoprotective drug. It is used to treat various liver diseases characterized by liver dysfunction or degenerative necrosis. It is hepatoprotective activity is unique and acts in diverse ways, including antioxidant and anti-inflammatory activity, cell permeability regulator and membrane stabilizer, stimulation of liver regeneration and inhibition of collagen fibre deposition that may lead to cirrhosis (Bahmani et al. 2015). SM can gradually restores the body functions of *A. ocellaris* adult fish and promotes food intake and growth. This also occurs in *Pseudoplatystoma fasciatum* and *Pangasianodon hypophthalmus*, which demonstrate better growth when SM is added (Abdel-Latif et al. 2023). SM is used in medical applications to reduce the activity of cytotoxic T lymphocytes and natural killer cells in the blood of patients with alcoholic liver disease (Deak et al. 1990). LC demonstrates hepatoprotective and antioxidant activity in animals (Burke et al. 2005). VR is also used as an alternative fishmeal source in

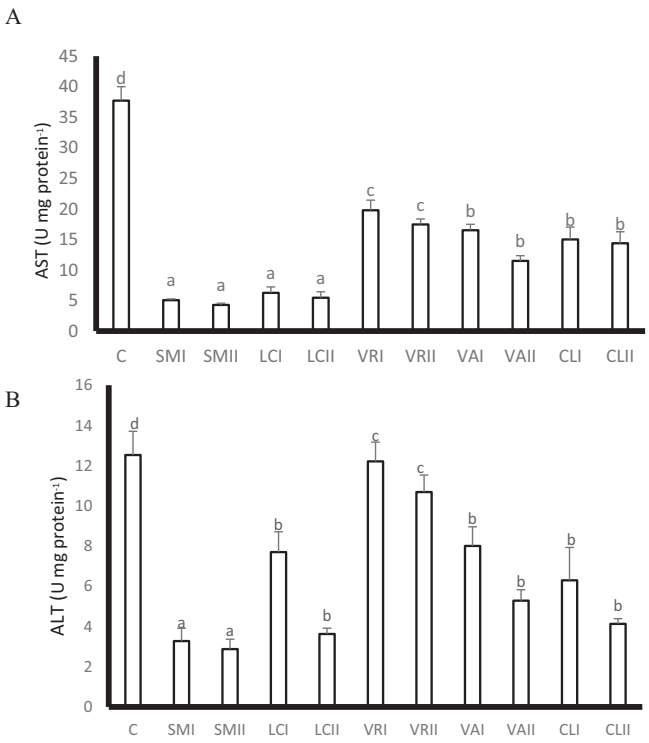


FIGURE 4 | Ammonia toxicity test (0.6 mg L^{-1}) for AST and ALT activity performance in fish serum after 72 h. Bars represent ±SD ($n = 15$ fish). The superscript English letters represent significant differences ($p < 0.05$). I: 1000, II: 2000 mg kg^{-1}. ALT, alanine aminotransferase; AST, aspartate aminotransferase; C, control group; CL, *Colix lacrymaiobi*; LC, *Lycium chinense*; SM, *Silybum marianum*; VA, *Vigna angularis*; VR, *Vigna radiate*. (A) Represents AST. (B) Represents ALT.

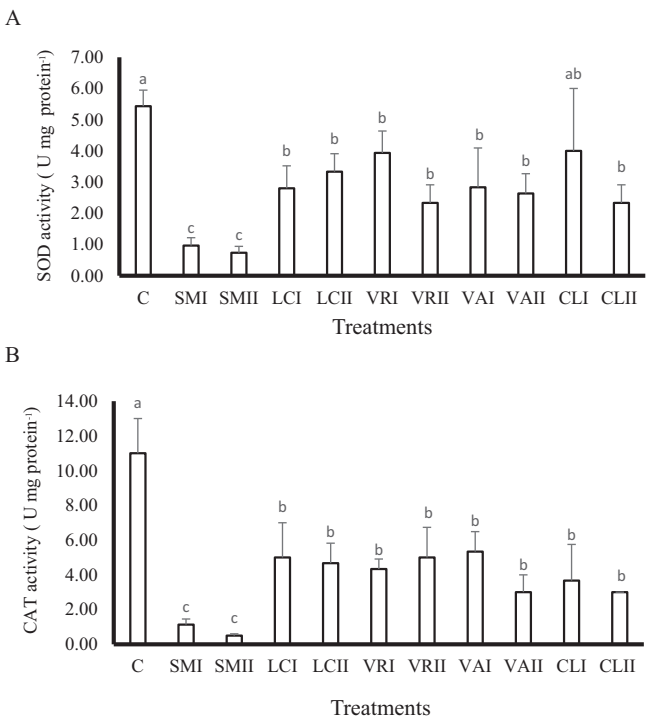


FIGURE 5 | Ammonia toxicity test (0.6 mg L^{-1}) for SOD and CAT activity performance in fish serum post 72 h. Bars represent ±SD ($n = 15$ fish). The superscript English letters represent significant differences ($p < 0.05$). I: 1000, II: 2000 mg kg^{-1}. C, control group; CAT, catalase; CL, *Colix lacrymaiobi*; LC, *Lycium chinense*; SM, *Silybum marianum*; SOD, superoxide dismutase; VA, *Vigna angularis*; VR, *Vigna radiate*. (A) Represents SOD. (B) Represents CAT.

aquaculture. Fifty per cent *V. radiate* powder was used for protein substitution testing. The growth, weight gain, feed conversion rate and survival of white shrimp were consistent with those of whole fishmeal feed (Mubarak 2005). VR contains antinutritional factors. Before use, it must undergo pretreatment methods, such as shelling, soaking, germination, cooking and high-pressure treatment, to reduce anti-nutritional factors, such as trypsin inhibitors, tannins and haemagglutinins (Mubarak 2005). Plants contain anti-nutritional factors that hinder absorption of fibres, such as protease inhibitors, phytates, glucosinolates, saponins tannins, lectins, oligosaccharides and non-starch polysaccharides, phytoestrogens, alkaloids, antigenic compounds, gossypols, cyanogens, mimosine and cyclopropenoid fatty acids (Francis et al. 2001) by fish. This may be one of the factors leading to *A. ocellaris* death. Therefore, except for the SM group, the survival rate of each treatment group was <60%.

4.2 | Liver Repair and Antioxidant

ALT and AST levels tended to increase when *A. ocellaris* was stressed with ammonia. Previous research by Deng et al. (2010) found that when tilapia were fed different concentrations of aflatoxin, the activities of AST and ALT increased with increasing aflatoxin concentration. This study found that SM reduced AST and ALT activity post ammonia toxicity testing ($p < 0.05$). Adding SM to *Oncorhynchus mykiss* feed can reduce peroxidase activity; increase lysozyme activity, complement activity, total protein and immunoglobulin levels; and enhance non-specific immunity (Ahmadi et al. 2012). The protective mechanism of silymarin against liver fibrosis involves the inhibition of the activation and proliferation of stellate cells and a reduction in the degree of liver fibrosis (Chen et al. 2012). In human trials, patients with hepatitis B received 160 mg of silymarin orally thrice a day. After 4 weeks, the results demonstrated that it significantly reduced AST and ALT activities in patients with hepatitis B (Torres et al. 2013). Cancer chemotherapeutic drugs (cisplatin and CDDP) and drugs that cause liver damage can be toxic when used at high doses. If SM is used simultaneously, it can reduce the AST and ALT activities in the blood of the patient (Mansour et al. 2006). Clinical studies have verified that the polysaccharides contained in LC have anti-ageing and lipid peroxidation effects. These can reduce the hydroxyproline content, the precursor of collagen in the liver, and have inhibitory effects on liver fibrosis (Chao et al. 2006; Wu et al. 2004). Adding SM seeds to *Oreochromis niloticus* feed also has the ability to regulate antioxidant enzymes, including SOD and CAT. It does not damage the liver tissue and intestines (Hassaan et al. 2019).

In the VR treatment group, ALT and AST activities were lower than those in the control group ($p < 0.05$). VR is planted in an environment containing chromium, which can enhance antioxidant defence and phytochelatins production to inhibit heavy metal poisoning (Diwan et al. 2010). Additionally, it can inhibit the oxidation of low-density lipoproteins in the human

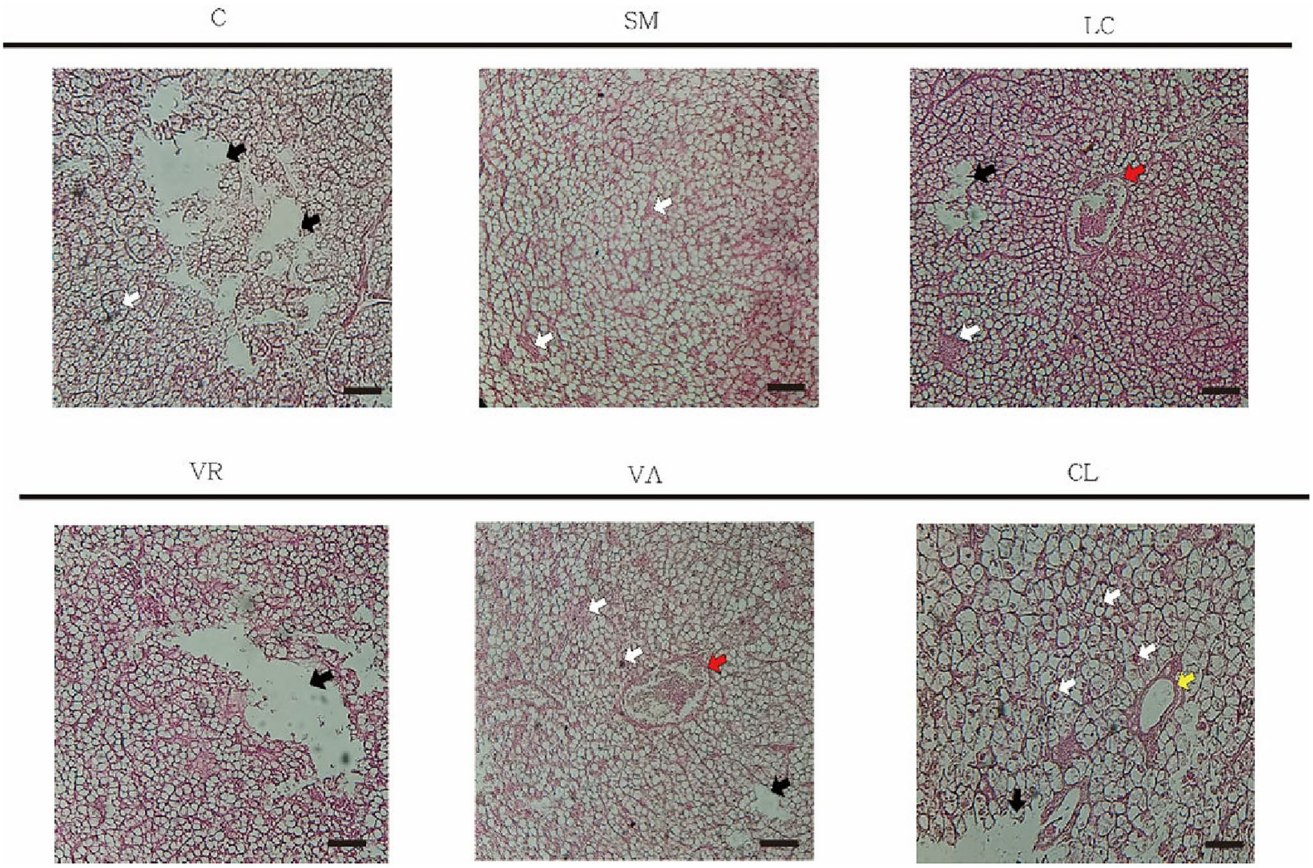


FIGURE 6 | Histopathological examination of liver in each treatment group. Liver cell nucleus (white arrows), vacuolated tissue (black arrows), fatty degeneration cells (red arrows) and Kupffer cells (yellow arrow). Bar represents 50 μm. C: The liver tissue produces vacuolated tissue, and severe fibrosis can be observed. The black arrows indicate vacuolated tissue; damage to these tissues, approximately 50–100 μm, is irreversible. Hepatocyte damage showed signs of extension and expansion. SM: The liver tissue and cells were intact, and no lesions were found. Hepatocytes with nuclei and lipid droplets were clearly observed in the liver tissue, but no fatty liver or vacuolated tissue of liver pathology was found. LC: A large number of fatty degeneration cells (60–100 μm) were found, also accompanied by vacuolated tissue (50–100 μm). Histological examination revealed not only vacuolation in the tissue but also fatty liver, indicating that liver damage impairs detoxification and subsequently leads to tissue damage. VR: Liver tissue produces severely vacuolated tissue. The presence of vacuolated tissue (>500 μm) in the tissue sections indicates severe liver damage, and the diffuse presence of this vacuolated tissue throughout the liver tissue suggests significant liver degeneration. VA: Fatty degeneration cells and vacuolated tissue were observed in liver tissue. The observed fatty degeneration cells, approximately 230 μm in size, are pathological fatty liver lesions specifically caused by fat accumulation in the liver, indicating that the liver's detoxification capacity is being threatened. The vacuolated tissue, intact and teardrop-shaped, is approximately 50–100 μm in size. CL: Vacuolated tissue and Kupffer cells (110 μm) can be observed. Tissue fibrosis can be observed. These are specialized resident macrophages in the liver, which have immune defence functions. They indicate that the liver is being attacked by toxins or has an inflammatory response and is attempting to repair the tissue. C, control group; CL, *Colix lacrymaiobi*; LC, *Lycium chinense*; SM, *Silybum marianum*; VA, *Vigna angularis*; VR, *Vigna radiate*.

body, which is helpful for removing free radicals and lowering cholesterol (Chung et al. 2011). VA is extracted with 50% alcohol. Its free radical scavenging effect was better than that of other types of beans, such as mung beans, black beans, soybeans and peas. Aged VA can increase the antioxidant capacity of animals without affecting digestive enzymes. This result demonstrates that the aqueous extract of VA has the effect of promoting the growth of Hep G2 cells and, therefore, may also promote the growth of normal liver cells (Chang et al. 2012).

4.3 | Liver Histology

Histological studies have found that after the liver is attacked by UIAN, it will cause the liver tissue to produce vacuolated tissue, causing serious harm to the liver. As ammonia is the end product of nitrogen metabolism, fish ammonia is usually excreted into the water through the gills. Ammonia poisoning leads to an increase in the accumulation of ammonia in the blood of fish. If the excretion of ammonia is reduced due to certain factors (such as excessive water pH, insufficient oxygen and damage to the gills), and the concentration of ammonia in the blood of fish increases to a high level, autointoxication will occur (Benli et al. 2008; Figueiredo-Silva et al. 2005). These toxins will enter the liver through blood circulation for metabolism and decomposition. This study found that vacuolated tissue was observed in the liver tissue in groups C, VA and CL, which indicates that liver cells have become fibrotic or necrotic. This kind of cell necrosis is irreversible damage (Ramadori et al. 2008). The distribution of Kupffer cells, a type of macrophage, has been

observed in CL. Kupffer cells can be observed in the liver when the liver tissue is damaged by inflammation (Ramadori et al. 2008). Previous studies have pointed out that if the liver is stimulated by microorganisms, dimethylnitrosamine, acetaminophen, alcohol and so forth, it will induce the activation of Kupffer cells (Sato et al. 2016). Studies have found fatty degeneration cells in both LC and LA. This may be caused by toxic drugs, accumulation of transitional heavy metals or hepatitis (Portincasa et al. 2005). Previous research has shown that fatty degeneration cells are often found in the liver of alcoholics, leading to alcoholic steatohepatitis (Portincasa et al. 2005). In addition, there is also non-alcoholic steatohepatitis, which is a chronic and progressive inflammatory reaction caused by the liver. Both of them can cause serious damage to liver tissue. Liver damage will not only affect the detoxification function but also cause metabolic syndrome, which will affect the changes in ALT and AST mentioned earlier (Sato et al. 2016). The results showed that when the clownfish ingested SM, the liver tissue intact, and no vacuolated tissue or Kupffer cells were found. SM may be used in feed to prevent and treat aflatoxin poisoning, reduce liver damage and improve metabolic function in the future. Further research can be conducted in the future.

CL can reduce the ALT and AST activities of *A. ocellaris*. They were significantly lower than those in the control group ($p < 0.05$). Previous studies have shown that long-term intake inhibits the activity of microsomal cytochrome P enzymes in the human liver. It increases the activity of glutathione and antioxidant enzymes in the lungs (Chung et al. 2011; Yao et al. 2011). The results of this experiment demonstrated that the SM I and SM II groups had the lowest AST and ALT activities. However, adding SM to the feed effectively reduced AST and ALT activities in *A. ocellaris*. It thereby improved the detoxification function of the liver and repaired damaged liver cells.

5 | Conclusions

Adding SM at 1000 or 2000 mg kg^{-1} concentration to the feed improved the ability of *A. ocellaris* to resist UIAN stress. It effectively reduced the activities of AST, ALT, SOD and CAT, thereby protecting the liver cells and increasing survival rate and growth. And it can reduce cell and tissue damage in the liver. The findings of this study can be used in large-scale marine fish aquaculture to improve ammonia resistance in fish. They can also be used to improve the detoxification ability of the liver and reduce the risk of fish poisoning or sudden death during aquaculture.

Author Contributions

Chih-Hung Pan: conceptualization, project administration, data curation, resources. **De-Sing Ding**: project administration, writing – review and editing, writing – original draft, validation. **You – Ren Lai**: formal analysis, supervision. **Wei – Ting Sun**: supervision, formal analysis.

Ethics Statement

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to and the appropriate ethical review committee approval has been received. The authors confirm that they have followed EU standards for the protection of animals used for scientific purposes.

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The authors declare no conflicts of interest.

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No data were used for the research described in the article.

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