INTRODUCTION

The Pacific white shrimp *Litopenaeus vannamei* is one of the pillar species in China’s aquaculture industry. However, with the rapid development of the shrimp breeding industry in recent years, the continuous expansion of the shrimp breeding area and scale has led to increased breeding intensity and the deterioration of the breeding environment. During the cultivation process, due to the high degree of intensification, high stocking density and high feeding amount, with the extension of the cultivation time, the contents of ammonia and harmful microorganisms in the aquaculture water gradually increase (Duan et al., 2018; Liu, 2004). Excess ammonia can reportedly reduce the immune ability of *Litopenaeus vannamei*, and the residual bait will lead to the accumulation of harmful microorganisms (Cui et al., 2017; Si et al., 2019). *Litopenaeus vannamei* can readily develop

Effects of medical herbs in Tian-Dong-Tang-Gan powder on non-specific immune responses and resistance to acute ammonia stress in *Litopenaeus vannamei*

Xiao-Dong Xie | Shu-Mian Zhou | Jing Cheng | Mei-Ling Yu | Ying-Yi Wei | Mei-Lan Mo | Ting-Jun Hu

College of Animal Science and Technology, Guangxi University, Nanning, PR China

Correspondence
Mei-Lan Mo and Ting-Jun Hu, College of Animal Science and Technology, Guangxi University, 530005, Nanning, P.R. China. Email: momelan@gxu.edu.cn; tingjunhu@gxu.edu.cn

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Abstract

Tian-Dong-Tang-Gan powder (TDTGP) is a newly developed immune enhancer in *Litopenaeus vannamei*. In vitro, TDTGP was non-toxic to haemolymph at 400 µg ml\(^{-1}\) and increased the activities of phenol oxidase (PO), acid phosphatase (ACP) and superoxide dismutase (SOD) after incubation with haemolymph for 8–48 h. In vivo, the activities of PO, SOD, ACP and alkaline phosphatase (AKP) in the shrimp haemolymph could be increased during the 28-day feeding trials. An acute ammonia stress test was performed after the feeding trials were terminated. We studied the immunological parameters and histology diagnosis. In the drug group, the immune index burst following acute ammonia exposure was higher than that in the negative control and control groups. Histological detection showed that the integrity of the hepatopancreas cells in the TDTGP groups was higher than that in the negative control group at 72 h after ammonia exposure. The results showed that feeding shrimp TDTGP for 28 days could improve the immunity and resistance to ammonia stress by *Litopenaeus vannamei* and protect the integrity of the hepatopancreas during ammonia stress. These findings showed that TDTGP can be developed and used as an immunoenhancer in *Litopenaeus vannamei*.

KEYWORDS
 acute ammonia stress, immunoenhancer, *Litopenaeus vannamei*, Tian-Dong-Tang-Gan powder
various diseases due to excess ammonia such as acute hepatopancreatic necrosis disease, white spot syndrome and those caused by the white spot syndrome virus, Taura syndrome virus, Vibrio harveyi and Vibrio damsela (Ananda Raja et al., 2017; Leu et al., 2013; Pang et al., 2019) in culture, resulting in large economic losses. At present, the prevention and treatment of these diseases primarily depend on antibiotics and chemicals, but the use of antibiotics and chemicals is increasingly restricted due to the shortcomings associated with drug residues, drug resistance, pollution and other concerns (Johansson et al., 2014; Valitalo et al., 2017).

Traditional Chinese medicine has been used for thousands of years as a medicine to prevent and treat diseases in China. Modern research shows that many traditional Chinese medicines and their extracts can improve immunity and can be used as alternatives to antibiotics to prevent and treat diseases of Litopenaeus vannamei, for example polysaccharides from Angelica sinensis (Pan et al., 2018), San-Huang-San (Zhai & Li, 2019) and rose myrtle Rhodomyrtus tomentosa seed extract (Dang et al., 2019). In traditional Chinese medicine, Asparagus cochinchinensis is used to nourish yin and moisten dryness. Modern research shows that Asparagus extract pregnane glycosides and polysaccharides have anti-oxidant and anti-neuroinflammatory properties (Cho & Yang, 2018; Jian et al., 2014; Valitalo et al., 2017).

In this study, according to early-stage exploration in the laboratory, the total polysaccharides in Asparagus cochinchinensis were primarily composed of Panax notoginseng R1, ginsenoside Rg1, ginsenoside Re, ginsenoside Rb1, ginsenoside Rd, and others, and the total saponins in Panax notoginseng can be used to treat inflammation and bleeding caused by internal and external injuries, among others, and it can improve cardiovascular activity function (Wang et al., 2016; Yang et al., 2014).

In this study, according to early-stage exploration in the laboratory, the total polysaccharides in Asparagus cochinchinensis were extracted by an alcohol alkali extraction method, and the total saponins in Panax notoginseng were extracted by an alcohol extraction method. After they were mixed in a specific proportion, an appropriate amount of water-soluble silica was added as the auxiliary material, and then, the extracts were evenly granulated, dried and comminuted into a powder named Tian-Dong-Tang-Gan powder (TDTGP). The total polysaccharide and total saponin contents of TDTGP were determined, followed by an in vitro immune test, a 28-day feeding test and an acute ammonia stress test to study the effects of TDTGP on the non-specific immunity of Litopenaeus vannamei.

2 | MATERIALS AND METHODS

2.1 | Herb sources and extraction

Asparagus cochinchinensis and Panax notoginseng were purchased from Nanning Traditional Medicine Market in Guangxi, China. Asparagus cochinchinensis was extracted twice in a water bath at 80°C for 2.5 h with 10% ethanol at pH = 9 in a 1:10 ratio after soaking for 1.5 h, combining the two 300 mesh and concentrate filtrates in boiling water and concentrating it all to a density of 1.2 g ml⁻¹, adding 5 times the volume of 95% ethanol for 24 h and discarding the supernatant, and precipitating and weighing it for later analysis. Panax notoginseng was extracted twice and concentrated to 1.15 g ml⁻¹ in a water bath at 90°C with 70% ethanol in a 1:10 ratio for 2 h. It was purified with D101 macroporous adsorption resin (Solarbio, M0041), concentrated in a water bath at 100°C and freeze-dried (Christ ALPHA 1-2 LD plus) to obtain the refined total saponins.

The total polysaccharides from asparagus, total saponins of notoginseng and the silica were mixed in a specific proportion. Last, after drying at 60°C, the 100 meshes were crushed and stored at room temperature for further study.

2.2 | Content determination

The total polysaccharide content was measured by anthrone sulphuric acid method. The standard curve for the polysaccharide content absorbance value was established with dextran as the standard, and the absorbance value of the sample was placed within the standard curve to calculate the polysaccharide content.

The total saponin content of the TDTGP was determined by high-performance liquid chromatography (HPLC, Waters e2695). The column is a Phenomenex Luna C18 chromatographic column (250 mm × 4.60 mm × 5 µm) and the HPLC method in terms of the flow rate (1 ml min⁻¹), column temperature (25°C), elution method (Table 1), wavelength (203 nm), mobile phase A (Acetonitrile) and mobile phase B (ultrapure water) were performed according to the Chinese Veterinary Medicine Code, with a resolution ginsenoside Rg1 and ginsenoside Re of more than 1.5. The quality standard curve was established with notoginsenoside R1, ginsenoside Rg1, ginsenoside Re, ginsenoside Rb1 and ginsenoside Rd standard (Purchased from National Drug Reference Standards of China). After selecting an appropriate amount of TDTGP and treating it with the same method as the standard product, we added it to the HPLC to detect the saponin content.

2.3 | Cell counting kit-8 (CCK-8) assay

The viability of the haemolymph cells was detected using a CCK-8 assay (Beyotime Biotechnology) in this research (Li et al., 2018). A
control group, Astragalus polysaccharide (APS, 100 μg ml⁻¹) group and TDTGP I to VI groups (25, 50, 100, 200, 400 and 800 μg ml⁻¹) were established. Healthy Litopenaeus vannamei shrimp were collected from a breeding farm for Litopenaeus vannamei in Guangxi, Guangxi, China. 75% alcohol cotton ball was used to disinfect the thoracic sinus of Litopenaeus vannamei, and the haemolymph was aseptically extracted from the thoracic sinus of the shrimp with a syringe containing 0.5 ml sterile 5% trisodium citrate anticoagulant. Mix several haemolymphs to centrifuge at 700 g for 10 min, discard the supernatant, add Dulbecco’s modified Eagle medium (DMEM; Life Technologies Corporation) with 10% foetal bovine serum (Wisent Inc) to the cell pellet and dilute to 2.2 × 10⁶ cells ml⁻¹, and 200 µl was added to each well of a 96-well plate (Thermo Fisher Scientific). The cells were cultured in a 5% CO₂ incubator at 28°C for 2 h. The culture medium for each group was changed for culture medium containing drugs. After 4, 8, 12, 24 and 48 h, the culture medium containing drugs was changed into 200 µl of 10% DMEM, and 20 µl of CCK-8 was added. The solution was incubated in a 37°C incubator for 4 h, and then, the absorbance was measured at a 450 nm wavelength with a multi-functional enzyme-labelling instrument (Tecan Infinite Pro2000) to calculate the cytotoxicity.

2.4 | Immune response in vitro

According to the cells of haemolymph viability results, the control group, APS group (100 μg ml⁻¹) and TDTGP I to IV groups (25, 50, 100 and 200 μg ml⁻¹) were established for the experiment, and each group had four repetitions. One ml of 2.2 × 10⁶ ml⁻¹ cells of haemolymph suspension was added to each hole in the 24-well plates and placed in an incubator at 28°C under 5% CO₂ for 2 h. The culture solution in each hole was replaced with one containing each group of drugs. The supernatant and cells were collected after 8, 12 and 24 h of co-culture. The supernatant was used to determine the PO, SOD, AKP and ACP activities.

2.5 | Experimental diets

Various drug-containing diets and a control diet for the experimental shrimp were prepared according to the feed formulation shown in Table 2. In brief, the basal diet was formulated to contain approximately 417.33 g kg⁻¹ crude protein and 72 g kg⁻¹ crude fat, which are sufficient to support the optimal growth of Litopenaeus vannamei.

| Ingredients                     | Control | Negative | APS    | TDTGP-1 | TDTGP-2 | TDTGP-3 |
|--------------------------------|---------|----------|--------|---------|---------|---------|
| Fish meal⁶ (g kg⁻¹)          | 200.0   | 200.0    | 200.0  | 200.0   | 200.0   | 200.0   |
| Wheat gluten⁵ (g kg⁻¹)       | 300.0   | 300.0    | 300.0  | 300.0   | 300.0   | 300.0   |
| Wheat meal⁵ (g kg⁻¹)         | 200.0   | 200.0    | 200.0  | 200.0   | 200.0   | 200.0   |
| Cellulose (g kg⁻¹)           | 180.0   | 180.0    | 178.0  | 178.0   | 176.0   | 172.0   |
| Fish oil (g kg⁻¹)            | 25.0    | 25.0     | 25.0   | 25.0    | 25.0    | 25.0    |
| Soybean oil (g kg⁻¹)         | 25.0    | 25.0     | 25.0   | 25.0    | 25.0    | 25.0    |
| Soybean phospholipids (g kg⁻¹)| 20.0  | 20.0     | 20.0   | 20.0    | 20.0    | 20.0    |
| Gelatin (g kg⁻¹)             | 20.0    | 20.0     | 20.0   | 20.0    | 20.0    | 20.0    |
| Choline chloride (g kg⁻¹)     | 10.0    | 10.0     | 10.0   | 10.0    | 10.0    | 10.0    |
| Vitamin mix⁷ (g kg⁻¹)        | 10.0    | 10.0     | 10.0   | 10.0    | 10.0    | 10.0    |
| Mineral mix⁸ (g kg⁻¹)         | 10.0    | 10.0     | 10.0   | 10.0    | 10.0    | 10.0    |
| APS (g kg⁻¹)                  | 0       | 0        | 2.0    | 0       | 0       | 0       |
| TDTGP (g kg⁻¹)               | 0       | 0        | 0      | 2.0     | 4.0     | 8.0     |

Proximate nutrient composition (as fed)

| Ingredients | Control | Negative | APS    | TDTGP-1 | TDTGP-2 | TDTGP-3 |
|-------------|---------|----------|--------|---------|---------|---------|
| Crude protein (g kg⁻¹) | 417.33  | 417.33   | 417.33 | 417.33  | 417.33  | 417.33  |
| Crude fat (g kg⁻¹)    | 72.0    | 72.0     | 72.0   | 72.0    | 72.0    | 72.0    |
| Crude ash (g kg⁻¹)    | 68.0    | 68.0     | 68.0   | 68.0    | 68.0    | 68.0    |
| Total energy (kJ g⁻¹) | 16.41   | 16.41    | 16.41  | 16.41   | 16.41   | 16.41   |

⁶Fish meal: crude protein, 688.7 g kg⁻¹ dry matter; crude fat, 78.2 g kg⁻¹ dry matter; wheat gluten: crude protein, 789.3 g kg⁻¹ dry matter; crude fat, 1.81 g kg⁻¹ dry matter; wheat meal: crude protein, 164.0 g kg⁻¹ dry matter; crude fat, 15.7 g kg⁻¹ dry matter.

⁷Vitamin mixture (mg kg⁻¹ diet): riboflavin, 45.0 mg; thiamine, 25.0 mg; vitamin K₃, 10.0 mg; inositol, 800.0 mg; pyridoxine hydrochloride, 20.0 mg; vitamin B12, 0.1 mg; calcium pantothenate, 60.0 mg; biotin, 1.3 mg; vitamin A, 32.0 mg; vitamin D, 5.0 mg; nicotinic acid, 200.0 mg; folic acid, 20.0 mg; vitamin E, 120.0 mg.

⁸Mineral mix (mg kg⁻¹ diet): KI, 0.8 mg; NaF, 2.0 mg; Fe₂(SO₄)₃, 80.0 mg; ZnSO₄, 55.0 mg; CoCl₂·6H₂O, 50.0 mg; CuSO₄·5H₂O, 10.0 mg; MgSO₄·7H₂O, 200.0 mg; NaCl, 100.0 mg; Ca(H₂PO₄)₂, 3000.0 mg.
Tian-Dong-Tang-Gan powder was supplemented separately in the basal diet at the expense of cellulose to obtain the final drug dose for the different experimental groups as follows: control (0 mg kg\(^{-1}\)), negative control (0 mg kg\(^{-1}\)), APS (2 g kg\(^{-1}\)), TDTGP-1 (2 g kg\(^{-1}\)), TDTGP-2 (4 g kg\(^{-1}\)) and TDTGP-3 (8 g kg\(^{-1}\)). The ingredients were grounded and sieved through a 300-μm mesh to obtain a fine powder. The powder was then mixed thoroughly with fish oil and then tap water was gradually added until a stiff dough was obtained. Later, the dough was extruded through a mincer, ripened at 70°C for 5 h, air-dried in the dark and then sieved into pellets. The feed pellets were stored in plastic bags at −20°C until use.

2.6 | Feeding experimental shrimp

Healthy \textit{L. vannamei} shrimp were collected from a breeding farm for \textit{Litopenaeus vannamei} in Guangxi, China. Individuals weighing 5.0 ± 0.5 g were released into individual experimental fiberglass tanks (200 L capacity) with three experimental groups (TDTGP-1 to TDTGP-3), a positive control group and a control group in triplicate (\(n = 40 \times 3 = 120\)) with a constant aeration system. The shrimp were maintained throughout the experiment at a constant level of salinity (10 ± 1.0‰), temperature (27 ± 1.0°C), pH (8.0 ± 0.2) and dissolved oxygen (above 5 ppm). Water exchange (30%) was performed daily until the end of the feeding experiment. Each treatment group was fed with the aforementioned diet four times daily.

2.7 | Acute ammonia exposure

At the end of the 28-day feeding experiment, we adjusted the water level for each group to 60 L, added the appropriate concentration of NH\(_4\)Cl solution to the other groups except the control group, adjusted the concentration of NH\(_4\)Cl to 100 mg L\(^{-1}\) (the actual ammonia molecular concentration was 2.10 mg L\(^{-1}\)), and measured the ammonia nitrogen concentration by indophenol blue method every 24 h and adjusted the ammonia nitrogen concentration of the experimental water in time, and we performed the ammonia stress experiment for 72 continuous hours.

2.8 | Histomorphology

After the acute ammonia exposure experiment, four shrimp from each tank were randomly collected for histomorphology analysis and fixed in a 4% formalin solution for 48 h following fixation, and the histomorphology samples were processed and stained with haematoxylin and eosin (H&E) using standard histological techniques (Howard et al., 2004) and examined for cell morphology under a light microscope (Olympus, DP72).

2.9 | Post-analysis after administration

Haemolymph was collected once every 7 days for the feeding experiment and once every 24 h during the ammonia stress experiment from each experiment using insulin syringes preloaded with 0.2 ml of anticoagulant (5% sodium citrate at pH 7) to avoid haemolymph clotting, and the samples were stored at −80°C for further analysis.

2.10 | Immunological parameters

The PO activity of the haemolymph samples was determined spectrophotometrically by recording the formation of dopachrome using \(\mu\)-dihydroxyphenylalanine (\(\mu\)-dopa) as a substrate. We used \(\mu\)-dopa as the substrate and modified it according to Söderhäll’s and Mason’s method (Mason, 1956; Söderhäll, 1983). A certain amount of \(\mu\)-dopa formulated into 0.01 mol L\(^{-1}\), sealed it and placed it in a water bath at 37°C for 5 h. After it was fully dissolved, we added 100 μl of 0.15 mol L\(^{-1}\) potassium phosphate buffer solution, 100 μl of the test supernatant and 0.01 mol L\(^{-1}\) \(\mu\)-dopa 100 μl in 96-well plates. The samples were shaken and mixed at room temperature. After that, the OD value of the reaction system at 490 nm was measured immediately, once every two minutes, continuously for 20 min. The increase in the OD value at 490 nm per minute was defined as an enzyme activity unit.

Acid phosphatase (ACP), alkaline phosphatase (AKP) and superoxide dismutase (SOD) were detected according to the method described in the kit from Nanjing Jiancheng Bioengineering Institute, Nanjing, China.

2.11 | Data analysis

One-way ANOVA was performed using SPSS software (SPSS version 22.0 for Windows). The means were compared at the 0.05 levels and with the subsequent post hoc multiple comparison with the Duncan test, and the results are presented in a histogram prepared in GraphPad Prism 6.0.

3 | RESULTS

3.1 | Content determination

The total polysaccharide content of every 1 g of TDTGP was 59.9% according to anthrone sulphuric acid analysis. The contents of no-toginsenoside R1, ginsenoside Rg1, ginsenoside Re, ginsenoside Rb1 and ginsenoside RD in every 1 g of TDTGP were 0.901%, 3.413%, 0.481%, 3.354% and 0.779%, respectively, according to HPLC analysis (Figures 1–3).

Howard et al., 2004
3.2 | Viability of haemolymph

The viability of the haemolymph can be improved by co-culture with the drugs and shrimp haemolymph in each dose group for 4, 12 and 24 h for which the effect was the best at 12 and 24 h (Figure 4). There was no effect at or below TDTGP 400 μg ml⁻¹, and the activity of each group gradually decreased; the decrease of TDTGP 800 μg ml⁻¹ was the most obvious (p < 0.05) when co-incubation was performed for 48 h (Figure 4). The results showed that TDTGP...
could enhance the activity of the shrimp haemolymph when it was incubated with the haemolymph for 24 h or less.

3.3 | Cell immune response in vitro

TDTGP can promote the activity of immune factors in cells of haemolymph (Figure 5). At 8 h after co-incubation, the activity of SOD in the supernatant of each drug group was significantly higher than that of the control ($p < 0.05$); the activity of PO in the supernatant of the TDTGP 200 µg ml$^{-1}$ group was significantly higher than that of the control ($p < 0.05$); the activity of AKP in the supernatant of TDTGP in the 50 µg ml$^{-1}$ group was significantly higher than that of the control ($p < 0.05$), and at 12 h after co-incubation, the activity of AKP in the supernatant of each dose group of TDTGP was significantly higher than that of the control ($p < 0.05$). The ACP activity was significantly higher in the control ($p < 0.05$).

3.4 | Feeding experiment

The PO, ACP and AKP activities first increased and then decreased at different time points after providing the shrimp with asparagine powder during the 28-day feeding experiment (Figure 6). On the 7th day, the PO activity in the haemolymph of the TDTGP-1 and TDTGP-2 groups was significantly higher than that of the control ($p < 0.05$), and the activity of ACP and AKP in the TDTGP-2 group was significantly higher than that of the blank control group.
On the 14th day of the feeding test, the SOD activities of APS, TDTGP-1 and TDTGP-3 were significantly higher than that of the control (p < 0.05). On the 28th day, the SOD activity in the TDTGP-1, TDTGP-2 and TDTGP-3 groups was significantly higher than that of the control (p < 0.05).

3.5 | Acute ammonia stress experiment

The changes in the immune parameters in the shrimp haemolymph under acute ammonia stress are shown in Figure 7. Over the 24 h of acute ammonia stress, the PO activity in the haemolymph of each ammonia stress model group was significantly higher than that of the control group (p < 0.05), and the PO activity in the haemolymph of the APS and TDTGP-2 groups was significantly higher than that of the negative control group (p < 0.05). The ACT activity in the TDTGP-3 group was significantly higher than that in the other groups (p < 0.05) at 24 h after ammonia stress (Figure 6), and there was no significant difference between the other groups and the control group (p > 0.05). At 48 h, the ACP activity of the negative group and each treatment group was significantly higher than that of the control group (p < 0.05). The activity of AKP in the TGTGP-2 group was significantly higher (p < 0.05) than that in the negative group at 48 h (p < 0.05).

3.6 Effects of TDTGP on the histomorphology of the acute ammonia stress test

The shrimp exhibited marked histological alterations in the hepatopancreas following acute ammonia stress test for 72 h. Compared to the control, negative, APS and TDTGP-1 groups, there was an evident separation between the myoepithelial layer and the epithelium of the shrimp hepatopancreas. Furthermore, the storage cells (R-cell), secretory cells (B-cell) and star-shaped polygonal structures of the lumen had disappeared and were necrotic. The epithelium layer was destroyed completely at the infected site (Figure 8b,c).

4 | DISCUSSION

Innate immunity was considered as the primary defence mechanism in crustaceans, and it includes both humoral components and cellular activities that are coordinated to eliminate foreign organisms that could be potentially hazardous to the host. In the crustacean, cellular defence was directly performed by the haemolymph, by
actors generally recognized as hyaline cells (HCs), semi-granular cells (SGCs) and large granular cells (GCs) (Kenneth Söderhäll, 1983) that are involved in the reactions of phagocytosis, encapsulation and nodule formation, etc (Holmblad & Söderhäll, 1999; Mapanao et al., 2018). In these cells, hyaline cells are the main phagocytic cells, while granular cells contain many secretory granules containing components of the proPO system. Semi-granular cells appear to be the most sensitive ones and react first during an immune response, by degranulating. These two cells main to take part in coagulation, release of agglutinins and/or synthesis of melanin. (Holmblad & Söderhäll, 1999; Mapanao et al., 2018; Ratcliffe & Rowley, 1979).

Humoral components include the activation and release of molecules stored within haemocytes, such as agglutinins, phenol oxidase (PO), antimicrobial peptides, acid phosphatase (ACP) and superoxide dismutase (SOD) (Holmblad & Söderhäll, 1999). SOD plays a crucial role in the phagocytosis process or antioxidant stress, and following hyaline cells phagocytosis, the infection pathogen can induce a reactive oxygen reaction to synthesise reactive oxygen intermediates (ROIs). However, the excessive accumulation of ROIs may induce oxidative stress in the host. In a normal physiological state, harmful effects of ROIs are effectively neutralized by the antioxidant defence system of organisms, which in general comprises enzymes like SOD, and SOD degrades excess \(O_2^-\), thereby inhibiting ROIs generation to prevent harm to the host (Chang et al., 2013; Holmblad & Söderhäll, 1999; Wu et al., 2016). Many studies have found that activated PO catalyses the stepwise oxidation of phenols to quinones, which polymerize non-enzymatically and form insoluble melanin deposits, then encapsulation pathogens. Therefore, the activity of PO has a positive correlation with shrimp disease resistance (Nappi et al., 1995). ACP is an important component of phagocytic lysosomes, and in the phagocytosis and encapsulation of haemocytes, phagocytic lysosomes have bactericidal action with the release of ACP. AKP is an important component of lysosomal enzymes, and it plays a role in invertebrate immune responses (Cheng, 1978, 1989; Kuo et al., 2019). Therefore, the activities of PO, SOD, ACP and AKP were used as indicators to evaluate the effect of TDTGP on the immune state and disease resistance in Litopenaeus vannamei.

There is no cell line of Litopenaeus vannamei; Toshiaki Itami (Toshiaki Itami et al., 1999) tried to culture the lymphoid organ cells and haemocytes of kuruma shrimp, Penaeus japonicus, and the fibroblast-like cells and epithelial-like cells can survive for 54 days. Sunil K (George & Dhar, 2010) cultured haemocytes in Grace’s insect medium supplemented with 10% (v/v) foetal bovine serum and 10% SME (v/v), resulting in the enhanced attachment and proliferation of the cells, and this culture could be maintained for 48 days and was sub-cultured four times. For this study, 10% DMEM was used to culture cells of haemolymph, which was able to survive for more than 7 days. In this experiment, cells of haemolymph were incubated with different concentrations of TDTGP for 24–72 h, and then, the activities of PO, SOD, AKP and ACP in the supernatant were detected. The results showed that the activities of PO, SOD, AKP and ACP in the cell supernatant of each administration group were slightly increased compared to the control group. Similarly, at different time points during the 28-day feeding experiment, the activity
of different enzymes rose at different time points, showing that the enzyme activity rose first and then gradually returned to normal, which is consistent with the report by Yu-Sheng Wu. Wu (Wu et al., 2016) found that the Litopenaeus vannamei oral different concentrations beta 1,3/1,6-glucan effectively enhanced O$_2^-$ production and phenoloxidase and superoxide dismutase activity, and oral administration with 0.2 g/kg of vitamin C presented beneficial nonspecific immune responses and enzyme activity. This shows that TDTGP can improve the nonspecific immunity of Litopenaeus vannamei like beta 1,3/1,6-glucan and vitamin C.

During the high-density culture of Litopenaeus vannamei, ammonia is the final product of organic matter and protein decomposition. Ammonia–N (>5 mg/L) and NH$_3$–N (>0.357 mg/L) will affect the expression of coagulation and immune genes (Chang et al., 2015; Frías-Espericueta et al., 2000). Chen (Chen et al., 1988) found that during the culture of Penaeus penicillatus, the highest ammonia content can reach 46 mg/L. Zhang (Zhang et al., 2018) found that long-term ammonia stress (46 mg/L) can lead to decreases in the total haemolymph, phagocytic activity and antibacterial activity, and Xiao et al. (2019) tested the effect of ammonia stress on the transcriptomics of Litopenaeus vannamei and found that the immune defence, cytokine reprogramming and antioxidant system of Litopenaeus vannamei changed after ammonia stress. In this study, we used a large dose (100 mg/L) of NH$_4$Cl, and it was twice the dose of 46 mg/L of ammonia concentration in normal aquaculture water, to establish an ammonia stress model to create an acute ammonia stress model within a short period of time. Following acute ammonia stress, the PO, ACP and AKP activities in the shrimp haemolymph first increased and then decreased gradually. The same trend has been observed in other studies. Zhang (Zhang et al., 2018) found that after ammonia exposure, the expression of nuclear factor kappa-b (NF-kB) and cAMP-response element-binding protein mRNA in Litopenaeus vannamei reached the maximum within 6 h, and then gradually returned to normal. The total haemocyte count, phagocytic activity and antibacterial activity decreased dramatically within 48 hours. The activity of phenoloxidase was increased slightly. Then, it was decreased significantly up to 48 h. However, in the 24th hour of ammonia stress, the activities of PO, SOD and AKP in the TDTGP-2 group and the activity of PO in the APS group were higher than those in the negative control group, which indicated that TDTGP and APS could increase the bursting capacity of the Litopenaeus vannamei immune system under stress.

Asparagus is the root of Asparagus cochinensis, its extract has no specific toxicity, and it has anti-inflammatory, anti-oxidation, anti-asthma and other effects (Jian et al., 2013; Lei et al., 2017; Son & Anh, 2013). Jun Young Choi used the fermentation products of Asparagus in an ovalbumin-induced asthma model and found that it has a good anti-asthma effect and can prevent airway inflammation and remodelling through the recovery of cholinergic regulation in the structural and inflammatory cells of the chronic asthma model (Choi et al., 2018). The total saponins in Panax notoginseng have numerous pharmacological effects, including cerebral vasodilation, blood dynamic invigoration, haemostasis, anti-inflammation, anti-oedema, anti-thromboembolism, anti-coagulation, anti-hyperlipidaemia and anti-hyperglycaemia effects (Yang et al., 2014). In this experiment, histopathological studies found that after 72 h of ammonia stress in the negative and APS groups, the number of cells in the hepatopancreas decreased, and even part of the structure disappeared. However, the hepatopancreas cells in each group fed with TDTGP could maintain their integrity, which may be due...
to the anti-inflammatory and anti-oxidation effects of *Asparagus* extract and the total saponins of *notoginseng*. In future studies, the mechanism of TDTGP to improve the anti-ammonia stress effect of *Litopenaeus vannamei* will be further studied.

## 5 | CONCLUSION

In this study, we addressed the immunoregulatory effect of asparagus powder on *Litopenaeus vannamei* from three perspectives: in vitro, in vivo and under acute ammonia stress. We found that asparagus powder can improve the activities of PO, ACP, AKP, SOD and other immune factors in *Litopenaeus vannamei* and can help cells resist acute ammonia stress by maintaining the integrity of the hepatopancreas cells. The results showed that the Tian-Dong-Tang-Gan powder could improve the immune ability of *Litopenaeus vannamei* and its ability to resist ammonia stress. This powder can be used as a new immune enhancer, but its exact mechanism requires further study. In addition, this study found that Astragalus polysaccharides had no protective effect on the hepatopancreas cells of *Litopenaeus vannamei* under ammonia stress, but it could enhance its immunity.

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## CONFLICT OF INTEREST

There are no conflict of interest on any front.

## AUTHOR CONTRIBUTIONS

Xiao-Dong Xie and Mei-lan Mo designed and performed research, analysed data and wrote the paper; Shu-Mian Zhou and Jing Cheng, and Mei-Ling Yu performed research and analysed data; Ying-Yi Wei revised and proved the paper; Ting-Jun Hu designed research analysed data and revised the paper.

## DATA AVAILABILITY STATEMENT

Some or all data, models, or code generated or used during the study are available from the corresponding author by request.

## REFERENCES

- Ananda Raja, R., Sridhar, R., Balachandran, C., Palanisammi, A., Ramesh, S., & Nagarajan, K. (2017). Pathogenicity profile of Vibrio parahaemolyticus in farmed Pacific white shrimp. *Penaeus vannamei*. *Fish & Shellfish Immunology*, 67, 368–381. https://doi.org/10.1016/j.fsi.2017.06.020
- Chang, C. C., Tan, H. C., & Cheng, W. (2013). Effects of dietary administration of water hyacinth (*Eichhornia crassipes*) extracts on the immune responses and disease resistance of giant freshwater prawn, *Macrobrachium rosenbergii*. *Fish & Shellfish Immunology*, 35(1), 92–100. https://doi.org/10.1016/j.fsi.2013.04.008
- Chang, Z. W., Chiang, P. C., Cheng, W., & Chang, C. C. (2015). Impact of ammonia exposure on coagulation in white shrimp, *Litopenaeus vannamei*. *Ecotoxicology and Environmental Safety*, 118, 98–102. https://doi.org/10.1016/j.ecoenv.2015.04.019
- Chen, J.-C., Liu, P.-C., Lin, Y.-T., & Lee, C.-K. (1988). Super intensive culture of red-tailed shrimp *Penaeus penicillatus*. *World Aquaculture Society*, 19, 127–131. https://doi.org/10.1111/j.1749-7345.1988.tb00940.x
- Cheng, T. C. (1978). The role of lysosomal hydrolases in molluscan cellular response to immunologic challenge. *Invertebrate Models for Biomedical Research*, 4, 59–71.
- Cheng, T. C. (1989). Immunodeficiency diseases in marine mollusks: Measurements of some variables. *Journal of Aquatic Animal Health*, 1(3), 209–216. https://doi.org/10.1577/1548-8667(1989)001<0209:didmm>2.3.co;2
- Cho, Y.-H., & Yang, M.-S. (2018). Antioxidant activity of gamma-irradiated *Asparagus cochinchinensis* (*Asparagi radix*) (Lour.) Murr. Extract and inhibition effect on lipid oxidation of emulsion-type pork sausage. *Korean Journal for Food Science of Animal Resources*, 38(6), 1196–1202. https://doi.org/10.5851/kosfa.2018.e51
- Choi, J., Kim, J. I., Park, J., Lee, M. I., Song, B. O., Park, J. I., Kang, M. I., Lee, H., Son, H., Hong, J., & Hwang, D. (2018). The anti-inflammatory effects of fermented herbal roots of *Asparagus cochinchinensis* in an ovalbumin-induced asthma model. *Journal of Clinical Medicine*, 7(10), 377. https://doi.org/10.3390/jcm7100377
- Cui, Y., Ren, X., Li, J., Zhai, Q., Feng, Y., Xu, Y., & Ma, L. (2017). Effects of ammonia-N stress on metabolic and immune function via the neuroendocrine system in *Litopenaeus vannamei*. *Fish & Shellfish Immunology*, 64, 270–275. https://doi.org/10.1016/j.fsi.2017.03.028
- Dang, L. T., Nguyen, H. T., Hoang, H. H., Lai, H. N. T., & Nguyen, H. T. (2019). Efficacy of rose myrtle *Rhodomyrtus tomentosa* seed extract against acute hepatopancreatic necrosis disease in Pacific whiteleg shrimp *Penaeus vannamei*. *Journal of Aquatic Animal Health*, 31(4), 311–319. https://doi.org/10.1002/aaah.10080
- Duan, Y., Wang, Y., Zhang, J., Sun, Y., & Wang, J. (2018). Dietary effects of succinic acid on the growth, digestive enzymes, immune response and resistance to ammonia stress of *Litopenaeus vannamei*. *Fish & Shellfish Immunology*, 78, 10–17. https://doi.org/10.1016/j.fsi.2018.04.008
- Frías-Esparciueta, M. G., Harfush-Melendez, M., & Páez-Osuna, F. (2000). Effects of ammonia on mortality and feeding of postlarvae shrimp *Penaeus vannamei*. *Bulletin of Environmental Contamination and Toxicology*, 65(1), 98–103. https://doi.org/10.1007/s001280000100
- George, S. K., & Dhar, A. K. (2010). An improved method of cell culture for marine bivalve mollusks and crustaceans. *Aquaculture*, 172, 111–123. https://doi.org/10.1016/j.aquaculture.2004.08.046
- Holmblad, T., & Söderhäll, K. (1999). Cell adhesion molecules and the neuroendocrine system in *Penaeus* spp. *Toxicology*, 65(1), 98–102. https://doi.org/10.1016/S0044-8486(98)00446-3
- Howard, D. W., Lewis, E. J., Keller, B. J., & Smith, C. S. (2004). Histological techniques for marine bivalve mollusks and crustaceans. 2nd ed.
