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Full Length Research Paper

Water decoction of *Astragalus membranaceus* enhances cellular immune response and disease resistance in spotted maigre

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The effect of dietary Chinese herb *Astragalus membranaceus* on the cellular immune response of spotted maigre (*Nibea albifiora*) was investigated. 300 spotted maigre with an average initial weight of 49.6 ± 5.5 g were selected and fed with different levels of water decoction of *A. membranaceus* (WDA) (0, 0.5, 1.0, 2.0 and 4.0%) for 4 weeks. Non-specific immune responses were measured after 0, 3, 7, 14, 21, 28 days of feeding. We then injected control and WDA treated fish with an intraperitoneal injection of *Vibrio vulnificus*, and recorded their mortality rate for 7 days post-infection. The results showed that the phagocytic index of phagocytes was significantly higher in 2% WDA treated group as compared to other groups (P<0.05). Whereas, the percentages of phagocytes were significantly higher in 0.5, 1.0 and 2.0% WDA groups as compared to 4.0% WDA and control groups (P<0.05). But lysozyme activities were significantly higher in the Astragalus-fed groups as compared to the control group (P≤0.05), and 2% group was the highest. Furthermore, 2% WDA-fed fish were significantly protected against *V. vulnificus* infestation (73.3% survival) as compared to 63.3, 53.3 and 36.7% survival in the 1 and 0.5% WDA-fed and control, respectively. In conclusion, dietary WDA may effectively enhance cellular immune response and disease resistance in spotted maigre against *V. vulnificus.*

Key words: *Astragalus membranaceus*, spotted maigre, non-specific immune response, *Vibrio vulnificus*.

INTRODUCTION

The spotted maigre (*Nibea albiflora*), a member of the family Sciaenidae, is distributed in China, Japan and Korea, and highly valued because of its taste and nutritional value (Xing et al., 2009). However, infectious diseases such as vibriosis are becoming a severe problem in culture (Wang et al., 2012). Chemotherapy is effectively used to control fish infections, but needs serious consideration regarding the establishment of antibiotic-resistant bacteria (Wang et al., 2012). One of the most promising methods for con- trolling diseases of aquatic animals is by strengthening their defense mechanisms through prophylactic administration of immunostimulants (Citarasu, 2010). Over the last decade, a lot of research has focused on the application of plant extracts to replace antibiotic growth promoters in terrestrial animal feeds (Rawling et al., 2009), such as Traditional Chinese Medicine (TCM).

In China, for thousands of years, many herbs have been used in humans to boost the immune system (Wang et al., 2009; Zhao et al., 2011), such as aromatic plants (e.g., ginger, curcuma and coriander) and herbal products

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Abbreviations: TCM, Traditional Chinese medicine; **WDA,** water decoction of *Astragalus membranaceus*; **PBS,** phosphate buffered saline; **PI,** phagocytic index; **PP,** percentage of phagocytes; **IL,** interleukin; **TNF**, tumor necrosis factor.

(e.g. roots, leaves and bark), essential oils (e.g., hydro-distilled volatile plant compounds) and oleoresins (extracts based on non aqueous solvents). Among the many herbs used in TCM, the root of *Astragalus membranaceus* has been used as an immune booster for nearly 2000 years (Wang et al., 2009; Cho et al., 2007), which harbors multiple therapeutic efficacies in humans and animals, such as polysaccharides, saponins, monosaccharides, flavonoid and alkaloid, together with choline, betaine, folic acid, various amino acids, mucoitin, gum, cellulose and 14 trace minerals including selenium, zinc and iron, which are essential micronutrients for man and other animals (Galina et al., 2009). More recently, such applications have began to demonstrate positive effects in many aquatic animals (Merrifield et al., 2009), which has been shown to have significant immuno-stimulatory effects, enhancing the non-specific immunity of fishes including carp (Yin et al., 2009; Xie et al., 2008), large yellow croaker (Jian and Wu, 2003) and tilapia (Yin et al., 2006; Ardó et al., 2008). Although, *A. membranaceus* had been shown to enhance the nonspecific immunity of some fishes, to our knowledge, there is no literature to date concerning the use of different levels of water decoction of *A. membranaceus* (WDA) as derivatives in diets for spotted maigre.

The aim of the current investigation was to assess the dietary inclusion of different levels of WDA on the non-specific humoral (lysozyme activity) and cellular (phagocytic activity) responses of spotted maigre (*N. albifiora*).

MATERIALS AND METHODS

Spotted maigre (49.6 ± 5.5 g) were provided by a commercial fish farm in China. Fish were kept in cement tanks (3 × 4 × 1.5 m) with a 12 m^3 recirculating water system and continuous aeration. Water temperature, pH and salinity were constant (27 ± 2°C, 8 to 8.5 and 20 to 22%, respectively) during the experimental period. Water flow was maintained at 20 $L \cdot min^{-1}$ with dissolved oxygen maintained at 80 to 90% of saturation.

WDA preparation

The plant, *A. membranaceus* was purchased from the TCM Hospital of Wenzhou City, Zhejiang Province. The roots were collected and washed in sterile distilled water. They were powdered after drying, and stored at -20°C until further use.

WDA was done by following the method of our previous paper (Wang et al., 2012). 10 g powder was exhaustively extracted with sterile distilled water, filtered through sterile muslin cloth, then the filtrate was collected and the solvent was removed using a rotary vacuum evaporator after allowed to stand for 30 min at room temperature. The resulting residue was collected after evaporation and resuspended in sterile distilled water at the desired concentrations concentrations (Yan et al., 2000).

Feed and experimental design

Normal balanced feed were purchased from Haima Feed company, Fuzhou, China, which were mixed with WDA on 0.5% (A group), 1% (B group), 2% (C group), 4% (D group) and 0% (E, control group), respectively. Then five mixtures were incorporated into the diets at the same crude powder rate and maintained at room temperature. For all feeds, moisture, crude ash, crude protein and crude lipid compositions were at the equivalent concentrations, respectively. Fish were divided into five groups (A to E, n = 60 for each group) and fed one of the diets twice a day for 4 weeks, respectively.

LD_{50} determination

Fish were anesthetized with tricaine methanesulfonate (MS-222) (Hangzhou Animal Medicine Factroy) at a concentration of 195 $\mu g \cdot ml^{-1}$. Experimental fish were intraperitoneally injected with *Vibrio vulnificus* on 1.0×10^3 to 1.0×10^8 CFU/fish, while the control fish were injected with 100 µL phosphate buffered saline (PBS). Ten fishes were used per dose. Mortality was monitored until 1 week post-infection. The experiment was repeated three times at the same time. The results were averaged and used to calculate the LD_{50} value, using the method of Reed and Muench (1938).

Serum and leucocytes separation

Fish were bled from the common cardinal vein using a 2-ml syringe on days 0, 3, 7, 14, 21, and 28 after the start of the experiment. Serum and leukocytes for assay were separated following the method of Yin et al. (2009).

Lysozyme activity

The lysozyme activity was measured using photoelectric colorimeter by following the method of Azza (2009) with slight modifications. In brief, a series of dilutions was prepared by diluting a standard lysozyme sample (Amresco, Switzerland) mixed with a *Micrococcus lysodeikticus* (ATCC NO. 1698) suspension for establishing a calibration curve. For this, 100 µl of standard solution or serum was added to 1800 µl of *Micrococcus* suspension. The changes in the extinction were measured at 640 nm immediately after adding the solution which contained the lysozyme (start of the reaction) and after 2 min incubation of the preparation under investigation at 28°C (end of the reaction after adding 100 µl $5mol \cdot l^{-1}$ KOH). The lysozyme content was determined using the calibration curve and the extinction measured.

Phagocytic activity

The phagocytic activity analysis was carried out following the method of Fujiki and Yano (1997) with slight modifications. In brief, leucocyte separations were added to 100 µl *Staphylococcus aureus* (type strain ATCC25923, $10^8 CFU \cdot ml^{-1}$) suspension and incubated for 2 h at 25°C. About 10 µl mixture was placed on a glass slide, and allowed to adhere for 20 min at 25°C. The slides were then rinsed with saline, fixed with methanol for 5 min, and then stained with Giemsa solution for 6 min. The percentage of phagocytes (PP) that ingested *S. aureus* and the number of *S. aureus* ingested per phagocyte were calculated by enumerating about 100 phagocytes under a microscope. Phagocytic activity was expressed as the phagocytic index (PI) according to Matsuyama et al. (1992).

Infestation with virulent pathogen

On day 28, fish from all groups were divided into 3 subgroups (1 to 3, n = 10 for each subgroup) and infested with *V. vulnificus* (2.6×10^6 CFU $fish^{-1}$, 100 µl), using an intraperitoneal injection. Dead fish were necropsied and liver samples homogenized and plated onto

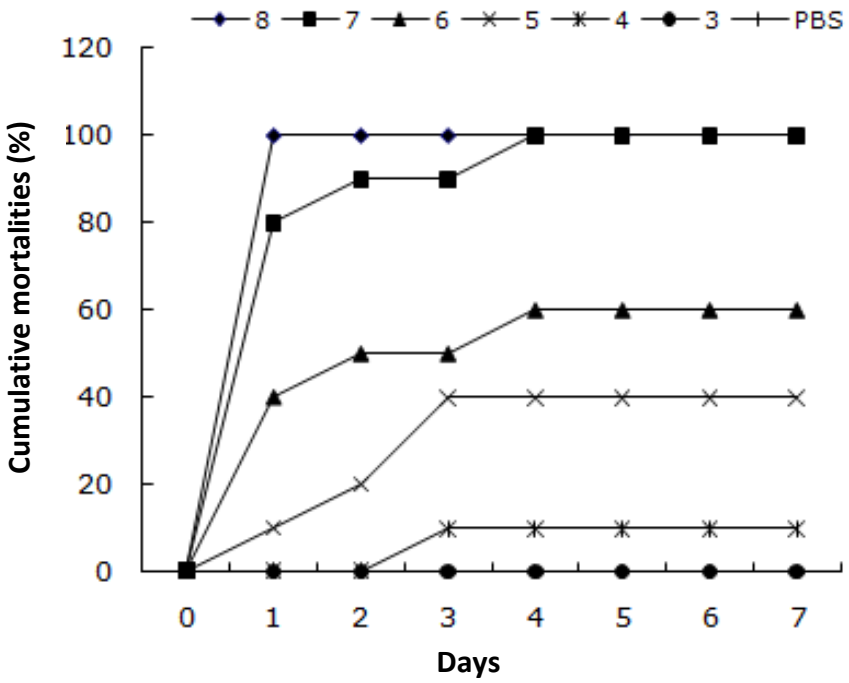

Figure 1. Cumulative mortalities after infestation with V. vulnificus in spotted maigre. ◆, ■, ▲, x, ж, ● and I indicated that fish were injected with V. vulnificus at 10^8, 10^7, 10^6, 10^5, 10^4 and 10^3 CFU/fish and PBS control, respectively.

ZOBELL 2216 E agar plates. Bacteria isolated from the fish were confirmed as *V. vulnificus* using conventional methods.

Statistical analysis

Statistical significance was determined by analysis of variance (ANOVA) using the Statistical Package for the Social Sciences (SPSS) program. Differences were considered significant at $P < 0.01$ or $P < 0.05$ by Duncan's multiple range test (Duncan, 1955).

RESULTS

The LD_{50} of *V. vulnificus*

Following infection with *V. vulnificus*, the fish were monitored daily for 1 week post infestation. Disease manifestations appeared between days 1 and 7 post infestation and included reduced activity, anorexia, convulsions and death. The data of the accumulative mortality are shown in Figure 1. Therefore, the 50% lethal dose (LD_{50}) value of *V. vulnificus* was calculated to be 2.6×10^5 CFU/fish.

Lysozyme activity

Spotted maigre fed with different concentrations of WDA for 4 weeks and measured activities of serum lysozyme are shown in Figure 2. The results showed that WDA can significantly increase ($P \leq 0.05$) the serum lysozyme activities after 7 days in all groups fed with different levels of WDA as compared to the control group in the same period. The highest lysozyme activities in serum samples were found in the 2% WDA group, followed by 1, 0.5, 4% and control groups. In addition, lysozyme activities were significantly higher ($P<0.01$) in the 2% group as compared to A and D groups in the whole observed period, while A, B and D groups were significantly higher ($P<0.01$) than control group.

Phagocytic activity

Phagocytic activity (including PP and PI) was significantly higher ($P \leq 0.05$) after 3 days in groups fed with herbs as compared to control in the same period (Tables 1 and 2). For PP (Table 1), there were no significant differences ($P>0.05$) among 0.5, 1 and 2% groups, but significantly higher ($P \leq 0.05$) than 4% and control groups in the whole observed period. For PI (Table 2), 2% group was significantly higher ($P \leq 0.05$) than other groups in the whole observed period.

Disease resistance

After 4 weeks of feeding, spotted maigre were infested with *V. vulnificus* and cumulative mortality was measured (Table 3). Control fish infested with *V. vulnificus*

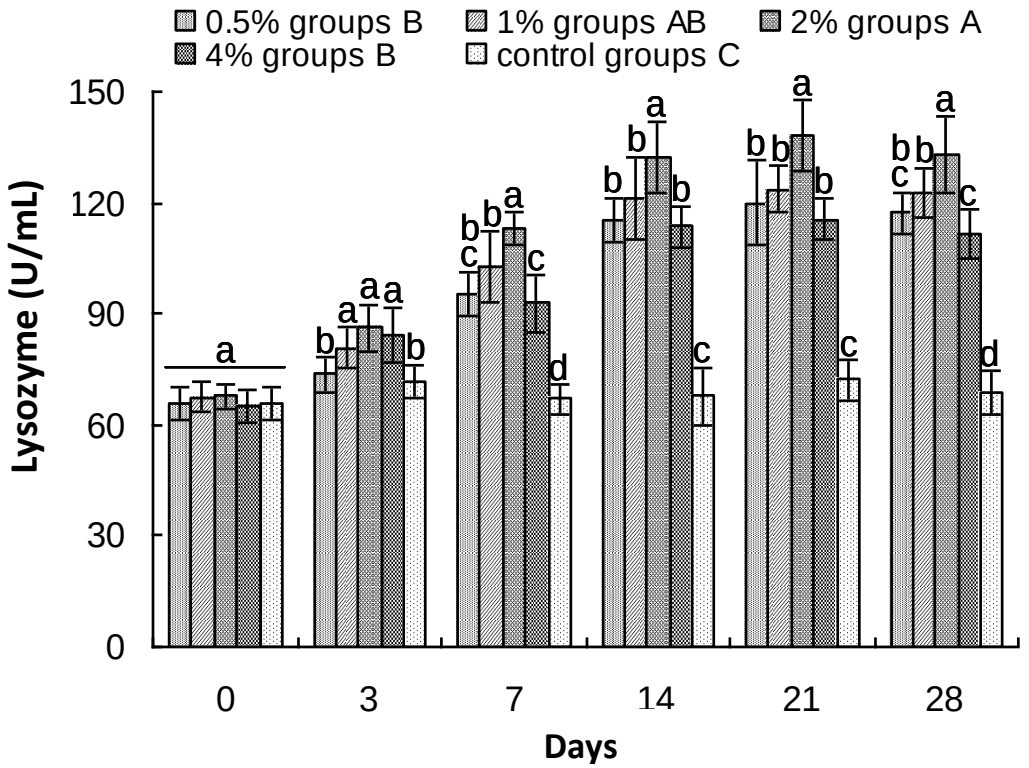

Figure 2. Changes in serum lysozyme activities in control group and in groups fed diets containing different concentrations of herbal extracts. Data are expressed as the mean ± SD of six fish. Significant differences from the control group are indicated by lowercase letters (P≤0.05, different groups in the same observed period) and uppercase letters (P<0.01, different groups in the whole observed period).

Table 1. PP of phagocytic cells in control group and groups fed diets containing different concentrations of WDA.

Day	PP of different groups				
	0.5% WDA[a]	1.0% WDA[a]	2.0% WDA[a]	4.0% WDA[b]	Control[b]
0	12.2±0.94[a]	12.4±0. 51[a]	12.1±0.79[a]	12.3±0.47[a]	12.2±0.68[a]
3	13.5±0.68[a]	13.8±0.75[a]	14.5±0.31[a]	14.2±0.86[a]	12.7±0.54[b]
7	15.7±1.11[a]	16.9±0.83[a]	18.7±0.91[a]	15.3±0.55[a]	12.5±0.76[b]
14	18.3±0.39[a]	19.0±0.63[a]	21.0±0.52[a]	18.0±0.71[a]	12.4±0.48[b]
21	20.8±1.18[a]	21.9±1.28[a]	23.9±1.25[a]	19.2±1.07[a]	12.5±0.59[b]
28	20.2±1.23[a]	20.3±1.11[a]	23.3±1.32[a]	18.3±1.08[a]	12.3±0.71[b]

WDA: Groups fed diets with different concentrations of WDA. Control group was fed diets without any supplementation. Data are expressed as the mean ± SD of six fishes. Significant differences are indicated by lowercase letters (P<0.05).

started to die 36 h post-infection and the survive rate of over 7-days treatment period was 36.7%. In all the treated groups, fish mortality occurred after 48 h post-infection, and survival rates of the 0.5, 1.0, 2.0 and 4.0% were significantly increased as compared to control by 53.3, 63.3, 73.3 and 70.0%, respectively (p<0.05). Furthermore, the highest survival rate was observed in the 2.0% group, which was significantly higher than that of 0.5, 1.0 and control groups (P<0.01).

DISCUSSION

A. membranaceus fed to the spotted maigre modulated the non-specific defence mechanism (Wang et al., 2012). The present results showed that fish fed with different

Table 2. PI of phagocytic cells in control group and groups fed diets containing different concentrations of WDA.

Day	PI of different groups				
	0.5% WDA[b]	1.0% WDA[b]	2.0% WDA[a]	4.0% WDA[b]	Control[b]
0	1.95±0.09[a]	1.96±0.14[a]	1.93±0.21[a]	1.95±0.13[a]	1.92±0.08[a]
3	2.05±0.08[a]	2.14±0.12[a]	2.35±0.18[a]	2.19±0.16[a]	1.98±0.14[b]
7	2.21±0.13[a]	2.39±0.16[a]	2.68±0.22[a]	2.46±0.29[a]	1.99±0.28[b]
14	2.41±0.09[a]	2.45±0.29[a]	3.25±0.35[a]	2.42±0.12[a]	1.99±0.07[b]
21	2.47±0.23[a]	2.57±0.27[a]	3.37±0.30[a]	2.37±0.13[a]	1.93±0.23[b]
28	2.36±0.14[a]	2.43±0.23[a]	3.26±0.31[a]	2.36±0.35[a]	1.92±0.13[b]

WDA: Groups fed diets with different concentrations of WDA. Control group was fed diets without any supplementation. Data are expressed as the mean ± SD of six fish. Significant differences are indicated by lowercase letters (P<0.05).

Table 3. Survival rates following different concentrations of WDA treatment and *V. vulnificus* infestation[1].

Group (%)	Subgroups (n = 10)[2]			Survival rate (%)[3]	Relative percent survival (%)[3]
	1	2	3		
0.5	5	5	6	53.3[c]	26.3[c]
1.0	7	6	6	63.3[b]	42.1[b]
2.0	7	7	8	73.3[a]	57.9[a]
4.0	6	8	7	70.0[a,b]	52.6[ab]
Control	3	4	4	36.7[d]	/[d]

[1]Fish were infected intraperitoneally; [2]number of survival fish/subgroup post infestation; [3]significant differences from the control group are indicated with lowercase letters (P<0.05).

concentrations of WDA significantly enhanced phagocytic activity of phagocytic cells and lysozyme activities in serum. Similar results were obtained in carp, large yellow croaker, yellow catfish, *Convict cichlid, Epinephelus tauvina* and tilapia, fed with a combination of *Astragalus* root or other Chinese Herbs (Jian and Wu, 2003; Bai et al., 2012; Punitha et al., 2008).

Bacteria such as *Vibrio* spp., *Aeromonas* spp. and *Edwardsiella* spp. are the most common pathogens of cultured fish, and cause major losses to the aquaculture industry in China and other places. Currently, *Astragalus* has been used as an immunostimulant in aquaculture, which can effectively protect fish against bacteria infections. In addition, it can be easily obtained, and the commercial production is not expensive because the preparation is simple as the use of highly purified products is not needed. Medicinal herbs as immunostimulants in aquaculture have received more attention in the last two decades not only for their immune stimulating functions but also for their growth promoting effects and little or no side effects (Harikrishnan et al., 2009).

The nonspecific immune system of fish is considered to be the first line of defence against invading pathogens, and is more important for fish than for mammals (Narnaware et al., 1994). In addition, the innate immune system also has humoral elements: the complement system, lysozyme, transferrin, agglutinins and precipitins (Magnadóttir, 2006). It has been reported that the main active component of *Astragalus* extracts is a poly-saccharide, which can modulate the functions of the immune cells including T cells, B cells, NK cells and macrophage (Yin et al., 2009; Ardó et al., 2008), and enhance the expression of cytokine genes, e.g. interleukin (IL)-1, IL-6 or tumor necrosis factor (TNF)-α (Song 2000), the nitric oxide production of these cells and the expression of the inducible nitric oxide synthase (iNOS) gene (Lee et al., 2005; Ardó et al., 2008).

The major components of the innate immune system are macrophages, monocytes, granulocytes and humoral elements, like lysozyme or complement system (Secombes and Fletcher, 1992; Magnadóttir, 2006). Therefore, phagocytosis and lysozyme activity are important indicators of immunostimulants. Results of this study indicated that suitable level of WDA could significantly enhance phagocytosis and lysozyme activity in spotted maigre. In this study, it was found that phagocytic activity of leucocytes was significantly increased in spotted maigre fed with different con-centrations of WDA as compared to the control. In addition, serum lysozyme levels were significantly elevated, and

activity was measured three days after starting the feeding with the diets containing the different concentrations of WDA. Such enhancement in lysozyme levels could also be correlated with enhanced phagocytic activity, whose enhancement on immune response of fish is well defined (Yin et al., 2009). All these results suggested that WDA include main active component of *Astragalus* extracts.

After the experimental infestation with *V. vulnificus*, all treated groups exhibited a significantly reduced mortality as compared to the control group. The best survival rate was observed in the group treated with 2% WDA, followed by 4, 1 and 0.5% WDA. A similar result was reported after feeding yellow catfish with *Astragalus* polysaccharides and infestation with *Aeromonas hydrophila* (Bai et al., 2012).

Overall, we showed that WDA could effectively enhance lysozyme activity and phagocytic activity of blood phagocytes. This suggests that WDA can be used to enhance disease resistance and as an immunostimulant to enhance cellular immune response of cultured spotted maigre. Furthermore, the use of such plants products as immunostimulants in aquaculture systems is not related to food safety and antibiotic-resistant bacteria isolates. The optimal *A. membranaceus* dietary concentration to add is 2% WDA.

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