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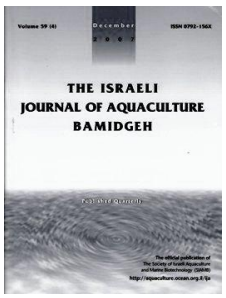


# Polysaccharides, Saponins, and Water Decoction of *Astragalus membranaceus* Significantly Enhance the Non-Specific Immune Response of Spotted Maigre (*Nibea albiflora*)

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**Abstract**

The effects of polysaccharides, saponins, and water decoction of the Chinese herb *Astragalus membranaceus* on the immune response of spotted maigre (*Nibea albiflora*) were investigated. Fish with an average initial weight of 49.6±5.5 g were fed a diet containing 2% extract for four weeks. The lysozyme and phagocytic activities of the fish were determined and compared with those of control fish fed an unsupplemented feed on days 0, 3, 7, 14, 21, and 28. Both activities were significantly higher in groups fed the *Astragalus* extract than in the control group ($p<0.01$) but there were no differences between the three *Astragalus*-fed groups ($p>0.05$). Most important, the *Astragalus*-fed groups were significantly protected against *Vibrio vulnificus* challenge compared to the control group ($p<0.01$). Our results indicatd that polysaccharides and saponins are main active components of *Astragalus* extracts and can enhance the non-specific immune response in spotted maigre.

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## 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. However, infectious diseases such as vibriosis are becoming a severe problem in culture (Lio-Po et al., 2009). Chemotherapy is effectively used to control fish infections, but arouses serious concern regarding the establishment of antibiotic-resistant bacteria (Wang and Lu, 2009). One of the most promising methods for controlling diseases of aquatic animals is by strengthening their immune system (Ardó et al., 2008).

In China, many herbs have been used for thousands of years to boost the immune system in humans (Guo et al., 2011). Over the last decade, research has focused on plant extracts (i.e., phytobiotics) from aromatic plants (e.g., ginger, curcuma, coriander) and herbal products (e.g., roots, leaves, bark), essential oils (e.g., hydro-distilled volatile plant compounds), and oleoresins (extracts based on non-aqueous solvents) to replace antibiotic growth promoters in terrestrial animal feeds (Bhuvaneswari and Balasundaram, 2006). More recently, such applications have begun to demonstrate positive effects in aquatic animals (Merrifield et al., 2009). Fishes, shrimps, and other aquatic animals fed diets containing certain Chinese herbs showed improved nonspecific immunity, such as bacteriolytic and lysozyme activity, NBT positive cells, and leukocyte function (Galina et al., 2009).

Among the many herbs used in traditional Chinese medicine, the root of *Astragalus membranaceus* has been used as an immune booster for thousands of years. *Astragalus membranaceus* contains 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). Most importantly, it has significant immunostimulatory effects, enhancing the nonspecific immunity of fishes including carp (Yin et al., 2009), large yellow croaker (Jian and Wu, 2003) and tilapia (Ardó et al., 2008). The aim of the current investigation was to assess the dietary inclusion of different *A. membranaceus* extracts on the nonspecific humoral (lysozyme activity) and cellular (phagocytic activity) responses of spotted maigre (*Nibea albifiora*).

## Materials and Methods

*Fish*. 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, 8-8.5, and 20-22%, respectively). The water flow was maintained at 20 l/min and dissolved oxygen at 80-90% saturation.

*$LD_{50}$ determination.* Fish were anesthetized with tricaine methanesulfonate (MS-222) (Hangzhou Animal Medicine Factory) at a concentration of 195 μg/ml and intraperitoneally injected with $1.0 \times 10^3$ to $1.0 \times 10^8$ CFU/fish of *Vibrio vulnificus*; control fish were injected with 10 mM phosphate-buffered saline. Ten fish were injected with each dose. Mortality was monitored for one week after infection. The experiment was repeated in triplicate. Results were averaged and used to calculate the $LD_{50}$ value according to the method of Reed and Muench (1938).

*Herbal extracts. Astragalus membranaceus* was procured from the TCM Hospital of Wenzhou City, Zhejiang Province. The roots were collected and washed in sterile distilled water, dried, powdered, and stored at -20°C until further use.

*Water decoction*. Water was decoction according to the methods of Natarajan et al. (2005) and Divyagnaneswari et al. (2007) with slight modifications. Ten grams of powdered *A. membranaceus* was exhaustively extracted with sterile distilled water, filtered through a sterile muslin cloth. The filtrate was collected and the solvent was removed using a rotary vacuum evaporator after being 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.

*Polysaccharide preparation.* Polysaccharides were extracted by the method of Yang et al. (2005) with slight modifications. In brief, powder was extracted three times using

boiling water, 20 ml filtrate was collected by concentration at 60°C, 17 ml alcohol was added, the mixture was allowed to stand for 24 h at room temperature, and the solvent was removed using a rotary vacuum evaporator. The filtrate paper was washed three times with 70% alcohol. The filtrate was collected and the solvent was removed using a rotary vacuum evaporator. The residue was dissolved, purified, and resuspended in sterile distilled water at the desired concentrations.

*Saponin preparation*. Saponins were extracted by the method of Gu et al. (2000) with slight modifications. Powder was extracted three times using 80% alcohol, diethyl ether, and n-butyl alcohol respectively. Extracted liquid was collected and the solvent was removed by evaporation using a rotary vacuum evaporator. The residue was dissolved, purified, and resuspended in sterile distilled water at the desired concentrations.

*Feed and experimental design.* Normal balanced feed (Haima Feed, Fuzhou, China) was mixed with a 2% water decoction of *A. membranaceus*, or with polysaccharides or saponins. The mixtures were incorporated into the diets at the same crude powder rate. The pelleted feed was maintained at room temperature. Fish were divided into four treatment groups (three diets plus one control; n = 60 for each group) and fed one of the diets twice a day for five weeks.

*Serum and leukocyte 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*. Lysozyme activity was measured using a photoelectric colorimeter following the method of Azza (2009) with slight modifications. In brief, changes in extinction were measured at 640 nm immediately after adding the lysozyme solution (the start of the reaction) and after 2-min incubation of the preparation at 28°C (end of the reaction after adding 100 µl of 5 mol/l KOH).

*Phagocytic activity.* Phagocytic activity was expressed as the percentage of phagocytes and the phagocytic index, analyzed following the method of Fujiki and Yano (1997) with slight modifications. In brief, *Staphylococcus aureus* (type strain ATCC25923) was substituted for baker's yeast in the phagocytosis test.

*Challenge with virulent pathogen.* On day 28, fish from each group were divided into three subgroups (n = 10 for each subgroup) and challenged with *Vibrio vulnificus* at 2.6 × $10^6$ CFU/fish, i.e., 100 µl), by intraperitoneal injection. Dead fish were examined and liver samples were homogenized and plated onto Zobell 2116 E agar plates. Bacteria isolated from the fish were confirmed as *V. vulnificus* using conventional methods.

*Statistical analysis.* Statistical significance was determined by ANOVA analysis. Differences were considered significant at $p<0.01$ or $p<0.05$.

## Results

*$LD_{50}$ of V. vulnificus.* Fish were monitored daily for one week after the *V. vulnificus* injection to determine $LD_{50}$. Disease manifestations appeared between days 1 and 7, and included reduced activity, anorexia, convulsions, and death. There were differences in survival between groups (Table 1) and the 50% lethal dose ($LD_{50}$) of *Vibrio vulnificus* was 2.6 × $10^5$ CFU/fish.

Table 1. Mortality of spotted maigre (*Nibea albiflora*) injected with *Vibrio vulnificus.*

| CFU/fish (1×) | Control | $10^2$ | $10^3$ | $10^4$ | $10^5$ | $10^6$ | $10^7$ |
|---|---|---|---|---|---|---|---|
| Mortality (%) | 0 | 0 | 0 | 10 | 40 | 60 | 100 |

*Phagocytic and lysozyme activity.* Phagocytic activity (percent phagocytes and phagocytic index) was significantly higher ($p<0.05$) in groups fed the herbal supplement than in the control (Table 2). Serum lysozyme activity was significantly higher ($p<0.05$) after three days in all groups fed the extracts than in the control (Fig. 1). The polysaccharide group had the highest lysozyme activity, followed by the water decoction, saponin, and control groups. Throughout the period, lysozyme activity was significantly higher ($p<0.01$) in the polysaccharide group than in the other groups, while the water decoction and saponin groups were significantly higher ($p<0.01$) than the control.

*Disease resistance.* Survival after bacterial challenge differed among groups (Table 3).

Table 2. Phagocytic activity of phagocytic cells in spotted maigre (*Nibea albiflora*) fed diets containing different kinds of *Astragalus membranaceus* extracts.

| Day | Phagocytic activity | *Astragalus membranaceus* extract | | | Control |
|---|---|---|---|---|---|
| | | Water decoction | Polysaccharides | Saponin | |
| 0 | Phagocytes (%) | 12.1±0.79 | 12.7±0.44 | 12.3±0.41 | 12.2±0.68 |
| | Phagocytic index | 1.93±0.21 | 1.95±0.07 | 1.93±0.38 | 1.92±0.08 |
| 3 | Phagocytes (%) | 14.5±0.81$^{a}$ | 14.8±0.57$^{a}$ | 15.1±0.65$^{a}$ | 12.7±0.54$^{b}$ |
| | Phagocytic index | 2.35±0.18$^{a}$ | 2.74±0.13$^{a}$ | 2.62±0.22$^{a}$ | 1.98±0.14$^{b}$ |
| 7 | Phagocytes (%) | 18.7±0.91$^{a}$ | 20.7±0.97$^{a}$ | 21.9±1.53$^{a}$ | 12.5±0.76$^{b}$ |
| | Phagocytic index | 2.68±0.22$^{a}$ | 3.18±0.13$^{a}$ | 2.78±0.04$^{a}$ | 1.99±0.28$^{b}$ |
| 14 | Phagocytes (%) | 21.0±0.52$^{a}$ | 21.0±1.12$^{a}$ | 21.4±1.32$^{a}$ | 12.4±0.48$^{b}$ |
| | Phagocytic index | 3.25±0.35$^{a}$ | 3.25±0.37$^{a}$ | 3.45±0.27$^{a}$ | 1.99±0.07$^{b}$ |
| 21 | Phagocytes (%) | 23.9±1.25$^{a}$ | 23.9±1.35$^{a}$ | 24.7±1.28$^{a}$ | 12.5±0.59$^{b}$ |
| | Phagocytic index | 3.37±0.23$^{b}$ | 3.37±0.33$^{b}$ | 3.87±0.42$^{a}$ | 1.93±0.23$^{c}$ |
| 28 | Phagocytes (%) | 23.3±1.32$^{a}$ | 23.3±2.13$^{a}$ | 25.3±1.41$^{a}$ | 12.3±0.71$^{b}$ |
| | Phagocytic index | 3.26±0.31$^{b}$ | 3.26±0.31$^{b}$ | 3.53±0.23$^{a}$ | 1.92±0.13$^{c}$ |

Data are means±SD of six fish. Superscripts indicate significant differences ($p$<0.01) between groups in the same observation.

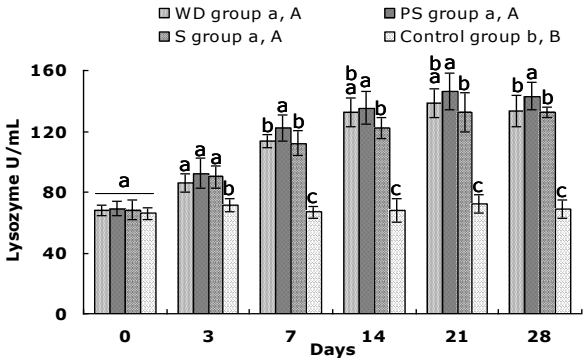


Fig. 1. Serum lysozyme activity in spotted maigre (*Nibea albiflora*) fed diets containing different kinds of extracts of *Astragalus membranaceus*: WD = water decoction, PS = polysaccharides, S = saponins. The control group was fed an unsupplemented diet. Data are means±SD of six fish. Significant differences from the control during the same observation are indicated by lowercase letters above the bars ($p$<0.05) and over the entire period by uppercase letters in the legend ($p$<0.01).

Table 3. Survival after challenged by *Vibrio vulnificus* of spotted maigre (*Nibea albiflora*) fed diets containing different kinds of *Astragalus membranaceus extracts.*

| Extract | No. dead fish per triplicate (n = 10) | | | Survival (%) | Relative survival (%)* |
|---|---|---|---|---|---|
| Water decoction | 3 | 3 | 2 | 73.3$^{a}$ | 57.9$^{a}$ |
| Polysaccharide | 3 | 3 | 4 | 66.7$^{ab}$ | 47.4$^{ab}$ |
| Saponin | 5 | 3 | 4 | 60.0$^{b}$ | 36.8$^{b}$ |
| Control | 7 | 6 | 6 | 36.7$^{c}$ | 0$^{c}$ |

Significant differences from the control group are indicated by superscripts ($p$<0.05).
* Relative survival = 1 - (mortality rate of treated groups/mortality rate of control group) × 100

## Discussion

The herbal extracts fed to the spotted maigre modulated nonspecific defense mechanisms. Fish fed different kinds of extracts from *A. membranaceus* had significantly enhanced lysozyme and phagocytic cellsactivity in the serum, similar to results in carp, large yellow croaker, yellow catfish, and tilapia fed a combination of *Astragalus* root or other Chinese herbs (Bai et al., 2012; Jian and Wu 2003).

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 elsewhere. Chinese herbs used as immunostimulants and adjuvants in fish vaccines offer an alternative to the drugs, chemicals, and antibiotics currently used in fish culture to control disease. Medicinal herbs also have growth promoting effects and little or no side effects (Abutbul et al., 2005; Bhuvaneswari, 2006; Harikrishnan et al., 2009).

The nonspecific immune system of fish is the first line of defense against invading pathogens, and is more important for fish than for mammals (Narnaware et al., 1994). The major components of the innate immune system (nonspecific) are macrophages, monocytes, granulocytes, and humoral elements such as lysozyme or the complement system (Magnadóttir, 2006). The nonspecific immune response is often reported as a function of macrophage activity such as phagocytosis and chemotaxis. Immunostimulants can be applied via injection, bath, or oral administration, the latter seems the most practicable (Harikrishnan et al., 2009). The innate immune system has humoral elements: the complement system, lysozyme, transferrin, agglutinins, and precipitins

(Magnadóttir, 2006). On many occasions, *A. membranaceus* extracts can enhance plasma lysozyme activity (Azza, 2009). Lysozyme is a cationic enzyme that breaks down β-1,4 glycosidic acids and N-acetyl glucosamine in the peptidoglucan of bacterial cell walls, which is correlated with the bactericidal activity of leukocytes (Bai et al., 2012). This action attacks mainly gram-positive bacteria and some gram-negative bacteria in conjunction with the complement (Alexander and Ingram, 1992). In our experiment, activity was significantly elevated three days after the start of feeding extract-containing diets, and protection against fish bacterial infection was increased, in correlation with an increment in serum lysozyme levels and phagocytic activity of the leukocytes. The enhancement in lysozyme levels could also be correlated with enhanced phagocytic activity (Yin et al., 2009).

Phagocytic cells are the most important cellular components of the innate immune system of fish in which phagocytosis is a primitive defense mechanism and an important characteristic of the nonspecific immune system (Galina et al., 2009). Oral administration of Chinese herbal extracts (*Rheum officinale, Andrographis paniculata, Isatis indigotica, Lonicera japonica*) increase phagocytosis of the white blood cells of carp (Yin et al., 2009). The main active component of *Astragalus* extracts is a polysaccharide that can modulate the function of immune cells including T-cells, B-cells, NK-cells, and macrophages, and enhance the expression of cytokine genes such as IL-1, IL-6, and TNF-α (Song et al., 2000), the nitric oxide production of these cells, and the expression of the inducible nitric oxide synthase (iNOS) gene (Bai et al., 2012). In this study, the phagocytic activity of the leukocytes was significantly increased in spotted maigre fed all three *Astragalus* extracts, suggesting that saponin is another main active component of *Astragalus* extracts.

After experimental challenge with *V. vulnificus*, all treated groups exhibited significantly reduced mortality compared to the control. The best survival rate was in the group treated with water decoction, followed with polysaccharides and saponins, similar to results with Nile tilapia fed Chinese herbs (*A. membranaceus* and *L. japonica*) and challenged with *A. hydrophila* (Ardó et al., 2008).

Our results show there the use of *Astragalus* extracts as immunostimulants in fish could significantly enhance lysozyme and phagocytic activity of blood phagocytes. *Astragalus* is easily obtained, and commercial production is inexpensive because preparation is simple and highly purified products are not needed. Further, the use of such plants products as immunostimulants in aquaculture systems creates no concern regarding food safety and antibiotic-resistant bacterial isolates.

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