

**EFFECTS OF TEN TRADITIONAL CHINESE HERBS ON IMMUNE RESPONSE
AND DISEASE RESISTANCE OF *SCIAENOPS OCELLATUS*
(ACTINOPTERYGII: PERCIFORMES: SCIAENIDAE)**

Tingshuang PAN^{1,3}, Maocang YAN^{2*}, Shaobo CHEN², and Xuepeng WANG⁴

¹ College of Fisheries, Huazhong Agricultural University, Wuhan, Hubei Province 430070, China

² Zhejiang Mariculture Research Institute, Zhejiang Key Laboratory of Exploitation and Preservation of Coastal Bio-resource, Wenzhou, Zhejiang Province 325005, China

³ Fisheries Institute, Anhui Academy of Agricultural Sciences, Hefei, Anhui Province 230031, China

⁴ College of Animal Science and Technology, Shandong Agricultural University, Taian, Shandong Province, 271018, China

Pan T., Yan M., Chen S., Wang X. 2013. Effects of ten traditional Chinese herbs on immune response and disease resistance of *Sciaenops ocellatus* (Actinopterygii: Perciformes: Sciaenidae). Acta Ichthyol. Piscat. 43 (1): 41–49.

Background. Traditional Chinese herbs (TCHs) are widely used for improving non-specific immunity of fish in aquaculture. Possible effects of many important TCH on red drum, *Sciaenops ocellatus* (Linnaeus, 1766), have not been adequately studied. The aim of the presently reported study was to investigate the effects of ten TCH on selected immunological- and haematological responses of red drum.

Materials and methods. Ten TCH preparations, consisting of selected parts of plants and fungi, known in medicinal practice as: *Astragalus membranaceus*, fructus forsythiae, *Polyporus umbellatus*, *Scutellaria baicalensis*, *Wolfiporia extensa*, rhizoma coptidis, radix glycyrrhizae, flos lonicerae japonicae, isatidis radix, and bupleuri radix were used in the experiment. A dose of 10 g of each TCH was decocted and concentrated, then mixed with the diets (mixed diets) at ratio of 2%. After 28 days of feeding, the fish were infected with *Vibrio splendidus* and observed for 14 days for possible mortalities. Leukocyte phagocytic activity and lysozyme activity (LZM) were measured on days 0, 3, 7, 14, 21, 28, and 35. Relative percentage survival (RPS) of each TCH treatment was also investigated after the fish exposure to *V. splendidus*.

Results. Red drum fed 2% doses of individual TCH extracts significantly ($P < 0.05$) increased its: phagocytic percentage (PP) (with the exception of flos lonicerae japonicae treatment), phagocytic index (PI) (with the exception of flos lonicerae japonicae- and bupleuri radix treatments), LZM (with the exception of *Wolfiporia extensa* treatment), and RPS (with the exception of *P. umbellatus*, *Wolfiporia extensa*-, radix glycyrrhizae-, flos lonicerae japonicae-, isatidis radix-, and bupleuri radix treatments). The indexes of PP, PI, and LZM, for all experimental groups, decreased quickly on day 35 (7 days after TCH diets were discontinued). On the other hand, PP values were significantly ($P < 0.05$) higher than in control (with the exception of flos lonicerae japonicae- and *Wolfiporia extensa* treatments). Also PI values were significantly higher ($P < 0.05$) than in control (with the exception of flos lonicerae japonicae-, *Wolfiporia extensa*-, and bupleuri radix treatments). LZM values exhibited no significant difference ($P > 0.05$) in all treatment groups compared with control. Most importantly, the *A. membranaceus*-, or *S. baicalensis*-, or fructus forsythiae-fed groups were significantly protected ($P < 0.01$) against *V. splendidus* challenge compared to control. RPS of *A. membranaceus*- and *S. baicalensis* groups were the highest, reaching 88.9% on day 28, followed by the fructus forsythiae group, whereas bupleuri radix- and *Wolfiporia extensa* treatments showed the lowest values.

Conclusion. *A. membranaceus*, *S. baicalensis*, and fructus forsythiae were effective in preventing red drum from acquiring the disease while challenged by *V. splendidus*.

Keywords: traditional Chinese herbs, *Sciaenops ocellatus*, *Vibrio splendidus*, immune function

INTRODUCTION

Red drum, *Sciaenops ocellatus* (Linnaeus, 1766), is an economically important sciaenid fish in the US and other

countries (Diamant 1998). In China, red drum culture trials have begun with the importation of eggs and fry from the US in the early 1990s. The species is now being an

* Correspondence: Dr Maocang Yan, Zhejiang Mariculture Research Institute, Zhejiang Key Laboratory of Exploitation and Preservation of Coastal Bio-resource, Wenzhou, 325005, China, phone: + 86 (0) 577 88210966, fax: + 86 (0) 577 88229314, e-mail: yanmaocang@126.com.

economically important sciaenid fish in the southern China. In recent years, red drum growers in China have encountered some difficulties with its management due to skin fester disease outbreaks and mortalities.

To prevent financial losses due to diseases, fish farmers must take sufficient preventive measures. Antibiotics and other chemotherapeutics used to control these diseases can result in the development of drug resistant pathogens, environmental pollution, and accumulation of residues in fish. Though vaccination is an effective prophylactic method for controlling fish diseases, vaccines are relatively expensive and they are very specific to particular pathogens (Sakai 1999).

The use of immunostimulants for the disease prevention in fish is considered as a promising alternative to vaccines (Anderson 1992). In our research we used traditional Chinese herbs (TCHs) to give early activation to the non-specific defence mechanisms and to elevate the specific immune response.

For thousands of years, TCH have been used as traditional medicine and immune booster for humans in China. Recently, growing interest has focused on the immune stimulating function of some TCH in aquaculture, and non-specific immunity can be improved by a single or mixed TCH (Luo 1997, Ardó et al. 2008, Yin et al. 2009). For example, in the serum of crucian carp phagocytosis and lysozyme activity increased when the fish were fed different TCH: *Rheum officinale*, *Andrographis paniculata*, *Isatis indigotica*, and *Lonicera japonica* (see Chen et al. 2003), NBT positive cells and lysozyme activities increased in sturgeon hybrid (*Acipenser ruthenus* × *A. baerii*) by feeding with TCH mixture (Jeny and Jeny 2002). And the lysozyme activity significantly increased and the cumulative mortality significantly decreased when bastard halibut, *Paralichthys olivaceus* (Temminck et Schlegel, 1846)—fed diet enriched with 0.1% and 1.0% monkey head mushroom, *Hericium erinaceum*—was challenged with *Philasterides dicentrarchi* (see Harikrishnan et al. 2011). In this study TCH extracts of: *Astragalus membranaceus*, fructus forsythiae, *Polyporus umbellatus*, *Scutellaria baicalensis*, *Wolfiporia extensa*, rhizoma coptidis, radix glycyrrhizae, flos loniceriae japonicae, isatidis radix, and bupleuri radix were chosen for the experiment because of their recorded ability to enhance the immune system (Lou et al. 2001, Chen et al. 2003, Jian and Wu 2003, Wang et al. 2006, Ardó et al. 2008, Yin et al. 2009, Yan et al. 2010).

In the presently reported study, red drum was fed with feed containing one of the ten TCH. Key factors of the non-specific immune response were measured during the experiment and at the end fish were challenged with *V. splendidus* to determine disease resistance in fish fed with these substances.

MATERIALS AND METHODS

Fish. Apparently healthy red drum, *Sciaenops ocellatus* (weight 63.7 ± 1.2 g), was maintained in 20 000 L concrete tanks with recirculation system in Zhejiang

Mariculture Research Institute, Wenzhou, Zhejiang province, China. Two weeks later, the fish were transferred into 2000 L fibreglass tanks in recirculation system of the institute for experiment. Water temperature 20–21°C, salinity of $26 \text{ g} \cdot \text{L}^{-1}$, and pH 8.5 were maintained during the experiment. Fish were fed with a formulated wet feed, produced in the experimental milling facility of the institute. The dissolved oxygen was maintained at 80%–90% of saturation.

Preparation of TCH extracts and supplementation diet. Ten TCH preparations were purchased locally. Those preparations, consisting of selected parts of plants and fungi, are known in medicinal practice as: *Astragalus membranaceus*; fructus forsythiae (fruits of *Forsythia* spp.); *Polyporus umbellatus*; *Scutellaria baicalensis*; *Wolfiporia extensa*; rhizoma coptidis (dried rhizomes of *Coptis chinensis*); radix glycyrrhizae (dried roots and rhizomes of *Glycyrrhiza glabra*); flos loniceriae japonicae (dried flowers of *Lonicera japonica*); isatidis radix (dried roots of *Isatis indigotica* = *Isatis tinctoria*); and bupleuri radix (dried roots of *Bupleurum chinense*). Hot-water extracts of the ten TCH were prepared based on the method described by Wu et al. (2010). The amounts of 10 g of the each TCH were added to 200 mL of deionized water, boiled for 0.5 h, filtered through a nylon fabric, then supplemented with 200 mL of deionized water and boiled for 0.5 h again, then filtered and boiled for the third time. The 600 mL filtrate of each TCH was concentrated to 50 mL and added to 490 g crushed formula feed (Fuzhou Haima Feed Co., Ltd). Control group received the same volume of 0.65% sodium chloride. The feed used was a regular, balanced feed, composed of 46% proteins, 22% carbohydrates, 3.5% lipids, and 10.2% ash. All ratios of the pelleted feed were stored in a refrigerator.

Experiment design. Fish were allocated into 11 groups (30 fish per group) in triplicate and fed diets twice a day for 4 weeks. Then the remained fish of all groups were fed the same formula feed (Fuzhou Haima Feed Co. Ltd) for 2 weeks.

Preparation of serum and blood cells. Blood samples (2 fish per group) were collected from the caudal vein without anaesthesia on days 0, 3, 7, 14, 21, 28, and 35 after start of feeding. A portion of sampled blood was placed in an Eppendorf tube to prepare serum for assaying lysozyme, the remaining blood was pipetted into another tube containing sodium heparinate and used to measure phagocytic activity of blood leukocytes.

Phagocytic activity. Phagocytic activity of blood leukocytes was determined by the method of Lou et al. (2001). To perform the essay, 100 μL blood was mixed with 100 μL *Staphylococcus aureus* (1.0×10^8 CFU $\cdot \text{mL}^{-1}$). The mixtures were incubated at 25°C for 60 min. Following incubation, a drop of mixture were smeared on one glass slide (5 slides per blood sample), then denatured by methanol, dyed by Giemsa for 1–1.5 h, water scrubbed, air dried, and observed with an oil immersion objective. Phagocytic percentage (PP) and phagocytic index (PI) were calculated by the following formulae:

$$PP = 100N_p \cdot N_t^{-1}$$

$$PI = N_A \cdot N_p^{-1}$$

where: N_p is the number of phagocytic leukocytes, N_t is the total number of leukocytes, N_A is the number of *S. aureus* in a leukocyte.

Lysozyme activity. Lysozyme activity was measured spectrophotometrically using the method described by Abd-El-Rhman (2009) with a minor modification. 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 μ L of standard solution or serum was added to 1800 μ L of *M. lysodeikticus* 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 a 2 min incubation of the preparation under investigation at 28°C (end of the reaction after adding 100 μ L 5 mol \cdot L⁻¹ KOH). The lysozyme content was determined using the calibration curve and the extinction measured. One unit (U) of lysozyme activity was defined as the amount of enzyme that decreases the absorbance by 0.001 min⁻¹mL⁻¹ serum.

Relative percent survival (RPS). *Vibrio splendidus* isolated from diseased red drum, and conserved in Zhejiang Mariculture Research Institute, Zhejiang Key Laboratory of Exploitation and Preservation of Coastal Bio-resource, Wenzhou, China. Fish were challenged intraperitoneally with LD₅₀ dose (0.2 mL PBS containing 1 \times 10⁸ cells) of live *V. splendidus* on day 28. The LD₅₀ dose was determined earlier by administering varying doses of *V. splendidus* to untreated fish groups (Reed and Muench 1938). Mortality was recorded for 14 days. From the organs of the just dead fish, *V. splendidus* was re-isolated to confirm the mortality due to the bacterial infection. The relative percentage survival (RPS) was calculated by the following formula:

$$RPS = 1 - 100M_t \cdot M_c^{-1}$$

where: M_t is percent mortality in treated groups and M_c is percent mortality in control group.

Statistical analysis. The data were expressed as arithmetic mean \pm standard error. Statistical analysis involved one-way analysis of variance (ANOVA) followed by Duncan's multiple pair comparison test (SPSS 13.0). Asterisk in the figures represent the significant difference at $P < 0.05$.

RESULTS

Phagocytic activity. The PP of leukocyte did not vary significantly ($P > 0.05$) at any time in control group. However, the PP activity significantly increased ($P < 0.01$) or ($P < 0.05$) when the fish were fed 2% *Astragalus membranaceus*-, or fructus forsythiae-, or *Scutellaria baicalensis*-, or isatidis radix-enriched diets or fed 2% *Polyporus umbellatus*-, or rhizoma coptidis-enriched diets on day 3 compared to control. The PP significantly increased when the fish were fed 2% *Wolfiporia extensa*-, or radix glycyrrhizae-, or bupleuri radix-enriched diets on day 7 compared to control. And the flos loniceriae japonicae group did not have significant variance during the 28 days experiment (Table 1).

During the experiment period, PI of control group was 1.96–2.50, and the *Astragalus membranaceus* group demonstrated the highest values of this parameter among all experimental groups, reaching 5.89 on day 21. PI of groups of *A. membranaceus*, fructus forsythiae, *Scutellaria baicalensis*, rhizoma coptidis, and isatidis radix had a significant difference ($P < 0.05$) on day 3, ($P < 0.01$) on day 7 compared to the control group. Groups of *Polyporus umbellatus*, *Wolfiporia extensa*, radix glycyrrhizae had a significant difference ($P < 0.05$) on day 14 compared to control group. And PI of flos loniceriae japonicae- or bupleuri radix-treated groups did not have significant difference ($P > 0.05$) compared to control during the 28-day experiment (Table 2).

Lysozyme activity. The plasma lysozyme activity significantly increased ($P < 0.05$) from day 7 on being fed *Astragalus membranaceus*-, or fructus forsythiae-, or *Scutellaria baicalensis*-, or rhizoma coptidis-, or radix glycyrrhizae-, or flos loniceriae japonicae-enriched diets when compared to control. All experimental groups significantly differed ($P < 0.05$) compared to control, except *Wolfiporia extensa* group on day 14 and this high lysozyme activity persisted until day 28. On day 35, the plasma lysozyme activity of all experiment groups did not significantly differed ($P > 0.05$) from control (Fig. 1).

Disease resistance. In this study, after the challenge with *V. splendidus*, the mortalities were significantly reduced in some groups compared to control (90%), and RPS had significantly differed ($P < 0.01$) among different TCH-treated groups. The highest RPS values amounted to: 88.9%, 88.9%, and 77.8% in groups fed *A. membranaceus*-, or *S. baicalensis*-, or fructus forsythiae-enriched diets, and the lowest relative percent survival was only 22.2% in groups fed *Wolfiporia extensa*-, or bupleuri radix-enriched diets when challenged with *V. splendidus*. The highest mortality of 90% was noted in control diet (Table 3). Fish began to die on day 1 in groups treated with *A. membranaceus*, *S. baicalensis*, rhizoma coptidis, radix glycyrrhizae, flos loniceriae japonicae, and bupleuri radix, on day 2 in groups treated with fructus forsythiae, *P. umbellatus*, *Wolfiporia extensa*, and isatidis radix after challenged with *Vibrio splendidus* (Table 3).

DISCUSSION

The immunostimulating effect of *Astragalus membranaceus*, fructus forsythiae, *Polyporus umbellatus*, *Scutellaria baicalensis*, *Wolfiporia extensa*, rhizoma coptidis, radix glycyrrhizae, flos loniceriae japonicae, isatidis radix, and bupleuri radix extracts on the marine fish, red drum, was investigated in this study. The presently reported results showed that experimental fish fed 2% doses of the TCH extracts significantly enhanced PP (with the exception of flos loniceriae japonicae), PI (with the exception of flos loniceriae japonicae and bupleuri radix), lysozyme activities (with the exception of *Wolfiporia extensa*), and RPS (with the exception of *P. umbellatus*, *W. extensa*, radix glycyrrhizae, flos loniceriae japonicae, isatidis radix, and bupleuri radix).

Table 1
Feeding supplementation effect of traditional Chinese herbs on phagocytic percentage of the blood leukocytes in *Sciaenops ocellatus*

Feed treatment group	Phagocytic percentage [%] after time elapsed [day]						
	0	3	7	14	21	28	35
<i>Astragalus membranaceus</i>	13.3 ± 0.54	19.6 ± 0.88**	21.3 ± 0.53**	27.6 ± 0.68**	28.7 ± 0.91**	25.4 ± 0.56**	24.9 ± 0.83*
fructus forsythiae	13.2 ± 1.02	18.1 ± 0.39**	23.4 ± 0.57**	28.9 ± 0.32**	29.4 ± 0.95**	30.4 ± 0.64**	24.4 ± 0.55*
<i>Polyporus umbellatus</i>	13.4 ± 0.91	17.3 ± 0.44*	19.4 ± 0.63**	22.0 ± 0.34**	21.6 ± 1.09**	20.7 ± 0.84**	18.8 ± 0.37*
<i>Scutellaria baicalensis</i>	13.1 ± 0.87	18.4 ± 0.24**	24.2 ± 0.88**	31.4 ± 0.49**	31.2 ± 0.74**	30.4 ± 0.91**	26.5 ± 0.35*
<i>Wolfiporia extensa</i>	13.3 ± 0.38	14.8 ± 0.14	16.6 ± 0.52*	17.5 ± 0.41*	17.9 ± 0.14**	16.8 ± 0.36*	14.1 ± 0.79
rhizoma coptidis	13.2 ± 0.43	27.7 ± 0.27**	31.1 ± 0.96**	31.3 ± 0.88**	31.2 ± 0.49**	29.6 ± 0.52**	26.2 ± 0.45*
radix glycyrrhizae	13.4 ± 0.65	16.7 ± 0.49	20.1 ± 0.57**	24.0 ± 0.92**	25.7 ± 0.65**	25.4 ± 0.49**	22.0 ± 0.74*
flos loniceræ japonicae	13.5 ± 0.61	16.8 ± 0.52	17.6 ± 0.63*	16.8 ± 0.39	16.1 ± 0.74	14.5 ± 0.85	13.3 ± 0.62
isatidis radix	13.5 ± 0.55	17.8 ± 0.37*	21.4 ± 0.65**	27.5 ± 0.48**	29.1 ± 0.39**	29.4 ± 0.37**	27.7 ± 0.83*
bupleuri radix	13.4 ± 0.91	16.2 ± 0.43	18.7 ± 0.76**	21.0 ± 0.58**	20.2 ± 0.58**	20.3 ± 0.92**	18.5 ± 0.58*
Control	13.4 ± 0.42	13.3 ± 0.87	13.6 ± 0.36	13.5 ± 0.73	13.6 ± 0.72	13.7 ± 0.86	13.8 ± 0.59

Values are mean ± standard error ($n = 30$ fish in each treatment); Data in the same column with asterisk are significantly different from the control diet indicated by one or two asterisks among different treatments; * Significant different from control; $P < 0.05$ (one-way ANOVA); ** Significant different from control; $P < 0.01$ (one-way ANOVA).

Table 2
Feeding supplementation effect of traditional Chinese herbs on phagocytic index of the blood leukocytes in *Sciaenops ocellatus*

Feed treatment group	Phagocytic index after time elapsed [day]						
	0	3	7	14	21	28	35
<i>Astragalus membranaceus</i>	1.97 ± 0.33	3.35 ± 0.21*	3.86 ± 0.36**	4.79 ± 0.18**	5.62 ± 0.29**	5.03 ± 0.43**	4.86 ± 0.32*
fructus forsythiae	1.94 ± 0.14	3.04 ± 0.25*	4.45 ± 0.42**	5.72 ± 0.19**	5.64 ± 0.16**	5.66 ± 0.61**	4.27 ± 0.44*
<i>Polyporus umbellatus</i>	1.95 ± 0.14	2.31 ± 0.11	2.63 ± 0.37	3.54 ± 0.19*	3.45 ± 0.56*	3.43 ± 0.31*	3.13 ± 0.45*
<i>Scutellaria baicalensis</i>	1.96 ± 0.14	2.98 ± 0.40*	4.21 ± 0.38**	5.87 ± 0.77**	5.89 ± 0.37**	5.76 ± 0.53**	5.01 ± 0.29*
<i>Wolfiporia extensa</i>	1.97 ± 0.09	2.24 ± 0.21	2.58 ± 0.10	3.15 ± 0.13*	3.11 ± 0.02*	3.09 ± 0.02*	2.67 ± 0.02
rhizoma coptidis	1.98 ± 0.04	2.87 ± 0.04*	3.79 ± 0.07**	4.37 ± 0.11**	4.57 ± 0.17**	4.38 ± 0.04**	3.68 ± 0.07*
radix glycyrrhizae	1.95 ± 0.33	2.44 ± 0.16	3.35 ± 0.23	3.65 ± 0.31*	4.02 ± 0.36*	3.89 ± 0.15*	3.04 ± 0.22*
flos lonicerae japonicae	1.96 ± 0.33	2.21 ± 0.24	2.36 ± 0.27	2.71 ± 0.22	2.67 ± 0.14	2.52 ± 0.17	2.31 ± 0.18
isatidis radix	1.96 ± 0.311	2.87 ± 0.16*	4.28 ± 0.32**	5.46 ± 0.36**	5.34 ± 0.32**	5.39 ± 0.34**	4.62 ± 0.24*
bupleuri radix	1.95 ± 0.33	2.45 ± 0.31	2.38 ± 0.23	2.35 ± 0.23	2.37 ± 0.23	2.36 ± 0.23	2.30 ± 0.26
Control	1.96 ± 0.14	1.97 ± 0.27	1.98 ± 0.16	2.03 ± 0.37	2.05 ± 0.22	1.98 ± 0.09	1.96 ± 0.33

Data in the same column with asterisk are significantly different from control; Values are mean ± SE ($n = 30$ fish in each treatment); * Significantly different from control ($P < 0.05$, one-way ANOVA); ** Significantly different from control ($P < 0.01$, one-way ANOVA).

Phagocytic cells are the most important cellular components of the innate immune system of fish (Ardó et al. 2008). Phagocytic activity is a primitive defence mechanism (Neumann et al. 2001) and an important characteristic of the non-specific immune system (Seeley et al. 1990). This

parameter usually shows an increase after oral administration of immunostimulants (Jeney et al. 1997). It is well known that fish treated with immunostimulants can increase phagocytosis (Sakai 1999). TCH extracts can enhance phagocytosis in various fish species (Galina et al. 2009, Yin

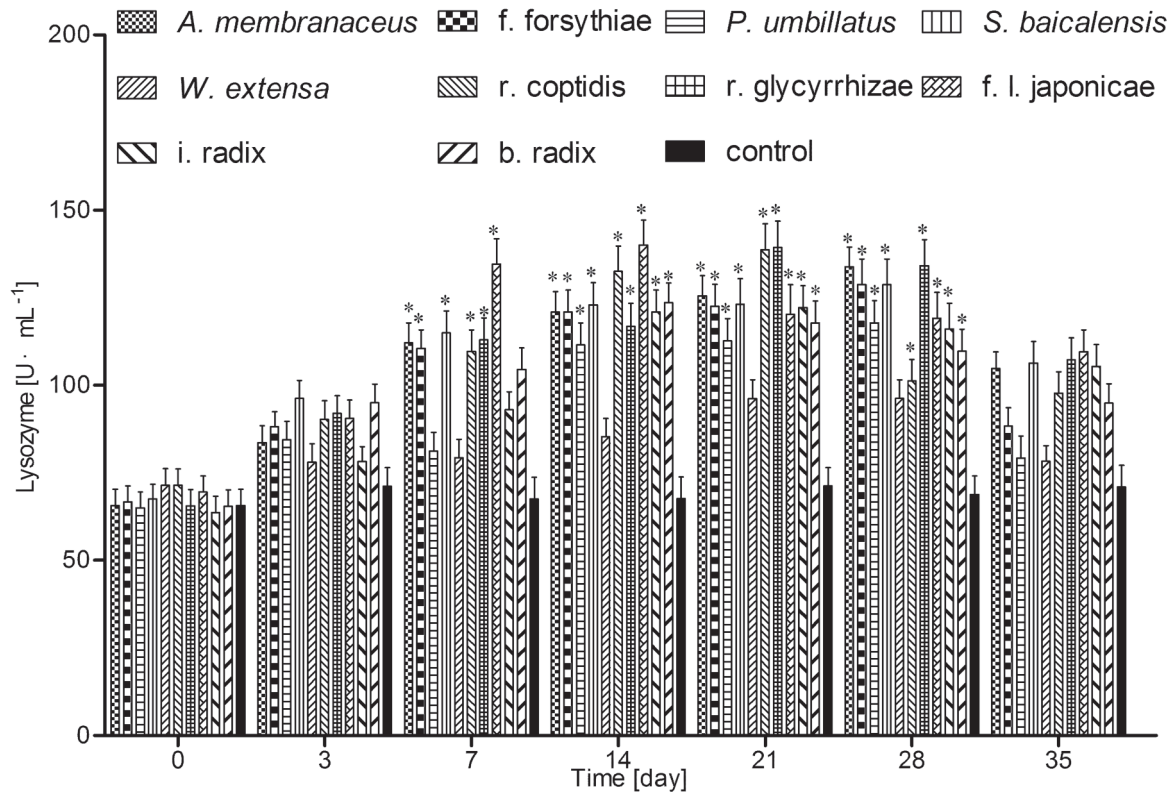


Fig.1. Effects of traditional Chinese herbs on serum lysozyme activity in *Sciaenops ocellatus*; * Significantly different from control ($P < 0.05$, one-way ANOVA); *A. membranaceus* = *Astragalus membranaceus*, *f. forsythiae* = fructus forsythiae, *P. umbellatus* = *Polyporus umbellatus*, *S. baicalensis* = *Scutellaria baicalensis*, *W. extensa* = *Wolfiporia extensa*, *r. coptidis* = rhizoma coptidis, *r. glycyrrhizae* = radix glycyrrhizae, *f.l. japonicae* = flos lonicer-ae japonicae, *i. radix* = isatidis radix, *b. radix* = bupleuri radix

Table 3

Relative percentage of survival (RPS) in *Sciaenops ocellatus* after being infected by *Vibrio splendidus*

Feed treatment group	N	Time [day]	No. dead	Mortality [%]	RPS [%]
<i>Astragalus membranaceus</i>	3 × 10	1	3	10	88.9**
fructus forsythiae	3 × 10	2	6	20	77.8**
<i>Polyporus umbellatus</i>	3 × 10	2	18	60	33.3
<i>Scutellaria baicalensis</i>	3 × 10	1	3	10	88.9**
<i>Wolfiporia extensa</i>	3 × 10	2	21	70	22.2
rhizoma coptidis	3 × 10	1	9	30	66.7*
radix glycyrrhizae	3 × 10	1	18	60	33.3
flos lonicer-ae japonicae	3 × 10	1	18	60	33.3
isatidis radix	3 × 10	2	18	60	33.3
bupleuri radix	3 × 10	1	21	70	22.2
Control	3 × 10	1	27	90	/

N = No. of fish challenged; Time indicates the day that fish began to die after challenged with *Vibrio splendidus*; No. dead = No. of dead fish; Mortality = cumulative mortality; Data in the same column with asterisk are significantly different; * Significantly different from other treatments ($P < 0.05$, one-way ANOVA); ** Significantly different from other treatments ($P < 0.01$, one-way ANOVA).

et al. 2009, Zhang et al. 2009, Huang et al. 2011, Ahmadi et al. 2012). Previous studies showed that polysaccharides and saponins are main active components of *Astragalus membranaceus*. The latter herb enhanced the non-specific immune response in yellow drum, *Nibea albiflora* (Richardson, 1846) (see Wang et al. 2012). Feeding Nile tilapia, *Oreochromis niloticus* (L.) with *A. membranaceus* and flos lonicerae japonicae alone or in combination significantly enhanced phagocytic and respiratory bursts activity of blood phagocytic cells of the fish and both herbs reduced the mortality following *Aeromonas hydrophila* infection (Ardó et al. 2008). Bastard halibut, *Paralichthys olivaceus*, fed diets containing TCH, including flos lonicerae japonicae, had a significantly higher ($P < 0.05$) superoxide dismutase (SOD) activities compared to control group, but the lysozyme activity and total protein contents were not significantly different ($P > 0.05$) from control (Wang et al. 2006). In our experiments, *A. membranaceus*, fructus forsythiae, *P. umbellatus*, *S. baicalensis*, rhizoma coptidis, isatidis radix extracts significantly enhanced the phagocytic activity of leukocytes isolated from red drum during the 28 days feeding, even as early as 3 days after start of feeding. This elevated activity was maintained during the entire experiment (in the group fed with feed containing *A. membranaceus*, or fructus forsythiae, or *Polyporus umbellatus*, or *Scutellaria baicalensis*, or rhizoma coptidis, or isatidis radix, or *Wolfiporia extensa*, or radix glycyrrhizae, or bupleuri radix) compared to the control group. On the other hand, no effect on phagocytic activity of leukocytes was found when fish were fed with 2% flos lonicerae japonicae extracts during the 28 days experiment. This suggests that some species of TCH are not capable to boost the phagocytic activities of the non-specific immunity of the fish.

Lysozyme level is a measurable humoral component of the non-specific defence mechanisms. Lysozyme is an enzyme that can hydrolyze b-1-4-glucosidic linkages between *N*-acetylmuramic acid and *N*-acetyl-D-glucosamine residues present in the mucopolysaccharide cell wall of a variety of bacterial pathogens. In the presently reported study, it was observed that the lysozyme activity of the red drum was enhanced by nine TCH (except *Wolfiporia extensa*). Significant difference of lysozyme activity in all treatment groups discontinued on day 35 (7 days after TCH-supplemented feeding stopped). This suggests that lysozyme activities are TCH dependent. Various authors have also reported similar observations of increased values of fish serum lysozyme after activation of the immune system with TCH (Yin et al. 2006, Ma et al. 2009, Zhang et al. 2009, Ahmadi et al. 2012).

It is important to estimate the increased protection in the treated fish to determine the efficacy of an immunostimulant. In this study, after challenge with *Vibrio splendidus*, all treated groups showed a reduced mortality compared to control. The highest RPS was observed in the group treated with *Astragalus membranaceus*, or *Scutellaria baicalensis*, or fructus forsythiae. Among the other groups, fish treated with rhizoma coptidis had

a higher RPS than the fish treated with *Polyporus umbellatus*, *Wolfiporia extensa*, radix glycyrrhizae, flos lonicerae japonicae, isatidis radix, or bupleuri radix. Survival rates of infected fish usually increase after treatment with immunostimulants (Sakai 1999), vaccines (Bakopoulos et al. 2003), or probiotics (Brunt et al. 2007). Feeding large yellow croaker, *Larimichthys crocea* (Richardson, 1846), with glucan reduced mortality after challenging with *Vibrio harveyi* (see Ai et al. 2007). A similar result was reported after feeding *Oreochromis niloticus* with *A. membranaceus*, flos lonicerae japonicae, and boron and subsequent infection with *Aeromonas hydrophila* (see Ardó et al. 2008). Fish began to die on day 1 or 2 in different TCH treated groups and the control after challenged with *V. splendidus*. RPS was the highest in *A. membranaceus*-, or *S. baicalensis*-, or fructus forsythiae treated groups (the fish began to die on day 1 or 2). Groups treated with other TCH had a low RPS, and the day that fish began to die was also on 1 or 2. Thus, we can conclude that the day that fish began to die had no correlation with RPS. This phenomenon can be found in the paper of Yin et al. (2009), who reported that *Cyprinus carpio* L. infected with *Aeromonas hydrophila* started to die after 36 h in the groups fed diets containing *Astragalus radix*, *Ganoderma lucidum*, and the combination of those two.

A relation between the phagocytic tests and the survival in the challenge was evident in the presently reported study. Significant difference ($P < 0.01$) of both the PP activity on day 3 and PI on day 7 can be found in the TCH-enriched diets compared to control. RPS also exhibited significant differences ($P < 0.01$ or $P < 0.05$) when the fish were challenged with *Vibrio splendidus*. This phenomenon may be helpful for animal welfare in future trials.

Feeding a mixture of Chinese herbs may result in a potential probiotic effect in the intestine, this can be confirmed by the higher feed conversion efficiency and lower feed conversion ratio of *Paralichthys olivaceus* fed diets containing TCH, including flos lonicerae japonicae (see Wang et al. 2006). The subsequent increased activity of phagocytic cells in the bloodstream, as well as increased lysozyme activity, can destruct ingested microorganisms, followed by a presumed positive effect of these increased activities.

To summarize, the results of our study showed that *Astragalus membranaceus*, *Scutellaria baicalensis*, and fructus forsythiae extracts alone could significantly enhance leukocyte phagocytic activity of plasma lysozyme activity. The three TCH alone, significantly decreased the mortality, and enhanced the RPS. Thus, it can be concluded that *A. membranaceus*, *S. baicalensis*, and fructus forsythiae extracts can be used as immunostimulants to enhance immune response and disease resistance of cultured red drum.

ACKNOWLEDGEMENTS

This research was supported by science and technology project of the Zhejiang Province (2007F10009), Innovation team construction and talent training project in

the Zhejiang Province (2012F20029), Youth talents project of the Zhejiang Province (2006R20001), Science and technology innovation team construction project of Wenzhou city (C20120004), Project of enrich sea with science and technology in Wenzhou city (S20070051), and Innovation team construction and talent training project in the Zhejiang Province (2009F20009).

REFERENCES

- Abd-El-Rhman A.M.M.** 2009. Antagonism of *Aeromonas hydrophila* by propolis and its effect on the performance of Nile tilapia, *Oreochromis niloticus*. *Fish and Shellfish Immunology* **27** (3): 454–459. DOI: 10.1016/j.fsi.2009.06.015
- Ahmadi K., Banaee M., Vosoghei A.R., Mirvaghefi A.R., Ataimehr B.** 2012. Evaluation of the immunomodulatory effects of silymarin extract (*Silybum marianum*) on some immune parameters of rainbow trout, *Oncorhynchus mykiss* (Actinopterygii: Salmoniformes: Salmonidae) *Acta Ichthyologica et Piscatoria* **42** (2): 113–120. DOI: 10.3750/aip2011.42.2.04
- Ai Q., Mai K., Zhang L., Tan B., Zhang W., Xu W., Li H.** 2007. Effects of dietary β -1, 3 glucan on innate immune response of large yellow croaker, *Pseudosciaena crocea*. *Fish and Shellfish Immunology* **22** (4): 394–402. DOI: 10.1016/j.fsi.2006.06.011
- Anderson D.P.** 1992. Immunostimulants, adjuvants, and vaccine carriers in fish: Applications to aquaculture. *Annual Review of Fish Diseases* **2**: 281–307. DOI: 10.1016/0959-8030(92)90067-8
- Ardó L., Yin G., Xu P., Váradi L., Szigeti G., Jeney Z., Jeney G.** 2008. Chinese herbs (*Astragalus membranaceus* and *Lonicera japonica*) and boron enhance the non-specific immune response of Nile tilapia (*Oreochromis niloticus*) and resistance against *Aeromonas hydrophila*. *Aquaculture* **275** (1–4): 26–33. DOI: 10.1016/j.aquaculture.2007.12.022
- Bakopoulos V., Volpatti D., Gusmani L., Galeotti M., Adams A., Dimitriadis G.J.** 2003. Vaccination trials of sea bass, *Dicentrarchus labrax* (L.), against *Photobacterium damsela* subsp. *piscicida*, using novel vaccine mixtures. *Journal of Fish Diseases* **26** (2): 77–90. DOI: 10.1046/j.1365-2761.2003.00438.x
- Brunt J., Newaj-Fyzul A., Austin B.** 2007. The development of probiotics for the control of multiple bacterial diseases of rainbow trout, *Oncorhynchus mykiss* (Walbaum). *Journal of Fish Diseases* **30** (10): 573–579. DOI: 10.1111/j.1365-2761.2007.00836.x
- Chen X., Wu Z., Yin J., Li L.** 2003. Effects of four species of herbs on immune function of *Carassius auratus gibelio*. *Journal of Fishery Sciences of China* **10** (1): 36–40.
- Diamant A.** 1998. Red drum *Sciaenops ocellatus* (Sciaenidae), a recent introduction to Mediterranean mariculture, is susceptible to *Myxidium leei* (Myxosporidia). *Aquaculture* **162** (1–2): 33–39. DOI: 10.1016/S0044-8486(97)00307-4
- Galina J., Yin G., Ardó L., Jeney Z.** 2009. The use of immunostimulating herbs in fish. An overview of research. *Fish Physiology and Biochemistry* **35** (4): 669–676. DOI: 10.1007/s10695-009-9304-z
- Harikrishnan R., Kim J.-S., Kim M.-C., Balasundaram C., Heo M.-S.** 2011. *Hericium erinaceum* enriched diets enhance the immune response in *Paralichthys olivaceus* and protect from *Philasterides dicentrarchi* infection. *Aquaculture* **318** (1–2): 48–53. DOI: 10.1016/j.aquaculture.2011.04.048
- Huang C.W., Lee T.T., Shih Y.C., Yu B.** 2011. Effects of dietary supplementation of Chinese medicinal herbs on polymorphonuclear neutrophil immune activity and small intestinal morphology in weanling pigs. *Journal of Animal Physiology and Animal Nutrition* **96** (2): 285–294. DOI: 10.1111/j.1439-0396.2011.01151.x
- Jeney G., Galeotti M., Volpatti D., Jeney Z., Anderson D.P.** 1997. Prevention of stress in rainbow trout (*Oncorhynchus mykiss*) fed diets containing different doses of glucan. *Aquaculture* **154** (1): 1–15. DOI: 10.1016/S0044-8486(97)00042-2
- Jeney G., Jeney Z.** 2002. Application of immunostimulants for modulation of the non-specific defense mechanisms in sturgeon hybrid: *Acipenser ruthenus* × *A. baerii*. *Journal of Applied Ichthyology* **18** (4–6): 416–419. DOI: 10.1046/j.1439-0426.2002.00405.x
- Jian J., Wu Z.** 2003. Effects of traditional Chinese medicine on nonspecific immunity and disease resistance of large yellow croaker, *Pseudosciaena crocea* (Richardson). *Aquaculture* **218** (1–4): 1–9. DOI: 10.1016/S0044-8486(02)00192-8
- Lou L., Chen X., Cai X.** 2001. Influence of creat (*Andrographis paniculata*) on the phagocytic activity of phagocytes in grass carp. *Journal of Southwest Agricultural University* **23** (1): 33–34.
- Luo R.** 1997. Induction of immunity substance in *Penaeus chinensis* by Chinese herbal medicine. *Oceanologia et Limnologia Sinica* **28** (6): 577–585.
- Ma A.-m., Yan M.-c., Chang W.-s., Xie Q.-l., Chen S.-b., Shan L.-z?** 2009. Effects of Chinese herbs on the growth and immune function in *Sciaenops ocellatus*. *Marine Sciences* **33** (12): 96–102.
- Neumann N.F., Stafford J.L., Barreda D., Ainsworth A.J., Belosevic M.** 2001. Antimicrobial mechanisms of fish phagocytes and their role in host defense. *Developmental and Comparative Immunology* **25** (8–9): 807–825. DOI: 10.1016/S0145-305X(01)00037-4
- Reed L.J., Muench H.** 1938. A simple method of estimating fifty per cent endpoints. *American Journal of Epidemiology* **27** (3): 493–497.
- Sakai M.** 1999. Current research status of fish immunostimulants. *Aquaculture* **172** (1–2): 63–92. DOI: 10.1016/S0044-8486(98)00436-0
- Seeley K.R., Gillespie P.D., Weeks B.A.** 1990. A simple technique for the rapid spectrophotometric determina-

- tion of phagocytosis by fish macrophages. *Marine Environmental Research* **30** (1): 37–41. DOI: 10.1016/0141-1136(90)90009-D
- Wang J.Q., Sun Y., Zhang J.** 2006. Effects of Chinese herb additives on growth, digestive activity and non-specific immunity in flounder *Paralichthys olivaceus*. *Journal of Fisheries of China* **30** (1): 90–96.
- Wang X., Yan M., Chai X., Wang Q.** 2012. Water decoction of *Astragalus membranaceus* enhances cellular immune response and disease resistance in spotted maigre. *African Journal of Biotechnology* **11** (55): 11851–11856. DOI: 10.5897/AJB12.1375
- Wu C.-C., Liu C.-H., Chang Y.-P., Hsieh S.-L.** 2010. Effects of hot-water extract of *Toona sinensis* on immune response and resistance to *Aeromonas hydrophila* in *Oreochromis mossambicus*. *Fish and Shellfish Immunology* **29** (2): 258–263. DOI: 10.1016/j.fsi.2010.04.021
- Yan M., Chen S., Shan L., Ma A., Xie Q., Dai S., Li S.** 2010. Selection of 22 kinds of Chinese herbal medicines on bacteriostasis in vitro on *Vibrio vulnificus* and *Vibrio splendidus*. *Journal of Hydroecology* **2**: 95–98.
- Yin G., Ardó L., Thompson K.D., Adams A., Jeney Z., Jeney G.** 2009. Chinese herbs (*Astragalus radix* and *Ganoderma lucidum*) enhance immune response of carp, *Cyprinus carpio*, and protection against *Aeromonas hydrophila*. *Fish and Shellfish Immunology* **26** (1): 140–145. DOI: 10.1016/j.fsi.2008.08.015
- Yin G., Jeney G., Racz T., Xu P., Jun X., Jeney Z.** 2006. Effect of two Chinese herbs (*Astragalus radix* and *Scutellaria radix*) on non-specific immune response of tilapia, *Oreochromis niloticus*. *Aquaculture* **253** (1–4): 39–47. DOI: 10.1016/j.aquaculture.2005.06.038
- Zhang G., Gong S., Yu D., Yuan H.** 2009. Propolis and Herba Epimedii extracts enhance the non-specific immune response and disease resistance of Chinese sucker, *Myxocyprinus asiaticus*. *Fish and Shellfish Immunology* **26** (3): 467–472. DOI: 10.1016/j.fsi.2009.01.011

Received: 25 November 2012

Accepted: 7 January 2013

Published electronically: 31 March 2013