Pakistan J. Zool., vol. 51(4), pp 1363-1369, 2019. DOI: http://dx.doi.org/10.17582/journal.pjz/2019.51.4.1363.1369

Houttuynia cordate Thunb Boosts the Non-Specific Immune Response and Enhances Resistance to Edwardsiellosis in the Olive Flounder (*Paralichthys olivaceus*)

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Article Information
Received 23 June 2018
Revised 11 August 2018
Accepted 30 August 2018
Available online 03 May 2019

Authors' Contribution
RL conceived and designed the experiments. XY performed the experiments. FY and SY analyzed the data. HL contributed reagents and materials. FY wrote the article.

Key words
Dietary supplementation, Edwardsiellosis, *Houttuynia cordata* Thunb, Immune enhancing, *Paralichthys olivaceus*.

ABSTRACT

Houttuynia cordata Thunb (HCT) has been widely used as traditional Chinese medicine in human and livestock for its antiviral and antibacterial activities. In this study we are investigating the effect of HCT in aquaculture animals. In this study, 300 olive flounders (*Paralichthys olivaceus*) were stochastically divided into four experimental groups: basal diet feeding group, 0.5% HCT continuous feeding group, 1% HCT feeding group, and 1% HCT interval feeding group. Three replicates were set for each treatment, and 25 fish were stocked on each replicate. The results of continuous feeding experiments showed that the continuous addition of 0.5% and 1% HCT feed for 28 days could very markedly increase peripheral leukocytes count, serum lysozyme activity, phagocytic rate, phagocytic index, respiratory burst activity of head kidney cells, compared with the basal feed group ($P<0.05$ or $P<0.01$). The challenge test results also showed that the 0.5% and 1% HCT feeding had higher immune protection against *Edwarsiella tarda* infection; and the relative protection ratios were 60% and 50%, respectively. It was also obtained that interval feeding experiments also significantly boost non-specific immunological function of *P. olivaceus* compared with continues feeding experiments, HCT application in *P. olivaceus* farming potentially enhances *P. olivaceus* resistance against *E. tarda* infection.

INTRODUCTION

Paralichthys olivaceus (also called olive flounder) is an important species of mariculture fish in China. However, intensive farming has caused continuous outbreaks of diseases, such us notorious streptococcosis, lactococcosis, and edwardsiellosis (Shin *et al*., 2006; Baeck *et al*., 2006; Griffin *et al*., 2013; Park *et al*., 2009; Pérez-Sánchez *et al*., 2011), which severely restricts the development of marine fish breeding industry. At present, the prevention and treatment of *P. olivaceus* disease are still dominated by chemical drugs, and the food safety problems caused are worrying. Immunostimulants have been used in aquaculture in fish, shrimp, and shellfishes. The practice has proven that they can effectively improve the immunity and disease resistance of aquatic animals.

Houttuynia cordata Thunb (HCT) is a member of Saururaceae and has long been used both as an edible vegetable and a kind of traditional Chinese medicine. It is mainly distributed in Asia including India and Korea (Lee *et al*., 2008; Bhattacharyya and Sarma, 2010; Yadav, 2011). HCT has already exhibited a wide range of pharmaceutical activities including anti-inflammatory, antibacterial, antiviral, immunologic, anticancer, and anti-oxidative effects (Yang and Jiang, 2009). Nevertheless, there is insufficient information on HCT application in marine fish, especially *P. olivaceus*. In this experiment, HCT superfine powder mixed with the *P. olivaceus* basal feed. Continuous feeding (feeding for 28 days) and interval feeding (feeding 14d, stopping 14d, feeding 14d again) were used. Then, the number of peripheral leukocytes and the phagocytic rate, phagocytic index, respiratory burst activity of head kidney cells, serum lysozyme activity and *E. tarda* challenge were measured and done. The effects of HCT on the immunity and disease resistance of *P. olivaceus* were investigated.

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0030-9923/2019/0004-1363 $ 9.00/0

Our results would provide a reference for the further application of HCT on *P. olivaceus* farming.

MATERIALS AND METHODS

Bait preparation

Shandong Sansuo Fish Feed Research Center produced and supplied the basic bait for *P. olivaceus*. Experimental HCT was obtained from Dalian Integrated Traditional Chinese and Western Medicine Hospital. After the HCT was superfine grinded, it was uniformly sprayed on the basic bait by mass fractions of 0%, 0.5%, and 1%, dry for 24 h and then stored for further experiments.

Experimental animals and design

The experimental *P. olivaceus* were purchased from Dalian He Sheng Feng Marine Product Farm. The mean weight is 80-100g, and the mean body length is 17.5-20 cm. The experimental fish were reared in a cylindrical water tank (150L) in the North Sea Aquaculture Laboratory of the Ministry of Agriculture of Dalian Ocean University, cultured in running water for 10 days observed. During the test, the water temperature was 13.0°C-19.0°C, and the oxygen was supplied continuously for 24 h. Fish was fed twice a day at 9:00 AM and 18:00 PM, and the feed rate was 3.0% of fish's body weight. Before the feeding, the fish feces were sucked out, and the bait was sucked out half an hour after feeding, and the feeding amount was adjusted in time according to the fish body weight and feeding conditions.

The experimental fish were stochastically divided into four experimental groups, and the basic bait was fed as the control group (Group I). The 0.5% and 1% HCT was fed continuesouly for 28 days to experimental Group II and Group III, respectively. Moreover, fish was administered with 1% HCT interval fed (feeding 14d, stopping 14d, feeding 14d again) as experimental Group IV. The experimental groups II and III were continuously fed for 28 d days, the control group (Group I) and the interval fed group (Group IV) were fed for 42 d. Each of the experimental group and the control group had three parallel groups of 25 fish per group.

Sample collection

These fish, each were collected on 7, 14, 21, and 28 d after the start of the experiment. The blood was collected from the tail vein with a medical syringe (1 mL) after disinfecting the fish body with an alcohol cotton ball. The extracted blood was gently poured into a sterilized test tube and placed on a slant at room temperature for 2 h, then placed in a 4°C freezer overnight. The next morning, tubes were centrifuged at 2000 rpm for 10 min to collect supernatant fluid, which was then sloved at -80°C for follow-up experiment. The sampling method of head kidney cells refers to Secombes (1990) and Lin *et al*. (2011).

Total leukocyte count (TLC)

The number of leukocytes was counted using Neubauer chamber by an optical microscope (Dotta *et al*., 2014).

Phagocytic activity and index

Yeast cells suspension for phagocytic activity detection was prepared, stained with 1% methylene blue, and washed with physiological saline repeatedly to make a 1.2×10^7/mL suspension of bacteria in a refrigerator for use. The 100 µL isolated 1.0×10^7 head kidney cells were taken and inoculated into a centrifuge tube. Then, 100 µL of stained yeast liquid was added dropwise, and incubated at 37°C for 30-60 min in the thermostat. After incubation, the mixed droplets were aspirated with a dropper, covered with a glass slide and observed with a high power microscope, and then calculated the phagocytic percentage (PP) and phagocytic index (PI).

Phagocytic Percentage (PP) =
(The number of cells involved in phagocytosis among every 100 head kidney cells /100) × 100%
Phagocytosis Index (PI) =
Total number of bacteria in phagocytic cells/number of phagocytic cells involved in phagocytosis

Respiratory burst activity

Secombes (1990) was adopted for respiratory burst activity. For this activity 100µL *P. olivaceus* head kidney cells were added to each well of 96 well microtiter plate centrifuge for 5 min, Supernatant was removed and then 100 µL of PMA was added to each well of head kidney cells, placed in water bath and incubated at 37°C for 0.5 h. Then 100 µL of 0.3% NBT was added to each well and incubated at 37°C for 0.5 h. After incubation, the 96-well plate was removed and centrifuged at 400 g for 5 min in a centrifuge. The supernatant was discarded, then methanol was added and fixed for 2 min. Then, it was washed three times with 70% methanol and allowed to air dry. After dried entirely, 120 mL of KOH (2M) and 160 mL of DMSO were added to it. After the complete reaction, the absorbance value was measured by Thermo Scientific Microplate Reader at 630 nm wavelength.

Lysozyme activity

The lysozyme (LSZ) activity was measured according to Ellis (1990).

Table I.- Effects of different concentrations of HCT on blood leukocytes.

Group	No. of leukocytes ($\times 10^7$ cells mL^{-1})					
	7	14	21	28	35	42 (Day)
I	1.95±0.27	1.99±0.13	2.05±0.08	2.03±0.17	2.02±0.14	2.01±0.03
II	2.15±0.23	2.79±0.11**	2.38±0.21*	2.35±0.16*	-	-
III	2.62±0.40**	2.60±0.15**	1.97±0.14	2.31±0.28*	-	-
IV	2.62±0.40**	2.60±0.15**	2.23±0.09*	2.45±0.06*	2.55±0.01**	2.34±0.16*

**, extremely significant difference ($P<0.01$); *, difference ($P<0.05$).

Challenge

After four weeks of feeding, the control group (Group I) and the continuous feeding Groups II and III were used as challenge experiments. The *E. tarda* (stored in our lab, Dalian Key Laboratory of Marine Animal Disease Control and Prevention) was used for the challenge. After amplification culture at 28°C to a bacterial suspension concentration of 2.2×10^8 cells/mL, 200 μL of bacterial suspension was injected intraperitoneally into each fish. Then, the observation was continued for 10 days, mortality and relative percentage survival (RPS) was calculated.

Statistical analysis

The test data obtained were expressed as the mean ± standard deviation (X ± SE), using the SPSS 17.0 analysis of variance, and the Duncan multiple tests. $P<0.05$ is significant difference, $P<0.01$ is the extremely significant difference.

RESULTS

Total leukocyte count (TLC)

Compared with the control group, TLC of *P. olivaceus* increased significantly after adding HCT to the basal diet (Table I). TLC of 0.5% HCT group (Group II) was first increased and then decreased in 28 days. Moreover, 1% HCT group (Group III) showed increasing trend first, decreasing next and then increasing after 28 days. On the 7th day, the TLC in Group II was not significant compared with the control group (Group I); while TLC in group III had significant differences ($P<0.01$). On the 14th day, both Group II and Group III had extremely significant differences with the control Group I ($P<0.01$). The result of interval feeding experiment showed that there was a slight decrease in TLC in 14 d intervals, then the TLC could still return to a higher level in another 14 d of continuous feed.

Phagocytic activity and index

The effect of HTC on the phagocytic percentage (PP) of head kidney cells of the *P. olivaceus* was shown in Figure 1. Within 28 days, the PP of head kidney cells in both Group II and III were significantly higher than that in Group I (control group) ($P<0.01$). Moreover, the interval-feeding group, from 21st day to 42nd day, the PP of head kidney cells in the HCT interval fed Group (IV) was also significantly higher than that of the control group (I) ($P<0.01$) (Fig. 1). Also, head kidney cells of the *P. olivaceus* gradually increased with time going. The phagocytosis index (PI) of Groups II, III, and IV were significantly higher than that in group I (Fig. 2).

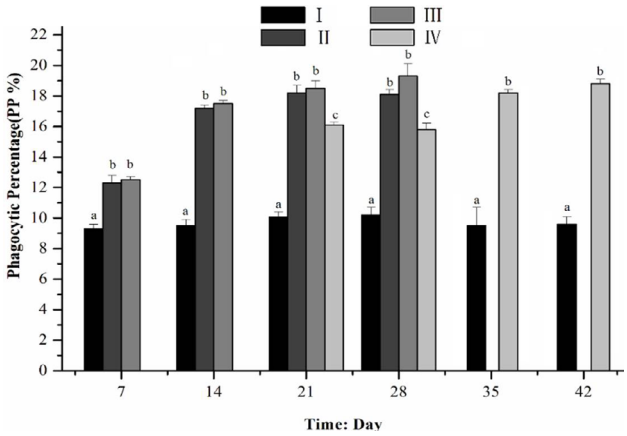

Fig. 1. Effect of HCT on PP of *P. olivaceus* head kidney cells. Data are expressed as mean ± SE. Means in the same column sharing the same superscript letter are not significantly different as determined by Duncan's test ($P < 0.05$).

Respiratory burst activity

Compared with the control group (I), the respiratory burst activity of head kidney cells markedly increased ($P<0.01$) in the experimental group (II, III), and both showed a trend of first increase and then decrease. The activity of respiratory burst in the kidneys of the *P. olivaceus* of Group II reached its maximum on the 21st day, while that of the Group III reached its maximum on the 14th day (Fig. 3). Also, the respiratory burst activity was significantly increased in the interval-fed Group IV compared with the control Group I ($P<0.01$) (Fig. 3).

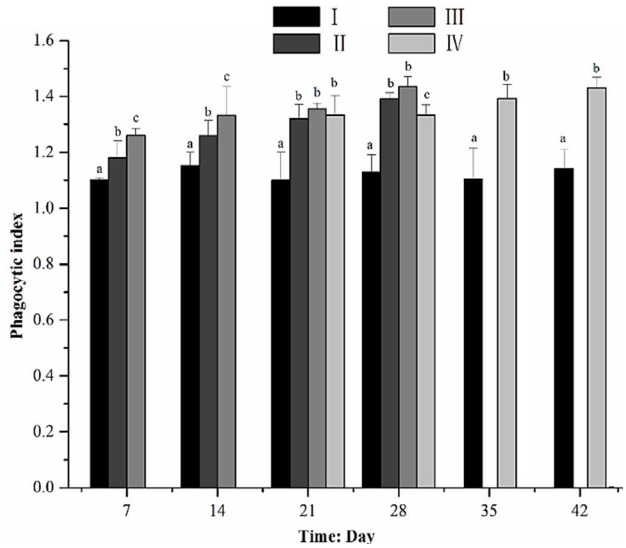

Fig. 2. Effect of HCT on PP of *P. olivaceus* head kidney cells. Data are expressed as mean ± SE. Means in the same column sharing the same superscript letter are not significantly different as determined by Duncan's test ($P < 0.05$).

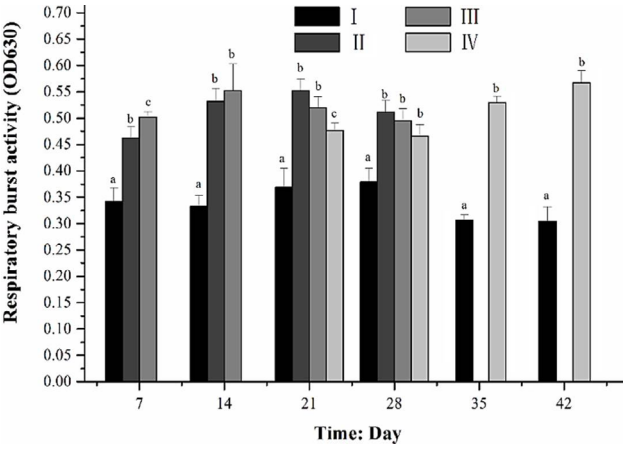

Fig. 3. Effect of HCT on respiratory burst activity of *P. olivaceus* head kidney cells. Data are expressed as mean ± SE. Means in the same column sharing the same superscript letter are not significantly different as determined by Duncan's test ($P < 0.05$).

Lysozyme activity

The lysozyme activity of each experiment showed a trend of increasing first and then decreasing. Compared with the control Group I, the lysozyme activity of the Group II was highest on the 14th day, and the Group III reached the highest on the 21st day. Moreover, interval feeding group (IV group) also showed higher activity than the control group (Fig. 4).

Challenge

The experimental results of *E. tarda* showed that the mortality rate of both groups II and III was significantly reduced; compared with the control group, the relative protection rates (RPS) of group II and group III was as high as 60% and 50%, respectively (Fig. 5).

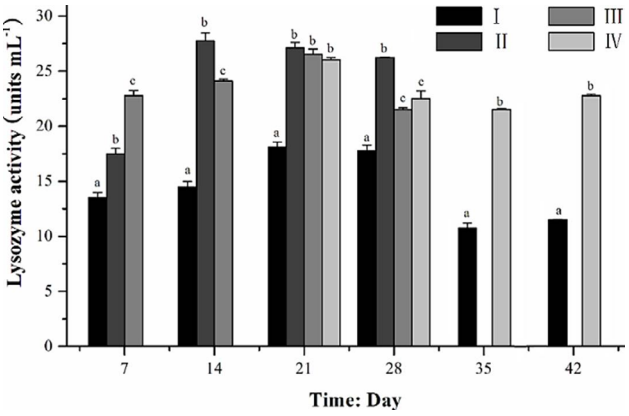

Fig. 4. Effect of HCT on lysozyme activity of *P. olivaceus* serum. Data are expressed as mean ± SE. Means in the same column sharing the same superscript letter are not significantly different as determined by Duncan's test ($P < 0.05$).

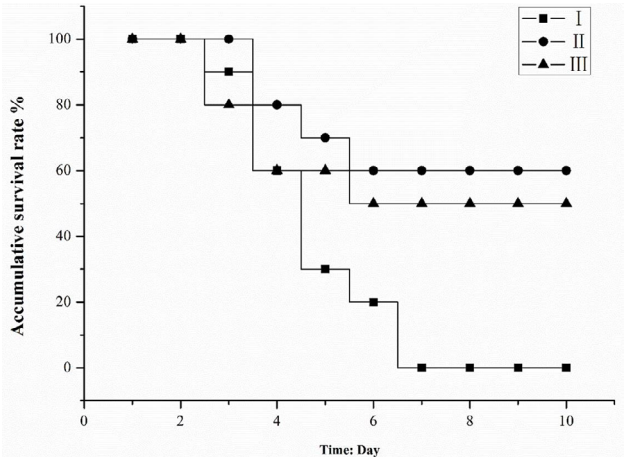

Fig. 5. *Edwarsiella tarda* challenge experiment.

DISCUSSION

Herbs, which have been used as medicine and human immunity intensifier for thousand years in China (Tan and Vanitha, 2004). The use of herbal compounds as immunostimulants has been increasing rapidly in aquaculture to avoid the indiscriminate use of hazardous antibiotics in recent years. To date, it has been proved that administration of herbs can improve the innate and

adaptive immune response of different freshwater or marine fish and shellfish against bacterial, viral and parasitic diseases (Citarasu, 2010; Harikrishnan *et al*., 2011). However, research about HCT as the immune boosters in flounder was very limited. In this study, we proved that the continuous addition of 0.5% and 1% HCT feed for 28 days could markedly boost innate immune response and enhanced resistance after *Edwarsiella tarda* challenge in *Paralichthys olivaceus*. However, the molecular mechanism of HCT's enhancement of *P. olivaceus* resistance is still a mystery that remains to be further unveiled by us. It is also the work we are advancing.

Peripheral leukocytes of fish have an important role in resisting the invasion of pathogenic organisms and maintaining body health. The amount of leukocytes in the blood is also directly related to the strength of the fish against pathogenic organisms. In this study, compared with the control group, the number of white blood cells in the 0.5% HCT group was significantly increased on the 14th and 21st, while the number of leukocytes in the 1% HCT group was significantly increased on the 7th and 14th days ($P<0.05$). This trend of increasing first and then decreasing, and all reached the highest on the 14th day, This is consistent with the findings of Zhou *et al*. (2006), the number of white blood cells in the peripheral blood was significantly increased with different doses of oxymatrine ($P<0.01$), and the number of white blood cells in the blood of each line of allogynogenetic crucian carp (*Carassius auratus*) showed an upward trend. The trend of decreasing after high was the highest on the 15th day.

Lysozyme is a hydrolase widely present in various body fluids, serum, and macrophages of fish. The activity of lysozyme is an important indicator to evaluate the immunity of fish. In this experiment, the serum lysozyme activity of the experimental group showed a trend of increasing first and then decreasing, and all of them reached the highest level on the 21st. The enzyme activity was significantly increased compared to the control group ($P<0.01$). This was compared with the results of Wang *et al*. (2007), who used 2% *Houttuyniae* to feed *Litopenaeus vannamei*. They showed that the lysozyme activity in the 28 days was significantly higher than that in the control group, showing a trend of increasing first and then decreasing, reaching the highest conclusion in the 4th day. Also, Dong and Liu (2012) have studied and reported that, with *Houttuynia* feeding Gold crucian carp for 30 d, the lysozyme activity of head kidney and spleen of experimental group lysozyme activity were significantly higher than the control group ($P<0.05$). This may also be consistent with the conclusion of Muona and Soivio (1992) reported that the addition of Houttuynia results in a significant increase in the number of leukocytes, a significant increase in phagocytosis, and an increase in lysozyme activity.

There is no doubt that the different HCT ingredients were with different biological functions. Many scholars have done much research on the active chemical ingredients and purification process of HCT. Volatile oil of HCT could be as a novel and selective COX-2 inhibitor with anti-inflammatory activity (Li *et al*., 2011), flavonoids exert anti-tumor effects (Fan *et al*., 2008; Xue *et al*., 2013), and chlorogenic acid and its derivatives had an antioxidation effects (Nuengchamnong *et al*., 2009). In this study, we used the whole dry HCT powder as a feed additive to enhance the immune responses in the *P. olivaceus*. Moreover, for this which one of these active ingredients playing an important role was a mystery waiting to be unraveled.

CONCLUSION

In conclusion, compared with the control group, both dietary supplements containing 0.5% and 1% HCT could significantly increase the non-specific immunity and disease resistance of the *P. olivaceus*. Therefore, HCT can be used as an immune enhancer for *P. olivaceus*. It is recommended to add 0.5% or 1% concentration. When HCT was added at a concentration of 1%, the method of interval feeding is more cost-saving and less labor than continuous feeding. Therefore, it is recommended that 1% concentration of HCT be suitable for interval feeding.

ACKNOWLEDGMENTS

Thanks for the financial support of National Natural Science Foundation Project (41706177) and Key Laboratory of Mariculture and Stock Enhancement in North China's Sea Open Project (2018-KF-17) to Dr. Ruijun Li.

Statement of conflict of interest

All authors declare that there is no conflict of interests regarding the publication of the manuscript.

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