Fish and Shellfish Immunology 93 (2019) 116–123

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Full length article

Hesperetin protects crayfish *Procambarus clarkii* against white spot syndrome virus infection

Xiyi Qian, Fei Zhu*

Zhejiang Provincial Engineering Laboratory for Animal Health Inspection and Internet Technology, College of Animal Science and Technology, Zhejiang Agriculture and Forestry University, Hangzhou, 311300, China

ARTICLE INFO

Keywords:
Hesperetin
WSSV
Innate immunity
Procambarus clarkii

ABSTRACT

Hesperetin is a natural flavanone compound, which mainly exists in lemons and oranges, and has potential antiviral and anticancer activities. In this study, hesperetin was used in a crayfish pathogen challenge to discover its effects on the innate immune system of invertebrates. The crayfish *Procambarus clarkii* was used as an experimental model and challenged with white spot syndrome virus (WSSV). Pathogen challenge experiments showed that hesperetin treatment significantly reduced the mortality caused by WSSV infection, while the VP28 copies of WSSV were also reduced. Quantitative reverse transcriptase polymerase chain reaction revealed that hesperetin increased the expression of several innate immune-related genes, including NF-kappaB and C-type lectin. Further analysis showed that hesperetin treatment plays a positive effects on three immune parameters like total hemocyte count, phenoloxidase and superoxide dismutase activity. Nevertheless, whether or not infected with WSSV, hesperetin treatment would significantly increase the hemocyte apoptosis rates in crayfish. These results indicated that hesperetin could regulate the innate immunity of crayfish, and delaying and reducing the mortality after WSSV challenge. Therefore, the present study provided novel insights into the potential therapeutic or preventive functions associated with hesperetin to regulate crayfish immunity and protect crayfish against WSSV infection, provide certain theoretical basis for production practice.

1. Introduction

Procambarus clarkii, commonly known as crayfish, is a species of freshwater crayfish, native to northern Mexico, and southern and southeastern United States [1]. This species had been introduced into many areas of China and had become an important economic species of freshwater aquaculture [2]. With the rapid development of crayfish pond culture and rice shrimp comprehensive breeding industry, the white spot disease of crayfish has become increasingly prominent. White spot disease is one of the most disastrous in shrimp culture, having reduced shrimp production extensively worldwide. Mortality rates are usually very high and cumulative mortality can reach 100% within 3–10 days from the onset of visible gross signs [3,4]. The clinical symptoms of white spot syndrome include loss of appetite, lethargy, and the appearance of white spots on the exoskeleton [5]. White spot disease had been reported from several Asian countries since the early 1990s, and then from South America and from the south-eastern coast of the USA [6–9].

White spot syndrome virus (WSSV) is a rod-shaped crustacean virus with a wide geographic distribution and host range, which belongs to the genus *Whispovirus* of the Nimaviridae family [10,11]. All the major species of cultivated penaeid shrimp can be naturally infected by this virus [6–9]. In addition to penaeid species, both natural and experimental infections have also been reported in caridean shrimp (*Macrobrachium rosenbergii*), crayfish (*Orconectes punctimanus*), wild crabs (*Calappa lophos, Portunus sanguinolentus*, *P. pelagicus*, *Charybdis* sp., *Helice tridens* and *Scylla serrata*), wild lobsters (*Panulirus* sp.), palaemonidian pest shrimp, krill (*Acetes* sp.), planktonic copepods and pupae of an ephydridian insect [12–14]. WSSV replicates in the host cells after infecting the host. Virus could not be eliminated by conventional drugs without damaging the host cells, there is no specific treatment for the viral disease. During the breeding process, disease prevention and control measures are mostly based on prevention. Once the white spot disease breaks out, the crayfish's mortality rate is extremely high, which leads to the farmers' blood loss [5]. Therefore, there is an urgent need to develop a therapeutic drug that is less harmful to crayfish cells and could effectively reduce the mortality rate after infected WSSV.

By consulting a large amount of literature, we found that hesperetin has multiple effects, such as anti-inflammatory, anti-cancer, anti-virus and so on. Hesperetin is a type of natural flavonoid that occurs mainly

* Corresponding author.
E-mail addresses: zhufei@zju.edu.cn, 20110050@zafu.edu.cn (F. Zhu).

https://doi.org/10.1016/j.fsi.2019.07.029
Received 21 May 2019; Received in revised form 5 July 2019; Accepted 11 July 2019
Available online 11 July 2019

in the form of glycoside hesperidin, which is the main flavonoid in lemons and oranges. It is known that hesperetin has an anti-oxidation effect. It was selected as a biological antioxidant to protect osteoblasts and was used as an auxiliary preparation for melanin in the treatment of type I diabetic bone disease [15]. Additional, hesperetin has an anti-inflammatory effect and can reduce the production of sebum, thereby effectively inhibiting the growth of acne. Hesperetin can also be used to protect the cardiovascular, protect the nerves, and reduce triglyceride content in the liver [16–19]. Hesperetin has an anti-cancer effect, and citrus flavonoid hesperetin and naringin have obvious inhibitory effects on the proliferation of human breast cancer cells in vitro [20]. Most importantly, hesperetin has antiviral properties, and has significant antiviral activity against the Chikungunya virus (CHIKV), reducing CHIKV replication efficiency and down-regulating the production of viral proteins involved in replication [21]. Hesperetin can reduce intracellular replication of herpes simplex virus type 1 (HSV-1), poliovirus type 1, parainfluenza virus type 3 (Pf-3), and respiratory syncytial virus (RSV) [22]. Hesperetin showed its inhibitory activity on Sindbis virus infection in vitro and were not cytotoxic on Baby Hamster cells 21 clone 15 (BHK-21) [23]. The intake of hesperetin in laying hens diet may exhibit positive effects and increased the ratio of yolk weight/egg weight and the blood serum superoxide dismutase (SOD) activity [24]. Based on this, we would explore whether hesperetin could effectively inhibit viral replication and reduce mortality in crayfish.

To elucidate whether hesperetin could be used as an effective antiviral additive for crayfish, we detecting the immunological indicators before and after treatment with hesperetin, as well as the immunological activity, VP28 copies of WSSV, mortality and apoptosis in crayfish after WSSV infection. In the present study, the results indicated that hesperetin could regulate the innate immunity, inhibit viral replication and reducing mortality of crayfish.

2. Materials and methods

2.1. Crayfish, hesperetin and pathogens preparation

Healthy crayfish (approximately 15 g and 10 cm each) were purchased from a crayfish breeding case in Wenzhou, China. In order to adapt the crayfish to the new environment, the crayfish were bred for a week before the experiment, they were kept in tanks with fresh water and fed with commercial pellet feed at 5% of body weight per day, the room temperature and water temperature were set at 25 °C constantly. The body weight of randomly selected individuals was recorded to calculate the average crayfish weight. Hemolymph and gill tissues from cultured crayfish were subjected at random to PCR detection with WSSV-specific primers to ensure that the crayfish were WSSV-free. Hesperetin (purity ≥ 98%) was purchased from Shanghai Yuanye Biotechnology Co. Ltd. (Shanghai, China). The commercial pellet feed is specifically used for crayfish. The preparation method of hesperetin additive feed is as follows: firstly, commercial pellet feed is crushed by disintegrator; secondly, crushed pellet feed is mixed with hesperetin in a certain proportion, added super-pure water to knead into 'dough', which is formed in dough press; finally, that is dried in an oven (40 °C). Oral use, hesperetin was added to commercial pellet feed for crayfish at a dose of 40, 50, and 60 mg/kg, stored at room temperature and kept dry.

The virus isolate, WSSV, used in this study originated from infected crayfish and was reserved at −80 °C as described previously [25]. 10 crayfish were randomly selected for WSSV challenge from the tanks, which were infected with WSSV. Prepared WSSV extract was diluted with sterilized PBS to a density of 1×10^5 WSSV copies per mL, each crayfish would receive 100 μL of diluted injection. Approximately four or five days later, crayfish infected with WSSV started to display various clinical signs including lethargy, reduced food consumption, reduced preening activities, loosening of the cuticle, and discoloration of the hepatopancreas. Before the crayfish died, partial appendages of each crayfish were taken for virus testing, and then the infected crayfish were stored in an ultra-low temperature freezer. In the virus challenge experiment mentioned in this article, we took out the infected crayfish from the ultra-low temperature freezer, thawed it, took its muscles, and made the meat batter. In manner of feeding attack, each crayfish was fed with minced meat, which was about the size of rice grains.

2.2. Hesperetin treatment and pathogen challenge

Crayfish were randomly transferred into separate tanks as different groups. In the hesperetin treatment experiment, crayfish were either fed with hesperetin additive feed every 24 h as the hesperetin group or commercial pellet feed as the control group. To determine the influence of hesperetin on crayfish innate immune signal pathways, the hemolymph of each group was collected 24 h post feeding to analyze gene expression. To determine the influence of hesperetin in the pathogen challenge process, after 72 h of hesperetin treatment, crayfish in each group were fed with minced meat of WSSV-infected crayfish. After the feeding, crayfish fed commercial pellet feed only, 40 mg/kg hesperetin additive feed only, 50 mg/kg hesperetin additive feed only, 60 mg/kg hesperetin additive feed only, commercial pellet feed for crayfish + minced meat, 40 mg/kg hesperetin additive feed + minced meat, 50 mg/kg hesperetin additive feed + minced meat, 60 mg/kg hesperetin additive feed + minced meat were placed back into separated tanks for further research. Crayfish fed with commercial pellet feed alone were defined as the control group and those fed with 40, 50, or 60 mg/kg hesperetin additive feed alone were defined as the 40 mg/kg hesperetin, 50 mg/kg hesperetin, or 60 mg/kg hesperetin groups, respectively. Crayfish fed with commercial pellet feed and minced meat were defined as the WSSV group. Crayfish fed with either 40, 50, or 60 mg/kg hesperetin additive feed and minced meat were defined as the WSSV + 40 mg/kg hesperetin, WSSV + 50 mg/kg hesperetin, or the WSSV + 60 mg/kg hesperetin groups, respectively. Based on the requirements of different analysis, crayfish samples were collected at different times after hesperetin or pathogen treatment.

To analyze mortality, eight groups of crayfish were kept in the tanks for 12 days and the populations were counted every 24 h. Dead crayfish were removed immediately, and aquatic water was replaced with clean water every day. To ensure the statistical accuracy in mortality analysis, every group contained 20 individuals. The mortality data was arranged and analyzed in Microsoft GraphPad 5.0. In the analysis of WSSV copies, three crayfish (as a technical repeat) from the control or WSSV challenge groups were collected at 24, 48, 72, and 96 h post-challenge with WSSV. In immune parameter analyze, at least three crayfish (as technical repeats) from each group were collected at 24 and 48 h after the challenge with pathogens. Experiments described above were all repeated three times.

2.3. WSSV replication analysis

The detection of WSSV copies was based on previous studies [26,27]. Briefly, DNA was extracted from the crayfish hemocytes of each sample, and TaqMan real-time quantitative PCR was performed. WSSV protein VP28-specific primers (5′-TTGGTTTCAGCCCGAGATT-3′ and 5′-CCTTGGTCAGCCCCTTGA-3′) and TaqMan fluorogenic probe (5′-FAM-TGCTGCCGTCTCCAA-TAMRA-3′) were applied. Thermal cycling was performed on an iCycle IQ5 real-time PCR detection system (Bio-RAD, USA).

2.4. Expression analysis by real-time quantitative PCR

Crayfish hemocytes were collected from each group 24 h after feeding. Total RNA was extracted from the hemocytes using an RNApure Tissue & Cell kit (CWBIO, China) according to the manufacturer's protocol. In total, 200 ng RNA was applied to cDNA reverse transcription using a ReverTra Ace qPCR RT Master Mix with a gDNA

Table 1
Real-time quantitative PCR primer sequences of immune signal pathways related genes in the hemocytes of *Procambarus clarkii*.

Primer Name	Primer Seqeunce (5′ to 3′)
β-Actin-F	ACCACTGCCGCCTCATCCTC
β-Actin-R	CGGAACCTCTCGTTGCCAATGG
Toll-like receptor-F	TTGCGTAGTGACTTGTGGAGC
Toll-like receptor-R	CTACTGTAACGCAGGCGATGG
NF-appa B–F	TAGTGCGTGATGATGGGTCTT
NF-kappa B-R	GCTGATTATGGAGGCAGAAAA
crustin 1-F	CCACAGATGGCAATCGGAGTC
crustin 1-R	AGGGAACGAACGCTGGAAAGT
C-type-lectin-F	ACTTTGCTAACGCCAATCCAC
C-type-lectin-R	CTACGCTGTCATCGACGAACC

Remover kit (Toyobo, Japan). The cDNA of each group was applied to SYBR Green real-time quantitative PCR immediately. A two-step RT-qPCR method was performed using a Bio-Rad Two-Color Real-Time PCR Detection System. The gene expression level was calculated with the $2^{-\Delta\Delta CT}$ method [28], the amplification cycle of β-actin was used as an internal control to calculate the relative expression level. Expression levels of genes of the control group were used as index 1.

Four innate immune pathway-related genes, Toll-like receptor (KP259728.1), NF-kappa B (KF662471.1), crustin 1 (GQ301201.1), and C-type-lectin (KC857544.1), were selected to predict the potential influence of hesperetin treatment on the innate immune system. The design and synthesis of the RT-qPCR primers were entrusted to the Generay Shanghai Company. The primer sequences are listed in Table 1.

2.5. Total hemocyte count, phenoloxidase activity, and superoxide dismutase activity

Three immune parameters, total hemocyte count (THC), phenoloxidase (PO) activity, and superoxide dismutase (SOD) activity were used to analyze the condition and immunity of the experimental crayfish. At 24 and 48 h post-pathogen challenge, crayfish hemolymph was collected from each group and combined 1:1 with an anticoagulant (20 mM EDTA, pH 5.6), the hemolymph and anticoagulant mixtures were kept on ice constantly. To determine total hemocyte count, hemolymph (100 μl) was withdrawn from the ventral sinus of individual crab into a 1 ml syringe containing 100 μl of 10% Methanal in 0.45 M NaCl and transferred to a microfuge tube. The hemocyte count was performed using a hemocytometer and defined as number of cells ml^{-1}, and the data presented as the total hemocyte count [29]. PO activity was quantified in the hemolymph mixture based on the formation of dopa chrome from the substrate L-3, 4-dihydroxyphenylalanine (L-DOPA), as described previously [30]. SOD activity was quantified in hemocytes isolated from 300 μL of the hemolymph mixture, according to the improved method described by Beauchamp and Fridovich [31]. Data were presented as measurements.

2.6. Apoptosis analysis

An apoptosis assay was conducted using Annexin V (Invitrogen, USA) according to an optimized method based on the manufacturer's protocol. It first verified that whether hesperetin affects crayfish cell apoptosis. The apoptosis rate of the control group and the 50 mg/kg hesperetin group was measured after feeding the commercial pellet feed and hesperetin additive feed for 24 h. Secondly, the apoptosis of the crayfish infected with WSSV after hesperetin treatment was confirmed. After feeding commercial pellet feed and hesperetin additive feed for 72 h, the apoptosis rate of WSSV group and WSSV +50 mg/kg hesperetin group was detected after WSSV challenge for 24 h. At 24 h post pathogen challenge, the hemolymph was drawn using 2 mL syringe with 20 mM of EDTA at a ratio of 1:1 and kept on ice. Samples were centrifuged at 800 × *g* for 5 min at 4 °C to collect hemocytes. After washing with sterilized PBS, counted and adjusted to a density of 3–5 × 10^6 cells/ml. hemocytes were resuspended in ice-cooled 1 × binding buffer with Annexin-V FITC and propidium iodide (PI), and incubated at room temperature for 15 min to stain. After staining, the samples were centrifuged to remove residual dye and applied to flow cytometry immediately to avoid cell death. The empty control, negative control, and positive control for threshold values were prepared simultaneously with experimental samples. The data were presented as means ± standard deviation (SD) derived from three independent experiments.

2.7. Statistical analysis

Quantitative data were expressed as mean ± standard deviation (SD). Data from three independent experiments were analyzed by one-way analysis of variance to calculate the means and standard deviations of the triplicate assays. Statistical differences were estimated using one-way ANOVA followed by least-significant differences (LSD) and Duncan's multiple range test. All statistics were measured using SPSS software version 19 (IBM, USA). And the level of 0.01 was used to indicate statistical significance ($P < 0.01$). All graphs were made using Microsoft GraphPad 5.0.

3. Results

3.1. Effects of hesperetin on the cumulative mortality of pathogen-challenged crayfish

Crayfish mortality and viral copy number were assessed to demonstrate the immunity of different groups. Under the experimental conditions, there was no death in the control and hesperetin only group (40, 50, and 60 mg/kg hesperetin; data not shown). In the WSSV challenge experiments, crayfish death was observed at 24 h in all groups. From day 4 to day 10, crayfish mortality in the WSSV group increased rapidly to 94.667% ($p = 0.0032$), and at day 12 after infection, crayfish mortality in the WSSV group reached 100%. When successively treated with 40 or 60 mg/kg hesperetin and WSSV, crayfish mortality was higher than that of 50 mg/kg hesperetin and WSSV-infected crayfish. Especially after 10 days, the mortality of 50 mg/kg hesperetin and WSSV group showed a significant difference from that observed in the control group ($p = 0.0032$), and the mortality reached only 55% (Fig. 1). A follow-up study with a 50 mg/kg hesperetin dose group was conducted based on the results of the crayfish mortality.

3.2. Effects of hesperetin on the WSSV copy number

The sampling time of the WSSV copy number experiment was determined by the crayfish mortality rate, which began on the 5th day after the pathogen challenge. Therefore, the sampling time was set at 0, 24, 48, 72 and 96 h. The WSSV copy number after WSSV treatment of each group reached 1 × 10^7 in 96 h. The WSSV copy number of the WSSV group was higher than that of WSSV +50 mg/kg hesperetin group at 24, 48 and 72 h. We suggest that hesperetin might have a certain inhibitory effect on viral replication, but the inhibition of crayfish mortality may not be achieved by the inhibition of the WSSV (Fig. 2).

3.3. Effects of hesperetin on the expression of immune pathway genes

Real-time reverse transcription polymerase chain reaction (RT-PCR) analysis of four immune-related genes showed that the expression of Toll-like receptor, crustin 1, NF-kappa B and C-type-lectin were down-regulated/up-regulated following treatment with 50 mg/kg hesperetin when compared with the control group ($p < 0.05$) (Fig. 3). These results suggested that hesperetin might induce certain immune pathways.

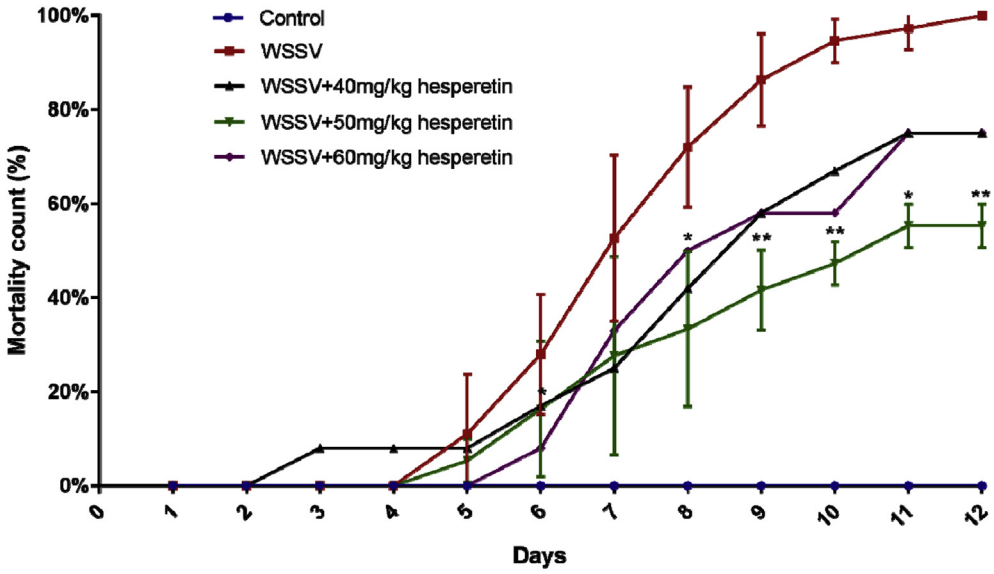

Fig. 1. Cumulative mortality of pathogen-challenged crayfish treated with/without hesperetin. Crayfish were treated with either formula feed, minced meat with WSSV, or WSSV + hesperetin. The formula feed only group was defined as the control. Each group contained at least 15 individuals. The mortality of crayfish in each group was recorded continuously once a day for 12 days. To represent mortality variation more clearly, hesperetin only group is not shown in the figure. The experiment was repeated three times.

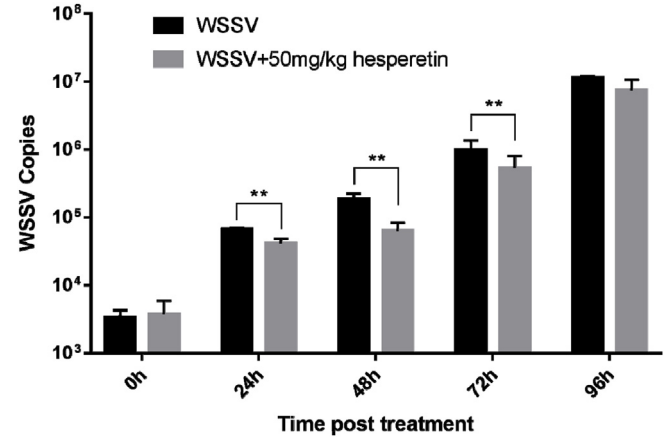

Fig. 2. WSSV copies were detected at 0, 24, 48, 72 and 96 h after the WSSV challenge. The experiment was repeated three times. Data are presented as the mean value with standard deviation. Asterisks mark the significance of difference between the pathogen group and pathogen + hesperetin group (* = P < 0.05, ** = P < 0.01), analyzed by a multiple *t*-test.

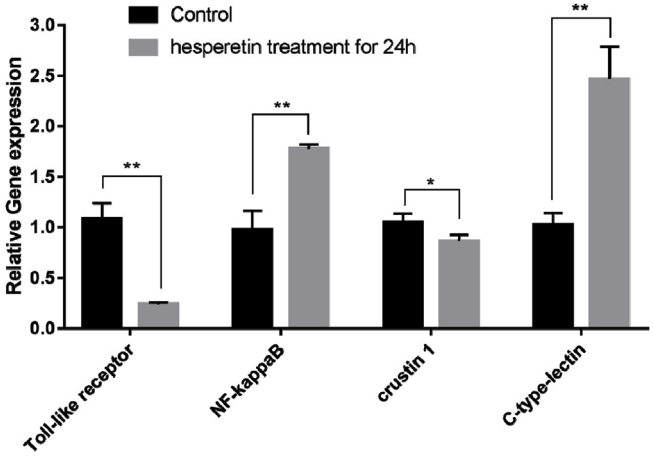

Fig. 3. Real-time quantitative PCR analysis of immune gene expression. The expression of four immune-related genes (Toll-like receptor, NF-kappaB, crustin1 and C-type-lectin) in the hemocytes of *Procambarus clarkii* were detected 24 h post 50 mg/kg hesperetin treatment. The amount of gene mRNA was normalized to the β-actin transcript level. These data are shown as means ± standard deviation of three separate individuals. The asterisks indicate a significant difference between 50 mg/kg hesperetin group and the control group when analyzed by a multiple *t*-test (* = P < 0.05, ** = P < 0.01).

3.4. Effects of hesperetin on immunological parameters

THC, PO activity, and SOD activity were measured at 24 and 48 h in the WSSV group, WSSV +50 mg/kg hesperetin group, control group, and 50 mg/kg hesperetin group. There was no significant difference in the THC of crayfish between the 50 mg/kg hesperetin group and the control group at 24 and 48 h (p = 0.162 at 24 h; p = 0.303 at 48 h) (Fig. 4a), and after the WSSV infection. The THC of the WSSV group was significantly higher than the control group at 48 h. When hesperetin was applied, crayfish THC increased to 1.41×10^6/mL at 24 h and 1.27×10^6/mL at 48 h, which were both higher than levels observed in the WSSV-infected groups (p = 0.0014 at 24 h; p = 0.0029 at 48 h) (Fig. 4b).

PO activity of 50 mg/kg hesperetin showed no difference when compared with that observed in the control group at 24 (0.8176 U) and 48 h (1.0598 U) (p = 0.3397 at 24 h; p = 0.1453 at 48 h) (Fig. 4c). In WSSV challenge experiments, PO activity decreased (0.5091 U) nearly 2-fold at 24 h (p < 0.0001), while PO activity increased at 48 h, but was still significantly lower than in the control group (p = 0.0055). When hesperetin was applied in WSSV challenge experiments, PO activity was significantly higher than that in the WSSV group (p = 0.0055 at 24 h; p = 0.0129 at 48 h) (Fig. 4d).

There was no significant difference in SOD activity between the 50 mg/kg hesperetin group at 24 h (1.1084 U) and the control group (1.0924 U). In addition, there was no significant difference in SOD activity at 48 h between the 50 mg/kg hesperetin group (1.2821 U) and the control group (1.2707 U) (p = 0.3911 at 24 h; p = 0.5304 at 48 h) (Fig. 4e). In the WSSV challenge experiment, there was no difference between the control group at 24 h (p = 0.3253), and the SOD activity decreased slightly from 1.0834 U to 1.0429 U at 48 h. When hesperetin was applied in the challenged groups, the SOD activity increased slightly in the WSSV challenge experiment, increasing to 1.1493 U at 24 h (p = 0.0386) and increasing to 1.0972 U at 48 h (p = 0.0244) (Fig. 4f).

3.5. Effects of hesperetin on the apoptosis of hemocytes

The hemocyte apoptosis rates in the presence or absence of 50 mg/

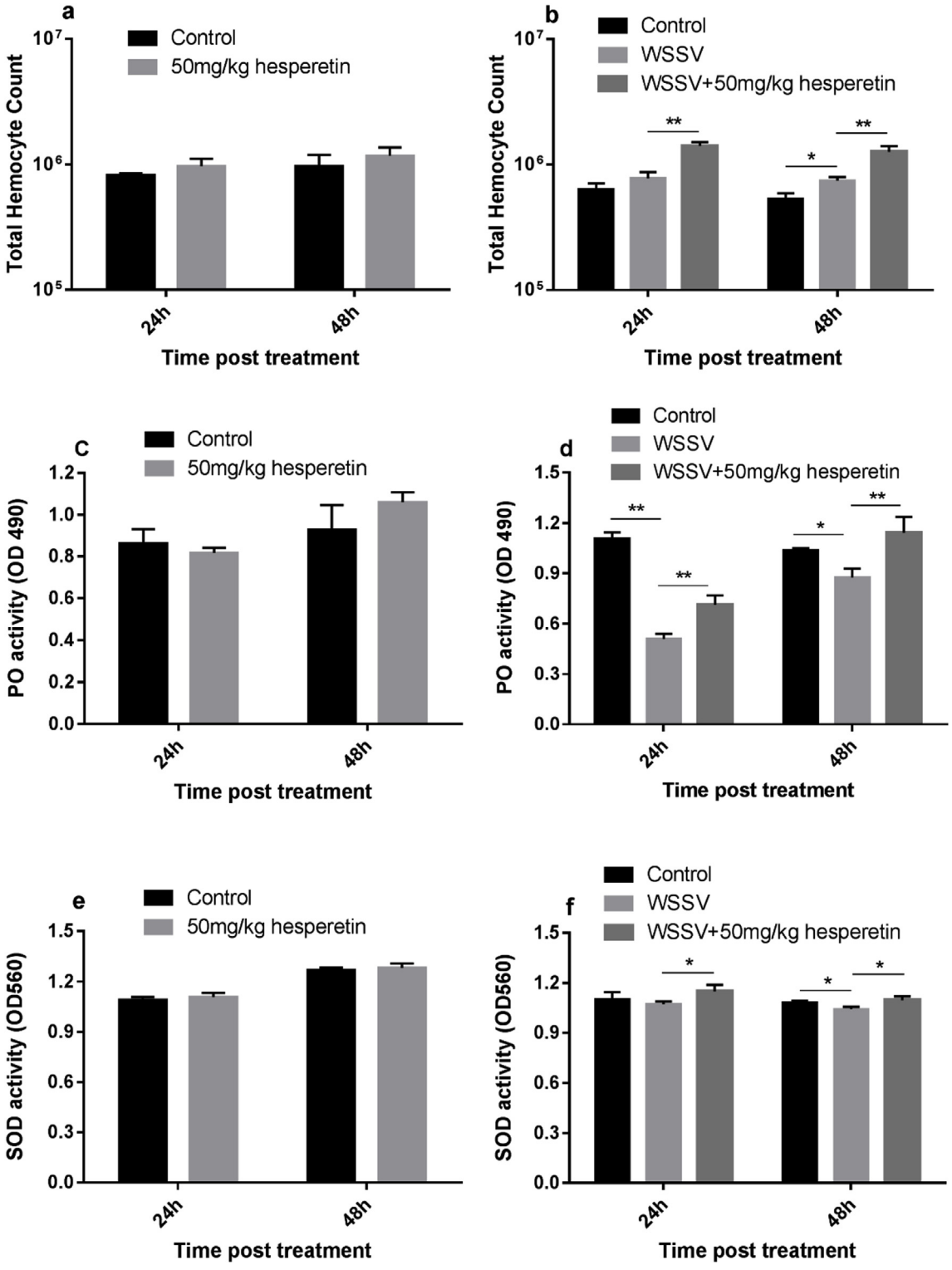

Fig. 4. The immune-related parameters measured in the challenge experiment. Crayfish were treated with formula feed (control), 50 mg/kg hesperetin, WSSV, or WSSV and 50 mg/kg hesperetin to compare the differences of three immune-related parameters, total hemocyte count (THC), phenoloxidase (PO) activity, and superoxide dismutase (SOD) activity before and after WSSV challenges, and the influence of hesperetin on crayfish immunity. For the co-treatment of WSSV and hesperetin, crayfish were challenged with WSSV 72 h after 50 mg/kg hesperetin treatment, that is, minced meat carrying WSSV was used instead of 50 mg/kg hesperetin. The variation of 50 mg/kg hesperetin on THC (a), proPO activity (c), and SOD activity (e) showed the influence of hesperetin treatment on healthy crayfish. (b), (d), and (f) show the influence of hesperetin on the WSSV infection process. Each treatment at each time point contained at least three crayfish individuals, data are represented by mean values and standard deviation. Significant differences are represented with asterisks (* = $P < 0.05$, ** = $P < 0.01$), and analyzed by a multiple *t*-test.

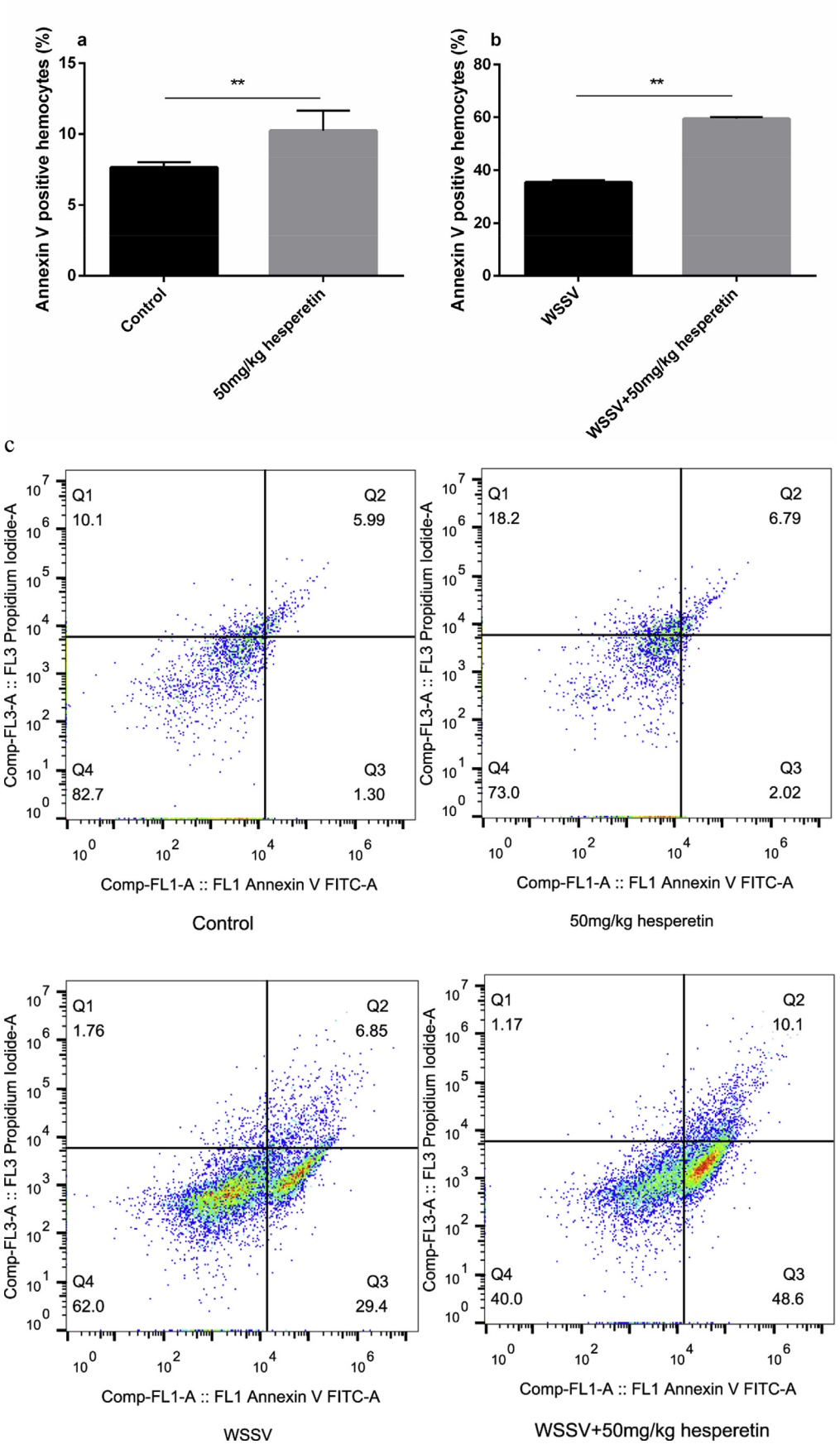

Fig. 5. Apoptosis analysis of hemocytes in hesperetin treated crayfish by flow cytometry. Crayfish were treated with formula feed or 50 mg/kg hesperetin, followed by a pathogen challenge of WSSV. At 24 h post pathogen challenge, the hemocyte samples of different treatments were collected and stained with Annexin-V FITC and propidium iodide (PI) for flow cytometry detection. Fluorescent 1-Annexin V (FL1-A5) represents apoptosis cells. Fluorescent 3-PI (FL3-PI) represents dead or damaged cells. The determination of the threshold was based on the empty control, negative control, and positive control. The percentage of Annexin V positive cells represents the apoptosis rate of the hemocyte sample. The apoptosis rate of control and hesperetin control (a), each column represents the mean value of three isolated repeats. (b) Apoptosis rate of challenged groups. Scatter plots of one of the challenged groups (c), Q1 represents cell fragments caused by centrifuge and resuspension, Q2 represents the late stage apoptosis, Q3 represents early stage apoptosis, Q4 represents normal cells. The apoptosis rate of the sample was determined by the total fluorescent intensity of Q2 and Q3.

kg hesperetin treatment were detected using flow cytometry. The apoptosis rate in the control group was approximately 7.645%, and the apoptotic rate of the 50 mg/kg hesperetin group was 10.23% (Fig. 5a), which was significantly higher than that of the control group ($p = 0.0377$), indicating that the hesperetin was cytotoxic to the blood cells of the crayfish. In these experiments, the treated crayfish blood cells were very fragile. Due to centrifugation, liquid impact associated with pipetting, and subsequent resuspension. We attempted to do our best to reduce the damage from external sources. In the WSSV-infected groups, the apoptosis rate increased to an average of 35.45%, and upon hesperetin treatment, this rate increased to 59.35% and showed significant variation from the WSSV group ($p < 0.0001$) (Fig. 5b).

4. Discussion

To protect the host from virus invasion, the host gradually evolves a series of antiviral mechanisms in response to virus attack. For crustaceans, the innate immunity is an important way to protect them from viral infection. In the present study, the results directly indicated that hesperetin can regulate the innate immune system of crayfish. Although hesperetin had been shown to influence the process of multiple viral infection, such as inhibited cleavage activity of the 3 C-like protease, which is the SARS-coronavirus 3C-like protease that mediates the proteolytic processing of replicase polypeptides 1a and 1b into functional proteins [32], inhibited enterovirus 17 infection [33], inhibited influenza A virus replication and so on [34]. This article firstly explored the possibility of hesperetin to resist WSSV infection, which is the main pathogen for most crustaceans. Based on the results of mortality, the mortality rate of hesperetin treatment groups was significantly lower than that of un-treated group, and the best dosage of hesperetin was 50 mg/kg. This result indicated that hesperetin could effectively reduce the mortality of crayfish after WSSV infection. The results of WSSV copy number showed that hesperetin could effectively inhibit viral replication, maybe by affecting the innate immunity of crayfish. Moreover, after feeding 50 mg/kg hesperetin additive for 24 h, we detected the expression levels of four important immune genes in crayfish, and found that the expression levels of Toll-like receptor and crustin 1 were significantly down-regulated, but the expression levels of NF-kappaB and C-type-lectin were significantly up-regulated. Proteins containing a C-type lectin domain have multiple functions including cell-cell adhesion, immune response to pathogens, and apoptosis [35,36]. C-type lectin plays a central role in host defense, and many members of this family are involved in viral infection, primarily by identifying virions and affecting their entry into host cells. C-type lectin, LvCTL1, in *Litopenaeus vannamei* has been shown to protect shrimps from WSSV in vitro and in vivo, and protection may be related to the interaction of LvCTL1 with several viral proteins [37]. The knockdown of crustin-like through RNAi inhibits WSSV replication in shrimp, and the mortality, WSSV copy number and expressions of WSSV immediate early genes (IE1, IE2, DNA polymerase, VP28) were both decreased [38]. To discover the effect of hesperetin on the innate immunity of crayfish, the immunological activity of crayfish fed hesperetin before and after WSSV challenge was tested.

THC is the total number of hemocytes in crayfish. Hemocyte is a very important immune tissue in the innate immunity of crustaceans. It is involved in cellular immunity and humoral immunity, and can directly recognize and engulf foreign pathogens. The results showed that hesperetin treatment significantly increased the total hemolymph counts of crayfish after the WSSV challenge. After treatment with hesperetin, PO activity was significantly up-regulated at 48 h, and pathogen challenge experiments showed that PO activity in crayfish treated with hesperetin for 48 h was also increased, indicating that hesperetin treatment promoted the expression of the proPO non-self-identification system. PO is the terminal enzyme of the proPO pathway, which converts tyrosine to dihydroxyphenylalanine (DOPA) and then to dopaquinone and melanin synthesis [39]. PO activity is very important for crustacean innate immune defense against invading pathogens [40]. In the WSSV challenge experiment, the SOD activity of the 50 mg/kg hesperetin group was higher than the WSSV group, indicated that hesperetin treatment significantly increased the SOD activity, which is very important for the stability and health of crayfish. Reactive oxygen species (ROS) released during the innate immune response can destroy bacteria or viruses; however, large amounts of ROS can also attack host proteins, lipids, and DNA nonspecifically, leading to cell damage [41]. To avoid ROS self-injury, SOD is activated as part of the antioxidant defense to remove superoxide anions and maintain homeostasis during the immune response [42]. The above results indicated that hesperetin seemed to reduce crayfish mortality mainly by enhancing innate immunity, but did not directly act on WSSV, although it was reported to inhibit viral replication.

It is well known that innate immunity plays an essential role in immune responses against viral infection in which apoptosis and phagocytosis are two important components [43]. During viral infection, a series of signaling pathways can be triggered in the host, leading to apoptosis of infected cells [44,45]. Apoptosis is a process of cell death caused by pre-existing death procedures triggered by internal and external factors, and it is also called programmed cell death [46]. NF-kappaB is closely related to apoptosis, which is involved in the transcriptional regulation of a variety of apoptosis-related genes and has a two-way effect of inhibiting and promoting apoptosis [47]. Whether or not infected with WSSV, hesperetin increase a little on the total hemocyte counts, and significantly increase the hemocyte apoptosis rates in crayfish. Due to the complexity of crayfish immune system, hesperetin may promote hemocyte apoptosis but have no cytotoxic to crayfish hemocytes. The apoptosis rate of the WSSV +50 mg/kg hesperetin group was significantly higher than that of the WSSV group, which may be related to the up-regulation of the NF-kappaB gene, and the WSSV copy number during this period is directly and effectively suppressed. These results showed that hesperetin treatment significantly increased apoptosis. This finding revealed that hesperetin may be an enhancer of apoptosis, although it could restrain mortality. In the study of hesperetin against CHIKV virus, hesperetin can effectively inhibit the replication of CHIKV in host cells through elicit the inhibitory effect against the activity of *Rluc* marker expressed by CHIKV [21]. In this study, according to the virus copy number, hesperetin can effectively inhibit the replication of WSSV in crayfish cells. Iin this study, hesperetin treatment has an impact on some immune parameters, and inhibiting WSSV replication maybe by regulating apoptosis.

In conclusion, our results showed that hesperetin treatment promoted the innate immunity of crayfish, especially the cellular immunity following viral infection, and the activity of several innate immune pathways. Additionally, hesperetin treatment inhibited pathogen replication and reduced mortality.

Acknowledgments

This work was financially supported by National Key Research and Development Program of China (2018YFD0500300) and Qianjiang talent program (QJD1602023).

References

[1] D.J. Harris, K.A. Crandall, Intragenomic variation within ITS1 and ITS2 of freshwater crayfishes (Decapoda: Cambaridae): implications for phylogenetic and microsatellite studies, Mol. Biol. Evol. 17 (2000) 284–291.
[2] D.M. Holdich, A review of astaciculture: freshwater crayfish farming, Aquat. Living Resour. 6 (1993) 307–317.
[3] H.Y. Chou, C.Y. Huang, C.H. Wang, H.C. Chiang, C.F. Lo, Pathogenicity of a baculovirus infection causing white spot syndrome in cultured penaeid shrimp in Taiwan, Dis. Aquat. Org. 23 (1995) 165–173.
[4] C. Wongteerasupaya, J.E. Vickers, S. Sriurairatana, G.L. Nash, P. Kanchanaphum, V. Boonsaeng, et al., A non-occluded, systemic baculovirus that occurs in cells of ectodermal and mesodermal origin and causes high mortality in the black tiger prawn *Penaeus monodon*, Dis. Aquat. Org. 21 (1995) 69–77.

[5] B. Verbruggen, L. Bickley, R. van Aerle, K.S. Bateman, G.D. Stentiford, E.M. Santos, et al., Molecular mechanisms of white spot syndrome virus infection and perspectives on treatments, Viruses 8 (2016) 1–30.
[6] K. Inouye, S. Miwa, N. Oseko, H. Nakano, T. Kimura, K. Momoyama, et al., Mass mortalities of cultured kuruma shrimp *Penaeus japonicus* in Japan in 1993: electron microscopic evidence of the causative virus, Fish Pathol. 29 (1994) 149–158.
[7] J. Huang, X.L. Song, J. Yu, C.H. Yang, Baculoviral hypodermal and hematopoietic necrosis-study on the pathogen and pathology of the explosive epidemic disease of shrimp, Mar. Fish. Res. 16 (1995) 1–10.
[8] C.V. Mohan, K.M. Shankar, S. Kulkarni, P.M. Sudha, Histopathology of cultured shrimp showing gross signs of yellow head and white spot syndrome during 1994 India epizootics, Dis. Aquat. Org. 34 (1998) 9–12.
[9] Y.G. Wang, M.D. Hassan, M. Shariff, S.M. Zamri, X. Chen, Histopathology and cytopathology of white spot syndrome virus (WSSV) in cultured *Penaeus monodon* from peninsular Malasia with emphasis on pathogenesis and the mechanism of white spot formation, Dis. Aquat. Org. 39 (1999) 1–11.
[10] C.M. Escobedo-Bonilla, V. Alday-Sanz, M. Wille, P. Sorgeloos, M.B. Pensaert, H.J. Nauwynck, A review on the morphology, molecular characterization, morphogenesis and pathogenesis of white spot syndrome virus, J. Fish Dis. 31 (2008) 1–18.
[11] J.H. Leu, F. Yang, X. Zhang, X. Xu, G.H. Kou, C.F. Lo Whispovirus, Curr. Top. Microbiol. Immunol. 328 (2009) 197–227.
[12] P.S. Chang, H.C. Chen, Y.C. Wang, Detection of white spot syndrome associated baculovirus in experimentally infected wild shrimp, crab and lobsters by in situ hybridization, Aquaculture 164 (1998) 233–242.
[13] P. Kanchanaphum, C. Wongteerasupaya, N. Sititidilokratana, V. Boonsaeng, S. Panyim, A. Tassanakajon, et al., Experimental transmission of white spot syndrome virus (WSSV) from crabs to shrimp *Penaeus monodon*, Dis. Aquat. Org. 34 (1998) 1–7.
[14] Q. Wang, L.M. Nunan, D.V. Lightner, Identification of genomic variations among geographic isolates of white spot syndrome virus using restriction analysis and southern blot hybridization, Dis. Aquat. Org. 43 (2000) 175–181.
[15] E.M. Choi, Y.H. Kim, Hesperetin attenuates the highly reducing sugar-triggered inhibition of osteoblast differentiation, Cell Biol. Toxicol. 24 (2008) 225–231.
[16] J. Cho, Antioxidant and neuroprotective effects of hesperidin and its aglycone hesperetin, Arch Pharm. Res. (Seoul) 29 (2006) 699–706.
[17] Y.R. Jin, X.H. Han, Y.H. Zhang, J.J. Lee, Y. Lim, T.J. Kim, et al., Hesperetin, a bioflavonoid, inhibits rat aortic vascular smooth muscle cells proliferation by arresting cell cycle, J. Cell. Biochem. 104 (2008) 1–14.
[18] S. Rainey-Smith, L.W. Schroetke, P. Bahia, A. Fahmi, R. Skilton, J.P. Spencer, et al., Neuroprotective effects of hesperetin in mouse primary neurons are independent of CREB activation, Neurosci. Lett. 438 (2008) 29–33.
[19] J.Y. Cha, Y.S. Cho, I. Kim, T. Anno, S.M. Rahman, T. Yanagita, Effect of hesperetin, a citrus flavonoid, on the liver triacylglycerol content and phosphatidate phosphohydrolase activity in orotic acid-fed rats, Plant Foods Hum. Nutr. 56 (2001) 349–358.
[20] F.V. So, N. Guthrie, A.F. Chambers, M. Moussa, K.K. Carroll, Inhibition of human breast cancer cell proliferation and delay of mammary tumorigenesis by flavonoids and citrus juices, Nutr. Cancer 26 (1995) 167–181.
[21] A. Ahmadi, P. Hassandarvish, R. Lani, P. Yadollahi, A. Jokar, S.A. Bakar, et al., Inhibition of chikungunya virus replication by hesperetin and naringenin, RSC Adv. 6 (2016) 69421–69430.
[22] T.N. Kaul, E. Middleton, P.L. Ogra, Antiviral effect of flavonoids on human viruses, J. Med. Virol. 15 (1985) 71–79.
[23] A. Paredes, M. Alzuru, J. Mendez, M. Rodríguez-Ortega, Anti-Sindbis activity of flavanones hesperetin and naringenin, Biol. Pharm. Bull. 26 (2003) 108–109.
[24] T.F. Lien, H.S. Yeh, W.T. Su, Effect of adding extracted hesperetin, naringenin and pectin on egg cholesterol, serum traits and antioxidant activity in laying hens, Arch. Anim. Nutr. 62 (2008) 33–43.
[25] F. Zhu, X. Zhang, The Wnt signaling pathway is involved in the regulation of phagocytosis of virus in *Drosophila*, Sci. Rep. 3 (2013) 2069.
[26] Z. Wang, F. Zhu, Minichromosome maintenance protein 7 regulates phagocytosis in kuruma shrimp *Marsupenaeus japonicas* against white spot syndrome virus, Fish Shellfish Immunol. 55 (2016) 293–303.
[27] B. Sun, Z. Wang, F. Zhu, The crustin-like peptide plays opposite role in shrimp immune response to *Vibrio alginolyticus* and white spot syndrome virus (WSSV) infection, Fish Shellfish Immunol. 66 (2017) 487–496.
[28] K.J. Livak, T.D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method, Methods 25 (2011) 402–408.
[29] K. Wongprasert, T. Rudtanatip, J. Praiboon, Immunostimulatory activity of sulfated galactans isolated from the red seaweed *Gracilaria fisheri* and development of resistance against white spot syndrome virus (WSSV) in shrimp, Fish Shellfish Immunol. 36 (2014) 52–60.
[30] Z. Zhao, C. Jiang, X. Zhang, Effects of immunostimulants targeting Ran GTPase on phagocytosis against virus infection in shrimp, Fish Shellfish Immunol. 31 (2011) 1013–1018.
[31] C.O. Beauchamp, I. Fridovich, Isozymes of superoxide dismutase from wheat germ, Biochim. Biophys. Acta 317 (1973) 50–64.
[32] C.W. Lin, F.J. Tsai, C.H. Tsai, C.C. Lai, L. Wan, T.Y. Ho, et al., Anti-SARS coronavirus 3C-like protease effects of *Isatis indigotica* root and plant-derived phenolic compounds, Antivir. Res. 68 (2005) 36–42.
[33] F.J. Tsai, C.W. Lin, C.C. Lai, Y.C. Lan, C.H. Lai, C.H. Hung, et al., Kaempferol inhibits enterovirus 71 replication and internal ribosome entry site (IRES) activity through FUBP and HNRP proteins, Food Chem. 128 (2011) 312–322.
[34] H.K. Kim, W.K. Jeon, B.S. Ko, Flavanone glycosides from *Citrus junos* and their anti-influenza virus activity, Planta Med. 67 (2001) 548–549.
[35] K. Drickamer, C-type lectin-like domains, Curr. Opin. Struct. Biol. 9 (1999) 585–590.
[36] A. Cambi, C. Figdor, Necrosis: C-Type lectins sense cell death, Curr. Biol. 19 (2009) 375–378.
[37] Z.Y. Zhao, Z.X. Yin, X.P. Xu, S.P. Weng, X.Y. Rao, Z.X. Dai, et al., A novel C-type lectin from the shrimp *Litopenaeus vannamei* possesses anti-white spot syndrome virus activity, J. Virol. 83 (2009) 347.
[38] B. Sun, Z. Wang, F. Zhu, The crustin-like peptide plays opposite role in shrimp immune response to *Vibrio alginolyticus* and white spot syndrome virus (WSSV) infection, Fish Shellfish Immunol. 66 (2017) 487–496.
[39] K. Sritunyalucksana, K. Söderhäll, The proPO and clotting system in crustaceans, Aquaculture 191 (2000) 53–69.
[40] A. Tassanakajon, K. Somboonwiwat, P. Supungul, S. Tang, Discovery of immune molecules and their crucial functions in shrimp immunity, Fish Shellfish Immunol. 34 (2013) 954–967.
[41] P.T. Schumacker, Reactive oxygen species in cancer: a dance with the devil, Cancer Cell 27 (2015) 156–157.
[42] A.I. Campa-Córdova, N.Y. Hernández-Saavedra, R.D. Philippis, F. Ascencio, Generation of superoxide anion and SOD activity in haemocytes and muscle of American white shrimp (*Litopenaeus vannamei*) as a response to beta-glucan and sulphated polysaccharide, Fish Shellfish Immunol. 12 (2002) 353–366.
[43] S. Akira, S. Uematsu, O. Takeuchi, Pathogen recognition and innate immunity, Cell 4 (2006) 783–801.
[44] L. Deng, T. Adachi, K. Kitayama, Y. Bungyoku, S. Kitazawa, S. Ishido, et al., Hepatitis C virus infection induces apoptosis through a Bax-triggered, mitochondrion-mediated, caspase 3-dependent pathway, J. Virol. 21 (2008) 10375–10385.
[45] L. Wang, B. Zhi, W. Wu, X. Zhang, Requirement for shrimp caspase in apoptosis against virus infection, Dev. Comp. Immunol. 6 (2008) 706–715.
[46] N.N. Danial, S.J. Korsmeyer, Cell death: critical control points, Cell 116 (2004) 205–219.
[47] J. Dutta, Y. Fan, N. Gupta, G. Fan, C. Gélinas, Current insights into the regulation of programmed cell death by NF-kappaB, Oncogene 25 (2006) 6800–6816.

浙江农林大学图书馆收录证明报告

《SCI-Expanded》检索结果（收录情况）

WEB OF SCIENCE™

Web of Science™ Core Collection

经检索《**Web of Science™ Core Collection**》，朱斐 发表的下述 **1** 篇论文被《**SCI－Expanded**》收录。（检索时间：**2019** 年 **11** 月 **7** 日）

第 1 条，共 1 条

标题: Hesperetin protects crayfish Procambarus clarkii against white spot syndrome virus infection

作者: Qian, XY (Qian, Xiyi); Zhu, F (Zhu, Fei)

来源出版物: FISH & SHELLFISH IMMUNOLOGY 卷: 93 页: 116-123 **DOI:** 10.1016/j.fsi.2019.07.029 出版年: OCT 2019

Web of Science 核心合集中的 "被引频次": 0

被引频次合计: 0

入藏号: WOS:000488998300014

PubMed ID: 31302287

地址: [Qian, Xiyi; Zhu, Fei] Zhejiang Agr & Forestry Univ, Coll Anim Sci & Technol, Zhejiang Prov Engn Lab Anim Hlth Inspect & Intern, Hangzhou 311300, Zhejiang, Peoples R China.

通讯作者地址: Zhu, F (通讯作者)，Zhejiang Agr & Forestry Univ, Coll Anim Sci & Technol, Zhejiang Prov Engn Lab Anim Hlth Inspect & Intern, Hangzhou 311300, Zhejiang, Peoples R China.

电子邮件地址: zhufei@zju.edu.cn

浙江农林大学图书馆

检索人：桑宇芳

2019 年 11 月 7 日