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# Evaluation on the antiviral activity of genipin against white spot syndrome virus in crayfish

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ABSTRACT

White spot syndrome virus (WSSV) is a serious epidemic pathogen of crustaceans and cause severe economic losses to aquaculture. However, no commercial drugs presently available to control WSSV infection. Genipin (GN) is a bioactive compound extracted from the fruit of *Gardenia jasminoides* and exhibits potential antiviral activity. In the study, the antiviral activity of GN against WSSV was investigated in crayfish *Procambarus clarkii* and in shrimp *Litopenaeus vannamei*. *In vitro* antiviral test showed that GN could inhibit WSSV replication in crayfish and in shrimp, and the highest inhibition on WSSV was over 99% when treatment with 50 mg/kg of GN for 24 h. *In vivo* antiviral test proved that GN could be used to treat and prevent WSSV infection. GN could also effectively protect crayfish from WSSV infection by reducing the mortality rate of WSSV-infected crayfish. Moreover, GN attenuated the WSSV-induced oxidative stress and inflammatory by upregulation the expression of antioxidant-related genes and downregulation the expression of inflammatory-related genes, respectively. Mechanically, GN inhibited WSSV replication at least via decreasing *STAT* (*signal transducer and activator of transcription*) gene expression to block WSSV immediate-early gene *ie1* transcription. Additionally, the inhibition of *BI-1* (*Bax inhibitor-1*) gene expression also played an important role in the suppression of WSSV infection. In conclusion, GN represented a potential therapeutic and preventive agent to block WSSV infection.

## 1. Introduction

As one of penaeid shrimp pathogens recognized by the World Organisation for Animal Health (OIE) [1], white spot syndrome virus (WSSV) is a severe viral disease of crustaceans and brings significant losses to aquaculture [2]. It can almost infect all crustaceans, including penaeid shrimp, crab and crayfish [3]. Due to the wide range of hosts and easy replication, it is hard to prevent and inhibit the spread of WSSV [4]. Although many studies have been implemented to investigate defensive measures against WSSV [5,6], no successful commercial drugs presently available for use in the shrimp culture industry. Thus, it is urgent to develop an effective measure for controlling WSSV infection.

At present, a lot of research has been carried out to study WSSV pathogenesis [7,8], but the pathogenesis remains unclear. However, signal transducer and activator of transcription (STAT) gene, a key part of the JAK-STAT pathway, is responsive to WSSV infection [9–13]. STAT can physically interact with WSSV immediate early (IE) protein IE1, and the activated STAT can enhance the transcription of IE gene *ie1* by binding to STAT-binding motifs *in vitro* and *in vivo* [14]. WSSV IE gene *ie1* during WSSV infection play a critical role in WSSV replication [15]. dsRNA silencing of *STAT* or *ie1* both reduce the WSSV replication [11,15].

Genipin (GN), an aglycone derived from an iridoid glycoside called geniposide, is a bioactive compound extracted from the fruit of *G. jasminoides* [16]. GN is known to be an excellent natural cross-linker, and possess anti-inflammatory, anti-oxidative and antiviral activities [17–21]. For example, as a natural cross-linker, GN could be used to synthesis of various biopolymers for drug delivery application due to the low toxicity and excellent biocompatibility [17,18]. As an effective antiviral agent, GN show antiviral activity on H1N1 by increasing binding affinity for the influenza M2 channel [22]. In addition, GN can also suppress Epstein-Barr virus (EBV) infection by activating EBV F promoter for EBV lytic activation in EBV-associated gastric carcinoma cells [21].

In the study, the antiviral activity of GN against WSSV was investigated in red swamp crayfish *Procambarus clarkii*. The survival rate of WSSV-infected crayfish and WSSV replication in crayfish were studied. Moreover, we also tested whether GN could inhibit WSSV replication in Pacific white leg shrimp *Litopenaeus vannamei*. The

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underlying mechanism by which GN inhibited WSSV replication was also explored. The present study will provide reference for the development of prevention and treatment of WSSV infection.

## 2. Materials and methods

### 2.1. Animal, virus, and natural compounds

Red swamp crayfish *P. clarkii* (approximately 12.74 ± 1.08 g each) were purchased from the Yangling market in Xianyang (China). Healthy shrimp *L. vannamei* (approximately 7.21 ± 0.73 g each) were collected from shrimp farm in Wenchang (China). The crayfish and shrimp were both stocked in water tanks at 25 °C and fed twice daily with commercial feed pellets. The crayfish and shrimp were randomly screened by PCR for WSSV detection as previous study [23], and WSSV-free healthy crayfish and shrimp were collected for the challenge experiments. In order to reduce the impact of exogenous factors, crayfish and shrimp were not fed during trials.

WSSV was kindly provided by Prof. Feng Yang (Third Institute of Oceanography, State Oceanic Administration, Xiamen, PR China), and propagated in crayfish as previously described [23]. For challenge experiment, WSSV inocula were diluted with sterilized TM buffer (100 mM Tris-HCl, 10 mM $MgCl_2$, pH 7.5), and $7.5 \times 10^7$ and $7.5 \times 10^4$ copies virus were intramuscularly injected into crayfish (100 μL) and shrimp (50 μL), respectively. GN (CAS No. 6902-77-8) was purchased from Nanjing spring & autumn biological engineering Co., Ltd. The structure of GN was confirmed by $^1H$, and $^{13}C$ NMR.

### 2.2. Safety evaluation of GN in crayfish

GN was dissolved in dimethyl sulfoxide (DMSO) and stored at 4 °C until used. GN was diluted to a serial of concentrations with TM buffer when used. The tested concentrations of GN were 50, 150 and 250 mg/kg, respectively. Crayfish were divided into two parts: TM (control) groups and GN (treatment) groups. The crayfish of TM groups were injected intramuscularly with TM buffer (100 μL). The crayfish of GN groups were injected with GN solution of different concentrations, respectively. Each treatment was in three replicates, and each replicate contained five crayfish. The crayfish was observed for 3 days and the death of crayfish was recorded daily.

### 2.3. Antiviral activity of GN in crayfish

To evaluate the antiviral activity of GN against WSSV in crayfish, qRT-PCR assay and survival rate assay were carried. For qRT-PCR assay, crayfish were randomly divided into three parts: (A) The mixtures of virus-GN were made first by mixing equal volumes of virus and GN and incubating for 0, 1, 2 or 3 h at 25 °C, and then were injected into crayfish, respectively. The tested concentrations of GN in part A were 6.25, 12.5, 25 and 50 mg/kg, respectively; (B) After GN solution treatment for 2, 6 or 24 h, crayfish would receive a second injection of WSSV solution, respectively. The tested concentrations of GN in part B were 50 mg/kg; (C) After WSSV treatment for 2, 6 or 24 h, crayfish would receive a second injection of GN solution, respectively. The tested concentrations of GN in part C were 50 mg/kg. Each part contained two groups: WSSV groups (positive controls) and GN/WSSV groups (treatment groups). Each treatment was in three replicates. Crayfish were randomly collected at different time after treatment according to the needs of different analyses.

For survival rate assay, crayfish were divided into three groups: TM groups (negative control), WSSV groups (positive control) and GN/WSSV groups (treatment groups, 50 mg/kg). For challenge, the mixtures of virus-GN were made first by mixing equal volumes of virus and GN solution and incubating for 0 h at 25 °C (the mixtures were not incubated at all), and then were injected into crayfish. Each group contained 30 crayfish. The mortality in each group were monitored continuously and recorded each day for 10 days.

### 2.4. Antiviral activity of GN in shrimp

To determine whether GN inhibited WSSV replication in shrimp, shrimp were injected with the mixtures of virus and GN (incubating for 0 h at 25 °C). After 24, 48 or 72 h, five shrimp were randomly collected and gill tissues were sampled and stored at −80 °C until used. The tested concentrations of GN were 50 mg/kg. Each group was in three replicates, and each replicate contained 30 crayfish.

### 2.5. WSSV replication analysis

Total genomic DNA was extracted from gill tissues using TIANamp Marine Animals DNA Kit (Tiangen, China) according to the manufacturer's instructions, and quantification of extracted DNA used NanoDrop spectrophotometer (ND-1000, NanoDrop Technologies Inc., Wilmington, DE, USA). WSSV genomic DNA copy numbers were quantified using qRT-PCR and standard curve as a previous study [23].

### 2.6. Expression analysis of WSSV genes and crayfish genes

Total RNA was extracted from gill tissues using RNAiso Plus reagent following the manufacturer's protocols. RNA was applied to cDNA synthesis using PrimeScript RT reagent Kit with gDNA Eraser (Takara, China). qRT-PCR of WSSV genes and crayfish genes was performed with CFX96 Real-Time PCR Detection System (Bio-Rad, USA) by using AceQ® qPCR SYBR® Green Master Mix (Vazyme, China) with the parameters based on a previous study [23]. The sequences of primers for qRT-PCR were shown in Table 1. Gene expression level was calculated with $2^{-\Delta\Delta Ct}$ method [24].

### 2.7. Statistical analysis

The data were presented as mean values ± standard deviation (SD), and statistical analyses were performed with SPSS 18.0 software (SPSS Inc., USA) using one-way ANOVA and post-hoc Tukey test to determine significance. $p$ value less than 0.05 was considered statistically significant.

**Table 1**
Sequences of primers for this study.

| Primers | Primer sequences (from 5′ to 3′) |
|---|---|
| ie1-RTF | GACTCTACAAATCTCTTTGCCA |
| ie1-RTR | TGCTGATAAACTCTTGAAGGAA |
| DNApol-RTF | CTCGCCAAAGTGAGTAGTGT |
| DNApol-RTR | CCTTGTTGATGGAGGTAGAA |
| VP28-RTF141 | AAACCTCCGCATTCCTGTGA |
| VP28-RTR141 | TCCGCATCTTCTTCCTTCAT |
| Pc-cMnSOD-RTF | GCCACCACTAAAATACGAGTA |
| Pc-cMnSOD-RTR | CCATTGAACTTTATAGCTGGTA |
| Pc-mMnSOD-RTF | CATCACTCCAAGCACCACC |
| Pc-mMnSOD-RTR | GAGCAAGGGATATAACAGTAC |
| Pc-CAT-RTF | CGACCATACACCGCTTCAC |
| Pc-CAT-RTR | TTTCAGGAATGCGTTCTCTATC |
| Pc-GST-RTF | ACTTAGAGACGGACTTCCAG |
| Pc-GST-RTR | CGAGGGCGAACTTCACGG |
| Pc-BI-1-RTF | TGCCATTACATCTTGGGTTCT |
| Pc-BI-1-RTR | CGACCTAATCCCATCTCAAGC |
| Pc-COX1-RTF | ATGGGATACCTCGACGTTATTC |
| Pc-COX1-RTR | GCAGGAGGATAAGAATGCTGT |
| Pc-COX2-RTF | GGTCATCAGTGATATTGAAGG |
| Pc-COX2-RTR | TCTAATAAACGGAACCCAGAC |
| Pc-STAT-RTF | TGGTAGTGAAGAGAGGTTGAG |
| Pc-STAT-RTR | CATTGTTTCCCATCTGTCC |
| Pc-18s-RTF | ACCGATTGAATGATTTAGTGAG |
| Pc-18s-RTR | TACGGAAACCTTGTTACGAC |

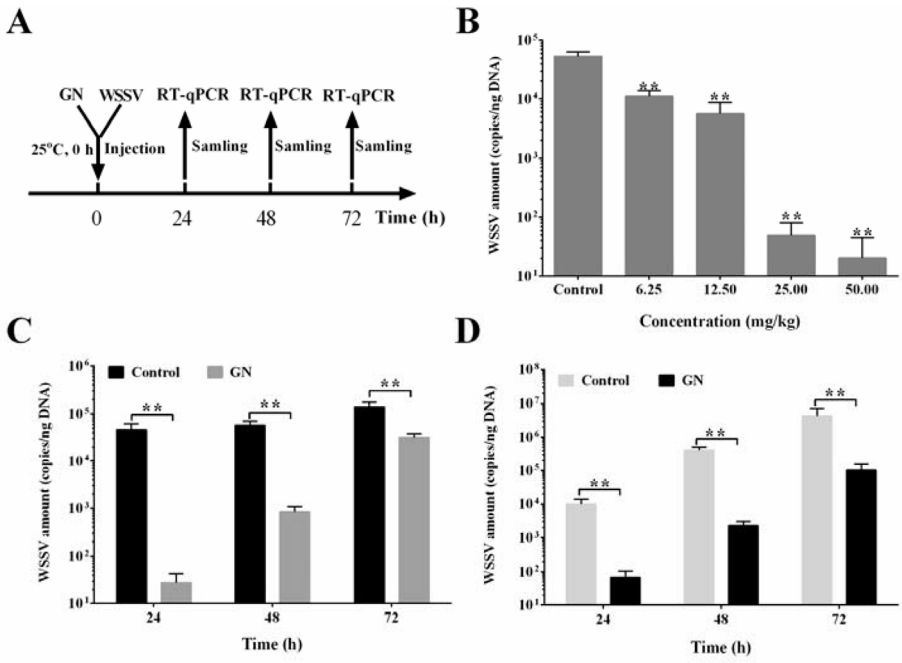


**Fig. 1.** GN inhibited WSSV replication *in vitro*. (A) Workflow of the experimental design followed in (B–D). (B) GN inhibited WSSV replication in crayfish *P. clarkii* at 24 h. (C) GN (50 mg/kg) inhibited WSSV replication in crayfish *P. clarkii* at 24, 48 and 72 h. (D) GN (50 mg/kg) inhibited WSSV replication in shrimp *L. vannamei* at 24, 48 and 72 h. Data were shown as mean ± SD (n = 5). Asterisks mark the significant difference between experimental data and control data (**$P < 0.01$).

## 3. Results

### 3.1. Safety evaluation of GN in crayfish

The information of GN was shown in Fig. S1, including the chemical structure, $^{1}$H NMR spectra, and $^{13}$C NMR spectra. Additionally, the safe concentrations of GN in crayfish was more than 250 mg/kg (data not shown).

### 3.2. GN blocks WSSV infection in vitro and in vivo

The dose effect of GN on WSSV replication were investigated. Crayfish were challenged with GN and WSSV as shown in Fig. 1A. From Fig. 1B, it could be found that WSSV copy numbers in crayfish were significantly reduced in the existence of GN compared to the control groups, and GN had a concentration-dependent inhibition on virus replication in crayfish at 24 h. The maximum inhibition rate was more than 99% after treatment with 50 mg/kg GN for 24 h. Hence, the test dose of 50 mg/kg GN is used in subsequent experiment.

The time effect of GN on WSSV replication were also studied. As shown in Fig. 1C, WSSV replication in crayfish were also significantly inhibited after treatment with 50 mg/kg GN from 24 h to 72 h. In addition, we also tested whether GN can inhibit WSSV replication in shrimp *L. vannamei*. Similarly, GN could significantly inhibit WSSV replication in shrimp (Fig. 1D). These results implied that GN indeed possessed *anti*-WSSV activity.

To explore whether GN could be used as a therapeutic or preventive agent to block WSSV infection in crayfish, the copy numbers of WSSV genome DNA were detected after different treatments with GN and WSSV. Firstly, we explore whether GN disturbed WSSV to recognize host cells, and the operation was shown in Fig. 2A. As shown in Fig. 2B, GN significantly inhibited WSSV replication, and the inhibition rate was all more than 99%. To investigate whether GN could be used for prevention of WSSV in crayfish, the second test was done (Fig. 3A), and the results were shown in Fig. 3B. The results indicated that GN could block WSSV replication and be used for prevention. Finally, we evaluate whether GN could inhibit WSSV multiplication in crayfish (Fig. 4A). As demonstrated in Fig. 4B, GN could inhibit virus multiplication and be used for therapy.

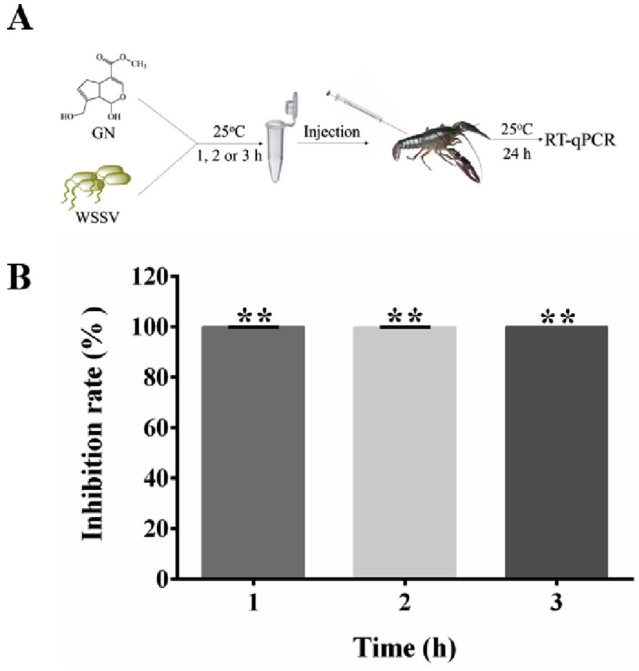


**Fig. 2.** The effect of GN on WSSV infectivity in crayfish. (A) Workflow of the experimental design followed in (B). (B) The inhibition rate of GN against WSSV replication. Inhibition rate was calculated with the formula: (control – treatment)/control × 100. The inhibition rate of WSSV groups was zero. Data were shown as mean ± SD (n = 5). Asterisks represent the significant difference between GN/WSSV and WSSV groups (**P < 0.01).

### 3.3. GN improves the survival rate of WSSV-infected crayfish

To prove the protective effect of GN against WSSV, the survival assay was studied in crayfish. The results were shown in Fig. 5. In TM groups, crayfish mortality was observed at 192 h, only 3.3%. In WSSV groups, crayfish death was observed after treatment with WSSV for 48 h. From 48 h to 96 h, crayfish mortality in WSSV groups increased rapidly from 26.7% to 83.3%, and reached 100% at 168 h post-infection. However, crayfish mortality was significantly lower in GN/WSSV

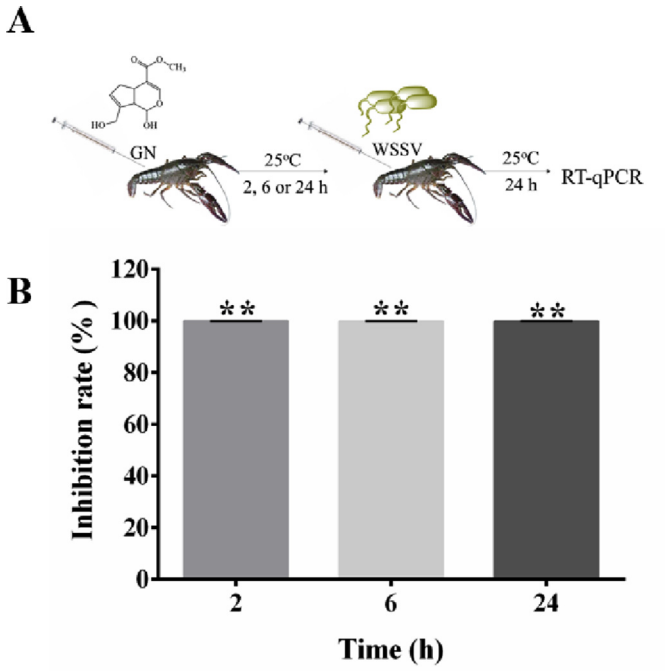


**Fig. 3.** The prevention of GN on WSSV infection in crayfish. (A) Workflow of the experimental design followed in (B). (B) The inhibition rate of GN against WSSV replication. Inhibition rate was calculated with the formula: (control – treatment)/control × 100. The inhibition rate of WSSV groups was zero. Data were shown as mean ± SD (n = 5). Asterisks represent the significant difference between GN/WSSV and WSSV groups (**P < 0.01).

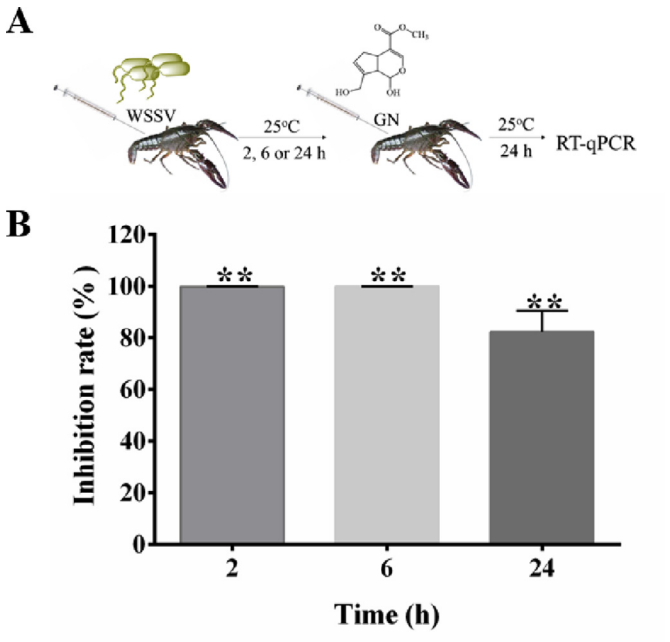


**Fig. 4.** The effect of GN on WSSV replication in crayfish. (A) Workflow of the experimental design followed in (B). (B) The inhibition rate of GN against WSSV replication. Inhibition rate was calculated with the formula: (control – treatment)/control × 100. The inhibition rate of WSSV groups was zero. Data were shown as mean ± SD (n = 5). Asterisks represent the significant difference between GN/WSSV and WSSV groups (**P < 0.01).

groups than that in WSSV groups, with 50.0% at 240 h. Crayfish death in GN/WSSV groups was first found at 96 h, which was 48 h later than WSSV groups. In addition, crayfish died more slowly in GN/WSSV groups than those in WSSV groups.

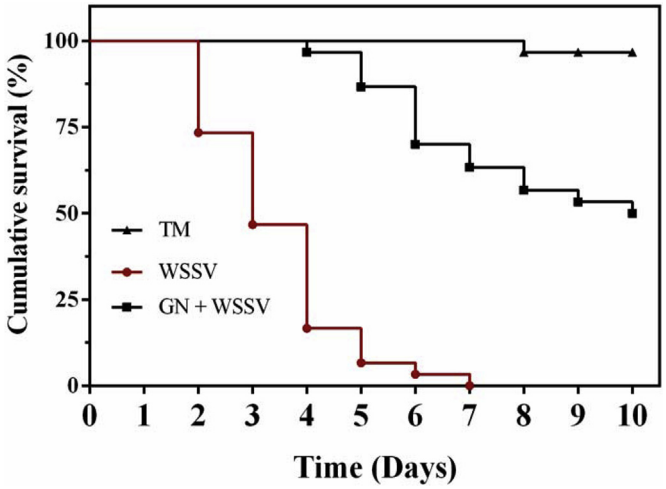


**Fig. 5.** Protective effect of GN on WSSV-infected crayfish. Crayfish were treated with GN/WSSV (50 mg/kg), WSSV and TM (control), respectively. Mortality of each group was recorded continuously once a day for 10 days (n = 30).

### 3.4. GN inhibits the expression of WSSV genes

To further prove the inhibitory effect of GN, the expression of WSSV genes was detected. Crayfish were treated with GN/WSSV or WSSV for 24 h, and the total RNA of gill tissues was extracted using RNAiso Plus reagent. As displayed in Fig. 6, significant downregulation of WSSV immediate-early (IE) gene *ie1*, early gene *DNA polymerase* (*DNApol*) and late gene *VP28* was observed in GN/WSSV treated crayfish.

### 3.5. GN regulates the expression of crayfish genes

The effects of GN on the expression of crayfish genes were tested. The antioxidant-related genes (*cMnSOD*, *mMnSOD*, *CAT* and *GST*), apoptosis-related genes (*Bax inhibitor-1* (*BI-1*)), antivirus-related genes (*STAT*) and inflammatory-related genes (*COX1* and *COX2*) of crayfish in GN/WSSV and WSSV groups were studied. From Fig. 7, it could be found that the antioxidant-related genes in GN/WSSV groups significantly increased than those in the control groups. The expression levels of *cMnSOD*, *mMnSOD*, *CAT* and *GST* were 5.01-, 3.40-, 5.13- and 4.28-fold higher in GN/WSSV groups than WSSV groups, respectively. However, significant downregulation of *BI-1*, *STAT*, *COX1* and *COX2*

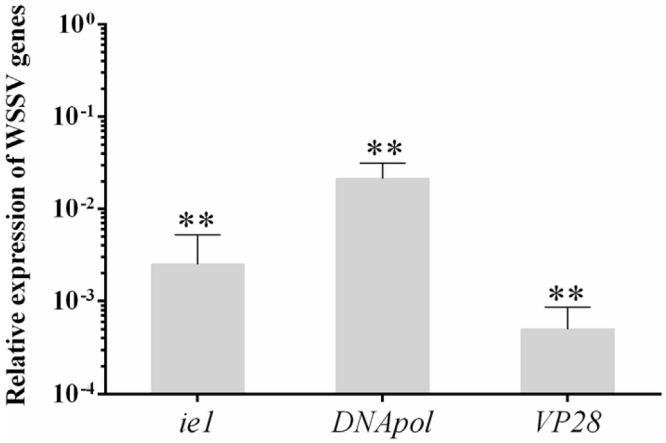


**Fig. 6.** GN inhibited WSSV genes expression in crayfish at 24 h. The crayfish treated with GN/WSSV (treatment) and WSSV (control), respectively. *18s* was served as an internal reference gene. Gene expression level was calculated with $2^{-\Delta\Delta Ct}$ method. The expression of control groups was one. Data were shown as mean ± SD (n = 5). Asterisks represent the significant difference between treatment and control groups (**P < 0.01).

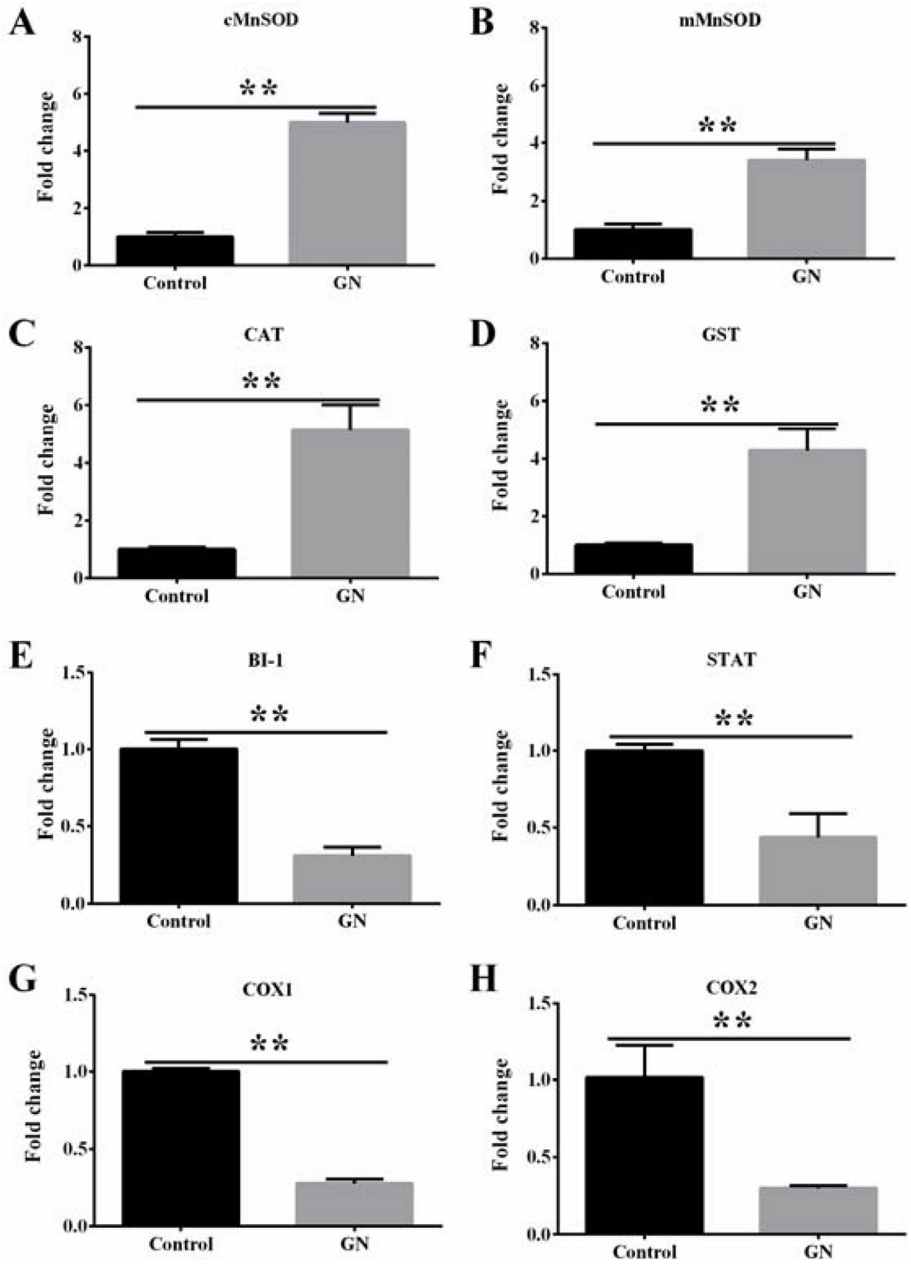


**Fig. 7.** GN regulated the expression of crayfish genes at 24 h. The crayfish treated with GN/WSSV (treatment) and WSSV (control), respectively. *18s* was served as an internal reference gene. Gene expression level was calculated with $2^{-\Delta\Delta Ct}$ method. Data were shown as mean ± SD (n = 5). Asterisks represent the significant difference between treatment and control groups (**$P < 0.01$).

was found in the GN/WSSV groups. The expression of *BI-1*, *STAT*, *COX1* and *COX2* in GN/WSSV groups were 0.31-, 0.43-, 0.27- and 0.30-fold that of WSSV groups, respectively.

## 4. Discussion

In shrimp farm, shrimp mortality is rapid and cumulative mortality is generally over 90% within 3 and 10 days after WSSV infection. Although a series of studies have been implemented to develop interventions, there are currently no commercial agents available for use in the shrimp culture industry. In this article, we investigated the antiviral effects of GN on WSSV infection and evaluated the underlying mechanisms of GN antivirus. Gill is regarded as a major target organ for WSSV replication [23,25,26], thus, we selected gill to detect the copy numbers of WSSV genome DNA and the expression of WSSV genes and crayfish genes in the study. For better quantify the antiviral activity and reduce the impact of exogenous factors, natural compound GN solution was administrated by intramuscular injection method.

Recently, we have reported that *G. jasminoides* can inhibit WSSV replication and improve the survival rate of WSSV-infected crayfish [23]. However, we don't know which ingredients of *G. jasminoides* have *anti*-WSSV activity. GN isolated from *G. jasminoides* is known to be an effective antiviral agent [27]. In the study, we found that GN could inhibit WSSV replication in crayfish and in shrimp by the experiment of incubation of GN and WSSV *in vitro* first and then injection. In addition, GN could also delay and reduce the cumulative mortality of WSSV-infected crayfish. These results were consistent with our previous studies [23]. Although the survival rate was similar to previous studies, the inhibitory effects of GN on WSSV replication were better than *G. jasminoides*. It might be due to the difference of the contents of GN, and these indicated that GN is one of the most important components of *G. jasminoides* to inhibit WSSV replication. GN could inhibit WSSV

replication, but we did not know whether GN could be used to treat and prevent WSSV infection. Thus, we carried out two different experiments to prove this. Firstly, to prove whether GN had a potential therapeutic function against WSSV replication, we carried out the experiment of WSSV treatment first and then GN treatment. Previous studies have shown that WSSV genes can be detected in shrimp after 2 h of WSSV infection, such as WSSV IE gene *ie1*, early gene *DNApol*, and late gene *VP28* [28]. At 24 h, WSSV replication is in the late stage of logarithmic growth and low mortalities or no mortalities were found in crayfish [23]. Thus, we selected these time (2, 6 and 24 h) for WSSV challenge before GN treatment. The results proved that GN could inhibit WSSV multiplication and had therapeutic efficacy. In addition, we implemented the experiment of pre-injection of GN in crayfish and then injection with WSSV to explore whether GN could prevent WSSV infection. Previous studies have found that the expression of immune-related genes of shrimp *Marsupeneaus japonicus* was up-regulation at 2 h post injection of epigallocatechin-3-gallate [29], indicating that shrimp innate immune signal pathways can be influenced after treatment with natural compound for 2 h. Thus, we chose to treat GN at least 2 h prior to WSSV challenge, and the results proved that GN could be used as a preventive against WSSV infection.

Although GN could inhibit WSSV replication in crayfish and shrimp, the potential mechanism by which GN inhibited WSSV replication was unknown. During infection by large DNA viruses, such as herpesviruses and baculoviruses, virus gene expression is modulated such that the IE genes are first transcribed and followed by the expression of the early and late genes [15]. Up to date, 21 IE genes have been identified from WSSV [14]. Although most of WSSV IE genes functions have not yet been investigated, many virus IE genes can encode multifunctional transcriptional regulators that positively and negatively regulated the expression of virus early and late genes [15]. Among these genes, *ie1* is the focus of investigations and it can be used as a transcription factor with transactivation, dimerization and DNA binding activities [30]. Additionally, dsRNA silencing of *ie1* expression also significantly blocked WSSV replication [15], indicating that *ie1* is important for viral lytic replication. Similarly, the expression of *ie1* gene was significantly decrease in GN treated WSSV-infected crayfish, indicating that GN could inhibit WSSV replication at least via reducing the expression of *ie1*. Although the downstream target genes of IE1 are still unclear, *ie1* silencing leaded to reducing the expression of WSSV early gene *DNApol* [15]. *DNApol* is essential for WSSV replication, and WSSV can encode its own DNA polymerase [31]. Interestingly, we also found that GN could inhibit the expression of *DNApol* in WSSV-infected crayfish in the study, further demonstrating GN blocked WSSV replication. In the cascade of viral regulatory events, late genes expression are usually dependent on the proper virus DNA replication of preceding stage [32]. For example, most EBV and KSHV late genes are strictly dependent on DNA replication from the lytic replication, and block of DNA replication reduce the transcription of late genes [32–34]. Thus, inhibition of WSSV genome DNA replication might decrease the expression of late genes. In the study, the expression of WSSV late gene *VP28* was significantly inhibited in GN/WSSV treated crayfish. All these results indicated that inhibition of *ie1* expression would block WSSV genome DNA replication and reduce the expression of early and late genes.

The JAK/STAT signaling pathway is highly evolutionarily conserved and it is found in species from *Dictyostelium discoideum* to human [35]. As a key component of innate immune system, the importance of the JAK/STAT signaling pathway in antiviral immunity has been widely demonstrated in vertebrates and invertebrates [12,36–38]. STAT, as a key component of the JAK/STAT signaling pathway, play an important role in the antiviral responses [10], and is usually the common target of viruses [14]. Some viruses can modulate STAT activity to promote viral replication. For example, KSHV (Kaposi's sarcoma-associated herpesvirus) IE protein ORF50 and latency-associated nuclear antigen could increase the transcriptional activity of STAT3 by direct binding [39,40]. Rabies virus P-protein could form interactions with STAT protein through interfaces on opposite sides of the C-terminal domain, which is important to lethal rabies disease [41]. Similarly, STAT is also a potential target of WSSV. In shrimp, WSSV infection could induce STAT activation, and the activated STAT would bind to WSSV *ie1* gene through STAT-binding motifs to enhance the expression of *ie1* [9,10,14]. Thus, dsRNA silencing of *STAT* expression would significantly reduce WSSV replication in shrimp [11], leading to inhibition of the transcript of *ie1*. Interestingly, the expression of *STAT* was inhibited in GN/WSSV treated crayfish in the study, indicating that GN inhibited WSSV replication at least via decreasing the expression of *STAT*. BI-1 protein is an ancient suppressor of cell death [23]. It is also an anti-apoptotic factor in crayfish [42]. A previous study found that BI-1 is conducive to WSSW replication in crayfish, and *BI-1* silencing could inhibit WSSV replication [42]. Thus, the decrease of *BI-1* expression would block WSSV replication. In this study, GN significantly inhibited the transcription of *BI-1* in WSSV-infected crayfish, suggesting that GN reduced WSSV replication at least by decreasing the expression of *BI-1*. In brief, these results indicated that GN could inhibit WSSV replication at least via reducing the expression of *STAT* and *BI-1*.

WSSV induced significant variations in the expression of the anti-oxidant-related genes, such as *SOD*, *CAT*, and *GST* in shrimp and *Portunus trituberculatus* [43,44]. Intracellular redox-cycling would be imbalance in cells when the cellular endogenous antioxidant defenses were disabled, and ROS generation is triggered by viruses [45]. As a result, WSSV infection caused oxidative stress by producing reactive oxygen species (ROS) in shrimp [46,47]. Oxidative stress usually leads to inflammatory responses and tissue injury in the host body [45]. Cells have antioxidants defenses mechanisms to balance ROS overproduction and oxidative stress, which includes nonenzymatic molecules and enzymes [44]. Inflammatory response is activated by cyclooxygenase via catalyzing the biosynthesis of prostaglandins from arachidonic acid [48]. Two primary genes encoded cyclooxygenase are constitutive gene (*COX1*) and inducible isoform (*COX2*) [48]. Previous studies have found that GN possesses anti-inflammatory and antioxidant activities [17,19]. Similarly, GN could upregulation the expression of anti-oxidant-related genes (*cMnSOD*, *mMnSOD*, *CAT* and *GST*) and down-regulation the transcript of inflammatory-related genes (*COX1* and *COX2*). These results indicated that GN possessed anti-inflammatory and antioxidant activities in WSSV-infected crayfish. Therefore, GN may prevent crayfish from WSSV-induced oxidative damage to improve the crayfish survival rate.

GN is known to be an excellent natural biological crosslinker for proteins [27]. GN is colourless, but reacts spontaneously with amino acids and proteins to form blue-black pigments [20]. It is also a non-toxic compound used in traditional medicine, and is safe in crayfish or shrimp in the study. However, we observed that the body color of shrimp changed to blue after treatment with GN. Thus, in addition to WSSV genes and crayfish genes regulation, it is possible that extracellular GN bind to WSSV attachment proteins such as VP28 leading to these proteins malfunction in recognizing cellular WSSV receptors such as calreticulin and Rab7, and finally prevents WSSV infection [2]. Additionally, GN might also bind to WSSV receptors and would cause to prevent WSSV receptors from WSSV attachment proteins. From these assumptions, we presented at least two possible mechanisms to generate antiviral activity by GN. One is to disturb WSSV transcriptional regulation and the other is to disturb WSSV recognition for host cells. However, these suggestions are demanded to test by further studies.

In conclusion, we identified GN as a potential antiviral agent against WSSV in crayfish and shrimp. GN could reduce WSSV replication *in vitro* and *in vivo* and improved the survival rate of WSSV-infected crayfish. GN possessed anti-oxidative and anti-inflammatory activities in crayfish. Mechanically, GN inhibited WSSV replication at least via decreasing *STAT* expression to block IE gene *ie1* transcription. Furthermore, the decrease of *BI-1* gene expression might also play an important role in the suppression of WSSV infection. This work provides theoretical references for the development of *anti*-WSSV agents.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2019.07.083.

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