Jurnal Medik Veteriner
DOI: 10.20473/jmv.vol8.iss2.2025.286–296
October 2025, Vol.8 No.2, 286–296
online at https://e-journal.unair.ac.id/JMV

Dietary Honeysuckle (*Lonicera japonica*) Extract Enhances Resistance to Infectious Myonecrosis Virus in Whiteleg Shrimp (*Litopenaeus vannamei*)

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Abstract

This study aimed to investigate the immune response of whiteleg shrimp (*Litopenaeus vannamei*) to prevent infectious myonecrosis virus (IMNV) infection by incorporating *Lonicera japonica* water extract into artificial feed. A total of 180 whiteleg shrimp were used in four treatment groups (triplicate) receiving different doses of *L. japonica* extract in their feed, including treatment A (1% w/w), B (2% w/w), C (3% w/w), and D (without supplementation/control). The dietary treatments were applied twice daily for two weeks. On the 15th day, the shrimp were injected intramuscularly at the fifth abdominal segment with IMNV isolates (100 µL/shrimp) and observed for seven days post-infection (dpi). The parameters observed included hemolymph profile, survival rate, gross clinical signs, and water quality. IMNV infection was confirmed through both external clinical signs and PCR tests. The results showed that the addition of *L. japonica* water extract to the feed improved the survival rate and immune responses of whiteleg shrimp following the IMNV challenge test. In conclusion, dietary supplementation of *L. japonica* water extract at a concentration of 2% w/w in artificial feed could serve as a preventive agent against IMNV infection in whiteleg shrimp.

Keywords: feed, natural medicine, non-specific immune response, shrimp health, viral infection

Received: March 8, 2025 **Revised:** April 17, 2025 **Accepted:** May 18, 2025

INTRODUCTION

Whiteleg shrimp (*Litopenaeus vannamei*) aquaculture is a high-value industry and one of the leading export commodities for Indonesia (Indonesia Ministry of Marine and Fisheries Affairs 2022). However, viral diseases pose significant threats to shrimp productivity, with Infectious Myonecrosis Virus (IMNV) being one of the most detrimental. IMNV is a non-enveloped double-stranded RNA (dsRNA) with icosahedral particles and 40 nm in diameter (Wan *et al.*, 2023). IMNV was first discovered in whiteleg shrimp farming in Brazil in 2003 and confirmed in Indonesian whiteleg shrimp farming in 2006 (Naim *et al.*, 2015). This disease causes substantial losses due to high mortality rates across all shrimp life stages (postlarvae, juveniles, and adults) (Prasad *et al.*, 2017) and induces physical deformities in infected shrimp, such as a "cooked" appearance, which severely reduces market value (Jithendran *et al.*, 2021). Efforts to prevent or treat this disease are therefore necessary.

Prevention of IMNV in whiteleg shrimp has been proven by administering synbiotics (Oktaviana *et al.*, 2014), and raising salinity to prevent outbreaks (Umiliana *et al.*, 2016). In recent years, efforts to control the disease have been encouraged by using herbal ingredients as they do not cause residues in the shrimp bodies and are environmentally friendly (Vijayaram *et al.*, 2022). Herbal products have an important role in improving the immune function of aquatic animals, including fish, shrimp, and crabs, and also effectively increase the antiviral,

antibacterial, and antiparasitic activity of the immune system (Zhu, 2020).

Several herbal products have been applied to enhance the immune response of shrimp (Citarasu *et al.*, 2022), such as *Paederia foetida* (Ismawati *et al.*, 2020), polyherbal formulations (Chandran *et al.*, 2016), and *Lonicera japonica* (Jiang *et al.*, 2022). Recent research suggests that *L. japonica* inhibits iridovirus (Liu *et al.*, 2020; Tang *et al.*, 2021). However, studies on its application against IMNV remain limited. This study aimed to evaluate the potential of dietary supplementation of *L. japonica* water extract to enhance immune responses in whiteleg shrimp and prevent IMNV infection.

MATERIALS AND METHODS

Ethical Approval

This study was approved by the Ethical Clearance Committee of Universitas Airlangga with certificate number 855/HRECC.FODN/II/2023.

Study Period and Location

This study was conducted from April to July 2023 at the Bioassay Laboratory and the Fish Health and Environmental Laboratory, Brackishwater Aquaculture Development Center, Situbondo, East Java, Indonesia.

Experimental Design

One hundred eighty healthy whiteleg shrimp (length 5 ± 0.35 cm and weight 4.5 ± 0.55 g) were obtained from the Nursery Unit of the Situbondo Brackish Water Aquaculture Fisheries Center, East Java, Indonesia. Shrimp were divided into 12 glass aquaria (15 L volume each, density 15 shrimp per aquarium) and acclimated for 14 days before treatment. Seawater (32-35 ppt salinity) was chlorinated and neutralized by sodium thiosulphate prior to use. The experiments were carried out in a completely randomized design (CRD) with 4 treatments (triplicate) concentrations of the dietary water extract of *L. japonica*, including (A) 1% w/w, (B) 2% w/w, (C) 3% w/w, and D (control) without the addition of water extract *L. japonica* for 14 days. The impact of dietary water extract of *L. japonica* was measured by analyzing shrimp mortality and external clinical signs in shrimp bodies (including muscle necrosis and a cooked appearance), confirmed by PCR, hemocyte profiles, and water quality parameters after IMNV infection for 7 days post infection (dpi). Water quality parameters, including pH (7.9 – 8.14), salinity (32 – 35 $^{0}/_{00}$), nitrite (NO_2) (0.046 – 0.321 ppm), and total ammonia nitrogen (TAN) (0.175 – 0.1895 ppm) were measured using standard methods weekly (Baird *et al.*, 2017).

L. japonica Water Extract Preparation

L. japonica flowers were collected from local farmers and extracted using distilled water following the method of Liu *et al.*, (2020). The extraction protocol involved macerating 25 g of dried flowers in 500 mL distilled water at 4°C for 12 hours, followed by boiling for 4 minutes. The resulting solution was filtered to obtain 100 mL of concentrated extract (250 mg/mL), which was immediately prepared for use. According to Shang et al. (2011), the aqueous extract of *L. japonica* contains bioactive compounds including essential oils, organic acids, flavones, iridoids, saponins, trace minerals, and unique components such as lonijaposides, shuangkangsu, and nicotinate. For feed incorporation, the extract was uniformly mixed with commercial feed using 2% egg white as a binder through spray application, followed by air-drying. The prepared medicated feed was stored in airtight glass containers at 4°C to maintain stability.

Dietary Supplementation of *L. japonica* and IMNV Challenge Test

Feeding was conducted twice a day at 07.00 AM and 07.00 PM, with a dosage of 5% biomass per aquarium per day. Waste food and feces were removed daily, and 60% of the water was replaced to maintain optimal conditions. Water quality sampling was performed weekly. On day 15, all surviving shrimp were injected intramuscularly in the fifth abdominal segment with IMNV isolate (0.1 mL/shrimp) and observed for seven days post infection (dpi). The IMNV isolate was obtained from the viral collection at the Fish Health and

Environmental Laboratory, Brackishwater Aquaculture Development Center, Situbondo, East Java, Indonesia. The isolate was prepared following Mai *et al*. (2019) and stored at -80°C. Clinical symptoms and mortality of whiteleg shrimp were observed daily for seven dpi.

Evaluation of the Survival Rate of Whiteleg Shrimp

The survival rate of whiteleg shrimp was determined by comparing the number of shrimp that remained alive after the challenge test to the total number present before the test, then expressing this value as a percentage. (Arianto *et al.*, 2023).

Hemocyte Quantification

The hemolymphs (100 µL) were collected from the base of the last walking leg of shrimp in each treatment group three times during the experimental period. Collection was performed using a syringe filled with 0.1 mL of anticoagulant, and the collected hemolymph was immediately stored on ice (cold temperature) to prevent hemocyte lysis before being used to calculate THC and DHC. Hemolymphs were sampled before the addition of immunostimulants (D0), before the challenge test (D14), and seven days post infection (7 dpi). The hemolymph-anticoagulant mixture was analyzed for total hemocyte count (THC) and differential hemocyte count following the methods previously described Kuo *et al.*, (2022).

IMNV Validation

IMNV detection was conducted from two moribund samples from every treatment. Sampling was performed on day 15 of feeding and again seven dpi after the challenge test. Validation was performed using a nested PCR method according to Mai *et al.*, (2019) in three-stage process: RNA extraction of the sample was extracted using IQ2000 *Manual of Diagnostic Tests for Aquatic Animals* (2021), followed by amplification, and electrophoresis. The primers used in the first PCR were 4587 forward primers: 5' – CGA – CGC – TGC – TAA – CCA – TAC – AA – 3' (328 bp) and 4914 reverse primers: 5' – ACT – CGG – CTG – TTC – GAT – CAA – GT – 3' (328 bp), while the nested PCR utilized 4725 nested forward primers: 5' – GGC – ACA – TGC – TCA – GAG – ACA – 3' (139 bp) and 4863 nested reverse primers: 5' – AGC – GCT – GAG – TCC – AGT – CTT – G – 3' (139 bp). The first PCR cycle consisted of 39 cycles (reverse transcription: 95°C, 2 minutes, denaturation 95°C, 45 minutes; annealing 60°C, 45 minutes; final extension 60°C, 7 minutes; and a final cycle 4°C). Nested PCR consisted of 39 cycles (reverse transcription: 95°C, 2 minutes; denaturation 95°C, 30 minutes; annealing 65°C, 30 minutes; final extension 72°C, 2 minutes; and a final cycle 4°C).

Data Analysis

The survival rate, THC, and DHC were analyzed using Analysis of Variance (ANOVA) with significance accepted at 95% using IBM SPSS v20. Tukey's test was applied if significant differences were detected ($p < 0.05$) to determine the most significant treatment. Clinical signs and water quality parameters were described using tables and figures.

RESULTS AND DISCUSSION

Survival Rate of Whiteleg Shrimp

The survival rate of whiteleg shrimp ranged from 5% to 20% after being treated with different concentrations of *L. japonica* (Figure 1). The survival rate in treatment A (5%) was not significantly different from that of treatment C (10%) ($p < 0.05$). In contrast, treatment B (20%) showed the highest survival rate among the treatments, and it was significantly different from the control group ($p < 0.05$).

Clinical Signs

Infected shrimp exhibited characteristic clinical signs before mortality. Notable observations included: (1) localized muscle necrosis in the fifth abdominal segment (Figure 2A, white arrows), (2) pleopod discoloration from white to orange (Figure 2A, yellow arrows), and (3) pale musculature at the IMNV injection site (Figure 2B, yellow arrow).

Table 1. Total hemocyte count (THC) of whiteleg shrimp administered *L. japonica* and challenged with IMNV. Different superscripts show significant differences ($p < 0.05$)

Treatments	THC ($\times 10^6$ cells/mL)		
	Before treatments (D0)	**After treatments (D14)**	**After challenge test (D21)**
A	34.9 ± 5.23^a	44.2 ± 0.28^a	60.9 ± 4.38^b
B	34.00 ± 4.81^a	84.90 ± 1.56^b	55.60 ± 5.94^a
C	34.30 ± 4.38^a	46.20 ± 0.85^a	59.30 ± 0.71^a
D	36.10 ± 0.71^a	33.90 ± 4.67^a	50.10 ± 10.89^a

(A) 1% w/w, (B) 2% w/w, (C) 3% w/w, D (control) without water extract *L. japonica*.

Table 2. Differential hemocyte count (DHC) of whiteleg shrimp before and after administration of *L. japonica*, and the effects after challenged with IMNV. Different superscripts show significant differences ($p < 0.05$)

Treatments	Hemocyte type	DHC ($\times 10^6$ cells/mL)		
		Before treatments (D0)	**After treatments (D14)**	**After challenge test (D21)**
A	Granular	18.50 ± 0.14^a	6.60 ± 1.98^d	9.90 ± 1.27^b
	Hyaline	17.60 ± 0.85^a	37.60 ± 1.70^a	51.00 ± 3.11^a
B	Granular	16.30 ± 2.40^a	55.90 ± 4.10^a	15.50 ± 12.59^a
	Hyaline	18.00 ± 1.98^a	29.00 ± 2.55^b	40.10 ± 6.65^b
C	Granular	16.90 ± 1.56^a	26.50 ± 2.40^b	7.90 ± 1.84^b
	Hyaline	17.10 ± 3.25^a	19.70 ± 3.25^c	51.40 ± 2.55^a
Control	Granular	16.90 ± 0.99^a	16.20 ± 0.57^c	13.50 ± 6.36^a
	Hyaline	18.00 ± 4.24^a	17.70 ± 4.10^c	36.60 ± 17.25^a

(A) 1% w/w, (B) 2% w/w, (C) 3% w/w, D (control) without water extract *L. japonica*.

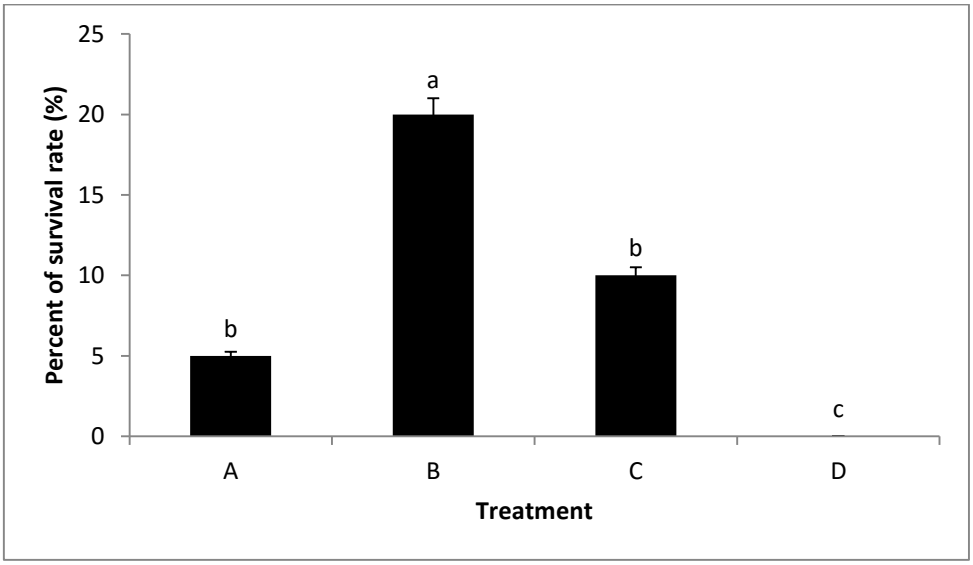

Figure 1. Survival rate of whiteleg shrimp after administration of *L. japonica* and challenge with IMNV. Treatment (A) 1% w/w, (B) 2% w/w, (C) 3% w/w, D (control) without water extract *L. japonica*. Different superscripts show significant differences ($p < 0.05$).

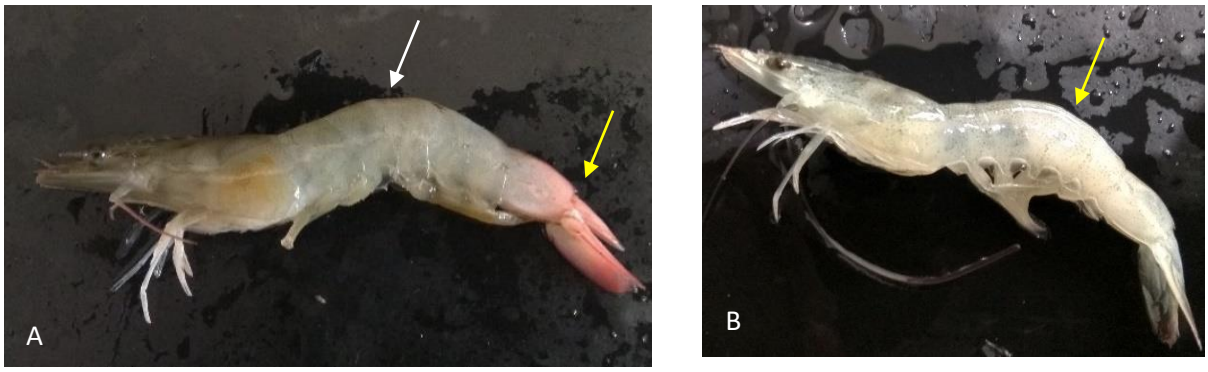

Figure 2. Shrimp infected with IMNV after the challenge test**.** White arrows in Figure A indicates muscle necrosis in the 5th abdominal segment, and the pleopod changes color from white to orange ("cooked" appearance) (yellow arrow). Shrimp muscles also display a pale color in the injection area of IMNV (yellow arrow in Figure B).

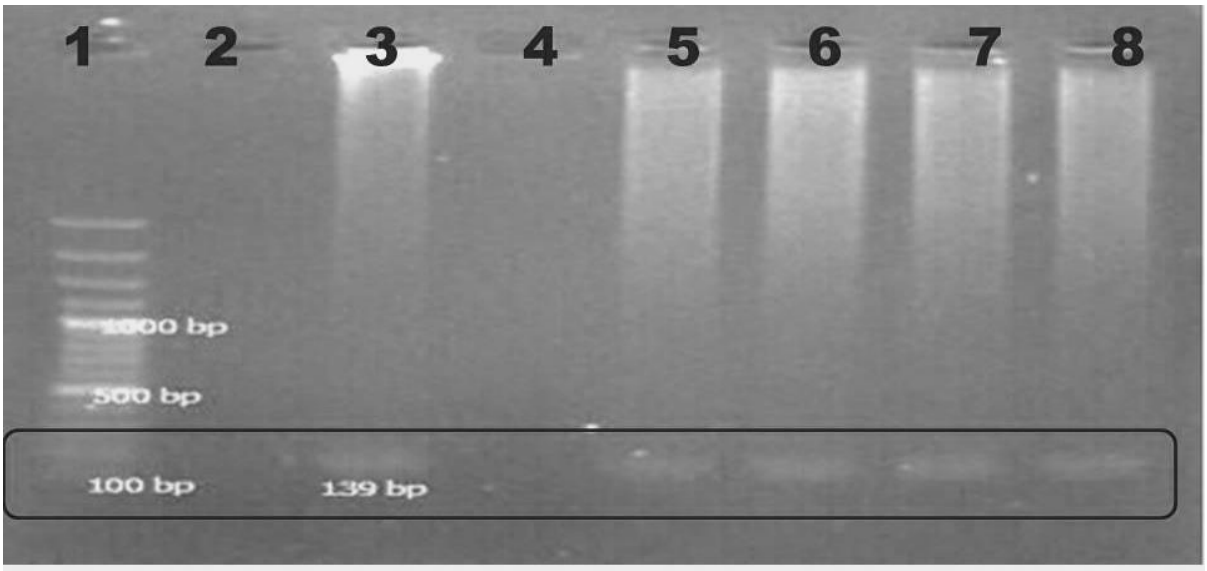

Figure 3. Agarose gel electrophoresis for qualitative estimation of IMNV's DNA fragmentation in whiteleg shrimp from different treatment groups. Lane 1 is DNA ladder; Lane 2 is negative control; Lane 3 is positive control (~139 bp); Lane 4 is DNA fragments of shrimp before the IMNV infection; Lanes 5–8 are treatment A, B, C, and D, respectively.

PCR Validation

PCR analysis confirmed IMNV infection status, with lane 4 showing negative results while lanes 5–8 exhibited clear positive bands (Figure 3), verifying viral infection in test groups.

Hemocyte Quantification

Table 1 shows the number of hemocytes in whiteleg shrimp before and after administering *L. japonica*, and the effects after the challenge test. In treatments A, B, and C, the total hemocyte count (THC) increased before and after the administration of *L. japonica* extract, with treatment B exhibiting the highest THC value ($p < 0.05$), while treatments A and C showed no significant difference. In contrast, the control group (treatment D) maintained relatively stable hemocyte counts, ranging from (36.10 ± 0.71) to (33.90 ± 4.67) × 10^6 cells/mL.

Following the IMNV challenge, the highest THC value was observed in treatment A, reaching (60.9 ± 4.38) × 10^6 cells/mL. Meanwhile, the THC value in treatment B declined and was not significantly different from those in treatments C and D ($p < 0.05$). The survival rate (SR) in treatment B was the highest at 20%, which was supported by the highest THC value recorded after 14 days of *L. japonica* extract

administration, reaching (84.90 ± 1.56) × 10^6 cells/mL. However, seven days post-infection, the THC value decreased, indicating the shrimp's response to the invading pathogen. In treatments A (SR 5%), C (SR 10%), and D (SR 0%), the THC value increased seven days after the challenge test. This increase can be attributed to the fact that hemocyte counts were measured in surviving shrimp, which likely retained a higher hemocyte count as a defense mechanism.

In the control treatment, the total number of hemocytes was relatively consistent (33.90 ± 4.67 × 10^6 cells/mL). The administration of *L. japonica* extract at a concentration of 2% (84.90 ± 1.56) × 10^6 cells/mL) resulted in the highest increase in THC ($p < 0.05$). Meanwhile, the maximum value of THC after IMNV challenges were found at a concentration of 1% w/w (60.9 ± 4.38 × 10^6 cells/mL) when compared with all treatments ($p < 0.05$).

Differential Hemocyte Count (DHC)

Table 2 reveals that the addition of *L. japonica* water extract (D14) improved the granular and hyaline cells in treatments B and C. DHC analysis after administration of *L. japonica* extract showed that there was an increase in the number of granules in treatments B and C, but a decrease in treatment A. Treatment B had the highest value (55.90 ± 4.10 × 10^6 cells/mL) followed by treatment C and control. The lowest was treatment A (6.60 ± 1.98 × 10^6 cells/mL). Meanwhile, the amount of hyaline in treatments A, B, and C increased with the highest value in treatment A, then treatments B and C. In treatment D (control), the amount of granular and hyaline was consistent.

After the IMNV infection (7 dpi), the number of granules in treatments B and C decreased, while in treatment A increased. Treatment B had the highest value (15.50 ± 12.59 × 10^6 cells/mL), followed by treatments A and C which were not significantly different ($p > 0.05$). The amount of hyaline in treatments A and C also increased and was not significantly different, followed by treatment B. In treatment D (control), the granular value decreased and the hyaline increased. This trend is attributed to the fact that shrimp samples collected from this group were survivors, leading to a relatively high hyaline count (36.60 ± 17.25) × 10^6 cells/mL. The highest granular and hyaline values were observed in treatment B, supporting its superior survival rate (SR).

Treatment B demonstrated the best performance with a survival rate (SR) of 20%. The differential hemocyte count (DHC) improved with the addition of *L. japonica* extract, leading to a threefold increase in granular cells, followed by a 30% decrease after the challenge test. Meanwhile, the hyaline cell count increased 1.5 times with *L. japonica* supplementation and continued to rise by another 1.5 times after the challenge test. Additionally, treatment C recorded the highest overall cell count compared to all other treatments.

Infectious myonecrosis virus (IMNV) was first identified as a deadly infection in whiteleg shrimp in America and was introduced to Indonesia around 2006 (Srisala *et al.*, 2021). Recent studies have revealed that herbal medications have a wide range of positive effects, including growth promotion, improved meat quality, disease resistance, anti-stress, immunostimulant, tonic, aphrodisiac, and antibacterial activities (Citarasu *et al.*, 2022). Our findings showed that the addition of *L. japonica* extract can increase the survival rate of whiteleg shrimp after being challenged with IMNV. The best results were observed in Treatment B, where supplementation with 2% w/w *L. japonica* water extract resulted in a survival rate of 20% after the challenge test. This improvement is likely attributed to the active compounds in *L. japonica* extract, which stimulate the shrimp's immune response. The *L. japonica* extract contains several active substances, including phenolic acids, essential oils, flavones, saponins, and iridoids (Tang *et al.*, 2021), which have a broad spectrum of antioxidant, anti-inflammatory, antiviral, anti-microbial, and hepatoprotective activities (Shang *et al.*, 2011). Furthermore, Yu *et al.* (2013) reported that iridoids have antiviral activity against several viruses including influenza and Cox virus. A similar study by Jiang *et al.* (2022) stated that the extract of *L. japonica* can increase

the immune response of crayfish (*Procambarus clarkia*). Liu *et al.* (2020) also reported that *L. japonica* water extract reduces grouper mortality in the iridovirus infection.

Shrimp mortality began on day one after the challenge test. IMNV infection of shrimp is distinguished by total necrosis of the shrimp's abdomen striated muscles. The entire abdomen region has a diffuse milky white opacity. A reddish-pink necrotic coloration was visible on the tail fan and in the last segment. The infected organisms displayed abnormal behavior, and floating near the water surface, showing reduced feeding activity and subsequently experiencing mortality (Jha *et al.,* 2021). Prasad *et al.* (2017) stated that this condition occurs since these abdominal regions have the highest metabolic activity, especially when oxygen is scarce and during hyperactive stress. Similar findings have been reported by Tang *et al.* (2005) as well as Poulos and Lightner (2006).

Dietary supplementation of *L. japonica* water extract has been shown to enhance shrimp's immune response. Crustacean hemocytes play an important role in the immune system against pathogens, including viruses, bacteria, fungi, protozoa, and metazoa (Kulkarni *et al.,* 2021). Hemocyte count also serves as an indicator to assess health conditions and immunity (Kumar *et al.,* 2023). This finding was similar to the results of Chen *et al*. (2013). The authors demonstrated that feeding *L. japonica* increases the THC value in tiger shrimp compared with shrimp not fed with *L. japonica*. Rahmaningsih *et al*. (2021) also reported that administering *Crescentia cujete*, an herbal medicine, increases the THC. THC also increases after feeding with *Chaetoceros ceratosporum* (Ekawati *et al.,* 2012). The active compounds in *L. japonica* water extract include luteolin, quercetin, polyphenol, bioflavonoids, and dicaffeoylquinic acid. Water extract of *L. japonica* also has broad pharmacological activities, such as antibacterial, antiviral, antioxidant, and anti-inflammatory activity (Tang *et al.,* 2008). Furthermore, polyphenols contained in *L. japonica* extract have anti-inflammatory effects and help increase macrophage activity (Park *et al.,* 2012). These active compounds were able to diffuse into the shrimp's body and influence the shrimp's immune system through an increase in the number of hemocytes (THC).

This study showed that the supplementation of water extract of *L. japonica* increases the number of hyaline cells and granular hemocytes, which are the important defense mechanism of shrimp. Hyaline cells are the first line of defense against pathogen infection (Van De Braak *et al.,* 2002; Fikri *et al*., 2022). They are the smallest active phagocytes among the three types of hemocytes: granular, semi-granular, and hyaline. Hyaline cells engage in phagocytosis in response to stimulation from peroxinectin and masquerade-like protein, both of which are involved in cell adhesion and opsonic functions (Kulkarni *et al*., 2021). Crustacean hemocytes are classified based on the presence of cytoplasmic granules, with hyaline cells involved in phagocytosis, semi-granular cells responsible for encapsulation, and granular cells aiding in proPO system activation and cytotoxic responses (Kumar *et al.,* 2023). Previous studies have reported similar findings in different crustaceans during bacterial infections. Jiang *et al.* (2022) observed immune responses in crayfish (*Procambarus clarkii*), while Chen *et al.* (2013) documented THC increases in *Penaeus monodon*. Zhao *et al.* (2018) also found comparable immune-enhancing effects in the Chinese mitten crab (*Eriocheir sinensis*), further supporting the role of *L. japonica* extract in strengthening crustacean immunity.

Higher dietary doses of *L. japonica* extract (3% w/w) resulted in a lower immune response compared with 2% w/w. Awad and Awaad, (2017) suggested that a higher dietary intake of herbs depresses the immune response, therefore it is necessary to determine the optimal dose and its interaction with the fish immune system. Certain components, including saponins and other secondary metabolites may act as immunosuppressors. Furthermore, Raja and Poochirian, (2015) stated that immunosuppressors can inhibit the activation of immunological defense mechanisms or diminish the production of their components, and return immune function to normal levels. A similar finding was reported in zebrafish (*Danio rerio*)

supplemented with *Origanum vulgare* extract and challenged with *Aeromonas hydrophila* (Rashidian *et al.*, 2021). Additionally, Abidin *et al.* (2022) found that a 2.5% *Moringa oleifera* leaf extract was more effective in enhancing the immune response of whiteleg shrimp against *Vibrio alginolyticus* infection compared to a higher dose of 5%.

IMNV has spread globally, and in Indonesia, it was first introduced in 2006 through infected broodstock (Andrade *et al.*, 2022). Mai *et al.* (2019) reported that the IMNV genotype from East Java, Indonesia, has a unique structure compared with other isolates abroad, likely due to pathogen genome mutations. The application of *L. japonica* at a dose of 2% w/w for 14 days was found to reduce shrimp mortality caused by IMNV infection by up to 20% compared to the control group, which experienced 100% mortality. These findings suggest that *L. japonica* extract has potential as a preventive agent against IMNV infection. However, additional experiments need to be performed before definitive conclusions can be drawn on the efficacy of *L. japonica* extract in controlling IMNV infection.

CONCLUSION

Dietary supplementation with *L. japonica* water extract improves the survival of whiteleg shrimp by up to 20% compared to the control group and enhances the non-specific immune response following IMNV infection. These findings suggest that *L. japonica* extract can serve as a promising natural immunostimulant, offering a sustainable strategy to strengthen disease resistance and support healthier shrimp production in aquaculture.

ACKNOWLEDGEMENTS

The authors are grateful to the Brackishwater Aquaculture Development Center, Situbondo, East Java, Indonesia, for providing all materials and equipment used in this study.

AUTHORS' CONTRIBUTIONS

SS, JK, MM, TT, and BS contributed to the conceptualization, methodology, supervision, validation, formal analysis, investigation, and provision of resources. DSB, MAP, and MFU were responsible for software development, data curation, formal analysis, writing of the original draft, reviewing and editing the manuscript, as well as visualization. AI and JYL contributed to writing, reviewing, and editing the manuscript, in addition to visualization and formal analysis. All authors have read and approved the final version of the manuscript.

COMPETING INTERESTS

The authors declare that they have no competing interests.

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