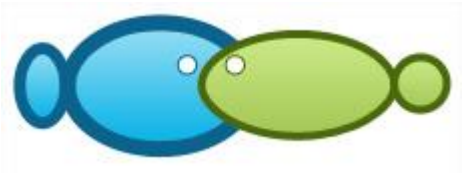

Dietary supplementation with *Phyllanthus urinaria* and *Terminalia catappa* enhances innate immunity and resistance to white spot syndrome virus in whiteleg shrimp (*Penaeus vannamei*)

Radma Abon Isnani, Hong Mong Huyen, Tran Thi My Duyen, Tran Thi Tuyet Hoa

College of Aquaculture and Fisheries, Can Tho University, Campus II, Xuan Khanh Ward, Ninh Kieu district, Can Tho City, Vietnam. Corresponding author: T. T. T. Hoa, ttthoa@ctu.edu.vn

Abstract. The present study was conducted to determine the effect of dietary supplementation of *Phyllanthus urinaria* and *Terminalia catappa* extracts on enhancing growth, innate immunity, and disease resistance of whiteleg shrimp *Penaeus vannamei*. A commercially available feed diet was incorporated with a concentration of 1% *P. urinaria* and 1% *T. catappa* extracts (experimental treatments), and the feed without herbal extract was considered the control treatment. The results showed that the dietary supplementation of 1% *P. urinaria* extract increased the daily weight gain and specific growth rate of shrimp ($p<0.05$). Dietary supplementation of 1% *P. urinaria* and 1% *T. catappa* extracts recorded a lower feed conversion ratio and had no effect on the survival rate of whiteleg shimp ($p>0.05$). The dietary supplementation of 1% *P. urinaria* and 1% *T. catappa* extracts significantly enhanced the shrimp non specific immune response after 4 weeks of herbal supplementation. The shrimp fed a diet containing 1% *T. catappa* extract showed significantly higher survival than those fed the control diet ($p<0.05$). Our results indicate that the supplementation of the herbal extracts in the diet resulted in beneficial effects in improving growth, immunity responses, and resistance of whiteleg shrimp against WSSV, specifically, at 1% *T. catappa* extract.

Key Words: growth performance, herbal extract, immune response, Penaeid, WSSV.

Introduction. Whiteleg shrimp *Penaeus vannamei* (Boone, 1931) is one of the most commercially important aquaculture species cultivated around the world that contributes to 80% of the world's total shrimp production (Anh et al 2019). The intensification of farming practices along with the environmental problems have resulted in stress causing disease outbreaks. In particular, the emergence of bacterial and viral disease outbreaks drives the shrimp farming industry to vulnerability, leading to serious losses of billions of dollars globally (Liu et al 2021). White spot syndrome virus (WSSV) is reported as the most devastating viral pathogen in cultured shrimp cultured, typically resulting in a reduction of stock by 80 to 100% (Shinn et al 2018).

The high susceptibility of shrimp to stress has forced farmers to focus on different biosecurity measures and the application of probiotics and antibiotics to prevent and treat diseases (Duc et al 2016). The application of antibiotics and other synthetic drugs has proved to be effective. However, excessive use has presented various disadvantages in both environmental and human health (Ben et al 2019; Serwecinska 2020). Moreover, vaccination or "tolerines" in aquaculture are known for their effectiveness in disease prevention, but they are costly and pathogen-specific (Miccoli et al 2021); therefore, an alternative is being used.

The use of herbal additives is promising, being a common source of therapeutics in aquaculture, since these products are safe, locally available, low cost, effective, and friendly prepared (Madhuri et al 2012). Herbal plants have a wide range of functions due to their active compounds like phenolic polyphenols, alkaloids, terpenoids, polypeptides,

flavonoids, tannins, saponins, steroids, pigments, and essential oils that are discussed by several studies to promote various biological activities such as: anti-stress, appetite stimulator, aphrodisiac, antifungal, antibacterial, antiviral, enhance immunity, and growth performance of farmed aquatic animals (Chakraborty & Hancz 2011; Madhuri et al 2012; Reverter et al 2017).

Phyllanthus urinaria (chamber bitter) and *Terminalia catappa* (tropical almond) are among the medicinal plants that can be used to enhance the growth performance, immune system and disease resistance of culture animals. Phytochemical screening of *P. urinaria* extract revealed the presence of flavonoids, terpenoids, tannins, phenolic acids, and lignans (Geethangili & Ding 2018). Moreover, saponin, tannins, flavonoids, triterpenoids, quinone and phenolic compounds were isolated as active components found in *T. catappa* leaves extract (Nugroho et al 2016). *T. catappa* leaves extract reportedly showed a strong radical scavenging action. Additionally, *T. catappa* extracts have been described as hypoglycaemic agents capable of regenerating pancreatic β-cells due to the presence of methanol and aqueous extracts (Nagappa et al 2003).

Previous research has reported the positive effects of *Phyllanthus amarus* and *T. catappa* extracts on the immune response and disease resistance of aquatic animals (Nhu et al 2020; Ngo et al 2020). To our knowledge, this study is the first to determine the effectiveness of *P. urinaria* and *T. catappa* extracts on the enhancement of resistance to WSSV in *P. vannamei*. This study was conducted to determine the immune parameters, growth performance, and disease resistance to WSSV of whiteleg shrimp fed a diet containing *P. urinaria* and *T. catappa* extracts.

Material and Method

Experimental shrimp. The study was conducted from September 2020 to January 2021 at the Department of Aquatic Pathology Laboratory, College of Aquaculture and Fisheries (CAF), Can Tho University, Vietnam. A stock of similar sizes of shrimp was acclimated to the laboratory condition for 2 weeks before the experimental period. During the acclimation period, shrimps were fed with a commercial diet, four times a day. Before the experiment, shrimps were randomly sampled for polymerase chain reaction (PCR) detection of *V. parahaemolyticus*, *V. harveyi,* and WSSV.

Herbal extract and diet preparation. The *T. catappa* (leaves) and *P. urinaria* (whole plant) were collected from the Mekong Delta, Vietnam. These herbal plants were washed, dried, and ground using a blender. The methanol extract was prepared by soaking the powdered plant materials in methanol with a ratio of 1:10 for 3 days at room temperatures. The extract was filtered and concentrated by using a rotary evaporator (Mariita et al 2011). The produced weight of the extracts obtained from *T. catappa* or *P. urinaria* in powder form was 12.94±0.34 g for 100 g and 7.29±0.26 g for 100 g, respectively.

Three diets were prepared by supplementing the standard diet (control diet), with 1% (*T. catappa*), and 1% (*P. urinaria*) kg^{-1} of feed. The dosage of herbal extracts was selected based on a previous study of Huyen et al (2020). The produced plant extracts were incorporated within the commercially available artificial pellet feed (CP 40%) at a concentration of 1% (10 g herbal extracts kg^{-1} of the commercial pellet). The herbal supplemented feeds and control feed were coated with squid oil (1%). The experimental diets were air-dried before being kept in plastic bags and stored at 4°C.

Feeding experiment. Shrimps with an initial weight of 0.61±0.08 g $individual^{-1}$ were randomly distributed and stocked in nine tanks (500 L), 70 shrimps $tank^{-1}$. Three treatments were designed for the experiment with control treatment (Control), 1% *T. catappa* treatment (1% TCE), and 1% *P. urinaria* treatment (1% PUE).

The shrimps in each treatment were fed with a control diet and herbal supplemented diets, four times a day (at 7:00, 11:00, 13:00, and 17:00 h) at the rate of 5% of body weight for 4 weeks. The shrimps were randomly weighed every week (10 shrimp per tank), and daily feed allocation was modified accordingly. Tanks were provided with continuous aeration, and 50% of the water was replaced weekly with

freshly dechlorinated water throughout 4 weeks feeding trial. To keep good hygienic conditions, uneaten food, fecal matter and dead shrimp were removed and siphoned out daily. During the feeding trial, water parameters were measured and maintained within normal values for the optimal growth of the shrimp: salinity (15‰), temperature (28-30°C), pH (7.7-8.3), dissolved oxygen (4.36 mg L^{-1}), ammonia (0.5-1 mg L^{-1}), nitrites (4.5-4.7 mg L^{-1}), and alkalinity (110 $CaCO_3$ mg L^{-1}), respectively.

Growth performance analysis. After 4 weeks of the feeding experiment, shrimps were starved for 24 h before the final sampling. Shrimps in each tank were individually weighed and counted to determine the growth and survival rate. Growth performance was measured as daily weight gain (DWG), specific growth rate (SGR), feed conversion ratio (FCR), survival rate (SR) and calculated based on the following equations:

Daily weight gain (DWG, g/day) = [final weight (g) − initial weight (g)]/ [time (40 days)]

Weight gain (Wg, %) = [final weight (g) − initial weight (g)]/ initial weight (g) × 100

FCR = feed intake (FI)/ (weight gain)

SGR (% day^{-1}) = [ln final weight (g) − ln initial weight (g)]/ [time (40 days)] × 100

Survival rate (SR, %) = [(Final no. of shrimp)/(Initial no. of shrimp)] × 100

Immunological parameters analysis. After 4 weeks of the feeding experiment, nine shrimps from each treatment were collected randomly for analysis of haematological and immunological parameters such as: total haemocyte count, differential haemocyte count, phenoloxidase activity, superoxide dismutase activity, and immune genes expression.

Total haemocyte count (THC). A volume of approximately 100 µL haemolymph was withdrawn from the pleopod base of the first abdominal segment of the shrimps with a sterile 1 mL syringe and gently mixed with 900 µL of sterile anticoagulant solution (trisodium citrate 30 mM, NaCl 338 mM, glucose 115 mM, EDTA 10 mM, pH 7). Then, 10 µL of the diluted haemolymph were immediately determined for THC using a Neubauer haemocytometer. THC on the haemocytometer was observed under a light microscope and the cells were counted (Le Moullac et al 1997).

Differential haemocyte count (DHC). The withdrawn haemolymph mixture was centrifuged at 5000 rpm for 5 min at 4°C, washed, and re-suspended with 200 µL of formalin-AS pH 4.6 (1:10). A haemocyte suspension was spread into glass slides, fixed in ethanol, stained with Giemsa for 30 min, and rinsed in acetone and xylene. The stained-glass slide was used for the identification and enumeration of granular cells; hyaline cells identification was based on the methods described by Le Moullac et al (1997); cells were observed and counted (200 cells $sample^{-1}$) under a light microscope.

Phenoloxidase (PO) activity. Total phenoloxidase activity was determined by using L-Dihydroxyphenylalanine (L-DOPA) (Hernandez-Lopez et al 1996). Haemolymph (100 µL) was collected from the ventral sinus of the shrimp and mixed with 900 µL of the sterile anticoagulant solution. Haemolymph withdrawn in anticoagulant was centrifuged at 2500 rpm for 20 min at 4°C, washed and re-suspended gently in 1000 µL Cacodylate Citrate buffer solution (pH 7). The samples were centrifuged again in the same conditions, washed, and re-suspended in a 200 µL Cacodylate Citrate buffer solution (pH 7). The suspension of 100 µL was incubated with 50 µL Trypsin solution (mL mg^{-1}) and Cacodylate Citrate buffer solution (pH 7) (control tube). A 50 µL aliquot of L-DOPA was added, then 800 µL Cacodylate Citrate buffer solution (pH 7), and measured using a spectrophotometer at 490 nm.

Superoxide dismutase (SOD) activity. Superoxide dismutase activity was determined according to the method described by Beauchamp & Fridovich (1971), using nitroblue tetrazolium (NBT) in the presence of riboflavin. Haemolymph (100 µL) was diluted in 500 µL buffer phosphate and centrifuged at 5000 g for 5 min at 4°C. The supernatant was heated up to 65°C for 5 min to acquire SOD crude extract. Then, a 150 µL of SOD crude extract was added to 50 µL nitroblue tetrazolium (NBT) reagent (0.1 mM EDTA, 13 µM methionine. 0.75 mM NBT and 20 µM riboflavin in 50 mM phosphate buffer, pH 7.8) and incubated for 2 min. The optical density was measured using a spectrophotometer at 560 nm.

RNA extraction, cDNA synthesis, and real-time PCR. The RNAzol reagent (Molecular Research Center, Inc. USA) was used to isolate total RNA from the haemolymph of shrimp, which was collected after 4 weeks of herbal supplementation.

A 1 µg total RNA sample was synthesized to cDNA according to the SensiFast cDNA synthesis kit (Bioline) following the manufacturer's procedures. The produced cDNA was used as a template to determine the relative expression of the lysozyme and penaeidin-3 in haemolymph samples with a real-time PCR (Biorad) and the gene specific primers listed in Table 1 with *β*-actin as an internal control to calculate fold change in the target genes (Wang et al 2008).

The PCR mix contains 1 × ROX qPCR Master Mix, cDNA template, 0.5 µM F primer, 0.5 µM R primer, and free water. The reaction times and cycling conditions were set at 95℃ for 3 min, followed by 95℃ for 15 sec, 40 cycles of 56°C for 30 sec and 72°C for 30 sec. The critical threshold (Ct) quantifies of the target genes were normalized with quantities (Ct) of *β*-actin using the $2^{-\Delta\Delta}$Ct method (Schmittgen & Livak 2008).

Table 1

Primers used for RT-qPCR in the present study

Gene	*Primer sequences (5′ to 3′)*	*GenBank #*	*Products (bp)*
Lysozyme	F: GGA CTA CGG CAT CTT CCA GA R: ATC GGA CAT CAG ATC GGA AC	AY170126	97
Penaeidin-3	F: CAC CCT TCG TGA GAC CTT TG R: AAT ATC CCT TTC CCA CGT GAC	Y14926	121
β-actin	F: CCA CGA GAC CAC CTA CAAC R: AGC GAG GGC AGT GAT TTC	AF300705	142

WSSV challenge test. After 4 weeks of the feeding experiment, the challenge experiment was carried out by immersion method (Huyen & Hoa 2015) at the lethal dose LD_{75}. The challenge experiment was conducted with four treatments and three replicates. Thirty shrimps from each treatment (10 shrimp per replicate) of the feeding experiment were challenged with WSSV (Po-control, 1% TCE, and 1% PUE), and one treatment served as a negative control (Ne-control, not being challenged to WSSV). During the 14 days of the challenge test, shrimps were fed with their corresponding treatment diets (twice a day) until the end of the challenge period. The negative and positive controls were fed with commercial feed diets. The dead shrimp, uneaten feeds, and waste were removed daily.

The clinical signs and mortality rate were observed and recorded daily. Moribund shrimp were collected for WSSV confirmation by the PCR method (Kimura et al 1996). Samples of nine shrimps per treatment were collected on the third day of the challenge test for analysis of haematological and immunological parameters: total haemocyte count, differential haemocyte count, phenoloxidase activity, superoxide dismutase activity, and immune genes expression.

Statistical analysis. Data were presented as mean±SD. A one-way analysis of variance (ANOVA; $p<0.05$) was used to calculate and compare the data. Duncan's test was used in multiple comparisons to assess the differences between treatment means at a 95%

confidence level. Comparison of differences between groups (after 4 weeks of herbal supplementation and after infection) was performed by using the Independent-Samples t-Test. Statistical significance of differences requires the p values to be less than 0.05.

Results and Discussion

Effects of dietary supplementation of herbal extracts on the growth performance and survival rate of experiment shrimp. The daily weight gain (DWG) and specific growth rate (SGR) were statistically found highest ($p<0.05$) in supplemented the 1% *P. urinaria* extract (PUE) treatment compared to the control treatment. The feed conversion ratio (FCR) was found lowest in supplemented treatment (at 1.11 for 1% TCE and 1.05 for 1% PUE), but it was not significantly different ($p>0.05$) to that of the control treatment (1.21). The survival rate did not show a statistically significant difference ($p>0.05$) among treatments (Table 2).

Table 2

Growth performance and survival of *Penaeus vannamei* after 4 weeks of herbal supplementation

Treatment	*DWG (g day^{-1})*	*SGR (% day^{-1})*	*FCR*	*Survival rate (%)*
Control	0.15 ± 0.02^{a}	6.07 ± 0.32^{a}	1.21 ± 0.01^{b}	86.36 ± 6.60^{a}
1% TCE	0.17 ± 0.03^{ab}	6.47 ± 0.44^{ab}	1.11 ± 0.11^{ab}	88.38 ± 0.87^{a}
1% PUE	0.19 ± 0.02^{b}	6.71 ± 0.36^{b}	1.05 ± 0.12^{ab}	86.36 ± 5.45^{a}

Note: DWG - daily weight gain; SGR - specific growth rate; FCR - feed conversion ratio; TCE - *T. catappa* treatment; PUE - *P. urinaria* treatment; values are means of three replicate groups ±SD; within a column, different superscripts represent significant differences ($p<0.05$).

Total haemocyte count (THC). After 4 weeks of herbal supplementation, the THC of shrimp was increased in the supplemented 1% TCE and 1% PUE treatments. The THC was recorded highest in 1% TCE and lowest in the control, the difference being statistically significant ($p<0.05$) among treatments (Table 3).

Differential haemocyte count (DHC). The DHC as presented by granular cell (GC) and hyaline cell (HC) of shrimp is presented in Table 3. The GC and HC of shrimp increased in the supplemented treatments of 1% TCE and 1% PUE. Similarly, the GC and HC were highest in 1% TCE and lowest in the control treatment, the difference being statistically significant ($p<0.05$) among the treatments.

Table 3

Immunological parameters of *Penaeus vannamei* after 4 weeks of herbal supplementation

Treatments	*THC*	*GC ($\times 10^6$ cells mL^{-1})*	*HC*	*PO (490 nm)*	*SOD (U mL^{-1})*
Control	14.73 ± 1.15^{a}	1.71 ± 0.41^{a}	13.02 ± 0.96^{a}	0.137 ± 0.016^{a}	1.089 ± 0.120^{a}
1% TCE	22.25 ± 1.03^{c}	3.12 ± 0.62^{c}	19.13 ± 0.53^{c}	0.178 ± 0.014^{b}	2.195 ± 0.385^{c}
1% PUE	19.52 ± 1.44^{b}	2.49 ± 0.41^{b}	17.03 ± 1.20^{b}	0.150 ± 0.017^{a}	1.455 ± 0.250^{b}

Note: THC - total haematocyte count; GC - granular cell; HC - hyaline cell; PO - phenoloxidase; SOD - superoxide dismutase; TCE - *T. catappa* treatment; PUE - *P. urinaria* treatment; values are means of three replicate groups ±SD; within a column, different superscripts represent significant differences ($p<0.05$).

Phenoloxidase (PO) activity. The PO activity after 4 weeks of herbal supplementation was highest in the shrimp fed with supplemented 1% TCE and the difference was statistically significant ($p<0.05$) compared to other treatments (Table 3).

Superoxide dismutase (SOD) activity. The SOD activity of shrimp increased in the experimental treatments, while it was the lowest in control. The differences were statistically significant ($p<0.05$) among treatments (Table 3).

Immune gene expression of lysozyme and penaeidin-3. To determine the transcriptional responses of shrimp *P. vannamei* to *P. urinaria* and *T. catappa* extracts supplemented diets, mRNA expressions of two immune-related genes were assessed. After 4 weeks of herbal supplementation, the mRNA expression of the lysozyme gene was highest in control, but it was not significantly different from that of 1% TCE (Figure 1A). On the other hand, the expression of penaeidin-3 was upregulated in the supplemented treatments compared to the control and the difference was statistically significant ($p<0.05$) (Figure 1B).

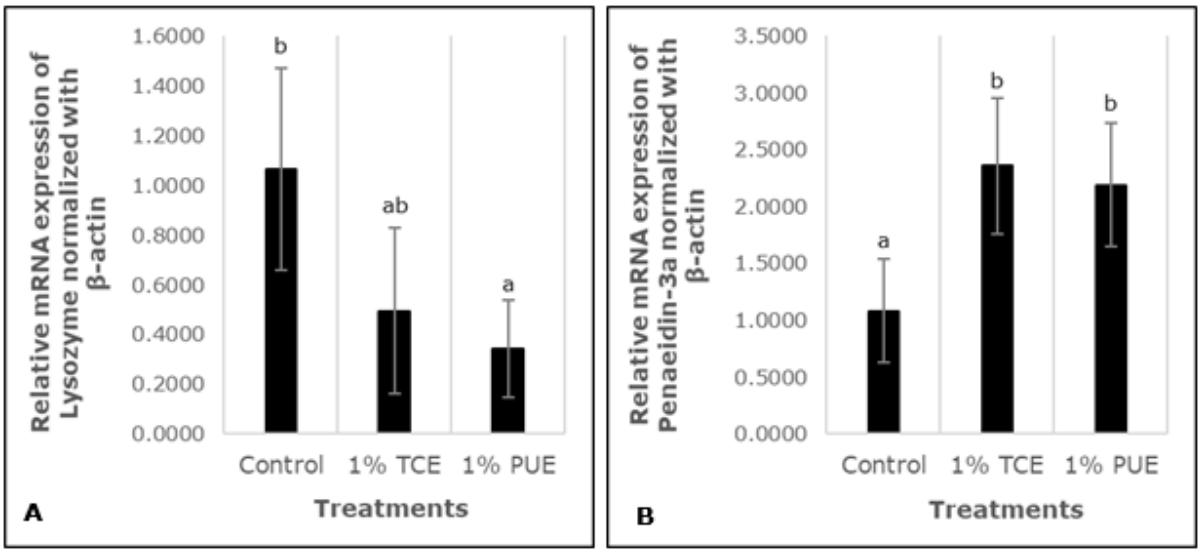

Figure 1. Relative mRNA expression of (A) lysozyme and (B) penaeidin-3 of shrimp *Penaeus vannamei* after 4 weeks of herbal supplementation; TCE - *T. catappa* treatment; PUE - *P. urinaria* treatment; The same superscripts among treatments indicate no significant differences ($p>0.05$).

Effects of dietary supplementation of herbal extracts on the resistance against white spot disease in experiment shrimp. The obtained cumulative mortality showed that the supplemented 1% TCE and 1% PUE extracts were significantly lower compared to the positive control during the 14 days of the challenge experiment (Figure 2). Specifically, the cumulative mortality of *P. vannamei* fed with supplemented diets of 1% TCE, 1% PUE, and Po-control were 53.33%, 63.33%, and 76.67%, respectively. A significant difference ($p<0.05$) was found in supplemented 1% TCE; however, 1% PUE results were not significantly different ($p>0.05$) from those of the positive control. Thus, the Ne-control showed no mortality.

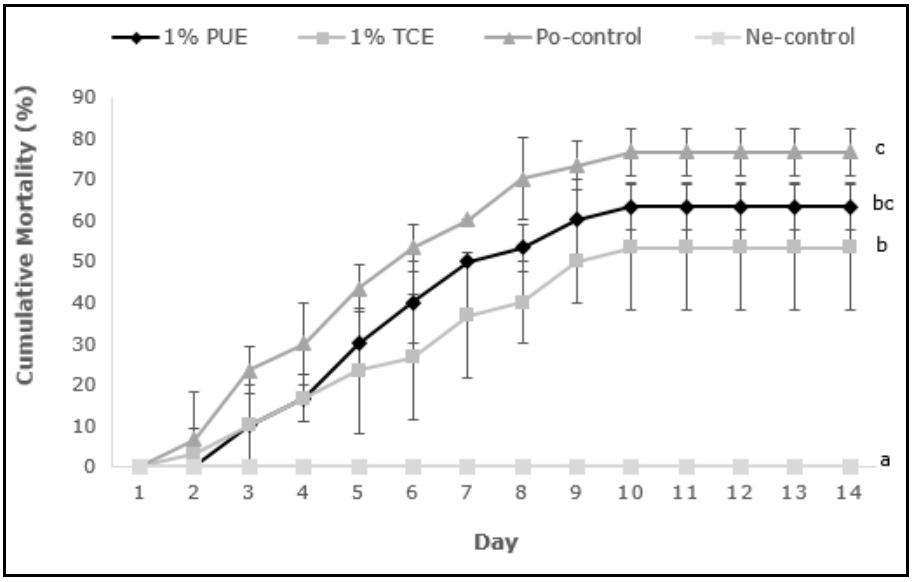

Figure 2. Cumulative mortality of *Penaeus vannamei* challenged with WSSV during the 14 days post-challenge; TCE - *T. catappa* treatment; PUE - *P. urinaria* treatment; Po-control - positive control; Ne-control - negative control. Different superscripts indicate significant differences ($p<0.05$).

During the 14 days of the challenge test, shrimp was observed and recorded for the presence of clinical signs. The shrimp in the treatments (Po-control, 1% TCE, and 1% PUE) challenged with WSSV showed clinical signs such as: white spots on the exoskeleton (Figure 3A), loosening of the cuticle, appetite loss and lethargy. The shrimp were also collected and the presence of WSSV was determined by the PCR method (Figure 3B). Figure 3B showed that challenged shrimp of Po-control, 1% TCE, and 1% PUE treatments (lanes 1, 2, and 3) obtained a bright positive band for WSSV (570 bp). On the other hand, the Ne-control shrimp (lane 4) was not experimentally infected with WSSV and did not show bright bands. The results concluded that the shrimp mortality during the challenge test was caused by WSSV.

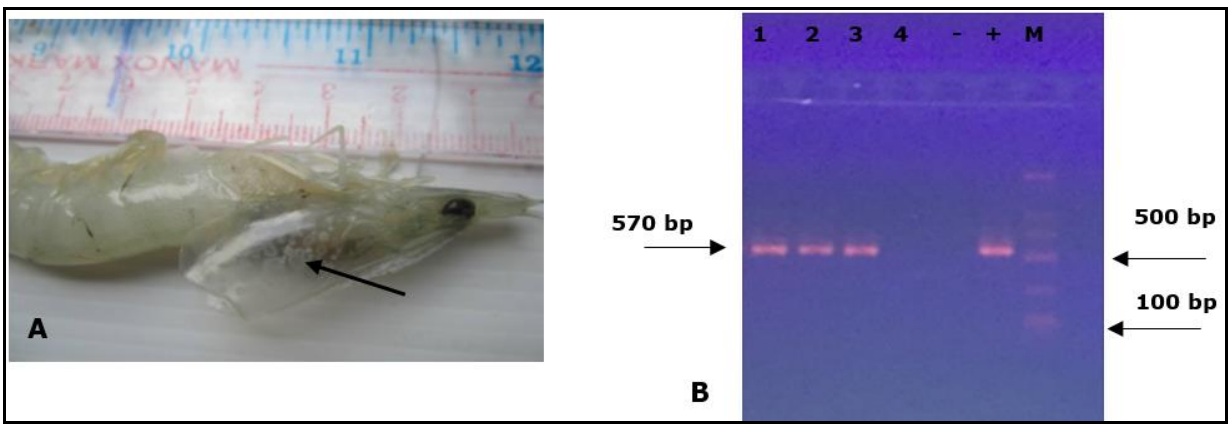

Figure 3. Clinical signs of the experimental shrimp post-infection with WSSV; (A) presence of white spots on the head exoskeleton (arrow); (B) PCR results of experimental shrimp post-infection with WSSV. Lane M: DNA ladder; Lane (+): positive control of PCR; Lane (-): negative control of PCR; Lane 1: positive control; Lane 2: 1% *T. catappa* treatment; Lane 3: 1% *P. urinaria* treatment; and Lane 4: negative control.

After infection, THC values significantly increased in all treatments. The 1% TCE recorded the highest value followed by 1% PUE and Po-control, the differences being statistically significant ($p<0.05$) among treatments (Table 4). Similarly, the GC increased in 1% TCE

and the difference was statistically significant ($p<0.05$) compared to the control treatments. The HC values increased significantly in all treatments (1% TCE, 1% PUE, Po-control), and a significant difference ($p<0.05$) was recorded among treatments. However, the Ne-control value was not significantly different from the Po-control.

After being challenged to WSSV, the PO values of shrimp after infection increased in all treatments (Table 4). A significantly higher PO value ($p<0.05$) was observed in shrimp receiving 1% TCE than in shrimp from other treatments. Nonetheless, the Ne-control recorded the lowest value, being different significantly ($p<0.05$) from the values recorded in other treatments. The SOD activity increased in the herbal supplemented treatments and differed significantly ($p<0.05$) compared to that of the control treatments (Table 4).

Table 4

Immunological parameters of *Penaeus vannamei* after infection

Treatments	*THC*	*GC (× 10^6 cells mL^{-1})*	*HC*	*PO (490 nm)*	*SOD (U mL^{-1})*
Ne-control	15.00±1.32^{a}	1.71±0.33^{a}	13.29±1.28^{a}	0.139±0.011^{a}	1.064±0.175^{a}
Po-control	15.93±1.38^{a}	2.62±0.49^{ab}	13.31±1.34^{a}	0.152±0.012^{b}	1.184±0.214^{a}
1% TCE	23.99±1.27^{c}	3.65±1.19^{c}	20.34±1.62^{c}	0.182±0.013^{c}	1.669±0.281^{b}
1% PUE	21.43±1.38^{b}	3.38±1.38^{bc}	18.05±0.93^{b}	0.164±0.011^{b}	1.493±0.295^{b}

Note: THC - total haematocyte count; GC - granular cell; HC - hyaline cell; PO - phenoloxidase; SOD - superoxide dismutase; TCE - *T. catappa* treatment; PUE - *P. urinaria* treatment; values are means of three replicate groups ±SD; within a column, different superscripts represent significant differences ($p<0.05$).

For the immune gene expression, the expression of lysozyme was downregulated in 1% TCE and Po-control, while upregulated in 1% PUE (Figure 4A). Thus, the Ne-control recorded the highest expression, but the difference was not significantly different ($p>0.05$) in all treatments. The expression of penaeidin-3 was significantly downregulated in all treatments and Po-control recorded the lowest expression, without being significantly different from other treatments (Figure 4B).

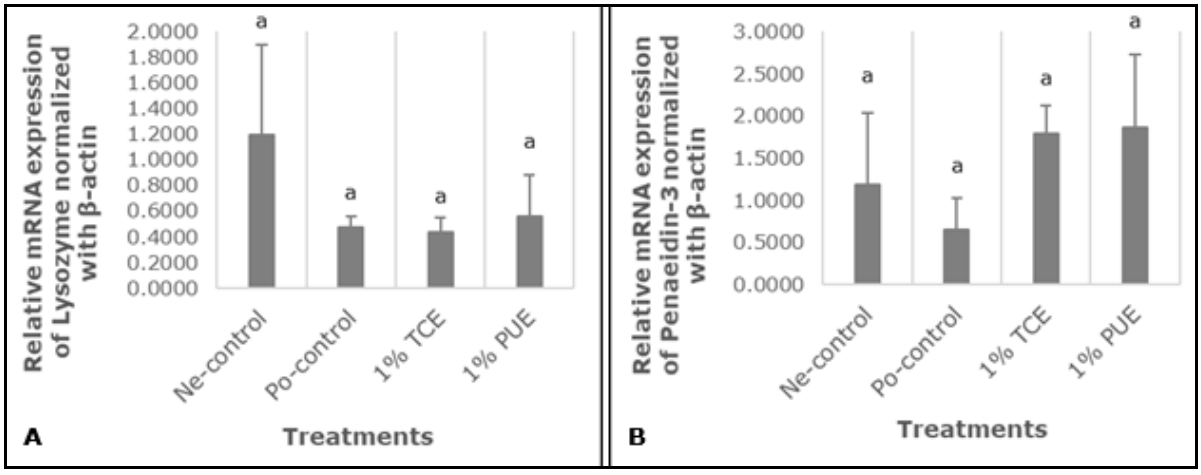

Figure 4. Relative mRNA expression of lysozyme (A) and penaeidin-3 (B) of experiment shrimp after WSSV infection. The same superscript letters indicate no significant differences ($p>0.05$); TCE - *T. catappa* treatment; PUE - *P. urinaria* treatment; Po-control - positive control; Ne-control - negative control.

Haematological parameters. The comparison of the haematological parameters of the non-infected shrimp (after 4 weeks of herbal supplementation) and shrimp infected with WSSV (after infection) was determined in Figure 5.

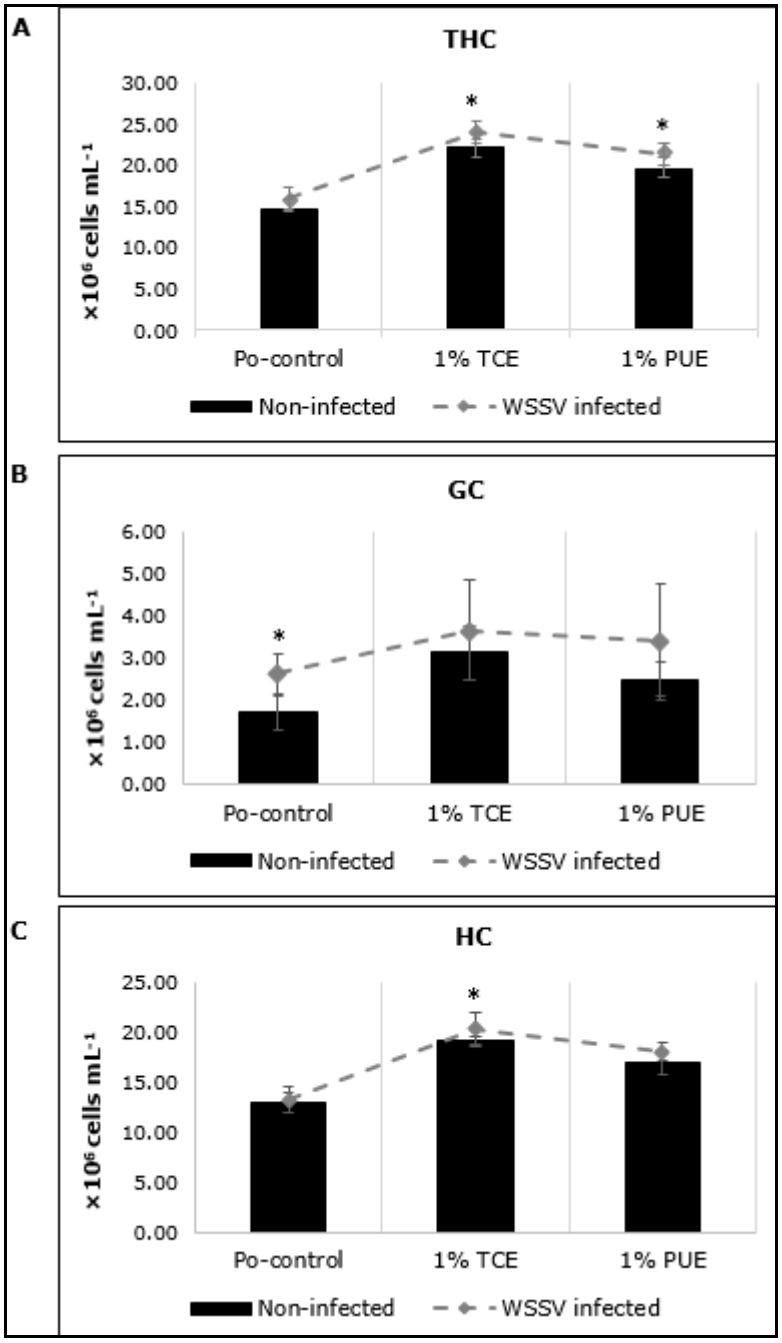

Figure 5. Haematological parameters; (a) total haemocyte count (THC); (b) granular cell (GC); (c) hyaline cell (HC) of *Penaeus vannamei* after 4 weeks of herbal supplementation and after WSSV infection; TCE - *T. catappa* treatment; PUE - *P. urinaria* treatment; Po-control - positive control; asterisks indicate a significant difference ($p<0.05$).

The THC values presented statistically significant differences ($p<0.05$) in the supplemented treatments of 1% TCE and 1% PUE compared to the Po-control. The GC values increased in supplemented treatments, but the differences were not statistically significant. However, Po-control showed a statistically significant difference ($p<0.05$) compared to the supplemented treatments. Thus, the HC values in 1% TCE were significantly different ($p<0.05$) compared to those of other treatments.

Immunological parameters. Figure 6 showed the comparison of immunological parameters of the non-infected shrimp (after 4 weeks of herbal supplementation) and shrimp infected with WSSV (3rd day after infection). The PO activity was significantly

different ($p<0.05$) in Po-control compared to that of the herbal supplemented treatments. Moreover, SOD activity showed a significant difference ($p<0.05$) in 1% TCE compared to that of other treatments, considering the activity decreased after the shrimp were infected with WSSV.

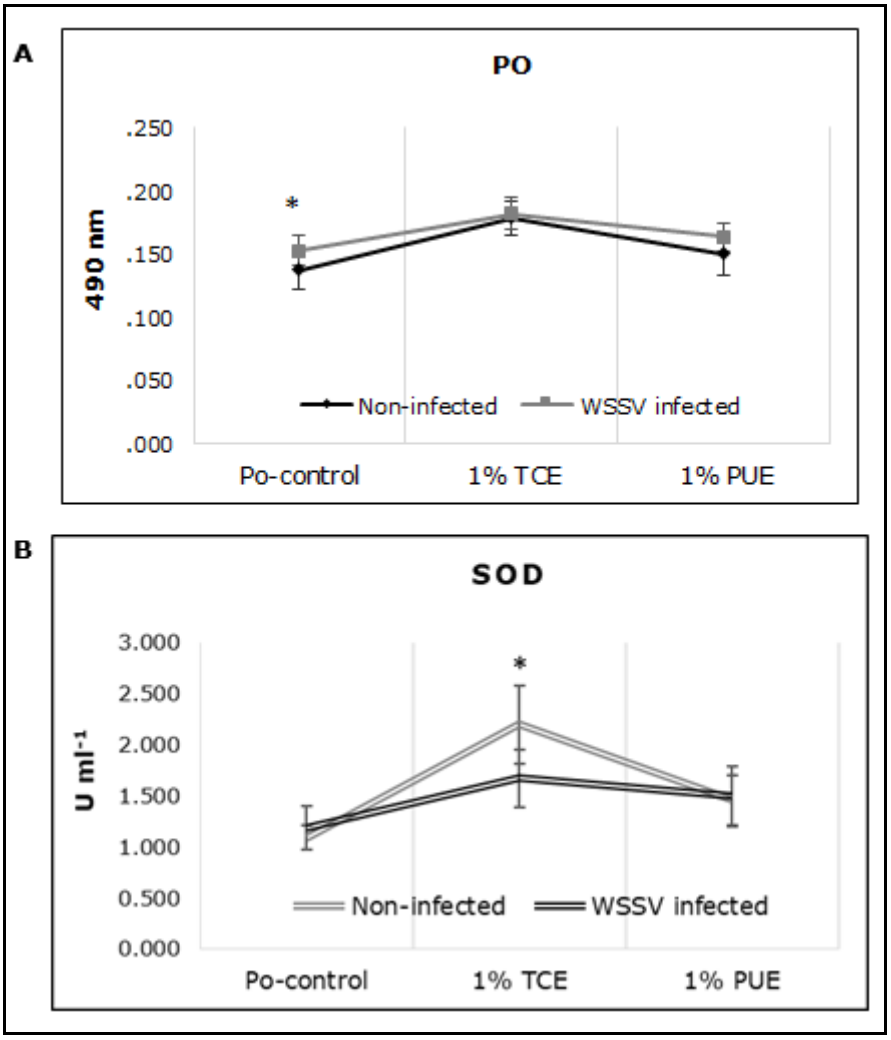

Figure 6. Phenoloxidase (PO) (A); and superoxide dismutase (SOD) (B) activities of *Penaeus vannamei* after 4 weeks of herbal supplementation and after infection; TCE - *T. catappa* treatment; PUE - *P. urinaria* treatment; Po-control - positive control; asterisks indicate a significant difference ($p<0.05$).

The comparison of the mRNA expressions of lysozyme and penaeidin-3 genes of the non-infected shrimp (after 4 weeks of herbal supplementation) and shrimp infected with WSSV (after infection) is presented in Figure 7. The expression of lysozyme and penaeidin-3 was downregulated in all treatments and no significant differences ($p>0.05$) were found.

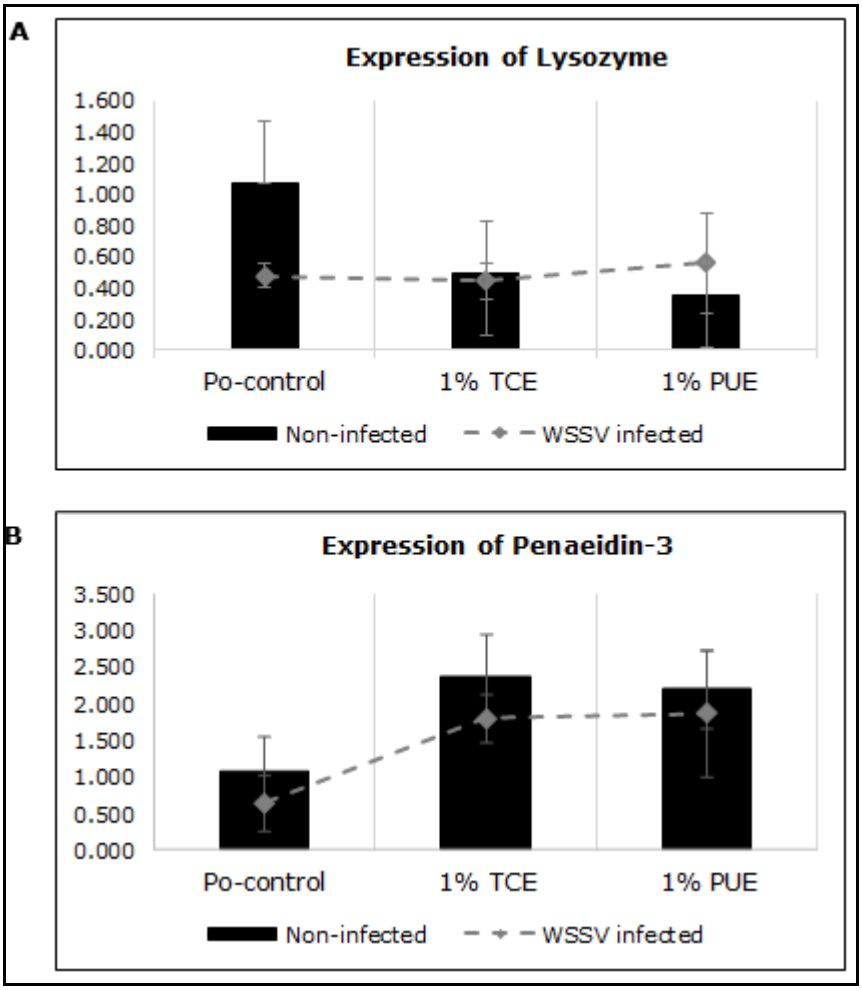

Figure 7. Relative mRNA expression of (A) lysozyme and (B) penaeidin-3 of shrimp *Penaeus vannamei* after 4 weeks of herbal supplementation and after infecting the groups. CE - *T. catappa* treatment; PUE - *P. urinaria* treatment; Po-control - positive control; the absence of an asterisk indicates no significant differences ($p>0.05$).

Several studies on the application of herbal plants in aquaculture have been described by various authors. Thus, findings proved that herbal extracts are effective in improving growth, survival, immune response and disease resistance of different species of cultured shrimp (Citarasu et al 2006; Rajasekar et al 2011; Prathomya et al 2019). The present study design to evaluate the effect of *T. catappa* and *P. urinaria* extracts supplementation on the growth and health status of *P. vannamei* provided similar results.

Accordingly, the growth rate is a measure of the extent of utilization of the food provided to the cultured animal. The present study found that the supplemented diet containing 1% *P. urinaria* extract performed better in growth (DWG and SGR), but the results were not significantly different to those of shrimp fed supplemented 1% *T. catappa* extract. The results were similar to a recent study where a diet containing *Phyllanthus amarus* extract (20 g kg^{-1}) recorded the highest weight gain and SGR in *L. vannamei* (Ngo et al 2020). However, supplementation of *P. urinaria* crude extract (10000 ppm) fed to shrimp showed a slightly higher growth performance and feed utilization (Charoendat & Koedprang 2019). Moreover, some species of *Phyllanthus* were also reported to improve growth performance of different cultured animals. The dietary supplementation of ethanol extract of *Phyllanthus emblica* significantly increases the SGR of tilapia (*Tilapia mossambicus*) (Sivagurunathan et al 2012), and *Phyllanthus niruri* (2%) powder enhanced the growth of *Cyprinus carpio* (Sunitha et al 2017). The FCR values obtained were lower when using supplemented treatments of 1% *T. catappa* and 1% *P. urinaria* extracts, but not significantly different to those of the control. Lower FCR means

a more efficacious utilization of the feed nutrients by the organism (Pholdaeng & Pongsamart 2010). This result is in agreement with the study of Ngo et al (2020), where the lowest FCR occurred when using *P. amarus* extract supplemented diet (20 g kg^{-1}), but it was not significantly different to the results of the control. Contrarily, the ethanol extract of *P. emblica* in the diet increased the FCR of tilapia (Sivagurunathan et al 2012). The survival rate in the present study did not record a significant difference among treatments. This finding was similar to a previous study on the dietary supplementation of *P. urinaria* crude extract (Charoendat & Koedprang 2019), and *Astragalus membranaceus*, *Codonopsis pilosula* and *Glycyrrhiza uralensis* extracts (Prathomya et al 2019) in shrimp. Additionally, the different results between studies on the effects of herbal supplementation on growth performance are thought to be attributable by types of herbal products, concentrations, and exposure period (Chang et al 2017). In this study, *P. urinaria* extracts were demonstrated to have a better growth promoter effect compared to other treatments. The supplemented herbal extracts were not toxic, as the recorded survival rate ranged between 86-88% and was not different from control treatment.

On the other hand, the lack of an adaptive immune system of shrimp and other kinds of crustaceans made them highly dependent on their non-specific immune system (Mekata et al 2010) to stand against environmental stresses and harmful pathogens (Cheng & Chen 2000). Shrimp immunity is based on a series of intricate defense processes that are mostly initiated and driven by haemocytes generated during an immune response (Soderhall & Cerenius 1992). THC and DHC have been utilized as valid predictors of stress in crustaceans (Lorenzon et al 2001; Urbina et al 2013). The present study showed that supplemented diets of 1% *P. urinaria* and 1% *T. catappa* extracts significantly enhanced the THC and DHC by increasing the number of hyaline and granular cells of the *P. vannamei* at both sampling times compared to the control. The increase in THC of shrimp might be due to the rapid maturation of haemocyte in the haematopoietic tissue following a discharge of new cells into the circulation system to maintain the haemocyte population (Sequeira et al 1996). Thus, dietary supplementation of *A. membranaceus*, *C. pilosula*, and *G. uralensis* had a positive effect on the THC and increased the granulocytes number in *L. vannamei* (Prathomya et al 2019). Similarly, higher THC was also observed in shrimp fed diets supplemented with herbal extracts from *Boerhaavia diffusa* (Chithambaran & David 2014) and *P. amarus* (Ngo et al 2020).

The phenoloxidase enzyme is part of the proPo-activating system and is responsible for the melanization process, which is frequently seen as a response to a pathogen within a crustacean's body cavity (Soderhall & Cerenius 1992). The PO activity isolated from shrimp of the supplemented diet with 1% *T. catappa* extract recorded the highest effect after the feeding trial and further increased the effect after infection challenge compared to other treatments. Similarly, Wongprasert et al (2014) found higher levels of PO activity after supplementation with sulfated galactans (SG) and gradually increased after WSSV inoculum. Thus, supplementation of *P. amarus* extracts in a diet was found to enhance the PO activity of *L. vannamei* in two feeding experiments (Ngo et al 2020). The *T. catappa* leaves extract was reported to improve the innate immune response of some cultured fish (Nugroho et al 2016; Yakubu et al 2020).

The SOD activity of shrimp groups with a supplemented diet of 1% *T. catappa* extract was enhanced compared to other treatments after the feeding trial. However, the SOD activity of shrimp decreased after the infection challenge. This result is also in agreement with Wongprasert et al (2014) with the addition of SG fed to shrimp. SOD is one of the main antioxidant enzymes that play an important role in shrimp's defense system by scavenging superoxide anion (Lin et al 2011). Superoxide anion has the function of killing invaded pathogens (Bogdan et al 2000). The herbal supplemented diets of the present study showed promising results on immune response i.e. THC, HC, GC, PO, and SOD activities of *P. vannamei*, which might be attributed to the presence of the bioactive compounds in plant extracts having biological activities. The presence of flavonoids in *T. catappa* extract is beneficial for boosting immune function; it is also an antioxidant enzyme that prevents cellular damage and improves the immunological competence of an organism (Saroja et al 2012).

Recent reports have studied the effect of immunostimulants extracted from several types of plants in enhancing lysozyme gene (Wang et al 2008) and penaeidin gene expression (Trejo-Flores et al 2018) in cultured shrimp. Lysozyme and penaeidin are important antimicrobial protein and peptide, respectively, produced by shrimp haemocytes. The lysozyme acts as a non-specific innate immunity molecule against invading bacterial pathogens (Wang et al 2008). The penaeidin gene is associated with local defense from the release of haemocytes and binds to cuticle surfaces of the shrimp (Destoumieux et al 1999). After 4 weeks of herbal supplementation, the expression of the lysozyme gene in diets with 1% *T. catappa* and 1% *P. urinaria* extracts was downregulated compared to the control. Our observation was in line with Wang et al (2008), who reported the response pattern of lysozyme as short-term up-regulation. Lysozyme gene expression was upregulated after 6 h of supplementation of β-1,3-glucan, then returned to the control level at 24 h and 72 h, and downregulated post 7 days of supplementation (Wang et al 2008). Contrarily, the mRNA expression of penaeidin-3 gene in supplemented groups was significantly upregulated compared to the control, suggesting that its early expression in haemocytes might be due to immunostimulant substances present in *T. catappa* and *P. urinaria*. Wang et al (2008) mentioned that the upregulation of penaeidin-3 gene in response to immunostimulation takes up the tank of energy and transcriptional machinery in haemocytes. The upregulation of this gene might cause competition with other genes in expression and result in slowing down or even stopping the transcription of other genes leading to the downregulation of the genes. Furthermore, the downregulation of lysozyme and penaeidin-3 after the infection challenge might be attributed to WSSV infection. Bangrak et al (2004) found a decrease of translationally controlled tumour protein (TCTP) in *P. monodon* after being subjected to high WSSV infection. The viral proteins negatively regulate the transcription of TCTP, where the infected haemocytes cause the necrobiosis pathway, resulting in the downregulation of survival genes. Hence, studies and information on the transcriptional responses of cultured shrimp and fish to *T. catappa* and *P. urinaria* extracts are still lacking. To fully understand the mechanism underlying the upregulation and downregulation of certain genes further research is necessary.

Survival after a certain pathogen challenge is considered a measure of disease resistance (Adrino et al 2012). The present study showed that shrimp fed with supplemented 1% *T. catappa* and 1% *P. urinaria* extracts increased protection and susceptibility to WSSV immersion compared to the control treatment. Following the challenge with WSSV, the lowest mortality was observed in the supplemented treatment of 1% *T. catappa* extract compared to the supplemented with 1% *P. urinaria* extract and the positive control. The positive control recorded the highest mortality of 77% after the infection challenge, which means that the immune parameters were reduced, decreasing its defense ability after some time (Yakubu et al 2020). Adrino et al (2012) explained that factors such as a stable water system and the volume of experimental containers might be resulting in the rapid progression of infection considering the lower amount of dissolved oxygen present. Thus, stress caused by low dissolved oxygen levels could make the shrimp more vulnerable to infection.

The *T. catappa* extract has been demonstrated in numerous studies to reduce the mortality rate of cultured fish and shrimp when exposed to fungi, parasites, bacteria, and viruses (Chitmanat et al 2005; Mohale et al 2009; Ikhwanuddin et al 2014). In particular, the percent survival of a supplemented treatment of 1% *T. catappa* extract in the present study is much lower (47%) compared to the study of Yakubu et al (2020) of 70% and 57% after dietary supplementation of *T. catappa* in red hybrid tilapia (*Oreochromis* sp.) against *Streptococcus agalactiae*. Based on the previous and present studies, *T. catappa* extract has antiviral activity and its incorporation in a diet significantly enhanced resistance and protection of the cultured shrimp and fish against WSSV. Moreover, the antiviral activity of several herbal plant extracts (such as *Cynodon dactylon*, *Aegle marmelos*, *Tinospora cordifolia*, *Picrorhiza kurooa, Eclipta alba*, *Momordica charantia*, *Pongamia pinnata*, *P. amarus*) were also reported in previous studies to enhance the immune response of cultured animals against WSSV (Citarasu et al 2006; Balasubramanian et al 2007; Rameshthangam & Ramasamy 2007; Sundaram et al

2016). The pathological signs recorded from infected shrimp were similar to the description of Sanchez-Paz (2010), which include the white spots appearing on an exoskeleton, appendages, and in the epidermis, reduced food consumption, loosening of the cuticle, lethargy, and red discolouration throughout the body.

Conclusions. The results indicate that the supplementation of the herbal extracts in the diet resulted in beneficial effects on growth, immunity responses, and resistance of *P. vannamei* against WSSV, specifically at 1% *T. catappa* extract. These findings might pave the way for discovering potential uses of *T. catappa* and *P. urinaria* extracts in aquaculture, as they had already been proven to be useful in treating various human illnesses. These findings may also help to develop a safe antiviral drug for preventing WSSV infection in shrimp culture.

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Conflict of Interest. The authors declare that there is no conflict of interest.

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Authors:
Radma Abon Isnani, College of Aquaculture and Fisheries, Can Tho University, Campus II, 3/2 St., 94115 Xuan Khanh Ward, Ninh Kieu District, Can Tho City, Vietnam, e-mail: phmarketingdiv@gmail.com
Hong Mong Huyen, College of Aquaculture and Fisheries, Can Tho University, Campus II, 3/2 St., 94115 Xuan Khanh Ward, Ninh Kieu District, Can Tho City, Vietnam, e-mail: hmhuyen@ctu.edu.vn
Tran Thi My Duyen, College of Aquaculture and Fisheries, Can Tho University, Campus II, 3/2 St., 94115 Xuan Khanh Ward, Ninh Kieu District, Can Tho City, Vietnam, e-mail: ttmduyen@ctu.edu.vn
Tran Thi Tuyet Hoa, College of Aquaculture and Fisheries, Can Tho University, Campus II, 3/2 St., 94115 Xuan Khanh Ward, Ninh Kieu District, Can Tho City, Vietnam, e-mail: ttthoa@ctu.edu.vn

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