BENEFICIAL IMPACTS ON IMMUNE RESPONSES AND DISEASE RESISTANCE OF WHITELEG SHRIMP (*Litopenaeus vannamei*) FED DIETARY HERBAL EXTRACTS

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https://doi.org/10.59411/jvrfxx80

How to Cite:
Mutunga, R. (2024). BENEFICIAL MPACTS ON IMMUNE RESPONSES AND DISEASE ESISTANCE OF WHITELEG SHRIMP (Litopenaeus vannamei) FED DIETARY HERBAL EXTRACTS. *Helix*, *5*(2). https://doi.org/10.59411/jvrfxx80

Abstract

*The present study was conducted to determine the effective herbal extracts of Phyllanthus urinaria and Combretum quadrangulare incorporated into the diets on the health of whiteleg shrimp (Litopenaeus vannamei). A commercially available feed diet was incorporated with a concentration of 1% P.urinaria, 1% C.quadrangulare,1% of a mixture of both 1% P. urinaria and 1% C. quadrangulare Kurz (experimental diets), and no herbal extract (control treatment). Shrimps **(**1.05±0.33 g**)** in triplicate groups were fed with the supplemented diets for 4 weeks in a 500 L tank with a stocking density of 70 shrimp/tank. Hematological and immunological parameters (Total hemocyte count, granular cells, hyaline cells, phenoloxidase activity-PO, and immune gene expressions) were evaluated after the feeding trial. The results showed that after 4 weeks of dietary herbal supplementation of 1% P. urinaria (PUE), and 1% mixture of P. urinaria and C. quadrangulare (PUE-CQE) significantly enhanced hematological and immunological parameters (total hemocyte count, granular and hyaline cells, phenoloxidase, lysozyme, and Penaeidin-3). After being challenged against V. parahaemolyticus, cumulative mortality of shrimp in treatment of a 1% mixture of P. urinaria and C. quadrangulare (PUE-CQE) was lower compared to the positive control at 43.6% and 66.7% respectively and the difference was statistically significant ($p<0.05$). Dietary herbal-supplemented treatments of 1% CQE and 1% PUE were slightly lower than the positive control at 55.6% and 59.5% respectively hence no significant difference ($p>0.05$). This shows that all the herbal treatments lowered the susceptibility and increased protection against V. parahaemolyticus compared to the positive control. In summary, the results indicate that P. urinaria and C. quadrangulare dietary herbal extracts have beneficial impacts on immune responses and disease resistance in whiteleg shrimp (Litopenaeus vannamei) against V. parahaemolyticus.*

Keywords: *Combretum quadrangulare*, disease resistance, innate immunity, *Phyllanthus urinaria*, *Litopenaeus vannamei*, *Vibrio parahaemolyticus*.

CHAPTER 1: INTRODUCTION

1.1 Problem statement

The most preferred seafood globally is whiteleg shrimp (*Litopenaeus vannamei*), thus, shrimp farming has expanded drastically since 1970s (De Silva *et al*., 2018), having an annual production of nearly 4 million metric tonnes in 2019 (Anh *et al*., 2019). Global shrimp and prawns trade represents a major source of foreign exchange earnings for many developing countries in Asia and Latin America; valued at USD 24.7 billion in 2020 (FAO, 2022). Although *L. vannamei* is not indigenous to Asia, it is currently mainly produced (about 80%) by Asian countries. The world aquaculture production of *L. vannamei* achieved over 5.8 million tonnes in 2020 (FAO, 2022).

Whiteleg shrimp is a tropical marine species cultivated across the world in different culture systems (Li *et al*., 2017). *L. vannamei* has desirable characteristics, such as prolific growth and wide demand in the global market. *L. vannamei* offers outstanding advantages, such as domestication and genetic selection for growth rate, disease resistance, and rapid maturation (De Silva *et al*., 2021). Its production has increased recently due to its high economic returns, but it has also been plagued by disease outbreaks. Compared to *P. monodon, L. vannamei* is the preferred choice for processing because of its higher meat yield (around 66%–68 (Liao and Chein,2011; El-saadony *et al*., 2022). Globally, resistance to pathogens is the main obstacle to the sustainable development of whiteleg shrimp farms (FAO, 2020).

Devastating shrimp pathogens, including *Enterocytozoon hepatopenaei* (EHP) and *Vibrio parahaemolyticus* (VPH) (Soto-Rodriguez *et al*., 2015), are the cause of significant economic losses to shrimp producers (Han *et al*., 2020). Because of culture intensification according to Thitamadee *et al*. (2016), the global shrimp farming industry has been affected by reoccurring infections. Acute hepatopancreatic necrosis disease (AHPND) is caused by a pathogenic isolate of *Vibrio parahaemolyticus* (VPH) (Joshi *et al* ., 2014), AHPND alone throughout several Asian states and in Mexico across the period of 2009 to 2016 was estimated to be US$ 23.58 billion (Hien *et al*., 2016).

The changing global regulatory controls and frameworks on the use of antibiotics in aquaculture have necessitated much interest in the development of functional aquafeeds (Ng *et al*., 2023). Reduction in the use of synthetic antibiotics is a priority due to the high incidence of resistant bacteria (Vibrio) in the whiteleg shrimp, (*L.vannamei)* with

recommendations to use therapeutic potential of herbal medicines and other natural alternatives (Syahidah *et al*., 2015; Macusi *et al*., 2022) as well use of functional feed additives to enhance shrimp growth and resistance to AHPND (Soowannayan *et al*., 2019; Kumar *et al*., 2020; Isnani *et al*., 2021) An increasing number of reports show the bactericidal activity of natural treatments in the aquaculture sector to fight pathogenic agents (Tang *et al*., 2020; Naylor *et al*., 2021). Studies have revealed that Vibrio bacteria causing AHPND have now shown antibiotic resistance to a wide range of antibiotics (Lai *et al*., 2015).

It is under this background this study was done to identify the beneficial impacts on immune responses and disease resistance of whiteleg shrimp-fed dietary herbal extracts. It should be noted that no previous research on using *Phyllanthus urinaria* Linn and *Combretum quadrangulare* Kurz has been done before. *P. urinaria* and *C. quadrangulare* Kurz are among the medicinal plants that can be used to enhance the growth performance, immune system, and disease resistance of cultured animals (Awad *et al.*, 2017; Sivakumar *et al*., 2017; Isnani *et al*., 2021; Tran *et al.*, 2021; Men *et al*., 2022).

This study is the first to determine the effectiveness of *P. urinaria* and *C. quadrangulare* Kurz extracts on the enhancement of resistance to *Vibrio parahaemolyticus* and acute hepatopancreatic necrosis disease in *L. vannamei.* This study was done to determine the immune parameters and disease resistance to acute hepatopancreatic necrosis disease of whiteleg shrimp fed on a diet containing *P. urinaria* Linn and *C. quadrangulare* Kurz extracts.

1.2 Research objectives

The study aimed to determine the effectiveness of diets supplemented with plant extracts on the health of whiteleg shrimp (*L. vannamei*).

1.3 Research contents

Effects of dietary supplementation of herbal extracts on innate immunity of whiteleg shrimp.

Effects of dietary supplementation of herbal extracts on the resistance against *Vibrio parahaemolyticus* in whiteleg shrimp.

CHAPTER 3: MATERIALS AND METHODS

3.1 Time and location

This study was conducted in the Fish pathology wet laboratory at the Faculty of Aquatic Pathology, College of Aquaculture and Fisheries, Can Tho University from September to December 2022.

3.2 Materials

- A system of 500-L composite tanks, glass tanks, air aerators, media for bacteria culture, formal, chlorine, and brine water.
- Whiteleg shrimp with mean bodyweight each **(**1.05±0.33 g) negative for *V. parahaemolyticus* were selected for the experiments.
- *P. urinaria* (whole plant) and *C. quadrangulare* (leaves) were collected in the Mekong delta.
- The commercial pellet (Grobest) containing 40% crude protein covered with/without squid oil and herbal extracts.
- *Vibrio parahaemolyticus* used for the challenge experiment was selected from the microbial collection of the College of Aquaculture and Fisheries, Can Tho University.

3.2.1 Herbal extracts preparation

The collected plants were washed, shade dried, chopped into small pieces, and crushed using a blender. The grounded materials were soaked in methanol with a ratio of 1kg: 10L for 3 days at room temperature. The herbal extracts were filtered through Whatman filter paper and concentrated by use of a rotary evaporator with a water bath set at 48^0 C to remove the solvents (Mariita *et al*., 2011).

Figure 3.1: Shade-dried leaves and sample herbal extract of *P. urinaria* and *C. quadrangulare* leaves.

3.2.2 Preparation of experimental diets

Four diets were prepared by supplementing the herbal extracts at 0% (Control), 1% (*P. urinaria*), 1% (*C. quandrangulare*), and 1% (mixed herbal extract, *P. urinaria,* and *C. quandrangulare*). The concentration of the herbal extracts was selected based on a previous study by Huyen *et al*. (2020). These herbal extracts (1%) were dissolved with DMSO (0.4g of herbal extract in 1mL DMSO) and were coated with commercial pellets, 20 % ethanol (of mixture of both DMSO and herbal extracts) was added to all treatments to increase the volume of herbal extract and applied in experimental feed using a dropper. These pellets were kept at room temperature for 30 minutes to allow absorption of the extract and evaporation of ethanol. Then, the pellets were coated with 2% squid oil (Chirawithayaboon *et al*., 2020) to prevent the dispersion of the extract into the water and reduce the smell. The experimental diets were kept in plastic bags at 4^{o}C for feeding trials after being air dried for 30 minutes.

3.3 Feeding experiment

3.3.1 Experimental design

The shrimps were acclimatized to the laboratory conditions for 3 days before starting the experiment. Whiteleg shrimp with a mean body weight of **(**1.05±0.33 g) were stocked in 500L tanks containing 400L of water. The stocking density was 70 shrimp/tank. There were 4 treatments with 3 replications. These treatments were identified as follows:

Treatment 1: Commercial pellet without herbal extract **(Control**)

Treatment 2: Commercial pellet supplemented with 1% *P. urinaria* **(PUE)**

Treatment 3: Commercial pellet supplemented with 1% *C. quadrangulare* Kurz **(CQE)**

Treatment 4: Commercial pellet supplemented with 1% of a mixture of both *P. urinaria* and *C. quadrangulare* Kurz **(PUE-CQE)**

The feeding regime with the experimental diets was 5%-7% of body weight for 4 weeks. The frequency of feeding was 4 times per day (7:00,11.00,14.00,18.00h).

3.3.2 Monitoring

Continuous aeration was provided in the rearing tanks in addition to the water replenishment by changing 50% of the water used weekly with chlorine-free water with salinity (15 ‰),

throughout the experimental feeding period. Dead shrimp, uneaten food, and fecal matter were removed through siphoning daily to maintain high standards of hygiene.

Water quality parameters were measured regularly and maintained as follows: Water temperature (27-30^{o}C), salinity (15 ‰), pH (8-8.5), and dissolved oxygen (4.75 mg L^{-1}), daily, were measured twice a day (morning -07.00h and evening -05.00h, every day), and alkalinity (110 CaCaCO3 mg L^{-1}) was monitored every week. These parameters were checked using portable Oxy-guard, and Sera Test Kits.

3.4 Analytical methods for immune parameters

After the 4-week of feeding experiment, nine shrimps from each treatment were randomly sampled for hematological and immunological parameters such as Total hemocyte count (THC), differential hemocyte count (DHC), Phenoloxidase activity (PO), Panaeidin (PEN) and Lysozyme (LSZ).

3.4.1 Total hemocyte count (THC)

Hemolymph (100µL of individual shrimp was withdrawn from the pleopod base of the first abdominal segment with a sterile 1-mL syringe and mixed gently with 900µL of sterilized anticoagulant (trisodium citrate 30mM, NaCl 338mM, glucose 115mM, EDTA 10 mph 7). A volume of 10 µL of the anticoagulant-hemolymph mixture was placed in a Neubauer hemocytometer to count the hemocyte under a light microscope at 40× and repeated 2 times (Le Moullac *et al*., 1997a).

3.4.2 Differential Hemocyte Count (DHC)

Anticoagulant-hemolymph mixture was used for the identification and enumeration of hyaline cells, semi-granular cells, and granular cells based on the methods described by Le Moullac *et al*. (1997b). To obtain smears, the hemolymph was centrifuged at 5000 rpm for 5 min at 4℃, the supernatant was discarded, and the pellet was rinsed once with the formalin-AS pH 4·6 (1:10) and resuspended gently in the same solution. A drop of the hemocyte suspension was spread on glass slides, fixed for 5 min in ethanol, air-dried, and stained with Giemsa for 30 min then, stained slides were rinsed in acetone, and xylene, then, were washed in distilled water, air-dried, and cells were observed and counted (200 cells/sample) under a light microscope ×100. DHC was calculated as the Density of each hemocyte (cell mL^{-1}) = Number of each hemocyte ×THC/200.

3.4.3 Phenoloxidase activity (PO)

L-Dihydroxyphenylalanine (L-DOPA) was used to measure total phenoloxidase activity (Hernandez-Lopez *et al*., 1996). Hemolymph (200 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℃. The supernatant was discarded, and the pellet was rinsed and suspended gently in 1000 µL Cacodylate Citrate buffer solution (pH 7), and slightly mixed. The samples were centrifuged again at the same condition, the supernatant discarded, and the pellet was rinsed in 200 µL Cacodylate Citrate buffer solution (pH 7), and slightly mixed. The suspension of 100 µL was incubated with 50 µL Trypsin solution (mL mg^{-1}) and Cacodyte Citrate buffer solution (pH 7) (control tube). A 50 µL aliquot of L-DOPA was added, then 800 µL Cacodylate Citrate buffer solution (pH 7.0) and measured using a spectrophotometer at 490 nm.

3.4.4 Immune-Related Gene Expressions

Expressions of the immune-related gene were conducted using target genes such as Penaeidin-3 (PEN), Lysozyme (LSZ), and Beta-actin as the reference gene.

3.4.4.1 RNA extraction

An RNAzol reagent was used to extract RNA from the hemolymph. 250µL of hemolymph was taken from an eppendorf tube containing 250 µL of cold RNAzol (Molecular Research Center, Inc. USA) and homogenized. 100 µL of distilled water was added to the eppendorf tube and centrifuged at 10,500 rpm for 15 minutes at 4°C. The supernatant (300µL) was then transferred to a new eppendorf tube containing 300 µL isopropanol and centrifuged for 10 minutes at 4°C at 10,500 rpm. The supernatant was discarded, and the pellet was maintained. Next, 200 µL of 75% ethanol was added, and the pellet was centrifuged at 3,600 rpm for 5 minutes at 4°C. The supernatant was then discarded. Twice in a row. The eppendorf tube was turned upside -down and the pellet was air-dried. After that, 100 µL of DEPC water was added, mixed, and stored at -80°C until use (Wang *et al*., 2008).

3.4.4.2 cDNA synthesis

The cDNA was obtained using the SensiFast cDNA synthesis kit (Bioline). Before use, the solutions were vortexed and centrifuged. The master mix was then prepared and gently by mixing DNase/RNase-free water, 5X TransAmp Buffer, reverse transcriptase, and 1 µL of RNA. Then, before adding the RNA template, an aliquot of the master mix was placed in each tube and incubated at 25°C for 10 minutes (primer annealing), 45°C for 15 minutes

(reverse transcription), 85°C for 5 minutes (inactivating), and cDNA was stored at -20°C until use (Schmittgen and Livak 2008).

Using a Real-time PCR cycler (Biorad), the generated cDNA was utilized as a template to assess the relative expression of lysozyme (LSZ) and penaeidin-3 (PEN) in hemolymph samples. and the gene-specific primers listed in Table 3.1 with *β*-actin as an internal control to calculate fold change in the target genes (Wang *et al*., 2008).

3.4.4.3 Real-time PCR

The concentration of cDNA was determined and diluted. The PCR master mix was then prepared and gently mixed, consisting of distilled water, 1x of ROX mix, 0.5 µM F primer, 0.5 µM R primer, and cDNA. After that, an aliquot of the master mix was added to each tube and incubated for 1 cycle at 95°C for 3 minutes (polymerase activation), 95°C for 15 seconds (denaturation), and 40 cycles at 56°C for 30 seconds (annealing) and 72°C for 30 seconds (extension). The critical threshold (Ct) quantifies of the target genes were standardized with quantities (Ct) of β-actin using the $2^{-\Delta\Delta}$Ct method (Schmittgen and Livak, 2008).

Table 3.1: 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	AY170126	97
	R: ATC GGA CAT CAG ATC GGA AC		
Penaeidin-3	F: CAC CCT TCG TGA GAC CTT TG	Y14926	121
	R: AAT ATC CCT TTC CCA CGT GAC		
β-actin	F: CCA CGA GAC CAC CTA CAAC	AF300705	142
	R: AGC GAG GGC AGT GAT TTC		

3.5 Challenge experiment

3.5.1 *Vibrio parahaemolyticus* challenge test

After 4 weeks of herbal supplementation, the challenge experiment was set up with 5 treatments,3 replicates per treatment, and 15 shrimps per tank. The shrimp feeding experiment was challenged with *V. parahaemolyticus* by immersion method (Tran *et al*.,

2013). Briefly, experimental shrimp were challenged by immersion in bacteria solution at a concentration that lethal dose of 50% of the experimental shrimp, and the dose of the bacteria was (1.4×10^7 CFU/mL). The negative control shrimp was immersed in nutrient broth. The challenged shrimp were fed with respective treatment diets twice a day throughout the challenge period. Removal of wastes and uneaten feed was conducted daily. The mortality rate was recorded daily for 14 days of the challenge test. Moribund shrimps were collected for *V. parahaemolyticus* confirmation by PCR method with primer sets adapted from Dangtip *et al*. (2015).

3.5.2 PCR method for detection of *V. parahaemolyticus*

Vibrio parahaemolyticus was isolated from the hepatopancreas of moribund shrimp using TCBS agar. Then, DNA was extracted from the isolated bacteria in the TCBS agar, then utilized for PCR to identify bacteria *V. parahaemolyticus* using chemical composition reaction and thermal cycling conditions performed according to the method of Dangtip *et al*. (2015). Electrophoresis results were recorded with a reader gel, based on the 100 bp DNA scale to determine the molecular weight, in which the sample had a line of 1269 bp (product PCR product step 1) and 230 bp (step PCR product 2) samples infected with *V. parahaemolyticus* and samples, not product line is negative, not infected with *V. parahaemolyticus*.

3.6 Data analysis

The data was analyzed using Microsoft Excel 2016, SPSS -21(SPSS Inc., Chicago, Illinois, USA). A one-way analysis of Variance (ANOVA<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 the 4-week herbal dietary feeding experiment and after infection was performed using the Independent-Sample t-Test. Statistical significance of the differences requires that the p values are less than 0.05.

CHAPTER 4: RESULTS AND DISCUSSIONS

4.1 Results

4.1.1 Effect of dietary supplementation of herbal extracts on immunological parameters of white leg shrimp

Following the four-week feeding trial, shrimp supplemented with 1% *P. urinaria*, 1% *C. quadrangulare*, and 1% of a mixture of both *P. urinaria* and C. *quadrangulare* Kurz (supplement treatments), and no herbal extract (control treatment) were evaluated for non-specific immune parameters such as THC, granular cell-GC, hyaline cell -HC, PO, and immune genes.

4.1.1 .1 Hematological Parameters

Total hemocyte count (THC)

Among the three supplement diets, THC value was obtained at the highest value in PUE treatment (1% *P. urinaria*) (0.068±0.026 x 10^5 cell/mL), followed by PUE-CQE treatment (1% mixture of *P. urinaria* and *C. quadrangulare*) (0.059±0.031 x 10^5 cell/mL), then CQE treatment (1% *C. quadrangulare*) at (0.040±0.021x 10^5 cell/mL). Notably, the CQE treatment (1% *C.quadrangulare*) exhibited lower THC compared with the control treatment. However, there was a significant difference ($p<0.05$) between 1% *P.urinaria* treatment and control as shown in Table 4.1.

Among the three supplement diets, HC was obtained at the highest value in PUE treatment (1% *P. urinaria*, followed by PUE-CQE treatment (1% mixture of *P. urinaria* and *C. quadrangulare*), then CQE treatment (1% *C.quadrangulare*. However, there was a statistically significant difference ($p<0.05$) between 1% PUE treatment and control as shown in Table 4.1.

Table 4.1: Hematological parameters of *L. vannamei* after being fed with supplemented diets of 1% *P. urinaria* extracts (PUE),1% *C. quadrangulare* extracts (CQE),1% mixture of *P. urinaria* and *C. quadrangulare* extracts (PUE-CQE), and no herbal extracts (control) after 4 weeks of herbal supplementation (× 10^5 cell/mL).

Treatment	**THC**	**GC**	**HC**
CONTROL	0.042±0.019[a]	0.009±0.005[a]	0.032±0.014[a]
1% PUE	0.068±0.026[b]	0.013±0.008[a]	0.055±0.020[b]

1% CQE	0.040±0.021[a]	0.007±0.004[a]	0.033±0.017[a]
1% PUE-CQE	0.059±0.031[ab]	0.011±0.006[a]	0.049±0.026[ab]

Values presented as mean ± S.D (n=9). Values on the same column with the same superscript ([a, ab, b]) indicate no significant difference p<0.05)

Differential Hemocyte Count (DHC)

Among the three supplement diets, the highest value for hyaline cells was recorded highest in PUE treatment (1% *P. urinaria*) (0.055±0.020 x 10^5 cell/mL), followed by PUE-CQE treatment (1% mixture of *P. urinaria* and *C. quadrangulare*) (0.049±0.026 x 10^5 cell/mL), then CQE treatment (1% *C. quadrangulare*) at (0.033±0.017 x 10^5 cell/mL). However, there was a statistically significant difference ($p<0.05$) between 1% PUE treatment and control as shown in Table 4.1.

Among the three supplement diets, GC was obtained at the highest value in PUE treatment (1% *P. urinaria*); PUE treatment (1% *P. urinaria*) (0.013±0.008 x 10^5 cell/mL), followed by PUE-CQE treatment (1% mixture of *P. urinaria* and *C. quadrangulare*) (0.011±0.006x 10^5 cell/mL), then CQE treatment (1% *C. quadrangulare*) at (0.007±0.004 x 10^5 cell/mL). Notably, the CQE treatment (1% *C. quadrangulare*) exhibited lower GC compared with the control treatment (0.009±0.005 x 10^5 cell/mL). However, there were no statistically significant differences ($p>0.05$) among the supplement treatments as shown in Table 4.1.

4.1.1.2 Immunological parameters

Phenoloxidase (PO) activity

The phenoloxidase activity of the shrimp after 4 weeks of dietary herbal supplementation has been shown in Table 4.2. The PUE treatment (1% *P. urinaria*), showed higher PO activity (0.081±0.029) compared to the other treatments, followed by a 1% mixture of *P. urinaria* and *C. quadrangulare* extracts (PUE-CQE) and (0.061±0.021), then 1% *C. quadrangulare* dietary herbal extract (CQE) Notably CQE treatment had a lower PO activity compared with control treatment (0.061±0.013) However, the difference was statistically significant ($p<0.05$) between 1% PUE treatment and 1% CQE treatment

Table 4.2: PO activity of *L. vannamei* after being fed with herbal dietary supplementation of 1% *P. urinaria* extracts (PUE),1% *C. quadrangulare* extracts (CQE),1% mixture of *P. urinaria* and *C. quadrangulare* extracts (PUE-CQE), and no herbal extracts (control) after 4 weeks.

Treatment	**PO (490nm)**
CONTROL	0.061 ± 0.013^{ab}
1% PUE	0.081 ± 0.029^{b}
1% CQE	0.056 ± 0.015a
1% (PUE -CQE)	0.061 ± 0.021^{ab}

Values presented as mean ± S.D (n=9). Values on the same column with the same superscript ($^{a, ab, b}$) indicate no significant difference among treatments (p<0.05).

Immune gene expressions -Lysozyme

To determine the transcriptional responses of *L. vannamei* to dietary herbal supplementation of *P. urinaria*, *C. quadrangulare*, and a mixture of both *P. urinaria* and *C. quadrangulare*, mRNA expressions of two-immune related genes (Lysozyme and Penaeidin -3) were assessed.

After 4 weeks of dietary herbal supplementation, the mRNA expression of Lysozyme was found to be upregulated after herbal dietary supplementation of 1% *P. urinaria* (PUE) as shown in Figure 4.1. Specifically, PUE treatment had higher Lysozyme expression PUE-(1.508±0.308), than control (1.005±0.126), CQE-(0.581± 0.132), and PUE-CQE (0.589±0.110) had lower than control. These expressions were significantly different (p>0.05)

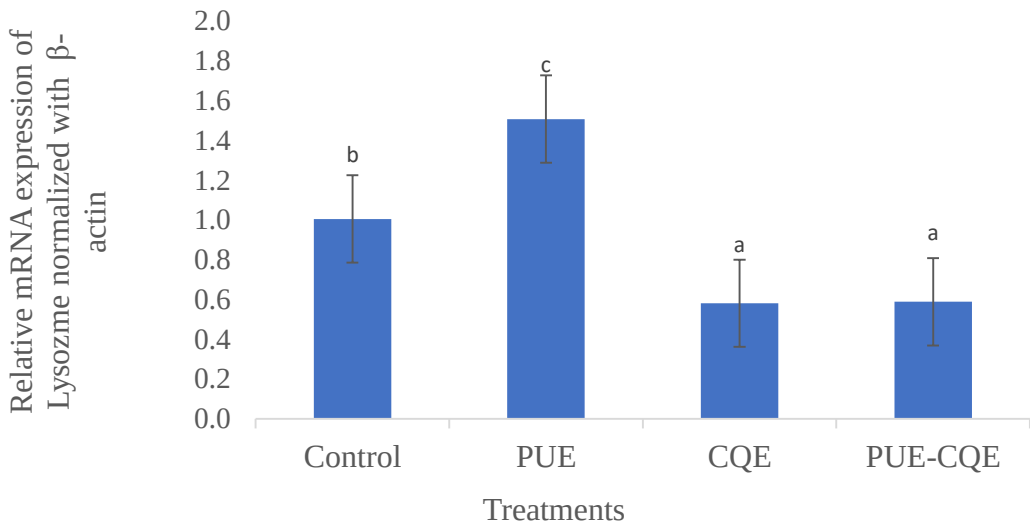

Figure 4.1**:** Relative mRNA expressions of Lysozyme of *L. vannamei*, after being fed with dietary herbal extracts of 1% *P. urinaria* extracts (PUE),1% *C. quadrangulare* extracts (CQE),1% mixture of *P. urinaria* and *C. quadrangulare* extracts (PUE-CQE), and no herbal extracts (control) after 4 weeks by RT- PCR. All the samples were normalized using β-actin expression as an internal control. Relative levels of lysozyme mRNA were analyzed by the $2^{-\Delta\Delta Ct}$ method (the Ct value of the Lysozyme gene minus the Ct value of the β-actin gene) method. Data are presented as mean) ± S.D (n=9).The same superscript letters (^{a,b,c}) indicate there was no significant difference among the treatments ($p>0.05$).

Immune gene expression -Penaeidin -3 The expression of penaeidin-3 after 4 weeks of dietary herbal supplementation was found upregulated in the supplemented treatments; 1% CQE-(0.617±0.128) and 1% mixture of PUE-CQE at (0.728±0.208),1%PUE had a slight increase of (1.231±0.355) than control (1.002±0.072) and the difference was found to be statistically significant ($p<0.05$), as shown in Figure 4.2.

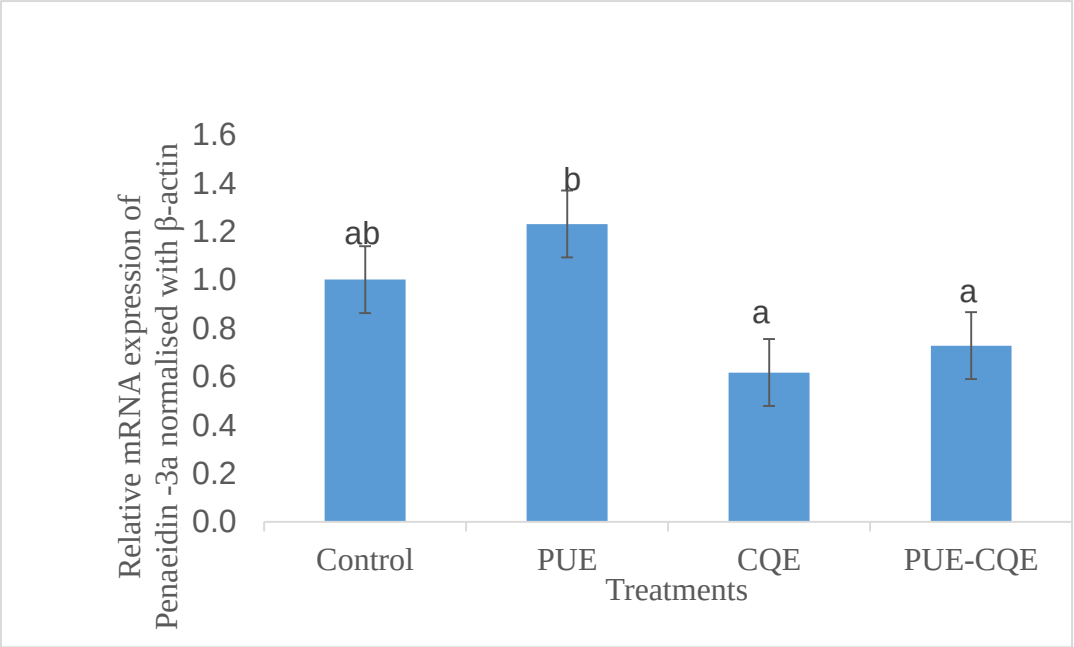

Figure 4.2: Relative mRNA expressions of penaeidin-3 of *L.vannamei*. Relative mRNA expressions of Penaeidin -3 of *L. vannamei* after being fed with dietary herbal extracts of 1% *P. urinaria* extracts (PUE),1% *C. quadrangulare* extracts (CQE),1% mixture of *P. urinaria* and *C. quadrangulare* extracts (PUE-CQE), and no herbal extracts (control) after 4 weeks by RT- PCR. All samples were normalized using β-actin expression as an internal control. Relative levels of Penaeidin -3 mRNA were analyzed by the $2^{-\Delta\Delta Ct}$ method (the Ct value of the Penaeidin -3 gene minus the Ct value of the β-actin gene) method. Data are presented as mean ± S.D (n=9). The same superscript letters (^{a,b,c}) indicate there was no significant difference among the treatments ($p>0.05$). After 4 weeks of dietary herbal supplementation indicate a significant difference except for 1% CQE treatment and 1% PUE-CQE ($p>0.05$).

4.1.2 Effects of dietary supplementation of herbal extracts on disease resistance against *Vibrio parahaemolyticus* in whiteleg shrimp

The shrimp's cumulative mortality in the dietary herbal-supplemented treatment of a 1% mixture of PUE-CQE was significantly lower compared to the positive control at 43.6% and 66.7% respectively and the difference was statistically significant ($p<0.05$). Dietary herbal-supplemented treatments of 1% CQE and 1% PUE were slightly lower than the positive control at 55.6% and 59.5% respectively hence no significant difference ($p>0.05$).

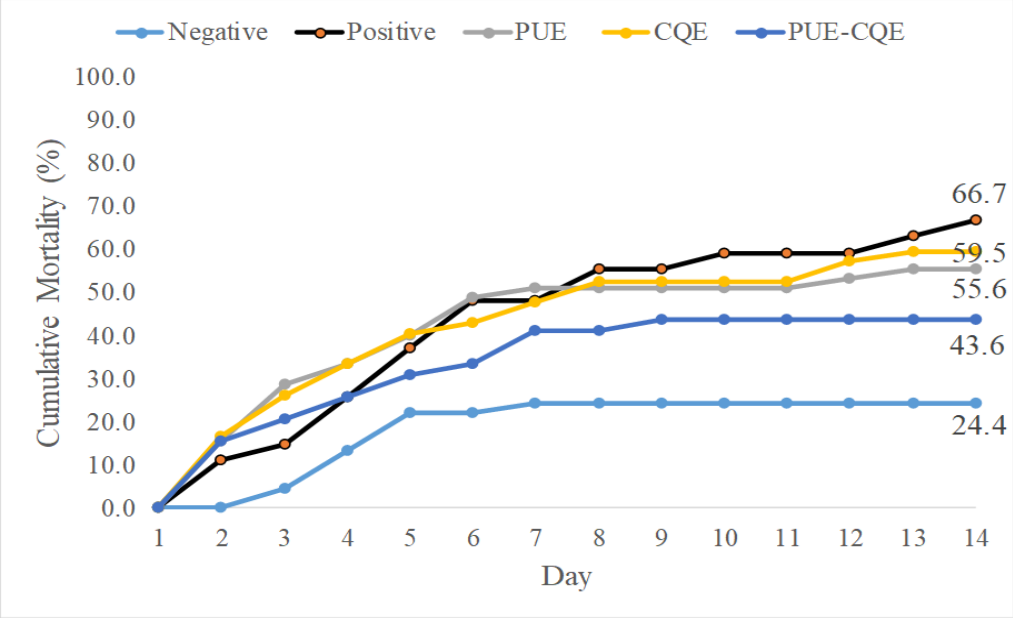

Figure 4.3: Cumulative mortality of *L. vannamei* challenged with VPH,14 days post-challenge fed 1% *P. urinaria* extracts (PUE),1% *C. quadrangulare* extracts (CQE),1% mixture of *P. urinaria* and *C. quadrangulare* extracts (PUE-CQE), and no herbal extracts (control).

During the 14 days challenge experiment, shrimps were observed, and recorded the clinical signs and sampled for PCR detection of challenged bacteria. The clinical signs observed were soft shells, empty gut, and pale color of the hepatopancreas in all treatments immersed with bacteria in Figure 4.4A as compared to Figure 4.4B showing healthy shrimp at the end of the dietary herbal supplementation experiment period.

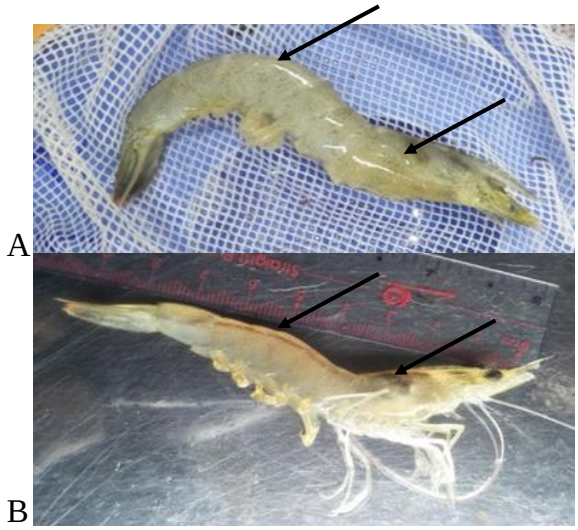

Figure 4.4: Signs of the experimental shrimp. A) Shrimp post-infection with VPH, pale, small hepatopancreas and empty gut (black arrow); B) Heathy shrimp at the end of the dietary herbal supplementation experiment period, gut visible with feed and clear hepatopancreas organ.

The experimental shrimp were also collected and detected for the presence of *V. parahaemolyticus* by PCR method (Figure 4.5). Figure 4.5 showed that the challenge shrimp of the supplement treatments (1% PUE; 1% CQE; 1% PUE-CQE) and positive treatment (lanes 1, 2, 3, and 4) were detected and obtained a bright positive band for *V. parahaemolyticus* at 230 bp. On the other hand, the negative control shrimp (lane 5) was not experimentally infected with *V. parahaemolyticus* and did not show bright bands. The results concluded that the shrimp mortality in the challenge test was caused by *V. parahaemolyticus*.

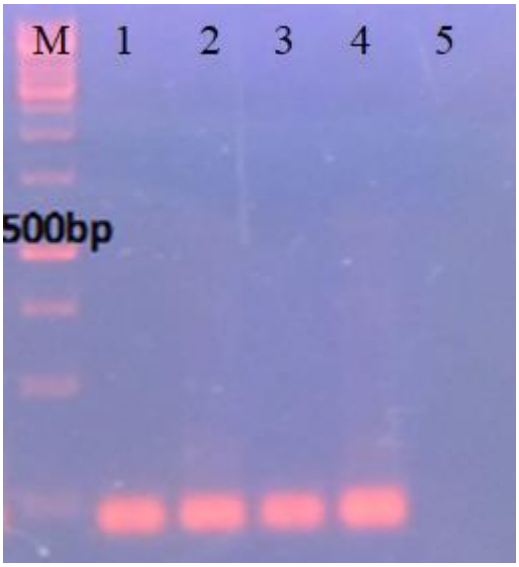

Figure 4.5: PCR results of the challenge test of the experimental shrimp post-infection with *Vibrio parahaemolyticus*. Lane M: DNA marker; and lane 1, 2, 3, 4, 5: shrimp DNA isolated from five treatments of the challenge experiments.

4.2. Discussion

The application of dietary herbal extracts in aquaculture has been described in various studies. Thus, findings proved that herbal extract is effective in the immune response and disease resistance of different species of cultured shrimp (Syahidah *et al*., 2015; Prathomya *et al*., 2019; Santhosh *et al*., 2023). The present study was designed to determine the effective

herbal extracts incorporated in the diets on the health of whiteleg shrimp (*L. vannamei)* with specificity on the use of *P. urinaria* and *C. quadrangulare* against *V.parahaemolyticus.* Despite crustaceans like shrimps not possessing a true adaptive immune system, they have well-orchestrated innate defense mechanisms based on non-self-recognition which provide a non-specific, yet highly effective, response to pathogen invasion, other biotic and abiotic stressors (Medzhitov and Janeway, 2000). They highly depend on their cellular and humoral components to render protection against invading pathogens (Kulkarni *et al*., 2021). Cellular immune responses include phagocytosis, nodulation, and encapsulation leading to melanization and are complemented by humoral responses such as secretion of clotting proteins, agglutinins, antimicrobial peptides (AMPs), proteinase inhibitors, and reactive intermediates of oxygen or nitrogen. Cellular and humoral immune responses are mainly produced by the hemolymph and hemocytes of shrimp (Tassanakajon *et al*., 2013).

As part of the immune system, shrimp hemocytes are responsible for inducing a range of cellular and humoral responses such as clotting and lysing pathogens as well as wound healing. According to the established classification, crustacean hemocytes are differentiated as hyaline cells, semi-granular cells (SGCs), and granular cells (GCs) (Lin and Söderhäll, 2011). Many biomarkers predominantly biochemical and cellular measures such as THC and DHC of hemolymph are considered to gauge immunosuppression and immunocompetence in shellfish and crustaceans in general. Such measures range from the immune cell (hemocyte) counts to enzymic activities and metabolite quantitation (Coates and Söderhäll, 2021). In the present study, the THC of shrimp in PUE treatment (1% *P.urinaria*) and PUE-CQE treatment (1%mixture of *P. urinaria* and C. *quadrangulare)* was higher than and CQE treatment (1% *C.quadrangulare*) and control treatment. However, the difference was statistically significant ($p<0.05$) between 1% *P.urinaria* and control treatments. Previous studies show that in *L. vannamei*, an increasing number of (THC and DHC) granular and hyaline cells might be caused by the rapid maturation of hemocytes after new cells are discharged to maintain the hemocyte pool (Sequeira *et al*., 1996; Johansson *et al*., 2000).

It has also been hypothesized that the decrease in THC is due to changes in shrimp's physiological conditions, such as increased susceptibility to bacterial infection (Ford *et al*., 1993). Pipe and Cole (1995) reported that a decrease in THC could be caused by cell lysis or an increase in cells from hemolymph to tissues. In addition, Söderhäll and Cerenius (1992) stated that phagocytosis is thought to be one of the most important mechanisms for cellular defense. Granular cells have a crucial role in phagocytosis due to their ability to kill intracellularly

(Maftuch *et al*., 2013). In Hose and Martin (1989), granular hemocytes are capable of phagocytosis of foreign material, though the frequency is less than that of smaller hemocytes. The percentage increase in a particular compartment can be due to the recruitment of cells from non-circulating compartments of the hemolymph, induced cellular proliferation, or rapid differentiation in response to antigenic challenge (Aladaileh *et al*., 2008). *Astragalus membranaceus, Codonopsis pilosula*, and *Glycyrrhiza uralensis* supplementation improved the THC and granulocyte count in *L. vannamei* (Prathomya *et al*., 2019). Supplementing shrimp with herbal extracts of Boerhaavia diffusa as well produced similar results of improving THC and granulocytes (Chithambaran and David,2014), *P.amarus* (Ngo *et al*., 2020), *P.urinaria* and *T.cattapa* enhanced innate immunity and THC in *P.vannamei* as reported by Isnani *et al*.(2021). White leg shrimp fed with dietary fed with *Gynura bicolor* extract also enhanced the non-specific immune response specifically through an increase in total hemocyte count. A study by Abidin *et al.* (2022) showed Moringa *oleifera* leaf extract enhanced *immune response, and resistance against Vibrio alginolyticus and V.parahaemolyticus in whiteleg shrimp* (*Penaeus vannamei*) where THC increased too. *Syzygium cumini* leaf powder enhanced the non-specific immunity of *L. vannamei* (Boone 1921) and defense against a virulent strain of *Vibrio parahaemolyticus* through enhancement of lysozyme and phagocytotic activity (Prabu *et al*., 2018). Dietary supplementation with *Salvinia Cucculata* increased THC in *L. vannamei* and improved innate immunity against *V.parahaemolyticus* (Santhosh *et al*., 2023). Also, *Ulva lactuta* extract increased total hemocyte count (THC) and phagocytic when given to *L. vannamei* (Suleman *et al*., 2018).

Invertebrates undergo melanization due to the action of phenoloxidase (PO) which is controlled by the phenoloxidase enzyme. The PO enzyme results from the activation of the proPO enzyme (Söderhäll and Cerenius, 1992; Johansson and Söderhäll, 1996), which plays an important role in the invertebrate immune system in allowing a rapid response to pathogen infection (Amparyup *et al*., 2013). ProPO activation, induced by pattern-recognition proteins (PRPs) that recognize microorganisms, triggers a serine protease cascade, eventually leading to the cleavage of the inactive proPO to the active PO that functions to produce the melanin and toxic reactive intermediates against invading pathogens (Rodríguez and Le Moullac, 2000). In this current study, the shrimp fed a 1% *P. urinaria* dietary herbal extract (PUE) and 1% mixture of *P. urinaria* and *C. quadrangulare* (PUE- CQE) showed higher PO activity compared to 1% *C. quadrangulare* dietary herbal extract (CQE) and control treatment. According to Chang *et al*. (2012), dietary administration of zingerone significantly increased

phenoloxidase levels in shrimp increasing their resistance to *Vibrio alginolyticus* in *L. vannamei*. Also immune, and antioxidant enzyme activities significantly improved in the treated groups than the untreated in a study conducted to determine the effects of dietary black garlic supplementation in *L.vannamei* and its resistance to *Vibrio parahaemolyticus* .*T.cattapa* herbal extract in a dietary feed of shrimp shown increased PO as reported by Isnani *et al*. (2021) in *L.vannamei* and fish as reported by Yakubu *et al*. (2020). Similarly, the Supplementation of *P.amarus* extract in a diet was found to enhance the PO activity of *L.vannamei* in two feeding experiments (Ngo *et al*., 2020).

This study evaluated the penaeidin-3 and lysozyme transcript regulation in shrimp fed with plant immunostimulants from *C.quadrangulare* and *P.urinaria.* Past reports on the effect of immunostimulant extracted from several plants enhanced lysozyme gene (El-Asely *et al* ., 2011; Chang *et al* ., 2012) and penaeidin gene expression (Sivagnanavelmurugan *et al* ., 2014; Isnani *et al*., 2021) in cultured shrimps. Lysozyme is an important antibacterial protein produced by shrimp hemocytes, within tissues of *L. vannamei* in response to a pathogen challenge (Burge *et al*., 2007) and it acts as a non-specific innate immunity molecule against invading molecules and can hydrolyze bacterial's cell wall (Prager and Jolles, 1996; Wang *et al*., 2008). After 4 weeks of dietary herbal supplementation, the mRNA expression of lysozyme was found to be upregulated after herbal dietary supplementation of 1% *P. urinaria* extracts (PUE), while 1% *C. quadrangulare* (CQE), and 1% mixture of both *P. urinaria* and *C. quadrangulare* was downregulated. Upregulated lysozyme gene expression was reported in red swamp crayfish (*Procambarus clarkii*) after being fed with dietary glycyrrhizic acid (Liu *et al*., 2021). Lysozyme levels were found to be significantly higher in *L. vannamei* after feeding on diets supplemented with Indian ginseng (*Withania somnifera*), which improved immune responses and disease resistance(Abdel-Tawwab *et al*., 2022). Dietary administration of *Gynura bicolor* water extract enhanced immune response by increasing lysozyme among other immune parameters against *Vibrio alginolyticus* and white spot syndrome virus in *L. vannamei* (Wu *et al*., 2015). Moreover, penaeidins are members of antimicrobial peptides originally isolated from *Litopenaeus vannamei* hemolymph. They are present in a stored form in the granulocytes and secreted after microbial stimulation (Song and Li, 2014). The penaeidin gene is associated with local defense from the release of hemocytes and binds to cuticle surfaces of the shrimp by directing granulocyte migration towards the inflammatory foci which assists in the inflammatory responses (Destoumieux *et al*., 2000) and they are also involved in the wound-healing process. penaeidin-positive granulocytes are functionally

similar to vertebrate neutrophils and are also the subtype of hemocytes that appear first in the wound tissue and release AMPs (Song and Li, 2014). After 4 weeks of dietary herbal supplementation, the expression of penaeidin-3 was found slightly upregulated in treatment 1%PUE compared to the other treatments, with no statistical significance. The upregulation of the penaeidin -3 gene in response to immunostimulant takes up the tank of energy and transcriptional machinery in hemocytes (Wang *et al*., 2008).In which the upregulation of this gene might cause resource competition with other genes in expression and result in slowing down and stopping the transcription of the other genes that appears the downregulation of the gene. Abdel *et al*. (2021) recorded an upregulated relative expression of panaeidin -4 in all treatments that were supplemented with *Sargassum polycystum* and nucleotides fed to *L.vannamei*. A study by Trejo-Flores *et al*. (2018) reported that mRNA expression of the Panaeidin 4 gene was significantly upregulated at 6hr and 12 hr after *P.vannamei* was fed Aloe vera. Yu-Ping-Feng polysaccharides (YPS) upregulated the penaeidin gene significantly in a study done by Su *et al*. (2020) on the immune response, intestinal microbiota, disease resistance, and growth performance of *L. vannamei*. In another study by Isnani *et al*. (2021), the expression of penaeidin-3 was upregulated in the supplemented treatments of *P.urinaria* and *T.catappa* compared to *P.vannamei*. Apple cider vinegar and propionic acid upregulated lysozyme and penaeidin -3 in L. *vannamei* in a study done by Pourmozaffar *et al*. (2017).

Under the attack of pathogens, aquatic animals with a good immune performance show low cumulative mortality and it has also been demonstrated that many natural herbs or their extracts can be used as prospective immunostimulants against pathogen infection in aquaculture (Huang *et al*., 2018). According to Andrino *et al*. (2012), survival after a challenge with certain pathogens is usually considered a measure of disease resistance. VP strains have different virulence as some of them are less virulent strains and do not induce 100% mortality, and mortality rates also rise more slowly than they do for the more virulent strains (Soto-Rodriguez *et al*., 2015). Also, the pathological signs recorded from infected shrimp are similar pathological signs as those recorded by Soto-Rodriguez *et al*. (2015) which include lethargy, empty gut, pale and aqueous hepatopancreas, expanded chromatophores, and erratic swimming (Hong *et al*., 2016). Additionally, environmental suppression by exposure to high concentrations of nitrite and ammonia inhibits the immune responses of *P. vannamei* and increases their susceptibility to *V. parahaemolyticus*, eventually leading to increased mortality (Ge *et al*. 2014). After the feed diet experiment shrimp were challenged with *V.parahaemolyticus* and observed for the mortality rate, the

positive control experiment showed the highest mortality of 66.7%, CQE -59.5%, PUE-55.6%, PUE-CQE-43.6%. This means the survival rate of the negative control was the highest at 75.6%, while the survival rate of the positive control experiment was the lowest at 33.3%. This illustrates that all the herbal treatments lowered the susceptibility and increased protection against VPH compared to the positive control. The positive control having the highest mortality compared with the supplemented diets depicts that the immunological parameters reduced their defense ability after some time. Additionally, *P. urinaria* crude extract has a slight effect on growth promotion, but it does not affect to increment in the survival rate of Pacific white shrimp as reported by Charoendat *et al*. (2019). Results from Isnani *et al*. (2021) showed a slightly higher survival rate after dietary supplementation with *P. urinaria* compared to the positive control after challenge infection with WSSV. In a study done to analyze antioxidant, and antibacterial activities of ethanol extract from *Combretum quadrangulare* collected in Vietnam, it was reported that *Combretum quadrangulare* Kurz contains bioactive compounds such as alkaloids, flavonoids, steroids, and triterpenoids, tannins, and phenolics. In addition to that, the extract from *Combretum quadrangulare* Kurz also can fight against the bacteria causing diseases in aquatic animals such as *Edwardsiella ictaluri, Aeromonas hydrophila, and Streptococcus agalactiae* (Men *et al*., 2022). A study by Tran *et al* . (2021) reported that *C. quadrangulare* methanol extracts possess a significantly strong and broad spectrum of antimicrobial activity against the three bacterial pathogens such as *E. ictaluri, A. hydrophila,* and *S. agalactiae* and recommended the plant for further research to use in combating fish bacterial diseases. From this study, the use of mixed dietary herbal extracts of PUE-CQE, increased the cumulative survival rate to 56.4%, higher than *P. urinaria* at 44.4 % and *C.quadrangulare* at 40.5%.

The present study has shown that incorporating *P. urinaria* and *C. quadrangulare* extracts in the diet was able to increase THC, DHC, expression of lysozyme and penaeidin-3, and resistance of *L.vannamei* against VPH. These findings might pave way for discovering potential uses of *C. quadrangulare* in aquaculture. These findings may also help in identifying the best type of *P.urinaria* plant to use, the green-stemmed or the purple red - stemmed. The findings may also help in developing a mixed PUE-CQE cheap, organic, safe, and readily available antiviral drug against VPH to prevent massive fatalities as well as huge losses witnessed when an outbreak occurs in the shrimp aquaculture industry.

CHAPTER 5:CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

The present study demonstrates dietary herbal supplementation of *Litopenaeus vannamei* with *P. urinaria* and *C. quadrangulare* can enhance innate immunity. Several hematological and immunological parameters were increased such as THC (for 1%PUE and 1% PUE-CQE), HC (for 1%PUE and 1% PUE - CQE), expression of lysozyme (for 1% PUE) and penaeidin-3 (for 1%PUE), and resistance of *L. vannamei* against bacteria, *V.parahaemolyticus*.

In summary, the results indicate that 1% of a mixture of *P.urinaria* and *C.quadrangulare* dietary herbal extracts have beneficial impacts on disease resistance in whiteleg shrimp (*Litopenaeus vannamei*) against *V.parahaemolyticus*.

5.2 Recommendations

The utilization of *P. urinaria* and *C. quadrangulare* extracts as sources of immunostimulant in disease prevention in shrimp culture.

Further studies need to be done on methanolic (*C. quadrangulare* and *P.urinaria*) extracts to determine their antibacterial activity against other disease-resistant pathogens in shrimp.

A comparative study to determine *P.urinaria* subspecies with a better performance against *V.parahaemolyticus* between the green stem plant and the purple-red plant.

REFERENCES

Abdel-Rahim, M., Bahattab, O., Nossir, F., Al-Awthan, Y., Khalil, R. H., and Mohamed, R. (2021). Dietary supplementation of brown seaweed and/or nucleotides improved shrimp performance, health status, and cold-tolerant gene expression of juvenile whiteleg shrimp during the winter season. *Marine Drugs*, *19*(3), 175.

Abdel-Tawwab, M., Abo Selema, T. A. M., Khalil, R. H., El-Sabbagh, N., Eldessouki, E. A. A., Fawzy, R. M., and Abd El-Naby, A. S. (2022). The growth performance, antioxidant and immune responses, and disease resistance of *Litopenaeus vannamei* fed on diets supplemented with Indian ginseng (*Withania somnifera*). *Fish and Shellfish Immunology*, *128*, 19–27.

Abidin, Z., Huang, H. T., Hu, Y. F., Chang, J. J., Huang, C. Y., Wu, Y. S., and Nan, F. H. (2022). Effect of dietary supplementation with *Moringa oleifera* leaf extract and *Lactobacillus acidophilus* on growth performance, intestinal microbiota, immune response, and disease resistance in whiteleg shrimp (*Penaeus vannamei*). *Fish and Shellfish Immunology*, *127*, 876-890.

Aladaileh, S., Nair, S. V, and Raftos, D. A. (2008). Effects of noradrenaline on immunological activity in Sydney rock oysters. *Developmental and Comparative Immunology*, *32*(6), 627–636.

Amoah, K., Liu, H., Dong, X. H., Tan, B. P., Zhang, S., Chi, S. Y., ... and Yang, Y. Z. (2021). Effects of varying dietary black garlic supplementation on the growth, immune response, digestive and antioxidant activities, intestinal microbiota of *Litopenaeus vannamei* and its resistance to *Vibrio parahaemolyticu*s infection. *Aquaculture Nutrition*, *27*(5), 1699-1720.

Amparyup, P., Charoensapsri, W., and Tassanakajon, A. (2013). Prophenoloxidase system and its role in shrimp immune responses against major pathogens. *Fish and Shellfish Immunology*,*34*(4),9901001.

Anderson, J. L., Diego Valderrama, and Jory, D. E. (2019). GOAL 2019: Global shrimp production review. *Global Aquaculture Advocate*, *November 2019*, 2017–2021.

Anderson, J. L., Valderrama, D., and Jory, D. (2017). Shrimp Production Review. Global Outlook for Aquaculture Leadership (GOAL): Dublin, Ireland, 2017. *Global AquacultureAlliance*,54. /2018/01/Global-Shrimp-Production-Data-Analysis-Dr.-

James-Anderson-GOAL-2017.pdf.

Andrino, K. G. S., Serrano, A. E., and Corre, V. L. (2012). Effects of Dietary Nucleotides on the Immune Response and Growth of Juvenile Pacific White Shrimp *Litopenaeus vannamei* (Boone, 1931). *Asian Fisheries Science*, *25*(2), 180–192.

Angela, C., Wang, W., Lyu, H., Zhou, Y., and Huang, X. (2020). The effect of dietary supplementation of *Astragalus membranaceus* and *Bupleurum chinense* on the growth performance, immune-related enzyme activities, and genes expression in white shrimp, *Litopenaeus vannamei*. *Fish and Shellfish Immunology*, *107*, 379-384.

Anh, N. T. N., An, B. N. T., Lan, L. M., and Hai, T. N. (2019). Integrating different densities of white leg shrimp *Litopenaeus vannamei* and red seaweed *Gracilaria tenuistipitata* in the nursery phase: effects on water quality and shrimp performance. *Journal of Applied Phycology*, *31*(5), 3223–3234.

Awad, E., and Awaad, A. (2017). Role of medicinal plants on growth performance and immune status in fish. *Fish and Shellfish Immunology*, *67*, 40–54.

Bachere, E. (2000). Shrimp immunity and disease control. *Aquaculture*, *191*(1–3), 3–11.

Battistella, S., Bonivento, P., and Amirante, G. A. (1996). Hemocytes and immunological reactions in crustaceans. *Italian Journal of Zoology*, *63*(4), 337–343.

Belton, B., Reardon, T., and Zilberman, D. (2020). Sustainable commoditization of seafood. *Nature Sustainability*, *3*(9), 677-684.

Bi, W. J., Li, D. X., Xu, Y. H., Xu, S., Li, J., Zhao, X. F., and Wang, J. X. (2015). Scavenger receptor B protects shrimp from bacteria by enhancing phagocytosis and regulating the expression of antimicrobial peptides. *Developmental and Comparative Immunology*, *51*(1), 10-21.

Boyd, C. E., D'Abramo, L. R., Glencross, B. D., Huyben, D. C., Juarez, L. M., Lockwood, G. S., McNevin, A. A., Tacon, A. G. J., Teletchea, F., Tomasso, J. R., Tucker, C. S., and Valenti, W. C. (2020). Achieving sustainable aquaculture: Historical and current perspectives and future needs and challenges. *Journal of the World Aquaculture Society*, *51*(3), 578–633.

Bulfon, C., Volpatti, D., and Galeotti, M. (2015). Current research on the use of plant-derived products in farmed fish. *Aquaculture Research*, *46*(3), 513-551.

Burge, E. J., Madigan, D. J., Burnett, L. E., and Burnett, K. G. (2007). Lysozyme gene expression by hemocytes of Pacific white shrimp, *Litopenaeus vannamei*, after injection with Vibrio. *Fish and Shellfish Immunology*, *22*(4), 327–339.

Chang, Y.-P., Liu, C.-H., Wu, C.-C., Chiang, C.-M., Lian, J.-L., and Hsieh, S.-L. (2012). Dietary administration of zingerone to enhance growth, non-specific immune response, and resistance to *Vibrio alginolyticus* in Pacific white shrimp (*Litopenaeus vannamei*) juveniles. *Fish and Shellfish Immunology*, *32*(2), 284–290.

Charoendat, U., and Koedprang, W. (2019). Effects of crude extract of *Phyllanthus urinaria* Linn. on growth performance, feed utilization, and survival rate of Pacific white shrimp (*Litopenaeus vannamei* Boone). *Rajamangala University of Technology Srivijaya Research Journal*, *11*(3), 519-527.

Chau, D. TM, Hoa, TTT, and Duyen, TTM (2020). Survey on the use of herbs in red tilapia (*Oreochromis sp*.) culture in the Mekong Delta. *Journal of Science Can Tho University*, *56* (6), 227-236.

Chen, M. Y., Hu, K. Y., Huang, C. C., and Song, Y. L. (2005). More than one type of transglutaminase in invertebrates? A second type of transglutaminase is involved in shrimp coagulation. *Developmental and Comparative Immunology*, *29*(12), 1003–1016.

Chirawithayaboon, P., Areechon, N., and Meunpol, O. (2020). Hepatopancreatic antioxidant enzyme activities and disease resistance of Pacific white shrimp (*Litopenaeus vannamei*) fed a diet supplemented with garlic (*Allium sativum*) extract. *Agriculture and Natural Resources*, *54*(4), 377–386.

Chisholm, J. R., and Smith, V. J. (1995). Comparison of antibacterial activity in the hemocytes of different crustacean species. *Comparative Biochemistry and Physiology Part A: Physiology*, *110*(1), 39-45.

Chithambaran, S., and David, S. (2014). Antiviral property and growth promoting potential of Punarnava, Boerhaavia diffusa in tiger prawn culture.

Coates, C. J., and Söderhäll, K. (2021). The stress–immunity axis in shellfish. *Journal of Invertebrate Pathology*, *186* (October), 107492.

Dangtip, S., Sirikharin, R., Sanguanrut, P., Thitamadee, S., Sritunyalucksana, K., Taengchaiyaphum, S., Mavichak, R., Proespraiwong, P., and Flegel, T. W. (2015).

AP4 method for two-tube nested PCR detection of AHPND isolates of *Vibrio parahaemolyticus*. *Aquaculture Reports*, *2* (November), 158–162.

Dao, N. L. A. (2022). Use of Vietnamese plant extracts in striped catfish (*Pangasianodon hypophthalmus*) farming and processing to improve the shelf life of fish fillets.

Dashtiannasab, A. (2020). White spot syndrome virus. In *Emerging and Reemerging Viral Pathogens* (pp. 717-728). Academic Press.

Dawood, M. A. O., Koshio, S., and Esteban, M. Á. (2018). Beneficial roles of feed additives as immunostimulants in aquaculture: a review. *Reviews in Aquaculture*, *10*(4), 950–974.

De Silva, B. C. J., Hossain, S., Wimalasena, S. H. M. P., Pathirana, H. N. K. S., and Heo, G. J. (2018). Putative virulence traits and antibiogram profile of *Aeromonas spp.* isolated from frozen white-leg shrimp (*Litopenaeus vannamei*) marketed in Korea. *Journal of Food Safety*, *38*(4), 1–10.

De Silva, M. L. I., Ranjula, S., Thanuja, M., Katuwawala, D., and Sumanapala, A. P. (2021). *Review on Impacts of Litopenaeus vannamei on Aquaculture*. *March*, 149–170.

De-La-Re-Vega, E., García-Orozco, K. D., Calderón-Arredondo, S. A., Romo-Figueroa, M. G., Islas-Osuna, M. A., Yepiz-Plascencia, G. M., and Sotelo-Mundo, R. R. (2004). Recombinant expression of marine shrimp lysozyme in Escherichia coli. *Electronic Journal of Biotechnology*, *7*(3), 12-13.

Destoumieux, D., Muñoz, M., Cosseau, C., Rodriguez, J., Bulet, P., Comps, M., and Bachère, E. (2000). Penaeidins, antimicrobial peptides with chitin-binding activity, are produced and stored in shrimp granulocytes and released after microbial challenge. *Journal of Cell Science*, *113*(3), 461–469.

Dhar, A. K., Cruz-Flores, R., Caro, L. F. A., Siewiora, H. M., and Jory, D. (2019). Diversity of single-stranded DNA containing viruses in shrimp. *VirusDisease*, *30*(1), 43–57.

Direkbusarakom, S. (1997). Effect of *Phyllanthus spp*. against yellow head baculovirus infection in black tiger shrimp, *Penaeus monodon*.

Direkbusarakom, S., Ezura, Y., Yoshimizu, M., and Herunsalee, A. (1998). Efficacy of Thai traditional herb extracts against fish and shrimp pathogenic bacteria. *Fish Pathology*, *33*(4), 437–441.

EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP). (2013). Guidance on the renewal of the authorization of feed additives. *EFSA Journal*, *11*(10), 3431.

El-Asely, A. M., Shaheen, A. A., Abbass, A. A., Inada, M., Okugawa, S., Tachikawa, Y., ... and Itami, T. (2011). Changes in the immune-related gene expression of kuruma shrimp *Marsupenaeus japonicus* in response to dietary inclusion of Macrophage Activating Chinese Herbs (MACH). In *Proceedings of the 4th Global Fisheries and Aquaculture Research Conference, the Egyptian International Center for Agriculture, Giza, Egypt, 3-5 October 2011* (pp. 295-303). Massive Conferences and Trade Fairs.

El-Saadony, M. T., Swelum, A. A., Ghanima, M. M. A., Shukry, M., Omar, A. A., Taha, A. E., ... and Abd El-Hack, M. E. (2022). Shrimp production, the most important diseases that threaten it, and the role of probiotics in confronting these diseases: a review. *Research in veterinary science*, *144*, 126–140.

European Commission, 2009. Commission Regulation (EC) No 710/2009 of 5 August 2009 Amending Regulation (EC) No 889/2008 Laying down Detailed Rules for the Implementation of Council Regulation (EC) No 834/2007, as Regards Laying down Detailed Rules on Organic Aquaculture Animal and Seaweed Production. Off. J. Eur. Union. 204, 15-34.

F. A. O. (2006). *State of world aquaculture 2006. FAO Fisheries Technical Paper No. 500*. *500*, 134 p.-134 p.

FAO. (2020). *World Fisheries and Aquaculture, FAO: Rome,2020*. https://www.fao.org/3/ca9229en/online/ca9229en.html#chapter-1_1.

FAO. (2022). COVID-19 pandemic impacts on Asia and the Pacific. In *COVID-19 pandemic impacts on Asia and the Pacific*.

FAO. (2022). The State of World Fisheries and Aquaculture 2022. Towards Blue Transformation. Rome, FAO.

Fernandes, S. (2019). *Halotolerant bacteria as probiotics in shrimp aquaculture* (Doctoral dissertation, Goa University).

Flegel, T. W. (1998). Advances in Shrimp Biotechnology Special Session on Shrimp Biotechnology 5th Asian Fisheries Forum the National Center for. In *Science and Technology*.

Flegel, T. W. (2006). Detection of major penaeid shrimp viruses in Asia, a historical perspective with emphasis on Thailand. *Aquaculture*, *258*(1–4), 1–33.

Ford, S. E., Kanaley, S. A., and Littlewood, D. T. J. (1993). Cellular responses of oysters infected with *Haplosporidium nelsoni*: changes in circulating and tissue-infiltrating hemocytes. *Journal of invertebrate pathology*, *61*(1), 49-57.

Ge, H., Li, J., Chen, P., Liang, Z., Ren, H., and Li, D. (2014). The immune response of *Litopenaeus vannamei* and its susceptibility to *Vibrio parahaemolyticus* under stress caused by ammonia nitrogen at different concentrations. *Fish Sci Prog*, *35*, 76-82.

Gianazza, E., Eberini, I., Palazzolo, L., and Miller, I. (2021). Hemolymph proteins: An overview across marine arthropods and molluscs. *Journal of Proteomics*, *245*, 104294.

Giri, S. S., Sen, S. S., Chi, C., Kim, H. J., Yun, S., Park, S. C., and Sukumaran, V. (2015). Effect of cellular products of potential probiotic bacteria on the immune response of *Labeo rohita* and susceptibility to *Aeromonas hydrophila* infection. *Fish and Shellfish Immunology*, *46*(2), 716–722.

Giulianini, P. G., Bierti, M., Lorenzon, S., Battistella, S., and Ferrero, E. A. (2007). Ultrastructural and functional characterization of circulating hemocytes from the freshwater crayfish *Astacus leptodactylus*: cell types and their role after in vivo artificial non-self-challenge. *Micron*, *38*(1), 49-57.

Han, J. E., Lee, S. C., Park, S. C., Jeon, H. J., Kim, K. Y., Lee, Y. S., Park, S., Han, S. H., Kim, J. H., and Choi, S. K. (2020). Molecular detection of *Enterocytozoon hepatopenaei* and *Vibrio parahaemolyticus*-associated acute hepatopancreatic necrosis disease in Southeast Asian *Penaeus vannamei* shrimp imported into Korea. *Aquaculture*, *517*, 734812.

Hien, N., Thi Lan Huong, N., Dinh Chuong, V., Thi Viet Nga, N., Hong Quang, P., Thi Viet Hang, B., Van, N., and Van Long, N. (2016). Status of acute hepatopancreatic necrosis disease (AHPND) and other emerging diseases of penaeid shrimps in Viet Nam. *Aquaculture Department, Southeast Asian Fisheries Development Center., 330*, 88–95.

Holland, M. C. H., and Lambris, J. D. (2002). The complement system in teleosts. *Fish and Shellfish Immunology*, *12*(5), 399–420.

Hong, P. N., and San, H. T. (1993). *Mangroves of Vietnam* (Vol. 7). Iucn.

Hong, X., Lu, L., and Xu, D. (2016). Progress in research on acute hepatopancreatic necrosis

disease (AHPND). *Aquaculture International*, *24* (2), 577–593.

Hose, J. E., and Martin, G. G. (1989). Defense functions of granulocytes in the ridgeback prawn *Sicyonia ingentis*. *Journal of Invertebrate Pathology*, *53*(3), 335-346.

Huang, H. T., Liao, Z. H., Wu, Y. S., Lin, Y. J., Kang, Y. S., and Nan, F. H. (2022). Effects of *Bidens alba* and *Plectranthus amboinicus* dietary supplements on nonspecific immune responses, growth, and resistance to *Vibrio alginolyticus* in white leg shrimp (*Penaeus vannamei*). *Aquaculture*, *546*, 737306.

Huang, H., Pan, L., Pan, S., and Song, M. (2018). Effects of dietary herbal formulae combined by Astragalus polysaccharides, chlorogenic acid, and allicin in different combinations and proportions on growth performance, non-specific immunity, antioxidant status, vibriosis resistance, and damage indexes. *Aquaculture Research*, *49*(2), 701–716.

Huyen, HM, Hai, TN, Hoa, TTT, and Viet, LQ (2020). Effect of herbal extracts on growth, nonspecific immunity, and disease resistance of white shrimp (*Penaeus vannamei*) to *Vibrio parahaemolyticus*. *Journal of Science Can Tho University*, *56* (5), 150-159.

Isnani, R. A., Huyen, H. M., Duyen, T. T. M., and Hoa, T. T. T. (2021). Dietary supplementation with *Phyllanthus urinaria* and *Terminalia catappa* enhances innate immunity and resistance to white spot syndrome virus in whiteleg shrimp (*Penaeus vannamei*). *AACL Bioflux*, *14*(6), 3566–3582.

Johansson, M. W., and Söderhäll, K. (1996). *The Prophenoloxidase Activating System and Associated Proteins in Invertebrates BT - Invertebrate Immunology* (B. Rinkevich and W. E. G. Müller (Eds.); pp. 46–66). Springer Berlin Heidelberg.

Johansson, M. W., Keyser, P., Sritunyalucksana, K., and Söderhäll, K. (2000). Crustacean haemocytes and hematopoiesis. *Aquaculture*, *191*(1–3), 45–52.

Joshi, J., Srisala, J., Truong, V. H., Chen, I. T., Nuangsaeng, B., Suthienkul, O., Lo, C. F., Flegel, T. W., Sritunyalucksana, K., and Thitamadee, S. (2014). Variation in *Vibrio parahaemolyticus* isolates from a single Thai shrimp farm experiencing an outbreak of acute hepatopancreatic necrosis disease (AHPND). *Aquaculture*, *428–429*, 297–302.

Kim, N. T. Q., Van Hien, H., Doan Khoi, L. N., Yagi, N., and Lerøy Riple, A. K. (2020). Quality management practices of intensive whiteleg shrimp (*Litopenaeus vannamei*) farming: A Study of the Mekong delta, Vietnam. *Sustainability (Switzerland)*, *12*(11).

Kongton, K., Phongdara, A., Tonganunt-Srithaworn, M., and Wanna, W. (2011). Molecular cloning and expression analysis of the interferon-γ-inducible lysosomal thiol reductase gene from the shrimp *Penaeus monodon*. *Molecular Biology Reports, 38*, 3463-3470.

Kulkarni, A., Krishnan, S., Anand, D., Kokkattunivarthil Uthaman, S., Otta, S. K., Karunasagar, I., and Kooloth Valappil, R. (2021). Immune responses and immunoprotection in crustaceans with special reference to shrimp. *Reviews in Aquaculture*, *13*(1), 431–459.

Kumar, R., Ng, T. H., and Wang, H. C. (2020). Acute hepatopancreatic necrosis disease in penaeid shrimp. A *Reviews in Aquaculture*, *12*(3), 1867–1880.

Kumar, V., Nguyen, D. V., Baruah, K., and Bossier, P. (2019). Probing the mechanism of VP AHPND extracellular proteins toxicity purified from *Vibrio parahaemolyticus* AHPND strain in germ-free Artemia test system. *Aquaculture*, *504*, 414–419.

Kumar, V., Roy, S., Behera, B. K., Bossier, P., and Das, B. K. (2021). Acute hepatopancreatic necrosis disease: Virulence, pathogenesis, and mitigation strategies in Shrimp aquaculture. *Toxins*, *13*(8), 1–28.

Lai, H. C., Ng, T. H., Ando, M., Lee, C. T., Chen, I. T., Chuang, J. C., ... and Wang, H. C. (2015). Pathogenesis of acute hepatopancreatic necrosis disease (AHPND) in shrimp. *Fish and shellfish immunology*, *47*(2), 1006-1014.

Le Cao, Q. (2007). The development and sustainability of shrimp culture in Viet Nam. *Species and system selection for sustainable aquaculture*, 283-294.

Le Moullac, G., Le Groumellec, M., Ansquer, D., Froissard, S., and Levy, P. (1997a). Hematological and phenoloxidase activity changes in the shrimp *Penaeus stylirostris* in relation with the moult cycle: Protection against vibriosis. *Fish and Shellfish Immunology*, *7*(4), 227–234.

Le Moullac, G., Le Groumellec, M., Ansquer, D., Froissard, S., and Levy, P. (1997b). Haematological and phenoloxidase activity changes in the shrimp *Penaeus stylirostris* in relation with the moult cycle: Protection against vibriosis. *Fish and Shellfish Immunology*, *7*(4), 227–234.

Li, E., Wang, X., Chen, K., Xu, C., Qin, J. G., and Chen, L. (2017). Physiological change and nutritional requirement of Pacific white shrimp *Litopenaeus vannamei* at low salinity. *Reviews in Aquaculture*, *9*(1), 57-75.

Li, S., and Li, F. (2020). The Anti-lipopolysaccharide Factors in Crustaceans. *Sub-cellular biochemistry*, *94*, 63–80.

Liao, I. C., and Chien, Y. H. (2011). The pacific white shrimp, *Litopenaeus vannamei*, in Asia: The world's most widely cultured alien crustacean. *In the wrong place-alien marine crustaceans: Distribution, biology, and impacts*, 489-519.

Lightner, D. V. (2011). Status of shrimp diseases and advances in shrimp health management. *Diseases in Asian Aquaculture VII. Fish Health Section, Asian Fisheries Society, Selangor, Malaysia*, 121-134.

Lightner, D. V., Redman, R. M., Pantoja, C. R., Tang, K. F. J., Noble, B. L., Schofield, P., ... and Navarro, S. A. (2012). Historic emergence, impact, and current status of shrimp pathogens in the Americas. *Journal of invertebrate pathology*, *110*(2), 174-183.

Lin, X., and Söderhäll, I. (2011). Crustacean hematopoiesis and the astakine cytokines. *Blood*, *117*(24), 6417–6424. https://doi.org/10.1182/blood-2010-11-320614.

Linga Prabu, S. Chandrasekar, K. Ambashankar, J. Syama Dayal, Sanal Ebeneezar, K. Ramachandran, M. Kavitha, P. Vijayagopal, Effect of dietary *Syzygium cumini* leaf powder on growth and non-specific immunity of *Litopenaeus vannamei* (Boone 1931) and defense against a virulent strain of *Vibrio parahaemolyticus,* Aquaculture, Volume 489,2018, Pages920, ISSN00448486.

Liu, F., Shao, G. Y., Tian, Q. Q., Cheng, B. X., Shen, C., Wang, A. M., ... and Yu, Y. B. (2021). Enhanced growth performance, immune responses, immune-related gene expression, and disease resistance of red swamp crayfish (*Procambarus clarkii*) fed dietary glycyrrhizic acid. *Aquaculture*, *533*, 736202.

Luu, Q. H., Nguyen, T. B. T., Nguyen, T. L. A., Do, T. T. T., Dao, T. H. T., and Padungtod, P. (2021). Antibiotics use in fish and shrimp farms in Vietnam. *Aquaculture Reports*, *20*, 100711.

Macusi, E. D., Estor, D. E. P., Borazon, E. Q., Clapano, M. B., and Santos, M. D. (2022). Environmental and Socioeconomic Impacts of Shrimp Farming in the Philippines: A Critical Analysis Using PRISMA. *Sustainability (Switzerland)*, *14*(5), 1–19.

Maftuch, Prasetio, E., Sudianto, A., Rozik, M., Nurdiyani, R., Sanusi, E., Nursyam, H., Fariedah, F., Marsoedi, and Murachman. (2013). Improvement of innate immune responses and defense activity in tiger shrimp (*Penaeus monodon* Fab.) by

intramuscular administration of the outer membrane protein *Vibrio alginolyticus*. *SpringerPlus*, *2*(1), 432.

Mariita, R. M., Ogol, C. K. P. O., Oguge, N. O., and Okemo, P. O. (2011). Methanol extract of three medicinal plants from samburu in northern Kenya shows significant antimycobacterial, antibacterial, and antifungal properties. *Research Journal of Medicinal Plant*, *5*(1), 54–64.

Medzhitov, R., and Janeway, C., Jr (2000). Innate immunity. *The New England journal of medicine*, *343*(5), 338–344

Men, T. T., Yen, H. K., Lan, H. T. C., Son, N. H., Khang, D. T., and Tuan, N. T. (2022). Phytochemical Screening and Evaluation of Antioxidant, Antibacterial Activities of Ethanol Extract from *Combretum quadrangulare* Collected in Vietnam. *International Journal of Pharma Medicine and Biological Sciences*, *11*(3), 59–64.

Naylor, R. L., Hardy, R. W., Buschmann, A. H., Bush, S. R., Cao, L., Klinger, D. H., and Troell, M. (2021). A 20-year retrospective review of global aquaculture. *Nature*, *591*(7851), 551-563.

Ng, W. K., Mong, M. L., and Abdul-Hamid, A. A. (2023). Dietary montmorillonite clay improved *Penaeus vannamei* survival from acute hepatopancreatic necrosis disease and modulated stomach microbiota. *Animal Feed Science and Technology*, 115581.

Ngo, H. V. T., Huang, H. T., Lee, P. T., Liao, Z. H., Chen, H. Y., and Nan, F. H. (2020). Effects of *Phyllanthus amarus* extract on nonspecific immune responses, growth, and resistance to *Vibrio alginolyticus* in white shrimp *Litopenaeus vannamei*. *Fish and Shellfish Immunology*, *107*, 1–8.

Ngoc, Q. N., Minh, T. N., Van, M. L., Hoang, D. P., and Trung, T. N. (2021, December). Chemical Composition and Antioxidant Activities of Extracts of *Combretum quadrangulare* Kurz Leaves Grown in An Giang Province, Vietnam. In *IOP Conference Series: Earth and Environmental Science* (Vol. 939, No. 1, p. 012060). IOP Publishing.

Nopsiri, W., Chansakaow, S., Putiyanan, S., Natakankitkul, S., and S Santiarworn, D. (2014). Antioxidant and anticancer activities from leaf extracts of four Combretum species from northern Thailand. *Chiang Mai University Journal of Natural Sciences*, *13*(2), 195-205.

Pipe, R. K., and Coles, J. A. (1995). Environmental contaminants influencing immune function in marine bivalve molluscs. *Fish and Shellfish Immunology*, *5*(8), 581–595.

Pourmozaffar, S., Hajimoradloo, A., and Miandare, H. K. (2017). Dietary effect of apple cider vinegar and propionic acid on immune-related transcriptional responses and growth performance in white shrimp, *Litopenaeus vannamei*. *Fish and shellfish immunology*, *60*, 65-71.

Prabu, D. L., Chandrasekar, S., Ambashankar, K., Dayal, J. S., Ebeneezar, S., Ramachandran, K., ... and Vijayagopal, P. (2018). Effect of dietary *Syzygium cumini* leaf powder on growth and non-specific immunity of *Litopenaeus vannamei* (Boone 1931) and defense against virulent strain of *Vibrio parahaemolyticus*. *Aquaculture*, *489*, 9-20.

Prager, E. M., and Jolles, P. (1996). Animal lysozymes c and g: an overview. *Exs*, *75*, 9-31.

Prathomya, P., Hussein, H. A., Tran, N. T., Duan, Y. H., Peng, S. H., Xu, Y., ... and Wang, G. T. (2019). Effects of dietary supplementation of *Astragalus membranaceus*, *Codonopsis pilosula*, and *Glycyrrhiza uralensis* extract mixture on growth performance, hematological parameters and hepatopancreatic performance in juvenile Pacific white shrimp (*Litopenaeus vannamei*). *Aquaculture Research*, *50*(9), 2707-2717.

Qin, Z., Babu, V. S., Wan, Q., Zhou, M., Liang, R., Muhammad, A., ... and Lin, L. (2018). Transcriptome analysis of Pacific white shrimp (*Litopenaeus vannamei*) challenged by *Vibrio parahaemolyticus* reveals unique immune-related genes. *Fish & shellfish immunology*, *77*, 164-174.

Ramírez-Castillo, E. R., Arellano-Martínez, M., Ceballos-Vázquez, B. P., González-Ocampo, H. A., Aguirre-Guzmán, G., and Luna-González, A. (2011). Immune response of Lion's paw Scallop *Nodipecten subnodosus* (Sowerby, 1835) Challenged with *Vibrio alginolyticus. Thai Journal of Veterinary Medicine*, *41*(4), 425–432.

Ringø, E. (2020). Probiotics in shellfish aquaculture. *Aquaculture and Fisheries*, *5*(1), 1-27.

Robinson, M. J., Sancho, D., Slack, E. C., LeibundGut-Landmann, S., and Sousa, C. R. E. (2006). Myeloid C-type lectins in innate immunity. *Nature immunology*, *7*(12), 1258-1265.

Roch, P. (1999). Defense mechanisms and disease prevention in farmed marine invertebrates. *Aquaculture*, *172*(1–2), 125–145.

Rodríguez, J., and Le Moullac, G. (2000). State of the art of immunological tools and health control of penaeid shrimp. *Aquaculture*, *191*(1), 109–119.

Roy, R., Singh, R. K., Jash, S. K., Sarkar, A., and Dilip Gorai. (2014). *Combretum quadrangulare* (Combretaceae): Phytochemical Constituents and Biological activity. *Indo-American Journal of Pharmaceutical Research*, *4*(8), 3416–3427.

Santhosh, P., Kamaraj, M., Saravanan, M., and Nithya, T. G. (2023). Dietary supplementation of *Salvinia cucullata* in white shrimp *Litopenaeus vannamei* to enhance the growth, nonspecific immune responses, and disease resistance to *Vibrio parahaemolyticus*. *Fish and shellfish immunology*, *132*, 108465.

Schmittgen, T. D., and Livak, K. J. (2008). Analyzing real-time PCR data by the comparative CT method. *Nature Protocols*, *3*(6), 1101–1108.

Sequeira, T., Tavares, D., and Arala-Chaves, M. (1996). Evidence for circulating hemocyte proliferation in the shrimp *Penaeus japonicus*. *Developmental and Comparative Immunology*, *20*(2), 97–104.

Shinn, A. P., Pratoomyot, J., Griffiths, D., Trong, T. Q., Vu, N. T., Jiravanichpaisal, P., and Briggs, M. (2018). Asian shrimp production and the economic costs of disease. *Asian Fish. Sci. S*, *31*, 29-58.

Shinn, A., Pratoomyot, J., Jiravanichapisal, P., Delannoy, C., Kijphakapanith, N., Paladini, G., and Griffiths, D. (2016). Counting the cost of the aquatic disease in Asia. *Aquac. Asia Pacific*, *12*, 14-18.

Sivagnanavelmurugan, M., Thaddaeus, B. J., Palavesam, A., and Immanuel, G. (2014). Dietary effect of *Sargassum wightii* fucoidan to enhance growth, prophenoloxidase gene expression of *Penaeus monodon*, and immune resistance to *Vibrio parahaemolyticus*. *Fish* and *Shellfish Immunology*, *39*(2), 439-449.

Sivakumar, A. T. and K. A. (2017). Synthesis, characterization, and antibacterial activity of silver nanoparticles from leaf extract of *Phyllanthus urinalia*. L. *European Journal of Biomedical and Pharmaceutical Sciences AND Pharmaceutical Sciences*, *4*(8), 544–533.

Smith, V. J., Brown, J. H., and Hauton, C. (2003). Immunostimulation in crustaceans: Does it really protect against infection? *Fish and Shellfish Immunology*, *15*(1), 71–90.

Söderhäll, K., and Cerenius, L. (1992). Crustacean immunity. *Annual Review of Fish*

Diseases, *2*, 3–23.

Song, Y. L., and Li, C. Y. (2014). Shrimp immune system - Special focus on penaeidin. *Journal of Marine Science and Technology (Taiwan)*, *22*(1), 1–8.

Soowannayan, C., Boonmee, S., Puckcharoen, S., Anatamsombat, T., Yatip, P., Ng, W. K., ... and Withyachumnarnkul, B. (2019). Ginger and its component shogaol inhibit Vibrio biofilm formation in vitro and orally protect shrimp against acute hepatopancreatic necrosis disease (AHPND). *Aquaculture*, *504*, 139-147.

Soto-Rodriguez, S. A., Gomez-Gil, B., Lozano-Olvera, R., Betancourt-Lozano, M., and Morales-Covarrubias, M. S. (2015). Field and experimental evidence of *Vibrio parahaemolyticus* as the causative agent of acute hepatopancreatic necrosis disease of cultured shrimp (*Litopenaeus vannamei*) in northwestern Mexico. *Applied and Environmental Microbiology*, *81*(5), 1689–1699.

Sritong, D., Hanbanjong, A., Isariyodom, S., Ingkagoon, A., and Sangvaranont, A. (2005). Anthelmintic efficacy of *Diospyros mollis*, *Combretum quadrangulare*, and *Euphorbia heterophylla* on adult *Ascaridia galli* in layers. In *Proceedings of 43rd Kasetsart University Annual Conference, Thailand, 1-4 February 2005. Subject: Animals* (pp. 323-328). Kasetsart University.

Su, C., Fan, D., Pan, L., Lu, Y., Wang, Y., and Zhang, M. (2020). Effects of Yu-Ping-Feng polysaccharides (YPS) on the immune response, intestinal microbiota, disease resistance, and growth performance of *Litopenaeus vannamei*. *Fish and Shellfish Immunology*, *105*(March), 104–116.

Subramanian, D., Jang, Y. H., Kim, D. H., Kang, B. J., and Heo, M. S. (2013). Dietary effect of *Rubus coreanus* ethanolic extract on immune gene expression in white leg shrimp, *Penaeus vannamei*. *Fish & shellfish immunology*, *35*(3), 808-814.

Suleman, S., Andayani, S., and Yuniarti, A. (2018). The Effect of *Ulva lactuta* Polysaccharide Extract on Total Haemocyte Count and Phagocytic Activity of *L. vannamei*. *Research Journal of Life Science*, *5*(3), 156-162.

Syahidah, A., Saad, C. R., Daud, H. M., and Abdelhadi, Y. M. (2015). Status and potential of herbal applications in aquaculture: A review. *Iranian Journal of Fisheries Sciences*, *14*(1), 27–44.

Tang, K.F.J., Bondad-Reantaso, M.G., Arthur, J.R., MacKinnon, B., Hao, B., Alday-Sanz,

V., Liang, Y., and D. (2020). Shrimp acute hepatopancreatic necrosis disease strategy manual. In *Shrimp acute hepatopancreatic necrosis disease strategy manual* (Vol. 1190, Issue December).

Tassanakajon, A., Somboonwiwat, K., Supungul, P., and Tang, S. (2013). Discovery of immune molecules and their crucial functions in shrimp immunity. *Fish and Shellfish Immunology*, *34*(4), 954–967.

Thien Nguyen Pharmaceutical Joint Company (Ed.). (2019, November 21). *Active ingredients that make the effect of Cao Diep Chau*. Thien Nguyen Pharmaceutical Joint Company. Retrieved January 30, 2023, from https://thiennguyen.net.vn/thanh-phan-hoat-chat-lam-nen-tac-dung-cua-cao-diep-ha-chau.html.

Thitamadee, S., Prachumwat, A., Srisala, J., Jaroenlak, P., Salachan, P. V., Sritunyalucksana, K., and Itsathitphaisarn, O. (2016). Review of current disease threats for cultivated penaeid shrimp in Asia. *Aquaculture*, *452*, 69-87.

Tran, L., Nunan, L., Redman, R. M., Mohney, L. L., Pantoja, C. R., Fitzsimmons, K., and Lightner, D. V. (2013). Determination of the infectious nature of the agent of acute hepatopancreatic necrosis syndrome affecting penaeid shrimp. *Diseases of Aquatic Organisms*, *105*(1), 45–55.

Tran, T. M. D., Nguyen, T. T., and Tran, T. T. H. (2021). In vitro antibacterial activity of several plant extracts against fish bacterial pathogens. *Can Tho University Journal of Science*, *13*, 106-112.

Trejo-Flores, J. V., Luna-González, A., Álvarez-Ruiz, P., Escamilla-Montes, R., Fierro-Coronado, J. A., Peraza-Gómez, V., Flores-Miranda, M. D. C., Diarte-Plata, G., and Rubio-Castro, A. (2018). Immune-related gene expression in *Penaeus vannamei* fed aloe vera. *Latin American Journal of Aquatic Research*, *46*(4), 756–764.

Walker, P. J., and Winton, J. R. (2010). Emerging viral diseases of fish and shrimp. *Veterinary research*, *41*(6).

Wang, Y.-C., Chang, P.-S., and Chen, H.-Y. (2008). Differential time-series expression of immune-related genes of Pacific white shrimp *Litopenaeus vannamei* in response to dietary inclusion of β-1,3-glucan. *Fish & Shellfish Immunology*, *24*(1), 113–121.

Wu, C.-C., Chang, Y.-P., Wang, J.-J., Liu, C.-H., Wong, S.-L., Jiang, C.-M., and Hsieh, S.-L. (2015). Dietary administration of *Gynura bicolor* (Roxb. Willd.) DC water extract

enhances immune response and survival rate against *Vibrio alginolyticus* and white spot syndrome virus in white shrimp *Litopeneaus vannamei*. *Fish and Shellfish Immunology*, *42*(1), 25–33.

Yakubu, Y., Talba, A. M., Chong, C. M., Ismail, I. S., and Shaari, K. (2020). Effect of *Terminalia catappa* methanol leaf extract on nonspecific innate immune responses and disease resistance of red hybrid tilapia against *Streptococcus agalactiae*. *Aquaculture Reports*, *18*, 100555.

Yap, W. G. (1999). Shrimp culture: a global overview. *SEAFDEC Asian Aquaculture*, *21*(4), 18–21.

Zacarias, S., Fegan, D., Wangsoontorn, S., Yamuen, N., Limakom, T., Carboni, S., Davie, A., Metselaar, M., Little, D. C., and Shinn, A. P. (2021). Increased robustness of postlarvae and juveniles from non-ablated Pacific whiteleg shrimp, *Penaeus vannamei*, broodstock post-challenged with pathogenic isolates of *Vibrio parahaemolyticus* (VpAHPND) and white spot disease (WSD). *Aquaculture*, *532*, 736033.

Zheng, W., and Chen, X. (2006). Cloning and expression analysis of interferon-γ-inducible-lysosomal thiol reductase gene in large yellow croaker (*Pseudosciaena crocea*). *Molecular immunology*, *43*(13), 2135-2141.