Effectiveness of *Wedelia chinensis* extract supplementation on the hepatopancreas histology of white-leg shrimp (*Penaeus vannamei)* experimentally infected with AHPND-causing *Vibrio parahaemolyticus*

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Abstract. This study aimed to evaluate the effects of *Wedelia chinensis* extract (WCE) supplementation on the histological changes of the hepatopancreas of white-leg shrimp (*Penaeus vannamei*) experimentally infected *with Vibrio parahaemolyticus* causing acute hepatopancreatic necrosis disease (AHPND). White-leg shrimp with an average weight of 1.5-2 g ind^{-1} was randomly placed into 12 fiber plastic tanks (50 shrimp per 120 L tank). They were fed with a basal diet (control) or with a basal diet supplemented with WCE at three different concentrations: 31.25 mg L^{-1}, 312.5 mg L^{-1}, 625 mg L^{-1} for the first 7 days, and then fed with the basal diet for the next 7 days, and repeated with the same experimental diets for the following 7 days. An experimental challenge with *V. parahaemolyticus* was introduced on day 14. Samples for histological analyses were collected on day 0, day 7, and day 14 after challenge. In the challenge test against *Vibrio parahaemolyticus* at day 8, histological changes of hepatopancreas (HP) of shrimp fed on WCE treatment were not observed, while shrimp fed basal diet clearly showed histopathological characteristics of AHPND such as pale or yellow HP, massive necrosis in tubules and the disappearance of star-shaped polygonal structures of the lumen. The results indicate that the WCE supplementation has positive effects on the shrimp's health and reduces the damage of the shrimp's hepatopancreas infected with AHPND-causing *Vibrio parahaemolyticus*.
Key Words: bacteria, herb, early mortality syndrome, penaeid shrimp, vibriosis.

Introduction. White-leg shrimp (*Penaeus vannamei*) is one of the main shrimp species in the world and contributes to over 50% of shrimp production globally (Halim & Juanri 2016; FAO 2020). Bacterial diseases caused by *Vibrio* spp. are one of the most important challenges in shrimp farming (Anderson et al 2019). Acute Hepatopancreatic Necrosis Disease (AHPND) is caused by *Vibrio* spp. (*V. parahaemolyticus*, *V. harveyi*, *V. owensii*, *V. campbellii* and *V. punensis*), which have plasmids carrying the PirA and PirB (Photorhabdus insect-related -Pir) toxin genes, which destroy the shrimp's hepatopancreatic system and can cause a mass mortality rate of up to 100% from 30 to 35 days after stocking (OIE 2019; Tang et al 2020). AHPND-infected shrimp show lethargy, loss of appetite, atrophy of the hepatopancreas (HP), swelling, pale color of HP, and empty intestines. In addition, histological analysis shows that the HP tubules have degenerated, rounding up and sloughing into their lumens at the early stage of infection, and presenting gradual proximal to distal deterioration, with significant rounding and massive sloughing of HP epithelial cells into the lumen at the acute phase (Lightner et al 2013; Tang et al 2020). The shrimp's HP is a gland system consisting of massive branched tubules with different types of epithelial cells, which are responsible for the functions of the liver and pancreas (Manan et al 2015). The shrimp's HP is highly sensitive to feed and chemicals incorporated in the feed, so it is considered an indicator illustrating the shrimp's health condition and the severity of health problems.

Presently, some herbs have been known to contain antibacterial ingredients, are environmentally friendly, and can be used in bacterial disease prevention in aquaculture (Cos et al 2006; Castro et al 2008; Zhu 2020). The herbs-incorporated feed can stimulate the appetite of aquatic animals, increase feed utilization efficiency, enhance the immune system and growth performance, leading to increased resistance to pathogens (Venketramalingam et al 2007; Citarasu 2010; Effendi et al 2022; Li et al 2022). Many herbs have been applied to enhance the non-specific immune system and growth promotion in fish or shrimp farming (Kirubakaran et al 2010; Chandran et al 2016).

Wedelia chinensis is a species of the Asteraceae family and is widely used in therapy, pharmaceutical products for humans. The flavonoids, sterols, and saponins found in *W. chinensis* have been defined as antibacterial, and anti-inflammatory agents, and play an important function in liver cell protection (Koul et al 2012). *W. chinensis* extract (WCE) also stimulates the activity of monocytes and lymphocytes leading to enhanced immune response against bacterial and viral agents (Koul et al 2012). Recently, WCE has been applied in the prevention of AHPND in white-leg shrimp and showed high potential functions in maintaining the shrimp's health and survival (Ngoc et al 2023).

This study was carried out to evaluate the health condition of *P. vannamei* through histopathology analysis of the HP of *P. vannamei* fed with a WCE incorporated diet, post-challenged with *V. parrahaemolyticus*.

Material and Method

Preparations of the W. chinensis extract incorporated diets. The WCE used for this study was obtained as in the study of Ngoc et al (2023). The extraction process of *W. chinensis* grass in the methanol solvent was processed by the method of Appiah et al (2003) and Hang et al (2018), with slight modifications and stored at -20^{o}C at the Laboratory of Fish Pathology, University of Agriculture and Forestry, Hue University, for further use. Four experimental diets were prepared: a basal (control) diet without supplementation of WCE, and three experimental diets containing WCE at the inclusion dose of 31.25 mg kg^{-1}, 312.5 mg kg^{-1}, and 625 mg kg^{-1} feed. The doses were used based on previous antimicrobial and toxicity tests (Ngoc et al 2023).

Bacterial preparation. A strain of *V. parahaemolyticus* NNP 001 was isolated from white-leg shrimp infected with AHPND in Thua Thien Hue, and identified as *V. parahaemolyticus* by PCR in 2019. This strain was stored at -20^{o}C in 20% glycerol at the Department of Aquatic Diseases, Faculty of Fisheries, University of Agriculture and Forestry, Hue University (Phuoc et al 2020). This strain was recovered on thiosulfate citrate bile salt sucrose (TCBS, Himedia, India) agar. A single colony on TCBS was collected and sub-cultured on tryptone soya broth medium (TSB, Himedia, India) supplemented with 2% NaCl and incubated in a GFL 3032 incubator (×150 rpm, GFL, Germany) at 28°C for 24 h. The overnight bacteria cultured was harvested by centrifugation at ×3000 rpm (KL04A-II, Kaida, China) for 20 minutes and diluted by sterilized saline water to achieve an OD_{600} of 1 (which corresponds to a density of 10^9 CFU mL^{-1}) using spectrophotometry at 600 nm (U2900, Hitachi, Japan). Serial 10-fold dilutions were made to achieve the bacterial concentration of 10^6 CFU mL^{-1}, which was used for experimental challenge in this study.

Shrimp source. White-leg shrimp juveniles with an average weight of 1.5±0.1 g were obtained from Phu Thuan hatchery, Thua Thien Hue province, Vietnam. The juveniles were quarantined to determine they were free of AHPND, white spot disease, and yellow head disease at the Veterinary Clinic of Thua Thien Hue. The shrimp were maintained in a 1000 L fiberglass tank at 27±2°C, pH 7.8–8, a salinity of 30–32‰, and dissolved oxygen at 6 mg L^{-1} for 2 weeks before conducting experiments. Shrimp were fed four times a day (8 am, 11 am, 2 pm, 5 pm) with a basal diet (INVE, Thailand) with protein >55%, lipid >9%, fiber <1.9%, moisture <8%. The uneaten feed, fecal matter, and dead shrimp were siphoned twice daily at 7 am and 6 pm. At the beginning of the experiment, the shrimp were checked for *Vibrio* by randomly sampling the HP of 5 shrimp, which was spread directly on TCBS agar, and bacterial growth was checked after a 24-hour incubation at 28°C (Phuoc et al 2020).

Experimental design. The experiment was designed with 4 treatments in triplicates (3 tanks per treatment) consisting of three experiment groups that were fed *W. chinensis* diets containing a single dose of 31.25 mg kg^{-1}, 312.5 mg kg^{-1}, and 625 mg kg^{-1} feed for the first 7 days. Then they were fed a basal diet for the next 7 days, and a repeat of the first 7 days followed (the same experimental diets). The control group was fed a basal diet. Shrimp were fed four times daily (8 am, 11 am, 2 pm, 5 pm) with the control or experimental diets at a rate of 3-5% of body weight. After the 21-day feeding regime, shrimp in all treatments were experimentally challenged with pathogenic *V. parahaemolyticus* (10^6 CFU mL^{-1}) by immersion for 30 minutes. 600 juvenile white-leg shrimp were allocated randomly into 16 fiberglass tanks (120 L, 50 shrimp per tank) with continuous aeration. During the experimental period, the water parameters were managed at the same conditions described above. Samples were collected from 3 shrimp from each treatment at 0, 7 and 14 days post-challenge (3 shrimp per tank; moribund shrimp was collected after challenge). Re-isolation of *V. parahaemolyticus* in moribund shrimp was confirmed by PCR.

Histopathological analysis. The histopathological analysis was done following the method of Lightner (1996). Briefly, the HP of shrimp in all treatments was sampled and preserved in Davidson's solution for histopathology analysis. The specimens for histopathological examination were processed following Lightner (1996). Sections were cut at 5 μm and stained with hematoxylin and eosin before being observed under the light microscope (Olympus CX23, Japan).

Statistical analysis. The histopathology was analyzed descriptively according to Tang et al (2020), with 3 phases of infection: the early phase of disease, acute phase and terminal phase of disease.

Results

Histological analysis of shrimp fed with W. chinensis extract. Shrimp in all experimental treatments fed with *W. chinensis* addition in feed showed active swimming with gut full of feed. The HP was dark in color and had a solid triangular shape (Figure 1A). These normal signs were observed in shrimp fed with the basal diet (Figure 1B).

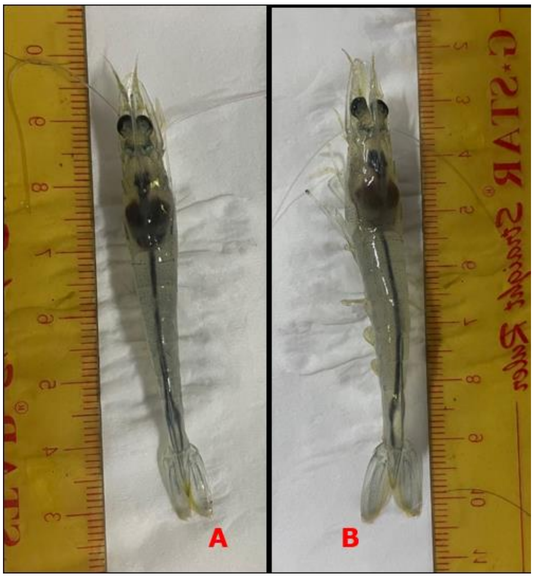

Figure 1. Healthy shrimp (*Penaeus vannamei*) with dark and sharp edges of hepatopancreas; the shrimp's guts are full of feed; A - shrimp fed with *Wedelia chinensis* supplementation in feed at the dose of 625 mg kg^{-1} feed; B - shrimp fed with basal feed.

The histological analysis showed normal hepatopancreatic tissues in both shrimp fed with herbal incorporated feed (Figures 2B, 2C, 2D) and shrimp fed with the basal diet (Figure 2A). No histology changes were observed in the shrimp of all treatments. Shrimp from the experimental groups and the control group showed normal tubular epithelium. The HP was enclosed by a thin membrane of connective tissue and each HP tubule had a lumen in the center that was surrounded by an epithelium. It is suggested that WCE was safe for shrimp.

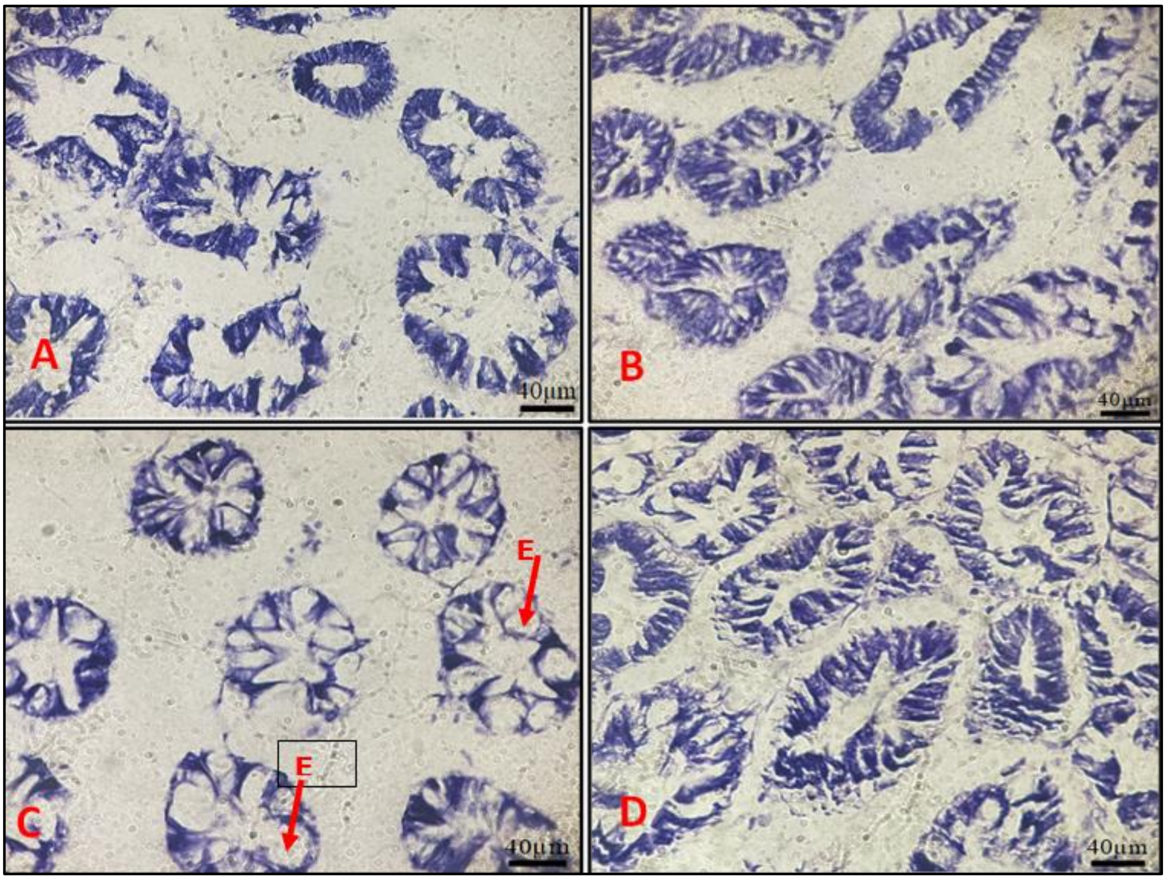

Figure 2. Normal hepatopancreas of healthy shrimp (*Penaeus vannamei*) showed the normal tubule with a lumen in the center and the presence of embryonic or Embryozellen cells (E): A - shrimp fed the basal diet; B, C, D - shrimp fed with *Wedelia chinensis* extract inclusion dose of 31.25 mg kg^{-1}, 312.5 mg kg^{-1}, and 625 mg kg^{-1} feed, respectively.

Histopathological analysis of shrimp fed with W. chinensis extract after experimental challenge with V. parahaemolyticus. After 7 days post-challenge with *V. parahaemolyticus*, shrimp in the experimental groups fed with herb-incorporated feed showed a full and dark colored HP with inverted gourd shape, and clear outline (Figure 3A). Their gut was full of feed (Figure 3A). Normal HP tubes with clear lumens were observed in the HP histology of these shrimp (Figure 4A). By contrast, the color of HP turned pale, with an unclear outline and empty gut was observed in shrimp fed with the basal diet (Figure 3B). The HP tubules were degenerated, rounded up and sloughed into their lumens, necrosis being present in the connecting tissue (Figure 4B).

After a 14-day challenge with *V. parahaemolyticus*, the shrimp in the herbal addition groups showed normal HP with a dark and brownish color (Figure 5A). No histology changes were observed in the HP of shrimp fed with *W. chinensis* at all three doses. Shrimp from these groups showed normal tubular epithelium (Figure 6).

Figure 3. Healthy shrimp (*Penaeus vannamei*) fed with *Wedelia chinensis* extract (A) and *Vibrio parahaemolyticus* infected shrimp fed with the basal diet (B).

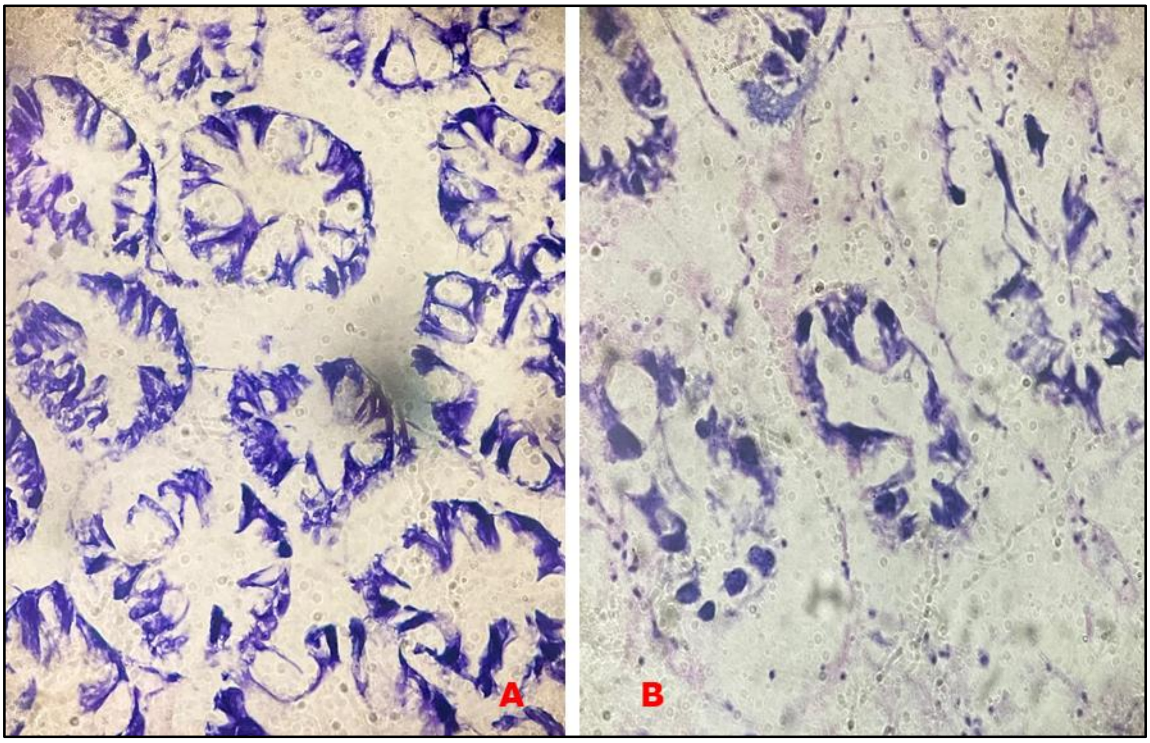

Figure 4. Normal hepatopancreas of shrimp (*Penaeus vannamei*) fed with *Wedelia chinensis* and degradation of hepatopancreas infected with *Vibrio parahaemolyticus* when fed a basal diet.

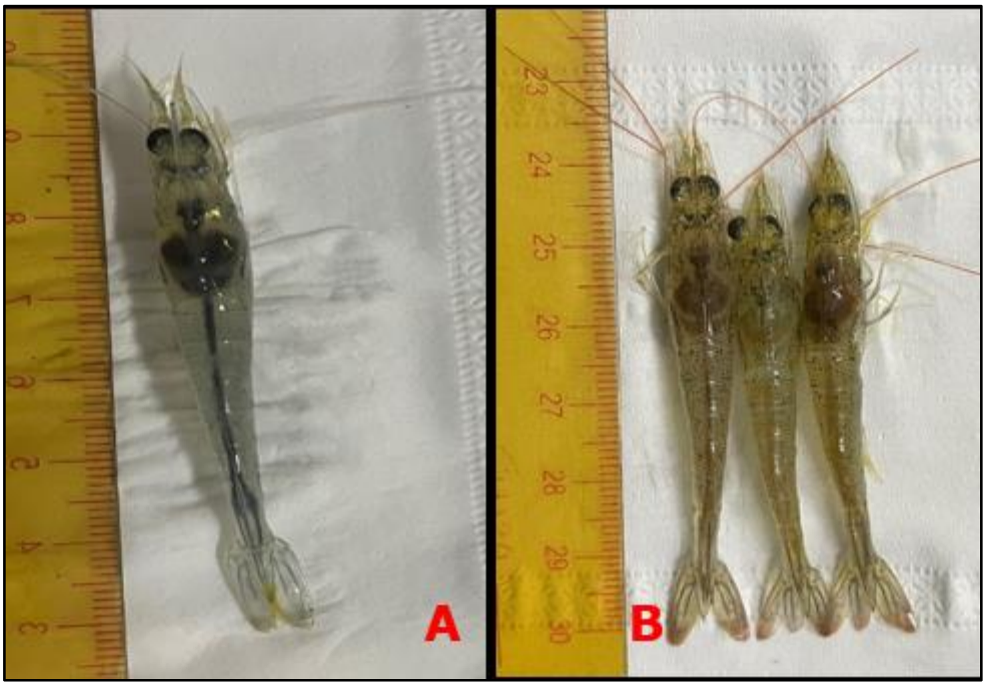

Figure 5. Healthy shrimp (*Penaeus vannamei*) with brownish color (A) when fed with *Wedelia chinensis* and infected shrimp with pale and yellow hepatopancreas (B) when fed with a basal diet at day 14 after challenge with *Vibrio parahaemolyticus.*

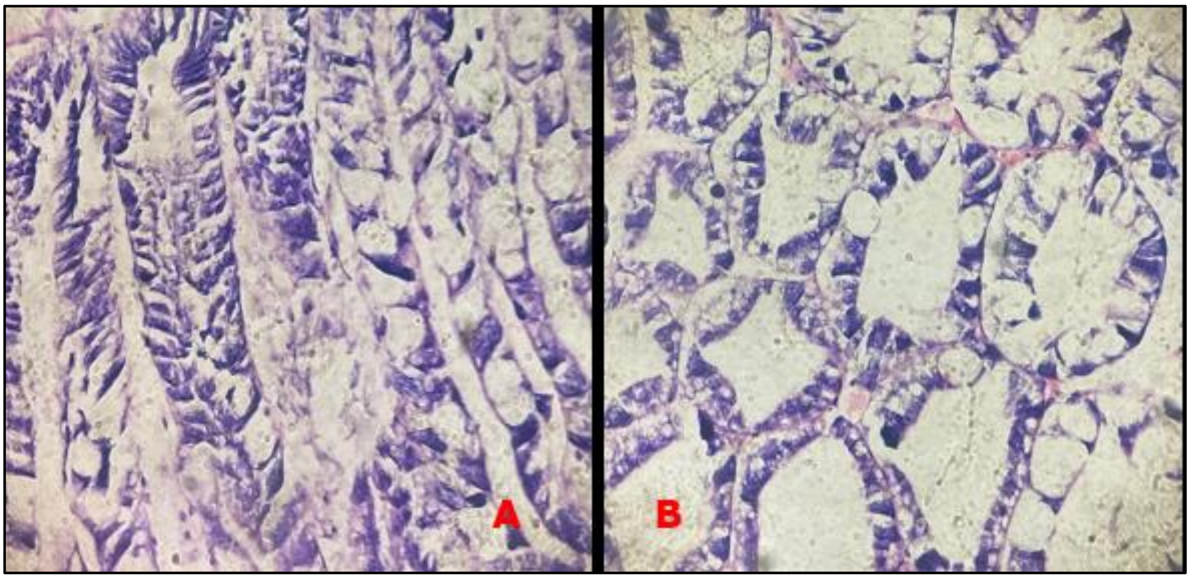

Figure 6. Healthy shrimp (*Penaeus vannamei*) with normal tubule lumen when fed with *Wedelia chinensis* at the dose of 312.5 mg kg^{-1} (A), or 625 mg kg^{-1} feed (B).

Shrimp in the control group showed pale HP and an empty gut (Figure 5B). The HP was less solid (Figure 5B). The HP tubule of unhealthy shrimp collapsed and degenerated. The necrosis of epithelial cells, karyomegally of the HP nuclei was observed in the HP tissue. AHPND pathology was characterized by the sloughing of undifferentiated epithelial cells of the hepatopancreatic tubule epithelia (Figure 7A). Huge aggregation of hemocytes was accompanied in the tubule lumens (Figure 7B).

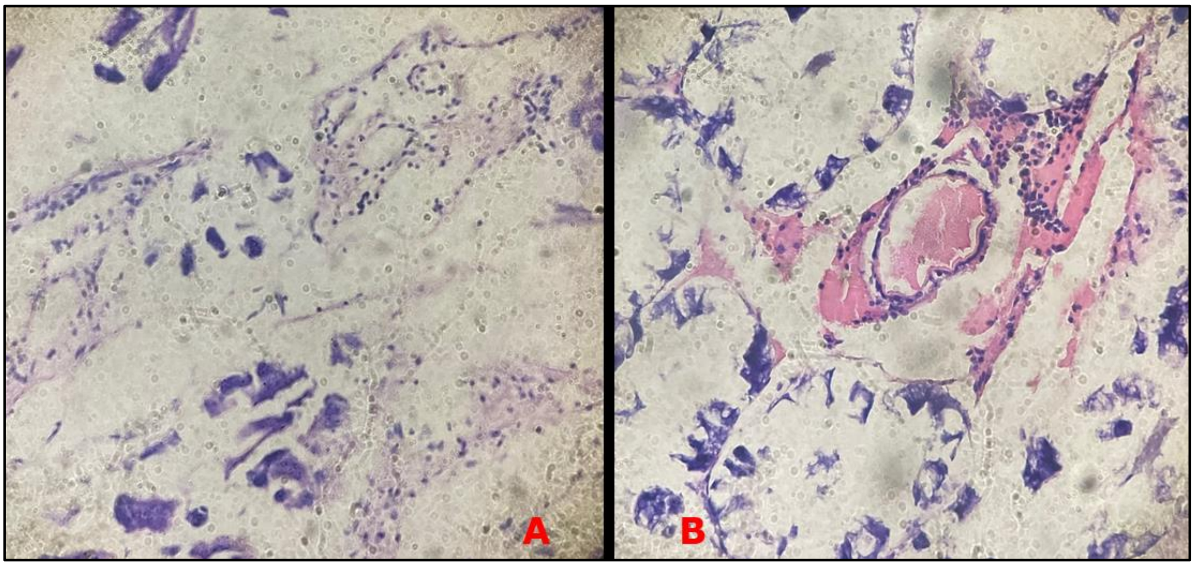

Figure 7. Massive destruction of hepatopancreas tubules (A); and there was extensive infiltration of hemocytes, along with some tubules showing potential presence of *Vibrio* bacteria extensive haemocytic infiltration (B).

Discussion. In shrimp, the HP is responsible for digestion (Manan et al 2015), and also plays an important role in immune and heat stress response (Sun et al 2014). However, the HP is easily damaged by toxins in diets, water pollution or *Vibrio* infection, especially *V. parahaemolyticus* (Khimmakthong & Sukkarun 2017). In this study, shrimp fed with a basal diet (without WCE added) and challenged with *V. parahaemolyticus* showed hepatopancreatic histopathological changes, characterized by atrophy of the hepatopancreatic tubules, massive degradation of hepatopancreatic cells sloughing to the lumen, and blood cells appearing around bacterial clusters in the necrotic area. In the terminal phase, there were severe necrosis and extensive haemocytic infiltration of the hepatopancreas tubules. In addition, the detachment of the epithelial cells from the membrane to the lumen and the inability to differentiate between the distinct types of cells was observed in this phase.

Observation of histopathological changes in the HP of shrimp infected with *V. parahaemolyticus* showed severe necrosis of the HP tubules. Histopathological sections also indicated detachment of the epithelial cells from the membrane to the lumen and the inability to differentiate between the distinct types of cells. In addition, severe HP necrosis limited the observation of lipid cells due to the cellular detachment caused by a bacterial infection, evidencing the formation of melanized haemocytic nodules in the middle part of the HP tubules. The histopathological changes in HP of shrimp infected with *V. parahaemolyticus* in this study were in line with those of Lightner et al (2012) and Flegel (2012). However, there are differences between the histopathology of HP from shrimp fed with WCE and those fed without it.

Shrimp administered *W. chinensis* in doses of 31.25, 312.5, and 625 mg kg^{-1} feed showed normality of the HP tissue with normal cells, normal nucleus cells, and tubules with lumens in the center, indicating the positive effect of WCE on the shrimp's health. However, the shape and the size of the HP of shrimp fed with the lowest dose of *W. chinensis* were not large, and unclear edges showed that the infection still happened in this group. The chemical content of WCE, such as flavonoids, sterols, saponins, etc. protects the HP of shrimp and reduces the infection with *V. parahaemolyticus*. The WCE particularly, and plant extract generally, also enhances the innate immune response of shrimp and stimulates the shrimp's growth performance (Koul et al 2012; Ngoc et al 2023). This study shows that the use of WCE in feed has the potential to improve the health status of vannamei shrimp.

Conclusions. Feeding shrimp with a basal diet mixed with WCE at concentrations of 31.25 312.5 and 625 mg L^{-1} did not affect hepatopancreatic structure and functions, and reduced the infection with *V. parahaemolyticus* in whiteleg shrimp.

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

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