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The effects of *Pandanus tectorius* leaf extract on the resistance of White-leg shrimp *Penaeus vannamei* towards pathogenic *Vibrio parahaemolyticus*

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ABSTRACT

Pandanus tectorius leaf extract effect on the White-leg shrimp *Penaeus vannamei* tolerance against *Vibrio parahaemolyticus* were investigated in this study. Thirty shrimp post-larvae measured at approximately 1 cm were exposed for 24 h to 0.5, 1, 2, 3, 4, 5 and 6 g/L leaf extract and subsequently observed for survival and immune-related genes expression (*Hsp70, ProPO*, peroxinectin, penaeidin, crustin and transglutaminase), followed by determination of their tolerance and histological tissue profiles upon *Vibrio* challenge. Survival of shrimps treated with 6 g/L of leaf extract improved by up to 95% to controls. Hsp70, crustin, and prophenoloxidase mRNA levels were observed to be 8.5, 10.4, and 1.5-fold higher, respectively. Histopathological analysis of the hepatopancreas and the muscle tissues revealed major tissue degeneration in *Vibrio*-challenged shrimps but not in shrimps primed with *P. tectorius* leaf extract. Of all the dose examined, the best pathogen resistance results were obtained with a 24 h incubation of shrimp in 6 g/L *P. tectorius* methanolic leaf extract. The tolerance towards *V. parahaemolyticus* might be associated with the increased regulation of *Hsp70*, prophenoloxidase and crustin upon exposure to the extract, all immune-related proteins essential for pathogen elimination in Penaeid shrimp. The present study primarily demonstrated that *P. tectorius* leaf extract is a viable alternative for enhancing *P. vannamei* post-larvae resistance against *V. parahaemolyticus*, a major bacterial pathogen in aquaculture.

1. Introduction

Marine shrimp farming is currently practised in at least 50 countries around the world, with major development centres in Asia and the Americas. Total production of marine shrimp was 5.51 million metric tonnes in 2017, valued at US$34.2 billion. In this context, White-leg shrimp, *Penaeus vannamei* is the main cultured Penaeid shrimp species in the world with 4.46 million metric tonnes, valued at US$26.7 billion [1]. Many obstacles occurred during shrimp farming, with diseases caused by viruses, bacteria and fungi being a major developmental threat. Disease outbreak causes mass mortality, resulting in major financial repercussions. Antibiotics were often used as a quick solution to treat or prevent shrimp diseases, but excessive use of these therapeutic and prophylactic agents has led to the spread of antibiotic-resistant pathogens. In addition, bioaccumulation of antibiotic residues can occur in shrimp tissues, resulting in serious human health consequences after consumption [2–5].

Realizing the downside of antibiotics, a number of bio-control strategies have been proposed to control diseases during shrimp culture. These include the use of microalgae, probiotic bacteria, bioflocs, and plant extracts rich in antimicrobial properties to control the proliferation of pathogens [6,7]. Application of these alternatives have been

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shown to enhance the general health and protect cultivated shrimp in the event of a pathogen attack [4,8]. In this context, many medicinal plants used in the treatment of human disease are capable of having immunoprophylactic and chemotherapeutic effects in aquatic animals, including shrimp [9–19]. They possess bioactive compounds and phytochemicals such as phenols, polyphenols, alkaloids, quinones, terpenoids, lectins, and polypeptides that are effective against pathogens that cause disease in aquatic organisms [20]. Thus, it is important to identify more plant species that are useful for the control of aquatic diseases.

P. tectorius is a Southeast Asian medicinal plant and they are used by locals to treat wide range of human disease including stomach spasm, headache and arthritis. They grow in sandy soil and are mainly found along the shore and beach. The leaves and fruits are rich in anti-inflammatory [21], anti-oxidant, anti-hyperglycemic, anti-hyperlipidemic [22], anti-cancer [23,24], anti-tumor [25,26], anti-viral [27], and anti-diabetic [28] properties, with qualitative phytochemical analysis result confirmed the presence of phenolics, flavonoids, terpenoid, steroids, saponins and glycosides as chemical constituents in *P. tectorius* extract [29,30]. An antibacterial kinetic analysis demonstrated that *P. tectorius* exhibits strong antibacterial activity [29]. Despite their potential therapeutic benefits, relatively few research have been undertaken to date on their efficacy in treating and preventing diseases in aquatic organisms. Awad et al. [31] discovered that feeding *P. tectorius* extract increased the expression of immune-related genes (TNF, LYZ2, IL-8, and CD-4) as well as a tumor suppressor gene (WT-1a) and protected rainbow trout *Oncorhynchus mykiss* from *Yersinia ruckeri* infection. Dietary supplementation of *P. tectorius* extract at 20 g/kg significantly improved weight gain, serum antioxidant parameters, immunity, and resistance of the common carp *Cyprinus carpio* against *Aeromonas hydrophila*, a common bacterial pathogen of fish [13].We demonstrated that a 24 h incubation of 2–6 g/L of *P. tectorius* fruit extract improved *P. vannamei* tolerance against *V. parahaemolyticus*, with survival increasing two-fold when the highest dose of extract was used [10]. The fruit extract pre-treatment increased the expression of several shrimp immune genes, including Hsp70, ProPO, peroxinectin, penaeidin, crustin, and transglutaminase, all of which increased in a dose-dependent manner. The mechanisms of action or the compounds derived from them that provide protection are however unknown [10].

In this study, we investigated the efficacy of *P. tectorius* leaf extract in improving *P. vannamei* tolerance to *V. parahaemolyticus*, the causative agent of vibriosis in Penaeid shrimp. The efficacy of this plant leaf extract in conferring shrimp immunity was determined by measuring the expression of Hsp70, prophenoloxidase, crustin, transglutaminase, peroxinectin, and penaeidin, all of which are immune proteins required for Penaeid shrimp resistance to pathogenic bacterial infection. Histology on the hepatopancreas and muscle assessed the degree and condition of tissue damage caused by *V. parahaemolyticus* challenge in shrimp exposed and not exposed to *Pandanus tectorius* leaf extract. The findings of this study may help to improve our understanding of the prophylactic value of this mangrove plant species to Penaeid shrimps and aid in the development of strategies to prevent and treat vibriosis in aquaculture.

2. Materials and methods

2.1. Maintenance of P. vannamei

P. vannamei post-larvae measuring approximately 1 cm were purchased from iSHARP Sdn. Bhd., Terengganu, and they were acclimatized for two weeks at 28 °C and 38 g/L salinity with continuous aeration before being used in the experiment. Post-larvae were raised at a density of 2000 individuals/L and fed live *Artemia* nauplii twice daily. The shrimp health status was monitored daily, and rearing water was changed once every two days to maintain water quality. Shrimps measured at approximately 1 cm were selected for experiments.

2.2. Preparation of P. tectorius leaf extract and assessment of the safety level

P. tectorius leaves collected from Setiu Wetlands, Terengganu were washed, cut into small pieces, air-dried, and stored at -80 °C for a day before lyophilization (EYELA FD-550, USA). Dried samples were ground to a fine powder, and 30 g of the powder was incubated in 300 mL of methanol for 24 h at room temperature (RT). The methanol extract was filtered through Whatsman No. 1 filter paper, concentrated in a vacuum with a rotary evaporator (BUCHI R-300, Switzerland), and stored at room temperature until further use [10].

The safety level of *P. tectorius* leaf extract was determined by exposing 30 shrimps separately for 24 h to 0.5, 1, 2, 3, 4, 5, and 6 g/L of extract, with non-exposed shrimps serving as controls. Shrimps were incubated in a closed water system, and the water was not changed during exposure. The number of dead and moribund animals was used to determine mortality [10]. The procedure was repeated twice, with each treatment carried out in triplicate.

2.3. V. parahaemolyticus challenge tests

In order to examine whether *P. tectorius* extracts could prime or induce protection of shrimp against pathogenic *Vibrio, P. vannamei* post-larvae exposed to the leaf extract at concentration as described in the previous section, were transferred to rearing water consisting *V. parahaemolyticus* for challenge tests, while shrimp not exposed to the extract served as the control group. For the challenge tests, 30 shrimp were exposed for 24 h to 1×10^6 cells/ml *V. parahaemolyticus*, the median lethal dose (LD50) of *V. parahaemolyticus* [10]. The *V. parahaemolyticus* used in this study were isolated from diseased shrimps in Vietnam [10,32]. The survival percentage was determined by counting the number of actively swimming animals, with moribund shrimps considered dead. The procedure was repeated twice, with each treatment carried out in triplicate.

2.4. Tissue profiles in hepatopancreas and muscle of V. parahaemolyticus-challenged shrimps

The hepatopancreas and muscle tissues of shrimps challenged with *V. parahaemolyticus* (with or without 24 h incubation at 6 g/L of *P. tectorius* leaf extract) and control shrimps (without leaf extract and *Vibrio* exposure) were fixed in Davison reagent for 48 h. Tissues were placed in 70% alcohol for 30 min before being transferred to a cassette and then to an automatic tissue processing machine (Leica™ 1020, Germany). The samples were then placed on a steel cassette and embedded in paraffin (melting point 54–56 °C). The paraffin blocks were allowed to freeze on a cold plate (Leica™ EG1150C, Germany) and then refrigerated overnight at 4 °C. The blocks were kept cool on a cold plate before being sectioned with an automated microtome (Leica™ RM2255 Microtome, Germany) at a thickness of 5 μm. Before staining with Harris hematoxylin and eosin, sample sections were mounted on albumin-coated glass slides (Sigma, USA). Slides were fixed with dibutylphthalate polystyrene xylene (DPX), and histological images were examined with a Leica™ DM LB2 Light Microscope (Germany) at 40x magnification and a Leica™ Image Analyzer System (Germany).

2.5. Hsp70 expression and densitometry analysis

Proteins were extracted from 100 mg of shrimp caudal muscle tissues exposed to different concentrations of leaf extract as previously mentioned [10]. Tissues were homogenized in cold Buffer K [33,34] and Protease Inhibitor Cocktail (Sigma-Aldrich Inc-P8849, USA) and centrifuged at 4 °C for 30 s. Prior to electrophoresis, the supernatant was transferred into a sterile Eppendorf tube and stored at -20 °C. For SDS polyacrylamide gel electrophoresis, 5 μL of homogenate was mixed in a 1:1 ratio with sample buffer and heated at 95 °C for 5 min. Ten (10) μL of

sample was added to each lane of 10% polyacrylamide gels, and electrophoresis was performed at 120 V for 15 min, followed by 150 V for 45 min. Until visualization, the protein gel was stained overnight with Coomassie Biosafe (BioRad Laboratories, USA).

Protein gel was transferred to a polyvinylidene fluoride transfer membrane (BioRadImmun-Blot™ PVDF, USA) for antibody probing for Western immunoblotting. The PVDF membrane was probed with a 1:5000 dilution of a mouse monoclonal antibody to Hsp70 (Clone 3A3, Thermo Scientific, USA). As a secondary antibody, a 1:2000 dilution of goat anti-Mouse IgG F(ab')2 polyclonal antibody conjugated with horse radish peroxidase (HRP) (Bioreagent-SAB-100 J, Stressgen, Canada) was used. 0.7 mM diaminobenzidine tetrahydrochloride dehydrate (DAB) was used to detect antibody-reactive protein [10,35,36]. The expression of Hsp70 in shrimp was then determined by densitometry analysis. A densitometer (GS-800 BioRad Laboratories, USA) was used to scan the protein blot, and the intensity of Hsp70 bands was determined using Quantity One software (BioRad Laboratories, USA) and presented as a fold difference.

2.6. Expression of immune-related genes in P. vannamei

RNA was extracted from 100 mg caudal muscle tissues of *V. parahaemolyticus*-challenged shrimps (with or without 6 g/L leaf extract exposure) and control shrimps using the TRIsure™ reagent (Bioline, UK) according to the manufacturer's protocol. Approximately 1 μg of RNA was converted into first strand cDNA using a cDNA synthesis package, (Bioline, UK). qRT-PCR was used to determine the expression of immune-related genes, as described by Anirudhan et al. [10]. Prior to qRT-pCR, the primer specificity was confirmed with NCBI blastn (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn). qRT-PCR was carried out in a CFX Connect™ System (Bio-Rad, USA) with a 2X SensiMix SYBR No-ROX package (Bioline, UK) containing forward and reverse primers targeting proPO, peroxinectin, penaeidin, crustin, haemocyanin, Hsp70, and ß-Actin (Table 1). The cycle threshold (CT) values were recorded by CFX Manager program software (BioRad, USA) and fold difference in quantity for each immune-related cDNA, relative to the β-actin gene, was calculated by the 2-ΔΔCt method [37].

2.7. Statistical analysis

Values of larval survival were ArcSin transformed to satisfy normality and homocedasticity requirements whenever necessary. Significant differences in terms of survival were investigated by performing one-way ANOVA, followed by Tukey test at a significance level of 0.05.

3. Results

3.1. P. tectorius leaf extract protected shrimps against V. parahaemolyticus

The survival percentage of *P. vannamei* exposed to various concentrations of *P. tectorius* leaf extract were shown in Fig. 1. When shrimps were incubated in varying doses of leaf extract for 24 h, survival was approximately 93%, which was comparable to the control. Shrimp exposed to *V. parahaemolyticus* had a 24 h median lethal concentration (LC_{50}) of 1.6×10^6 cells/ml (Fig. 2). The survival of shrimps incubated with *P. tectorius* leaf extract was increased in a dose-dependent manner following a LD_{50} *V. parahaemolyticus* challenge, with survival reaching 95% when the leaf extract concentration was increased to 6 g/L, the highest dose examined in this study (Fig. 2).

3.2. P. tectorius leaf extract upregulated Hsp70 and immune genes expression

The expression of Hsp70 increased in a dose-dependent manner when shrimps were exposed to *P. tectorius* leaf extract. Hsp70 levels in shrimp tissues increased from 1.7 to 16.5-fold, with the latter occurring at 6 g/L of leaf extract (Table 2).

Quantification of immune-related genes expression by qRT-PCR revealed a 8.5, 10.4 and 1.5-fold increase in Hsp70, crustin and prophenoloxidase mRNA respectively, whereas augmentation of peroxinectin, penaeidin, and transglutaminase mRNA were unapparent upon exposure to the highest concentration of *P. tectorius* leaf extract (Table 3).

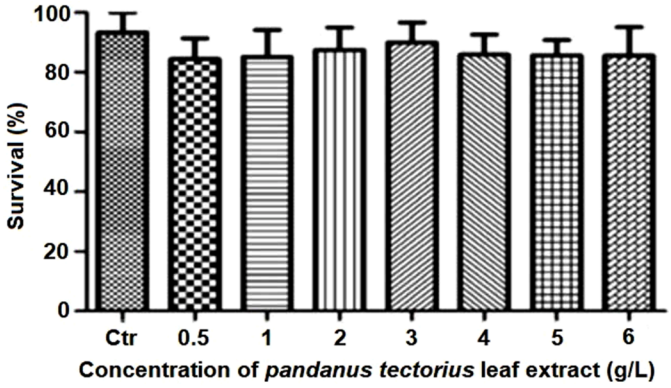

Fig. 1. Survival of *P. vannamei* after 24 h exposure to different concentrations of *Pandanus tectorius* leaf extract. Ctr, control (*P. vannamei* not exposed to leaf extract); Treatments, 0.5 to 6 g/L of leaf extract. Error bars are standard error.

Table 1
Primer sequences, size (base pairs) and melting temperatures (Tm) used to amplify immune-related genes (Hsp70, crustin, prophenoloxidase, penaeidin, peroxinectin and transglutaminase) of *P. vannamei*.

Primers	Accession #	Sequence	Size bp	Tm (°C)
Hsp70 (F)	EF495128	5′-CCTCCAGGACTTCTTCAACG-3′	144	63.0
Hsp70 (R)		5′-GGTCACGTCCAACAGCAAC-3′		
Crustin (F)	AF430076	5′-ACGAGGCAACCATGAAGG-3′	141	62.7
Crustin (R)		5′-AACCACCACCAACACCTAC-3′		
Prophenoloxidase (F)	AY723296	5′-CGGTGACAAAGTTCCTCTTC-3′	122	61.5
Prophenoloxidase (R)		5′-GCAGGTCGCCGTAGTAAG-3′		
Penaeidin-3a (F)	Y14926	5′-CACCCTTCGTGAGACCTTTG-3′	121	63.0
Penaeidin-3a (R)		5′-AATATCCCTTTCCCACGTGAC-3′		
Peroxinectin (F)	AF188840	5′-CGAAGCTTCTTGCAACTACCA-3′	56	63.8
Peroxinectin (R)		5′-GCAGGCTGATTAAACTGGCTT-3′		
Transglutaminase (F)	EF081004	5′-GAGCTTCAAGATCGAGGATCGA-3	79	64.6
Transglutaminase (R)		5′- GCTGGTGTTCGTAGCGGTTATC-3		
β-actin (F)	AF300705	5′-CCACGAGACCACCTACAAC-3′	142	62.7
β-actin (R)		5′-AGCGAGGGCAGTGATTTC-3′		

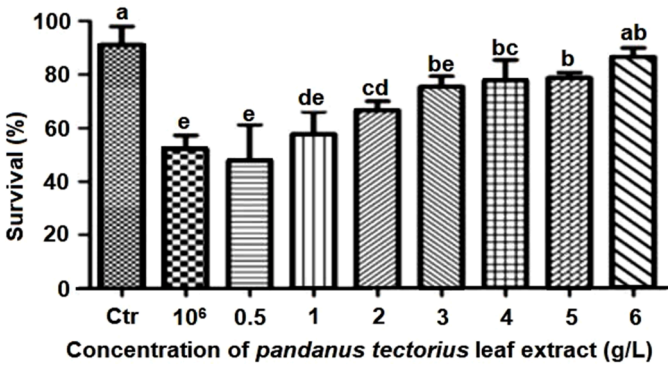

Fig. 2. Survival of *P. vannamei* after pre-treatment with *P. tectorius* leaf extract and 24 h exposure to 1 × 10^6 cells/ml *V. parahaemolyticus*. Ctr, control (*P. vannamei* not exposed to leaf extract); 10^6, shrimps challenged with *V. parahaemolyticus* alone (1 × 10^6 cells/ml); Pre-treatments, 0.5–6 g/L of leaf extract. Error bars are standard error.

Table 2
The relative density (RD) of Hsp70 expression in *P. vannamei* incubated 24 h with *P. tectorius* leaf extract. The relative Hsp70 quantity was calculated based on the RD of non-exposed control, with a relative value of 1. Asterisks (*) indicate protein up-regulation.

Treatment	Adjective Volume (RD)	Relative Quantity
Control	9.0	1.0
0.5 g	15.7	1.7*
1 g	17.4	1.9*
2 g	19.8	2.2*
3 g	26.7	2.9*
4 g	40.9	4.5*
5 g	122.5	13.6*
6 g	147.8	16.5*

Table 3
The relative fold-difference of immune-related genes of *P vannamei* upon 24 h incubation in 6 g/L *Pandanus tectorius* leaf extract. The value represents the fold changes of mRNA expression (normalized against internal control β-actin) relative to the control value, which are the samples from shrimp without any treatment. * represents significance difference between treatment and control ($P < 0.05$). The data shown are representative of two independent experiments with similar expression patterns.

Immune related genes	Relative fold-difference
Hsp70	8.4 ± 4.0*
Crustin	10.4 ± 2.0*
ProPO	1.5 ± 1.0
Transglutaminase	-1.0 ± 0.5
Peroxinectin	-1.1 ± 0.5
Penaeidin	-1.1 ± 0.5

3.3. *P. tectorius leaf extract prevented histological degeneration in V. parahaemolyticus-challenged shrimps*

Histological examination of hepatopancreas and muscles of shrimps treated with *Pandanus tectorius* leaf extract and then exposed to *V. parahaemolyticus* are shown in Figs. 3 and 4, respectively. Similar to positive control group (shrimps un-exposed to *P. tectorius* or *V. parahaemolyticus*), shrimps exposed to the leaf extract and subsequently challenged with *Vibrio* had intact tubules and tubules of lumen. However, the negative control group (shrimps challenged with *V. parahaemolyticus* without *P. tectorius* treatment) displayed enlarged hepatopancreatic nuclei, degenerated hepatopancreatic tubules and decreased number of blassenzelen, fibrillenzene and restzellen epithelial cells (Fig. 3).

The muscle structure of control shrimps appeared normal, without tissue lesion or necrosis. Vacuolization increased in the lining of muscle cells and lumen epithelium of the positive control group, while haemolytic infiltration and loss of muscle tissue structure in shrimp samples not exposed to *P. tectorius* leaf extract occurred upon *V. parahaemolyticus* challenge (Fig. 4).

4. Discussion

In this study, exposure to methanolic *P. tectorius* leaf extracts enhanced the survival of *P. vannamei* against pathogenic *V. parahaemolyticus*. When the maximal amount of extract (6 g/L) was employed, *P. tectorius* leaf extract enhanced *P. vannamei* tolerance against *V. parahaemolyticus* better than fruit extract, with survival percentage increased by 15% in the median lethal dose challenge [10]. How this plant extract contributes to the enhanced tolerance in shrimp is uncertain, but several possibilities exist.

P. tectorius is high in phenolic acids, flavonoids, and tannins, and it can behave similarly to other medicinal plants against *V. parahaemolyticus* ([38]; Andriani et al., 2015). These antimicrobial and antioxidant compounds enter the shrimp's gut or circulatory system and prevent virulent bacterial growth by either chemically interfering with the synthesis or function of vital components [39] or circumventing their ability to conventionally resist antibacterial compounds [40–42, 39,43,44]. In fact, many plants, including *P. tectorius*, contain antimicrobial substances that are effective at destroying pathogenic bacteria such as *Vibrios*. These include *Acalypha indica, Adhatoda vasica, Anacardium occidentale, Azadirachta indica, Lawsonia inermis, Psidium guajava, Rosa damascena* and *Tridax procumbans* extracts of which have been shown to be effective against *V. anguillarum, V. alginolyticus*, and *V. harveyi*, all of which are typical bacterial pathogen of *Penaeus vannamei* [18]. On other examples, methanolic extracts of *Murraya koenigii, Psoralea corylifolia* and *Quercus infectoria* protected shrimps from *V. harveyi, Pseudomonas aeruginosa* and *Staphylococcus aureus*. In these studies, improved survival was associated with a substantial reduction in bacterial load, suggesting a significant pathogen inhibition in the host [45].

On other aspect, *P. tectorius* can protect shrimps against *V. parahaemolyticus* by activating the immune system. Short-term exposure to the leaf extract was shown to increase Hsp70 accumulation in *P. vannamei*. Hsp70 mediates the activation of the prophenoloxidase (proPO) system [46] and in this study, the number of ProPO mRNA was increased approximately 1.5-fold after exposure to *P. tectorius* leaf extract. Despite the low expression level, there might be a rise in ProPO activity. The proPO system is essential in antibacterial immunity because it produces melanin and cytotoxic intermediates that help in bacterial sequestration and killing, preventing severe tissue and organ damage during infection [47,48]. Hsp70 also stimulates the production of cytokines, which are secreted by activated immunocytes or matrix cells to inhibit pathogens. Cytokines occur in shrimp and play a variety of immunostimulatory functions, including the Fas receptor (Fas), platelet factor 4 (PF4), and interleukin-22 (IL-22), all of which are essential in obscuring viral replication [49].

As shown in this study, protection against *V. parahaemolyticus* of shrimps with *P. tectorius* leaf extract may also be offered by induction of crustin, an important group of antimicrobial peptides (AMP) for *P. vannamei*. Exposing shrimp to 6 g/L of leaf extract resulted in a 10.4-fold increase in the amount of crustin mRNA. Crustins are immune proteins with antimicrobial activity against Gram-positive and Gram-negative bacteria, including *Vibrio* [50,51]. In this context, Arockiaraj et al. [52] demonstrated that recombinant crustin protein from the freshwater giant prawn *Macrobrachium rosenbergii* (MrCrs) has the ability to agglutinate *A. hydrophila, E. coli, Edwardsiella tarda, V. parahaemolyticus, V. alginolyticus* and *Pseudomonas sp*. and other Gram-positive bacteria. The bacterial agglutination activities of recombinant MrCrs protein recognized lipopolysaccharides in bacterial

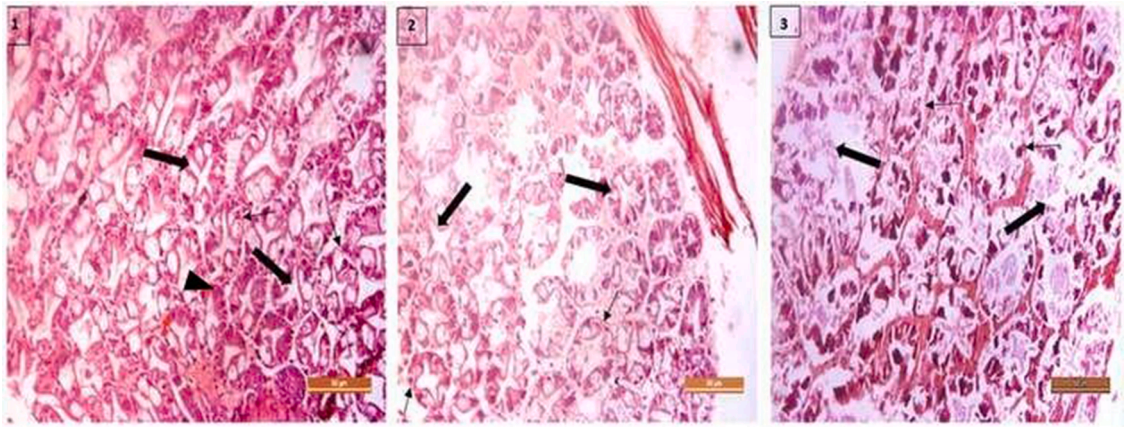

Fig. 3. Histological section of shrimp hepatopancreas. 1. Hepatopanceatic section of control shrimp showing healthy tubule (black thin arrow), tubule lumen (black wide arrow) and epithelial cells lining the tubules (black arrowhead). 2. Hepatopancreatic session of shrimp treated with leaf extract and then challenged with *V. parahaemolyticus* showing intact tubule (black thin arrow) and tubule lumen (black wide arrow). 3. Hepatopancreatic tubules from shrimp challenged with *V. parahaemolyticus* showing distended tubule lumen (black wide arrow) and distorted hepatopancreatic structure (back thin arrow).

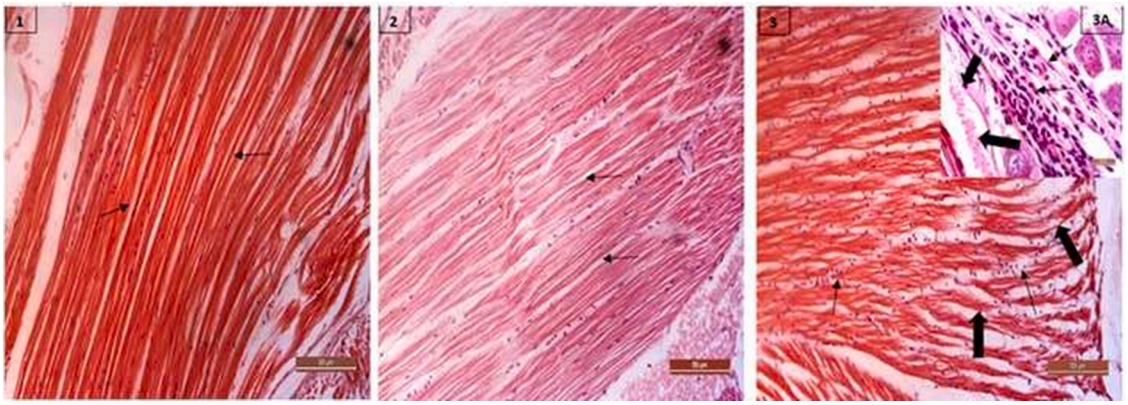

Fig. 4. Histological session of shrimp muscle. 1. Normal muscle from the control shrimp with muscle fibres intact (black arrow), no lesion and necrosis. 2. Muscle structure of shrimp treated with leaf extract and then challenged with *V. parahaemolyticus* showing no muscle necrosis and hemocytic infiltration. 3. Multifocal area of extensive coagulative necrosis and fragmentation of muscle fibres observed on *Vibrio*-challenged shrimps. (3A) Hyalinization and fragmentation of muscle fibres (wide arrow) along with hemocytic infiltration (thin arrow). Extensive loss of muscle.

cell walls and bound on its substrate, immobilizing the invading bacteria and inhibiting further tissue invasion. They are also important components of shrimp hemocytes, as they are needed for apoptosis and phagocytosis [53]. Similar immunoregulatory capabilities were discovered for a type II crustin (LvCrustinB) that was strongly synthesized in the muscle and epithelium of L. *vannamei*, suppressing V. parahaemolyticus following infection [54]. According to the pattern of transglutaminase, peroxinectin, and penaeidin expression, protection against *V. parahaemolyticus* in shrimp exposed to *P. tectorius* leaf extract was unlikely to be linked to these immune proteins. The exact mechanism by which *P. tectorius* protects shrimps against pathogens is unknown, although dietary supplementation with this plant extract for 2 weeks were demonstrated to boost the non-specific immune response and increased resistance to *Y. ruckeri* infection in rainbow trout. The highest recommended dose of 2% significantly increased lysozyme activity, myeloperoxidase level, and various important immune related genes such as TNF, LYZ2, IL-8, and CD-4 in the head kidney of rainbow trout [31]. These findings clearly suggested that *P. tectorius* contains compounds that can stimulate the immune system of fish, and that this may also occur in shrimp, as seen in our work.

AHPND is caused by *V. parahaemolyticus* that have acquired a plasmid encoding $PirA^{vp}/PirB^{vp}$ [55,56]. Upon infection, *V. parahaemolyticus* colonizes the shrimp gut by producing binary toxins such as TOXA (12.7 kDa) and TOXB (50.1 kDa) that resembles binary insecticidal toxins from *Photorhabdus* species, the *Photorhabdus* insect related (Pir) toxins; PirA and PirB. These toxins cause cell sloughing and damage to the hepatopancreas, eventually leading to organ failure and death [48,57,58]. Aside from the hepatopancreas, AHPND toxin was found to be toxic to the hemocytes and stomach [59]. In this study, the hepatopancreas of shrimp that were not exposed to *P. tectorius* leaf extract showed enlargement of the hepatopancreatic nuclei, collapse of the hepatopancreatic tubules, a lack of Blassenzelen, fibrillenzen, restzellen epithelial cells and sloughing, all of which are typical histopathological conditions of AHPND. Other pathological signs include necrosis, loss of structure, atrophy of tubule epithelial cells, vacuolation, rounding and sloughing of cells into the lumen, as well as inflammation [60]. Inflammation in infected tissues is a natural defense mechanism that occurs in response to injury, either locally or systemically within the challenged organism [61,62]. Interestingly, exposing shrimp to *P. tectorius* leaf extract prior to *V. parahaemolyticus* challenge was able to mitigate the hepatopancreas's adverse effects. Histology showed that these shrimp hepatopancreas have massive compact ducts and blind ending tubules, with each consisting of a single layer of epithelial cells when challenged with *V. parahaemolyticus*. The hepatopancreas structure was intact and there was no sloughing, observations comparable to the non-challenge shrimp sample (control). These findings suggest that *P. tectorius* leaf extract play a role in protecting shrimp against tissue injury, which may be explained by the presence of many antioxidative

compounds in this plant. *P. tectorius* is primarily rich in caffeoylquinic acids [63,64], vitamins (such as vitamins C, E and b-carotene) [65–67], isopentenyl, dimethylallyl acetate and cinnamate [68] and these residual antioxidative compounds deposit of the *P. tectorius* leaf extract in the shrimp tissue can reduce toxin accumulation to non-lethal levels for shrimps [41,69] The anti-inflammatory properties linked to these phytochemicals ([70,71]; Andriani et al., 2015) may be the reason for less adverse histopathological effects in *Vibrio*-challenged shrimp tissues.

Overall, incubating shrimp for 24 h in 6 g/L *P. tectorius* methanolic leaf extract is beneficial because it may increase immunological status and pathogenic bacteria tolerance during culture. Future studies should focus on finding phytochemical substances in the plant extracts and investigating their mechanistic activities during infection. Understanding how *P. tectorius* methanolic extracts improves tolerance may aid in the development of strategies to protect *P. vannamei* and possibly other Penaeid shrimp species against vibriosis while also reducing antibiotic use in farming.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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