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Research Article

Evaluating the Protective Immunological Effects of *Pandanus tectorius* Leaf Extract Against Pathogenic *Vibrio campbellii* Using Gnotobiotic Brine Shrimp Model System

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This study investigated the protective effects of *Pandanus tectorius* leaf extract (PLE) on the tolerance of the brine shrimp *Artemia franciscana* against the pathogen *Vibrio campbellii*. Axenic *Artemia* nauplii were hatched and exposed to PLE for 2 h, after which their resistance to *V. campbellii* was examined. Protective responses were evaluated by measuring nauplii survival and changes in the expression of key immune-related genes, such as heat shock protein 70 (*hsp70*), *hsp60*, *hsp90*, high mobility group box 1 (*hmgb1*), prophenoloxidase (*proPO*), and transglutaminase (*tgase*) upon *V. campbellii* challenge. Additionally, a *Vibrio* colonization assay was conducted to assess PLE's antimicrobial potential. The results revealed that 2 h pretreatment with PLE at a concentration of 1 g/L significantly improved survival against *V. campbellii*. Immune-related gene expression was induced at different time points (e.g., 12–24 h) during the *Vibrio* challenge and the colonization assay confirmed PLE's antimicrobial properties. The observed protection could likely be due to a combination of immune gene activation, antioxidant activity, and the antimicrobial effect of the extract. This study highlights the significance of PLE by indicating its potential to serve as a protective agent in aquaculture, enhancing the resilience of aquatic organisms against biotic stressors like vibrios.

Keywords: antimicrobial properties; antioxidant properties; axenic *Artemia* nauplii; immune-related genes; *Pandanus tectorius* leaf extract; *V. campbellii*

1. Introduction

Shrimp farming is a highly profitable industry and over the past two decades, the sector has experienced rapid expansion to provide humans with high-quality animal protein worldwide [1]. However, the growth of shrimp aquaculture has brought many challenges, such as sustainability, economic viability, and diseases [2]. One of the most significant challenges is the occurrence of diseases caused by bacterial pathogens belonging to *Vibrio* genus, such as *V. campbellii* and *V. parahaemolyticus*, which have led to massive production and tremendous socio-economic losses [3–5]. These *Vibrio* species, including *V. campbellii*, are opportunistic pathogens that cause infection in penaeid shrimps and other marine vertebrates and invertebrates when other stressors (e.g., caused by environmental factors) compromise the host's defense system [4, 6, 7]. A specific

strain of *V. campbellii* has been reported to obtain a approximately 70 kb plasmid, responsible for causing a serious acute hepatopancreatic necrosis disease (AHPND), causing huge losses in shrimp, with an estimated annual financial impact of more than USD 1 billion [8, 9]. To control vibriosis in farmed shrimps, traditional approaches, such as the use of antibiotics, have commonly been used. However, the overuse of antibiotics has resulted in causing negative effects on both human health and the environment [10, 11]. Owing to this, there is a global awareness of reducing antibiotic use in shrimp farming, both as a growth promotor and as an antimicrobial and exploring alternative strategies for promoting shrimp health and growth.

Over the past few years, increasing attention has been paid to the use of natural bioactive components derived from plant sources as a promising alternative to traditional chemotherapeutics [12–14]. The growing interest in utilizing plant-derived bioactive compounds in aquaculture animals is driven by several reasons: (i) Natural bioactive components are often considered more eco-friendly, reducing the risk of pollution, and harm to ecosystems. (ii) Many of these compounds have multifunctional properties that include immune-stimulation activities, antioxidants, antimicrobial, and antistress properties [14–16]. These multifunctional properties facilitate the organisms in improving their overall health and resistance towards disease without causing negative effects as done by chemotherapeutics. (iii) They are widely available and cost-effective, making them suitable as dietary supplements for farmed aquatic animals [17].

Pandanus tectorius, commonly known as thatch screw pine or Hala tree, is a mangrove plant native to Southeast Asia, including Malaysia [12, 18]. The leaves, fruits, and roots of the *Pandanus* plant are rich in bioactive components with numerous biological properties, such as anti-inflammatory, antioxidant, and antimicrobial effects [19, 20]. Due to these properties, extracts from *P. tectorius* have been used for centuries for treating a wide range of human diseases [18, 19]. In recent years, relatively few studies were conducted to explore whether *P. tectorius* extract could be used to control bacterial diseases in farmed fish and shrimps [12, 13, 21]. For example, Awad et al. [21] fed rainbow trout *Oncorhynchus mykiss* a diet supplemented with *P. tectorius* leaf extract (PLE) for 2 weeks to study protective immune responses of trout to *Yersinia ruckeri* infection. The results showed that the PLE significantly protected the trout from *Y. ruckeri* infection, which was associated with a marked increase in the expression of immune-related genes, such as TNF, LYZ2, IL-8, and CD-4 as well as a tumor suppressor gene (WT-1a). In another study, *Penaeus vannamei* shrimp pretreated with *Pandanus* fruit extract for 24 h showed increased tolerance to *V. parahaemolyticus* challenge [12], with elevated expression of immune genes heat shock protein 70 (*hsp70*), prophenoloxidase (*proPO*), *peroxinectin*, *penaeidin*, *crustin*, and *transglutaminase* (*tgase*) in a dose-dependent manner. In a subsequent study, the same authors found that pretreatment with PLE significantly improved the survival of *P. vannamei* post larvae (PL) challenged with *V. parahaemolyticus*, with survival rates reaching up to 95% compared to the control. This improvement was accompanied by marked increases in *hsp70*, *crustin*, and *proPO* expression levels by 8.5-fold, 10.4-fold, and 1.5-fold, respectively [13].

Given that *V. parahaemolyticus* and *V. campbellii* both belong to the Harveyi clade, it was hypothesized that PLE could offer protection to farmed shrimp against *V. campbellii*-mediated diseases. This study uses the axenic brine shrimp *Artemia* as a model system for shrimp to examine if prior exposure of *Artemia* to PLE can provide prophylactic effects against *V. campbellii*. Additionally, we explored the mode of action of PLE by examining its impact on key defense-related genes in *Artemia*.

2. Materials and Methods

2.1. Bacteria Strain and Culture Conditions. Vibrio campbellii strain LMG21363, stored at −80°C with 30% glycerol, was grown at 28°C for 24 h on Marine Agar (Difco Marine Agar 2216, USA) and then to log phase in Marine Broth (Difco Marine Broth 2216, USA) by incubation at 28°C under constant agitation. This strain was originally isolated from the lymphoid organ of diseased penaeid shrimp [22]. Bacterial cells were harvested by centrifugation at $5000 \times g$ for 15 min. The bacteria pellets were rinsed several times with filtered autoclaved seawater (FASW) using an autoclave machine (HICLAVE HVE-50 autoclave; Hirayama Manufacturing Corporation, Japan) before use in the *Vibrio* challenge assays. The bacterial cell density was determined spectrophotometrically at 600 nm, assuming that an optical density of 1.0 corresponds to 1.2×10^9 colony-forming units (CFU) per mL [23–25].

2.2. Preparation of Methanolic PLE. The leaves of *P. tectorius* were collected from the coastal area of Setiu Wetlands, Terengganu, Malaysia, and processed at the Natural Product Laboratory of the Institute of Climate Adaptation and Marine Biotechnology (ICAMB), University Malaysia, Terengganu. The leaves were cut into small pieces to facilitate lyophilization using a freeze dryer (EYELA FD-550, 123 USA). The lyophilized leaf samples were ground into a fine powder and a total of 30 g of the leaf powder was mixed with 300 mL of methanol (100%) and left for 48 h in a closed round-bottom flask at room temperature for extraction. The methanolic extract was filtered through a Whatman No. 1 filter paper and concentrated using a rotary evaporator (BUCHI R-300, Switzerland) at a pressure of 337 mbar and temperature of 42°C. The paste obtained after the evaporation was stored in a refrigerator (4–7°C) before use for the experiments. An organic solvent comprising 99% ethanol was used to improve the solubility of the leaf extract [12, 13].

2.3. Axenic Artemia Hatching. The process of hatching *Artemia* cysts and collecting axenic *Artemia* nauplii was conducted following the procedure previously standardized [23, 24]. High-quality *A. franciscana* cysts from the Great Salt Lake, Utah, USA (INVE Aquaculture Malaysia, Puchong), were used for hatching. Approximately 2 g of cysts were hydrated in 89 mL of distilled water for 1 h. The hydrated cysts were decapsulated using 3.3 mL NaOH (32%) and 50 mL NaOCl (50%). The decapsulation process was stopped after about 2 min by adding 50 mL $Na_2S_2O_3$ (10 g/L). The decapsulated

cysts were thoroughly washed using sterile seawater (30 g/L), suspended in a 1-L glass bottle containing sterile seawater and further incubated for 28 h with constant illumination of approximately 27 μE/m^2·s. The axenic nauplii were collected after 28 h of incubation at instar stage II, when their mouths were open to ingest feed particles. After 28 h of incubation, the sterility of the hatched *Artemia* nauplii was verified by spread plating hatching water (500 μL) on marine agar and then incubating at 28°C for 5 days [26, 27]. All the tools and utensils used in the hatching process were autoclaved at 121°C for 20 min. Aeration was provided through a 0.2 μm filter during hydration, decapsulation, and hatching period. All manipulations were carried out under a laminar flow hood to maintain the sterility of the cysts and nauplii. Any experiments starting with nonsterile nauplii were discarded.

2.4. Artemia Experimental Exposure. The potential toxic effect of PLE on *Artemia* nauplii was determined as described previously [13, 27]. Hatched *Artemia* nauplii at developmental stage II were collected, counted volumetrically, and then, an equal number of nauplii was transferred to 50 mL sterilized glass bottles containing 40 mL of sterile seawater. The axenic nauplii were pretreated with increasing concentrations of extract (1, 2, 3, 4, 5, and 6 g/L) for a fixed time of 2 h at 28°C. They were then repeatedly washed with FASW to remove the extract residues and allowed to recover for another 2 h at 28°C. After the recovery period, a group of 20 nauplii were transferred to 50-mL falcon tubes containing 20 mL of sterile seawater. Two control groups were maintained: one consisting of *Artemia* nauplii not exposed to the extract and ethanol (negative control) and the other exposed only to the ethanol (ethanol control). Four replicates were maintained for both the treatment and control groups. The toxicity of the extract was determined after 48 h recovery period by counting the number of survived nauplii, as previously described [13, 27].

2.5. Bacterial Challenge Assay. Two separate *Artemia* challenge test assays were conducted. In the first test, the dose-response relation of PLE was determined as described in the above section. After a recovery period, the nauplii were exposed to *V. campbellii* challenge at a concentration of 10^6 CFU/mL [27, 28]. The survival of the nauplii was assessed after 48 h of the challenge. Non-pretreated nauplii challenged with *V. campbellii* (positive control) or left unchallenged (negative control) were used as control groups. Additionally, *Artemia* pretreated only with ethanol and challenged with *V. campbellii* was also used as one of the control groups. Each treatment and control group were maintained in four replicates. The experiment was repeated once to confirm the reproducibility.

2.6. Mode of Action of PLE. To determine whether the extract has antioxidant or pro-oxidant properties, a mode of action study was conducted using the procedure previously described [27]. The axenic *Artemia* nauplii were pretreated with either an optimized dose of PLE (selected from the dose-response study), a combination of antioxidant enzymes superoxide dismutase (75 units) and catalase (10 mg/L), or a combination of PLE and a mixture of antioxidant enzymes. Nauplii without PLE pretreatment (negative control) and those exposed only to ethanol (ethanol control) served as controls. Positive control I and II correspond to the challenged groups of the negative and ethanol controls, respectively. The nauplii were counted, distributed into sterile 50-mL falcon tubes, and then, challenged with *V. campbellii* (10^6 CFU/mL) as described in the dose-response study. The survival of the nauplii was recorded after 48 h of challenge.

TABLE 1: Specific primers (forward and reverse) used for qRT-PCR.

Gene/primer name	Primer sequences (5′-3′) (F/R)	Reference
β-actin	agcggttgccatttcttgtt ggtcgtgacttgacggactatct	[29]
α-tubulin[b]	cgaccataaaagcgcagtca ctacccagcaccacaggtctct	[30]
hsp70	cgataaaggccgtctctcca cagcttcaggtaacttgtccttg	[31]
hsp60	aattgcgggctctttacgctc aacgcgaacgatcatctcagc	[32]
hsp90	ggtgtgggtttctattctgc gcagcagattcccacaca	[33]
hmgb1	agaggcgggaaaggaagc cccacaccaagaccaggttg	[34]
proPO	tctgcaaggaggatttaagga tgactgacaaaggagatgggac	[34]
tgase	tctctccgtgtctctccaaaag ccccacaagaagcatctgaag	[29]

2.7. Immune Gene Expression Analysis Using Quantitative Real-Time PCR (qRT-PCR). After pretreating axenic *Artemia* nauplii with an optimized dose of PLE, the conditioned nauplii, were challenged with *V. campbellii* at 10^6 CFU/mL. Samples containing 0.1 g of live nauplii were collected from all the groups before *Vibrio* challenge (0 h) and at 6, 12, 24, and 48 h postchallenge. The samples were stored at −80°C for gene expression analysis. Each treatment was carried out in triplicate. Total RNA extraction from the nauplii was performed using the TRIsureTM kit, according to the manufacturer's protocol (Bioline, UK). The quality and quantity of the extracted RNA were determined using Nanodrop 2000 spectrophotometer (Thermo Scientific, USA). The cDNA was synthesized using 2 μg of RNA and a cDNA synthesis kit (Bioline, UK). The qRT-PCR was performed in a CFX Connect System (Bio-Rad, USA) using a 2x SensiMix SYBR No-ROX kit (Bioline, UK) with specific forward and reverse primers for the genes of interest: *hsp70*, *hsp90*, *hsp60*, *proPO*, *tgase*, high mobility group box 1 (*hmgb1*), and *β-actin* and *α-tubulin*[b] were used (Table 1). The latter two genes *β-actin* and *α-tubulin*[b] were used as reference (housekeeping) genes. The amplification involved an initial denaturation at 95°C for 2 min, followed by 40 cycles at 95°C for 30 s, 60°C for 1 min, and 72°C for 1 min. The melting curve was at 55–95°C with a heating rate of 0.10°C/s and a continuous fluorescence measurement and cooling was done at 4°C. The quantification was performed to confirm the amplification of a single product. The cycle threshold (Ct) values were determined using CFX Manager software (Bio-Rad, USA). The fold difference for each of the immune-related genes relative to *β-actin* and *α-tubulin*[b] was

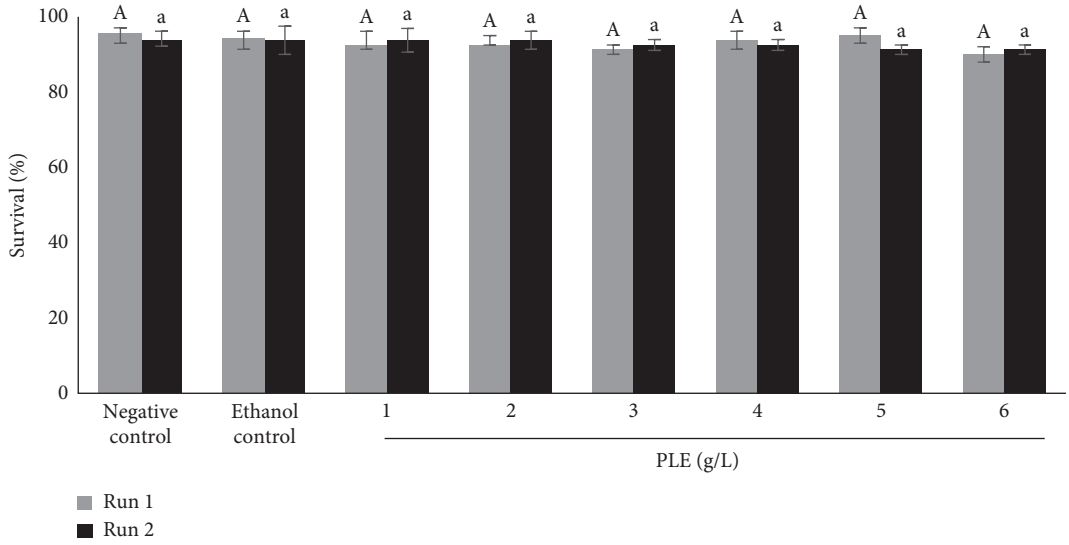

FIGURE 1: Toxic effect of PLE on axenic *Artemia* nauplii over 48 h. PLE was directly added to the *Artemia* rearing water at indicated concentrations for 2 h and subsequently, the nauplii were allowed to a 2 h recovery period. The survival was counted after 48 h. Nauplii that did not PLE pretreatment (negative control) and those that received only the diluting solvent ethanol (ethanol control) served as controls. Error bars represent the standard error of four replicates. Experiments were repeated once indicated as run 1 and run 2. Bars with the same alphabet letters (capital and small letters for run 1 and run 2, respectively) are not different significantly ($p > 0.05$).

calculated using the relative expression software tool developed by Pfaffl [35]. The primer efficiency test was also performed for all the used primers to ensure that the amplification efficiencies were near or above 90% [36].

2.8. V. campbellii Colonization in Artemia nauplii. The *Artemia* nauplii were pretreated with an optimized dose of PLE (1 g/L) and were sampled at 6 h after challenge with *V. campbellii*. The sampled nauplii were then rinsed with autoclaved nine-salt solution (NSS; 17.6 g/L NaCl, 1.47 g/L Na_2SO_4, 0.08 g/L, $NaHCO_3$, 0.25 g/L KCl, 0.04 g/L KBr, 1.87 g/L $MgCl_2$, 0.41 g/L $CaCl_2$, 0.008 g/L $SrCl_2$, and 0.008 g/L H_3BO_3). Ten nauplii, from each treatment and control group, were homogenized using a sterilized motor and pestle using 10 mL of NSS as the solution. The homogenates were then transferred to falcon tubes, and tenfold serial dilutions were prepared using the NSS. Bacterial culture plates were prepared using thiosulfate–citrate–bile salts–sucrose agar, a selective media for vibrios. Spread plating was done and the culture plates were incubated at 28°C for 24 h before counting the colonies. Each treatment and control was performed in triplicate. This assay was conducted using the procedure described by Baruah et al. [27].

2.9. Statistical Analysis. The survival data were tested for their normality and homogeneity of variance before the analysis was done using the one-way ANOVA followed by Duncan's multiple range tests from the statistical software Statistical Package (SPSS) for the Social Sciences version 27.0. The immune gene expressions were presented as fold expressions relative to housekeeping genes. Student's *t*-test was employed to determine the significant difference in *Vibrio* counts between the control and PLE-pretreated groups. The significant level for all the analyses was set at $p < 0.05$. The graphical illustrations were done using GraphPad Prism 8.0.2 and Microsoft Excel 2019.

3. Results

3.1. Toxicity of PLE on Artemia nauplii. Axenic nauplii were exposed to increasing doses of the PLE extract for 2 h, followed by a 2 h recovery period as described in the methodology section. The survival of the *Artemia* nauplii in both the control and treated groups was above 90%. Additionally, no significant differences were observed between the control and treatment groups (Figure 1).

3.2. Impact of PLE on the Survival of Axenic Artemia nauplii Challenged With V. campbellii. As shown in Figure 2, pretreatment with the lowest dose (0.05 g/L) did not significantly protect the *Vibrio*-challenged *Artemia* compared to the ethanol control. However, doses ranging from 1 g/L to 6 g/L protected *Artemia* against *Vibrio* challenge. Among the doses tested, the highest survival of 78% was observed in the group treated with 2 g/L, compared to 45% in the ethanol-treated control group. The survival at 1 g/L was 76%, which was not significantly different from the survival rate at 2 g/L. Therefore, 1 g/L was selected as the optimum dose for subsequent studies.

3.3. Mode of Action of the PLE on the Protection of Axenic Artemia nauplii. In the absence of a *Vibrio* challenge, neither PLE nor the antioxidant enzyme mixture significantly affects the survival of *Artemia* nauplii. However, after being challenged with *V. campbellii*, the survival of the untreated control nauplii decreased from 77% to 49%. Pretreatment with PLE at 1 g/L increased the survival of the *Vibrio*-challenged *Artemia* nauplii from 49% to 77%. When *Artemia* nauplii were pretreated with both PLE and an antioxidant enzyme mixture, there survival was 78%, which was not significantly different from those treated with PLE alone. The survival of challenged nauplii pretreated only with the antioxidant enzyme mixture did not significantly differ from that of the non-pretreated

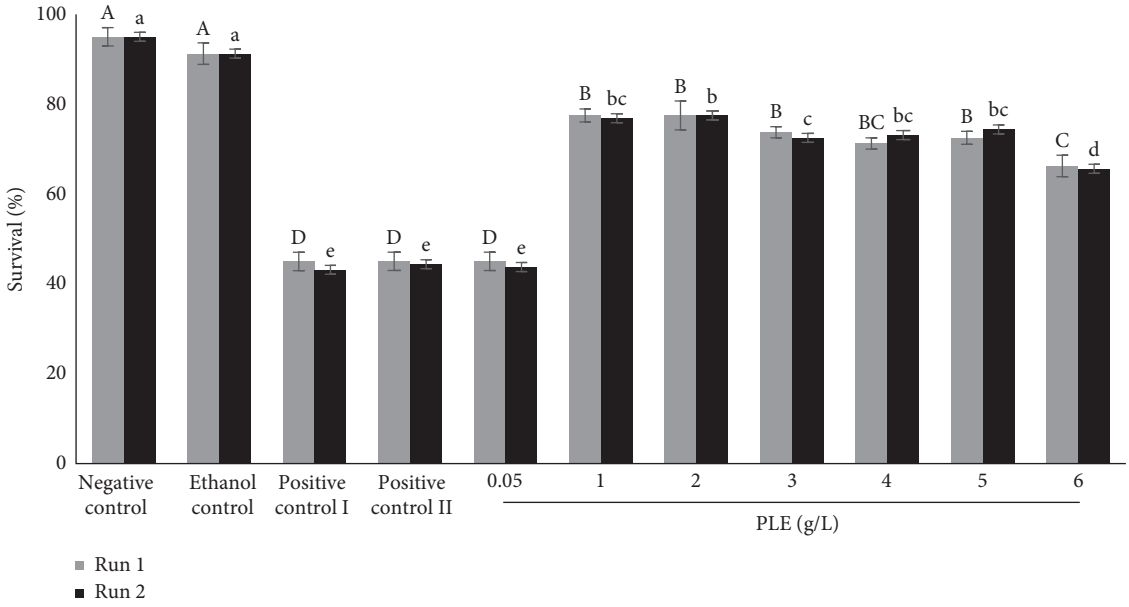

FIGURE 2: Impact of PLE treatment on the survival of axenic brine shrimp nauplii after 48 h challenge with *Vibrio campbellii* LMG21363. Unchallenged nauplii that did not receive PLE pretreatment (negative control) and those that received only the diluting solvent ethanol (ethanol control) served as controls. Positive control I and II are challenged groups of the negative and ethanol control groups, respectively. Error bars represent the standard error of four replicates. Experiments were repeated once indicated as run 1 and run 2. Bars with the same alphabet letters (capital and small letters for run 1 and run 2, respectively) are not different significantly ($p > 0.05$).

challenged larvae (positive control), suggesting that the enzymes alone did not offer protection against *V. campbellii* (Figure 3).

3.4. Impact of PLE on the Expression of Immune-Related Genes in Artemia. As shown in Figure 4a, after a 2-h recovery period (0 h), the expression level of *hsp70* in PLE-treated larvae increased significantly by 3.2-fold compared to the control. At 6 h postchallenge (Figure 4b), the *hsp70* expression level in the PLE-exposed group challenged with *V. campbellii* (PLE + *Vibrio*) showed no significant difference from the unexposed challenged group or the control. However, the PLE-exposed group that was not challenged (PLE) continued to have significantly higher *hsp70* expression levels (4.5-fold) at this time point. At 12 h, there was no significant upregulation of the *hsp70* gene in PLE-exposed groups (i.e., PLE and PLE + *Vibrio*) compared to the control and the *Vibrio*-challenged group. At 24 h, the PLE-exposed groups (i.e., PLE and PLE + *Vibrio*) exhibited a significant increase in the *hsp70* expression levels (9-fold and 7.3-fold, respectively) relative to the control and *Vibrio*-challenged groups. However, at 48 h postchallenge, a significant increment in the *hsp70* level was not observed in the PLE-exposed groups. We also compared *hsp70* expression levels in *Artemia* challenged with only *V. campbellii* to those in the other three experimental groups at all time points. It is worth noting that the *Vibrio*-challenged *Artemia* that did not receive PLE exposure showed no significant increase in *hsp70* at any of the tested time points.

Before challenge (0 h), *Artemia* exposed to PLE exhibited a 2.4-fold increase in the expression level of *hsp60* relative to the corresponding control (Figure 4a). At 6 h (Figure 4c), there was no significant increase in *hsp60* expression in the PLE-exposed groups (i.e., PLE or PLE + *Vibrio*). At 12 and 24 h, the PLE-exposed group in the absence of *Vibrio* challenge exhibited a 6.5-fold and 5.1-fold increase in the *hsp60* expression levels, respectively ($p < 0.05$). However, this significant increase in the *hsp60* level was not observed in the PLE-exposed group in the presence of *Vibrio* challenge. At 24 h, the PLE-exposed group challenged with *Vibrio* had a 2.7-fold higher hsp60 expression level than the *Vibrio*-challenged *Artemia* group, though this difference was not significant. Similarly, at 48 h postchallenge, the PLE-exposed *Artemia* challenged with *Vibrio* tended to have a relatively higher (2.4-fold) level of *hsp60* compared to the respective control group which did not receive PLE exposure. However, the level of *hsp60* in PLE treated group was significantly lower compared to its respective control.

No significant upregulation of *hsp90* was observed at 0 and 6 h time points (Figure 4a,d). There was also no significant difference in *hsp90* expression levels among the different experimental groups at 12 h postchallenge. The *hsp90* gene in the PLE-exposed group challenged with *Vibrio*, however, tends to show an increase in the expression level (2.5-fold higher than *Vibrio*-challenged group; $p > 0.05$). At 24 h, the increase in the *hsp90* level in PLE-exposed groups (i.e., PLE and PLE + *Vibrio*) was significant (2.5-fold and 6.4-fold for PLE and PLE + *Vibrio*, respectively) to their corresponding experimental groups. The same treatment group at 48 h postchallenge had a 5.4-fold significantly higher ($p > 0.05$) *hsp90* level than the respective untreated *Vibrio*-challenged group.

Similar to what was observed for *hsp90*, the expression levels of *hmgb1* gene in *Artemia* nauplii pretreated with PLE before the *Vibrio* challenge (0 h) did not increase significantly relative to the control (Figure 4a). At 6 h postchallenge, no

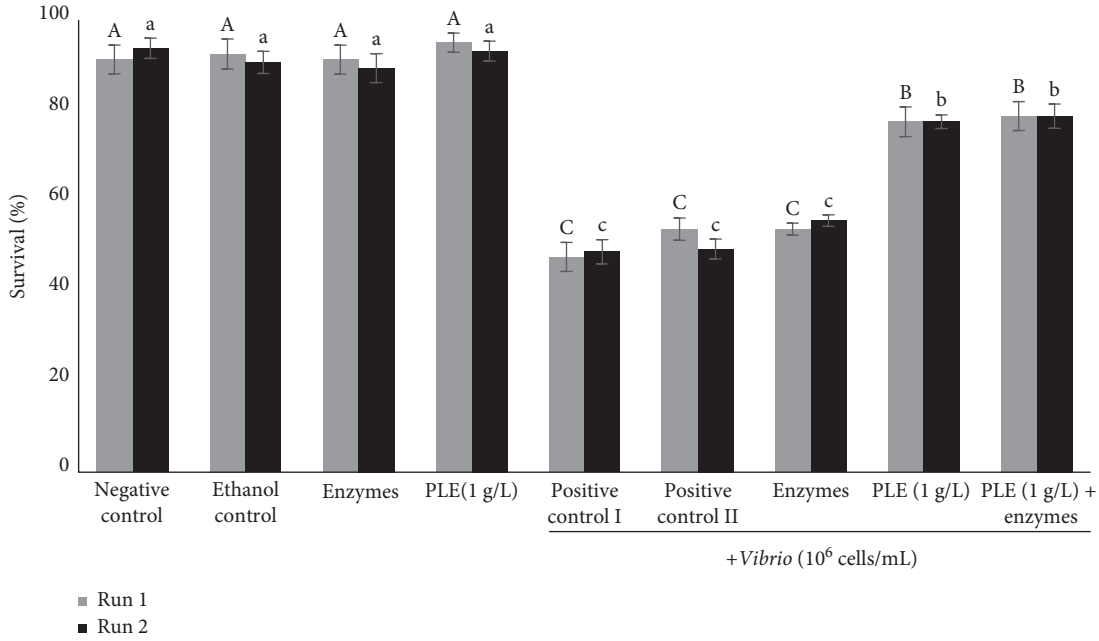

FIGURE 3: Percentage (%) of survived *Artemia* nauplii after 48 h of challenge with *Vibrio campbellii*. The nauplii were pretreated with PLE and/or a mixture of antioxidant enzymes at indicated conditions and then challenged with *V. campbellii*. Nauplii that did not PLE pretreatment (negative control) and those that received only the diluting solvent ethanol (ethanol control) served as controls. Positive control I and II are challenged groups of the negative and ethanol control groups, respectively. Error bars represent the standard error of four replicates. Experiments were repeated once indicated as run 1 and run 2. Bars with the different alphabet letters (capital and small letters for run 1 and run 2, respectively) are not different significantly ($p < 0.05$, Duncan's multiple range test).

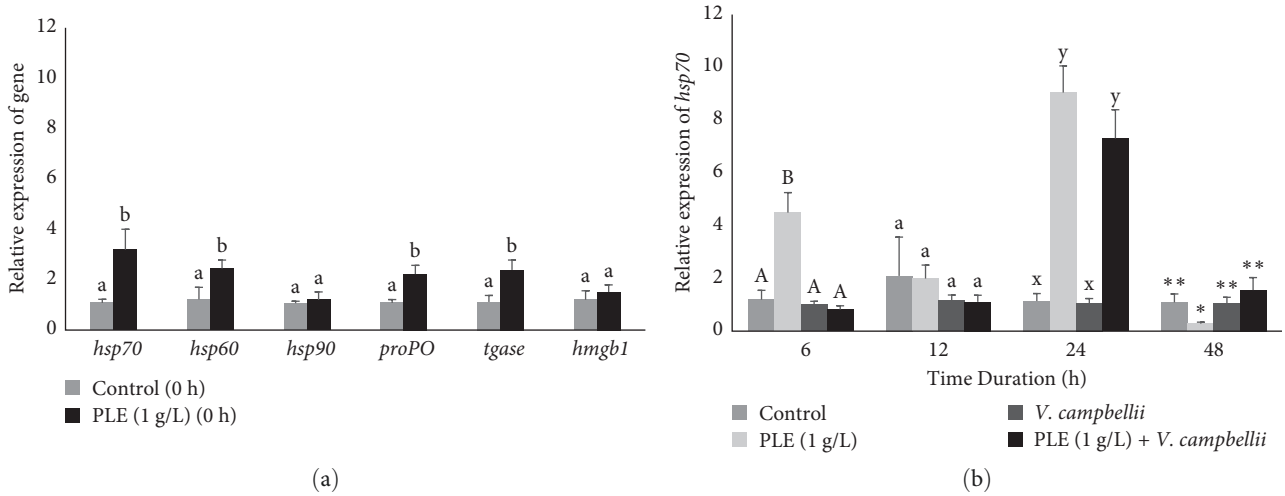

FIGURE 4: Continued.

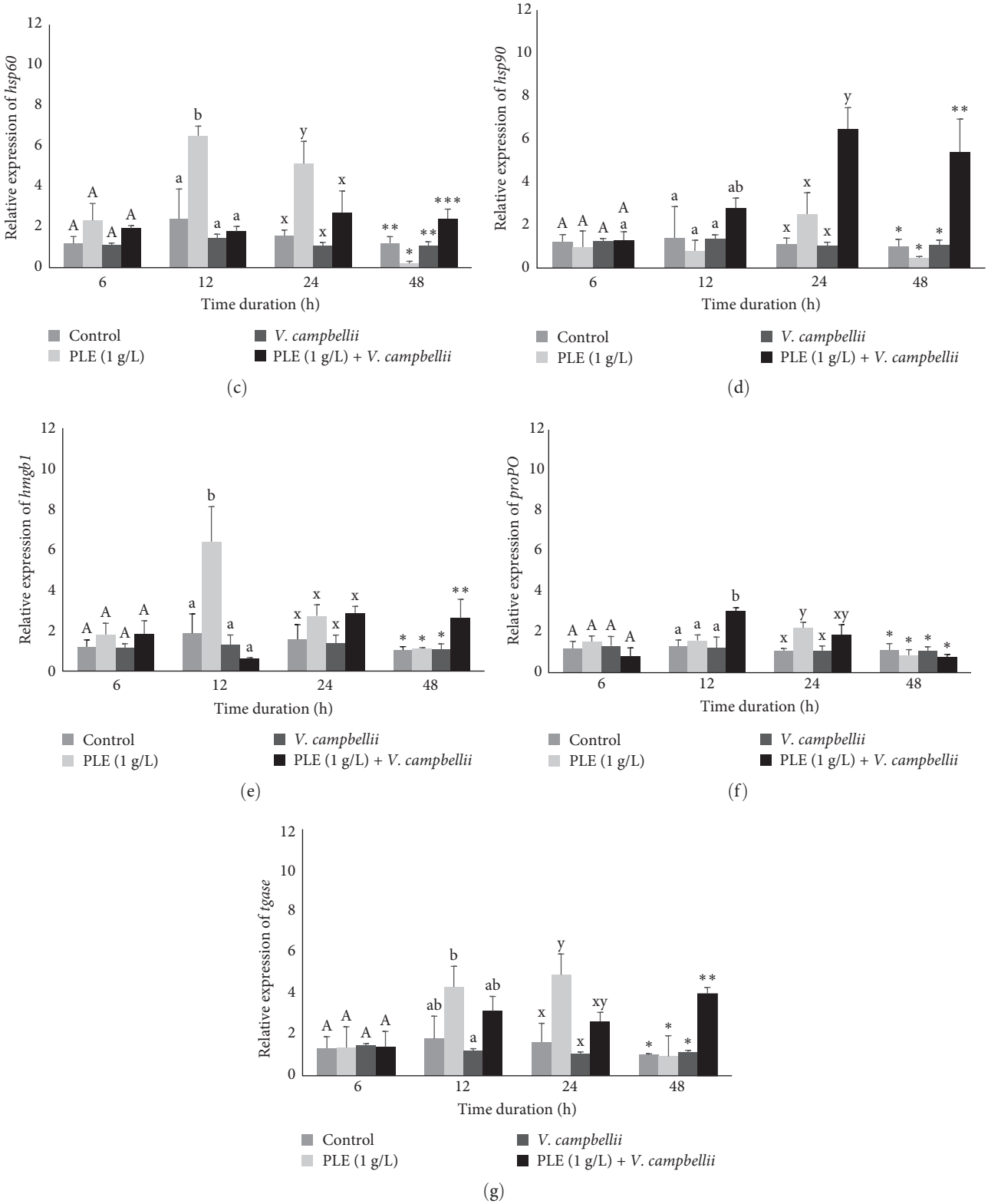

FIGURE 4: Expression of (a) the heat shock protein *hsp70*, (b) *hsp60*, (c) *hsp90*, (d) high mobility group box 1 (*hmgb1*), (e) prophenoloxidase (*proPO*), and (f) transglutaminase (*tgase*) genes in axenic *Artemia* nauplii. The nauplii were pretreated with PLE and then challenged with *V. campbellii* as described in the methodology section. Samples were collected for the gene expression assays before the challenge (0 h) and at 6, 12, 24, and 48 h of *Vibrio* challenge. Error bars indicate the standard errors of three biological replicates. Different alphabet letters under each gene in (a) indicate significant differences (*t*-test; $p < 0.05$). Different alphabet letters or symbols at each time point in (b–f) indicate significant differences (Duncan's multiple range test; $p < 0.05$).

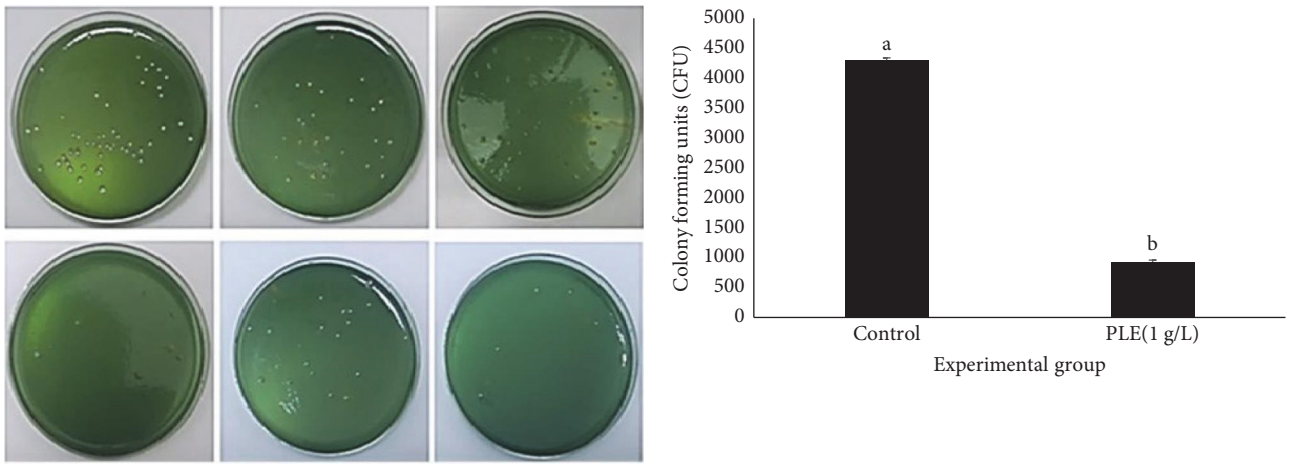

FIGURE 5: (a) Colony formation and (b) numbers on a log scale of *V. campbellii* in *Artemia* nauplii. The culture plates in the first row show the control sample and the second row shows the treatment (PLE) sample. Error bars represent the standard error of three replicates. Different letters indicate significant differences (*t*-test; $p < 0.05$).

significant changes in the expression level were observed in nauplii pretreated with the PLE compared with the untreated groups. However, at a 12 h time point, PLE-exposed *Artemia* not challenged with *Vibrio* exhibited a significant 6.4-fold increase in the relative abundance of the *hmgb1* gene. This response, however, was not reflected in the PLE-exposed group that were challenged with *Vibrio*. At 24 h, no significant difference in the expression level was observed between the PLE-exposed group and those that were not exposed to the extract. However, at 48 h time point, the *hmgb1* gene expression level in PLE-exposed *Artemia* challenged with *Vibrio* was significantly higher (2.6-fold) than the other experimental groups (Figure 4e).

Before the challenge, the PLE-exposed *Artemia* had a 2.2-fold higher *proPO* level than the corresponding control (Figure 4a). However, at 6 h, no significant difference in the *proPO* level was observed between the experimental groups. At 12 h, the PLE-exposed group in the presence of *Vibrio* challenge exhibited a significant increase in the *proPO* gene expression level (threefold) compared to the other experiment groups. The *proPO* level in the PLE-exposed *Artemia* challenged with *Vibrio* was still higher compared to the control and *Vibrio*-challenge group at 24 h. However, the difference was not significant ($p > 0.05$). *Artemia* that received PLE exposure in the absence of *Vibrio* challenge had significantly higher upregulation of *proPO* (2.2-fold) than the control and *Vibrio*-challenged *Artemia*. At 48 h, no significant difference in the *proPO* level was observed between the experimental groups (Figure 4f).

As shown in Figure 4a, before the *Vibrio* challenge (0 h), there was no significant upregulation of the *tgase* gene in PLE-exposed groups compared to the unexposed groups. At 12 h, the PLE-exposed groups appeared to have higher expression levels of *tgase* compared to the unexposed group, but the difference between the groups was not prominent ($p > 0.05$) unlike the PLE-exposed group at 24 h, which exhibited a significant increase in the expression level of *tgase* gene (4.9-fold). Moreover, at 48 h the nauplii challenged with *Vibrio* following exposure to PLE showed significant expression of *tgase* (four-fold) compared to other experimental groups (Figure 4g).

3.5. Impact of PLE on the Vibrio Colonization in Artemia. The average cell number was significantly reduced by approximately fivefold in the PLE pretreated group (0.96×10^3 CFU/mL) compared to the control group (4.3×10^3 CFU/mL) as depicted in Figure 5.

4. Discussion

This study investigated whether PLE could induce resistance in shrimp to fight against pathogenic *V. campbellii*. To address this, we used axenic *Artemia* as an experimental model system (Figure 6). Maintaining a germ-free condition was crucial to eliminate potential interference from microbial communities within the experimental system and/or associated with the host. This setup enabled us to focus solely on the interaction between the host and the pathogen under study. Additionally, such controlled conditions allowed us to distinguish the specific effects of the extract on the host organism, rather than any effects on the pathogen itself [27, 28].

During aquaculture operations, the safety and well-being of farmed shrimps are critical. When considering the application of plant extracts or their components, it is of paramount importance that the extracts demonstrate a high protective index (PI), which is a measure of the safety margin of a disease-control agent. The PI is calculated by comparing the dose of an extract that causes toxicity with the dose that provides a protective effect [37]. To assess this, we carried out a toxicity assay in a germ-free environment, which was followed by a challenge experiment. Our findings indicated that PLE did not cause an adverse effect on *Artemia* nauplii at least at the tested doses. Similarly, an earlier study showed no toxicity in *P. vannamei* PL exposed to 6 g/L of PLE extract for 24 h [13], despite differences in exposure duration (24 h) and experimental conditions.

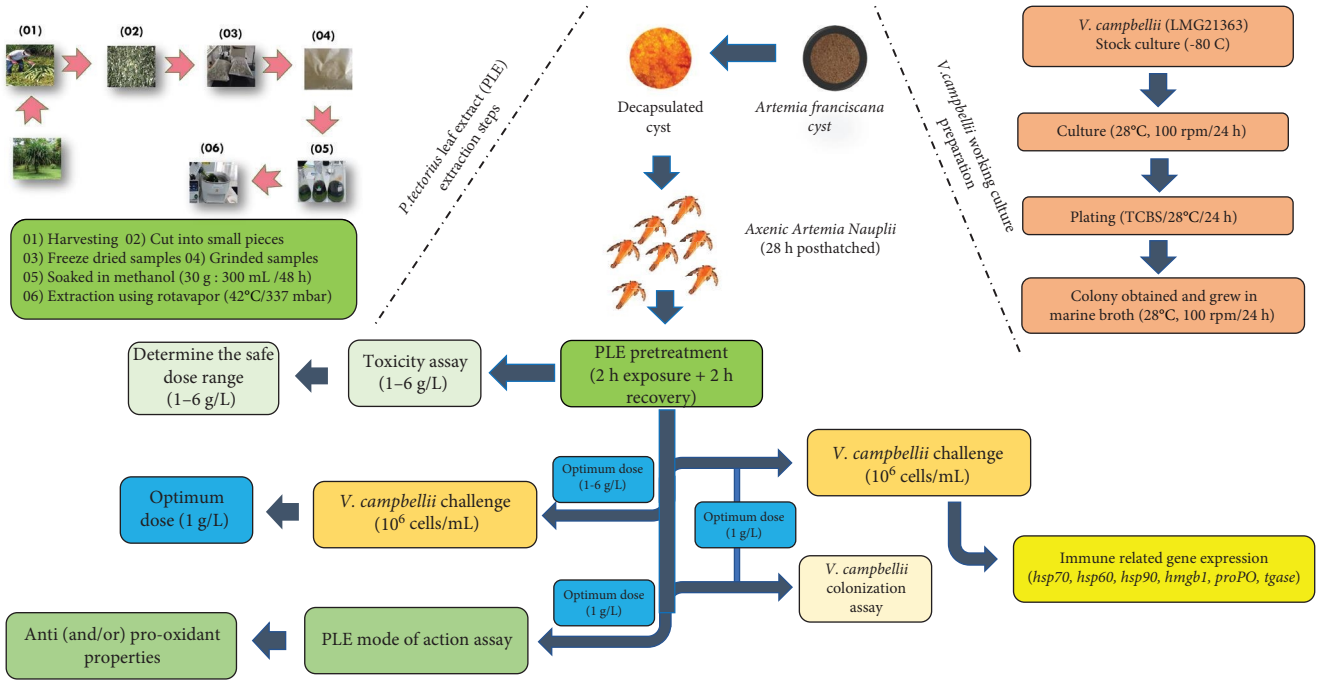

FIGURE 6: Graphical illustration of the pretreatment study conducted using axenic *Artemia* nauplii to investigate the prophylactic effect of PLE.

Furthermore, our study demonstrated that pretreatment with PLE led to significant protection of *Artemia* nauplii against *V. campbellii*. This protective effect of the extract was noticeable in the dose range starting from a minimum of 1 g/L up to 6 g/L. Based on the survival results from the challenge tests, we selected 1 g/L as the optimum protective dose to conduct further studies to unravel the mechanisms behind the protective effects of PLE against *V. campbellii*. We selected the lowest effective dosage of the extract to ensure a higher PI. Our results align with those of Anirudhan et al. [13], who also reported significant improvements in the survival of *P. vannamei* PLs after 24 h of exposure to PLE in the dose range of 2–6 g/L, followed by a challenge with pathogenic *V. parahaemolyticus* (10^6 CFU/mL).

Our subsequent experiments aimed at gaining more insight into the protective effects of the PLE against *Vibrio* infection. Several plant extracts or phenolic compounds were reported to cause protective effects on organisms through their prooxidant actions, releasing reactive oxygen species (ROS; e.g., hydrogen peroxide and superoxide anion), within the host cells and/or in the culture medium [28, 29, 38]. For instance, in the experimental animal as well as in the rearing medium, the generation of ROS has contributed to the generation of resistance in the animal towards pathogenic vibrios [24, 39]. Given this evidence, we questioned whether the *Vibrio*-protective effect of PLE observed in our study could be attributed to the generation of ROS by the extract. To address the question, we conducted additional in vivo survival studies, as described in Figure 3 legends. We hypothesized that if ROS produced by PLE were responsible for the protective effect, then, neutralizing these ROS with a cocktail of antioxidant enzymes would eliminate the protection conferred by PLE. However, our results suggested no strong association between PLE's protective effect against *V. campbellii* and ROS generation by the extract. This conclusion was drawn from our observation that the addition of antioxidant enzymes did not neutralize the protective effect of PLE. The following two possible explanations arise from these results: First, it is likely that the mode of action of PLE in inducing protective responses does not involve pro-oxidant activity, but rather other mechanisms, such as eliciting immune responses. Second, while we used antioxidant enzymes doses based on previous studies [27, 38], these doses may not have been sufficient to fully neutralize any ROS generated by PLE. Further studies focusing on the effects of PLE on immune response and the pro-oxidant/antioxidant defense mechanisms are needed to better understand how PLE confers protection against *Vibrio*. It is important to highlight that we employed an indirect approach to assess ROS generation. Further research is required to confirm the extract's prooxidant effects through direct ROS measurements.

Pandanus tectorius leaves are rich in essential oils and phenolic compounds [17, 19, 40]. They were shown to exhibit antibacterial, antioxidant, anti-inflammatory, and immune-stimulating activities [18, 19, 21, 41, 42]. For instance, in the farmed shrimp *P. vannamei*, pretreatment of the shrimp for 24 h with a methanolic extract of *P. tectorius* fruit or leaf resulted in a marked increase in the resistance of the shrimp against *V. parahaemolyticus* challenge [12, 13]. The protective effect was associated with a significant upregulation of the key innate immune genes, including *hsp70*, *proPO*, *peroxinectin*, *penaeidin*, *crustin*, and *tgase*. Additionally, the leaf extract was found to reduce histopathological damage in the

hepatopancreas of shrimp caused by *V. parahaemolyticus* infection [13]. It is, however, noteworthy to mention that those studies did not examine the temporal expression profile of the immune genes after pretreatment and subsequent exposure of the shrimps to the pathogen. Exploring this aspect could provide further insights into the protective mechanisms of *Pandanus* extracts.

Similar to the shrimp *P. vannamei*, the brine shrimp *Artemia* relies solely on its innate immune system to defend against invading pathogens, including vibrios [36, 43–45]. Given that *Artemia* was pretreated with the extract before being subjected to the *Vibrio* challenge, we hypothesized that the host's defense system was primarily responsible for the observed protection against the pathogen. We, therefore, focused on the immune responses in *Artemia*, particularly the hsp belonging to the 70 kDa Hsp (Hsp70) [28, 46, 47]. Hsp70s are highly conserved proteins and can be either constitutively expressed or induced under various conditions. Functionally, Hsp70s are molecular chaperones involved in protein biogenesis and protein homeostasis in the cells under both normal and stressful conditions [48, 49]. These functions include the folding of nascent proteins, protein translocation, assembly, disassembly, refolding, degradation of denatured proteins due to stress, and other cellular dysfunctions improving the survival of normal and diseased cells [38, 50, 51]. Additionally, Hsp70 is known to contribute to protective immune responses against many diseases, including vibriosis, as demonstrated in several in vitro and in vivo experimental models [29, 45, 52, 53]. For instance, in *Artemia*, early induction of Hsp70 by Hsp-inducing agents, like cactus extract [24, 26] and plant-derived compounds (e.g., carvacrol [38], phloroglucinol [28], and pyrogallol [27]) has been shown to significantly enhance resistance to stress caused by vibrios. These studies suggest that Hsp70-mediated immune responses play a crucial role in improving the resistance of the animal. In our study, we observed an early increase in *hsp70* expression in *Artemia* exposed to PLE. However, this increment in *hsp70* expression level was not seen during the first 12 h postchallenge, but at 24 h postchallenge, a significant increase was observed, which again dropped to normal level at 48 h postchallenge. This result suggests that PLE-pretreated *Artemia* responds to the *Vibrio* challenge with a stochastic expression of *hsp70* to provide protection against *V. campbellii*. Similarly, hsps *hsp60* and *hsp90* also exhibited stochastic expression patterns in PLE-pretreated *Artemia* subsequently challenged with *V. campbellii*. Both Hsp60 and Hsp90 are ATP-dependant, high molecular weight proteins involved not only in protein homeostasis but also in modulating immune responses through various pathways [54]. Based on this evidence, it can be suggested that the enhanced resistance of PLE-pretreated *Artemia* to *V. campbellii* challenge is, at least in part, due to the induction of a constellation of hsps, which strengthen the defense system of the organism.

The involvement of Hsps in generating resistance in *Artemia* challenged with *Vibrio* may represent one of the several underlying mechanisms. Since crustaceans like *Artemia* lack an adaptive immune system, unlike vertebrates, they likely rely on a range of other potent innate immune effectors to defend against bacterial infections [44, 55]. To investigate this further, we examined three vital genes: *hmgb1*, *proPO*, and *tgase*, which encode for immune effector proteins HMGB1, phenoloxidase, and TGase, respectively. These genes have been shown to play essential roles in *Vibrio* resistance in both *P. vannamei* and *Artemia* [12, 13, 27, 44]. HMGB1 is a highly conserved protein associated with chromatin, responsible for stabilizing nucleosome formation and regulating transcription [56]. However, the functional role of HMGB1 is not solely mediated by its ability to bind to DNA. In fact, evidence suggest that this DNA chaperone can get released into the extracellular environment and can instigate the host immune system to mount a nonspecific biological response at the site of infection or injury caused, for instance, by stress agents [45]. Given the link between *hmbg1* induction and infection stress, as well as its correlation with organism survival [44, 45], we further analyzed the expression profile of *hmgb1* in PLE-pretreated *Artemia*. Our results showed significant upregulation of *hmgb1* in PLE-pretreated *Artemia* in response to *V. campbellii* challenge, particularly at the latter stage of the challenge, that is, at 48 h time point. Notably, this increased response in the *hmgb1* level coincided with higher survival of *Artemia* in the pretreated group. Taken together, these findings suggest that, in addition to *hsp60*, *hsp70*, and *hsp90*, *hmgb1* is also induced in *Artemia* in response to exposure to PLE and these molecular chaperones through an unexplored cascade of biochemical and immunological reactions might have contributed to the protection of *Artemia* against *V. campbellii*. Our results align with an earlier report that points towards the critical role of HMGB1 in protecting brine shrimp against *Vibrio* infection caused by pathogenic vibrios [36, 44, 45].

The *proPO* and *tgase* genes are two major components of the humoral innate immune system in invertebrates known for their protective roles against infectious agents [31, 57]. Phenoloxidase provides its protective effect by cuticular melanization, sclerotization, wound healing, encapsulation, and eventual killing of the pathogens [57]. On the contrary, TGase exerts its effects through the hemolymph (blood) coagulation mechanism, preventing the loss of hemolymph through injuries in the exoskeleton, and the subsequent entry and proliferation of microbes throughout the body [58]. Earlier studies on the role of Hsps and HMGB1 in crustaceans, including *Artemia*, have shown that these proteins are involved in initiating humoral innate immune responses, rendering the animals more resistant to bacterial diseases [36, 44]. For example, the induction of Hsp70 by cactus extract or plant-derived pyrogallol compound has been found to stimulate *proPO* and *tgase* in *Artemia* [27, 29]. Similarly, the injection of recombinant Hsp70 in *P. vannamei* was shown to induce the expression of *proPO1*, *proPO2*, and *tgase1* [59]. Our results showed a significant increase in the expression of *hsp70* and *hsp60*, along with *proPO* and *tgase*, immediately following the recovery period following PLE exposure. However, after the challenge, elevated *proPO* level was detected in the PLE exposed group at 12 h, which returned to the basal level at 24 and 48 h postchallenge. Meanwhile, *tgase* levels in PLE-pretreated *Artemia* remained elevated at 12, 24, and 48 h postchallenge. Interestingly, these patterns correlate largely with enhanced resistance of *Artemia* to *V. campbellii*. A notable observation from this study was the marked reduction

in *V. campbellii* colonization in PLE-pretreated *Artemia*. Previous reports have documented the antibacterial activities of PLE against various bacterial pathogens, including vibrios, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus* [19, 30]. Although the *Artemia* were rinsed repeatedly to remove PLE before the recovery period, residual extract likely contributed to inhibiting the colonization of *V. campbellii* through its antibacterial effects. We suggest that the observed decrease in *Vibrio* colonization, along with improved survival, may be attributed to both the antibacterial properties of PLE and its role in enhancing the immune system.

5. Conclusions

In conclusion, our results provide compelling in vivo evidence suggesting that PLE could offer prophylactic benefits against pathogenic *V. campbellii*. The protective effects observed in this study appear to be linked to the generation of protective innate immune responses, especially through the regulation of *hsps*, *hmgb1*, *proPO*, and *tgase* expression. These results add new information about the functional roles of PLE and advance our knowledge of this compound as a potential antimicrobial agent for controlling *V. campbellii*-mediated diseases in farmed shrimps. Further validation studies should be carried out to confirm these findings and assess the practical applications of PLE in shrimp aquaculture settings.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Ethics Statement

This study utilized an invertebrate model, *Artemia* spp., for experimental purposes. As invertebrates are not subject to the same ethical regulations as vertebrates, no specific ethical approval was required for the use of these organisms in the research. However, all experiments were conducted following established guidelines to ensure the welfare of the animals.

Conflicts of Interest

The authors declare no conflicts of interest.

Author Contributions

Patabandi PPSK, Yeong Yik Sung, and Kartik Baruah designed the experiments. Patabandi PPSK and Kartik Baruah wrote the paper. Patabandi PPSK and Parisa Norouzitallab organized and typeset the images. Patabandi PPSK, Mohamad Asmidar, and Yosie Andriani helped with sample collection and data presentation. Patabandi PPSK, Mohamad Asmidar, and Yosie Andriani performed the majority of the experiments and analyzed the data. Patabandi PPSK, Mohamad Asmidar, Yosie Andriani, Parisa Norouzitallab, Yeong Yik Sung, and Kartik Baruah helped revise the manuscript. Parisa Norouzitallab, Yeong Yik Sung, and Kartik Baruah supervised the study. Patabandi PPSK drafted the original paper. All authors have read and approved the final manuscript. Kartik Baruah, Yeong Yik Sung, and Parisa Norouzitallab shared the senior authorship.

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