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α-Phellandrene enhances the immune response and resistance against *Vibrio alginolyticus* in white shrimp (*Litopenaeus vannamei*)

Chih-Chung Wu[a], Chia-Ling Lin[b], Chun-Yung Huang[b], Shuchen Hsieh[c], Chun-Hung Liu[d], Shu-Ling Hsieh[b,*]

[a] Department of Food and Nutrition, Providence University, Taichung, 43301, Taiwan
[b] Department of Seafood Science, National Kaohsiung University of Science and Technology, Kaohsiung, 81157, Taiwan
[c] Department of Chemistry, National Sun Yat-sen University, Kaohsiung, 80424, Taiwan
[d] Department of Aquaculture, National Pingtung University of Science and Technology, Pingtung, 91201, Taiwan

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ABSTRACT

Innate immunity and resistance against *Vibrio alginolyticus* in white shrimp, *Litopenaeus vannamei*, that received α-phellandrene were examined. The results indicated that the percent survival of shrimp receiving 4, 8, and 12 μg g^{-1} α-phellandrene was significantly higher than that of control shrimp after 72 h ($p < 0.05$). In a separate experiment, the phenoloxidase (PO), respiratory bursts, superoxide dismutase (SOD), and phagocytic and lysozyme activity of *L. vannamei* receiving 8 and 12 μg g^{-1} α-phellandrene were significantly higher than those of the other groups upon challenge with *V. alginolyticus* at 24–60, 36–60, 12–60, 12–72 and 48–72 h, respectively. However, no significant differences in the total haemocyte counts (THC) of *L. vannamei* receiving any dose of α-phellandrene and of control shrimp were observed at 12–72 h. The expression (mRNA transcripts) of the immune genes prophenoloxidase (proPO), LPS- and β-1,3-glucan-binding protein (LGBP) and peroxinectin (PE) of shrimp receiving α-phellandrene at 8 and 12 μg g^{-1} significantly increased after challenge with *V. alginolyticus* for 72 h ($p < 0.05$). We conclude that the immune ability and resistance against *V. alginolyticus* infection increased in *L. vannamei* receiving > 4 μg g^{-1} α-phellandrene. These results indicated that α-phellandrene plays an important role in the innate immunity of white shrimp.

1. Introduction

Some naturally occurring plants have various benefits for aquaculture, including growth promotion, enhancement of disease resistance, promotion of digestive enzyme activity, improved immunity, and improved meat quality [1]. Moreover, plants represent a source of relatively safe, inexpensive, biodegradable, biocompatible treatments that are safe for the environment [2]. In recent years, many studies have applied plants and plant extracts as immunomodulators and growth promoters in aquafeeds due to their positive effects on adaptive immunity and the prevention of diseases in crustaceans; for example, *Toona sinensis* [3], *Petalonia binghamiae* [4], *Gynura bicolor* [5], *Syzygium cumini* [6], and *Argemone mexicana* [7] have been used for white shrimp (*Litopenaeus vannamei*); *Cynodon dactylon* has been used for *Macrobrachium rosenbergii* [8]; and *Cynodon dactylon*, *Aegle marmelos*, *Tinospora cordifolia*, *Picrorhiza kurroa*, *Eclipta alba* [9], *Cardiospermum halicacabum*, *Agati grandiflora*, *Justicia tranquebariensis* [10] and *Eclipta erecta* [11] have been used for *Penaeus monodon*.

Plants contain many active ingredients, including polysaccharides, alkaloids, flavonoids, phenolics, essential oils, organic acids, and tannins [12]. Notably, α-phellandrene, 5-isopropyl-2-methyl-1,3-cyclohexadiene, is a cyclic monoterpene present in essential oils of many dietary spices and herbs [13]. Studies have shown that α-phellandrene is enriched in the essential oils of *Monodora myristica* (Gaertn.) Dunal, *Schinus molle* L., *Schinus terebinthifolius* Raddi, *Anethum graveolens*, *Moringa oleifera* Lam., *Diplotaenia damavandica*, and *Piper nigrum* [14–19]. In particular, the essential oils of *M. myristica*, *Schinus molle* L., and *Schinus terebinthifolius* Raddi contain high α-phellandrene concentrations of 53% [14], 46.52, and 34.38% [15], respectively. Previous studies have reported that α-phellandrene does not exhibit cytotoxic effects [20] and has demonstrated antibacterial [21,22], antifungal [23], antioxidant [24], hypolipidaemic [16], antinociceptive [20,25], and anticancer [13,26–28] effects. According to previous studies, α-phellandrene may also have an effect in chemoprevention and

* Corresponding author. Department of Seafood Science, National Kaohsiung University of Science and Technology, 142 Haijhuan Rd., Nanzih District, Kaohsiung City, 81143, Taiwan.
E-mail address: slhsieh@nkust.edu.tw (S.-L. Hsieh).

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Table 1
Effects of α-phellandrene on the percent survival of white shrimp (*Litopenaeus vannamei*) challenged with *Vibrio alginolyticus*.

α-PA	*V. alginolyticus*	No.	Survival rate (%) after challenge (h)					
(μg/g)	(CFU/shrimp)	Shrimp	12	24	36	48	60	72
Unchallenged control	Saline	30	100	100	100	100	100	100
Challenged control	2×10^5	30	93.3 ± 3.3^a	86.7 ± 8.9^b	73.3 ± 5^b	66.7 ± 6.6^b	63.3 ± 3.3^c	50.0 ± 5.8^c
4	2×10^5	30	100 ± 0^a	96.7 ± 3.3^a	93.3 ± 3.3^a	76.7 ± 3.5^{ab}	76.7 ± 3.5^b	66.7 ± 6.6^b
8	2×10^5	30	100 ± 0^a	100 ± 0^a	93.3 ± 3.3^a	83.3 ± 6.4^a	83.3 ± 6.4^a	83.3 ± 6.4^a
12	2×10^5	30	100 ± 0^a	100 ± 0^a	93.3 ± 3.3^a	83.3 ± 6.4^a	83.3 ± 6.4^a	83.3 ± 6.4^a

Data with differ markers among different treatment are significant difference at the same time ($p < 0.05$). Values are the mean ± SE (n = 30 shrimp in each treatment).

chemotherapy. However, the immunostimulating effects of α-phellandrene on shrimp have not been reported.

White shrimp (*L. vannamei*) is one of the most widely cultivated shrimp worldwide, and aquaculture production of *L. vannamei* in Taiwan alone reached 12,376 metric tons in 2017 [29]. However, the rapid development of intensive and industrialized shrimp aquaculture systems has been affected by the occurrence and spread of various infectious diseases such as vibriosis, which is caused by the Gram-negative bacteria *Vibrio* spp. Vibriosis is a major disease in white shrimp aquaculture that causes widespread mortality in farming operations [30]. Therefore, enhancement of immunity and disease resistance in white shrimp has become critical to the sustained growth of the shrimp aquaculture industry.

Plant products have been reported to have antibacterial and antiparasitic properties in fish and shellfish aquaculture [31]. For example, a previous study [32] showed that tilapia (*Oreochromis mossambicus*) intraperitoneally injected with water extracts of purple fruited pea eggplant (*Solanum trilobatum*) presented 27% reduced mortality when challenged with *A. hydrophila* compared to that of the control groups. In other studies, Asian seabass (*Lates calcarifer*) fed with a *Azadirachta indica* (neem) leaf-supplemented diet showed significantly increased phagocytic activity, superoxide anion production, serum lysozymes, serum bactericidal activity, and serum anti-protease activity throughout the experimental period compared with those of the control group [33]. However, studies on the effect of α-phellandrene in shrimp are limited.

This study aimed to determine the impacts of α-phellandrene application on pathogen infections and the immune responses of *L. vannamei*. Immune parameters including THC, PO, RBs, SOD, and the phagocytic and lysozyme activity of *L. vannamei* were examined. In addition, haemocyte morphology and immune gene expression (proPO, LGBP, and PE mRNA transcripts) of *L. vannamei* were monitored. Finally, the susceptibility of *L. vannamei* to *V. alginolyticus* following treatment with α-phellandrene was also monitored.

2. Materials and methods

2.1. Shrimp

L. vannamei shrimp averaging 5.3 ± 0.8 g (mean ± SD) were supplied by the Department of Aquaculture, National Pingtung University of Science and Technology. Upon arrival, they were acclimated to laboratory conditions for seven days in indoor fibreglass-reinforced plastic tanks and fed commercial diets (Shye-Yih, Kaohsiung, Taiwan). A proximate analysis of the commercial diet showed 35% crude proteins, 5% crude lipids, 16% ash, and 44% carbohydrates (nitrogen-free extract and crude fiber). All shrimp were fed twice daily with a formulated shrimp diet (Shinta Feed Company, Pingtung, Taiwan). The water was maintained at a temperature of 25 ± 1 °C throughout the experimental period, with a pH of 7.8–8.0 and a salinity of 28‰.

2.2. Preparation of Vibrio alginolyticus

V. alginolyticus were cultured on tryptic soy agar (TSA; supplemented with 2% NaCl, Difco, Detroit, MI, USA) for 24 h at 28 °C and then transferred to 10 mL of tryptic soy broth (TSB; supplemented with 2% NaCl, Difco) for 24 h at 28 °C. The broth culture was centrifuged at 7155 × *g* for 20 min at 4 °C. The supernatant was removed, and the bacterial pellet was re-suspended in saline solution (0.85% NaCl) at a density of 10^7 colony-forming units (CFU) mL^{-1} for the susceptibility test [34].

2.3. Experimental design

α-Phellandrene was purchased from Sigma Chemical Co. (St. Louis, MO). A total of two experiments were conducted. The first experiment aimed to determine the susceptibility of *L. vannamei* to *V. alginolyticus*, with treatment and control groups comprising 10 shrimp each in triplicate. On one day, *L. vannamei* were individually injected into the ventral sinus of the cephalothorax with 1, 2, or 3 mg mL^{-1} of α-phellandrene solution (approximately 20 μL) to achieve doses of 4, 8, or 12 μg g^{-1} body weight, respectively. On day two, a challenge test was conducted by injecting 10 μL of bacterial suspension (1×10^7 CFU mL^{-1}) resulting in 2×10^5 CFU $shrimp^{-1}$ through the ventral sinus of the cephalothorax. Shrimp that did not receive α-phellandrene treatment received *V. alginolyticus* at 2×10^5 CFU $shrimp^{-1}$, which served as the challenged controls, while shrimp that received saline only (10 μL) served as the unchallenged controls (Table 1). There were therefore five treatments in total, and the treatment and control groups comprised 10 shrimp, each in triplicate, for a total of 30 shrimp per treatment. A total of 150 shrimp (5 groups × 30 shrimp) were used for the present study. Shrimp survival was observed after 12, 24, 36, 48, 60 and 72 h. Each aquarium was provided with continuous aeration. In each aquarium, 75% of the water was changed every day to remove impurities and maintain water quality.

The second experiment aimed to study non-specific immune responses (the levels of THC, PO, RBs, SOD, and phagocytic and lysozyme activity), gene expression (the mRNA transcript levels of proPO, LGBP, and PE) and haemocyte morphology. Shrimp were individually injected into the caudal vein with different doses of α-phellandrene on the first day. On the second day, shrimp were injected with 2×10^5 CFU $shrimp^{-1}$ through the ventral sinus of the cephalothorax. Shrimp that received saline only (10 μL) served as the control group (no α-phellandrene and bacterial suspension). In total, there were four treatments (control, 4, 8, and 12 μg g^{-1}) with six sampling times (12, 24, 36, 48, 60, and 72 h), and eight shrimp were used for each treatment and time. Therefore, a total of 192 shrimp (4 treatments x 6 times × 8 shrimp) were used for the present study. In addition, eight shrimp that received no treatment served as the initial group (0 h).

2.4. Determination of immune parameters

A drop of anticoagulant-haemolymph mixture (100 μL) was placed

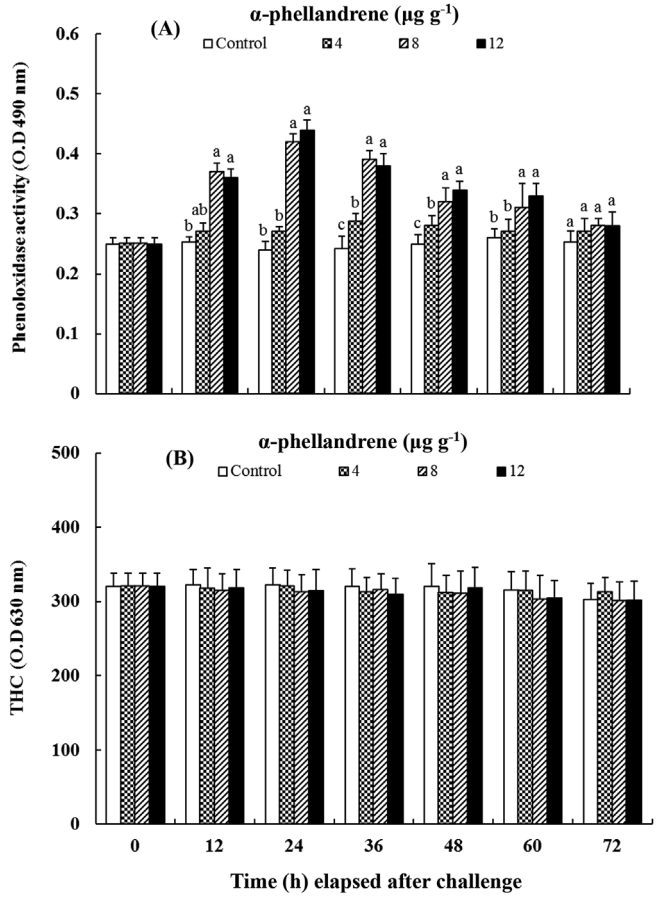

Fig. 1. Mean (± SE) phenoloxidase (PO) (A) and total haemocyte count (THC) (B) of *Litopenaeus vannamei* that received α-phellandrene at 4, 8, and 12 μg g^{-1} and of control shrimp after challenge with *Vibrio alginolyticus* at 12, 24, 36, 48, 60, and 72 h. Each bar represents the mean value from eight determinations with the standard error (± SE). Data at the same exposure time with different letters significantly differed ($p < 0.05$ among treatments).

in a haemocytometer to measure THC using an inverted phase-contrast microscope (Olympus IX 71, Tokyo, Japan) according to the procedures of Wu et al. [5]. PO is the terminal enzyme in the prophenoloxidase (proPO) system, and it acts as both a recognition component and an effector component of the arthropod defence system. PO activity was measured using the spectrophotometric method described by Hernández-López et al. [35]. The shrimp PO activity was measured as the optical density of dopachrome formation in 50 μl of haemolymph. Moreover, RB activity was measured by the reduction of nitro blue tetrazolium (NBT) to formazan as a reflection of superoxide anion (O_2^-) production, as described by Song and Hsieh [36]. SOD activity was measured with a Ransod kit (Randox, Crumlin, UK) using the method described by Wu et al. [5]. Phagocytic activity was analysed following the method described by Chang et al. [37] and was defined as the phagocytic rate (PR) of *V. alginolyticus*: PR = [(phagocytic haemocytes)/(total haemocytes)] 100%. Lysozyme activity was determined following the procedures of Yeh and Chen [38] and was defined as the amount of sample causing a decrease in absorbance of 0.01 per min.

2.5. Quantification of shrimp immune gene expression

Haemolymph samples were collected at 0, 12, 24, 36, 48, 60, and 72 h. Immune gene expression was quantified by quantitative real-time reverse-transcription-polymerase chain reaction (qRT-PCR). RNA was isolated from the haemocyte pellets using an Ultraspec™-II RNA Isolation System (Biotecx Laboratories, TX, USA) following the manufacturer's instructions. Haemocyte pellets were obtained and reverse transcription (RT) was accomplished using the method described by Hsieh et al. [39]. To quantify shrimp immune gene expression, specific primer pairs were designed as follows: proPO forward primer, 5′-GCCTTGGCAACGCTTTCA-3′ and reverse primer, 5′-CGCGCATCAG TTCAGTTTGT-3’ [40]; LGBP forward primer, 5′-CATGTCCAACTTCGC TTTCAGA-3′ and reverse primer, 5′-ATCACCGCGTGGCATCTT-3’ [40]; PE forward primer, 5′-TGGACCTCGCGGGAGAT-3′ and reverse primer, 5′-GACCGATAGCCACCATGCTT-3’ [41]; and β-actin forward primer, 5′-GAGCAACACGGAGTTCGTTGT-3′ and reverse primer, 5′-CATCACC AACTGGGACGACATGGA-3’ (GenBank accession no. AF300705).

A SYBR Green I real-time RT-PCR assay was performed using an ABI PRISM™ 7900 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Data analysis for RT-PCR was performed with SDS software ver. 2.0 (Perkin-Elmer Applied Biosystems).

2.6. Examination of haemocyte morphology

A JEOL JSM-6380 SEM 7000F scanning electron microscope (SEM) (JOEL/Tokyo/Japan) was used to examine morphological changes in haemocyte shape and protrusive surface structures [42]. Haemolymph was drawn from each group of shrimp using a sterile 1-ml syringe and immediately dropped onto a coverslip. Proteins in the haemolymph were washed out with anticoagulants and fixed in a 2.5% glutaraldehyde solution (Sigma, 25%) for 1 h. Dehydration was achieved gradually (2 × 15 min in 50% ethanol (v/v), 2 × 15 min in 70% ethanol (v/v), 2 × 15 min in 80% ethanol (v/v), 2 × 15 min in 90% ethanol (v/v), and 2 × 20 min, 60 min, and overnight in absolute ethanol). Finally, the cells were prepared on gold substrates and platinum-coated conductive substrates (JEOL, JFC-1600). SEM images were obtained at an accelerating voltage of 10 kV.

2.7. Statistical analysis

A multiple comparisons (Tukey's) test was used to examine significant differences among treatments using SAS computer software (SAS Institute, Cary, NC, USA). Percentage data (from the susceptibility study) were normalized using an arcsine transformation prior to analysis. Statistically significant differences required that $p < 0.05$.

3. Results

3.1. Effect of α-phellandrene on the susceptibility of L. vannamei to V. alginolyticus

All unchallenged control shrimp survived. However, death began to occur after 12 h in challenged shrimp. After 24–72 h of challenge, the percent survival of shrimp that received α-phellandrene at 4, 8, and 12 μg g^{-1} was significantly higher than that of challenged control shrimp ($p < 0.05$) (Table 1). The percent survival at 72 h was 66.7%, 83.3%, and 83.3% for shrimp that had been injected with α-phellandrene at 4, 8, and 12 μg g^{-1}, respectively.

3.2. Effect of α-phellandrene on the immune parameters of L. vannamei exposed to V. alginolyticus

At 24–60 h, the PO activity of shrimp that received α-phellandrene at 8 and 12 μg g^{-1} was significantly higher than that of control shrimp ($p < 0.05$). However, at 12–72 h, no significant differences in PO activity were observed among shrimp that received α-phellandrene at 8 and 12 μg g^{-1} (Fig. 1A). No significant differences were observed in THCs among shrimp that received α-phellandrene at 4, 8, and 12 μg g^{-1} and control shrimp, either at the beginning of the experiment or at 72 h following treatment (Fig. 1B).

At 12–72 h, the RB activity of shrimp that received α-phellandrene at 4, 8 and 12 μg g^{-1} was significantly higher than that of control

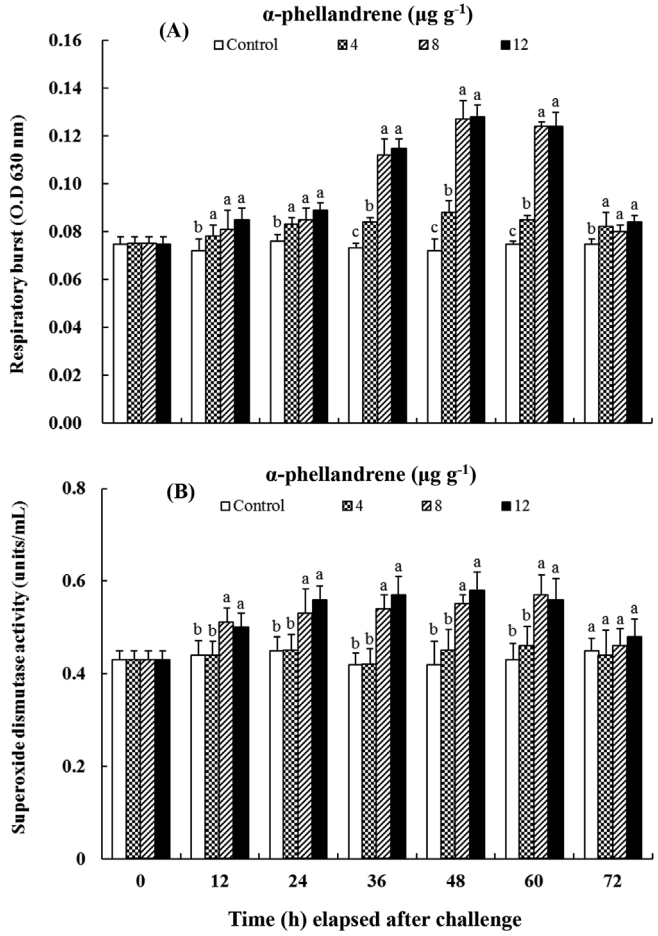

Fig. 2. Mean (± SE) respiratory burst activity (A) and superoxide dismutase (SOD) activity (B) of *Litopenaeus vannamei* that received α-phellandrene at 4, 8, and 12 μg g^{-1} and of control shrimp after challenge with *Vibrio alginolyticus* at 12, 24, 36, 48, 60, and 72 h. See Fig. 1 for statistical information.

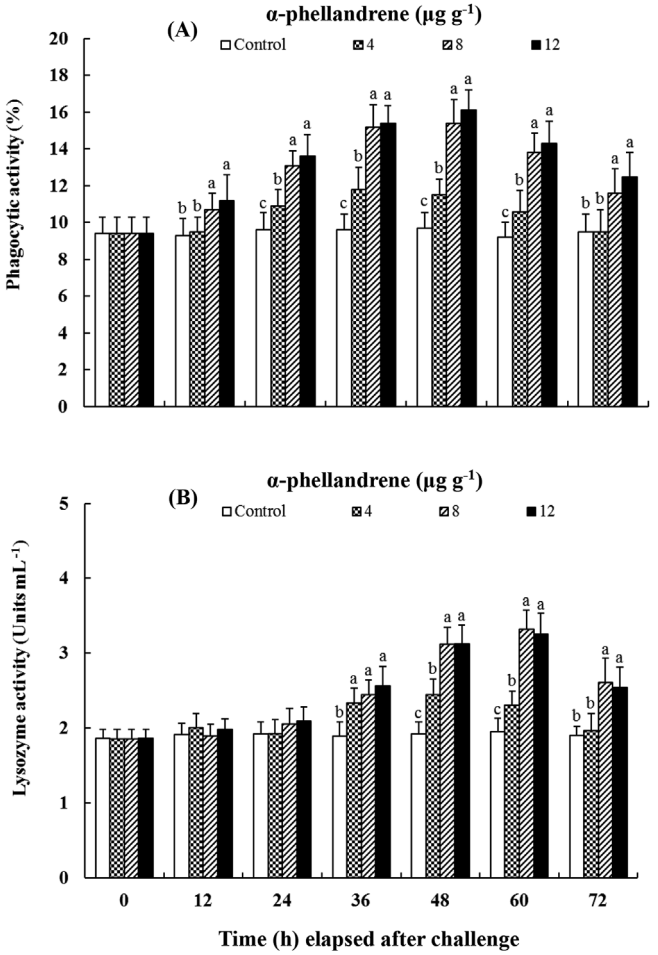

Fig. 3. (A) Phagocytic activity and (B) lysozyme activity of *Litopenaeus vannamei* that received α-phellandrene at 4, 8, and 12 μg g^{-1} and of control shrimp after challenge with *Vibrio alginolyticus* at 12, 24, 36, 48, 60, and 72 h. See Fig. 1 for statistical information.

shrimp ($p < 0.05$) (Fig. 2A). The SOD activity of shrimp that received α-phellandrene at 8 and 12 μg g^{-1} was significantly higher than that of control shrimp at 12–60 h (Fig. 2B). However, no significant differences in SOD activity were observed among shrimp that received the α-phellandrene and control shrimp at 72 h (Fig. 2B).

At 24–60 h, the phagocytic activity of shrimp that received α-phellandrene at 4, 8 and 12 μg g^{-1} was significantly higher than that of control shrimp ($p < 0.05$) (Fig. 3A). Moreover, at 36–60 h, the lysozyme activity of shrimp that received α-phellandrene at 4, 8 and 12 μg g^{-1} was significantly higher than that of control shrimp ($p < 0.05$) (Fig. 3B). However, the phagocytic activity and lysozyme activity of shrimp that received α-phellandrene at 8 and 12 μg g^{-1} were significantly higher than those of control shrimp at 72 h ($p < 0.05$) (Fig. 3).

3.3. Effect of α-phellandrene on the immune gene expression of L. vannamei exposed to V. alginolyticus

The effects of α-phellandrene on immune gene expression are presented in Fig. 4. Furthermore, the mRNA expression of shrimp immune genes that received α-phellandrene at 4, 8, and 12 μg g^{-1} was measured using qRT-PCR prior to and at 12, 24, 36, 48, 60, and 72 h following *V. alginolyticus* challenge. At 12–72 h, the proPO mRNA expression levels of shrimp that received α-phellandrene at 8 and 12 μg g^{-1} were significantly higher than those of control shrimp ($p < 0.05$) (Fig. 4A). Shrimp that received α-phellandrene at 8 and 12 μg g^{-1} had approximately 3.65- and 3.78-fold higher expression levels of proPO mRNA than those of control shrimp at 48 h. Following *V. alginolyticus* challenge, the LGBP mRNA expression levels of shrimp that received α-phellandrene at 8 and 12 μg g^{-1} were significantly higher than those of the control shrimp at 12–72 h. However, significantly higher LGBP mRNA expression levels were expressed by shrimp that received α-phellandrene at 4 μg g^{-1} than by control shrimp at 48–72 h ($p < 0.05$) (Fig. 4B). At 48–72 h, the PE mRNA expression levels of shrimp that received α-phellandrene at 4, 8, and 12 μg g^{-1} were significantly higher than those of control shrimp ($p < 0.05$) (Fig. 4C). No significant differences in the PE mRNA transcripts of shrimp were observed among the four treatments at 0–36 h. Shrimp that received α-phellandrene at 8 and 12 μg g^{-1} had approximately 3.32- and 3.50-fold higher expression levels of PE mRNA than those of control shrimp at 72 h.

3.4. Effect of α-phellandrene on the haemocyte morphology of L. vannamei exposed to V. alginolyticus

An SEM was used to study the changes in haemocyte shape and surface structure of *L. vannamei* following *V. alginolyticus* challenge. The challenged haemocytes exhibited flattening, shrinkage and pseudopodia, unlike the control haemocytes. Furthermore, haemocytes that received α-phellandrene at 8 and 12 μg g^{-1} exhibited less flattening and pseudopodia than did the challenged haemocytes (Fig. 5).

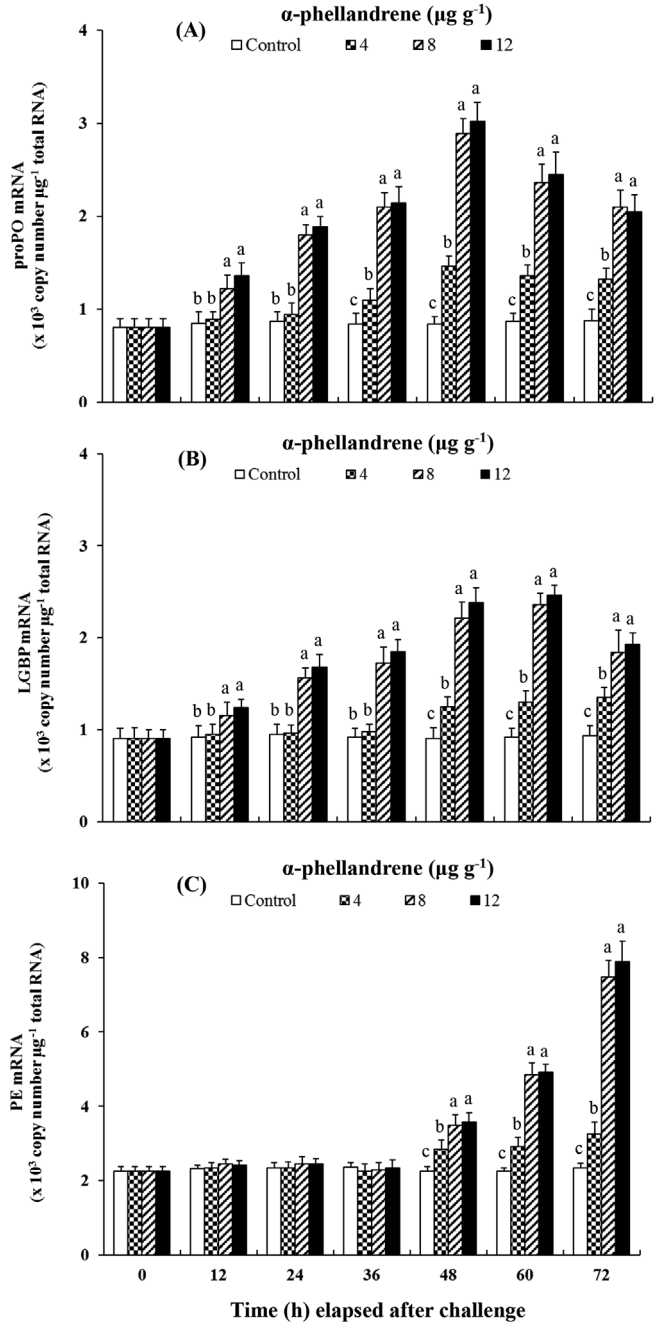

Fig. 4. Real-time RT-PCR analyses of prophenoloxidase (ProPO) expression (A), lipopolysaccharide- and b-glucan-binding protein (LGBP) expression (B), and peroxinectin (PE) expression (C) in haemocytes of white shrimp, *Litopenaeus vannamei*, that received α-phellandrene at 4, 8, and 12 μg g^{-1} and of control shrimp after challenge with *Vibrio alginolyticus* at 12, 24, 36, 48, 60, and 72 h. See Fig. 1 for statistical information.

4. Discussion

Many studies have demonstrated the successful use of essential oils as immunostimulants in aquaculture. For example, cinnamon oil (*Cinnamomum verum*) has the potential to control *Streptococcus iniae* infection in Nile tilapia [43]. Moreover, effective components such as thymol and terpinene present in the oils can stimulate fish immunity [44]. Furthermore, diets supplemented with thyme (*Zataria multiflora*) essential oil have the potential to enhance the innate immune system of carp under temperature stress [45]. *Argemone mexicana* seeds (which contain argemone oil) inhibit viral multiplication and immune system stimulation in *L. vannamei* [7].

Notably, α-phellandrene is a cyclic monoterpene that can be extracted from the essential oils of herbs. Previous studies have indicated that α-phellandrene possesses many useful functions, including 1) antibacterial function via the inhibition of the growth of *Escherichia coli*, *Salmonella choleraesuis*, *Bacillus subtilis* [21], *Arthrobacter protophormiae*, *Micrococcus luteus*, *Rhodococcus rhodochrous*, and *Staphylococcus aureus* [22]; 2) antifungal function via the inhibition of the growth of *Aspergillus flavus* [23]; 3) antioxidant activity via the reduction of aflatoxin-induced oxidative stress in male rats [24] and the chemoprevention of aflatoxin production [23]; 4) antinociceptive activity, possibly involving the glutamatergic, opioid, nitrergic, cholinergic and adrenergic systems [20]; and 5) anticancer effects via potent inhibitory activity against lung carcinoma cells [26], human breast and prostate tumour cells [27] and human liver tumour cells [13,28]. In the present study, following challenge with *V. alginolyticus*, the percent survival of white shrimp was significantly higher in all experimental groups than that of the challenge controls, with the lowest cumulative mortality observed in shrimp that received 4, 8, and 12 μg g^{-1} α-phellandrene. This is the first study to use α-phellandrene application against pathogen infection in shrimp. This study suggests that α-phellandrene at ≥ 4 μg g^{-1} reduces mortality against pathogenic challenges in *L. vannamei*. Furthermore, at 24–72 h with 8 and 12 μg g^{-1} α-phellandrene, there was no variation in the immune response. From the results, it can be confirmed that there is no increase in effect at doses higher than 8 μg g^{-1}.

In shrimp, innate immunity is considered the primary defence mechanism and includes both cellular and humoural responses to pathogens [46]. The cellular responses are mediated by haemocytes and include phagocytosis, cytotoxicity, encapsulation, nodule formation, cell adhesion and the haemolymph clotting mechanism [47]. However, humoural responses involve the prophenoloxidase-activating cascade and immune-related proteins within haemocytes, such as lysozymes, antimicrobial peptides, and lectins [48]. Various pathogen-associated molecular patterns, such as lipopolysaccharide, peptidoglycan, and β-1,3- glucans, can active the prophenoloxidase (proPO) system, leading to the conversion of inactive proPO stored in haemocytes into active PO, which then triggers the production of melanin and toxic reactive intermediates with antibacterial and bactericidal activities [49]. The present study indicated that the LGBP and proPO mRNA expression levels of shrimp that received α-phellandrene at 8 and 12 μg g^{-1} were significantly higher than those of control shrimp at 12–72 h. In addition, the PO activity of shrimp that received α-phellandrene at 8 and 12 μg g^{-1} increased within 12 h of treatment. Moreover, the PO activity of shrimp that received α-phellandrene at 8 and 12 μg g^{-1} was significantly higher than that of control shrimp at 12–60 h. These results additionally suggest that shrimp that received α-phellandrene can up-regulate LGBP and proPO mRNA by increasing PO activity to enhance their resistance against *V. alginolyticus*.

In addition, the proPO system is activated to release PE, which is a multifunctional protein possessing the biological activity of peroxidase [50]. In the present study, the PE mRNA transcript levels of shrimp that received α-phellandrene at 8 and 12 μg g^{-1} increased significantly at 48–72 h. In a previous study, the PE transcript significantly increased at 24–48 h in mud crab (*Scylla serrata*) injected with *V. alginolyticus* and peptidoglycan [51]. *L. vannamei* injected with water extract of *G. bicolor* exhibited upregulation of PE expression, which effectively activated an immune response at 24–96 h post injection [39]. Dietary administration of *Panax ginseng* and *G. bicolor* extract increased peroxinectin mRNA expression in *L. vannamei* [5,52], which suggests that α-phellandrene is likely involved in a defence response to exterior pathogen invasion and adhesion.

Phagocytosis is an important cellular defence mechanism, whereas clearance efficiency is an important humoural defence mechanism in crustaceans [53]. Previous studies have indicated that herbal formulae can enhance the phagocytic capacity in *L. vannamei* [54]. For example, Harikrishnan et al. [55] reported that *Solanum nigrum* extract improves

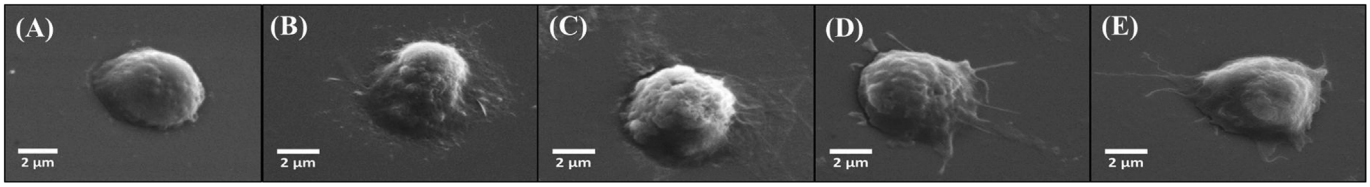

Fig. 5. Effects of different concentrations of α-phellandrene on cell surface morphology in white shrimp (*Litopenaeus vannamei*) haemocytes after challenge with *V. alginolyticus* at 72 h as determined with a scanning electronic microscope (SEM). SEM images from the haemocytes of control shrimp (A), *V. alginolyticus*-only shrimp (B), α-phellandrene 4 μg g^{-1} shrimp (C), α-phellandrene 8 μg g^{-1} shrimp (D) and α-phellandrene 12 μg g^{-1} shrimp (E) (10,000 X).

the phagocytic activity in *P. monodon* against *V. harveyi.* Moreover, lysozyme activity has been revealed as a first-line innate immune defence in crustaceans that functions in the prevention of the incursion of detrimental bacterial pathogens [6]. Many studies have demonstrated that the ingestion of immunostimulants, such as guava leaves [55], zingerone [37], *Solanum nigrum* extract [56], poly-herbal formulas [57], and *Syzygium cumini* extract [6] elevates lysozyme activity in shrimp. In the present study, the results indicated that the phagocytic activity and lysozyme activity of shrimp that received α-phellandrene at 8 and 12 μg g^{-1} were significantly higher than those of control shrimp at 72 h. This suggests that α-phellandrene induces the innate immunity of *L. vannamei* to enhance their resistance to diseases by activating phagocytosis and lysozyme activity.

RBs have been widely used to evaluate the defence ability of hosts against pathogens [58]. However, the excessive accumulation of reactive oxygen ions (ROIs) is extremely toxic to host cells, and the damaging effects of ROIs are minimized by various enzymatic antioxidants. The present study indicated that the RB activity of shrimp that received α-phellandrene at 4, 8, and 12 μg g^{-1} was significantly higher than that of control shrimp at 12–60 h. Similarly, the SOD activity of shrimp that received α-phellandrene at 8 and 12 μg g^{-1} was significantly higher than that of control shrimp at 12–60 h. This suggests that α-phellandrene potentially mediates the production of microbicidal components through the RB activity of haemocytes during pathogen infection. Moreover, α-phellandrene can enhance antioxidative mechanisms to defend against oxidative damage by ROIs in shrimp, as SOD is an antioxidative enzyme that scavenges superoxide anions (O_2^-) and hydrogen peroxide (H_2O_2) [59]. A previous study showed that the RB and SOD activity of *L. vannamei* increased when the shrimp were fed diets supplemented with *Syzygium cumini,* zingerone, *Gynura bicolor* extract, and *Rubus coreanus* ethanolic extract [6,37,60,61].

THCs can reflect shrimp immune ability [62], and there was no significant difference in THCs between shrimp that received α-phellandrene and control shrimp in the present study. However, the phagocytic activity of shrimp that received α-phellandrene at 8 and 12 μg g^{-1} was significantly higher than that of control shrimp at 72 h. These results suggest that α-phellandrene enhances the immunity and disease resistance of shrimp mainly by phagocytosis rather than by increasing the THC.

To clarify the effect of α-phellandrene on haemocytes, we studied haemocyte morphology. The present study indicated, for the first time, that the haemocytes of shrimp exhibit flattening, shrinkage, and pseudopodia following *V. alginolyticus* challenge. However, shrimp that received α-phellandrene at 8 and 12 μg g^{-1} exhibited reduced flattening and pseudopodia in their haemocytes compared to the challenged haemocytes. Therefore, α-phellandrene could protect and maintain haemocyte shape and surface structure.

In conclusion, the present study indicated that α-phellandrene can enhance immunity (as indicated by PO, RB, SOD, phagocytic activity and lysozyme activity) and upregulate LGBP, ProPO, and PE gene expression. In addition, *L. vannamei* that received α-phellandrene at ≥ 4 μg g^{-1} exhibited increased resistance against *V. alginolyticus* following 24–72 h of challenge. These results suggest that α-phellandrene can effectively enhance shrimp immunity and disease resistance against the pathogen *V. alginolyticus* through cellular and humoural immune responses.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2018.11.013.

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