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Kiam wood, *Cotylelobium lanceotatum*, extract as a natural antimicrobial agent: protecting Pacific white shrimp, *Penaeus vannamei*, against vibriosis

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Vibriosis, caused by *Vibrio parahaemolyticus*, is a major bacterial disease in shrimp aquaculture, often managed with antibiotics that contribute to antimicrobial resistance and environmental concerns. This study investigated the antimicrobial properties of Kiam wood (*Cotylelobium lanceotatum*) extract and its potential as a dietary supplement to enhance the disease resistance of Pacific white shrimp, *Penaeus vannamei*. Kiam wood extracts were prepared using ethanol-water mixture at different ratios, and their antimicrobial activity was evaluated against *V. parahaemolyticus*. The water extract (KWE) exhibited the strongest anti-*Vibrio* activity as indicated by the widest clearance zone (15.65 mm), with a minimum inhibitory concentration (MIC) of 256 µg/mL and a minimum bactericidal concentration (MBC) of 512 µg/mL. Further assays demonstrated that KWE effectively inhibited biofilm formation and restricted bacterial motility at 512 µg/mL (MBC). Scanning electron microscopic images revealed significant cell-wall damages in treated bacteria as shown by membrane disruption and pore formations. Liquid chromatography-mass spectrometry (LC-MS) analysis identified Amuresins D ($C_{42}H_{30}O_9$), Pauciflorol A ($C_{24}H_{32}O_9$), Vaticasides A ($C_{48}H_{42}O_{14}$), Vaticanols B ($C_{56}H_{42}O_{12}$), and Cotylelophenol B ($C_{24}H_{30}O_{10}$) as key bioactive compounds. For the *in vivo* trials, *P. vannamei* was firstly infected by feeding them a *V. parahaemolyticus*-impregnated diet (1.5×10^4 CFU/g) for two days, followed by a 15-day feeding period with KWE-supplemented diets at 2MBC (1 mg/g). Shrimp-fed KWE diets showed a significant reduction in intestinal *Vibrio* loads, enhanced immune responses (total hemocyte count, semi-granulocyte, and granulocyte levels), and improved survival rates. Notably, the KWE diet achieved the highest survival rate (85%), compared to 52% in the control group. These findings highlight KWE as a promising natural antimicrobial agent with immunostimulatory properties, offering a sustainable alternative to synthetic antibiotics for managing vibriosis in shrimp aquaculture. Further studies should explore its mode of action and long-term effects on shrimp health and aquaculture environments.

Keywords KWE, Antimicrobial, Vibriosis, Medicinal plant, Immune system

The aquaculture industry, particularly shrimp farming, is a crucial contributor to global food security and economic development. Among the various species cultivated, the Pacific white shrimp (*Penaeus vannamei*) is one of the most important species due to its high market demand and adaptability to diverse farming conditions[1].

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However, the shrimp farming industry faces significant challenges, particularly from bacterial infections, which can lead to severe economic losses[2]. One of the most notorious pathogens affecting shrimp farming is *Vibrio parahaemolyticus*, a bacterium responsible for acute hepatopancreatic necrosis disease (AHPND), which has caused devastating impacts on shrimp production worldwide[3,4]. The bacterial pathogen also triggers the emergence of white feces disease (WFD). These diseases are nowadays considered the most significant threats to shrimp aquaculture industries[5].

The conventional approach to manage bacterial infections in aquaculture has largely relied on the use of synthetic antibiotics[6]. Despite being effective, the overuse and misuse of these antibiotics have raised concerns regarding the development of antibiotic-resistant bacterial strains, environmental contamination, and residue accumulation in cultured animals, posing risks to both human health and the environment[7,8]. These challenges have driven the search for alternative, eco-friendly solutions that can mitigate bacterial infections without the associated drawbacks of synthetic antibiotics. One promising approach is the use of medicinal plant extracts with anti-bacterial properties[3,9].

Medicinal plants have long been used across various cultures for their therapeutic properties, and recent research has highlighted their potential as natural alternatives to antibiotics in aquaculture[10]. These plants contain bioactive compounds with antimicrobial activities, making them promising candidates for protecting shrimp from bacterial infections. Due to their diverse array of bioactive compounds, medicinal plants are increasingly recognized as sustainable and safer alternatives to conventional antibiotics for disease management in aquaculture[11]. Several studies have explored the antibacterial potential of medicinal plant extracts against common aquaculture pathogens[9,12,13]. For example, extracts from *Anadendrum microstachyum* and *Selaginella plana* have demonstrated inhibitory effects against pathogens such as *Edwardsiella ictaluri* and *Streptococcus agalactiae*. Furthermore, aromatic plants such as *Cumin cyminum* and *Pimenta dioica* extracts have shown direct antimicrobial activity against aquatic pathogens and have beneficial impacts on the intestinal microbiome, nutrient utilization, metabolism, oxidative stress, and immune responses in both fish and shrimp[9,13]. Despite these promising findings, the effectiveness of plant extracts against specific aquaculture pathogens, such as *V. parahaemolyticus*, remains relatively underexplored. For instance, Kiam wood (*Cotylelobium lanceotatum*), traditionally used as a natural preservative by submerging it in sugar palm sap to inhibit spoilage microbes[14], is known for its strong antimicrobial and antioxidant properties[15]. However, its potential antimicrobial benefits in aquaculture, particularly against *V. parahaemolyticus*, have to be thoroughly investigated.

This study aimed to fill existing gaps by evaluating the antimicrobial properties of Kiam wood extract against *V. parahaemolyticus* and its potential for treating Vibrio infections in Pacific white shrimp. Using a combination of microbiological assays, scanning electron microscopy (SEM), liquid chromatography-mass spectrometry (LC-MS), and *in vivo* trials, this research seeked to clarify the mechanisms behind the antimicrobial action of the plant extract. Additionally, the active compounds in the extract were identified and assesses for its impact on the immune system of Pacific white shrimp. The findings were expected to provide a potential alternative for preventing and treating vibriosis, particularly infections caused by *V. parahaemolyticus* in shrimp farming.

Results

Antimicrobial activities.

The antimicrobial activity of Kiam wood extracts using ethanol-water mixtures at different ratios toward *V. parahaemolyticus* was expressed by inhibition zone, minimum inhibitory concentration (MIC), and minimum bactericidal concentration (MBC). Among the tested extracts, the water-based Kiam wood extract (KWE) exhibited the strongest anti-*Vibrio* effect, as indicated by the widest inhibition zone (15.65 ± 0.49 mm), Table 1.

However, the difference in the inhibition zone was not reflected in the MIC and MBC values, as no significant differences were observed ($p > 0.05$). The MIC of KWE prepared using the ethanol-water mixtures at various ratios against *V. parahaemolyticus* was recorded at 256 µg/mL, while the MBC was 512 µg/mL (2 MIC). Since

Extracts	*V. parahaemolyticus*		
	CZ (mm)	MIC (µg/mL)	MBC (µg/mL)
KWE	15.65 ± 0.49^{a}	256	512
KWE-60%Eth	$13.15 \pm 0.49^{b, c}$	256	512
KWE-70%Eth	$12.50 \pm 0.85^{b, c}$	256	512
KWE-80%Eth	12.20 ± 0.00^{c}	256	512
KWE-99%Eth	12.60 ± 0.57^{c}	256	512
PS	$14.30 \pm 0.14^{a, b}$	16	128

Table 1. Antimicrobial activities including clearance zones, minimum inhibitory concentration, and minimum bactericidal concentration of Kiam wood extracted with ethanol-water mixtures at different ratios against *V. parahaemolyticus*. KWE refers to Kiam wood extract prepared with water; while KWE-60%Eth, KWE – 70%Eth, KWE – 80%Eth, and KWE-99%Eth indicate extract obtained using ethanol at concentrations of 60%, 70%, 80%, and 99% respectively. Potassium sorbate (PS) was used as the positive control. The clearance zone (CZ) represents the diameter of the inhibition area (mm). Values are presented as mean ± standard deviations of two replicates. MIC refers to the minimum inhibitory concentration, and MBC denotes the minimum bactericidal concentration for the extracts tested.

all KWEs had an MBC/MIC ratio of less than 4, they were classified to show bactericidal effect against *V. parahaemolyticus*.

Inhibition of biofilm formation

The results demonstrated that KWE effectively inhibited biofilm formation in the tested bacterial pathogen (Fig. 1). Biofilm inhibition increased significantly with augmenting concentrations of KWE ($p < 0.05$). At 2 MIC (MBC), KWE reduced biofilm formation by 67%, followed by 64% at MIC, 43% at 1/2MIC, and 19% at 1/8MIC compared to control. These findings highlight the potential of KWE as a promising anti-biofilm agent for controlling vibriosis in Pacific white shrimp farmings.

Effect on motility

KWE significantly reduced the motility of *V. parahaemolyticus*, as evidenced by a smaller diameter of the ring-shaped colony structure in KWE-treated samples compared to the control (Fig. 2). When KWE was applied at concentrations of 2MIC, MIC, 1/2MIC, 1/4MIC, and 1/16MIC, the colony diameters were significantly reduced to 3.77, 9.40, 12.20, 12.53, 12.80, and 12.87 mm, respectively, compared to controls (23 mm) (Fig. 2a). Additionally, KWE inhibited the swimming activity of *V. parahaemolyticus*, as shown by the significantly shorter crawling distance. Both swimming and swarming motilities were inhibited by KWE in a concentration-dependent manner, in which the strongest effect was observed at 2MIC, followed by MIC, 1/2MIC, 1/4MIC, and 1/16MIC (Fig. 2b). At higher concentrations, KWE particularly impaired bacterial movement, thus potentially limiting the spread of *V. parahemolyticus*.

Scanning electron microscopic images

The impact of KWE treatment on bacterial morphology was examined using a scanning electron microscope at a 50,000x magnification. Exposure to KWE at 2MBC caused significant structural damage to the cell walls of *V. parahaemolyticus* (Fig. 3b). Treated bacterial cells displayed noticeable distortion, a roughened surface with visible pores, and severe cell wall damage (Fig. 3b and c), in contrast to the smooth, intact structure observed in untreated cells (Fig. 3a). These morphological alterations likely led to nutrient leakage, disrupting bacterial metabolism and ultimately resulting in cell lysis.

Phenolic compounds in water-based Kiam wood extract

The analysis revealed the presence of various phenolic compounds in the water-based Kiam wood extract. Table 2 presents the phenolic compounds detected in the KWE. Notably, the negative ion mode of the LC-MS exhibited greater sensitivity than the positive ion mode, likely due to its enhanced ability to generate negative ions, which are particularly effective in profiling phenolic and polyphenolic components. The phenolic compounds were characterized based on their potential molecular formulas, m/z values, and relative abundances.

The primary compound identified was pauciflorol A ($C_{42}H_{32}O_9$), followed by vaticasides A ($C_{48}H_{42}O_{14}$), amurensins D ($C_{42}H_{30}O_9$), vaticanols B ($C_{56}H_{42}O_{12}$), cotylelophenols B ($C_{42}H_{30}O_{10}$), and (-)-trans-epsilon-viniferin ($C_{28}H_{22}O_6$). In the positive ion mode, amurensins D was the most abundant compound, followed

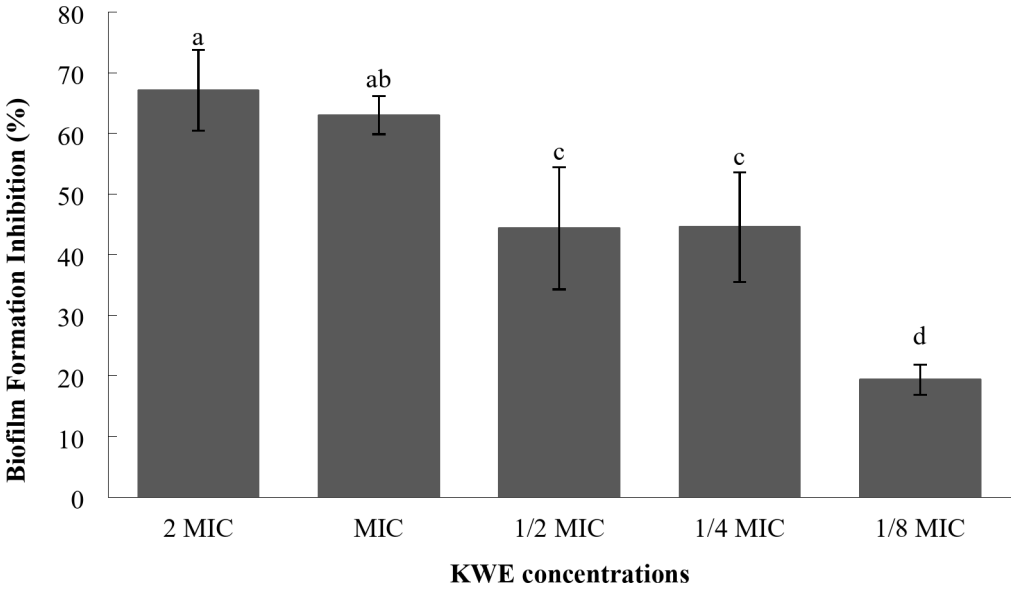

Fig. 1. Inhibitory effects of Kiam wood extract (KWE) at different concentrations on the biofilm formation of *V. parahaemolyticus* ATCC17802 after 48 h. Bars represent standard deviation. Different lowercase letters on the bars denote significant differences between treatments ($p < 0.05$). Data are presented as means ± standard deviation ($n = 3$).

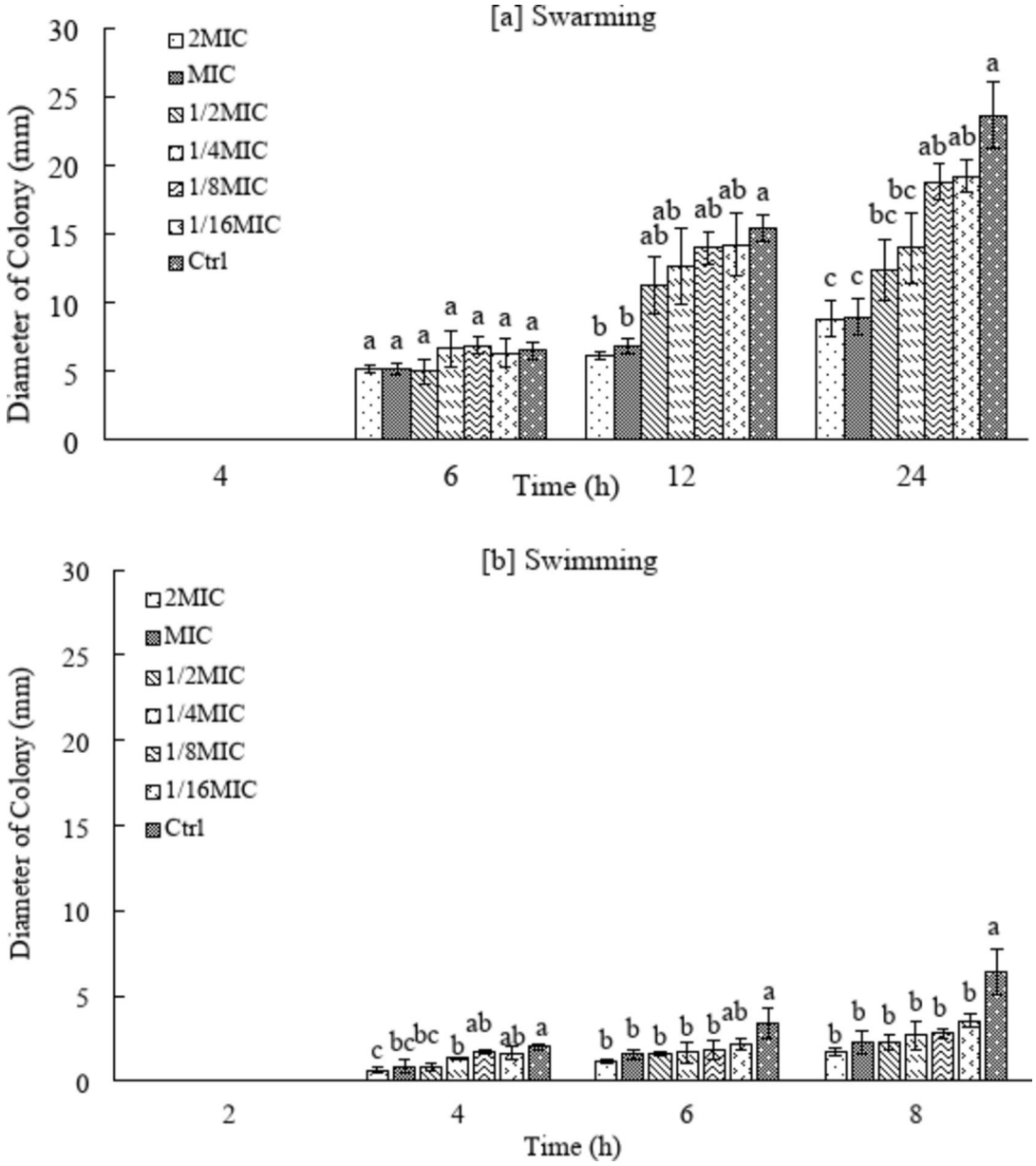

Fig. 2. Effects of different concentrations of KWE on the swarming and swimming motility of *V. parahaemolyticus* ATCC17802. Bars represent standard deviations. Different letters on the bars indicate statistically significant differences ($p < 0.05$) within the sme time point. Data are represented as means ± standard deviations ($n = 3$).

by (-)-trans-epsilon-viniferin and cotylelophenol B. Table 2 provides detailed information on the phenolic compounds identified in the water-based Kiam wood extract.

Survival rate of *V. parahemolyticus*-infected Pacific white shrimps

Water-based Kiam wood extract positively influenced the survival rate of Pacific white shrimp after being infected by *V. parahaemolyticus*. As shown in Fig. 4, shrimps fed a diet with KWE at 2MBC (1 mg/g diet) exhibited a higher survival rate (92%) throughout the experimental period compared to the positive control (89%) and the negative control (43%). At the beginning of the experiment (Day 0), no mortality was observed across all treatment groups (negative control [C-], positive control [C+], and KWE treatment). However, by day 6, the survival rate of the *V. parahaemolyticus*-infected group dropped to 90%, showing a significant reduction ($p < 0.05$). The survival rate in this group continued to decrease sharply, reaching 58% by day 9, 51% by day 12, and 43% by day 15. In contrast, KWE-treated and negative control groups maintained a 100% survival rate until day 9. By day 12, the survival rate in the KWE treatment group slightly decreased to 97%, and further to 92% by day 15. Similarly, survival in the negative control group, dropped to 97% on day 12 and 89% on day 15. Statistical analysis revealed significant differences ($p < 0.05$) between the positive control (C+) and both negative control

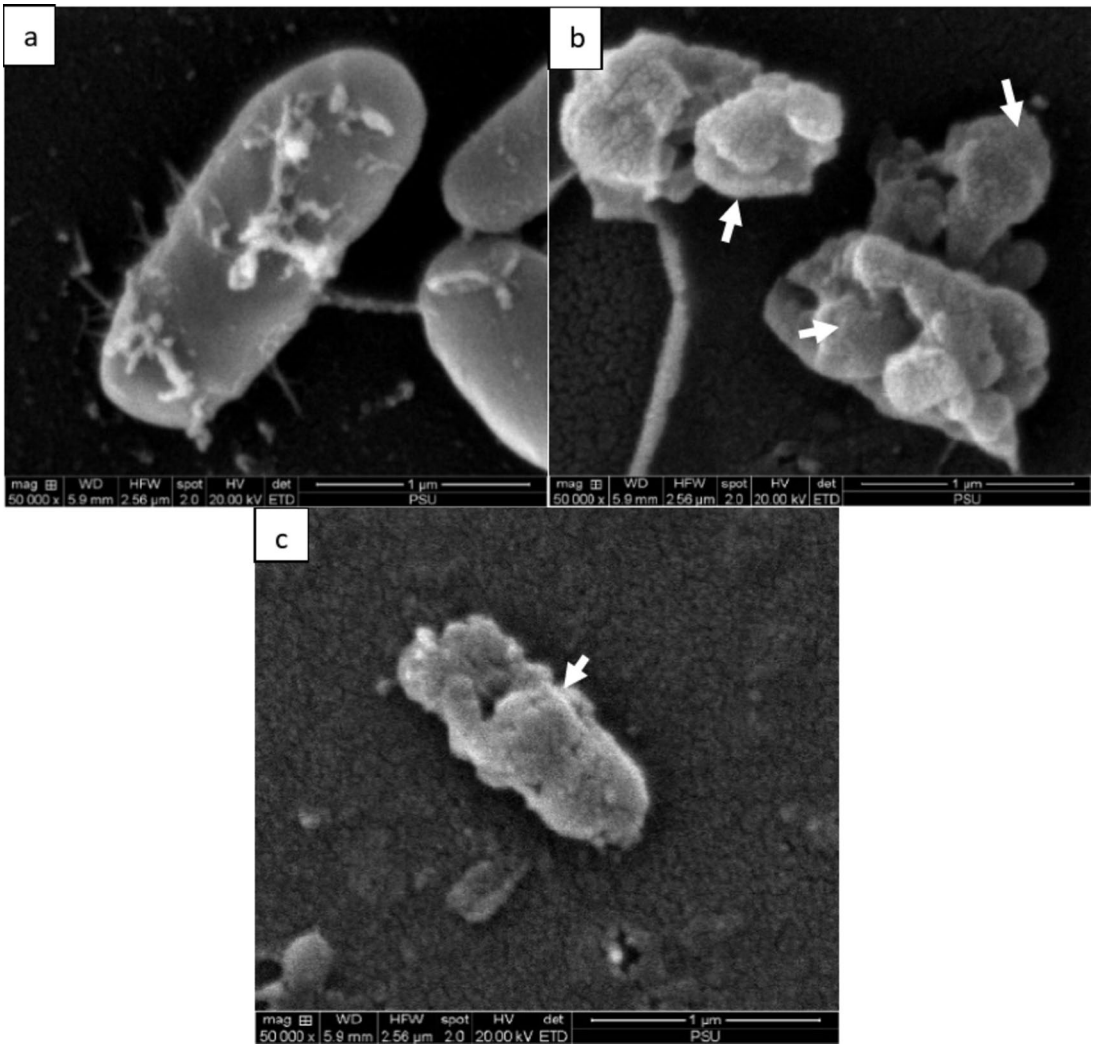

Fig. 3. Scanning electron microscopy images of *Vibrio parahaemolyticus* ATCC17802 at 50,000x magnification. (**a**) untreated bacterial cells serving as the negative control, (**b**) bacterial cells treated with 2MBC of water-based Kiam wood extract, (**c**) bacterial cell treated with 2x MBC of potassium sorbate as the positive control. Arrows indicate damaged bacterial cells resulting from KWE or potassium sorbate treatment.

(C-) and KWE-treated groups ($p < 0.05$). These results suggest that the water-based Kiam wood extract provided an effective protection against *V. parahaemolyticus* infection, thereby improving the survival rate of Pacific white shrimp.

Total *Vibrio* counts

At the beginning of the study (day 0), total *Vibrio* counts in the intestines of all shrimp groups were below 100 CFU/g. Following pathogen challenge through feeding with *V. parahaemolyticus*-impregnated diets for two days, *Vibrio* counts in both positive control (C+) and Kiam wood extract (KWE) treatment groups significantly increased, exceeding 2.0×10^4 CFU/g intestine. In contrast, the total *Vibrio* count in the negative control (C-) group remained consistently below 100 CFU/g throughout the study.

After the introduction of the KWE-supplemented diet, total vibrio counts in the KWE groups declined significantly throughout the study, with the count dropping to 5.0×10^3 CFU/g by day 3 and further declining to 2.5×10^3 CFU/g by days 9 and 12. A slight increase to 3.4×10^3 CFU/g was observed by day 15. In contrast, total *vVibrio* counts in the positive control (C+) group showed a substantial surge from 86 CFU/g on day 0 (before the pathogen challenge) to 2.06×10^4 CFU/g on day 1 (after the pathogen challenge). *Vibrio* populations remained elevated throughout the study, peaking at 2.14×10^4 CFU/g on day 6, before gradually declining to 1.24×10^4 CFU/g by day 15. Meanwhile, in the C- group, total *Vibrio* counts fluctuated modestly between 40 CFU/g and 98 CFU/g, indicating minimal *Vibrio* proliferation in the absence of pathogen exposure.

These findings suggest that KWE treatment effectively controlled the *Vibrio* population after the initial spike, highlighting its potential in managing *Vibrio* infections in shrimp. The C- group served as a baseline, showing low *Vibrio* levels without significant exposure, while the C + group illustrated the rapid proliferation in the absence of intervention (Fig. 5).

KWE					
Retention time (min)	**Identified compounds**	**Formula**	**Mass (g/mol)**	**m/z**	**Abundance (×10^5)**
Positive mode					
8.94	(-)-trans-epsilon-viniferin	$C_{28}H_{22}O_6$	454.14	454.14	0.89
16.71	Amurensins D	$C_{42}H_{30}O_9$	678.19	678.19	3.59
16.89	Cotylelophenol F	$C_{35}H_{26}O_9$	590.16	590.16	0.03
17.89	Vaticasides A	$C_{48}H_{42}O_{14}$	842.26	842.26	0.11
Negative mode					
8.88	(-)-trans-epsilon-viniferin	$C_{28}H_{22}O_6$	454.14	454.14	0.17
13.98	Vaticasides A	$C_{48}H_{42}O_{14}$	842.26	842.26	21.82
15.87	Lyoniresinol	$C_{22}H_{28}O_8$	420.18	420.18	0.07
16.67	Pauciflorol A	$C_{42}H_{32}O_9$	680.20	680.20	53.70
16.82	Cotylelophenol F	$C_{35}H_{26}O_9$	590.15	590.16	0.11
16.90	Cotylelophenols B	$C_{42}H_{30}O_{10}$	694.18	694.18	2.95
17.24	Vaticasides B	$C_{62}H_{52}O_{17}$	1068.32	1068.32	0.26
17.68	Amurensins D	$C_{42}H_{30}O_9$	678.19	678.19	0.92
17.85	Vaticanols B	$C_{56}H_{42}O_{12}$	906.27	906.27	3.25

Table 2. LC-MS profile of compounds in water-based Kiam (*Cotylelobium lanceotatum*) wood extract (KWE).

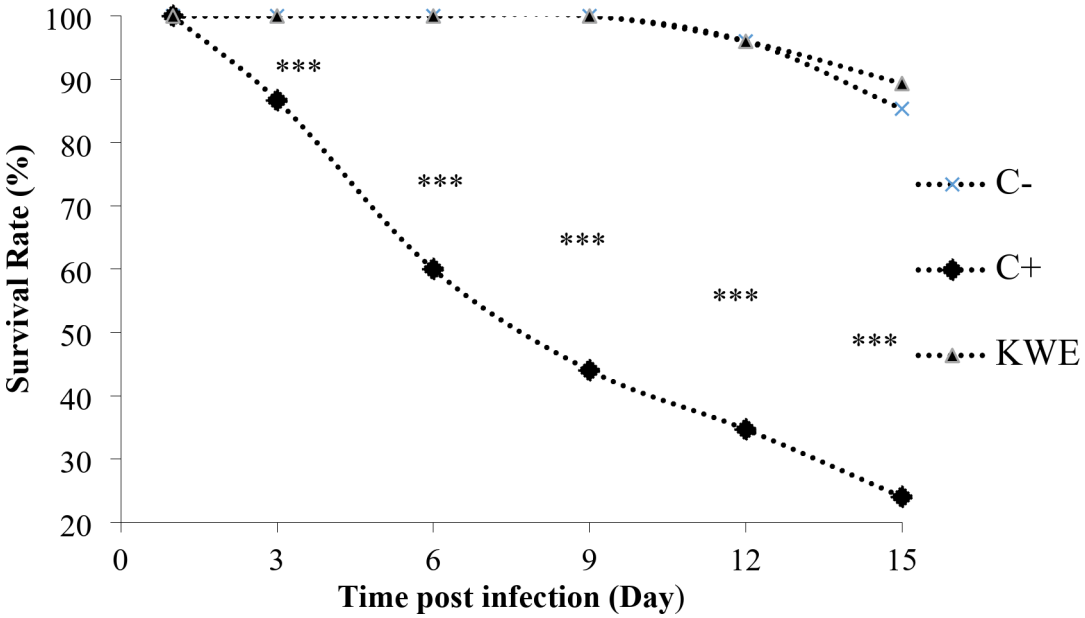

Fig. 4. The survival rate of the Pacific white shrimps infected with *V. parahemolyticus* followed by feeding with a diet supplemented with Kiam wood extract at 1 mg/g feed. C-: Negative control (NaCl 0.85%), where shrimp were fed a normal diet without any pathogen challenge. The study included three groups: a negative control (C-) where shrimp were fed a normal diet without pathogen challenge, a positive control (C+) where shrimp were challenged with a bacterial pathogen but received no KWE supplementation, and a treatment group (KWE) where shrimp were challenged with the pathogen and fed a diet mixed with KWE. Values represent the mean survival rate. Bars represent the standard deviation of four replicates. Asterisk (*) denotes a significant difference at $p < 0.001$.

Total hemocyte counts (THC)

Figure 6 illustrates the hemocyte cell counts of Pacific white shrimp infected with *V. parahaemolyticus* over 15 days. The administration of KWE resulted in a significant increase in hemocyte cells in the infected shrimp, compared to the control groups ($p < 0.05$). At the initial stage of the experiment (day 0), hemocyte counts in KWE-treated shrimp ranged from 8.56 to 11.20×10^5 cells/mL. Following the challenge test, these counts increased progressively, reaching 15.50 to 18.50×10^5 cells/mL on day 3, 18.00 to 20.70×10^5 cells/mL on day 6, 24.60 to 26.20×10^5 cells/mL on day 9, 28.70 to 32.40×10^5 cells/mL on day 12. By day 15, hemocyte counts further increased from 39.54 to 45.90×10^5 cells/mL, the highest count among all experimental groups. A similar increasing trend was observed in the positive control group, where hemocyte counts rose from 9.32×10^5 cells/mL

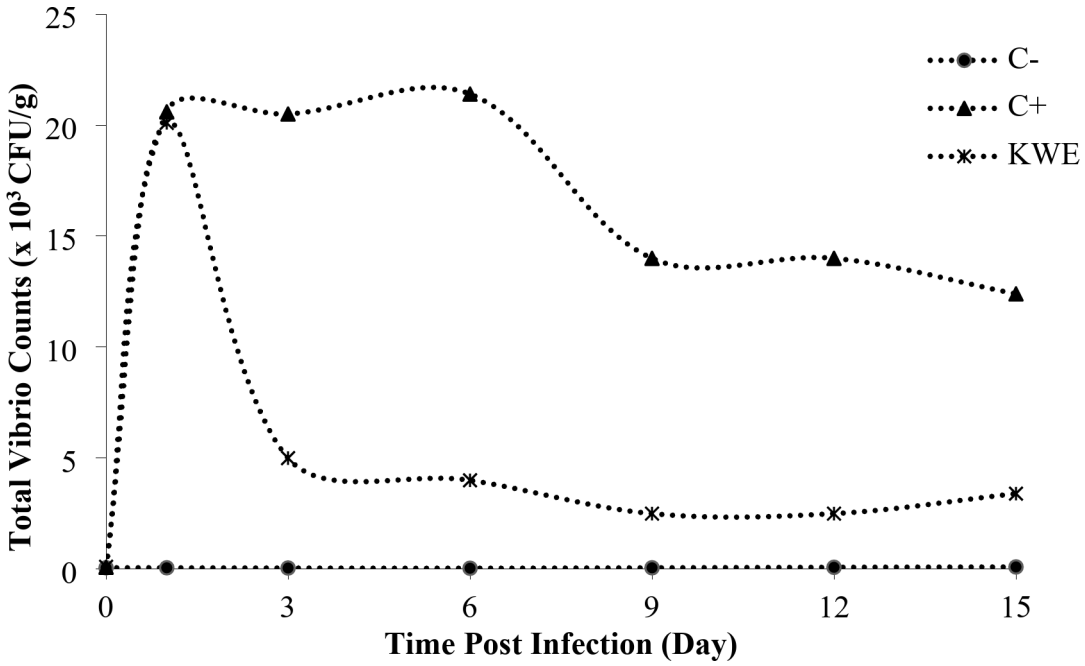

Fig. 5. Total *Vibrio* count in shrimp intestines measured at different sampling points (days 0, 1, 3, 6, 9, 12, and day 15 post-infection). C- (negative control): shrimps fed a normal diet without any pathogen exposure (NaCl 0.85% NaCl treatment). C+ (Positive control): shrimps challenged with *V. parahaemolyticus* but received no KWE supplementation. KWE treatment: shrimps challenged with *V. parahaemolyticus* and fed a diet supplemented with KWE.

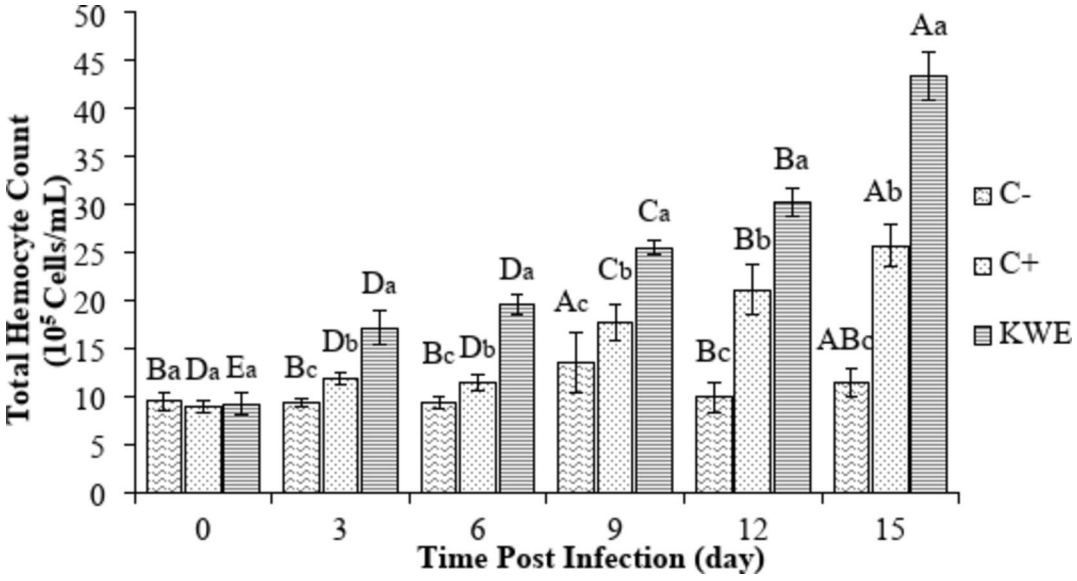

Fig. 6. The total hemocyte counts of Pacific white shrimp over 15 days, with measurements taken on days 0, 6, 9, 12, and day 15. The data presented are the average values, and the error bars represent the standard deviations of four replicates. Statistical analysis indicated significant differences ($p < 0.05$) between treatments, with lowercase letters denoting comparisons within the same sampling period and uppercase letters indicating comparisons of the same treatments across different sampling periods. The study included three groups: a negative control (C-) where shrimp were fed a normal diet without pathogen challenge, a positive control (C+) where shrimp were challenged with a bacterial pathogen but received no supplementation, and a treatment group (KWE) where shrimp were challenged with the pathogen and fed a diet mixed with KWE.

on day 0 to 12.02×10^5 cells/mL on day 3, 11.50×10^5 cells/mL on day 6, 17.82×10^5 cells/mL on day 9, 21.16×10^5 cells/mL on day 12, and 25.78×10^5 cells/mL on day 15. In contrast, the negative control group exhibited a more variable pattern, with hemocyte counts increasing from 8.90×10^5 cells/mL on day 0 to 10.20×10^5 cells/mL on day 3 and 10.00×10^5 cells/mL on day 6. By day 9, counts rose to 13.62×10^5 cells/mL but subsequently declined to 10.50×10^5 cells/mL on day 12 before reaching 11.46×10^5 cells/mL on day 15. Overall, KWE administration resulted in the most substantial increase in hemocyte counts, suggesting its potential to enhance the immune response of Pacific white shrimp against *V. parahaemolyticus* infection.

Semi-granulocyte cells

Figure 7 illustrates the semi-granulocyte cell counts in Pacific white shrimp treated with the KWE over 15 days. The highest differential hemocyte count (DHC) for semi-granulocyte cells was observed in the negative control group (C−) on day 9, reaching 62.81%, while the lowest value was recorded in the positive control group (C+) on day 12, at 22.72%. At the beginning of the experiment (day 0), no significant differences ($p > 0.05$) were observed in DHC values among the treatment groups, with C − at 59.19%, C + at 59.31%, and KWE-treated shrimp at 57.35%. However, by day 6, significant differences ($p < 0.05$) emerged, with C − maintaining a higher DHC value (58.20%) compared to KWE-treated shrimp (46.96%) and C+ (30.35%). On day 9, all groups exhibited significant differences, with C − at 62.81%, KWE at 48.25%, and C + at 23.06%. By day 12, the highest semi-granulocyte count was again observed in C− (55.96%), followed by KWE-treated shrimp (47.58%), while C + had the lowest value (22.72%). On day 15, significant differences persisted, though the gap between C− (58.81%) and KWE-treated shrimp (52.14%) became lower compared to C+ (31.06%). Overall, while semi-granulocyte counts fluctuated in the KWE-treated group, they consistently remained higher than those observed in the positive control group, suggesting a potential immunomodulatory effect of KWE.

Granulocyte cells

Figure 8 presents the granulocyte cell counts in Pacific white shrimp treated with Kiam wood extract (KWE) over 15 days. The highest granulocyte count was recorded in the positive control group (C+) on day 9, reaching 67.78 log cells/mL, while the lowest count was observed in the negative control group (C−) on the same day, at 30.17 log cells/mL. At the start of the experiment (day 0), no significant differences ($p > 0.05$) were observed in granulocyte counts among the treatment groups, with C − at 33.95 log cells/mL, C + at 33.39 log cells/mL, and the KWE-treated group at 35.37 log cells/mL. By day 6, granulocyte counts increased significantly in C+ (60.87 log cells/mL), which was markedly higher than both C− (34.05 log cells/mL) and the KWE-treated group (47.00 log cells/mL) ($p < 0.05$). On day 9, significant differences were observed across all groups, with C − at 30.17 log cells/mL, C + at 67.33 log cells/mL, and KWE-treated shrimp at 45.27 log cells/mL. This trend continued on day 12, where granulocyte counts remained significantly different among treatments: C− (36.98 log cells/mL), C+ (67.78 log cells/mL), and KWE (45.35 log cells/mL). By day 15, similar patterns were observed, with C − at

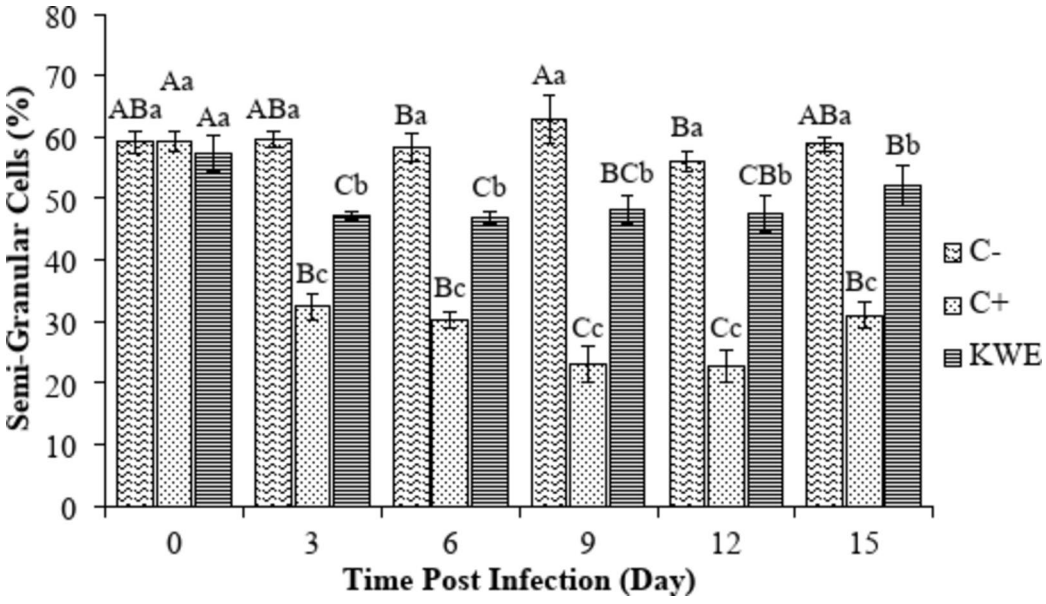

Fig. 7. Semi-granulocyte cells of Pacific white shrimp quantified on days 0, 6, 9, 12, and day 15. The data presented are the average values, and the error bars represent the standard deviations of four replicates. Statistical analysis indicated significant differences ($p < 0.05$) between treatments, with lowercase letters denoting comparisons within the same sampling period and uppercase letters indicating comparisons of the same treatments across different sampling periods. The study included three groups: a negative control (C-) where shrimp were fed a normal diet without pathogen challenge, a positive control (C+) where shrimp were challenged with a bacterial pathogen but received no supplementation, and a treatment group (KWE) where shrimp were challenged with the pathogen and fed a diet mixed with KWE.

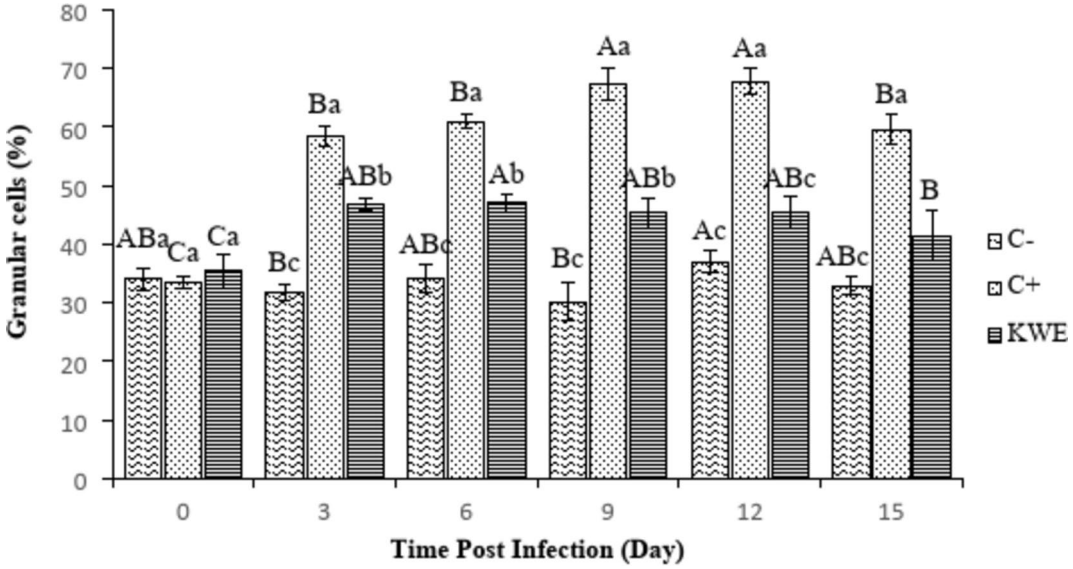

Fig. 8. Granulocyte cells of Pacific white shrimp quantified at multiple time points during the study period (day 0, 6, 9, 12, and day 15). The data presented are the average values, and the error bars represent the standard deviations of four replicates. Statistical analysis indicated significant differences ($p < 0.05$) between treatments, with lowercase letters denoting comparisons within the same sampling period and uppercase letters indicating comparisons of the same treatments across different sampling periods. The study included three groups: a negative control (C-) where shrimp were fed a normal diet without pathogen challenge, a positive control (C+) where shrimp were challenged with a bacterial pathogen but received no supplementation, and a treatment group (KWE) where shrimp were challenged with the pathogen and fed a diet mixed with KWE.

32.80 log cells/mL, C + at 59.54 log cells/mL, and the KWE-treated group at 41.35 log cells/mL. Overall, while granulocyte counts in the KWE-treated shrimp were consistently lower than those in the positive control, they remained significantly higher than in the negative control, suggesting that KWE might enhance the granulocyte response in Pacific white shrimp.

Hyaline cells

Figure 9 illustrates the hyaline cell counts in Pacific white shrimp treated with the KWE over 15 days. By the end of the observation period (day 15), the highest hyaline cell count was recorded in the positive control group (C+), reaching 9.47%, while the lowest count was observed in the KWE-treated group on day 3, at 6.04%. At the start of the study (day 0), no significant differences ($p > 0.05$) were detected among the treatment groups, with hyaline cell counts ranging from 6.86% in the negative control (C−) to 7.30% in the positive control (C+). By day 6, the hyaline cell count in the KWE-treated shrimp (6.04%) was significantly lower than in both control groups ($p < 0.05$). On day 9, the C + group exhibited the highest hyaline cell count (9.60%), which was significantly greater than that of the C- (7.02%) and KWE-treated (6.49%) groups. This trend persisted on day 12, with the C + group maintaining a significantly higher hyaline count (9.50%) compared to C- (7.07%) and KWE-treated shrimp (7.08%), although there was no significant difference between the latter two groups. By day 15, the hyaline count remained significantly higher in the C+ (9.47%) and C− (8.40%) groups compared to the KWE treatment (6.51%) ($p < 0.05$). Overall, these findings suggest that KWE administration had a limited effect on increasing hyaline cell counts compared to the positive control, indicating that KWE might not significantly enhance this particular immune parameter in Pacific white shrimp.

Discussion

Water-based Kiam wood extract exhibited stronger antimicrobial activity against *V. parahaemolyticus*, a major pathogen in shrimp aquaculture, compared to the extract using ethanol at different concentrations as solvents. Several factors might contribute to the result. First, the difference in solvent polarity likely plays a crucial role. Water is a highly polar solvent, whereas ethanol is less polar. The antimicrobial compounds in Kiam wood extract appear to be highly polar, making them more soluble in water than in ethanol. As a result, water extraction yields a higher concentration of bioactive compounds, leading to stronger antimicrobial activity[16]. Second, the chemical interactions between the solvent and bioactive compounds may influence extraction efficiency. Ethanol can sometimes modify or react with certain compounds during extraction, potentially diminishing their antimicrobial properties. In contrast, water is generally less reactive, helping to preserve the structural integrity and bioactivity of sensitive compounds[17,18]. Third, water extraction may recover a broader spectrum of bioactive compounds that act synergistically to enhance antimicrobial activity[19]. LC-MS analysis confirmed the presence of several key bioactive compounds in Kiam wood extract, including Amuresins D, Pauciflorol A, Vaticasides A, Vaticanols B, and Cotylelophenol B. These compounds contain multiple hydroxyl (-OH) groups, characteristic

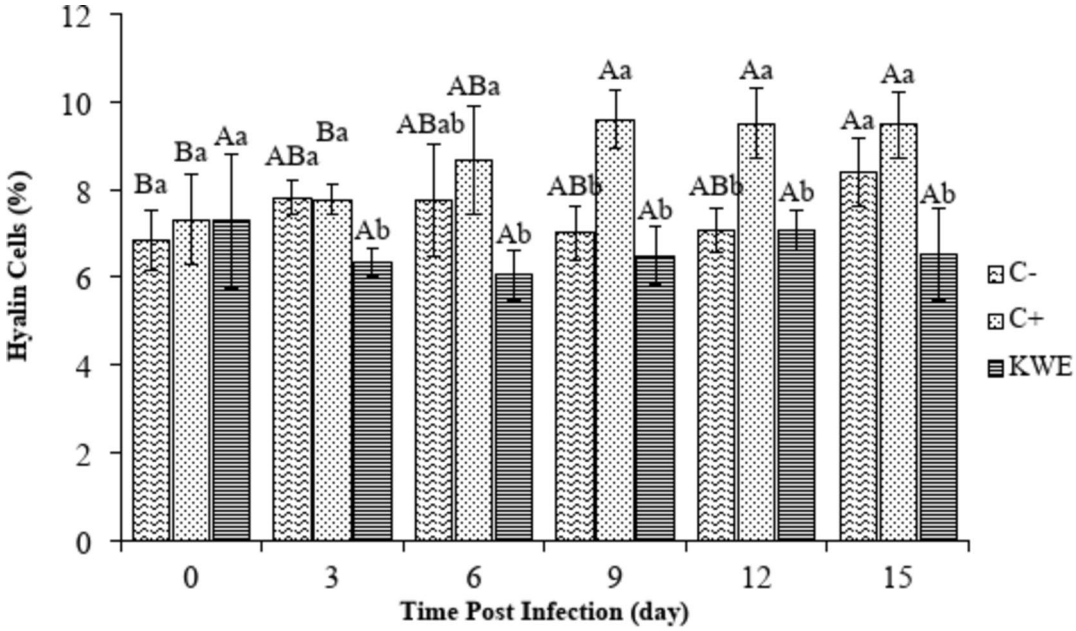

Fig. 9. Hyaline cell counts in Pacific white shrimp quantified on days 0, 6, 9, 12, and day 15. The data presented are the average values, and the error bars represent the standard deviations of four replicates. Statistical analysis indicated significant differences ($p < 0.05$) between treatments, with lowercase letters denoting comparisons within the same sampling period and uppercase letters indicating comparisons of the same treatments across different sampling periods. The study included three groups: a negative control (C-) where shrimp were fed a normal diet without pathogen challenge, a positive control (C+) where shrimp were challenged with a bacterial pathogen but received no supplementation, and a treatment group (KWE) where shrimp were challenged with the pathogen and fed a diet mixed with KWE.

of polyphenols and stilbenoids, which contribute to their high polarity and water solubility. While ethanol can extract certain bioactive compounds effectively, it may fail to extract others, thereby reducing the overall synergistic effect. This observation aligns with previous studies highlighting the influence of solvent polarity on extraction efficiency. Borges, et al.[19], demonstrated that different solvents extract bioactive compounds with varying degrees of antimicrobial potency, with water being particularly effective for highly polar compounds. Similarly, Nguyen, et al.[20], found that water was the most efficient solvent for extracting polar phenolics from *Phyllanthus amarus*, and Skalicka-Woźniak, et al.[21] reported that solvent polarity significantly impacts the extraction of flavonoids from plant matrices. Overall, the superior antimicrobial activity observed in water-based Kiam wood extract underscores the importance of solvent selection in optimizing bioactive compound extraction. These findings suggest that water is a preferable extraction solvent for obtaining polar antimicrobial compounds from Kiam wood, offering potential applications in shrimp disease management and sustainable aquaculture practices.

Kiam wood extract exhibited a minimum inhibitory concentration (MIC) of 256 µg/mL and a minimum bactericidal concentration (MBC) of 512 µg/mL against *V. parahaemolyticus*. These values indicate that KWE outperforms several previously reported plant-derived antimicrobials targeting Vibrio species. For instance, Jain, et al.[22] reported that *Ziziphus mauritiana* leaf extracts exhibited a MIC of 2.5 mg/mL (2500 µg/mL) and an MBC of 4.0 mg/mL (4000 µg/mL) against *V. parahaemolyticus*, which are significantly higher than those of KWE. This suggests that KWE is at least ten times more effective in inhibiting and eliminating *V. parahaemolyticus*, highlighting its superior antibacterial potential. Several factors may contribute to this enhanced efficacy, including differences in phytochemical composition, bioactive compound concentration, and extraction techniques. Cowan[23] provided a comprehensive overview of the major classes of plant-derived antimicrobial compounds, emphasizing that their efficacy often results from synergistic interactions among bioactive constituents. Similarly, Urzúa, et al.[24] discussed how specific structural features of bioactive compounds contribute to their antibacterial activity. In the case of Kiam wood extract, LC-MS analysis identified key bioactive compounds, including Amuresins D ($C_{42}H_{30}O_9$), Pauciflorol A ($C_{24}H_{32}O_9$), Vaticasides A ($C_{48}H_{42}O_{14}$), Vaticanols B ($C_{56}H_{42}O_{12}$), and Cotylelophenol B ($C_{24}H_{30}O_{10}$). These compounds, belonging to the polyphenol and stilbenoid classes, are well-documented for their antimicrobial properties. Olmedo-Juárez, et al.[25] similarly demonstrated the antibacterial effects of methyl gallate and gallic acid in *Caesalpinia coriaria* fruit, reinforcing the role of specific phytochemicals in antimicrobial activity. The synergistic interactions among these bioactive compounds in KWE likely enhance its overall effectiveness against *V. parahaemolyticus*. Moreover, structural features of bioactive compounds play a critical role in their antibacterial potency. Quaglio, et al.[26] demonstrated that the acidic group at C18 in the tetracyclic ent-beyerene scaffold contributes to antibacterial activity. Similarly, Lee, et al.[27] identified flavaspidic acids from *Dryopteris crassirhizoma* as potent antibacterial agents, further

supporting the importance of compound structure in antimicrobial efficacy. The strong bacteriostatic and bactericidal effects observed in KWE suggest its potential as a natural alternative to conventional antibiotics in aquaculture. These findings are particularly promising for the development of sustainable disease management strategies, reducing dependence on synthetic antibiotics and mitigating the risk of antimicrobial resistance in shrimp farming. Further research is warranted to evaluate KWE's efficacy under *in vivo* conditions and explore its potential for integration into aquaculture disease prevention programs.

Mechanisms of antimicrobial action of Kiam wood extract

Kiam wood extract exerted its antimicrobial effects by disrupting the cell wall and membrane integrity of *V. parahaemolyticus*, as confirmed through scanning electron microscopic analysis. Treated bacterial cells exhibited severe membrane deformation, pore formation, and eventual lysis, indicating a significant loss of structural integrity. This mode of action aligns with the well-established antimicrobial mechanisms of plant-derived polyphenols, which compromise bacterial cell walls, increase membrane permeability, and interfere with intracellular metabolic pathways. The resulting membrane disruption ultimately leads to bacterial cell death, a key strategy employed by many natural antimicrobial compounds. Gomes, et al.[28] highlighted the critical role of membrane permeabilization in facilitating access to intracellular biomolecules, underscoring the necessity of membrane integrity for bacterial survival. Similarly, Xiong, et al.[29] demonstrated that modifications in cell wall components, such as mycolic acids in *Mycobacterium*, can influence membrane permeability and overall cell viability, reinforcing the relationship between cell wall integrity and antimicrobial susceptibility.

In Vivo disease mitigation and immune enhancement

Beyond its bactericidal effects, KWE also inhibited biofilm formation and bacterial motility at 512 µg/mL, targeting two crucial virulence factors of *V. parahaemolyticus*. Biofilms serve as protective environments that enhance bacterial resistance to antimicrobial agents, making infections more persistent in aquaculture. Li, et al.[30] described how the outer membrane of Gram-negative bacteria, including *V. parahaemolyticus*, acts as a formidable barrier against many antimicrobial agents. KWE's ability to disrupt this barrier and prevent biofilm formation represents a significant advantage, particularly in shrimp farming, where biofilms contribute to disease outbreaks and treatment failures. Traditional antibiotics often struggle to penetrate biofilms, making bacterial infections difficult to control. By preventing biofilm establishment, KWE provides a promising natural alternative for managing *V. parahaemolyticus* infections in aquaculture. Furthermore, Stirke, et al.[31] emphasize the importance of effective biofilm prevention strategies, noting that some microorganisms can recover from membrane permeabilization induced by pulsed electric fields. This underscores the significance of KWE's dual mechanism, both disrupting membrane integrity and inhibiting biofilm formation, in combating *V. parahaemolyticus* infections. Given these multifaceted antimicrobial properties, KWE holds strong potential as a sustainable alternative to conventional antibiotics in shrimp farming, contributing to improved disease management and reduced reliance on synthetic antimicrobial agents.

Protective effects of kiam wood extract in *Penaeus vannamei*

Beyond its direct antibacterial activity, Kiam wood extract (KWE) exhibited significant protective effects in *P. vannamei* when incorporated into the diet. Shrimp fed a diet supplemented with KWE at twice the minimum bactericidal concentration (2MBC; 1 mg/g diet) for 15 days showed notable benefits, including reduced intestinal *Vibrio* loads, enhanced immune responses, and improved survival rates. The significant reduction in intestinal *Vibrio* populations aligns with KWE's demonstrated *in vitro* efficacy against *V. parahaemolyticus*, confirming its antimicrobial potential in a biological system. A balanced gut microbiome is crucial for shrimp health, as microbial dysbiosis can impair immune function and increase disease susceptibility[32]. The observed decline in pathogenic bacteria suggests that KWE contributes to maintaining intestinal microbial balance, thereby supporting overall shrimp health.

In addition to its antimicrobial effects, KWE supplementation stimulated the shrimp's immune system, as evidenced by increased total hemocyte counts (THC), granulocyte levels, and semi-granulocyte levels. Hemocytes play a vital role in pathogen recognition, phagocytosis, and antimicrobial peptide production, making their proliferation a key indicator of enhanced immune defense. Li and Xiang[33]provide a detailed overview of immune signaling pathways in shrimp, suggesting that KWE may activate these pathways similarly to other immunostimulants. While the precise immunomodulatory mechanisms of KWE remain to be elucidated, its potential role in modulating immune-related genes or reducing oxidative stress warrants further investigation[34]. The most notable outcome of KWE supplementation was the significant increase in shrimp survival rates. Shrimp receiving the KWE-enriched diet achieved an 85% survival rate, compared to 52% in the control group. This substantial improvement highlights KWE's potential as a functional feed additive for disease prevention in shrimp aquaculture. Enhancing disease resistance is a major challenge in shrimp farming[35], and dietary interventions, including plant-derived bioactives, have been shown to improve shrimp immunity and survival[36]. Given its dual antimicrobial and immunostimulatory properties, KWE represents a promising alternative to conventional disease management strategies, offering a sustainable approach to reducing mortality in shrimp farming.

Immunomodulatory effects of Kiam wood extract on hyaline cell populations

The observed reduction in hyaline cell proportions following KWE supplementation warrants further investigation, particularly given the complexity of the shrimp immune system. While hyaline cells play a crucial role in innate immunity, their decline does not necessarily indicate immunosuppression. Instead, it may reflect a shift in hemocyte populations, favoring granulocytes and semi-granulocytes, potentially as a response to

KWE. This shift could be driven by KWE's influence on immune cell differentiation, proliferation, or apoptosis, mechanisms that warrant further exploration. Previous studies have demonstrated that environmental stressors can modulate shrimp immunity, leading to alterations in hemocyte composition and gene expression[34]. Similarly, Pope, et al.[37] emphasized the complexity of immune regulation in shrimp and the potential for modulation through bioactive compounds.

A comparative analysis of KWE's effects with other immunostimulants or environmental stressors could provide valuable insights into its immunoregulatory role. For instance, Rahmaningsih and Andriani[38] investigated the immunostimulatory effects of Majapahit fruit powder on shrimp using in silico methods, offering a potential framework for evaluating KWE's impact. Further research is essential to determine whether the observed shifts in hemocyte populations enhance disease resistance or represent an immunological trade-off. Lillehammer, et al.[35] underscored the importance of understanding immune modulation in shrimp, given their reliance on innate immunity. A deeper mechanistic understanding of KWE's immunomodulatory effects will be critical for assessing its suitability as an immunostimulant and optimizing its application in aquaculture.

Future prospects and research directions

These findings highlight the potential of KWE as a sustainable alternative to traditional antibiotics for managing vibriosis in shrimp aquaculture. Its dual function as an antimicrobial and immunostimulant addresses two major challenges in shrimp farming: effective disease management and the growing concerns over antibiotic resistance and environmental contamination. The increasing demand for non-antibiotic disease prevention strategies in aquaculture is required. Dawood, et al.[39] underscored the significance of KWE as a promising candidate for integration into shrimp feed. To fully realize KWE's potential, further research is needed to elucidate its precise mechanisms of action, particularly in modulating shrimp immune responses and inhibiting *V. parahaemolyticus*. Establishing the optimal dosage for commercial application is crucial to ensure a balance between efficacy and cost-effectiveness. Additionally, long-term studies should assess its impact on shrimp health, growth performance, and microbiota stability to confirm its safety and sustainability in aquafeeds. While KWE has demonstrated strong antibacterial and immunostimulatory properties, further investigation is required to identify the specific bioactive compounds responsible for these effects. A deeper understanding of these compounds will help optimize extraction methods and refine formulation strategies for large-scale applications. Overall, KWE presents a promising plant-based alternative to synthetic antibiotics in shrimp aquaculture, contributing to more sustainable disease management practices. However, comprehensive validation through controlled trials remains essential to ensure its efficacy, safety, and feasibility for widespread adoption in commercial shrimp farming.

In conclusion, this study demonstrates that water-based Kiam wood extract is a promising natural antimicrobial and immunostimulant for managing *V. parahaemolyticus* infections in *P. vannamei*. KWE exhibited strong antibacterial activity with an MIC of 256 µg/mL and an MBC of 512 µg/mL, effectively inhibiting bacterial growth, biofilm formation, and bacterial motility. Scanning electron microscopic images confirmed its bactericidal effects, while LC-MS analysis identified Amuresins D and Pauciflorol A as key bioactive compounds contributing to its antimicrobial properties. I*n vivo* trials further validated KWE's protective role, as it significantly enhanced immune responses and increased survival rates of shrimp challenged with *V. parahaemolyticus*. These findings highlight KWE's dual function as both an antimicrobial agent and an immunostimulant, offering a sustainable alternative to synthetic antibiotics in aquaculture. The integration of KWE into shrimp feed could help mitigate the growing concerns of antibiotic resistance and environmental contamination in shrimp farming. However, further research is needed to optimize dosage, evaluate its long-term effects on shrimp health and microbiota, and assess its potential for large-scale commercial application in aquaculture.

Materials and methods

Kiam wood extraction

Kiam wood, harvested from a plantation in Phatthalung Province, Thailand, was first cut into pieces and then dried in an oven at 50 °C for 18 h. The dried wood pieces were ground using a colloid mill and further processed with an AY46 blender to achieve particles less than 80 mesh in size. This powdered material was then homogenized in ethanol at varying concentrations (0, 60, 70, 80, and 99.60%), using a 1:15 powder-to-solvent ratio at 10,000 rpm for 2 min. Each homogenized mixture was stirred overnight at ambient temperature and filtered through the Whatman No. 1 filter paper. The filtrates were concentrated at 40 °C using a rotary evaporator. To remove any remaining ethanol, the extracts were purged with nitrogen gas for 10 min and then freeze-dried for 3 days. The dried extracts were further powdered using a mortar, packaged in a zip-lock bag, and stored in a desiccator for subsequent analysis.

Determination of antimicrobial activity

Preparation of pathogen inoculum

The antimicrobial activity of Kiam Wood Extract (KWE) was evaluated against the *Vibrio parahaemolyticus* ATCC17802. The bacterial cultures utilized in this investigation were obtained from the Faculty of Agro-Industry at Prince of Songkla University. Each strain was propagated in Tryptic Soya Broth (TSB, CM0129 OXOID) supplemented with 3% sodium chloride (KA465, KEMAUS) and incubated at 37 °C for 24 h. Following incubation, the cell density was standardized to match the 0.5 McFarland turbidity standard, which corresponds to approximately 1.5×10^8CFU/mL[40].

Disk-diffusion assay

KWE powder was dissolved in 2% dimethyl sulfoxide (DMSO, D5879 SIGMA ALDRICH) to achieve a final concentration of 500 mg/mL. The solution was then sterilized by passing it through a 0.22 µm syringe filter

(SARTORIUS, Minisart). Ten microliters of this solution was pipetted onto a 6 mm sterilized paper disc, resulting in a concentration of 5 mg KWE per disc. The KWE-impregnated discs were air-dried for 15 min and stored at 4 °C until needed. To screen for antimicrobial properties, the disk diffusion method described by Ngamsurach and Praipipat[41] was employed against the targeted pathogen (*V. parahaemolyticus* ATCC17802). Bacterial inoculum, previously adjusted to a 0.5 McFarland standard, was streaked onto Mueller-Hinton agar (MHA, M391, HiMedia) using sterile cotton swabs. The KWE-impregnated paper discs were then placed on the bacterial plates in duplicate and incubated at 37 °C for 18 h. As controls, 10 µL of 2% DMSO served as the negative control, while potassium sorbate at 5 mg per disc was used as the positive control. Following the 18-hour incubation, the diameters of the inhibition zones were measured using a Vernier caliper (Series 530, Mitutoyo, Japan).

Evaluation of minimum inhibitory concentration and minimum bactericidal concentration

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were assessed using a serial micro-dilution technique involving two-fold dilutions, based on the approach by Sallau, et al.[42], with some modifications. KWE was serially diluted in Mueller-Hinton broth (MHB, M391, HiMedia) containing 3% NaCl, using 96-well plates to achieve ten different concentrations in 50 µL volumes. Each concentration was tested in triplicates. The bacterial suspension was adjusted to a 0.5 McFarland standard using 0.85% NaCl solution and then diluted with MHB to reach approximately 1.5×10^5 CFU/mL. Each well was inoculated with 50 µL of this bacterial suspension. The final concentrations of KWE ranged from 4 to 4096 µg/mL. Potassium sorbate at the same concentrations as KWE served as the positive control. After incubating the plates at 37 °C for 15 h, 20 µL of 0.09% (w/v) resazurin solution was added to each well, followed by an additional 3 h incubation. The MIC was determined by visually inspecting the wells for a color change from blue to pink; the lowest KWE concentration without a color change was recorded as the MIC value. To determine the MBC, 10 µL suspension from wells showing no color change was cultured on Mueller-Hinton agar (MHA, M391-HiMedia), and the plates were incubated at 37 °C for 24 h. The MBC was defined as the lowest concentration of KWE that resulted in no bacterial growth on the MHA after incubation.

Inhibition of biofilm formation

The KWE with the highest antimicrobial activity was selected for testing of biofilm formation inhibition. The ability of KWE to inhibit biofilm formation of spoilage and pathogenic bacteria was performed according to a protocol of Lopes, et al.[43] with slight modifications. In brief, *V. parahaemolyticus* ATCC17802 was cultured in TSB comprising 3% NaCl and was used with incubation at 37 °C for 24 h. Thereafter, cell concentration was adjusted to 0.5 MacFarland standard with 0.85% NaCl solution and then diluted with TSB or TSB with 3%NaCl medium to obtain ~ 1.5×10^5 CFU/mL. The inoculum (100 µL) was added to a 96-well flat bottom plate and mixed with KWE at 4MIC, 2MIC, MIC, and 1/2MIC, followed by incubation at 37 °C for 48 h. Then, planktonic cells and medium were removed, washed with phosphate buffer saline (PBS; pH 7.4) three times, and dried at room temperature for 30 min. Afterward, 200 µL of 0.1% (w/v) crystal violet solution was added to each well, followed by incubation at room temperature (~ 25 °C) for 45 min. Then, the crystal violet (CV) was discarded, washed with PBS three times, and dried at room temperature overnight. CV was solubilized using 200 µL of 95% ethanol for 10 min and the absorbance was measured at 570 nm. Inhibition of biofilm formation (IBF) was calculated according to Equation below.

$$IBF = \frac{(C_+ - C\_) - (AbsT - C\_)}{(C_+ - C\_)} \times 100$$

C_+ is the OD_{570} of CV-stained biofilm without extract; $C\_$ is the OD_{570} of CV-stained without bacteria; AbsT is the OD_{570} of CV-stained biofilm with extract.

Anti-motility assay

Swimming and swarming motilities were assayed using the method outlined by Buatong, et al.[44], with minor modifications. A 2 µL aliquot of overnight *V. parahaemolyticus* ATCC17802 culture was inoculated at the center of swarming soft agar (0.5% agar, 30 g/L TSB) and swimming soft agar (0.2% agar, 30 g/L TSB), both containing 5 g/L NaCl. These agars were supplemented with KWE at concentrations of 2MIC, MIC, 1/2MIC, 1/4MIC, and 1/8MIC. Potassium sorbate at the same concentrations served as controls. After incubation at 37 °C, the diameters of the colonies were measured at 2, 4, 6, 8, and 10 h for swimming motility and at 8, 12, and 24 h for swarming motility.

Scanning electron microscopy

The cell morphology of untreated and KWE-treated bacteria was examined using a scanning electron microscope, following the protocol described by Sinlapapanya, et al.[45]. *V. parahaemolyticus* ATCC17802 was treated with KWE at a concentration of 4MIC. Potassium sorbate at the same concentration (4MIC) was used as a positive control. Bacterial cells grown in culture medium alone served as the negative control.

Identification of phenolic compounds

The identification of compounds in KWE was conducted using a Chromatograph-Quadrupole Time-of-Flight Mass Spectrometer (Agilent 1290 Infinity LC-6540, Agilent Technologies, Waldbronn, Germany). The system was equipped with a Diode Array Detector (DAD), an Agilent Poroshell 120 EC-C18 column (4.6 × 150 mm, 2.7 µm), an autosampler, and a binary pump. The column compartment was set at 35 °C for chromatographic separation. The autosampler injected 1 µL of samples, with a flow rate of 0.2 mL/min and a maximum pressure

of 1,000 bar. The mobile phase consisted of water with 0.1% formic acid (solvent A) and acetonitrile with 0.1% formic acid (solvent B) in a 95:5 ratio. Mass spectrometry was performed using dual ESI ion sources with a range of 100–1000 m/z. Instrument settings included a capillary voltage of 3500 V, a gas temperature of 300 °C with a flow of 11 L/min, a fragment voltage of 135 V, a skimmer voltage of 65 V, a nebulizer gas pressure of 45 psi, and an Octopole RF peak of 750 V. Both positive and negative mode scans were conducted. Data processing was carried out using Personal Compound Database and Library (PCDL) and Agilent Mass Hunter Workstation software (Qualitative Analysis, version B.08.00). The mass and molecular formula of selected peaks were determined using the LC-MS tool system and the electrospray source system (ESI), which provided reliable mass information from parent ions and source-induced dissociation fragments.

Evaluation of the efficacy of Kiam Wood Extract in treating shrimp infected with *V. parahaemolyticus*

KWE-supplemented feed was prepared by evenly incorporating the extract into commercial shrimp feed pellets, following the method described by Dewi, et al.[46], with slight modifications. In brief, 51.2 mg KWE (MBC) was firstly mixed with 20 mg feed binder (progol), and dissolved in 25 mL of distilled water to ensure uniform distribution. This solution was then evenly sprayed onto 100 mg of feed pellets. For the control feed, the same amount of distilled water and binder was applied without KWE. The KWE-supplemented feed was air-dried at room temperature to remove excess moisture while preserving the bioactive compounds in KWE. Once dried, the feed was placed in airtight containers and stored in 4 °C to maintain its stability and effectiveness throughout the feeding trial.

A total of 500 Pacific white shrimps (*Penaeus vannamei*), weighing 0.8 g and 54 mm in length, were obtained from PT. Windu Raya Hatchery in Situbondo, East Java, Indonesia. The shrimps were transported in aerated plastic bags to the Aquaculture Laboratory at the Faculty of Fisheries and Marine, Universitas Airlangga. In the laboratory, the shrimps were initially acclimated in a 500 L fiber tank at a stocking density of 1 shrimp per liter for 5 days. They were fed at 5% of their body weight per day, divided into three meals. After the acclimatization period, the shrimps were fasted for 24 h and then transferred to experimental aquaria at a stocking density of 1 shrimp per liter on day 0. They were subsequently divided into three treatment groups:

T1: Negative control negative in which shrimp were fed with normal diets without bacterial infection.

T2: Positive control in which shrimp were firstly infected with *V. parahaemolyticus* at a final concentration of 1.5×10^4 CFU/gr and fed with a normal diet.

T3: Shrimps were infected with *V. parahaemolyticus* and afterward fed a KWE-impregnated diet at a final concentration of 512 µg/g diet (MBC for *V. parahaemolyticus*).

The bacterial infection was induced by feeding the shrimps with a diet containing *V. parahaemolyticus* at a final concentration of 1.5×10^4 CFU/g for 2 days. Infection was monitored through clinical symptoms such as reduced feeding, pale hepatopancreas, increased mortality, and the production of white feces. On the third day (day 3), shrimps in the negative control groups (T1 and T2) were switched to a basal diet, while shrimps in group T3 were fed a diet mixed with KWE at a final concentration of 512 µg/g diet. During the experiment, the survival rate, immune responses (including total hemocyte count and differential hemocyte count), and total Vibrio count in the shrimp's intestine were assessed.

Survival rate (SR)

Survival rate was calculated by counting the number of shrimps at the beginning of an experimental period and the final number of living shrimp at the end of the experimental period. Then SR was calculated with the following formula:

$$SR\ (\%) = \frac{number\ of\ living\ shrimps\ at\ the\ end\ of\ experimental\ period}{number\ of\ living\ shrimp\ at\ the\ begining\ of\ experimental\ period} \times 100$$

Total vibrio count (TVC)

TVC was carried out by collecting 1 g of intestinal tract and mixed in 9 ml 0.85% NSS solution. Thereafter, the mixture was serially diluted from $10^{-1} - 10^{-6}$. 100 µl solution from each dilution tube was spread on duplicate TCBS agar, and incubated at 28 °C for 24 h. Bacterial colonies that grew and showed good colony separation were enumerated and calculated to have TVC values.

Total hemocyte counts (THC)

THC was enumerated on days 0, 3, 6, 9, 12, and 15 of the culture periods according to a protocol of Ekawati et al. (2012) with slight modifications. Two healthy shrimp were collected from each aquarium, and hemolymph was derived using a 1 ml syringe previously filled with anticoagulant with a ratio of 1:1. One-hundred µl of the mixture was dropped on the surface of a hemocytometer and covered using a cover glass. The number of hemolymph cells was observed and counted under a binocular microscope with a 40x magnification. The data obtained was recorded and calculated according to the following formula:

$$\mathrm{THC} = \frac{\sum \text{number of counted cells}}{\sum \text{Total number of cells}} \times 25 \times \frac{1}{Haemocytometer\ volume} \times \mathrm{FP}$$

where: THC is total hemocyte counts (cells/ml), and FP is dilution factor.

Differential hemocytes count (DHC)

DHC assay was carried out on days 0, 3, 6, 9, 12, and 15 of the culture period according to a protocol of Widanarni, et al.[47] with some modifications. In brief, 100 µL of hemolymph collected from shrimp was dropped on an object glass, air dried, and fixed with 100% methanol for 10 min. The preparations were air dried after being fixed using 100% methanol and stained by immersing them in 10% Giemsa solution for 15 min. The preparations were air-dried, washed in running water for 30 s, and allowed to dry. The preparations were observed using a light microscope with a 40x magnification and were differentiated according to their type, namely hyaline, semi-granulocyte, and granulocyte cells (Abdi, 2022). DHC calculation formula is shown below:

$$\text{Haemocyte counts } (\%) = \frac{\text{Number of each haemocyte cell(Hialin)}}{\text{Total haemocyte}} \times 100$$

Throughout the experiment, water quality parameters—including pH, temperature, dissolved oxygen (DO), and salinity—were monitored daily and maintained at optimal levels for Pacific white shrimp culture. The pH was kept within the range of 7.5 to 8.5, the temperature was at 28 –33 °C, dissolved oxygen (DO) levels were maintained above 4 mg/L, and salinity was held between 33 and 34 ppt (SNI 8037.1:2014).

Statistical analysis

Data were analyzed statistically using analysis of variance (ANOVA). Any significant different values between treatments were further analyzed using Tukey's multiple range tests at confidence limits of 95% ($p < 0.05$). Analysis was done with the help of SPSS software (22.0 version).

Data availability

Data are however available from the authors upon reasonable request to the corresponding author.

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M.A., J.B, W.T, S.A.R., G.P., S.B. Experimental design of the study, writing manuscript, proofread. M.A., J.B, W.T, S.A.R., G.P., S.B. data collections, M.A., J.B, W.T, S.A.R., G.P., S.B. Data analysis. M.A., J.B, W.T, S.A.R., G.P., S.B metagenomics analysis, drafting manuscript, submission. All authors have read and approved the final manuscript.

Declarations

Competing interests

The authors declare no competing interests.

Additional information

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