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Ethanolic extract of red seaweed *Gracilaria fisheri* and furanone eradicate *Vibrio harveyi* and *Vibrio parahaemolyticus* biofilms and ameliorate the bacterial infection in shrimp

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ABSTRACT

Bacteria respond to host immunity for their proliferation and survival by cell-cell communications such as biofilm formation, bioluminescence, and secreting virulence factors. In the biofilm form, bacteria are more resistant to various antimicrobial treatments and withstand the host's immune system. The approaches of deciphering biofilm formation for treating bacterial infections are therefore highly desirable. Recently, we have reported that the ethanolic extract of the red seaweed *Gracilaria fisheri* (*G. fisheri*) enhanced immune activities and inhibited growth of the luminescent bacteria *Vibrio harveyi* in shrimp. We undertook the present research study in order to evaluate and compare the effectiveness of the ethanolic extract from *G. fisheri* and furanone, a known biofilm inhibitor, in inhibiting the formation of clinically important Vibrio biofilms. The results showed that sub-lethal concentrations of both the ethanolic extracts (5, 10 and 100 μg ml^{-1}) and furanone (5 μM) inhibited biofilm formation by *V. harveyi* and *Vibrio parahaemolyticus* and also light production (luminescence) in *V. harveyi*. It is known that *V. harveyi* mediated light production via autoinducer AI-2 pathway, we further determined whether the inhibitory effect of the extract was involved the AI-2 signaling. The bioluminescence assay was conducted in an AI-2 deletion mutant *V. harveyi*. Supplementation of the AI-2 containing media with the extract or furanone impaired the light production in the mutant *V. harveyi* suggesting that the extract interfered AI-2 mediated light production similar to furanone. *In vivo* challenge study showed that the low concentrations (Sub MICs) of the ethanolic extract and furanone decreased bacterial adhesion and colonization in the surfaces of stomach lumen, down-regulated expression of a virulence factor, and protected shrimp against mortality from *V. harveyi* and *V. parahaemolyticus* infection. In conclusion, the present results suggest a potential application of the low concentrations of the ethanolic extract of *G. fisheri* as an efficient approach for treating biofilm-associated Vibrio diseases in aquacultures.

1. Introduction

The cell-cell conversation or quorum sensing (QS) mechanism allows bacteria to act communally, which permits them to assess their population density via the production, launch, and perception of small diffusible molecules referred to as autoinducers (AIs) for coordinating the expression of essential genes for adapting the group behaviors to various environments [1]. When a certain cell population or AIs concentration is reached, the transcription of certain genes is altered. Many pathogenic bacteria utilize cell-to-cell communication or quorum sensing as a regulatory mechanism for their pathogenicity, as an example, bioluminescence, biofilm formation, antibiotics resistance and production of virulence factors [2]. In recent years a number of studies have exploited the bacteria quorum sensing system as a target for treatment of bacterial infections in place of conventional therapeutic strategies such as bactericidal and bacteriostatic procedures [3] since this strategy disrupted only the communication mechanism between the bacteria without killing the individual cells thereby reducing the rate at which antibiotic resistance develops during the treatment [4].

Vibrio harveyi is a luminescent bacterium most significantly responsible for massive mortalities of aquaculture marine organisms both in natural populations and farmed stocks around the world [5]. Pathogenicity of *V. harveyi* infection includes colonization, adhesion of the pathogen to host surface where it multiplies and establish biofilm;

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invasion of host organs by expression of virulence factors to initiate disease; disperse of the pathogen and disease transmission to neighbouring host [6]. A biofilm is formed by bacterial cells attachment to surfaces, micro-colonies formation and further maturation into biofilms, their structural basis are composed of proteins, nucleic acids, lipids and exopolysaccharides [7], and these components protect bacteria from the environment and host immunity [8]. Since these processes are important for bacterial survival by providing protection against host defense mechanism and persistence of *V. harveyi* and its infection, therefore, a search for compounds that can prevent or destroy biofilms has gained increased attention.

Targeting the QS system of the bacteria infection in shrimp by plant extracts was recently reported by Yatip and colleagues [9]. They showed that the extract from the fermented soybean or Notto inhibited vibrio biofilms formation and decreased the mortality of shrimp infected with *V. harveyi*. Furthermore, halogenated furanones produced by red seaweed *Delisea pulchra* showed protection against the infection of *V. harveyi* in brine shrimp *Artemia franciscana* by disruption of the bacterial AI-2 QS signal [10]. Previously, our group has demonstrated that the ethanolic extract from red seaweed *Gracilaria fisheri* (*G. fisheri*) stimulated shrimp immunity and protected shrimp mortality by *V. harveyi* infection [11]. Regarding this property, the ethanolic extract from *G. fisheri* is now being used as a diet supplementation to protect against bacterial activity in some shrimp culture farms. However, whether it has anti-QS properties has not been explored. In the present study, we tested the hypothesis that the ethanolic extract from *G. fisheri* not only enhance immunity but may notably interfere with the quorum sensing of the bacteria. We evaluated the effects of the sub-lethal concentrations of the ethanolic extract, comparison to furanone, on the prevention of biofilm formation by *V. harveyi* and *V. parahaemolyticus* which are the causative agents of vibriosis and early mortality syndrome in shrimp, respectively. Additionally, we herein demonstrate the inhibitory effects of the ethanolic extract on the light production and expression of a virulence factor of *V. harveyi*. The results from this study provide preliminary information on anti-biofilms effect of the ethanolic extract from *G. fisheri,* as a pre-requisite for its potential therapeutic use as an alternative antimicrobial from a natural source.

2. Materials and methods

2.1. Ethanolic extract of G. fisheri

G. fisheri were collected from the Coastal Aquaculture Research and Development Center Regional Center, Surat Thani, Thailand. The ethanolic extract was prepared according to the method described previously [11]. Briefly, the dry seaweed was crushed into powder using a blender, freeze-dried, and stored in polythene bags before extraction. To prepare the extract, the seaweed powder (30 g) was extracted with 500 ml ethanol (100%) using a Soxhlet apparatus for 24 h and then evaporated in a rotatory evaporator at 60 °C until dried. The extracts were then stored at −20 °C until use. The yield of the ethanolic extract was found to be 3.5%. A stock solution of the extract (100 mg ml^{-1}) was prepared in sterile Milli-Q water.

Furanone (C-30), [(5Z)-4-bromo-5-(bromomethylene)-2(5H)-furanone] was purchased from Sigma-Aldrich (Buchs, Switzerland). Furanone was dissolved in absolute ethanol to a concentration of 50 mg ml^{-1} and stored at −20 °C. The stock was further diluted in media to a working solution.

2.2. Bacterial strains and growth conditions

V. harveyi (strains BB120 [12] and 1114 [13]) were obtained from the American Type Culture Collection (ATTC). *V. parahaemolyticus* (strains 3HP, 5HP) [14] were obtained from the Center of Excellence for Shrimp Molecular Biology and Biotechnology, Mahidol University, respectively. The lyophilized cells were either reconstituted in Muller-Hinton's broth (MHB) or marine broth or tryptic soy broth (TSB) and the cultures were resuscitated under aerobic conditions at 30 °C and 250 rpm to reach exponential growth. The cell concentration of 10^8 colony-forming units (CFU) ml^{-1} was routinely estimated by spectrophotometric turbidity measurement at 600 nm using a spectrophotometer and by CFU counts on tryptic soy agar (TSA) or Marine Agar 2216 (Difco: BD Biosciences, Franklin Lakes, NJ, USA).

2.3. Minimal inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs)

The MICs of prepared extract and furanone were determined for each bacterial strain using the broth dilution method as described by Valgas and colleagues [15]. An equal volume of each bacterial strain culture (1×10^8 CFU ml^{-1}) was added to MHB containing different concentrations of the extract (two fold serially diluted in conical tubes) and furanone and incubated at 30 °C for 24 h. The control was bacteria grown without the extract. Bacterial growth was determined from the turbidity using a spectrophotometer. MIC was notably described as the concentration of the extract or furanone that totally inhibited the growth of the bacteria. To determine the minimum bactericidal concentration (MBC), an aliquot of 20 µl broth was incubated at 30 °C for 24 h, and the colony counted. The minimum concentration of the sample at which the bacterial colony was visibly reduced to 99.9% was considered as the MBC.

2.4. Effect of sub-lethal concentrations of the ethanolic extract and furanone on V. harveyi and V. parahaemolyticus biofilms formation

2.4.1. Determination of the bacterial growth

Different strains of bacteria were inoculated in optimal medium with or without sub-lethal concentrations of the ethanolic extracts (5, 10 and 100 µg ml^{-1}) or furanone (5 µM). The cultures were grown overnight at 30 °C in a shaking incubator at 250 rpm, and the OD600 nm was measured every 2 h using a microplate reader (Versamax, USA), up to 24 h. The bacterial growth curve was plotted. Sub-lethal concentrations of the extract that did not interfere with the growth of the bacteria were determined and used in the subsequent biofilms assessment experiments.

2.4.2. Assessment of biofilm biomass formation

The inhibition of bacterial biofilm formation was investigated using the crystal violet assay according to O'Toole [16]. A single colony of each bacterial strain was grown in MHB media at 30 °C overnight with shaking, and the cultures diluted (1:100) into fresh MHB supplemented with 3% NaCl and 1% glycerol. Culture OD was measured and adjusted to 0.1 with the medium, after which 180 µl of the diluted culture were plated into a round bottom 96-well plate. In the treatment groups, 20 µl of furanone (5 µM) or different sub-lethal concentrations of the ethanolic extract were added into each well (final concentrations of 5, 10 and 100 µg ml^{-1}), and in the control group the same amount of extract diluent was added at similar final concentrations, and the plate incubated for 24 h at 30 °C without shaking. Each group had 8 replicates. After the medium was discarded, the plate was gently washed twice by submerging under water, and incubated with 0.3% crystal violet aqueous solution at room temperature for 15 min. The plate was washed to remove unattached bacteria, and then air dried. The crystal violet stained biofilm was solubilized by adding 220 µl of 33% acetic acid in water to each well and the plate was incubated at room temperature for 15 min. The solubilized crystal violet solution was transferred to a new flat-bottom ELISA reader plate, and the OD was measured at 535 or 600 nm. Acetic acid (33%) in water was used as a blank. The percentage of biofilm biomass inhibition was determined using the following equation:

Percentage inhibition of biofilm biomass = [Control OD -Test OD/

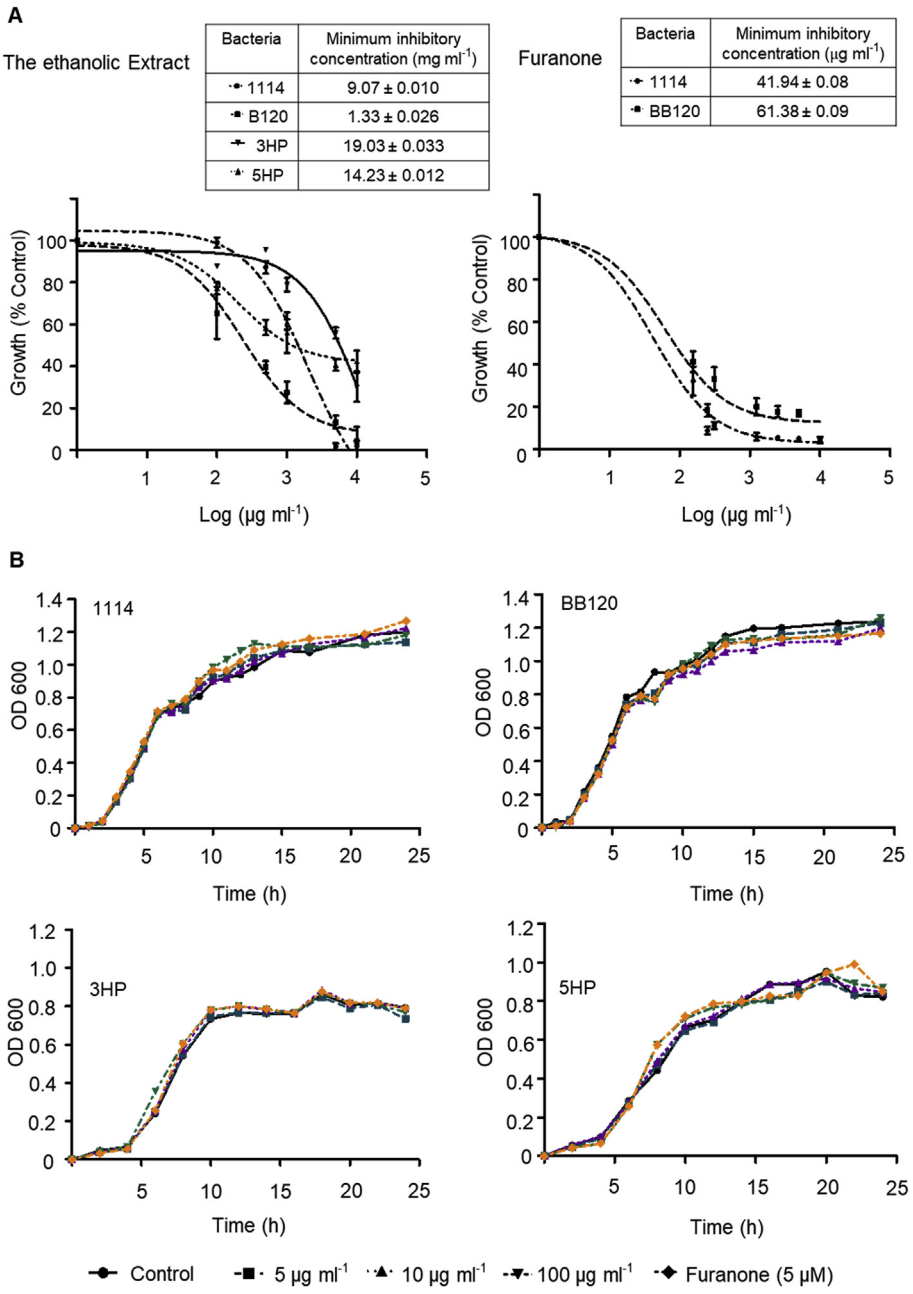

Fig. 1. The effects of the various concentrations and sub-lethal concentrations of the ethanolic extract or furanone against the bacteria growth
(A) Log dose–response curves showing the ethanolic extracts and furanone decreased the growth rate of the different strains of the bacteria. The MICs of the ethanolic extract against *V. harveyi* (1114 and BB120 strains) and *V. parahaemolyticus* (3HP and 5HP strains) are shown. (B) The growth curves of *V. harveyi* strains 1114, BB120 and *V. parahaemolyticus* strains 3HP, 5HP treated with or without the sub-lethal concentrations of the ethanolic extracts (5, 10 and 100 μg ml^{-1}) or furanone (5 μM) for 24 h. The results showed that sub-lethal concentrations of ethanolic extract did not disturb the growth rate of the bacteria.

Control OD] x 100

2.4.3. Confocal laser scanning microscopy (CLSM)

In order to visualize the bacterial biofilms formed the biofilms were grown on glass coverslips. The diluted bacterial culture (1 ml), prepared as above, was plated into a 24-well plate, each well containing a glass coverslip. Aliquots of 100 μl of furanone (5 μM) or the ethanolic extracts (final concentrations of 5 and 10 μg ml^{-1}) or the extract diluent (water) were added to the treated wells and control wells, respectively. After incubation for 24 h at 30 °C without shaking, the bacterial biofilm on the coverslips was stained with wheat germ agglutinin (WGA-Alexa Fluor® 488, green color) and non-viable bacteria were stained with propidium iodide (PI, red color). The biofilm on the coverslips was visualized under a confocal laser scanning microscope (Model FV1000 710; OLYMPUS). Images and the 3D model construction were obtained using LSM confocal software (FV10-ASW 4).

In order to determine if the extract disrupts mature biofilms, biofilms were grown on coverslips for 3 days. The mature biofilm was incubated with 20 μl of the ethanolic extracts (final concentrations of 5 and 10 μg ml^{-1}) for 1 h at 30 °C and biofilm integrity visualized under CLSM.

2.5. Bioluminescence assay

To study whether the ethanolic extract and furanone were able to interfere *V. harveyi* cell-cell communication, the bioluminescence assay was carried out as reported previously [17] using the wild type *V. harveyi* strain BB120 [12]. *V. harveyi* were cultured overnight at 30 °C with aeration in the autoinducer bioassay medium AB2746 (AB medium) and distributed in a 96-well plate to an OD_{600} of approximately 0.05 with fresh medium, then incubated with the ethanolic extracts or furanone for 4 h at 30 °C with shaking. After incubation, luminescence was measured using a microplate reader (Wallac model 1409, PerkinElmer). Bioluminescence was expressed as the percentage of control.

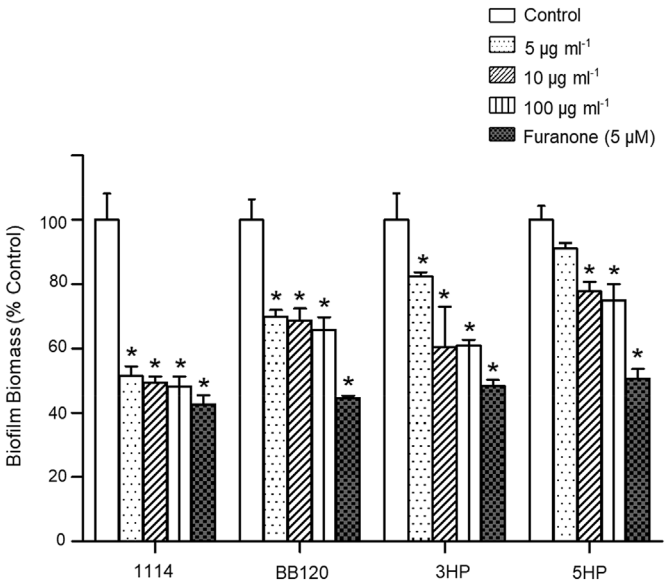

Fig. 2. The ethanolic extract and furanone decreased the production of biofilm biomass. Biofilm biomass of *V. harveyi* (strains 1114 and BB120) and *V. parahaemolyticus* (strains 3HP and 5HP) after treatment with the different sub-lethal concentrations of the ethanolic extracts or furanone using the crystal violet method. Data are presented as the percentage of biofilm biomass compared to that of control (mean ± SD of triplicate experiments).

To determine whether the ethanolic extract and furanone interfere AI-2 signaling, the *V. harveyi* mutant strain BAA-1119 which produces only autoinducer AI-2 was used as an inducer strain [18,19]. BAA-1119 is the mutant luxM deletion *Vibrio harveyi* (luxM produces autoinducer HAI-1), thus this strain contains only luxS that produces AI-2. The *V. harveyi* mutant strain BAA-1121 (containing AI-2 receptor, does not produce AI-2), was used as a reporter strain [20]. In this assay, the cell-free supernatant of BAA-1199 was used as a source of exogenous AI-2 to stimulate light production in BAA-1121. BAA-1119 cells were grown overnight in AB medium and the cell-free supernatant (containing AI-2) was collected by centrifugation at 12,000g for 5 min. The supernatant was passed through 0.22 μm Millipore filters (Millipore, Bedford, USA) before use [12]. *V. harveyi* BAA-1121 was grown overnight in AB medium, diluted to an $OD_{600\ nm}$ of 0.05 in fresh medium and plated into a 96-well plate. BAA-1121 cells were then treated with the cell-free supernatant of BAA-1119 (1:10 dilution) plus the ethanolic extracts (5 and 10 μg ml^{-1}) or cell-free supernatant of BAA-1119 plus furanone for 4 h at 30 °C with shaking. The bacterial luminescence was measured using a microplate reader (Wallac model 1409, PerkinElmer). The BAA-1121 treated with the cell-free supernatant of BAA-1119 only was set as a positive control and BAA-1121 cells treated with fresh medium only was set as a negative control.

2.6. Bacterial challenge assay

2.6.1. Preparation of bacteria enriched artemia

Bacteria *(V. harveyi, V. parahaemolyticus)* were cultured overnight and the bacterial suspensions were adjusted to an absorbance of 1.0 at OD 600 nm (approximately 1×10^8 CFU ml^{-1}). One-hundred grams of artemia (200 artemia) were allowed to filter feed on the bacterial suspension (10 ml) for 30 min. The pre-washed artemia (n = 10) were examined for bacterial concentration by spreading on TCBS agar and bacterial colonies counted. There were approximately 1.4×10^2 CFU of bacteria per artemia.

2.6.2. Challenge assay

Healthy adult white leg shrimp, *Penaeus vannamei* (averaged 15 g body weight) were obtained from a commercial shrimp farm in Samutsakorn, Thailand. The shrimp were cultured in circular tanks (58x92 × 160 cm plastic-lined tank), 20 ppt salinity and fed twice daily with artemia at 10% body weight/meal. Shrimp were divided into 5 groups (n = 30), a control and four bacterial-challenge groups. The control group was shrimp fed with normal artemia. The bacterial-challenge groups comprised (1) shrimp fed with normal artemia, (2) shrimp fed with 50 μg ml^{-1} ethanolic extract enriched artemia, (3) shrimp fed with 100 μg ml^{-1} ethanolic extract enriched artemia, and (4) shrimp fed with furanone (5 μM). Each treatment was performed in triplicate. After 7 days of feeding, artemia infected with *V. harveyi* or *V. parahaemolyticus* were fed to the shrimp (10 infected artemia/shrimp). Shrimp mortalities were observed for 5 days after the bacterial challenge. All shrimp and artemia were conducted according to the ethical guidelines of animal experimentations and supervised by the ethical committees of Mahidol University–Institutional Animal Care and Use Committee (MUSC–IACUC: MUSC61-016-418).

2.6.3. Scanning electron microscopy (SEM)

Shrimp were divided into the experimental groups as above. The shrimp (n = 5) from each treatment group were collected at 24 h after infection. The stomach samples were collected from shrimp received bacterial infection via oral route while the carapace samples were collected from shrimp received bacterial infection via immersion (1×10^6 CFU L^{-1}). The tissues were processed according to the standard method of SEM. Adhesion and colonization of *V. harveyi* into the epithelial surfaces of the stomach and carapace of shrimp were observed under a scanning electron microscope (Hitachi SU3500, Japan). The shrimp samples were also collected to determine the bacterial colony count and expression of a virulence gene.

2.6.4. Bacteria colony counting

Gut were collected from shrimp (n = 5) using the sterile scissors, then washed 3 times with 1xPBS and liquefied in 100 ml of 1xPBS. Thiosulfate citrate bile salt sucrose (TCBS) agar (Oxoid Ltd., Basingstoke, England) was a selective agar for Vibrio spp. Fifty microliters of sample solution were spread on TCBS agar plates then incubated at 37 °C for 24 h. The colonies of bacteria were counted by observing green or yellow-green colonies on the agar.

2.6.5. Expression of a quorum sensing virulence gene

The stomach and gut from each experimental group were used to determine expression of the virulence gene, *VopD* (T3SS, virulence factor) by quantitative real-time polymerase chain reaction (RT-qPCR). The total RNA was extracted using the Trizol reagent (Invitrogen, CA, USA) according to the manufacturer's instruction. The RNA samples were stored at −80 °C.

cDNA was transcribed from the RNA (1 μg) using the RevertAid First strand cDNA synthesis kit (Thermo scientific, USA) according to the manufacturer's instruction. The *VopD* gene and the *rpoA* genes (an internal control) were amplified by RT-qPCR using the synthesized cDNA, a SYBR-green detection system (Biorad, CA, USA), and the specific primer pairs as follow: *rpoA* forward 5′-CGTAGCTGAAGGCAAAGA TGA-3′, *rpoA* reverse 5′-AAGCTGGAACATAACCACGA-3'; *VopD* forward 5′- TGAGCAACAGGTTCTGCAAC-3′, *VopD* reverse 5′- GCGACTTCTGC CTTGATTTC-3'. Duplicate RNA samples from three independent experiments were transcribed, and amplification the CT value (cycle threshold) was set at 40 cycles using the Bio-Rad CFX Manager software (Biorad). Values were normalized with reference to *rpoA* and relative changes in transcript levels were calculated using the comparative CT method.

2.7. Statistical analysis

All experiments were performed in triplicate (three independent experiments). The data are presented as mean ± SD and analyzed by one-way ANOVA followed by Turkey's multiple comparison test using

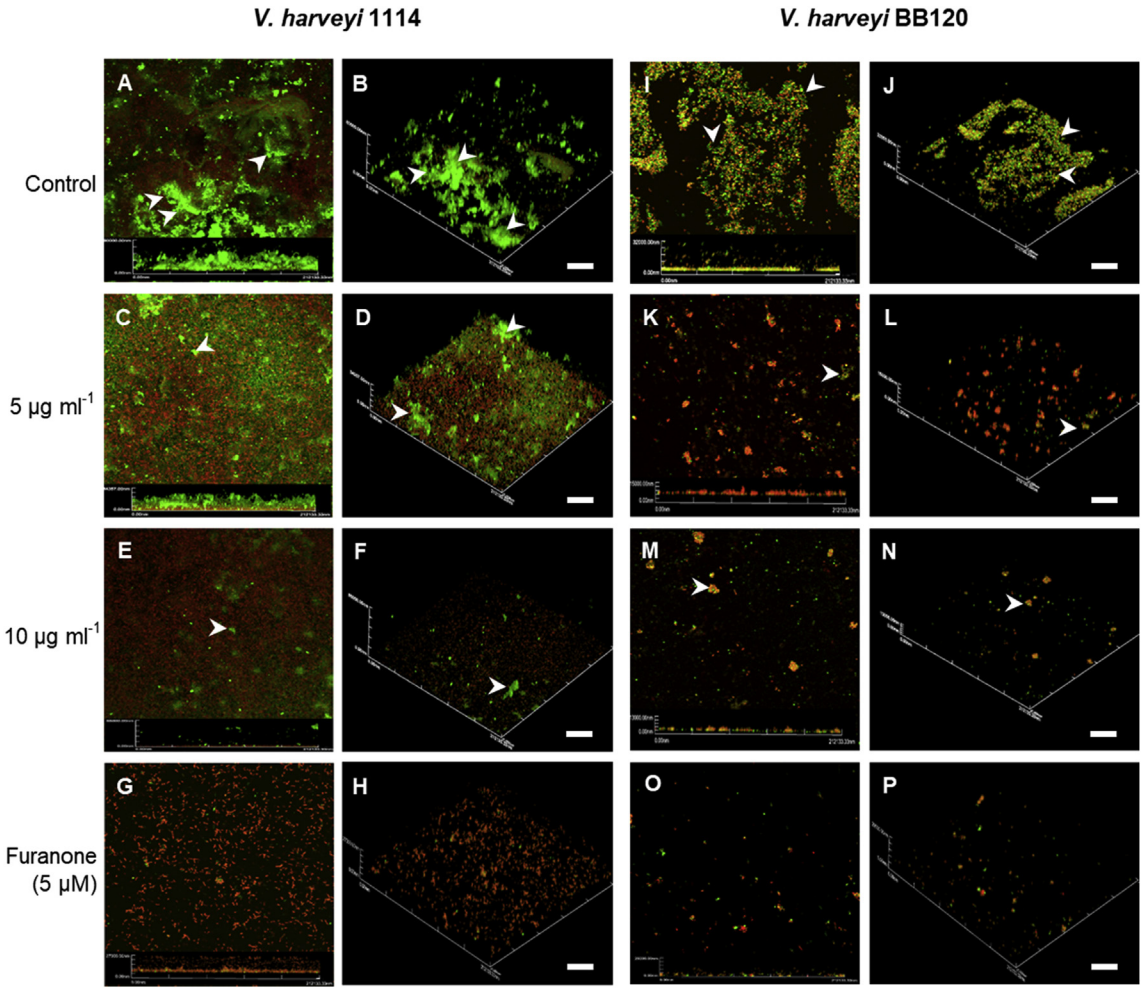

Fig. 3. Representative CLMS images of *V. harveyi* biofilms after treatment with the ethanolic extracts or furanone. Biofilms were grown for 4 h then treated with the ethanolic extracts for 24 h. The CLMS images showing anti-biofilm activity of the ethanolic extract against biofilm formation by *V. harveyi* strains 1114 and BB120. Control untreated, A and B (1114); I and J (BB120). Treatment with 5 μg ml^{-1} ethanolic extract, C and D (1114); K and L (BB120). Treatment with 10 μg ml^{-1} ethanolic extract, E and F (1114); M and N (BB120). Treatment with furanone, G and H (1114); O and P (BB120). Green represents biofilms (WGA stained; white head arrow). Red represents dead bacteria (PI stained). Left, top down and side views; Right, 3D representation of the corresponded biofilms. Scale bar = 20 μm.

the GraphPad Prism program version 5 (GraphPad software, CA). Statistically significant difference was required at p-value less than 0.05.

3. Results

3.1. In vitro antibacterial activity of G. fisheri ethanolic extract

MICs and MBCs of the ethanolic extract against the four bacterial strains tested were in the range of 1.33–19.03 mg ml^{-1} whereas the MICs of furanone were 41–61 μg ml^{-1} (Fig. 1A.). Among them, the wild type strain *V. harveyi* BB120 showed the greatest sensitivity to the ethanolic extract. The bacteria treated with either furanone or the ethanolic extract showed significantly ($p < 0.001$) decreased growth rate from control (Fig. 1A). However, treatments of the bacteria with the sub-MIC concentrations of the ethanolic extracts (5, 10 and 100 μg ml^{-1}) or furanone (5 μM) showed no inhibitory effect on the growth of the bacteria and bacterial cell viability (Fig. 1B), suggesting that these low concentrations of the ethanolic extract and furanone were suitable for studying the effects on QS of the bacteria.

3.2. The low concentrations of the ethanolic extract decreased biofilm biomass produced by V. harveyi and V. parahaemolyticus

The inhibitory effect of the sub-MIC concentrations of the ethanolic extract and furanone on the quorum sensing regulatory system was evaluated by determining the biofilm development by the bacteria. The results revealed that biofilm inhibition by the ethanolic extract was concentration-dependent. Treatment with the sub-MIC concentrations of the ethanolic extracts (5, 10 and 100 μg ml^{-1}) or furanone decreased biofilm biomasses of *V. harveyi* 1114 to 51.55, 49.43, 49.23 and 42.60% of control, respectively; *V. harveyi* BB120 biofilm biomasses were decreased to 70.00, 68.72, 65.88 and 44.67% of control, respectively. Treatment with the sub-MIC concentrations of the ethanolic extracts (5, 10 and 100 μg ml^{-1}) decreased biofilm biomasses of *V. parahaemolyticus* 3HP to 82.55, 60.41 and 60.87% of control, respectively; *V. parahaemolyticus* 5HP biofilm biomasses were decreased to 91.18, 77.87 and 75.10% of control, respectively (Fig. 2).

3.3. Confocal laser scanning microscopy (CLSM)

Since the sub-MIC concentrations of ethanolic extract inhibited biofilm biomass, we further evaluated their anti-biofilm formation by CLSM analyses. The results are as follows: untreated *V. harveyi* 1114 showed a thick mat of biofilm (coverslips stained with WGA, green

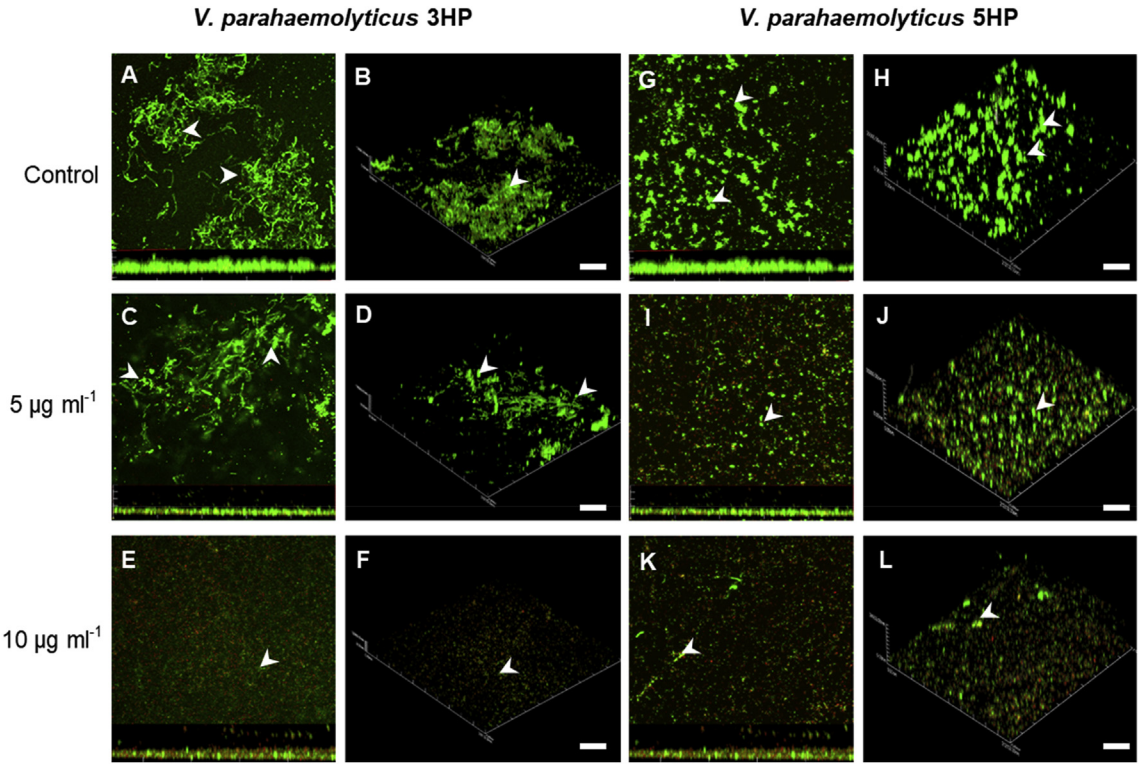

Fig. 4. Representative CLMS images of *V. parahaemolyticus* biofilms after treatment with the ethanolic extracts. Biofilm was grown for 4 h and then treated with the ethanolic extracts for 24 h. The CLMS images showing inhibitory effect of the ethanolic extracts against biofilm formation by *V. parahaemolyticus* strains 3HP and 5HP. Control untreated, A and B (3HP); G and H (5HP). Treatment with 5 μg ml^{-1} ethanolic extract, C and D (3HP); I and J (5HP). Treatment with 10 μg ml^{-1} ethanolic extract, E, F (3HP); K, L (5HP). Green represents biofilms and live bacteria (WGA stained; white head arrow). Red represents dead bacteria (PI stained). Left, top down and side views; Right, 3D representation of the corresponded biofilms. Scale bar = 20 μm.

color) (Fig. 3A–B); *V. harveyi* 1114 cultured with the ethanolic extracts (5 and 10 μg ml^{-1}) showed a dose-dependent reduction in the thickness of biofilm, noticeable changes architecture of the biofilm and increasing the amount of dead cells (stained with PI, red color) (Fig. 3C–F) similar to those treated with furanone (Fig. 3G–H). *V. harveyi* BB120 treated with the extract or furanone also showed similar results (Fig. 3I-P). *V. parahaemolyticus* 5HP (Fig. 4A–F) and *V. parahaemolyticus* 3HP (Fig. 4G-L) cultured with the ethanolic extracts (5 and 10 μg ml^{-1}) clearly showed a dose-dependent reduced thickness of biofilm formation on the coverslips in a trend similar to their corresponding biofilm biomasses (Fig. 2). Mature biofilm (produced over 3 days) treated with the ethanolic extracts for 1 h became less dense and dis-aggregated when compared with control. The shape of biofilm changed from thick matt to pisiform-like shape with the lower height of the films (Fig. 5). These findings suggest that the ethanolic extract not only reduces the quantity of biofilms but also apparently disrupts the architecture of the mature biofilms produced by *V. harveyi*.

3.4. Effect of low concentrations of the ethanolic extract on bioluminescence in V. harveyi

The effect of the ethanolic extract on cell-cell communication of the bacteria was evaluated by bioluminescence assay. The wild type *V. harveyi* strain BB120 and reporter mutant strain *V. harveyi* BAA-1121 (normally no light emission) were used. The strain BAA1119 (produce AI-2 autoinducer) was used to induce the bioluminescence of BAA-1121 in the absence or presence of the ethanolic extract or furanone. As expected, no light was produced in the control BAA-1121 culture. Both the extract and furanone inhibited light production of the wild type strain BAA-1116. Furthermore, the BAA-1121 cultured with the cell-free supernatant from BAA-1119 showed a high luminescent intensity production. When *V. harveyi* BAA-1121 were co-treated with the cell-free supernatant plus the ethanolic extracts (5 and 10 μg ml^{-1}) there was a reduction in light emission in a dose dependent manner. A similar reduction occurred with furanone co-treatment cultures (Fig. 6). Thus, the results provide evidence that the ethanolic extract and furanone significantly inhibited light production by interfering with the auto-inducer AI-2-mediated bioluminescence in *V. harveyi*.

3.5. Low concentrations of the ethanolic extract protected shrimp mortality induced by Vibrio spp. infection

Shrimp pre-treated with sub-MIC concentrations of the ethanolic extract or furanone and challenged with the bacteria showed significantly higher survival rates compared to control (no pre-treatment). Shrimp fed with the 100 μg ml^{-1} ethanolic extract showed the highest survival rates, 83.0 ± 2.3% and 93.3 ± 4.1% against *V. harveyi* and *V. parahaemolyticus*, respectively. Similar results were found in shrimp treated with furanone. The survival rates were 86.0 ± 0.5% and 90.0 ± 1.5% against *V. harveyi* and *V. parahaemolyticus*, respectively (Fig. 7). These results suggest that sub-MIC concentrations of the ethanolic extract or furanone could decrease the mortality of shrimp *P. vannamei* orally inoculated with *V. harveyi* and *V. parahaemolyticus*.

3.6. Low concentrations of the ethanolic extract ameliorated the bacterial count, bacterial adhesion and colonization in shrimp gut

In shrimp orally inoculated with the bacteria only, at 6 h after inoculation, many bacterial colonies were found adhesion into the gut luminal surface. At 24 h after inoculation, the number of bacteria decreased, however, a substantial number of bacterial colonies remained in the gut lumen. Whereas the ethanolic extract and furanone treated shrimp showed less number of the bacterial colonies in the gut lumen at both 6 h and 24 h after inoculation compared to bacterial control

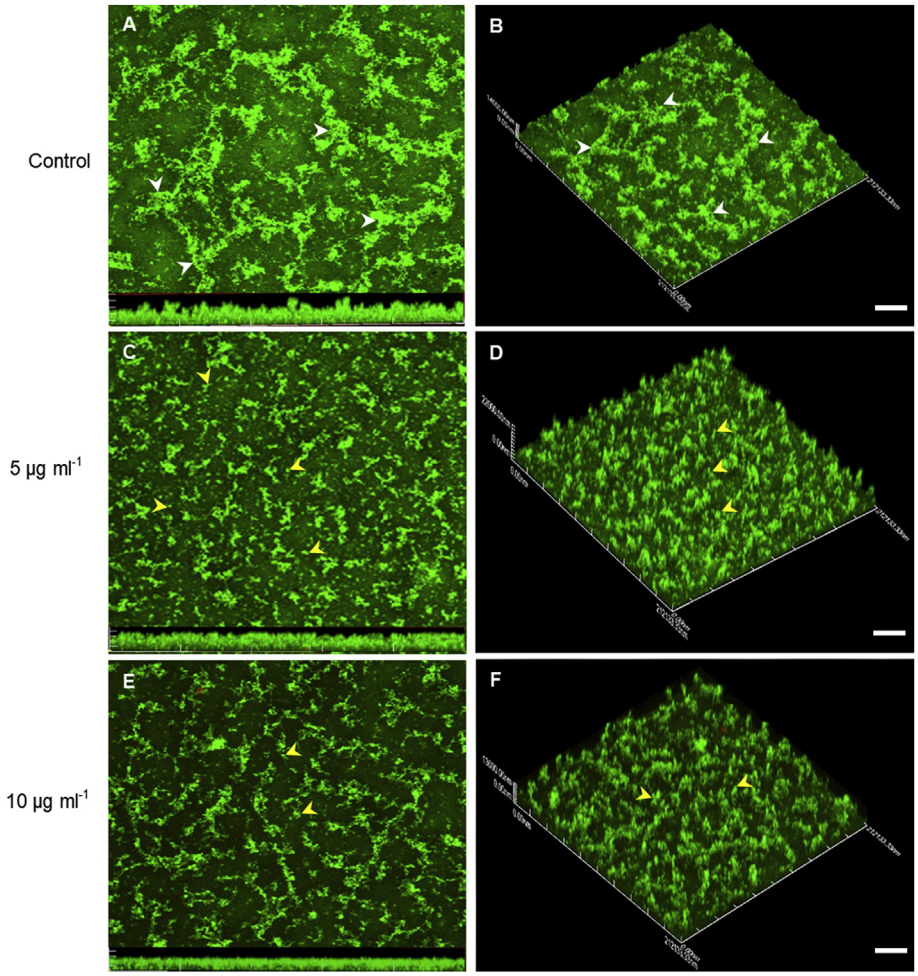

Fig. 5. Representative CLMS images of mature *V. harveyi* biofilms after treatment with the ethanolic extracts. Biofilms were grown for 3 days and treated with the ethanolic extracts for 1 h. The CLMS images showing effect of the ethanolic extracts on the mature biofilms of *V. harveyi*. Control untreated, A and B. Treatment with 5 μg ml^{-1}, C and D. Treatment with 10 μg ml^{-1}, E and F. Green represents biofilms and live bacteria (WGA stained). White head arrows indicate thick and large biofilms. Yellow head arrows indicate thin and dispersing biofilms. Left, top down and side views; Right, 3D representation of the corresponded biofilms. Scale bar = 20 μm.

shrimp (Fig. 8).

SEM was carried out to examine the bacterial adhesion into the luminal surface of the stomach and carapace of shrimp. The luminal surface of the stomach in control shrimp showed the peritrophic membrane (PM) and food particle (FP) without bacterial cells (Fig. 9A). At 24 h after inoculation, the bacterial control group showed bacteria embedded in the peritrophic membrane and food particles. A massive number of rod shaped bacteria of a single morphotype firmly attached to the surfaces of stomach lumen. The attached bacteria showed pili-like structures (P) connecting with each other (Fig. 9B). Whereas shrimp fed with the ethanolic extract showed less bacteria adhesion to stomach (Fig. 9C). Similar results were observed in the shrimp fed with furanone; less number of bacteria adhesion to the PM surface (Fig. 9D). Furthermore, the bacterial control shrimp after challenge via the bacteria immersion showed the bacteria adhesion and packed on the carapace surfaces with the presence of pili-like structures (Fig. 10A). Whereas the ethanolic extract and furanone treated shrimp showed bacteria loosely attached to the carapace surfaces (Fig. 10B–C). These findings were correlated well with the less colony counting and lower mortality rates in the shrimp fed with the ethanolic extract or furanone.

3.7. Low concentrations of the ethanolic extract down-regulated VopD gene expression

The expression of virulence factor of *V. harveyi*, *VopD* gene was determined in shrimp fed with the ethanolic extracts and furanone by q-RT-PCR after oral inoculation for 24 h compared to A subunit (*rpoA*) mRNA, an internal control. The results showed that shrimp treatment with the extracts or furanone showed decreased expression of *VopD* compared to the infected control shrimp. However, furanone treatment shrimp induced a lower level of *VopD* expression than the ethanolic extract (Fig. 11).

4. Discussion and conclusion

Vibrio species like *V. harveyi* and *V. parahaemolyticus* present a serious problem of infection in aquatic ecosystems. One key factor for bacterial survival is their ability to form biofilms which is a preferred way for bacteria to improve growth and survival by providing access to nutrients and protection from predators or antimicrobial compounds [21]. Therefore, new therapeutic means to control bacterial biofilm growth either by diminishing resistance or by altering the micro-organism's defensive capability are urgently required.

G. fisheri are found in the gulf of Thailand and most commonly used in shrimp ponds as a source of nutrient and wastewater treatment. Shrimp co-cultured with *G. fisheri* seem to be healthier with less bacterial infection than those cultured without the seaweed. This effect may be caused by the metabolites that are released from *G. fisheri* or by the components of *G. fisheri* itself. Our previous study showed that the ethanolic extract of *G. fisheri* exhibited anti-bacterial activities and stimulated the immune response in shrimp [11]. In this study, we examined the ethanolic extract and compared it with furanone for the anti-biofilm activity. First it was determined that the ethanolic extract showed MICs ranging from 1.33 to 19.03 mg ml^{-1} against *V. harveyi* and *V. parahaemolyticus* which was in agreement with our previous study [11]. We further demonstrated that the ethanolic extract at sub-MIC concentrations did not disturb the growth of free-living (planktonic) cells or bacterial cell viability, thereby assuring that the effect of

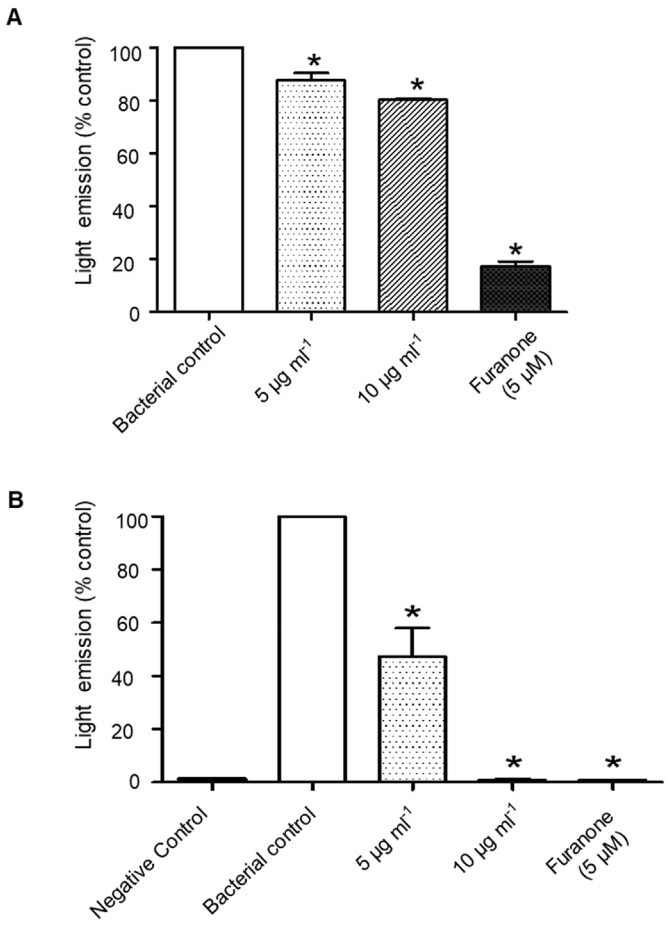

Fig. 6. Effect of the ethanolic extract on the bioluminescence in *V. harveyi*. (A) The light emission (% of control) in the *V. harveyi* BB120 determined at 4 h after the additions of 5 and 10 µg ml^{-1} of the ethanolic extract or furanone (5 µM). (B) Interference of the ethanolic extract with *V. harveyi* AI-2 communication was determined by bioluminescence assay. Cell-free supernatant from BAA-1119 that contained AI-2 signal only was used to stimulate the light emission in the reporter mutant strain *V. harveyi* BAA-1121. Light emission produced by *V. harveyi* BAA-1121 was reduced in the presence of the ethanolic extracts or furanone. Data are presented as the percentage of light emission compared to that of control (mean ± SD of triplicate experiments). * indicates a statistically significant difference ($p < 0.05$) from the control.

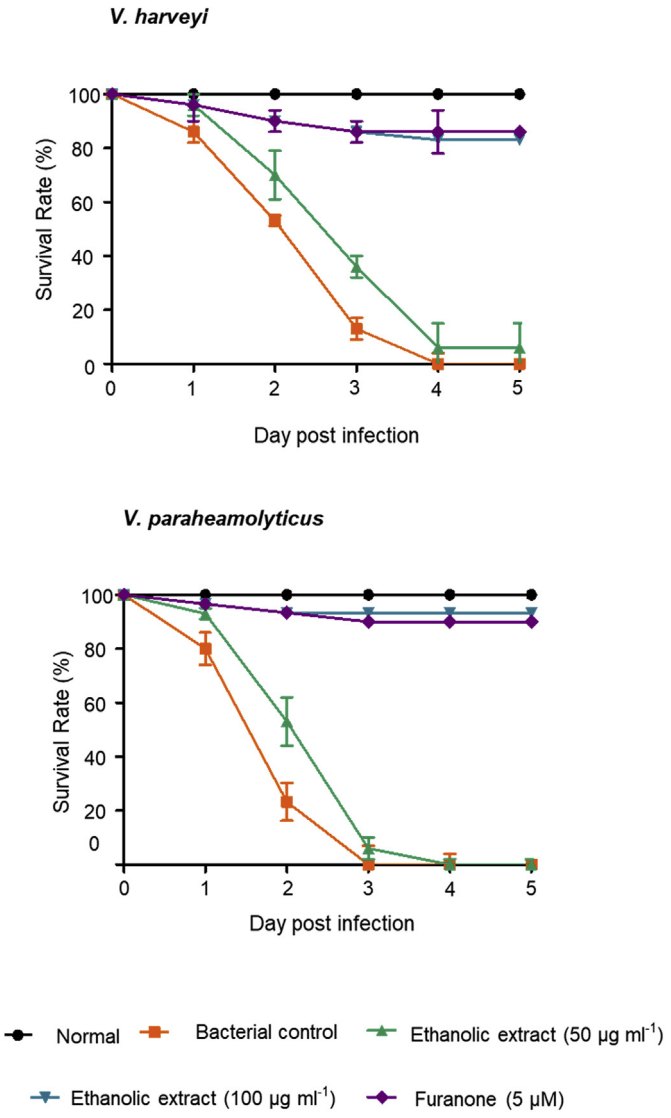

Fig. 7. Survival rates of shrimp fed with the ethanolic extract or furanone and challenged with *V. harveyi* or *V. parahaemolyticus*. Data are presented as mean ± SD of triplet experiments. The results showed the ethanolic extracts or furanone improved the survival rates in shrimp infected with *V. harveyi* or *V. parahaemolyticus*.

sub-MIC concentrations of the extract in the subsequent biofilms and bioluminescence assays was not a result of an anti-microbial (killing) effect.

V. harveyi bacteria treated with sub-MIC concentrations of the ethanol extracts (5, 10 and 100 µg ml^{-1}) or furanone (5 µM) showed significant biofilm biomass reduction. Moreover, image analysis of the characteristics of biofilms architecture using CLSM contrasted different features between the control and treatment groups. After exposure to the ethanolic extracts and furanone, the biofilms became loosely distributed with a thinner matt and showed an abundance of dead bacterial cells suggesting that they inhibited biofilms development. Similar changes were reported when *V. harveyi* were treated with low doses of citrus flavonoid [17]. Halogenated furanone from *Delisea pulchra* inhibited the biofilms formation of *Escherichia coli* [22], *Bacillus subtilis* [23] *Aeromonas hyrdrophila* [24] and *V. harveyi* [25]. These compounds caused deterioration of biofilms and cells released from the biofilms became susceptible to antimicrobial treatments [26]. At the initial stage of biofilms formation, the planktonic cells adhere to a surface and this result in rapid growth of the bacteria. Therefore, we considered that preventing bacterial adhesion in the initial stage would be a prudent way to inhibit biofilms formation since once the mature biofilms are formed it would make the bacteria become much more resistant [27,28]. In this study we demonstrated that the ethanolic extract could prevent biofilms formation as early as at the initial attachment stage and were able to destroy mature biofilms, this would provide for a more effective strategy of the extract. Additionally, we found the ethanolic extract could prevent bacterial attachment on the carapace surface of shrimp.

Bioluminescence is a product of cell-cell communication that plays important roles in bacterial infection. In this study, we revealed that the low concentrations of ethanolic extract inhibited light production similar to furanone. In addition, the bioluminescence assay using the *V. harveyi* mutant strains suggests that the ethanolic extract and furanone may mediate inhibition of the bioluminescence production via interfering autoinducer AI-2 signaling in the bacteria. The result of furanone was in agreement with a previous study that it disrupted the AI-2 signaling in *V. harveyi* [29]. Furthermore, a recent report demonstrated that furanone disrupted the QS in *V. harveyi* by decreasing the DNA-binding activity of the quorum sensing master regulator LuxR [30]. To elucidate the underlined QS inhibition of the ethanolic extract on a

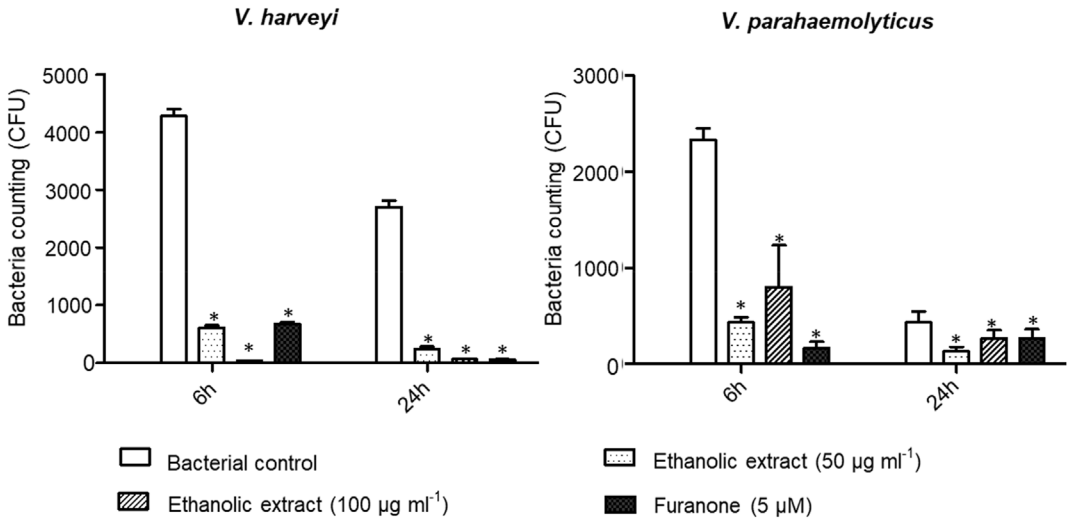

Fig. 8. The effect of the ethanolic extract and furanone on the bacterial colony count in gut at 6 and 24 h after oral inoculation. Shrimp fed with the ethanolic extracts or furanone showed decreased number of *V. harveyi* or *V. parahaemolyticus* colonies in the gut lumen. Data are presented as mean ± SD of triplet experiments.

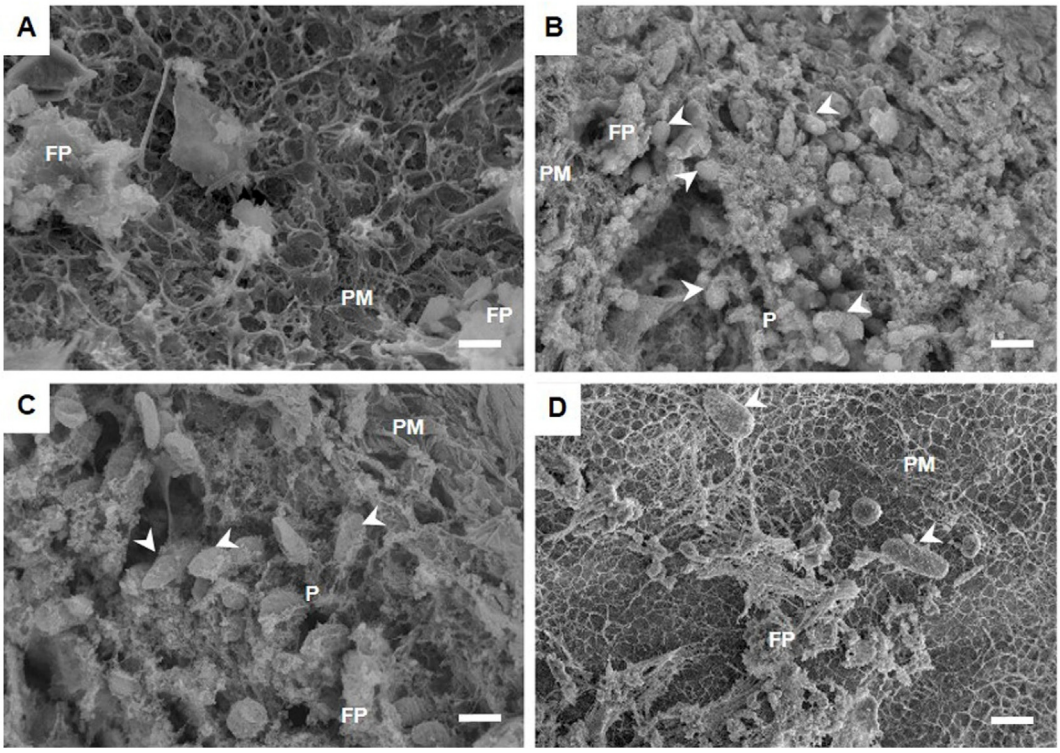

Fig. 9. Representative SEM micrographs showing *V. harveyi* adhesion and colonization into the stomach luminal surface of the *P. vannamei* after fed with the bacteria enriched artemia for 24 h. (A) Non-treated control shrimp showed the surface of peritrophic membrane (PM) and food particle (FP) with no bacteria adhesion. (B) Infected control shrimp showed numerous bacterial cells (white head arrow) with numerous pili-like structure (P) connecting to either the adjacent bacteria and the surface. Bacteria attached, packed and colonized in the digestive tract peritrophic membrane (PM). Shrimp fed with the ethanolic extract (C) or furanone (D) and received bacteria showed less number of bacteria adhesion, pili structures and colonization. Scale bar = 5 μm.

molecular level in *V. harveyi* requires further research.

The inhibitory effects of the ethanolic extract and furanone on biofilm biomass and bioluminescence in the *in vitro* experiments were correspondence with the *in vivo* challenge experiments. The SEM results revealed that shrimp fed with the ethanolic extract and followed with *V. harveyi* oral inoculation had less bacteria adhesion to the gut luminal surface which data corresponded to the low number of the bacterial colony counting. Furthermore, the less biofilms formed and the down-regulation of *VopD* gene by the ethanolic extract were associated with the lowered shrimp mortality following *V. harveyi* infection. The effect of furanone in the present study was in accordance with the previous report that furanone at concentration similar to that needed to block quorum sensing could protect the *Artemia* nauplii mortality from *V. harveyi* infection [29].

It has been shown that in the gut of shrimp, bacteria attach, colonize and form biofilms on the surfaces of the intestinal lumen, particularly the stomach, where the bacteria are protected from antimicrobial agents and the host immune system, contributing to the resistance to

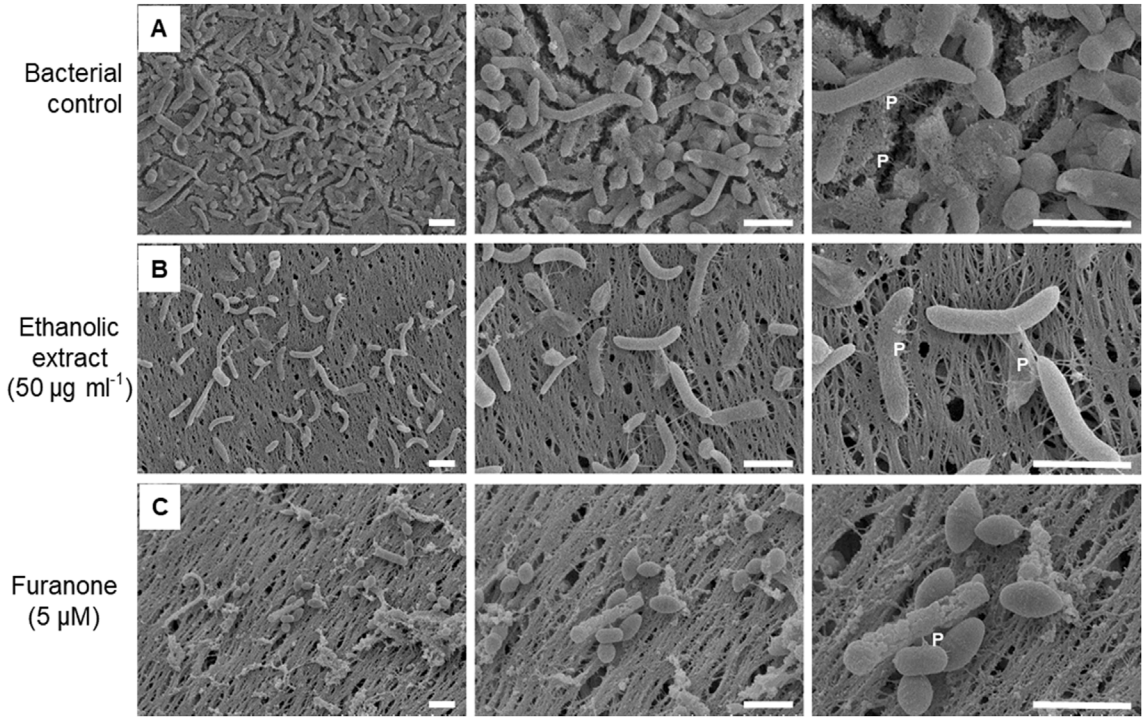

Fig. 10. Representative SEM micrographs showing adhesion and colonization of *V. harveyi* onto the carapace surface of the *P. vannamei* after immersion with the bacteria for 24 h. (A) Infected control shrimp showed numerous bacterial cells attached on the carapace surfaces with thick matrix, extensive pili-like structures (P) connecting between bacteria and bacterial colonization. Shrimp received bacteria and the ethanolic extract (B) or furanone (C) showed less number of attached bacteria and pili-like structure (P). The middle and left columns are higher magnifications of the correspondent treatments. Scale bar = 2 µm.

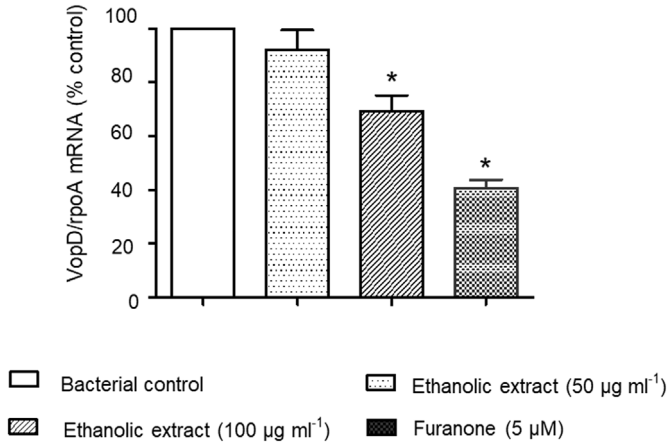

Fig. 11. The expression of virulence *VopD* gene in gut lumen in the shrimp fed with the ethanolic extract or furanone. The expression of *VopD* gene is presented in relative to *rpoA* mRNA. Data are presented as mean ± SD of triplet experiments. * indicates a statistically significant difference ($p < 0.05$) from the control.

treatments, and they use virulent factors and enzymes to infect the host [31]. Bacterial biofilms release extracellular polymeric substances that act as a barrier to both effectors of the immune system and antimicrobial agents [32]. Indeed, biofilms infection is rarely resolved by the host unless the biofilms are destroyed. Based on the current data we propose that the low concentrations of the ethanolic extract, once in the gut lumen, it prevented bacterial adhesion to the gut surface and, thus, biofilms formation was restricted. Therefore, it is reasonable to predict that the perturbation of biofilms formation by the ethanolic extract would increase the susceptibility of biofilm cells to shrimp haemocytes-bacteria released from the biofilms were then eliminated by the host immune defense. The enhanced immune response along with the less virulence factor released resulted in increased shrimp survival rate. The present data correlate well with our previous report [11] that the ethanolic extract of *G. fisheri* enhanced the shrimp immune activities such as haemocyte counts, the proPO and superoxide dismutase activities. Additional studies are needed to further understanding host immune-Vibrio biofilms interaction. Taken together, our results showed that the ethanolic extract and furanone at sub-MIC concentrations interfered quorum-sensing, in a lesser extent, bioluminescence and the biofilm formation activities of *V. harveyi*. These anti-QS activities of the ethanolic extract could conceivably be improved through opportune bioactivity guided fractionation of the extract, which may uncover promising bioactive QS inhibitors in the extract.

In conclusion, this study is the first to demonstrate the anti-QS inhibition property of the ethanolic extract from *G. fisheri* which shows a similar function as the known QS inhibitor, furanone. The ethanolic extract may provide a viable alternative treatment of bacterial infections associated with biofilms formation, in particular prevention of *V. harveyi* and *V. parahaemolyticus* infections for the aquaculture industry.

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