

# The Potential of Galangal (*Alpinia galanga* Linn.) Extract against the Pathogens that Cause White Feces Syndrome and Acute Hepatopancreatic Necrosis Disease (AHPND) in Pacific White Shrimp (*Litopenaeus vannamei*)

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## Abstract

White feces syndrome and acute hepatopancreatic necrosis disease (AHPND) are serious diseases that have recently been noted in Pacific white shrimp (*Litopenaeus vannamei*). *Vibrio* bacteria and 6 species of fungi (*Aspergillus flavus*, *A. ochraceus*, *A. japonicus*, *Penicillium* sp., *Fusarium* sp., and *Cladosporium cladosporioides*) were isolated from shrimp naturally infected with white feces syndrome. Antibiotics have been used to treatment the disease for many years, but these have been ineffective and have resulted in drug residue problems for the shrimp industry. In this study, an alternative method was tested for its efficacy in controlling these pathogens. The crude extract of galangal (*Alpinia galanga* Linn.), an herbal medicine, inhibited the growth of 8 vibrio species of the pathogen, *V. parahaemolyticus* (EMS/AHPND) in particular. The results also showed that 0.5 mg/ml of the galangal extract was a concentration that produced the strongest inhibition of the fungi *A. ochraceus*. Naturally infested shrimp *L. vannamei* were fed 2 and 4% (v/w) portions of the herb extract for 12 days and their progress was compared with that of a control group (no herb extract). At the end of the feeding trial, the numbers of total *Vibrio* spp. and the incidence of fungi infestation in the hepatopancreas and intestines of treated shrimp were significantly lower than that in the control group ( $P < 0.05$ ). Furthermore, the survival rates for the treatment groups, after injections with *V. parahaemolyticus* (EMS/AHPND), were significantly higher than that of the control group ( $P < 0.05$ ). Based on these results, we can report that the galangal extract has antimicrobial properties that are applicable as bio-medicinal agents against white feces syndrome and AHPND. Therefore, in the future this herb should be an alternative to chemotherapeutic agents that are being used in the shrimp industry.

**Keywords:** galangal, *Alpinia galanga* Linn., *Vibrio* spp., *V. parahaemolyticus* EMS/AHPND, fungi, *Litopenaeus vannamei*

## 1. Introduction

Disease outbreaks caused by viruses, bacteria and protozoa are important disease agents within the shrimp aquaculture, a major industry in Southeast Asia, because they can lead to serious economic losses for long periods of time. These bacterial disease pathogens have grown rapidly due to global warming, which has stressed the shrimp population by reducing immunity and enhancing the infection rates. In 2009, the first reports of an emerging disease in *Penaeus* sp. shrimp was initially named Early Mortality Syndrome (EMS) in China. This disease was later referred to as Acute Hepatopancreatic Necrosis Disease (AHPND). In 2011, EMS/AHPND occurred frequently in Vietnam, which led to the isolation of the causative agent, *V. parahaemolyticus* (Tran et al., 2013). In 2012, AHPND had a major impact on shrimp farmers in Thailand and Southeast Asian who sustained heavy losses in production (Flegel, 2012; NACA-FAO, 2011). Outbreaks of disease reported in Thailand during 2010-2011 called White feces syndrome were found mostly in Pacific white shrimp (*L. vannamei*). Infestations of Gregarine protozoa and *Vibrio* bacteria have been found that caused loose shells,

decreased appetite, retarded growth, and finally sporadic mortality of the shrimp in grow out ponds. Typical symptoms of the disease can be noted by white feces floating on the water surface in the rearing pond (Tangtrongpiros, 2010). Several reports have shown that vibrio bacteria including *V. alginolyticus*, *V. cholerae*, *V. parahaemolyticus*, *V. vulnificus*, *V. mimicus*, and *V. harveyi* are the important pathogens, as these are always isolated from diseased *L. vannamei* (Lighter, 1993; Vandenberghe et al., 1999; Montero & Austin, 1999). Bacterial pathogens can cause problems that range from growth retardation to sporadic mortalities and mass mortalities of shrimp. From this point of view, vibrio bacteria represent the greatest bacterial threat to the shrimp industry. When shrimp are under stressful conditions, such as when they are reared in a high-density setting, their immunity is lowered. Under those conditions, vibrio bacteria easily attack, which leads to the occurrence of disease (Ruangpan et al., 1997; Sung et al., 2001; Karunasagar et al., 2004; Lee et al., 2009). Some aquatic fungi are also opportunistic pathogens. Fusariosis or black gill disease is caused by *Fusarium* spp., and it may affect all developmental stages of penaeid shrimp (Karunasagar et al., 2004). Shrimp farmers have few choices to control the diseases that threaten their shrimp production. Many types of drugs and chemicals have been used to treat vibrio bacteria, and this has created a residual drug problem that has led to the spread of drug-resistant bacterial strains in the shrimp industry. To solve these problems, medicinal herbs have been introduced as alternative methods for the control and treatment of bacterial diseases, because herbs are known to have anti-parasite and antimicrobial effects. Galangal (*Alpinia galanga* Linn.) is one of the typical herbs that can be used for the treatment of diseases in shrimp, and it is available year-round at a low cost. This herb is rich in phenolic compounds such as flavonoids and phenolic acids (Mayachiew & Devahastin, 2008). The essential oil of the crude galangal extract is responsible for its antimicrobial activity (de Pooter et al., 1985; Turker et al., 2002; Oonmetta-aree et al., 2006; and, Rao et al., 2010). Therefore, we tested the potential efficacy of an ethanol galangal crude extract to inhibit and reduce the number of *Vibrio* spp. bacteria and fungi in natural infestations of white feces syndrome and AHPND in Pacific white shrimp (*L. vannamei*).

## 2. Materials and Methods

### 2.1 Preparation of Galangal-Ethanol Extract

Fresh galangal rhizomes were purchased from a local market in Chanthaburi, Thailand. The rhizomes were sliced into thin layers and dried at room temperature in a tray dryer followed by heating at 45 °C for 24 h. After drying, they were ground into powder using an electric blender (Philips, Cucina, Thailand). Ten grams of the galangal powder were suspended in 100 ml of ethanol, and then let stand at room temperature overnight. After filtration through filter paper (No. 1, Whatman International Ltd., Maidstone, UK), the extract was dried in a rotary evaporator (Rotary Evaporator BUCHI R-114, Vacuum pump BUCHI B-169 Switzerland) (Oonmetta-aree et al., 2006).

### 2.2 Isolation and Identification of Bacteria

Pathogenic *Vibrio* species that included *V. cholerae*, *V. parahaemolyticus*, *V. parahaemolyticus* (EMS/AHPND), *V. fluvialis*, *V. vulnificus*, *V. alginolyticus*, *V. mimicus*, and *V. harveyi* were isolated from diseased Pacific white shrimp (*L. vannamei*) obtained from grow-out ponds in Chanthaburi Province, using the Ruangpan & Kitao (1991) method. The isolate was streaked on Thiosulfate Citrate-Bile Salt Sucrose Agar (TCBS Agar, Difco USA) for selective isolation of *Vibrio*, and cultivated at 30 °C for 18-24 h. The *Vibrio* species was identified according to a scheme developed by Colwell (1984) using API 20E kits (ATB System, BioMérieux, France) and the PCR technique for the detection of *V. parahaemolyticus* (EMS/AHPND) (Joshi et al., 2014).

### 2.3 Bacterial Inoculum

The isolate was streaked on TCBS agar and incubated at 30 °C for 18–24 h. A pure colony was streaked on Trypticase Soy Agar (TSA, Difco, USA), and incubated at 30 °C for 18–24 h. Several colonies of bacteria were inoculated into a 1.5% sterile sodium solution, which was mixed well with a vortex mixer. The bacterial suspension was tested using McFarland Standard No. 0.5 ( $\times 10^8$  cfu/ml) then diluted to  $\times 10^6$  cfu/ml for antibacterial activity testing.

### 2.4 Disc Diffusion Test

The efficacy of herbs for the growth inhibition of *Vibrio* was done using an agar disc diffusion assay and efficacy testing method developed by Oonmetta-aree et al. (2006). The *Vibrio* inoculums ( $\times 10^6$  cfu/ml) were spread on Mueller Hinton agar (Difco, USA) supplemented with 1.5% NaCl (MHA–1.5 % NaCl), and then kept for about 5 min to allow the surface of the agar to dry. Each herb extract (80  $\mu$ l) was applied to a paper disc ( $\Phi$ 8 mm, Advantec, Tokyo, Japan) then both sides were dried in a sterile laminar flow. The paper disc was then placed onto an MHA–1.5% NaCl plate inoculated with *Vibrio*, followed by incubation at 30 °C for 18-24 h. The efficacy of herbs for the growth inhibition of *Vibrio* was estimated by measuring the diameter of the clear zone surrounding the herbal disc.

### 2.5 Minimum Inhibitory Concentration (MIC) Test

One milliliter of *Vibrio* ( $\times 10^6$  cfu/ml) was inoculated into liquid Mueller Hinton broth containing 1.5% NaCl (MHB–1.5 % NaCl). Each herbal extract (0.25 g/ml) was diluted with ethanol using a 2-fold dilution method. In this method, 160  $\mu$ l of each dilution was dropped onto a paper disc ( $\Phi$ 8 mm, Advantec, Tokyo, Japan), followed by drying in a sterile laminar flow. Then the paper disc was placed into a MHB–1.5% NaCl liquid medium and incubated at 30 °C for 18–24 h. The MIC of ethanol extract was regarded as the lowest concentration of the extract in the liquid medium that would permit no turbidity of the tested microorganism (Oometta-aree et al., 2006).

### 2.6 Minimum Bactericidal Concentration (MBC) test

The tubes that showed no turbidity of the bacteria and the last tubes showing turbidity from the MIC test were used for further MBC testing. Then, 0.1 ml of culture medium used in the MIC test was spread onto TCBS Agar (Difco, USA), incubated at 30 °C for 18–24 h, and the formations of colonies were counted. The MBC was the lowest concentration of herbal extract that formed less than 20 colonies, corresponding to an inhibition of the bacterial growth at 99.9%, or more (Oometta-aree et al., 2006).

### 2.7 Efficacy of the Galangal Extract against Pathogenic Fungi

#### 2.7.1 Fungi Isolation and Identification

The samples of naturally infected shrimp were collected from 50 grow-out ponds. Artificial feed was simultaneously collected for use in rearing shrimp samples. Fungi were isolated from the hepatopancreas and intestines of diseased shrimp and artificial feed were cultured on Glucose – Yeast in seawater (GYS) agar then incubated at 25–27 °C for 2–5 days (Khomvilai, 2006). Several isolation samples were streaked on GYS agar until the fungus had formed a pure culture. Then the isolations were identified by the National Center for Genetic Engineering and Biotechnology, Thailand, as described by Klich (2002), Raper & Fennell (1965) and by Samson et al. (1994 & 1995).

#### 2.7.2 Fungi growth Inhibition Test

The galangal crude extract was dissolved with sterilized seawater in 8 dilutions: 0.0005, 0.005, 0.05, 0.5, 1, 2, 4 and 8 mg/ml. Agar plates of pure fungus were cultured for normal growth. Each fungus colony was cut into a 1 cm diameter using a cork border and was immersed in each galangal extract dilution for 3 replications. The treatment group was exposed to each extract dilution for 30 minutes, then incubated at 25 °C, and the control group was immersed in sterilized seawater. After the fungi had been completely immersed, the fungi plugs were cleaned twice in sterilized seawater and placed on GYS agar followed by incubation at 25–27 °C. After incubation for 2 days, the fungi colony diameters from each plate were measured. The efficacy of the crude extract against growth inhibition was established by comparing the diameters of the colony radii of the fungal mycelia of the treatment and control groups, which was calculated as follows:

$$\text{Colony radius growth rate (\%)} = \frac{(\text{colony radius of the treated fungus})}{(\text{colony radius of the control})} \times 100$$

$$\text{The growth inhibition of fungi (\%)} = 100 - \text{Colony radius growth rate (\%)}$$

### 2.8 Effect of the Galangal Extract against *Vibrio* spp. Bacteria and Fungi in *L. vannamei*

#### 2.8.1 Test Diets

The galangal extract was dissolved in ethanol to a concentration of 0.25 g/ml, then was mixed with commercial pellet feed (CP feed, Thailand) at 2 (5 g/kg diet) and 4% (10 g/kg diet). For the control group, no herbal extract was added. The feed was kept at room temperature for 30 min to allow the absorption of the extract and the evaporation of the ethanol. The control diet was also absorbed with ethanol, and then evaporated. Next, the pellets were coated with squid fish oil (Agrithai And Development Co., Ltd., Thailand) at 10 g/kg feed to prevent the dispersion of the galangal extract in water and to reduce the smell of the extract. The feed was then dried at room temperature. These tested diets were prepared for shrimp each day. The feeding rate was 3.0% of the shrimp body weight.

#### 2.8.2 Treatment of Pathogenic *Vibrio* Bacteria and Fungi

About nine hundred white feces syndrome diseased shrimp, *L. vannamei*, with average body weight  $10.7 \pm 1.6$  g were obtained from a shrimp farm in Chanthaburi Province, Thailand. The samples were acclimatized for 3 days in 3 plastic tanks, each with a capacity of 500 L. Then, the number of *Vibrio* spp. bacteria and fungi in the hepatopancreas and intestines of 15 shrimp were examined and recorded. Experimental aquariums (90 L capacity) were filled with 70 L of chlorine-treated 29 ppt seawater. Flow-through water equipped with an aeration system was

arranged. Each group of 25 shrimp was sampled from the stocked tanks and was transferred into the individual experimental aquariums for 5 replications of treatment and the control group. The shrimp were fed the test diet 3 times a day for 12 days. Excess feed and waste was removed before each feeding. The parameters of the water were maintained for optimal quality. Three shrimp were sampled at 1, 3, 5, 7, 10, and 12 days from each aquarium after the feeding trials. The hepatopancreas and intestines of the shrimp were examined for *Vibrio* spp. and fungi via the spread-plate method.

$$\text{Incidence of fungi infestation (\%)} = \frac{\text{The number of infested shrimp}}{\text{Total number of observed shrimp}} \times 100$$

## 2.9 Effect of the Extract on Disease Resistance Caused by *Vibrio* spp.

### 2.9.1 Preparation of the Bacterial Samples

The pathogenic *V. parahaemolyticus* (EMS/AHPND) was isolated from infected shrimp. This bacterium was identified based on a scheme established by Colwell (1984) using API 20E kits (ATB System, BioMérieux, France). Identification of *V. parahaemolyticus* (EMS/AHPND) was accomplished via the PCR technique (Joshi et al. 2014). The isolate was grown on Thiosulfate Citrate Bile Sucrose Agar (TCBS Agar, Difco USA) incubated at 30 °C for 18-24 h. Then a pure colony was streaked on Trypticase Soy Agar (TSA Difco, USA), and incubated at 30 °C for 18-24 h. Heavy streaks of isolated bacteria were inoculated into a 0.85% sterile sodium solution, which was then mixed well via a vortex mixer. The bacterial suspensions were tested using McFarland Standard No. 0.5 (10<sup>8</sup>cfu/ml) then diluted to 10<sup>5</sup>cfu/ml for the antibacterial activity test.

### 2.9.2 Resistance Test

The water in the 90 L aquariums was managed according to the method mentioned above. Triplicate aquariums were prepared for each group. Ten shrimp (10.7 ± 0.8 g/shrimp) were transferred to each aquarium for 2 treatment groups (2 and 4% of the extract diet) and 1 control group (0% of the extract diet). The shrimp were fed test diets 3 times a day for 12 days. After the shrimp were fed a treated diet for 12 days, disease resistance tests were conducted via the injection of 100 µl of bacterial suspension, *V. parahaemolyticus* (2.85 ± 0.63 X 10<sup>5</sup> cfu/ml), into the abdominal segment. After injection, shrimp were fed a commercial diet twice a day for 15 days. Disease symptoms and mortality rates were observed and recorded daily; the surviving shrimp were counted, and statistical analysis was calculated after the end of the experiment.

## 2.10 Statistical Analysis

A multiple comparison (Pair wise Comparison Test: Fisher's LSD) test was used to examine the significant differences (P<0.05) among treatments and control groups using SYSTAT VERSION 5.0.

## 3. Results

### 3.1 Antibacterial Activities of Galangal Extract

Table 1. Growth Inhibition zones, Minimum Inhibitory Concentration (MIC) and Minimum bactericidal Concentration (MBC) by the galangal extract to 8 species of *Vibrio* spp.

<i>Vibrio</i> spp.	Growth inhibition zone (mm.)		MIC	MBC	Sensitivity of bacteria
	Galangal	Ethanol	(mg/ml)	(mg/ml)	MBC/MIC
<i>V. cholerae</i>	31.0 ± 0.5	0 ± 0	0.15	0.15	1
<i>V. parahaemolyticus</i>	29.8 ± 0.8	0 ± 0	1.25	2.50	2
<i>V. parahaemolyticus</i> (EMS/AHPND)	18.0 ± 2.3	0 ± 0	2.50	5.00	2
<i>V. fluvialis</i>	28.0 ± 0.7	0 ± 0	0.31	0.63	2
<i>V. vulnificus</i>	27.6 ± 0.5	0 ± 0	1.25	1.25	1
<i>V. alginolyticus</i>	27.1 ± 0.8	0 ± 0	0.63	2.50	4
<i>V. mimicus</i>	27.6 ± 0.5	0 ± 0	0.31	0.63	2
<i>V. harveyi</i>	21.3 ± 2.1	0 ± 0	2.50	5.00	2

Note: Growth inhibition zone (mm.); Resistant: ≤ 9 mm; Intermediate: ≥ 10 – 13 mm; Susceptible: ≥ 14 mm (Lorian, 1995 in Oonmetta – aree et.al., 2006), Data in table, Average ± SD.

As reported by Canillac & Mourey (2001), if the MBC/MIC ratio is found to be less than or equal to 4, the strain is considered to be susceptible; on the other hand, if this ratio is greater than 4, the strain is considered to be tolerant.

The results showed a growth inhibition zone surrounding the galangal extract disc of all species' bacteria. The MBC and MIC of 2 bacterial species, *V. cholerae* and *V. vulnificus*, showed the highest degree of sensitivity. The other 5 species, *V. parahaemolyticus*, *V. parahaemolyticus* (EMS/AHPND), *V. fluvialis*, *V. mimicus*, and *V. harveyi*, showed intermediate sensitivity, and *V. alginolyticus* showed low sensitivity among the bacteria tested. The results of the antibacterial activity of the galangal extract in this study showed most of the species of *Vibrio* spp. to be sensitive (Table 1).

### 3.2 Fungi Isolation and Identification

Pathogenic fungi were isolated from *L. vannamei*, feces, and artificial feed. All fungi were identified by the 6 species shown in Table 2.

Table 2. The 6 species of fungi isolated from the hepatopancreas and intestines of shrimp naturally infected with white feces syndrome *L. vannamei*, and from artificial feed

Fungi species	Source of isolated
<i>Aspergillus flavus</i>	Hepatopancreas, intestine, and artificial feed
<i>Aspergillus ochraceus</i>	Hepatopancreas and artificial feed
<i>Aspergillus japonicus</i>	White feces and artificial feed
<i>Penicillium sp.</i>	Hepatopancreas and intestine
<i>Fusarium sp.</i>	Hepatopancreas, intestine, and artificial feed
<i>Cladosporium cladosporioides</i>	Intestine

### 3.3 Efficacy of the Extract against Pathogenic Fungi

Table 3 shows the efficacy of the galangal extract on 6 species of fungi. The galangal extract at a concentration of 0.5 mg/ml represented the strongest inhibition of *A. ochraceus*. A concentration of 1 mg/ml inhibited the growth of *Penicillium sp.*; *Fusarium sp.*, and *C. cladosporioides*. Two species, *A. flavus* and *A. japonicus*, were least susceptible to the galangal extract (8 mg/ml).

Table 3. Efficacy of the galangal extract on the growth inhibition of fungi (%) isolated from an infection of white feces syndrome pathogen *L. vannamei* and from artificial feed

Extract Concentration (mg/ml)	The growth inhibition of fungi (%)					
	<i>A. flavus</i>	<i>A. ochraceus</i>	<i>A. japonicus</i>	<i>Penicillium sp.</i>	<i>Fusarium sp.</i>	<i>C. cladosporioides</i>
0.0005	6.12 ± 5.77	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	6.67 ± 6.29
0.005	16.33 ± 2.89	6.82 ± 2.50	18.64 ± 9.59	0.00 ± 0.00	4.23 ± 1.48	17.05 ± 11.25
0.05	48.98 ± 14.43	18.18 ± 9.28	15.25 ± 0.00	16.67 ± 0.00	27.95 ± 12.58	40.00 ± 3.14
0.5	56.73 ± 2.89	100.00 ± 0.00	25.42 ± 4.79	56.23 ± 15.89	42.35 ± 19.49	51.11 ± 6.29
1	77.55 ± 31.75	100.00 ± 0.00	37.29 ± 1.15	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00
2	77.55 ± 31.75	100.00 ± 0.00	57.63 ± 11.98	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00
4	82.66 ± 49.06	100.00 ± 0.00	55.93 ± 9.59	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00
8	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00

Note: Data in table, Average ± SD.

### 3.4 Effect of the Galangal Extract against *Vibrio* spp. and Fungi

#### 3.4.1 Health Status of the Naturally infected Shrimp

The hepatopancreas and intestines of shrimp from a grow-out pond that were infected with white feces syndrome were observed, and 4 species of fungi were found including *C. cladosporioides*, *Penicillium sp.*, *A. japonicus*, and *Fusarium sp.* In addition, a rather high incidence of *Vibrio* spp. were also isolated from the same samples (100.0 ± 0.0 %). The total counts from the hepatopancreas and intestines were as high as 386.73 ± 323.73 X 10<sup>3</sup> cfu/g and 426.46 ± 168.21 X 10<sup>5</sup> cfu/g, respectively. The dominant numbers of *Vibrio* spp. isolated in the feces were green and yellow colonies.

### 3.4.2 Effect of the Galangal Extract on the Total Number of *Vibrio* spp.

The totals for the number of *Vibrio* spp. in the hepatopancreas and intestines of infested *L. vannamei* shrimp fed the galangal extract (treated diet) were significantly less than that for the control group ( $P < 0.05$ ). Figure 1 lists the numbers after 3 days in the hepatopancreas (A) and 5 days in the intestines (B) after feeding with the treated diet. In contrast, the total number of *Vibrio* spp. in the hepatopancreas (A) and intestines (B) of the control group had increased through 12 days (Figure 1). No green colonies of *Vibrio* spp. were found in either of the groups fed 2 or 4% treated diets.

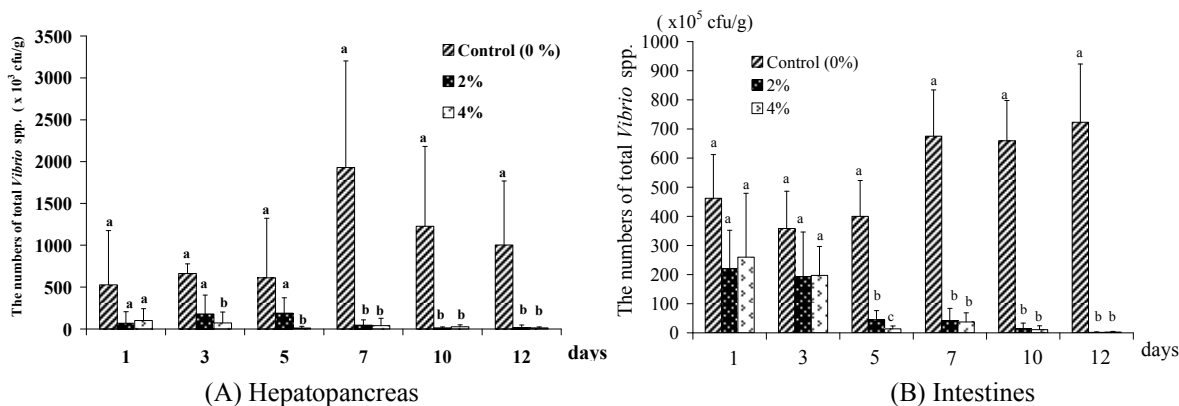


Figure 1. The total number of *Vibrio* spp. in (A) hepatopancreas and (B) intestines of infested *L. vannamei* fed the 2 and 4% galangal extract, as well as the control (0%), for 12 days. Data are the means  $\pm$  SD; different letters for the time interval indicates a significant difference ( $P < 0.05$ )

### 3.4.3 Effect of the Galangal Extract on Pathogenic Fungi

The incidence of fungi infestation in the hepatopancreas and intestines of infested *L. vannamei* shrimp fed the crude galangal extract (treated diet) was significantly lower than that in the control group ( $P < 0.05$ ). Also, no fungi could be found in the hepatopancreas and intestines of white shrimp after feeding with the treated diet for 10 to 12 days (Figure 2 (A) and (B)).

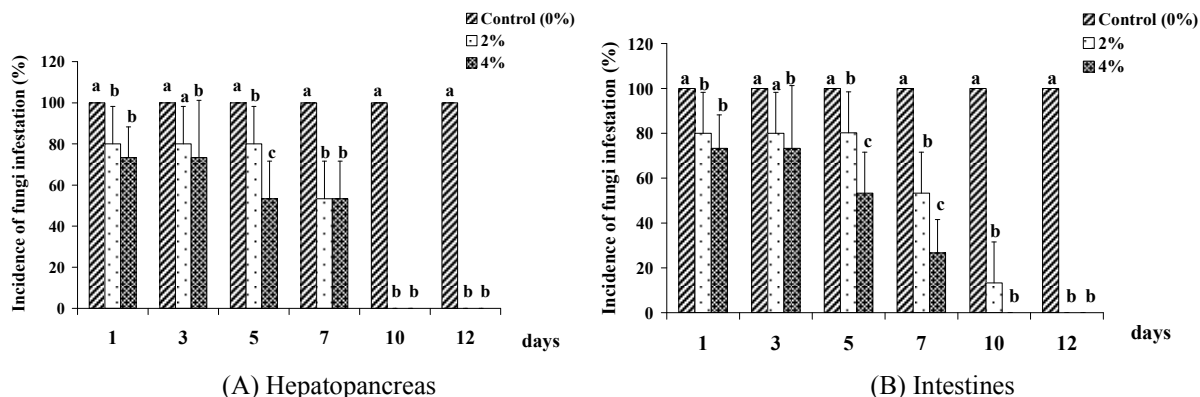


Figure 2. Incidence of fungi infestation (%) in the hepatopancreas (A) and intestines (B) of infested *L. vannamei* fed galangal extract at 2 and 4%, along with that of the controls (0%), for 12 days. Data are the means  $\pm$  SD; different letters for the time interval indicates a significant difference ( $P < 0.05$ )

### 3.5 Effect of the Galangal Extract on Disease Resistance Challenged by *V. parahaemolyticus* (EMS/AHPND)

The survival rates for shrimp fed 2 and 4% galangal extract were  $73.3 \pm 5.8\%$  and  $83.3 \pm 5.8\%$ , respectively. Only  $16.7 \pm 5.8\%$  of the survival shrimp were from the control group (0% galangal extract). The results differed significantly between the treated groups and the control group ( $P < 0.05$ ). Obviously, shrimp fed the galangal extract at 4% had the highest survival rate (Figure 3).

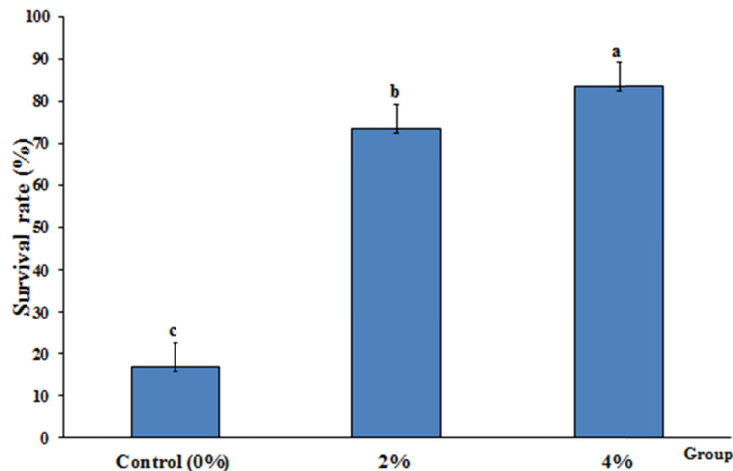


Figure 3. The survival rate of *L. vannamei* shrimp fed diets containing 2 and 4% of galangal extract (treated diet) and 0% (control) following 12 days of feeding trials prior to being challenged with *V. parahaemolyticus* (EMS/AHPND). Different letters indicate a significant difference ( $P < 0.05$ )

#### 4. Discussion

Investigation into the bacteria that are important and common in the aquatic environment of EMS/AHPND has been used to develop a safe and environmentally friendly product in shrimp aquaculture via alternative methods to control and treat vibrios diseases. The essential oil of crude galangal extract is a candidate for such a product since many researchers have documented its antimicrobial activities (de Pooter et al., 1985; Thomas et al., 1996; Turker et al., 2002; Oonmetta-areea et al., 2006; Tachakittirungrod & Chowwanapoonpohn, 2007; Vuddhakul et al., 2007; Mayachiew & Devahastin, 2008; Latha et al., 2009; Rao et al., 2010). Canillac & Mourey (2001) reported that strains of vibrios with an MBC/MIC ratio of less than or equal to 4 are considered to be susceptible to the extract, and if the ratio is greater than 4 a strain is considered to be tolerant of the extract. Based on our results, the MBC/MIC ratios showed that 8 species of *Vibrio* were sensitive to the extract. The results also revealed that the extract inhibited the growth of *V. harveyi*. Also, the ethanol galangal extract in this study was considered to be antibacterial against *V. parahaemolyticus* (EMS/AHPND), which is a causative agent of a recently recognized serious disease in Southeast Asia (Lightner et al., 2012; NACA FAO, 2011; Bondad-Reantaso et al., 2012). Ethanol extract of galangal containing 1'-acetoxyeugenol acetate (ACA) as a main ingredient could inhibit the growth of Gram-positive bacteria such as *Staphylococcus cerevisiae*, *S. epidermidis*, *S. aureus* and *Bacillus cereus*, but it did not inhibit the growth of Gram-negative bacteria such as *Salmonella spp.*, *Escherichia coli*, and *Enterobacter aerogenes* (Oonmetta-aree et al., 2006). The ingredients in this herb, 1'-acetoxychavicol acetate, ACA and eugenol, are effective for the treatment of inflammation and fungal diseases on human skin, and they inhibit the growth of *Escherichia coli* (De Pooter et al., 1985; Kubo et al., 1991; Norajit et al., 2007; Natta et al., 2008; Chudiwal et al., 2010). Furthermore, *V. parahaemolyticus* growth is inhibited by the chloroform extract of fresh galangal root, but not by the methanol extract (Vuddhakul et al., 2007).

These studies were carried out to determine the fungal species that are isolated from the hepatopancreas and intestine of white feces syndrome *L. vannamei* and from artificial feed. Among these species, *Fusarium* sp. were observed more frequently than the others. The study was focused on the degree that galangal crude extract inhibited the growth of these pathogenic fungi. De Silava et al. (2011) isolated 18 fungi species from *L. vannamei*, and among those, *Aspergillus flavus* and 2 species of *A. parasiticus* were able to produce aflatoxin B1. Contamination of *F. moniliforme* (Li et al., 1994) and *A. flavus* (Lightner, 1993) was reported in raw materials used to produce commercial pellet feed. Yulia (2005) previously reported that the ethanol extract of galangal rhizomes was the most effective in reducing spore germination of the fungi. Furthermore, this extract also inhibited the colony areas of *F. moniliforme*, *A. flavus* and *A. niger* (Handajani & Purwoko, 2008). This study's results also showed the growth inhibition of fungi by crude galangal extract. The study found fungi that produce aflatoxin to be few in number in artificial feed, which may be due to moisture retention during transport or storage on the farm. To prevent fungi in feed, it should be kept in a dry place and it should not be expired.

Our tests on the inhibition of *Vibrio* spp. and fungi *in vitro* and *in vivo* returned similar results. The infected shrimp fed the galangal extract diets had a lower number of *Vibrio* spp. and fungi compared with the control group. It is

remarkable that the galangal extract could be used to inhibit growth and reduce the number of *Vibrio* spp. and fungi that cause white feces syndrome and AHPND in shrimp. In the present study, after shrimp were fed a diet mixed with 2 and 4% of the extract with ethanol, from day 1 the numbers of *Vibrio* spp. in the hepatopancreas and intestines were lower than in the control group. Remarkably, galangal extract can be used to inhibit the growth and reduce the number of *Vibrio* spp. and fungi to prevent white feces syndrome. Moreover, resistance in *L. vannamei* against *V. parahaemolyticus* causing AHPND occurred when the shrimp were fed a diet mixed with galangal extract at 2 or 4% for 12 days. Our results also showed a higher survival rate (83.3%) of the treatment group (fed with 4% galangal extract) than the control group (feeding with 0% extract). The results revealed the efficiency of the extract, which has ability to inhibit *V. parahaemolyticus* (EMS/AHPND), and it may stimulate the immune system in the tested shrimp (Chaweepeak et al., 2015). Therefore, shrimp fed galangal extract diet are healthier than the control group, and shrimp fed a galangal extract diet could resist infection well. For several years, antibiotics and some hazardous chemicals have been used to treat pathogenic agents in the aquaculture industry, particularly in intensive shrimp-farming systems. The development of resistant bacteria and residue in shrimp caused a major obstacle to trade and had a negative impact on the environment as well as on consumer health. The use of herbal medicines is a viable alternative to the overuse of antibacterial agents. The main advantage of herbal agents is that the crude extract contains a mixture of compounds such as phenols, acids, esters, and aldehydes, to which bacteria are unlikely to develop resistance. A synthetic agent that contains a single compound, however, is easier for bacterial strains to resist (Abraham et al., 1997). Based on the results of the present study, we recommend alternative methods for control of the disease that causes white feces syndrome and AHPND in the shrimp industry by using galangal crude extract, which has proven to be effective and safe for the environment as well as for consumers.

## 5. Conclusion

This investigation was focused on the efficacy of galangal extract for treatment of the pathogenic organisms that cause white feces syndrome and AHPND in Pacific white shrimp, *L. vannamei*. Ethanol galangal extract exhibited the highest potential for *Vibrio* spp. and fungi reduction. The highest percentage (4%) of ethanol extract was most effective in reducing all species of the pathogens of *V. parahaemolyticus* (EMS/AHPND). Therefore, galangal extract should be used as an antimicrobial for white feces syndrome and AHPND therapeutics in Pacific white shrimp *L. vannamei* and in shrimp cultures. This alternative method could assist in reducing the impact of antibiotic or chemical residue in shrimp products as well as helping to reduce the presence of resistant bacterial strains in the environment.

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