Journal of Agricultural Science; Vol. 7, No. 9; 2015
ISSN 1916-9752 E-ISSN 1916-9760
Published by Canadian Center of Science and Education

Effect of Galangal (*Alpinia galanga* Linn.) Extract on the Growth Rate and Resistance to *Vibrio harveyi* and White Spot Diseases in Pacific White Shrimp (*Litopenaeus vannamei*)

Tidaporn Chaweepack[1], Chutima Khomvilai[2], Surachart Chaweepack[1] & Kaeko Kamei[3]

[1] Chanthaburi Coastal Fisheries Research and Development Center, Department of Fisheries, Muang, Chanthaburi, Thailand

[2] Marine Shrimp Research Institute, Department of Fisheries, Chatuchak, Bangkok, Thailand

[3] Department of Biomolecular Engineering, Kyoto Institute of Technology, Kyoto, Japan

Correspondence: Tidaporn Chaweepack, Chanthaburi Coastal Fisheries Research and Development Center, Department of Fisheries, Muang, Chanthaburi 22000, Thailand. Tel: 66-394-579-878. E-mail: tidaporn2513@gmail.com

Received: April 20, 2015 Accepted: June 25, 2015 Online Published: August 15, 2015

doi:10.5539/jas.v7n9p117 URL: http://dx.doi.org/10.5539/jas.v7n9p117

Abstract

The anti-microbial activity of galangal (*Alpinia galanga* Linn.) is well known. In this study, the feeding of galangal crude extract was investigated for its effect on preventing the infectious diseases *Vibrio harveyi* and white spot syndrome virus in Pacific white shrimp (*Litopenaeus vannamei*). A commercial diet mixed with galangal ethanol extract was fed to shrimp for 1 or 2 months. In the first month of feeding, the growth rate of the galangal extract diet group was lowered compared with that of the control diet group, possibly because the shrimp required time to acclimatize to the galangal diet. After 2-months of feeding, the growth of the shrimp in terms of body weight, specific growth rate and survival rate of the galangal diet group did not differ significantly ($P > 0.05$) from that of the control diet group. The clearance ability was evaluated by counting the bacterial cells in the hemolymph of shrimp injected with *V. harveyi* in the abdomenal segment. The number of *V. harveyi* in the hemolymph of the galangal diet group was significantly lower than that in the control diet group ($P < 0.05$), indicating the higher clearance ability of the galangal diet group. The oral administration of galangal extract enhanced the resistance of Pacific white shrimp against *V. harveyi* and white spot syndrome diseases, as demonstrated by the significantly higher survival rate of the galangal diet group. These results suggested that galangal is useful as an alternative to chemotherapeutic treatment to solve the problems created by residual antibiotics in shrimp.

Keywords: galangal, infection, shrimp, survival rate, *Vibrio*, white spot syndrome virus

1. Introduction

The shrimp aquaculture industry continues to face infectious diseases caused by bacteria and viruses, which accounts for major economic losses in many countries. Vibriosis in shrimp caused by *Vibrio* spp. is considered a significant bacterial disease and common infectious problem (Ruangpan & Kitao, 1991; Ruangpan et al., 1997; Sung et al., 1999). Antibiotic-resistant *V. harveyi* is known to cause mass mortality, retarded growth and deformities in penaeid shrimp around the world (Karunasagar, 1994; Liu et al., 1997; Lavilla-Pitogo et al., 1998; Ruangpan, 1998; Austin, 2010). Sung et al. (2001) confirmed that these infectious diseases are related to increases in the vibrio population in shrimp pond water. Some *Vibrio* spp. are opportunistic pathogenic bacteria that become problematic when shrimp are under stressful conditions or have low immunity. When shrimp are reared in high density, vibrio bacteria attack and cause diseases, which can lead to shrimp mortality. Vibrio bacteria such as *V. alginolyticus*, *V. cholerae*, *V. parahaemolyticus*, *V. vulnificus*, *V. mimicus*, and *V. harveyi* were isolated from diseased *Litopenaeus vannamei* (Vandenberghe et al., 1999; Montero & Austin, 1999). White spot disease (WSD) has been responsible for huge economic losses in the shrimp culture industry worldwide, because of massive rates of mortality in a total crop within 3–10 days (Lightner, 1996). WSD is caused by an extremely virulent DNA virus known as white spot syndrome virus (WSSV). It has a wide host range and targets various tissues (Chang et al., 1996; Lightner, 1996; Lo et al., 1996; Kou et al., 1998; Maeda et al., 1998). The principal

clinical sign of WSD is the appearance of white spots in the exoskeleton and epidermis of shrimp (Peinado-Guevara & Lo'pez-Meyer, 2006).

Many chemical substances and antibiotics have been used to control vibrio diseases, but these can cause residual problems in shrimp. To solve these problems, medical herbs have been introduced for control and treatment of these bacterial and virus diseases (Yu et al., 2008; Olmedo Sanchez et al., 2009; Velmurugan & Citarasu, 2010; Velmurugan et al., 2010; Sankar et al., 2011, Direkbusarakom et al., 1998). In addition, herbal bio-medicinal active ingredients with antimicrobial capabilities can also be used to promote the growth and survival rates of shrimp, and the tonic can improve immune systems. The utilization of medical herbs and herbal bio-medicinal active ingredients will reduce the cost and side effects caused by synthetic compounds, while it is also eco-friendly. Hence, this alternative herbal biomedicine has proven to be effective in shrimp aquaculture operations (Citarasu, 2010). Traditional herbs such as galangal (*Alpinia galanga* Linn.) were studied to determine if and how they can control shrimp diseases. Galangal belongs to the Zingiberaceae family and can be obtained at fresh markets in Thailand at a low price. The galangal extract contains 1'-acetoxychavicol acetate (ACA) as a main ingredient that can inhibit gram positive bacteria such as *Staphylococcus cerevisiae, S. epidermidis* and *S. aureus* (Oonmetta–aree et al., 2006), and can decrease inflammation and inhibit the growth of *Escherichia coli*, fungal pathogens and ringworm (Chudiwal et al., 2010). Latha et al. (2009) found that ACA in the galangal extract shows antimicrobial activity on many forms of drug-resistant bacteria.

This is the first study to examine the effect of the oral administration of galangal crude extract for its clearance ability and its ability to promote resistance to the *V. harveyi* and WSSV diseases in Pacific white shrimp.

2. Materials and Methods

2.1 Galangal Extract

Fresh galangal rhizomes were purchased from a local market in Chanthaburi, Thailand. An ethanol extract of galangal was prepared according to a procedure established by Oonmetta-aree et al. (2006). Briefly, 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, the rhizomes were ground into powder using an electric blender (Philips, Cucina, Thailand). Ten grams of the powder was suspended in 100 ml of ethanol, and then left to 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). Thus, galangal ethanol extract was obtained.

Galangal dried powder (500 g) was extracted with methanol three times. The first extraction was done with 1 L of methanol for 5 h at room temperature, the second with 1.5 L of methanol overnight and the final at 55 °C for 4 h. The supernatant was collected by centrifugation and dried with a rotary evaporator (IWAKI, Japan). The obtained methanol extract was suspended in 200 ml water and partitioned twice with diethyl ether (150 ml/time) to obtain a diethyl ether extract layer (PE) and a water layer (PW). Each layer was filtered and dried using a rotary evaporator, freeze dried and then stored at 4 °C until used.

2.2 Shrimp Sample

L. vannamei was obtained from the JR hatchery, Trad province, Thailand. Negative infections of the post-larvae (PL12) with WSSV (Cybeles^{TM} WSSV, Germany), taura syndrome virus (Navarro et al., 2009), infectious hypodermal and haematopoietic necrosis virus (Tang et al., 2007) or yellow-head virus (Cowley et al., 2004) were confirmed via PCR. Healthy *L. vannamei* weighing between 5–15 g were used for the experiments. The post-larvae stock was reared in a 15 m^3 concrete pond and fed 3 meals/day. The feeding rate was adjusted to 2.2–3.5% of shrimp weight. Seawater chlorinated with 20–25 mg/l calcium hypochlorite was changed twice a week after vigorous aeration. In the feeding trial, excess food and waste matter was removed daily before changing the water. Water parameters such as temperature (27–28 °C), salinity (26–30‰), amount of dissolved oxygen (6.50 ± 0.02 mg/l), and pH (7.8–8.1) were maintained throughout the experimental period.

2.3 Vibrio harveyi

Pathogenic *V. harveyi* was isolated from the hepatopancreas of diseased *L. vannamei*, which had been found in grow-out ponds in Chanthaburi province, Thailand, and identified according to schemes established by Colwell (1984) using API 20E kits (ATB System, BioMérieux, France). The isolate was cultured on TCBS agar at 30 °C for 24 h, and then single colonies were picked up and cultured on Tryptricase Soy Agar (TSA, Difco, USA) at 30 °C for a further 24 h. Several colonies were then suspended into a 0.85% sterile sodium chloride solution and mixed well using a vortex mixer. The bacterial suspensions were tested using a McFarland standard No. 0.5 (10^8 cfu/ml), and then diluted to 10^{-4} to 10^{-6} colony-forming units (cfu)/ml for experimentation. To determine the cfu,

the diluted bacterial suspensions were cultured on three TCBS agar plates, and then bacterial colonies were counted. The pathogenicity of *V. harveyi* was evaluated according to the challenge it presented to juvenile shrimp (LD_{50} value, 10^4 cfu/g body weigh).

2.4 WSSV

Pacific white shrimp infected with WSSV were collected from shrimp farms in Chanthaburi, Thailand. The WSSV infections were checked using nested PCR with WSSV determination and screening kits (Cybeles™ WSSV, Germany; OIE Manual). The gills of diseased shrimp were homogenized, suspended in phosphate buffered saline (PBS), and centrifuged at 3000 × g for 20 min at 4 °C. The supernatant was stored at -20 °C after filtration through a 0.45 μm filter. The presence of WSSV in the filtrate was also confirmed by nested PCR before use.

2.5 Disc Diffusion Test

V. harveyi ($\times 10^6$ cfu/ml) was spread onto Mueller Hinton agar (Difco, USA) supplemented with 1.5% NaCl. Onto a paper disc (8 mm, Advantec, Tokyo, Japan), 80 μl of ethanol or methanol galangal extract (0.45 g/ml), or 80 μl of PW (0.49 g/ml) or PE (0.60 g/ml) was applied. After drying, the paper disc was put onto Mueller Hinton agar–1.5% NaCl inoculated with *V. harveyi* ($\times 10^6$ cfu/ml), and then incubated at 30 °C for 18–24 h. The anti-*Vibrio* activity of galangal extract was evaluated by measuring the diameter of the clear zone that formed around the disc (Oometta-aree et al., 2006).

2.6 Minimum Inhibitory Concentration (MIC) Test and Minimum Bactericidal Concentration (MBC) Test

The MIC of galangal ethanol extract was determined according to a procedure established by Oometta-aree et al. (2006). A 2-fold serial dilution (160 μl) of galangal extract was dropped onto a paper disc (8 mm, Advantec, Tokyo, Japan). After drying, the paper disc was placed into a 1 milliliter suspension of *V. harveyi* ($\times 10^6$ cfu/ml) in liquid Mueller Hinton broth containing 1.5% NaCl, and incubated at 30 °C for 18–24 h. The MIC of galangal extract was determined as the lowest concentration of the extract in a liquid medium that would permit no turbidity of *V. harveyi*.

The tubes in which a culture medium showed no turbidity of the bacteria and the last tubes showing turbidity in the MIC test were used for further MBC testing. The 0.1 ml culture medium used in the MIC test was spread onto TCBS Agar (Difco, USA) and incubated at 30 °C for 18–24 h, and the colonies were then counted. The MBC was the lowest concentration of galangal extract that would form less than 20 colonies, corresponding to the inhibition of the bacterial growth at 99.9%, or more.

2.7 Diet Containing Galangal Ethanol Extract

Galangal ethanol extract was dissolved again with ethanol at 0.25 g/ml and mixed with commercial pellet feed (CP feed, Thailand) to make 1, 1.5, 2, 3, and 5% diets (v/w pellet) that contained 0.25, 0.375, 0.5, 0.75 and 1.25%, respectively, of galangal ethanol extract (w galangal ethanol extract/w pellet). For the control diet, ethanol was mixed with commercial pellets. These pellets were kept at room temperature for 30 min to allow absorption of the extract and evaporation of the ethanol. Next, the pellets were coated with squid oil (Agrithai And Development Co., Ltd., Thailand) at 10 g/kg of each pellet to prevent the dispersion of the extract into water and reduce the smell of the extract, followed by complete drying at room temperature. This preparation was done daily to insure fresh diets.

2.8 Effect of a Galangal Ethanol Extract Diet on the Growth and Survival Rate

Thirty 200-L plastic tanks equipped with aeration and flow-through water systems were filled with 80 L of seawater. Sixty shrimp weighing between 5.9 and 6.2 g were transferred from stock to each 200-L tank for the 5 galangal diet groups (0.25, 0.375, 0.5, 0.75, and 1.25% [w galangal ethanol extract/w pellet]) and the control diet group. Each group comprised 5 replications. The shrimp were fed 3 times a day for either 30 or 60 days. A group of 30 shrimp from each diet group was measured for body weight every month. The specific growth rate (SGR) (Immanuel, 2004) was calculated using Equation (1).

$$SGR\ (\%) = (\ln w_0 - \ln w_t)/t \times 100 \quad (1)$$

Where w_0 is the initial body weight, w_t is the final body weight, and t is the feeding period.

2.9 Effect of Galangal Extract Diet on Clearance Ability

Clearance ability expressed as percentage inhibition (PI) was evaluated by counting the number of *V. harveyi* in a hemolymph 3 hours after the injection of *V. harveyi* into the shrimp. The suspension (100 μl) of *V. harveyi* (1-month feeding group, $4.5 \pm 0.7 \times 10^6$; 2-months feeding group, $8.7 \pm 2.3 \times 10^6$ cfu/ml) was injected into the

abdomenal segment. Three hours after injection, a hemolymph of 30 shrimp from each diet group was collected from the ventral sinus, and diluted with saline solution to produce serial 2-fold dilutions. Twenty µl of each hemolymph dilute was dropped onto duplicate TCBS plates and then incubated at 30 °C for 24 h. The colonies that formed were counted in order to determine the cfu. The PI was calculated using Equation (2) (Adams, 1991).

$$PI\ (\%) = 100 - [(\text{cfu in test diet group})/(\text{cfu in control group})] \times 100 \quad (2)$$

2.10 Effect of Galangal Extract Diet on V. harveyi Disease Resistance

The shrimp injected with *V. harveyi* described in subhead 2.9 were reared exclusively with a commercial diet twice a day with no galangal extract. The disease symptoms and mortality were recorded. After 14 days of feeding, the surviving shrimp were counted.

2.11 In vitro Determination of the Antiviral Activity of Galangal Extract

Ten shrimp (average weight, 10.5 ± 0.8 g), which had been fed a normal commercial diet without galangal extract, were transferred to each aquarium, and triplicate aquariums were assigned to each group. Three concentrations of galangal ethanol extract, 5, 50 and 500 µg/ml, were prepared with 0.85% NaCl. Ten µl of a viral suspension of a median infectious dose (ID_{50}) and 90 µl of galangal extract were mixed, and then maintained at 27–28 °C for 3 h. Then, the mixture (100 µl) was injected intramuscularly into the shrimp. The positive control group was injected with a mixture that included the suspension (10 µl of viral suspension (ID_{50}) and 90 µl of 0.85% NaCl). The negative control group was injected with a mixture of 10 µl of PBS and 90 µl of 0.85% NaCl. The injected shrimp was fed a commercial diet without galangal extract twice a day for 14 days, and disease symptoms and mortality were recorded. The surviving shrimp were counted, and statistical analysis was conducted at the end of the experiment. Nested PCR was conducted to check the dead and surviving shrimp to confirm WSSV infections.

2.12 In vivo Determination of Antiviral Activity of Galangal Extract

Healthy shrimp with an average body weight of 11.7 ± 1.3 g were fed 3 times/day with either the galangal extract diet (0.61 or 1.25% [w/w]) or the normal commercial diet. After 14 days of the feeding trial, 30 shrimp from each diet group were injected intramuscularly with WSSV at the LD_{50} concentration, and another 30 shrimp were injected with PBS as a negative control. Then, the shrimp were fed a normal commercial diet twice a day, and disease symptoms and rates of mortality were recorded. The surviving shrimp were counted for 14 days and statistical analysis was conducted. A nested PCR of the shrimp was performed to confirm the rates of WSSV infection.

2.13 WSSV Detection

2.13.1 First-Step PCR

DNA was extracted from the gills of shrimp using DNAzol reagent (invitrogen). The amplification of WSSV DNA was performed via WSSV Determination using a screening kit (Cybeles^{TM} WSSV, Germany) with a 50 µl reaction mixture containing 1 µl of the WSSV DNA template, 6.50 µl of first step Master Mix solution, 0.25 µl of WSSV Taq Mix, and 42.25 µl of sterile water. The amplification profile was carried out as follows: denaturation at 94 °C for 2 min, followed by 20 cycles of denaturation at 94 °C for 20 sec, annealing at 55 °C for 30 sec, and elongation at 72 °C for 40 sec before final elongation at 72 °C for 5 min.

2.13.2 Nested-PCR

Ten µl of the first-step PCR product was mixed with 40 µl of Nested-PCR master mix (26.6 µl of Nested-PCR Blue Mix solution, and 13.4 µl sterile water). The amplification profile was set by denaturation at 94 °C for 2 min, followed by 35 cycles of denaturation at 94 °C for 20 sec, annealing at 55 °C for 30 sec, elongation at 72 °C for 30 sec, and elongation at 72 °C for 5 min. Thereafter, the products were analyzed by 1.5% agarose gel electrophoresis, then stained in ethidium bromide solution and visualized by UV transillumination. The WSSV amplification was expected to yield a specific PCR product of 497, 425, 328, and 256 bp, respectively. All reactions, including a positive control, were required to yield a product of 154 bp as the PCR Control. The positive control lane should contain at least fragments of 256 bp and 154 bp (as PCR internal control) to confirm the quality of the template and successful reaction. The sensitivity was increased by using a Nested PCR that could detect a quantity of as few as 20 copies of WSSV with a Detection Limit of 2 copies/reaction.

2.14 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 the SYSTAT VERSION 5.0.

3. Results

3.1 Antibacterial Activities of Galangal Extracts

Table 1 shows the growth-inhibition zones of *V. harveyi* formed by galangal crude extract. The antibacterial activities of methanol extract were partitioned in both the water (PW, 0.49 g/ml) and the diethyl ether fractions (PE, 0.6 g/ml), and PE showed a higher activity than that of PW. The *V. harveyi* showed an intermediate bacteria (MBC/MIC) sensitivity, as shown in Table 2. These results show that *V. harveyi* can be considered susceptible to the crude galangal extract.

Table 1. Growth inhibition of *V. harveyi* by galangal extracts

Extract	Growth inhibition zone (mm)				
	Galangal	Ethanol	Methanol	Sterile water	Diethyl Ether
Crude ethanol extract	21.3 ± 2.1	0 ± 0	−	0 ± 0	−
Crude methanol extract	25.7 ± 1.2	−	0 ± 0	0 ± 0	−
PW	13.0 ± 0.0	−	−	0 ± 0	−
PE	28.7 ± 1.5	−	−	0 ± 0	0 ± 0

Note. Growth inhibition of *V. harveyi* was assayed by growth inhibition zone (mm). Growth inhibition zone: Resistant, ≤ 9 mm; Intermediate, ≥ 10 – 13 mm; Susceptible, ≥ 14 mm. (Lorian, 1995; Oonmetta-aree et al., 2006). Data in Table is shown as average ± SD.

Table 2. MIC and MBC of *V. harveyi* by galangal extracts

Galangal extract	MIC (mg/ml)	MBC (mg/ml)	Sensitivity of bacteria (MBC/MIC)
Crude ethanol extract	2.25	4.50	2
Crude methanol extract	2.25	4.50	2

Note. According to a report by Canillac and Mourey (2001), if the MBC/MIC ratio is smaller than or equal to 4, the strain is considered to be susceptible; on the other hand, if this ratio is larger than 4, the strain is considered to be tolerant.

3.2 Effect of Galangal Extract on the Growth and Survival Rates of Shrimp

Table 3 shows the effect of the oral administration of the galangal ethanol extract on growth in terms of body weight and SGR, as well as the survival rate of *L. vannamei*. After 1 month of feeding, the growth rate of the control group was significantly higher than that of the galangal diet group ($P < 0.05$). However, there was no significant difference ($P > 0.05$) in weight, SGR and survival rate between the galangal diet and control groups after the 2-month feeding.

Table 3. Body weight, SGR and survival rate of *L. vannamei* groups fed a galangal extract diet and a control diet during a 1-month and 2-months feeding trial

Item	Galangal extract content in diet (% [w/w])					
	0	0.25	0.375	0.5	0.75	1.25
Initial weight (g)	6.2 ± 0.2 [a]	6.1 ± 0.2 [a]	5.9 ± 0.4 [a]	6.0 ± 0.3 [a]	6.0 ± 0.1 [a]	6.0 ± 0.5 [a]
	(5.2 – 6.5)	(5.9 – 6.5)	(5.8 – 6.8)	(5.3 – 6.3)	(5.3 – 6.3)	(5.8 – 6.1)
Weight at 1-month (g)	12.7 ± 1.6[a]	11.5 ± 1.7 [b]	12.1 ± 2.1 [ab]	10.7 ± 1.5 [c]	11.3 ± 1.7 [b]	11.6 ± 1.6 [b]
	(9.6 – 14.7)	(6.8 – 15.6)	(6.7 – 15.9)	(7.8 – 13.5)	(8.6 – 16.2)	(8.5 – 15.1)
SGR at 1-month (%)	2.5 ± 0.4[a]	2.1 ± 0.5[bc]	2.3 ± 0.6[ab]	1.9 ± 0.5[c]	2.1 ± 0.5[b]	2.2 ± 0.5[b]
	(1.6 – 3.0)	(0.4 – 3.2)	(0.4 – 3.3)	(0.9 – 2.3)	(1.3 – 3.3)	(1.2 – 3.1)
Weight at 2- months (g)	21.9 ± 2.7 [a]	21.7 ± 3.0 [a]	21.8 ± 3.1 [a]	21.0 ± 2.6 [a]	20.1 ± 2.7 [a]	21.5 ± 2.2 [a]
	(14.0 – 29.0)	(12.0 – 27.0)	(14.0 – 30.0)	(16.0 – 29.0)	(14.0 – 27.0)	(16.0 – 28.0)
SGR at 2 months (%)	2.1 ± 0.1[a]	2.1 ± 0.1 [a]	2.2 ± 0.2 [a]	2.1 ± 0.1 [a]	2.0 ± 0.1 [a]	2.2 ± 0.1 [a]
	(1.4 – 2.6)	(1.1 – 2.2)	(1.4 – 2.7)	(1.7 – 2.7)	(1.4 – 2.6)	(1.7 – 2.6)
Survival rate at 2-months (%)	70.0 ± 8.0 [a]	62.0 ± 4.7 [a]	64.0 ± 8.9 [a]	68.0 ± 2.7 [a]	66.0 ± 2.2[a]	72.0 ± 8.4 [a]
	(65 – 80)	(58 – 70)	(60 – 80)	(65 – 80)	(65 – 80)	(65 – 80)

Note. Data are shown as the average ± SD and range (Minimum – Maximum). The significant difference ($P < 0.05$) is indicated by a different superscript letter within the same row.

3.3 Effect of Galangal Extract Diet on Bacterial Disease

3.3.1 Clearance Ability

V. harveyi was injected into the abdomenal segments of test shrimp, and then the number of *V. harveyi* in the hemolymph was counted 3 hours post-injection. The clearance ability was evaluated by the percentage inhibition (PI) of *V. harveyi* calculated using equation 2 with the results shown in Figures 1A and 1B. Figure 1A shows that the number of *V. harveyi* in the hemolymph of the 0.375% galangal diet group was higher than that in the 0.25% galangal diet group. However, the statistical analysis shown in Figure 1C indicates that the difference between the two groups was not significant. The oral administration of galangal extract was effective in enhancing the clearance ability of shrimp, as shown in Figure 1C. Among the 1-month galangal diet groups, shrimp fed the 0.5, 0.75, and 1.25% (w/w) galangal extract diets showed significantly higher clearance ability compared with the 0.25 and 0.375% (w/w) galangal diet groups. The shrimp fed galangal extract for 2 months showed high values for the PI of *V. harveyi* (90–100%), with a clearance ability that did not significantly differ according to the concentration of the galangal extract in the diet.

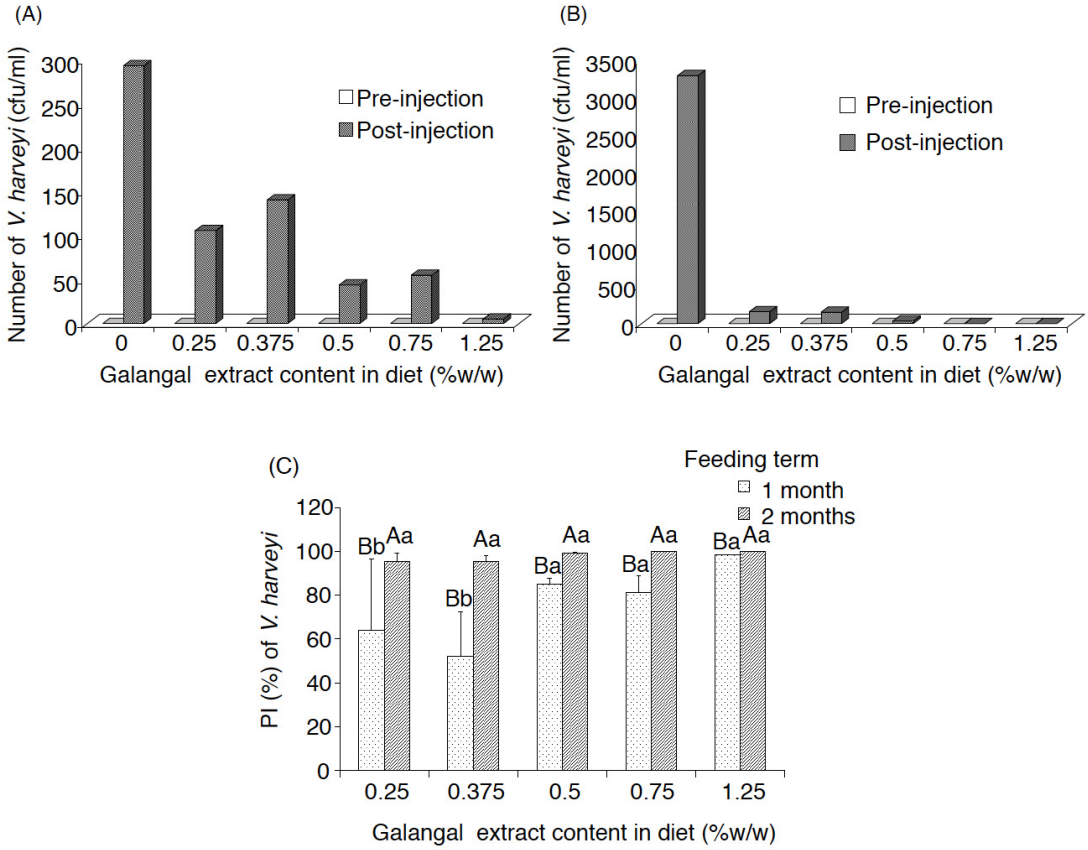

Figure 1. Clearance ability of *L. vannamei* fed galangal extract for 1 or 2 months. *V. harveyi* (1-month feeding group, $4.5 \pm 0.7 \times 10^6$; 2-months feeding group, $8.7 \pm 2.3 \times 10^6$ cfu/ml) was injected into the abdomenal segment, and the number of *V. harveyi* in the hemolymph at 3 hours post-injection was counted (A, 1-month feeding; B, 2-month feeding). PI of *V. harveyi* was calculated from the data in (A) and (B), and is shown in (C). The significant differences ($P < 0.05$) are indicated by a different capital letter within the same group and by a different lowercase letter within the same time interval

3.3.2 Resistance against Bacterial Disease

A challenge test was carried out after 1 and 2 months of feeding. Shrimp mortality was recorded daily for 14 days after an injection of *V. harveyi*. Figure 2 shows that the survival rate of shrimp was enhanced significantly by the administration of a galangal diet compared with the control diet group ($P < 0.05$), and the effect was dependent on the galangal content in the diet.

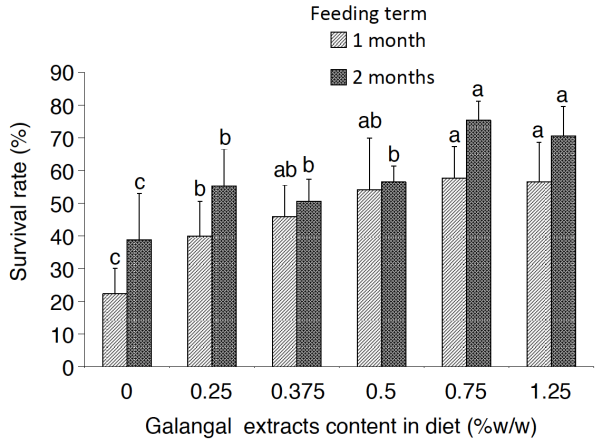

Figure 2. Challenge test of shrimp with *V. harveyi*. The survival rate of *L. vannamei* fed a galangal extract diet for 1 or 2 months was monitored for 14 days after a challenge with *V. harveyi*. The different letters for the same time interval indicate a significant difference ($P < 0.05$)

3.4 In vitro Determination of Antiviral Activity

To determine the antiviral activity of galangal ethanol extract against WSSV, the extract was incubated with WSSV for 3 hours. Then, the mixture was injected intramuscularly into the shrimp, and the survival rate was monitored. The results in Figure 3A show that when shrimp were pre-incubated with galangal extract, and then injected with WSSV, their survival rate was significantly higher compared with the control shrimp group. The dead shrimp in all groups, and the shrimp that survived an injection with WSSV only and those that survived the injection of a mixture of WSSV and galangal extract (0.045 μg/g body weight), were shown to be WSSV positive by nested PCR. The shrimp that survived the injection of WSSV after being pre-incubated with galangal extract at a rate of 0.45 μg/g were 55.56 ± 9.62% WSSV positive, but those pre-incubated with galangal extract at a rate of 4.5 μg/g body weight were not WSSV positive.

3.5 Effect of Galangal Extract Diet on Virus Disease

Shrimp samples fed the control diet or a galangal extract diet of 0.61 and 1.25% (w/w) for 14 days were injected with WSSV, and reared with a normal diet for a further 14 days. The survival rate of the galangal extract diet groups was significantly higher than that of the control groups (Figure 3B). The negative control, which had not been injected with WSSV, showed a survival rate as high as 100% through 14 days of the experiment. All of the dead shrimp fed a galangal extract diet and the control groups were WSSV positive according to the nested PCR test. The control group shrimp that survived the WSSV challenge were nonetheless found to have an infection rate of 100%, while the shrimp fed diets composed of 0.61 and 1.25% galangal extract showed no infection.

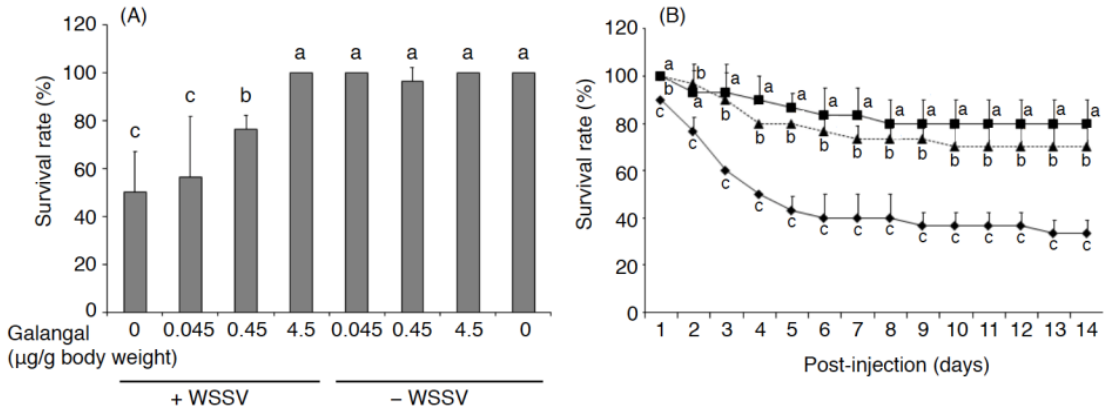

Figure 3. (A) Antiviral activity of galangal extract against WSSV. After a 3-hour pre-incubation of galangal extract with WSSV at LD_{50}, the mixture was injected intramuscularly into shrimp. The survival rate was monitored for 14 days. Different letters at the same time interval indicate a significant difference ($P < 0.05$). (B) Effect of galangal extract diet on infection from WSSV. After 14 days of feeding a 0.61% (triangle) or 1.25 % (w/w) (square) galangal-containing diet or a control diet (diamond), shrimp were intramuscularly injected with WSSV at LD_{50}. Shrimp from all groups were cultured with a control diet without galangal extract for a further 14 days, and the survival rate was monitored. Different letters at the same time interval indicate a significant difference ($P < 0.05$)

4. Discussion

A number of studies have reported the antimicrobial activity of galangal extract (de Pooter et al., 1985; Turker & Usta, 2006; Oonmetta-areea et al., 2006; Tachakittirungrod & Chowwanapoonpohn, 2007; Vuddhakul et al., 2007; Mayachiew & Devahastin, 2008; Latha et al., 2009; Rao et al., 2010). The main constituent of the extract has been identified as 1'-acetoxychavicol acetate (Oonmetta-aree et al., 2006). 1,8-Cineole, *β*-bisabolene, *β*-caryophyllene and *β*-selinene (Mayachiew & Devahastin, 2008), 5-hydroxymethyl furfural, and benzyl alcohol (Rao et al., 2010) have also been identified as components of galangal. Chudiwal et al. (2010) showed anti-food-borne bacteria activity of *A. galangal* rhizomes and its component, trans-*p*-coumaryl diacetate. Natta et al. (2008) found that lipophilic compounds soluble in ethanol from galangal rhizome possess anti-microbial properties that are similar to those of essential oils. With the large number of different chemical compounds that are contained in galangal extract, its mechanism can affect multiple bacterial cell target sites such as the cell wall, the cell membrane, and the mitochondrial membrane (Burt, 2004; Oonmetta-aree et al., 2006). Another advantage for using galangal extract is that it can suppress the generation of resistant bacteria, unlike synthetic agents that contain a single compound that is easily resisted by bacteria. For example, *V. harveyi* found in diseased shrimp are resistant to most chemotherapeutic agents used in aquaculture operations (Abraham et al., 1997).

To gauge the utility of galangal extract when used in a shrimp aquaculture, this study examined the effect of the oral administration of galangal extract on shrimp growth and survival rates, as well as the anti-microbial effect against *V. harveyi* and WSSV. As shown in Table 1, a 1-month feeding of galangal extract lowered the growth rate of shrimp. However, following 2 months of feeding, the growth rate was recovered and the shrimp showed no significant difference in growth rate, SGR or the survival rate between the galangal diet group and the control diet group. We could not measure the feeding amounts, because galangal was dispersed during feeding. However, the results suggest that the lower growth rate in the first month feeding trial might have been caused by the reduced consumption of an unfamiliar diet with a strong smell. The galangal diet should be improved with a smell that will be more attractive for shrimp, and a feeding period of at least 2-months is necessary to allow shrimp to acclimate to a galangal diet.

The galangal diet group showed a higher ability for the clearance of bacteria from the hemolymph at 3 h post-injection of *V. harveyi*. As the number of bacteria in the hemolymph decreased, the survival rate was enhanced in the galangal diet group compared with that in the control groups. This study also demonstrated the anti-viral activity of galangal extract and a significantly higher survival rate from WSSV infection for the galangal diet group. These results indicate that shrimp aquaculture could become more profitable with the suppression of infectious diseases through the use of galangal extract diets. This research introduces the use of

galangal rhizomes as an alternative to chemicals or antibiotics to ensure that shrimp aquaculture will be conducted in a clean environment and will produce a safe, high-quality product for consumers.

Acknowledgements

This research was supported by the Department of Fisheries (DOF), Agriculture Research Development Agency (ARDA), Ministry of Agriculture and Cooperatives of Thailandand and the Kyoto Institute of Technology (KIT), Japan.

References

Abraham, T., Manley, J. R., Palaniappan, R., & Devendran, K. (1997). Pathogenicity and antibiotic sensitivity of luminous *Vibrio harveyi* isolated from diseased penaeid shrimp. *Journal of Aquaculture in the Trop*ics*, 12*(1), 1-8.

Adams, A. (1991). Response of penaeid shrimp to exposure to *Vibrio* species. *Fish Shellfish Immunol., 1*, 59-70. http://dx.doi.org/10.1016/S1050-4648(06)80020-3

Austin, B. (2010). Vibrios as causal agents of zoonoses. *Veterinary Microbiology, 140*, 310-317. http://dx.doi.org/10.1016/j.vetmic.2009.03.015

Burt, S. (2004). Essential oils: their antibacterial properties and potential applications in foods − A review. *International Journal of Food Microbiology, 94*, 223-253. http://dx.doi.org/10.1016/j.ijfoodmicro.2004.03.022

Canillac, N., & Mourey, A. (2001). Antibacterial activity of the essential oil of *Picea excels* on *Listeria*, *Staphylococcus aureus* and coliform bacteria. *Food Microbiology, 18*, 261-268. http://dx.doi.org/10.1006/fmic.2000.0397

Chang, P. S., Lo, C. F., Wang, Y. C., & Kou, G. H. (1996). Identification of white spot syndrome associated aculovirus (WSBV) target organs in the shrimp *Penaeus monodon* by in situ hybridization. *Diseases of Aquatic Organisms, 27*, 131-139. http://dx.doi.org/10.3354/dao027131

Chudiwal, A. K., Jain, D. P., & Somani, R. S. (2010). *Alpinia galangal* Wild. − An overview on phyto-pharmacological properties. *Indian Journal of Natural Products and Resources, 1*(2), 143-149.

Citarasu, T. (2010). Herbal biomedicines: A new opportunity for aquaculture industry. *Aquaculture International, 18*, 403-414. http://dx.doi.org/10.1007/s10499-009-9253-7

Colwell, R. R. (1984). *Vibrios in the Environment*. Wiley, New York.

Cowley, J. A., Cadogan, L. C., Wongteerasupaya, C., Hodgson, R. A. J., Boonsaeng, V., & Walker, P. J. (2004). Multiplex RT-nested PCR differentiation of gill-associated virus (Australia) from yellow head virus (Thailand) of *Penaeus monodon*. *Journal of Virological Methods, 117*, 49-59. http://dx.doi.org/10.1016/j.jviromet.2003.11.018

de Pooter, H. L., Omar, M. N., Coolaset, B. A., & Schamp, N. M. (1985). The essential oil of greater galanga (*Alpinia galanga*) from Malaysia. *Phytochemistry, 24*, 93-96. http://dx.doi.org/10.1016/S0031-9422(00)80814-6

Direkbusarakom, S., Rungpan, L., Ezura, Y., & Yoshimizu, M. (1998). Protective efficacy of *Clinacanthus nutans* on yellow-head disease in black tiger shrimp (*Penaeus monodon*). *Fish Pathology, 33*, 401-404. http://dx.doi.org/10.3147/jsfp.33.401

Immanuel, G., Sivaram, V. C. V., Palavesam, A., & Marian, M. P. (2004). Effect of butanolic extracts from terrestrial herbs and seaweeds on the survival, growth and pathogen (*Vibrio parahaemolyticus*) load on shrimp *Penaeus indicus* juveniles. *Aquaculture, 236*, 53-65. http://dx.doi.org/10.1016/j.aquaculture.2003.11.033

Karunasagar, I., Pai, R., Malathi, G. R., & Karunasagar, I. (1994). Mass mortality of *Penaeus monodon* larvae due to antibiotic-resistant *Vibrio harveyi* infection. *Aquaculture, 128*, 203-209. http://dx.doi.org/10.1016/0044-8486(94)90309-3

Kou, G. H., Peng, S. E., Chiu, Y. L., & Lo, C. F. (1998). Tissue distribution of white spot syndrome virus (WSSV) in shrimp and crabs. In T. W. Flegel (Ed.), *Advances in shrimp biotechnology* (pp. 267-271). National Centre for Genetic Engineering and Biotechnology, Bangkok, Thailand.

Latha, C., Shriram, V. D., Jahagirdar, S. S., Dhakephalkar, P. K., & Rojatkar, S. R. (2009). Antiplasmid activity of 1'-acetoxychavicol acetate from *Alpinia galanga* against multi-drug resistant bacteria. *Journal of*

Ethnopharmacology, 123, 522-525. http://dx.doi.org/10.1016/j.jep.2009.03.028

Lavilla-Pitogo, C. R., Leano, E. M., & Paner, M. G. (1998). Mortalities of pond-cultured juvenile shrimp, *Penaeus monodon*, associated with dominance of luminescent *vibrios* in the rearing environment. *Aquaculture, 164*, 337-349. http://dx.doi.org/10.1016/S0044-8486(98)00198-7

Lightner, D. V. (1996). A handbook of shrimp pathology and diagnostic procedures for diseases of cultured penaeid shrimp. *World Aquaculture Society*. Baton Rouge, Louisiana.

Liu, P. C., Lee, K. K., & Chen, S. N. (1997). Susceptibility of different isolates of *Vibrio harveyi* to antibiotics. *Microbios, 91*, 175-180.

Lo, C. F., Ho, C. H., Peng, S. E., Chen, C. H., Hsu, H. C., Chiu, Y. L., ... Kou, G. H. (1996). White spot syndrome baculovirus (WSBV) detected in cultured and captured shrimp, crabs and other arthropods. *Diseases of Aquatic Organisms, 27*, 215-225. http://dx.doi.org/10.3354/dao027215

Maeda, M., Itami, T., Furumoto, A., Hennig, O., Imamura, T., Kondo, M., ... Takahashi, Y. (1998). Detection of penaeid rod-shaped DNA virus (PRDV) in wild-caught shrimp and other crustaceans. *Fish Pathol, 33*(4), 373-380. http://dx.doi.org/10.3147/jsfp.33.373

Mayachiew, P., & Devahastin, S. (2008). Antimicrobial and antioxidant activities of Indian gooseberry and galangal extracts. *LWT - Food Science and Technology, 41*, 1153-1159. http://dx.doi.org/10.1016/j.lwt.2007.07.019

Montero, A. B., & Austin, B. (1999). Characterization of extracellular products from an isolate of *Vibrio harveyi* recovered from diseased post-larval *Penaeus vannamei* (Bonne). *Journal of Fish Diseases, 22*, 377-386. http://dx.doi.org/10.1046/j.1365-2761.1999.00189.x

Natta, L., Orapin, K., Krittika, N., & Pantip, B. (2008). Essential oil from five Zingiberaceae for anti food-borne bacteria. *International Food Research Journal, 15*, 337-346.

Navarro, S. A., Tang, K. F. J., & Lightner, D. V. (2009). An improved Taura syndrome virus (TSV) RT-PCR using newly designed primers. *Aquaculture, 293*, 290-292. http://dx.doi.org/10.1016/j.aquaculture.2009.05.003

Olmedo Sanchez, J. A., Curiel Flores, A., & Orozco Hernandez, J. R. (2009). The Effect of a Herbal Growth Promoter Feed Additive on Shrimp Performance. *Research Journal of Biological Sciences, 4*(9), 1022-1024.

Oonmetta-aree, J., Suzuki, T., Gasaluck, P., & Eumkeb, G. (2006). Antimicrobial and action of galangal (*Alpinia galanga* Linn.) on *Staphylococus aureus*. *LWT - Food Science and Technology, 39*, 959-965. http://dx.doi.org/10.1016/j.lwt.2005.06.015

Peinado-Guevara, L. I., & Lo'pez-Meyer, M. (2006). Detailed monitoring of white spot syndrome virus (WSSV) in shrimp commercial ponds in Sinaloa, Mexico by nested PCR. *Aquaculture, 251*, 33-45. http://dx.doi.org/10.1016/j.aquaculture.2005.05.022

Rao, K., Ch, B., Narasu, L. M., & Giri, A. (2010). Antibacterial activity of *Alpinia galangal* (L) Willd crude extracts. *Applied Biochemistry and Biotechnology, 162*, 871-884. http://dx.doi.org/10.1007/s12010-009-8900-9

Ruangpan, L. (1998). Luminous bacteria associated with shrimp mortality. In T. W. Flegel (Ed.), *Advances in shrimp biotechnology* (pp. 205-211). National Center for Genetic Engineering and Biotechnology, Bangkok.

Ruangpan, L., & Kitao, T. (1991). Vibrio bacteria isolated from black tiger shrimp *Penaeus monodon* Fabricius. *Journal Fish Diseases, 14*, 383-388. http://dx.doi.org/10.1111/j.1365-2761.1991.tb00836.x

Ruangpan, L., Thanasomwang, V., & Sangrungrauge, K. (1997). Bacteria in black tiger shrimp pond farming development systems. *The proceeding of 35th Kasetsart university annual conference, Thailand* (pp. 3-10).

Sankar, G., Elavarasi, A., Sakkaravarthi, K., & Ramamoorthy, K. (2011). Biochemical changes and growth performance of black tigher shrimp larvae after using *Ricinus commuunis* extract as feed additive. *International Journal of PharmTech Research, 3*(1), 201-208.

Sung, H. H., Hsu, S. F., Chen, C. K., Ting, Y. Y., & Chao, W. L. (2001). Relationships between disease outbreaks in cultured tiger shrimp (*Penaeus monodon*) and composition of *Vibrio* communities in pond water and shrimp hepatopancreas during cultivation. *Aquaculture, 192*, 101-110.

http://dx.doi.org/10.1016/S0044-8486(00)00458-0

Sung, H. H., Tsai, F. U., Ting, Y. Y., & Chao, W. L. (1999). Changes in the composition of *Vibrio* communities in pond water during tiger shrimp (*Penaeus monodon*) cultivation and in the hepatopancreas of healthy and diseased shrimp. *Journal of Experimental Marine Biology and Ecology, 236*, 261-271. http://dx.doi.org/10.1016/S0022-0981(98)00206-8

Tachakittirungrod, S., & Chowwanapoonpohn, S. (2007). Comparison of antioxidant and antimicrobial activities of essential oils from *Hyptis suaveolens* and *Alpinia galangal* growing in Northern Thailand. *Chiang Mai University Journal of Natural Sciences, 6*, 31-42.

Tang, K. F. J., Navarro, S. A., & Lightner, D. V. (2007). PCR assay for discriminating between infectious hypodermal and hematopoietic necrosis virus (IHHNV) and virus-related sequences in the genome of *Penaeus monodon*. *Diseases of Aquatic Organisms, 74*, 165-170. http://dx.doi.org/10.3354/dao074165

Turker, A., & Usta, C. (2006). Biological activity of some medicinal plants sold in Turkish health – Food stores. *Biotechnology & Biotechnological Equipment, 20*, 105-113. http://dx.doi.org/10.1080/13102818.2006.10817386

Vandenberghe, J., Verdonck, L., Robles-Arozarena, R., Rivera, G., Blland, A., Balladares, M., ... Swings, J. (1999). Vibrios associated with *Litopenaeus vennamei* larvae, postlarvae, broodstock, and hatchery probionts. *Applied and Environmental Microbiology, 65*, 2592-2597.

Velmurugan, S., & Citarasu, T. (2010). Effect of herbal antibacterial extracts on the gut floral changes in Indian white shrimp *Fenneropenaeus indicus*. *Romanian Biotechnological Letters, 15*(6), 5709-5717.

Velmurugan, S., Punitha, S. M. J., Baru, M. M., Selvaraj, T., & Citarasu, T. (2010). Indian herbal medications to replace antibiotics for shrimp *Penaeus monodon* post larvae. *Journal of Applied Aquaculture, 22*, 230-239. http://dx.doi.org/10.1080/10454438.2010.500589

Vuddhakul, V., Bhoopong, P., Hayeebilan, F., & Subhadhirasakul, S. (2007). Inhibitory activity of Thai condiments on pandemic strain of *Vibrio parahaemolyticus*. *Food Microbiology, 24*, 413-418. http://dx.doi.org/10.1016/j.fm.2006.04.010

Yu, M. C., Li, Z. J., Lin, H. Z., Wen, G. L., & Ma, S. (2008). Effects of dietary *Bacillus* and medicinal herbs on the growth, digestive enzyme activity, and serum biochemical parameters of the shrimp *Litopenaeus vannamei*. *Aquaculture International, 16*, 471-480. http://dx.doi.org/10.1007/s10499-007-9159-1