Ph.D Thesis

Regulation of infectious diseases of Pacific white shrimp (*Litopenaeus vannamei*) by galangal (*Alpinia galanga* Linn.) extract

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This thesis is dedicated to my great motherland, Thailand.

Contents

Abbreviations

ACA	1’-acetoxyeugenol acetate
AHPND	Acute Hepatopancreatic Necrosis Disease
cDNA	complementary Deoxyribonucleic Acid
cfu	colony forming unit
cMnSOD	cytosolic manganese superoxide dismutase
CMNV	Covert Mortality Nodavirus
^{13}C-NMR	Carbon13- Nuclear magnetic resonance
DNA	Deoxyribonucleic Acid
dNTP	Deoxy nucleotide triphosphate
EMS	Early Mortality Syndrome
ESI-MS	Negative-ion electro-spray-ionization mass spectra
^{1}H-^{13}C	Proton-Carbon-13
HMBC	Heteronuclear multiple bond coherence spectroscopy
HMQC	Heteronuclear multiple quantum coherence
^{1}H-NMR	Proton nuclear magnetic resonance
HPLC	High Performance Liquid Chromatography
HPV	Hepatopancreatic Parvovirus
IC_{50}	The half maximal inhibitory concentration
ID_{50}	The half Infectious dose or Infectious Dose with 50% endpoint
IHHNV	Infectious Hypodermal and Hematopoietic Necrosis Virus
IMNV	Infectious Myonecrosis Virus
LD_{50}	The half lethal Dose or Lethal Dose 50 with 50% endpoint
LPS	Lipopolysaccharides

LSZ	Lysozyme
MBC	Minimum Bactericidal Concentration
MBV	Monodon Baculovirus
MIC	Minimum Inhibitory Concentration
mRNA	messenger Ribonucleic Acid
MSGS	Monodon Slow Growth Syndrome
NHP	Necrotizing Hepatopancreatitis
OD	Optical Density
OIE	Office International des Epizooties
PNE3	Penaeidin-3
PO	Phenoloxidase
ProPO	Prophenoloxidase
RNAi	Ribonucleic acid interference
RT-PCR	Reverse-transcription polymerase chain reaction
SEMBV	Systemic Ectodermal and Mesodermal Baculovirus
SGR	Specific growth rate
SPF	Specific Pathogen-Free
TEM	Transmission Electron Microscopy
TGase	Transglutaminase
TOCSY	Two-dimensional total correlation spectroscopy
TSV	Taura syndrome virus
WSSV	White spot syndrome virus
YHV	Yellow head virus

ABSTRACT

In this study, galangal extract was tested for its efficacy for controlling shrimp pathogens, using Pacific white shrimp (*Litopenaeus vannamei*) which is the main product of penaeid shrimp. We discovered that the galangal (*Alpinia galanga* Linn.), ethanol extract suppressed the growth of 8 pathogenic *Vibrio* species, *V. parahaemolyticus* (EMS/AHPND) in particular. We also found that antibacterial compounds of the galangal extract were *trans*-*p*-hydroxy cinnamaldehyde, *trans*-*p*-acetoxy cinnamic alcohol and *trans*-*p*-coumaryl diacetate with the half maximal inhibitory concentrations (IC_{50}) against *V. harveyi* of 0.0740 ± 0.0095, > 5 and 0.7200 ± 0.0190 µmol/ml, respectively.

A commercial diet mixed with galangal extract was fed to shrimp. 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 shrimp were significantly lower than that in the control group. Furthermore, the survival rates for the treatment groups, after injections with *V. parahaemolyticus* (EMS/AHPND), *V. harveyi* or white spot syndrome virus were significantly higher than that of the control group. The number of *V. harveyi* in the hemolymph of the galangal diet group was significantly lower than that in the control diet group, indicating the higher clearance ability of the galangal diet group.

The intramuscular injection of either galangal extract or *trans*-*p*-coumaryl diacetate to Pacific white shrimp showed significant increases in the relative expression level of the six immune-related genes compared with a control group. Furthermore, by the oral administration of galangal extract, similar inducible effects of the expression of immune-related genes in shrimp were obtained, which led to an enhanced survival rate from *V. harveyi* infection. Based on these results, the galangal extract might be useful for the treatment of shrimp diseases in the aquaculture industry. Moreover, this studies can solve the problem of antibiotic residual in shrimp product and make it safe for consumers.

General Introduction

Introduction

Aquaculture is very important because it came as a substitute for aquatic product capturing from natural source is reduced, but demand for aquaculture products is rapidly increasing (Fig.1) Shrimp aquaculture is one part of fisheries culture that is rapid industrialization. In 2011, shrimp aquaculture production of the world was the highest volumes as 4.0 Million MT (Fig. 2). Therefore, in the present shrimp is considered one of the world's important industries have high economic value, because the shrimp culture industry consists of several important elements, such as Broodstock, larviculture (hatcheries and nursery), grow out pond, processing and distribution of shrimp production, as well as the feed industry, chemical and medical, biological substances, including supplements. These activities have been continued growth based on the development of the shrimp industry.

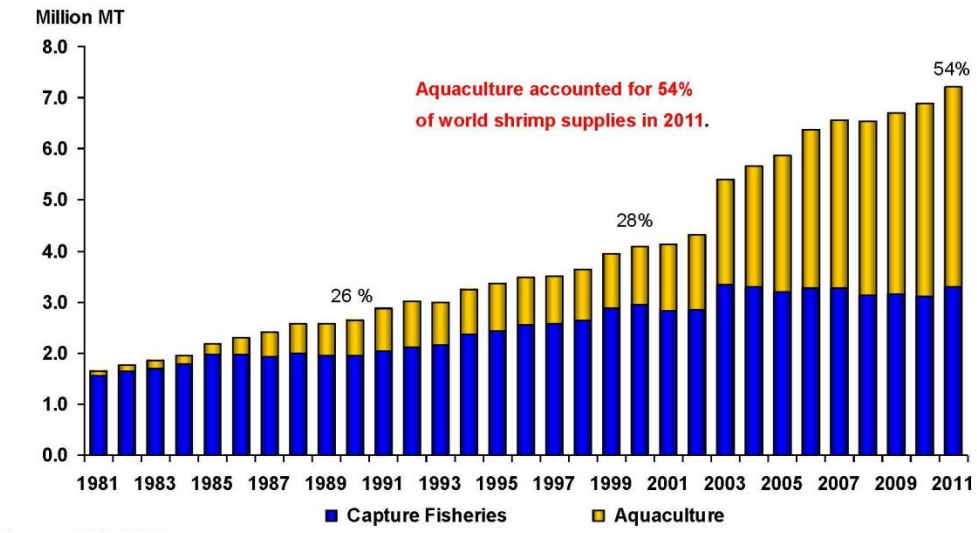


Fig. 1 Trends in shrimp aquaculture production of the world compared to capture (FAO, 2013), http://gaalliance.org/wp-content/uploads/2015/04/goal13-anderson.pdf

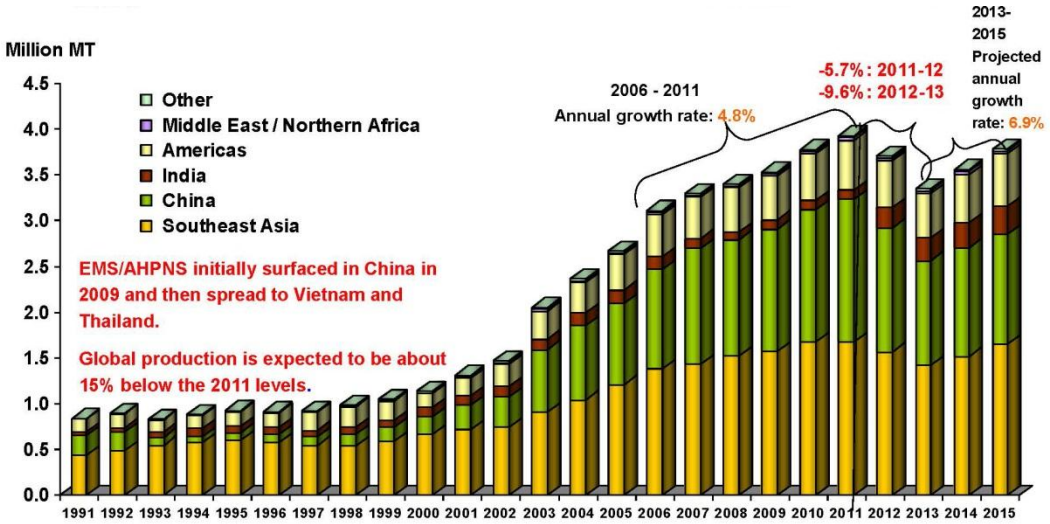


Fig. 2 Major of the world countries with shrimp aquaculture production in 1991-2015 (FAO, 2013), http://gaalliance.org/wp-content/uploads/2015/04/goal13-anderson.pdf

The Pacific white shrimp *Litopenaeus vannamei* is the main product of penaeid shrimp. The countries culture *L. vannamei* are mainly in Asia and South America (Fig. 2). The rapid development of productivity in shrimp culture became problems are cumulative impacts, one of the problems is that the occurrence of epidemic diseases. Diseases have emerged as a major constraint to the sustainable growth of shrimp aquaculture industry. Shrimp diseases have caused significant losses in production and jobs, reduced earning, export restrictions, failure and closing of business and decreased the confidence of consumers (1). Shrimp can be threatened by a protozoan, fungal, bacterial and viral pathogens, but viral and bacterial diseases cause major troubles in shrimp farming (2). Apart from diseases caused by viruses, those caused by bacteria are also major problems. The bacterial diseases due mainly to *Vibrio* spp. species are often associated with low survival rates in a hatchery or grow out conditions. Several outbreak diseases such as WSD, YHV, TSV, IMNV, Vibriosis, AHPND in marine shrimp production reduced yields and the farmer use of antibiotics to prevent and treat disease in shrimp that, the problem causing residue in the shrimp. The use and occasional misuse of antibiotics in shrimp farming has led to multiple drug resistant pathogens and sometimes even to the rejection of farmed shrimp by importing countries

(3). However, extensive use of these drugs has resulted in an increase of drug resistant bacteria.

The current solutions in the marine shrimp disease will focus on preventing and managing environmentally in a friendly way so that they do not affect the consumer, such as using probiotic, immune stimulant and use herbal extract is safe and friendly to the environment. The alternative herbal bio-medicinal products in the aquacultural operations, which have the characteristics of growth promoting ability and tonic to improve the immune system, act as appetite stimulators. They increase consumption, induce maturation, and have the antimicrobial capability and also antistress characteristics that will be of immense use in the culture of shrimps and other fin fishes without any environmental and hazardous problems. Herbal compounds such as phenolics, polyphenols, alkaloids, quinones, terpenoids, lectines and polypeptides have been shown to be very effective alternatives to antibiotics and other synthetic compounds (4). This research results prompted this investigation into the application of herbs in the shrimp aquaculture. Therefore, the antimicrobial activity of the extract of Zingiberaceae against *Vibrio* species, fungi, and WSSV is yet to be clarified as well as identify the structure of antibacterial active ingredients. However, 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*) and we focused on examining the impact of galangal-ethanol extracts on the expression levels of the immune-related genes of Pacific white shrimp. The results of this thesis might be the regulation of infectious diseases of shrimp.

LITERATURE REVIEWS

Pacific white shrimp (*Litopenaeus vannamei*)

The biology of Pacific white shrimp

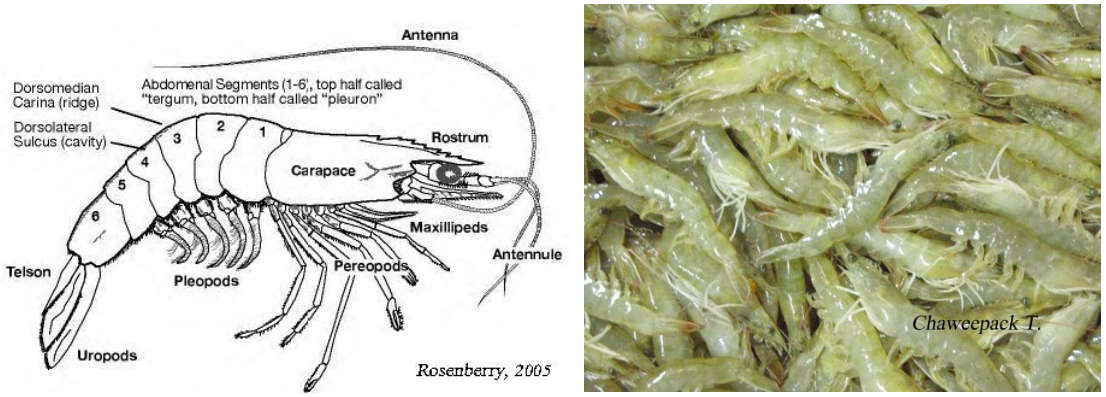


Fig. 3 External anatomy of *Litopenaeus vannamei* (5)

Penaeid shrimps are within the largest phylum in the animal kingdom, the Arthropoda. Arthropods have an exoskeleton including rigid cuticle, chitin, and proteins, which cover the whole animal (6). The subphylum Crustacean contains around 42,000 species, including lobsters, crabs, shrimp, pill bugs, krill, barnacles, water fleas, brine shrimp, copepods, and ostracods. Shrimp, crayfish, lobsters, and crabs are a member of the order Decapoda, which is a part of the class Malacostraca. *L. vannamei* is a decapod crustacean that belongs to the family Penaeidae. *L. vannamei* is a member of the genus *Penaeus*, which is distinguished by the presence of the teeth on both the upper and lower margin of the rostrum, and by the lack of setae on the body (7). The external anatomy of *L. vannamei* is characterised by a cephalothorax with a characteristic hard rostrum (Fig.3). Most internal organs like gills, heart, lymphoid organ, haepatopancreas, and stomach are located in the cephalothorax while the muscles concentrate in the abdomen. In the head region, antennules and antennae perform sensory functions. In the thorax region, the maxillipeds are the first three pairs of

appendages, modified for food handling, and the remaining five pairs are the walking legs (pereiopods). Five pairs of swimming legs (pleopods) are found on the abdomen (5).

Taxonomy

All farming shrimps include *Penaeus vannamei* (or *Litopenaeus vannamei*) under the Penaeidae family are referred to as Penaeids.
(http://www.fao.org/fishery/culturedspecies/Penaeus_vannamei/en).

Phylum: Arthropoda
Subphylum: Crustacea
Class: Malacostraca
Subclass: Eumalacostraca
Superorder: Eucarida
Order: Decapoda
Suborder: Dendrobranchiata
Superfamily: Penaeoidea
Family: Penaeidae
Genus: Penaeus
Subgenus: Litopenaeus
Genus: Litopenaeus
Species: *Litopenaeus vannamei* (Boone, 1931)

White leg shrimp (En, FAO Name; *Litopenaeus vannamei*, formerly *Penaeus vannamei*), also known as Pacific white shrimp, is a variety of prawn of the eastern Pacific Ocean commonly caught or farmed for food. Distribution: Eastern Pacific: from Sonora, Mexico, south to northern Peru. It is restricted to areas where the water temperature remains above 20 °C (68 °F) throughout the year. Habitat: Depth 0 to 72 m. Bottom mud. Marine (adults), and estuarine (juveniles) (Fig. 4). Size: Maximum total length 230 mm, maximum carapace length 90 mm. The rostrum is moderately long, with 7–10 teeth on the dorsal side and 2–4 teeth on the ventral side.

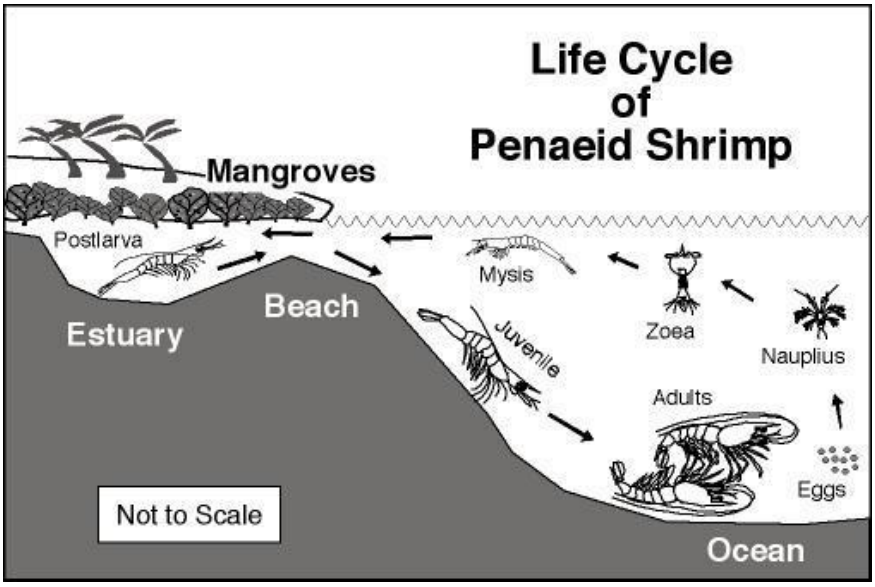


Fig. 4 The life cycle of penaeid shrimp (5) http://www.shrimpnews.com

Shrimp Aquaculture

During the 20th century, *L. vannamei* was an important species for Mexican inshore fishermen, as well as for trawlers further offshore. In the late 20th century, the wild fishery was overtaken by the use of aquaculture; this began in 1973 in Florida using prawns captured in Panama. In Latin America, the culture of *L. vannamei* showed peaks of production during the warm El Niño years, and reduced production during the cooler La Niña years, due to the effects of the disease (http://www.fao.org/fishery/culturedspecies/Litopenaeus_vannamei/en). Production of *L. vannamei* is limited by its susceptibility to various diseases, including white spot syndrome, Taura syndrome, infectious hypodermal and haematopoietic necrosis, baculoviral midgut gland necrosis and *Vibrio* infections.

Shrimp Aquaculture in Thailand

The major cultured shrimp producing countries in Asia are China, Thailand, Indonesia, India, and Vietnam (Fig. 5). Thailand has been the world's leading exporter of cultivated shrimp since the mid-90's; in recent year is one in five of leader exporter in the world (8). Thailand is the leader of shrimp industry group of the world. Thus, areas and climate environment of this locate is a suitable and shrimp farmer has more experience than the other countries. Intensive shrimp farming in Thailand was started since the mid-1980s, Tiger shrimp (*P. monodon*) culture is main exporter production from 1980 – 2002. Development is semi-intensive farm to intensive farm that is high density and aeration system. Shrimp culture usually produces high-value products for export. The main shrimp cultured species were giant tiger prawn (*P. monodon*), Pacific white shrimp (*L. vannamei*). Shrimp farming has been practiced in Thailand for more than 30 years but developed and expanded very rapidly during the mid-1980s, supported by the technological breakthrough in shrimp feed development and successful production of larvae in 1986. Three distinct types of shrimp farming can be distinguished in Thailand, namely extensive farming, semi-intensive farming and intensive farming. Extensive farming is the original shrimp culture system that cultures shrimp in large areas using the traditional methods of tidal exchange of water and natural seed supply. This extensive system yields mainly the banana shrimp (*P. merguiensis*), but production is unreliable. Semi-intensive shrimp farming became popular with the successful production of both banana and giant tiger prawn in 1972. Farms are usually 3-5 ha in size with a reservoir, from which the water is pumped into the main rearing ponds. Predators are removed before shrimp larvae are stocked. Artificial feed is supplied to increase production. The extensive and semi-intensive systems are currently not very practical and very few are operated today. The semi-intensive system shifted to the Taiwanese-style intensive farming system after the successful development of commercial giant tiger prawn seed production in 1986. The ponds are stocked at densities of 50-100 larvae per m^2 and fed with high-quality artificial feed at least 4-5 times a day. Because of the heavy feed, the pond becomes very anaerobic within 100 days and has to be well aerated using paddlewheels and air/oxygen injectors to keep the oxygen levels above 5 ppm. Production can be as high

as 15 tons/ha/crop (9, 10). The present Department of Fisheries (DOF), Thailand has been encouraged standard farm to shrimp farmer including GAP (Good Aquaculture Practice), Compartment (Biosecure) and Organic standard farm for sustainable shrimp aquaculture (11).

Recently, coastal aquaculture started with the introduction of intensive and super-intensive culture technologies and has today become the most successful regarding income. The culture of Pacific white shrimp (*L. vannamei*) has recently increased rapidly due to its fast growth rate and high market demand. Pacific white shrimp cultured in Thailand started from 2002 to the present. The years 2002 – 2006 were relatively short space white shrimp largely replaced black tiger shrimp as the preferred species in Thailand. Support from a vertically integrated major firm, an extensive foundation of learning networks within the industry, and early profitability made the scaling-up and embedding of the experiment with white shrimp very rapid once the formal ban on import of exotic brood stock was lifted. Moreover, the relative production cost is lower than with black shrimp (12).

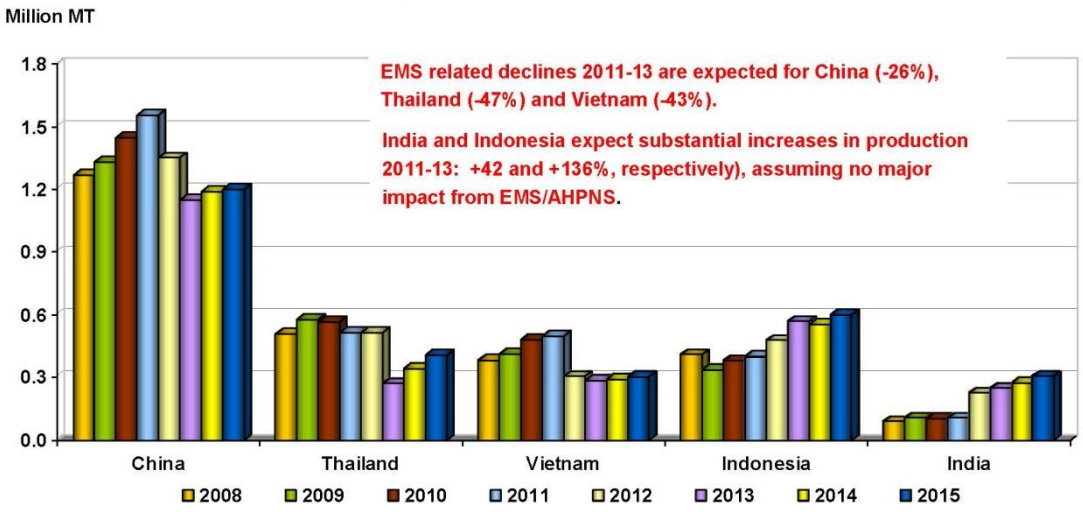


Fig. 5 Shrimp aquaculture production in Asia: major producer countries in 2008 - 2015 (FAO, 2013; GOAL, 2013), http://gaalliance.org/wp-content/uploads/2015/04/goal13-anderson.pdf

Shrimp production of Thailand had been increased from 23,566 ton to about 309,862 ton at the end of the year 2000 (Fig. 6). This is because a tropical climate of Thailand creates an ideal suitability for intensive marine shrimp farming (13). However, a rapid growth in shrimp production has also negatively impacted on its sustainability indicating by the outbreak of YHV (Yellow Head Virus) since 1992 and SEMBV (Systemic Ectodermal and Mesodermal Baculovirus), also known as white spot baculovirus), which is the most serious pathogen since 1994 (14). During 2002, Monodon Slow Growth Syndrome (MSGS) was reported throughout shrimp growing areas of Thailand and figures indicated that annual production volume was reduced by approximately 36% (15). The cause of this slow growth was not determined, but laboratory trails suggested that a filterable infectious agent was involved (16). This is the most significant shrimp disease outbreak in Thailand which ended the era majority of *P. monodon* farming in Thailand in 2004. Pacific white shrimp (*L. vannamei*) was introduced to farm in Thailand in 2003 when the *P. monodon*. This species has shown a good adaptation to the tropical climate and intensive farming in Thailand indicating the change of the majority of farmed shrimp and the increase of shrimp production to nearly 600,000 ton during 2009-2010 (Fig 6).

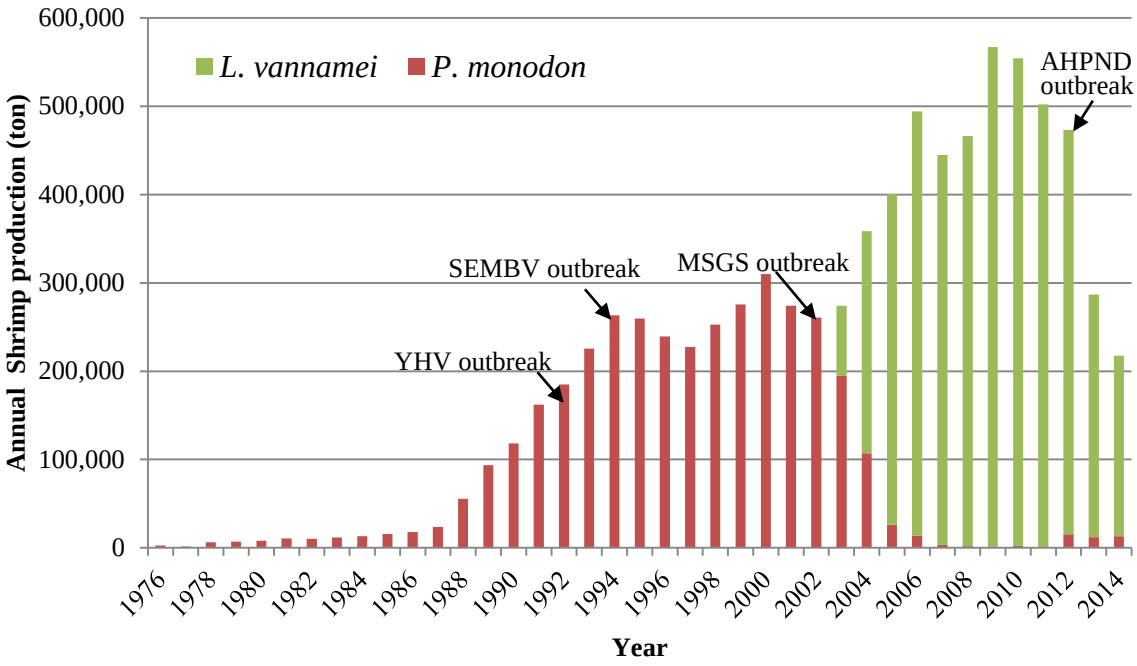


Fig. 6 Annual shrimp production in Thailand from 1976-2014 indicating the year of major disease outbreak and the transition period from the majority of *Penaeus monodon* to the majority of *Litopenaeus vannamei* (17).

Since the first found of EMS/AHPND (18), Thailand lost its potential of farmed shrimp (Fig. 6). This outbreak has been impacted to the shortage of raw materials, an increase in production cost and fluctuation of farm gate price especially of the farming in the coastal area that significantly impacts to the sustainability of shrimp industry in Thailand (17).

Shrimp immune system

The immune system is important in the defense of life from the pathogens and foreign substance. In crustaceans, only innate immunity without immunological memory exists (19). Innate immunity includes physical barriers, humoral and cellular responses. While invading pathogens gain entry into the body of the shrimp, they encounter a complex system of innate defense mechanism involving cellular and humoral responses. The rigid and wax covered cuticle in crustaceans serves as a mechanical barrier against pathogen invasion (19, 20). Factually, there is an overlap between cellular and humoral defense, since hemocytes are an important source of many humoral factor and many humoral molecules affect hemocyte function (21, 22). The cellular immune responses include apoptosis, encapsulation, phagocytosis, clotting and nodule formation, whereas the humoral responses include the prophenoloxidase (proPO) system, the clotting cascade and a wide array of antimicrobial peptides (23, 24).

Shrimp haemocytes keep important roles in the host immune response. They stimulate defense system using different mechanisms against various infectious organisms (Fig.7). Besides controlling the invasion of foreign organisms, haemocytes participate in recognition, phagocytosis, melanization, cytotoxicity, nodulation, encapsulation and communication between cells (25). By far, several types of haemocytes such as hyaline cells (HCs), semi-granular cells (SGCs), and granular cells (GCs) have been classified in decapods crustaceans that classification mainly based on their morphology such as size of cells, size of nucleus, and the presence of cytoplasmic granules (26, 27). Differences types of haemocytes have different functionalities in defense reactions. Hyalinocytes with small size and contain no or few granules are chiefly involved in phagocytosis and also in coagulation by releasing transglutaminase (25, 28) that can change coagulogen into dissolved coagulin. They also produce reactive oxygen intermediates (ROIs) after phagocytosis (29). Semi-granular cells, which have many eosinophilic granules, are active in encapsulation and have a limited function in phagocytosis. These cells contain the prophenoloxidase activating system (proPO system). Granular cells with numerous big eosinophilic granules participate in storage

and release of the prophenoloxidase (proPO) activating system and cytotoxicity (26, 27, 29, 30).

The innate immune mechanism allows *L. vannamei* to respond to common antigens on the cell surfaces of bacterial, fungal, and viral invasions. This defense mechanism includes many factors such as antimicrobial peptides, prophenoloxidases, clotting factors, lectins, complement factors, protease inhibitors, and other humoral factors, which were found mainly in hemolymph plasma and haemocytes (23). There are two steps involved in the defensive response. The first process is recognition and the other is activation in the crustacean defense system. The first process and essential process is the recognition of invading micro-organisms by the hemocytes and plasma proteins (31). The invertebrate immune system presumably recognizes large groups of pathogens, represented by fixed common molecular patterns, rather than fine structures, specific for particular microbes (32). Several types of recognition protein are called pattern recognition proteins (PRPs) that are highly conserved in evolution (33, 34). After binding of the PRP ligand with microbial component, a second site becomes active for cellular binding. Hemocyte activation is generated after this second binding step (31). Both SGCs and GCs can be induced to degranulate by foreign molecules. The SGC is the first hemocyte type to react to foreign particles in vivo and they respond by degranulation, releasing the proPO system from their granules into the plasma (35). The open circulatory system demands a rapid and efficient defense, in which the proteolytic cascades play an important role (36). The hemocytes are involved in the synthesis, storage and upon activation discharge of proenzymes and substrates of the clotting and proPO cascades (32, 36). These activated hemocytes are also induced to release other proteins that are related to defensive responses. The proteins include multifunctional peroxinectin, some AMPs and some defense-related enzyme such as lysozyme, superoxide dismutase. The hyaline cells also can be activated to have the phagocytic ability (37).

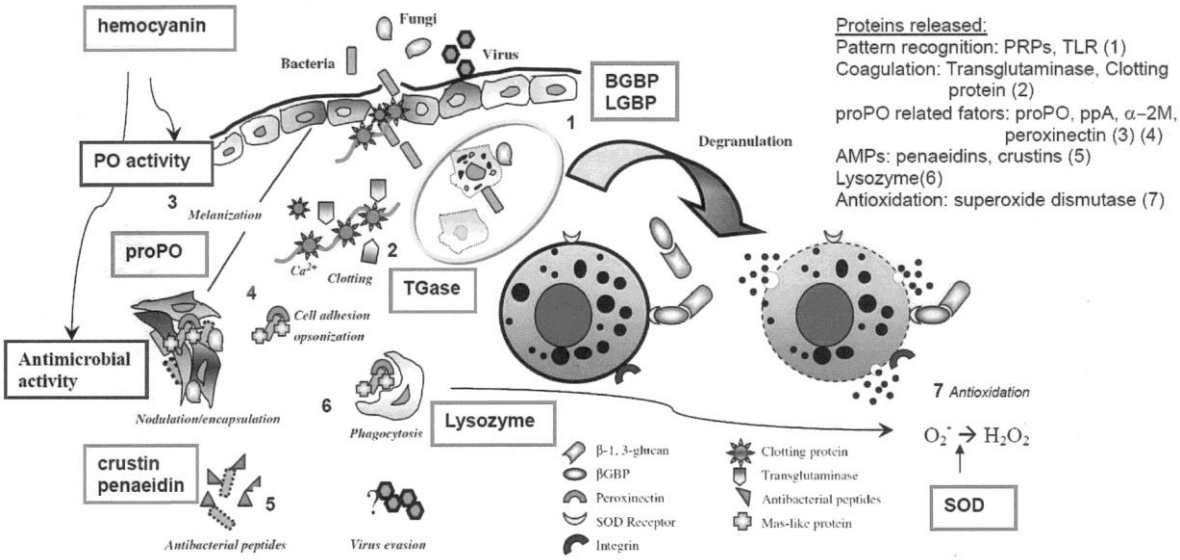


Fig. 7 Once pathogens gain entry into the hemocoel of the host, they must encounter a complex system of innate defence mechanisms in decapod crustaceans (modified from Jiravanichpaisal *et al*., 2006 *In*: Wang, 2007) (37).

Pattern Recognition Proteins (PRPs)

The crustaceans use PRPs to recognize and respond to microbial intruders through the signature molecules on the surface of the intruders. A number of invertebrate PRPs have been isolated and characterized. According to the molecules they recognize, the PRPs are grouped as ß-1, 3-glucan binding proteins (BGBP); lipopolysaccharide (LPS)-binding proteins (LBP): LPS and ß-1, 3-glucan binding proteins (LGBP) and peptidoglycan-binding proteins (PGBP). BGBP is a component of the immune system in arthropods and has been purified from three crustaceans. The complete sequence of the Pacific white shrimp BGBP has also became available recently (38). It was recently shown that Pacific white shrimp high-density lipoprotein (HDL) and the ß-1, 3-glucan binding proteins (BGBP) are identical, implying the protein has dual function: lipid transport and involvement in the defense system (39). LGBP cDNA clones were obtained from the hemocyte and hepatopancreas of *L. vannamei* and LGBP tanscript in hemocyte of *L. vannamei* increased in 3 and 6 h after *Vibrio alginolyticus* injection (40).

The prophenoloxidase and phenoloxidase activities

Granulocytes are responsible for the synthesis, storage and secretion of the proPO system, which is activated by fungal β-glucans, PG and LPS. These molecules induce the granulocyte secretion of inactive proPO granules and their transformation (cascade reaction) to proPO enzyme. An enzyme involved in this important process is phenoloxidase (PO). PO is synthesized as a zymogen, prophenoloxidase (proPO), which can be activated by specific proteolysis. A cascade of serine proteinase and other factor has been recognized to control the activation of proPO into PO and is known as proPO activation system (PAS) (36, 41, 42). The production of toxic quinone intermediates and O-quinones by phenoloxidase is an initial step in the biochemical cascade of melanin biosynthesis, and is also important in cuticular scleotization, wound healing, and in the encapsulation of foreign materials for host defense. Melanization prevents or retards the growth of intruders because highly reactive and toxic quinone intermediates are produced when melanin formed (41, 43). Peroxynectin is a proPO system associated factor that creates cellular adhesion and acts as a peroxidase. This molecule is synthesized and stored by the granulocytes and activated upon cell secretion. Hemocyte's transmembrane receptors are responsible for the peroxynectin cell adhesion, hemocyte dispersion, phagocytosis, encapsulation, nodule formation and agglutination which resulted in peroxide activation and the invading agent destruction (41). The first invertebrate proPO gene was cloned from the freshwater crayfish *Pacifastacus leniusculus* (44). Since then, about 20 arthropod proPO have been cloned, including the tiger shrimp (45) and the Pacific white shrimp (46).

Antioxidant system and superoxide dismutase (SOD)

Antioxidant factors protect the shrimp from the cytotoxic effects caused by the cellular metabolism and oxidative stress generated by the disequilibrium of the reactive oxygen intermediates (ROIs), stress tests had been done in marine organisms (47). The study detected an increase in the levels of the antioxidant enzymes and immune system molecules pointing out the important role of the antioxidant enzymes as the immune response modulators. ROIs and reactive nitrogen intermediates (RNIs) are generated in

phagocytic vacuoles. These molecules are capable of crossing the cell barrier and damaging the neighbouring cells (48). To prevent this damage, antioxidant defense strategies have been developed including enzymatic substance (catalase, glutathione peroxidase (GPx), and SOD) and nonenzymatic components (ascorbate, β-carotene, flavonoids,α-tocopherol and vitamin E), which may neutralize the ROIs or repair the molecular damage done to the cell (48). Superoxide dismutases (SODs) are essential antioxidant enzymes that occur in virtually all oxygen-respiring organisms. SODs are one of the main defense mechanisms against oxidative stress caused by pollution, infections, hypoxia, hyperoxia, temperature and immunostimulants (49) They are classified into three distinct groups depending on the metal content: manganese SOD (mitochondria and prokaryotes), iron SOD (bacteria and plants) and copper-zinc SOD (eukaryotic cytosol).extracellular SOD (ecSOD) is constitutively present at the hemocyte surface and is a peripheral protein. Physical interaction with a peroxidase appears to be a novel function for an SOD. SOD may mediate or regulate cell adhesion and/or phagocytosis. By producing hydrogen peroxide, it may also provide substrate for peroxidase and localize an efficient microbicidal attack. The complete cDNA clone from *L.vannamei* was established recently (50). After ß-glucan and sulfate polysaccharide immersion, the SOD activity in hemocyte and muscle of juvenile white shrimp was greatly enhanced (51).

The Clotting system

The clotting mechanism is an essential defense response of crustaceans that entraps foreign material and prevents loss of hemolymp and to immobilization of invading pathogens (52, 53). In crustaceans, the coagulation process is regulated by clotting proteins (coagulogens) and compartmentalized cellular factors within circulating cells. Clotting proteins in plasma are converted to covalently joined polymers by a Ca++ dependent transglutaminase secreted by the hemocytes (54). Transglutaminase (TGase) are important for blood coagulation and post-translation remodeling of proteins. The TGase-dependent clotting reaction in crayfish is induced when TGase is released from hemocytes or tissues, becomes activated by Ca^{2+} in plasma and starts to crosslink the clotting protein molecules found in the plasma, to

from large aggregates (55, 56). TGases are Ca dependent enzymes capable of forming covalent bonds between the side chains of free lysine and glutamine residues on certain proteins. The cellular clotting proteins can be activated by LPS or β-1, 3-glucan, and are related to the proPO activation system (57).

Antimicrobial peptides (AMPs)

The major AMPs are represented by the three cationic peptide families: penaeidins, crustins, and antilipopolysaccharide factors (ALFs) are comprised of multiple classes or isoforms and possess antibacterial and antifungal activities against different strains of bacteria and fungi. Shrimp AMPs are primarily expressed in circulating hemocytes, which is the main site of the immune response, and hemocytes expressing AMPs probably migrate to infection sites to fight against pathogen invasion (58). As the incidence of microbial resistance to existing antibiotics increase, AMPs are a promising resource for therapeutic and pharmaceutical alternatives. Some AMPs have been clinically tested against multidrug-resistant strains of bacteria (59). Indeed, most AMPs are produced as early as the nauplii developmental stage to protect shrimp larvae from infections. The information available on antimicrobial activities indicates that these shrimp AMPs have potential therapeutic applications in the control of disease problems in aquaculture (60).

Penaeidins

Penaeidin, a family of AMPs, The three peptides PEN2-1, PEN2-2 and PEN-3 were initially isolated from the hemocytes of the pacific white shrimp, *L. vannamei* (61). Penaeidins are synthesized and stored in the granulocyte, and present Gram-positive antibacterial and antifungal activities (27, 61, 62). They also display weak antibacterial activity in vitro against Gram-negative strains including *Vibrionaceae* species, a family of bacteria commonly associated with shrimp mortalities. (63)

Crustins

In shrimp, most crustins are constitutively expressed in the hemocytes but not in the hepatopancrease (64, 65, 66). However, in response to pathogens, crustin exhibits different expression patterns. The transcripts of *L. vannamei* crustin isoform I was downregulated at 12 to 24 h post-injection of *Vibrio alginolyticus,* whereas the isoform P transcript levels remained unchanged (67). The expression level of crustin-like peptide from *P. monodon* increased more than five-fold at 24 h postchallenge with *Vibrio harveyi* and returned to the normal level within 72 h (66).

Lysozyme

Lysozymes (EC 3.2.1.17) are hydrolytic enzymes, characterized by their ability to cleave the β-(1,4)-glycosidic bond between *N*-acetylmuramic acid and *N*-acetylglucosamine in peptidoglycan, the major bacterial cell wall polymer. The widely recognized function of lysozymes is their contribution to antibacterial defense but, additionally, some lysozymes (belonging to different types) are known to function as digestive enzymes (68). In addition to their activity against Gram-positive bacteria, some insect c-type lysozymes are antibacterial against Gram-negative bacteria. Moreover, other antimicrobial proteins and peptides that are also induced by bacterial infection can broaden the antibacterial spectrum of lysozyme through synergistic effects. In insects, examples of such components are cecropins, defensins and attacinlike proteins, all known to affect bacterial cell membranes (69, 70). Therefore, together with these molecules, at least the c-type lysozyme is likely to play an important role in the insect's defense against bacteria. Recently, the c-type lysozyme cDNA was cloned and characterized from the *L. vannamei* (71). The presence of lysozyme was confirmed directly from shrimp hemocytes and mRNA was detected in hemocytes by RT-PCR. The enzymatic activity of shrimp lysozyme was detected in hemocyte lysate, but not on plasma, indicating that lysozyme is expressed, translated and stored inside the cells (37).

Immunostimulants

Iinnate immune system has been found in invertebrates. Several antigens (vibrio cells, yeast glucans or their derivatives) have been experimentally tested to elucidate the innate immune mechanisms in shrimp (72, 73). Astanxhantins, chitosan, fucoidan, β1-3 glucan, herbal extracts, laminaria, LPS, PG, saponins, and vitamin C are the main antigens experimentally tested in shrimp (74). These substances can be administered by injection, immersion, bioencapsulation, per os intubation, and in the feed in marine organisms (75). The results suggested that they can be an important element in the control of disease. The application of antibiotic or other chemicals to culture ponds is expensive and undersirable because it risks contamination of both the environment and the final product. Application of various compounds to boost or stimulate the innate immune system of cultured shrimp has been widely accepted as a good alternative to enhance shrimp health and fight epidemics. There is also increased variability on the consistency and effectiveness of immunostimulants on disease resistance in crustaceans. The effect may depend upon the severity and frequency of the infection, the mode of application and the presence of other stressors. Disease being a complex process, overdependence on immunostimulants to prevent disease shall be avoided as their effectiveness will depend upon the water quality and the presence of various other stressors, which are likely to increase the susceptibility of the animal to infectious disease. Duration of protection in crustaceans is like to be shorter and repeated application timed prior to infection will be required to optimize the effectiveness of these products (76). Immunostimulants have been proven to be safer than chemotherapeutics and their range of efficacy is wider than vaccination (72). Immunostimulants are particularly suitable for booting immature immune systems and effective against a number of opportunistic and secondary pathogens. With a detailed understanding of the limitations of immunostimulants, they may become powerful tools to control diseases (77).

Diseases of Pacific white shrimp

The recent years, shrimp aquaculture industry has the high production in farming systems and the concomitant with the recognition of disease as a primary intervention limiting shrimp aquaculture production worldwide. The principal economically important disease of Pacific white shrimp includes those with bacterial, viral, fungal, protozoa and noninfectious (toxic and nutritional). Infectious diseases are caused by viruses, bacteria, fungi and parasites. The bacteria is *Vibrio* spp., the viruses such as WSSV, YHV, TSV, IHHNV, IMNV, CMNV, and BP are known to be important disease agents in cultured shrimp. Bacterial disease due primarily to species of *Vibrio* spp. are among the most economically significant disease problems in the shrimp aquaculture, with known to cause vibriosis diseases in all of penaeids shrimp. Although once thought to be of little importance, parasitic gregarines are emerging as importance, parasitic gregarines are emerging as important parasites (2) and *Enterocytozoon hepatopaenei* (EHP) (78) in pond culture penaeids. Many shrimp farms as well as the hatcheries have faced viral and bacterial infections in which polymicrobial infections have caused significant economic loss (79). It is estimated that approximately 60% of disease losses in shrimp aquaculture have been caused by viral pathogens and 20% by bacterial pathogens. Since 2009, increasing losses with *L. vannamei* in China, Vietnam and Thailand are associated with acute hepatopancreatic necrosis disease (AHPND) caused by *Vibrio parahaemolyticus*$_{\text{AHPND}}$. Vibriosis (*V. harveyi*, *V.parahaemolyticus*$_{\text{AHPND}}$ and *Vibrio* spp.) and WSSV are considered as the serious pathogens more than fungi and parasite. It affected shrimp producing countries of the world.

Infectious Pacific white shrimp diseases

Bacterial diseases

Vibriosis

Bacterial diseases may cause a range of problems ranging from mass mortalities to growth retardation and sporadic mortalities in shrimp aquaculture. Vibriosis is one of the major diseases in shrimp aquaculture causing devastating mortality in the farm (80). *Vibrio* spp. are aquatic bacteria that are widely distributed in fresh water, estuarine and marine environments. The genus Vibrio from the family Vibrionaceae belongs to the class Gammaproteobacteria and contain 463 recognized species of gram-negative rods, which are widely distributed in the estuarine and marine environments (81). In general, *Vibrio* spp. tolerate a wide range of salinities and tend to be more common in warm waters, notably when temperatures exceed 17 ^{o}C (82). There are indications that vibrios play a role in nutrient cycling in aquatic environments by taking up the dissolved organic matter, and they may provide essential polyunsaturated fatty acids to the aquatic food web (Thompson et al., 2004). In the past decade, various Vibrio species have been reported to cause mortalities of cultured penaeids (83, 84, 85). *Vibrio* spp. are the most important bacterial pathogens of shrimp. The researchers (86) claimed that the vibriosis caused 85% mortality in white leg shrimp larvae in America and infected various types of aquatic animals at 100% mortality of the cultured aquatic animals. Mass mortality, slow growth, and deformity of shrimp cause major economic losses in shrimp aquaculture. The outbreaks of these diseases have led to the near or total collapse of the shrimp farming industry throughout the world. Although viral infections typically have more deleterious effects on shrimp farm stocks, vibriosis can also cause mass mortalities of farmed shrimps. Vibriosis in shrimps caused by *Vibrio* spp. is considered to be a significant yet common infectious problem. *Vibrio alginolyticus* is predominantly present in all larval stages and is associated with healthy nauplius and zoea stages. *V. alginolyticus* was found to be associated with larvae with the zoea 2 syndrome and the mysis mold syndrome, while different *Vibrio* species (*V. alginolyticus* and *V. harveyi*) are associated with the bolitas syndrome. *V. harveyi* is associated with diseased postlarvae, juveniles, and broodstock. *V. parahaemolyticus*, *Photobacterium*

damselae, and *V. mimicus* are associated with juvenile and adult stages (87). Bacterial diseases, mainly due to Vibrio, have been reported in penaeid shrimp culture systems implicating at least 14 species and they are *V. harveyi, V. splendidus, V. parahaemolyticus, V. alginolyticus, V. anguillarum, V. vulnificus, V. campbelli, V. fischeri, V. damsella, V. pelagicus, V. orientalis, V. ordalii, V. mediterrani, V. logei* etc. (88, 89, 90).

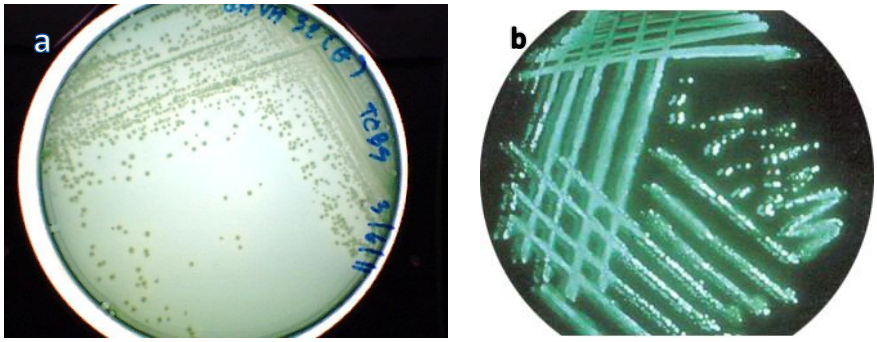
Vibrio harveyi

V. harveyi is found in marine environments (mainly in tropical locations). This bacteria may be either free-living or in symbiosis with marine life like their close relations, *V. fischeri*. *V. harveyi's* 16s RNA classifies them in the Proteobacteria phylum, and their bioluminescence (Fig. 8) places them in the Vibrionaceae family. (http://microbewiki.kenyon.edu/index.php/Vibrio_harveyi), Non-sporulating rods (0.5 microns x 2 microns) help to maintain their structure while polar flagella (Fig. 9) whose sheath is an extension of their outer membrane help the bacteria to move. *V. harveyi* produces an enzyme (luciferase) that generates light seen in their characteristic bioluminescent capabilities, whose functioning is dependent on the cell concentration, and works via quorum sensing.

V. harveyi is a Gram-negative, facultative anaerobic bacteria with non-sporulating rods and polar flagella. As a Gram-negative bacteria, it has an thin inner layer of peptidoglycan, surrounded by a periplasmic space, which is surrounded by a thick peptidoglycan layer called the outer membrane and is more permeable than the plasma membrane. The outer membrane is composed of lipids, lipoproteins, and lipopolysaccharides. The polar flagella of the bacteria help it to move in a run and tumble motion while employing chemotaxis (moving towards chemical attractants and away from chemical repellants). As a facultative anaerobe, these bacteria will thrive most in environments with oxygen, but are able to survive in environments lacking oxygen as well. The most distinctive characteristic of *V. harveyi* (and all bacteria of the vibrionaceae family) is their ability for bioluminescence. This occurs through quorum

sensing—a way for the cells to communicate with each other. The bioluminescence is dependent on the concentration of the cells because the necessary enzyme (luciferase) will only be produced in high enough cell concentrations via a series of chemical reactions induced by autoinducers (AI) In addition to bioluminescence, this bacteria also controls some virulence factors, sporulation, and conjugation. (http://genome.wustl.edu/genome.cgl?GENOME=Vibrio%20harveyi).

During the past several years, penaeid shrimps have suffered problems of infectious diseases due to vibriosis such as *V. harveyi* (84), *V. harveyi*, a marine Gram-negative organism with a single polar flagellum, has been recognized as a significant pathogen of marine vertebrates and invertebrates. The disease attributed to *V. harveyi* is initially distributed to the seawater aquaculture of China, Australia, India, Indonesia, Thailand, Philippines, Taiwan and other countries and regions and brought large financial losses (91, 92). Several factors are known to contribute to the virulence of *V. harveyi* that include proteases, hemolysins, phospholipases, siderophores, cytotoxins, and lipopolysaccharide (84, 92, 93, 94). Quorum sensing and bioluminescence have also been associated with the virulence of *V. harveyi* (95). Antibiotic - resistant *V. harveyi* is now known to cause mass mortalities, slow growth and deformity of shrimp in penaeid shrimp farms across the world (92, 96, 97, 98, 99). The researchers confirm that these infectious diseases are related with the increasing of Vibrio population in the shrimp pond water. Some *Vibrio* spp. are opportunistic pathogen bacteria when shrimp are in the stress condition or have low immunity especially as well as they are rearing with high density, those vibrio bacteria will attack and lead to the disease and may cause mortality to the shrimp. Mass mortality in post-larvae of shrimp due to antibiotic-resistant *V. harveyi* has been reported by various researchers (96, 100, 101, 102). Some farms in the South Asia use antibiotics to control *Vibrio harveyi*, a responsible pathogen for luminous vibriosis. However, the antibiotic-resistant strain was found recently in many shrimp farms. Not only emergences of antibiotic-resistant bacteria but also antibiotic residues in food-producing animals pose health concerns in humans and animals.



Vibrio harveyi www.Thailandshrimp.com

Fig. 8 Green colony on TCBS agar (a), Luminescence colony of *V. harveyi* (b)

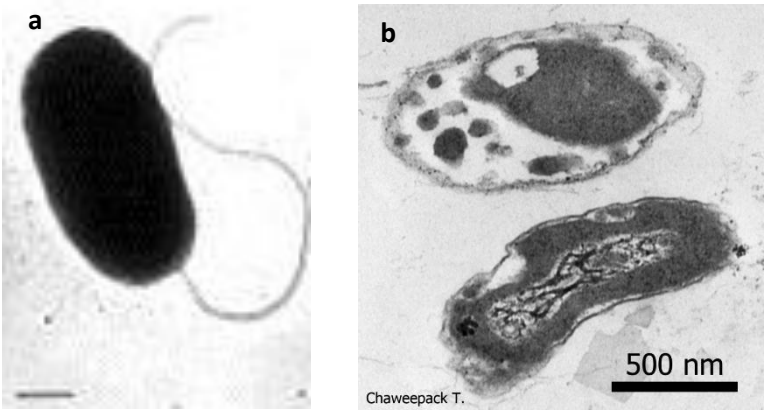
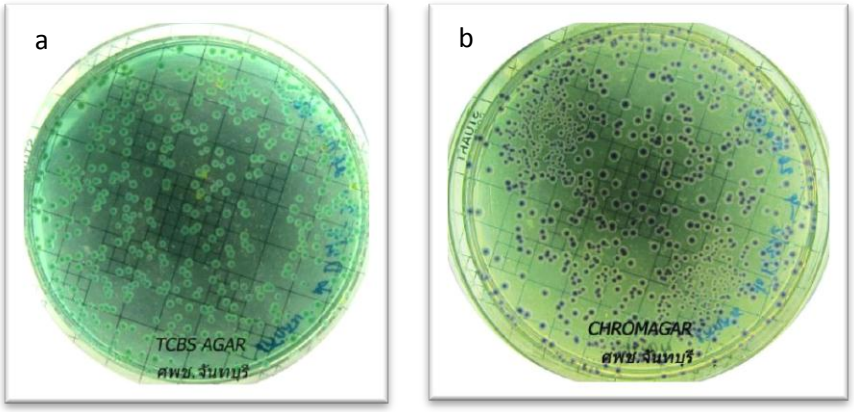


Fig. 9 The cellular structure of *V. harveyi*, whose polar flagella help to facilitate quorum sensing by allowing it to move rapidly towards an attractant (other *V. harveyi* cells) and establish high cell densities, http://en.citizendium.org/wiki/File:V_harveyi_structure.jpg (a), TEM of *V. harveyi* (b).

Acute hepatopancreatic necrosis disease (AHPND)

Early Mortality Syndrome (EMS) was ascribed to specific isolates of *Vibrio parahaemolyticus* that caused hepatopancreatic necrosis disease (AHPND) (18). *V. parahaemolyticus*, this strain contains the specific plasmids that induce in toxins. AHPND is characterized by severe atrophy of the shrimp hepatopancreas (HP) that exhibits unique histopathology at the acute stage of the disease, consisting of massive sloughing of HP epithelial cells in the absence of bacteria or other pathogens (www.enaca.org). AHPND was first reported in China in 2009 (103) followed by Malaysia in 2010 (103), Vietnam in 2011 (18), Thailand in 2012 (104, 105) and Mexico in 2013 (106). Susceptible species are *L. vannamei*, *P. monodon* and *P. chinensis* (103, 107). The genome of *V. parahaemolyticus* (AHPND) from Thailand and Mexico have a plasmid harboring virulent genes encoding type IV pili proteins and conjugal transfer proteins (106, 108) The plasmid of *V. parahaemolyticus* AHPND strains contains a region that encodes homologues *Photorhabdus* Pir toxins (109) with are insecticidal toxins. Isolation of *V. parahaemolyticus* AHPND strains had been used CHROMagar Vibrio and TCBS agar media for fast detection, and the colony of *V. parahaemolyticus* on agar had violet and green color, respectively (Fig. 10, 11).

The disease affects both *P. monodon* and *L. vannamei* and is characterized by mass mortalities (reaching up to 100% in some cases) during the first 20-30 days of culture (post-stocking in grow-out ponds). Clinical signs observed include slow growth, corkscrew swimming, loose shells, as well as pale coloration. Affected shrimp also consistently show an abnormal hepatopancreas (shrunken, small, swollen or discolored) (Fig. 12). The progressive dysfunction of the HP results from lesions that reflect degeneration and dysfunction of the tubule epithelial cells that progress from proximal to distal ends of HP tubules. This degenerative pathology of HP is highly suggestive of a toxic etiology, but anecdotal information suggests that disease spread patterns may be consistent with an infectious agent (103, 107).



(a) TCBS agar (b) CHROMagar

Fig. 10 The colony of *V. parahaemolyticus* AHPND showed the green color on TCBS agar (a) and the violet color on CHROMagar (b).

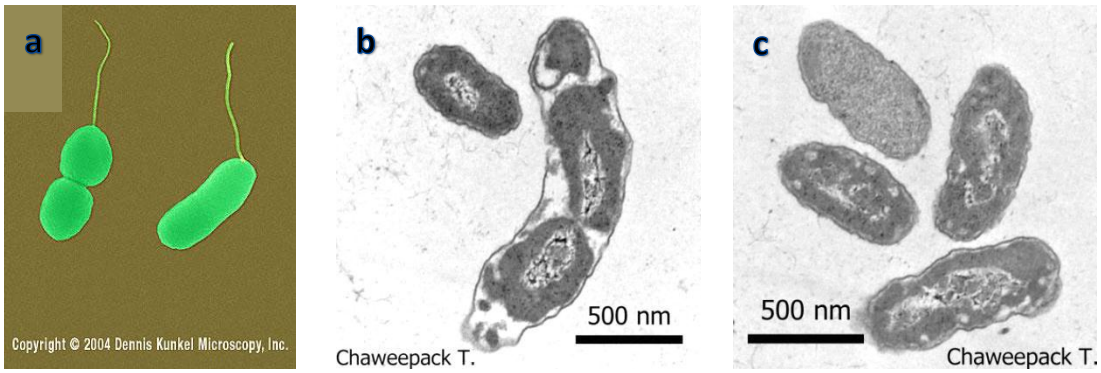


Fig. 11 The cellular structure of *V. parahaemolyticus*, whose polar flagella help to facilitate quorum sensing by allowing it to move rapidly towards an attractant (other *V. parahaemolyticus* cells) and establish high cell densities (a), (http://www.nadidem.net/k/Vib/pages/Vibrio%20parahaemolyticus_jpg.htm), TEM of *V. parahaemolyticus* AHPND (b, c).

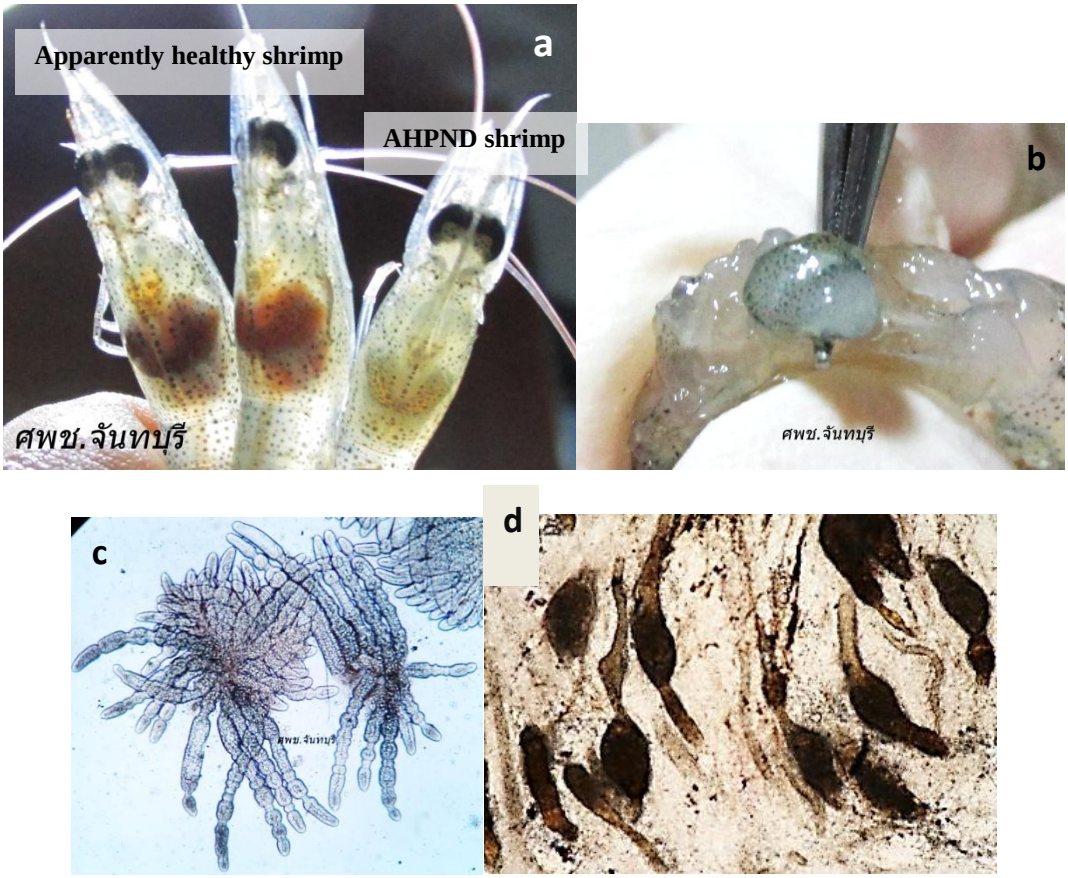


Fig. 12 Comparison between the apparently healthy with AHPND shrimp (a), The pale coloration of hepatopancrease AHPND shrimp (b), Affected shrimp also consistently show an abnormal hepatopancreas (shrunken, small, swollen, discolored or melanization) (c, d).

Viral Disease

More than 20 viruses have been reported to infect marine shrimp (110). Seven viral pathogens of marine shrimp are currently listed by the World Organization for Animal Health (OIE) as causing notifiable aquatic animal diseases, and two are under study for listing (111). Production of *L. vannamei* is limited by its susceptibility to various diseases, including white spot syndrome, Taura syndrome, infectious hypodermal and haematopoietic necrosis, baculoviral midgut gland necrosis, IMNV and *Vibrio* infections (111).

White Spot Disease

White Spot Disease (WSD) is a viral infection of penaeid shrimp. The disease is highly lethal and contagious, killing shrimps quickly. Outbreaks of this disease have wiped out within a few days the entire populations of many shrimp farms throughout the world. Viral diseases along the way have caused billions of dollars of losses for shrimp farmers. The virus has a wide host range, is highly virulent and leads to mortality rates of 100% within days in the case of cultured penaeid shrimps. Most of the cultured penaeid shrimps (*Penaeus monodon*, *Marsupenaeus japonicus*, *Litopenaeus vannamei*, and *Fenneropenaeus indicus*) are natural hosts of the virus. Several non-penaeid shrimps were also found to be severely infected during experimental challenges. Many crustaceans like crabs (*Scylla spp.*, *Portunus spp.*), spiny lobsters (*Panulirus spp.*), crayfish (*Astacus spp.*, *Cherax spp.*) and freshwater shrimp (*Macrobrachium spp.*) are reported to be infected with variable severities depending on the life stage of the host and presence of external stressors (temperature, salinity, bacterial diseases, pollutants). Clinical signs of WSSV include a sudden reduction in food consumption, lethargy, loose cuticle and often reddish discolouration, and the presence of white spots of 0.5 to 2.0 mm in diameter on the inside surface of the carapace, appendages and cuticle over the abdominal segments (Fig. 13). (https://en.wikipedia.org/wiki/White_spot_syndrome).

Fig. 13 White spot disease in infected shrimp (1) (www.daff.gov.au), white spots on carapace (2).

The WSD is caused by a family of related viruses subsumed as the White Spot Syndrome Baculovirus complex (WSSV) (http://nis.gsmfc.org) and the disease caused by them as white spot syndrome (WSS) (2). The first reported epidemic due to this virus is from Taiwan in 1992. However, reports of losses due to white spot disease came from China in 1993.Where it led to a virtual collapse of the shrimp farming industry. This was followed by outbreaks in Japan and Korea in the same year, Thailand, India, and Malaysia in 1994, and by 1996 it had severely affected East Asia and South Asia. In late 1995, it was reported in the USA, 1998 in Central and South America, 1999 in Mexico' in 2000 in the Philippines, and in 2011 in Saudi Arabia. Currently, it is known to be present in all shrimp-growing regions except Australia. (https://en.wikipedia.org/wiki/White_spot_syndrome). WSSV is a rod-shaped, double-stranded, DNA virus, and the size of the enveloped viral particles have been reported to be 240–380 nm long and 70–159 nm in diameter and nucleocapsid core is 120–205 nm long and 95–165 nm in diameter. The virus has an outer lipid bilayer membrane envelope, sometimes with a tail like an appendage at one end of the virion. The nucleocapsid consists of 15 conspicuous vertical helices located along the long axis; each helix has two parallel striations, composed of 14 globular capsomers, each of which is 8 nm in diameter (112). The complete DNA sequence of WSSV genome has been assembled into a circular sequence of 292,967 bp (113). It encodes 531 putative

open reading frames. One of the proteins – WSSV449 – has some similarity to host protein Tube and can function like Tube by activating the NF-κB pathway (114). Transmission of the virus is mainly through oral ingestion and water-borne routes in farms (horizontal transmission) and vertical transmission (from infected mother prawns) in the case of shrimp hatcheries. The virus is present in the wild stocks of shrimp, especially in the coastal waters adjacent to shrimp farming regions in Asian countries, but mass mortalities of wild shrimps are yet to be observed. In the host, WSSV infects a wide variety of cells from the ectodermal and mesodermal origin. Histological changes are seen in the gill epithelium, antennal gland, haematopoietic tissue, nervous tissue, connective tissue and intestinal epithelial tissue. Infected cells have prominent intranuclear occlusions that initially stain eosinophilic, but become basophilic with age; hypertrophied nuclei with chromatin margination; and cytoplasmic clearing (115). Pathogenesis involves widespread tissue necrosis and disintegration. The chemical composition of the spots is similar to the carapace, calcium forming 80–90% of the total material and it is suggested to have derived from abnormalities of the cuticular epidermis (116).

White feces syndrome disease

White feces syndrome disease in Pacific white shrimp is polymicrobial diseases which co-infection of gregarine protozoa, fungi, EHP and Vibrio bacteria has been described of shrimp culture and outbreaks in Thailand during the years 2010 to 2011. The disease occurs under different soil conditions, and it results in deteriorated water quality. Peak mortality rates are seen in the face of extremely low oxygen (<3.0 mg/L)/low alkalinity (<80 ppm) levels. Early disease indications appear in both control feed trays and at water surface, where abundant floating white feces are observed (Fig. 14) Gregarine protozoa and Vibrios bacteria have been highly effected to Pacific white shrimps which were loose shell, decrease in appetite, high feed conversion rate, slow growth, sporadic mortalities throughout the culture and white stool was found floating at the surface of water volume in growth out pond are the typical symptoms of the disease. The histopathological examination reveals hemocyte encapsulation with nodules and melanization of the hepatopancreas. Both a group of *Vibrio* spp. bacteria

and parasitic protozoa known as gregarins have been related with the disease. The following *Vibrio* species have been found in the fecal analyses *V. parahaemolyticus, V. fluvialis, V. alginolyticus, V. mimicus,* Gregarins found in white feces of infected shrimp belong to the *Nematopsis* genus (Fig. 14) (117, 118). And another opinion of framers caused from fungal toxin in commercial pellet feed. Co-infection of many *Vibrio* strains was noticed in red disease syndrome of *P. monodon.* The syndrome is characterized by the reddening of the shrimp body. *V. harveyi, V. parahaemolyticus, V. fluvialis* and *Vibrio* sp. were isolated from diseased shrimp in which *V. harveyi* and *V. parahaemolyticus* showed the most dominant species. Performing shrimp challenge tests with the isolated *V. parahaemolyticus* and *V. harveyi*, the researcher concluded that these bacterial strains can produce the characteristic red discoloration in healthy shrimp (119).

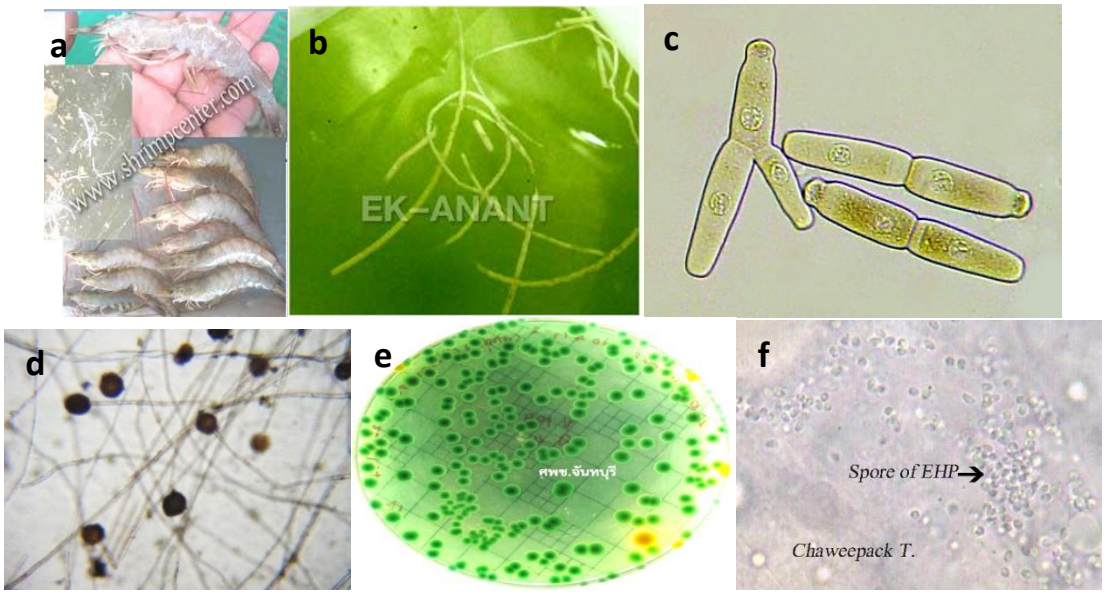


Fig. 14 White feces of infected shrimps (a), White feces floating on the surface of water in shrimp pond (b), *Nematopsis* sp. Gregarine protozoa (c), Fungi (d), *Vibrio* spp. (e), Spores of EHP (Microsporidian) (f).

The antibiotic problem with the marine shrimp

Marine shrimp farming industry as aquaculture production industry that is important to Thailand economy. Development of a system for the industry, which has been high-density shrimp culture. Highlighted by accelerating growth, the increased feeding, waste water is accumulated in pond management, as well as disease prevention system made more difficult. Contribute to the risk of disease and the use of antimicrobial drugs to control diseases in intensive shrimp farm. The use of antimicrobial drugs mixed with feed, which often can dissolve in water wells and contaminate the environment by causing bacterial disease may cause various types of bacteria that live in and the environment antimicrobial resistant drug development. A problem of residues in shrimp that is the big problem is Thai national. Respectively, and occurred during the following period. In early 2002 the Office of Commercial Affairs Minister in Vienna stated that the supermarket of Austria has announced the termination of release shrimp from Thailand, tropical, including Vietnam and Indonesia, which had been detected in shrimp production from third countries with chloramphenicol drug contamination. It also has a surveillance plan, submitted to random shrimp from Thailand strict as well. Therefore, shrimp imports into Europe are going to be more difficult. The procedure is as follows. The Ministry of Agriculture Cooperative, Thailand, General Issue, 1998, announced is not allowed to import and use of chloramphenicol, as objects added to animal feed and production factor. Animals fed the dust release and check the adulterated chloramphenicol drug in food animals rigorous. Antibiotic residues in shrimp measures to solve the problems of the fisheries department are as follows. Educating, the public relations with the media and training seminars to educate. Production at the farm and the facility to create a strong understanding of the prevention and control of residues of antibiotics to farmers and those involved in business continuity, such as cold storage exporter Manufacturers Association, then created the network control and monitoring product quality. However, at present, Good Aquaculture Practices and Code of Conduct systems applied by most of shrimp farmers may help to overcome problems and lead to produce high quality and safety food production. For antibiotics used in shrimp, aquaculture was widespread such as oxytetracycline (OTC) and oxolinic acid (OA), an antimicrobial agent is allowed to

legally particular OTC is a pharmaceutical substance CODEX registered for use in the manufacture of marine and terrestrial animals for use as human food but in the past decade, quinolones and the combination of sulfadiazine and trimethoprim because more popular in Asian shrimp farming (120, 121). Cumulatively, the USA Food and Drug Administration's (FDA) refusals of shrimp in 2015 substantially exceed the agency's actions over the prior thirteen years. As the Fig. 15 below indicated the 377 shrimp refusals in just the first nine months of 2015. FDA refusals of shrimp for antibiotics increase in October; agency has refused more Indian shrimp entry lines for antibiotics.

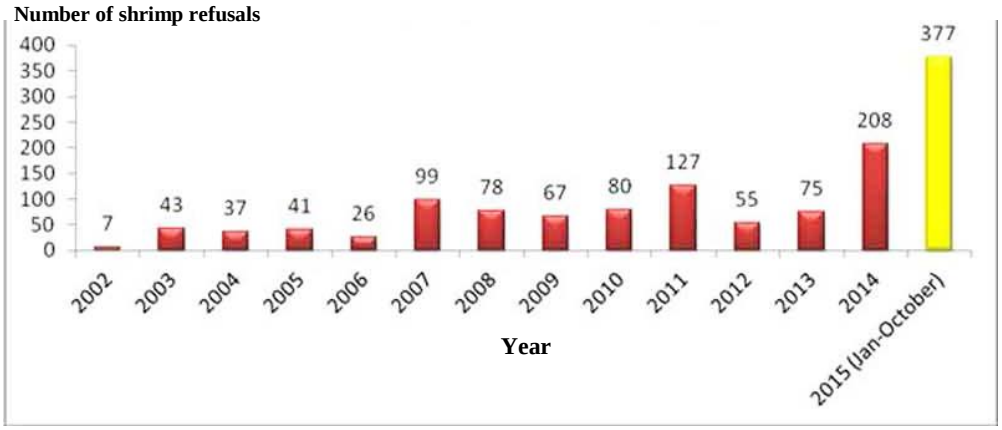


Fig. 15 Shrimp product; FDA refusals of shrimp entry lines for veterinary drug residues (https://www.shrimpnews.com).

Many types of drugs and chemicals have been used to treat Vibrio bacteria, and this has created a residual drug problem that was led to the spread of drug-resistant bacterial strains in the shrimp industry. *Vibrio* spp. bacteria were isolated from the diseased shrimp have been reported in bacterial resistance to erythromycin, streptomycin, penicillin, ampicillin, amoxicillin, kanamycin, tetracycline, oxytetracycline, trimethoprim-sulfamethoxazole, flofenicol and chloramphenicol (122, 123, 124). The conclusion of the cultured shrimps can be vehicles of vibrios resistant to β-lactam and tetracycline. Thus, the emergence of resistant bacteria to antibacterial drugs may be indicative of the indiscriminate use of these drugs in the cultivation of aquatic organisms (125). Antibiotics are widely used to treat infectious diseases in

aquaculture, but the emergence of antibiotic resistance in previously susceptible bacterial populations is a very serious threat and now a major public health issue. Also, different procedures for disease control such as vaccination, bacterial identification, and antimicrobial susceptibility test should be emphasized for more effective management such as medical herbs, probiotic, and reduction density and feeding of shrimp for control and prevention bacterial diseases in aquaculture.

Herbal biomedicines

Immunostimulants plant extract have been used to enhance immune system and disease resistance of shrimp. Hormones, antibiotics, vitamins and several other chemicals have been tested in aquaculture operations for various remedies. Even though they give positive effects, they cannot be recommended due to their residual and other side effects. The alternative herbal bio-medicinal products in the aquacultural operations, that have the characteristics of growth promoting ability and tonic to improve the immune system, act as appetite stimulators. They increase consumption, induce maturation, and have antimicrobial capability and also antistress characteristics that will be of immense use in the culture of shrimps and other fin fishes without any environmental and hazardous problems. Herbal compounds such as phenolics, polyphenols, alkaloids, quinones, terpenoids, lectines and polypeptides have been shown to be very effective alternatives to antibiotics and other synthetic compounds. The present paper is presented after a careful review of more than 50 herbal plants for their biological effects such as growth promotion, immunostimulation, antistress, antibacterial, antifungal, antivirals, appetite stimulators and aphrodisiac (4). Herbs may be another way for treating bacterial disease in aquatic animals because it is natural product which may prevent the resistance of bacteria (126). Currently, herbs have been studied for prevention and treatment for many diseases of aquatic animals. Direkbusarakom *et al.* (1998) (127) found that *Clinacanthus nutans* extract could inhibit Yellow-head Virus (YHV) in black tiger shrimp (*P. monodon*). Guava (*Psidium guajava*) extract was recommended for treatment of some fish and shrimp pathogenic agents. Some herbs are cheap and available in many humid tropical countries such as

China, India, Vietnam, Indonesia, and Thailand, where diversified flora are abundant. Moreover, nearly both cultures, ancient and recent, from Persia, Arabia, China, India and Korea have used plants as source of medicine for human and animals.

Galangal (*Alpinia galanga* Linn.)

Alpinia galanga (Linn.) or greater galangal is known as "*Kha"* in Thailand. The rhizome is a common ingredient in Thai curries and soups (Tom Yum and Tom Kha). It is an annual crops herb cultivated for its aromatic and medicinal rhizome plant.

The biology of galangal

Alpinia galanga, a plant belongs to family "Zingiberaceae" commonly known as galangal, is a herb used in cooking, especially in Thai and Indonesian cuisines. The plant grows from rhizomes in clumps of stiff stalks up to 2 m in height with long, abundant leaves that bear red fruit. It is distributed in India, Nepal, China, and Southeast Asia. (https://en.wikipedia.org/wiki/Alpinia_galanga). The herb growing in a simple, terminal spike, the petals white, with deep red veining distinguishing the lip petal. The branched pieces of the rhizome are from 1 ½ to 3 inches in length and seldom more than ¾ inch thick. They are cut while fresh, and the pieces are usually cylindrical, marked at short intervals by narrow, whitish, somewhat raised rings, which are the scars left by former leaves. They are dark reddish-brown externally, and the section shows a dark centre surrounded by a wider, paler layer, which becomes darker in drying. Their odour is aromatic, and their taste pungent and spicy (http://www.botanical.com/botanical/mgmh/g/galang01.html).

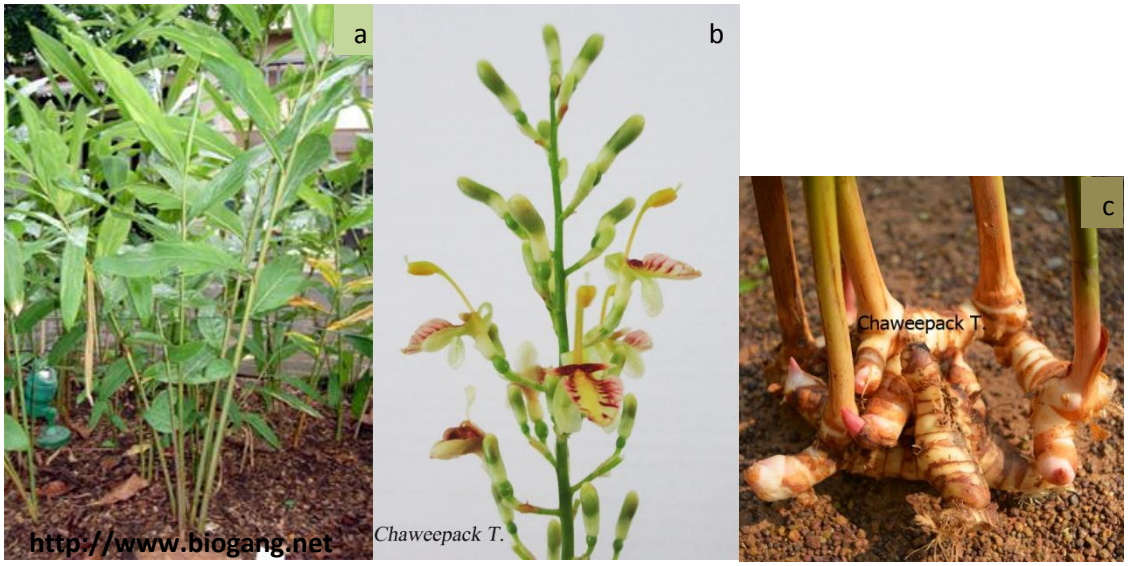


Fig. 16 The *Alpinia galanga* plants (a), flowers (b) and rhizomes (c).

Properties and activities of galangal extract

Chemical constituents

The rhizome of galangal contains tannins and flavonoids, some of which have been identified as kaempferide, galangin is a flavonol, a type of flavonoid and alpinin. (https://en.wikipedia.org/wiki/Alpinia_galanga). Rhizomes yield an essential oil containing methyl cinnamate, cineole and d-pinene and sesquiterpenoids. The researchers (128) isolated 18 monoterpenes of which α-pinene (22.5%), β-pinene (36.7%) and limonene (13.8%) were the major compounds. Charles *et al*. (1992) (129) characterised twelve compounds by GC/MS in *A. galanga*. The major compound was myrcene: 94.51% in rhizome and 52.34% in leaves. Fresh rhizome yielded 18 monoterpenoids of which pinene, and limonene as major compounds and 17 oxygen containing monoterpenoids with cineol, terpinen-4-o1, and terpineol as minor compounds (130). The rhizome oils contained limonene (3.5-3.7%), 1,8-cineole (30.2-33.0%), camphor (5.0-14.0%), alpha-terpineol (2.3-9.3%), alpha-fenchyl acetate (1.1-12.7%) and (E)-methyl cinnamate (2.6-5.3%) (131, 132). Essential oils such as mono-,

sesquiterpene and cinnamate derivatives were found (Jirovetz *et al.* 2003) in various parts of the plant. Its use in sweet goods, dressings and personal care products is due to the presence of the pungent compound, 1′-acetoxychavicol acetate (ACA) (133). The Malaysian *A. galanga* showed weak activity compared with the Thai sample, and this was shown to due to the relatively high amounts of 1'-acetoxychavicol acetate present in the Thai sample (http://www.readbag.com/hillgreen-pdf-alpinia-galanga).

Pharmacological activities

The galangal rhizomes are bitter, acrid, thermogenic, aromatic, nervine tonic, stimulant, revulsive, carminative, stomachic, disinfectant, aphrodisiac, expectorant, broncho-dilator, febrifuge, tonic. The rhizomes extract has been found to possess various therapeutic activities such as anti-inflammatory, analgesic, antiallergic, antifungal, antibacterial, antidiabetic, antivirous, antiulcer, anticancer, antioxidant, antiamoebic, antidermatophytic, immunostimulating. Rhizome is diuretic and hypothermic. Rhizome spray in ether, over space, showed high to knock down values against houseflies. Alcohol (50%) extract of the rhizome is anti-amphetaminic. Unani physicians consider it good for impotence (134, 135, 136, 137, 138, 139).

Antimicrobial Activity

The extracts of galangal rhizomes were more effective against the tested microorganisms, for their antibacterial activity in vitro against different multi-resistant Gram positive and Gram negative bacteria on *Staphylococcus aureus* and *Klebsiella pneumonia, Bacillus subtilis, Streptococcus faecalis, Escherichia coli, Proteus vulgaris, Salmonella enteritidis, Saccharomyces cerevisiae* and fungi as *Aspergillus niger*. The antiplasmid activity of crude acetone extract of the galangal rhizomes exhibited against *Salmonella typhi, E. coli* and vancomycin resistant *Enterococcus faecalis*. The various galangal extracts showed significant antibacterial and antifungal properties (136, 140, 141, 142, 143). The active fractions of galangal extract were found that 1'-Acetoxychavicol acetate, 1'-acetoxyeugenol acetate and 1'-hydroxychavicol acetate which was active against the seven fungi tested (144). The chloroform extracts galangal

had pronounced antifungal activity in AIDS patients against *Cryptococcus neoformans* and *Microsporum gypseum*, but exhibited weak activity against *Candida albicans* (135). The endophytic actinomycetes activity of roots of galangal against phytopathogenic fungi and tested against *Candida albicans* and phytopathogenic fungi, *Colletotrichum musae* and *Fusarium oxysporum*, The strain identified as *Streptomyces aureofaciens* CMUAc130 was the most effective in antifungal activity amongst those investigated (145). The antifungal activities of aroma components from galangal such as linalool, geranyl acetate and 1,8-cineole against some fungi in the Saprolegniaceae (146).

Galangin has been shown to have *in vitro* antibacterial and antiviral activity. The flavonol also inhibits the growth of breast tumor cells *in vitro* (https://en.wikipedia.org/wiki/Alpinia_galanga). The 1'*S*-1'-acetoxychavicol acetate (ACA) isolated from *A. galanga* rhizomes extract inhibited Rev transport at a low concentration by binding to chromosomal region maintenance a and accumulating full-length HIV-1 RNA in the nucleus, resulting in a block in HIV-1 replication in peripheral blood mononuclear cells (147).

Anti-inflammatory activity

The several reported that anti-inflammatory activity of galangal extract such as; the 80% aqueous acetone extract of the rhizomes of *A. galanga* was found to inhibit release of beta-hexosaminidase, as a marker of antigen-IgE-mediated degranulation in RBL-2H3 cells (148). Total alcoholic extract (TAE) and total aqueous extract (TAQ) from *A. galanga* rhizomes were evaluated in acute (carrageenan-induced paw oedema; M1) and sub-acute (cotton-pellet-induced granuloma; M2) rat models (149, 150).

Antioxidant activity

Studies conducted on the antioxidant activity of *A. galanga* were focused on the ethanolic extracts of rhizomes (134). The ethanolic extract showed the highest DPPH free radical scavenging ability as well as the highest oxygen radical absorbance capacity (ORAC) value when compared to the water extract and the essential oil (151).

The other properties

The properties of anti-diabetic activities from the extract of rhizome of galangal was found in normal rabbits, powdered rhizome and its methanol and aqueous extracts significantly lowered the blood glucose (152). The researchers (153) had been reported that antidiabetic and anti-inflammatory activities from the phenolic and methanolic. Extract of rhizome of *A. galanga*. Immunostimulating activity of the hot water-soluble polysaccharide extracts of *A. galanga* was tested for their immunostimulating activity in mice. (154). Cytotoxicity activity of isolated pinocembrin (5,7-dihydroxyflavanone) from *A. galanga* that showed cytotoxicity against a variety of cancer cells including normal lung fibroblasts with relative nontoxicity to human umbilical cord endothelial cells (http://www.readbag.com/hillgreen-pdf-alpinia-galanga).

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The specific objectives and the thesis outline are as follows:

Chapter 1: **Identification of an antibacterial active substance from the Zingiberaceae family herbs and show antimicrobial activity against *Vibrio* isolated from marine shrimp**

The present study was conducted to investigate the antimicrobial activities of ethanol extracts of the aforementioned 3 Zingiberaceae plants, galangal, cardamom, and fingerroot, against 7 species of common pathogenic *Vibrio* bacteria isolated from marine shrimp as well as identify the structure of an antibacterial active ingredient.

Chapter 2: 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*)

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*).

Chapter 3: 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*)

This research aims to investigate the efficiency of Galangal (*Alpinia galanga* Linn) extract against *V. harveyi as well as determine* the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC). This is the first study to examine galangal (*A. galanga* Linn.) crude extract indifference concentrations to test the effectiveness of galangal crude extract on growth inhibition, survival rate**,** clearance ability, detection resistance of the *V. harveyi* bacterial disease and White spot disease treatment in Pacific white shrimp (*L. vannamei*).

Chapter 4: Effect of galangal (*Alpinia galanga* Linn.) extract on the expression of immune-related genes and *Vibrio harveyi* resistance in Pacific white shrimp (*Litopenaeus vannamei*)

The study was focused on examining the impact of galangal-ethanol extracts and *trans-p*-coumaryl diacetate on the expression levels of the immune-related genes of Pacific white shrimp. Also, the effect of galangal extract administration on the survival rate of Pacific white shrimp from *Vibrio harveyi* infection was investigated.

Chapter 5: **Conclusions**

CHAPTER 1

Identification of an antibacterial active substance from the Zingiberaceae family herbs and show antimicrobial activity against *Vibrio* isolated from marine shrimp

Abstract

Antimicrobial activities against pathogenic *Vibrio* bacteria isolated from marine shrimp were investigated for Thai herbs belonging to *Zingiberaceae* family: Galangal, Cardamom and Fingerroot. Ethanol extract of galangal rhizome exhibited the highest growth inhibition of 7 tested *Vibrio* species, while fingerroot extract showed no antimicrobial activity. The antibacterial active compounds of the galangal extract were *trans-p*-hydroxy cinnamaldehyde, *trans-p*-acetoxy cinnamic alcohol and *trans-p*-coumaryl diacetate which were identified by HPLC and NMR. The half maximal inhibitory concentration (IC_{50}) of the antibacterial active compounds against *V. harveyi* was found at 0.074 ± 0.0095, > 5 and 0.720 ± 0.0190 µmol/ml, respectively. The minimum inhibitory concentration (MIC) of *trans-p*-coumaryl diacetate against 7 tested *Vibrio* species were found at 0.24 mg/ml. This study suggests that galangal extract might be useful for the treatment of Vibrio disease in the aquaculture industry.

1.1 Introduction

Vibrio species are the main pathogenic bacteria that cause several diseases to marine shrimp that are farmed both in hatcheries and grow-out pond systems (27) (28) (2). *Vibrio harveyi* is the major pathogen in shrimp hatcheries, and it causes luminescent disease with a high mortality (14). Pathogenic *Vibrio,* which includes *V. alginolyticus, V. harveyi, V. parahaemolyticus, V. fluvialis,* and *V. damsela,* were isolated from the hepatopancreas of black tiger shrimp (17) (26). The main causative bacteria in *Litopenaeus vannamei* were identified as *V. alginolyticus, V. cholerae, V. parahaemolyticus, V. vulnificus, V. mimicus* and *V. harveryi* (20) (32).

Antibiotics and some hazardous chemicals have been used in the treatment of pathogenic bacteria in the aquaculture industry particularly in intensive shrimp-farming systems. However, development of antibiotic resistance has become a major obstacle to trade as a result of the negative impact on consumer health. One of the alternatives is a biological control method that uses herbal medicines and probiotics. There has been some research on the application of Thai herbs to control infectious diseases in shrimp and fish. Guava (*Psidium guajava* L.) leaf and Mara-keenok (*Momordica charantia*) inhibited the growth of 10 pathogenic *Vibrio* strains most effectively among 7 kinds of herbs (9). Thai herbs belonging to the Zingiberaceae family such as turmeric, galangal (*Alpinia galanga* Linn Stuntz), cardamom (*Elettaria cardamomum*) and fingerroot (*Boesenbergia pandurata* (Roxb.) Schltr) are known as medicinal and culinary herbs. Several researchers have reported the antibacterial activity of cardamom extract against some pathogenic bacteria, both Gram-positive and Gram-negative (15) (10) (13) (12). Antimicrobial activity of extracts from the Zingiberaceae family, such as galangal, ginger, turmeric and fingerroot, against some pathogenic bacteria have been reported (24) (33) (25). 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*, while it did not inhibit the growth of Gram-negative bacteria such as *Salmonella spp., Eschericia coli, and Enterobacter aerogenes* (23). The ingredients in these herbs, 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* (8) (15) (22) (21) (6).

This research results prompted this investigation into the application of herbs in the aquaculture industry. However, the antimicrobial activity of the extract of Zingiberaceae against *Vibrio* species is yet to be clarified. The present study showed the antimicrobial activities of ethanol extracts of the aforementioned 3 Zingiberaceae plants, galangal, cardamom, and fingerroot, against 7 species of common pathogenic *Vibrio* bacteria isolated from marine shrimp as well as identify the structure of an antibacterial active ingredients.

1.2 Materials and Methods

1.2.1 Preparation of herb extract

Ripe roots or rhizomes of galangal, cardamom, and fingerroot were purchased at 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^{o}C for 24 h. After drying, each herb was grounded into powder using an electric blender (Philips, Cucina, Thailand). Ten grams of the each herb powder were suspended in 100 ml of ethanol or methanol, 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) (23).

The extraction yield (in %) was calculated using the ratio of the final yield of dried extract (g)/10 g of original dry galangal samples and converted to a percentage (%), formula in the parenthesis [(final dried extracts/10 g of original dry sample)*100].

1.2.2 Preparation of bacteria

Seven pathogenic *Vibrio* species, *V. cholerae, V. parahaemolyticus, V. fluvialis, V. vulnificus, V. alginolyticus, V. mimicus, and V. harveyi* were isolated from diseased shrimps harvested in the grow-out ponds in Chanthaburi Province Thailand, according to the method of (27). Two independent isolates of each *Vibrio* species, one from black tiger shrimp (*Penaeus monodon*) and the other from Pacific white shrimp (*Litopenaeus vannamei),* were used in this study. The isolate was streaked on TCBS agar (Difco,

USA) for selective isolation of *Vibrio*, and cultivated at 30ºC for 18–24 h. The *Vibrio* species was identified according to the schemes of Colwell (1984) and using API 20E kits (ATB System, BioMérieux, France).

In order to make bacterial inoculum, a pure colony streaked on Trypticase Soy Agar (TSA, Difco, USA), and cultivated at 30ºC for 18–24 h. Bacteria taken from a single colony was suspended into 1.5% sterile sodium chloride solution, mixed well by vortex mixer, and then estimated the number of bacteria using McFarland Standard No. 0.5 ($\times 10^{8}$ cfu/ml). The suspension was diluted to $\times 10^{6}$ cfu/ml for used antibacterial activity test.

1.2.3 Disc diffusion test

In order to estimate the efficacy of herbs for the growth inhibition of *Vibrio*, an agar disc diffusion assay was performed. 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 µl) was applied to a paper disc (Φ8 mm, Advantec, Tokyo, Japan), then both sides were dried in a sterile laminar flow. The paper disc was then put onto an MHA–1.5% NaCl plate inoculated with *Vibrio*, followed by incubation at 30 ºC for 16-18 h. The efficacy of herbs for growth inhibition of *Vibrio* was estimated by measuring the diameter of the clear zone surrounding the herbal disc based on the procedure established by (23).

1.2.4 Minimum Inhibitory Concentration (MIC) test

The MIC of galangal ethanol extract was determined based on the (23) procedure. 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 galangal extract partition was diluted with ethanol using a 2-fold dilution method. For the method, 160 µl of each dilution was dropped onto a paper disc (Φ8 mm, Advantec, Tokyo, Japan), followed by drying in a sterile laminar flow. Then the paper disc was put into MHB–1.5% NaCl liquid medium and incubated at 30 ºC for 18-24 h. The MIC of ethanol extract was regarded as the lowest concentration of the extract in a liquid medium that would permit no turbidity of the tested microorganism.

1.2.5 Minimum Bactericidal Concentration (MBC) test

The tubes in which culture medium had not showed turbidity of the bacteria and the last tubes with turbidity in MIC test were used for next MBC test. The 0.1 ml of culture medium used in MIC test was spread onto TCBS Agar (Difco, USA), incubated at 30ºC for 18–24 h and then colonies formed were counted. The MBC was the lowest concentration of herbal extract which forms less than 20 colonies, corresponding to inhibit the bacterial growth at 99.9 % or more.

1.2.6 Effect of galangal ethanol extract on cell of V. harveyi

V. harveyi was culture in TSB supplemented with 1.5 % NaCl at 30ºC for 18–24 h. Twenty microliters of the bacterial suspension were transferred to 3ml of TSB. Filter paper discs (Φ 8 mm, Advantec, Tokyo, Japan) were infiltrated with 160 ml of the extract and air-dried for 2 h. One paper disc was submerged into one test tube of the 4 h culture of bacterial suspension. This suspension was incubated at 30ºC for 18–24 h. Bacterial cells were collected by centrifugation at 10,000 g for 5 min. They were fixed in the mixture of 3 % glutaraldehyde solution at 4ºC for 2 h. (23). The method of Transmission Electron Microscopy (TEM) was modified from Horiuchi et al. (2001). Cells were washed three times with 0.1 mol/l phosphate buffer solution, pH 7.2 and post-fixed with 2 g OsO4/100 ml solution in 0.1 mol/l phosphate buffer solution at room temperature overnight. The pellets were embedded in 2 g/100 ml melted agar and then dehydrated through a serial concentration of ethanol (35, 50, 70, 95, 100 ml in 100 ml distilled water, respectively, for 15 min). They were infiltrated in a propylene oxide for 15 min twice, working mixture (propylene oxide: Spurr' resin, 1:1) for 30 min, followed by Spurr' resin for 60 min. They were kept in 100% Spurr' resin and polymerized in a hot air oven at 60ºC for 72 h. The polymerized samples were sliced with an ultramicrotome (MTX 75500, RMC, USA) and observed using a transmission electron microscope (HITACHI, HT7700, Japan).

1.2.7 Purification and Identification of galangal methanol extract compound

The galangal 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 at room temperature overnight, and the final at 55^{o}C for 4 h. The supernatant was collected by centrifugation then combined and dried in a rotary evaporator (IWAKI, Japan). The residual was then suspended in 200 ml of water and

was extracted twice with diethyl ether (150 ml/time). The ether fraction was dried via a rotary evaporator and further lyophilized. The antibacterial active substances were purified from the ether fraction by a high-performance liquid chromatograph (L-2130, Hitachi, Japan) equipped with an Inertsil ODS-3 column (10 × 250 mm, 5 μm, GL Science, Tokyo, Japan). The sample was eluted with a 120-min linear gradient of the mobile phase consisting of 1% acetonitrile containing 0.1% trifluoroacetic acid (TFA) and 99% acetonitrile containing 0.1% TFA at a flow rate of 3 ml/min. The sample was monitored at 260 nm. The antibacterial active substances were identified by nuclear magnetic resonance spectroscopy (Bruker, AV-600) and mass analysis (Bruker, micro-TOF).

1.2.8 Identification of galangal methanol extract compound

Spectrometric measurements

The structure of some antibacterial active ingredients from major galangal methanol extract was identified by ^{1}H-NMR, ^{13}C-NMR.

For the acquisition of nuclear magnetic resonance (NMR) spectra, 1~2 mg of the sample were dissolved in 400 μl of a DMSO-d_4 solution. All NMR spectra were recorded on a Bruker AV-600 spectrometer (Bruker Biospin K.K., Yokohama, Japan) at 25^{o}C. The transmitter frequencies were 600.19 and 150.9 MHz for ^{1}H and ^{13}C respectively. Two-dimensional total correlation spectroscopy (TOCSY), ^{1}H-^{13}C heteronuclear multiple quantum coherence spectroscopy (HMQC) and ^{1}H-^{13}C heteronuclear multiple bond coherence spectroscopy (HMBC) experiments were performed using standard Bruker software (XWINNMR, Bruker Biospin K.K., Yokohama, Japan). The chemical shifts were referenced to the internal standard tetramethylsilane (TMS). Negative and Positive-ion electro-spray-ionization mass spectra (ESI-MS) were obtained using a Bruker microTOF system (Bruker Biospin K.K., Yokohama, Japan). The sample was injected directly into the MS system using a pump without a separation column.

1.2.9 Determination of half maximal (50%) inhibitory concentration (IC_{50})

The galangal extracts from HPLC was screened for antibacterial activity using a modification of a previously described microdilution assay (Piccolomini *et al.,* 1997) The test was performed in 96-well at bottomed plates with every well containing a total volume of 200 μL consisting of 150 μL medium (BTSB (Bacto Tryptic Soy Broth) +

1.5% NaCl), 50 µL inoculum, 30 µL 1.5% NaCl and 20 µL test sample in different concentrations. Instead of 20 µL test sample only the 1.5% NaCl was used in the negative control and bacterial suspension in the positive control. The plates were incubated at 30 ºC for 24 h while shaking at 115 rpm (Orbital Shaker BL 4236, Edwards Instrument). The ether layer fraction, samples were prepared so that the final concentration of 500µg/ml. Then, a microplate reader (MTP-120 type, CORONA ELECTRIC MTP) at a wavelength of 492 nm using, it was measured turbidity. The evaluation of the antibacterial activity that used Half maximal (50%) inhibitory concentration (IC50).

The growth inhibition of the bacteria caused by the tested samples was calculated as follows:

Test: a = OD test sample (medium, inoculum, 1.5% NaCl and test sample dissolved in water, ethanol or DMSO)

Negative control: b = OD solvent (medium, inoculum and solvent (1.5% NaCl solution)

% inhibition: $c = (1 - a/b)*100$

1.2.10 Investigate structure-activity relationships of galangal from antimicrobials.

The compounds were used that showed below.

1. cinnamaldehyde (Wako Pure Chemical Industries, Ltd.)
2. cinnamyl alcohol (Wako Pure Chemical Industries, Ltd.)
3. *trans*-cinnamic acid (Wako Pure Chemical Industries, Ltd.)
4. cinnamyl acetate (Wako Pure Chemical Industries, Ltd.)
5. *o* - hydroxy cinnamic acid (Tokyo Kasei Kogyo Co., Ltd.)
6. *m* - hydroxy cinnamic acid (Wako Pure Chemical Industries, Ltd.)
7. *p* - hydroxy cinnamic acid (Wako Pure Chemical Industries, Ltd.)
8. benzyl cinnamate (Wako Pure Chemical Industries, Ltd.)
9. *p* - hydroxybenzaldehyde (Nacalai Tesque, Inc.)
10. vanillin (Tokyo Kasei Kogyo Co., Ltd.)
11. 4-methoxy cinnamaldehyde (Tokyo Kasei Kogyo Co., Ltd.)
12. α-methyl cinnamaldehyde (Tokyo Kasei Kogyo Co., Ltd.)

13. 3-phenylpropion aldehyde (Tokyo Kasei Kogyo Co., Ltd.)
14. 4-dimethylamino cinnamaldehyde (Tokyo Kasei Kogyo Co., Ltd.)
15. 4-fluoro cinnamaldehyde (Tokyo Kasei Kogyo Co., Ltd.)

In these compounds, 1000 µg / ml to create a dilution series of up to 2^8 as a reference which were subjected to antimicrobial activity measured in the same manner as in subhead 2.9. The results were calculated IC_{50} for each compound based.

1.3 Results

1.3.1 Antibacterial activities of 3 herbal extracts

The antimicrobial efficacy of 3 herbal extracts against 7 species of *Vibrio* was estimated by the diameter of the clear zone around the paper disc put on a *Vibrio*-inoculated agar plate in a disc diffusion assay. As shown in Table 1, galangal ethanol extract formed the largest clear zone on all *Vibrio*-inoculated plates. This result indicated that, among the 3 herbs, galangal extract was the most efficient at inhibiting the growth of the *Vibrio* species. *V. cholera*e was the most susceptible to the galangal extract among the *Vibrio* species tested while *V. harveyi* was the least susceptible. Cardamon extract showed a smaller effect on the growth for all tested *Vibrio* isolates, and fingerroot extract had no effect.

1.3.2 The galangal extraction yield

Extraction final yield of galangal with methanol was significantly more produced yield than galangal extracted with ethanol and galangal powder was immersed for 72 hours with methanol was no significantly immersed for 24 hours (Table 2).

1.3.3 Effect of galangal ethanol extract on cell of V. harveyi

The effects of galangal extract on *V. harveyi* cells that has been changed on both the cell walls and the intracellular components of cells. From Fig 1. a-b showed normal cells of *V. harveyi*, therefore it has a cell wall that consists of two membranes. The outer membrane of cell wall was destroyed and showed thin layer of cytoplasmic membrane that made the cell swelling because of the changes to the cell osmosis (Fig 1. c). Figs. c-d showed the intracellular components of cells, such as nucleic acids, proteins, ribosomes were coagulated. The cell wall was destroyed and cell membrane being split resulting in a release of cytoplasm and nucleus materials (Fig 1. d).

1.3.4 Identification of the galangal extract compound

The results of methanol extraction and distribution than the yield in the ether layer fraction. The antibacterial activity measured for each peak obtained by separation by the subsequent reverse phase HPLC, the antibacterial activity was observed in the three locations of the peaks (PEAK 1, PEAK 2, PEAK 3) (Fig. 2A). The major of antibacterial active ingredients compound of the galangal extract were *trans-p*-hydroxy cinnamaldehyde, *trans-p*-acetoxy cinnamic alcohol and *trans-p*-coumaryl diacetate (Fig. 2C) which were identified by HPLC and NMR.

1.3.5 Antibacterial activities ingredients compound of the galangal extract

The major of antibacterial active compound of the galangal extract was *trans-p*-coumaryl diacetate (Fig.2). The minimum inhibitory concentration (MIC) of *trans-p*-coumaryl diacetate against 7 tested *Vibrio* species were found at 0.24 mg/ml (Table 3). From these studies, 7 species of *Vibrio* were considered to be susceptible (MBC/MIC) on the *trans-p*-coumaryl diacetate that was isolated from the crude galangal extract. The antibacterial active compounds of the galangal extract were *trans-p*-hydroxy cinnamaldehyde, *trans-p*-acetoxy cinnamic alcohol and *trans-p*-coumaryl diacetate. The half maximal inhibitory concentration (IC_{50}) of the antibacterial active compounds against *V. harveyi* was found at 0.074 ± 0.0095, > 5 and 0.720 ± 0.0190 µmol/ml, respectively (Table 4). The studies *V. harveryi* were considered to be susceptible on the crude galangal extract.

1.3.6 Antimicrobial activity of similar compounds

For the 15 types of compounds that are similar to the resulting anti-microbial material, the results of the measurement of the antibacterial activity about the IC50 were summarized in Table 5.

1.4 Discussion

In Southeast Asia, especially the use of herbal plants regularly every day. Herbal family Zingiberaceae used as food and medicine. In this study, the ethanol herb extracts from some rhizomatous members of the Zingiberaceae family namely galangal, cardamom, and fingerroot were used to test for antimicrobial activities that the galangal extract was severely inhibited 7 *Vibrio* spp. test. The researchers (5) have been reported, 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. From our results, MBC/MIC ratios of galangal extracts were less than 4. Therefore, it was concluded that all 7 *Vibrio* species were considered to be sensitive to these extracts. The present study revealed a microbiocidal effect of the galangal extract on Gram-negative *Vibrio*. However, The (23) reported that Galangal extract suppressed the growth of most Gram-positive bacteria, but had no effect on Gram-negative bacteria such as *Salmonella* sp., *Enterobacter aerogenes*, and *Psuedomonas aeruginosa*. Furthermore, *V. parahaemolyticus* inhibited the growth by chloroform extract of fresh galangal root, but not by methanol extract (34). The reason for this difference from the results found in the present study is not clear but may have been caused by a difference in extraction methods. The Vuddhakul group (34) used methanol to extract fresh rhizomes from galangal. In the present study, however, ethanol was used for extraction after drying at 45 ºC. This might indicate that the extraction method is important when using the galangal extract as an antimicrobial. The interesting on essential oil has been increased. The essential oil of the crude galangal extract is responsible for its antimicrobial activity (8) (30) (23) (31) (29) (34) (19) (16) (25). The main advantage of herbs agent is that the crude extracts contain a mixture of compounds like phenols, acids esters and aldehydes for which it is difficult to develop resistance by bacteria unlike the synthetic that contain a single compound because *V. harveyi* is resistant stain from diseased shrimp that are resistant to most of the chemotherapeutic agents used in aquaculture systems (1). The result of this study was revealed that the effective of growth inhibition of the galangal extract which can inhibit *V. harveyi* and susceptible on this extract. Furthermore, there were identified to *trans*-*p*-coumaryl diacetate. Later on, the main constituents of the extracts were identified as D, L-1’-acetoxychavicol acetate

(23), 1, 8-cineole, *β*-bisabolene, *β*-caryophyllene and *β*-selinene (19), 5-hydroxymethyl furfural and benzyl alcohol (25). The researahers (6) studied on *A. galanga* rhizomes and isolated trans-*p*-coumaryl diacetrate. Galangal herb was also very interested by (21), they studied clued extracted and found the lipophilic compounds, soluble in ethanol that have the property of essential oils. Since the large number of different chemical compounds presented in this crude extract, therefore, its mechanism can affect multiple target site against the bacterial cells such as enables them to partition in the lipids of the cell membrane and mitochondria, rendering them permeable and leading to leakage of cell contents (4). The extract also inhibits gram positive bacteria, *S. aureus* by destroying external cell wall and break internal cell wall into many pieces. Furthermore, it can change the value of pH and protein in the bacterial cell which made them loss protoplasm and ion, these depend on bacterial concentration and period of the experiment (23). *V. harveyi*, therefore it has a cell wall that consists of two membranes: an outer membrane made up of lipopolysaccharides and an inner cytoplasmic membrane. In between these is a periplasmic space housing a peptidoglycan layer (https://microbewiki.kenyon.edu/index.php/Vibrio_harveyi, 2007). The results from TEM showed that the galangal extract might affect a cell wall, the cell osmolarity, ribosomes and cell coagulation compounds of *V. harveyi*. The structural analogs compound 15 types that are resulting anti-microbial on *V. harveyi* in this experiment. The *trans-p*-hydroxy cinnamaldehyde with a double bond leading to the aldehyde group, in the comparison of *p*-hydroxybenzaldehyde having no double bonds, found that the antimicrobial activity of *trans -p*-hydroxy cinnamaldehyde was strong, the aldehyde group leading double bond has been suggested that to enhance the antibacterial activity. Encouraged to compare between a compound which *trans-p*-hydroxy cinnamaldehyde having a phenolic hydroxyl group bonded to the benzene ring and the cinnamaldehyde, in the comparison of the *trans*-cinnamic acid and *p*-hydroxy cinnamic acid, respectively. The *trans-p*-hydroxy cinnamaldehyde and *p*-hydroxy cinnamic acid with the group was strong, phenolic hydroxyl groups have been shown likely to enhance the antimicrobial activity. The *trans-p*-acetoxy cinnamic alcohol and *trans-p*-coumaryl diacetate has an antibacterial effect against *V. harveyi*, this antibacterial mechanism can be considered to be related with trans-p-acetoxy cinnamic alcohol and trans-p-coumaryl diacetate is acetate in both (ester of acetic acid). Acetate neutralization and membrane

gradient, act as an organic acid to denature the protein in the cell (11). Antimicrobial activity of acetic acid is related to pH, taking the undissociated has become a major source of antimicrobial activity. When the ester was dissolved in a solution, undissociated acid because intracellular pH is higher than the extracellular pH will penetrate the layer to the cell membrane lipids in bacteria and yeast, it is possible to emit a proton (3) (7) (18).

1.5 Conclusion

Galangal extract showed microbiocidal activity against *Vibrio* pathogens isolated from shrimp. The active substance which has mechanism action in antibacterial, 7 tested *Vibrio* species, was identified as *trans-p*-coumaryl diacetate. The antibacterial active compounds of the galangal extract against *V. harveyi* were *trans-p*-hydroxy cinnamaldehyde, *trans-p*-acetoxy cinnamic alcohol and *trans-p*-coumaryl diacetate. From the results, we can use this galangal for treatment diseases alternative of using antibiotic which lead to drug residue and resistance. Since galangal is a cheap and common plant found in tropical areas, it has great potential for effective application as an antimicrobial for the biological control of farmed shrimp and fish.

Table 1 Efficacy of 3 herbs for growth inhibition against 7 species of *Vibrio* spp. was isolated from marine shrimp. Diameter of clear zone around paper disc subjected herbal ethanol extract on bacteria-supplemented agar plate was represented by average ± SD.

Bacterial Species	Diameter of growth inhibition zone (mm)*				
	Galangal	Cardamom	Fingerroot	ethanol	Water
V. cholerae	31.0 ± 1.0	10.0 ± 0.0	0	0	0
V. parahaemolyticus	29.7 ± 1.2	0	0	0	0
V. fluvialis	28.0 ± 1.0	13.0 ± 0.7	0	0	0
V. vulnificus	27.3 ± 0.6	10.5 ± 0.7	9.0 ± 0.0	0	0
V. alginolyticus	27.3 ± 0.6	10.0 ± 0.0	0	0	0
V. mimicus	27.3 ± 0.6	8.8 ± 0.4	0	0	0
V. harveyi	21.3 ± 2.1	10.5 ± 0.7	0	0	0

*Resistant, ≤ 9 mm; Intermediate, ≥ 10 – 13 mm; Susceptible, ≥ 14 mm (Lorian 1995 *In* Oonmetta-aree et al. (2006)).

Table 2 Effect of difference solvent on the galangal extractions yield (%)

Immersed Times	The yield (%) of galangal extracts	
	Ethanol	Methanol
24 hours (1 day)	$9.32 \pm 0.28^{\mathbf{Bb}}$	$16.80 \pm 0.95^{\mathbf{Aa}}$
72 hours (3 days)	$12.10 \pm 0.46^{\mathbf{Ba}}$	$16.70 \pm 0.30^{\mathbf{Aa}}$

Note: Significant difference (P<0.05) was indicated by different superscript big letter within the same row and different superscript small letter within the same column. Data in table were Average ± SD.

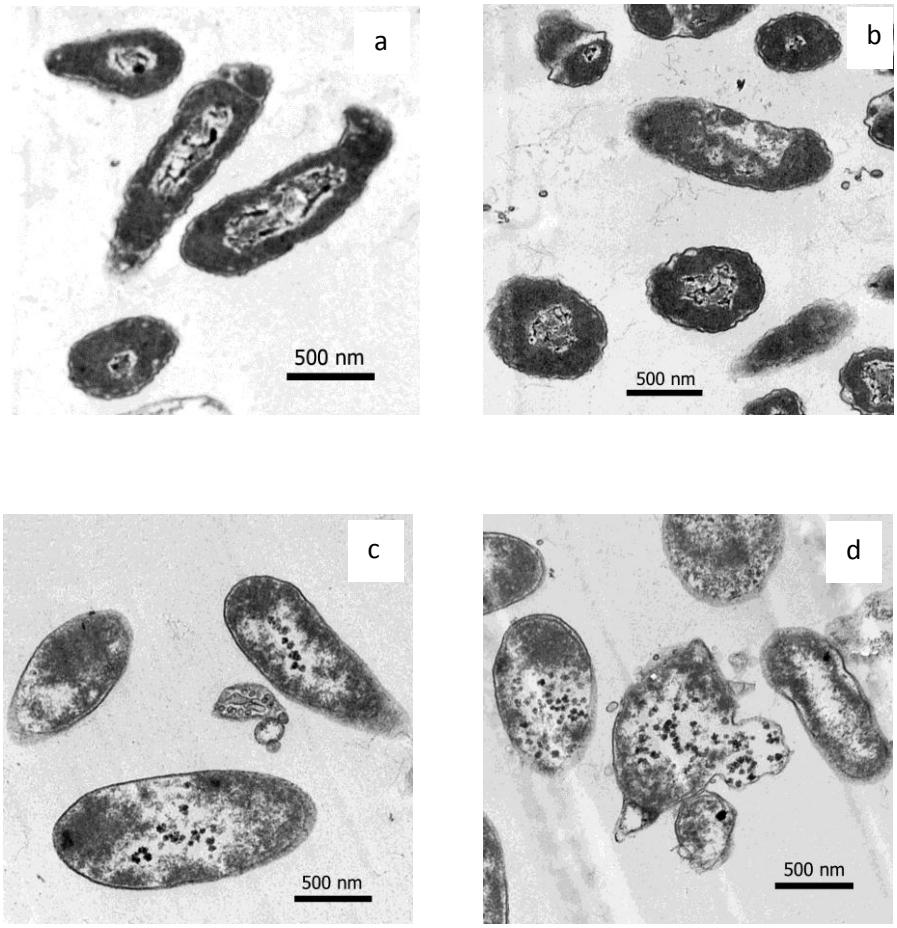

Fig. 1 TEM of cytological effect of galangal ethanol extract on *V. harveyi*; (a and b) Control (ethanol treatment); (c and d) galangal extract treatment after 20 h incubation.

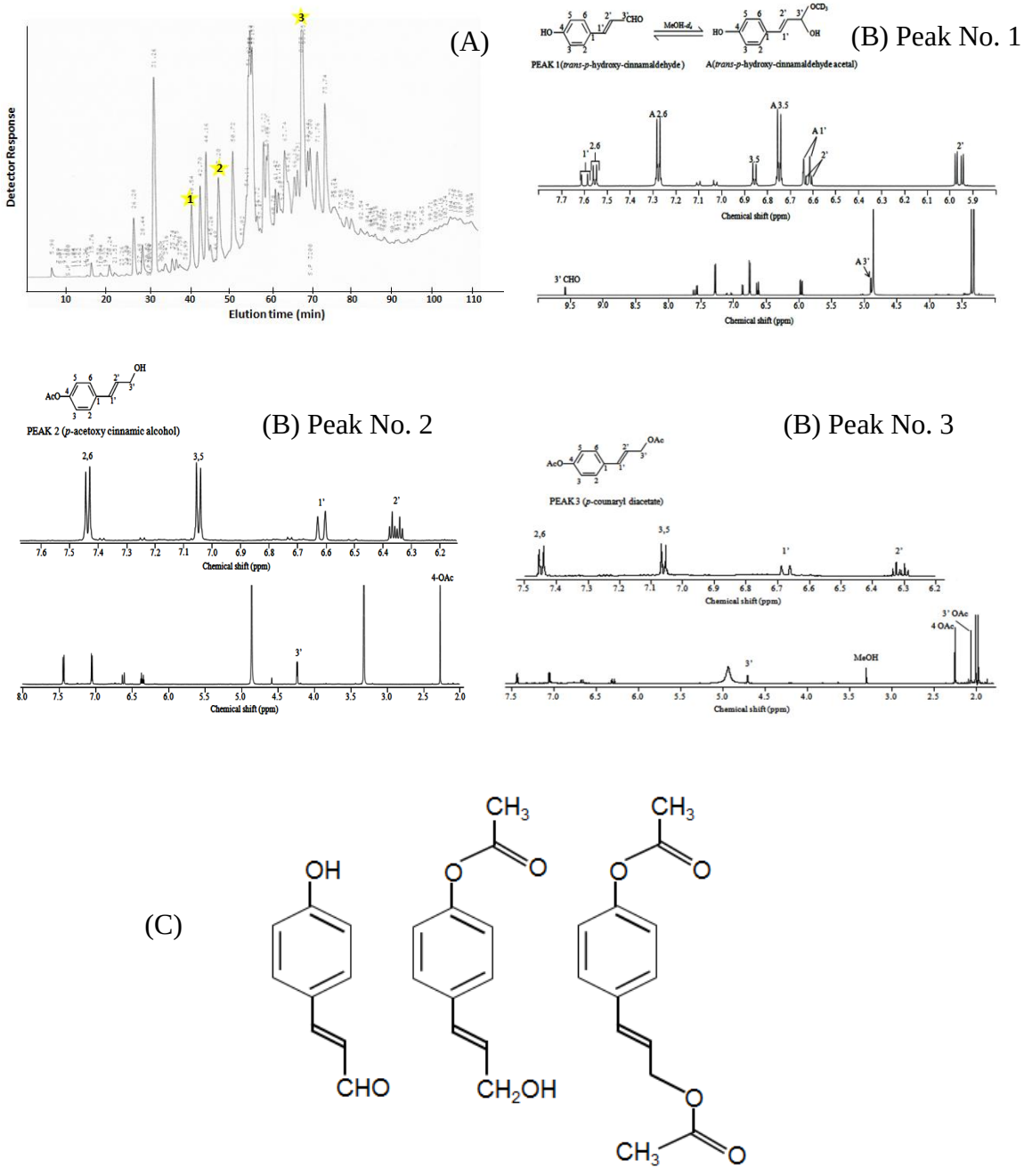

Fig. 2 (A) HPLC chromatographic analysis (B) Mass Specta analysis of the methanol galangal extract (C) The antibacterial active compounds of the galangal extract were (Peak No. 1) *trans-p*-hydroxy cinnamaldehyde, (Peak No. 2) *trans-p*-acetoxy cinnamic alcohol and (Peak No. 3) *trans-p*-coumaryl diacetate detected from the peak of HPLC analysis.

Table 3 MIC and MBC of the antibacterial active ingredients *Trans-p*-coumaryl diacetate isolated from the galangal extract against 7 species *Vibrio* spp. were isolated from marine shrimp.

Vibrio Species	MIC (mg/ml)	MBC (mg/ml)	Sensitivity of bacteria (MBC/MIC)
V. cholerae	0.24	0.32	1.3
V. parahaemolyticus	0.24	0.48	2.0
V. fluvialis	0.24	0.48	2.0
V. vulnificus	0.24	0.40	1.6
V. alginolyticus	0.24	0.64	2.6
V. mimicus	0.24	0.40	1.6
V. harveyi	0.24	0.48	2.0

Note: As reported by Canillac and 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.

Table 4 Half maximal (50%) inhibitory concentration **(**IC_{50}**)** of the antibacterial active ingredients isolated from galangal extract against *V. harveyi*.

The antibacterial active ingredients	IC_{50} (µmol/ml)
Trans-p-hydroxy cinnamaldehyde	0.0740 ± 0.0095
Trans-p-acetoxy cinnamic alcohol	>5
Trans-p-coumaryl diacetate	0.7200 ± 0.0190

Table 5 Half maximal (50%) inhibitory concentration **(**IC_{50}**)** of the analogous compounds of structure-activity relationships of galangal from antimicrobials against *V. harveyi*.

The analogous compounds	IC_{50} (µmol/ml)
4-methoxy cinnamaldehyde	2.97±0.56
α-methyl cinnamaldehyde	2.88±0.19
3-phenylpropion aldehyde	3.01±0.11
4-dimethylamino cinnamaldehyde	not active
4-fluoro cinnamaldehyde	1.13±0.40
Cinnamaldehyde	1.73±0.24
m-hydroxy cinnamic acid	2.50±0.06
p-hydroxybenzaldehyde	0.92±0.11
trans-cinnamic acid	2.86±0.29
trans-o-coumaric acid	1.51±0.52
cinnamyl alcohol	2.94±0.59
p-hydroxy cinnamic acid	1.91±0.36
cinnamyl acetate	not active
benzyl cinnamate	not active
Vanillin	1.36±0.21

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CHAPTER 2

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*)

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.

2.1 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* (37). In 2012, AHPND had a major impact on shrimp farmers in Thailand and Southeast Asian who sustained heavy losses in production (23) (9). 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 (35). 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* (19) (22) (39). 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 (30) (33) (12) (17). Some aquatic fungi are also opportunistic pathogens. Fusaruosis or black gill disease is caused by *Fusarium* spp., and it may affect all developmental stages of penaeid shrimp (12). 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 (21). The essential oil of the crude galangal extract is responsible for its antimicrobial activity (7) (26) (38) (27). 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*).

2.2 Materials and Methods

2.2.1 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 ^{o}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) (26).

2.2.2 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 (29). The isolate was streaked on Thiosulfate Citrate-Bilesalt Sucrose Agar (TCBS Agar, Difco USA) for selective isolation of *Vibrio*, and cultivated at 30 ºC for 18-24 h. The *Vibrio* species was identified according to a scheme developed by (6) using API 20E kits (ATB System, BioMérieux, France) and the PCR technique for the detection of *V. parahaemolyticus* (EMS/AHPND) (11).

2.2.3 Bacterial Inoculum

The isolate was streaked on TCBS agar and incubated at 30^{o}C for 18–24 h. A pure colony was streaked on Trypticase Soy Agar (TSA, Difco, USA), and incubated at 30^{o}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.

2.2.4 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 (26). 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 µl) was applied to a paper disc (Φ8 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 ºC 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.

2.2.5 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 µl of each dilution was dropped onto a paper disc (Φ8 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 30ºC 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 (26).

2.2.6 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 30ºC 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 (26).

2.2.7 Efficacy of the Galangal Extract against Pathogenic Fungi

2.2.7.1 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^{o}C for 2-5 days (13). 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 (14), (28) and by (31) (32).

2.2.7.2 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 ^{o}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 ^{o}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:

$$\text{Colony radius growth rate (\%)} = \frac{(\text{colony radius of the treated fungus})}{(\text{colony radius of the control})} x100$$

The growth inhibition of fungi (%) = 100 – Colony radius growth rate (%)

2.2.8 Effect of the Galangal Extract against Vibrio spp. Bacteria and Fungi in L. vannamei

2.2.8.1 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.

2.2.8.2 Treatment of Pathogenic Vibrio Bacteria and Fungi

About nine hundred white feces syndrome diseased shrimp, *L. vannamei*, with average body weight 10.7 ± 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.

$$\text{Incidence of fungi infestation (\%)} = \frac{\text{The number of infested shrimp}}{\text{Total number of observed shrimp}} x100$$

2.2.9 Effect of the Extract on Disease Resistance Caused by Vibrio spp.

2.2.9.1 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 (6) using API 20E kits (ATB System, BioMérieux, France). Identification of *V. parahaemolyticus* (EMS/AHPND) was accomplished via the PCR technique (11). The isolate was grown on Thiosulfate Citrate Bile Sucrose Agar (TCBS Agar, Difco USA) incubated at 30 ^{o}C for 18-24 h. Then a pure colony was streaked on Trypticase Soy Agar (TSA Difco, USA), and incubated at 30 ^{o}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^{8}cfu/ml) then diluted to 10^{5}cfu/ml for the antibacterial activity test.

2.2.9.2 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^{5} 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.

2.2.10 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.

2.3 Results

2.3.1 Antibacterial Activities of Galangal Extract

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).

2.3.2 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.

2.3.3 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).

2.3.4 Effect of the Galangal Extract against Vibrio spp. and Fungi

2.3.4.1 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* spp. 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 10^3 cfu/g and 426.46 ± 168.21 X 10^5 cfu/g, respectively. The dominant numbers of *Vibrio* spp. isolated in the feces were green and yellow colonies.

2.3.4.2 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.

2.3.4.3 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)).

2.3.5 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 ± 5.8% and 83.3 ± 5.8%, respectively. Only 16.7 ± 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).

2.4 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 (34) (7) (36) (26) (38) (40) (21) (16) (27). (3) 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 (23) (2) (20). 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* (26). 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* (7) (15) (25) (24) (5). Furthermore, *V. parahaemolyticus* growth is inhibited by the chloroform extract of fresh galangal root, but not by the methanol extract (40).

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. was observed more frequently than the others. The study was focused on the degree that galangal crude extract inhibited the growth of these pathogenic fungi (8) 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* (18) and *A. flavus* (18) was reported in raw materials used to produce commercial pellet feed (41) 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* (10). This study's results also showed the growth inhibition of fungi by crude galangal extract. The study found fungi that produce alflatoxin 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 (4). 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 stains to resist (1). 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.

2.5 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.

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

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

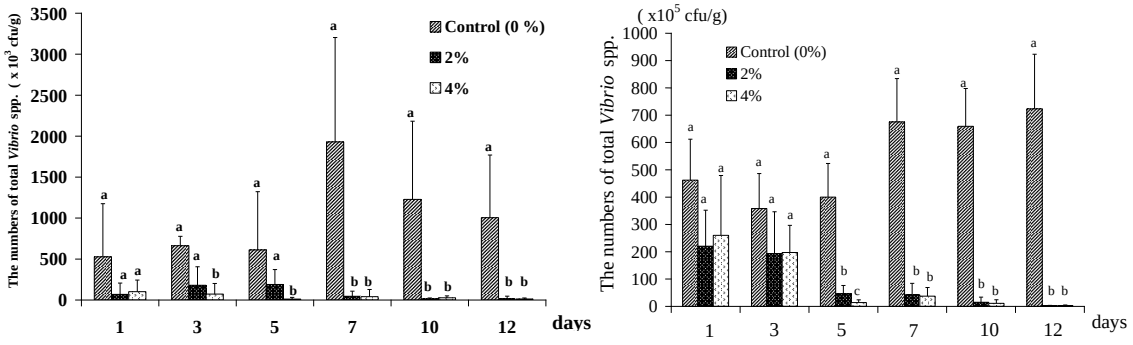
Table 2 The 6 species of fungi isolated from the hepatopancreas and intestines of shrimp naturally infected with white feces syndrome *L. vannamei*, and from artificial feed

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

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

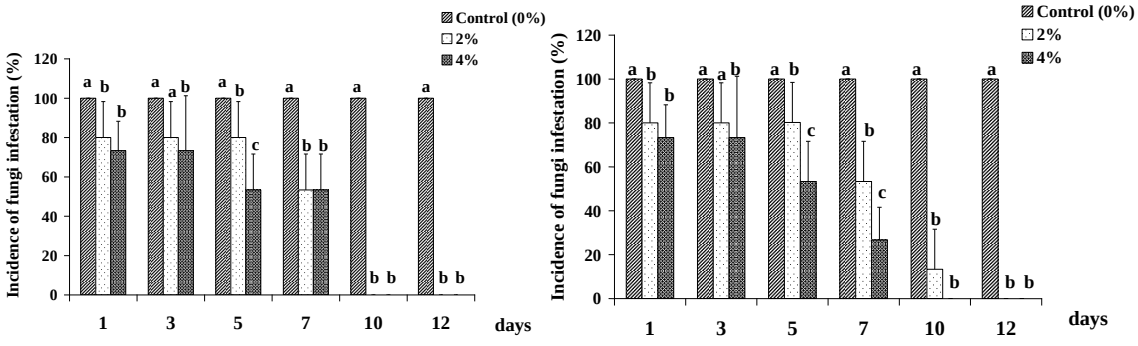
Extract Concentration	The growth inhibition of fungi (%)					
(mg/ml)	*A. flavus*	*A. ochraceus*	*A. japonicus*	*Penicillium* sp.	*Fusarium* sp.	*C. cladosporioides*
0.0005	6.1 ± 5.7	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	6.7 ± 6.3
0.005	16.3 ± 2.8	6.8 ± 2.5	18.6 ± 9.6	0.0 ± 0.0	4.2 ± 1.5	17.1 ± 11.3
0.05	48.9 ± 14.4	18.2 ± 9.3	15.3 ± 0.0	16.7 ± 0.0	27.9 ± 12.6	40.0 ± 3.1
0.5	56.7 ± 2.8	100.0 ± 0.0	25.4 ± 4.8	56.2 ± 15.9	42.4 ± 19.5	51.1 ± 6.3
1	77.5 ± 31.7	100.0 ± 0.0	37.3 ± 1.2	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0
2	77.5 ± 31.7	100.0 ± 0.0	57.6 ± 12.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0
4	82.6 ± 49.1	100.0 ± 0.0	55.9 ± 9.6	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0
8	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0

Note: Data in table, Average ± SD.



(A) Hepatopancreas (B) Intestines

Fig. 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 ± SD; different letters for the time interval indicates a significant difference (P<0.05)



(A) Hepatopancreas (B) Intestines

Fig. 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 ± SD; different letters for the time interval indicates a significant difference (P<0.05)

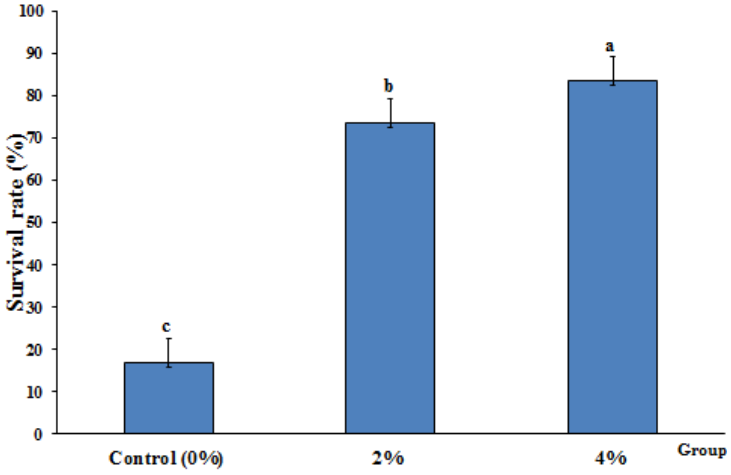


Fig. 3 The survival rate of *L. vannamei* shrimp fed diets containing 2 and 4% of galangal extract (treated diet) and 0% (control) following 12 days of feeding trials prior to being challenged with *V. parahaemolyticus* (EMS/AHPND). Different letters indicate a significant difference ($P<0.05$)

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CHAPTER 3

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*)

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.

3.1 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 (30) (31) (34). Antibiotic-resistant *V. harveyi* is known to cause mass mortality, retarded growth and deformities in penaeid shrimp around the world (14) (19) (17) (29) (3). (33) 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* (23) (38). 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 (18). 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 (5) (18) (20) (15) (21). The principal clinical sign of WSD is the appearance of white spots in the exoskeleton and epidermis of shrimp (27).

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 (12) (41) (10) (39) (40) (32). 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 (7). 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* (26), and can decrease inflammation and inhibit the growth of *Escherichia coli*, fungal pathogens and ringworm (6). The researchers (16) 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.

3.2 Materials and Methods

3.2.1 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 (26). 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.

3.2.2 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 (25), infectious hypodermal and haematopoietic necrosis virus (36) or yellow-head virus (9) 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.

3.2.3 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 (8) 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).

3.2.4 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^{TM} 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.

3.2.5 Disc Diffusion Test

V. harveyi (×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* (×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 (26).

3.2.6 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 (26). 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* (×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.

3.2.7 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.

3.2.8 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) (13) was calculated using Equation (1).

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

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

3.2.9 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) (2).

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

3.2.10 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.

3.2.11 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.

3.2.12 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.

3.2.13 WSSV Detection

3.2.13.1 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.

3.2.13.2 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.

3.2.14 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

3.3 Results

3.3.1 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.

3.3.2 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.

3.3.3 Effect of Galangal Extract Diet on Bacterial Disease

3.3.3.1 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.

3.3.3.2 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.

3.3.4 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.

3.3.5 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.

3.4 Discussion

A number of studies have reported the antimicrobial activity of galangal extract (11) (26) (37) (35) (22) (16) (28). The main constituent of the extract has been identified as 1'-acetoxychavicol acetate (26). 1,8-Cineole, *β*-bisabolene, *β*-caryophyllene and *β*-selinene (22), 5-hydroxymethyl furfural, and benzyl alcohol (28) have also been identified as components of galangal. The results (6) showed anti-food-borne bacteria activity of *A. galangal* rhizomes and its component, trans-*p*-coumaryl diacetate. The researchers (24) 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 (4) (26). 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 (1).

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.

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.

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

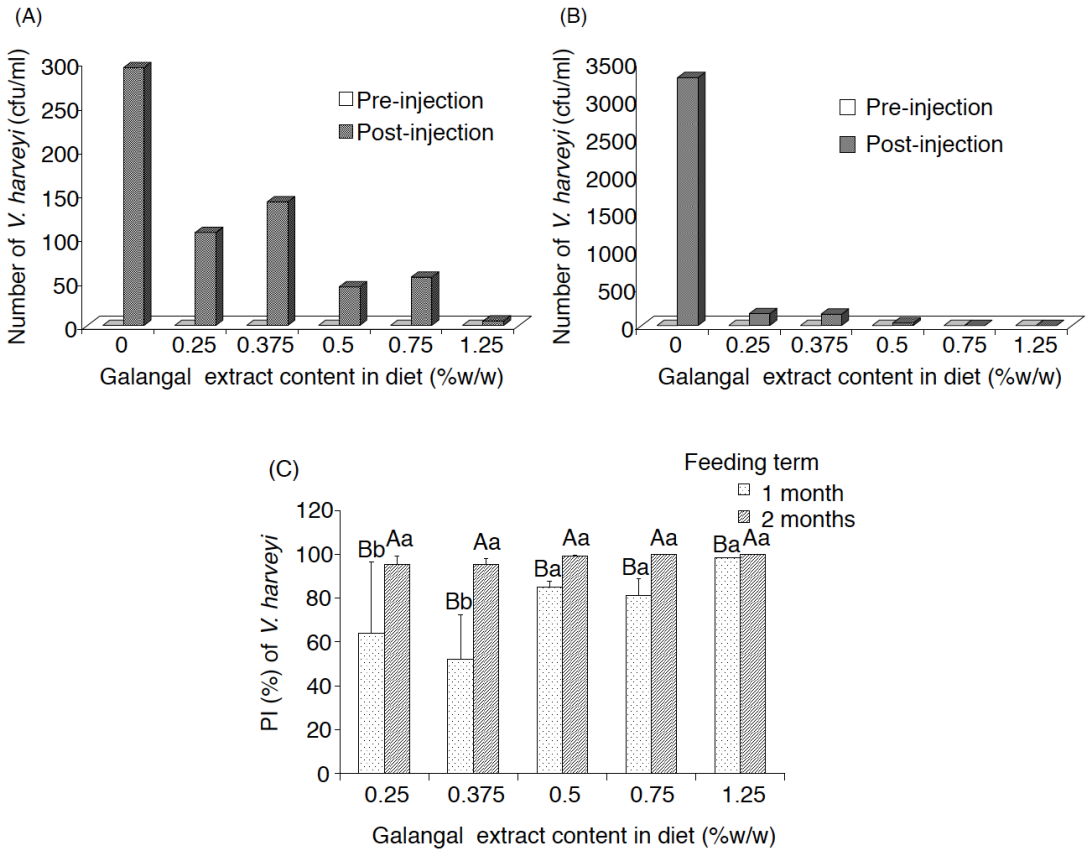

Fig. 1 Clearance ability of *L. vannamei* fed galangal extract for 1 or 2 months. *V. harveyi* (1-month feeding group, $4.5 \pm 0.7 \times 10^6$; 2-months feeding group, $8.7 \pm 2.3 \times 10^6$ cfu/ml) was injected into the abdomenal segment, and the number of *V. harveyi* in the hemolymph at 3 hours 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

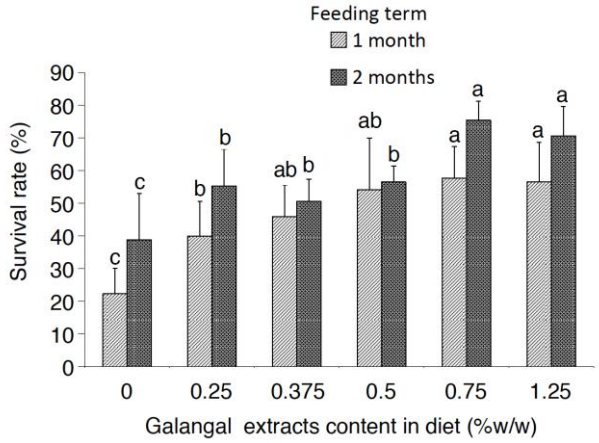

Fig. 2 Challenge test of shrimp with *V. harveyi*. The survival rate of *L. vannamei* fed a galangal extract diet for 1 or 2 months was monitored for 14 days after a challenge with *V. harveyi*. The different letters for the same time interval indicate a significant difference ($P < 0.05$)

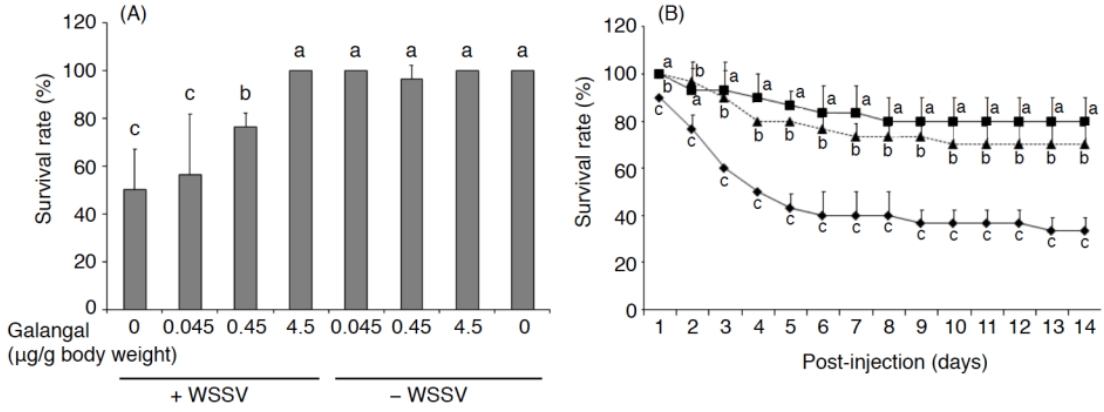

Fig. 3 (A) Antiviral activity of galangal extract against WSSV. After a 3-hour pre-incubation of galangal extract with WSSV at LD_{50}, the mixture was injected intramuscularly into shrimp. The survival rate was monitored for 14 days. Different letters at the same time interval indicate a significant 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 ($P < 0.05$)

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CHAPTER 4

Effect of galangal (*Alpinia galanga* Linn.) extract on the expression of immune-related genes and *Vibrio harveyi* resistance in Pacific white shrimp (*Litopenaeus vannamei*)

Abstract

Some plant and herb extracts reportedly possess antimicrobial activities and also have the ability to enhance the nonspecific immune system of shrimp, thereby promoting disease resistance. In this study, a natural herb, galangal (*Alpinia galanga* Linn.), was used to prevent infectious disease in cultured shrimp, and the effects of galangal–ethanol extract on the expression of the immune-related genes in the shrimp were analyzed via a reverse-transcription polymerase chain reaction of mRNA in circulating hemocytes. Following the intramuscular injection of either galangal extract or *trans-p*-coumaryl diacetate that had been isolated from galangal rhizome, Pacific white shrimp (*Litopenaeus vannamei*) showed significant increases in the relative expression ratio of the six immune-related genes compared with a control group. Furthermore, following the oral administration of galangal extract, similar inducible effects of the expression of immune-related genes in the Pacific white shrimp were obtained, which led to an enhanced survival rate from *Vibrio harveyi* infection. Thus, this study revealed that both galangal extract and *trans-p*-coumaryl diacetate stimulated the immune system response, thereby promoting resistance to *V. harveyi* infection in Pacific white shrimp.

4.1 Introduction

Shrimp aquaculture is one of the fastest growing food production sectors and provides important income to industrial agriculture. However, disease outbreaks have caused serious economic loss in several countries. Bacterial diseases cause problems ranging from growth retardation to sporadic mortality and mass mortality. *Vibrio* is the most devastating of the bacterial pathogens for all species of shrimp, and vibriosis is a major disease in shrimp aquaculture that causes widespread mortality in farming operations (20). When shrimp are stressed or experience low immunity, particularly when they are reared in conditions of high density, they are easily infected by vibrio bacteria, which leads to disease (36) (35) (40) (18) (19). Reports have shown that vibrio bacteria such as *Vibrio alginolyticus, V. cholerae, V. parahaemolyticus, V. vulnificus,V. mimicus*, and *V. harveyi* have been found in Pacific white shrimp (*Litopenaeus vannamei*) (28) (44). In the shrimp farm industry, proper health management dictates an improved understanding of the molecular mechanisms that control the immune responses in shrimp.

The discovery of several shrimp antimicrobial peptides such as lysozymes, penaeidins and crustins has suggested a prime importance for these immune-effector molecules (41). The antibacterial and antiviral activities of lysozyme protein have been demonstrated in shrimp (14) (24). The penaeidins and crustins in *L. vannamei* have been described as proteins belonging to an antibacterial peptide family with similar sequences but different sizes (45). Superoxide dismutase (SOD) is one of the main defense mechanisms against oxidative stress caused by pollution, infection and immunostimulants (30). Phenoloxidase (PO) is an enzyme associated with the synthesis of melanin and exists in hemolymph as a precursor of PO, prophenoloxidase (proPO). ProPO is activated to PO from when it reacts with bacterial lipopolysaccharide (LPS) (38). Transglutaminase (TGase) is known to be involved in blood coagulation and in a conserved defense mechanism among invertebrates. Thus, it is an important component of the shrimp immune response and is involved in the regulation of some immune-related genes, particularly antimicrobial peptides such as crustin and lysozyme (12).

Galangal (*Alpinia galanga* Linn.) is rich in phenolic compounds such as flavonoids and phenolic acids (26), and various physiological activities of galangal

extract and its components have been reported. The hydroxychavicol acetate from galangal rhizomes may be beneficial for treating inflammatory immune disorders caused by the extravagant activation of Th1-mediated immune responses (27). Hot-water extracts of galangal containing soluble polysaccharide are known to stimulate the immune activity in mice-an effect on the reticulo-endothelial system and an increased number of peritoneal exudate cells, and spleen cells (1). *Trans-p*-Coumaryl diacetate isolated from galangal has displayed antioxidant and anti-inflammatory activities (48) (5).

Herbs and some spices are known to have antiparasitic and antimicrobial properties, which can be used alternatively as methods for the treatment of infectious diseases in cultured shrimp. The essential oil of crude galangal extract is the ingredient that is responsible for its antimicrobial activity (10) (32) (43) (34). We also have identified *trans-p*-coumaryl diacetate from galangal methanol extract as one of the anti-microbial compounds (not published). Meanwhile, some medicinal plant extracts are known to act as immunostimulants that could modulate the immune response to protect fish, shellfish and shrimp from infectious diseases (4) (16) (47) (13). However, the immunostimulating effects of galangal on shrimp have not been reported. The present study was focused on examining the impact of galangal-ethanol extracts and *trans-p*-coumaryl diacetate on the expression levels of the immune-related genes of Pacific white shrimp. Also, the effect of galangal extract administration on the survival rate of Pacific white shrimp from *Vibrio harveyi* infection was investigated.

4.2 Materials and methods

4.2.1 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^{o}C for 24 h. After drying, they were grounded 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). The galangal extract was dissolved in dimethyl sulfoxide (DMSO) as stock for intramuscular injections into shrimp, and it was dissolved in ethanol for use in oral administration.

4.2.2 Isolation of trans-p-coumaryl diacetate from galangal rhizome

The galangal 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 at room temperature overnight, and the final at 55^{o}C for 4 h. The supernatant was collected by centrifugation then combined and dried in a rotary evaporator (IWAKI, Japan). The residual was then suspended in 200 ml of water, and was extracted twice with diethyl ether (150 ml/time). The ether fraction was dried via a rotary evaporator and further lyophilized. The *trans-p*-coumaryl diacetate was purified from the ether fraction by a high-performance liquid chromatograph (L-2130, Hitachi, Japan) equipped with an Inertsil ODS-3 column (10 × 250 mm, 5 µm, GL Science, Tokyo, Japan). The sample was eluted with a 120-min linear gradient of the mobile phase consisting of 1% acetonitrile containing 0.1% trifluoroacetic acid (TFA) and 99% acetonitrile containing 0.1% TFA at a flow rate of 3 ml/min. The sample was monitored at 260 nm. The *trans-p*-coumaryl diacetate was identified by nuclear magnetic resonance spectroscopy (Bruker, AV-600) and mass analysis (Bruker, micro-TOF).

4.2.3 Shrimp samples

The post-larvae (PL12) of *L. vannamei* shrimp were obtained from JR hatchery, Trad Province, Thailand. Polymerase chain reaction (PCR) confirmed a negative infection with white spot syndrome virus (23) (31), taura syndrome virus (29), infectious hypodermal and haematopoietic necrosis virus (42) and yellow-head virus (8). The stock of post-larvae was reared in a 15 m^3 concrete pond, fed 3 meals/day, and the water was changed twice a week. Healthy *L. vannamei* shrimp with a body weight of 10 –15 g were used for the experiments.

In the present study, the seawater was chlorinated with 20 –25 mg/l calcium hypochlorite and then de-chlorinated by vigorous aeration before use. Flow-through water equipped with an aeration system was supplied. In the feeding trial, excess food and waste matter was removed each day before changing the water. The water parameters such as temperature (27 ± 2.0^{o}C), salinity (30 ± 0.0 ‰), dissolved oxygen

6.50 ± 0.02 mg/l, and pH (7.8 – 8.0) were maintained throughout the experimental period.

4.2.4 Intramuscular administration of galangal extract and trans-p-coumaryl diacetate

Healthy shrimp with an average body weight of 11.0 ± 0.8 g were transferred from the stock to 200-l experimental (plastic) tanks filled with 180 L of seawater. Each experimental group contained 3 tanks (15 shrimp/tank). Each shrimp was injected intramuscularly with galangal extract at 5 or 50 µg/g body weight or *trans-p*-coumaryl diacetate at 5 µg/g body weight. In all treatment groups, the samples dissolved in 100 µl of 0.1% DMSO–0.85% NaCl were injected. The shrimp of the control groups were injected with either 0.1% DMSO–0.85% NaCl or 0.85% NaCl. Before injection (at 0 h) and at 6, 24, 48, 72 and 120 h post-injection, 500 µl of hemolymph was collected from the ventral sinus of each shrimp to determine the expression level of immune-related genes. This experiment was performed in triplicate.

4.2.5 Oral administration of galangal extract

The galangal extract dissolved in ethanol was mixed with commercial pellet feed (CP feed, Thailand) at concentrations of 2.5, 5 and 10 g galangal extract/kg pellet feed. 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 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.

Healthy shrimp (average body weight, 13.3 ± 1.2 g) from each diet group were cultured in 200-l tanks (20 shrimp/tank, each diet group contained 8 tanks) with 180 L of seawater. The shrimp were fed a diet containing galangal extract 3 times/day. The control diet group was given pellet feed without galangal extract. The feeding rate per meal was 3 – 3.5% of the shrimps' weight. At 1, 3, 5, 7, 10, and14 days of feeding, six shrimp were selected from each diet group, and 500 µl of hemolymph was collected from the ventral sinus of each of these shrimp to determine the expression level of immune-related genes.

4.2.6 Preparation of bacteria

The pathogenic *V. harveyi* was isolated from infected *L. vannamei*, and the species of *Vibrio* spp. were identified using API 20E kits (ATB System, BioMérieux, France) (7). The isolate was streaked on Thiosulfate Citrate-Bilesalt Sucrose Agar (TCBS Agar, Difco USA), and incubated at 30^{o}C for 18–24 h. A pure colony was streaked on Trypticase Soy Agar (TSA, Difco, USA), and incubated at 30^{o}C for 18 – 24 h. The picked culture was suspended in sterile 0.85% NaCl and diluted to an optical density of 0.11 at 610 nm (McFarland Standard No. 0.5). This suspension contained approximately 10^8 colony-forming units (cfu)/ml. The pathogenicity of *V. harveyi* was confirmed via challenge studies in juvenile shrimp (LD_{50} value 10^5 cfu /g body weight).

4.2.7 Effect of galangal extract on vibrio resistance and on the immune-related gene expression in shrimp

Fifteen shrimp in a 70-l experimental glass aquarium filled with 50 l of seawater were fed a galangal extract diet (0, 2.5, 5 or 10 g galangal extract/kg feed) for 7 or 14 days. The shrimp were then infected with *V. harveyi* by an injection of 100 µl of bacterial suspension into the abdomen segment (5.3×10^7 cfu/ml for the 7-day feeding group and 6.3×10^7 cfu/ml for the 14-day feeding group). After the injection of bacteria, the shrimp were fed a commercial diet without galangal extract twice a day for a further 10 days, during which the disease symptoms and the survival rates were monitored. Prior to injection (at 0 h), and at 3, 24, and 48 h post-injection, hemolymph (500 µl from each) of three shrimp from each diet group was collected from the ventral sinus for immune genes expression analysis.

4.2.8 Analysis of the expression levels of immune-related genes

Six immune-related genes commonly found in shrimp were selected for analysis of expression levels: prophenoloxidase (proPO), cytosolic manganese superoxide dismutase (cMnSOD), transglutaminase (TGase), lysozyme (LSZ), penaeidin-3 (PEN), and crustin. The expression levels were detected via reverse-transcription polymerase chain reaction (RT-PCR) of the mRNA in circulating hemocytes (45) (32). Briefly, the hemolymph of the shrimp (500 µl/shrimp) was collected from the ventral sinus, mixed with 1,000 µl of 10% (w/v) sodium citrate as an anticoagulant, and then centrifuged immediately at 3,000×*g* and 4^{o}C for 10 min to obtain the hemocytes. The total hemocyte RNA was extracted using Trizol reagent (Invitrogen). One microgram was used as a

template for the first-strand cDNA synthesis using a cDNA Synthesis Kit (Invitrogen). An amplification of each representative immune gene transcript was performed in a 50 µl reaction mixture containing 2 µl of the cDNA template, 0.2 mM of each dNTP, 1U of *taq* DNA polymerase (Invitrogen), 0.4 µM of the primer pairs as shown in Table 1, and 2.0 mM of $MgCl_2$. The amplification was carried out as follows: predenaturation at 94^{o}C for 5 min, followed by 25 cycles of denaturation at 94^{o}C for 30 s, annealing at 60^{o}C for 30 s and extension at 72^{o}C for 60 s followed by a final extension at 72^{o}C for 10 min. Five microliters of each PCR product were analyzed via 1.5% (w/v) agarose gel electrophoresis followed by staining in ethidium bromide solution with visualization by UV transillumination. The intensity of each target band was detected using a Gel Documentation System (Digimage System, Model DI-01, Major Science, Taiwan), and was further quantified using Genetools analysis software (Vision works software, UVP, UK). The expression levels were calculated relative to that of beta-actin transcripts.

4.2.9 Statistical analysis

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

4.3 Results

4.3.1 The expression levels of the immune-related genes in shrimp injected intramuscularly with galangal extract or trans-p-coumaryl diacetate

A number of Pacific white shrimp were injected with galangal-ethanol extract or *trans-p*-coumaryl diacetate, and then the expression levels of six immune-related genes in the hemocytes, proPO, cMnSOD, TGase, lysozyme, penaeidin and crustin in circulating hemocytes were analyzed via RT-PCR. As shown in Fig. 1, the relative mRNA expression rations of all these genes were increased in both treatment groups. Interestingly, the significant up-regulation was observed from different times by genes. In shrimp which had been injected with galangal extract at 50 μg/g body weight, the expression levels of ProPO, cMnSOD, lysozyme and penaeidin were up-regulated significantly at 24 h post-injection, whereas TGase and crustin responded later and significant up-regulation was observed at 48 h post-injection. After up-regulation of the immune-related genes in the treatment groups, the significant higher mRNA levels than those measured in the control groups were maintained till the end of experiment at 120 h post-injection ($P<0.05$).

4.3.2 The expression levels of the immune-related genes in shrimp fed with galangal extract

Galangal-ethanol extract was orally administered to Pacific white shrimp for 14 days, and then RT-PCR analysis for six immune-related genes was carried out using hemocytes. The relative mRNA expression ratio of all analyzed transcripts of the administered group was significantly higher than that of the control group throughout the 14-day feeding trial (Fig. 2, $P<0.05$). Although the expression level of the six genes was lowered by the feeding of galangal extracts of 2.5 and 5 g/kg diet on the first day, it was stimulated and became higher than that of the control group from the third day. The diet with the higher concentration of galangal extract (10 g/kg) stimulated the expressions of the six immune genes on the first day.

4.3.3 The expression levels of the immune-related genes in shrimp after a V. harveyi challenge

Pacific white shrimp that had been fed galangal extract for 7 days were injected with *V. harveyi*, and then fed a control diet without galangal extract in order to analyze the expression level of immune-related genes. The mRNA expression levels of proPO, cMnSOD, TGase, lysozyme and penaeidin transcripts in the circulating hemocytes were decreased at 3 h post-injection of *V. harveyi* in both the group fed galangal extract diet for 7 days and the control diet group (Fig. 3). By 24 h post-injection, the expression levels of proPO, cMnSOD and lysozyme had reb*oun*ded to pre-injection levels (at 0 h), and those of TGase and penaeidin had rebounded by 48 h. The expression of crustin had decreased slightly 24 h after injection, but had then rebounded by 48 h. The relative expression ratios of the six immune-related genes of the group fed galangal extract diet for 7 days were significantly higher than those of the control diet group at all the time points tested throughout the experimental period (Fig. 3).

The expressions of six immune-related genes in the hemocytes of shrimp that had been fed a galangal extract diet for 14 days were also analyzed after injection with *V. harveyi.* The expression level of all tested genes had decreased at 3 h post-injection. However, the galangal extract diet groups showed a significantly higher level of gene expression by 24 h after injection compared with that of the control diet group, even though the galangal extract diet was changed to the control diet after infection (Fig. 4).

4.3.4 The survival rate from infection with V. harveyi

After galangal extract had been administered orally to Pacific white shrimp for either 7 or 14 days, *V. harveyi* was injected to monitor survival rates for 10 days. After infection, the galangal diet was changed to a control diet without galangal extract to avoid the antimicrobial activity of galangal. In all groups, shrimp was died in the first 24-h post-injection of *V. harveyi* as shown in Fig. 5. The survival rate of the group fed galangal extract was significantly higher than control group in both feeding periods (7 and 14 days). And the effect was largest at 5 g/kg diet. However, the survival rate of the

group fed galangal extract diet was significantly higher than that of the group fed control diet during the 10 days after the injection of *V. harveyi* ($P<0.05$).

4.4 Discussion

Several previous reports have shown that plant and herb extracts enhance non-specific immune systems and disease resistance in shrimp. Mixed artificial diets with the methanolic extract of five different herbal medicinal Indian plants have enhanced immunostimulation and reduced the viral load in giant tiger prawns (*Penaeus monodon*) (6). A diet containing *Solanum nigrum* extract enhanced the immunity and disease resistance of *P. monodon* against *V. harvei* (13). Zingerone supplementation in Pacific white shrimp feed increased immunity and disease resistance against *V. alginolyticus* (3). This study is the first, however, to investigate the immunostimulant activities of galangal extract in shrimp. In the present study, intramuscular administration of galangal extract to Pacific white shrimp stimulated the expression level of immune-related genes such as proPO, cMnSOD, TGase, lysozyme, penaeidin, and crustin (Fig.1). Further, *trans-p*-coumaryl diacetate also stimulated the six immune-related genes in Pacific white shrimp in a similar manner to that observed with galangal extract. Because we have identified this compound as one of the anti-bacterial compounds from galangal rhizome (not published), this result suggests that galangal and *trans-p*-coumaryl diacetate could work as both an antibacterial and an immunostimulant in shrimp.

By injection of *V. harveyi*, the relative expression ratio of six immune-related genes was decreased within 3 h in both the group fed galangal extract diet and group fed control diet groups (Figs. 3 and 4). This was likely caused by *Vibrio* injection, because injection of *trans-p*-coumaryl diacetate did not show such suppressive effects on the expression of immune-related genes. Furthermore, these results of *Vibrio* injection are consistent with reports showing that the lysozyme expression in *L. vannamei* after injection with *V. campbellii* resulted in a decreased lysozyme signature, as seen in tissues and circulating hemocytes during the early stages of infection (4 h) (2). *L. stylirostris* infected with *V. penaeicida* showed lower lysozyme, TGase and PNE3 mRNA levels compared with those in the unchallenged shrimp at 12 h post-injection

(8), and the penaeidin mRNA level was decreased in the circulating haemocyte of *P. vannamei* in the first 3 h after microbial challenge (11). However, differing results have shown that a direct injection of *V. harveyi* resulted in a more rapid increase in lysozyme expression (39).

In the present study, the galangal extract diet groups showed higher expression levels of 6 immune-related genes compared with the control diet group. This was also observed after *V. harveyi* injection, even though the galangal diet was changed to the control diet. Also, the survival rate of the galangal diet groups was significantly higher than that of the control diet group. A significant increase in the mortality of shrimp with crustin depleted by RNAi has been observed following injection with low pathogenic *V. penaeicida* (37), and the expression of crustins in crayfish has also shown a response to *Aeromonas hydrophila* infection (17). TGase silencing caused a down-regulation of the crustin and lysozyme expression, and TGase-depleted samples were found to have lower hemocyte and higher total bacterial counts in the haemolymph of kuruma shrimp (12). The mortality rate of shrimp after infection with *V. penaecida* and white spot syndrome virus was significantly higher in TGase depleted and in clotting protein-depleted animals, indicating the essential role of these two molecules in shrimp immune protection against microbial infection (25). The gene silencing of proPO in a crustacean, *Pacifastacus leniusculus* indicated that enhanced PO activity is correlated with both increased survival time and enhanced bacterial clearance (22). Together with these reports, our results indicate that the immune system of Pacific white shrimp was stimulated by a galangal diet, which equated to an increased resistance to bacterial infection.

Furthermore, the present study revealed that the haemocytes of shrimp fed galangal extract had a significantly increased SOD gene expression level. This suggests that galangal extract enhances the antioxidative mechanism in shrimp, because SOD is an antioxidative enzyme that scavenges superoxide anions (O^{-}_{2}) and hydrogen peroxide (H_2O_2) (14). The increased SOD activity and increased transcript of SOD have been also observed in *P. monodon* after receiving a diet containing sodium alginate at 2.0 g kg^{-1} for 5 months (21).

The expression of immune-related genes in galangal diet shrimp compared with the control diet group was distinctly different at 3 h post-injection of Vibrio. However, the expression level of these genes was suppressed at this point, maybe due to the effects by injection of Vibrio. These might suggest that total hemocyte number (THC) at this point was decreased by vibrio injection and its decrease was suppressed in galangal diet group compared with control diet group. In order to clarify the effects of galangal diet on the expression of immune-related genes, the evaluation of THC is still remained.

In the present study, a 5 g/kg galangal extract diet stimulated the immune-related gene expression and enhanced the infection survival rate to a greater extent than a 10 g/kg galangal extract diet. The reason for this was not clear, but perhaps the10 g/kg-diet was an overdose and the immunostimulation by galangal has an optimum dose that triggers a maximum immune response.

4.5 Conclusion

The galangal extract that was used in this study considerably enhanced the relative expression ratio of shrimp immune-related genes and increased the bacterial disease resistance of Pacific white shrimp. These results suggest that galangal extract might have the potential for application in bacterial disease control in shrimp aquaculture. Galangal crude extract could be recommended as a substitute for antibiotics in shrimp culture with advantages that would include a reduction in drug residue problems.

Table 1 The seven immune-related genes of the Pacific white shrimp (*L. vannamei*) and their corresponding PCR primers used for RT-PCR analysis (46).

Gene	Primer (forward/reverse sequence)	GenBank #
Prophenoloxidase (proPO)	5'-GGAATTGTTTTACTACATGCATCAGC-3'	AY723296
	5'-GGAACAAGTCATCCACGAGCTT-3'	
Cytosolic manganese superoxide dismutase (cMnSOD)	5'-GAGAAGAAGTTGGCTGAGCT-3'	AY486424
	5'-ATGTTGGGTCCAGAAGATGG-3'	
Transglutaminase (TGase)	5'-CAACCTGGAGGTTCACAAGC-3'	BE188522
	5'-CAAAGCTCTYGGKAAATACG-3'	
Lysozyme (LSZ)	5'-TTCGGGAAGTGCGAATTCG-3'	AY170126
	5'-AATGGAAACCCTTGGTGAC-3'	
Penaeidin-3 (PEN)	5'-AGCCTCACCTGCAGAGACCA-3'	Y14926
	5'-AATCAGGATCRCAGKCTCTTCAC-3'	
Crustin	5'-ATTCTGTGCGGCCTCTTTAC-3'	AF430076
	5'-ATCGGTCGTTCTTCAGATGG-3'	
Beta-actin	5'-TGTGTGACGACGAAGTAGCC-3'	AF300705
	5'-TGGTCGTGAAGGTGTAACCA-3'	

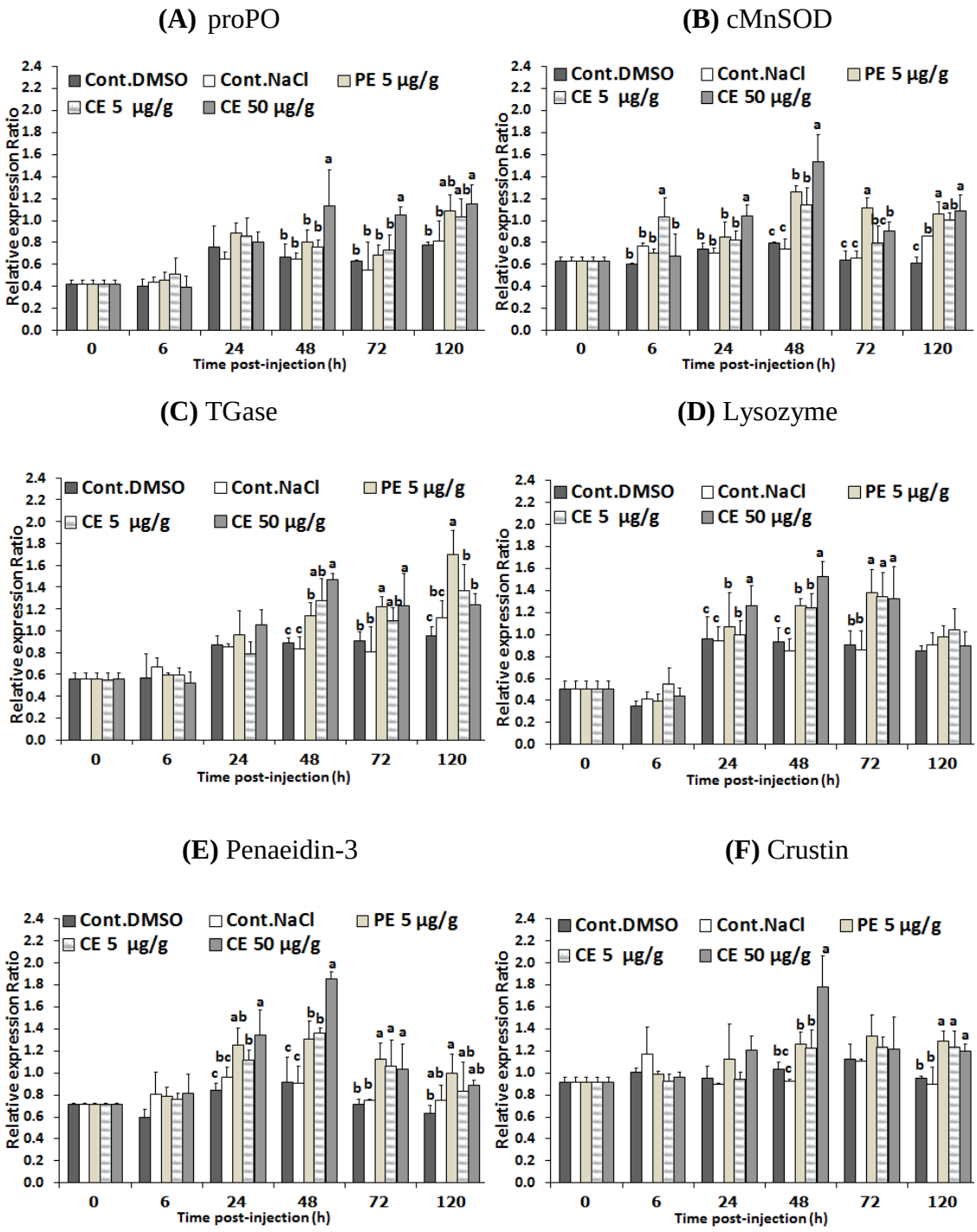

Fig. 1 Relative expression ratio of immune-related genes in hemocytes of shrimp injected with galangal extract or *trans-p*-coumaryl diacetate. Pacific white shrimp were injected intramuscularly with 0.1% DMSO in 0.85% NaCl (control), 0.85%NaCl (control), galangal-ethanol extract (CE) at 5 and 50 µg/g body weight or *trans-p*-coumaryl diacetate (PE) at 5 µg/g body weight. Before injection (0 h) and at 6, 24, 48, 72, and 120 h post-injection, hemocytes were collected and analyzed the expression of (**A**) proPO, (**B**) cMnSOD, (**C**) TGase, (**D**) Lysozyme, (**E**) Penaeidin-3 and (**F**) Crustin mRNAs by RT-PCR. Values are reported as the mean ± SD, n = 3. The mean values with different letters (at each time point) indicate significant difference (P<0.05).

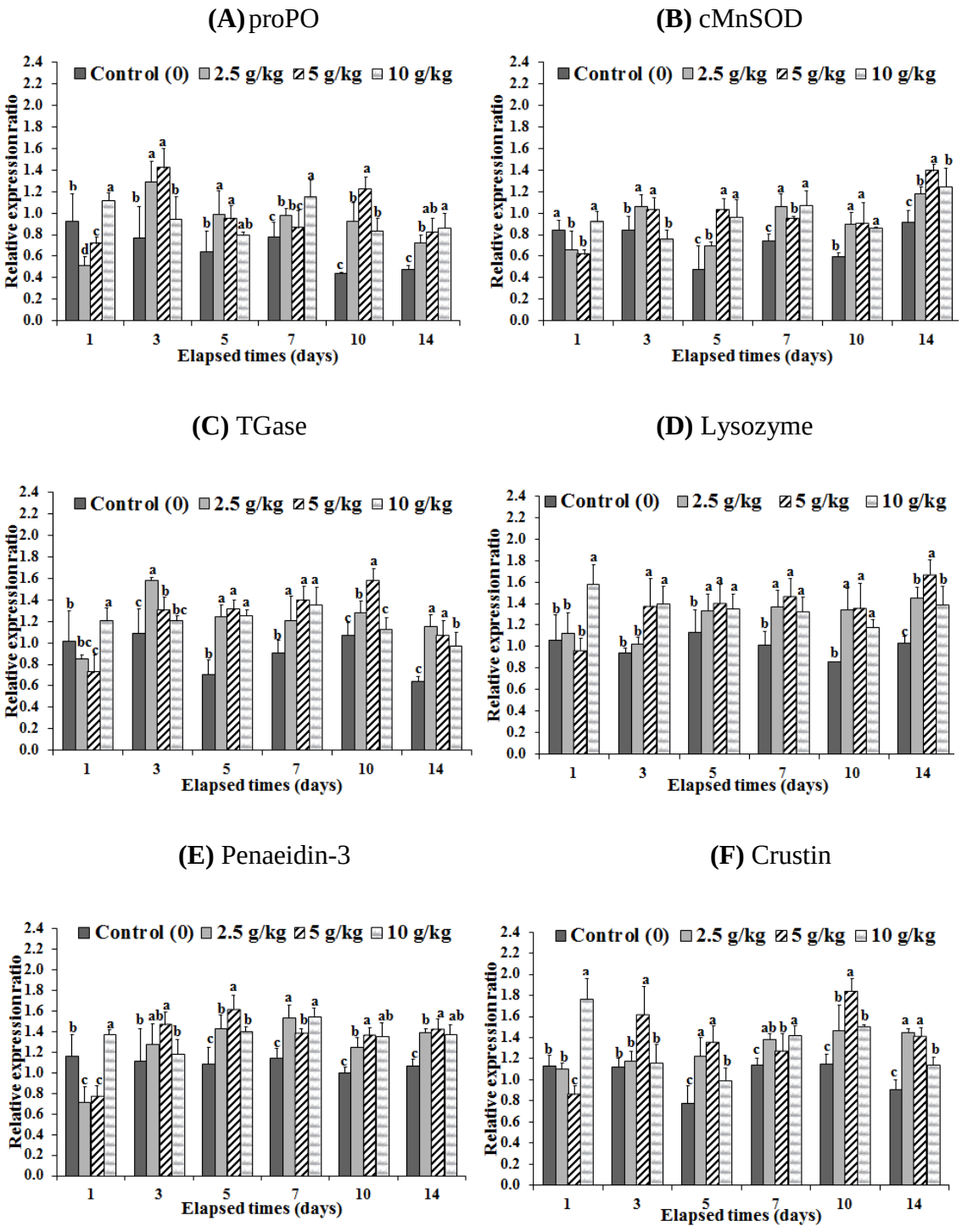

Fig. 2 Relative expression ratio of immune-related genes in hemocytes of shrimp fed galangal extract-supplemented diets for 14 days. Pacific white shrimp were orally administered a commercial diet (control, 0 g/kg diet) or a diet containing galangal-ethanol extract at 2.5, 5, and 10 g/kg diet. At 1, 3, 5, 7, 10, and 14 days of feeding trials, hemocytes were collected and analyzed for the expression of (**A**) proPO, (**B**) cMnSOD, (**C**) TGase, (**D**) Lysozyme, (**E**) Penaeidin-3 and (**F**) Crustin mRNAs by RT-PCR. Values are reported as the mean ± SD, n = 6. The mean values with different letters (at each time point) indicate significant difference (P<0.05).

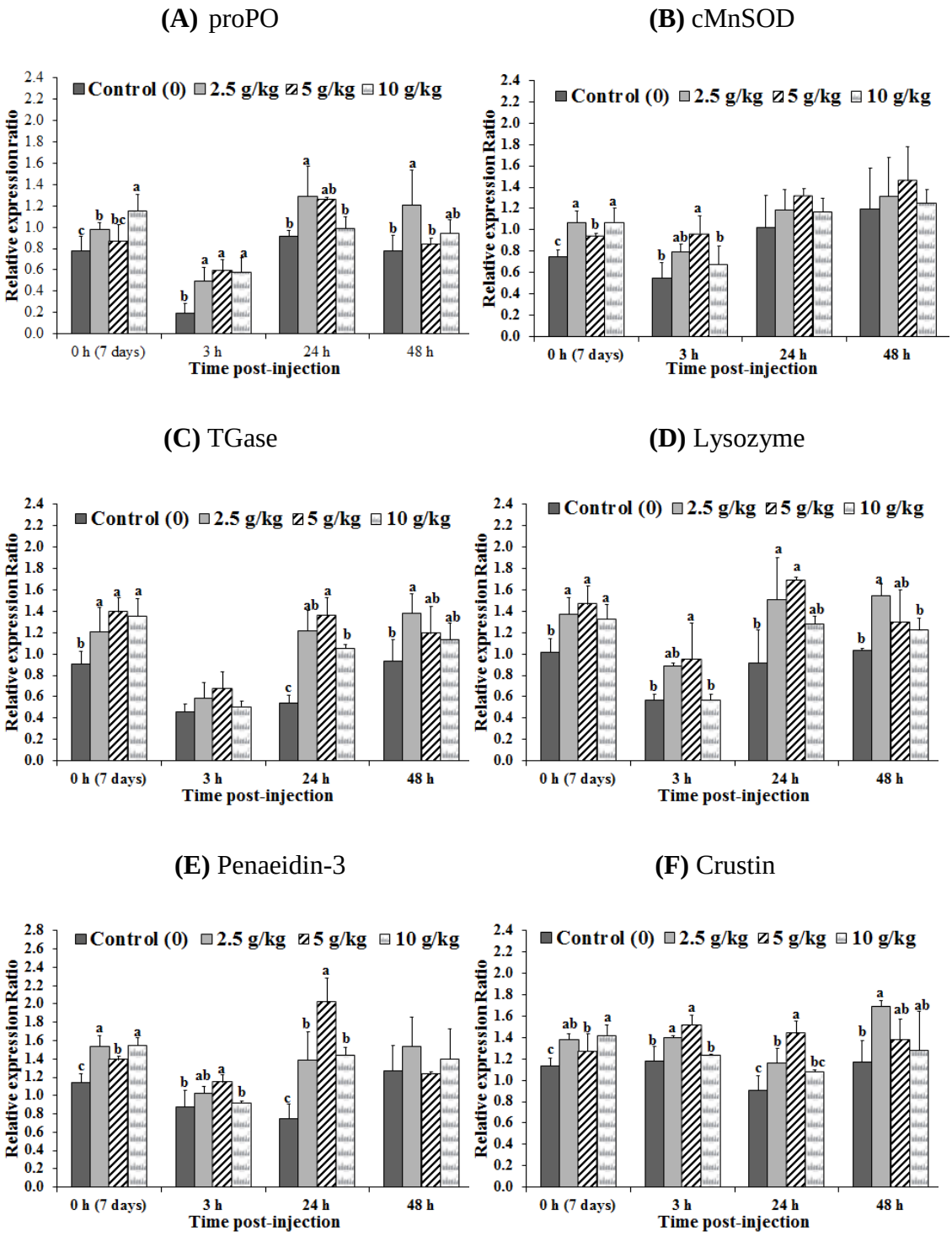

Fig. 3 Relative expression ratio of immune-related genes in hemocytes of shrimp, which had fed a 7-day galangal diet and was injected with *V. harveyi*. Pacific white shrimp were fed for 7 days a diet containing galangal extract (GE, 0, 2.5, 5 and 10 g/kg diet). Thereafter, shrimp were injected with *V. harveyi* and fed a diet without galangal extract. Before injection (0 h) and at 3, 24, and 48 h post-injection of *V. harveyi*, hemocytes were collected and analyzed for the expression of (**A**) proPO, (**B**) cMnSOD, (**C**) TGase (**D**) Lysozyme, (**E**) Penaeidin-3 and (**F**) Crustin mRNAs by RT-PCR. Values are reported as the mean ± SD, n = 3. The mean values with different letters (at each time point) indicate significant difference (P<0.05).

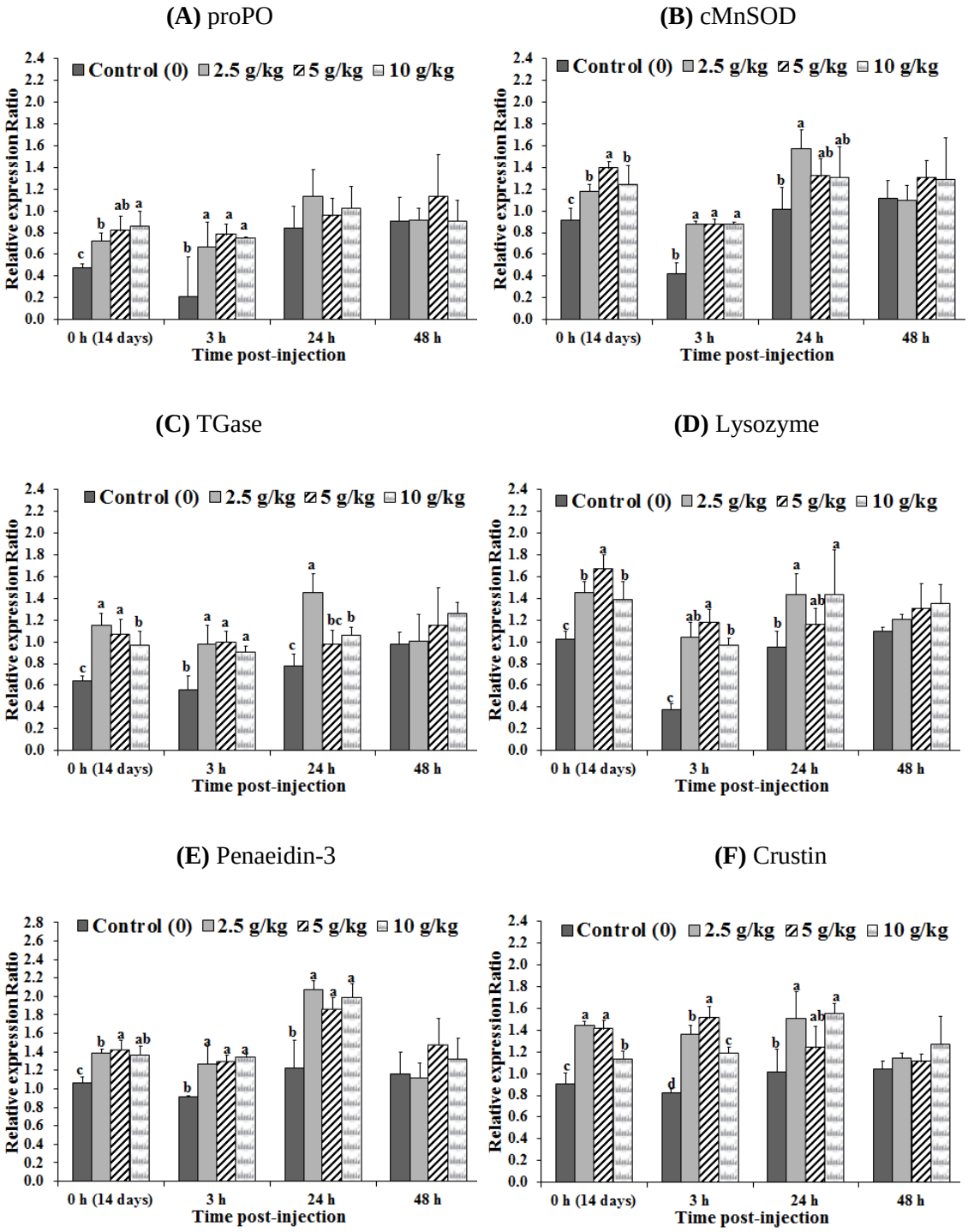

Fig. 4 Relative expression ratio of immune-related genes in hemocytes of shrimp, which had fed a 14-day galangal diet and was injected with *V. harveyi*. Pacific white shrimp were fed for 14 days with a diet containing galangal extract (GE, 0, 2.5, 5 and 10 g/kg diet). Thereafter, shrimp were injected with *V. harveyi* and fed a diet without galangal extract. Before injection (0 h) and at 3, 24, and 48 h post-injection of *V. harveyi*, hemocytes were collected and analyzed for the expression of (**A**) proPO, (**B**) cMnSOD, (**C**) TGase, (**D**) Lysozyme, (**E**) Penaeidin-3 and (**F**) Crustin mRNAs by RT-PCR. Values are reported as the mean ± SD, n = 3. The mean values with different letters (at each time point) indicate significant difference (P<0.05).

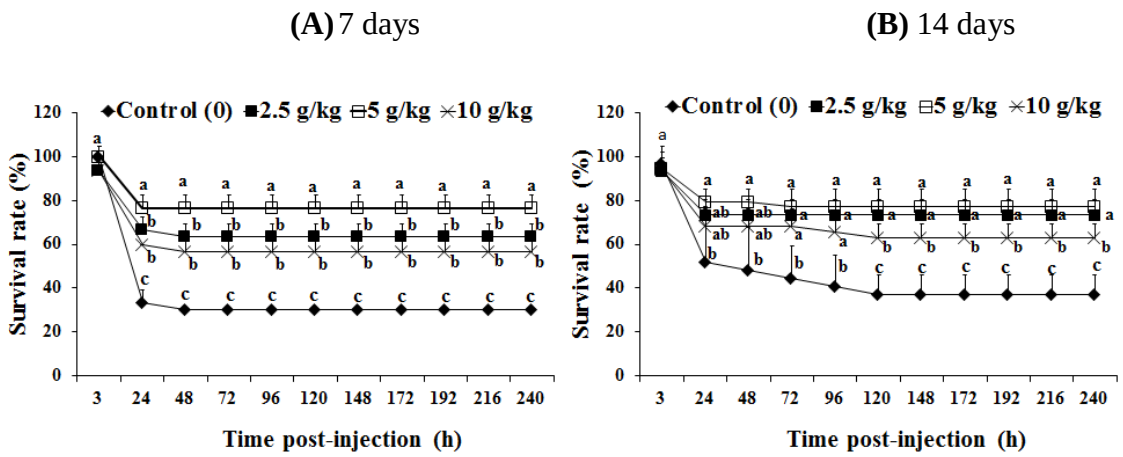

Fig. 5 Survival rate (%) of shrimp fed galangal extract-supplemented diets after *V. harveyi* infection. Pacific white shrimp were fed a diet containing galangal extract (GE, 0, 2.5, 5 and 10 g/kg diet) for **(A)** 7 days and **(B)** 14 days. Thereafter, shrimp were injected with *V. harveyi* and fed a diet without galangal extract for 10 days more. Values are reported as the mean ± SD, $n = 3$. The mean values with different letters (at each time point) indicate significant difference ($P<0.05$).

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CHAPTER 5

Conclusions

Conclusions

The antibiotic administration has prevented microbial infection in shrimp aquaculture-derived products with limited success. Recently, drug-resistant bacteria have become a global problem, urging for the efficient development of alternative control strategies to improve food quality and safety. The alternative approach of using medical herbs or their products, as bioagents for the treatment or prevention of infectious diseases, has gained interest. The studies have successfully substantiated of the galangal extract with ethanol for the prevention and control of Pacific white shrimp (*L. vannamei*) diseases. We had found that the galangal (*A. galanga*) extract showed microbiocidal activity against the eight species of *Vibrio* pathogens including *V. cholerae*, *V. vulnificus*, *V. parahaemolyticus*, *V. parahaemolyticus* (EMS/AHPND), *V. fluvialis*, *V. mimicus*, *V. harveyi*, and *V. alginolyticus*. The active antibacterial compounds of the galangal extract against *V. harveyi* were *trans*-*p*-hydroxy cinnamaldehyde, *trans*-*p*-acetoxy cinnamic alcohol, and *trans*-*p*-coumaryl diacetate. This study's results also showed the growth inhibition of the six species of fungi including *A. ochraceus*, *A. flavus*, *A. japonicas*, *Penicillium* sp. *Fusarium* sp., *C. cladosporioides*, and WSSV that caused serious virus pathogens by crude galangal extract.

The oral administration was focused on the efficacy of the commercial diet mixed with the crude galangal extract for the treatment of the pathogenic organisms that cause white feces syndrome, AHPND, *V. harveyi* and WSSV in Pacific white shrimp, *L. vannamei*. Ethanol galangal extract exhibited the highest potential for *Vibrio* spp. and fungi reduction of infected white feces syndrome shrimp. Furthermore, the survival rates for the galangal diet groups, after injections with *V. parahaemolyticus* (EMS/AHPND), *V. harveyi* or white spot syndrome virus were significantly higher than that of the control group. The number of *V. harveyi* in the hemolymph of the galangal diet group was significantly lower than that in the control diet group, indicating the higher clearance ability of the galangal diet group. The growth of the shrimp regarding body weight and specific growth rate of the galangal diet group did not differ significantly from that of the control diet group after two months feeding. However, the strong smell of galangal extract might have been caused by the reduced consumption. The galangal diet should

be improved with a smell that will be more attractive for shrimp. The both of crude galangal extract and trans-*p*-coumaryl diacetate that was used in this study considerably enhanced the relative expression ratio of six immune-related genes in the shrimp hemocytes such as proPO, cMnSOD, TGase, lysozyme, penaeidin and crustin and increased the bacterial disease resistance of Pacific white shrimp.

These results suggest that galangal extract might have the potential for application in the disease control in shrimp aquaculture. Galangal crude extract could be recommended as a substitute for antibiotics in shrimp culture with advantages that would include a reduction in drug residue problems. Obviously, we concluded that all of the results were conducted to investigate the effect of optimum dose for prevention and treatment of *Vibrio* spp, *V. harveyi*, *V. parahaemolyticus* AHPND, fungi, WSSV and stimulated the immune system response of Pacific white shrimp (*L. vannamei*). The optimum dose of the crude galangal extract could recommend to shrimp farmer is 0.25-0.50 % (w/w) or 2.5 – 5 g/kg diet, time to take the extract 7 – 10 days. This alternative method could assist in reducing the impact of antibiotic or chemical residue in shrimp products as well as helping to decrease the presence of resistant bacterial strains in the environment.

List of Publications

1. **Tidaporn Chaweepack**, Boonyee Muenthaisong, Surachart Chaweepack & Kaeko Kamei. 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*). *International Journal of Biology* (2015) 7(3): 8-17. doi: 10.5539/ijb.v7n3p8.

2. **Tidaporn Chaweepack**, Chutima Khomvilai, Surachart Chaweepack & Kaeko Kamei. 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*). *Journal of Agricultural Science*; (2015) 7(9): 117-128. doi: 10.5539/jas.v7n9p117.

3. **Tidaporn Chaweepack**, Surachart Chaweepack, Boonyee Muenthaisong, Lila Ruangpan, Kei Nagata, Kaeko Kamei. Effect of galangal (*Alpinia galanga* Linn.) extract on the expression of immune-related genes and *Vibrio harveyi* resistance in Pacific white shrimp (*Litopenaeus vannamei*). *Aquaculture International* (2015) 23(1):385–399. doi: 10.1007/s10499-014-9822-2.

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