ประสิทธิภาพของยาปฏิชีวนะและโพลีฟีนอลต่อเชื้อวิบริโอพาราฮีโมลัยติคัส
ซึ่งแยกได้จากกุ้งขาวแปซิฟิค ระหว่างการระบาดของกลุ่มอาการตายด่วนในประเทศไทย

นายตรัน ฮือ ตินห์

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ลิขสิทธิ์ของจุฬาลงกรณ์มหาวิทยาลัย

EFFICACY OF ANTIBIOTICS AND POLYPHENOLS AGAINST *VIBRIO PARAHAEMOLYTICUS*

ISOLATED FROM PACIFIC WHITE SHRIMP DURING EARLY MORTALITY SYNDROME

OUTBREAK IN THAILAND

Mr. Tran Huu Tinh

A Thesis Submitted in Partial Fulfillment of the Requirements

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Thesis Title	EFFICACY OF ANTIBIOTICS AND POLYPHENOLS AGAINST *VIBRIO PARAHAEMOLYTICUS* ISOLATED FROM PACIFIC WHITE SHRIMP DURING EARLY MORTALITY SYNDROMEOUTBREAK IN THAILAND
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ตรัน ฮือ ตินห์ : ประสิทธิภาพของยาปฏิชีวนะและโพลีฟีนอลต่อเชื้อวิบริโอพาราฮีโมลัยติคัส ซึ่งแยกได้จากกุ้งขาวแปซิฟิค ระหว่างการระบาดของกลุ่มอาการตายด่วนในประเทศไทย (EFFICACY OF ANTIBIOTICS AND POLYPHENOLS AGAINST *VIBRIO PARAHAEMOLYTICUS* ISOLATED FROM PACIFIC WHITE SHRIMP DURING EARLY MORTALITY SYNDROMEOUTBREAK IN THAILAND) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ผศ. น.สพ. ดร. ชาญณรงค์ รอดคำ, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ศ. ดร. วราภรณ์ วุฒฑะกุล, 48 หน้า.

Vibrio parahaemolyticus (VP) เป็นแบคทีเรียที่ก่อให้เกิดความเสียหายทางเศรษฐกิจ ต่ออุตสาหกรรมการผลิตกุ้งในหลายประเทศ ความพยายามที่จะใช้ยาปฏิชีวนะในการควบคุมการติด เชื้ออาจทำให้เกิดปัญหาการดื้อยาปฏิชีวนะของเชื้อแบคทีเรียขึ้นได้ การศึกษานี้มีวัตถุประสงค์เพื่อที่ จะหาความไวรับของ VP ทั้งที่ก่อโรคและไม่ก่อโรคในกุ้งต่อยาปฏิชีวนะ จำนวน 8 ชนิดและโพลีฟี- นอล (polyphenols) ซึ่งเป็นสารสกัดจากพืชที่เป็นอีกทางเลือกในการยับยั้งและทำลายเชื้อแบคทีเรีย จำนวน 4 ชนิด เชื้อ VP ในการศึกษานี้แยกได้จากกุ้งขาว (pacific white shrimp, *Litopenaeus vannamei*) ที่เพาะเลี้ยงในภาคกลางและภาคใต้ของประเทศไทย จากนั้นนำมาพิสูจน์เชื้อด้วยวิธีการ ทดสอบจากลักษณะฟีโนไทป์และอณูชีววิทยา VP ไอโซเลท (isolate) ที่ก่อโรคในกุ้ง (Acute Hepatopancreatic Necrosis Disease, AHPND-VP) ได้รับการยืนยันด้วยวิธี PCR ที่มี toxin gene เป็น gene เป้าหมายความไวรับของเชื้อต่อยาปฏิชีวนะและโพลีฟีนอล ตรวจสอบโดยวิธี broth microdilution ผลของโพลีฟีนอลต่อ VP ถูกนำไปตรวจสอบต่อด้วยวิธี time-kill curve ผลการ ทดลองแสดงให้เห็นว่า VP ที่แยกได้จำนวน 96 ไอโซเลททั้งที่ก่อโรคและไม่ก่อโรคในกุ้งดื้อต่อ ampicillin และ amoxicillin ในความเข้มข้นที่สูงและในอัตราการดื้อที่สูงมาก อย่างไรก็ตาม VP ที่ แยกได้ทั้งหมดยังคงไวต่อยาปฏิชีวนะชนิดอื่นๆที่นำมาทดสอบ โพลีฟีนอลทั้งหมดที่นำมาทดสอบ แสดงประสิทธิภาพในการต่อต้านเชื้อ VP ทั้งหมดที่แยกได้ อย่างไรก็ตามเฉพาะ pyrogallol เท่านั้น ที่แสดงประสิทธิภาพสูงสุด นอกจากนี้ยังพบว่าประสิทธิภาพในการต่อต้านเชื้อ VP ของ pyrogallol ขึ้นอยู่กับเวลาและปริมาณที่ใช้ จากผลการศึกษาทั้งหมดสรุปได้ว่ายาปฏิชีวนะทุกชนิด ยกเว้น ampicillin และ amoxicillin ยังคงมีประสิทธิภาพสูงต่อเชื้อ VP ทั้งหมดที่แยกได้ทั้งที่ก่อโรคและ ไม่ ก่อโรคในกุ้ง ส่วน pyrogallol คือโพลีฟีนอลที่มีประสิทธิภาพสูงที่สุดในการต่อต้าน VP ทั้งหมดที่ แยก ได้ทั้งที่ก่อโรคและไม่ก่อโรคในกุ้งเมื่อเปรียบเทียบกับโพลีฟีนอลอื่นๆ จากการศึกษาในครั้งนี้

ภาควิชา	พยาธิวิทยา	ลายมือชื่อนิสิต	
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TRAN HUU TINH: EFFICACY OF ANTIBIOTICS AND POLYPHENOLS AGAINST *VIBRIO PARAHAEMOLYTICUS* ISOLATED FROM PACIFIC WHITE SHRIMP DURING EARLY MORTALITY SYNDROMEOUTBREAK IN THAILAND. ADVISOR: ASST. PROF. DR. CHANNARONG RODKHUM, CO-ADVISOR: PROF. DR. VARAPORN VUDDHAKUL, 48 pp.

Vibrio parahaemolyticus (VP) is an emerging pathogen causing vast economic losses in shrimp production. Using antibiotics to control disease may have resulted antibiotics resistance. This study aimed to investigate susceptibility of VP to 8 antibiotics, and 4 polyphenols, potential alternative against bacterial species. VP were isolated from Pacific white shrimp (*Litopenaeus vannamei*) in central and southern parts of Thailand, and identified by phenotypic-based and molecular-based methods. Pathogenic isolates (Acute Hepatopancreatic Necrosis Disease, AHPND-VP) were confirmed by PCR targeting toxin gene. Susceptibility to antibiotics and polyphenols was determined by broth microdilution method. Effects of polyphenols on VP was further evaluated by time-kill curve. The results showed that all VP isolates were resistant to ampicillin, and amoxicillin at high concentrations, but susceptible to 6 other antibiotics. Polyphenols demonstrated antimicrobial effects on VP isolates. However, pyrogallol exhibited outstanding activity compared to others. Further investigation proved that pyrogallol possessed time and dose dependent bactericidal activity on VP isolates. In conclusion, all tested antibiotics except ampicillin and amoxicillin have high potential against VP isolates. Additionally, pyrogallol showed highest efficacy against VP isolates among polyphenols.

Department: Veterinary Pathology   Student's Signature ....

Field of Study: Veterinary Pathobiology   Advisor's Signature ....

Academic Year: 2014   Co-Advisor's Signature ....

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LIST OF ABBREVIATIONS

AHPND	Acute hepatopancreatic necrosis disease
CIT	Citrate test
EMS	Early mortality syndrome
MBC	Minimal bactericidal concentration
MHB	Mueller-Hinton broth
MIC	Minimal inhibitory concentration
MOT	Motility test
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
RPM	Round per minute
TBE	Tris-borate-EDTA
TCBS	Thiosulfate-citrate-bile salts-sucrose
TSA	Tryptic soy agar
TSB	Tryptic soy broth
VP	Voges–Proskauer test
WFS	White feces syndrome

CHAPTER I. INTRODUCTION

1.   Importance and rationale

Vibrio parahaemolyticus is an important marine fish and shellfish pathogen. This bacteria can cause disease in human via two routes, ingestion of contaminated seafood or direct contact with open wound which rarely lead to death in some cases (Roland, 1970; Barker and Gangarosa, 1974; Bisha et al., 2012). It naturally inhabits in sediment, water, and aquatic organisms (Kaneko and Colwell, 1973). Recently, a new highly pathogenic strain of *V. parahaemolyticus* was identified as the causative agent of acute hepatopancreatic necrosis disease (AHPND) in shrimp culture (Tran et al., 2013). This emerging disease has caused great economic losses in many countries, especially in Asia (Flegel, 2012). In addition, it can cause secondary infections in white feces syndrome (WFS), and subsequently increase mortality of culture shrimp (Flegel, 2012; Sriurairatana et al., 2014).

Traditionally, antibiotics such as oxytetracycline and norfloxacin are applied when bacterial infections in fish and shellfish occur (Shaw et al., 2014). However, using antibiotics in aquaculture is no longer recommended due to a number of reasons. The application of antibiotics would not only destabilize microbiota, it has also proven ineffective in treating fish and shellfish infected with *Vibrio* spp. such as *V. harveyi* and closely related bacteria including *V. parahaemolyticus* (De Schryver et al., 2014). Using antibiotics to control bacterial infection in aquatic animals has created selective pressure for the development of resistant strains of *Pseudomonas* sp., *Escherichia coli*, *Enterococcus* spp., *Vibrio* spp. (Le et al., 2005; Di Cesare et al., 2013). In the emerging AHPND *V. parahaemolyticus* strains, antimicrobial resistance was also detected (Kongrueng et al., 2014). In addition, horizontal transfer of resistance genes among bacterial species can make the problem even more complicated (Gao et al., 2012; Shah et al., 2014). Moreover, the presence of antibiotic residues in environment and

aquaculture products is an important threat to public health (Zong et al., 2010; He et al., 2012). Therefore, new tactics for controlling bacterial infections in aquaculture are urgently needed in order to make the industry more sustainable (De Schryver et al., 2014).

For the control of bacterial diseases in aquaculture, a number of alternatives to antibiotics have been proposed. Multidisciplinary strategies include improvement of health of host, optimization of water quality, and killing or inhibiting pathogens by phage therapies or natural products (Defoirdt et al., 2011). Polyphenols are plant-derived products that are commonly found in fruits, vegetables, and plant-derived beverages (Daglia, 2012). These products are well-known for their antioxidant properties due to the ability to scavenge free radicals (Bravo, 1998). In addition, many polyphenols have been proved to have bactericidal effects to both Gram-negative, and Gram-positive bacterial species, including a few human pathogenic *V. parahaemolyticus* strains (Nagayama et al., 2002; Taguri et al., 2004). Because of their wide bacterial spectrum polyphenols can be potential alternatives to antibiotics in controlling *V. parahaemolyticus* in Pacific white shrimp. Recently, it has been demonstrated that the susceptibility to antibiotics of AHPND *V. parahaemolyticus* strains was slightly different from that of non-AHPND strains (Kongrueng et al., 2014). Therefore, scientific evidence showing the efficacy of polyphenols against both AHPND and non-AHPND *V. parahaemolyticus* is needed to evaluate the potential of polyphenols in controlling vibriosis in shrimp farms.

2.   Research questions

- What are antibiotic resistance patterns of *V. parahaemolyticus* isolates from central and southern provinces of Thailand?

- Do polyphenols have high efficacy against both AHPND and non-AHPND *V. parahaemolyticus*?

- Are there any differences in resistance patterns of AHPND and non-AHPND *V. parahaemolyticus* isolates?

3.   Hypothesis

We hypothesized that *V. parahaemolyticus* recovered from Pacific white shrimps in eastern and southern parts of Thailand resisted many commonly used antibiotics at high levels. Besides, resistance pattern of AHPND and non-AHPND isolates are different from each other. Some polyphenols have high bactericidal activities against both AHPND and non-AHPND *V. parahaemolyticus* isolates *in vitro*.

4.   Objectives of study

This study aimed to evaluate antimicrobial susceptibility of *V. parahaemolyticus* isolated from Pacific white shrimps in eastern and southern parts of Thailand, and investigate *in vitro* anti-bacterial activities of four polyphenol products against both AHPND and non-AHPND *V. parahaemolyticus*.

5.　　Conceptual framework

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┌─────────────────────────────────────────────────────────────────────┐
│                                                                       │
│              Live Pacific white shrimps from eastern and southern     │
│                           parts of Thailand                           │
│                                    │                                  │
│                                    ▼                                  │
│              V. parahaemolyticus isolation                            │
│              -   from stomach and hepatopancreas                      │
│              -   inoculated on TCBS agar                              │
│                                    │                                  │
│                                    ▼                                  │
│                        Bacterial identification                       │
│              ┌─────────────────┴──────────────────┐                  │
│              ▼                                     ▼                  │
│     Biochemical tests                      Molecular method          │
│     (Alsina and Blanch, 1994)              (Kim et al., 1999)         │
│     -  Gram staining, catalase, oxidase    -  PCR targeting toxR gene │
│     -  Decarboxylase, VP, salinity, etc.   -  Positive band: 368bp    │
│              └─────────────────┬──────────────────┘                  │
│                                    ▼                                  │
│                    V. parahaemolyticus isolates                       │
│                                    │                                  │
│                                    ▼                                  │
│              Identification of AHPND and non-AHPND                     │
│              V. parahaemolyticus (Sirikharin R. et al., 2014)         │
│              - PCR targeting toxin gene   - Positive band: 336bp      │
│              ┌─────────────────┴──────────────────┐                  │
│              ▼                                     ▼                  │
│   Susceptibility to antibiotics         Susceptibility to polyphenols │
│   -  Micro-dilution method (MIC, MBC)   -  Micro-dilution method      │
│      determination (CLSI, 2006)            (MIC, MBC) determination   │
│   -  Isolates: 56 AHPND and 40            (CLSI, 2006)               │
│      non-AHPND V. parahaemolyticus      -  Isolates: 56 AHPND and 40  │
│   -  Antibiotics: ampicillin,              non-AHPND                  │
│      amoxicillin, enrofloxacin,            V. parahaemolyticus        │
│      oxolinic acid, florfenicol,        -  Polyphenols: pyrogallol,   │
│      oxytetracycline, trimethoprim/        rutin, syringic acid,      │
│      sulfamethoxazole (1:19)               vanillic acid             │
│              │                                     │                  │
│              ▼                                     ▼                  │
│   Antimicrobial susceptibility profile  Time-kill curve assay         │
│                                         (Punam, 2007)                 │
│                                         -  Polyphenol: 1 most         │
│                                            effective agent            │
│                                         -  Isolates: 1 AHPND and      │
│                                            1 non-AHPND                │
│                                                    │                  │
│                                                    ▼                  │
│                                         Scanning electron microscope  │
│   In vitro efficacy of polyphenols ◄─── (Kawai and Yamagishi, 2009)   │
│                                         -  Polyphenol: 1 most         │
│                                            effective agent            │
│                                         -  Isolates: 1 AHPND          │
│                                         -  Conditions: 4x MIC, 6 hours│
│                                                                       │
└─────────────────────────────────────────────────────────────────────┘
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CHAPTER II. LITERRATURE REVIEW

1.   *Vibrio parahaemolyticus*

Vibrio parahaemolyticus is a halophilic, Gram-negative bacterial species. It is motile, facultatively anaerobic, able to grow at 43°C, and failed to grow in 10% NaCl (Barker and Gangarosa, 1974). It was first isolated in 1953 in Japan from patient with food poisoning (Zen-Yoji et al., 1965). After its initial identification, this bacterial pathogen later on was also reported to cause food-related infection in other many countries such as USA, England, Thailand, and Philippines (Molenda et al., 1972; Peffers et al., 1973; Sanyal et al., 1973). Infected patients exhibited diarrhea, abdominal pain, nausea, vomiting, headache, fever, chill, acute gastroenteritis (Zen-Yoji et al., 1965; Barker and Gangarosa, 1974). Besides, *V. parahaemolyticus* can infect people with open wound in contact with contaminated water (Roland, 1970). Human pathogenic strains exhibit hemolytic activity called Kanagawa phenomenon, while environmental strains are non-hemolytic (Miyamoto et al., 1969; Barker and Gangarosa, 1974). There are two distinct hemolysins in *V. parahaemolyticus*, thermostable direct hemolysin (TDH) and TDH-related hemolysin (TRH) (Sakurai et al., 1974).

V. parahaemolyticus is sensitive to cold, low salinity condition (Kampelmacher et al., 1970). Therefore, incidence of this pathogen is correlated with water temperature. It can be found in sediment, where water temperature is stable, more frequently than in water (Baross and Liston, 1970). Besides, the occurrence of *V. parahaemolyticus* is higher in summer than in winter (Kaneko and Colwell, 1973) . The temperature range of 14-19°C was found to be critical in the annual cycles of *Vibrio* (Kaneko and Colwell, 1973). Samples collected from lagoons contained more *V. parahaemolyticus* than those from ocean (Bockemuhl and Triemer, 1974). Among three major human pathogenic *Vibrio* species, occurrence of *V. parahaemolyticus* in sea food product was highest (31.1% of samples) followed by *V. vulnificus* (12.6%) and

V. cholerae (0.6%) (Robert-Pillot et al., 2014). *V. parahaemolyticus* was detected in effluents of wastewater (Khouadja et al., 2014).

2. Acute hepatopancreatic necrosis disease

Acute hepatopancreatic necrosis disease (AHPND), also referred to as early mortality syndrome (EMS) is the most recent, serious bacterial infection to cause great economic losses in shrimp cultivation of Asian countries (Sriurairatana et al., 2014). This disease first occurred in China in 2009 and then was reported in many other countries including Vietnam (2010), Malaysia (2011), Thailand (2012), and Mexico (2013) (Nunan et al., 2014; Sriurairatana et al., 2014). Economic loss for Vietnam in 2010 alone would exceed USD 75 million (Flegel, 2012). Within 5 months of 2013, estimated reduction of shrimp export in Thailand was approximately 34% (Kongrueng et al., 2013). .Estimated losses to the Asian shrimp culture sector amount to USD 1 billion (De Schryver et al., 2014). Not until 2013, the causative agent of AHPND was identified to be *V. parahaemolyticus* (Tran et al., 2013).

AHPND could affect both Pacific white shrimp (*Penaeus vannamei*) and black tiger shrimp (*Penaeus monodon*) within the first 35 days of stocking (Tran et al., 2013; Joshi et al., 2014). It is uniquely characterized with massive, medial sloughing of shrimp hepatopancreatic cells caused by a presently unidentified toxin(s) of the pathogenic bacteria in digestive system of shrimp (Sriurairatana et al., 2014). AHPND can lead to secondary bacterial infections, or be accompanied by white feces syndrome (WFS), and increase the mortality (Flegel, 2012; Sriurairatana et al., 2014). When disease occurs, the mortality of culture shrimp is up to 100% in most cases (De Schryver et al., 2014).

A number of studies on genetics of AHPND-causing *V. parahaemolyticus* have been conducted to better understand mechanisms rendering high pathogenicity of this pathogen. Four plasmids were detected in pandemic strain, including one large extra-chromosomal plasmid that encodes a homolog to the insecticidal *Photorhabdus*

insect-related binary toxin PirAB (Gomez-Gil et al., 2014; Yang et al., 2014). Many of the genes in AHPND-causing strain are phage-related, and/or have not been previously reported, while these genes were not detected in non-pandemic strain (Gomez-Jimenez et al., 2014; Kondo et al., 2014). These fragments encode type IV pilus/type IV secretion system, homologues of cholera toxin and conjugal transfer proteins, which suggests that it is located on a plasmid (Kondo et al., 2014; Yang et al., 2014). Besides, these strains also possessed several pathogenicity mechanisms including five iron acquisition and seven secretion systems (Gomez-Gil et al., 2014).

3.   Antibiotics use in aquaculture

Antibiotic used in shrimp farming is a major problem because it causes negative impact on human health through contact dermatitis or development of resistant human pathogens (Gräslund and Bengtsson, 2001; Holmström et al., 2003). Antibiotics such as erythromycin (macrolide class), which inhibits protein synthesis, is effective against Gram-positive and some Gram-negative bacteria, and is often used in shrimp hatcheries in south-east Asia (Gräslund and Bengtsson, 2001). Interestingly, antibiotics such as rifampicin, chloramphenicol, furazolidone and nifurpirinol, some of which were prohibited for usage in food animals in European Union due to their potential carcinogenicity, but were extensively used in the Philippines (Primavera et al., 1993). Among antibiotics, oxytetracycline (tetracyclines group) is probably the most used antibiotic in aquaculture (Gräslund and Bengtsson, 2001). A wide-range survey on the application of chemicals in aquaculture in Asian countries including Bangladesh, China, Thailand, and Vietnam reported the use of at least 20 antibiotics (Rico et al., 2013). For treating vibriosis, a number of different groups of antibiotics, including tetracyclines, fluoroquinolones, cephalosporins, and aminoglycosides can be effective (Shaw et al., 2014). Antibiotics were also used not for treatment of bacterial infection, but as prophylactic agents, which can be a source of antibiotic resistance in human bacterial pathogens (Cabello, 2006). However, the current information on the use of chemicals and biological products applied by Asian farmers is very limited (Rico et al., 2013).

In Thailand, oxolinic acid, norfloxacin, and sulfadiazine (sulfonamides group) potentiated with trimethoprim were antibiotics often used in shrimp farming (Gräslund and Bengtsson, 2001). About 74% interviewed farmers in a study used antibiotics in tetracyclines (tetracycline and oxytetracycline), quinolones (oxolinic acid, norfloxacin, enrofloxacin, ciprofloxacin) and sulphonamides (sulphamethazine) groups in shrimp pond management (Holmström et al., 2003). Chloramphenicol and gentamycin were sometimes applied (Holmström et al., 2003). A study detecting contamination of antibiotics in aquatic environment in Thailand showed that contamination of norfloxacin was highest among fluoroquinolones group (Takasu et al., 2011). A survey conducted recently demonstrated a decline in the use of antibiotics in shrimp culture in Thailand. In this study, about 2.9% Thai shrimp farmers informed that they applied amoxicillin and norfloxacin antibiotics for treatment of disease (Rico et al., 2013).

Introduction of antibiotics to aquatic environment creates selective pressure which could promote the development of resistant bacteria. Resistance to quinolones and tetracyclines groups by *Aeromonas* spp. and *Vibrio* spp. isolated from shrimp culture in Asian countries were demonstrated (Defoirdt et al., 2011). Recently, *E. coli* of aquatic origin showed resistance to ampicillin, tetracycline, and sulphamethoxazole/trimethoprim (Rocha Rdos et al., 2014). With regards to *V. parahaemolyticus*, phenotypic resistance against ampicillin and polymycin B was detected before the heavy use of antibiotics (Chatterjee et al., 1970; Roland, 1970). In a recent research on *V. parahaemolyticus* isolates from oysters, low susceptibility was detected only to ampicillin (81%; MIC > 16 μg/ml) (Han et al., 2007). Later on, high percentage of resistance of *V. parahaemolyticus* to ampicillin (90%), and amikacin (60%) was reported. Besides, resistance to both antibiotics was 50%, and there was increased intermediate resistance to ciprofloxacin (de Melo et al., 2011). Environmental bacterial samples demonstrated susceptibility to antibiotics recommended for treating *Vibrio* infections, but showed intermediate resistance to chloramphenicol (96% of *V. parahaemolyticus*) and penicillin (68%) (Shaw et al., 2014). Resistance to ampicillin,

tetracycline, doxycycline, and nalidixic acid was recently encountered in *V. parahaemolyticus* isolates from shrimps in Malaysia (Banerjee et al., 2012; Hua and Apun, 2013).

4.   Polyphenols

Polyphenols make up one of the most numerous and widely distributed groups of substances, with more than 8000 phenolic structures currently known in the plant kingdom (Bravo, 1998). These compounds are secondary metabolites, and are produced in response to stress (Citarasu, 2012). They act as antioxidants by scavenging reactive oxygen species (ROS), which produce oxidative stress and can adversely affect many cellular processes (Itoh et al., 2009). In addition, polyphenols serve as a defense against attack by microorganisms (Citarasu, 2012). Their protection against bacteria is a result initially of antioxidant, the ability to scavenge free radicals and chelate metals, and different enzymes inhibition, interaction with signal transduction pathways and cell receptors (Daglia, 2012).

Antimicrobial effect of polyphenols has long been reported. These plant extracts demonstrated effects against *Shigella*, *Streptococcus mutans*, *V. cholerae* O1, and *Helicobacter pylori* (Batista et al., 1994; Vijaya et al., 1995; Borris, 1996; Yoshida et al., 2000). A research on 10 polyphenols against 4 different bacterial genera including *Staphylococcus*, *Escherichia*, *Salmonella*, and *Vibrio* showed that there was no clear correlation between Gram-staining and bacterial susceptibility to polyphenols (Taguri et al., 2006). However, another research demonstrated greater antimicrobial effect of these plant extracts to Gram-positive bacteria (*Bacillus cereus, Listeria monocytogenes, Staphylococcus aureus*) than to Gram-negative bacteria (*Escherichia coli, and Salmonella anatum)* (Shan et al., 2007). Investigation of antimicrobial effect of 18 plant species against *V. parahaemolyticus* showed promising results (Yano et al., 2006). Recent researches with aquatic bacteria also demonstrated antimicrobial effect of polyphenols. Polyphenols showed antimicrobial action against piscine *Aeromonas*

salmonicida, *Aeromonas hydrophila* and *Edwardsiella tarda*, and when two polyphenols were mixed together, the mixture demonstrated synergistic effect *in vitro* (Prasad et al., 2014). Polyphenols extracted from Thai medicinal plant have been proven to have bactericidal effects against *Streptococcal* bacteria isolated from tilapia (Pirarat et al., 2013).

Mechanisms of antibacterial activities of polyphenols are not clear; however, it is hypothesized that polyphenols physically kill bacteria by absorbing to bacterial cell wall, or generating hydrogen peroxide (Taguri et al., 2006). Polyphenols possessing the ability to form soluble polyphenol-protein complexes, may adhere to bacterial cell wall and disturb external receptor (Perumal Samy and Gopalakrishnakone, 2010). Polyphenols may lyse cell wall, block protein synthesis and DNA synthesis, and inhibit enzyme secretions (Campos et al., 2009; Citarasu, 2012). Their antibacterial activities are also attributed to the ability of microbial virulence factor suppression and synergistic effects with antibiotics (Daglia, 2012). Some polyphenols showed quorum-sensing inhibitory activity which renders the inability of expression of bacterial virulence factors (Defoirdt et al., 2013; De Schryver et al., 2014).

4.1. Pyrogallol

Pyrogallol is an organic compound with the formula $C_6H_3(OH)_3$. It can be found in citrus plant, mango (Karimi et al., 2012; Cheema and Sommerhalter, 2015). Crude extract of bitter orange bloom containing pyrogallol demonstrated anti-inflammatory and anti-cancer activities in *in vitro* experiments with cell line (Karimi et al., 2012). *In vivo* studies showed that this substance protected brine
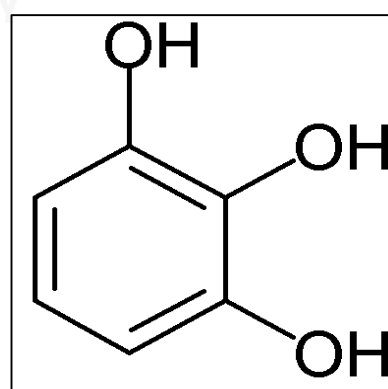

Figure 1. Chemical structure of pyrogallol

shrimp and river prawn against pathogenic *V. harveyi* due to its apparent quorum sensing inhibitory ability (Defoirdt et al., 2013). Among many different polyphenols

extracted from mango, pyrogallol showed highest polyphenol oxidase activities which involves in wound healing, pathogen defense (Cheema and Sommerhalter, 2015).

4.2. Rutin

Rutin is the most abundant phenolic compounds extracted from apple (Fratianni et al., 2011). Apple extract containing rutin showed *in vitro* antimicrobial activity against both Gram-positive and Gram-negative bacteria. Rutin can also be found in citrus plant, mango (Karimi et al., 2012; Cheema and Sommerhalter, 2015). Its anti-inflammatory and anti-cancer activities were
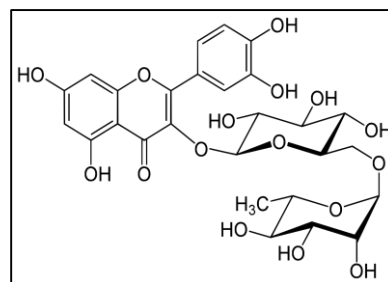
Figure 2. Chemical structure of rutin

demonstrated in cell line experiments (Karimi et al., 2012). Olive leaves extract containing rutin reduced microbial load in peeled un-deveined shrimp (Ahmed et al., 2014). In experimental challenge with *Aeromonas hydrophila*, tilapia previously injected with extract containing rutin showed significantly higher survival rate than control group injected with phosphate buffered saline (Wu et al., 2010). *In vivo* experiments with Pacific white shrimp, crude extract containing rutin, and rutin alone significantly increased the immune ability and resistance of the host against *V. alginolyticus* (Hsieh et al., 2008; Hsieh et al., 2013).

4.3. Syringic acid

Syringic acid is a phenolic acid, having basic structure of C_6C_1 (Bravo, 1998). It can be extracted from palm, avocado, grape, and mushroom (Gálvez et al., 1994; Pacheco-Palencia et al., 2008; Itoh et al., 2009; Oboh et al., 2014). Syringic acid extracted from mushroom showed hepatoprotective effect, decreased cytokine levels, immune-mediated liver inflammation by free radical-scavenging activities in mice challenge model (Itoh et al., 2009). This
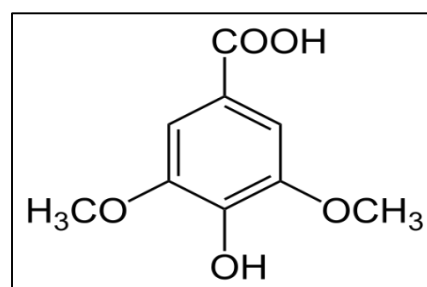
Figure 3. Chemical structure of syringic acid

phenolic acid is among many other acids in extracts of apple, which possesses *in vitro* antimicrobial activity against *Bacillus cereus*, and *Escherichia coli,* but not *Staphylococcus aureus* (Fratianni et al., 2011). This activity is explained by its ability to inhibit quorum sensing (Fratianni et al., 2011; Kalinowska et al., 2014). Syringic acid can be found in myrtle, a medicinal plant endemic to the Mediterranean area (Aleksic and Knezevic, 2014). Extracts of this plant exerted antibacterial effect on some pathogenic bacteria, particularly *Staphylococcus aureus* and *Vibrio cholerae.*

4.4.   Vanillic acid

Vanillic acid is a phenolic acid that possesses similar chemical structure, and can be found in the same source of plants as syringic acid (Gálvez et al., 1994; Pacheco-Palencia et al., 2008; Itoh et al., 2009; Oboh et al., 2014). However, its chemical structure has less hydroxyl groups which can cause the difference in antimicrobial spectrum (Taguri et al., 2006). *In vivo* experiments also proved that this

Figure 4. Chemical structure of vanillic acid

phenolic acid has hepatoprotective effect (Itoh et al., 2009). Extracts of mushroom containing vanillic acid demonstrated bactericidal effect against pathogenic bacteria such as *Micrococcus luteus, Pseudomonas aeruginosa,* especially Gram-positive species (Nowacka et al., 2014).
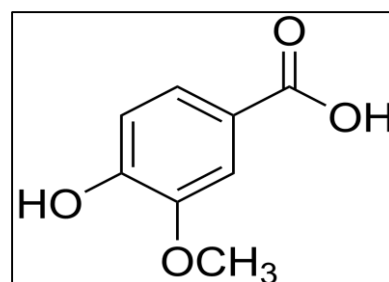
CHAPTER III. MATERIALS AND METHODS

1.   Bacterial isolation and identification

1.1.   Bacterial isolation

A number of 5-10 live shrimp samples were collected from each pond of different shrimp farms in the eastern and southern parts of Thailand where AHPND had been reported. Shrimp samples were kept in aerated plastic bags filled with pond water, and transported to the laboratory of Department of Veterinary Microbiology, Faculty of Veterinary Science, Chulalongkorn University where they were dissected to separate intestine and hepatopancreas. Shrimp samples were immersed in ice water for stunning, and externally sterilized with 70% ethanol. Bacterial samples were taken from intestine and hepatopancreas using sterile loop, and streaked on thiosulfate-citrate-bile salts-sucrose (TBCS) agar (Difco^{TM}, USA), a selective medium routinely used for isolation of *Vibrio* species. After the incubation period of 24 hours at room temperature (30°C), non-sucrose fermenting colonies presumptively considered as *V. parahaemolyticus* were selected. Three colonies from each plate were subculture on tryptic soy agar (TSA) (Difco^{TM}, USA) supplemented with 1% sodium chloride (NaCl) for further identification.

1.2.   Biochemical identification

The bacterial isolates were subjected to Gram staining, oxidase, catalase, and motility tests. Confirmation using biochemical tests including arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, citrate, D-glucosamine utilization, Voges-Prokauer, and growth in 8% NaCl (Alsina and Blanch, 1994) were performed. This scheme had been proven to be more reliable than commercially available test kits API 20E and API 20NE (Croci et al., 2007). A summary of biochemical characteristics of *V. parahaemolyticus* is shown in Table 1.

1.3.   DNA extraction

Genomic DNA was extracted by boiling as previously described with some modifications (Croci et al., 2007). Isolates were grown in tubes containing tryptic soy broth (TSB) (Difco^{TM}, USA) supplemented with 1% NaCl for 24 hours at 30°C. One milliliter of bacterial culture were centrifuged for 3 minutes. The pellet were suspended in 200µl pure water, and boiled at 100°C for 10 minutes. Another centrifugation was performed at 9,000 RPM for 5 minutes to obtain the supernatant. The supernatant was diluted with distilled water at the ratio of 1:10, and stored at -20°C until use.

Table 1. Biochemical characteristics of *V. parahaemolyticus*

Gram	Oxidase	Catalase	Motile	ADH	LDC	ODC	Citrate	D-glucosamine	VP	8% NaCl
-	+	+	+	-	+	+	+	+	-	+

1.4.   Molecular identification

Bacteria isolates were confirmed as *V. parahaemolyticus* by polymerase chain reaction (PCR) targeting the species-specific *toxR* gene using the following nucleotide sequences   5'-GTCTTCTGACGCAATCGTTG-3'   and   5'-ATACGAGTGGTTGCTGTCATG-3' (Kim et al., 1999). PCR was performed in 25µl mixture containing 2µl of previously extracted DNA templates, 2µl of each primer, 6.5µl of pure water, and 12.5µl of MasterMix (Promega, USA). The temperature condition included 5 minutes of denaturation at 94°C, followed by 20 cycles of 1 min at 94°C, 1.5 min at 63°C, and 1.5 min at 72°C, and 5 min of final extension at 72°C. *V. parahaemolyticus* DMST21243 was used as positive control of the experiment. The reactions were performed in thermal cycler (Life Express, China). PCR products were visualized in gel electrophoresis which is described later on in this document. Products showing DNA band of approximately 368 bp were positive for *V. parahaemolyticus*.

1.5.   Identification of AHPND *V. parahaemolyticus*

In order to identify AHPND *V. parahaemolyticus* (*V. parahaemolyticus* believed to cause AHPDN), PCR procedure using AP3 primer pair was performed (Sirikharin et al., 2014). The primer pair, 5'-ATGAGTAACAATATAAAACATGAAAC-3' and 5'-GTGGTAATAGATTGTACAGAA-3' targets to the toxin-encoding nucleotide sequence of AHPND *V. parahaemolyticus*. The 25µl reaction mixture contained 12.5µl of MasterMix, 6.5µl of DNase-free water, 2µl of DNA template, and 2µl of each primer. The thermal protocol included 5 min of denaturation at $94^{o}C$, thirty cycles of $94^{o}C$ for 30 sec, $53^{o}c$ for 30 sec, and $72^{o}C$ for 40 sec, and 5 min of final extension at $72^{o}C$. DNA template of AHPND *V. parahaemolyticus* isolate obtained from Center of Excellence for Shrimp Molecular Biology and Biotechnology (Centex Shrimp, Bangkok, Thailand) was used as positive control.

1.6.   Gel electrophoresis

Agarose gel mixed with redsafe (iNtRON Biotechnology, Korea) was prepared at concentration of 1%, in 0.5X tris-borate-EDTA (TBE) buffer. Two microliters of PCR products were loaded in gel electrophoresis for 30 min. One microliter of DNA ladder (Promega, USA) was run parallel as molecular weight marker. The gel was visualized under UV transilluminator (Syngene, USA).

1.7.   Bacterial stock

V. parahaemolyticus were grown overnight in TSB supplemented with 3% NaCl at room temperature. The culture was mixed with glycerol to final concentration of 20% of the total volume, and kept at $-80^{o}C$ as bacterial stock culture. Sixty-six isolates of *V. parahaemolyticus* (including 47 AHPND, and 19 non-AHPND strains) were obtained from culture collection of the Department of Microbiology, Faculty of Science, Prince of Songkla University, Thailand. These isolates had been isolated from shrimp farms in southern part of Thailand during outbreak of AHPND (or early mortality syndrome), and already identified by molecular methods (Kongrueng et al., 2014).

2. **Susceptibility of *V. parahaemolyticus* to antibiotics and polyphenols**

Susceptibility to 8 antibiotics and 4 polyphenols (Table 2) of 56 AHPND and 40 non-AHPND *V. parahaemolyticus* isolates were evaluated using minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) assays (CLSI, 2006b). All antibiotics, except for florfenicol, were allowed for use in aquaculture by Department of Fisheries in Thailand.

2.1   Bacterial suspension

V. parahaemolyticus isolates were culture overnight at 30°C on TSA supplemented with 1% NaCl. Suspension of pure colonies in normal saline (0.85% NaCl) was adjusted to 0.5 McFarland standard corresponding to 10^{8}CFU/ml. The standardized suspension was then 1:100 diluted with 2x Mueller Hinton Broth (MHB) (Difco^{TM}, USA) supplemented with 2% NaCl to obtain the working concentration of 10^{6}CFU/ml, since *V. parahaemolyticus* is an obligate halophilic strain.

2.2   Drug preparation

Eight antibiotics and four polyphenols were purchased from Sigma-Aldrich Company (St. Louis, MO, USA). Stock solution of antibiotics and polyphenols were prepared with the appropriate solvents and diluents (Table 2). Two-fold dilution was performed to obtain the highest concentration of 1024 μg/ml and the lowest concentration of 0.125 μg/ml. Stock solutions were stored in -20°C until used.

2.3   Minimal inhibitory concentration (MIC) assay

A volume of 100μl of bacterial suspension was loaded to each well of the 96-well plate. The same volume of antimicrobial agents of each concentration were orderly loaded to the well after that. The negative growth control well contained only 200μl of NaCl-supplemented MHB, while the positive growth control well was filled with 100μl of bacterial suspension and 100μl of corresponding diluent. *Escherichia coli* ATCC 25922 strain was used as quality control. After 1-day incubation at 28 ± 2°C, the

lowest concentration showing no visible bacterial growth was interpreted as minimal inhibitory concentration (MIC). *Escherichia coli* ATCC 25922 strain was tested in parallel for quality control. Each experiment was performed in duplicate.

Table 2. List of antibiotics and polyphenols used in this study

Types	Names	Solvents	Diluents
Antibiotics	Ampicillin	Phosphate buffer, pH 8.0, 0.1 mol/l	Phosphate buffer, pH 6.0, 0.1 mol/l
	Amoxicillin	Phosphate buffer, pH 6.0, 0.1 mol/l	
	Oxolinic acid	½ volume of water, then 1 mol/l NaOH drop-wise to dissolve	Water
	Enrofloxacin		
	Oxytetracycline	100% methanol	
	Florfenicol	95% ethanol	
	Trimethoprim/ Sulfamethoxazole (1:19)	- 0.05 mol/l hydrochloric acid, 10% final volume - ½ volume of water, minimal amount of 2.5 mol/l NaOH to dissolve	
Polyphenols	Pyrogallol (98%)	Water	
	Syringic acid (95%)	Water	
	Vanillic acid (97%)	Water	
	Rutin (94%)	DMSO 10%	DMSO 10%

2.4   Minimal bactericidal concentration (MBC) assay

To determine MBC, a loopful from each MIC assay well, showing no visible bacterial growth, was streaked on NaCl-supplemented TSA, and incubated for another 24 hours at $30^{\circ}C$. The lowest concentration of antimicrobial agents that did not allow any bacterial growth was interpreted as MBC. Each experiment was done in duplicate.

3.   Time-kill curve of polyphenol

The bactericidal activity of the most potential polyphenol was investigated, following a previously described (Punam, 2007). Briefly, one AHPND and one non-AHPND *V. parahaemolyticus* isolates that were inhibited at MIC_{90} of the polyphenol were selected for this experiment. The bacterial suspension of these two isolates were prepared as described above to obtain the concentration of 10^6CFU/ml.

Stock solution of the polyphenol was diluted with appropriate diluent to 4x, 2x, and 1x of MIC_{90}. Equal volumes (5 ml) of bacterial suspension and polyphenol at each dilution were mixed together in sterile experimental tube. The control tube was prepared by mixing equal volumes of bacterial suspension and corresponding diluent of the polyphenol. At 0h after mixing, 100µl of mixture from each tube was serially 10-fold diluted with normal saline. From each of these dilutions, 100µl was spread on TSA supplemented with 1% NaCl, and incubated overnight to determine the number of viable cells. The remaining mixtures were incubated at $28 \pm 2^{o}C$ with shaking at 180rpm. Colonies enumeration was continued at 2, 4, 6, 8, 12, 16, and 24h after that. These experiments were done in duplicate.

4.   Effect of pyrogallol on bacterial cell

The effect of pyrogallol on bacterial cell wall of *V. parahaemolyticus* was investigated by scanning electron microscope, following previous report (Kawai and Yamagishi, 2009). One AHPND *V. parahaemolyticus* isolate was prepared as in time-kill experiment, and then exposed to pyrogallol at 4x MIC (512 µg/ml) for 6 hours, the duration at which a 3 log_{10} reduction of viable cells was observed. In control culture, the isolate was exposed to normal saline, instead of pyrogallol in the same duration.

After 6 hours, cells were harvested by centrifugation at 13,000 rpm for 20 minutes. Cell pellets were washed with PBS three times to eliminate residue of pyrogallol. Pyrogallol-treated and control cell pellets were kept separately in PBS and sent for photographing with scanning electron microscope.

5.   Data analysis

MIC, and MBC were analyzed by descriptive analysis using basic functions in Microsoft Office Excel. Time-kill curve data were analyzed by plotting log_{10} CFU/mL versus time. A reduction of 3 log_{10} CFU/ml of the original inoculum was considered as bactericidal. MIC_{50} and MIC_{90} of antimicrobial agents were determined by using WHONET software (http://www.who.int/drugresistance/whonetsoftware/).

CHAPTER IV. RESULTS

1. Bacterial isolation and identification

From October-2013 to August-2014, shrimp samples were collected totally 5 times in 3 provinces of Thailand, including Chanthaburi (2), Nakhonpathom (1), and Ratchaburi (2) where mass mortality of culture white leg shrimps was reported. From primary bacterial cultures on TCBS, a number of 49 isolates were suspected to be *V. parahaemolyticus*, and were subculture (Figure 5) for further identification. The results of biochemical tests (Figure 6) and PCR with species-specific *toxR* primers (Figure 7) confirmed that 30 isolates were *V. parahaemolyticus*.

Figure 6. Colony morphology of
V. parahaemolyticus on 1% NaCl TSA
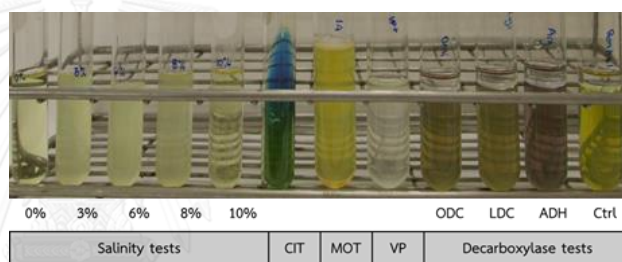
Figure 5. Biochemical identification of *V. parahaemolyticus*

Second PCR procedure confirmed that only 9 out of 30 *V. parahaemolyticus* isolates recovered from central provinces contained toxin gene (positive with AP3 primers) (Figure 8). These isolates were called AHPND isolates, while AP3-negative isolates were designated as non-AHPND *V. parahaemolyticus*. The number of AHPND *V. parahaemolyticus* isolated from Chanthaburi, Nakhonpathom, and Ratchaburi was

Figure 8. Gel electrophoresis of products of toxR primers
Lane M: DNA marker,   Lanes 1-7: representative samples
Lane (--): Negative-control, Lane (+): Positive-control

Figure 7. Gel electrophoresis of products of AP3 primers
Lane M: DNA marker, Lanes 1-7: representative samples
Lane (--): Negative-control, Lane (+): Positive-control

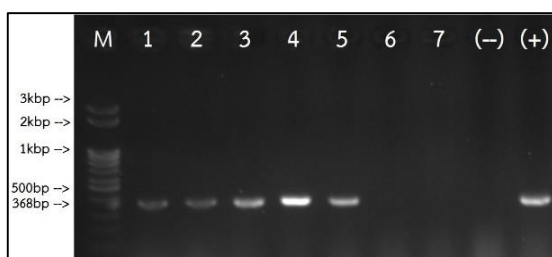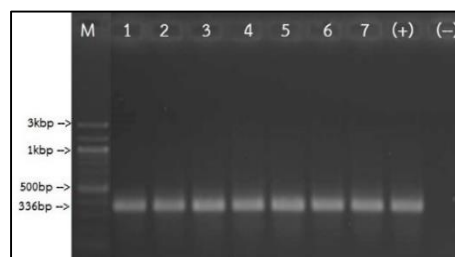2, 3, and 4 isolates, respectively. Noticeably, AHPND isolates were found in all provinces where shrimp samples were collected.

The other 66 isolates of *V. parahaemolyticus*, obtained from culture collection of Faculty of Science, Prince of Songkla University, were isolated from shrimp farms in Pattani and Songkhla provinces, southern part of Thailand, during outbreak of AHPND in this area (Kongrueng et al., 2014). Forty-seven isolates of which were AP3-positive. A summary of number of isolates and their geographic origins is illustrated in Table 3.

Table 3. Geographic origins of 96 *V. parahaemolyticus* isolates in this study

Geographic origins	AHPND	Non-AHPND	Total
Central provinces	9	21	30
Southern provinces	47	19	66
Total	56	40	96

2.   Susceptibility of *V. parahaemolyticus* to antibiotics and polyphenols

2.1   Susceptibility to antibiotics

MIC of the quality control strain *E. coli* ATCC 25922 to antibiotics were in the acceptable ranges (Appendix 1). Currently, there is no recommended interpretive criteria (resistant, intermediate, or susceptible breakpoints) of antimicrobial agents for aquatic pathogens, therefore MIC and MBC for *V. parahaemolyticus* isolates were reported directly (CLSI, 2006b). Percent of *V. parahaemolyticus* isolates that were susceptible to each MIC, MBC of 8 antibiotics and 4 polyphenols was summarized in Table 4. Among 8 antibiotics, ampicillin and amoxicillin were most resisted by *V. parahaemolyticus*. MICs and MBCs of these two agents were ≥32 µg/ml for all isolates.

Other antibiotics including florfenicol, oxytetracycline, oxolinic acid, and enrofloxacin showed high antimicrobial effects on *V. parahaemolyticus*. MICs and MBCs of these antimicrobial agents against 100% of the isolates were ≤ 8 µg/ml. Currently, MIC breakpoints data for these antibiotics against *V. parahaemolyticus* are not available. However, similar MICs of other agents in the same groups were considered

susceptible (i.e chloramphenicol, ≤ 8 µg/ml; tetracycline, ≤ 4 µg/ml; ciprofloxacin, ≤ 1 µg/ml) (CLSI, 2006a). MICs of trimethoprim/sulfamethoxazole (1:19) ranged from 1.2/0.06 to 38/2 µg/ml, which is still in the susceptible range. However, MBCs varied in wide range, and exceeded 304/16 µg/ml for some isolates. The lowest MIC and MBC were obtained from enrofloxacin (MIC, MBC values ranging from 0.25-1 µg/ml).

2.2   Susceptibility to polyphenols

Among four polyphenols used in this study, syringic acid and rutin demonstrated lowest effects against *V. parahaemolyticus*. All of the isolates were able to grow even when they were exposed to these agents at concentrations >512 µg/ml. Higher concentrations of syringic acid and rutin (≥ 1024 µg/ml) resulted in coagulation of the substances in the solution. Therefore, susceptibility of *V. parahaemolyticus* to these agents at concentrations above 512 µg/ml was not determined.

Vanillic acid showed antibacterial effect on *V. parahaemolyticus*. MICs and MBCs of 1024-2048 µg/ml were able to inhibit 100% of the isolates. Pyrogallol showed the strongest activity against *V. parahaemolyticus*. MIC values of this substance were in the range of 32-256µg/ml. MBC of pyrogallol against all isolates was in the same range as MIC, but the percent of isolates in higher concentration was higher.

2.3   Comparison of susceptibility of AHPND and non-AHPND isolates

V. parahaemolyticus was divided to AHPND and non-AHPND groups, and their susceptibilities to antibiotics and polyphenols were shown in Tables 5 and 6. MIC range, MIC_{50}, and MIC_{90} of antimicrobial agents to both *V. parahaemolyticus* groups were summarized in Table 7. Basing on MIC_{90}, we compared the susceptibility between two *V. parahaemolyticus* groups.

There were differences in MIC ranges of antimicrobials against AHPND and non-AHPND groups. Specifically, MIC ranges of ampicillin and amoxicillin against AHPND group were lower than those against non-AHPND group. MIC range of rutin and syringic acid was the same among AHPND and non-AHPND. MIC range of enrofloxacin against

AHPND was higher than that of non-AHPND group. However, MIC_{90} of these antimicrobials was not different between AHPND and non-AHPND groups.

Similarly, MIC ranges of other antimicrobials against AHPND and non-AHPND groups showed some differences. MIC ranges of oxolinic acid, oxytetracycline, trimethoprim/sulfamethoxazole (1:19), and pyrogallol against AHPND group were lower than those against non-AHPND group. MIC ranges of florfenicol and vanillic acid against AHPND isolates was the same as those against non-AHPND isolates. Nevertheless, the results of MIC_{90} showed that AHPND group was more sensitive to all of these antimicrobials than non-AHPND group.

Table 4. Percent distribution of MIC, MBC against 96 *V. parahaemolyticus* isolates

Conc. (µg/ml)	Antibiotics														Polyphenols							
	Ampicillin		Amoxicillin		Enrofloxacin		Oxolinic acid		Florfenicol		Oxytetracycline		Sulfa/Trim*		Pyrogallol		Rutin		Syringic acid		Vanillic acid	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
2048	-	-	-	-													-	-	-	-	13.54	54.17
1024	-	-	-	-													-	-	-	-	86.46	45.83
>512	83.33	85.42	61.46	71.88													100	100	100	100		
512	15.63	13.54	25.00	21.88																		
256	1.04	1.04	8.33	4.17											2.08	12.50						
128	1.04		2.08	1.04											14.58	34.38						
64			1.04	1.04											72.92	45.83						
32			2.08										-	-	10.42	7.29						
>16														8.33								
16														2.08								
8							2.08	2.08		6.25	2.08	4.17		7.29								
4							1.04	1.04	11.46	23.96	4.17	10.42		7.29								
2							1.04	3.13	51.04	48.96	4.17	26.04	1.04	3.13								
1					1.04	4.17	1.04	3.13	37.50	20.83	25.00	18.75	7.29	17.71								
0.5					14.58	23.96	54.17	72.92			40.63	35.42	16.67	19.79								
0.25					84.38	71.88	40.63	17.71	-	-	23.96	5.21	27.08	22.92								
0.12					-	-	-	-	-	-	-	-	35.42	9.38								
0.06					-	-	-	-	-	-	-	-	12.50	2.08								

Notes: (*) MIC, MBC are concentrations of trimethoprim in trimethoprim/sulfamethoxazole (1:19)

(-) susceptibility is not determined at these concentrations

Table 5. Percent distribution of MIC, MBC against AHPND *V. parahaemolyticus* (*n* = 56)

Conc (µg/ml)	Antibiotics														Polyphenols							
	Ampicillin		Amoxicillin		Enrofloxacin		Oxolinic acid		Florfenicol		Oxytetracycline		Sulfa/Trim*		Pyrogallol		Rutin		Syringic acid		Vanillic acid	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
2048	-	-	-	-													-	-	-	-	8.93	55.36
1024	-	-	-	-													-	-	-	-	91.07	44.64
>512	71.43	75.00	51.79	67.86													100		100			
512	26.79	23.21	28.57	23.21														100	100	100		
256		1.79	12.50	5.36												5.36						
128	1.79		1.79	1.79											7.14	42.86						
64			1.79	1.79											83.93	44.64						
32			3.57										-	-	8.93	7.14						
>16														1.79								
16																						
8										5.36		1.79		10.71								
4									7.14	14.29	3.57	8.93		8.93								
2							1.79		57.14	55.36	3.57	30.36										
1					1.79	5.36		1.79	35.71	25.00	32.14	26.79	1.79	16.07								
0.5					14.29	23.21	62.50	89.29			42.86	25.00	12.50	28.57								
0.25					83.93	71.43	35.71	7.14			17.86	7.14	42.86	25.00								
0.12					-	-	-	-	-	-	-	-	32.14	5.36								
0.06					-	-	-	-	-	-	-	-	10.71	3.57								

Notes: (*) MIC, MBC are concentrations of trimethoprim in trimethoprim/sulfamethoxazole (1:19)

(-) susceptibility is not determined at these concentrations

Table 6. Percent distribution of MIC, MBC against non-AHPND *V. parahaemolyticus* (n = 40)

Conc (µg/ml)	Antibiotics														Polyphenols							
	Ampicillin		Amoxicillin		Enrofloxacin		Oxolinic acid		Florfenicol		Oxytetracycline		Sulfa/Trim*		Pyrogallol		Rutin		Syringic acid		Vanillic acid	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
2048	-	-	-	-													-	-	-	-	20.00	52.50
1024	-	-	-	-													-	-	-	-	80.00	47.50
>512	100	100	75.00	77.50													100	100	100	100		
512			20.00	20.00																		
256			2.50	2.50											2.50	22.50						
128			2.50												27.50	22.50						
64															57.50	47.50						
32													-	-	12.50	7.50						
>16														17.50								
16														5.00								
8							5.00	5.00		7.50	5.00	7.50		2.50								
4							2.50	2.50	17.50	37.50	5.00	12.50		5.00								
2								5.00	42.50	40.00	5.00	20.00	2.50	7.50								
1						2.50	2.50	2.50	40.00	15.00	15.00	7.50	15.00	20.00								
0.5					15.00	25.00	42.50	50.00			37.50	50.00	22.50	7.50								
0.25					85.00	72.50	47.50	32.50			32.50	2.50	5.00	20.00								
0.12									-	-	-	-	40.00	15.00								
0.06									-	-	-	-	15.00									

Notes: (*) MIC, MBC are concentrations of trimethoprim in trimethoprim/sulfamethoxazole (1:19)

(-) susceptibility is not determined at these concentrations

Table 7. MIC$_{50}$ and MIC$_{90}$ of *V. parahaemolyticus* groups

Antimicrobials	VP (96)			AHPND (56)			Non-AHPND (40)		
	MIC Range	MIC50	MIC90	MIC Range	MIC50	MIC90	MIC Range	MIC50	MIC90
Ampicillin	128 - >512	>512	>512	128 - >512	>512	>512	>512	>512	>512
Amoxicillin	32 - >512	>512	>512	32 - >512	>512	>512	128 - >512	>512	>512
Enrofloxacin	0.25 - 1	0.25	0.5	0.25 - 1	0.25	0.5	0.25 - 0.5	0.25	0.5
Oxolinic acid	0.25 - 8	0.5	0.5	0.25 - 2	0.5	0.5	0.25 - 8	0.5	1
Florfenicol	1 - 4	2	4	1 - 4	2	2	1 - 4	2	4
Oxytetracycline	0.25 - 8	0.5	1	0.25 - 4	0.5	1	0.25 - 8	0.5	2
Trimethoprim/Sulfamethoxazole (1:19)*	0.06 - 2	0.25	0.5	0.06 - 1	0.25	0.5	0.06 - 2	0.12	1
Pyrogallol	32 - 256	64	128	32 - 128	64	64	32-256	64	128
Rutin	>512	>512	>512	>512	>512	>512	>512	>512	>512
Syringic acid	>512	>512	>512	>512	>512	>512	>512	>512	>512
Vanillic acid	1024 - 2048	1024	2048	1024 - 2048	1024	1024	1024 - 2048	1024	2048

Notes: (*) MIC, MBC are concentrations of trimethoprim in trimethoprim/sulfamethoxazole (1:19)

3.   Time-kill curve of pyrogallol

Among four polyphenols, pyrogallol showed highest antibacterial effect on *V. parahaemolyticus* (lowest MIC, MBC values). MIC_{90} of this substance was 128 µg/ml. Therefore, bactericidal activity (time-kill curve) of this substance was investigated at 1x, 2x, and 4x MIC, with one isolate from each of AHPND and non-AHPND groups, which were inhibited at 128 µg/ml of pyrogallol.

In control experiment in which both bacterial isolates were exposed to water, the number of bacteria was increasing, from initial culture of approximately 5×10^{5} CFU/ml to maximum of 10^{14} CFU/ml after 24h of incubation.

In experiment with AHPND isolate, after exposure to pyrogallol, the number of viable cells in the mixture was decreasing. All bacterial cells were killed at 12, 8, and 8 hours of incubation after being exposed to 1x, 2x, and 4x MIC of pyrogallol, respectively.

With regard to non-AHPND isolate, the changes in number of viable cells showed the same trend as of AHPND isolate. Cells started to die gradually when being in contact with pyrogallol. Pyrogallol was able to kill all non-AHPND cells in the mixture after 12, 12 and 8 hours of exposure to this agent at 1x, 2x, and 4x MC, respectively.
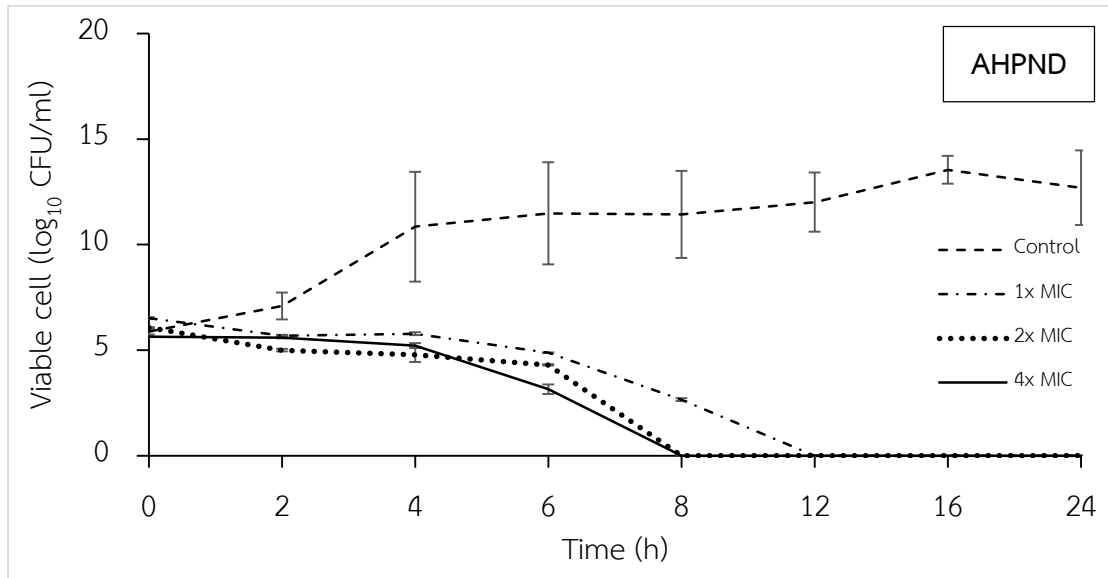
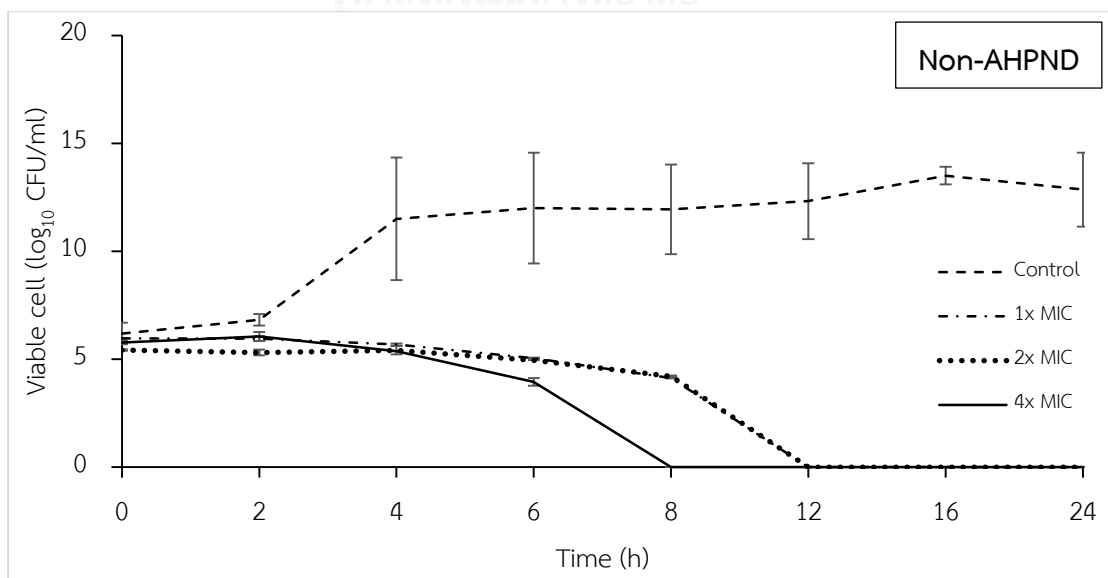Figure 9. Time-kill curve of pyrogallol against AHPND *V. parahaemolyticus*

Figure 10. Time-kill curve of pyrogallol against non-AHPND *V. parahaemolyticus*

4.   Effect of pyrogallol on bacterial cell

Scanning electron micrographs of *V. parahaemolyticus*, taken at 10,000x magnification, are shown in Figure 11. In control experiment where *V. parahaemolyticus* was not treated with pyrogallol, bacterial cells remained their normal morphology (Figure 11. A). Besides, the presence of tiny coccoid cells were still observable.

Micrograph of pyrogallol-treated cells showed disruption of the majority of bacterial cells (Figure 11. B). The disrupted cells and tiny coccoid cells are undistinguishable. In conducted experimental conditions (512 µg/ml of pyrogallol, 6 hours), a few cells remained intact, but prolonged exposure to pyrogallol, up to 8 hr, killed all bacterial cells.
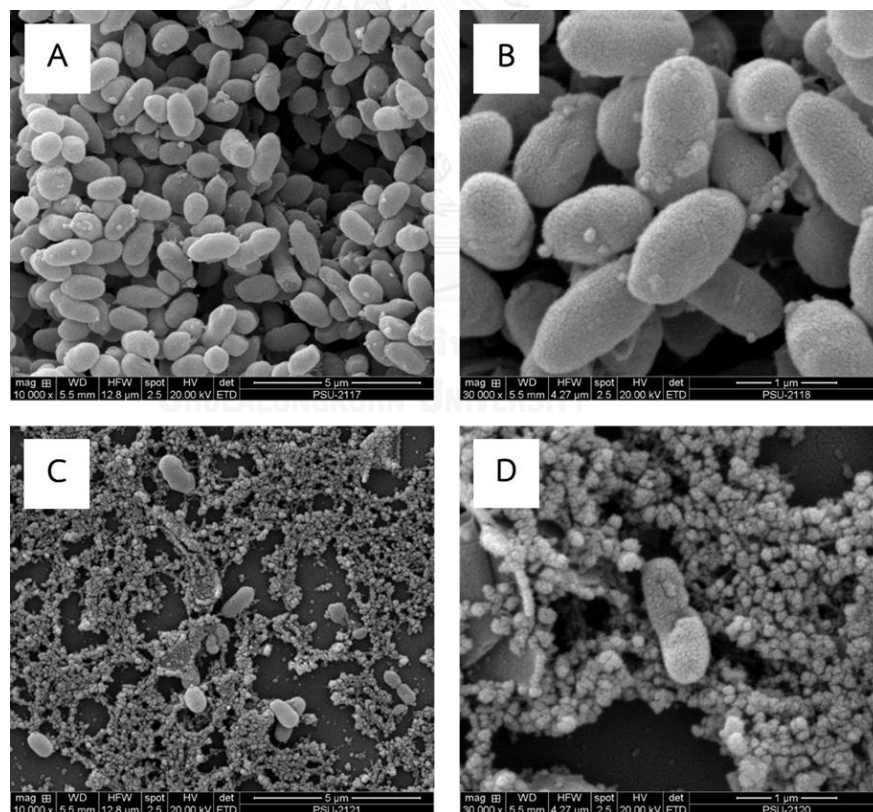
Figure 11. Scanning electron micrographs of *V. parahaemolyticus*

A. Cells in control (x10,000); **B.** Cells in control (x30,000); **C**. Cells treated with pyrogallol, 512 µg/ml, 6 hours (x10,000); **D**. Cells treated with pyrogallol, 512 µg/ml, 6 hours (x30,000)

CHAPTER V. DISCUSSION AND CONCLUSIONS

1.   Discussion

Although *V. parahaemolyticus* is a marine bacterial flora, its massive damages to shrimp culture have been recently emphasized (Flegel, 2012). In addition, this bacteria is a threat to human health which attracts attention of many scientists (Thongjun et al., 2013; Okoh et al., 2015). At ideal conditions, *V. parahaemolyticus* can duplicate every 8-9 minutes, which is the fastest replicating bacteria (Daniels et al., 2000). Thus, the bacteria can reach infectious dose in a few hours, even with a small starting number. The concentration of *V. parahaemolyticus* is high in sediment, compared to shrimp and water (de Jesus Hernandez-Diaz et al., 2015). In filter-feeder such as oysters and clams, *vibrios* concentrate in the gut where they can multiply (Okoh et al., 2015). Kongrueng and colleagues (2014) demonstrated that *V. parahaemolyticus* can be obtained more from intestines than from hepatopancreas. Therefore, investigation of AHPND *V. parahaemolyticus* in shrimp farms should be performed with shrimp intestine.

Vibrio species are susceptible to most antibiotics. For example, susceptibility of *Vibrio* isolates from United States with ampicillin, cefotaxime, ceftazidime, chloramphenicol, ciprofloxacin, gentamicin, imipenem, tetracycline revealed only ampicillin resistance (81%; MIC > 16 g/ml) (Han et al., 2007). Resistance to ampicillin and high susceptibility to most antibiotics tested were also detected in India, Italy, Malaysia, and Mexico (Ottaviani et al., 2013; Reyhanath and Kutty, 2014; Sahilah et al., 2014; de Jesus Hernandez-Diaz et al., 2015). In Thailand, *V. parahaemolyticus* was susceptible (MIC ≤ 8 µg/ml) to chloramphenicol and florfenicol (Tipmongkolsilp et al., 2006). Susceptibility to these two antibiotics in our study showed similar results, suggesting that resistance to chloramphenicol group has not yet developed. A recent study showed that AHPND *V. parahaemolyticus* isolates were resistant to ampicillin and erythromycin, whereas they were susceptible to tetracycline, chloramphenicol,

sulphamethoxazole/trimethoprim, gentamycin and norfloxacin (Kongrueng et al., 2014). In general, data on antimicrobial susceptibility of this bacterium are incomprehensive, and often limited to antibiotics used for treatment in human, which are not allowed for use in Thai aquaculture.

In this study, multi-drug resistance of *V. parahaemolyticus* was not detected. However, this phenomenon drastically elevated from 8.6% (2004-2010) to 22.93% (2011-2013; p < 0.05) in Mexico (de Jesus Hernandez-Diaz et al., 2015). In addition, multi-drug resistant strains were encountered more often from water samples than from shrimps in India (Reyhanath and Kutty, 2014). In China, more than half of *V. parahaemolyticus* isolates (*n* = 87) showed multi-drug resistance to at least 3 antibiotics, and mechanisms of which are related to the presence of resistance genes, and/or mutations in targeted genes (Jiang et al., 2014). Plasmid-mediated resistance genes were similar among bacteria species, suggesting the transfer of these genes in bacterial community are possible (Aedo et al., 2014).

Although many antibiotics showed high *in vitro* efficacy against bacteria in our study, new methods to control bacterial infections are still being investigated. Researches on beneficial bacteria for probiotic candidates showed potential application of *Bacillus* spp. in controlling vibriosis in mud crab (Wu et al., 2014). Bacteriophages were also examined for their ability to inhibit food and waterborne bacterial pathogens, including *V. parahaemolyticus* (Jun et al., 2014a; Jun et al., 2014b; Tskhvediani et al., 2014). Besides, screening for bioactive natural compounds were also done. Polyhydroxybutyrate biopolymer, produced by Gram-positive *Brevibacterium casei*, showed antiadhesive activity against *vibrios*, including *V. vulnificus, V. fischeri, V. parahaemolyticus, V. alginolyticus,* and *V. harveyi* (Kiran et al., 2014). The sponge belonging to genus *Haliclona* contained antimicrobial compounds which have notable effect on *V. parahaemolyticus* (Hoppers et al., 2015).

Researches on antimicrobial effects of polyphenols have been extensively conducted. For example, *V. parahaemolyticus* was tested with 18 plant species, some of which showed promising results (Yano et al., 2006). Most of these studies were conducted with crude extracts which often contain many different substances, including syringic acid and pyrogallol (Carvajal et al., 2012). Therefore, the role of active ingredients was unclear. In this study, we investigated the effect of pyrogallol, rutin, syringic and vanillic acid separately on *V. parahaemolyticus*, and only pyrogallol showed satisfying result.

The antimicrobial effects of polyphenols are often unpredictable and species-specific. When tested 10 polyphenols against with 4 different bacterial genera, the results showed no clear correlation between Gram-staining and bacterial susceptibility to polyphenols (Taguri et al., 2006). Methanol extract of *Vitex negundo* leaf (500 µg/mL) killed *V. cholerae* and *V. parahaemolyticus* after 1 hour, but not *V. mimicus* after 16 hours (Kamruzzaman et al., 2013). Pyrogallol previously showed protection against *V. harveyi* (Defoirdt et al., 2013). In this study, pyrogallol also demonstrated antimicrobial effect on *V. parahaemolyticus*. On the contrary, Hsieh and colleagues (2008) proved that rutin had inhibitory activity against *V. alginolyticus* when administered to *Litopenaeus vannamei* at 10 µg/ml, but in this study, could not inhibit the growth of *V. parahaemolyticus* even at 512 µg/ml.

Pyrogallol is the major substance in extract of many plant species (Gopi et al., 2015; Khatua et al., 2015). It is soluble in water, and can go through rapid autoxidation in the presence of oxygen, which subsequently produces peroxides and hydro peroxides (Marklund and Marklund, 1974). When observed under scanning electron microscope, pyrogallol-free treatment showed tiny coccoid cell sticking to intact *V. parahaemolyticus* cells, an interesting phenomenon called as formation of budding which was previously reported (Coutard et al., 2007). There was a massive cell disruption in pyrogallol-treated experiment. The bactericidal effect of pyrogallol was

attributed to peroxide production resulting from the autoxidation of the compound (Defoirdt et al., 2013).

2. Conclusions

Our study re-confirmed the presence of AHPND *V. parahaemolyticus* in the central and southern provinces of Thailand. Ampicillin and amoxicillin are not suitable for controlling *V. parahaemolyticus*. Other antibiotics including enrofloxacin, oxolinic acid, oxytetracycline, florfenicol, and trimethoprim/sulfamethoxazole (1:19) showed high potency *in vitro* against *V. parahaemolyticus*, and may be effective for treating this bacterial pathogen in shrimp.

Three polyphenols, including rutin, syringic and vanillic acids showed low potency against *V. parahaemolyticus*. Due to bactericidal effect, pyrogallol is the most potential among the four polyphenols examined. Effect of pyrogallol is dose and time-dependent. It causes *V. parahaemolyticus* cell disruption at 512 µg/ml, and completely kill all cells after 8 hours.

3. Advantages of study

This study provides a collection of (AHPND and non-AHPND) *V. parahaemolyticus* isolates which can be used for further studies on this pathogen. In addition, antimicrobial susceptibility of both non-AHPND and emerging AHPND *V. parahaemolyticus* isolated from Pacific white shrimp (*Litopenaeus vannamei*) in central and southern parts of Thailand has been demonstrated. The present study also confirms the potential of polyphenols in controlling *V. parahaemolyticus*, and demonstrates the effect of pyrogallol on *V. parahaemolyticus* cell.

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APPENDIX

Appendix 1. Acceptable quality control ranges of MICs (µg/ml) for *Escherichia coli* ATCC 25922 when tested at 28 ± 2°C after 24 to 28 hours (CLSI, 2006)

Antimicrobial agent	*Escherichia coli* ATCC 25922
Ampicillin	2 – 16
Enrofloxacin	0.008 – 0.03
Florfenicol	4 - 16
Oxolinic acid	0.06 – 0.025
Oxytetracycline	0.5 – 2
Trimethoprim/sulfamethoxazole (1:19)	0.03/0.6 – 0.25/4.8

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