UTILIZATION OF ORGANIC BANANA PEEL EXTRACT FOR ENHANCING IMMUNE RESPONSE OF GIANT FRESHWATER PRAWN (*Macrobrachium rosenbergii*)

THANYARAT NAKSING

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE PROGRAM IN APPLIED BIOLOGY
FACULTY OF SCIENCE AND TECHNOLOGY
RAJAMANGALA UNIVERSITY OF TECHNOLOGY THANYABURI
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Thesis Title	Utilization of Organic Banana Peel Extract for Enhancing Immune Response of Giant Freshwater Prawn (*Macrobrachium rosenbergii*)
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Program	Applied Biology
Thesis Advisor	Mr. Atsadawut Areesirisuk, Ph.D.
Academic Years	2020

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Date 9 Month March Year 2021

หัวข้อวิทยานิพนธ์	การใช้สารสกัดเปลือกกล้วยอินทรีย์เพื่อเพิ่มการตอบสนองภูมิคุ้มกันของกุ้งก้ามกราม (*Macrobrachium rosenbergii*)
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บทคัดย่อ

งานวิจัยนี้มีวัตถุประสงค์เพื่อ (1) ศึกษาคุณสมบัติของสารออกฤทธิ์ทางชีวภาพ และระบุลักษณะองค์ประกอบทางพฤกษเคมีของสารสกัดเปลือกกล้วยอินทรีย์จำนวน 6 ชนิด ได้แก่ กล้วยหอมทอง กล้วยน้ำว้า กล้วยไข่ กล้วยหักมุก กล้วยเล็บมือนาง และกล้วยหอมไต้หวัน (2) เพื่อหาสภาวะในการสกัดที่เหมาะสม และ (3) เพื่อตรวจสอบผลของการฉีดสารสกัดเปลือกกล้วยอินทรีย์และการใช้สารสกัดเปลือกกล้วยอินทรีย์เป็นสารเสริมอาหารต่อภูมิคุ้มกันของกุ้งก้ามกราม (*Macrobrachium rosenbergii*)

อันดับแรก การออกฤทธิ์ทางชีวภาพของสารสกัดเปลือกกล้วยอินทรีย์ ได้แก่ ปริมาณฟีนอลิกทั้งหมด (total phenolic content; TPC) ปริมาณสารต้านออกซิเดชัน (antioxidant content) และฤทธิ์ต้านอนุมูลอิสระด้วยวิธี ferric-reducing antioxidant power (FRAP) ได้รับการตรวจวัด ผลการทดลองพบว่าสารสกัดเปลือกกล้วยไข่อินทรีย์ให้ ค่า TPC และ FRAP สูงสุด สารสกัดเปลือกกล้วยหักมุกอินทรีย์สามารถยับยั้งเชื้อ *Aeromonas hydrophila* และ *Staphylococcus aureus* ได้ สเปคตรัมสเปกโทรสโกปีอินฟราเรดแบบฟูเรียร์ทรานส์ฟอร์ม (Fourier-transform infrared spectroscopy; FTIR) แสดงสารประกอบพฤกษเคมีปฐมภูมิและทุติยภูมิหลายชนิด สารประกอบหลัก 4 ชนิด ได้แก่ กรดอะซิติก (acetic acid) กรดฟอร์มิก (formic acid) 1,2-เบนซินิไดออล, 3-เมทิล- (1,2-benzenediol,3-methyl-) และ 4-ไฮดรอกซี-2-เมทิลอะซิโตโฟน (4-hydroxy-2-methylacetophone) ซึ่งถูกตรวจวัดด้วยเครื่องแก๊สโครมาโทรกราฟี-แมสสเปคโทรมิเตอร์ (gas chromatography-mass spectrometer; GC-MS) แสดงคุณสมบัติการต้านออกซิเดชัน และกิจกรรมการยับยั้งเชื้อก่อโรค สารสกัดเปลือกกล้วยหอมทองอินทรีย์สามารถยับยั้งเชื้อก่อโรคในสัตว์น้ำ *A. hydrophila* และให้ผลได้ของการสกัดสูงสุด (extraction yield) ดังนั้นเปลือกกล้วยหอมทองจึงถูกนำไปศึกษาสภาวะที่เหมาะสมในการสกัด การทดลองถัดไปเป็นการศึกษาสภาวะที่เหมาะสมในการสกัดสารจากเปลือกกล้วยอินทรีย์ โดยสภาวะในการสกัดที่เหมาะสม คือ การใช้สารละลายเมทานอล 50 เปอร์เซ็นต์โดยปริมาตร อุณหภูมิ 100 องศาเซลเซียส เป็นเวลา 10 นาที ภายใต้สภาวะนี้ได้ปริมาณฟีนอลิกทั้งหมด (total phenolic content) และผลได้ของการสกัด (extraction yield) สูงสุดเท่ากับ 10.44 มิลลิกรัมสมมูลย์ของกรดแกลลิกต่อกรัมของเปลือกกล้วยอินทรีย์แห้ง (mg GAE/ g DM) และ 33 เปอร์เซ็นต์ โดยน้ำหนักต่อปริมาตร ตามลำดับ สารสกัดเปลือกกล้วยหอมทองอินทรีย์นี้ยังสามารถยับยั้งเชื้อ *A. hydrophila* ที่ความเข้มข้นต่ำสุดที่สามารถ

ยับยั้งได้ (minimum inhibitory concentration; MIC) เท่ากับ 312.50 ไมโครกรัมต่อแผ่น (µg/disc) การทดลองสุดท้ายผลของสารสกัดเปลือกกล้วยอินทรีย์ต่อภูมิคุ้มกันของกุ้งก้ามกรามถูกตรวจสอบด้วยการฉีดสารโดยตรงและการเสริมในอาหารเพาะเลี้ยง ผลการทดลองแสดงว่าการฉีดสารสกัดเปลือกกล้วยอินทรีย์โดยตรงสามารถเพิ่มภูมิคุ้มกัน กิจกรรมการกลืนกินเชื้อก่อโรค (phagocytic activity) และลดความอ่อนแอของกุ้งก้ามกรามได้ นอกจากนี้อาหารที่เสริมด้วยสารสกัดเปลือกกล้วยอินทรีย์และผงโปรไบโอติกยังสามารถส่งเสริมระบบภูมิคุ้มกัน ลดระยะเวลาในการแข็งตัวของเลือดกุ้งก้ามกราม และต่อต้านเชื้อก่อโรค *A. hydrophila* ได้หลังจากให้อาหารเป็นวลา 90 วัน

ผลการวิจัยโดยรวมแสดงให้เห็นว่าสารสกัดเปลือกกล้วยหอมทองอินทรีย์เป็นสารเสริมอาหารที่น่าสนใจสำหรับส่งเสริมภูมิคุ้มกันของกุ้งก้ามกรามและต่อต้านเชื้อก่อโรค *A. hydrophila* นอกจากนี้การใช้เปลือกกล้วยในการเพาะเลี้ยงสัตว์น้ำยังสามารถเพิ่มมูลค่าของเปลือกกล้วย และลดภาระในการกำจัดของเสียในสิ่งแวดล้อมได้

คำสำคัญ: สารสกัดเปลือกกล้วยอินทรีย์ ฤทธิ์ทางชีวภาพ กุ้งก้ามกราม (*Macrobrachium rosenbergii*) ภูมิคุ้มกัน

Thesis Title	Utilization of Organic Banana Peel Extract for Enhancing Immune Response of Giant Freshwater Prawn (*Macrobrachium rosenbergii*)
Name-Surname	Miss Thanyarat Naksing
Program	Applied Biology
Thesis Advisor	Mr. Atsadawut Areesirisuk, Ph.D.
Academic Year	2020

ABSTRACT

This research aimed to: (1) study the bioactive compound properties and specify the phytochemical constituents of organic banana peel extract (BPE) from six types of organic banana cultivars, including Kluai Homthong, Kluai Namwa, Kluai Kai, Kluai Hukmook, Kluai Lebmuernang, and Kluai Homtaiwan, (2) optimize the optimum extraction condition, and (3) investigate the effect of organic BPE injection and the use of organic BPE as a dietary supplement to immunity of giant freshwater prawn (*Macrobrachium rosenbergii*).

Firstly, the biological activities of organic BPE, i.e., total phenolic content (TPC), antioxidant content, and ferric-reducing antioxidant power (FRAP) were measured. The result was found that the organic BPE of Kluai Kai provided the highest TPC and FRAP. Organic BPE of Kluai Hukmook could inhibit *Aeromonas hydrophila* and *Staphylococcus aureus*. The Fourier-transform infrared spectroscopy (FTIR) spectrum exposed diverse compounds of primary and secondary phytochemical compounds. Four main constituents which were determined by gas chromatography-mass spectrometer (GC-MS) were acetic acid, formic acid, 1,2-benzenediol,3-methyl-, and 4-hydroxy-2-methylacetophone. The compounds exhibited antioxidant properties and antipathogenic activity. The BPE of Kluai Homthong could inhibit aquatic pathogen *A. hydrophila* and provided the highest extraction yield. Thereby, banana peel (BP) of Kluai Homthong was chosen to study the optimum extracting condition. The next experiment was to study the optimum extracting condition of organic BPE. The optimum extracting condition was 50 % v/v of methanol solution at 100 °C for 10 minutes. Under this condition, the maximum TPC and extraction yield were provided for

10.44 mg equivalent of gallic acid per gram of dried material (mg GAE/g DM) and 33 % w/v, respectively. This organic BPE was also able to inhibit *A. hydrophila* at a minimum inhibitory concentration (MIC) of 312.50 µg/disc. Finally, the effects of organic BPE on the immunity of *M. rosenbergii* were investigated by directly injecting the extract and supplementation of the extract in a cultured diet. The result presented that the direct injection of organic BPE could increase immunity and phagocytic activity and decrease the susceptibility of *M. rosenbergii*. In addition, the diet supplemented with organic BPE and probiotic powder could also enhance the immune system, reduce the coagulation time of *M. rosenbergii*, and increase resistance to pathogen *A. hydrophila* after 90 days of feeding.

Overall findings have shown that organic BPE was an interesting supplement for enhancing the immunity of giant freshwater prawn and resistance against *A. hydrophila*. Furthermore, the use of banana peel (BP) in aquaculture could increase the value of BP and reduce the burden of its waste disposal in the environment.

Keywords: organic banana peel extract, biological activity, giant freshwater prawn (*Macrobrachium rosenbergii*), immunity

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Thanyarat Naksing

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List of Abbreviations

µL	Micro Liter
BP	Banana Peel
BPE	Banana Peel Extract
CFU/mL	Colony forming Unit per Milliliter
DHC	Different Hemocyte Count
DM	Dry Matter
FCR	Feed Conversion Ratio
FRAP	Ferric Reducing Antioxidant Power
FSBM	Fermented Soybean Meal
FTIR	Fourier Transform Infrared Spectroscopy
g	Gram
GAE	Gallic Acid Equivalent
GC-MS	Gas Chromatography Mass Spectrometry
GCs	Granular cells
h	Hour
HCs	Hyaline Cells
L	Liter
M	Molar
mg	Milligram
mg/L	Milli Gram Per Liter
min	Minute
mL	Milliliter
mM	Milli Mole
mm^3	Cubic Microliter
nm	Nanometer
PCI	Peel Color Index
PLG	Percentage Length Gain
PP	Probiotic Powder
proPO	Prophenoloxidase

List of Abbreviations (continued)

PWG	Percentage Weight Gain
rpm	Round Per Minute
SBM	Soybean Meal
sec	Second
SGCs	Semigranular Cells
SGR	Specific Growth Rate
SR	Survival Rate
SSF	Solid State Fermentation
THC	Total Hemocyte Count
TPC	Total Phenolic Content
TVC	Total Viable Cell Count
v/v	Volume by Volume
w/v	Weight by Volume

CHAPTER 1
INTRODUCTION

1.1 Background and statement of the problems

Aquaculture has become the fastest-growing food-producing sector and contributes significantly to the global food supply, food security, and national economic development [1]. Presently, the prawn culture (*Macrobrachium rosenbergii*) is developing and very popular due to the continuously increasing consumption requirement. Farmers, therefore increasing the amount of prawn culture. Resulting in an intense population and increase the infection the disease of prawns. The prawns were impacted by the epidemic from viruses and bacteria [2], which have caused effect economic losses. The farmers are necessary must apply the antibiotic during the culture to reduce the infection of pathogens, promote the immune system and growth rate. However, the excessive use and misuse of antibiotics have resulted in antibiotic residuals in food and the spread of antibiotic-resistant pathogens in the aquatic environment. In addition, nowadays, consumers more attention to safe food consumption and organic food production. For solving this problem, this research was to develop feeding additives to increase prawn cultivation performance.

The plant extracts have been reported to antipathogenic in aquatic because there are many bioactive compounds, especially phenolic compounds, a substance found in many plants such as vegetables, fruits, and herbs. It has antioxidant properties and stimulates the immune in aquatic animals. Banana is belonging to the family *Musaceae*. It's a trendy fruit globally and is consumed as a staple food in many countries and grown worldwide constitutes the fifth most important agricultural food crop in terms of world trade ranking next to rice, wheat, and maize in terms of its importance as a food crop. It's a rich source of important phytonutrients, including vitamins and phenolic compounds. It's also notably enriched with minerals, such as phosphorus, sodium, potassium, calcium, magnesium, iron, copper, zinc, and manganese. It's contained several bioactive compounds, such as phenolic, carotenoids, biogenic amines, and phytosterols, which are highly desirable in the diet as they exert many positive effects on human health and well-being.

Phenolic compounds are known for their antioxidant properties. It's an important secondary metabolite [3]. Many of these compounds have antioxidant activities and are useful in protecting the body against various oxidative stress. In the past, the banana was effectively used to treat various diseases, including reducing the risk of many chronic degenerative disorders. The waste peel of banana or plantains poses the problem of disposal without causing environmental pollution in the countries of the processing industries of banana and plantains. In the previous report, total phenolic compounds have more abundant in the peel, which was consistent with the antioxidant activity. Significant quantities of banana peels, equivalent to 40 % of the total weight of fresh banana, are generated as waste. Banana peels are the major by-product obtained during the processing to show that they are a good source of polyphenol, carotenoids, and other bioactive compounds that process various beneficial effects on human health. Different components have activities like antimicrobial, antioxidant, anti-inflammatory, and anticancer. Banana peel (BP) is rich in phytochemical compounds, mainly antioxidants. The total amount of phenolic compounds in banana (*Musa acuminata* Colla AAA) peel ranges from 0.90 to 3.0 g/100 g dry weight [4]. The antioxidant capacity of bananas was reported to increase during fresh maturity and to have a higher capacity level than other plants [5]. Banana is a plant that contains the highest phenolic compounds in the peel, which has antioxidant properties and antipathogenic and promotes the immune system in prawns [2]. Accordingly, this study aimed to investigate the immune parameters and growth performance of giant freshwater prawn (*M. rosenbergii*) and resistance against *A. hydrophila* using direct injection method and dietary feeding with organic banana peel extract (BPE).

1.2 Purpose of the study

In this thesis, the purpose of the study is to extend the concept of the previous works and to generalize new concepts which are:

1.2.1 To examine the phenolic compound and antioxidant activity of various organic BPE.

1.2.2 To study the antipathogenic activity of various organic BPE.

1.2.3 To identify the bioactive compound of various organic BPE.

1.2.4 To optimize the extraction condition of phenolic compound in organic BPE.

1.2.5 To investigate the effect of organic BPE injection and organic BPE supplemented feeding on immune parameters, resistance against *A. hydrophila*, and growth performance of giant freshwater prawn (*M. rosenbergii*).

1.3 Scope of thesis

The scope of this research is the study of the phenolic compound extraction from six species of organic BP i.e Kluai Homthong, Kluai Namwa, Kluai Kai, Kluai Hukmook, Kluai Lebmuernang and Kluai Homtaiwan. Thereafter, the optimization of organic BPE by using one factor at a time contains three parameters include the concentration of methanol, extraction time, and temperature. To obtain the highest phenolic compound, have antioxidant properties and inhibit the pathogens in freshwater prawn. The bioactive compound of organic BPE examines by GC-MS and FTIR. Finally, the efficiency of organic BPE on the immune and growth performance of the giant freshwater prawn is investigated by injection and feeding.

1.4 Expectation of thesis

This thesis, I have the scope and limitations of studying which are concerned to the previous works which are:

1.4.1 Known the total phenolic content, antioxidant and antipathogenic activity of various organic BPE.

1.4.2 Known the type and properties of the bioactive compound at various organic BPE.

1.4.3 Known the optimum condition of the highest phenolic compound extraction of organic BPE.

1.4.4 Known the effect of immune and growth performance in giant freshwater prawn after direct injection and feeding with organic BPE.

1.4.5 Added the value of organic banana to be a novel prawn feed.

1.4.6 Increased the income of farmers (prawn and banana farming).

1.4.7 Delivered food safety to farmer and customer.

CHAPTER 2
LITERATURE REVIEWS

2.1 Banana

Banana is in the family Musaceae genus Musa**.** The banana is accounted among the most well-known fruit containing high nutritional value [6]. It is usually known as dessert cooking. Generally, banana can be consumed fresh or further processed into many types of products, such as foods, beverages, functional foods [7]. Currently, banana has gained attention in their use as an ingredient for functional foods because of the low digestibility of starch and non-starch composition of banana bringing it a great choice of food ingredient.

Banana from tropical countries is distributed to South Asia and currently cultivated worldwide [8]. The world's fruit production in 2013 statistic reported that banana sharing 16.8 % of the market, followed by 11.4 % from apple and orange [9]. The last 20 years, from 1993 to 2013, the production of banana worldwide has been increasing from approximately 46 million tons to 105 million tons [9] . Asia has been accounted as the largest banana-producing continent, 57.3 % of the world production [9]. India is in the first rank country with 722 thousand hectares cultivated area and 26.51 million tons products annually in Asia. The increase in banana production due to demand growth and the widening of cultivated areas and productivity increment.

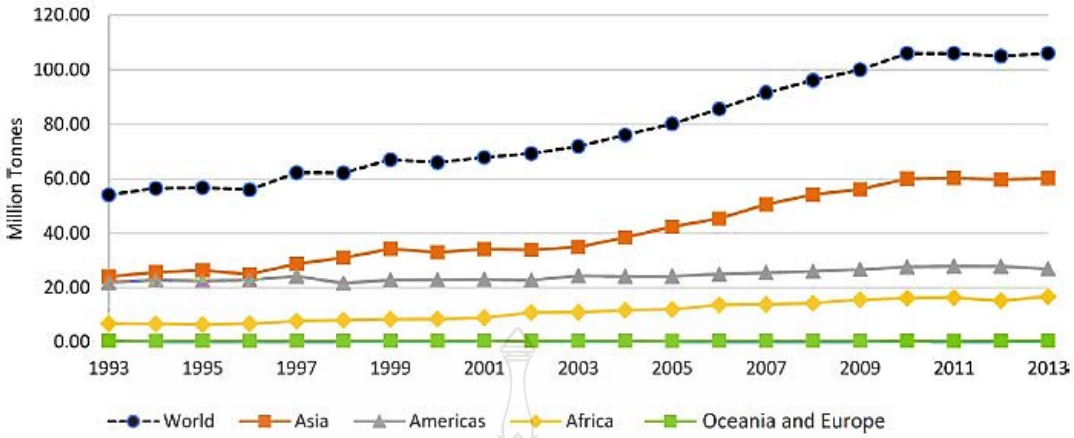

Figure 2.1 Banana world production by years

Source: FAOSTAT. 2013 [9]

2.2 Bioactive Compound in Banana Fruit

The secondary metabolites from plant are well-known bioactive compounds in their therapeutic potential by assisting in the antioxidant activities. Many kinds of fruits and vegetable contained phenolics and carotenoids as their main phytochemicals which are related to human health. Bananas, like other relevant fruit, have received particular attention in their bioactive compound incorporate in fruit and peel. Phenolics, carotenoids, flavonoids, and biogenic amines have been found in both raw and ripened banana as well as phytosterols in pulp, even at low levels [4]. These bioactive compounds found in bananas have a greater antioxidant level than other herbs and vegetables.

2.2.1 Phenolic compound

The hydroxyl group in aromatic ring of phenolic compounds acts as antioxidants. Hydroxybenzoic acids and hydroxycinnamic acids are, two classes of phenolic compounds found in plant materials, act as a precursor to other phenolic compounds synthesizing. The major group of phenolic compounds plentifully found in plants are simple phenolics, phenolic acids, flavonoids, coumarins, stilbenes, tannins, lignans, and lignins [10]. The phenolics present several health benefits, including antibacterial, anti-inflammatory, and antimutagenic activities. Various phenolic components are considered in the banana pulp and peel, such as gallic acid, catechin, epicatechin, anthocyanins, and tannins. The banana contains large amounts of total

phenolic compounds and flavanols. Flavonols, including quercetin, myricetin, kaempferol, and cyanidin, are the primary groups of flavonoids discovered in bananas. Many researchers have recorded the health advantages of flavonoids in bananas. As protective scavengers against free radicals and reactive oxygen-derived from oxygen, flavonoids represent disease-responsible species (ROS) in different conditions [4].

2.2.2 Carotenoids

Carotenoids are the natural pigments that are most widely distributed and show red, orange, and yellow colors. Two main subclasses of carotenoids are carotenes and xanthophylls, based on their structures. Carotenoid-rich food intake enhances immunity and decreases the risk of human diseases. Carotenoids compounds having about 600 substances in the group. Some combinations are the vitamin A precursors, which have high antioxidant activity to decrease ROS [11]. Consumption of fruits having carotenoids can induce the immune and decrease the risk of various diseases [12]. The banana cultivars can be produced and consumed by the emerging country met the vitamin A deficiency problem [13].

2.2.3 Biogenic amines

Biogenic amines are nitrogenous substant constructed by amino acid decarboxylation or aldehyde amination and ketones. The amines generally present in microorganisms, plants, and animals. It has been shown to contain in BP and pulp. The serotonin content of banana pulp ranged from 8-50 µg/g. Serotonin contributes to feelings of well-being and happiness. It also contains large amounts of dopamine and norepinephrine during ripening. Dopamine highly increases during the changing from unripe to ripening stage [5].

2.2.4 Phytosterols

Phytosterols are naturally occurring steroid alcohols having the steroid structure with a hydroxyl group at position number 3 and a side chain at position number 17, usually containing one or more double bonds in the steroid skeleton. They are obtained from many sources, and especially commercial sources include seed oils like corn, soybean, and rapeseed oil at typical levels of 0.1–1 % [4].

2.3 Banana Peel

Banana is primarily produced annually for 102 million fresh fruit tones [9, 14]. Therefore approximately 36 million tons of BP is wasted per year. Most of the BP is usually removed into landfills or general waste [15]. However, the BP waste has been reported as a potential material for further utilization. The BP has been traditionally used in medicine to treat various ailments [10, 11]. It has been discovered to contain high dietary fiber and phenolic compounds [12]. Moreover, the material has been demonstrated to exhibit potent antioxidant capacity, antimicrobial and antibiotic properties [13, 14]. For these reasons, BP is a valuable material for the nutraceutical and pharmaceutical industries.

2.4 Phenolic Compound in Banana Peel

Phenolic compounds (PC) are derived from a broad group of compounds originating from plants' secondary metabolism found in various natural sources such as fruits, vegetables, tea, wine, and honey. The phenol has been identified and described for 500 different polyphenols in over 400 products, many of them found in fruits. An aromatic ring bearing one or more hydroxyl groups is present in the PC molecular structure and can be a simple or polymerized molecule. PCs are classified into several groups based on their structural features, while phenolic acids, flavonoids, and non-flavonoids are the main groups [20]. Beneficial bioactivities of PC are related to their chemical characteristics, particularly the presence of an aromatic structure and multiple hydroxyl groups able to donate electrons or hydrogen atoms, neutralizing free radicals and other reactive oxygen species (ROS) [21].

2.5 Antioxidant Activity

The antioxidant-rich in a banana contributes to their physiological defense against oxidative and free-radical-mediated reactions, leading to stable radicals after scavenging in the biological systems [22]. Reactive oxygen species (ROS) produced in the cells during oxidation can damage nucleic acids, proteins, and lipids. Many people regularly consume bananas, and the bioactive compounds present in them have significant antioxidant activities, which effectively protected the body against various oxidative

stresses [23]. Many bioactive compounds with antioxidant and chelating properties have been identified in banana [23, 24, 25]. Among these, the most abundant antioxidants in a banana are phenolics, carotenoids, and ascorbic acid [26, 27, 28].

2.6 Utilization of Banana Peel

2.6.1 Traditional medicine

BP has been used to treat various diseases [29]. In addition, the BP can be used as an alternative to treat skin warts, while rubbing the inside of the BP is an effective remedy to minimize swelling and discomfort following mosquito bites [17].

Unripe peel has also been used to treat diarrhea [17]. The green BP was used to treat diarrheal problems [30]. BP exhibits anti-acid effects on stomach ulcers. Leucocyanidin has been shown to substantially increase the thickness of the mucous membrane layer of the stomach, a flavonoid in the BP [17]. BP is a source of prebiotic of bacteria in the intestinal tract [31]. Therefore, the BP is an excellent material for medicinal uses once treated and processed optimally.

2.6.2 Livestock feed

BP contains a high content of major nutritional components accounting for 91.50 % of the dry weight (lipids, proteins, and carbohydrates). The indigestible fiber also contains in BP [8]. In addition, various mineral components were observed in the banana peel. Potassium is the most dominant, accounting for approximately 55.23–63.52 mg/kg [32]. Potassium has been reported to be associated with regulating body fluids and maintaining normal blood pressure [17]. In addition, the low levels-antinutritive compounds have been denoted in the peel, such as hydrogen cyanide, a poisonous substance, which was lower than the safe limit (0.5–3.5 mg/g) [33].

2.6.3 Bio-substrate

BP has been used as a bio-substrate because it contains high cellulose levels [34]. For instance, the material has been used to produce fermentation products [35, 36]. BP has also been used as a substrate to cultivate edible mushrooms, wine, xylitol, and alternative current traditional sweetener [37, 38].

2.6.4 Other uses

BP has been used as an excellent absorber for several heavy metals and synthetic dyes [36, 39, 40]. BP is also a good source of pectin. Extractable pectin from both banana and plantain peels is higher than the other fruit peels [41]. Several studies have reported using the BP to isolate pectin for further application [42]. The BP was also used to extract aromatic compounds like a banana flavor [43]. Finally, the BP was used to make flour, which is then used as a carbohydrate source (mainly dietary fiber) or thickening agent in the food industry [44, 45]. Green banana starch provides good properties for several applications. Banana starch is considered marginally superior to maize starch, which gives a high market value [37].

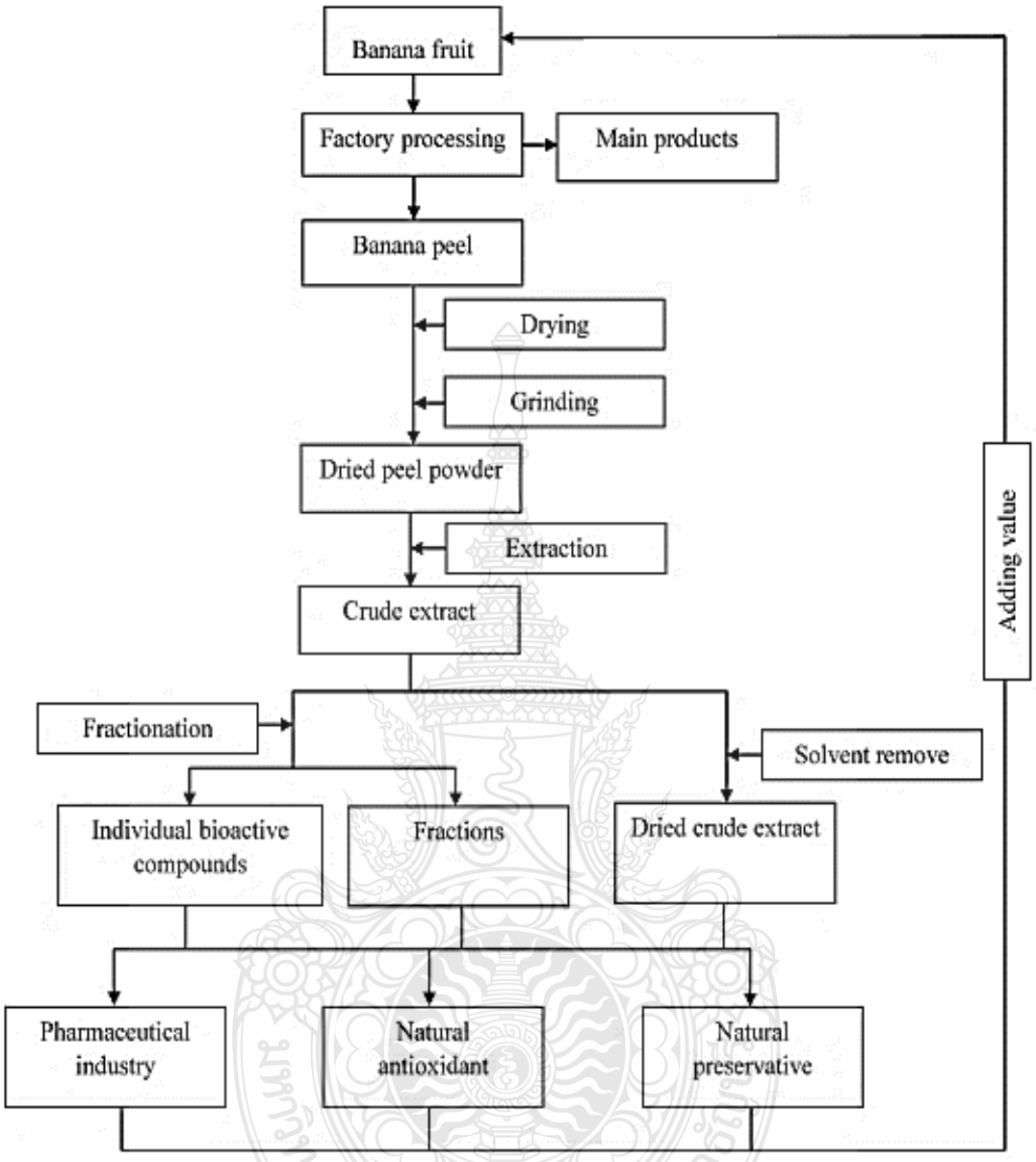

Figure 2.2 The trend for recovery and utilization of BP

Source: Vu et al. 2018 [46]

2.7 Probiotic

Probiotic is a Latin and Greek-derived word, meaning “for life”, a definition of probiotics as substances secreted by one microorganism that stimulate the growth of another [47, 48]. In 2002, A joint FAO/WHO panel described probiotics as live microorganisms that provide a health benefit to the host when administered in appropriate quantities [49]. Most probiotics are Gram-positive bacteria, including lactic acid bacteria (LAB) bacteria, but a few molds and leaves can also be used as probiotics to enhance food safety and consumer health by preventing and reducing pathogenic bacteria [50]. Probiotics are beneficial bacteria in that they favorably alter the intestinal microflora balance, inhibit the growth of harmful bacteria, promote good digestion, boost immune function and increase resistance to infection. Other physiological benefits of probiotics include removing carcinogens, cholesterol-lowering, immune-stimulating and allergy lowering effect, synthesis and enhancing the bioavailability of nutrients, and alleviating lactose intolerance [51].

2.7.1 Probiotics in the animal feed industry

Over the last two decades, there has been an increasing concern of consumers regarding the safety, quality of animal products, and environmental issues. Therefore, the purpose of the use of feed additives is to increase digestive and production performance by reducing the prevalence of pathogens and reducing the environmental impact of livestock [52]. The use of probiotics, which can be an alternative to chemotherapeutics and pharmaceuticals and can work individually or in combination to support the host and its efficiency in various ways, is an important approach to fulfilling these objectives, such as:

2.7.1.1 Inhibition of pathogens by the manufacture of antagonistic compounds, competition for binding sites, competition for nutrients and alteration of pathogens' enzymatic operation.

2.7.1.2 Immuno-stimulation and increased natural resistance to infectious diseases in the gastrointestinal tract and against cancer.

2.7.1.3 Nutritional advantages, such as improving the digestibility of food and feed by developing exoenzymes such as phytase, amylase, lactase, etc.

2.7.1.4 Increased milk yield and quality, meat quality and improved egg production.

Over the last decade, several studies on probiotics in aquaculture have been reported. Nevertheless, some possible benefits linked to the administering of probiotics have already been suggested as: (1) competitive exclusion of pathogenic bacteria (2) source of nutrients and enzymatic contribution to digestion (3) direct uptake of dissolved organic material mediated by the bacteria (4) enhancement of the immune response against pathogenic microorganisms and (5) antiviral effects [53].

Some species are suggested as biological control agents in aquaculture that belong to the lactic acid bacteria and yeast bacteria (Table 2.1).

Table 2.1 Probiotic applied in prawn cultivation

Probiotics	Used on	References
Bacillus sp. *Bacillus* sp. s11 *Lactobacillus* sp.	*Penaeus monodon*	[54]
Phaffia rhodozoma *Saccharomyces cerevisiae* *Saccharomyces exiguous*	*Litopenaeus vannamei*	[54]
Bacillus p64 *Vibrio hepatarius* *Vibrio* sp. *Vibrio* p62 *Vibrio* p63 *Pseudomonas* sp.	*Penaeus vanamei*	[53]
Bacillus cereus	*Penaeus monodon*	[55]
Lactobacillus plantarum	*Litopenaeus vannamei*	[56]
Bacillus subtilis *Bacillus licheniformis*	*Litopenaeus vannamei*	[57]

2.8 Immunostimulants

Immunostimulants cause or increase the activity of some of their components by stimulating the immune system. Immunostimulants can be used by injection, bathing, or orally. It will decrease the dose necessary for the vaccine. Therefore, it is the choice to antibiotics, which would improve the immune system of cultured organisms and effectively counter the attack of pathogens [58].

Natural plant products provide different activities, i.e., anti-stress, antipathogenic, and immune inducer. The plant materials are broadly used in aquacultural industries for preventing pathogenic diseases as an eco-friendly material [58].

2.9 Mode of Plants Action as Immunostimulants in Aquaculture

Plants have been used for thousands of years as immunostimulants. They have potential in aquaculture as natural and innocent compounds as an alternative to antibiotics. Immunoprophylactic is easy to use, and they are friendly with the environment and have few side effects. Plants demonstrate their key properties to the host immune system as growth promoters, immune enhancers, antibacterial and antiviral agents, stimulating elements of appetite, and anti-stress. Phenolic, polyphenolic, alkaloid, quinone, terpenoid, lectin, and polypeptide compounds are found in many plants, several of which are useful alternatives to antibiotics, pesticides, vaccines, and other synthetic compounds [59].

2.9.1 Improve the innate immune response

Several plants showed antimicrobial activity and enhanced growth and immune system of various aquatic. Therefore, there is a growing interest in using plants as immunostimulants in aquaculture. The use of immunostimulants increases interest in boosting the defense mechanisms and protecting aquatic from infectious diseases. The major components of the innate immune system are macrophages, monocytes, granulocytes, and humoral elements, like lysozyme. Many active components are available, such as polysaccharides, alkaloids, or flavonoids [59].

2.9.2 Enhance antimicrobial activity

The antimicrobial properties of plants and their active compounds are reported. Against both gram-positive and gram-negative bacteria, plant extracts have the

greatest antibacterial activity. They can also be used to treat serious diseases caused by viruses, fungi, and parasites. Several plant-extracted compounds inhibited gram-positive and gram-negative growth. In the host immune system, immunostimulants function as antiviral agents. Plant active compounds are known to inhibit or block the transcription of the virus to decrease replication in the host cells and increase non-specific immunity. Plant extracts have been used in the treatment of lymphocytic disease viruses and parasitic diseases such as myxobolasis, trichodinosis, gyrodactylosis, argulosis, and aquatic scuticocliates [59].

2.9.3 Demonstrate promising antibiotics

The plants can be used as profitable antibiotics after challenging with pathogens, the survival rates of infected fish priority fed various immunostimulants, vaccines, and probiotics [59].

2.9.4 Enhance the growth, feed utilization, and nutrient digestibility

Plants have been proven as promoters of growth. It increases digestive enzymes and enhances the survival and growth rates of aquatic animals. When diets supplemented with plant extract were fed to the prawns, the amount of vitamin C and E in the hepatopancreas, both the sodium and potassium levels, and the muscle of freshwater prawns increased [59].

2.10 Giant Freshwater Prawn (*Macrobrachium rosenbergii*)

M. rosenbergii is a large freshwater prawn native to the Indo-West Pacific from northwest India to Vietnam, Philippines, New Guinea, and northern Australia. It has been distributed into many countries for aquaculture.

2.10.1 Taxonomic and biological features

M. rosenbergii is the largest prawn in *Macrobrachium* species. The maximum recorded size for males and females is 33 cm and 29 cm, respectively. The cephalothorax consists of 5 indistinct segments in the head and eight in the thoracic region. The abdomen has six distinct segmented movable terga. Each abdominal segment has a pair of biramous pleopods (swimming legs), eyes, Cephalothorax with 2 pairs of antennae, 3 pairs of jaws, 3 pairs of maxillipids, 5 pairs of walking legs. The second and largest abdominal tergum laterally overlaps the first and the third. Dorsal surface of

abdominal carapace smooth and rounded. The rostrum is slender and curved upwards. The rostrum extends beyond the antennal scale and has 11-14 dorsal teeth and 8-10 on the ventral side [60].

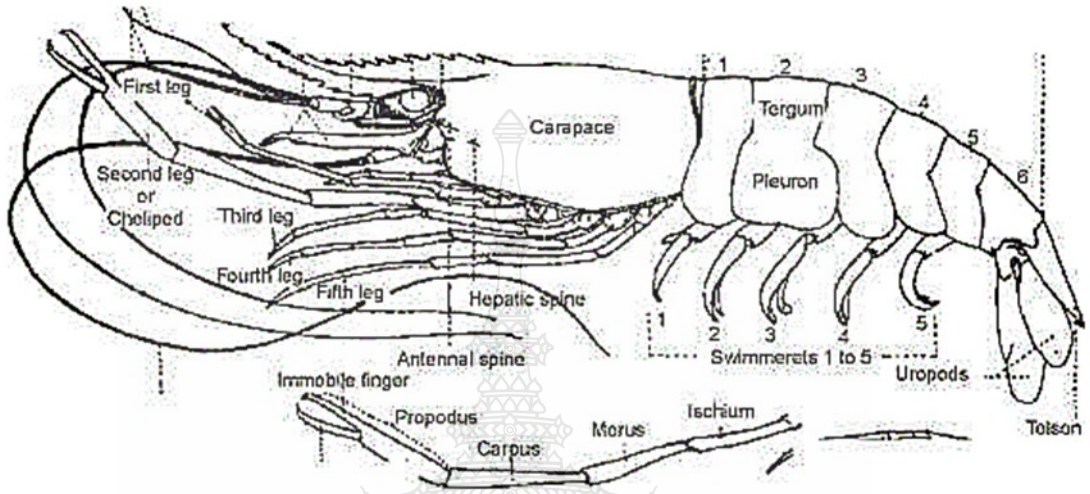

Figure 2.3 The external taxonomy of *M. rosenbergii*
Source: Jayachandran. 2001 [60]

The taxonomic tree was following;

Domain: Eukaryota
Kingdom: Metazoa
Phylum: Arthropoda
Subphylum: Crustacea
Class: Malacostraca
Subclass: Eumalacostraca
Order: Decapoda
Suborder: Natantia
Unknown: Palaeomonoidea
Family: Palaemonidae
Genus: Macrobrachium
Species: *Macrobrachium rosenbergii*

2.10.2 Coloration

The colors of *M. rosenbergii* are very dependent on the place that prawns are found, but the body can be greenish-grey. In small individuals, delicate striping on the cephalothorax can be seen but these markings are not apparent in tank-reared specimens. The chelipeds of dominant males are bright blue but more yellowish in non-dominant males and females. The ventral side is pale and translucent [60].

Figure 2.4 Giant freshwater prawn (*M. rosenbergii*)

Source: Jayachandran. 2001 [60]

2.11 Shrimp Aquaculture

Aquaculture is growing more rapidly than the food of animal-producing sector. This sector has resulted in increasing requirements for vast aquatic areas. The disease from the pathogen is one of the most troubling for aquaculture. In the development of the aquaculture industry, disease is the single most significant limiting factor. Aquaculture practices encounter many challenges, and one of the most devastating problems is disease outbreaks caused by microbial pathogens. The control disease uses chemotherapeutics and antibiotics, which leads to residual problems in the surrounding environment affecting higher animals and humans [61]. Diseases have caused enormous economic losses and have become the most significant issue that affects the growth of shrimp. In the shrimp aquaculture industry, bacterial infections have caused tremendous economic losses and prevented sustainable growth in the international economy. Viruses and Diseases bacteria are major pathogens that contribute to over 80 % of shrimp disease, while fungi and parasites are much lower. The mechanisms for host defense

against successful strategies to avoid disease outbreaks and invading pathogens are important. Shrimp rely on the innate immune system to protect themselves against disease outbreaks from a variety of microbes across cellular and humoral immune responses by detecting and attacking them [61].

2.12 The Immune System in Crustaceans

Hematopoiesis is a complex process. The different blood cells are formed and released from hematopoietic tissues of extensive invertebrates. Among the vertebrate blood cells, granulocytes, monocytes, and macrophages are mainly involved in innate immune responses and tissue repair. Blood cells usually are referred to as hemocytes. Hematopoiesis in invertebrates provides a basic model system to study the regulation of the blood cells of the innate immune system due to their lack of oxygen-carrying erythrocytes and blood cells of the lymphoid lineage involved in adaptive immune defense [62]. Crustaceans have an open circulatory system where the hemolymph distributes nutrients, oxygen, hormones, and cells. The hemocytes (circulating cells) could be functionally analogous to vertebrate leukocytes because they are mainly involved in non-self-matter recognition and elimination, and downstream coagulation [63].

2.13 Hemocytes in Crustaceans and Role of Immunity

Three main types of hemocytes were identified in most decapod crustaceans: hyaline cells (HCs), semigranular cells (SGCs), and granular cells (GCs). The HCs are small and do not contain granules, or very few, and may act as phagocytes (Figure 2.5A). The SGCs have a variable number of small eosinophilic granules and are involved in early recognition, coagulation, extent, and phagocytizes. It is the main hemocyte type involved in the encapsulation of microorganisms. Encapsulation reactions are usually followed by melanization and the proPO system (Figure 2.5B). However, GCs are densely packed with large eosinophilic granules and release their contents through exocytosis on activation, which are the key reservoirs for the proPO system (Figure 2.5C). Apart from the proPO system, the granules contain different antimicrobial peptides, seven various

proteinase inhibitors, ten and the cell adhesion/degranulating factor peroxinectin, a homologue of vertebrate myeloperoxidase [62].

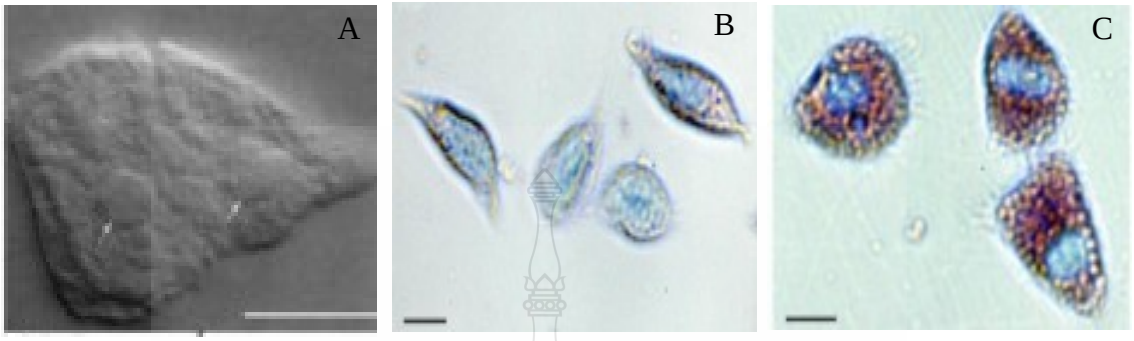

Figure 2.5 Hemocytes of crustaceans; Hyaline cells (A), Semigranular cells (B), and Granular cells (C)

Source: Lin and Söderhäll. 2011 [62]

2.14 Defense Mechanisms in Crustaceans

2.14.1 Pattern recognition proteins of crustaceans

Microorganisms have molecules in their different cell walls, such as lipopolysaccharides (LPSs) or peptidoglycans (PGNs) in bacterial cell walls and β-1,3-glucans in fungal cell walls, which are not found in other multicellular organisms. The proPO system, through proteolytic processes and phagocytosis, could be achieved by specific interaction with cellular receptors or by opsonization, which facilitate phagocytosis by crustacean hemocytes [63].

2.14.2 Lectins

The important characteristic of invertebrate immunity is distinguishing between the self and non-self-particles. Molecules with a recognizing function have been obtained in unicellular organisms. These proteins are considered functional precursors of antibodies and constituted as groups of proteins generically denominated lectins. Lectins have the property of recognizing specifically the carbohydrates from the membrane or surface of cells and, consequently, can induce agglutination of these cells or lead to diverse cellular events, such as phagocytosis acting as opsonins [63].

2.14.3 Melanization

The melanization pathway is activated by the prophenoloxidase (proPO) system, which is a principal innate immune response in shrimp. The binding of PRPs to corresponding microbial cell wall components activates the serine proteinase cascade which subsequently activates the final proteinases, called proPO-activating enzymes (PPAEs). The PPAEs induce the inactive proPOs to active POs, leading to the initiation of melanin formation [63].

2.14.4 Prophenoloxidase system

The Prophenoloxidase (proPO) system is an important encapsulating mechanism of many higher invertebrates. The proPO is the zymogen form of phenoloxidase (PO) enzyme and has a molecular mass of 70–80 kDa. In more primitive animals, proPO is found either in free form in the hemolymph or deposited in the exoskeleton. When microbial LPS, glucans, or peptidoglycans are present, the coagulation cascade is triggered and/or changes to Ca^{2+} concentration or pH occur that activate a series of serine proteases present in the hemolymph (Figure 2.6). These activated serine proteases induce proPO to generate activated PO (molecular mass of 60–70 kDa) [64].

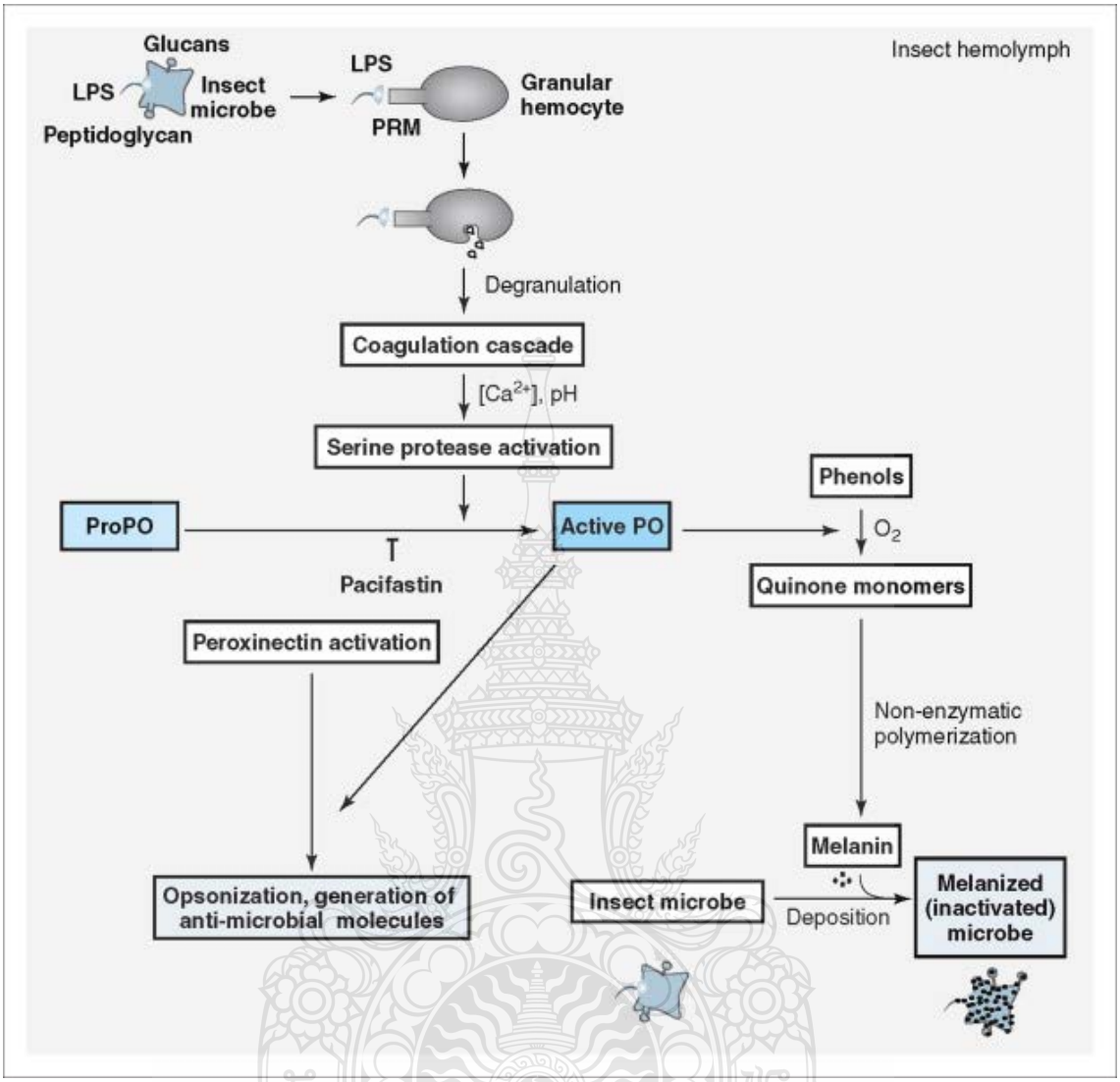

Figure 2.6 The proPO system in invertebrates

Source: Mak and Saunders. 2006 [64]

2.14.5 Phagocytosis

Phagocytosis is a process carried out by hemocytes (phagocytes) that can recognize and ingest non-self-molecules such as bacteria, spores, or senescent cells of the own organism. This immunity process has also been preserved during evolution. Some authors suggest that this mechanism is a precursor of vertebrate innate immunity [63]. Phagocytic cells are the most important cellular components of the innate immune system of animals. Phagocytes also produce toxic oxygen forms during a process called the respiratory burst. Phagocytic activity is a primitive defense mechanism and an important characteristic of the animal immune system. This parameter usually shows an increase after oral administration of immunostimulants [59].

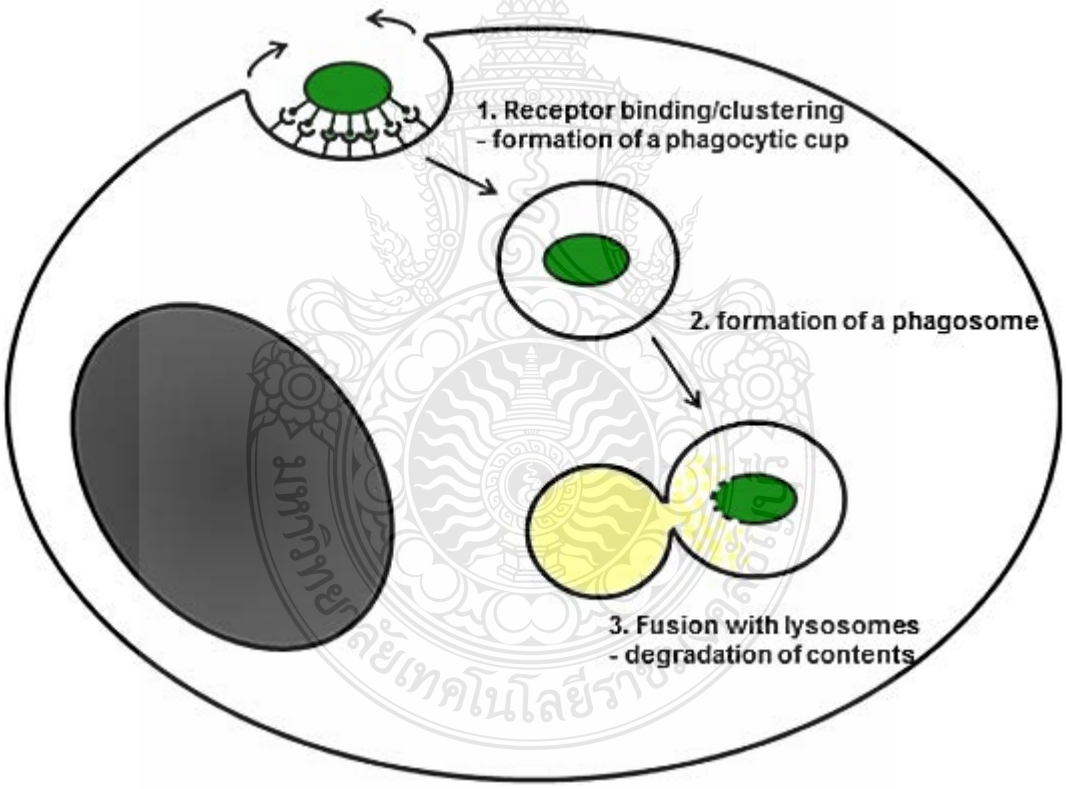

Figure 2.7 Three stages of phagocytosis; receptor binding and formation of a phagocytic cup, pinching-off and formation of a discreet phagosome and fusion with lysosomes

Source: Vazquez et al. 2009 [63]

2.14.6 Encapsulation

Encapsulation is a multicellular response to eliminate foreign particles that humoral mechanisms cannot destroy. This process kills pathogens or, at least, restricts their movement and growth in the hemocoel cavity. In vitro assays on encapsulation in *Astacus leptodactylus* showed semi-granular hemocyte aggregation and the presence of adhesive factors surrounding particles larger than 10 mm. in diameter [63].

2.14.7 Clottable protein

Clottable proteins, which are defense molecules in invertebrates possess multifunctional properties to prevent hemolymph loss in case of injury. Moreover, these molecules favor recognition in some crustaceans. Hemolymph clotting is based on the direct transglutaminase-mediated cross-linking of clottable proteins in the presence of Ca^{2+} [63].

Table 2.2 Effector defense mechanisms in crustaceans [63]

Defense mechanisms	Cellular population involved	Targets
proPO	Semigranulocytes, hemocytes with big refractile granules	Bacteria and fungi
Antimicrobial proteins	Hemocytes with granules	Bacteria and fungi
Phagocytosis	Hyalinocytes, semigranulocytes	Bacteria and microorganisms <10 μm
Encapsulation	Semigranulocytes hemocytes with big refractile granules	Fungal spore and yeast, organism >10 μm
Lectins	Hyalinocytes, semigranulocytes, and hemocytes with refractile granules	Distinguish between the self and non-self particles, inducing agglutination and phagocytosis
Clottable protein	Clottable proteins from hemocytes	Bacteria and fungi

2.15 Reviews of the Literature

Chiu et al. [56] have reported the supplementation of probiotic (*Lactobacillus plantarum* 7-40) in white shrimp feed (*Litopenaues vannamei*) at different viable cell numbers of probiotic. The pathogen, *Vibrio alginolyticus*, was injected into prawn to examine the immune responses of prawn. The results showed that prawn fed with 10 CFU/kg probiotic reduced 20 percent of mortality rate compared with the control diet after 1-week pathogen injection. There were also substantial increases in phenoloxidase (PO), superoxide dismutase (SOD) and prophenoloxidase (proPO) production in probiotic-fed prawns.

Mordi et al. [65] studied the oils extraction from two varieties of Nigeria banana peels by methanol. The crude methanolic-extract was studied the phytochemical, which revealed steroids, saponin, terpenoids, anthraquinones, and tannins. The extracted oils were effective against some bacteria. The chemical constituents of the oils were identified

and characterized by GC-MS. The fatty acids (stearic, palmitic, oleic and linoleic acids) and their methyl esters (2,3-dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one,5-(hydroxymethyl)-2-furancarboxyaldehyde, cyclododecane, dibutyl phthalate, β-sitosterol, sesamin, and epi-sesamin) were found and defined the biological and medicinal activity.

Rattanavichai and Cheng [2] evaluated the hot water extracts of banana peel, *Musa acuminata*, antibacterial activity to pathogens from aquatic animals, and immunostimulant potential, disease resistance and anti-hypothermal stress in giant freshwater prawn, *Macrobrachium rosenbergii*. Banana peel extract (BPE) has shown strong activity against 1 Gram-positive and 3 Gram-negative pathogens, including *Lactococcus garvieae*, *Vibrio alginolyticus*, *Photobacteria damsella*, and *Vibrio parahemolyticus* by disk diffusion method. The lowest dosages of inhibition against *L. garvieae*, *P. damsella*, *V. parahemolyticus* and *V. alginolyticus* were 31.25, 62.5, 125.0 and 250 mg/disc, respectively.

Muang-rat et al. [66] examined the total phenolic compounds extraction from dried Kluai Homthong peels using the subcritical solvent extraction method. The factors affecting total phenolic compounds extraction were investigated, such as the type of solvents, the ratio of dried Kluai Homthong and solvent, extraction temperatures, and extraction time. The results showed that the mixture of water-ethanol ratio 1: 1 was a suitable solvent for extraction. The highest amount of total phenolic compounds were obtained from dried Kluai Homthong peels. The total phenolic compounds were decreased when increasing extraction temperature more than 100 °C and incubation time longer than 15 min. The ratio of dried Kluai Homthong peels to water-ethanol solvent was suitable at 1.25 g/mL. The total phenolic extract concentration that could inhibit 50 % of DPPH was 1.40 mg/ mL.

Rattanavichai and Cheng [67] studied the effect of prawns feeding with diets containing banana peels extract to *M. acuminata* at 0, 1, 3, and 6 g/ kg after 120 days. The results showed that the dietary BPE at 6.0 g/ kg could promote the growth, anti-hypothermal stress, and enhance immunity and resistance against *L. garvieae* in *M. rosenbergii*.

Mohan et al. [68] reported the effect of dietary supplementation of *Ganoderma lucidum* (GLP) on growth, innate immune responses, and disease resistance of the freshwater prawn against *A. hydrophila* infection with different concentration of GLP for 15 days. The results showed that the GLP at 1.5 g/kg could increase survival, growth performance, immunity, and decreased mortality of fed prawns.

Lee et al. [69] investigated the immune parameters and signaling pathways of innate immune responses of *L. vannamei*, resistance to *V. alginolyticus*, and tolerance to hypothermia following injection with the hot-water extract of fresh cacao pod husks (CPHs). The result demonstrated that shrimp were injected with fresh CPHs extract at 40 μg/ shrimp was enhanced immunological and physiological responses. In addition, shrimp could be protected from *V. alginolyticus* and tolerance to hypothermal stress.

CHAPTER 3
MATERIALS AND METHODS

3.1 Materials

3.1.1 Instruments

3.1.1.1 24- well microplate (Thermo Scientific, China)

3.1.1.2 96-well microplate (Thermo Scientific, China)

3.1.1.3 Airstone

3.1.1.4 Aluminums foil (Renolds metal, Thailand)

3.1.1.5 Analytical balance (PL3002, Mettler Toledo, Thailand)

3.1.1.6 Aquarium air pump (8000, Sonic, China)

3.1.1.7 Autoclave (NB-1080, N-BIOTEK, Korea)

3.1.1.8 Beaker (Pyrex, USA)

3.1.1.9 Caps and septum (National Scientific, USA)

3.1.1.10 Centrifuge (3-18KS, Sigma, Germany)

3.1.1.11 Centrifuge tube (Isolab, Germany)

3.1.1.12 Cylinder (Isolab, Germany)

3.1.1.13 Disposable syringe (Nipro, Thailand)

3.1.1.14 Duran bottle (Duran, Germany)

3.1.1.15 Electronic grinder (MX-AC400, Panasonic, Japan)

3.1.1.16 Erlenmeyer flask (Pyrex, USA)

3.1.1.17 Filter paper (Whatman, United Kingdom)

3.1.1.18 Fourier-transform infrared spectroscopy (FTIR) (iD7 ATR, Thermo scientific, China)

3.1.1.19 Gas chromatography (GCMS-3.1.18 QP2010 SE, Bara scientific, Japan)

3.1.1.20 Glass aquarium

3.1.1.21 Glass vial (National Scientific, USA)

3.1.1.22 Hemocytometer (BOECO, Germany)

3.1.1.23 Hand tally counter (OfficeMate, Thailand)

3.1.1.24 Hot air oven (Thermotec 2000 oven, CONTHERM, Neuenstein)

3.1.1.25 Hypodermic needle (Nipro, Thailand)

3.1.1.26 Incubator (JSGI-250T, JSR, Thailand)

3.1.1.27 Insulin terumo syringe (Nipro, Thailand)

3.1.1.28 Light microscope (Olympus, Japan)

3.1.1.29 Micropipette (Gilson, USA)

3.1.1.30 Microplate reader (EZ Read 2000, Biochrom, USA)

3.1.1.31 Microscope slide and cover glass

3.1.1.32 Microtube (ExtraGene, Taiwan)

3.1.1.33 Oxygen line

3.1.1.34 Plastic plate (Hycon plastic, Thailand)

3.1.1.35 Pond

3.1.1.36 Precision balance (K N science innovation Co., Lyd, Thailand)

3.1.1.37 Spectrophotometry (DR 6000, HACH, Germany)

3.1.1.38 Sterile cotton stick (Extra plus, Thailand)

3.1.1.39 Sterile water for injection (A.N.B. Labaratories Co., Ltd, Thailand)

3.1.1.40 Strainer

3.1.1.41 Straining cloth

3.1.1.42 Test tube (Pyrex, USA)

3.1.1.43 Timer

3.1.1.44 Vacuum rotary evaporator (B-300 Base, BUCHI, China)

3.1.1.45 Volumetric flask (Favorit, Malaysia)

3.1.1.46 Water bath (FALC, Italy)

3.1.2 Chemical reagents and culture media

3.1.2.1 2,2-diphenyl-1-picrylhydrazyl (DPPH) (SRL, India)

3.1.2.2 2,4,6-Tri(2-pyridy)-s-triazine (TPTZ) (ACROS organics, Belgium)

3.1.2.3 Absolute ethanol (C_2H_5OH) for HPLC-plus-gradient (CARLO ERBA, Thailand)

3.1.2.4 Acetic acid Glacial (CH_3COOH) AR grade (QRec, New Zealand)

3.1.2.5 Agar (HIMEDIA, India)

3.1.2.6 Banana peel extract

3.1.2.7 Beef extract (SRL, India)

3.1.2.8 Bio-Rad Protein Assay Kit (BIO-RAD, United Kingdom)

3.1.2.9 Bovine serum albumin (BSA) (SIGMA, Germany)

3.1.2.10 Calcium chloride ($CaCl_2$) (UNIVAR, USA)

3.1.2.11 Ethylene diamine tetra acetic acid; EDTA ($C_{10}H_{16}N_2O_8$) (RCI Lacscan, Thailand

3.1.2.12 Folin-ciocalteu's phenol reagent (LOBAChemie, India)

3.1.2.13 Gallic acid (SIGMA, Germany)

3.1.2.14 Hydro chloric acid 37 % Grade AR (QRec, New Zealand)

3.1.2.15 Iron (III) chloride RPE ($FeCl_3$) (RCI Labscan, Thailand)

3.1.2.16 Methanol (CH_3OH) AR Grade (VWR international, USA)

3.1.2.17 Methanol (CH_3OH) HPLC grade (RCI Labscan, Thailand)

3.1.2.18 Peptone (SRL, India)

3.1.2.19 Prawn meal (CPF, Thailand)

3.1.2.20 Soda water (SINGHA Corporation, Thailand)

3.1.2.21 Sodium acetate ($C_2H_3NaO_2$) (UNIVAR, USA)

3.1.2.22 Sodium carbonate ($NaCO_3$) (LOBA Chemie, India)

3.1.2.23 Sodium chloride (NaCl) (UNIVAR, USA)

3.1.2.24 Sodium citrate ($C_2H_3NaO_2$) (KEMAUS, Australia)

3.1.2.25 Sodium di-hydrogen phosphate ($NaH_2PO_4 \cdot 2H_2O$) (UNIVAR, USA)

3.1.2.26 Sodium hydrogen phosphate (Na_2HPO_4) (UNIVAR, USA)

3.1.2.27 Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) (ACROS organics, Belgium)

3.2 Microorganisms

3.2.1 *Aeromonas hydrophila*

3.2.4 *Lactobacillus plantarum* 7-40

3.2.3 *Staphylococcus aureus*

3.2.4 *Vibrio parahaemolyticus* TISTR 1596

3.3 Animal

3.3.1 Giant freshwater prawn (*Macrobrachium rosenbergii*)

3.4 Methods

3.4.1 The extraction of phenolic compounds from organic BP

3.4.1.1 Selection of organic BP having high phenolic content and related properties

(1) Location of organic banana farm and harvesting

The organic banana was cultured by the organic process and farming at the Fungkajorn Garden Organic Farm, Nong Suea, Pathum Thani Province, Thailand, which located at GPS: 14°10'12.6"N 100°48'00.4"E.

(2) Preparation of organic BP

The six most common banana strains found in Thailand; Kluai Kai, Kluai Homthong, Kluai Homtaiwan, Kluai Lebmuernang, Kluai Hukmook, and Kluai Namwa were collected in the raw stage. The raw banana was matured in a plastic box until the peel color index (PCI) reached a level of 7, colored yellow with brown speckles [70]. Maturated banana fruits were cleaned with tap water, rinsed twice to remove the dust and other extraneous materials adhering to them, and soaked in soda water for 5 min [2]. The washed BP was separated, cut into small pieces, and hot air-dried at 50 ℃ until moisture content decreased to 10 % approximately.

The dried BP was ground into a powder with an electronic grinder and stored at 4 ℃ for further experiments.

(3) Extraction of organic BP

Ten grams of dried BP powder was added with 100 mL of 50 % v/v methanol. The extraction was performed at 55 ℃ for 120 min in a water bath with continuously stirred. The mixture was left and precipitated at room temperature. The sample was centrifuged to collect the supernatant at 5,000 rpm for 20 min. The methanol in obtained supernatant was then removed using a vacuum rotary evaporator until completely evaporating. The sticky crude extracted was collected from the extraction bottle by redissolved with H_2O and kept at -20 ℃ for the next experiments.

3.4.1.2 Optimization of organic BP extraction process

The BP which containing the highest total phenolic content, antioxidant and antipathogenic activity was selected to optimize the extracting condition. Three independent parameters of the optimization experiment were methanol concentration, extraction time, and temperature. The experiments were performed with one factor at a time technique with triplicates.

The independent parameters consist of the following:

(1) Concentration of methanol was 0, 50, and 100 % v/v

(2) Extraction time was 10, 20, and 30 minutes

(3) Temperature was 50, 75, and 100 ℃

After that, the optimum condition of organic BP was investigated the bioactive compound properties (total phenolic content, antioxidant activity and ferric reducing antioxidant power), antipathogenic activity and phytochemical (GC-MS and FTIR), respectively.

3.4.1.3 Scaling up of organic BP extraction and powdering

One hundred grams of dried BP powder were extracted following the previous optimal extracting condition. Then, the mixture was separated by centrifugation at 5000 rpm at 4 ℃ for 20 min. The supernatant was evaporated by a vacuum rotary evaporator, lyophilized and stored at -20 ℃. Moreover, the total phenolic content (TPC), antioxidant activity, ferric reducing antioxidant power (FRAP),

antipathogenic activity, and phytochemical were also investigated. The experiments were triplicates.

3.4.2 Bioactive compound properties assay

The amount of total phenolic content in crude organic BPE was measured by modifying the method of Singleton and Rossi [71]. The antioxidant activity was determined by the DPPH method according to Blios [72]. The ferric reducing antioxidant power (FRAP) was evaluated following Benzie and Strain [73].

3.4.2.1 Total phenolic and antioxidant

(1) Total phenolic content (TPC)

The amount of total phenolic content was determined by a modified method of Singleton and Rossi [71]. The gallic acid was used as a standard. Briefly, 200 µL of organic BPE was taken in the test tube and added 1 mL of 10 % v/v Folin-ciocalteu, and incubated for 5 min. Finally, 0.8 ml of 7.5 % Na_2CO_3 was mixed and incubated in dark condition for 30 min. The absorbance is measured at 765 nm. The results were then calculated as mg of gallic acid equivalents (GAE) per g of dry matter (mg GAE/g DM).

(2) Antioxidant activity

The antioxidant activity was measured using the DPPH cording to Blios [72]. The trolox was used as a standard. The 20 µL of sample was mixed with 180 µL of DPPH solution and then incubated in dark condition room temperature for 20 min. The absorbance was measured at 517 nm. The results were expressed as µg trolox equivalents per g of dry matter (mg TE/g DM).

(3) Ferric reducing antioxidant power (FRAP)

The FRAP of the organic BPE assay was carried out according to the procedure of Benzie and Strain [73]. The reaction was performed using 300 mM of acetate buffer (pH 3.6), 10 mM TPTZ with 40 mM HCl, and 20 mM $FeCl_3 \cdot 6H_2O$. The working FRAP reagent was prepared by mixing acetate buffer pH 3.6, TPTZ and $FeCl_3 \cdot 6H_2O$ at the ratio of 10:1:1. Gallic acid was used as a standard. Twenty µL of BP extract and 180 µl of FRAP reagent are mixed in a 96-well microplate and incubated at room temperature for 30 min with light avoidance. The intensity of color

was measured at 593 nm. The FRAP was determined as mg gallic acid equivalents per g of dry matter (mg GAE/g DM).

3.4.3 Antipathogenic activity

The antimicrobial activity of crude organic BPE was analyzed by disc diffusion method [2] on the following pathogens: *A. hydrophila*, *S. aureus* and, *V. parahaemolyticus*. The pathogen was spread on nutrient agar (NA). The sterilized paper disc was soaked in BPE and put on the NA plate smeared with a pathogen. The water and methanol were experimented as control and incubated at 37°C for 24 h. The antimicrobial activity of BPE was observed by the clear zone and reported as positive (+) or negative (-) results.

3.4.4 Phytochemical analysis

3.4.4.1 GC-MS analysis

The phytochemical components were detected by gas chromatography-mass spectroscopy (GC-MS) (GC-MSQP2010 SE, Shimadzu). The chromatography column was the DB-5MS (30 m x 0.25 mm i.d., 0.25 µm film thickness). The injector temperature was operated at 220 °C. The oven temperature was programmed at 45 °C, held for 5 min, increased to 60 °C at 2 °C/min, increased to 220 °C at 3 °C/min, and held for 10 min. The carrier gas was helium. The detector was mass spectrometry.

3.4.4.2 FTIR analysis

The FTIR (iD7ATR, Thermo Scientific) was performed to specify the functional groups of phytochemicals of different crude organic BPEs. The translucent sample discs were prepared by mixing 10 mg KBr salt and 1 mg freeze-dried powder of different BPE. The mixtures were packed in FTIR spectroscope (Shimadzu, Japan, 4 cm^{-1} resolution), having a frequency range from 4000 to 400 cm^{-1}.

3.4.5 Injection of organic BPE on the immune parameters of *M. rosenbergii*

3.4.5.1. Experimental prawn

Prawn, *M. rosenbergii* used in this study were obtained from Kalasin University, Kalasin province, Thailand and were acclimated at 28 ± 1 °C in laboratory cement tanks, pH 7.0-7.5 and fed with a control diet twice daily for two weeks before experimentation.

3.4.5.2 *A. hydrophila* stock culture

In this study, *A. hydrophila* was used as a pathogen. It was kept at -80 °C was cultured in nutrient broth containing 1 % w/v of NaCl. The strain was centrifuged at 10,000 rpm at 4 °C for 10 min. The pellet was washed twice with 0.85 % w/v of NaCl before using in challenge experimentation. The bacterial pellet was re-suspended in sterile water for injection for susceptibility and phagocytic activity. The viable cell number of the pathogen was 6.8×10^7 CFU/mL.

3.4.5.3 Experimental design

Injection of organic BPE to *M. rosenbergii* was conducted to study the immune response and mortality. Ten prawns were tested and performed in triplicate. Twelve of the aquarium glass tank (size of 15 L) containing 10 L of aerated freshwater were operated in this study. Ten prawns were added to each aquarium glass tank. During the experiments, prawns were fed twice daily with a commercial prawn diet, and the water temperature was maintained at 28 ± 1.0 °C. The four different organic BPE dosages were directly injected into prawns for 0 (control), 6, 12, and 24 µg of organic BPE per gram prawn. The sterile water was used as an experimental control. Six prawns (6.1 ± 2.6 g) per replicate were randomly collected to determine the immune response after 1, 3, and 6 days of the organic BPE injection. The prawn in the first, second, and third experiment was sampled to determine the total hemocyte count (THC) and different hemocyte count (DHC), coagulation time, and total protein, respectively. The fourth and fifth experiments were tested with BPE injection with a prawn weight of about 7.5 ± 0.1 g, and a challenge by the pathogen. The challenged phagocytic activity and mortality were measured, respectively.

3.4.5.4 Collection of hemolymph

The hemolymph was individually withdrawn from the ventral sinus cavity for each experimental group of prawns using a 1 mL sterile disposable syringe (26-guage needle) mixed with anticoagulant solution. (0.8 g sodium citrate and 0.34 g EDTA in 100 mL of distilled water, at pH 7.45 with the osmolality adjusted to 490 mOsm/kg with NaCl)

3.4.5.5 Susceptibility experiment

Prawns (7.5 ± 1.0 g) were injected with 20 μL of the organic BPE dissolved in sterile water. The organic BPE solution was directly delivered into the ventral sinus of the cephalothorax of the prawn for 6, 12, and 24 μg/g prawn. Prawn injected with an equal volume of sterile water for injection served as the challenged control. After injection, the prawn was held in a 15 L aquarium glass tank containing 10 L of aerated freshwater at 28 ± 1 °C. Subsequently, challenge tests were conducted by injecting 20 μL of *A. hydrophila* suspension into the ventral sinus of the cephalothorax. The experiments were comprised of ten prawns per replicate and observed the susceptibility on 6^{th} day

3.4.5.6 Immune response parameters

(1) Total hemocyte count (THC) and Different hemocyte count (DHC)

One hundred microliters of hemolymph were placed into sterilized microtubes containing an anticoagulant buffer of 0.9 mL (0.8 g sodium citrate and 0.34 g EDTA in 100 mL distilled water, at pH 7.45 with the osmolality adjusted to 490 mOsm/kg with NaCl) for the THC and DHC. A mix of the anticoagulant hemolymph mixture was placed on a hemocytometer to measure the THC and DHC using a light microscope (Olympus, Japan) and counted manually in all 25 squares (0.1 mm^3) and calculated by using the formula;

THC (x 10^6 cell/mL) = counted cells x 50 x 1000 x dilution factor

(2) Coagulation time

The coagulation time of hemolymph was measured using the method of Jussila et al. [74]. Briefly, 270 µL of hemolymph mixed with 30 µL of 20 mM $CaCl_2$ in a sterilized microtube and continuously turned up and down in slow motion every 5 sec. The movement was continually repeated until the coagulated hemolymph was observed, and the time was recorded.

(3) Total protein

The protein concentration of hemolymph was quantified with the method described by Bradford [75] using a Bio-Rad protein assay Kit (Bio-Rad Laboratories, USA) using bovine serum albumin (BSA) as the standard. The absorbance was measured at 595 nm and expressed as mg/L.

3.4.5.7 Phagocytic activity of prawns injected with organic BPE

Twenty microliters of organic BPE solution were directly injected into the ventral sinus of prawns. The prawns were then held in a separate aquarium glass tank containing 10 L of water at 28 ± 0.5 °C for 2 h. Subsequently, the pathogenic suspension was inoculated to the prawns. Two hundred microliters of hemolymph were collected and mixed with 200 µL of an anticoagulant solution for measuring the phagocytic activity describing by Li et al. [76]. The phagocytic activity was defined as the phagocytic rate (PR) as follows;

$$\text{PR (\%)} = \frac{\text{(phagocytic hemocytes)}}{\text{(total hemocytes)}} \times 100$$

3.4.6 Production of prawn feed contained organic BPE and probiotic powder

3.4.6.1 Microorganisms and starter preparation

Lactobacillus plantarum 7-40 [56] was used as a probiotic strain in this study. The probiotic was cultured in de Man, Rogosa and Sharpe (MRS) broth and kept at -80°C as a master stock. One milliliter of *L. plantarum* 7-40 was reactivated in 30 mL of MRS broth and incubated at 37 °C for 24 h. Subsequently, the cultured 7-40 strain was scaled up by transferring to 200 mL of MRS broth and incubated at the

previous condition. The culture was carried out as a starter for probiotic powder (PP) production.

3.4.6.2 Production of probiotic powder

The probiotic strains were grown separately in soybean meal (SBM) with solid-state fermentation (SSF). The SBM was mixed with water (ratio of 1:1) and sterilized at 121 °C for 15 min. The probiotic starter was inoculated into sterilized SBM, thoroughly mixed, and incubated at 37 °C for 24 hr. Subsequently, the fermented SBM (FSBM) was dried at 40 °C until the moisture content was lower than 10 % dry basis in a hot-air oven. The dried FSBM was then grounded by a grinder with 80 mesh size. The viable cell count (TVC) of PP was approx. 7.0 log CFU/g. The PP was stored at 4 °C before use in the next experiment.

3.4.6.3 Production prawn feed pellet mixed probiotic powder

The commercial prawn feed pellet (brand of CP 9093s) was used for feeding. Feed pellet was mixed with organic BPE and PP following Table 3.1. Each diet formula was dried in the air-drying cabinet until the moisture content was around 10 % dry basis.

Table 3.1 The mixing ratio of prawn feed pellet, organic BPE, and PP

Ingredients (g)	Control	Formula 1	Formula 2	Formula 3
Prawn feed pellet	1000	1000	1000	1000
PP[a,b]	-	-	10	10
Organic BPE[c]	-	6	-	6

[a]Probiotic powder, [b]TVC was 10^7 CFU/ kg, and [c]Banana peel extract

3.4.7 Examination of prawn culture with BP extract and probiotic powder supplementation

The prawns, *M. rosenbergii* from a farm in Kalasin province, were cultured in the laboratory for acclimating at 27-28 °C, acidity in the range of 7.0-7.5 for two weeks. During the acclimatization period, the prawn was fed with a control diet (commercial prawn feed pellet) twice daily before the experiment began. The prawns

were transported in a pond of 1000 L containing 700 L of chlorine-free water. The cultivation pond was aerated continuously throughout cultivation. Each experiment used 50 prawns per pond, conducted triplicate, and feeding for 90 days. The prawn was fed twice a day with a prawn feed pellet, and performed with 3 to 5 % of the weight prawn. The specific growth rate (SGR), survival rate (SR), and feed conversion ratio (FCR) were examined once time a month. The different feed pellet was provided to the prawn twice a day (Table 3.1). At 90 days, the prawns were transported to glass aquaria of 20 L added 15 L of the chlorine-free water with continuous aeration. The six prawns per replicate were chosen and conducted in triplicate. The prawn immunities included THC, DHC, coagulation time, and total protein, were determined. Finally, the prawns were challenged with *A. hydrophila* for phagocytic activity evaluation.

3.4.7.1 The growth performance parameters

The growth performance of the prawns was estimated using the following formula [67]:

$$\text{Percentage of weight gain} = \frac{\text{final body weight-initial body weight}}{\text{initial body weight}} \times 100$$

$$\text{Percentage length gain} = \frac{\text{final body length-initial body length}}{\text{initial body length}} \times 100$$

$$\text{Specific growth rate} = \frac{\log w_2 - \log w_1}{t} \times 100$$

where w_1 = initial weight (g)

w_2 = final initial (g)

t = duration of experiment in days

$$\text{Survival rate} = \frac{\text{number of live prawns}}{\text{number of prawns introduced}} \times 100$$

$$\text{Feed conversion ratio} = \frac{\text{feed intake (g)}}{\text{weight gain (g)}}$$

3.4.8 Statistical analysis

The results were presented as the mean ± standard deviation (SD) of triplicate experiments. Significant differences were determined by one-way analysis of variances (ANOVA) and comparing the investigation by Duncan multiple range test (DMRT). The data were normalized using logarithm transformation before study at $p < 0.05$ and performed by SPSS version 15.

3.5 Venue of the Study

3.5.1 Biotechnology Laboratory, Division of Biology, Faculty of Science and Technology, Rajamangala University of Technology Thanyaburi, Thanyaburi, Pathum Thani, Thailand.

3.5.2 Department of Fisheries Technology, Faculty of Agricultural Technology, Kalasin University, Kalasin, Thailand.

CHAPTER 4
RESULTS AND DISCUSSIONS

4.1 Extraction of Organic Banana Peel

The characteristics of fresh, hot air-dried, and powdered organic BP were illustrated in Figure 4.1(A)-4.1(C). The color of fresh organic BP changed from yellow to black and brown after hot air-drying, and powdering processes, respectively. Table 4.1 indicates the weight of BP before and after hot air-drying in the oven at 50 °C for 48 h. The moisture content of dried organic BP ranged from 5.2 to 7.3 % (wet basis), which depended on the thickness of each type. The moisture decreased from the surface of samples through capillary action. The unbound water was transported from the surface via material capillaries [77]. Next, the organic BP powder was extracted by 50 % v/v of methanol. Results showed that the maximum extraction yield was obtained from the BP of Kluai Homthong, for 3.50 g/10 g DW.

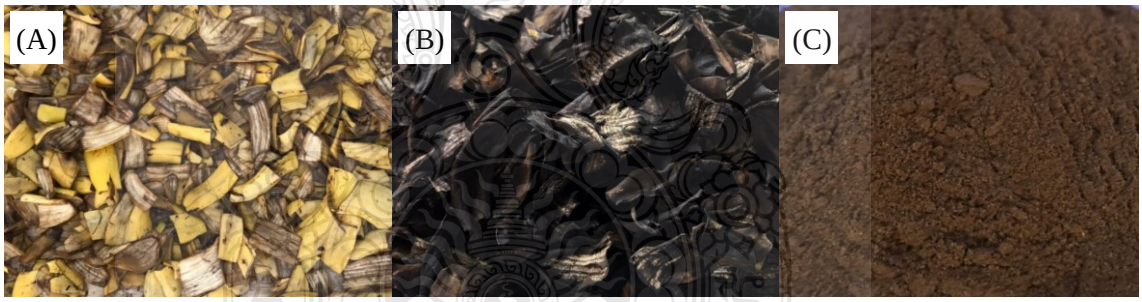

Figure 4.1 The characteristics of fresh organic BP (A), hot air-dried organic BP (B), and powdered organic BP (C)

Table 4.1 The weight of organic BP, moisture content, and extraction yield

Type of banana	Weight (g)		Moisture content (%)	Extraction yield (g/10 g DW)
	Fresh peel	Dry peel		
Kluai Kai	1,035.72	228.40	6.98	2.05^{bc}
Kluai Homthong	2,756.71	506.53	5.29	3.50^{a}
Kluai Homtaiwan	3,736.36	111.08	6.91	1.73^{cd}
Kluai Lebmuernang	1,027.68	120.21	4.99	2.09^{b}
Kluai Hukmook	5,902.21	615.22	7.30	1.49^{de}
Kluai Namwa	1,418.13	179.04	7.31	1.32^{e}

The different letters present significantly different at $\alpha \leq 0.05$

4.2 Antioxidant Assay of Organic BPE

4.2.1 Total phenolic content (TPC)

The TPC of obtained crude organic BPE is presented in Figure 4.2A. The results show that Kluai Kai provided the highest TPC for 7.82 ± 0.64 mg GAE/ g DM, followed by Kluai Homthong, Kluai Homtaiwan, Kluai Lebmuernang, and Kluai Hukmook (5.58 ± 0.60, 3.42 ± 0.50, 2.47 ± 0.05 and 2.12 ± 0.07 mg GAE/ g DM), respectively. While Kluai Namwa gave the lowest TPC for 1.38 ± 0.31 mg GAE/ g DM. These results demonstrated that the TPC depended on the type of banana. Gonzalez-Montelongo et al. [78] measured the total phenolic content of organic BPE, extracted by 50 % v/v of methanol at 55 °C for 120 min. The TPC was obtained for 16 and 17 mg GAE/g DW from two different varieties of Gruesa and Grande Naine (*M. acuminata* Colla AAA) [78]. This result suggests that the quantity and quality of phenolic compounds from plants are influenced by geographical origin, plant genetics, cultivar, soil composition, growing conditions, state of maturity, post-harvest processing, among other drying, and extraction methods [3, 4].

4.2.2 Antioxidant content

The amount of antioxidant content was determined by the DPPH radical-spectrophotometric method, according to Blios [72]. The maximum amount of antioxidant content of organic BPE was noticed in Kluai Kai (7.15 ± 1.30 mg TE/g DM), followed by Kluai Homthong, Kluai Homtaiwan, Kluai Namwa, and Kluai Lebmuernang (5.52 ± 0.05, 1.94 ± 0.17, 1.22 ± 0.24, and 1.02 ± 0.12 mg TE/ g DM), respectively (Figure 4.2B), while the minimum antioxidant content was achieved with Kluai Hukmook (0.97 ± 0.07 mg TE/ g DM). The antioxidant content of organic BPE in this study was lower than that of Gruesa and Grande Naine (9.4 and 8.8 mg TE/g DW, respectively) due to the difference in species and extraction method [78]. The antioxidant compounds were widely known to prevent or inhibit oxidative reduction in the human body. Moreover, antioxidants can retard aging, prevent coronary heart disease, cancer, and neurodegenerative disorders related to oxidative stress, caused by reactive oxygen species (ROS) [4]. Polyphenols are antioxidant compounds in a plant's innate defense system; the antioxidant content is synthesized under extreme conditions, i.e., temperature alterations, UV contact, and pathogenic infections [81].

4.2.3 Ferric reducing antioxidant power (FRAP)

Figure 4.2C presents the amount of FRAP of BPEs, analyzed with the method used by Benzie and Strain [73]. The maximum FRAP was received from the organic BPE of Kluai Kai for 2.74 ± 0.13 mg GAE/ g DM, followed by Kluai Homthong and Kluai Homtaiwan. However, the FRAP of Kluai Homtaiwan, Kluai Lebmuernang, Kluai Hukmook, and Kluai Namwa was not significantly different. Moreover, the FRAP of organic BPE allows reductants to be electron donors, which is able to convert them into more stable constituents and terminate the free radical reaction [82].

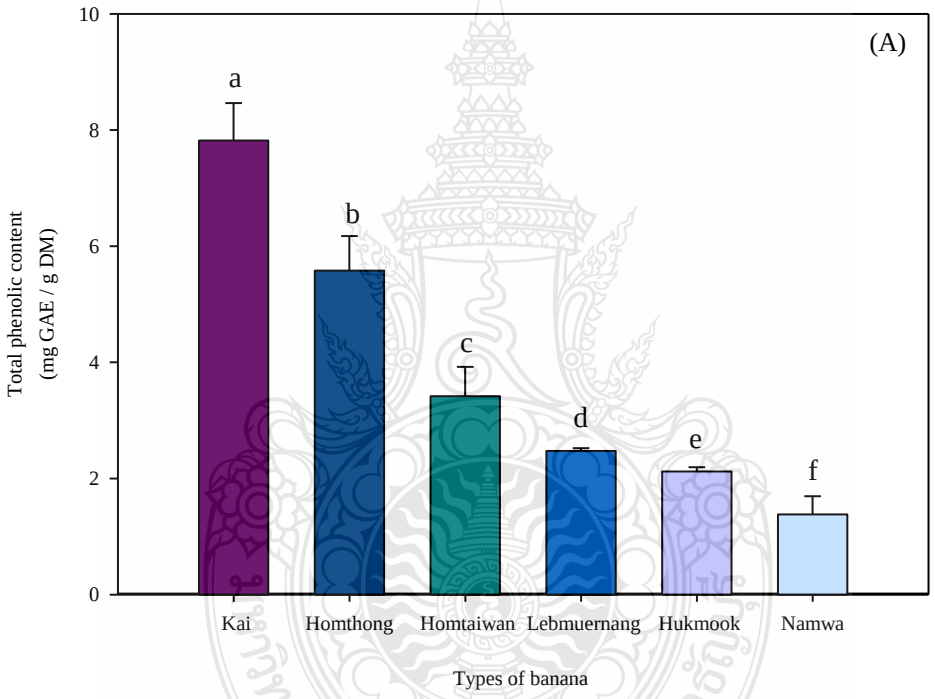

Figure 4.2 Total phenolic content (A) of organic BPEs. Mean values with different superscripts (a, b, c, d, e, and f) made a significant difference ($\alpha < 0.05$) with the error bar ± SD.

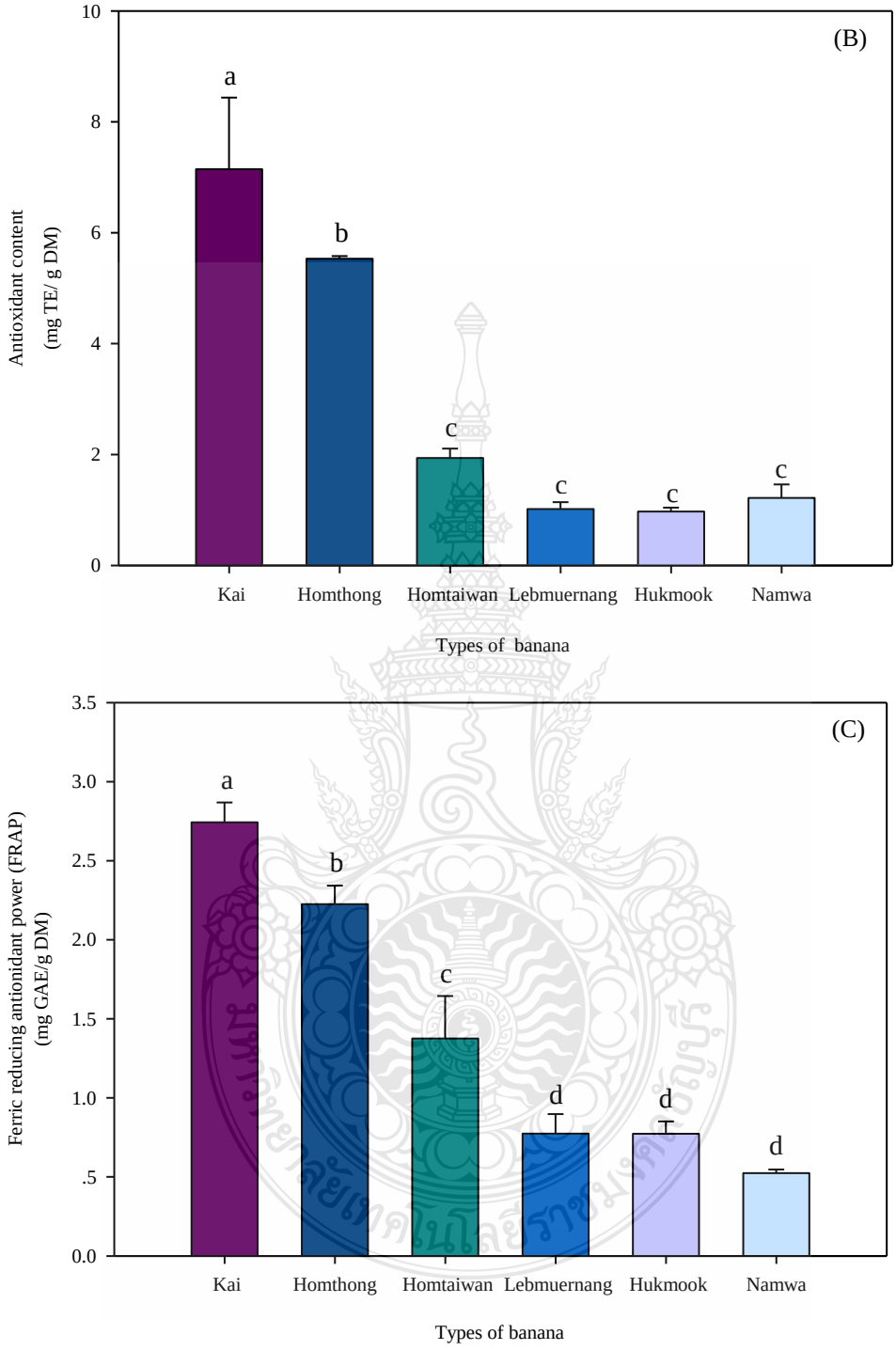

Figure 4.2 Antioxidant content (B) and ferric reducing antioxidant power (C) of organic BPEs. Mean values with different superscripts (a, b, c, and d) made a significant difference ($\alpha < 0.05$) with the error bar ± SD. (Continued)

4.3 Antipathogenic Activity

Three pathogens of aquatic animals were chosen to indicate the antimicrobial activity in this study. The inhibited zone of a pathogen by organic BPEs is presented in Table 4.2. The results demonstrate that *A. hydrophila* was inhibited by all organic BPEs except that of Kluai Kai. In addition, the organic BPE of Kluai Hukmook could also inhibit *S. aureus*, while *V. parahaemolyticus* could not be inhibited with any organic BPEs. It has been suggested that the antipathogenic activity of organic BPEs is related to the type of banana. The previous study reported on the antibacterial activity of BP (Musa, AAA cv. Cavendish), which shows that the BP used ethyl acetate to extract the inhibited *Bacillus subtilis, Bacillus cereus, Salmonella enteritidis, Escherichia coli,* and *S. aureus* [83]. However, Kluai Kai was showed the highest TPC, antioxidant content, and FRAP. However, the antipathogenic substances were lower than in Kluai Homthong. Normally, plant extracts have antioxidant and antibacterial properties. The hydroxyl group of the phenolic compound can have an inhibitory effect on target bacteria. Gram-negative bacteria are more sensitive than gram-positive bacteria, with differences in their cell wall structures. The gram-positive bacteria have a thick multilayer peptidoglycan cell wall, which is an obstacle to environmental materials, including natural matter and antibiotics. In contrast, the cells of gram-negative bacteria have single peptidoglycan in the outer layer. As such, gram-negative bacteria have a penetrability barrier lower than gram-positive bacteria [84].

Furthermore, gram-negative bacteria have a low resistance to physical disruption due to a weak cell wall structure. In a previous study, the extract of pomegranate peels, yellow lemon peels, orange peels, and BP containing phenolic compounds was responsible for excellent antimicrobial activities [84, 85, 86]. However, the modification of outer membrane permeability and porin mutation increase a resistant ability in gram-negative bacteria. On the other hand, this important layer is absent in gram-positive bacteria. Thereby, gram-negative bacteria can be more resistant to antibiotics and cause health problems than gram-positive ones [87].

Table 4.2 The antipathogenic inhibition of organic BPEs

Type of banana	Pathogen inhibition		
	A. hydrophila	*S. aureus*	*V. parahaemolyticus*
Kluai Kai	Negative	Negative	Negative
Kluai Homthong	Positive	Negative	Negative
Kluai Homtaiwan	Positive	Negative	Negative
Kluai Lebmuernang	Positive	Negative	Negative
Kluai Hukmook	Positive	Positive	Negative
Kluai Namwa	Positive	Negative	Negative

4.4 Phytochemicals in Organic BPE

4.4.1 GC-MS analysis

GC-MS was used to investigate the phytochemical composition in crude organic BPE of six banana types. The results of numerous organic BPEs by GC-MS contained 1 to 3 main chemical constituents. The different components of BPEs would be affected by the species of banana and cultivation conditions, maturity, and extraction method [46]. The main constituents were identified from the data of various organic BPEs and shown in Table 4.3, 4.4 and Figure 4.3. The major compounds were acetic acid, formic acid, 1,2-benzenediol,3-methyl-, and 4-hydroxy-2-methylacetophone. These major compounds have various biological activities, such as antibacterial, antifungal, and antioxidant properties. The acetic acid, formic acid, and 4-hydroxy-2-methylacetophone were the antibacterial and antifungal properties [13, 14, 15]. The 4-hydroxy-2-methylacetophone in *Musa acuminata* Colla demonstrated the inhibition on several bacteria strains: *Bacillus* spp., *Staphylococcus aureus*, *Pseudomonas* spp., *E. coli*, *Streptococcus* spp., *Klebsiella* spp., and *Proteus* spp. [65]. Acetic acid was shown to have good antibacterial activity against microorganisms, while formic acid functioned as an important intermediate in chemical synthesis, an antibacterial and preservative agent in livestock feed. The 1,2- benzenediol,3-methyl- is one of the phenolic compounds in the flavan-3-ol group, which also has antioxidant activity [13, 14].

Table 4.3 Bioactive compound from organic BPEs using GC-MS

Type of banana	Compounds*		Area (%)
	Common name	IUPAC	
Kluai Kai	Ethanoic acid	Acetic acid	43.61
	Methanoic acid	Formic acid	3.57
Kluai Homthong	Ethanoic acid	Acetic acid	49.94
	Methanoic acid	Formic acid	7.73
	3-methylcatechol	1,2- Benzenediol,3-methyl-	1.28
Kluai Homtaiwan	Ethanoic acid	Acetic acid	38.78
Kluai Lebmuernang	Ethanoic acid	Acetic acid	48.25
	3-methylcatechol	1,2- Benzenediol,3-methyl-	1.00
Kluai Hukmook	Ethanoic acid	Acetic acid	5.66
	2-methyl-5-(1-methylethyl) phenol	4-Hydroxy-2-methylacetophone	3.79
Kluai Namwa	Ethanoic acid	Acetic acid	25.64

*Only phytochemical constituents present biological activities were reported

Table 4.4 Name and properties from organic BPEs of GC-MS

Compounds	Common name	IUPAC
1	Ethanoic acid[a]	Acetic acid[a]
2	Methanoic acid[a]	Formic acid[a]
3	3-methylcatechol [b]	1,2- benzenediol,3-methyl-[b]
4	2-methyl-5-(1-methylethyl) phenol[a]	4-hydroxy-2-methylacetophone[a]

[a]Anti-pathogen and [b]Antioxidant activity

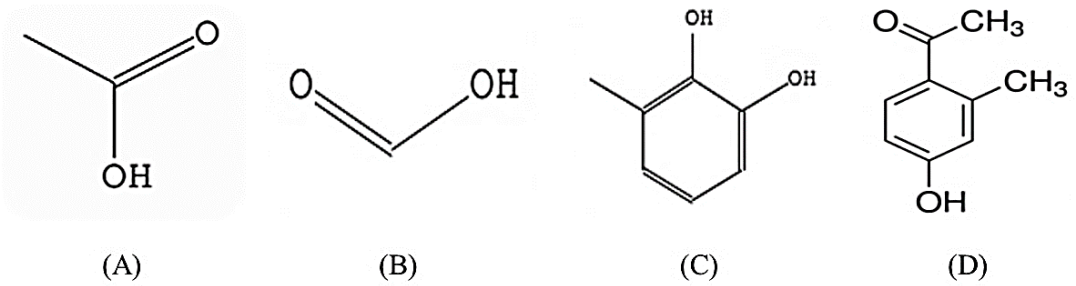

Figure 4.3 Structure of bioactive compounds identified from organic BPEs of GC-MS; acetic acid (A), formic acid (B), 1,2-benzenediol,3-methyl-, (C) and 4-hydroxy-2-methylacetophone (D)

4.4.2 FTIR analysis

FTIR measurements were taken to identify the major functional groups of the organic BPE. The infrared spectra of Kluai Kai, Kluai Homthong, Kluai Homtaiwan, Kluai Lebmuernang, Kluai Hukmook, and Kluai Namwa were similar to each other, conveying that they contained similar functional groups (Figure 4.4 and Table 4.5). The strong peaks, at frequencies between 3,328 and 3,344 cm^{-1}, were assigned to O–H stretching, which represents the free hydroxyl group of the polymer: lignins and BP pectins [90], phenols, [91] and polysaccharides [92]. At these frequencies, it was attributed to the presence of O–H stretching of a carboxylic group from the two main constituents of GC-MS: acetic acid and formic acid. The distinctive peak at around 1,636 cm^{-1} was assigned to the stretching C=C ring and the COO- antisymmetric stretching of aromatic and carboxylate ions, [91] and amides' C=C stretching present in phytoconstituents of organic BPE [93]. Gnanasambandam and Proctor [94] found the stronger bands between 1,640 and 1,620 cm^{-1} to be an important region that identified and quantified pectin samples. The prominent peak of ester linkage of the carboxylic group from lignin or hemicellulose appears at around 1,730 cm^{-1}, normally found in organic BPE; this disappeared, which might be due to the chemical treatment of BPs [95]. The weak absorption band between 1,395 to 1,412 cm^{-1} was characteristic of C=C in aromatic rings and COO- symmetric stretching of carboxylate ions [17, 20, 22]. The IR spectrum of organic BPE of Kluai Kai, Kluai Homthong, and Kluai Lebmuernang

exhibited C-O stretching and C-C stretching between 1,058 to 1,061 cm^{-1}, indicating the existence of cellulose and phenol [17, 23]. Lu et al. [98] specified the wavenumber between 950 and 1,200 cm^{-1} as the main functional group of carbohydrates, whereas [99] found that the pectin of BP was the "fingerprint" region between 800 to 1,300 cm^{-1}. The FTIR absorption peak of six organic BPEs indicates the existence of functional groups, like hydroxyl and carboxyl, in agreement with organic BPEs [19, 26, 27, 28].

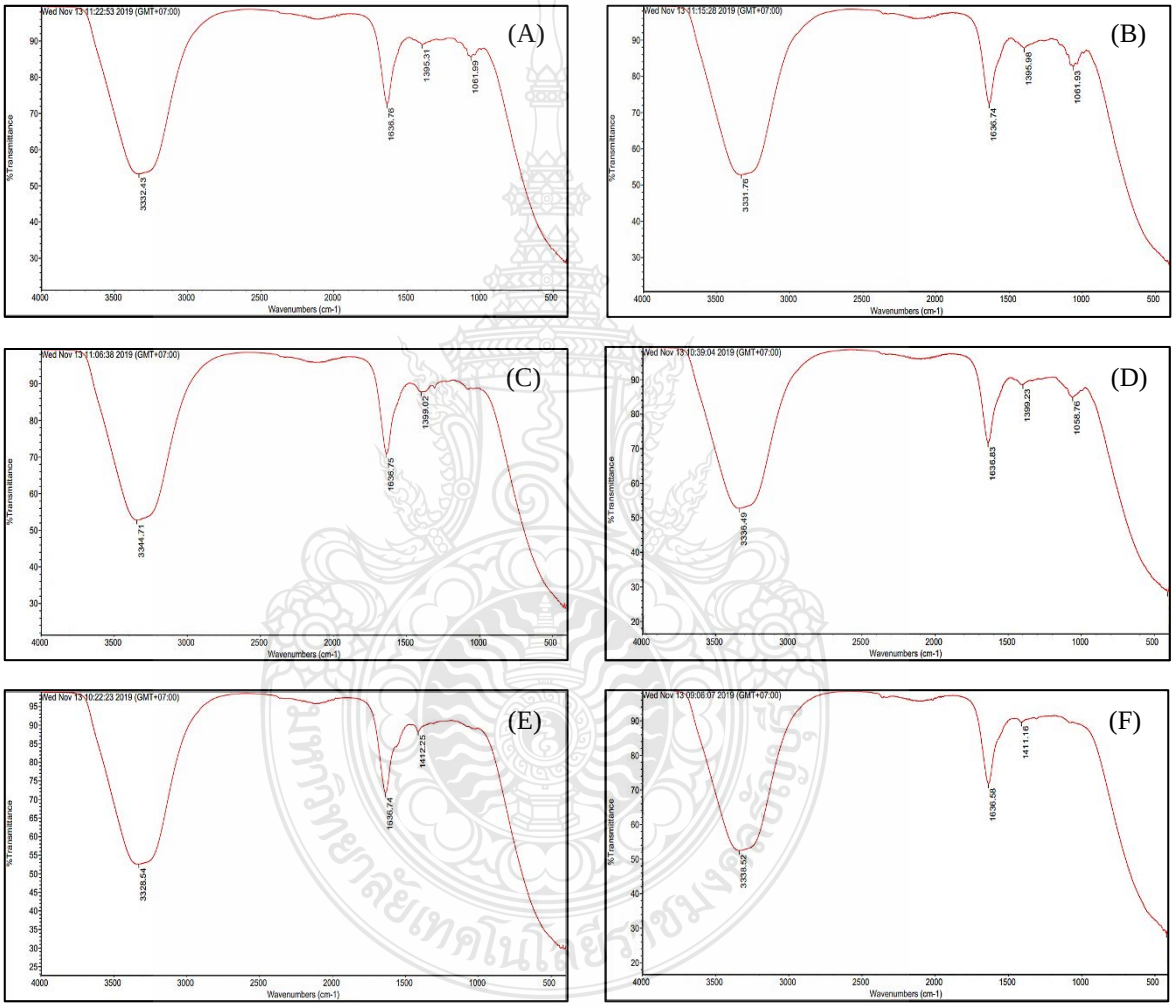

Figure 4.4 FTIR-Spectrum of different organic BPEs of Kluai Kai (A), Kluai Homthong (B), Kluai Homtaiwan (C), Kluai Lebmuernang (D), Kluai Hukmook (E) and Kluai Namwa (F)

Table 4.5 The functional group of organic BPEs from FTIR

Type of banana	Functional group
Kluai Kai	- OH stretching - (C=C) stretching and (N-H) bending - COO-symmetric stretching and CHC bending - Alcohol C-O stretching, C-C stretching and C-N stretching
Kluai Homthong	- OH stretching - (C=C) stretching and (N-H) bending - COO-symmetric stretching and CHC bending - Alcohol C-O stretching, C-C stretching and C-N stretching
Kluai Homtaiwan	- OH stretching - (C=C) stretching and (N-H) bending - COO-symmetric stretching and CHC bending
Kluai Lebmuernang	- OH stretching - (C=C) stretching and (N-H) bending - COO-symmetric stretching and CHC bending - Alcohol C-O stretching, C-C stretching and C-N stretching
Kluai Hukmook	- OH stretching - (C=C) stretching and (N-H) bending - C-C aromatic, Asymmetric bend methyl (C-H) and CH_2 scissoring
Kluai Namwa	- OH stretching - (C=C) stretching and (N-H) bending - C-C aromatic, Asymmetric bend methyl (C-H) and CH_2 scissoring

4.5 Optimization of Organic BPE

In this experiment, the extracting condition of the phenolic compound of organic BP was optimized. The optimum condition was studied by one factor at a time. The studied factors were the concentration of methanol, temperature, and extraction time. The TPC was the main response variable for optimization following by the antipathogenic activity, GC-MS, and FTIR analysis, respectively.

The effect of methanol concentration on the TPC extraction from organic BPE was investigated. The highest TPC was obtained for 10.61 ± 0.55 mg GAE/ g DM when extraction with 50 % v/v of methanol (Table 4.6). The solvent concentration has resulted from the polar because it can extract substances by solubilization [102]. Then, the effect of temperature was studied, and the results showed in Table 4.6. The results reported that the extraction temperature presented a significant impact on the TPC of BPE at $\alpha < 0.05$. The maximum TPC was provided for 10.27 ± 0.70 mg GAE/g DM at 100 °C of extraction temperature. This result was suggested that the increase in temperature of solid-liquid extraction could enhance the yield of extractant due to the rise of the diffusion coefficient and mass transfer [103]. Finally, the impact of extraction time on the amount of TPC was tested. The highest TPC from BPE was achieved for 11.32 ± 0.55 mg GAE/ g DM at 10 min of extraction time (Table 4.6). The results showed that the extraction for a long time would decrease the TPC in BPE. The extraction time has to influence solubility and mass transfer in the molecule of bioactive compounds. Moreover, prolonged extraction time increases the cogency of oxidation, epimerization, and degradation of bioactive compounds. Hence, the extended extraction time may inappropriate for phenolic compound extraction [104].

Table 4.6 Factors of optimum extraction and the total phenolic content from organic BPE

Parameters	Experimental level	Total phenolic content* (mg GAE/ g DM)
Concentration of methanol (% v/v)	0	6.50 ± 0.41^{b}
	50	10.61 ± 0.55^{a}
	100	7.07 ± 0.66^{b}
Temperature (°C)	50	8.29 ± 0.84^{b}
	75	8.84 ± 0.62^{b}
	100	10.27 ± 0.70^{a}
Extraction time (min)	10	11.32 ± 0.55^{a}
	20	8.41 ± 0.52^{b}
	30	8.59 ± 1.38^{b}

*Mean values with different superscripts (a and b) made a significant difference ($\alpha \leq 0.05$) with the error bar ± SD.

4.5.1 Antipathogenic activity

The effect of optimized BPE concentration on antipathogenic activity was investigated by the disc diffusion method (Table 4.7 and Figure 4.5). *A. hydrophila* was chosen as the critical pathogen in prawn farming. Different concentrations of the organic BPE were showed antipathogenic activity. The increasing BPE concentration could enhance the antipathogenic activity. The result suggested that the high concentration of BPE increased the bioactive compounds and provided a high level of antipathogenic activity. The antipathogenic activity was likely from the effect of phenolic compounds in organic BPE. The phenolic compound against the microorganism by degrading the lipid and inhibited the process of membrane biosynthesis. The disruption to the cell membrane would decrease the osmosis-barrier ability [105].

Table 4.7 The antipathogenic activity of the organic BPE

BPE concentration (µg/disc)	Results
Control	-
20000	++++
10000	++++
5000	++++
2500	+++
1250	+++
625	++
312.5	+
156.25	-
78.125	-

++++: Very high, +++: High, ++: Moderate, +: Trace, and -: Not detected

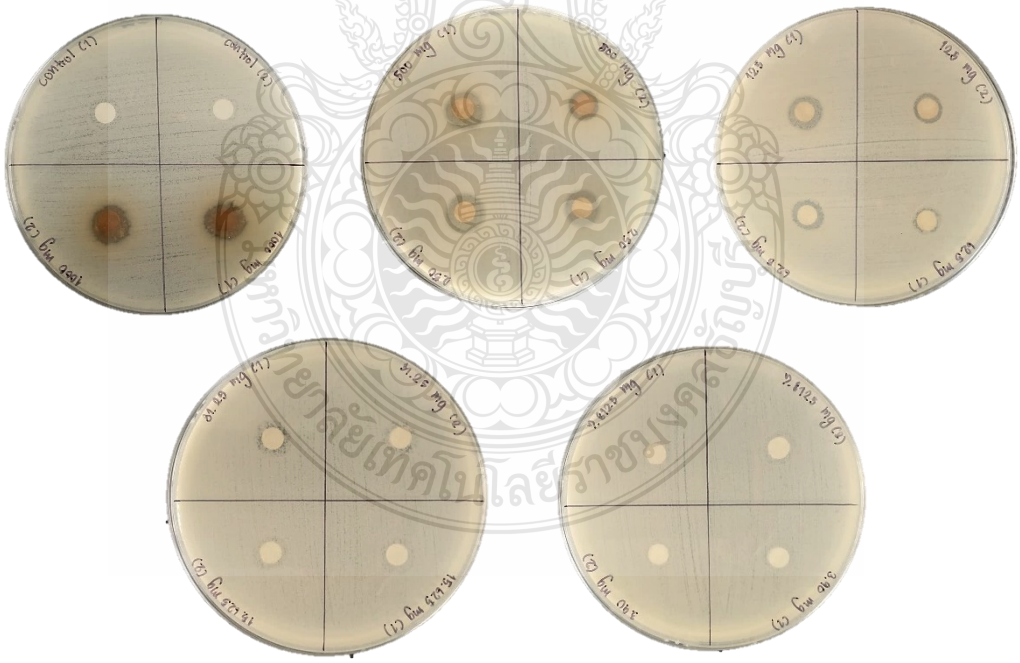

Figure 4.5 Zone of pathogen growth inhibition by organic BPE at various concentration

4.5.2 GC-MS analysis

The important phytochemicals in organic BPE obtained from the optimum condition were shown in Table 4.8. The biological function of phytochemicals was divided into two main groups of antioxidant and antipathogenic activities. These components were similar to the phytochemical found in the methanolic extract of soybean. Their function was the antioxidant and antimicrobial activity such as 1,2-cyclopentanedione, 2,4-dihydroxy-2,5-dimethyl-3(2H)-furan-3-one, 2H-pyran-2,6(3H)-dione, and 2-methoxy-4-vinylphenol [106].

Table 4.8 The phytochemicals in organic BPE extracting by optimum condition

Properties	Phytochemicals
Antioxidant	1,2-cyclopentanedione
	2,4-dihydroxy-2,5-dimethyl-3(2H)-furan-3-one
	2H-pyran-2,6(3H)-dione
	2(3H)-furanone, dihydro-4-hydroxy-
	2-methoxy-4-vinylphenol
Antipathogenic	2-propenoic acid
	2-propenoic acid, 2-methyl-, oxiranylmethyl ester
	1,2-cyclopentanedione, 3-methyl-
	Furaneol
	α-acetobutyrolactone
	4H-pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-
	2-furancarboxaldehyde, 5-(hydroxymethyl)-
	2-methoxy-4-vinylphenol

The previous research showed that these phytochemicals could promote the activity of hemolymph and decrease the coagulation time. In addition, antipathogenic activity was also given from these components, which could improve phagocytosis and reduce the mortality of *M. rosenbergii*.

4.5.3 FTIR analysis

FTIR spectrum of organic BPE of optimum condition was illustrated in Figure 4.6. The spectrum, peaks at 3279.21 cm^{-1} were defined as the O–H stretching [90], demonstrated the free hydroxyl group of the polymer: lignins and pectins [90], phenols [91], and polysaccharides [92]. Lipid, fatty acid, protein, alkaloids, lignin, and carboxylate ions were assigned at 2928.50 and 1389.95 cm^{-1} [107]. The wavelength at 1583.77 cm^{-1} authorized to the lignin polymer [100]. The absorption band at 1030.33 cm^{-1} was assigned to C-O , C-C and C-N stretching of phenol and polysaccharide [17, 23]. Whereas the peak number between 817-922 cm^{-1} corresponded to the amine groups [40]. Finally, the IR spectrum of 515.47 was characterized by the haloalkanes (C–Cl stretching) [97].

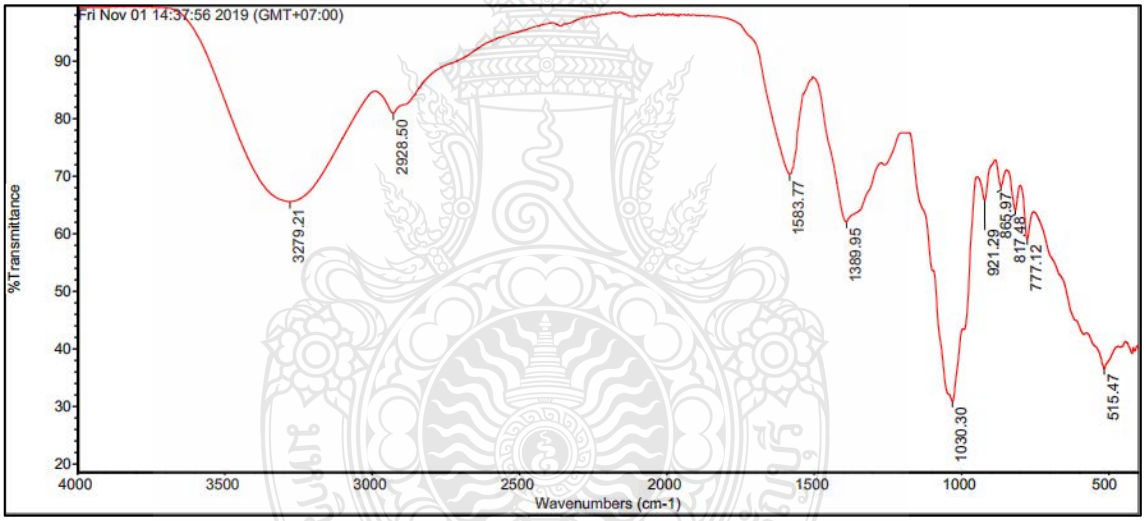

Figure 4.6 FTIR spectrum of organic BPE at optimum condition

4.6 Scaling Up Extraction

The optimum condition of BP extraction was performed as scaling up for ten times of previous studies. The biological activity of BPE was evaluated (results showed in Table 4.9). The results demonstrated that TPC, DPPH, FRAP, and total solids were not significantly different from previous studies (initial scale). Therefore, the upscale extraction of organic BP can increase production capacity to respond to future utilization demand.

Table 4.9 Biological activities of organic BPE between initial and up scale extraction

Extraction scale	TPC[a]	DPPH[b]	FRAP[a]	Total solids[c]
Initial scale extraction	10.44	6.41	4.31	0.33
Up scale extraction	10.58	6.46	4.84	0.25

a= mg GAE/g DM, b= mg TE/g DM and c = grams

4.7 Injection of Organic BPE on the Immune Parameters of *M. rosenbergii*

4.7.1 Effect of the organic BPE injection on the immune parameters of *M. rosenbergii*

The health protection of prawns is one of the most reasons for the success of aquaculture. Prophylactic chemotherapy or antibiotics was used to regulate the disease and disease in aquaculture. However, the use of antibiotics increases the number of resistant bacteria and presents residues in the aquaculture organisms that could be harmful to both humans and the aquatic environment [108]. According to Bricknell and Dalmo [109] , the immune system must be considered an immunostimulant is a naturally occurring compound that modulates the immune system by increasing resistance of the host against diseases that in most circumstances are caused by pathogens. For several years in aquaculture, immunostimulants are used as feed additives. Several studies have documented the application of immunostimulants to aquaculture to increase immunity and pathogens resistance. Immunostimulants can improve disease resistance to pathogens in crustaceans by enhancing oral or oral non-specific safety mechanisms and safe for the

environment and human health [58]. In this study, the immunological parameters such as THC, DHC, coagulation time, and total protein were analyzed from the hemolymph of prawns that received organic BPE at 0, 6, 12, and 24 µg/g prawn after injected at 1, 3, and 6 days.

4.7.1.1 Total hemocyte count (THC)

The THC was significantly ($p<0.05$) higher in organic BPE than in control of each sampling after injecting at 1, 3, and 6 days (Figure 4.7). In context, the significant elevation of THC was received organic BPE at 12, 24, and 6 µg/g prawn were obtained $5.23 \pm 0.70 \times 10^6$, $10.42 \pm 5.27 \times 10^6$ and $17.24 \pm 2.21 \times 10^6$ cell/mL, respectively. The primary mediators of immune systems in invertebrates, hemocytes perform important immune functions, including phagocytic activities and pathogens, that entangle the body with defensive activity against pathogens. Circulating hemocytes and crustacean THCs play a critical role in controlling physiological functions, including metabolism by carbohydrates, protein, amino acid transportation and storage, hemolymphatic coagulation, and phagocytosis [37, 38]. The THC increase increased the survival rate of prawns and enhanced the transport and metabolism of nutrients [69]. The hemocyte count of crustaceans depends on several factors, such as infectious agents and environmental stress [112]. The injection of cacao pod husks (CPHs) extract into *L. vannamei* was showed a significant increase in the total hemocyte count after shrimp were injected with fresh CPHs extract at 40 µg/shrimp on the first day [69]. The injection of the hot-water extract of BP (*M. acuminata*) to *M. rosenbergii* at 1-6 µg/g prawn increases THC from 3 to 6 days [2]. *M. rosenbergii* was fed by *G. Lucidum* dietary, THC was considerably higher than the control group at 1.5 g/kg [68].

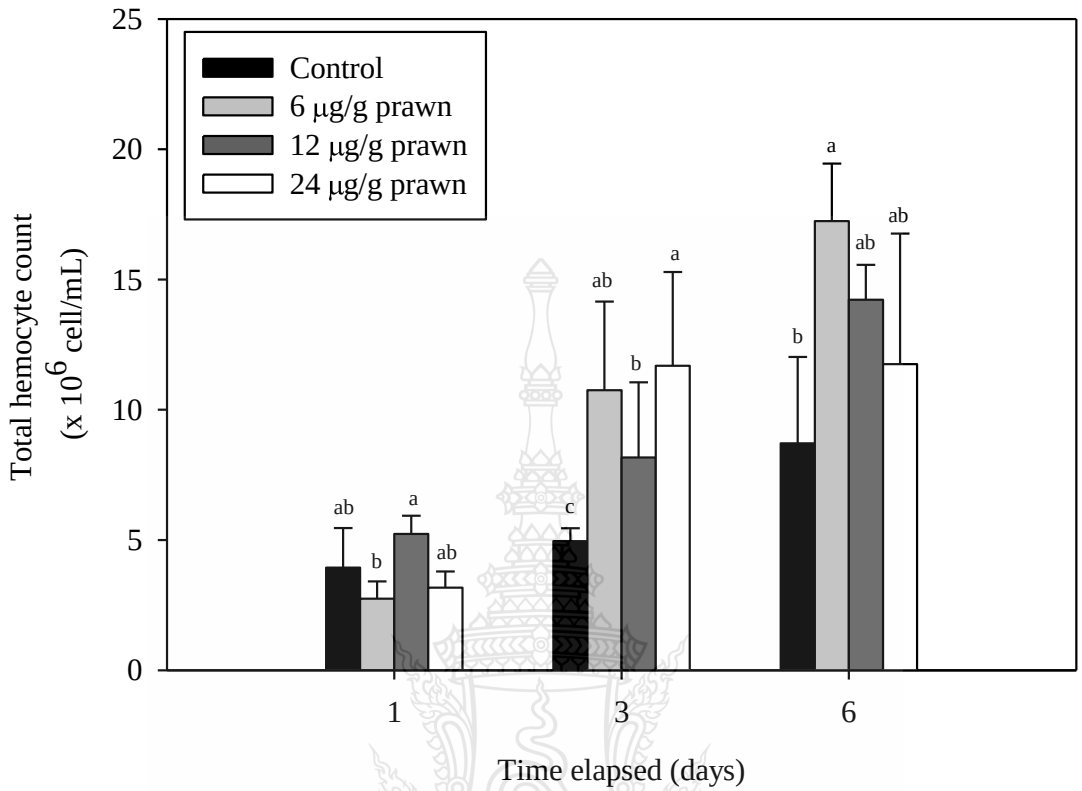

Figure 4.7 Total hemocyte count (THC) of *M. rosenbergii* received organic BPE at 0, 6, 12, and 24 µg/g prawn. The bar represents the mean with a standard deviation value (±SD). The different capital presents a significant difference at $p<0.05$ on the same day.

4.7.1.2 Difference hemocyte count (DHC)

The hyaline cells were significantly ($p<0.05$) higher in organic BPE than in control of each sampling after injecting at 3 and 6 days. Prawns were received organic BPE at 6 µg/g prawn showed the highest of the hyaline cell were obtained $2.72 \pm 1.96 \times 10^6$ and $11.46 \pm 4.26 \times 10^6$ cell/mL, respectively (Figure 4.8). Semi-granular cells were significantly ($p<0.05$) higher in organic BPE than in the control of each sampling after 1, 3, and 6 days of injection. In context, the significant elevation of semi-granular cell was received organic BPE at 12, 6, and 6 µg/g prawn were obtained $0.6 \pm 0.22 \times 10^6$, $0.88 \pm 0.41 \times 10^6$ and $0.67 \pm \times 10^6$ cell/mL at 1, 3 and 6 days of experiment, respectively (Figure 4.9). Granular cells were significantly ($p<0.05$) higher in organic BPE than the control of all sampling after injection at 1, 3, and 6 days. A significant elevation of the granular cell was obtained for prawns fed with organic BPE at 12, 24, and 12 µg/g of prawn for $5.09 \pm 1.70 \times 10^6$, $10.78 \pm 5.34 \times 10^6$ and $8.28 \pm 3.09 \times 10^6$ cell/mL, respectively (Figure 4.10). Each cell types are active in defense reactions and the hyaline cells are mainly concerned with phagocytosis [113]. The semi-granular cells are the active cells in encapsulation, early non-self-recognition, melanization, and coagulation. They respond to the β-1,3-glucan-binding protein (βGBP) complexed with glucans by degranulation and release of the proPO activity were cell-adhesive and opsonic protein, peroxinectin from storage granules [113]. The granular cells were contained small and large cells. They were produced in hematopoietic tissues (HPTs) that collaborate in the storage and release of the prophenoloxidase proPO system, melanization, antimicrobial peptides, and cytotoxicity [62]. *L. vanamei* fed with *G. tenuistipitata* extract after 14 days able to increase the THC. Specifically, both hyaline and granular cells were increased the mototic cells and mitotic index of HPTs supported the circulation of hemocytes [114]. *M. rosenbergii* were received the hot water extract of banana (*M. acuminata*) peel at 1-6 µg/g prawn by injection. The result showed the increase of hyaline and granular cells from 3 to 6 days [2]. Therefore, the injection of organic BPE can motivate diffusion of the hemocyte in HPTs and promote mature granular cells, which increases the THC of prawns.

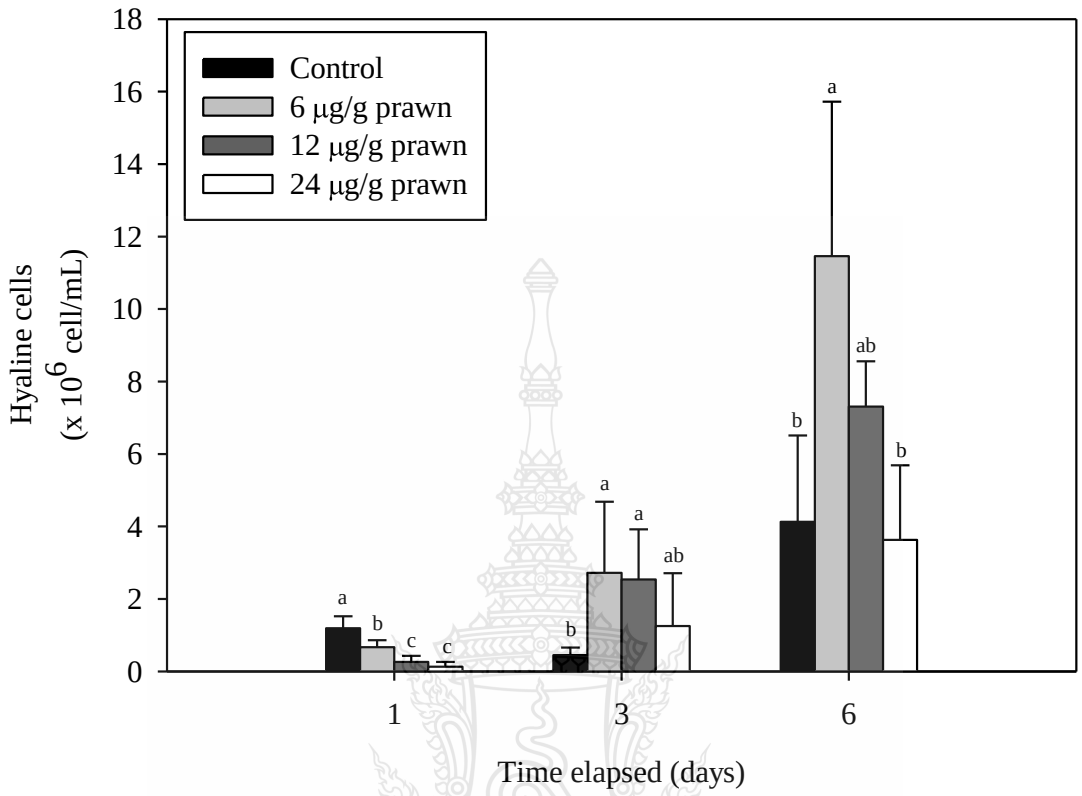

Figure 4.8 Hyaline cells of *M. rosenbergii* received organic BPE at 0, 6, 12, and 24 µg/g prawn. The bar represents the mean with a standard deviation value (±SD). The different capital presents a significant difference at $p<0.05$ on the same day.

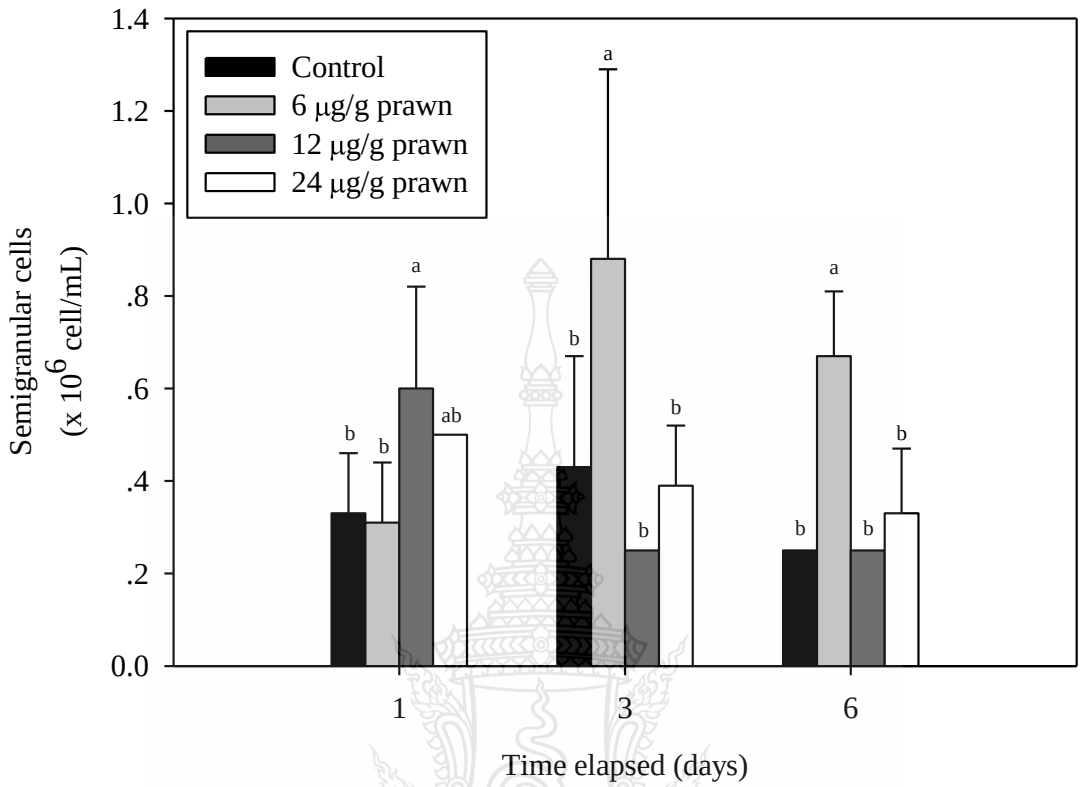

Figure 4.9 Semigranular cells of *M. rosenbergii* received organic BPE at 0, 6, 12, and 24 μg/ g prawn. The bar represents the mean with a standard deviation value (±SD). The different capital presents a significant difference at $p<0.05$ on the same day.

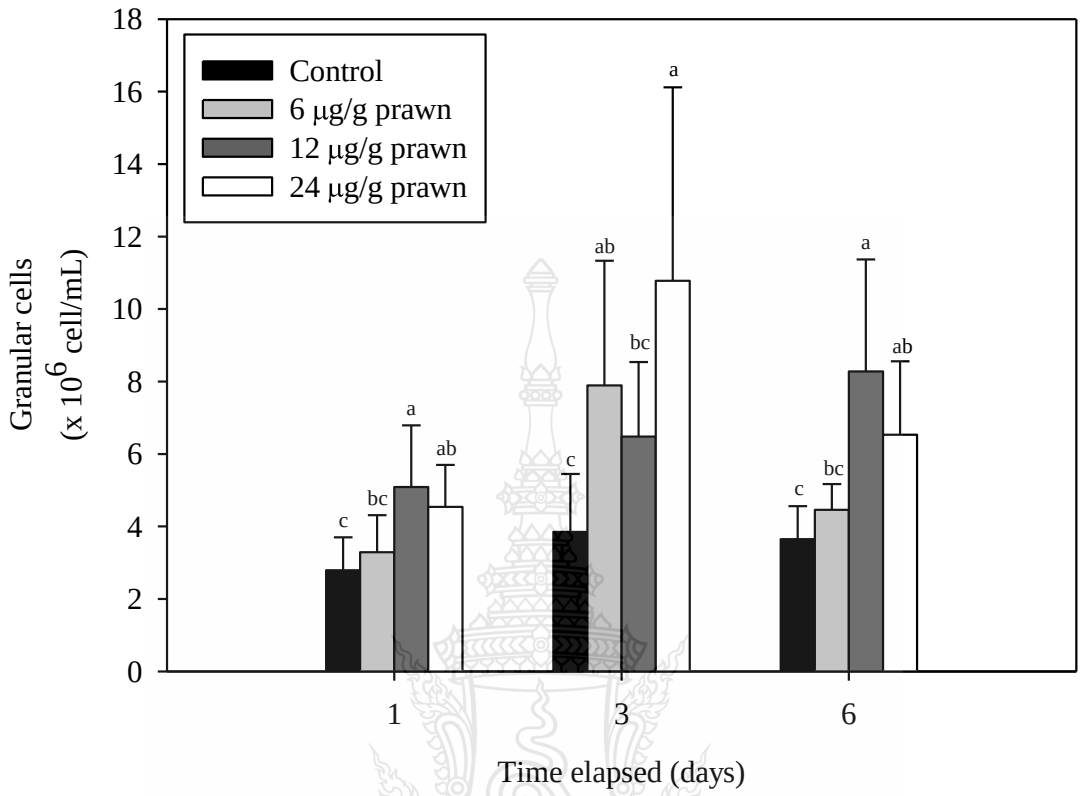

Figure 4.10 Granular cells of *M. rosenbergii* received organic BPE at 0, 6, 12, and 24 µg/g prawn. The bar represents the mean with a standard deviation value (±SD). The different capital presents a significant difference at $p<0.05$ on the same day.

4.7.1.3 Coagulation time

The result showed that coagulation time was significantly decreased in organic BPE compared with control each all sampling after injected at 1, 3, and 6 days. In context, the significant elevation of coagulation time was received organic BPE at 24, 6, and 6 µg/g prawn were obtained 40.34 ± 7.59, 41.53 ± 10.04 and 45.22 ± 3.01 sec, respectively. (Figure 4.11). In crustaceans, hemolymph coagulation time is the innate immune response and triggered by the release of the clotting proteins (CPs) in transglutaminase activity (TG). It leads to preventing leakage of hemolymph and protects against invaders pathogen entirely. The body prevents blood loss during injury and wound healing [46, 47]. The coagulation time is s simple parameter to evaluate the performance of immune stimulants of crustacean [117]. *M. rosenbergii* were injected with the BPE at 1-6 µg/g prawn can reduce hemolymph coagulation time [2]. Prawns fed with GLP supplemented diets at 1.5 g/kg showed a significant decrease in hemolymph coagulation time when compared with the control group. This result suggested that GLP had expedited the transglutaminase activity for decreased hemolymph coagulation time [118].

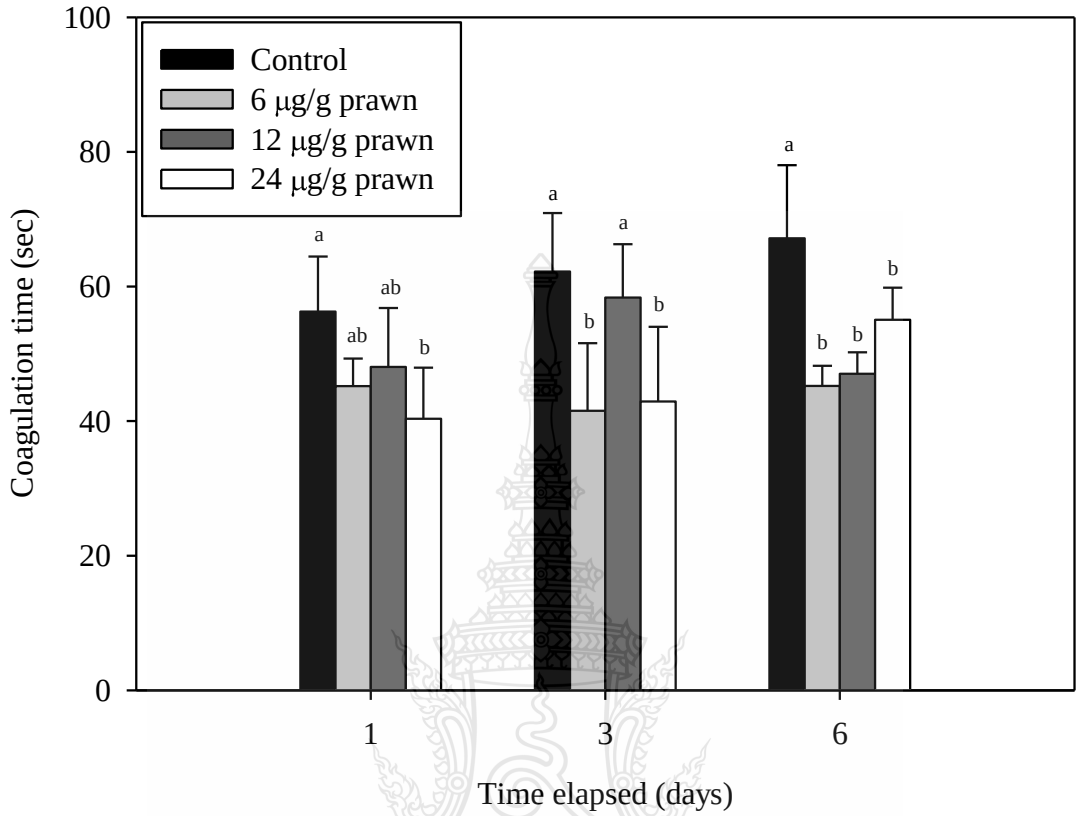

Figure 4.11 Coagulation time of *M. rosenbergii* received organic BPE at 0, 6, 12, and 24 μg/g prawn. The bar represents the mean with a standard deviation value (±SD). The different capital presents a significant difference at $p<0.05$ on the same day.

4.7.1.4 Total protein

The total protein was significantly ($p<0.05$) higher in organic BPE than in control after injection at 3 and 6 days. The results showed that the total protein of prawn, which received organic BPE at 6 and 24 µg/g prawn was 2.29 ± 0.01 and 3.36 ± 0.02 mg/L, respectively (Figure 4.12). The protein in hemolymph has a potent innate immune response and promotes the innate immune function efficacy of crustaceans [63]. The concentration of protein in the blood is an index to the health of prawns. The molting has decreased the protein in hemolymph and created subsequently [119]. The increase in hemolymph protein improves stress responses through hemocyte regeneration and maturation of hemocyte precursors in hematopoietic tissue. Hence, *M. rosenbergii* has been injected with BPE to explain the hypothesis that may cause an early recovery of immune responses [2].

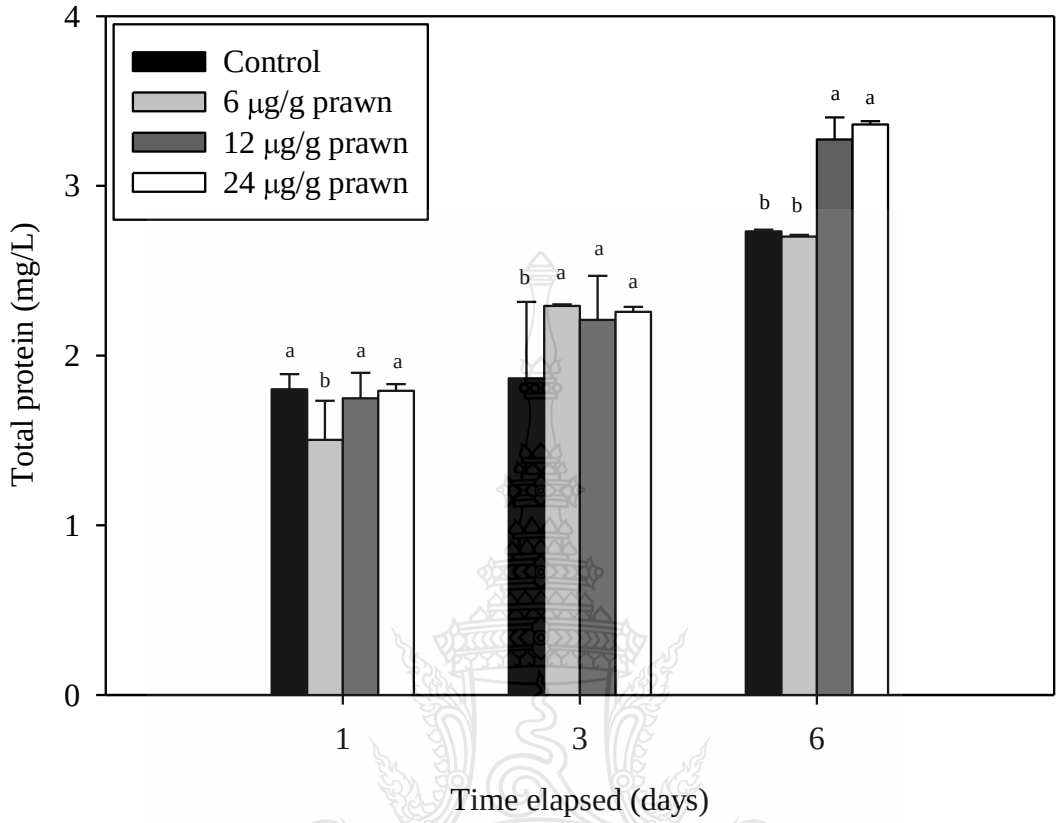

Figure 4.12 Total protein of *M. rosenbergii* received organic BPE at 0, 6, 12, and 24 μg/g prawn. The bar represents the mean with a standard deviation value (±SD). The different capital presents a significant difference at $p<0.05$ on the same day.

4.8 Effect of Organic BPE Injected to *M. rosenbergii* on the Phagocytic Activity

The phagocytic activity of prawns injected with organic BPE was significantly higher than the control group. Prawns were received organic BPE at 6, 12, and 24 µg/g prawn were obtained 82.00 ± 5.05, 81.48 ± 8.35 and 79.65 ± 9.88 %, respectively (Figure 4.13). The infection of bacterial and viral to prawn is the principal issue in the large-scale prawn cultivation industry. It's can easily trespass into the body cavity, and these exotic encroaches are obliterated by phagocytosis, which was a crucial cellular defense mechanism [120]. H_2O_2 and superoxide anion (O_2^-) from hemocytes present the bactericides and prevent the pathogens [121]. Phagocytosis is a major cellular prevention mechanism of an organism, while clearance efficiency is a principal mechanism in crustaceans [111]. Several previous studies have verified that plant phenolics, polysaccharides, proteoglycans, and flavonoids can enhance phagocytosis to prevent infectious microbes in various fish and crustaceans [58, 118]. The injection of norepinephrine (NE) at 50.0 *p*mol/prawn can increase the phagocytic activity in *M. rosenbergii* after 2 h challenged with *L. garvieae* [122]. Lee et al. studied the phagocytic activity in *L. vannamei*. The results suggested that the phagocytic activity was increased after injected with fresh CPH extract at 40 µg/ shrimp for 3 days [69].

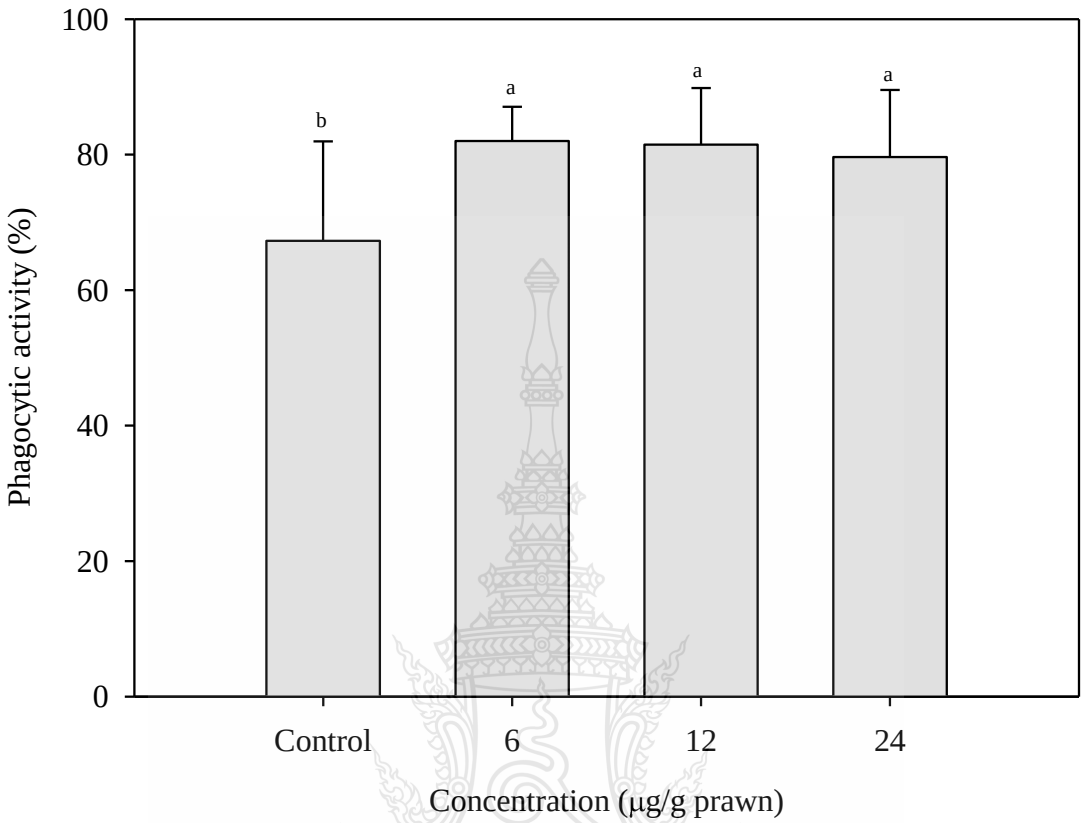

Figure 4.13 Phagocytic activity of *M. rosenbergii* received organic BPE at 0, 6, 12, and 24 µg/g prawn. The bar represents the mean with a standard deviation value (±SD). The different capital presents a significant difference at $p<0.05$.

4.9 Susceptibility of Organic BPE Injected Prawn to *A. hydrophila*

The unchallenged prawn was injected with the sterilized water in this study. Simultaneously, the challenge test of prawns received the organic BPE at 6, 12, and 24 µg/g prawn. The result showed that the cumulative mortality of unchallenged prawns was significantly higher than the challenged prawn after 144 h. The unchallenged prawn received organic BPE at 6.0, 12.0, and 24.0 µg/g prawn presented the accumulative mortality for 20.0, 20.0, and 10.0 %, respectively (Figure 4.14). The previous study has reported the influence of *W. somnifera* extract on the innate immune response of *M. rosenbergii* inhibit *A. hydrophila*. The result demonstrated that the lowest cumulative mortality was 40 %, respectively [123]. Both *M. rosenbergii*, which were fed with the *Morinda citrifolia* leaf extract (HMLE) [124] and BPE [67] at 0.6 and 6 g/kg challenged with *L. garvieae* after 144 h. The results showed a significant decrease in cumulative mortality were 66.7 and 40.0 %, respectively.

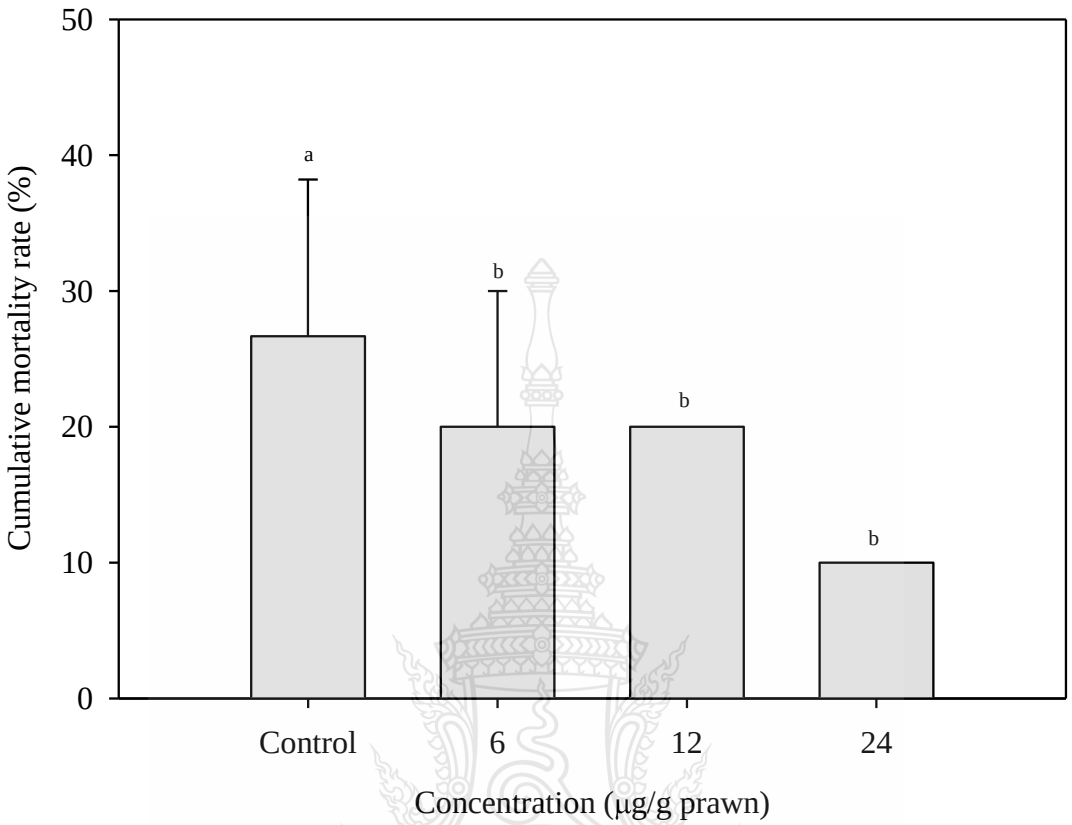

Figure 4.14 Cumulative mortality of *M. rosenbergii* received organic BPE at 0, 6, 12, and 24 µg/g prawn at the 6th day of the experiment. The bar represents the mean with a standard deviation value (±SD). The different capital presents a significant difference at $p<0.05$.

This study reported that the immunity of *M. rosenbergii* was affected by directly injected organic BPE. However, the different organic BPE concentrations provided a similar immunity effect on tested prawns. It suggested that the suitable concentration for the next experiment was 6 µg/g prawn, which sufficient to promote the immune system of prawns.

4.10 Feeding of Organic BPE and Probiotic Powder on the Growth Performances and Immune Parameters of *M. rosenbergii*

This experiment studied the dietary supplement containing organic BPE and PP for giant freshwater prawns. The growth measurements were determined as the percentage of weight gain (PWG), percentage of length gain (PLG), specific growth rate (SGR), survival rate (SR), and feed conversion ratio (FCR). The immunity parameters were contained total hemocyte count (THC), difference hemocyte count (DHC), coagulation time, and total protein. The experiments were divided into four experiments, control, organic BPE (formula 1), PP (formula 2) and organic BPE mixed PP (formula 3).

4.10.1 Effect of organic BPE and probiotic powder feeding supplement on the growth performances of *M. rosenbergii*

This study explored the effect of dietary feeding containing organic BPE and probiotic on the growth and immunity of giant freshwater prawns in constructed aerated-pond. The results were illustrated in Figure 4.15-4.19. The average initial weight and initial length of prawns for this experiment were 4.12 ± 0.92 g and 7.8 ± 0.47 cm, respectively. The growth performances include percentage weight gain and percentage length gain of a control diet, and organic BPE was insignificantly different ($p>0.05$). Nevertheless, there were significantly elevated with PP (formula 2), and organic BPE mixed PP (formula 3) all 90 days (Figure 4.15 and Figure 4.16). The specific growth rate of prawns fed with organic BPE (formula 1) and PP (formula 2) was showed significantly higher than with prawns fed with a control diet and organic BPE mixed PP (formula 3) (Figure 4.17). The survival rate of prawns fed with organic BPE (formula 1) and organic BPE mixed PP (formula 3) supplement diet was significantly higher than the control diet and PP (formula 2) (Figure 4.18). The different diets showed that the FCR

of prawns was not significantly different after 90 days of feeding (Figure 4.19). In conclusion, the specific growth rate (SGR) and survival rate (SR) for 30 days were significantly different with a control group. Hence, the organic BPE could promote the health of prawns in the acclimatization and initial stage of cultivation. In aquaculture, plant extract has been widely used to prevent diseases by regulating pathogenic bacteria, improving immunity, increasing the survival and growth rate of aquatic animals [58]. In the previous study, the diet supplemented with *Gracilaria fisheri* to FCR of prawns has been investigated. The result found that the FCR of *M. rosenbergii* increased after being fed with a high concentration of *G. fisheri* extract, which decreased digestion efficiency in prawns [125].

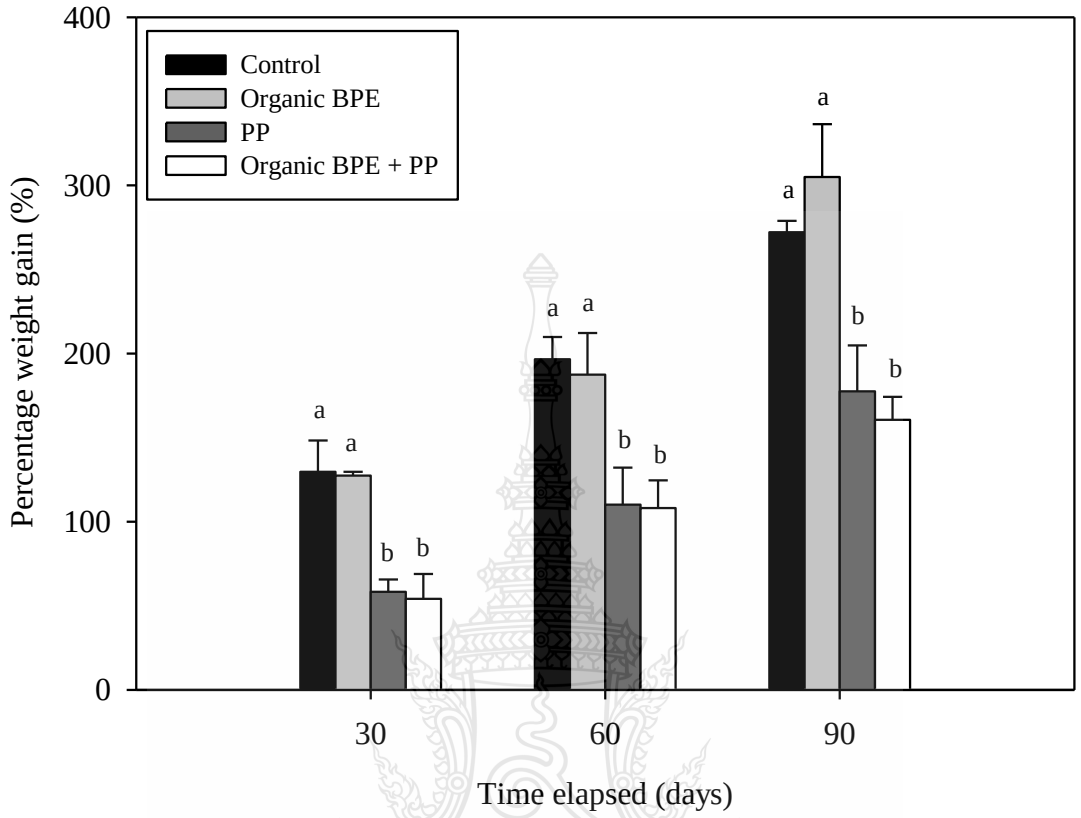

Figure 4.15 Percentage weight gain of *M. rosenbergii* fed with a control diet, organic BPE, PP, and organic BPE mixed PP. The bar represents the mean with a standard deviation value (±SD). The different capital presents a significant difference at $p<0.05$ on the same day.

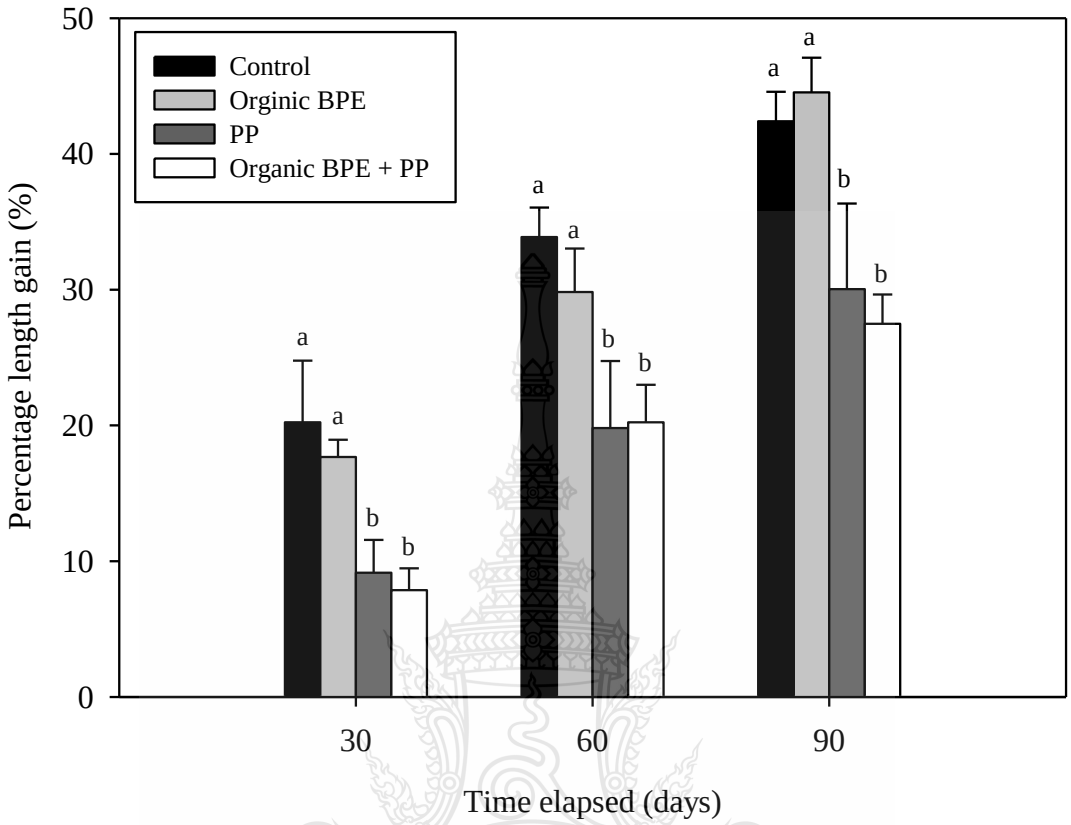

Figure 4.16 Percentage length gain of *M. rosenbergii* fed with a control diet, organic BPE, PP, and organic BPE mixed PP. The bar represents the mean with a standard deviation value (±SD). The different capital presents a significant difference at $p<0.05$ on the same day.

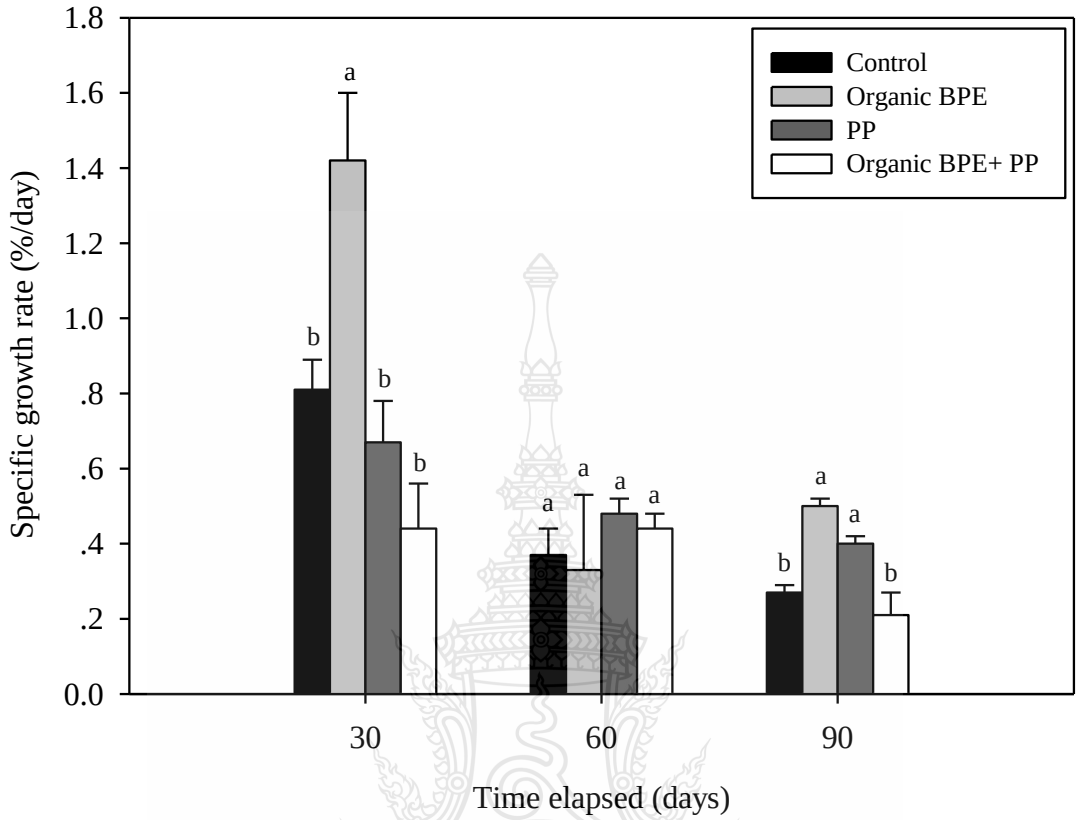

Figure 4.17 Specific growth rate of *M. rosenbergii* fed with a control diet, organic BPE, PP, and organic BPE mixed PP. The bar represents the mean with a standard deviation value (±SD). The different capital presents a significant difference at $p<0.05$ on the same day.

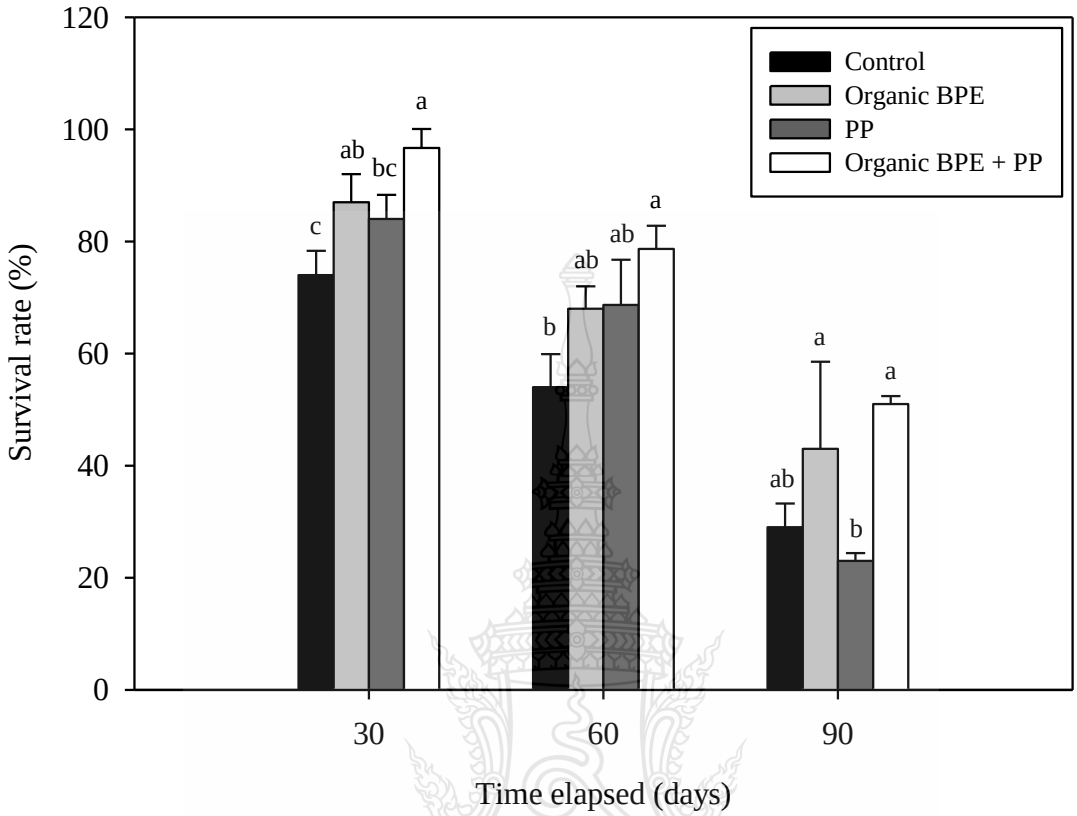

Figure 4.18 Survival rate of *M. rosenbergii* fed with a control diet, organic BPE, PP, and organic BPE mixed PP. The bar represents the mean with a standard deviation value (±SD). The different capital presents a significant difference at $p<0.05$ on the same day.

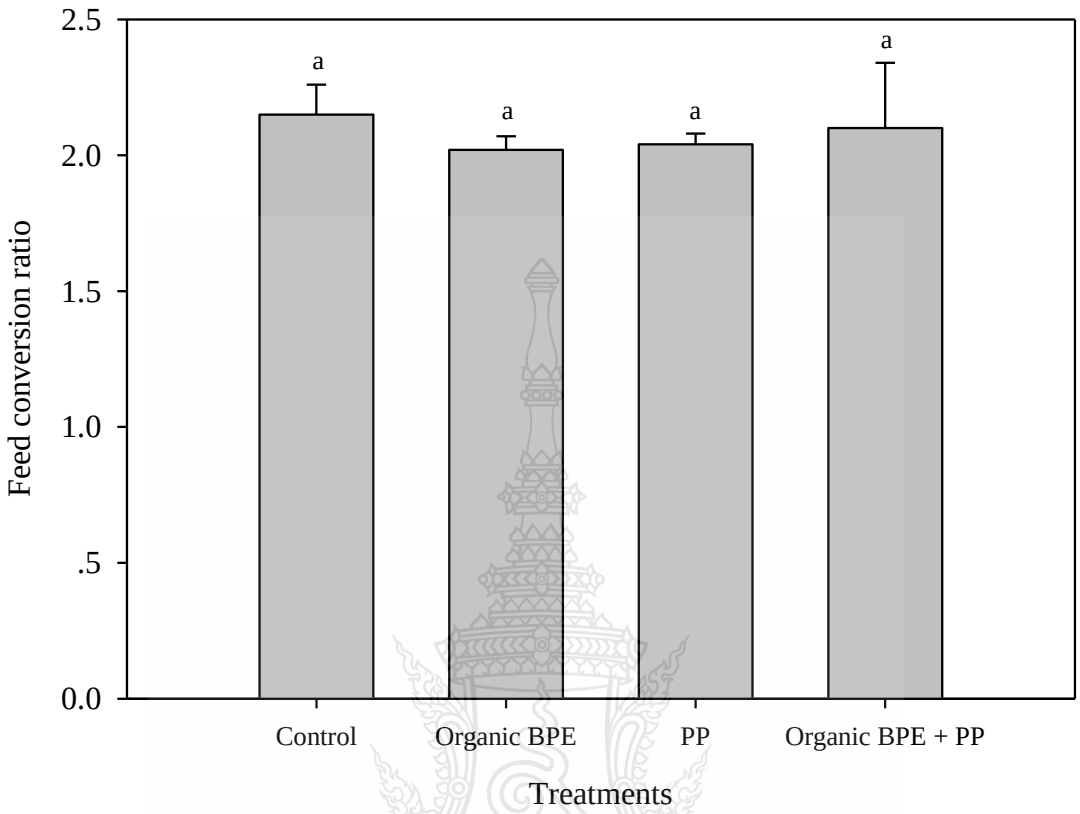

Figure 4.19 Feed conversion ratio of *M. rosenbergii* fed with a control diet, organic BPE, PP, and organic BPE mixed PP after 90 days. The bar represents the mean with a standard deviation value (±SD). The different capital presents a significant difference at $p<0.05$.

Probiotics are one of the most prevalent dietary supplements for pathogenic control, disease prevention, and health promotion in aquatic animals. The organic acids (butyric acid and lactic acid) produced from probiotics can reduce the pH level and inhibiting the growth of pathogens [1]. In addition, probiotics could improve the water quality of aquaculture [126]. The use of probiotics in aquaculture for dietary supplements can also enhance aquatic animal production efficiency [127].

4.10.2 Effect of organic BPE and probiotic powder feeding supplement on the immune parameters of *M. rosenbergii*

The prawns fed with a diet supplementing organic BPE presented the highest THC for 21.15 x 10^6 ± 9.01 cell/mL at 90 days (Figure 4.20). In comparison, the THC of prawns fed with other diets was not significantly different. The DHC (includes hyaline cells, semigranular cells, and granular cells) of prawns after feeding with a different formula diet was exhibited in Figure 4.21-4.23. The result denoted that feeding the diet supplementing organic BPE provided the highest hyaline cells among the other diet for 13.08 x 10^6 ± 4.62 cell/mL. In comparison with another diet, the obtained hyaline cells were not significantly different (Figure 4.21). The number of semigranular cells was not significantly different from other formulas after 90 days of feeding (Figure 4.22). The highest granular cells were 12.94 x 10^6 ± 3.32 cell/mL when the diet supplementing organic BPE mixed probiotic was fed (Figure 4.23). This result suggested that the hemocyte is the beginning mechanism to defense against disease in aquatic animals. At the same time, phagocytosis is involved with hyaline cells. The semigranular cells enhanced encapsulation, melanization, and coagulation. The granular cells induced prophenoloxidase system in crustaceans [62].

The coagulation time obtained from all feeding formulas was similar, but it was significantly shorter than the control diet (Figure 4.24). In addition, the total protein in all studies was also comparable. This result denoted that the protein content of prawns was not affected by the supplemented diet after 90 days of cultivation (Figure 4.25). The proteins were the essential components in the plasma of blood [59]. The protein concentration in the hemolymph states the health of prawns. The molting reduced the hemolymph protein and subsequently produced [119]. The hemolymph protein is related to the coagulation time. The expressed protein could prevent the

hemolymph, protect invaders pathogen entirely and regulate blood loss during injury and wound healing [115, 116].

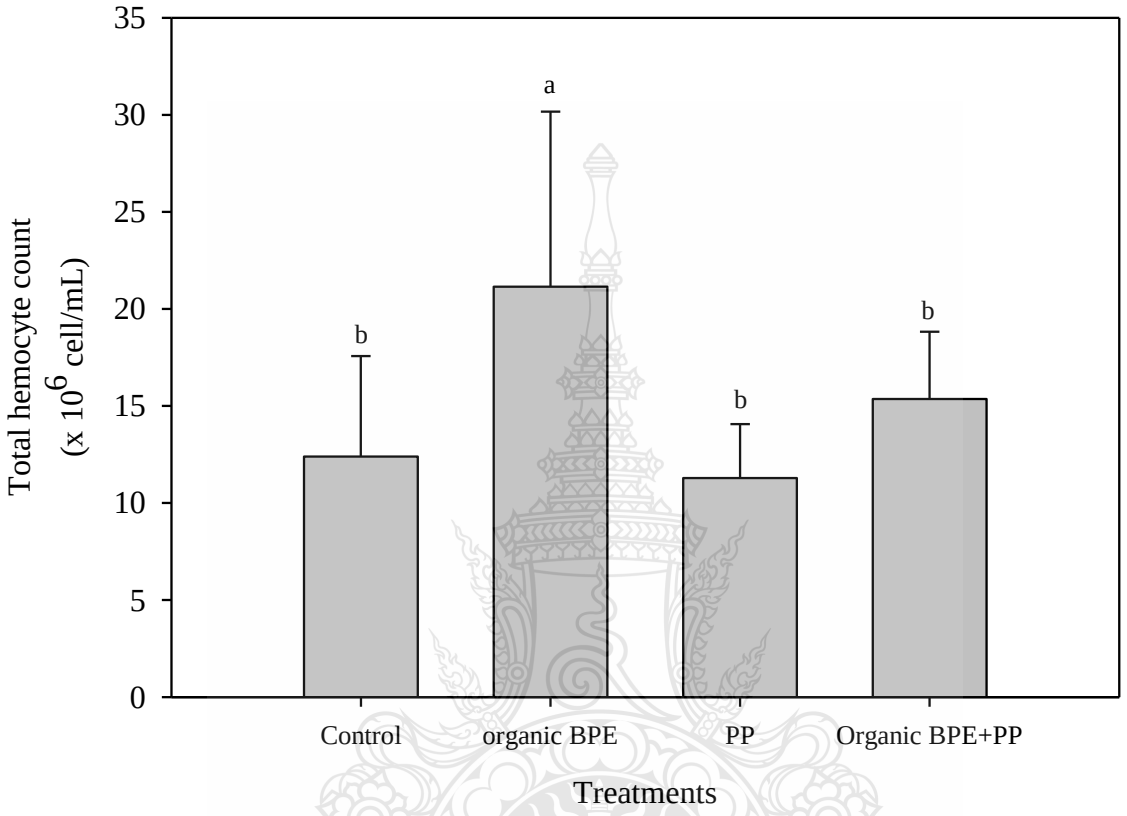

Figure 4.20 Total hemocyte count of *M. rosenbergii* fed with a control diet, organic BPE, PP, and organic BPE mixed PP after 90 days. The bar represents the mean with a standard deviation value (±SD). The different capital presents a significant difference at $p<0.05$.

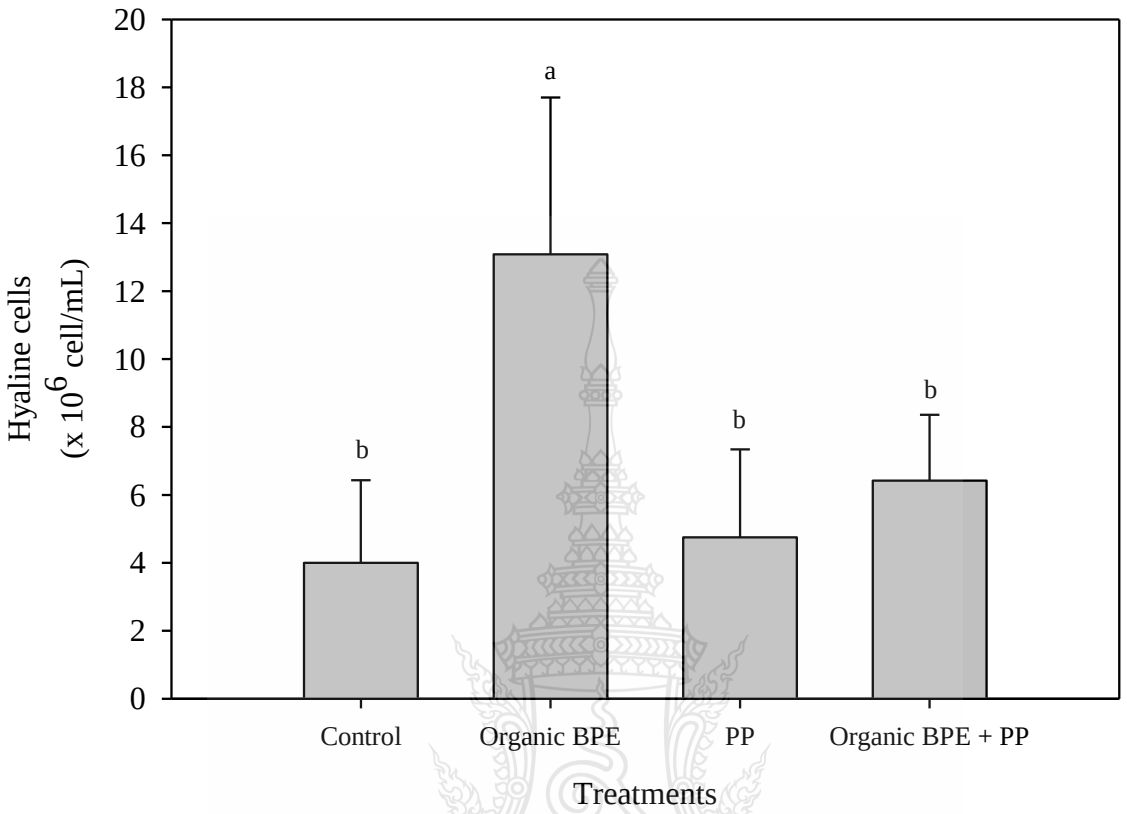

Figure 4.21 Hyaline cells of *M. rosenbergii* fed with a control diet, organic BPE, PP and organic BPE mixed PP, after 90 days. The bar represents the mean with a standard deviation value (±SD). The different capital presents a significant difference at $p<0.05$.

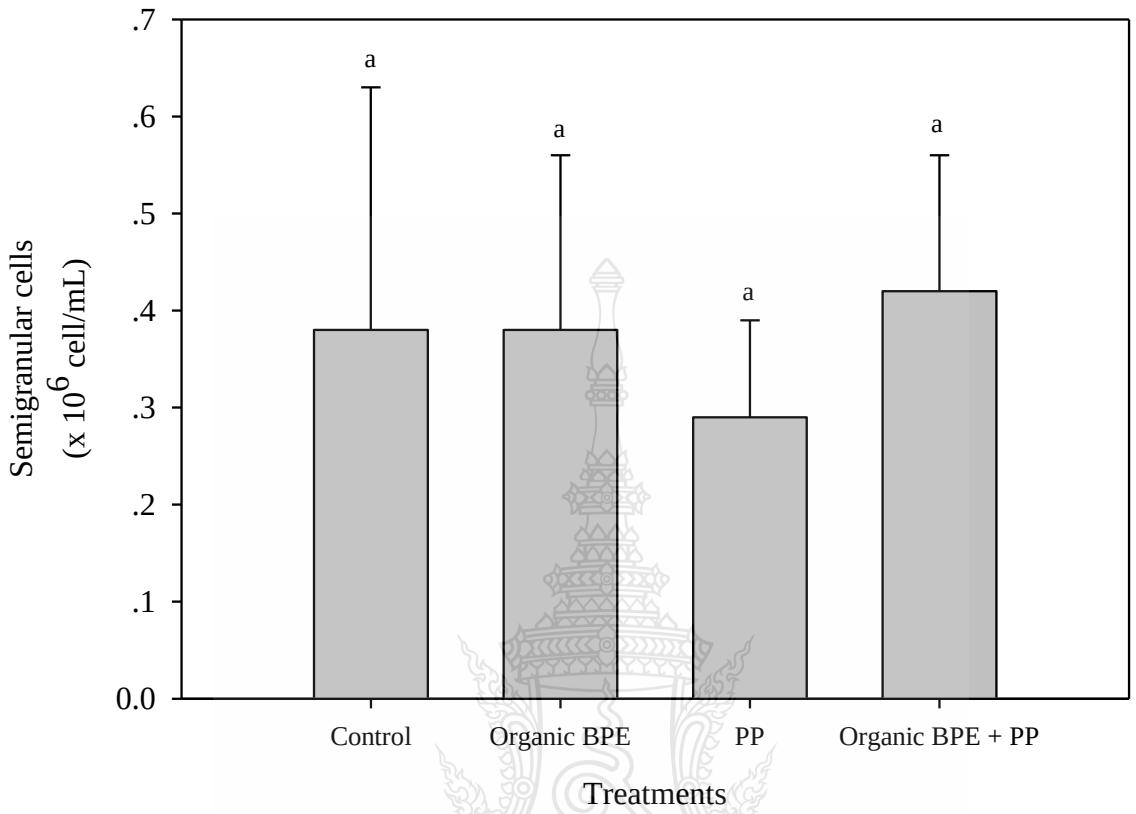

Figure 4.22 Semigranular cells of *M. rosenbergii* fed with a control diet, organic BPE, PP, and organic BPE mixed PP after 90 days. The bar represents the mean with a standard deviation value (±SD). The different capital presents a significant difference at $p<0.05$.

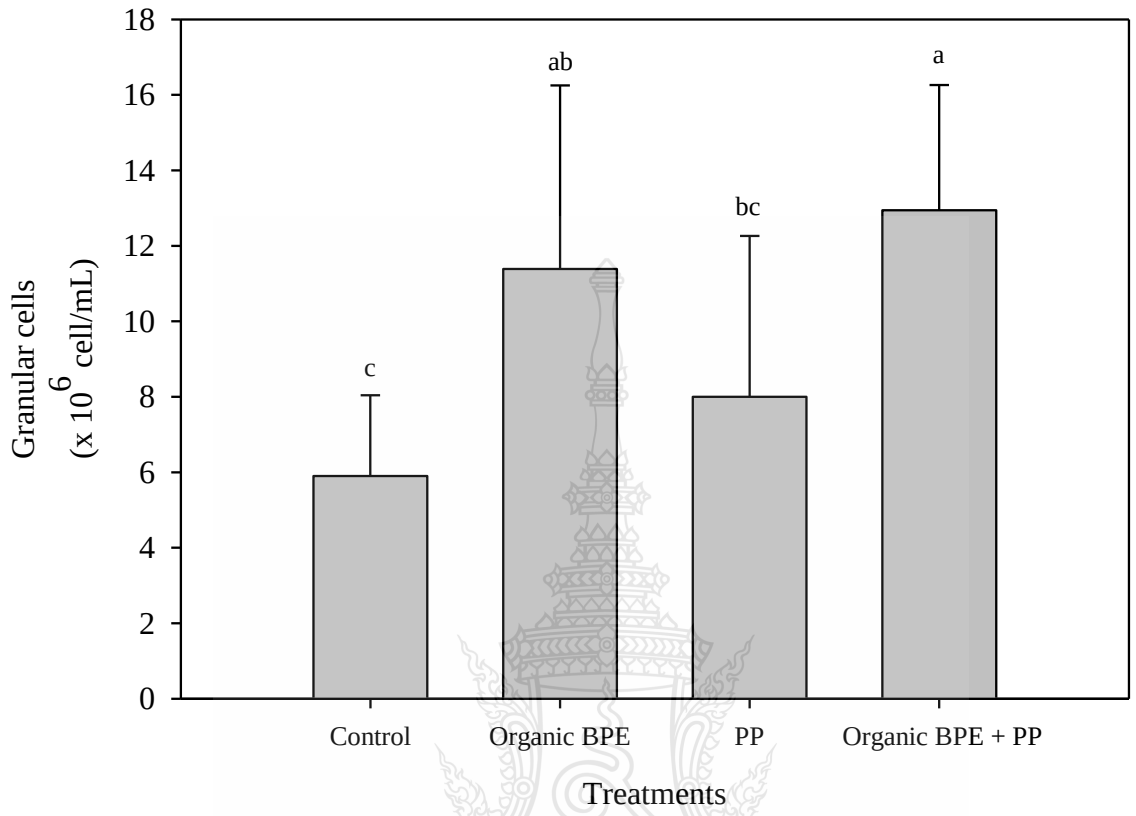

Figure 4.23 Granular cells of *M. rosenbergii* fed with a control diet, organic BPE, PP, and organic BPE mixed PP after 90 days. The bar represents the mean with a standard deviation value (±SD). The different capital presents a significant difference at $p<0.05$.

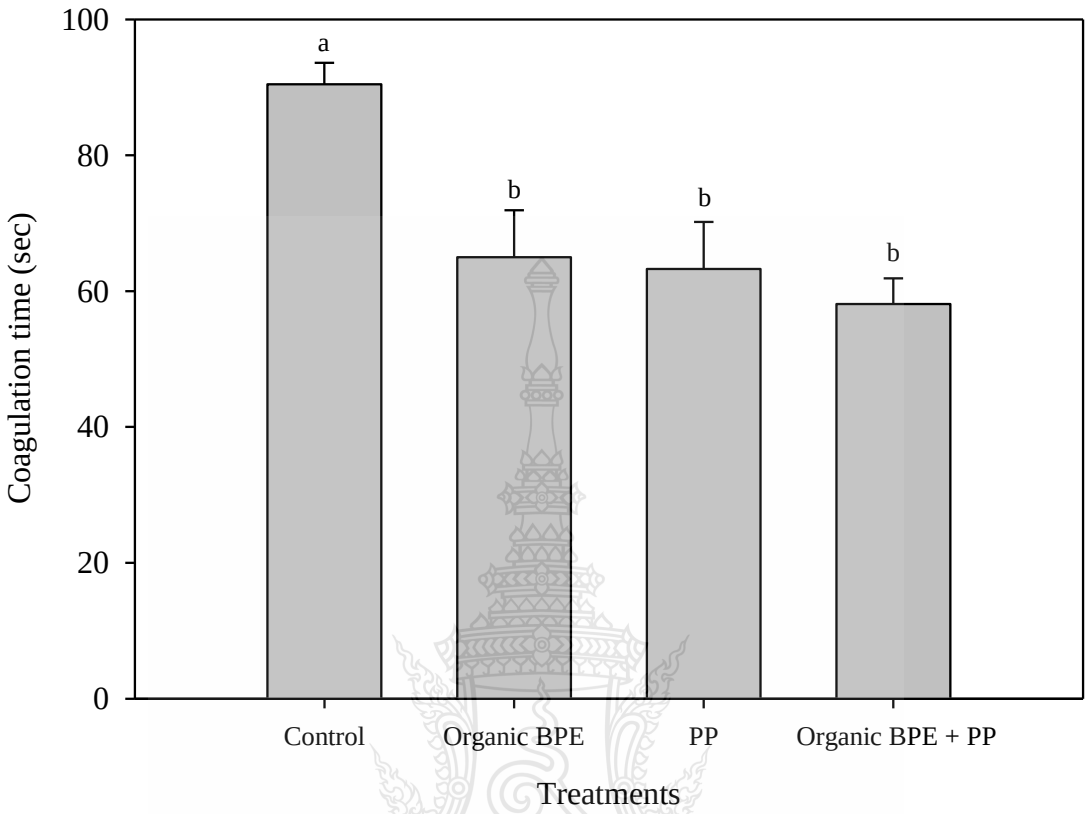

Figure 4.24 Coagulation time of *M. rosenbergii* fed with a control diet, organic BPE, PP, and organic BPE mixed PP after 90 days. The bar represents the mean with a standard deviation value (±SD). The different capital presents a significant difference at $p<0.05$.

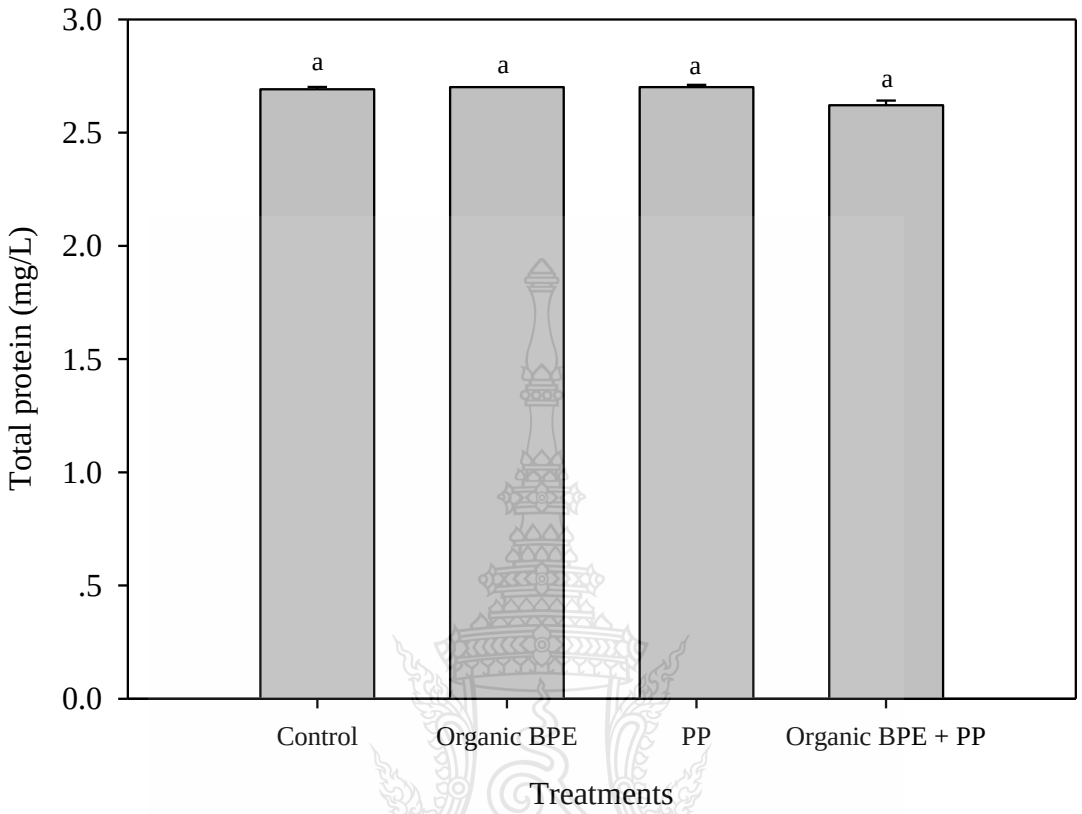

Figure 4.25 Total protein of *M. rosenbergii* fed with a control diet, organic BPE, PP, and organic BPE mixed PP after 90 days. The bar represents the mean with a standard deviation value (±SD). The different capital presents a significant difference at $p<0.05$

4.11 Effect of Organic BPE and Probiotic Powder Fed into *M. rosenbergii* on the Phagocytic Activity

The phagocytic activity of prawns was determined in this study. The experiments found that the phagocytic activity of the control diet was 85.00 ± 2.10 %, which significantly lower than other experimented diets (Figure 4.26). After 90 days of feeding with supplemented diets, the phagocytic activity was approximately 89 %.

Presently, to support the wellbeing of prawns, a diet supplemented with probiotics and plant extract has been performed. It could protect the pathogen and stimulate the immunity of animals by a phagocytic mechanism of hyaline cells. Phagocytosis is a primitive vital mechanism to enable the immune system related to the toxic oxygen during the respiratory burst (RBs) process after feeding the dietary supplement to aquatic animals [59].

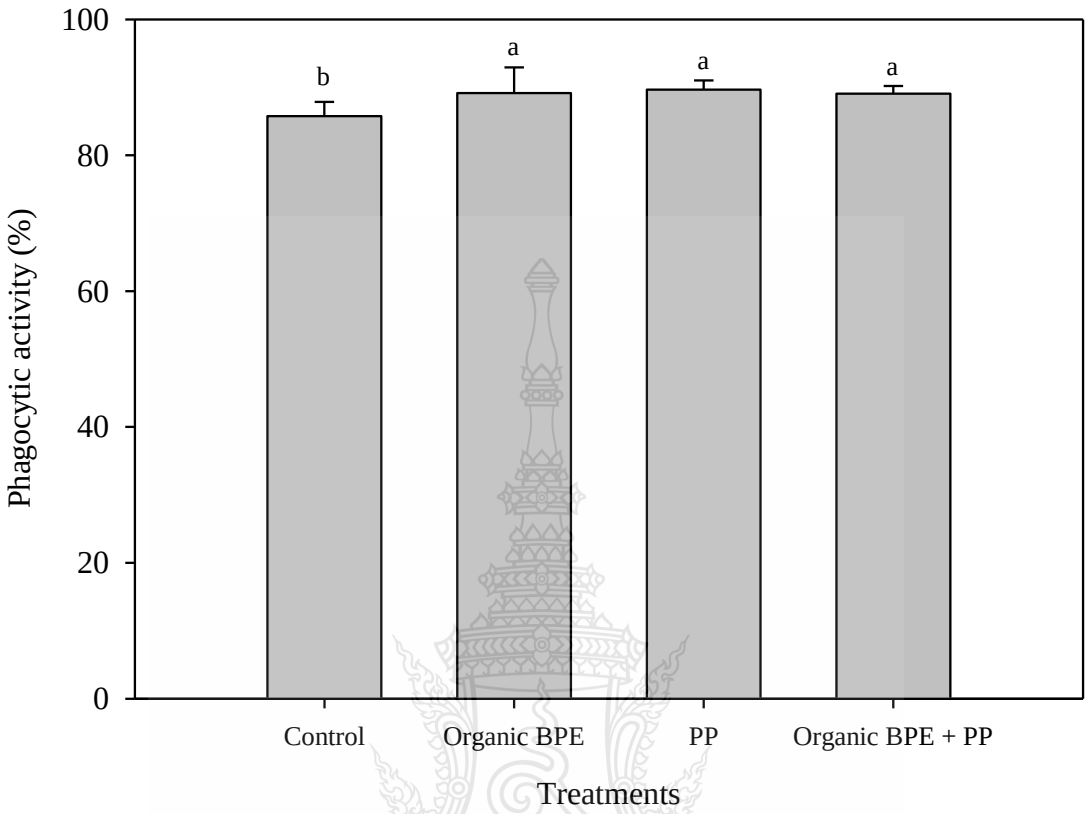

Figure 4.26 Phagocytic activity of *M. rosenbergii* fed with a control diet, organic BPE, PP, and organic BPE mixed PP after 90 days. The bar represents the mean with a standard deviation value (±SD). The different capital presents a significant difference at $p<0.05$.

CHAPTER 5
CONCLUSIONS

Six species of BP from an organic farm in Thailand, i.e., Kluai Homthong, Kluai Namwa, Kluai Kai, Kluai Hukmook, Kluai Lebmuernang, and Kluai Homtaiwan were studied. Firstly, the research exposed the bioactive compound property and identified the phytochemical in organic BPEs. The organic BPEs showed a difference in TPC, antioxidant activity, FRAP, and antipathogenic activity among all organic BPE types. Moreover, antipathogenic activity was also affected by the kind of banana. The acetic acid, formic acid, 1,2-benzenediol,3-methyl-, and 4-hydroxy-2-methylacetophone were the main phytochemicals present antioxidant activity and antipathogenic activity. Consideration of large-scale extraction feasibility, Kluai Homthong was then chosen to optimize the extracting condition due to it provided the maximum extraction yield. The result indicated that the extraction by 50 % v/v of methanol at 100 °C for 10 minutes provided the highest TPC, antioxidant activity, and FRAP. The dominant phytochemical components in organic BPE performed the antioxidant and antimicrobial properties under the optimized extracting condition. The injection of organic BPE could induce the immune parameters and resistance against pathogen and reduced the mortality of *M. rosenbergii*. The dietary supplementation of organic BPE and probiotic powder could also enhance the specific growth rate and survival rate at 30 days of cultivation. It also could induce the immune system of *M. rosenbergii*, and resistance against *A. hydrophila*. Hence, this study suggests that the organic BPE was an attend dietary supplement of *M. rosenbergii* to enhance growth performance in the initial cultivation stage and induce immunity throughout cultivation.

Future works

1. The extraction process should be developed to use on a commercial scale.

2. The shelf life and storage condition of BPE should be studied to maintain the phytochemical components and biological activity to perform as a commercial product.

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APPENDIX A
Standard Calibration Curve

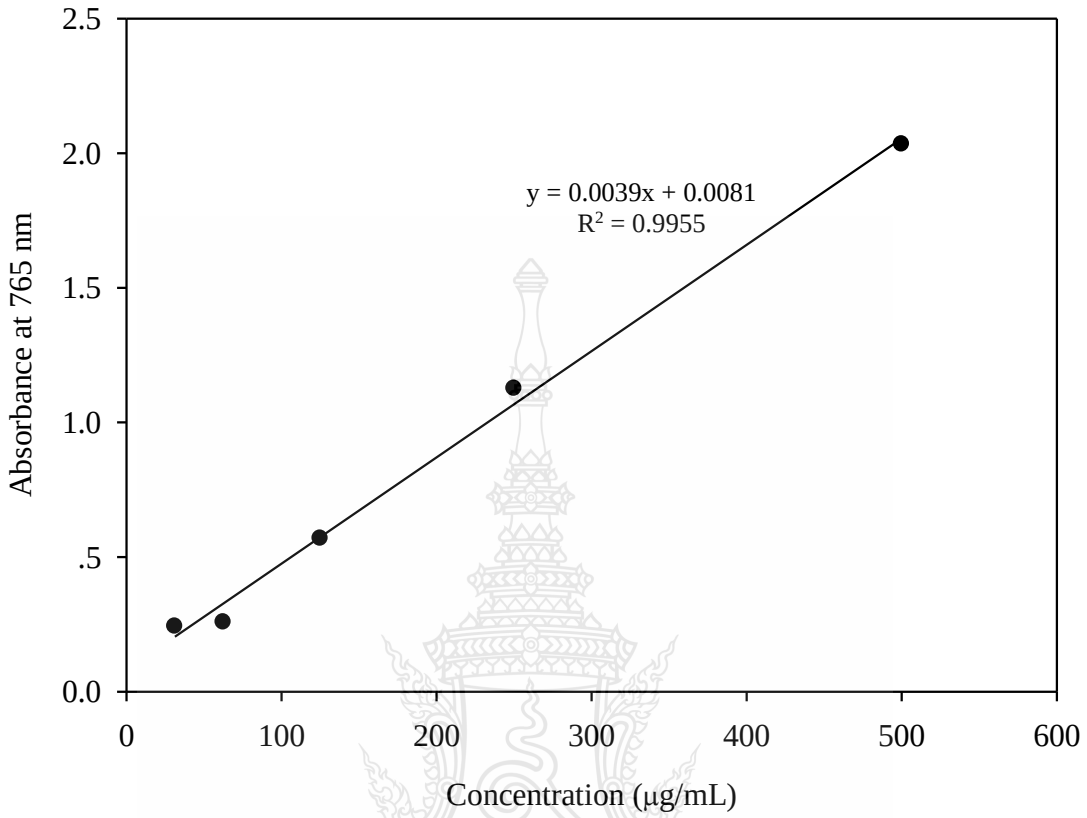


Figure A1 Standard calibration curve between Gallic acid and the absorbance at 765 nm of total phenolic content (TPC)

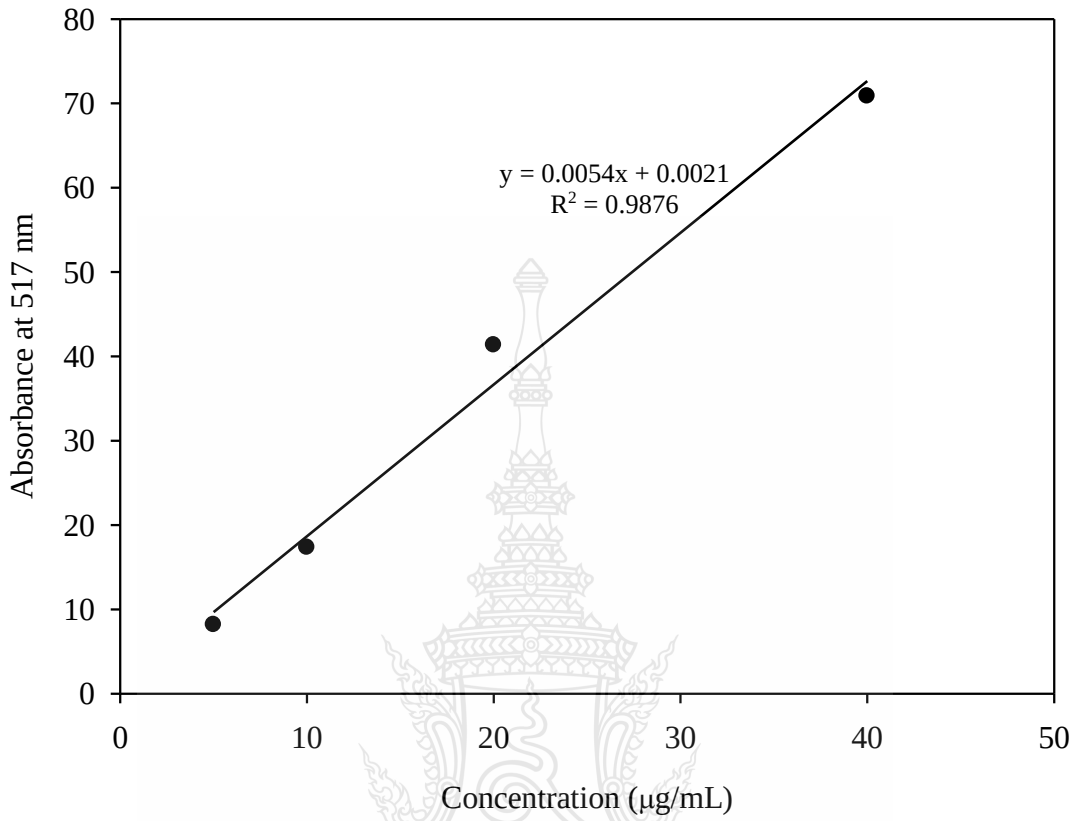


Figure A2 Standard calibration curve between Trolox and the absorbance at 517 nm of antioxidant content (DPPH)

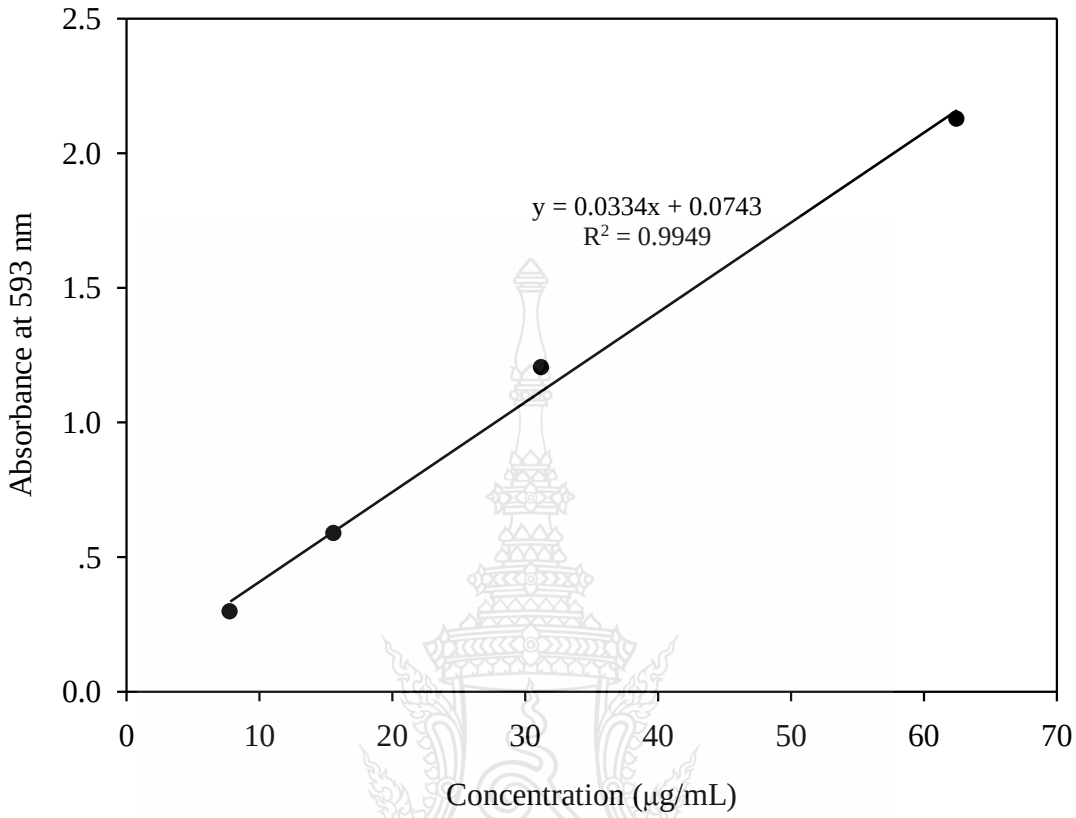


Figure A3 Standard calibration curve between gallic acid and the absorbance at 593 nm of ferric reducing antioxidant power (FRAP)

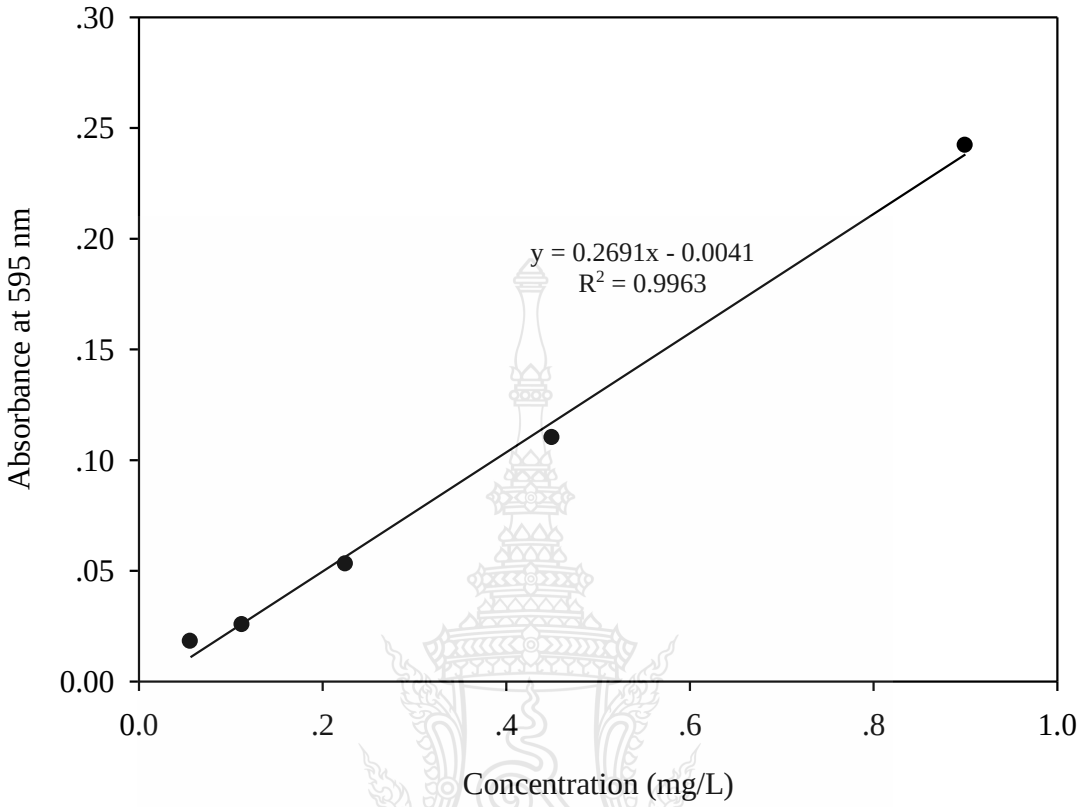
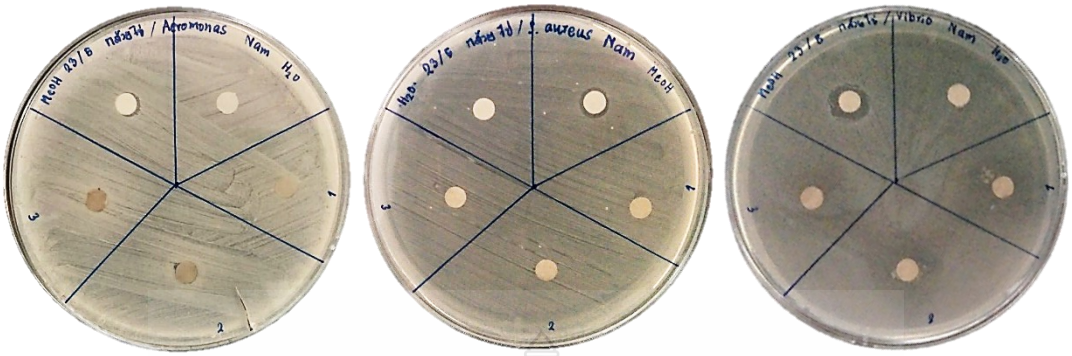


Figure A4 Standard calibration curve between Bovine serum albumin and the absorbance at 595 nm of total protein

APPENDIX B

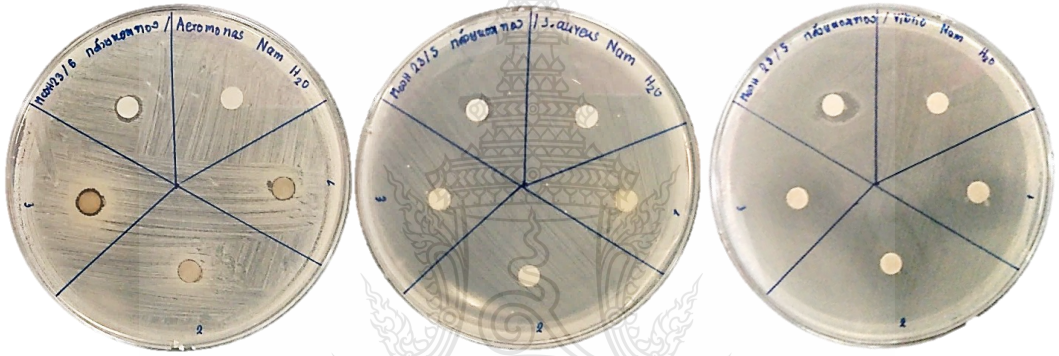
Pathogenic Inhibited Zone of Organic BPEs



(A) (B) (C)

Figure B1 Inhibition zone of banana peel extract (Kluai Kai)

(A) : *A.hydrophila*, (B) : *S. aureus*, and (C) : *V. parahaemolyticus*



(A) (B) (C)

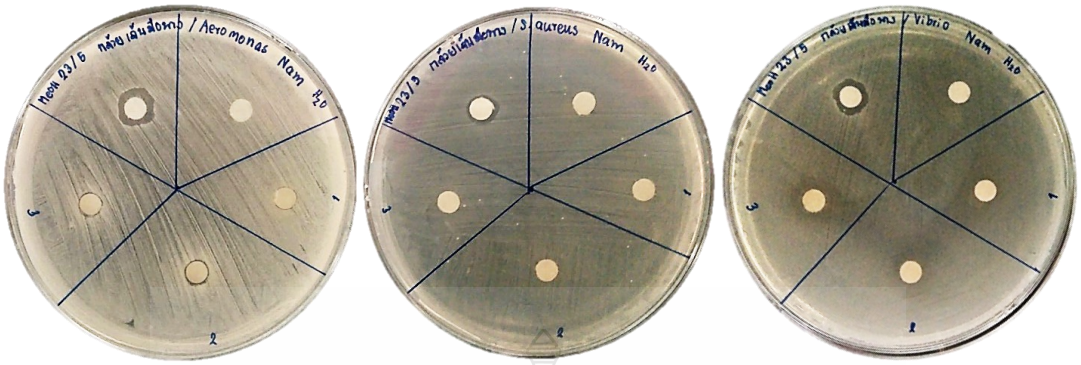
Figure B2 Inhibition zone of banana peel extract (Kluai Homthong)

(A) : *A.hydrophila*, (B) : *S. aureus*, and (C) : *V. parahaemolyticus*

(A) (B) (C)

Figure B3 Inhibition zone of banana peel extract (Kluai Homtaiwan)

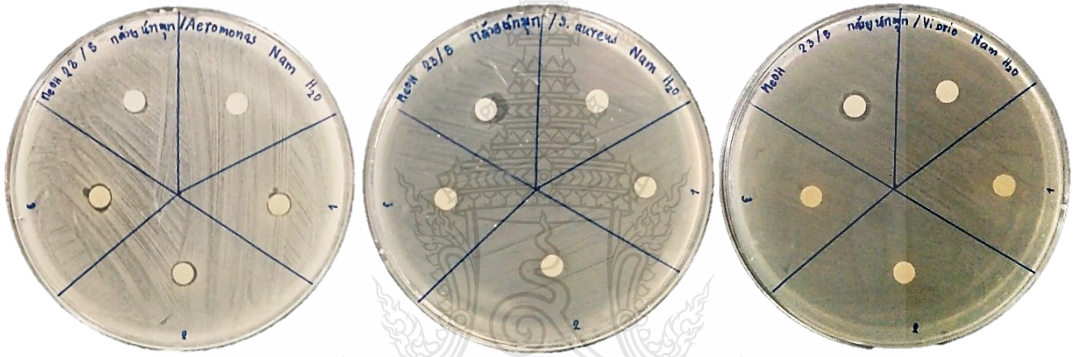
(A) : *A.hydrophila*, (B) : *S. aureus*, and (C) : *V. parahaemolyticus*



(A) (B) (C)

Figure B4 Inhibition zone of banana peel extract (Kluai Lebmuernang)

(A) : *A.hydrophila*, (B) : *S. aureus*, and (C) : *V. parahaemolyticus*



(A) (B) (C)

Figure B5 Inhibition zone of banana peel extract (Kluai Hukmook)

(A) : *A.hydrophila*, (B) : *S. aureus*, and (C) : *V. parahaemolyticus*

(A) (B) (C)

Figure B6 Inhibition zone of banana peel extract (Kluai Namwa)

(A) : *A.hydrophila*, (B) : *S. aureus*, and (C) : *V. parahaemolyticus*

APPENDIX C

Chromatogram of BPEs from GC-MS

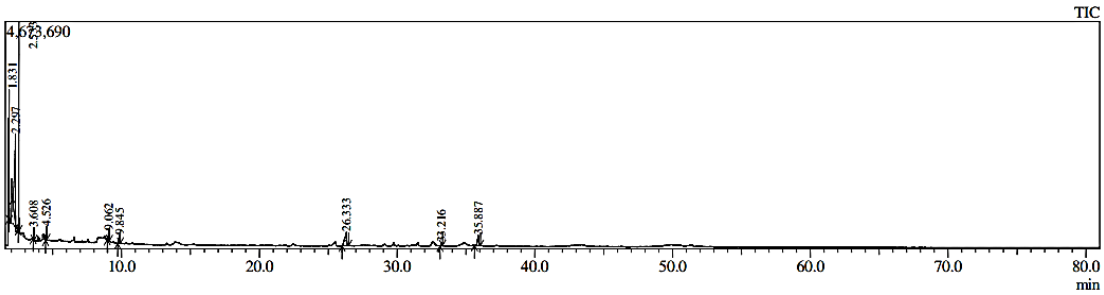


Figure C1 GC-MS chromatogram of the methanolic extract of Kluai Kai

Table C1 The compounds of the methanolic extract of Kluai Kai

Peak	Area (%)	Name
1	3.89	Pentane, 2-methyl-
2	3.9	Pentane, 3-methyl-
3	8.43	n-Hexane
4	4.74	Cyclopentane, methyl-
5	3.57	Formic acid
6	1.86	Cyclohexane
7	43.61	Acetic acid
8	28.79	2-Propanone, 1-hydroxy-
9	1.21	2,3-Butanediol

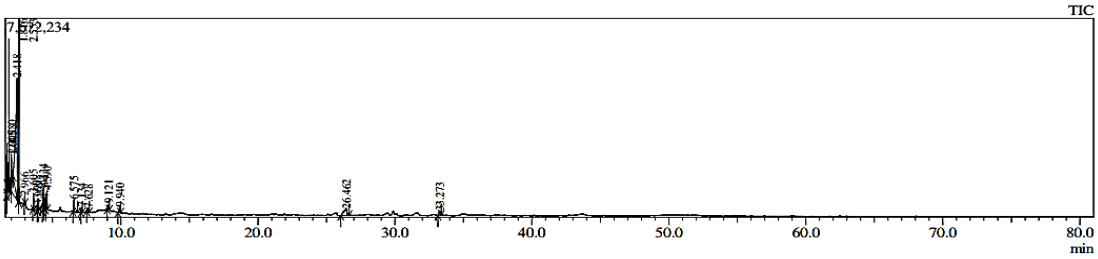


Figure C2 GC-MS chromatogram of the methanolic extract of Kluai Homthong

Table C2 The compounds of the methanolic extract of Kluai Homthong

Peak	Area (%)	Name
1	0.83	Acetic acid
2	0.92	2,3-Butanediol, [S-(R*,R*)]-
3	7.73	Formic acid
4	1.48	Acetaldehyde, hydroxy-
5	49.94	Acetic acid
6	14.67	2-Propanone, 1-hydroxy-
7	0.41	Acetoin
8	1.16	2-Propenoic acid, methyl ester
9	0.92	1-Hydroxy-2-butanone
10	0.43	2-Propanone, 1-hydroxy-
11	3.95	2,3-Butanediol, [R-(R*,R*)]-
12	0.92	Propanoic acid, 2-oxo-, methyl ester
13	3.56	2,3-Butanediol, [R-(R*,R*)]-
14	1.71	2-Furanmethanol
15	0.71	2-Propanone, 1-(acetyloxy)-

Table C2 The compounds of the methanolic extract of Kluai Homthong (continued)

Peak	Area (%)	Name
16	0.98	1,3-Dioxolane-2-methanol, 2,4-dimethyl-
17	1.43	Butyrolactone
18	1.74	1,2-Cyclopentanedione
19	5.23	4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-
20	1.28	1,2-Benzenediol, 3-methyl-

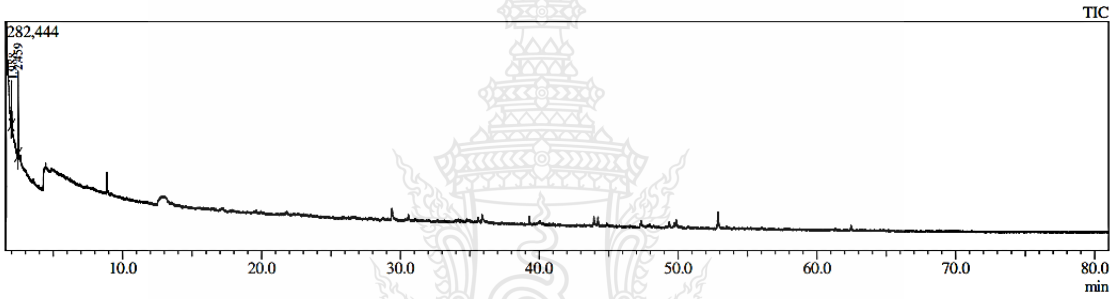


Figure C3 GC-MS chromatogram of the methanolic extract of Kluai Homtaiwan

Table C3 The compounds of the methanolic extract of Kluai Homtaiwan

Peak	Area (%)	Name
1	38.78	Acetic acid
2	61.22	2-Propanone, 1-hydroxy-

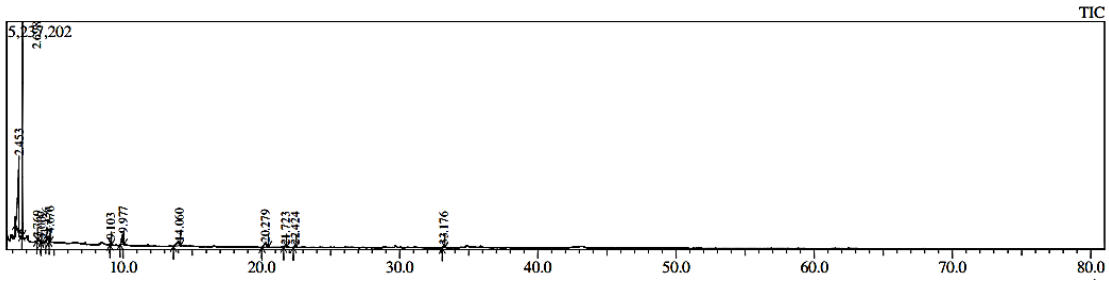


Figure C4 GC-MS chromatogram of the methanolic extract of Kluai Lebmuernang

Table C4 The compounds of the methanolic extract of Kluai Lebmuernang

Peak	Area%	Name
1	48.25	Acetic acid
2	25.64	2-Propanone, 1-hydroxy-
3	0.32	2-Propenoic acid, methyl ester
4	0.62	1-Hydroxy-2-butanone
5	1.04	2,3-Butanediol, [R-(R*,R*)]-
6	1.55	2,3-Butanediol, [S-(R*,R*)]-
7	1.35	Butyrolactone
8	6.8	1,2-Cyclopentanedione
9	6.3	2-Hydroxy-gamma-butyrolactone
10	5.08	Acetic acid, 3,4-dihydroxy-3-methyl-butyl ester
11	0.94	Acetic acid, 3,4-dihydroxy-3-methyl-butyl ester
12	1.1	Cyclopropyl carbinol
13	1	1,2-Benzenediol, 3-methyl-

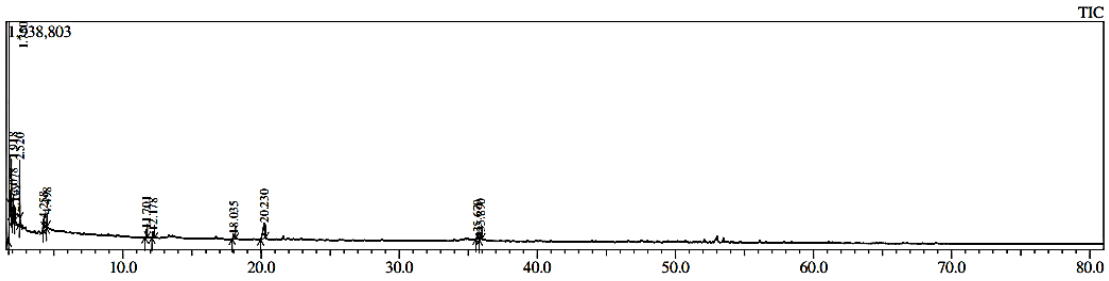


Figure C5 GC-MS chromatogram of the methanolic extract of Kluai Hukmook

Table C5 The compounds of the methanolic extract of Kluai Hukmook

Peak	Area (%)	Name
1	33.82	Pentane, 2,4-dimethyl-
2	8.06	Cyclopentane, methyl-
3	5.66	Acetic acid
4	1.27	Cyclohexane
5	13.19	2-Propanone, 1-hydroxy-
6	1.99	2,3-Butanediol, [R-(R*,R*)]-
7	2.53	2,3-Butanediol, [R-(R*,R*)]-
8	3.28	Propanoic acid, 2-oxo-
9	2.86	Propanoic acid, 2-oxo-
10	3.02	2(3H)-Furanone, dihydro-3-hydroxy-4,4-dimethyl-, (.+/-.)-
11	16.17	4-Butoxy-2-butanone
12	4.36	5-Amino-1-pentanol, N,O-diacetyl-
13	3.79	4-Hydroxy-2-methylacetophenone

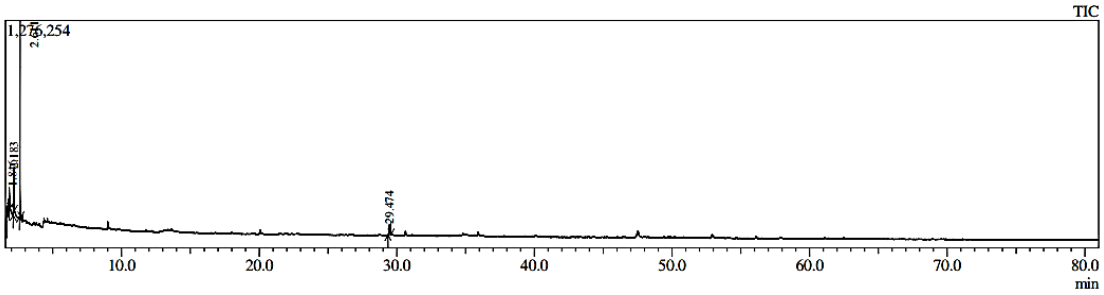


Figure C6 GC-MS chromatogram of the methanolic extract of Kluai Namwa

Table C6 The compounds of the methanolic extract of Kluai Namwa

Peak	Area (%)	Name
1	4.74	Methylamine, N,N-dimethyl-
2	25.64	Acetic acid
3	54.5	2-Propanone, 1-hydroxy-
4	15.11	Isosorbide

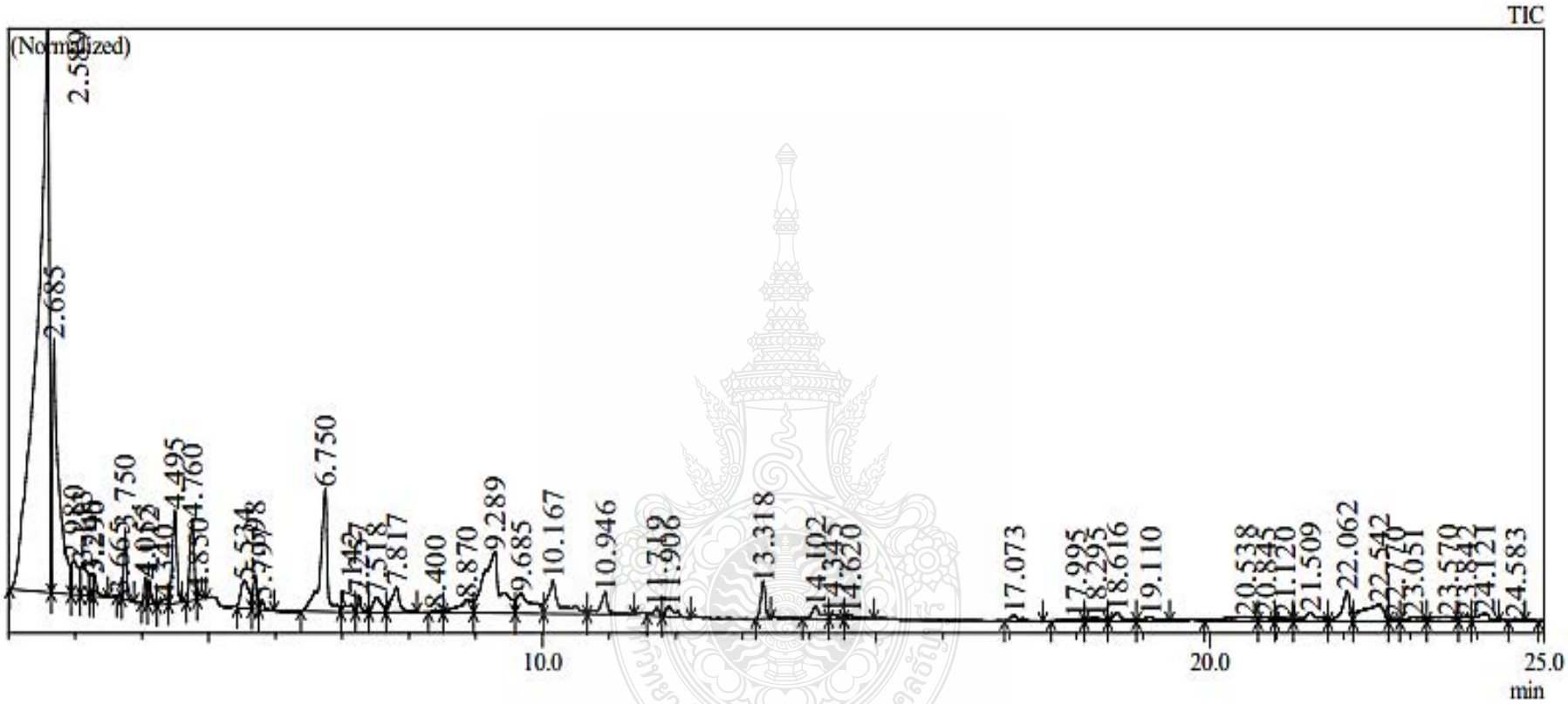


Figure C7 GC-MS chromatogram of the organic BPE obtained from the optimum condition

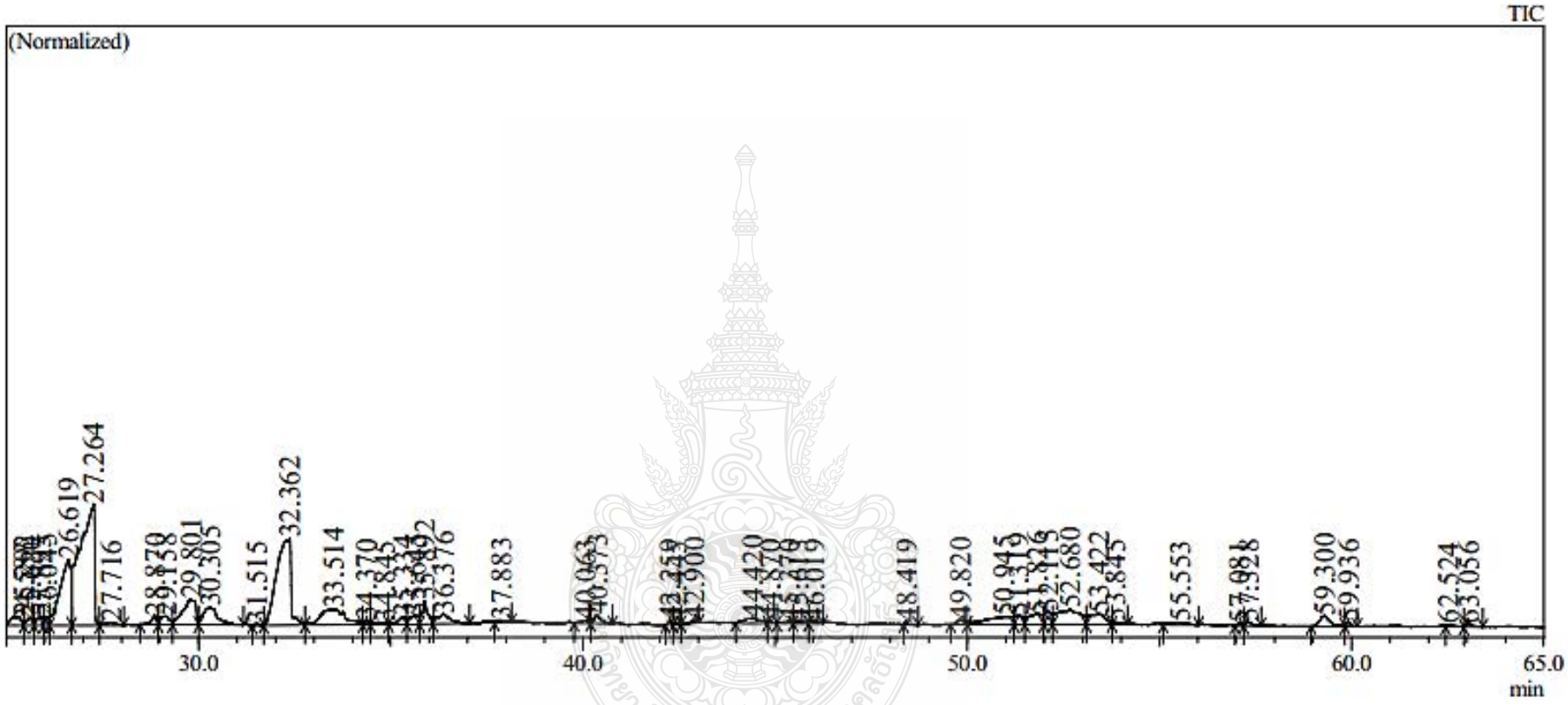


Figure C7 GC-MS chromatogram of the organic BPE obtained from the optimum condition (continued)

Table C7 The compounds of the organic BPE obtained from the optimum condition

Peak	% Area	Name
1	0.2	Propanal, 2-methyl-
2	5.76	2-Propanone, 1-hydroxy-
3	0.74	Acetoin
4	0.25	2-Propenoic acid
5	0.61	2-Propenoic acid, methyl ester
6	0.24	1-Hydroxy-2-butanone
7	0.24	Acetic anhydride
8	0.12	1-Propanol, 2-amino-, (.+/-.)-
9	1.47	2,3-Butanediol, [R-(R*,R*)]-
10	0.1	(S)-5-Hydroxymethyl-2[5H]-furanone
11	0.8	Furfural
12	0.44	1,2-Propanediol, 1-acetate
13	0.14	p-Dioxane, 2,5-divinyl-
14	0.2	2-Propanone, 1-(acetyloxy)-
15	0.44	4-Cyclopentene-1,3-dione
16	0.71	Butanoic acid, 2-ethyl-3-oxo-, methyl ester
17	0.06	2-Pentanone, 3-methyl-
18	0.66	2-Propenoic acid, 2-methyl-, oxiranylmethyl ester
19	1.08	2-Methylene cyclopentanol
20	1.37	1,2-Cyclopentanedione
21	0.15	2-Furanmethanol, 5-methyl-

Table C7 The compounds of the organic BPE obtained from the optimum condition (continued)

Peak	% Area	Name
22	0.29	2-Furancarboxaldehyde, 5-methyl-
23	0.57	2,4-Dihydroxy-2,5-dimethyl-3(2H)-furan-3-one
24	0.38	2H-Pyran-2,6(3H)-dione
25	0.16	α -d-Erythro-hex-2-enopyranoside, ethyl 2,3-dideoxy-
26	0.19	1,2-Cyclopentanedione, 3-methyl-
27	0.28	Pentanoic acid, 4-oxo-
28	0.2	2,5-Hexanedione
29	0.46	3(2H)-Furanone, 4-hydroxy-5-methyl-
30	0.41	Methyl 2-furoate
31	0.82	2,5-Dimethylfuran-3,4(2H,5H)-dione
32	1.2	Furaneol
33	0.24	2,3-Dimethylfumaric acid
34	0.16	α -Acetobutyrolactone
35	0.33	2-Furancarboxylic acid
36	0.06	2-Cyclopenten-1-one, 3-ethyl-2-hydroxy-
37	0.54	2(3H)-Furanone, 5-heptyldihydro-
38	0.3	3-Acetoxydodecane
39	4.32	4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-
40	0.47	2(3H)-Furanone, dihydro-4-hydroxy-
41	1.98	4H-Pyran-4-one, 3,5-dihydroxy-2-methyl-
42	1.58	Pentanoic acid, 2-isopropoxyphenyl ester

Table C7 The compounds of the organic BPE obtained from the optimum condition (continued)

Peak	% Area	Name
43	0.05	4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-
44	2.34	1,2,3-Propanetriol, 1-acetate
45	0.35	1,2-Benzenediol, 4-methyl-
46	0.48	2-furancarboxaldehyde, 5-(hydroxymethyl)-
47	0.81	2-Methoxy-4-vinylphenol
48	0.81	Cyclooctane-1,2,5-trione
49	0.16	1,2-Benzenediol
40	0.38	(2-Methylpyrazol-3-yl)-methanol
51	0.11	2,2,3,3-Tetramethylcyclopropanecarboxylic acid, 4-methylphenyl ester
52	0.08	Ethyl N-(o-anisyl) formimidate
53	0.89	Oxazole, 2-butyl-5-ethyl-4-methyl-
54	0.49	7-Methoxy-6-methylbenzo[d] [1,3] dioxol-4-ol
55	2.01	Isosorbide Dinitrate
56	1	Tetrahydro-4H-pyran-4-ol
57	0.06	2-Cyclohexen-1-one, 4-hydroxy-3,5,6-trimethyl-4-(3-oxo-1-butenyl)-
58	0.09	4,6-Diamino-O-cresol
59	0.12	Methyl-β-D-thiogalactoside
60	0.18	Cyclodecasiloxane, eicosamethyl

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