STUDY THE EFFICIENCY OF *Phellinus linteus* EXTRACT ENCAPSULATION IN CHITOSAN NANOPARTICLES FOR COSMECEUTICAL APPLICATIONS

BY

MISS PAPASSORN KINGKLAO

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF MASTER OF SCIENCE (BIOTECHNOLOGY)
DEPARTMENT OF BIOTECHNOLOGY
FACULTY OF SCIENCE AND TECHNOLOGY
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ENTITLED

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was approved as partial fulfillment of the requirements for
the degree of Master of Science (Biotechnology)

on July 6, 2018

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Thesis Title	STUDY THE EFFICIENCY OF *Phellinus linteus* EXTRACT ENCAPSULATION IN CHITOSAN NANOPARTICLES FOR COSMECEUTICAL APPLICATIONS
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Degree	Master of Science (Biotechnology)
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Academic Years	2017

ABSTRACT

The efficiency of *Phellinus linteus* extract encapsulation in nanochitosan for cosmeceutical applications has been studied in this research. The *P. linteus* extracts were PL-EH, PL-E1, PL-E2, PL-W, PL-A and PL-N. PL-EH had the highest total phenolic contents (TPC) and total flavonoid contents (TFC) were 610.92±7.611 mg GAE/g crude extract and 756.75±1.283 mg Que/g crude extract, respectively. Whereas, PL-W had the highest total polysaccharide content of 167.96±0.061 mg Glu/g crude extract. However, PL-EH and PL-E1 revealed the high antioxidant activity, IC_{50} at 2.04 µg/mL and 2.07 µg/mL, respectively, with no statistically significant difference level at 0.05. On the other hand, PL-N showed efficiently antibacterial activities against *Bacillus subtilis, Enterobacter aerogenes, Staphylococcus aureus* and *Escherichia coli* via Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC). PL-EH, PL-E1 and PL-N were analyzed by HPLC and founded that all contained gallic acid, quercetin caffeic acid, (2)-epicatechin, *p*-coumaric acid, benzoic acid and myricetin.

PL-EH, PL-E1 and PL-N were selected for following encapsulation efficiency and releasing efficiency study. The encapsulation efficiencies of PL-EH in nanochitosan, nanoliposome and nano-double particles were 96.19, 48.31 and 75.59 %, respectively. While encapsulation efficiencies of PL-E1 were 94.89, 32.20 and 61.98 and PL-N were 95.15, 38.90 and 43.73 %, respectively. Therefore, nanochitosan provided the best encapsulation efficiencies, following with nano-double particles for all extracts. Non-accumulation and accumulation releasing of PL-EH, PL-E1 and PL-N, the highest efficiencies were sorted by nanoliposome, nano-double particles and nanochitosan.

From all results, PL-EH was selected for further cosmeceutical production. The product model was tested the properties of antioxidant, antibacterial and preliminary allergy test. The results showed that this product model was able to reduce more free radical effects (55% DPPH inhibition), inhibit bacteria and non-allergy effect to the volunteers.

Keywords: *Phellinus linteus*, antioxidant, antimicrobial, nanoparticles encapsulation and cosmeceutical product

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Miss Papassorn Kingklao

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LIST OF ABBREVIATIONS

Symbols/Abbreviations	Terms
PL-EH	*P. linteus* were extracted with 95% ethanol at 80°C
PL-E1	*P. linteus* were extracted with 95% ethanol in Round 1 at 25°C
PL-E2	*P. linteus* were extracted with 95% ethanol in Round 2 at 25°C
PL-W	*P. linteus* was extracted with water at 95°C
PL-A	*P. linteus* extracted with a 1% solvent, aluminum oxalate (1% $(NH_4)_2C_2O_4$) at 95°C
PL-N	*P. linteus* extracted with 1.25M sodium hydroxide/0.05% sodium borohydride (1.25MNaOH/0.05%$NaBH_4$) at 25°C
GAE	Gallic Acid Equivalent
QE	Quercetin Equivalent
GE	Glucose Equivalent
HPLC	High-Performance Liquid Chromatography
TEM	Transmission Electron Microscope
DLS	Dynamic Light Scattering

CHAPTER 1
INTRODUCTION

1.1 Background and Signification of Research Problem

Extracts from nature are interested. Medicinal plants have been widely use. Generally focused on food, medicine and cosmetics mostly. At present, showed a lot of diseases can be treated or symptom relief by using natural extracts. Common diseases and the use of natural extracts to treat diseases such as diabetes, heart disease and cancer, etc. (Chonlada, *et al* 2013). The beauty of your skin with products and cosmeceuticals is gaining increasing popularity. Cosmetics industry product development is remarkable and has expanded dramatically.

Phellinus linteus in the Phellinus genus and Hymenochaetaceae family. Research on mushrooms showed that *Phellinus linteus* contains important substance. The bioactive compounds of *Phellinus linteus* mainly include substances such glucan, a polymer of glucose that can be found in life generally. Glucan can be classified into two types according to the bond connecting the molecules of glucose include alpha glucan (α-glucan) and beta-glucan (β-glucan) (Suwit and Siriwan, 2010). β-glucan has the ability to stimulate the immune system, anti-tumor and antiviral compounds also have the ability to against free radicals. It is used to treat cancer patients. Reduce toxicity in the liver and improves the ability to destroy or reduce the effects of free radicals. (Ajith and Janardhanan, 2002 and 2003)

One technique to increase the efficiency of drug delivery. The active ingredient is a substance or bringing them to the store (encapsulation) is a wizard to control the release of the substances to a target. It also can help reduce the harmful substances. In the event of excessive amounts of the target. It also allows the active ingredient can be absorbed better. At present, chitosan, a substance that gets attention and is commonly used as a material. Since it is derived from natural substances. It is compatible with life, in addition, non-toxic as well. When the nano-encapsulation technology was applied to the storage of these substances. Further optimize the delivery and release of substances that reduce the amount required.

Therefore, in this study will investigate the encapsulation of substances extracted from *Phellinus linteus* in nanoparticles such as chitosan, the properties of the extracts as well as efficiency of releasing, in order to develop cosmeceutical product.

1.2 Objectives of Research

1.2.1 To study the suitable *Phellinus linteus* extractions

1.2.2 To investigate the activities of *Phellinus linteus* extracts

1.2.3 To study the efficiency of encapsulation and releasing of *Phellinus linteus* extracts in various type of nanoparticles

1.2.4 To develop cosmeceutical product from the *Phellinus linteus* extracts with suitable nanoparticle

1.3 Expected Benefits

1.3.1 Suitable crude extract from *Phellinus linteus*

1.3.2 Effectively encapsulation method for active ingredients of *Phellinus linteus* extract in nanoparticles

1.3.3 Cosmeceuticals product of *Phellinus linteus* extract

1.4 Work Place

1.4.1 Department of Biotechnology, Faculty of Science and Technology, Thammasat University

1.4.2 Department of Chemistry, Faculty of Science and Technology, Thammasat University

1.4.3 Central Scientific Instrument Center (CSIC), Faculty of Science and Technology, Thammasat University

CHAPTER 2
REVIEW OF LITERATURE

2.1 *Phellinus linteus*

2.1.1 General information

The shape is grayish brown. The width is about 3-20 centimeters, length about 5-30 centimeters and thick about 1.5-15 centimeters.

Phellinus linteus has a half-circle growth. The mushroom will grow together and bonded to the same. The growth is going on; the flowers are large and thick. The skin is rough, cracked and very hard like a bark.

Figure 2.1: The characteristics of the *Phellinus linteus* mushroom.
(Retrieved on 29 June 2016, Reference: http://thaiphimaan.co.th/?page_id=60)

The spore of mushroom is 4.5-6 × 5.4-7 millimeters in size and oval shape. Smooth and thick surface. Spores are reddish brown. It grows in trees and timber. It can be found all over the region of Thailand.

2.1.2 Scientific classification of *Phellinus linteus*

Table 2.1: Scientific classification of *Phellinus linteus*

Scientific classification	
Scientific name	*Phellinus linteus*
Kingdom	Fungi
Phylum	Basidiomycota
Class	Basidiomycetes
Order	Hymenochaetales
Family	Hymenochaetaceae
Genus	*Phellinus*
Species	*P. linteus*

(Retrieved on 29 June 2016, Reference: https://en.wikipedia.org/wiki/Phellinus_linteus)

2.2 Free Radicals and Antioxidants

(Retrieved on 29 June 2016, Reference: http://kb.psu.ac.th/psukb/bitstream/2553/2906/7/241434_ch1.pdf)

2.2.1 Free Radicals

Free radicals mean unstable molecules due to lack of electrons. It is necessary to find other electrons to catch to stabilize. Another molecule that has been scrambled electrons is a problem. Molecules structure has change and do not work. This causes serious problems. It can destroy important components of cells around the area, such as protein, fat, carbohydrate, DNA, etc. If the formation of free radicals will cause cellular violence mechanism of disease such as inflammation, cancer, tuberculosis, inflammatory diseases, etc. Causes of free radicals are caused by various internal and external factors.

2.2.1.1 Internal factors

(1) The reactive oxidation reactions such as oxidation of fat.

(2) The oxidation reactions with enzymes as the catalyst.

There are two types of enzymes that stimulate the formation of free radicals in the body.

- Xanthine oxidase (XO) an important role in the purine base process by catalyzing the conversion of hypoxanthine to xanthine and the conversion of xanthine to uric acid. Formed as a super oxide radical ($O2^{\bullet-}$)

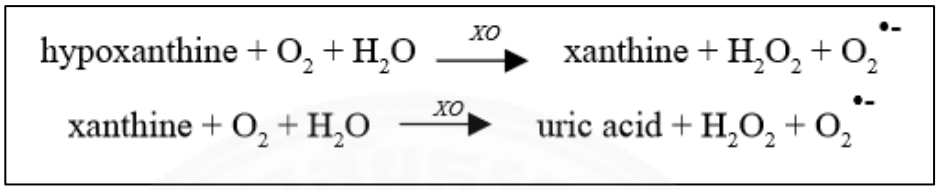

Figure 2.2: Function of xanthine oxidase

- Lypoxigenase (LOX) accelerates the oxidation of unsaturated fatty acids (polyunsaturated fatty acid). Enzymes are iron (Fe^{2+}) is a component. It acts to draw hydrogen atoms from fatty acids. And add oxygen to fatty acids, resulting in hydroperoxide. This will break down the radicals of fatty acids.

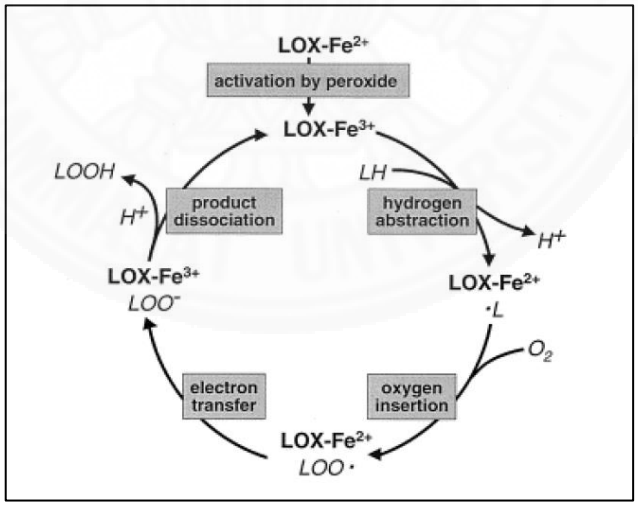

Figure 2.3: Function of lypoxigenase (LOX) in fatty acid oxidation.

(3) The process of removal of foreign matter of white blood cells.

White blood cells use oxygen molecules to produce a super oxidant ($O_2^{\bullet-}$) by the NADPH oxidase enzyme on the outer lining of white blood cells.

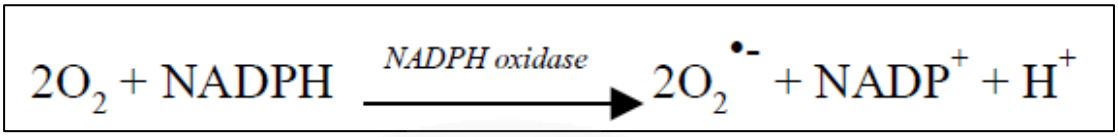

Figure 2.4: Super Oxide ($O_2^{\bullet-}$)

The pigment of white blood cells (granule) contain myeloperoxidase causes radical hypochlorous (hypochlorus, $HOCl^{\bullet}$) is a microorganism.

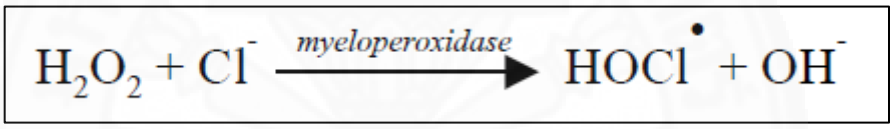

Figure 2.5: Hypochlorite formation reaction (hypochlorus, HOCl •)

(4) Transition metal

There are two types of transition metal: iron (Fe^{2+}) and copper (Cu^{2+}). Hydroxyl (OH) from superoxide ($O_2^{\bullet}$) and hydrogen peroxide (H_2O_2) in Fenton's reaction.

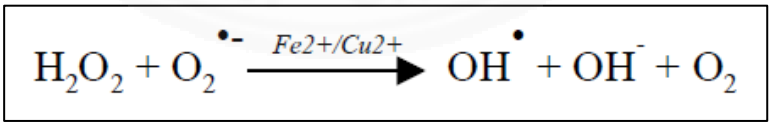

Figure 2.6: The reaction creates oxidative radicals, hydroxyl ($OH^{\bullet}$).

2.2.1.2 External factors

(1) Medicine; some drugs can generate free radicals within the body.

(2) Radiation such as X-ray, gamma ray, etc.

(3) Tobacco smoke consists of nitric oxide (NO), nitrogen dioxide (NO_2) and peroxynitrite ($ONOO^-$). Oxygen ($O_2^{\bullet-}$) forms within the cell.

(4) Ozone can be transformed into hydroxyl radicals by UV light.

2.2.2 Antioxidants

Antioxidants are substances such as enzymes and others. To slow down or prevent the oxidation of the substrate. Destruction of the molecule that triggers free radicals is a mechanism of the antioxidant system. This may be due to the enzyme system or not the enzyme system. Normally, antioxidants are available in two forms: substances found in the body and substances found in food.

Free radicals in our body are divided into two types.

(1) Enzyme substance

- Superoxide Dismutase: SOD
- Catalase: CAT
- Glutathione Peroxidase: GPX
- Glutathione Reductase: GR
- Glutathione S-Transferase: GST

(2) Non-enzymatic antioxidants

- Glutathione
- Lipoic acid
- Ceruloplasmin
- Albumin
- Transferrin
- Haptoglobin
- Hemopexin
- Uric Acid

Non-enzymatic antioxidants in foods

- Tocopherols
- Carotenoids

- Ascorbic Acid
- Stearoids
- Ubiquinone
- Thiols
- Imosine
- Taurine
- Pyruvate
- Gallic Acid
- Flavonoids
- Trolox
- BHT
- BHA

2.3 Nanotechnology

(Retrieved on 29 June 2016, Reference: http://www.nanotec.or.th/th/?p=1137)

Technology refers to applications that involve managing the creation of synthetic materials or devices at the level of atoms. Small molecules or components in the range of about 1 to 100 nanometers, which will result in materials or devices. There are new functions. It has special properties, physically, chemically and biologically, to benefit and increase economic value.

The term “nano” is derived from the Greek word nanos, which means “dwarf” or “small”. It means one billionth of a measure.

The word "nanometer" comes from the words "nanos" and "meters." So the term nanometer means the unit of measure. "One billionths of a meter" is One nanometer (1 nm) = one billionth of a meter

$$\frac{1}{1000000000} = 10^{-9} \text{ meters}$$

One nanometer is smaller than the human hair diameter of about eight thousand to one hundred thousand times the size of the smallest human that

can be seen with the naked eye is about 10,000 nanometers. Cannot see with the eye or even a normal microscope. So, scientists will be able to see a small nanometer scale. The microscopic microscope, or microscope, was created to be used in the study and research particularly nanotechnology.

2.4 Encapsulations

(Retrieved on 29 June 2016, Reference: http:// e-book.ram.edu/e-book/f/FY463(50)/FY463-4.pdf)

Encapsulation is the process by which the substance or mixture of substances is coated, sealed, or encapsulated with other substances. The substance is coated or held but sometimes it can be solid particles or gas. The term material is called wall material, carrier, membrane, shell or coating.

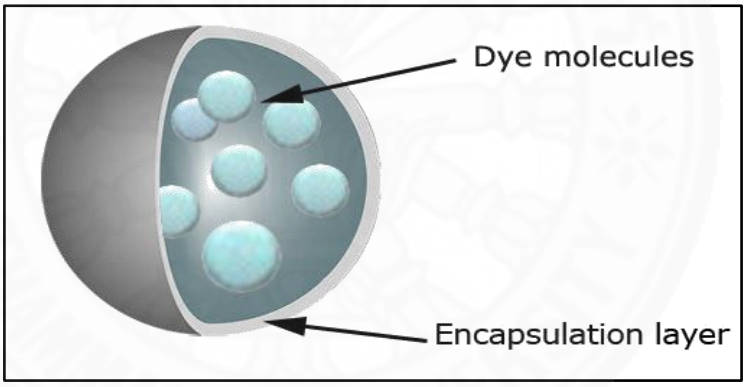

Figure 2.7: Structure of microcapsules

2.4.1 Encapsulation techniques

Quarantine can be done in several ways. The method is widely used in Industrial applications such as preservative, spray drying and extrusion. (Berthain *et al*., 1996; Goubet *et al*., 1998). Other techniques are Spray chilling and cooling, Coacervation, Fluidized Bed Coating, Liposome Entrapment, Inclusion Complexation, Chitosan Storage, and Freeze Drying.

2.5 Nanoparticles

Nanoparticles of a substance when designed to be less than 100 nanometers in diameter, new features will be created or increase the level of the original, such as titanium dioxide and zinc dioxide.

It can be seen through the nanoscale but still qualifies. The sunscreen is absorbed and is therefore used to make sunscreen or other chemicals. The nanoparticles can be used to make paints, coatings or paint the car. Including as a component in clothing. Cosmetic products that contain nanoparticles can penetrate the skin more deeply, it is used to make anti-aging cream.

In addition to the invention of nanoparticles based on existing chemicals. The nanoparticles are also integrated with other things. Creating nanocomposite materials to catalyze the industry. Development of nanocomposite plastic film that has the ability to block some gas passages and steam to be used for packaging to extend the freshness of vegetables and fruits and increase the value of exports. Capacities or medical instruments used to kill bacteria, viruses, or water logging.

2.6 Chitosan

Chitosan, or deacetylated chitin, is a copolymer composed of glucosamine and N-acetylglucosamine. More than 90% of the glucosamine is a derivative of chitin produced by the alkaline reaction. Get rid of acetyls to make the molecule smaller. Chitosan is composed of the amino group ($-NH_2$) and the hydroxyl group (-OH) that can react with other substances to convert to other derivatives.

2.6.1 Acid Solubility Properties of Chitosan

(1) Low solubility, such as formic acid, acetic acid, salicylic acid and protic acid.

(2) Moderate dissolution, such as citric acid, citric acid, tartaric acid, sulfuric acid and hydrochloric acid.

(3) Good solubility, such as oxalic acid, succinic acid and benzoic acid.

2.6.2 Benefits of Chitosan

2.6.2.1 Medical

(1) Chitosan is a good substance that can be used in many medical forms can be prepared in the form of gel tablets, sponges, pellets, capsules and tablets.

(2) Chitosan and derivatives used to prevent tooth decay, such as ethanol, glycoconjugate, chitin, methyl chitin, sulfate, chitosan and phospholipidase chitin. Inhibits the capture and formation of bacteria on teeth that cause tooth decay.

2.6.2.2 Agriculture

(1) The seed coating plant disease, insect and microbial spoilage and extend the shelf life of seeds.

(2) Accelerate the growth of plants as a root stimulating hormone.

(3) Used for soil improvement whether it is salty soil, soil pH and add nutrients in the soil.

2.6.2.3 Medicine

Chitosan is used as an ingredient in pharmaceutical products. It is used to prevent the degradation of the stomach. The drug release control or a drug delivery system into blood circulation.

2.6.2.4 Food Industry

(1) Use as a supplementary food that can provide energy. And reduce the amount of LDL cholesterol, cholesterol and triglycerides in the blood well. By catching with the fat to reduce intestinal absorption, chitosan is used as a dietary supplement to lose weight.

(2) Film, food packaging By using polyethylene plastic film, there are disadvantages that spoil food quickly. Due to moisture retention. But

the film from chitosan could extend well beyond food. Because it can transfer moisture from food to the outside better.

(3) An additive in juices. The addition of chitosan improves the efficiency of the fining agent and controls the pH of the fruit juice.

2.6.2.5 Cosmetics

(1) The properties of chitosan can absorb water well. It is a thin film covering the skin to prevent moisture loss of the skin. Including antimicrobial activity. It is used as a mixture of many kinds of cosmetics such as facial powder, facial soap, toothpaste, shampoo, sunscreen, skin cream, hair dye, hair dye etc.

(2) The properties of chitosan can absorb. And bind to organic compounds such as fatty substances such as heavy metals, including color, so popular has applied for a water filter or absorb pollutants in the sewage system.

2.6.3 Chitosan production process

Production Chitosan is a process similar to the production of chitin. It was pure chitin. After that chitin is used as a precursor in the production of chitosan. Removal of Acetyl groups of chitin (Demineralization). Removal of acetyl groups can be by hydrolysis with acid. But this method is not popular. The efficiency of the removal is also inferior to that of alkaline disposal. Sodium hydroxide is commonly used in the reaction. Then rinse with water and the dried product is complete. The efficient production process looks at the residuals of the remaining acetyls.

2.6.4 The dissolution of chitosan

Chitosan is composed of many amino groups (polyamine) which reacts with the organic acid and some inorganic acids. And a salt at pH 6.5 reaction was from among amine of chitosan into protons from acids and a polycarbonate, polysaccharide containing cationic (RNH^{3+}) with a salt of chitosan

water dissolved. For example, acetate, formate, glycolate, lactate, glyoxylate, malate, pyruvate and ascorbate. Chitosan will not dissolve in neutral or alkaline solutions.

2.7 Liposomes

(Retrieved on 29 June 2016, Reference: http://www.pharmacy.mahidol.ac.th/th/knowledge/article/112/การพัฒนาอนุภาคนาโนและระบบนำส่งตอนที่1)

Liposome is a particle that is smaller than submicron. It is a round bag of fatty substances. These fatty acids are amphipathic. The amphipathic group is polar, hydrophobic, and hydrophobic, which are mostly fatty phospholipids both natural and synthetic. Such as phosphatidyl-choline (lecithin), phosphatidyl-ethanolamine, phosphatidyl-glycerol and phosphatidyl-ionositol.

When mixed into aqueous solution. Molecules of fat phospholipids can be arranged in layers in contrast to the water molecules in the water solution. The molecular structure consists of both polar, hydrophilic (polar) and hydrophobic (nonpolar). When placed in water, it is arranged by bringing the polar or charged part facing the water molecule, while at the same time removing the non-polar part facing the non-polar part of the same molecule. It is a row of fat molecules stacked together as a double layer or lipid bilayer.

If the liposomes are lipid bilayer only. It is classified as a unilamellar bilayer vesicles (ULVs). If the liposome has more than one lipid bilayer (With a layer of water between the two walls) is classified as a type of liposomes is multilamellar bilayer vesicles (MLVs) Drugs or hydrophilic substances are trapped in the polarized layer. Individuals, drugs, or important hydrophilic substances are inserted into the lipid bilayer. Generally, drugs or hydrophobic substances are trapped in the liposomes more than those who do not like water.

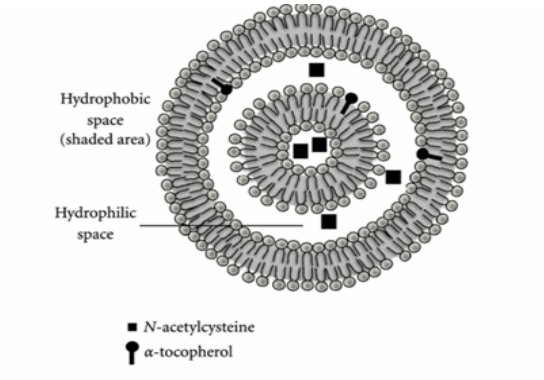

Figure 2.8: Liposomes

2.8 Review of literature

Chonlada., *et al* (2013) studied the extraction *Phellinus rimosus* with various solvents to extract the alkaloid has the highest % yield is 20.82%, extraction with ethanol (18.5%) and water extraction (14.5%), respectively. In addition, the antioxidant activity by DPPH assay showed that the highest antioxidant extract (EC_{50} lowest) was the extract of *Phellinus rimosus* extracted with ethanol (8.26±1.40 µg/mL), water extraction (20.12±3.65 µg/mL) and extraction of alkaloids (94.15±9.08 µg/mL), respectively. In comparison to Ascorbic acid (5.02±0.46 mg/mL). The antioxidant activity was determined by the FRAP assay. The highest antioxidant extract (Fe II equivalent) was found in the extract. Extraction with ethanol extraction (0.33±0.017 mmol/mg extracts), extracted with water (0.11±0.01 mmol/mg extract) and extracted alkaloid (0.13±0.01 mmol/mg extract), respectively, compared with Ascorbic acid (0.34±0.02 mmol/mg extract).

Table 2.2: The antioxidant activity by DPPH assay and FRAP assay extract of *Phellinus rimosus* (n = 8).

Antioxidant activity assay	Solvents Extraction		
	Ethanol	Water	Alkaloid
DPPH assay : EC_{50} (µg/mL)	8.26±1.40	20.12±3.65	94.15±9.08
FRAP assay (Fe II equivalent, mmol/mg extract)	0.33±0.017	0.11±0.01	0.13±0.01

Namfon., *et al* (2014) analyzed the properties of inhibiting oxidation of crude extracted from water, methanol and ethyl acetate from mushrooms, *Thaeogyroporus porentosus*, *Amanita princes, Russula virescens, Russula nigricans,* and *Russula emetic*. The antioxidant activity was higher than that of the standard Trolox with an IC_{50} lower than that of the standard Trolox (IC_{50} = 2.2430 mg/mL). Water extraction have IC_{50} is 2.4500, 0.1260, 3.0241, 0.3686 and 0.0339 mg/mL, extraction with methanol is 1.8615, 0.0177, 0.2904, 0.3862 and 0.5641 mg/mL and extracted ethyl acetate is 0.2013, 0.0073, 0.0244, 0.2391 and 0.1146 mg/mL, respectively.

The results showed that the ethyl acetate extracts has the highest antioxidant activity ($p<0.05$) and that the *Amanita princes* extracts in the ethyl acetate had antioxidant activity. The maximum IC_{50} was 0.0073 mg/mL.

Table 2.3: The antioxidant activity of the extracts of the mushroom samples in 5 different solvents, water, methanol and ethyl acetate.

Mushroom	Solvents Extractions		
	Water	Methanol	Ethyl acetate
Thaeogyroporus porentosus	2.4500	1.8615	0.2013
Amanita princes	0.1260	0.0177	0.0073
Russula virescens	0.0339	0.5641	0.1146
Russula nigricans	3.0241	0.2904	0.0244
Russula emetic	0.3686	0.3862	0.2391

DPPH radical scavenging activity (IC_{50}, mg/mL)

IC_{50} of Trolox standard = 2.2430 mg/mL

Sasamol., *et al* (2015) studied the total phenolic content, total tannins content and total flavonoids content in crude extract, *Pseuderanthemum palatiferum* leaves and stems. Total phenolic content were 1.80 and 1.64 mg of gallic acid/g of sample. Total tannins content were 1.85 and 1.68 mg of tannic acid/g of sample and total flavonoid content were 40.52 and 40.71 mg of rutin acid/g of sample, respectively. When comparing the total phenolic content, total tannin content and total flavonoids content in crude extracts, leaves and stems. No significant differences ($p > 0.05$).

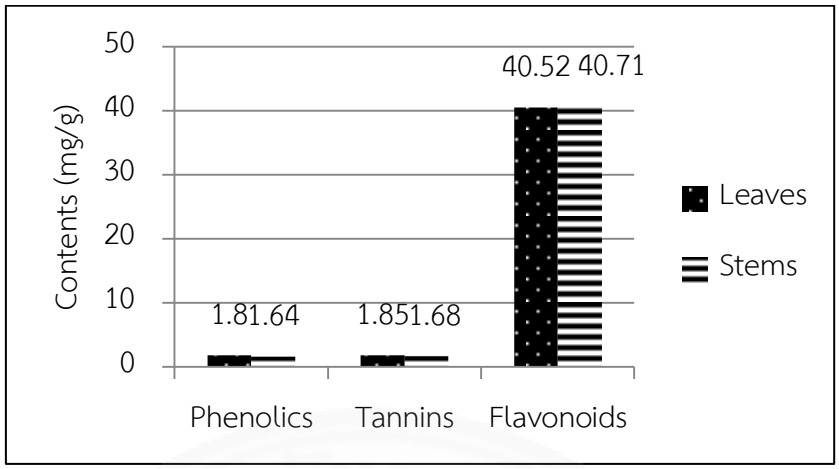

Figure 2.9: Total phenolic content, total tannins content and total flavonoids in crude extract, *Pseuderanthemum palatiferum* leaves and stems

Sasamol., *et al* (2015) studied the toxicity tests on breast cancer cells and colon cancer extract of the *Pseuderanthemum palatiferum* leaves show in the **table. 2.4** and **2.5**

Table 2.4: The results of the study on the toxicity of breast cancer cells of crude leaves extract.

Concentration (µg/mL)	Percentage survival of cancer cells. (mean)	Standard Deviation (mean)	IC_{50}±SD (µg/mL)
1000	41	4	593±12
500	53	5	
250	73	4	
125	83	7	
62.5	91	11	
31.25	96	0.3	
15.63	103	1	
7.81	101	4	

The results showed that the crude extract at 7.81-1,000 µg/mL was found to have MCF-7 cell lines with an IC_{50}±SD of 593±12 µg/mL.

Table 2.5: The results of the study on the toxicity of colon cancer cells of crude extract

Concentration (µg/mL)	Percentage survival of cancer cells. (mean)	Standard Deviation (mean)	IC_{50}±SD (µg/mL)
500	40	5	445±45
250	82	4	
125	87	7	
62.5	92	11	
31.25	96	0.3	
15.63	97	1	
7.81	100	4	
500	40	5	

The results showed that the crude extract at 7.81-1,000 µg/mL was found to have Caco2 cell lines with an IC_{50}±SD of 445±45 µg/mL.

Sasamol., *et al* (2015) studied the toxicity tests on breast cancer cells and colon cancer extract of the *Pseuderanthemum palatiferum* stems show in the **table. 2.6** and **2.7**

Table 2.6: The results of the study on the toxicity of breast cancer cells of crude leaves extract.

Concentration (µg/mL)	Percentage survival of cancer cells (mean)	Standard Deviation (mean)	IC_{50}±SD (µg/mL)
5000	71	16	>5000
2500	81	7	
1250	88	12	
625	83	10	
312.5	85	1	
156.25	95	2	
78.13	96	4	
39.06	91	4	

Pseuderanthemum palatiferum stems concentration 39.06-5,000 µg/mL no toxicity to human breast cancer cells (MCF-7 cell lines), with IC_{50}±SD values over 5,000 µg/mL.

Table 2.7: The results of the study on the toxicity of colon cancer cells of crude leaves extract.

Concentration (µg/mL)	Percentage survival of cancer cells (mean)	Standard Deviation (mean)	IC_{50}±SD (µg/mL)
1000	20	16	620±94
500	74	7	
250	89	12	
125	93	10	
62.5	98	1	
31.25	93	2	
15.63	98	4	
7.81	97	4	

The results showed that the crude extract at 7.81-1,000 µg/mL was found to have Caco2 cell lines with an IC_{50} ± SD of 620±94 µg/mL.

Mohammadi., *et al* (2015) studied the Nanoencapsulation of *Zataria multiflora* essential oil preparation and characterization with enhanced antifungal activity for controlling *Botrytis cinerea*, the causal agent of gray mold disease.

Zataria Multiflora essential oil that Nanoencapsulation have antifungal activity of Botrytis cinerea than essential oil non- Nanoencapsulation show in **Figure 2.10** and **2.11**

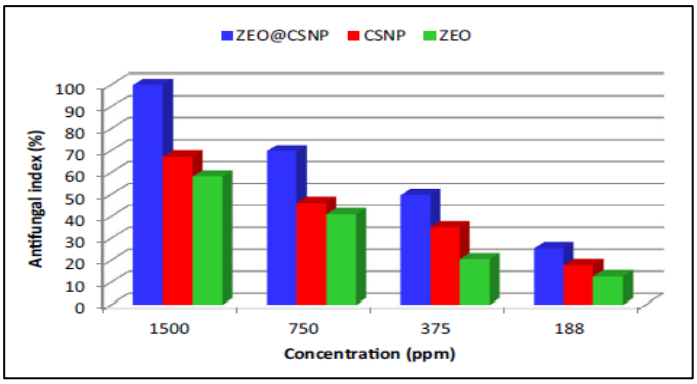

Figure 2.10: *Zataria Multiflora* essential oil that Nanoencapsulation and non-Nanoencapsulation antifungal activity of *Botrytis cinerea*

Figure 2.11: Comparative study of gray mold antimicrobial activity in strawberry fruit between the controls nanochitosan at 1500 ppm and essential oil nanoencapsulation were collected at 1500 ppm, respectively.

CHAPTER 3
RESEARCH METHODOLOGY

Materials and equipment

3.1.1 Reagents

(1) Ethanol (C_2H_5OH, Assay 98%, RCI Labscan, Thailand)

(2) Methanol (CH_3OH, Assay 98%, RCI Labscan, Thailand)

(3) Dimethyl sulfoxide : DMSO ($(CH_3)_2SO$, ACS grade, Merck, Germany)

(4) Distilled water (dH_2O,)

(5) Folin & Ciocalteu's phenol reagent (Merck, Germany)

(6) 2,2-Diphenyl-1-picrylhydrazyl (DPPH, Sigma-Aldrich, Germany)

(7) Tripolyphosphate (TPP)

(8) Sulfuric acid (H_2SO_4, Assay 98%, AR grade, RCI Labscan, Thailand)

(9) Resazurin (Dye content 93%, certified by the Biological Stain Commission, Sigma-Aldrich, Germany)

(10) Lecithin (Scharlau, Spain)

(11) Quercetin (Sigma-Aldrich, Germany)

(12) Aluminium chloride ($AlCl_3$, Ajax Finechem, Australia)

(13) Sodium carbonate (Na_2CO_3, Ajax Finechem, Australia)

(14) Sodium nitrite ($NaNO_2$, Ajax Finechem, Australia)

(15) Sodium hydroxide (NaOH, Ajax Finechem, Australia)

(16) Glucose (Ajax Finechem, Australia)

(17) Gallic acid (Sigma-Aldrich, Germany)

(18) Phenol (C_6H_5OH, Extra Pure, QRëC™, Thailand)

(19) Seafresh Chitosan Powder (95% DAC, 80 Mesh, Seafresh Chitosan (Lab) Company Limited, Thailand)

3.1.2 Microorganisms

(1) *Bacillus subtilis*

(2) *Staphylococcus aureus* (ATCC 25923)

(3) *Escherichia coli* (ATCC 25922)

(4) *Enterobacter aerogenes*

3.1.3 Equipments

(1) UV-VIS Spectrophotometer

UV-VIS Spectrophotometer (Genesys 10S UV-VIS, Thermo Scientific, USA) used for measuring the amount of substances in the analysis stage of the properties of mushroom extract. Determine the total phenolic contents, total flavonoid contents and total polysaccharide contents. Analysis of antioxidant activity and measure the amount of microorganisms.

Figure 3.1: UV-VIS Spectrophotometer (Thermo Scientific, USA)

(2) Transmission Electron Microscope (TEM)

Transmission Electron Microscope (TEM 1400) at The Scientificand Technological Research Equipment Centre (STREC), Chulalongkorn University. Used to analyze the morphology and the size of the nanoparticles.

Figure 3.2: Transmission Electron Microscope (TEM 1400)

(3) High Performance Liquid Chromatography (HPLC)

High Performance Liquid Chromatography (Shimadzu, Japan) and LabSolutions for analysis Data System. Analysis of the compounds and the content of mushroom extract at Central Scientific Instrument Center (CSIC), Faculty of Science and Technology, Thammasat University

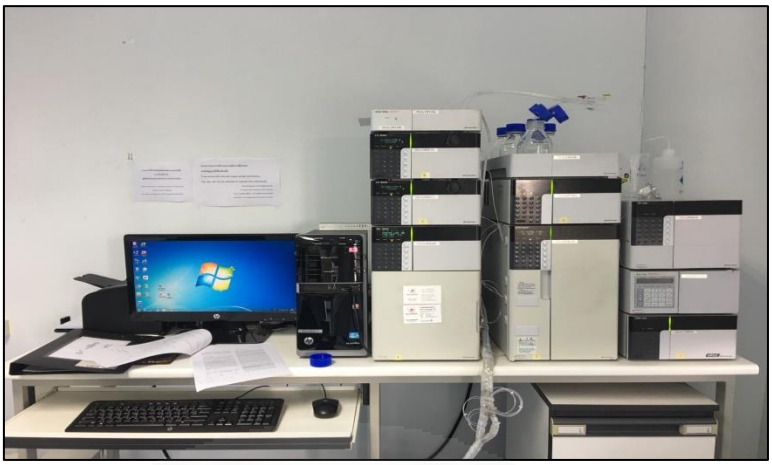

Figure 3.3: High Performance Liquid Chromatography (Shimadzu, Japan)

(4) Nano Particle Analyzer, Dynamic light scattering (DLS)

Nano particle analyzer (SZ-100, HORIBA Scientific, Japan) Measure the size of the nanoparticles. At the nanometer level.

Figure 3.4: Nano particle analyzer (SZ-100, HORIBA Scientific, Japan)

(5) Rotary Evaporator

Rotary Evaporator (BUCHI R II, United Kingdom) used for evaporation of solvents.

Figure 3.5: Rotary Evaporator (BUCHI R II, United Kingdom)

3.2 Methodology

3.2.1 Extractions

3.2.1.1 95% Ethanol extraction at 80°C (PL-EH)

(1) 20 grams of *P. linteus* was mixed with 300 mL 95% ethanol.

(2) Shaken on a shaker at 120 rpm at 80°C for 24 hours.

(3) Filter the liquid with filter paper.

(4) Remove the liquid by evaporating the solvent with a rotary evaporator.

(5) Weighing of the crude extract (Mark that: PL-EH) and to analyze the properties in the next step.

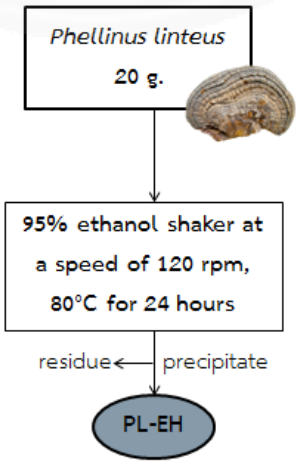

Figure 3.6: 95% Ethanol extraction at 80°C

3.2.1.2 95% Ethanol extraction at 25°C (PL-E1)

(1) 20 grams of *P. linteus* was mixed with 300 mL 95% ethanol.

(2) Shaken on a shaker at 120 rpm at 25°C for 24 hours.

(3) Filter the liquid with filter paper.

(4) Remove the liquid by evaporating the solvent with a rotary evaporator.

(5) Extract with 95% ethanol one more time as above.

(6) Extract from 95% ethanol solvent extraction rounds 1 (Mark that: PL-E1) and 2 (Mark that: PL-E2).

(7) Weighing of the crude extract and to analyze the properties in the next step.

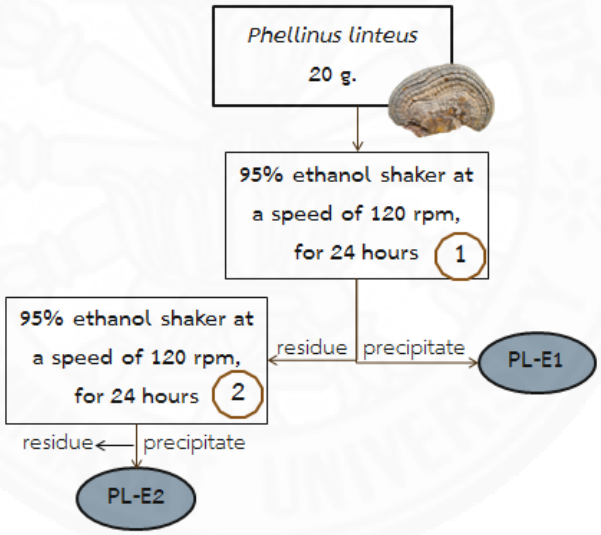

Figure 3.7: 95% ethanol extraction at 25°C

3.2.1.3 Water extraction at 95°C (PL-W), 1% Aluminium oxalate at 95°C (PL-A) and 1.25M Sodium hydroxy/0.05% Sodium borohydride at 25°C (PL-N) as successively

(1) 20 grams of *P. linteus* was defatted with petroleum ether for 6 hours, 2 times. And then mixed with 300 mL of water.

(2) Shaken on a shaker at 120 rpm at 95°C for 8 hours, 2 times.

(3) Filter the liquid with filter paper.

(4) The liquid to be precipitated with 95% ethanol. Then centrifuged at 8,000 rpm at 4 ° C for 15 min. (Mark that: PL-W)

(5) The residue was extracted with 1% aluminum oxalate at 95 ° C for 8 hours, 2 times.

(6) Filter the liquid with filter paper.

(7) The liquid to be precipitated with 95% ethanol. Then centrifuged at 8,000 rpm at 4 ° C for 15 min. (Mark that: PL-A)

(8) The residue was extracted with 1.25M Sodium hydroxy /0.05% Sodium borohydride at 25 ° C for 3 hours, 2 times

(9) Filter the liquid with filter paper.

(10) The liquid to be precipitated with 95% ethanol. Then centrifuged at 8,000 rpm, 4 ° C for 15 min. (Mark that: PL-N)

(11) The crude extract of 3 solvents (PL-W, PL-A and PL-N)

(12) Weigh the crude extract and to analyze the properties in the next step.

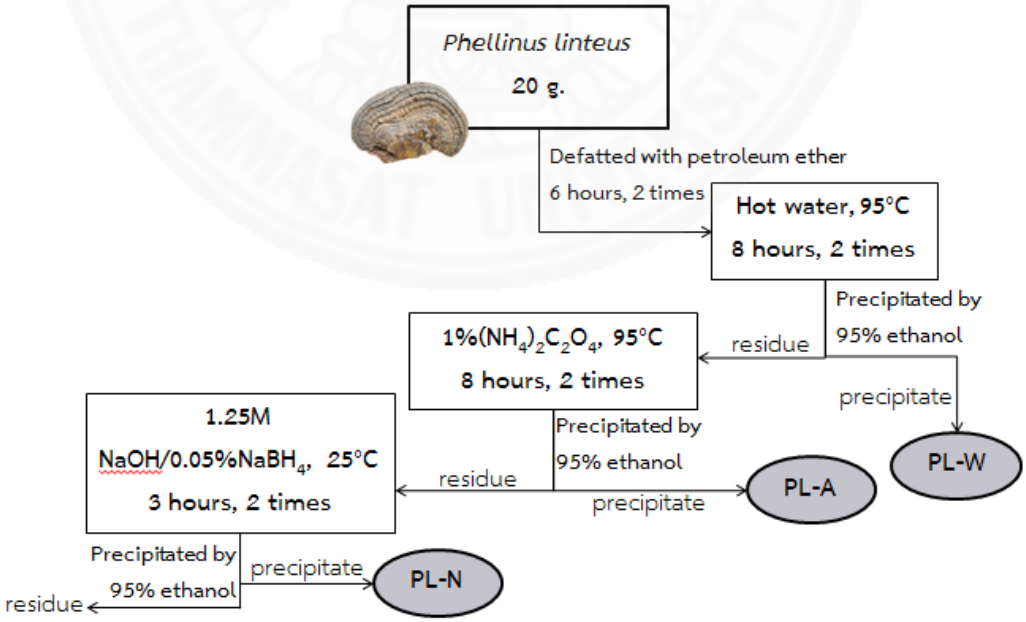

Figure 3.8: Water extraction at 95°C, 1% Aluminium oxalate at 95°C and 1.25M Sodium hydroxy/0.05% Sodium borohydride at 25°C

3.2.2 Determination of the *Phellinus linteus* extract compounds

3.2.2.1 Total Phenolic Contents analyzed by the modified method Folin-Ciocalteu colorimetric method described by Wolfe *et al*. (2003)

(1) Add 125 µL of *P. linteus* extracts in a 500 µL distilled water and add Folin-Ciocalteu reagent 125 µL to the mixture. Stand for 6 minutes.

(2) Add 7% sodium carbonate solution of 1250 µL and add 1000 µl of distilled water. Set aside at room temperature for 90 minutes.

(3) UV-VIS Spectrophotometer at 760 nm wavelength, the values was compared with Gallic Acid standard graphs, expressed as mg of Gallic Acid Equivalent in 1 g of Gallic Acid Equivalent/g crude extract.

3.2.2.2 Total Flavonoid Contents using Colorimetric Method described by Wolfe *et al*. (2003)

(1) Add 250 µL of the crude extract to a 1250 µL distilled water tube and add 5% of 75 µL of sodium nitrite for 5 minutes.

(2) Add 150 µL of 10% aluminum chloride to leave for 6 minutes.

(3) Add 500 µL of 1M sodium hydroxide and 275 µL distilled water.

(4) UV-VIS Spectrophotometer at the 510 nm wavelength, Quercetin is calculated as mg equivalent of Quercetin in 1 g Quercetin/g crude extract

3.2.2.3 Total Polysaccharide Contents using by Phenol-Sulfuric acid method

(1) Add 600 µL of crude extract to 500 ml of 5% phenol, and then shake well.

(2) Add 3 mL of concentrated sulfuric acid quickly, stand for 10 minutes, and then shake well, for 30 minutes.

(3) UV-VIS Spectrophotometer at 490 nm wavelengths, Glucose is calculated as mg equivalent of the Glucose Standard in 1 g of Glucose/g crude extract.

3.2.3 Analysis activities of the *Phellinus linteus* extract compounds

3.2.3.1 The activity of antioxidant (Radical Scavenging Capacity Assay) and IC_{50} described by Karagozler *et al*. (2008)

(1) Various concentrations of crude extract with methanol and add 2 mL of each extract. Add 0.08 mM of DPPH solution 2 mL and shake for 30 minutes.

(2) UV-VIS Spectrophotometer at the wavelength of 515 nm, the absorbance was calculated as % radical scavenging activity.

$$\text{DPPH (\%)} = \left(\frac{\boldsymbol{A_0 - A_1}}{\boldsymbol{A_0}}\right) \times \mathbf{100}$$

A_0 = the absorbance of control

A_1 = the absorbance of the sample

(3) % Radical scavenging activity to the graph to calculate IC_{50} (when IC_{50} is the concentration of the extract, the % radical scavenging activity decreased by 50%).

3.2.3.2 The activity of antimicrobial using by Minimum Inhibitory Concentration method (MIC) and Minimum Bactericidal Concentration method (MBC)

(1) Take the crude extract into a 96-well plate with various concentrations of Nutrient broth (NB). The hole 11 is a negative control variable (No sample) and the 12^{th} hole is a positive control variable. (only NB).

(2) Take the microorganisms to be diluted in Normal saline. UV-VIS Spectrophotometer at the wavelength of 600 nm value of 0.080-0.100 or 0.5 Mcfarland. The pipette is placed into a 96 well, except for the 12th hole.

(3) Incubated at 37 ° C for 24 hours.

(4) After incubation, the resazurin solution was placed in each hole of 96 well plate 10 µL per well and incubated at 37°C for 2 hours.

(5) Recorded MIC (Minimal Inhibitory Concentration) from the first hole at lowest concentration. (Color: purple/dark blue).

(6) For the MBC or Minimal Bactericidal Concentration, streak the positive of resazurin (purple/dark blue) and incubate at 37°C for 24 hours. See the lowest concentration of growth.

3.2.4 Separation of the *Phellinus linteus* extract compounds

HPLC were compared with standard. Preliminary databases to identify the type of crude extract described by Chen *et al*. (2001)

Column type: ZORBAX Eclipse XDB-C18 (4.6×150 mm, 5µm)
Wavelength detected: 254 nm and 280 nm
Solvent A: water–acetic acid 97:3, (v/v)
Solvent B: methanol

Table 3.1: HPLC solvent gradient elution program

Time (min)	Solvent B (%)	Flow rate (mL/min)
0	0	1.0
10	10	1.0
40	70	1.0
44	0	1.0
47	0	1.0

3.2.5 Nano Chitosan Encapsulation

(1) 0.05% (w/v) Chitosan was mixed with crude extract (PL-EH at 1.0 mg/mL, PL-E1 at 0.5 mg/mL and PL-N at 3.0 mg/mL)

(1) Add 0.75% tripolyphosphate solution at a ratio of 200: 40 slowly under stirring with a stirrer.

(2) The solution was to sonicate for 5 minutes.

(3) Centrifugation at 8,000 rpm, 4° C for 15 minutes.

(4) The suspension to analyze the releasing efficiency, shape and size of nanoparticles, and developing cosmeceutical product.

(5) The supernatant to analyze the encapsulation efficiency.

3.2.6 Nano-double particles Encapsulation

(1) Selected phospholipid was mixed with crude extract (PL-EH at 1.0 mg/mL, PL-E1 at 0.5 mg/mL and PL-N at 3.0 mg/mL)

(2) The solvent removed by rotary evaporation at 45° C to obtain a thin lipid film.

(3) Thin lipid film was slowly hydrated using 0.05% (w/v) Chitosan.

(4) The solution was to sonicate for 5 minutes.

(5) Centrifugation at 8,000 rpm, 4° C for 15 minutes.

(6) The suspension to analyze the releasing efficiency, shape and size of nanoparticles, and developing cosmeceutical product.

(7) The supernatant to analyze the encapsulation efficiency.

3.2.7 Nano Liposome Encapsulation

(1) 3% (w/v) Lecithin was mixed with crude extract (PL-EH at 1.0 mg/mL, PL-E1 at 0.5 mg/mL and PL-N at 3.0 mg/mL)

(2) The solvent removed by rotary evaporation at 45° C to obtain a thin lipid film.

(3) Thin lipid film was slowly hydrated using 10mM phosphate buffer (pH7).

(4) The solution was to sonicate for 5 minutes.

(5) Centrifugation at 8,000 rpm, 4° C for 15 minutes.

(6) The suspension to analyze the releasing efficiency, shape and size of nanoparticles, and developing cosmeceutical product.

(7) The supernatant to analyze the encapsulation efficiency.

3.2.8 Determination of encapsulation efficiency and releasing efficiency

3.2.8.1 Encapsulation efficiency

(1) The supernatant from (3.2.5), (3.2.6) and (3.2.7) use for determine Total Phenolic Contents by Folin-Ciocalteu colorimetric method.

(2) Calculate from equation

$$\text{Encapsulation efficiency (\%EE)} = \left(\frac{\textbf{Total agent} - \textbf{Free agent}}{\textbf{Total agent}}\right) \times 100$$

3.2.8.2 Releasing efficiency

Non Accumulation Releasing efficiency

(1) The suspension of nanoparticle that encapsulate with crude extract (PL-EH, PL-E1 and PL-N) shaken on a shaker at 180 rpm and centrifuge at 8,000 rpm, 4° C for 15 minutes.

(2) The supernatant from (1) use for determine Total Phenolic Content by Folin-Ciocalteu colorimetric method.

(3) The precipitate from (1) that add DI water and shaken on shaker at 180 rpm

(4) Repeat the same procedure for 0 hour, 6 hours, 12 hours, 18 hours, 24 hours (day1), 48 hours (day2), 72 hours (day3), 96 hours (day4), 120 hours (day5), 144 hours (day6), 168 hours (day7), 192 hours (day8), 216 hours (day9) and 240 hours (day10).

(5) Control is the suspension of nanoparticles that non encapsulate.

(6) Calculate from equation

$$\text{Releasing efficiency (\%RE)} = \left(\frac{\textbf{Free agent}}{\textbf{Total agent}}\right) \times 100$$

Accumulation Releasing efficiency

(1) The suspension of nanoparticle that encapsulate with crude extract (PL-EH, PL-E1 and PL-N) shaken on a shaker at 180 rpm and centrifuge at 8,000 rpm, 4° C for 15 minutes.

(2) The supernatant from (1) use for determine Total Phenolic Content by Folin-Ciocalteu colorimetric method.

(3) Repeat the same procedure for 0 hour, 6 hours, 12 hours, 18 hours, 24 hours (day1), 48 hours (day2), 72 hours (day3), 96 hours (day4), 120 hours (day5), 144 hours (day6), 168 hours (day7), 192 hours (day8), 216 hours (day9) and 240 hours (day10).

(4) Control is the suspension of nanoparticles that non encapsulate.

(5) Calculate from equation

$$\text{Releasing efficiency (\%RE)} = \left(\frac{\textbf{Free agent}}{\textbf{Total agent}}\right) \times 100$$

3.2.9 Characterization of the *Phellinus linteus* extracts and encapsulated nanoparticles

3.2.9.1 Analysis of the shape of nanoparticles.

- Transparent electron microscope Transmission Electron Microscope (TEM)

3.2.9.2 Analysis of the size of nanoparticles

- Dynamic light scattering (DLS)

3.2.10 Development cosmeceutical product

The best crude extracts were encapsulated and release in nanoparticles. To tested the properties of product with the preliminary as antioxidant and antimicrobial including the appearance of serum products.

3.2.10.1 The activity of antioxidant (Radical Scavenging Capacity Assay) and IC_{50} described by Karagozler *et al*. (2008)

(1) 2 mL of product control and product. Add 0.08 mM of DPPH solution 2 mL and shake for 30 minutes.

(2) UV-VIS Spectrophotometer at the wavelength of 515 nm, the absorbance was calculated as % radical scavenging activity.

3.2.10.2 The activity of antimicrobial using by Minimum Inhibitory Concentration method (MIC) and Minimum Bactericidal Concentration method (MBC)

(1) Take the crude extract into a 96-well plate with various concentrations of nutrient broth (NB). The hole 11 is a negative control variable (No sample) and the 12^{th} hole is a positive control variable. (only NB).

(2) Take the microorganisms to be diluted in Normal saline. UV-VIS Spectrophotometer at the wavelength of 600 nm value of 0.080-0.100 or 0.5 Mcfarland. The pipette is placed into a 96 well, except for the 12th hole.

(3) Incubated at 37 ° C for 24 hours.

(4) After incubation, the resazurin solution was placed in each hole of 96 well plate 10 µL per well and incubated at 37 ° C for 2 hours.

(5) Recorded MIC (Minimal Inhibitory Concentration) from the first hole at lowest concentration. (Color: purple/dark blue).

(6) For the MBC or Minimal Bactericidal Concentration, streak the positive of resazurin (purple/dark blue) and incubate at 37°C for 24 hours. See the lowest concentration of growth.

CHAPTER 4
RESULTS AND DISCUSSION

4.1 Percentage of *P. linteus* extract

P. linteus is a famous medicinal polypore used throughout China, Japan, and Korea with a long history. Its first medicinal usage was recorded in an oldest Chinese medicinal book *Shennong's compendium of material medica* written by '*sanger*' (ear of *Morus alba L.*) in Han dynasty 2000 years ago (Chen *et al*., 2016 and Wu *et al*., 2003). Active components of *P. linteus* contained including flavones, coumarins, ergosterols, agaricic acids, fatty acids and triterpenes etc. (Hui *et al*., 2009 and Qi *et al*., 2010). Therefore, *P. linteus* was selected for the study of substances, activities and development for applications as well.

20 grams of the *P. linteus* were extracted with 95% ethanol (300 ml) and shaken on a shaker at 120 rpm for 24 hours at 80°C, and fill 95% ethanol again for 24 hour at room temperature (PL-EH) is 1.74 grams of a percent yield is 3.48%. And the *P. linteus* were extracted with 95% ethanol and then shaken at 120 rpm for 24 h at 25°C. *P. linteus* extracted with a solvent, 95% ethanol in round 1 at 25°C (PL-E1) is 0.32 grams of a percent yield is 1.6%. Finally, the *P. linteus* were extracted with 95% ethanol in round 2 at room temperature (PL-E2), 0.09 grams of a percent yield is 0.45%. **(Table 4.1)**

Table 4.1: The percent yield of PL-EH, PL-E1 and PL-E2 the extract of *P. linteus*

Crude extract	Grams of extraction (g.)	% Yield
PL-EH	1.74	3.48%
PL-E1	0.32	1.60%
PL-E2	0.09	0.45%

In this extraction for preliminary propoties of polysaccharide, three different extraction, namely hot water,1% ammonium oxalate $(NH_4)_2C_2O_4$, and 1.25 M

sodium hydroxide(NaOH)/sodium borohydride ($NaBH_4$) solutions according to research of Wang *et al*., 2014

20 grams of the *P. linteus* was extracted with water at 95°C and then precipitated with 95 percent ethanol (PL-W) was 2.64 grams of a percent content is 13.2%. Then remove the residue of *P. linteus* extracted with a 1% solvent, aluminum oxalate (1% $(NH_4)_2C_2O_4$) at a temperature of 95°C and precipitation fluid with a solution of 95% ethanol to extract (PL-A) is 16.97 grams of a percent content is 84.75%. Finally, the residue was extracted with 1.25M sodium hydroxide/0.05% sodium borohydride (1.25MNaOH/0.05%$NaBH_4$) at 25°C. And liquid precipitation with a solution of 95% ethanol to extract (PL-N) is 24.86 grams of a percent content is 124.3%. **(Table 4.2)**

Table 4.2: The percent content of PL-W, PL-A and PL-N the extract of *P. linteus*

Crude extract	Grams of extraction (g.)	% Content
PL-W	2.64	13.20 %
PL-A	16.97	84.75 %
PL-N	24.86	124.30 %

Calculate the results as % content because the content was precipitation by 95% ethanol, resulting in a very high percentage.

4.2 Determination of the *P. linteus* extract compounds

4.2.1 Total Phenolic Contents of *P. linteus* extract

Mushrooms contain bioactive compounds and chemical compounds. (Ferreira, Barros, & Abreu, 2009). Biological properties, especially phenolic acid, from mushrooms is an antitumor (Heleno, Ferreira, Calhelha, Esteves, & Queiroz, 2014; Vaz, Almeida, Ferreira, Martins, & Vasconcelos, 2012), antimicrobial (Alves *et al*., 2013), and antioxidant (Piazzon *et al*., 2012).

Total Phenolic Contents were analyzed by Folin-Ciocalteu colorimetric method and measured by UV-VIS Spectrophotometer at the wavelength of 760 nm is

the same as the standard graph of Gallic acid. The results showed that the mg of Gallic Acid Equivalent in 1 g extract (mg GAE/g crude extract). The extract of *P.linteus* extracted with 95% ethanol solvent at 80 ° C, 95% ethanol round 1 at 25°C and 95% ethanol round 2 at 25°C was 610.92±7.611, 455.49±7.611 and 401.36±2.250 mg GAE/g crude extract, respectively. **(Table 4.3)**

Table 4.3: The Total Phenolic Contents of *P. linteus* extract (PL-EH, PL-E1 and PL-E2)

Crude extract	Total Phenolic Content (mg GAE/g crude extract)
PL-EH	610.92±7.611
PL-E1	455.49±7.611
PL-E2	401.36±2.250

Assays were performed in triplicates. Values are expressed as means±SD.

Table 4.3 shows that PL-EH had the highest total phenolic contents of 610.92±7.611 mg GAE/g crude extract, followed by PL-E1 and PL-E2 of 455.49±7.611 and 401.36±2.250 mg GAE/g crude extract, respectively. And PL-EH, PL-E1 and PL-E2 were significantly different at the 0.05 level in One way ANOVA , LSD and DUNCAN.

The extract of *P.linteus* extracted with water at 95°C, 1%$(NH_4)_2C_2O_4$ at 95°C and 1.25M NaOH/0.05%$NaBH_4$ at 25°C was 122.52±0.021, 28.50±0.224 and 46.52±0.177 mg GAE/g crude extract, respectively. **(Table 4.4)**

Table 4.4: The Total Phenolic Contents of *P. linteus* extract (PL-W, PL-A and PL-N)

Crude extract	Total Phenolic Content (mg GAE/g crude extract)
PL-W	122.52±0.021
PL-A	28.50±0.224
PL-N	46.52±0.177

Assays were performed in triplicates. Values are expressed as means±SD.

Table 4.4 shows that PL-W had the highest total phenolic contents of 122.52±0.021 mg GAE/g crude extract, followed by PL-N and PL-A of 46.52±0.177 and 28.50±0.224 mg GAE/g crude extract, respectively. And PL-W, PL-A and PL-N were significantly different at the 0.05 level in One way ANOVA, LSD and DUNCAN.

Homogeneous Subsets

TPC

	CrudeSample	N	Subset for alpha = 0.05					
			1	2	3	4	5	6
Duncan[a]	PL_A	3	28.50075					
	PL_N	3		46.52135				
	PL_W	3			122.52336			
	PL_E2	3				401.36386		
	PL_E1	3					455.48520	
	PL_EH	3						610.92169
	Sig.		1.000	1.000	1.000	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

4.2.2 Total Flavonoid Contents of *P. linteus* extract

Total Flavonoid Contents were analyzed by Colorimetric method and measured by UV-VIS Spectrophotometer at the wavelength of 510 nm is the same as the standard graph of Quercetin. The results showed that the mg of Quercetin Equivalent in 1 g extract (mg QE/g crude extract). The extract of *P.linteus* extracted with 95% ethanol solvent at 80 ° C, 95% ethanol round 1 at 25℃, 95% ethanol round 2 at 25℃ was 756.75±1.283, 574.19±2.670, 531.44±1.959 mg QE/g crude extract, respectively. **(Table 4.5)**

Table 4.5: The Total Flavonoid Contents of *P. linteus* extract (PL-EH, PL-E1 and PL-E2)

Crude extract	Total Flavonoid Contents (mg QE/g crude extract)
PL-EH	756.75±1.283
PL-E1	574.19±2.670
PL-E2	531.44±1.959

Assays were performed in triplicates. Values are expressed as means±SD.

Table 4.5 shows that PL-EH had the highest total flavonoid contents of 756.75±1.283 mg QE/g crude extract, followed by PL-E1 and PL-E2 of 574.19±2.670 and 531.44±1.959 mg QE/g crude extract, respectively. And PL-EH, PL-E1 and PL-E2 were significantly different at the 0.05 level in One way ANOVA, LSD and DUNCAN.

The results shows that the mg of Quercetin Equivalent in 1 g extract (mg QE/g crude extract). The extract of *P. linteus* extracted with water at 95°C, 1%$(NH_4)_2C_2O_4$ at 95°C and 1.25M NaOH/0.05%$NaBH_4$ at 25°C was 126.43±0.789, 43.64±0.564 and 67.60±1.140 mg QE/g crude extract, respectively. **(Table 4.6)**

Table 4.6: The Total Flavonoid Contents of *P. linteus* extract (PL-W, PL-A and PL-N)

Crude extract	Total Flavonoid Contents (mg QE/g crude extract)
PL-W	126.43±0.789
PL-A	43.64±0.564
PL-N	67.60±1.140

Assays were performed in triplicates. Values are expressed as means±SD.

Table 4.6 shows that PL-W had the highest total flavonoid contents of 126.43±0.789 mg QE/g crude extract, followed by PL-N and PL-A of 67.60±1.140 and 43.64±0.564 mg QE/g crude extract, respectively. And PL-W, PL-A and PL-N were significantly different at the 0.05 level in One way ANOVA, LSD and DUNCAN.

Homogeneous Subsets

TFC

	CrudeSample	N	Subset for alpha = 0.05					
			1	2	3	4	5	6
Duncan[a]	PL_A	3	43.64286					
	PL_N	3		67.60448				
	PL_W	3			126.43307			
	PL_E2	3				531.43505		
	PL_E1	3					574.18927	
	PL_EH	3						756.74982
	Sig.		1.000	1.000	1.000	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Total Phenolic Contents and Total Flavonoids Contents of *P.linteus* extracted with 95% ethanol solvent at 80°C, 95% ethanol round 1 at 25°C and 95% ethanol round 2 at 25°C gave the same result in PL-EH, followed by PL-E1 and PL-E2.

Comply with the research of Phadungkit., *et al* (2016), it was found that the total phenolic and flavonoid content of the *Anaxagorea luzonensis* extract with different solvents. The results are consistent, methanol extracts showed the highest phenolic content and most flavonoids.

4.2.3 Total Polysaccharide Contents of *P. linteus* extract

Total Polysaccharide Contents were analyzed by Phenol-Sulfuric Acid method and measured by UV-VIS Spectrophotometer at wavelength of 490 nm is the same as the standard graph of Glucose. The results showed that the mg of Glucose Equivalent in 1 g extract (mg GE/g crude extract). The extract of *P.linteus* extracted with 95% ethanol solvent at 80°C, 95% ethanol round 1 at 25°C, 95% ethanol round 2 at 25°C was 141.33±1.350, 109.35±0.295, 113.43±1.284 mg GE/g crude extract, respectively. **(Table 4.7)**

Table 4.7: Total Polysaccharide Contents of *P. linteus* extract (PL-EH, PL-E1 and PL-E2)

Crude extract	Total Polysaccharide Contents (mg GE/g crude extract)
PL-EH	141.33±1.350
PL-E1	109.35±0.295
PL-E2	113.43±1.284

Assays were performed in triplicates. Values are expressed as means±SD.

Table 4.7 shows that PL-EH had the highest total polysaccharide contents of 141.33±1.350 mg GE/g crude extract, followed by PL-E2 and PL-E1 of 113.43±1.284 and 109.35±0.295 mg GE/g crude extract, respectively. And PL-EH, PL-

E1 and PL-E2 were significantly different at the 0.05 level in One way ANOVA, LSD and DUNCAN.

Total Polysaccharide Contents different from Total Phenolic Contents and Total Flavonoid Contents. Due to polysaccharide is carbohydrates, phenols and flavonoids are compounds with benzene rings and hydroxyl groups (-OH group)

The extract of *P. linteus* extracted with water at 95°C, 1%$(NH_4)_2C_2O_4$ at 95°C and 1.25M NaOH/0.05%$NaBH_4$ at 25°C was 167.96±0.061, 29.88±0.050 and 25.94±0.021 mg GE/g crude extract, respectively. **(Table 4.8)**

Table 4.8: Total Polysaccharide Contents of *P. linteus* extract (PL-W, PL-A and PL-N)

Crude extract	Total Polysaccharide Contents (mg GE/g crude extract)
PL-W	167.96±0.061
PL-A	29.88±0.050
PL-N	25.94±0.021

Assays were performed in triplicates. Values are expressed as means±SD.

Table 4.8 shows that PL-W had the highest total polysaccharide contents of 167.96±0.061 mg GE/g crude extract, followed by PL-A and PL-N of 29.88±0.050 and 25.94±0.021 mg GE/g crude extract, respectively. And PL-W, PL-A and PL-N were significantly different at the 0.05 level in One way ANOVA, LSD and DUNCAN.

Homogeneous Subsets

TPoC

	CrudeSample	N	Subset for alpha = 0.05					
			1	2	3	4	5	6
Duncan[a]	PL_N	3	25.93833					
	PL_A	3		29.88067				
	PL_E1	3			109.35400			
	PL_E2	3				113.43533		
	PL_EH	3					141.32633	
	PL_W	3						167.95900
	Sig.		1.000	1.000	1.000	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

In addition, contained including coumarins, ergosterols, agaricic acids, fatty acids, triterpenes, aromatic acids, amino acids, xylose oxidases, urease, catalases, esterases, sucrases, lactase cellulase enzymes, etc. (Hui *et al*., 2009 and Qi *et al*., 2010) Furthermore, compositions in fruiting bodies and mycelia are also differential. (Chen *et al*., 2016)

4.3 Analysis activities of the *P. linteus* extract compounds

4.3.1 The activities of antioxidant with DPPH assay

Natural products with antioxidant activity may be used to help the human to reduce oxidative damage. Many natural antioxidants have already been isolated from different plant (Shon *et al*., 2003).

The antioxidant activities of the *P. linteus* extract. To determine the concentration of 50 percent antioxidant the *P. linteus* extract (IC_{50}), the *P. linteus* was extracted with 95% ethanol at 80°C, 95% ethanol round 1 and 95% ethanol round 2 of antioxidant activity was reduced by 50% at concentration of 2.04, 2.07 and 3.60 µg/mL, respectively. **(Table 4.9)**

Table 4.9: The activities of antioxidant with DPPH assay, IC_{50} (PL-EH, PL-E1 and PL-E2)

Crude extract	IC_{50}
PL-EH	2.04 (µg/mL)
PL-E1	2.07 (µg/mL)
PL-E2	3.60 (µg/mL)

Table 4.9 revealed that PL-EH is lowest concentration that can inhibit by 50% at 2.04 µg/mL. Secondary, the PL-E1 and PL-E2 can inhibit by 50% at a concentration of 2.07 and 3.60 µg/mL, respectively. From One way ANOVA, LSD and DUNCAN. Show PL-EH and PL-E1 was not significantly different at the 0.05 level.

Homogeneous Subsets

Concentration

Duncan[a]

CrudeSample	N	Subset for alpha = 0.05	
		1	2
PL-EH	3	.00204317	
PL-E1	3	.00206585	
PL-E2	3		.00366758
Sig.		.407	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

In a previous study, the ethanol extract was found to notably scavenge the stable radical DPPH with an EC_{50} of 22.07 µg/mL and inhibit lipid peroxidation with an IC_{50} of 48.45 µg/mL in a concentration dependent manner. (Song *et al*., 2003)

P. linteus extract was extracted with water at 95°C at 0.30 mg/mL , 1% $(NH_4)_2C_2O_4$) at 95°C at 0.30 mg/mL and 1.25M NaOH/0.05% $NaBH_4$ at 25°C at 0.30 mg/mL of crude sample have the highest antioxidant activity was 14.63%, 20.49% and 28.61%, respectively. IC_{50} cannot be determined due to the higher concentration of sediment, resulting in a decrease in % Inhibition. Therefore, it show a results % inhibition at 0.30 mg/mL. **(Figure 4.1, 4.2 and 4.3)**

P. linteus extract was extracted with water at 95°C at 0.30 mg/mL have the lowest % inhibition of antioxidant activities was 14.63% and at 0.60 mg/mL have the % inhibition of antioxidant activities was 63.96%.**(Figure 4.1)**

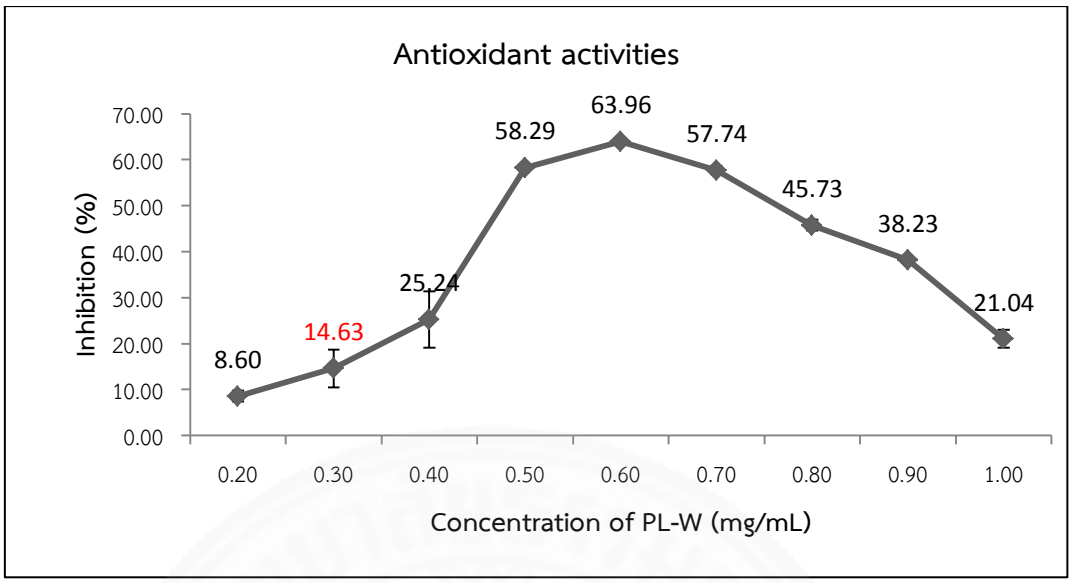

Figure 4.1: The activities of antioxidant with DPPH assay, % Inhibition of *P. linteus* extract was extracted with water at 95°C (PL-W)

P. linteus extract was extracted with 1% $(NH_4)_2C_2O_4$) at 95°C at 0.30 mg/mL have the highest %inhibition of antioxidant activities was 20.49%. **(Figure 4.2)**

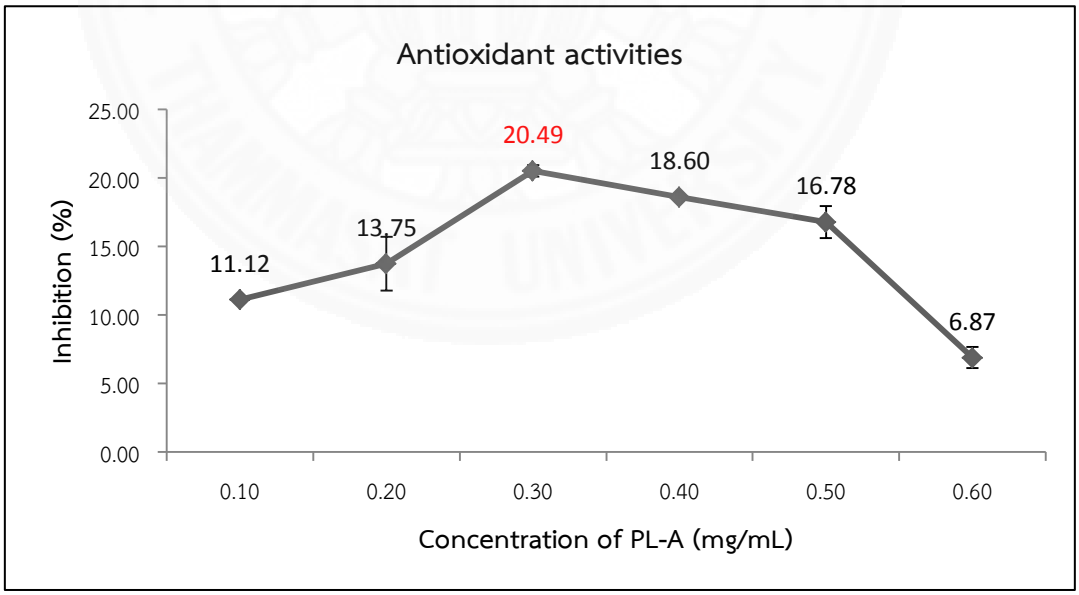

Figure 4.2: The activities of antioxidant with DPPH assay, % Inhibition of *P. linteus* extract was extracted with1% $(NH_4)_2C_2O_4$) at 95°C (PL-A)

P. linteus extract was extracted with 1.25M NaOH/0.05% $NaBH_4$ at 25°C at 0.30 mg/mL have the highest %inhibition of antioxidant activities was 28.61%. **(Figure 4.3)**

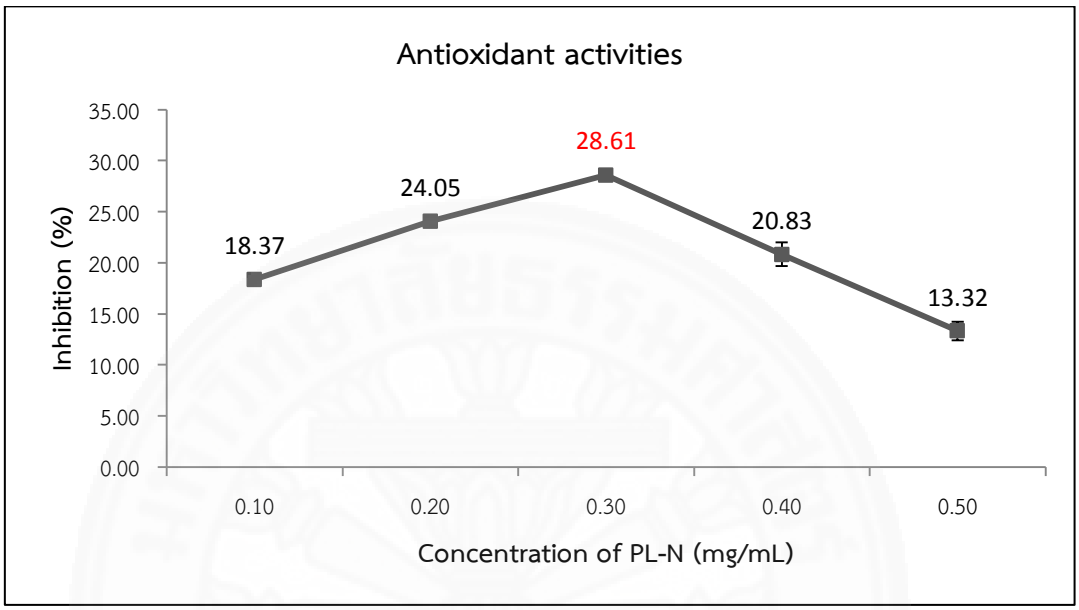

Figure 4.3: The activities of antioxidant with DPPH assay, % Inhibition of *P. linteus* extract was extracted with 1.25M NaOH/0.05% $NaBH_4$ at 25°C (PL-N)

Table 4.10: The activities of antioxidant with DPPH assay, % Inhibition (PL-W, PL-A and PL-N)

Crude extract	% Inhibition
PL-W (0.30 mg/mL)	14.63±4.116
PL-A (0.30 mg/mL)	20.49±0.421
PL-N (0.30 mg/mL)	28.61±0.243

At 0.30 mg/mL, the *P. linteus* was extracted with 1.25M NaOH/0.05% $NaBH_4$ at 25°C have the highest %inhibition of antioxidant activities was 28.61%. Followed by 1% $(NH_4)_2C_2O_4$) at 95 ° C and water at 95°C was 20.49% and 14.63%, respectively.

In a previous study, (Wang et al., 2014) reported the antioxidant activity of *P. linteus* was affected by the molecular weight, monosaccharide compositions and chemical structures of the polysaccharides.

From analysis of the Total Phenolic Contents, Total Flavonoid Contents and Antioxidant activity were found to be consistent. The *P. linteus* extract contains a lot of Total Phenolic Contents and Total Flavonoid Contents as well. It also has the best antioxidant as well. (Isuntea and Wongkrajang., 2015)

4.3.2 The activity of antimicrobial by MIC and MBC method

P. linteus has been experimentally demonstrated to possess antitumor, immuno-modulatory, anti-inflammatory, anti-oxidant, anti-hyperlipidemic, antimicrobial and anti-diabetic activities (Song *et al*., 2003; Nakamura *et al*., 2004; Kim *et al*., 2004; Inagaki *et al*., 2005; Kim *et al*., 2007)

The Minimum Inhibitory Concentration (MIC) antimicrobial analysis, the lowest concentration of the *P. linteus* was extracted with 95% ethanol at 80°C (PL-EH), 95% ethanol round 1 (PL-E1) and 95% ethanol in the round 2 (PL-E2) with concentration of 32 µg/mL can inhibit the bacteria *B. subtilis*, *E. aerogenes, S. aureus* and *E. coli* **(Table 4.11)**

Table 4.11: The activity of antimicrobial by Minimum Inhibitory Concentration method (PL-EH, PL-E1 and PL-E2)

Crude extract (µg/mL)	***B. subtilis***	***E. aerogenes***	***S. aureus***	***E. coli***
PL-EH	32	32	32	32
PL-E1	32	32	32	32
PL-E2	32	32	32	32
Penicillin	✓	✖	✓	✖
Vancomycin	✓	✓	✓	✓

The Minimum Bactericidal Concentration (MBC) antimicrobial analysis, the lowest concentration of the *P. linteus* was extracted with 95% ethanol at 80°C (PL-EH) can kill the bacteria *B. subtilis*, *E. aerogenes, S. aureus* and *E. coli* with concentration of 32, 128, 64 and 128 µg/mL, respectively.

The lowest concentration of the *P. linteus* was extracted with 95% ethanol round 1 (PL-E1) can kill the bacteria *B. subtilis*, *E. aerogenes, S. aureus* and *E. coli* with concentration of 128 µg/mL.

The lowest concentration of the *P. linteus* was extracted with 95% ethanol round 2 (PL-E2) can kill the bacteria *B. subtilis*, *E. aerogenes* and *E. coli* with concentration of 128 µg/mL and 64 µg/mL can kill the *S. aureus*. **(Table 4.12)**

Table 4.12: The activity of antimicrobial by Minimum Bactericidal Concentration method (PL-EH, PL-E1 and PL-E2)

Crude extract (µg/mL)	***B. subtilis***	***E. aerogenes***	***S. aureus***	***E. coli***
PL-EH	32	128	64	128
PL-E1	128	128	128	128
PL-E2	128	128	64	128

The antimicrobial activities should be MIC and MBC identical or similar (not more than one or two concentrations; MBC / MIC ≤4). The *P. linteus* was extracted with 95% ethanol at 80°C (PL-EH) had the ratio MBC:MIC is 1, 4, 2 and 4 of bacteria *B. subtilis*, *E. aerogenes, S. aureus* and *E. coli*, respectively.

The *P. linteus* was extracted with 95% ethanol round 1 (PL-E1) had the ratio MBC:MIC is 4 of bacteria *B. subtilis*, *E. aerogenes, S. aureus* and *E. coli*.

The *P. linteus* was extracted with 95% ethanol in the round 2 (PL-E2) has the ratio of MBC:MIC is 4, 4, 2 and 4 of bacteria *B. subtilis*, *E. aerogenes, S. aureus* and *E. coli*, respectively. **(Table 4.13)**

Table 4.13: The ratio of Minimum Bactericidal Concentration: Minimum Inhibitory Concentration (PL-EH, PL-E1 and PL-E2)

Crude extract (MBC:MIC)	***B. subtilis***	***E. aerogenes***	***S. aureus***	***E. coli***
PL-EH	1	4	2	4
PL-E1	4	4	4	4
PL-E2	4	4	2	4

The Minimum Inhibitory Concentration (MIC) antimicrobial analysis, the lowest concentration of the *P. linteus* was extracted with water at 95°C (PL-W) at concentration of 32, 32, 64 and 32 µg/mL can inhibit the bacteria *B. subtilis*, *E. aerogenes, S. aureus* and *E. coli*, respectively.

The lowest concentration of the *P. linteus* was extracted with 1%$(NH_4)_2C_2O_4$ at 95°C (PL-A) at concentration of 32, 32, 64 and 16 µg/mL can inhibit the bacteria *B. subtilis*, *E. aerogenes, S. aureus* and *E. coli*, respectively.

And the lowest concentration of the *P. linteus* was extracted with 1.25M NaOH/0.05%$NaBH_4$ at 25°C (PL-N) at concentration of 32, 32, 64 and 32 µg/mL can inhibit the bacteria *B. subtilis*, *E. aerogenes, S. aureus* and *E. coli*, respectively. **(Table 4.14)**

Table 4.14: The activity of antimicrobial by Minimum Inhibitory Concentration method (PL-W, PL-A and PL-N)

Crude extract (µg/mL)	***B. subtilis***	***E. aerogenes***	***S. aureus***	***E. coli***
PL-W	32	32	64	32
PL-A	32	32	64	16
PL-N	32	32	64	32
Penicillin	✓	✘	✓	✘
Vancomycin	✓	✓	✓	✓

The Minimum Bactericidal Concentration (MBC) antimicrobial analysis, the lowest concentration of the *P. linteus* was extracted with water at 95°C (PL-W) can kill the bacteria *B. subtilis*, *E. aerogenes, S. aureus* and *E. coli* with concentration of 64, 128, 64 and 32 µg/mL, respectively.

The *P. linteus* was extracted with 1%$(NH_4)_2C_2O_4$ at 95°C (PL-A) can kill the bacteria *B. subtilis*, *E. aerogenes, S. aureus* and *E. coli* with concentration of 64 µg/mL.

The *P. linteus* was extracted with 1.25M NaOH/0.05%$NaBH_4$ at 25°C (PL-N) and kill the bacteria *B. subtilis*, *E. aerogenes, S. aureus* and *E. coli* with concentration of 32, 64, 64 and 64 µg/mL**. (Table 4.15)**

Table 4.15: The activity of antimicrobial by Minimum Bactericidal Concentration method (PL-W, PL-A and PL-N)

Crude extract (µg/mL)	***B. subtilis***	***E. aerogenes***	***S. aureus***	***E. coli***
PL-W	64	128	64	32
PL-A	64	64	64	64
PL-N	32	64	64	64

The antimicrobial activities should be MIC and MBC identical or similar (not more than one or two concentrations; MBC / MIC ≤4). The *P. linteus* was extracted with water at 95°C (PL-W) had the ratio MBC:MIC is 2, 4, 1 and 1 of bacteria *B. subtilis*, *E. aerogenes, S. aureus* and *E. coli*, respectively.

The *P. linteus* was extracted with 1%$(NH_4)_2C_2O_4$ at 95°C (PL-A) had the ratio MBC:MIC is 2, 2, 1 and 4 of bacteria *B. subtilis*, *E. aerogenes, S. aureus* and *E. coli*, respectively.

The *P. linteus* was extracted with 1.25M NaOH/0.05%$NaBH_4$ at 25°C (PL-N) had the ratio MBC:MIC is 1, 2, 1 and 2 of bacteria *B. subtilis*, *E. aerogenes, S. aureus* and *E. coli*, respectively. **(Table 4.16)**

Table 4.16: The ratio of Minimum Bactericidal Concentration: Minimum Inhibitory Concentration (PL-W, PL-A and PL-N)

Crude extract (MBC:MIC)	***B. subtilis***	***E. aerogenes***	***S. aureus***	***E. coli***
PL-W	2	4	1	1
PL-A	2	2	1	4
PL-N	1	2	1	2

From the antimicrobial activity of all the extracts (PL-EH, PL-E1, PL-E2, PL-W, PL-A and PL-N), The *P. linteus* extract was extracted with 1.25M NaOH/0.05%$NaBH_4$ at 25℃ (PL-N) was an effective antibacterial extract. The ratio of MBC: MIC of bacteria *B. subtilis, E. aerogenes, S. aureus* and *E. coli* was 1, 2, 1 and 2, respectively, less than the *P. linteus* extract was extracted with 95% ethanol at 80℃ (PL-EH), 95% ethanol round 1 (PL-E1), 95% ethanol round 2 (PL-E2) , water at 95℃ and 1%$(NH_4)_2C_2O_4$ at 95℃ (PL-A).

In a previous study, *P. linteus* methanol extract showed a good antibacterial effects (MICs, 63–125 Ag/ml) against methicillin-resistant *S. aureus* for all tested strains. (Hur *et al*., 2004)

And the methanolic extract also revealed the highest potential, considering MIC or MBC values it gave higher activity than ampicillin for all bacteria (*Staphylococcus aureus, Bacillus cereus, Micrococcus flavus, Listeria monocytogenes, Pseudomonas aeruginosa, Salmonella typhimurium, Escherichia coli, Enterobacter cloacae*) and than streptomycin (except in the case of *S. aureus*). (Reis *et al*., 2014)

From the above analysis, the sample is divided into 3 groups.

1. The group is a very significant amount Total Phenolic Contents, Total Flavonoid Contents and Total Polysaccharide Contents of the *P. linteus* comprising extracted by

- 95% ethanol at 80℃ (PL-EH)

- 95% ethanol round 1 (PL-E1)
- 95% ethanol round 2 (PL-E2)

All pair difference is significant at the 0.05 level in One way ANOVA and LSD therefore the *P. linteus* were extracted with 95% ethanol solvent at 80°C (PL-EH) is the highest compounds for next experiment.

2. The antioxidant group consisted of the *P. linteus* comprising extracted by

- 95% ethanol at 80°C (PL-EH)
- 95% ethanol round 1 (PL-E1)
- 95% ethanol round 2 (PL-E2)

The *P. linteus* were extracted with 95% ethanol solvent at 80°C (PL-EH) and 95% ethanol round 1 (PL-E1) was lowest concentration that can inhibit by 50% at 2.04 µg/mL and 2.07 µg/mL, respectively. And not significantly different statistically at the 0.05 level in One way ANOVA. So choose both types of extracts.

3. The antimicrobial activity group consisted of *P. linteus* comprising extracted by

- Water at 95°C (PL-W)
- 1%$(NH_4)_2C_2O_4$ at 95°C (PL-A)
- 1.25M NaOH/0.05%$NaBH_4$ at 25°C (PL-N)

From **(Table 4.16 and 4.17)** the ratio of Minimum Bactericidal Concentration: Minimum Inhibitory Concentration. The *P. linteus* was extracted with 1.25M NaOH/0.05% $NaBH_4$) at 25°C (PL-N) has the ratio of MBC: MIC is 1, 2, 1 and 2 of bacteria *B. subtilis, E. aerogenes, S. aureus and E. coli*, respectively.

Antimicrobial substances are considered as bactericidal agents when tha ratio MBC:MIC ≤ 4 and bacteriostatic agent when the ratio MBC:MIC > 4. For most of the extracts tested, the ratio MBC:MIC was ≤ 4 against the bacteria strains used, suggesting that these extracts may be classified as bactericidal agents. (Gatsing *et al*., 2010

Table 4.17: The summary of the substances and activities from *P. linteus*

Results	PL-EH	PL-E1	PL-E2	PL-W	PL-A	PL-N
%Yield and %Contents	3.48%	1.6%	0.45%	13.2%	84.75%	124.3%
Total Phenolic Content (mg GAE/g crude extract)	610.92	455.49	401.36	122.52	28.50	46.52
Total Flavonoid Content (mg QE/g crude extract)	756.75	574.19	531.44	126.43	43.64	67.60
Total Polysaccharide Content (mg GE/g crude extract)	141.33	109.35	113.43	167.96	29.88	25.94
Inhibition of free radicals	IC_{50}=2.04 (µg/mL)	IC_{50}=2.07 (µg/mL)	IC_{50}=3.60 (µg/mL)	14.63 (%)	20.49 (%)	28.61 (%)
MIC (µg/mL)						
B. subtilis	32	32	32	32	32	32
E. aerogenes	32	32	32	32	32	32
S. aureus	32	32	32	64	64	64
E. coli	32	32	32	32	16	32
MBC (µg/mL)						
B. subtilis	32	128	128	64	64	32
E. aerogenes	128	128	128	128	64	64
S. aureus	64	128	64	64	64	64
E. coli	128	128	128	32	64	64
MBC:MIC						
B. subtilis	1	4	4	2	2	1
E. aerogenes	4	4	4	4	2	2
S. aureus	2	4	2	1	1	1
E. coli	4	4	4	1	4	2

4.4 Separation of the *Phellinus linteus* extract compounds

4.4.1 High-Performance Liquid Chromatography

The grouping of the extract into 3 groups was the *P. linteus* were extracted with 95% ethanol solvent at 80°C (PL-EH), 95% ethanol round 1 (PL-E1) and 1.25M NaOH/0.05% $NaBH_4$) at 25°C (PL-N) for analysis by High-Performance Liquid Chromatography.

A HPLC method was developed for the separation and determination of flavonoid and phenolic antioxidants in *P. linteus* extract (PL-EH, PL-E1 and PL-N) was compared with Gallic Acid and Quercetin standard.

Results of extraction and analysis of HPLC extracts were compared with standard chromatograms. The retention time analysis with standard chromatograms shows that Gallic Acid has a retention time of 3.731 minutes at a wavelength of 254 and 280 nm. As shown in **Figure 4.4 and 4.5**.

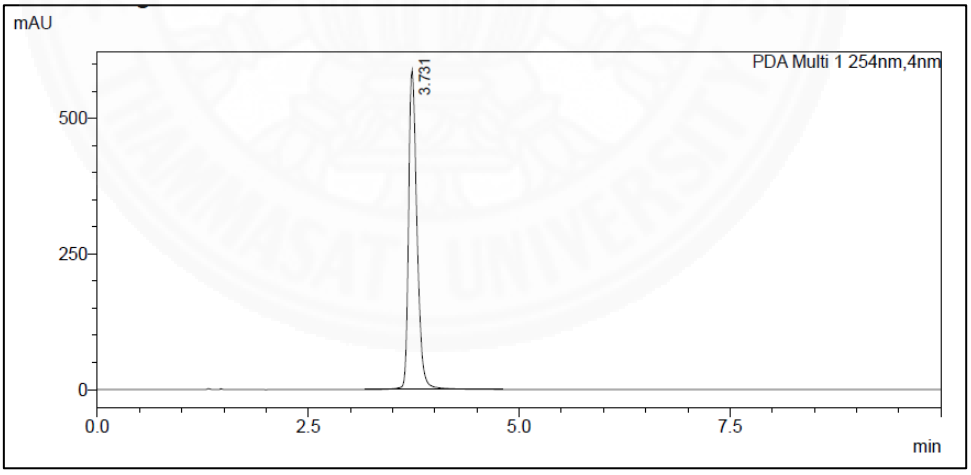

Figure 4.4: Chromatogram of High-Performance Liquid Chromatography of Gallic Acid at a wavelength of 254 nm.

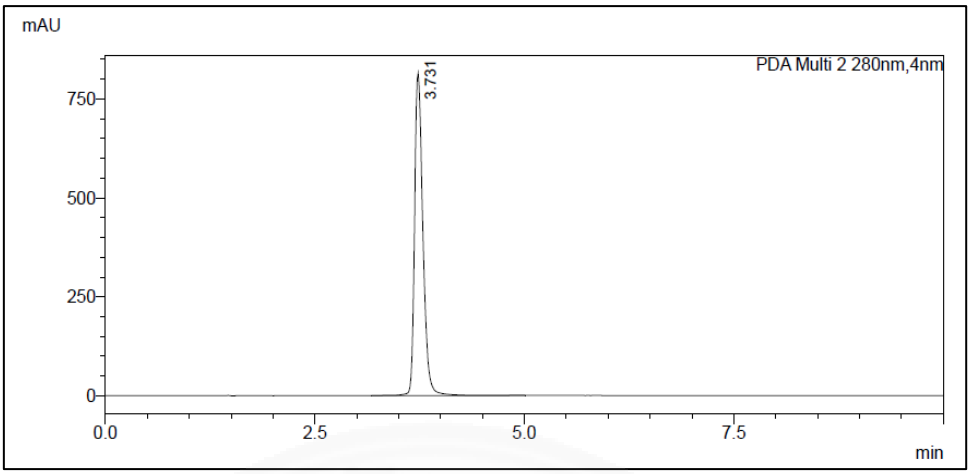

Figure 4.5: Chromatogram of High-Performance Liquid Chromatography of Gallic Acid at a wavelength of 280 nm.

The retention time analysis with standard chromatograms shows that Quercetin has a retention time of 31.1576 minutes at a wavelength of 254 and 280 nm. As shown in **Figure 4.6 and 4.7.**

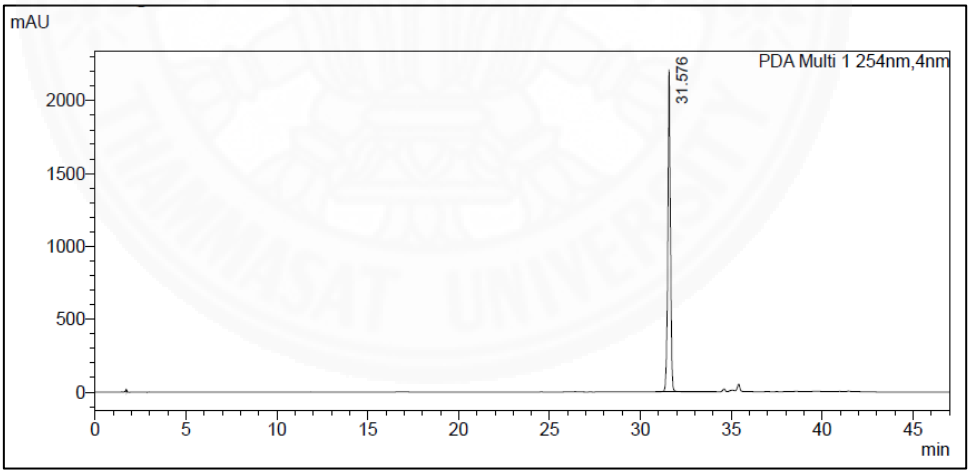

Figure 4.6: Chromatogram of High-Performance Liquid Chromatography of Quercetin at a wavelength of 254 nm.

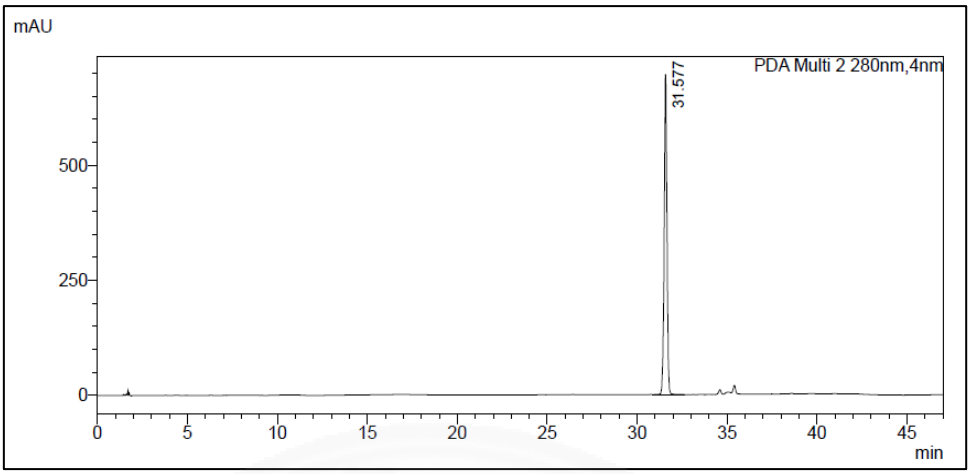

Figure 4.7: Chromatogram of High-Performance Liquid Chromatography of Quercetin at a wavelength of 280 nm.

Chromatogram of the *P. linteus* were extracted with 95% ethanol solvent at 80°C (PL-EH) has the estimated retention time at a wavelength of 254 and 280 nm. As shown in **Figure 4.8 and 4.9**

There may be substance that corresponds to the retention time of the standard substance, is 31.202 minutes, which is Quercetin. And may be defined as (2)-Epicatechin, *p*-Coumaric acid, Benzoic acid, Myricetin and Quercetin, due to the same HPLC system. (Chen *et al*., 2001)

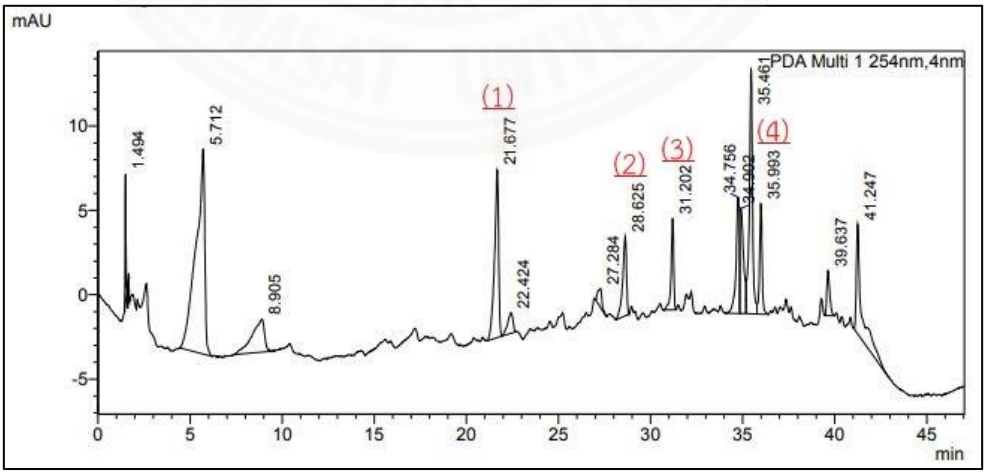

Figure 4.8: Chromatogram of High-Performance Liquid Chromatography of the extract was extracted with 95% ethanol at 80°C (PL-EH) at a wavelength of 254 nm. (1=(2)-Epicatechin, 2=Benzoic acid, 3=Myricetin and 4=Quercetin)

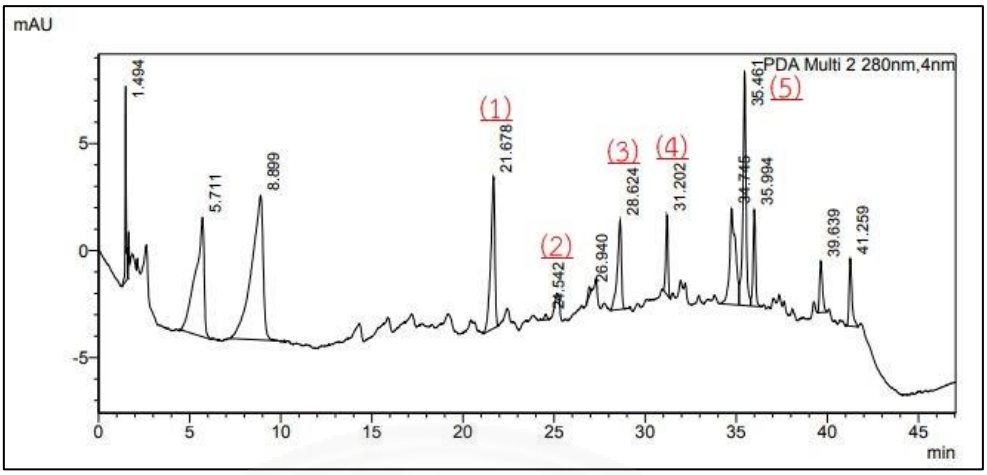

Figure 4.9: Chromatogram of High-Performance Liquid Chromatography of the extract was extracted with 95% ethanol at 80°C (PL-EH) at a wavelength of 280 nm. (1=(2)-Epicatechin, 2=*p*-Coumaric acid, 3=Benzoic acid, 4=Myricetin and 5=Quercetin)

Chromatogram of the *P. linteus* were extracted with 95% ethanol round 1 (PL-E1) has the estimated retention time at a wavelength of 254 and 280 nm. As shown in **Figure 4.10 and 4.11**

There may be an important substance that corresponds to the retention time of the standard substance, is 31.209 minutes, which is Quercetin. And may be defined as Caffeic acid, (2)-Epicatechin, *p*-Coumaric acid, Benzoic acid, Myricetin and Quercetin, due to the same HPLC system. (Chen *et al*., 2001)

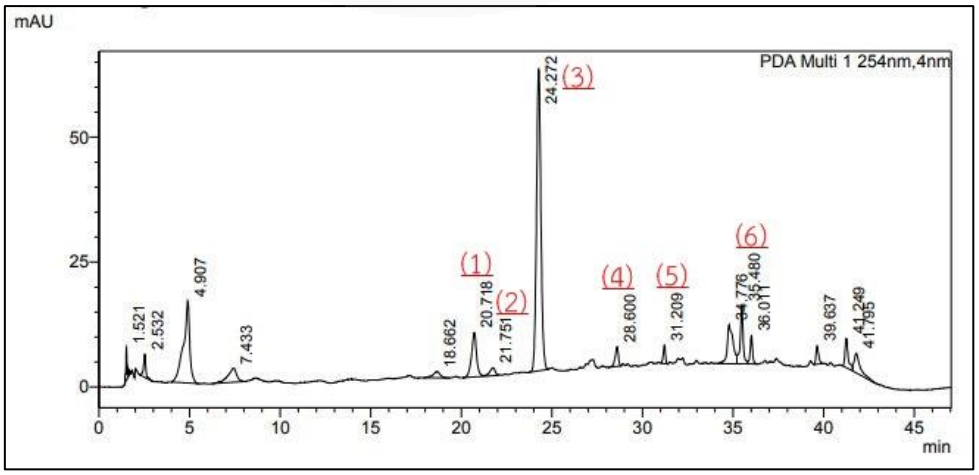

Figure 4.10: Chromatogram of High-Performance Liquid Chromatography of the extract was extracted with 95% ethanol round 1 at 25°C (PL-E1) at a wavelength of 254 nm. (1=Caffeic acid, 2=(2)-Epicatechin, 3=*p*-Coumaric acid, 4=Benzoic acid, 5=Myricetin and 6=Quercetin)

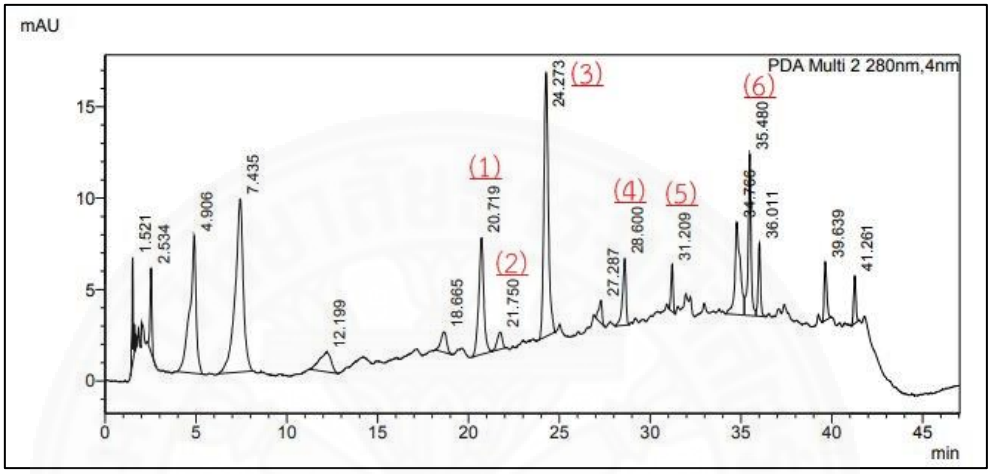

Figure 4.11: Chromatogram of High-Performance Liquid Chromatography of the extract was extracted with 95% ethanol round 1 at room temperature (PL-E1) at a wavelength of 280 nm. (1=Caffeic acid, 2=(2)-Epicatechin, 3=*p*-Coumaric acid, 4=Benzoic acid, 5=Myricetin and 6=Quercetin)

Chromatogram of the *P. linteus* were extracted with aluminum oxalate (1% $(NH_4)_2C_2O_4$) at 95°C (PL-N) has the estimated retention time at a wavelength of 254 and 280 nm. As shown in **Figure 4.12 and 4.13**

As a result of retention time, it was not possible to identify standard Gallic Acid and Quercetin.

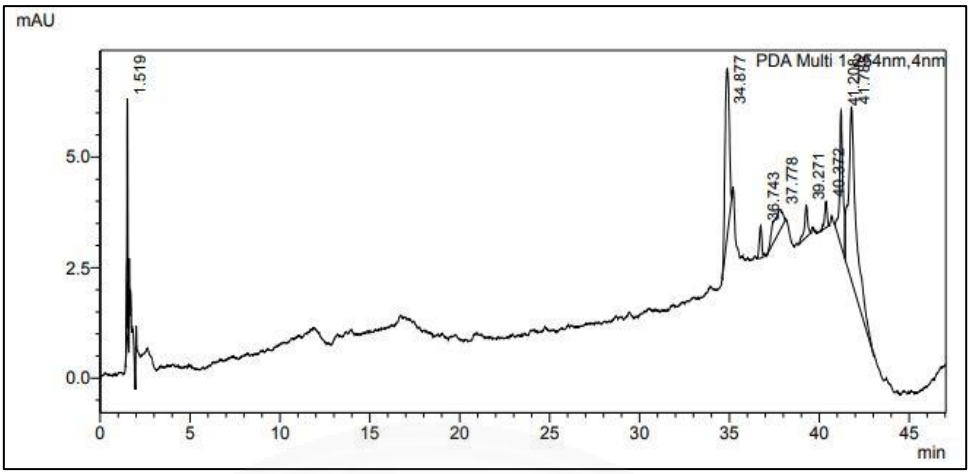

Figure 4.12: Chromatogram of High-Performance Liquid Chromatography of the extract was extracted with aluminum oxalate (1% $(NH_4)_2C_2O_4$) at 95°C (PL-N) at a wavelength of 254 nm.

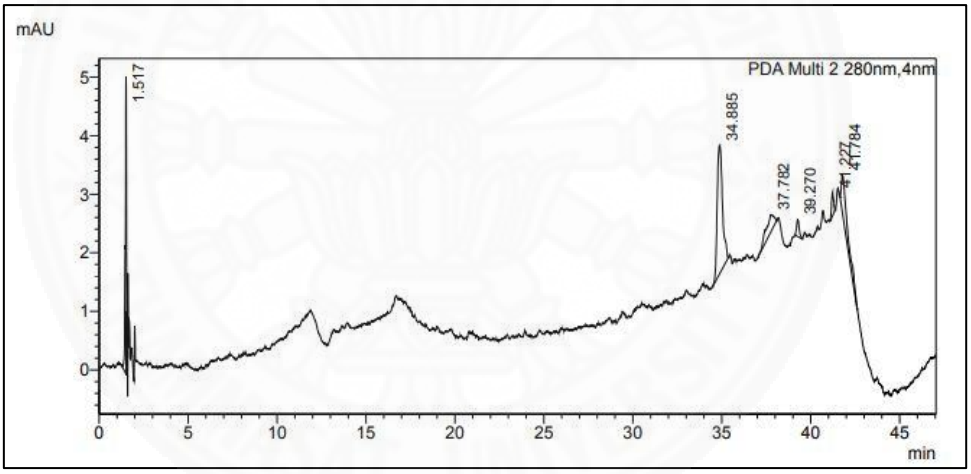

Figure 4.13: Chromatogram of High-Performance Liquid Chromatography of the extract was extracted with aluminum oxalate (1% $(NH_4)_2C_2O_4$) at 95°C (PL-N) at a wavelength of 280 nm.

From the retention time of all three extracts, the *P. linteus* were extracted with 95% ethanol solvent at 80°C (PL-EH), 95% ethanol round 1 at 25°C (PL-E1) and 1.25M NaOH/0.05% $NaBH_4$ at 95°C (PL-N). Retention time may be defined as Caffeic acid, (2)-Epicatechin, *p*-Coumaric acid, Benzoic acid, Myricetin and Quercetin, due to the same HPLC system. (Chen *et al*., 2001)

And polysaccharide can determine by HPLC but use different system and column. The polysaccharides from *P. linteus* contain mainly glucose 78.88%. There are also rhamnose, mannose, arabinose, galactose and xylose use HPLC post-column system with TSK gel SCX, equilibrated with 0.35 M borate buffer (pH 7.6) and flow rate was 0.6 ml/min. (Suabjakyong *et al*., 2015)

4.5 Determination of encapsulation efficiency and releasing efficiency

The results of the extraction encapsulate in chitosan nanoparticles at a concentration of 0.05% (w/v) (Sornsiri and Na Nakorn, 2011) and concentration of the extract is extracted with a solvent such that *P.linteus* was extracted with 95% ethanol at 80°C (PL-EH) was 0.001, 0.010, 0.050, 0.100, 0.500, 1.000 and 1.500 mg/mL **(Figure 4.14)**

It was found that the extract concentration of 1.00 mg/mL was highest in the nanochitosan at 93.49%. So PL-EH at a concentration of 1.00 mg/mL to encapsulate in nano-double particles and nanoliposome.

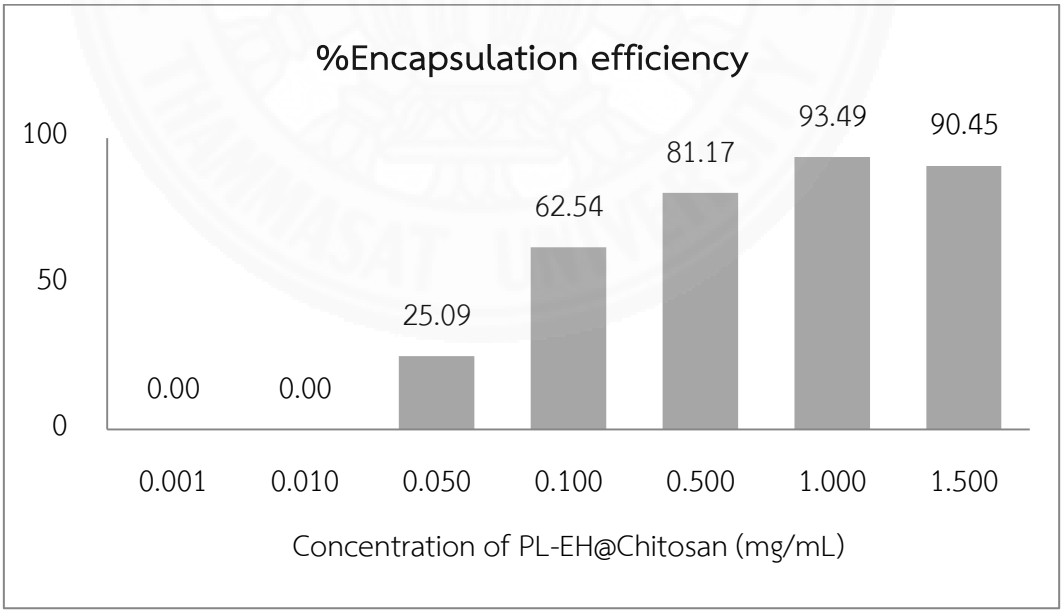

Figure 4.14: The extract was extracted with 95% ethanol at 80°C (PL-EH) was 0.001, 0.010, 0.050, 0.100, 0.500, 1.000 and 1.500 mg/mL enacapsulated in nanochitosan.

The results of the extraction encapsulate in chitosan nanoparticles at a concentration of 0.05% (w/v) and concentration of the extract is extracted with a

solvent such that *P. linteus* was extracted with 95% ethanol round 1 at 25°C (PL-E1) was 0.001, 0.010, 0.050, 0.100, 0.500 and 1.000 mg/mL **(Figure 4.15)**

It was found that the extract concentration of 0.500 mg/mL was highest in the nanochitosan at 90.74%. So PL-E1 at a concentration of 0.50 mg/mL to encapsulate in nanoliposome and nano-double particles.

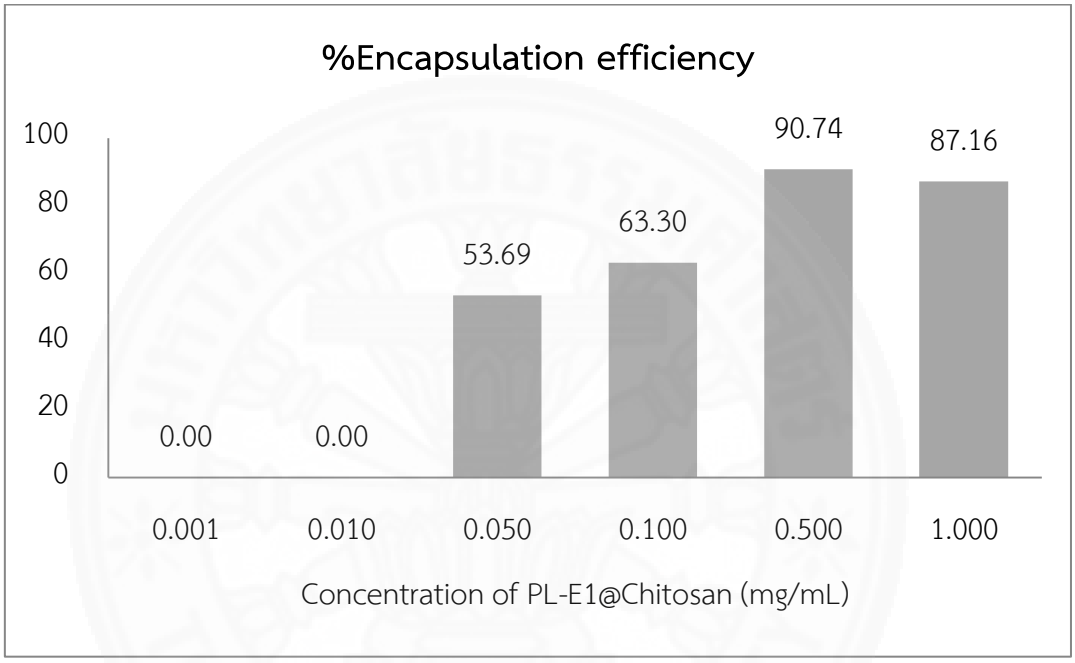

Figure 4.15: The extract was extracted with 95% ethanol round 1 at room temperature (PL-E1) was 0.001, 0.010, 0.050, 0.100, 0.500 and 1.000 mg/mL enacapsulated in nanochitosan.

The results of the extraction encapsulate in chitosan nanoparticles at a concentration of 0.05% (w/v) and concentration of the extract is extracted with a solvent such that *P. linteus* was extracted with aluminum oxalate (1% $(NH_4)_2C_2O_4$) at 95°C (PL-N) was 0.001, 0.010, 0.100, 0.500, 1.000, 1.500, 2.000 3.000 and 4.000 mg/mL **(Figure 4.16)**

It was found that the extract concentration of 3.000 mg/mL was highest in the nanochitosan at 90.74%. So PL-N at a concentration of 3.000 mg/mL to encapsulate in nanoliposome and nano-double particles.

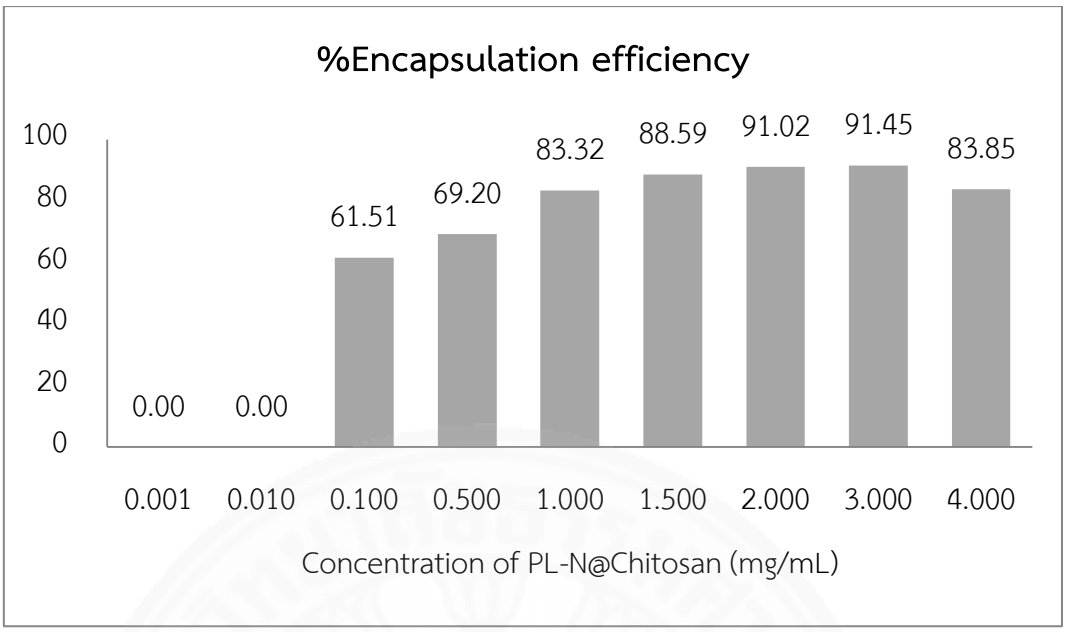

Figure 4.16: The extract was extracted with aluminum oxalate (1% $(NH_4)_2C_2O_4$) at 95°C (PL-N) was 0.001, 0.010, 0.100, 0.500, 1.000, 1.500, 2.000 3.000 and 4.000 mg/mL enacapsulated in nanochitosan.

An analysis of the efficiency of three different types of extracted (PL-EH, PL-E1 and PL-N) solutions extracts comparison of encapsulate of the *P. linteus* extract in various nanoparticles. 1). Encapsulate in nanochitosan. 2). Encapsulate in nanoliposome. 3). Encapsulate in nano-double particles.

% Encapsulation efficiency of the *P. linteus* was extracted with 95% ethanol at 80°C (PL-EH) in nanochitosan, nanoliposome and nano-double particles is 96.19, 48.31 and 75.59%, respesctively.

% Encapsulation efficiency of the *P. linteus* was extracted with 95% ethanol round 1 (PL-E1) in nanochitosan, nanoliposome and nano-double particles is 94.89, 32.20 and 61.98 %, respesctively.

% Encapsulation efficiency of the *P. linteus* was extracted with 1% $(NH4)_2C_2O_4$) at 95°C (PL-N) in nanochitosan, nanoliposome and nano-double particles is 95.15, 38.90 and 43.73 %, respesctively. (**Figure 4.17)**

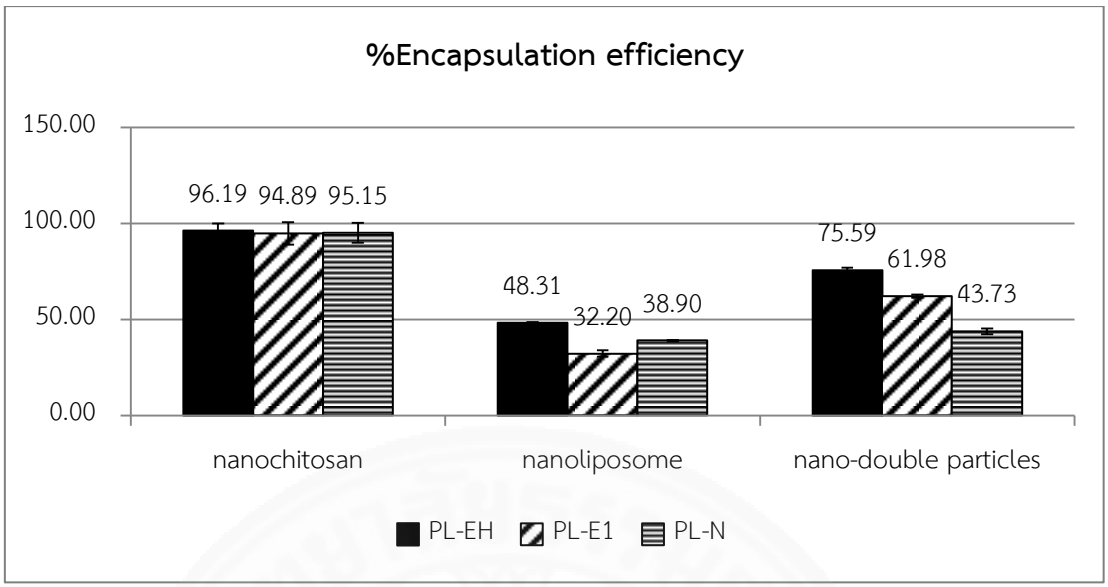

Figure 4.17: % encapsulation efficiency in nanochitosan, nanoliposome and nano-double particles

All of the *P. linteus* (PL-EH, PL-E1 and PL-N) have the highest % encapsulation efficiency that encapsulate in nanochitosan followed by nano-double particle and nanoliposome. In a previous study, the encapsulation efficiency of liposomes containing GSE was 88.2 ± 4.7% and an encapsualtion efficiency of 99.5 ± 2.3% was found for liposomes coated with chitosan. (Monika *et al*., 2016) It can be seen that chitosan can help to encapsulate extract better than only liposome.

The analysis of encapsulation efficiency in nanoparticles of various types already. Then the performance analysis released extracts. It is divided into non accumulative release and accumulative release.

Non accumulation release of the *P. linteus* was extracted with 95% ethanol at 80°C (PL-EH) in nanochitosan, nanoliposome and nano-double particles for 10 days. The conclusion that PL-EH encapsulated in nanoliposome can release most of the substance at 33.33% **(Figure 4.18)**

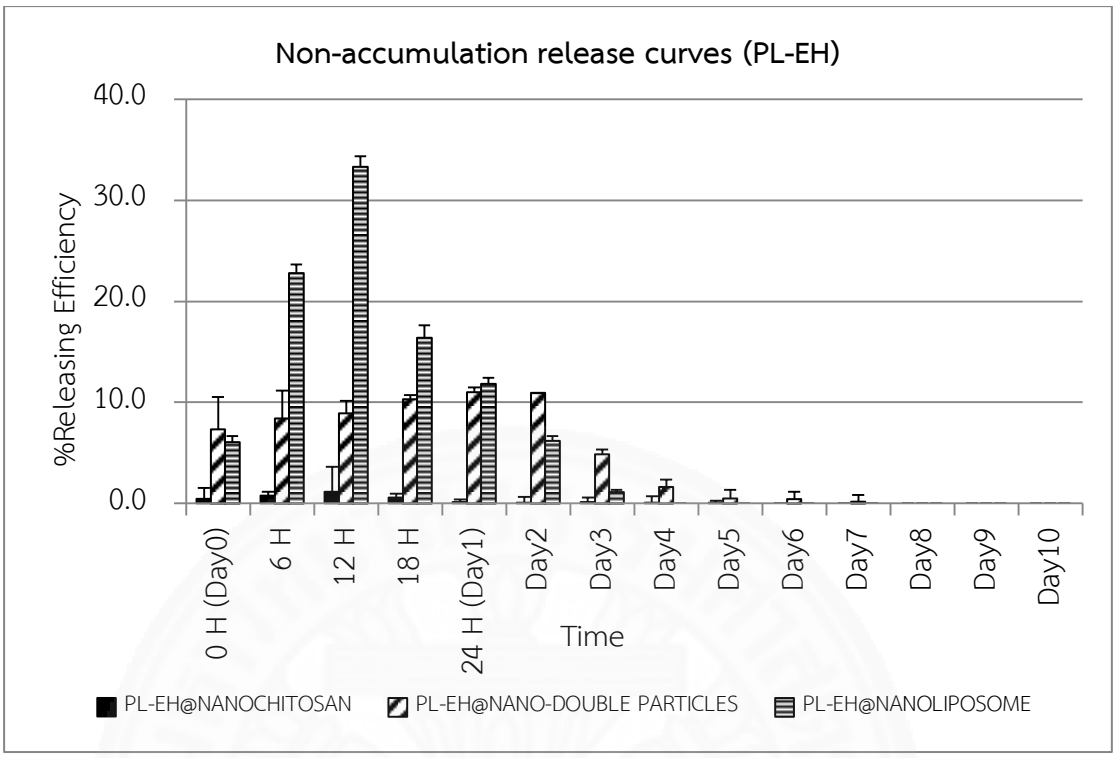

Figure 4.18: Non-accumulation release curves (PL-EH)

From the **Figure 4.18** show non-accumulation release of the *P. linteus* was extracted with 95% ethanol at 80℃ (PL-EH) in nanochitosan, nanoliposome and nano-double particles for 10 days. The conclusion that PL-EH encapsulated in nanoliposome can release most of the substance; followed by encapsulation in nano-double particles and nanochitosan.

Non-accumulation release of the *P. linteus* was extracted with 95% ethanol round 1 at 25℃ (PL-E1) in nanochitosan, nanoliposome and nano-double particles for 10 days. The conclusion that PL-E1 encapsulated in nanoliposome can release most of the substance at 35.58% **(Figure 4.19)**

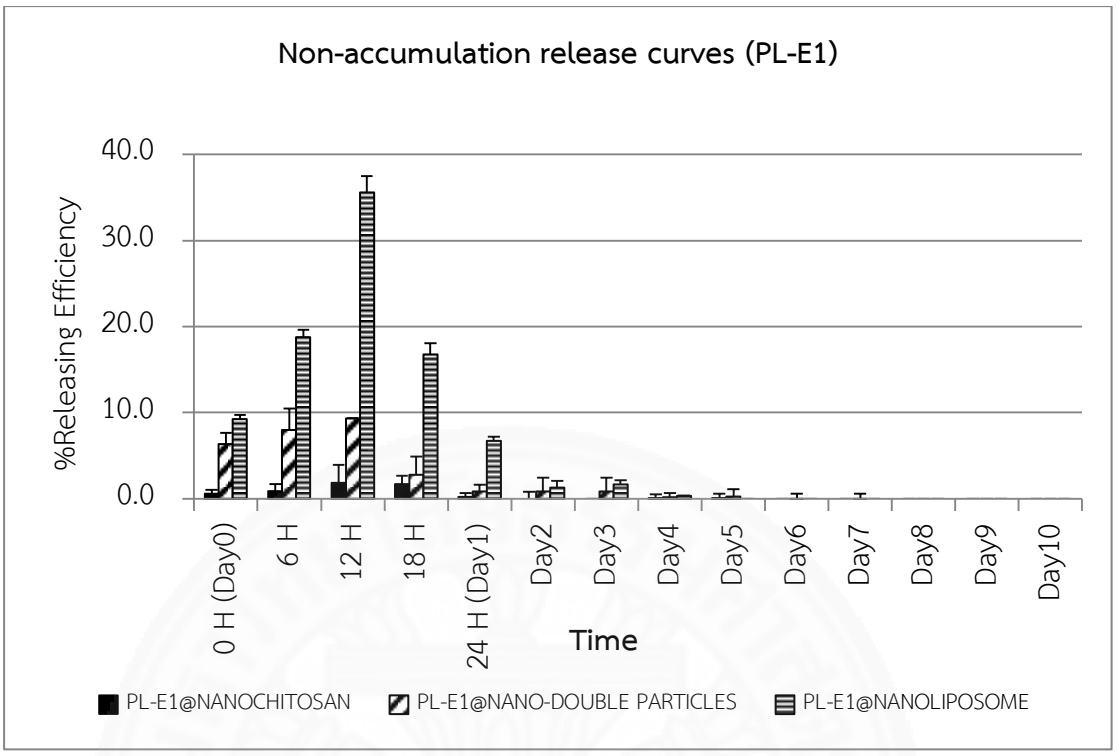

Figure 4.19: Non-accumulation release curves (PL-E1)

From the **Figure 4.19** non-accumulation release of the *P. linteus* was extracted with 95% ethanol round 1 (PL-E1) in nanochitosan, nanoliposome and nano-double particles for 10 days. The conclusion that PL-E1 encapsulated in nanoliposome can release most of the substance; followed by encapsulation in nano-double particles and nanochitosan.

Non-accumulation release of the *P. linteus* was extracted with aluminum oxalate (1% $(NH_4)_2C_2O_4$) at 95°C (PL-N) in nanochitosans, nanoliposome and nano-double particles for 10 days. The conclusion that PL-N retained in nano-double particles can release of the substance at 22.28% but PL-N encapsulated in nanoliposome can release most of substance and longer period, and last is encapsulation in nanochitosan. **(Figure 4.20)**

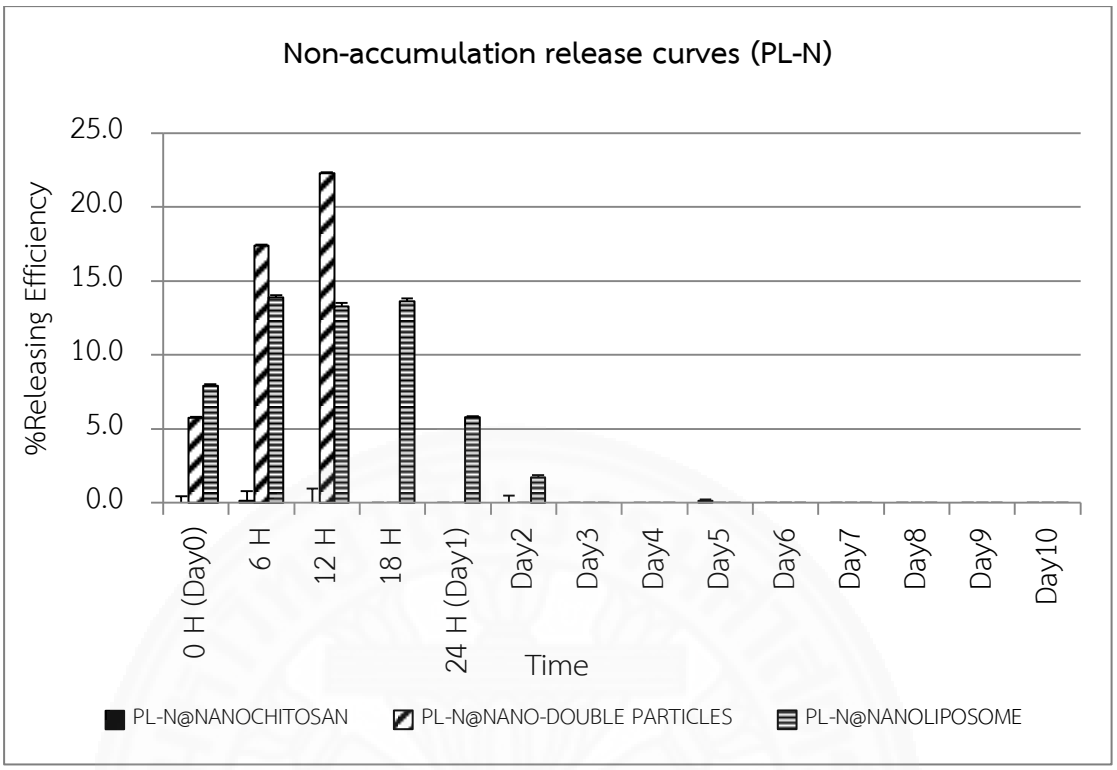

Figure 4.20: Non-accumulation release curves (PL-N)

From **Figure 4.20** non-accumulation release of the *P. linteus* was extracted with aluminum oxalate (1% $(NH_4)_2C_2O_4$) at 95°C (PL-N) in nanochitosan, nanoliposome and nano-double particles for 10 days. The conclusion that PL-N encapsulated in nano-double particles can release highest of the substance but PL-N encapsulated in nanoliposome can release most of substance and longer period. And the last is encapsulation in nanochitosan.

All of the *P. linteus* extract (PL-EH, PL-E1 and PL-N) that encapsulate in nanoliposome have the highest % releasing efficiency, followed by encapsulate in nano-double and nanochitosan. (nanoliposome>nano-double>nanochitosan)

Accumulation release of the *P. linteus* was extracted with 95% ethanol at 80°C (PL-EH). It was found that PL-EH in chitosan could release the substance less than other storage. It will increase from 0H at 0.352% until 18H starts releasing steadily until Day10. The highest % release on Day8 is 1.820%

PL-EH retention in the nanoliposome can release more substances than others. It will increase from 0H to 11.779% until 24H, releasing it permanently until Day10. The highest % release date was Day6 in 52.861%.

PL-EH retention in nano-double particles is less likely to release the substance than nanoliposome retention, increasing from 0H 6.768% until 24H, releasing slowly until Day10. The highest was Day4, 39.752%. **(Figure 4.21)**

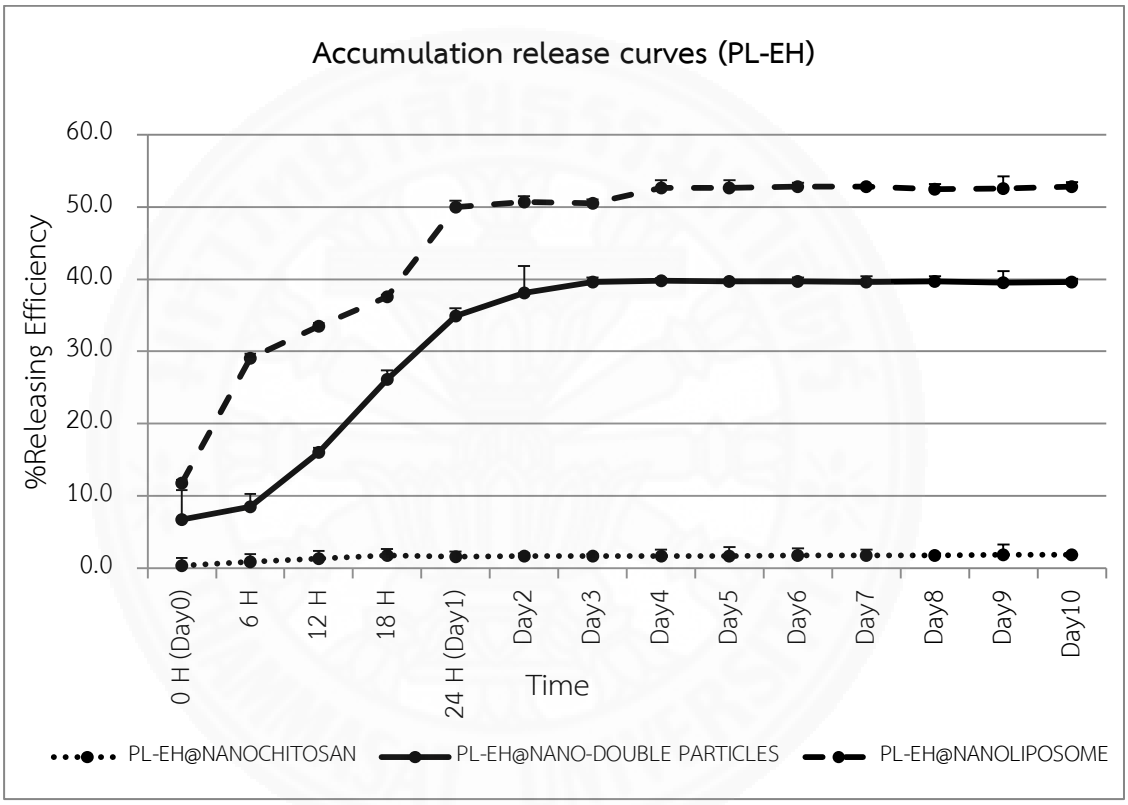

Figure 4.21: Accumulation release curves (PL-EH)

From **Figure 4.21**, the *P. linteus* was extracted with 95% ethanol at 80°C (PL-EH) that encapsulated in nanoliposome can release most of substance. Followed by PL-EH encapsulated in nano-double and nanochitosan.

Accumulation release of the *P. linteus* was extracted with 95% ethanol round 1 at 25°C (PL-E1). It was found that PL-E1 in nanochitosan can release the substance less than other retention. It will increase from 0H to 3.575% until 12H, releasing it permanently until Day10. The highest % release date is Day 8 = 10.449%.

PL-E1 in nanoliposome can release more substances than others. It will increase from 0H to 10.371% until Day2 starts releasing steadily until Day10. The highest % release date is Daytime 52.007%.

PL-E1 retention in nano-double particles is less likely to release the substance than nanoliposome retention, increasing from 0H at 6.746% until 18H begins to release steadily until Day10. The highest were Day6 and Day9 at 17.643%. **(Figure 4.22)**

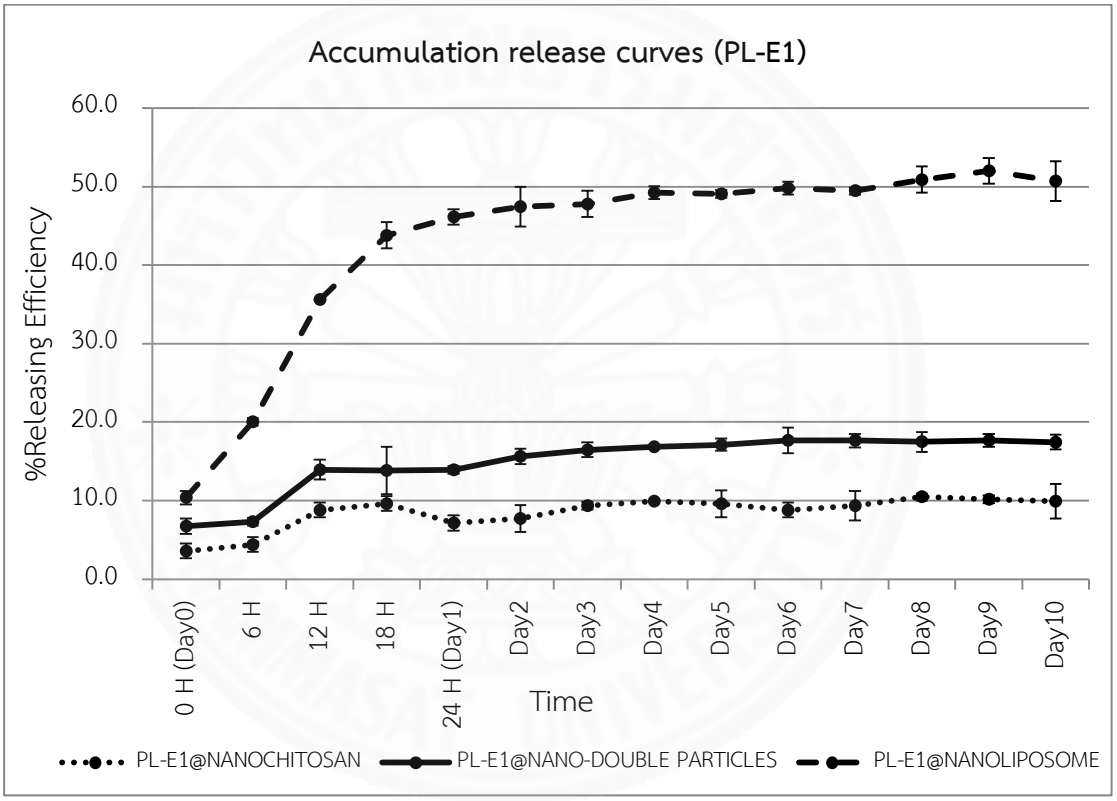

Figure 4.22: Accumulation release curves (PL-E1)

From **Figure 4.22**, the *P. linteus* was extracted with 95% ethanol round 1 at 25°C (PL-E1) that encapsulated in nanoliposome can release most of substance, followed by PL-E1 encapsulated in nano-double and nanochitosan.

Accumulation release of the *P. linteus* was extracted with aluminum oxalate (1% $(NH_4)_2C_2O_4$) at 95°C (PL-N). It was found that PL-N in nanochitosan can release the substance less than other types of retention. It will increase from 0H at

0.464% until 12H, releasing it permanently until Day10. The highest % release at Day8 is 2.322%

PL-N retention in the nanoliposome can release more substances than others. It will increase from 0H to 6.122% until Day8 starts releasing steadily until Day10. The highest % release on Day10 is 63.469%

PL-N retention in nano-double particles is less likely to release the substance than nanoliposome retention, increasing from 0H 0.953% until Day2 begins to release steadily until Day10. The highest was at Day4 at 49.569% **(Figure 4.23)**

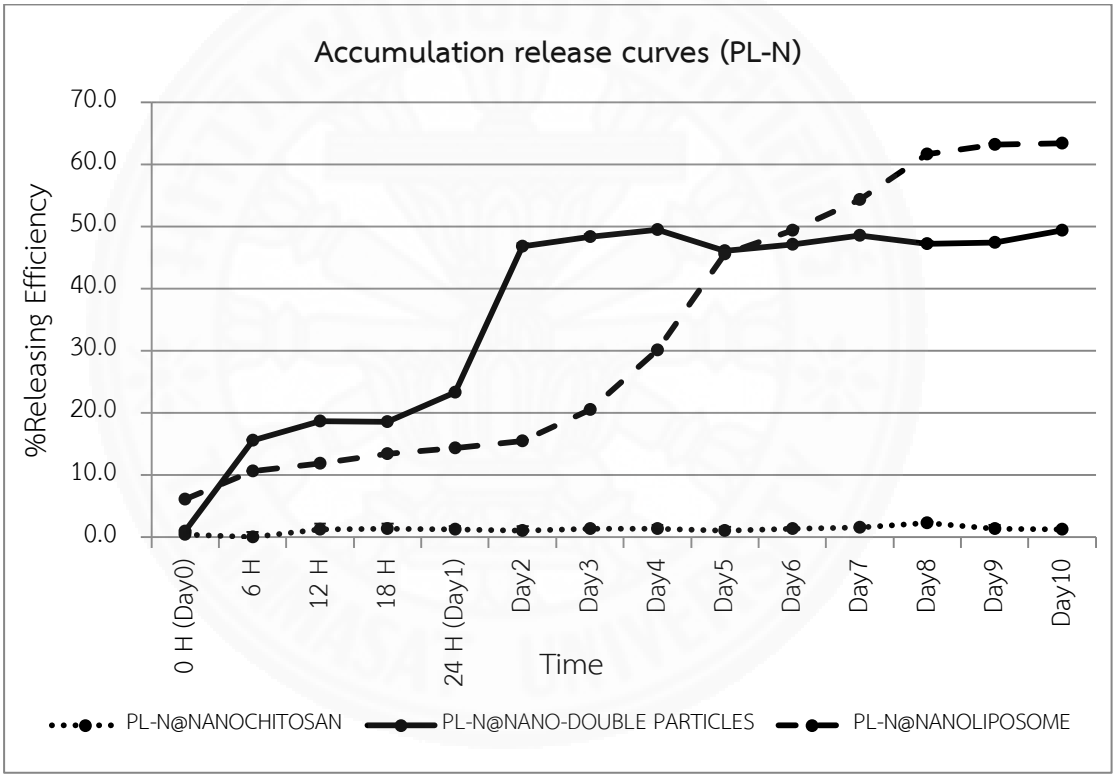

Figure 4.23: Accumulation release curves (PL-N)

From **Figure 4.23**, the *P. linteus* was extracted with aluminum oxalate (1% $(NH_4)_2C_2O_4$) at 95℃ (PL-N) that encapsulated in nano-double particles and nanoliposome can release most of substance, followed by PL-EN encapsulated in nanochitosan.

In a previous study, investigated the release of acetylsalicylic acid from uncoated and carboxymethyl chitin-coated liposomes and showed that the coated

liposomes yielded significantly lower release rates than the uncoated liposomes. (Dong and Rogers., 1991) This mean that only nanoliposome gives % releasing efficiency over nano-double particle. The degree of continuity and cohesiveness in the structure of particles and in the polymer coating as a physical barrier towards diffusing solute plays an important role in the release behavior (Dong & Rogers, 1991)

Conclusions: The encapsulations of the extract in nanochitosan have the highest % encapsulation efficiency, but have the lowest % releasing efficiency. And the encapsulations extract in nanoliposome have the highest % releasing efficiency, but have the lowest % encapsulation efficiency. So the encapsulations of the extract in nano-double particle have % encapsulation efficiency and releasing efficiency in both performance levels. It means that % encapsulation efficiency and releasing efficiency, not less and not too much to be developed into cosmeceutical applications. And from Phetdee *et al*., 2008 it was found that increasing chitosan concentration resulted in better encapsulation efficiency and the release rate slows when compared with the encapsulation of only nanoliposome particles. Chitosan can be binding onto many other derivatives by cross-linking and hard to broken or scarcely. (Bansal *et al*., 2011)

The structure of phospholipids creates compartments within the liposome bilayer that may contain exclusively lipid or aqueous solutions. This characteristic makes liposomes a versatile tool for the encapsulation of compounds. (Hayley Rutherford., 2011) The cause liposome entrapped substance less than chitosan. And release more than chitosan. Because the structure of the liposome is round and may be easier to break than the chitosan. These two polymers have different advantages and disadvantages. It can be used together. By encapsulated particulate double.

In the selection of extracts and encapsulation types, both % encapsulation efficiency and % releasing efficiency were used. The researchers selected the *P. linteus* was extracted with 95% ethanol at 80°C (PL-EH extract) encapsulated in nano-double particles because PL-EH had more substances and more bioactivities than another extracts, and nano-double particles had % encapsulation efficiency and % releasing efficiency appropriate.

4.6 Characterization of the *Phellinus linteus* extracts and encapsulated nanoparticles

4.6.1 Analysis of the shape of nanoparticles.

- **Transmission Electron Microscope (TEM)**

From the analysis of Transmission Electron Microscope, the *P. linteus* was extracted with 95% ethanol at 80 ° C (PL-EH) in nano-double particles. The size of the nanoparticles is 270.59 nm and spherical. **(Figure 4.24)** It can be seen that the particle size analysis is large. It may be due to the clustering of the dispersed particles. The nano-double particles may be viewed as small particles that aggregate into particles. It can be concluded that nanoparticles are smaller in size. According to (Monika *et al*., 2016) the study of TEM images of layers coated with chitosan were calculated to 36.9 nm containing GSE and 13.2 nm without GSE. It can be concluded that the nano-double particle size is less than 270.59 nm.

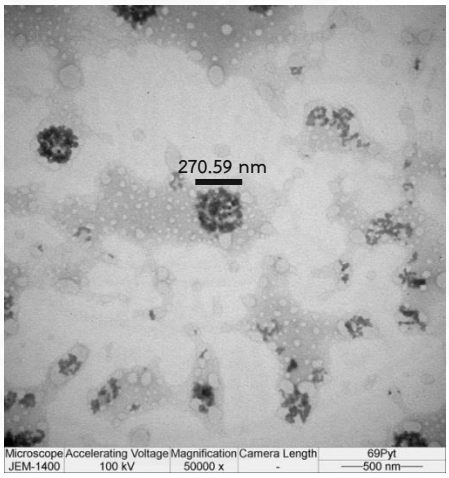

Figure 4.24: Shape of PL-EH encapsulate in nano-double particles

4.6.2 Analyze the size of nanoparticles At the nanometer level

- **Dynamic light scattering (DLS)**

The particle that PL-EH encapsulate in nano-double particles was 213.1 nm, Z-Average: 369.4 nm and PI: 0.439 **(Figure 4.25)**

The reason that the size of nanoparticles obtained by DLS analysis is not close to the size of nanoparticles analyzed by TEM. This is due to the

coalescence of the nanoparticles so that the analyzed particles are relatively large. The DLS analysis revealed that the PI value or the distribution index was too high. The PI value was less than or equal to 0.05, so the nanoparticles were not well distributed. Particle size results from polydispersity index (PI). If the polydispersity index is very high. This will result in the size of the particle is unstable or large because of the clustering. (Baalousha and Lead., 2018)

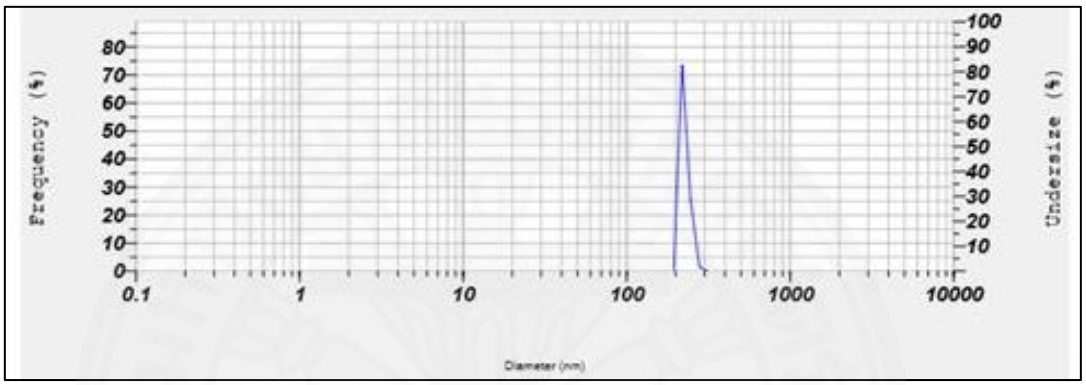

Figure 4.25: Size of PL-EH encapsulate in nano-double particles

4.7 Development cosmeceutical

4.7.1 The activity of antioxidant

The analysis of the antioxidant properties by % DPPH inhibition of the antioxidant products have 55%, Product control have antioxidant 5.98% and *P. linteus* were extracted with 95% ethanol solvent at 80°C (PL-EH) at 0.5 mg/mL have antioxidant 29.46% **(Figure 4.26)**

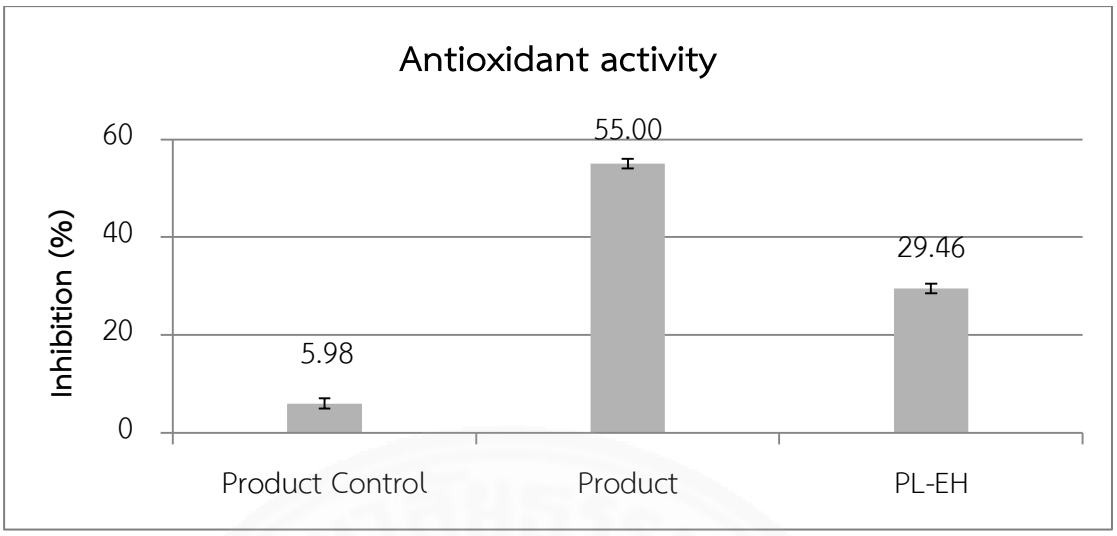

Figure 4.26: The antioxidant activities of Product Control (Hydroxyethyl Cellulose, Glycerol and DI water), Product (Hydroxyethyl Cellulose, Glycerol, DI water and PL-EH in nano-double particles), PL-EH (0.5 mg/mL)

From **Figure 4.26** show the antioxidant properties of the product showed 55% antioxidant activity, more than that of the extract and product control.

Due to the high concentration of 0.5 mg / ml of PL-EH, the decrease in inhibition comparing with PL-EH encapsualted in nano-double particle will result in higher inhibition. The particles will slow down the release of the substance and the extract is gradually released from the nanoparticles. Therefore, it is concluded that the PL-EH encapsulated in nano-double particle that have the effect of antioxidant more than the PL-EH do not have been encapsulated in nanoparticles.

4.7.2 The activity of antimicrobial

The inhibitory activity of the microorganism was found that the product was able to inhibit only one *B.subtilis* and glycerol as an ingredient in the product can inhibit bacterial infection anywhere is *B. subtilis, E. aerogenes, S. aureus* and *E. coli.* **(Table 4.18)**

Table 4.18: The activity of antimicrobial by MIC method

Sample for MIC	*B. subtilis*	*E. aerogenes*	*S. aureus*	*E. coli*
Penicillin	✓	✗	✓	✗
Vancomycin	✓	✓	✓	✓
DI water	✗	✗	✗	✗
Glycerol	✓	✓	✓	✓
Hydroxyethyl Cellulose	✗	✗	✗	✗
Product Control (Hydroxyethyl Cellulose, Glycerol and DI water)	✗	✗	✗	✓
Product	✓	✗	✗	✗

After testing the effect of killing the microbial products. The product cannot kill *B. subtilis, E. aerogenes, S. aureus* and *E. coli* and 100% glycerol can kill all microorganisms are *B. subtilis, E. aerogenes, S. aureus and E. coli* but glycerol in product control cannot kill all microorganisms are *B. subtilis, E. aerogenes, S. aureus and E. coli,* It may be because glycerol in product control has a lower concentration (40% glycerol). **(Table 4.19)**

Table 4.19: The activity of antimicrobial by MBC method

Sample for MIC	*B. subtilis*	*E. aerogenes*	*S. aureus*	*E. coli*
Penicillin	✗	✗	✓	✗
Vancomycin	✓	✓	✓	✓
DI water	✗	✗	✗	✗
Glycerol	✓	✓	✓	✓
Hydroxyethyl Cellulose	✗	✗	✗	✗
Product Control (Hydroxyethyl Cellulose, Glycerol and DI water)	✗	✗	✗	✗
Product	✗	✗	✗	✗

From **Table 4.19** show that Glycerol as ingredients in the product can inhibit and kill all microorganisms are *B. subtilis, E. aerogenes, S. aureus and E. coli.*

4.7.3 Allergy skin test

The volunteers were applied the product to the hand skin and have left for 15 minutes and then have been observed redness or inflammation of the skin. The tests found that 10 people do not have allergic reactions, itching, or redness of the skin after trying this product.

The herbal extract have antioxidants such as licopene, coenzyme Q, glutathione, selenium, zinc, bioflavonoids, polyphenols and others have been reported to possess substantial protective effects on UV-induced skin inflammation, oxidative stress and DNA damage (Poljsak *et al*., 2013). The extract reduces the harmful effects of free radicals and oxidative stress and improving prevention and treatment of photoaging and chronological aging of the skin. (Dragana *et al*., 2014) Factors influencing skin reactivity to an allergen include the amount of allergen injected; the number, degree of sensitization. (S. Dreborg and A. Frew, 2018)

CHAPTER 5
CONCLUSIONS AND RECOMMENDATIONS

Conclusions

The crude extract of *P. linteus* was isolated from 6 samples.

PL-EH had the highest Total Phenolic Contents and Total Flavonoid Contents were as follows: 610.92±7.611 mg GAE/g crude extract and 756.75±1.283 mg QE/g crude extract, respectively. PL-W had the highest total polysaccharide content of 167.96±0.061 mg GE/g crude extract.

PL-EH and PL-E1 have the best antioxidant activities. The IC_{50} was PL-EH at 2.04 µg/mL and PL-E1 at 2.07 µg/mL are antioxidant. The results showed that there was no statistically significant difference at 0.05 level.

PL-N was effective in inhibiting and killing bacteria *B. subtilis, E. aerogenes, S. aureus and E. coli* from MBC: MIC of 1, 2, 1 and 2, respectively.

PL-EH, PL-E1 and PL-N were analyzed for HPLC. Retention time may be defined as (2) -Epicatechin at 21.03 min, *p*-Coumaric acid at 24.20 min, Sinapic acid at 26.25 min, and Benzoic acid at 28.27 min, due to the same HPLC system. (Chen *et al*., 2001)

Analysis of encapsulation efficiency and releasing efficiency it was found that the % encapsulation efficiency of the *P. linteus* was extracted with 95% ethanol at 80°C (PL-EH) in nanochitosan, nanoliposome and nano-double particles was 96.19, 48.31 and 75.59 %, respesctively.

% Encapsulation efficiency of the *P. linteus* was extracted with 95% ethanol round 1 (PL-E1) in nanochitosan, nanoliposome and nano-double particles was 94.89, 32.20 and 61.98 %, respectively.

The encapsulation efficiency of the *P. linteus* was extracted with 1% ($(NH_4)_2C_2O_4$) at 95°C (PL-N) in nanochitosan, nanoliposome and nano-double particles was 95.15, 38.90 and 43.73 %, respesctively.

Non-accumulation release was found that all 3 extracts were encapsulated in the particles in the order of % RE that nanoliposome > nano-double particles > nanochitosan

Acumulate release was found that all 3 extracts were stored in the particles in the order of % RE that nanoliposome > nano-double particles > nanochitosan

Characterization of the *P. linteus* extracts and encapsulated nanoparticles. The *P. linteus* was extracted with 95% ethanol at 80℃ (PL-EH) in nanoliposome > nano-double particles > nanochitosan. The size of the nanoparticles with a size of 270.59 nm and spherical. And PL-EH encapsulate in nanoliposome > nano-double particles > nanochitosan was 213.1 nm, Z-Average: 369.4 nm and PI: 0.439 from Dynamic light scattering (DLS)

The activity of microorganism. It was found that the product was able to inhibit only one *Bacillus subtilis* and glycerol as an ingredient in the product of bacteria *Bacillus subtilis, Enterobacter aerogenes, Staphylococcus aureus and Escherichia coli.*

The product cannot kill bacteria *Bacillus subtilis, Enterobacter aerogenes, Staphylococcus aureus and Escherichia coli* and glycerol as ingredients in the product can kill all microorganisms are bacteria *Bacillus subtilis, Enterobacter aerogenes, Staphylococcus aureus and Escherichia coli.*

Finally, The analysis of the antioxidant properties of the antioxidant products have a 55%, Product control can have antioxidant 5.98% and *P. linteus* were extracted with 95% ethanol solvent at 80 ° C (PL-EH) at 0.5 mg/mL have antioxidant 29.46%

Recommendations

From this study, knowledge and understanding in the application of nanotechnology to the development of cosmeceutical products. Should be studied to test the stability of the product and test for human skin cell toxicity for more efficient products. This can be applied in the future. It also adds value to the product.

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APPENDICES

APPENDIX A

Preparation of the solvent used in the research

1. **1% (w/v) Aluminium oxalate ; $(NH_4)_2C_2O_4$**
    - Dissolve 1.0 grams of Aluminium oxalate in 50 milliliter of distilled water
    - And adjust the volume with distilled water to complete 100 milliliter

2. **1.25 M Sodium hydroxy/0.05% Sodium borohydride ; 1.25 M NaOH/0.05% $NaBH_4$**
    - Dissolve 5.0 grams of Sodium hydroxyl in 50 milliliter of distilled water
    - And adjust the volume with distilled water to complete 100 milliliter
    - Dissolve 0.05 grams of Sodium borohydride in 50 milliliter of distilled water
    - And adjust the volume with distilled water to complete 100 milliliter
    - 1.25 M Sodium hydroxyl 50 milliliter add in 0.05% Sodium borohydride 50 milliliter

3. **(1:1) Folin-Ciocaltue reagent**
    - 10 milliliter Folin-Ciocaltue reagent dilute with 10 milliliter distilled water

4. **7% (w/v) Sodium carbonate ; Na_2CO_3**
    - Dissolve 7.0 grams of Sodium carbonate in 50 milliliter of distilled water

- And adjust the volume with distilled water to complete 100 milliliter

5. 5% (w/v) Sodium nitrite ; $NaNO_2$

- Dissolve 5.0 grams of Sodium nitrite in 50 milliliter of distilled water
- And adjust the volume with distilled water to complete 100 milliliter

6. 10% (w/v) Aluminium chloride ; $AlCl_3$

- Dissolve 5.0 grams of Aluminium chloride in 50 milliliter of distilled water
- And adjust the volume with distilled water to complete 100 milliliter

7. 1M Sodium hydroxyl ; NaOH

- Dissolve 4.0 grams of Sodium hydroxyl in 50 milliliter of distilled water
- And adjust the volume with distilled water to complete 100 milliliter

8. 0.08 mM DPPH

- Dissolve 0.0197 grams of DPPH in 20 milliliter of methanol
- And adjust the volume with methanol to complete 50 milliliter

9. Nutrient Broth

- Dissolve 8 grams of Nutrient Broth in 500 milliliter of distilled water
- And adjust the volume with distilled water to complete 1000 milliliter
- Adjust the pH to 7.0±0.2

10. Nutrient Agar

- Dissolve 8 grams of Nutrient Broth in 500 milliliter of distilled water
- Add 15 grams of agar
- And adjust the volume with distilled water to complete 1000 milliliter
- Adjust the pH to 7.0±0.2

11. 0.18% (v/v) Resazurin

- Dilute 0.18 milliliter of Resazurin with distilled water to complete 100 milliliter

12. 0.05% (w/v) Chitosan, 1000 milliliter

- Dissolve 0.5 grams of chitosan in deionised water 200 milliliter (Stir-Overnight)
- Add 400 milliliter acetic acid (to complete 40% acetic acid) (Stir-Overnight)
- And adjust the volume with deionised water to complete 1000 milliliter

13. 3.0% (w/v) Lecithin

- Dissolve 3.0 grams of lecithin in ethanol 50 milliliter
- And 50 milliliter of chloroform to complete 100 milliliter

APPENDIX B

Data

Total Phenolic Contents

Table B1: Total Phenolic Contents of PL-EH, PL-E1 and PL-E2

Sample	OD 760 nm			TPC (mgGAE/gCrude)			Average	STDEV
	Repeat 1	Repeat 2	Repeat 3	Repeat 1	Repeat 2	Repeat 3		
PL-EH	0.477	0.468	0.466	619.581	607.891	605.293	610.922	7.6109
PL-E1	0.353	0.355	0.344	458.516	461.114	446.826	455.485	7.6109
PL-E2	0.308	0.311	0.308	400.065	403.962	400.065	401.364	2.2498

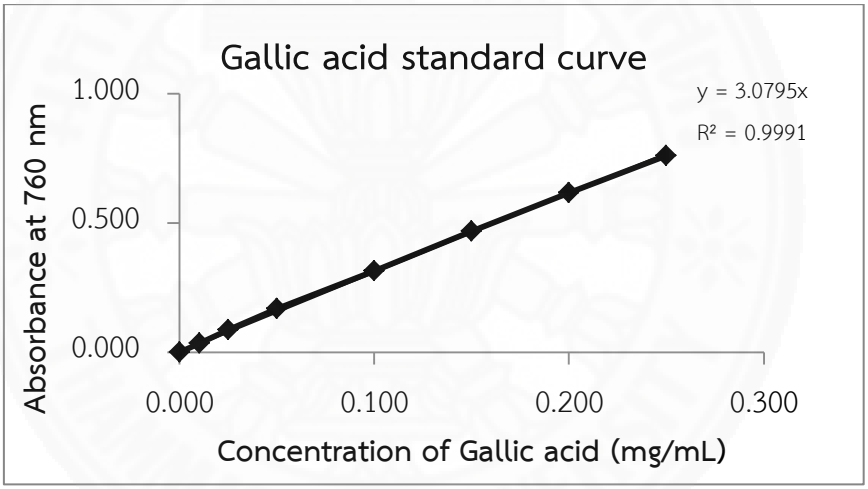


Figure B1: Gallic acid standard curve for calculated Total Phenolic Contents of PL-EH, PL-E1 and PL-E2

Table B2: Total Phenolic Contents of PL-W, PL-A and PL-N

Sample	OD 760 nm			TPC (mgGAE/gCrude)			Average	STDEV
	Repeat 1	Repeat 2	Repeat 3	Repeat 1	Repeat 2	Repeat 3		
PL-W	0.3418	0.3417	0.3418	122.535	122.499	122.535	122.523	0.0207
PL-A	0.0790	0.0802	0.0793	28.322	28.752	28.429	28.501	0.2239
PL-N	0.1292	0.1301	0.1300	46.318	46.641	46.605	46.521	0.1768

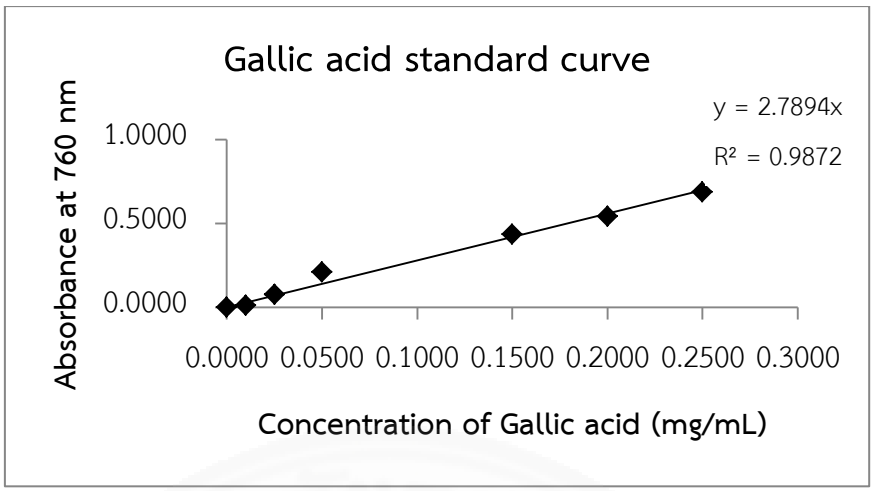


Figure B2: Gallic acid standard curve for calculated Total Phenolic Contents of PL-W, PL-A and PL-N

Total Flavonoid Contents

Table B3: Total Flavonoid Contents of PL-EH, PL-E1 and PL-E2

Sample	OD 510 nm			TFC (mgQE/gCrude)			Average	STDEV
	Repeat 1	Repeat 2	Repeat 3	Repeat 1	Repeat 2	Repeat 3		
PL-EH	0.589	0.59	0.591	755.467	756.750	758.032	756.750	1.2826
PL-E1	0.45	0.446	0.447	577.182	572.052	573.334	574.189	2.6700
PL-E2	0.414	0.413	0.416	531.008	529.725	533.573	531.435	1.9592

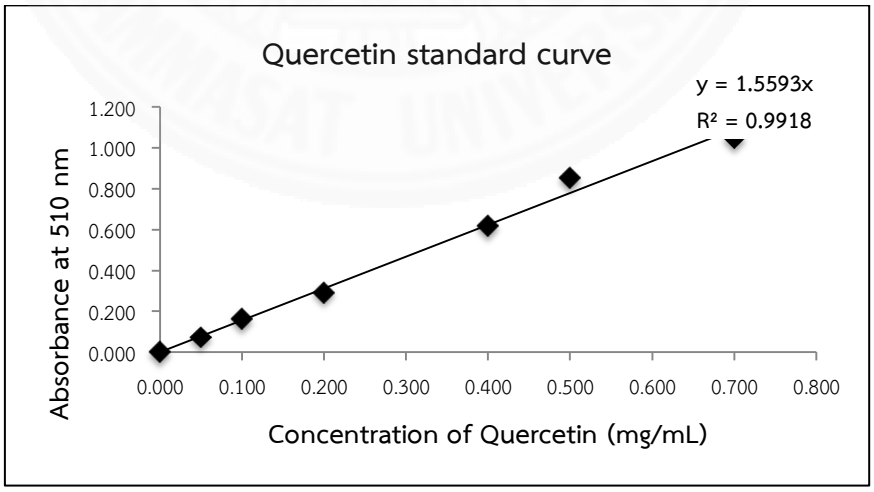


Figure B3: Quercetin standard curve for calculated Total Flavonoid Contents of PL-EH, PL-E1 and PL-E2

Table B4: Total Flavonoid Contents of PL-W, PL-A and PL-N

Sample	OD 510 nm			TFC (mgQE/gCrude)			Average	STDEV
	Repeat 1	Repeat 2	Repeat 3	Repeat 1	Repeat 2	Repeat 3		
PL-W	0.195	0.196	0.197	125.573	126.605	127.121	126.433	0.7886
PL-A	0.067	0.069	0.067	43.170	44.267	43.492	43.643	0.5638
PL-N	0.107	0.104	0.104	68.917	67.045	66.852	67.604	1.1404

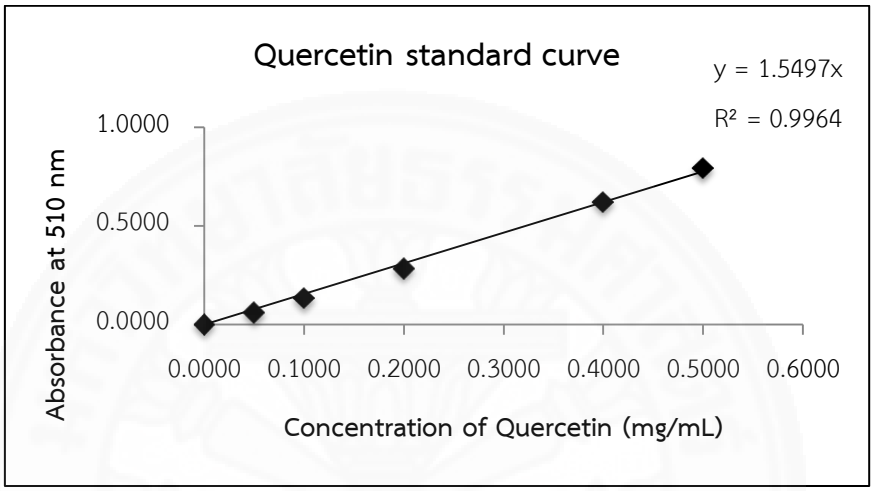


Figure B4: Quercetin standard curve for calculated Total Flavonoid Contents of PL-W, PL-A and PL-N

Total Polysaccharide Contents

Table B5: Total Polysaccharide Contents of PL-EH, PL-E1 and PL-E2

Sample	OD 490 nm			TPoC (mgGE/gcrude)			Average	STDEV
	Repeat 1	Repeat 2	Repeat 3	Repeat 1	Repeat 2	Repeat 3		
PL-EH	0.276	0.275	0.280	140.816	140.306	142.857	141.327	1.3499
PL-E1	0.214	0.215	0.214	109.184	109.694	109.184	109.354	0.2946
PL-E2	0.225	0.222	0.220	114.796	113.265	112.245	113.435	1.2840

Table B6: Total Polysaccharide Contents of PL-W, PL-A and PL-N

Sample	OD 490 nm			TPoC (mgGE/gcrude)			Average	STDEV
	Repeat 1	Repeat 2	Repeat 3	Repeat 1	Repeat 2	Repeat 3		
PL-W	1.361	1.361	1.360	167.988	168.000	167.889	167.959	0.0609
PL-A	0.242	0.243	0.242	29.852	29.938	29.852	29.881	0.0499
PL-N	0.210	0.210	0.210	25.926	25.963	25.926	25.938	0.0214

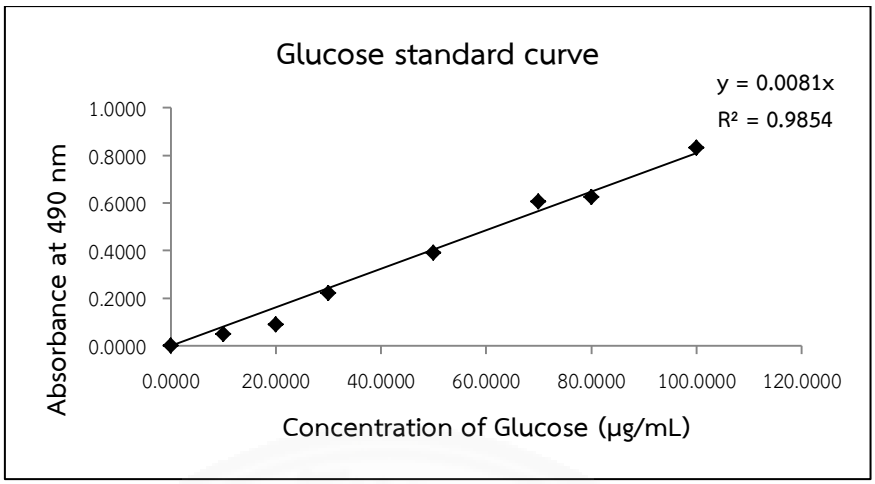


Figure B5: Glucose standard curve for calculated Total Polysaccharide Contents of PL-EH, PL-E1, PL-E2, PL-W, PL-A and PL-N

Antioxidant activities

Table B7: % Inhibition antioxidant activities of PL-EH

Concentration of PL-EH (mg/ml)	OD 515 nm			%DPPH			Average	STDEV
	Repeat 1	Repeat 2	Repeat 3	Repeat 1	Repeat 2	Repeat 3		
0.0005	0.192	0.196	0.197	16.52	14.78	14.35	15.22	1.1503
0.0014	0.146	0.150	0.148	36.52	34.78	35.65	35.65	0.8696
0.0032	0.072	0.070	0.069	68.70	69.57	70.00	69.42	0.6641
0.0045	0.045	0.047	0.047	80.43	79.57	79.57	79.86	0.5020
0.0068	0.041	0.041	0.040	82.17	82.17	82.61	82.32	0.2510

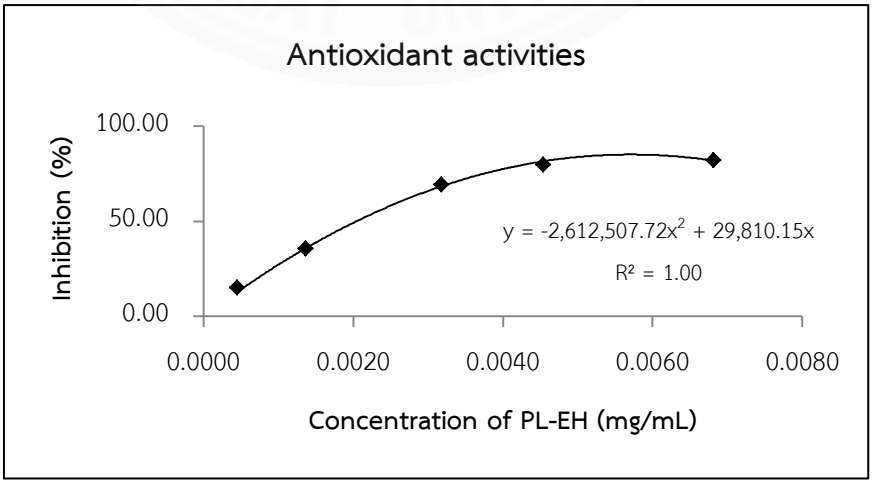


Figure B6: Antioxidant activities curve and equation for calculated for IC_{50} value of PL-EH

Table B8: % Inhibition antioxidant activities of PL-E1

Concentration of PL-E1 (mg/ml)	OD 515 nm			%DPPH			Average	STDEV
	Repeat 1	Repeat 2	Repeat 3	Repeat 1	Repeat 2	Repeat 3		
0.0001	0.217	0.228	0.229	6.87	2.15	1.72	3.58	2.8577
0.0002	0.216	0.212	0.212	7.30	9.01	9.01	8.44	0.9912
0.0010	0.185	0.193	0.190	20.60	17.17	18.45	18.74	1.7345
0.0029	0.056	0.053	0.055	75.97	77.25	76.39	76.54	0.6556

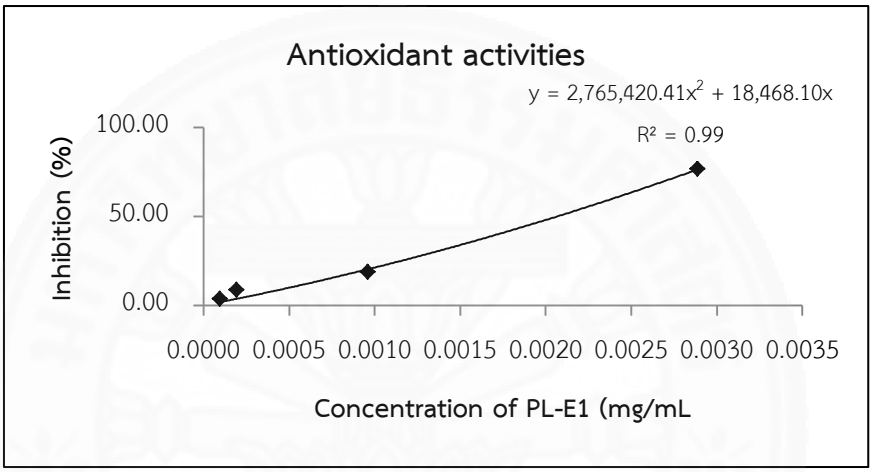


Figure B7: Antioxidant activities curve and equation for calculated for IC_{50} value of PL-E1

Table B9: % Inhibition antioxidant activities of PL-E2

Concentration Of PL-E2 (mg/ml)	OD 515 nm			%DPPH			Average	STDEV
	Repeat 1	Repeat 2	Repeat 3	Repeat 1	Repeat 2	Repeat 3		
0.0010	0.191	0.202	0.197	20.08	15.48	17.57	17.71	2.3044
0.0019	0.175	0.170	0.172	26.78	28.87	28.03	27.89	1.0530
0.0029	0.139	0.139	0.144	41.84	41.84	39.75	41.14	1.2078
0.0039	0.112	0.100	0.126	53.14	58.16	47.28	52.86	5.4447
0.0048	0.082	0.093	0.087	65.69	61.09	63.60	63.46	2.3044
0.0058	0.077	0.079	0.077	67.78	66.95	67.78	67.50	0.4831
0.0077	0.053	0.052	0.054	77.82	78.24	77.41	77.82	0.4184

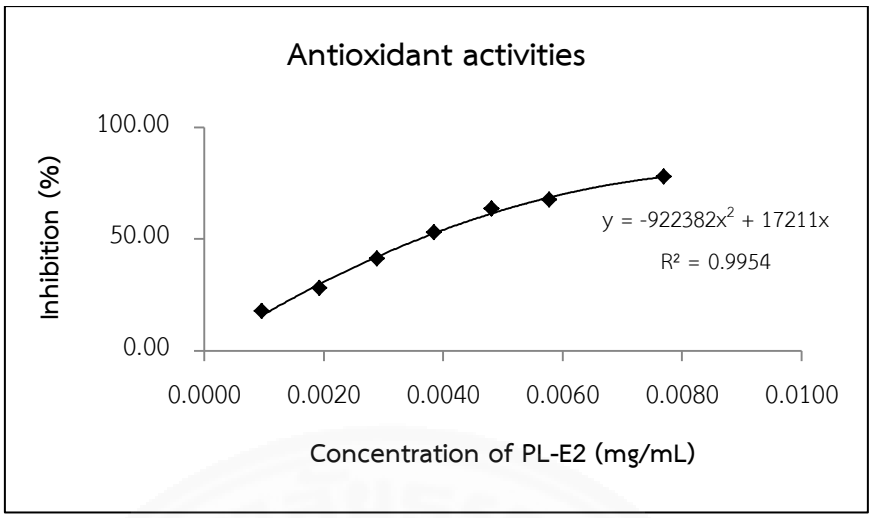


Figure B8: Antioxidant activities curve and equation for calculated for IC_{50} value of PL-E2

Antimicrobial activities

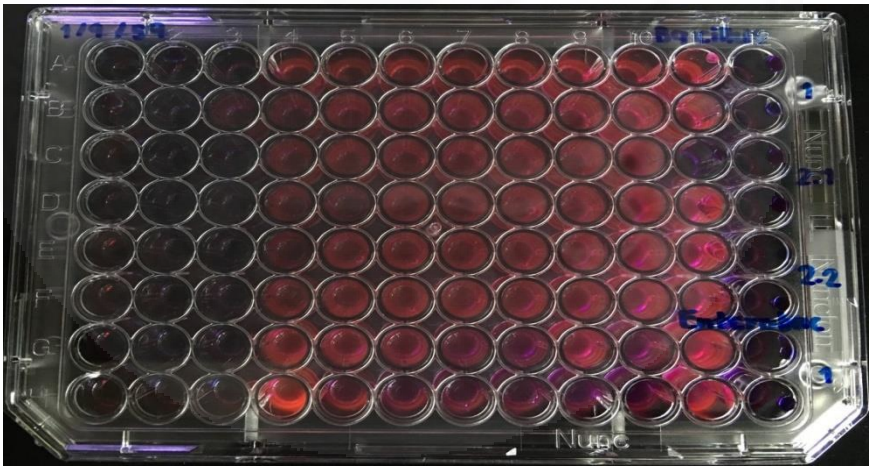


Figure B9-B11: Result of the activity of antimicrobial by Minimum Inhibitory Concentration method (PL-EH, PL-E1 and PL-E2)

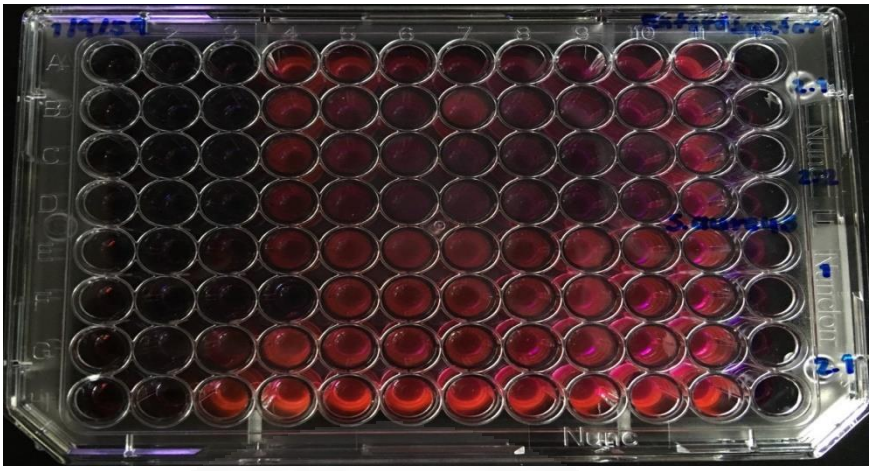


Figure B9-B11: Result of the activity of antimicrobial by Minimum Inhibitory Concentration method (PL-EH, PL-E1 and PL-E2)

Figure B9-B11: Result of the activity of antimicrobial by Minimum Inhibitory Concentration method; MIC (PL-EH, PL-E1 and PL-E2)

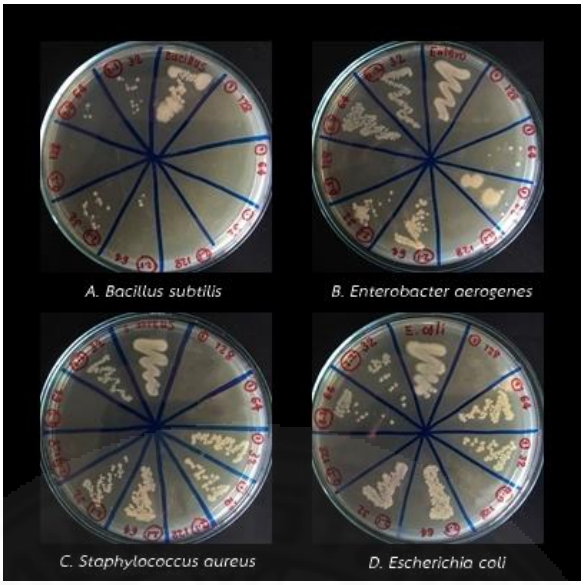


Figure B12: Result of the activity of antimicrobial by Minimum Bactericidal Concentration method; MBC (PL-EH, PL-E1 and PL-E2)

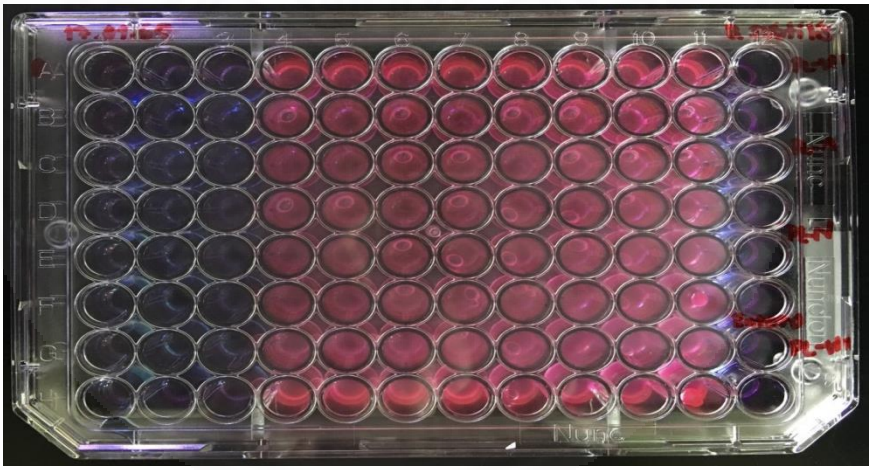


Figure B13-B15: Result of the activity of antimicrobial by Minimum Inhibitory Concentration method; MIC (PL-W, PL-A and PL-N)

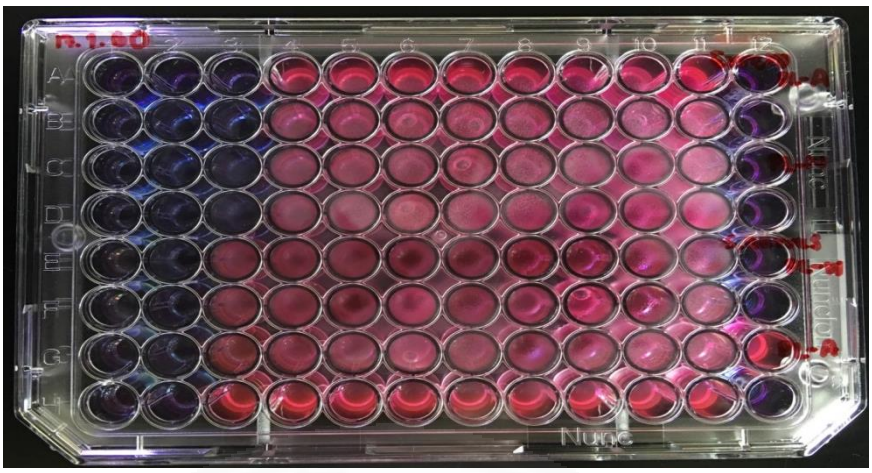


Figure B13-B15: Result of the activity of antimicrobial by Minimum Inhibitory Concentration method; MIC (PL-W, PL-A and PL-N)

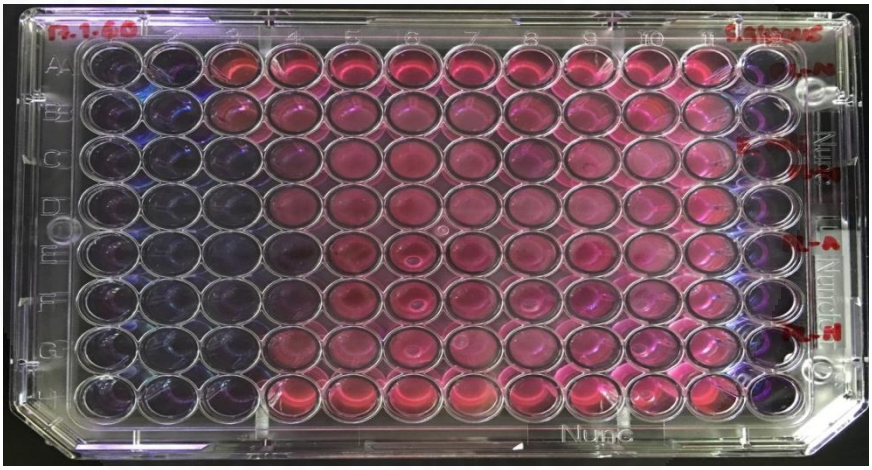


Figure B13-B15: Result of the activity of antimicrobial by Minimum Inhibitory Concentration method; MIC (PL-W, PL-A and PL-N)

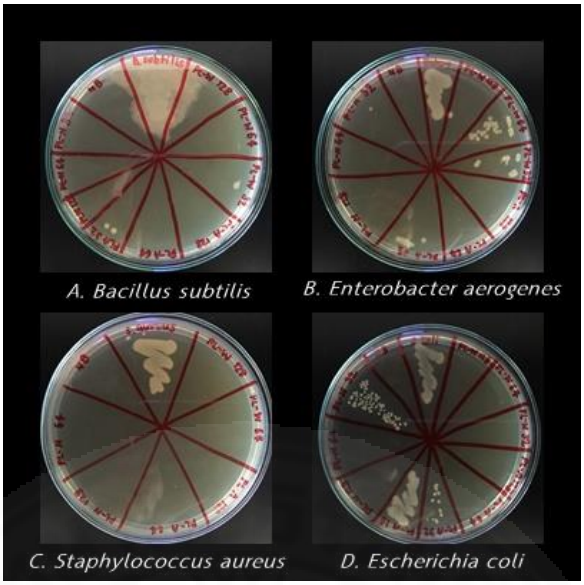


Figure B16: Result of the activity of antimicrobial by Minimum Bactericidal Concentration method; MBC (PL-W, PL-A and PL-N)

High-Performance Liquid Chromatography

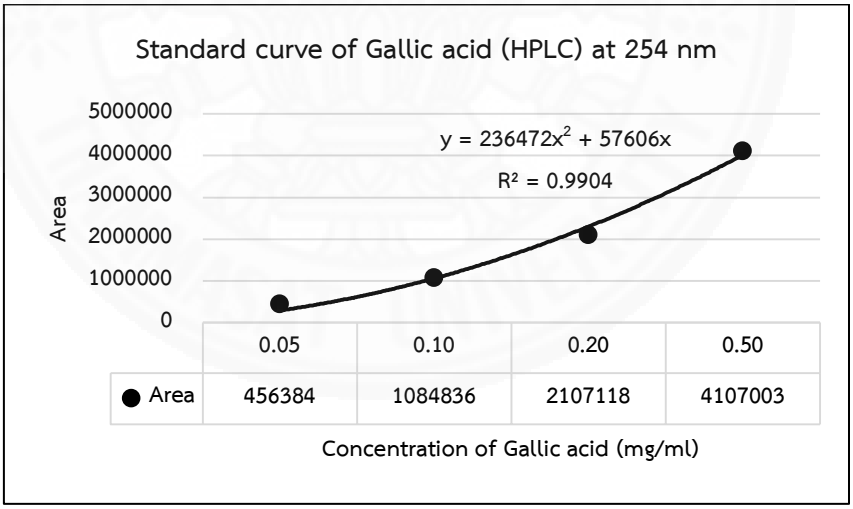


Figure B17: Standard curve of Gallic acid (HPLC) at 254 nm

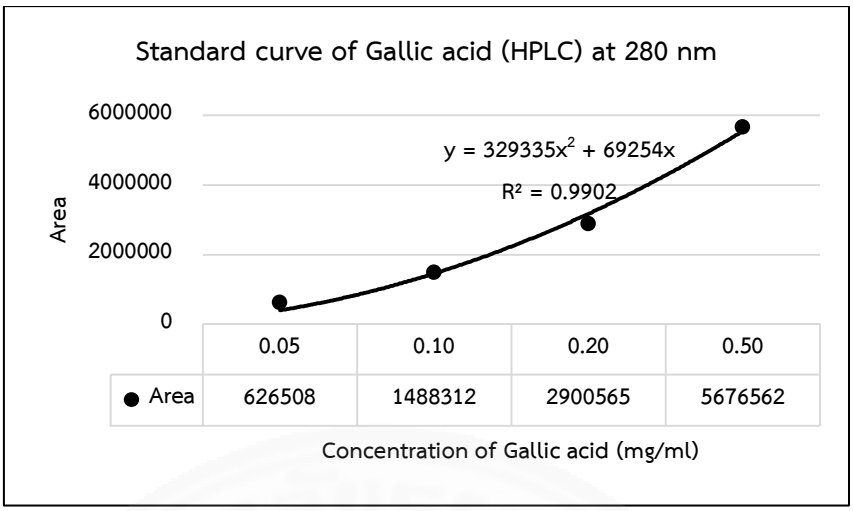


Figure B18: Standard curve of Gallic acid (HPLC) at 280 nm

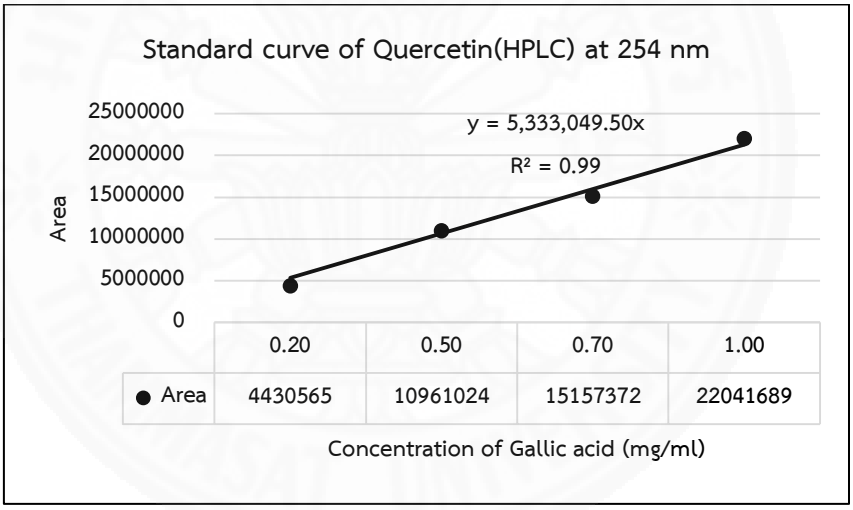


Figure B19: Standard curve of Quercetin (HPLC) at 254 nm

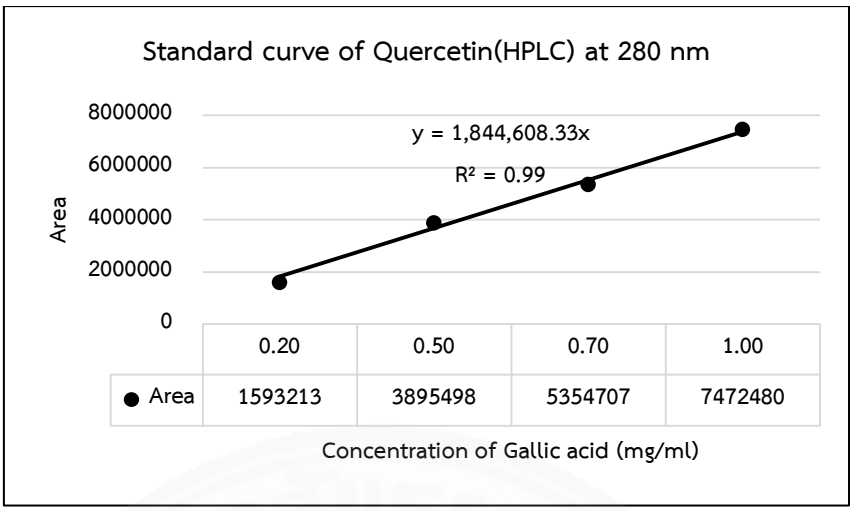


Figure B20: Standard curve of Quercetin (HPLC) at 280 nm

Encapsulation Efficiency

Table B10: Encapsulation Efficiency of PL-EH at various concentrations in chitosan nanoparticles

0.05% (w/v) Chitosan	PL-EH (mg/mL)	Not encapsulated (mgGAE/gCrude)	%Encapsulation Efficiency
	0.001	1591.850	
		2188.793	0
		994.906	
	0.010	1790.831	
		1333.174	0
		238.777	
	0.050	278.574	
		59.694	25.09
		119.389	
	0.100	103.4702	
		83.5721	62.54
		41.7861	
	0.500	39.000	
		33.031	87.1672
		6.367	
	1.000	30.046	
		4.975	93.4859
		4.776	
	1.500	51.204	
		6.367	90.4459
		0.796	

Table B11: Encapsulation Efficiency of PL-E1 at various concentrations in chitosan nanoparticles

0.05% (w/v) Chitosan	PL-E1 (mg/mL)	Not encapsulated (mgGAE/gCrude)	%Encapsulation Efficiency
	0.001	3581.662	
		5969.436	0
		3382.681	
	0.010	835.721	
		218.879	0
		437.759	
	0.050	87.552	
		51.735	53.69
		71.633	
	0.100	91.5314	
		29.8472	63.30
		45.7657	
	0.500	26.266	
		10.745	90.74
		5.174	
	1.000	46.164	
		10.148	87.16
		2.189	

Table B12: Encapsulation Efficiency of PL-N at various concentrations in chitosan nanoparticles

0.05% (w/v) Chitosan	PL-N (mg/mL)	Not encapsulated (mgGAE/gCrude)	%Encapsulation Efficiency
	0.001	3183.699	
		397.962	0
		0.000	
	0.010	119.389	
		0.000	0
		0.000	
	0.100	17.9083	
		0.000	61.51
		0.000	
	0.500	6.765	
		4.776	69.20
		2.786	
	1.000	4.975	
		1.393	83.32
		1.393	
	1.500	3.582	
		1.061	88.59
		0.663	
	2.000	2.487	
		0.895	91.02
		0.796	
	3.000	2.056	
		0.995	91.45
		0.929	
	4.000	1.642	
		2.935	83.85
		2.935	

Table B13: Encapsulation Efficiency of crude extract in nanochitosan, nano-double particles and nanoliposome

Nanoparticles	Crude extract (mg/mL)	Not encapsulated (mgGAE/gCrude)	%Encapsulation Efficiency
Nanochitosan	PL-EH at 1.00	39.796	96.19
		6.765	
Nano-double particles		186.430	75.59
		171.994	
Nanoliposome		377.397	61.51
		381.522	
Nanochitosan	PL-E1 at 0.50	42.184	94.89
		4.378	
Nano-double particles		187.895	61.98
		182.148	
Nanoliposome		377.397	32.20
		323.966	
Nanochitosan	PL-N at 3.00	3.980	95.15
		0.531	
Nano-double particles		20.026	43.73
		19.273	
Nanoliposome		21.448	38.90
		21.223	

Releasing Efficiency

Table B14: Releasing Efficiency of PL-EH in nanochitosan, nano-double particles and nanoliposome

Time	Non – Acuumulate Releasing (%)			Acuumulate Releasing (%)		
	Nano chitosan	Nano-double particles	Nano liposome	Nano chitosan	Nano-double particles	Nano liposome
0H	0.470	7.368	6.067	0.352	6.768	11.779
6H	0.763	8.396	22.811	0.920	8.525	29.046
12H	1.174	8.948	33.333	1.292	16.012	33.526
18H	0.587	10.311	16.378	1.722	26.117	37.604
24H (Day1)	0.157	11.014	11.830	1.585	34.889	50.010
Day2	0.039	10.919	6.204	1.644	38.123	50.669
Day3	0.137	4.869	1.118	1.663	39.578	50.552
Day4	0.078	1.657	0.000	1.703	39.752	52.673
Day5	0.039	0.527	0.000	1.703	39.676	52.667
Day6	0.000	0.452	0.000	1.742	39.720	52.861
Day7	0.000	0.201	0.000	1.722	39.591	52.829
Day8	0.000	0.000	0.000	1.800	39.671	52.478
Day9	0.000	0.000	0.000	1.820	39.519	52.524
Day10	0.000	0.000	0.000	1.820	39.588	52.789

Table B15: Releasing Efficiency of PL-E1 in nanochitosan, nano-double particles and nanoliposome

Time	Non – Acuumulate Releasing (%)			Acuumulate Releasing (%)		
	Nano chitosan	Nano-double particles	Nano liposome	Nano chitosan	Nano-double particles	Nano liposome
0H	0.562	6.375	9.255	0.664	6.746	10.371
6H	0.919	7.983	18.790	0.817	7.299	20.049
12H	1.890	9.362	35.584	1.634	13.938	35.584
18H	1.736	2.750	16.788	1.788	13.826	43.780
24H (Day1)	0.204	0.819	6.752	1.328	13.894	46.147
Day2	0.000	0.828	1.277	1.430	15.635	47.433
Day3	0.000	0.828	1.642	1.736	16.463	47.795
Day4	0.051	0.184	0.365	1.839	16.827	49.258
Day5	0.102	0.276	0.000	1.788	17.101	49.060
Day6	0.000	0.092	0.000	1.634	17.643	49.793
Day7	0.000	0.092	0.000	1.736	17.637	49.443
Day8	0.000	0.000	0.000	1.941	17.462	50.894
Day9	0.000	0.000	0.000	1.890	17.643	52.007
Day10	0.000	0.000	0.000	1.839	17.447	50.718

Table B16: Releasing Efficiency of PL-N in nanochitosan, nano-double particles and nanoliposome

Time	Non – Acuumulate Releasing (%)			Acuumulate Releasing (%)		
	Nano chitosan	Nano-double particles	Nano liposome	Nano chitosan	Nano-double particles	Nano liposome
0H	0.000	5.745	7.925	0.464	0.953	6.122
6H	0.155	17.390	13.878	0.000	15.650	10.612
12H	0.000	22.283	13.265	1.238	18.692	11.905
18H	0.000	0.000	13.605	1.393	18.606	13.435
24H (Day1)	0.000	0.000	5.782	1.238	23.352	14.388
Day2	0.000	0.000	1.701	1.084	46.891	15.510
Day3	0.000	0.000	0.000	1.393	48.429	20.578
Day4	0.000	0.000	0.000	1.393	49.569	30.136
Day5	0.155	0.000	0.000	1.084	46.154	45.612
Day6	0.000	0.000	0.000	1.393	47.173	49.388
Day7	0.000	0.000	0.000	1.548	48.590	54.320
Day8	0.000	0.000	0.000	2.322	47.234	61.701
Day9	0.000	0.000	0.000	1.393	47.481	63.265
Day10	0.000	0.000	0.000	1.238	49.392	63.469

APPENDIX C

Statistical Analysis

ANOVA

Total Phenolic Contents

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	892681.524	5	178536.305	8853.342	.000
Within Groups	241.992	12	20.166		
Total	892923.516	17			

Figure C1: The Total Phenolic Contents in one way ANOVA data that has least one pair difference is significant at the 0.05 level.

ANOVA

Total Flavonoid Content

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1416627.070	5	283325.414	114452.744	.000
Within Groups	29.706	12	2.475		
Total	1416656.776	17			

Figure C2: The Total Flavonoid Contents in one way ANOVA data that has least one pair difference is significant at the 0.05 level.

ANOVA

Total Polysaccharide Contents

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	50915.163	5	10183.033	17142.079	.000
Within Groups	7.128	12	.594		
Total	50922.292	17			

Figure C3: The Total Polysaccharide Contents in one way ANOVA data that has least one pair difference is significant at the 0.05 level

Multiple Comparisons

Dependent Variable: TPC

LSD

(I) CrudeSample	(J) CrudeSample	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
PL_EH	PL_E1	155.436489*	3.666604	.000	147.44764	163.42533
	PL_E2	209.557829*	3.666604	.000	201.56898	217.54667
	PL_W	488.398324*	3.666604	.000	480.40948	496.38717
	PL_A	582.420934*	3.666604	.000	574.43209	590.40978
	PL_N	564.400332*	3.666604	.000	556.41149	572.38918
PL_E1	PL_EH	-155.436489*	3.666604	.000	-163.42533	-147.44764
	PL_E2	54.121340*	3.666604	.000	46.13250	62.11018
	PL_W	332.961836*	3.666604	.000	324.97299	340.95068
	PL_A	426.984445*	3.666604	.000	418.99560	434.97329
	PL_N	408.963843*	3.666604	.000	400.97500	416.95269
PL_E2	PL_EH	-209.557829*	3.666604	.000	-217.54667	-201.56898
	PL_E1	-54.121340*	3.666604	.000	-62.11018	-46.13250
	PL_W	278.840496*	3.666604	.000	270.85165	286.82934
	PL_A	372.863105*	3.666604	.000	364.87426	380.85195
	PL_N	354.842503*	3.666604	.000	346.85366	362.83135
PL_W	PL_EH	-488.398324*	3.666604	.000	-496.38717	-480.40948
	PL_E1	-332.961836*	3.666604	.000	-340.95068	-324.97299
	PL_E2	-278.840496*	3.666604	.000	-286.82934	-270.85165
	PL_A	94.022609*	3.666604	.000	86.03377	102.01145
	PL_N	76.002008*	3.666604	.000	68.01316	83.99085
PL_A	PL_EH	-582.420934*	3.666604	.000	-590.40978	-574.43209
	PL_E1	-426.984445*	3.666604	.000	-434.97329	-418.99560
	PL_E2	-372.863105*	3.666604	.000	-380.85195	-364.87426
	PL_W	-94.022609*	3.666604	.000	-102.01145	-86.03377
	PL_N	-18.020602*	3.666604	.000	-26.00945	-10.03176
PL_N	PL_EH	-564.400332*	3.666604	.000	-572.38918	-556.41149
	PL_E1	-408.963843*	3.666604	.000	-416.95269	-400.97500
	PL_E2	-354.842503*	3.666604	.000	-362.83135	-346.85366
	PL_W	-76.002008*	3.666604	.000	-83.99085	-68.01316
	PL_A	18.020602*	3.666604	.000	10.03176	26.00945

*. The mean difference is significant at the 0.05 level.

Figure C4: The Total Phenolic Contents in LSD data that all pair difference is significant at the 0.05 level.

Multiple Comparisons

Dependent Variable: TFC

LSD

(I) CrudeSample	(J) CrudeSample	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
PL_EH	PL_E1	182.560551*	1.284648	.000	179.76154	185.35956
	PL_E2	225.314778*	1.284648	.000	222.51577	228.11378
	PL_W	630.316751*	1.284648	.000	627.51774	633.11576
	PL_A	713.106968*	1.284648	.000	710.30796	715.90597
	PL_N	689.145341*	1.284648	.000	686.34633	691.94435
PL_E1	PL_EH	-182.560551*	1.284648	.000	-185.35956	-179.76154
	PL_E2	42.754227*	1.284648	.000	39.95522	45.55323
	PL_W	447.756200*	1.284648	.000	444.95719	450.55521
	PL_A	530.546417*	1.284648	.000	527.74741	533.34542
	PL_N	506.584790*	1.284648	.000	503.78578	509.38380
PL_E2	PL_EH	-225.314778*	1.284648	.000	-228.11378	-222.51577
	PL_E1	-42.754227*	1.284648	.000	-45.55323	-39.95522
	PL_W	405.001973*	1.284648	.000	402.20297	407.80098
	PL_A	487.792190*	1.284648	.000	484.99318	490.59120
	PL_N	463.830563*	1.284648	.000	461.03156	466.62957
PL_W	PL_EH	-630.316751*	1.284648	.000	-633.11576	-627.51774
	PL_E1	-447.756200*	1.284648	.000	-450.55521	-444.95719
	PL_E2	-405.001973*	1.284648	.000	-407.80098	-402.20297
	PL_A	82.790217*	1.284648	.000	79.99121	85.58922
	PL_N	58.828590*	1.284648	.000	56.02958	61.62760
PL_A	PL_EH	-713.106968*	1.284648	.000	-715.90597	-710.30796
	PL_E1	-530.546417*	1.284648	.000	-533.34542	-527.74741
	PL_E2	-487.792190*	1.284648	.000	-490.59120	-484.99318
	PL_W	-82.790217*	1.284648	.000	-85.58922	-79.99121
	PL_N	-23.961627*	1.284648	.000	-26.76063	-21.16262
PL_N	PL_EH	-689.145341*	1.284648	.000	-691.94435	-686.34633
	PL_E1	-506.584790*	1.284648	.000	-509.38380	-503.78578
	PL_E2	-463.830563*	1.284648	.000	-466.62957	-461.03156
	PL_W	-58.828590*	1.284648	.000	-61.62760	-56.02958
	PL_A	23.961627*	1.284648	.000	21.16262	26.76063

*. The mean difference is significant at the 0.05 level.

Figure C5: The Total Flavonoid Contents in LSD data that all pair difference is significant at the 0.05 level.

Multiple Comparisons

Dependent Variable: TPoC

LSD

(I) CrudeSample	(J) CrudeSample	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
PL_EH	PL_E1	31.972333*	.629305	.000	30.60120	33.34347
	PL_E2	27.891000*	.629305	.000	26.51986	29.26214
	PL_W	-26.632667*	.629305	.000	-28.00380	-25.26153
	PL_A	111.445667*	.629305	.000	110.07453	112.81680
	PL_N	115.388000*	.629305	.000	114.01686	116.75914
PL_E1	PL_EH	-31.972333*	.629305	.000	-33.34347	-30.60120
	PL_E2	-4.081333*	.629305	.000	-5.45247	-2.71020
	PL_W	-58.605000*	.629305	.000	-59.97614	-57.23386
	PL_A	79.473333*	.629305	.000	78.10220	80.84447
	PL_N	83.415667*	.629305	.000	82.04453	84.78680
PL_E2	PL_EH	-27.891000*	.629305	.000	-29.26214	-26.51986
	PL_E1	4.081333*	.629305	.000	2.71020	5.45247
	PL_W	-54.523667*	.629305	.000	-55.89480	-53.15253
	PL_A	83.554667*	.629305	.000	82.18353	84.92580
	PL_N	87.497000*	.629305	.000	86.12586	88.86814
PL_W	PL_EH	26.632667*	.629305	.000	25.26153	28.00380
	PL_E1	58.605000*	.629305	.000	57.23386	59.97614
	PL_E2	54.523667*	.629305	.000	53.15253	55.89480
	PL_A	138.078333*	.629305	.000	136.70720	139.44947
	PL_N	142.020667*	.629305	.000	140.64953	143.39180
PL_A	PL_EH	-111.445667*	.629305	.000	-112.81680	-110.07453
	PL_E1	-79.473333*	.629305	.000	-80.84447	-78.10220
	PL_E2	-83.554667*	.629305	.000	-84.92580	-82.18353
	PL_W	-138.078333*	.629305	.000	-139.44947	-136.70720
	PL_N	3.942333*	.629305	.000	2.57120	5.31347
PL_N	PL_EH	-115.388000*	.629305	.000	-116.75914	-114.01686
	PL_E1	-83.415667*	.629305	.000	-84.78680	-82.04453
	PL_E2	-87.497000*	.629305	.000	-88.86814	-86.12586
	PL_W	-142.020667*	.629305	.000	-143.39180	-140.64953
	PL_A	-3.942333*	.629305	.000	-5.31347	-2.57120

*. The mean difference is significant at the 0.05 level.

Figure C6: The Total Polysaccharide Contents in LSD data that all pair difference is significant at the 0.05 level.

ANOVA

Concentration

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.000	2	.000	2678.087	.000
Within Groups	.000	6	.000		
Total	.000	8			

Figure C7: The antioxidant in one way ANOVA data that has least one pair difference is significant at the 0.05 level.

Multiple Comparisons

Dependent Variable: Concentration

LSD

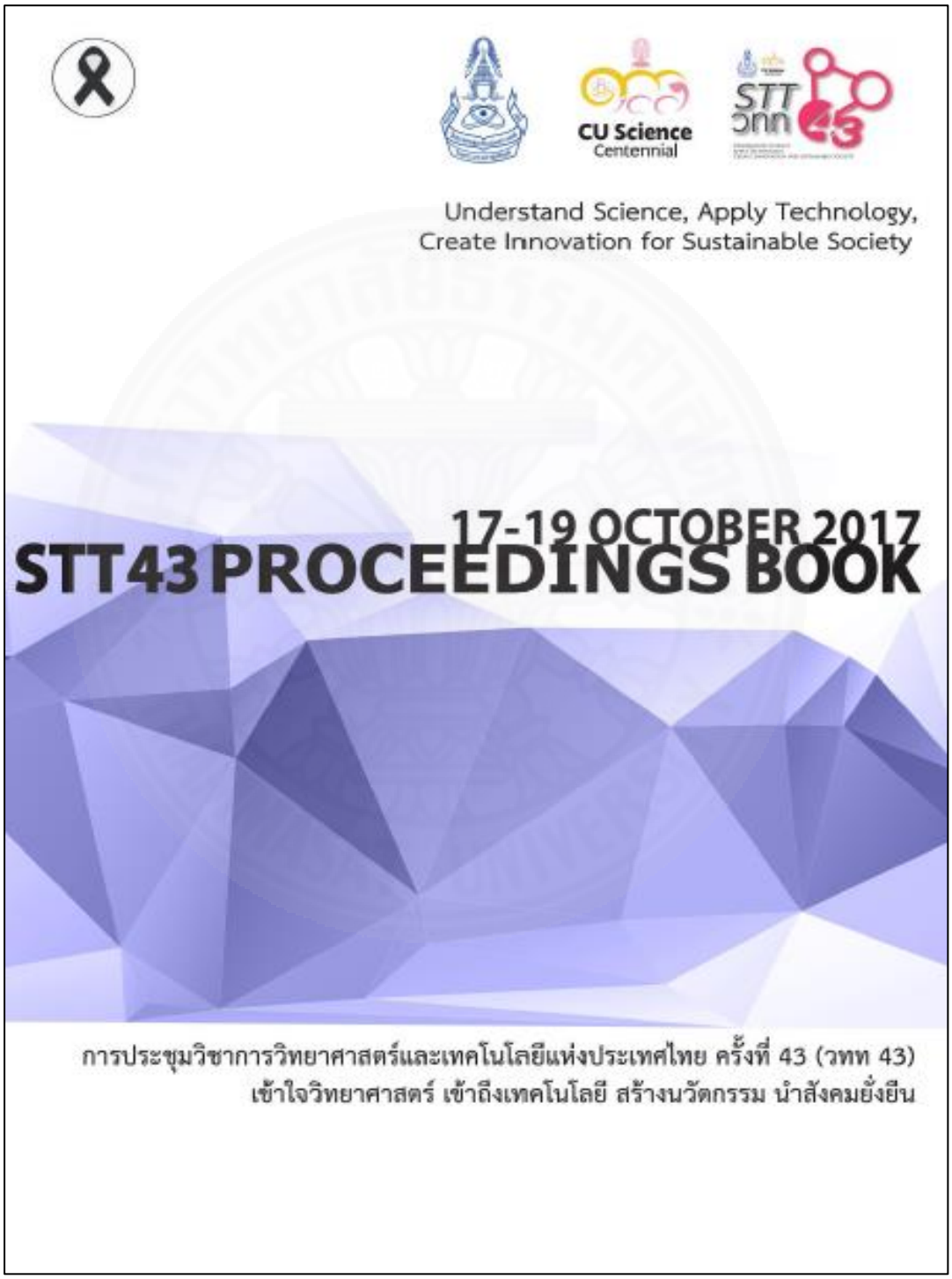
(I) CrudeSample	(J) CrudeSample	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
PL-EH	PL-E1	-.000022680	.000025452	.407	-.00008496	.00003960
	PL-E2	-.001624417*	.000025452	.000	-.00168670	-.00156214
PL-E1	PL-EH	.000022680	.000025452	.407	-.00003960	.00008496
	PL-E2	-.001601737*	.000025452	.000	-.00166402	-.00153946
PL-E2	PL-EH	.001624417*	.000025452	.000	.00156214	.00168670
	PL-E1	.001601737*	.000025452	.000	.00153946	.00166402

*. The mean difference is significant at the 0.05 level.

Figure C8: The antioxidant in LSD data that one pair difference is significant at the 0.05 level.

APPENDIX D

Publications



PROCEEDINGS BOOK

The 43rd Congress on Science and Technology of Thailand (STT43)

"Understand Science, Apply Technology, Create Innovation for Sustainable Society"

October 17-19, 2017
Venue: Chaloem Rajakumari 60 Building
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Organized by:
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Chulalongkorn University

A_018_PF: THE ANTIOXIDANT AND ANTIBACTERIAL ACTIVITIES OF *Phellinus linteus* CRUDE EXTRACT

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Abstract: This study investigated the extraction of *Phellinus linteus* using different extraction methods and solvents. Determination of total phenolic content using Folin-Ciocalteu reagent method, total flavonoid content using the colorimetric method and total polysaccharide content using phenol-sulfuric acid method. Antioxidant activity with an IC_{50} value using DPPH assay and antimicrobial activity using MIC and MBC assay. PL- E1 had the highest total phenolic content and total flavonoid content 455.4852 ± 0.006 mg GAE/g of dried sample and 574.1893 ± 0.002 mgQUE/g of dried sample, respectively and had the lowest concentration of 1.931 µg/mL to decrease 50% of antioxidant. PL-W has the highest total polysaccharide content 694.1156 ± 0.0005 mgGlucose/g of dried sample. The results of MIC and MBC showed that *P. linteus* was able to inhibit and slaughter *B. subtilis, E. aeruges, S. aureus* and *E. coli.*

Introduction: Extracts from nature are interested. Medicinal plants have been widely use. Generally focused on food, medicine and cosmetics mostly. At present, showed a lot of diseases can be treated or symptom relief by using natural extracts. Common diseases and the use of natural extracts to treat diseases such as diabetes, heart disease and cancer, etc. [1]. Oxidative stress, induced by free radicals, is believed to cause a variety of diseases, including cancer, heart disease, atherosclerosis, inflammation, carcinogenesis, Parkinson's and Alzheimer's diseases, and aging [2]. It has been reported that many of the antioxidants found are phenols and flavonoids. *Phellinus linteus* in the *Phellinus* genus and *Hymenochaetaceae* family. Research on Mushrooms show that *P. linteus* contains important substance. Polysaccharides represent a major class of bioactive constituents of *P. linteus* contributing to the health effects and pharmacological activities such as anticancer and antioxidation [3].

In this research, to study the extracted substances from *P. linteus* mushroom and to investigate the various activities in term of medical, further, applications.

Methodology:

Materials and chemicals: The fruiting body of *P. linteus* crushed to a powder and storage without humidity. All chemicals and solvents were of laboratory grade and used without further purification.

Preparation of P. linteus extract: P. linteus powder was extracted with 95% ethanol and shaking at a speed of 120 rpm at 25°C for 18 h. Then, filter with filter paper. The residue was extracted with 95% ethanol again. The aqueous extract was evaporating solvent by rotary evaporator was PL-E1 and PL-E2.

And another extract was treated with petroleum ether twice for 6 h to remove lipids and pigments. Then, extracted by hot water, 1% $(NH4)_2C_2O_4$, and 1.25 M NaOH/0.05% $NaBH_4$ extraction. The pretreated mycelium powder was extracted with distilled water at 95•C twice for 8 h. After centrifuged, the residue was further extracted with 1% $(NH_4)_2C_2O_4$ solution (w/v) at 95•C twice for 8 h. The residue obtained from 1% $(NH_4)_2C_2O_4$ extraction procedure was extracted with 1.25 M NaOH/0.05%$NaBH_4$ aqueous solution at room temperature twice for 3 h. Then the aqueous extracts were concentrated and precipitated with 4 volumes of 95% ethanol at 4°C overnight, followed by centrifugation (5000 rpm, 10 min) was PL-W, PL-A and PL-N. [4]

Determinations of the P. linteus extract compounds

Total phenolic content using Folin-Ciocalteu reagent method.: The total phenolic content was determined by the Folin–Ciocalteu method. 125 µL of crude extract were made up to 500 µL with distilled water, mixed thoroughly with 125 µL of Folin–Ciocalteu reagent for 6 min, followed by the addition of 1250 µL of 7% (w/v) sodium carbonate. The mixture was allowed to stand for a further 90 min and absorbance was measured at 760 nm. The total phenolic content was calculated from the calibration curve, and the results were expressed as mg of GAE/g of dried sample. [5]

Total flavonoid content using the colorimetric method: The total flavonoid content of crude extract was determined by the colorimetric method. 250 µL of crude extract were made up to 1250 µL with distilled water mixed thoroughly with 75 µL of 5% sodium nitrite solution for 5 min, followed by the addition of 150 µL of 10% aluminium chloride solution for 6 min. Then, 500 µL of 1 mol/L sodium hydroxide solution were added, 275 µL with double-distilled water and absorbance was measured at 510 nm. The total flavonoid content was calculated from the calibration curve, and the results were expressed as mg of QuE/g of dried sample. [5]

Total polysaccharide content using phenol-sulfuric acid metho: The total polysaccharide content of crude extract was determined by the phenol-sulfuric acid method. 600 µL of crude extract were made up to 500 µL with 5% phenol solution, mixed thoroughly with 3 mL of sulfuric acid for 10 min, stand for 30 min and absorbance was measured at 490 nm. The total polysaccharide content was calculated from the calibration curve, and the results were expressed as mg of glucose/g of dried sample.

Analysis activities of the Phellinus linteus extract compounds

The activity of antioxidant [6]: The activity of antioxidant using DPPH assay with an IC_{50} value. Crude extract were diluted with methanol to create solutions of varying concentration. Mixed with 2 mL of DPPH reagent, stand for 30 min and absorbance was measured at 517 nm. % DPPH inhibition was calculated by equation (1) below,

$$(1)\ \%\ \text{DPPH inhibition} = \left(\frac{A_B - A_S}{A_B}\right) \times 100$$

While, A_B = Absorbance of Blank
A_S = Absorbance of Sample

The concentration on preparing the above to create relationships with the percentage of inhibition by DPPH. Then, select linear in the range of 50 percent inhibition.

The activity of antimicrobial [7]: The activity of antimicrobial using Minimum Inhibitory Concentration (MIC).

All culture (*Bacillus subtilis*, *Enterobacter aerogenes*, *Staphylococcus aureus* and *Escherichia coli*) are adjusted with saline to give a turbidity equivalent to the McFarland 0.5 standard using 600 nm by spectrophotometer. The absorbance will be 0.08–0.10. Then, inoculum prepared above is diluted at ratio 1:200 in nutrient broth.

50 µl of nutrient broth was added to each well of the sterile 96-well microplates. 50 µl of sample were added to each well of the first row. Then 50 µl were removed from the first row and mixed five times with the broth in the corresponding well of the next row to tenth row. 50 µl of each bacterial suspension were added to each well except for twelfth row (control). The microplates were sealed with parafilm and incubated at 37 °C for 24 h. 10 µl of 0.18% resazurin solution were added to each well of microplates. Incubated at 37 °C for 3 h. If a result is blue or purple represents the sample can inhibit the bacteria but if the result is pink that can not inhibit the bacteria.

The activity of antimicrobial using Minimum Bactericidal Concentration (MBC) method.

Steak microorganisms from each well that show a result is a blue or purple on a plate with nutrient agar. Incubated at 37 °C for 24 h. The MBC was recorded as the lowest concentration without bacterial growth.

Results and Discussion:

Total Phenolic, flavonoid and polysaccharide content: The total phenolic content, total flavonoid content and total polysaccharide content of *P. linteus* mushroom calculated from the calibration curve.

Table 1 show that total phenolic content , total flavonoid content and total polysaccharide content of *P. linteus*.PL-E1 had the highest total phenolic content of 455.4852±0.006 mgGAE/g of dried sample and total flavonoid content of 574.1893±0.002 mgQuE/g of dried sample. PL-W had the highest total polysaccharide content of 694.1156±0.0005 mgGlucose/g of dried sample.

Table 1: Show the content of phenolic, flavonoid and polysaccharide in *P. linteus*

P. linteus extraction	Phenolic Content (mg GAE/g of dried sample)	Flavonoid Content (mg QuE/g of dried sample)	Polysaccharide Content (mg Glucose/g of dried sample)
95% Ethanol, 25°C_1 (PL-E1)	455.4852±0.006	574.1893±0.002	109.3537±0.001
95% Ethanol, 25°C_2 (PL-E2)	401.3639±0.002	531.4350±0.002	113.4354±0.003
Hot water, 95°C (PL-W)	122.5234±0.001	126.4331±0.001	694.1156±0.001
1%$(NH_4)_2C_2O_4$, 95°C (PL-A)	28.5008±0.001	43.6429±0.001	123.4864±0.000
1.25M NaOH/0.05%$NaBH_4$, 25°C (PL-N)	46.5214±0.001	67.6045±0.002	107.1939±0.000

The activity of antioxidant

Table 2: Show the antioxidant activity by DPPH assay in *P. linteus*

P. linteus extraction	Inhibition of free radicals
95% Ethanol_1 (PL-E1)	IC_{50} = 1.931 (µg/mL)
95% Ethanol_2 (PL-E2)	IC_{50} = 4.087 (µg/mL)
Hot water, 95°C (PL-W)	IC_{50} = 0.529 (mg/mL)
1%$(NH_4)_2C_2O_4$, 95°C (PL-A)	0.30 mg/mL = 20.49 ± 0.002(%)
1.25M NaOH/0.05%$NaBH_4$, 25°C (PL-N)	0.30 mg/mL = 28.61 ± 0.001(%)

Table 2 show that IC_{50} of antioxidant calculated from the equation (1). PL-E1 was the best crude extract to decrease 50% of antioxidant at the concentration 1.931 (µg/mL)

It has been reported that the presence of antioxidants such as phenolics, flavonoids, tannins and proanthocyanidins in plants [8]. Therefore, the results from Table 1 showed that PL-E1 had the highest total phenolic content (455.4852±0.006 mgGAE/g of dried sample) and total flavonoid content (574.1893±0.002 mgQuE/g of dried sample) which corresponds to IC_{50} value of antioxidant activities (Table 2) to decrease 50% of antioxidant at the concentration 1.931 (µg/mL)

The relationship between the antioxidant activities on total phenolic and flavonoid content in PL-E1, PL-E2, PL-W, PL-A and PL-N, thus reflecting that the active antioxidants may be phenolic and flavonoids.

The activity of antimicrobial by MIC and MBC method

Table 3 : The activity of antimicrobial by Minimum inhibitory concentration method

P. linteus extraction (µg/mL)	*B. subtilis*	*E. aerogenes*	*S. aureus*	*E. coli*
95% Ethanol_1 (PL-E1)	32	32	32	32
95% Ethanol_2 (PL-E2)	32	32	32	32
Hot water, 95°C (PL-W)	32	32	64	32
1%$(NH_4)_2C_2O_4$, 95°C (PL-A)	32	32	64	16
1.25M NaOH/0.05%$NaBH_4$, 25°C (PL-N)	32	32	64	32

Based on the Minimum Inhibitory Concentration (MIC) antimicrobial assay to determine the lowest concentration of *P. linteus* with antimicrobial activity, (Table 3) the lowest concentration of PL-

E1, PL-E2, PL-W, PL-A and PL-N that are resistant to *B. subtilis* and *E. aerogenes* have a concentration of 32 (µg/mL). The lowest concentration of PL-E1and PL-E2 that are resistant to *S. aureus* have a concentration of 32 (µg/mL). And the lowest concentration of PL-A that are resistant to *E. coli* have a concentration of 16 (µg/mL).

And the result of Minimum Bactericidal Concentration method (MBC) to determine the lowest concentration of *P. linteus* that killing bacterial. (Table 4) The lowest concentration of PL-N killing *B. subtilis,* has a concentration of 32 (µg/mL). The lowest concentration of PL-A and PL-N killing *E. aerogenes* has a concentration of 64 (µg/mL). The lowest concentration of PL-E2, Pl-W, PL-A and PL-N killing *S. aureus* has a concentration of 64 (µg/mL). The lowest concentration of Pl-W killing *E. coli* has a concentration of 32 (µg/mL).

4 : The activity of antimicrobial by Minimum bactericidal concentration method

***P. linteus* extraction** (µg/mL)	***B. subtilis***	***E. aerogenes***	***S. aureus***	***E. coli***
95% Ethanol_1 (PL-E1)	128	128	128	128
95% Ethanol_2 (PL-E2)	128	128	64	128
Hot water, 95°C (PL-W)	64	128	64	32
1%$(NH_4)_2C_2O_4$, 95°C (PL-A)	64	64	64	64
1.25M NaOH/0.05%$NaBH_4$, 25°C (PL-N)	32	64	64	64

Conclusion: The results of the *P. linteus* extract compounds, antioxidant activity and bacterial in *P. linteus* showed that PL-E1 had the highest total phenolic content and total flavonoid content 455.4852 ± 0.006 mg GAE/g of dried sample and 574.1893 ± 0.002 mgQuE/g of dried sample, respectively. Which varied according to antioxidant activity. PL-E1 was lowest in concentration of 1.931 µg/mL. to inhibit free radicals at 50 percent (IC_{50}). Furthermore, PL-W has the highest total polysaccharide content 694.1156 ± 0.0005 mg Glucose /g of dried sample.

As a result of the minimum inhibitory concentration (MIC) antimicrobial assay and minimal bactericidal concentration method (MBC), P. linteus was able to inhibit and slaughter *B. subtilis E. aeruges S. aureus* and *E. coli.*

All of these results. It was found that natural antioxidant could be used in pharmaceutical compositions. And further medical treatment in the future. And it can also be used as a substitute for synthetic antioxidants for safe use in the food and medical industries.

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Awards	- Gold Medal with Mention and Special Awards in topic "Nanobioactive particles for cosmeceutical products" at 11th International Warsaw Invention Show (IWIS 2017) (9-11 October 2017)
Publications	- Kingklao, P., Boonyuen, S., Wipatanawin, A., Na Nakorn, P. (2017) THE ANTIOXIDANT AND ANTIBACTERIAL ACTIVITIES OF *Phellinus linteus*

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