DEVELOPMENT OF ANTI-ACNE SOAP CONTAINING *MIMOSA PUDICA* L. EXTRACT

TANYARAT SUTTI

A Thesis Submitted to the Graduate School of Naresuan University
in Partial Fulfillment of the Requirements
for the Master of Science in (Cosmetic Sciences)
2021

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By TANYARAT SUTTI
has been approved by the Graduate School as partial fulfillment of the requirements
for the Master of Science in Cosmetic Sciences of Naresuan University

Oral Defense Committee

..... Chair
(Associate Professor Onanong Pringsulaka, Ph.D.)

..... Advisor
(Professor Jarupa Viyoch, Ph.D.)

..... Co Advisor
(Assistant Professor Atchariya Yosboonruang, Ph.D.)

..... Internal Examiner
(Associate Professor Tasana Pitaksuteepong, Ph.D.)

Approved

.....
(Associate Professor Krongkarn Chootip, Ph.D.)

Dean of the Graduate School

Title	DEVELOPMENT OF ANTI-ACNE SOAP CONTAINING *MIMOSA PUDICA* L. EXTRACT
Author	TANYARAT SUTTI
Advisor	Professor Jarupa Viyoch, Ph.D.
Co-Advisor	Assistant Professor Atchariya Yosboonruang, Ph.D.
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ABSTRACT

Mimosa pudica L. or sensitive plant is a pantropical weed that could be found in Asian countries including Thailand. This plant belongs to Fabaceae family and could be used as a traditional medicine to treat diarrhea, insomnia, headache, fever, and skin conditions. Some studies reported the presence of various bioactive compounds in this plant especially phenolic compounds. In this study, the investigation of total phenolic content and its antioxidant potential of *M. pudica* L. extract (MPE) were performed using Folin-Ciocalteu method and DPPH assay, respectively. Moreover, its antibacterial susceptibility against *Straphylococcus aureus* (*S. aureus*), *Straphylococcus epidermidis* (*S. epidermidis*) and *Cutibacterium acnes* (*C. acnes*) were also determined using disk diffusion method, and minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were determined using standard broth dilution technique. The results showed that, MPE which soaked in 95% ethanol (MPE_{95E}) exhibited the highest percent yield (16.64 %w/w), with highest amount of phenolic compounds (137.73 ± 0.69 mg GAE/g) and exhibited the highest free radical scavenging activity with IC_{50} value of 22.15 ± 0.03 µg/mL as compared with other samples, MPE soaked in water (MPE_{water}) and 70% ethanol (MPE_{70E}). Furthermore, the MIC of MPE_{95E} against both *S. aureus* and *S. epidermidis* were 7.5 mg/mL, while *C. acnes* was 30 mg/mL. In addition, MBC of MPE_{95E} against both *S. aureus* and *S. epidermidis* were 30 mg/mL, while *C. acnes* was more than 30 mg/mL, respectively. These results suggested that *M. pudica* L.

could be a potential source of antioxidant with free radical scavenging and antibacterial activity which will be useful for development of anti-acne cosmetic products. MPE_{95E} was selected to use as an active ingredient to develop soap bar formulation. MPE beads using encapsulation technique and MPE solutions using solubilizer were prepared to improve solubility of the extract. Then, glycerin soap bars containing MPE were developed. Anywise, glycerin soap bars containing MPE beads were shown uneven beads dispersed in that soaps, but soaps containing MPE solutions were homogeneously the whole bar with green color of extract. Finally, MPE beads, MPE solutions, and glycerin soap bars containing MPE solutions were used for stability testing. The results suggested that MPE alginate beads dispersed in medium were more stable than without medium. MPE solutions were shown not changed in viscosity at room temperature, while a bit higher and lower viscous liquid were observed at 4°C and 45°C, respectively. However, the results of stability test of glycerin soap bars containing MPE exhibited homogeneous with green color, enough foam volume, and good foam stability. The pH values of these formulations were in the range of 9.21 - 9.38 with minimum corrosive value. All results were acceptable according to the criteria of Thai Community Product Standards 665/2553 (glycerin soap bar), Ministry of Industry, Thailand. And most importantly, glycerin soap bar containing MPE showed effectively against *S. aureus*, *S. epidermidis*, and *C. acnes*. It could be suggested that this product would be attractive in daily life using for everyone in the future.

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I have graduated.

TANYARAT SUTTI

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CHAPETER I

INTRODUCTION

The rationale of study

The worldwide cosmetic industry has largely developed, and its growth continues to be driven essentially by consumers expectation of innovative products, as well as efficacy and quality. However, rising awareness about personal health and safety also led the consumers to look forward to safer cosmetics free of harmful chemicals. Hence, the dramatically increasing global trade in natural ingredients sourced from vegetable, mineral or marine renewable sources has perfectly illustrated this important trend in the past few years (Kerdudo et al., 2016). The predominance of such natural ingredients is largely due to the various roles they can play in a single cosmetic formula, acting as active ingredients (e.g., moisturizers), excipients (e.g., surfactants), additives (e.g., preservatives), etc.

Beauty is the important thing to people for a long time, from the past to the present. It is not restricted by gender or age. Nowadays, people pay more attention to health and beauty, which includes a healthy body shape and skin that is free of dark spots, dry or oily skin, uneven skin tone, and particularly acne. The problem of acne is regarded as one of the main problems of the skin, acne vulgaris, a chronic inflammatory disorder in adolescents consists of the pilosebaceous units (Lalla, Nandedkar, Paranjape, & Talreja, 2001). It might cause disfiguration and permanent scarring and has an adverse effect on emotion, which might lead to withdrawal from society, social phobias, and clinical depression (Duru & Orsal, 2021). The four main pathogenic factors in the development of acne are cornification of the pilosebaceous, duct increased sebum production, inflammatory activity, and disorders of the microflora including *Staphylococcus aureus* (*S. aureus*) and *Staphylococcus epidermidis* (*S. epidermidis*), gram positive facultative aerobic bacterium which frequently found on the skin. They are the opportunistic pathogens which usually involve in superficial infection within the sebaceous units (Truter, 2009). In general, the severity of skin disorders increases with age and time.

In additionally, *Cutibacterium acnes* (*C. acnes*), formerly *Propionibacterium acnes*, is an anaerobic, non-motile, and non-sporing gram positive bacterium that colonizes in the follicular duct (Knobler, O'Connor, Lemon, & Najafi, 2004). It generates mild local inflammation by producing neutrophil chemotactic factors. Accordingly, neutrophils get attached to the acne lesions and constantly release inflammatory mediators such as reactive oxygen species (ROS) (Leyden, 1997; Nand, Drabu, & Gupta, 2012). *C. acnes* or others bacterium with keratinocytes leading to ROS production. Keratinocytes are known to produce ROS upon exposure to toxic compounds especially ultraviolet radiations. These toxic ROS can also play an important role as a messenger in the induction of several biological responses. It becomes necessary to use antioxidants as free radical scavengers for removing ROS to deduct cell damage that occurs during acne inflammation (Halliwell, 1991). The immunochemical pathways underlying the initiation and propagation of inflammation in acne are complex and still being elucidated but might involve *C. acnes*. This bacterium might activate Toll-like receptors (TLR), components of the innate immune system, in both very early (comedogenic) and late (inflammatory) acne lesions (Tanghetti, 2013).

Natural antioxidants are increasingly popular in the medical, dietary supplement, and cosmetic industries. Additionally, it has surpassed synthetic antioxidants such as alpha hydroxy acids (AHA) and beta hydroxy acids (BHA) in popularity (Zhenbin, Zhongli, Haile, & Griffiths, 2011).

Mimosa pudica L. (MP), also known as sensitive plant, shy plant, touch-me-not, or Maiyarap in Thai, is a pantropical weed that could be found in Asian countries including Thailand. This creeping annual plant belongs to the family of Fabaceae and is mostly recognized by its rapid movement in response to touch or vibration. Some studies showed the presence of various bioactive compounds in this plant such as alkaloids, tannins, flavonoids, steroids, phenols, glycosides, terpenoids, anthraquinones and saponins. (Ankit et al., 2008; Zhenbin et al., 2011). Nevertheless, phenolic compounds are frequently reported. Medicinal plants contain several phytochemicals (secondary metabolites) could possess antioxidant and antimicrobial properties (Nand et al., 2012; Tunna et al., 2015).

The extracts from different parts of MP exhibited antibacterial activity with inhibitory effects against gram positive bacteria such as *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Klebsiella pneumoniae*, *Streptococcus pneumoniae*, *Bacillus cereus,* and *Bacillus subtilis* as well as gram negative bacteria such as *Escherichia coli*, *Salmonella typhi*, *Pseudomonas aeruginosa*, *Neisseria cinerea,* and *Proteus vulgaris* (Debashisha, Santosh, & Gouri, 2012; Mohan, Anand, & A, 2011; Thoa, Nam, & Minh Nhat, 2015). Nevertheless, effect of MP extracts on *C. acne* inhibition have not been reported.

Cleaning is important step to clean skin before using skin care products. Glycerin is a humectant that helps retain moisture in the skin and is used in a variety of skin care products due to its moisturizing properties. Glycerin soap bars are extremely gentle on the skin and could be used on the face. It has been classified as a safe ingredient for cosmetics and food applications. The European Union's (EU) cosmetics regulations impose no restrictions on the use of glycerin, as it is one of the safest ingredients that could be used in skin care products. Proper hygiene practices involving the use of bar soap, or a body cleanser could aid in the reduction of acne with mild to moderate skin lesion severity (Xu et al., 2020).

Nowadays, MP is still used in traditional medicine. In Thailand, we have MP tea which is One Tambon One Product (OTOP). Studies on the various properties of MP from weed in the field might be increased the value to this plant and might also be had a higher value to Thai community products. Additionally, MP has been granted patents in South Korea and Spain for its anti-aging properties (Jung-xiang & Lin, 2019; Nguyen & Cousy, 2017), while the phenolic compound in Mimosa has been registered in the United States (Jon Anderson et al., 2016). However, the last one is not pudica spicies; rather, it is tenuiflora, another member of the same genus. As a result, a cosmetic product containing *Mimosa tenuiflora* has been introduced to the market. Additionally, no product containing MP extract (MPE) has been developed to date. Herein, we have studied on total phenolic content, antioxidant activity, and antibacterial susceptibility of MPE to use as an active ingredient for anti-acne soap against acne causing bacteria.

Objectives of the study

1. To determine total phenolic content and antioxidant activity of MPE
2. To determine antibacterial susceptibility of MPE against *S. aureus* and *S. epidermidis*, and *C. acnes*
3. To develop glycerin soap bar formulation containing MPE

Expected output of the study

This study might contribute scientific knowledge about MP, a pantropical weed, thereby increasing value of this plant. The finished product, anti-acne glycerin soap bar containing MPE, might be suitable for all skin types especially acne-prone skin and could be used for everyone in daily life.

Scope of the study

In this study, the stems and leaves of MP were extracted using maceration technique with water and ethanol. Then, total phenolic content, antioxidant activity and antibacterial susceptibility of MPE against *S. aureus*, *S. epidermidis*, and *C. acnes* were determined. Following that, soap bar formulations containing MPE were developed. Then, glycerin soap bar containing MPE were used for stability testing. Finally, the finished product was evaluated for its physical, chemical, and biological properties.

CHAPTER II

LITERATURE REVIEW

Acne

Our skin, the largest organ of the body, hosts a complex skin microbiome with a lot of microorganisms. The microbiome is an integral part of the defense barrier and maintaining a healthy balance of commensals and pathogens might help prevent cutaneous disorders and, potentially, systemic disease. There are three main functions of skin: protection, regulation, and sensation. Skin is covered with tiny holes called hair follicles, pilosebaceous follicles or pores. Follicles contain sebaceous oil glands, that produce sebum, an oil which helps keep hair and skin moisturized (Figure 1 (A)).

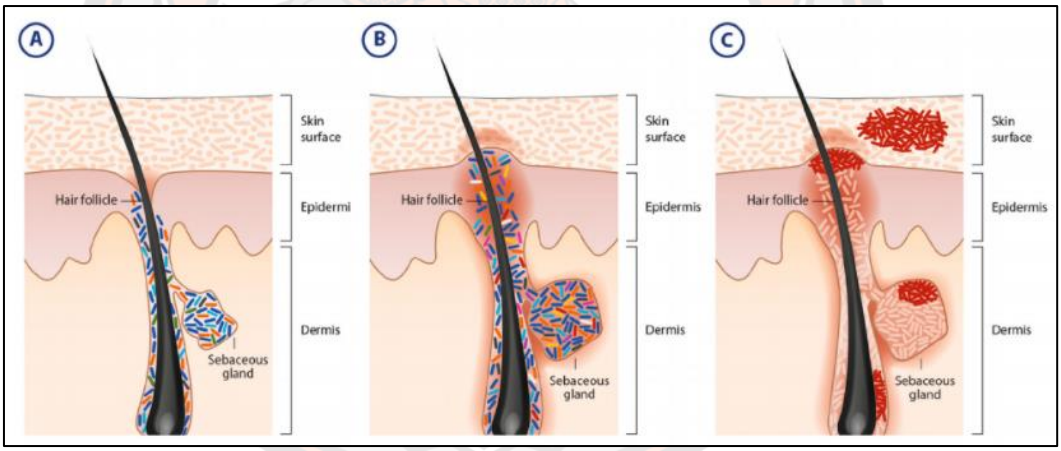

Figure 1 Acne formations

(A) Normal healthy skin, (B) Acne-induced dysbiosis, (C) Acne vulgaris

Source: (Brandwein, Steinberg, & Meshner, 2016)

Normally, dead skin cells rise to the surface of pores and are shed by the body. Hormones can cause excessive oil production during puberty. It clumps with dead skin cells inside the pore, forming a sticky plug that is referred to as "acne". *C. acnes* strains that form biofilms could be found on a variety of skin appendages, including the skin surface, sebaceous gland, hair follicle, and pore itself (Figure 1 (C)).

The main factor involved in the pathogenesis of acne is the abnormality in colonization of normal flora, specifically *S. aureus* and *S. epidermidis*, gram positive facultative aerobic organism (Figure 1 (B)). In additionally, *C. acnes* (Figure 2) which is present on normal skin, colonizes the duct of the sebaceous follicle, triggering an innate immune response and causing the comedone to progress from noninflammatory to inflammatory papule, pustule, or nodule (Figure 3). *C. acnes* might trigger an innate immune reaction via TLRs activation in both very early (comedogenic) and late (inflammatory) acne lesions. Colonization of *C. acnes* activates monocyte TLR2, which results in the production of IL-8 and IL-12, the primary proinflammatory cytokines produced by monocytes in response to invading gram positive organisms (J. Y. Lee et al., 2003; Wiesner & Vilcinskas, 2010). Notably, fatty acids found in sebum might act as endogenous regulators of TLR signaling, potentially influencing the effects of *C. acnes* mediated by TLRs (Tanghetti, 2013).

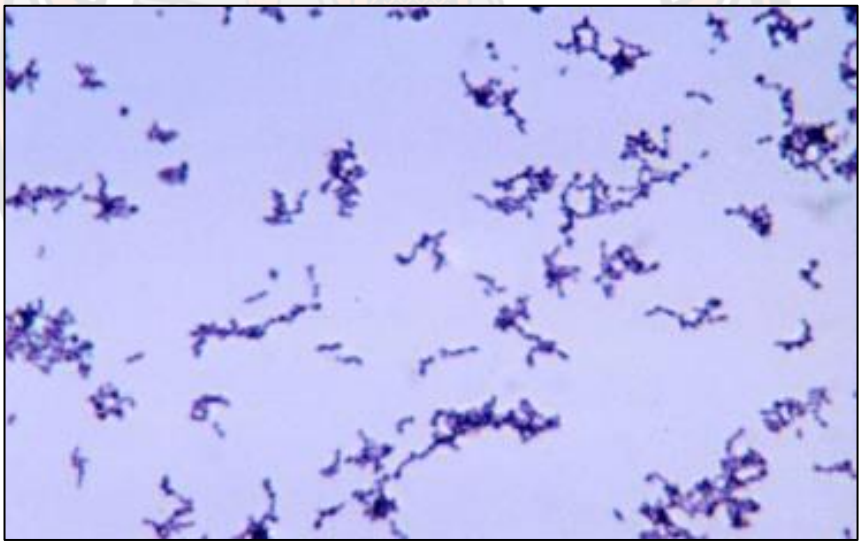

Figure 2 *Cutibacterium acnes* (formerly *Propionibacterium acnes*) gram staining

Source: (Jayashantha, 2015)

Acne primarily affects males and females during puberty but can occur at any age. Nearly 80% of adolescents and young adults are affected, it frequently persists into adulthood, and it could result in scarring and hyperpigmentation (Tanghetti, 2013).

Acne is one of the most prevalent multifactorial chronic inflammatory diseases of the pilosebaceous follicles involving androgen induced sebaceous hyperplasia, altered follicular keratinization, hormonal imbalance, immune hypersensitivity, and cause of bacterial namely *S. aureus*, *S. epidermidis* and especially *C. acnes* in the follicular canal (Holmberg et al., 2009; Sinha, Srivastava, Mishra, & Yadav, 2014).

According to the lesion type, acne could be classified into non-inflammatory acne (comedones; whitehead, blackhead) and inflammatory acne (papule, pustule, and nodule) (Nand et al., 2012; Rathi, 2011). The distribution of acne relates to the highest density of pilosebaceous units. It is distributed over face, forehead, neck, upper chest, shoulders, and back. The severity of this skin condition generally worsens with age and passage of time.

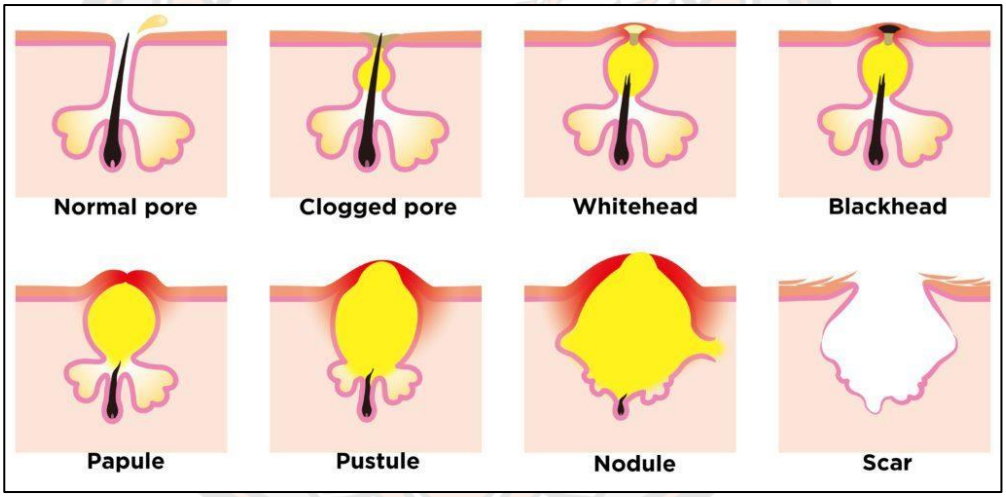

Figure 3 Type of acne

Source: https://skinlycious.com/types-stages-acne-treatment-options/

Dermatologists grade acne into four categories, according to the severity, which is shown in Figure 4 (Layton, 2005; Truter, 2009):

Grade 1 Mild Acne

It is the least severe of the four types of acne. This type of acne is characterized by comedones (blackheads), which typically appear on the nose, and a few papules, which are small, red breakouts that typically appear on the cheeks. These breakouts are brief and typically occur infrequently.

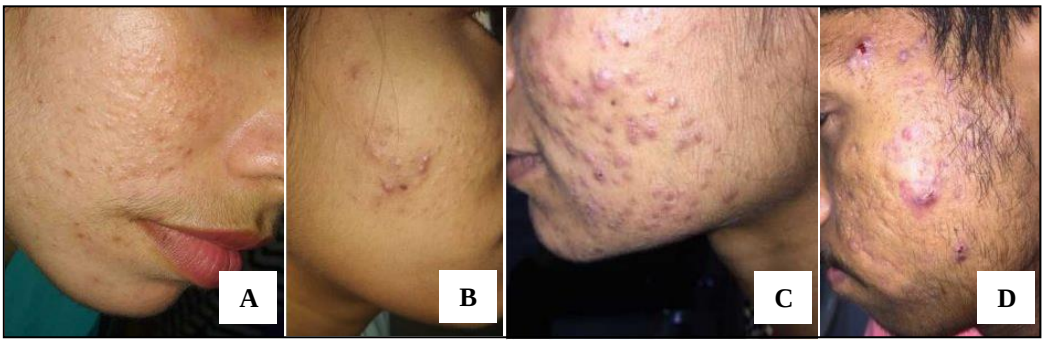

Figure 4 Stage of acne

(A) Mild Acne, (B) Moderate Acne, (C) Severe Acne, (D) Cystic Acne

Source: https://tibot.ai/symptoms/4-stages-of-acne-and-its-treatment/

Grade 2 Moderate Acne

Additionally, there are blemishes. Apart from the T zone, lesions can manifest themselves anywhere on the face. Numerous whiteheads, also known as closed comedones, are present on the skin. They take the form of elevated white or yellowish dots. When squeezed, white material will emerge. A white head is surrounded by redness as a result of mild inflammation and occasionally mild swelling of the area. Apart from whiteheads, papules and pustules are also referred to as pimple.

Grade 3 Severe Acne

The inflammation is obvious, and the face would be covered in papules and pustules. Due to the proximity of the lesions, they can spread and merge into crops. This results in skin damage, and even in the absence of squeezing, scarring can occur once the wound has healed. In severe acne, the infection has penetrated deep into the skin. There would be increased facial redness and mild swelling.

Grade 4 Cystic Acne

This stage is very severe. The blemishes are large. They can occur not only on the face, but also neck, shoulders, back, and sometimes arms. They are deep and firm to touch. Cysts can resemble a boil or a large blister. The size of a cyst might be about half of centimeter in diameter. Additionally, there are nodules, which are firm or hard bumps. When the face is touched, it is possible to experience pain. Scarring is extremely common in cystic acne due to the depth of the lesions.

Nonetheless, the most frequently prescribed topical medications for acne are oral and topical comedolytics and antibiotics. However, these comedolytics and antibiotics have several adverse effects on skin such as, skin irritation, peeling, burning, photosensitization, and abnormal skin pigmentation (Lalla et al., 2001). As a result, treating acne with natural cosmetics or natural cosmetic innovations might be considered a solution to this problem.

***Mimosa pudica* Linn**

Mimosa pudica L. (MP), also known as sensitive plant, shy plant, touch-me-not, or Maiyarap in Thai, is a pantropical weed that could be found in Asian countries including Thailand (Figure 5). This creeping annual plant belongs to the family of Fabaceae and is mostly recognized by its rapid movement in response to touch or vibration. Seismonatic sensitivity refers to the ability of the leaves to close and reopen within minutes of stimulation (Volkov, Foster, & Markin, 2010).

Figure 5 *Mimosa pudica* L. (Maiyarap)

Due to the abundance of valuable secondary metabolites found in MP, it is primarily used in traditional medicine to treat diseases such as diabetes, obesity, urinary infections, hepatitis, and cancer (Muhammad, Hussain, Jantan, & Bukhari, 2015). Numerous studies have demonstrated that this plant contains a variety of bioactive compounds, including alkaloids, tannins, flavonoids, steroids, phenols, glycosides, terpenoids, anthraquinones, and saponins (Ankit et al., 2008; Chitra, Athira, & Anitha, 2012; Debashisha et al., 2012; Mohan et al., 2011; Thoa et al., 2015).

The content of bioactive compounds in MP extracts varies according to the extraction method used. Antibacterial activity has been demonstrated for extracts from various parts of MP (Table 1).

Table 1 Studies of antimicrobial activity in Mimosa pudica L. extracts

Part of use	Solvent of extraction	Bioactive compounds	Microorganisms
Stem (Ankit et al., 2008)	Hexane, Dichloromethane, Methanol	Not reported	*E. coli*, *S. aureus*, Ampicillin resistant *E. coli*, *B. cereus*, *B. subtilis*, *P. aeruginosa*
Leaves (Mohan et al., 2011)	Water, Methanol	Alkaloids, Flavonoids	*E. coli*, *S. aureus*, *B. subtilis*, *P. aeruginosa*, *K. pneumonia*, *P. vulgaris*, *C. albicans*, *A. niger*
Root (Debashisha et al., 2012)	Petroleum ether	Alkaloids, Steroids	*S. typhi*, *E. coli*, *P. aeruginosa*, *P. vulgaris*, *N. cinerea*, *S. epidermidis*, *S. aureus*, *S. mutans*, *S. pneumonia*, *B. subtilis*, *C. albicans*, *A. flavus*
	Ethyl acetate	Phenolic compounds and Tannins, Flavonoids, Triterpenoids	
	Methanol	Phenolic compounds and Tannins, Glycosides, Saponins	
Whole plant (Chitra et al., 2012)	Methanol	Alkaloids, Tannins, Flavonoids, Steroids, Phenols, Glycosides, Terpenoids, Anthraquinones	*B. subtilis*, *S. aureus*, *K. pneumoniae*, *P. fluorescens.*
	Water	Steroids, Phenols, Glycosides, Terpenoids	
Leaves and Stem (Thoa et al., 2015)	Water, Ethanol	Flavonoids	*E. coli*, *S. aureus*, *B. cereus*, *S. typhi*

Antibacterial properties have previously been demonstrated in a few other species of the genus Mimosa (Chitra et al., 2012). Dichloromethane and methanolic extracts of MP stems can inhibit *S. aureus*, *B. cereus*, *B. subtilis*, *E. coli*, and *P. aeruginosa*. These extracts also showed no significant toxicity towards brine shrimps, suggesting that their activities are specific to bacteria (Ankit et al., 2008). Several plants, which are high in tannins and other antimicrobial compounds, have been shown to possess antimicrobial activity against a variety of microorganisms (Mohan et al., 2011).

An extract of MP leaves and stem showed inhibitory effect against gram positive bacteria such as *S. aureus, K. pneumoniae, B. cereus*, and *B. subtilis* as well as gram negative bacteria such as *E. coli, S. typhi, P. aeruginosa, P. vulgaris*, and *P. fluorescens.* (Chitra et al., 2012; Mohan et al., 2011; Thoa et al., 2015).

Additionally, ethanolic extract of MP leaves, which contains approximately three times the amount of flavonoids found in aqueous extracts, demonstrated greater inhibitory effects on bacteria than aqueous extracts, implying that flavonoids are the active compounds responsible for the antibacterial activity (Thoa et al., 2015).

The presence of biologically significant secondary metabolites such as alkaloids, steroids, phenolic compounds, tannins, flavonoids, triterpenoids, glycosides, and saponins was determined through preliminary phytochemical screening of various solvent extracts of MP roots (Debashisha et al., 2012). MP root extracts in petroleum ether, ethyl acetate, and methanol have been shown to inhibit both gram positive and gram negative bacteria, including *S. epidermidis*, *S. aureus*, *S. pneumoniae*, *B. subtilis*, *S. typhi*, *E. coli*, *P. aeruginosa*, *P. vulgaris*, and *N. cinerea*. However, ethyl acetate MP root extract inhibited bacteria and fungi more effectively than the other two extracts (petroleum ether and methanol).

Amongst the gram positive and gram negative bacteria, gram positive bacterial strains were more susceptible to the extracts as compared to gram negative bacteria. This could be explained by the fact that these two groups differ in their cell wall component structure. The differences in the cell walls of gram positive and gram negative bacteria have a significant effect on bacteria's susceptibility (Kashef, Huang, & Hamblin, 2017).

Gram positive bacteria have thick, porous peptidoglycan layers surrounding their cytoplasmic membrane, whereas gram negative bacteria have an outer membrane surrounded by a thinner peptidoglycan layer, which contains the cytoplasmic membrane (Figure 6).

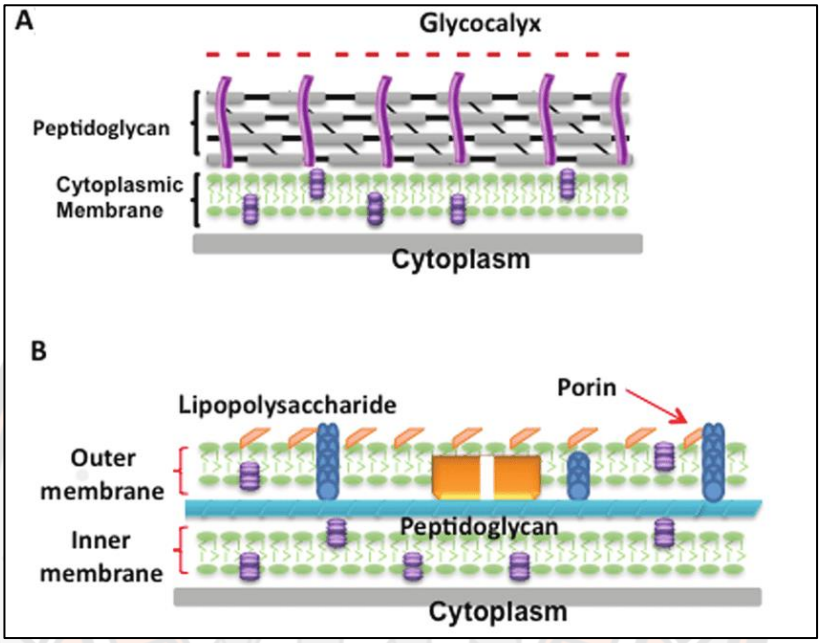

Figure 6 Cell wall structures of gram positive (A) and gram negative (B) bacteria

Source: (Kashef et al., 2017)

The development of antimicrobial drugs has focused on five bacterial targets: cell wall synthesis, protein synthesis, ribonucleic acid (RNA) synthesis, deoxyribonucleic acid (DNA) synthesis, and intermediary metabolism (Hooper, 2001). Table 2 summarizes the general antimicrobial mechanisms of action of several bioactive compounds. The various bioactive compounds found in plants can act as antioxidants and antimicrobials (Nand et al., 2012; Tunna et al., 2015).

Therefore, the purposes of this study are, to determine antioxidant activity, total phenolic content, and antibacterial activity of MPE. MP from traditional plants might represent a new source of biologically active antimicrobial agents, establishing a scientific foundation for the use of plant metabolites in therapeutic antimicrobials and allowing for further pharmacological evaluation.

Table 2 The general antimicrobial mechanism of actions

Compounds	Actions
Alkaloids	Interfere with the virulence gene regulatory systems such as quorum sensing (Thoa et al., 2015)
Tannins	Form irreversible complexes with highly rich protein resulting in the inhibition of cellular protein synthesis (Kaur, Kumar, Thippenahalli, & Kaur, 2011)
Flavonoids	Inhibit spore germination of pathogens (Thoa et al., 2015)
Saponins	Increases cell permeability of bacterial cell membrane, causing subsequent leakage of ions, ATP, nucleic acids, and amino acids (Arabski, Wegierek-Ciuk, Czerwonka, Lankoff, & Kaca, 2012)
Terpenoids	Damage the cytoplasmic membrane of bacterial cells through its hydrophobic nature (Trombetta et al., 2005)
Phenolic groups	Damage the integrity of bacterial cell wall, cause severe structural damage and significant morphological alteration (Shan, Cai, Brooks, & Corke, 2007)

Encapsulation

Nowadays, the cosmetics and personal care industries rely heavily on delivery systems and encapsulations. They provide an ideal and unique carrier system for active ingredients, enabling controlled and targeted release, isolation and protection of active compounds, increased stability and efficacy, safe administration, masking of undesirable properties of active ingredients such as odor and solubility, and enhancement of the tactile and visual appearance of a variety of cosmetic and personal care products.

Encapsulation is the protective technology of encapsulating solid, liquid or gas materials into particles. It has been widely used of application in medicine, cosmetics, food, textile, and advanced materials (Campos et al., 2013; Dubey, Shami, & Rao, 2009). The unique advantage of encapsulation lies in that the core material is completely coated and isolated from external environment. (Hawkins, Wolf, Guyard, Greenberg, & Dayan, 2005).

Encapsulation is the process of permanently or temporarily enclosing a material containing an active ingredient in another material. This results in small capsules or spheres with a variety of useful properties, as illustrated in Figure 7. Capsules have membrane-wall structures with an aqueous or oily core that serves as a reservoir for the bioactive material, whereas spheres have matrix systems that disperse the bioactive material throughout the particles (Khalil et al., 2017).

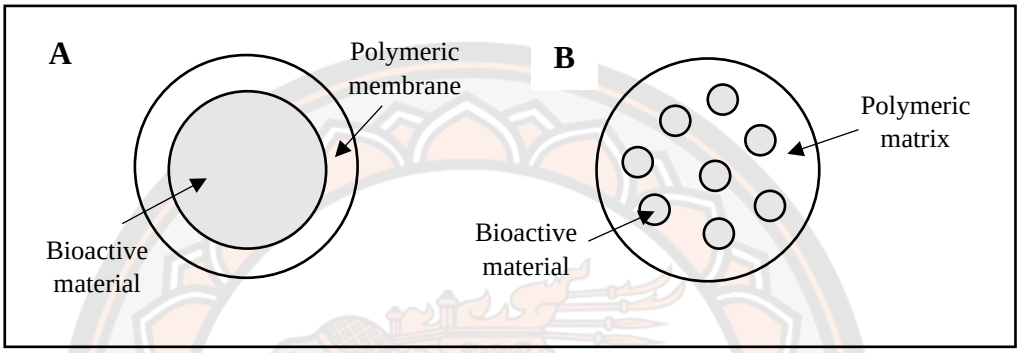

Figure 7 Scheme of capsule (A) and sphere (B)

Adapted from: (Bastos & Santos, 2015; Khalil et al., 2017)

The pharmaceutical and cosmetic industries are constantly on the lookout for novel delivery systems that will allow for the safe incorporation of many novel and sensitive active ingredients into cosmetic products. The development of new delivery systems might make it easier and more straightforward to use and develop critical emulsion systems. Encapsulation has the potential to deliver active ingredients in some difficult systems, such as those containing glycolic acid, alpha hydroxy acids, or salicylic acid, or those containing a high alcohol content or that contain critical water-in-oil or silicone emulsions. They can be used to deliver active ingredients into the skin in a safe, targeted, effective, and painless manner, to protect fragrances or volatile compounds from evaporation, to protect compounds such as antioxidants from oxidation, to protect active ingredients from degradation caused by heat, light, and moisture, and to control the rate of release (Martins, Rodrigues, Barreiro, & Rodrigues, 2011; Pardeike, Hommoss, & Müller, 2009; Rosen, 2005; Soest, 2007).

In addition, this technique could be used in cosmetic applications such as production of shower and bath gels, lotions and creams, hair products, sunscreens and tanning creams, makeup, perfumes, soaps, exfoliants, toothpastes and more. Encapsulation might contribute to the advancement of the cosmetics and personal care industries by bringing innovation and enabling the production of high-value-added products that response to human needs and desires (Andre, Marc, & Howard, 2014; Suraweera et al., 2014).

Polysaccharides (gums, starches, and celluloses), proteins (gelatin, casein, and soy proteins), lipids (waxes, paraffin, and oils), and synthetic polymers are the most frequently used polymeric materials in cosmetics (Bastos & Santos, 2015). The encapsulation technique and shell material are determined by the product's intended use, taking physical and chemical stability, concentration, required particle size, release mechanism, and manufacturing costs (Henriques Mota et al., 2017). The encapsulation technique was chosen in this study to protect antioxidants from oxidation and to protect MPE from degradation caused by the heat generated during the glycerin soap base melting step.

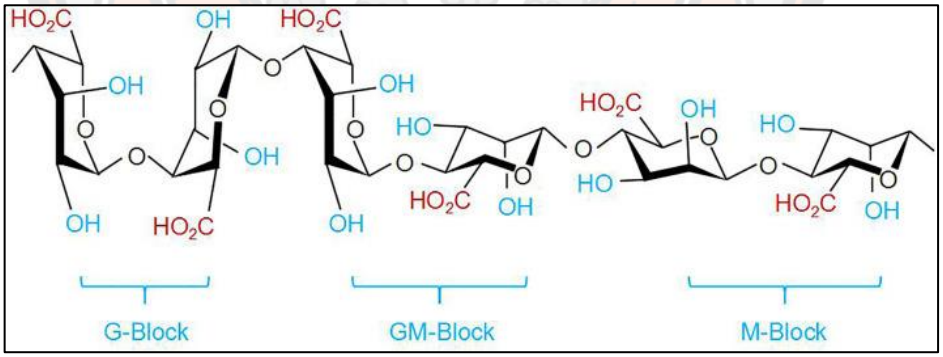

Figure 8 Alginate structure

Source: (Guo, Wang, Qin, Shen, & Peng, 2020)

Alginate is a natural polymer which is extracted from brown algae, water soluble, biocompatibility, low toxicity, relatively low cost, and mild gelation. Thereby, biopolymer and biodegradable polymer, alginate, is an encapsulating material with greater interest for this study.

It is composes of alternating blocks of 1-4 α-L-guluronic and β-D-mannuronic acid residues which showed in Figure 8 (K. Y. Lee & Mooney, 2012). The mechanism of gelation process involves guluronic residues with the specific chelation of Ca^{2+} (Segale, Giovannelli, Mannina, & Pattarino, 2016). Due to its high absorbency, this polymer can form a hydrophilic gel and incorporate more than 98% of water content in weight. From previous studies, researchers usually used 1-2% sodium alginate in the formulations depends on their purposes (Henriques Mota et al., 2017; Rajmohan & Bellmer, 2019).

Solubilizer and co solvent

Olive oil has been extensively used in cosmetics over time. Its free form, which increases the need for other formulation compounds that improve its mechanical and textural properties (Weisberg & Baumann, 2021). The main antioxidants in olive oil are phenolic compounds, which intervene by interrupting the chain reaction, while producing peroxides by free radical inactivation. Olive oil is frequently used in dermatology to treat skin conditions such as acne, psoriasis, and seborrhea eczema due to its antioxidant properties (Henriques Mota et al., 2017).

PEG-40 hydrogenated castor oil and PEG-7 glyceryl cocoate are nonionic surfactant that enable the combination of incompatible ingredients, gently cleanse and soothe the skin, create foam in cleansing products, and give products a consistent, thoroughly blended feel. In some studies, these surfactants were used to increase the rate of dissolution of poorly soluble drugs (Vervaet, Baert, & Remon, 1994). They are water and oil soluble and have historically been used to emulsify and solubilize oil-in-water formulations. The safety assessment of PEGylated oils in cosmetics concluded that they are safe to use at concentrations up to 100% (Burnett et al., 2014).

From the above reasons, olive oil, PEG-40 hydrogenated castor oil, and PEG-7 glyceryl cocoate were used as the solubilizers and co solvents in this study.

Glycerin soap base

Glycerin is a humectant that helps retain moisture in the skin and is used in a variety of skin care products due to its moisturizing properties. The EU cosmetics regulations impose no restrictions on the use of glycerin, as it is one of the safest ingredients that can be used in skin care products (Becker et al., 2019).

Glycerin soap base (transparent soap chip) is extremely gentle on the skin and is ideal for use on the face. It has been classified as a safe cosmetic ingredient (Figure 9). The distinct advantage of soap bases is that they have already been through the traditional soap-making process and are therefore ready to use; simply melt the base and pour it into a mold, allowing it to set.

Figure 9 Glycerin soap base (transparent soap chip)

Source: https://www.aliexpress.com/item/4000519129671.html

It is regarded as one of the most moisturizing soap varieties. Due to this soap's versatility, it is both moisturizing and safe for all skin types. The benefits can assist in maintaining clean and moisturized skin.

In this study, the criteria of glycerin soap bar were followed by Thai Community Product Standards 665/2553 (glycerin soap bar) that the soap bar should be clear with pH value in the range of 8 - 10 and provide bubbles when dissolved in water with minimum corrosive value.

CHAPTER III

RESEARCH METHODOLOGY

Chemicals and reagents

1. Brain Heart Infusion Agar (HiMedia Laboratories Pvt Ltd., Mumbai, India)
2. Brain Heart Infusion Broth (HiMedia Laboratories Pvt Ltd., Mumbai, India)
3. Sodium alginate (analytical grade, Merck, Darmstadt, Germany)
4. Calcium chloride (dihydrate, Ajax-Finechem (Univar), USA)
5. Clindamycin (CT0064B, Oxiod, Hampshire, UK)
6. Deionized water (LabScan Asia Co. Ltd., Bangkok, Thailand)
7. 2, 2-Diphenyl-1-picrylhydrazyl (Sigma-Aldrich, St. Louis, Missouri, USA)
8. Dimethyl sulfoxide (Lot: BCBR6170V, Sigma, Munich, Germany)
9. Ethanol (analytical grade, Merck, Darmstadt, Germany)
10. Fulin Ciocaltue's reagent (analytical grade, Merck, Darmstadt, Germany)
11. Gallic acid (Sigma-Aldrich, Chemie Gmbh, Munich, Germany)
12. Glycerin (GGC, Bangkok, Thailand)
13. L-ascorbic acid (Lot. 529150113, Poch, Sowiñskiego, Poland)
14. Mueller Hinton Agar (HiMedia, Dindori, Mumbai, India)
15. Olive oil (extra virgin, Bertolli, Mizkan America, Inc., USA)
16. PEG-7 Glyceryl Cocoate (BASF Care Creations, Florham Park, USA)
17. PEG-40 Hydrogenated Castor oil (Esteem Industries Pvt. Ltd., Mumbai, India)
18. Propylene glycol (GGC, Bangkok, Thailand)
19. Sodium Bicarbonate (analytical grade, Ajax Finechem, Auckland, New Zealand)
20. Soyabean Casein Digest Agar (HiMedia Laboratories Pvt Ltd., Mumbai, India)
21. Soyabean Casein Digest Medium (HiMedia Laboratories Pvt Ltd., Mumbai, India)
22. Tetracycline (CT0054B, Oxoid, Hampshire, UK)

23. Transparent soap base, glycerin (Batch no. 27102020-T1, TP-GR-001, Thailand)
24. Tween 20 (Srichand United Dispensary Co., Ltd., Bangkok Thailand)

Equipments

1. Anaerobic jar (Oxiod, Hampshire, UK)
2. Blender (HR2020, Philips, Amsterdam, Netherlands)
3. Colorimetric Spectrophotometer (MiniScan EZ 4500L, HunterLab, Virginia)
4. Hot air oven (UFP800DW, Memmert, Schwabach, Germany)
5. Incubator (VO400cool, Memmert, Schwabach, Germany)
6. Microplate Reader (BioTek Instruments, BioStack Ready, USA)
7. Magnetic stirrer (Heidolph, MR3001, ITS group, Bangkok, Thailand)
8. pH meter (Mettler Toledo, S20-k, Mettler-Toledo International Inc., Ohio, USA)
9. Rotary evaporator (R-200, Buchi, Postfach, Switzerland)
10. Rotary shaker (SK3PO, CTL, California, USA)
11. Ultrasonic Cleaner (Elma, Stuttgart, Germany)
12. UV-VIS Spectrophotometer (Shimadzu UV-1800, Kyoto, Japan)
13. Water bath (model LWB-211A, Daihom Lab Tech Co., Ltd., Korea)

Methodology

Collection and preparation of plant material

The fresh stems and leaves of MP were collected from the field around Naresuan University, Phitsanulok, Thailand. They were washed thoroughly in running tap water to remove the surface microflora and other adherent dirt (Debashisha et al., 2012; Mohan et al., 2011). After that, the samples were cut into small pieces and dried in hot air oven at 40 - 45 °C for 48 h. Then, the dried samples were pulverized in a mechanical grinder to obtain coarse powder. The plant powder was kept in sealed package to avoid humidity, heat, and light at room temperature.

During the flowering season, the entire mature MP was harvested to create a voucher herbarium specimen. It was then authenticated by a botanist and kept at Naresuan University, Phitsanulok, Thailand.

Plant extraction

The 150 g of dried MP powders were extracted with 1 L of different 3 solvents: water, 70% ethanol, and 95% ethanol, by using maceration technique. The samples were shaking at 200 rpm at room temperature for 72 h in triplicate (Debashisha et al., 2012; Mohan et al., 2011). The supernatants were filtrated through a qualitative filter paper (Whatman® grade 1) in a Buchner funnel prior to evaporation by using rotary evaporator. Then, MPEs were kept at 4 °C and the percent yield of crude extracts were calculated.

Determination of total phenolic content of MPE

The total phenolic content of MPEs were quantified by using Folin-Ciocalteu method. Gallic acid in ethanol at various concentrations were used for standard curve construction. MPEs were prepared at concentration of 1 mg/mL in ethanol. After that, 10 µL of gallic acid at various concentrations and MPEs were added into 96-well plate, and then added 130 µL of water and 10 µL of Folin-Ciocalteu's reagent. After being shaken for 5 min, 100 µL of 7 %(w/v) sodium bicarbonate solution were added. Then, the mixtures were incubated in the dark for 30 min prior to measurement of the absorbance at 750 nm by using microplate reader. The total phenolic content was calculated as gallic acid equivalents (GAE) from the calibration curve using the following formula (Singleton & Rossi, 1965):

$$GAE = \frac{C_1 \times V}{m}$$

Where: GAE is total content of phenolic compound in mg/g

C_1 is the conc. of gallic acid established from the calibration curve in mg/mL

V is the volume of extract in mL

m is weight of plant extract in g

Determination of antioxidant activity of MPE

The screening of the free radical scavenging activity of MPEs were accomplished by using DPPH assay in comparison to L-ascorbic acid. Various concentrations of MPEs and L-ascorbic acid were dissolved in ethanol.

Then, 75 µL of the mixture was added to 96-well plate, followed by adding 150 µL of 2, 2-Diphenyl-1-picrylhydrazyl (78.8 µg/mL DPPH solution). The mixture was left standing in the dark for 30 min at room temperature. Then, the absorbance of the remaining of DPPH was measured by using microplate reader at wavelength of 515 nm. Radical scavenging activity of MPEs and L-ascorbic acid were expressed as the inhibition percentage (%R_s) and calculated using the following formula:

$$\%R_s = \frac{A_c - A_s}{A_c} \text{ X } 100$$

Where: A_c is the absorbance of the DPPH without the sample.

A_s is the absorbance of the DPPH with the sample.

IC_{50}, an equivalent concentration to give the 50% effect, were determined by log-probit analysis of the samples (Bandoniene, Murkovic, Pfannhauser, Venskutonis, & Gruzdiene, 2002).

Antibacterial susceptibility testing of MPE

A biosafety and biosecurity certificate of this study was approved by Naresuan University (NUIBC MI 63-01-03).

S. aureus (DMST 8840), *S. epidermidis* (DMST 15505), and *C. acnes* (DMST 14916) were obtained from the Department of Medical Sciences, Ministry of Public Health, National Institute of Health of Thailand. Fresh cultures of the isolated bacteria were maintained on Mueller Hinton Agar (MHA) for *S. aureus* and *S. epidermidis*, and Brain Heart Infusion Agar (BHA) for *C. acnes*.

Antibacterial susceptibility was carried out using disk diffusion method (Balouiri, Sadiki, & Ibnsouda, 2016). Firstly, 100 µL of tested bacteria suspensions (final inoculums were $1.5x10^5$ CFU/mL) were spread on their nutrient agar plates. Then 20 µL of the MPEs were dropped into the disks (2, 4, 10 mg/disk), left absorbed, placed on agar plates of each bacteria stain, and incubated at 37 °C for 24 h (*S. epidermidis* and *S. aureus*) and for 48 h with anaerobic jar (*C. acnes*).

Antibacterial susceptibility of extracts was determined by measuring the diameter of a clear distinct zone surrounding the disk in terms of millimeter.

The re-suspend solvent of MPEs, 2% Tween 20 with 2% DMSO solution, was used as a negative control. Tetracycline (30 µg) was used as a positive control for *S. epidermidis* and *S. aureus*, while 2 µg clindamycin was used for *C. acnes*. The minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) of MPEs were determined using standard macro-broth dilution technique (Luis Esaú et al., 2019).

The quality control of antibacterial susceptibility testing was determined to verify if the susceptibility results were accurate. For routine, antibiotics were used in antimicrobial determination for the quality control of organisms according to EUCAST (European Committee on Antimicrobial Susceptibility Testing). An acceptable test values for the control strains must be within the published range.

Development of soap bar formulation containing MPE

For this study, glycerin soap base was used for preparation of soap bar formulation. This method was divided into two parts: improving the dissolution of MPE by using encapsulation technique or using solubilizer and co solvent, then adding MPE into soap formulation (Figure 10).

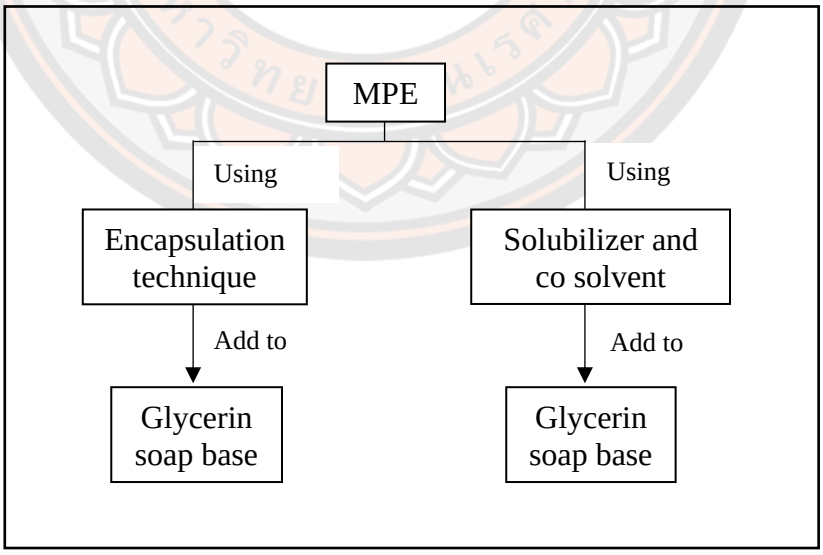

Figure 10 Schematic diagram of MPE soap bar formulation preparation

MPE beads preparation using encapsulation technique

Calcium alginate beads were prepared by an extrusion/external gelation method. Briefly, emulsions of the mixtures between water phase and oil phase were formed (Table 3). Then, MPE was added into the mixture after the temperature was dropped to 40 - 45 °C. After that, the emulsions were extruded through a fine syringe (21-gauge, diameter 0.81 mm) into 100 mL of 0.05 M calcium chloride solution under magnetic stirring for 15 min. The resulting solution was filtered to obtain gelled and hydrated beads (Henriques Mota et al., 2017).

Table 3 Emulsions preparation

Ingredients		**Uses (In formula)**
Trade name	**INCI name**	
Water phase		
Deionized water	-	Solvent
Sodium alginate	Sodium alginate	Encapsulating agent
Glycerin	Glycerin	Humectant
Oil phase		
Olive oil	Olive oil	Co solvent
Cremophor EL	PEG-40 Hydrogenated castor oil	Solubilizer
Cetio® HE	PEG-7 Glyceryl cocoate	Solubilizer
MPE	-	Active ingredient

Whereupon loading capacity and encapsulation efficiency of MPE were determined by indirect method. First, the maximum absorption of MPE for measuring the MPE content was chosen from the scanning at a wavelength of 200 - 800 nm using UV/VIS spectrophotometer. Then, 100 mg of MPE was dissolved in ethanol in a 25 mL volumetric flask. The solution was ultrasonically treated for 30 min and filtered through a 0.8 µm microporous membrane.

Of the resulting filtrate, the absorbance at collected wavelength was measured and the MPE calibration curve was prepared (Song, He, & Ping, 2017). After that, unloaded MPE which dissolved in calcium chloride solution was collected to measure. Then, the MPE concentration was calculated from calibration curve. The experiments were performed in triplicate.

Encapsulation efficiency (%EE) was calculated as:

$$\frac{\text{Total weight of MPE added - unloaded MPE}}{\text{Total weight of MPE added}} \text{ X } 100$$

MPE loading capacity (%LC) was calculated as:

$$\frac{\text{Total weight of MPE added - unloaded MPE}}{\text{Total weight of MPE beads}} \text{ X } 100$$

MPE solutions preparation using solubilizer and co solvent

The MPE solutions was prepared by dissolved MPE with PEG-7 glyceryl cocoate as a solubilizer, and mixed with PEG-40 hydrogenated castor oil, and olive oil to increase the viscosity. For this method, MPE was added directly to the solutions. So that, the concentrations were not required to measure later.

Soap bar formulation containing MPE

Glycerin soap bars which bought from cosmetic company were melted until soften, then the soaps were allowed to cool down at 40 - 45 °C before pouring into the molds. After that, MPE beads were dispersed in soap formulas and allowed to dry before de-molding them. By the way, MPE solutions were mixed directly with soaps after cooling down. Then, the soaps were poured into the molds and allowed to dry as the same process of the first method.

Stability testing

Stability studies were performed according to ICH guidelines (Ostrove, 2016).

Stability testing of MPE beads and MPE solutions

MPE solutions were contained in the bottles. In addition, the MPE beads containers were separated into 2 groups, only MPE beads, and MPE beads dispersed in propylene glycol (PG). All of samples were stored into 3 conditions in the dark for the stability test, 4 °C, room temperature (RT), and 45 °C for 30 days. The physical properties, such as color and shape, were observed and compared between day 0 and day 30.

Stability testing of MPE soap bar

The finished products, glycerin soap bar containing MPE, were stored into 3 conditions in the dark for the stability test, 4 °C, RT, and 45 °C for 30 days. At day 0 (D0) and day 30 (D30), the physical, chemical, and biological properties, such as pH, color, foamability, corrosion, and antibacterial activity of all samples were tested. The soap bar criteria were followed by Thai Community Product Standards 665/2553, glycerin soap bar.

pH values

MPE soap bar was cut into small pieces. Then, 0.4 g of the sample was dissolved in 9.6 g distilled water. After that, pH value of MPE soap bar was determined using pH meter (Khaosaad, 2008).

Color measurement

The color of MPE soap bar was measured using reflected-color measurement spectrophotometer, and the results were expressed as L*a*b* value which L* is lightness, a* is red/green value, and b* is blue/yellow value, respectively.

Foamability

Foamability and foam stability were evaluated by recording foam height (mL) and foam retention (min), respectively. MPE soap bar was weighed (20 g) and cut into small pieces. Then, the sample was shaking in 50 mL distilled water at 200 rpm at 30 °C for 5 min. After that, 30 mL of soap solutions was prepared in 100 mL cylinder and shook up and down 5 times, then flash foam was recorded. The sample was left standing for 2 min, after that foam drainage was recorded, respectively (Khaosaad, 2008).

Corrosion

The MPE soap bar (20 g) was shaking in 50 mL distilled water at 200 rpm at 30 °C for 5 min. Then, the solutions were poured out and wiped the soap bar to dry, then MPE soap bar was weighed. After that, the soap bar was shaking again for 10, 15, and 30 min, respectively. The remaining soap bar was weighed, and percent corrosion was calculated using the following formula (Khaosaad, 2008):

$$\% \text{ Corrosion} = \frac{W_b - W_a}{W_b} \text{ X } 100$$

Where: W_b is weight of soap bar before shaking

W_a is weight of soap bar after shaking

Antibacterial activity

MPE soap bar solutions were prepared by dissolve soap bar in distilled water, then antibacterial activity of 10 %(w/w) MPE soap bar solutions was determined using disk diffusion method (Suntararak et al., 2014) act in accordance with antibacterial susceptibility testing of MPE topic.

Statistical analysis

All analyses were done at least in triplicate, and these values were then showed as mean values along with their standard derivations (± SD) (Truong et al., 2019).

CHAPTER IV

RESULTS AND DISCUSSION

Collection and preparation of plant material

A voucher herbarium specimen of MP was made and authenticated by a botanist with an accession number of 004666. It was then kept at Naresuan University Herbarium (PNU Herbarium), Department of Biology, Faculty of Science, Naresuan University.

Plant extraction

The total weight of stem and leaves of MP was 1102.3 g and became 454.5 g after drying and pulverizing. The first evaluation step in chemical-based assay of MPE was to select the operating solvent used in extraction to achieve the optimized MPE. The percent yields and physical characteristics of MPEs were shown in Table 4 and Figure 11.

Table 4 The percent yields and physical characteristics of MPEs

Samples	Yield (%, w/w)	Physical characteristics
MPE_{water}	8.62	Dark brown viscous liquid
MPE_{70E}	10.39	Dark brownish green viscous liquid
MPE_{95E}	16.64	Dark green, high viscous liquid

From Table 4, The percent yield of MPE soaked with water (MPE_{water}) was 8.62 %(w/w), while MPE soaked with 70% ethanol (MPE_{70E}) and MPE soaked with 95% ethanol (MPE_{95E}) were 10.39 and 16.64 %(w/w), respectively. Extraction is the primary method for obtaining bioactive compounds from biomass materials. The extraction process is designed to maximize the amount of target compounds and to produce extracts with the highest biological activity possible. The extraction yield of the resulting extract is influenced by both the extraction technique and the extraction solvent (Kaur et al., 2011; Thoa et al., 2015).

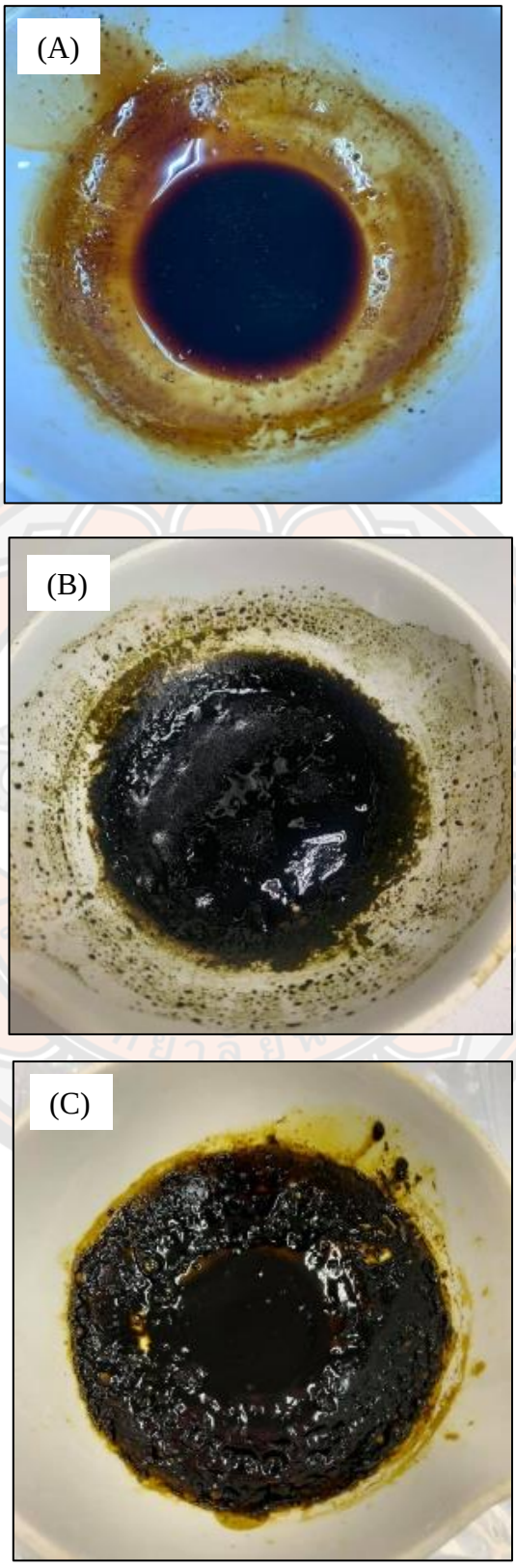

Figure 11 Physical characteristics of MPEs

(A) MPE_{water}, (B) MPE_{70E}, (C) MPE_{95E}

The physical characteristic of MPE_{water} was dark brown viscous liquid, while MPE_{70E} was dark brownish green viscous liquid. For MPE_{95E}, the extract was dark green with the highest viscous liquid and showed the highest percent yield as compared to other extracts (Figure 11).

Determination of total phenolic content of MPE

Researchers have focused their attention on secondary metabolites from plants due to their potential preventive and therapeutic effects on human health. Polyphenolic compounds such as phenolic acids, glycosides, tannins, and flavonoids are secondary metabolites which are very important constituents of plants. In this experiment, total phenolic content of MPEs were determined using Folin-Ciocalteu's reagent and expressed as mg of gallic acid equivalents (GAE)/g extract (Table 5).

Table 5 Total phenolic content of MPEs

Samples	Total phenolic content (mg GAE/g)
MPE_{water}	30.63 ± 0.51
MPE_{70E}	95.04 ± 0.24
MPE_{95E}	137.73 ± 0.69

From Table 5, the total phenolic content (TPC) of aqueous and ethanolic MPE were calculated. The TPC of MPE_{water} was 30.63 ± 0.51 mg GAE/g, while MPE_{70E} and MPE_{95E} were 95.04 ± 0.24 and 137.73 ± 0.69 mg GAE/g, respectively. From the results, MPE_{95E} showed the highest amount of phenolic contents as compared to other extracts. This could be due to the difference in the polarity of the solvents used for extraction. Many solvents, including water, methanol, ethanol, acetone, and other organic solvents, have been used for extracting bioactive compounds from the plant material. Due to the variety of bioactive compounds contained in plants and their differing solubility properties in different solvents, the optimal solvent for extraction depends on the plant materials, and the compounds that are to be isolated (Truong et al., 2019).

Determination of antioxidant activity of MPE

Interestingly, the level of antioxidant activity appears to be related to the amount of TPC present. It is possible to attribute differences in the antioxidant activity of solvents to differences in their phenolic content (Debashisha et al., 2012; Mohan et al., 2011; Muhammad et al., 2015; Thoa et al., 2015).

In general, the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity is measured by reducing the stable, violet DPPH radical to the yellow DPPH-H. The degree of colour change depends on the free radical scavenging activity of the tested samples through their hydrogen-donating ability (Bandoniene et al., 2002). In this experiment, L-ascorbic acid was used as a positive control.

Table 6 Free radical scavenging activity of MPEs

Samples	Maximum inhibition percentage (%R_s)	DPPH assay (IC_{50}, µg/mL)
MPE_{water}	91.67	64.02 ± 0.01
MPE_{70E}	93.47	39.14 ± 0.03
MPE_{95E}	94.09	22.15 ± 0.03
L-ascorbic acid	94.98	5.26 ± 0.01

The effectiveness of the MPEs and L-ascorbic acid as antioxidant and free radical scavenging were compared (Table 6). The concentration required for a 50% inhibition of DPPH (IC_{50}) was then calculated by plotting the percentage of residual DPPH against the sample concentration. The sample with a strong antioxidant activity provides high percentage of radical scavenging activity or with a low IC_{50}. The maximum inhibition percentage (%R_s) of MPE_{water} at prepared concentration, 1000 µg/mL, was 91.67 and produced IC_{50} values of 64.02 ± 0.01 µg/mL, while %R_s of MPE_{70E} and MPE_{95E} were 93.47 (IC_{50} values 39.14 ± 0.03 µg/mL) and 94.09 (IC_{50} values 22.15 ± 0.03 µg/mL), respectively. From the results, MPE_{95E} exhibited highest free radical scavenging activity as compared to other extracts. Additionally, %R_s of L-ascorbic acid, at prepared concentration, 100 µg/mL, was 94.98 and produced IC_{50} values of 5.26 ± 0.01 µg/mL. All samples showed weak antioxidant activity as compared to the standard antioxidant.

Ascorbic acid, or vitamin C, has been demonstrated to be an effective antioxidant in many experiments *in vitro* (Nand et al., 2012; Sutti, Viyoch, & Yosboonruang, 2020; Truong et al., 2019; Zhang, Yuan, Zhou, Zhou, & Yang, 2011). The overall consequence of these antioxidant activities is the beneficial control of lipid peroxidation of cellular membranes including those surrounding as well as within intracellular organelles in human (Bendich, Machlin, Scandurra, Burton, & Wayner, 1986). Due to its antioxidant property, ascorbic acid has been used as an active ingredient in many skin care products, such as, serums, day creams, night creams, facial masks, etc.

Anywise, medicinal plants contain several phytochemicals could be possessed antioxidant and antimicrobial properties (Nand et al., 2012; Tunna et al., 2015). Microbial are responsible for a range of human diseases. The study of natural compounds with antioxidant and antimicrobial properties continues to be a rapidly growing field of research.

Antibacterial susceptibility testing of MPE

The antibacterial susceptibility of MPEs were shown in Table 7. The ethanolic extracts (MPE_{70E} and MPE_{95E}) exhibited higher antibacterial property than aqueous extract (MPE_{water}). In contrast, aqueous extract exhibited no resistant activity against selected bacteria of all test concentration. The results were corresponded to previous reports (Muhammad et al., 2015; Thoa et al., 2015).

Both ethanolic extracts at the concentration of 2 mg/disk showed no resistant activity of all bacteria stains while 4 mg/disk demonstrated the inhibition zone between 6.67 - 8.33 mm against the selected bacteria. In comparison to other samples, the highest test concentration, 10 mg/disk, demonstrated the largest inhibition zone. From the results, MPE_{95E} showed more effectively antibacterial susceptibility than other samples. The inhibition zone of MPE_{95E} was 10.33 ± 0.58 mm for *C. acnes* at 10 mg/disk, while *S. aureus* and *S. epidermidis* were 13.67 ± 0.58 and 7.67 ± 0.58 mm, respectively, at the same concentration.

However, the inhibition zones of the samples in this experiment were weak in comparison to the positive control and were smaller than those observed in the other studies listed in Table 1. These results suggested that different part of the plants,

different extraction solvents, and different extraction technique resulting in differences in their biological property. Additionally, ethanol is a nontoxic, biodegradable, and biocompatible solvent that is capable of extracting certain phytochemicals from plants. As a result of these considerations, ethanol was frequently chosen as an extraction solvent in the cosmetic industry.

Table 7 The antibacterial susceptibility testing of MPEs.

Name of the Microorganisms		Concentration of MPEs (mg/disk)	Zone of inhibition (mm)		
			MPE_{water}	MPE_{70E}	MPE_{95E}
S. aureus		2	NA	NA	NA
		4	NA	6.67 ± 058	10.33 ± 0.58
		10	NA	9.33 ± 0.58	13.67 ± 0.58
S. epidermidis		2	NA	NA	NA
		4	NA	NA	7.00 ± 0.00
		10	NA	7.33 ± 0.58	7.67 ± 0.58
C. acnes		2	NA	NA	NA
		4	NA	NA	8.33 ± 0.58
		10	NA	7.00 ± 0.00	10.33 ± 0.58
Positive control (30 µg tetracycline)	*S. aureus*	-	29.00 ± 1.00		
	S. epidermidis	-	19.00 ± 1.00		
(2 µg clindamycin)	*C. acnes*	-	28.33 ± 0.58		
Negative control (2% Tween 20 and 2% DMSO solution)	All stain types	-	NA		

Note: NA is no activity detected at test concentrations

Furthermore, there was no inhibitory effect against selected bacteria of negative control. For the routine quality control, positive control (30 µg tetracycline) against *S. aureus*, showed 29.00 ± 1.00 mm in zone of inhibition. These results were in the range of 23 - 30 mm according to the standard (EUCAST, 2020). While, inhibition zone of 30 µg tetracycline against *S. epidermidis* showed 19.00 ± 1.00 mm represented in the range of resistance (19 - 21 mm) breakpoint scale (EUCAST, 2021). However, 2 µg clindamycin against *C. acnes* showed 28.33 ± 0.58 mm but disk diffusion criteria for antibacterial susceptibility testing of *C. acnes* have not yet been defined in EUCAST. Nevertheless, these results were in accordance with other studies on *C. acnes* (Luangnarumitchai, Lamlertthon, & Tiyaboonchai, 2007).

From the results of three methods above, determination of TPC, antioxidant activity, and antibacterial susceptibility testing of MPEs, the MPE_{95E} showed the highest percent yield (16.64 %w/w) with highest amount of phenolic contents (137.73 ± 0.69 mg GAE/g), exhibited the highest free radical scavenging activity with IC_{50} value of 22.15 ± 0.03 µg/mL, and showed more effectively against *S. aureus*, *S. epidermidis*, and *C. acnes* than other samples. Then, only MPE_{95E} was selected to determine MIC and MBC (Table 8).

Table 8 MIC and MBC of MPE_{95E} against *S. aureus*, *S. epidermidis* and *C. acnes*

Microorganism	MIC (mg/mL)	MBC (mg/mL)
S. aureus	7.5	30
S. epidermidis	7.5	30
C. acnes	30	>30

The MIC of MPE_{95E} against both *S. aureus* and *S. epidermidis* were 7.5 mg/mL, while *C. acnes* was 30 mg/mL. In addition, MBC of MPE_{95E} against both *S. aureus* and *S. epidermidis* were 30 mg/mL, while *C. acnes* was more than 30 mg/mL. Because of MPE solubility limitation, the highest concentration of MPE which can be soluble in broth medium was 30 mg/mL. The results suggested that MPE more effectively against Staphylococcus stains than *C. acnes*, an aerobic bacterium. In addition to the relevance of TPC and antioxidant activity, some studies reported a highly positive link between the amount of phenolic contents, antioxidant

activity, and antibacterial activity in extracts (Shan et al., 2007). Antioxidants might provide resistance to oxidative stress through a variety of mechanisms, including scavenging free radicals and inhibiting lipid peroxidation. Moreover, phenolic groups can damage the integrity of bacterial cell wall, cause severe structural damage and significant morphological alteration (Muhammad et al., 2015; Tunna et al., 2015; Zhang et al., 2011). Many chemical profiling and characterizations of MP were reported (Ijaz, Shoaib Khan, Anwar, Talbot, & Walsh, 2019; Tunna et al., 2015; Zhang et al., 2011). Besides, MPE in this experiment was crude extract which might be contained the various of bioactive constituents such as terpenoids, flavonoids, glycosides, alkaloids, quinines, phenols, tannins, saponins, and coumarins. Due to their broad antimicrobial mechanism of action, which is detailed in Table 2, the compounds might interact cooperatively with microbial cells. The compound that are typically found in MP ethanolic or methanolic extracts are alkaloids that can interfere with the virulence gene regulatory systems in microorganisms (Thoa et al., 2015), and tannins that can form irreversible complexes with highly rich protein resulting in the inhibition of cellular protein synthesis in microbial cell (Kaur et al., 2011).

Development of soap bar formulation containing MPE

Form previous experiments, MPE_{95E} was selected to use as an active ingredient to develop soap bar formulation. Encapsulation technique was selected to protect compounds such as antioxidants from oxidation, and to protect from degradation caused by heat in glycerin soap base melting step, because MPE beads can add to soap base after temperature was dropped. First, MPE emulsions were prepared (Table 9). Then, the emulsions contain sodium alginate were extruded through a fine syringe to calcium chloride solution.

In this experiment, alginates could be cross-linked by external gelation method (diffusion method). The cations diffuse from the external medium into the interior of alginate phase to form the hydrogel beads. The active ingredient, MPE, was mixed with the alginate solution, and then the solutions was extruded dropwise into calcium chloride solution with cross-linking cations to form gelation (Rajmohan & Bellmer, 2019). The mechanism of this gelation process involves guluronic residues with the specific chelation of Ca^{2+} (Segale et al., 2016).

Table 9 MPE emulsions preparation

Ingredients	Formulations (%w/w)		
INCI name	**Rx 1**	**Rx 2**	**Rx 3**
Water phase			
Deionized water	Add to 100	Add to 100	Add to 100
Sodium alginate	2	1	1.5
Glycerin	10	10	10
Oil phase			
Olive oil	10	10	10
PEG-40 Hydrogenated castor oil	12	12	12
PEG-7 Glyceryl cocoate	8	8	8
MPE	4	4	4

The different ingredient between Rx 1, Rx 2, and Rx 3 was the percentage of sodium alginate in emulsions formulations. From the previous studies, researchers usually used 1 - 2% sodium alginate in the formulations depends on their purposes (Henriques Mota et al., 2017; Rajmohan & Bellmer, 2019).

Figure 12 MPE beads

However, Rx 1, which contained the highest concentration of sodium alginate in this experiment (2%w/w), made it difficult to extrude emulsions through a syringe due to the high viscosity, while Rx 2, which contained the lowest concentration of sodium alginate in this experiment (1%w/w), demonstrated a low viscous liquid and did not form beads when extruded through a syringe. It takes the shape of a line and cannot be formed into a bead. Therefore, the appropriate concentration of sodium alginate in this experiment was 1.5%w/w in emulsions formulation (Rx 3). These emulsions can be easily extruded through a syringe and can be formed spheroid in shape with green color from MPE (Figure 12).

In this system, alginate cross-linking was achieved using calcium chloride. The content of M and G units in alginates affects its physical properties. Alginates with various sources would have specific M-G contents, and consequently, different physical properties (Guo et al., 2020; K. Y. Lee & Mooney, 2012).

Therefrom, percent loading capacity and percent encapsulation efficiency of MPE were determined by indirect method. The wavelength which MPE can be absorbed was 665 nm. After data collection using UV/VIS Spectrophotometer, the MPE calibration curve was prepared. The linearity relation was expressed as y = 0.0775x - 0.0018 with the coefficient determination (R squared) of 0.9979. Then, percent encapsulation efficiency and percent loading capacity were calculated (Table 10). The results showed that percent encapsulation efficiency of MPE was 82.07 ± 1.50 and percent loading capacity of MPE was 3.74 ± 0.61, respectively.

Table 10 MPE loading capacity and encapsulation efficiency

Sample	%EE	%LC
MPE	82.07 ± 1.50	3.74 ± 0.61

Encapsulation efficiency showed an idea about the percentage of MPE that were successfully entrapped/adsorbed into beads, and loading capacity showed MPE content in beads after separating from the medium (calcium chloride). Thus, 82.07% of MPE was entrapped in the beads and 3.74% of the beads weight was composed of MPE (each 1 mg beads contains 0.0374 mg MPE).

For MPE solutions preparation using solubilizer and co solvent, the physical characteristic of MPE solutions showed more liquid than pure crude extract and showed dark green formula from MPE (Figure 13).

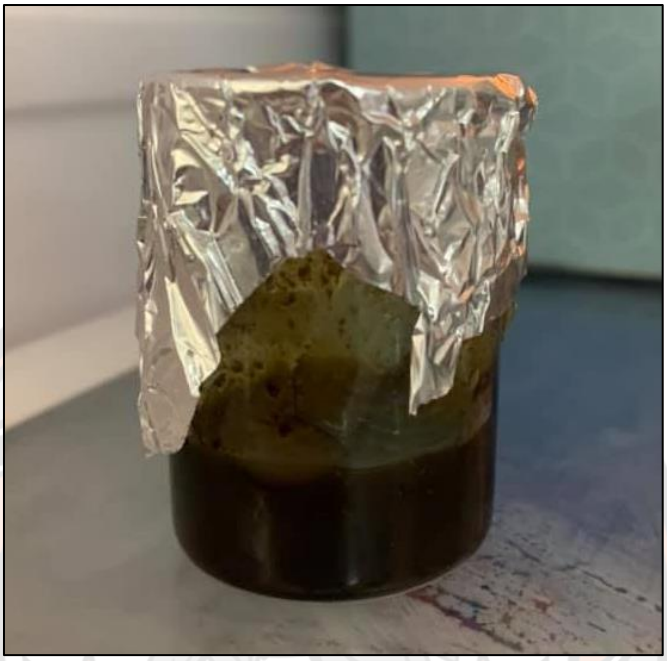

Figure 13 MPE solutions

In this formulation, PEG-40 hydrogenated castor oil and PEG-7 glyceryl cocoate function as solubilizers. They are nonionic surfactant which can combine immiscible ingredients, gently cleanse, soothe the skin, create foam in cleansing products, and offer a consistent thoroughly blended feel to products. Some studies used these surfactants to enhance the dissolution rate of poorly soluble drugs (Vervaet et al., 1994). Therewith, olive oil acts as co solvent in this formulation. The main antioxidants of olive oil are the phenolic compounds, which can produce peroxides by free radical inactivation (Henriques Mota et al., 2017). Due to its antioxidant properties, olive oil is currently used in topical applications for the treatment of several skin conditions, including dry skin, itch, acne, and inflammation (Weisberg & Baumann, 2021).

Then, MPE soap bar formulations were developed using glycerin soap base. An appearance of glycerin soap bars after the addition of both MPE beads and MPE solutions were shown in Figure 14.

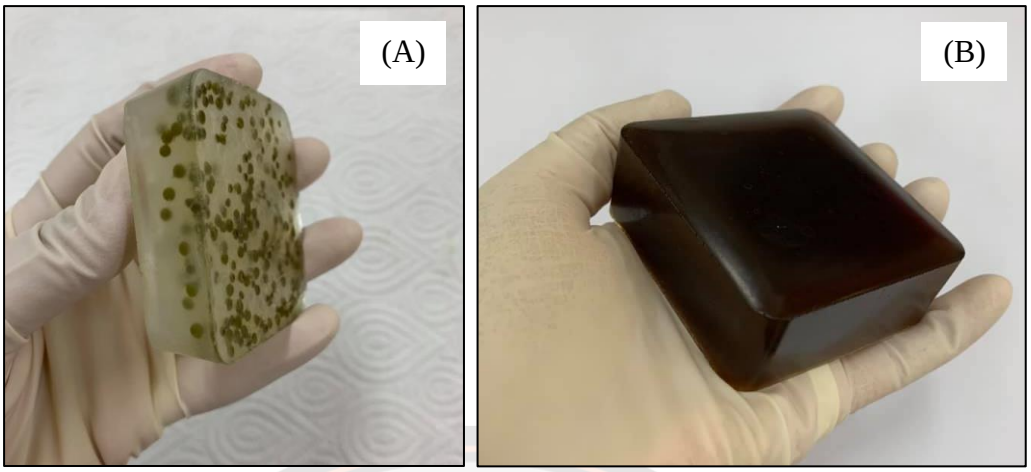

Figure 14 Glycerin soap bar containing MPE
(A) Soap bar containing MPE beads (B) Soap bar containing MPE solutions

Glycerin soap bar containing MPE beads showed uneven beads dispersed in the soap (Figure 14 (A)). Beads were cracked after soap bar was left to dry. This problem showed discontinuous active ingredient, MPE, in solid formulation.

Generally, in aqueous solutions, soaps are alkaline which neutralize the body's protective acid mantle, acting as barriers against microorganisms in the process (Oyekunle, Ore, Ogunjumelo, & Akanni, 2021). Many researchers focused on the development of calcium alginate beads as controlled drug delivery systems (Rajmohan & Bellmer, 2019; Segale et al., 2016; Tahtat et al., 2013). In addition, the limitation of alginate is ion-leaching leading to instability (David, Day, & Shikanov, 2016) and alginate is a pH sensitive, stable in acidic pH, but it swells and starts dissolving in alkaline pH (Tahtat et al., 2013). For these reasons, it is advocated that beads cannot be added to solid alkaline soaps. According to another study, there are many different types of alginate beads that contain multiple polymers to improve the stability of beads in formulations, such as calcium alginate-chitosan beads, calcium alginate-xanthan gum beads, and calcium alginate-maltodextrin beads (Segale et al., 2016; Sharmeen et al., 2019). The copolymer-polymer bonding might result in stronger beads, as well as an increase in the loading capacity and encapsulation efficiency of the beads. However, the stability testing of MPE beads were observed, because the results might be useful for future study.

Regardless, glycerin soap bar containing MPE solutions showed homogeneously the whole bar with green color from extract (Figure 14 (B)). The results demonstrate that PEG-40 hydrogenated castor oil and PEG-7 glyceryl cocoate can combine immiscible ingredients, MPE, to enhance the dissolution of this poorly soluble extract. Moreover, olive oil has the ability to increase the viscosity of these solutions. These enable the combination of ingredients, and it is easier to incorporate MPE solutions into soap formulations than it is to incorporate pure crude extract. Accordingly, only this formulation, glycerin soap bar containing MPE solutions, was selected to test the stability testing of finished product.

Stability testing

The stability testing of MPE beads and MPE solutions were evaluated by visual inspection and compared between day 0 and day 30 (Table 11).

Table 11 Stability testing of MPE beads and MPE solutions

Samples	**Changes of appearance at day 30**		
	4 °C	**RT**	**45 °C**
MPE beads (only)	Smaller size	Smaller and harder beads	Very small and hard beads
MPE beads (in PG)	Not changed	Green color leak in PG	Green color leak in PG, and PG turn to green
MPE solutions	Semisolid solution	Not changed	Lower viscous solutions

By the naked eye, an appearance of beads at day 0 and day 30 were compared. After 30 days, size of MPE beads which kept only beads in containers were smaller and harder than day 0 with irregular shape for all conditions. While MPE beads in PG were not changed in shape and size at 4 °C but at RT and 45 °C, the green color from beads were leak in PG and greener as higher temperature. In addition, MPE solutions were not changed in viscosity at RT, but showed a bit higher viscous liquid when it was kept at 4 °C and lower viscous liquid at 45 °C. However, neither the MPE beads nor the MPE solutions contained preservatives.

Although MPE beads might could not be added to soap bar formulation, stability of MPE beads were tested because these results can be used for other studies. PG was used as a medium to hold beads in containers. It is a viscous, colorless, and odorless liquid. Many pharmaceutical drugs utilize PG as a solvent and carrier including capsule preparation (PubChem, 2021). The results suggested that MPE alginate beads dispersed in medium were more stable than without medium. In addition, many studies used other polymers to form beads, or coat beads with co-polymer to improve the stability, such as xanthan gum, maltodextrin, and especially chitosan. (Segale et al., 2016; Tahtat et al., 2013). Chitosan is a biocompatible, biodegradable, and nontoxic linear polysaccharide. The two polymers of calcium alginate-chitosan beads form the polyelectrolyte complex via ionic interaction between carboxyl residues of alginate and amino residues of chitosan, caused an increase of the mechanical resistance of beads structure (Segale et al., 2016).

Stability testing of glycerin soap bars containing MPE were then performed. The results of physical and chemical such as appearance, pH, color, foamability, and corrosion were shown in Table 12.

Table 12 Stability testing of MPE glycerin soap bar

Parameter	**Glycerin soap bar containing MPE (mean±SD)**			
	D0	**4 °C**	**RT**	**45 °C**
		D30		
Homogeneity	Homogeneous	Homogeneous	Homogeneous	Homogeneous
Appearance	Green solid	Green solid	Green solid	Green solid
pH	9.21 ± 0.01	9.25 ± 0.01	9.31 ± 0.02	9.38 ± 0.02
Color (L*,a*,b*)	(14.97,0.50,1.95)	(14.94,0.44,2.01)	(13.34,0.45,2.55)	(13.79,0.45,2.02)
Foam height (ml)	42.33 ± 2.52	40.67 ± 2.52	42.67 ± 2.08	41.33 ± 2.08
Foam retention (min)	29.67 ± 2.52	26.67 ± 2.08	29.33 ± 2.52	32.00 ± 3.00
Corrosion (%)	1.17 ± 0.04	1.20 ± 0.03	1.79 ± 0.52	2.43 ± 0.24

The results exhibited that glycerin soap bars containing MPE were homogeneous with green color, had enough foam volume, and good foam stability. The pH values of these formulations were in the range of 9.21 - 9.38 with minimum corrosive value. All conditions in this experiment produced slightly different results. These results were corresponded to previous study (Meepripruk, Phongosot, Yotpanya, Chuajeen, & Buadee, 2018) and were acceptable according to the criteria of Thai Community Product Standards 665/2553 (glycerin soap bar), the soap bar should be clear with pH value in the range of 8 - 10, and provide bubbles when dissolved in water with minimum corrosive value. (Khaosaad, 2008).

In addition, the results of biological properties, antibacterial activity of MPE soap bar, using disk diffusion method were shown in table 13.

Table 13 Antibacterial activity of MPE soap bar

Name of the Microorganisms		**Zone of inhibition (mm)**			
		D0	**4 °C**	**RT**	**45 °C**
				D30	
S. aureus		9.33 ± 0.58	10.00 ± 0.58	9.33 ± 0.58	9.33 ± 0.58
S. epidermidis		8.67 ± 0.58	8.33 ± 0.58	7.33 ± 0.58	8.00 ± 1.00
C. acnes		8.00 ± 1.00	8.33 ± 0.58	8.33 ± 0.58	8.67 ± 0.58
Positive control (30 µg tetracycline)	*S. aureus*	29.00 ± 1.00		28.67 ± 0.58	
	S. epidermidis	19.00 ± 1.00		19.00 ± 1.00	
(2 µg clindamycin)	*C. acnes*	28.33 ± 0.58		28.33 ± 0.58	
Negative control (DW)	All stain types	No activity		No activity	

An antibacterial activity against *S. aureus*, *S. epidermidis*, and *C. acnes* were determined. The results at all conditions demonstrated zone of inhibition between 7.33 - 10.00 mm against the selected bacteria. Furthermore, negative control and positive controls were according to previous topic, antibacterial activity of MPE.

Although, MPE soap bar showed effectively against the selected bacteria, but the inhibition zone of MPE soap bar was weak in comparison to the positive control. Besides, inhibition zone of MPE soap bar was decreased as compared to pure crude MPE. This could be due to the MPE soap bar being dissolved in distilled water to a concentration of 10% w/w prior to testing. MPE should be reduced in quantity and its constituents, bioactive compounds, might also be reduced. Additionally, no preservatives were used in this formulation.

In addition, soap formulations contain many ingredients. Alkaline helps to break down bacteria cell wall and surfactants could be disrupted the lipid-lipid, lipid-protein and protein-protein interactions due to its antibacterial activity (Shehadul, Aryasomayajula, & Ponnambalam, 2017). Furthermore, the combination of ingredients in cosmetic product industrial must contain preservative to prevent degradation by bacteria. Multiple active ingredients can be combined in formulations to increase efficiency and quality of product. However, the amount of ingredient in the formula must comply with the law.

Suggestions and recommendations for future study

Due to the alkaline pH and solidity of MPE alginate beads, they cannot be added to soap bars or solid formulations. However, alginate beads might be added to an acidic pH formulation, such as a serum or gel, to help retain the beads. In addition, coating beads with a co-polymer, such as chitosan, might increase the mechanical resistance and stability of the beads structure.

However, further investigation such as bioactive compounds profiling of MP, toxicity of MPE, or others biological and chemical properties of MPE should be investigated. Moreover, *in vivo* and clinical studies on the application of this plant might be considered to improve the efficiency and reliability of products.

CHAPTER V

CONCLUSIONS

In this study, MPE_{95E} exhibited the highest percent yield (16.64 %w/w), with highest amount of phenolic compounds (137.73 ± 0.69 mg GAE/g) and exhibited the highest free radical scavenging activity with IC_{50} value of 22.15 ± 0.03 µg/mL as compared with other samples. Thereby, the MPE_{95E} could be the suitable extract to further experiment, it presented highest activity as compared to the other extracts. Also, MPE_{95E} exerted the activity of bactericidal against *S. aureus*, *S. epidermidis* and *C. acnes*.

The traditional plants, *Mimosa pudica* L. might represent new source of antimicrobial agents with biologically active which could establish a scientific base for the use of plant metabolites in the therapeutic antimicrobials and further pharmacological evaluation.

MPE solutions preparation using solubilizer and co solvent were selected to develop soap bar formulation. Although MPE beads cannot be added to soap bar formulation, some studies of bead formulations were tested. Because of these results might be useful for other studies, such as, foam, or serum formulation that can dispersed beads in their formula. The results of stability test of glycerin soap bars containing MPE exhibited homogeneous with green color, had enough foam volume, and good foam stability. The pH values of these formulations were in the range of 9.21 - 9.38 and had minimum corrosive value. All results were acceptable according to the criteria of Thai Community Product Standards 665/2553, Ministry of Industry, Thailand, which glycerin soap bar should be clear with pH value in the range of 8 - 10 and provide bubbles when dissolved in water with minimum corrosive value. Most importantly, glycerin soap bar containing MPE showed effectively against *S. aureus*, *S. epidermidis*, and *C. acnes.*

It could be suggested that this formulation would be attractive in daily life using for everyone in the future.

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APPENDIX

APPENDIX A COLLECTION AND PREPARATION OF PLANT MATERIAL

Figure 15 A voucher herbarium specimen of MP

APPENDIX B DETERMINATION OF TOTAL PHENOLIC CONTENT OF MPE

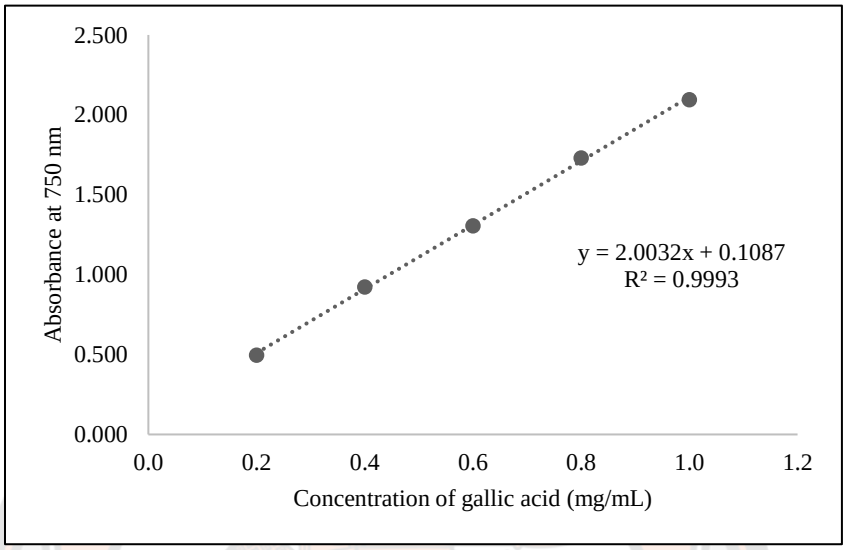

Figure 16 Calibration curve of the gallic acid standard

APPENDIX C DETERMINATION OF ANTIOXIDANT ACTIVITY OF MPE

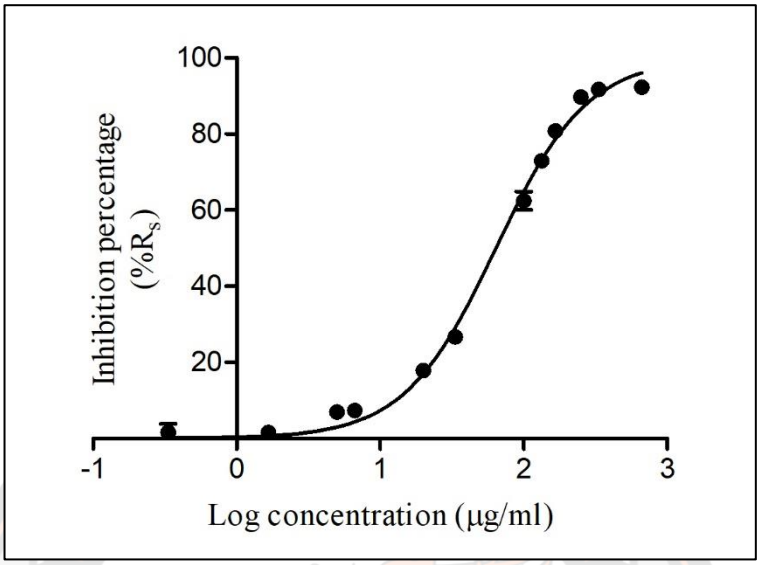

Figure 17 The ability of antioxidant activity of MPE_{water}

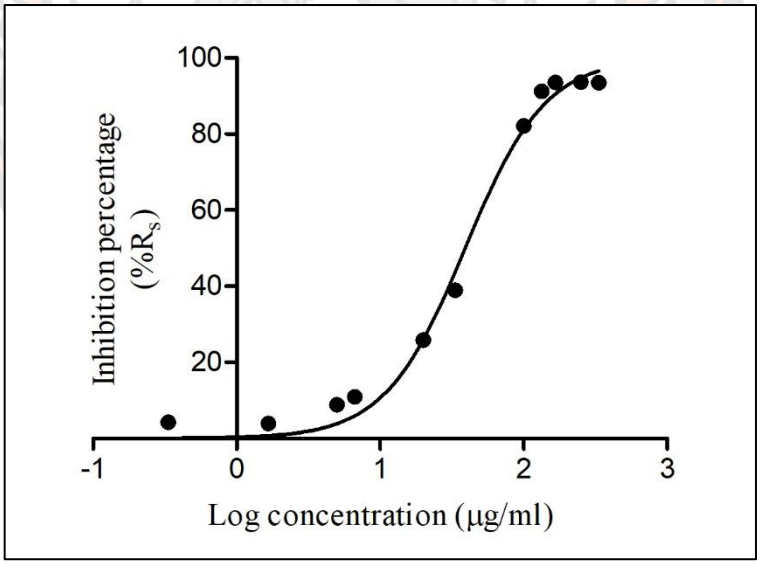

Figure 18 The ability of antioxidant activity of MPE_{70E}

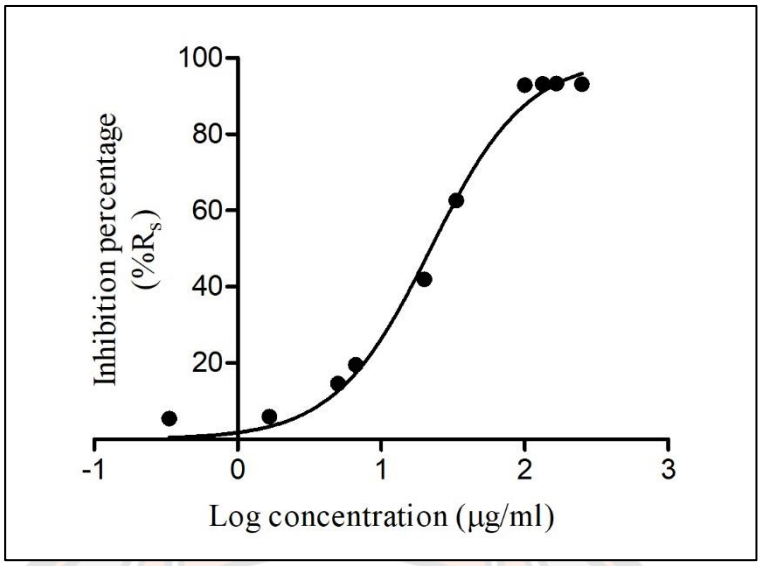

Figure 19 The ability of antioxidant activity of MPE$_{95E}$

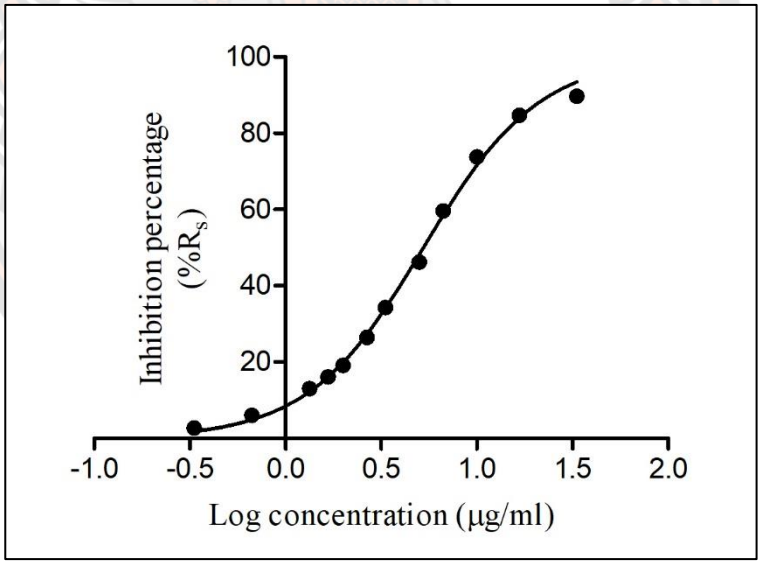

Figure 20 The ability of antioxidant activity of L-ascorbic acid

APPENDIX D ANTIBACTERIAL SUSCEPTIBILITY TESTING OF MPE

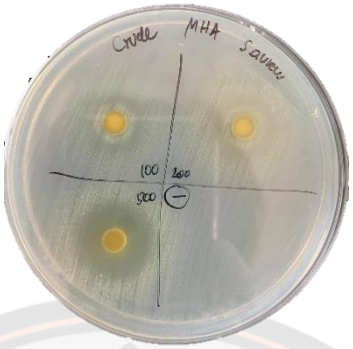

Figure 21 The diameter of clear zone of MPE against S. aureus

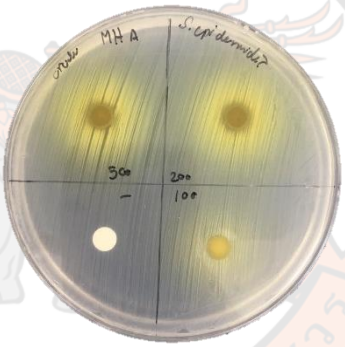

Figure 22 The diameter of clear zone of MPE against S. epidermidis

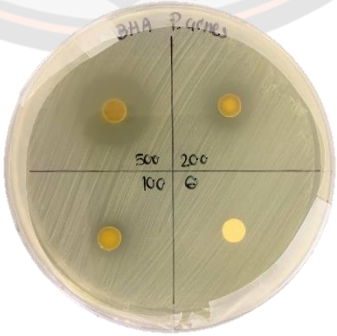

Figure 23 The diameter of clear zone of MPE against C. acnes

APPENDIX E DEVELOPMENT OF SOAP BAR FORMULATION CONTAINING MPE

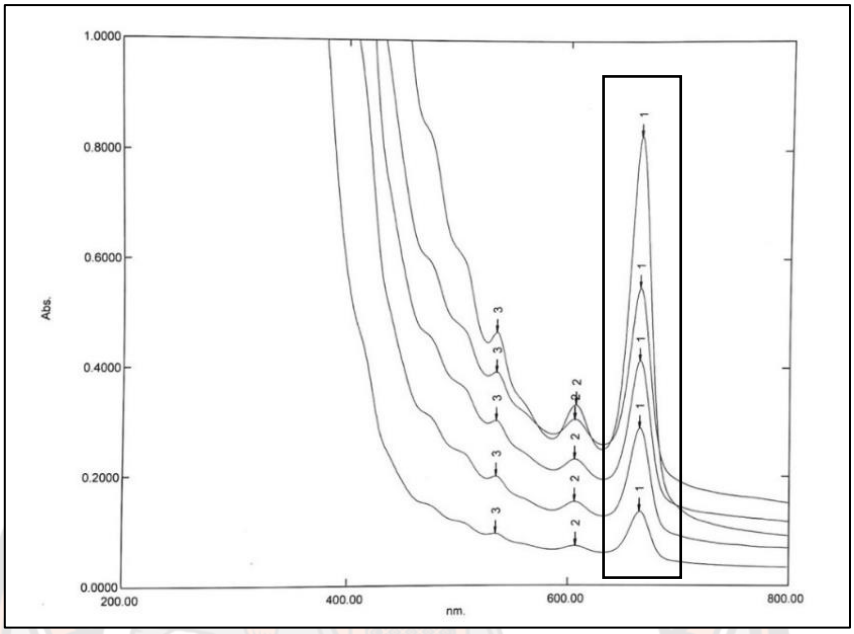

Figure 24 Spectrum peak of MP

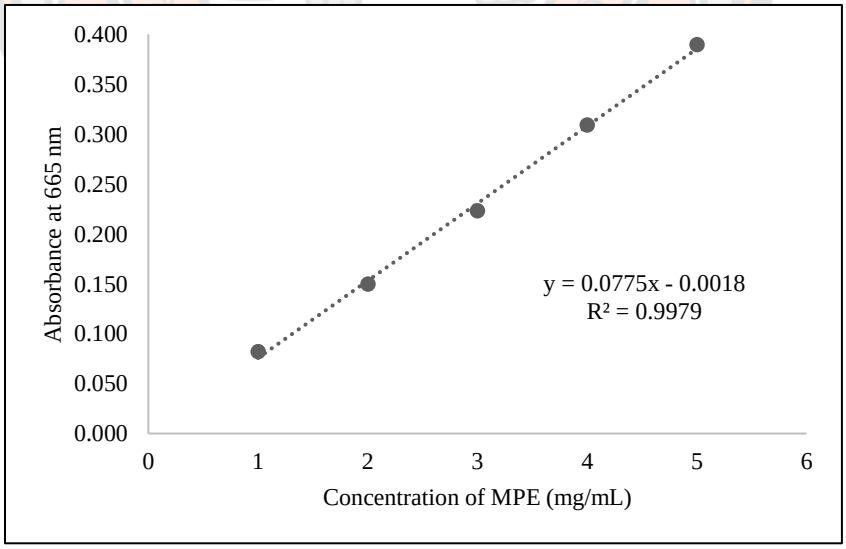

Figure 25 MPE calibration curve

BIOGRAPHY

Name-Surname Tanyarat Sutti

Date of Birth

Address

Education Background B.Sc. Medical Science (Second honors), Naresuan University, Thailand

Publication Jannoey, P., Channei, D., Kotcharerk, J., Pongprasert, W., & Sutti, T. (2016). Identification of some volatile compounds in rice infestation with brown planthopper (BPH). NU. International Journal of Science, 13(2), 49-61.

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