HERBAL COMPOSITES FILM PRODUCTION FOR INFLAMED ACNE TREATMENT

BY

MISS PIMONWAN PATIMANUKUL

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

HERBAL COMPOSITES FILM PRODUCTION FOR
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was approved as partial fulfillment of the requirements for
the degree of Master of Science (Biotechnology)

on July 7, 2016

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ABSTRACT

Acne is a chronic skin disease caused by the inflammation of sebaceous glands around hair follicles, where could be seen as red blisters, pustules, and scar on the face. These are the effects of bacteria especially *Staphylococcus aureus* and *Propionibacterium acnes* infection. Many investigations have been done on acne and scar treatments using Thai herbs as anti-bacterial agents for *S. aureus* and *P. acnes*. This research had developed thermoplastic starch films from cassava starch supplemented with herbal extracts from mangosteen pericarps (MP) and centella (CL), which consisted of three main parts; Part (1) Thermoplastic starch preparation, Part (2) MP and CL extraction, formulations and their anti-microbial, anti-oxidant properties, and also total phenolic contents, and Part (3) Preparation of thermoplastic starch films added with selected formulation of MP-CL extracts and the study of extracts releasing profiles and disk diffusion assay of selected formulation.

Part (1), Thermoplastic starches were prepared from cassava starch (ST), glycerol (GLY), and sodium alginate (AL). At first stage, the films were prepared only

with cassava starch plasticized with glycerol, and measured the mechanical properties of stickiness (SN), adhesiveness (AN), and cohesiveness (CN). The resulted revealed that the ratio of 30:70 (w/w) of ST, polymer part, and GLY, plasticizer part, exhibited high SN, AN, and CN values of 54.31±3.40 g, 0.86±0.06 g.sec, and 0.34±0.03 mm, respectively, at *p*-value < 0.05. But the appearance of these films revealed that the wrinkle and unsteady films instead.

Thus, to improve the quality of the films both appearance and high mechanical properties, sodium alginate (AL) was then added into the polymer part, 30% w/w of the ratio, while GLY, plasticizer part was kept constant at 70% w/w. The ratios between ST and AL in polymer part were varied at 10% (w/w) interval from 100:0 to 0:100 of ST:AL ratio. Ten newly prepared composite films were done on SN, AN, and CN measurement. The results showed that the films with polymer ratio (ST:AL) of 90:10 gave the high SN, AN, and CN values of 68.31±0.62 g, 1.65±0.05 g.sec, and 0.55±0.01 mm, respectively, at *p*-value < 0.05, with steady and non-wrinkle films. These films were selected to be supplemented with herbal extracts before conducting the third part of this study, extracts releasing profiles of the invented films.

Part (2), in this part, MP and CL were extracted by maceration and Soxhlet methods, respectively, and prepared for 7 MP-CL formulations by varying and combining MP and CL extracts at 7 different ratios (w/w), MG0, MG2, MG4, MG5, MG6, MG8, and MG10. These formulations were then measured the anti-oxidant activities, total phenolic contents (TPC), and anti-microbial activities. The anti-oxidant activity was done by using DPPH scavenging assay comparing to the standard ascorbic acid. It was found that MG8 gave the highest anti-oxidant activity with 17.10 µg/mL of IC_{50} and 69.99±4.67% of radical scavenging among five formulations, MG2 to MG8. However, its activity was lower than that of MP extract alone (MG10), 14.80 µg/mL of IC_{50} and 75.93±12.16% of radical scavenging, while CL extract alone (MG0) exhibited the lowest anti-oxidant activity of 61.70 µg/mL of IC_{50} and 31.79±10.54% of radical scavenging. The TPC results also confirmed the effectiveness of MP over the CL, as

the TPC for MP and CL was found to be 5,179.64±542.66 and 2,418.81±123.10 mg GAE (Gallic Acid Equivalent)/g extract, respectively.

This study also investigated on the anti-microbial activities of all MP-CL formulations by Minimal Inhibitory Concentration (MIC) using broth micro-dilution method. These tests were done to compare the formulations with gentamycin solution against *S. aureus* DMST 8840 and *P. acnes* DMST 14916. It was found that MG8 and MG10 had the highest anti-microbial activity with MIC values of 1.95 and 15.63 µg/mL against *S. aureus* and *P. acnes*, respectively. The MP-CL formulation MG8 was then selected to add into the anti-acne composite films mentioned in Part (1).

Part (3), the composite films supplemented with MG8 were then carried out with 480 minutes herbal extracts releasing profiles. It was found that the selected films gave the cumulative releasing content of 4.74±0.26% (w/w) at 30 minutes and remained approximately constant after that. The disk diffusion assay showed that the inhibition effect against *S. aureus* DMST 8840 and *P. acnes* DMST 14916 exhibited the large inhibition zone of 17.56±0.20 and 14.89±0.11 mm, respectively.

Keywords: Acne Vulgaris, *Garcinia mangostana*, *Centella asiatica*, Thermoplastic starch

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Miss Pimonwan Patimanukul

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LIST OF ABBREVIATIONS

Symbols/Abbreviations	Terms
α	Alpha
β	Beta
δ	Delta
ε	Epsilon
η	Eta
γ	Gamma
κ	Kappa
λ	Lambda
θ	Theta
µg	Microgram
µL	Microliter
AL	Alginate (Sodium alginate)
AN	Adhesiveness
BHA	Brain Heart Infusion agar
BHI	Brain Heart Infusion
CFU	Colony Forming Unit
CL	Centella
CN	Cohesiveness
g	Gram
GAE	Gallic Acid Equivalent
GLY	Glycerol
HPLC	High Performance Liquid Chromatography
IC_{50}	Inhibitory Concentration 50 percentages
I.D.	Inner Diameter
mAU	Milli Absorbance Unit
mbar	Millibar

MG	Mangosteen
mg	Milligram
MHA	Mueller Hinton agar
MHB	Mueller Hinton broth
MIC	Minimum Inhibitory Concentration
min	Minute
mL	Milliliter
mm	Millimeter
MP	Mangosteen pericarp
nm	Nanometer
PBS	Phosphate Buffer Saline
pH	Potential of hydrogen ion
S.D.	Standard deviation
sec	Second
SN	Stickiness
ST	Cassava starch
w/v	Weight by volume
w/w	Weight by weight

CHAPTER 1
INTRODUCTION

1.1 Motivation, Problem, and Solution

Inflamed acne is a common skin disease, mostly found in 85 percentages of young people (1). The main cause of inflamed acne is *Propionibacterium acnes*, which is obligate anaerobic bacteria, and performs as an immunostimulator by producing a variety of biologically active molecules and enzymes (2). This leads to the development of inflammatory acne, causing irritation and scar (3). It has been known that substances in some Thai herbs, such as alpha-mangostin found in *Garcinia mangostana* could inhibit *P. acnes* (4) and asiaticoside found in *Centella asiatica* also had a significant wound healing activity (5).

Key questions arose: "*What could be the easiest and most effective way to prevent or to alleviate the adverse effect of inflamed acne? And how to make it?*"

Then a tentative answer was *"it should be a small thin and clear film embedded with certain herbal extracts. The steps are as follows (a) to investigate thermoplastic starch films production (b) to extract the active compounds from mangosteen pericarp and centella, and generates their formulas (c) to combine the selected formulation with the films."* The final product will consist of main ingredients of (a) the films, cassava starch, glycerol, and sodium alginate, and (b) the selected Thai medicinal herb extracts. This product will be another choice for inflamed acne treatment.

1.2 Objectives

1.2.1 To prepare and characterize the invented film, which composed of cassava starch, glycerol, and sodium alginate, to meet the desired physical properties.

1.2.2 To extract the active compounds from mangosteen pericarp and centella, and to find the suitable formulation for their mixture.

1.2.3 To prepare the films added with the selected formula extraction.

1.2.4 To investigate releasing profiles of the films.

1.3 Scope of study

This research consists of three main parts.

1.3.1 Preparation and characterization of cassava starch and sodium alginate composite films plasticized with glycerol, where the films would be used as anti-inflamed and wound healing materials. The prepared films were then characterized their physical properties by performing the adhesive test using Texture Analyzer. The conditions that gave high stickiness, high adhesiveness, and high cohesiveness, were then selected to prepare the thermoplastic starch films added with formulated extracts addition.

1.3.2 Mangosteen pericarp and centella were extracted by using suitable solvents and extraction methods to obtain the crude extracts. The extracts were then formulated to determine their synergistic effects on biological activities including total phenolic compound content (Folin-Ciocalteu method), anti-oxidant activity (DPPH assay), and anti-microbial activity (Microbial dilution method). The results were used to select the suitable formulation. Subsequently, this herbal formulation was used to prepare the thermoplastic-starch films added with herbal extracts.

1.3.3 In the preparation of the herbal films, an appropriate portion of the extracts would be added into the films in order to inhibit the proliferation of the

infectious bacteria. Lastly, the films were investigated releasing profiles by using Franz diffusion cell under simulating condition.

CHAPTER 2
REVIEW OF LITERATURE

In this chapter, the background of 4 important parts, acne vulgaris, mangosteen, centella, and thermoplastic starch, are explained.

2.1 Acne vulgaris

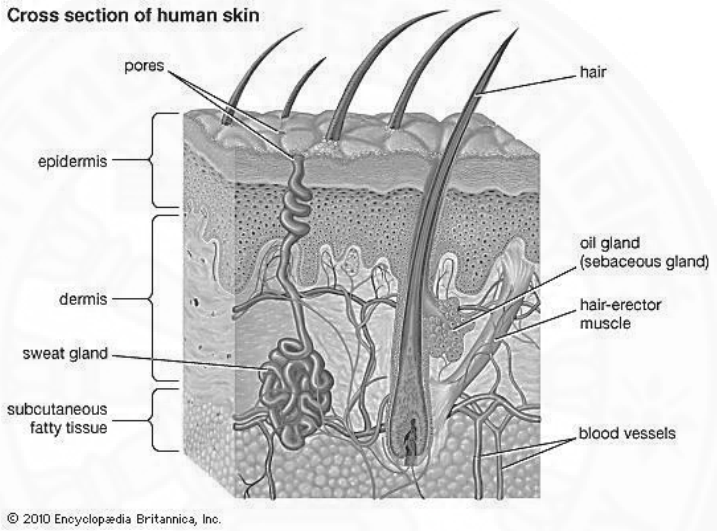

Figure 2.1 Normal Pilosebaceous Unit (6)

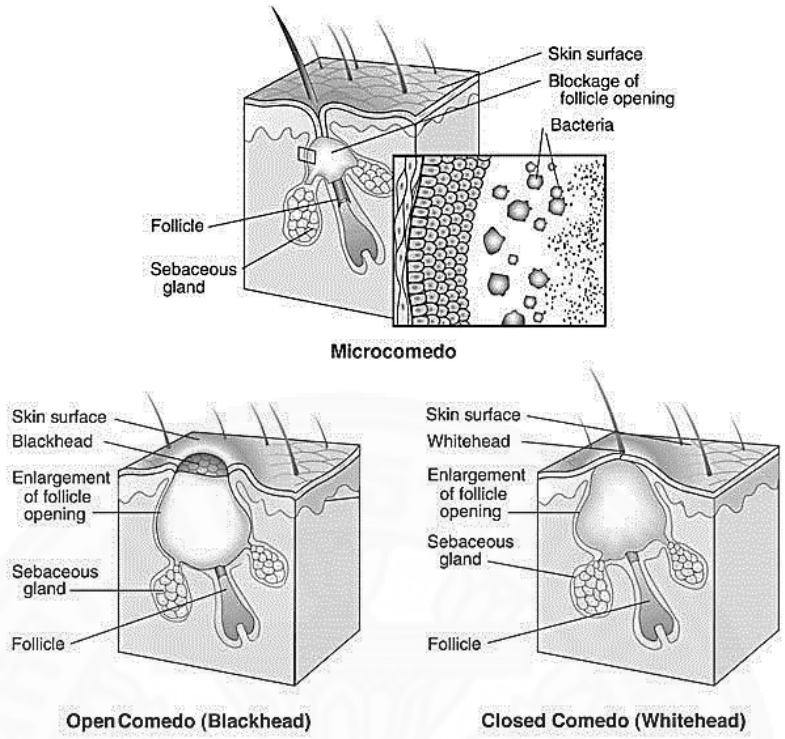

Figure 2.2 Type of Lesion for Acne Vulgaris in Pilosebaceous Unit (6)

Acne vulgaris is the inflammation of pilosebaceous gland. It can be called as comedones, papules, pustules, cysts, nodules, and often scars. The affected areas can be the largest oil glands, especially face and back. The pathogenesis of acne can be caused by hormonal imbalance, bacterial infection, and cosmetic application (4).

2.1.1 The cause of acne vulgaris

It has been believed that acne vulgaris could happen with four different causes.

2.1.1.1 Abnormalities in pilosebaceous gland

Acne is the abnormality of pilosebaceous gland that leads to Sebum overproduction. Pilosebaceous gland is controlled by androgen, the hormone secreted from testicle and adrenal gland. Androgen is the hormone which could

have an effect on pilosebaceous gland more than estrogen, causing more chance of acne from stimulating sebum over production (7). The most important hormone in androgen group is testosterone which can be converted to dihydrotestosterone by iso-enzyme 5 α-reductase type 1 (8), leads to the oversize of pilosebaceous gland produces more sebum. From previous reports, patients with acne disease could find 5 α-reductase at higher level and also high numbers of androgen receptors in pilosebaceous gland (9). The secreted sebum consists of many types of triglycerides, they are wax, esters, squalene, cholesterol, and cholesterol esters. When sebum tube has been observed it could find *Propionibacterium acnes*, which could produce lipase for digesting triglycerides and converted it to free fatty acids and glycerol (10) causing acne and pilosebaceous gland inflammation (11), (12).

2.1.1.2 Abnormalities in keratin production

Acne could be caused from keratin production and then clogged and accumulated around pilosebaceous gland (13). This was found in animal model study, when exogenous free fatty acids could induce acne in animal model (14), as important free fatty acid, linoleic acid, was lower than the normal condition with no acne. An increase in peroxide level also confirmed the over production of keratin from pilosebaceous gland was caused by *P. acnes* (15).

2.1.1.3 Bacterial infection

A numbers of bacteria can be the cause of acne, a human normal flora such as *P. acnes* is an example. This bacteria can live in pilosebaceous gland and skin around face, back, and body (2). *P. acnes* is gram positive, rod shape as seen in Figure 2.3 and Figure 2.4. It is anaerobic bacteria (16), with circular colony of 1-2 millimeter diameter (17).

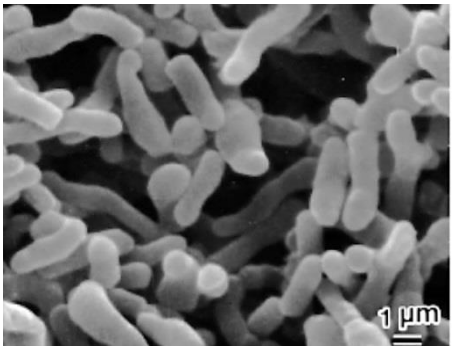

Figure 2.3 The Shape of *Propionibacterium acnes* (18)

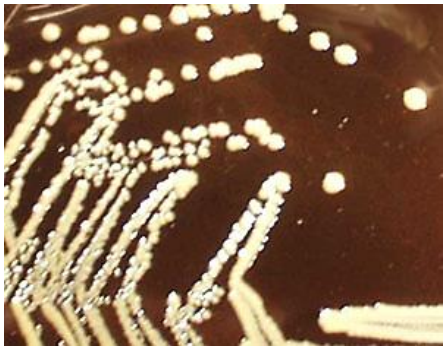

Figure 2.4 The Colony of *Propionibacterium acnes* (https://microbiology2009.wikispaces.com)

P. acnes can produce acids such as propionic acid and acetic acid from glucose. *P. acnes* can digest sebum from pilosebaceous gland and produce free fatty acids by lipase. Apart from lipase, *P. acnes* can produce protease, hyaluronidase, including chemotactic factors, which have an influence on inflammatory development of acne (19).

2.1.1.4 Inflammatory of skin

As mentioned earlier, *P. acnes*, lives in pilosebaceous gland, can produce low molecular weight chemotactic factors, which then moved to follicular epithelium and induce neutrophils to secrete lysosomal enzymes and reactive oxygen species causing the inflammation of the tissue, whereas free fatty acids can be cytotoxic co-factor with chemotactic factors of the tissue inflammation (2).

2.1.2 Acne treatment

In general, there are four methods of acne treatment, they are decrease of sebum, use vitamin A to decrease comedone production, decrease of bacteria causing acne, *P. acnes*, and alleviate the inflammation.

2.1.2.1 Decrease of sebum

The use of cyproterone acetate as anti-androgen could decrease sebum production and reduce the size of pilosebaceous gland. The use of this drug must be under the doctor suggestion (3), (20), (21), (22).

2.1.2.2 Reduce the occurrence of comedone

Drug in this group is vitamin A acid. It is used to decrease the comedone production and increase the proliferation of pilosebaceous gland epithelium to close comedone, or whitehead, or to open comedone, or blackhead. This can prevent pilosebaceous gland inflammation, stimulate blood, and eliminate papule and nodule (20), (23).

2.1.2.3 Decrease of *Propionibacterium acnes*

Benzoyl peroxide is the popular drug in this group, it is by product of coal tar. It is oxidizing agent against *P. acnes*, which could maintain the sebum production and to losing the comedone. 5% of benzoyl peroxide could inhibit *P. acnes* (20).

Using anti-biotics used for applying to the skin are tetracycline, erythromycin, and clindamycin, and for oral route are tetracycline, erythromycin, minocycline, and ampicillin.

2.1.2.4 Alleviation of inflammation

The agent used in this group is glucocorticoid which has an action on anti-inflammation and anti-androgen. This has been generally used for cystic acne (24).

Dapsone is nonsteroidal, which has been used in strong cystic acne for anti-inflammation and anti-androgen, whereas ibuprofen has a highly anti-inflammation. Isotretinoin has been only used in strong nodulocystic acne (20).

2.2 Mangosteen

Mangosteen, Figure 2.5 has been known widely as medicinal plant especially its pericarp extract, which has been used as anti-bacteria.

Figure 2.5 Mangosteen (http://www.thewisegardener.com)

Scientific name:	*Garcinia mangostana* Linn.
Common name:	*Mangostana garcinia*
General name:	English; mangosteen., France; mangoustan.
Family:	Guttiferae

2.2.1 Get to know mangosteen

Mangosteen is commonly known as “mung-kut” and has been raised to be the “Queen of Fruit”. Its origin was in Southeast Asia (25), it has been used in Asian traditional medicines for treatment of wounds, skin infections, and inflammation acne as examples. It contains high amounts of xanthones, such α-mangostin, which is the main component and other bioactive substances where tannins, flavonoids, and polyphenolics compounds are examples, its chemical structure shown in Figure 2.6. Many reports revealed that extracts from *G. mangostana* gave many medicinal properties, they were anti-oxidants, anti-inflammatory, and anti-allergy (26). Moreover, it was known to promote high anti-

microbial activity against bacteria related to acne inflammation, *P. acnes* (27), and also used as a raw material in cosmetics and drug preparations in preventing or treating acne (28).

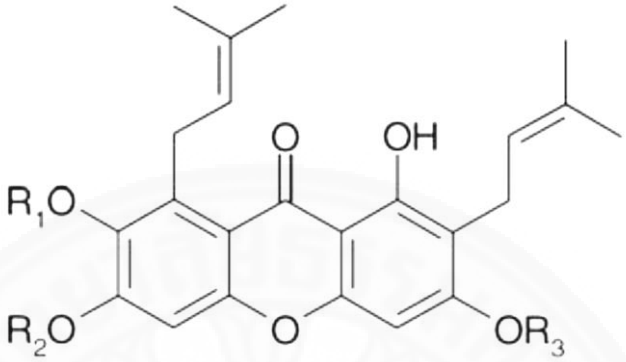

Figure 2.6 Chemical Structure of Mangostin
(http://www.gentcare.com/products_detail/&productId=2084.html)

Molecular formula:	$C_{24}H_{26}O_6$
Molecular weight:	410.47
Melting point:	181.6-182.6 degrees Celsius
Solubility:	In alcohol, ether, acetone, ethyl acetate, and Chloroform (29), (30)

2.2.2 Application of mangosteen

The study of Chomnawang *et al*. in 2005 revealed that 13 crude extracts from 19 medicinal plants crude extracts, which were tested for anti-microbial activities, could inhibit the growth of *P. acnes*, and *G. mangostana* did show the greatest anti-microbial effect (4). The work of Gubelin *et al*. in 2006 revealed that the anti-microbial susceptibility of 53 strains of *P. acnes* isolated from skin specimens of inflammatory acne patients, at the Clinical Hospital University of Chile was tested for acne treatment. All isolates were found to have good response to penicillin, minocycline, and norfloxacin, while erythromycin and clindamycin resistance was

found in 3.8 and 1.9 percentages isolates, respectively. Moreover, resistance to lymecycline, which was intermediate to tetracycline and doxycycline was also found in one isolate (31). In 2008, Leursuwannakit *et al.* reported their study of anti-microbial activity against *P. acne* and *S. aureus* ATCC 25923 of mangosteen crude extract obtained by maceration with 95 percentages ethanol comparing to those of rosemary and clary sage. The results revealed that the Minimal Inhibitory Concentration (MIC) of mangosteen extract was 4.88 micrograms per milliliter for both *P. acnes* and *S. aureus* while clary sage and rosemary extracts gave the MIC for both bacteria of 78.13 and 156.25 micrograms per milliliter, respectively (32).

Vishnu *et al.* reported their study in 2010 that *G. mangostana* could be used a phytomedicine in South East Asia for the treatment of trauma, diarrhea and skin infections. Anti-microbial activity of *G. mangostana* extract powder was then used in determining the MIC by micro-dilution broth technique. It was tested against *Staphylococcus aureus*, *Staphylococcus albus*, *Micrococcus luteus*. The MIC of the extract powder for *S. aureus*, *M. luteus,* and *S. albus* were 200, 50, and 50 micrograms per milliliter, respectively (33). Also in 2010, Pojjananukit and Kajornsheeppanngam, studied the anti-microbial activity three herbal plant extracts, mangosteen, turmeric, and gotu kola against *P. acnes* and *S. aureus.* The results showed that the MIC of mangosteen, turmeric and gotu kola crude extracts that could inhibit *P. acnes* were 12.5, 25, and 200 milligrams per milliliter, respectively and inhibit *S. aureus* were 6.25, 12.5, and 200 milligrams per milliliter, respectively. For Minimal Bactericidal Concentration (MBC) values of mangosteen, turmeric, and gotu kola extracts against *P. acnes* were 25, 50, and 200 milligrams per milliliter, respectively, against *S. aureus* were 12.5, 25, and 200 milligrams per milliliter, respectively. It can be concluded that mangosteen crude extract could be inhibit *P. acnes* and *S. aureus* with the lowest concentration (34).

2.3 Centella

Centella, Figure 2.7, has been known widely as medicinal plant especially its shoot extract, which has been used as wound healing.

Figure 2.7 Centella
(http://www.narniacream.com, http://www.liekr.com/post_138116.html)

Scientific name:	*Centella asiatica* Linn.
Common name:	Asiatic penywort
General name:	English; centella.
Family:	Umbelliferae

2.3.1 Introduction to centella

Centella asiatica has been known as a medicinal plant used since prehistoric periods. Its remedy was well documented in South East Asia for centuries. However, *C. asiatica* has been located in between traditional and modern, scientifically oriented, medicine. The active compound of this tropical plant was characterized, as shown in Figure 2.8, and had the clinical effects in chronic venous diseases, and wound healing disorders treatments (35). In Thailand, it is widely known as “Buabok”(36). Centella contains several active constituents, where the most important are asiaticoside, centelloside, madecassoside, and asiatic acid.

Furthermore, centella also contains other essential components such as, volatile oils, flavonoids, tannin, phytosterol, amino acids, and sugars (37).

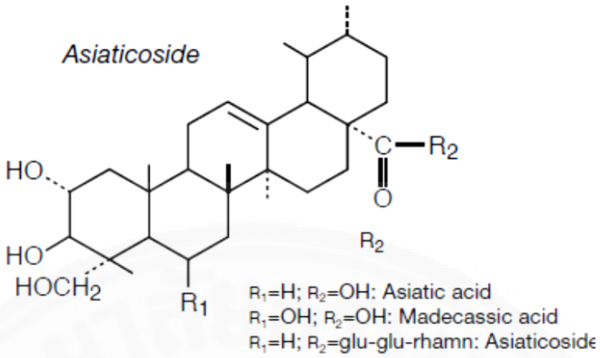

Figure 2.8 Chemical Structure of Asiaticoside (38)

Molecular formula:	$C_{48}H_{78}O_{19}$
Molecular weight:	959.12
Melting point:	230-233 degrees Celsius
Solubility:	In water, ethanol

2.3.2 Application of centella

In 1996, Suguna *et al.* studied the effect on wound healing of the *C. asiatica* extracts on rat dermal wound using topical administration. The results revealed that *C. asiatica* had the actions on wound repair, such as increasing cellular proliferation and collagen synthesis at the wound site causing an increase in collagen content of granulation tissues. The active compound of treated wounds were found to speed up the epithelialization rate, comparing to the controlled wounds (39). Sunilkumar *et al*. in 1998 studied the formulation types of *C. asiatica* extracts such as ointment, cream, and gel, it revealed that gel formulation was the best formula, when it was applied topically, thrice daily for 24 days on the open wounds in rats. It could increase cellular proliferation and collagen synthesis at the wound site, which help increase healing rate, comparing to the control wounds (40). From Shukla *et al.*

in 1999 study, it revealed the effect of asiaticoside, essential extracts from *C. asiatica,* on the levels of certain anti-oxidants in the wound to explain the role of asiaticoside in inducing wound healing. After applying asiaticoside, 0.2 percentages, topical, twice daily for 7 days, to excision-type cutaneous wounds in rats, which led to the increase enzymatic and non-enzymatic anti-oxidants. The results showed a several fold decrease in lipid peroxide levels, in terms of thiobarbituric acid reactive active compound. This could be concluded that asiaticoside could enhance the anti-oxidant levels at an initial stage of healing, which was the important in the healing process (5).

The results of healing study on the effects of *C. asiatica* extract and asiaticoside on acetic acid induced gastric ulcers (kissing ulcers) in rats revealed that it could reduce the size of ulcers at 3 and 7 days in a dose-dependent manner (41).

2.4 Thermoplastic starch

Starch is a natural polymer which is a part of plants. They can be rice starch, corn starch, or cassava starch, these depend on the difference of amylose and amylopectin ratios (Table 2.1), where amylose and amylopectin structures are shown in Figure 2.9 and Figure 2.10. Starches have some properties that are similar to the thermoset plastic but could be degraded naturally. Normally, when thermoplastic starch was prepared it seems to crumble before to melt because the chains of starch mostly have the hydrogen bonds which have limitation properties to use. As a results, it can be modified by mixing with plasticizer before heating, and when it has completed the process, it will get a thermoplastic starch (42).

Table 2.1 Amylose and Amylopectin Contents from Starch (43)

Type of Starch	Amylose (percentage)	Amylopectin (percentage)
Rice Starch	17	83
Glutinous Rice Starch	0-2	98-100
Cassava Starch	17	83

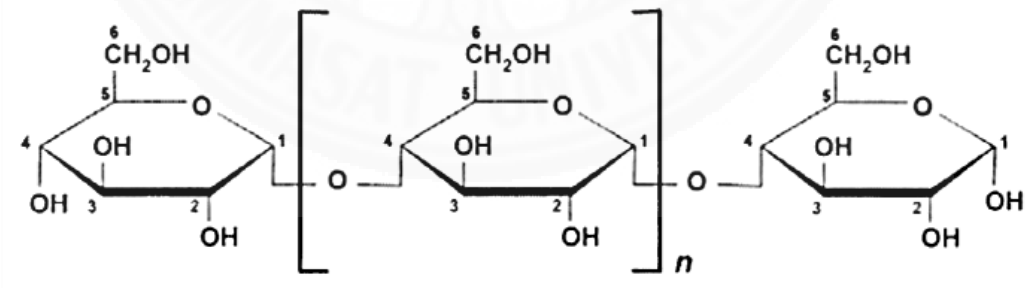

Figure 2.9 Chain of Glucose in Amylose
(http://structures-of-biomolecules.weebly.com/carbohydrates.html)

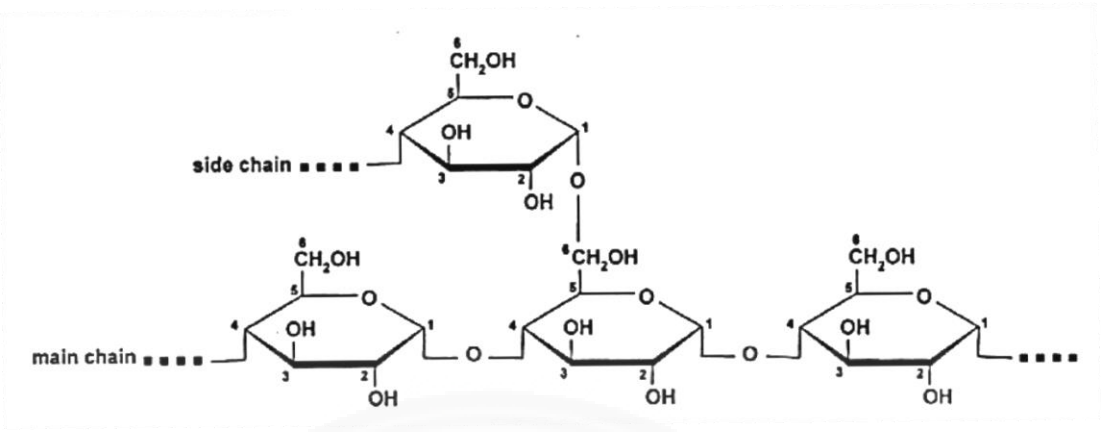

Figure 2.10 Chain of Glucose in Amylopectin
(http://www.handprintingguiderajasthan.in/appendix-2-chemicals-in-pre-treatment)

Plasticizer added into thermoplastic starches could assist the decrease of glass transition temperature (Tg) and assisted starch crumble from heat and shear force. Furthermore, plasticizer could help thermoplastic starch modification by reducing the re-crystallization, which then had the influence on mechanical properties. Plasticizers have a role in forming hydroxyl group (-OH) into starch molecule to assist incorporate between starch molecules (44). The plasticizer frequently used in forming thermoplastic starch was glycerol,

Figure 2.11, which was added 20 to 40 percentage by weight, approximately (45), (46). Moreover, many researches had used glycerol as plasticizer to increase the plasticity and improve the mechanical properties of biopolymer or bio composites. Because of many hydroxyl groups, small molecular size, and high boiling point, glycerol have always been used to enhance the flexibility of thermoplastic starch film (47), (48), (49). However, sodium alginate was also used to substitute into the synthetic polymer, where starch was blended with another polysaccharide, to investigate the processing conditions, and to evaluate the properties of the resulting materials. Sodium alginate, Figure 2.12, is a linear polysaccharide obtained from marine algae. Its structure composed of (1,4)-β-D-mannuronate and (1,3)-α-L-guluronate residues. It was chosen to use in biopolymer, because of its structural features, which contained carboxylic acid groups in every

repeating unit. These characteristics were expected to help an improvement on the compatibility and the mechanical properties of the biopolymers contained sodium alginate (50).

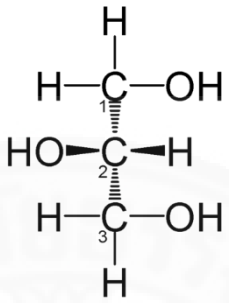

Figure 2.11 Chemical Structure of Glycerol
(https://commons.wikimedia.org/wiki/File:Sn-Glycerol.png)

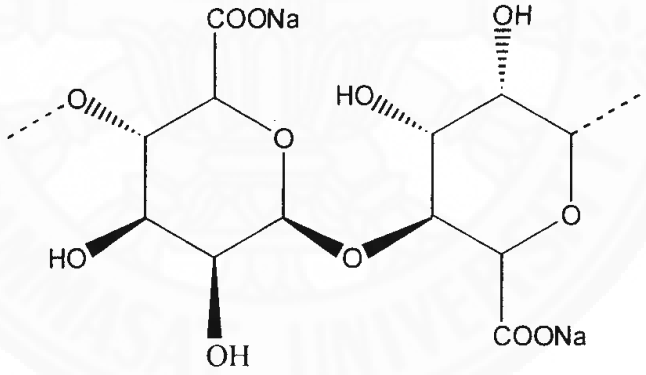

Figure 2.12 Chemical Structure of Sodium Alginate
(https://www.google.ch/patents/WO2001037802A1?cl=en)

Andres *et al*. in 2008 found that the effect of sodium alginate addition in glycerin plasticized corn starch process could cause a significant decrease in plasticization energy and in steady state torque, when sodium alginate content was increased from 0 to 15 percentages. Furthermore, the tendency of the blends to become more rubbery was found when sodium alginate content was increased,

confirming by Differential Scanning Calorimetry (DSC), where the glass transition temperature showed 20 degrees Celsius decrease when sodium alginate content was increased from 0 to 15 percentages, while Scanning Electron Microscopy (SEM) revealed a decrease of the granular starch structures when sodium alginate was increase in the blends (51).

CHAPTER 3
RESEARCH METHODOLOGY

3.1 Thermoplastic starch preparation

3.1.1 Preparation of cassava starch composite films plasticized with glycerol

Cassava starch which was obtained from Pla-Mungkorn trade mark, Tong-chan factory, Chonburi purchased from Talaad Thai, Pathum Thani and glycerol was purchased from Sigma-Aldrich, United State of America. Cassava starch was dried in a hot air oven at 60 degrees Celsius under atmospheric pressure for 24 hours with 3 replications before used (52). The cassava starch and glycerol ratios for films preparation were shown in Table 3.1.

Table 3.1 Cassava Starch and Glycerol Contents

Cassava starch (percentage (w/w))	Glycerol (percentage (w/w))
30	70
40	60
50	50
60	40
70	30

This ingredient were mixed with water and stirred at 100 degrees Celsius for 30 minutes. Subsequently, the mixture was poured into the 6 well muffin tray (diameter 5 centimeters each well) to obtain the 30 milliliters per well and then the mixture was slowly evaporated in a hot air oven at 60 degrees Celsius under atmospheric pressure for 36 hours to prepare the film without the entrapped air bubble. The sample was prepared with 3 replications. After that, the film was kept in

the silica gel desiccator to balance the absorbed moisture before characterized the mechanical properties. The adhesive test was done by using the Texture Analyzer apparatus that equipped with a 30 kilograms loading cell (TA-XT2i, Stable Micro Systems, Surrey, United Kingdom) as seen in Figure 3.2, which the characterization was performed at room temperature under ambient moisture. During the measurement, the film was put on the platform and measured by p/0.25s probes (Figure 3.1). Subsequently, the suitable condition of films was selected by high stickiness (SN), adhesiveness (AN), and cohesiveness (CN) and steady film.

Figure 3.1 p/0.25s Probes
(http://texturetechnologies.com/texture-analysis/Probes-Fixtures.php)

Figure 3.2 TA-XT2i, Stable Micro Systems, Surrey, United Kingdom
(http://texturetechnologies.com/texture-analysis/Probes-Fixtures.php)

Subsequently, the SN, AN, and CN values of each condition were collected to calculate in SigmaPlot program. In addition, the SN, AN, and CN values of the condition were statistically compared to determine the difference of each condition. Then SN, AN, and CN values were averaged and calculated the standard deviation (SD). The statistical difference was analyzed by the One-Way ANOVA tool on SPSS for windows with the Tukey's HSD test. The significant difference was noted at 95 percentages confidence interval.

3.1.2 Preparation of cassava starch/sodium alginate composite films plasticized with glycerol

Sodium alginate was purchased from Sigma-Aldrich in the form of alginic acid sodium salt, from brown algae. The film contents were prepared by varying between cassava starch and sodium alginate, the ratio shown in Table 3.2

Table 3.2 Cassava Starch and Sodium Alginate Contents

Cassava starch (percentage (w/w))	Sodium Alginate (percentage (w/w))
0	100
10	90
20	80
30	70
40	60
50	50
60	40
70	30
80	20
90	10
100	0

This ingredient were mixed by water and stirred by magnetic stirrer at 100 degrees Celsius for 30 minutes. Subsequently, the mixture was poured into the tray (diameter 5 centimeters) to obtain the 30 milliliters per well, then the mixture was slowly evaporated in a hot air oven at 60 degrees Celsius under atmospheric pressure for 36 hours to prepare the film without the entrapped air bubble. The sample was prepared with 3 replications. The film was kept in the silica gel desiccator to balance the absorbed moisture before characterized the mechanical properties.

The adhesive test was done by using the Texture Analyzer apparatus that equipped with a 30 kilograms loading cell (TA-XT2i, Stable Micro Systems, Surrey, United Kingdom), which the characterization was performed at room temperature under ambient moisture. During the measurement, the film was put on the platform and measured by p/0.25s probes. Subsequently, the suitable condition of films was selected by high SN, AN, and CN and steady film.

Subsequently, SN, AN, and CN values of each condition were collected to calculate in SigmaPlot program. In addition, the SN, AN, and CN values of the condition were statistically compared to determine the difference of each condition. The SN, AN, and CN values were averaged and calculated the standard deviation (SD). The statistical difference was analyzed by the One-Way ANOVA tool on SPSS for windows with the Tukey’s HSD test. The significant difference was noted at 95 percentages confidence interval.

3.2 Herbal extract preparation

3.2.1 Herbal extractions

Mangosteen pericarp and centella were separately dried at 45 degrees Celsius for 72 hours and then ground into powder. For mangosteen pericarp extraction, maceration extraction was performed by soaking the powder (5 grams) in 95 percentages ethanol (30 milliliters) at room temperature for 2 days (4). After that, it was filtered through Whatman No.1 filter paper to collect the filtrate, and the remaining residue was macerated again with the same condition for 7 days. The filtrate of both macerations were combined before drying using rotary evaporator under 100 mbar at 45 degrees Celsius until obtain the viscous crude extract. The extract was then dried in hot air oven at 45 degrees Celsius until obtain a constant dry weight. This method gave a high α-mangostin content.

For centella extraction, the powder (30 grams) was extracted by using Soxhlet extraction apparatus with 80 percentages ethanol (300 milliliters) (53), (54). The extractions were done for 30 cycles at boiling temperature of the solvent. This method was derived of high asiaticoside contents. The crude extract was filtered through Whatman No.1 filter paper before drying as the processes described above.

3.2.2 Formulation preparations

The extracts from mangosteen pericarp and centella were dissolved in absolute ethanol to obtain 1 milligram per milliliter stock solution. It was coded as MP and CL, respectively. The extract stock solutions were mixed together with various ratios to prepare 7 formulations namely MG0, MG2, MG4, MG5, MG6, MG8, and MG10 (Table 3.3). Subsequently, the 7 formulations were conducted to scan wavelength by spectrophotometer.

Table 3.3 Formulation Contents

Codes	Mangosteen pericarp extract (percentage (w/w))	Centella extract (percentage (w/w))
MG0	0	100
MG2	20	80
MG4	40	60
MG5	50	50
MG6	60	40
MG8	80	20
MG10	100	0

3.2.3 Formulation characterization

The 7 formulations were prepared and dissolved in releasing buffer to obtain standard curve of the concentrations of 1, 0.8, 0.6, 0.4, and 0.2 milligrams per milliliter.

3.2.4 The extracts preparation for HPLC analysis

The mangosteen extract was dissolved in methanol, and was filtered through a 0.45 micrometers filter before use. Standard solution of alpha-mangosteen was prepared as also.

Experiments were conducted using Nexera, Shimadzu, Japan, on HPLC system consisting of a LC-30AD pump, SIL-30AC auto-sampler, SPD-M30A photodiode array detector. LabSolutions software was utilized for instrument control, data collection, and data processing. Chromatographic column tested were Kromasil C_{18} (150 x 4.6 millimeters I.D., 5 micrometers, Waters). The mobile phases consisted of A: water and B: 90 percentages acetonitrile, 10 percentages methanol. All separations were carried out at a flow rate of 0.5 milliliters per minute, the column temperature was set at 30 degrees Celsius and a wavelength of 324 nanometers. The injection volume for all the samples and standard was set at 10 microliters (55).

The centella extract was dissolved in methanol, and was filtered through a 0.45 micrometers filter before use. Standard solution of asiaticoside was prepared as also.

Experiments were conducted using Nexera, Shimadzu, Japan, on HPLC system consisting of a LC-30AD pump, SIL-30AC auto-sampler, SPD-M30A photodiode array detector. LabSolutions software was utilized for instrument control, data collection, and data processing. Chromatographic column used was Kromasil C_{18} (150 x 4.6 millimeters I.D., 5 micrometers, Waters). The mobile phases consisted of A: 75 percentages water and B: 25 percentages acetonitrile. All separations were carried out at a flow rate of 1 milliliter per minute with column temperature of 30 degrees Celsius and wavelength of 203 nanometers. 10 microliters injection volume was applied for all samples and standard (56).

3.2.5 DPPH assay

The formulations were characterized the anti-oxidant activity by using modified DPPH assay (57). Before doing the reactions, 100 microliters of the samples, which were MG0, MG2, MG4, MG5, MG6, MG8, and MG10, were 2-fold serial diluted in 96-well plates from the 1^{st} well to the 11^{th} well to prepare the concentrations of 500, 250, 125, 62.5, 31.3, 15.6, 7.8, 3.9, 2.0, 0.1, and 0.05 micrograms per milliliter. The 12^{th} well was reserved for the control, no extract added. 100 microliters of 0.2 millimolars DPPH stock solution was then added into each well to begin the reaction. The plates were incubated for 30 minutes in the dark before measured the absorbance at 520 nanometers using microplate reader instrument (BioTek, PowerWave XS2, USA). Each sample was done in triplicate and standard ascorbic acid (AA) was used as the standard anti-oxidant. The percentage of radical scavenging inhibition was calculated by using Equation 3.1. Subsequently, the radical scavenging values of each sample were collected to calculate the inhibitory concentration 50 percentages (IC_{50}) using Standard Curves Analysis tool in SigmaPlot program. In addition, the radical scavenging values of the formulations, at 31.3 micrograms per milliliter, were statistically compared to determine the difference of each

formulation. Radical scavenging values were averaged and calculated the standard deviation (S.D.). The statistical difference was analyzed by the One-Way ANOVA tool on SPSS for windows with the Tukey's HSD test. The significant difference was noted at 95 percentages confidence interval.

Radical scavenging (percentage) = 100 (C - S) / C

Equation 3.1 Radical Scavenging Calculation; C is the Absorbance of DPPH without Sample and S is the Absorbance of DPPH with Sample.

3.2.6 Total phenolic assay

The herbal extracts, MP and CL, were determined the total phenolic contents (TPC) using Folin-Ciocalteu method (58). Before starting the reaction, Folin-Ciocalteu (FC) reagent and sodium carbonate (Na_2CO_3) solution were diluted with deionized water to 10 and 7 percentages (w/v), respectively. 30 microliters of the extract stock solutions were pipetted into the 1stwell of row A and E, diluted with 120 microliters of absolute ethanol and then serially diluted two fold from the 1st to the 10th well to obtain concentrations of 100, 50, 25, 12.5, 6.3, 3.1, 1.6, 0.8, 0.4, and 0.2 micrograms per milliliter, respectively. Subsequently, 150 microliters of FC reagent was added and mixed. After leaving in the dark for 5 minutes, it was then added with sodium carbonate solution. The reaction mixtures were incubated at room temperature in the dark for 30 minutes. The reactions were done in triplicate. The absorbance of the reaction was measured at 765 nanometers using micro-plate reader. The gallic acid (GA) standard was prepared with the same processes performed above and the reactions were done with the same conditions. The calibration curve was plotted to get the slope for gallic acid equivalent (GAE) calculation and TPC values were expressed as mg GAE per gram extract.

3.2.7 Anti-microbial assay

3.2.7.1 Microbial strain

The bacteria causing acne is Gram-positive bacteria: *Propionibacterium acnes* DMST 14916 and *Staphylococcus aureus* DMST 8840 from Department of Medical Sciences (DMSC) were cultured in Brain Heart Infusion agar (BHA) and Mueller Hinton agar (MHA), respectively, and *P. acnes* was grown in anaerobic conditions. Sub-cultured were done on a fresh appropriate agar plate at 24 hours for *S. aureus* and 72 hours for *P. acnes*. Then 3 colonies of *S. aureus* were transferred into the glass tube containing 5 milliliters of Mueller Hinton broth (MHB), and incubated at 37 degrees Celsius and shaking at 150 rounds per minute for 2 hours, while 3 colonies of *P. acnes* were transferred into the glass tube containing 5 milliliters of Brain Heart Infusion (BHI), and incubated at 37 degrees Celsius and shaking at 150 rounds per minute for 2 hours at anaerobic conditions prior to anti-microbial activity assay by using gentamicin, known as the effective anti-microbial drugs against bacteria, as positive control.

3.2.7.2 Minimal Inhibitory Concentration (MIC)

MIC was performed by broth micro-dilution method as previously reported with some modifications (59). The inoculum was adjusted to 0.5 McFarland standards and diluted with sterile MHB for *S. aureus* and BHI for *P. acnes* at 1:200 to give a final concentration of 5×10^5 CFU per milliliter. Serial two-fold dilutions of each crude extract are prepared. The 50 microliters of each concentration of formulations extracts solution and 50 microliters of the inoculums were added into 96 well micro plates. The well number 2 to 11 were performed the 2-fold serial dilution to obtain the extract concentration of 1,000.00, 500.00, 250.00, 125.00, 62.50, 31.25, 15.63, 7.81, 3.91, 1.95, and 0.98 micrograms per milliliter, respectively. The sterile plates and covers were sterile, then shaken to mix the contents using a plate shaker and incubated at 37 degrees Celsius for 18 and 48 hours for *S. aureus* and *P. acnes*, respectively. *P. acnes* was also done at anaerobic conditions.

MIC of the tested samples were detected after the addition of 10 microliters of resazurin (blue compound, 7-hydroxy-3H-phenoxazin-3-one 10-oxide) and incubated at 37 degrees Celsius for 2 hours. The result is interpreted by the change of resazurin color. MIC value is the lowest dilution of crude extract solution that can inhibit microorganism by generating blue color of resazurin. The assay was repeated in triplicate. The suitable formulations extract would be selected and loaded in the films.

3.3 Extract formulation loaded films preparation

3.3.1 Releasing profiles investigation

The prepared films were cut into a round shape with 1 centimeter diameter, and then kept in silica gel desiccator at room temperature. This study used modified Franz diffusion cells to investigate the releasing rate of the extract from the films at 37 degrees Celsius. The selected extracts were conducted and released through the cellophane membrane, while phosphate buffer saline pH 5.5 (PBS pH 5.5) was used as a receiving medium. This arrangement was set to simulate the human skin condition. The releasing was investigated for 480 minutes, while the phosphate buffer with the releasing formulations were collected for 500 microliters every 15 minutes from 1 to 60 minutes, collected every 30 minutes from 60 to 180 minutes, and collected every 60 minutes from 180 to 480 minutes. Every collected samples were replaced with fresh PBS pH 5.5 in the equal volume. The released extracts was determined using spectrophotometer with the optimum wave length of each formulation, where the releasing concentrations were calculated using the prepared standard curve.

3.3.2 Disc diffusion assay

The agar disc diffusion method was used to determine the anti-microbial efficiency of the films against *P. acnes* DMST 14916 and *S. aureus* DMST 8840. Bacterial strains grown and the inoculums were adjusted to 0.5 McFarland standards. Each inoculum was applied on MHA for *S. aureus* and BHA for *P. acnes*. Paper discs of 1 centimeter diameter containing 6.25 milliliters of suitable formulations was placed on the surface of the prepared agar plates and incubated at 37 degrees Celsius for 24 and 72 hours for *S. aureus* and *P. acnes*, respectively, *P. acnes* were incubated at anaerobic conditions, while plain filter papers (Whatman No.1) and filter paper containing 20 microliters of 0.1 milligrams per milliliter gentamicin were used

as a negative control and a positive control, respectively. Inhibition zones were then observed and measured (60).

CHAPTER 4
RESULTS AND DISCUSSION

4.1 Thermoplastic starch preparation

4.1.1 Preparation of cassava starch composite films plasticized with glycerol

The adhesive test was performed to derive the mechanical properties of stickiness (SN), adhesiveness (AN), and cohesiveness (CN) of the starch composite films. The results exhibited that the increase of glycerol (GLY) content significantly increased the values of SN, AN, and CN of the films as shown in Table 4.1 and Figure 4.1. But the increase in GLY contents caused the wrinkle appearance of the films and not steady, this could be caused by the rich in hydroxyl groups of GLY, as it acted as a plasticizer. It was important to reduce the biopolymer intermolecular forces, to improve the mechanical characteristics of the films, film extensibility is an example (61). As a result, the mechanical properties, unsteadiness, and the wrinkle appearance of the films had to be improved.

The solution was done through the addition of sodium alginate (AL) into the film, it was reported in previous research as it could behave as a plasticizer and becoming another polymer ingredient in a thermoplastic (62). With another reason, AL was also chosen because of its structural features, which contain carboxylic acid groups in every repeating unit that has a structure configuration. These characteristics could improve the compatibility and mechanical properties of the polymers blended (63).

Table 4.1 Stickiness, Adhesiveness, and Cohesiveness analyzed from adhesive test of cassava starch composite films plasticized with glycerol

Glycerol content (percentage)	Stickiness (gram)	Adhesiveness (gram per second)	Cohesiveness (millimeter)
30	7.61 ± 0.43^{a}	$0.22\pm0.02^{\alpha}$	0.28 ± 0.01^{I}
40	33.95 ± 4.09^{b}	$0.32\pm0.05^{\alpha,\beta}$	0.29 ± 0.01^{I}
50	41.91 ± 0.95^{c}	$0.41\pm0.04^{\beta}$	0.30 ± 0.01^{I}
60	43.01 ± 0.78^{c}	$0.57\pm0.02^{\gamma}$	0.31 ± 0.00^{I}
70	54.31 ± 3.40^{d}	$0.86\pm0.06^{\delta}$	0.34 ± 0.03^{II}

(Different symbols including a, b, c, and d represent significant difference at 95% confidence interval)

(Different symbols including α, β, and γ represent significant difference at 95% confidence interval)

(Different symbols including I and II represent significant difference at 95% confidence interval)

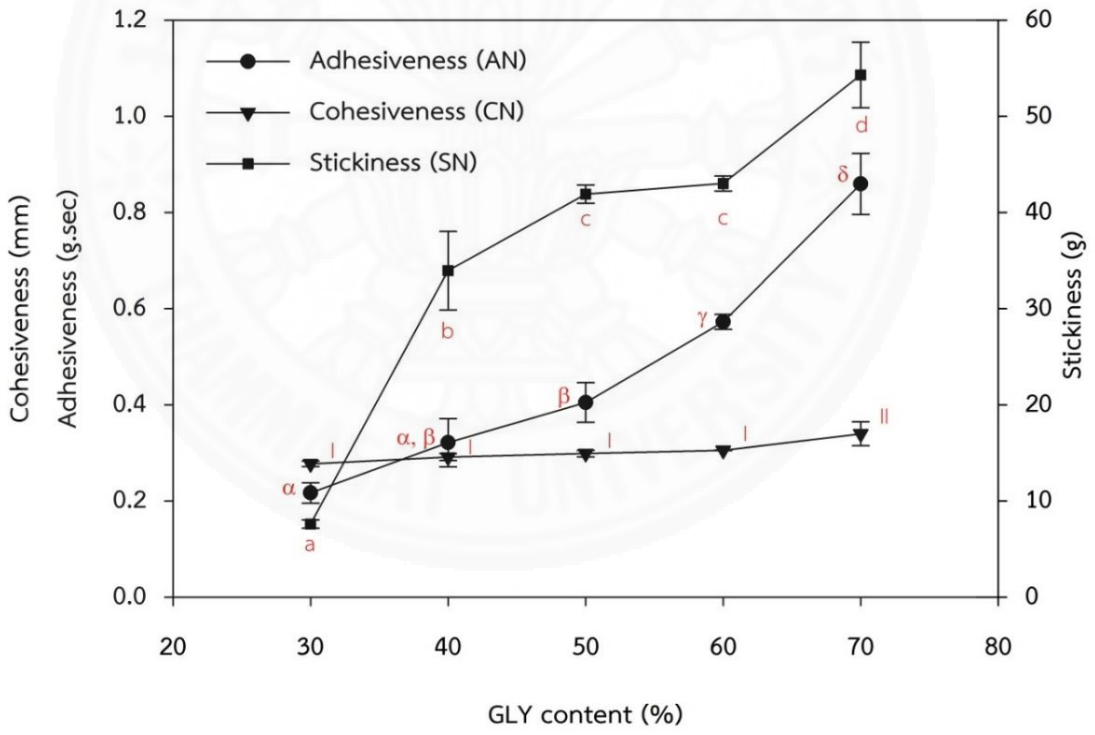

Figure 4.1 Stickiness, Adhesiveness, and Cohesiveness analyzed from adhesive test of cassava starch composite films plasticized with glycerol

4.1.2 Preparation of cassava starch/sodium alginate composite films plasticized with glycerol

The adhesive test was performed to derive the mechanical properties including SN, AN, and CN. The results revealed that the increase in AL content significantly increased the SN, AN, and CN values of the films as shown in Table 4.2 and Figure 4.2. And the increase of AL contents also increased the hardness and decreased the brittle of films. This might be due to the interaction between COO^- groups in AL and –OH groups in cassava starch, through the hydrogen bonds (62), (64). It could be seen that the addition of AL into starch/glycerol blends could increase the action of plasticizer in disrupting the occurring of starch granules. The effects happened in mechanical, thermal, and processing properties also indicated that the highly free sodium alginate oligomers were acting as a secondary plasticizer. This new plasticizer system which was glycerin solvated sodium alginate should be more efficient in disrupting the bonds holding together in the starch granules (51), (65).

Table 4.2 Stickiness, Adhesiveness, and Cohesiveness analyzed from adhesive test of cassava starch/sodium alginate composite films plasticized with glycerol

Alginate content (percentage)	Glycerol content (percentage)	Stickiness (gram)	Adhesiveness (gram per second)
0	49.99±0.73^{a}	1.45±0.03$^{\alpha}$	0.52±0.02^{I}
10	68.31±0.62^{b}	1.65±0.05$^{\beta}$	0.55±0.01^{II}
20	48.51±0.60^{a}	0.77±0.00$^{\gamma}$	0.40±0.00^{III}
30	47.67±1.48^{a}	0.74±0.02$^{\gamma}$	0.34±0.03^{IV}
40	40.89±0.81^{c}	0.66±0.04$^{\delta}$	0.32±0.00$^{IV, V}$
50	39.20±1.06^{c}	0.55±0.02$^{\varepsilon}$	0.31±0.00$^{IV, V}$
60	28.87±0.76^{d}	0.48±0.00$^{\varepsilon, \eta}$	0.31±0.00$^{IV, V}$
70	24.16±0.62^{e}	0.45±0.01$^{\eta, \theta}$	0.30±0.01^{V}
80	21.40±0.72^{f}	0.38±0.01$^{\theta, \kappa}$	0.30±0.00^{V}
90	16.75±0.52^{g}	0.36±0.01$^{\kappa, \lambda}$	0.29±0.01^{V}
100	15.10±0.77^{g}	0.29±0.01$^{\lambda}$	0.29±0.01^{V}

(Different symbols including a, b, c, d, e, f, and g represent significant difference at 95% confidence interval)

(Different symbols including $\alpha, \beta, \gamma, \delta, \varepsilon, \eta, \theta, \kappa$, and λ represent significant difference at 95% confidence interval)

(Different symbols including I, II, III, IV, and V represent significant difference at 95% confidence interval)

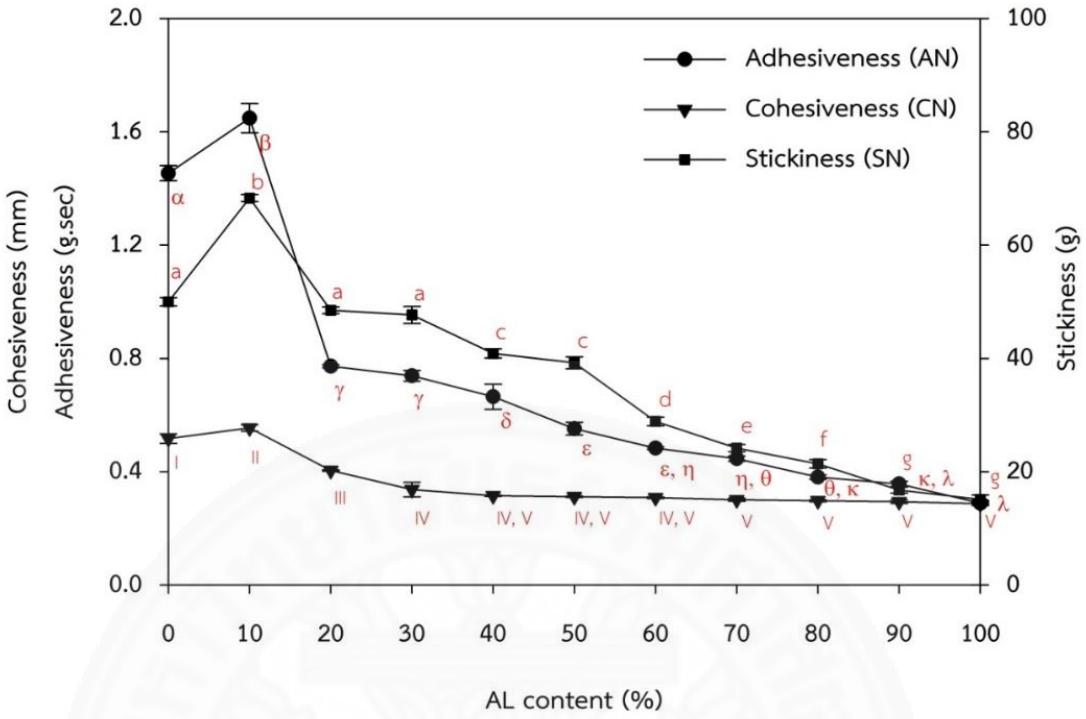

Figure 4.2 Stickiness, Adhesiveness, and Cohesiveness analyzed from adhesive test of cassava starch/sodium alginate composite films plasticized with glycerol

4.2 Herbal extract preparation

4.2.1 Herbal extraction

In extraction the 500 grams of dried mangosteen pericarp, the maceration method was chosen, while the extraction of 220 grams centella, the Soxhlet method was chosen. This was the results of preliminary studies for choosing the most appropriate methods of each kind of herbs selected in this research. The yields of the crude extracts from mangosteen pericarp and centella was shown in the Table 4.3.

Table 4.3 Percentage yield of ethanol crude extract of mangosteen pericarp and centella

Herbs	Weight of herbs (Gram)	Weight of extracts (Gram)	Yield of extract (Percentage)
Mangosteen pericarp	500	91.20	18.24
Centella	220	60.30	27.41

4.2.2 Formulation preparations

The preparation of extract formulations was shown in Table 3.3. They were determined to scan wavelength from 250-700 nanometers, when samples of 1 milligram per milliliter were diluted 200X with releasing buffer. The method was validated at 204, 280, and 320 nanometers. The wavelength of 280 nanometers was chosen to investigate the releasing profile of the selected formulation film because the 280 nanometers could measure the active compound in the extract formulations.

4.2.3 Formulations characterization

The selected formulation of the extracts was carried out its standard curve of 0 to 120 micrograms per milliliter with 20 micrograms per milliliter intervals by diluting with the releasing buffer. The results shown in Figure 4.3, with the relation of 0.015 between concentrations (X axis) and absorbance of 280 nanometers (Y axis) at R-square of 0.995, it would be used for releasing profile calculation.

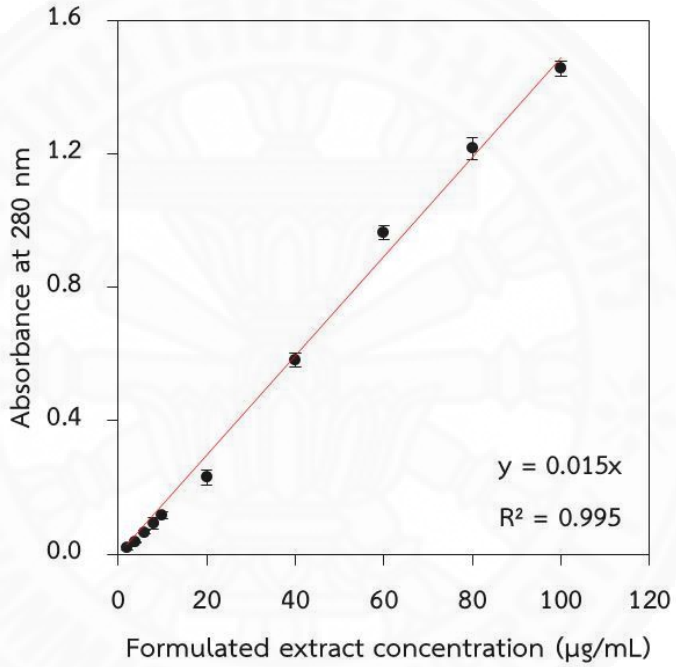

Figure 4.3 Standard curve of formulated extract at 280 nanometers

4.2.4 The extracts preparation for HPLC analysis

The extracts of mangosteen pericarp and centella were dissolved in methanol with concentration of 0.1 and 1 milligrams per milliliter. The results shown in Figure 4.4 and Figure 4.5 were from HPLC chromatogram of mangosteen pericarp and centella extracts.

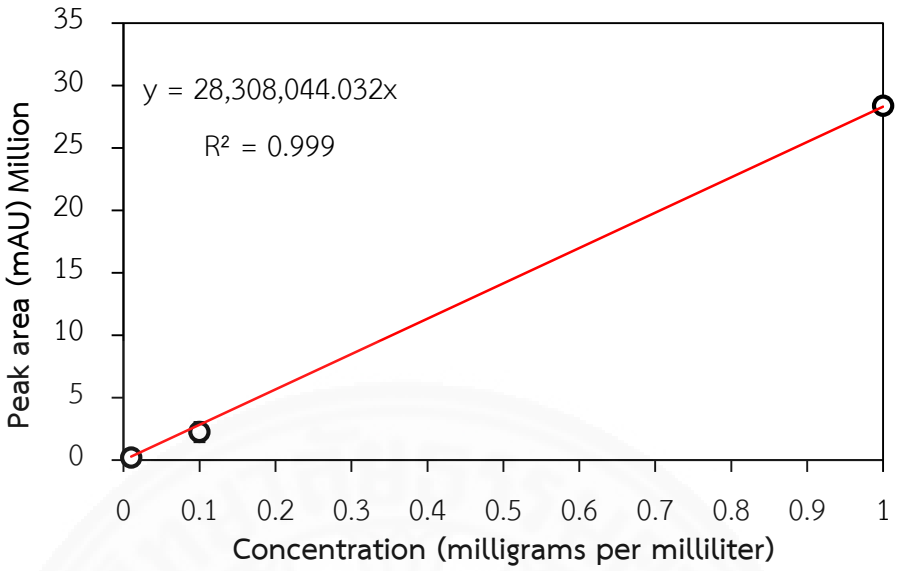

Figure 4.4 Peak area of mangosteen pericarp extract

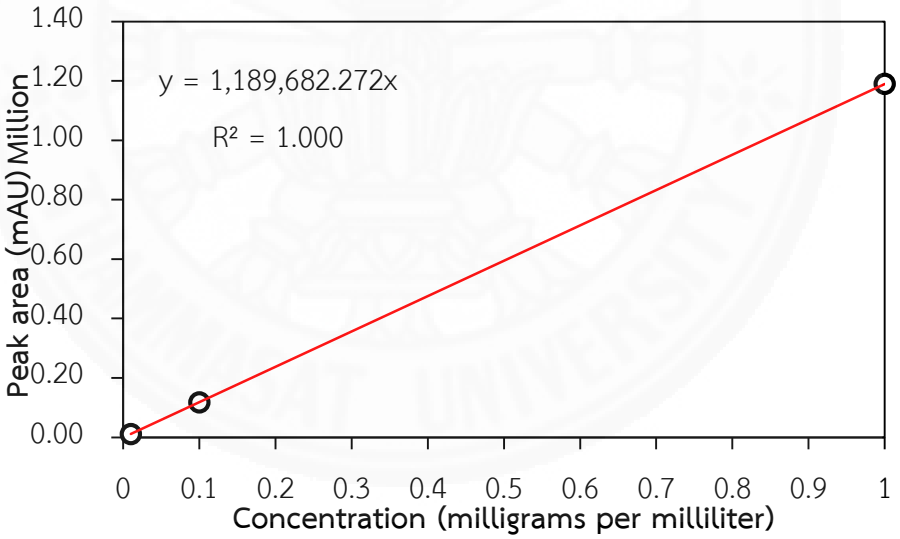

Figure 4.5 Peak area of centella extract

In this study the 1 milligram per milliliter concentration of the α-mangostin from mangosteen pericarp extract and α-mangostin standard were dissolved in methanol and prepared for HPLC analysis. From the chromatograms, it was seen that the peaks of both α-mangostins were found in very close retention

time of 24.648 and 24.664 minutes for α-mangostin in extract and in standard, respectively.

This study also found that asiaticoside in centella extract and asiaticoside standard at similar concentration of 1 milligram per milliliter dissolved in methanol and prepared for HPLC analysis had the similar retention time on chromatogram. Asiaticoside from centella extract and asiaticoside standard showed the retention time of 12.916 and 12.982 minutes, respectively. From the HPLC results shown in Table 4.4, it revealed that 1 milligram per milliliter of mangosteen pericarps extract and centella extract had the yield of α-mangostin and asiaticoside at 18.88 and 1.57 percentages by weight, respectively.

Table 4.4 Yield of α-mangostin and asiaticoside in their herbal extracts

Substance (1 milligram per milliliter)	**Yield of extract (milligram per milliliter)**
α-mangostin in mangosteen pericarp extract	0.192
α-mangostin standard	1.017
Asiaticoside in centella extract	0.022
Asiaticoside stadard	1.404

4.2.5 DPPH assay

Anti-oxidant activities of formulations MG0 to MG10 were shown in Table 4.5 and Figure 4.6. It could be seen that the inhibitory concentrations at 50 percentages or IC_{50} were increase from MG10 to MG0 and the radical scavenging concentrations were decrease from MG10 to MG0. The increase of IC_{50} and decrease of radical scavenging followed the decrease in MG content. When compare only the extracts, it was found that MG was more effective in anti-oxidation than CL. Among 7 herbal formulations, MG8 was the most effective formulation (17.1 micrograms per milliliter of IC_{50} and 69.99±4.67 percentages of radical scavenging).

However, although MG10 obtained the best anti-oxidant property but it lacked of wound healing activity, as it had no centella extracts in its ingredients. Thus, the formulation to be chosen for further film development was MG8 as it contained centella extract for wound healing, and its anti-oxidant activity was the second high from MG10 (66).

Table 4.5 Inhibitory concentration 50 percentages (IC_{50}) of herbal formulations

Codes	Inhibitory concentration at 50 percentages (IC_{50}) (microgram per milliliter)	Radical scavenging at 31.3 micrograms per milliliter (percentage)	Total phenolic content at 5 micrograms per milliliter (mg GAE per gram extract)
MG10	14.8	75.93±12.16 $^{\alpha}$	5,179.64±542.66
MG8	17.1	69.99±4.67 $^{\alpha, \beta}$	-
MG6	20.0	62.37±7.14 $^{\alpha, \beta}$	-
MG5	22.7	58.93±15.59 $^{\alpha, \beta}$	-
MG4	27.1	52.87±9.83 $^{\alpha, \beta}$	-
MG2	36.8	41.55±9.26 $^{\beta, \gamma}$	-
MG0	61.7	31.79±10.54 $^{\gamma}$	2,418.81±123.10
AA	3.0	-	-

(Different symbols including α, β, and γ represent significant difference at 95% confidence interval)

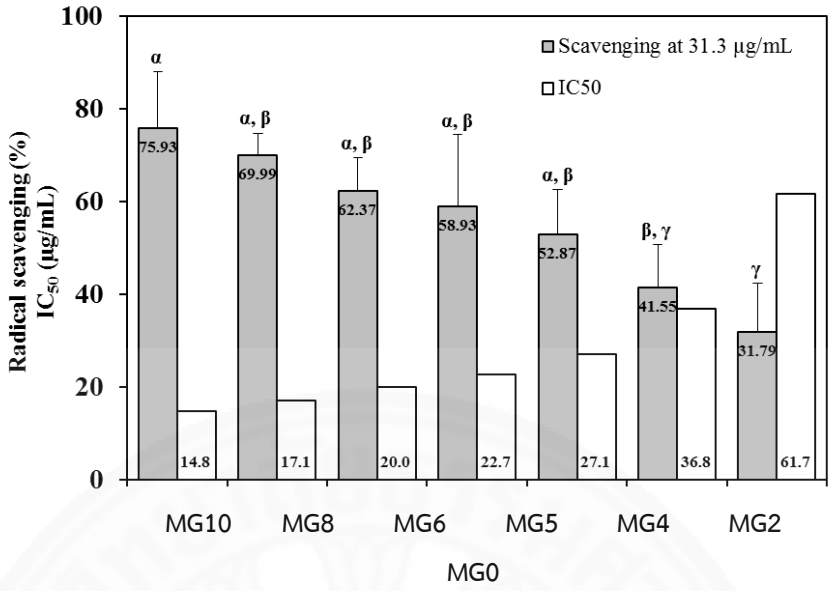

Figure 4.6 The IC_{50} and Radical Scavenging at 31.3 micrograms per milliliter of herbal formulations

4.2.6 Total phenolic assay

For TPC determination, also shown in Table 4.5, revealed that MG10 which held the highest TPC content of 5,179.64±542.66 milligrams GAE per gram extract, had TPC almost two times higher than that of MG0, which had TPC only 2,418.81±123.10 milligrams GAE per gram extract. These results confirmed MG10 formulation was the most effective in anti-oxidant activity (66).

4.2.7 Anti-microbial assay

The minimal inhibitory concentration of formulation extract on acne causative bacteria *Propionibacterium acnes* and *Staphylococcus aureus* was determined by the used of the standard Minimal Inhibitory Concentration (MIC) method. From Figure 4.7 the formulations, which obtained the best MIC values were MG10 and MG8 against *P. acnes* DMST 14916 and *S. aureus* DMST 8840. Their MIC values for *P. acnes* and *S. aureus* were 15.63 and 1.95 micrograms per milliliter, for

MG10 and MG8, respectively. The experimental results also indicated that the inhibitory effect of the bacteria may possess high synergetic effect on anti-microbial activities.

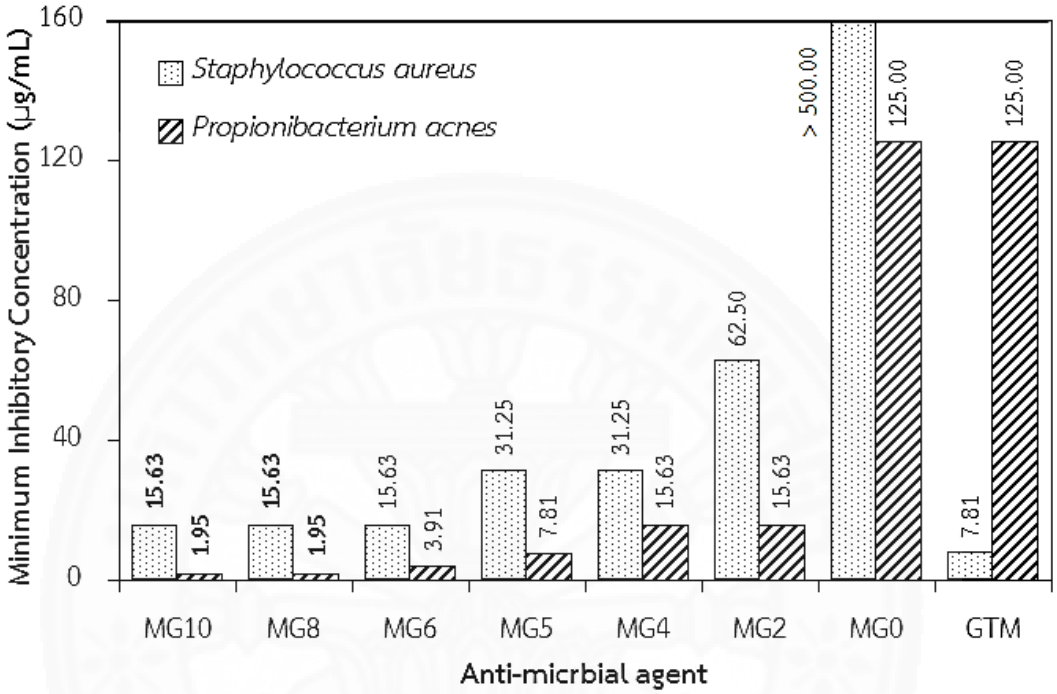

Figure 4.7 The inhibition of formulated extracts and gentamicin against *Propionibacterium acnes* DMST 14916 and *Staphylococcus aureus* DMST 8840

4.3 Preparation of formulations loaded films and releasing test

After the most suitable formulation was selected, and blended into the films. The releasing profile was observed and anti-microbial activity of the released contents was then investigated.

4.3.1 Releasing profiles investigation

In this study, after the cassava starch/sodium alginate composite films plasticized with glycerol and loaded with selected formulation extract was prepared by using the similar methods explained in Table 3.1. The releasing investigation was then carried out only the suitable formulation extracts, MG8, the results showed that the extract contained in the films were continuously released at different rates of 0.67 micrograms per milliliter per minute (1 to 15 minutes), 0.1 micrograms per milliliter per minute (15 to 30 minutes) until its accumulated concentration stayed approximately constant, 11.5 micrograms per milliliter, at 40 minutes.

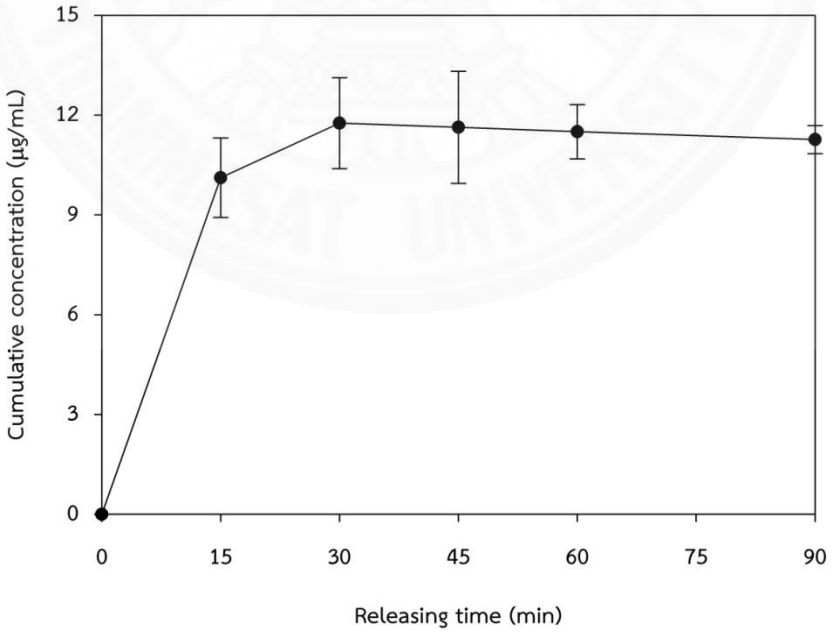

Figure 4.8 Releasing profile of the starch/sodium alginate composites films added with MG8 formulation extracts

4.3.2 Disc diffusion assay

The anti-microbial study of the releasing content of MG8 added composite films were tested against acne causative bacteria by disc diffusion method. The results are shown in Table 4.6.

Table 4.6 Disc diffusion assay of the releasing content of MG8 composite films

Extracts	Inhibition zone (millimeter)	
	P. acnes	*S. aureus*
Formulation MG8	1.489±0.105	1.756±0.201
Gentamicin	1.367±0.115	2.233±0.153

From Table 4.6, the results revealed that the content released from the MG8 composite films showed inhibitory effect against *P. acnes* DMST 14916 and *S. aureus* DMST 8840 with the large inhibition zone of 1.489±0.105 millimeters and 1.756±0.201 millimeters, respectively, where MG8 composite films showed larger inhibition zone on *P. acnes* and smaller inhibition zone on *S. aureus* when comparing to gentamicin, which gave clear zone of 1.367±0.115 and 2.233±0.153 millimeters on testing with *P. acnes* and *S. aureus*, respectively.

CHAPTER 5
CONCLUSIONS AND RECOMMENDATIONS

5.1 Thermoplastic starch preparation

In this part of study, the composite films were prepared from cassava starch plasticized with glycerol. It was found that when glycerol content was increased, it significantly increased the values of stickiness (SN), adhesiveness (AN), and cohesiveness (CN) of the films. Although the highest values were found at starch:glycerol ratio of 30:70, with SN, AN, and CN values were 54.31±3.40 g, 0.86±0.06 g.sec, and 0.34±0.03 mm, respectively (p-value<0.05), but the wrinkle or unsteadiness of films could be seen instead.

To improve the mechanical properties and unsteadiness of the films, sodium alginate was then added to the ingredients as an additional plasticizer for thermoplastic films. The cassava starch/sodium alginate composite films plasticized with glycerol were then tested for their mechanical properties, SN, AN, and CN. The results revealed that increasing of AL content could significantly increase SN, AN, and CN of the films. At starch:sodium alginate ratio of 90:10, SN, AN, and CN values were at the highest of 68.31±0.62 g, 1.65±0.05 g.sec, and 0.55±0.01 mm, respectively (p-value<0.05). The steadiness of the films could also be seen.

Then the suitable condition in preparing films was shown in polymer ratio of polymer:glycerol at 30:70, while starch:sodium alginate ratio was 90:10. It was then explained as (ST 90:AL 10) 30:GLY 70. This formula was then added with the selected herbal extracts formulation, MG8, to investigate the releasing profile.

5.2 Herbal extract preparation

Anti-oxidant activities of MG10 and MG8 were found to be the first and second lowest of inhibitory concentration 50 percentages (IC_{50}) and the first and second highest of radical scavenging values. Total phenilic content (TPC) determination shown in Table 4.5, also confirmed the highest TPC value obtained in MG10 of 5,179.64±542.66 mg GAE per gram extract, which was related to the mangosteen extracts in the formulations.

However, the efficiency of the formulations and the extracts on anti-oxidant activities, IC_{50} and radical scavenging values were ordered to be MG10, MG8, MG6, MG5, MG4, MG2, and MG0, from lowest to highest and highest to lowest, respectively. It was found that MG had more influence on anti-oxidation than that of CL, but it was important to have CL extracts in the formulation for wound healing property addition. As a results, MG8 with its 17.1 micrograms per milliliter of IC_{50} and 69.99±4.67 percentages of radical scavenging values was then selected to add into the composite film.

The minimal inhibitory concentration of extract formulations on acne causative bacteria *Propionibacterium acnes* and *Staphylococcus aureus* was determined by using standard Minimal Inhibitory Concentration (MIC) method. All formulations and extracts, MG10, MG8, MG6, MG5, MG4, MG2, and MG0, were investigated by broth micro-dilution method. MG8 was found to be the most effective formulation against *P. acnes* DMST 14916 and *S. aureus* DMST 8840. Its MIC values against both bacteria were 15.63 and 1.95 micrograms per milliliter, respectively, comparing to the MIC values of gentamicin against both bacteria were 7.81 and 125 micrograms per milliliter, respectively.

Moreover, this study revealed that the combination of extract was equivalent, not the combination in their anti-microbial activity. This also indicated that the inhibitory effect of these bacteria might possess high synergetic effect on anti-microbial activities.

5.3 Preparation of extract formulation loaded films

The cassava starch/sodium alginate composite films plasticized with glycerol added with extract formulation MG8 were prepared for observing the releasing profile. The films were continuously released at different rates of 0.67 micrograms per milliliter per minute (1 to 15 minutes), 0.1 micrograms per milliliter per minute (15 to 30 minutes) until its accumulated concentration stayed approximately constant of 11.5 micrograms per milliliter, at 40 minutes.

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APPENDICES

APPENDIX A
REAGENT PREPARATION

A.1 Phosphate buffered saline solution pH 5.5 (PBS pH 5.5)

- **Stock solution preparation**

  - Potassium dihydrogen phosphate solution (136.1 grams per liter)

- Potassium dihydrogen phosphate (KH_2PO_4)	13.61	grams
- Distilled water to	100	milliliters

  - Disodium hydrogen phosphate solution (358.1 grams per liter)

- Disodium hydrogen phosphate (Na_2HPO_4)	35.81	grams
- Distilled water to	100	milliliters

  - Saline solution (10X)

- Sodium chloride (NaCl)	8	grams
- Potassium chloride (KCl)	0.2	grams
- Distilled water to	100	milliliters

- **PBS pH 5.5 preparation**

9.64 milliliters of potassium dihydrogen phosphate solution, 0.36 milliliters of disodium hydrogen phosphate solution, and 10 milliliters of saline solution were mixed together. Then, total volume of the solution was adjusted with distilled water to be 100 milliliters.

A.2 Releasing buffer solution

- **Stock solution preparation**

  - Potassium dihydrogen phosphate solution (136.1 grams per liter)

- Potassium dihydrogen phosphate (KH_2PO_4)	13.61	grams
- Distilled water to	100	milliliters

  - Disodium hydrogen phosphate solution (358.1 grams per liter)

- Disodium hydrogen phosphate (Na_2HPO_4)	35.81	grams
- Distilled water to	100	milliliters

  - Saline solution (10X)

- Sodium chloride (NaCl)	8	grams
- Potassium chloride (KCl)	0.2	grams
- Distilled water to	100	milliliters

  - Absolute ethanol

- **Releasing buffer preparation**

9.64 milliliters of potassium dihydrogen phosphate solution, 0.36 milliliters of disodium hydrogen phosphate solution, 10 milliliters of saline solution, and 10 milliliters of absolute ethanol were mixed together. Then, total volume of the solution was adjusted with distilled water to be 100 milliliters.

A.3 Reagent for DPPH radical scavenging assay

- **Stock solution preparation**

- DPPH solution (0.2 millimolars)

- 2,2-diphenyl-1-picrylhydrazyl (DPPH)	7.89	milligrams
- Absolute ethanol to	100	milliliters

A.4 Reagent for determination of total phenolic content

- **Stock solution preparation**

- Folin-Ciocalteu's solution (0.2 molars)

Folin-Ciocalteu's reagent of 0.2 Molars concentrations was diluted 10-fold with distilled water.

- Sodium carbonate solution (75 grams per liter)

Sodium carbonate (Na_2CO_3)	7.5	grams
Distilled water to	100	milliliters

A.5 Gentamicin solution (10 milligrams per milliliter)

40 milligrams per milliliter gentamycin sulfate was four-time diluted into 10 milligrams per milliliter by sterile medium that was used for cultivation of the bacteria. The solution was kept at 4 degrees Celsius avoids light.

A.6 Resazurin solution (10 milligrams per milliliter)

20 milligrams of resazurin sodium salt was dissolved in sterile distilled water and was then mixed together. To sterile the solution, it was filtered through a 0.22 micrometers syringe filter. The solution was kept at 4 degrees Celsius avoids light.

APPENDIX B

MEDIUM PREPARATION

A.1 Brain Heart Infusion

Composition (per liter):

Commercial Brain Heart Infusion ..... 37.0 grams

Commercial Brain Heart Infusion was dissolved in distilled water, and the volume was adjusted with distilled water to be 1.0 liter. After that, it was autoclaved at 121 degrees Celsius under 15 psi pressures for 20 minutes. The medium was stored at 4 degrees Celsius.

A.2 Brain Heart Infusion agar

Composition (per liter):

Commercial Brain Heart Infusion agar..... 52.0 grams

Commercial Brain Heart Infusion agar was dissolved in distilled water, and the volume was adjusted with distilled water to be 1.0 liter. After that, it was heated to melt the agar. Lastly, it was autoclaved at 121 degrees Celsius under 15 psi pressures for 20 minutes. The medium was stored at 4 degrees Celsius.

A.3 Mueller Hinton agar

Composition (per liter):

Commercial Mueller Hinton agar	38.0	grams

Commercial Mueller Hinton agar was dissolved in distilled water, and the volume was adjusted with distilled water to be 1.0 liter. After that, it was heated to melt the agar. Lastly, it was autoclaved at 121 degrees Celsius under 15 psi pressures for 20 minutes. The medium was stored at 4 degrees Celsius.

A.4 Mueller Hinton broth

Composition (per liter):

Commercial Mueller Hinton broth	21.0	grams

Commercial Mueller Hinton broth was dissolved in distilled water, and the volume was adjusted with distilled water to be 1.0 liter. After that, it was autoclaved at 121 degrees Celsius under 15 psi pressures for 20 minutes. The medium was stored at 4 degrees Celsius.

BIOGRAPHY

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Publications

1. Patimanukul P, Oontawee S, Mustafa Z, Phonprapai C, Itharat A. Anti-oxidant efficiency of mangosteen pericarp and centella leaf mixture formulation. Paper presented at The 6th International Conference on Natural Products for Health and Beauty (NATPRO 6), KhonKhean, Thailand.