DEVELOPMENT OF POLYMERIC NANOPARTICLES LOADED WITH *Sesbania grandiflora* EXTRACT FOR ANTIBACTERIAL ACTIVITY IN SILKWORM INFECTION MODEL

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DOCTOR OF PHILOSOPHY
IN PHARMACY

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THIS THESIS HAS BEEN APPROVED TO BE A PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY IN PHARMACY

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หัวข้อดุษฎีนิพนธ์	การพัฒนานาโนพอลิเมอร์ที่มีสารสกัดแคเพื่อให้ฤทธิ์ต้านเชื้อแบคทีเรียในต้นแบบหนอนไหมที่ติดเชื้อ
ผู้เขียน	นางสาวพิมพ์พร อนันตวรสกุล
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บทคัดย่อ

วัตถุประสงค์ของการศึกษาวิจัยวิทยานิพนธ์นี้ เพื่อพัฒนานาโนพอลิเมอร์ที่กักเก็บสารสกัดแคที่มีฤทธิ์รักษาโรคติดเชื้อแบคทีเรียโดยใช้หนอนไหมเป็นต้นแบบสัตว์ทดลอง

การติดเชื้อแบคทีเรียเป็นหนึ่งในปัญหาหลักของสุขภาพ ที่มีสาเหตุจากเชื้อแบคทีเรียก่อโรค วิธีการรักษาอาการติดเชื้อแบคทีเรียดังกล่าวจะใช้ยาทางเคมีหรือยาปฏิชีวนะ ปัจจุบันยาปฏิชีวนะหลายชนิดที่ใช้รักษาอาการติดเชื้อจากแบคทีเรียก่อโรคมีประสิทธิภาพลดลง เนื่องจากปัญหาของเชื้อดื้อยา การกระตุ้นการผลิตของสารพิษ และผลข้างเคียงที่เกิดจากการใช้ยา ดังนั้นในการวิจัยเพื่อหาสารออกฤทธิ์สารใหม่เพื่อแก้ปัญหาการดื้อยาจึงเป็นประเด็นที่ยังคงท้าทาย

สำหรับวิธีการประเมินความสามารถในการยับยั้งของสารออกฤทธิ์ในหลอดทดลอง อาจยังไม่เพียงพอ เนื่องจากไม่ได้แสดงผลถึงเภสัชจลศาสตร์และเภสัชพลศาสตร์ในร่างกาย ดังนั้นในการทดสอบก่อนทางคลินิกในตัวอย่างสัตว์ทดลอง จึงมีความจำเป็นสำหรับการประเมินฤทธิ์รักษาของสารออกฤทธิ์ที่จะพัฒนาในลำดับต่อไป อย่างไรก็ตาม การใช้สัตว์เลี้ยงลูกด้วยนมเพื่อเป็นตัวอย่างสัตว์ทดลองนั้นมีปัญหาในเรื่องของราคาและจริยธรรม ดังนั้นสัตว์ไม่มีกระดูกสันหลังอย่างตัวหนอนไหมจึงถูกเสนอให้ใช้เป็นสัตว์ทดลองต้นแบบเพื่อศึกษาผลการรักษาของตัวอย่างยา

การใช้ประโยชน์ของพืชสมุนไพรสำหรับการรักษาโรคหรืออาการผิดปกติเป็นวิธีที่น่าสนใจสำหรับแพทย์ทางเลือก เนื่องจากสารธรรมชาติที่อยู่ในพืชนั้นสามารถช่วยฟื้นฟูสภาพให้ปกติและปลอดภัยต่อร่างกาย ในพื้นที่เขตร้อนชื้นมีพืชสมุนไพรหลายชนิดที่ถูกนำมาใช้ในการรักษาโรคต่างๆ แคเป็นหนึ่งในพืชไทยที่นำมาใช้ในทางอาหารและยาพื้นบ้าน สารสกัดจากเปลือกแคมีฤทธิ์ในการ

ยับยั้งการเจริญของเชื้อแบคทีเรียก่อโรคได้หลายชนิด รวมทั้งเชื้อแบคทีเรียดื้อยา นอกจากนี้สารสกัดดังกล่าวไม่มีความเป็นพิษต่อตัวหนอนไหม อีกทั้งสามารถช่วยเพิ่มการอยู่รอดของตัวหนอนไหมที่ติดเชื้อแบคทีเรียได้ ประสิทธิภาพของสารสกัดสำหรับยับยั้งการเจริญของเชื้อแบคทีเรียเป็นผลมาจากการเสริมฤทธิ์กันของสารธรรมชาติหลายๆชนิดที่อยู่ในสารสกัด โดยเฉพาะกรดแกลลิก ที่เป็นตัวบ่งชี้ทางชีวภาพของสารประกอบฟีนอลิกที่มีความสามารถในการยับยั้งโรคติดเชื้อ อย่างไรก็ตามสารสกัดดังกล่าวมีข้อจำกัดสำหรับการใช้ประโยชน์ทางเภสัชกรรมและทางการแพทย์ เนื่องจากความสามารถในการละลายในน้ำต่ำและความไม่คงสภาพ รวมทั้งรูปแบบของสารสกัดไม่สะดวกต่อการนำไปใช้

เมื่อกล่าวถึงการพัฒนานาโนเทคโนโลยี มีตัวนำส่งนาโนหลายชนิดที่ถูกเสนอเพื่อพัฒนาสมบัติของสารออกฤทธิ์ตั้งต้น โดยการเพิ่มพื้นที่ผิวต่อปริมาตรจะส่งผลถึงสมบัติของอนุภาคนาโนอย่างมีนัยสำคัญ ประโยชน์ของอนุภาคนาโนที่ถูกใช้ในระบบนำส่งคือมีความคงสภาพสูง มีความสามารถในการออกฤทธิ์สูง สามารถนำไปใช้กักเก็บสารได้ทั้งสารที่ชอบน้ำและสารที่ไม่ชอบน้ำ และสามารถปรับทางการให้ยาได้หลากหลาย ตัวนำส่งนาโนที่เป็นพอลิเมอร์ลิก ไมเซลล์ เป็นหนึ่งในอนุภาคนาโนพอลิเมอร์ ที่เพิ่มความสามารถในการละลายน้ำและความคงสภาพของสารออกฤทธิ์ พอลิเมอร์ลิก ไมเซลล์ ถูกสร้างจากพลูโรนิก พอลิเมอร์ (พอลอกซาเมอร์) ซึ่งเป็นสารที่ได้รับการอนุมัติจากสถาบันมาตรฐานในการประยุกต์ใช้ในทางเภสัชกรรมและการแพทย์ พอลิเมอร์ลิก ไมเซลล์ ที่กักเก็บสารสกัดเปลือกแคถูกเตรียมด้วยวิธีทินฟิล์มไฮเดรชั่น และประเมินอนุภาคนาโนที่ได้โดยประเมินฤทธิ์ยับยั้งการเจริญของแบคทีเรียและสมบัติทางกายภาพ พลูโรนิก ที่นิยมใช้ทั่วไปสองชนิด ได้แก่ พลูโรนิก เอฟ68 และ พลูโรนิก เอฟ127 ถูกนำมาใช้ในการสร้างพอลิเมอร์ลิก ไมเซลล์ ที่กักเก็บสารสกัดแคชนิดของพลูโรนิกและสัดส่วนของสารสกัดต่อพอลิเมอร์ จะถูกเลือกโดยประเมินจากผลของประสิทธิภาพการกักเก็บ โฟตอนคอรีเลชัน สเปกโตรสโคปี สมบัติการปลดปล่อย และฤทธิ์ทางชีวภาพ

พอลิเมอร์ลิก ไมเซลล์ ที่ประกอบด้วยสารสกัดและพลูโรนิก เอฟ68 ในอัตราส่วน 1:3 เป็นพอลิเมอร์ลิก ไมเซลล์ ที่เหมาะสมที่สุดโดยกักเก็บสารสกัดได้มากที่สุด อนุภาคมีขนาดเล็ก และรูปร่างกลม การศึกษาสมบัติทางความร้อนและสถานะทางกายภาพ ช่วยยืนยันได้ว่าสารสกัดแคสามารถถูกกักเก็บในส่วนแกนกลางของพอลิเมอร์ลิก ไมเซลล์ ที่มีโครงสร้างแบบอสัณฐาน ซึ่งช่วยเพิ่มการละลายของสารสกัดได้ นอกจากนี้การศึกษาการละลายในน้ำของสารสกัดแคที่กักเก็บในไมเซลล์แสดงการเพิ่มขึ้นของการละลายของสารสกัด โดยให้ค่าการละลายเทียบกับกรดแกลลิกเท่ากับ 76.11 ± 0.22 มก./มล. ซึ่งค่าการละลายเพิ่มขึ้นประมาณ 18 มก./มล. เมื่อพิจารณาฤทธิ์ในการยับยั้งการเจริญของแบคทีเรีย สารสกัดแคที่ถูกกักเก็บในพอลิเมอร์ลิก ไมเซลล์ แสดงฤทธิ์ในการยับยั้งการเจริญของเชื้อ

แบคทีเรียได้ โดยให้ค่าความเข้มข้นต่ำสุดที่สามารถยับยั้งและทำลายเชื้อแบคทีเรียได้เท่ากับ 0.50 มก./มล. ต่อเชื้อสแตปฟิโลค็อกคัส ออเรียส การศึกษาในสัตว์ทดลอง อนุภาคไมเซลล์ที่กักเก็บสารสกัดแสดงการเพิ่มการอยู่รอดของตัวหนอนไหมที่ติดเชื้อแบบแปรผันตามความเข้มข้นของสารออกฤทธิ์ โดยไม่มีความเป็นพิษ การศึกษาการปลดปล่อยใน 48 ชั่วโมง พบว่าการปลดปล่อยสารสกัดแคจากอนุภาคของพลูโรนิก ไมเซลล์ มีการปลดปล่อยแบบรวดเร็วในช่วงแรก จากนั้นจึงปลดปล่อยแบบช้าๆ ในสภาวะของระบบชีวภาพ-กายภาพ นอกจากนี้ พอลิเมอร์ลิก ไมเซลล์ ยังแสดงให้เห็นถึงความคงตัวทางจลนศาสตร์ที่ดีในสภาวะเร่งอุณหภูมิ อีกทั้งยังเพิ่มความคงสภาพของสารสกัดแคที่ถูกกักเก็บในพอลิเมอร์ลิก ไมเซลล์ โดยรักษาลักษณะทางกายภาพและปริมาณของสารออกฤทธิ์ได้ใกล้เคียงกับสภาวะเริ่มต้น การศึกษานี้แสดงให้เห็นถึงความสำเร็จของการกักเก็บสารสกัดแคในพอลิเมอร์ลิก ไมเซลล์ โดยเพิ่มสมบัติทางการละลายน้ำและความคงสภาพได้ นอกจากนี้สารสกัดแคที่ถูกกักเก็บในพอลิเมอร์ลิก ไมเซลล์ ช่วยเพิ่มการอยู่รอดของตัวหนอนไหมที่ติดเชื้อได้ โดยปราศจากความเป็นพิษ อนุภาคนาโนที่ถูกพัฒนาจึงมีแนวโน้มที่ดีในการใช้เป็นสารออกฤทธิ์ทางชีวภาพ สำหรับการลงทุนเพิ่มเติมในการทดลองทางคลินิก

Dissertation Title	Development of Polymeric Nanoparticles Loaded with *Sesbania grandiflora* Extract for Antibacterial Activity in Silkworm Infection Model
Author	Ms. Pimporn Anantaworasakul
Degree	Doctor of Philosophy (Pharmacy)
Advisory Committee	Assoc. Prof. Dr. Siriporn Okonogi — Advisor Lect. Dr. Srikanjana Klayraung — Co-advisor Lect. Dr. Kanlaya Jumpatong — Co-advisor

ABSTRACT

The aim of this research thesis study was to develop the polymeric nanoparticles loaded with *Sesbania grandiflora* extract which be able to treat bacterial infectious diseases using silkworms as an animal model.

Bacterial infection is one of major health problems that is caused by pathogenic bacteria. The mainstay of treatment the bacterial infections is using chemical drugs and antibiotics. Nowadays, many antibiotics have lost effectiveness against common pathogenic bacterial infections because of drug-resistance problems, stimulation of toxin production, and undesirable side effects. Therefore, the research for new finding substances to overcome these antibiotic-resistances is still the challenging issues.

For evaluating the inhibitory capacity of antibacterial agent *in vitro* study is not enough because it does not reveal pharmacokinetics and pharmacodynamics in body. Therefore, pre-clinical tests in animal models are essential for evaluating the therapeutic effect of active agent candidate for further development. However, the using of mammal model has some problems as involved with costly and ethical issues. Thus, invertebrates such as silkworms have been proposed as animal model for the study of therapeutic effects of drug candidates.

The utilization of medicinal plant for treatment disorder is interesting route for alternative medicine due to the natural compounds as existing in the plant which

promote to recuperation and safe in the body. In tropical regions, there are several medicinal plants which are applied for treatment of many diseases. *Sesbania grandiflora* is one of Thai plants as used for food and folk medicine. The extract of *S. grandiflora* bark exposed the most potential antibacterial action. It possessed broad spectrum antibacterial activity against pathogenic strains including drug-resistant strains. Besides, this plant extract was non-toxic to the silkworm body, also prolonged survivability of bacterial infected silkworms. The efficacy of the extract for antibacterial activity was resulted from synergistic effect of many phytochemical compounds in the extract particularly gallic acid as a biomarker of phenolic compounds that have ability to inhibit infectious diseases. However, this extract was limited for utilization in pharmaceutical and medicinal fields due to its low aqueous solubility and instability, also the extract form was not convenient for using.

Regarding to nanotechnology development, there are different types of nanocarrier which offer improving properties of original agents by increasing of surface area to volume ratio that results to properties of the nanoparticles significantly. The advantages of nanoparticles which are used in delivery systems are high stability, high capacity, feasible incorporation of hydrophilic and hydrophobic agents, and variable routes of administration. Polymeric micellar nanocarriers is one of polymeric nanoparticles which improve the aqueous solubility and stability of active agent. Polymeric micelles are created from Pluronic polymer (Poloxamer) which are approved by standard institute for applying in pharmaceutical and medicinal fields. The polymeric micelles loaded with *S. grandiflora* bark extract was prepared by thin-film hydration method and evaluated the obtained nanoparticles for antibacterial activity and physical properties. Two most commonly Pluronic such as Pluronic F68 and Pluronic F127 were used for creating polymeric micelles as entrapped with *S. grandiflora* extract. The Pluronic type and proportion of the extract to polymer was selected by estimating the results of entrapment efficiency, photon correlation spectroscopy, releasing property, and biological activity.

The polymeric micelles which composed of the extract and Pluronic F68 at a ratio of 1:3 was the most optimal polymeric micelles that revealed the highest entrapment efficiency with small particle size as exposing spherical shape. The studies of thermal

behavior and physical state were confirmed that *S. grandiflora* extract could be loaded in the micellar core of Pluronic polymer as forming amorphous state that increased the solubility of the extract. Moreover, the study of aqueous solubility of *S. grandiflora* extract as loaded in the micelles showed increasing solubility of the extract with Gallic acid equivalent (GAE) value of 76.11 ± 0.22 mg/mL, it was increased approximately 18 mg/mL. According to biological activity, *S. grandiflora* extract loaded in polymeric micelles demonstrated antibacterial action with MIC and MBC of 0.50 mg/mL against *Staphylococcus aureus*. *In vivo* study, the micelles entrapped with the extract presented the extending survival of infected silkworm in a dose dependent manner without toxicity. A release study was monitored for 48 h, the release of *S. grandiflora* extract from Pluronic micelles exhibited primarily rapid release after that sustain release in physiological system. In addition, the polymeric micelles established a good kinetic stability at accelerated temperatures, also enhancing stabilization of *S. grandiflora* extract as loaded in polymeric micelles with conserving of physical appearance and content of active compound which similarly the primitive state. The present study suggested that *S. grandiflora* extract was prosperously loaded in polymeric micelles which improved aqueous solubility and stability properties. Furthermore, *S. grandiflora* extract loaded polymeric micelles prolonged the survivability of the infected silkworms without any toxicity. The developed nanoparticles are a promising candidate with good biological activity for further investment in clinical trials.

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LIST OF ABBREVIATIONS

ATCC	American Type Culture Collection
BE	Butanol extract
C	Celsius
CE	Chloroform extract
CFU	Colony forming unit
CMC	Critical micelle concentration
Da	Dalton
DI	Deionized
DIZ	Diameter of inhibition zone
DMSO	Dimethyl sulfoxide
DMST	Department of Medical Sciences Thailand
DSC	Differential scanning colorimeter
Ea	Activity energy
ECE	Ethanol crude extract
ED_{50}	Median effective dose
EE	Encapsulation efficiency
EE	Ethyl acetate extract
FCE	Fractionated crude extract
g	Gram
GAE	Gallic acid equivalent
h	Hour
HE	Hexane extract
HPLC	High performance liquid chromatography
IC_{50}	Median inhibitory concentration
J	Joule
k	Kilo
k_{obs}	Reaction rate constant
LC	Loading capacity

LIST OF ABBREVIATIONS (CONTINUED)

LC_{50}	Median lethal concentration
LD_{50}	Median lethal dose
Log	Logarithm
MBC	Minimum bactericidal concentration
ME	Methanol extract
mg	Milligram
MHA	Mueller-Hinton Agar
MHB	Mueller-Hinton Broth
MIC	Minimum inhibitory concentration
min	Minute
mL	Milliliter
mm	Millimeter
MRSA	Methicillin-resistant *Staphylococcus aureus*
MW	Molecular weight
nm	Nanometer
NZ	No inhibition zone
PBS	Phosphate buffer solution
PCS	Photon correlation spectroscopy
PdI	Polydispersity index
PEO	Poly(ethylene oxide)
PF68	Pluronic F68 (Poloxamer 188)
PF127	Pluronic F127 (Poloxamer 407)
PPE	Partial purified extract
PPO	Poly(propylene oxide)
R	Gas constant
r^2	Correlation coefficient
rpm	Revolutions per minute
S	Frequency factor

LIST OF ABBREVIATIONS (CONTINUED)

SD	Standard deviation
SGE	*Sesbania grandiflora* extract
SGE-PF68	*Sesbania grandiflora* extract loaded polymeric micelles of Pluronic F68
SGE-PF127	*Sesbania grandiflora* extract loaded polymeric micelles of Pluronic F127
T	Absolute temperature
TEM	Transmission electron microscopy
temp	Temperature
TLC	Thin layer chromatography
TSA	Tryptic Soy Agar
TSB	Tryptic Soy Broth
$t_{1/2}$	Half–life
VRE	Vancomycin-resistant *Enterococcus*
w/w	Weight by weight
XRD	X–ray diffraction

LIST OF SYMBOLS

%	Percent
°	Degree
λ_{max}	The maximum wavelength
μ	Micro
θ	Theta

ข้อความแห่งการริเริ่ม

1. วิทยานิพนธ์นี้ได้นำเสนอวิธีการใหม่ในการใช้หนอนไหมไทยเป็นต้นแบบสัตว์ทดลอง สำหรับการประเมินฤทธิ์รักษา สัตว์ทดลองที่นิยมใช้อย่างเช่นสัตว์เลี้ยงลูกด้วยนมโดยเฉพาะหนู มักมีปัญหาในเรื่องของค่าใช้จ่ายในการเลี้ยงดูแล ตลอดจนขั้นตอนวิธีการทดลอง รวมทั้งปัญหาของจริยธรรมในสัตว์ทดลอง เพื่อเป็นการหลีกเลี่ยงปัญหาเหล่านี้ การใช้สัตว์ไม่มีกระดูกสันหลังอย่างเช่นแมลงหรือแมงจึงถูกเสนอขึ้น อย่างไรก็ตามแมลงหรือแมงบางชนิดนั้น ไม่มีความเหมาะสมในการศึกษาทางเภสัชพลศาสตร์ เนื่องจากมีขนาดตัวที่เล็กเกินไป ดังนั้นการเลือกใช้ชนิดของสัตว์จึงมีความสำคัญ ซึ่งการใช้ตัวหนอนไหมเป็นสัตว์ทดลองต้นแบบสามารถทำการศึกษาได้ทั้งการประเมินฤทธิ์รักษารวมทั้งการศึกษาทางเภสัชพลศาสตร์ของสารออกฤทธิ์ เชิงคุณภาพและเชิงปริมาณ โดยมีค่าใช้จ่ายต่ำในการดูแลและการทดลอง ตลอดจนไม่มีปัญหาในเรื่องของจริยธรรมต่อสัตว์ทดลอง

2. เพื่อเพิ่มประสิทธิภาพของสารออกฤทธิ์ในการนำไปใช้ประโยชน์ นาโนเทคโนโลยีจึงถูกนำมาประยุกต์ใช้เพื่อเตรียมสารออกฤทธิ์ให้อยู่ในรูปของพอลิเมอร์ลิก จากการศึกษาแสดงการเพิ่มขึ้นของความสามารถในการละลายในน้ำ และความคงสภาพของสารออกฤทธิ์ เมื่อถูกกักเก็บในพอลิเมอร์ลิก ไมเซลล์

STATEMENTS OF ORIGINALITY

1. Thai silkworm as an animal model was applied in this research for evaluation of therapeutic effect. Although mammal especially mice or rats are commonly used animal model, it is costly, complicated experimental design including the concern of animal research ethics. To avoid these problems, using of invertebrate e.g. insect, has been proposed. However, some species of insect are not suitable for pharmacodynamics study since their body sizes are too small. Therefore, the selection of animal type is necessary. There are several advantages of silkworm as experimental animal model i.e. it could be performed both therapeutic evaluation and pharmacodynamics study of active agents with low cost and absence of ethical issue.

2. In order to improve the efficacy of active agent, nanotechnology was applied to prepare active agent loaded in polymeric micelles. The remarkable observation was the increase of aqueous solubility and stability of the active agent entrapped in polymeric micelles.

CHAPTER 1

Introduction

1.1 Historical Background

Bacterial infection is one of major health problems. Most bacterial infection is caused by pathogenic bacteria existing in the environment, and in the body. Severe bacterial infection causes human morbidity and mortality. Treatment of bacterial infection is commonly done by using chemical antibiotic drugs and antibiotics [1]. These materials kill or inhibit the growth of bacteria. A antibiotics have been used increasingly because they are available not only in the hospitals but also in drug stores all over the country without any prescriptions. Recently, drug resistance of pathogenic bacteria to various antibiotics has been reported [2]. Treatment of the infection caused by those drug-resistant bacterial strains has been improved using higher dose or longer treatment course or even using a combination of different drugs. However, these treatments cause more harmful than preferable effects because it is higher risk of drug toxicity to the patients [3, 4]. Therefore, the research for new substances which are potential and patient friendly to overcome these antibiotic-resistant problems is needed. Alternative antimicrobial drugs for contending successful treatment of bacterial infectious diseases are being developed.

The World Health Organization (WHO) recognizes that approximately 80% of population in developing countries is dependent on traditional medical plants as a source of drug [5]. It is, therefore, important to create strategies, guidelines and standards of botanical medicines [6]. Plants have been increasingly used for treatment of various diseases, many of them are rich of various secondary metabolites which possess antibacterial properties and could serve as alternative, effective, cheap, and safe substances for treatment of bacterial infections [7]. Medicinal plants have been used for treatment of infectious diseases since 3000 BC [8]. Traditional remedies particularly the

Chinese and Indian remedies are well known of using different medicinal plants containing phytochemical compounds which expected to inhibit the growth of bacteria. Nowadays, more researches targeted on antibacterial substances from natural sources, high potential antibacterial constituents in plants have been investigated. It has been reported that some of the medicinal plants such as lemongrass [9-12], basil [10-13], galangal [10, 12, 14, 15], turmeric [11-15], clove [11, 12], garlic [12, 13, 16], and onion [12, 13, 16] present antibacterial activity. In Thailand, there are varieties of medicinal plants which possess antibacterial properties. The biodiversity of Thai plants could therefore be a valuable source for novel antibacterial agent [17].

Sesbania grandiflora Linn., commonly known as Vegetable Hummingbird in English, Agathi in Tamil or Kae in Thai [6], is a medicinal plant belongs to family Fabaceae. This plant is found in various regions of South-Eastern Asian countries and India [18]. *S. grandiflora* have been used as herbal medicine to treat various disorders [19]. This plant has been known to have antioxidant [20], antidiabetic, antitumorigenic [20], and antimicrobial activities [21, 22]. Moreover, the previous studies reported that *S. grandiflora* showed more biological activities such as anti-inflammatory [23], anxiolytic [24], anticonvulsive [24], wound healing [25], antifungal, and antibacterial activities [26]. The present study was aimed to prepare the extraction of *S. grandiflora* and focused on evaluation of the antibacterial activity of the extracts in order to get the natural high potential antibacterial compounds. However, the preliminary study indicated that the highest potential fraction of *S. grandiflora* extract showed practically insoluble in water and less stability. An organic solvent was required to prepare a clear solution. The effective extracts showed rapid degradation upon standing for few months which should be further investigated on the factors affecting the degradation. To overcome the low solubility and the instability problems of *S. grandiflora* extracts, nanotechnology will be applied in this study.

Nanotechnology has been used to improve the disadvantageous properties such as less water solubility and instability of many drugs and natural active compounds [27]. It was reported that the nanoparticles containing drug or any active compounds could deliver the active agent to the target organ [28, 29]. The tiny size of nanoparticles results in high surface area and high potential to avoid rapid clearance due to phagocytes.

Polymerization is one of the most effective nanotechnological techniques to prepare drug loading nanoparticles. The polymeric nanoparticles can be created using various kinds of natural or synthetic polymers. The obtained nanoparticle size is usually in nanometer range. Recently, it has been reported that polymeric nanoparticles from biodegradable and biocompatible polymers are good candidate for carrier to delivery active agents because of being well adsorbed and non-toxicity [30]. Nowadays, there are many kinds of polymers which are used for nanoparticles preparation. One promising example of polymeric nanoparticles is represented by a class of poloxamer block copolymer, commercially known as Pluronics. Pluronics consist hydrophilic poly(ethylene oxide) (PEO) chain and hydrophobic poly(propylene oxide) (PPO) chain that arranged to tri-block structure like PEO-PPO-PEO [31]. Pluronics have different types depend on a number of PEO and PPO in polymer chains. However, various Pluronics show slightly different properties. Pluronics also have important characteristic depending on concentration, at low concentration which is below critical micellization concentration (CMC), Pluronics present molecularly in solution whereas at higher concentration above critical points, micellization of polymer will be processed to obtain polymeric micelles.

Polymeric micelles are generally formed in a spherical shape with hydrophobic cores and hydrophilic shield in the aqueous solution [32]. These dynamic systems are used for the systemic delivery of water-insoluble drugs. The drug can be incorporated within the hydrophobic cores as linked covalently to component molecules of the micelles. Polymeric micelles have approached an excellent novel drug delivery system because of high loading capacity, stability in physiological conditions, slow rate dissolution, high accumulation of drug at target site, and possible functionalization of end group for conjugation with targeting ligands [33].

For fully evaluation of antibacterial activity of certain antibacterial agents, only the *in vitro* study is not sufficient because it does not reveal toxicity and pharmacodynamics in body. Therefore, pre-clinical tests in animal models are essential for evaluating the therapeutic effect of the active agent candidate for further development. Mammals such as mice and rats are normally used as animal model of therapeutic evaluation of antibiotics. However, the use of a large number of mammals for drug development is costly and highly problematic with relate to ethic in animal [34,

35]. To avoid these problems, the use of invertebrate animals such as *Romalea microptera* [36], *Caenorhabditis elegans* [37], and *Drosphila melanogaster* [38] as infected animal model has been proposed. However, there are limitation of these models due to their body size which is too small to use in pharmacodynamics studies. To overcome these limitations, larvae of silkworms (*Bombyx mori*) are used as an animal model. The silkworm larvae have a number of advantages as experimental animals as it be generated in a short period of time and are easily maintained and genetically tractable in laboratories [39-41]. Moreover, the silkworms are easily handled during injection of bacteria and active agents with syringes because of their slow movement. The body size of silkworm is large enough for isolation of hemolymph and organ, which are essential for pharmacodynamics study of active agents in animal bodies. Therefore, the present study, silkworms were used as an animal model in the antibacterial evaluation of *S. grandiflora* extract.

In summary, the scope of the present study are to prepare *S. grandiflora* extract which is high potential for inhibition of pathogenic bacteria. To enhance the water solubility of the extract by loading in Pluronic block copolymer micelles. The major active compound of the extract was investigated for being a marker compound and standardinzation of the extract. The physicochemical properties and releasing behavior from the polymeric micelles as well as the degradation of the extract entrapped in the polymeric micelles were evaluated. Comparative antibacterial activities among various kinds of extract loaded polymeric micelles were performed *in vitro* and *in vivo* studies.

1.2 Objectives

1.2.1 To evaluate the antibacterial activity of *S. grandiflora* extract

1.2.2 To study the toxicity and the therapeutic effect of *S. grandiflora* extract

1.2.3 To investigate the major bioactive compound of *S. grandiflora* extract

1.2.4 To enhance solubility and stability of *S. grandiflora* extract by polymeric micelles

1.2.5 To examine the physicochemical properties and biological activities of the *S. grandiflora* extract loaded polymeric micelles

1.3 Literature Review

1.3.1 Infectious disease

Infectious diseases are disorders caused by microorganisms which exist in an environment everywhere such as air, soil and water, also in a body. Some infectious diseases occur by passing from person to person via touching, eating, drinking, breathing, kissing or sexual contacting, transmitting via animals and insect bites, and acquiring via ingesting contaminated food, water or being exposed to microorganisms in the environment. The symptoms of infectious diseases are varied range from mild to severe level. It depends on the microorganisms which cause the infection. In severe case, the infection can kill human and spread to more people. The human infectious diseases have still been a public health problem particularly in tropical countries [42].

One of the severe infectious diseases is bacterial infection which is major health problems. The bacterial infection is caused by pathogenic bacteria existing in the environment, and inside the body such as digestive, respiratory, and genitourinary tracts. Common bacterial infections include pneumonia, diarrhea, urinary tract infections, and skin disorders.

The main treatment of the bacterial infections is done by using antibacterial agents which are classified into two types; one is chemical antimicrobial group and the other is antibiotics group [1]. The role of these drugs is to kill or inhibit the growth of bacteria. The effectiveness of these drugs depends on drug distribution, mechanism of action, site of infection, immune status of host, and resistance factors of bacteria [1, 43]. Comparison of both groups of antibacterial agents and the antibiotics have been used increasingly in the treatment of bacterial infection. Later, many antibiotics have lost efficacy for treatment of bacterial infections due to increasing drug resistance [44, 45]. The use of antibiotics as indiscriminate, inappropriate, and prolonged is main cause of antibiotic-resistance [46, 47]. In addition, the synthetic drugs are not only expensive and inadequate for treatment disease but also causing adverse effects and side effects such as eliminate normal flora bacteria which are non-pathogen and helpful bacteria, allergy in people who are allergic to antibiotics, shock in people who are gained over-dose, autoimmune disease, decreased platelets, kidney injury, liver injury, drug-drug

interaction, and death [43]. Therefore, searching of novel substances which are potential and safe for patients is needed to combat bacterial infectious diseases.

1.3.2 Silkworm infection model

At present, the evaluation of the therapeutic effects of antibiotics *in vivo* study is important as it indicates the results of toxicity and pharmacodynamics in body. Animal models are valuable for evaluating bacterial infectious diseases. Mammals, such as monkey, dog, rabbit, marmot, particularly mice and rat are normally used as animals model for examination of the pharmacodynamics of antibiotics including evaluation of the therapeutic effects of antibiotics. However, the use of mammal model has some problems. Pharmacokinetics study in a large number of drug candidates at initially stages for drug development is hardly performed, due to the highly financial cost needed to nurture of mammals in laboratory. It is also pointed out that experiment on mammals concerns ethical issue [34, 35]. The latter point is coming to be serious problems, thus the drug development is slow down in industrialized countries [48].

To overcome these problems, using of invertebrate as animal models, which can be used as a large numbers with low costs, is desired [49]. There have been previous reports of some invertebrate animals for infected animal model such as the nematode *C. elegans*, the insect *D. melanogaster*, and the amoeba *Dictyostelium discoideum*, has been possessed [40]. Particularly, *C. elegans* and *D. melanogaster* are used for identifying host proteins concerned with immune systems because their genetic have been tractable constructed [50]. However, these models have a limitation that their body sizes are too small for the studies of pharmacodynamics. The manipulations must be performed under a microscope. So, they are hardly injected with a precise volumes of samples into the body fluid. This technique is essential for quantitative evaluation of antibacterial agents on therapeutic effects.

Silkworms have advantages as animal model for studying the pharmacological effects of antibiotics. There are not only solving of ethical issue but also having greater advantages than other invertebrate animals. The body size of silkworm as the 5^{th} instar larvae is large enough to handle. Sample solutions of pathogens and drug can be injected into the hemolymph or intramidgut of the silkworm by syringes with needles. The principal trick of injecting technique is observation the transferring body color of

silkworm as shown in Figure 1.1. When silkworm is inoculated slightly with red ink solution into blood, the whole body immediately changes to red color, due to circulatory system. On the other hands, when the silkworm is inoculated deeply into the midgut, no change of the body color because the red ink is dispersed throughout the midgut [41]. These characteristics is applied to evaluation therapeutic effects of antibiotics.

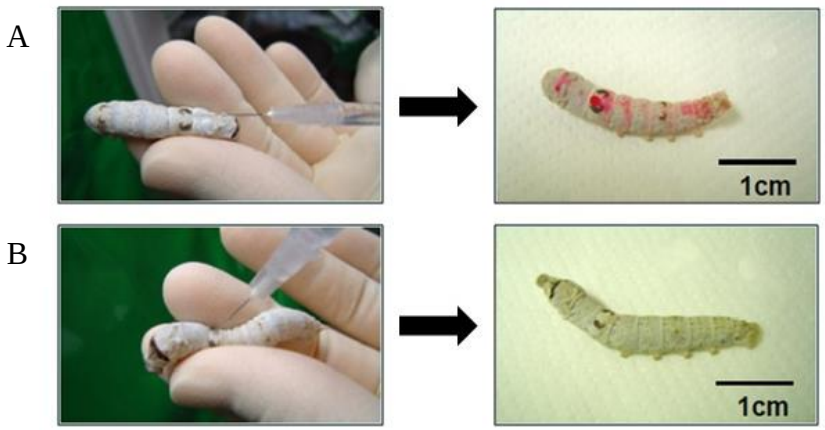


Figure 1.1 Technique of injection into hemolymph (A) and midgut (B) [59].

In addition, various tissues which are reliable for multiplying bacteria and drug metabolism of silkworm larva can be isolated, this technique is allowed for evaluating of pharmacodynamics of sample agents. The hemolymph of silkworm that is injected with pathogenic bacteria can be easily collected and counted the bacterial cell number in the hemolymph. So, it can be closely monitored proliferation process of the pathogenic bacteria in silkworms [51]. For the midgut study, it can be isolated for drug transport assays. The sample is injected into the midgut and passes through membrane which incubated in a suitable buffer, it will be detected in the buffer [52].

Recently, genome study of the silkworm was completed by Japanese and Chinese research groups [53, 54]. The reverse genetic method, RNA interference, was established in silkworms [55, 56]. The gained information based on the genome and RNA interference method will simplify the study of host factors that involved in infectious processes. In previous study, Hamamoto et al. reported establishment of infection models of silkworm with pathogenic bacteria and true fungi (Figure 1.2) [57]. They demonstrated that the values of ED_{50} and LD_{50} could be determined as revealed similar by between silkworms and mammals [58]. They also presented metabolic

pathways with cytochrome P450s and conjugation enzymes in silkworms model [52]. The results of antibiotics absorptions from intestine in silkworms and mammals are similar [59]. These reports suggest that mechanisms of pharmacokinetics of chemicals between models of silkworms and mammals are correlated.

A B C

Figure 1.2 Silkworm model of bacterial infection; normal growing silkworms of control group (A), dead silkworms of infectious group (B), and healed silkworms of treatment group (C) [59].

1.3.3 *Sesbania grandiflora* Linn.

Figure 1.3 *Sesbania grandiflora* Linn.

Sesbania grandiflora Linn. belongs to Fabaceae family. *S. grandiflora*, commonly known as Agati, is found and cultivated in many countries particularly Asian countries such as India, Indonesia, the Philippines, Malaysia, Myanmar, and Thailand. *S. grandiflora* is a loosely branching tree about 5-12 m high. Its leaves are pinnate compound 20-30 cm long with 20-40 leaflets in pairs of 2.5-3.5 cm long. Flowers are large, 7-9 cm long, white with calyx 15-22 mm long. The standard has dimensions up to 10.5 x 6 cm. Pods are linear, 20-60 cm long and thin, 7-8 mm wide, with broad sutures containing 15-50 seeds (Figure 1.3) [18].

The phytochemical compositions from leaves, stems, barks, flowers, pods, and roots have been previously reported. *S. grandiflora* is a source of various secondary metabolites as responsible for therapeutic effects. Therefore, several parts of *S. grandiflora* are used as medical herbs in many folk remedies in the tropical regions of the globe [60]. The previous study of the preliminary phytochemical screening of *S. grandiflora* extracts revealed the presence of chemical constituents such as galactommannans, linoleic acid, beta-sitosterol, and carbohydrate [61-63]. The major constituent in *S. grandiflora* are phenolic compounds (Figure 1.4) presented in several groups of saponins, tannins, flavonoides, coumarins, steroids, and triterpenes. But, alkaloids are generally found in the form of traces.

There are previous studies of the phytochemical, pharmacological, and phytopharmaceutical aspects of *S. grandiflora*. The bark contains tannin and gum [64]. The seeds contain saponin and sesbanimide. This compound is found to be cancer inhibitor [65]. The flowers yield amino acid, kaempferol, methyl ester, phenolic acids, and vitamin [66]. Wagh et al. [18] reviewed that natural gums have been widely explored as pharmaceutical excipients due to biocompatible, cheap, and commonly available, thus they are attractive substituent for costly semi-synthetic and synthetic excipients. Sesbania gum which was available locally in large quantities had been explored by some studies as pharmaceutical excipients. The seed contains galactomannan which was established as a potential binding agent in pharmaceutical formulation. *S. grandiflora* was extensively studied by different scientists for its phytopharmacological potentials especially from the leaves, flowers, and seeds; but the root, bark, and fruits are less researched in details.

S. grandiflora is used as traditionally medicinal plant for the treatment of multifactorial diseases like leprosy, gout [67], rheumatism, cancer, liver disorder [68], inflammation, ocular diseases [69], epilepsy, and anemia [24]. It also reported having many activities e.g. anti-inflammatory, analgesic, antipyretic [18], hypolipidemic [70], antibacterial [71], free radical scavenging [72], antiulcer [73, 74], antiurolithiatic [75], hepatoprotective [76], and chemo preventive [77] activities. The leaves of *S. grandiflora* have been reported having anxiolytic and anticonvulsant effects [24], also exhibit anti-urolithiatic and antioxidant properties [22]. Ramesh et al. [78] presented that *S. grandiflora* leaves restrain cigarette smoke-induced oxidative damage in liver and kidney of rats. It was revealed that chronic cigarette smoke exposure increased oxidative stress and the aqueous suspension of *S. grandiflora* had a protective effect against oxidative damage through an antioxidant effect [70]. The barks of *S. grandiflora* are used for treatment of small pox, scabies, ulcers in mouth, and alimentary canal infantile disorders of stomach. The flowers of *S. grandiflora* have been reported having antimicrobial activity [26, 79]. Solis [80] studied antibacterial and antibiotic properties of the leguminous plants. The results showed that *S. grandiflora* extract gave a clear wide inhibitory zone to *Candida albicans*. Subramanian et al. [81] extracted the roots of *S. grandiflora* with petroleum ether, chloroform, methanol, and water by soxhlet extraction, It was found that all *S. grandiflora* extracts exhibited significant antibacterial and antifungal activity. Sheikh et al. [25] reported that flower ethanolic extract showed greater wound healing contracting ability. It shows hypolipidemic property, *S. grandiflora* extract decreases the levels of serum cholesterol, phospholipid, triglycerides, LDL, and VLDL while the extract increases the level of serum HDL [82].

Although *S. grandiflora* presents several bioactive compounds, these compounds of *S. grandiflora* may be limited for utilization in pharmaceutical and medicinal fields. According to the preliminary of this study, *S. grandiflora* extract showed insoluble in water. In addition, the extracts was unstable after a few months storage at room temperature. Moreover, the use of extract form is not convenient nor easy to control. To overcome these problems, nanotechnology was applied for improving properties of *S. grandiflora* extracts.

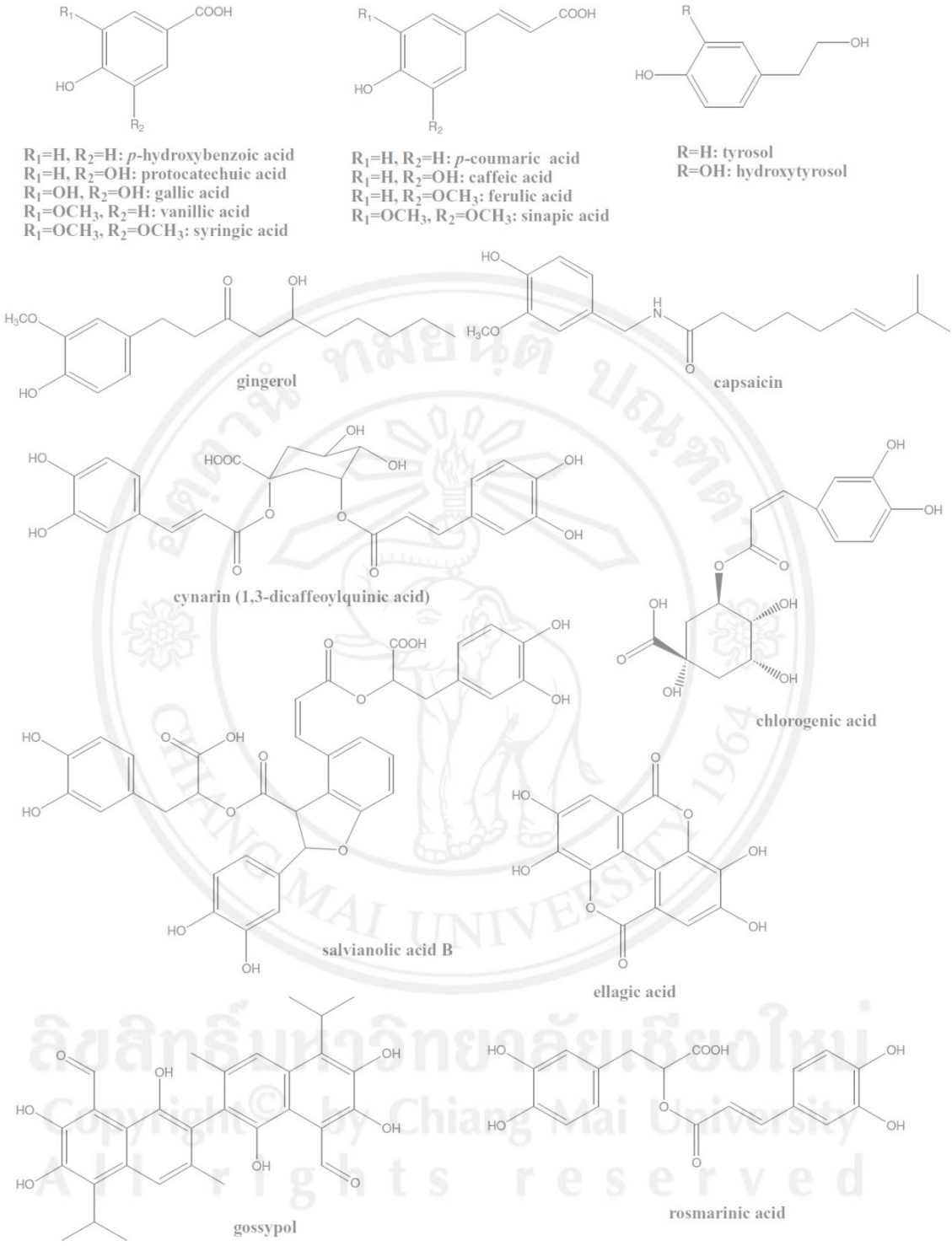


Figure 1.4 Chemical structures of common phenolic acids and analogs from medicinal plants [116].

1.3.4 Polymeric nanoparticles

Currently, nanotechnology is emerging as a new interdisciplinary field combining biology, chemistry, physics, and material science. The nanotechnology is well known to apply for making particles size in a level of part per billion, nanoparticles has size range from 1-1000 nm. There are many advantageous characteristics of nanoparticles such as high surface to volume ratio, homogeneous particles size distribution, possibility of facile surface modification, good stability and easy preparation [83]. Applications of nanoparticles are widely used in many fields such as material science, biology, dentistry, medicine, and pharmacy particularly in drug delivery system. The nanoparticles as nano-scaled drug carriers have the subjects of extensive research in the last few decades to optimize the delivery of conventional drugs, recombinant proteins, vaccines, and nucleic acids [84]. These systems alter the kinetics, body distribution, and drug release of associated drugs. Furthermore, nanoparticles display relatively biocompatible, capable of co-existence with living tissue or organism by low toxic and immunologic effect, and biodegradable, capable of being decomposed by natural processes [85]. Nanoparticles can also promote tissue or cell site-specific delivery of drugs and decrease undesired side effects [86]. Moreover, the nanoparticles can be freeze-dried so they are more stable than other colloidal systems for extending shelf life as long-term storage. Nanoparticles can be prepared by several techniques. One of the potential techniques is polymerization in order to obtain polymeric nanoparticles.

Polymeric nanoparticles are solid particles with the size between 10-1000 nm, although most of those reported that the size display a diameter ranging from 50 to 350 nm [85, 87]. Polymers which are used for drug delivery formulation must be chemically inert, nontoxic, and free of leachable impurities. Also, the polymers have an appropriate physical structure with less side effects. The significantly advantages of using polymeric nanoparticles in drug delivery are improving drug solubility and stability as well as increasing drug payload [88]. Polymers which are used for preparing nanoparticles can be natural or synthetic polymers [89]. The examples of natural polymers are albumin, alginate, chitosan, collagen, gelatin, haemoglobin, and starch. The advantages of using natural polymers include their low cost, biocompatibility, and aqueous solubility. However, there are limitations of using the natural polymers because of the presence of impurities, batch-to-batch variability, poor batch-to-batch

reproducibility, a tendency to degrade, and generally low hydrophobicity [90]. For the synthetic polymers, there are a number of polymers e.g. poly(lactic acid) (PLA), poly(glycolic acid) (PGA), poly(lactide-co-glycolide) (PLGA), polycaprolactone (PCL), poly(alkylcyanoacrylate) (PACA), and polyisobutylcyanoacrylate (PBCA). The advantages of synthetic polymers are more reproducible and capable to prepare with the desired degradation rates, molecular weights, and copolymer compositions.

According to the techniques of polymeric nanoparticles preparation, there are several processes to create polymeric nanoparticles such as emulsion polymerization, emulsification-solvent evaporation, and so on. Emulsion polymerization of alkylcyanoacrylates without irradiation or an initiator was first introduced to design nanoparticles for *in vivo* delivery of drugs [91]. PACA nanoparticles, which are characterized by a negative surface charge, can be commonly prepared by the anionic emulsion polymerization process. Polymerization takes place in micelles and is initiated by anionic ions [92]. The stabilizers, e.g. polysorbates, poloxamers, dextran, cyclodextrin, poly(ethylene glycol-) (PEG), and poly(ethylene glycol tertoctyphenyl ether) are often added to the aqueous polymerization medium [93]. Therefore, the type and concentration of the stabilizer are important factors for controlling the particles size, the surface properties, drug release, and the uptake of nanoparticles by organisms [93]. The anionic polymerization of alkyl cyanoacrylates initiated by organic bases, tertiary amines, and phosphines has been inferred to involve a zwitterionic mechanism. Thus, in this case, the very negative surface charge of PACA nanoparticles might be attributed to adsorption of anions from the aqueous polymerization medium [94]. For dispersion of preformed polymers, there are several methods to prepare various nanoparticles from different polymers such as PLA, PLG, PLGA, and PLC [94]. Common methods in use are emulsification-solvent evaporation technique [95], double or multiple emulsion technique [96], solvent displacement (polymer deposition) method [97, 98], spontaneous emulsification/solvent diffusion method (ESD) [96], salting-out [99], critical fluids [100], spray drying [101], ionic gelation/ coacervation [97, 101], reverse micellar method [100], and emulsion-droplet coalescence method [100]. The advantages of using this strategy, compared to polymerization of monomers, are the avoidance of cross-reaction of monomer or initiator with drug molecules, the prevention of the possible presence of residual toxic reagents or by products (oligomers, monomers) and

the ability to work with polymers with known molecular weight (in polymerization this parameter cannot be controlled precisely) [100]. Sulfobutylated poly(vinyl alcohol)-*graft*-poly(lactide-co-glycolide)s (SB-PVA-*g*-PLGA) nanoparticles as derivatized-PLGA nanoparticles were prepared utilizing the solvent displacement technique. The sulfobutyl negatively charged groups, oriented toward the outer aqueous phase resulting in increased negative zeta-potential, thus enabled optimized adsorption of a cationic antigen tetanus toxoid with the maximum loading rate achieved using the highest degree of sulfobutyl substitution [98].

Recently, Ravi Kumar et al. [102] developed a new emulsion-diffusion-evaporation technique for the preparation of cationic modified PVA chitosan PLGA nanoparticles that can bind DNA readily by electrostatic interaction, avoiding possible contact of the plasmid with organic solvents during the particle preparation process.

Calvo et al. reported hydrophilic chitosan (CS) nanoparticles were prepared by ionic gelation method [103]. The preparation method involved reversible physical cross-linking by electrostatic interaction between a mixture of two aqueous phases, the first one contained chitosan and a diblock copolymer of ethylene oxide (EO) and the other contained a polyanion sodium tripolyphosphate (TPP). These nanoparticles have shown high association with proteins, as well as oligonucleotides [104].

Different kinds of polymer and different techniques of nanoparticles preparation present different forms of polymeric nanoparticles such as polymeric nanocapsules (polymer shell), polymeric nanosphere (polymer matrix), and polymeric micelles. The polymeric nanocapsules are vesicular heterogeneous system in which the drug is confined to the inner cavity composed of oil or an aqueous core surrounded by a thin polymer membrane while the polymeric nanospheres are matrix homogenous systems in which the drug is dispersed within the polymer throughout the solid sphere [105]. The polymeric micelles are supramolecule self-assemblies of synthetic macro-molecules in which individual amphiphillic block copolymers are held together by noncovalent interactions [106]. They are composed of a hydrophobic core which serves as reservoir for hydrophobic drugs and hydrophilic shell which affects their pharmacokinetic behavior [107]. The defining properties of micelles include critical micellization concentration (CMC), aggregation number, size, and shape. The polymer which forms

the block of micelles can be classified into amino acids, polyesters, and poloxamers [33].

Poloxamer is a synonym of polyethylene-propylene glycol copolymer and the trade names are Synperonics, Kolliphor, and Pluronic. Poloxamer is a non-ionic tri-block copolymers. The block copolymers consist of two hydrophilic chains of poly(ethylene oxide) (PEO) chains that sandwiched one hydrophobic poly(propylene oxide) (PPO) chain, tri-block structure: PEO-PPO-PEO (Figure 1.5A) [31]. A number of PEO and PPO are varied. Different lengths of polymer block give rise to different poloxamer which identified as Poloxamer 124 (Pluronic L44), Poloxamer 188 (Pluronic F68), Poloxamer 237 (Pluronic F87), Poloxamer 338 (Pluronic F108), and Poloxamer 407 (Pluronic F127). The properties of various poloxamers are slightly different such as hydrophilic-lipophilic balance (HLB) because the amphiphilic character of these copolymers exhibit surfactant properties including ability to interact with hydrophobic surfaces and biological membranes. Poloxamer block copolymers are formed to micelles by self-assembly when the concentration in aqueous solution is above CMC [108]. The core of the micelles consists of hydrophobic PPO block that incorporate various therapeutic reagents as water-insoluble drugs (Figure 1.5B), while the PEO shell convinces that the micelles remain in dispersed state and decrease undesirable drug interactions with cells and proteins [109]. Incorporation of hydrophobic drugs into poloxamer micelles can increase solubility and metabolic stability [31].

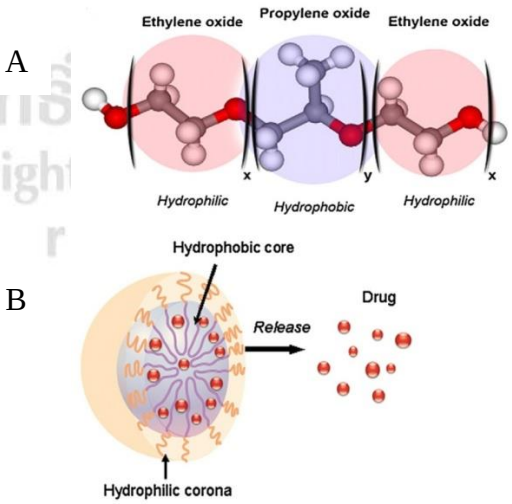


Figure 1.5 Poloxamer block copolymer structure (A) and micelle with water-insoluble drug (B) [31].

Futhermore, poloxamer possess a broad spectrum of biological activities so it is one of the most potential drug targeting systems as resulting in a remarkable impact on the emergent field of nanomedicine [31]. The previous reports presented that the poloxamer block copolymer enhance significantly the bioavailability of various antibacterial and antifungal agents including the biological activity of these agents with respect to many microorganisms [110-113]. Figure 1.6 presents the applications of nanoparticles as antibacterial agents in human health [114].

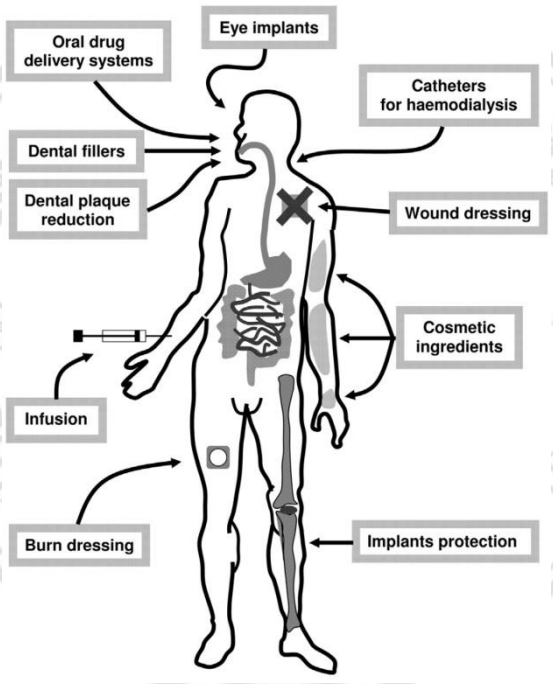


Figure 1.6 Medicinal applications of nanoparticles as antibacterial agents in human health [114].

1.4 Theory and/or Principle

The use of medicinal plant for treatment disorder is interesting way for alternative medicine [115] as there are many phytochemical compounds which can relieve ailment [116]. However, using of natural compounds in medicinal plants is restricted, especially water-insoluble and instability. Recently, nanotechnology has been applied to improve the disadvantageous properties of the natural compounds which exhibit biological activities [27, 28]. Up to date, there are several examples of using nanoparticles in

medical applications (Figure 1.7). Preparation of bioactive agents loaded in polymeric micelles is one of potential techniques to enhance aqueous-solubility and increase stability [31]. The water-insoluble agents can be incorporated within hydrophobic cores of polymeric micelles, thus drug loading capacity is increased. Also, the micelles can be dispersed in aqueous medium by shell of hydrophilic groups when the concentration of polymer in aqueous solution is above CMC [108, 109]. According to biological activities of active agents in polymeric micelles, the investigations are performed not only *in vitro* study but also *in vivo* study via animal model. The results of *in vivo* study indicated toxicity and pharmacodynamics in body that respond to therapeutic effects of the test substances. Animals which are chosen for testing model should be appropriate to biological assays. At present, *S. grandiflora* was extracted and determined for inhibitory action against pathogenic bacteria. Besides, therapeutic effect of the extracts were evaluated by silkworm infection model. The extract showed antibacterial action and therapeutic effect against pathogenic bacteria, however, there were some problems of its water-insolubility and instability. Hypothesis of this present study is that the polymeric micelles loaded with *S. grandiflora* extract might enhance aqueous solubility and increase stability of the extract, and also exhibit antibacterial activity as well as the therapeutic effect. *S. grandiflora* extract-loaded polymeric micelles provide the novel carrier which are effective to treat infectious diseases for further pharmaceutical and clinical trials.

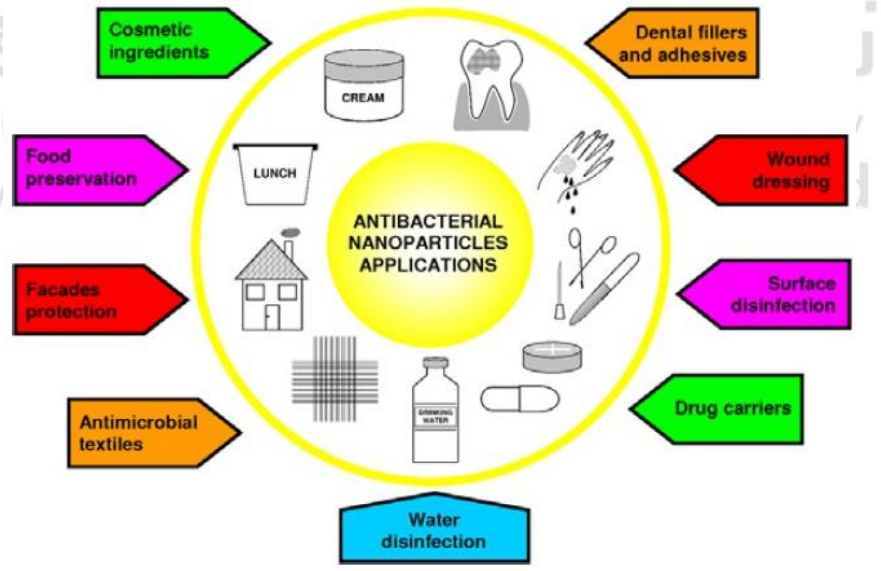
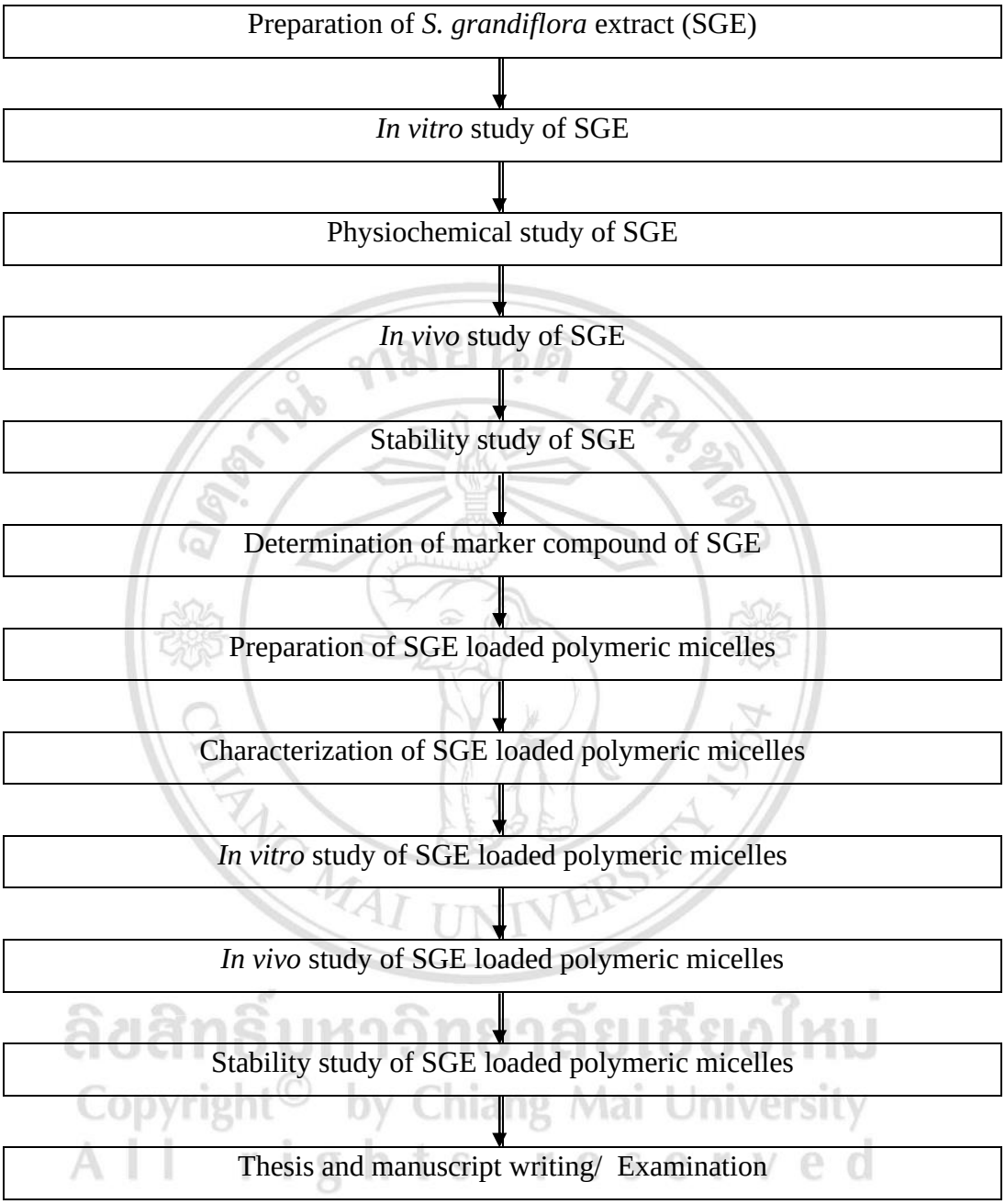


Figure 1.7 The versatility of antibacterial nanoparticles practical applications [114].

1.5 Scope of the Study



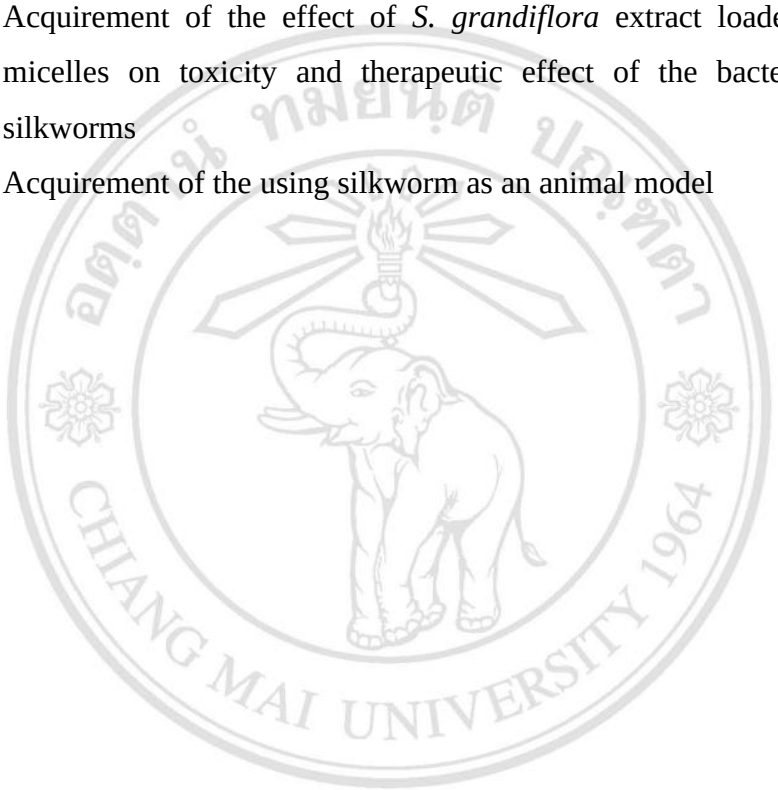
1.6 Expecting Benefit and/or Goal

1.6.1 Acquirement of the kinetic degradation of the bioactive compound in *S. grandiflora* extract

1.6.2 Acquirement of the preparation techniques of *S. grandiflora* extract loaded polymeric micelles in order to enhance the solubility and stability of the extract

1.6.3 Acquirement of the effect of *S. grandiflora* extract loaded polymeric micelles on toxicity and therapeutic effect of the bacterial infected silkworms

1.6.4 Acquirement of the using silkworm as an animal model



CHAPTER 2

Materials and Methods

2.1 Chemicals

2.1.1 Acetic anhydride $(CH_3CO)_2O$) (Merck, Germany)

2.1.2 Ammonium hydroxide (NH_4OH) (Merck, Germany)

2.1.3 Chloroform ($CHCl_3$) (Analytical grade, Merck, Germany)

2.1.4 Dimethyl sulfoxide (DMSO) (Analytical grade, Merck, Germany)

2.1.5 Disodium hydrogen phosphate (Na_2HPO_4) (Sigma-Aldrich, USA)

2.1.6 Ethanol, absolute (EtOH) (Analytical grade, Merck, Germany)

2.1.7 Ether (Analytical grade, Merck, Germany)

2.1.8 Ethyl acetate (Analytical grade, Merck, Germany)

2.1.9 Ferric chloride ($FeCl_3$) (Sigma-Aldrich, USA)

2.1.10 Gallic acid (Analytical grade, Sigma-Aldrich, USA)

2.1.11 Genlatin (Sigma-Aldrich, USA)

2.1.12 Glacial acetic acid (Analytical grade, Merck, Germany)

2.1.13 Hydrochloric acid (HCl) (Analytical grade, Merck, Germany)

2.1.14 Hexane (Analytical grade, Merck, Germany)

2.1.15 Methanol (MeOH) (Analytical grade, Merck, Germany)

2.1.16 Methanol (HPLC grade, Merck, Germany)

2.1.17 Pluronic® F68 (PF68) (Sigma-Aldrich, USA)

2.1.18 Pluronic® F127 (PF127) (Sigma-Aldrich, USA)

2.1.19 Potassium chloride (KCl) (Sigma-Aldrich, USA)

2.1.20 Potassium dihydrogen phosphate (KH_2PO_4) (Sigma-Aldrich, USA)

2.1.21 Potassium hydroxide (KOH) (Sigma-Aldrich, USA)

2.1.22 Silica gel 60 (0.063-0.200 mm) (Merck, Germany)

2.1.23 Sodium chloride (NaCl) (Sigma-Aldrich, USA)

2.1.24 Sodium hydroxide (NaOH) (Merck, Germany)

2.1.25 Sodium sulfate (Na_2SO_4) (Merck, Germany)

2.1.26 Sulfuric acid (H_2SO_4) (Analytical grade, Merck, Germany)

2.1.27 TLC Aluminium sheet Silica gel 60 F_{254}, layer thickness 210 μm (Merck, Germany)

2.2 Instruments

2.2.1 Analytical balance (Sartorius AC210, Scientific Promotion, Germany)

2.2.2 Centrifugation (Avanti 30, Beckman, USA)

2.2.3 Dialysis membrane, Molecular cut off 12000-14000 KDa (Cellu Sep T1, USA)

2.2.4 Differential scanning colorimeter (DSC) (SII, Seiko Instruments, Japan)

2.2.5 Freeze dryer (Advantage, Vertis, USA)

2.2.6 High performance liquid chromatography apparatus (HPLC) (Hewlett Packard series 1100, Agilent Technology, USA and Water 2695 Alliance, Waters Corporation, USA)

2.2.7 High performance liquid chromatography (HPLC) column (250 mm× 4.0 mm id, 5 μm, Agilent Technology, USA)

2.2.8 Incubator (Model 332, National Application Co., USA)

2.2.9 Magnetic stirrer (Variomag telesystem, Scientific Promotion, Germany)

2.2.10 Micropipette (Eppendrof, USA)

2.2.11 Microplate UV/Vis spectrophotometer (Model 680, Bio Rad, Japan)

2.2.12 Multimode detector (DTX 880, Beckman Coulter, USA)

2.2.13 Multiple point stirrer (Thermo scientific, McQueen Labs, USA)

2.2.14 pH meter (pH Level 2, Inolab, Germany)

2.2.15 Photon correlation spectrophotometer (PCS) (Zetasizer, Malvern Instruments, UK)

2.2.16 Preparative HPLC column (250 mm × 20 mm id, 5 μm) (Senshu Scientific, Japan)

2.2.17 Refrigerator (Sanyo, Japan)

2.2.18 Rotary vacuum evaporator (N100, Eyela, Japan)

2.2.19 Sonicator (Transsonic T460/H, Elma, Germany)

2.2.20 Transmission electron micrometer (TEM) (JEM-2200FS, Jeol, Japan)

2.2.21 Ultraviolet-Visible spectrophotometer (UV-2450, Shimadzu, Japan)

2.2.22 UV viewer (Chromato-VUE® C-70G, USA)

2.2.23 Vortex meter (Model 1291, Lab-line Instrument, Inc., USA)

2.2.24 X-ray diffractometer (Miniflex II destop, Rigaku, Japan)

2.2.25 Zorbax SB-C18 guard column (25 mm × 4.6 mm id, 5 μm) (Agilent Technology, USA)

2.3 Plant materials

S. grandiflora used in this study was collected from Chiang Mai, a northern province in Thailand. This plant was identified in accordance with typical characteristics including branching tree, pinnate leaves, white flowers, and linear pods containing many seeds. The voucher specimen collection of *S. grandiflora* as a herbarium was performed. In order to increase the assurance of the initial identification, the specimen could be identified by comparison with other previous *S. grandiflora* herbarium specimens.

2.4 Experiment

2.4.1 Extract preparation

1) Ethanol crude extract (ECE)

S. grandiflora was carefully sorted into various parts i.e. leaves, branches, barks and heartwood (core). Each part was cut into small pieces and dried at 50°C for 72 h. The dried materials of each part was ground into powder and extracted by maceration with 95% ethanol (50 g of dried powder sample per 500 mL of solvent) for two days, at room temperature, then filtered through Whatman No.1 filter paper. The filtrate was collected and the residue was macerated again with 95% ethanol in the same manner for two times. Finally, the solvent of total filtrate was removed under vacuum at 40°C using a rotary evaporator. The obtained ECE of each part was stored at 4°C for screening of antibacterial activity.

2) Fractionated crude extract (FCE)

The dried powder part of *S. grandiflora* which ECE showed the highest potential antibacterial activity was selected and macerated in a sequence of organic solvents with increasing the polarity i.e. hexane and followed by ethyl acetate, butanol, and methanol, respectively. The dried powder of 200 g per 2000 mL of solvent, was macerated for two days, at room temperature, then filtered through Whatman No.1 filter paper. The filtrate was collected and the residue was macerated again with the same solvent as the same manner for two times. The residue after the third filtration was dried at room temperature to ensure the prior solvent had removed completely. The dried residue was further macerated in another solvents in the same manner as the previous one. The filtrates of the same solvent was pooled together, then the solvent was removed under vacuum at 40°C using a rotary evaporator The FCE obtained from each solvent was stored at 4°C for further study.

2.4.2 Characterization of *S. grandiflora* extracts

The *S. grandiflora* extracts which presented potential antibacterial activity was chosen to indentify UV fingerprint using Ultraviolet-Visible spectrophotometer by scanning in a spectrum mode at the range of 800 to 200 nm. The spectrum peak which showed the highest absorption was expressed at the maximum wavelength (λ_{max}).

2.4.3 Phytochemical screening

ECE of the *S. grandiflora* part with distinctive antibacterial activity was chosen to identify the phytochemical compounds presented by different tests as follows.

1) Alkaloids test

1.1) Primary test

Alkaloids was investigated by precipitating reagents, e.g. Dragendorff's reagent (solution of potassium-bismuth iodide, $KBiI_4$), Mayer's reagent (solution of potassium-mercuric iodide, K_2HgI_4), Wagner's reagent (solution of potassium triiodide, KI_3), Marme's reagent (solution of potassium-cadmium iodide, K_2CdI_4), and Kraut's reagent (solution of potassium-bismuth iodide, modified Dragendorff's reagent).

Firstly, ECE was dissolved in ethanol with 1% HCl and stirred until completely dissolved, then filtered through Whatman No.1 filter paper for primary test. The acidic extract solution was placed into different test tubes. The alkaloidal precipitating reagents were dropped into each test tube. Positive results of the sample containing alkaloids showed turbidity and/or precipitation.

1.2) Confirming test

Acidic extract solution from primary test preparation was purified by partition with ether using separating funnel. The mixing solution was swirled slowly and sat at room temperature to separate ether phase. An aqueous phase was added with NH_4OH to obtain basic solution (alkaline solution), then ether was added and swirled gently to extract unionized base. The unionized base solution was existed in ether phase. The ether phase was separated and collected, then the aqueous phase was partitioned with ether again as same manner. After that, ether phase was pooled and ether was removed using evaporating dish on hot plate until the solution was nearly dry. The drying off solution was added with 1% HCl then stirred until it was completely dissolved. The acidic extract solution was filtered and filled into test tubes, followed by dropping alkaloid testing reagents as mentioned in primary test. If there was precipitate, it could be concluded that there was primary, secondary, or tertiary alkaloids in the extract. The aqueous phase which was basic extract solution was gradually added with 1% HCl until acidic solution was obtained. This phase was filtered and tested with alkaloid testing reagents. If there was precipitate, it could be concluded that there was quaternary alkaloids or amine oxide in the extract.

2) Glycosides test

Glycosides is a molecule which a sugar group bonded to another functional group by glycosidic bond. The sugar group is known as glycogen while the non-sugar group is known as aglycone or genin part of the glycoside. The glycogen many consist of a single molecule (monosaccharide) or several molecules (oligosaccharide) sugar. However, the using of glycoside for biochemistry and pharmacology has many applications, it depends on type of aglycone. Therefore, glycosides are classified

according to chemical nature of the glycone, also there are different methods for glycoside identification.

ECE was dissolved in ethanol with 10% HCl then refluxed for 30 min and cooled down at room temperature, then partitioned with ether using separating funnel. The aqueous phase was collected and partitioned with ether twice as same manner. Next, ether phase was pooled and added with anhydrous Na_2SO_4 for absorbing the excess water. The obtained solution both ether phase and aqueous acidic phase were investigated to identify the type of glycoside as follows.

2.1) Anthracene/Anthraquinone glycoside

Anthracene or anthraquinones was investigated by Borntrager's test. The test solution in ether phase was heated to increase concentration. After cool down, 25% NH_4OH solution was added into the test ether solution then shaken to stimulate reaction. The changing color of NH_4OH phase of test solution was observed. If the NH_4OH phase showed red color, it represented a positive result which indicated there was emodol group which was composition of anthraquinone glycoside in the test sample.

2.2) Coumarin glycoside

Coumarin was investigated by Coumarin's test. Solvent of the test solution in ether phase was removed solvent by heating. Then, dried sample was dissolved with hot DI water. The obtained test aqueous solution was divided into two parts in test tubes. The first test tube was added with 10% NH_4OH solution and the other was untreated as a control. Both test tubes were placed under UV lamp, the fluorescence of test solution was observed. If the test solution with NH_4OH appeared blue or green fluorescence under UV light, it indicated a positive result of conjugation of coumarin ring in the test sample.

2.3) Sterol glycoside/Triterpene glycoside

Sterol glycoside or triterpene glycoside was investigated by Liebermann Burchard's test. Solvent of the test solution in ether phase was removed by heating. Then, dried sample was dissolved in acetic anhydried with $CHCl_3$. The obtained sample

solution in test tube was added with H_2SO_4 by dropping slowly along the inner wall of the tube. The color of ring in the middle of two phases was observed at 5, 15, and 30 min. If the color of ring began red or reddish brown then changed to violet and finally green or blue-green, it pointed to steral glycoside resided in the test sample. It the results showed that the color of ring was initially red or reddish brown and continuously turned to violet, it indicated triterpene glycoside in the test sample.

2.4) Cardiac glycoside

Cardiac glycoside was investigated by Kadde's test. Solvent of the test solution in ether phase was removed using evaporating dish by heating. Then, dried sample was dissolved in methanol and added KOH solution in ethanol with 1% 3,5-dinitrobenzoic acid in ethanol. After that, the sample solution was boiled on water bath. The color of solution was observed, if the solution was violet, it revealed cardiac glycoside in the test sample.

2.5) Saponin glycoside

Saponin glycoside was investigated by froth test. The test solution in ether phase was evaporated by heating. The residue was dissolved in DI water and shaken with vortex. The bubble was observed, if there was froth after shaking, it indicated the presence of saponin glycoside in the test sample.

2.6) Flavonoid glycoside

Flavonoid glycoside was investigated by Shibata's test. Solvent of the test solution in ether phase was removed by heating. The obtained residue was dissolved with 50% methanol on water bath. After that, a small magnesium metal sheet was put in the test solution, then concentrated HCl was dropped on magnesium sheet. The color of test solution was observed, if the test solution showed red color, it represented there was flavonal compounds in the test sample. While orange color referred to the presence of flavanone compounds.

2.7) Anthocyanine

Anthocyanine was investigated by an acidic-alkaline test. Solution of 25% NH_4OH was added to the aqueous of the test solution in order to adjust pH to neutral and basic solution, respectively. Color changing of solution was observed, if the test solution was red in acidic solution, and changed to violet in neutral solution then changed to blue or green in basic solution, it implied the presence of anthocyanine.

3) Phenolic and tannin test

Phenolic compounds was investigated by ferric chloride test, and tanin by gelatin test.

Firstly, ECE was dissolved in 95% ethanol and removed the organic solvent using evaporating dish on hot plate until the solution was sticky. The sticky solution was added with hot water and stirred to cool down. After that the test extract solution was added with a few drops of 10% NaCl solution in order to salt out the impurity except tanin. The test solution was filtered using a buchner funnel through Whatman No.1 filter paper. The obtained clear solution was placed into different test tubes. Then, the reagents were dropped into each test tube comparison with untreated as a control. In the first test, 1% $FeCl_3$ solution was dropped into test solution. Unless there were any reaction, it could be referred that the test sample did not contain the phenolic compounds and tannin. But, it had phenolic compounds and/or tannin if there was precipitation with $FeCl_3$. Next test, 1% gelatin solution and 1% gelatin in saline solution was dropped into other test solution. If there were reaction with $FeCl_3$ and gelatin, it could be indicated that there was phenolic compounds and/or tannin in the test sample. However, there were several positive results up to type of tannin and phenolic compounds. If the result gave a green or blue solution with $FeCl_3$ but non-responding with gelatin, it meant that the sample included only phenolic compounds. If the result showed a blue-green or dark green nearly black solution with $FeCl_3$ and precipitating with gelatin in saline, it implied that there was catechol, subgroup of tannin. Whereas, the result rivaled a dark solution after adding $FeCl_3$ and precipitating with gelatin in saline, it indicated that there was pyrogallol or gallic tannin, subgroup of tannin, in the test sample.

2.4.4 *In vitro* antibacterial activity

1) Culture preparation

There were various test pathogenic bacteria e.g. standard strains of *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923, *Salmonella typhi* DMST 5784, and *Shigella sonnei* DMST 561. Clinical strains of *Bacillus cereus, Bacillus subtilis, Enterobacter cloacae, Enterococcus faecalis, Escherichia coli, Listeria monocytogenes, Pseudomonas aeruginosa, Serratia marcescens, Staphylococcus aureus, Staphylococcus epidermidis*, *Streptococcus agalactiae*, *Streptococcus pneumoniae*, *Streptococcus pyogenes*, and *Streptococcus sanguinis*. Sensitive strain such as methicilin sensitive *Staphylococcus aureus* (MSSA1). Resistant strains such as methicilin resistant *Staphylococcus aureus* strains (MRSA3, MRSA4, MRSA6, MRSA8, MRSA9, MRSA11, and MRSA12) and vancomycin resistant *Enterococcus faecalis* EF5 (VRE). They were sub-cultured individually on fresh Tryptic Soy Agar (TSA) and incubated overnight at 37°C, then suspended in normal saline (0.9% NaCl), and diluted to a McFarland turbidity standard No. 0.5. The bacterial suspension was used for antibacterial activity test.

2) Screening of antibacterial activity

Agar well diffusion method was used to evaluate the antibacterial activity. Briefly, using the bacterial suspension obtained from a 100-fold dilution of McFarland turbidity standard No. 0.5 was inoculated in melted TSA medium and poured into sterile plate. Then, the well of 6 mm diameter was performed. The test ECE of each part extract was dissolved in sterile 50% ethanol. Fifty µl of these sample solutions were delivered into the well and the same volume of sterile 50% ethanol was used as a negative control. The inoculated plates were incubated overnight at 37°C. After incubation, the diameter of inhibition zone (DIZ) was measured and expressed in millimeters to evaluate the antibacterial activity of the sample.

3) Minimum inhibitory concentration (MIC) assay

3.1) Agar dilution method

The standard agar dilution method was used to determine MIC of the test FCE of *S. grandiflora* bark which showed the highest DIZ according to the agar well diffusion method. The test extracts were done as follows. The test FCE of each sequent extracts was dissolved in sterile 50% ethanol and serially diluted to two-fold with 50% ethanol. One mL of each concentration of the test solution was added to 9 mL of melted Mueller-Hinton Agar (MHA) media and poured into a sterile plate. The concentration of each FCE in the agar plates was in the range of 0.63-20.00 mg/mL. Equivalent amounts of sterile distilled water and sterile 50% ethanol was used as controls. Ten µL of each test pathogenic bacteria suspension with turbidity equivalent to McFarland standard No. 0.5 which then diluted 100-fold was inoculated into each agar dilution plate. The inoculated plates were incubated overnight at 37°C. Then growth inhibition of the bacteria was detected.

The MIC was defined as the lowest concentration of the FCE samples which exhibited complete inhibition of bacterial growth.

3.2) Broth dilution method

The broth dilution method was used to determine MIC of the test extracts which showed the highest activity from the agar plate method. The test was performed as follows. The sample was firstly dissolved in sterile solvent. The sample solution obtained was added to Mueller-Hinton Broth (MHB). This sample solution was serially diluted to two-fold with MHB. The bacterial suspension obtained from 100-fold dilution of McFarland turbidity standard No. 0.5 was inoculated into the sample broth dilution. The test sample was incubated overnight at 37°C. Negative control was prepared with un-inoculated bacterial suspension and positive control was contained inoculated bacterial suspension without the test samples. After incubation overnight at 37°C, the dilution tests were checked turbidity visually to detect growth inhibition of the bacteria. The growth end point was determined by comparing the amount of growth in different concentrations of the test samples with that in the negative control. Acceptable growth must occur in the positive control.

The MIC was defined as the lowest concentration of the samples which was able to inhibit any visible bacterial growth.

4) Minimum bactericidal concentration (MBC) assay

To determine MBC, the broth dilution method was slightly modified. The sample was taken from the tested of the MIC assays, the concentrations which showed no visible turbidity were observed by streaking on freshly prepared TSA plates. The streaked plates were incubated overnight at 37°C. Later, the plates were detected decease of the bacteria.

The MBC was defined as the lowest concentration of the samples with no bacteria growth on the surface of the medium in the plates.

5) Kinetics of bacteria killing

Kinetic of bacteria killing was investigated by a broth dilution method. The adjusted bacterial suspension to a McFarland turbidity standard No. 0.5 was added to MHB with the test sample at the same concentration of MBC and incubated for an appropriate times at 37°C, to achieve a logarithmic growth phase. All cultures contained approximately $1x10^6$ CFU/mL at initial time. The actual initial concentration was determined by ten-fold serial dilution with normal saline of the inoculums then plated the serial dilutions on TSA to calculate the logarithm of colony forming units per milliliter (log CFU/mL). At indicated time points, the sample was removed and serially diluted with normal saline. The log CFU/mL in the culture at each time point was determined by spreading on freshly TSA plates and incubating overnight at 37°C. After that, the colonies were counted and the resulting CFU/mL was used to provide an estimate of the number of viable cells. Result of killing kinetic study was expressed as the difference between the log CFU/mL at the indicated time point and the log CFU/mL of the inoculums at initial time. Bactericidal activity was defined as decreasing the original inoculums after 24 h of incubation.

2.4.5 Solubility study of *S. grandiflora* extracts

The ECE and FCE which showed the highest potential antibacterial activity were selected to study the solubility in various solvents. The studied solvents were acetone, butanol, DMSO, ethanol, ethyl acetate, hexane, and water. Each solvent was gradually added into the exact amount of the extract and stirred until the extract was completely soluble. The exact amount of solvent that completely dissolved the extract by naked eyes observation was recorded and determined solubility in term of approximate solubility according to Table 2.1.

Table 2.1 Definition of solubility [117].

Descriptive term	Solubility (Part of solvent required to dissolve 1 part of solute)
Very soluble	Less than 1
Freely soluble	1 - 10
Soluble	10 - 30
Sparingly soluble	30 - 100
Slightly soluble	100 - 1000
Very slightly soluble	1000 - 10000
Practically insoluble	More than 10000

2.4.6 Stability study of *S. grandiflora* extracts

The ECE and FCE which showed the highest potential antibacterial activity were selected to study the stability in closed container with light protection at various temperatures of 4°C, room temperature, and 45°C for 90 days. The physical appearance was observed by naked eyes observation and *in vitro* antibacterial activity was determined by broth dilution assay against *S. aureus* ATCC 25923.

2.4.7 *In vivo* study of *S. grandiflora* extracts

Silkworm larvae were used as an animal model of toxicity evaluation and therapeutic effect of test samples.

1) Effect of diets on growth development in silkworm study

Fourth instar silkworm larvae were divided into two groups; the first group was fed with fresh mulberry leaves and the other group was fed with artificial diet. All of silkworms were kept in a safety cabinet at room temperature. The mulberry leave or artificial diet was given to silkworms everyday for 13 days. The morphology and body weight of silkworms were observed by visualization and measure using an analytical balance during the period of study.

2) Silkworm toxicity study

Fifth instar silkworm larvae were fed on the 1st day and reared overnight. The test sample was dissolved in normal saline. The sample solution was injected into the hemolymph using disposable plastic syringes with a 27-gauge needle. After one day, the survival rate of injected silkworms was recorded and the median lethal concentration (LC_{50}) also the median lethal dose (LD_{50}) were determined from the survival curve. The LC_{50} value was determined as the concentration of tested samples at which 50% of the injected silkworm larvae were dead 1 day later. The LD_{50} value was determined as the amount of tested samples per body weight of silkworm at which 50% of the injected silkworm larvae were dead 1 day later.

3) Killing effect of bacteria in silkworm study

Fifth instar silkworm larvae were divided into 6 groups according to concentration of bacterial suspension and control groups. The test silkworm of each group was injected with bacterial suspension as a concentration of 10^6, 10^7, 10^8, and 10^9 cells/mL. The positive control group was injected with normal saline without bacteria, and no treatment group was done for negative control group. The injection was performed using a 27-gauge needle pushed into the hemolymph through dorsal surface of the silkworm. All of injected silkworms were kept in a safety cabinet and observed the survival rate in later.

4) Therapeutic effect in silkworm larvae study

Fifth instar silkworm larvae were injected with bacterial suspension and test sample solution. Suspension of the bacteria in normal saline was injected in to the silkworm hemolymph. Then, the test sample which dissolved in normal saline was injected into the hemolymph immediately after injection of the pathogens. After injection, silkworms were placed in a safety cabinet. On the next days, the survival rates of tested silkworms were observed and measured for determining a median inhibitory concentration (IC_{50}). The IC_{50} value was determined as the concentration of test samples in the larval blood at which 50% of the injected silkworm larvae survive 2 days later. Moreover, a median effective dose (ED_{50}) was determined. The ED_{50} value was determined as the amount of tested sample per body weight of silkworm required for 50% survival under conditions in which more than 90% of silkworms were killed by pathogens.

2.4.8 Marker compound determination

The FCE which showed the highest antibacterial activity and therapeutic effect was selected to isolate by chromatography technique.

1) Quick column chromatography

Quick column chromatography was used to isolate different compounds of *S. grandiflora* extract. The isolation condition was performed by sintered glass column containing silica gel 60 as adsorbent and mixture of hexane:ethyl acetate and ethyl acetate:methanol in gradient composition with increasing polarity as eluent. The *S. grandiflora* extract was dissolved in hexane:ethyl acetate at a ratio of 95:5 and mixed with silica gel, then dried and put in the sintered glass column containing silica gel. Silica gel was put on the sample layer again and covered with cotton. After that, the extract was eluted by gradient eluent and eluting rate was increased by pump. Each collected fraction from the column was combined by similar TLC pattern and concentrated by rotary evaporator. The obtained fractions were tested for antibacterial activity against *S. aureus* by broth dilution method. The fraction which showed the

highest antibacterial activity was selected to test primary phytochemical compounds including glycoside, phenolic, and tannin in order to get the bioactive compound group.

2) Partial purified extract (PPE)

The FCE which showed the highest antibacterial activity and therapeutic effect was chosen to isolate and test for antibacterial activity. In case, the highest potential fraction of the FCE were quite semi-polar or polar compound, the extract was increased purification via extracting the impurities as non-polar compounds in previously. The dried powder part of *S. grandiflora* which exhibited the highest potential antibacterial activity was macerated with gradient of organic solvents by increasing the polarity when start from non-polar solvent in order to eliminate impurities of the extract like hexane and chloroform, then followed by semi-polar until polar solvent such as ethyl acetate, butanol, and methanol, respectively. The maceration, filtration, till evaporation the solvent was done as the same manner in the previous extract preparation. The obtained PPE for each solvent was compared with the FCE by TLC pattern as the same condition, including evaluated antibacterial activity.

3) Preparative column chromatography

Normal-phase column chromatography was used to isolate extract of PPE which exposed the highest antibacterial activity. The condition of isolation was used silica gel 60 as adsorbent and mixture of chloroform:methanol in gradient composition with increasing polarity as eluent. Each fraction collected from the column was evaporated by vacuum centrifugal evaporator. The obtained fractions were dissolved in DI water and tested for antibacterial activity against *S. aureus* by broth dilution method.

4) Preparative HPLC

The PPE which revealed the strongest antibacterial activity was dissolved in methanol and isolated by preparative HPLC using a Senshu Pak Pegasil ODS SP100 column. The mobile phase was a linear gradient from 10% to 100% of methanol for 30 min, and sustain in eluting with 100% methanol for 15 min at a flow rate of 9 mL/min. The eluents were detected by a photodiode array detector (PDA) and collected every 2 min. Each collected fraction was evaporated by vacuum centrifugal evaporator and

dissolved in DI water for determination of antibacterial activity against *S. aureus* by broth dilution method.

5) Qualitative HPLC analysis

Fingerprint analysis of PPE which demonstrated the strongest antibacterial activity was performed by HPLC using a Hypersil ODS column and gradient eluent of solvent A (1% acetic acid in water) and solvent B (methanol). The eluent gradient program started from 100% of solvent A for 1 min then changed to 70% and 40% at 10 and 20 min, respectively. Finally, the eluent composition was put back to 100% of solvent A at 25 min for 5 min in order to the next run. The eluent was detected by an UV/VIS detector at wavelength of 280 nm using a flow rate of 1 mL/min. The chromatogram of the PPE was compared with that of different standard phenolic compounds injected in the same condition of HPLC and sample preparation that dissolved in methanol (HPLC grade) and filtered through a 0.22 μm filter membrane before injection.

For confirming determination of marker compound in PPE, the standard phenolic compound as a marker compound and PPE were compared different chromatograms which got from various isocratic conditions of HPLC study. The variation of isocratic conditions was varied ratios of solvent A to solvent B and adsorption wavelengths for eluent detection.

6) Quantitative HPLC analysis

Quantification analysis of PPE which exhibited the highest antibacterial activity was executed by HPLC using a Hypersil ODS column and isocratic eluent of solvent A (1% acetic acid in water) and solvent B (methanol) which were an optimal condition as obtained from various isocratic eluent of the previous study. The content of major compound in the PPE was determined by comparison to the calibration of standard marker compound.

2.4.9 Development of polymeric micelles loaded with *S. grandiflora* extracts

1) Preparation of *S. grandiflora* extract-loaded polymeric micelles

The PPE of *S. grandiflora* bark extract which exposed the highest antibacterial activity was prepared to *S. grandiflora* extract (SGE) loaded polymeric micelles by thin-film hydration method. The stock solution of SGE (20 mg/mL) and Pluronic polymer (20 mg/mL) were dissolved in ethanol. Required amount of stock solutions was transferred to round-bottom flasks in order to vary proportion of SGE to polymer. The solvent was evaporated under reduced pressure using rotary vacuum evaporation to obtain a thin film of SGE-polymer, then the film was further dried over night at room temperature to remove any residual. After that, the film was hydrated with distilled water and dispersed with ultrasound sonicator to form SGE incorporated into micelle. Non-incorporated SGE was separated by centrifugation of the micelle suspension at 5000 rpm for 20 min and filtered through a 0.45 μm filter membrane, and solution of SGE polymeric micelles was obtained. The blank micelles were prepared with the same manner, but without SGE.

2) Evaluation of *S. grandiflora* extract-loaded polymeric micelles

2.1) Entrapment efficiency and loading capacity

The amount of SGE loaded inside the polymeric micelles was determined using HPLC by comparison to the calibration of marker standard compound. The polymeric micelles was firstly disintegrated with methanol (HPLC grade) and detected via HPLC determination. The qualitative HPLC analysis was carried out on an Agilent system using a Hypersil ODS column (4.0 x 250 mm, 5 μm) and mixed solvents of 1% acetic acid in water and methanol at a ratio of 80:20 with injection volume of 10 μL. The eluent was detected by an UV/VIS detector at wavelengths of 280 nm using a flow rate of 1 mL/min for 15 min. The entrapment efficiency (% EE) and loading capacity (% LC) were calculated by following equation:

$$\%\ \mathrm{EE} = \frac{\text{amount of loaded SGE}}{\text{total amount of feeding SGE}} \times 100$$

$$\% \text{ LC} = \frac{\text{amount of loaded SGE}}{\text{total amount of feeding polymer and SGE}} \times 100$$

2.2) *In vitro* antibacterial activity

The bacterial activity of SGE loaded polymeric micelles and blank polymeric micelles comparison with free SGE as non-incorporated in the micelles was evaluated by broth micro-dilution assay against bacterial standard strain of *S. aureus* ATCC 25923. The samples of polymeric micelles were serially diluted two-fold with MHB, and added the bacterial suspension which gained from a 100-fold dilution of McFarland turbidity standard No. 0.5 into the micelles dilution. The test micelles were incubated overnight at 37°C, then observed turbidity to detect growth inhibition of the bacteria. The minimum concentration of the test samples which could inhibit the bacterial growth was recorded as the MIC value. The samples which showed inhibitory activity were determined minimum bactericidal concentration (MBC) by streaking on freshly prepared TSA plates. The streaked plates were incubated overnight at 37°C, then observed the decease of bacteria. The lowest concentration of the test samples with no bacteria growth on the surface of the TSA plates was recorded as the MBC value.

2.3) *In vivo* study

The toxicity of SGE loaded polymeric micelles and blank polymeric micelles was investigated using a silkworm model. The test micelles were dispersed in normal saline, then separated the non-entrapment by centrifugation and filtration through a 0.45 μm filter membrane. The micelles solution was diluted into various concentrations with normal saline. The different dilution of test micelles were injected into the hemolymph of silkworms. The injected silkworms were monitored on the survivability for 1 day after injection.

The therapeutic effect of SGE loaded polymeric micelles was evaluated using a silkworm infected model. The suspension of *S. aureus* ATCC 25923 as concentration of McFarland turbidity standard No. 0.5 in normal saline was injected firstly into the silkworm hemolymph, then the SGE micelles solution was injected sequentially into the hemolymph of silkworm. The injected silkworms were placed in safety-cabinets and their survival was observed for 2 days after injection.

2.4) Particle size, size distribution, and zeta potential

The particle size, size distribution, and zeta potential of the obtained polymeric micelles were measured by dynamic light scattering method using photon correlation spectrophotometer (PCS). The micelles were dispersed in deionized water and measured at a fixed angle of 173° at 25°C.

2.5) Morphology

The shape of SGE loaded polymeric micelles and blank polymeric micelles was visualized by transmission electron microscopy (TEM) operating at an accelerating voltage of 200 kV. The polymeric micelles were dispersed using ultra sonicator for 5 min and dropped on the surface of a carbon coated copper grid, then the grid was dried by leaving overnight at room temperature in desiccators before TEM investigation.

2.6) Thermal behavior

The thermal behavior of lyophilized SGE loaded polymeric micelles and blank polymeric micelles comparison with freeze dried of SGE was investigated by differential scanning calorimeter (DSC). Weigh approximate 5-7 mg of each sample and sealed in an aluminum pan with hole, then heated from 25°C to 200°C at a heating rate constant of 10°C/min under nitrogen gas at constantly flow rate of 20 mL/min. An empty pan was used as a reference. The thermogram of samples was analyzed for melting point and thermal reaction.

2.7) Crystallography

The possible crystallinity of lyophilized SGE loaded polymeric micelles and blank polymeric micelles comparison to freeze dried of SGE was investigated by mean of X-ray diffractrometry (XRD). Each sample was smeared on the cell and registered at Bragg angle (2θ) in a range of 5° to 60° with counting time of 0.015° at a scanning rate of 12°/min. The XRD diffractogram of samples was analyzed for crystallinity.

2.8) Solubility study

The solubility of SGE loaded polymeric micelles in comparison with SGE was dissolved in water and dispersed with ultrasound sonicator for 30 min. The obtained dispersion was centrifuged at 5000 rpm for 20 min, the supernatant was filtered through 0.45 μm Millipore membrane to remove the insoluble compound. The filtrate was mixed with MeOH (HPLC grade) prior to HPLC analysis as previously described.

2.9) Release study

The *in vitro* release study of SGE from polymeric micelles was investigated by dialysis bag technique. The thin film of SGE loaded polymeric micelles was dispersed in phosphate buffer solution (PBS), pH 7.4 using ultrasound sonicator. Non-incorporated SGE was separated by centrifugation and filtration through a 0.45 μm filter membrane. One milliter of the SGE loaded polymeric micelles in PBS pH 7.4 was placed into a pre-swollen dialysis membrane (molecular weight cut off 12 kDa) and sealed with dialysis clip. Then the packed dialysis bag was immersed into 50 mL of the release medium of PBS pH 7.4 under constant stirring at 200 rpm, 37°C. At predetermined time intervals, 5 mL of the release medium of each sample was drawn and replaced with the same volume of fresh medium. The content of marker compound of SGE was quantified by UV-Visible spectrophotometer at 280 nm. The releasing medium of the PBS pH 7.4 and micelles without SGE were use as blank controls of free SGE and SGE loaded polymeric micelles, respectively.

2.10) Stability study

2.10.1) Accelerated stability analysis

The influence of accelerated temperature on the degradation of free form SGE as dissolved in 50% ethanol and micelles form as dissolved in water was investigated. The samples were incubated at different temperatures of 50°C, 60°C, 70°C, 80°C, and 90 °C for 8 h. The samples were withdrawn at different time intervals and determined the remained amount of SGE by HPLC analysis as previously described.

2.10.2) Storage stability analysis

The stability of free SGE and SGE loaded polymeric micelles was evaluated with aqueous solution form in storage condition. The test samples were stored in closed container with light protection at various temperatures of 4°C, room temperature, and 45°C for 90 days. Time-dependent change in physical appearance and remained SGE content during the storage period were monitored. The physical appearance was observed by naked eyes and the amount of SGE was investigated by HPLC analysis.

2.5 Statistical analysis

The obtained data were statistically analyzed by SPSS statistic 17.0 software. The mean value was determined for significance at $p<0.05$ by ANOVA and Tukey's Multiple as a post hoc test. According to silkworm experiment, Log-rank test was performed by Prism5 for Mac OS X (GraphPad Software, Inc.). Difference at $p<0.05$ was considerated significant. The results were expressed as mean ± standard deviations (SD) of at least 3x independent experiments.

CHAPTER 3

Results and Discussion

3.1 Plant material

S. grandiflora was collected from Chiang Mai, a northern province of Thailand and identified in accordance with typical characteristics including branching tree, pinnate leaves, white flowers, and linear pods as containing many seeds. The voucher specimen collection of *S. grandiflora* used in the present study has been deposited in the herbarium of Faculty of Pharmacy, Chiang Mai University, Chiang Mai, Thailand (no. 023207).

3.2 Ethanol crude extract (ECE)

3.2.1 Preparation of ECE

The ECE of several parts of *S. grandiflora* were extracted by maceration method with 95% ethanol. Leaf extract gave the highest yield of 23.28% (w/w) followed by bark extract of 13.33% (w/w). The yield values and appearances of each extract were shown in Table 3.1.

Table 3.1 Percentage yields and appearances of ECE.

ECE	Yield (% w/w)	Appearance
Leaf extract	23.28	Sticky dark-green extract
Branch extract	8.59	Sticky dark-brown extract
Bark extract	13.33	Sticky dark-brown extract
Heartwood extract	3.70	Sticky brown extract

3.2.2 Screening of antibacterial activity of ECE

The ECE of several parts of *S. grandiflora* were first by screened for their antibacterial activity by agar well diffusion method against standard strains of *E. coli* ATCC 25922 and *S. aureus* ATCC 25923 which represented Gram negative and Gram positive bacteria, respectively. Gentamicin with concentration of 75 μg/mL was used as a positive control. The DIZ of each ECE with concentration of 100 mg/mL against the *E. coli* and *S. aureus* were shown in Table 3.2. It was found that the ECE of bark extract exhibited the strongest inhibitory activity against both strains with DIZ of 9.4 mm and 13.7 mm for *E. coli* and *S. aureus*, respectively whereas the ECE of leaf extract did not show inhibition zone. Therefore, the bark was selected to prepare FCE for further studies.

Table 3.2 DIZ of growth inhibition of ECE from each part of *S. grandiflora* and gentamicin against *E. coli* and *S. aureus* by agar well diffusion method.

ECE	Diameter of inhibition zone, DIZ (mm) [a, b]	
	E. coli ATCC 25922	*S. aureus* ATCC 25923
Leaf extract	NZ [c]	NZ [c]
Branch extract	7.8 ± 0.3	10.7 ± 1.0
Bark extract	9.4 ± 0.1	13.7 ± 0.6
Heartwood extract	NZ [c]	8.9 ± 0.2
Gentamicin	16.3 ± 0.9	23.1 ± 2.5

[a] No bacterial growth in the negative control.

[b] Data are presented as mean ± SD (n = 3).

[c] NZ represents no inhibition zone.

3.2.3 UV spectrum of ECE

The ECE from the bark of *S. grandiflora* which presented the strongest antibacterial activity against *E. coli* and *S. aureus* was identified UV spectrum as shown in Figure 3.1. The λ_{max} of ECE was 276.00 nm.

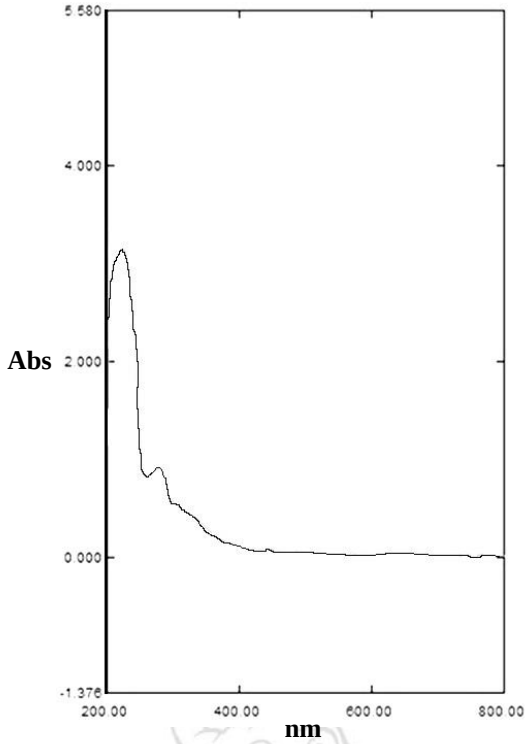


Figure 3.1 The UV spectrum of ECE from *S. grandiflora* bark.

3.2.4 Phytochemical screening of ECE

The ECE from the bark of *S. grandiflora* which presented the highest potential antibacterial activity against *E. coli* and *S. aureus* was preliminary phytochemical screened to specify chemical constituents. The phytochemical testing included alkaloids, glycoside, phenolics, and tannins were determined. The results of phytochemical screening of ECE in *S. grandiflora* bark were as follows.

1) Alkaloids

Alkaloids in the ECE of *S. grandiflora* bark was checked with alkaloidal precipitating reagents such as Dragendorff's reagent, Mayer's reagent, Wagner's reagent, Marme's reagent, and Kraut's reagent. It is also compared with alkaloid standards i.e. strychnine, atropine, and scopolamine HBr, and blank controls i.e. de-ionized water (DI water) and 95% ethanol. Turbidity or precipitation with specific reagents indicated the presenting of alkaloids. The alkaloids test of the ECE was shown in Table 3.3. The ECE of *S. grandiflora* bark gave negative results with all alkaloid testing reagents. There

were not turbidity or precipitations with any reagents. This results indicated that alkaloids were not phytochemical compositions in this plant extract.

Table 3.3 Alkaloids test of ECE from *S. grandiflora* bark, alkaloid standards, and controls with alkaloidal precipitating reagents.

Sample	Tests					
	Dragen-dorff	Mayer	Wagner	Marme	Kraut	Control
ECE	-	-	-	-	-	-
Strychnine [a]	+	+	+	+	+	-
Atropine [a]	+	-	-	-	+	-
Scopolamine HBr [a]	+	+	-	-	+	-
DI water [b]	-	-	-	-	-	-
95% EtOH [b]	-	-	-	-	-	-

(+) define as positive result and (-) define as negative result.

[a] Alkaloid standards of alkaloids test.

[b] Blank controls of alkaloids test.

2) Glycosides

Various types of glycosides in the ECE of *S. grandiflora* bark was examined by several tests as follows.

Anthracene or anthraquinone glycoside was determined by Borntrager's test. The sample was tested with NH_4OH solution. The results revealed that there was red color in NH_4OH phase, which indicated anthraquinone glycoside was consisted in the extract.

Coumarin glycoside was determined by Coumarin's test. The sample was added with 10% NH_4OH solution and fluorescence detected under UV light. It exhibited blue fluorescence under UV light at 365 nm, which implied the presence of coumarin glycoside in the extract.

Sterol glycoside or triterpene glycoside were determined by Liebermann Burchard's test. The sample solution was ascertained a ring color between sample phase and H_2SO_4 phase at several time periods (5, 15, and 30 min), i.e. a reddish brown at 5 min, and changed to violet at 15 to 30 min, triterpene glycoside was a composition in the extract.

Cardiac glycoside was determined by Kadde's test. The sample was tested with KOH solution and 1% 3,5-dinitrobenzoic acid, then a violet color occurred. It referred that the presence of cardiac glycoside in the extract.

Saponin glycoside was determined by froth test. After shaking the test solution, froth appeared on the top surface of solution. It implied that saponin glycoside was comprised of the extract.

Flavonoid glycoside was determined by Shibata's test. The reaction was observed after dropping HCl on magnesium sheet which existed in test solution. No change of reaction was seen, it indicated no flavonoid glycoside stood in the extract.

Anthocyanine was determined by an acidic-alkaline test. The sample was adjusted from acid to neutral and continued to base. The results showed red color in acidic solution without color change when pH increased. This outcome indicated absence of anthocyanine in the extract.

All results of glycosides examination were concluded in Table 3.4. The ECE of *S. grandiflora* bark consist of several glycosides such as anthraquinone glycoside, coumarin glycoside, triterpene glycoside, cardiac glycoside, and saponin glycoside.

Table 3.4 Glycosides test of ECE from *S. grandiflora* bark and controls.

Sample	Tests						
	Ant	Cou	Ste/Tri	Car	Sap	Fla	Antho
ECE	+[b]	+[c]	+[d]	+[e]	+[f]	-	-
DI water[a]	-	-	-	-	-	-	-
95% EtOH[a]	-	-	-	-	-	-	-

(+) define as positive result and (-) define as negative result.

Ant, Anthracene/Anthraquinone glycoside; Cou, Coumarin glycoside; Ste/Tri, Sterol glycoside/Triterpene glycoside; Car, Cardiac glycoside; Sap, Saponin glycoside; Fla, Flavonoid glycoside; Antho, Anthocyanine.

[a] Blank controls of glycoside test.

[b] Positive result of red solution.

[c] Positive result of blue fluorescence under UV light.

[d] Positive result of reddish brown ring turning to violet ring.

[e] Positive result of violet solution.

[f] Positive result of froth.

3) Phenolic and tannin

Phenolic and tannin in the ECE of *S. grandiflora* bark was investigated by ferric chloride and gelatin tests. Positive results revealed color changing solution with $FeCl_3$ and precipitating with gelatin. The results were shown in Table 3.5, green solution appeared after adding $FeCl_3$ and precipitation with gelatin in saline. This results interpreted that there were phenolic compounds and catechol, subgroup of tannin in the extract.

Table 3.5 Phenolic and tannin test of ECE from *S. grandiflora* bark and controls by ferric chloride and gelatin tests.

Sample	Tests			
	Ferric chloride	Gelatin	Gelatin in saline	Control
ECE	+ [b]	+ [c]	+ [c]	-
DI water [a]	-	-	-	-
95% EtOH [a]	-	-	-	-

(+) define as positive result and (-) define as negative result.

[a] Blank controls of phenolic and tannin test.

[b] Positive result of green solution.

[c] Positive result of precipitation.

3.3 Fractionated crude extract (FCE)

3.3.1 Preparation of FCE

The FCE of *S. grandiflora* bark which represented the strongest antibacterial activity was extracted by maceration method with a sequence of organic solvents of increasing the polarity. The methanol extract gave the highest yield of 2.40% (w/w)

Table 3.6 Percentage yields and appearances of FCE.

FCE	Yield (% w/w)	Appearance
Hexane extract	0.23	Sticky yellowish-brown extract
Ethyl acetate extract	1.75	Sticky dark-brown extract
Butanol extract	0.75	Sticky dark-brown extract
Methanol extract	2.40	Sticky brown extract

followed by ethyl acetate extract of 1.75% (w/w). The yield values and appearances of each extract were shown in Table 3.6.

3.3.2 Antibacterial activity of FCE

Antibacterial activity of various FCE from *S. grandiflora* bark were examined against ten pathogenic bacteria by agar dilution method. MIC of each FCE against several bacterial strains were shown in Table 3.7.

Table 3.7 MIC of FCE from *S. grandiflora* bark against different microorganisms by agar dilution method.

Microorganisms	MIC of FCE (mg/mL) [a]			
	Hexane extract	Ethyl acetate extract	Butanol extract	Methanol extract
Gram positive bacteria				
B. cereus	> 20.00	2.50	2.50	2.50
E. cloacae	> 20.00	5.00	20.00	> 20.00
E. faecalis	20.00	5.00	5.00	10.00
S. aureus	10.00	2.50	2.50	5.00
S. epidermidis	20.00	2.50	2.50	10.00
Gram negative bacteria				
E. coli	> 20.00	5.00	20.00	> 20.00
P. aeruginosa	> 20.00	10.00	20.00	20.00
S. marcescens	> 20.00	10.00	20.00	> 20.00
S. typhi	> 20.00	5.00	2.50	10.00
S. sonnei	> 20.00	5.00	5.00	20.00

[a] No bacterial growth in the negative control.

The results revealed that the FCE of ethyl acetate extract possessed the strongest antibacterial activity with the lowest MIC values against all tested strains, followed by butanol extract. Both FCE were selected for confirming antibacterial activity by broth dilution method against some bacterial strains which were weak to FCE of the ethyl acetate extract and the butanol extract from agar dilution assay.

The antibacterial activity of ethyl acetate and butanol extracts of *S. grandiflora* bark were determined against pathogenic bacteria by broth dilution method. To confirm the activity of both extracts, some microorganisms were used. Representative Gram positive bacteria were *B. cereus*, *E. faecalis*, *S. aureus*, and *S. epidermidis* whereas Gram negative bacteria were *S. typhi* and *S. sonnei*. The MIC and MBC values by broth dilution method of both extracts were shown in Table 3.8.

Table 3.8 MIC and MBC of ethyl acetate and butanol extracts from *S. grandiflora* bark against some different microorganisms by broth dilution method.

Microorganisms	MIC of FCE (mg/mL) [a]		MBC of FCE (mg/mL) [a]	
	Ethyl acetate extract	Butanol extract	Ethyl acetate extract	Butanol extract
Gram positive bacteria				
B. cereus	1.25	1.25	1.25	1.25
E. faecalis	5.00	5.00	5.00	5.00
S. aureus	2.50	2.50	2.50	2.50
S. epidermidis	2.50	2.50	2.50	2.50
Gram negative bacteria				
S. typhi	1.25	1.25	2.50	2.50
S. sonnei	2.50	2.50	5.00	5.00

[a] No bacterial growth in the negative control.

The MIC values determined by broth dilution method were equal or less than those obtained by the agar dilution method. The results indicated that MBC values of both extracts against Gram positive bacteria were equal to MIC values on corresponding strains, but MBC values of the both against Gram negative bacteria were more than MIC values on corresponding strains. Regarding bacterial cell wall, the wall of Gram positive bacteria contains a thick layer of peptidoglycane as attached with teichoic acids that are usual to the Gram positive cell wall [118]. The wall of Gram negative bacteria are more complex structure and chemically components, it composes of a thin layer of peptidoglycane and covers the surface with outer membrane which comprises lipopolysaccharides [119]. The structure of cell wall displays an important role in protection the cell from osmotic rupture and mechanical damage.

3.3.3 Kinetics of bacteria killing of FCE

The killing kinetic effect of FCE which were high potential antibacterial activity, ethyl acetate extract and butanol extract with a concentration at MBC, were studied against four pathogenic bacteria. The strains of *E. faecalis* and *S. aureus* were used as a model of Gram positive bacteria whereas *S. typhi* and *S. sonnei* were used as a model of Gram negative bacteria. The bactericidal kinetics was investigated by time course experiments, number of surviving bacteria was measured as defining Log CFU/mL.

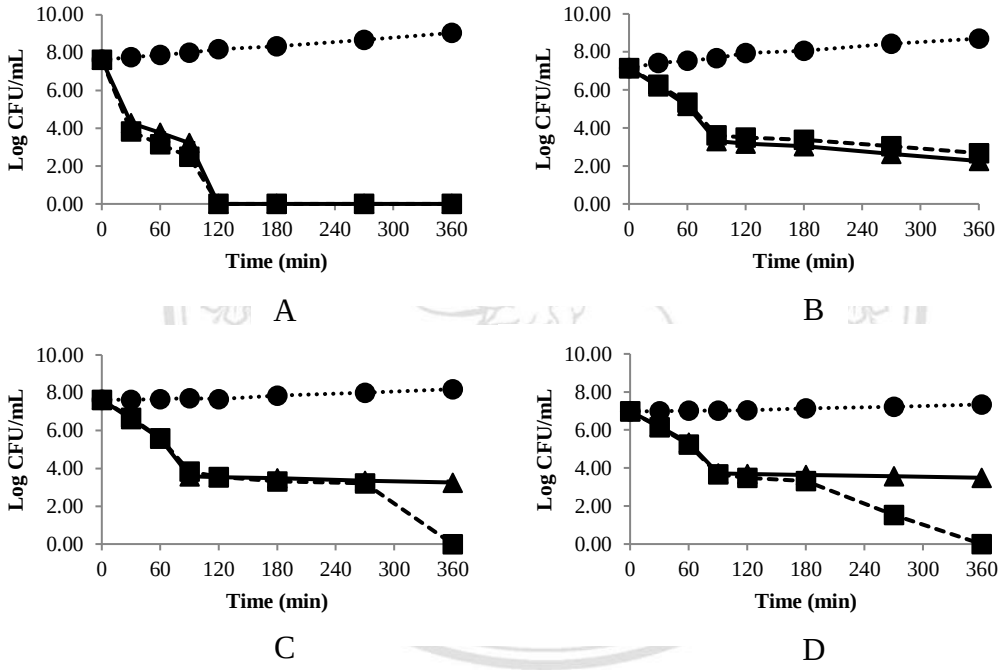


Figure 3.2 Killing kinetic curves of *E. faecalis* (A), *S. aureus* (B), *S. typhi* (C), and *S. sonnei* (D) between control (l) and FCE of ethyl acetate extract (s) and butanol extract (n) from *S. grandiflora* bark.

The results possessed that both extracts showed a similar killing rate against the tested Gram positive bacteria. *E. faecalis* was destroyed more than 50% of cells within 30 min (Figure 3.2A) and *S. aureus* was ruined more than 50% of cells within 90 min (Figure 3.2B). Similarly, *S. typhi* and *S. sonnei* were defeated more than 50% of cells within 90 min. However, butanol extract killed the tested Gram negative bacteria faster than ethyl acetate extract and completed destruction within 6 h (Figure 3.2C and Figure 3.2D). It

could be seen that butanol extract had more declared killing rate on Gram negative than ethyl acetate extract. The killing kinetics of both extracts on Gran positive was not distinguishable.

3.3.4 UV spectrum of FCE

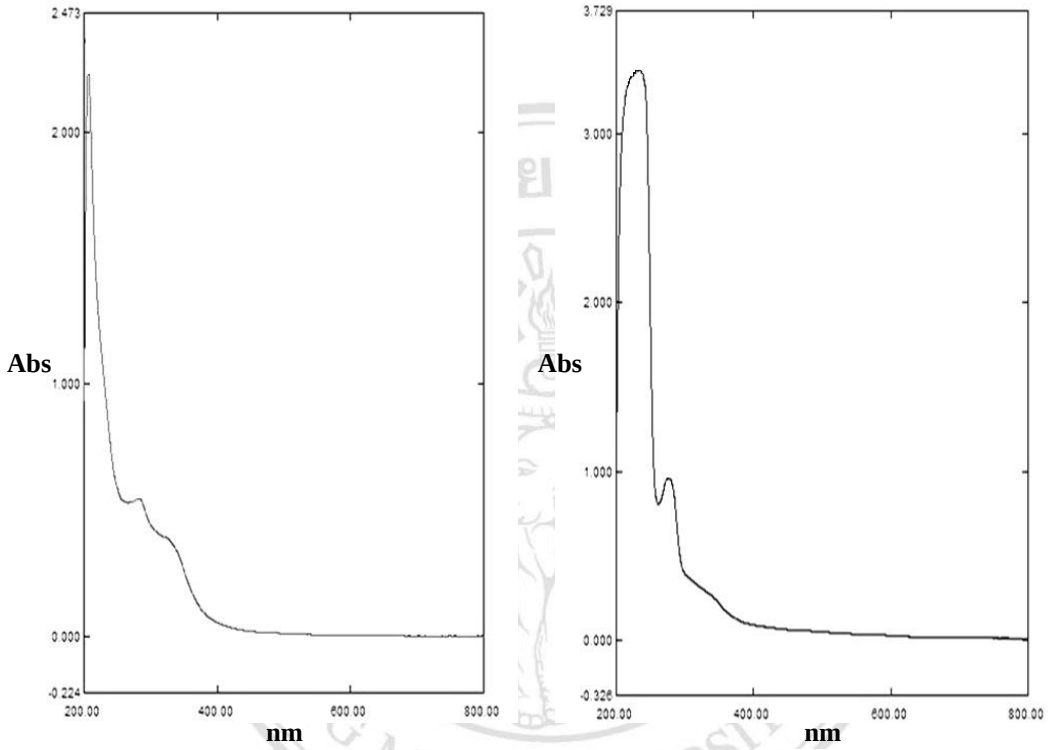


Figure 3.3 The UV spectrum of FCE of the ethyl acetate extract (A) and the butanol extract (B) from *S. grandiflora* bark.

The ethyl acetate extract and butanol extract of *S. grandiflora* bark which presented high potential antibacterial activity were identified as the UV spectrum shown in Figure 3.3. The λ_{max} of FCE of the ethyl acetate extract and the butanol extract were 281.20 and 275.40 nm, respectively.

3.4 Solubility study of *S. grandiflora* extracts

The solubility of ECE and FCE of ethyl acetate extract and butanol extract which were high potential antibacterial activity, were studied in various solvents. The results were shown in Table 3.9. The ECE was sparingly soluble in DMSO and ethanol. The

FCE of ethyl acetate extract was soluble in ethyl acetate; sparingly soluble in acetone; slightly soluble in butanol, DMSO, and ethanol. The FCE of butanol extract was slightly soluble in DMSO, and ethanol; very slightly soluble in butanol and water. All extracts could dissolve in DMSO and ethanol, but they were insoluble in hexane.

Table 3.9 Solubility of *S. grandiflora* extracts in various solvents.

Solvents	Solubility		
	ECE	FCE of ethyl acetate extract	FCE of butanol extract
Acetone	> 10000 [e]	61.60 [b]	> 10000 [e]
Butanol	> 10000 [e]	178.00 [c]	3752.91 [d]
DMSO	60.91 [b]	180.28 [c]	128.36 [c]
Ethanol	64.82 [b]	130.54 [c]	186.47 [c]
Ethyl acetate	> 10000 [e]	23.37 [a]	> 10000 [e]
Hexane	> 10000 [e]	> 10000 [e]	> 10000 [e]
Water	> 10000 [e]	> 10000 [e]	2001.25 [d]

[a] Descriptive term of soluble.

[b] Descriptive term of sparingly soluble.

[c] Descriptive term of slightly soluble.

[d] Descriptive term of very slightly soluble.

[e] Descriptive term of practically insoluble.

3.5 Stability study of *S. grandiflora* extracts

The stability of ECE and FCE of ethyl acetate extract and butanol extract which revealed potential antibacterial activity, were studied in closed container with light protection at various temperatures of 4°C, room temperature, and accelerated temperature (45°C) for 90 days. The physical appearance and *in vitro* antibacterial activity were determined by naked eyes observation and broth dilution assay, respectively. All extracts were dissolved in DMSO and diluted with sterile DI water. The feature of all extracts at different storage temperatures for time periods were reported in Table 3.10. The appearances of all extracts were brown suspension and did

not change upon each storage temperatures and time periods except that the butanol extract at 45°C for 30, 60, and 90 days was changed to dark-brown suspension.

Table 3.10 Physical appearance of *S. grandiflora* extracts at various temperatures for 30, 60, and 90 days.

Extract	Keeping condition	Physical appearance		
		30 days	60 days	90 days
ECE	4°C	Brown suspension	Brown suspension	Brown suspension
	room temp	Brown suspension	Brown suspension	Brown suspension
	45°C	Brown suspension	Brown suspension	Brown suspension
FCE of ethyl acetate extract	4°C	Brown suspension	Brown suspension	Brown suspension
	room temp	Brown suspension	Brown suspension	Brown suspension
	45°C	Brown suspension	Brown suspension	Brown suspension
FCE of butanol extract	4°C	Brown suspension	Brown suspension	Brown suspension
	room temp	Brown suspension	Brown suspension	Brown suspension
	45°C	Dark-brown suspension	Dark-brown suspension	Dark-brown suspension

Regarding the antibacterial activity, MIC and MBC values of each extract at various temperatures for different time periods were determined as presented in Figure 3.4. They were increased according to the length of storage time. The results exposed that the extracts was less effective upon standing for few months with temperature independent in this study. However, if concentrations of the extract were performed more frequently, the effective for antibacterial activity of the extract as storage at

different temperatures might be differenced. It might be mentioned that effective of the extract was slightly depend on temperature dependent.

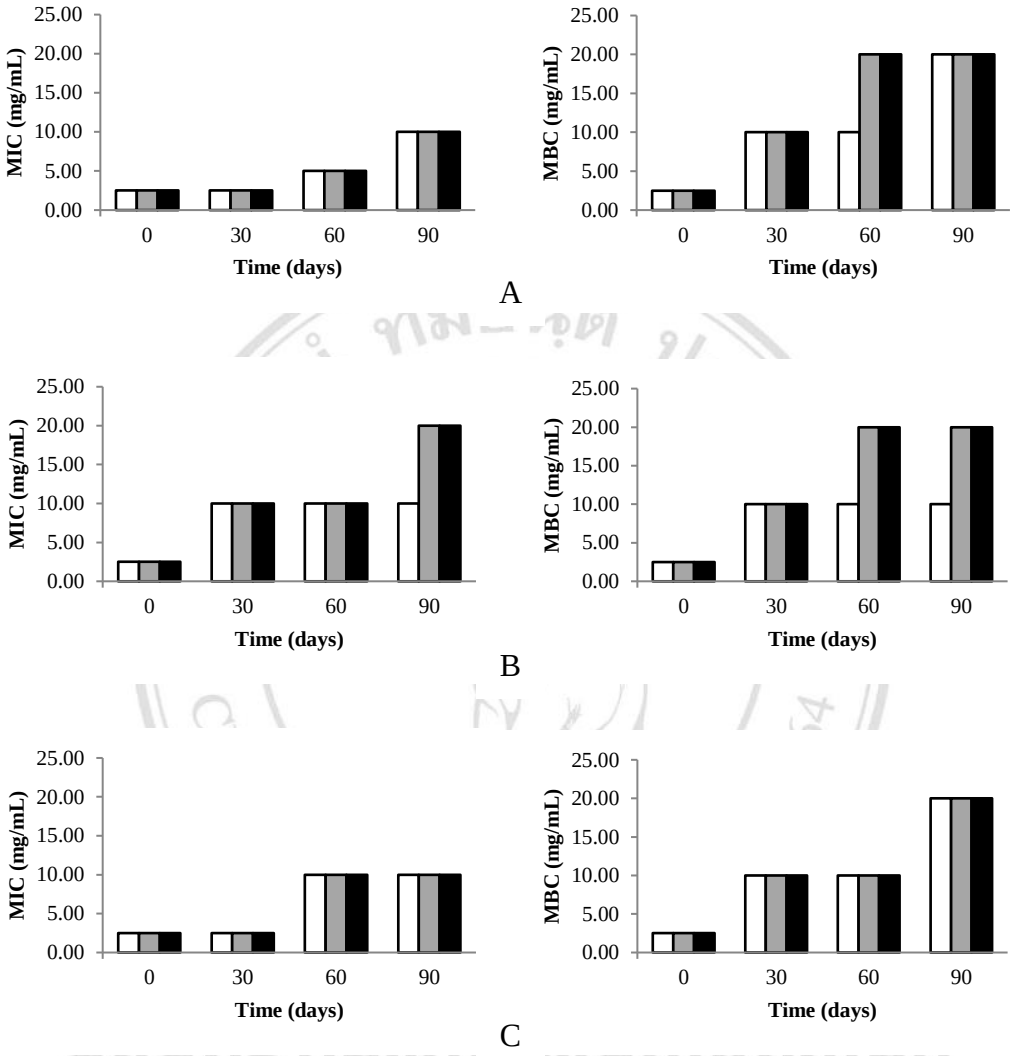


Figure 3.4 MIC (left) and MBC (right) values of ECE (A) and FCE of the ethyl acetate extract (B) and the butanol extract (C) from *S. grandiflora* bark which were kept at various temperatures of 4°C (□), room temperature (▒), and 45°C (■) for 30, 60, and 90 days against *S. aureus*.

3.6 Toxicity and therapeutic effect of *S. grandiflora* extracts

3.6.1 Effect of diets on silkworm growth

The results of two different diets; mulberry leaves and artificial diet, which fed to silkworm demonstrated slightly difference in growth as shown in Figure 3.5. It was

noted that during the first 6 days as the fourth instar period, the morphology and body weight of silkworms in both groups was insignificantly different. Regarding to in the fifth instar period which started from day 7, the silkworms as fed with mulberry leaves revealed increasing body weight more rapidly than the silkworms as fed with artificial diet. It could be explained that the development of most insects is directly or indirectly controlled by hormones [120], it can be partly regulated by exogenous factors such as food intake [121]. However, the silkworms for using as an animal model of toxicity and therapeutic effect evaluation were used the stage of the fifth instar larvae. The fifth instar silkworm were fed on the 1st day only and reared overnight in order to prepare for *in vivo* evaluation. The silkworms were injected with test solution, after that the silkworms were observed visually survival without feeding diet.

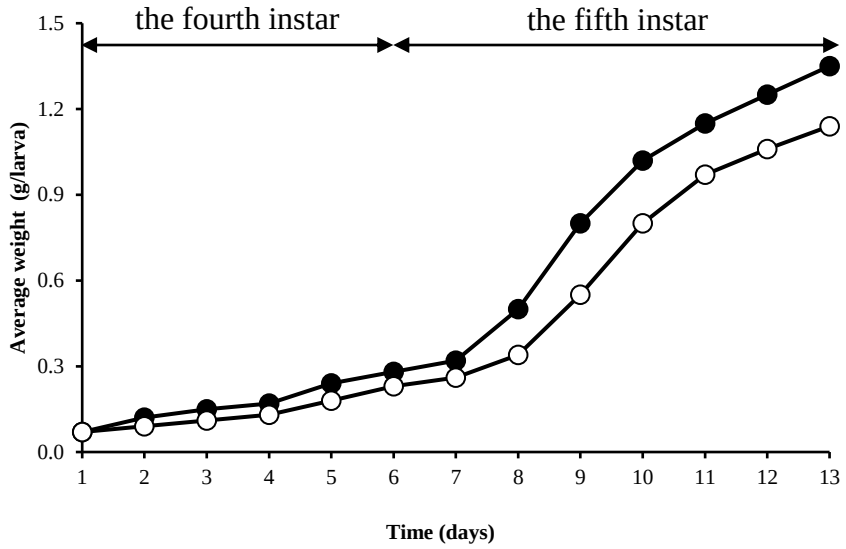


Figure 3.5 Average body weight of the silkworms as fed with mulberry leaves (●) and artificial diet (○) as gained everyday for 13 days since the first day of the fourth instar larvae.

3.6.2 Toxicity study

Use of silkworms as animal model to evaluate the toxicity of *S. grandiflora* extracts was performed in this study. The extracts were dissolved in DMSO and diluted with normal saline. Figure 3.6 represented the injected silkworm died due to the toxicity of test sample. The survival rates of silkworm after injected with test samples were

Figure 3.6 Death of silkworms due to toxicity of poisonous sample.

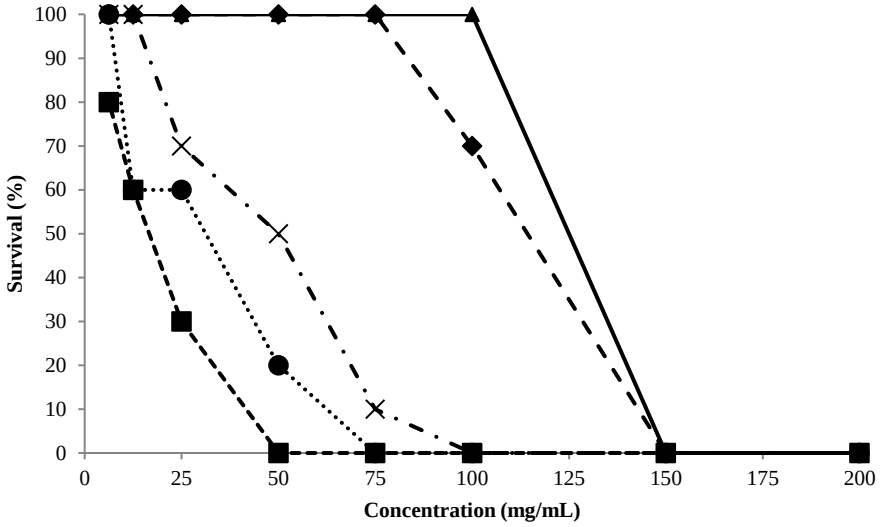


Figure 3.7 Survival rates of silkworms after injected with ECE (l) and FCE of hexane extract (u), ethyl acetate extract (s), butanol extract (n), and methanol extract (5) from *S. grandiflora* bark.

Table 3.11 LC_{50} and LD_{50} values of *S. grandiflora* extracts in silkworm.

Extract	LC_{50} (mg/mL)	LD_{50} (mg/g larva)
ECE	31.25	1.56
FCE of hexane extract	116.67	5.83
FCE of ethyl acetate extract	125.00	6.25
FCE of butanol extract	16.67	0.83
FCE of methanol extract	44.44	2.22

in Figure 3.7. The toxicity of the test samples were expressed as LC_{50} and LD_{50} values as shown in Table 3.11. It was revealed that FCE of hexane extract and ethyl acetate extract were low toxicity to silkworm. The LC_{50} and LD_{50} values of these extracts were higher than 100 mg/mL and 5 mg/g larva, respectively.

3.6.3 Killing effect of bacteria evaluation

The standard strain of *S. aureus* ATCC 25923 was used as pathogenic bacteria for killing the silkworm. Survival rate of silkworm decreased with an increase in bacterial concentration and time after injection as shown in Figure 3.8. It was found that bacterial concentration as 10^6 cells/mL could not kill silkworms, all silkworm in this group could survive as if a positive control groups which injected with normal saline without bacteria and a negative control groups which nothing injected. While bacterial concentration of 10^7 cells/mL could kill 30% of infected silkworms after 2 days injection. More than half of silkworm which infected with 10^8 cells/mL bacterial concentration were killed on day 2 at time of 36 h after injection, all silkworm were killed within 48 h later. Whereas, 10^9 cells/mL bacterial concentration killed all of infected silkworm on day 1 after injection. Therefore, bacterial suspension at concentration of 10^8 cells/mL was used as pathogenic suspension in the evaluation of therapeutic effect in silkworn infected model. Figure 3.9 showed the silkworm died due to *S. aureus* infection.

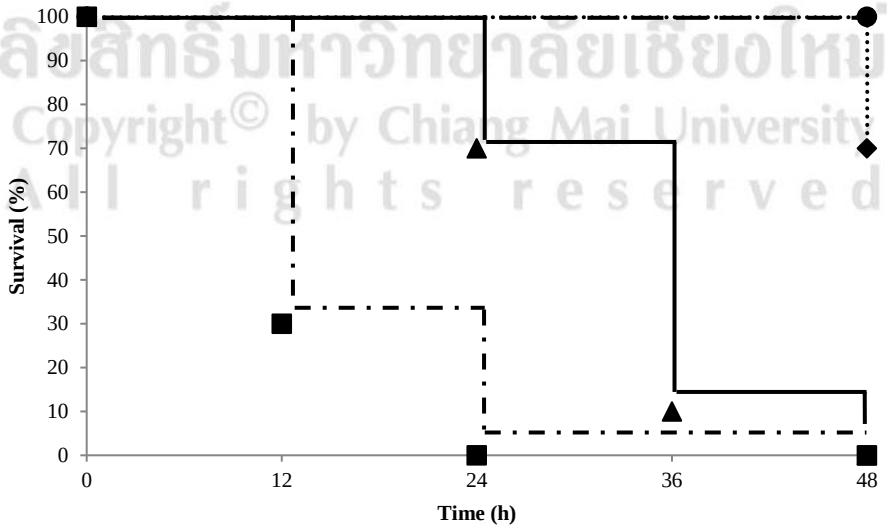


Figure 3.8 Survival rates of silkworms after injected with bacterial suspension as a concentration of 10^6 (l), 10^7 (u), 10^8 (s), and 10^9 (n) cells/mL.

Figure 3.9 Death of silkworms due to bacterial infection.

3.6.4 Therapeutic effect evaluation

Therapeutic effect of *S. grandiflora* extracts to *S. aureus* infected silkworms were executed in this study. The survival of silkworms was observed 2 days after injection of bacteria suspension and the test sample. Figure 3.10A and Figure 3.10B represented non-therapeutic effect and therapeutic effect of test samples on infected silkworm, respectively.

A B

Figure 3.10 Infection and therapy using silkworm model: non-therapeutic effect (A) and therapeutic effect (B).

The injected silkworms with bacteria suspension without the test sample administration which killed more than 90% were used as the control of this experiment. The therapeutic effect of test solution after injection of bacterial suspension and the test

samples to silkworms were exhibited in Figure 3.11. FCE of ethyl acetate extract increased surviving number of silkworm depending on the amount of sample whereas other test samples did not accord with concentration or dose dependent. These results revealed that FCE of ethyl acetate extract and methanol extract prolonged survivability of infected silkworms with IC_{50} and ED_{50} values of 2.08 mg/mL and 0.10 mg/g larva, respectively for ethyl acetate extract, also 10.00 mg/mL and 0.50 mg/g larva, respectively for methanol extract.

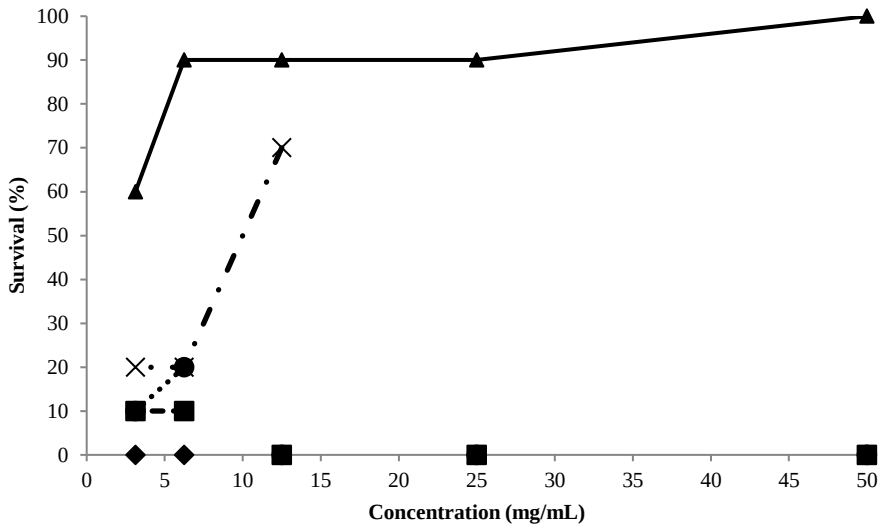


Figure 3.11 Therapeutic effect of silkworms after injected with bacterial suspension and test sample; ECE (l) and FCE of hexane extract (u), ethyl acetate extract (s), butanol extract (n), and methanol extract (5) from *S. grandiflora* bark.

3.7 Marker compound determination

3.7.1 Quick column chromatography

The FCE of ethyl acetate extract which exposed the most potential antibacterial activity and therapeutic effect without toxicity, was isolated by column chromatography with silica gel 60 as adsorbent and mixture of hexane:ethyl acetate and ethyl acetate:methanol with increasing gradient polarity as eluent. TLC pattern of the different eluates were checked under visible light and UV light at 254 and 365 nm, those with similar patterns were combined and concentrated using rotary evaporator. There were different 20 patterns from total 44 eluates, thus the obtained fractions had 20

fractions; fraction 1 (F1) to fraction 20 (F20). All fractions were determined for antibacterial activity against *S. aureus* by broth dilution assay. Table 3.12 presented yield, MIC and MBC values of each fraction. The results indicated that although F13 gave yield of 3.79% (w/w) which was lower than F4, F10, F18, and F20, it revealed the most potential antibacterial activity because of the lowest MIC and MBC values.

Table 3.12 Percentage yields, MIC, and MBC values of different fractions of FCE of ethyl acetate extract against *S. aureus* by broth dilution method.

Fraction	Yield (% w/w)	MIC (mg/mL) [a]	MBC (mg/mL) [a]
F1	0.82	2.50	> 5.00
F2	0.34	> 5.00	> 5.00
F3	0.43	> 5.00	> 5.00
F4	7.05	1.25	> 5.00
F5	1.66	0.63	2.50
F6	1.46	0.63	> 5.00
F7	1.17	2.50	> 5.00
F8	2.31	1.25	2.50
F9	1.51	1.25	> 5.00
F10	4.39	1.25	2.50
F11	1.80	1.25	> 5.00
F12	1.85	0.63	2.50
F13	3.79	0.63	1.25
F14	1.93	1.25	2.50
F15	2.65	2.50	> 5.00
F16	0.41	2.50	> 5.00
F17	2.14	2.50	> 5.00
F18	5.05	2.50	> 5.00
F19	0.94	2.50	> 5.00
F20	7.96	2.50	> 5.00

[a] No bacterial growth in the negative control.

F13 showed the highest antibacterial activity with MIC and MBC values of 0.63 and 1.25 mg/mL, respectively. Therefore, F13 was selected to examine phytochemical

compounds. This fraction exposed positive results of coumarin glycoside and phenolic compounds as shown in Table 3.13 and Table 3.14, respectively. It could be assumed that coumarin and phenolic were bioactive compounds in FCE of ethyl acetate extract from *S. grandiflora* bark. Mostly, the natural compounds which possess broad bioactivities were phenolic compounds [122, 123].

Table 3.13 Glycosides test of F13 from ethyl acetate extract of *S. grandiflora* bark and controls.

Sample	Tests						
	Ant	Cou	Ste/Tri	Car	Sap	Fla	Antho
F13	-	+ [b]	-	-	-	-	-
DI water [a]	-	-	-	-	-	-	-
95% EtOH [a]	-	-	-	-	-	-	-

(+) define as positive result and (-) define as negative result.

Ant, Anthracene/Anthraquinone glycoside; Cou, Coumarin glycoside; Ste/Tri, Sterol glycoside/Triterpene glycoside; Car, Cardiac glycoside; Sap, Saponin glycoside; Fla, Flavonoid glycoside; Antho, Anthocyanine.

[a] Blank controls of glycoside test.

[b] Positive result of blue fluorescence under UV light.

Table 3.14 Phenolic and tannin test of F13 from ethyl acetate extract of *S. grandiflora* bark and controls by ferric chloride and gelatin tests.

Sample	Tests			
	Ferric chloride	Gelatin	Gelatin in saline	Control
F13	+ [b]	-	-	-
DI water [a]	-	-	-	-
95% EtOH [a]	-	-	-	-

(+) define as positive result and (-) define as negative result.

[a] Blank controls of phenolic and tannin test.

[b] Positive result of green solution.

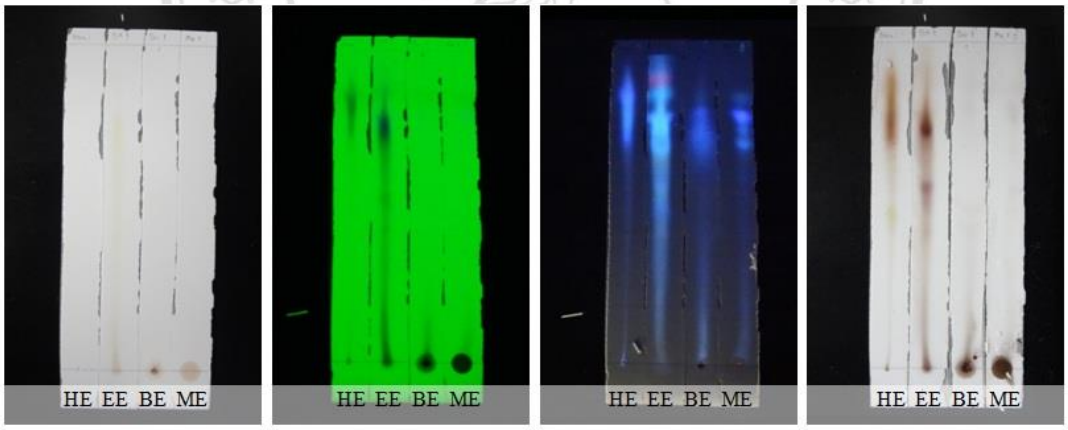
3.7.2 Partial purified extract (PPE)

The PPE of *S. grandiflora* bark was extracted to eliminate impurities as non-polar part by maceration method with gradient of organic solvents by increasing the polarity. The yield values and appearances of each PPE were shown in Table 3.15. Methanol extract gave the highest yield of 2.78% (w/w) followed by chloroform extract of 1.45% (w/w). Other extracts gave less that 1.00% (w/w) yield.

Table 3.15 Percentage yields and appearances of PPE.

PPE	Yield (% w/w)	Appearance
Hexane extract	0.23	Sticky yellowish-brown extract
Chloroform extract	1.45	Sticky dark-brown extract
Ethyl acetate extract	0.27	Sticky dark-brown extract
Butanol extract	0.72	Sticky dark-brown extract
Methanol extract	2.78	Sticky brown extract

A



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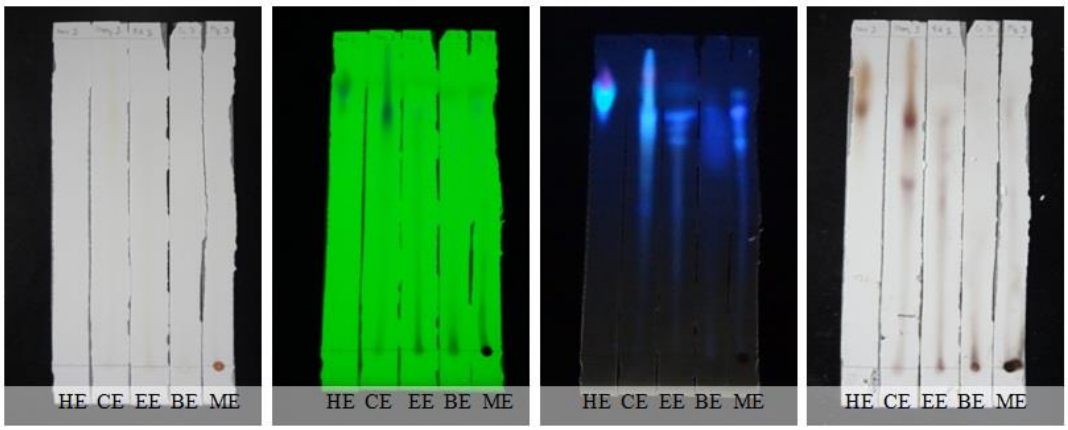


Visible light UV 254 nm UV 365 nm 10% H_2SO_4

Figure 3.12 TLC patterns of different FCE (A) and different PPE (B) in condition of $CHCl_3$:MeOH as a ratio of 3:1 under visible light and UV light at 254 and 365 nm, also 10% H_2SO_4 spraying with heating.

TLC pattern of various FCE in prior extraction and the various obtained PPE were compared at the same experiment condition i.e. 3:1 ratio of $CHCl_3$:MeOH. The TLC patterns were observed under visible light and UV light at 254 and 365 nm, also spraying with 10% H_2SO_4 and heating on hot plate as shown in Figure 3.12. TLC patterns obtained from hexane extract (HE), butanol extract (BE), and methanol extract (ME) of both FCE and PPE were similar. Comparison between TLC pattern of FCE of ethyl acetate (EE) and TLC patterns of PPE of chloroform extract (CE) and ethyl acetate (EE), the pattern of EE of FCE was alike pattern of combination of CE with EE of PPE. It revealed that non-polar compounds in EE of FCE could be isolated by chloroform due to several compounds as presented bands in TLC pattern of the EE were identical bands in TLC pattern of the CE and EE of PPE. According to antibacterial activity evaluation, the different extracts of FCE and PPE inhibited the growth of *S. aureus* as shown in Table 3.16. It was interesting that the EE of PPE presented higher antibacterial action than the EE of FCE with low MIC and MBC values. This could be because impurities in the extract were eliminated, the activity of purer extract would be better. It is concluded that the impurity in EE of FCE could be extracted by chloroform. Thus the EE of PPE exhibited higher activity.

Table 3.16 MIC and MBC of different FCE and different PPE from *S. grandiflora* bark against *S. aureus* by broth dilution method.

Extract	FCE		PPE	
	MIC (mg/mL) [a]	MBC (mg/mL) [a]	MIC (mg/mL) [a]	MBC (mg/mL) [a]
Hexane extract (HE)	10.00	> 10.00	10.00	> 10.00
Chloroform extract (CE)	No	No	2.50	2.50
Ethyl acetate extract (EE)	2.50	2.50	0.63	0.63
Butanol extract (BE)	2.50	2.50	5.00	5.00
Methanol extract (ME)	5.00	5.00	1.25	1.25

no define as absence of the extract.

[a] No bacterial growth in the negative control.

Table 3.17 MIC of different PPE from *S. grandiflora* bark against several pathogenic bacteria by broth dilution method.

Microorganisms	MIC (mg/mL) [a]		
	Hexane extract	Chloroform extract	Ethyl acetate extract
Gram positive bacteria			
MSSA1 [b]	50.00	100.00	1.56
MRSA3 [c]	25.00	25.00	1.56
MRSA4 [c]	50.00	50.00	1.56
MRSA6 [c]	25.00	25.00	1.56
MRSA8 [c]	50.00	50.00	1.56
MRSA9 [c]	100.00	25.00	1.56
MRSA11 [c]	50.00	25.00	1.56
MRSA12 [c]	50.00	50.00	1.56
B. cereus JCM 20037	25.00	25.00	0.78
B. subtilis JCM2499	50.00	50.00	0.78
E. faecalis EF1	25.00	100.00	1.56
E. faecalis EF5 (VRE) [d]	25.00	50.00	0.39
L. monocytogenes	100.00	50.00	3.12
S. agalactiae	100.00	50.00	1.56
S. pneumoniae	100.00	100.00	1.56
S. pyogenes	1.56	6.25	1.56
S. sanguinis	3.12	1.56	1.56
Gram negative bacteria			
E. coli w3110	100.00	200.00	6.25
P. aeruginosa PA01	100.00	200.00	3.12

[a] No bacterial growth in the negative control.

[b] MSSA define as methicilin sensitive *Staphylococcus aureus.*

[c] MRSA define as methicilin resistant *Staphylococcus aureus.*

[d] VRE define as vancomycin resistant *Enterococcus faecalis*.

It revealed that the extracts of BE and ME were toxic to silkworms in the prior studies. Therefore, the extracts of HE, CE, and EE were chosen for investigation antibacterial activity against different pathogenic strains including sensitive and resistant strains by broth dilution assay in further study. The different PPE exposed antibacterial activity against a broad spectrum of pathogenic bacteria as shown in Table 3.17. The HE and CE exhibited low antibacterial activity with the MIC values of 1.56-100.00 mg/mL and 1.56-200.00 mg/mL, respectively. While EE possessed the strongest antibacterial activity against several strains with low MIC values of 0.39-6.25 mg/mL. Interestingly, inhibitory capacity of EE to drug-resistant bacteria such as MRSA and VRE was approximately equal to some antibiotic e.g. gentamicin and vancomycin.

3.7.3 Preparative column chromatography

The PPE of ethyl acetate extract which presented the highest antibacterial activity, was fractionated by column chromatography with silica gel 60 as adsorbent and mixture of chloroform:methanol with increasing gradient polarity as eluent. Antibacterial activity against *S. aureus* of different eluates of 6 fractions were examined by broth dilution assay. The results were compared to crude extract. Table 3.18 presented yield, MIC and MBC values of crude extract and each of fraction.

Table 3.18 Percentage yields, MIC and MBC values of PPE of ethyl acetate extract (EE) and different fractions against *S. aureus* by broth dilution method.

Sample	Yield (% w/w)	MIC (mg/mL) [a]	MBC (mg/mL) [a]
Crude extract			
EE	100	0.50	0.50
Isolated fraction			
F1	0.05	> 0.02	> 0.02
F2	14.55	8.00	16.00
F3	2.30	1.00	2.00
F4	1.65	2.00	2.00
F5	0.10	> 0.04	> 0.04
F6	0.05	> 0.02	> 0.02

[a] No bacterial growth in the negative control.

This results indicated that fraction 2 (F2) which eluted with chloroform:methanol at a ratio of 4:1 gave the highest yield of 14.55% (w/w) whereas fraction 3 (F3) which eluted with chloroform:methanol at a ratio of 3:2 gave strong antibacterial activity with MIC and MBC values of 1.00 and 2.00 mg/mL, respectively. The antibacterial activity of test samples was expressed in term of recovery activity as shown in Figure 3.13. However the recovery activity of sum total different fractions was less than activity of the PPE of ethyl acetate extract (EE) as crude extract.

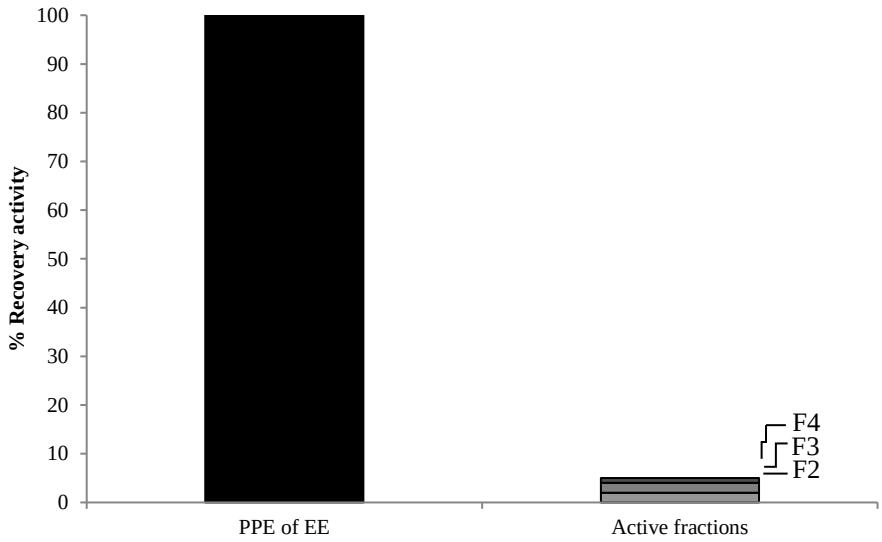


Figure 3.13 Recovery antibacterial activity of PPE of the ethyl acetate extract (EE) (left) and different active fractions from the preparative column chromatography (right); fraction 2, fraction 3, and fraction 4 against *S. aureus*.

3.7.4 Preparative HPLC

The PPE of ethyl acetate extract, which revealed the strongest antibacterial activity, was separated by preparative HPLC using a Senshu Pak Pegasil ODS SP100 column as stationary phase and a linear gradient from 10% to 100% of methanol as mobile phase. Various fractions were collected every 2 min for antibacterial activity determination. The results of preparative HPLC demonstrated that the PPE of ethyl acetate extract consisted of many weak antibacterial compounds as shown in Figure 3.14. There were four active fractions such as fraction 9, fraction 16, and fraction 17

which contributed 5% recovery activity to each fraction, also fraction 18 which attributed 20% recovery activity for antibacterial activity against *S. aureus*.

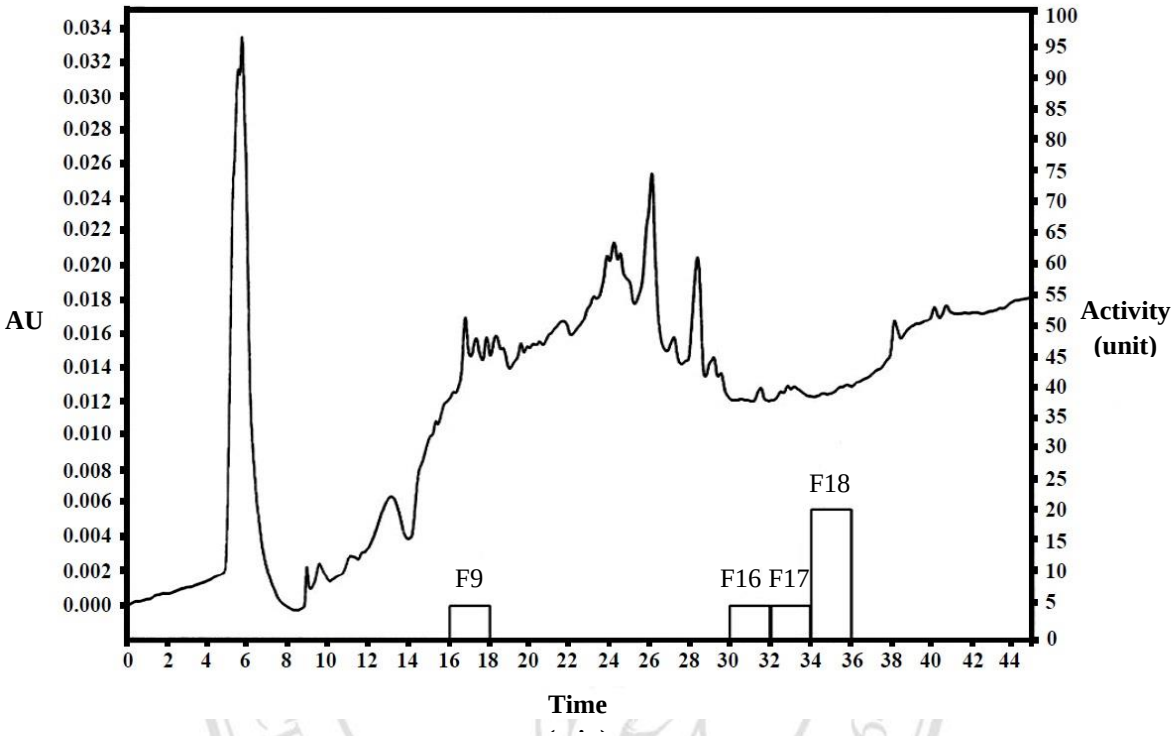


Figure 3.14 Preparative HPLC consequences of PPE of the ethyl acetate extract detected at 210 nm (left axis) and antibacterial activity against *S. aureus* (right axis) of each 2 min eluent fractions.

Comparison of recovery activity of the PPE of ethyl acetate extract as crude extract, entirely collected eluent of PPE of ethyl acetate extract as whole fraction, and all fractions combining of PPE of ethyl acetate extract as sum total different fractions on antibacterial activity against *S. aureus* were presented in Figure 3.15. This results indicated that synergistic effect of different phytochemical compounds as existed in plant extract exhibited the strong antibacterial activity [21, 124]. This effect suggested that combining several active agents gives stronger activity than the individual active compound [26].

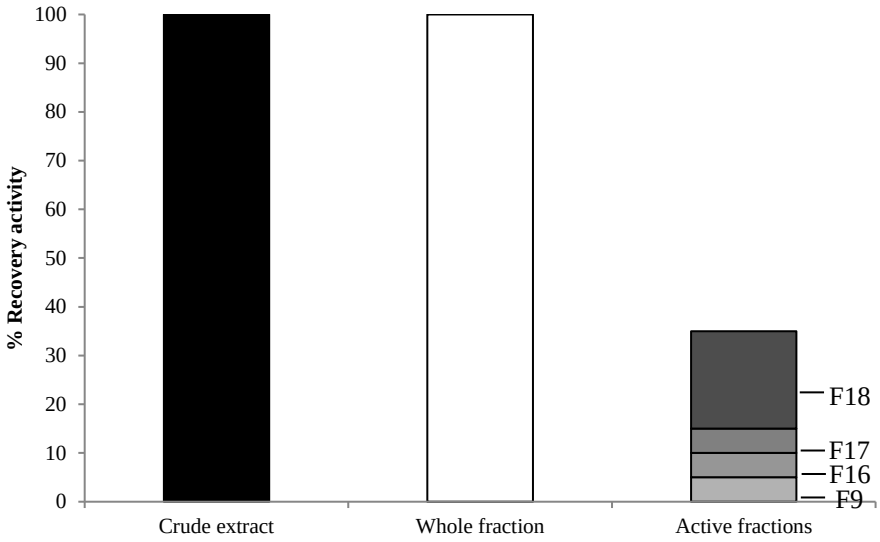


Figure 3.15 Recovery antibacterial activity of PPE of the ethyl acetate extract (EE) as crude extract (left), entirely collected eluent fractions which separated from PPE of EE as whole fraction (middle), and all combining fractions which got individually each 2 min eluent fractions that separated from PPE of EE as different active fractions (right); fraction 9 (F9, 16-18 min), fraction 16 (F16, 30-32 min), fraction 17 (F17, 32-34 min), and fraction 18 (F18, 34-36 min) against *S. aureus*.

3.7.5 HPLC analysis

Table 3.19 HPLC data of standard phenolic compounds.

Peak no	Phenolic compound	Retention time (min)	Area (mAU*s)	Hight (mAU)
1	Gallic acid	6.166	2697.62	205.86
2	Catechin	11.379	599.88	68.56
3	Vanilic acid	12.897	1404.21	163.03
4	Caffeic acid	13.294	2585.30	294.89
5	Syringin	13.629	2879.51	314.61
6	Coumaric acid	15.720	4525.72	458.61
7	Naringic acid	17.921	2735.18	226.32
8	Ellagic acid	18.964	842.26	52.84
9	Trans-cinnamic acid	21.231	8490.81	838.03
10	Quercetin	21.933	898.44	57.54

The marker compound in PPE of the ethyl acetate extract was identified by comparing HPLC chromatograms with standard phenolic compounds. Several standard phenolic compounds at each concentration of 100 µg/mL were eluted by gradient HPLC at different retention times as reported in Table 3.19 and Figure 3.16A.

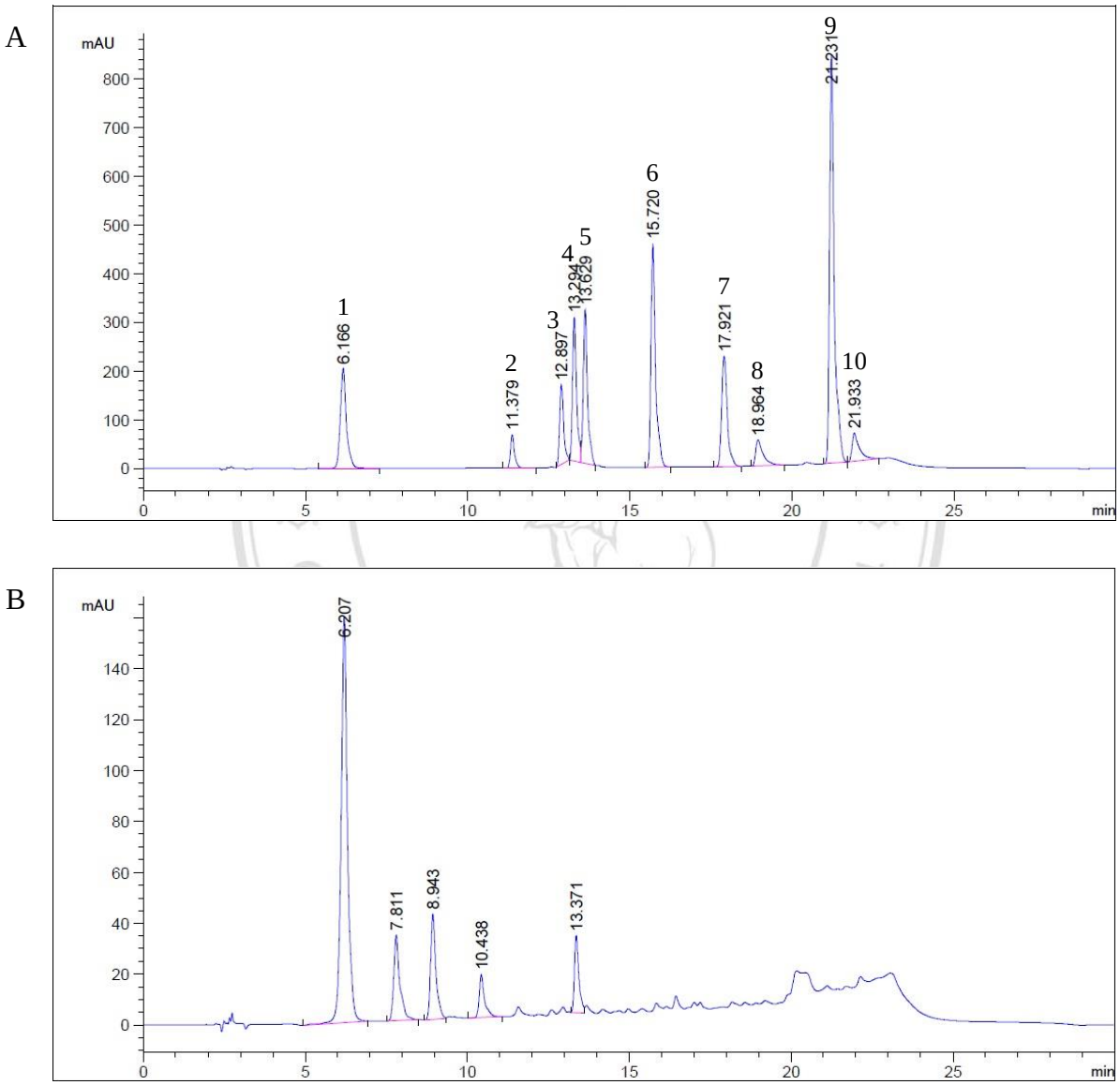


Figure 3.16 HPLC chromatograms of standard phenolic compounds (Peaks: 1. Gallic acid; 2. Catechin; 3. Vanilic acid; 4. Caffeic acid; 5. Syringin; 6. Coumaric acid; 7. Naringic acid; 8. Ellagic acid; 9. Trans-cinnamic acid; 10. Quercetin) (A) and PPE of the ethyl acetate extract (B) at 280 nm.

The HPLC fingerprints of ten standard phenolic compounds could be obtained using gradient eluents of 1% acetic acid in water and methanol for 30 min run time as

detected at 280 nm when each of these compounds were analyzed individually. The chromatograms of all these standard compounds as mixed together presented the same sequence of elution and retention time with the combined chromatogram of individual compound when used the same eluting condition. HPLC chromatogram of PPE of the ethyl acetate extract which eluted with the gradient condition gave a major peak at retention time of 6.207 min as shown in Figure 3.16B. This peak corresponded with the peak of standard gallic acid which eluted at retention time of 6.166 min as proved in same eluting detection. This evidence agreed with the previous studies which reported that gallic acid had been shown to possess inhibitory activity against human pathogenic bacteria such as *Staphylococcus aureus* and *Corynobacterium accolans* also human pathogenic yeast such as *Candida albicans* [125].

The confirming determination of marker compound in PPE of the ethyl acetate extract was evidenced by comparing HPLC chromatograms with standard gallic acid as various isocratic eluting conditions. The variation of eluting ratios between 1% acetic acid in water and methanol was detected at 280 nm. The different chromatograms of PPE of the ethyl acetate extract provided a major peak as accordant peak of gallic acid in the same run condition as shown in Figure 3.17. The eluted peaks of gallic acid at isocratic condition of 1% acetic acid in water and methanol at several ratios of 90:10, 87.5:12.5, 85:15, 82.5:17.5, and 80:20 were shown at 4.022, 3.674, 3.487, 3.076, and 2.883 min, respectively. While, the major eluted peaks of the extract when eluting with 1% acetic acid in water and methanol at the ratio of 90:10, 87.5:12.5, 85:15, 82.5:17.5, and 80:20 were shown at 4.053, 3.623, 3.448, 3.104, and 2.885 min, respectively. In addition, variation of adsorption wavelength as eluted with 1% acetic acid in water to methanol at a ratio of 80:20 was confirmed that the main peak in PPE of the ethyl acetate extract was similar to the peak of gallic acid as shown in Figure 3.18. The eluted peak of gallic acid as eluted with this condition which detected at 210, 254, 280 nm was shown at 2.997, 2.986, and 2.980 min, respectively. While, the eluted major peak of the extract as the same run which detected at 210, 254, 280 nm was 3.011, 3.002, and 2.976 min, respectively. However, the eluted peaks of both gallic acid and the extract could not be detected at 360 nm. The results suggested that the major compound in PPE of the ethyl acetate extract was gallic acid, a common phenolic compound which possesses inhibitory action against pathogenic microorganisms [126, 127].

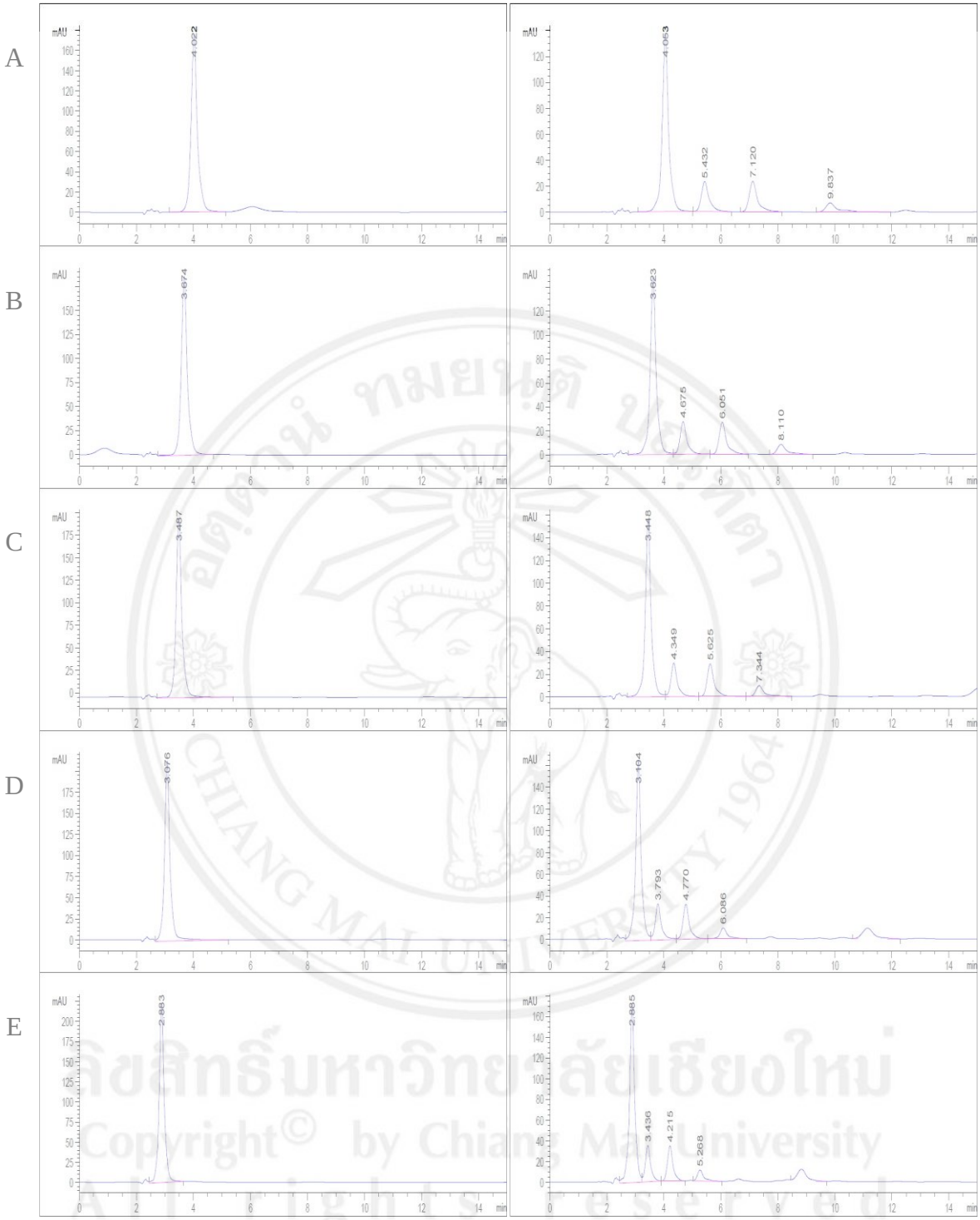


Figure 3.17 HPLC chromatograms of standard gallic acid (left) and PPE of the ethyl acetate extract (right) as eluted with 1% acetic acid in water to methanol at different ratios of 90:10 (A), 87.5:12.5 (B), 85:15 (C), 82.5:17.5 (D), and 80:20 (E) at 280 nm.

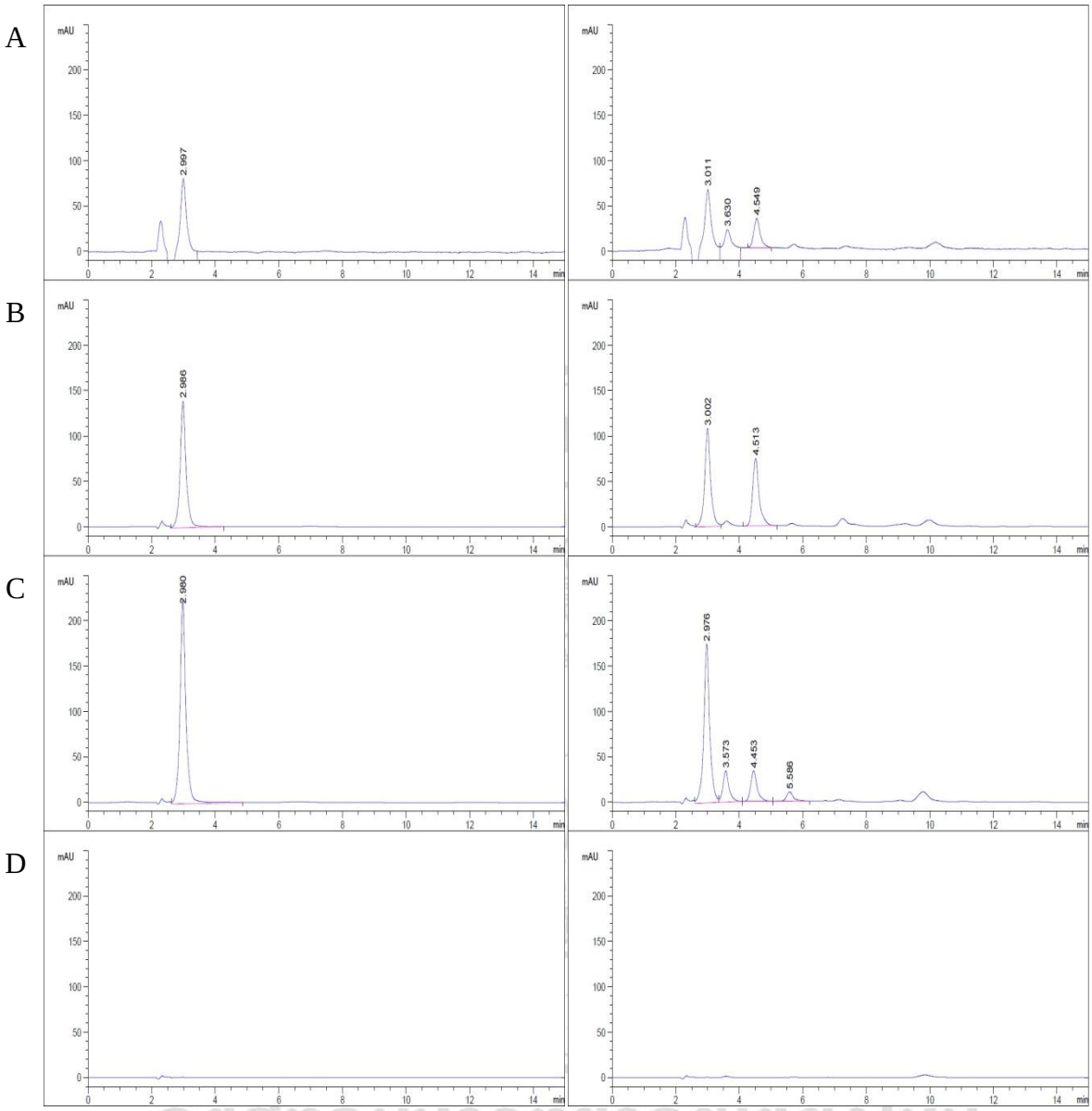


Figure 3.18 HPLC chromatograms of standard gallic acid (left) and PPE of the ethyl acetate extract (right) as eluted with 1% acetic acid in water to methanol at a ratio of 80:20 at different adsorption wavelengths of 210 nm (A), 254 nm (B), 280 nm (C), and 360 nm (D).

The HPLC consequences were reconcile the primary phytochemical test and antibacterial evaluation as prior studies. As phenolic compound was one of bioactive compounds in *S. grandiflora* bark extract, therefore, gallic acid was used as a marker compound of PPE of the ethyl acetate extract in the next studies.

The content of gallic acid in PPE of the ethyl acetate extract was determined by comparison with calibration curve of standard gallic acid as a marker compound. Isocratic eluting of 1% acetic acid in water to methanol at a ratio of 80:20 was HPLC condition and detecting wavelength at 280 nm. The concentration range of calibration curve was performed from 10 to 1000 μg/mL as shown in Figure 3.19. The determination of gallic acid content in PPE of the ethyl acetate extract indicated that the gallic acid content in extract was approximately 83.15 ± 2.12 mg per g extract.

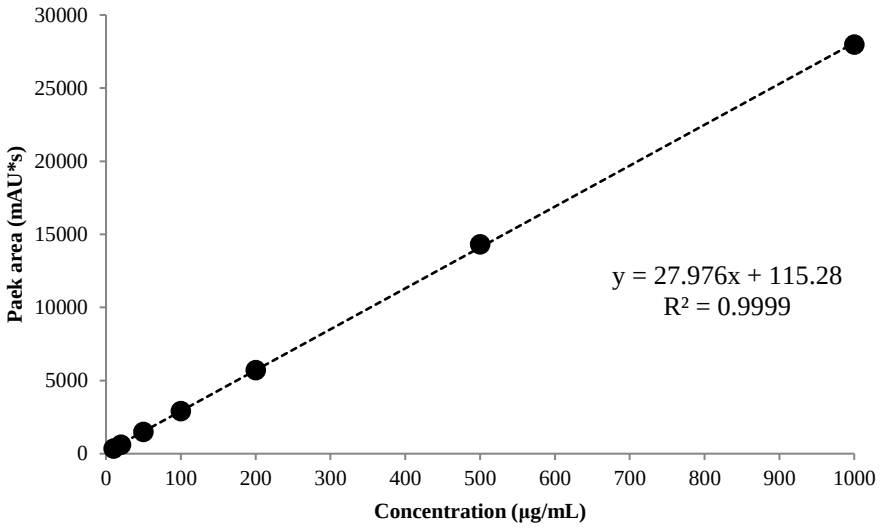


Figure 3.19 Calibration curve of standard gallic acid as a marker compound of PPE of the ethyl acetate extract.

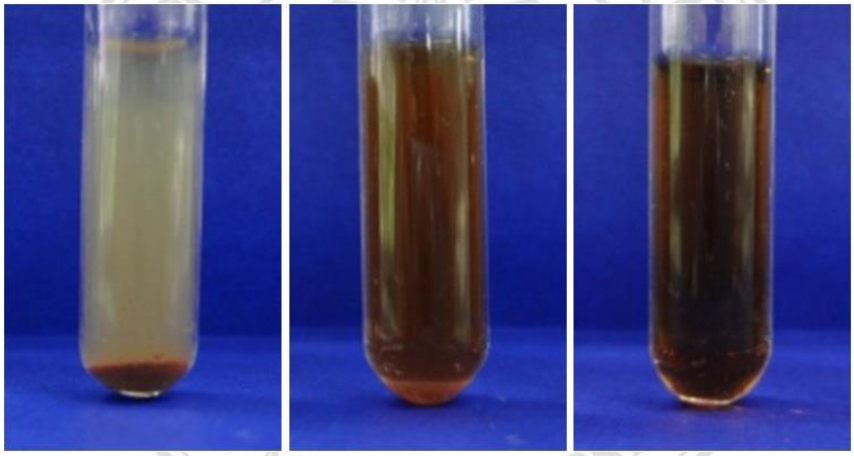
3.8 Development of polymeric micelles loaded with *S. grandiflora* extracts

The PPE of the ethyl acetate extract from *S. grandiflora* bark which demonstrated the highest broad spectrum antibacterial activity against pathogenic strains and resistant strains, also had therapeutic effect of infected silkworm without toxicity was selected to develop further for polymeric micelles loaded *S. grandiflora* extract (SGE).

3.8.1 Preparation of SGE loaded polymeric micelles

The SGE loaded polymeric micelles prepared from Pluronic F68 (SGE-PF68) and Pluronic F127 (SGE-PF127) were formulated by thin-film hydration method. Appearance of the SGE loaded polymeric micelles compared to non-incorporated micelles in aqueous solution. The SGE as non-incorporated micelles was observed by

adding 1 mL water into 10 mg of SGE as shown in Figure 3.20A. It indicated that solubility of SGE was a practically insoluble property in water. However, the appearance of SGE loaded polymeric micelles of both PF68 and PF127 at the same concentration of SGE were transparent liquid solution without any precipitate as shown in Figure 3.20B and Figure 3.20C, respectively. The color of these SGE loaded polymeric micelle systems for all ratios of SGE to polymer was brown due to the color of SGE whereas that of the blank micelles which contained only Pluronic micelles of the both polymers without the entrapped SGE was colorless. These consequences demonstrated that the polymeric micelles of both PF68 and PF127 could extremely enhance aqueous solubility of SGE.



A B C

Figure 3.20 Appearance of SGE as non-incorporated in micelles (A) and SGE loaded in micelles of SGE-PF68 (B) and SGE-PF127 (C) in aqueous solution.

3.8.2 Comparison of Pluronic type and SGE to polymer ratio

1) Entrapment efficiency and loading capacity

The entrapment efficiency and loading capacity of SGE in polymeric micelles were investigated by determining the content of gallic acid as a biomarker compound of the extract as expressed in Table 3.20. Both PF68 and PF127 showed efficiency to SGE but in different level. The SGE could be entrapped in polymeric micelles of PF68 higher than that of PF127 in all ratios. It was noted that the highest entrapment efficiency of SGE loaded in both polymers was obtained from the ratio of 1:5 which was 98.05 ± 0.32% and 92.41 ± 0.76% for PF68 and PF127, respectively. While the

loading capacity of these micelles was 1.48 ± 0.00% and 1.39 ± 0.01% for PF68 and PF127, respectively. These results were in agreement with the previous report that entrapment efficiency and loading capacity depend on the kind of the entrapped substance, polymer type, and proportion of the substance to polymer [128].

Table 3.20 Entrapment efficiency and loading capacity of SGE-PF68 and SGE-PF127 as prepared at different ratios of SGE to polymer.

Polymer type	SGE:Polymer ratio (w/w)	Entrapment efficiency (% w/w)	Loading capacity (% w/w)
Pluronic F68	1:1	95.41 ± 1.11	6.78 ± 0.08
	1:5	98.05 ± 0.32	1.48 ± 0.00
	1:10	97.75 ± 1.03	0.74 ± 0.01
Pluronic F127	1:1	90.17 ± 0.85	6.41 ± 0.06
	1:5	92.41 ± 0.76	1.39 ± 0.01
	1:10	91.62 ± 0.84	0.70 ± 0.01

2) Antibacterial activity

The *in vitro* antibacterial activity of free SGE as non-incorporated in the polymeric micelles, SGE loaded polymeric micelles, and blank polymeric micelles were investigated against *S. aureus* by broth micro-dilution assay. The MIC and MBC values of each sample were determined as shown in Figure 3.21. SGE-PF68 in all ratios of SGE to polymer gave stronger inhibitory effect than that of SGE-PF127, also served

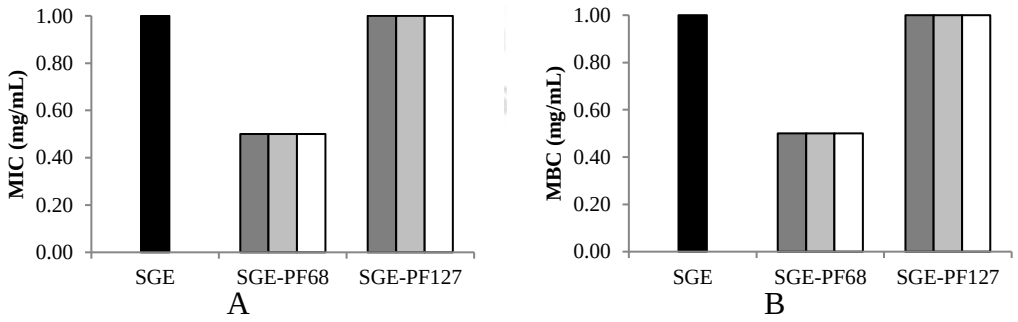


Figure 3.21 MIC (A) and MBC (B) values of free SGE (■) and SGE-PF68 and SGE-PF127 as prepared at different ratios between SGE to polymer of 1:1 (■), 1:5 (■), and 1:10 (□) against *S. aureus*.

stronger antibacterial action than free SGE. Meanwhile, the blank polymeric micelles of both PF68 and PF127 did not show any antibacterial effect. The inhibitory effect of SGE loaded in PF68 micelles was higher than that loaded in PF127 micelles. This result was considered to be due to higher entrapment efficiency of SGE in micelles of PF68 than that of PF127 micelles. Therefore, loading SGE in PF68 micelles is obviously better than in PF127 micelles. The comparison between free SGE and SGE loaded polymeric micelles in aqueous solution revealed that the SGE-PF68 provides higher efficacy of antibacterial activity than free SGE. This effect might be considered that the enhanced cellular uptake of polymeric micelles entrapped antibacterial substance could effectively improve the antibacterial effect due to the direct action by contact of micelles with bacteria and diffusion of released antibacterial agents to bacteria located sites [88].

3) Toxicity

The toxicity of blank polymeric micelles and SGE loaded polymeric micelles comparison to free SGE which non-incorporated in the polymeric micelles were evaluated using silkworms as animal model. The survival of silkworm after 1 day injected with the polymeric micelles were shown in Figure 3.22. The results were found that the feeding amount of SGE in this study served survival of all injected silkworm that indicated the SGE as dissolved in DMSO-normal saline solution (1:9) at a concentration of 10 mg/mL was safety for silkworms. The polymeric micelles of both PF68 and PF127 with and without SGE demonstrated some extent of toxicity to silkworm when high polymer concentration was used. At a ratio of SGE to polymer of 1:10 of blank polymeric micelles and SGE loaded polymeric micelles of both PF68 and PF127 affected to kill silkworms whereas at the ratios of 1:1 and 1:5 were safe to injected silkworm because all of injected silkworm survive after injection of these systems. The results pointed that when polymer content was increase as in a ratio of 1:10, the blank polymeric micelles that without SGE loading demonstrated toxicity to silkworms. The polymeric micelles of PF127 revealed higher toxic than that of PF68. The micelles as loaded with SGE showed higher toxic than that of polymer alone. It was noticed that the SGE-PF127 demonstrated approximately two times higher toxic than SGE-PF68. Many types of Pluronic polymers are presently available on market and

suggest for utilization in drug delivery systems [129]. This study suggest that the use of Pluronics to the body should be careful as it was found that toxicity of these polymers depend on type and used amount of polymer [130]. From these results, it could be concluded that PF68 was appropriate to be used for further study with SGE regarding to its higher potential than PF127 on high entrapment efficiency of SGE, high antibacterial activity, and low toxicity.

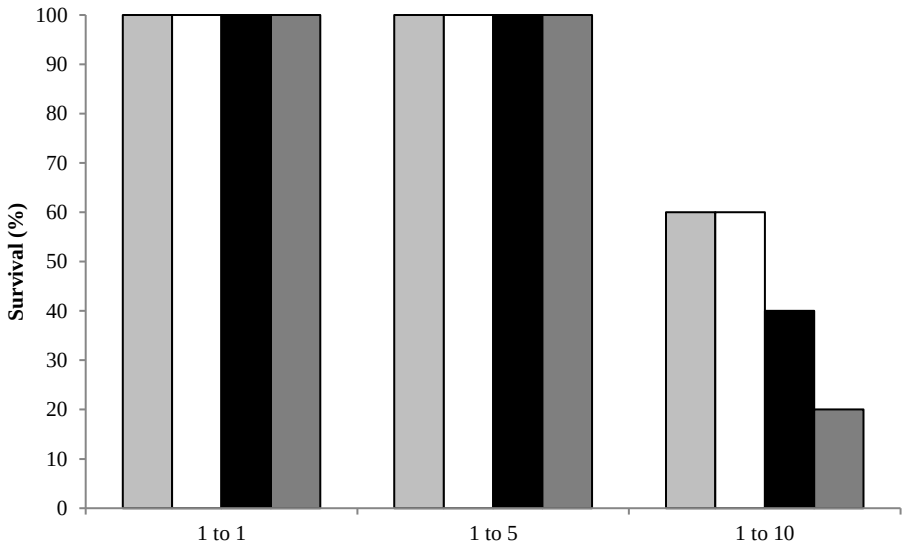


Figure 3.22 Survival of injected silkworm with blank polymeric micelles of PF68 (▒) and PF127 (□) comparison with SGE-PF68 (■) and SGE-PF127 (■) as prepared at different ratios between SGE to polymer of 1:1, 1:5, and 1:10.

4) *In vitro* release

The *in vitro* release profiles of SGE from polymeric micelles of PF68 and PF127 at a ratio of SGE to polymer of 1:5 were investigated by dialysis method in physiological conditions (PBS pH 7.4, 37°C) as shown in Figure 3.23. The release profiles of SGE from both micelles presented rapid release in during the first 2 h with release rate of 419.70 ± 0.43 μg/h for SGE-PF68 and 385.79 ± 0.87 μg/h for SGE-PF127, then continue to slow rate as the sustained release over prolonged time. In finally at 48 h, SGE from PF68 micelles was released with 89.47 ± 4.82% while SGE from PF127 micelles was released with 81.06 ± 0.27%. Comparison the relative release profiles of SGE from both the polymeric micelles, the SGE from PF68 micelles was

released faster than that from PF127 micelles due to a great interaction between SGE and PF127 micelles [131].

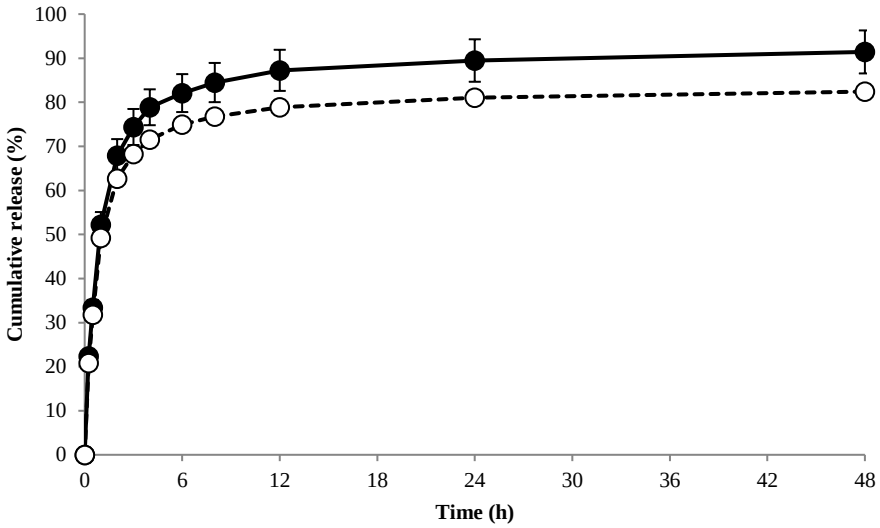


Figure 3.23 *In vitro* release of SGE from polymeric micelles of PF68 (●) and PF127 (○) as a ratio of 1:5 in physiological condition (PBS pH 7.4, 37°C).

3.8.3 Evaluation of SGE loaded polymeric micelles of Pluronic F68 (SGE-PF68)

1) Entrapment efficiency and loading capacity

Regarding to the comparison of PF68 and PF127 for preparing SGE loaded polymeric micelles as various ratios of SGE to polymer, PF68 showed the most suitable for entrapment of SGE. Moreover, it was found that the toxicity occurred when high amount of polymer was used while lower portion of polymer such as 1:1 and 1:5 were regarded as safe. Therefore, high ratio of PF68 were avoided. The SGE-PF68 was prepared with the ratios of SGE to polymer of 1:1, 1;2, 1:3, 1:4, and 1:5 by thin-film hydration method. The entrapment efficiency and loading capacity of SGE in polymeric micelles as prepared SGE to polymer ratios in range of 1:1 to 1:5 which non-toxicity to silkworm were shown in Table 3.21. Among all ratios, the ratio of 1:3 exhibited the highest entrapment efficiency of 99.63 ± 0.19% and loading capacity of 2.48 ± 0.00%. It was expected that micellar incorporation was resulted from the forces of assemble attraction between alkyl groups in Pluronic polymer and phenyl groups in SGE which compose of phenolic acid as a bioactive compound [132]. The entrapment efficiency

and loading capacity depend on kind of drug, solvent media, type of polymer, and proportion of drug to polymer since interactions of drug-drug, drug-solvent, and drug-polymer [133].

2) Particle size, size distribution, and zeta potential

Particle mean size, size distribution which expressed as polydispersity index (PdI), and zeta potential of SGE loaded polymeric micelles were investigated by PCS. These characteristic of the obtained micelles were shown in Table 3.21. The mean size of SGE-PF68 was in the range of 19.14 ± 0.24 to 47.59 ± 0.39 nm. The increasing polymer proportion resulted that the size of micelles was shrunken. This result indicated that size of polymeric micelles associated with not only the length of PEO and PPO chains of polymer but also the proportion of the entrapped substance or polymer which used to prepare the micelles [129, 132].

The SGE-PF68 micelles in all ratios showed narrow size distribution with PdI less than 0.20 nm. The result revealed that the developed micelles were highly monodispersed, particularly those in 1:3 ratio which showed the lowest PdI of 0.126 ± 0.008 nm. However, in ratio of 1:5 was observed the largest size distribution with PdI of 0.451 ± 0.029 nm. This results suggested that some micelles aggregation occurred in ratio of 1:5.

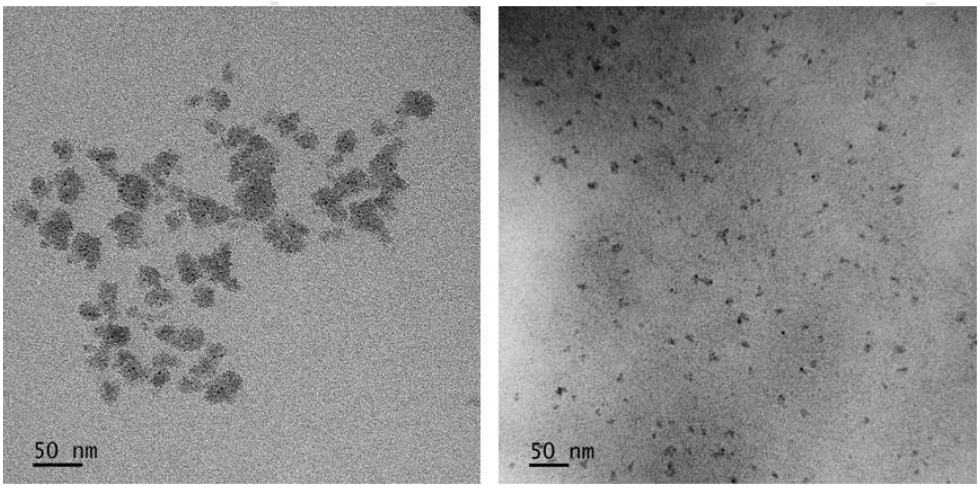
Table 3.21 Entrapment efficiency, loading capacity, particle mean size, polydispersity index (PdI), and zeta potential of SGE-PF68 as prepared at different ratios of SGE to polymer.

SGE:Polymer ratio (w/w)	Entrapment efficiency (% w/w)	Loading capacity (% w/w)	Size (nm)	PdI	Zeta potential (mV)
1:1	95.41 ± 1.11	6.78 ± 0.08	47.59 ± 0.39	0.128 ± 0.029	-34.20 ± 0.10
1:2	98.03 ± 0.40	3.61 ± 0.01	27.78 ± 0.32	0.173 ± 0.019	-39.57 ± 0.25
1:3	99.63 ± 0.19	2.48 ± 0.00	24.95 ± 0.34	0.126 ± 0.008	-41.53 ± 0.15
1:4	96.06 ± 0.60	1.80 ± 0.01	22.29 ± 0.46	0.163 ± 0.009	-35.37 ± 0.35
1:5	98.05 ± 0.32	0.74 ± 0.01	19.14 ± 0.24	0.451 ± 0.029	-33.83 ± 0.06

Zeta potential was an important parameter as represent the surface charge of polymeric micelles which influence to interaction behavior of the particles on the storage stability. Normally, the particles aggregation was occurred when the absolute value of zeta potential of the particles was too low due to sufficient electric repulsion or steric barriers between each other. The zeta potential of SGE-PF68 in all ratios were negative charge with absolute values of zeta potential were more than 30 mV. The results of these values meant that the SGE loaded PF68 micelles were stable against aggregation effect, especially the SGE-PF68 micelles as a ratio of 1:3 presented the highest zeta potential with the value of mV indicating that these micelles were the most stable [134].

3) Morphology

The morphology of polymeric micelles of both blank PF68 and SGE-PF68 were observed by TEM. The TEM images presented that the polymeric micelles of blank PF68 and SGE-PF68 were spherical shape as shown in Figure 3.24. It can be monitored that the particles size of SGE loaded polymeric micelles as measured via TEM images was accorded with that determined by light scattering technique using photon correlation spectrophotometer (PCS). The slightly different size between both techniques was resulted from different measuring, the particle size which evaluated using PCS was measured average size of the micelles as taking into account possible agglomeration that different from size classes [135].



A **B**

Figure 3.24 TEM images of polymeric micelles of SGE-PF68 (A) and blank PF68 (B) as prepared at a ratio between SGE to polymer of 1:3.

4) Thermal behavior

The thermal behavior of SGE-PF68 and blank PF68 were investigated in comparison with that of free SGE which non-incorporated in the polymeric micelles by DSC as shown in Figure 3.25. The thermograms of each sample gave the exothermic peak as approximately at 119°C (ΔH = -151 J/g), 56°C (ΔH = -123 J/g), and 52°C (ΔH = -127 J/g) for SGE, blank PF68, and SGE-PF68, respectively. The characteristic peak of SGE was not observed in SGE loaded polymeric micelles. The results was in line with the previous report that presented the absence peak of drug when it was loaded in the nanoparticles [136]. These evidence suggested that SGE could be loaded in the micellar core of Pluronic polymer which improved the aqueous solubility of SGE.

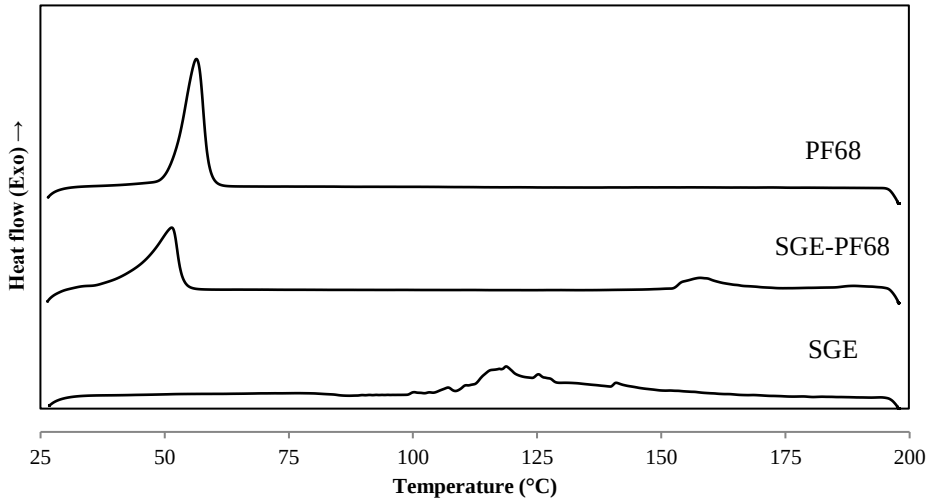


Figure 3.25 DSC thermogram of blank PF68, SGE-PF68, and SGE.

5) Crytallography

The physical state (crystalline or amorphous) of SGE-PF68 and blank PF68 were observed in comparison with that of free SGE which non-incorporated in the polymeric micelles by XRD as shown in Figure 3.26. XRD pattern of free SGE served a prominent characteristic peak at 2θ of 16° which indicated crystalline in nature. While XRD spectrum of blank PF68 presented two diffraction peaks which were crystalline in nature at 2θ of 19° and 23° as resulted from presence of PEO groups in the polymers [137]. The XRD profiles of SGE-PF68 was a parallel to XRD patterns of PF68 polymer.

These results confirmed that the SGE could be dispersed throughout the micellar polymer as forming amorphous state that improved the aqueous solubility of SGE.

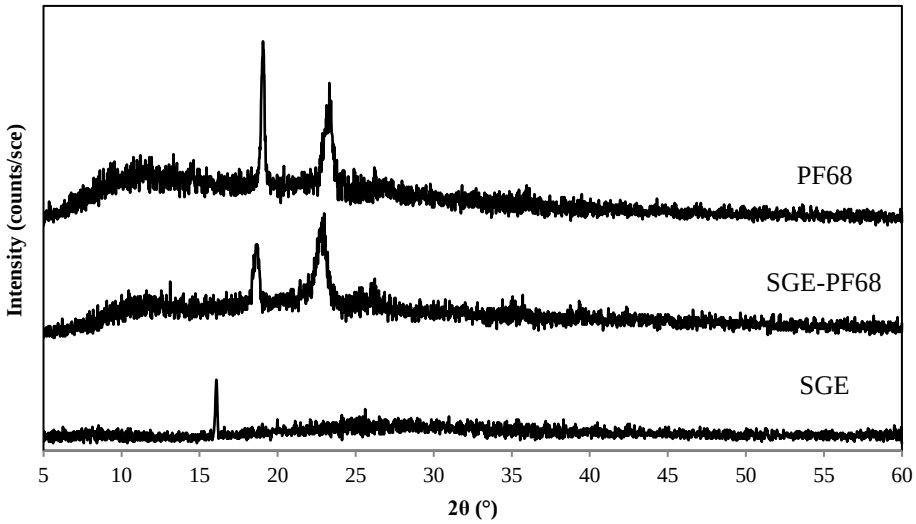


Figure 3.26 XRD pattern of blank PF68, SGE-PF68, and SGE.

6) Biological activity

The biological activity of SGE-PF68 at a ratio between SGE to polymer of 1:3 were evaluated in comparison with that of free SGE by *in vitro* antibacterial activity and *in vivo* therapeutic effect using blank micellar polymer of PF68 as a control.

The *in vitro* antibacterial assay, the SGE-PF68 demonstrated antibacterial activity with MIC and MBC values of 0.50 mg/mL against *S. aureus* by broth dilution assay. The inhibitory effect of the SGE micelles was revealed stronger than that of free SGE approximately two times. This test indicated to antibacterial capacity of SGE had not been adversely affected by micelles formulation. While blank PF68 as equivalent polymeric concentration had not presented inhibitory action on the visible growth of bacteria.

The *in vivo* assay was performed to evaluate toxicity and therapeutic effect of test samples using silkworm model. The silkworms were accepted for using as animal model, there was several evidences which used this model for studying therapeutic effect of antibacterial agent [51, 57]. The toxicity of prior study indicated that the polymeric micelles as high portion of polymer were toxic to silkworm. Therefore, the

therapeutic effect evaluation of this study used the Pluronic polymer as a mild concentration to silkworms.

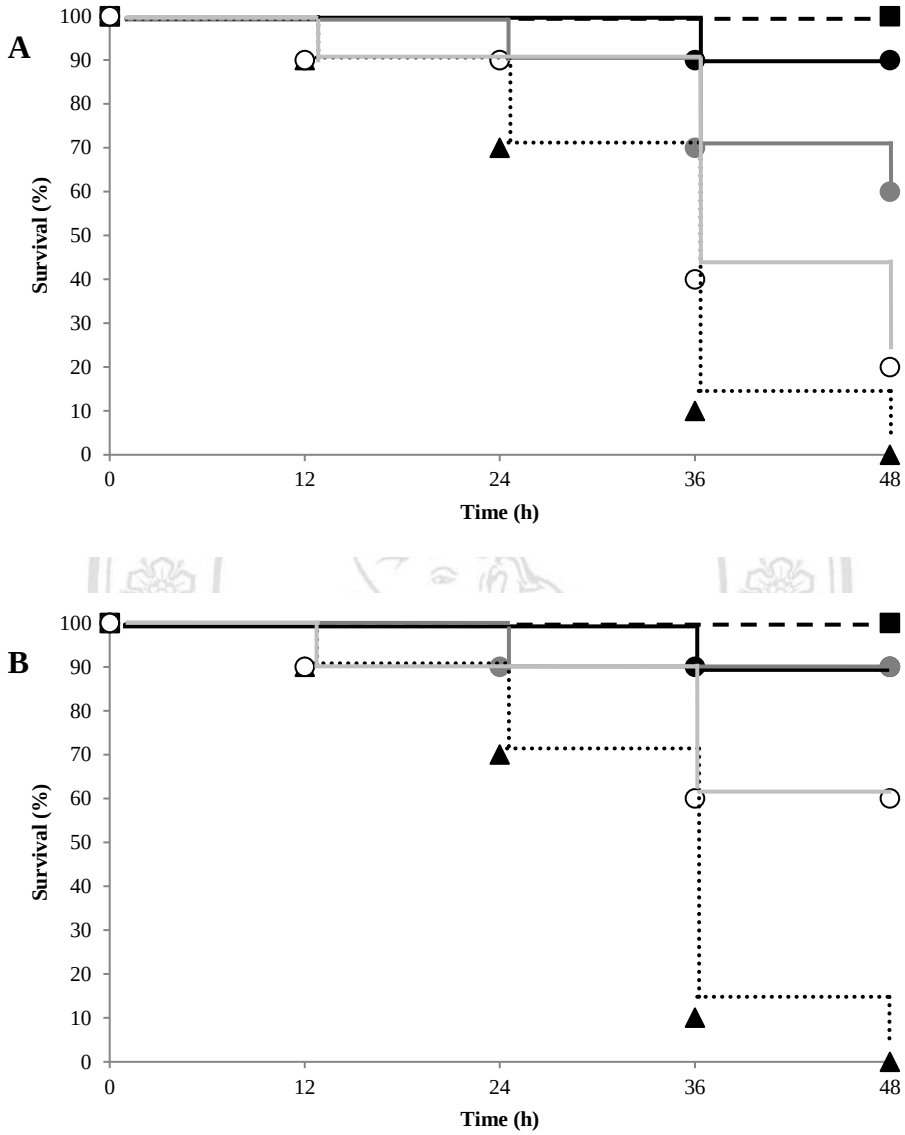


Figure 3.27 Survival rate of silkworm infected with *S. aureus* and injected with free SGE (A) and SGE-PF68 (B) at different SGE concentrations of 2.50 (○), 5.00 (●), and 10.00 mg/mL (●). The positive control expressed as infected silkworms without any treatment (s) and the negative control expressed as non-infected silkworms which injected with saline (■).

The survival rate of free SGE and SGE-PF68 micelles as different concentration of 2.50, 5.00, and 10.00 mg/mL comparison with group of infected silkworms without

any treatment (positive control) and another group of non-infected silkworms which injected with saline (negative control) were shown in Figure 3.27. The treatment with SGE and SGE-PF68 at a concentration of 10.00 mg/mL were demonstrated similarly therapeutic effect which revealed 90% of survival silkworms. However, the survival rates of these samples at concentration of 2.50 and 5.00 mg/mL presented different therapy. The SGE-PF68 micelles served an enhancing survival of infected silkworms when compared with the free SGE. The free SGE at a concentrations of 2.50 and 5.00 mg/mL exhibited the survival of 20% and 60%, respectively while these concentrations of SGE-PF68 provided increasing survival with existent infected silkworm of 60% and 90%, respectively.

The silkworms were sensitive to bacterial infection since all of infected silkworm as non-treatment could not survive more than 48 h. Whereas the infected silkworms which treated with free SGE and SGE-PF68 could prolong survivability in a dose dependent manner as shown in Figure 3.28. Survival curves represented plotting survival silkworm after infected 48 h versus SGE concentration. The increasing concentration of SGE and SGE-PF68 resulted in the survival of infected silkworms also increasing. These results exposed therapeutic effect of SGE to infected silkworms with IC_{50} and ED_{50} values of 8.95 mg/mL and 0.45 mg/g larva, respectively for free SGE, also 4.38 mg/mL and 0.22 mg/g larva, respectively for SGE-PF68 micelles. It indicated that the therapeutic effect of SGE was significantly enhanced approximately two times

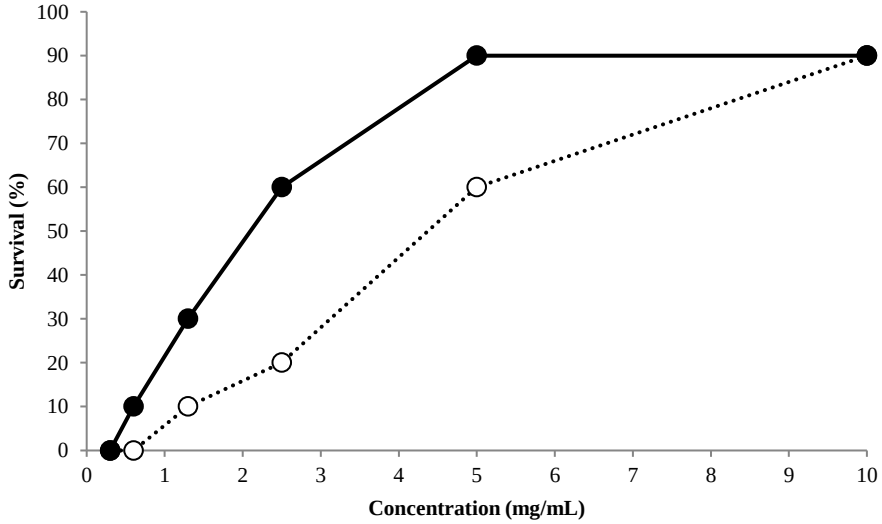


Figure 3.28 Therapeutic effect of free SGE (○) and SGE-PF68 (●) on silkworm infected with *S. aureus*.

by SGE-PF68. This result could be concluded that SGE-PF68 at a ratio of 1:3 was suitable for further study in mammals and clinical study in human body.

7) Solubility study

The solubility of SGE and SGE-PF68 were determined in aqueous solution by measuring the content of gallic acid which a biomarker compound of SGE as expressed in term of gallic acid equivalent (GAE). The SGE in polymeric micelles of PF68 could be dissolved in water increasingly with GAE as 76.11 ± 0.22 mg/mL while the solubility of SGE found GAE as 58.12 ± 1.88 mg/mL. The improvement dissolution could be attributed to the amorphous state of the Pluronic micelles as along with the emulsifying and solubilizing properties [138].

8) *In vitro* release study

The kinetic for *in vitro* release of SGE from PF68 micelles was taken by dialysis method under sink conditions of physiological medium (PBS pH 7.4 at 37°C). The SGE-PF68 at a ratio of 1:3 was used to investigate in comparison with free SGE as non-incorporated in the polymeric micelles. The release profiles were demonstrated in Figure 3.29. It was found that SGE was spontaneously release from the polymeric micelles in the physiological condition. The cumulative release profiles were noted that

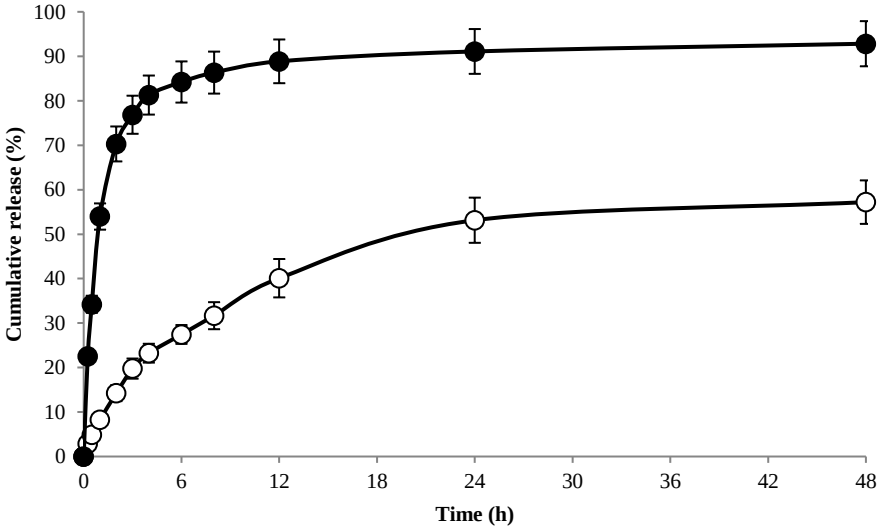


Figure 3.29 *In vitro* release of free SGE (○) and SGE from PF68 micelles as a ratio of 1:3 (●) in physiological condition (PBS pH 7.4, 37°C).

more than 50% SGE was released from the polymeric micelles within 1 h and over 80% within 4 h. For free SGE, more than 24 h was taken to receive 50% SGE release. The slow release rate of free SGE might be due to the limitation of SGE solubility in the aqueous system. This solubilized effect was considered to be due to the PEO chains in PF68 that can increase the interaction of water molecules with the loaded SGE in the core of micelles [139, 140]. These results expressed that the polymeric micelles of PF68 could enhance SGE properties not only the solubility but also the release behavior. This enhancement promoted SGE-PF68 to be suitable system for antibacterial action.

9) Stability study

9.1) Accelerated temperature condition

The influence of temperature between 50-90°C on degradation kinetic of SGE in PF68 micelles was compared with that of free SGE by measuring the remained GAE content. The relative between percentage of SGE remaining concentration in free form and micelles form versus incubated time were shown in Figure 3.30. The degradation curves revealed that an increasing temperature resulted in an increasing degradation rate of SGE. These results showed that the %SGE remaining in PF68 micelles keeping at all accelerated temperatures was significantly higher than SGE in free form, it was indicated that SGE loaded in polymeric micelles was high chemical stable. Kinetic degradation of SGE in aqueous system was found to follow the first order reaction. The linear regression after plotting log SGE concentration versus time was obtained for all

Table 3.22 Linear regression correlation (r^2) and the first order degradation rate constant (k) of SGE in free form and micelles form keeping at different accelerated temperatures for 60 min.

Accelerated temperatures (°C)	SGE		SGE-PF68	
	r^2	k (min^{-1})	r^2	k (min^{-1})
50	0.945	1.4×10^{-3}	0.969	4.0×10^{-4}
60	0.901	2.0×10^{-3}	0.971	7.0×10^{-4}
70	0.816	2.5×10^{-3}	0.958	9.0×10^{-4}
80	0.883	3.6×10^{-3}	0.925	1.2×10^{-3}
90	0.941	6.3×10^{-3}	0.937	1.4×10^{-3}

systems and the stability parameters during the first 60 min of storage were shown in Table 3.22. It was obviously seen that the kinetic degradation rate of SGE was retarded approximately ten times after it was loaded in PF68 micelles.

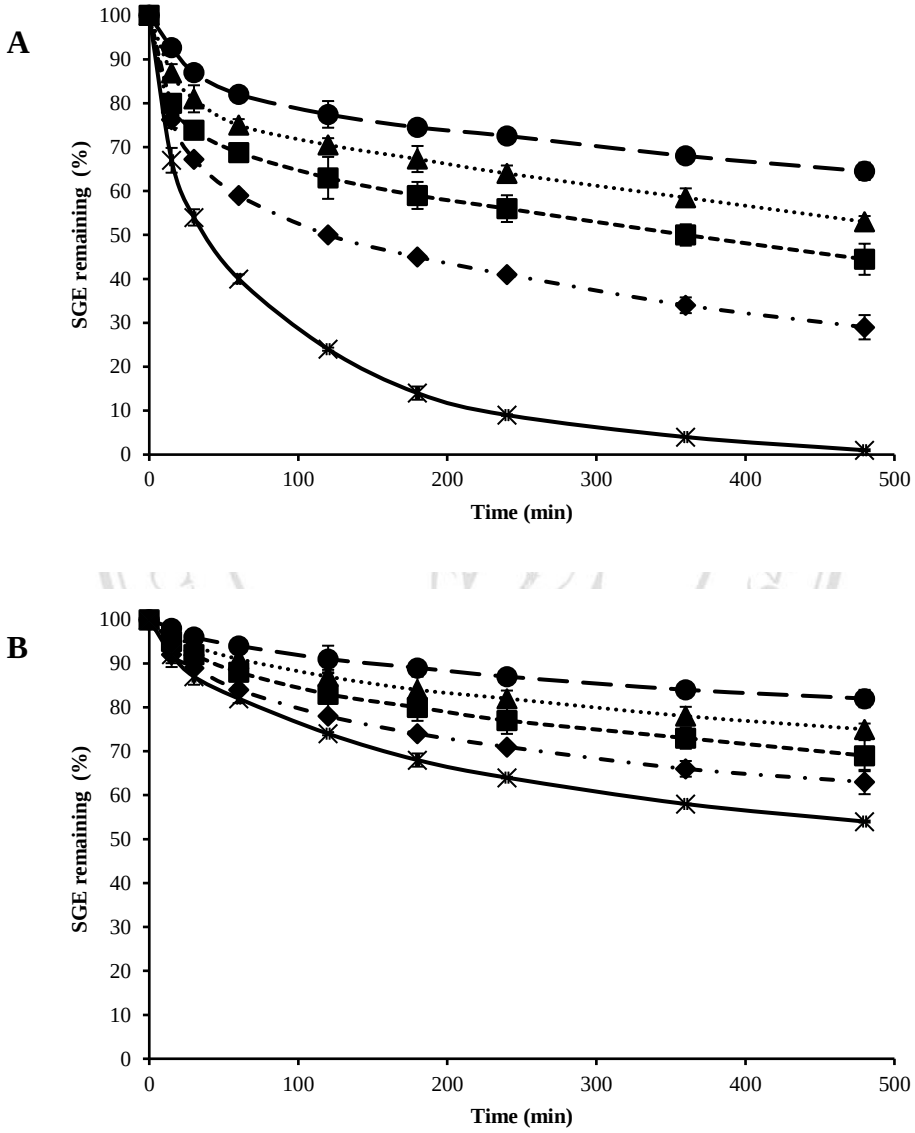


Figure 3.30 Percentage of SGE remaining concentration in free SGE (A) and SGE-PF68 (B) as prepared at a ratio between SGE to polymer of 1:3 at different accelerated temperatures of 50°C (●), 60°C (s), 70°C (n), 80°C (u), and 90°C (5) for 480 min.

The results suggested that the degradation of SGE in aqueous system could be explained by first-order kinetics reaction of Arrhenius plot, which presented a linear

relationship between log k and 1/T as shown in Figure 3.31. The Arrhenius equation is a following equation: $k_{obs} = Se^{-Ea/RT}$ where k_{obs} is the reaction rate constant (h^{-1}), S is the frequency factor (h^{-1}), Ea is the activity energy (J/mol), R is the gas constant (8.314 J/mol K), and T is the absolute temperature (K).

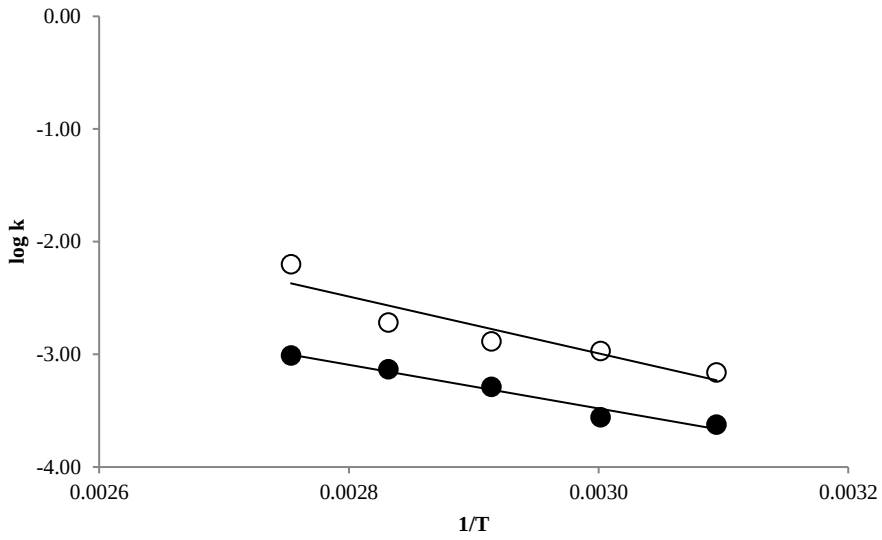


Figure 3.31 Arrhenius plot for degradation of SGE in free SGE (○) and SGE-PF68 (●).

The degradation kinetic of SGE loaded in polymeric micelles also followed the first-order kinetics reaction. The degradation rate of the first-order reaction could be considered from the slope of a straight line that is the high slope mean to the rapid degradation rate. The calculates values of experimental kinetic parameters of SGE degradation were exhibited in Table 3.23. The k_{obs} of SGE in free form was higher than that of SGE in micelle form approximately two times, therefore half-life ($t_{1/2}$) of SGE-PF68 was approximately two times longer than that of which non-loaded in micelles.

Table 3.23 Experimental first order kinetic parameters and correlation coefficient (r^2) of the degradation of SGE in free form and micelles form.

Sample	Ea (kJ/mol)	k_{obs} (h^{-1})	$t_{1/2}$ (h)	r^2
SGE	48.31 ± 1.68	0.0079 ± 0.0009	88.96 ± 11.17	0.8701
SGE-PF68	37.08 ± 0.27	0.0041 ± 0.0002	169.32 ± 8.59	0.9699

These results expressed that the polymeric micelles of PF68 could enhance kinetically stable in aqueous solution due to the stabilization effect of PEO chains in PF68 [130].

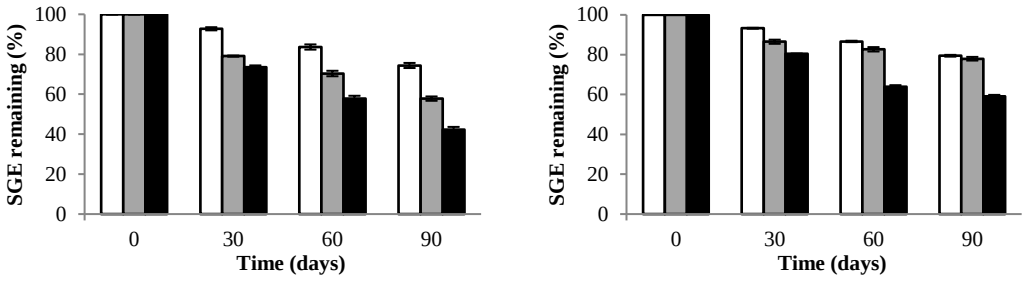
9.2) Storage condition

The storage stability was carried out in different temperatures of 4°C, room temperature, and 45°C for 90 days in order to estimate SGE stability after loaded in PF68 micelles comparison with SGE as non-entrapped in polymeric micelles, also find the suitable storage condition for SGE-PF68. The physical appearance and the remaining of SGE in free SGE and SGE-PE68 were determined by naked eyes observation and HPLC analysis, respectively. All samples were dissolved in aqueous solution and separated the insoluble compound by centrifuged and filtered through 0.45 μm Millipore membrane. The feature of SGE and SGE micelles at different storage temperatures for several time periods were observed as shown in Table 3.24. The aspect of SGE was brown suspension. After extended time of keeping, precipitation was occurred in storage condition of 45°C for 30 days and room temperature for 60 days. Whereas, the feature of SGE-PF68 was brown solution. It did not change any aspects when extended time of keeping at different temperatures. The appearance was still represented same as the original aspect. According to the SGE remained content during the storage period, the GAE of SGE and SGE-PF68 were measured by HPLC analysis. The start concentration as a 100% of SGE in free form and micelles form was found to be 58.12 ± 1.88 mg/mL and 76.11 ± 0.22 mg/mL, respectively.

Table 3.24 Physical appearance of SGE and SGE-PF68 in storage condition at various temperatures for 30, 60, and 90 days.

Sample	Keeping condition	Physical appearance		
		30 days	60 days	90 days
SGE	4°C	Brown suspension	Brown suspension	Brown suspension
	room temp	Brown suspension	Brown precipitate	Brown precipitate
	45°C	Brown precipitate	Brown precipitate	Brown precipitate
SGE-PF68	4°C	Brown solution	Brown solution	Brown solution
	room temp	Brown solution	Brown solution	Brown solution
	45°C	Brown solution	Brown solution	Brown solution

The percentages of SGE remaining concentration of SGE and SGE-PF68 at different storage condition were shown in Figure 3.32. The stability of SGE decreased as a time-dependent manner. The amount of SGE in free form was decreased to 74.39 ± 1.27, 57.79 ± 1.08, and 42.22 ± 1.42 % at 4°C, room temperature, and 45°C, respectively after 90 days. While the SGE content in the micelles was remained up to 79.47 ± 0.44, 77.98 ± 0.92, and 59.11 ± 0.72 % at 4°C, room temperature, and 45°C, respectively for 90 days. These results were confirmed that the polymeric micelles could enhance stabilization effect not only maintaining the physical appearance but also keeping up the content of active compound by protecting the SGE as loaded in the micelles from mechanisms of hydrolysis and biotransformation [141]. The suitable storage condition for SGE-PF68 was recommended that the SGE micelles should be reserved in closed container with light protection at lower than or equal to room temperature for 90 days. The remaining content of active compound was closed up 80%.



A B

Figure 3.32 Percentage of remaining SGE (A) and SGE-PF68 (B) as prepared at a ratio between SGE to polymer of 1:3 which were kept in storage condition at various temperatures of 4°C (□), room temperature (▒), and 45°C (▪) for 30, 60, and 90 days.

CHAPTER 4

Conclusion and Suggestion

This study focused on development of polymeric micelles loaded with *Sesbania grandiflora* extract for antibacterial activity in silkworm infection model. Ethyl acetate extract from the bark of *S. grandiflora* provided potential antibacterial activity against several pathogenic strains including drug-resistant strains. Moreover, it was not only non-toxic to the silkworm but also prolonged survivability of silkworm infected with pathogenic bacteria, *Staphylococcus aureus*. The efficacy of antibacterial activity was affected from synergism of many compounds in the extract particularly phenolic compounds. The results of chromatography analysis indicated that the marker compound was gallic acid, which contained approximately 83.15 ± 2.12 mg per g of the extract. Gallic acid was one of the main active compounds as existing in the extract that represented biological activities. However, the limitation of low aqueous solubility and instability resulted in inconvenient for use in pharmaceutical and medicinal fields. Nanotechnology base on polymeric nanoparticles was applied to create a nano-carrier to overcome these problems. Pluronic F68 and Pluronic F127 were used to form polymeric micelles. The *S. grandiflora* extract was entrapped into polymeric micelles by thin-film hydration technique. These micelles were appropriate strategy for *S. grandiflora* extract in treatment antibacterial infection. The polymeric micelles as loaded the extract presented effective inhibitory action against pathogenic bacteria both *in vitro* and *in vivo* studies also non-toxic to silkworm as used for animal model. The polymeric micelles of Pluronic which been a nano-carriers for *S. grandiflora* extract was evaluated entrapment efficiency and loading capacity as different ratios of the extract to polymer for obtaining the optimal polymeric micelles. The micelles which composed of *S. grandiflora* extract and Pluronic F68 at the ratio of 1:3 revealed the highest entrapment efficiency with 99.63 ± 0.19 % (w/w). The physical properties of Pluronic micelles loaded *S. grandiflora* extract were evaluated particle mean size, size

distribution, and zeta potential by dynamic light scattering technique also monitored shape of the nanoparticles by TEM. The *S. grandiflora* extract loaded polymeric micelles exhibited spherical shape with size in range nanometer and absolute values of zeta potential as more than 30 mV. The thermal behavior and physical state of the *S. grandiflora* extract loaded polymeric micelles were observation by techniques of DSC and XRD, respectively. The evidences of DSC and XRD confirmed that the *S. grandiflora* extract could be dispersed throughout the micellar core of Pluronic polymer as forming amorphous state that improved the aqueous solubility of the extract. The biological assays were determined by *in vitro* and *in vivo* studies. The polymeric micelles loaded with *S. grandiflora* extract presented *in vitro* antibacterial activity and *in vivo* therapeutic effect with a dose dependent manner. Moreover, the extract micelles could increase survival of infected silkworms when compared with the free extract as non-incorporated in the polymeric micelles. The aqueous solubility of *S. grandiflora* extract which showed GAE value of 58.12 ± 1.88 mg/mL, could be increased when the extract was entrapped in polymeric micelles, the GAE value of extract micelles up to 76.11 ± 0.22 mg/mL. The study of *in vitro* release of *S. grandiflora* extract from polymeric micelles exposed rapid release in the first time and continue to sustained release of the active compound in physiological conditions. The polymeric micelles could protect degradation of the extract as loaded in the micelles at the accelerated temperatures. The stabilization of *S. grandiflora* extract as entrapped in polymeric micelles was enhanced by Pluronic polymer, the physical appearance and the content of active compound were maintained as closed to the original state. The present study suggested that the *S. grandiflora* extract was successfully loaded in polymeric micelles which increase solubilization and stabilization of the extract. It might be a promising nanocarriers for further development in clinical trials.

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LIST OF PUBLICATIONS

1. **Pimporn Anantaworasakul**, Hiroshi Hamamoto, Kazuhisa Sekimizu, Siriporn Okonogi. Physiological comparison between mulberry (Morus alba L.) leaves diet and artificial diet on growth development as well as antibiotic therapeutic response of silkworms. Australian Journal of Crop Science. 2013;7:2029-2035.

2. **Pimporn Anantaworasakul**, Hiroshi Hamamoto, Kazuhisa Sekimizu, Siriporn Okonogi. *In vitro* antibacterial activity and *in vivo* therapeutic effect of *Sesbania grandiflora* in bacterial infected silkworms. Pharmaceutical Biology. 2017;55:1256-1262.

3. **Pimporn Anantaworasakul**, Siriporn Okonogi. Encapsulation of *Sesbania grandiflora* extract in polymeric micelles to enhance its solubility, stability, and bacterial activity. Journal of Microencapsulation. 2017;34:73-81.

APPENDIX

Medium and buffer preparation

1. Preparation of Tryptic Soy Agar (TSA)

1. Prepared 30 g of Tryptic Soy Broth (TSB)
2. Added 15 g of agar
3. Dissolved TSB and agar in DI water and adjusted volume to 1,000 mL

2. Preparation of Mueller-Hinton Agar (MHA)

1. Prepared 42 g of Mueller-Hinton Broth (MHB)
2. Added 15 g of agar
3. Dissolved MHB and agar in DI water and adjusted volume to 1,000 mL

3. Preparation of Mueller-Hinton Broth (MHB)

1. Prepared 42 g of Mueller-Hinton Broth (MHB)
2. Dissolved MHB in DI water and adjusted volume to 1,000 mL

4. Preparation of phosphate buffer solution (PBS), pH 7.4

1. Prepared 1.44 g of Na_2HPO_4, 8 g of NaCl, 0.24 g of KH_2PO_4, and 0.2 g of KCl
2. Mixed the chemical agents as mention in No.1
3. Dissolved the mixture in DI water
4. Adjusted pH to 7.4 with NaOH or HCl
5. Adjusted volume to 1,000 mL

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Publications

Journal

Anantaworasakul P., Klayraung S., Okonogi S. Antibacterial activities of *Sesbania grandiflora* extracts. Drug Discoveries & Therapeutics. 2011;5(1):12-17.

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Presentations

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Anantaworasakul P., Okonogi S. Development of polymeric micelles containing potential plant extract for solubility, stability, and activity enhancement. 4th Congress on Innovation in Drug Delivery (IDD2016), 26th - 28th September 2016, Antibes-Juan-les-Pins, France. (poster presentation)