ANTI-MICROBIAL, ANTI-INFLAMMATORY AND ANTI-ESTROGENIC ACTIVITIES OF "SA-TRI-LHANG-KLOD" REMEDY FOR POSTPARTUM CARE

BY

MISS KHWANCHANOK MOKMUED

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE IN APPLIED THAI TRADITIONAL MEDICINE

FACULTY OF MEDICINE

THAMMASAT UNIVERSITY

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ABSTRACT

Sa-Tri-Lhang-Klod (STK) is Thai traditional medicine preparation, is used for care during postpartum phase. It has been used for postpartum care on excrete lochia, blood tonic, uterine involution and improving blood circulation. Sa-Tri-Lhang-Klod remedy comprises 17 plant ingredients consists of roots of *Maclura cochinchinensis* (Lour.) Corner, stems of *Artocarpus heterophyllus* Lam., rhizomes of *Curcuma comosa* Roxb., stems of *Caesalpinia sappan* Linn., vines of *Piper ribesioides* Wall., roots of *Plumbago indica* Linn., flowers of *Piper longum* Linn., roots of *Angelica sinensis* (Oliv.) Diels., stems of *Salacia chinensis* Linn., fruits of *Piper nigrum* Linn., roots of *Piper samentosum* Linn., flowers of *Carthamus tinctorius* Linn., flowers of *Jasminum sambac* Ait., flowers of *Mimusops elengi* Linn., flowers of *Mesua ferrea* Linn., flowers of *Mammea siamensis* Kosterm., and flowers of *Nelumbo nucifera* Gaertn. The taste of these preparation is hot and spicy. Sa-Tri-Lhang-Klod remedy and some plant ingredients in this remedy have been investigated for antioxidant, cytotoxicity and anti-inflammatory. However, for Sa-Tri-Lhang-Klod remedy, there is no report to study on antimicrobial, anti-inflammatory (COX-2) and

estrogenic effect. Therefore, the objective of this study was to investigate the antimicrobial, anti-inflammatory (COX-2) and estrogenic effect of STK extracts and plant ingredients.

STK and its plant ingredients were extracted by maceration in 95% ethanol, 50% ethanol and decoction in water. All extracts of 20 samples were tested for, the first, antimicrobial activity against *Staphylococcus aureus* (ATTC 25923), *Escherichia coli* (ATTC 25922), and *Candida albicans* (ATTC 90028) by disc diffusion method. Minimum inhibitory concentration (MIC), and minimum bactericidal concentration (MBC) of all extracts were measured by broth dilution method. The results found that the inhibition zone of STK95 for inhibit *S. aureus* and *C. albicans* values 9.33±0.58 and 7.33±0.58 mm, respectively. STK50 and STW can inhibit *S. aureus* at 10.00±0.00 mm and 7.00±0.00 mm. And the ethanolic extract of *C. sappan* exhibited antimicrobial activity against *S. aureus* and *E. coli* with the highest inhibition zone of 22.67±2.32 mm and 10.33±0.58 mm, respectively. 95% ethanolic extract of *P. indica* exhibited the highest antimicrobial activity against *C. albican* with the highest inhibition zone of 13.00±0.00 mm. 95% ethanolic extract of *M. siamensis* showed bacteriacidal activity against *S. aureus* with MIC and MBC of 0.019 mg/ml. Moreover, the MIC and MBC values for 95% ethanolic extract of *C. sappan* against *E. coli* and *C. albican* were observed to be same values (1.25 mg/ml and 0.625 mg/ml, respectively).

For the anti-inflammatory, the 95% ethanolic extract (STK95) showed anti-inflammatory activity of inhibitory effect on cyclooxygenase-2 enzyme (Cox-2) in RAW 264.7 cells with IC_{50} of 3.15±0.30 µg/ml. However, it was less than prednisolone (positive control) with IC_{50} value of 1.34±0.03 µg/ml. While, the 95% exthanolic extract of *M. siamensis* showed the higher anti-inflammatory activity than prednisolone with IC_{50} value of 0.08±0.005 µg/ml.

For the estrogenic and anti-estrogenic activities against E2-enhance T47D and MCF-7 cell proliferation found STK95, STK50 and STW showed anti-estrogenic activity. STK50 showed strong inhibition against T47D and MCF-7 cells at low concentration with iEqE1 values < 0.01 µM which it against more antiestrogenic effect than tamoxifen (iEqE1=1.79 and 84.56 µM).

The 95% ethanolic extract of *M. siamensis* was chosen to separate by Vacuum Liquid Chromatography (VLC) because it showed good activity in many bioassays. It was separated by vacuum liquid chromatography to be 5 fractions; Hexane (MS-1), Hexane:Chloroform (MS-2), Chloroform (MS-3), Chloroform:Methanol (MS-4) and Methanol (MS-5). MS-4 fraction which showed the highest activities was isolated active compound by using column chromatography. The component of coumarin was isolated. It showed the anti-inflammatory in RAW 264.7 cells (COX-2) values 47.27±2.00 µg/ml and had no toxicity.

In conclusion, STK95, STK50 and STKW showed the moderate of antimicrobial. *M. siamensis* showed very high biological in antimicrobial and anti-inflammatory. And the all extract of remedy showed the good of anti-estrogenic activity. However, the results of the present study can partially support the use of STK, a remedy in Thai traditional medicine for protection of postpartum infection, anti-inflammation after delivery and for increasing lactation. Moreover, these results related to Thai traditional medicine and to support the using these remedy in the lists of The National List of Essential Herbal Medicine (NLEM) of Thailand.

Keywords: Anti-microbial, Anti-inflammatory, Estrogenic effect, Sa-Tri-Lhang-Klod remedy, Postpartum Care

หัวข้อวิทยานิพนธ์ ฤทธิ์ต้านจุลชีพ ฤทธิ์ต้านการอักเสบ และฤทธิ์ต้านเอสโตรเจนของตำรับยาสตรีหลังคลอดเพื่อใช้ดูแลหญิงหลังคลอด

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บทคัดย่อ

ตำรับยาสตรีหลังคลอดในบัญชียาหลักแห่งชาติเป็นยาที่ใช้ในการดูแลหญิงหลังคลอด ช่วยในการขับน้ำคาวปลา บำรุงเลือด ช่วยให้มดลูกเข้าอู่เร็วในหญิงหลังคลอด ซึ่งในตำรับยาสตรีหลังคลอดประกอบด้วยสมุนไพร 17 ชนิด ได้แก่ แก่นแกแล (*Maclura cochinchinensis* (Lour.) Corner), แก่นขนุน (*Artocarpus Heterophyllus* Lam.), เหง้าว่านชักมดลูก (*Curcuma comosa* Roxb.), แก่นฝาง (*Caesalpinia sappan* Linn.), แก่นสะค้าน (*Piper ribesioides* Wall.), ราก เจตมูลเพลิงแดง (*Plumbago indica* Linn), ดอกดีปลี (*Piper longum* Linn.), รากโกฐเชียง (*Angelica sinensis* (Oliv.) Diels), แก่นกำแพงเจ็ดชั้น *(Salacia chinensis* Linn), ผลพริกไทย (*Piper nigrum* Linn.), รากช้าพลู (*Piper samentosum* Linn), ดอกคำฝอย (*Carthamus tinctorius* Linn.), ดอกมะลิ (*Jasminum sambac* Ait.), ดอกพิกุล (*Mimusops elengi* Linn), ดอกบุนนาค *(Mesua ferrea* Linn.), ดอกสารภี (*Mammea siamensis* Kosterm.) และ ดอกเกสรบัวหลวง (*Nelumbo nucifera* Gaertn.) รสยาสมุนไพรในตำรับยาสตรีหลังหลอดส่วนใหญ่เป็นรสร้อน โดยตำรับและสมุนไพรบางชนิด เคยมีงานวิจัยศึกษาถึงฤทธิ์ทางชีวภาพที่เกี่ยวข้องกับสรรพคุณของตำรับ เช่น การทดสอบฤทธิ์ต้านอนุมูลอิสระ ฤทธิ์ความเป็นพิษต่อเซลล์ และฤทธิ์ต้านการอักเสบ เป็นต้น แต่ตำรับยาสตรีหลังคลอด ยังไม่เคยพบการรายงานฤทธิ์ต้านเชื้อจุลชีพ ฤทธิ์ต้านการอักเสบแบบเฉียบพลัน และฤทธิ์ต้านฮอร์โมนเอสโตรเจนของสารสกัดจากพืชสมุนไพรเดี่ยวในตำรับและสารสกัดของตำรับยาสตรีหลังคลอด

วิธีการศึกษา โดยนำสมุนไพรตัวเดี่ยวและตำรับยาสตรีหลังคลอด สกัดด้วยวิธีการต่างๆ ได้แก่ สารสกัดชั้นน้ำที่สกัดด้วยวิธีการต้มน้ำ สารสกัดชั้นเอทานอลที่สกัดด้วยวิธีการหมัก 95% และ 50% เอทานอล และทำให้สารสกัดแห้งด้วยเครื่องระเหยแห้ง นำสารสกัดทุกชั้นที่สกัดได้ 20 ตัวอย่าง มาทดสอบฤทธิ์ทางชีวภาพ คือ ฤทธิ์ต้านเชื้อจุลชีพต่อเชื้อแบคทีเรีย 2 สายพันธุ์ที่เกี่ยวข้องกับระบบสืบพันธุ์ ได้แก่ เชื้อ *Staphylococcus aureus* (ATTC 25923), *Escherichia coli* (ATTC 25922) และเชื้อรา *Candida albicans* (ATTC 90028)

การศึกษาฤทธิ์ต้านเชื้อจุลชีพด้วยวิธี disc diffusion เพื่อหาความยาวของเส้นผ่านศูนย์กลางของบริเวณที่สารสามารถยับยั้งเชื้อได้ (Inhibition zone) และ เพื่อหาความค่าความเข้มข้นต่ำที่สุดที่สามารถยับยั้งการเจริญเติบโตของเชื้อจุลชีพ (mimimum inhibition concentration หรือ MIC) และค่าความเข้มข้นต่ำที่สุดที่สามารถฆ่าเชื้อจุลชีพได้ (minimum bactericidal concentration หรือ MBC) พบว่า สารสกัดตำรับยาสตรีหลังคลอดชั้น 95% เอทานอล (STK95) มีฤทธิ์ยับยั้งเชื้อแบคทีเรีย *S. aureus* และเชื้อรา *C. albicans* โดยมีเส้นผ่าศูนย์กลาง inhibition zone เท่ากับ 9.33±0.58 และ 7.33±0.58 มิลลิเมตร ตามลำดับ ส่วนสารสกัดชั้น 50% เอทานอล (STK50) มีฤทธิ์ยับยั้งเชื้อแบคทีเรีย *S. aureus* โดยมีเส้นผ่าศูนย์กลาง inhibition zone เท่ากับ 10.00±0.00 มิลลิเมตร และสารสกัดชั้นน้ำ (STW) มีฤทธิ์ยับยั้งเชื้อแบคทีเรีย *S. aureus* โดยมีเส้นผ่าศูนย์กลาง inhibition zone เท่ากับ 7.00±0.00 มิลลิเมตร สารสกัดฝางชั้น 95% เอทานอล มีฤทธิ์ยับยั้งเชื้อแบคทีเรีย *S. aureus* และ *E. coli* ได้ดีที่สุดโดยมีค่าเส้นผ่าศูนย์กลางของ inhibition zone เท่ากับ 22.67±2.31 และ 10.33±0.58 มิลลิเมตร ตามลำดับ ส่วนสารสกัดเจตมูลเพลิงแดงชั้น 95% เอทานอล ฤทธิ์ยับยั้งเชื้อรา *C. albican* มากที่สุด โดยมีเส้นผ่าศูนย์กลาง inhibition zone เท่ากับ 13.00±0.00 มิลลิเมตร

การศึกษาฤทธิ์ต้านจุลชีพเพื่อหาความค่าความเข้มข้นต่ำที่สุดที่สามารถยับยั้งการเจริญเติบโตของเชื้อจุลชีพ (mimimum inhibition concentration หรือ MIC) และค่าความเข้มข้นต่ำที่สุดที่สามารถฆ่าเชื้อจุลชีพได้ (minimum bactericidal concentration หรือ MBC) พบว่าสารสกัดชั้น 95% เอทานอลมีฤทธิ์ยับยั้งเชื้อแบคทีเรีย *S. aureus* และเชื้อรา *C. albicans* โดยมีค่า MIC เท่ากับ 0.625 และ 2.5 มิลลิกรัมต่อมิลลิลิตร และมีค่า MBC เท่ากับ 0.625 และ 2.5 มิลลิกรัมต่อมิลลิลิตร เช่นกัน สารสกัดชั้น 50% เอทานอลมีฤทธิ์ยับยั้งเชื้อ *S. aureus* โดยมีค่า MIC และ MBC เท่ากับ 1.25 และ 1.25 มิลลิกรัมต่อมิลลิลิตร ตามลำดับ ส่วนสารสกัดชั้นน้ำของตำรับยาสตรีหลังคลอดไม่มีฤทธิ์ยับยั้งเชื้อทั้ง 3 สายพันธุ์ สารสกัดสารภีชั้น 95% เอทานอล มีฤทธิ์ยับยั้งเชื้อ *S. aureus* มากที่สุด โดยมีค่า MIC และ MBC เท่ากับ 0.019 และ 0.019 มิลลิกรัมต่อมิลลิลิตร ตามลำดับ

การทดสอบฤทธิ์ต้านการอักเสบ โดยวิธีดูการการยับยั้งเอนไซม์ไซโคออกซีจีเนส-ทู (cyclooxygenase-2) จากเซลล์ RAW 264.7 เมื่อถูกกระตุ้นด้วย LPS พบว่า สารสกัดตำรับยาสตรีหลังคลอดชั้น 95% เอทานอล (STK95) มีฤทธิ์ในการต้านการอักเสบที่มีการยับยั้งเอนไซม์ไซโคออกซีจีเนส-ทู (COX-2) ที่ IC_{50} เท่ากับ 3.15±0.30 ไมโครกรัมต่อมิลลิลิตร ขณะที่เพรดนิโซโลน ซึ่งเป็นสารมาตรฐาน มีค่า IC_{50} เท่ากับ 1.34±0.03 ไมโครกรัมต่อมิลลิลิตร ในขณะที่สารสกัดดอกสารภีชั้นเอทานอล มีฤทธิ์ในการต้านการอักเสบดีกว่าเพรดนิโซโลน โดยสามารถยับยั้งเอนไซม์ไซโคออกซีจีเนส-ทู (COX-2) ได้ที่ IC_{50} เท่ากับ 0.08±0.005 ไมโครกรัมต่อมิลลิลิตร อีกทั้งไม่มีความเป็นพิษต่อเซลล์อีกด้วย

การทดสอบฤทธิ์ต่อฮอร์โมนเอสโตเจน ซึ่งดูการเจริญเติบโตของเซลล์ MCF-7 และ T47D พบว่าสารสกัด 95% เอทานอล 50% เอทานอล และสารสกัดชั้นน้ำ มีฤทธิ์เพิ่มการยับยั้งการเจริญเติบโตของเซลล์ทั้งสองชนิด หรือฤทธิ์ต้านฮอร์โมนเอสโตรเจน โดยสารสกัด 95% เอทานอลของตำรับ (STK95) สารสกัด 50% เอทานอลของตำรับ (STK50) และสารสกัดชั้นน้ำของตำรับ (STW) มีฤทธิ์ต้านฮอร์โมนเอสโตเจนในระดับรุนแรง (Strong) ต่อเซลล์ MCF-7 และ T47D โดยมีเปอร์เซ็นการยับยั้งที่ 99% ที่ความเข้มข้น (iEqE1) น้อยกว่า 0.1, 0.01 และ 0.01 ไมโครโมลลาร์ ตามลำดับ และไม่มีสารสกัดชนิดใดที่มีฤทธิ์เพิ่มการเจริญเติบโตของเซลล์ หรือมีฤทธิ์เอสโตรเจน

สารสกัดสารภีชั้น 95% เอทานอล ซึ่งเป็นส่วนประกอบในตำรับยาสตรีหลังคลอดที่มีฤทธิ์ทางชีวภาพที่ดีจึงได้ถูกเลือกมาทำการแยกแบบหยาบด้วยวิธี Vacuum Liquid Chromatography (VLC) โดยเริ่มจากตัวทำละลาย Hexane, Hexane:Chloroform (1:1 v/v), Chloroform, Chloroform:Methanol (1:1 v/v) และ Methanol ได้เป็นสารสกัด MS-1, MS-2, MS-3, MS-4, MS-5 ตามลำดับ นำ MS-4 มาแยกหาองค์ประกอบทางเคมี ด้วยการแยกคอลัมน์โครมาโทกราฟฟี สารประกอบที่ได้เป็นสารกลุ่มคูมาริน โดยเมื่อนำสารดังกล่าวไปทดสอบฤทธิ์การต้านการอักเสบ โดยดูการยับยั้งเอนไซม์ไซโคออกซีจีเนส-ทู (cyclooxygenase-2) จากเซลล์ RAW 264.7 พบว่ามีค่า IC_{50} เท่ากับ 47.27±2.00 ไมโครกรัมต่อมิลลิลิตร โดยไม่มีความเป็นพิษต่อเซลล์

จากผลการทดลองพบว่า สารสกัดชั้น 95% เอทานอล (STK95) สารสกัดชั้น 50% เอทนอลของตำรับ (STK50) และสารสกัดชั้นน้ำ (STKW) มีฤทธิ์ต้านเชื้อแบคทีเรียและเชื้อราได้ในระดับหนึ่ง ส่วนสมุนไพรเดี่ยวในตำรับที่มีฤทธิ์ต้านเชื้อแบคทีเรียและเชื้อราได้ดีที่สุดคือ สารสกัดสารภีชั้น 95% เอทานอล นอกจากนี้ยังมีฤทธิ์ต่อการต้านการอักเสบได้ดีเช่นกัน ส่วนฤทธิ์การต้านฮอร์โมนเอสโตรเจนในสารสกัดทั้ง 3 ชั้นของตำรับ มีฤทธิ์ในการต้านฮอร์โมนเอสโตเจนได้ดีอีกด้วย จากผลการทดลองข้างต้นสามารถนำมาสนับสนุนการใช้ยาสตรีหลังคลอดในบัญชียาหลักแห่งชาติในหญิงหลัง

คลอด เพื่อลดอาการแทรกซ้อนหลังคลอด เช่น การติดเชื้อ การอักเสบ รวมทั้งช่วยเพิ่มการหลั่งน้ำนมในหญิงหลังคลอดอีกด้วย

คำสำคัญ: ฤทธิ์ต้านเชื้อจุลชีพ, ฤทธิ์ต้านการอักเสบ, ฤทธิ์ต่อฮอร์โมนเอสโตเจน, ตำรับยาสตรีหลังคลอด, การดูแลหญิงหลังคลอด

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Miss Khwanchanok Mokmued

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LIST OF ABBREVIATIONS AND SYMBOLS

Abbreviations / Symbols	**Terms**
ATCC	American type culture collection
BSA	Bovine serum albumin
$CHCl_3$	Chlorofrom
Cm	Centimeter
cm^3	Cubic centimeter
CO_2	Carbondioxide
conc.	Concentration
COX-1	Cyclooxygenase-1
COX-2	Cyclooxygenase-2
DI	Deionized water
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethy sulfoxide
e^-	Electron
EC_{50}	Concentration causing 50% effective activity
e.g.	Example gratia, for example
ELISA	Enzyme-linked immunosorbent assay
et al.	Et alii, a, d colleagues
etc.	Et cetera, amd other things
EtOH	Ethanol
EtOAc	Ethylacetate
FBS	Fetal bovine serum
g	Gram
g/l	Gram per liter
g/ml	Gram per milliliter
h	Hour
H_2O	Water

LIST OF ABBREVIATIONS AND SYMBOLS (CONTINUED)

Abbreviations / Symbols	Terms
HCl	Hydrochloric acid
IC_{50}	Concentration causing 50% inhibition effect
i.e.	Id est, that is
LPS	Lipopolysaccharide
m	Meter
M	Molar (concentration)
MBC	Minimal bactericidal concentration
MEM	Minimum essential medium eagle
MeOH	Methanol
mg	Milligram
MHA	Mueller Hinton Agar
MHB	Mueller Hinton Broth
MIC	Minimum inhibition concentration
min	Minute
ml	Millimeter
mm	Milliliter
MMC	Minimum microbacterial concentration
mg/ml	Milligram per milliliter
MTT	Thiazolyl blue tetrazolium bromide or 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-*2H*-tetrazolium bromide
NA	Nutrient agar
NaCl	Sodium chloride
$NaHCO_3$	Sodium bicarbonate
NaOH	Sodium hydroxide
ng	Nanogram

LIST OF ABBREVIATIONS AND SYMBOLS (CONTINUED)

Abbreviations / Symbols	Terms
ng/ml	Nanogram per milliliter
NI	No inhibition zone
nm	Nanometer
NO	Nitric oxide
NO_2^-	Nitrite
NOS	Nitric oxide synthase
O_2	Oxygen
°C	Degree celsius
OD	Optical density
PBS	Phosphate buffer saline
PGE2	Phostraglandin E2
PGEs	Phostraglandins
pH	Log concentration of (H^+)-1
pm	Picomolar
RAW 264.7	Murine macrophage leukemia
Rpm	Revolution per minute
RPMI-1640	Roswell Park Memorial Institute 1640
RPMI -PR	Roswell Park Memorial Institute-PR
SEM	Standard error of mean
TLC	Thin Layer Chromatography
UV	Ultraviolet
VLC	Vacuum Liquid Chromatography
w/v	Weight by volume
w/w	Weight by weight
%	Percent
>	More than
<	Less than

LIST OF ABBREVIATIONS AND SYMBOLS (CONTINUED)

Abbreviations / Symbols	Terms
=	Equal
/	Per
&	And
α	Alpha
β	Beta
γ	Gamma
µg	Microgram
µl	Microliter
µg/µl	Microgram per microliter
µM	Micromolar

CHAPTER 1
INTRODUCTION

1.1 General introduction

Life of Postpartum has changed both physically, mentally and socially. Postpartum has been known as a period of emotional well-being session. Medications, support, lifestyle modification and psychotherapy are very helpful and can prevent seriously life after pregnancy. After birth, the body of maternal will starts to recover and rest that have many physical to change. For example, vaginal has discharge called lochia that is the tissue and blood in uterus during pregnancy, hormone levels surge that the body begins to increase the volume of milk. After childbirth are a dangerous time for mother and newborn infant. The more than 500,000 women who died each year due to complications of pregnancy and childbirth. Infection and bleeding of maternal after childbirth was many maternal deaths. Suitable care in the after childbirth could prevent the great majority of these deaths. A Care in the period after birth is critical not only for survival but also to the future of mothers and newborn babies. This period that determine their well-being and potential for a healthy future after postpartum (WHO, 2008).

The studied before showed that many postpartum women suffering or symptoms of physical include perineal pain, wound infection, urinary tract problems, constipation, pyrexia from infection, pain from breast engorgement and emotional disorders such as postpartum blue and depression. While some of discomfortable could simple treated but others could become severe symptom if not identified or treated effectively, that leading toward a long-term debilitation, illness or death.

A Thai traditional medicine with folk doctors have been using to treat for woman after childbirth that has to lie by the fire called “Yoo-Fai”. The principle in Thai traditional medicine for Yoo-Fai is a processing to restore the health’s maternal after childbirth, the decline or deterioration of physical during pregnancy. Because during pregnancy, mother’s body will change which hormones and the circulatory system. These processing have many methods for mother to reactivate body such as a

massage for relaxing muscle, lying near an open fire for warmth the body, steam bath with herbal and “Tub-Mhor-Kluer” to treat the amniotic fluid which the uterus contraction normally.

For Thai traditional medicine, there has a remedy for postpartum to promote their health called "Sa-Tri-Lhang-Klod (STK)" which in the lists of The National List of Essential Herbal Medicines (NLEM). Its also used for postpartum care for excrete lochia, blood tonic and blood circulation. However, alternative medicine is now commonly used for postpartum care because generally, it is believed that herbal medicine is safe and has less side-effects than chemical medicine. The use of medicinal plants in women's health related conditions such as female fertility, birth control, pregnancy, childbirth, postpartum healthcare and lactation, including infant care, have been documented for various ethnic groups. In Thai traditional medicine in Thai National List of Essential Medicine, Sa-Tri-Lhang-Klod remedy was used to treat symptoms of women. It was used for treatment such as treating irregular menstruation, amenorrhea, stimulating blood circulation, menopause, postmenopausal, blood tonic, anti-inflammation in postpartum and astringent.

In the previous research showed that the 95% ethanolic extract of Sa-Tri-Lhang remedy has a good anti-inflammatory and antioxidant values by DPPH assay and good cytotoxicity against HeLa and MCF-7. And the 50% ethanolic of Sa-Tri-Lhang-Klod remedy exhibited little anti-inflammatory effect by the inhibition of NO production. (Inprasit, 2014). In Kabchoeng hospital, Surin province, Thailand studied the effect of postpartum herbal formula of Sa-Tri-Lhang-Klod remedy showed in the group that used Sa-Tri-Lhang-Klod remedy for care symptoms of after birth’s woman, there was a peritoneal wound, lactation, pain, breast engorgement and defecation got more health scores than the control group significantly at p-value 0.05. Morover in the group that used Sa-Tri-Lhang-Klod remedy was a change odor and color of amniotic fluid faster than the control group especially (Meepradit, 2011).

Sa-Tri-Lhang- Klod is used for care during postpartum phase (National List of Essential Medicines, 2013). Sa-Tri-Lhang-Klod consists of roots of *Maclura cochinchinensis* (Lour.) Corner, stems of *Artocarpus heterophyllus* Lam., rhizomes of *Curcuma comosa* Roxb., stems of *Caesalpinia sappan* Linn., vines of *Piper*

ribesioides Wall., roots of *Plumbago indica* L., flowers of *Piper longum* Linn., roots of *Angelica sinensis* (Oliv.) Diels, stems of *Salacia chinensis* Linn, fruits of *Piper nigrum* Linn., roots of *Piper samentosum* Linn, flowers of *Carthamus tinctorius* Linn., flowers of *Jasminum sambac* Ait., flowers of *Mimusops elengi* Linn, flowers of *Mesua ferrea* Linn., flowers of *Mammea siamensis* Kosterm., and flowers of *Nelumbo nucifera* Gaertn. The individual plants have long been used in Thai traditional medicine as stimulate blood circulation, blood tonic, anti-inflammation and astringent in postpartum (National List of Essential Medicines, 2013).

This remedy consists of seventeen plants. There is no report of anti-microbial activity, anti-inflammatory and anti-estrogenic activity of this preparation, but the previous studies reported that some herb and formula extracts showed cytotoxic activity, anti-oxidant and anti-inflammatory. Therefore, the objectives of this study were to investigate the antimicrobial activity, anti-inflammatory and anti-estrogenic activity of Sa-Tri-Lhang-Klod remedy extracts and each of its plant ingredients against three types of microbial such as *staphylococcus aureus, Escherichia coli* and *Candida albicans,* anti-inflammatory by cyclooxygenase-2 enzyme inhibition (Cox-2) and anti-estrogenic activity. These results will support the use of this remedy in Thai traditional medicine for infection and inflammation in postpartum.

1.2 Objectives

1.2.1 Overall aims

The overall aims of this research are to study the antimicrobial, anti-inflammatory and estrogenic effects of ethanolic and aqueous extracts of Sa-Tri-Lhang-Klod remedy and its plant ingredients related to postpartum care. And to isolate pure compound from *Mammea siamensis* Kosterm.

1.2.2 Specific aims

1.2.2.1 To investigate the antimicrobial activity of the ethanolic and aqueous extracts of Sa-Tri-Lhang-Klod remedy and its plant ingredients.

1.2.2.2 To investigate the anti-inflammatory activity on

cyclooxygenase-2 enzyme inhibition (cox-2) induced by lipopolysacaccharide in RAW 264.7 cell lines of ethanolic and aqueous extracts of Sa-Tri-Lhang-Klod remedy and its plant ingredients.

1.2.2.3 To investigate the anti-estrogenic effect activity of the ethanolic and aqueous extracts of Sa-Tri-Lhang-Klod remedy and its plant ingredients.

1.2.2.4 To isolate compound from *Mammea siamensis* Kosterm.

CHAPTER 2
REVIEW OF LITERATURE

2.1 Defining of Postpartum Period

The “postpartum” and “postnatal” are sometimes used interchangeably. The postpartum period (also called the puerperium) definitions after the birth of the placenta. Usually a timing about one hour after that moment is part of childbirth; during that time the immediate care of the mother and the infant take place, as described in the WHO reports care in normal birth and Essential newborn care. There is a smooth transition between childbirth and the postpartum period. “Postpartum period” or “puerperium” are officially defined. However, WHO has mean the first 28 days after childbirth as the neonatal period. The postpartum period is intended to end 6 weeks after birth. In many countries, where often the first 40 days. After birth are considered a time of recovery for the mother and her newborn infant. After delivery about six weeks, the body of the woman will have returned to the normally as well as the non-pregnant state (WHO, 1996).

The postpartum period has been a critical changing time for a woman, newborn and family that include a physiological, emotional and social. Poor quality care reduces opportunities for health promotion and for the early detection and adequate management of problems and disease.

2.2 Maternal Health

Pre-pregnant section has regained the uterus and vagina. During pregnancy has physiological changing, such as increased cardiac output and blood volume, increased extracellular fluid and changes in the composition of the blood. The rapid disappearance of placental hormones after delivery, and the begin of lactation have caused harsh endocrinological changes in the first weeks, but after six weeks a steady state has been acquired. However, the pre-pregnant state has completely returned: lactation usually continues, often the menstrual cycle has not yet

normalized, and sexual activity may not have been resumed yet. Contraception, though an important need, may be problematic for many couples at this time. For the infant, the age of six weeks is not a decisive turning point in his or her life, but the continuation or discontinuation of breastfeeding is directly related to the social and economic activities of the mother and her choice of contraceptive method. Although in this report attention is mainly focused on the first six weeks postpartum, it is fully recognized that the life of the woman and her baby is a continuum, and discussions will be extended to the following weeks and months where appropriate.

2.3 Problem of Postpatum Women

2.3.1 Infections

Urinary tract infections frequently occur during the postpartum period. During pregnancy stasis in the urinary tract and asymptomatic bacteriuria contribute to the occurrence of infections, during labour the bladder is sometimes catheterized, and finally urinary retention postpartum predisposes to infection. Cystitis and pyelonephritis occur in the puerperium, and should be treated adequately by antibiotics. The task of the caregiver is to diagnose and treat a urinary tract infection in time. One of the diagnostic tools is measuring the body temperature: fever is often a sign of genital infection, but sometimes it indicates the onset of pyelonephritis.

Factors for puerperal genital infections are prolonged labour, prelabour rupture of the membranes, frequent vaginal examination, internal (vaginal) electronic fetal monitoring and caesarean section (Gibbs 1980). Although caesarean section is the most important risk factor the role of poorly observed rules of cleanliness and an unhygienic environment should not be overlooked. In Caesarean section the risk of genital infection is clearly increased compared to vaginal delivery (Gibbs 1980, Simpson 1988). The most important causative agents of genital infections are *E. coli*, *streptococci*, anaerobic microorganisms like Bacteroides, and gonococci. *Chlamydia trachomatis* often causes a genital infection with relatively mild symptoms, but later a localized peritonitis may occur, with perihepatitis and obstructed fallopian tubes. The clinical picture of genital infections is fairly uniform. Fever (temperature >38.0°C) is

the main clinical symptom. Often no other symptoms are present, and a source of infection cannot be found. Sometimes the uterus is tender. Elevated temperature (>38°C) during labour ("chorioamnionitis") is always an alarming sign and is often followed by serious postpartum infections. In the literature fever after exclusion of other causes is generally considered to be the most important criterion of endometritis, or probably more correctly, metritis (Cunningham et al 1997). The treatment of (endo)metritis recommended in the literature and by WHO is antibiotics and, if required by the general condition, referral to the first referral level (WHO 1995d).

One of the most dangerous causative agents of puerperal sepsis and the concomitant maternal mortality is the Group A Streptococcus (GAS) or Streptococcus pyogenes. It was the main cause of childbed fever in Europe in the 19th century. Since then the virulence of GAS seems to have diminished, but in recent years a new period of increased virulence has arrived (Gaworzewska & Colman 1988, Swingler *et al* 1988).

2.3.2 Perineal Pain

In Egypt 2.1% of the women reported dyspareunia after childbirth. Sleep & Grant (1987) also found that 15% of women experienced dyspareunia up to three years after a normal delivery. In the Grampian study perineal pain was reported in 22% after 8 weeks, and in 10% after 2-18 months. This long-term complaint after >2 months was present in 4% of multiparae and in 16% of primiparae; in only 7% after a spontaneous vaginal delivery, and in 30% after assisted vaginal deliveries. Sleep (1984) found that 8% of women experienced perineal pain 12 weeks after a normal delivery (compared with 7% after >2 months in the Grampian study).

2.3.3 Breast Problems

In the Grampian study 33% of all women experienced breast problems in the first 2 weeks postpartum, and 28% in the weeks thereafter. This may be an underestimation, because some of the women may have considered these problems as baby feeding problems. Apart from overt mastitis, a relatively rare condition, these problems may have comprised engorgement, and sore, cracked, bleeding or inverted nipples. Breast problems are often cited as the reason for stopping breastfeeding, and

breastfeeding rates might improve if effective care could be given for these problems. The majority of such problems can be prevented by routines and practices which support breastfeeding, and skilled help to establish breastfeeding in the early postpartum period.

2.4 General data of remedy called Sa-Tri-Lhang-Klod

Sa-Tri-Lhang-Klod, a multi-herbal Thai traditional medicine preparation, is used for care during postpartum phase (National List of Essential Medicines, 2013). Sa-Tri-Lhang-Klod remedy comprises 17 herbs in 2 sections, first section has double the herb weight (10 g) of second section (5 g). The first section consists of roots of *Maclura cochinchinensis* (Lour.) Corner, stems of *Artocarpus heterophyllus* Lam., rhizomes of *Curcuma comosa* Roxb., stems of *Caesalpinia sappan* Linn., vines of *Piper ribesioides* Wall., roots of *Plumbago indica* L., flowers of *Piper longum* Linn., roots of *Angelica sinensis* (Oliv.) Diels, stems of *Salacia chinensis* L. And second section consists of fruits of *Piper nigrum* Linn., roots of *Piper samentosum* L., flowers of *Carthamus tinctorius* Linn., flowers of *Jasminum sambac* Ait., flowers of *Mimusops elengi* L., flowers of *Mesua ferrea* Linn., flowers of *Mammea siamensis* Kosterm., and flowers of *Nelumbo nucifera* Gaertn. The individual plants have long been used in Thai traditional medicine as stimulate blood circulation, blood tonic, anti-inflammation and astringent in postpartum (National List of Essential Medicines, 2013).

2.5 Biological activities of Sa-Tri-Lhang- Klod remedy and its ingredients

2.5.1 *Angelica sinensis* (Oliv.) Diels (UMBELLIFERAE)

Figure 2-1 *Angelica sinensis* (Oliv.) Diels (UMBELLIFERAE)
(https://www.samunpri.com)

Common names: Kot-chiang (Thai)

Family: UMBELLIFERAE

Botanical characteristics:

Angelica sinensis (Oliv.) Diels is a herbaceous plant, root of *Angelica sinensis* (Oliv.) Diels as well-known Chinese herbal medicine. There are the perennial herb (80–150 cm), leaves tridigitato-pinnate divided, petioles expand tubular sheath, flowers white compound umbel, fruit longelliptic lateral angular with wide wings (How, 1982).

Part used: Root

Chemical constituents:

The chemical constituents such as alkyl phthalides (ligustilide, 6,7epoxyligustilide, angelicide, butylidenephthalide, butylphthalide, 2,4 dihydrophthalicanhydride), terpenes (β-cadinene, carvacrol and cis-β-ocimene); phenylpropanoids (ferulic acid, coniferyl ferulato); benzenoids (valerophenone-o-carboxylic acid and vanillic acid); and coumarins (angelol G, angelicone and umbelliferone) have been isolated and identified from *Angelica sinensis* (Oliv.) Diels (Hon *et al*., 1990; Dung *et al*., 1996; Lin *et al*., 1998). Polysaccharide fractions of low relative molecular mass have also

been reported (Wang and Zhu, 1996; Chen *et al*., 2001). Its main essential component, Z-ligustilide, other phthalides and ferulic acid arethe biologically active components of *A. sinensis*.

Traditional used:

The root of *Angelica sinensis (*Oliv.) Diels (Apiaceae), known as Dong quai in Chinese, is one of the most important traditional Chinese medicines, used for tonifying the blood and treating female menstrual disorders (dysmenorrhea, amenorrhea, irregular menstruation) and also menopausal symptoms (Zhu, 1987). It is also used for the treatment of anemia, hypertension, chronic bronchitis, asthma, rheumatism and cardiovascular diseases (Hardy, 2000).

2.5.2 *Artocarpus heterophyllus* Lam. (MORACERE)

Figure 2-2 *Artocarpus heterophyllus* Lam. (MORACERE)

(http:// qsds.go.th/colorsilkDb/show/knowledgeshow.com)

Common names: Khanun (Thai)

Family: MORACERE

Botanical characteristics:

Artocarpus heterophyllus Lam. is one of the most significant trees in tropical homegardens and perhaps the most widespread and useful tree in the important genus Artocarpus. It is a medium-size evergreen tree typically reaching 8–25 m (26–82 ft) in height that is easily recognized by its fruit, the largest among cultivated plants. The succulent, aromatic, and flavorful fruit is eaten fresh or preserved in myriad ways (Baliga *et al*., 2011).

Part used: Stem

Chemical constituents:

The studies of *Artocarpus heterophyllus* Lam. have shown that contains many classes of compounds such as carotenoids, flavonoids, volatile acids sterols and tannins, and that their concentration changes with the variety (Arung, Shimizu, & Kondo, 2007; Chandrika, Jansz, & Warnasuriya, 2004; Ong *et al*., 2006). The bark from the stem also contains betullic acid and two new flavone pigments, cycloheterophyllin, triterpenic compounds like cycloartenyl acetate, cycloartenone, heterophylol and tannin (Barik, Bhaumik, & Kundu, 1997; Prakash *et al*., 2009). The leaves and stem are also reported to contain sapogenins, cycloartenone, cycloartenol, β-sitosterol and tannins (Prakash *et al*., 2009).

They have three flavonoids: artocarpanone, artocarpin and cycloartocarpin in extracts from A. heterpohyllus heartwoods (Septama and Panichayupakaranant, 2016).

Traditional used:

Artocarpus heterophyllus Lam. is of great importance in the various folk and traditional system of medicine in Asia. Reports suggest that almost all parts of the jackfruit tree are of use in the preparations of various Ayurvedic and Yunani medicines (Saxena, Bawa, & Raju, 2009). The decoction of seeds or bark is supposed to help in digestion while ripe fruits may be used as a natural laxative (Hossain & Nath, 2002). An ash produced by burning bark is supposed to heal abscesses and ear problems (Gupta & Tandon, 2004).

2.5.3 *Caesalpinia sappan* Linn. (LEGUMINOSAE)

Figure 2-3 *Caesalpinia sappan* Linn. (LEGUMINOSAE)

(http://www.kasetorganic.com/wp-content/uploads/2013/11/fang-1.jpg.)

Common names: Fang-Sen (Thai)

Family: LEGUMINOSAE

Botanical characteristics:

Caesalpinia sappan L. is a plant of Leguminosae family, commonly known as Brazil or Sappan wood and distributed in Southeast Asia and its dried heartwood has been used as traditional ingredient of food or beverages (Toegel *et al*., 2012). The important part of *Caesalpinia sappan* L. is the heartwood which is pale red, hard, heavy with even and fine structure (Saenjum *et al*., 2010).

Part used: Stem

Chemical constituents:

Chemical constituent's investigation of sappan wood resulted in the various structural types of phenolic components including xanthone, coumarin, chalcones, flavones, homoisoflavonoids, and brazilin *etc*. Brazilin is the major compound naturally occurring in the CS heartwood and is used as a red dye for histological staining (Bae, 2005).

Traditional used:

The heartwood is traditionally used in Indian Ayurveda and Chinese folk medicine. In Thailand, it is mostly used as coloring agent in beverage, food, garment and cosmetics (Saenjum *et al*., 2010). A decoction of heartwood is used in Namya-utai solution which has anti-thirst and cardiotonic properties. In Northern Thailand, especially in Chiang Mai, Nan and Lampang province, *Caesalpinia sappan* L.

heartwood decoction is used as anti-inflammatory agent for the treatment of traumatic disease and arthritis. The Northern Thai community has a long history of using decoction of *Caesalpinia sappan* L. heartwood for local consumption including health promotion and disease treatment. In Ayurveda, the heartwood is used for vitiated conditions of pitta which includes skin rashes, burning sensations, peptic ulcers, excessive body heat, heartburn and indigestion. It also used as blood purifier and in treatment of wounds, diarrhea, epilepsy, diabetes etc. *Caesalpinia sappan* L. heartwood is also used to reduce pain and swelling caused by external injuries and improvement of complexion (Srilakshmi, Vijayan& Raj, 2010). In traditional Chinese medicine, brazilin is used for treatment of increased blood circulation, promotes menstruation and exhibit analgesic and anti-inflammatory potentials (China Pharmacopoeia Commission, 2010). Brazilin have been reported to possess various biological activities including antibacterial, anti-inflammatory, anti-allergic and antioxidant (Xu&Lee, 2004).

2.5.4 *Carthamus tinctorius* Linn. (COMPOSITAE)

Figure 2-4 *Carthamus tinctorius* Linn. (COMPOSITAE)

(http://cdn.xl.thumbs.canstockphoto.com/canstock28903901.jpg)

Common names: Khamfoi (Thai)

Family: COMPOSITAE

Botanical characteristics:

Carthamus tinctorius Linn. is a highly branched, herbaceous, thistle-like annual plant. It is commercially cultivated for vegetable oil extracted from the seeds. Plants are 30 to 150 cm (12 to 59 in) tall with globular flower heads having yellow, orange, or red flowers. Each branch will usually have from one to five flower heads containing 15 to 20 seeds per head. Safflower is native to arid environments having seasonal rain.

Part used: Flower

Chemical constituents:

It is known that Oleic acid has desirable characteristics as frying stability and mild flavor, while the linoleic acid reduces the cholesterol level in the blood (Wilson *et al*., 2006). Additionally, the effects of safflower extracts as anticoagulant, antitumor, antihypertensive, antioxidant, neuroprotective, liver protectant, and inhibitor of melanin production (Fan *et al*., 2009).

Traditional used:

For a long time *C. tinctorius* has been used in traditional medicines as a purgative, analgesic, antipyretic and an antidote to poisoning. It is a useful plant in painful menstrual problems, post-partum hemorrhage and osteoporosis. In Thailand has used for rea to reduce blood cholesreol and anti-hypertention. (Arpornsuwan *et al*., 2010)

2.5.5 *Curcuma comosa* Roxb. (ZINGIBERACERE)

Figure 2-5 *Curcuma Comosa* Roxb. (ZINGIBERACERE)

(http:// www.dreamstime.com)

Common names: Wan-Chak-Motlook (Thai)

Family: ZINGIBERACERE

Botanical characteristics:

Curcuma comosa Roxb. (Zingiberales: Zingiberaceae) commonly known as Wan Chak Mod Look which mean uterus tightening, belongs to Zingiberaceae family, is used in folk medicine to relieve postpartum uterine inflammation (Suksamrarn *et al*., 2008).

Part used: Rhizome

Chemical constituents:

As part of our continuing chemical studies on Thai medicinal plants, six diarylheptanoids were isolated and their structures were characterized (Suksamrarn *et al*., 2008).

Traditional used:

Curcuma comosa Roxb. is an indigenous Thai herb which is usually used as a food ingredient but it is also used in traditional folk medicine for the treatment of uterine inflammation (Boonmee *et al*., 2011). *Curcuma comosa*, commonly known as ‘‘Waan Chak Mod Look” in Thai language, has been used as a food ingredient, to medically treat postpartum uterine bleeding and as an aromatic stomachic. This plant, and especially the rhizomes, has been found to harbour various biological properties, such as oestrogenic (Winuthayanon *et al*., 2009). That is an indigenous medicinal plant in the Zingiberaceae family which has traditionally been

used for treatment of postpartum uterine bleeding and inflammation in Thailand (Weerachayaphorn *et al*., 2010).

2.5.6 *Jasminum sambac* Ait. (OLEACERE)

Figure 2-6 *Jasminum sambac* Ait. (OLEACERE)

(http://www.rak-sukapap.com)

Common names: Ma-Li (Thai)

Family: OLEACERE

Botanical characteristics:

Jasmine (Jasminum) is a genus containing approximately 600 species of small trees and vines in the Family Oleaceae. These glabrous twining shrubs are widely cultivated in gardens and easily found in forests throughout tropical Asia and warm temperate regions in Europe and Africa (Kunhachan *et al*., 2012).

Part used: Flower

Chemical constituents:

Flowers yield an essential oil rich in linalool, benzyl acetate, *cis*-jasmone and sesquiterpene. Indole, pyridine and nicotine derivatives have been reported in this plant. Tetrameric iridoid glycoside, sambacosides A, E and F, are present in the leaves. The glycosides, β-primeveroside and β-rutinoside, have been isolated from the flower buds (Ghani, 2003).

Traditional used:

The plants are used for the treatment of insanity, weakness of sight and affections of the mouth. Roots are used as an emmenagogue. The dried leaves soaked in water are used as a poultice in indolent ulcers. The flowers act as a lactifuge; bruished flowers applied to breasts to arrest secretion of milk in puerperal state in cases of threatened abscess. It is used against indolent and Brest tumour. In khagrachari leaves are used for menstrual disorders (Yusuf *et al.,* 2009). EtOH (50%)

extract of aerial parts is CNS depressant and hypotensive. Leaf extract possesses antibacterial properties (Asolkar *et al*., 1992).

2.5.7 *Maclura cochinchinensis* (Lour.) Coener (MORACERE)

Figure 2-7 *Maclura cochinchinensis* (Lour.) Coener MORACERE

(http://www.dnp.go.th/botany.com)

Common names: Kaelae (Thai)

Family: MORACERE

Botanical characteristics:

Maclura cochinchinensis is a branched thorny shrub, scrambling or even becoming a climber. It produces stems up to 10 metres long, and up to 15cm in diameter, with long thorns on the nodes. A famous dye plant in Indonesia, where it has long tradition of use as in batik. The wood is gathered from the wild leading to the plant becoming rare in parts of its range due to over-exploitation (Sasaki *et al*., 1972).

Part used: Stem

Chemical constituents:

Phytochemical studies have reported the isolation of a number of xanthones, including tetraoxygenated xanthones with mono- or di-C5 derived substituents, 2 which show cytotoxicity, anti-microbial activity, and anti-inflammatory activity (Hou *et al*., 1992).

Traditional used:

M. cochinchinensis heartwood has been used in Thai traditional medicine for the treatment of chronic fever, skin infection and abnormality of the lymph node (Kasipan, 1979).

2.5.8 *Mammea siamensis* Kosterm (GUTTIFERAE)

Figure 2-8 *Mammea siamensis* Kosterm (GUTTIFERAE)

(http://kornpanot302.blogspot.com/2013/09/mammea-siamensis-kosterm.html.com)

Common names: Sa-Ra-Phi (Thai)

Family: GUTTIFERAE

Botanical characteristics:

Mammea siamensis (Miq.) T. Anders. (*Ochrocarpus siamensis* Anders.), a small, evergreen tree up to 15 m tall and 10-30 cm in diameter, is native to Myanmar, Thailand, Laos, Cambodia and Vietnam (Kostermans, 1961).

Part used: Flower

Chemical constituents:

The genus Mammea of the family Guttiferae is reported to contain various types of xanthones. Previously, two phenylcoumarins, 6-butyryl-5-hydroxy-4-phe- R, R2 R3 nylseselin and 6-butyryl-5,7-dihydroxy-8-(3,3-dime- 1 OCH3 H H thylallyl)-4-phenylcoumarin, were isolated from the flowers of *M. siamensis* (Thebtaranonth, 1981). Previous phytochemical studies on the flower of *M. siamensis* have led to the isolation of several unique coumarins, 3–5 some of which have potential pharmacological and therapeutic properties (Ngo *et al*., 2010).

Traditional used:

The flowers of this plant have been used for a heart tonic in Thai traditional medicine (Wuthithammawech, 1997).

2.5.9 *Mesua ferrea* Linn. (GUTTIFERAE)

Figure 2-9 *Mesua ferrea* Linn. (GUTTIFERAE)

(http://www.123rf.com/photo_37619040_indian-rose-chestnut-herb-on-white-background.html.com)

Common names: Bunnak (Thai)

Family: GUTTIFERAE

Botanical characteristics:

Mesua ferrea Linn. is a medium to large sized tree that can attain a height between 18 and 30 m, with reddish-brown to grey colored bark that peels off in thin flakes, the wood is extremely hard. The leaves are simple, lanceolate, acute, and leathery, covered in a waxy bloom below, red when young, oppositely arranged, 7 to 13 cm long by 2 to 4 cm wide. The flowers are white with a floral fragrance, up to 7.5 cm in diameter, with numerous golden-colored stamens shorter than the length of the petals, the style is twice as long as the stamens, borne singly or in pairs, axillary or terminal (Dassanayake, 1980).

Part used: Flower

Chemical constituents:

M. ferrea include mesuaferrone-A & B, mesuaferrol, mesuanic acid, amyrin and sitosterol present in the stamen while it is reported that seeds contain essential oils, xanthones and coumarins (Dennis *et al*., 1998)

Traditional used:

The Ayurvedic Pharmacopoeia of India recommends the use of the plant in gout, hemorrhagic disorders and diseases of the urinary bladder. *Mesua ferrea* Linn. is a rare plant which is traditionally being used for its antiseptic, anti-

inflammatory, blood purifier, anthelmintic, cardiotonic, diuretic, expectorant, antipyretic, purgative, antiasthmatic, antiallergic and several other effects (Chahar *et al*., 2013)**.**

2.5.10 *Mimusops elengi* L. (SOPOTACERE)

Figure 2-10 *Mimusops elengi* L. (SOPOTACERE)

(http://keys.trin.org.au/key-server/data/media/Html/taxon/Mimusops_elengi.htm.com)

Common names: Phikul (Thai)

Family: SOPOTACERE

Botanical characteristics:

Mimusops elengi L. belongs to the Saponaceae family. It is a large evergreen ornamental tree about 50 ft in height with white flowers and the corolla preserves its fragrance even after drying (Rout, 2010).

Part used: Flower

Chemical constituents:

The major compounds in ethanol extract and headspace of flowers were identified as p-methylanisole (1.33, 9.94%), methyl benzoate (3.82, 13.40%), methyl salicylate (1.88, 0.48%), 2-phenyl ethyl acetate (2.55, 7.16%), benzyl alcohol (1.75, 4.41%), 2-phenyl ethyl alcohol (38.79, 37.80%), nerolidol (3.19, 1.46%), methyl-E-cinnamate (3.63, 6.83%), 3-hydroxy-4-phenyl-2- butanoate (4.74, 2.85%), E-cinnamyl alcohol (13.72,0.85%) and 2-phenyl ethyl benzoate (1.13, 0.37%), respectively (Rout, 2010).

Traditional used:

The bark and fruits of this plant are used in the treatment of diarrhoea and dysentery. Rinsing mouth with bark decoction is believed to strengthen the gums, reduce inflammation, prevent bleeding of gums, and to stop bad breath caused by pyorrhoea and dental caries. Different parts of the plant have also been reported for

anti-microbial, anti-ulcer, anti-anxiety, anti-oxidant, anti-hyperglycaemic, anti-hyperlipidemic, anti-helminthic, anti-inflammatory and anti-pyretic properties (Purnima *et al*., 2010).

2.5.11 *Nelumbo nucifera* Gaertn. (NELUMBONACERE)

Figure 2-11 *Nelumbo nucifera* Gaertn. (NELUMBONACERE)

(http://www.uniprot.org/taxonomy/4432.com)

Common names: Bua luang (Thai)

Family: NELUMBONACERE

Botanical characteristics:

Nelumbo nucifera Gaertn. (Fam: Nymphaeaceae) is a large aquatic herb with stout creeping yellowish white colored rhizomes. Every part of the plant has distinct name and almost all parts are used medicinally supplying one or more drugs (Tomita *et al*., 1962)

Part used: Flower

Chemical constituents:

Germs and rhizomes of the lotus were focused on the antioxidant activity of phenolic compound, carotene, alkaloid and saponins (Yen *et al*., 2015).

Traditional used:

Every part of this plant is used; the roots are a popular food or are found in lists of ingredients for traditional Chinese cuisine; the seed, leaves, flowers, stamens, embryos, and rhizomes also have much more pharmacological effects, reported that *Nelumbo nucifera* is effective in treating antipyretic, anti-hypoglycemia and excretory system disease. (Mukherjee *et al*., 1996).

2.5.12 *Piper longum* Linn. (PIPERACERE)

Figure 2-12 *Piper longum* Linn. (PIPERACERE)

(http://www.daivivarnashram.com/index.php/ /pippalikhand.com)

Common names: Di-Pli (Thai)

Family: PIPERACERE

Botanical characteristics:

P. longum is a small shrub with a large woody root and numerous creeping, jointed stems that are thickened at the nodes. The leaves are alternate, spreading, without stipules and with blades varying greatly in size (Rastogi, 1993).

Part used: Fruit

Chemical constituents:

The fruit contains a large number of alkaloids and related compounds, the most abundant of which is piperine, followed by methyl piperine, pipernonaline, piperettine, asarinine. The essential oils of the fruit are a complex mixture. Excluding the volatile piperine, the three major components are caryophyllene, pentadecane (both about 17.8%), and bisaboline (11%). Others include thujone, terpinolene, zingiberene, p-cymene, pmethoxyacetophenone, dihydrocarveol, and vitamins A and E (Rastogi, 1993).

Traditional used:

In thai traditional medicine is used as a carminative, element tonic and antidiarrheal.

2.5.13 *Piper Nigrum* Linn. (PIPERACERE)

Figure 2-13 *Piper Nigrum* Linn. (PIPERACERE)

(http://hashmidawakhana.co.in/piper-nigrum-linn.html.com)

Common names: Prikthai (Thai)

Family: PIPERACERE

Botanical characteristics:

Piper nigrum L. (*P. nigrum*) is one of the best-known species of Piperaceae family. The plant will climb 20 or more feet, but for commercial purposes it is restricted to 12 feet. It is a perennial with a round, smooth, woody stem, with articulations, swelling near the joints and branched; the leaves are entire, broadly ovate, acuminate, coriaceous, smooth, with seven nerves; colour dark green and attached by strong sheath-like foot-stalks to joints of branches. Flowers small, white, sessile, covering a tubular spadix; fruits globular, red berries when ripe, and surface coarsely wrinkled (Kalu *et al*., 2011).

Part used: Fruits

Chemical constituents:

Piperine, which is identical in composition to morphia, volatile oil, a resin called Chavicin. Its medicinal activities depend mainly on its pungent resin and volatile oil, which is colourless, turning yellow with age, with a strong odour, and not so acrid a taste as the peppercorn; it also contains starch, cellulose and colouring. (Kalu *et al*., 2011).

Traditional used:

It is commonly used as a spice all over the world and also possesses pharmacological properties and thus used in traditional system of medicine, such as

Ayruvedic and Unani medicine for the treatment of various diseases such as fever, pain and inflammation (Doucette *et al*., 2013).

2.5.14 *Piper ribesioides* Wall. (PIPERACERE)

Figure 2-14 *Piper ribesioides* Wall. (PIPERACERE)

(http://www.jiaogulan4u.com/sakarn.html.com)

Common names: Sa-Kan (Thai)

Family: PIPERACERE

Botanical characteristics:

Piper ribesioides is an evergreen, climbing shrub producing very stout stems. The plant is sometimes gathered from the wild for local use as a medicine and food flavouring. It is said to be occasionally cultivated for its fruits, which contain an oil that is used as a spice.

Part used: Fruits

Chemical constituents: -

Traditional used:

In Thai traditional medicine, the stem is used as a carminative, antiflatulant and element tonic.

2.5.15 *Piper samentosum* Roxb. (PIPERACERE)

Figure 2-15 *Piper samentosum* Roxb. (PIPERACERE)

(http://www.canstockphoto.com/images-photos/roxb.html)

Common names: Cha-Phlu (Thai)

Family: PIPERACERE

Botanical characteristics:

Piper sarmentosum Roxb. (family Piperaceae) or locally known by the Thailand as Cha-Phlu. It is a glabrous, creeping terrestrial herbaceous plant with aromatic odour and pungent taste, and widely distributed in the tropical and sub-tropical regions of the world, such as the Asian and South-East Asia regions (Rukachaisirikul *et al*., 2004).

Part used: Leaves

Chemical constituents:

Phytochemical investigations of *Piper sarmentosum* Roxb. have led to the isolation of several classes of physiologically active compounds such as alkaloids, amides, pyrones, dihydrochalcones, flavonoids, phenylpropanoids, lignans and neolignans (Parmar *et al*., 1997).

Traditional used:

Piper sarmentosum Roxb. (Piperaceae), that fruits are used in Thailand as an expectorant (Pongboonrod, 1976).

2.5.16 *Plumbago indica* L. (PLUMBAGINACERE)

Figure 2-16 *Plumbago indica* L. (PLUMBAGINACERE)

(http://www.mpbd.info/plants/plumbago-indica.php.com)

Common names: Chettamun phloeng Daeng (Thai)

Family: PLUMBAGINACERE

Botanical characteristics:

A less branched rambling herb. Leaves oblong, attenuate and slightly obtuse upwards, short-cuneate at the base. Flowers bright red, 3-5 cm long, forming very long terminal and axillary slender, lax spikes, reaching up to 60 cm. Calyx red, short, cylindric, along the ribs covered with stipitate glands (Ghani, 2003).

Part used: Roots

Chemical constituents:

Root and bark contain a strong antimicrobial napthoquinone, plumbagin, sitosterol glycoside, tannin, glucose and organic acids. Aerial parts contain 6-hydroxyplumbagin, plumbagin, sitosterol, stigmasterol and campesterol. A new bi-naphthaquinone, roseanone and the known naphthaquinones, droserone, elliptinone and zucylanone, have been isolated from the roots and physcion-β-D-glucopyranoside (Ghani, 2003; Rastogi & Mehrotra, 1993).

Traditional used:

Roots are abortifacient; used in hepatitis, dyspepsia, flatulence, piles, leucoderma, leprosy and anasarca; locally as vesicant in rheumatism, paralytic affections and enlarged glands. Root contains an active principle “Plumbagin” which have got the antifertility properties (Yusuf *et al.,* 2009).

2.5.17 *Salacia chinensis* (CELASTRACERE)

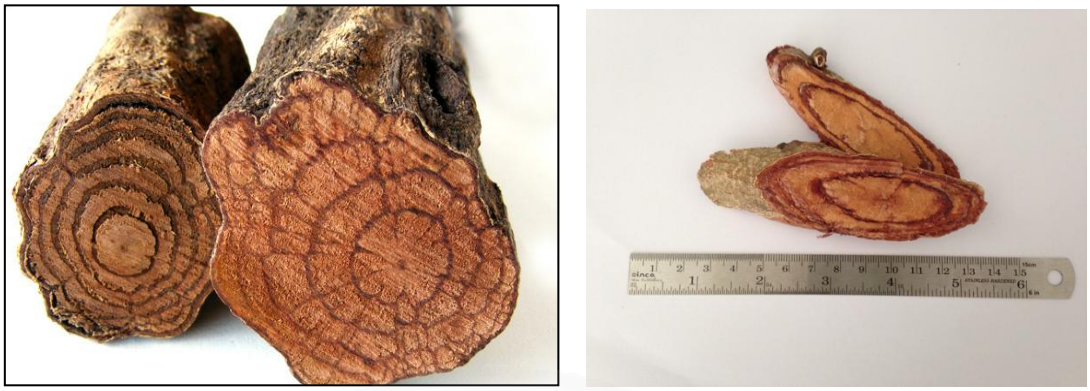


Figure 2-17 *Salacia chinensis* (CELASTRACERE)

(http://www.thaicrudedrug.com/main.php?action=viewpage&pid=21.com)

Common names: Kamphaeng Chet Chan (Thai)

Family: CELASTRACERE

Botanical characteristics:

Salacia chinensis L. which belongs to the family Celestraceae (spike-thorn family), is a small erect or straggling tree or large woody, climbing shrub (Deokate, 2012).

Part used: Stem

Chemical constituents:

S. chinensis roots have biologically active compounds such as triterpenes, phenolic compounds, glycosides and colouring agents which show various medicinal properties (Deokate, 2012).

Traditional used:

Root bark was used in the treatment of gonorrhoea, rheumatic, tonic, blood purifiers, amenorrhea, dysmonorrhoea, asthma, skin diseases and ear disease (Singh, 2010)

2.6 Biological activities of Sa-Tri-Lhang- Klod remedy and its ingredients

Table 2-1 Biological activities of *Angelica sinensis* (Oliv.) Diels

Scientific Name	Activity	Part of used/Bioactive compounds	Detail on biological	References
Angelica sinensis (Oliv.) Diels	Anti-inflammatory	Root	Exhibited anti-inflammatory activity on RAW 264.7 murine macrophage leukemia cells with an IC_{50} value of 12.52 µg/ml	Makchuchit *et al*., 2010
	Antimicrobial	Root	The ethanolic extract showed strong antibacterial activity at all tested concentrations (1.25, 2.5, and 5 µg/ml) to *Escherichia coli*, *Staphylococcus aureus*, and *Shigella Castellani* and Chalmers.	Han and Guo, 2012

Table 2-1 Biological activities of *Angelica sinensis* (Oliv.) Diels (continued)

Scientific Name	Activity	Part of used/Bioactive compounds	Detail on biological	References
Angelica sinensis (Oliv.) Diels (cont.)	Estrogenic Effect	Root	The extract of the root of *A. sinensis*, at a dose of 300 mg/kg, provoked a significant modification of the vaginal smear in 67% of the treated rats.	Circosta *et al*., 2006

Table 2-2 Biological activities of *Artocarpus heterophyllus* Lam.

Scientific Name	Activity	Part of used/Bioactive compounds	Detail on biological	References
Artocarpus heterophyllus Lam.	Anti-inflammatory	Artocarpesin, Norartocarpetin and Oxyresveratrol	That bioactive compounds decreased the LPS (1 μg/ml)-induced generation of nitric oxide in the RAW 264.7 cells and the effect was dose dependent (0–50 μM). The optimal effect was observed for artocarpesin followed by norartocarpetin and oxyresveratrol and at none toxic concentrations.	Fang *et al*., 2008

Table 2-2 Biological activities of *Artocarpus heterophyllus* Lam. (continued)

Scientific Name	Activity	Part of used/Bioactive compounds	Detail on biological	References
Artocarpus heterophyllus Lam. (cont.)	Anti-inflammatory	Artocarpesin	Artocarpanone also exhibited significant inhibitory effect on lipopolysaccharide-induced production of NO and iNOS protein expression in RAW 264.7 cells with an IC_{50}=35.9 µg/ml	Wei *et al*., 2005

Table 2-2 Biological activities of *Artocarpus heterophyllus* Lam. (continued)

Scientific Name	Activity	Part of used/Bioactive compounds	Detail on biological	References
Artocarpus heterophyllus Lam. (cont.)	Anti-inflammatory	leaves	In the disc diffusion, the aqueous extract possessed the highest inhibitory activity against *S. aureus* (15 mm), *E. faecalis* (14 mm), *S. typhimurium* (13 mm) and *E. coli* (11.3 mm), while the ethyl acetate fraction showed the highest inhibition against *S. enterica* (13 mm) and the aqueous fraction against *L. monocytogenes* (15 mm).	Loizzo *et al*., 2010

Table 2-3 Biological activities of *Caesalpinia sappan* Linn.

Scientific Name	Activity	Part of used/Bioactive compounds	Detail on biological	References
Caesalpinia sappan Linn.	Anti-inflammatory	Photosappanin A deoxysappanchalcone	Photosappanin A and 3 - doxysappanchalcone showed strong inhibitory activities toward the LPS-induced NO production in macrophage RAW.264.7 cells, with IC_{50} = 12.5 and 8.1 µg/ml	Min *et al*., 2012
	Antimicrobial	Stem	The ethanolic extract against *Pseudomonas aeruginosa* (34 mm), *Staphylococcus aureus* (31 mm), *Candida albicans* (20 mm), Escherichia coli (15 mm),	Srinivasan, 2012

Table 2-3 Biological activities of *Caesalpinia sappan* Linn. (continued)

Scientific Name	Activity	Part of used/Bioactive compounds	Detail on biological	References
Caesalpinia sappan Linn. (cont.)	Antimicrobial	Stem	against *Aspergillus niger* (14 mm)	Srinivasan, 2012

Table 2-4 Biological activities of *Jasminum sambac* Ait.

Scientific Name	Activity	Part of used/Bioactive compounds	Detail on biological	References
Jasminum sambac Ait.	Antimicrobial	Flowers	They against *Escherichia coli* (MTCC-443) strain. The determined minimum inhibitory concentration (MIC) by tube dilution technique. They reported that MIC of 31.25, 7.8, 15.62, and 15.62 μL/mL for natural oil, synthetic oil, synthetic blend, and linalool, respectively.	Rath *et al*., 2008

Table 2-5 Biological activities of *Curcuma comosa* Roxb.

Scientific Name	Activity	Part of used/Bioactive compounds	Detail on biological	References
Curcuma comosa Roxb.	Anti-inflammatory	Rhizomes	The concentrations of 0.1, 0.5 and 1 μM significantly decreased LPS-induced NO and PGE_2 production in a concentration-dependent manner. Parallel to the decreases in NO and PGE_2 production was a reduction in the expression of inducible NO synthase (iNOS) and cyclooxygenase 2 (COX-2) as measured by mRNA and protein levels.	Thampithak *et al*., 2009

Table 2-5 Biological activities of *Curcuma comosa* Roxb. (continued)

Scientific Name	Activity	Part of used/Bioactive compounds	Detail on biological	References
Curcuma comosa Roxb. (cont.)	Estrogenic Effect	Rhizomes	The hexane extract of *C. comosa* rhizomes was found to have an estrogenic-like action in rats.	Piyachaturawat *et al*., 1995

Table 2-6 Biological activities of *Mammea siamensis* Kosterm

Scientific Name	Activity	Part of used/Bioactive compounds	Detail on biological	References
Mammea siamensis Kosterm	Anti-inflammatory	Flowers	The 95% ethanolic extract show anti-inflammatory effect by inhibition NO production (IC_{50}=74.62±8.77µg/ml).	Makchuchit, 2010
	Antimicrobial	Flowers	The 95% ethanolic extract inhibited *S. aureus* with inhibition zone value of 10 mm. by disc diffusion, MIC value of 1.25 mg/ml and MBC value 1.25 mg/ml.	Sattaponpan, 2011

Table 2-7 Biological activities of *Mesua ferrea* Linn.

Scientific Name	Activity	Part of used/Bioactive compounds	Detail on biological	References
Mesua ferrea Linn.	Anti-inflammatory	Flowers	The 95% ethanolic extract show anti-inflammatory effect by inhibition NO production (IC_{50}=26.23±3.42µg/ml).	Makchuchit, 2010

Table 2-8 Biological activities of *Mimusops elengi* Linn

Scientific Name	Activity	Part of used/Bioactive compounds	Detail on biological	References
Mimusops elengi Linn	Antimicrobial	Flowers	The 95% ethanolic extract inhibited *S. aureus* with inhibition zone value of 9 mm. by disc diffusion, MIC value of 0.62 mg/ml and MBC value 0.62 mg/ml.	Sattaponpan, 2011
	Anti-inflammatory	Flowers	The 95% ethanolic extract show anti-inflammatory effect by inhibition NO production (IC_{50}=69.24±5.30 µg/ml).	Makchuchit, 2010

Table 2-9 Biological activities of *Nelumbo nucifera* Gaertn.

Scientific Name	Activity	Part of used/Bioactive compounds	Detail on biological	References
Nelumbo nucifera Gaertn.	Antimicrobial	Flowers	The aqueous extract inhibited *S. aureus* with inhibition zone value of 8 mm. by disc diffusion, MIC value of 5 mg/ml and MBC value 10 mg/ml.	Sattaponpan, 2011
	Antimicrobial	Flowers	The 95% ethanolic extract inhibited S. *aureus* with inhibition zone value of 9 mm. by disc diffusion, MIC value of 10 mg/ml and MBC value 10 mg/ml.	Sattaponpan, 2011

Table 2-10 Biological activities of *Piper longum* Linn.

Scientific Name	Activity	Part of used/Bioactive compounds	Detail on biological	References
Piper longum Linn.	Anti-inflammatory	Fruits	A marked anti-inflammatory activity of *P. longum* fruit decoction has been reported using carrageenaninduced rat edema.	Kumar *et al*., 2005

Table 2-11 Biological activities of *Piper Nigrum* Linn.

Scientific Name	Activity	Part of used/Bioactive compounds	Detail on biological	References
Piper Nigrum Linn.	Antimicrobial	Fruits	Petroleum ether and ethyl acetate extracts of *P. longum* were found to exert antimicrobial effects against various microorganisms.	Ali *et al*., 2007
	Anti-inflammatory	Fruits	The ethanolic extract inhibited LPS-actived NO production in RAW 264.7 cells (IC_{50} = 32.0 µg/ml)	Kakatum *et al*., 2011

CHAPTER 3
RESEARCH METHODOLOGY

3.1 Materials

3.1.1 Plant materials

Sa-Tri-Lhang-Klod is a multi-herbal Thai traditional medicine preparation that comprises 17 herbs. In Table 3-1 shown Thai name, part used and voucher specimens that were obtained at the herbarium of Southern Center of Thai Medicinal Plants at Faculty of Pharmaceutical science, Prince of Songkla University, Songkhla, Thailand.

Table 3-1 Plant of Sa-Tri-Lhang remedy

Botanical name	Family	Plant collected from	Voucher specimen number	Part of used
Angelica sinensis (Oliv.) Diels	UMBELLIFERAE	China	SKP199010901	Root
Artocarpus heterophyllus Lam.	MORACEAE	NakhonRatchasima	SKP117010801	Stem
Caesalpinia sappan Linn.	LEGUMINOSAE	Bangkok	SKP098031901	Stem
Carthamus tinctorius Linn.	COMPOSITAE	Chiang Mai	SKP051032001	Flower
Curcuma comosa Roxb.	ZINGIBERACEAE	Phetchabun	SKP201030301	Rhizome
Jasminum sambac Ait.	OLEACEAE	NakhonPathom	SKP129101901	Flower
Maclura cochinchinensis (Lour.) Corner.	MORACEAE	PrachuapKhiri Khan	SKP117130301	Stem
Mammea siamensis Kosterm.	GUTTIFERAE	Ratchaburi	SKP083131901	Flower
Mesua ferrea Linn.	GUTTIFERAE	Ratchaburi	SKP083130601	Flower

Botanical name	Family	Plant collected from	Voucher specimen number	Part of used
Mimusops elengi Linn.	SAPOTACEAE	Ratchaburi	SKP171130501	Flower
Piper longum Linn.	PIPERACEAE	Chanthaburi	SKP146160301	Fruit
Piper Nigrum Linn.	PIPERACEAE	Chanthaburi	SKP146161401	Fruit
Piper ribesioides Wall.	PIPERACEAE	Sakhonnakhon	SKP146161801	Stem
Piper samentosum Linn.	PIPERACEAE	Ratchaburi	SKP146161901	Root
Plumbago indica Linn.	PLUMBAINACEAE	Bangkok	SKP148160901	Root
Salacia chinensis Linn.	CELASTRACEAE	Ratchaburi	SKP044190301	Stem

3.2 List of chemicals and reagents used in the study

3.2.1 Preparation of plant extracts

95% ethanol, commercial grade (C.M.J. Anchor company, Thailand)

Distilled water (Milford, USA)

3.2.2 Antimicrobial activity

3.2.2.1 Disc diffusion method

Dimethyl sulfoxide [$(CH_3)_2SO$] (DMSO) (RCI Labscan, Thailand)

Distilled water (Milford, USA)

Mueller Hinton Agar (Difco, USA)

Mueller Hinton Broth (Difco, USA)

Nutrient Agar (Difco, USA)

Sabouraud Dextrose Agar (Difco, USA)

3.2.2.2 Microtiter plated-based assay

Dimethyl sulfoxide [$(CH_3)_2SO$] (DMSO)	(RCI Labscan, Thailand)
Distilled water	(Milford, USA)
Mueller Hinton Agar	(Difco, USA)
Mueller Hinton Broth	(Difco, USA)
Nutrient Agar	(Difco, USA)
Resazurin sodium salt	(Sigma-Aldrich, USA)
Sabouraud Dextrose Agar	(Difco, USA)

3.2.3 *In vitro* anti-inflammatory assay

3.2.3.1 COX-2 inhibitory effects

Dimethyl sulfoxide [$(CH_3)_2SO$] (DMSO)	(RCI Labscan, Thailand)
Distilled water	(Milford, USA)
Fetal bovine serum (FBS)	(Biochem, Germany)
Hydrochloric acid (HCl)	(Univar, Australia)
Isopropanol	(RCI labscan, Thailand)
Lipopolysaccharide from *E. coli* (LPS)	(Sigma, USA)
N-(1-Naphthyl) ethylenediamine dihydrochloride	(Sigma, USA)
Penicillin-Streptomycin (P/S)	(Sigma, USA)
Phosphate buffered saline (PBS)	(Amresco, USA)
Phosphoric acid 85% (H_3PO_4)	(Sigma, USA)
Prednisolone $\geq$ 90%	(Sigma, USA)
RPMI medium 1640	(Gibco, USA)
Sodium bicarbonate ($NaHCO_3$)	(BHD, England)
Sodium hydroxide (NaOH)	(Univar, Australia)
Sulfanilamide ($H_2NC_6H_4SO_2NH_2$)	(Sigma, USA)
Trypan blue 0.4%	(Gibco, USA)
Trypsin-EDTA	(Gibco, USA)

3.2.3.2 MTT assay

Thiazolyl blue tetrazolium bromide (MTT)	(Sigma, USA)

3.2.4 Estrogenic activity assay

Dimethyl sulfoxide [$(CH_3)_2SO$] (DMSO)	(RCI Labscan, Thailand)
Distilled water	(Milford, USA)
Fetal bovine serum (FBS)	(Biochem, Germany)
Isopropanol	(RCI labscan, Thailand)
N-(1-Naphthyl) ethylenediamine dihydrochloride	(Sigma, USA)
Penicillin-Streptomycin (P/S)	(Sigma, USA)
Phosphate buffered saline (PBS)	(Amresco, USA)
Phosphoric acid 85% (H_3PO_4)	(Sigma, USA)
RPMI medium 1640 (-PR)	(Gibco, USA)
Sodium bicarbonate ($NaHCO_3$)	(BHD, England)
Sodium hydroxide (NaOH)	(Univar, Australia)
Sulfanilamide ($H_2NC_6H_4SO_2NH_2$)	(Sigma, USA)
Thiazolyl blue tetrazolium bromide (MTT)	(Sigma, USA)
Trypan blue 0.4%	(Gibco, USA)
Trypsin-EDTA	(Gibco, USA)

3.2.5 Phytochemical investigation of compound isolated

3.2.5.1 Column chromatography

Hexane, $(CH_3(CH_2)_4CH_3)$ Analytical grade	(RCI Labscan, Thailand)
Chloroform, $(CHCl_3)$ Analytical grade	(RCI Labscan, Thailand)
Methanol, (CH_3OH) Analytical grade	(RCI Labscan, Thailand)
Ethyl acetate, $(CH_3COOC_2H_5)$ Analytical grade	(RCI Labscan, Thailand)
Acetone, (CH_3COCH_3)	(RCI Labscan, Thailand)
Silica Gel 60 (0.063-0.200 mm)	(Merck, Germany)
Silica Gel 60 (0.040-0.063 mm)	(Merck, Germany)
TLC silica gel 60 F254	(Merck, Germany)
Anisaldehyde, $(C_8H_8O_2)$	(Fluka, Switzerland)
Sulfuric acid, (H_2SO_4)	(Merck, Germany)

3.3 List of Instruments used in the studies

75 cm^2 plastic tissue culture flasks	(Costar Corning, USA)
25 cm^2 plastic tissue culture flasks	(Costar Corning, USA)
96-well plate flat, bottom with lid	(Costar Corning, USA)
96-well plate flat, bottom without lid	(Costar Corning, USA)
Analytical balance	(Mettler Toledo, Switzerland)
Autoclave	(Hirayama, Japan)
Biomedical freezer	(Thermo Scientific, USA)
Buchner Funnel	(Schott Duran, Germany)
Centrifugation	(Beckman Coulter, USA)
Centrifugation	(Boeco, Germany)
Centrifuge tube 15, 50 mL	(Costar Corning, USA)
CO_2 humidified incubator	(Shel lab, USA)
Column (5.5×90 cm, glass)	(Becthai, Thailand)
Cryogenic tube 2 mL	(Costar Corning, USA)
Densitometer	(Grant, United Kingdom)
Disposable pipette 1, 2, 5, 10, 25 mL	(Costar Corning, USA)
Environmental chamber	(Termaks, Norway)
Eppendroff	(Costar Corning, USA)
Filter paper (125 mm ∅)	(Whatman, USA)
Filter unit (0.22 µm, radio-sterilized)	(Millipore, Ireland)
Freezer	(Sanyo, Japan)
Glass bottles	(Schott Duran, Germany)
Glasswares	(Schott Duran, Germany) (Pyrex, USA)
Haemocytometer	(Boeco, Germany)
UV-vis detector SpectroMonitor 4100	(LDC Analytical, USA)
Hot air oven	(Memmert, Germany)
Hot plate	(Thermolyne, USA)
Incubated tabletop orbital shaker	(Thermo Scientific, USA)

Inverted microscope	(Nikon, USA)
Laminar air flow	(Boss tech, Thailand)
Liquid nitrogen tank	(Taylor-Wharton, USA)
Lyophilizer	(Lyophilization Systems Inc, USA)
Membrane filters with pore-size ratings of 0.22 microns	(Sartorius, Germany)
Micropipettes 1-20 μL, 1-200 μL, 100-1000 μL	**(**Gilson, USA)
Microplate reader	(Bio Tek, USA)
Multi-channels pipette	(Costar Corning, USA)
Paper discs (0.6 cm diameter)	(Whatman, USA)
Petri dish	(Biomed, Thailand)
pH buffer	(Thermo Scientific, USA)
pH meter	(WTW inolab, Germany)
Pipette tips	(Costar Corning, USA)
Pipette boy	(Integra biosciences, Switzerland)
Reagent reservoir (Sterile)	(Costar Corning, USA)
Rota evaporator	(Buchi, Switzerland)
Sonicator	(Elma, Germany)
Syringe	(Nipro, Thailand)
Syringe filters	(Nipro, Thailand)
Syringe Filter Nylon (13 mm, 0.45 um)	(GAT, Thailand)
Vacuum pump	(Rocker, Taiwan)
Vortex mixer	(Scientific industries, USA)
Water bath	(Memmert, Germany)
Water purification machine	(Elga, United Kingdom)

3.4 Methods

3.4.1 Preparation of crude extracts

The parts of these plants were weighed and mixed to Sa-Tri-Lhang-Klod remedy follow to NLEM and extracts were obtained by two methods, that is maceration and decoction. The crude extracts of each plant be also achieved from Miss Janjira Inprasit and were kept in freezer (-20 ^{o}C) until use.

3.4.2 Antimicrobial activities

Antibacterial and antifungal activities were screened by the disc diffusion assay. The MIC and the MBC were determined using the micro-dilution technique.

Microbial strains

The microbial species *Staphylococcus aureus* (ATTC 25923), *Escherichia coli* (ATTC 25922) and *Candida albicans* (ATTC 90028) were used for this study. The cultures of bacteria were maintained at 4 ◦C though out the study and used as stockcultures. The cultivation medium is Mueller Hinton Agar (MHA) as base medium for the screening of the anti-microbial activity. Mueller-Hinton Broth (MHB) was used for preparation of inoculums. The antimicrobial test was performed in triplicate. Gantamicin and Ampicillin were used as positive control for bacteria and Amphotericin B for fungus, Dimethylsulphoxide (DMSO) was used as negative control.

Preparation of inoculums

Each type of microorganism was streaked on a non-inhibitory NA plate to obtain isolated colonies and incubated at 37 ^{o} C for 18-24 hours for bacteria and 36-48 hours for fungi. One single colony was selected with an inoculating loop, and transferred into 3 ml of Mueller-Hinton Broth (MHB) and incubated in a shaking incubator at 37 ^{o} C for 2 hours. The turbidity of bacteria was adjusted to 0.5 McFarland standards ($1.5x10^{8}$ CFU/ml) by Mueller-Hinton Broth (MHB)

Preparation of test disc

The 95% ethanol extracts were dissolved in Dimethylsulfoxide (DMSO) to a final concentration 500 mg/ml and the aqueous extracts were dissolved in

distilled water to a final concentration of 100 mg/ml and filtered with Millipore filter 0.22 µm. Then 10 µl of extracts were applied to sterilized paper discs (6 mm in diameter)

3.4.2.1 Disc diffusion method

Principle

Disc diffusion method determined the antimicrobial that test *in vitro* susceptibility. The principle of disc diffusion method is the filter paper disc impregnated with an extract solution or chemical that is placed into the agar. The extract solution or chemical determined the size of the area infiltration around the disc. If an organism is placed on the agar it is not grow in the area around the disc if it is susceptible to the chemical. This area of no growth around the disc is known as a "zone of inhibition". The plate is incubated under standardised conditions following Clinical and Laboratory Standards Institute (CLSI) guidelines (Clinical and Laboratory Standards Institute, 2011). Zone of inhibition is formed through the interaction between the growth-inhibiting substance diffusing through a nutrient agar gel and the increasing population of the sensitive organism with which the gel has been inoculated (Mayr-Harting, 1947). The rate of diffusion of the antimicrobial through the agar is dependent on the diffusion and solubility properties of the drug in *Mueller- Hinton agar* (Bauer *et al*., 1966) and the molecular weight of the antimicrobial compound. Larger molecules will diffuse at a slower rate than lower molecular weight compounds (Hudzicki, 2009). Following incubation, the diameter of this zone is measured and the results are interpreted as resistant, intermediate, or susceptible using standard guidelines (e.g. CLSI M100).

The size of the inhibition zone indicates the degree of resistance, and might also give important information about the resistance mechanism and the resistance genes involved. In addition, the disc diffusion method can be used for determination of MIC values that provided the necessary reference curves for conversion of inhibition zones into MIC values.

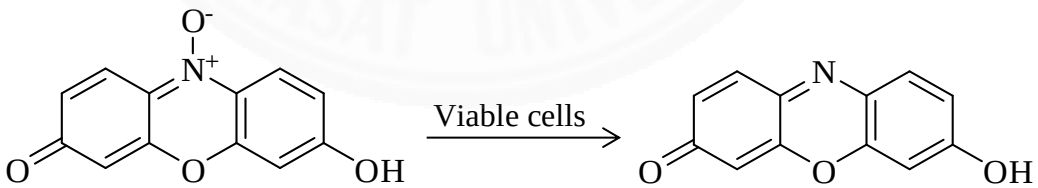
Procedure

This method used to screen antimicrobial activity of the extracts, as described by Lorian, 1996. Filter paper discs (6 mm in diameter) were impregnated with 10 µl of the extract (conc. 500 µg/ml). Air-dried discs were placed on inoculated Mueller-Hinton agar (MHA) surface (for bacteria) and Sabouraud Dextrose agar (SDA) surface (for the fungus). Positive controls in the present study Gentamicin and Amphotericin B (conc. 1 mg/ml). These plates incubated at 37°C for 24 h for bacteria and 30°C for 48 h for the fungus. The zone of inhibition was calculated by measuring the diameter of the inhibition zone. Three different fixed directions measured to triplicate data and the average value calculated.

3.4.2.2 Determination of minimum inhibitory concentration (MIC)

Principle

Six microorganisms were performed by measuring the minimal inhibitory concentrations (MIC in mg of extract/mL). The experiment was described by the method of Sarker *et al*., 2007. Broth microdilution assay was used to determine the MIC. This test was performed in 96 well microplates sterile. This technique was using resazurin to determination of the Minimum inhibitory concentration (MIC). Resazurin is an oxidation-reduction indicator used for the evaluation of cell growth, particularly in various cytotoxic activity assays (McNicholl *et al*., 2006).



Resazurin Resorufin

Figure 3-1 Reaction scheme of resazurin reduction to resorufin by live cells (Ge Wu, 2010)

Resazurin can penetrate cells, where it is reduced to resorufin, probably as the result of the action of several different redox enzymes in mitochondria, cytosol and microsome. The fluorescent resorufin then diffuses from cells and back into the surrounding medium. The ability of different cell types to

reduce reazurin to resorufin varies depending on the metabolic capacity of the cell and the length of incubation with resazurin. For experiment, an incubation period of 1 to 4 h. is adequate. Resazurin in normal assay solution is dark blue in color. Reduction of resazurin by viable cells produces red fluorescent resorufin (Ge Wu, 2010).

Procedure

The minimal inhibitory concentration (MIC) values were determined by microdilution assay. This experiment was described by the method of Sarker et al., 2007. The cultures were prepared from 18-24 h cultures of *Bacillus subtilis*, *Staphylococcus aureus, Escherichia coli* and *Salmonella Typhi* and form 36-48 h cultures of *Candida albicans* in broth cultures. The MIC was defined as the lowest concentration of the compound to inhibit the growth of microorganisms. This technique utilizes the microdilution method in a 96 well microplate. Briefly, 50µL of extract was diluted in two-fold dilution in the well. The culture was diluted with Mueller-Hinton broth (MHB) medium to 0.5 McFarland standard using densitometer. After that, the cultures were diluted in 1/200. Then, the culture (50 µL) was added in the well. After 16-18 h, 10 µL of resazurin solution was added in each well and incubated for 2 h. The minimum inhibitory concentration (MIC) was defined as the lowest concentration of the extract that prevent color change in the 96 well microplates. The lowest concentration value was determined in triplicate and expressed in mg/ml.

The minimum inhibititory concentration (MIC) assay is a technique used to measure the lowest effective concentration of extracts described by the method of Sarker *et al*., 2007.The cultures will be prepared from 18-24 hours cultures of *Staphylococcus aureus*, *Escherichia coli* and from 36-48 h cultures of *Candida albicans* in broth cultures. The MIC is defined as the lowest concentration of the compound to inhibit the growth of microorganisms. This technique utilizes the microdilution method in a 96-well microplate. Concisely, 50 µl of extract is diluted in two-fold dilution in the well. The culture will be diluted with Mueller-Hinton broth (MHB) medium to 0.5 McFarland standard using densitometer, and 50 µl will be added into each well and thoroughly mixed. After 16-18 h at37°C, 10 µl of resazurin solution will be added in each well and incubated further for 2 hours.

MIC is defined as the lowest concentration of the extract that prevents a color change in the 96-well microplate. The minimal bactericidal concentration (MBC) is defined as the lowest concentration of the compound to kill the microorganisms. The MBC is determined by sub culturing the test dilution (used in MIC) onto fresh solid medium and incubated further for 24 hours.

3.4.2.3 Determination of minimum bactericidal concentration (MBC)

Principle

The minimal bactericidal concentration (MBC) is defined as the lowest concentration of an antimicrobial agent that kill at least 99.9% of the initial inoculums (Vasanthakumari, 2007). The minimum bactericidal concentration was determined on the selected extracts in the 96 well plates from MIC determination. The absence of growth in the medium was an indication of antimicrobial activity at that concentration while the presence of growth suggested non-activity and the minimal inhibitory concentration (MIC) value.

Procedure

For MBC was determined after taking the MIC values immediately. The MBC was taken a row of all the wells with no visible growth in them and transferred to the agar plates with quadrants. The plates were incubated at 37°C for 24 hours, and then bacterial growth was evaluated. Last quadrant (i.e. with the lowest concentration of plant extract) showing no growth was taken as the MBC. The samples were tested in triplicate and the values were recorded as mg/mL (Taylor *et al*., 1983; ESCMID, 2000).

3.4.3 *In vitro* anti-inflammatory activity assay for effect on cyclooxygenase-2 enzyme inhibition (Cox-2)

Principle

This assay was modified to evaluated the PGE_2 levels in the supernatants of macrophage were measured by competitive immunoassay (EIA; Cayman Chemicals, Ann Arbor, MI) after 8 h of incubation with different stimuli as recommended by the manufacturer. When indicated, the COX inhibitors NS398 (5 mM) and valeryl salicylate (1 mM) (Cayman Chemicals) or the iNOS inhibitor NG-

monomethyl-Larginine monoacetate (L-NMMA; 500 mm) (Alexis, San Diego, CA) were used. Statistically significant differences were identified using the unpaired Student's t test. Values of p 5 0.01 were considered statistically significant.

Procedure

For this assay, cells growing as monolayer in 75 cm^3 flask washed with Phosphate-buffered saline (PBS) free of magnesium and calcium. The PBS decanted and cell detached with 0.25 % trypsin-EDTA to make a single cell suspension. Then,3 ml of medium added to flask to stop working trypsin-EDTA working. Next, the cell pallet obtained by centrifugation (1,500 rpm, 5 minutes), supernatant removed, and 10 ml of fresh medium added and cells mixed to make a single cell suspension. The viable cells counted by trypan blue exclusion in haemocytometer. After that, cells diluted with medium to give final concentration of $1x10^6$ cells/ml for RAW264.7. One hundred microliters of these cell suspensions seeded in 96-well plate and incubated at 37 ^oC at 5% CO_2 atmosphere with 95% humidity for 24 hours. At the end of the incubation time, the medium replaced with fresh medium containing 2 ng/ml of LPS (Lipopolysaccharide) together with test samples at various concentrate levels and then incubated at 37 ^oC 5% CO_2 atmosphere with 95% humidity for 24 hours. Then, the 96-well plate include with this kit is supplied ready to use.

Addition of the Reagents

1.EIA Buffer

Add 100 µl EIA Buffer to NSB wells. Add 50 µl EIA Buffer to B_0 wells.

2. Prostraglandin E_2 EIA standard

Add 50 µl from tube to both of the lowest standard wells.

3.Samples

Add 50 µl of sample per well. Each sample should be assayed at a minimum of two dilutions. Each dilution should be assay in duplicate.

4.Prostaglandin E_2 AChE Tracer

Add 50 µl to each well except the TA and the Blk wells.

5.Prostaglandin E2 Monoclonal Antibody

Add 50 µl to each well except TA and the Blk wells.

Table 3-2 Pipetting summary

Well	EIA Buffer	Standard/Sample	Tracer	Antibody
Blk	-	-	-	-
TA	-	-	5µl	-
NSB	100 µl	-	50 µl	-
B0	50 µl	-	50 µl	50 µl
Std/Sample	-	50 µl	50 µl	50 µl

And then incubation of the plate; Cover each plate with plastic film and incubate 18 hours at 4 ^{0}C. Reconstitute Ellman's Reagent immediately before use (20 ml of reagent is sufficient to develop 100 wells). Empty the wells and rinse five time with wash buffer.and then add 200 µl of Ellman'sReagent to each well, add 5 µl of tracer to the TA wells. In addition, cover the plate with plastic film and shaker incubator at 37 ^{0}C for 4 hours. Then remove the plate cover and read the plate at a wavelength between 405 and 420 nm. The percentage of inhibition was calculated by using the follow equation:

$$\text{Inhibition (\%)} = \frac{A-B}{A-C} \text{x } 100$$

A-C: NO_2^- concentration (µM)

[A: LPS (+), sample (-); B: LPS (+), sample (+); C: LPS (-), sample (-)]

3.4.3.1 MTT assay

Principle

MTT assay is a laboratory test and a standard colorimetric assay (an assay which measures changes in color) for measuring cellular proliferation (cell growth). It is used to determine cytotoxicity of potential medicinal agents and other toxic materials.

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole) reduced by metabolically active cells, in part by the action of dehydrogenase enzymes, to generate reducing equivalents such as NADH and NADPH. The resulting intracellular purple formazan can be solubilized. A solubilization solution (usually either dimethyl sulfoxide or a solution of the detergent sodium dodecyl sulfate in dilute hydrochloric acid) is added to dissolve the insoluble purple formazan product into a colored solution. The absorbance of this colored solution can be quantified by measuring at a certain wavelength (usually between 500 and 600 nm) by a spectrophotometer (Predoi *et al*., 2008).

This reduction takes place only when mitochondrial reductase enzymes are active, and therefore conversion is directly related to the number of viable (living) cells. When the amount of purple formazan produced by cells treated with an agent is compared with the amount of formazan produced by untreated control cells, the effectiveness of the agent in causing death of cells can be deduced, through the production of a dose-dependence (Mosmann, 1983).

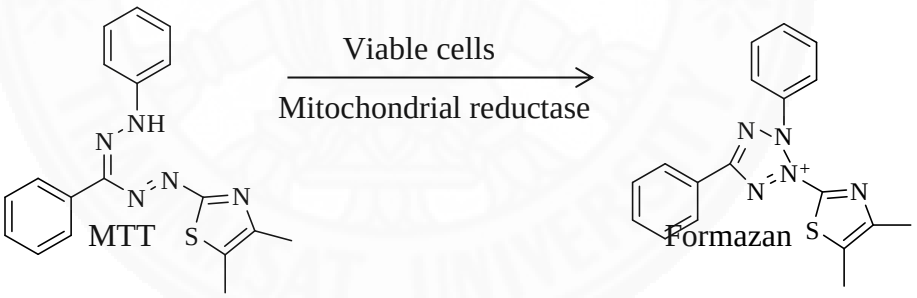


Figure 3-2 Reaction scheme of MTT reduction by live cells.

Yellow MTT is reduce to insoluble purple formazan in the mitochondria of live cells. A solubilization solution is added to the sample to dissolve the insoluble formazan product into a colored solution (Ge Wu, 2010).

Procedure

Cytotoxicity was determined using the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) colorimetric method. After 48 h, the cells were incubated with test samples, and MTT solution (5 mg/mL of MTT in PBS) was added to the wells. After 2 h incubation, the medium was removed, and 100 µL

of isopropanol containing 0.04M HCl was then added to dissolve the formazan production in the cells. The optical density (OD) of formazan solution was measured with a microplate reader at 570 nm. The test simples were considered to be cytotoxic when the optical density of the sample-treated group was less than 70% of that in the control (vehicle-treated) group. The percentage survival of the cell growth at each extract is calculated as:

$$\text{The Percentage of Survival} = \left(\frac{\text{Abs. Sample}}{\text{Abs. Control}}\right) \times 100$$

All determinations were carried out in triplicate and determined using the 3-(4,5-dimethyl-2-thiazolyl)-2, 5-diphenyl-*2H*-tetrazolium bromide (MTT) colorimetric method. Briefly, after 24 hours incubation with test sample, 10 µl of MTT solution (5 mg/ml in PBS) added to the wells and incubated at 37 ^{o}C in at 5% CO_2 atmosphere with 95% humidity for 2 hours. After that, medium will be removed. Isopropranol containing 0.04 M HCl added to dissolve the formazan production in the cells. Formazan solution measured the optical density with a microplate reader at 570 nm. The test compounds considered to cytotoxic when the optical density of the sample treated group less than 70 % of the vehicle-treated group.

3.4.4 *In vitro* assay for Estrogenic activity assay (Umehara *et al*., 2009)

3.4.4.1 Cell proliferation assay

Estrogenic activity (cell proliferation assay) was assessed by the protocol described previously (Luecha *et al*., 2009). Alamar blue reagent was used to determine cell concentrations, and fluorescence were measured at 570 nm with excitation at 530 nm.

3.4.4.2 Estrogen activity

Estrogenic assay was conducted according to the procedure for the cell proliferation assay with minor modifications. MCF-7 or T47D cells seeded at a density of 1 x 10^4 cells/well in 96 well plates in 90 µl of 5% DCC-treated, FBS-supplemented RPMI phenol red-free medium. A 5 µl portion of each test compound

added to each well with concentrations ranging from 2 to 2000 µM used as positive controls. Alamar Blue reagent added to the wells and left in the room temperature for 3 hr. The plate measured by fluorescence 590 nm.

3.4.4.3 Anti-estrogen activity

Anti-estrogenic assay also was conducted according to the procedure for the cell proliferation assay with minor modifications. MCF-7 or T47D cells seeded at a density of $1x10^4$ cells/well in 96 well plates in 90 µl of 5% DCC-treated, FBS-supplemented RPMI phenol red-free medium. Then, 5 µl of estradiol (E_2) at a concentration of 20 nM added into each well, and the plates incubated at 37 ^{O}C with 5% CO_2 for 1 hour. A 5 µl of portion of each test compound were added to each well with concentrations ranging from 2 to 2000 µM and incubated in a CO_2 incubator for 96 hours. In all experiments, 5 µl of serially diluted tamoxifen at concentrations from 2 to 200 µM are used as positive controls. Antagonistic effects of samples are evaluated from the cell populations, and iEqE values of each sample ($iEqE_{50}$, $iEqE_{10}$ and $iEqE_1$) are determined for the required concentrations to inhibit the E_2 effect ($iEqE_{50}$, $iEqE_{10}$ and $iEqE_1$, the concentration suppressing the E_2 effect to the equivalant level of 50; 10; and 1pM, respectively), When samples constantly suppress E_2 activity to the level less than 10 or 50 pM through the concentration tested, they are categorized as strong (S) or mild (M), respectively.

3.4.5 Phytochemical investigation of compound isolated

3.4.5.1 Bioassay-guided fractionation

Active fractions are fractionated using a bioassay guided fractionation. In bioassay-guided fractionation, a crude mixture is fractionated into its fraction components using chromatographic procedures, followed by biological evaluation (bioassay) of each fraction. Only fractions which display biological activity in the bioassay are selected for further fractionation. The cycle of fractionation and testing and further fractionation is repeated until a pure compound with the desired activity is isolated (Rimando *et al*., 2001).

Bioassay-guided fractionation is a procedure used for finding active compounds or markers of the extract. In this research, the ethanolic extract was chromatographed by vacuum liquid chromatography (VLC) on silica gel 60 using

gradient elution with solvent, hexane, hexane:chloroform (1:1), chloroform, chloroform:methanol (1:1) and methanol, respectively. Each fraction was concentrated by evaporator. The collected fractions were monitored and combined according to similar TLC chromatogram characteristic.

3.4.5.2 Isolation and structural characterization procedures

(1) Vacuum liquid chromatography (VLC)

The 95% ethanolic extracts of Sa-Tri-Lhang-Klod (8.019 grams) was separated by vacuum liquid chromatography (VLC) on silica gel 60 (code 1.07734.2500) using gradient elution with solvent, hexane (1,000 ml), hexane: chloroform (1:1, 1,000 ml), chloroform (2,500 ml), chloroform: methanol (1:1, 1,000 ml) and methanol (1,000 ml), respectively. Each fraction was concentrated to dryness by evaporator. The collected fractions were monitored and combined according to similar TLC chromatogram characteristics.

(2) Column chromatography (CC)

The fraction which showed the highest activity was chosen to separate by column chromatography. The extract was dried and mixed with silica gel as stationary phase. The silica gel column was eluted with polarity gradient of solvents manner with mixture, hexane: ethylacetate, ethylacetate, ethylacetate: methanol and methanol, respectively. The solvent was collected for 10 ml/tube. The collected fractions were examined by TLC chromatogram and detected with anisaldehyde spray. The structure of isolated pure compound was identified by TLC chromatogram characteristic and proton Nuclear Magnetic Resonance spectrum.

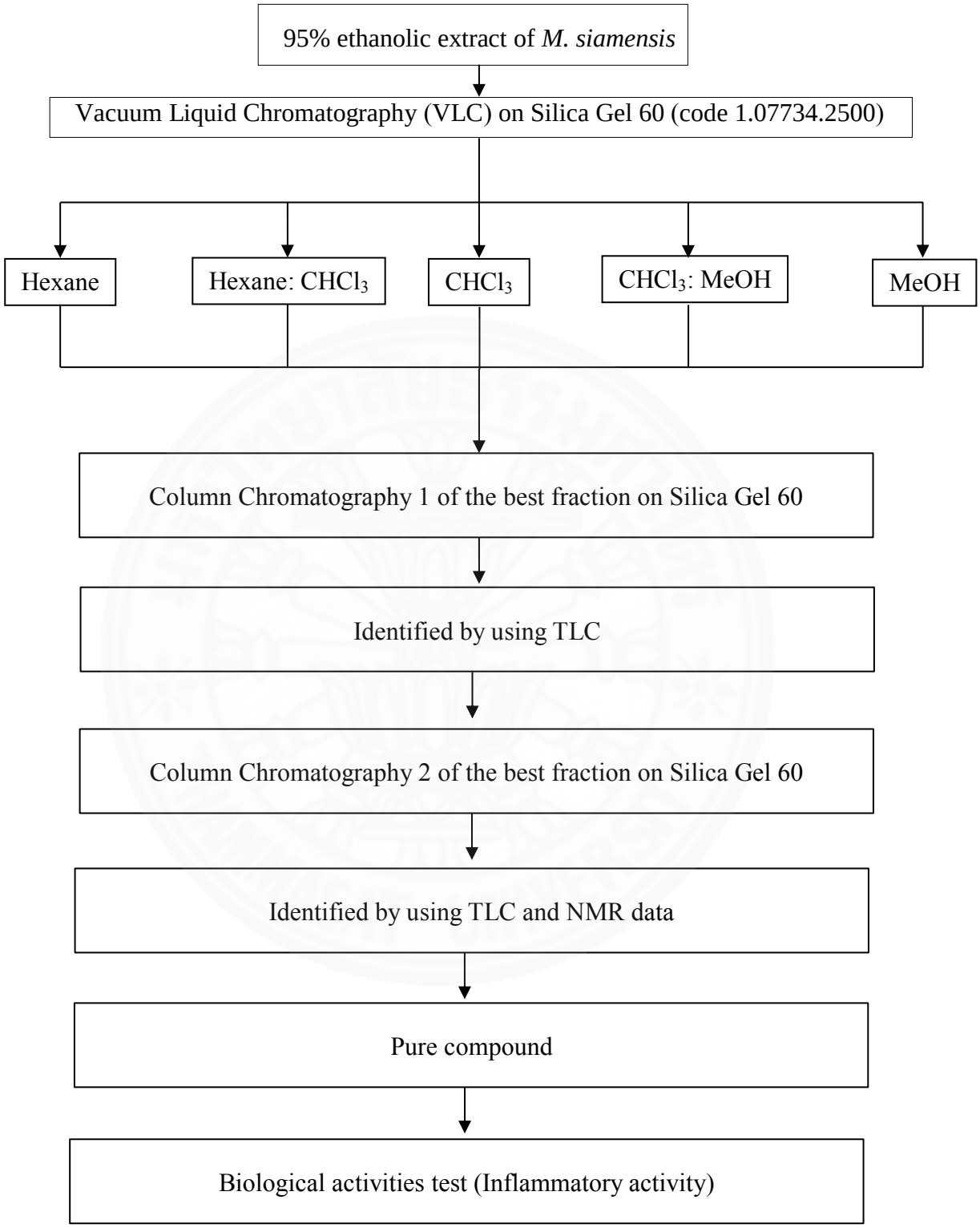


Figure 3-3 Fractionation of 95% ethanolic extract of *M. siamensis*

3.4.6 Statistic analysis

For statistical analysis, the values were expressed as mean ± standard error of mean (SEM) of three independent samples in triplicate. The data of antimicrobial assay by disc diffusion method is expressed as mean ± standard deviation (SD) from triplicate in three different fixed directions. The IC_{50} was calculated using the GraphPad Prism 4.03. Linear regression to correlate between total phenolics as well as total flavonoid with antioxidant activities was carried using Excel 2007 and analysis at p-value< 0.05 using GraphPad Prism 4.03.

CHAPTER 4
RESULTS AND DISCUSSION

4.1 Antimicrobial activities

4.1.1 Disc diffusion method

All extracts were tested for their antimicrobial activity by disc diffusion method to determine the inhibition zone against one-gram positive bacterium (*Staphylococcus aureus* ATTC 25923), one-gram negative bacterium (*Escherichia coli* ATTC 25922) and one fungus (*Candida albicans* ATTC 90028) that relate with infection complication in woman after childbirth or postpartum woman. The results of disc diffusion method are summarized in the following **Table 4-1.**

Staphylococcus aureus is a commensal bacterium and a human pathogen. Approximately 30% of the human population is colonized with *S. aureus.* At the same time, it is a majority cause of bacteremia and infective endocarditis (IE) as well as osteoarticular, skin and soft tissue, pleuropulmonary, and device-related infections (Wertheim, 2005). The results found that the most ethanolic extracts of Sa-Tri-Lhang-Klod remedy and its plant ingredients could against one-gram positive bacterium, one-gram negative bacterium and one fungus with inhibition zone of 22.67±2.31 mm, 10.33±0.58 mm and 11.33±1.16 mm, respectively. The result of Sa-Tri-Lhang-Klod remedy has never been reported. This study is the first report on antimicrobial activity by disc diffusion assay.

The ethanolic extract of *C. sappan* (CSE) showed the highest antimicrobial activity against these two bacteria and one fungus with inhibition zone at 22.67±2.31 mm, 10.33±0.58 mm and 11.33±1.16 mm, respectively. The aqueous extract of Sa-Tri-Lhang-Klod remedy (STKA) was the only one of all the aqueous extracts which showed antimicrobial activity against the positive bacterium with little inhibition zone at 7.00±0.00 mm.

Sa-Tri-Lhang-Klod remedy and its all plant ingredients showed antimicrobial activity against *S. aureus* with the range of inhibition zone at 9 to 23 mm. The 95% ethanolic extracts of Sa-Tri-Lhang-Klod showed antimicrobial activity against *S. aureus* with inhibition zone at 9.33±0.58 mm. The ethanolic extract of *C. sappan* showed the highest activity with inhibition zone at 22.67±2.31 mm. The result of *C. sappan* have been many report on antimicrobial activity by using disc diffusion method. The ethanolic extract of *P. ribesioides* (PRE) showed the lowest activity with inhibition zone at 8.33±1.16 mm. Thus, all extracts, Sa-Tri-Lhang-Klod remedy and its all plant ingredients showed antimicrobial activity lower than gentamicin (positive control).

Escherichia coli is gram-negative bacilli belong to the family Enterobactriaceae and the tribe Escherichia (Edward and Ewing, 1972). *E. coli* normally live in the intestines of people and animals. Most *E. coli* are harmless and actually are an important part of a healthy human intestinal tract. However, some *E. coli* are pathogenic, can cause urinary tract infections, respiratory illness, diarrhea, pneumonia, and other illnesses (Myron, 2010). Sa-Tri-Lhang-Klod remedy and its all plant ingredients showed antimicrobial activity against *E. coli* with the range of inhibition zone at 7 to 12 mm. Ethanolic and aqueous extracts of Sa-Tri-Lhang-Klod remedy had no activity against *E. coli.* However, there are three ethanolic extracts of plant ingredients namely *C. sappan* (CSE), *P. nigrum* (PNE) and *P. indica* (PIE) that showed antimicrobial activity against *E. coli* low inhibition zone at 10.33±0.58 mm, 8.33±0.58 mm amd 8.67±0.58 mm, respectively. Nevertheless, they exhibited lower antimicrobial activity than (gentamycin positive control) with inhibition zone at 23.00±0.00 mm.

Candida albican is fungal pathogen in the normal flora in humans. It can found in mouth, intestine and vagina (Berman, 2002). The 95% ethanolic extracts of Sa-Tri-Lhang-Klod remedy (STK95) showed against *C. albican* with inhibition zone at 7.33±0.58 mm. However, there are four ethanolic extracts of plant ingredients namely *C. sappan* (CSE), *M. siamensis* (MSE), *P. nigrum* (PNE) and *P. indica* (PIE) which showed antimicrobial activity against *C. albican* with low power inhibition zone at 11.33±1.16 mm, 9.67±0.58 mm, 8.33±0.58 mm and 13.00±0.00 mm,

respectively. Nevertheless, they showed lower antimicrobial activity against this fungi than amphotericin B (positive control) with inhibition zone at 19.00±0.00 mm.

Table 4-1 Antimicrobial activity of the ethanolic and aqueous extracts of Sa-Tri-Lhang-Klod remedy and its plant ingredients by disc diffusion method (mean ± SD) (n=3)

Sample	Code	Inhibition zone (mm)		
		S. aureus	***E. coli***	***C. albican***
A. sinensis	ASE	NI	NI	NI
A. heterophyllus	AHE	11.67±0.58	NI	NI
C. sappan	CSE	22.67±2.31	10.33±0.58	11.33±1.16
C. tinctorius	CTE	NI	NI	NI
C. comosa	CCE	NI	NI	NI
J. sambac	JSE	NI	NI	NI

NI = No Inhibition zone

Table 4-1 Antimicrobial activity of the ethanolic and aqueous extracts of Sa-Tri-Lhang-Klod remedy and its plant ingredients by disc diffusion method (mean ± SD) (n=3) (continued)

Sample	Code	Inhibition zone (mm)		
		S. aureus	***E. coli***	***C. albican***
M. cochinchinensis	MCE	12.00±0.00	NI	NI
M. siamensis	MSE	10.00±0.00	NI	9.67±0.58
M. ferrea	MFE	9.00±0.00	NI	NI
M. elengi	MEE	NI	NI	NI
N. nucifera	NNE	NI	NI	NI
P. longum	PLE	9.67±0.58	NI	NI

NI = No Inhibition zone

Table 4-1 Antimicrobial activity of the ethanolic and aqueous extracts of Sa-Tri-Lhang-Klod remedy and its plant ingredients by disc diffusion method (mean ± SD) (n=3) (continued)

Sample	**Code**	Inhibition zone (mm)		
		S. aureus	***E. coli***	***C. albican***
P. nigrum	PNE	NI	8.33±0.58	8.33±0.58
P. ribesioides	PRE	8.33±1.16	NI	NI
P. samentosum	PSE	NI	NI	NI
P. indica	PIE	20.00±0.00	8.67±0.58	13.00±0.00
S. chinensis	SCE	10.00±0.00	NI	NI
Sa-Tri-Lhang-Klod 95% ethanolic extract	STK95E	9.33±0.58	NI	7.33±0.58

NI = No Inhibition zone

Table 4-1 Antimicrobial activity of the ethanolic and aqueous extracts of Sa-Tri-Lhang-Klod remedy and its plant ingredients by disc diffusion method (mean ± SD) (n=3) (continued)

Sample	Code	Inhibition zone (mm)		
		S. aureus	***E. coli***	***C. albican***
Sa-Tri-Lhang-Klod 50% ethanolic extract	STK50E	10.00±0.00	NI	NI
Sa-Tri-Lhang-Klod aqueous extract	STKA	7.00±0.00	NI	NI
Gentamycin	Gen	22.00±0.00	23.00±0.00	NT
Amphotericin B	Amp	NT	NT	19.00±0.00

NI = No Inhibition zone, NT = Not Test

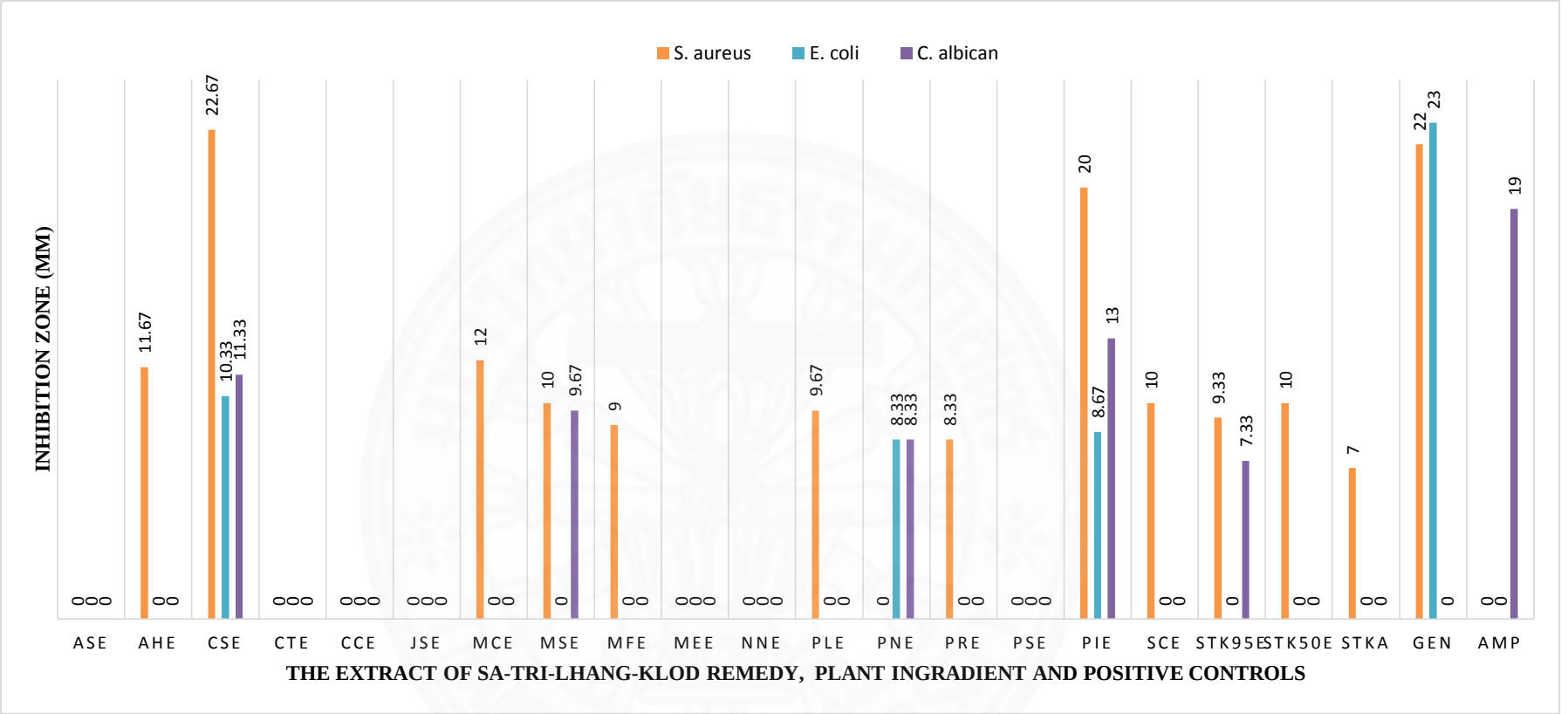


Figure 4-1 Antimicribial activity of Sa-Tri-Lhang-Klod remedy and its plant ingradient extracts by different extraction method against three microorganisms by disc diffusion method

4.1.2 Measurement of minimum inhibitory concentrations (MIC) and minimum bactericidal concentration (MBC)

All extracts were tested for antimicrobial activity by measurement of minimum inhibitory concentrations (MIC) which one-gram positive bacterium (*Staphylococcus aureus* ATTC 25923), one-gram negative bacterium (*Escherichia coli* ATTC 25922) and one fungus (*Candida albicans* ATTC 90028) to confirmed in their antimicrobial activities by disc diffusion test. The MIC of the extracts was the lowest concentration of extracts that did not permit any turbidity of the tested microorganism (Lorian, 1995; Lennette *et al*., 1991). MIC and MBC values of all extracts are shown in **Table 4-2**.

The 95% ethanolic extracts of Sa-Tri-Lhang-Klod (STK95) remedy showed antimicrobial activity against *Staphylococcus aureus* and *Candida albicans* with MIC values of 0.62 and 2.5 mg/ml, respectively. The 95% ethanolic extracts of Sa-Tri-Lhang-Klod (STK95) had no activity against *Escherichia coli*. The 50 % ethanolic extracts of Sa-Tri-Lhang-Klod (STK50) remedy showed antimicrobial activity against *Staphylococcus aureus* with MIC values of 1.25 mg/ml and MBC values 1.25 mg/ml. However, the aqueous extract of Sa-Tri-Lhang-Klod remedy had no activity against all bacteria and fungi.

The results showed the MIC values against microbial ranged between 0.019 to 2.5 mg/ml. The ethanolic extract of *M. siamensis* (MSE) showed the highest antimicrobial activity against *S. aureus* with MIC values of 0.019 mg/ml and MBC values 0.019 mg/ml. While the ethanolic extract of *A. heterophyllus* (AHE), *C. sappan* (CSE), *M. cochinchinensis* (MCE), M. *ferrea* (MFE), *P. longum* (PLE), *P. ribesioides* (PRE), *S. chinensis* (SCE) showed moderate antimicrobial activity against *S. aureus* with range of MIC value of 0.039 to 5 mg/ml.

The previous study were determined the antimicrobial activity against *Staphylococcus aureus* that the ethanolic extract of *Angelica sinensis* (Han *et al*., 2012), *Artocarpus heterophyllus* (Septama *et al.,* 2015), *Caesalpinia sappan* (Kim *et al*., 2014), *Mammea siamensis, Nelumbo nucifera* (Sattaponpan *et al*., 2011), that the same on present study. This present that *Mammea siamensis* study showed higher antimicrobial activity against *Staphylococcus aureus with* MIC 0.039 mg/ml.

Caesalpinia sappan (Kim *et al*., 2014) and *Plumbago indica* had against all of microbial on this study that had inhibition zone and minimum inhibitory concentration on *S. aureus, E. coli* and *C. albicans.* The present study is the first report on antimicrobial activity of the Sa-Tri-Lhang-Klod remedy. All of extracts had less potential for antimicrobial activity than Gentamicin and Amphotericin B. On the other hand, the 95% ethanolic extracts of Sa-Tri-Lhang-Klod remedy had anti-microbial effect against *S. aureus, E. coli* and *C. albicans*. Thus, this result can support using this remedy in Thai traditional medicine for postpartum care and protect from infection.

Table 4-2 Minimum inhibitory concentration (MIC) of the ethanolic and aqueous extracts of Sa-Tri-Lhang-Klod remedy and its plant ingredients (n=3)

Sample	Code	Minimum Inhibitory Concentration (MIC) /Minimum Bactericidal Concentration (MBC)		
		S. aureus	***E. coli***	***C. albican***
A. sinensis	ASE	NI	NI	NI
A. heterophyllus	AHE	0.156/0.156	NI	NI
C. sappan	CSE	0.156/0.156	1.25/2.5	0.625/0.625
C. tinctorius	CTE	NI	NI	NI
C. comosa	CCE	NI	NI	NI
J. sambac	JSE	NI	NI	NI

NI = No Inhibition zone

Table 4-2 Minimum inhibitory concentration (MIC) of the ethanolic and aqueous extracts of Sa-Tri-Lhang-Klod remedy and its plant ingredients (n=3) (continued)

Sample	Code	Minimum Inhibitory Concentration (MIC) /Minimum Bactericidal Concentration (MBC)		
		S. aureus	***E. coli***	***C. albican***
M. cochinchinensis	MCE	0.625/0.625	NI	NI
M. siamensis	MSE	0.019/0.019	NI	NI
M. ferrea	MFE	0.125/0.125	NI	NI
M. elengi	MEE	NI	NI	NI
N. nucifera	NNE	NI	NI	NI
P. longum	PLE	1.25/1.25	NI	NI

NI = No Inhibition zone

Table 4-2 Minimum inhibitory concentration (MIC) of the ethanolic and aqueous extracts of Sa-Tri-Lhang-Klod remedy and its plant ingredients (n=3) (continued)

Sample	Code	Minimum Inhibitory Concentration (MIC) /Minimum Bactericidal Concentration (MBC)		
		S. aureus	***E. coli***	***C. albican***
P. nigrum	PNE	0.039/0.039	1.25/1.25	1.25/1.25
P. ribesioides	PRE	1.25/1.25	NI	NI
P. samentosum	PSE	NI	NI	NI
P. indica	PIE	2.5/2.5	2.5/2.5	1.25/1.25
S. chinensis	SCE	1.25/1.25	NI	NI
Sa-Tri-Lhang-Klod 95% ethanolic extract	STK95E	0.625/0.625	NI	2.5/2.5

NI = No Inhibition zone

Table 4-2 Minimum inhibitory concentration (MIC) of the ethanolic and aqueous extracts of Sa-Tri-Lhang-Klod remedy and its plant ingredients (n=3) (continued)

Sample	Code	Minimum inhibitory concentration (MIC) /Minimum Bactericidal Concentration (MBC)		
		S. aureus	***E. coli***	***C. albican***
Sa-Tri-Lhang-Klod 50% ethanolic extract	STK50E	1.25/1.25	NI	NI
Sa-Tri-Lhang-Klod aqueous extract	STKA	NI	NI	NI
Gentamycin	Gen	0.125 /0.125(µg/ml)	NT	NT
Amphotericin B	Amp	NT	NT	1/1 (µg/ml)

NI = No Inhibition zone, NT = Not Test

4.2 Anti-inflammatory activity

Anti-inflammatory activities of ethanolic and aqueous extracts of Sa-Tri-Lhang-klod remedy and its ingredients were tested by measuring their inhibitory effect on cyclooxygenase-2 enzyme (Cox-2) release from RAW 264.7 cell lines using ELISA test kit were shown in **table 4-4**. Measurment of cytotoxicity was performed by MTT assay. All results were shown in **table 4-4**.

For the Sa-Tri-Lhang-Klod remedy extracts, the 95% ethanolic extract (STK95) showed anti-inflammatory activity of inhibitory effect on cyclooxygenase-2 enzyme (Cox-2) with IC_{50} of 3.15±0.30 µg/ml which is higher than the 50% ethanolic extract (STK50) with IC_{50} values of 13.60±0.27 µg/ml and the aqueous extract (STKA) had no measurable activity (IC_{50}>100 µg/ml). However, there were less than prednisolone (positive control) with IC_{50} value of 1.34±0.03 µg/ml.

There were sixteen extracts of plant ingredients exhibited anti-inflammatory activity of inhibitory effect on cyclooxygenase-2 enzyme (Cox-2) were shown in **table 4-5**. The exthanolic extract of *M. siamensis* (MSE) showed the highest anti-inflammatory activity with IC_{50} value of 0.08±0.005 µg/ml. The second highest anti-inflammatory activity was the exthanolic extract of *C. comosa* with IC_{50} 1.37±0.08 µg/ml. The third was the exthanolic extract of *A. heterophyllus* (AHE) which showed IC_{50} value of 4.57±0.08 µg/ml. There were only three the exthanolic extract of *J. sambac* (JSE), *N. nucifera* (NNE), *S. chinensis* (SCE) had no measurable activity (IC_{50}>100 µg/ml).

Table 4-3 Inhibitory effect of the ethanolic extract of Sa-Tri-Lhang-Klod remedy and its plant ingredients on LPS-induced cyclooxygenase-2 enzyme (Cox-2) from RAW 264.7 cell lines (n=2) and percentage of viable cells at various concentrations by MTT assay (n=3)

Sample	**Code**	**%Inhibition at various concentrations (µg/ml)/ (percentage of viable cells at various concentrations)(mean ± SEM)**								
		100	**50**	**30**	**10**	**1**	**0.1**	**0.01**	**0.001**	**0.0001**
A. sinensis	ASE	70.73±2.98 (2.64±11.27)	71.82±3.25 (-13.89 ±12.52)	51.08±1.76 (-10.55±18.025)	29.13±2.85 (-16.89±6.43)	NT	NT	NT	NT	NT
A. heterophyllus	AHE	NT	61.38±5.56 (61.62±6.69)	184.68 ±3.12 (-25.10±4.26)	121.14 ±14.91 (-64.41±5.10)	23.31±0.54 (-30.30±3.89)	NT	NT	NT	NT
C. sappan	CSE	NT	NT	NT	NT	NT	NT	NT	NT	NT
C. tinctorius	CTE	57.10±3.25 (-54.78±2.50)	68.02±6.50 (-45.40±2.05)	50.27±0.95 (-53.58±2.43)	21.95±2.44 (-51.61±2.56)	NT	NT	NT	NT	NT
C. comosa	CCE	NT	NT	NT	NT	NT	NT	NT	NT	NT

NT = Not Tested

Table 4-3 Inhibitory effect of the ethanolic extract of Sa-Tri-Lhang-Klod remedy and its plant ingredients on LPS-induced cyclooxygenase-2 enzyme (Cox-2) from RAW 264.7 cell lines (n=2) and percentage of viable cells at various concentrations by MTT assay (n=3) (continued)

Sample	Code	%Inhibition at various concentrations (µg/ml)/ (percentage of viable cells at various concentrations)(mean ± SEM)								
		100	**50**	**30**	**10**	**1**	**0.1**	**0.01**	**0.001**	**0.0001**
J. sambac	JSE	4.06 ±2.18 (-47.77 ± 2.73)	NT	NT	NT	NT	NT	NT	NT	NT
M. cochinchinensis	MCE	87.53±0.27 (-43.77±2.42)	63.14±1.08 (-54.63±2.81)	42.41±0.68 (-49.80±3.38)	31.17±0.54 (-46.91±7.62)	NT	NT	NT	NT	NT
M. siamensis	MSE	NT	NT	NT	NT	50.79±2.85 (11.79±1.04)	66.61±0.47 (24.63±1.23)	43.83±0.32 (33.92±1.81)	41.93±0.47 (5.00±4.68)	38.77±0.32 (3.41±2.09)
M. ferrea	MFE	NT	NT	139.70±0.41 (14.01±5.17)	54.88±0.95 (12.57±2.28)	16.22±1.93 (-10.33±2.14)	10.04±0.19 (-23.05±3.22)	NT	NT	NT
M. elengi	MEE	50.14±2.71 (-27.43±2.03)	46.75±2.03 (-23.62±1.58)	31.03±2.03 (-26.26±2.06)	12.47±4.07 (-30.45±1.27)	NT	NT	NT	NT	NT

NT = Not Tested

Table 4-3 Inhibitory effect of the ethanolic extract of Sa-Tri-Lhang-Klod remedy and its plant ingredients on LPS-induced cyclooxygenase-2 enzyme (Cox-2) from RAW 264.7 cell lines (n=2) and percentage of viable cells at various concentrations by MTT assay (n=3) (continued)

Sample	**Code**	**%Inhibition at various concentrations (µg/ml)/ (percentage of viable cells at various concentrations)(mean ± SEM)**								
		100	**50**	**30**	**10**	**1**	**0.1**	**0.01**	**0.001**	**0.0001**
N. nucifera	NNE	12.03±2.65 (14.70±1.19)	NT	NT	NT	NT	NT	NT	NT	NT
P. longum	PLE	NT	NT	NT	NT	NT	NT	NT	NT	NT
P. nigrum	PNE	NT	NT	NT	NT	NT	100.71±1.03 (22.08±2.54)	42.01±0.24 (15.82±6.28)	35.52±1.82 (7.62±1.40)	39.87±0.32 (8.82±0.82)
P. ribesioides	PRE	NT	NT	NT	NT	NT	NT	NT	NT	NT
P. samentosum	PSE	NT	NT	NT	NT	NT	NT	NT	NT	NT

NT = Not Tested

Table 4-3 Inhibitory effect of the ethanolic extract of Sa-Tri-Lhang-Klod remedy and its plant ingredients on LPS-induced cyclooxygenase-2 enzyme (Cox-2) from RAW 264.7 cell lines (n=2) and percentage of viable cells at various concentrations by MTT assay (n=3) (continued)

Sample	**Code**	**%Inhibition at various concentrations (µg/ml)/ (percentage of viable cells at various concentrations)(mean ± SEM)**								
		100	**50**	**30**	**10**	**1**	**0.1**	**0.01**	**0.001**	**0.0001**
P. indica	PIE	NT	NT	NT	NT	NT	NT	NT	NT	NT
S. chinensis	SCE	27.97±2.66 (-57.56±1.72)	NT	NT	NT	NT	NT	NT	NT	NT
Sa-Tri-Lhang-Klod 95% ethanolic extract	STK95E	87.74±0.48 (17.70±1.96)	NT	101.68±8.89 (-19.02±7.38)	93.03±4.57 (-29.74±9.65)	34.13±0.96 (-6.83±2.68)	NT	NT	NT	NT
Sa-Tri-Lhang-Klod 50% ethanolic extract	STK50E	90.26±0.12 (-10.23±2.73)	NT	69.23±4.33 (-10.71±1.76)	41.59±0.24 (-15.49±2.64)	12.50±1.92 (15.45±1.67)	NT	NT	NT	NT
Sa-Tri-Lhang-Klod aqueous extract	STKA	8.59±0.78 (-13.22±1.56)	NT	NT	NT	NT	NT	NT	NT	NT

Table 4-3 Inhibitory effect of the ethanolic extract of Sa-Tri-Lhang-Klod remedy and its plant ingredients on LPS-induced cyclooxygenase-2 enzyme (Cox-2) from RAW 264.7 cell lines (n=2) and percentage of viable cells at various concentrations by MTT assay (n=3) (continued)

Sample	**Code**	**%Inhibition at various concentrations (µg/ml)/ (percentage of viable cells at various concentrations)(mean ± SEM)**								
		100	**50**	**30**	**10**	**1**	**0.1**	**0.01**	**0.001**	**0.0001**
Prednisolone	PN	NT	93.20±3.79 (0.83±2.21)	NT	87.74±1.50 (-5.71±1.58)	89.16±1.82 (22.16±1.59)	81.57±0.87 (-54.29±1.08)	52.93±1.34 (-51.23±4.17)	NT	NT

NT = Not Tested

Table 4-4 IC_{50} values of plant extracts for LPS-induced cyclooxygenase-2 enzyme (Cox-2) from RAW 264.7 cell lines

Sample	Code	Inhibition of COX2 production	
		% inhibition at conc. 100 µg/ml	IC_{50}±SEM (µg/ml)
A. sinensis	ASE	70.73±2.98	29.25±1.43
A. heterophyllus	AHE	61.38± 5.56	4.57±0.08
C. sappan	CSE	NT	19.13±0.72
C. tinctorius	CTE	57.10±3.25	29.87±0.77
C. comosa	CCE	NT	1.37 ± 0.08
J. sambac	JSE	4.06 ±2.18	>100
M.cochinchinensis	MCE	87.53±0.27	37.76±0.79
M. siamensis	MSE	118.75±5.63	0.08±0.005
M. ferrea	MFE	111.09±0.16	8.38±0.68
M. elengi	MEE	50.14±2.71	58.69±6.53
N. nucifera	NNE	12.03±2.65	>100
P. longum	PLE	NT	5.22 ± 0.53
P. nigrum	PNE	98.28±0.78	12.72±0.79
P. ribesioides	PRE	NT	10.88±8.88
P. samentosum	PSE	NT	11.79±3.20
P. indica	PIE	NT	7.96 ± 2.44
S. chinensis	SCE	27.97±2.66	>100
Sa-Tri-Lhang-Klod remedy extracts	STK95E	87.74±0.48	3.15±0.30
	STK50E	90.26±0.12	13.60±0.27
	STKA	8.59±0.78	>100
Prednisolone (conc. 50 µg/ml)	PN	93.20±3.79	0.065±0.003

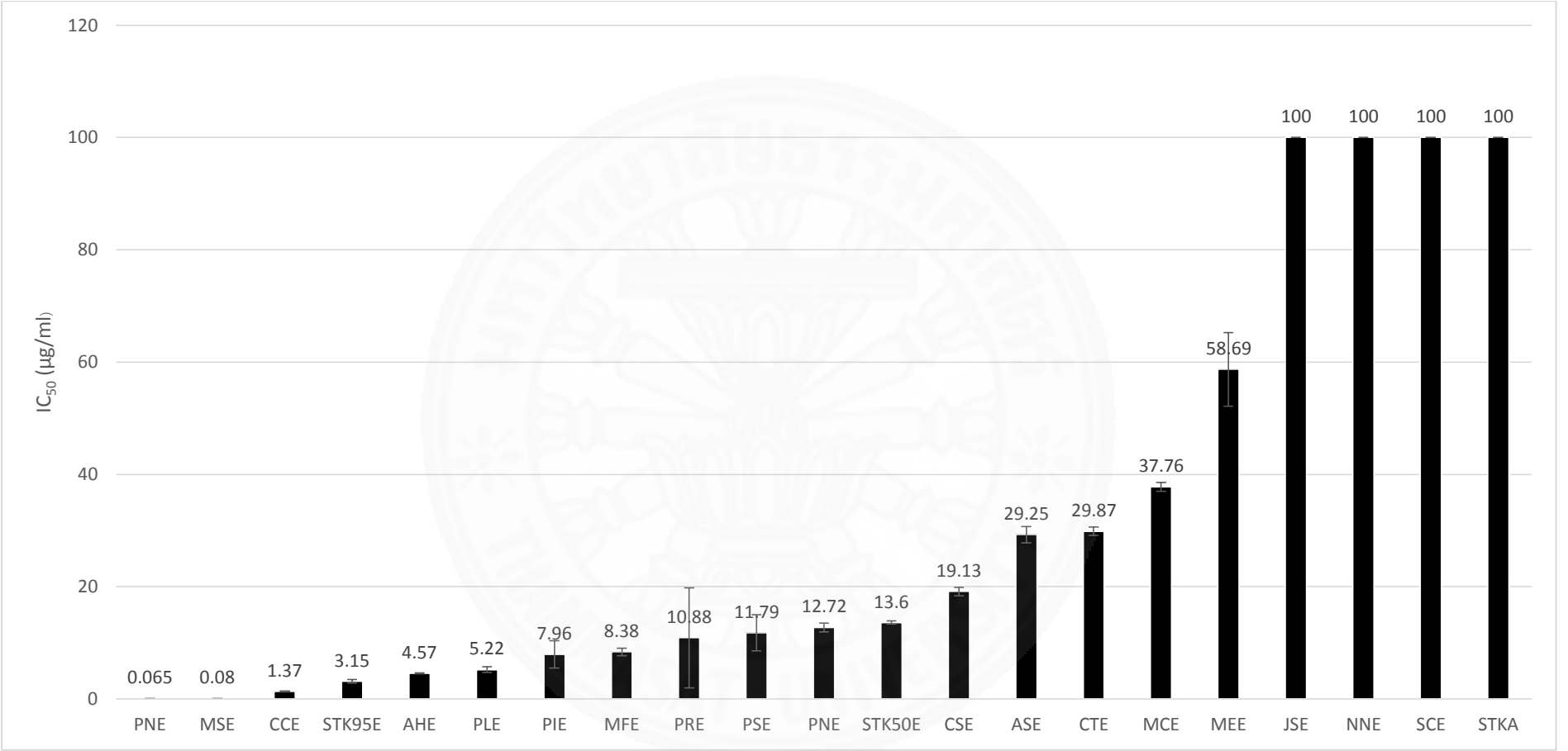


Figure 4-2 IC_{50} (µg/ml) values of Sa-Tri-Lhang-Klod remedy and its plant ingredients for LPS-induced cyclooxygenase-2 enzyme (Cox-2) from RAW 264.7 cell lines (n=2)

4.3 Estrogen activity

Estrogenic activity of ethanolic and aqueous extracts of Sa-Tri-Lhang-klod remedy and its ingredients were tested using estrogen-responsive breast cancer cell lines; MCF-7 and T47D with increasing concentrations at 0.01, 0.1, 1 and 10 µM, and their stimulatory activity on cell proliferation was determined by comparison with a positive control, estradiol (E_2) at concentrations ranging from 1 to 100 pM.

Anti-estrogenic activity based on MCF-7 and T47D cell proliferation**.** Estradiol (E_2) at 100 pM was used initially to enhance cell proliferation, and each compound was tested at 0.01, 0.1, 1, and 10 µM (Table 2). Tamoxifen was used as a positive control in this assay, and it suppressed E2-enhanced cell proliferation nearly completely (iEqE1) for both cells at the concentration of lower than 10 µM. They showed cell proliferation stimulating activity in **figure 4-3, figure 4-4** and inhibitory activities of Sa-Tri-Lhang-Klod remedy against E2-Enhanced MCF-7 and T47D cell proliferation in **table 4-4**

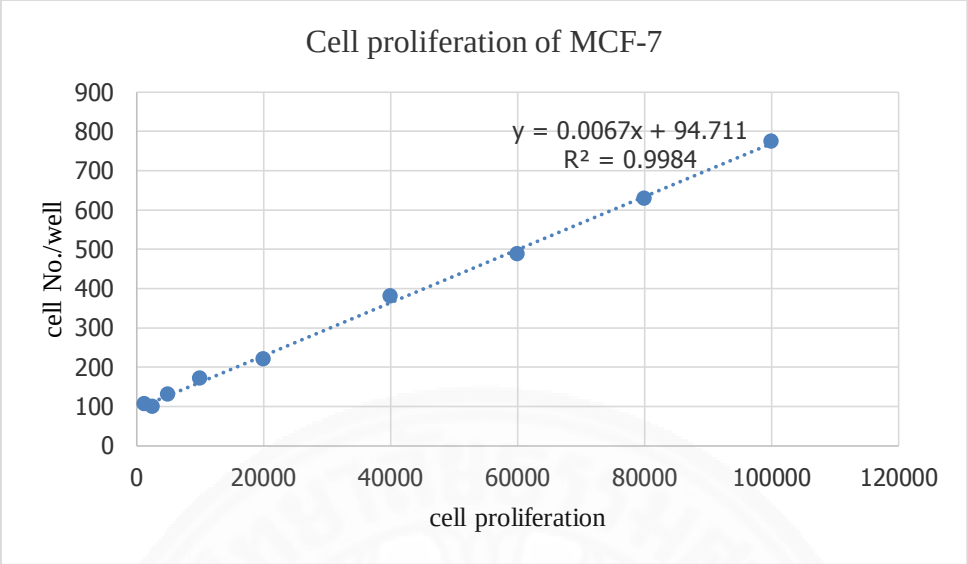


Figure 4-3 Cell proliferation activity of MCF-7 cells

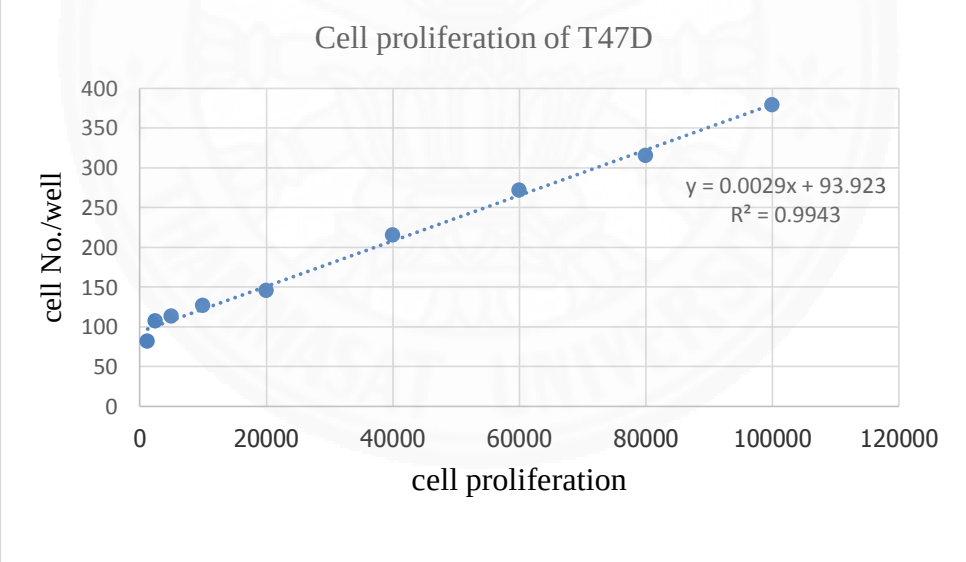


Figure 4-4 Cell proliferation activity of T47D cells

Table 4-5 Inhibitory activities of Sa-Tri-Lhang-Klod remedy against E2-Enhanced MCF-7 and T47D cell proliferation

Sample	Anti-estrogenic activity							
	MCF-7				T47D			
	iEqE50	iEqE10	iEqE1	Inhibition level	iEqE50	iEqE10	iEqE1	Inhibition level
STK50	59.72	3.65	<0.01		5.95	<0.01	<0.01	Strong
STK95	19.61	3.17	<0.1		<0.1	<0.1	10.8	
STKW	21.19	27.07	25.39		1.36	<0.01	<0.01	Strong
Tamoxifen	1.49	1.75	1.79		<0.1	<0.1	84.56	

4.4 Fractionation

M. siamensis (MS) that showed the good values in many bioilogical such as antimicrobial and anti-inflammatory activities, was chosen to separation by Vacuum Liquid Chromatography (VLC). MS (8.02 g) was sequentially eluted with five solution systems as follow: Hexane (MS-1, 1,000 ml), Hexane:Chloroform (MS-2), Chloroform (MS-3, 1,000 ml), Chloroform: Methanol (MS-4, 3,000 ml) and Methanol (MS-5, 2,000 ml). Each fraction was concentrated to dryness by evaporator and the percentage of yield calculated. These extracts were to dryness by evaporator and the percentage of yield (W/W) of these extract is showed in **Table 4-6**.

Table 4-6 Percentage of yield of fraction from the ethanolic extract of *Mammea siamensis* separated by VLC

Fractions	Code	Weight (g)	%Yield(w/w)
Hexane	MS-1	0.07	0.96
Hexane: Chloroform	MS-2	0.08	0.94
Chloroform	MS-3	0.10	1.29
Chloroform: Methanol	MS-4	3.17	39.47
Methanol	MS-5	2.46	30.71

The 5 fractions were tested by TLC analysis using 1 systems as; chloroform: methanol (1:1) that showed spots detected from UV absorption at short wave 254 and 366 nm. Anisaldehyde spray reagent was used with heating at 120 ºC.

Code of fractions

MS-1: Hexane

MS-2: Hexane: Chloroform

MS-3: Chloroform

MS-4: Chloroform: Methanol

MS-5: Methanol

4.5 Isolation and structural characterization procedures

4.5.1 Isolation of compounds from the ethanolic extract of *Mammea siamensis* Kosterm.

The fraction of MS-4 which showed the highest weight was chosen for separation by column chromatography. Fraction MS-4 (3.16 g) was dried and mixed with silica gel 60 (code 1.09385.2500). The column chromatography was performed using 200 g silica gel in column, eluting with hexane: EtOAC (4:1 v/v) 1,000 ml and increasing polarity as follows; 500 ml of each fraction for hexane: EtOAC (4:1, 3:2). 250 ml for EtOAC: hexane (2:3, 1:4) and 200 ml for EtOAC. 10 ml/tube of each fraction was collected and examined by thin layer chromatography (TLC), with detection of compounds using anisaldehyde spraying reagent. Fractions with similar TLC chromatogram were combined and dried. Seventeen separate combined fractions were obtained from pooling fractions; CM1, CM2, CM3, CM4, CM5, CM6, CM7, CM8, CM9, CM10, CM11, CM12, CM13, CM14, CM15, CM16 and CM17. Fraction CM14 (387.07 mg) was dried to separate and used the column chromatography was performed using 100 g silica gel in column, eluting with $CHCl_3$: MeOH (4:1 v/v) 500 ml and increasing polarity as follows; 300 ml of each fraction for $CHCl_3$: MeOH (4:1, 3:2). 200 ml for $CHCl_3$: MeOH (2:3, 1:4) and 100 ml for MeOH. Compound (2.7 mg) was obtained as a yellow solid. Purity was comfirmed by chromatography using three TLC solvent systems. The structure of the isolated compound was established by comparing the ^{1}H and ^{13}C NMR (Nuclear Magnetic Resonance).

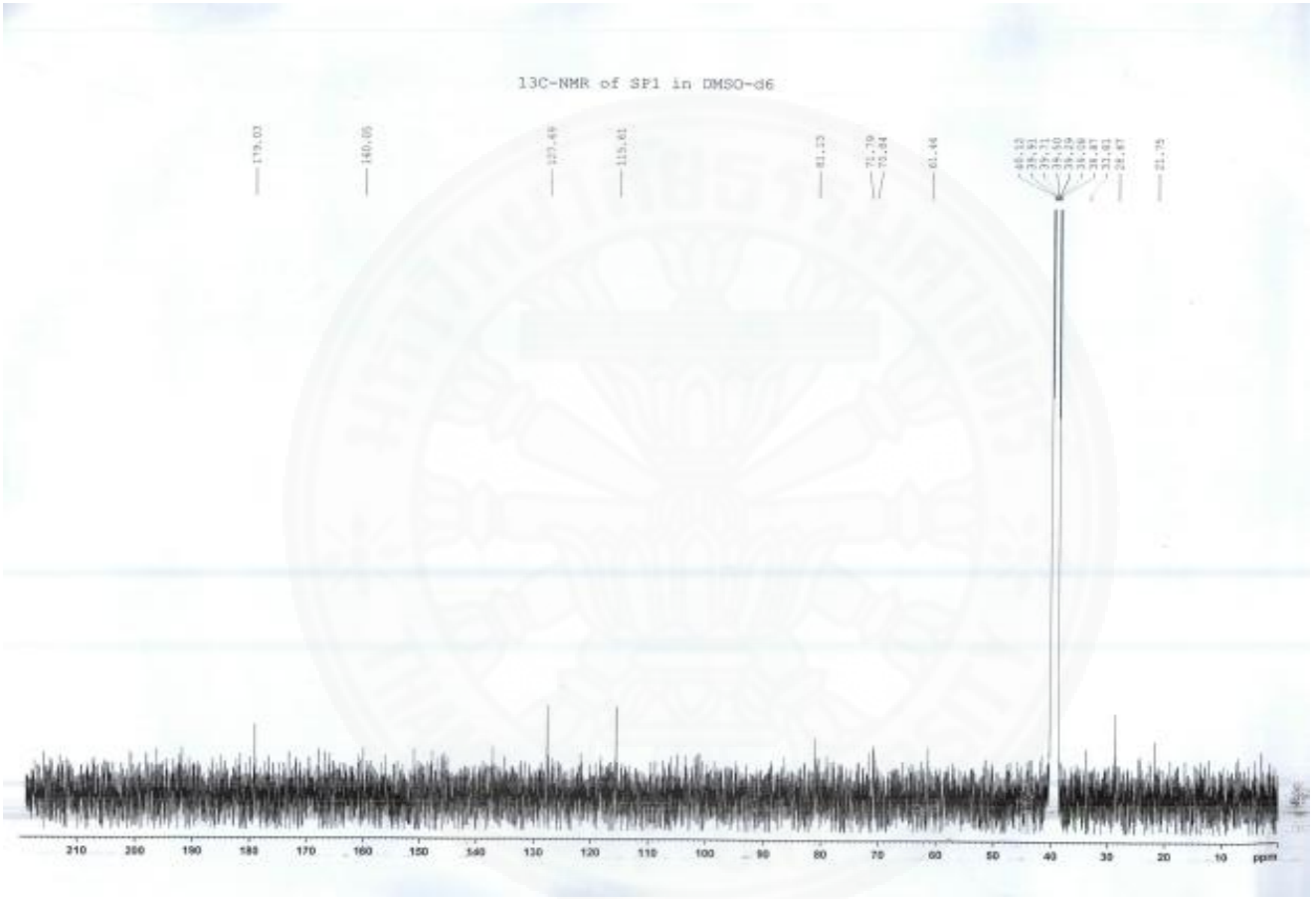


Figure 4-5 ^{1}H-NMR Spectrum of SP1 in DMSO (400 MHz, in DMSO-d6)

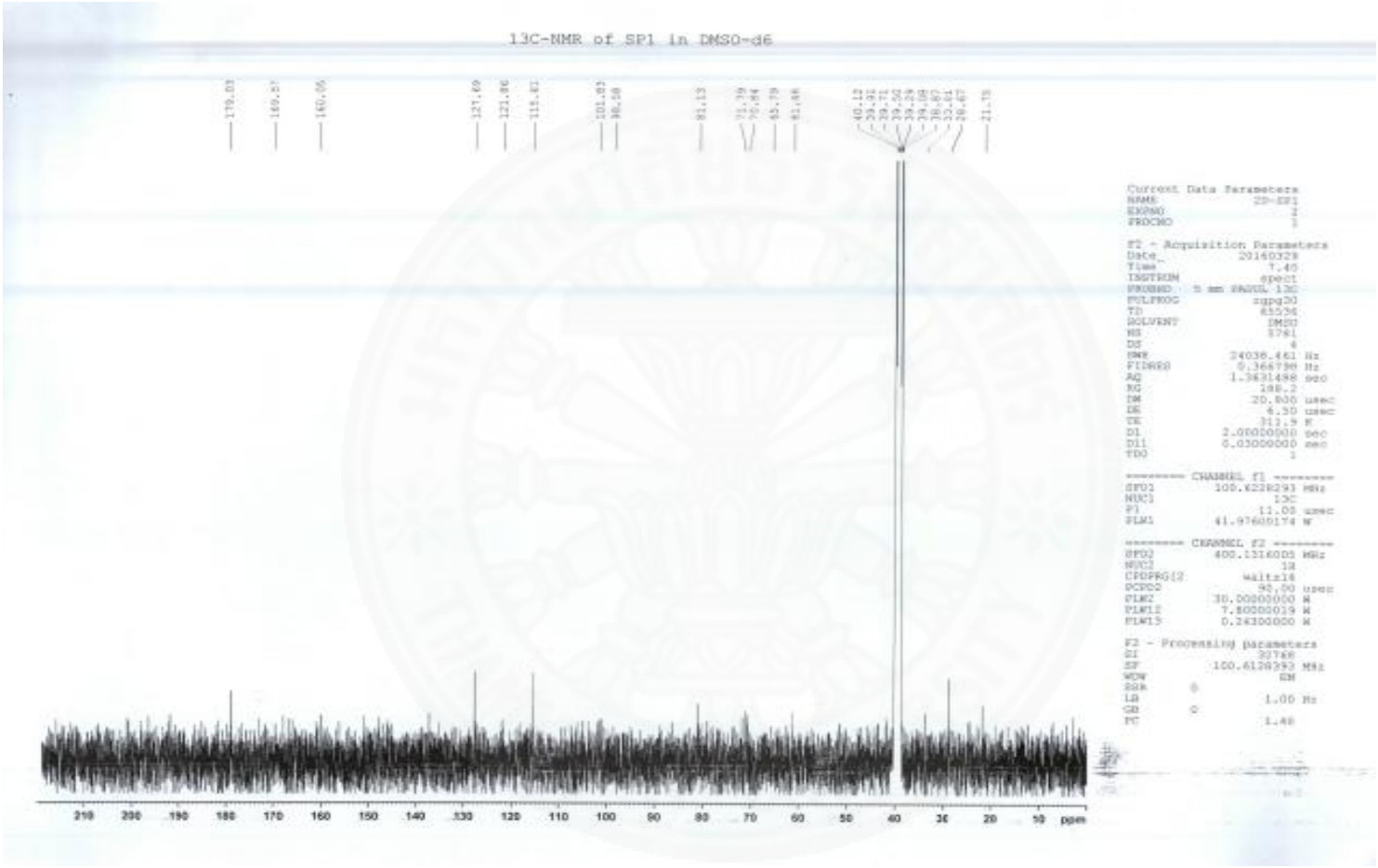


Figure 4-6 ^{13}C-NMR Spectum of SP1 in DMSO (400 MHz, in DMSO-d6)

4.6 Biological activity of isolated compound

The study compound of separation mixture component which was isolated from the ethanolic extract of *M. siamensis* (SP1) exhibited found the moderate of the inhibitory effect on LPS-induced cyclooxygenase-2 enzyme (COX-2) from RAW 264.7 cell lines with IC_{50} values of 47.27±2.00 µg/ml and it not toxic to RAW 264.7 cell lines showed in **Table 4-7**.

Table 4-7 Inhibitory effect of the ethanolic extract of SP1 that isolated from *Mammea siamensis* Kosterm. on LPS-induced cyclooxygenase-2 enzyme (COX-2) from RAW 264.7 cell lines (n=2) and percentage of viable cells at various concentrations by MTT assay (n=3)

Sample	**Code**	**%Inhibition at various concentrations (µg/ml)/ (percentage of viable cells at various concentrations) (mean ± SEM)**					
		100	**50**	**10**	**1**	**0.1**	**IC_{50}**
component of coumarin	SP1	66.18±0.49 (10.83±2.11)	52.82±0.86 (6.83±1.21)	10.42±0.86 (5.10±2.21)	-18.75±0.86 (15.71±1.68)	-33.09±907 (12.16±1.59)	47.27±2.00

CHAPTER 5
CONCLUSIONS AND RECOMMENDATIONS

Sa-Tri-Lhang-Klod remedy which consists of seventeen plants has been used for care during postpartum phase as stimulate blood circulation, blood tonic, anti-inflammation and astringent in postpartum (National List of Essential Medicines, 2013). The flavor of Sa-Tri-Lhang-Klod remedy is hot and spicy because most of the ingredients are spices. That consist of thai medicine plants as follow: roots of *Maclura cochinchinensis* (Lour.) Corner, stems of *Artocarpus heterophyllus* Lam., rhizomes of *Curcuma comosa* Roxb., stems of *Caesalpinia sappan* Linn., vines of *Piper ribesioides* Wall., roots of *Plumbago indica* L., flowers of *Piper longum* Linn., roots of *Angelica sinensis* (Oliv.) Diels, stems of *Salacia chinensis* L., fruits of *Piper nigrum* Linn., roots of *Piper samentosum* L., flowers of *Carthamus tinctorius* Linn., flowers of *Jasminum sambac* Ait., flowers of *Mimusops elengi* L., flowers of *Mesua ferrea* Linn., flowers of *Mammea siamensis* Kosterm., and flowers of *Nelumbo nucifera* Gaertn.

There is no report for anti-microbial activity, anti-inflammatory and anti-estrogenic activity of this preparation, but the previous studies reported that some herb and formula extracts showed cytotoxic activity, anti-oxidant and anti-inflammatory. Therefore, the objectives of this study are to investigate the antimicrobial activity, anti-inflammatory and anti-estrogenic activity of Sa-Tri-Lhang-Klod remedy extracts and each of its herb ingredients against three types of microbial such as *staphylococcus aureus, Escherichia coli* and *Candida albicans,* anti-inflammatory by cyclooxygenase-2 enzyme inhibition (COX-2) and anti-estrogenic activity.

The result showed that the ethanolic extract of *C. sappan* exhibited the highest inhibition zone of *S. aureus* and *E. coli* (22.67±2.32 mm and 10.33±0.58 mm), respectively and the exthanolic extract of *P. Indica* exhibited antimicrobial activity against *C. albican* with the highest inhibition zone (13.00±0.00 mm). The MIC and MBC of the exthanolic extract of *M. siamensis* showed the highest inhibitory activity against *S. aureus* (0.019 mg/ml). Moreover, MIC of *C. Sappan* showed the highest

inhibitory activity against *E. coli* and *C. albican* (1.25 mg/ml and 0.625 mg/ml), respectively. All of extracts had less potential for antimicrobial activity than Gentamicin and Amphotericin B. On the other hand, the 95% ethanolic extract of STK95 had anti-microbial effect against *S. aureus* and *C. albicans*.

In summary, the ethanolic extract of Sa-Tri-Lhang-Klod remedy showed antimicrobial activity against *Staphylococcus aureus* that cause of postpartum infection. In addition, the ethanolic extract of some plant such as *Caesalpinia sappan* and *Mammea siamensis* exhibited good antimicrobial activity. These results support the use of this remedy in Thai traditional medicine for pyrexia and infection in postpartum.

Investigation of anti-inflammatory activity of inhibitory effect on cyclooxygenase-2 enzyme (Cox-2), the Sa-Tri-Lhang-Klod remedy extracts, the 95% ethanolic extracts (STK95) showed anti-inflammatory activity of inhibitory effect on cyclooxygenase-2 enzyme (Cox-2) (IC_{50} = 3.15±0.30 µg/ml) which is higher than the 50% ethanolic extract (STK50) (IC_{50} = 13.60±0.27 µg/ml) and the aqueous extract (STKA) had no measurable activity (IC_{50}>100 µg/ml). However, there were less than prednisolone (IC_{50} = 1.34±0.03 µg/ml).

There were sixteen extracts of plant ingredients exhibited anti-inflammatory activity of inhibitory effect on cyclooxygenase-2 enzyme (Cox-2). The exthanolic extract of *M. siamensis* (MSE) showed the highest anti-inflammatory activity (IC_{50} = 0.08±0.005 µg/ml). The second highest anti-inflammatory activity was *C. comosa* with (IC_{50} = 1.37 ±0.08 µg/ml). The third was *A. heterophyllus* (AHE) which (IC_{50} value of 4.57±0.08 µg/ml). There were three extracts namely *J. sambac* (JSE), *N. nucifera* (NNE), *S. chinensis* (SCE) had no measurable activity (IC_{50}>100 µg/ml).

The estrogenic and anti-estrogenic activities against E2-enhance T47D and MCF-7 cell proliferation found STK95, STK50 and STW showed anti-estrogenic activity. STK50 showed strong inhibition against T47D and MCF-7 cell at low concentration with iEqE1 values <0.01 µM and against more than tamoxifen (iEqE1= 1.79 and 84.56 µM). Moreover, STK95 showed high cytotoxic activity against HeLa, SKOV-3 and MCF-7. And *Caesalpinia sappan* (CSE), *Curcuma comosa* (CCE) and

Mammea siamensis (MSE) also showed very high cytotoxic activity against HeLa, SKOV-3 and MCF-7 (Inprasit, 2014).

M. siamensis was chosen to separate by Vacuum Liquid Chromatography (VLC) because their showed good activity in many bioassays. It has 5 fractions; Hexane (MS-1), Hexane:Chloroform (MS-2), Chloroform (MS-3), Chloroform: Methanol (MS-4) and Methanol (MS-5). MS-4 was used to isolated active compounds by using column chromatography. The component of cumarin was isolated. Their showed the anti-inflammatory in RAW 264.7 cells (COX-2) and had no toxicity against RAW 264.7 cells.

In conclusion, 95% ethanolic extract of ST (STK95) displayed good value of the anti-inflammatory, antioxidant by DPPH assay and cytotoxic activities (HeLa and MCF-7), but had no antioxidant activity by NBT reduction assay. 50% ethanolic and water extracts of ST (STK50 and STKW) had no biological activity, except ST50 exhibited less anti-inflammatory effect on the inhibition of NO production. The ethanolic extract of *Caesalpinia sappan* (CSE), *Curcuma comosa* (CCE) and *Mammea siamensis* (MSE) showed very high biological activities. (Inprasit, 2014). In summary, STK95, STK50 and STKW showed the moderate of antimicrobial. The ethanolic extract of some plant such as *Caesalpinia sappan* (CSE) and *Mammea siamensis* (MSE) exhibited good antimicrobial and anti-inflamatory activities. In Addition, all extract of remedy also showed the good of anti-estrogenic activity. However, the results of the present study can partially support the use of STK, a remedy in Thai traditional medicine for protection of postpartum infection, anti-inflammation after delivery and for increasing lactation. Moreover, these results related to Thai traditional medicine and to support the using these remedy in the lists of The National List of Essential Herbal Medicine (NLEM) of Thailand to use in postpartum women so this study will be very useful to provide this information for further research in the development of herbal medicine for used for postpartum care.

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APPENDICES

APPENDIX A

Chemical Reagent for Laboratory Experiments

1.Reagent for Antimicrobial activity

1.1 Nutrient Agar (NA)

NA powder	28 g
Deionized water	1000 ml

Suspend 28 g of NA powder in 1000 ml of deionized water, mix well and sterilize in autoclave at 121 °C for 15 minutes. Allow it to cool but not solidify, pour NA into each sterile plastic petri dish and replace the lid immediately. Place NA plates on a counter top to cool and set at room temperature and kept at 2-8 ºC.

1.2 Mueller Hinton Agar (MHA)

MHA powder	38 g
Deionized water	1000 ml

Suspend 38 g of MHA powder in 1000 ml of deionized water, mix well and sterilize in autoclave at 121 °C for 15 minutes. Allow it to cool but not solidify, pour MHA into each sterile plastic petri dish and replace the lid immediately. Place MHA plates on a counter top to cool and set at room temperature and kept at 2-8 ºC.

1.3 Mueller Hinton Broth (MHB)

MHA powder	21 g
Deionized water	1000 ml

Suspend 38 g of MHB powder in 1000 ml of deionized water, mix well and dispense 5 ml portion of MHB into 18 x 150 mm tube then sterilize in autoclave at 121 °C for 15 minutes.

1.2 Sabourand Dextrose Agar (SDA)

SDA powder	65 g
Deionized water	1000 ml

Suspend 65 g of SDA powder in 1000 ml of deionized water, mix well and sterilize in autoclave at 121 °C for 15 minutes. Allow it to cool but not solidify, pour SDA into each sterile plastic petri dish and replace the lid immediately. Place SDA plates on a counter top to cool and set at room temperature and kept at 2-8 ºC.

1.3 Resazurin solution

Resazurin	1 mg
Sterile distilled water	1000 µl

The resazurin solution was prepared by dissolving 1 mg in 1000 µl of Sterile distilled water. The resazurin was sterillzed by fillration though a 0.2 µm filter and keep it at 4 ºC for 1-2 weeks and protected from the light.

2. Reagent for Anti-inflammatory activity on cyclooxygenase-2 enzyme inhibition (cox-2)

2.1 ELISA buffer solution

ELISA buffer concentration (10X)

Diluted the content of one vial of ELISA buffer concentrate (10x) with 90 ml of ultra-pure water (stored at 4 ºC)

2.2 Wash buffer solution

Wash buffer concentrate (400x)	5 ml
Ultra-pure water	2000 ml
Polysorbate 20	1 ml

Dilute the wash buffer to a total volume of 2000 ml with ultra-pure water and add 1 ml of Polysorbate 20(stored at 4 ºC)

2.3 Prostaglandin E2 AChE Tracer solution

Reconstituted PGE_2 AChE tracer 100 dtn with 6 ml of EIA buffer

(stored at 4 ºC)

2.4 Ellman's Reagent

Reconstituted PGE_2 Ellman's Reagent100 dtn with 20 ml of ultra-pure water (stored at 4 ºC)

2.5 Reagent for MTT assay

MTT solution (5 mg/ml)

3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide or Thiazolyl blue tetrazolium bromide 200 mg

PBS 40 ml

(Wrapped in foil and stored at 4 ºC)

0.04 M HCl in Isopropanol

HCl 0.83 ml

Adjust volume with Isopropanol to 250 ml

3. Reagent for Anti-estrogenic activity

3.1 RPMI-1640

RPMI-1640 power medium is dissolve in 500 ml sterile water. Add 2.4 g os sodium bicarbonate and dilute to 100 ml with sterile water. And filter through 0.2 micron membrane filter and keep in sterile bottle.

The complete media is mixture of 400 ml of RPMI-1640, 4 ml fetal bovine serum-supplement RPMI phenol red-free medium and 4 ml of 5% DCC-treated. The medium was store at 4 ºC.

3.2 Alamar Blue reagent

4.Reagent for cell culture

4.1 Dulbeco's modified eagle medium (DMEM)

13.4 g of Dulbeco's modified eagle powder medium is dissolve in 500 ml sterile water. Add 3.7 g of sodium bicarbonate and dilute to 1000 ml with sterile water. Adjust pH to 7.2-7.4 with 10% sodium hydroxide or 10 % hydrochloric acid and filter through 0.2micron membrane filter and keep in sterile bottle.

The complete media is mixture of 400 ml of Dulbeco's modified eagle medium (DMEM), 40 ml fetal bovine serum and 4 ml penicillin/streptomycin. The medium was stored at 4 ºC

4.2 Minimum essential medium (MEM)

9.5 g of Minimum essential powder medium is dissolve in 500 ml sterile water. Add 2.2 g of sodium bicarbonate and dilute to 1000 ml with sterile water. Adjust pH to 7.2-7.4 with 10% sodium hydroxide or 10 % hydrochloric acid and filter through 0.2micron membrane filter and keep in sterile bottle.

The complete media is mixture of 400 ml of Minimum essential medium (MEM), 40 ml fetal bovine serum, 4 insulin and 4 ml penicillin/streptomycin. The medium was stored at 4 ºC

4.3 RPMI medium 1640

10.4 g of RPMI medium 1640 powder medium is dissolve in 500 ml sterile water. Add 2.7 g of sodium bicarbonate and dilute to 1000 ml with sterile water. Adjust pH to 7.2-7.4 with 10% sodium hydroxide or 10 % hydrochloric acid and filter through 0.2micron membrane filter and keep in sterile bottle.

The complete media is mixture of 400 ml of RPMI medium 1640 medium (RPMI), 40 ml fetal bovine serum, 4 ml insulin and 4 ml penicillin/streptomycin. The medium was stored at 4 ºC

4.4 10% Hydrochoric acid (HCI)

Conc. HCl (37%)	27 ml
Distilled water to	100 ml

4.5 10% Sodium hydroxide (NaOH)

NaOH	10 g
Distilled water to	100 ml

4.6 Fetal bovine serum (FBS)

Slowly thaw the FBS (inactive), heat 56 ºC, 60 mins (Aliqout, stored at -20 ºC)

4.7 Penicilin-Streptomycin (P/S)

Slowly thaw the frozen P/S, 37 ºC, till completely thawed (Aliquot, store at -20 ºC)

4.8 Phosphate buffer saline (PBS)

PBS	1 tablet
Distrilled water to	100 ml

Sterile by autoclave 121 ºC, 15 min and stored in 4 ºC

4.9 Trypsin-EDTA

Slowly thaw the frozen 0.5% trypsin-EDTA, 37 ºC, 60 mins till completely thawed (Aliquot, store at -20 ºC)

APPENDIX B

ยาสตรีหลังคลอด

ยาต้ม (รพ.)

สูตรตำรับ

ในยา 130 กรัม ประกอบด้วย

1. แก่นแกแล แก่นขนุน ว่านชักมดลูก แก่นฝางเสน เถาสะค้าน รากเจตมูลเพลิงแดง ดอกดีปลี โกฐเชียง เถากำแพงเจ็ดชั้น หนักสิ่งละ 10 กรัม
2. พริกไทยล่อน รากช้าพลู ดอกคำฝอย ดอกมะลิ ดอกพิกุล ดอกบุนนาค ดอกสารภี เกสรบัวหลวง หนักสิ่งละ 5 กรัม

คำแนะนำ

ขับน้ำคาวปลา บำรุงเลือด ช่วยให้มดลูกเข้าอู่เร็วในหญิงหลังคลอด

ขนาดและวิธีใช้

นำยาใส่น้ำพอท่วม ต้มด้วยไฟปานกลาง นานครึ่งชั่วโมง นำเฉพาะส่วนน้ำมารับประทาน ครั้งละ 250 มิลลิลิตร วันละ 3 ครั้ง ก่อนอาหาร หรือดื่มแทนน้ำ

รับประทานติดต่อกัน 1 สัปดาห์หรือจนกว่าน้ำคาวปลาจะหมด แต่ไม่เกิน 15 วัน

ข้อห้ามใช้

ห้ามใช้ในหญิงตกเลือดหลังคลอด หญิงตั้งครรภ์ และผู้ที่มีไข้

คำเตือน

- ควรระวังในการรับประทานร่วมกับยาในกลุ่มสารกันเลือดเป็นลิ่ม (anticoagulant) และยาต้านการจับตัวของเกล็ดเลือด (antiplatelets)
- ควรระวังการใช้ยาในผู้ป่วยที่แพ้ละอองเกสรดอกไม้

APPENDIX C

NMR SPECTRUM OF ISOLATED COMPOUNDS

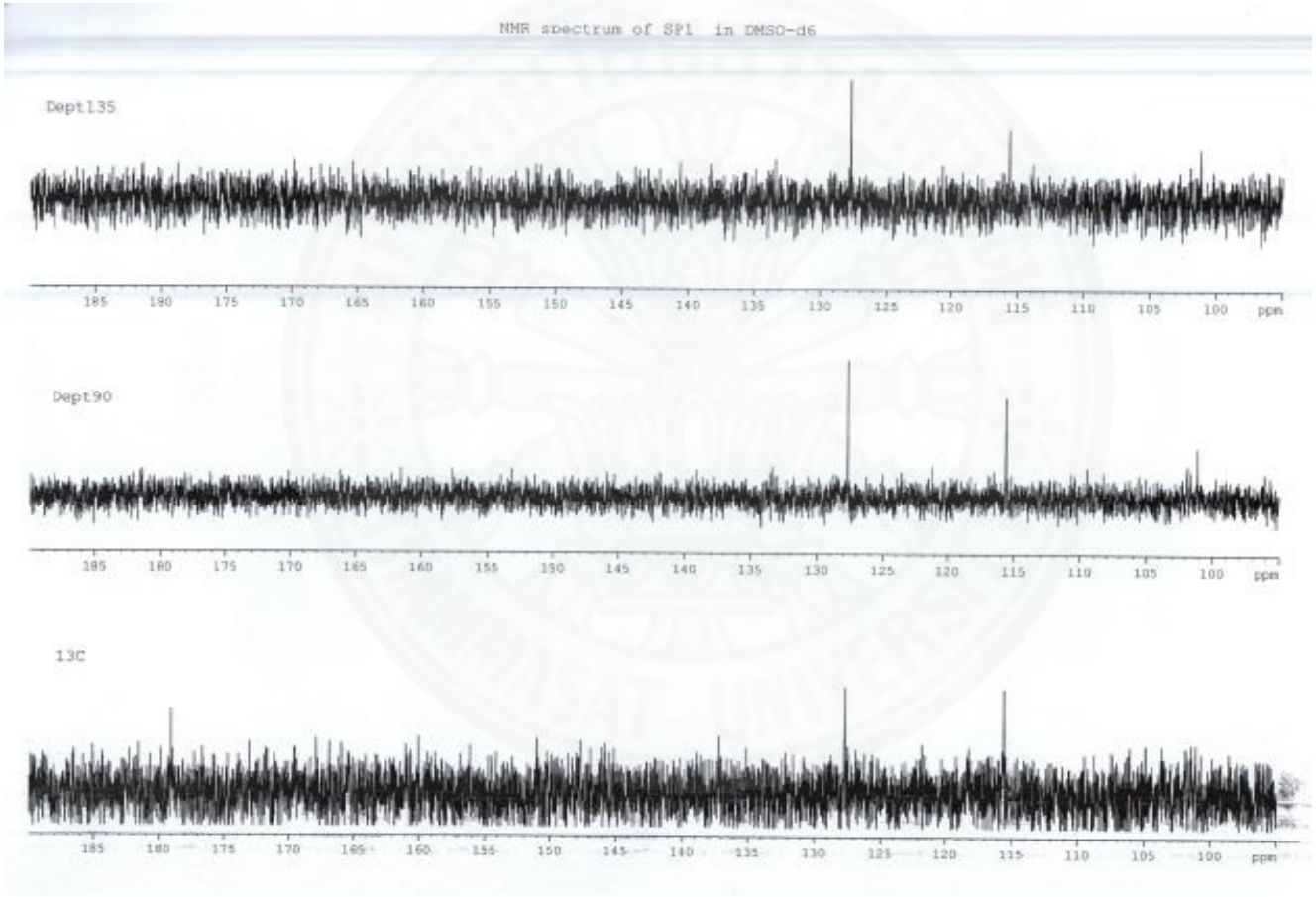

Figure A1 NMR Spectrum of SP1 (400 MHz, in DMSO-d6)

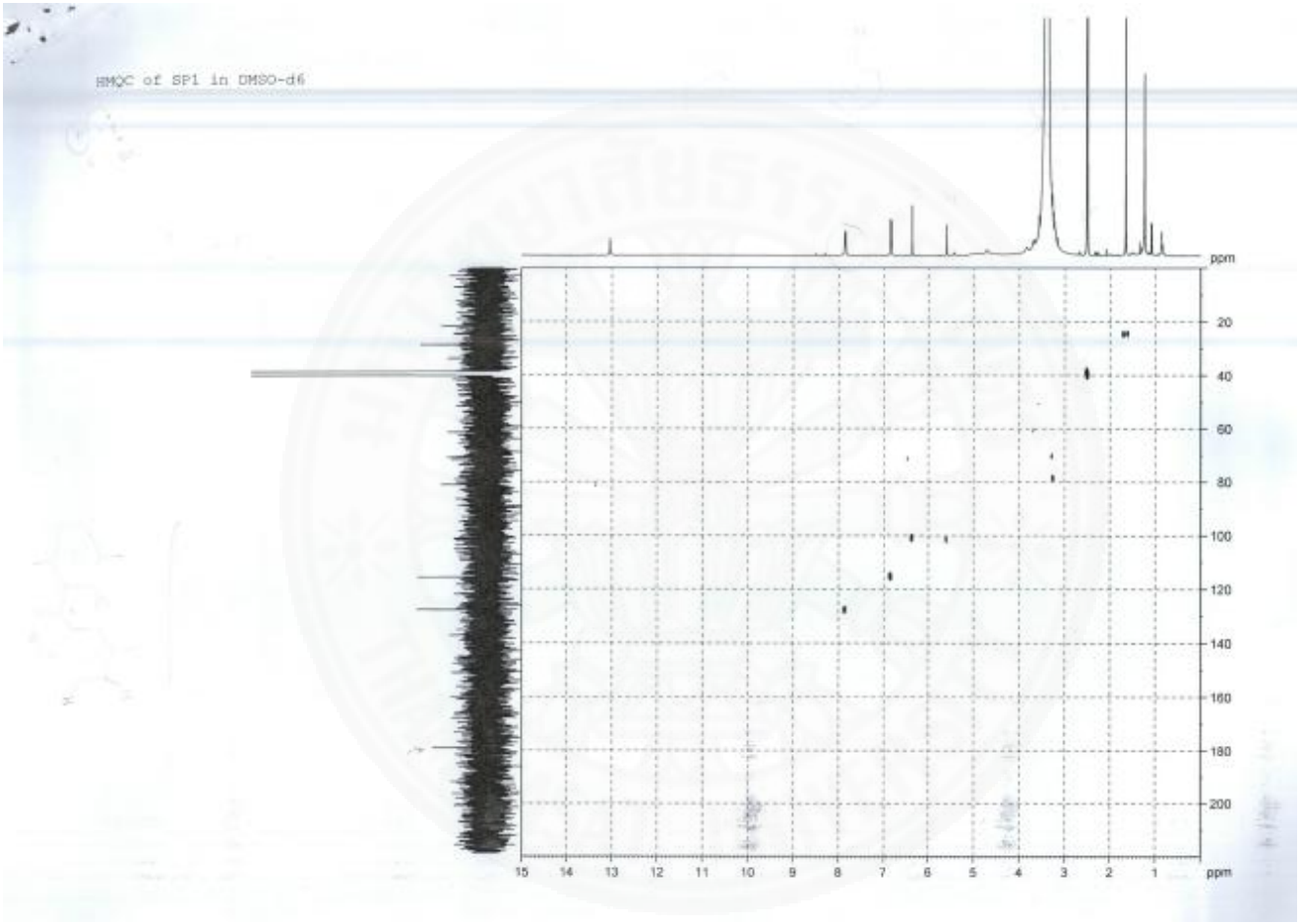

Figure A2 HMQC of SP1 (400 MHz, in DMSO-d6)

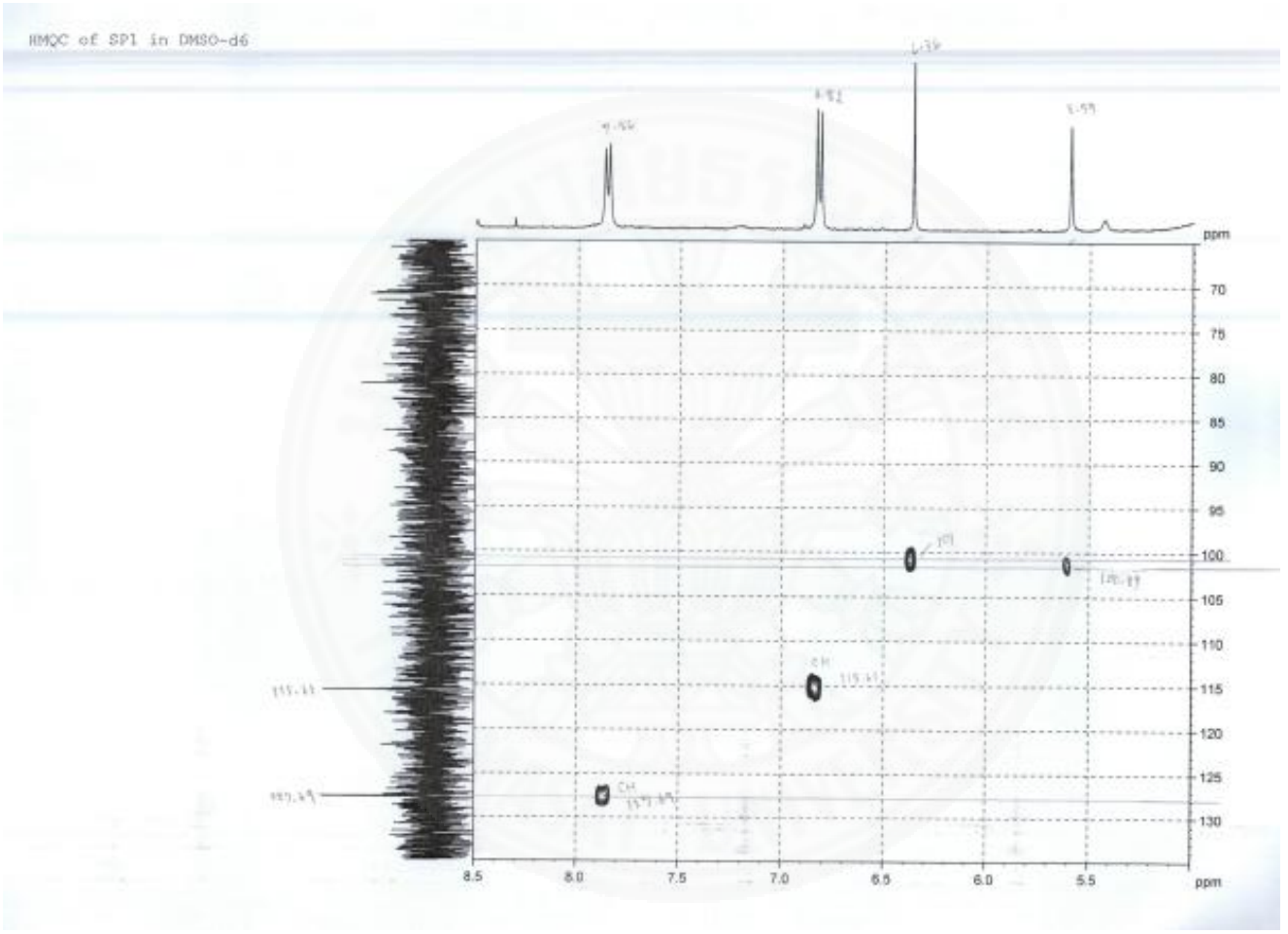

Figure A3 HMQC of SP1 (400 MHz, in DMSO-d6)

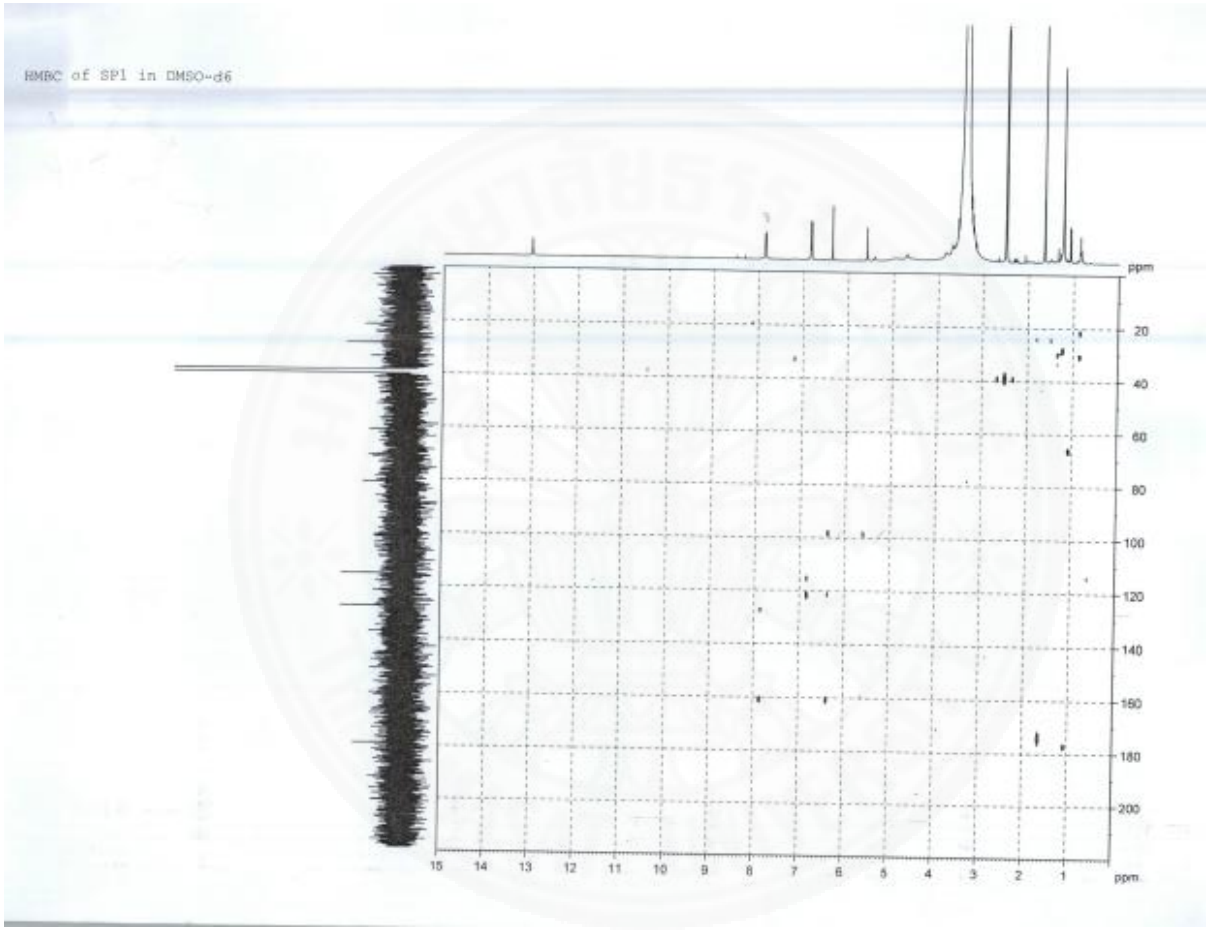

Figure A4 HMBC of SP1 (400 MHz, in DMSO-d6)

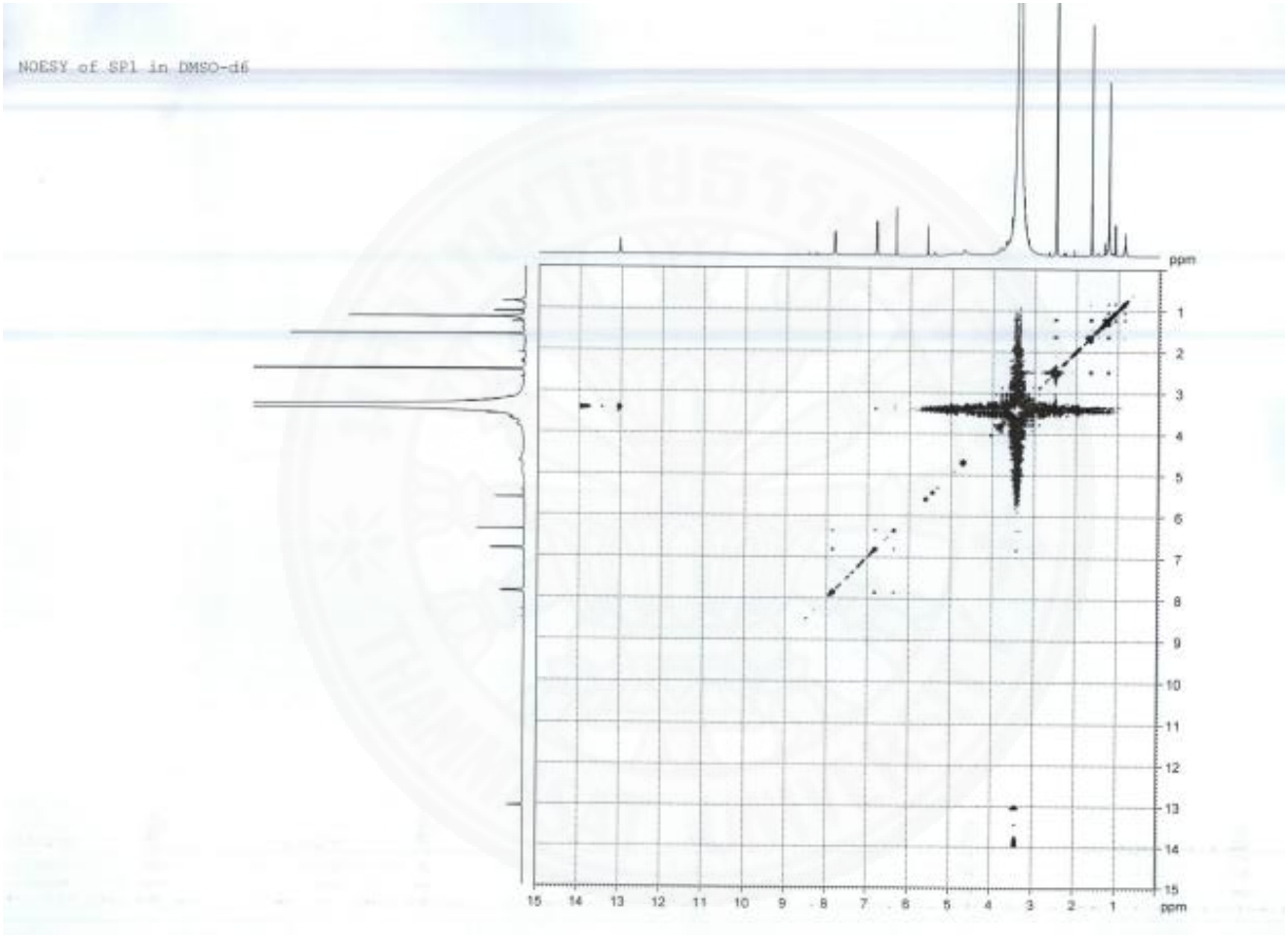

Figure A5 NOESY of SP1 (400 MHz, in DMSO-d6)

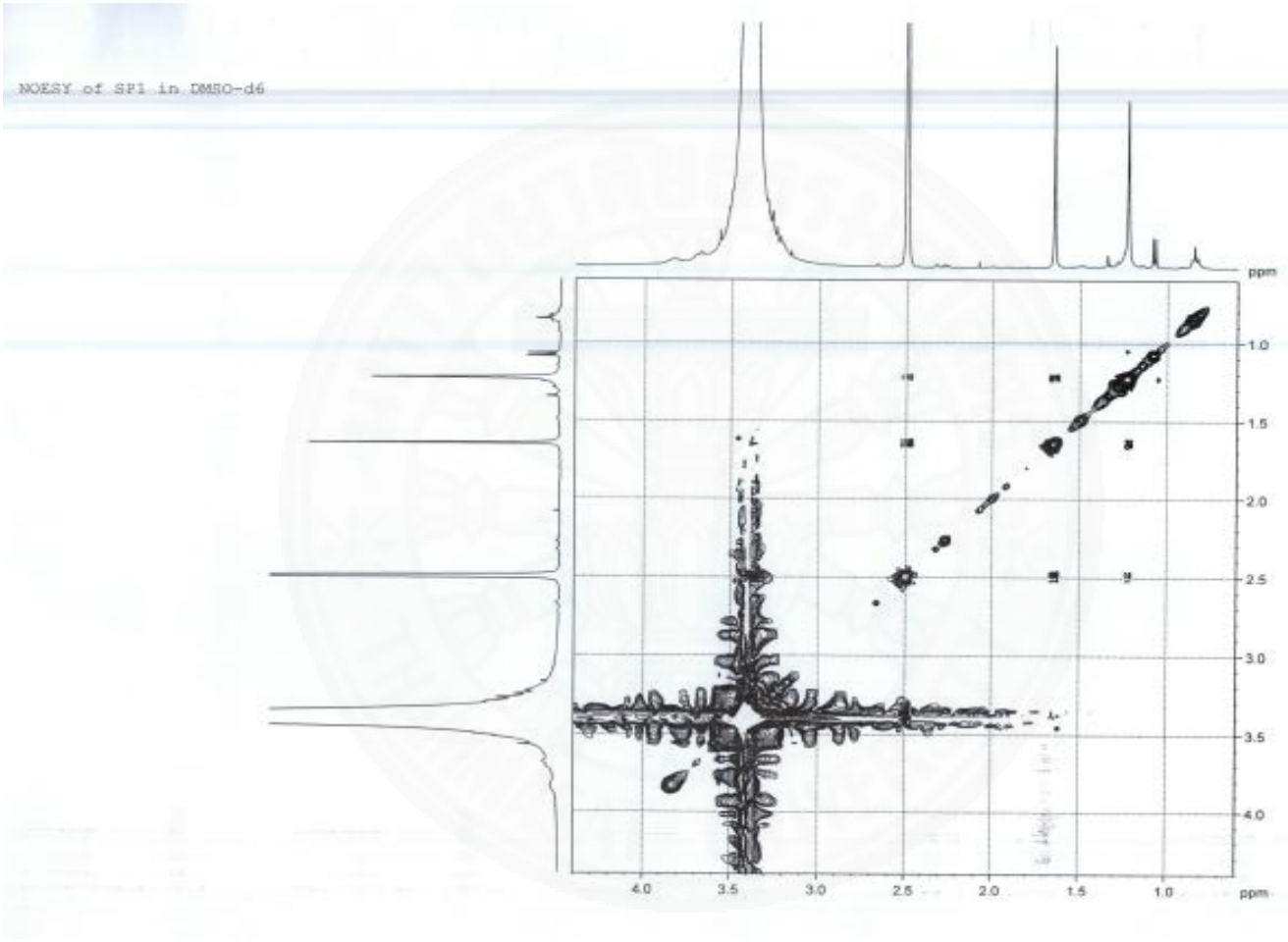

Figure A6 NOESY of SP1 (400 MHz, in DMSO-d6)

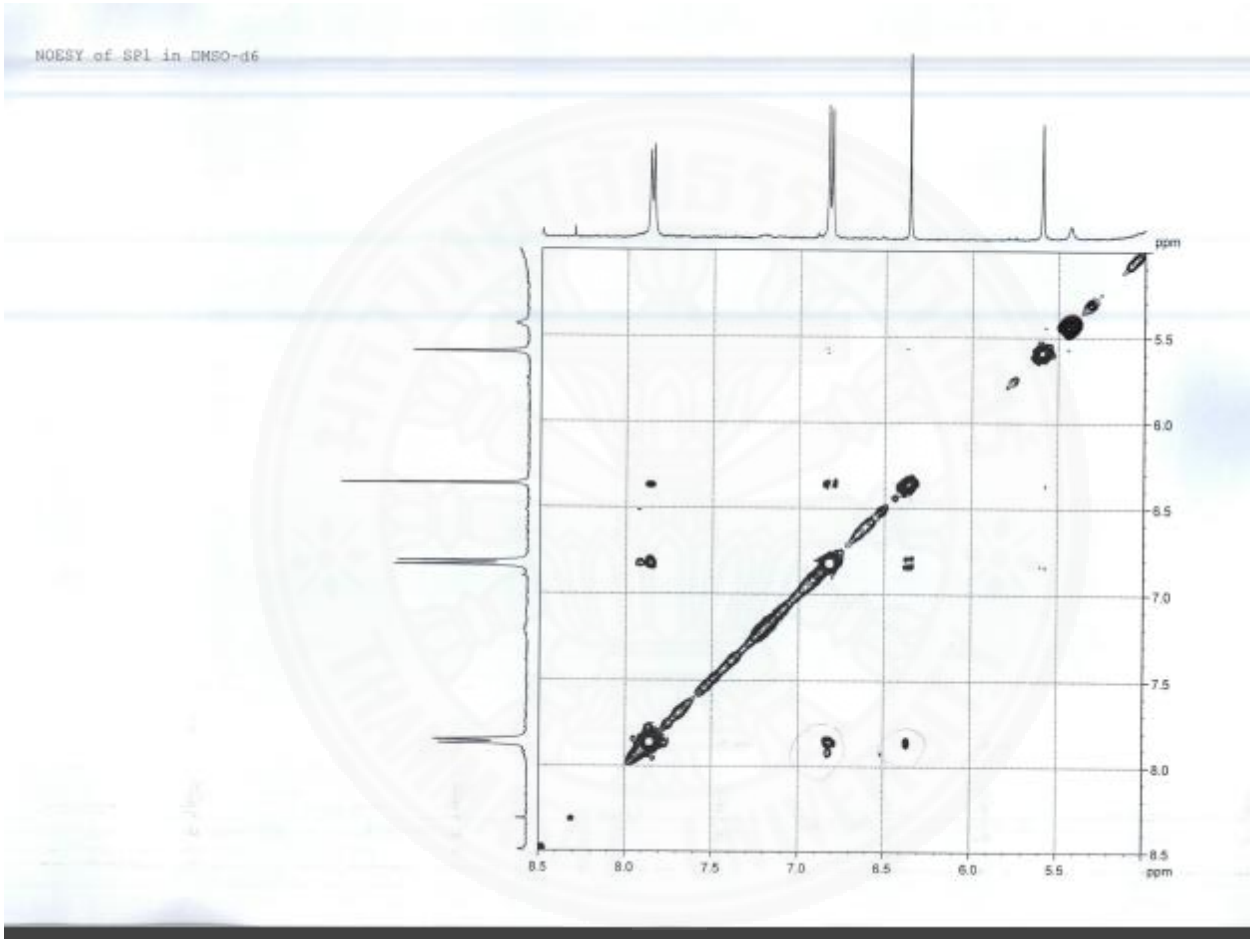

Figure A7 NOESY of SP1 (400 MHz, in DMSO-d6)

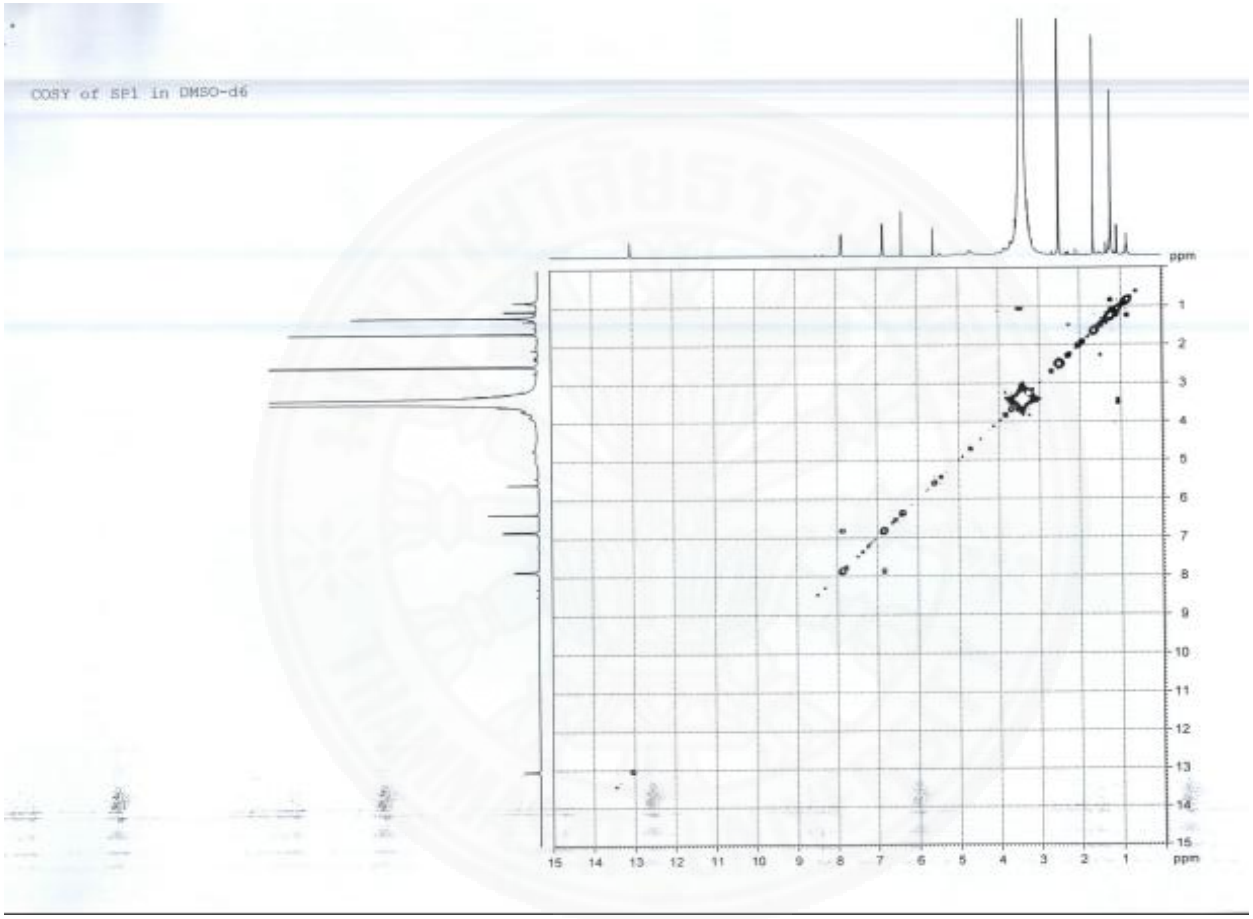

Figure A8 COSY of SP1 (400 MHz, in DMSO-d6)

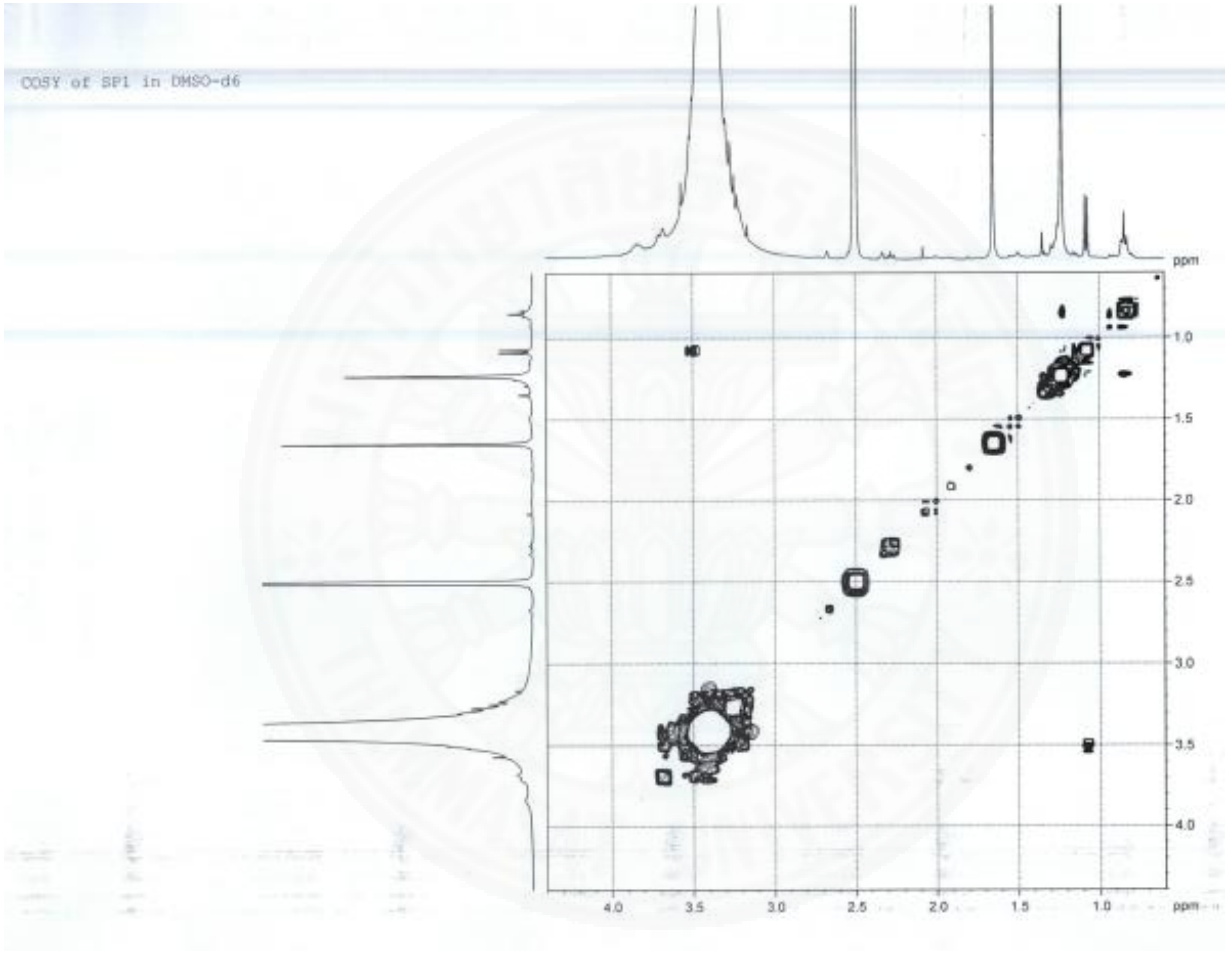

Figure A9 COSY of SP1 (400 MHz, in DMSO-d6)

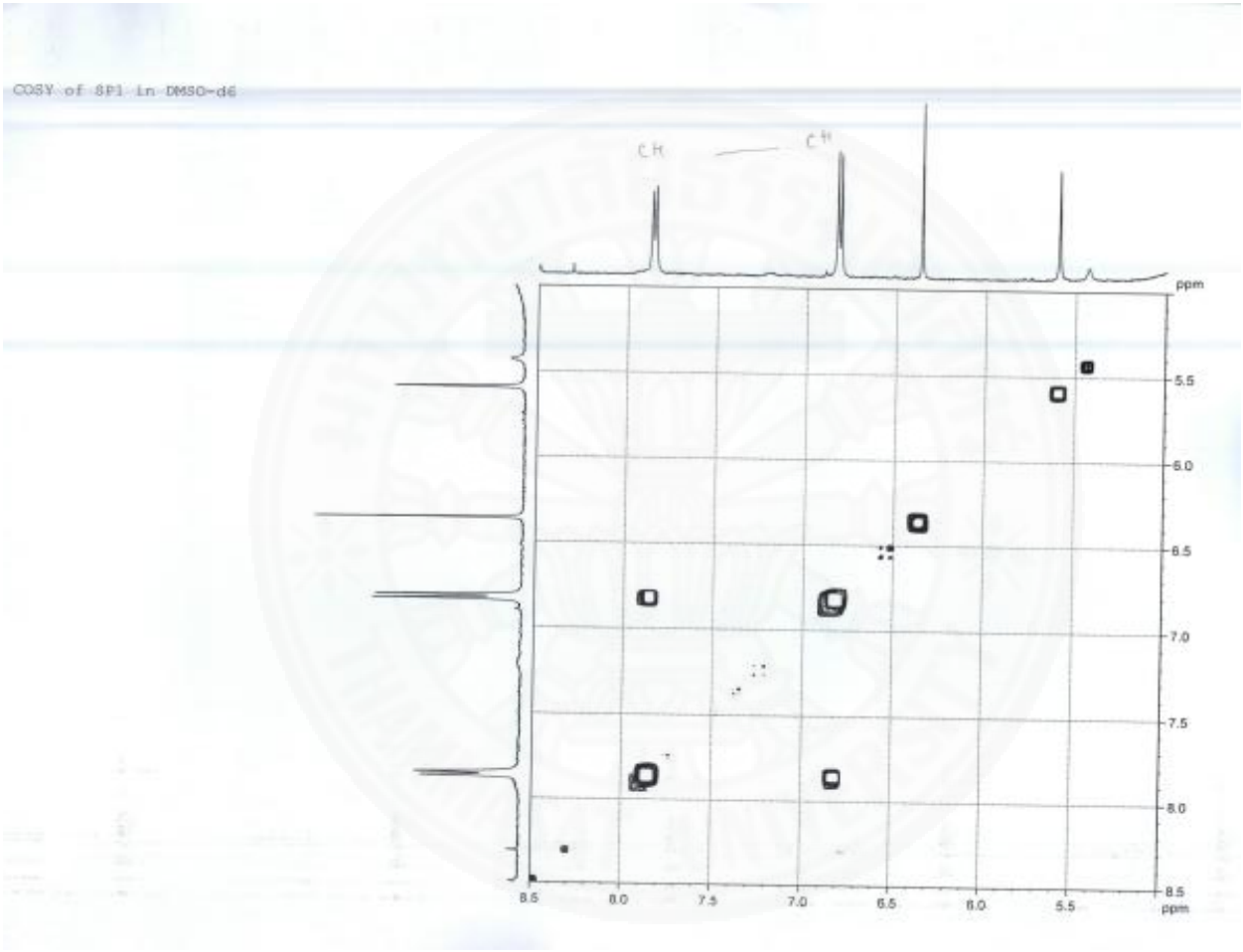

Figure A10 COSY of SP1 (400 MHz), in DMSO-d6

BIOGRAPHY

Name	Miss Khwanchanok Mokmued
Date of Birth	April 4, 1992
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	2010-2013: Bachelor of Applied Thai Medicine, Faculty of Medicine, Thammasat University

Publications and Proceedings

Mokmued, K., Ruangnoo, S., and Itharat, A. (2017) Anti-inflammatory of the ethanolic extract of Thai traditional postpartum remedy (Sa-Tri-Lhang-Klod) and plant ingredients. *Thammasat Medical Journal*. (In press).

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