MYOMETRIAL PHYSIOLOGY IN GESTATIONAL DIABETIC RATS AND EFFECTS OF SELECTED ANTI-DIABETIC THAI MEDICINAL PLANTS ON MYOMETRIAL CONTRACTILITY

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A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Biomedical Sciences

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สรีรวิทยาของกล้ามเนื้อเรียบมดลูกในหนูเบาหวานขณะตั้งท้อง และผลของสมุนไพรไทยบางชนิดที่มีฤทธิ์รักษาเบาหวานต่อการหดตัวของกล้ามเนื้อเรียบมดลูก

นางสาวศศิธร เกิดสุขนิรันดร์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy

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ศศิธร เกิดสุขนิรันดร์ : สรีรวิทยาของกล้ามเนื้อเรียบมดลูกในหนูเบาหวานขณะตั้งท้องและผลของสมุนไพรไทยบางชนิดที่มีฤทธิ์รักษาเบาหวานต่อการหดตัวของกล้ามเนื้อเรียบมดลูก (MYOMETRIAL PHYSIOLOGY IN GESTATIONAL DIABETIC RATS AND EFFECTS OF SELECTED ANTI-DIABETIC THAI MEDICINAL PLANTS ON MYOMETRIAL CONTRACTILITY) อาจารย์ที่ปรึกษา : รองศาสตราจารย์ สัตวแพทย์หญิง ดร.ศจีรา คุปพิทยานันท์, 290 หน้า.

รางจืด (*Thunbergia laurifolia* Lindl.) และหญ้าดอกขาว (*Cyanthillium cinereum* (L.) H. Rob.) มีประโยชน์ในการรักษาโรคเบาหวานทางการแพทย์แผนโบราณทั่วโลก จากการทบทวนวรรณกรรมมีข้อมูลทางวิทยาศาสตร์น้อยมากที่แสดงถึงผลของพืชดังกล่าวต่อสรีรวิทยาของมดลูกที่เกิดขึ้นในการเป็นเบาหวานขณะตั้งท้อง การศึกษาในครั้งนี้มีวัตถุประสงค์เพื่อศึกษาสรีรวิทยาของกล้ามเนื้อเรียบมดลูกในหนูเบาหวานขณะตั้งท้องเปรียบเทียบกับผลของยารักษาเบาหวานมาตรฐาน ผลของสารสกัดเอทานอลจากใบรางจืดและสารสกัดเอทานอลจากต้นหญ้าดอกขาวต่อการหดตัวของกล้ามเนื้อเรียบมดลูกของหนูขณะตั้งท้องโดยศึกษาในหลอดทดลอง ทดสอบผลของพืชทั้งสองชนิดต่อประสิทธิภาพระบบสืบพันธุ์ของแม่และผลกระทบต่อลูกในหนูเบาหวานขณะตั้งท้องโดยศึกษาในตัวสัตว์ ภาวะเบาหวานถูกเหนี่ยวนำด้วยสเตรปโตโซโทซิน (60 มิลลิกรัมต่อกิโลกรัม) ผลการทดสอบสารพฤกษเคมีเบื้องต้นด้วยแก๊สโครมาโทกราฟฟีและลิควิดโครมาโทกราฟฟี พบว่าสารสกัดใบรางจืดและสารสกัดต้นหญ้าดอกขาวมีสารประกอบฟีนอลิกสูง สำหรับการศึกษาในหลอดทดลองทำโดยการเก็บตัวอย่างมดลูกในวันที่ 21 ของการตั้งท้องในหนูที่ไม่ได้เป็นเบาหวานขณะตั้งท้องและหนูที่เป็นเบาหวานขณะตั้งท้องโดยทดสอบการหดตัวของกล้ามเนื้อเรียบมดลูก ผลการศึกษาพบว่าการหดตัวของกล้ามเนื้อเรียบมดลูกของหนูที่เป็นเบาหวานขณะตั้งท้องมีความบกพร่องของการหดตัวตามธรรมชาติ การตอบสนองต่อสภาวะโปแทสเซียมสูงและต่อฮอร์โมนออกซิโทซินลดลง และพบการปรับเปลี่ยนรูปแบบการหดตัวต่อนิเฟดิพิน Y27632 และเวิร์ตมานินลดลง สำหรับการหดตัวของกล้ามเนื้อเรียบมดลูกของหนูที่ไม่ได้เป็นเบาหวานขณะตั้งท้องพบว่ายาไกลเบนคลาไมด์มีผลกระตุ้นการหดตัวเป็นการชั่วคราวในช่วงแรกตามด้วยการคลายตัวเป็นระยะเวลานาน ยาเมทฟอร์มินมีผลเล็กน้อยต่อการหดตัวและยาอินซูลินมีผลกระตุ้นการหดตัว ซึ่งผลเหล่านี้พบตรงกันข้ามในหนูที่เป็นเบาหวานขณะตั้งท้องโดยมีการคลายตัวของกล้ามเนื้อเรียบมดลูกลดลงต่อยาไกลเบนคลาไมด์ มีการกระตุ้นการหดตัวต่อยาเมทฟอร์มินและมีการหดตัวที่แรงขึ้นต่อยาอินซูลิน สารสกัดใบรางจืดและสารสกัดต้นหญ้าดอกขาวแสดงฤทธิ์ยับยั้งการหดตัว

คุณสมบัติแบบไบเฟสิคโดยมีการกระตุ้นการหดตัวในช่วงเริ่มต้นตามด้วยการคลายตัวเป็นระยะเวลานานพบในสารสกัดต้นหญ้าดอกขาว ซึ่งเป็นผลเนื่องมาจากกลไกการออกฤทธิ์ของพืชทั้งคู่เกี่ยวข้องกับการยับยั้งการไหลแคลเซียมเข้าในเซลล์และยับยั้งการหลั่งแคลเซียมจากซาโคพลาสมิกเรติคิวลัมภายในเซลล์ ทำให้ความเข้มข้นของแคลเซียมภายในเซลล์ลดลงและพบว่าฤทธิ์ของพืชทั้งคู่ลดลงในหนูที่เป็นเบาหวานขณะตั้งท้อง สำหรับการศึกษาในตัวสัตว์ทำโดยการป้อนสารสกัดใบรางจืดและสารสกัดต้นหญ้าดอกขาวในปริมาณต่ำ (50 มิลลิกรัมต่อกิโลกรัม) และปริมาณสูง (500 มิลลิกรัมต่อกิโลกรัม) ให้กับหนูที่เป็นเบาหวานขณะตั้งท้อง ในช่วงวันที่ 7 ถึงวันที่ 21 ของการตั้งท้อง ผลการศึกษาพบว่าสารสกัดใบรางจืดและสารสกัดต้นหญ้าดอกขาวมีคุณสมบัติลดระดับน้ำตาลในเลือดได้โดยมีการฟื้นฟูกลุ่มเซลล์ของตับอ่อน มีคุณสมบัติลดระดับไขมันในเลือดที่สูงขึ้นได้ มีคุณสมบัติในการป้องกันการทำลายของตับได้และสามารถลดระดับเอนไซม์ในตับที่สูงขึ้นได้ด้วยการสร้างโครงสร้างของเนื้อตับใหม่ นอกจากนี้ยังไม่พบความเป็นพิษต่อแม่หนูและไม่พบผลกระทบต่อลูกในท้อง เป็นผลเนื่องจากน้ำหนักตัวที่เพิ่มขึ้นของแม่หนู และยังพบว่าภาวะการแท้งลูก ภาวะลูกโตช้าในขณะตั้งท้อง และภาวะลูกตายขณะคลอดมีอัตราลดลง ทั้งยังพบการเจริญเติบโตของลูกในท้องเพิ่มขึ้นด้วยการฟื้นฟูโครงสร้างของมดลูกและมีการปรับเปลี่ยนองค์ประกอบของเซลล์มดลูก ซึ่งผลเหล่านี้มีความใกล้เคียงกับผลของยาเมทฟอร์มิน (100 มิลลิกรัมต่อกิโลกรัม) ผลของสารสกัดใบรางจืดและสารสกัดต้นหญ้าดอกขาวในการศึกษาครั้งนี้สนับสนุนผลการรักษาในทางการแพทย์แผนโบราณโดยไปส่งเสริมการทำหน้าที่ของมดลูกในภาวะเบาหวานขณะตั้งท้องให้ดีขึ้นหลายประการ

สาขาวิชาปรีคลินิก ลายมือชื่อนักศึกษา Sasitorn Kerdsuknirund

ปีการศึกษา 2563 ลายมือชื่ออาจารย์ที่ปรึกษา Sajeem Kupittayanant

SASITORN KERDSUKNIRUND : MYOMETRIAL PHYSIOLOGY IN GESTATIONAL DIABETIC RATS AND EFFECTS OF SELECTED ANTI-DIABETIC THAI MEDICINAL PLANTS ON MYOMETRIAL CONTRACTILITY. THESIS ADVISOR : ASSOC. PROF. SAJEERA KUPITTAYANANT, Ph.D. (DVM), 290 PP.

GESTATIONAL DIABETIC MELLITUS/ *THUNBERGIA LAURIFOLIA* L./ *CYANTHILLIUM CINEREUM* (L.) H. ROB./ UTERINE CONTRACTION/ MATERNAL REPRODUCTIVE PERFORMANCES/ FETAL OUTCOMES

Thunbergia laurifolia Lindl. and *Cyanthillium cinereum* (L.) H. Rob. have been taken advantage of diabetic remedies in traditional medicine worldwide. There were a few data in the literature demonstrated the alteration of these plants on the uterine physiology in gestational diabetes mellitus (GDM). The aims of the present study were to demonstrate uterine smooth muscle physiology in GDM, examine the effects of ethanolic *T. laurifolia* leaves extract (TLE) and *C. cinereum* whole plant extract (CCE) on isolated pregnant rats' myometrium *in vitro* study by comparing with the effects of diabetic standard drugs and evaluate plant efficacies on maternal reproductive performances and fetal outcomes in GDM rats' *in vivo* study. GDM was induced by streptozotocin (STZ 60 mg/kg). Phytochemical screening, GC-MS, and LC-MS showed that TLE and CCE contained high phenolic compounds. For *in vitro* study, the uterine sample was collected on day 21 of gestation in non-gestational diabetic rats (Non-GD) and gestational diabetic rats (GD) for examining uterine contractility. The results implied that uterine contractility of GD was impaired in spontaneous activities,

maximal response to high K^+, and oxytocin (OT) was decreased and force production to nifedipine, Y27632, and wortmannin (WT) was also decreased. In non-GD, glibenclamide (GLB) promoted the temporary uterine contractility followed by prolonging relaxation whereas metformin (MET) slightly altered spontaneous contraction and insulin stimulated myometrial contraction. These effects were reversed in GD by decreasing the relaxation to GLB, slightly stimulating activity to MET, and producing stronger force to insulin. TLE and CCE showed tocolytic activity. A biphasic effect pattern with initial stimulation following by the prolonged relaxation was found in CCE. Their mechanisms involved the inhibition of calcium influx and calcium release from sarcoplasmic reticulum, resulting in a decrease in intracellular calcium concentration in GD. For *in vivo* study, low (50 mg/kg) and high (500 mg/kg) dosages of TLE and CCE were administrated to GD from day 7 to 21 of gestation. TLE and CCE had a hypoglycemic property with the pancreatic islet restoration, hypolipidemic property by decreasing the higher level of lipid profiles, and hepatoprotective property by diminishing the higher level of liver enzymes with hepato-architecture reconstruction. In addition, there was no developmental toxicity in maternal rats and no deleterious effects in fetuses caused by maternal body weight improvement. These implied that miscarriages, intrauterine growth restriction, and stillbirth were reduced and that fetal development was strengthened with the uterine structural regeneration and modified cellular compositions. These effects were similar to the effects of MET (100 mg/kg). In conclusion, the findings showed that TLE and CCE treatment supported their traditional uses by improving various physiological functions in GDM.

School of Preclinical Sciences Student's Signature Sasitorn Kerdsuknirund

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LISTS OF ABBREVIATIONS

$0Ca^{2+}$ solution	=	calcium-free solution
a.u.	=	absorbance unit
ADA	=	american diabetes association
ALP	=	alkaline phosphatase
ALT	=	alanine transaminase
AMPK	=	5'-AMP-activated protein kinase
ANOVA	=	analysis of variance
APA	=	appropriate for pregnancy age
AST	=	aspartate transaminase
ATP	=	adenosine triphosphate
AUC	=	area under the contraction
BaF_2	=	barium fluoride
BK	=	calcium-sensitive potassium channels
BW	=	body weight
c/s	=	cesarean section
Ca^{2+}	=	calcium ion
$CaCl_2$	=	calcium chloride
CAT	=	catalase
cGMP	=	cyclic guanosine monophosphate
CICR	=	calcium induced calcium release pathway

LISTS OF ABBREVIATIONS (Continued)

Cl^-	=	chloride ion
CL	=	corpora lutea
cm	=	centimeter
CO_2	=	carbon dioxide
COX	=	cyclooxygenase
CRL	=	crown rump length
DAG	=	diacylglycerol
DM	=	diabetes mellitus
DNA	=	deoxyribonucleic acid
EC	=	excitation-contraction coupling
EC_{50}	=	a half-maximum effective concentration
EFS	=	electrical field stimulation
ESI	=	electrospray ionization
FA	=	formic acid
FDA	=	US food and Drug Administration
FFAs	=	free fatty acids
FPG	=	fasting plasma glucose
FSH	=	follicle stimulating hormone
FTIR	=	fourier transform infrared spectroscopy
g	=	gram
GC-MS	=	gas chromatograph-mass spectrometry

LISTS OF ABBREVIATIONS (Continued)

GDM	=	gestational diabetes mellitus
GLB	=	glibenclamide
GLUT	=	glucose transporter
GnRH	=	gonadotropin-releasing hormone
GPCRs	=	G-protein couple receptors
GPx	=	glutathione peroxidase
GST	=	glutathione S-transferase
h	=	hour
H&E	=	hematoxylin and eosin
H_2SO_4	=	sulphuric acid
HbA1C	=	glycated hemoglobin
hCG	=	human chorionic gonadotropin
HCL	=	hydrochloric acid
HDL	=	high-density lipoprotein cholesterol
HEPES	=	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
hPL	=	human placental lactogen
i.p.	=	intraperitoneal injection
i.v.	=	intravenous injection
IC_{50}	=	a half-maximum inhibitory concentration
IDDM	=	insulin-dependent diabetes mellitus
IFG	=	impaired fasting glucose

LISTS OF ABBREVIATIONS (Continued)

IGT	=	impaired glucose tolerance
IP_3	=	inositol triphosphate
IR	=	infrared spectra
IRs	=	insulin receptors
IRSs	=	insulin receptors substrates
K^+	=	potassium ion
K_{ATP}	=	ATP-sensitive potassium channels
KCl	=	potassium chloride
Kg	=	kilograms
L	=	liter
LC-MS	=	liquid chromatography-mass spectrometry
LDL	=	low-density lipoprotein cholesterol
LH	=	luteinizing hormone
LPA	=	large for pregnancy age
m	=	meter
M	=	mole
MCT	=	mercury-cadmium-telluride
MET	=	metformin
mg	=	milligrams
mg/dL	=	milligrams per deciliter
$MgSO_4$	=	magnesium sulphate

LISTS OF ABBREVIATIONS (Continued)

min	=	minute
ml	=	milliliter
MLC	=	myosin light chain
MLCK	=	myosin light chain kinase
MLCP	=	myosin light chain phosphatase
mM	=	millimole
mmol/L	=	millimoles per liter
n	=	number of samples
N_2	=	nebulizer gas
Na^+	=	sodium ion
NaCl	=	sodium chloride
NGT	=	normal glucose tolerance
NIDDM	=	non-insulin-dependent diabetes mellitus
nM	=	nanomole
nm	=	millimeter
NO	=	nitric oxide
NOAEL	=	no observable adverse effect level
OCT	=	optimal cutting temperature
OECD	=	organization for economic co-operation and development
OGTT	=	oral glucose tolerance test
OT	=	oxytocin

LISTS OF ABBREVIATIONS (Continued)

P_4	=	progesterone
PG	=	prostaglandins
PGDM	=	pregestational diabetes mellitus
PGE_2	=	prostaglandin E_2
PI3K	=	phosphatidylinositol-4,5-bisphosphate 3-kinase
PIH	=	pregnancy-induce hypertension
PIP_2	=	phosphatidylinositol 4,5-biphosphate
PIP_3	=	phosphatidylinositol triphosphate
PKA	=	protein kinase A
PKC	=	protein kinase C
PLC	=	phospholipase C
PMCA	=	plasma membrane calcium-ATPase
PPAR	=	peroxisome proliferator-activated receptors
ROCs	=	receptor operated calcium channels
ROS	=	reactive oxygen species
rpm	=	revolutions per minute
RyRs	=	ryanodine receptors
S.D.	=	standard error
S.E.M	=	standard error of mean
SERCA	=	sarcoplasmic reticulum calcium-ATPase
SOCs	=	store-operated calcium channels

LISTS OF ABBREVIATIONS (Continued)

SOD	=	superoxide dismutase
SPA	=	small for pregnancy age
SR	=	sarcoplasmic reticulum
STZ	=	streptozotocin
SUR	=	sulfonylurea receptor
T1DM	=	type 1 diabetes mellitus
T2DM	=	type 2 diabetes mellitus
TC	=	total cholesterol
TNF	=	tumor necrosis factor
TRI	=	triglyceride
µg	=	microgram
µl	=	microliter
µM	=	micromole
µm	=	micrometer
μm^2	=	square micrometer
U/L	=	unit per liter
VOCs	=	voltage operated calcium channels
WHO	=	world health organization
WT	=	wortmannin

CHAPTER I

INTRODUCTION

Pregnancy in women with diabetes is complicated the increases risk of obstetric and neonatal complications, morbidity, and mortality. The higher risk of late fetal loss, perinatal death, and stillbirth are shown in preexisting gestational diabetes mellitus (GDM) mothers. The incidence of emergency cesarean section (c/s) delivery and maternal-fetal complications are significantly increased in GDM which compared with normal pregnancy, resulting from the impairment of myometrial contractility. In addition, several classes of anti-diabetic drugs and a large number of medicinal plants have been widely used during pregnancy which primary attempt to control diabetes. However, there were fewer studies exhibited the diabetic alteration and the effects of these herbal supplementations on the general function of uterine smooth muscle physiology during gestation. Therefore, this thesis aimed to tested the uterine function in gestational diabetes, as well as the bioactive phytoconstituents from *Thunbergia laurifolia* (L.) and *Cyanthillium cinereum* (L.) H. Rob., the traditional anti-diabetic plants, and demonstrated its interfering patterns on maternal reproductive performances and pregnancy-related reproductive behaviors including contractile response, associated tissue morphology, maternal and fetal outcomes in chemical agent-induced diabetes during gestation in laboratory animal models.

1.1 Diabetes mellitus

World Health Organization (WHO, 1998) defines diabetes mellitus as a metabolic disorder of multiple etiologies characterized by chronic hyperglycemia with disturbances of carbohydrate, protein, and fat metabolism resulting from defects in insulin secretion, insulin action, or both. American Diabetes Association (ADA, 2010) suggested there are many different types of diabetes with an individual type depends on the presence at the time of diagnosis and it is so important that understanding the pathogenesis and effective treatment is a crucial role for diabetic patients. ADA diabetes guidelines in 2016 recommended that there are 4 general classification forms of diabetes as follow:

1) Type 1 diabetes mellitus (T1DM) or insulin-dependent diabetes mellitus (IDDM) is the complete insulin deficiency caused by autoimmune destruction of the insulin-producing pancreatic β-cells which eliminates the production of insulin and can diagnose at any age but mostly develops in children (5-10% of all diagnosed diabetes) (Atkinson et al., 2014; Gillespie, 2006).

2) Type 2 diabetes mellitus (T2DM) or non-insulin-dependent diabetes mellitus (NIDDM) is commonly found in adults (90% of all diagnosed diabetes) and represents insulin resistance resulting from the body cannot produce enough insulin or inability to respond to the action of insulin. The pathogenesis of T2DM reveals the reductions in both insulin sensitivity and insulin action along with hyperglycemia. All of these factors are contributed to the progression from normal glucose tolerance (NGT) to impaired glucose tolerance (IGT) and ultimately diabetes (Weyer et al., 1999).

3) Gestational diabetes mellitus (GDM) represents various degrees of hyperglycemia with onset or first recognition during pregnancy, whether or not the

condition persisted after pregnancy. Glucose intolerance occurs normally during pregnancy, particularly in the 3rd trimester. Nearly 90% of all pregnancies are complicated by diabetes and associated with adverse maternal and neonatal outcomes which increased the rate of morbidity and mortality (White, 1949).

4) Other categories of glucose regulation are diabetes that cannot classify into a single class, for example, maturity-onset diabetes of the young cause form genetic defects of pancreatic function, the high blood glucose level in the pregnant diabetic woman after delivery who prefer to T2DM, progressive exocrine pancreas injuries, drugs or chemicals induced diabetes, β-cell destruction from the certain viruses' infection, and immune-mediated diabetes (WHO, 1998).

1.2 Diabetes and pregnancy

Diabetes in pregnancies can modify belong to the epidemiological and clinical purposes that simply recommended by the WHO and in association with the National Diabetes Data Group (NDDG) as pregestational diabetes mellitus (PGDM), GDM, and any types of DM occurring first in pregnancy (Krishna Murthy et al., 2002).

1.2.1 Pregestational diabetes mellitus

The PGDM population is varied in its basic ethnic characteristics. For example, two-thirds of type 1 PGDM and one-third of type 2 PGDM are found in the French population. In contrast, two-thirds of type 2 PGDM and only one-third of type 1 PGDM are reported in the American population. The Mexican population exhibits 90% of type 2 PGDM and 10% of type 1 PGDM (Forsbach-Sánchez et al., 2005). A recent systemic review reported that the severe glycemic disturbance in pregnant women with T2DM is less than those with T1DM whereas a worse perinatal and

neonatal mortality complicated in T2DM more than T1DM, indicating that T2DM in pregnancy is a serious condition (Balsells et al., 2009). Non-pregnant women with diabetes or intermediate hyperglycemia or impaired fasting glucose (IFG) or IGT are defined PGDM which is considered diabetes in pregnancy by WHO standard criteria for diagnosed diabetes guidelines in 2014.

The clinical classification method to access diabetes in pregnancy is based on age at the onset of diabetes, duration of the disease, and the presence or absence of atherosclerotic, vascular disease, and renal complications which were established by Priscilla White's researched to predict the course of treatment for the diabetic pregnancy and the chance of newborn survival (White, 1949). This classification is useful for pregnancy prognosis with dramatically decrease the percent of fetal survival along with the severity of maternal diabetes such as 100%, 67%, 48%, 32%, 13%, and 3% of fetal survival in class A, B, C, D, R, and F, respectively (Forsbach-Sánchez et al., 2005) as described in Table 1.1.

Table 1.1 White's classification of two classes of diabetes during pregnancy modified by Pedersen's hypothesis (Pederson, 1968).

Class	Descriptions
A_1	Gestational diabetes with normal fasting plasma glucose (FPG) and postprandial plasma glucose
A_2	Gestational diabetes with FPG > 105 mg/dL, or 2-h postprandial plasma glucose > 120 mg/dL, or 1-h postprandial plasma glucose > 140 mg/dL
B	Overt diabetes developing at age-onset ≥ 20 years and duration < 10 years
C	Overt diabetes developing at age-onset 10-19 years or duration 10-19 years
D	Overt diabetes developing at age-onset < 10 years or duration ≥ 20 years or background retinopathy or hypertension
R	Overt diabetes with proliferative retinopathy or vitreous hemorrhage
F	Overt diabetes with nephropathy with proteinuria > 500 mg/day
RF	Overt diabetes with criteria for both R and F classes coexists
H	Overt diabetes with arteriosclerotic heart disease
T	Overt diabetes with prior renal transplantation

Class A_1 requires diet alone; Class A_2-T requires diet combine with insulin therapy; Class R, F, RF, H, and T have no criteria for age at onset or duration of diabetes, but usually exhibited long term complications.

1.2.2 Gestational diabetes mellitus

Approximately, there are 2-7% of all pregnant women complicated by diabetes worldwide. The prevalence of GDM in the United State population is increased

as high as 9.2% and affected 1-14% of all pregnancies annually (DeSisto et al., 2014). Nearly 90% of all pregnancies refer to GDM (ADA, 2010). In addition, a 60% reduction in insulin sensitivity and hyperinsulinemia in normal pregnancy are developed in second and third trimesters with contributed to inadequate insulin reserve and promote clinical hyperglycemia or GDM (Catalano, 2014; Ryan, 2002). Pregnant women who having hyperglycemia have been reported as 90-95% of diabetes-free in a standard 2-h 75g oral glucose tolerance test (OGTT) after giving birth, 4-9% of T2DM diagnosis in 6-12 weeks, more than 20% are IGT or IFG or both as prediabetes, 30% present metabolic syndrome in 36 months, 50% ultimately T2DM in 5 years and 2.6-70% increased cumulative risk over past 10 years (Inturrisi et al., 2011). The Hyperglycemia and Adverse Pregnancy Outcome (HAPO study, 2008) also suggest that the risk of adverse maternal, fetal and neonatal outcomes continuously increased depend on maternal hyperglycemia in 24-28 weeks, even within normal pregnancy.

Previous WHO (2014) diagnostic criteria for GDM and ADA (2014) are based on the risk of adverse pregnancy outcomes with needed to screen and prevent adverse outcomes. GDM should be diagnosed at any time during pregnancy with initial screening risk factors followed by glucose testing as soon as possible. Silently undiagnosed T2DM or undetected GDM in the first parental visit should be retested using standard criteria undertaken 24-28 weeks of gestation in pregnant women who have not previously known diabetes. However, one-third to one-half of GDM fails to screen the high-risk factors. Recently, the screening test consists of high-risk and low-risk characteristics of GDM patients. Furthermore, GDM women should screen for persistent DM at 6-12 weeks postpartum, using OGTT and non-pregnant standard

criteria. For a long time, women with GDM history should have lifelong careening for the development of DM or pre-DM at least every 3 years (Krishna Murthy et al., 2002).

1.2.3 Diagnosis criteria for diabetes mellitus

Various screening tests for diabetic patients are used by direct measuring the level of glucose in the bloodstream. According to the ADA guidelines in 2010, overnight FPG levels and 2-h values in OGTT measure the body's ability to properly handle the excess glucose presented after high glucose drinking is primarily performed. Moreover, the basic provisional diagnosed criteria for diabetes possibly starts with the classic symptoms of diabetes (especially, polyuria, polydipsia, and uncertain weight loss) are shown with casual plasma glucose concentration at any time of day without regard to time since last meal ≥ 200 mg/dL (11.1 mmol/L), followed by overnight at least 8-h FPG level ≥ 126 mg/dL (7.0 mmol/L), or OGTT ≥ 200 mg/dL using a 75-g glucose loading by WHO protocol. Both IFG level between 100-125 mg/dL (5.6-6.9 mmol/L) and IGT level between 140-199 mg/dL (7.8-11.1 mmol/L) are increased risk factors for future diabetes as considering to prediabetes (ADA, 2010). Recently, an International Expert Committee (2009) also recommended the A1C test based on the attachment of glucose to hemoglobin within 3 months of life expectancy. The A1C test is reported in the percentage of the average blood glucose level over the past 3 months with no fasting and can apply any time of the day. Diabetes should be diagnosed when A1C is ≥ 6.5%, prediabetes is between 5.7-6.4%. Nowadays, ADA and WHO suggest the GDM recommendations with different diagnostic criteria will identify different magnitudes of maternal hyperglycemia, maternal and fetal risk as FPG > 126 mg/dL if any degree of hyperglycemia absent, GDM women with high-risk characteristics should follow OGTT in one-step or two-step strategies as seen in Table 1.2.

Table 1.2 The guidelines for the diagnosis of hyperglycemia in pregnancy following ADA recommendations (2014).

One-step strategy (IADPSG consensus)			
75-g OGTT	Performed in the morning, after an overnight fast at least 8 hours Glycemic targets for GDM are met by any values exceeded • Fasting: ≥ 92 mg/dL (5.1 mmol/L) • 1-h: ≥ 180 mg/dL (10 mmol/L) • 2-h: ≥ 153 mg/dL (8.5 mmol/L)		
Two-step strategy (NIH consensus)			
Step 1: 50-g OGTT	Non-fasting • 1-h ≥ 140 mg/dL (7.8 mmol/L) *		
Step 2: 100-g OGTT	Fasting Glycemic targets for GDM are met at least 2 values exceeded		
		Carpenter/Coustan	or NDDG
	• Fasting	95 mg/dL (5.3 mmol/L)	105 mg/dL (10 mmol/L)
	• 1-h	180 mg/dL (10 mmol/L)	190 mg/dL (10.6 mmol/L)
	• 2-h	155 mg/dL (8.6 mmol/L)	165 mg/dL (9.2 mmol/L)
	• 3-h	140 mg/dL (7.8 mmol/L)	145 mg/dL (8 mmol/L)

IADPSG = International Association of Diabetes and Pregnancy Study Groups, NIH = National Institutes of Health, NDDG = National Diabetes Data Group, *The American College of Obstetricians and Gynecologists (ACOG) suggests a lower threshold of 135 mg/dL (7.5 mmol/L) in higher prevalence of GDM, some experts also recommend 130 mg/dL (7.2 mmol/L).

1.2.4 A diabetogenic state of pregnancy

Several physiologic alterations in the period of normal pregnancy are affected glucose homeostasis, resulting in insulin resistance, hyperinsulinemia, and mild post-prandial hyperglycemia which are mediated by the enhanced pancreatic β-cells function and the alteration of the counter-regulatory hormones from placental secretions. For example, progesterone reduced the expression of the insulin receptor, leading to inhibited insulin-induced glucose transporter 4 (GLUT4) translocation and decreased glucose uptake. High estrogen level in normal pregnancy is diminished insulin sensitivity. Likewise, human placental lactogen (hPL) is generated lipolysis and free fatty acids (FFAs) in adipocyte tissues which increased glucose uptake as glycogen storage in the mother. In recent years, two other factors, tumor necrosis factor α (TNFα) and leptin can interrupt the signaling of insulin and propose as mediators of insulin resistance during normal pregnancy, particularly in late pregnancy, as well as, serum cortisol and growth hormone. On the other hand, normoglycemia can be present after delivery by decrease the level of counter-regulatory hormones but may increase the prevalence of developing T2DM in the mother. However, the maternal adaptation of insulin resistance serves as a progressive condition to meet the maternal and fetal demands of nutrition and rapid fetal growth (Inturrisi et al., 2011; Sonagra et al., 2014).

Basal and postprandial glucose metabolisms are changed throughout pregnancy because of the maternal insulin concentrations, starting at the end of the first trimester that defined as an anabolic state of fat stored and 15-20% insulin sensitivity enhancement (Lain and Catalano, 2007). In addition to maternal hyperglycemia during advanced gestation, impairment of intercellular performance in the offspring may correlate with the increasing demand of fetuses for amino acids and glucose utilization

as a catabolic state. About 15-30% of maternal hepatic glucose production by glycogen depletion and lipolysis are found in late third pregnancy with fetal growth accelerated. Amino acids are transported to fetal circulation with 3-4 folds of maternal concentration. Fetal feeding is accompanied by maternal fasting and feeding state. Glucose is transported by facilitated diffusion from GLUT1 across the placenta to the fetus, but the insulin is not across the placenta. Maternal glucose is stimulated insulin synthesis and secretion in 9-12 weeks of gestation with a decreased requirement of maternal insulin. The reasons for this reduction are not well explained. The fetal β-cells activity is responded to the level of maternal blood glucose and amino acids with fetal hyperglycemia or hypoglycemia and hyperinsulinemia, resulting in the increased incidence of adverse pregnancy outcomes (Inturrisi et al., 2011; Krishna Murthy et al., 2002) as a summary diagram in Figure 1.1.

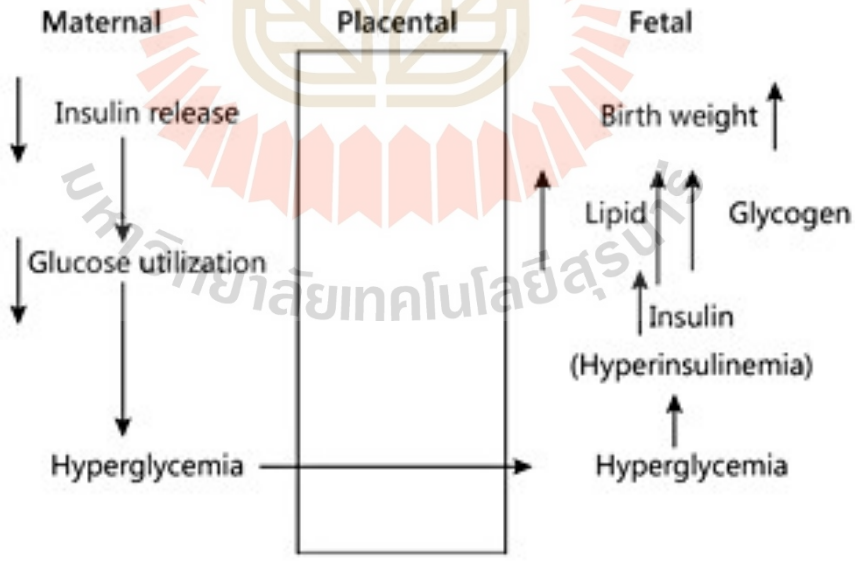

Figure 1.1 Diagram of maternal hyperglycemia modified by Pedersen's hypothesis (Pederson, 1967).

1.3 Uterus and its function

Female reproductive organs have consisted of vagina, uterus, fallopian tubes, and ovaries which are responsible for the physiologic changes of menstruation, pregnancy, and menopause. Structurally, the uterus (womb) is a dynamic hollow muscular and pear-shaped organ that located above the vagina, behind the bladder, and in front of the rectum in the pelvic cavity. The upper part of the uterus is the corpus that connects laterally to uterine tubes and the narrow lower part is the cervix that connects distally to the vagina. The simple uterus with a single chamber for the development of a single embryo is found in humans. In contrast, the duplex uterus with two uterine horns jointed together and open into the vagina is commonly formed in some animals which is available for multiple embryos per litter. In addition, the uterus consists of the following two main layers, the inner layer is the endometrium and the outer thick muscular layer is the myometrium. The coating thin layer outside the uterus is the perimetrium or serosa. In detail, the endometrium is a well-differentiated single-layered columnar epithelium with a cell-rich connective tissue (stroma) that surrounds the uterine glands. The myometrium is made up of uterine volume, consists of bundles of smooth muscle fibers, and intermixes with blood vessels, lymphatic vessels, and nerves. The main function is the modulation of the regular spontaneous contraction. Both layers are played an important role in many functions under the cyclic influence of ovarian sex steroid hormone alterations throughout menstruation, pregnancy, and parturition (Aguilar and Mitchell, 2010; Naftalin and Jurkovic, 2009).

1.3.1 Female reproductive cycle

Briefly, the 28 days average range of the menstrual cycle is regulated by cyclic hormonal changes. The hypothalamic-pituitary-gonadal axis is the reproductive hormonal axis which generates gonadotropin-releasing hormone (GnRH), in turn, stimulates the synthesis and release of gonadotropic hormones such as follicle-stimulating hormone (FSH) and luteinizing hormone (LH) which impacts the sex-sensitive end organs such as the testis in male, the ovary in female, and controlled by biofeedback system. The 4 main phases of the menstrual cycle are 1) Menstruation, 2) Follicular or proliferative phase, 3) Ovulation, and 4) Luteal phase or secretory phase (Farage et al., 2009) as seen in Figure 1.2.

Menstruation is defined as the elimination of the thickened endometrium of the uterus through the vagina as menstrual bleeding; generally, lasts between 4-6 days. The first half of the cycle is dominated by the follicular or proliferative phase, starts on the first day of menstruation to the end of ovulation. FSH will stimulate primary follicular growths and induces estrogen secretion by controlling aromatase action in granulosa cells with an increase in estrogen receptor and size of endometrial glands. In the mid-late phase, LH also encourages the production of androgens by theca cells, passes onto granulosa cells, and then converts to estrogen. The increased level of estrogen is an inhibition to GnRH and sustains decrease levels of FSH and LH. The continued follicular growth build-up the thicken endometrium and one of the follicles matures into an egg called the Graafian follicle. Peak estrogen level exerts positive feedback on the LH surge for ovulation followed by a rapid decline. At ovulation, the Graafian follicle will swell and rupture with fluid and oocyte released. Oocyte enters the uterine tube by muscular contraction. A typical egg stays in the fallopian tube only

around 24 hours for fertility, if not it will degrade. In the second half of the luteal phase after ovulation, the granulosa cells, theca cells, and remaining tissues are converted into the corpus luteum (CL) that promotes a large amount of progesterone synthesis and secretion with a small among of estrogen secretion. Progesterone causes endometrium changes into the secretory phase, dilation of tortuous glands, and coiled blood vessels that are prepared for the implantation of a fertilized egg. Peak progesterone level exerts in 5-7 days post-ovulation with negative feedback to GnRH, result in dramatically diminished FSH and LH level. After 2 weeks around day 21, CL begins involute with both progesterone and estrogen levels declined, then the thickened endometrium is disintegrated for the next menstruation (Lim and Wang, 2010).

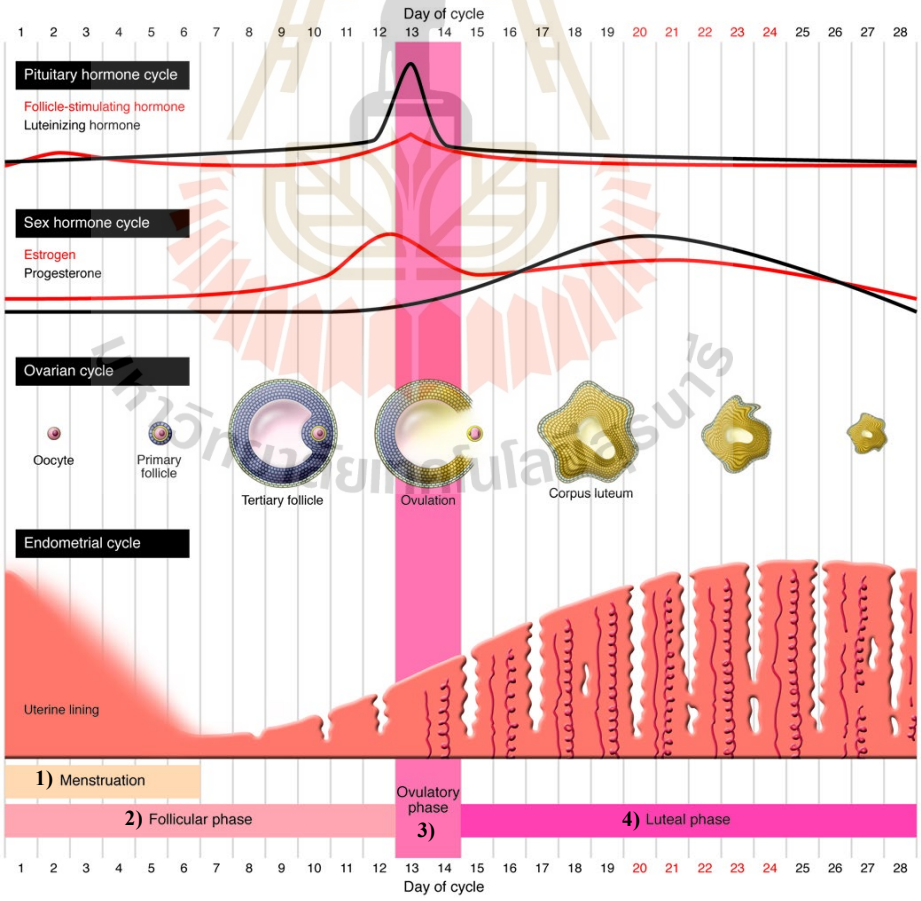

Figure 1.2 The changes during the menstrual cycle (Lim and Wang, 2010).

1.3.2 Estrous cycle

In rodents, the female reproductive status undergoes the estrous cycle same as the menstruation in humans. Rats and mice typically have spontaneous ovulate in 4-5 days of the reproductive cycle throughout their life. The fluctuation in hormone levels during the cycle is altered by the types and number of cells present in the vagina cytology. The relative ratio of cell types in vagina smears can identify the various stages of the estrous cycle (McLean et al., 2012).

The estrous cycle is divided into 4 stages as seen in Figure 1.3: 1) Proestrus is exhibited animals will come on the heat with most highly receptive time for mating at the end stage and the next following day, vagina smears' cells are exhibited dense oval nucleated epithelial cells, 2) Estrous is in the heating time, irregular-shaped cornified squamous epithelial cells can detect, 3) Metestrus is presented in the absence of conception when estrus changes in the reproductive tract subside, cells are fragmented cornified cells with small darker stained leukocytes and 4) Diestrous is demonstrated reproductive tract will prepare for implantation. Another one, anestrous is a non-breeding season with quiescent of the reproductive organ, cells are predominated leukocytes, reduced cornified cells, and in turn presence of nucleated epithelial cells. After the fourth postnatal week, the onset of puberty in females occurs following the pre-ovulatory increase in estradiol levels and LH surge in 8-9 days before ovarian maturation in the first proestrus stage. Ovulation occurs in 10-12 hours during the night of the estrous stage. Progesterone levels start to rise and a small surge in estradiol levels in response to CL activation during the metestrus stage in non-mated females. Finally, circulating progesterone peak and enter into the diestrus stage. The

regression of CL causes a sharp decline in the progesterone level (McLean et al., 2012; Westwood, 2008).

The estrous cycle is characterized by morphological changes in ovaries, uterus, and vagina (Westwood, 2008). The structural changes are observed in the vaginal epithelium of female rats during the estrous cycle are induced by estrogen and progesterone are presented in Table 1.3.

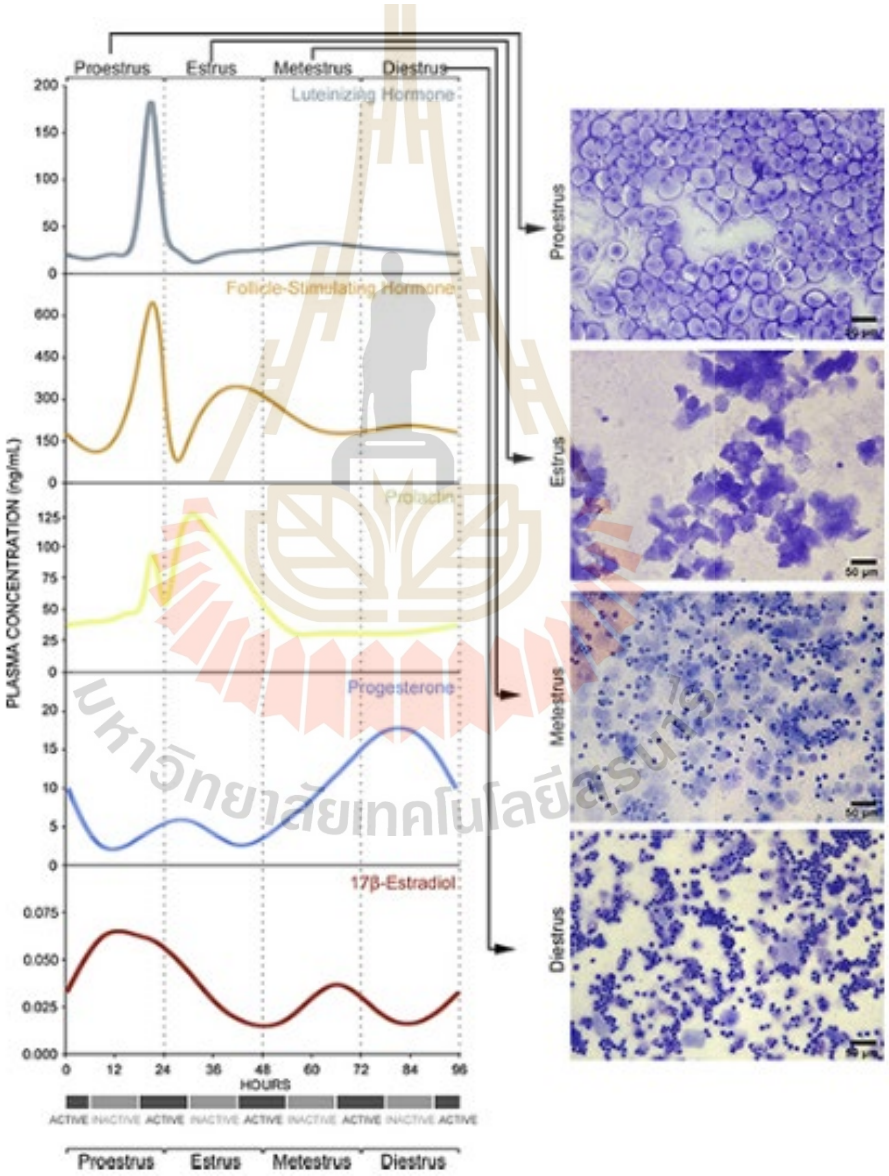

Figure 1.3 The fluctuation in hormone level and vagina smear cytology in 4 days estrous cycle (McLean et al., 2012).

Table 1.3 Summary of the defining histological features of the rat female reproductive tract during the estrous cycle (Westwood, 2008).

Phase	Vagina	Uterus	Ovaries
Diestrous	Start defined by epithelium at the lowest level with variable leukocyte infiltration. Subsequent epithelial proliferation and thickening (no clear stratum granulosum) with a reduction in leukocyte infiltration.	Small, avascular, slit-like lumen. Lined by low columnar epithelium. Initially few mitoses, but some increase during a phase. Only occasional degenerate cells. Stromal edema at the end of the stage.	Large CL. May be finely vacuolated. Fibrous tissue formation in the central cavity.
Proestrous	Mitotic figures are present. Occasional polymorphs. Little if any degeneration or desquamation. Formation of stratum granulosum (defines start), superficial mucoid layer, and stratum corneum progressively. At end of the stage, fully cornified and generally showing a superficial mucoid layer with some desquamation of mucoid cells.	The epithelium is cuboidal to columnar. Mitoses present in epithelial cells with little or no degeneration and little inflammatory cell infiltration. Dilatation, particularly toward the end of the stage.	CL often degenerates. Cytoplasmic vacuoles are generally present. Fibrous tissue proliferation in the central cavity.
Estrous	Progressive shedding of superficial mucoid and cornified layers. Reduction in height of epithelium. Cell debris present. Loss of mitotic figures. Progressive leukocyte infiltration.	The start of estrus is defined by the appearance of notable degeneration/ necrosis of epithelial cells, glands generally first. Loss of mitotic activity. Leukocyte infiltration. Dilatation may persist in late estrus.	Degenerate CL is often present. Some small CL with basophilic cell cytoplasm, central fluid-filled cavity, and no fibrous tissue.
Metestrous	Start defined by a virtually complete detachment of the cornified layer. Continued desquamation with loss of stratum granulosum and upper germinativum. Leukocyte infiltration.	Continued degeneration of endometrial epithelial cells. Return of mitotic activity; both (mitotic activity and degeneration) are seen together.	CL may still contain a fluid cavity. Smaller than at diestrus. Slightly basophilic cells. Generally devoid of fibrous tissue.

1.3.3 Myometrial force production

Uterine contractions are stimulated by uterine myocytes which are control by 4 important parameters including frequency, amplitude, duration, and direction of propagation. The uterus can generate spontaneous contractions without any agonist stimulation such as hormonal or nervous input and manifest both tonic and phasic contractions in various conditions (Wray et al., 2001). The normal contraction of uterine smooth muscle is phasic with a relatively short-lasting and fast-relaxing period between itself. On the other hand, the weak intensity and irregular pattern are found in early gestation to maintain the conceptus, while the regular pattern with the strong contractions has been adapted during the time of labor (Al Otaibi, 2014).

Actually, in the Ca^{2+}-Calmodulin-MLCK pathway, it is well known that the contraction of myometrial cells is a direct consequence of membrane depolarization in the excitation-contraction (EC) coupling process that triggered by an action potential, same as many other smooth muscles (Garfield and Maner, 2007). Intracellular calcium (Ca^{2+}) concentration is the key regulatory factor to induced uterine contraction which is generated from 2 sources: 1) Ca^{2+} entry through the opening of voltage-operated Ca^{2+} channels (VOCs) which is almost entirely via L-type Ca^{2+} channels and receptor-operated Ca^{2+} channels (ROCs), and 2) release from an internal Ca^{2+} stored, the sarcoplasmic reticulum (SR) via inositol triphosphate (IP_3) channels by its second messenger and the ryanodine receptors (RyRs) by Ca^{2+} itself activation in the Ca^{2+} induced Ca^{2+} release (CICR) pathway. Principally, free Ca^{2+} ions influx into the cell and bind to the calmodulin protein that activates the myosin light chain kinase (MLCK) to initiate the myosin light chain (MLC_{20}) phosphorylated, subsequent cross-bridge cycling with significant interaction between myosin and actin filaments, hydrolysis of

ATP by myosin ATPase activity and promote the uterine contraction in the Ca^{2+}-Calmodulin-MLCK pathway (Kupittayanant et al., 2002; Matthew et al., 2004; Wray, 2007).

Furthermore, EC coupling in myometrium undergoes 2 main mechanisms: electrochemical and pharmacomechanical coupling. In electrochemical coupling, the transient increase in intracellular Ca^{2+} concentration can be modulated by several ion channels involved in membrane potential regulation. The excitability of myocytes is defined by a large movement of sodium (Na^{+}), calcium (Ca^{2+}), and chloride (Cl^{-}) ions into the cytoplasm with the movement of potassium (K^{+}) ions outward the cytoplasm which generates the action potential, membrane depolarization, and then consequent opening of VOCs in contractile process. However, the membrane is permeable to K^{+} ion down its gradients and activation of Ca^{2+}-sensitive K^{+} (BK) channels that cause the blockade of VOCs, limiting Ca^{2+} entry, inducing stored-depletion while sparks store-operated Ca^{2+} channels (SOCs) at the membrane and refilling Ca^{2+} in the internal stored. For pharmacomechanical coupling, agonists activation on the specific receptors of G-protein coupled receptors (GPCRs) on the plasma membrane results in the opening of ROCs and VOCs. Besides, GPCRs are also stimulated phospholipase C (PLC) activity to cleave phosphatidylinositol 4,5-biphosphate (PIP_2) into diacylglycerol (DAG) and IP_3. Subsequently, IP_3 binds to its receptors in the SR membrane and thereby releases Ca^{2+} from the internal stored with elevated cytosolic Ca^{2+} concentration (Aguilar and Mitchell, 2010; Al Otaibi, 2014; Wray, 2007) as shown in Figure 1.2(A).

Another pathway, the free Ca^{2+} activator can control smooth muscle contraction as the non-Ca^{2+}-Calmodulin-MLCK pathway in Figure 1.2(A). The potent second messenger, DAG activates PKC along with or without Ca^{2+} that initiates the

contraction-promoting effect of PKC by phosphorylates specific proteins such as L-type Ca^{2+} channels or cross-bridge cycling regulated protein. Similarly, the tonic force of contractile is responded to the mechanism of Ca^{2+} sensitization by the inhibition of the enzymatic activity of myosin light chain phosphatase (MLCP) which promoting the phosphorylated state of myosin light chain, and by the regulation of MLCP in Rho kinase activity (Webb, 2003).

1.3.4 Myometrial force relaxation

Differently, smooth muscle relaxation is followed by the removal of the contractile stimulus as Ca^{2+} withdrawal from the Ca^{2+}-Calmodulin-MLCK pathway and the direct inhibition of the contractile mechanisms such as dephosphorylate activity of MLCP, decrease Ca^{2+} concentration in the response of the closer VOCs and ROCs, and Ca^{2+} efflux mechanisms initiated through a plasma membrane Ca^{2+}-ATPase (PMCA), SR Ca^{2+}-ATPase (SERCA) and Na^{+}/Ca^{2+} exchanger (Webb, 2003) as shown in Figure 1.2(B).

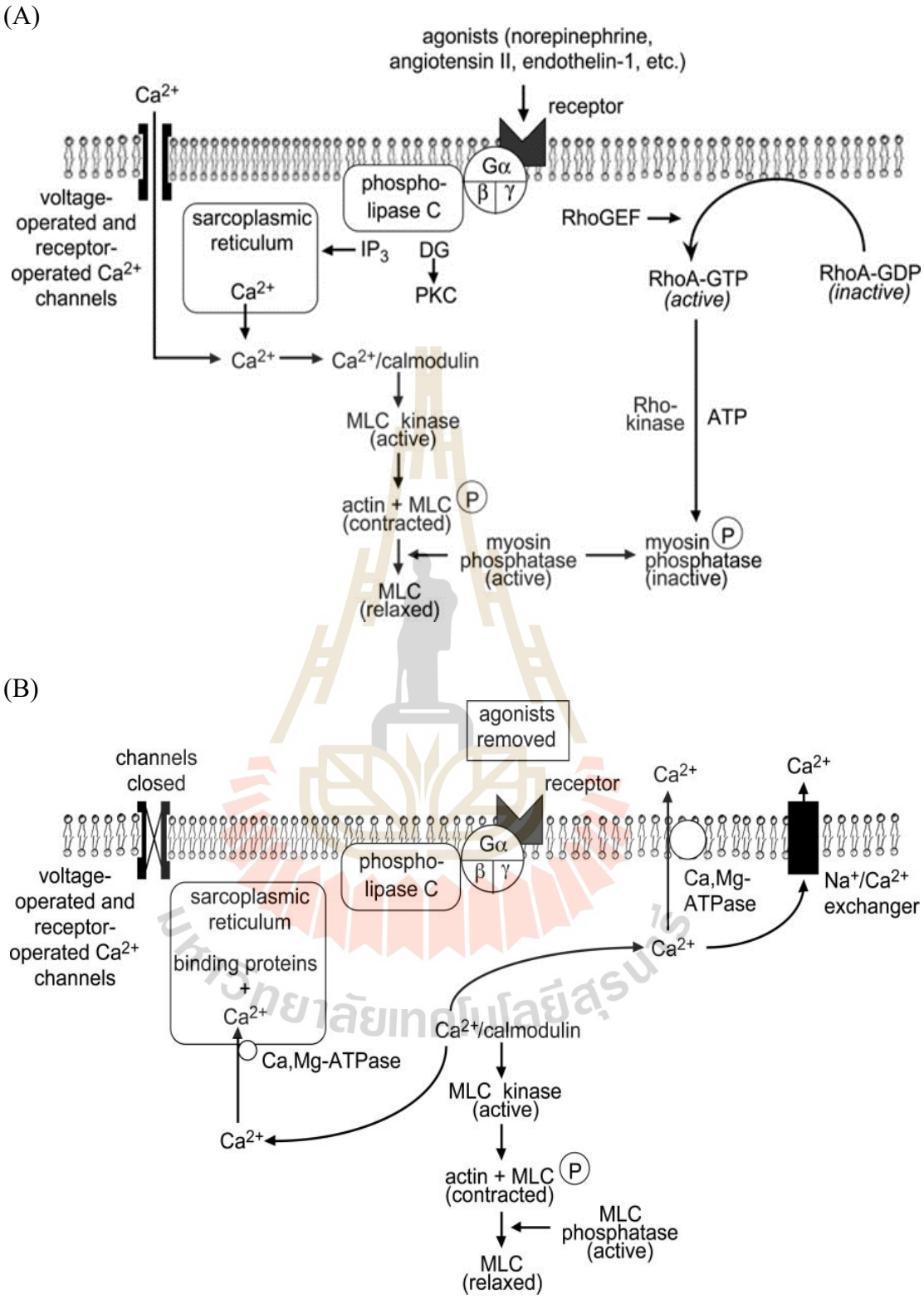

Figure 1.4 Schematic diagram showing the mechanism of uterine smooth muscle contraction (A) and relaxation (B) (Webb, 2003).

1.3.5 Pregnancy and parturition

For fertile women, fertilization usually occurs after spermatozoa from the male travel into the fallopian tube and fertilizes the egg within 24-48 hours. Embryonic implantation in the endometrium of the uterus will happen 8-10 days after ovulation and fertilization as the result of pregnancy. The different period of gestation is exhibited in various species which contribute to maternal, fetal, and environment factors (Taylor and Badell, 2011).

The regulation of uterine activity during pregnancy can be divided into 4 distinct physiologic phases (Asgari Safdar et al., 2013). To start with phase 0: Inhibitors active during pregnancy, the placenta will promote human chorionic gonadotropin (hCG) which can influence of increasing steroid hormones production around 3 weeks of gestation (progesterone and estrogen). Peak hCG is found during the 10^{th} gestational weeks and gradually declines in the third trimester. CL also dramatically increases progesterone, relaxin, and estrogen production that necessary for the establishment and maintenance of early pregnancy. However, CL generally undergoes involution around the 7^{th} gestational week and the production of progesterone will shift to the developing placenta during the luteal-placental transition period. Progesterone is known to inhibit uterine contractions and decrease prostaglandin formation to suppose the fetal growth in the stretched uterus and prevent abortion in early pregnancy. Similarly, progesterone is also promoted prolactin secretion which serves to regulated fluid and electrolyte flux through fetal membranes under the influence of increasing estrogen and acts in lactation postpartum. Estrogen regulates the vasodilators' function by the principle up-regulation of uterine blood flow and contributes to mammary gland development. Relaxin hormone ripens the cervix, softens the pubic symphysis, and synergies with

progesterone and nitric oxide (NO) to maintain uterine quiescence during pregnancy (Tal et al., 2015; Taylor and Badell, 2011).

Subsequently, phase 1: Myometrial activation to trigger parturition, the quiescent uterus with dyssynchronous contractions is turned to the muscular components' activation that is resulting in regular phasic uterine contraction. This phase is characterized by the increased expression of estrogen, activation of myometrial receptors for prostaglandins (PG) and oxytocin (OT), stimulation of specific ion channels, and formation of gap junction, both of which allow for effective coordination of uterine contraction (Asgari Safdar et al., 2013; Kota et al., 2013). Afterward, phase 2: Stimulatory phase following the activation, the uterus becomes activated in response to estrogen dominance, up-regulate OT receptors, increased PG synthesis, decrease NO activity, and increased gap junction that essential for the propagation of action potential. Myometrial functions are enhanced by elevate Ca^{2+} metabolism and augment uterine blood flow according to the increased level of endothelin. Likewise, the initiation of labor contributes to the alteration of connective tissues and a reduction in collagen stabilization, lead to cervical connective tissues softening and dilation to allow the passage of the fetus from the uterus (Kota et al., 2013). Eventually, phase 3: The process of involution associate with the uterus will attempt to involute after delivery with a return to its normal size and condition. This is mediated primarily by OT (Asgari Safdar et al., 2013).

1.4 Diabetes and anti-diabetic agents on the reproductive function

Recently, some evidence suggests that poor myometrial contractility may be an important independent factor found in pregnancies complicated diabetes. The underlying mechanism is related to reduced Ca^{2+} channel expression, decreased intracellular Ca^{2+} signaling, diminished response of OT, and gradual loss of muscle mass compared with non-diabetic patients (Al-Qahtani et al., 2012). In addition, the electrical field stimulation (EFS)-evoked contraction during pregnancy of isolated uterine rings is declined in normal rats and continuously low sensitivity in diabetic rats (Spiegl et al., 2009). Notably, the ultrastructural study of smooth muscle cells in diabetic mice revealed that diabetes affects the organization of the muscle layers, the contractile apparatus, and the cell proliferation profile in the first adaptation phase of the pregnant myometrium in a time-sensitive manner (Favaro et al., 2010).

Nevertheless, the management of diabetes during pregnancy recommends nutrition counseling to control weight gain and achieve glycemic targets within 2 weeks from nutritional therapy alone, if not the insulin therapy in form of multiple injections or short-acting bolus insulin should be initiated, especially T1DM. Obese and T2DM women with extreme insulin resistance are required about 100 and 300 units of insulin a day until the end of pregnancy. Alternatively, oral glucose-lowering agents such as the sulfonylureas insulin-sensitizing agents (glibenclamide: GLB) or the biguanide insulin-sensitizing agents (metformin: MET) are commonly used and relatively safe in pregnancy for glycemic control (Dornhorst, 2005). GLB can bind to its specific receptor in islet cells, stimulate insulin secretion with normal onset within 8 hours after ingestion, and promote hypoglycemia prolonged for several days. On the other hand, MET also suppresses gluconeogenesis by inhibiting hepatic glucose production,

enhancing sensitivity to insulin-sensitive tissues, and decreasing intestinal absorption of glucose which is not caused by hypoglycemia, but may generate severe lactic acidosis (Waring, 2012). GLB has the same effect as insulin with a higher increased risk of neonatal hypoglycemia, high fetal birth weight, and macrosomia (Zeng et al., 2014). Seriously for GDM, GLB should not be used if insulin or MET is available (Balsells et al., 2015; Poolsup et al., 2014).

Insulin also affects the force-producing pathway in potassium chloride (KCl)-induced myometrial contractions in humans and rats (Kuznetsova et al., 2005), and enhances the OT-induced myometrial contractions in rats (Goldraij et al., 1979). Further, GLB is a well-known inhibitor of K^+ channels opening that has been reported in the prevention of both high K^+ depolarization and agonist-induced contraction, and decreasing cytosolic Ca^{2+} concentration, but no effect on the force of contraction in normal aortic smooth muscle cells (Yoshitake et al., 1991). The antagonism of several K^+ channels opener by GLB suggests its actions may involve a blocker of an ATP-sensitive K^+ channel in the non-pregnant uterus (Piper et al., 1990), in contrast with insulin that has no antagonistic effect (Downing and Hollingsworth, 1991). In non-diabetes non-pregnant rats, spontaneous uterine contractility has not interfered with or not inhibited by MET (Kelany et al., 2016). Additionally, MET has no significant effect on the contractile pattern of spontaneous or agonist-induced contractility *in vitro* (Hehir and Morrison, 2012).

1.5 Traditional anti-diabetic supplementations

According to the traditional use of herbal medicines in diabetes, numerous scientific researches have proven the efficacy of herbal therapy belonging to the origin of the country such as Africa (Ssenyange et al., 2015), China (Xie et al., 2011), India (Grover et al., 2002) and Mexico (Andrade-Cetto and Heinrich, 2005) with the different parts used such as seed, roots, stem, bark, leaves, bulb, whole plant, aerial part, fruit, and flower, based on their active phytoconstituents showing anti-diabetic action including triterpenes, flavonoids, sterols, coumarins, saponins, phenolics, polysaccharides, and alkaloids (Patel et al., 2012). There is a 56% distribution of therapeutic herbal plants in Asia continents (C. H. Chan et al., 2012). In Thailand, decoction, powdered drugs, tea, and fresh-eaten herbal plants are used for oral ingestion (Phumthum and Balslev, 2018). *Thunbergia laurifolia* (L.) and *Cyanthillium cinereum* (L.) H. Rob. are among those plants that have also been recommended in Thai medicines for treating diabetes and potentially useful for various therapeutic activities (Cheeptham and Towers, 2002; Chuakul et al., 2002).

The overall anti-diabetic properties of *Thunbergia laurifolia* (L.) (*T. laurifolia*) in the *Thunbergia* genus of the climbing plant of the Acanthaceae family have been summarized and reviewed in the literature (Kosai et al., 2015). In detail, the aqueous extract of *T. laurifolia* leaves exhibited hypoglycaemic activity as it decreased high blood glucose levels in diabetic rats for 15 days-treatment and potentially recovered some of the β-cells destruction which implied that its leaves may contain insulin-like or certain substances for the regenerative process of β-cells and the secretion of insulin (Aritajat et al., 2004). Flavonoids-isolated from *T. laurifolia* extract, apigenin and delphinidin can act as an insulin-sensitizing agent as well as can ameliorate the

diabetes-related complications in diabetic rats (Akcilar et al., 2015; Jin et al., 2009). Aqueous extract from *T. laurifolia* has possessed the hypoglycemia-associated hepatoprotective activity against ethanol-induced liver injury (Pramyothin et al., 2005). Moreover, *T. laurifolia* has been shown a 99.05% availability for the competitive inhibitor of alpha-amylase activity, the carbohydrate digestive enzymes in the small intestine, resulting in the reduction of blood glucose level in diabetic rats and has a beneficial effect in the treatment of diabetes (Jaiboon et al., 2011).

In 2014, *Cyanthillium cinereum* (L.) H. Rob. (*C. cinereum*) is preferred an updated scientific name of *Vernonia cinerea* (L.) Less (*V. cinerea*) which regarded as synonyms (Borah, 2014). *C. cinereum* in the *Cyanthillium* (*Vernonia*) genus is a woody shrub plant of the Asteraceae family and many medicinal properties have been announced in literature with their synonyms of *V. cinerea* (Shelar et al., 2014). For its anti-diabetic properties, vernolide, a sesquiterpene lactones have been exhibited anti-diabetic properties and may potentially be useful for the treatment of diabetes by suppressed the key hepatic gluconeogenic enzymes, glucose-6-phosphatase and increased GLUT 4 translocations as found in aqueous *V. colorata* leaves extract (Sy et al., 2005), methanolic *V. glaberrima* leaves extract (Abdullahi et al., 2015), and ethanolic *V. amygdalina* leaves extract (Ong et al., 2011). The ethanolic sesquiterpene lactones-isolated from a whole plant extract of *V. cinerea* has been shown a significant decrease in blood glucose level in a dose dependent manner and may stimulate the pancreatic secretion of insulin to exert hypoglycemic activity in diabetic rats (Choudhary et al., 2013). Methanolic extract of stem bark and leaves of *V. cinerea* exhibited anti-hyperglycemic property due to their restoration of the pancreatic function

(Haque et al., 2013). Aqueous *V. cinerea* extract has been used as herbal supplementation in diabetic patients (Abas et al., 2015).

Traditional uses of both plants in the treatment of several medical conditions are also mentioned in Table 1.4.

Table 1.4 Ethnopharmacological uses of *T. laurifolia* and *C. cinereum* according to its different parts used.

	T. laurifolia		***C. cinereum***	
Biological activities	Part used	References	Part used	References
Anti-diabetic activity	L	Aung et al. (2020)	L	Alara et al. (2018)
	L, S	Hongsing et al. (2018)	WP	Choudhary et al. (2013)
Analgesic and antipyretic activity	L	Nanna et al. (2017)	WP	Gupta et al. (2003)
Anti-inflammatory activity	L	Kwansang et al. (2015)	A	Pillai et al. (2018)
	L	Wonkchalee et al. (2012)	F	Latha et al. (1998)
			WP	Fadillah and Santoso (2019)
Anti-oxidant activity	L	Junsi et al. (2020) and E. W. C. Chan et al. (2012)	WP	Pratheeshkumar and Kuttan (2011) and Guha et al. (2009)
			R, S, L	Goggi and Malpathak (2017)
			L	Swetha Bindu and Prathibha (2018)
Anti-poisoning activity	L	Tangpong and Satarug (2010)	L	Gokilaveni et al. (2006)
Anti-malarial activity	L	Khobjai et al. (2014)	L	Ngbolua et al. (2011)
			R, L, S	Panda and Luyten (2018)
Hepatoprotective activity	L	Palipoch et al. (2019)	L	Leelaprakash et al. (2011)
Anti-drug addition	L	Thongsaard and Marsden (2002)	WP	Promputta et al. (2012)

Plant parts used: stem (S), roots (R), leaves (L), flower (F), whole plant (WP), and aerial part (A).

Table 1.4 Ethnopharmacological uses of *T. laurifolia* and *C. cinereum* according to its different parts used (continued).

	T. laurifolia		***C. cinereum***	
Biological activities	Part used	References	Part used	References
Anti-cancer activity	L	Jetawattana et al. (2015)	L, S	Youn et al. (2014)
			WP	S S and Joseph (2020)
Nephroprotective activity	L	Chattaviriya et al. (2020)	WP	Hiremath and Jalalpure (2016)
Detoxifying activity	L	Rojsanga et al. (2015)	WP	Pratheeshkumar and Kuttan (2012)
Anti-microbial activity	L, S	Wiyakrutta et al. (2004)	WP	Sonibare et al. (2016)
Anti-bacterial activity	L	Oonsivilai et al. (2007)	L	Sahayaraj et al. (2015)
			L, R	Tantengco et al. (2016)
			A	Dharani et al. (2018)
Larvicidal efficacy	L	Chungsamarnyart et al. (1994)	L	Arivoli et al. (2011)
Anti-nociceptive activity	L	Boonyarikpunchai et al. (2014)	WP	Thiagarajan et al. (2014)
Anti-diarrhoeal activity	L, S	Tangjitman et al. (2015)	WP	Senthil Nagaraj and Venkateswarlu (2013)
Anti-spasmodic activity			L	Swetha Bindu et al. (2018)
Smoking cessation			WP	Leelarungrayub et al. (2010) and Thongkhao et al. (2020)

Plant parts used: stem (S), roots (R), leaves (L), flower (F), whole plant (WP), and aerial part (A).

1.6 Aims

To date, there were fewer studies demonstrated the effect of gestational diabetes on the general function and uterine smooth muscle physiology, as well as, there were little data in the literature about the effects of these plants, whether *T. laurifolia* or *C. cinereum* have uterotonic or tocolytic activity on the uterus is not clear. The alteration of these supplementations on pregnancy, especially in diabetic conditions, has not yet been investigated. Thus, the aims of this thesis were therefore to investigate:

1) the phytochemical compounds in ethanolic *T. laurifolia* leaves (TLE) and *C. cinereum* whole plant (CCE) extracts by GC-MS and LC-MS method
2) the underlying mechanisms of contractile pathways of gestational diabetes myometrium and the effects of GLB, MET, and insulin on uterine contraction *in vitro*
3) the effects of TLE and CCE on uterine contraction during spontaneous contraction, high K^+ depolarization, and OT-induced contraction *in vitro*
4) the effects of TLE and CCE on blood biochemistry, lipid profiles, liver enzymes, maternal reproductive performances, and fetal outcomes parameters in streptozotocin (STZ)-induced gestational diabetic rats
5) the effects of TLE and CCE on the histological and ultrastructural study of maternal liver, pancreas, and uterus in STZ-induced gestational diabetic rats

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CHAPTER II

GENERAL MATERIALS AND METHODS

This chapter will explain a description of the equipment, materials, and methods utilized for this thesis. More specific details in each experiment are presented in each of the chapter.

2.1 Plants preparations

2.1.1 Plant materials collection

Leaves of *Thunbergia laurifolia* (L.) *(T. laurifolia*) and fresh whole plant of *Cyanthillium cinereum* (L.) H. Rob. *(C. cinereum*) were collected from Pak Kran district (Ayutthaya province, Thailand) under natural conditions in November 2015. Voucher specimens were deposited and identified at the Royal Forest Department of Thailand, Bangkok, Thailand.

2.1.2 Plant extraction

Leaves of *T. laurifolia* and fresh whole plant of *C. cinereum* were collected, washed well, oven-dried for 2 days at low temperature (< 40ºC), and then ground to the powders. The powder of *T. laurifolia* leaves (1.2 kg) and *C. cinereum* whole plant (1.2 kg) were weighted, extracted with 70% ethanol (4 L) by a Soxhlet extraction method for 6 hours, and filtered using a Whatman® No.41 filter paper. Then, the extracts were evaporated to dryness under reduced pressure at a low temperature (< 40ºC) by a rotary

evaporator and lyophilized by a lyophilizer. The extracts were stored in the closed container at -20°C until use.

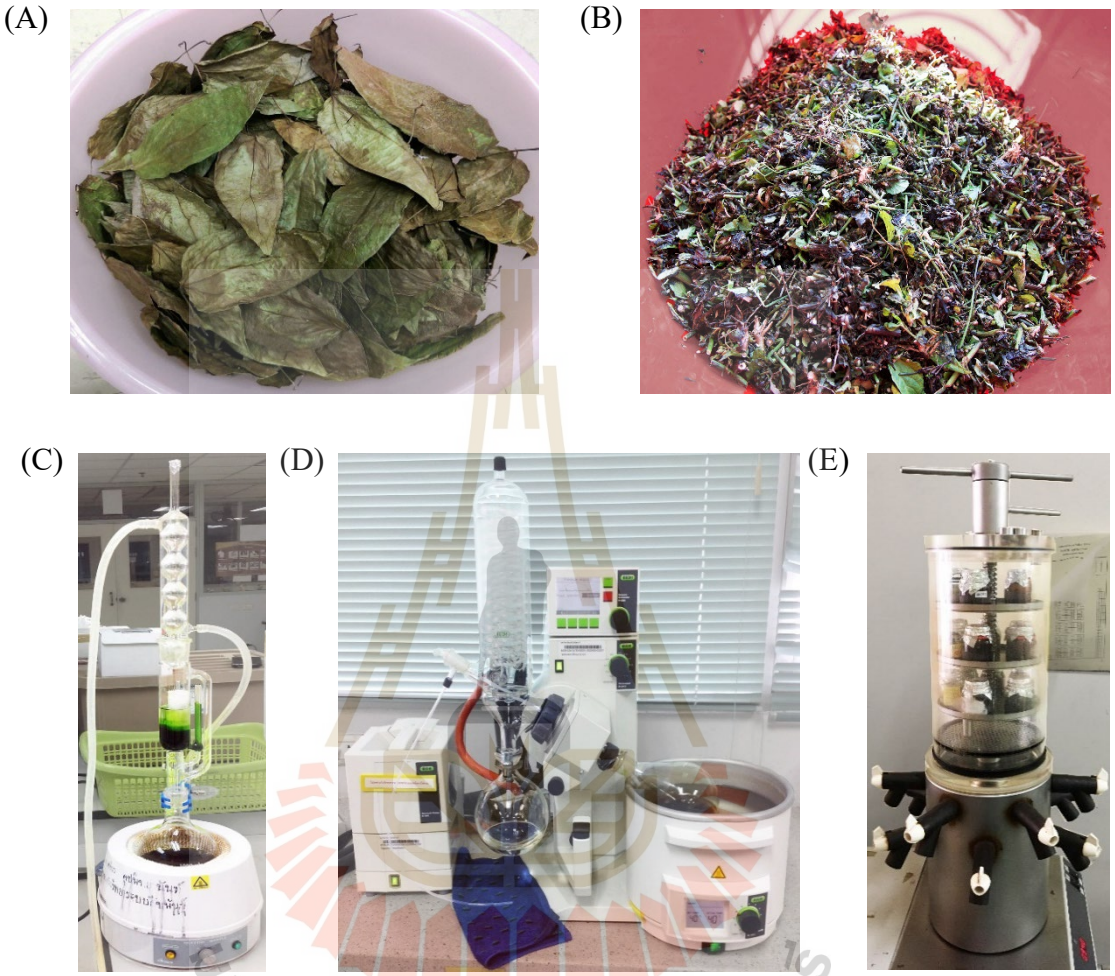

Figure 2.1 The plant materials and the apparatus used in the extraction process; (A) Dried leaves of *T. laurifolia*, (B) Dried whole plant of *C. cinereum*, (C) Soxhlet extractor (Borosil®, India), (D) Rotary evaporator (Buchi Co. Ltd., Thailand) and (E) Lyophilizer (Labconco®, Bacthai Bangkok Equipment & Chemical Co. Ltd., Thailand).

2.1.3 Preliminary phytochemical analysis

The extracts were prepared for the preliminary phytochemical screening, using the standard qualitative procedures as previously described (Sharma et al., 2013;

Tiwari et al., 2011), to identify the presence or absence of various classes of phytochemical constituents including alkaloids, flavonoids, tannin, saponin, and phytosterols. Moreover, the plant extracts were analyzed for the quantitative phytochemical screening by gas chromatograph-mass spectrometry (GC-MS) (Haber et al., 2001) and liquid chromatography-mass spectrometry (LC-MS) analysis (Junsi et al., 2017).

2.2 Animal preparations

2.2.1 Animal ethics and regulations

The animal care was followed by the guidelines of the committee on Care and Use of Laboratory Animal Resources, National Research Council of Thailand. The procedures of the experiments were approved by the advice of the Institutional Animal Care and Use Committee, Suranaree University of Technology, Nakhon Ratchasima, Thailand. The approval animal ethics number was 11/2557.

2.2.2 Animal housing

Female Wistar rats (250-300 g) were individually housed in 24 x 15 x 15 cm^3 cages maintained under 12-h light-dark illumination cycles at a constant temperature of 25 ± 0.5ºC and relative humidity 45-50%. Rats were fed with a standard laboratory food containing 0.8% calcium (CP. Co. Ltd., Thailand) and free access to water *ad libitum*. The number of animals used was explained in each experiment.

2.2.3 Vaginal cytology

Vaginal smears from adult female Wistar rats were examined to classifying the vaginal epithelial cells during various stages of the estrous cycle with the exhibition of leukocytes, nucleated cells, or cornified epithelium cells. Smears of the vaginal were

performed daily in the morning (between 9.00-10.00 a.m.) by gently inserting the disposable transfer pipettes filled with normal saline (0.9% NaCl) into the vagina, flushing in and out, and then dropping the secretion onto the microscope slides. The slides were stained by Methylene blue dripping and observed using the light microscope (20x) (Parhizkar et al., 2011). The presence of cornified epithelium cells was used as an indicator of the heating time.

2.2.4 Mating and conception

After vaginal smear examination, adult female rats which entered into the proestrous phase were selected to mating with a fertile male rat (2 females: 1 male) overnight stay. The following morning, the findings of the mucous plug of spermatozoa in vagina smears were designed as gestational day 0. The mating procedure was consisted of 15 consecutive days, approximately 3 estrous cycles. Besides, unmated female rats were considered to be infertile and excluded from the study.

2.2.5 Induction of diabetes mellitus

The experimental models of pregnant diabetes in rodents by the use of chemicals were performed as shown in Table 2.1 (Jawerbaum and White, 2010). The single dose of STZ 60 mg/kg BW was dissolved in 0.1 M cold citrate buffer solution (8 ml of 0.1 M citric acid mixed with 92 ml of 0.1 M sodium citrate solution, 100 ml buffer), pH 6.5, and prepared freshly before immediately used within 10 min by intraperitoneal injection (*i.p.*) on day 5 of gestation. The blood samples were obtained from a tail vein puncture and glucose levels were monitored 2 days after diabetic induction by a glucometer (Accu-Chek® Performa, Thailand). Diabetes was defined as hyperglycemia equal to or higher than 200-300 mg/dL as described in previous studies (Spiegl et al., 2009).

Table 2.1 The most frequently used models in diabetic pregnancy by the use of chemicals in rodents (Jawerbaum and White, 2010).

Chemicals	Experimental model	Phenotypes
Streptozotocin	1 dose, 40-45 mg/kg *i.v.* or 50-75 mg/kg *i.p.* given to adult rats several days before mating	Severe diabetes
Streptozotocin	1 dose, 200-240 mg/kg *i.p.* given to adult mice several days before mating	Severe diabetes
Streptozotocin	1 dose, 45 mg/kg to rats, or 100 mg/kg *i.v.* to mice given several days before mating and insulin administration until day 1 of gestation	Severe diabetes
Streptozotocin	1 dose, 15-65 mg/kg *i.v.* or *i.p.* given to rats during pregnancy	Mild/Severe diabetes
Streptozotocin	3 consecutive doses, 75-90 mg/kg *i.v.* or *i.p.* given to mice prior to mating	Mild/Severe diabetes
Alloxan	given to mice (300 mg/kg) prior to induced superovulation and mating /or given to rats (40 mg/kg *i.p.*) during pregnancy	Mild/Severe diabetes

Route: intravenous injection (*i.v.*), intraperitoneal injection (*i.p.*)

2.3 Uterine contractility investigations

Pregnant Wistar rats (250-300 g) were collected at term (19^{th}-21^{st} day of gestation). Myometrial strips were dissected and prepared for *in vitro* study. The end of myometrial strips was attached to metal hooks and another end was fixed to a transducer (ADInstruments Pty Ltd., Spain) in the organ bath apparatus that containing the physiologic Krebs' solution at 37ºC, pH 7.4. The strip was allowed to contract spontaneously under a resting tension of 1 g. An equilibration time of 30 min was applied for all tissues before the application of any chemical study. Change in isometric force was measured with the PowerLab system software (ADInstruments Pty Ltd., Australia). The amplifier electrical signal from a force-displacement transducer was converted to a digital signal and recorded on a computer using the Chart software as seen in Figure 2.2. (Kupittayanant et al., 2002). More details were described in chapters IV and V.

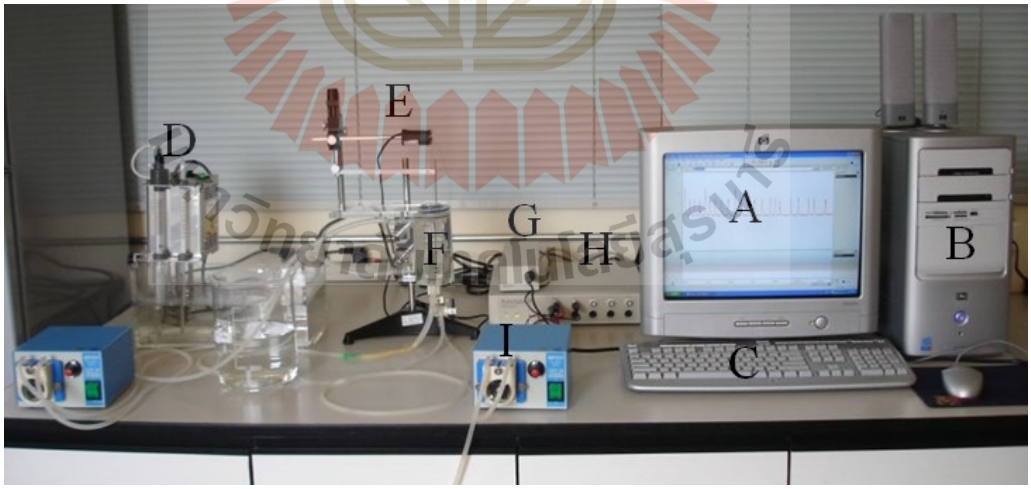

Figure 2.2 The equipment used for tension measurement; (A) Monitor, (B) CPU, (C) Keyboard, (D) Thermostat, (E) Transducer, (F) Organ bath chamber, (G) Bridge Amp, (H) PowerLab, and (I) Peristaltic pumps (Kupittayanant et al., 2002).

2.4 Tissue histological investigations

The sacrificed animals were quickly dissected. Tissues were removed, washed in physiological solution, and fixed in 10% neutral formalin. The routine paraffin technique for hematoxylin and eosin (H&E) staining was performed and examined for light microscope observation. Images of tissues cross-section were taken using a Nikon Eclipse 80i upright microscope (Hollywood International Ltd., Thailand) and Cell^D imaging software (Olympus, EforL International Co., Ltd., Thailand) (Qadori, 2011; Thanamool et al., 2013). More detail was described in chapter VII.

2.5 Spectrophotometric analysis

The uterus was dissected from sacrificed animals, washed in physiological solution, and immediately put on dry-ice after devascularized to avoid morphological distortion and damage. The frozen section was obtained from the cryostat sectioning technique and mounted on barium fluoride (BaF_2) windows (13 mm diameter x 1 mm thick), air-dried, and stored in a desiccator until used. Spectra data were collected at an infrared (IR) spectroscopy and imaging beamline (Beamline 4.1 IR spectroscopy and imaging) at the Synchrotron Light Research Institute (Public Organization), Nakhon Ratchasima, Thailand as seen in Figure 2.3. (Thumanu et al., 2014). More detail was described in chapter VII.

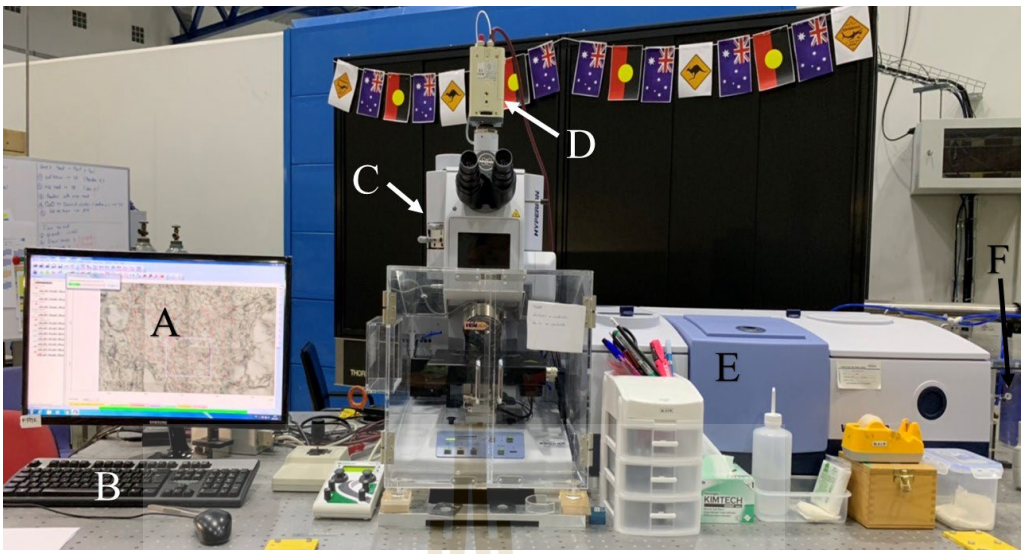

Figure 2.3 The equipment used for spectrophotometric analysis; (A) Monitor, (B) Keyboard, (C) Hyperion 2000 microscope, (D) Mercury-cadmium-telluride (MCT) detector, (E) Vertex 70 spectrometer, and (F) The entrance of the Synchrotron light via matching unit (Bruker Switzerland AG, Co. Ltd., Thailand) (Thumanu et al., 2014).

2.6 Chemicals

All chemicals were purchased from Sigma® and Merck, Singapore. All stock solutions were prepared and stored by the guideline of the producer. A standard physiological saline Krebs' solution was contained compositions (mM): NaCl: 154.0; KCl: 5.4; $CaCl_2$: 2.0; $MgSO_4$: 1.2; glucose: 8.0; HEPES: 10.0. A Ca^{2+} free or $0Ca^{2+}$ solution was excluded $CaCl_2$ and added ethylene glycol tetraacetic acid or EGTA (1 mM) in normal Krebs' solution instead. The standard agonists and antagonists used were prepared in Krebs' solution. For example, a high K^+ or KCl (40 mM) solution was made by isosmotic replacement of NaCl with KCl in normal physiological saline Krebs' solution. OT, hormone stimulation of uterine contraction was dissolved in distilled

water and used at a concentration of 10 nM. (Kupittayanant et al., 2001; Kupittayanant et al., 2002; Longbottom et al., 2000). Other specific chemicals were described in each chapter.

2.7 Statistical analysis

All data were calculated as mean ± standard error of mean (S.E.M). Tension measurements in uterine contractility investigations were exhibited as a percentage of control of contractions (i.e. the control assumes 100%) including maximum tension development of each contraction, the integral contraction (total tension developed in each contraction), amplitude, frequency, and duration of contraction. All data were evaluated using Microcal Origin software. Significance between with and without any chemical exposure in the same strips was tested using a paired student *t*-test. The percent response change was compared between experimental groups. Significance between groups was tested using an unpaired student *t*-test.

Analysis of variance (ANOVA) followed by Tukey's *post hoc* test was used to compare the mean values of the maternal glycemia, body weight, food consumption, lipid profile, liver enzymes, gravid uterus, number of corpora lutea, number of implantations, number of live and dead fetuses, number of resorptions, pre-implantation loss rate percentage, post-implantation loss rate percentage, fetal weight, placental weight, placental index, crown rump length, pancreatic islets' area percentage, relative interstitial space percentage, relative muscle fiber percentage, and FTIR's absorbance units. A Chi-square test was used to calculate SPA, APA, and LPA fetus percentage. SPSS statistical analysis software (SPSS Inc, USA) was used to analyze the data. The

statistical significance interval is considered as $P < 0.05$ for all data and "n" represented as the number of samples from a different animal.

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CHAPTER III

PRELIMINARY PHYTOCHEMICAL ANALYSIS OF *THUNBERGIA LAURIFOLIA* (L.) AND *CYANTHILLIUM CINEREUM* (L.) H. ROB. ETHANOLIC EXTRACTS AND THEIR THERAPEUTIC EFFICACIES

3.1 Abstract

Thunbergia laurifolia (L.) (*T. laurifolia*) or Rang Jeud, belongs to the family Acanthaceae and *Cyanthillium cinereum* (L.) H. Rob. (*C. cinereum*) or Ya Dok Khao, belongs to the family Asteraceae are commonly growing in tropical areas of Thailand. The ancient traditional medical purposes of these plants have been widely utilized such as herbal teas, fermentation, aroma, or fresh consume. Many previous studies were also revealed their anti-hyperglycemic activity for treating diabetes. This experiment aimed to determine the qualitatively and quantitatively phytochemical constituents from *T. laurifolia* leaf ethanolic extracts (TLE) and *C. cinereum* whole plant ethanolic extracts (CCE). The qualitative phytochemical screening was performed by color changes observation according to alkaloids, flavonoids, tannins, saponins, and sterols detection. The GC-MS and LC-MS analysis were also represented the quantitative phytochemical constituents of these plant extracts depend on the retention time and mass spectra. As a result, alkaloids, flavonoids, phenols and tannins, sterols and terpenoids, and reducing sugars were presented in both TLE and CCE. In contrast,

saponins were not found in both extracts. The GC-MS analysis of TLE showed the presence of phenolic compounds and aromatic acids (0.65-14.91%: benzoic acid, 4-vinyl phenol, 5-hydroxymethylfurfural, 4-methylindoline, 2-furanmethanol, 9,9-dimethyl-9,10-dihydroanthracene, megastigmatrienone, indole, 7,9-di-tert-butyl-1-oxaspiro (4,5)deca-6,9-diene-2,8-dione, dihydroactinidiolide, and phenylethyl alcohol), a flavonoids (4H-Pyran-4-one,2,3-dihydro-3,5-dihydroxy-6-methyl: 17.41%), a chromone (3-Methyl-2-Methyoxymethyl-4-Chromone: 1.23%), terpenoids (0.52-3.65%: (-)-loliolide, neophytadiene, and dehydro-β-ionone) and 13 unknown compounds. Nevertheless, the LC-MS analysis was selected to identify the phenolic and flavonoid compounds in TLE and the rosmarinic acid as the bioactive compound was represented (36.9 mg/g extract). Furthermore, GC-MS analysis of CCE showed the high rich of terpenoids (0.27-32.36%: lupeol, β-amyrin, β-amyrin acetate, neophyte diene, lupenone, and hexahydrofarnesyl acetone). Additionally, some phenolic compounds and aromatic acids (0.17-0.85%: benzoic acid, 2,6,10,14,18-pentamethyl-2,6,10,14,18-eicosapentaene, phthalic acid, and 10s,11s-himachala-3(12),4-diene), phytosterols (1.18-2.68%: β-sitosterol and campesterol), alkaloids (0.32-18.48%: 2-methyl-2,3,4,5,6,7-hexahydro-1H-2-benzazonine and β-carboline,7-methoxy-1,2-dimethyl) and fatty acyls (0.17-0.77%: palmitic acid and palmitic acid ethyl ester) have been detected with 10 unknown compounds. The highest compounds in TLE were benzoic acid and 4H-Pyran-4-one,2,3-dihydro-3,5-dihydroxy-6-methyl. Similarly, lupeol, β-amyrin, and β-carboline,7-methoxy-1,2-dimethyl was higher considered in CCE.

3.2 Introduction

The use of plants during pregnancy is a common practice worldwide which plays a significant role during pregnancy, birth, and post-partum care. A large number of medical plants of traditional use have already tested and confirmed their possible benefits for various therapeutic uses. Many diabetic pregnant women report the use of popular medical plants to treat diabetes because of the high cost of the anti-diabetic drug. *Thunbergia laurifolia* (L.) (*T. laurifolia*) and *Cyanthillium cinereum* (L.) H. Rob. (*C. cinereum*; under the synonym *Vernonia cinerea* L.) are widely used in the alternative therapy that has the potential for treating diabetes on several experimental reports (Aritajat et al., 2004; Choudhary et al., 2013).

In addition, many phytoconstituents were found belonging to the *Thunbergia* species. For example, phenols, alkaloids, and flavonoids have been shown the anti-bacterial and anti-fungal activity (Jeeva et al., 2011) and proanthocyanidin tannin has been potent anthelmintic activity in *T. grandiflora* (Kabir et al., 2015). Interestingly, the aqueous leaves extract of *T. laurifolia* has been shown the anti-diabetic potency against alloxan-induced diabetic rats (Aritajat et al., 2004). Some novel phytoconstituents in *T. laurifolia* have purified and identified its biological actions such as rosmarinic acid is potent anti-nociceptive, anti-inflammatory effects and anti-oxidant activity in ethanolic leaves extract (Boonyarikpunchai et al., 2014; Suwanchaikasem et al., 2014), similarly to Oonsivilai et al. (2008) who suggested that apigenin, caffeic acid, gallic acid, chlorophyll derivatives, and lutein are found in all aqueous, ethanol and acetone leaves extraction. Higher concentrations of phenolic (aromatic) and glycoside compounds showed the cytoprotective effect in aqueous leaves extract (Ruangyuttikarn et al., 2013). High phenolic content and antioxidant activity prevented

learning deficit and memory loss of mice's brains in aqueous leaves extract (Phyu and Tangpong, 2013).

Due to the ethnomedicinal use for traditional medicine in diabetes belonging to a large *Cyanthillium* (Synonym: *Vernonia)* species, the hypoglycemic effects are widely reported in aqueous and ethanolic leaves extract containing a high rich of polyphenols such as dicaffeoyl-quinic acid, 1,5-dicaffeoyl-quinic acid, chlorogenic acid, and luteolin-7-O-glucoside in *V. amygdalina* (Adikwu et al., 2010; Ong et al., 2011), ethanolic seeds extract with epoxy acid or vernolic acid in *V. anthelmintica* (Fatima et al., 2009; Rao et al., 2010), acetonic leaves extract of *V. colorata* due to vernolide, 11β,13-dihydrovernolide and vernodalin (Rabe et al., 2002; Sy et al., 2005) and aqueous leaves extract of *V. galamensis* contains saponins, glycosides, carbohydrates, flavonoids and alkaloids (Autamashih et al., 2011). Moreover, Alara et al. (2018) reported mainly phenolic acids, phenolic aldehyde, and flavonoids are found in ethanolic leaves extract of *V. cinerea* which is related to anti-diabetic and anti-oxidative activity and Choudhary et al. (2013) suggested that sesquiterpene lactone with hirsutinolide type may have a similar mechanism to glibenclamide. Luteolin-isolated from the whole plant *V. cinerea* extract shown an anti-inflammatory activity (Abeysekera et al., 1999). Several major sesquiterpenes lactones-isolated from *V. cinerea* extract are known to have many bioactivities such as vernolide A, B, and 8α-tigloyloxy-hirsutinolide-13-O-acetate have been shown cytoprotective effect and anti-apoptosis activity (Khay et al., 2012; Kuo et al., 2003; Pratheeshkumar and Kuttan, 2011), vernolide D potent anti-malarial activity (Chea et al., 2007) and 8α-hydroxyhirsutinolide against inflammatory condition (Youn et al., 2012).

Besides, medical plants exhibit a wide range of biologically active compounds. Previous researches on phytochemistry constituents revealed their therapeutic properties as the plant's activities which depending on the area of research in individual-environment variables. However, the native *T. laurifolia* and *C. cinereum* which grown in the Central region of Thailand had never been identified and there is no scientific data to prove the traditional uses. Therefore, the aim of this chapter was designed to qualitatively and quantitatively investigate the phytochemical constituents of *T. laurifolia* leaves, and *C. cinereum* whole plant ethanolic extracts which were collected in the Central region of Thailand. As the result, the therapeutic efficacy of these plants under investigation might be the actual value of folkloric remedies.

3.3 Materials and methods

3.3.1 Plant identification

Leaves of *T. laurifolia* and fresh whole plant of *C. cinereum* were collected from Central of Thailand in November 2015. A voucher herbarium specimen was examined to verify and identity by the botanist at the Royal Forest Department of Thailand, Bangkok, Thailand.

Thunbergia laurifolia (L.): BKF No.193577

Cyanthillium cinereum (L.) H. Rob.: BKF No.193578

3.3.2 Phytochemical analysis

Preliminary phytochemical screening

Small amounts of dried *T. laurifolia* ethanolic extract (TLE) and *C. cinereum* ethanolic extract (CCE) about 2 g were dissolved in 20 ml of distilled water, which prepared for the stock solution. The identification of the presence or absence of various

classes of phytochemical constituents in the preliminary phytochemical screening was performed using standard qualitative procedures as previously described (Gopalasatheeskumar et al., 2017; Tiwari et al., 2011).

The test for alkaloids was carried out by adding 4 ml of aqueous extract in 1 ml 1% HCL (Hydrochloric acid). Then 2 ml of the mixture was taken separately in 2 test tubes. For Mayer's test in the first tube, few drops of Mayer's reagent (Potassium mercuric iodide) were added in one tube and the formation of a yellow-cream colored indicates the presence of alkaloids. As well as, Wagner's test in the second tube, few drops of Wagner's reagent (Iodine in potassium iodide) were added and the formation of reddish-brown colored indicates the presence of alkaloids.

The test for flavonoids was carried out by using the alkaline reagent test and the Shinoda test. For the alkaline reagent test, about 2 ml of the stock solution was treated with a few drops of sodium hydroxide solution. The formation of an intense yellow-colored, which becomes colorless from the addition of dilute HCL, indicates the presence of flavonoids. Shinoda test was also used to test for flavonoids and was carried out by dissolved 0.4 g extract in 4 ml of 95% ethanol. Few drops of concentrated HCL were added followed by 3 pieces of magnesium ribbon dropped. The formation of magenta-colored indicates the presence of flavonoids.

The test for saponins was carried out by using the froth test. About 2 ml of the stock solution was made up with distilled water to 20 ml and then shaken in a test tube for 15 min. The formation of a 1 cm stable layer of foam indicates the presence of saponins.

The test for phenolic compounds and tannins was carried out by using the ferric chloride test and gelatin test. For the ferric chloride test, about 2 ml of the stock

solution and a few drops of 2% ferric chloride solution was added. The formation of brownish-green or blue-black colored indicates the presence of tannin. For the gelatin test, about 2 ml of the stock solution was treated with 1 ml 1% gelatin solution containing 10% sodium chloride solution. The formation of a white-colored indicates the presence of tannins.

The test for sterols and terpenoids was carried out by using the Salkowski's test. About 2 ml of the stock solution was treated with 2 ml chloroform and filtered. Then the filtered was treated with few drops of concentrate solution of sulphuric acid (H_2SO_4), shaken well, and allowed to sand. The appearance of golden yellow-colored indicates the presence of triterpenes.

Gas chromatography-Mass spectrometry (GC-MS) analysis

The plant extracts were subjected to quantitative phytochemical screening by GC-MS analysis (Haber et al., 2001).

An Agilent Technologies 7890A gas chromatography, coupled with an Agilent Technologies 5975C (EI) mass spectrometer was used for the establishment of a fingerprint of crude extracts. The separation was performed on the HP-5MS column, 0.25 mm ID x 30 m x 0.25 mm coating thickness. The temperature of the column was programmed from 50-300ºC at 10ºC/min. The injector temperature and the detector temperature were 250ºC. Helium was used as carrier gas with a constant flow rate of 1.0 µl/min. All separated compounds were identified from the recorded mass spectra by compared with the mass spectra from the NIST and Wiley Libraries.

Liquid chromatography-Mass spectrometry (LC-MS) analysis

Selected phenolic compounds and flavonoids were determined by LC-MS analysis (Junsi et al., 2017). The plant extract was dissolved in absolute ethanol (30 mg/

2 ml) and prepared for quantitative analysis.

LC-MS analysis was performed on the Dionex Ultimate 3000 UHPLC system (Dionex, USA) coupled with electrospray ionization (ESI) tandem mass spectrometer (micrOTOF-Q II) (Bruker, Germany). The injection volume for all samples was 5 µl. The separation was achieved using a Zorbax SB-C18 (250 mm × 4.6 mm × 3.5 µm (Agilent Technologies, USA)), and thermostated at 35°C, with a flow rate of 0.8 ml/min of mobile phase which included deionized water containing 0.1% formic acid (FA) as solvent A and acetonitrile containing 0.1% FA as solvent B. The gradient elution was performed using the following solvent gradient: starting with 30% solvent B and holding until 5 min, increasing to 80% solvent B at 30 min, and holding until 38 min, reducing to 30% solvent B in 2 min and holding until the run ending at 45 min. The eluted components were ionized by an ESI source and were detected in the mass scanning mode, in the range of 50 to 1,500 m/z at negative ion polarity. The nebulizer gas (N_2) was 2 Bar, drying gas was 8 L/min, dry heater temperature was 180°C, and the capillary voltage was 4.5 kV.

The LC-QTOF data were collected and processed by Compass 1.3 software (Bruker, Germany). The target phenolic and flavonoid compounds were identified and quantified with Bruker QuantAnalysis Version 2.0 SP 5 software. The calibration curves were constructed from peak areas of different concentrations of the reference standard (from 1 to 20 µg/ml), and the concentration of targeted compounds was calculated based on the equation for linear regression obtained from the calibration curves.

3.3.3 The yield of TLE and CCE calculation

The powder of TLE (1.2 kg) and CCE (1.2 kg) were extracted with 70% ethanol in a Soxhlet apparatus for 6 hours. The extracts' solvents were filtered, evaporated, lyophilized, and finally stored at -20°C until use. The percent yields of the extracts were calculated using the following formula:

$$\text{Percent yield} = \left(\frac{W_{crude\ extract}}{W_{dried\ plant}}\right) \times 100$$

$W_{crude\ extract}$ is the mean weight of the crude extract.

$W_{dried\ plant}$ is the mean weight of the dried plant.

3.4 Results

3.4.1 Botanical profile of *T. laurifolia*

- Kingdom: Plantae
- Division: Tracheophyta
- Class: Magnoliopsida
- Family: Acanthaceae
- Genus: *Thunbergia*
- Species: *Thunbergia laurifolia*
- Other names: *Thunbergia grandiflora* var. *laurifolia* (Lindl.) Benoist and *Thunbergia harrisii* Hook.f.
- Common names: Laurel clock vine, Blue trumpet vine (English), Kar-Tuau (Malayalam), Rang Jeud, Rang Yen (Thai)

T. laurifolia is a woody climbing shrub plant that upright to 15 m tall, small heart-shaped up to 6 cm with dark green leaves, trumpet-shaped with bluish-purple

petals flowers in Figure 3.1(A)-(C), and belonging to the large family of Acanthaceae which popularly known as Rang Jeud in Thai. Various parts of *T. laurifolia* are utilized as traditional medicine and the other species from the same genus as *T. grandiflora* and *T. harissi* have been reported to relieve symptoms of several diseases in Central and southern Africa, Asia and Central America (Kosai et al., 2015). Bioactivity compounds isolated from *T. laurifolia* are presented in Table 3.1.

3.4.2 Botanical profile of *C. cinereum*

- Kingdom: Plantae
- Division: Tracheophyta
- Class: Magnoliopsida
- Family: Asteraceae (Compositae)
- Genus: *Cyanthillium*
- Species: *Cyanthillium cinereum*
- Synonym name (common): *Vernonia cinerea*
- Other names: *Cacalia cinerea* (L.) Kuntza, *Conyza cinerea* L. *Vernonia laxiflora* L., *Vernonia parviflora* Reinw ex Blume, *Vernonia rhomboidea* Edgew
- Common names: Little Ironweed (English), Sahadevi (Sanskrit and Hindi), Kukshim (Bengali), Puvamkurunnel (Malayalam), Ya Dok Khao, Ya Mor Noi, Ya La Oong, Ya Sam Wan (Thai)

C. cinereum is a perennial grass plant in the family Asteraceae (Compositae) and widely distributed in East and West Africa as well as in India, South America, and Asia. The roadsides plant is grown up to 1 meter in height with small oval-irregularly

shallow teeth various shape of green leaf. A group of flowers is composed of pink-violet colored flowers with small white hairy fruits in Figure 3.1(D)-(F). Ya Dok Khao is used in different traditional medicines worldwide which different parts are exhibited various therapeutic values (Toyang and Verpoorte, 2013). Phytochemical studies in *C. cinereum* are shown in Table 3.2.

(A) (B) (C)

(D) (E) (F)

Figure 3.1 Morphology of *T. laurifolia* whole plant (A), leaves (B), inflorescence flowers (C), and *C. cinereum* whole plant (D), leaves (E), and inflorescence flowers (F).

Table 3.1 Bioactive compounds in previous reported that have isolated from *T. laurifolia*.

Plant extraction	**Major of phytochemicals (references)**
Aerial part	
Methanol extract	8-epi-grandifloric acid, 3'-O-β-glucopyranosyl-stilbericoside, Benzyl β-glucopyranoside, Benzyl β-(2′-O-β-glucopyranosyl)-glucopyranoside, Grandifloric acid, (E)-2-hexenyl-β-glucopyrano-side, Hexanol-β-glucopyrano side, 6-C-gluco-pyranosyl apigenin, and 6,8-di-C-gluco-pyranosyl apigenin (Kanchanapoom et al., 2002)
Leaves	
Aqueous extract	Gallic acid, caffeic acid, protocatechuic acid, apigenin, and apigenin glucosides (Oonsivilai et al., 2007)
	β-Sitosterol, stigmasterol, α-spinasterol, apigenin, caffeic acid, gallic acid, protocatechuic, and lutein (Chuthaputti, 2010)
	Phenolic and glycoside compounds (Phyu and Tangpong, 2013; Ruangyuttikarn et al., 2013)
Acetone, ethanol	Chlorophyll A and B, pheophorbide a, pheophytin a, and lutein (Oonsivilai et al., 2008)
extract	Rosmarinic acid (Boonyarikpunchai et al., 2014; Suwanchaikasem et al., 2014)

Table 3.2 Bioactive compounds in previous reported that have isolated from *C. cinereum*.

Plant extraction	**Major of Phytochemicals (references)**
Whole plant	
Not reported	β-amyrin, lupeol, β-sitosterol, stigmasterol, and α-spinasterol (Venkateswara, 1962)
	luteolin, luteolin 7-O-glucoside, luteolin 4'-O-glucoside, chlorogenic acid, 3,5-dicaffeoylquinic acid, 3,4-dicaffeoylquinic acid, 4,5-dicaffeoylquinic acid, methyl caffeate, and gallic acid (Abeysekera et al., 1999)
Aqueous extract	vernolide C, vernolide D, 8α-tigloyloxy-hirsutinolide-13-O-acetate, 8α-epoxymethacryloyloxy-hirsutinolide-13-O-acetate, 8α-tigloyloxyhirsutinolide, hirsutinolide-13-O-acetate, piptocarphin D, and 8α-(4-hydroxymethacryloyl oxy)-hirsutinolide-13-O-acetate (Chea et al., 2007)
Aqueous, methanol, dichloromethane extract	8α-tigloyloxy-hirsutinolide-13-O-acetate (Khay et al., 2012)
Methanol extract	alkaloids, flavonoids, and triterpenoids (Bashar et al., 2014)
	n-hexadecanoic acid, 1,2 benzenedicarboxylic acid disoocty ester, squalence, caryophyllene oxide, guaiol, 3,7,11,15-tetramethyl-2-hexadecen-1-01, decanoic acid ethyl ester, 9,12-octadecanoic acid(z-z)-, and octadecanoic acid (Abirami and Rajendran, 2012)
Ethanol extract	glycosides, esters, flavonoids, steroids, tannins, and terpenoids (Sesquiterpene lactones) (Choudhary et al., 2013)
Aerial part	
Aqueous extract	β-caryophyllene, δ-cadinene, γ-amorphene, cis-β-guaiene, premnaspirodiene, and 9-epi-β-caryophyllene (Joshi, 2014)
Methanol extract	lupeol, 12-oleanen-3-ol-3ß-acetate, stigmasterol, and β-sitosterol (Haque et al., 2012)

Table 3.2 Bioactive compounds in previous reported that have isolated from *C. cinereum* (continued).

Plant extraction	**Major of Phytochemicals (references)**
Stem	
Aqueous extract	epicatechin gallate, epicatechin, epigallocatechin gallate, myricetin, nitrate, and nitrite (Ketsuwan et al., 2017)
Ethanol extract	vernolide A and B (Kuo et al., 2003)
Root	
Petroleum ether extract	α-amyrin, α-amyrin acetate, β-amyrin, β-amyrin acetate, δ-amyrin acetate, and 3β-acetoxyurs-13(18)-ene (Misra et al., 1984)
Flower	
Aqueous extract	epigallocatechin gallate, kaempferol, myricetin, quercetin, and nicotine (Ketsuwan et al., 2017)
Hexane extract	8α-tigloyloxyhirsutinolide, 8α-tigloyloxyhirsutinolide-13-O-acetate, 8α-(2-methylacryloyloxy)-hirsutinolide-13-O-acetate, 8α-(2-methylacryloyloxy)-1α-methoxyhirsutinolide-13-O-acetate, vernolide-B, hirsutinolide-13-Oacetate, and vernolide-A (Youn et al., 2012)
Leaves	
Aqueous extract	epicatechin gallate, epicatechin, epigallocatechin gallate, catechin, myricetin, quercetin, nitrate, nitrite, and nicotine (Ketsuwan et al., 2017)
Ethanol extract	phenolic acids, phenolic aldehyde, and flavonoids (Alara et al., 2018) alkaloids, phenols, tannins, steroids, glycosides, flavonoids, carbohydrates, and terpenoids (Varsha et al., 2015)
Petroleum ether extract	alkaloids, tannins, saponins, and glycosides (Varsha et al., 2015)

3.4.3 The yield of TLE and CCE

The TLE was a sticky yellow to dark greenish powder with 6.27% extraction yields and the CCE was a sticky yellow to dark brownish powder with 6.95% extraction yields.

3.4.4 Preliminary phytochemical screening of TLE and CCE

The qualitative constituents of the leaf ethanoic TLE and the whole plant ethanoic CCE are presented in Table 3.3. Both plant extracts showed various active compounds that may enhance medical activity as in many ethnomedical reports. Alkaloids, flavonoids, phenolic compounds and tannins, sterols and terpenoids, and reducing sugars were presented in both plant extracts. In contrast, saponins were not found in both extracts.

Table 3.3 Preliminary qualitative phytochemical screening of TLE and CCE.

Phytochemical compounds	**Tests**	**TLE**	**CCE**
Alkaloids	Mayer's test	+	+
	Wagner's test	+	+
Flavonoids	Alkaline reagent test	+	+
	Shinoda test	+	+
Saponins	Foam test	-	-
Phenolic compounds and Tannins	Ferric chloride test	+	+
	Gelatine test	+	+
Sterols and Terpenoids	Salkowski's test	+	+
Reducing sugars	Benedict test	+	+

(-) defines as a negative reaction and (+) defines as a positive reaction

3.4.5 GC-MS and LC-MS analysis of TLE

The quantitative identification of the chemical compositions of TLE was analyzed using the GC-MS technique. The results showed that TLE composed of 16 known compounds including 11 richness compounds in phenolic compounds and aromatic acids as benzoic acid (14.91%), 4-vinylphenol (2.15%), 5-hydroxymethylfur fural (1.93%), 4-methylindoline (1.81%), 2-furanmethanol (1.59%), 9,9-dimethyl-9, 10-dihydroanthracene (1.41%), megastigmatrienone (1.23%), indole (1.08%), 7,9-di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione (0.99%), dihydroactinidiolide (0.98%) and phenylethyl alcohol (0.65%). A flavonoid (4H-Pyran-4-one,2, 3-dihydro-3,5-dihydroxy-6-methyl: 17.42%), a chromone (3-Methyl-2-Methyoxymethyl-4-Chro mone: 1.23%) and three terpenoids including loliolide (3.65%), neophytadiene (1.82%), dehydro-β-ionone (0.52%) and 13 unknown compounds were detected. The most prominent compounds in TLE were benzoic acid and 4H-Pyran-4-one,2,3-dihydro-3,5 -dihydroxy-6-methyl. The chemical compositions of the TLE are shown in Table 3.4 and the GC-MS chromatogram is demonstrated in Figure 3.2.

As mentioned in the literature reviews, the novel biologically actives such as phenolic and flavonoid compounds of TLE were identified by the LC-MS technique. Rutin, rosmarinic acid, quercetin, and kaempferol were used as reference standards based on the other previous scientific reports. Interestingly, only rosmarinic acid was presented in the TLE but other compounds were not found. The rosmarinic acid content was 36.9 mg/g extract, the detail is shown in Table 3.5 and the LC-MS chromatogram is demonstrated in Figure 3.3.

Table 3.4 The phytochemical constituents of TLE detected by GC-MS.

Peak	Identified compound	Molecular formula	Retention time (min)	Peak area (%)
1	2-Furanmethanol	$C_5H_6O_2$	4.641	1.593
2	Unknown		7.299	1.271
3	Unknown		8.568	5.029
4	Phenylethyl Alcohol	$C_8H_{10}O$	10.817	0.651
5	4H-Pyran-4-one,2,3-dihydro-3,5-dihydroxy-6-methyl-	$C_6H_8O_4$	11.767	17.415
6	4-Vinylphenol	C_8H_8O	13.725	2.149
7	5-Hydroxymethylfurfural	$C_6H_6O_3$	13.983	1.927
8	Unknown		14.135	0.548
9	Indole	C_8H_7N	15.779	1.075
10	4-Methylindoline	$C_9H_{11}N$	16.114	1.808
11	Unknown		19.622	22.621
12	Unknown		19.981	0.801
13	Dehydro-β-Ionone	$C_{13}H_{18}O$	20.536	0.516
14	Unknown		20.692	0.230
15	Unknown		21.236	1.858
16	Dihydroactinidiolide	$C_{11}H_{16}O_2$	21.847	0.981
17	Unknown		22.713	0.919
18	Megastigmatrienone	$C_{13}H_{18}O$	22.972	1.226
19	Unknown		23.731	2.040

Table 3.4 The phytochemical constituents of TLE detected by GC-MS (continued).

Peak	Identified compound	Molecular formula	Retention time (min)	Peak area (%)
20	Benzoic acid	$C_{10}H_{12}O_3$	24.048	14.910
21	9,9-Dimethyl-9,10-dihydroanthracene	$C_{16}H_{16}$	25.425	1.410
22	Unknown		26.560	2.057
23	3-Methyl-2-Methyoxymethyl-4-Chromone	$C_9H_6O_2$	26.739	1.234
24	(-)-Loliolide	$C_{11}H_{16}O_3$	27.257	3.651
25	Unknown		27.657	5.922
26	Unknown		28.558	0.854
27	7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9 diene-2,8-dione	$C_{17}H_{24}O_3$	29.977	0.988
28	Neophytadiene	$C_{20}H_{38}$	33.900	1.817
29	Unknown		40.653	2.500

Table 3.5 The phenolic and flavonoid compounds of TLE detected by LC-MS.

Peak	Identified compound	Retention time (min)	Quantitation mass (m/z)	Contents (mg/g extract)
1	Rutin	8.3	609	ND
2	Rosmarinic acid	14.6	359	36.9
3	Quercetin	19.4	301	ND
4	Kaempferol	23.2	285	ND

ND = not detected

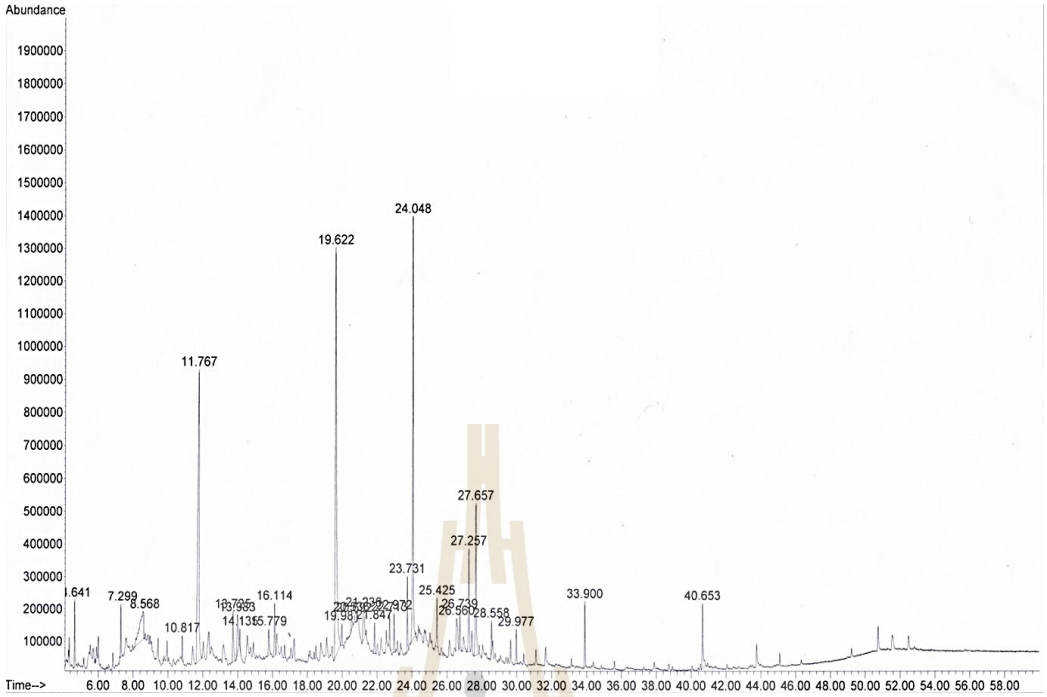

Figure 3.2 GC-MS chromatogram of TLE.

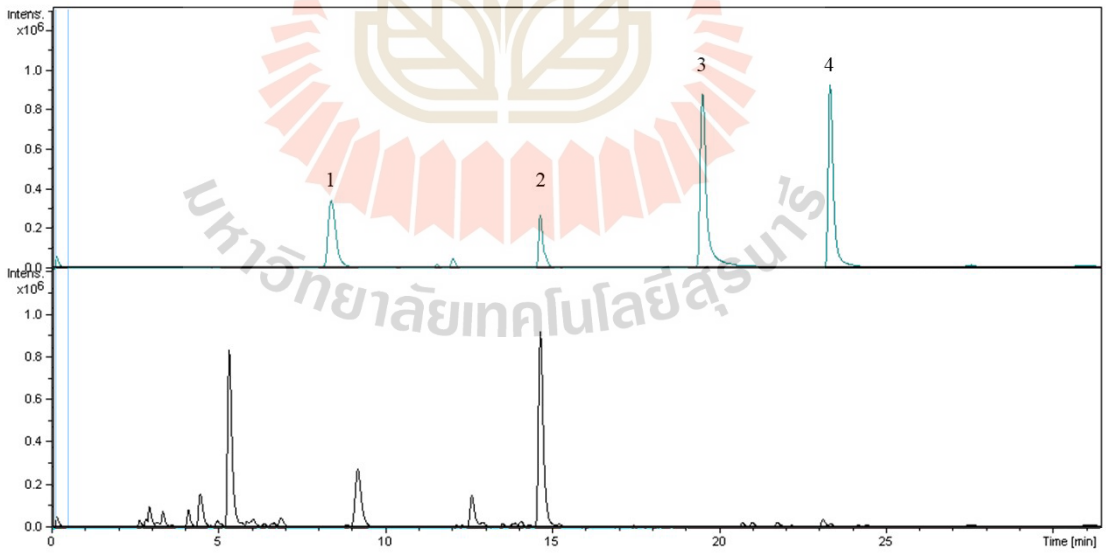

Figure 3.3 LC-MS chromatogram of TLE (lower panel) and selected reference standards (upper panel) including rutin (1), rosmarinic acid (2), quercetin (3), and kaempferol (4).

3.4.6 GC-MS analysis of CCE

The active compounds in CCE were determined by GC-MS and the result showed that 6 higher triterpenoids were presented such as lupeol (32.36%), β-amyrin (16.39%), β-amyrin acetate (7.47%), neophytadiene (7.19%), lupenone (1.27%) and hexahydrofarnesyl acetone (0.272%). Four phenolic compounds and aromatic acids are found including benzoic acid (0.85%), 2,6,10,14,18-pentamethyl-2,6,10,14,18-eicosapentaene (0.26%), phthalic acid (0.23%) and 10s,11s-himachala-3(12),4-diene (0.17%). Two phytosterols (β-Sitosterol: 2.68% and campesterol: 1.18%), two alkaloids (2-methyl-2,3,4,5,6,7-hexahydro-1H-2-benzazonine: 18.48% and β-carboline,7-methoxy-1,2-dimethyl: 0.316%), two fatty acyls (palmitic acid: 0.768% and palmitic acid ethyl ester: 0.172%) and 10 unknown compounds were presented. The highest prominent compounds were lupeol, 2-methyl-2,3,4,5,6,7-hexahydro-1H-2-benzazo nine, and β-amyrin in CCE. The chemical compositions CCE are shown in Table 3.6 and the GC-MS chromatogram is demonstrated in Figure 3.4.

Table 3.6 The phytochemical constituents of CCE detected by GC-MS.

Peak	Identified compound	Molecular formula	Retention time (min)	Peak area (%)
1	10s,11s-Himachala-3(12),4-diene	$C_{15}H_{24}$	20.733	0.173
2	Unknown		23.130	0.248
3	Benzoic acid	$C_{10}H_{12}O_3$	24.026	0.848
4	Unknown		28.633	0.280
5	Hexahydrofarnesyl acetone	$C_{18}H_{36}O$	28.740	0.272
6	Palmitic acid	$C_{16}H_{32}O_2$	31.138	0.768
7	Palmitic acid ethyl ester	$C_{18}H_{36}O_2$	31.762	0.172
8	β-Carboline, 7-methoxy-1,2-dimethyl	$C_{14}H_{14}N_2O$	32.379	0.316
9	Neophytadiene	$C_{20}H_{38}$	33.908	7.192
10	Unknown		40.222	0.336
11	Unknown		40.522	0.320
12	Phthalic acid	$C_{24}H_{38}O_4$	40.944	0.233
13	2,6,10,14,18-pentamethyl-2,6,10,14,18-eicosapentaene	$C_{25}H_{42}$	45.089	0.255
14	Unknown		46.392	0.302
15	Campesterol	$C_{28}H_{48}O$	50.395	1.179
16	Cholesta-6,22,24-triene,4,4-dimethyl-	$C_{29}H_{46}$	50.745	6.423
17	Unknown		51.001	0.271
18	β-Sitosterol	$C_{29}H_{50}O$	51.590	2.676
19	Unknown		51.750	0.381

Table 3.6 The phytochemical constituents of CCE detected by GC-MS (continued).

Peak	Identified compound	Molecular formula	Retention time (min)	Peak area (%)
20	β-amyrin	$C_{30}H_{50}O$	52.223	16.394
21	Lupenone	$C_{30}H_{48}O$	52.494	1.269
22	Lupeol	$C_{30}H_{50}O$	52.987	32.363
23	β-amyrin acetate	$C_{32}H_{52}O_2$	53.664	7.470
24	2-methyl-2,3,4,5,6,7-hexahydro-1H-2-benzazonine	$C_{13}H_{19}N$	54.533	18.480
25	Unknown		56.094	0.363
26	Unknown		56.024	0.447
27	Unknown		56.453	0.570

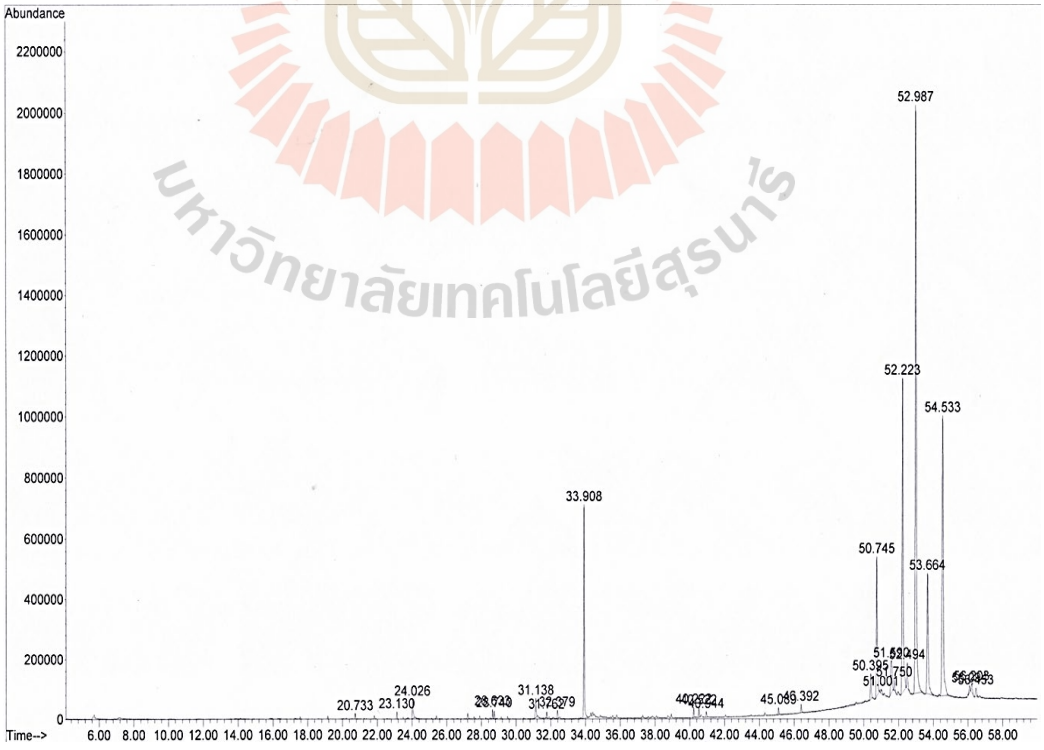

Figure 3.4 GC-MS chromatogram of CCE.

3.5 Discussion

Natural compounds may be alternatively used for the treatment of diabetes. Many herbals and their biomolecules have been studied in the literature for anti-diabetic potential, whereas their mechanisms of action are not completely understood (Coman et al., 2012). In this study, the primary phytochemical screening, GC-MS, and LC-MS analysis were revealed the presence of the various pharmaceutically phytochemical compounds of *T. laurifolia* leaves and *C. cinereum* whole plant ethanolic extracts.

During the primary phytochemical screening, phytochemical constituents as alkaloids, flavonoids, tannin, phenolic compounds, terpenoids, sterols, and reducing sugar were detected in both crude extracts, same as the results in Chuthaputti (2010), Oonsivilai et al. (2007), Phyu and Tangpong (2013) and Ruangyuttikarn et al. (2013) for TLE, and Varsha et al. (2015), Bashar et al. (2014) and Haque et al. (2012) for CCE.

The phytochemistry from GC-MS analysis of TLE had higher pyran derivatives (4H-Pyran-4-one,2,3-dihydro-3,5-dihydroxy-6-methyl; 17.41%) and benzoic acid (14.91%) which were similar to the report from Kanchanapoom et al. (2002). Pyran derivatives have various biological activities including anti-microbial (Khafagy et al., 2002), anti-viral (McCord et al., 1976), anti-tumor activity (Hong et al., 2011), sex pheromone (Shi et al., 1995), agrochemicals in cosmetics (Kumar et al., 2015) and glucose-lowering activity (Kumar et al., 2009). Another one, benzoic acid derivatives have been commonly revealed their biological activities as anti-microbial properties (Friedman et al., 2003) and anti-inflammatory of skin irritation in cosmetics (Ray et al., 2016). Dietary intake of sodium benzoate (the sodium salt of benzoic acid) could be used as the adjunctive treatment of schizophrenia and early psychotic disorders (Glue et al., 2014; Ryan et al., 2017). The benzoic acid derivatives administration has been

shown the potent anti-oxidant activity (Gayathri and Kannabiran, 2012). The polyphenols compounds were higher to 28.72% in TLE, same as previously reported from Phyu and Tangpong (2013) which may due to the higher anti-oxidant properties in this extract. Moreover, some phytoconstituent active compounds in *T. laurifolia* were not detected by GC-MS in this study, it may be due to the differentiation based on their general characteristics of this plants including global distribution, climate exposure, and ripening season which was mentioned in the review article from Sultana et al. (2015). Therefore, the LC-MS was also selected to specific the bioactive compounds of TLE instead and rosmarinic acid (36.9 mg/g extract) was higher represented which maybe act as an important bioactive compound and this result was similar to the study from Boonyarikpunchai et al. (2014) and Suwanchaikasem et al. (2014). Rosmarinic acid is an ester of caffeic acid and a natural polyphenolic compound in herbal plants. The biological effects of rosmarinic acid have been confirmed by various scientific reports such as anti-inflammatory (Colica et al., 2018), anti-allergic effects (Osakabe et al., 2004), anti-oxidant capacity and anti-microbial effect (Benedec et al., 2015), and anti-apoptosis activity (Gao et al., 2005), as well as anti-hyperglycemic activity and enhanced insulin sensitivity (Runtuwene et al., 2016).

Furthermore, the GC-MS analysis has been shown the highest natural terpenoids in CCE including lupeol, β-amyrin, β-amyrin acetate, and lupenone. This finding was similar to the study from Venkateswara (1962), Misra et al. (1984), and Haque et al. (2012). Beneficial health effects of lupeol have been reported such as cataract treatment (Asha et al., 2016), skin damage treatment (Malinowska et al., 2019), anti-carcinogenesis with ameliorate inflammation process (Saleem, 2009), and prevented free radicals production (Gupta et al., 2012). Previous researches have been

found that β-amyrin and β-amyrin acetate have various possibility effect such as anti-depressant effect (Aragão et al., 2006), analgesic and anti-inflammatory activities (Akihisa et al., 2010; Aragão et al., 2008), anti-hyperglycemic and hypolipidemic effects (Santos et al., 2012), anti-oxidant and free radical scavenging effects (Fabiyi et al., 2012; Sunil et al., 2014), anti-nociceptive effect (Holanda Pinto et al., 2008) and erectile function enhancement (Watcho et al., 2012). The therapeutic studied of lupenone has been shown anti-diabetic and anti-inflammatory effects (Xu et al., 2018; Xu et al., 2014). The bioactive phytosterols; campesterol and β-sitosterol have been detected in this CCE as similar to the previous reported from Venkateswara (1962). Various biological activities of phytosterols and their derivatives have been reported in several experiments which may improve health benefits such as anti-cancer agent (Shahzad et al., 2017), anti-inflammatory activity (Hu et al., 2017), safety used in eye makeup and a moisturizing product (Fernandes and Cabral, 2007) and acted as anti-diabetic and cholesterol-lowering agents (Jones et al., 1997). Relevant literature in the largest distribution of *Vernonia* genus has been collected by Toyang and Verpoorte (2013) which provided the different bioactive compounds among *Vernonia* genus depending on each folk ethnomedicine uses and extraction methodology. Sesquiterpene lactones (vernolide derivatives) are not detectable in this extract, hence the special and specific extraction process may be verifying. However, the terpenoids were the highest potential in this extract (64.96%), regardless of other sesquiterpenoids, triterpene, or triterpenoids and the higher detection of phytosterols compounds.

These phytochemicals might be indicating the importance of both *T. laurifolia* and *C. cinereum* for several medicinal purposes. The isolated phytoconstituents from TLE and CCE have been proven in the management of several illnesses and clinical

usage. Interestingly, in TLE phytoconstituents, pyran and rosmarinic acid have been shown the glucose lowering activity and insulin sensitivity improvement (Kumar et al., 2009; Runtuwene et al., 2016). Rosmarinic acid has been described in the arachidonic acid inhibition which involves in various signaling pathways as in uterine intracellular signaling (Chen, 2010; Gamaro et al., 2011), as well as the coronary artery smooth muscle relaxation caused by the inhibition of calcium influx was found in 5-Hydroxymethylfurfural (Wölkart et al., 2017). Likewise, β-amyrin, β-amyrin acetate, lupenone, campesterol, and β-sitosterol have been revealed their anti-hyperglycemic and anti-lipidemic properties (Jones et al., 1997; Santos et al., 2012; Xu et al., 2018; Xu et al., 2014). Moreover, β-amyrin and β-amyrin acetate exhibited the relaxant properties against an agonists-induced contraction in reproductive erectile function (Watcho et al., 2012). β-sitosterol has been proven the uterotonic activity by inhibited calcium entry on the uterine contractile pattern (Promprom et al., 2010). Lupeol can inhibit cAMP-PKA signaling pathways which may take an alteration on the relaxation process in the uterus (Hasmeda et al., 1999). These relevant reviews were offered the value of these herbal potentials. Therefore, there is no evidence demonstrating the pharmaceutical interventions of these plants on the female reproductive alteration along with their well-known anti-diabetic properties, the effects of these herbal plants on uterine contraction in gestational diabetic rats will be concentrated in the next experiment.

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CHAPTER IV

MYOMETRIAL PHYSIOLOGY

IN GESTATIONAL DIABETIC RATS

4.1 Abstract

Poor myometrial activity may play an important role in pregnancy complicated diabetes or gestational diabetes mellitus (GDM) which affects severely maternal health status and fetal complications. The incidence of cesarean section has been higher in maternal diabetes. Several classes of anti-diabetic drugs (glibenclamide: GLB, metformin: MET, and insulin) are used to control diabetes with several adverse effects that have been reported. Nevertheless, there are less observational and interfering researches in the mechanical characteristics of uterine smooth muscle contraction in pregnancy complicated diabetes. The aims of this experiment were investigated the uterine smooth muscle physiology in term of general contractility patterns (I), in the response to glibenclamide (II), metformin (III), and insulin (IV) on spontaneous contraction, comparison between non-gestational diabetic (Non-GD) and gestational diabetic (GD) rat uteri on late gestation. Uterine strips from GD have significantly manifested the reduction in spontaneous activity, abolished the maximal response to KCl ($P < 0.01$) and OT-induced contractility ($P < 0.05$), and decreased contractile response to the modulation of force production by nifedipine (L-type Ca^{2+} channels blocker), Y27632 (Rho-associated kinase blocker) and WT (MLCK inhibitor). For

anti-diabetic drugs tested, various concentration dependency was performed and there was demonstrated that GLB (IC_{50} value; 27.19 μM) facilitated the impermanent uterine mobility with prolonging relaxation due to the incomplete K_{ATP} channel closure, significantly found in non-GD ($P < 0.01$), MET (IC_{50} value; 417.51 μM) had been shown a small alteration on spontaneous contraction with no statistically different in both groups ($P > 0.05$), insulin (EC_{50} value; 4.12 μM) had been stimulated the uterine contraction, significantly higher found in GD ($P < 0.01$). In conclusion, the main Ca^{2+}-Calmodulin-MLCK pathway and Ca^{2+} sensitization in uterine smooth muscle function were altered in diabetic uteri, indicating uterine dysfunction in late diabetic gestation. Different actions of anti-diabetic drugs generally mediated in the pregnant uterus which GLB was potent temporary mobility with prolonging relaxation, MET had no alteration, whereas insulin had a stimulation property. Diabetic uteri were reversed these actions by attenuated the relaxation to GLB, variation response to MET, and stronger force production to insulin. In women with GDM, the treatment with ordinary anti-diabetic drugs (GLB, MET, and insulin) appears to be associated with various enhancements of uterine contractility which may relate to the onset of labor.

4.2 Introduction

Preterm birth, ineffective labor, post-partum hemorrhage, and various mother and child complications result from uterine excitation-contraction dysfunction under different physiological conditions and its response ability on membrane potential, intracellular Ca^{2+} transients and stimuli actions are selective to determine the myometrial activities with the variant types of action potentials adaptation are seen during late gestation (Testrow et al., 2018). Generally, the control of uterine smooth

muscle activity was able to maintain quiescence throughout pregnancy, changes to weak irregular contractions at midterm, and then turn to strong regular contractions at term approaches to mediate parturition (Bengtsson et al., 1984). Ovarian steroid hormone regulation of myometrial quiescence during pregnancy is linked with the circulating levels progesterone (P_4) which in turn directly inhibited contractile genes, and shift to its level withdrawal and estrogen activation leads to the initiation of labor at the end of pregnancy which increasing the expression of the contraction-associated proteins, as resulting increased Ca^{2+} influx into myometrial cells (Ilicic et al., 2020). However, the myometrial sensitivity at rest during pregnancy and contractile initiation during parturition are incompletely understood.

The preterm delivery is tended significantly higher in GDM with the length of gestation alteration (Köck et al., 2010). The risk of spontaneous preterm delivery is correlated to glucose levels during pregnancy (Hedderson et al., 2003). The reasons for preterm delivery were a large population and 38% associated with preeclampsia in GDM (Sibai et al., 2000). Moreover, GDM is associated with an increased frequency of maternal complications and the need for cesarean section (Krishna Murthy et al., 2002). The rate of cesarean section (c/s) became higher in pre-existing maternal diabetes (Ehrenberg et al., 2004), but the true reasons for the high cesarean section rate were not established. Therefore, poor myometrial contractility has an important role linked to c/s and promoted a 6-fold risk of post-partum hemorrhage in gestational diabetes compared with normal pregnancy (Dunne et al., 2003). Some studies have been manifested these relations *in vitro* experiments and the conditions that pre-existing diabetes may affect the myometrial functions were unclear (Franchi et al., 1988; Jawerbaum et al., 1996; McMurtrie et al., 1985). Review evidence indicated the anti-

diabetic agents used in pregnancy appears to be associated with few adverse complications (Nicholson and Baptiste-Roberts, 2011). Therefore, the uterine contractile responses on late gestation in gestational diabetic myometrium and the force development to diabetic standard drugs were investigated in organ bath experiments.

4.3 Materials and methods

4.3.1 Myometrial tissue preparations and tension measurement

Pregnant Wistar rats (250-300 g) were assigned into 2 groups: non-gestational diabetic rats (Non-GD) and gestational diabetic rats (GD). The mating procedure and diabetic induction were described in 2.2.4 and 2.2.5, respectively. Term pregnant rats were sacrificed by CO_2 asphyxia on the 19^{th}-21^{st} days of pregnancy. The uterine horn was removed, weighed, and immediately washed in the physiologic Krebs' solution for *in vitro* study in physiological uterine contraction.

The longitudinal muscular strips were cut and separated from the endometrium (1-2 mm x 0.5 mm x 10 mm) which attached at each end to metal hooks and another end was fixed to a transducer in the organ bath apparatus that containing the physiologic Krebs' solution (37ºC, pH 7.4) with tension measurements recorded. The spontaneous contraction was allowed under 1g resting tension. All strips were allowed to contract spontaneously with 30 min-equilibration times before the application of any chemical study.

4.3.2 Effect of diabetes on force production in the Ca^{2+}-Calmodulin-MLCK pathway

Several pathways can be generated uterine force which involves the main Ca^{2+}-Calmodulin-MLCK pathway. The force produced on spontaneous contraction,

high K^+ depolarization-induced contraction, and oxytocin-induced contraction were evaluated.

(A) Spontaneous activity

To investigate the general alteration of diabetes on the spontaneous pattern in the process of arise intracellular Ca^{2+} concentration via L-type Ca^{2+} channel activation in Ca^{2+}-Calmodulin-MLCK pathway during pregnancy, the strips were allowed to contract spontaneously under normal Krebs' solution at least 30 min until stable contractility. The tension was recorded in both non-GD and GD.

(B) High K^+ depolarization (KCl)

To investigate the effect of diabetes on force production during voltage-operated Ca^{2+} channel opening in response to membrane depolarization using a high KCl solution, strips were allowed to stable spontaneous contraction and the last 30 min was taken as the control (100% contraction). The strips were stimulated by high KCl (40 mM) for 30 min and then replaced by Krebs' solution with spontaneous contractions resumed. The tension was recorded in both non-GD and GD.

(C) Oxytocin-induced contraction

To investigate the effect of diabetes on the modulation of OT stimulated IP_3-induced contractility pathway and enhances its sensitivity due to free Ca^{2+} withdrawal, OT is a well-known hormone stimulant in smooth muscle cells by triggers Ca^{2+} release from internal stores to initiates a stronger contraction and its sensitivity is enhancing in Ca^{2+}-free solution ($0Ca^{2+}$ solution). In normal Krebs' solution, OT (10 nM) was added to the strips after stable contraction for 30 min followed by Krebs' solution washed off. In $0Ca^{2+}$ solution, strips were explored to $0Ca^{2+}$ solution for 15 min after spontaneous contraction and oxytocin was applied to the strips in the

continued presence of $0Ca^{2+}$ solution for 15 min followed by Krebs' solution washed off. Tension during oxytocin stimulation in normal Krebs' solution and oxytocin in the presence of $0Ca^{2+}$ solution of both non-GD and GD were recorded.

4.3.3 Effect of diabetes on Ca^{2+} transient in the non-Ca^{2+}-Calmodulin-MLCK pathway modulation

In addition, alternative pathways have been modulated the protein cascade which related to contractile activity as well. To investigate the effect of diabetes depends on the Ca^{2+}-Calmodulin-MLCK pathway, the contractile response in the presence of cascades inhibitors; L-type Ca^{2+} channels, Rho-associated kinase, and MLCK inhibitors were examined.

(D) L-type Ca^{2+} channels inhibitors

To investigate the effect of diabetes during the absence of external Ca^{2+} condition, a blocker of Ca^{2+} entry via L-type Ca^{2+} channel was used. Nifedipine (10 μM) was applied to the strips after stable contraction for 30 min and then washed off with normal Krebs' solution. The tension was recorded in both non-GD and GD.

(E) Rho-associated kinase inhibitors

To investigate the effect of diabetes on the Ca^{2+} sensitization in the Rho-associated kinase pathway, a selective potent ATP-competitive ROCK components inhibitor, Y27632 (10 μM) was used. In the control condition, Y27632 was applied to the strips for 30 min and washed off with Krebs' solution in both non-GD and GD. The tension was recorded in both non-GD and GD.

(F) Myosin light chain kinase (MLCK) inhibitors

To investigate the effect of diabetes on the complements in the Ca^{2+}-calmodulin-MLCK pathway. Wortmannin (WT, 4 μM), a well-known selective

inhibitor of protein cascade enzyme in the MLCK pathway was added after stable contraction, allowed for 30 min, and returned to normal Krebs' solution. Additionally, the effects of WT on the contractile response in Ca^{2+} influx by high KCl solution or Ca^{2+} release from internal stores by OT were taken. Strips were explored to KCl solution for 30 min after spontaneous contraction and wortmannin was applied to the strips in the continued presence of KCl for 30 min, and then wash off. The experiment in the exposure to OT was performed the same as KCl procedure. The tension of both non-GD and GD were recorded during WT alone and in the presence of KCl and OT.

4.3.4 Effect of selected anti-diabetic drugs on uterine force modulation

Many pregnant women showed that at least one or more drugs have been used during their gestation following the US food and Drug Administration (FDA) X category. The selected anti-diabetic agents which were commonly used in diabetic pregnant women; GLB, MET, and insulin were examined.

(G) Oral anti-diabetic drugs; GLB and MET

To investigate the effect of commonly anti-diabetic agents on the pregnant uterine contractility, the concentration dependency of GLB (10, 20, 30, 40, 50, and 60 μM) and MET (100, 200, 300, 400, 500, and 600 μM) were tested with the gradually increased concentration cumulatively for 30 min intervals to the pregnant myometrial strips after the spontaneous period and washed out with Krebs' solution. The contractile responses in each concentration were recorded. The contractile response in AUC of GLB and MET were used to determine the median effective concentration whatever a half-maximum stimulation (the effective concentration: EC_{50}) or a half-maximum inhibition (the inhibitory concentration: IC_{50}) in a nonlinear curve fitting program, Microcal Origin Software (Sebaugh, 2011). The optimal concentration from

EC_{50} or IC_{50} of GLB and MET were tested in 30 min-spontaneous contractions and tension was recorded in both non-GD and GD.

(H) Pancreatic hormone (Insulin)

Insulin is the first requirement in pregnancy complicated by diabetes or when oral glucose-lowering drugs are not achieved glycemic control. As a selected anti-diabetic agent, the concentration dependency of insulin (1 to 5 μM) was tested the same as GLB and MET procedure. The contractile responses in each concentration were recorded. As the priority agent in diabetic pregnancy, the optimal concentration from EC_{50} or IC_{50} of insulin was tested in spontaneous contraction for 30 min. The tension was recorded in both non-GD and GD.

4.3.5 Chemicals and physiological solutions

All analytical grade of chemicals were purchased from Sigma® and Merck, Singapore and prepared as describes in 2.6. Y27632; a selective inhibitor of ROCK was dissolved in distilled water and used at a concentration of 10 μM. WT, an inhibitor of MLCK was dissolved in DMSO and used at a concentration of 4 μM. Nifedipine, an inhibitor of the L-type Ca^{2+} channel was dissolved in absolute ethanol and used at a concentration of 10 μM. The EC_{50} or IC_{50} concentration of GLB, MET, and insulin was dissolved in Krebs' solution. All stock solutions were prepared and stored following the guideline of the producer.

4.3.6 Statistical analysis

The data were analyzed using Microcal Origin software. Spontaneously uterine contractility was exhibited as a percentage of contractions including the integral of force (area under the contraction, AUC) which was assessed as the ability of uterine contraction, amplitude (g), frequency (contraction/min), and duration (peak/min). The

contractility responses to any exposure of drugs or chemicals are compared with the general spontaneous contraction as estimated 100%. The percent response change was calculated and compared between non-GD and GD. Data are presented as means ± standard error of mean (S.E.M) and "n" represents the number of uterine samples, each one from a different animal. Significance was tested using an appropriately paired student *t*-test in the same strip and using unpaired student *t*-test compared between groups. *P* value < 0.05 taken to be significant.

4.4 Results

4.4.1 Effects of diabetes on spontaneous contraction in pregnant myometrium

The phasic continued spontaneous contractions are depended on the efflux of Ca^{2+} with the activation in the MLCK pathway. The alteration of diabetes on the stable uterine contractions pattern in 30 min is shown in Figure 4.1. Contractile parameters included frequency, amplitude, single peak duration, and AUC are summarized in Table 4.1. The contractile response in non-GD was 0.53 ± 0.04 contraction/min for frequency, 1.25 ± 0.16 g for amplitude, 1.95 ± 0.14 min in single peak duration, and 588.20 ± 78.20 for AUC, which these parameters were assumed as control (100%) and used to calculate the percentage change between groups. In GD, there was a significantly decreased frequency of the contractions to 0.42 ± 0.04 contraction/min (-21.35%), reduced amplitude of the contractions to 0.82 ± 0.08 g (-34.00%), as well as prolonged duration of the contraction to 2.56 ± 0.25 min/peak (+31.54%), together these changes resulting in the mean integral force was significantly decreased to 351.83 ± 36.65 (-40.19%), compared with non-GD ($P < 0.05$).

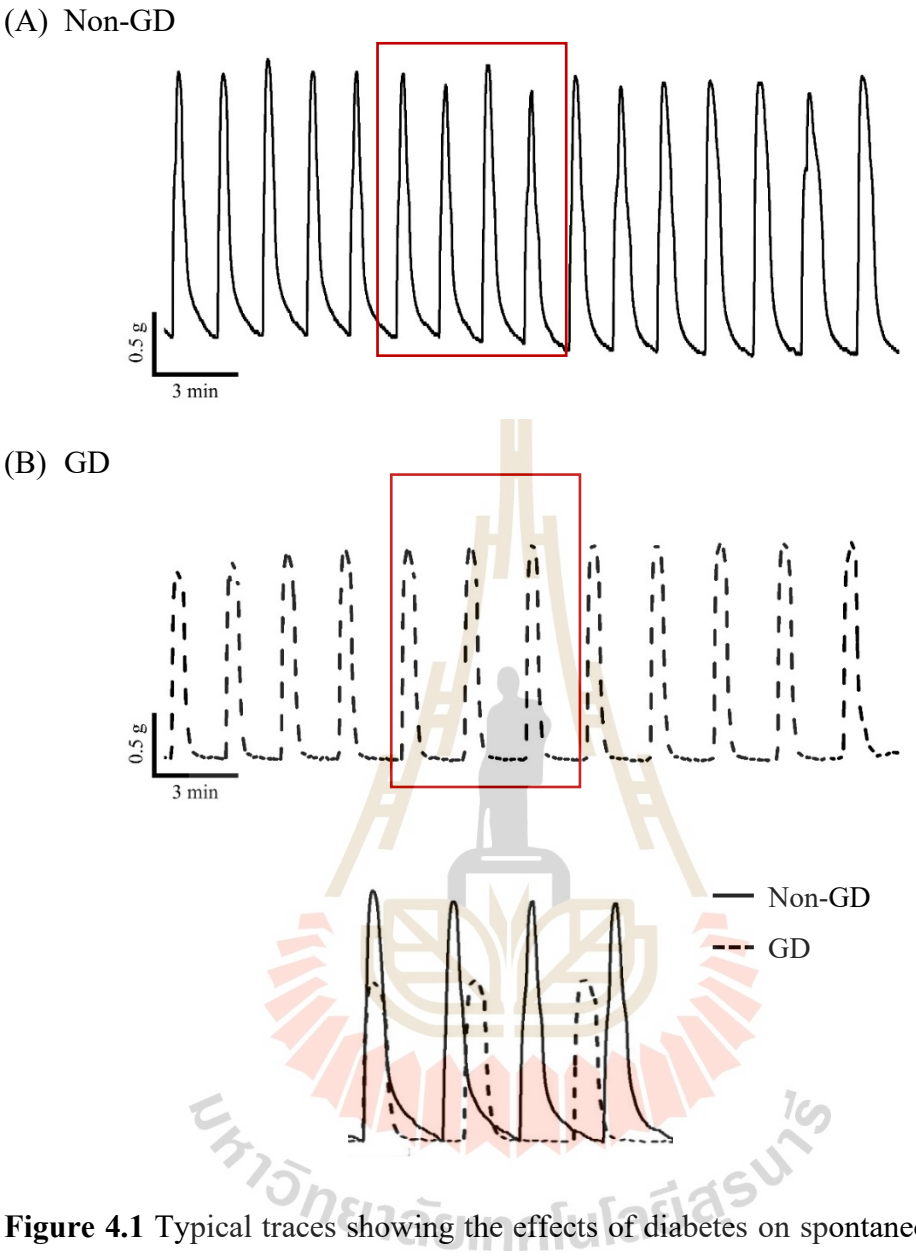


Figure 4.1 Typical traces showing the effects of diabetes on spontaneous contraction pattern in non-GD (A) and GD (B). Strips were allowed to contract spontaneously for 30 min and frequency, amplitude, and single peak duration and AUC of contraction were observed (n = 12).

Table 4.1 The spontaneous contractile activity in non-GD and GD at term.

Contractile parameters	Non-GD	GD	%Change	n
Frequency (contraction/min)	0.53 ± 0.04	0.42 ± 0.04*	-21.35%	12
Amplitude (g)	1.25 ± 0.16	0.82 ± 0.08*	-34.00%	12
Duration (min/peak)	1.95 ± 0.14	2.56 ± 0.25*	+31.54%	12
AUC	588.20 ± 78.20	351.83 ± 36.65*	-40.19%	12

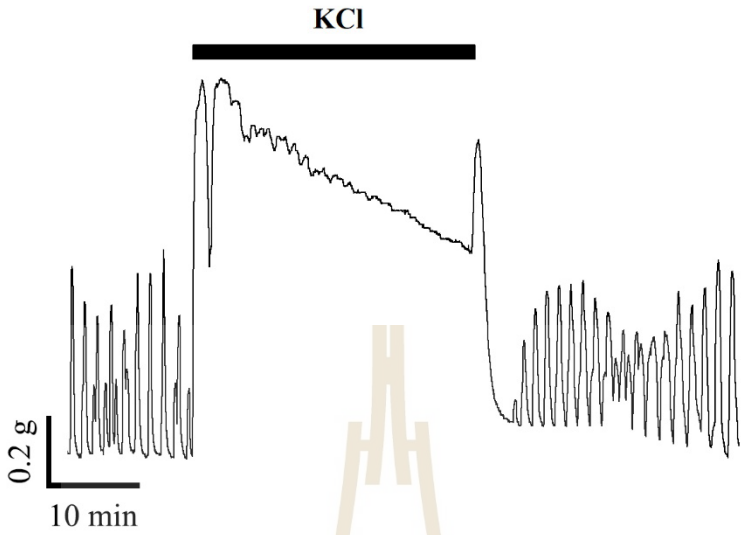
Data are expressed in mean ± S.E.M. and n represented uterine sample from a different animal. The *P*-values for frequency, amplitude, duration, and AUC of GD is significantly different from the non-GD using unpaired student *t*-test. The percentage change in GD was calculated using the value in non-GD assume as 100%. (*$P < 0.05$).

4.4.2 Effects of diabetes on KCl and OT-induced contraction in the presence of external Ca^{2+} and $0Ca^{2+}$ solution

High K^+ depolarization-induced contraction

The tonic force was rose and stabilized during membrane depolarization by KCl solution until return to normal Krebs' solution in both experimental groups. The mean AUC was also significantly increased in non-GD (535.66 ± 6.80%) and decreased in GD (375.22 ± 17.03%), compared with spontaneous control, 100%. The contractile response to KCl in GD was significantly dropped as 29.95% less than non-GD ($P < 0.01$) as seen in Figure 4.2.

(A) Non-GD



(B) GD

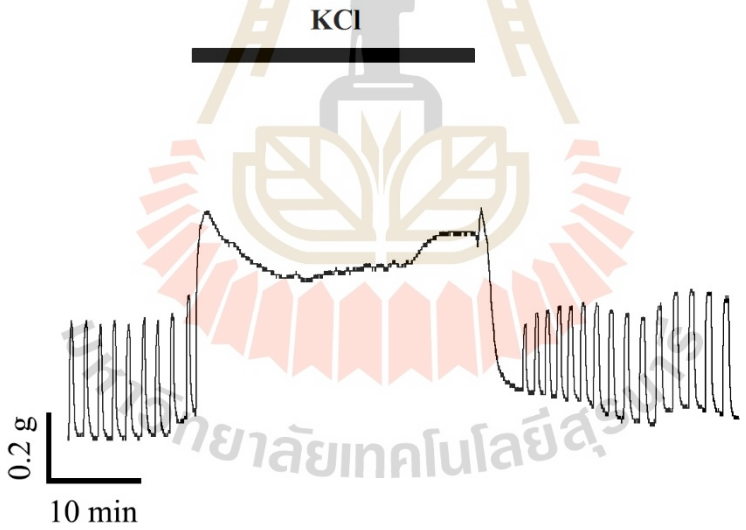


Figure 4.2 Typical traces showing the uterine response to the high K^+ depolarization-induced contraction in non-GD (A) and GD (B). A significant reduction of the contractile response in AUC was observed in GD (n = 4).

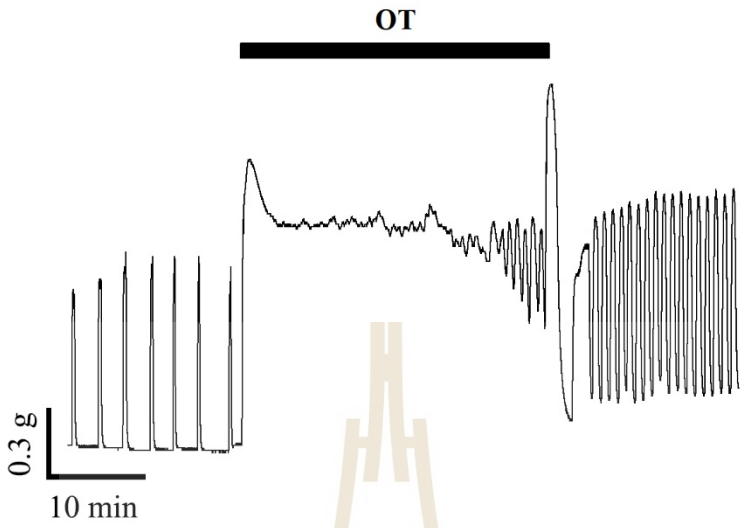
Oxytocin-induced contraction

The application of OT had triggered the myometrium in both experimental groups. A significant increase in the mean AUC in non-GD was 429.66 ± 22.90% with the prolonged strong tonic contraction after exposure to the OT. Additionally, the transient tonic force was rose in GD at the beginning as well and then turned over to the phasic contraction as seen in Figure 4.3. The mean AUC in GD was significantly lower, 319.10 ± 25.45% as compared with spontaneous control (100%) with 25.73% reduced response activity compared with non-GD ($P < 0.05$).

Moreover, under the $0Ca^{2+}$ solution, OT had produced a small tonic force during the external Ca^{2+} withdrawals. As seen in Figure 4.4, the mean AUC of the triggered contraction by OT in non-GD was 42.23 ± 9.28% and little contraction in GD was 14.20 ± 1.21% which was a 66.36% response reduction compared with non-GD ($P > 0.05$).

The contractile response of agonists-induced uterine contraction was summarized in Table 4.2.

(A) Non-GD



(B) GD

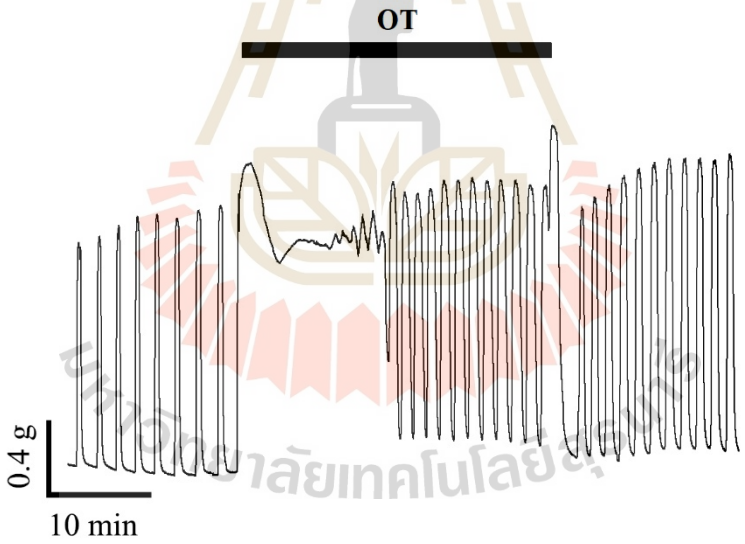
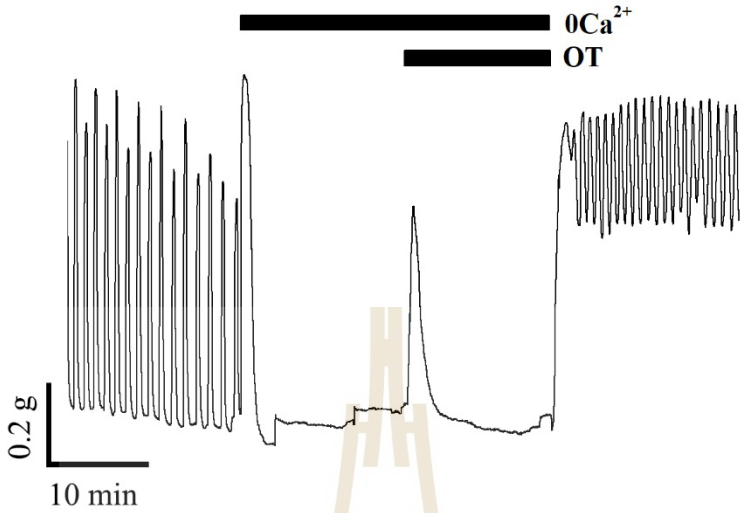


Figure 4.3 Typical traces showing the uterine response to the oxytocin-induced contraction in the presence of external Ca^{2+} in non-GD (A) and GD (B). A significant reduction of the contractile response in AUC was observed in GD (n = 4).

(A) Non-GD



(B) GD

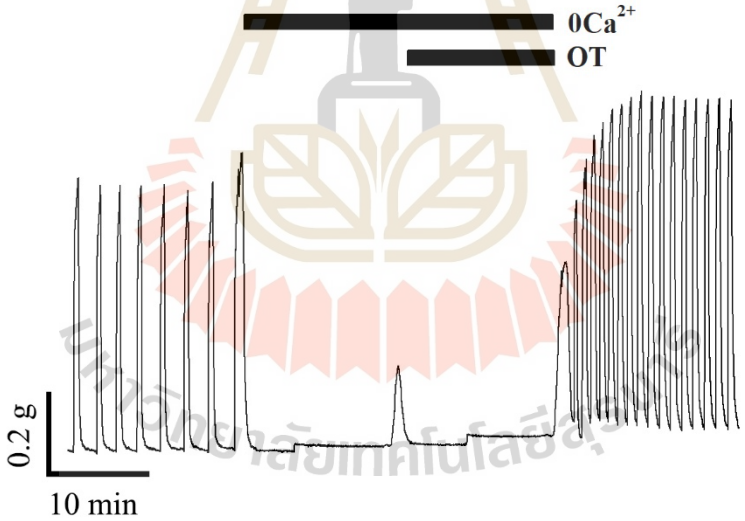


Figure 4.4 Typical traces showing the uterine response to the oxytocin-induced contraction under the $0Ca^{2+}$ solution in non-GD (A) and GD (B). A reduction of the contractile response in AUC was observed in GD (n = 4).

4.4.3 Effects of diabetes on nifedipine, Y27632, and wortmannin on spontaneous contraction

Nifedipine

Nifedipine had blocked the entry of external Ca^{2+} on the L-type Ca^{2+} channel which is the primary cascade for initiating the contraction. After the application of nifedipine, the frequency and amplitude were directly disappeared to the basal line in both experimental groups. Both integral forces were abolished and no significant difference was observed between groups ($P > 0.05$) (Figure 4.5).

Y27632

The inhibitory effect occurred during the application of the Rho-kinase inhibitor, Y27632. Myometrial contractility was slightly deceased in both experimental groups, 94.01 ± 2.56% in non-GD and 74.13 ± 7.76% in GD with a 21.15% response reduction compared with non-GD ($P > 0.05$) as shown in Figure 4.6.

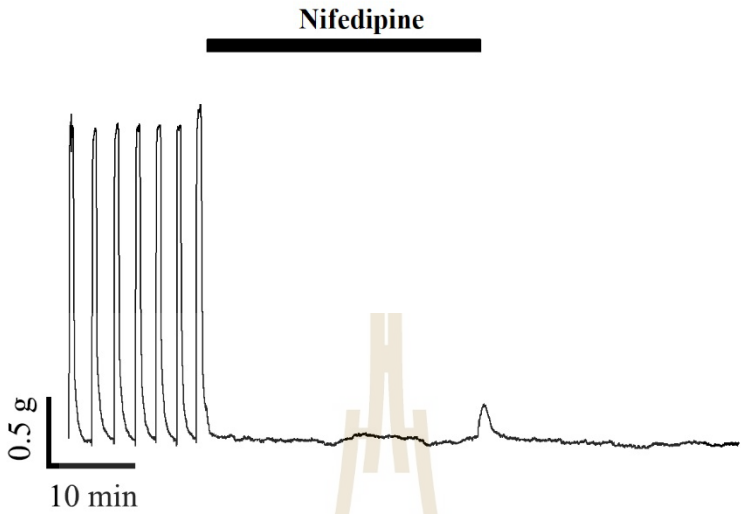
Wortmannin

WT has gradually diminished the uterine spontaneous contractility in both experimental groups (Figure 4.7). After applied WT, the amplitude of both experimental groups was reduced and the average response time was 22.70 ± 2.75 min in non-GD and 19.54 ± 4.36 min with 13.92% faster response time in GD, no significantly different between both reaction times to wortmannin ($P > 0.05$). As a result, the AUC in both experimental groups were abolished as 57.58 ± 14.99% in non-GD and 50.59 ± 10.89% with 12.15% declined the responsibility in GD. A significantly different was not observed between experimental groups ($P > 0.05$).

The application of WT following the exposure to KCl solution had been shown that the contractility was also declined due to the effect of WT after the sustained

tonic contraction induced by KCl solution (Figure 4.8). The AUC in both experimental groups was reduced to 38.25 ± 5.68% in non-GD and 33.39 ± 4.13% with a 12.73% decreased response in GD ($P > 0.05$). Furthermore, the result in WT during the exposure to OT in both experimental groups was presented in Figure 4.9. The slight reduction in AUC was greater in both experimental groups which were 32.45 ± 3.27% in non-GD and 35.19 ± 5.62% with 8.46% higher in GD. There was no significant difference between the experimental groups ($P > 0.05$). Data on the contractile response in the specific inhibitors were summarized in Table 4.2.

(A) Non-GD



(B) GD

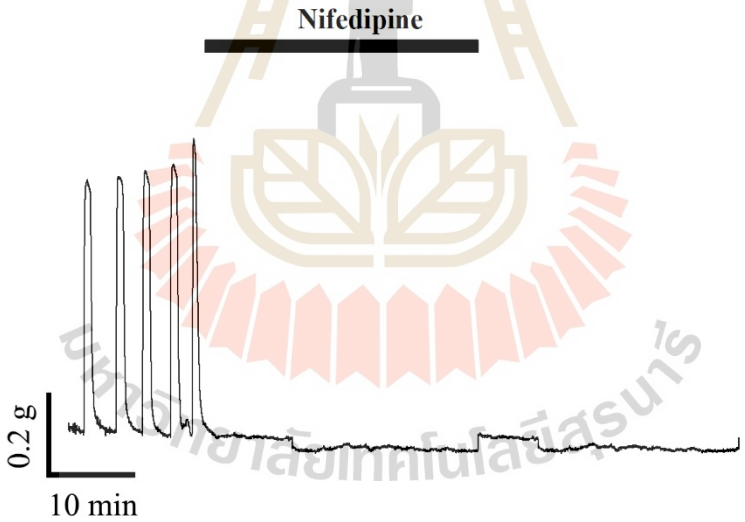
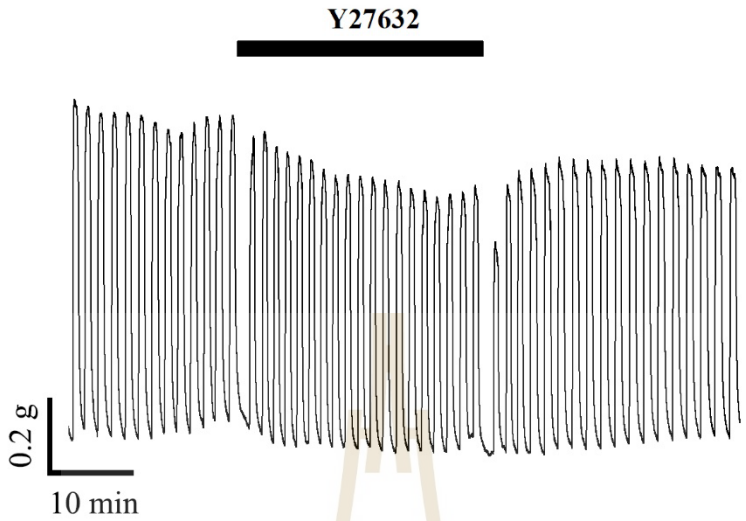


Figure 4.5 Typical traces showing the uterine response to nifedipine in non-GD (A) and GD (B). The inhibitory response during the application of nifedipine was observed in both experimental groups (n = 4).

(A) Non-GD



(A) GD

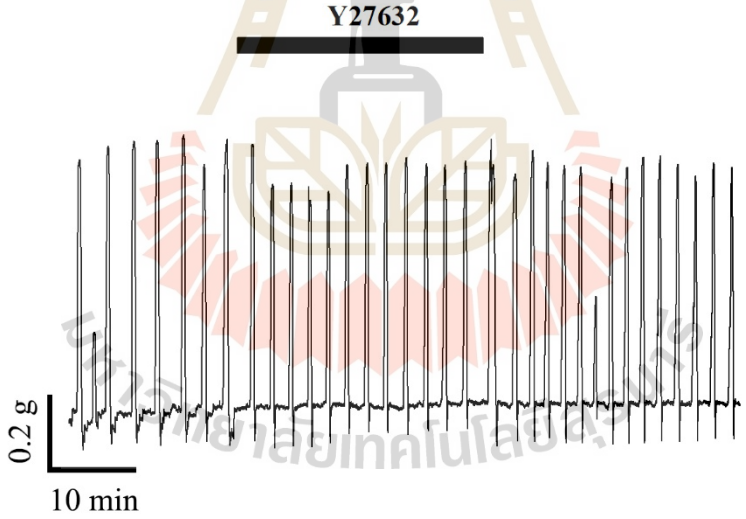
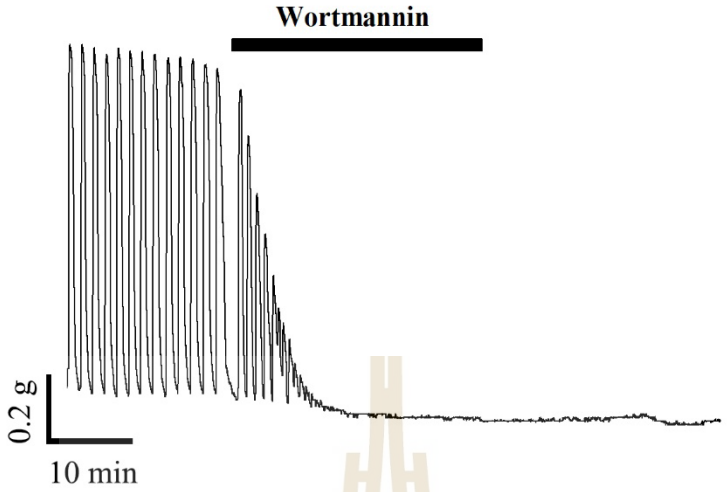


Figure 4.6 Typical traces showing the uterine response to Y27632 in non-GD (A) and GD (B). A significantly reduction of the contractile response in AUC was observed in both experimental groups (n = 5).

(A) Non-GD



(A) GD

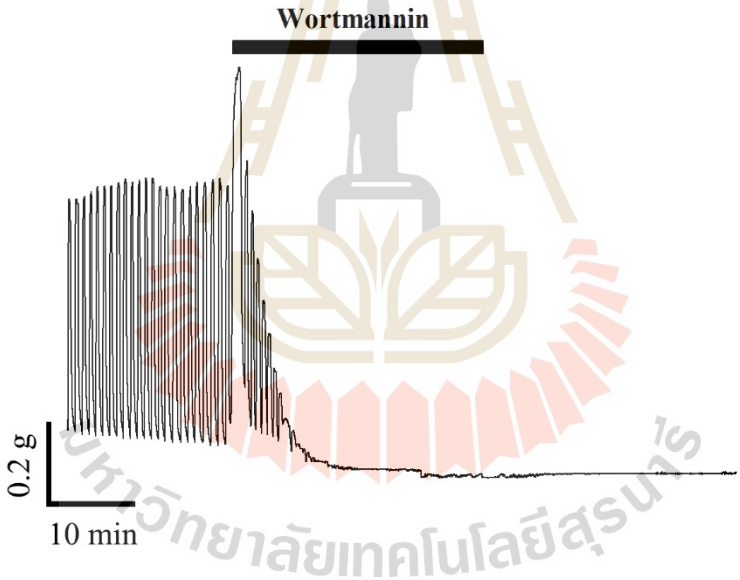
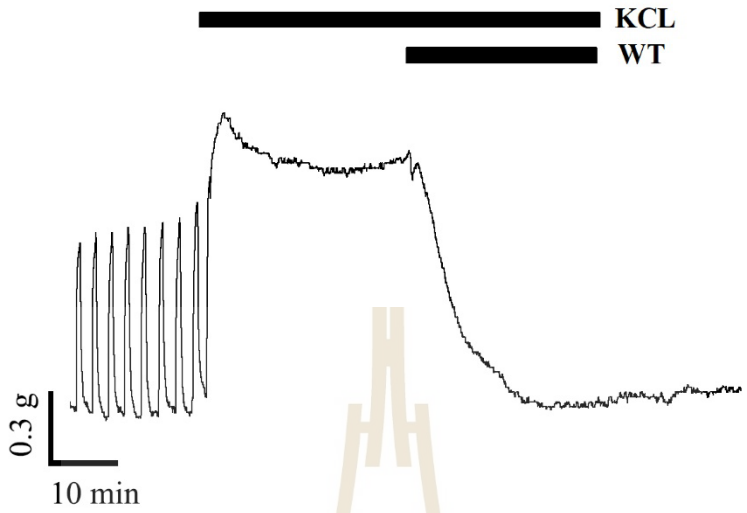


Figure 4.7 Typical traces showing the uterine response to wortmannin alone in non-GD (A) and GD (B). A significant reduction of the contractile response in AUC was observed in both experimental groups (n = 5).

(A) Non-GD



(B) GD

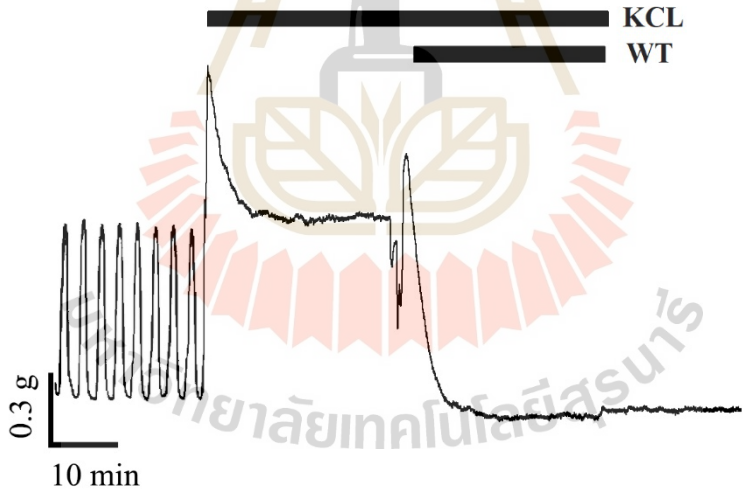
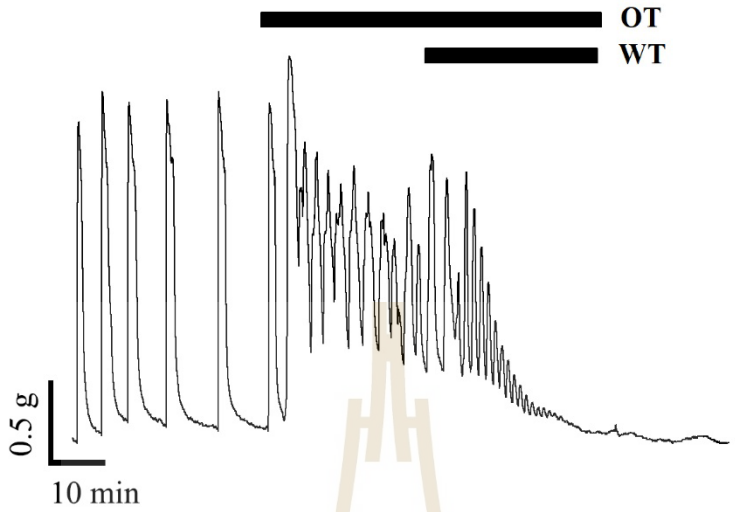


Figure 4.8 Typical traces showing the uterine response to wortmannin during the exposure to KCl solution in non-GD (A) and GD (B). KCl had initiated a tonic force and a reduction of the contractile response in AUC after wortmannin applied was observed in both experimental groups (n = 5).

(A) Non-GD



(B) GD

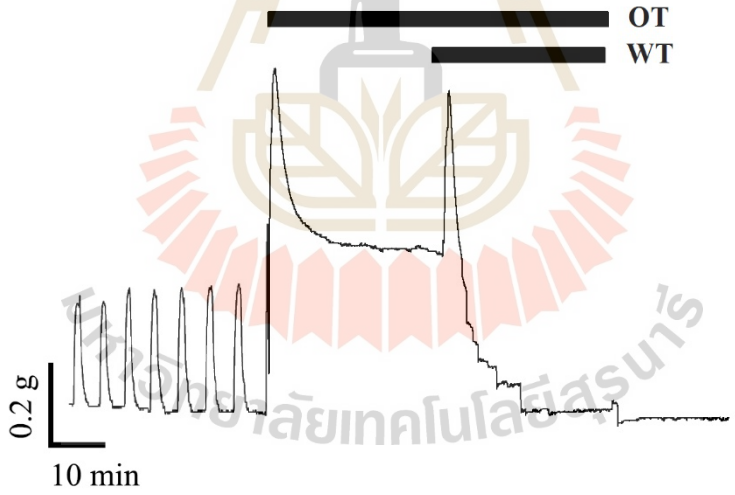


Figure 4.9 Typical traces showing the uterine response to wortmannin during the exposure to OT in non-GD (A) and GD (B). The strong phasic contraction was originated by OT and thereby a reduction of the contractile response in AUC after wortmannin applied was observed in both experimental groups (n = 5).

Table 4.2 The general contractile response in the several pathways modulation in non-GD and GD.

Tested substances	AUC (%)			
	Non-GD	GD	% Change	n
Spontaneous control	100	100		
Agonists-induced contraction				
High K^+	535.66 ± 6.80$^{\#\#}$	375.22 ± 17.03$^{\#\#}$	-29.95%**	4
Oxytocin	429.66 ± 22.90$^{\#\#}$	319.10 ± 25.45$^{\#\#}$	-25.73%*	4
Oxytocin in $0Ca^{2+}$	42.23 ± 9.28$^{\#\#}$	14.20 ± 1.21$^{\#\#}$	-66.36	4
Selective inhibitors				
Nifedipine	0 ± 0.00	0 ± 0.00	0%	4
Y27632	94.01 ± 2.56	74.13 ± 7.76$^{\#}$	-21.15%	5
Wortmannin	57.58 ± 14.99$^{\#}$	50.59 ± 10.89$^{\#}$	-12.15%	5
Wortmannin in KCl	38.25 ± 5.68$^{\#\#}$	33.39 ± 4.13$^{\#\#}$	-12.73%	5
Wortmannin in OT	32.45 ± 3.27$^{\#\#}$	35.19 ± 5.62$^{\#\#}$	+8.46%	5

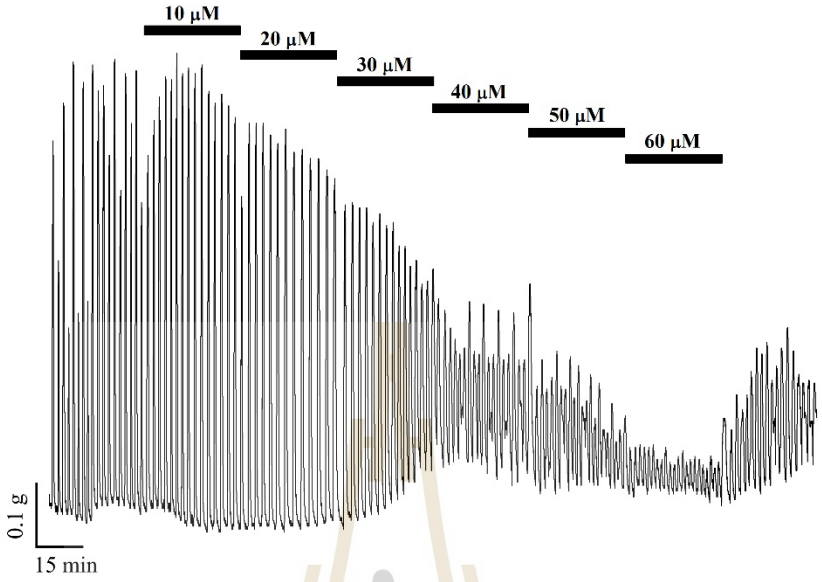
Data are expressed in mean ± S.E.M. and n represented uterine sample from a different animal. Data are not shown in nifedipine. The *P*-values for contractile response in AUC in both experimental groups were significantly different from the spontaneous control, 100% in the same uterine strips (paired student *t*-test, $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$) and a significantly different was found between non-GD and GD (unpaired student *t*-test, $*P < 0.05$ and $**P < 0.01$). The percentage change in GD was calculated using the value in non-GD assume as control; 100%.

4.4.4 Concentration dependency of GLB, MET, and insulin on myometrial contraction

Concentration dependency of GLB

After the equilibration period, the addition of increasing concentrations of GLB at 10, 20, 30, 40, 50, and 60 µM was decreased AUC, amplitude, and frequency at the initial concentration of 10 µM with a cumulative concentration in pregnant myometrium samples. At the highest concentration of 60 µM, all contractile parameters were significantly reduced from 99.10 ± 3.75% to 32.05 ± 8.60% AUC, 94.03 ± 6.06% to 8.64 ± 4.66% amplitude and 92.05 ± 3.59% to 26.83 ± 23.89% frequency, compared with each mean control, 100% ($P < 0.05$). Interestingly, the elevated baseline was presented at the higher concentration. The small phasic spontaneous contractions are resumed after returned to the physiologic Krebs' solution at the end of the experiment, as seen in Figure 4.10(A). This result was indicated that the possible mechanism of GLB in uterine contractility in pregnant rats displayed the relaxant effect on spontaneous contraction which marked depression of AUC, amplitude, and frequency with the cumulative concentration. The AUC was used to define the IC_{50} concentration of GLB, which was 27.19 µM in Figure 4.10(B). Hence, this concentration was used in the reminder throughout the experiments.

(A)



(B)

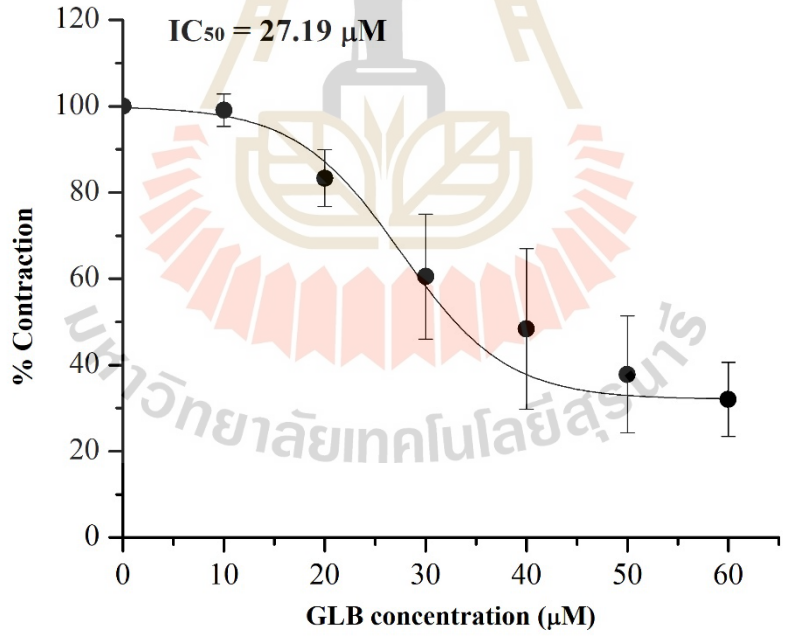
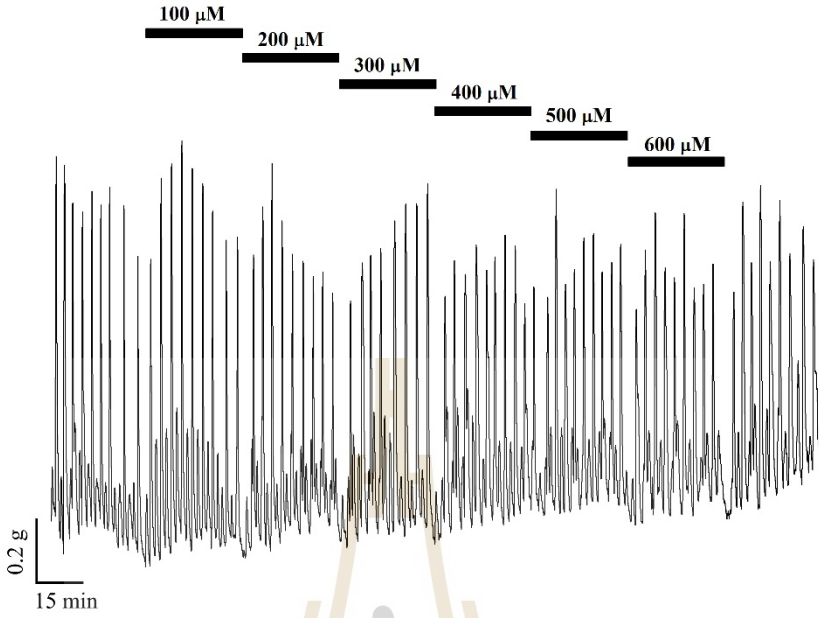


Figure 4.10 The concentration dependency of GLB (10 to 60 µM) on the contractile response in isolated pregnant rat myometrium; typical traces of force production (A) and the concentration-response curves for AUC (B). The symbol represents a mean. Vertical lines represent standard errors of the means (n = 5 for each concentration).

Concentration dependency of MET

The cumulative concentration of MET (100, 200, 300, 400, 500, and 600 μM) was applied to the pregnant myometrium strips as shown in Figure 4.11(A). As a result, the contractile response in AUC was varied after the exposure to MET. There was a slightly dropped at the initial concentration of 100 μM (97.09 ± 9.96%), then slightly evoked at the concentration of 200 μM (104.29 ± 16.81%) and continued decreasing with the cumulative concentration manner (88.93 ± 22.51% at the highest concentration). In contrast, amplitude and frequency were reduced at the starting concentration of 100 μM to the highest concentration of MET administration (reduced from 92.41 ± 14.51% to 54.80 ± 22.33% amplitude and reduced from 87.55 ± 3.16 to 56.63 ± 16.13% frequency). The responsibility in AUC, amplitude, and frequency were not significantly different from each mean control, 100% ($P > 0.05$). The reversible contractile response was returned to normal after wash off with Krebs' solution. The administration of metformin did not enhance the myometrial contractility in the pregnant rat model. An AUC at the maximum concentration-response (200 to 600 μM) was calculated for the IC_{50} concentration of MET and there was 417.51 μM since it was gently facilitated the myometrial contractility during the concentration cumulative manner in Figure 4.11(B). Thus, the concentration of 417.51 μM was used throughout further experiments.

(A)



(B)

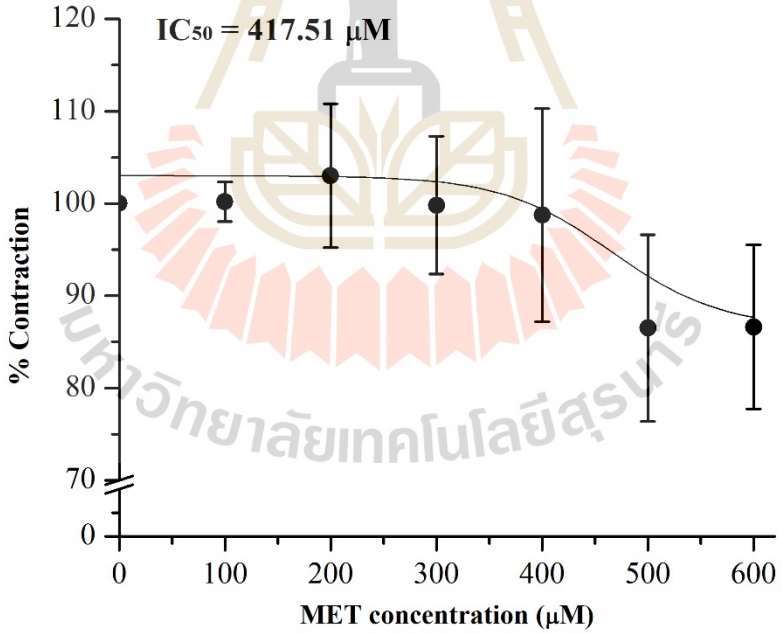
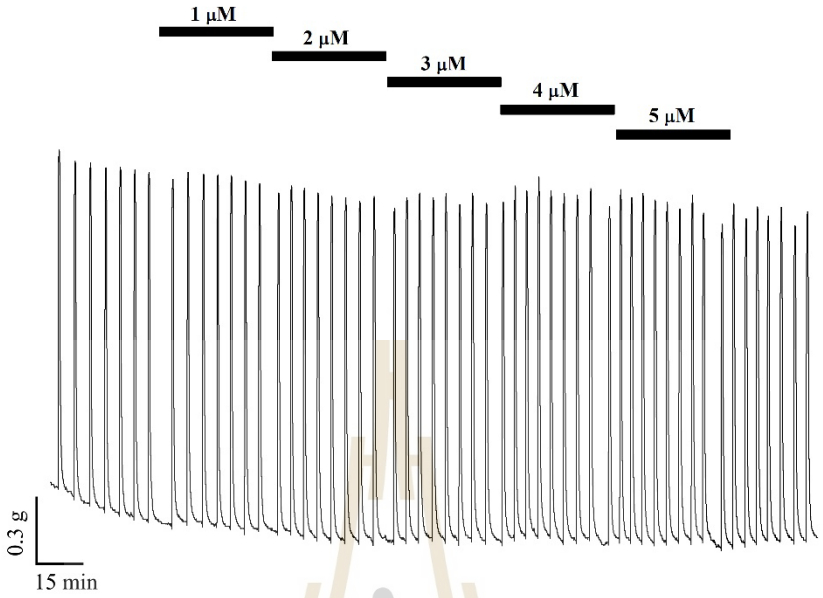


Figure 4.11 The concentration dependency of MET (100 to 600 µM) on the contractile response in isolated pregnant rat myometrium; typical traces of force production (A) and the concentration-response curves for AUC (B). The symbol represents a mean. Vertical lines represent standard errors of the means (n = 5 for each concentration).

Concentration dependency of insulin

The contractile response after the cumulative concentration of insulin addition (1, 2, 3, 4, and 5 μM) slightly oscillated in both AUC and amplitude of the uterine contractility. At the starting concentration of 1 μM, the AUC was slightly increased to 105.99 ± 2.65% followed by a small dropped to 104.52 ± 6.82% at the concentration of 2 μM and then continually increased after the concentration of 3 μM with a significant highest increase at the concentration of 5 μM until the wash off (increased from 108.61 ± 10.15% to 131.48 ± 11.12% AUC, $P < 0.05$). Frequency and amplitude had irregularly arisen with no significantly different from the mean control, 100% ($P > 0.05$). After the end of the experiments, the contractile response was still reversed, as shown in Figure 4.12(A). Noticeably, the highest concentration of 5 μM insulin had able to stimulated the uterine contractility in the pregnant rat. An AUC at the maximum concentration-response (2 to 5 μM) was calculated for the EC_{50} concentration of insulin and there was 4.12 μM in Figure 4.12(B). Therefore, this concentration of 4.12 μM was used throughout further experiments.

(A)



(B)

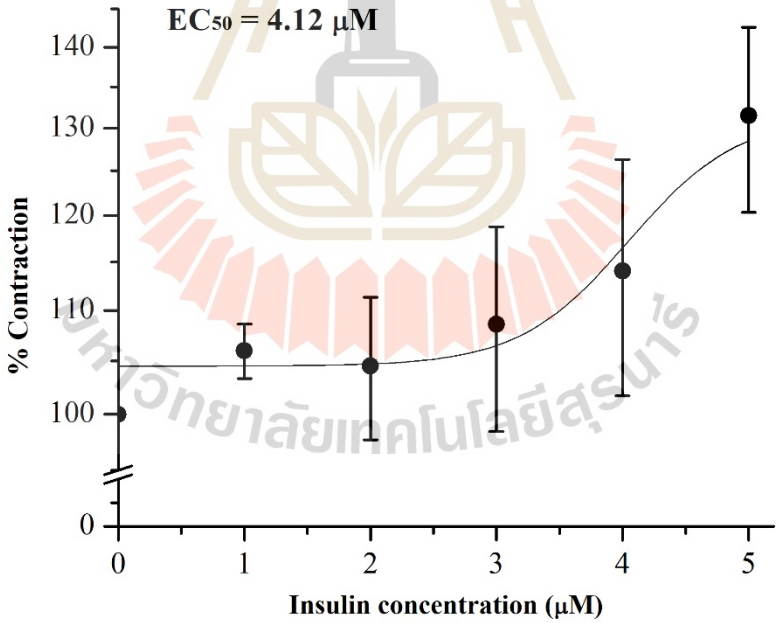


Figure 4.12 The concentration dependency of insulin (1 to 5 µM) on the contractile response in isolated pregnant rat myometrium; typical traces of force production (A) and the concentration-response curves for AUC (B). The symbol represents a mean. Vertical lines represent standard errors of the means (n = 5 for each concentration).

4.4.5 Effect of GLB, MET, and insulin on spontaneous contraction in non-GD and GD

GLB administration on spontaneous contraction

The single concentration of GLB (27.19 μM) had administrated to the spontaneous contraction in non-GD and GD during 30 min period as seen in Figure 4.13. The contractile activity in AUC and frequency in non-GD were slightly increased with no significantly different from control (100%, $P > 0.05$). The baseline force was shifted to higher, whereas the amplitude was significant reduced to 82.19 ± 1.06%, compared with control (100%, $P < 0.01$). On the other hand, the reduction of AUC, amplitude, and frequency was found in GD and no baseline shifted had been observed. For a 30 min period, the decreased amplitude in GD (95.13 ± 3.14%) was significantly different from the decreased amplitude in non-GD ($P < 0.05$). In addition, the reduction of amplitude with the evoked baseline force in non-GD was similar to the concentration dependent study of GLB and it can imply that GLB had a greater effect in non-GD more than GD, as well as the contractile sensitivity to GLB, may reduce in GD.

MET administration on spontaneous contraction

The spontaneously contractile response in non-GD and GD during 30 min under the exposure of the single concentration of MET (417.51 μM) was shown in Figure 4.14. The AUC and frequency in non-GD were slightly decreased with a small increased amplitude which no significantly different from the control (100%, $P > 0.05$). Conversely, the contractile response in GD during the exposure to MET had reversed these contractile parameters by increasing AUC and frequency while decreasing amplitude, but there was no significantly different from control (100%, $P > 0.05$), as well as there was no significant difference between group ($P > 0.05$). Additionally, this

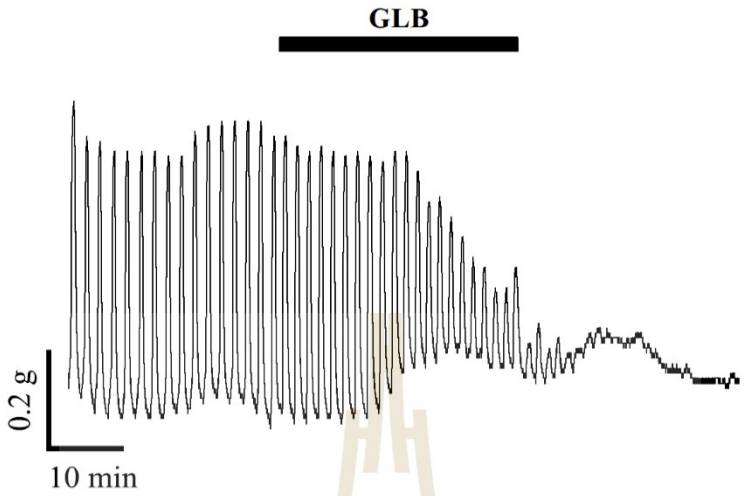
result had supported the potential used of MET with no interfered on the myometrial contractility in pregnant rats, but the stimulant activity in the integral force along with the increased frequency of myometrial in GD may further mention.

Insulin administration on spontaneous contraction

The contractile response in a 30 min period under the exposure to the single concentration of insulin (4.12 μM) in both non-GD and GD were presented in Figure 4.15. There was obviously increased contractile response in both non-GD and GD. The AUC, amplitude and frequency in non-GD were rose, but no significant different from control (100%, $P > 0.05$), in contrast, the AUC (131.49 ± 2.63%) and amplitude (117.30 ± 3.56%) in GD were significant increased from control (100%, $P < 0.01$). This result was demonstrated that GD shows a stronger contractile response during the exposure to insulin.

The effects of GLB, MET and insulin administration on spontaneous contraction in non-GD and GD are summarized in Table 4.3.

(A) Non-GD



(B) GD

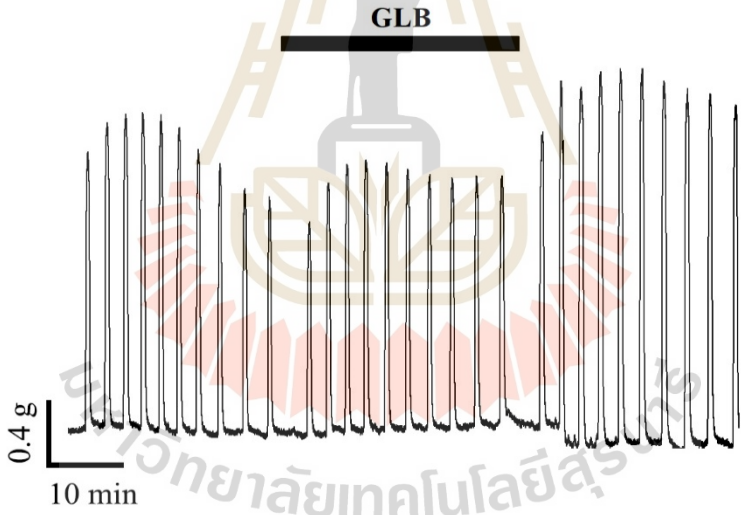
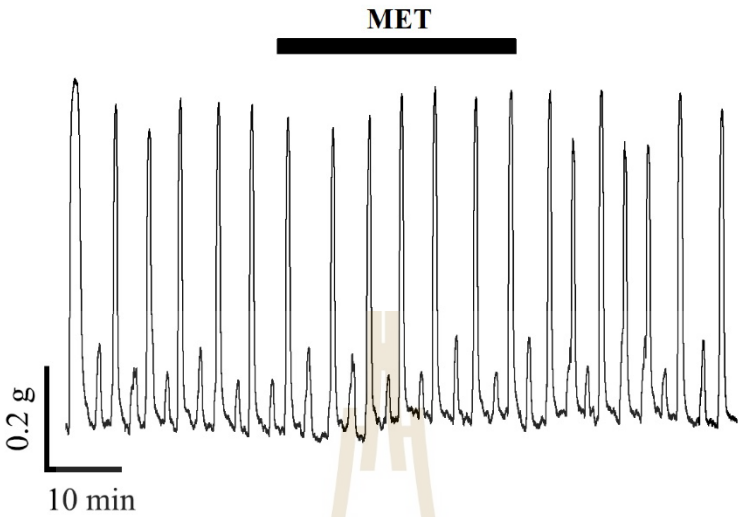


Figure 4.13 Typical traces showing the uterine spontaneous response in the presence of GLB in non-GD (A) and GD (B). A significant difference in the contractile response was compared during spontaneous control (100%) and tested period (GLB) within the same sample and compared between groups (n = 3).

(A) Non-GD



(B) GD

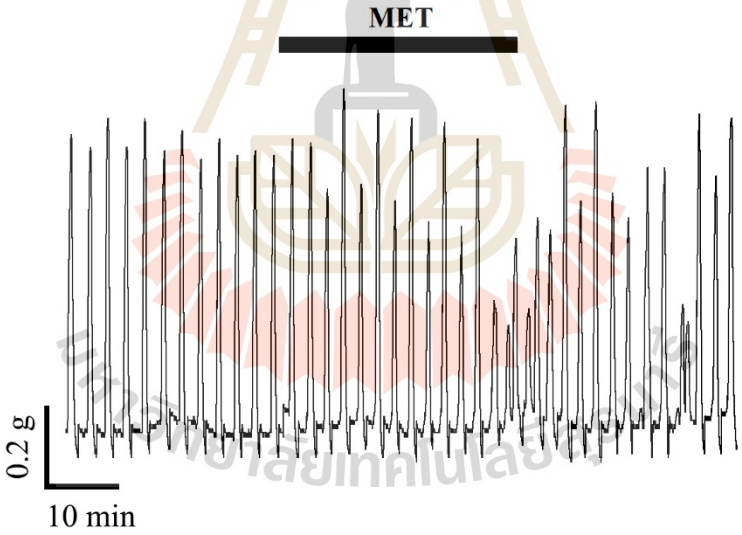
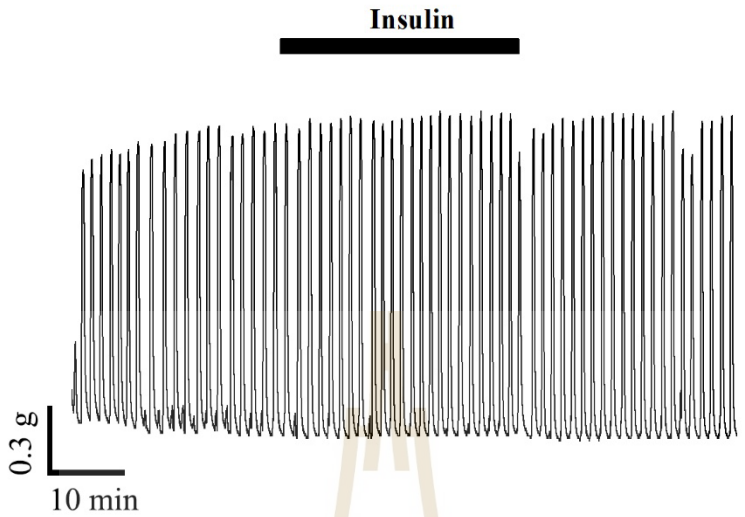


Figure 4.14 Typical traces showing the uterine spontaneous response in the presence of MET in non-GD (A) and GD (B). A significant difference in the contractile response was compared during spontaneous control (100%) and tested period (MET) within the same sample and compared between groups (n = 3).

(A) Non-GD



(B) GD

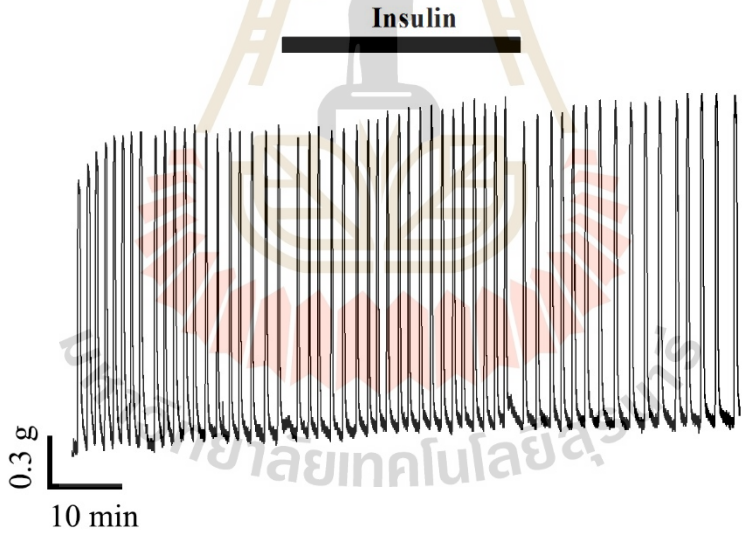


Figure 4.15 Typical traces showing the uterine spontaneous response in the presence of insulin in non-GD (A) and GD (B). A significant difference in the contractile response was compared during spontaneous control (100%) and tested period (insulin) within the same sample and compared between groups (n = 5).

Table 4.3 The effect of anti-diabetic drugs (GLB, MET, and insulin) on spontaneous contraction in non-GD and GD.

Tested substances	Non-GD			GD			
	AUC (%)	Amplitude (%)	Frequency (%)	AUC (%)	Amplitude (%)	Frequency (%)	n
Control	100	100	100	100	100	100	
GLB	109.08 ± 11.29	82.19 ± 1.06$^{\#\#}$	104.27 ± 14.88	94.01 ± 4.61	95.13 ± 3.14*	90.09 ± 6.66	3
MET	98.67 ± 8.72	103.36 ± 5.27	89.53 ± 2.46	104.20 ± 2.48	92.93 ± 7.38	107.75 ± 7.75	3
Insulin	113.70 ± 7.19	109.73 ± 4.56	100.92 ± 5.01	131.49 ± 2.63$^{\#\#}$	117.30 ± 3.56$^{\#\#}$	106.51 ± 7.06	5

Data are expressed in mean ± S.E.M. (n = 5 for each concentration). The *P* values for AUC, amplitude, and frequency of contractile response during the exposure to anti-diabetic drugs (GLB, MET, and insulin) in both experimental groups were significantly different from the spontaneous control (100%) in the same uterine strips (pair student *t*-test; $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$) and significantly different in each AUC, amplitude, and frequency between non-GD and GD (unpair student *t*-test; $*P < 0.05$ and $**P < 0.01$).

4.5 Discussion

Uterine regulation remains relatively in a state of quiescent throughout the gestation period. The powerful rhythmic contractions are mediate at the end of pregnancy to initiating parturition with quite diverse mechanisms. The primary cascades are involved in the signaling in the myometrium caused by various hormones action related to intracellular Ca^{2+} concentration. The myometrial dysfunction causes several important clinical disorders which are complicated by diabetes. This studied attempted to demonstrate the myometrial function responsiveness in diabetic pregnancy and in the treatment of common hypoglycemic agents in pregnant rat uteri during late gestation.

In this study, the GD had been manifested the contractile reduction in spontaneous activity in the main Ca^{2+}-Calmodulin-MLCK pathway including frequency, amplitude, and AUC with the prolonged duration through the L-type Ca^{2+} channels activation. Additionally, the maximal force production was also decreased regardless of whether the membrane depolarization activating Ca^{2+} channels by KCl or GPCR activating IP_3-induced Ca^{2+} release by OT hormones when compared to the contractile response in non-GD. These results suggested that the impaired uterine contractility based on the main Ca^{2+}-Calmodulin-MLCK pathway have been found in diabetic pregnancy and are in agreement with Al-Qahtani et al. (2012) who suggested the uterine contractility may be impaired in diabetic pregnant patients considering to the reduction of Ca^{2+} channel expression, intracellular Ca^{2+} signals, and muscle mass, as well as McMurtrie et al. (1985) who reported the maximal contractile responses induced by OT, was significantly smaller in diabetic uteri which correlated to the differences in the uterine myometrial structure. However, Spiegl et al. (2009) found the

higher expression of OT receptors on late gestation in the diabetic myometrium of rats which contributed to the increased risks of premature uterine contractions. Thus, no clinical data to elucidate the mechanism of uterine respond to OT whether downregulated oxytocin receptors and it signaling or not.

Additionally, the modulation in the non-Ca^{2+}-Calmodulin-MLCK pathway had been shown that force production was completely inhibited in both groups caused by nifedipine, a L-type Ca^{2+} channels blocker. Together with Y27632 (Rho-associated kinase pathway) and WT (MLCK inhibitor), the contractile response was diminished in both groups with no significant difference between groups, but the reduction was a trend to higher in GD. On another way, the ability of WT modulated during membrane depolarization by KCl and agonist-induced by OT had been shown that the contractile response was gradually reduced and no significance was observed between groups. This finding was determined that diabetic pregnancy interrupted the contractile response in the main Ca^{2+}-Calmodulin-MLCK pathway, as well as Ca^{2+} sensitization interfering with MLCK activity in non-Ca^{2+}-Calmodulin-MLCK pathway. Thus, these were less investigation on the non-Ca^{2+}-Calmodulin-MLCK pathway modulation in diabetic pregnant myometrial function, hence some finding on Ca^{2+} sensitization has been reported in other diabetic muscles. For example, the protein contents of MLC and MLCK and phosphorylation of MLC were decreased and myofibrillar activity was depressed in diabetic cardiomyopathy rats (Liu et al., 1997). The MLCK expression in gastrointestinal smooth muscles was decreased in diabetic rats (Hu and Feng, 2012) and detrimental changes of Ca^{2+} sensitization were developed in diabetic gastroparesis patients (Li et al., 2018).

Numerous studies have been revealed the various effect of oral anti-diabetic agents (GLB and MET) on uterine contractility. The major effect of GLB is a well-known directly closure of ATP-sensitive K^+ channels (K_{ATP}) cause depolarization on the pancreatic β-cells membrane, which in turn stimulates Ca^{2+} influx and subsequently improve metabolic control by an increase insulin secretion in human (Ligtenberg et al., 1997) and animals (Sokolovska et al., 2012). Previous studies demonstrated that K_{ATP} channels are expressed in various tissues and mediated its effect in aortic (Yoshitake et al., 1991), vascular (Zhang et al., 1991) and uterine smooth muscle cells (Piper et al., 1990). Concentration dependency of GLB also exhibited a gradually decreased amplitude continuing with some baseline elevation in the increased cumulative concentration manner on the uterine contractile response in the non-diabetic pregnant uterus. GLB administration at IC_{50} concentration of 27.19 μM also exhibited a half maximal inhibition. The slightly increased AUC and frequency coupled with the significantly decreased amplitude caused by GLB had higher potential in non-GD, in contrast with the slight reduction of AUC, amplitude, and frequency was found in GD. Thus, the contractile facilitation by GLB was permanent in non-diabetic pregnancy on late gestation and may potent more incomplete closing K_{ATP} channel due to the presence of total relaxation with decreased amplitude. The efficacy of GLB was attenuated in diabetic pregnancy during the active labor initiation. As mentioned above, Lovasz et al. (2011) have been explained that GLB facilitated the uterine contractility during normal laboring time by non-selectively suppressed the expression of sulfonylurea receptor (SUR) subunits which are related to K_{ATP} channels. SURs are sensitive to the binding of utero-relaxant compounds and SUR expression is downregulated in late pregnancy to enhanced contractility. Moreover, Stephan et al. (2006) indicated the inhibitor

binding transduction into channel closure by GLB has different in the affinity of the K_{ATP} channel on various smooth muscle.

For anti-diabetic drug; MET, its intercellular process has been involved 5'-AMP-activated protein kinase (AMPK) signaling pathway which could inhibit MLCK activity and suppressed MLCP phosphorylation in smooth muscle cells, leading to the relaxation effect as reported in aortic smooth muscle (Sung and Choi, 2012) and membrane repolarization with Ca^{2+} influx reduction in arterial smooth muscle (Chen et al., 1997). Thus, the intercellular Ca^{2+} sensitivity of force in those studies has not been mentioned. In uterine smooth muscle cells, the spontaneous uterine contractility has not interfered or not inhibited by MET in non-diabetes non-pregnant rats (Kelany et al., 2016), and no significant alteration whether on spontaneous activity or during the oxytocin-induced contractility in non-diabetic pregnant human myometrium biopsies at term (Hehir and Morrison, 2012). However, no evidence evaluated any significant effect on the contractile response in diabetic pregnancy's sample. The cumulative additions concentration of MET in non-diabetic pregnant contractile response in this study has been shown a small reduction in all parameters (AUC, amplitude, and frequency) with non-statistically different from spontaneous control, which suggested the effect of MET is not affected to the uterine contraction even in the highest concentration in non-diabetic pregnant myometrium. The uterine contractile response in diabetic pregnancy during the late gestation after the single IC_{50} concentration of MET exposure (417.51 μM) has been exhibited a rose in the mean integral force together with the slightly increased frequency in the spontaneous contraction, but no significantly different from spontaneous control and between groups, which implied that the variation in the response to MET is found in the diabetic pregnant uterus.

Rowan et al. (2008) showed the higher rate of spontaneous preterm labor in GDM achieved metformin treatment whilst many researchers found no significant difference in a literature review by Singh et al. (2015). This study was revealed that MET might be exerted stimulant activity in diabetic pregnancy during late gestation and its mechanism requires further investigation.

During the quiescence period, insulin plays a role in the proliferation, metabolism, and fetal growth in pregnancy, wherewith bind to its receptors (IRs) and phosphorylates IR substrates (IRSs) on tyrosine kinase receptor, that induced the upregulation of phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K)/AKT signaling pathway, its second messenger, PI triphosphate (PIP_3) is generated which mediated the glucose uptake by GLUT4 translocation (Cartee and Wojtaszewski, 2007; Neirijnck et al., 2019). The higher expression of PI3K, increased AKT activation and IRSs gene polymorphism are influenced in STZ-induced diabetic mice (Hami et al., 2016) and women with GDM (Fallucca et al., 2006). Leturque et al. (1987) have been reported that there was an increased 30-50% of maternal glucose utilization in conceptus tissues in late gestation, responsibility for the development of plasma insulin levels, Korgun et al. (2003) found the expression of IRs in myometrial smooth muscle is regulated the utero-placenta environment for fetal and placental growth. For uterine smooth muscle, the phosphatidylinositol signaling pathway is important for agonist-stimulated phasic myometrial contractions, caused by the cytosolic Ca^{2+} concentration transients (Ruzycky and Crankshaw, 1988). PI3Ks have been reported in various tissues whereas PI3Ks class II (C2α and C2β) are highly expressed in myometrial cells during the normal parturition which regulated Rho activation in RhoA-Rho kinase-MLCP pathway, in response to augmented uterine force (Sarker et al., 2019). The higher

glucose uptake found in the period of myometrial relaxation after a sustained incomplete tetanic contraction in the non-pregnant uterus was related to sensitive insulin response whereas absolute tetanic contraction was found in a late pregnant uterus with no longer insulin sensitivity (Rodriguez-Candela et al., 1962). Moreover, insulin also induced hypercontractile activity in bovine airway smooth muscle (Schaafsma et al., 2007) and affects the sensitivity of the relaxin signaling pathway in T1DM pregnant myometrium (Kuznetsova et al., 2005). Corresponding to the results in the current study, insulin was slightly induced the contractile response during the cumulative concentration dependency, starting at the concentration of 3 μM to the highest concentration of 5 μM. The EC_{50} of insulin (4.12 μM) was induced the contractile response in both non-GD and GD, incidentally, there was no significant difference between non-GD and control (100%), but the AUC and amplitude were significantly increased in GD. This result was demonstrated that insulin was triggered the little uterine force production in non-diabetic pregnancy, but the higher force production was found in diabetic pregnancy which may relate to the level of insulin sensitivity during the end of pregnancy. This condition might lead to a higher prevalence of preterm birth with insulin treatment in diabetic pregnancy, as previously reported by Balani et al. (2009) and Mesdaghinia et al. (2013).

The myometrial function was altered in diabetic pregnancy on late gestation. The hypoglycemic agents (GLB, MET, and insulin) exerted its effect on uterine smooth muscle cells, but no interfered in MET treatment and the different myometrial responsibility to anti-diabetic agents were mentioned in diabetic pregnancy.

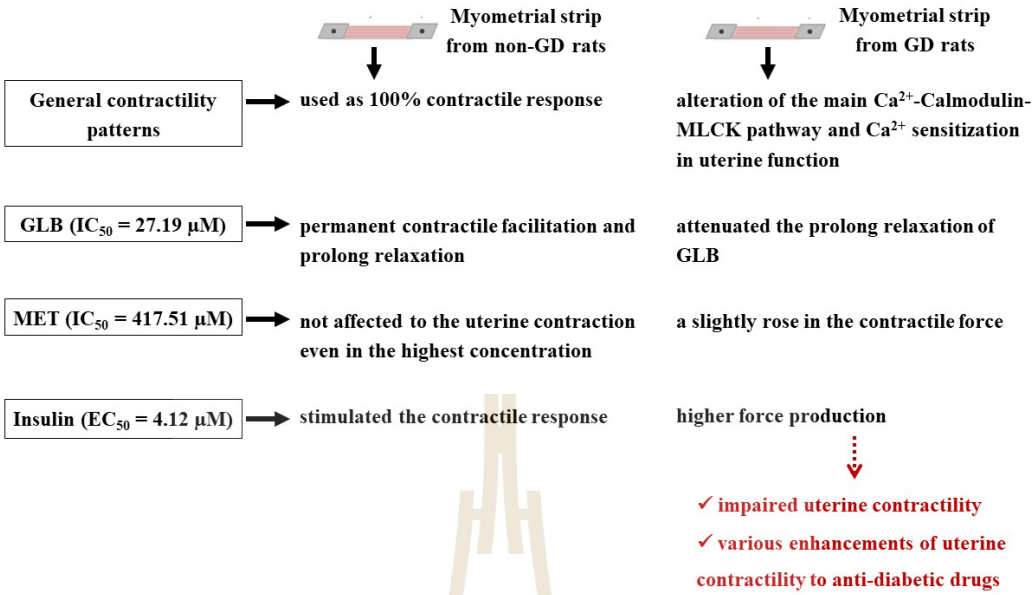


Figure 4.16 The diagram shows the uterine contractile responses in gestational diabetes mellitus and the effects of anti-diabetic drugs (GLB, MET, and insulin) on uterine contractility in non-gestational diabetic and gestational diabetic rat myometrium.

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CHAPTER V

THE EFFICACY OF *THUNBERGIA LAURIFOLIA* (L.) AND *CYANTHILLIUM CINEREUM* (L.) H. ROB. EXTRACTS ON UTERINE CONTRACTIONS

5.1 Abstract

The duration and severity of the chronic hyperglycemia condition are the major sources of morbidity and mortality. The most common malformations are higher rates in maternal diabetes. Severe hypoglycemia, gastrointestinal symptoms, and abnormal fetal development which increased incidence of the secondary complications are profound as adverse events in the use of modern anti-diabetic drugs. Anti-diabetic Thai medicinal plants, *Thunbergia laurifolia* (L.) and *Cyanthillium cinereum* (L.) H. Rob. are commonly used as an alternative therapy for diabetes treatment in many previous reported. Hence, their efficacy and safety used of these plants along with their hypoglycemic properties on uterine function in GDM women are still lacking. The aims of this experiment were to evaluate the ethanolic extract of *T. laurifolia* leaves (TLE) and *C. cinereum* whole plant (CCE) on uterine spontaneous contraction (I) and in combination with agonist-induced contractions; KCl (II) and OT (III), compared between isolated myometrium on late gestation of non-gestational diabetic (Non-GD) and gestational diabetic (GD) rat. As a result, the concentration dependency (0.5-2 mg/ml) of both plant extracts have exhibited a trend to having muscle relaxant activity

with the cumulative concentration manner. The spontaneous uterine contractility was significant inhibited by TLE (IC_{50}: 1.19 mg/mL), the significant reduction was still active continuing in the combination with KCl and OT-mediated contractility in both groups ($P < 0.05$). In contrast with the CCE application, a biphasic effect with the modified uterine contractility was found in the concentration dependent experiment, clarify stimulation at the low concentration, and turned to incomplete inhibition at the high concentration. The CCE (IC_{50}: 1.50 mg/mL) promoted transient contraction following by prolonging relaxation and significantly found in both groups ($P < 0.05$). The slight relaxation was potent in the combination with KCl ($P > 0.05$), but significant acted on OT-mediated contractility in both groups ($P < 0.01$). This finding was indicated that TLE and CCE have been shown a tocolytic activity on non-gestational diabetic myometrium during spontaneously contraction, KCl, and OT-induced contractions, the related mechanisms have been involved in the inhibition of Ca^{2+} influx and Ca^{2+} release from internal storage, subsequently decreased intercellular Ca^{2+} concentration. The tocolytic effect of these anti-diabetic plants was diminished in gestational diabetic myometrium as compared to non-diabetic conditions. *T. laurifolia* leave and *C. cinereum* whole plant ethanolic extract extracts may be useful in traditional prevention of premature labor during late gestation in both non-diabetic and diabetic pregnancy.

5.2 Introduction

Uterotonics and tocolytic drugs available to control the pregnant state as recommended by WHO (2015, 2018). The mechanisms of uterotonics were to initiate delivery, slowly accumulate progression of labor and stimulate placental delivery in

postpartum, whereas tocolytic drugs are warranted to stopping preterm labor as summarized in Arrowsmith et al. (2010). Natural therapies are attempted in alternative medicine for beneficial effects on pregnancy which aims to reduce the harmful side effects from conventional medicines and verify their efficacy and safety for pregnant women (Tiran, 2003). Among medicinal herbs in Thailand, *Thunbergia laurifolia* (L.) and *Cyanthillium cinereum* (L.) H. Rob. (Synonym: *Vernonia cinerea* L.) have pharmacological effects from their active constituents due to anti-diabetic activity (Aritajat et al., 2004; Choudhary et al., 2013).

Previous experimental evidence of both plants phytoconstituents suggested that flavonoids (apigenin, delphinidin, quercetin, chlorogenic acid, caffeic acid, gallic acid, and protocatechuic acid) have potent relaxing effects on the contractility of various smooth muscles such as vascular smooth muscle cells by increasing the NO production via protein kinase G signaling cascade (Jin et al., 2009; Martin et al., 2003; Suzuki et al., 2006; Suzuki et al., 2002), suppression of bladder's movement by MLCK signaling pathway regulation (Liu et al., 2011), reduction of gastric tone in the stomach (Amira et al., 2008) and enhanced estrogenic activity or anti-estrogenic activity by the distribution of estrogen receptors in the uterus (Breinholt et al., 2000; Chalopin et al., 2010) with no interfered on cytosolic-free Ca^{2+} concentration (Martin et al., 2003), depending on the differenced flavonoids potency. Moreover, coumaric acids, ferulic acid, and caffeic acid are observed to increased serum estradiol levels (Zych et al., 2009). Luteolin may contribute to vasodilatory effects by protein kinase C (PKC) inhibition or decreased Ca^{2+} uptake (Duarte et al., 1993) and possess the inhibitory effect of vascular endothelial growth factor-induced angiogenesis by blockade of PI3K activity (Bagli et al., 2004), as well as exhibited estrogenic properties in the uterus by

increasing uterine weight, uterine diameter and thickness of the endometrium along with slightly anti-estrogenic activity as the same effect of apigenin (Hiremath et al., 2000). Plant sterols (β-sitosterol and stigmasterol) might be stimulated the synthesis of endogenous estradiol, up-regulated estrogen as well as progesterone receptors which promoted the growth of uterine endometrium, enhanced implantation process, and maintained gestation (Salah et al., 2002). The uterotonic agents from plant-isolated for modulation of uterine contraction with estrogenic activity have previously been reported the presence of β-sitosterol (Promprom et al., 2010) and glycoside (grandifloric acid) (Gruber and O'Brien, 2011). The rich sources of triterpenoid saponins (lupeol) from plants with its principal active constituents are employed to heal reproductive system disorders such as the uterus and ovary inflammation (Gallo and Sarachine, 2009). Rutin is a well-known antagonize prostaglandin E_2 (PGE_2) in a non-competitive manner that exerts the relaxant action in the duodenum (Altinkurt and Öztürk, 1987).

Nevertheless, the effects of *T. laurifolia* ethanolic leave extract (TLE) and *C. cinereum* ethanolic whole plant extract (CCE) have not been reported as their mechanism of action on the uterus. Thus, the uterine smooth muscle activities related to the effect of TLE and CCE were evaluated on isolated pregnant rat uterus in normal and diabetic conditions.

5.3 Materials and methods

5.3.1 Measurement of isometric contraction

Uterine strips were collected and prepared the same as described in 4.3.1 from non-gestational diabetic rats (Non-GD) and gestational diabetic rats (GD).

Myometrial strips were perfused with Krebs' solution (37ºC, pH 7.4) in organ bath experiments for tension measurement.

5.3.2 Experimental Protocols

The concentration dependency of TLE and CCE on the isolated pregnant myometrium

The concentration range of 0.5, 1, 1.5, and 2 mg/mL of TLE and CCE were completely dissolved in Krebs' solution and then were added to the strips after 30 min equilibration time in a cumulative increased concentration manner. The rhythmic contraction was observed and used as the standard 100% spontaneous contraction. The pattern changed in uterine activity during the exposure to each plant extracts were recorded. The EC_{50} or IC_{50} concentration of each plant extracts were calculated from AUC in Microcal Origin Software (Sebaugh, 2011).

Effect of TLE and CCE on spontaneous contraction and during the exposure to KCl and OT

The EC_{50} or IC_{50} concentration of both plant extracts were used in 30 min period of spontaneous contraction and during the presence of agonists-induced contraction (KCl and OT). The tension was recorded in both non-GD and GD during plant extracts alone and in the combination with KCl and OT.

5.3.3 Chemicals and physiological solutions

The analytical chemicals grade was purchased from Sigma® and Merck, Singapore. KCl and OT were performed as describes in 2.6. The EC_{50} or IC_{50} concentration of TLE and CCE were dissolved in physiologic Krebs' solution and stored by the guideline of the producer.

5.3.4 Statistical analysis

Spontaneously uterine contractility was used as 100% of contractions (AUC, amplitude, and frequency) and compared to the contractility responses from TLE and CCE application whether added alone or in the combination of KCl and OT. The contractility responses changed as response change (%) between non-GD and GD. Data presented in means ± S.E.M with the number of uterine samples (n) from a different animal. Paired and unpaired student *t*-test was tested the significant difference in the same strip and between groups, respectively with a P value < 0.05.

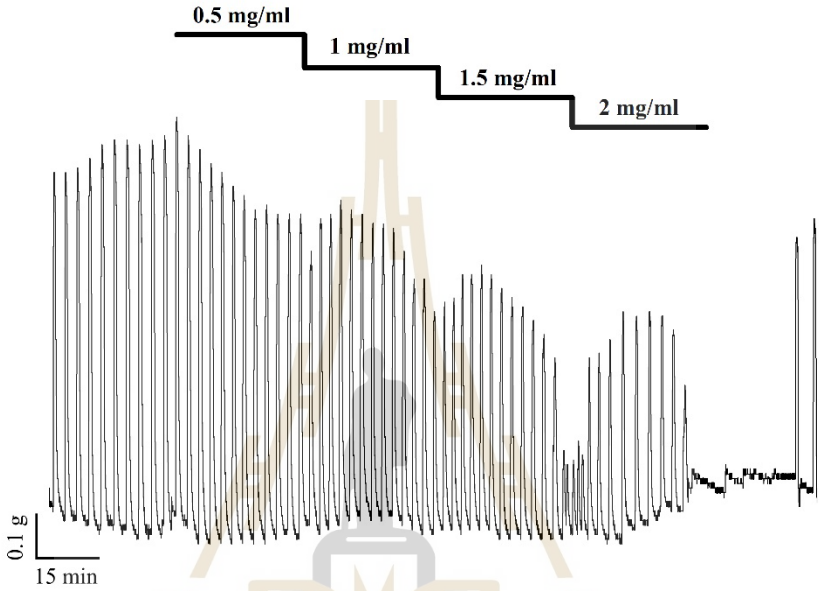
5.4 Results

5.4.1 Concentration dependency of TLE on myometrial contraction

Concentration-dependent (0.5, 1, 1.5, 2 mg/mL) of TLE with an increasing cumulative concentration manner was applied to the myometrial strips as seen in Figure 5.1(A). The AUC after the application of 0.5 mg/mL TLE were increased to 120.95 ± 17.11%, and then dramatically declined to 95.56 ± 5.41% at the concentration of 1 mg/mL, 68.06 ± 13.13% at the concentration of 1.5 mg/mL and 33.80 ± 5.69% at the concentration of 2 mg/mL, respectively. Similarly, a fluctuation pattern has been found in frequency after TLE was applied, frequency was highest increased to 119.75 ± 8.00% at the concentration of 1 mg/mL and then continuedly declined to 93.46 ± 23.96% at the concentration of 1.5 mg/mL and 36.85 ± 21.09% at the concentration of 2 mg/mL, respectively. Conversely, the amplitude was starting to decrease as the increasing cumulative concentration (decreased from 98.66 ± 3.17% to 40.98 ± 12.79%). A significant inhibitory effect in all AUC, amplitude, and frequency were detected at the highest concentration of 2 mg/mL (P < 0.05). Moreover, the spontaneous contraction

can initiate after washed out with Kreb solution. The IC_{50} concentration of TLE at the maximum concentration-response (0.5 to 2 μM) was calculated from AUC and 1.19 mg/mL was taken as seen in Figure 5.1(B). The concentration of 1.19 mg/mL was used throughout further experiments.

(A)



(B)

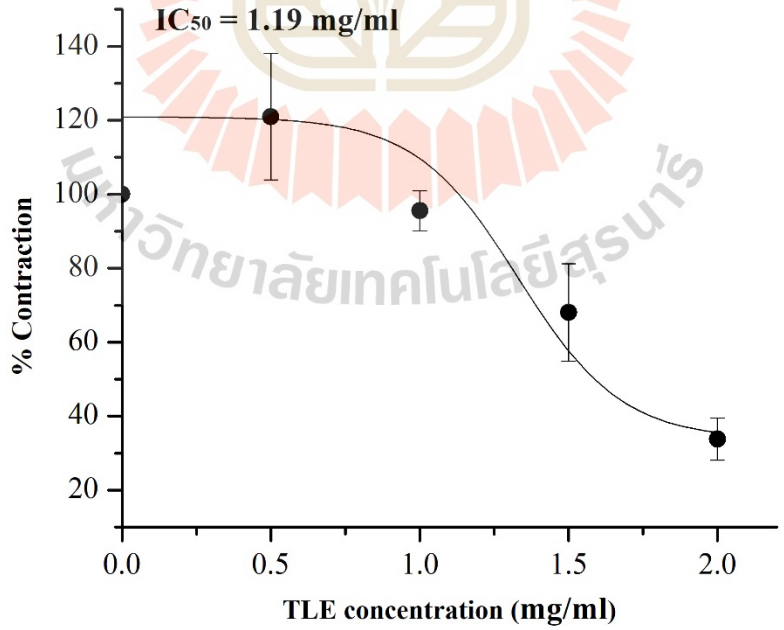


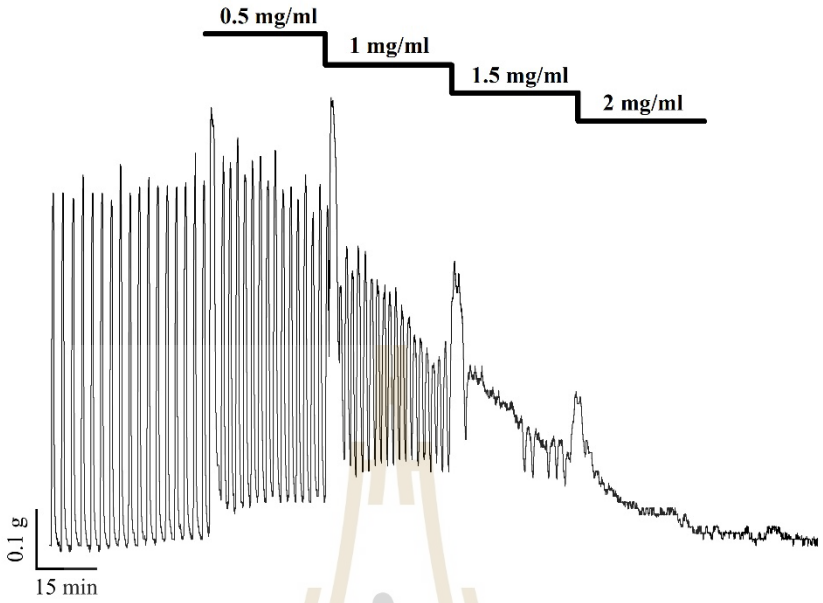
Figure 5.1 The concentration dependency of TLE (0.5 to 2 mg/mL) on the contractile response in isolated pregnant rat myometrium; typical traces of force production (A)

and the concentration-response curves for AUC (B). The symbol represents the mean. Vertical lines represent standard errors of the means (n = 5 for each concentration).

5.4.2 Concentration dependency of CCE on myometrial contraction

The cumulative concentration-dependent of CCE at the concentration of 0.5, 1, 1.5, 2 mg/mL was added to the myometrial strips as seen in Figure 5.2(A). As a resulted, the AUC and frequency during the exposure to CCE at the starting concentration of 0.5 and 1 mg/ml were significantly highest increased to 190.43 ± 14.58% and 206.68 ± 24.30% AUC, 141.01 ± 14.40% and 164.10 ± 29.81% frequency, respectively ($P < 0.05$) with elevating baseline was observed, and then significant gradually reduced with a cumulative concentration manner as reduced to 62.74 ± 11.11% AUC and 40.85 ± 31.56% frequency at the highest concentration of 2 mg/mL CCE. Amplitude was already significantly decreased as the increasing cumulative concentration (decreased from 108.73 ± 4.46% at the concentration of 0.5 mg/mL to 21.92 ± 6.31% at the concentration of 2 mg/mL, $P < 0.01$). This result was revealed the biphasic property of CCE on the spontaneous contraction of isolated pregnant rats' myometrium. The IC_{50} concentration of CCE was calculated from AUC at the maximum concentration-response (1 to 2 μM) and 1.50 mg/mL was taken as seen in Figure 5.2(B). The concentration of 1.50 mg/mL was used throughout further experiments.

(A)



(B)

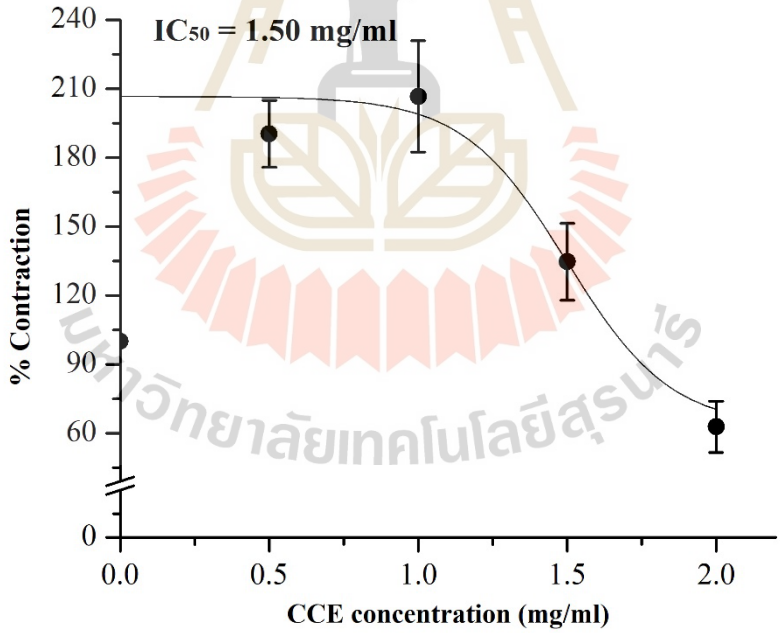


Figure 5.2 The concentration dependency of CCE (0.5 to 2 mg/mL) on the contractile response in isolated pregnant rat myometrium; typical traces of force production (A) and the concentration-response curves for AUC (B). The symbol represents the mean. Vertical lines represent standard errors of the means (n = 5 for each concentration).

5.4.3 Effect of TLE and CCE on spontaneous contraction in non-GD and GD

TLE administration on spontaneous contraction

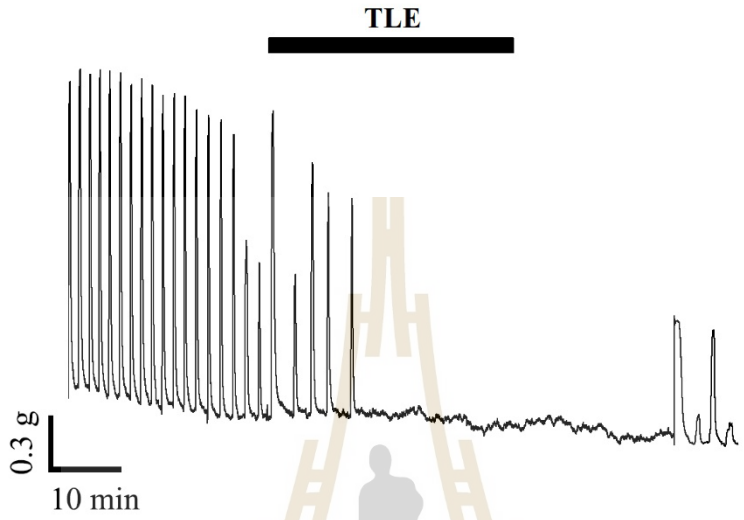
The single concentration of TLE (1.19 mg/ml) was administrated to the spontaneous contraction in 30 min period in non-GD and GD as seen in Figure 5.3. The reduction of contractile response was manifestly in both non-GD and GD. A significantly decreased in non-GD was 37.61 ± 4.07% for AUC, 61.81 ± 10.31% for amplitude and 51.03 ± 14.39% for frequency, compared from control (100%, $P < 0.01$). Similarly, a reduction in GD was 76.01 ± 7.79% for AUC, 73.92 ± 4.18% for amplitude, and 97.33 ± 10.19% for frequency, compared to control (100%). As the result, the decreased AUC and amplitude in GD have been shown significantly different from the non-GD ($P < 0.05$). To conclude, the tocolytic effect of TLE on contractile response was powerful in non-GD and diminished effect in GD.

CCE administration on spontaneous contraction

Furthermore, the spontaneous contraction over 30 min period during the exposure to the single concentration of CCE (1.50 mg/ml) in non-GD and GD has been shown in Figure 5.4. Noticeably, the contractile response was potently increased in both non-GD and GD. There was a significantly increased in AUC (128.54 ± 7.90%) and frequency (133.68 ± 11.06%), however, the amplitude was significantly reduced to 81.86 ± 5.99% in non-GD, compared to control (100%, $P < 0.05$). Similarly, there was significantly increased in AUC (152.84 ± 14.90%, $P < 0.05$) and frequency (131.66 ± 16.65%) with decreased amplitude (91.39 ± 8.88%) in GD, compared from control (100%). The contractile response to the biphasic activity of CCE was effective in both groups, the AUC and amplitude have remained higher in GD, but no significantly different from non-GD which seemed as more considerable to stimulant activity in GD.

The effects of TLE and CCE on spontaneous contraction in non-GD and GD are summarized in Table 5.1.

(A) Non-GD



(B) GD

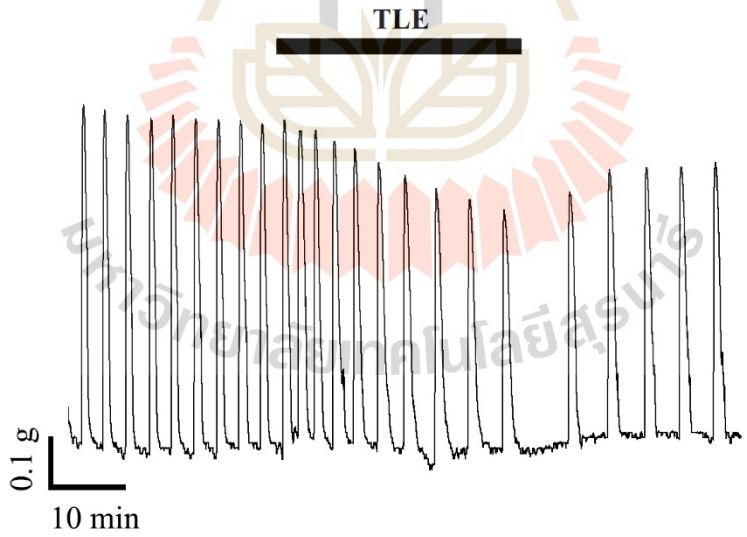
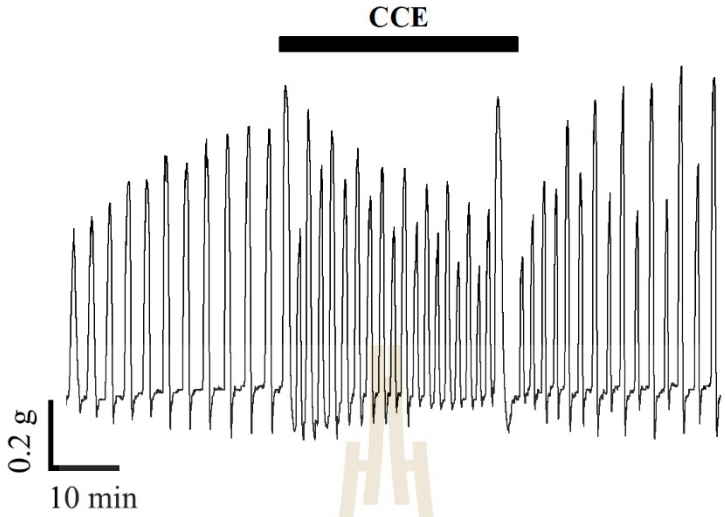


Figure 5.3 Typical traces showing the uterine spontaneous response in the presence of TLE in non-GD (A) and GD (B). A significantly different of the contractile response was compared during spontaneous control (100%) and tested period (TLE) within the same sample and compared between groups (n = 5).

(A) Non-GD



(B) GD

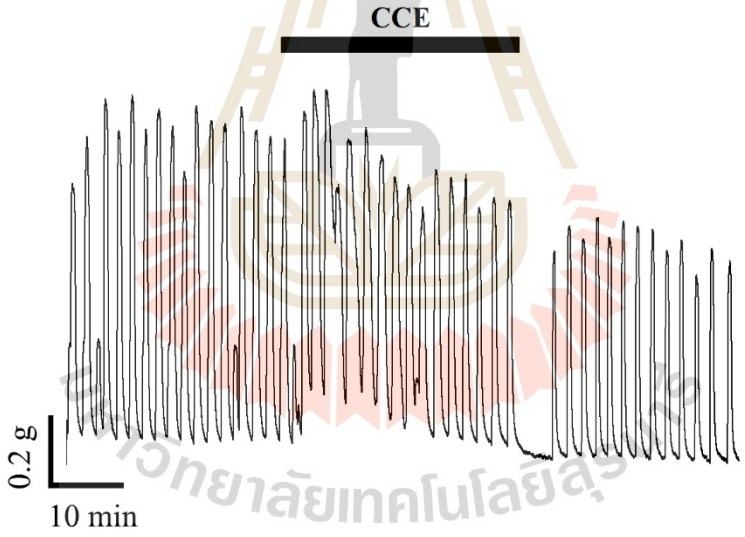


Figure 5.4 Typical traces showing the uterine spontaneous response in the presence of CCE in non-GD (A) and GD (B). A significantly different of the contractile response was compared during spontaneous control (100%) and tested period (CCE) within the same sample and compared between groups (n = 5).

Table 5.1 The effect of anti-diabetic plants (TLE and CCE) on spontaneous contraction in non-GD and GD.

Tested substances	Non-GD			GD			
	AUC (%)	Amplitude (%)	Frequency (%)	AUC (%)	Amplitude (%)	Frequency (%)	n
Control	100	100	100	100	100	100	
TLE	$37.61 \pm 4.07^{\#\#}$	$61.81 \pm 10.31^{\#}$	$51.03 \pm 14.39^{\#}$	$76.01 \pm 7.79^{\#}$ **	$73.92 \pm 4.18^{\#\#}$	97.33 ± 10.19*	5
CCE	$128.54 \pm 7.90^{\#}$	$81.86 \pm 5.99^{\#}$	$133.68 \pm 11.06^{\#}$	$152.84 \pm 14.90^{\#}$	91.39 ± 8.88	131.66 ± 16.65	5

Data are expressed in mean ± S.E.M. (n = 5 for each concentration). The *P* values for AUC, amplitude, and frequency of contractile response during the exposure to anti-diabetic plants (TLE and CCE) in both experimental groups were significantly different from the spontaneous control (100%) in the same uterine strips (pair student *t*-test; $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$) and significantly different in each AUC, amplitude, and frequency between non-GD and GD (unpair student *t*-test; $*P < 0.05$ and $**P < 0.01$).

5.4.4 Effect of TLE and CCE on the contractile response in the continued presence of KCl and OT in non-GD and GD

Further experiments were performed on whether the plant extracts (TLE and CCE) could enhance the highly coordinated contractile response during the labor process by using agonists-induced uterine contractility.

TLE administration in the continued presence of KCl and OT

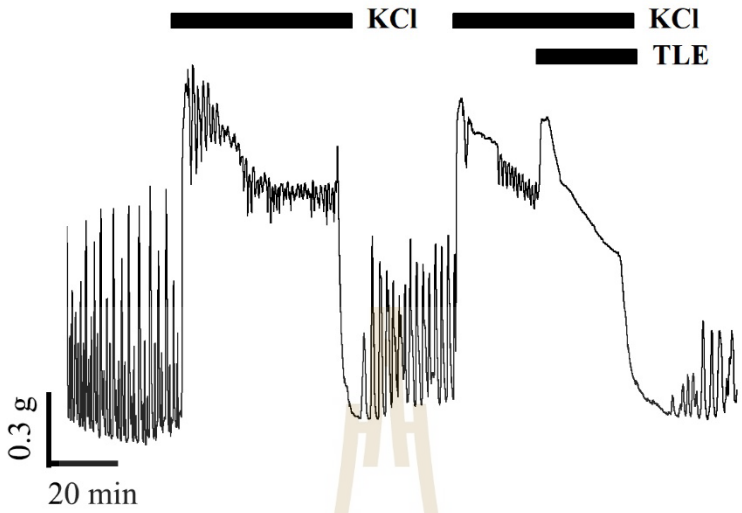
During the depolarization-induced Ca^{2+} influx by the KCl solution, the tonic force was well-generated in both non-GD and GD (Figure 5.5). Application of the TLE was able to diminish the uterine force which significantly dropped to 68.74 ± 3.28% in non-GD ($P < 0.01$) and 68.94 ± 6.52% in GD ($P < 0.05$), compared with each KCl alone (100%). The percent reduction during the TLE application was not different between groups (+0.29%, $P > 0.05$). This indicates that the alterations in the membrane depolarization by TLE was not different between non-diabetic pregnancy and diabetic pregnancy.

Similarly, a force was rose during the applications of OT in both non-GD and GD, as seen in Figure 5.6. After the exposure to TLE, a significant force reduction was found which was dropped to 64.47 ± 4.09% in non-GD ($P < 0.01$) and 73.56 ± 7.44% in GD ($P < 0.05$), compared with each OT alone (100%). There was no significant difference between the percent reduction between groups (+14.10%, $P > 0.05$). Conversely, the potential of OT under the tocolytic effect of TLE was shown in Figure 5.7. The contractile response was continued to reduce after the exposure to TLE in both groups. When OT was added, the force was significantly remained as 48.60 ± 8.84% in non-GD and 79.32 ± 3.06% in GD, compared with each TLE alone (100%, $P < 0.01$). The remained force in GD was significantly higher than non-GD (+63.19%, $P < 0.05$).

This result was suggested that the tocolytic effect of TLE was altered the Ca^{2+} release from the sarcoplasmic reticulum and the stronger effect was initiated in non-diabetic pregnancy whereas impaired effect in diabetic pregnancy.

The tocolytic effect of TLE in the continued presence of KCl and OT were summarized in Figure 5.8.

(A) Non-GD



(B) GD

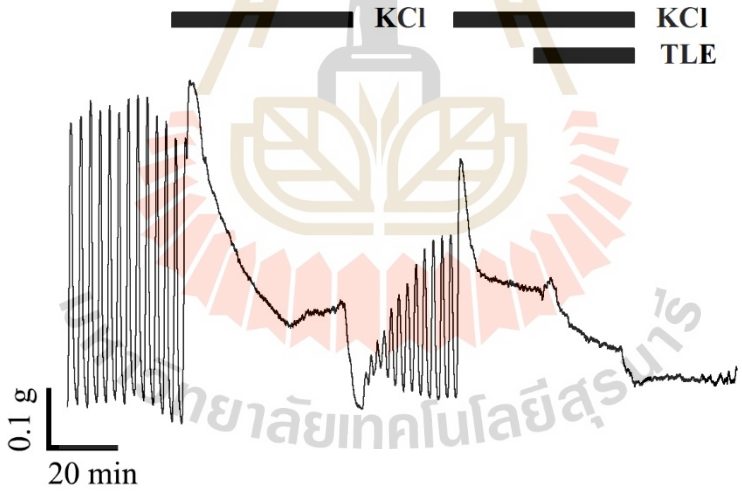
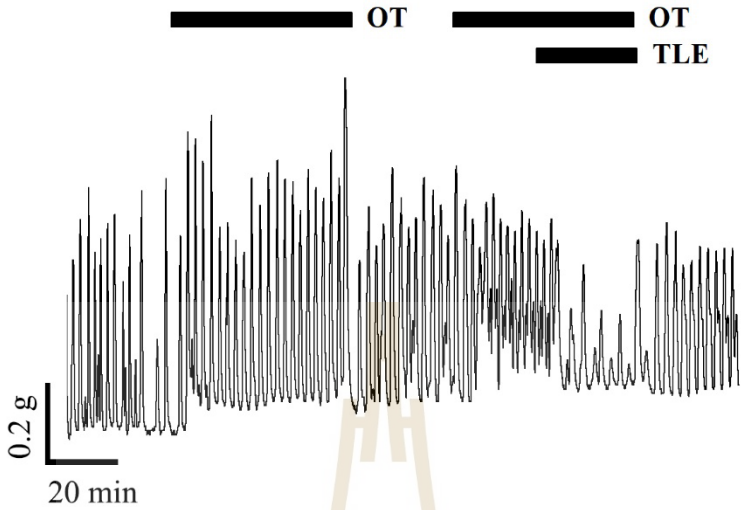


Figure 5.5 Typical traces showing the uterine response to TLE in the presence of KCl in non-GD (A) and GD (B). TLE was added after the exposure to KCl. The contractile response in the tested period (KCl + TLE) was compared to the time control (KCl alone, 100%), n = 4.

(A) Non-GD



(B) GD

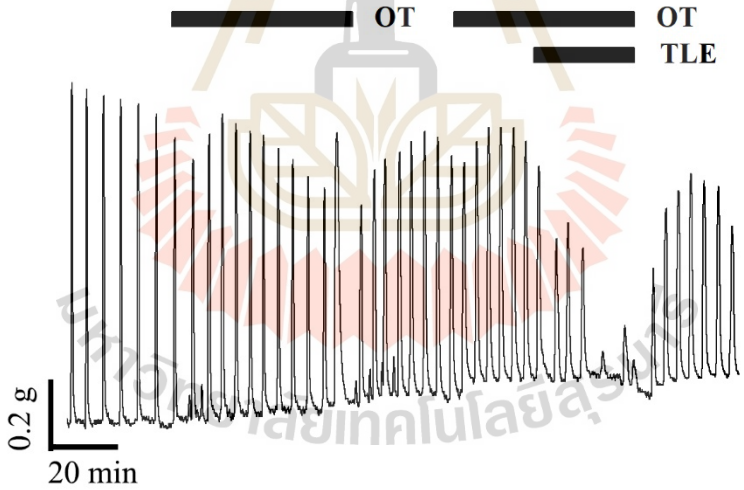
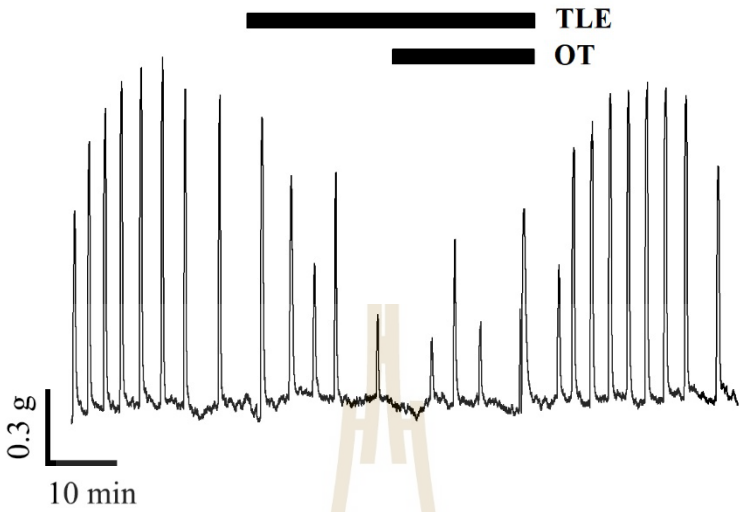


Figure 5.6 Typical traces showing the uterine response to TLE in the presence of OT in non-GD (A) and GD (B). TLE was added after the exposure to OT. The contractile response in the tested period (OT + TLE) was compared to the time control (OT alone, 100%), n = 4.

(A) Non-GD



(B) GD

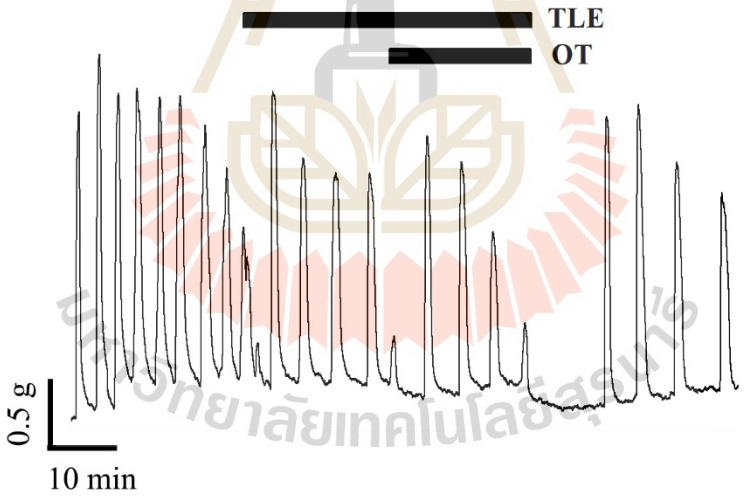


Figure 5.7 Typical traces showing the uterine response to TLE in the presence of OT in non-GD (A) and GD (B). TLE was added before the exposure to OT. The contractile response in the tested period (TLE + OT) was compared to the time control (TLE alone, 100%), n = 4.

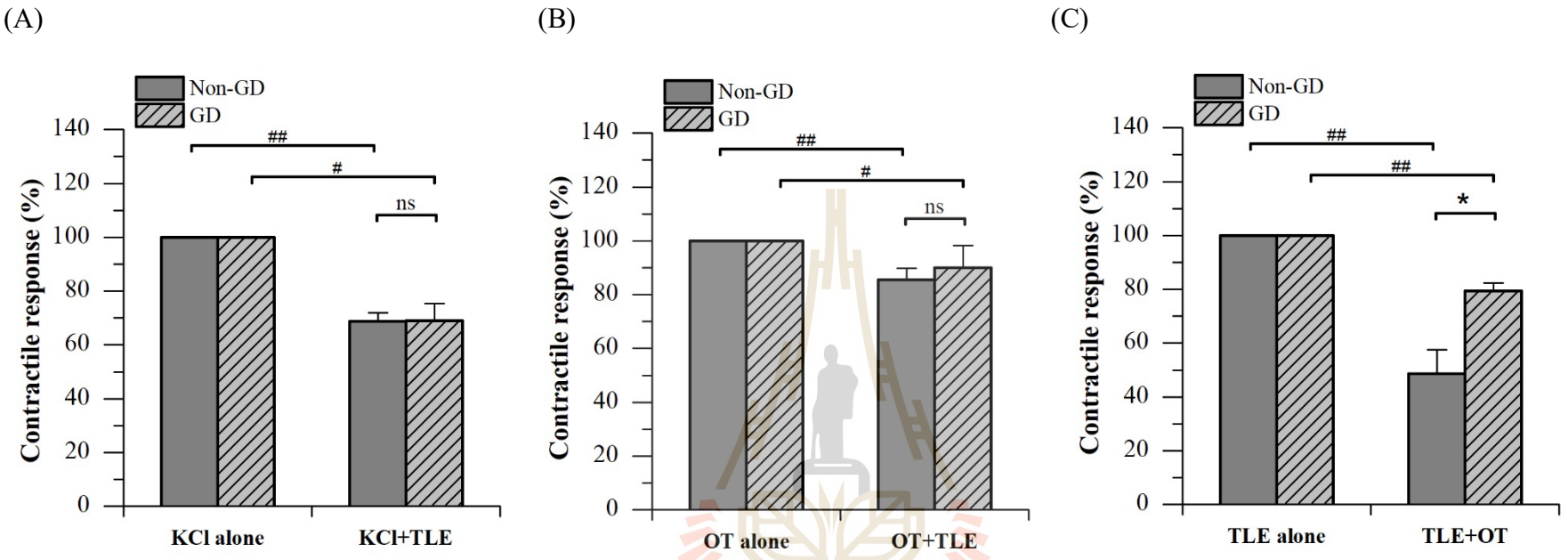


Figure 5.8 The effect of TLE in the continued presence of agonists-induced uterine contraction in non-GD and GD. TLE was added during the combination of KCl (A) and in the combination of OT by added after OT (B) and added before OT (C). Data are express as means ± S.E.M. A significantly different of the contractile response in the tested period (agonists + TLE) was compared to the time control, 100% (agonists alone or TLE alone, pair student *t*-test; $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$) and significantly different between groups (unpair student *t*-test; $^{*}P < 0.05$ and $^{**}P < 0.01$); ns = not significant.

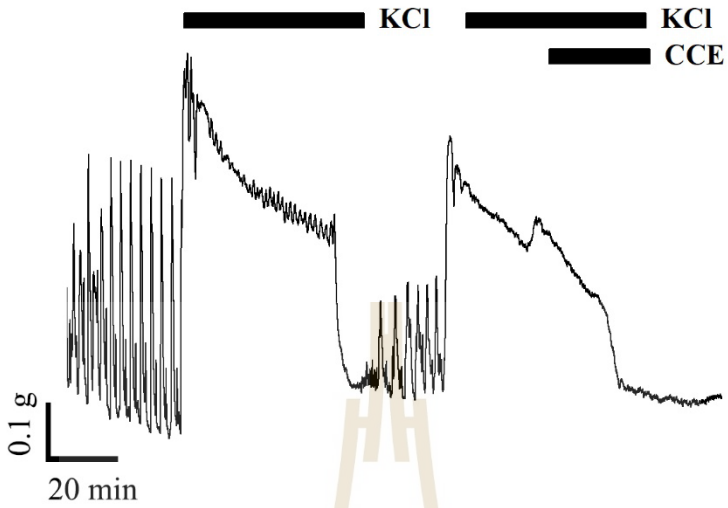
CCE administration in the continued presence of KCl and OT

The tonic force was produced during the application of the KCl solution in both non-GD and GD (Figure 5.9). The sustained force was gradually decreased due to the exposure to the biphasic effect of CCE, there was 82.65 ± 5.29% in non-GD ($P < 0.05$) and 87.93 ± 5.88% in GD from each KCl alone (100%). There was not different between groups (+6.39%, $P > 0.05$). Besides, non-diabetic pregnancy and diabetic pregnancy exhibited the non-different in membrane depolarization altered by CCE.

Additionally, the application of CCE in the continued presence of OT has been facilitated the uterotonic effect in both non-GD and GD with a trend to decrease amplitude (Figure 5.10). The AUC was significant increased to 131.31 ± 9.77% in non-GD ($P < 0.05$) and 162.81 ± 3.15% in GD ($P < 0.01$), compared with each OT alone (100%). The higher AUC in GD was significantly different from non-GD (+23.99%, $P < 0.05$). Inversely, the combination of OT after the uterine force activated by CCE was shown in Figure 5.11. Force was generated with increased frequency and gradually decreased amplitude by CCE in both groups. When OT was added, the force significantly remained which was 57.79 ± 3.01% in non-GD and 61.91 ± 4.08% in GD, compared with each CCE alone (100%, $P < 0.01$). The remained force in GD was higher than non-GD, but no significant difference between groups (+7.13%, $P > 0.05$). Excitingly in diabetic pregnancy, the uterine force has been promoted due to the uterotonic effect of CCE and more potent in the combination of OT which was higher than non-diabetic pregnancy as it has stronger sensitivity to the stimulant agents.

The uterotonic effect of CCE in the continued presence of KCl and OT were summarized in Figure 5.12.

(A) Non-GD



(B) GD

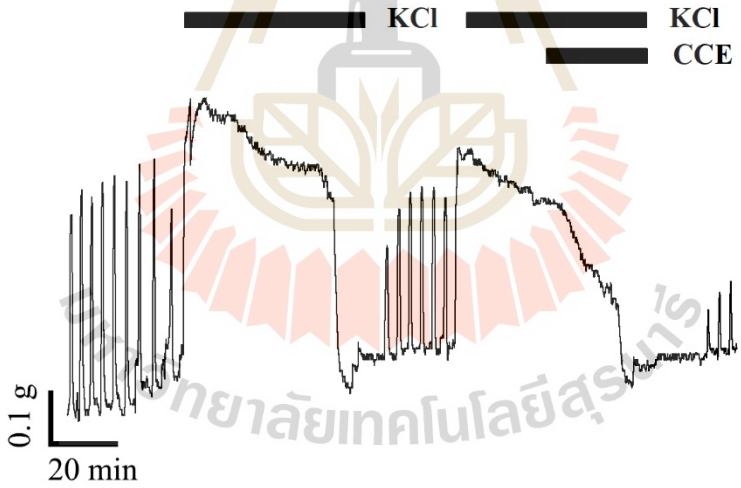
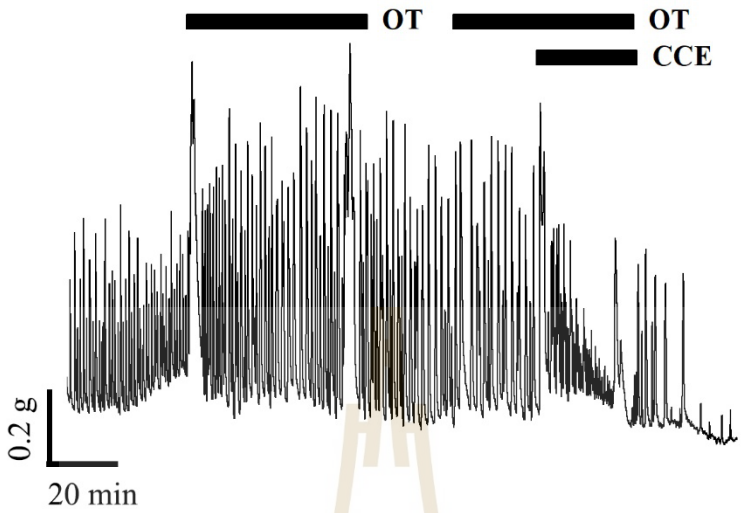


Figure 5.9 Typical traces showing the uterine response to CCE in the presence of KCl in non-GD (A) and GD (B). CCE was added after the exposure to KCl. The contractile response in the tested period (KCl + CCE) was compared to the time control (KCl alone, 100%), n = 4.

(A) Non-GD



(B) GD

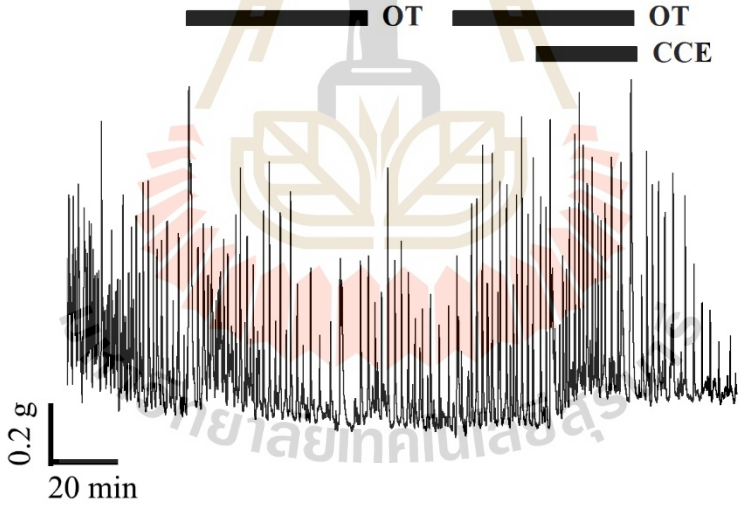
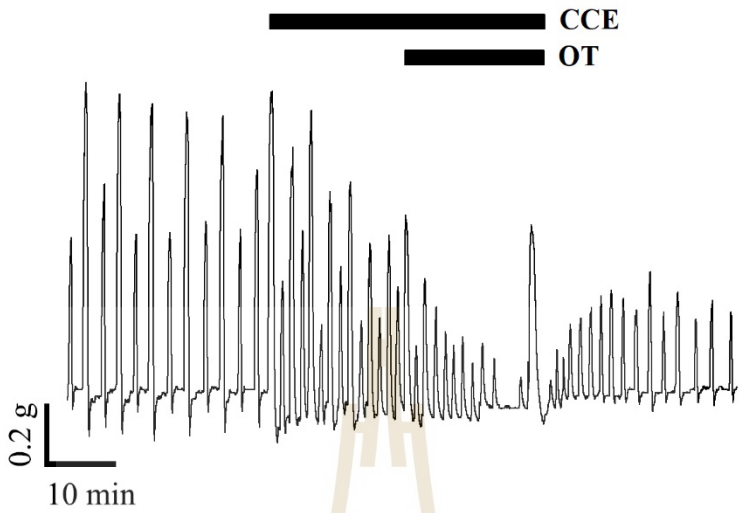


Figure 5.10 Typical traces showing the uterine response to CCE in the presence of OT in non-GD (A) and GD (B). CCE was added after the exposure to OT. The contractile response in the tested period (OT + CCE) was compared to the time control (OT alone, 100%), n = 4.

(A) Non-GD



(B) GD

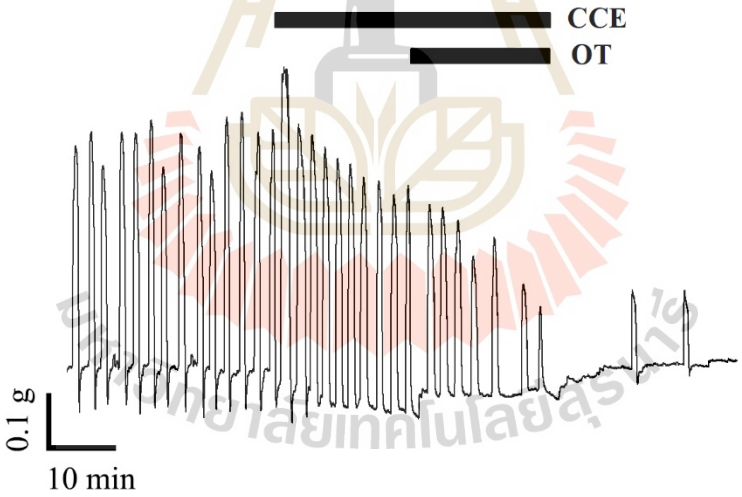


Figure 5.11 Typical traces showing the uterine response to CCE in the presence of OT in non-GD (A) and GD (B). CCE was added before the exposure to OT. The contractile response in the tested period (CCE + OT) was compared to the time control (CCE alone, 100%), n = 4.

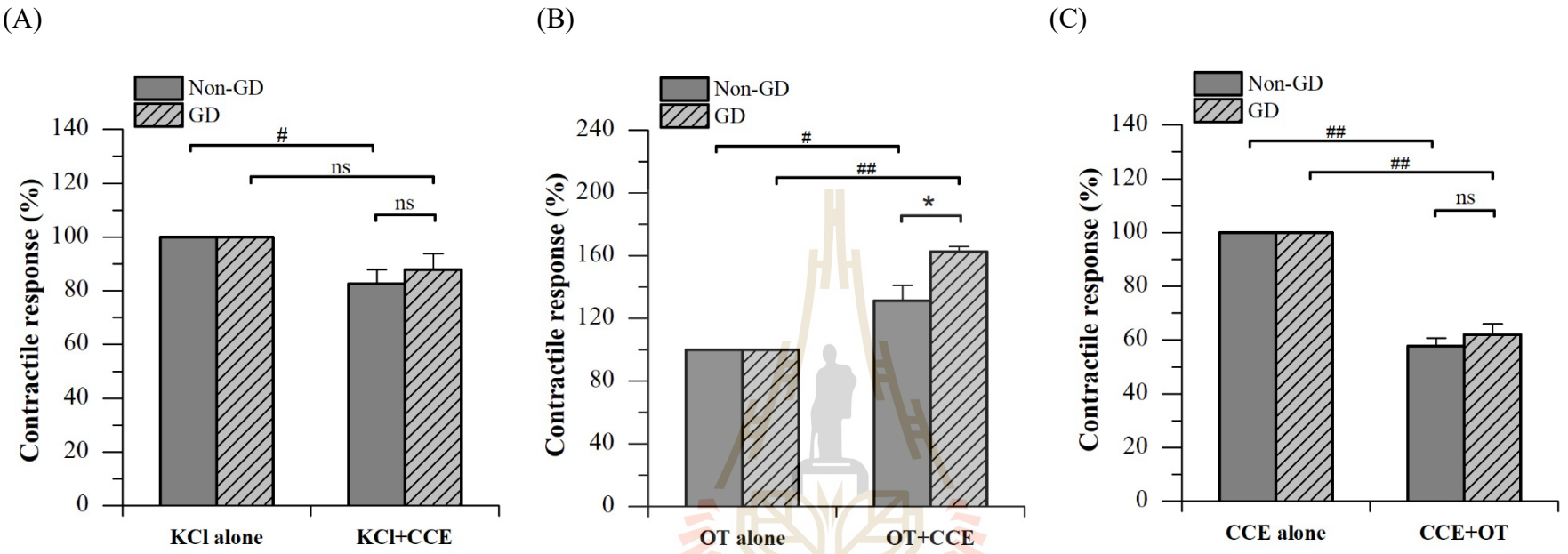


Figure 5.12 The effect of CCE in the continued presence of agonists-induced uterine contraction in non-GD and GD. CCE was added during the combination of KCl (A) and in the combination of OT by added after OT (B) and added before OT (C). Data are express as means ± S.E.M. A significantly different of the contractile response in the tested period (agonists + CCE) was compared to the time control, 100% (agonists alone or CCE alone, pair student *t*-test; $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$) and significantly different between groups (unpair student *t*-test; $^{*}P < 0.05$ and $^{**}P < 0.01$); ns = not significant.

5.5 Discussion

Nowadays, there is no data in the literature about the pharmacologic property in the uterotonic or tocolytic activity of *T. laurifolia* or *C. cinereum* on the uterus. Therefore, the alteration of these plants during pregnancy, especially in diabetic conditions has been investigated in this experiment.

Several medicinal plants in Acanthaceae family is responsible for non-specific smooth muscle relaxant property in various tissues such as blood vessels, trachea, intestine and uterus (Adeyemi et al., 1999; Bafor et al., 2019; Dimo et al., 2007; Moreira Leal et al., 2017). Interestingly, the different fractions from *Acanthus montanus* have been shown a biphasic effect on both uterine relaxation and contraction (Asongalem et al., 2013; Foyet et al., 2006). However, there was no evidence to support the compensatory biological effect of genus *Thunbergia* on the uterine smooth muscle alteration during gestation due to its popular use in diabetes treatment.

In this study, the biphasic action on contractile parameters was initiated as a transient contraction in low concentration dependency (0.5 mg/mL) followed by a prolonged relaxation in the cumulative concentration (1-2 mg/mL) during the TLE concentration dependency application. Furthermore, the IC_{50} of TLE (1.19 mg/mL) has been shown a stronger relaxant effect in non-GD and degraded in GD. This result was indicated that TLE has a potent tocolytic effect in some alteration on uterine smooth muscle cells. During the agonists-induced in the laboring process, an inhibitory effect of TLE on K^+ channels was found in both non-GD and GD when exposure to KCl and there were similar. This alteration caused by TLE has interacted with Ca^{2+} influx through L-type voltage-operated Ca^{2+} channel which activated by the depolarization and this interaction was not altered by diabetes in pregnant myometrium. The

application of TLE after OT-mediated maximal intracellular Ca^{2+} release was also produced the reduction of integral force in both non-GD and GD with similar reduction was seen. Moreover, OT was not completely promoted uterine contraction during the continued application of TLE in both non-GD and GD and the inhibitory effect was statistically weakened in GD. This also suggested that TLE exerted the suppression effect with a possible antagonist to IP_3 and its downstream signaling of OT. Taken together, the excessive intracellular Ca^{2+} concentration was abolished from both the L-type Ca^{2+} channel and intracellular stores as demonstrated the ability to preventing preterm labor, and this tocolytic activity attenuated in diabetic pregnancy. It may result from some dominance of the TLE constituents found in various medicinal plants which are attributed to the inhibitory process. Higher abundant as 17.42% of pyran derivative (4H-Pyran-4-one,2,3-dihydro-3,5-dihydroxy-6-methyl-) was found in this study, same as the highest abundant components in *Bryophyllum pinnatum* and *Stachytarpheta jamaicensis* (Ololade et al., 2017; Uchegbu et al., 2017), their tocolytic abilities were revealed in human myometrium (Gwehenberger et al., 2004) and rat uterus (Amaechina and Bafor, 2016) by modified spontaneous contractile pattern and inhibited of the oxytocin signaling pathway. For other TLE constituents, Wölkart et al. (2017) had been confirmed that the inhibition of L-type Ca^{2+} channels were mediated by 5-Hydroxymethylfurfural in coronary arteries relaxation. Chromone derivative in *Evolvulus linarioides* shown the tocolytic effect against OT-induced uterine contraction in female rats (Pereira et al., 2016). Indole alkaloid also reported as a major active compound in *Nauclea latifolia* (Abreu and Pereira, 1998) and *Himatanthus lancifolius* (Rattmann et al., 2005), their anti-abortifacient properties were disrupted the agonists-induced uterine contractions on the isolated non-pregnant rats' uterus (Nworgu et al.,

2010) and were dependent on both voltage- and receptor-operated Ca^{2+} channels due to the external Ca^{2+} uptake (Rattmann et al., 2005). Moreover, a natural phenolic compound, rosmarinic acid has been well recognized to inhibit the cyclooxygenase (COX1 and COX2) enzymes in a specific pathway, resulting in reduced prostaglandin biosynthesis from arachidonic acid (Chen, 2010; Gamaro et al., 2011). Likewise, Slater et al. (1999) suggested the greater uterine COX2 expression and Vane and Williams (1973) reported the higher prostaglandin production during the day of delivery which contributed to the cervix ripening and fetus expulsion, potentially activated by OT. However, Fuchs et al. (1976) found that the inhibition of COX activity or prostaglandin production is indirectly related to the myometrial contractions and its primary effect is a postulate, so the prostaglandin synthesis inhibitors drugs are well-used as tocolytics. Additionally, Hirst et al. (2005) exhibited the specific inhibitor to membrane prostaglandin receptors had a beneficial effect on the prevention of preterm birth. Hence, there was no evidence that demonstrates the role of COX enzymes or prostaglandin synthesis inhibition mediated-mechanism on uterine contractility.

Due to the various pharmacological activities in the largest Asteraceae family, the dual effect in partial agonists at low potency and non-competitive antagonists at high potency on isolated rat intestine was found in *Mikania cordifolia* (Colares et al., 2013) and *Calendula officinalis* (Bashir et al., 2006). The different action depending on the variety of the extraction and target tissues, as seen in non-specific smooth muscle, *Bidens pilosa* has potent both uterotonic (Frida et al., 2007) and vasorelaxant activity (Dimo et al., 2006). The phytochemical compound, kaurenoic acid isolated from *Aspilia mossambicensis* shown the agonist uterine contraction (Page et al., 1992), whereas isolated from *Viguiera robusta* shown the antagonist vascular contraction (Tirapelli et

al., 2002). For the genus *Cyanthillium* (Synonym: *Vernonia*), the popular traditional uses of *V. amygdalina* have been stimulated the uterine motility (IJEH et al., 2011) and vasorelaxant properties by Ca^{2+} channels blockage and reduced Ca^{2+} released from SR (Ch'ng et al., 2017). *V. glabra* and *V. cinerea* promoted spasmodic response on the intestine smooth muscle in rabbit and rat (Achola et al., 1996; Pandey et al., 2012). These relevant pharmacological effects may be due to the different presence of bioactive compounds in the plant extract.

As present in this study, the uterine stimulation or relaxation was mediated due to the application of CCE on isolated pregnant rat myometrium. This activity was observed in the concentration dependent experiment that the increased AUC, amplitude, and frequency in phasic contraction with the elevated baseline were significantly found at low concentration dependent (0.5-1 mg/ml) administration of CCE, whereas all parameters were significantly turned to inhibitory phase as the presence of incomplete relaxation at high concentration dependent (1.5-2 mg/ml) with the significantly decreased amplitude starting reduced at the concentration of 1 mg/ml. It was implied that the CCE altered the Ca^{2+} influx and then enhancement of Ca^{2+} utilization, subsequently produced incomplete relaxant activity associated with a concentration-dependent manner. Next, the IC_{50} of CCE (1.50 mg/mL) has been promoted a transient Ca^{2+} influx with significantly increased AUC and amplitude, and then following by the prolong incomplete relaxation in non-GD and stronger potential in GD with the elevated baseline was presented. This result was revealed the modifying pattern of spontaneous contraction on isolated pregnant myometrium by *C. cinereum* ethanolic extract. Furthermore, to verified the alteration to K^+ channels, the application of CCE in the continued exposure to KCl has been shown the reduction of contractile

response in both non-GD and GD with no significant difference when compared to each KCl alone and between groups. This indicated that the external Ca^{2+} influx was fully activated by KCl and was partially blockage by CCE and this reduction was not altered in diabetic pregnancy. The administration of CCE under the OT stimulation has been significantly exhibited some stimulant effect in the integral force with elevated baseline and gradually decreased amplitude in both non-GD and GD which higher force was found in GD. It seems like the extract was acted on the OT signaling pathway which reduced the response to OT while increased the utilization of intracellular Ca^{2+} concentration. To demonstrated the possible effect on OT receptor, its cascades, or the IP_3 receptor on SR, the CCE was generated an incomplete relaxation ability with gradually reduced amplitude in both non-GD and GD. After OT was added, the contractile response has still remained significantly reduced (compared with CCE alone) and the ability to triggered Ca^{2+} release from SR by OT was not significantly different between groups. This result was elucidated the possible action of CCE may be acted as a biphasic effect on OT signaling pathway whether bind to their receptors, mediated their cascades, or actioned on the IP_3 signaling pathway. OT cannot promote the powerful contraction under the effect of CCE and diabetic pregnancy has abolished the response to CCE. However, the alteration on these specifical cascades should be confirmed by further investigation or it could be explained in previous researches about the effect of phytoconstituents found in CCE. Terpenoids, lupeol (32.36%), β-sitosterol (2.68%), and lupenone (1.27%) are exhibited in this extract and these compounds are commonly found in *Sorbus* genus (Lee and Lee, 1999) with a various mode of action has been reported such as strong vasodilation in *S. commixta* and weaker action in *S. aucuparia* which possess Ca^{2+} influx inhibition through nitric oxide-cGMP signaling

pathway (Bujor et al., 2019; Kang et al., 2005). The spasmodic effect was found in *Albizia chinensis*, *Streblus asper*, *Asteracantha longifolia* containing high rich of lupeol and β-sitosterol which produce powerful smooth muscle contractions and useful for reproductive problems treatment (Ambasta et al., 1992; Lipton, 1959; Rastogi et al., 2006; Sharmin et al., 2014; Singh et al., 2015). Lupeol show specific inhibitors of cAMP-PKA, but it does not inhibit MLCK activity (Hasmeda et al., 1999). β-sitosterol promote uterotonic activity by inhibited K^+ channels and SERCA with increasing calcium entry on L-type Ca^{2+} channels and involving MLCK pathway (Promprom et al., 2010), and the changed basal contraction tone is resulted from SERCA inhibition and increase in intracellular Ca^{2+} concentration (Kupittayanant et al., 2002). High rich of β-amyrin was found in this CCE (16.39%) and presented in *Clerodendrum inerme* which stimulated the uterine motility in the pregnant uterus, but not sustained prolong effect following by the incomplete relaxation with the basal tone elevation and amplitude reduction as closely similar to the effect of CCE (Parveen et al., 2010; Sharaf et al., 1969). Higher composition of palmitic acid in *Nigella sativa* (Nickavar et al., 2003), *Pentanisia prunelloides* (Kaido et al., 1997), and *Saraca indica* (Kashima and Miyazawa, 2012) exerted the inhibition of uterine spontaneous movement, abolished the maximum response to OT and failed to possess OT-like activity (Aqel and Shaheen, 1996; Mitra et al., 1999; Yff et al., 2002).

Biological testing of the ethanolic extract of anti-diabetic plants used in this experiment (*T. laurifolia* leaves and *C. cinereum* whole plant) possessed the tocolytic effect with the different mechanisms on pregnant uterine activity *in vitro*.

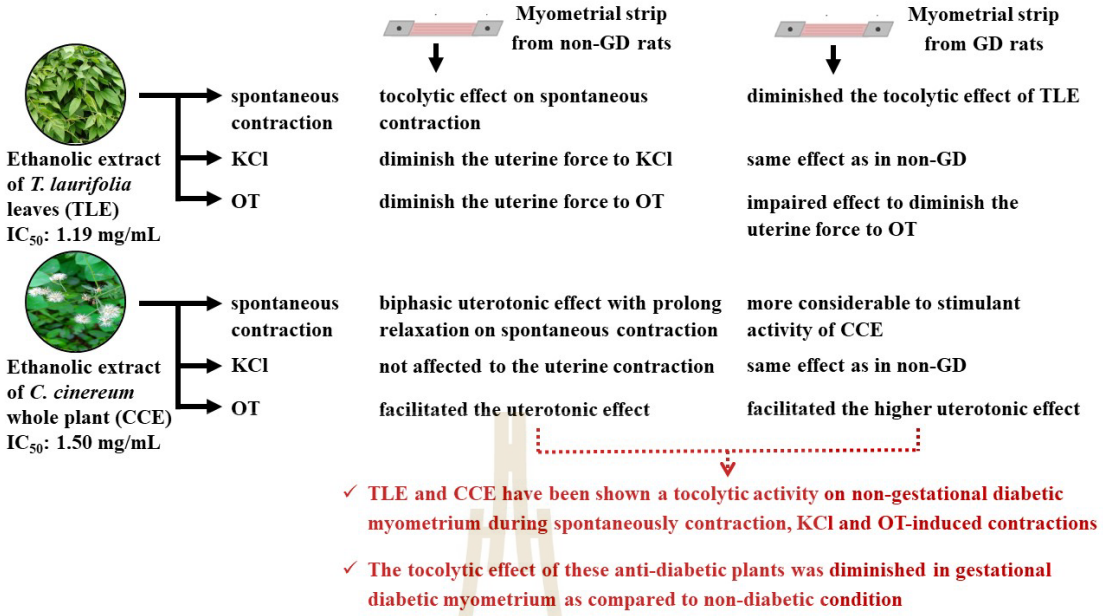


Figure 5.13 The diagram shows the effects of TLE and CCE on the uterine spontaneous response, in the presence of KCl and OT-induced contraction in non-gestational diabetic and gestational diabetic rat myometrium.

5.6 References

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CHAPTER VI

EFFECTS OF SELECTED TRADITIONAL ANTI-DIABETIC PLANTS ON BLOOD ANALYSIS, MATERNAL REPRODUCTIVE PERFORMANCES AND FETAL OUTCOMES IN PREGNANT DIABETIC RATS

6.1 Abstract

Diabetes during pregnancy or gestational diabetes mellitus (GDM) is one of the serious metabolic disorders which affects severely maternal health status, and fetal complications. Herbal supplementations have a beneficial effect on reducing the glycemic state and long-term safety effect in diabetic patients. This experiment was to investigate the oral administration with *Thunbergia laurifolia* leave (TLE) and *Cyanthillium cinereum* whole plant (CCE) ethanolic extract on biochemical parameters, maternal reproductive performances, and fetal outcomes in experimental non-diabetic and STZ-induced diabetic pregnant rats. Pregnant Wistar rats were randomly divided into group 1 = non-gestational diabetic control rats (Non-GD) and group 2 = gestational diabetic control rats (GD) along with five treated subgroups including group 3 (MET) = received metformin at a dose of 100 mg/kg, group 4 (LDTLE) and group 5 (HDTLE) = received with *T. laurifolia* extract at a dose of 50 and 500 mg/kg, respectively, group 6 (LDCCE) and group 7 (HDCCE) = received with *C. cinereum* extract at a dose of 50 and 500 mg/kg, respectively. Diabetes was induced by STZ (60 mg/kg i.p.) injection

on day 5th of pregnancy. All treatments were performed orally once daily throughout pregnancy after diabetes was confirmed on day 7th of pregnancy. Maternal glycemic status, body weight gain, and food intake were weekly recorded. On day 21st of pregnancy, maternal lipid profiles, liver enzymes, a gravid uterus with its contents, and fetal samples were assessed. A significant elevated maternal hyperglycemia, reduced weight gain, altered lipid metabolism, impaired maternal reproductive outcomes, and ineffective fetal-placental development leading to decreased numbers of live fetuses that were presented in gestational diabetic control rats. Treatment with TLE and CCE in gestational diabetic rats have potently mediated higher glycemic level on the late gestation (I), a trend to maternal weight gain improvement on the mid and late gestation (II), no alteration on maternal food intake throughout pregnancy (III), reduced higher level of total cholesterol, triglyceride, LDL whilst increased HDL level (IV), diminished higher level of AST, ALT and ALP (V), closed to the effect of MET. The diverse modulation of maternal reproductive performances and fetal outcomes were presented. The TLE treatment showed a possible decreasing trend in the number of dead fetuses, the ability to prevented pre-implantation loss rates, post-implantation loss rates, and increased the proportion of APA fetuses (VI), same as the effect of MET while CCE treatment showed only the decreasing trend in the number of dead fetuses and higher APA fetuses proportion (VII) with no alteration on resorptions in both dose-dependent administrations. These data suggest that *T. laurifolia* and *C. cinereum* achieved better outcomes in the management of gestational diabetic mellitus and safety potential along with its anti-hyperglycemic activities, anti-hyperlipidemic activities, and cytoprotective effects, probably to the effect of metformin. An effective therapeutic supplementation during diabetic pregnancy of *T. laurifolia* provides the prevention of

miscarriages, intrauterine growth restriction, stillbirth, and supported fetal development, and *C. cinereum* provides the protection of stillbirth and enhanced fetal development.

6.2 Introduction

Hyperglycemia and insulin dysfunction are an important cause of maternal and fetal complications in any types of diabetic pregnant women with greatly influenced by the severity of maternal diabetes (Krishna Murthy et al., 2002). Identically, 8% in uncomplicated diabetic pregnancies (as uncontrolled diabetes during the first 8 weeks of pregnancy) are presented the congenital malformations from organogenesis processes in the first trimesters with potentially fetal loss through early spontaneous abortion, as well as premature rupture of membranes or premature labor that the delivery occurring before 37 weeks of gestation as reported 11% of all pregnancies, same as the births before 32 weeks that dramatically increased rate of neonatal deaths (Lain and Catalano, 2008). In the second half term of pregnancy, around 5-10% of all pregnancies are developed pregnancy-induced hypertension (PIH) and classified as preeclampsia or transitional gestation hypertension which caused various metabolic abnormalities such as dyslipidemia and hyperuricemia. Same as the risks of vascular disease is a 30% increase with difficultly to control blood pressure and severity in pregnant women who have prolonged nephropathy. Congenital malformations in diabetic mothers are frequently associated with polyhydramnios as excessive amniotic fluid with high incidence for spontaneous onset of labor or preterm labor (Forsbach-Sánchez et al., 2005; Krishna Murthy et al., 2002; Ryan, 2002). The rate of macrosomia has been reported 14% in normal pregnancy, 29% in untreated women with GDM, and

10% in treated GDM women with related many complications (Moses et al., 2000). The higher rates of perinatal death or stillbirth resulted from fetal infection in mothers with diabetes (Dunne et al., 2003). Evers et al. (2004) found that pre-eclampsia, macrosomia, breech presentation, and fetal distress caused an emergency cesarean section.

Natural compounds are commonly found in plants consumed in the dietary supplement that attempt to control diabetes and attenuate its complications during pregnancy (Illamola et al., 2020). *Thunbergia laurifolia* (*T. laurifolia*) leaves exhibited the hypoglycemic activity with recovered β-cells destruction and increased insulin secretion by insulin-like or certain substances (Aritajat et al., 2004), reduced blood glucose level by a competitive inhibitor of alpha amylase activity (Jaiboon et al., 2010). Furthermore, stem-bark and leaves of *Cyanthillium cinereum* (*C. cinereum*, synonym of *Vernonia cinerea*) have been potent anti-hyperglycemic properties with restored the pancreatic function and increased insulin output or decreased in the intestinal absorption of glucose in diabetic rats (Haque et al., 2013). Traditionally herbal medicines are used to ameliorate the diabetes-related complications in diabetic rats (Abas et al., 2015; Bin Sayeed et al., 2013).

Therefore, it is important to study the positive or negative efficacy of these anti-diabetic plants in diabetes associated with pregnancy that may alter in the reproductive performances and health behavior during pregnancy including blood chemicals, maternal reproductive outcomes and, fetal anomaly incidence from the non-diabetic and streptozotocin-induced diabetic pregnant rats.

6.3 Materials and methods

6.3.1 The administration doses of MET, TLE, and CCE

The safety evaluation was performed by acute toxicity testing by oral gavage with a fix-dose procedure using 3 animals of both sexes per each dose administered as following the Organization for Economic Co-operation and Development (OECD) guidance document 423 (2002).

A toxicity test of MET in animals given 200, 600, 900, or 1200 mg/kg/day up to 13 weeks is performed by Quaile et al. (2010), they found that some clinical signs of toxicity were found at a dose higher than 900 mg/kg/day in female and 600 mg/kg/day in male. Therefore, a dose lower than 600 mg/kg/day are potent glucose metabolism and the no observable adverse effect level (NOAEL) was found at 200 mg/kg/day, based on the body weight changed.

The acute toxicity study of *T. laurifolia* and *C. cinereum* (Synonym: *V. cinerea*) has been previously done by Cowawintaweewat et al. (2011) and Choudhary et al. (2013), respectively. The plant extracts are administered at a single dose of 500, 1000, 2000 mg/kg for a total of 14 days. They found that there is no sign of toxicity, no adverse behavior, no change in physical activities, and no clinical death in animals up to 2000 mg/kg/day.

For animal welfare concerning pregnant animals in the OECD guidance document 415 (1983) for reproductive toxicity study, MET administration at a dose of 100 mg/kg/day ($1/2^{th}$ of NOAEL) was designed for the maximum testing. Due to its low toxicity in both plants studied, a limit level of no adverse effects is expected at a dose of 1000 mg/kg/day. Hence, the dose of 500 mg/kg ($1/2^{th}$) was selected to the

maximum testing and the 50 mg/kg ($1/20^{th}$) was defined as the minimum testing on pregnant animals with considered to the animal health status in this study.

6.3.2 Animal and chemical exposures

The Mating procedure and the conception of female Wistar rats (250-300 g) were well-described in 2.2.4. The diabetic induction was described in 2.2.5, briefly, streptozotocin (60 mg/kg BW) in citrate buffer was injected on day 5^{th} of pregnancy. Two days after injection, small blood was dropped on the glucometer. Gestational diabetic rats were selected for the experimental procedure when hyperglycemia was found (glucose level was higher than 300 mg/dL), whereas non-gestational diabetic rats were received only citrate buffer injection.

Additionally, all pregnant rats were randomly assigned to 7 experimental groups. Group 1 (Non-GD) was non-gestational diabetic untreated rats who received the vehicle (Tween 80 in distilled water, 10% v/v). Group 2 (GD) was untreated gestational diabetic rats who received the vehicle (Tween 80 in distilled water, 10% v/v). Group 3 (MET) was gestational diabetic rats treated with metformin (100 mg/kg BW) and served as anti-diabetic drug control. Group 4 (LDTLE) was gestational diabetic rats treated with a low dosage (50 mg/kg BW) of *T. laurifolia* extract. Group 5 (HDTLE) was gestational diabetic rats treated with a high dosage (500 mg/kg BW) of *T. laurifolia* extract. Group 6 (LDCCE) was gestational diabetic rats treated with a low dosage (50 mg/kg BW) of *C. cinereum* extract. Group 7 (HDCCE) was gestational diabetic rats treated with a high dosage (500 mg/kg BW) of *C. cinereum* extract. The treatments were given orally once a day by gavage (intragastric route) in 15 consecutive days (from day 7^{th} to 21^{st} of gestation). The maximum volume administered was 1 mL/rat/day. The experiment protocols have been shown in Table 6.1.

Table 6.1 The treatment for experimental groups.

Groups	Treatment and Dosage	Route	Duration
Non-GD	Non-gestational diabetic rats; vehicle (1 ml/rat/day)	Orally	15 days
GD	Gestational diabetic rats; vehicle (1 ml/rat/day)	Orally	15 days
MET	Gestational diabetic rats; metformin (100 mg/kg BW/day)	Orally	15 days
LDTLE	Gestational diabetic rats; low dosage of *T. laurifolia* extract (50 mg/kg BW/day)	Orally	15 days
HDTLE	Gestational diabetic rats; high dosage of *T. laurifolia* extract (500 mg/kg BW/day)	Orally	15 days
LDCCE	Gestational diabetic rats; low dosage of *C. cinereum* extract (50 mg/kg BW/day)	Orally	15 days
HDCCE	Gestational diabetic rats; high dosage of *C. cinereum* extract (500 mg/kg BW/day)	Orally	15 days

6.3.3 Glycemic monitor

Maternal blood glucose levels and body weight were determined on day 0, 7th, 14th, 21st of pregnancy. A small drop of maternal blood was collected by the tail vein tipping method and the glycemic level was determined using an Accu-Chek Performa glucometer with glucose strips (Roche Diagnostics).

6.3.4 Lipid profiles analysis

Maternal rats were euthanized by CO_2 asphyxia on day 21st of pregnancy. The blood samples were collected at the end of the experiment by cardiac puncture and were immediately allowed to clot. The serum samples were separated by centrifuging (4°C) the blood at 1500 rpm for 10 min and were stored at -80°C until used for the determination of biological parameters. Total cholesterol (TC), triglyceride (TRI), high-density lipoprotein (HDL cholesterol), and low-density lipoprotein (LDL cholesterol) concentrations were measured by the end-point reaction method using Mindray BS-120 blood chemistry analyzer (Mindray Bio-Medical Electronics Co., Ltd., China).

6.3.5 Liver transaminases activity

Serum aspartate transaminase (AST), alanine transaminase (ALT), and alkaline phosphatase (ALP) were investigated from maternal rats' blood serum. Mindray BS-120 blood chemistry analyzer (Mindray Bio-Medical Electronics Co., Ltd., China) was used to analyze these liver enzymes with the kinetic reaction method.

6.3.6 Outcome of pregnancy measurement

During the experiment, the vaginal bleeding of maternal rats was daily observed until the term. On day 21st of gestation, Maternal rats were laparotomy under CO_2 asphyxia. The gravid uterus containing fetuses with their placentas were removed

and weighed. The corpora lutea (CL), implantations (fertilization), resorptions (embryonic deaths), live (survival) and dead (stillbirth) fetuses were carefully dissected for subsequent counting. Pre-implantation loss rates and post-implantation loss rates were calculated as follow:

$$\text{Pre-implantation loss rates (\%)} = \frac{no.of\ CL - no.of\ implantations}{no.of\ CL} \times 100$$

$$\text{Post-implantation loss rates (\%)} = \frac{no.of\ implantations - no.of\ livefetuses}{no.of\ implantations} \times 100$$

A number of total resorptions were used to detecting miscarriage. Fetuses and placentas were taken off from the uterus and weighted for placental index calculation which is the early markers for preeclampsia disorder, as the following equation:

$$\text{Placental index} = \frac{Placental\ weight}{Fetal\ weight} \times 100$$

All fetuses were weighted for classifying fetal growth. The mean birth weight of non-gestational diabetic control pups was classified as appropriate for pregnancy age (APA) and who weighted was at least 1.0 standard error (S.D.) above the mean birth weight of non-gestational diabetic control pups was classified as large for pregnancy age (LPA) which characterizing macrosomia. In contrast, who weighted was at least 1.0 S.D. below the mean birth weight of non-gestational diabetic control pups was classified as small for pregnancy age (SPA) which indicating intrauterine growth restriction. The crown rump length (CRL) of pups was measured as the maximum length from the top of the head (crown) to the base of the buttocks (rump) by using a measuring tape in a centimeter scale for the embryonic size determination (Damasceno et al., 2002b; Kiss et al., 2009).

6.3.7 Statistical analysis

The data of maternal glycemia, body weight, food consumption, lipid profile, liver enzymes, gravid uterus, number of corpora lutea, number of implantations, number of live fetuses, number of resorptions, pre-implantation loss rate percentage, post-implantation loss rate percentage, fetal weight, placental weight, placental index, and crown rump length are reported in mean ± standard error of the mean (S.E.M.). One way ANOVA followed by Turkey's *post hoc* test in SPSS Statistics for Windows, version 17.0 (SPSS Inc., Chicago, Illinois, USA) was used to compare the mean values among the experimental groups. Fetal growth classification for SPA, APA, LPA are shown in the proportions using the Chi-square test. A *P* value of less than 0.05 is indicated as statistically significant.

6.4 Results

6.4.1 Effects of TLE and CCE on maternal glycemia, body weight, and food consumption

As shown in Table 6.2, the glycemic level of non-GD was under 200 mg/dL throughout pregnancy whilst the glycemic level was higher than 300 mg/dL in all gestational diabetic rats ($P < 0.05$), which found on early gestation (day 7th of pregnancy). Hyperglycemia was continuing to developed in all gestational diabetic rats until term. Compared to the GD group, the treatment with MET, TLE and CCE were significantly reduced the glycemic level in gestational diabetic rats on the late gestation (day 21st of pregnancy) ($P < 0.05$), but did not interfere on the mid-gestation (day 14th of pregnancy). The strong glucose-lowering effect was found in high dosage treatment of TLE (decreased from 528.25 ± 22.59 to 430.75 ± 11.02 mg/dL) and high dosage

treatment of CCE (decreased from 565.50 ± 19.50 to 417.75 ± 32.38 mg/dL), which these glycemic levels were possibly closed to the effect of MET (decreased from 563.00 ± 17.45 to 405.00 ± 27.38 mg/dL, $P < 0.05$).

The maternal body weight gain did not change on the early gestation among the experimental groups, but it was significantly lower weight gain in all gestational diabetic rats on mid- and late gestation (compared with non-GD, $P < 0.05$). Treatment with MET, TLE, and CCE have slightly increased the maternal body weight gain on mid- and late gestation which reached near non-GD with non-significant (compared with GD, $P > 0.05$). Thus, both dosages of TLE treated (50 and 500 mg/kg BW/day) and high dosage of CCE treated (500 mg/kg BW/day) were exhibited the highest effective to improve the maternal body weight gain during late gestation (increased to 334.00 ± 11.22 g, 356.00 ± 2.45 g and 336.00 ± 8.12 g, respectively), same as the value from the MET treatment (340.00 ± 7.07 g), as shown in Table 6.3.

Food consumption in all gestational diabetic rats was not different from non-GD during the entire pregnant period ($P > 0.05$, Table 6.4).

Table 6.2 Glycemic levels of experimental pregnant rats from day 0 to 21st of pregnancy.

Treatment groups (dose, mg/kg BW)		Glycemic levels (mg/dL)			
		Day 0	**Day 7**	**Day 14**	**Day 21**
Non-GD	Non-gestational diabetic rats	102.25 ± 1.84	106.50 ± 0.87[a]	96.00 ± 1.58[a]	156.25 ± 5.12[a]
GD	Gestational diabetic rats	103.00 ± 1.68	432.00 ± 27.77[b]	575.75 ± 14.34[b]	587.75 ± 12.25[c]
MET	Metformin (100)	100.75 ± 1.93	484.25 ± 17.38[b]	563.00 ± 17.45[b]	405.00 ± 27.38[b]
LDTLE	*T. laurifolia* extract (50)	102.25 ± 1.03	467.75 ± 21.66[b]	546.75 ± 31.53[b]	451.75 ± 29.02[b]
HDTLE	*T. laurifolia* extract (500)	103.75 ± 3.28	490.25 ± 26.07[b]	528.25 ± 22.59[b]	430.75 ± 11.02[b]
LDCCE	*C. cinereum* extract (50)	100.00 ± 1.47	481.75 ± 23.70[b]	584.00 ± 16.00[b]	465.25 ± 42.86[b]
HDCCE	*C. cinereum* extract (500)	104.75 ± 1.25	454.25 ± 18.31[b]	565.50 ± 19.50[b]	417.75 ± 32.38[b]

Data are expressed in mean ± S.E.M. (n = 4 per group) and analyzed by one-way ANOVA, followed by Turkey's *post hoc* test.

Means with different superscripted letters in the same column indicate statistical significance ($P < 0.05$).

Table 6.3 Body weight of experimental pregnant rats from day 0 to 21st of pregnancy.

Treatment groups (dose, mg/kg BW)		Body weight (g)			
		Day 0	**Day 7**	**Day 14**	**Day 21**
Non-GD	Non-gestational diabetic rats	266.00 ± 8.12	278.00 ± 5.83^{ab}	312.00 ± 10.20^{b}	374.00 ± 17.49^{b}
GD	Gestational diabetic rats	258.00 ± 5.83	260.00 ± 5.48^{ab}	276.00 ± 5.10^{a}	314.00 ± 4.00^{a}
MET	Metformin (100)	266.00 ± 6.78	274.00 ± 7.48^{ab}	282.00 ± 4.90^{a}	340.00 ± 7.07^{ab}
LDTLE	*T. laurifolia* extract (50)	268.00 ± 5.83	272.00 ± 3.74^{ab}	290.00 ± 7.07^{ab}	334.00 ± 11.22^{ab}
HDTLE	*T. laurifolia* extract (500)	270.00 ± 3.16	280.00 ± 3.16^{b}	290.00 ± 3.16^{ab}	356.00 ± 2.45^{ab}
LDCCE	*C. cinereum* extract (50)	258.00 ± 3.74	256.00 ± 4.00^{a}	276.00 ± 4.00^{a}	320.00 ± 7.07^{a}
HDCCE	*C. cinereum* extract (500)	268.00 ± 5.83	266.00 ± 4.00^{ab}	288.00 ± 5.83^{ab}	336.00 ± 8.12^{ab}

Data are expressed in mean ± S.E.M. (n = 5 per group) and analyzed by one-way ANOVA, followed by Turkey's *post hoc* test.

Means with different superscripted letters in the same column indicate statistical significance ($P < 0.05$).

Table 6.4 Food consumption of experimental pregnant rats from day 0 to 21st of pregnancy.

Treatment groups (dose, mg/kg BW)		Food consumption (g)			
		Day 0	**Day 7**	**Day 14**	**Day 21**
Non-GD	Non-gestational diabetic rats	0.00 ± 0.00	12.26 ± 1.88	14.04 ± 0.96	16.05 ± 1.22
GD	Gestational diabetic rats	0.00 ± 0.00	9.54 ± 1.31	12.71 ± 0.83	17.00 ± 0.95
MET	Metformin (100)	0.00 ± 0.00	11.86 ± 0.77	13.43 ± 1.66	18.28 ± 0.72
LDTLE	*T. laurifolia* extract (50)	0.00 ± 0.00	9.57 ± 1.39	12.50 ± 1.02	17.76 ± 0.69
HDTLE	*T. laurifolia* extract (500)	0.00 ± 0.00	10.43 ± 1.27	14.71 ± 1.49	18.90 ± 1.68
LDCCE	*C. cinereum* extract (50)	0.00 ± 0.00	9.86 ± 1.70	12.71 ± 1.05	15.81 ± 1.08
HDCCE	*C. cinereum* extract (500)	0.00 ± 0.00	13.43 ± 0.97	17.14 ± 1.15	18.43 ± 0.98

Data are expressed in mean ± S.E.M. (n = 5 per group) and analyzed by one-way ANOVA, followed by Turkey's *post hoc* test.

No statistical significance in food consumption between experimental groups ($P > 0.05$).

6.4.2 Effects of TLE and CCE on serum lipid profiles

At term, serum concentrations of TC were significant higher in GD group (141.40 ± 6.10 mg/dL), compared with non-GD (101.00 ± 3.74 mg/dL, $P < 0.05$), whereas the TC serum concentrations were significantly reduced in MET treated group (106.80 ± 2.29 mg/dL), low and high dosages of TLE treated (LDTLE: 114.80 ± 7.28 mg/dL and HDTLE: 109.60 ± 5.96 mg/dL) and high dosage of CCE treated (112.00 ± 2.49 mg/dL), compared with GD group ($P < 0.05$), as seem in Figure 6.1(A).

In the GD group, serum concentrations of TRI were significantly marked increase to 913.00 ± 51.22 mg/dL, approximately 4-fold compared with non-GD (265.80 ± 36.96 mg/dL, $P < 0.05$). The administration of low and high dosages of both TLE and CCE have significantly reduced the TRI serum concentrations in gestational diabetic rats (LDTLE: 593.20 ± 65.96 mg/dL, HDTLE: 590.00 ± 109.20 mg/dL, LDCCE: 580.40 ± 43.28 mg/dL and HDCCE: 570.20 ± 43.22 mg/dL) and the most significant reduction was found in MET treated group, 429.60 ± 79.77 mg/dL (compared with GD, $P < 0.05$) as seen in Figure 6.1(B).

Gestational diabetic rats treated with MET showed the highest serum HDL concentrations (34.24 ± 4.47 mg/dL), compared with the lowest serum HDL concentrations found in GD group (15.58 ± 1.69 mg/dL, $P < 0.05$). The gestational diabetic rats treated with TLE and CCE showed the elevating in the serum HDL concentrations (LDTLE: 20.90 ± 3.00 mg/dL, HDTLE: 28.82 ± 4.30 mg/dL, LDCCE: 24.02 ± 2.35 mg/dL and HDCCE: 23.64 ± 4.07 mg/dL) which were closely to the normal level in non-GD group (26.94 ± 2.97 mg/dL), as seem in Figure 6.1(C).

Diabetes caused significant arise in serum LDL concentrations in GD group up to 44.58 ± 8.70 mg/dL, compared to non-GD (19.38 ± 3.13 mg/dL, $P < 0.05$). In

MET treatment, there was a significant reduction in serum LDL concentrations to 21.96 ± 6.31 mg/dL, as well as the same results found in TLE and CCE treated group (LDTLE: 26.34 ± 1.28 mg/dL, HDTLE: 26.48 ± 1.12 mg/dL, LDCCE: 24.84 ± 4.37 mg/dL and HDCCE: 23.10 ± 5.36 mg/dL, compared with GD, $P < 0.05$), as seem in Figure 6.1(D).

Gestational diabetic rats were significantly increased TC, TRI, and LDL levels, but decreased HDL level as compared to non-GD. The administration with MET, TLE, and CCE had significantly reduced TC, TRI, and LDL serum concentrations with increased HDL serum concentrations. The highest dosage (500 mg/kg BW) of both TLE and CCE showed the highest beneficial reduction, closely to the effects in MET treated group.

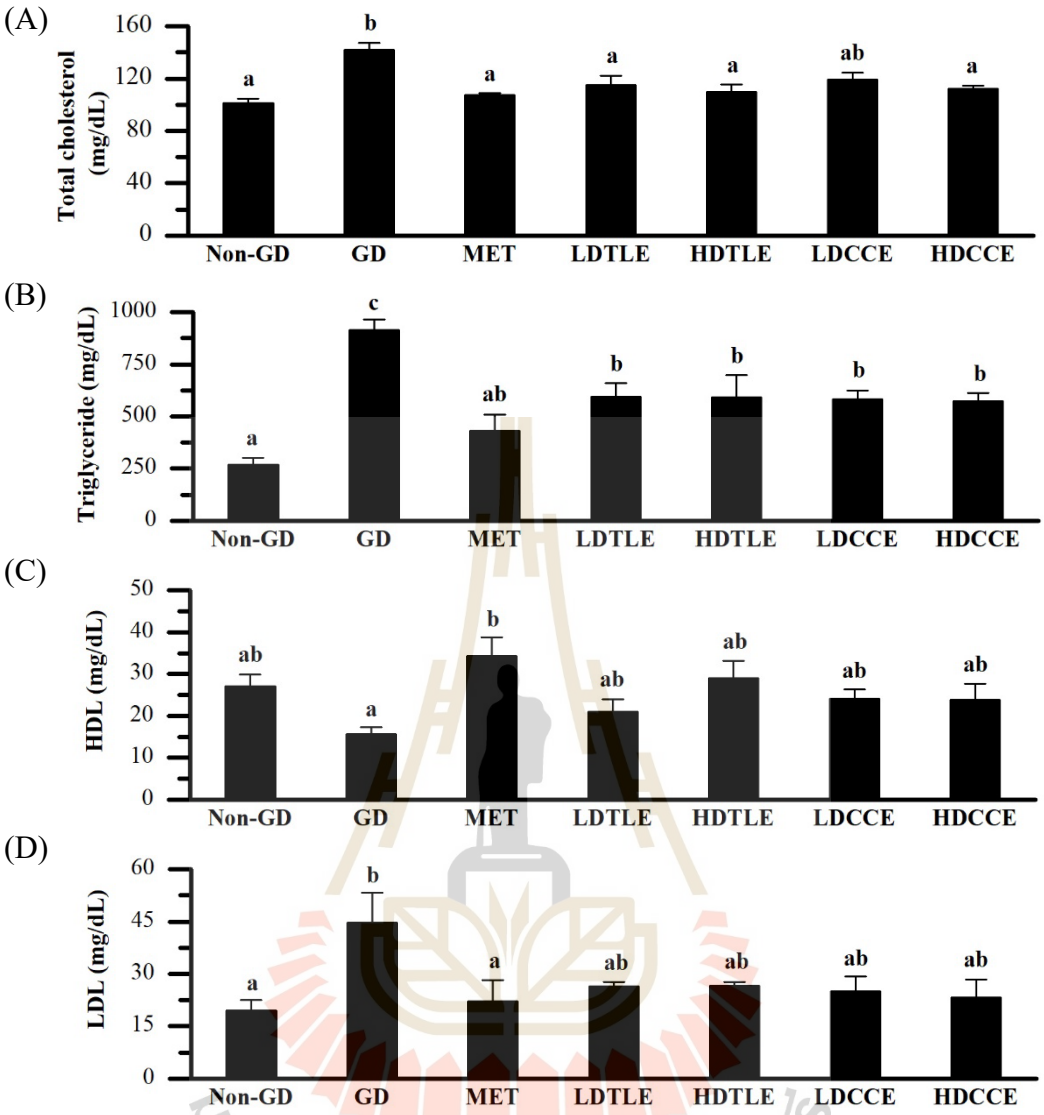

Figure 6.1 Effects of anti-diabetic plant extracts (TLE and CCE) and metformin on lipid profiles, serum TC level (A), serum TRI level (B), serum HDL level (C), serum LDL level (D) in experimental pregnant rats. Data express as mean ± S.E.M (n = 5 per group). Data were analyzed by one-way ANOVA, followed by Turkey's *post hoc* test. Groups bearing the different superscripted letters on the bar indicate statistical significance between the groups ($P < 0.05$). Non-GD = non-gestational diabetic control; GD = gestational diabetic control; MET = 100 mg/kg BW/day metformin treated; LDTLE = 50 mg/kg BW/day TLE treated; HDTLE = 500 mg/kg BW/day TLE treated; LDCCE = 50 mg/kg BW/day CCE treated; HDCCE = 500 mg/kg BW/day CCE treated.

6.4.3 Effects of TLE and CCE on serum liver enzymes

At the end of the experiments, serum levels of hepatic transaminases activity (AST, ALT, and ALP) were measured. Serum AST level was significantly increased in GD (103.40 ± 9.15 U/L), compared with non-GD (63.60 ± 4.03 U/L, $P < 0.05$). Administration of MET, TLE, and CCE to gestational diabetic rats resulted in the reduction in serum level of AST (MET: 71.60 ± 6.76 U/L, LDTLE: 89.00 ± 11.11 U/L, HDTLE: 83.20 ± 5.74 U/L, LDCCE: 73.80 ± 6.09 U/L and HDCCE: 72.40 ± 3.41 U/L). The higher reduction was detected in MET treated group and high dosage of both TLE and CCE treated, as shown in Figure 6.2(A).

Additionally, serum ALT level was rose to 66.40 ± 6.87 U/L in GD which was significantly different from non-GD (28.40 ± 1.17 U/L, $P < 0.05$). In gestational diabetic rats treated with MET, TLE and CCE were also decreased the serum ALT level (MET: 40.60 ± 3.88 U/L, LDTLE: 46.80 ± 7.23 U/L, HDTLE: 41.00 ± 4.06 U/L, LDCCE: 49.20 ± 7.19 U/L and HDCCE: 42.60 ± 4.43 U/L). As compared with GD, the significant reduction was found in MET and the high dosage of the TLE treated group ($P < 0.05$) in Figure 6.2(B).

Similarly, serum ALP level was significantly higher in GD (219.80 ± 49.77 U/L), compared to non-GD (69.40 ± 4.52 U/L, $P < 0.05$). MET, TLE, and CCE treatment were also reduced the serum ALP level as MET: 100.60 ± 8.49 U/L, LDTLE: 144.40 ± 19.79 U/L, HDTLE: 118.00 ± 16.56 U/L, LDCCE: 122.00 ± 9.66 U/L, and HDCCE: 120.80 ± 23.40 U/L. A significant reduction was detected in MET treated group, compared with GD ($P < 0.05$) as seen in Figure 6.2(C).

Hepatic transaminases activity of AST, ALT, and ALP were increased in all gestational diabetic rats compared to non-GD. Low and high dosages treatment of both

TLE and CCE have potentially reduced the levels of hepatic transaminases activity, similarly with the effect of standard MET.

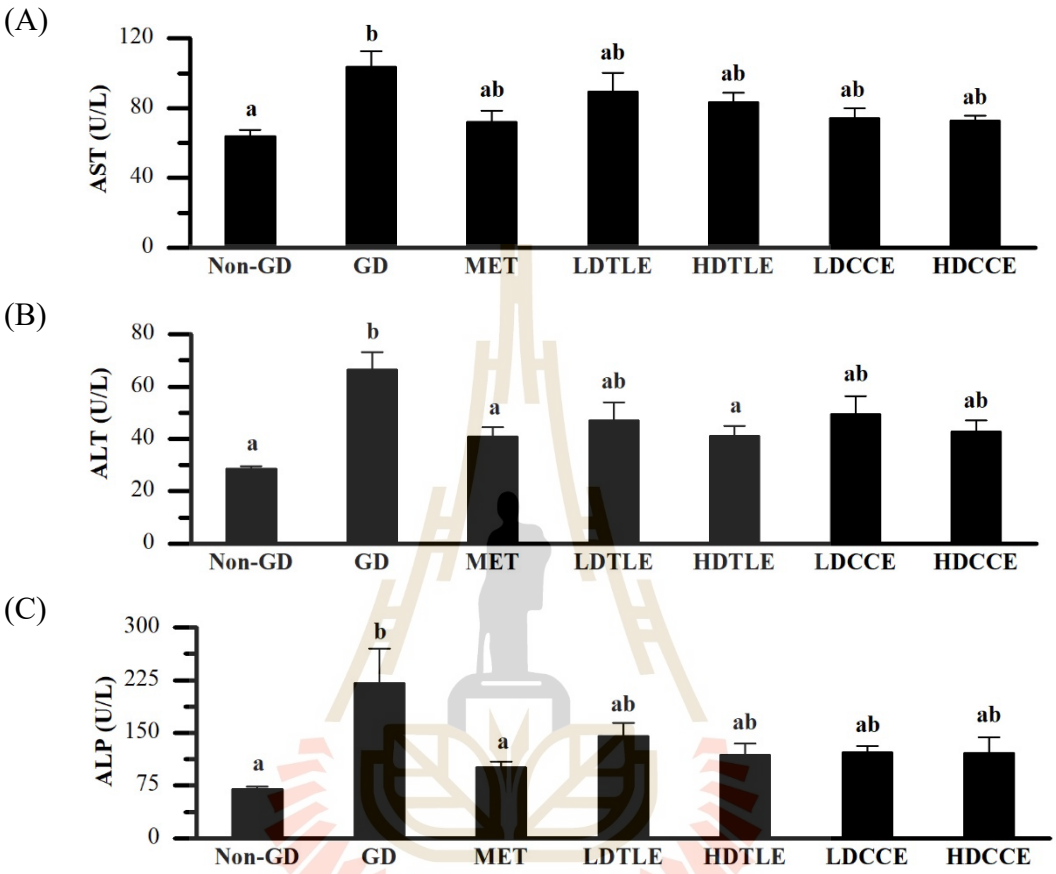

Figure 6.2 Effects of anti-diabetic plant extracts (TLE and CCE) and metformin on serum hepatic transaminases activity, AST (A), ALT (B), and ALP level (C) in experimental pregnant rats. Data express as mean ± S.E.M (n = 5 per group). Data were analyzed by one-way ANOVA, followed by Turkey's *post hoc* test. Groups bearing the different superscripted letters on the bar indicate statistical significance between the groups ($P < 0.05$). Non-GD = non-gestational diabetic control; GD = gestational diabetic control; MET = 100 mg/kg BW/day metformin treated; LDTLE = 50 mg/kg BW/day TLE treated; HDTLE = 500 mg/kg BW/day TLE treated; LDCCE = 50 mg/kg BW/day CCE treated; HDCCE = 500 mg/kg BW/day CCE treated.

6.4.4 Effects of TLE and CCE on maternal reproductive performances and fetal outcomes

Some gestational diabetic rats showed the spotting during mid-gestation while there is no spotting in non-gestational diabetic rats until term. Additionally, all parameters on maternal reproductive performances are presented in Table 6.3.

As compared to non-GD ($P < 0.05$), the GD group showed a significant lowest reduction of gravid uterus weighted (43.43 ± 3.04 g) and a number of live fetuses (8.00 ± 1.00) and a number of implantation (10.40 ± 1.12). Conversely, MET treatment was significantly increased the gravid uterus weighted (54.84 ± 1.16 g), as well as enhanced a number of live fetuses and a number of implantation but no significant difference from GD. The treatment with both dosages of TLE and CCE has been protected the diabetic status closed to the effect of MET, especially in the high dosage treatment such as significantly increased gravid uterus weight (HDTLE: 72.37 ± 1.95 g and HDCCE: 54.35 ± 1.70 g), a number of live fetuses (HDTLE: 13.20 ± 0.73 and HDCCE: 9.80 ± 0.20) and a number of implantation (HDTLE: 14.40 ± 0.40 and HDCCE: 12.00 ± 0.71), compared to GD. A number of corpora lutea were tended to not different among experimental groups, but the higher value in a high dosage of TLE and CCE (HDTLE: 15.00 ± 0.32 and HDCCE: 14.20 ± 0.37). Alternatively, there was no significant difference in a number of dead fetuses and a number of resorptions ($P > 0.05$), but the high value was found in all gestational diabetic rats. Interestingly, the high dosage of TLE and CCE could reduce the number of dead fetuses, but could not reduce the number of resorptions. Based on these results, both pre-implantation loss rates and post-implantation loss rates were tended to higher in GD and no difference among groups. The treatment of MET and high dosage of TLE had a possibly lowered

pre-implantation loss rate and post-implantation loss rate which is not be seen in both CCE treatments in Figure 6.3(A) and 6.3(B).

The fetal weight in GD was significantly reduced (3.27 ± 0.09 g), while placental weight was significantly increased (0.65 ± 0.03 g), compared to non-GD ($P <$ 0.05). As compared to GD, MET treated has no effect on fetal and placental weight, as well as the value in the placental index. In TLE and CCE treatment, fetal weight was increased in the high dosage treatment of both TLE and CCE (HDTLE: 3.49 ± 0.05 g and HDCCE: 3.45 ± 0.08 g), similarly to the reduction in placental weight (HDTLE: 0.57 ± 0.01 g and HDCCE: 0.59 ± 0.02 g) with no statistically different from GD. Notably, a significant lowest placental weight was found in the low dosage treatment of CCE (0.51 ± 0.01 g). Consequently, the placental index was significantly higher in all gestational diabetic rats, compared to non-GD ($P < 0.05$). A significant reduction was found in high dosage TLE and low dosage CCE treatment (HDTLE: 16.64 ± 0.56 and LDCCE: 15.81 ± 0.78), compared to GD ($P < 0.05$). Moreover, CRL was significant decreased in GD (3.59 ± 0.03 cm), compared to non-GD ($P < 0.05$). The significant increased CRL was detected in a high dosage of both TLE and CCE treatments (HDTLE: 3.78 ± 0.02 cm and HDCCE: 3.74 ± 0.04 cm) with the same effect of MET (3.73 ± 0.03 cm), compared to GD ($P < 0.05$).

For fetal classification, all gestational diabetic rats also had higher SPA and lower APA while LPA is absented in this study. Treatment with MET may not affect the fetuses classified but TLE and CCE treatment may enhance the proportion of fetuses classified by increased the APA and decreased the SPA rates with dose dependent manner, especially in high dosage treatment, as presented in Figure 6.3(C).

Table 6.5 Maternal reproductive performances of experimental pregnant rats at term pregnancy.

	Treatment groups (dose, mg/kg BW)	Gravid uterus (g)	No. of corpora lutea	No. of implantation	No. of live fetuses	No. of dead fetuses
Non-GD	Non-gestational diabetic rats	73.54 ± 0.54^{c}	12.80 ± 0.49^{a}	11.80 ± 0.49^{ab}	11.40 ± 0.40^{bc}	0.00 ± 0.00
GD	Gestational diabetic rats	43.43 ± 3.04^{a}	13.00 ± 0.55^{ab}	10.40 ± 1.12^{a}	8.00 ± 1.00^{a}	0.20 ± 0.20
MET	Metformin (100)	54.84 ± 1.16^{b}	12.80 ± 0.58^{a}	12.20 ± 0.49^{ab}	10.00 ± 0.32^{ab}	0.20 ± 0.20
LDTLE	*T. laurifolia* extract (50)	48.57 ± 1.72^{ab}	13.60 ± 0.68^{ab}	10.60 ± 0.81^{a}	9.20 ± 0.58^{ab}	0.40 ± 0.24
HDTLE	*T. laurifolia* extract (500)	72.37 ± 1.95^{c}	15.00 ± 0.32^{b}	14.40 ± 0.40^{b}	13.20 ± 0.73^{c}	0.00 ± 0.00
LDCCE	*C. cinereum* extract (50)	48.86 ± 1.36^{ab}	12.60 ± 0.24^{a}	11.00 ± 0.84^{a}	9.20 ± 0.58^{ab}	0.20 ± 0.20
HDCCE	*C. cinereum* extract (500)	54.35 ± 1.70^{b}	14.20 ± 0.37^{ab}	12.00 ± 0.71^{ab}	9.80 ± 0.20^{ab}	0.00 ± 0.00

Data are expressed in mean ± S.E.M. (n = 5 per group) and analyzed by one-way ANOVA, followed by Turkey's *post hoc* test. Means with different superscripted letters on the same column indicate statistical significance ($P < 0.05$).

Table 6.5 Maternal reproductive performances of experimental pregnant rats at term pregnancy (continued).

	Treatment groups (dose, mg/kg BW)	No. of resorptions	Fetal weight (g)	Placental weight (g)	Placental index	CRL (cm)
Non-GD	Non-gestational diabetic rats	0.40 ± 0.40	4.73 ± 0.08^{c}	0.56 ± 0.01^{ab}	12.10 ± 0.35^{a}	3.96 ± 0.03^{d}
GD	Gestational diabetic rats	2.40 ± 1.03	3.27 ± 0.09^{ab}	0.65 ± 0.03^{c}	20.37 ± 1.25^{de}	3.59 ± 0.03^{ab}
MET	Metformin (100)	1.67 ± 0.32	3.34 ± 0.06^{ab}	0.62 ± 0.02^{bc}	19.03 ± 0.82^{cde}	3.73 ± 0.03^{c}
LDTLE	*T. laurifolia* extract (50)	1.00 ± 0.63	3.07 ± 0.06^{a}	0.63 ± 0.02^{bc}	20.88 ± 0.78^{e}	3.50 ± 0.02^{a}
HDTLE	*T. laurifolia* extract (500)	1.20 ± 0.49	3.49 ± 0.05^{b}	0.57 ± 0.01^{abc}	16.64 ± 0.56^{bc}	3.78 ± 0.02^{c}
LDCCE	*C. cinereum* extract (50)	1.60 ± 0.40	3.36 ± 0.08^{ab}	0.51 ± 0.01^{a}	15.81 ± 0.78^{b}	3.68 ± 0.03^{bc}
HDCCE	*C. cinereum* extract (500)	2.20 ± 0.58	3.45 ± 0.08^{b}	0.59 ± 0.02^{abc}	17.59 ± 0.74^{bcd}	3.74 ± 0.04^{c}

Data are expressed in mean ± S.E.M. (n = 5 per group) and analyzed by one-way ANOVA, followed by Turkey's *post hoc* test. Means with different superscripted letters on the same column indicate statistical significance ($P < 0.05$).

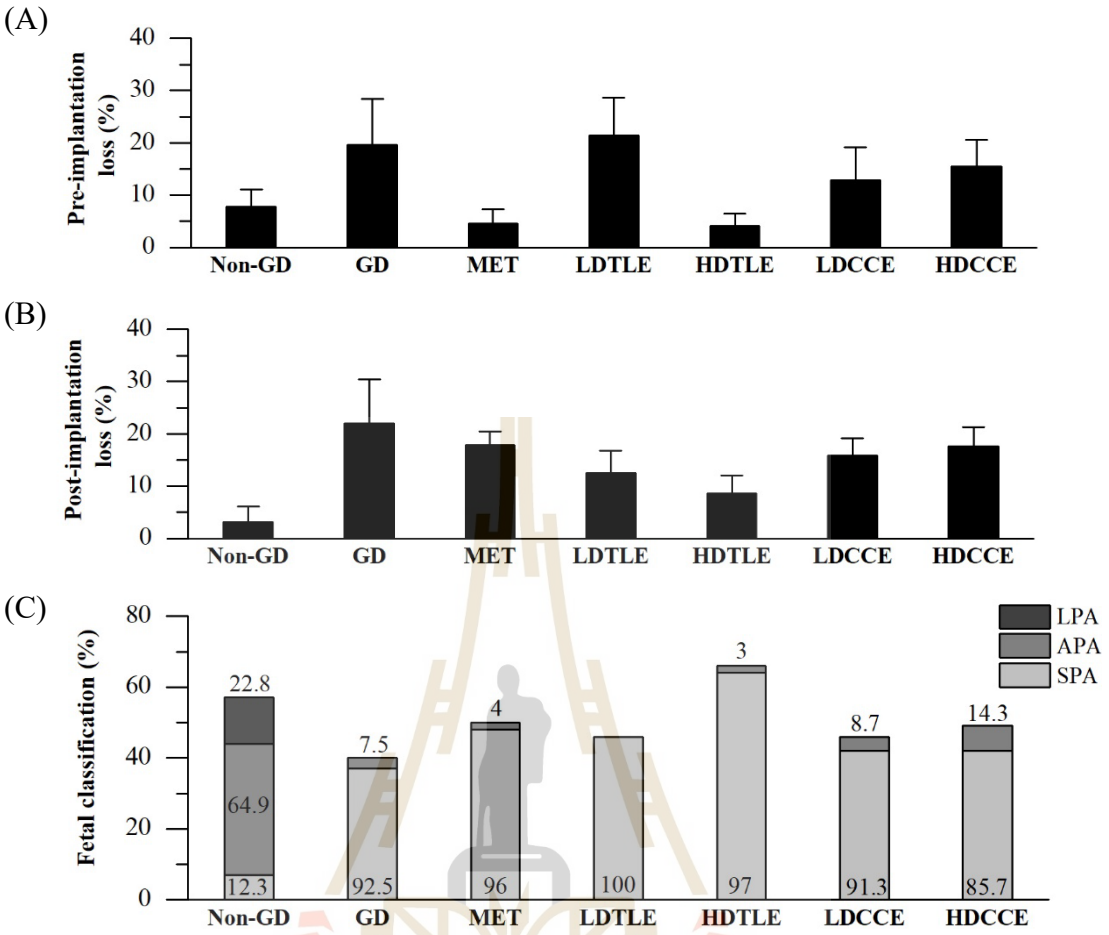

Figure 6.3 Effects of anti-diabetic plant extracts (TLE and CCE) and metformin on maternal reproductive performances, pre-implantation loss rate (A), post-implantation loss rate (B) and fetal classification (C) in experimental pregnant rats. Data express as mean ± S.E.M and percent (n = 5 per group). Data were analyzed by one-way ANOVA, followed by Turkey's *post hoc* test. No statistical significance in pre-implantation loss rates and post-implantation loss rate between experimental groups ($P > 0.05$). Percentage of fetal classified as small (SPA), appropriate (APA) and large (LPA) for the pregnancy age. Non-GD = non-gestational diabetic control; GD = gestational diabetic control; MET = 100 mg/kg BW/day metformin treated; LDTLE = 50 mg/kg BW/day TLE treated; HDTLE = 500 mg/kg BW/day TLE treated; LDCCE = 50 mg/kg BW/day CCE treated; HDCCE = 500 mg/kg BW/day CCE treated.

6.5 Discussion

Several physiologic alterations in a period of pregnancies can be affected by the glucose homeostasis resulting in insulin resistance, hyperinsulinemia, mild post-prandial hyperglycemia which is mediated by the enhanced pancreatic β-cells function and the alteration of the counter-regulatory hormones from placental secretions. The maternal homeostasis alterations are commonly found in the diabetic pregnant state with developed to gestational diabetes mellitus (Damasceno et al., 2002a). Hence, many medicinal plants will be tested for their anti-diabetic action to solved diabetic problems. Therefore, this experiment attempted to demonstrate the hypoglycemic activity, lipid alteration, and maternal reproductive outcome related to the major components of TLE and CCE, compared with a standard anti-diabetic agent (MET) in the diabetic pregnant rat model.

The hyperglycemia on mid-gestation after STZ injection and the hyperlipidemia on late gestation in all gestational diabetic rats in this study were indicated the model of the diabetogenic state in pregnant rats. Abdel-Reheim et al. (2014) and López-Soldado and Herrera (2003) have been revealed that STZ administration during pregnancy in animals produced severe diabetes with the glucose levels above 400 mg/dL, altered lipid metabolism, insulin action, and fetal alterations which the degree of diabetogenic response is due to the dose-dependent response to STZ administration. Some studies on STZ-induced diabetic pregnant rats have been reported the average TC level of 150-250 mg/dL, TRI level of 225-1100 mg/dL, HDL level of 15-50 mg/dL, and LDL level of 120-170 mg/dL, as shown in Afiune et al. (2017) and Braga et al. (2013). The diabetogenic state in pregnant rats in this study is not different from the previous research findings. The administration of both TLE and CCE has been

significant potent hypoglycemic effects in gestational diabetic rats on the late gestation, especially in both high dosages of plant treatment (500 mg/Kg BW/day, $P < 0.05$) whist did not alter on early or mid-gestation. Additionally, TLE and CCE treatment were also potent hypolipidemic effects by significantly decreased TC and TRI with elevated HDL and reduced LDL level, particularly in both high dosages of plant treatment at term ($P < 0.05$). These reductions were similar to the hypoglycemic effect from standard MET treatment which may due to the reduction in hepatic gluconeogenesis and increases peripheral glucose uptake (Scarpello and Howlett, 2008).

Additionally, hyperglycemia is the main sign of DM caused by the defectively coordinated function of muscle, adipose, and liver tissues in insulin-resistant conditions (Kawahito et al., 2009). The major cellular mechanism for glucose transport is insulin-stimulated PI3K and AMPK pathway to suppressing glucose production in the liver and increasing glucose uptake in muscle and adipocytes (Huang and Czech, 2007). For more details, Aritajat et al. (2004) explained that the hypoglycemic activity was found in diabetic rats after *T. laurifolia* leaf extracts administration and its leaf may contain insulin-like substances, as well as enhances insulin-sensitizing hormone by increasing lipid storage in adipocytes (Rocejanasaroj et al., 2014). In the same way, some researches with *C. cinereum* extract treatment also shown the significant reduction of blood glucose level in diabetic animal (Choudhary et al., 2013; Haque et al., 2013) which due to increased PI3K/Akt activity for glucose transport and activated AMPK pathway for inhibition of glucose production in the liver, skeletal muscle, and adipose tissue for enhancing insulin sensitivity (Naowaboot et al., 2018). Results from our GC-MS and LC-MS study found that the pyran, benzoic acid derivatives, and rosmarinic acid were detected in the TLE, as well as terpenoids and phytosterols were detected in

this CCE. To illustrate their bioactive constituents of these compounds, pyran derivatives have been shown their possible anti-hyperglycemic activity and anti-dyslipidemic activity by suppressing the key hepatic gluconeogenic enzymes; glucose-6-phosphatase, glycogen phosphorylase, and α-glucosidase inhibition in STZ-induced diabetic rats (Kumar et al., 2009). Benzoic acid derivatives reduced lipid peroxidation in STZ-induced diabetic rats (Gayathri and Kannabiran, 2012). Rosmarinic acid administration can reverse the STZ and HFD induced decrease in GLUT4 expression in skeletal muscle as it can ameliorate hyperglycemia and insulin sensitivity (Runtuwene et al., 2016) by a greater α-glucosidase inhibitory activity (Zhu et al., 2014). Moreover, terpenoids detection in CCE, lupeol possess increased plasma insulin level and decreased the glycated hemoglobin (HbA1C) levels, corresponding with the β-cell protein damages prevention, pancreatic regeneration, and the minimization of the free radicals production in STZ-induced diabetes rats (Gupta et al., 2012). The oral efficacy of β-amyrin might be reduced glucose absorption from the gut, glucose produced inhibition in hepatic tissue, and increased glucose uptake by muscle and adipose tissue with manifested a hypolipidemic effect in STZ-induced diabetic mice (Santos et al., 2012). The management of diabetes by lupenone and β-sitosterol administration caused a significant reduction in FPG and HbA1C levels in diabetic rats (Xu et al., 2014). Additionally, dietary phytosterols (campesterol and β-sitosterol) are correlated with TC and LDL cholesterol reduction with acts as a cholesterol-lowering agent (Jones et al., 1997).

There were slightly increases in maternal body weight gain and food intake after TLE and CCE treatment at term in this study, this suggested there were no signs of maternal toxicity and safety used to management of diabetic pregnancy. Aruna et al.

(2012), Chivapat et al. (2009), and Choudhary et al. (2013) explored the safety evaluation of *T. laurifolia* and *C. cinereum* extract with no alteration in body weight, food consumption, behavior, and general health of experimental animals.

Besides, Liberati et al. (2004) suggested the reference value of AST and ALT and ALP activities in pregnant rats as 41-149 U/L, 31-86 U/L, and 46-127 U/L, respectively, compared to nonmated rats. Shekhar and Diddi (2015) explained that serum AST and ALT remain unchanged during pregnancy while ALP may be elevated to 300% and their ranges are changed due to the illness. Chatila and West (1996) elucidated the increases in the intracellular distribution of liver glycogen in response to glycogen synthesis with a mild to moderate increase in aminotransferases activities caused by the association of hyperglycemia and liver injury in diabetic patients. The medical records in Erdoğan et al. (2014) have been suggested that ALP was significantly higher, same as Yarrington et al. (2016) found the 4-fold increased ALT level in GDM patients. Zafar et al. (2009) reported the AST and ALT levels were significantly rose which may be due to the cellular damage in the liver caused by STZ-induced diabetic rats. Coupled with the results in this study on the liver enzymes, serum AST, ALT, and ALP levels in all gestational diabetic rats were significantly increased due to the STZ-induced hepatotoxicity state in rats' livers, compared to non-gestational diabetic rats. Besides, TLE and CCE treatment have been shown a significant reduction in all serum liver enzymes. The higher reduction was found in both high dosages of plant treatment that is probably close to the effect of MET. Chan and Lim (2006), Oonsivilai et al. (2008), and Pramyothin et al. (2005) suggested that *T. laurifolia* leaves extract contained the high anti-oxidant activities and phenolic contents with a significant reduction of AST and ALT levels in liver injury as its possess the

hepatoprotective activity. Many previous evidences supported a strong anti-oxidant potential against a different type of free radicals and prevent the overproduction of reactive species in the cell injury from the TLE's phytoconstituents as benzoic acid derivatives (Gayathri and Kannabiran, 2012; Velika and Kron, 2012) and rosmarinic acid (Nicolai et al., 2016; Zhu et al., 2014). Despite the use of *C. cinereum*, the ethanolic extract did not produce any toxicity in the hepatic parameters as AST and ALT levels are not changed in the chronic toxicity study (Aruna et al., 2012). Moreover, Abubakker (2018) found the significantly increased activities of anti-oxidants including superoxide dismutase (SOD), catalase (CAT), peroxidise, and glutathione S-transferase (GST) in the methanolic extract, whereas the significant higher activities of glutathione peroxidase (GPx) in the ethanolic extract which higher rich of vitamin A, C, E, and total phenols. For CCE's terpenoids, its antioxidant capabilities and cytotoxicity potentials have been possessed free-radical scavenging activity by lupeol fraction (Santiago and Mayor, 2014; Sunitha et al., 2001), strong suppressive effect on lipid peroxidation by β-amyrin (Sunil et al., 2014), cytotoxic activities by β-amyrin acetate fraction (Fabiyi et al., 2012) and reduced the reactive oxygen species (ROS) production by lupenone (Xu et al., 2018). Similarly, CCE's phytosterols, the radical-scavenging antioxidants of β-sitosterol, and campesterol are accelerated the lipid peroxidation as reported by Yoshida and Niki (2003) and Yeap et al. (2016).

To summarizes, these relevant literature experiments were also supported the hypoglycemic and hypolipidemic properties of several types of bioactive constituents in TLE and CCE with did not provoke maternal toxicity in the gestational animal model. Additionally, the beneficial effect of TLE and CCE administrations showed strong anti-oxidant potential as the reduction of lipid peroxidation, confirming the existence of

hepatoprotective effect against diabetes-induced oxidative stress in gestational diabetic rats.

Further investigation was accessed the maternal reproductive performances related to diabetic condition compared among MET, TLE, and CCE treatment. Probably, the adverse pregnancy outcomes including cesarean delivery, pregnancy-induced hypertension, shoulder dystocia, and fetal macrosomia were also significantly higher in the GDM women (Srichumchit et al., 2015). The ability of diabetic rats to maintain pregnancy has been reported by Lawrence and Contopoulos (1960). In details, ovaries from the diabetic rats contained no mature follicles with only small to small-medium sized follicles were present, and uterine weighed is less than non-diabetic animals with the same age. The dioestrus prolongation and a bloody discharge were observed in diabetic animals on the 8^{th} day of gestation, indicating the early resorption while fetal death and resorption occurred about the 10^{th} day of gestation. Correspondingly, as compared to non-gestational diabetic rats, the present study found the highest number of resorptions, pre- and post-implantation loss rates leading to the significant decreased gravid uterus weight and a number of live fetuses and increased number of dead fetuses in untreated gestational diabetic rats ($P < 0.05$) as referenced to Eriksson et al. (2003) and Saito et al. (2010), who suggested the intrauterine death and abortion are presented in uncontrolled diabetic women and animals. Embryogenesis is constricted caused by hyperglycemia (Fraser et al., 2007).

There was no alteration in the number of corpora lutea along with a notable trend to the decreased number of implantation found in untreated gestational diabetic rats which indicated the perinatal growth restriction in association with the progesterone secretion related to the CL and implantation (Papp et al., 2015). The Resorptions and

rate of pre-implantation loss were possible higher in untreated gestational diabetic rats which are defined to the fertilization failure related to the cellular abnormalities and enhanced inflammatory cytokines induced by oxidative stress in severely maternal diabetes (Damasceno et al., 2011; Moley et al., 1998). The abortifacient effect as vaginal bleeding is found during the mid-gestation in untreated gestational diabetic rats coupled with the higher post-implantation loss rate, consequently, embryonic development was delayed and decreased survival vitality (Bequer et al., 2018).

As compared to untreated gestational diabetic rats, The MET was significant increased gravid uterus weight ($P < 0.05$) and tended to a slightly increased number of implantation and number of live fetuses while decreased number of resorptions but not reaching significance, and no altered on the number of corpora lutea and a number of dead fetuses. A tend to reduce the pre-implantation loss and slightly decreased of post-implantation loss was found after MET administration with no significantly different. Metformin exerts the moderation of maternal metabolic dysfunction to improve the outcomes of pregnancy by decreasing cytokine signaling and productions (Desai et al., 2013) and mediated of oxidative stress (Esteghamati et al., 2012).

For TLE treatment, the especially high dosage was significant increased gravid uterus weight, number of corpora lutea, number of implantation, and number of live fetuses ($P < 0.05$) with a decreasing trend in a number of dead fetuses and the ability to prevented pre-implantation loss rates and post-implantation loss rates, not including resorptions. In contrast, gravid uterus weight was significantly increased in CCE treated group (high dosage, $P < 0.05$) whereas an increasing trend in the number of corpora lutea, number of implantation, number of live fetuses, compared to untreated gestational diabetic rats, as well as, a decreasing trend in the number of dead fetuses,

but does not contribute to the protection of the resorptions, pre-implantation loss rates, and post-implantation loss rates. Additionally, the dose-dependent of TLE treatment has a significantly beneficial effect to improve maternal reproductive performances as potent the ability to the prevention of miscarriages interfering with the viability of the fetuses while CCE treatment was tended to only decrease the incidence of fetal death with increased maternal weight gain. These effects from plant extracts may cause by the hypoglycemic and anti-oxidant properties of their phytoconstituents as mentioned previously.

The assessment of fetal outcomes have been exhibited that the placental weight and index were significantly higher in all gestational diabetic rats ($P < 0.05$), indicating the inappropriate growth between the placenta and fetal which increase the incidence of perinatal death (Matsuda et al., 2018), as a result of a significant reduction in fetal weight (compared to non-gestational diabetic rats, $P < 0.05$). The higher placental weight is associated with 5% increasing risk factors for serious neonatal outcomes (Hutcheon et al., 2012). Thus, the placental dysfunction is involved in the intrauterine growth restriction affecting fetal growth and the long-term complication of the offspring (Gaccioli et al., 2013). The severity of infant complications is mentioned in maternal diabetes, particularly LPA and macrosomia (Pantham et al., 2015). In contrast, the SPA fetuses are commonly presented in diabetic rats at term (Giavini et al., 1986), same as the higher proportion of SPA with laking LPA fetuses were found in untreated gestational diabetic rats in this study which demonstrated the placental dysfunction, combination with the fetal growth restriction and earlier congenital abnormality (Baken et al., 2017) as a significant reduction of fetal CRL ($P < 0.05$).

On the other hand, MET was significantly increased fetal CRL, compared to untreated gestational diabetic rats ($P < 0.05$) but not quite significant to slightly increasing fetal weight, reduced placental weight, and index, as well as attenuating the proportion of APA fetuses. Glueck et al. (2001) reported the rate of spontaneous abortion in the first-trimester was reduced from 73% to 10% in metformin therapy without evident fetal teratogenicity and maternal side effects, the growth of the fetus are continuing due to placental ability (reduced placental index) to balance fetal demand until term (Gaccioli et al., 2013).

As compared to untreated gestational diabetic rats, the significantly increased CRL ($P < 0.05$) and a slight tendency to increase fetal weight in both dose-dependent of TLE and CCE treatments. Only a probable trend to decrease placental weight and a significant decreased placental index ($P < 0.05$) were detected in TLE treatment, without deteriorating by CCE treatment with a dose-dependent manner. The proportion of APA fetuses were increased in the dose-dependent of both TLE and CCE treatments. These changes are related to the placental efficiency and function in the existence of maternal-fetal exchange (Gaccioli et al., 2013).

To conclude that TLE and CCE administered to gestational diabetic rats are an attempt to control the hyperglycemia with the difference among its influence on maternal reproductive performances and fetal or placental development. The prevention of miscarriages, intrauterine growth restriction, and stillbirth was found in the high dosage of TLE treatment, the same as promoting the development of fetuses. On the contrary, the high dosage of CCE treatment was only prohibited stillbirth and encouraged fetal development.

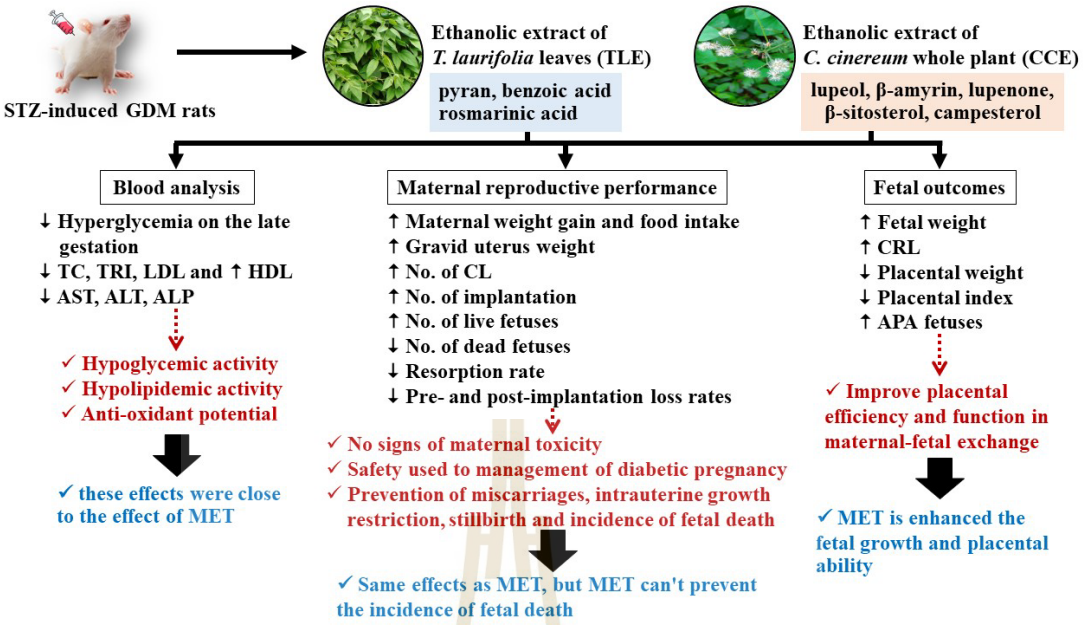

Figure 6.4 The diagram shows the effects of TLE and CCE on maternal blood analysis, reproductive performances, and fetal outcomes in gestational diabetic rats.

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CHAPTER VII

IMPACTS OF SELECTED TRADITIONAL ANTI-DIABETIC PLANTS ON ULTRASTRUCTURAL STUDY IN PREGNANT DIABETIC RATS

7.1 Abstract

Diabetes in pregnancy was the independent risk factor for cesarean section, accompany with macrosomia, fetal distress syndrome, and poor myometrial function. Perinatal death or stillbirth is highly found in diabetic mothers. The developing pregnant complications are associated with an increased risk of GDM development. Oxidative stress in a hyperglycemic state leading to the structural tissues damaged and dysfunction with serious metabolic complications progression. Traditional plant medicines have been shown the potential roles in the management of diabetes with its ability to the restoration of diabetes-induced damaged tissue architecture, specifically on the morphology of pancreatic β-cells and enhanced insulin secretion. Despite the fact on the anti-hyperglycemic activity of *Thunbergia laurifolia* and *Cyanthillium cinereum*, this experiment was to demonstrate the possible protective effect of ethanolic *T. laurifolia* leaves (TLE) and *C. cinereum* whole plant (CCE) extracts on histological examination of the liver, pancreas, uterus and molecular structural changes of the uterus in STZ-induced diabetic pregnant rats. Seven equal groups of 35 pregnant rats were randomized into group 1 served as non-gestational diabetic rats (Non-GD), group 2

comprised STZ-induced gestational diabetic rats (GD), group 3 was gestational diabetic animals treated with metformin (MET), group 4 and 5 were gestational diabetic animals treated with low dosage TLE (LDTLE) and high dosage TLE (HDTLE), group 6 and 7 were gestational diabetic animals treated with low dosage CCE (LDCCE) and high dosage CCE (HDCCE). All treatments were given once daily by gavage after STZ injection until term. Pregnant rats were sacrificed on day 21st of pregnancy. Sections of liver, pancreatic and, uterine samples were taken for light microscopic examination and spectrophotometric studies. Histological analysis of liver tissue obtained from STZ-induced gestational diabetic rats has been developed lipid accumulation, inflammatory cell infiltration, degenerative hepatocytes, and dilated sinusoids. The pancreatic islets were damaged and reduced in size with marked loss of granules. The degenerative changes in the uterus were found as a thin endometrium and loose irregular arrangement of both longitudinal and circular myometrial layers with increased interstitial space, as compared to non-GD. Attractively, the restoration of liver morphology, the protective architecture of pancreatic islets and, the well-reorganization of uterine smooth muscle returned to normal after treated with both TLE and CCE extracts, notably with a high dosage treatment, compared to MET. Ultrastructure study of the gestational diabetic uterus had an increase in the lipid contents, a decreased in protein contents, and an alteration in all phospholipids, nucleic acids, glycogen and other carbohydrates contents with a trend to increase ester functional groups and no statistically changed in amide III collagen vibrations. Both plant extracts regenerated these uterine structural damages in cellular morphology by correlating to the IR spectral evaluation. In conclusion, *T. laurifolia* and *C. cinereum* have a protective activity on damaged tissue against STZ-induced gestational diabetic rats in a dosage-dependent manner. Spectral findings on

uterine tissue have been identified as the essential alteration at the molecular levels which may enhance its traditional use in various physiological functions in diabetic pregnancy.

7.2 Introduction

The prevalence of adverse perinatal outcomes including morbidity, mortality, live births with a congenital anomaly in pregestational diabetes has been increasing 50% in the past 10 years (Bell et al., 2008). Dunne et al. (2003) suggested that infants are at a higher risk for stillbirth (2-fold), perinatal mortality (2.5-fold), an early death (3.5-fold), 1-year prolong mortality (6-fold), and congenital malformation (11-fold) in diabetic mothers' population. Besides, the fetal distress, macrosomia, and obesity contributed to the increased cesarean birth rates in gestational diabetes, around 51% of emergency cesarean section arise from prolonged labor and failed induction or obstructed labor (Evers et al., 2004). The excessive bleeding with a cesarean birth influenced the oxygen saturation and oxidative stress (Torres-Cuevas et al., 2017), particularly the direct altered Ca^{2+} mobilization in smooth muscle cells (Inazu et al., 1991). Prolonged elevated plasma glucose level leads to toxicity in the cells known as glucose toxicity, the highly susceptible to excess glucose enters into the cells (hyperglycemic damage) due to equilibrate between intracellular and extracellular glucose environment (Campos, 2012). Hyperglycemia-induced overproduction of oxidative stress contributes to the metabolic disturbances' characteristic and dysfunction in various organs such as vessels (Sena et al., 2018), kidney (Daenen et al., 2019), heart (Senoner and Dichtl, 2019), liver (Zhu et al., 2012), pancreatic β-cells (Wang and Wang, 2017), non-pregnant and pregnant uterus (Silveira et al., 2018).

Many life-threatening diseases of macro- and microvascular complications are associated with long term untreated diabetes (Chawla et al., 2016).

A diabetogenic agent, streptozotocin (STZ) has been widely used in various laboratory animals, resulting in ultrastructural damage of pancreatic β-cells necrosis (Aughsteen, 2000). The abnormal rate of protein and fat metabolism are caused by fatty acids accumulated in the liver after STZ administration (Morral et al., 2007). The lipotoxicity is leading to cell dysfunction or cell death and a greater developing risk of metabolic diseases, especially insulin resistance (Herpen and Schrauwen-Hinderling, 2008). Marked increase lipid droplets, myofibrillar degeneration, atherosclerotic plaque formation, and degenerative changes of coronary vasculature were apparent found in STZ-induced diabetic myocardium (Seager et al., 1984). Various reproductive systems and performances are impacted by STZ-induced diabetes in both male and female organs (Omolaoye et al., 2018; Valdes et al., 1990). Chronic hyperglycemia during gestation affected the uterine environment and maternal-fetal exchange that causing multiple disruptions in dramatic changes in smooth muscle function and vaginal structure (N. N. Kim et al., 2006), abnormal embryos development at the earliest gestational stages (Diamond et al., 1989) and altered the reproductive functions of adult offspring (Spadotto et al., 2012). Higher cholesterol content is critically inhibited the uterine force generation, contributing to the difficulties in labor (Smith et al., 2005).

Moreover, ultrastructure alterations in rat myometrium such as cell differentiation are related to their contractile responsiveness to essential reproductive hormones (S. M. Kim et al., 2006; McMurtrie et al., 1985). Histological studies of Tariq et al. (2015) found many lipid-containing dense-cored granules in the epithelial cells of the endometrium, deranged myocytes and fewer myofibrils in the myometrium of

diabetic rat uteri. Favaro et al. (2010) suggested that uterine atrophy and myofibril alteration were correlated with impaired uterine functions in diabetic pregnancy.

Consistent with the previous studies, the protective effect against tissues damage related to the normal structural integrity were found in the STZ-induced diabetic rats fed with various alternative herbal plants such as fenugreek (Abou El-Soud et al., 2007), *Aloe Vera* (Noor et al., 2008), peppermint (Abdellatief et al., 2017) or black tea (Manikandan et al., 2009). *Thunbergia laurifolia* (L.) and *Cyanthillium cinereum* (L.) H. Rob. (Synonym: *Vernonia cinerea* L.) extract exhibited numerous biological activities, especially the anti-hyperglycemic effect (Aritajat et al., 2004; Haque et al., 2013), anti-oxidant activity (Abubakker, 2018; Palipoch et al., 2013), anti-hyperlipidemic agents (Naowaboot et al., 2018; Sompong et al., 2016), and anti-inflammatory effects (Junsi and Siripongvutikorn, 2016; Latha et al., 1998) in several degenerative toxicity diseases.

As shown in previous chapters, *T. laurifolia* and *C. cinereum* extract exhibited the tocolytic activity in the uterine contraction together with hypoglycemic, hypolipidemic, and lower hepatic transaminase activities. Thus, the effectiveness of *T. laurifolia* and *C. cinereum* extract on histopathological investigations of liver, pancreas, and uterus induced by STZ in gestation rats were carried on and its impact on uterine ultrastructural changes in Fourier transform infrared (FTIR) spectroscopy. The histopathology was used to evaluate the microscopic examination of sampled whole tissues to the clinical manifestation of disease and then FTIR spectroscopy was used to determine the structural determination in the coordinates of molecule components in the adaptation of the biological samples (Gurcan et al., 2009; Petibois et al., 2009).

7.3 Materials and methods

7.3.1 Experimental animals

Fertile female rats were mated and prepared vaginal smears for conception (see details in 2.2.4). Intraperitoneal injection of 60 mg/kg BW STZ was used to induce diabetes during pregnancy (see details in 2.2.5). Experimental pregnant rats were described in 5.3.2. In detail, the control group was non-gestational diabetic rats who were given a vehicle (Non-GD). The diabetic control group was gestational diabetic rats who were given a vehicle (GD). Alternative five groups of gestational diabetic rats were administered with the anti-diabetic drug and anti-diabetic plants including metformin treatment at a dose of 100 mg/kg (MET), TLE treatment at a dose of 50 and 500 mg/kg (low dose: LDTLE and high dose: HDTLE, respectively) and CCE treatment at a dose of 50 and 500 mg/kg (low dose: LDCCE and high dose: HDCCE, respectively). All treatments were continued for 15 gestation days (day 7^{th}-21^{st} of gestation).

7.3.2 Histological studies on the liver, pancreatic, and uterine tissues

After sacrificed with CO_2 asphyxia on the 21^{st} day of gestation, a sample of the maternal liver, pancreas, and uterine horn was immediately fixed in 10% neutral formalin followed by dehydration in ascending grades of alcohol and embedding in hard paraffin for hematoxylin and eosin (H&E) processes as described in (El-Kordy and Alshahrani, 2015; Thanamool et al., 2013). In detail, sections of 5 µm thickness were cut by the microtome, mounted on clear clean slides, and allowed to dry overnight. Then, sections were warmed (15ºC) for 1 hour and deparaffinized in four changes in xylene, 10 min each, followed by hydrated 1 min through a descending series of ethanol, each of 100%, 95%, 75%, and 50% respectively, and then held in distilled

water. Next, staining protocol was performed using hematoxylin for 4 min, rinsed in distilled water, decolorized with lithium carbonate solution, and rinsed with distilled water again following a 1 min wash in 70% ethanol. Subsequently, slides were counterstained with eosin for 1 min and then dehydrated. Coverslips were mounted with a pre-mount for nucleus and cytoplasm investigation by a light microscope at 4x and 20x magnifications. The quantification of pancreatic islets' H&E staining area was measured by circling islets' area in square micrometer unit (μm^2), as well as, the relative proportion of interstitial space and muscle fiber from total uterine H&E staining area was analyzed by a selected color threshold, using ImageJ software, version 1.52 (Komura and Ishikawa, 2018).

7.3.3 Spectrophotometric studies on uterine tissue

Freezing uterine horn samples were embedded in optimal cutting temperature compound (OCT compound) for the processing of cryostat sectioning at -25ºC. Then, optimal thickness sections of 5 μm were transferred to the BaF_2 optical window for the application of infrared spectroscopy. Spectra were acquired with a Vertex 70 FTIR spectrometer (Bruker Optics, Ettlingen, Germany) connected with an IR microscope (Hyperion 2000, Bruker) with a mercury-cadmium-telluride (MCT) detector (over 4000 to 800 cm^{-1} measurement range) cooled with liquid nitrogen. The microscope stage was placed in a specially designed box with dry air purged. The 36x objective of transmission mode, aperture size (10 × 10 μm^2), and spectral resolution (6 cm^{-1}) with 64 scans co-added were corrected. Baseline corrections and vector-normalized spectra were used to the minimum point of linear correction and representative averaged spectra of samples, respectively. Average spectra were calculated from individual tissue sections which generated a group average spectrum. Secondary derivative spectra were

created by 9 smoothing points of the Savitsky-Golay algorithm from the group average spectrum in which the overlapping spectra were interpreted. The quantification of band area in the absorbance unit (a.u.) was measured using the integration method corresponding to the center of the height of each band following reference intensities. Spectral acquisition and instrument control were performed using Opus software, version 7.5 (Bruker Optics Inc, Ettlin-Gen, Germany) (Thumanu et al., 2014).

7.3.4 Statistical analysis

All data are reported in the mean ± standard error of the mean (S.E.M.). ANOVA followed by Turkey's *post hoc* test was used to compare the mean values using SPSS Statistics for Windows, version 17.0 (SPSS Inc., Chicago, Illinois, USA). A probability level of less than 5% ($P < 0.05$) was taken to be statistically significant.

7.4 Results

7.4.1 Effects of TLE and CCE on liver histology

The liver of the rat is multilobulated, approximately 5% of mass to the total body weight. The superior surface is fixed in the diaphragm and abdominal wall which divides into the right and left lobes by the falciform ligament. The concave inferior surface is attached to the stomach, duodenum, the superior part of the pancreas, and the right kidney (Martins and Neuhaus, 2007). Under liver architecture, the parenchymal cells are hepatocytes having nuclei and non-parenchymal cells are inflammatory cells (Kupffer cells), fat-storing cells (stellate cells or lipid droplets), and endothelial cells. Round or cuboidal hepatocytes are arranged in cords and radiated from the central vein which separated by sinusoidal space in turn containing stellate cells, long with portal

triad carries small branches of the portal vein, artery, and bile duct to provide the components of liver lobules (Baratta et al., 2009).

The normal hepatic architecture of non-GD has been shown in Figure 7.1(A) with a normal of oval central vein surrounded by the normal shape of hepatocytes with round nuclei and line up with hepatic cords (sinusoids). Some inflammatory cells and lipid droplets were seen. In GD, the degeneration and irregular-shaped of hepatocytes were observed with enlarging and deformities of nuclei. The dilated and scattered sinusoids (increased intercellular space) have been found with inflammatory cell infiltration and increased lipid droplets in Figure 7.1(B). The treatment with both TLE and CCE were alleviated the pathological damage demonstrating the restoration and rearrangement of the size and shape of the hepatic cells with a well distinct cytoplasm and nuclei, compared with GD. The intercellular spaces were decreased, same as inflammatory cell infiltration with dose dependency in Figure 7.1(D), 7.1(E), 7.1(F), and 7.1(G), respectively. These improvements were indicated a restoration of normal liver architecture and greatly observed in the gestational diabetic rats received both high dosage treatment (500 mg/kg BW) which these observations were close to the effect of MET in Figure 7.1(C).

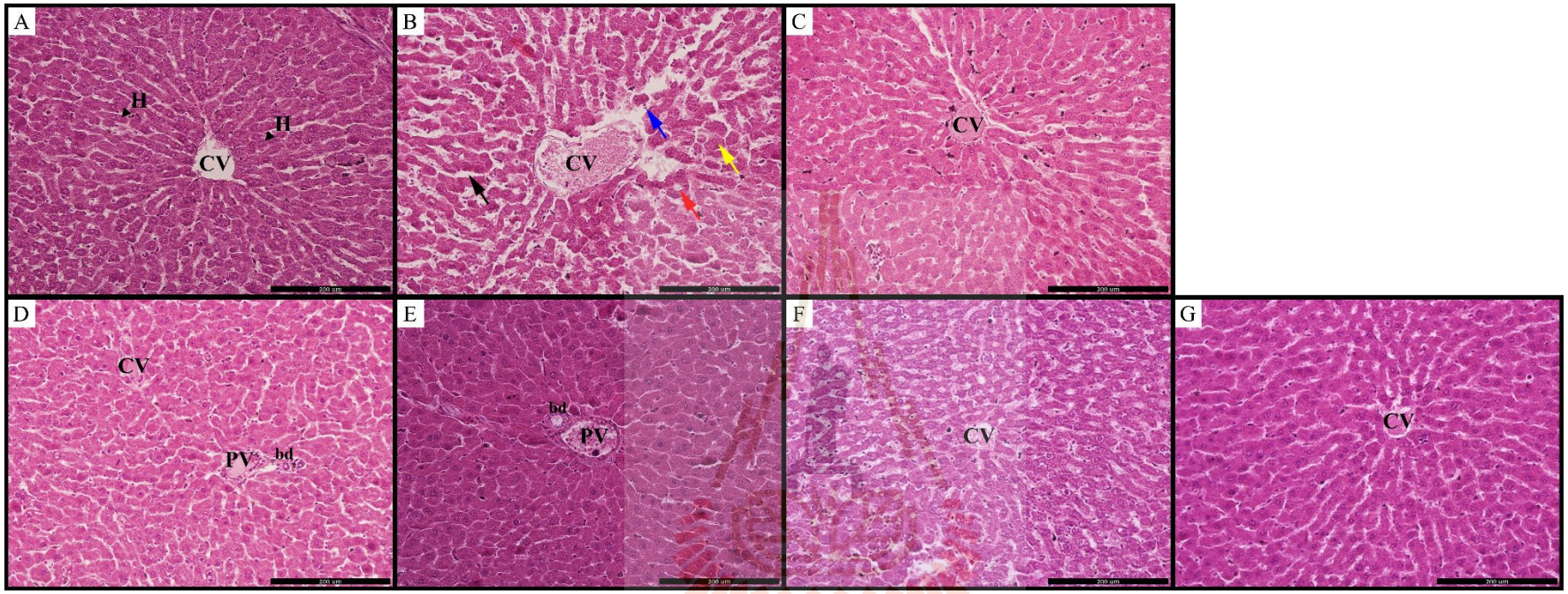

Figure 7.1 Histomorphology of H&E staining of liver sections of non-GD and GD treated by various treatments along the pregnant period. A represents the typical hepatocytes in non-gestational diabetic rat; B represents the increased intercellular space (black arrows) pathological damage in gestational diabetic rat received vehicle control; C represents metformin treatment (100 mg/kg BW/day); D represents low dosage TLE treatment (50 mg/kg BW/day); E represents high dosage TLE treatment (500 mg/kg BW/day); F represents low dosage CCE treatment (50 mg/kg BW/day); G represents high dosage CCE treatment (500 mg/kg BW/day). Sections showing central vein region (CV), portal vein area (PV), hepatocytes (H), bile ducts (bd), enlarged nuclei (yellow arrow), inflammatory infiltration (blue arrow), and lipid droplets (red arrow). The bar represents 200 µm.

7.4.2 Effects of TLE and CCE on pancreatic histology

In the abdominal cavity, the rat pancreas is a large, flat, white to a pink-colored organ embedded in mesenteric fat tissue. The large portion of the pancreas is attached and located under and attached to the spleen. Alternatively, the head portion of the pancreas is attached to the proximal duodenum associated ducts and duodenum papillae. The rat's pancreas is divided into lobules which formed dense accumulations of exocrine glands (acini cells) surrounding close to the islets of Langerhans, the endocrine portion. The large interlobular islet and enlarged acinar surrounding cells can see in humans as well as in pregnant rats. Almost rat's islets are located in interlobular spaces near the interlobular duct with unevenly distributed throughout the pancreas. Human and rodent's islets have consisted of 4 main endocrine cells; beta cells (secrete insulin), alpha cells (secrete glucagon), delta cells (secrete somatostatin), and PP or F cells (secrete pancreatic polypeptide). In rat's islets composed primarily of beta cells predominate which is located central, other cells are located peripheral (Tsuchitani et al., 2016).

The histological images of the pancreas with the islets are demonstrated in Figure 7.2. The representative images of H&E staining of the pancreas showed the large, bright, and normal structure of islets cells, compact and regular shape arrangement of pancreatic cells, and uniformly distribution of intercellular spaces in non-GD in Figure 7.2(A). On the other hand, GD demonstrated a significantly reduced size with degenerative changes of the islets cells, resulting from STZ-induced toxicity in islets' DNA damage. The pancreatic cells showed the varying shape and irregular distribution in Figure 7.2(B). The MET treatment has been shown the minimal pathological changes which define as the islets' regeneration in Figure 7.2(C). The

treatment with TLE and CCE (low and high dosages) were also marked improved islets morphology as seen in Figure 7.2(D), 7.2(E), 7.2(F), and 7.2(G), respectively.

The quantification H&E staining of islets' area in pancreases is summarized in Figure 7.3. The area of islets in GD was significantly reduced to 6056.02 ± 1112.04 μm^2, compared with non-GD (37157.59 ± 6932.83 μm^2, $P < 0.05$). As compared to GD, the islets' area of gestational diabetic rats administrated with MET was significantly highest increased to 24715.95 ± 979.98 μm^2. The administration of both dosages of TLE has also improved the islets' area up to 16039.03 ± 1808.18 μm^2 in LDTLE and 19559.93 ± 3559.89 μm^2 in HDTLE, respectively. Moreover, the treatment with of both dosages of CCE has enhanced the islets' area by increasing to 21407.43 ± 1976.21 μm^2 in LDCCE and 22070.91 ± 3407.64 μm^2 in HDCCE, respectively.

To conclude, the high dosage of both anti-diabetic plant extracts showed the efficacy of pancreatic islets recovery in experimental diabetic pregnant rats during gestation, but the value was lower than the possible effect of MET treatment. Noticeably, the high dosage of CCE is also closed to the MET treatment.

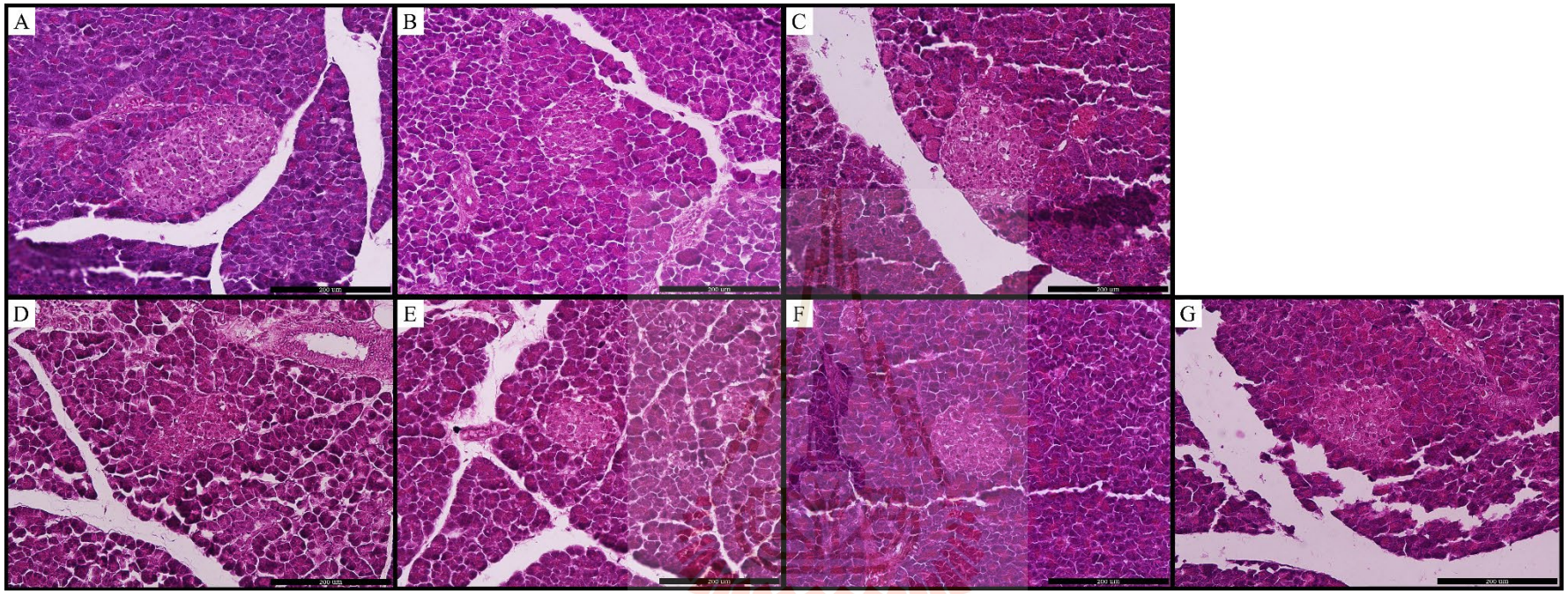

Figure 7.2 Histomorphology of H&E staining of pancreatic islets cells of non-GD and GD treated by various treatments along the pregnant period. A represents the typical islets cells in non-gestational diabetic rat's pancreas; B represents the atrophic pattern of islets cells in gestational diabetic rat received vehicle control; C represents metformin treatment (100 mg/kg BW/day); D represents low dosage TLE treatment (50 mg/kg BW/day); E represents high dosage TLE treatment (500 mg/kg BW/day); F represents low dosage CCE treatment (50 mg/kg BW/day); G represents high dosage CCE treatment (500 mg/kg BW/day). The bar represents 200 μm.

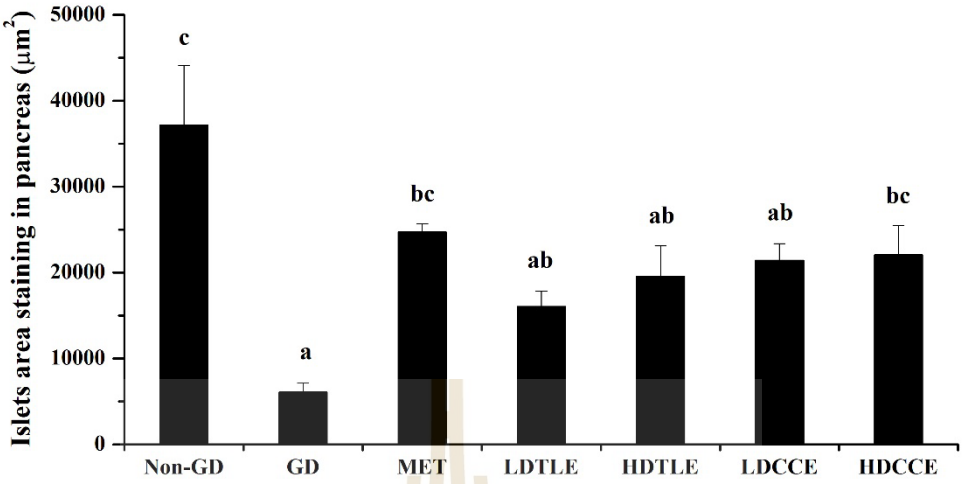

Figure 7.3 Effects of anti-diabetic plant extracts (TLE and CCE) and metformin on islets' area quantification of H&E staining in experimental pregnant rats. Data are expressed as mean ± S.E.M (n = 4). Data were analyzed by one-way ANOVA, followed by Turkey's *post hoc* test ($P < 0.05$). Groups bearing the different superscripted letters on the bar indicate statistical significance between the groups. Non-GD = non-gestational diabetic control; GD = gestational diabetic control; MET = 100 mg/kg BW/day metformin treated; LDTLE = 50 mg/kg BW/day TLE treated; HDTLE = 500 mg/kg BW/day TLE treated; LDCCE = 50 mg/kg BW/day CCE treated; HDCCE = 500 mg/kg BW/day CCE treated.

7.4.3 Effects of TLE and CCE on uterine histology

The wall of the uterus comprises 3 different tissue layers: an inner epithelial-lined mucosa (endometrium), muscular layers (myometrium), and outer serosa (perimetrium). The endometrial mucosa consists of simple columnar epithelial cells lining on the uterine cavity that extends into the branched tubular gland within the endometrial stroma, the loosely arranged reticular connective tissue containing vascular

and large numbers of inflammatory cells. Smooth muscle fibers in the myometrium are divided into the inner circular layer and the outer longitudinal layer which are separated by the stratum vasculosum, a loose with highly vascular connective tissue (Boyd et al., 2018). The uterine wall section of the gestational rat was thinner than the non-gestational rat. The longitudinal and circular muscular bundles were widely separated by interstitial tissues in the stratum vasculosum due to the enlarged and distended uterus as seen in Figure 7.4.

The uterus cross-sections of non-gestational diabetic rats, gestational diabetic rats, and gestational diabetic treated rats are exhibited in Figure 7.5. Transverse sections of non-GD in this experiment showed the widely endometrial layer with the different luminal epithelium of the endometrial mucosa resting on the surface of the uterine lumen. The bulk stromal layer of endometrium was also observed and run in longitudinal folds. The uterine glands were presented with normal columnar epithelial cells. Myometrium was thick with the longitudinal and circular fibers were regularly dense and well organized. The small interstitial space has appeared with blood vessels that are relatively seen in Figure 7.5(A). The section of GD has demonstrated the thinner stromal layer of the endometrium with shortening luminal surface epithelium. The degeneration of the uterine gland was shown and inflammatory cells can be seen. Sparse longitudinal and circular myometrial fibers were presented with loose irregular fibers arrangement. The larger interstitial space was seen with rarely blood vessel infiltration in Figure 7.5(B).

Oral administration of MET during the gestation period remarkably developed all structures of the uterus as illustrated by an increasing the epithelial thickness of the endometrium. Myometrial smooth muscle fibers including longitudinal

and circular were reorganized and expanded which indicated by the reduction of interstitial space between layers as seen in Figure 7.5(C). Furthermore, the histological findings of the uterus in the anti-diabetic plants' treatment (TLE and CCE) were enhanced the uterine structure which was dependable on the dosage of the extracts used. The higher dose of both extracts (500 mg/kg BW/day) appeared the greater level of thickening of the endometrial layer and the reorganization of myometrial muscular fibers in Figure 7.5(D), 7.5(E), 7.5(F), and 7.5(G), respectively).

The proportion of relative interstitial space and muscle fiber cross-section area from the total histological staining between experimental groups are summarized in Figure 7.6. In details, the interstitial space was significantly higher to 44.04 ± 1.32%, and fiber area was significantly lower to 52.66 ± 2.36% in GD, compared to non-GD (interstitial space: 30.00 ± 1.69% and fiber area: 70.00 ± 1.69%, $P < 0.05$). Standard anti-diabetic MET can reduce the interstitial space to 36.03 ± 2.41% and increase fiber area to 63.97 ± 2.41%. Additionally, the low dosage treatment of TLE (LDTLE) was also enhanced the uterine structure by decreasing interstitial space to 37.70 ± 2.26% and improving fiber area to 62.30 ± 2.26%, but the greater result was seen in high dosage treatment (HDTLE) which was reduced interstitial space to 32.65 ± 2.89% and increased fiber area to 67.35 ± 2.89%. For low dosage treatment of CCE (LDCCE), the interstitial space was decreased to 43.17 ± 3.03% and the fiber area was up to 56.83 ± 3.03%. The high dosage treatment of CCE (HDCCE) has been shown great results which were reduced interstitial space to 39.31 ± 2.02 % and elevated fiber area to 60.69 ± 2.02 %.

To conclude, both anti-diabetic plant extracts showed the powerful reduction of uterine interstitial space and improvements of smooth muscle fiber area in

experimental diabetic pregnant rats during gestation. Treatment with TLE and CCE, both low and high dosages has produced results similar to MET treatment, especially the high dosage administration.

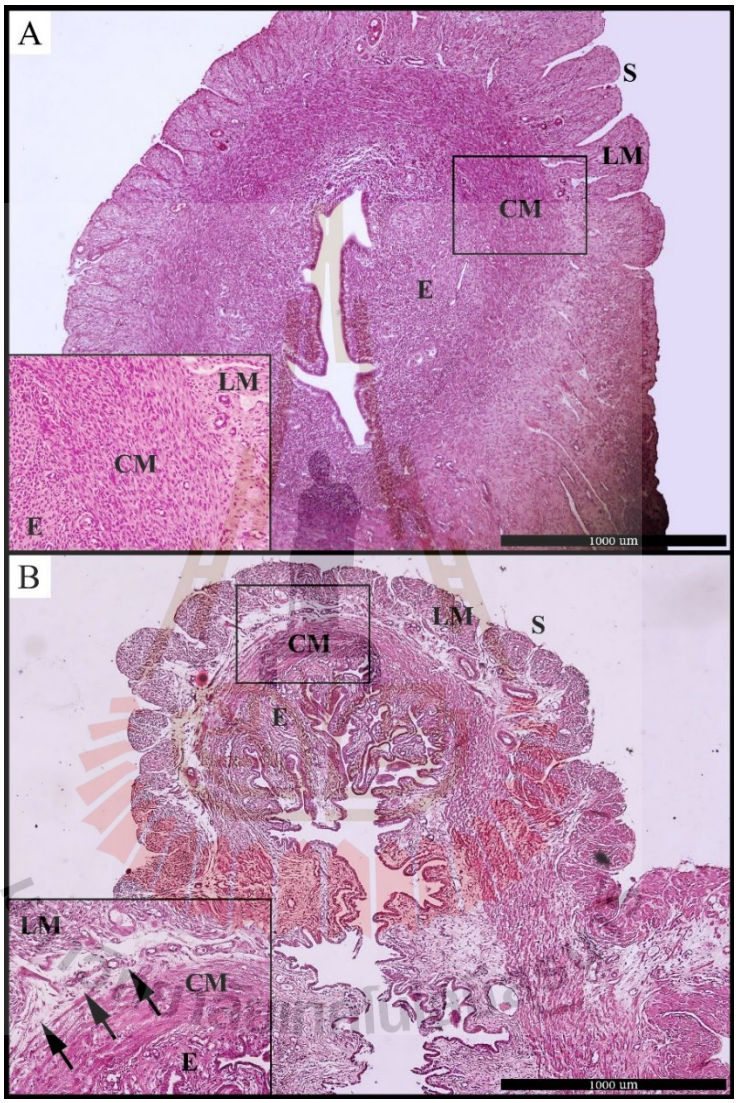

Figure 7.4 Histomorphology of H&E staining on the uterine horn of experimental non-pregnant and late pregnant rats. Sections showing three layers of uterine wall including endometrium (E), circular layer (CM), and longitudinal layer (LM) of myometrium and serosa (S) of non-pregnant rat (A) and late pregnant rat (B). Interstitial space (arrows) between myometrial circular and longitudinal layers was seen in the late pregnant rat caused by the uterine distension. The bar represents 1000 µm.

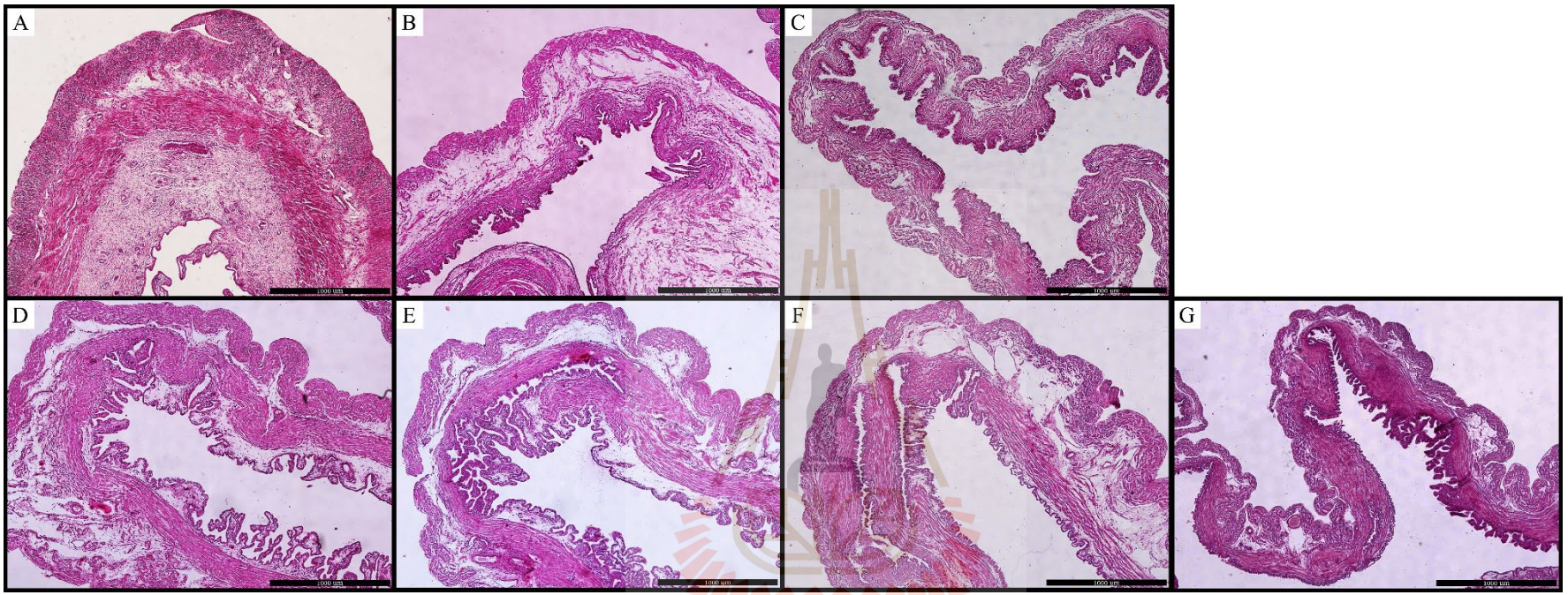

Figure 7.5 Histomorphology of H&E staining on the uterine horn of non-GD and GD treated by various treatments along the pregnant period. A represents the typical uterine structure in non-gestational diabetic rat's uterus; B represents the atrophic pattern of the uterus in gestational diabetic rat received vehicle control; C represents metformin treatment (100 mg/kg BW/day); D represents low dosage TLE treatment (50 mg/kg BW/day); E represents high dosage TLE treatment (500 mg/kg BW/day); F represents low dosage CCE treatment (50 mg/kg BW/day); G represents high dosage CCE treatment (500 mg/kg BW/day). The bar represents 1000 μm.

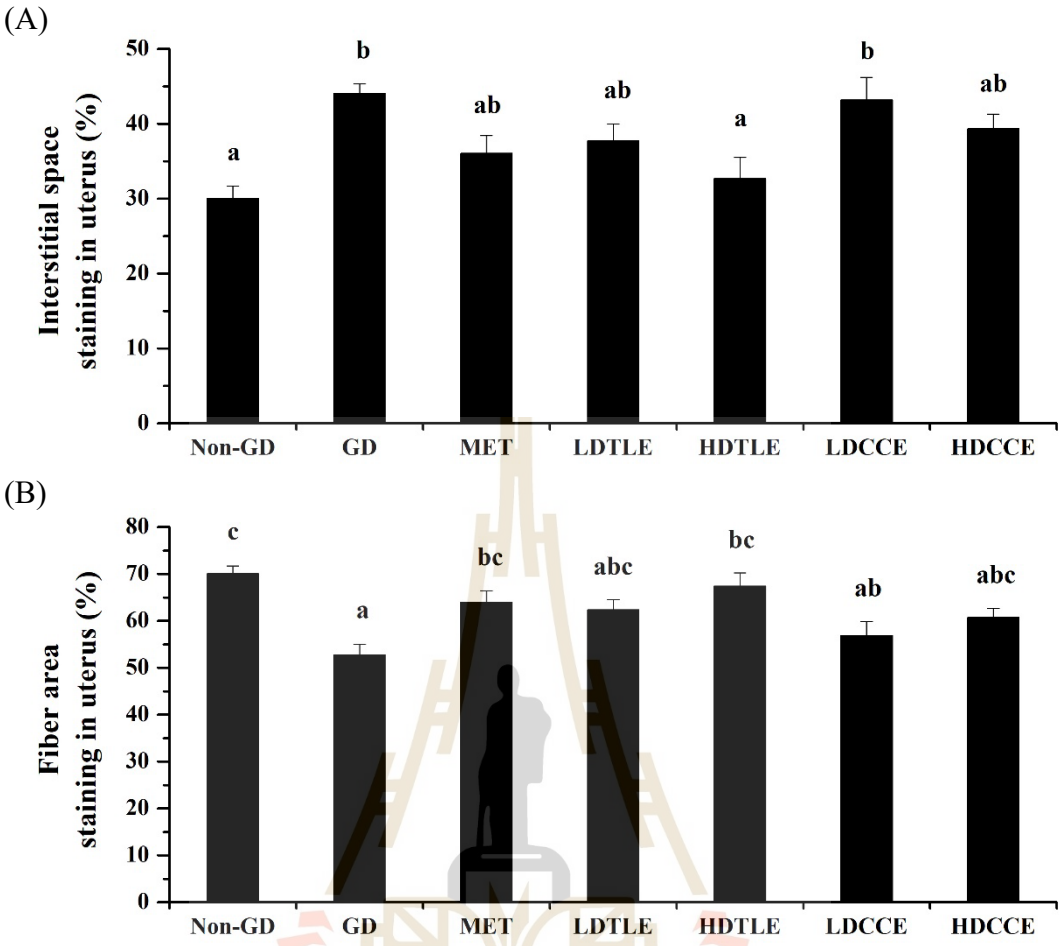

Figure 7.6 Effects of anti-diabetic plant extracts (TLE and CCE) and metformin on the proportion of relative interstitial space (A) and fiber area (B) quantification of H&E staining in experimental pregnant rats. Data express as mean ± S.E.M (n = 5). Data were analyzed by one-way ANOVA, followed by Turkey's *post hoc* test ($P < 0.05$). Groups bearing the different superscripted letters on the bar indicate statistical significance between the groups. Non-GD = non-gestational diabetic control; GD = gestational diabetic control; MET = 100 mg/kg BW/day metformin treated; LDTLE = 50 mg/kg BW/day TLE treated; HDTLE = 500 mg/kg BW/day TLE treated; LDCCE = 50 mg/kg BW/day CCE treated; HDCCE = 500 mg/kg BW/day CCE treated.

7.4.4 Effects of TLE and CCE on uterine spectrophotometric study

Interesting in FTIR imaging morphology, the fatty acyl chains are detected from 3020 to 2800 cm^{-1}, proteins are detected from 1700 to 1500 cm^{-1} and phosphorylated carbohydrates are detected from 1200 to 900 cm^{-1} as determined by Petibois et al. (2009). Relative quantification of structural determination such as lipids, proteins, carbohydrates, and other biological molecules from the histopathological image of uterine sections in this study was detected by FTIR spectroscopy. The main eight distinct absorption bands of FTIR spectra were observed and classified on their characteristic absorption as seen in Table 7.1, as well as the averages of integrated peak areas of these main absorption bands were shown in Table 7.2.

As a result, the change in fatty acyl chains in the C–H stretching region (2973-2844 cm^{-1}) based on the deformations of CH_2 and CH_3 functional groups of lipids and amino acids were detected in gestational diabetic rats. In detail, there was a significant increase in the integrated areas of C–H stretching in GD (0.004534 ± 0.000244 a.u.), compared with non-GD (0.003099 ± 0.000050 a.u., $P < 0.05$). As compared to GD, MET treatment significantly reversed this conformation changed of CH_2 and CH_3 functional groups from diabetic conditions (0.003699 ± 0.000161 a.u.). Additionally, the administration of TLE and CCE has a decreasing trend in the integrated areas of C–H stretching in gestational diabetic rats with no reach to the significant level, except the high dosage treatment of CCE was exhibited the significantly different (0.003535 ± 0.000200 a.u., $P < 0.05$).

The region between 1747 and 1507 cm^{-1} is possessed by the ester functional groups and protein secondary structures (α-helical and β-sheet structure of amide I and amide II bands). The intensities of ester functional groups were not significant altered

among experimental groups ($P > 0.05$), but the higher integral areas are found in all gestational diabetic rats and a trend to the highest value in GD. The intensities of amide I was significantly diminished (0.007133 ± 0.000142 a.u., $P < 0.05$) and a trend to decreasing in amide II bands (0.004232 ± 0.000071 a.u.) in GD, compared with non-GD (0.007733 ± 0.000097 a.u. and 0.004633 ± 0.000105 a.u., respectively). As compared to GD, the improvement of amide I and amide II bands' intensities were represented in MET with non-significant to GD. The treatment with both dosages of TLE were significantly increased the intensities of amide I (LDTLE: 0.007865 ± 0.000133 a.u. and HDTLE: 0.008265 ± 0.000067 a.u.) and amide II (LDTLE: 0.004766 ± 0.000112 a.u. and HDTLE: 0.005366 ± 0.000078 a.u.), compared to GD ($P < 0.05$). Both dosages of CCE treatment were also increased amide I and amide II bands' intensities with a weak trend toward significance, but an increased amide II integral area was reached statistical significance in the high dosage treatment of CCE (0.004667 ± 0.000087 a.u., $P < 0.05$).

The absorption bands of C–H bending (mainly lipids and amino acids) and amide III collagen vibrations are featured in the region between 1472 and 1273 cm^{-1}. The average spectra belonging to the C–H bending in GD was marked arise from CH_2 and CH_3 scissoring of lipids and amino acids (0.002768 ± 0.000086 a.u.), probably not experimentally significant from non-GD (0.002432 ± 0.000052 a.u., $P > 0.05$). As compared to GD, there was a significantly reduced the C–H bending in both dosages of TLE treatment (LDTLE: 0.002401 ± 0.000097 a.u. and HDTLE: 0.002265 ± 0.000067 a.u.) and high dosage of CCE treatment (0.002400 ± 0.000084 a.u.), same as the reduction in MET treated (0.002366 ± 0.000078 a.u.). For the vibrations of the collagen

amide III backbone (C–N stretching), the intensity was not markedly significant between experimental groups ($P > 0.05$).

The region of 1259-927 cm^{-1} is mainly attributable to the PO_2^- stretching of phospholipids and nucleic acids. This intensity was significant higher in GD (0.002834 ± 0.000102 a.u.), compared to non-GD (0.002166 ± 0.000133 a.u., $P < 0.05$). A significant reduced PO_2^- stretching intensity was found in gestational diabetic rats administered both dosages of TLE treatment (LDTLE: 0.002299 ± 0.000135 a.u. and HDTLE: 0.002167 ± 0.000114 a.u.) and high dosage of CCE treatment (0.002200 ± 0.000089 a.u.), similarly to the MET treated (0.002134 ± 0.000113 a.u.), compared to GD ($P < 0.05$). Additionally, the band for C–O stretching vibrations of glycogen and other carbohydrates such as oligosaccharides and polysaccharides are also dominated along this region. The C–O stretching vibrations tended to arise in GD (0.000165 ± 0.000055 a.u.), with not close significant to non-GD (0.000000 ± 0.000000 a.u., $P > 0.05$). There was a significant reduction of the C–O stretching vibrations found in a high dosage of CCE treatment (0.000000 ± 0.000000 a.u.), correspondingly to the MET treated (0.000066 ± 0.000044 a.u., compared to GD). Likewise, a reduction trend was found in both dosages of TLE treatment, but not significant reached ($P > 0.05$).

Table 7.1 The band assignments of major absorptions in FTIR microspectroscopic study in 4000 to 800 cm^{-1} regions based on literature (Bozkurt et al., 2007; Jerônimo et al., 2012; Petibois et al., 2009; Severcan et al., 2003).

Peak no.	Wavenumber (cm^{-1})	Definition of spectral assignment
1	2973-2844	C–H stretching of lipids and amino acids
2	1747-1723	Ester functional groups in lipids (C=O stretching)
3	1683-1620	Amide I: α-helical and β-sheet structure (protein C=O stretching)
4	1560-1507	Amide II α-helical structure (protein N–H bending, C–N stretching)
5	1472-1327	C–H bending of lipids and amino acids
6	1318-1273	Amide III collagen vibrations
7	1259-1071	Nucleic acids and phospholipids vibrations (PO_2^- asymmetric and symmetric stretching)
8	1071-927	Glycogen, oligosaccharides, glycolipids, and other carbohydrates vibrations (C–O stretching)

Table 7.2 Changes in the band area of the main functional groups in the 4000 to 800 cm^{-1} regions of experimental pregnant rats.

	Treatment groups	**Peak no.**			
	(dose, mg/kg BW)	**1**	**2**	**3**	**4**
Non-GD	Non-gestational diabetic rats	$0.003099 \pm 0.000050^{a}$	0.000033 ± 0.000033	$0.007733 \pm 0.000097^{c}$	$0.004633 \pm 0.000105^{ab}$
GD	Gestational diabetic rats	$0.004534 \pm 0.000244^{c}$	0.000334 ± 0.000122	$0.007133 \pm 0.000142^{ab}$	$0.004232 \pm 0.000071^{a}$
MET	Metformin (100)	$0.003699 \pm 0.000161^{ab}$	0.000099 ± 0.000050	$0.007534 \pm 0.000056^{bc}$	$0.004517 \pm 0.000053^{ab}$
LDTLE	*T. laurifolia* extract (50)	$0.004067 \pm 0.000204^{bc}$	0.000066 ± 0.000044	$0.007865 \pm 0.000133^{cd}$	$0.004766 \pm 0.000112^{b}$
HDTLE	*T. laurifolia* extract (500)	$0.004100 \pm 0.000193^{bc}$	0.000100 ± 0.000071	$0.008265 \pm 0.000067^{d}$	$0.005366 \pm 0.000078^{c}$
LDCCE	*C. cinereum* extract (50)	$0.004066 \pm 0.000120^{bc}$	0.000334 ± 0.000122	$0.006949 \pm 0.000196^{a}$	$0.004450 \pm 0.000141^{ab}$
HDCCE	*C. cinereum* extract (500)	$0.003535 \pm 0.000200^{ab}$	0.000066 ± 0.000044	$0.007349 \pm 0.000101^{abc}$	$0.004667 \pm 0.000087^{b}$

Data are expressed in mean ± S.E.M. (n = 10 spectra per group) and analyzed by one-way ANOVA, followed by Turkey's *post hoc* test. Means with different superscripted letters in the same row indicate statistical significance ($P < 0.05$).

Table 7.2 Changes in the band area of the main functional groups in the 4000 to 800 cm^{-1} regions of experimental pregnant rats (continued).

	Treatment groups	Peak no.			
	(dose, mg/kg BW)	5	6	7	8
Non-GD	Non-gestational diabetic rats	$0.002432 \pm 0.000052^{abc}$	0.000000 ± 0.000000	$0.002166 \pm 0.000133^{a}$	$0.000000 \pm 0.000000^{a}$
GD	Gestational diabetic rats	$0.002768 \pm 0.000086^{c}$	0.000000 ± 0.000000	$0.002834 \pm 0.000102^{b}$	$0.000165 \pm 0.000055^{ab}$
MET	Metformin (100)	$0.002366 \pm 0.000078^{ab}$	0.000000 ± 0.000000	$0.002134 \pm 0.000113^{a}$	$0.000066 \pm 0.000044^{a}$
LDTLE	*T. laurifolia* extract (50)	$0.002401 \pm 0.000097^{ab}$	0.000000 ± 0.000000	$0.002299 \pm 0.000135^{a}$	$0.000132 \pm 0.000054^{ab}$
HDTLE	*T. laurifolia* extract (500)	$0.002265 \pm 0.000067^{a}$	0.000000 ± 0.000000	$0.002167 \pm 0.000114^{a}$	$0.000132 \pm 0.000054^{ab}$
LDCCE	*C. cinereum* extract (50)	$0.002684 \pm 0.000107^{bc}$	0.000000 ± 0.000000	$0.002484 \pm 0.000133^{ab}$	$0.000334 \pm 0.000122^{b}$
HDCCE	*C. cinereum* extract (500)	$0.002400 \pm 0.000084^{ab}$	0.000000 ± 0.000000	$0.002200 \pm 0.000089^{a}$	$0.000000 \pm 0.000000^{a}$

Data are expressed in mean ± S.E.M. (n = 10 spectra per group) and analyzed by one-way ANOVA, followed by Turkey's *post hoc* test. Means with different superscripted letters in the same row indicate statistical significance ($P < 0.05$).

7.5 Discussion

According to the metabolic dysfunction caused by STZ-induced diabetes, the histological morphology of the liver by light microscopic examination in this experiment has been revealed the development of pathological damage in experimental diabetic rats. The excess fat accumulation (lipids droplets) and inflammatory infiltrates are generated the degradation in the hepatocytes which characterized by the vacuolation of the cell, variance in the size of the nuclei, and dilated sinusoidal spaces, subsequently induced fatty liver disease, inflammation, and necrosis (Mohamed et al., 2016) which in turn elevated serum biomarkers of liver failure (Ozer et al., 2008). The hyperglycemia, free radicals' production, and anti-oxidant capacity are the main mechanisms for diabetic tissue damage and pathogenesis of diabetic complications (Giacco and Brownlee, 2010). The liver observation in this study showed that the treatment with TLE and CCE at the high dosages (500 mg/kg BW) and MET (100 mg/kg BW) administered to gestational diabetic rats had a restoration of the degenerative changes by reducing lipids droplets, hepatocyte vacuolation, connective tissues rearrangement and reconstruction of the hepato-architecture of the maternal liver structure. It was demonstrated that the hepatoprotective effects of TLE and CCE administration on hepatic oxidative damage induced by diabetes in pregnant condition, in agreement with its free radical scavenging activity and anti-oxidant effects (Goggi and Malpathak, 2017; Thinh et al., 2017) which these effects appear to be similar to hepatocellular improvements of metformin therapy (Loomba et al., 2009). The beneficial actions of TLE and CCE administration on liver tissue oxidative damage might be due to its phytoconstituents containing polyphenol-rich compounds in TLE or terpenoid-rich compounds in CCE which these bioavailability studies in oxidative

stress have been generally reviewed by Vitaglione et al. (2004) and Wojtunik-Kulesza et al. (2018).

The large consequence reduced in the size and number of granules of islet morphology with high blood glucose levels has appeared in 60 mg/kg STZ-induced diabetic rats during pregnancy in this experiment. The shrinking of the islets cells was observed in untreated gestational diabetic rats which exhibiting the degenerative and necrotic changes in cells. Accordingly, Mythili et al. (2004) have been revealed that the degree of islet cells damage is depended on the dose of STZ used, including 30 mg/kg STZ served minimally structural changes of islet cells swelling, 40 mg/kg STZ induced slight destruction of islets and 50 mg/kg STZ affected the most ultrastructural changes such as degranulation and showed signs of cell death which promoted necrosis, subsequently inhibiting the synthesis and release of insulin. The treatment with TLE and CCE had significantly prevented the histological changes in pancreas pathology with dose dependency, returning to the normal regeneration as shown in the increased islets' area staining. This was suggested that the TLE and CCE can promote the rehabilitation of islets of Langerhans with restructuring pancreatic cells and may enhance insulin secretion. The potential of *T. laurifolia* treatment had an effective restoration of pancreatic β-cells activity and other important pancreatic enzymes for hyperglycemic management (Aritajat et al., 2004; Sompong et al., 2016). Hence, this is the first findings on the possible protective activity of *C. cinereum* (Synonym: *V. cinerea*) against pancreatic islets damage in STZ-induced diabetic rats. Therefore, the pancreatic β-cells regeneration and a gradual release of insulin have been found in diabetic animals fed with the species in the same genus of *V. amygdalina* (Atangwho et al., 2010) and *V. anthelmintica* (Arya et al., 2012).

Histopathological investigations of uterine tissue in untreated diabetic pregnant rats have been shown the thinner layer of endometrium and loosely irregular arrangement of the myometrium, resulting in the higher relative interstitial space while lower relative muscle fiber contents. The degenerative of uterine tissues with inflammatory cells diffusion is caused by oxidative stress and insulin resistance (Alchalabi et al., 2016; Hu et al., 2019), associated with the structure-function relationships by decreased the number of myofilament (McMurtrie et al., 1985), attenuated its function (Al-Qahtani et al., 2012) and altered in cellular compositions (Gam et al., 2018) as previously reported.

In association with the application of FTIR spectroscopy, the infrared spectrum's area of C–H stretching and C–H bending vibrations containing in fatty acids were significantly increased while the protein bands of Amide I and amide II were significantly decreased in the untreated diabetic pregnant uterus. On the other hand, the ester functional groups had a trend to highest in the untreated diabetic pregnant uterus with non-significant among experimental groups, as well as the collagen amide III vibrations. An increase in the order of lipid contents together with a decrease in the protein conformations has corresponded to the accumulation of lipids in the diabetic uterus, same as the dynamics amount of protein and lipid contents in diabetic heart tissue (Toyran et al., 2006), liver tissue (Severcan et al., 2000) and skeletal muscle (Bozkurt et al., 2010). Likewise, a arise in PO_2^- stretching in phospholipids and nucleic acids vibrations and C–O stretching in glycogen and various carbohydrates vibrations were indicated a presence of a large amount of lipid, glycolipid and glycogen accumulation within the cytoplasm, causing the disturbance of carbohydrate and lipid metabolism which suggested by Toyran et al. (2006). Nevertheless, the unstable lipid

metabolism and higher lipid accumulation are reported in diabetic cardiac myocytes due to the severely damaged and breakdown of the mitochondria (Toyran et al., 2006), whereas no mitochondrial dysfunction with increased lipid content in obese myometrial cells (Gam et al., 2017). Hall (1975) showed that maternal lipid droplets are increased in early pregnancy and storing inform of triacylglycerols for energy utilization in developmental processes, probably presenting in uterine epithelial and myometrial cells with extracellular protein deposition. The cellular accumulation of intracellular lipids is related to structural changes and functional decline, resulting in organ dysfunction and ineffectiveness reproduction (Garris et al., 1986; Gerasimova et al., 2019).

Metformin modulated the networks of signaling pathways by inhibiting insulin resistance-induced uterine inflammation in the expression of inflammation-related genes to abnormalities reproductive prevention with resulting in uterine morphological recovery (Zhang et al., 2017) and either directly or indirectly restoration of uterine function (Elia et al., 2009), supporting to this study, the structures of the uterus were developed in diabetic pregnant rats treated with MET. The luminal epithelial cells remained cuboidal in the endometrium and muscular layers were restructured which leads to interstitial space reduction and fiber area encouragement. For a previous FTIR study, Shams et al. (2019) showed the possible interactions of metformin in molecular compositions' characteristic band at 3369 and 3294 cm^{-1} (primary amine N–H stretching vibration), band at 3155 cm^{-1} (secondary amine stretching), and band at 1622 and 1567 cm^{-1} (C=O stretching, C–N stretching and N–H bending of proteins). Conversely, these absorption bands of pure MET were not defined in this study, the efficacy of MET for the treatment of GDM in the important spectra detection on uterine tissue was investigated. Uterine components had a significantly reversed on the higher

lipid contents in C–H stretching and C–H bending vibrations, phospholipids and nucleic acids in PO_2^- stretching region, glycogen and various carbohydrates vibrations in C–O stretching region, as well as a trend to improved protein contents (Amide I and II) in MET treated-diabetic pregnant uterus. Additionally, a decreased trend in the area of the absorption bands in C–H stretching and C–H bending in TLE and CCE treatment were observed which indicating the decreased lipid accumulation in gestational diabetic uterine tissue, comparable with markedly increased protein content (amide I and amide II) and enhanced carbohydrate and lipid metabolism by the reduction in PO_2^- stretching and C–O stretching vibrations integral areas.

Hence, no evidence illustrated the alterations of macromolecular contents in gestational diabetic uterine tissue due to MET or anti-diabetic plant treatment using FTIR spectroscopy. By considering, metformin therapy exerts the normalization of lipid peroxidation in addition to abnormal lipid and cholesterol metabolism along with serum hypolipidemic and hypotriglyceridemic activities due to lipoprotein homeostasis in plasma and tissues (Anurag and Anuradha, 2002). The administration of *T. laurifolia* significantly induced the peroxisome proliferator-activated receptors (PPARγ) gene expression, in the activating of lipid and carbohydrate metabolism (Rocejanasaroj et al., 2014) and linking to the mediators of lipotoxicity in the influence of gestation (Froment et al., 2006). The lower level of lipid peroxide in the liver, plasma, and spleen with the prevention of harmful effects in free radical-induced tissue damage was associated with *V. cinerea* extract treatment (Kumar and Kuttan, 2009; Latha et al., 1998).

Several traditional herbal plants markedly displayed anti-hyperglycemic, anti-hyperlipidemic, and anti-oxidant properties against the STZ-induced diabetic rats'

model, particularly *T. laurifolia* and *C. cinereum*. The histological and ultrastructural changes of liver, pancreatic islet, and uterine tissues caused by diabetes are related to the defective reproductive performances throughout gestation. Hence, the multiple therapeutic effects of these plants extract had protecting liver damage, preventing the pancreatic islet destruction, and improving reproductive performances as a potential herbal medicine in gestational diabetic therapy.

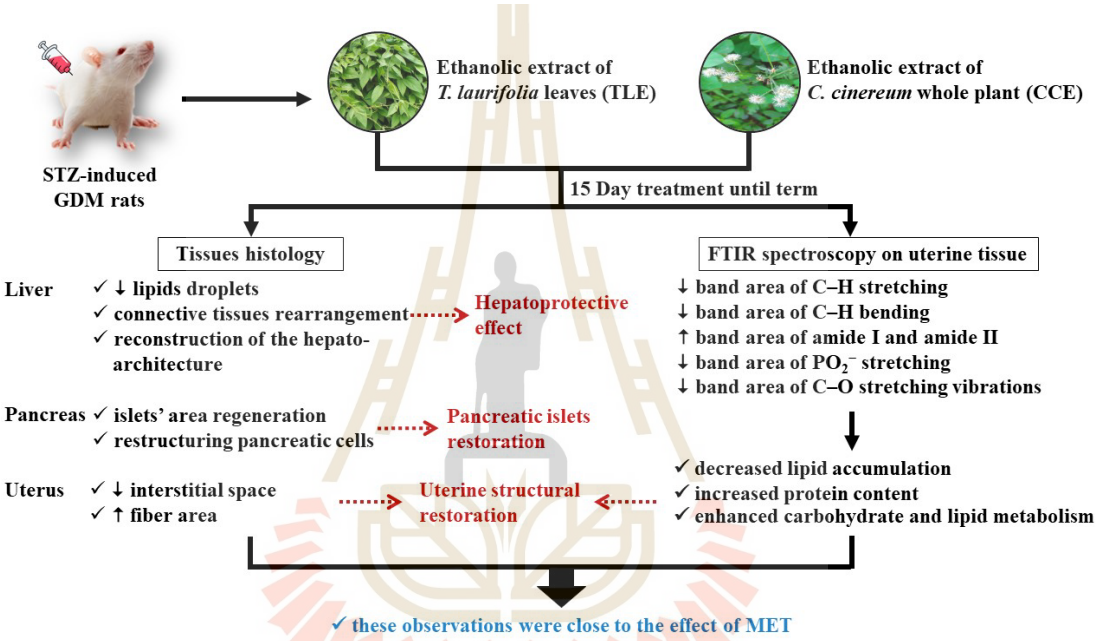

Figure 7.7 The diagram shows the effects of TLE and CCE on tissue histology and FTIR spectroscopy in gestational diabetic rats.

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CHAPTER VIII

CONCLUSIONS

Pregestational diabetes had a significant disturbance in the emergency cesarean section which was up to 67.4%, reported in 2010-2011 by the Confidential Enquiry into Maternal and Child Health (CEMACH) for National Health Service (NHS) maternity statistics. Moreover, CEMACH showed that 56% of all cesarean section was critical in an emergency more than elective cesarean section, increased 2.5-fold risk of infection in pregestational diabetes (Evers et al., 2004; Takoudes et al., 2004), promoted thrombosis that obstruction to blood flow and initiated the risk of post-partum hemorrhage (Dunne et al., 2003). Severe bleeding related to the smooth muscle functions such as hypertension, gastroparesis, constipation, and retention of urine in diabetic patients (Fleischhacker et al., 1999; Forrest et al., 2005; Inazu et al., 1991; Pfaffman et al., 1982). Even though some recent studies have been manifested these relations *in vitro* experiments, there were fewer studies exhibited the effect of diabetes on the general function and uterine smooth muscle physiology (Franchi et al., 1988; Jawerbaum et al., 1996; McMurtrie et al., 1985), it is difficult to predict whether pre-existing diabetes can disturb the myometrial functions or not.

Hypoglycemia is commonly found as primary toxicity in anti-diabetic agents' treatment. Kim-Katz (2012) and Waring (2012) reported that clinical presentation of severe hypoglycemia with impaired elimination of the drugs by hepatic or renal insufficiency, severe lactic acidosis, and permanent neurologic sequelae are commonly

complicated in glibenclamide (GLB), metformin (MET), and insulin treatment, respectively. *Thunbergia laurifolia* L. (*T. laurifolia*) and *Cyanthillium cinereum* (L.) H. Rob. (*C. cinereum*) are popular Thai medicinal plants that possess anti-diabetic activity. Several constituents including alkaloids, flavonoids, glycosides, triterpenoid saponins, sterols, and tannins have been isolated from these species (Haque et al., 2012; Kanchanapoom et al., 2002). Some of the constituents have been examined their effects on the contractility of various smooth muscle cells. For example, some flavonoids established relaxant activity on vessels, stomach, bladder, intestine, and uterus smooth muscle cells (Altinkurt and Öztürk, 1987; Amira et al., 2008; Breinholt et al., 2000; Jin et al., 2009; Liu et al., 2011). In addition, some sterols acted as uterotonic or tocolytic agents on the uterus (Gruber and O'Brien, 2011).

The principal aim of this thesis was to determine the physiology of uterine smooth muscle in gestational diabetic condition with an emphasis on the mechanisms of the uterine contraction and the response of hormone-induced contractility, which may answer the question of why diabetic pregnancies have a high cesarean section rate, and to evaluate the effect of *T. laurifolia*, and *C. cinereum* on uterine smooth muscle contractility in gestational diabetes condition compared with the effects of diabetic standard drugs, associated with the effects of these plants on fetal and maternal adverse outcomes in diabetic pregnancy. The significant findings can conclude as follows.

8.1 Phytochemical compositions of TLE and CCE

Thunbergia and *Cyanthillium* specimens were obtained from Ayutthaya province, Thailand, and were identified by the botanist at the Royal Forest Department, Bangkok, Thailand, which is *T. laurifolia* from Acanthaceae and *C. cinereum* from Asteraceae.

For phytochemical screening, alkaloids, flavonoids, phenolic compounds, tannins, sterols, terpenoids, and reducing sugars are presented in both TLE and CCE. The result from GC-MS and LC-MS showed the different constituents were found in the plant extract. The identical majority constituents of both plant extracts were phenolic compounds such as rosmarinic acid in TLE, lupeol, β-amyrin, β-amyrin acetate, and β-sitosterol in CCE. The distinct flavonoid (4H-Pyran-4-one,2,3-dihydro-3,5-dihydroxy-6-methyl) was found in TLE whereas alkaloids (2-methyl-2,3,4,5,6,7-hexahydro-1H-2-benzazonine) was found in CCE. The present investigation demonstrates that the ethanolic extract of *T. laurifolia* leaves and *C. cinereum* whole plant are considered the fundamental of phenolic compounds and various other constituents which may have several medicinal activities.

Besides, the qualitative and quantitative phytochemical data in phytochemical screening, GC-MS, and LC-MS of TLE and CCE displayed the existence of bioactive constituents as shown in Table 8.1.

Table 8.1 The summary table of phytochemical, GC-MS, and LC-MS screening of *T. laurifolia* and *C. cinereum* extract with ethanol.

	***T. laurifolia* (TLE)**			***C. cinereum* (CCE)**	
Group of phytochemicals	**Phytochemical screening**	**GC-MS**	**LC-MS**	**Phytochemical screening**	**GC-MS**
Alkaloids	✓	–	–	✓	✓
Flavonoids	✓	✓	–	✓	–
Saponins	–	–	–	–	–
Phenolic compounds and Tannins	✓	✓	✓	✓	✓
Sterols and Terpenoids	✓	✓	–	✓	✓
Reducing sugars	✓	–	–	✓	–

(–) indicates absence and (✓) indicates presence.

8.2 The possible mechanisms of TLE and CCE

Gestational diabetic rats were received the low (50 mg/kg BW) and high (500 mg/kg BW) dosages of both TLE and CCE once daily on day 7th of pregnancy until term, accompanied by the previously toxicity test with OCDC guideline. The results showed that the hypoglycemic activity of both TLE and CCE occurred in the late gestation, especially in both high dosages treatment. This may due to the restoration of islets tissue morphology from STZ-induced islets cells damaged. Moreover, the hypolipidemic properties were presented after TLE and CCE treatment by decreased TC, TRI, and LDL with increased HDL level, according to the hepato-architecture reconstruction in the maternal liver conforming the anti-oxidant properties of phytochemical constituents from the plant extracts as well as a decrease in liver enzymes (AST, APT and ALP). These findings suggest that the effects of both plant extracts were not different from the standard metformin for controlling the high blood glucose level, abnormal lipid levels, and elevated liver transaminase levels in gestational diabetic rats.

Various parameters in maternal reproductive performances and fetal outcomes were firstly evaluated in this study which accesses the potentials useful of these plants on GDM. Based on the present animal study, TLE and CCE administration were non-toxic in gestational diabetic rats. For maternal reproductive performances' indices, TLE has a potent effect on miscarriages, intrauterine growth restriction, and stillbirth prevention by decreasing the number of dead fetuses, pre-implantation loss rates, and post-implantation loss rates whereas CCE had a trend to produce the prevention of fetal death. Correspondingly to the fetal outcomes' indices, the placental-fetal circulation is affected by TLE and CCE via increasing weight and length of fetuses while decreasing

weight of placentas as the increased risk of APA fetuses, which indicated the maternal-placental-fetal-unit improvement.

Moreover, the uterine responsibility during the exposure to TLE showed the possible tocolytic activity whether on spontaneous contraction, KCl and, OT-induced contraction. Its tocolytic effect on KCl-induced membrane depolarization is not altered by diabetes, but it seems to be affected on the OT-induced contraction by diminished the response to oxytocin (OT) and weakened tocolytic effect in gestational diabetic myometrium. Moreover, the possible biphasic uterine responsibility is also found in the application of CCE on uterine tissues. The transient stimulant activity with prolonged relaxation was observed in the spontaneous contraction, suggesting the modified spontaneous contraction pattern with a tocolytic effect and this response was stronger in gestational diabetic myometrium. The effect of CCE on KCl-induced contraction was not mediated by diabetes, but its potentially act on OT-induced contraction. The initial effect of CCE may be increased the utilization of intracellular Ca^{2+} concentration with tocolytic activity as a competitive antagonist to OT on it signaling pathway due to the inability to generate the maximal contractile response under the action of CCE. Correspondingly, the uterine histology and FTIR spectroscopy in the gestational diabetic model relate to the TLE and CCE treatment in this study revealed the probable mediators of lipotoxicity by increasing muscle fiber contents and decreasing interstitial space. Furthermore, the decreased lipid contents, increased protein contents, and enhanced phospholipids, nucleic acids, glycogen, and various carbohydrates contents are found in the FTIR spectroscopy on uterine samples as well. These alterations are linked to uterine function improvement in gestational diabetic rats. Likewise, the potential use of TLE has convenient for gestational diabetic therapy in conditions

associated with the prevention of premature labor or hypermobility of the uterus because of its tocolytic activity, noting that the traditional use of CCE must be aware in gestational diabetic therapy because of its biphasic activity on the uterine responsibility. The possible mechanisms of these plants were summarized in Table 8.2.

Table 8.2 The summary of possible mechanisms of *T. laurifolia* and *C. cinereum* extract in GDM.

Parameters	***T. laurifolia* (TLE)**		***C. cinereum* (CCE)**	
	Active compounds	**Mode of action**	**Active compounds**	**Mode of action**
Blood glucose, lipid and, liver enzymes levels	pyran derivatives benzoic acid rosmarinic acid	↓hepatic gluconeogenesis ↓lipid peroxidation ↑GLUT4 translocation ↓hepatic gluconeogenesis	Lupeol β-amyrin lupenone, β-sitosterol, campesterol	↑insulin level ↑pancreatic regeneration ↓free radicals' production ↓glucose absorption ↓glucose production ↓lipid profiles ↓ Fasting blood glucose ↓lipid profiles
Maternal reproductive performances and fetal outcomes	all active compounds	✓no sign of toxicity ↓miscarriages ↓intrauterine growth restriction ↓stillbirth ↑placental-fetal efficiency	all active compounds	✓no sign of toxicity ↓stillbirth ↑placental-fetal efficiency
Uterine contraction on spontaneous contraction, KCl, and OT-induced contraction	pyran derivatives chromone derivative indole alkaloid	✓modified the spontaneous contractile pattern ↓OT signaling pathway ↓OT signaling pathway ↓agonists signaling pathway	lupeol β-sitosterol β-amyrin	↓relaxation pathway ↑intracellular Ca^{2+} concentration initial uterine mobility and elongate relaxation

Table 8.2 Summary of possible mechanisms of *T. laurifolia* and *C. cinereum* in GDM (continued).

Parameters	***T. laurifolia* (TLE)**		***C. cinereum* (CCE)**	
	Active compounds	**Mode of action**	**Active compounds**	**Mode of action**
	rosmarinic acid	↓cyclooxygenase enzymes in SMCs	palmitic acid	↑ uterine movement ↓ maximum response to OT, and OT-like activity
Uterine ultrastructure alteration	all active compounds	↓interstitial space ↑muscle fiber ↓lipid contents ↑protein contents ↓phospholipids and nucleic acids ↓glycogen and various carbohydrates contents	all active compounds	↓interstitial space ↑muscle fiber ↓lipid contents ↑protein contents ↓phospholipids and nucleic acids ↓glycogen and various carbohydrates contents

8.3 Future research

The effectiveness of *T. laurifolia* and *C. cinereum* in this study showed the maternal anti-hyperglycemic, anti-hyperlipidemic and hepatoprotective properties along with the prevention of miscarriages, intrauterine growth restriction, and stillbirth which may associate with the potential tocolytic activity in STZ-induced gestational diabetes mellitus. This resulted was clearly exhibited that these plant treatments are not caused adverse effects for mother and fetus in diabetic pregnancy. However, further studies may include the targeted mechanism of tocolytic acts in the uterine signaling pathway to verify their ability to preterm birth or hypermobility prevention in diabetic pregnancy. Moreover, the underlying mechanisms of these plants should be encouraging in the human model for further experiments.

8.4 References

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