ผลของว่านผักปัง (*Talinum paniculatum* (Jacq.) Gaertn.) สกัดต่อการทำหน้าที่ของระบบสืบพันธุ์หนูแรทเพศเมีย

นางสาวแคทรียา ธนะมูล

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
สาขาวิชาชีวเวชศาสตร์
มหาวิทยาลัยเทคโนโลยีสุรนารี
ปีการศึกษา 2555

EFFECTS OF *TALINUM PANICULATUM* (JACQ.) GAERTN. EXTRACTS ON REPRODUCTIVE FUNCTIONS IN FEMALE RAT

Catthareeya Thanamool

A Thesis Submitted in Partial Fulfillment of the Requirements for the

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THE EFFECTS OF *TALINUM PANICULATUM* (JACQ.) GAERTN. EXTRACTS ON REPRODUCTIVE FUNCTIONS IN FEMALE RAT

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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แคทรียา ธนะมูล : ผลของว่านผักปัง (*Talinum paniculatum* (Jacq.) Gaertn.) สกัดต่อการทำหน้าที่ของระบบสืบพันธุ์หนูแรทเพศเมีย (THE EFFECTS OF *TALINUM PANICULATUM* (JACQ.) GAERTN. EXTRACTS ON REPRODUCTIVE FUNCTIONS IN FEMALE RAT) อาจารย์ที่ปรึกษา : รองศาสตราจารย์ สัตวแพทย์หญิง ดร.ศจีรา คุปพิทยานันท์, 187 หน้า.

ว่านผักปัง (*Talinum paniculatum* (Jacq.) Gaertn.) หรือ “โสม จาวา” เป็นพืชสมุนไพรที่เชื่อว่ามีความสามารถในการบรรเทาความรุนแรงของโรคหลายชนิด โดยรวมถึง ความผิดปกติของระบบสืบพันธุ์ มีรายงานว่า ว่านผักปัง ประกอบด้วยสารไฟโตสเตอรอลที่สำคัญหลายชนิด ดังนั้น วัตถุประสงค์ของการศึกษานี้ คือ ศึกษาฤทธิ์การเป็นสมุนไพรของสารสกัดจากรากและใบของว่านผักปัง (100 และ 1,000 มก. ต่อ กก. น้ำหนักตัว ต่อวัน) และสารที่เกี่ยวข้อง คือ ไฟตอล (ไฟตอลมาตรฐาน 500 มก. ต่อ กก. น้ำหนักตัว ต่อวัน) โดยศึกษาผลต่อ 1) ระดับฮอร์โมนเอสโตรเจน ระดับฮอร์โมนลูทีไนซิง และระดับชีวเคมีของเลือด (คลอเลสเตอรอลรวม ไตรกลีเซอไรด์ ไลโปโปรตีนชนิดความหนาแน่นสูง ไลโปโปรตีนชนิดความหนาแน่นต่ำ และอัลคาไลน์ฟอสฟาเตสรวม) 2) อวัยวะสืบพันธุ์ (ช่องคลอด มดลูกและเต้านม) 3) ความสมบูรณ์พันธุ์ และ 4) การหดตัวของมดลูกในหนูแรทเพศเมียเต็มวัย ผลการศึกษาพบว่า ว่านผักปังประกอบด้วยสารไฟโตสเตอรอล 5 ชนิด แอลฟาโทโคเฟอรอล และไฟตอลที่ได้จากคลอโรฟิลล์ โดยหลังจากสิ้นสุดช่วง 42 วันของการทดลอง การป้อนสารสกัดว่านผักปังและไฟตอลมาตรฐานให้กับหนูตัดรังไข่ ส่งผลกระตุ้นให้เกิดการตอบสนองในลักษณะที่เป็นฤทธิ์คล้ายเอสโตรเจน โดยบ่งชี้จากการเปลี่ยนแปลงของเนื้อเยื่อผนังช่องคลอด การเพิ่มขึ้นของระดับเอสโตรเจน น้ำหนักสัมพัทธ์ของมดลูก น้ำหนักสัมพัทธ์ของเนื้อเยื่อเต้านม รวมถึงการเพิ่มความหนาตัวของผนังช่องคลอด มดลูก และระบบท่อของเต้านม การป้อนสารสกัดจากใบของว่านผักปังในปริมาณสูงและไฟตอลมาตรฐาน สามารถลดระดับของฮอร์โมนลูทีไนซิงลงมาสู่ระดับปกติ เมื่อเปรียบเทียบกับกลุ่มหนูตัดรังไข่ควบคุม ($P < 0.05$) นอกจากนี้ การป้อนสารสกัดจากใบให้กับหนูตัดรังไข่ ยังส่งผลให้ระดับอัลคาไลน์ฟอสฟาเตสรวมลดลงอย่างมีนัยสำคัญ ($P < 0.05$) ซึ่งถือเป็นข้อบ่งชี้เบื้องต้นว่า สารสกัดจากใบของว่านผักปังมีฤทธิ์ในการป้องกันกระดูก ถึงแม้ว่าสารสกัดว่านผักปังจะไม่มีประสิทธิภาพในการลดปริมาณคลอเลสเตอรอลรวม แต่อย่างไรก็ตาม สารสกัดว่านผักปังยังสามารถออกฤทธิ์ในเชิงบวกต่อระดับไตรกลีเซอไรด์ และสัดส่วนระหว่างระดับไลโปโปรตีนชนิดความหนาแน่นสูงต่อระดับไลโปโปรตีนชนิดความหนาแน่นต่ำอย่างมีนัยสำคัญ ($P < 0.05$) สารสกัดว่านผักปังมีประสิทธิภาพในการต้านความสมบูรณ์พันธุ์ ในขณะที่ไฟตอลมาตรฐาน มีฤทธิ์ในการต้านความสมบูรณ์พันธุ์แบบอ่อน เมื่อ

เปรียบเทียบกับกลุ่มหนูท้องควบคุม (P = 0.35) นอกจากนี้การศึกษาผลของสารสกัดว่านผักปังต่อการทำงานของมดลูกหนูไม่ตั้งท้อง แสดงให้เห็นว่าสารสกัดว่านผักปังจากรากและใบมีประสิทธิภาพในการยับยั้งการหดตัวโดยธรรมชาติ ในลักษณะที่ขึ้นอยู่กับปริมาณของสารที่ให้ โดยสารสกัดจากรากและใบมีฤทธิ์ยับยั้งการหดตัวของมดลูกในรูปแบบที่คล้ายคลึงกัน แต่สารสกัดจากรากมีประสิทธิภาพสูงกว่าสารสกัดจากใบ ซึ่งบ่งชี้จากค่าความเข้มข้นของสารสกัดทั้งสองชนิดที่สามารถยับยั้งการหดตัวโดยธรรมชาติได้ร้อยละ 50 (สารสกัดจากราก 0.23 มก. ต่อ มล. และ สารสกัดจากใบ 1.67 มก. ต่อ มล. ตามลำดับ) สารสกัดทั้งสองยังมีฤทธิ์ในการยับยั้งการหดตัวของมดลูกในสภาวะกระตุ้น จากสิ่งกระตุ้น ได้แก่ สารละลายโพแทสเซียมคลอไรน์ความเข้มข้นสูง เบย์ เค 8644 และออกซีโตซิน (P < 0.05) โดยกลไกที่เกี่ยวข้องต่อการยับยั้งการหดตัวของมดลูก อาจเกิดจากการขัดขวางการไหลของแคลเซียมผ่านประตูแคลเซียมที่ผนังเซลเข้าสู่เซล ขัดขวางการไหลของแคลเซียมออกจากซาโคพลาสมิกเรติคูลัม และรบกวนสัญญาณการหดตัวของมดลูกนอกเหนืออิทธิพลของแคลเซียม โดยส่งผลให้ระบบการหดตัวของมดลูกลดความไวต่อแคลเซียม ดังนั้น จากข้อมูลทั้งหมดสามารถสรุปได้ว่า ว่านผักปังเป็นพืชสมุนไพรที่มีคุณค่าทางการแพทย์สูง กลไกการออกฤทธิ์โดยส่วนใหญ่เป็นไปได้ว่า เกิดจากฤทธิ์คล้ายเอสโตรเจนที่เหนี่ยวนำจากสารสำคัญ ได้แก่ สารในกลุ่มของไฟโตสเตอรอล และไฟตอล

สาขาวิชาเภสัชวิทยา ลายมือชื่อนักศึกษา _____

ปีการศึกษา 2555 ลายมือชื่ออาจารย์ที่ปรึกษา _____

ลายมือชื่ออาจารย์ที่ปรึกษาร่วม_____

CATTHAREEYA THANAMOOL : THE EFFECTS OF *TALINUM PANICULATUM* (JACQ.) GAERTN. EXTRACTS ON REPRODUCTIVE FUNCTIONS IN FEMALE RAT. THESIS ADVIOSOR : ASSOC. PROF. SAJEERA KUPITTAYANANT, Ph.D. (DVM), 187 PP.

PHYTOSTEROLS/ RAT/ ESTROGEN/ CHOLESTEROL/ VAGINA/ UTERUS/ MAMMARY GLAND/ CONTRACTION/ CALCIUM

Talinum paniculatum (Jacq.) Gaertn. (*T. paniculatum*) or "Som Java" is a medicinal plant which is claimed to alleviate diverse arrays of ailments including reproductive disorders. The plant has been reported to contain important phytosterols. Therefore, the purposes of this study were to explore the medicinal properties of *T. paniculatum* root and leaf extracts (100 and 1,000 mg/kg BW/day), and related compound-phytol (standard-phytol; 500 mg/kg BW/day) by observing their effects on 1) estradiol, luteinizing hormone (LH), and blood biochemistry (total cholesterols (TC), triglycerides (TG), high-density lipoprotein (HDL), low-density lipoprotein (LDL), and total alkaline phosphatase (tALP)); 2) reproductive organs (vagina, uterus and mammary tissues); 3) fertility; and 4) uterine contraction in adult female rats. The results showed that *T. paniculatum* contained 5 phytosterols, α-tocopherol and chlorophyll derived-phytols. After the 42-day oral feeding period with the extracts and standard-phytol, these compounds possessed estrogenic activity as indicated by inducing vaginal cornification, rising in estradiol level, relative uterine weight, relative mammary weight, and increasing proliferative changes in vagina, uterus, and mammary ductular tissues in ovariectomized (OVX) rats. A reduction in LH to the

physiological level was occurred by orally treated a high dosage of leaf extract and standard-phytol. The OVX rats treated by leaf extract exerted bone protective effect which was reflected by lowering tALP level ($P < 0.05$). The extracts were not effective for the reduction of TC level, but they exhibited positive effects on the concentration of TG, and HDL/LDL ratio ($P < 0.05$). The extracts also exhibited potent anti-fertility activity while standard-phytol showed mild anti-fertility activity compared to pregnant control rats ($P = 0.35$). The study of the extracts on non-pregnant uterine contractility exemplified that the extracts produced dose-related tocolytic actions on spontaneous contractions. The relaxation pattern after cumulative exposure to root extract was similar but more potent than that of leaf extract as indicated by IC_{50} concentration of each extract (root at 0.23 mg/mL and leaf at 1.67 mg/mL). The extracts also produced tocolytic activity during agonist exposures including high KCl solution, Bay K8644, and oxytocin ($P < 0.05$). The probable contractile mechanisms might be involved with the blockade of Ca^{2+} influx via L-type Ca^{2+} channels, Ca^{2+} efflux from sarcoplasmic reticulum, and interruption of Ca^{2+}-independent pathways that might reduce the sensitivity of contractile system to Ca^{2+}. These findings illustrated that *T. paniculatum* will be a highly beneficial medicinal plant that encourages favorable therapeutic properties. The possible mechanisms are mainly due to its estrogenic action of presented phytochemical constituents including phytosterols and phytols.

School of Pharmacology
Academic Year 2012

Student's Signature_____
Advisor's Signature_____
Co-advisor's Signature _____

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LIST OF ABBREVIATIONS

ANOVA	=	analysis of variance
apoB	=	apoprotein B
AUC	=	area under the contraction
bALP	=	bone specific-alkaline phosphatase
Bay K8644	=	calcium channel agonist
BMD	=	bone mineral density
BW	=	body weight
°C	=	degree Celsius
Ca^{2+}	=	calcium ion
$[Ca]_i$	=	cytoplasmic Ca^{2+} concentration
$CaCl_2$	=	calcium chloride
CaM	=	calmodulin
cAMP	=	cyclic adenosine monophosphate
Cd	=	caldesmon
cGMP	=	cyclic guanosine monophosphate
cm	=	centimetre
CO_2	=	carbon dioxide
Cp	=	calponin
CYP7A1	=	cholesterol-7-alpha-hydroxylase
DAG	=	diacylglycerol

LIST OF ABBREVIATIONS (Continued)

E_2	=	17β-estradiol
EGTA	=	ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid
ER	=	estrogen receptor
ERs	=	estrogen receptors
ERα	=	estrogen receptor alpha
ERβ	=	estrogen receptor beta
ELCIA	=	electrochemiluminescence
FSH	=	follicle-stimulating hormone
g	=	gram
GC/MS	=	gas chromatography-mass spectrometry
GnRH	=	gonadotropin-releasing hormone
H_2SO_4	=	sulphuric acid
HEPES	=	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HDL	=	high-density lipoprotein
HMG-CoA	=	3-hydroxy-3-methylglutaryl-coenzyme A
hr	=	hour
HRT	=	hormone replacement therapy
IGF-1	=	insulin like growth factor-1
KCl	=	potassium chloride
kDa	=	kilodalton

LIST OF ABBREVIATIONS (Continued)

IP_3	=	inositol (1, 4, 5)-triphosphate
IP_3R	=	inositol (1,4,5)-trisphosphate receptor
IU	=	international unit
LH	=	luteinizing hormone
LDL	=	low-density lipoprotein
MAP	=	mitogen-activated protein
mg/kg BW/day	=	milligram per kilogram body weight per day
min	=	minute
mL	=	milliliter
mm	=	millimeter
mM	=	millimolar
mIU/mL	=	milli-international unit per milliliter
mg/dL	=	milligram per deciliter
MLC_{20}	=	20-kDa regulatory side chain of myosin light chain
MLCK	=	myosin light chain kinase
MLCP	=	myosin light chain phosphatase
n	=	number of sample
NaCl	=	sodium chloride
NO	=	nitric oxide
NCX	=	sodium/calcium exchanger
OVX	=	ovariectomy

LIST OF ABBREVIATIONS (Continued)

pg/mL	=	picogram per milliliter
PKA	=	protein kinase A
PKC	=	protein serine/threonine kinase C
PMCA	=	plasma membrane Ca^{2+}-ATPase
PPARα	=	peroxisome proliferator-activated receptor alpha
ROK	=	rho-associated kinase
rpm	=	revolutions per minute
RyR	=	ryanodine receptor
RXR	=	retinoid X receptor
%RM	=	percentage of relative mammary weight
%RU	=	percentage of relative uterine weight
S.E.M	=	standard error of the mean
SERCA	=	SR Ca-ATPase
SR	=	sarcoplasmic reticulum
tALP	=	total alkaline phosphatase
TC	=	total cholesterol
TG	=	triglyceride
µg	=	microgram
µg/kg BW/day	=	microgram per kilogram body weight per day
µm	=	micrometer
VOCC	=	voltage gate calcium channels

LIST OF ABBREVIATIONS (Continued)

v/v	=	volume per volume
ZIP	=	zipper-interaction protein

CHAPTER I

INTRODUCTION

Sex hormones are crucial elements to nourish woman's sexual health. They systematize menstruation, fertility, sex drive and menopause. Sex hormones dysfunction leads to life-threatening conditions including menopausal symptoms, genitourethral atrophy, osteoporosis, cardiovascular diseases, nerve disorders, diabetes, or cancers. Despite these complications can be treated by classical synthetic hormones, but they are still controversy because of undesirable side effects. These solutions are of special concern to communities worldwide, hence, lead to an urgent need for alternate and more effective treatments. Plant-based remedies are gaining considerable attention due to their fecundity therapeutic values. Scientists attempt to elucidate the phytochemical composition of medicinal plants contributing to the uses of plants in the treating of sex-hormone related conditions. Therefore, this thesis explored the plant *Talinum paniculatum* (Jacq.) Gaertn. which have been claimed to be pharmacologically active on female reproductive functions.

1.1 Female Reproductive Capacity

During the female mammals' fertile phase, the gametes are limited throughout the life span and the copulation with fertile male can lead to conception. Conversely, the end of fertility (non-fertile phase or menopause), is traditionally indicated by the permanent end of menstruation or gamete release. In primate, including human, the

menstrual cycle is orchestrated by two key hormones; the estrogen and progesterone. When ovulation occurs, the rates of progesterone also increase to support pregnancy.

The length of menstrual cycle has been known to correlate with the underlying endocrine milieu, as well as, her potential for fertility. The menstrual cycle is made up of many changes that take place inside the body. These changes prepare a woman's body for pregnancy each month. Irregular menstrual cycles can also be present during puberty (Deligeoroglou and Tsimaris, 2010) and years before menopause (Mackey, 2009).

In the reproductive years, regular menstrual cycle is around 28 days. In follicular phase, inhibin, activin, follistatin, and insulin-like factors are responsible for regulating follicular growth. During the first day of the follicular phase, granulosa cells are the first to respond to rising follicle-stimulating hormone (FSH) levels. Follicles secrete androstenedione, which is aromatized into estradiol. Enhanced estradiol production stimulates granulose cell development through inhibin, which results in a drop of the FSH production and prevents the additional development of follicles. On the other hand, luteal phase is also called the secretory phase or progestational phase, and it starts after ovulation. If pregnancy does not occur, an increase in FSH level is observed 3-4 days before the luteal-follicular transition. This hormone is concomitant with lysis of the corpus luteum (CL) along with an associated decrease in progesterone and estrogen levels. In addition, the level of FSH generally drops to the lowest level near the end of luteal phase and rise to the highest level during the early follicular phase.

The final period or menopause occurs when the follicle cannot produce hormones. Normally, the ovary is endowed with a finite number of eggs and the

menopause represents the loss of any further eggs within the ovarian tissue. Signs of gradual decrease in number of follicles and rising in the proportion of poor quality oocytes in elderly female occur. The exponential decrease starts from the age of 40 years until the ovary is unable to keep up with its normal function in the neuroendocrine axis (Lambalk et al., 2009). The consequences of ovarian aging, dysfunction, or estrogen deprivation have many phenotypic effects on tissue regeneration and maintenance.

The most significant hormonal changes during menopausal period include a decrease in early cyclic inhibin B and in anti-Mullerian hormone (AMH) levels. The decline in inhibin B results in an increase in FSH, which appears to be an important factor in the maintenance of estrogen concentration until later in reproductive life. In the postmenopausal period, FSH levels are markedly raised, whereas inhibin B and AMH are undetectable (Burger et al., 2007).

Hormonal changes during menopause were proven to raise cardiovascular disease (CVD) risk. Estrogen has a direct effect on vasodilatation (Farhat, Abi-Younes and Ramwell, 1996), an indirect long-term effect consisting of modulation of the response to endothelial damage and atherosclerotic changes in vessels (Baker et al., 2003). In addition, it also has an influence on levels of serum lipoproteins and triglycerides. Postmenopausal women possess higher levels of triglycerides, total cholesterol (TC) and low density lipoprotein (LDL) when compared with the premenopausal women (de Aloysio et al., 1999).

A number of studies have shown that oral estrogen therapy results in a significant decrease of TC and LDL concentrations, but it produces an increase in high density lipoprotein (HDL) concentration. According to kinetic studies on the

effect of estrogen on lipoprotein metabolism, estrogen may increase the rate of LDL catabolism by various mechanisms. For example, induced the expression of hepatic LDL receptor (Walsh et al., 1999), increased apoprotein B (apoB) catabolism by LDL-receptor-independent pathways, removal of desialylated LDL by transcytosis and accelerated the conversion of hepatic cholesterol to bile acid (Karjalainen et al., 2000). Furthermore, estrogen induces nitric oxide synthase and improves lipoprotein metabolism, which are fundamental mechanisms in promoting a healthy arterial tree (Shaul, 1999).

Since estrogen plays a vital role in variety of female functions, a decline of its production is primarily responsible for the changes women experience during and after menopause. Altered and declining of hormonal levels may affect the entire body, particularly estrogen deficiency. Virtually all body tissues, especially genital system to include vulva, vagina and uterus have estrogen receptors (ERs) that indicated their responsiveness to this hormone (Wang, Eriksson and Sahlin, 2000).

1.2 Female Reproductive Cycles

The reproductive cycles of mammals are influenced by a number of hormones, including FSH, Luteinizing hormone (LH), estrogen, testosterone, and progesterone. Additionally, reproductive cycles are influenced by environmental factors such as day length or nutritional status.

In the mammalian species, the female has either a menstrual cycle or estrous cycle. The menstrual cycle is a series of physiological changes that can occur in fertile females. Overt menstruation (where there is blood flow from the uterus through the vagina) occurs primarily in humans and non-human primate such as chimpanzees.

Other placental mammal species undergo estrous cycles, in which the endometrium is completely reabsorbed by itself (covert menstruation) at the end of reproductive cycle.

In primate, menstrual cycle can be divided into several different phases, including menstrual phase (menstruation), follicular (proliferative) phase, ovulatory phase, and luteal (secretory) phase. It is stimulated by gradually increasing amounts of estrogen in the follicular phase, discharges of blood (menses) slow then stop, and the lining of the endometrium. In this phase, folliculogenesis occurs under the influence of a complex interplay of hormones, mainly FSH. During mid-cycle (24-36 hr after the LH surges), the dominant follicle releases an ovum, or egg in an event called ovulation. After ovulation, the egg only lives for 24 hr or less without fertilization while the remains of the dominant follicle in the ovary become a CL. CL has a primary function of producing large amounts of progesterone. Under the influence of progesterone, the endometrium changes to prepare for potential implantation of an embryo to establish a pregnancy. If implantation does not occur within approximately 2 weeks, the CL will involute, causing rapidly drops in levels of both progesterone and estrogen. These hormones drop lead the uterus to shed its lining and egg in a process termed menstruation (Figure 1.1).

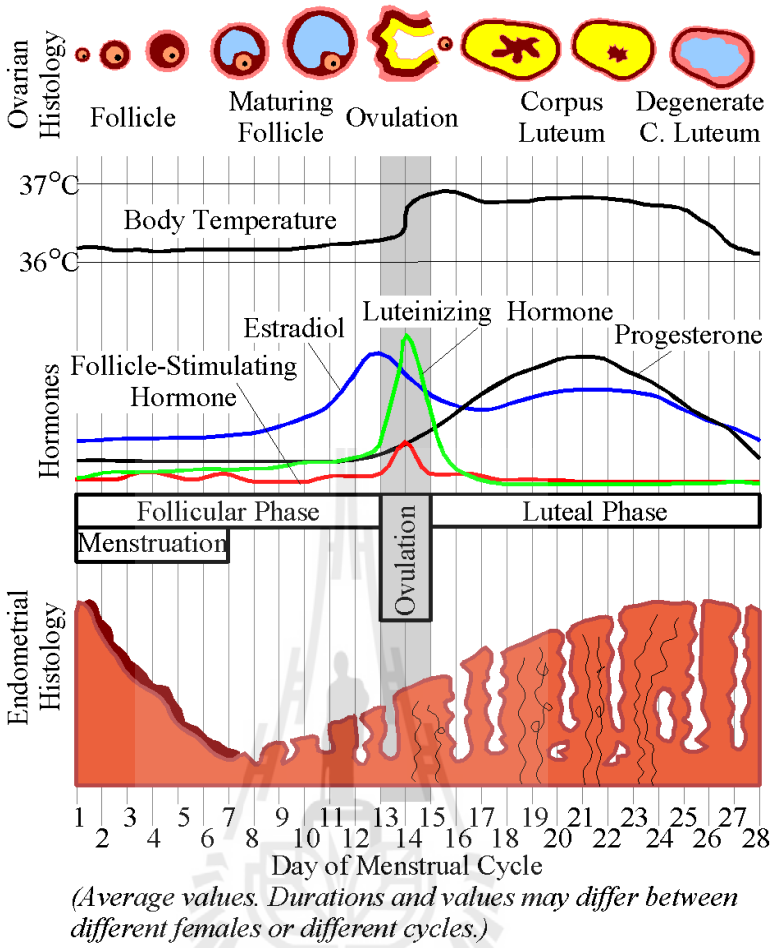

Figure 1.1 The relation of reproductive hormones, ovarian cycle, endometrium, and basal body temperature changes throughout the normal menstrual cycle (Marieb and Katja, 2007).

In non-primate animals, the estrous cycle starts after puberty in sexually mature females and is interrupted by anestrus phase or pregnancy. Typical estrous cycles continue until death. The estrous cycle is classically divided into 4 phases; diestrus, proestrus, estrus, and metestrus. The dominant hormones in various stages of the cycle have shown in Figure 1.2.

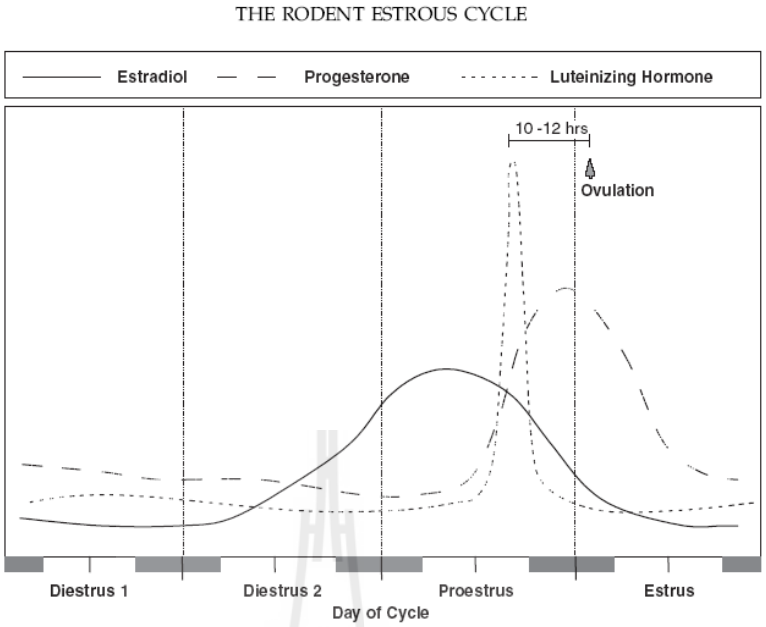

Figure 1.2 Schematic pattern of the 4-day estrous cycle in the rat. The estrous pattern is depicting serum estradiol and progesterone concentrations as they relate in time to the surge of luteinizing hormone (LH). Ovulation will typically occur during the early morning hour of estrus, approximately 10-12 hr after the rise in LH. Shaded blocks at the base of the figure indicate the dark portion of a 14:10 hr light:dark photoperiod (Goldman, Murr and Cooper, 2007).

For the simplification and indication of which structures predominate throughout the cycle, these stages may be grouped into the follicular phase (proestrus and estrus) or the luteal phase (diestrus and anestrus or metestrus). In laboratory animal such as rat, the estrous cycle has 4 days interval that contains 4 distinct stages and can be followed by vaginal cytology. Furthermore, the CL morphology can be used to support the staging procedure.

Table 1.1 summarizes the characteristics of the vaginal, uterine, and ovarian morphology during the 4 estrus stages seen in the normally cycling adult rat.

Table 1.1 Summary of the defining histological features of the rat female reproductive tract during estrous cycle (Westwood, 2008).

Phase	Vagina	Uterus	Ovaries
Diestrus	Start defined by epithelium at lowest level with variable leukocyte infiltration. Subsequent epithelial proliferation and thickening (no clear stratum granulosum; SG) with reduction in leukocyte infiltration.	Small, avascular, slit-like lumen. Lined by low columnar epithelium. Initially a few mitoses, but some increase during phase. Only occasional degenerate cells.	Large corpora lutea (CL). May be finely vacuolated. Fibrous tissue formation in central cavity
Proestrus	Mitotic figures present. Occasional polymorphs. Little if any degeneration or desquamation. Formation of SG, superficial mucoid layer and stratum corneum (SCr) progressively. At end of stage, fully cornified and generally showing superficial mucoid layer with some desquamation of mucoid cell.	Epithelium cuboidal to columnar. Mitoses present in epithelial cells with little or no degeneration and little inflammatory cell infiltration. Dilatation, particularly toward end of stage.	CL often degenerates. Cytoplasmic vacuoles generally present. Fibrous tissue proliferation in central cavity.

Table 1.1 Summary of the defining histological features of the rat female reproductive tract during estrous cycle (Westwood, 2008) (continued).

Phase	Vagina	Uterus	Ovaries
Estrus	Progressive shedding of superficial mucoid and cornified layers. Reduction in height of epithelium. Cell debris present. Loss of mitotic figures. Progressive leukocyte infiltration.	Start of estrus defined by appearance of notable degeneration/necrosis of epithelial cells, glands generally first. Loss of mitotic activity. Leukocyte infiltration. Dilatation may persist to late estrus.	Degenerate CL often present. Some small CL with basophilic cell cytoplasm, central fluid-filled cavity, and no fibrous tissue.
Metestrus	Start defined by virtually complete detachment of cornified layer. Continued desquamation with loss of SG and upper stratum germinativum. Leukocyte infiltration.	Continued degeneration of endometrial epithelial cells. Return of mitotic activity; both (mitotic activity and degeneration) seen together.	CL may still contain fluid cavity. Smaller than at diestrus. Slightly basophilic cells. Lack of fibrous tissue.

1.3 Pregnancy and Parturition

In most mammals, the egg(s) is ovulated approximately midway through the menstrual or estrous cycle. During copulation, spermatozoa are released by the male into the vagina and travel via the uterus to the oviducts where the egg may become fertilized. The length of gestation is different in various species and it can be varied by maternal, fetal or environmental factors.

Classically, progesterone is a key hormone necessary for maintenance of pregnancy. It is produced by the CL and placenta. The CL persists throughout gestation period in almost all mammals except horse. Progesterone impedes the uterine smooth muscle contraction and decreases prostaglandin formation, both of which allow the fetus to grow with the expanding uterus.

Estrogen also increases during pregnancy. It is generally accepted that estrogen is necessary for the maintenance of pregnancy. Among other functions, estrogen increases uterine blood flow. Prolactin, the hormone that allows for lactation postpartum, also increases throughout pregnancy, and its production is thought to be stimulated by increasing level of estrogen.

When the egg is fertilized and subsequently implants within the uterus, by day 10 after ovulation, a glycoprotein molecule unique to pregnancy is now present in the blood. In human, this protein is referred to human chorionic gonadotropin (hCG) that is secreted by syncytiotrophoblast. The role of hCG is to stimulate progesterone biosynthesis by luteal cells of the CL. In human, plasma level of hCG rises to its highest between the 9^{th} and 14^{th} weeks of pregnancy and then begin to decline gradually, reaching a nadir at approximately 20 weeks of gestation. It has been suggested that hCG keeps the CL function that required for the development of the

conceptus. Moreover, estrogen and progesterone productions are taken over by the placenta and fetal adrenal glands for ensuring maintenance of pregnancy. In other placenta-dependent species such as sheep and primates (in which the CL regresses well before term), a decrease in particular placental steroids may be the key to the parturition process.

Parturition is triggered by the fetus and is completely complex interaction of endocrine, neural, and mechanical factors. The uterine prostaglandins participate in the initiation of delivery of the fetus at term by actions at the number of target sites. Furthermore, estrogen enhances uterine excitability by various mechanisms that include the increasing endometrial prostaglandins synthetase activity, the up-regulating of myometrial and endometrial oxytocin (OT) receptors, and an increasing of OT production and secretion by the neurohypophysis. These mechanisms are facilitated by the rising in estrogen : progesterone ratio (de Ziegler et al., 1998).

1.4 Mechanism of Uterine Contraction and Relaxation

The functional cell of the uterus is the myocyte, which is a homogenous cell type. Certainly, it is responsible for generation of contraction force, passage of action potentials, and control of contractility. Uterus is spontaneously active and produces normal spontaneous contraction without any stimulation. This implies that the uterus can impulsively contract without agonist stimulation, and also exhibit both tonic and phasic contraction (Wray et al., 2001).

1.4.1 Mechanism of Uterine Contraction

As in other types of smooth muscles, the uterine myocytes is well established that a calcium ion (Ca^{2+}) is an essential activator for contraction. In general, the

smooth muscle contraction can be achieved by the increasing in free cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_i$) and enhances the binding of Ca^{2+} to calmodulin (CaM). A Ca-CaM complex activates myosin light chain kinase (MLCK), which further phosphorylates the 20-kDa regulatory side chain of myosin light chains (MLC_{20}). This phosphorylation reaction subsequently triggers cross-bridge cycling of actin and myosin to generate the contraction (Matthew, Shmygol and Wray, 2004). The mechanisms which modulate the uterine contraction may generate through two main mechanisms: electrochemical and pharmacomechanical mechanism.

Electrochemical Mechanism

This mechanism depends on the changing of membrane potential to operate the uterine contractility. Uterine contraction is activated by membrane depolarization that opens voltage gate Ca^{2+} channels (VOCC) (Garfield and Maner, 2007) or potassium channel (Miyoshi, Urabe and Fukiwara, 1991), allowing extracellular Ca^{2+} influx into cell and trigger the contraction. This depolarization may be stimulated by plasma membrane oscillator. This action generates the periodic pacemaker depolarization responsible for the action potentials that drive force production (Berridge, 2008).

Pharmacomechanical Mechanism

This mechanism is independent from the alteration in membrane potential. It is based on the activation of guanine nucleotide binding protein (G-protein) and secondary messenger by different agonists (hormones neurotransmitter or drugs), resulting in muscle contraction. This activation enhances the releasing of Ca^{2+} from an internal store, the sarcoplasmic reticulum (SR) via inositol (1,4,5)-trisphosphate receptor (IP_3R) or ryanodine receptor (RyR) (Kupittayanant, Lucklas and Wray,

2002). Finally, MLC_{20} is phosphorylated by MLCK due to the activating by Ca^{2+}-CAM complex.

Indeed, Ca-mediated pathway has been acknowledged as the principle mechanism by which regulates MLC phosphorylation and contraction. Recently, the researchers have demonstrated that some biochemical activators can induce smooth muscle contraction without the raising of $[Ca^{2+}]_i$ concentration (Himpens, Kitazawa and Somlyo, 1990; Rembold, 1990; Ishine et al., 1992). This phenomenon is characterized as a Ca^{2+} sensitization in smooth muscle contraction. Some studies elucidated that this Ca^{2+}-independent contraction occurs mainly through the inhibition of MLC phosphatase (MLCP) and involves the monomeric GTP-protein RhoA. This signaling is known as Rho/Rho-associated kinase (ROK) pathway which prefers to produce tonic force rather than phasic contraction (Somlyo and Somlyo, 2003). Kupittayanant , Burdyga and Wray (2001) also demonstrated that uterine contraction activated by Rho/ROK pathway can be accomplished without the changing of $[Ca^{2+}]_i$. Together, these evidences indicated that the Rho/ROK pathway may not imperative for modulation of force development in the myometrium under physiological condition.

The stimulation of Rho through G-protein couple receptor leads to the stimulation of ROK. Activated ROK further phosphorylates the myosin binding-subunit of MLCP. The action of Rho/ROK cascade results in the inhibition of MLCP activity and parallel modulation the MLCK activity that convergent enhances contractile activity (Lartey and López Bernal, 2009).

A protein serine/threonine kinase C (PKC) is proposed to be a candidate in Ca^{2+} sensitization in smooth muscle. It is activated by diacyl dlycerol (DAG) and has

been reported to increase uterine contractility. DAG can also hydrolyse diacyl and monoacylglycerol, resulting in the production of arachidonic acid that further inhibits MLCP (Gong et al., 1992). PKC can trigger the releasing of Ca^{2+} from SR via activating IP_3R, and also directly phosphorylates the MLCP (CPI17) that causes contraction (Eto et al., 1997). Mitogen-activated protein (MAP) kinase is known to be linked to the signal cascade between the reactions of PKC. To date, the signaling of MAP activation is still unclear. MAP kinase is activated by some agonists such as oxytocin or the high KCl-induced depolarization, hence the contractile cascades enhancement (Katoch and Moreland, 1995; Nohara et al., 1996).

The actin-binding proteins caldesmon (Cd) and calponin (Cp) are known to inhibit acto-myosin ATPase that results in the prevention of acto-myosin interaction in myocytes (Winder and Walsh, 1993; Alahyan, Marston and Mezgueldi, 2006). The phosphorylation of Cd and Cp by PKC leads them to conformational changes, and both no longer inhibit acto-myosin interaction. These consequence interactions lead to the initiation of contraction process (Gerthoffer and Pohl, 1994). Furthermore, some investigators have reported a zipper-interaction protein (ZIP) kinasse may modulate smooth muscle contraction by phosphorylating the MLC_{20} in a Ca^{2+}-CaM independent pathway (Murata-Hori et al., 1999; Niiro and Ikebe, 2001).

1.4.2 Mechanism of Uterine Relaxation

During relaxation, the main goal is to reduce the free $[Ca^{2+}]_i$. There are two mechanisms which responsible for this alteration: 1) the removing of cytoplasmic Ca to the extracellular compartment via Na/Ca exchanger (NCX) or plasma membrane Ca-ATPase (PMCA), and 2) refilling the internal Ca^{2+} stores by the action of SR Ca^{2+}

ATPase (SERCA pump). The depletion of $[Ca^{2+}]_i$ leads to inhibition of Ca-CaM-dependent MLCK activity and MLC_{20} phosphorylation (Wray et al., 2003).

Myosin light chain phosphatase (MLCP) is the key enzyme for controlling the whole mechanism of smooth muscle relaxation. MLC_{20} is dephosphorylated by MLCP, modulates the resetting of contractile system and relaxation occurs. The alternative pathways which modulate smooth muscle relaxation may involve with some protein kinase enzymes. Relaxation is enhanced by increasing cyclic adenosine monophosphate (cAMP) dependent protein kinase A (PKA). This enzyme increase cAMP level and also deactivates MLCK. As a result, it decreases the affinity of the Ca^{2+}-CaM complex. They also inhibit Ca mobilization and RhoA/ROK activity which leads to increases MLCP activity and induces MLC_{20} dephosphorylation, consequently muscle relaxation (Murthy, 2005).

Additionally, nitric oxide (NO) is becoming more fascinated, since it causes uterine relaxation by the activation of guanlylate cyclase to produce cyclic guanosine monophosphate (cGMP). The pharmacological mechanism of cGMP mediated decrease in $[Ca^{2+}]_i$, includes: 1) hyperpolarization by opening potassium channels (White et al., 2000); 2) reduces Ca^{2+} influx with a constant membrane potential (Anwer et al., 1992); and 3) increases Ca^{2+} efflux and/or Ca^{2+} sequestration (Rembold, 1995).

1.5 *Talinum paniculatum* (Jacq.) Gaertn.

Talinum paniculatum (Jacq.) Gaertn. (*T. paniculatum*) belongs to Portulaceae family that is native to the Southern United States, the Caribbean, Mexico, Central and South America. *T. paniculatum* is commonly called “Jewel of Opar”, “flame

flower" or "Baby's-breath". *T. paniculatum* can be found in Indonesia by the name of "Som Java". It categorizes as a wild type plant, but generally can be locally throughout Thailand and known as "Wan Pag Pang" or Som Thai. Naturally, *T. paniculatum* is the herb, which can reach a height of 100 cm, and have a well-developed root system. It is a deciduous perennial herb with lignified stems and succulent bright green leaf. Its flowers are in terminal panicles, small and pink colored.

The Thai herbal repository reported that *T. paniculatum* root is known as a tonic remedy that promotes fertility and rectifies the gynecological problems like irregular in menstrual cycle. The leaf have the galactogue, anti-inflammatory effects and able to cure ulcer. In addition, the leaf can also encourage healthy body, and increase appetite (Petprai et al., 1996).

Recent research regarding the sows supports the traditional use of *T. paniculatum* leaf as a dietary supplement, promoting the reproductive productivity and physical health. It has been demonstrated that *T. paniculatum* leaf-treated group significantly increase in litter sizes and weight gain after weaning when compared with control group (Salakit, Jungsamanyat and Salakit, 1990).

The phytochemical compositions of *T. triagulare* and *T. portulacifolium*, the plants that belong to Portulaceae family, were carried out. Alkaloids, flavonoids, saponins and tannins, were present in *T. triangular* leaf extract. The valuable pharmaceutical properties in *T. triangulare* leaf extract may be attributed to the presence of their bioactive compounds such as anti-oxidant, anti-inflammatory, and cardioprotective activities (Aja et al., 2010). The investigation of *T. portulacifolium*

leaf extract showed the evidence of anti-oxidant and antidiabetic properties in a dose-dependent manner (Babu et al., 2009).

Manuhar, Yachya and Kristanti (2012) demonstrated that the root of *T. paniculatum* is the richest source of steroidal saponins and can be used in many medicinal purposes. The root extract of *T. paniculatum* can improve mice libido higher more than Korean ginseng's root extract in the condition of low testosterone (Winarni, 2007). The previous primary phytochemical analysis showed the chemical components in *T. paniculatum* root extract are similar with the Chinese and Korean ginseng (*Panax ginseng*), and this plant is usually used as a substitute for ginseng (Yulia, Wientarsih and Razief, 2005).

1.6 Phytoestrogens

Phytoestrogens are plant-derived compounds that, because of their structural similarity with mammalian estrogen, may display both estrogenic and anti-estrogenic effects. There are 3 major classes of phytoestrogens; isoflavones, coumestans (coumestrol), and lignans. Isoflavones are found in high concentration in soybean, soybean products (e.g. tofu) and red clover. Lignans are mainly found in flaxseed. The main dietary source of coumestrol is legumes; however low levels have been reported in brussel sprouts and spinach.

All phytoestrogens are diphenolic compounds with structural similarities to natural and synthetic estrogens. They have the capability of binding to the estrogen β receptor (ER_β), but with a weak affinity compared to estradiol. The metabolism of phytoestrogens in human is complex: once ingested, lignans are transformed by the intestinal microflora and converted to hormone-like compounds. On the other hand,

isoflavones (which are present in soy as glycosides) are initially hydrolyzed by glucosidases of the intestinal bacteria and then metabolized to glucuronide conjugates in the intestine and liver. Thus, the bioavailability of phytoestrogens depends on the intestinal microflora (Borrelli and Ernst, 2010).

As mentioned above, phytoestrogens' structure is similar to those mammalian ERs; compete with ERs for its cellular response. Human and rat ERs exist as two subtypes; ER_{α} and ER_{β}, which exhibit specific tissue localization and levels of expression. ER_{α} is more abundant than ER_{β} in female reproductive tissue, while ER_{β} is mainly distributed in bone tissue. Phytoestrogens can proceed as both estrogenic and antiestrogenic actions that depend on their affinity to ERs (Heldring et al., 2006).

Phytoestrogens estrogenic activity was first explored in postmenopausal women by Wilcox et al. (1990). They illustrated that soy supplemented diet significantly improvements in vaginal cell maturation index. Numerous scientific data confirm that phytoestrogens improve the vaginal dryness and other reproductive organs disorder by the activation of ER_{α} (Suetsugi et al., 2003; Harris et al., 2005; Chrzan and Bradford, 2007).

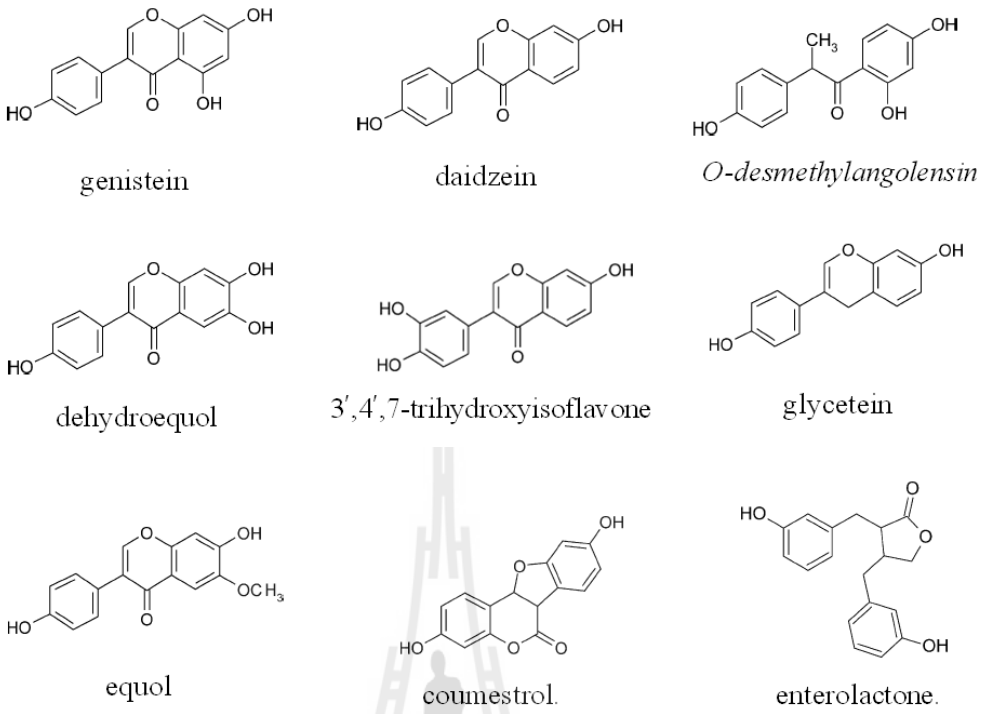

Figure 1.3 Structure of important phytoestrogens (Knight and Eden, 1995).

Epidemiological and experimental studies imply that the consumption of a phytoestrogen-rich diets may have beneficial effects on reproductive health and have a lowering the risk of various type of cancers. There is also suggested that phytoestrogens may prevent or alleviate other estrogen-related diseases such as cardiovascular diseases, osteoporosis, and cognitive problems (Carlson et al., 2008).

The activation of ERs also exhibits the positive estrogenic effects on non-reproductive tissues such as bone or cardiovascular system. The consumption of phytoestrogens- rich diet has been proven to improve bone remodeling on both human and rodent ovariectomy model. Many studies indicate that phytoestrogens could be the ideal candidates for treatment of osteoporosis due to their ability to stimulate osteoblastic activity and inhibit osteoclast formation (Chiechi and Micheli, 2005; Liu et al., 2008). The effects of phytoestrogens on bone metabolism markers may closely

resemble that of other estrogen mechanisms. These mechanisms include promotion of calcium absorption, and increase insulin like growth factor-1 (IGF-1), which is known to modulate osteoblastic activity. Additionally, phytoestrogens possess positive effects on some bone biomarkers by increasing the bone formation activity (e.g. specific bone alkaline phosphatase or osteoblastic activity marker), but suppressing bone turnover markers (e.g. total alkaline phosphatase, plasma/serum osteocalcin, urinary pyridinoline and deoxypyrodinoline cross links) (Setchell, Brown and Lydeking-Olsen, 2002; Setchell and Lydeking-Olsen, 2003; Burali et al., 2010).

In contrast, phytoestrogens have also been reported to protect against various types of cancers, especially breast and endometrium cancers due to their antiestrogenic activity. The mechanism by which phytoestrogens exert their antiestrogenic activity is still unclear. The proposed mechanism may be due to the presence of these low-affinities, low-potency ligands for ERs can reduce the effect of potent endogenous estrogens when they are present in sufficient quantities. This action results to the net effect of antagonizing the estrogen-responsive system which is used for the treatment and prevention of some hormone-related cancers (Katzenellenbogen and Muthyala, 2003).

Lower in the TC concentration has been shown to reduce cardiovascular diseases risks. Consuming plant sterols (sitostanol, sitosterol, stigmasterol and campesterol) exert healthy benefits to the heart by altering lipid metabolism in various animal and human studies (Heinemann, Leiss, and van Bergmann, 1986; Ling and Jones, 1995). The studies demonstrated that dietary intake of phytoestrogen-rich diets showed a significant reduction in TC, LDL, and triglyceride with an elevation in HDL levels (Jayagopal et al., 2007; Osma, Ayad and El-Mahdy, 2011). Several

mechanisms of plant sterols effective on cholesterol concentration may include: 1) formation of a nonabsorbable complex with cholesterol; 2) altering the size and/or stability of the micelles; 3) interferences with cholesterol esterification in the mucosal cell; 4) interacting with protein receptors which are required in cholesterol absorption (Rong, Ausman and Nicolosi, 1997; Sunita and Pattanayak, 2011).

1.7 Phytols and Phytanic Acid

Phytols are terpenes alcohol (3,7,11,15-tetramethylhexadec-2-en-1-ol) that are naturally partial of chlorophyll molecule. They are universally found in green vegetables or plants. Only ruminants can obtain phytols from plant-chlorophyll that breakdown by their gastrointestinal flora. The release of phytols from chlorophyll takes place effectively by ruminant's bacterial gut (Hansen, 1966) that further accumulated in relatively high quantity in meat, milk or fat in the form of phytanic acid (Brown et al., 1993).

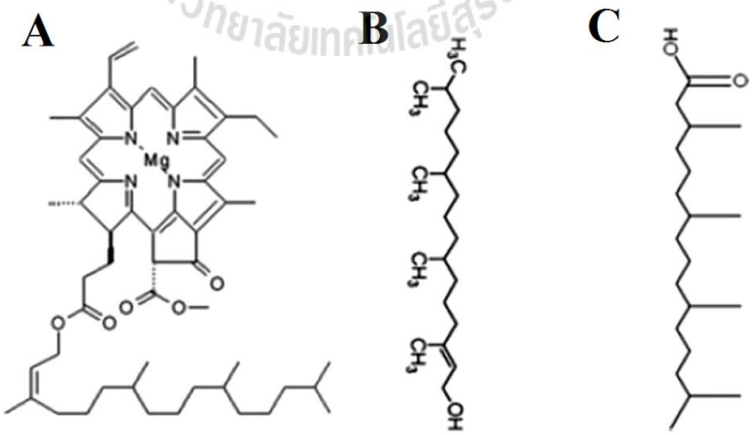

Figure 1.4 The structure of chlorophyll (A), phytol (B) and phytanic acid (Hellgren, 2010).

Phytols as well as other terpenoids have been found to influence on normal biological processes. Plant terpenoids are cytotoxic to tumor cells that lead them to be as valuable chemotherapeutic or chemo-preventive compounds (Mo and Elson, 2004; Thoppil and Bishayee, 2011). Additionally, they are reported to be involved with cellular function, defense, and communication in some biosynthetic pathways. These beneficial outcomes may be implicated with hypocholesterolemic action, defense or repellents some toxins, and in communications as hormones in aggressions and alarm pheromones (Harrewijn et al., 2001, Pickett and Gibson, 1983).

To date, it is well accepted that dietary phytols are the potential precursor of phytanic acid both in experimental animals and in human. In mammals, free phytols are rapidly absorbed in small intestine and subsequently converted to phytanic acid by hepatic enzyme (Mize et al., 1966). The administration of phytols-enriched diet results in an increase of phytols metabolites; phytanic acid and pristanic acid in tissues and plasma (Van den Brink et al., 2004). It has previously been reported that the orally administration of whole green vegetables to mammal, only less than 5% of the total phytols content was absorbed into the intestinal lymph. Thus, dairy products and meat from ruminants is the major sources of phytanic acid for human (Baxter, 1968).

Phytanic acid is known to be the natural agonist of and the peroxisome proliferator-activated receptor α (PPARα) which regulates carbohydrate metabolism (Keller et al., 1993). It also activates retinoid X receptor (RXR) that appears critical to regulating important aspects of lipid metabolism (Lefebvre, Benomar and Staels, 2010). The activation of both PPARα and RXR alternatively regulate estrogen responsiveness genes that apparently regulate gene expression in their target organs

(Nuñez et al., 1997). Therefore, this has been used as a model to study the effects of the accumulation of phytols metabolites on fatty acid metabolism, in particular via the activation these receptors. These data emphasize that the potential uses of phytanic acid or its precursor molecules as natural products may be beneficial for treatment of various ailments (Chowdhury and Ghosh, 2012).

1.8 Aims

The aims of this thesis were to explore the effects of *T. paniculatum* extracts and its related-component, phytol on: 1) reproductive hormones (estrogen and LH), and blood biochemistry (low-density lipoprotein (LDL), high-density lipoprotein (HDL), triglycerides and total alkaline phosphatase (tALP)); 2) female reproductive organs (vagina, uterus and mammary tissues); 3) the anti-fertility activity; and 4) uterine contraction in adult female rats.

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CHAPTER II

GENERAL MATERIALS AND METHODS

This chapter provides a general description of materials and methods utilized to perform the experiments in this thesis. More specific details in each study are documented in each of the fowwing chapter.

2.1 Plant Materials

2.1.1 Plant Collection and Identification

T. paniculatum samples were collected from the northeastern area of Thailand; where they grew under natural conditions during the month of November 2010. Voucher specimen was identified and deposited at the Royal Forest Department of Thailand, Bangkok, Thailand (BKF174387).

2.1.2 Plant Extraction

Root or leaf powder (10 g) was extracted separately with refluxing methanol in a Soxhlet apparatus for 12 h. The extracts were filtrated, evaporated under a reduced pressure at low temperature in a rotary evaporator, and dried by a lyophilizer. The dried extracts were stored at -20°C until use.

2.1.3 Phytochemical Screening

The plant extracts were subjected to both qualitative and quantitative phytochemical screenings to identify the various classes of phytochemical constituentsThe qualitative phytochemical analysis of the plant extracts were carried

out by using standard procedures as previously described (Tiwari et al., 2011). The tested compounds included alkaloids, flavonoids, tannins, saponins and phytosterols.

The quantitative phytochemical screening of the plant extracts were analyzed by GC-MS (A Agilent Technologies 7890A gas chromatograph, coupled with an Agilent Technologies 5975C (EI) mass spectrometer). The separation was performed on an HP-5MS column; 30 m x 0.25 mm ID x 0.25 mm film thickness. The temperature of the column was programmed from 50°C to 300°C at the rate of 10°C /min. The injector's temperature and the detector's temperature were 250°C. Helium gas was used as the carrier gas with a constant flow rate of 1.0 µL/min. All separated compounds were identified from the recorded mass spectra by comparing the mass spectra from the NIST and Wiley libraries.

2.2 Animal Protocols

2.2.1 Animal Ethics

Animal care, environmental conditions and used were followed the guidelines of Laboratory Animal Resources, National Research Council of Thailand. The procedures of the experiment were approved by the Institutional Animal Care and Use Committee, Suranaree University of Technology, Nakhon Ratchasima, Thailand.

2.2.2 Housing

Female Wistar rats were individually house in 24 x 15 x 15 cm cages under a 12:12-hr light-dark illumination cycles, at a constant temperature of 25 $\pm$ 0.5°C and 45-50% humidity. All rats were fed with the standard laboratory food containing 0.8% calcium (CP. Co. Ltd, Thailand). *Ad libitum* of water were also provided.

2.2.3 Bilateral Ovariectomy

Adult female Wistar rats (200-250 g) were anesthetized using thiopental (25-35 mg/Kg BW/day) intraperitoneally, and bilaterally ovariectomy (OVX) was operated via paralumbar incision just caudal to the 13^{th} rib. The sham-operated rats were subjected to sham surgery, but the ovaries were not removed (Shih, Wu and Lin, 2001). Rats were administered the antibiotic (amoxicillin 250 mg/Kg BW/day, orally). After 14 days of endogenous hormonal decline (Tanee et al., 2007), the OVX rats were subjected into each study as the animal model of sex hormone-depleted experiment (Wu et al., 2005).

2.2.4 Vaginal Cytology

Vaginal smear was performed to examine cellular differentiation and to evaluate the presence of leukocytes, nucleated cells, or cornified cells. Vaginal smear samples were collected between 9:00-10:00 am daily by gently inserting the tip of dropper into the vagina, flushing normal saline (0.9% NaCl) in and out, and placing the fluid onto microscope slides and stained by Methylene blue dripping (Parhizkar et al., 2011). The appearance of cornified cells was used as an indicator of estrogenic activity

2.2.5 Tissue Histological Preparations

Horns of uterus, vagina and mammary tissue were fixed in 10% formalin solution and cut into short segments using the paraffin technique. Sections of 5 μm thicknesses were cut and stained using routine hematoxylin and eosin method. All organs were observed and measured on hematoxylin and eosin stained slides, and 3 randomly chosen areas of the section were measured per slide. Images of organ cross-sections were taken using a Nikon Eclipse 80i Upright microscope (Hollywood

International Co., Ltd., Thailand) and Cell^D imaging software (Olympus, EforL International Co., Ltd., Thailand). The number, thickness, size of the organs and epithelial lining were analyzed by using Image J v1.41 software (Cordial et al., 2006).

2.2.6 Mating

In order to the investigation of *T. paniculatum* extracts' efficacy to produce anti-fertility activity, pregnant rats were used (Mukhram et al., 2012). Adult virgin female rats (200-250 g) in the proestrous stage were selected and left overnight with proven fertile male (1 female : 1 male). The rats that showed thick clumps of spermatozoa in vaginal smears were separated and designated as 1^{st} day pregnancy.

2.2.7 Myometrium Tissue Preparation and Tension Measurement

In order to investigate the uterine contractility from *T. paniculatum* extracts in non-pregnant rats, the rat was humanely sacrificed by CO_2 asphyxia; the uterus was dissected and immediately immersed in physiological Krebs' solution (pH 7.4). The uterine strip was cut into longitudinal strip (1-2 mm x 0.5 mm x 10 mm) and attached at each end to the metal hooks. Another hook was fixed to a transducer (ADInstruments Pty Ltd., Spain) in the organ bath that containing Krebs's solution at 37°C. The strip was allowed to contract spontaneously under a resting tension of 1g. An equilibration time of 30 min was applied for all tissues before the application of any chemical study. The change in isometric force was measured during 5-7 min with PowerLab system software (ADInstruments Pty Ltd., Australia). The electrical signal from the transducer was amplified and converted to a digital signal and recorded on a computer using Chart software (Kupittayanant, Burdyga and Wray, 2001).

2.3 Chemicals

17ß-estradiol (E8875) and standard-phytol (139912) were purchased from Sigma-Aldrich Chemical Co. (St.Louis, MO, USA). All solvents and chemicals used were of analytical grade and obtained from Sigma® and Merck®. All stock solutions were prepared and stored in accordance with the guideline of the producer.

2.4 Statistical Analysis

In chapter III-VI, statistical differences analysis between groups were performed by analysis of variance (ANOVA) followed by Scheffe's *post hoc* test using SPSS windows program version 11 (SPSS Institute, Inc., Chicago, IL, USA). A probability level that was less than 5% ($P < 0.05$) was considered statistically significant. All data were expressed to the mean value $\pm$ standard error of the mean (S.E.M.) and "*n*" represented as the number of animal in each experimental group.

In chapter VII, all data were expressed as percentage of control of contractions (i. e. the control is 100%). Parameters that were measured include maximum tension developed on each contraction, the contraction integral (total tension developed in each contraction), duration of contraction and frequency. All data were evaluated using Microcal Origin Software and were then presented as mean $\pm$ S.E.M.. The differences between control and treatment groups were analyzed by student *t*-test, and "*n*" represented as the number of uterine sample from a different animal.

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CHAPTER III

PHYTOCHEMICAL SCREENING OF *TALINUM PANICULATUM* (JACQ.) GAERTN. METHANOLIC ECTRACTS AND THEIR POSSIBLE THERAPEUTIC VALUES

3.1 Abstract

Talinum paiculatum (Jacq.) Gaertn. (*T. paniculatum*) or "Som Java*"*, belongs to the family Portulacaceae. It commonly grows in tropical and subtropical areas including, Thailand. This plant has long been used in the traditional medicine from ancient time for diverse arrays of purposes. The aims of this study were attempted to qualitatively and quantitatively determine the phytochemical constituents from the two different parts of *T. paniculatum* methanolic extracts (root and leaf). The qualitative phytochemical analysis was conducted to detect alkaloids, flavonoids, tannins, saponins and phytosterols by observing the changes of the extract's color. The GC/MS analysis was also performed for the qualitative phytochemical constituents of the plant extracts. The results showed that alkaloids, tannins, flavonoids and phytosterols were observed in both parts of the plant while only saponins were found in the root extract. The GC/MS analysis of the root extract showed the presence of 5 phytosterols which were β-sitosterol (17.37%),

stigmasterol (4.23%), stigmastan-3-ol (4.10%), stigmast-22-en-3-ol (1.84%) and campesterol (1.56%), respectively. 12 known compounds that included fatty acids (0.50%-11.32%) and 2 unknown compounds were detected. The leaf extract showed the presence of 4 phytosterols which were β-sitosterol (10.60%), stigmastanol (2.76%), stigmasterol (0.85%) and campesterol (0.80%). 11 known compounds: phytols (69.32%), α-tocopherol (0.99%), fatty acids (0.43-3.41%) and 2 unknown compounds were also identified.

3.2 Introduction

Plants are the natural sources that produce the richest phytochemical constituents with precise therapeutic values. Since the bioactive phytochemical compounds were isolated and identified, many of them are used as the active ingredients of the modern medicine or as the compounds for natural drug improvement. Plants produce an extraordinarily wide range of over 500,000 low molecular mass natural products which are known as secondary metabolites. These secondary compounds such as alkaloids, flavonoids, phenolic compounds, tannins, saponins or phytosterols are recognized to produce a definite physiologic action in human body or able to treat various disease conditions (Edeoga, Okwu and Mbaebie, 2005).

Talinum paniculatum (Jacq.) Gaertn. (*T. paniculatum*) or “Som Java” is one of the plants in Portulacaceae family that contains notable medicinal properties (Thomas, 2008). *T. paniculatum* is a wild deciduous perennial herb with well-developed root system. It is naturally grown around the world, including Thailand with the local name of Wan Pak Pang. In Thailand, the locals consume the leaf as vegetable

supplement and the roots as reproductive tonic. Preparation of *Talinum* spp. has long been used in traditional medicine, particularly in the treatment of type-2 diabetes, inflammatory skin problems, gastrointestinal disturbance, general weakness and reproductive disorders (Shimoda et al., 2001; Pak et al., 2005). Steroidal saponins are active constituents found in the root, while only tannins can be detected in the leaf. (Yulia, Wientarsih and Razief, 2005). Additionally, Filho and colleagues (2010) isolated and reported that compesterol, β-sistosterol, stigmasterol could be extracted from the leaf of *T. paniculatum.*

However, the plants in *Talinum* spp. are recognized for some of their constituents and biological properties. The native *T. paniculatum* which grown in Thailand had never been identified and there is no scientific data to prove the traditional uses. The aim of this study was designed to qualitatively and quantitatively investigate the phytochemical constituents of *T. paniculatum* root and leaf methanolic extracts. The knowledge of the chemical constituents of this plant is valuable, not only for the discovery of therapeutic agents, but also for further evaluating the actual value of folkloric remedies.

3.3 Materials and Methods

3.3.1 Plant Collection and Identification

The fully grown plants, *T. paniculatum* (50-80 cm in height), were collected from the northeastern area of Thailand in November, 2010. A voucher specimen (BKF174387) was deposited and identified by Botanist at the Royal Forest Department of Thailand, Bangkok, Thailand.

3.3.2 Plant Extraction

The powder of root or leaf (10 g) was extracted separately by refluxing methanol in a Soxhlet apparatus for 12 h. The extracts were concentrated in a rotary evaporator, dried by a freeze dryer and finally stored at -20°C until use. The % yield of the extract was calculated using the following formula:

$$\% \text{ yield} = \left(W_{\text{crude extract}}/W_{\text{dried plant}}\right) x\ 100$$

$W_{\text{crude extract}}$ is the mean weight of crude extract and $W_{\text{dried plant}}$ is the mean weight of dried plant.

3.3.3 Phytochemical Analysis

Preliminary Phytochemical Analysis

The plant extracts were subjected to preliminary phytochemical screening with various qualitative chemical tests to identify the presence or absence of various classes of phytochemical constituents using standard qualitative procedures as previously described (Tiwari et al., 2011).

The test for alkaloids was carried out by adding 0.5 g aqueous extract in 5 mL 1% HCl, boiled and filtered. Then Mayer's reagent was added. Formation of a yellow colored precipitate (potassium mercuric iodide) indicates the presence of alkaloids.

The test for tannins was carried out by adding 0.5 g aqueous extract in 5 mL 1% gelatin solution containing sodium chloride. Formation of white precipitate indicates the presence of tannins.

The test for flavonoids was carried out by using alkaline reagent test. The extracts were treated with few drops of sodium hydroxide solution. Formation of intense yellow color, which becomes colorless from the addition of dilute acid, indicates the presence of flavonoids.

The test for saponins was carried out by using froth test. The extracts were diluted with distilled water to 20 mL and were shaken in a graduated cylinder for 15 minutes. Formation of 1 cm layer of foam indicates the presence of saponins.

The test for phytosterols was carried out by using Salkowski's test. The extracts were treated with chloroform and filtered. The filtrates were treated with few drops of concentrated solution of sulphuric acid (H_2SO_4), shaken and allowed to stand. Appearance of golden yellow color indicates the presence of phytosterols.

GC/MS Analysis

The quantitative phytochemical screenings of the crude extracts were performed using GC-MS (A Agilent Technologies 7890A gas chromatograph, coupled with an Agilent Technologies 5975C (EI) mass spectrometer). The separation was performed on an HP-5MS column; 30 m x 0.25 mm ID x 0.25 mm film thickness. The temperature of the column was programmed from 50°C to 300°C at 10°C /min. The injector temperature and the detector temperature were 250°C. Helium was used as the carrier gas with a constant flow rate of 1.0 µL/min. All separated compounds were identified from the recorded mass spectra by comparing the mass spectra from the NIST and Wiley libraries.

3.4 Results

3.4.1 Botanical Profile of *T. paniculatum*

Kingdom: Plantae

Division: Magnoliophyta

Class: Magnoliopsida

Family: Portulacaceae

Genus: Talinum

Species: *Talinum paniculatum* (Jacq.) Gaertn

Other name: *Talinum paiculata* (Jacq.) Gaertn

Common name: Jewels of Opar, Som Java, Wan Pak Pang, Som Thai

3.4.2 Description of *T. paniculatum*

T. paniculatum belongs to the Portulacaceae family. It has a world-wide distribution and commonly grows in tropical and subtropical areas. It is a wild deciduous perennial herb with tuberous rooted (Figure 3.1A). Habit is upright from 1-3 feet tall by 2 feet wide. It has fleshy, delicate and waxy green leaf (Figure 3.1B). The stems or branches are terminated by a multiflower panicle or modified panicle. The flowers' shade are hot pink color which hold by sinewy flower stalks and carried in airy panicles from late spring to summer.

(A) **(B)**

Figure 3.1 General morphology of *T. paniculatum* root (A), stem and leaf (B).

3.4.3 The Yield of *T. paniculatum* Extracts

The root extract was a yellowish to brownish power, whereas the leaf extract was a greenish sticky mass. The yields of the root and leaf extracts were 6.67%, and 9.62%, respectively.

3.4.4 Preliminary Phytochemical Constituents of *T. paniculatum* Root and Leaf Extracts

The qualitative estimation of primary phytochemical constituents of the root and leaf methanolic extracts is summarized in Table 3.1. The results revealed the presence of various active medical compounds in the *T. paniculatum* extracts. The results showed that alkaloids, tannins, flavonoids and phytosterols were observed in both parts of the plant while only saponins were found in the root extract. These compounds may be responsible for several medicinal activities of *T. paniculatum* (Table 3.1).

3.4.5 GC/MS Analysis

The phytochemical components of the plant extracts are present in Table 3.2 and Table 3.3. The GC/MS analysis of the root extract showed the presence of 5 phytosterols which were β-sitosterol (17.37%), stigmasterol (4.23%), stigmastan-3-ol (4.10%), stigmast-22-en-3-ol (1.84%) and campesterol (1.56%), respectively. 12 known compounds were fatty acids (0.50%-11.32%) and 2 unknown compounds were detected (Figure 3.2).

The leaf extract showed the presence of 4 phytosterols which were β-sitosterol (10.60%), stigmastanol (2.76%), stigmasterol (0.85%) and campesterol (0.80%). 11 known compounds; phytols (69.32%), α-tocopherol (0.99%), fatty acids (0.43-3.41%) and 2 unknown compounds were identified (Figure 3.3).

Table 3.1 Preliminary phytochemical analysis of *T. paniculatum* extracts and their reported medicinal activities. (-) indicates as negative reaction and (+) indicates as positive reaction.

Secondary Metabolites	Root	Leaf	Examples	Medicinal Activity	References
Alkaloids	+	+	Berberine, coptisine, Piperine, Palmatine, Tetrahydropalmatine	Antimicrobial (bacteria, fungi, protozoa), Antihelmintic activity, Antidiarrhea, Antioxidant, Antiinflammatory activity, Antidepressant, Renoprotective agent , Anticancer , Antimutagenic, Hepatoprotetive activity, Cardioprotective activity	Singh et al., 2010 Tiwari et al., 2011 Lu et al., 2012
Flavonoids	+	+	Chrysin, Quercetin, Rutin, Kaempferol, Cyadinin, Genistein, Diadzien	Antimicrobial, Antidiarrhea, Antioxidant, Antiatherosclerotic, Antiplatelet aggregation, Antithrombogenic, Antiviral, Antiulcerative, Antiinflammaroty, Antiarthritis, Antiosteoporotic, Antileukemic activity	Hodex, Trefil and Stiborova, 2002 Cazarolli et al., 2008 Patel, 2008

Table 3.1 Preliminary phytochemical analysis of *T. paniculatum* extracts and their reported medicinal activities. (-) indicates as negative reaction and (+) indicates as positive reaction (continued).

Secondary Metabolites	Root	Leaf	Examples	Medicinal Activity	References
Saponins	+	-	Ginsenosides, Vina-ginsenosides-R5 and -R6	Antifungal, Antidiarrhea, Hepatoprotective, Antidiabetics, Antitumor/cancer, Sexual impotence, Adaptogenic	Nocerino, Amato and Tzzo, 2000 Tsuzuki et al., 2007 Man et al., 2010 Tiwari et al., 2011
Tannins	+	+	Totarol, Ellagitannin, Gallotamine, Gallic Acid, Hexahydroxydiphenic acid	Antimicrobial, Anthelmintic, Antidiarrhea, Cardioprotectant, Antihypertensive, Antitumor, Antiviral, Antiinflammatory, Antiulcerative	Liu et al., 2003 Okuda, 2005 Souza et al., 2007 Tiwari et al., 2011

Table 3.1 Preliminary phytochemical analysis of *T. paniculatum* extracts and their reported medicinal activities. (-) indicates as negative reaction and (+) indicates as positive reaction (continued).

Secondary Metabolites	Root	Leaf	Examples	Medicinal Activity	References
Phytosterols	+	+	B-sitosterol, Stigmasterol, Campesterol, Coumestrol, Brassicasterol, Daidzen, Genistein, Formononetin, Equol, Diosgenin	Antidiarrhea, Cardioprotective, Cholesterol lowering agents, Antidiabetics, Anti-inflammatory, Anti-bacterial, Antifungal, Antiulcerative, antitumor/cancer, Antiosteoporotic, Reproductive enhancer, Anti-fertility, Aphrodisiac, Immunostimulant	Rao and Koratkar, 1997 Bouic and Lamprecht, 1999 Gabay et al., 2010 Tiwari et al., 2011 Hörmann et al., 2012 Mbambo, Odhav and Mohanloll, 2012

Table 3.2 The phytochemical constituents of *T. paniculatum* root extract were detected by GC/MS.

Peak	Identified Compound	Retention time (min)	Peak area (%)
1	Cycloesanone	5.568	0.50
2	Benzoic acid	23.164	5.45
3	Palmitic acid	30.171	11.04
4	Palmitic acid ethyl ester	30.745	2.08
5	Linoleic acid	32 .605	0.50
6	Oleic acid	32.721	1.59
7	Cis,cis-Linoleic acid	33.326	4.01
8	9-octadecenoic acid, (E)	33.431	6.76
9	9,12-ocladecadienoic acid, ethyl ester	33.813	2.15
10	Ethyl oleate	33.918	1.36
11	Bis (2-ethylhexyl) adipate	37.805	11.32
12	Unknown	39.052	21.54
13	Palmitic acid beta-mono glyceride	39.505	1.31
14	Campesterol	49.353	1.56
15	Stigmasterol	49.754	4.23
16	Stigmast-22-en-3-ol (3α, 5α, 22E, 24XI)	49.875	1.84
17	β-sitosterol	50.484	17.37

Table 3.2 The phytochemical constituents of *T. paniculatum* root extracts were detected by GC/MS (continued).

Peak	Identified Compound	Retention time (min)	Peak area (%)
18	Stigmastan-3-ol (3β, 5α, 24S)	50.604	4.10
19	Unknown	52.342	1.19

Table 3.3 The phytochemical constituents of *T. paniculatum* leaf extract were detected by GC/MS.

Peak	Identified Compound	Retention time (min)	Peak area (%)
1	Hexadecanoic acid, methyl ester	29.431	0.42
2	Palmitate	30.151	0.79
3	Cis-phytol	32.519	22.52
4	Methyl 5,12-octadecadienoate	32.513	0.61
5	Elaidic acid	32.728	0.99
6	Trans-phytol	32.994	46.80
7	Linolenic acid	33.419	0.84
8	Hexanedioic acid, ethylhexyl ester	37.803	3 .41
9	Unknown	39.049	6.59
10	Glycerol 1-monopalmitate	39.504	0.30
11	Squalene	44.166	0.32
12	α-tocopherol	48.203	0.99
13	Campesterol	49.358	0.80
14	Stigmasterol	49.755	0.85
15	Unknown	49.876	0.44
16	β -sitosterol	50 .499	10.60
17	Stigmastanol	50.613	2.76

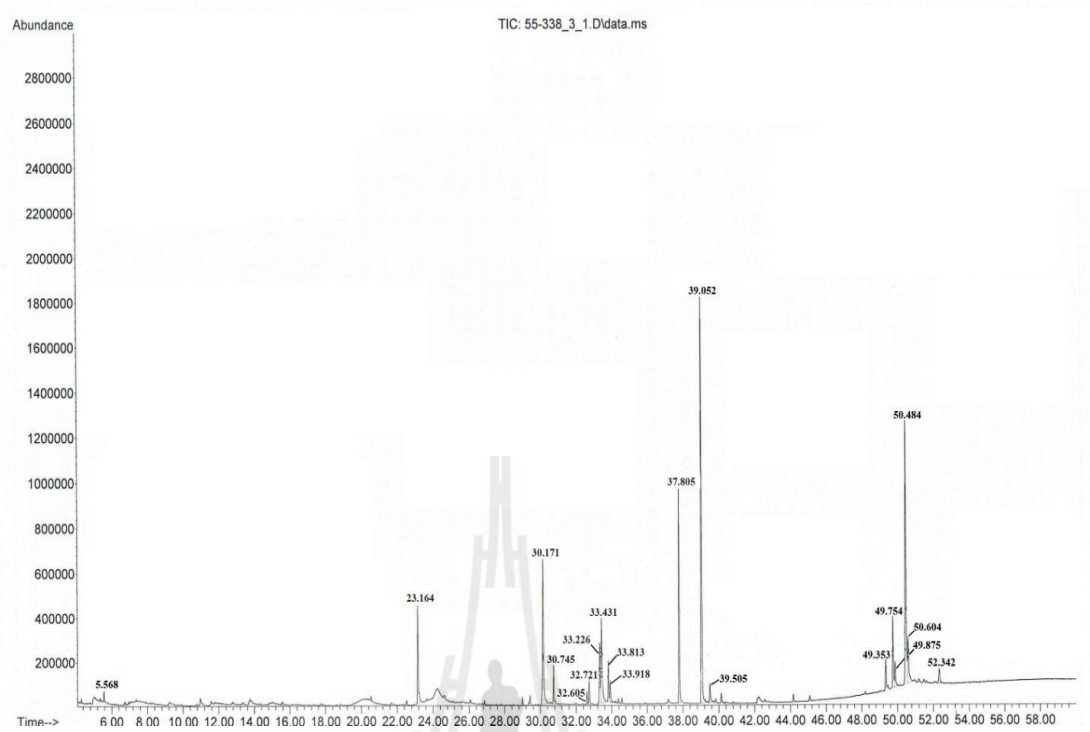

Figure 3.2 GC/MS chromatogram of *T. paniculatum* root extract.

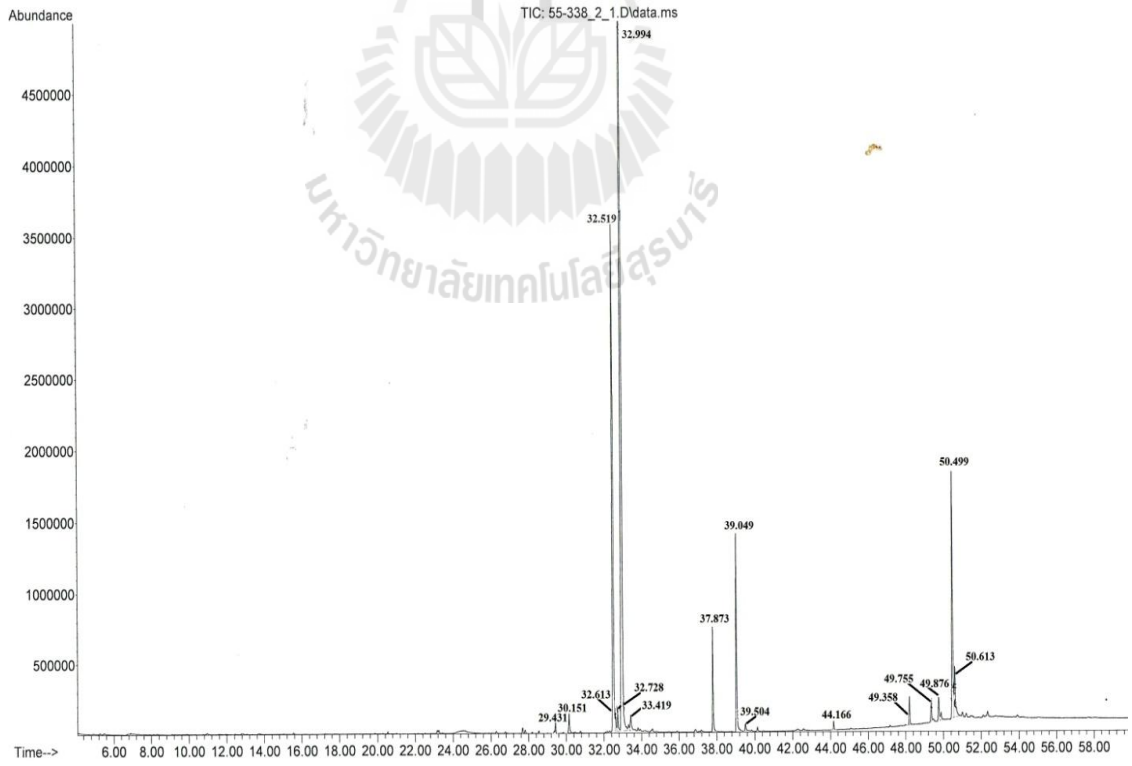

Figure 3.3 GC/MS chromatogram of *T. paniculatum* leaf extract.

3.5 Discussion

Different plants with broad range of nutritional and therapeutic values were used in many parts of the word as herbal medicines. The plants provide an excellent bio-resource for novel chemical entities for synthetic drugs (Ncube, Afolayan and OKoh, 2008). Traditional uses of medicinal plants are attracting the attention of the pharmaceutical and scientific communities, and evidence has demonstrated the promising potential of plant extracts that promote healthy function of human's health and wellbeing. This involves the isolation and identification of secondary metabolites produced by plants and their use as active principles in medicinal preparations (Taylor et al., 2001).

In this study, the primary phytochemical screening and GC-MS analysis proved that *T. paniculatum* extracts are pharmaceutically significant due to the presence of the various medicinally phytochemical compounds and secondary plant metabolites. Although their specific roles were not being investigated in this study, these compounds were reported to elicit the medicinal activity and physiological activity (Table 3.1). The GC-MS analysis of this plant showed the presence of key bioactive compounds, especially phytoserols and α-tocopherol (Table 3.2-3.3). These have significant therapeutic values regarding to relieve the complications from many hormone-related or metabolic diseases. Furthermore, phytols (both cis- and tran-forms) were abundant in *T. paniculatum* leaf extract. It acts as anti-itching agent (Ryu et al., 2010), anticancer agent (Wei et al., 2011), immunostimulant (Chowdhury and Ghosh, 2012) and able to elevate the neurotransmitter GABA levels in central nervous system (Bang, 2002). Structurally, phytols are diterpene terpenoids and usually used to synthesize the vitamin K, vitamin E and other tocopherol (Gömöry, 2010). It has

been reported that phytols also exhibited the insecticidal and antihelmintic or antiseptic activity (Anand and Gokulakrishnan, 2012). Therefore, *T. paniculatum* leaf may be used as antiseptic drug. Based on this study, it is suggested that *T. paniculatum* would be a highly beneficial medicinal plant for managing various ailments. It can be noteworthy to explore this plant for further pharmacological interventions.

3.6 References

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CHAPTER IV

EFFECTS OF *TALINUM PANICULATUM* (Jacq.) GAERTN. EXTRACTS IN FEMALE REPRODUCTIVE HORMONES, TOTAL ALKALINE PHOSPHATASE AND LIPID PROFILES

4.1 Abstract

Talinum paniculatum (Jacq.) Gaertn. (*T. paniculatum*) contains valuable phytosterols and medicinal secondary metabolites such as alkaloids, tannins, flavonoids and saponins. This plant is extensively used in Asian traditional medicine as a medicinal supplement. However, there is no conclusive scientific data to support this practice. This study was conducted to explore the potential medicinal property of *T. paniculatum* extracts and standard-phytol on female reproductive hormones, total alkaline phosphatase (tALP) and lipid profiles in adult bilaterally ovariectomized (OVX) rat. OVX rats were randomly divided into seven groups, and an additional group was sham-operated rats. OVX and sham-operated groups were orally treated with sesame oil as vehicle controls. The other six groups were treated with different treatments consisted of the groups which treated by a positive standard control of 17ß-estradiol (10 µg/Kg BW/day), standard-phytol (500 mg/Kg BW/day), and *T. paniculatum* root or leaf extract at two different doses (100 and 1,000 mg/Kg. BW

/day) for 42 consecutive days. The results showed thesignificantly rise in serum estradiol level could be observed in rats treated by standard E_2 and 1,000 mg/Kg BW/day of the leaf extract. The significant negative effect between estradiol and LH could be observed in sham-operated, standard E_2, standard-phytol and 1,000 mg/Kg BW/day of the leaf extract treated rats ($P < 0.05$). Administrations of *T. paniculatum* extracts could reduce serum tALP, and leaded to dose-dependency manner. However, only the rats treated by *T. paniculatum* leaf extracts significantly reduced serum tALP as compared to OVX negative control rats ($P < 0.05$). Administration of *T. paniculatum* extracts and standard-phytol trended to decrease the serum levels of total chlolesterol (TC). In contrast, the rats treated by 1,000 mg/Kg BW/day of root or leaf extracts (1,000 mg/Kg BW/day) significantly produced hypotriglyceremic actions ($P < 0.05$). Interestingly, *T. paniculatum* extracts and standard-phytol showed the positive effects on lipid profile as indicated by increasing HDL/LDL ratio.

4.2 Introduction

Estrogen plays a critical pharmacological role in many bodily targets that is not only in the reproductive system, but also in the other organs such as brain, cardiovascular and musculoskeletal systems. The pathogenic declining of the estrogen level is directly related to the cessation of ovarian follicular activity, and women will encounter menopausal conditions which contribute to the gradual decline in fertility and continue to natural sterility. Estrogen is the predominant factor that influences on the hypothalamic-pituitary system. After menopause, the negative feedback from ovarian steroid hormones eradicated, hence, raises the gonadotropins and gonadotropin releasing hormone (GnRH) concentrations (Alexandris et al., 1997).

The administration of estrogenic substances or phytoestrogens can modulate their concentrations down to physiological levels (Romanowicz, Misztal and Barcikowski, 2004; Trisomboon et al., 2007).

The reducing of estrogen level has been proven to enhance the progression of osteoporosis which subsequence to susceptibility to fragile bones and fractures. Osteoporosis is considered to be a major cause of morbidity and mortality in postmenopausal women. Since estrogen is directly associated with the growth and maturation of the bones, the depletion of circulating estrogen concentration contributes to the increasing of bone turnover (Jagtap, Ganu and Nagane, 2011; Susanto, 2011). Metabolic changes in the bone tissue can be also determined by some of bone turnover markers. Total alkaline phosphatase (tALP) is the enzyme which is accepted to be the preliminary parameter to evaluate the bone turnover rate and correlated with the increasing of tALP activity after menopause (Garnero et al., 1994).

Cardiovascular diseases also exert a significant burden on postmenopausal women's life. The evidences show that menopausal women are accompanied by an unfavorable characteristic of cardiovascular risk factors such as the significant increase in total cholesterol (TC), low density lipoprotein (LDL)-cholesterol and triglycerides (de Aloysio et al., 1999). The treatment with estrogen, 3-hydroxy- 3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitor or statin decreases these parameters (Lemay et al., 2001).

The hormone replacement therapy (HRT) has the benefit that can reduce or prevent the menopausal osteoporosis and cardiovascular diseases risk factors. Unfortunately, long term use of HRT extensively increases the risk of uterine, cervical, endometrial or breast cancer. Therefore, it would be the most beneficial to

discover a natural or safer dietary substances which possess the positive effect to postmenopausal women in order to minimize the side effect from HRT.

Due to the effectiveness and less complication of the estrogenic plants over HRT, the public became aware of its efficiency and have searched for the alternative from the classical synthetic estrogen (Wuttke et al., 2002). These plants have biological active substance(s) which can interact with the estrogen receptors (ERs) in various target organs and act as agonist or antagonist via ER-dependent signaling pathways (Kuiper, 1998).

Talinum paniculatum (Jacq.) Gaertn. (*T. paniculatum*) has been commonly consumed as a vegetable in local food of many countries in Asia that includes Thailand. *T. paniculatum* root has been reported to have a medicinal property similar to the root of Korean Ginseng or Panax ginseng; *T. paniculatum* usually employed as their substitute (Komatsu et al., 1982). As the traditional medicine, the root has been documented as a tonic remedy to promote fertility and to rectify the gynecological problems (Yulia Wientarsih and Razief, 2005). The leaf can be applied as supplement that increases milk production in sow, enhances anti-inflammatory responses, cures ulcer, and improves physical health (Petprai et al., 1996). Furthermore, β-sistosterol has been found in *T. paniculatum* leaf extract (Filho et al., 2010). The *T. paniculatum*'s efficacies on woman health, however, have not been elucidated; particularly no scientific data was found on its effect on female reproductive system and lipid profile. Consequently, this study was aimed to clarify if *T. paniculatum* extracts possess any estrogenic activity on the female reproductive hormones, bone and lipid metabolisms during the status of ovarian hormone exhausted condition. The experiment was designed to determine the major active constituent of the leaf

(phytols) and the effects of intermediated term supplementation of *T. paniculatum* extracts on serum estradiol, bone turnover marker (tALP) and lipid profiles in ovariectomized (OVX) rats. In addition, chlorophyll-derived phytols, mainly found in *T. paniculatum* (Filho et al., 2010), can act as the natural pro-agonist of retinoid X receptor (RXR) and the peroxisome proliferator-activated receptor α (PPARα) which activate estrogen responsiveness genes (Nuñez et al., 1997) the agent was, therefore, used in this study.

4.3 Materials and Methods

4.3.1 Animals and Chemical Exposures

17ß-estradiol (E8875) and standard-phytol (139912) were purchased from Sigma-Aldrich Chemical Co. (St.Louis, MO, USA). All solvents and chemicals used were of analytical grade and obtained from Sigma® and Merck®.

Animal care, environmental conditions and use were followed the guidelines of Laboratory Animal Resources, National Research Council of Thailand. The procedures of the experiment were approved by the Institutional Animal Care and Use Committee, Suranaree University of Technology, Nakhon Ratchasima, Thailand.

In order to induce menopausal condition, female Wistar rats (200-250g) were bilaterally OVX following the standard rodent ovariectomy procedure. A period of 14 days was allowed for wound to heal and acclimatization prior to treatment.

Rats were assigned into eight groups. Sham operated and OVX control groups were given 10% tween 80 combined with sesame oil, served as vehicle control. The third group was treated with 17ß-estradiol (10 μg/Kg BW/day) as positive control. The fourth group was treated with 500 mg/Kg BW/day of standard-phytol; the major

constituents of leaf extract. The fifth and sixth groups were treated with the low and high dosage (100 and 1,000 mg/Kg BW/day, respectively) of *T. paniculatum* root. The seventh and eighth groups were also treated with the low and high dosage (100 and 1,000 mg/Kg BW/day, respectively) *T. paniculatum* leaf extract. The experiment was continued for 42 consecutive days. The experimental regimen has been shown in Table 4.1.

4.3.2 Female Reproductive Hormones, Serum Lipids and Total ALP Analysis

The blood samples were collected at the end of the experiment by cardiac puncture. The rats were fasted for 12 hr before the blood collection and were euthanized by CO_2 asphyxia. Blood samples were centrifuged (4°C) at 1500 g for 15 min to separating the serum. The serum samples were stored at -80°C until assay was performed.

Serum estradiol and leuteinizing hormone (LH) concentrations were analyzed by the Electrochemiluminescence immunoassay (ECLIA) on Elecsys and cobas e immunoassay analyzer (Roche Diagnostics, USA). Assay procedures were followed the instructions supplied by manufacturer (Ausmanas et al., 2007).

Serum levels of tALP, total cholesterol (TC), triglyceride, HDL and LDL concentrations were measured by using Enzymatic color test and assayed by OLYMPUS analyzer (Olympus Life and Material Science, Germany).

4.3.3 Statistical Analysis

All data were expressed to the mean value $\pm$ standard error of the mean (S.E.M.). Statistical analysis of difference was carried out by analysis of variance (ANOVA) followed by Scheffe's *post hoc* test using SPSS windows program version

11 (SPSS Institute, Inc., Chicago, IL, USA). A probability level less than 5% ($P < 0.05$) was considered statistically significant.

Table 4.1 Treatment regimen for the experiment.

Group	Treatment and Dosage	Route	Duration
1. Sham operated control	Vehicle (1 mL/rat/day; 10% v/v Tween 80 in sesame oil)	Orally	42 days
2. OVX vehicle control	Vehicle (1 mL/rat/day; 10% v/v Tween 80 in sesame oil)	Orally	42 days
3. OVX E_2 control	17ß-estradiol (10µg/Kg BW/day)	Orally	42 days
4. OVX	Standard-phytol (500 mg/Kg BW/day)	Orally	42 days
5. OVX	Root extract (100 mg/Kg BW/day)	Orally	42 days
6. OVX	Root extract (1,000 mg/Kg BW/day)	Orally	42 days
7. OVX	Leaf extract (100 mg/Kg BW/day)	Orally	42 days
8. OVX	Leaf extract (1,000 mg/Kg BW/day)	Orally	42 days

4.4 Results

4.4.1. Effect of *T. paniculatum* Extracts on Serum Estradiol, LH and tALP Levels.

To explore the influents of the plant extracts and its major compound (standard-phytol) treatment on pituitary function, the experiment was designed to investigate serum hormone profiles, including estradiol and LH concentrations as illustrated in Figure 4.1

Compared with OVX control rats, all the groups that were treated with *T. paniculatum* extracts and standard-phytol illustrated the evidence of rising in serum estradiol concentrations (n = 5). The statistical significant elevation of mean serum estradiol values can be observed in the groups which treated by the highest dosage of the leaf extract (1,000 mg/Kg BW/day; 70.46 $\pm$ 6.03 pg/mL), but it still was lower than sham-operated (109.89 $\pm$ 4.01 pg/mL) and standard E_2 treated groups (128.61 $\pm$ 9.53 pg/mL), respectively ($P < 0.05$). On the other hand, serum LH level rose approximately 10-fold upon OVX-received vehicle compared to sham-operated rats (Sham: 0.38 $\pm$ 0.02 mIU/mL; OVX: 3.63 $\pm$ 0.55 mIU/mL). In this case, the significantly negative effect between estradiol and LH could be observed in sham, standard E_2 , standard-phytol and the high dosage of the leaf extract treated groups ($P < 0.05$).

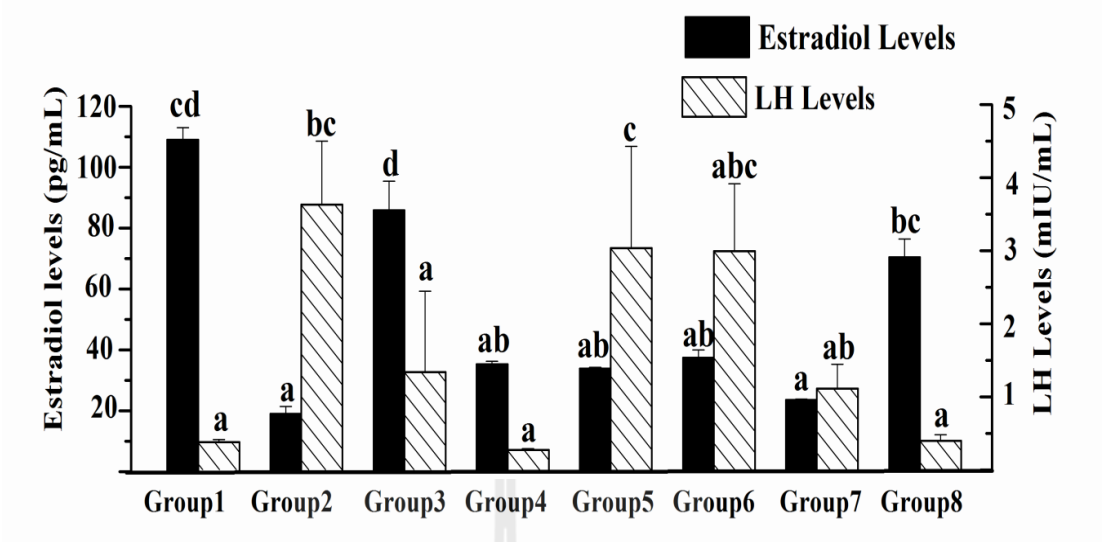

Figure 4.1 Effects of *T. paniculatum* extracts and standard-phytol on serum estradiol and LH levels in adult female Wistar rats. Data express as mean ± S.E.M. (n = 5). Data were analyzed by one-way ANOVA, followed by Scheffe's *post hoc* test. Groups bearing the different superscripted letters on the bar indicate statistical significance between the groups ($P < 0.05$). Group 1= Sham operated control; Group 2 = OVX control; Group 3 = Standard E_2 control (17β-estradiol 10 µg/Kg BW/day); Group 4 = Standard main component (standard-phytol 500 mg/Kg BW/day); Group 5 = *T. paniculatum* root extract (100 mg/Kg BW/day); Group 6 = *T. paniculatum* root extract (1,000 mg/Kg BW/day.); Group 7 = *T. paniculatum* leaf extract (100 mg/Kg BW/day); Group 8 = *T. paniculatum* leaf extract (1,000 mg/Kg BW/day).

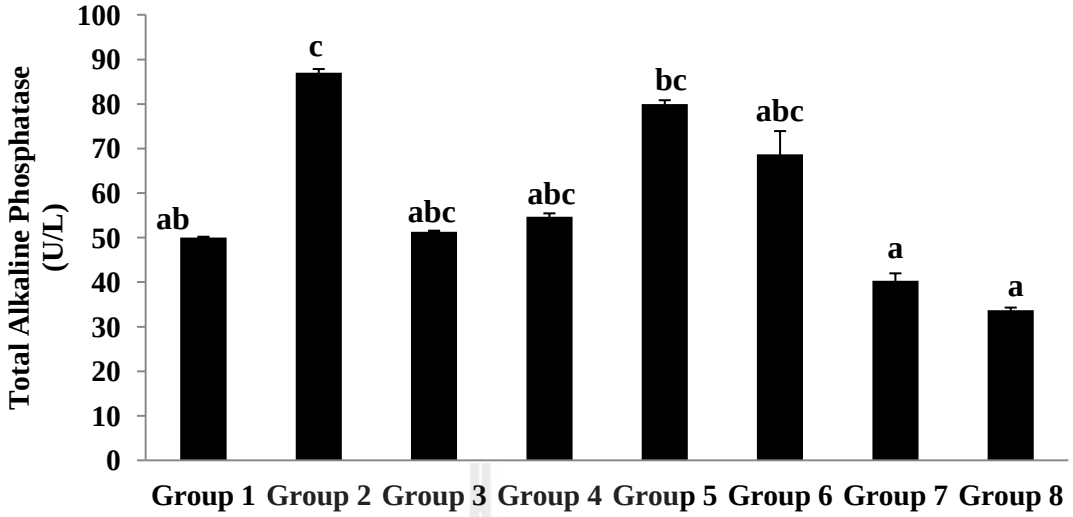

Figure 4.2 Effects of *T. paniculatum* extracts and standard-phytol on serum tALP level in adult female Wistar rats. Data express as mean ± S.E.M. (n = 5). Data were analyzed by one-way ANOVA, followed by Scheffe's *post hoc* test. Groups bearing the different superscripted letters on the bar indicate statistical significance between the groups ($P < 0.05$). Group 1= Sham operated control; Group 2 = OVX control; Group 3 = Standard E_2 control (17β-estradiol 10 µg/Kg BW/day); Group 4 = Standard main component (standard-phytol 500 mg/Kg BW/day); Group 5 = *T. paniculatum* root extract (100 mg/Kg BW/day); Group 6 = *T. paniculatum* root extract (1,000 mg/Kg BW/day.); Group 7 = *T. paniculatum* leaf extract (100 mg/Kg BW/day); Group 8 = *T. paniculatum* leaf extract (1,000 mg/Kg BW/day).

At the end of the experiment, as expected, ovariectomy induced a rise in serum tALP level due to lack of protectively effect from endogenous circulating estradiol. In contrast, the concentrations of serum tALP in all treated-rats showed the inclination decreased when compared to OVX received vehicle control. The groups that were administered by the leaf extracts showed a considerable declined in the

tALP level as a dose-dependent manner compared to the OVX control group ($P < 0.05$, Figure 4.1).

4.4.2. Effect of *T. paniculatum* Extracts on Serum Lipid Profile

At the end of the experiment, the significantly reduce the serum TC level could be investigated in only sham-operated control groups. As shown in Table 4.2, the oral administration of standard E_2, *T. paniculatum* extracts, as well as, standard-phytol did not affect the serum TC when compared with vehicle control group ($P > 0.05$). The prominent hypotriglyceridemic effect can be observed in 1,000 mg/Kg BW/day both *T. paniculatum* root and leaf extracts treated rats by comparing with OVX negative control ($P < 0.05$).

Ovariectomy could enhance the serum LDL concentration (14.80 $\pm$ 1.04 mg/dL) when compared to sham-operated control rats (5.20 $\pm$ 0.34 mg/dL). In addition, the groups that were treated by standard E_2, standard-phytol and the leaf extracts significantly have a decrease in the LDL level ($P < 0.05$). All plant extracts treated groups and sham-operated control showed a substantial elevating in serum HDL level (Table 4.3).

The groups that were treated with standard E_2, plant extracts and sham-operated control showed the evidence of rising in HDL/LDL ratio as a concentration dependent characteristic (Figure 4.3); this ratio was decreased in OVX control group. As comparing to OVX control, the significantly increasing in HDL/LDL ratio were observed in sham, standard E_2 and 1,000 mg/Kg BW/day of leaf extract treated groups ($P < 0.05$; $n = 5$).

Table 4.2 Hypolipidemic effects of *T. paniculatum* extracts and standard-phytol in adult female Wistar rats.

Group	Treatment and Dosage	Total Cholesterol (mg/mL)	Triglyceride (mg/mL)
1. Sham operated control	Vehicle control (1 mL/rat/day)	58.80 ± 3.36[a]	63.60 ± 1.93[a]
2. OVX control	Vehicle control (1 mL/rat/day)	114.80 ± 7.12[b]	92.80 ± 5.39[b]
3. OVX E_2 control	17ß-estradiol (10 µg/Kg BW/day)	49.80 ± 6.15[a]	70.80 ± 4.63[ab]
4. OVX	Standard-phytol (500 mg/Kg BW/day)	92.40 ± 3.42[b]	80.80 ± 4.41[ab]
5. OVX	Root extract (100 mg/Kg BW/day)	108.00 ± 0.45[b]	68.20 ± 6.40[ab]
6. OVX	Root extract (1,000 mg/Kg BW/day)	101.60 ± 3.73[b]	65.60 ± 2.99[a]
7. OVX	Leaf extract(100 mg/Kg BW/day)	109.80 ± 3.29[b]	71.80 ± 3.70[ab]
8. OVX	Leaf extract (1,000 mg/Kg BW/day)	102.60 ± 4.48[b]	64.60 ± 1.08[a]

All values are expressed as mean ± S.E.M. of 5 rats in each group (n = 5).

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

Table 4.3 The effects of *T. paniculatum* extracts and standard-phytol on serum HDL and LDL levels in adult female Wistar rats.

Group	Treatment and Dosage	HDL (mg/dL)	LDL (mg/dL)
1. Sham operated control	Vehicle (1 mL/rat/day)	71.00 ± 0.54^{b}	5.20 ± 0.34^{a}
2. OVX control	Vehicle (1 mL/rat/day)	40.00 ± 2.73^{a}	14.80 ± 1.04^{d}
3. OVX E_2 control	17ß-estradiol (10 µg/Kg BW/day)	62.80 ± 3.41^{b}	4.80 ± 0.44^{a}
4. OVX	Standard-phytol (500 mg/Kg BW/day)	64.60 ± 4.01^{b}	7.80 ± 0.33^{ab}
5. OVX	Root extract (100 mg/Kg BW/day)	78.20 ± 3.56^{b}	12.00 ± 0.63^{cd}
6. OVX	Root extract (1,000 mg/Kg BW/day)	81.40 ± 3.05^{b}	11.40 ± 0.73^{bcd}
7. OVX	Leaf extract (100 mg/Kg BW/day)	72.60 ± 1.54^{b}	8.80 ± 0.66^{abc}
8. OVX	Leaf extract (1,000 mg/Kg BW/day)	73.00 ± 1.50^{b}	7.00 ± 0.63^{a}

All values are expressed as mean $\pm$ S.E.M. of 5 rats in each group ($n = 5$).

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

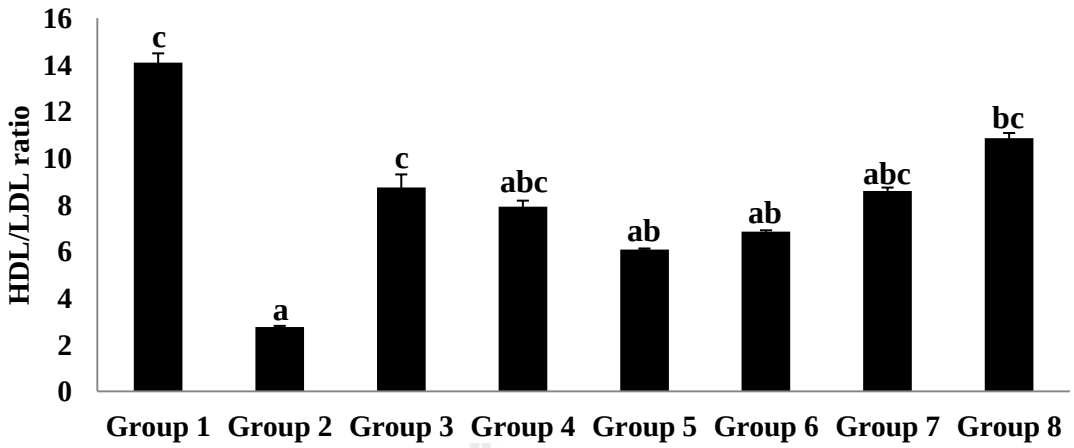

Figure 4.3 Effects of *T. paniculatum* extracts and standard-phytol on HDL/LDL ratio in adult female Wistar rats. Data express as mean ± S.E.M. (*n* = 5). Data were analyzed by one-way ANOVA, followed by Scheffe's *post hoc* test. Groups bearing the different superscripted letters on the bar indicate statistical significance between the groups ($P < 0.05$). Group 1= Sham operated control; Group 2 = OVX control; Group 3 = Standard E_2 control (17β-estradiol 10 μg/Kg BW/day); Group 4 = Standard main component (standard-phytol 500 mg/Kg BW/day); Group 5 = *T. paniculatum* root extract (100 mg/Kg BW/day); Group 6 = *T. paniculatum* root extract (1,000 mg/Kg BW/day); Group 7= *T. paniculatum* leaf extract (100 mg/Kg BW/day); Group 8 = *T. paniculatum* leaf extract (1,000 mg/Kg BW/day).

4.5 Discussions

Compares to the classical synthetics drugs, the public's interest in natural products' effectiveness against aging or hormone-dependent diseases has noticeably increased in the past decade. Menopause is the condition with estrogen drops from the circulating physiological level and increased to the high risk of various cancers and metabolic diseases. Hormone-related cancers, osteoporosis and cardiovascular

diseases are the major problems for postmenopausal women that have chronic exposure to HRT. This experiment attempted to demonstrate the estrogenic activity of the *T. paniculatum* extracts and its major component as related with these complications by examining hormonal, tALP and lipid behavioral responses in OVX rat model.

Natural estrogenic substance(s) possibly exert its hormonal effects through direct or indirect signaling. In vitro study, it is plausible that they may modulate estrogen levels through signaling via pregnane X receptor (PXR; also known as the steroid and xenobiotic receptor) binding then induces the expression of CYP3A4 which plays a major role in the hydroxylation of both estrone and estradiol (Jacob, Nolan and Hood, 2005; Ricketts et al., 2005). The results exhibited the circulating estradiol level in OVX rats that received vehicle control extensively reduced from the mean value of the sham-operated control rats on over 42-days experimental period. The oral consumption of *T. paniculatum* extracts from the root and leaf, especially at the high dose of leaf extract (1,000 mg/Kg BW/day), were sufficient to improve the serum estradiol level that almost equally to sham-operated control and standard E_2 treated group. Interestingly, the consumption of standard-phytol also showed the tendency to increase in serum estradiol concentration in OVX rats. This evidence illustrated that the plant extracts and standard-phytol possess the favorable estrogenic activity in female reproductive hormonal system.

The abolition of ovarian hormones signal to anterior hypothalamus leads to the rising of the gonadotropins level during menopausal period can be observed. The inhibitory effect of the estrogenic substances and phytoestrogen on the hypothalamic axis has widely been proven in many mammalian species (Whitten and Patisaul,

2001). Düker and colleges (1991) clarified that consuming of *Cimicifuga racemosa*'s rhizome extract for eight weeks can significantly reduce the LH level in menopausal women and OVX rats by modulating via ERs. In addition, other similar studies with rats supported that neonatal exposure to phytoestrogen results in a failure to show LH surges in response to estrogen (Mcgarveyey et al., 2001; Böttner et al., 2006). The current experiment demonstrated that the groups which were administered by standard E_2, standard-phytol and all the high dosage of leaf extract can turn the LH down to the physiological level when compared with the OVX control. This verification emphasizes that the leaf extract and its main component (phytols) may at least having effects on the hypothalamic level.

Osteoporosis is the aged-related condition, characterized by reduced bone mass and disruption of bone architecture, resulting in increased bone fragility and increased fracture risk. The depletion of serum estradiol level during menopausal period also directly associates with the severity of bone disease. Unfortunately, in the menopausal women and OVX rats, number of vitamin D receptors in jejunum also diminishes and leads to reduce intestinal calcium absorption. For this reason, they suffer more severing progression of osteoporosis (Chan, Chiu and Atkins, 1984).

Bone mineral density (BMD) is the gold standard to estimate bone health quality and usually used as a primary indicator of the risk to osteoporotic fractures. However, BMD measurements provide a clinical representation of bone mineral status; it cannot be used to evaluate the slight bone metabolic changes. Biochemical markers, by contrast, can be used to assess the bone's health and appear to be sensitive enough to determine the dynamic change of bone metabolism (Maïmoun et al., 2005). Serum tALP is the non-specific enzyme which can be produced by many

tissues including liver, intestine, mammary glands placenta, bone and leukocytes; more than 90% of tALP activity is also related to bone and liver isoenzyme (Mahjoub and Roudsari, 2012). Estrogen is considered to be the maintainer of bone remodeling and metabolism. The elevation of net bone lost and biochemical markers such as specific bone-ALP (bALP) and tALP are generally recognized in postmenopasal women and OVX animals. The current data support that in sham-operated and OVX received E_2 standard control showed the reduction of tALP levels compared to OVX control. Surprisingly, the OVX rats administered by the leaf extract exert bone protective effect which is primary reflected by the lowering in the level of tALP ($P < 0.05$). However, our study needs to provide other bone markers such as serum bALP, ostecalsin, and/or carboxyl-terminal propeptide of type I procollagen to confirm the protective effect of the plant extracts and standard-phytol on bone metabolic condition (Singer and Eyre, 2008). Phytoestrogens might have beneficial effects on bone metabolism by modulating the ER_β rather than ER_α both in *vitro* and in *vivo* (Arjmandi, 2001; Setchell and Lydeking-Olsen, 2003). The oral consumption of dietary phytoestrogens such as soybean and genistein demonstrated to have positive effect on the bone in OVX rats. Other human studies revealed that isoflavone-rich soy protein diets could improve the value of bone turnover markers and preventing bone loss as measured from BMD and content (Picherit et al., 2000).

Pathogenic changing in lipid metabolism is regularly seen in menopausal women, which are characterized by overall shift toward more alteroscerogenic lipid profile and metabolic syndrome. Koskovà and colleagues (2009) reported that the triglycerides and LDL cholesterol increased and ratio of HDL/TC decreased with age, most significantly in menopause ($P < 0.001$). Several data elucidated that estrogen has

a direct effect on an indirect long-term effect consisting of modulation of the response to endothelial damage and atherosclerotic changes in vessels (Baker et al., 2003). Our results confirm that in OVX control rats showed significantly elevated levels of serum TC at the end of experiment as compared to sham-operated control group. On the other hand, the treatment with standard E_2 could significantly decreased TC levels in serum. The results indicated that standard E_2 treatment protected OVX rats against increased TC levels caused by ovariectomy, while the plant extracts were not effective to reduce the serum TC. Although the plant extract did not affect the serum TC, they astoundingly showed the positive effects on the serum concentration of triglyceride, HDL and LDL as demonstrated in Table 4.2-4.3.

The active constituents of *T. paniculatum* extracts responsible for hypolipedemic and hypotriglyceremic actions are not clearly known. However, these activities may be mediated by one or more of the following compounds identified in *T. paniculatum* root and leaf extracts that have been previously reported as a medicinal properties (as shown in Chapter 2).

The tender hypocholesterolemic activity of *T. paniculatum* extracts may be due to a variety of mechanisms, for example: a) inhibition of HMG-CoA reductase, b) activation of cholesterol-7-alpha-hydroxylase (CYP7A1), which converts cholesterol into bile acids, and/or c) inhibition of intestinal cholesterol absorption due to formation of complexes with compounds such as glycosides and saponins (Amin Riyad, Abdul Ghani Abdul-Salam and Suleiman., 1998; Yokogoshi and Oda, 2002; Chen et al., 2004). A reduction in triglyceride levels may be due to the decreased lipogenesis, increased lipolytic activity by inhibition of hormone-sensitive lipase (Al-

Shamaony, Al-Khazraji and Twaij, 1994) or the lipogenic enzymes (Pari and Venkteswaran, 2004), and/or activation of lipoprotein lipase (Ahmed et al., 2001).

The beneficial association between phytoestrogens and lipoproteins has not been clearly elucidated. The mechanism responsible for the increase in HDL level is by hormone-induced reduction in hepatic endothelial lipase and HDL-degradation enzyme which leads to increase in the plasma HDL level (Lobo, 1991). Phytoestrogens have been reported to have hypolipidemic effect by the reduction of gonadal steroid biosynthesis through effects on cholesterol availability or the activity of the side chain cleavage enzyme. In addition, they may act as a binding to steroids in the intestine and excreting them into feces (Maclachy and Kraak, 1999). Other proposed mechanisms are accordance with enhance thyroid function by changes in T4 level (Forsythe, 1995; Wroblewski-Lissin and Cooke, 2000), up-regulation of hepatic LDL cholesterol receptors (Kurowska Jordan and Spence, 1997), increased apoprotein B (apoB) catabolism by LDL-receptor-independent pathways, removal of desialylated LDL by transcytosis and accelerated the conversion of hepatic cholesterol to bile acid (Karjalainen et al., 2000), decreased zinc to copper ratios (Edman, 2000), and activation of the estrogen ERs (Kuiper et al., 1998).

Base on this study, the use of *T. paniculatum* extracts may have an advantage for hormone replacement therapy in the case of the ovarian exhausted. The estrogen-like effects of *T. paniculatum* extract and the major component in the leaf (phytols) on blood biochemistry index reveal possible treatment to postmenopausal complications such as vasomotor symptoms, osteoporosis and metabolic risk diseases.

4.6 References

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CHAPTER V

THE ESTROGENIC ACTIVITY OF *TALINUM PANICULATUM* (JACQ.) GAERTN. ECTRACTS IN OVARIECTOMIZED RATS

5.1 Abstract

Talinum paniculatum (Jacq.) Gaertn. (*T. paniculatum*) is commonly used in Asian traditional medicine as a reproductive enhancement. This plant has been reported to contain some phytosterols that may affect the reproduction system. However, there is no conclusive data to support this practice. Therefore, the comparative estrogenic activity of *T. paniculatum* extracts with17ß-estradiol (10 µg/Kg BW/day) was studied in adult bilaterally ovariectomized (OVX) rats (200-250 g). OVX rats were randomly divided into seven groups, and an additional group were sham-operated rats (n = 5). OVX and sham-operated groups were orally treated with sesame oil as vehicle controls. The other six groups were treated with different treatments consisted of a positive standard control of 17ß-estradiol (10 µg/Kg BW/day), standard-phytol (500 mg/Kg BW/day), *T. paniculatum* root or leaf extract at two different doses (100 and 1,000 mg/Kg BW/day) for 42 consecutive days. Estrogenic activity was evaluated by determining the vaginal cornification, relative uterine weight (%RU), relative mammary weight (%RM), and their

histoarchitecture changes. The results showed that standard-phytol, *T. paniculatum* root and leaf extracts significantly increased the vaginal cornification ($P < 0.001$). Compared to OVX control, a dose dependency response of %RU, %RM, proliferative changes in vagina, uterus, and mammary ductular tissue were observed in OVX treated with standard-phytol and *T. paniculatum*.

5.2 Introduction

The reproductive disorder has always been the critical issue over women's life span, and it leads to natural medications dependency. Using medicinal herbs or phytoestrogenic substances as a substitute is not as persuasive in the estrogenic property as the classical synthetic estrogen, but they are safer in terms of avoiding undesirable side effects. *Talinum paniculatum* (Jacq.) Gaertn. (*T. paniculatum*) or "Som Java" is one of the plants in Portulacaceae family that contains notable medicinal properties. *T. paniculatum* is a wild deciduous perennial herb with well-developed root system. It is naturally grown around the world, including Thailand with the local name of Wan Pak Pang. In Thailand, the locals consume the leaf as vegetable supplement and the roots as reproductive tonic. Preparation of *Talinum* spp. has long been used in ancient folk medicine, particularly in the treatment of type-2 diabetes, inflammatory skin problems, gastrointestinal disturbance, general body weakness and reproductive disorders (Shimoda et al., 2001; Pak et al., 2005). The root has active constituents such as steroidal saponins and tannins while only tannins can be detected in the leaf (Yulia, Wientarsih and Razief, 2005). Additionally, Filho and colleagues (2010) isolated and reported that compesterol, β-sistosterol, stigmasterol

and high amount of chlorophyll-phytols could be extracted from the leaf of *T. paniculatum.*

To our knowledge, the plants in *Talinum* spp. were recognized for their some constituents and biological properties, however, *T. paniculatum* still lacks of scientific data to clarify its estrogenic property. Therefore, this study was designed to evaluate the estrogenic activity of *T. paniculatum* extracts by observing estrogen-responsiveness parameters. The observation would include the relative of uterus or mammary weight to body weight, vaginal cornification and the histological structure changes in female reproductive organs by using the rodent ovariectomy (OVX) as an animal model of menopause (Wu et al., 2005). It has been reported that chlorophyll-phytols, mainly found in *T. paniculatum* (Filho et al., 2010), can act as the natural pro-agonist of retinoid X receptor (RXR) and the peroxisome proliferator-activated receptor α (PPARα). The activation of these receptors are further able to trigger estrogen responsiveness genes (Nuñez et al., 1997), therefore the agent was added into this study.

5.3 Materials and Methods

5.3.1 Animals and Chemical Exposures

17ß-estradiol (E8875) and standard-phytol (139912) were purchased from Sigma-Aldrich Chemical Co. (St.Louis, MO, USA). All solvents and chemicals used were of analytical grade and obtained from Sigma® and Merck®.

Animal care, environmental conditions and use were followed the guidelines of Laboratory Animal Resources, National Research Council of Thailand. The

procedures of the experiment were approved by the Institutional Animal Care and Use Committee, Suranaree University of Technology, Nakhon Ratchasima, Thailand.

Experiment was conducted on female Wistar rats weighing between 200-250 g. All rats, except in sham-operated control group, were bilaterally OVX following standard rodent ovariectomy procedure. A period of 14 days was allowed for wound healing and acclimatization prior to treatment.

Rats were randomized into eight groups of five animals, and the estrogenic activity from plant extracts were being compared with standard 17β-estradiol (E_2) as a positive control. Sham-operated and OVX control groups were given 10% (v/v) Tween 80 combined with sesame oil, served as vehicle controls, and the third group was treated with E_2 (10 μg/kg/day) as a positive control. The fourth group was treated with 500 mg/Kg BW/day of standard-phytol (Arnhold, Elmazar and Naũ, 2002). The fifth and sixth groups were treated with the low and high dosage (100 and 1,000 mg/Kg BW/day, respectively) of *T. paniculatum* root. The seventh and eighth groups were also treated with the low and high dosage (100 and 1,000 mg/Kg BW/day, respectively) of *T. paniculatum* leaf extract. The experiment was continued for 42 consecutive days.

5.3.2 Determination of Body Weight and Relative Uterus or Mammary Weight

Rats were humanely sacrificed at the end of the experiment by CO_2 asphyxia. All connective tissues were removed prior to wet weight recording. Body weight, horns of uteri and inguinal mammary tissues weights were recorded. Relative uterus and mammary weights were calculated by this following formula:

$$\text{Relative uterus or mammary weight (\%)} = \frac{\text{uterus or mammary gland weight (g)x 100}}{\text{body weight (g)}}$$

5.3.3 Vaginal Cornification Assay

Vaginal smear was performed to examine cellular differentiation and to evaluate the presence of leukocytes, nucleated cells, or cornified cells. Vaginal smear samples were collected between 9.00 AM and 10.00 AM daily by gently inserting the tip of dropper into the vagina, flushing normal saline (0.9% NaCl) in and out, and placing the fluid onto microscope slides, and stained by Methylene blue dripping (Parhizkar et al., 2011). The appearance of cornified cells was used as an indicator of estrogenic activity and percentage of cornified cells was evaluated using the following formula:

$$\text{Percentage of cornified cell (\%)} = \frac{\text{cornified cells x 100}}{\text{cornified cells+nucleated cells+leucocytes}}$$

5.3.4 Histological Analysis

Histological Staining Preparation

The horns of rat uteri, vagina and mammary tissue were cut into short segments using the paraffin technique. Sections of 5 µm thicknesses were cut and stained using routine hematoxylin and eosin method. Briefly, tissue sections were dehydrated in an ascending ethanol series (75%, 85%, 95% and 100%, 1 hr each) and the sections were removed into pure xylene for 2 min. The sections were then embedded with xylene:paraplast (3:1 and 1:1, 15 min each) and followed by the pure paraplast for 1 hr. Post-embedded tissues were cut approximately 5µm with microtome and these sections were moved into water bath for incubation (60ºC). The tissues were mounted on slides in the slide warmer at 60ºC and then immersed into pure xylene two times (5 and 2 min, respectively). Next, tissues were hydrated in a descending ethanol series (100%, 95%, 70%, 30%, distilled water, 2 min each) and stained with eosin for 1 min. Finally, the slide was immersed into 95% ethanol (3

min) then later 100% ethanol (1 min). Next, the slide was simultaneously immersed into pure xylene (5 min) and then covered with cover slip after xylene clearing for light microscopic (LM) study.

Morphometry of the Reproductive Organs Histology

All reproductive organs were observed and measured on hematoxylin and eosin stained slides at 4X, 10X, 20X or 40X magnifications. 3 randomly chosen areas of the section were measured per slide. Images of uterine, vagina and mammary gland cross-sections (n = 3) were taken using a Nikon Eclipse 80i Upright microscope (Hollywood International Ltd., Thailand) and Cell^D imaging software (Olympus, EforL International Co., Ltd., Thailand). The number, thickness, size of the organs and epithelial lining were analyzed by using Image J v1.41 software (Cordial et al., 2006).

5.3.5 Statistical Analysis

All data are expressed to the mean value $\pm$ standard error of the mean (S.E.M.). Statistical analysis of the difference was carried out by analysis of the variance (ANOVA), and followed by Scheffe's *post hoc* test using SPSS windows program version 11 (SPSS Institute, Inc., Chicago, IL, USA). A probability level less than 5% ($P < 0.05$) was considered statistically significant.

5.4 Results

5.4.1 Body Weight and Relative Uterus and Mammary Weight Changes

14 days after the ovariectomy, the means of initial body weight were not different among the groups as before the experiment started. All rat groups did not

show any data of acute toxicity of abnormal clinical signs or death during the 42 days treatment period.

At the end of the experiment, the results showed that bilateral OVX enhanced sustainable increased in final body weight while decreased the percentages of relative uterine weight (%RU) in relation to sham-operated control group. The final body weight after 42 days of the experimental treatment significantly elevated in the OVX control compared with that in the sham-operated control group ($P < 0.05$). In contrast, standard-phytol and plant treated groups showed slightly lower weight compared with the OVX control groups.

The macroscopic observation of on the characteristic of the uterus demonstrated the bilateral ovariectomy considerably changed the wet weight and morphology. Table 5.1 shows the mean %RU in each treatment group. The %RU of OVX vehicle control group was significantly reduced by approximately 2- to 3-fold when compared with sham-operated control ($P < 0.05$). Feeding with standard-phytol (500 mg/Kg BW/day), root extracts (both 100 and 1,000 mg/Kg BW/day) and low dosage of leaf extract (100 mg/Kg BW/day) did not show significant effects on the %RU as compared to OVX vehicle control. The group treated by E_2, high dosage of leaf extract (1,000 mg/Kg BW/day) and sham-operated control showed significant increases in %RU (0.41 ± 0.01, 0.33 ± 0.00 and 0.37 ± 0.01, respectively; $P < 0.05$, $n = 5$).

The mammogenic effect of the plant extracts and standard-phytol on mammary tissue was also evaluated by observing the percentage of relative mammary wet weight (% RM) in OVX rats. Atrophic mammary mass was present in OVX-received vehicle control rats. The administration of E_2 intensely increased %RM and

it was also greater than sham-operated control ($P < 0.001$). Experimental data showed that the treatment of OVX rats for 42 days with the high dosage of root and leaf extracts (1,000 mg/Kg BW/day) prevented mammary tissues regression. They exhibited the significantly greater %RM than those in the OVX negative control group ($P < 0.05$). Additionally, rats fed by standard-phytol (500 mg/Kg BW/day) and low dosage of leaf and root extract (100 mg/Kg BW/day) showed slightly increases in %RM compared with OVX rats fed with vehicle control (Table 5.1).

5.4.2 Vaginal Cornification

Estrogenic activity of *T. paniculatum* extracts and standard-phytol were evaluated through the vaginal cytology by comparing to standardized E_2 administration as the positive control. Cornified cells could not be observed in the vaginal smear obtained from all OVX rats at 14 days after the operation. This result confirmed the menopausal pattern with atrophic vaginal epithelium in OVX rats as characterized by vaginal smear consisting of parabasal cells, leukocytes and nucleated epithelial cells.

All vaginal smears obtained from OVX and sham-operated rats are shown in Figure 5.1. During the experimental period, the vaginal smear of OVX-received vehicle control did not show any vaginal cornification, whereas, the regular estrous cycle can be observed in sham-operated control. The persistent estrous stage was detected in the group-treated with E_2, standard-phytol and all doses of *T. paniculatum* extracts for both root and leaf. The mean percentage of vaginal cornification obtained from the plant extract treated groups significantly increased as a dose dependent manner, but they were lesser than standard-phytol and positive E_2 control groups ($P < 0.05$).

Standard-phytol, the high dose of root and leaf extracts (1,000 mg/Kg BW/day) provoked a significant differentiation of vaginal epithelial cells to the exfoliated cornified cells in the smear (42.77 $\pm$ 1.56%, 39.46 $\pm$ 0.63% and 39.35 $\pm$ 1.76%, respectively) when compared to the OVX control (00.00 $\pm$ 0.00%). The oral administration of *T. paniculatum*'s root and leaf extracts at the dose of 100 mg/Kg BW/day for 42 days could slightly induce the cornification in OVX rats (26.29 $\pm$ 0.77% and 21.49 $\pm$ 0.54%) as shown in Table 5.2.

Figure 5.1 demonstrates the changing of vaginal cytology at 21-d of experimental period. From sham-operated control rats during pro-estrous period, the vaginal smear mainly contained growing and maturing vaginal epithelial cells which included some intermediated (I) and superficial or cornified (Co) cells. The OVX group treated with vehicle control showed only parabasal (P) cells and lymphocytes (L). The greatest amounts of cornified cells were found from the group treated with E_2 at the dose of 10 μg/Kg BW/day. The smear from standard phytol-treated rats exhibited high amount of intermediated and cornified cells. All groups received *T.paniculatum* leaf and root extract (100 and 1,000 mg/Kg BW/day) illustrated the cornified cells with lesser than E_2 and standard phytol-treated groups. Furthermore, E_2 treated group illustrated the cornified cells within 3 days after E_2 administration and showed the persistent feature of estrous condition until the end of the experiment. In all plant extracts treated groups, the cornified cells initially presented at day 4 and 5 after treatment at the dose of 100 and 1,000 mg/Kg BW/day, respectively (data not shown).

Table 5.1 Effect of *T. paniculatum* extracts and standard-phytol on body weight changes.

Group	Treatment and Dosage	Initial Body Weight (g)	Final Body Weight (g)
1. Sham-operated control	Vehicle (1 mL/rat/day; 10% v/v Tween 80 in sesame oil)	204.00±3.03^{a}	257.60±1.80^{bc}
2. OVX control	Vehicle (1 mL/rat/day; 10% v/v Tween 80 in sesame oil)	205.00±3.61^{a}	266.00±2.17^{c}
3. OVX E_2 control	17ß-estradiol (10 µg/Kg BW/day)	201.00±2.86^{a}	233.00±2.79^{a}
4. OVX	Standard-phytol (500 mg/Kg BW/day)	207.00±2.97^{a}	260.00+1.87^{bc}
5. OVX	Root extract (100 mg/Kg BW/day)	204.00±3.63^{a}	258.00±0.94^{bc}
6. OVX	Root extract (1,000 mg/Kg BW/day)	206.00±3.11^{a}	255.60±1.25^{bc}
7. OVX	Leaf extract (100 mg/Kg BW/day)	200.00±2.55^{a}	258.00±1.14^{bc}
8. OVX	Leaf extract (1,000 mg/Kg BW/day)	204.00±2.28^{a}	244.60±2.86^{bc}

All values are expressed as mean ± S.E.M. of 5 rats in each group (n = 5).

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

Table 5.2 Effect of *T. paniculatum* extracts and standard-phytol on relative uterus and mammary weight changes (n = 5).

Group	Treatment and Dosage	Relative Uterine Weight (%RU)	Relative Mammary Weight (%RM)
1. Sham-operated control	Vehicle (1 mL/rat/day; 10% v/v Tween 80 in sesame oil)	0.37 ± 0.01^{b}	0.54 ± 0.01^{d}
2. OVX vehicle control	Vehicle (1 mL/rat/day; 10% v/v Tween 80 in sesame oil)	0.17 ± 0.02^{a}	0.22 ± 0.01^{a}
3. OVX E_2 control	17ß-estradiol (10 µg/Kg BW/day)	0.41 ± 0.01^{b}	0.86 ± 0.01^{e}
4. OVX	Standard-phytol (500 mg/Kg BW/day)	0.20 ± 0.01^{a}	0.23 ± 0.00^{ab}
5. OVX	Root extract (100 mg/Kg BW/day)	0.15 ± 0.01^{a}	0.21 ± 0.01^{a}
6. OVX	Root extract (1,000 mg/Kg BW/day)	0.14 ± 0.01^{a}	0.30 ± 0.01^{bc}
7. OVX	Leaf extract (100 mg/Kg BW/day)	0.16 ± 0.01^{a}	0.28 ± 0.01^{abc}
8. OVX	Leaf extract (1,000 mg/Kg BW/day)	0.33 ± 0.00^{b}	0.35 ± 0.01^{c}

All values are expressed as mean ± S.E.M. of 5 rats in each group (n = 5).

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

Table 5.3 Effect of *T. paniculatum* extract and standard-phytol on vaginal cornification in OVX rats, 42 days treatment period.

Group	Treatment and dosage	Cornified cell (%)		
		Day 1	Day 7	Day 42
1. Sham-operated control	Vehicle			
	(1 mL/rat/day; 10% v/v Tween 80 in sesame oil)	37.95±2.32^{b}	32.54±0.90^{c}	21.08±0.90^{b}
2. OVX vehicle control	Vehicle (1 mL/rat/day; 10% v/v Tween 80 in sesame oil)	00.00±0.00^{a}	00.00±0.00^{a}	00.00±0.00^{a}
. OVX E_2 control	17ß-estradiol (10 µg/Kg BW/day)	00.00±0.00^{a}	34.15±1.46^{c}	56.04±1.46^{d}
4. OVX	Standard-phytol (500 mg/Kg BW/day)	00.00±0.00^{a}	37.02+1.56^{c}	42.77±1.56^{c}
5. OVX	Root extract (100 mg/Kg BW/day)	00.00±0.00^{a}	15.86±1.73^{b}	26.29±0.77^{b}
6. OVX	Root extract (1,000 mg/Kg BW/day)	00.00±0.00^{a}	26.27±0.64^{bc}	39.45±0.63^{c}
7. OVX	Leaf extract (100 mg/Kg BW/day)	00.00±0.00^{a}	16.30±0.84^{b}	21.50±0.54^{b}
8. OVX	Leaf extract (1,000 mg/Kg BW/day)	00.00±0.00^{a}	18.94±0.60^{b}	39.35±1.76^{c}

All values are expressed as mean ± S.E.M. of 5 rats in each group (n = 5).

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

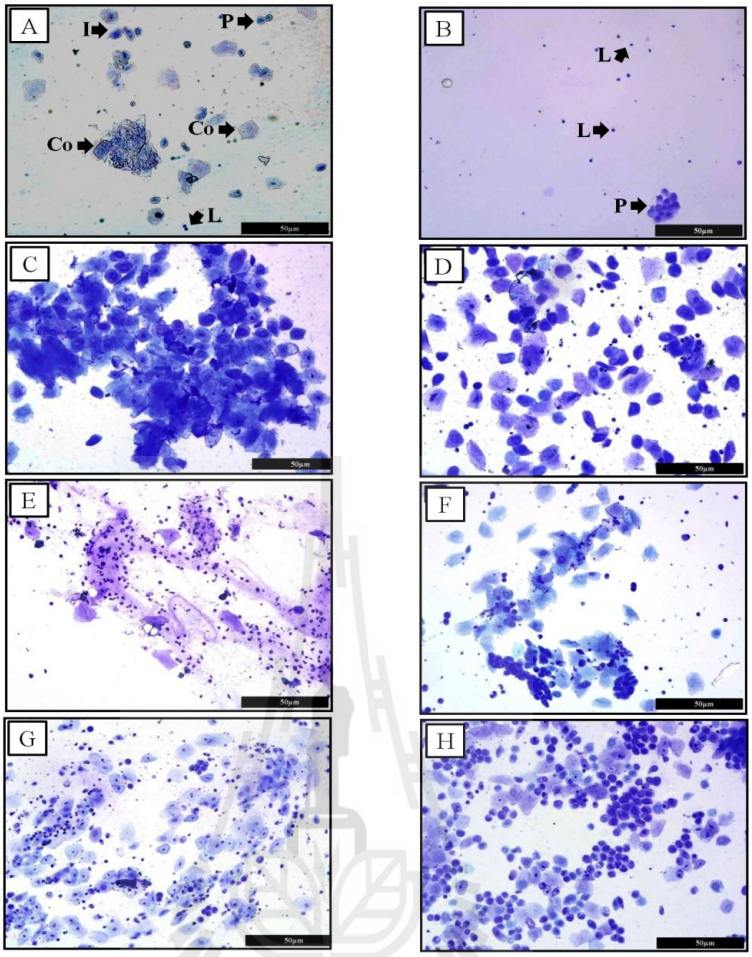

Figure 5.1 Photographic of methylene blue staining on vaginal smear from the rats at 21 days of the experimental period. A represents Sham-operated control during proestrous; B represents OVX control; C represents standard drug control (17β-estradiol 10µg/Kg BW/day); D represents standard component (standard-phytol 500 mg/Kg BW/day); E represents *T. paniculatum* root extract (100 mg/Kg BW/day); F represents *T. paniculatum* root extract (1,000 mg/Kg BW/day); G represents *T. paniculicata* leaf extract (100 mg/Kg BW/day); H represents *T. paniculatum* leaf extract (1,000 mg/Kg BW/day). Note; Co represents as superficial or cornified cell, I represents as intermediated cell, L represents as leucocytes and P represents as parabasal cell (Bars represent 50 µm).

5.4.3 Histological Observation of Female Reproductive Organs

Vaginal Histologic Observation

The representative vaginal histology is demonstrated in Figure 5.2, showing intensive atrophic vaginal epithelial lining in OVX rats received vehicle control. The responsiveness of the vaginal epithelial thickness to *T. paniculatum* extracts depended on the quantity of the plant extracts which were fed to OVX rats. The groups treated by E_2, standard-phytol, plant extracts and sham-operated control showed the proliferative changes of the vaginal epithelial layers. The administration of 1,000 mg/Kg BW/day of root and leaf extracts generated more response than the dose of 100 mg/Kg BW/day.

In addition, the morphological changes of the vaginal epithelial cell types were determined. In OVX control group, the eradication of ovarian hormones stimulation caused an atrophy of the vaginal epithelium which characterized by poorly inactive epithelial lining. This layer consisted of one to two shrivel cuboidal or flattened squamous cell type with a diminutive mucous cells (Figure 5.2B).

The typical estrogenic pattern, a keratinized stratified squamous epithelium was outstanding exhibited in sham-operated and positive E_2 treated rats (Figure 5.2A and 5.2C). This area was covered by high amount of mucous cells. A similar epithelial feature was also found in the OVX rats treated with standard-phytol at the dose of 500 mg/Kg BW/day. For OVX rats treated with 1,000 mg/Kg BW/day of root and leaf extract (Figure 5.2E and 5.2H), the histological feature of the vaginal sections demonstrated a thickening keratinized stratified squamous epithelium that was almost comparable to the standard E_2 and standard-phytol treated rats. The basal layer (stratum basale) of *T. paniculatum* extract-treated groups were established by a

pseudo-stratified low columnar mucous cells, thus, they were more developed than OVX control group (Figure 5.3B) that composed of only one or two of undefined cuboidal epithelial cells.

The vaginal epithelial layer in each section was also measured. OVX noticeably reduced the thickness of the vaginal epithelial layer (8.26 $\pm$ 0.57µm) compared with the sham-operated control group (34.00 $\pm$ 0.90µm) ($P < 0.001$). All rats which were fed by plant extracts and standard-phytol showed the evidence of vaginal epithelial expansion. The greatest response of the vaginal thickness was found in the group which treated by standard-phytol for 500 mg/Kg BW/day (27.30 $\pm$ 0.71µm). A dose-dependent increase of the epithelial layer thickness was observed in rats supplemented with elevating the dose of *T. paniculatum* extracts. As a result, oral feeding by 1,000 mg/Kg BW/day of the leaf extract (26.49 $\pm$ 0.33µm) restored the epithelial thickness as comparable to the positive E_2 treated rats (25.53 $\pm$ 0.62µm) ($P = 0.47$). The treatment with 100 mg/Kg BW/day of leaf extract showed the lesser response (16.72 $\pm$ 0.33µm) compared to 1,000 mg/Kg BW/day. The groups that were fed by the root extracts (100 and 1,000 mg/Kg BW/day) exhibited less effective than the leaf extract treated groups compared to the same dose (13.49 $\pm$ 0.88µm and 20.97 $\pm$ 0.45µm, respectively).

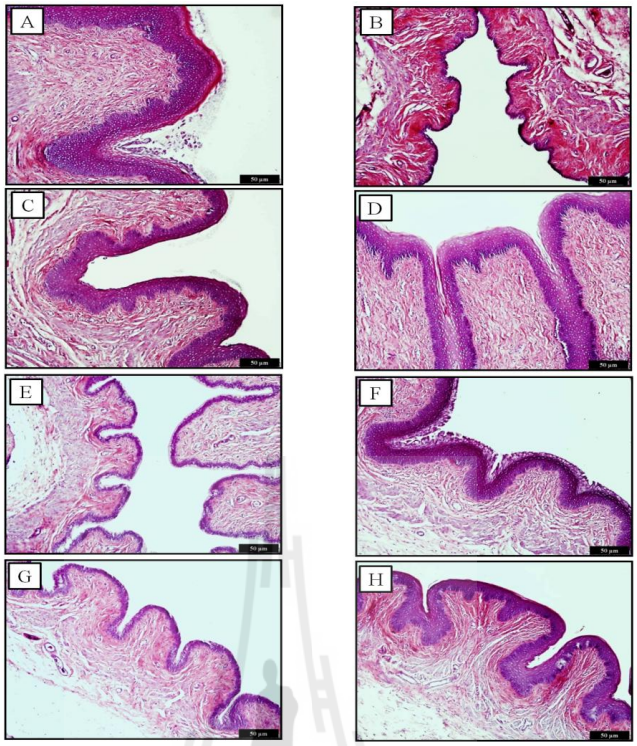

Figure 5.2 Representative images of hematoxylin and eosin staining of vaginal tissue from the rats after 42 days experimental period. A represents the keratinization and thickening of the vaginal epithelial layer in sham-operated control during pro-estrous. The vaginal layer mainly composted by 4 layers; stratum basale, stratum spinosum, statrum granulasum and statrum corneum which covered by the packed flattened cornified cells; B represents the atrophic pattern of vaginal epithelial lining in OVX rat control showing undeveloped vaginal epithelial surface comprised by atrophic cuboidal/undefined flattened cells; C represents E_2 control (17β-estradiol 10 µg/Kg BW/day); D represents standard-phytol (500 mg/Kg BW/day); E represents *T. paniculatum* root extract (100 mg/Kg BW/day); F represents *T. paniculatum* root extract (1,000 mg/Kg BW/day); G represents *T. paniculatum* leaf extract (100 mg/Kg BW/day); H represents *T. paniculatum* leaf extract (1,000 mg/Kg BW/day) (Bars represent 50 µm).

Uterine Histological Observation

The uterine wall comprises of three distinct tissue layers: a tunica mucosa (endometrium), tunica muscularis (myometrium), and tunica serosa (perimetrium). Only the endometrium was described in this study.

Endometrium:

The endometrium is an inner layer of the uterus when defined with light microscope. The histological section of sham-operated uterus was bright and bulky. The thickening endometrial surface consisted of surface epithelium and the lamina propia mucosa associated with numerous tubular endometrial glands supported by a thick vascular stroma. The columnar type could be observed in surface epithelium (Figure 5.3A).

The transverse sections of OVX control uterus showed a narrow atrophic endometrium with the evidence of endometrial hypoplasia. As shown in Figure 5.6B, the uterine sections of OVX control in this experiment are dense and thin. The histological feature elicit typical atrophic feature with the thinning of endometrial layer. This layer contained atrophied uterine glands and poor vascularity which covered by low cuboidal epithelial cells in the luminal surface. The surface epithelium was covered with shorten simple columnar and inactive cuboidal types (Figure 5.3B).

Oral administration of E_2 at the dose of 10 μg/Kg BW/day for 42 days remarkably stimulated the size and all structures of the uterus as illustrated by an increasing in endometrial thickness, well developed uterine gland and more vascularity. The bulky epithelial layer was well developed which indicated by the columnar cell type. As illustrated in Figure 5.3D, standard-phytol treatment (500 mg/Kg BW/day) slightly enhanced both on uterine size and thickening of the

endometrial area. Furthermore, the histological findings of the uterus in this group demonstrated varying extent of endometrial thickening, which was dependable to the dosage of the extracts used; i. e. the higher dose brings greater degree of thickening and proliferation of the endometrial layer (Figure 5.3E, 5.3F, 5.5G and 5.3H). There was no significant change of endometrial proliferation in the groups treated by both dosages of the root (100 and 1,000 mg/Kg BW/day) and 100 mg/Kg BW/day of leaf extract. The treatment with high dose of leaf extract (1,000 mg/Kg BW/day) was potentially stimulated the histological architecture of the uterus as illustrated by well-developed glands and thickening of endometrial layer. The uterotrophic data described that the high dosage of leaf extract estrablished the most effective effect to induce endometrial development in OVX rats which greater than E_2 and standard-phytol treated rats.

Endometrial gland:

In sham-operated uteri, the glandular profiles covered with height of the simple columnar epithelium were observed. Some glandular sections were lined with pseudo-stratified epithelium. The light microscopic observations depicted numerous branching endometrial glands in this group. On the other hand, the small, closed, and non-branching endometrial glands were found in OVX-receive vehicle uterine section. The general morphology of cell structure did not notably differ between the sham-operated versus OVX control. Including its sizes, the number and distribution of glands were greatly condensed in OVX uteri. All structures were hypertrophic and hyperplastic from the treatment with E_2; the sizes, number and distribution of uterine glands were intensively observed. In addition, the treatment by standard-phytol and *T. paniculatum* extracts showed evidence of the uterotrophic properties as illustrated by

increasing the numbers of uterine glands compared to OVX-received vehicle control. The oral feeding by 1,000 mg/Kg BW/day of the leaf extracts revealed the most effective to prevent the regression of the uterus (Figure 5.4).

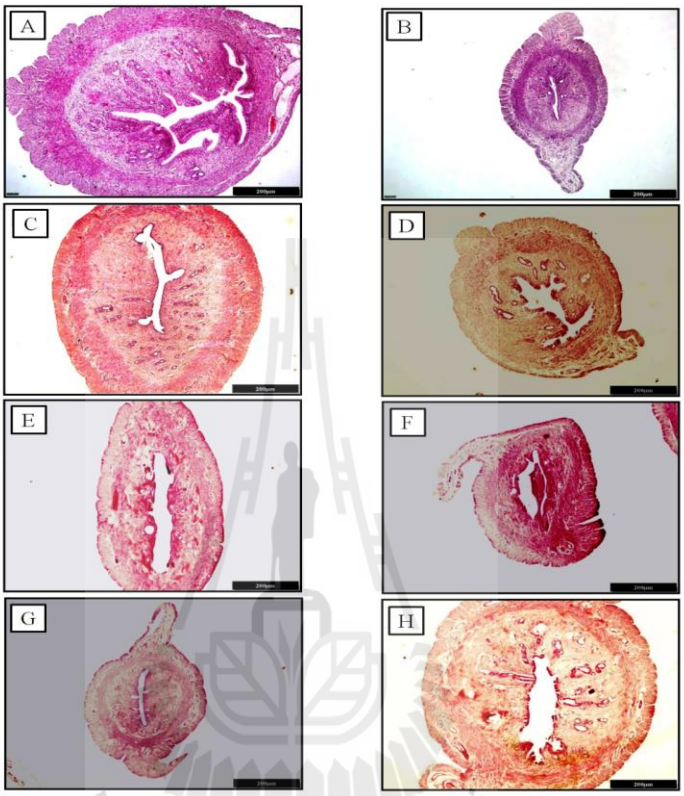

Figure 5.3 Representative images of hematoxylin and eosin staining on uterine histomorphology of the sham-operated and OVX rats treated by various treatments for 42 days. A represents the representative of estrogenic effect demonstrates the in sham-operated rat's uterus during estrous period; B represents the atrophic pattern of uterus in OVX rat received vehicle control; C represents E_2 control (17β-estradiol 10 µg/Kg BW/day); D represents standard-phytol (500 mg/Kg BW/day); E represents *T. paniculatum* root extract (100 mg/Kg BW/day); F represents *T. paniculatum* root extract (1,000 mg/Kg BW/day); G represents *T. paniculatum* leaf extract (100 mg/Kg BW/day); H represents *T. paniculatum* leaf extract (1,000 mg/Kg BW/day) (Bars represent 200 µm).

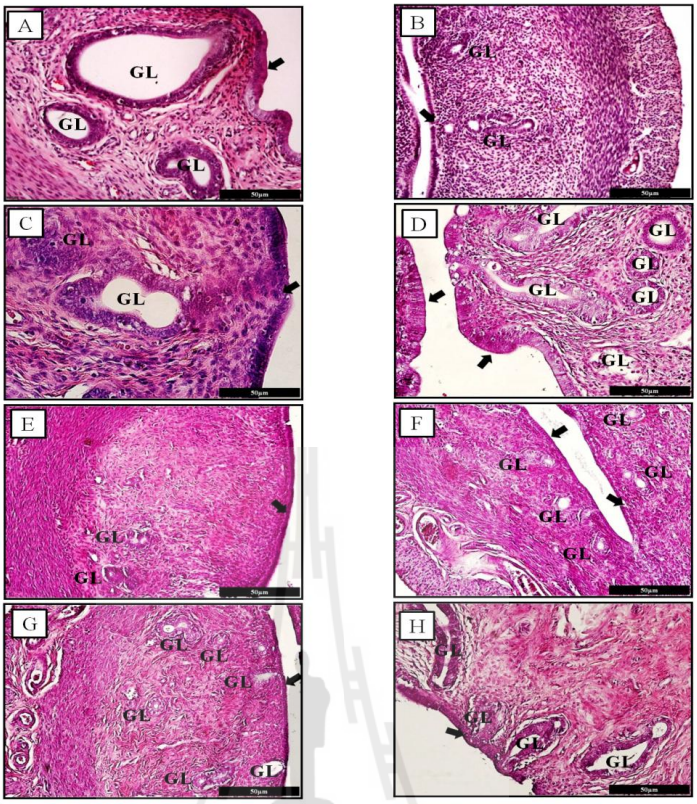

Figure 5.4 Representative images of hematoxylin and eosin staining on endometrial gland (GL) and surface epithelium (arrow) histomorphology of the sham-operated and OVX rats treated by various treatments for 42 days experimental peroid. A represents the representative of estrogenic effect demonstrates the tall columnar surface epithelium in sham-operated control rat during pro-estrous period; B represents the atrophic pattern of uterus in OVX rat received vehicle control. The surface epithelium comprised by atrophic cuboidal or undefined flattened cells; C represents standard E_2 control (17β-estradiol 10 µg/Kg BW/day); D represents standard-phytol (500 mg/Kg BW/day); E represents *T. paniculatum* root extract (100 mg/Kg BW/day); F represents *T. paniculatum* root extract (1,000 mg/Kg BW/day); G represents *T. paniculatum* leaf extract (100 mg/Kg BW/day); H represents *T. paniculatum* leaf extract (1,000 mg/Kg BW/day). (Bars represent 50 µm).

Mammary Tissue Histological Observation

Figure 5.5 shows histological observation of representative mammary tissues from one animal per treatment group. The revolution of overall mammary development and epithelial duct proliferation were considerably different in the microscopic features indicated by the number and the mammary duct organization. Proliferation of mammary mass was decreased around 40.24% in OVX rats compared to sham-operated control rats (0.22 $\pm$ 0.01 vs. 0.54 $\pm$ 0.01%) ($P < 0.001$) and it corresponded to reduced total mammary duct per section (15.33 $\pm$ 0.69). The histological section in sham-operated control showed more complex branching mammary epithelial duct, and the section from OVX-received vehicle control was almost completely absent in the number. In addition, the uterine glands obtained from OVX control consisted of one or two major ducts with limited branching. The degree of ductular formation revealed to be related to the abundance of parenchymal tissue presented, which leads to a decrease in the total %RM. OVX rats received E_2 had substantially much more parenchymal tissues and large mammary gland containing the secretory fluid. The supplementation with standard-phytol notably induced ductular structure; however, the secretory fluid was undetectable. Moreover, the mammary duct formation was present in all rats which were treated by the plant extracts. The partial extended of ductular formation was observed in OVX rats administered by both dosages of root extract and 100 mg/Kg BW/day of leaf extract.

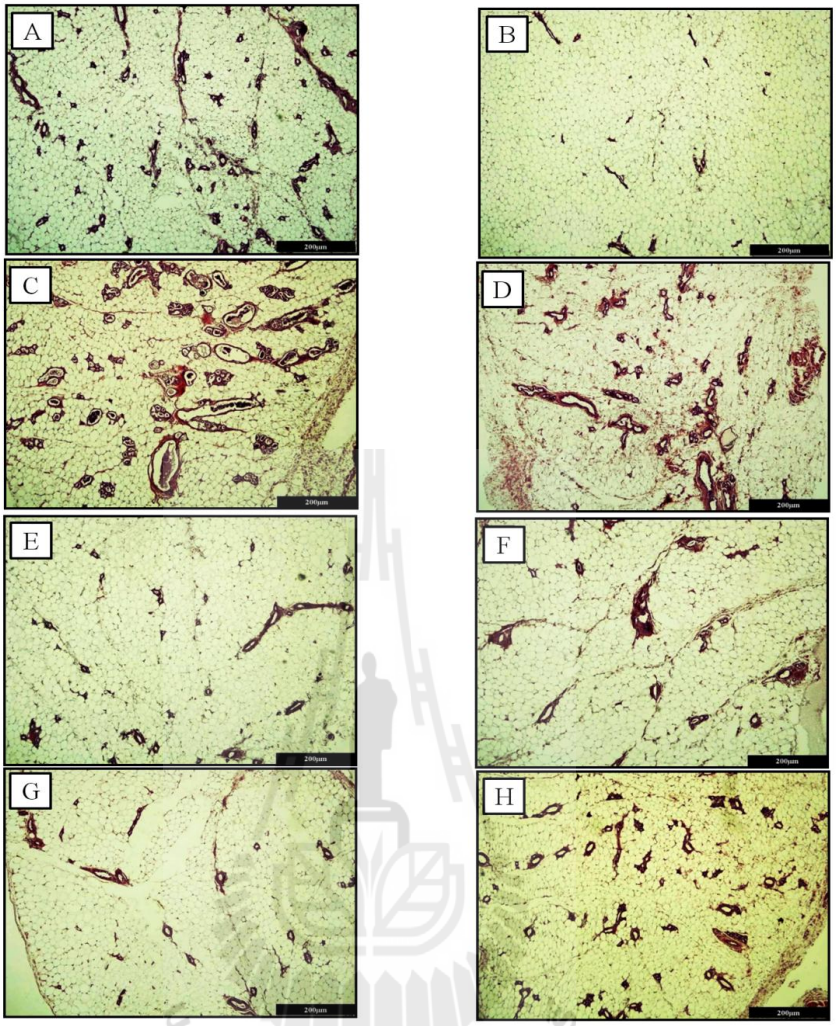

Figure 5.5 Representative images of hematoxylin and eosin staining on mammary tissue preparations from the sham-operated and OVX rats treated by various treatments for 42-days treatment period. A represents sham-operated control; B represents OVX control; C represents standard E_2 control treated rat (17β-estradiol 10 µg/Kg BW/day); D represents standard-phytol (500 mg/Kg BW/day); E represents *T. paniculatum* root extract (100 mg/Kg BW/day); F represents *T. paniculatum* root extract (1,000 mg/Kg BW/day); G represents *T. paniculatum* leaf extract (100 mg/Kg BW/day); H represents *T. paniculatum* leaf extract (1,000 mg/Kg BW/day)

(Bars represent 200 µm).

5.5 Discussion

Female reproductive organs undergo numerous physiological and biochemical changes that depend on the ovarian steroid hormones. The physiological level of ovarian steroid hormones are associated not only the initiation, but also in the developmental process of reproductive system (Rasier et al., 2006; Wilhelm, Palmer and Koopman, 2007). The gonadal ablation or bilateral ovariectomy causes a decrease in these hormones, hence directly affect to their target organs.

Genital atrophy and mammary gland regression are the common clinical presentations that occur after the cessation of ovarian function. These symptoms are the serious problems in postmenopausal women and lead to decrease their life quality. Exogenous hormone supplementation induces the intrinsic hormonal equilibrium and affects the normal physiology through gross morphological, histological and biochemical modifications. Despite the report of some phytosterols in the plant of Portulacaceae family have been described (Ahmad and Beg, 2001), there was no scientific information which elucidated the estrogenic activity of *T. paniculatum* extracts. The present experiment attempted to evaluate the estrogenic activity of *T. paniculatum* root and leaf extracts and to compare their effects with standard E_2 and standard-phytol using OVX rat model.

The underlying mechanisms by which phytoestrogenic substances exert their effects on body composition and obesity after menopause are still unclear; they are acknowledged to have the beneficial effects on body fat distribution and lipid metabolism (Cederroth et al., 2007). The current experimental data exhibited the elevation of final body weight of OVX rats that received vehicle control might be due to overiectomy-induced hyperphagia and decreased energy expenditure. The

treatment with E_2 prospectively reversed these effects. According to mild rising of the final weight in the groups which treated by standard-phytol and *T. paniculatum* extracts, the results suggested that these compounds did not have any statistically effect on the body weight.

It is well known that the estrous cycle undergoes physiological and biochemical changes under the influence of reproductive hormones which leads to dynamic changes of the uterus and vagina. Estrogen is the vital hormone that enhances uterine growth and vaginal cornification response by genomic or non-genomic pathways. Subsequently, it increases uterine weight and the keratinization of the vagina. Due to the lack of estrogen level from the removal of ovaries, the uterus and vagina become atrophy (Diel, 2002).

The rat vaginal wall provides an excellent model to determine the estrogenic activity of the estrogenic substances that recognize as a simple, sensitive, and inexpensive method (Parhizkar et al., 2011). Vaginal cytology assay is a practical technique which firstly conducted by Cook et al. in 1933. The estrogenic-like compounds have been clarified to have an effect on vaginal epithelium differentiation (Laws et al., 2000; Burton and Wells, 2002). They affect the vaginal epithelium by changing it into squamous cell and later shading into lumen. The current experiment was confirmed menopausal stage in OVX rats by monitoring the cellular differentiation in vaginal smears for 14 consecutive days, and none of these rats were cycling.

The cornification and keratinization were observable in the rat vaginal smears following the treatment of *T. paniculatum* extracts within the first week after experimenting. This indicated that the estrogenic effects of these compounds occurred

by short-term consumption. Additionally, the dose dependent increases in percentage of vaginal cornification induced by the extracts of *T. paniculatum* revealed its estrogenic activity. Surprisingly, the treatment with standard-phytol also enhanced estrogenic effects which indicated by the differentiation of vaginal epithelial cells.

To support the evidence of estrogenic activity exerted by *T. paniculatum* extracts and standard-phytol, the experiment was designed to further investigate both quantitative and qualitative changes of the uterus. The current experimental data revealed that the oral treatment of *T. paniculatum* leaf extract (1,000 mg/Kg BW/day) and standard-phytol had a significant effect by increasing %RU. The result was strongly confirmed by the greatest response in the uterine histological section showing the thickening of endometrial area, well developing of luminal epithelial surface and uterine glands.

The mammary tissues are profoundly endocrine-sensitive organs that rely on ovarian steroids and other hormonal signals for its proper growth and differentiation (Hansen and Bissell, 2000; Mukhina et al., 2006). Therefore, there were subjected to estrogenic action. In order to evaluate if the estrogenic compound would cause proliferation in this study, we observed the increasing of mammary weight, quantity and characteristic of the duct system in all samples from OVX and sham-operated control groups. Mammary tissue was underdeveloped in the OVX control rats, and a few collapsed terminal mammary ducts were detected. The oral administration of estrogen extremely enhanced both gross and histological structure as indicated by increasing %RM and luminal structure formation containing the secretion. The dose of 100 mg/Kg BW/day of both root and leaf extracts (100 and 1,000 mg/Kg BW/day) had slightly effects on mammary weight and morphology compared to the dose of

1,000 mg/Kg BW/day. Furthermore, mammary tissue of OVX treated by standard-phytol at the dose of 500 mg/Kg BW/day showed high amount of developing luminal histoachitecture without secretion, but its quantity was still lower than sham-operated and OVX receiving standard E_2 control.

The study demonstrated that gonadal ablation in rats can directly decrease sex steroid hormones, thus having direct effects on female reproductive organs. Estrogen can induce vaginal cornification indicating the estrous stage, and the full cornification requires the higher surging of circulating estrogen level (Safranski, Lamberson and Keisler, 1993). Buchanan and colleagues (1998) demonstrated that the proliferation of vaginal epithelium was interceded indirectly through estrogen receptor-α (ERα), which mediated by estrogen-induced cornification and stratification.

Estrogen is also documented to induce uterine growth response by non-genomic action which associates with increases in vascular permeability, water imbibitions, and cellular infiltration (Rockwell et al., 2002). In this study, the primary source of estrogen was terminated due to the removal of ovaries. The appearance of vaginal cornification, the increasing of the parameters in uterotrophic, and mammogenic assay attributed to describe the estrogenic effect of *T. paniculatum* extracts; which confirmed by their histological features. However, phytoestrogens mainly bind to estrogen receptor-β (ER_β) with more affinity compared to ER_α, but they still generate their estrogenic activity through ER_β (Kuiper et al., 1997). This evidence could be supported the possible mechanism by which *T. paniculatum* extracts produced estrogenic activity in OVX rats.

Chlorophyll-derived phytols is the precursor of phytanic acid; the natural agonist of RXR and PPARα (Goldstein et al., 2003; Heim et al., 2002). The activation

of these receptors can modulate the estrogen responsiveness genes, consequently stimulates the ERs activity at their target tissues (Björnström and Sjöberg, 2005). The current data demonstrated that treatment by standard-phytol (500 mg/Kg BW/day) to OVX rats could activate only the histoarchitecture of the genital and mammary tissues, but were not able to enhance the gross morphological changes of these organs. Based on our findings, the estrogenic parameters of the OVX rats treated by leaf extract showed more effective response than the root extract had in the same dose. These findings are probably due to the synergic effect between the phytosterols and phytols in the leaf extract. Additionally, sesame oil could not cause the statistical elevation in these estrogenic parameters. This study also suggested that sesame oil could possibly account as an appropriate vehicle control to measure the estrogenic activity of the estrogenic compounds.

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CHAPTER VI

THE ANTI-FERTILITY ACTIVITY OF *TALINUM PANICULATUM* (JACQ.) GAERTN. EXTRACTS ON PREGNANT RATS

6.1 Abstract

Talinum paniculatum (Jacq.) Gaertn. (*T. paniculatum*) is extensively used in Asian traditional medicine to manage the reproductive system. However, there is no conclusive evidence to support this practice. The aim of this study was to determine the validity of antifertility effect of *T. paniculatum* and its related compound-phytols as compared with17ß-estradiol (E_2), which was studied in proven fertile female rats. Pregnant rats were randomly separated into 7 groups (n = 5). Group 1 received the vehicle (Tween 80 in sesame oil, 10% v/v) and served as vehicle control. Group 2 was treated with 17β-estradiol (10 μg/Kg BW/day) as a positive E_2 control. Group 3 was treated with 500 mg/Kg BW/day of standard-phytol. Group 4-7 were treated with different doses of *T. paniculatum* root and leaf extracts (100 and 1,000 mg/Kg BW/day, respectively). The results showed that all of the extract dosages produced antiimplantation activity and early abortifacient activity in a dose dependent manner ($P<0.05$). In contrast, the oral administration of standard-phytol could not exhibit the

significantly antiimplantation and early abortifacient activity when compared to vehicle control rats ($P > 0.05$). The potent antifertility activity were observed in all dosage of *T. paniculatum* root (47.11 $\pm$ 5.34% and 71.65 $\pm$ 4.42%, respectively) and leaf extracts (81.21 $\pm$ 7.06% and 97.29 $\pm$ 6.24%%, respectively) ($P < 0.05$). Likewise, the oral feeding of standard-phytol showed mild antifertility activity compared to vehicle control ($P = 0.35$). In conclusion, the results suggested that *T. paniculatum* root and leaf extracts possess a potent antifertility effect in pregnant rats.

6.2 Introduction

Fertility control is a critical issue for women worldwide. About 1% of pregnant women loss their lives due to unintended pregnancy or hazardous abortion procedures (Glasier et al., 2006). Generally, the severe adverse effects such as depression, gastrointestinal disturbance, massive painful uterine contraction, systemic illness, permanent infertility or death are frequently reported regarding women who utilized synthetic drugs or steroid contraceptions (O'Connell, Davis and Kerns, 2007; Sanchez-Criado, Tebar and Padron, 1997). Although herbal contraceptives could never reach the level of classical contraceptive pills, they are commonly cheaper and are safer in terms of undesirable side effects. Hence, there is a need for suitable medicinal plants with antiimplantation and abortifacient activity that could be both safe and effective to control pregnancy.

Talinum paniculatum (Jacq.) Gaertn. (*T. paniculatum*) or Som Java is recognized as having various medicinal properties (Thomas, 2008). The plant is a wild deciduous perennial herb with well-developed root system. The medicinal-prepared *Talinum* spp. has long been used in folk medicine; particularly in the

treatment of type-2 diabetes, inflammatory skin problems, gastrointestinal disturbance, general weakness and reproductive disorders (Shimoda et al., 2001; Pak et al., 2005). The root has active constituents such as steroidal saponins and tannins. Meanwhile, only tannins can be detected in the leaf (Yulia, Wientarsih and Razief, 2005). Additionally, Filho and colleagues (2010) reported that campesterol, β-sistosterol, stigmasterol could be extracted from the leaf of *T. paniculatum*. Despite these traditional medicinal properties, no scientific data has been carried out regarding to antifertility activity of the plant *T. paniculatum*. Therefore, this study was designed to evaluating the antifertility activity of *T. paniculatum* root and leaf extracts in female Wistar rats.

6.3 Materials and Methods

6.3.1 Animals

Pregnant Wistar rats (200-250g) were used for antifertility activity evaluation. All procedures involving animals were performed in accordance with the guidelines of the Committee on Care and Use of Laboratory Animal Resource, National Research Council of Thailand. The experiments performed on rats were conducted under strict compliance with the advice of the Institutional Animal Care and Use Committee, Suranaree University of Technology, Nakhon Ratchasima, Thailand.

6.3.2 Anti-Fertility Activity Evaluation

The experimental protocols were designed by evaluating the antiimplantation activity and abortifacient activity as previously described by Mukhram and coworkers (2012). Briefly, virgin female rats in the proestrous stage were selected and placed

overnight with proven fertile male. The rats that showed thick clumps of spermatozoa in vaginal smears was separated and designated as 1^{st} day pregnancy.

Pregnant rats were randomly separated into 7 groups of 5 rats per group. Group 1 received the vehicle (Tween 80 in sesame oil, 10% v/v) and served as control. Group 2 was treated with 17β-estradiol (10 μg/Kg BW/day) as a positive E_2 control. Group 3 was treated with 500 mg/Kg BW/day of standard-phytol. Group 4-7 were treated with different doses of *T. paniculatum* root and leaf extacts (100 and 1,000 $mg.kg^{-1}$ BW, respectively). All groups were orally administered the vehicle and plant extracts during 1^{st}-7^{th} day of pregnancy. On the 8^{th} day, the bilateral laparotomy was carried out under surgical stage of anesthesia (pentobarbital sodium 15 mg/Kg BW/day) in sterile conditions. The numbers of implantation sites and corpora lutea in ovaries were observed in order to evaluate the antiimplantation activity. The lateral abdomens were sutured and rats were positioned in the cages for recovery. The vehicle and plant extracts were further treated for 7 days (9^{th}-14^{th} day of pregnancy). On the 15^{th} day, pregnant rats were scarified to evaluating the early abortifacient activity. A treatment regimen is shown in Figure 6.1.

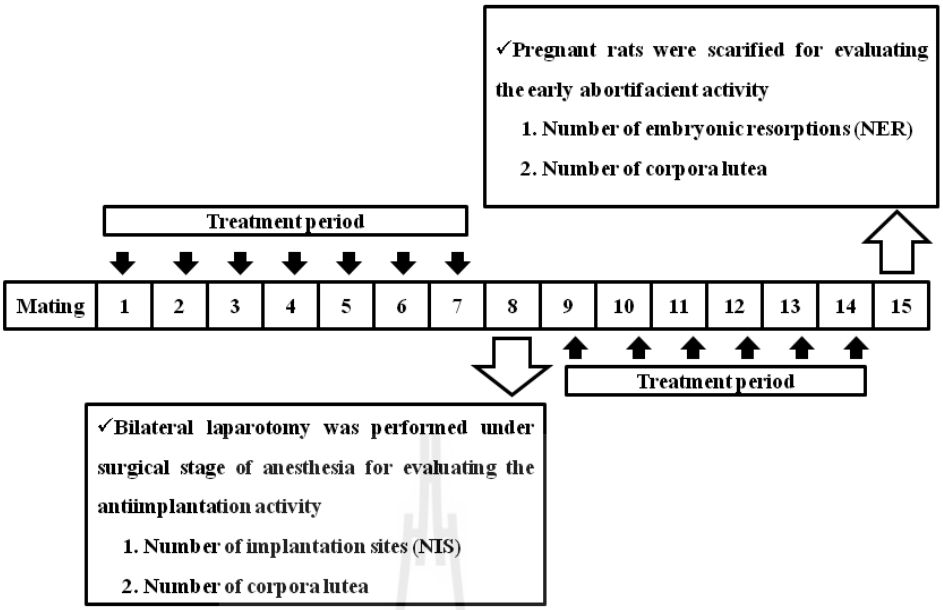

Figure 6.1 The treatment regimen for anitifertility evaluation of *T. paniculatum* extracts and standard-phytol.

The percentages of antiimplantation and early abortifacient activities were calculated. The summation of antiimplantation and early abortifacient activity gives percentage anti-fertility activity of the tested materials. The calculation formulas are shown below:

Anti-implantation activity (%) = 100 -$\left(\frac{No.of\ implantations}{No.of\ copora\ lutea}\right)$ X 100

Abortifacient activity (%) = $\left(\frac{No.of\ resorptions}{No.of\ copora\ lutea}\right)$ X 100

Anti-fertility activity (%) = $\%Antiimplantation\ activity + \%Abortifacient\ activity$

6.3.3 Statistical Analysis

All data are expressed to the mean value $\pm$ standard error of the mean (S.E.M.). Statistical analysis of difference was carried out by analysis of variance (ANOVA) followed by Scheffe's *post hoc* test using SPSS windows program version

11 (SPSS Institute, Inc., Chicago, IL, USA). A probability level less than 5% ($P < 0.05$) was considered statistically significant.

6.4 Results

6.4.1 Effect of *T. paniculatum* Extracts on Number of Implantation Sites and Number of Embrypic Resorptions

The effect of standard-phytol, *T. paniculatum* root and leaf extracts on number of implantation sites (NIS) and number of embrypic resorptions (NER) are summarized in Table 6.1. As expected, the treatment by standard E_2 possessed significantly negative effects on pregnant rats as indicated by the most lowered in NIS but highest in NER.

Compared to vehicle control pregnant rats, the oral administration of 1,000 mg/Kg BW/day of root extract and all dosages of leaf extracts significantly decreased in NIS but increased in NER. Alternatively, the treatment by standard-phytol did not have any statistically effect on NIS and NER when compared with the vehicle control pregnant rats.

Table 6.1 Effect of *T. paniculatum* extracts and standard-phytol on number of implantation sites (NIS) and number of embrypic resorptions (NER) in female Wistar rats.

Group	Treatment and Dosage	Number of Implantation Sites (NIS)	Number of Resorptions (NER)
1. Pregnant Control	Vehicle control (1mL/rat/day)	12.20 ± 1.39^{d}	0.00 ± 0.00^{a}
2. Pregnant	17ß-estradiol (10 µg/Kg BW/day)	0.80 ± 0.37^{a}	11.00 ± 0.71^{d}
3. Pregnant	Standard-phytol (500 mg/Kg BW/day)	10.60 ± 1.17^{cd}	1.20 ± 0.37^{ab}
4. Pregnant	Root extract (100 mg/Kg BW/day)	10.40 ± 0.68^{bcd}	3.60 ± 0.60^{bc}
5. Pregnant	Root extract (1,000 mg/Kg BW/day)	8.40 ± 0.24^{bcd}	5.20 ± 0.58^{c}
6. Pregnant	Leaf extract (100 mg/Kg BW/day)	6.60 ± 0.24^{bc}	4.60 ± 0.68^{c}
7. Pregnant	Leaf extract (1,000 mg/Kg BW/day)	6.00 ± 0.84^{b}	5.20 ± 0.40^{c}

All values are expressed as mean $\pm$ S.E.M. of 5 rats in each group ($n = 5$).

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

6.4.2 Effect of *T. paniculatum* Extracts on Anti-implantation and Early Abortifacient Activity

Among the tested groups, the oral supplementation of standard E_2 elicited the most potent effects on anti-implantation and early abortifacient activities in pregnant rats. In contrast, mild anti-implantation and early abortifacient activities were observed in pregnant rats that were treated by standard-phytol, as compared to vehicle control rats ($P > 0.05$).

Anti-implantation and early abortifacient activity dose dependencies responded to *T. paniculatum* extracts on pregnant rats are illustrated in Table 6.2. With an increase in the dose of both root and leaf extracts (100 and 1,000 mg/Kg BW/day), the percentage of Anti-implantation activity were significantly increased as evidenced by decreasing of the number of implantation site on 8th day of pregnancy ($P < 0.05$). Moreover, *T. paniculatum* extracts also produced a significantly early abortifacient activity which indicated from the implantation scars in the uterine horn on 15th day of pregnancy (Figure 6.2).

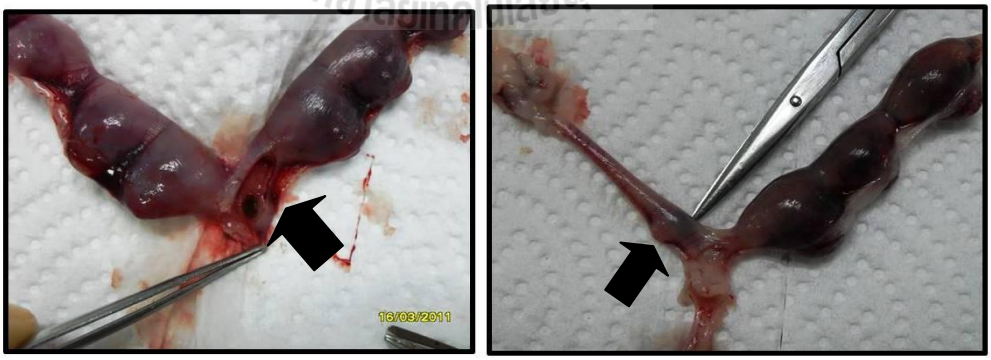

Figure 6.2 The 15th day of pregnant uteri show embryonic resorption scars (arrow) after the oral administration of *T. paniculatum* root extract (1,000 mg/Kg BW/day; left) and *T. paniculatum* leaf extract (1,000 mg/Kg BW/day; right) for 15 consecutive days.

6.4.3 Effect of *T. paniculatum* Extracts on Anti-Fertility Activity

Among the different dosage of the plant extracts, the significantly dose dependent effect of anti-fertility activity was observed ($P < 0.05$). Compared with vehicle control rats, the percentage of anti-fertility activity of *T. paniculatum* root extracts at the dose of 100 and 1,000 mg/Kg BW/day were found to be 47.11 $\pm$ 5.34% and 71.65 $\pm$ 4.42% respectively; whereas the percentage of anti-fertility activity of *T. paniculatum* leaf extracts at the dose of 100 and 1,000 mg/Kg BW/day were found to be 81.21 $\pm$ 7.06% and 97.29 $\pm$ 6.24%. The oral administration of standard-phytol at the dose of 500 mg/Kg BW/day showed mild anti-fertility, but the standard-phytol could not exhibit the statistical difference when compared with the vehicle control rats ($P = 0.35$).

Table 6.2 Anti-fertility activity of *T. paniculatum* extracts and standard-phytol in pregnant Wistar rats.

Group	Treatment and Dosage	Anti-implantation Activity (%)	Early-abortifacient Activity (%)	Anti-fertility Activity (%)
1. Pregnant Control	Vehicle control (1mL/rat'day)	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
2. Pregnant	17ß-estradiol (10 µg/Kg BW/day)	91.89 ± 4.09^{d}	$96.33 + 2.26^{e}$	$188.22 + 6.12^{e}$
3. Pregnant	Standard-phytol (500 mg/Kg BW/day)	9.43 ± 3.22^{ab}	$11.60 + 3.75^{ab}$	$21.02 + 6.80^{ab}$
4. Pregnant	Root extract (100 mg/Kg BW/day)	18.97 ± 2.21^{b}	28.15 ± 4.28^{bc}	47.11 ± 5.34^{bc}
5. Pregnant	Root extract (1,000 mg/Kg BW/day)	27.28 ± 2.31^{bc}	44.38 ± 3.26^{cd}	71.65 ± 4.42^{cd}
6. Pregnant	Leaf extract (100 mg/Kg BW/day)	41.44 ± 3.79^{c}	39.77 ± 4.12^{cd}	81.21 ± 7.06^{d}
7. Pregnant	Leaf extract (1,000 mg/Kg BW/day)	45.42 ± 5.66^{c}	51.57 ± 2.27^{d}	97.29 ± 6.24^{d}

All values are expressed as mean $\pm$ S.E.M. of 5 rats in each group ($n = 5$).

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

6.5 Discussion

The estrogenic substances are recognized to restrain pregnancy by affecting the equilibrium of reproductive hormones to regulate the hypothalamus-pituitary-gonadal axis (Laurence and Bacharach., 1964; Havranex et al., 1973). Any disturbance in the level of these hormones may cause infertility by affecting the ovulation, implantation and obstructing the uterine milieu (Hughes et al., 1991; McGarvey et al., 2001; Abu and Uchenda, 2011).

The large consumption of estrogenic substances, as well as, phytoesterogens can enhance the luteolytic activity (Shibeshi et al., 2006). They also increase the sensitivity of the uterus responding to prostaglandins, which leads to the failure of implantation and increase the abortion rate (Woclawek-Potocka et al., 2005).

In this study, the evaluation of the anti-fertility of *T. paniculatum*'s extracts and standard-phytol was conducted for both times before and after implantation process. As results, *T. paniculatum* extracts enhanced the anti-implantation activity as a dose dependent manner, whereas standard-phytol did not showed the statistically significant effect in pregnant rats. *T. paniculatum* extracts also affected the conceptous after implantation period as illustrated by the elevation of abortifacient activity. Among the tested groups, the group that was treated with *T. paniculatum* leaf extract at the dose of 1,000 mg/Kg BW/day exhibited the most potent in an anti-fertility activity, which evidenced by the decreasing of implantation sites and increasing of abortifacient activity.

Additionally, the treatment by standard-phytol (500 mg/Kg BW/day) to pregnant rats enhanced non-statistical anti-fertility effects. Our current data are concur with the Arnhold, Elmazar and Nau's study (2002) which demonstrated that phytols

(or their derivative-phytanic acid) did not potentiate to produce the teratogenic activity in pregnant rats.

The classical effects of estrogenic compounds, such as vaginal cornification and uterotrophic and mammogenic actions, are used to detect and confirm the property of anti-fertility substances (Ahirwar, Ahirwar and Kharya, 2010). The results in chapter 5 supported that standard- phytol brings estrogenic activity which strongly confirms an anti-fertility property in pregnant rats. Therefore, the observed mild anti-fertility effects of standard-phytol may be due its estrogenic activity by indirectly activates estrogen responsiveness genes via RXR and PPARα (Goldstein et al., 2003; Heim et al., 2002).

As shown in chapter 3, the GC/MS analysis of *T. panicaulatum* crude extracts showed the non-steroidal phytoestrogens such as campesterol, β-sitosterol, stigmasterol, stigmastan-3-ol, stigmast-22-en-3-ol and stigmastanol. These phytosterols claimed to possess estrogenic activity due to their affinity to estrogen receptors and produce infertility in animals (Dane and Patil, 2012; Suryawanshi, 2011). In conclusion, the anti-fertility activity of *T. paniculatum* root and leaf extracts is mainly due to the estrogenic activity of the presented phytosterols.

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CHAPTER VII

THE EFFECTS OF *TALINUM PANICULATUM* (JACQ.) GAERTN. EXTRACTS ON NON-PREGNANT RAT UTERINE CONTRACTILITY

7.1 Abstract

Talinum paniculatum (*T. paniculatum*) has long been used in Thai herbal recipes because of its various therapeutic properties. *T. paniculatum* is believed to be beneficial for female reproductive system by inducing lactation and restoring uterine functions after post-partum. Although, the plant has been claimed to influence the female reproductive system, but there is no scientific data regarding to clarify the effects on the uterus to support its therapeutic relevance. Therefore, the purpose of this study was to investigate the effects of *T. paniculatum* root and leaf extracts on uterine contractility and their possible mechanism(s) on adult female virgin rats (200-250 g). The rats were humanly euthanatized by CO_2 asphyxia and uteri removed. Isometric force was measured in strips of longitudinal myometrium (1-2 mm x 0.5 mm x 10 mm) in organ bath containing physiological Krebs' solution maintained at 37°C, pH 7.4. The effects of *T. paniculatum* root and leaf extracts at certain concentration level (IC_{50}; 0.23 mg/mL and 1.67 mg/mL, respectively) on spontaneous contraction and agonist-induced contraction such as high KCl (40 mM) solution, Bay K8644 (1 μM), and oxytocin (10 nM) were observed. All values were

analyzed by Paired student *t*-test. A probability level less than 5% ($P < 0.05$) was considered statistically significant. The results showed that spontaneous uterine contractile activity was found to be dose-dependency inhibited by the extracts ($n = 5$). The application of root extract produced more potent effects than that of the leaf extract. The extracts significantly inhibited the contraction induced by high KCl solution ($P < 0.01$) ($n = 5$). In Bay K8644 and oxytocin studies, the extracts significantly relaxed the uterus in a time-dependent manner ($P < 0.05$) ($n = 5$). The extracts also potentially inhibited oxytocin-induced contraction in the absence of external Ca^{2+} ($n = 7$). Interestingly, both extracts potentially diminished the tonic force-induced by OT in the presence of high KCl solution. Taken together, the data implied that *T. paniculatum* extracts produces tocolytic effects on both spontaneous and agonist-induced contractions. The possible mechanisms may be due to the blockade of Ca^{2+} influx via L-type Ca^{2+} channel, Ca^{2+} efflux from internal store and interruption of Ca-independent pathways that might reduce the sensitivity of contractile system to Ca^{2+}.

7.2 Introduction

Preterm delivery is one of the ailments faced by pregnant women. Approximately 28% of these premature babies die within first week after birth (Lawn, Wilczynska-Ketende and Cousens, 2006). Factors possibly contributing to but not completely explaining this unwanted outcome, the most important involves a breakdown in the normal uterine quiescence with a short-circuiting or overwhelming of the normal parturition cascade (Giles and Bisits, 2007). A few medications are used clinically as uterine relaxant or “tocolytics”, including magnesium sulphate,

indometacin, β_2-adrenergic receptor agonists, atosiban, progesterone, prostaglandin synthesis inhibitors, nitric oxide donors and calcium (Ca^{2+}) channel blockers (Vermillion and Landen, 2001). These drugs are designed to restrain the contractions of uterus. However, there is still controversy about their effectiveness and long-term safety, especially to fetus (Kim and Shim, 2006). These considerations have not been favorable by patients in developing countries. Therefore, the using of plants or plant products became more recognized. Many herbs or plants' families were accommodated to optimistic the uterine physiology. One of interested plant is *Talinum paniculatum* (Jacq.) Geartn. (*T. paniculatum*), which has been traditionally acclaimed as a female reproductive rehabilitation (Setyowati and Wardah, 2011). Preparation of *Talinum* spp. has long been used in ancient folk medicine, particularly in the treatment of type-2 diabetes, inflammatory skin problems, gastrointestinal disturbance, general body weakness and reproductive disorders (Pak et al., 2005; Shimoda et al., 2001). Although, this plant has been reported to influence the female reproductive system, but there is no scientific data regarding to clarify the effects on uterus to support its therapeutic values. Thus, this study attempted to investigate the effects of *T. paniculatum* root and leaf extracts on uterine contraction and its possible mechanism(s).

7.3 Materials and Methods

7.3.1 Chemicals and Physiological Solutions

All chemicals were obtained from Sigma-Aldrich Chemical Co. (St.Louis, MO, USA). The solvents and chemicals used were of analytical grade and obtained from Sigma® and Merck®.

The physiological Krebs's solution (pH 7.4) was prepared in accordance with the following composition (mM): NaCl: 154.0; KCl: 5.4; $CaCl_2$: 2.0; $MgSO_4$: 1.2; glucose: 8.0; 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES): 10.0. Ca^{2+}-free physiological Krebs' solution was prepared by omitting $CaCl_2$ and adding 1 mM ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid (EGTA) (Kupittayanant, Lucklas and Wray, 2002). High KCl (40 mM) solution was made by iso-osmotic replacement of NaCl (Noble and Wray, 2002). Oxytocin was dissolved in distilled water and used at the final concentration of 10 nM to produce a phasic contraction (Kupittayanant, Lucklas and Wray, 2002). Bay K8644, the L-type Ca^{2+} channel agonist; S-(–)-1,4-dihydro-2,6-dimethyl-5-nitro-4-[2-(trifluoromethyl)-phenyl]-3pyridine carboxylic acid methyl ester, was dissolved in absolute ethanol and used at the final concentration of 10 μM (Kupittayanant, Kupittayanant and Suwannachat, 2008).

T. paniculatum root (0.23 mg/mL) or leaf (1.67 mg/mL) extract was used and directly dissolved in physiological solution.

7.3.2 Animal Procedures

Animal care, environmental conditions and uses followed the guidelines of Laboratory Animal Resources, National Research Council of Thailand. The procedures of the experiment were approved by the Institutional Animal Care and Use Committee, Suranaree University of Technology, Nakhon Ratchasima, Thailand.

7.3.3 Isolated Uterine Preparation and Tension Measurement

Adult female virgin rats (200-250 g) were euthanatized by CO_2 asphyxia. Uteri were isolated and immediately placed in Krebs's solution (37°C, pH 7.4). The uterine strips (1-2 mm x 0.5 mm x 10 mm) were attached at each end to metal hooks,

and another hook was fixed to a transducer (AD Instruments Pty Ltd, Spain) in the organ bath that contain Krebs' solution (37°C, pH = 7.4). An equilibrium time of 30 min was applied for all tissues before the application of any standard chemicals. The isometric force respond was measured during 10-30 min with PowerLab system software and recorded with a force-displacement transducer connected to a computer using Chart software. The relaxation was expressed as a percentage of the contractile activity reduction induced by each agonist.

7.3.4 Experimental Protocols

Dose dependency of the extracts

The uterine strips were allowed to equilibrate in the bathing medium for about 30 min. The rhythmic contractions were observed and used as the initial 100% spontaneous contraction. The concentration-response effects of *T. paniculatum* on spontaneous contraction were tested with root or leaf extracts with an increased concentration in a cumulative manner (0.1 to 0.5 mg/mL and 0.5 to 2.5 mg/mL, respectively) for 30 min intervals. The median inhibition concentration (IC_{50} values; a concentration which produce 50% of the maximum inhibition of the area under the contraction; AUC) was calculated by using a nonlinear curves fitting program, Microcal Origin Software (Vergara-Galicia et al., 2010), and the concentration at IC_{50} value of each extract was used.

Effects of the extracts on high KCl-induced contraction:

To determine effects of the extracts on contractile response to high KCl (40 mM) solution, a controlled contractile response was stimulated to the plateau stage. After the maximum contraction-induced by high KCl solution was achieved, the uterine strips were treated with the extracts in the presence of high KCl solution. At

the end of the experiment, the bathing solution was replaced by Krebs' solution and tension was monitored. In addition, the experiments were done the other way round. Briefly, the extract was applied in physiological Krebs' solution and then high KCl solution was added, in the continued presence of the extracts.

For either high KCl solution or extracts exposure, the experiment duration was performed at least 20 min. The percentage inhibition of original response was calculated to indicate the inhibitory action of the extracts.

Effects of the extracts on Bay K8644-induced contraction

To investigate whether the relaxation effects of *T. paniculatum* extracts were dependent upon external Ca^{2+} entry through voltage L-type Ca^{2+} channels, Bay K8644 (the L-type channel agonist) was used. In this study, Bay K8644 (1 μM) was applied and the plant extract was added with the presence of Bay K8644. Next, Bay K8644 was further applied to the organ bath. For each tested substances, the experiment duration was performed at least 30 min. The inhibition percentage of original response was calculated, indicating inhibitory action of the extracts.

Effects of the extracts on oxytocin (OT) -induced contraction

a) In normal Ca^{2+} Krebs's solution: To determine the contractile response of uterus to OT, after the equilibrium stage, the uterine strip was induced by OT (10 nM). In next stage, with the presence of OT, the extract was added and OT was subsequently applied to the bath. For either OT or extracts exposure, the experiment duration was performed at least 30 min. The percentage inhibition of original response was calculated indicating inhibitory action of the extracts.

b) In Ca^{2+}-free EGTA solution: To determine the effect of the extract on intracellular Ca release, the extracts were determined in Ca-free EGTA solution. After

the equilibrium period, the uterine strips were applied with the extracts in organ bath containing Ca-free EGTA solution for 10 min. In the continued presence of the extract and Ca-free EGTA solution, OT was later added to the organ bath for 10 min.

c) In the present of high KCl: The uterine strip was induced to the plateau stage by the high KCl solution. The solution in the bath was further replaced by high KCl containing OT (10 nM) and equilibrated for 10 min. After the maximum contraction-response to high KCl containing OT was achieved, the extract was subsequently applied. At the end of the experiment, the bathing solution was replaced by Krebs' solution and the tension was monitored.

7.3.5 Chemicals

All chemicals were purchased from Sigma®, Singapore. The measurement of tension was made whilst the tissue was continually perfused with physiological Krebs' solution (control; 100%). All stock solutions were prepared and stored in accordance with the guideline of the producer.

7.3.6 Statistical Analysis

Contractility endpoints were area under the contraction (AUC), amplitude and frequency. Relaxation was expressed as a percentage of inhibition of the maximal contraction obtained by adding the standard chemicals and extracts. All data are expressed as the mean ± standard error of the mean (S.E.M.) of 5-7 preparations (*n*) from different animals. The data were evaluated using Microcal Origin Software, and the differences between control and treatment groups were analyzed by paired student *t-test*. Probability values of less than 0.05 ($P < 0.05$) were considered statistical significant.

7.4 Results

7.4.1 Concentration-Response Effects of *T. paniculatum* Extracts on Spontaneous Contraction

To investigate the relaxation effects of extracts, the cumulative increases in concentration of root (0.1-0.5 mg/mL) or leaf (0.5-2.5 mg/mL) extract were added into the organ bath after the 30 min equilibrium period and used as an initial contraction base line control (100%). Both of root and leaf extracts produced an extensive dose-related inhibition of the spontaneous contractions. The examples of these experimental trace are shown in Figure 7.1. At each concentration, both extracts significantly decreased the AUC and amplitude, whereas frequency of the contractions increased. The relaxation pattern of the uterus after the cumulative application of the root is similar but more potent than that of leaf extract. The IC_{50} concentrations of the extracts were 0.23 and 1.67 mg/mL, respectively (Figure 7.2). Hence, these IC_{50} concentrations were used in the remainder of the experiments.

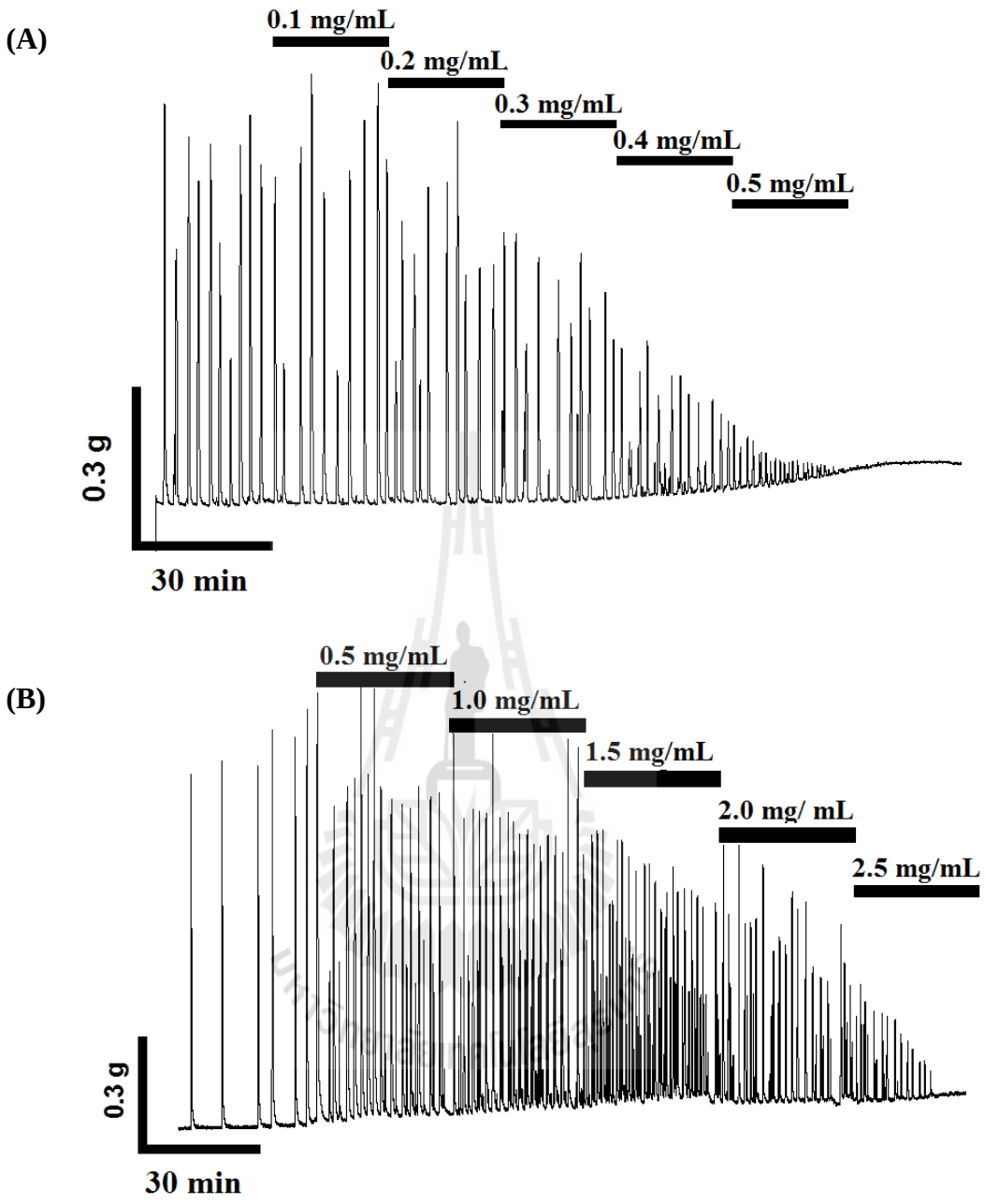

Figure 7.1 The effects *of T. paniculatum*'s root (A) and leaf (B) extracts on spontaneous contraction. The cumulative inhibitory responses are shown.

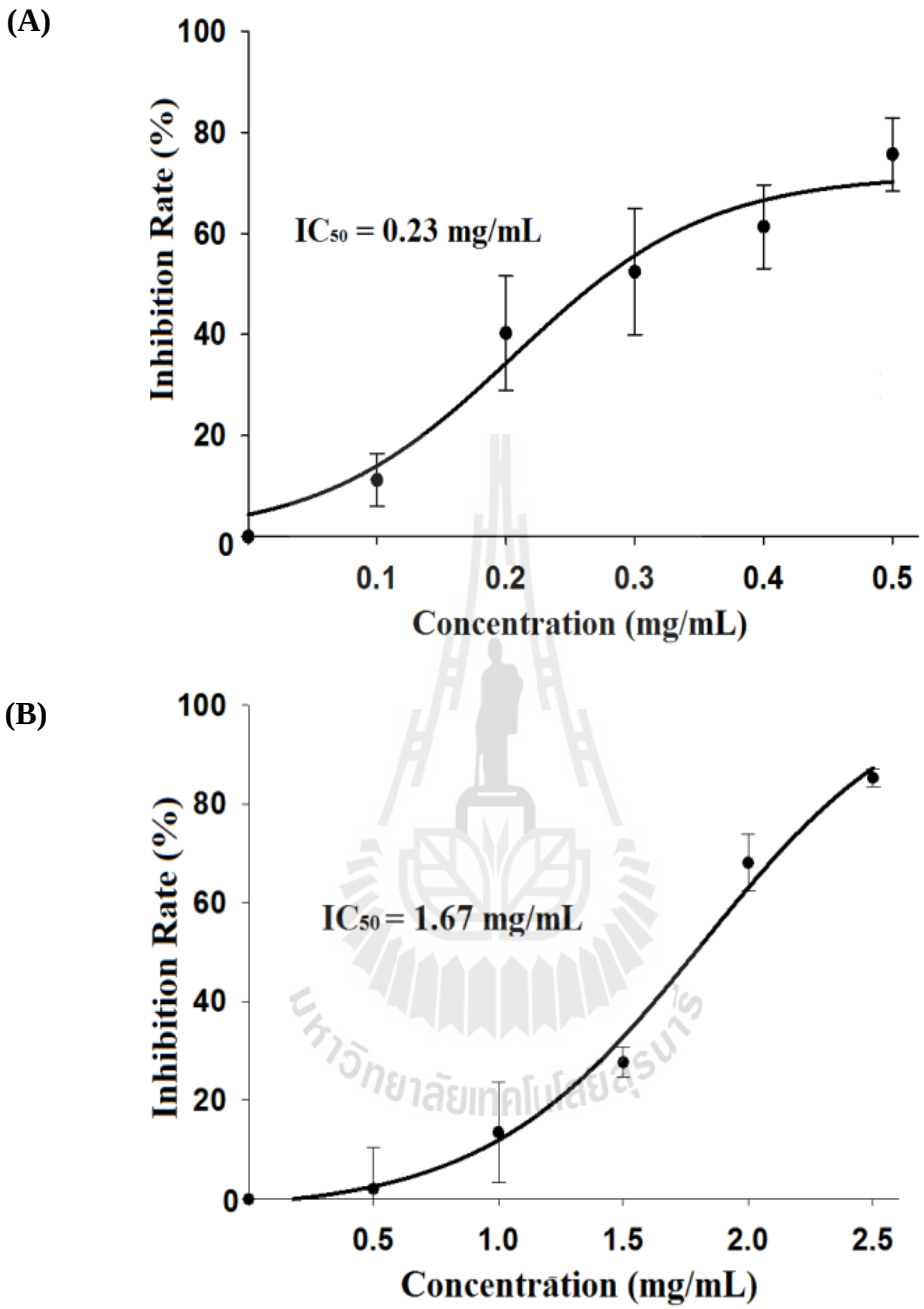

Figure 7.2 Dose-response curves of the root (A) and leaf (B) extracts on uterine contractile activity. The IC_{50} of the root and leaf extracts were 0.23 mg/mL and 1.67 mg/mL, respectively. Vertical bars represent the S.E.M. (n = 5).

7.4.2 Effects of *T. paniculatum* Extracts on Spontaneous Contraction

The application of *T. paniculatum* extracts at the IC_{50} concentrations to isolated uterine strips significantly diminished the AUC and amplitude of force compared with control ($P < 0.05$). The AUC means obtained by the root and leaf extracts were significantly decreased to 66.51 $\pm$ 12.54% and 69.05 $\pm$ 9.63%, respectively ($P < 0.01$). Figure 7.3 demonstrated both extracts produced the potent tocolytic activity to isolated uterine strips. After the tested period, the uterine strips were later washed by Krebs' solution. The results showed that the rhythmic spontaneous contractions of the uterus were reversed. Thus, this finding indicated that the tocolytic effects produced by either root or leaf extract were reversible. The effects of *T. paniculatum* extracts on spontaneous contraction are summarized in Table 7.1.

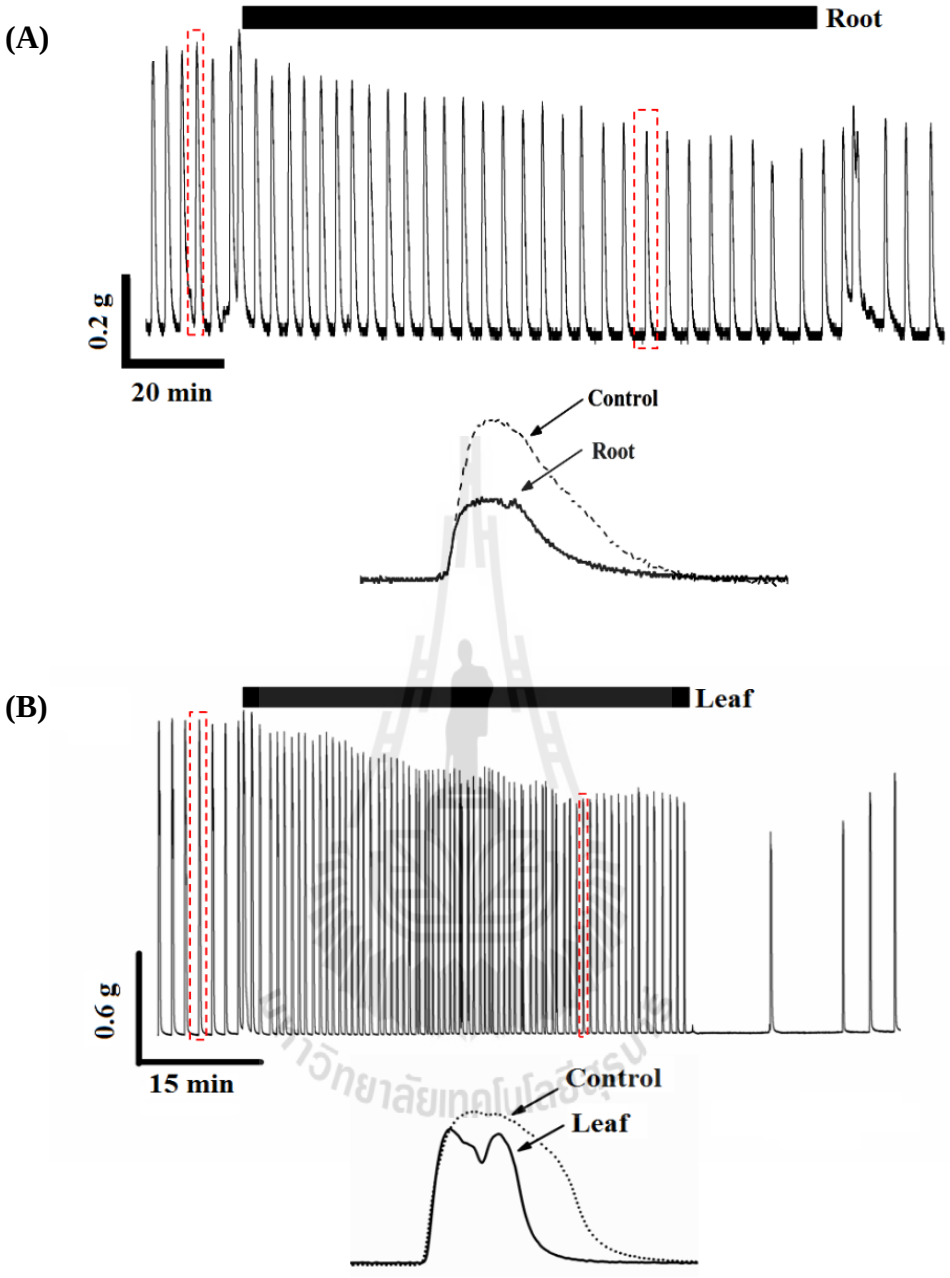

Figure 7.3 The trace representations of *T. paniculatum*'s root (A) and leaf (A) extracts on spontaneous contraction. At the IC_{50} concentration, both extracts significantly exhibited the tocolytic effects on the isolated uterine strips (n = 5).

Table 7.1 The summarization of the effects of *T. paniculatum*'s extracts at the concentration of IC_{50} value on spontaneous contraction.

Tested substances	AUC (%)	Amplitude (%)	Frequency (%)	*n*
Root extract				
Control	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	5
Root extract	77.09 ± 6.33**	76.26 ± 2.11**	84.59 ± 2.54**	5
Recovery	76.51 ± 6.97**	84.59 ± 2.55**	81.32 ± 3.59**	5
Leaf extract				
Control	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	5
Leaf extract	71.51 ± 8.24**	72.59 ± 9.39*	182.83 ± 27.29*	5
Recovery	68.22 ± 7.66*	95.21 ± 5.44	57.86 ± 17.08*	5

The *P*-values for AUC, amplitude and frequency of *T. paniculatum r*oot and leaf extracts achieved are significantly different from the base line control (*$P < 0.05$ and **$P < 0.01$). Mean ± S.E.M. are given; "*n*" represented as the number of uterine sample from a different animal.

7.4.3 Effects of *T. paniculatum* Extracts on High KCl-Induced Contraction

The depolarization of membrane potentially induces myometrial contraction by opening L-type Ca^{2+} channels and may activate other regulatory cascades (Parkington et al., 1999). Therefore, the study further investigated whether the extract affected Ca^{2+} influx; an experiment was performed to determine the effects of extracts on contractile responses to depolarization- induced Ca^{2+} influx in high KCl (40 mM) solution.

The results demonstrated that high KCl solution generated the sustained contraction in isolated uterine strips. Applications of the extracts were able to diminish the force of the uterine strips. As shown in Figure 7.4, 20 min after the root or leaf extract applications, the force of contraction had dropped to 56.53 $\pm$ 8.14% and 43.92 $\pm$ 8.31% of control force development, respectively ($P < 0.01$). This indicates that alterations in the of membrane potential are involved the process of the extracts induced uterine relaxation.

When the order of the solution was switched (Figure 7.5), the strips could not produce equivalent force as induced by high KCl solution alone ($P < 0.001$). The integral forces of contraction after the incubation of the root or leaf extracts, and subsequently high KCl solution were fallen to 27.74 $\pm$ 8.12% and 33.12 $\pm$ 6.28%, respectively.

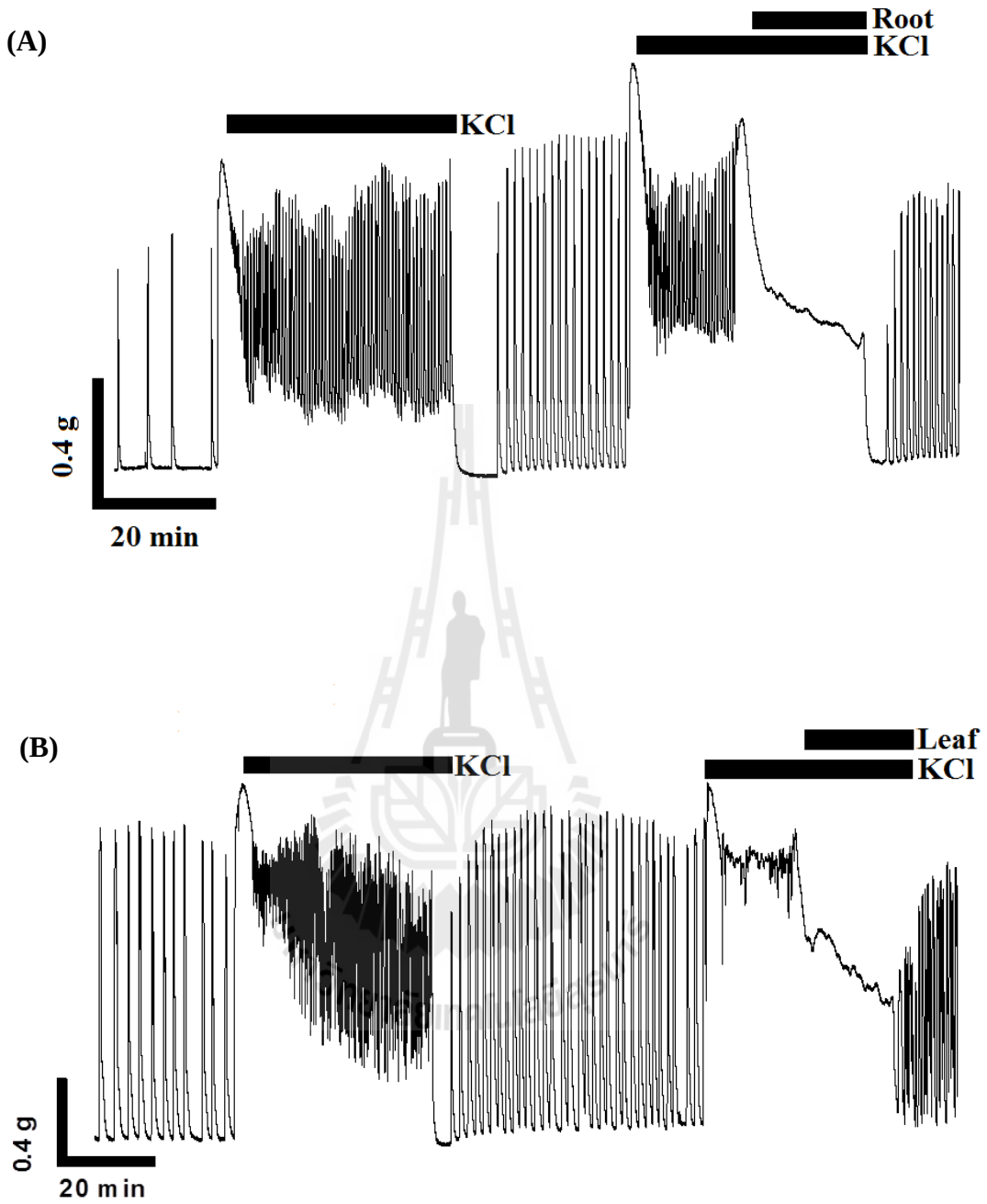

Figure 7.4 The samples of the experimental trace of the uterine responded-contraction of KCl-induced force affected by *T. paniculatum* root (A) and leaf (B) extracts. The responses were compared to those of the time control (high KCl alone; 100%) and the tested period (high KCl + plant extract) (*n* = 5).

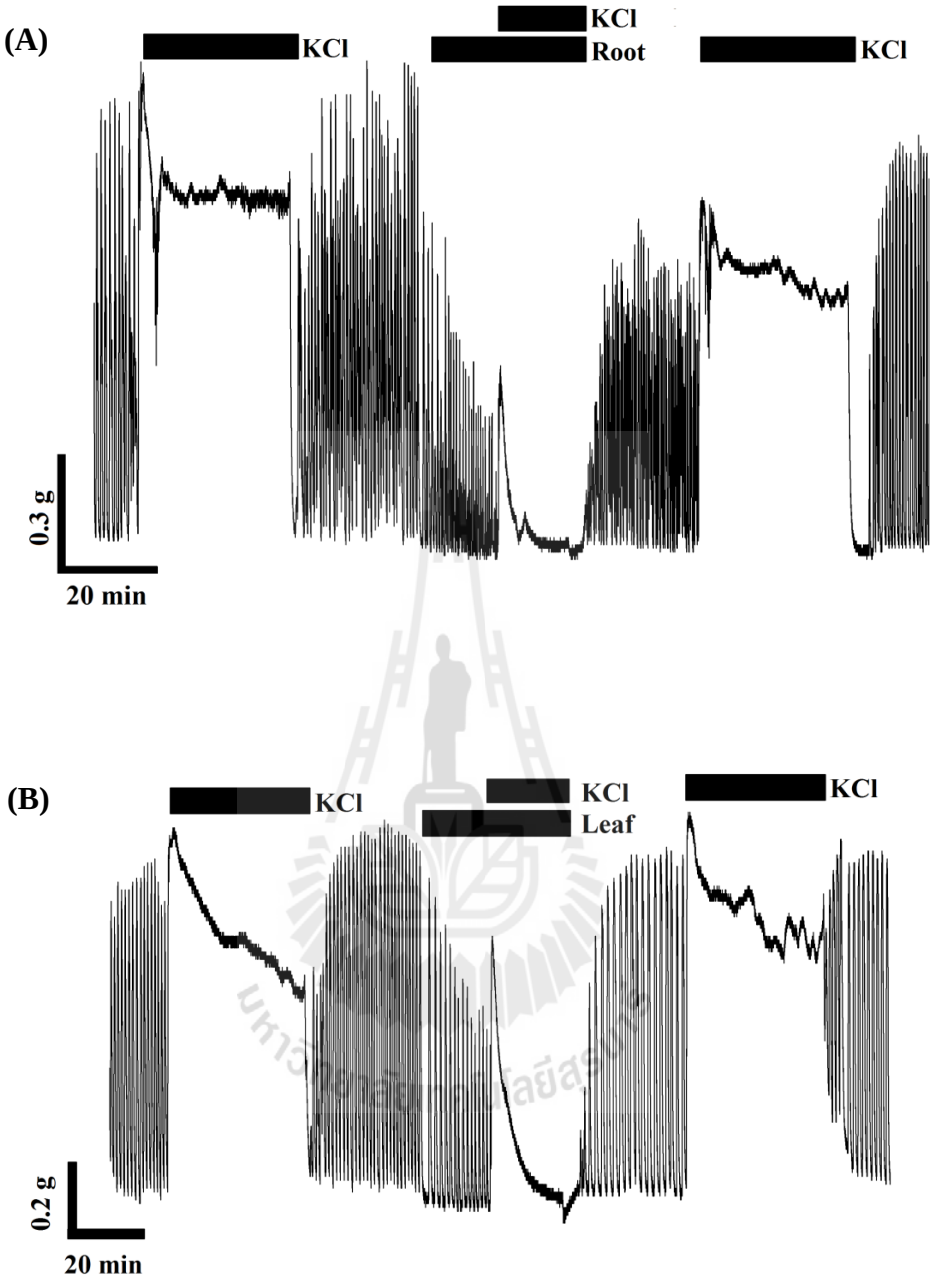

Figure 7.5 The samples of the experimental trace of the inhibition of force produced by *T. paniculatum*'s root (A) and leaf (B) extracts which later-applied by high KCl solution. The responses were compared to those of the time control (high KCl alone; 100%) and the tested period (high KCl + plant extract) (n = 5).

7.4.4 Effects of *T. paniculatum* Extracts on L-type Ca^{2+} Channels Agonist (Bay K8644)-Induced Uterine Contraction

The uterine force directly relied on the influx of external Ca through the L-type Ca^{2+} channels (Matthew, Shmygol and Wray, 2004). The further experiment was designed to investigate whether the plant extracts would produce a tocolytic effects that might be involved with the external Ca^{2+} entry via L-type Ca channels. In order to achieve this, the L-type Ca^{2+} channels were activated by Bay K8644 (1 μM) and the contractile responses to the extracts were observed.

The results showed that pretreatment of the uterine strips with Bay K8644 produced a significant increase in the contractile amplitude and frequency compared with spontaneous contraction. The addition of *T. paniculatum* extracts in the continued presence of Bay K8644 significantly produced persuasive tocolytic effects which indicated by a marked decrease in AUC and force contraction amplitude (Table 7.2). As illustrated in Figure 7.6-7.8, the contractile activities were reversed by adding Bay K8644, but the AUC and amplitude of the contractions could not completely return to the spontaneous control level (n = 5).

Table 7.2 The effects of *T. paniculatum*'s root (A) and leaf (B) extracts in the presence of the L-type Ca channels activator (Bay K8644).

Tested substances	AUC (%)	Amplitude (%)	Frequency (%)	*n*
Root extract				
Control	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	5
Bay K8644	125.33 ± 3.03**	121.03 ± 3.41**	111.26 ± 3.23**	5
Bay K8644+Root extract	71.70 ± 14.57	75.02 ± 8.98*	119.16 ± 7.08*	5
Leaf extract				
Control	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	5
Bay K8644	135.81 ± 11.99*	118.38 ± 6.15*	112.16 ± 5.46	5
Bay K8644+Leaf extract	56.77 ± 11.11**	68.35 ± 4.86**	116.93 ± 8.96	5

The *P*-values for AUC, amplitude and frequency of *T. paniculatum r*oot and leaf extracts achieved are significantly different responses from the control (*$P < 0.05$ and **$P < 0.01$). Mean ± S.E.M. are given. "*n*" represented as the number of uterine sample from a different animal.

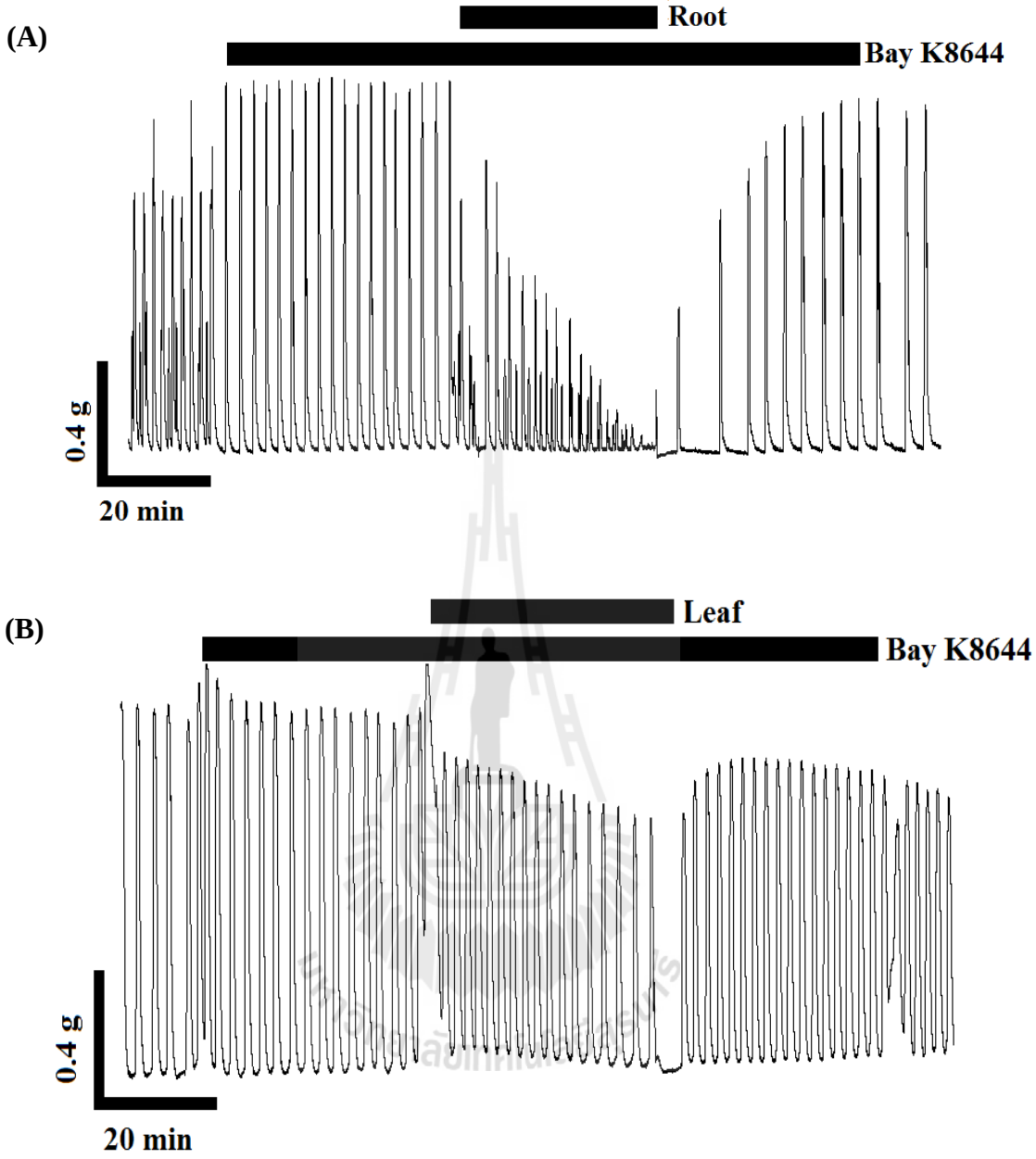

Figure 7.6 The trace representations of the effects of *T. paniculatum* root (A) and leaf (B) extracts in the presence of the L-type Ca channels activator (Bay K8644). The extracts show time-dependent relaxations effect where Bay K8644 was added in a continued presence of the root (A) or leaf (B) extract (n = 5).

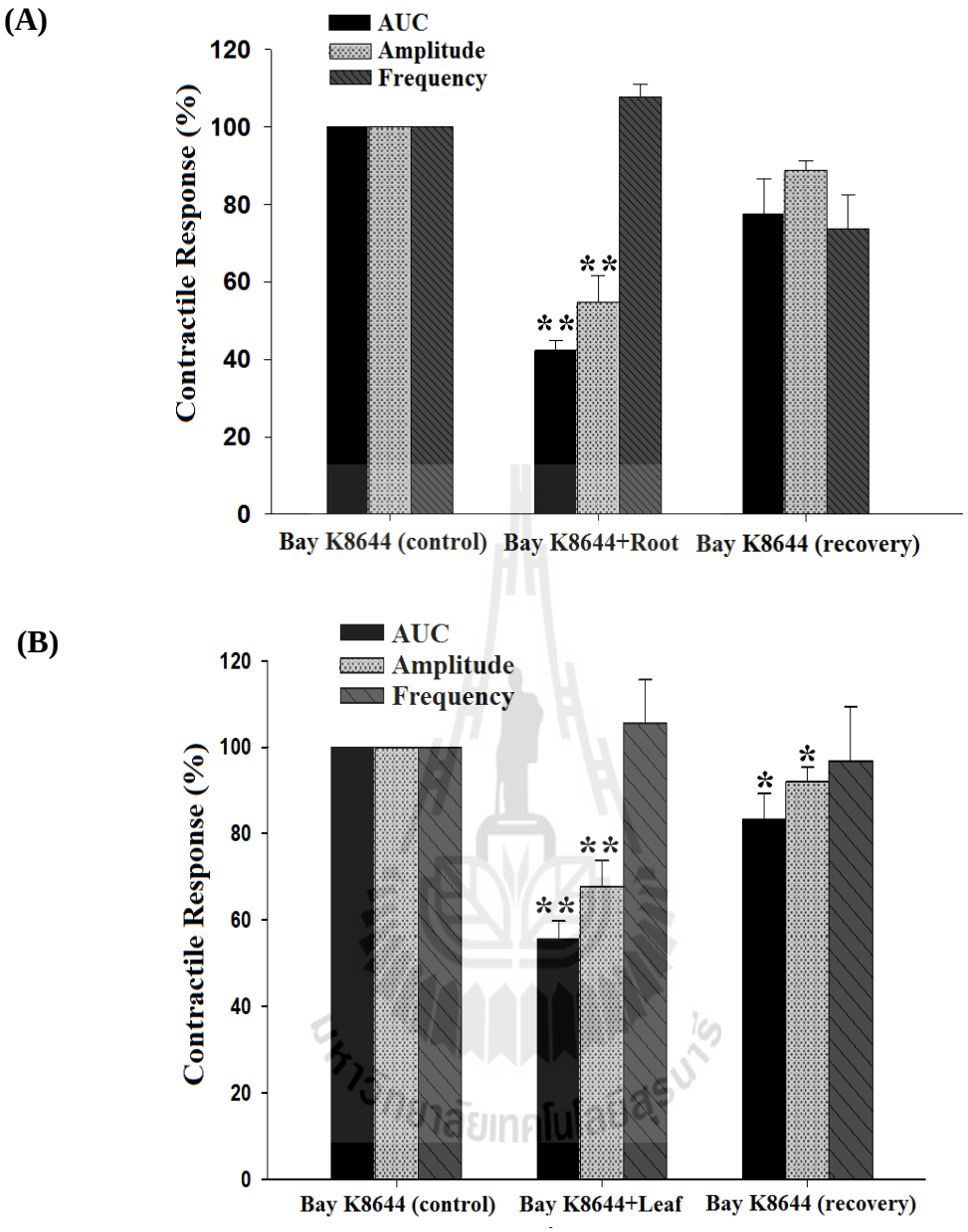

Figure 7.7 Inhibition of L-type Ca^{2+} channels agonist-induced contractions of isolated rat uterine strips by *T. paniculatum* root (A) and leaf (B) extracts. The responses were compared to those of the time control (Bay K8644 alone; 100%). Bars represent mean contractile responses (mean ± S.E.M.) of 5 experiments (n = 5). The asterisks indicates significantly differences than the time control (*P < 0.05 and **P < 0.01).

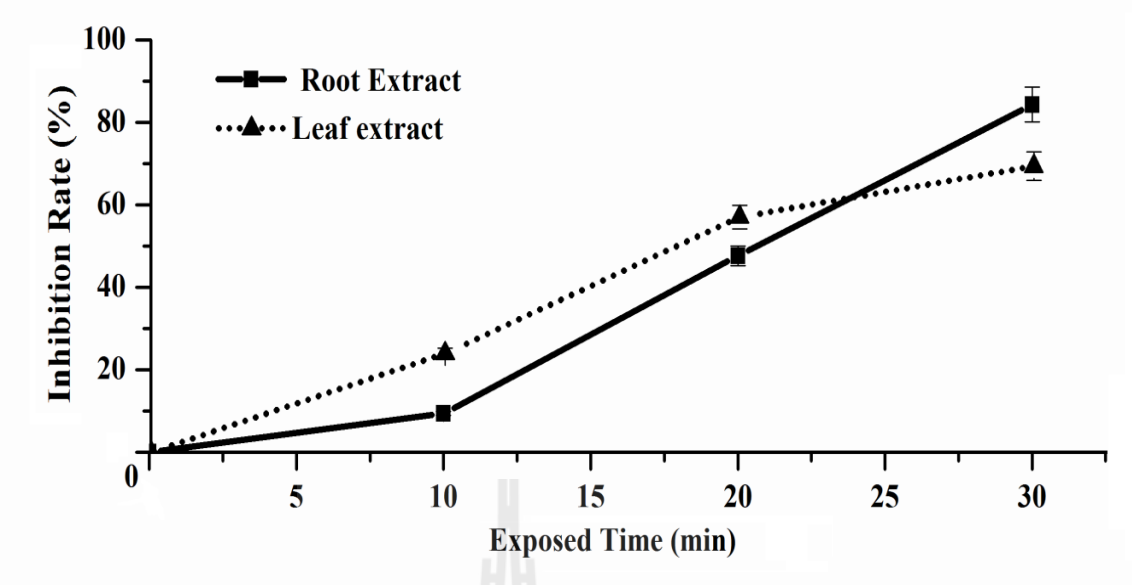

Figure 7.8 The inhibition rates of Bay K8644-induced contractions of isolated rat by *T. paniculatum*'s root and leaf extracts. The contractile responses were compared between the time control (Bay K8644 alone; 0% inhibition rate) and the tested period (Bay K8644 + plant extract). There were no significant differences of inhibition rate between root and leaf extracts compared at 10, 20, and 30 min ($P > 0.05$). The points are mean and the vertical bars show the S.E.M. ($n = 5$).

7.4.5 Effects of *T. paniculatum* Extracts on Oxytocin-Induced Uterine Contraction

Oxytocin (OT) is a neurohypophysial hormone that stimulates uterine contraction by operating through its receptors. The activation of OT receptor triggers of a large number of intracellular pathways by increased intracellular Ca^{2+} concentration, enhanced myosin light chain phosphorylation and production of prostaglandins; subsequently increased myometrial contractions (Kawamata et al., 2007). To examine whether the extracts could decreases OT-evoked contraction. Thus, OT (10 nM) was added either in the absence or presence of Ca^{2+} solution.

In Ca^{2+} containing solution, isolate uterine strips showed regular spontaneous rhythmic contractions within 30 min of the equilibration period. Contractile response of the uterine strip to OT application showed augmentation of the contractile activity compared to control ($P < 0.05$).

The sample of experimental traces is shown in Figure 7.9 and summarized data are demonstrated in Table 7.3. Following the test period, the extract significantly decreased the AUC and amplitude of rat uterine smooth muscle (Figure 7.10, $P < 0.001$), and the force of contraction was time dependency diminished when the extracts were applied (Figure 7.11).

7.4.6 Effects of *T. paniculatum* Extracts on Oxytocin-Induced Uterine Contraction in the Absence of External Ca^{2+}

Another experiment was designed to elucidate the effects of the extracts on the release of Ca^{2+} from intracellular stores; the responses to OT were performed in the absence of the extracts in Ca-free EGTA solution (Kupittayanant, Lucklas and Wray, 2002). The results demonstrated that in Ca^{2+}-free EGTA solution, OT (10 nM) produced a diminutive force as long as it was present, indicating that OT is able to release Ca^{2+} from the SR. Upon return to control Krebs' solution, spontaneous rhythmic contraction recurred. After the later equilibrium period, the solution was replaced by the combination of Ca^{2+}-free EGTA solution and *T. paniculatum* extract. Both root and leaf extracts potentially inhibited the contractile activity by completely abolished tonic contraction of the uterus during OT exposure (100% inhibitory action, $n = 7$) (Figure 7.12).

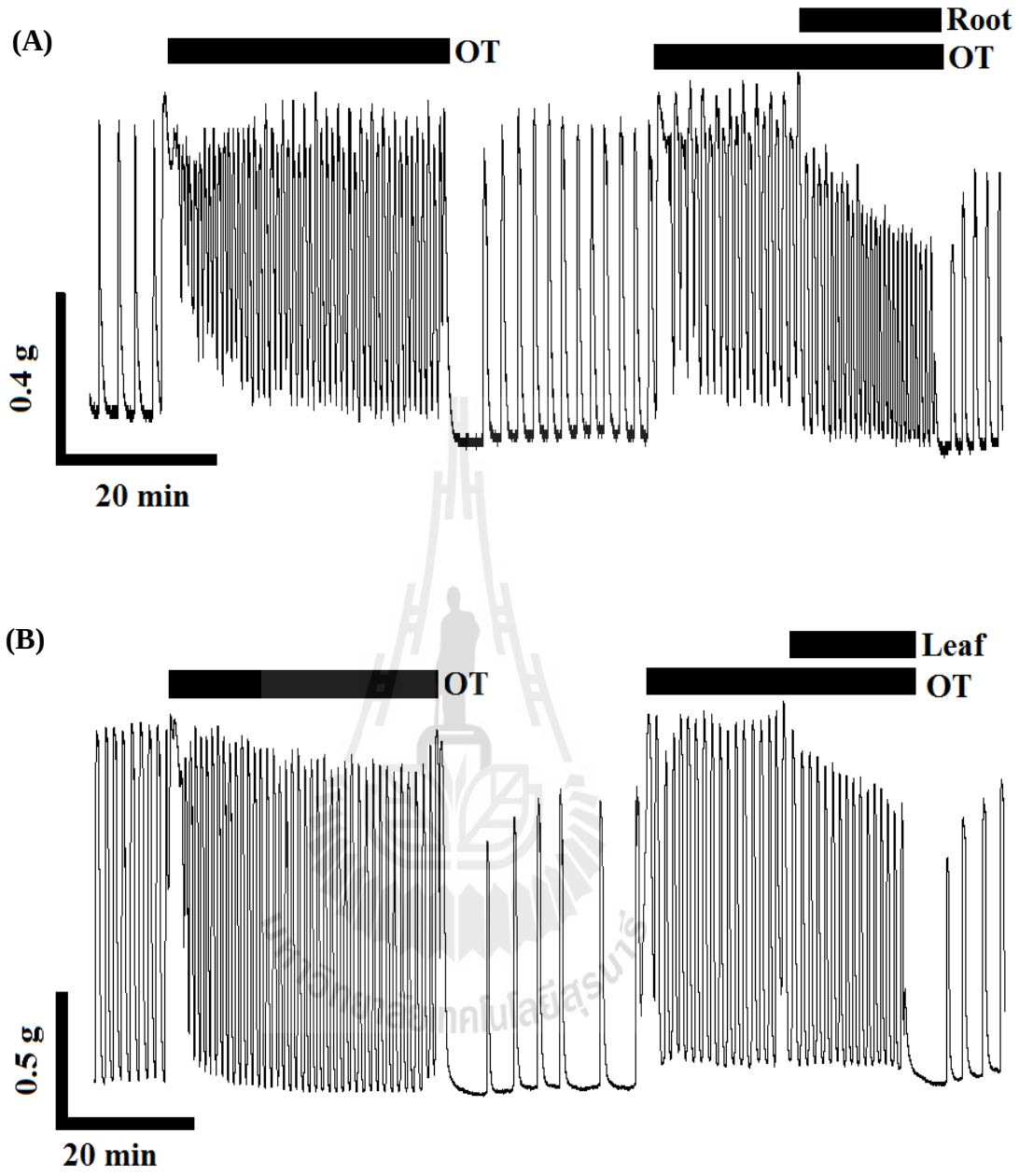

Figure 7.9 The trace representation of the time dependency inhibition of oxytocin (OT)-induced contractions of isolated rat uterus in normal Ca^{2+} Krebs's solution by *T. paniculatum*'s root (A) and leaf (B) extracts (n = 5).

Table 7.3 The effects of *T. paniculatum* root and leaf extracts in the presence of oxytocin.

Tested substances	AUC (%)	Amplitude (%)	Frequency (%)	*n*
Root extract				
Control	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	5
Oxytocin	269.30 ± 13.05**	109.82 + 3.79*	128.40 ± 7.57**	5
Oxytocin +Root extract	97.06 + 9.22	59.49 + 9.96**	133.66 ± 3.83**	5
Leaf extract				
Control	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	5
Oxytocin	179.42 ± 11.42***	114.03 ± 6.64	121.02 ± 8.59*	5
Oxytocin +Leaf extract	135.58 ± 10.86*	86.16 ± 11.29	104.00 ± 8.09	5

The *P*-values for AUC, amplitude and frequency of *T. paniculatum r*oot and leaf extracts achieved are significantly different from base line control (*$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$). Mean ± S.E.M. are given. "*n*" represented as the number of uterine sample from a different animal.

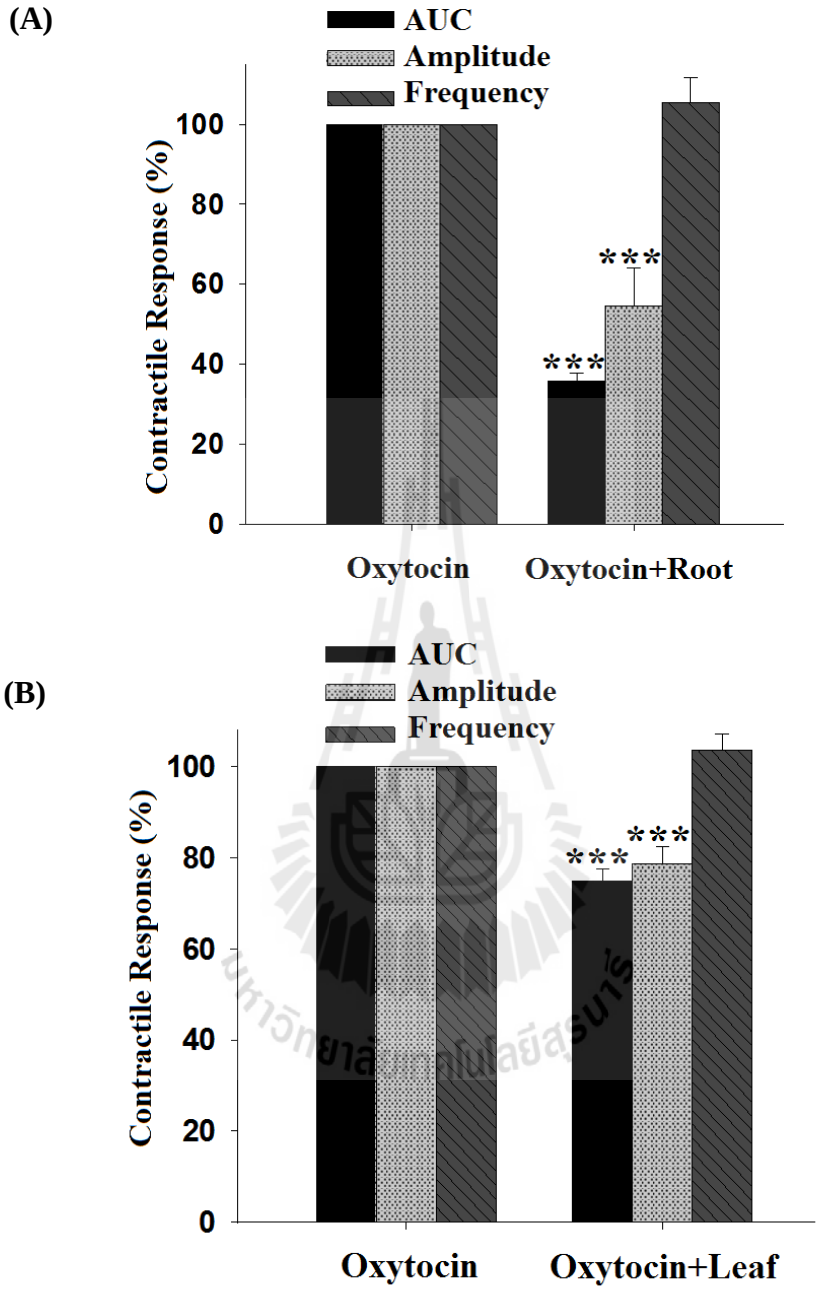

Figure 7.10 Inhibition of oxytocin (OT)-induced contractions of isolated rat uterine strips by *T. paniculatum*'s root (A) and leaf (B) extracts. The responses were compared to those of the control (Oxytocin alone). Bars represent mean responses (mean ± S.E.M.) of 5 experiments (n = 5). The asterisks indicate a significant decrease in the determined parameters compared with control (***$P < 0.001$).

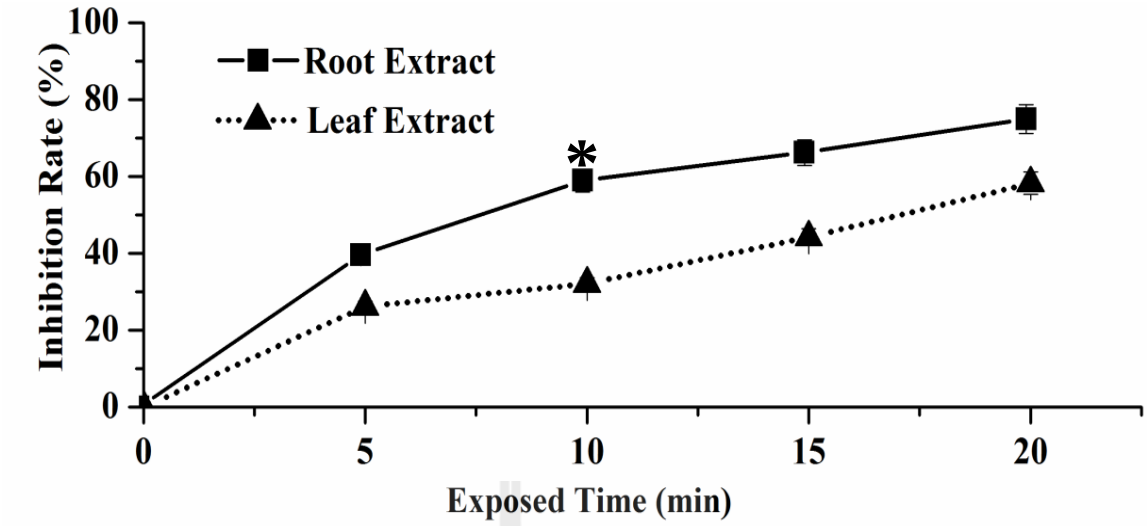

Figure 7.11 The inhibition rate of oxytocin (OT)-induced contractions of isolated rat by *T. paniculatum* root and leaf extracts. The contractile responses were compared between the control (OT alone; 0% inhibition rate of AUC) and the tested period (OT + plant extract). The asterisk indicates significant differences within a specific time point (10, 20, and 30 min) between the root and leaf extracts ($P < 0.05$). The points are mean and the vertical bars show the S.E.M. ($n = 5$).

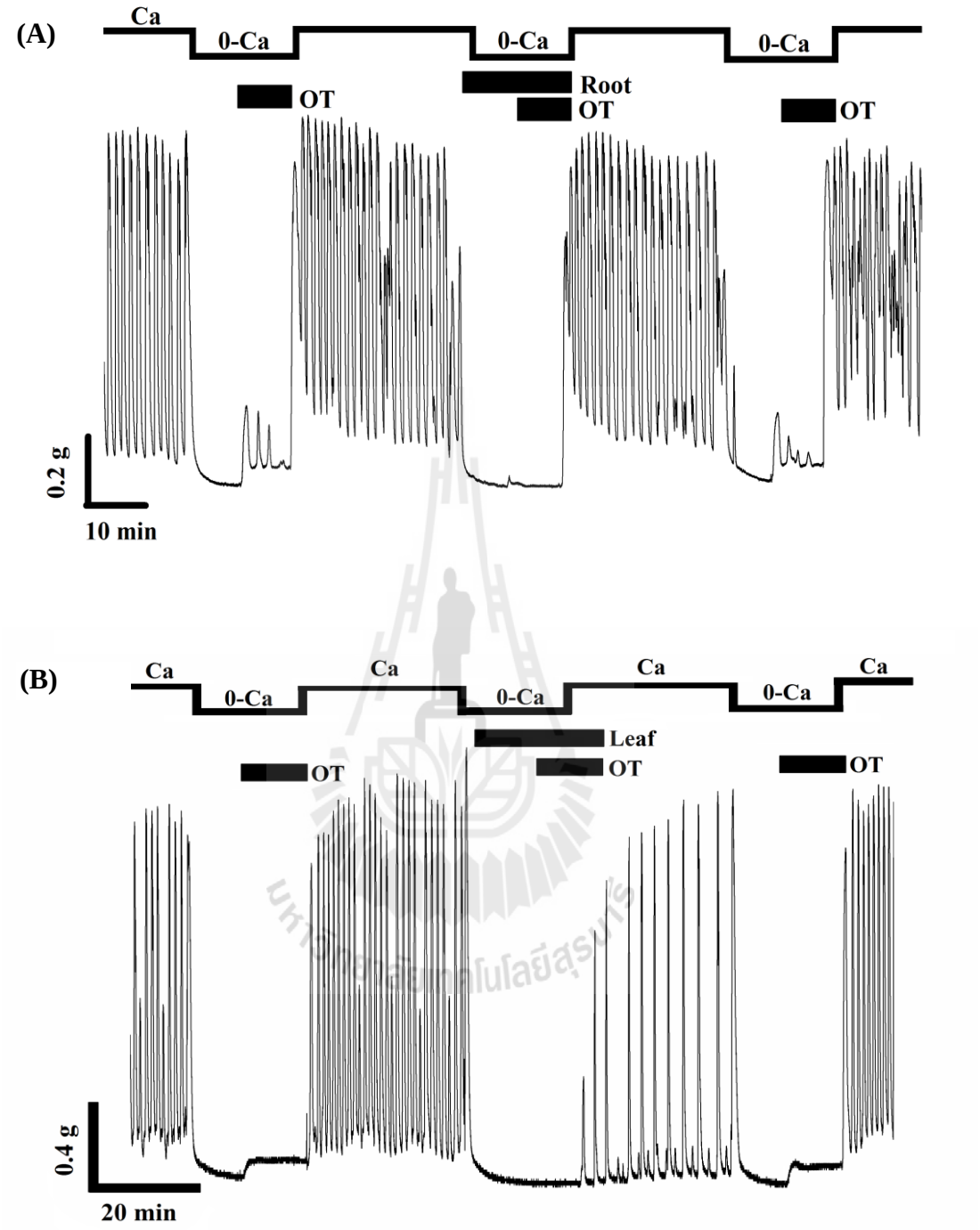

Figure 7.12 The trace representations of the inhibition of oxytocin (OT)-induced contractions of isolated rat uterus in Ca^{2+}-free EGTA containing solution by *T. paniculatum*'s root and leaf extracts (*n* = 7).

7.4.7 Effects of *T. paniculatum* Extracts on Oxytocin-Induced Uterine Contraction with the Presence of High KCl Solution

Extracellular high potassium concentration depolarizes myocyte followed by contraction. It has been reported that high potassium-induced contraction is involved with the opening of L-type voltage dependent Ca^{2+} channels (L-type VOCCs). The application of OT in during plateau state of high KCl-induced contraction also generates a tonic force which resulted from the release Ca^{2+} from sarcoplasmic reticulum (SR). In addition, the production of force by OT in high KCl solution may be due to the modulation of MLCP activity through rho-associated kinase (ROK) pathway. Recently, Kupittayanant and coworkers (2001) had demonstrated that ROK activated by OT produced a greatly increase in force without changing in intracellular Ca^{2+} concentration. It implies that this contraction occur through the Ca-independent pathway.

As shown in Figure 7.13, the application of OT (10 nM) in the continued presence of high KCl solution produced a tonic force contraction. The later-added of the root or leaf extract produced a noticeably drop in force ($P < 0.01$). Immediate reductions in uterine force after root or leaf extract exposure were approximately 16.88 $\pm$ 6.05% and 14.87 $\pm$ 3.95% changed from integral force control, respectively (integral force control was the maximum contraction induced by OT in the presence of high KCl solution (100%), $n = 5$).

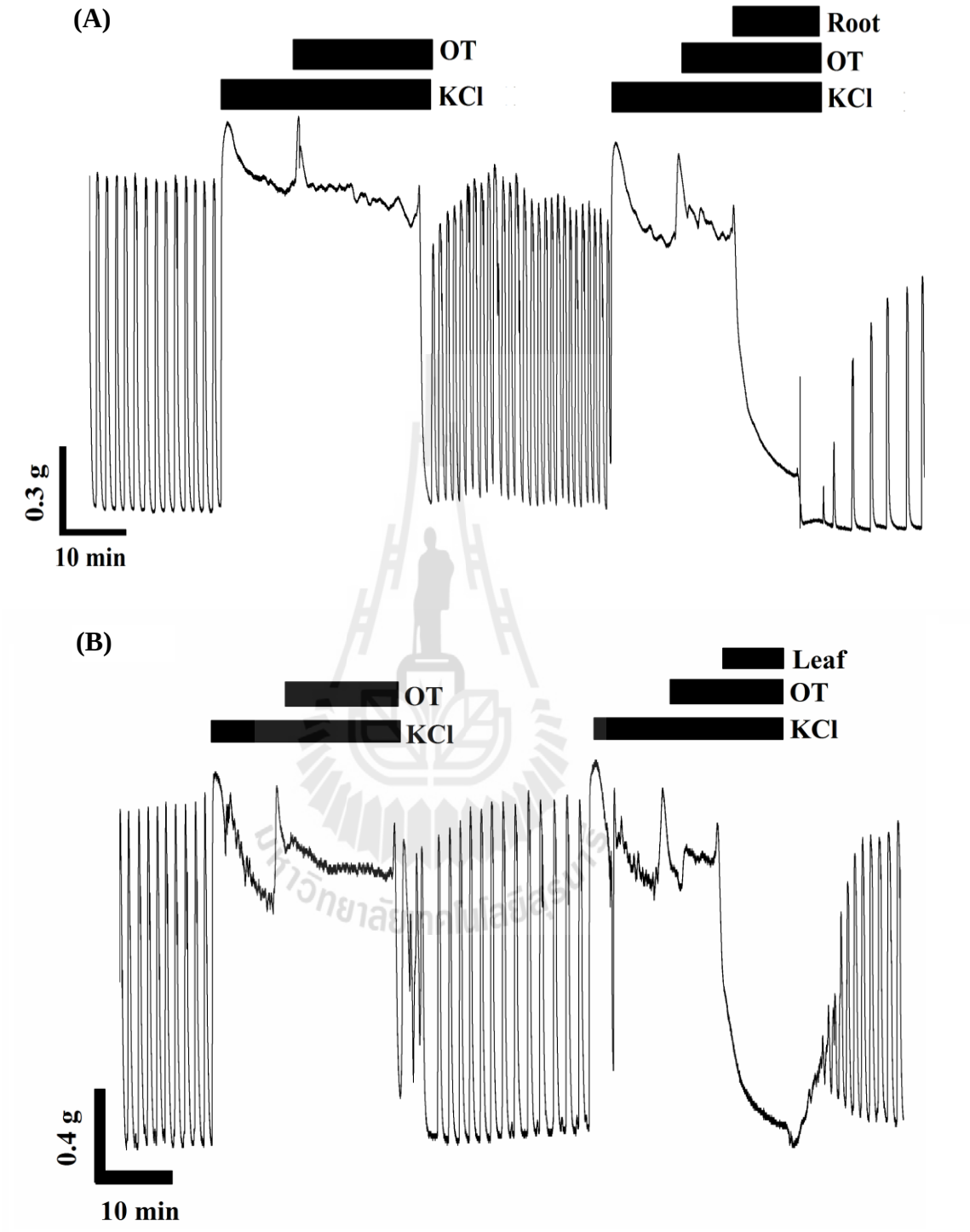

Figure 7.13 The trace representations of oxytocin (OT)-induced uterine contractions in the presence of high KCl solution which produced by *T. paniculatum*'s root (A) and leaf (B) extracts (n = 5).

7.5 Discussion

Cytoplasmic Ca^{2+} plays an essential role in modulating uterine contractions. Many studies clarified that the elevation of the cytoplasmic Ca^{2+} concentration can be managed by extracellular Ca^{2+} and intracellular (sarcoplasmic reticulum or SR) sources (Wray, 2007). Any substances inducing Ca influx from extracellular spaces, or increasing intracellular Ca^{2+} released by SR into the cell and binding to calmodulin, can activate the myosin light chain kinase (MLCK). Hence, this initiates the phosphorylation and subsequent cross-bridge cycling (Matthew et al., 2004; Noble et al., 2009). Furthermore, the depolarizing of membrane potential by high KCl solution could exert tonic force of contraction, whereby this contraction is due to direct Ca^{2+} influx through L-type VOCCs (Maggi and Giuliani, 1995).

The results of primary phytochemical screening in chapter 2 illustrated that *T. paniculatum* extracts mainly contain alkaloids, flavonoids and phytosterols. Numerous studies elucidated that these metabolites plant posse a potent relaxation activity to various types of smooth muscle. Zhang et al. (2012) demonstrated total alkaloids in *Buxus microphylla* leaf extracts significantly relaxed thoracic aorta vascular smooth muscle by suppressing influx of extracellular Ca^{2+} via VOCCs and receptor-operated Ca^{2+} channel. Comparable to the findings of this study, Calixto et al. (1984) described that the alkaloids from *Phyllanthus sellowianus*, extract exhibited the antispasmodic activity in rat uterus more than that in aortic ring and ileum smooth muscle. Additionally, the other alkaloids also reported to reduce KCl-induced Ca^{2+} influx in neuroblastoma cells (Matsumoto et al., 2005).

Several effects of flavonoids on smooth muscle contraction have already been clearly described. Genistein and quercetin inhibit the vascular contractile activity

induced by noradrenalin or serotonin (Di Salvo et al., 1993). They also reduce the spontaneous or agonist-induced contractions in ileum smooth muscle (Herrera, Marhuenda and Gibson, 1992; Hollenberg, 1994; Yang Saifeddine and Hollenberg, 1992). Possible mechanisms include protein kinase inhibition (Hollenberg, 1993, Srivastava, 1985), the raising in intracellular cAMP (Landolfi, Mower and Steiner, 1984; Buxton, 2004), the inhibition of Ca influx (Di Salvo et al., 1993) and decreasing protein kinase C activity (Duarte et al., 1994; Webb, 2003). These evidences could support their relaxation effects on smooth muscle contraction.

Plant phytosterols and their derivatives are greatly acknowledged to affect on female reproductive system. These compounds exhibit inducible or inhibitory activity in uterine contraction based on the difference of their structures or multifactorial actions which impacted by plant crude extracts. Phytosterols and saponins also act as inhibitors of SR CaATPase and potassium ion channels that induce the contractile activity (Bao et al., 2006; Promprom et al., 2010). In contrast, some plant sterols were reported to be a uterine relaxant. Hsia and colleagues (2008) demonstrated that fractionated phytosterols extracted from Adlay (*Coix lachryma-jobi L. var. ma-yuen Stapf*.) hull could inhibit rat uterine contraction by blocking external Ca influx, subsequently decrease in intracellular Ca^{2+} concentration. Okunrobo, Nwagwuogbe and Bafor (2012) reported that extracted saponins and alkaloids from *Pentaclethra macrophylla* produced significant inhibition of OT by blocking β-receptors and potassium channels in uterine smooth muscle. These data indicated that the tocolytic effects of plant extracts may be due to the cholinergic and Ca^{2+} antagonist activities of their present phytosterols (Gilani, Aftab and Ahmed, 1994; Revuelta, Cantabrana and Hidalgo, 1997).

The outcome of this study illustrated that *T. paniculatum* extracts produced significant tocolytic effects on spontaneous contractions in a concentration-dependent way. The imperative effects of *T. paniculatum* extracts on uterine contractile activity might partially mediate through the antagonizing via L-type Ca^{2+} channels indicated by the antagonistic action of *T. paniculatum* extracts on Bay K8644 (L-type Ca^{2+} Channels agonist), high KCl and OT. These evidences suggest that the extracts may contain inhibitory effect on either the L-type voltage dependent Ca^{2+} channels or reduce the sensitivity of contractile system to Ca^{2+}.

Exposure of the uterine strips to high KCl solution provokes an increasing in intracellular Ca^{2+} concentration by depolarizing membrane potential, resulting to the opening of L-type Ca channels, and hence contraction. Some of Ca^{2+} channel antagonists can abolish the high KCl-induced contraction (Grasa et al., 2004; Gharib Naseri and Yahyavi, 2007). The applications of *T. paniculatum* extracts to uterine strips were able to decrease the force with the presence of high KCl solution. This evidence proposed that *T. paniculatum* extracts exhibit the ability to block a Ca entry.

The current model of Ca^{2+} sensitization in smooth muscle contraction is accepted to be completely associated with G protein-coupled receptor (GPCR) activation (Somlyo and Somlyo, 2003). Various agonists including KCl can produce the smooth muscle contraction by coupling with GPCR, and relaxant agents can generate the opposite effect to cause Ca^{2+} desensitization (Ratz et al., 2005; Ratz and Miner, 2009). As the uterine strip was incubated with *T. paniculatum* extracts and subsequently high KCl solution, the strips could not produce the integral force as induced by high KCl solution alone. This finding indicated that *T. paniculatum* extracts fabricate the Ca^{2+} desensitization.

OT enhances uterine contractility by activating the L-type VDCCs and OT receptor (Vrachnis et al., 2011). This receptor is connected to the G-protein couple receptor which further also by activation of phospholipase C and increasing inosital 1,4,5-triphosphate (IP_3) production followed by promotion of calcium release from SR that leads to myometrial contraction (Sanborn et al., 1998). The present study illustrated that *T. paniculatum* extracts significantly reduced the integral force of contractions. This data supported that *T. paniculatum* extracts may partially disrupted the Ca^{2+} entry via G-protein signaling pathway. Clear abolished of OT-evoked tonic force were also observed when the extracts were added in Ca^{2+}-free EGTA solution. Thus, it is implied that some part of the tocolytic action induced by *T. paniculatum* extracts may also be involved with the Ca enlistment from SR.

As mentioned above, OT-induce contraction in the presence of high KCl solution is not only generated by Ca^{2+}-dependent pathways, but also Ca^{2+}-independent pathways by the activation via ROK cascade (Janssen et al., 2004). The activation of ROK affects on MLCP activities which modulating the uterine contractile activity. However, the contraction triggered by ROK is more imperative in promoting force during tonic force rather than phasic contractions (Kupittayanant, Burdyga and Wray, 2001). In addition, the inhibition of ROK by Y-27632 during the tonic contraction causes significant decreased in force without changing in intracellular Ca^{2+} concentration. This evidence alternatively implies that tonic contraction generated by ROK activation can occur at constant in Ca^{2+} concentration or by the Ca^{2+} sensitization (Somlyo and Somlyo, 1998). Based on this theory, when the uterine strips were incubated with OT in the presence of high KCl solution, this was suggested that intracellular Ca^{2+} concentration ascend to the maximum due to the

continuous activation of store-operated Ca^{2+} entry, SR Ca release and L-type Ca^{2+} channels. In this condition, ROK mediated MLCP activity may be under the influence of OT activity (Mitchell et al., 2013). *T. paniculatum* extracts exposures during this circumstance caused potential decreases in tonic force. This result expresses that *T. paniculatum* extracts produce tocolytic activity, and may be involved with the inhibition of the ROK pathways.

Fascinatingly, all the effects produced by the extracts on the isolated uteri were reversed by constant replacement of the physiological Krebs' solution. The result indicated that tocolytic effects of *T. paniculatum* extracts to the uterus were reversible.

In conclusion, this study on rat uterus provide the primary evidences that *T. paniculatum* root and leaf extracts produce tocolytic effects on both spontaneous and agonist-induced contractions. The possible mechanism(s) may be due to the blockade of Ca influx via L-type Ca^{2+} channels, Ca^{2+} efflux from internal store, and the interfering of ROK pathway that might reduce the sensitivity of contractile system to Ca^{2+}. The alteration of Ca oscillation, however, should be specifically confirmed by further studies using electrophysiological methods to elucidate authenticated Ca^{2+} mobilization in the uterine cells. Finally, the potent inhibitory effects of the extracts on Bay K8644 and OT induced contraction could substantiate the medicinal use of *T. paniculatum* to treat preterm labor or in abnormal hyper-contractility of the uterus.

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CHAPTER VIII

CONCLUSIONS

The uses of medicinal plants as the original source for medicine and as foundation for primary health care are becoming more common. However, only 25% of plant species are actively being prescribed as a medicinal plant (WHO, 2011). This practice is the greatest challenge in exploring novel medicinal plants and to discover their therapeutic values. *Talinum paniculatum* (Jacq.) Gaertn. (*T. paniculatum*) or "Som Java" belongs to Portulaceae family and locally grown throughout Thailand. This plant is recognized as having various medicinal properties such as the treatment of type-2 diabetes, inflammatory skin problems, gastrointestinal disturbance, general weakness and reproductive tonic (Pak et al, 2005; Manuhara, Yachya and Kristanti, 2012; Shimoda et al, 2001; Thomas, 2008). Although the plant has been reported to influence the reproductive system, there is no scientific data to clarify the effects on the female reproductive system to support its therapeutic significance. Therefore, this study focused on investigating the physiological effects of *T. paniculatum*'s extracts, and its related-component, phytol, on female reproductive functions.

There were four main purposes which included their effects on: 1) reproductive hormones (estrogen and LH), and blood biochemistry (low-density lipoprotein (LDL), high-density lipoprotein (HDL), triglycerides and tALP); 2) female reproductive organs (vagina, uterus and mammary tissues); 3) the anti-fertility

activity; and 4) uterine contraction in adult female rats. The major findings can be concluded as follows.

8.1 Phytochemical Compositions of *T. paniculatum* Root and Leaf Extracts

T. paniculatum belongs to the Portulacaceae family which widely grows in Thailand in the name of Wan Pak Pang and kob luj xeeb (Tichachart, 2004). This plant has long been used in the traditional medicine for diverse arrays of purposes (Shimoda et al., 2001; Pak et al., 2005). The research was designed to investigate the preliminary phytochemical, GC/MS analysis of *T. paniculatum*'s root and leaf extracts. The results showed that alkaloids, tannins, flavonoids and phytosterols were observed in both parts of the plant while only saponins were found in the root extract. The GC/MS analysis of the root extract revealed the presence of 5 phytosterols which were β-sitosterol (17.37%), stigmasterol (4.23%), stigmastan-3-ol (4.10%), stigmast-22-en-3-ol (1.84%) and campesterol (1.56%), respectively. Twelve known compounds that included fatty acids (0.50%-11.32%) and 2 unknown compounds were detected. The leaf extract showed the presence of 4 phytosterols which were β-sitosterol (10.60%), stigmastanol (2.76%), stigmasterol (0.85%) and campesterol (0.80%), respectively. Eleven known compounds, phytols (69.32%), α-tocopherol (0.99%), fatty acids (0.43-3.41%) and 2 unknown compounds were also identified.

8.2 Effects of *T. paniculatum*'s Extracts in Female Reproductive Hormones, Total Alkaline Phosphatase and Lipid Profile

T. paniculatum contains valuable phytosterols and medicinal secondary metabolites which may cure or manage various ailments (Filho et al., 2010; Yulia, Wientarsih and Razief, 2005). This study explored the potential medicinal properties of *T. paniculatum*'s extracts with two different dosages (100 and 1,000 mg/Kg BW/day) and its major component (standard-phytol 500 mg/Kg BW/day) on female reproductive hormones, total alkaline phosphatase (tALP) and lipid profiles. The experiment was designed to study in bilaterally ovariectomized rats (OVX) in 42-day treatment periods. The results demonstrated that oral administration of both root and leaf extracts to OVX rats produced to dose-dependency increase in serum estradiol level. In addition, OVX rats that were treated by standard-phytol and high dose of leaf extract (1,000 mg/Kg BW/day) showed significant negative effects between estradiol and luteinizing hormone. The oral administration by both dosages of *T. paniculatum* leaf extract to OVX rat significantly reduced serum tALP. Additionally, the treatment of *T. paniculatum*'s (both root and leaf) extracts and standard-phytol showed the positive effects on lipid profile as indicated by the tendency decrease of total cholesterol level and the significant increase in HDL/LDL ratio.

8.3 The Estrogenic Activity of *T. paniculatum*'s Extracts in Ovariectomized Rat

T. paniculatum is commonly used in Asian traditional medicine as a reproductive enhancement (Manuhara, Yachya and Kristanti, 2012). This plant has

been reported to contain some phytosterols that certainly affect the reproduction system (Edeoga, Okwu and Mbaebie, 2005). Thus, the estrogenic activity of chlorophyll derived-phytols and different dosages of *T. paniculatum*'s root and leaf extracts (100 and 1,000 mg/Kg BW/day) were performed in adult bilaterally ovariectomized rat as an experimental model of estrogen-depleted-patient (Wu et al., 2005). The results exhibited that the oral supplementation of standard-phytol, *T. paniculatum*'s root and leaf extracts possess the potent estrogenic effects in OVX as indicated by a dose dependency optimistic augmentation of various estrogen-responsiveness tissues revolution including vaginal cornification, increasing in the relative uterus and mammary weights. They also encourage the histoachitecture rehabilitation of vagina, uterus and duct system of mammary tissues.

8.4 The Anti-fertility of *T. paniculatum*'s Extracts in Pregnant Rats

T. paniculatum has been claimed to manage the reproductive system due to the presence of its phytosterols (Filho et al., 2010). The large consumption these compounds can enhance the luteolytic activity (Shibeshi et al., 2006) and disturbance in the level of reproductive hormones, hence cause infertility (Hughes et al., 1991; McGarvey et al., 2001; Abu and Uchenda, 2011). The aim of this study was to determine the validity of anti-fertility effect of *T. paniculatum*'s extracts and its related compound-phytol in pregnant rats. The results demonstrated that *T. paniculatum*'s extracts enhanced the dose dependency anti-implantation and abortifacient activities, whereas standard-phytol did not show the statistically significant effect in pregnant rats. Thus, this implied that *T. paniculatum*'s root and leaf extracts exhibited potent anti-fertility activity in pregnant rats.

8.5 Effects of *T. paniculatum*'s Extracts on Non-Pregnant Rat Uterine Contractility

T. paniculatum is believed to be beneficial for a female reproductive system by inducing lactation and restoring uterine functions after post-partum (Salakij, Jungsamanyat and Salakit, 1990). This study was attempted to investigate the effects of *T. paniculatum* root and leaf extracts on uterine contractility in non-pregnant rats. The results exemplified that *T. paniculatum*'s extracts possessed extensive dose-related inhibition of the spontaneous contractions. The relaxation pattern of the uterus after the cumulative application of the root was similar but more potent than that of leaf extract as indicated by the IC_{50} concentration of each extract (root : 0.23 mg/mL and leaf : 1.67 mg/mL). They impeded the tocolytic activity during agonist exposures including high KCl solution, Bay K8644 (specific Ca^{2+} channels activator), and oxytocin. These data suggested that the possible mechanisms may be due to the blockade of Ca^{2+} influx via L-type Ca channels. They also inhibited the force production during OT exposure in Ca^{2+}-free solution. This notion could explain that *T. paniculatum*'s extracts inhibited *Ca* efflux from internal store, and might interrupted the mechanism of Ca^{2+}-independent pathways that consequently reduced the sensitivity of contractile system to Ca^{2+}.

In summary, the significant phytochemical components in *T. paniculatum*'s root and leaf extracts are phytosterols (β-sitosterol, stigmastanol, stigmasterol stigmastan-3-ol, stigmast-22-en-3-ol and campesterol) and chlorophyll-derived phytols that are responsible for the existing medicinal activities report. The crude extracts of this plant and major compound (phytols) exhibit the potent estrogenic

activity by improving estrogen homeostasis on either their target sites and/or hypothalamic level.

The restoration of circulating estradiol may be due to the stimulation of endogenous estradiol production or mimic estrogenic activity induced by the presented phytosterols or xenoestogens. These compounds significantly influence the reproductive organs and other involved-systems, especially lipid and bone.

The physiological effects of *T. paniculatum*'s extracts are orchestrated by the activations on both classical genomic and rapid non-genomic effects that involve the intracellular estrogen receptors (ERs) and membrane bounded receptors, respectively. This system occurs in response to their phytosterols and signals integrated emanating from phytols signaling pathways (Björnström and Sjöberg, 2005; Goldstein et al., 2003; Heim et al., 2002). These convergent-activated receptors end and extensively lead to the estrogen responsive systems alternation including reproductive, lipid and bone systems. The major mechanisms of action of *T. paniculatum*'s extracts are proposed in Figure 8.1.

These findings clearly explain that *T. paniculatum*'s extracts and phytols notably possess positive property that mainly affect on female reproductive functions, particularly restoring the circulating estradiol levels in the animal model of estrogen-depleted condition.

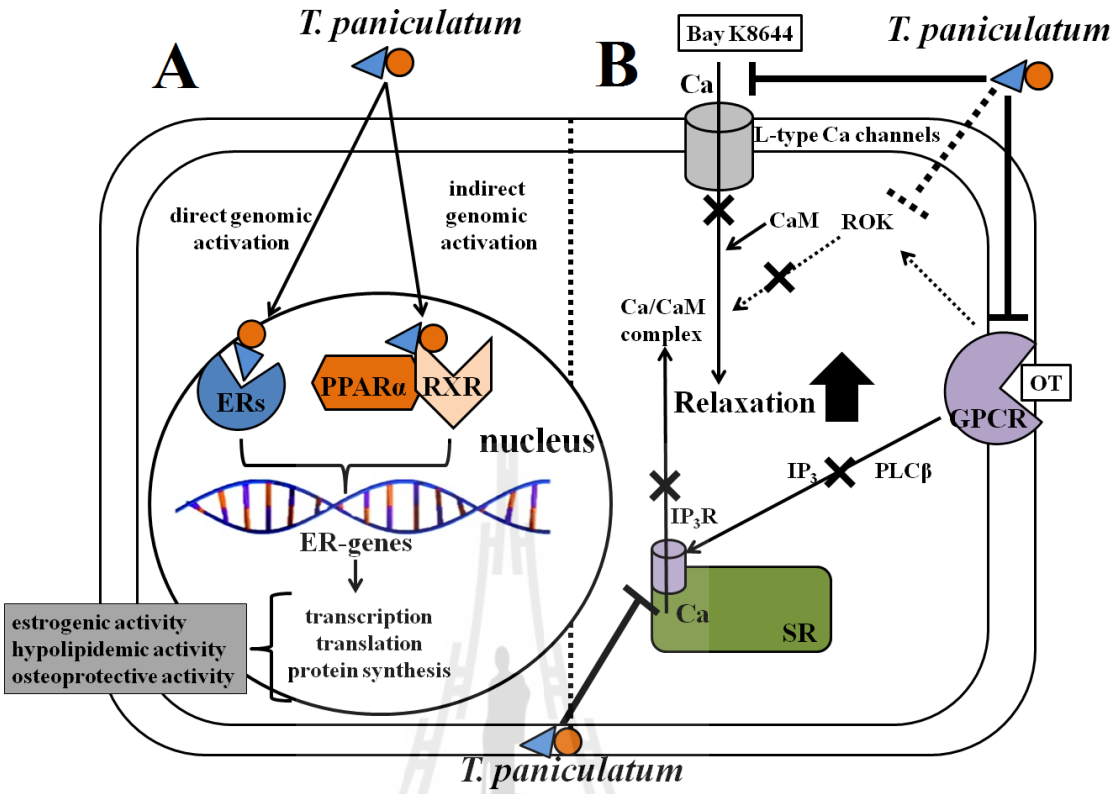

Figure 8.1 Schematic representation of the genomic (A) and non genomic (B) pathways modulated by *T. paniculatum*'s extracts on their target organs. *T. paniculatum*'s extracts may directly bind to estrogen receptors (ERs), resulting in dimerization and activation of gene transcription. *T. paniculatum's extracts* also indirectly activate estrogen responsive gene (ER-gene) via the activation of peroxisome proliferator-activated receptor α (PPARα) and retinoid X receptor (RXR) that apparently regulate gene expression. On the other hand, *T. paniculatum's extracts* also exhibit tocolytic activity via rapid nongenomic signaling pathways. *T. paniculatum*'s extracts prominently block L-type Ca^{2+} channels which were partially reverse by Bay K8644, a specific L-type Ca^{2+} channels activator. *T. paniculatum*'s extracts may also interrupt oxytocin and/or G-protein coupling receptor's (GPCR) signaling cascade which involves with phospholipase C β (PLCβ) and IP_3 system, hence diminish the activity of Rho kinase (ROK) pathway.

8.6 Future Investigation

The results noticeably suggested that *T. paniculatum* is a therapeutic plant with extraordinarily beneficial for managing various ailments. However, the studies were conducted in an animal model; it would be noteworthy to explore this plant for further physiological interventions in a human model.

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