EFFECTS OF *MORINGA OLEIFERA* LAM. SEED OIL ON FEMALE OVARIECTOMIZED RATS

Piyathida Kusolrat

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ผลของน้ำมันเมล็ดมะรุม (*Moringa oleifera* Lam.) ต่อหนูแรทตัดรังไข่เพศเมีย

นางปิยะธิดา กุศลรัตน์

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

Thesis Examining Committee

Rungrudee Srisawat
(Asst. Prof. Dr. Rungrudee Srisawat)
Chairperson

Sajeera Kupittayanant
(Assoc. Prof. Dr. Sajeera Kupittayanant)
Member (Thesis Advisor)

(Asst. Prof. Dr. Nopparat Buddhakala)
Member

G. Eumkeb.
(Assoc. Prof. Dr. Griangsak Eumkeb)
Member

Ratchadaporn .O.
(Asst. Prof. Dr. Ratchadaporn Oonsivilai)
Member

(Prof. Dr. Sukit Limpijumnong)
Vice Rector for Academic Affairs and Innovation

(Prof. Dr. Santi Maensiri)
Dean of Institute of Science

ปิยะธิดา กุศลรัตน์ : ผลของน้ำมันเมล็ดมะรุม (*Moringa oleifera* Lam.) ต่อหนูแรทตัดรังไข่เพศเมีย (EFFECTS OF *MORINGA OLEIFERA* LAM. SEED OIL ON FEMALE OVARIECTOMIZED RATS) อาจารย์ที่ปรึกษา : รองศาสตราจารย์ สัตวแพทย์หญิง ดร.ศจีรา คุปพิทยานันท์, 188 หน้า.

มะรุม (*Moringa oleifera* Lam.) เป็นพืชที่มีการนำมาใช้ในยาแผนโบราณอย่างแพร่หลาย จากรายงานการศึกษาที่ผ่านมาแสดงให้เห็นว่าน้ำมันเมล็ดมะรุมมีองค์ประกอบสำคัญเป็นสารในกลุ่มไฟโตเอสโตรเจนหลายชนิด โดยเฉพาะอย่างยิ่งไฟโตสเตอรอล การศึกษานี้มีวัตถุประสงค์เพื่อศึกษาฤทธิ์การเป็นเอสโตรเจนของน้ำมันเมล็ดมะรุมต่อการหดตัวของมดลูกหนูแรทเพศเมียในหลอดทดลอง และศึกษาฤทธิ์การเป็นเอสโตรเจนในหนูแรทเพศเมียที่เหนี่ยวนำให้อยู่ในภาวะพร่องฮอร์โมนโดยการตัดรังไข่ต่อการเปลี่ยนแปลง 1) อวัยวะระบบสืบพันธุ์ 2) ลิปิด โปรไฟล์ ค่าชีวเคมีในเลือดและปัสสาวะ และ 3) ความหนาแน่นกระดูกและปริมาณเนื้อกระดูก โดยศึกษาในหนูแรท 2 กลุ่ม คือ กลุ่มแรกได้รับน้ำมันเมล็ดมะรุมวันที่สามภายหลังจากตัดรังไข่ และกลุ่มที่สองได้รับน้ำมันเมล็ดมะรุมภายหลังจากตัดรังไข่หกสัปดาห์ โดยเทียบผลที่ได้กับการได้รับฮอร์โมนเอสโตรเจนสังเคราะห์ ทำการทดลอง เป็นระยะเวลาหกสัปดาห์ ผลการวิเคราะห์องค์ประกอบน้ำมันเมล็ดมะรุมด้วยวิธีแก๊สโครมาโทกราฟี พบว่าน้ำมันเมล็ดมะรุมประกอบด้วยไฟโตสเตอรอลสำคัญหลายชนิด ได้แก่ บีตา ซิโตสเตอรอล แคมพีสเตอรอล และสติกมาสเตอรอล โดยผลการศึกษาฤทธิ์ของน้ำมันเมล็ดมะรุมต่อการหดตัวของมดลูกพบว่าน้ำมันเมล็ดมะรุมมีฤทธิ์ทำให้มดลูกหดตัว โดยมีฤทธิ์สูงสุดที่ 400 ไมโครลิตร ใน 100 มิลลิลิตร และกลไกที่ไปกระตุ้นการการหดตัวอาจเกี่ยวข้องกับกลไกการออกฤทธิ์ซึ่งไม่ได้เกิดจากการมีฤทธิ์เป็นเอสโตรเจนแต่เกิดจากการเพิ่มการหดตัวซึ่งเกิดจากการเข้าสู่เซลล์ของแคลเซียมผ่านแอลไทป์แคลเซียมชาแนลและการหลั่งแคลเซียมจากซาโคพลาสมิคเรติคูลัม ส่วนการทดสอบฤทธิ์การเป็นเอสโตรเจนในการทดลองทั้งสองกลุ่ม พบว่าน้ำมันเมล็ดมะรุมแสดงฤทธิ์คล้ายเอสโตรเจน โดยบ่งชี้จากการเปลี่ยนแปลงของเนื้อเยื่อบุผนังช่องคลอด ทำให้มดลูกมีน้ำหนักเพิ่มขึ้น เหนี่ยวนำให้เยื่อบุมดลูกหนาขึ้น เพิ่มระดับเอสตราไดออล เพิ่มระดับคลอเรสเตอรอลชนิดความหนาแน่นสูง และลดคลอเรสเตอรอลชนิดความหนาแน่นต่ำ นอกจากนี้ยังพบว่าค่าชีวเคมีเลือดและปัสสาวะ ได้แก่ อัลคาไลน์ ฟอสฟาเทส แคลเซียม ฟอสฟอรัส ของหนูที่ถูกชักนำให้อยู่ในสภาวะพร่องฮอร์โมนสามารถกลับคืนมาสู่สภาวะปกติเมื่อให้น้ำมันมะรุมในขนาด 0.25 และ 0.50 มิลลิลิตรต่อ 100 กรัม น้ำหนักตัวต่อวัน นอกจากนี้น้ำมันเมล็ดมะรุมมีแนวโน้มที่จะเพิ่มความหนาแน่นและปริมาณเนื้อกระดูก จากผลการศึกษาทั้งหมดอาจกล่าวได้ว่าน้ำมันมะรุมมีฤทธิ์การเป็นเอสโตรเจน โดยการออกฤทธิ์เป็นได้ทั้งแบบที่ไม่ผ่านและผ่านยีน โดยผลจากการแสดงฤทธิ์คล้ายเอสโตรเจนของน้ำมันมะรุมนี้จะเป็นประโยชน์และเป็นแนวทางต่อการ

นำไปใช้ในการป้องกันและรักษาโรคสำคัญที่เกิดจากภาวะพร่องฮอร์โมน เช่น โรคไขมันและหลอดเลือด โรคกระดูกพรุน ในสตรีวัยหมดประจำเดือน

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ปีการศึกษา 2558 ลายมือชื่ออาจารย์ที่ปรึกษา _____

PIYATHIDA KUSOLRAT : EFFECTS OF *MORINGA OLEIFERA* LAM. SEED OIL ON FEMALE OVARIECTOMIZED RATS. THESIS ADVISOR : ASSOC. PROF. SAJEERA KUPITTAYANANT, Ph.D. (DVM), 188 PP.

MORINGA OLEIFERA LAM./ PHYTOESTROGENS/ PHYTOSTEROLS/ RAT/ CONTRACTION/ CHOLESTEROL/ UTERUS/ CALCIUM/ BONE

Moringa oleifera Lam. has been used in the traditional medicine passed down for centuries in many cultures around the word. Moringa seed oil has been reported to contain phytoestrogens including phytosterols. Therefore, the purposes of this study were to study the estrogenic effects of *M. oleifera* Lam. seed oil (MSO) on isolated rat uterine contraction and their estrogenic activity in female ovariectomized (OVX) rats by observing the effects on 1) reproductive organs 2) lipid profiles, blood and urine biochemistry and 3) bone mineral density [BMD] and bone mineral content [BMC]. The underlying mechanisms of the oil were investigated. Rats were divided into two major study groups, group 1; OVX rats were orally administered with MSO for 6 weeks starting from day 3 after ovariectomy and group 2; OVX rats were administered with MSO for 6 weeks starting from day 60 after ovariectomy. The effects of MSO were comparable with standard estradiol. The results of GC-MS analysis revealed that MSO contained major phytosterols including β-sitosterol, campesterol and stigmasterol. The examination of physiological effects of MSO on uterine contractility exhibited that MSO was uterotonic as it increased spontaneous contraction with a maximum effect of 400 μL/100 mL. The possible mechanisms of action were not due to its estrogenic effects, but increasing contraction via Ca^{2+} entry on L-type calcium channel and

sarcoplasmic reticulum Ca^{2+}. The estrogenic activities evaluation in two studies indicated that MSO oral feeding showed estrogenic like activity as indicating by inducing vaginal cornification, increasing relative uterine weight and epithelium cell height. MSO increased serum estradiol level, increased high-density lipoprotein cholesterol and decreased low-density lipoprotein cholesterol, restored bone biomarkers such as alkaline phosphatase, calcium and phosphorus of OVX rats to normal levels when received MSO 0.25 and 0.50 mL/100g BW/day. In addition, MSO had a tendency to increase BMD and BMC. In summary, these findings reveal that MSO has estrogenic activities and the modes of action can occur through both non-genomic and genomic pathways. It therefore, may be beneficial in the prevention and treatment of menopausal disorders such cardiovascular diseases and osteoporosis.

School of Preclinic

Academic Year 2015

Student's Signature P. Kaosrat

Advisor's Signature Sajeera Kupittayanant

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LISTS OF ABBREVIATIONS

ALP	=	alkaline phosphatase
ANOVA	=	analysis of variance
ATP	=	adenosine triphosphate
AUC	=	area under the contraction
BMC	=	bone mineral content
BMD	=	bone mineral density
BW	=	body weight
°C	=	degree Celsius
Ca^{2+}	=	calcium ion
$[Ca]_i$	=	cytoplasmic Ca^{2+}concentration
$CaCl_2$	=	calcium chloride
CAD	=	Coronary artery disease
CaM	=	calmodulin
cAMP	=	cyclic adenosine monophosphate
Cd	=	caldesmon
cGMP	=	cyclic guanosine monophosphate
CL	=	corpus lutium
cm	=	centimeter
CO_2	=	carbon dioxide
Cp	=	calponin

LIST OF ABBREVIATIONS (Continued)

Ca-CaM	=	calcium-calmodulin
CPA	=	cyclopiazonic acid
DAG	=	diacylglycerol
E_1	=	estrone
E_2	=	17β-estradiol
E_3	=	estriol
EGTA	=	ethylene glycol-bis (2-aminoethylether)-N,N,N′,N′-tetraacetic acid
ER	=	estrogen receptor
ER_s	=	estrogen receptors
ER_α	=	estrogen receptor alpha
ER_β	=	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-C	=	high-density lipoprotein cholesterol
hr	=	hour
HRT	=	hormone replacement therapy

LIST OF ABBREVIATIONS (Continued)

KCl	=	potassium chloride
kDa	=	kilodalton
IGF	=	insulin-like growth factor
IL	=	interleukin
IU	=	international unit
LH	=	luteinizing hormone
IP_3	=	inositol (1, 4, 5)-triphosphate
LDL-C	=	low-density lipoprotein cholesterol
MAP	=	mitogen-activated protein
MAPK	=	mitogen-activated protein kinase
µg/kg BW/day	=	microgram per kilogram body weight per day
mg/dL	=	milligram per deciliter
$MgSO_4$	=	magnesium sulphate
min	=	minute
mIU/mL	=	milli-international unit per milliliter
mL	=	milliliter
mL/100g BW/day	=	milliliter per 100 gram body weight per day
MLC_{20}	=	20-kDa regulatory side chain of myosin light chain
MLCK	=	myosin light chain kinase
MLCP	=	myosin light chain phosphatase
mm	=	millimeter

LIST OF ABBREVIATIONS (Continued)

mM	=	millimolar
MW	=	molecular weight
n	=	number of sample
NaCl	=	sodium chloride
nM	=	nanomolar
NO	=	nitric oxide
OPG	=	osteoprotegerin
OVX	=	ovariectomy
PDE	=	phosphodiesterases
pg/mL	=	picogram per milliliter
PKA	=	protein kinase A
PKC	=	protein serine/threonine kinase C
pM	=	picomolar
PMCA	=	plasma membrane Ca^{2+}-ATPase
PPAR	=	peroxisome proliferator-activated receptor
rpm	=	revolutions per minute
RANKL	=	receptor activator of nuclear factor kappa-B ligand
ROCs	=	receptor-operated channels
%RU	=	percentage of relative uterine weight
S.E.M	=	standard error of the mean
SERCA	=	SR Ca-ATPase
SR	=	sarcoplasmic reticulum

LIST OF ABBREVIATIONS (Continued)

TC	=	total cholesterol
TEA	=	tetraethylammonium
TG	=	triglyceride
TNF	=	tumornecrosis factor
µg	=	microgram
µl	=	microliter
µm	=	micrometer
VOCC	=	voltage gate calcium channels
v/w	=	volume per weight

CHARPTER I

INTRODUCTION

Women's health and menopause is a rapidly expanding field of medical practice and scientific investigation. It is a field of great social importance and impact. Although, pharmacological options for prevention and treatment of postmenopausal exist, the incidence of postmenopausal symptoms remains high. Estrogen deficiency can also lead to longer-term health issues such as cardiovascular disease and osteoporosis. For many years, menopausal therapy with estrogen and hormone replacement was prescribed as a form of preventive medicine, but this practice ceased after severe side effects came to light. Nowadays, evidence for the potential health benefits of phytoestrogens is increasing. Natural alternative therapies, such as diet modification and phytotherapy are perceived to be safe and have little or no side effects. While pharmacological agents are available to treat postmenopausal symptoms, many non-pharmacological treatment options are also available. Thus, it is necessary to continue research the effects of phytoestrogens from novel herbs. Therefore, this thesis focuses on the effects and mechanisms of *Moringa oleifera* seed oil on menopause using laboratory animal models.

1.1 Female reproductive cycles (Human and rat)

The uterine or menstrual cycle is a series of cyclic changes in the endometrium that occur on a monthly basis in response to changes in levels of ovarian

hormones. The typical human reproductive menstrual cycle encompasses a 28-day timeframe (Figure 1.1). During the ovarian cycle, growth and development of the follicle is driven by two gonadotrophic hormones: Follicle Stimulating Hormone (FSH) and Luteinizing hormone (LH). Both FSH and LH are secreted by the anterior pituitary and are under the control of gonadotrophin releasing hormone (GnRH) secreted by the hypothalamus. FSH and LH stimulate follicle growth. As the follicle grows, thecal cells secrete estrogens. Rising levels of estrogen in the plasma have a negative feedback effect on the anterior pituitary inhibiting output of FSH and LH. However, this negative feedback is only transient and as levels of estrogen increase they begin to have a positive effect on the hypothalamic-pituitary axis resulting in a burst of LH and, to a lesser extent, FSH. This sudden burst of LH and FSH stimulates completion of meiosis I in the primary oocyte and is also believed to be involved in stimulating synthesis of enzymes involved in bulging of the ovarian wall.

After ovulation, LH promotes the transformation of the ruptured graafian follicle into the corpus luteum (CL). LH stimulates the corpus luteum to secrete progesterone and estrogen. Progesterone and estrogen from the corpus luteum have a negative feedback effect on the anterior pituitary and inhibit FSH and LH production (Figure 1.2). This prevents development of new follicles. As LH levels fall, the corpus luteum begins to degenerate. Levels of progesterone and estrogen fall. FSH and LH are again produced by the anterior pituitary and a new cycle begins. Although the length of the follicular phase varies greatly between females, the length of the luteal phase is usually constant. Menstrual phase can be divided into three phase as follows.

Menstrual phase (1-5 days)

The functional layer of the endometrium becomes detached from the uterine wall and this results in bleeding. Blood loss is usually between 50-150 mL. Detached tissues and blood pass through the vagina as the menstrual flow.

Proliferative phase (days 6-14)

As levels of estrogen increase the endometrium begins to proliferate and thicken, tubular glands and spiral arteries form. Estrogen also stimulates the synthesis of progesterone receptors in endometrial cells. Ovulation occurs at the end of this phase.

Secretory phase (days 15-28)

Rising levels of progesterone produced by the corpus luteum act on the endometrium stimulating the enlargement of glands which begin secreting mucus and glycogen in preparation for implantation of the fertilized ovum. If fertilization does not occur, the corpus luteum degenerates, progesterone levels fall and the endometrium degenerates. The cycle starts again with the first day of menstrual flow (Fox, 1996).

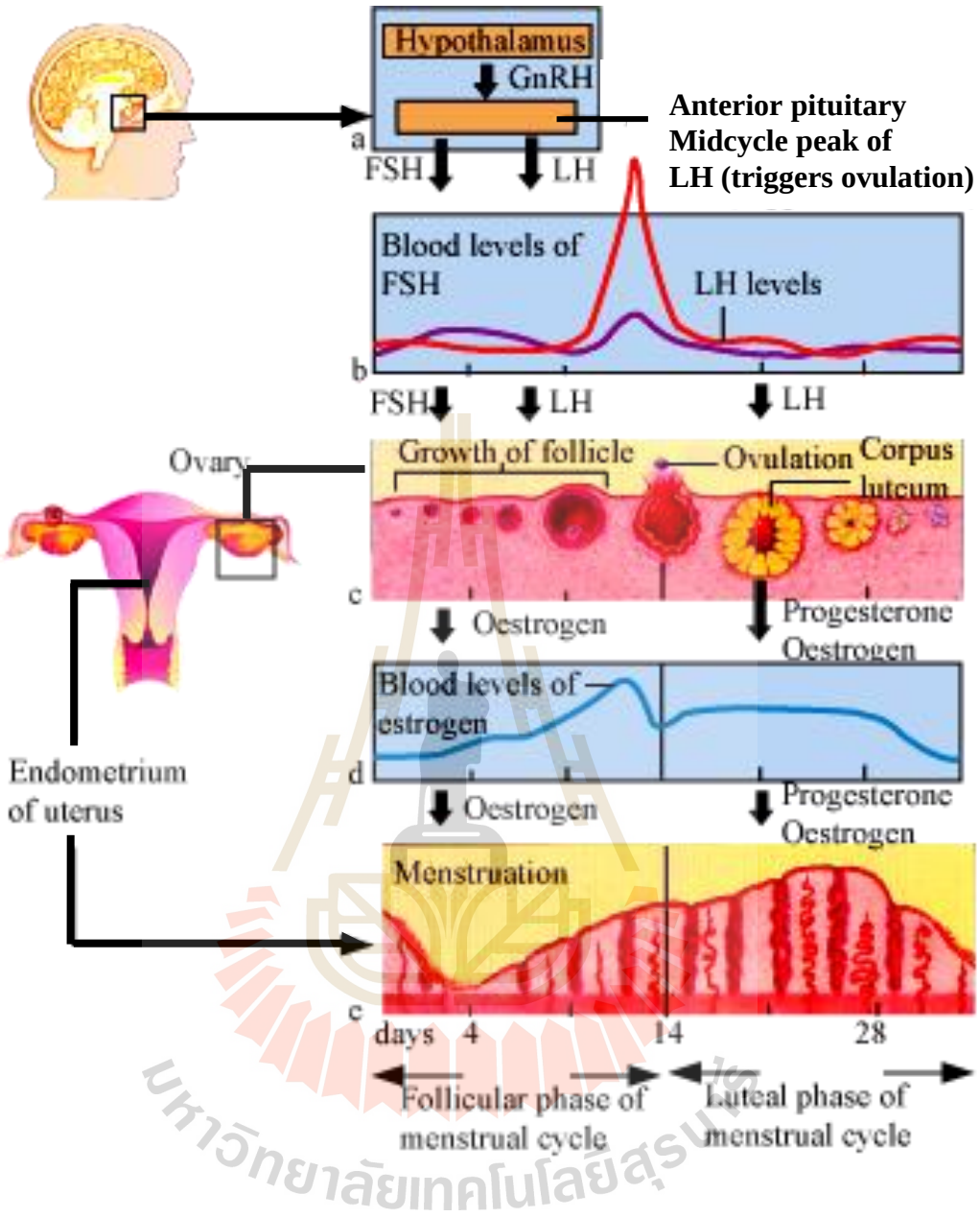

Figure 1.1 The relation of reproductive hormones, ovarian cycle and endometrium changes throughout the normal menstrual cycle (Widmaier et al., 2008).

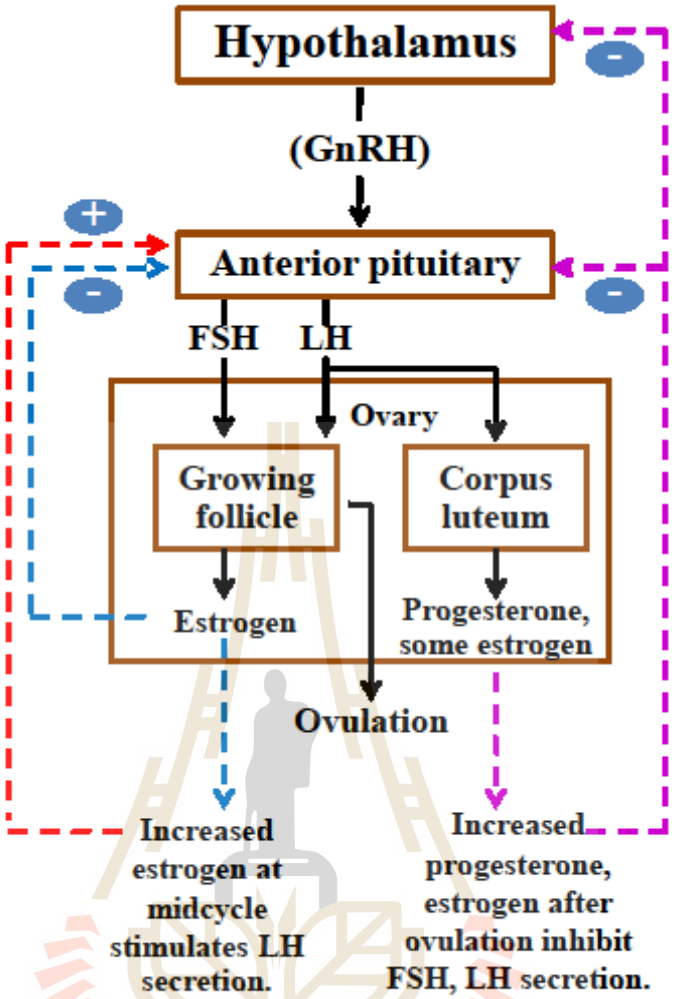

Figure 1.2 Illustrations of hypothalamic-pituitary-ovarian axis and control of the menstrual cycle. The first feedback loop is the blue one. In this loop, FSH and LH were secreted to start the development of the follicle. These two hormones work together to start estrogen secretion. The estrogen has a negative feedback on FSH, which causes it to stop being secreted. The second feedback loop is shown in red. This occurs with the increased amount of estrogen that is experienced at the midpoint in the cycle. This increased amount of estrogen causes a positive feedback to occur on the LH cells in the pituitary. LH secretion will rise, and ovulation occurs. The final feedback loop is the purple. This is another negative feedback. After ovulation, the

follicle cells are transformed into the corpus luteum. The corpus luteum secretes estrogen and progesterone. This buildup of progesterone and estrogen further increases the formation of the endometrial lining. These hormones work together and send a second negative feedback to inhibit the release of FSH and LH. This causes the corpus luteum to deteriorate, slowing the production of estrogen and progesterone. The drop in these hormones signals menstruation (Di, 2003).

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 (Figure 1.3). The dominant hormones in various stages of the cycle have shown in Figure 1.4.

In contrast with the human cycle, the rat cycle is much shorter, consisting of 4 to 5 days. Progesterone increases sharply beginning early in the postovulation phase (i.e., diestrus) on day 2 and drops sharply in late diestrus on day 2. At approximately noon of the start of the follicular phase (i.e., proestrus), estrogen levels markedly surge, causing a rapid peaking of LH and FSH between about 4 PM and 6 PM of proestrus and an increased progesterone secretion. As in humans, the gonadotropin surge triggers ovulation. All these hormones return to baseline levels when ovulation occurs (i.e., estrus) on day 4. Finally there is a brief temporary peak of estradiol the evening of estrus.

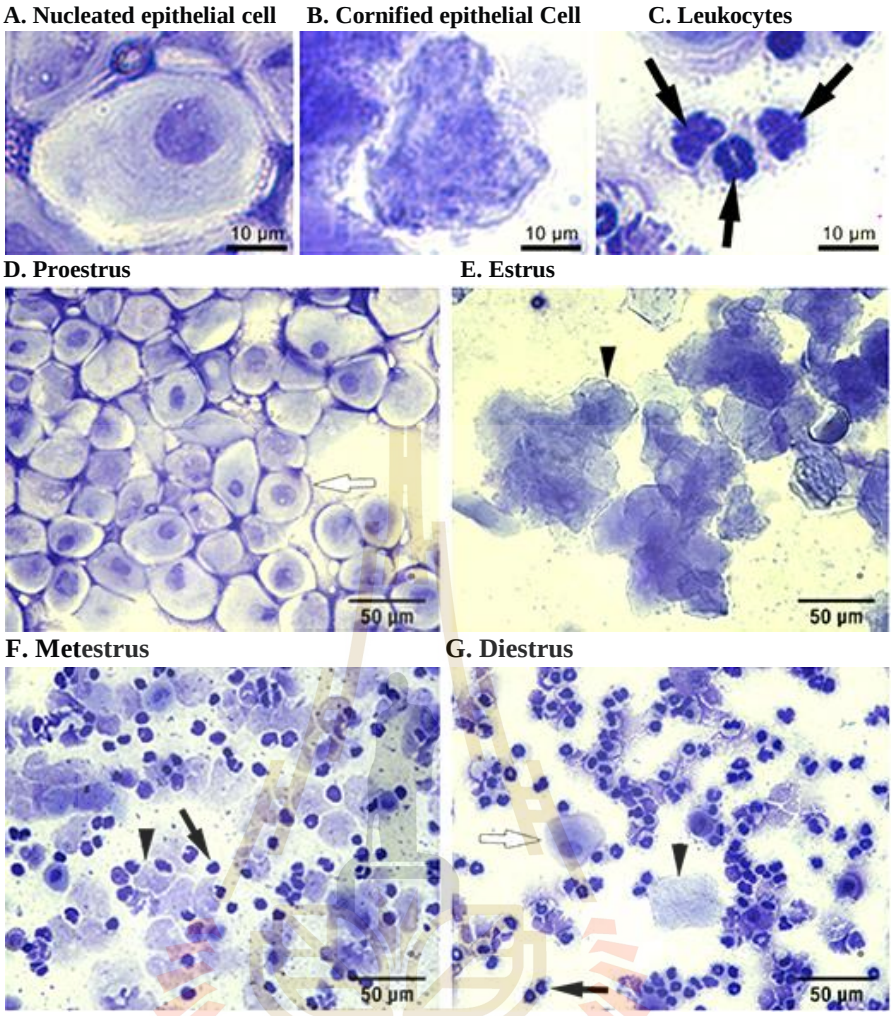

Figure 1.3 Representative hematoxylin stained from vaginal lavage at each phase of the estrous cycle in rat. Three main cell types are detected in vaginal smear samples: (A) nucleated epithelial cells, (B) cornified squamous epithelial cells, and (C) leukocytes. The ratio of these cell types present in the smear can be used to identify mice in (D) proestrus, (E) estrus, (F) metestrus, or (G) diestrus as described in representative results. Black arrow heads in E, F and G point to representative cornified squamous epithelial cells. Black arrows in C, F and G point to representative leukocytes. White arrows in D and G highlight representative nucleated epithelial cells (McLean, 2012).

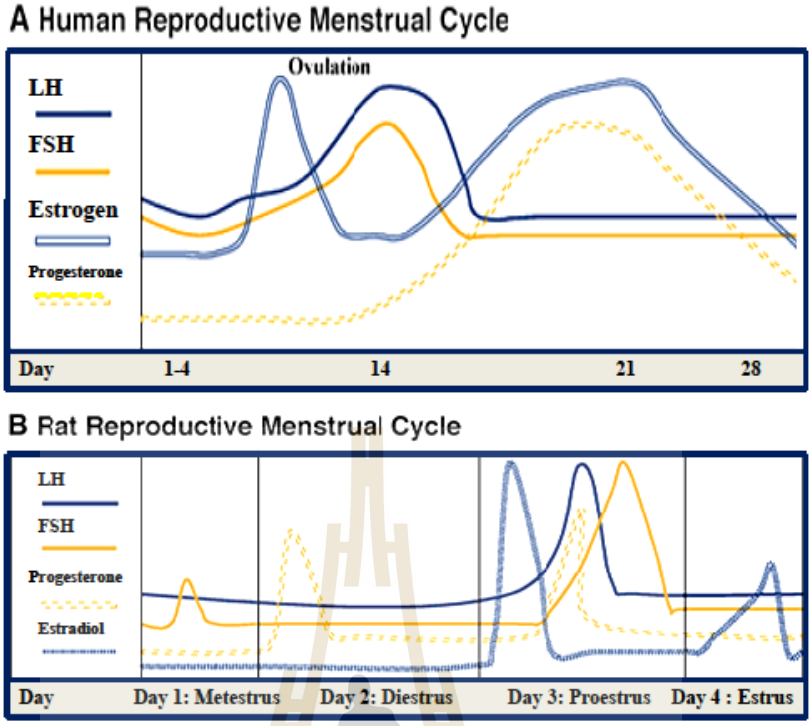

Figure 1.4 Schematic comparative endocrinology of the 28-day human reproductive menstrual cycle and the 4-day estrous cycle in the rat. The estrous pattern is presenting serum estradiol and progesterone concentrations as they relate in time to the surge of luteinizing hormone (McLean, 2012).

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.

1.2 Mechanism of uterine contraction and relaxation

The uterus is a hollow muscular organ situated deep within the female pelvic cavity. The smooth muscle contained within it (myometrium) is able to produce regular spontaneous contractions without any hormonal or nervous input (Wray, 2003). The non-pregnant uterus is not a quiescent organ as some may thought and it can produce contractions to facilitate the journey of sperms to the fallopian tubes and to help expel the shed inner lining of the uterus (endometrium) during menstruation (Otaibi, 2014).

Myometrial contractions are dependent on interaction between smooth muscle contractile proteins actin and myosin. Arrays of thick protein filaments containing myosin interact with arrays of thin filaments containing actin, leading to reversible cross-bridges. It is believed that the myosin head rotates due to conformational changes in the protein structure, leading to a pulling on the actin filament to generate tension and produce a relative spatial displacement (Lye and Challis, 2001). The energy needed for myometrial contractions comes from adenosine triphosphate (ATP) hydrolysis (Carsten and Miller, 1987).

An increase in intracellular calcium ($[Ca^{2+}]_i$) concentration leads to activation of myosin light chain kinase (MLCK) by the calcium-calmodulin (Ca-CaM) complex. MLCK phosphorylates the 20 kDa light chains of myosin (MLC_{20}) facilitating interaction of myosin with actin, resulting in contraction (Wray, 1993; Word, 1995). Relaxation of the myometrium may occur as a result of reduction in the phosphorylation of MLC_{20} caused by a decrease in MLCK activity. MLCK activity could be reduced as a result of a fall in $[Ca^{2+}]_i$ levels leading to a decrease in Ca-CaM levels. The activity of MLCK is also modulated by a cyclic adenosine monophosphate

(cAMP)-dependent protein kinase (A-kinase). The A-kinase phosphorylates MLCK causing it to have weak affinity for Ca-CaM complex. This results in dephosphorylation of myosin and myometrial relaxation (Adelstein and Eisenberg, 1980; Walsh and Hartshorne, 1982). cAMP is also capable of modulating myometrial contractility by reducing $[Ca^{2+}]_i$ levels either by increasing intracellular uptake of Ca^{2+} or by enhancing Ca^{2+} efflux from the myocyte.

Myosin light chain phosphatase (MLCP) dephosphorylates MLC_{20}, leading to relaxation. This action is negatively regulated by the small G-protein rhoA-rho-associated kinase and protein kinase C (PKC) pathways and positively regulated by cyclic guanosinemonophosphate (cGMP) (Savineau and Marthan, 1997; Sanborn, 2001). A number of actin-binding proteins that could also regulate myometrial contractions are present in smooth muscles. These proteins include caldesmon (Cd) and calponin (Cp). Cd and Cp reduces actin-myosin interactions by binding to actin in a manner that decreases actomyosin-ATPase activity (Gerthoffer and Pohl, 1994). Decrease in myometrial actomyosin-ATPase activity would result in inhibition of uterine contraction. Moreover, phosphorylation of Cd and Cp reduces their affinities for actin thereby hindering their ability to inhibit actomyosin-ATPase activity. Cd is phosphorylated by p38 mitogen-activated protein kinase (p38 MAPK), p21-activated kinase (PAK) and PKC. Cp is also phosphorylated by PKC (Horowitz et al., 1996).

An increase in $[Ca^{2+}]_i$ to initiate myometrial contraction can occur through several mechanisms such as:

1) Intracellular release of Ca^{2+} (e.g. from the sarcoplasmic reticulum);

2) Inhibition of Ca^{2+} efflux; and

3) Increase in extracellular Ca^{2+} influx.

Depending on the stimulus, one or more of these events may occur during myometrial contraction (Lye and Challis, 2001).

The extracellular Ca^{2+} concentration is higher than the $[Ca^{2+}]_i$. Although there is electrochemical gradient, calcium does not rush into the cell at rest because of the low calcium permeability of the cell membrane (Carsten and Miller, 1987). Calcium enters the cell through voltage gate calcium channels (VOCC) or receptor-operated channels (ROCs). Regulation of Ca^{2+} channels appears to be an important mechanism by which contractants and relaxants mediate their effects in smooth muscle (Sanborn et al., 1994). VOCs respond to changes in membrane potential. Based on their activation kinetics and sensitivity to pharmacological agents, VOCs in smooth muscles were further sub-classified as L type (slow or long-acting) and T type (fast or transient) (Nelson et al., 1990). ROCs on the other hand respond to binding of certain agonists (e.g. prostaglandins and noradrenaline) to their receptors leading to increased extracellular Ca^{2+} influx. However, ROCs are less well-characterized (Sanborn et al., 1994). Calcium channel blockers produce adequate uterine relaxation and some of them have been used in the management of preterm labour (Csapo et al., 1982) and primary dysmenorrhoea (Forman et al., 1982). Agonist-receptor interaction (e.g. oxytocin-oxytocin receptors) could also cause activation of phospholipase C (PLC) which leads to hydrolysis of inositol phosphate and results in the release of inositol-1,4,5, triphosphate (IP_3). IP_3 causes the release of $[Ca^{2+}]_i$ from the endoplasmic or sarcoplasmic reticulum (Lye and Challis, 2001) resulting in the cascade of events that culminate in myometrial contractions. The other product of phosphoinositide

hydrolysis is diacyl glycerol (DAG). In addition to activating PKC, DAG could also be a substrate for PG and eicosanoid biosynthesis (Ca^{2+} efflux through the cell membrane participates in the relaxation of a contracted muscle. This energy-requiring transport is mediated by two major membrane-linked Ca^{2+} exporting systems) (Kosterin et al., 1994):

1. Mg^{2+}, ATP-dependent Ca^{2+} pump, which utilizes the energy of ATP hydrolysis for Ca^{2+} transport against its electrochemical gradient.

2. Na^{+}-Ca^{2+} exchanger, which uses the energy of Na^{+} gradient for active Ca^{2+} transport.

Ca^{2+} efflux mechanisms are inhibited by oxytocin, and PGs. Inhibition of Ca^{2+} efflux will prolong the elevation of $[Ca^{2+}]_i$ thereby promoting uterine contraction (Wray, 1993). Hence, inhibition of Ca^{2+} efflux by oxytocin, and PGs could play a role in their oxytocic activity. cAMP levels are regulated by adenylcyclase and phosphodiesterases (PDEs). Adenylcyclase mediates cAMP synthesis while PDEs causes cAMP hydrolysis. Some inhibitors of myometrial contractions (e.g. β-adrenergic agonist and relaxin) increases cAMP level possibly through an increase in adenylcyclase activity (Lye and Challis, 2001). On the other hand, inhibitors of PDEs relax the uterus by reducing cAMP or cGMP hydrolysis (Agha and Taha, 2001). Nitric oxide (NO) donors are also capable of relaxing uterine contractions signifying that NO may act as an endogenous regulator of myometrial activity. The mechanism of NO-induced myometrial relaxation is not fully known however, NO-induced increase in cGMP could play a role in its relaxant effect on the uterus (Lye and Challis, 2001; Adebiyi, 2004) (Figure 1.5).

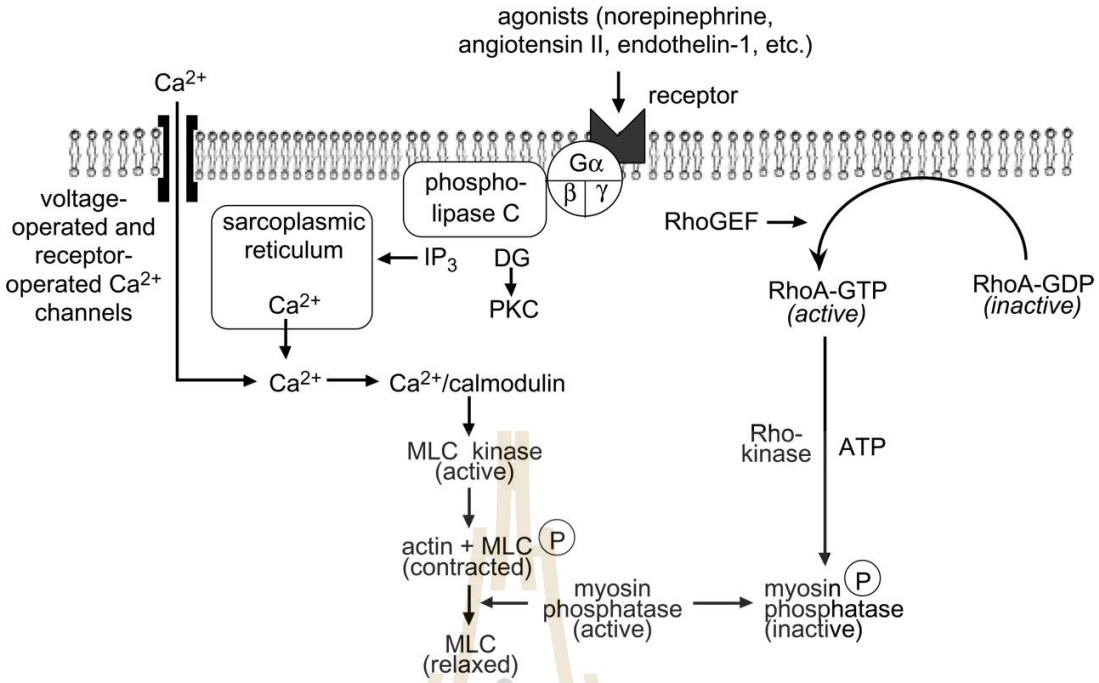

Figure 1.5 Regulation of smooth muscle contraction. Various agonists (neuro-transmitters, hormones, etc.) bind to specific receptors to activate contraction in uterine smooth muscle. Subsequent to this binding, the prototypical response of the cell is to increase phospholipase C activity via coupling through a G protein. Phospholipase C produces two potent second messengers from the membrane lipid phosphatidylinositol 4,5-bisphosphate: diacylglycerol (DG) and inositol 1,4,5-trisphosphate (IP_3). IP_3 binds to specific receptors on the sarcoplasmic reticulum, causing release of activator calcium (Ca^{2+}). DG along with Ca^{2+} activates PKC, which phosphorylates specific target proteins. PKC has contraction-promoting effects such as phosphorylation of Ca^{2+} channels or other proteins that regulate cross-bridge cycling. Activator Ca^{2+} binds to calmodulin, leading to activation of myosin light chain kinase (MLC kinase). This kinase phosphorylates the light chain of myosin, and, in conjunction with actin, cross-bridge cycling occurs, initiating shortening of the smooth muscle cell. However, the elevation in Ca^{2+} concentration within the cell is

transient, and the contractile response is maintained by a Ca^{2+}-sensitizing mechanism brought about by the inhibition of myosin phosphatase activity by Rho kinase. This Ca^{2+}-sensitizing mechanism is initiated at the same time that phospholipase C is activated, and it involves the activation of the small GTP-binding protein RhoA. Upon activation, RhoA increases Rho kinase activity, leading to inhibition of myosin phosphatase. This promotes the contractile state, since the light chain of myosin cannot be dephosphorylated (Clinton Webb, 2003).

1.3 Menopause

The word “menopause” was used for the first time in 1816 by Gardanne (Wilbush, 1979). The menopause is defined as the time after a woman's menstrual periods have ceased (12 months after a woman's final menstrual period). It is associated with an estrogen deficiency and can cause an increase in vasomotor symptoms (hot flushes), genitourinary symptoms (vaginal dryness, sexual dysfunction, frequent urinary tract infections, urinary incontinence), and musculoskeletal symptoms (joint pain) as well as sleep and mood disturbance. Cessation of ovarian cycles after menopause brings about a major change in female hormonal homeostasis. The postmenopausal woman estrogen levels drop from 0.3-2.6 nM to about 0.1 nM (Setiawan et al., 2006). Among a variety of physiological changes due to the decreased estrogen levels, the most detrimental consequence is loss of bone mass and development of osteoporosis (NAMS, 2006).

Ninety-five percent of estradiol (E_2) is produced by the ovaries premenopausally and only 5 percent from fat cells. After menopause, estrone is partially converted to E_2 but this amounts to much less E_2 than was available

premenopausally (Steiner et al., 2003). As hormone levels decrease, a number of symptoms may emerge, although their presentation and severity varies greatly from woman to woman. The most common menopause symptoms are hot flashes, depression, insomnia, vaginal dryness, irritability, mood swings and headaches.

The process of the menopausal transition appears to take about a decade. The earliest signs of this transition are (1) shorter menstrual cycles by 2-3 days, and (2) infertility. After birth, the number of oocytes continuously decreases. At puberty, 1 million oocytes are left. This number decreases to 0.3 million by the age of 20 years. Menopause is marked by the exhaustion of the ovarian supply of oocytes (McKinlay et al., 1992).

The most significant source of estrogen after the menopause when ovarian function fails is the weaker estrogen estrone, which is produced in fat cells. A significantly smaller amount of E_2 relative to the premenopausal state is produced by the conversion of estrone (McEwen, 2002). The menopause can therefore be defined as a period of low effective estrogen. Kessler et al. (1993) indicate that the rate of depression among women continues to decline in the postmenopausal age group, suggesting that estrogen deficiency is not a risk factor for depression during this period. This contradicts results of a compilation by Arpels (1996) who looked at published literature and was able to establish a correlation between low levels of estrogen in women and dysfunction in mood/memory and cognition (Lobo, 1997). Sustained low levels of estrogen after premature ovarian failure and natural or surgical menopause (in the chronic phase), which may produce signs of physical decline, including:

Vasomotor symptoms

One of the most obvious symptoms of estrogen deficiency is the instability of body temperature resulting in hot flashes and sweating. Hot flashes result from vasodilation of the skin capillaries, and typically affect the chest and facial areas. Skin temperature increases one to two degrees Celsius. Episodes can differ in frequency and severity, usually lasting between 0.5 minutes and ten minutes (Greendale, 1999). Hot flashes usually disappear spontaneously; about 25-50% of women will continuously suffer from symptoms for up to five years following menopause (Al-Azzawi, 2001).

Urogenital symptoms

The loss of estrogen affects the reproductive tract. This loss results in vaginal dryness, leading to atrophy of the endometrium, cervix, and vagina. The bladder and urethra are also affected by the reduction of estrogen resulting in urinary incontinence, increased frequency of cystitis, and dysuria (Ratner and Ofri, 2001).

Cardiovascular system

Estrogen deficiency results in several changes in the cardiovascular system. Coronary artery disease (CAD) is the leading cause of death in American women. The risk of CAD increases significantly after menopause, suggesting that estrogen may play a protective role in preventing heart disease (Ratner and Ofri, 2001). Other factors contributing to the increased risk of cardiovascular disease due to estrogen deficiency after menopause include hypertension, increased total cholesterol and triglyceride levels, and increases in coagulation factors (Matthews et al., 1989).

Bone Metabolism

Osteoclastic activity significantly increases within six to eight weeks after loss of estrogen. As a result, several biochemical changes in bone metabolism may occur. These changes include reduction in serum osteocalcin levels, increase in urinary hydroxyproline: creatinine ratio, increase in urinary calcium, and reduction in intestinal absorption of calcium (Al-Azzawi, 2001).

1.4 Estrogen and its functions

Estrogens are hormones best known for their roles in female reproductive physiology. In mammals, there are three primary endogenous estrogens: Estrone (E_1), β-estradiol (E_2), and estriol (E_3). Each has different potencies and physiological roles. β-estradiol, the most potent, is the predominant estrogen in premenopausal women, secreted mainly by the ovaries. Estrone, less potent than β-estradiol, is the main estrogen in postmenopausal women, synthesized in adipose tissue from adrenal precursors. Estriol, the weakest of the three natural estrogens, is produced in large quantities by the placenta. It does not appear to affect the bones, breast, brain, heart, and other organs the way estrogen does, and is therefore not likely to ameliorate cognitive and mood disorders (Coelingh Bennink, 2004).

In the developing CNS, estrogens are crucial in determining gender dimorphism. The gender-specific brain is produced by the epigenetic action of gonadal hormones at critical periods of brain development (Carrer and Cambiasso, 2002). While the female brain morphology develops in the absence of testicular secretions, irrespective of chromosomal sex, many of the masculinizing actions of androgen on the brain require conversion of testosterone to estrogen. The diversity of

estrogen effects on the adult brain implies a role beyond the control of reproductive function, since estrogen signaling is known to influence memory, motor activity, and mood (McEwen and Alves, 1999). Moreover, as the brain ages, it undergoes biochemical and structural changes regulated by estrogen (Thakur and Sharma, 2006).

In addition to their role in reproduction, estrogens influence a number of systems in females, including the mammary gland the cardiovascular system, and bone. E_2 is synthesized in the granulose cells of mature ovary through aromatization of testosterone supplied by the theca cells. The rate-limiting enzyme in E_2 synthesis is aromatase, a number of P_{450} family. Synthesis of estrogens also occurs in adipose tissue, skeletal muscle, skin, hair follicles, and bone. As illustrated by the increased risk of fractures in post-menopausal women and decreased risk with hormone replacement therapy (Pinkerton and Santen, 1999), estrogens are important for maintenance of female bone mass.

Estrogens also exert a protective effect on the cardiovascular system, possibly accounting for the low incidence of heart disease in women of reproductive age. After menopause the risk of cardiovascular disease increases and at 60 years of age or older the sexes converge in incidence of heart disease. In adult men endogenous estrogens are produced by the adrenals, testes, and adipose tissue. The role of estrogen production and ERs in males has historically been unclear, but testicular response to exogenous estrogens in men includes an increase in Leydig cell number, atrophy of seminiferous tubules, and hyperplasia of rete testes (Ciocca and Roig, 1995). Steroidogenesis and proper development of Leydig cells are impaired in rats treated with exogenous E_2 (Abney, 1999). ERs are present in all fetal reproductive organs in

male mice. Furthermore, ER_{α} is present in seminal vesicles, epididymis, and nonhyperplastic prostate in men.

The blood concentration of estrogens varies throughout the life of the human female. Estrogen levels are less than 10 pM in prepubescent girls (Bay et al., 2004), and increase throughout puberty as pituitary hormones stimulate production in the ovaries (Apter, 1997). In the adult premenopausal woman, plasma estrogen levels fluctuate around 0.3-2.6 nM. After menopause, estrogen levels decrease to levels below 0.1 nM (Setiawan et al., 2006). Estrogens induce many physiological changes in the mammalian female reproductive tract and mammary glands. The effects of estrogens on mammalian female physiology have been studied extensively in the ovariectomized (OVX) adult mouse model, and such findings are outlined in this section as they relate to human physiology.

At the cellular level, estrogen promotes cell proliferation and inhibits apoptosis in the uterus and vagina (Berman et al., 1998). Epithelial cell layers regress and/or decrease in height after ovariectomy, while estrogen treatment of OVX mice increases uterine epithelial height and vaginal epithelial thickness after a few days of estrogen treatment (Suzuki et al., 1996). In the uterus, the columnar epithelial cells become taller with a concomitant increase in cytoplasmic volume. The epithelial cells proliferate in response to estrogen causing overcrowding and a pseudostratified appearance. In the vagina, the single squamous epithelial layer of the OVX mouse becomes a multi-cell layer after estrogen treatment.

If there is no pregnancy, estrogen and progesterone withdrawal initiates the human endometrium into a degenerative phase, ending in menstruation (Brosens and Gellersen, 2006). In the OVX mouse model, the uteri and vagina regress after

ovariectomy while estrogen treatment increases their wet weight in as few as 3 days of treatment (Gordon et al., 1986). This uterotrophic effect has been used as the standard bioassay for testing estrogenic action of compounds (Odum et al., 1997).

Fluctuations in estrogen levels are encountered in various phases and intervention related to a woman's reproductive life, including within an ovulatory menstrual cycle, postpartum, during lactation, and periods of unpredictable ovulation (puberty, perimenopause). Sudden estrogen withdrawal, fluctuating estrogen, and sustained estrogen deficit may induce menopausal symptoms in women (Arpels, 1996).

1.5 Phytoestrogens

Phytoestrogens are defined by the British Working Group on Phytoestrogens of the Committee of Toxicity of Chemicals in Food, Consumer Products and the Environment of the Food Standards Agency (FSA, 2003) as any plant substance or metabolite that induces biological responses in vertebrates and can mimic or modulate the actions of endogenous estrogens usually binding to estrogen receptors (Bakker, 2004). There are four main groups of phytoestrogens: isoflavones (genistein, daidzein, glicetin, formononetin, biochanin A and equol, an isoflavone metabolite), flavones (quercetin and campherol), coumestans (cumestrol), and lignans (enterolactone, enterodiol) (Strauss et al., 1999).

Phytoestrogens are non-steroidal compounds present in plants. These compounds are structurally similar to natural estrogens, such as 17β-estradiol, allowing them to bind with estrogen receptors and thereby to induce biologically detectable effects (Navarro, 2005). Their name comes from the roots phyto = plant +

oestrous = estrous + gen = to generate. Although phytoestrogens can mimic the effects of estradiol (E_2), their effects are not necessarily identical, since phytoestrogen can produce estrogenic or antiestrogenic effects (Yildiz, 2005). These compounds compete with endogenous steroids, so the balance between estrogenic and antiestrogenic activity is determined by the phytoestrogen-estrogen ratio. This might explain why estrogenic effects of phytoestrogens predominate in livestock, whose estradiol plasma concentrations are relatively low (15 pg/mL). In comparison, antiestrogenic effects are reported mainly in humans, in which estrogen plasma levels are relatively high (50-400 pg/mL; Adlercreutz et al., 1991).

When comparing the chemical structures of phytoestrogens to 17β-Estradiol, it can be seen the key structural elements that enable phytoestrogens to bind with estrogen receptors and display estradiol-like effects. These are the phenolic ring and the distance between two hydroxyl groups. The chemical structures of 17β-Estradiol and those of some phytoestrogens are shown in Figure 1.6.

Phytoestrogens exert their effects via multiple mechanisms, they interact with both ER_α and ER_β, thereby inducing weak estrogenic or antiestrogenic actions (Mitchell et al., 2001). The key structural elements that enable phytoestrogens to bind with high affinity to estrogen receptors and display estradiol-like effects are 1) the phenolic ring that is indispensable for binding to the estrogen receptor 2) the ring of isoflavones mimicking a ring of estrogens at the receptor binding site 3) low molecular weight, similar to estrogens (MW = 272) 4) the distance between two hydroxyl groups in the isoflavone nucleus is similar to that in estradiol and optimal hydroxylation pattern (Yildiz, 2005).

Phytoestrogens have lower affinity for estrogen receptors (ER) than estradiol (E_2), and most of them exhibit a higher affinity for ER_β than for ER_α (Turner et al., 2007) by approximately 30 fold. Other phytoestrogens, such as resveratrol, bind to ER_β and ER_α with comparable affinity, but with a 7,000-fold lower activity than E_2. In comparison, E_2 recruits the co-regulators of both types of receptors in a non - selective way (An et al., 2001). The ligand-receptor complex generated is capable of inducing transcriptional activity (Kuiper et al., 1998) (Figure 1.7).

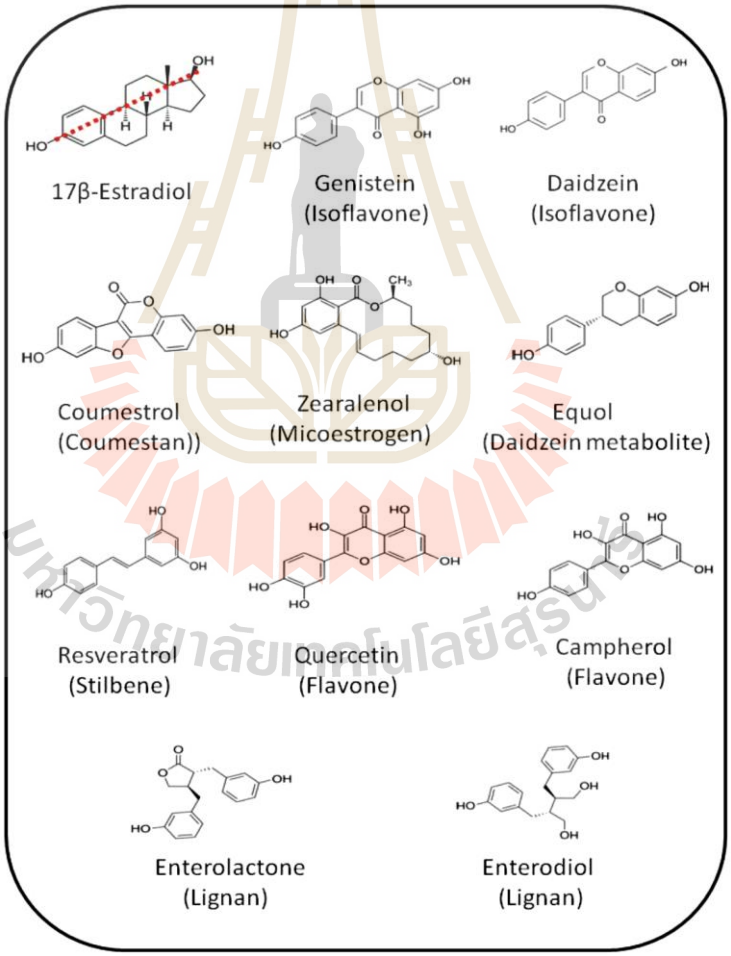

Figure 1.6 17β-Estradiol and some examples of phytoestrogens (Retana-Mairquez et al., 2012).

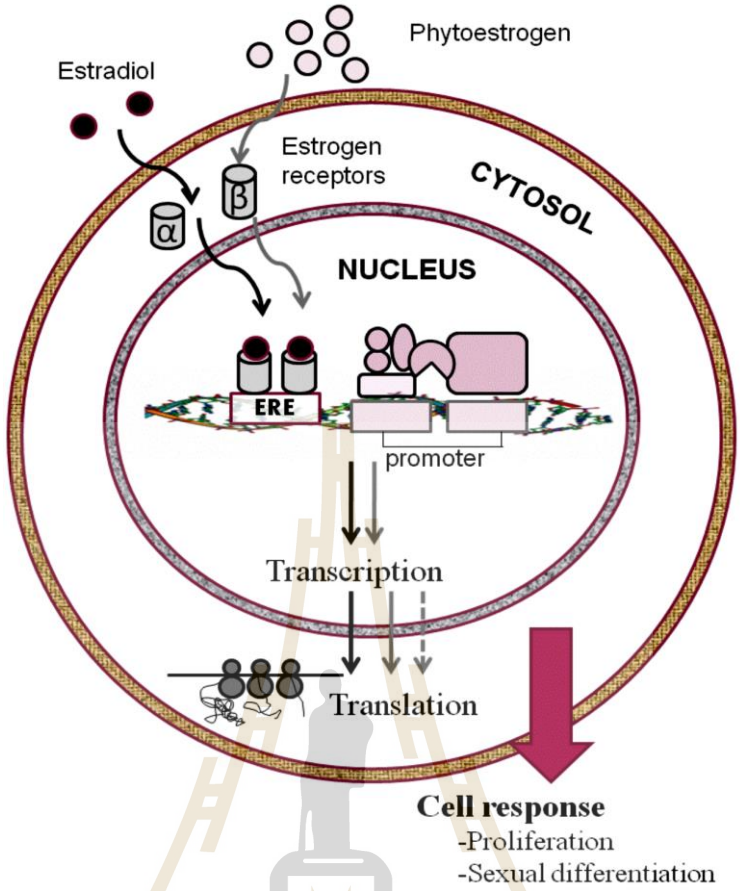

Figure 1.7 Phytoestrogens have weaker estrogenic activity than estradiol. They exhibit higher affinity for ER_β than for ER_α but with a lower activity than E_2. ERE = estrogen response elements. Black lines: estradiol mechanism of action. Grey solid and discontinuous lines: phytoestrogens mechanisms of action (Retana-Mairquez et al., 2012).

Global consumption of phytoestrogens has increased both in animals and human beings due to the augmented use of legumes in animal diets and the increase of vegetarian diets in some human populations (Patisaul et al., 2010). Even though the general opinion and that of clinicians toward phytoestrogens is mainly positive, many phytoestrogens are now recognized as endocrine disruptor compounds (EDCs). EDCs

are natural or synthetic compounds that may alter hormonal function by numerous mechanisms, including: 1) direct stimulation or inhibition of the endocrine system; 2) mimicking or blocking the body response to endogenous steroid hormones; or 3) altering the biosynthesis, secretion, transportation, binding, action, degradation or elimination of endogenous hormones that are responsible for the maintenance of homeostasis, reproduction, development and/or behavior (Phillips and Tanphaichitr, 2008).

Although, HRT is less effective in treating various menopausal symptoms and may actually increase the risk of cancer; the use of phytoestrogens derived herbs cannot be blindly advocated due to the incomplete understanding and insufficient evidence regarding their potential health effects. Thus, developing an understanding of the effects of phytoestrogens from plants as opposed to human estrogens also holds great promise for further research. The extent to which any of these specific mechanisms are involved in mediating the physiological actions of novel phytoestrogen is still requiring considerable study to develop effective biomedical understanding and therapeutic application.

1.6 Moringa plant

The plant family Moringaceae consists of 12-14 species belonging to only one genus, Moringa. Almost all Moringa species are native to India, from where they have been introduced into several countries of the tropics. Moringa is an important food commodity as all plant parts such as leaves, flowers, fruits, and immature pods can be used as a highly nutritive vegetable. These are commonly consumed in India,

Pakistan, Philippines, Thailand, Hawaii, and many parts of Africa (Siddhuraju and Becker, 2003; Abdulkarim et al., 2005).

In addition, moringa is believed to have multiple medicinal qualities. For example, the barks, roots, leaves and flowers of moringa tree are used in traditional medicine and folk remedies in many countries (Anwar et al., 2007; Singh and Kumar, 1999). The seeds of moringa are one of the best natural coagulants, possess antimicrobial properties and are also effectively utilized for treatment and purification of highly turbid water (Mormitsu et al., 2000). The seeds also contain high quality oil that can be used in cooking, cosmetics and lubrication (Abdulkarim et al., 2005).

Almost all the parts of this plant: root, bark, gum, leaf, fruit (pods), flowers, seed and seed oil have been used for various ailments in the indigenous medicine of South Asia, including the treatment of inflammation and infectious diseases along with cardiovascular, gastrointestinal, hematological and hepatorenal disorders (Singh and Kumar, 1999; Anwar et al., 2007). The healing properties of moringa oil, have been documented by ancient cultures. Moringa oil has tremendous cosmetic value and is used in body and hair care as a moisturizer and skin conditioner. Moringa oil has been used in skin preparations and ointments since Egyptian times (Ramachandran et al., 1980; Mishra et al., 2011).

The characteristics of moringa oil can be highly desirable especially with the current trend of replacing polyunsaturated vegetable oils with those containing high amounts of monounsaturated acids (Corbett, 2003). The composition of the sterols of moringa oil mainly consists of campesterol, stigmasterol, β-sitosterol, Δ5-avenasterol and clerosterol accompanied by minute amounts of 24-methylenecholesterol, Δ7-campestanol, stigmastanol and 28-isoavenasterol (Tsaknis et al., 1999; Anwar and

Bhanger, 2003; Anwar et al., 2005). Previous studies on *Moringa oleifera* have been focused on its medicinal uses and nutritional aspects of the tree parts; however, little or no studies have been done on the oil properties. Therefore, in this thesis some physiological and biochemical properties such as uterotonic effects and estrogenic activities using female Wistar rats as animal model were determined.

1.7 Aims

The effects and mechanisms of *Moringa oleifera* seed oil (MSO) on menopause using laboratory animal models. Thus the aims of this thesis were therefore to evaluate:

1) the bioactive compounds in MSO by GC-MS method

2) the effects of MSO on uterine contraction using *in vitro* assays

3) the estrogenic properties of MSO on reproductive tissue in ovariectomized (OVX) rats

4) the effects of MSO on lipid profiles, bone biomarkers, and reproductive hormones in OVX rats

5) the effects of MSO on bone mineral density (BMD) and bone mineral content (BMC) in OVX rats.

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CHARPTER II

GENERAL MATERIALS AND METHODS

The aim of this thesis was to investigate the estrogen-like effects of *M. oleifera* seed oil on prevention or relief menopausal symptoms using ovariectomized (OVX) rat model. The study was conducted in both the Reproductive Physiology Laboratory, Suranaree University of Technology and Primate Research Unit, Department of Biology, Faculty of Science, Chulalongkorn University. This chapter describes the general details of major mater materials and methods used in this research. More details of the study will appear in each chapter of the thesis.

2.1 Chemicals

All the chemicals, reagents, and solvents used in the assay protocols were of analytical grade. They were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA) and Merck Ltd (Darmstadt, D-64271, Germany). All stock solutions were prepared and stored in accordance with the guideline of the protocol.

2.2 Preparation of plant materials

The moringa samples were collected from Lopburi province, Thailand. The specimens were identified and authenticated by the Royal Forest Department, Bangkok, Thailand. The plant samples were deposited at the herbarium with reference

number BKF186485. The seed dried samples were extracted by cold pressed method (detailed in Chapter III) and kept at 4°C till used for the assay.

2.3 Preparation of experimental animals

2.3.1 Selection of animals and housing

Adult female Wistar rats (weighing 200-250 g) were used. They were purchased from the Animal House, Suranaree University of Technology, Nakhon Ratchasima Thailand. All animals were housed in an environmentally controlled animal care with constant temperature (25 ± 0.5°C), humidity (45-50%) and light dark (12:12) cycled. They were fed with a standard laboratory rat diet contained 0.8% calcium (CP. Co. Ltd, Thailand), and supplied *ad libitum* drinking water.

2.3.2 Procedure for ovariectomy

The adult female Wistar rats were weighed and subjected to complete bilateral ovariectomy following the method used by Shih et al. (2001). Briefly, animals were exposed to light anesthesia with 40 mg/kg body weight sodium pentobarbital (given intraperitoneally) with spontaneous ventilation. A small incision was made on the dorsal side just above and parallel to the lumbrical vertebrae. The ovary from both sides beneath the fat pads was removed one at a time and the incision was stitched immediately. To perform the sham operation, ovaries were exteriorized and replaced to create a stress similar to that obtained by a bilateral ovariectomy. Rats were orally administered the antibiotic (amoxicillin 250 mg/kg BW/day) for 5 days.

2.3.3 Animal ethics

Care and use of animals in this thesis followed the Guide for the Care and Use of Laboratory Animals, the National Research Council of Thailand. All the

experiments in this thesis were conducted in accordance with the advice of the Institutional Animal Care and Use Committee, Suranaree University of Technology (SUT), Thailand.

2.4 Experimental design

The following described experimental design that involved in the studying of estrogen-like effects of *M. oleifera* seed oil (MSO) on prevention or relief menopausal symptoms using OVX rat model. The experiments were carried out in both *in vitro* and *in vivo* which were divided into major five parts as follows:

Part 1 was designed to examine phytochemical screening of MSO using standard chemical detection methods and GC-MS (detailed in Chapter III).

Part 2 was designed to determine the stimulation/relaxation effects of MSO on non-pregnant rat uterus using the tissue organ bath system (detailed in Chapter IV).

Part 3 was designed to evaluate estrogenic activity of MSO by evaluating uterine weight measurements, vaginal cornification, qualitative morphologic analysis using hematoxylin and eosin staining of uterine sections (detailed in Chapter IV).

Part 4 was designed to elucidate the effect of MSO on lipid profile (Total cholesterol, triglyceride, high density lipoprotein and low density lipoprotein), some biochemical data of blood and urine, and female reproductive hormone levels in OVX rats (detailed in Chapter VI).

Part 5 was designed to study the effect of MSO on bone properties using peripheral Quantitative Computed Tomography (pQCT) (detailed in Chapter VII).

It should be noted that part 3-5 were performed in OVX rats that assigned to two different series, the first group is preventive study and the second group is

recovery study. Each experiment series consisted of five sub-groups similarly as follows.

Group 1: Sham-operated control rats receiving vehicle (1% Tween 80 in water).

Group 2: Ovariectomized rats receiving vehicle (1% Tween 80 in water).

Group 3: Ovariectomized rats receiving MSO 0.25 mL/100g BW/day.

Group 4: Ovariectomized rats receiving MSO 0.50 mL/100g BW/day.

Group 5: Ovariectomized rats receiving 17β-estradiol 10 µg/kg BW/day.

In the administration process, preventive protocol study started on day 3 after ovariectomy, while in the recovering protocol one, it started on day 60 after ovariectomy. Overall of the procedures are shown in Figure 2.1.

2.5 *In vitro* study

2.5.1 Phytochemical screening

MSO extracts were qualitatively screened by different chemical tests for the identifying the basic chemical constituents present in the extract. The standard chemical tests for presence of alkaloids, tannins, flavonoids, saponins, and phytosterols were performed to get a preliminary idea of the chemical constituents (Thimmaiah, 2004; Harborne, 2007; Trease, 2008; Muthuboopathi, 2013; Goyal, 2014). Moreover, MSO were analyzed for the constituent by GC-MS (Thanamool et al., 2013). The details of each method can be found in Chapter III.

2.5.2 Measurements of uterine contraction

Uteri were removed from rats and cut into longitudinal strips. Muscle strips of 5 mm long were sliced from the uterine horns and mounted vertically in an organ bath

containing Krebs' solution, which was maintained at 37°C. The change in isometric force was measured with Power Lab system (AD Instruments 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, 2002).

2.6 *In vivo* study

2.6.1 Cornification assay

Vaginal smear samples were collected between 8.00 AM to 10.00 AM by inserting the tip of a medicine dropper into the vagina, flushing saline in and out and placing the fluid onto microscope slides and stained with suitable dye. The vaginal epithelium cells were observed and classified under a light microscope at a 20x magnification. The vaginal smears were classified by their cytology (Malaivijijnond et al., 2006; Parhizkar et al., 2011). In this study, the appearance of cornified cells (or the majority of cornified cells) were used as an indicator of estrogenic activity.

2.6.2 Assay for serum and urine biochemistry

Total serum cholesterol, high density lipoprotein cholesterol (HDL-C), low density lipoprotein cholesterol (LDL-C) and triglyceride concentration in serum were measured using diagnostic kits (Raichem). The measurements of lipid profiles were made using Reflotron; Roch Diagnostics GmbH (Promprom et al., 2010).

The levels of serum calcium (S-Ca), serum phosphorus (S-P) and serum alkaline phosphatase (ALP) activity were measured on an automatic analyzer (Ciba-Corning 550, USA) using a diagnosticreagent kit for the *in vitro* determination. The urine calcium (U-Ca), phosphorus (U-P) and creatinine (Cr) concentrations were analyzed by the same method as the serum samples (Zhang et al., 2012).

The levels of 17β-estradiol (E_2) and luteinizing hormone (LH) were analyzed by the Electrochemiluminescence rat immunoassay (ECLIA) on Elecsys and cobas e immunoassay analyzers (Roche Diagnostics, USA). Assay procedures were followed the instructions supplied by manufacturer (Lijuan et al., 2011).

2.6.3 Evaluation of bone quality

Bone mineral density and content were measured using pQCT in the research M mode (XCT Research SA^+, Stratec Medizintechnik GmbH, Germany). This instrument was supported by Primate Research Unit, Department of Biology, Faculty of Science, Chulalongkorn University. The determination was performed in each bone slice using XCT-5.50E software (Stratec Medizintechnik GmbH, Germany).

2.6.4 Histological examination

The horns of rat uteri and mammary tissue were cut into short segments and using the paraffin section method. Sections of 5 μm thickness were cut and stained using routine hematoxylin and eosin (H & E) method. Briefly, uterine strips were dehydrated in an ascending ethanol series (75%, 85%, 95%, and 100% 1 hr. each) and then removed the sections into pure xylene for 2 min. The sections were embedded with xylene:paraplast (3:1 and 1:1, 15 min each) and then followed by the pure paraplast for 1 hr. Post embedded tissues were cut approximately 5 μm with microtome and these sections were moved into bath for incubation (60°C). The tissues were mounted on slides in the slide warmer at 60°C and then immersed into pure xylene two times (5 min 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 were immersed into 95% ethanol

(2-3 min) and 100% ethanol (1 min), pure xylene (5 min) and then covered with cover slip after xylene clearing for light microscopic (LM) study.

2.7 Statistical analyses

Statistical analyses were performed to detect differences among groups for serum and urine levels of each parameter. Differences among groups were determined by one-way ANOVA followed by the Tukey's *post-hoc* test (SigmaStat: Systat Software Inc, Version 3.5, Chicago, IL, USA).

For estrogenic activity evaluation experiments, the statistical analysis of differences between groups were performed using one way ANOVA (The SPSS, a statistical analysis program, version 17).

For tension measurements, all data were expressed as percentage of control of contraction (i.e. the control is 100%). Measured parameters included maximum tension developed in each contraction, duration of contraction and frequency. All data were evaluated using Microcal Origin Software and the differences between control and treatment groups were analyzed by student *t*-test. Probability values of less than 0.05 or 0.01 ($P < 0.05$, $P < 0.01$) were considered statistically significant.

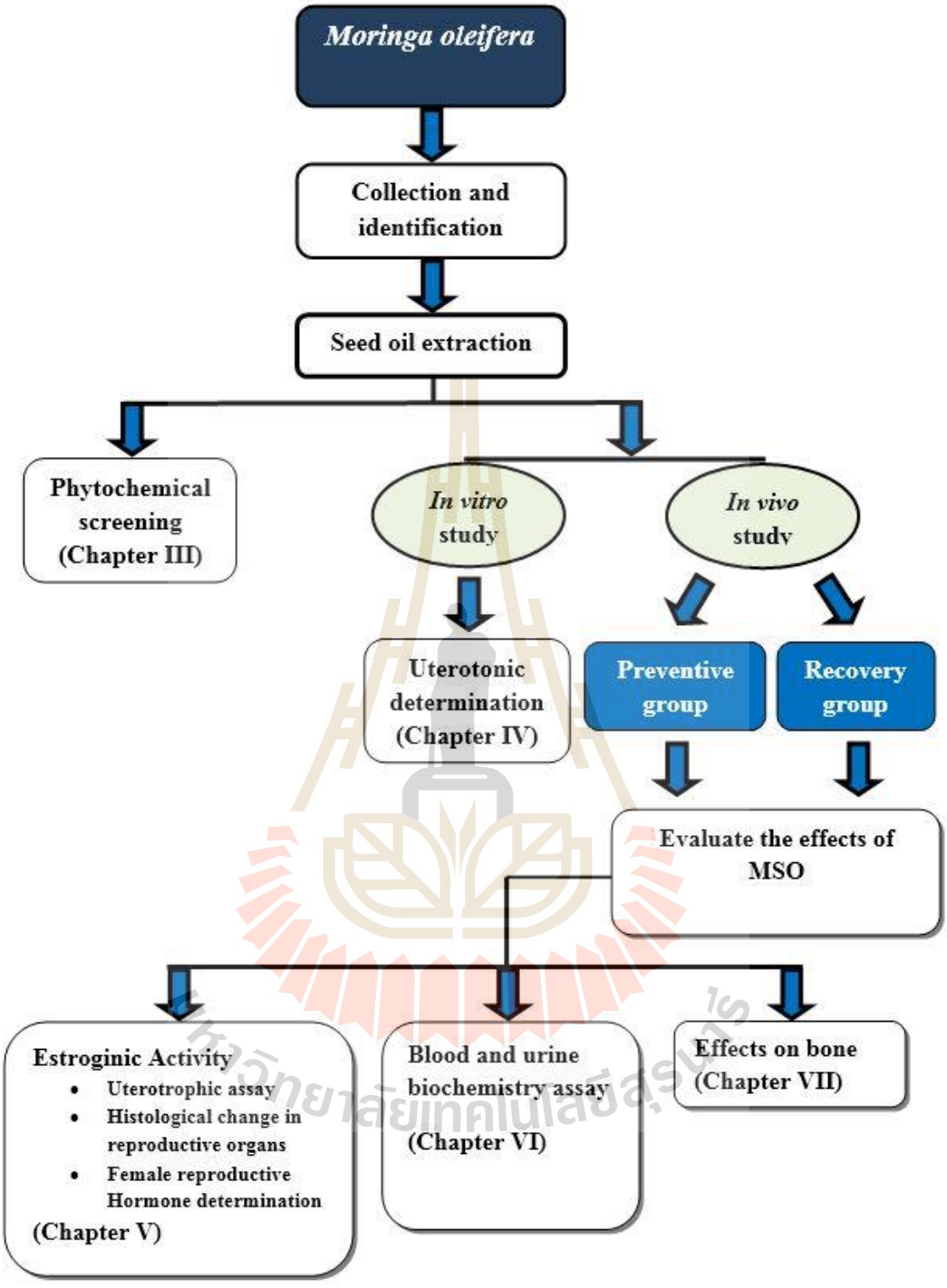

Figure 2.1 Scientific approaches.

2.8 References

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CHAPTER III

SCREENING OF BIOACTIVE COMPOUNDS IN *MORINGA OLEIFERRA* LAM. SEED OIL

The exploratory search for pharmacological activities in medicinal plants will continue to be an important step in the development of novel compounds from natural products. In the present chapter, the bioactive constituents of MSO that were related to medicinal properties were determined.

3.1 Abstract

Moringa oleifera Lam. (syn. *M. pterygosperma* Gaertn.) is a highly valued plant, distributed in many countries of the tropics and subtropics. Various parts of this plant are reported to the treatment of different ailments in the indigenous system of medicine, particularly in South Asia. The oil from *M. oleifera* seeds was extracted using cold press extraction and the chemical compositions were determined using gas chromatography and mass spectrometry (GC-MS) detector. Results showed that oil yield was 7.6% (v/w). A total of 17 compounds were found containing 16 known and 1 unknown compounds. The important substances found in the MSO could be divided into three groups as follows: 1) sterols including β-sitosterol, stigmasterol, campesterol, stigmast-4-en-3-oneand cholest-5-en-3-ol; 2) fatty acids including seven saturated fatty acids (cis-9-hexanoic acid, n-hexanoic acid, 6-octanoic acid, cis-13-octadecanoic acid, 9-octadecanoic acid, stearic acid and 2-hydroxyethyl ester) and

three unsaturated fatty acids (vaccinic acid, oleic acid, and oleamide) and 3) α-tocopherol. The compounds found in the present study were similar to those found in the previous study indicating that the oil from *M. oleifera* can be used as potential sources of various substances with potential medicinal value.

3.2 Introduction

Moringa is known as a miracle tree worldwide. There are about 13 species belonging to family Moringaceae consist of *M. oleifera*, *M. arborea*, *M. borziana*, *M. concanensis*, *M. drouhardii*, *M. hildebrandtii*, *M. longituba*, *M. ovalifolia*, *M. peregrine*, *M. pygmaea*, *M. rivae*, *M. ruspoliana*, *and M. stenopetala.* The most widely known species *M. oleifera* is referred to as the "drumstick tree" or the "horse radish tree" (Anwar and Bhanger, 2003). In Thailand, *M. oleifera* is locally known as "Ma-rum" and is grown and cultivated all over the country.

In nutritional and medicinal view, almost every part of plant has value for food. The leaves, flowers, pods and seeds are consumed by humans as nutritious vegetables in some countries (Makkar and Becker, 1997). These are commonly consumed in India, Pakistan, Philippines, Thailand, Hawaii, and many parts of Africa (Anwar et al., 2003; Siddhuraju et al., 2003). A number of medicinal properties attributed to different parts of *Moringa* have been recognized by both Ayurvedic and Unani systems of medicines (Mughal et al., 1999).

The various parts of this tree have been reviewed in the past by many research groups. One of the earliest reports on the possible phytochemical constituents of *M. oleifera* was made by Kerharo (1969) that reported the stem bark contains two alkaloids, namely moringine and moringinine. Additional some investigations found

quercetin, kaempherol, rhamnetin, isoquercitrin and kaempferitrin from ethanolic extract of *M. oleifera* flower (Faizi et al., 1994; Ruckmani et al., 1998; Siddhuraju and Becker, 2003). Furthermore, studied by Tsaknis et al. (1999) also indicated that phytosterols are the dominant component of *M. oleifera* seed oil. Recently, Zhang and colleagues (2011) demonstrated that two carotenoids, epicatechin and o-coumaric acidare the chief bioactive compounds in leaves extracts of *Moringa* responsible for its antioxidant activity.

Until now, a full characterization of the oil produced from the seeds of *M. oleifera* has not been many reported. In the present study, phytochemical screening method and high performance liquid chromatography were used to examine the dominant compound in cold press oil of *Moringa* seeds. Not only to define chemical composition in this plant, but the data obtained from this study also provided fundamental information regarding the phytochemical constituents of oil from the seeds of *M. oleifera*, thus promotes their potential medicinal applications.

3.3 Materials and methods

3.3.1 Collection and authentication of plant

The moringa plant was collected in 2012 from Lopburi Province, Thailand. Completed leave flower and pod of *M. oleifera* were used for herbarium specimen preparation. The genus and species of *M. oleifera* were confirmed by the botanists at The Royal Forest Department, Bangkok, Thailand and specimen deposited for future references. Particularly, the seeds of *M. oleifera* were removed from the pods, washed in distilled water and dried in an open space. The drying procedures were carried out with care to prevent contamination from extraneous substances.

3.3.2 Cold press oil extraction

The total of 10 kg of the dried seeds that completed were used for oil produced by cold press method (Babatunde et al., 2011). The seeds were mechanical extraction by a simple hydraulic hand press (Friend energy limited partnership, Thailand) with a max pressure of 300 kg/cm^2, temperature below 60°C. The oil obtained was filtered and the yield of oil extracted was determined by volume per kg dried seed weight. The oil was kept in bottles under refrigeration (0-4°C) for further analysis. The cold press moringa seed oil was named as MSO throughout the thesis.

3.3.3 An overviewr of phytochemistry of *M. oleifera*

In this review, bibliographic investigation was carried out during April 2012-May 2014, by analyzing classical text books, peer reviewed papers, and scientific databases. Peer reviewed articles were gathered consulting the databases ProQuest Medical Library, ProQuest Dissertations & Theses Global, UpToDate, SCOPUS, PUBMED and Google scholar.

3.3.4 Determination of chemical constituents

Phytochemical examinations were carried out for the extracts using standard methods as follows.

3.3.4.1 Preliminary phytochemical screening

The presence of various plant constituents in the MSO was determined by preliminary phytochemical screening with the chemical standard methods as following (Thimmaiah, 2004; Harborne, 2007; Trease, 2008; Muthuboopathi, 2013; Thanamool et al., 2013).

***Detection of alkaloids*:** The qualitative detection of alkaloids was detected by Mayer's test with Mayer's reagent (Potassium Mercuric Iodide). Briefly,

the extracts were dissolved individually in dilute hydrochloric acid and filtered and the filtrates were treated with Mayer's reagent. Formation of a yellow colored precipitate indicates the presence of alkaloids.

***Detection of tannins*:** Gelatin solution (1%) containing sodium chloride was added to the extracts. Formation of white precipitate indicates the presence of tannins.

***Detection of flavonoids*:** Extracts were treated with a few drops of sodium hydroxide solution. Formation of intense yellow color, which becomes colorless on the addition of dilute acid, indicates the presence of flavonoids.

***Detection of saponins*:** Extracts were diluted with distilled water to 20 mL and this was shaken in a graduated cylinder for 15 min. Formation of 1 cm layer of foam indicates the presence of saponins.

***Detection of phytosterols*:** Extracts were treated with chloroform and filtered. The filtrates were treated with a few drops of conc. sulphuric acid, shaken and allowed to stand. Appearance of golden yellow color indicates the presence of triterpenes.

3.3.4.2 Gas chromatography coupled with a mass spectrometer (GC-MS) analysis

GC-MS is the primary method for phytoestrogen analysis, which involved extensive purification procedures for either plants or physiological samples before analysis (Wang et al., 2002). The determination of the chemical composition was carried out using a modified of the method described by Carpenter (1979) and Lalas et al. (2002). MSO were analyzed for the constituent by GC-MS. A Hewlett-Packed 6890 gas chromatograph, coupled with a Hewlett-Packed 5973N mass

spectrometer were used for the establishment of fingerprint of MSO. The separation were performed on HP-5MS column, 0.25 mmi.d. x 30 m, 0.25 µm coating thickness. The temperature of column programmed from 150-230°C at 6°C min^{-1}, 257-278°C at 10°C and 278-300°C at 50°C min^{-1} and 70°C min^{-1}, respectively. The injector temperature and the detector temperature were 300°C. Helium were used as carrier gas with a constant flow rate of 1.0 ml min^{-1}. The mass spectrometer were operated at 70 eV, scan range 20-450 amu. All separated compounds were identified from the recorded mass spectra by comparison with the mass spectra from the NIST and Wiley Libraries.

3.3.5 Reagents

All of the reagents (analytical and HPLC grade) were obtained from Sigma Chemicals Company Co. (St. Louis, MO, U.S.A), and the standard solutions for the determination of tocopherols were purchased from Merck Ltd. (Darmstadt, Germany), and sterols standards from Larodan AB (Malmo, Sweden).

3.4 Results

3.4.1 Plant identification

The plant specimens were correctly identified and authenticated by botanists at The Royal Forest Department, Bangkok, Thailand. Herbarium specimens were also deposited with reference number BKF 186485 as detailed below.

Taxonomic classification

Kingdom : Plantae

Division : Magnoliophyta

Class : Magnoliopsida

Order : Capparales

Family : Moringaceae

Genus : *Moringa*

Species : *Oleifera*

Botanical description

M. oleifera is a small, fast-growing evergreen or deciduous tree that usually grows as high as 9 m, with a soft and white wood and corky and gummy bark. The details of each part of the plant as follows (Negi, 1977; Ramachandran et al., 1980; Palanisamy et al., 1985; Mishra et al., 2011 and Pandey et al., 2011).

The leaves are longitudinally cracked, 30-75 cm long main axis and its branch jointed, glandular at joints, leaflets are glabrous and entire. The leaflets are finely hairy, green and almost hairless on the upper surface, paler and hairless beneath, with red-tinged mid-veins, with entire (not toothed) margins, and are rounded or blunt-pointed at the apex and short-pointed at the base (Figure 3.1A).

The flower of *Moringa* is yellowish-white in colour, slender, with hairy stalks in spreading or dropping auxiliary clusters (Panicle). The flower is scented, bisexual and measures about 10-25 cm long. Individual flowers are set in a basal cup (Hypanthum) that is 3 mm long and are approximately 0.7 to 1 cm long and 2 cm broad with five unequal yellowish-white, thinly veined, spatulate petals, five stamens with five smaller sterile stamens (Staminode) and a pistil composed of one celled ovary and slender style (Figure 3.1B).

The pods are pendulous linear three-sided with nine longitudinal ridges, usually 20-50 cm long, but seldom up to 1m or longer and 2.0 to 5.0 cm broad. Each

pod usually contains up to 26 seeds which are dark green during their development and take up to 3 months to mature after flowering (Figure 3.1C).

The seeds of moringa measure about 1 cm in diameter with three whitish papery wings on the angles. Seed weight differ among varieties ranging from 3000 to 9000 seeds per kg and about 270 g to 300 g for 1000 seeds (Figure 3.1D).

Figure 3.1 Morphology of *M. oleifera* (A) leaves (B) flower (C) pod and (D) seed.

3.4.2 The yield of MSO

The extracted oil was liquid at room temperature. The yield of the oil extract was 7.6% (v/w) of dried seeds material. MSO was yellow-brown in colour and hygroscopic when left outside the freezer for a long time. The extract was not

completely soluble in water at very high concentrations. However, it was soluble in standard solvents such as ethanol, methanol, hexane and tween 80.

3.4.3 Previous reports of phytochemicals from *M. oleifera*

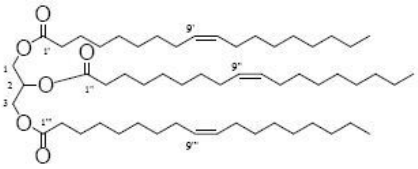
Based on the previous studies, many articles are reviewed for searching phytochemical constituents of this plant. The evidences show that each parts of *M. oleifera* (leaves, flower, pod, seed and root) contains active compounds that relevant to pharmacological and medicinal used. The detail has also been summarizes as below (Table 3.1, Figure 3.2).

Table 3.1 Previous reports of chemical compositions from the various parts of *M. oleifera*.

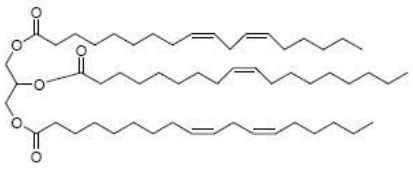
Part of Plant	Method extraction	Major of Phytochemicals	References
Stem bark	Ethanol extract	Moringine, moringinine	Kerharo, 1969
Stem	Ethanol extract	Vanillin, β-sitosterol, β-sitostenone, 4-hydroxymellin and octacosanoic acid	Faizi et al., 1994
Leave	Ethanol extract	Niazirin, niazirinin , quercetin, kaempferol flavonoids, phenolics and carotenoids	Faizi et al., 1994 Makkar and Becker, 1996 Murakami et al., 1998 Siddhuraju and Becker, 2003 Anwar et al., 2005 Zhang et al., 2011
Flower	Ethanol extract	Amino acids, sucrose, D-glucose, alkaloids, quercetin, kaempferat, potassium, calcium, rhamnetin, isoquercitrin and kaempferitrin	Faizi et al., 1994 Ruckmani et al., 1998 Siddhuraju and Becker, 2003

Table 3.1 Previous reports of chemical compositions from the various parts of *M. oleifera* (Continued).

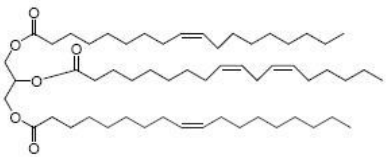
Part of Plant	Method extraction	Major of Phytochemicals	References
Pod	Ethanol extract	Niaziridin, niazirind-galactose, 6-O-Me-D-galactose, D-galacturonicacid, l-arabinose, and l-rhamnose	Shanker et al., 2007 Roy et al., 2007
Seed	Haxane extract, Ethanol extract	4 (α-L-rhamnosyloxy)-benzyl isothiocyanate, Niazimicin, 3-*O*-(6′-*O*-oleoyl-β-D-glucopyranosyl)-β-sitosterol, β-sitosterol-3-*O*-β-D-glucopyranoside, niazirin, β-sitosterol, and glycerol-1-(9-octadecanoate)	Eilert et al., 1981 Villasenor et al., 1989 Guevara et al., 1999 Bennett et al., 2003
Seed oil	Ethanol extract, Haxane extract, cold press	campesterol, stigmasterol, β-sitosterol, Δ5-avenasterol, clerosterol, 24-methylenecholesterol, Δ7-campestanol, stigmastanol and 28-isoavenasterol	Tsaknis et al., 1999 Lalas and Tsaknis, 2002 Anwar and Bhanger, 2003 Anwar et al., 2005 Babatunde et al., 2011
Root	Ethanol extract	4-(alpha-l-rhamnopyranosyloxy)-benzylglucosinolate and benzylglucosinolate.	Bennett et al., 2003



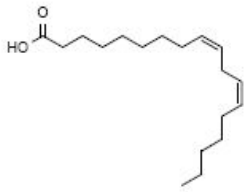
1, 2, 3 - triolein



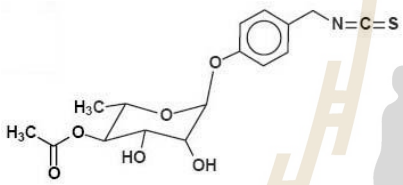
1,3 - dilinoleoyl-2-olein



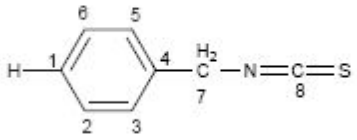
1, 3-dioleoyl-2-linolein



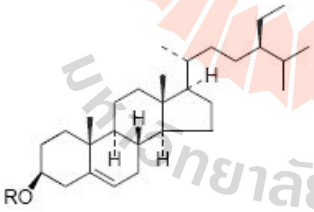
Linoleic acid



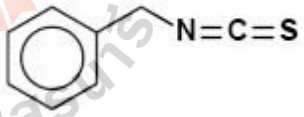
4-(4'-*O*-acetyl-α-L-rhamnopyranosyloxy)benzyl isothiocyanate



Isothiocyanatomethylbenzene

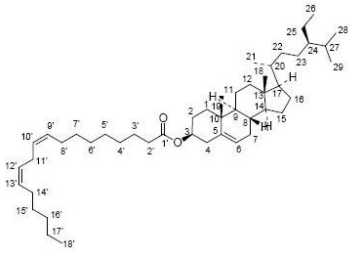


R=H → β-sitosterol

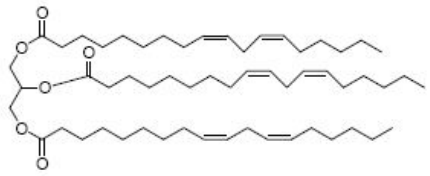


Benzyl isothiocyanate

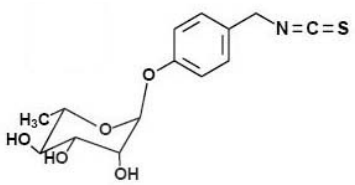
Figure 3.2 Structures of some phytoconstituents isolated from *M. oleifera* (Anwar et al., 2007; Mishra et al., 2011).



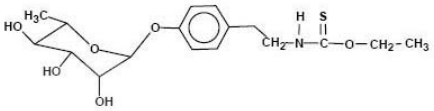
Linoleic sitosteroate



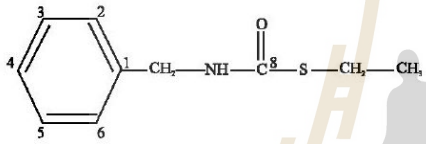
1, 2, 3-trilinolein



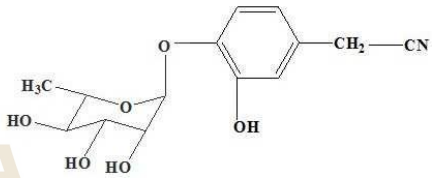
4-(α-L-rhamnopyranosyloxy) benzyl isothiocyanate



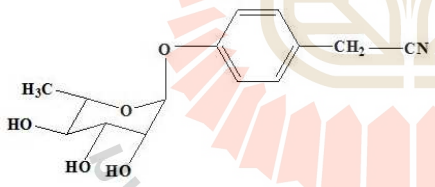
Niazimicin



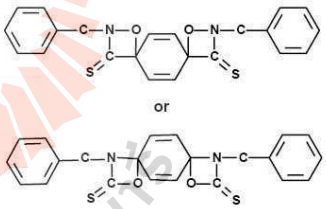
Aglycone of Deoxy-Niazimicine (N-benzyl, S-ethyl thioformate)



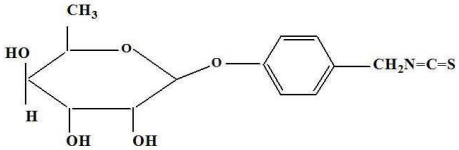
Niaziridin



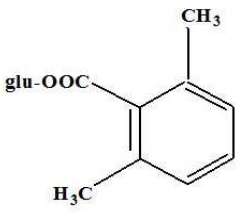
Niazirin



Pterygospermin



4-(α-L-rhamnosyloxy) benzyl isothiocyanate



Moringyne

Figure 3.2 Structures of some phytoconstituents isolated from *M. oleifera* (Anwar et al, 2007 and Mishra et al., 2011) (Continued).

3.4.4 Phytochemical constituents of MSO

Phytochemical screening was conducted in order to identify the chemical nature of active principles especially in the group of phytoestrogens. Preliminary qualitative analysis of the extracts revealed the presence of phytosterols, flavonoids and trace amounts of alkaloids in MSO (Table 3.2).

Table 3.2 Preliminary phytochemical screening of *M. oleifera* seed oil.

No.	Constituent	Results
1	Alkaloids	+
2	Phytosterols	+
3	Saponins	-
4	Tannins	-
5	Flavonoids	+

GC-MS Analysis

Results of the GC-MS analysis of the oils are shown in Table 3.2, where the components are listed in order of their elution from the HP-5MS column. A total of 17 compounds were identified, which composed of 16 known compounds and 1 unknown compound. The substances found in the MSO were sterol groups which were β-sitosterol (3.46%), stigmasterol (1.91%), campesterol (1.90%), stigmast-4-en-3-one (0.32%) and cholest-5-en-3-ol (0.84%). In addition, α-tocopherols and oleic acid were detected to 0.25% and 0.30%, respectively. The details of chemical compositions in MSO are represented in Figure 3.3 and Table 3.3, respectively.

Table 3.3 Percentage composition of the MSO detected by GC-MS.

	Compound name	**Peak area (%)**	**Retention time (min)**
1	Cis-9-Hexanoic acid	0.731	29.943
2	N-Hexanoic acid	7.533	30.459
3	6-Octanoic acid	69.859	34.103
4	Cis-13-Octadecanoic acid	6.136	34.301
5	9-Octadecanoic acid	1.812	34.525
6	Cis-Vacenic acid	0.818	37.046
7	Oleic acid	0.298	37.310
8	9-octadecenamide	2.048	37.400
9	Stearic acid	0.772	40.602
10	2-hydroxyethyl ester	0.954	42.399
11	Stigmast-4-en-3-one	0.320	44.605
12	α-tocopherol	0.251	48.351
13	Campesterol	1.901	49.525
14	Stigmasterol	1.905	49.928
15	β-sitosterol	3.458	50.668
16	Cholest-5-en-3-ol	0.841	50.883
17	Unknown	0.268	52.565

RT = retention time on HP-5 capillary column.

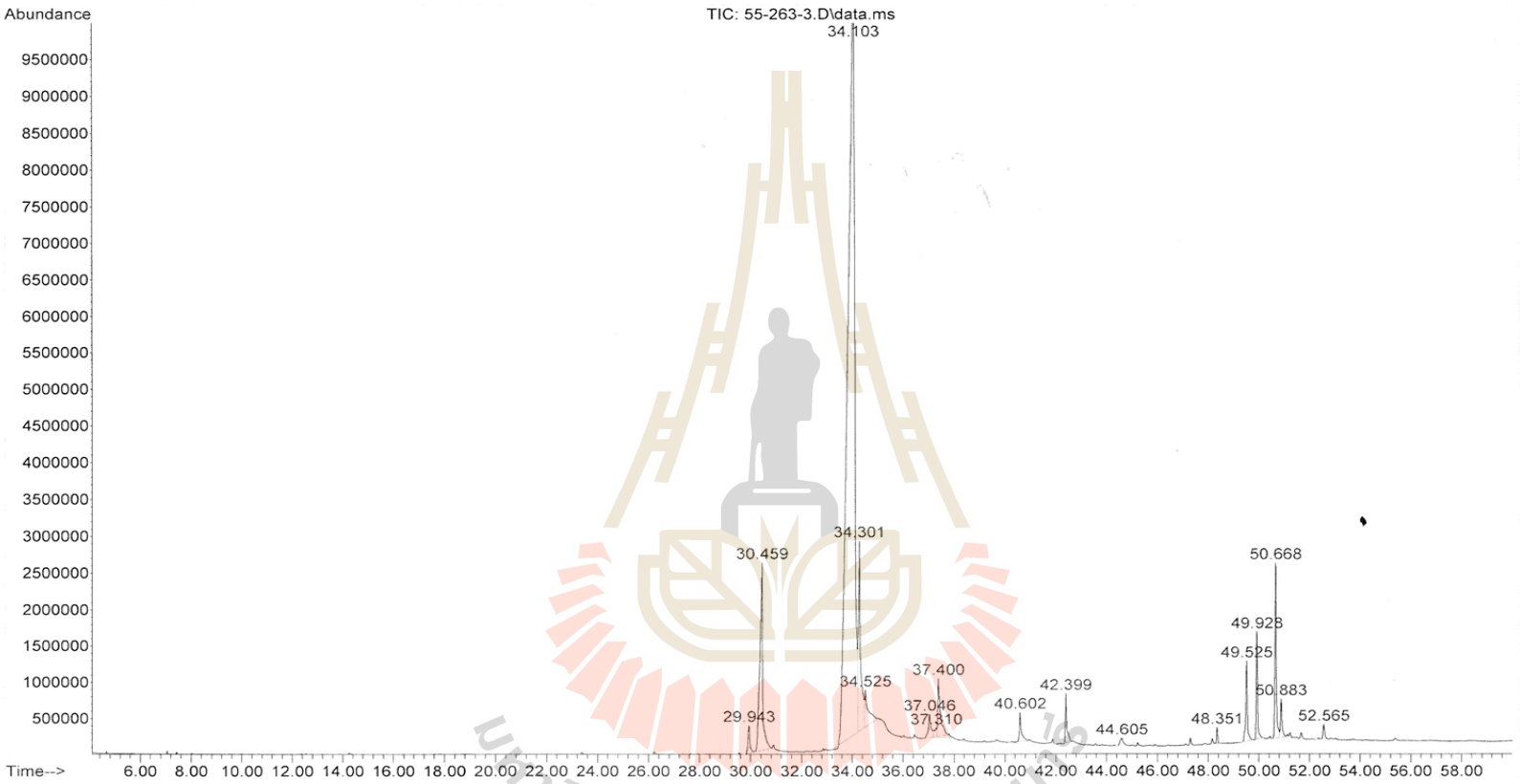


Figure 3.3 GC-MS chromatogram of MSO.

3.5 Discussion

M. leifera is an important medicinal plant which the various parts of the plant have been widely used for human medication. Literature review on this plant clearly explained its various traditional uses as enormously for instance antispasmodic, stimulant, abortifacient, antimicrobial, anti-inflammatory, antioxidant, anticancer, antifertility, hepatoprotective, cardiovascular, antiulcer, analgesic, wound healing, anticonvulsant, antiallergic and anthelmintic activities (Anwar et al., 2007; Mishra et al., 2011; Zhang et al., 2011). Many research attempted to find the important constituents which is beneficial to the development of medicinal and pharmaceutical used in the future.

According to Irvine (1961); Burkill (1985) Abdulkarim et al. (2005) and Ayerza et al. (2011) the yield of mature seed oil extraction was 22-72% (v/w). However, the results from this study show that the yield of the MSO was 7.6% (v/w) lower than previously reports. This may be due to several factors including, the differences in variety of plant, cultivation climate, ripening stage, the harvesting time of the seeds and the extraction method used (Srivastava et al., 2013). Especially, the oil extraction in this study using cold press method that applied a small hydrolic hand press with a capacity of seed input is less than 10 kg per time. It may be possible that the amount of oil extracted from this process was found to be lower than that obtained by solvent extraction and/or enzyme extraction (Abdulkarim et al., 2005). However, the amount of oil extracted at this level is more sufficient to be used in the all of the trial.

Subsequently, when the oil was analyzed with preliminary qualitative phytochemical screening method it revealed the presence of alkaloid, flavanoids and

phytosterols compound, corresponding with the results from the GC-MS detected. The data show three major group of chemicals including:

1) sterols containing campesterol, stigmasterol, β-sitosterol, stigmast-4-en-3-one and cholest-5-en-3-ol which were similar to the values reported for *M. oleifera* oil from Kenya and Pakistan (Tsaknis et al., 1999; Anwar et al., 2005) and to another. Although there are different in the sterols type as proposed by Lalas and Tsaknis (2002) which characterized composition of the *M. oleifera* seeds oil variety Periyakulam 1 from India with gas-liquid chromatography method. They found various sterol such as campesterol, stigmasterol, β-sitosterol, and Δ^5-avenasterol, among which *β*-sitosterol was the most predominant, accompanied with trace or few amounts of cholesterol, brassicasterol, clerosterol, Δ^7-avenasterol, campestanol, 24-methylenecholesterol, ergostadienol, stigmastanol, 28-isoavenasterol, and $\Delta^{7,14}$-stigmastanol. As a result of this any difference may be depend on the process of oil extraction, method and instrument that used to determination of the chemical compositions.

2) fatty acids containing 5 saturated fatty acids and 5 unsaturated fatty acids particular, two *ω*-9 mono-unsaturated acids, [*cis*-9-octadecenoic (oleic acid)] and [9-octadecenamide (oleic acid amide)] one *ω*-7 mono-unsaturated acid (*cis*-11-octadecenoic acid (vaccenic acid). Previous study, Vlahov et al. (2002) also detected these chemicals in *M. oleifera* seed oil by using 13^C NMR spectroscopy method. Other prominent fatty acids in *M. oleifera* seed oil that have been reported include palmitic, stearic, arachidic and behenic acids for the solvent and enzyme-extracted oils, respectively (Abdulkarim et al., 2005). Oils with high amounts of monounsaturated (oleic type) fatty acids are desirable due to an association with

decreased risk of coronary heart disease (Mensink et al., 1990; Aldulkarim et al., 2007). Furthermore, oxidative stability of *M. oleifera* seed oil is higher than in other oils rich in oleic acid, such as high-oleic sunflower, meadowfoam, macadamia, hybrid safflower, safflower, almond and apricot oils (Kleiman et al., 2006).

3) tocopherol containing α-tocopherol while other studies in most vegetable oils contain α -, γ - and δ - tocopherols (Bourgeois and Czornomaz, 1982). Therefore, tocopherols present in *M. oleifera* seed oil were expected to offer some protection during storage and processing (Tsaknis et al., 1999), which possess antioxidant properties (Lalas and Tsaknis, 2002).

It is interesting that MSO as a rich source of various phytochemical might be medicinally important and/or nutritionally valuable. Although, some phytochemicals have been substantially reported the importance. However, the present data indicate that the composition of MSO cultivated in Thailand is similar to those which are reported from Kenya (Tsaknis et al., 1999) Pakistan, India (Lalas et al., 2002; Anwar et al., 2005), and Argentina (Ayerza, 2011). Additional studies with appropriates techniques may be necessary to further characterize and screen the valuable compounds present in the plant to achieve lead molecules in the search of novel herbal drugs. Moreover, there is no evidence showing the effects of MSO which related the female reproduction, at least in part of uterine contraction alteration. In the next experiment, therefore, the effects of this oil on rat uterine contraction will be focused.

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CHAPTER IV

EFFECTS OF *MORINGA OLEIFERA* LAM. SEED OIL ON ISOLATED RAT UTERUS

Various parts of moringa and their active constituents are known to possess diverse biological activity. However, little is known scientifically about the uterotonic potential of *Moringa oleifera* seed oil (MSO). Therefore, the aim of this chapter was to investigate the effects of MSO on uterine contraction using *in vitro* assays.

4.1 Abstract

Moringa oleifera is one of the many plants used in herbal preparations in worldwide including Thailand to relieve problems associated with gynaecological conditions of menstruation and menopause. Nevertheless, the effects of *M. oleifera* seeds oil (MSO) on uterine smooth muscle have not been reported. Thus, this study was to elucidate the effects of MSO and its mode of action on isolated rat uterus. The experiments were carried on organ bath experiments using rat uterine strips in physiological Krebs' solution maintained at pH 7.4, temperature of 37°C and gasses with O_2. It was found that cumulative concentrations of the extract (50-400 µL/ 100 mL) increased tension of uterine spontaneous contractions; dose-dependently ($P < 0.01$) with an EC_{50} value of 93.90 µL/100 mL. To evaluate the mechanisms of MSO, the extracts were compared against drug or standard solutions of oxytocin, high-KCl solution, nifedipine (L-type calcium channel inhibitor), wortmannin

(myosin light chain kinase (MLCK) inhibitor), tetraethylammonium (TEA; calcium-activated potassium channels inhibitor), cyclopiazonic acid (CPA; sarcoplasmic reticulum calcium-ATPase (SERCA) inhibitor), and fulvestrant (estrogen receptor antagonist). Force produced in the presence of MSO was abolished by nifedipine and wortmannin. The oil also potentially activated oxytocin-induced contraction in the absence of external Ca^{2+}. In addition, MSO significantly stimulated the contraction induced by high-KCl solution ($P < 0.01$). MSO induced significant amounts of force in the absence of extracellular calcium, which could be blocked by CPA. Furthermore, the actions of MSO were not blocked by the estrogen receptor blocker, fulvestrant. In conclusion, this study provides the first evidence that MSO possess uterotonic effects. The induction of myometrial contraction may involve non-estrogenic effects and may relate to the inhibition of K^+-channel and SERCA pump and/or increase intracellular $Ca^{2+}[Ca^{2+}]_i$ via L-type Ca^{2+} channel and MLCK activation. In conclusion, this herb may also be used to develop as a natural uterotonic agent on inducing labour and treatment for post-partum haemorrhage.

4.2 Introduction

The WHO recognizes phytomedicine as a valuable and readily available resource for Primary Health Care and it has endorsed their safe and effective use (Tilburt and Kaptchuk, 2008). The use of phytomedicine to relieve problems associated with gynaecological conditions of menstruation and menopause, to support health during pregnancy and to facilitate childbirth is common amongst many traditional cultures. Most often the biological effects elicited by these remedies are due to biomolecules that primarily act on the uterus. The nature of these actions may

involves the modulation uterine contractions at labour, resulting in either the stimulation (uterotonic) or inhibition (tocolytic) of myometrial muscle contractions (Gruber and Brien, 2010).

Abnormal uterine contractions encompass a wide range of clinical conditions, ranging from minor aggravation to life threatening emergencies. Depending on the condition, the primary objective of pharmacological control of uterine activity is either to induce or reduce myometrial contractions (Mitchell, 1998). Based on traditional reports, people are thus increasingly willing to manage their medical needs by using complementary and alternative medicines like herbs. Among the medicinal plants that are commonly used in management of various conditions is *M. oleifera*.

Almost every part of the moringa tree can be used as food, or has some other beneficial property. Among these properties it has been reported that *M. oleifera* contains calcium, amino acids and other chemical components that are known to affect muscle contraction (Ray, 2003; Gilani et al., 2006). The different parts of the tree (leaves, roots, bark, flowers, and seeds) are used in traditional medicine in the various countries where it is found (Anwar et al., 2007). This herb has been reported in the management of cardiovascular disease such as hypertension and in inducing abortion by women in India (Nath et al., 1992; Faizi et al., 1995).

Furthermore, *M. oleifera* are reportedly used for treatment of painful uterus, inducing uterine contractions, management of retained placenta and post partum bleeding among other conditions and have thus been found useful in obstetric care (Kamatenesi-Mugisha, 2002). Traditionally women use herbal preparations for the process of labour. Herbs are also prescribed by traditional birth attendants and healers

for various reasons including increasing chances of having twins, correcting a mal-positioned fetus, anaemia in pregnancy among others (Azriani et al., 2007).

Phytochemicals in *M. oleifera* leaf has been purposed to be a good source of nutrition and naturally organic health supplement that can be used in many therapeutic ways (Fahey, 2005) and rich in biologically active carotenoids, tocopherols and vitamin C that have health-promoting potential in maintaining a balanced diet and preventing free-radical damage. In addition, moringa leaves are also considered a rich source of minerals, polyphenols, flavonoids, alkaloid, and protein (Lako et al., 2007).

The moringa seeds contain much of plant edible oil which utilized as a frying oil can be a healthy alternative to other commonly used oils such as palm and soybean oil when comparing fatty acid composition. Oils with high amount of monounsaturated (oleic type) fatty acids are desirable due to an association with decreased risk of coronary heart disease. MSO was also found to contain high levels of β-sitosterol (up to 50%), stigmasterol (17%), and campesterol (15%), α-,γ-,δ-tocopherol which detected up to levels of 105, 39, and 77 mg/kg of oil, respectively (Tsaknis et al., 1999). Despite its wide use by communities around the world, there is inadequate scientific information available on the actual physiological/ pharmacological effects on uterine muscles. Thus, the aim of this chapter was to evaluate the effects of MSO on uterine contraction.

4.3 Materials and methods

4.3.1 Experimental animals

Female Wistar rats weighing 200-250 g were housed in a controlled environment with temperature kelp at 25 ± 0.5°C, relative humidity between 45-50%, 12 hr. light-dark cycle and had free access to rodent food pellet and tap water *ad libitum*. The experiments carried out on animals were approved by the Institutional Animal Care and Use Committee, Suranaree University of Technology (SUT), Thailand.

4.3.2 Preparation of plant materials

Plant preparation and oil extraction were as described in Chapter III.

4.3.3 Physiological solutions and test substances

Krebs' solution

The purpose of the tissue-bathing media was to provide suitable physiological milieu for optimal tissue responses. The physiological Krebs' solution compositions of (in millimoles per liter): 154 NaCl; 5.4 KCl; 1.2 $MgSO_4$; 8 glucose; 2 $CaCl_2$ and 10 N-[2-hydroxyethyl] piperazine-N'-[2-ethanesulfonic acid] (HEPES) were used. The media were maintained at pH of 7.4, temperature of 37°C and gassed with O_2.

Drugs/ test substances

All chemicals were purchased from Sigma Chemical Company, Singapore. Other drugs used for the investigation of physiological pathways were as described below. The stock solutions of the drugs/test substances stored at -20°C were thawed and reconstituted to appropriate concentrations with suitable solvents prior to the experiments. Nifedipine, an inhibitor of L-type Ca^{2+} entry was dissolved in

ethanol at a concentration of 10 mM (Shmigol, Eisner and Wray, 1998). Wortmannin, an inhibitor of MLCK, was dissolved in dimethyl sulfoxide (DMSO) at a concentration of 4 µM (Longbottom et al., 2000). Tetraethylammonium (TEA), an inhibitor of calcium-activated potassium channels was dissolved in distilled water at a concentration of 5 mM. Cyclopiazonic acid (CPA), an inhibitor of the SERCA pump was dissolved in DMSO at a concentration of 20 µM (Kupittayanant, Luckas and Wray, 2002). Fulvestrant, an estrogen receptor antagonist was dissolved in DMSO at a concentration of 1 µM (Buzdar, 2008). These working solutions were diluted to the desired concentrations with Krebs'solution.

Preparation of MSO working solutions

Working solution of MSO was dissolved in 5% Tween 80 with ratio 1:3, and the final concentration of Tween 80 in buffer solution was less than 0.1%, which had no effect on spontaneous contractions and the contractions evoked by stimuli.

4.3.4 Measurement of isometric contraction

Uterine tissue preparation

The rats were euthanatized by CO_2 asphyxia and uteri were rapidly removed and placed to a Krebs' solution. The two uterine horn segments were cleaned free fat and connective tissues and cut into longitudinal strips (approximately 1-2 mm x 0.5 mm x 10 mm).

The tissue organ bath system

The tissue was then placed vertically in an organ bath containing solution with Krebs' solution, giving a pH of 7.4 and maintained temperature at 37°C. Uterine strip was suspended between two stainless steel hocks; one of the hocks was fixed to the chamber wall while the other was attached to an isometric force

transducer (AD Instruments Pty, Spain) connected to Power Lab system software (AD Instruments, Pty Ltd., Australia) for recording and analysis of tissue responses.

Elucidation of physiological effects:

1. the effects of MSO on spontaneous uterine contraction:

After mounting, preparations were allowed to equilibrate until the spontaneous phasic contraction became stable (30 min). The tissue was then challenged with the MSO into the bathing solution in a cumulative increase in concentration manner (50, 100, 200, 400 µL/100 mL) for 30 min intervals. The EC_{50} (concentration causing half maximal contraction) value for the extract was determined.

2. the effects of MSO on KCl-induced uterine contractions:

In other sets of experiments, after equilibration period in Krebs' solution, the uterine strips were stimulated with KCl (40 mM) for 40 min and then washed 20 min later KCl was then added into bathing solution, and 20 min later MSO was incubated for 20 min in the continued presence of KCl. At the end of experiment, the bathing solution was replaced by Krebs' solution and tension monitored up to 30 min. The percentage of original response was calculated.

3. the effects of MSO on oxytocin-induced uterine contraction in calcium-free solution:

Uterine strip was incubated with 0-Ca containing 1 mM EGTA solution for 15 min, then 10 nM oxytocin was added to stimulate the release of Ca^{2+} from sarcoplasmic reticulum (SR) for 15 min. The tissue was washed and next stage, MSO was applied to the bath solution for 15 min and then challenge by oxytocin the continued presence of MSO. The effects of MSO with oxytocin were recorded.

4. **the effects of MSO on uterine contraction in the presence of calcium antagonist (nifedipine):**

To investigate uterotonic activity of MSO if it was dependent upon external Ca^{2+} entry through voltage-gated L-type Ca^{2+} channel, nifedipine was applied to the tissue organ bath for 15 min and then MSO added, in the continued presence of nifedipine. In addition, the experiments were done the other way round. Conversely, MSO was applied for 30 min and then nifedipine added, in the continued presence of the extract.

5. **the effects of MSO on uterine contraction in the presence of MLCK inhibitors (wortmanin):**

After rhythmic contractions of uterine strips for 30 min, wortmannin was applied to the tissue organ bath for 30 min to evaluate the effects of MSO on Ca-calmodulin-MLCK. Then MSO was applied, in the continued presence of wortmannin. In addition, the experiments were done the other way round. In the other hand, MSO was applied for 30 min and then wortmannin added, in the continued presence of the extract.

6. **the effects of MSO on uterine contraction in the presence of K^+ channel inhibitor (TEA):**
7. **the effects of MSO on uterine contraction in the presence of sarco/endo-plasmic reticulum Ca $^{2+}$-ATPase (SERCA) pump inhibitor (CPA):**
8. **the effects of MSO on uterine contraction in the presence of estrogen receptor inhibitor (fulvestrnt):**

Detailed for the study 6-8, they were used the same protocol as above for wortmannin.

4.3.5 Statistical analysis

All responses were recorded isometrically and analyzed on a computer using Chart solfware (AD Instruments, Pty Ltd., Australia). The results were expressed as the mean ± S.E.M. (standard error of the mean). The EC_{50} (concentration which produced 50% of maximum response) were computed for each concentration-response experiment. Contractility endpoints were amplitude, frequency and area under the contraction (AUC). Contraction was expressed as a percentage of stimulation of the maximal contraction obtained by adding the standard chemicals and extracts. The data were evaluated using Microcal Origin 8.0 (Microcal Software Inc., USA), and the differences between control and treatment groups were analyzed by student *t-test*. *P*-values less than 0.05 were considered significant.

4.4 Results

4.4.1 Effects of MSO on spontaneous uterine contraction

The cumulative increases in concentration of MSO (50, 100, 200, or 400 µL/100 mL were added into organ bath after the 30 min equilibium period and used as a control (100%). In all preparations (5 strips from 5 animals) MSO had stimulating effect on spontaneous contractions. At each concentration, it produced a significant ($P < 0.01$) increase in amplitude and AUC (Figure 4.1, Table 4.1), whereas frequency of the contraction decreased ($P < 0.01$). In figure 4.2, demonstrates EC_{50} value of 93.90 µL/100 mL and then this concentration was used in the all remainder of the experiments.

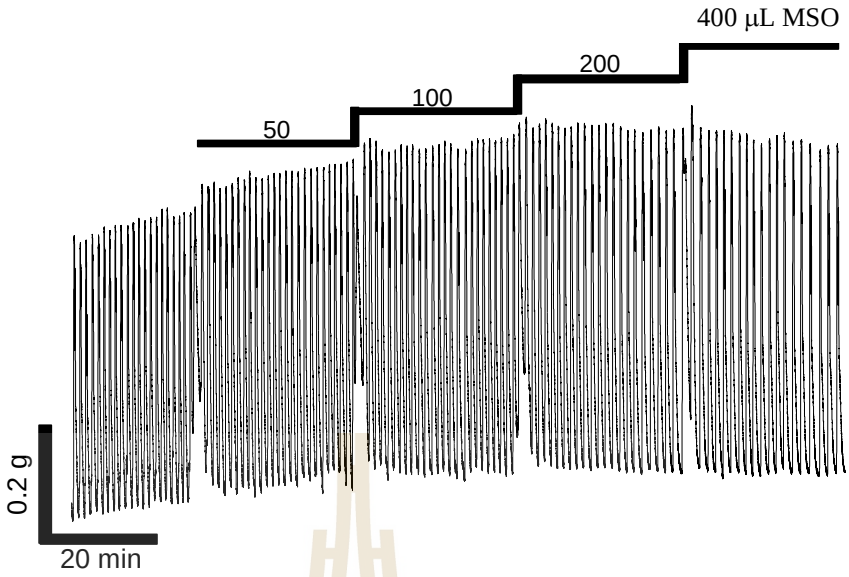

Figure 4.1 The effects of MSO on spontaneous contraction. The cumulative stimulation responses are shown.

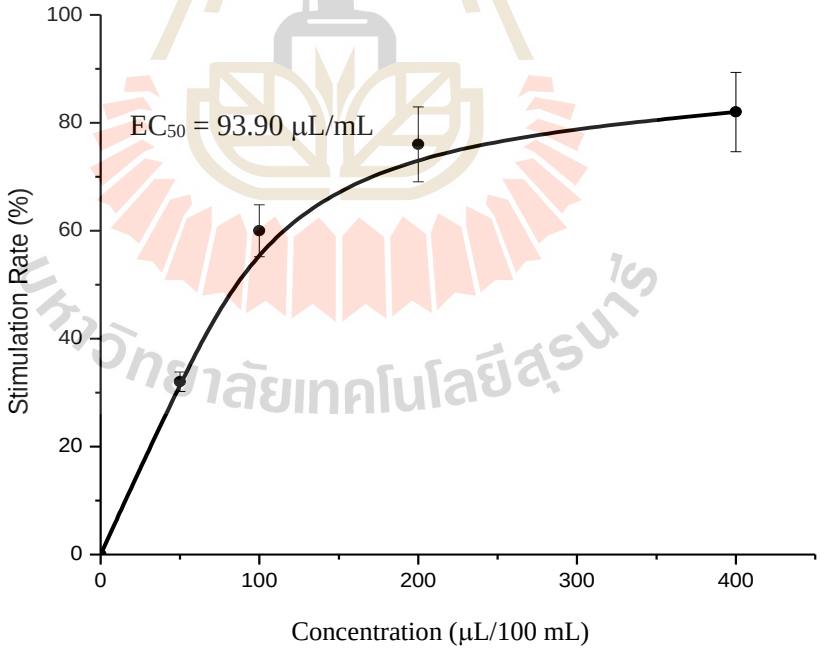

Figure 4.2 The rate of stimulation from the increase in amplitude of spontaneous contraction induced by 50-400 µL/100 mL. Values are expressed as means ± S.E.M (n = 5). N is the number of animals from which tissues were taken.

Table 4.1 The effects of MSO at various concentrations on spontaneous contraction.

	Amplitude (% Mean ± S.EM.)	**Frequency (% Mean ± S.E.M.)**	**AUC (% Mean ± S.E.M.)**	**n**
0 (control)	100	100	100	5
50	116.89 ± 1.58**	82.28 ± 5.16**	123.68 ± 10.24	5
100	126.29 ± 6.29**	80.21 ± 6.16**	131.86 ± 16.12	5
200	139.13 ± 11.93**	72.70 ± 5.92**	147.58 ± 10.70**	5
400	139.29 ± 12.31**	74.84 ± 4.55**	155.08 ± 5.54**	5

The *P*-value for amplitude, frequency and AUC of MSO treated are significantly different from the control (**$P < 0.01$). Mean ± S.E.M. are given; n is number of animals.

4.4.2 Effects of MSO on high KCl-induced contraction

Addition of high KCl (40 mM) solution to tissue organ bath for the first time caused a strong contraction of the isolated rat uterus. The results demonstrated that high KCl solution generated the sustained tonic contraction in uterine strips. As shown in Figure 4.3, 20 min after the high KCl applications, AUC had significantly increased to 229.74 ± 4.71% and after adding MSO multiplied up to 259.93 ± 15.74% compared of control force development (100%) ($P < 0.01$). The response was shown in Figure 4.4.

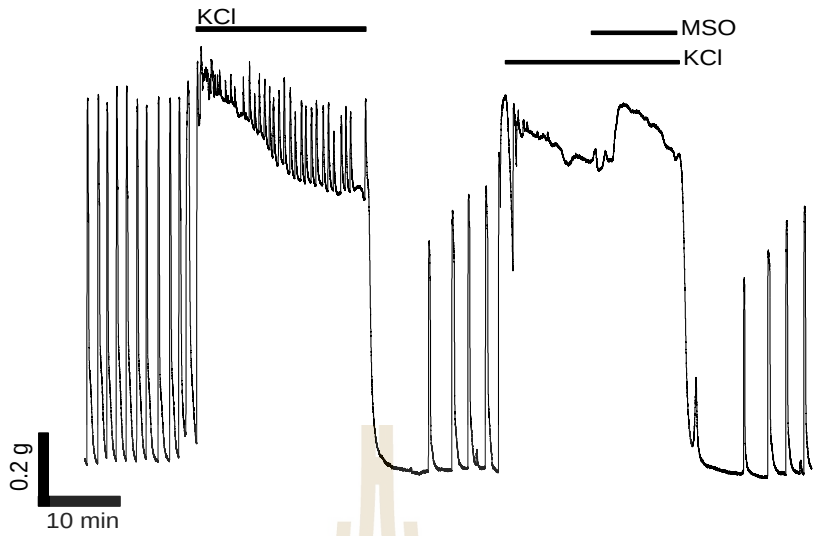

Figure 4.3 Representative tracings showing the effects of MSO on uterine contraction induced by KCl. The responses were compared of the time control (KCl alone; 100%) and the tested period (KCl + MSO) (n = 5).

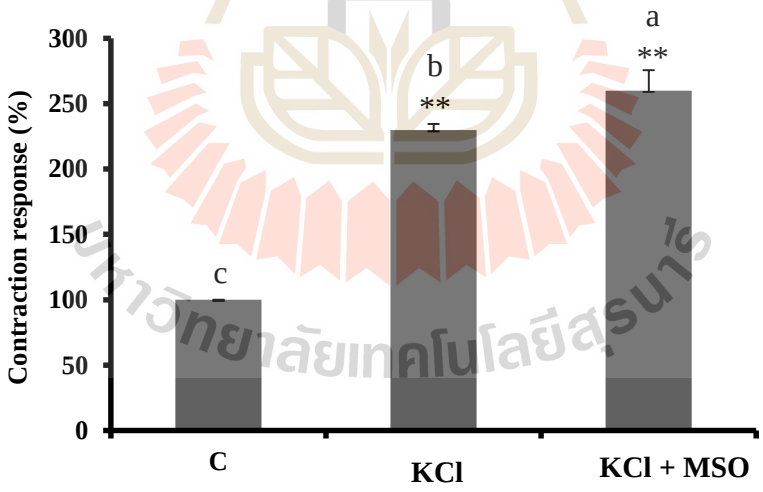

Figure 4.4 A graph showing the effects of MSO on uterine contraction induced by KCl using AUC as a parameter of investigation. The responses were compared of the time control (KCl alone; 100%) and the tested period (KCl + MSO) (n = 5). Bars represent means ± S.E.M. (n = 5). ** indicate a significant ($P < 0.01$). Values that do not share the same superscript letters are significantly different ($P < 0.05$).

4.4.3 Effects of MSO on oxytocin-induced uterine contraction in the absence of external Ca^{2+}

This experiment was carried out to examine the effects of MSO on contraction in the absence of extracellular calcium. Figure 4.5 shows a representative trace of phasic contractions that were abolished upon changing from Krebs' solution to zero-Ca^{2+} EGTA solutions. In continued presence of zero-Ca^{2+}solution, oxytocin produced a small tonic force. Thereafter, MSO was added in the continued presence of zero-Ca^{2+}solutionand it caused a stimulation of the little contraction compared to a control.

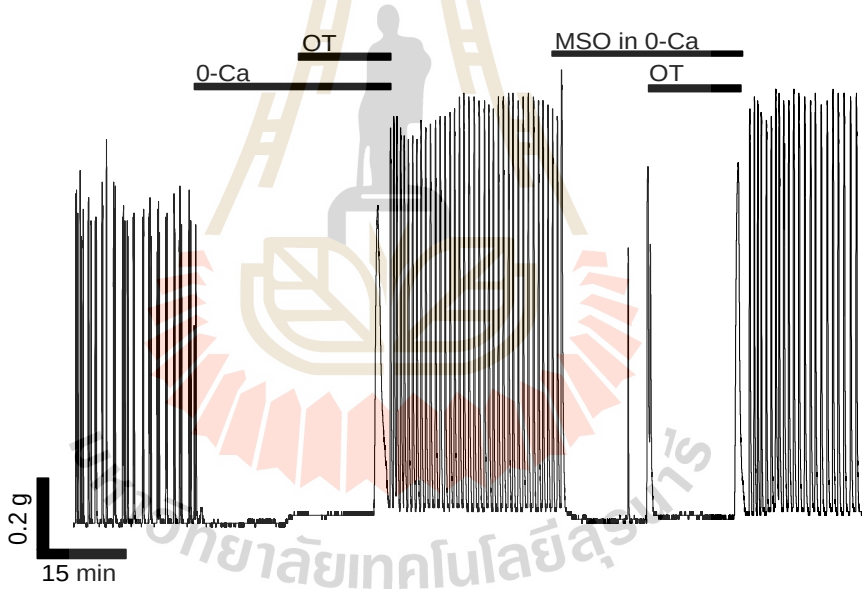

Figure 4.5 The effects of MSO on oxytocin-induced uterine contraction in the absence of external Ca^{2+} (n = 5).

4.4.4 Effects of MSO on uterine contraction in the presence of calcium antagonist on isolated rat uterus

The entry of Ca^{2+} into the cells through the voltage-gated Ca^{2+} channel is one of the crucial factors in the generation of smooth muscle contraction. This experiment was applied to examine the effects of MSO to uterine contraction when L-type Ca^{2+}channel was blocked with an antagonist (nifedipine). As shown in Figure 4.6A, an application of 10 µM nifedipine in the presence of MSO rapidly inhibited force and then abolished. Thereafter, basal force did not return to control levels, but remained somewhat elevated and oscillatory. Conversely, when MSO was added after application of nifedipine, spontaneous force was abolished and the MSO could elicit transient force with oscillatory in nature (Figure 4.6B).

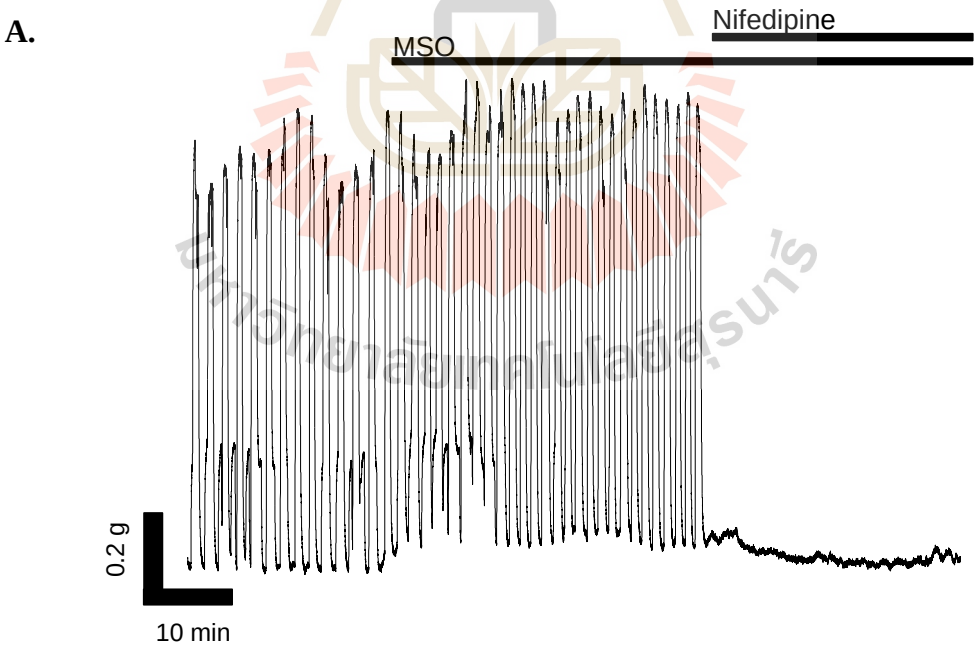

Figure 4.6 The effects of MSO on uterine contraction in the presence of L-type Ca^{2+}channel inhibitor. Nifedipine was added before (A) and after (B) MSO (n = 5).

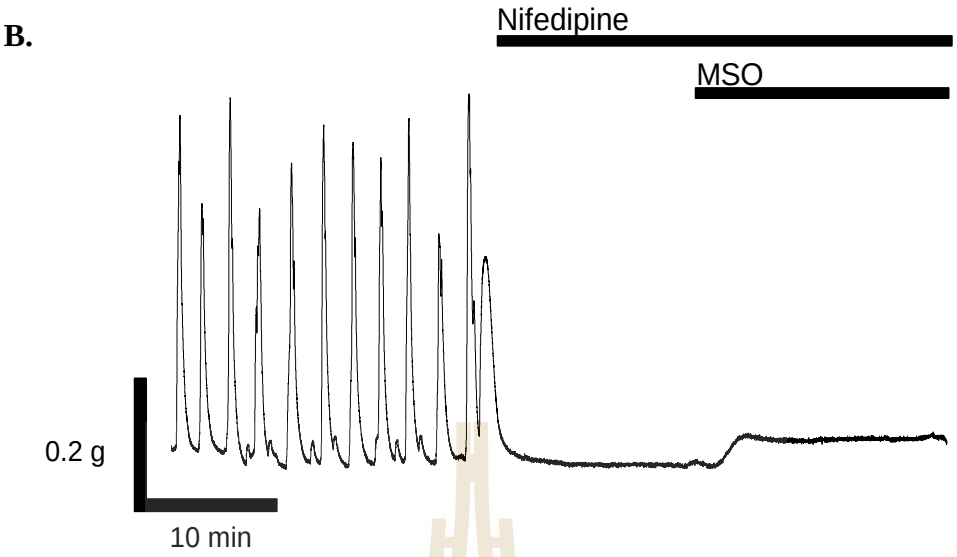

Figure 4.6 The effects of MSO on uterine contraction in the presence of L-type Ca^{2+}channel inhibitor. Nifedipine was added before (A) and after (B) MSO (n = 5) (Continued).

4.4.5 Effects of MSO on uterine contraction in the presence of MLCK inhibitors

The effects of MSO on uterine contraction in the presence of wortmanin, the MLCK inhibitor, were investigated. The effects are shown in Figure 4.7A and 4.7B. When MSO was applied in the continued presence of wortmanin, it gradually reduced force. A significant reduction occurred after 10 min (mean amplitude of contraction 79.51 ± 1.92% compared to 100% control), and by 30 min AUC were significant decreased to 3.71 ± 1.04% ($P < 0.01$). In addition, MSO consistently increased basal force, even in the presence of wortmanin and the response represents in Figure 4.7.

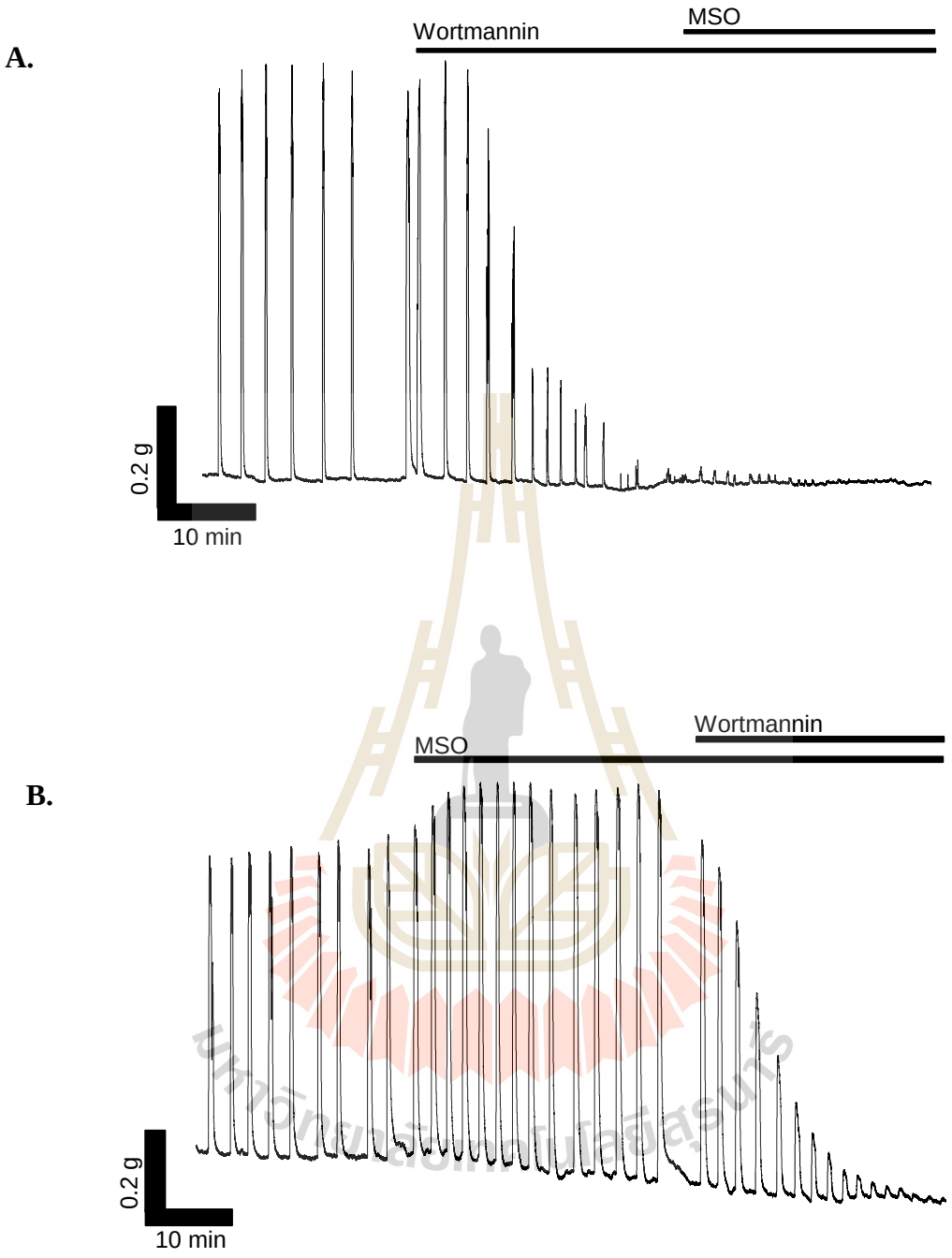

Figure 4.7 The effects of MSO on uterine contraction in the presence of the MLCK inhibitor. Wortmannin was added before (A) and after (B) MSO (n = 4).

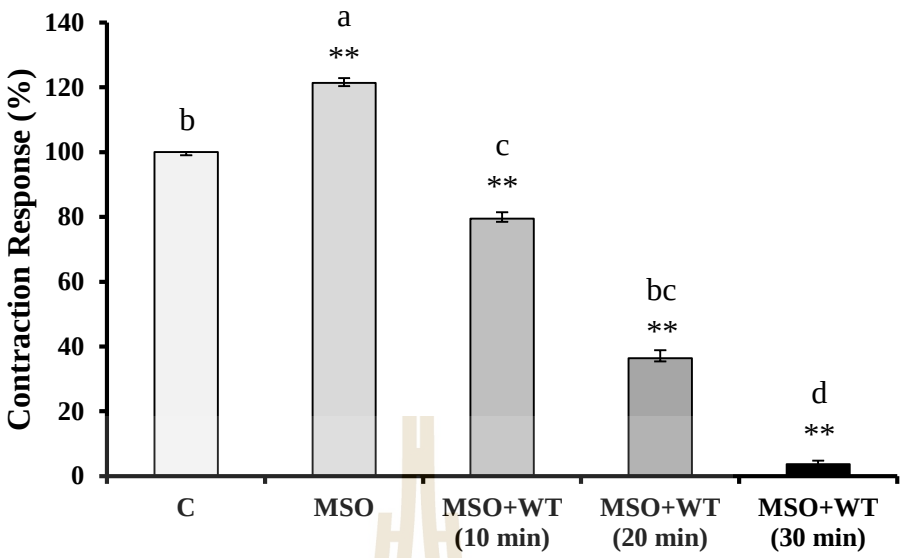

Figure 4.8 A graph showing the effects of MSO on uterine contraction in the presence and absence of wortmannin using AUC as a parameter of investigation. Bars represent means ± S.E.M. (n = 4). ** indicate a significant ($P < 0.01$). Values that do not share the same superscript letters are significantly different ($P < 0.05$).

4.4.6 Effects of MSO in the presence of K^+ channel inhibitor

Application of 5 mM TEA produced a significant increase in amplitude of the uterine contraction to 113.38 ± 7.06% ($P < 0.05$, n = 3); compared with the control (100%). After MSO was added in the continued presence of TEA the amplitude was significantly increased. Similarly, MSO and MSO combined with TEA produced a significant increase in amplitude of uterine contraction to 125.89 ± 6.54% and 129.66 ± 5.47%, respectively ($P < 0.05$, n = 3) compared with the control (100%). The effects are summarized in Table 4.2 and typical effect is shown in Figure 4.9A and 4.9B.

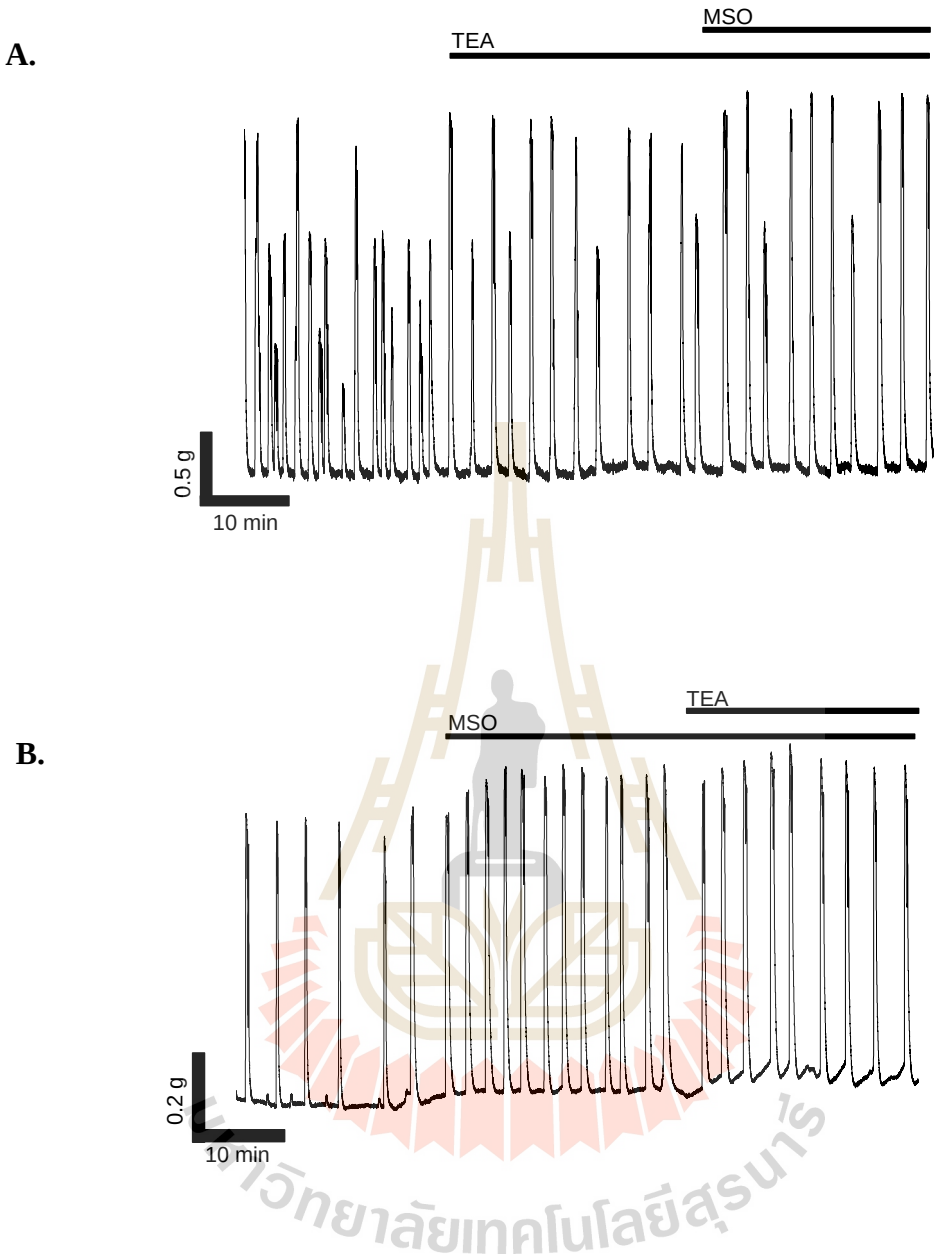

Figure 4.9 The effects of MSO in the presence of the K^+ channel inhibitor. TEA was added before (A) and after (B) MSO (n = 3).

Table 4.2 The effects of MSO on uterine contractions in the presence of the K^+ channel inhibitor.

	Amplitude (% Mean ± S.E.M.)	**Frequency (% Mean ± S.E.M.)**	**AUC (% Mean± S.E.M.)**	**n**
MSO (after)				
control	100	100	100	3
TEA	113.38 ± 7.06*	101.89 ± 3.37	116.11 ± 4.41	3
TEA + MSO	110.76 ± 3.26*	103.07 ± 10.40	164.58 ± 3.39*	3
MSO (before)				
control	100	100	100	3
MSO	125.89 ± 6.54*	88.42 ± 8.30*	175.52 ± 6.75*	3
MSO + TEA	129.66 ± 5.47*	100.15 ± 10.96	181.97 ± 2.55*	3

The *P*- value for amplitude, frequency and AUC of MSO treated are significantly different from the control ($P < 0.05$). Mean ± S.E.M. are given; n is number of animals.

4.4.7 Effects of MSO on uterine contractions in the presence of SERCA pump inhibitor, CPA

When CPA was added to the tissue bath solution, it increased the amplitude, frequency and AUC of contraction, compared with control (100%). Appling of MSO in the continued presence of CPA still induced amplitude, frequency and AUC (115.82 ± 7.42%, 118.12 ± 12.96% and 111.30 ± 3.75%, respectively), but the amplitude trend to decrease when compared of CPA alone. Nevertheless, these effects were not significant. Furthermore, addition of MSO in the continued presence of CPA produced a significant increase in amplitude, frequency and AUC of uterine

contraction (n = 4) when compared to the control. All effects of MSO on uterine contraction in the presence of SERCA pump inhibitor are summarized in Table 4.3 and a typical effect is shown in Figure 4.10.

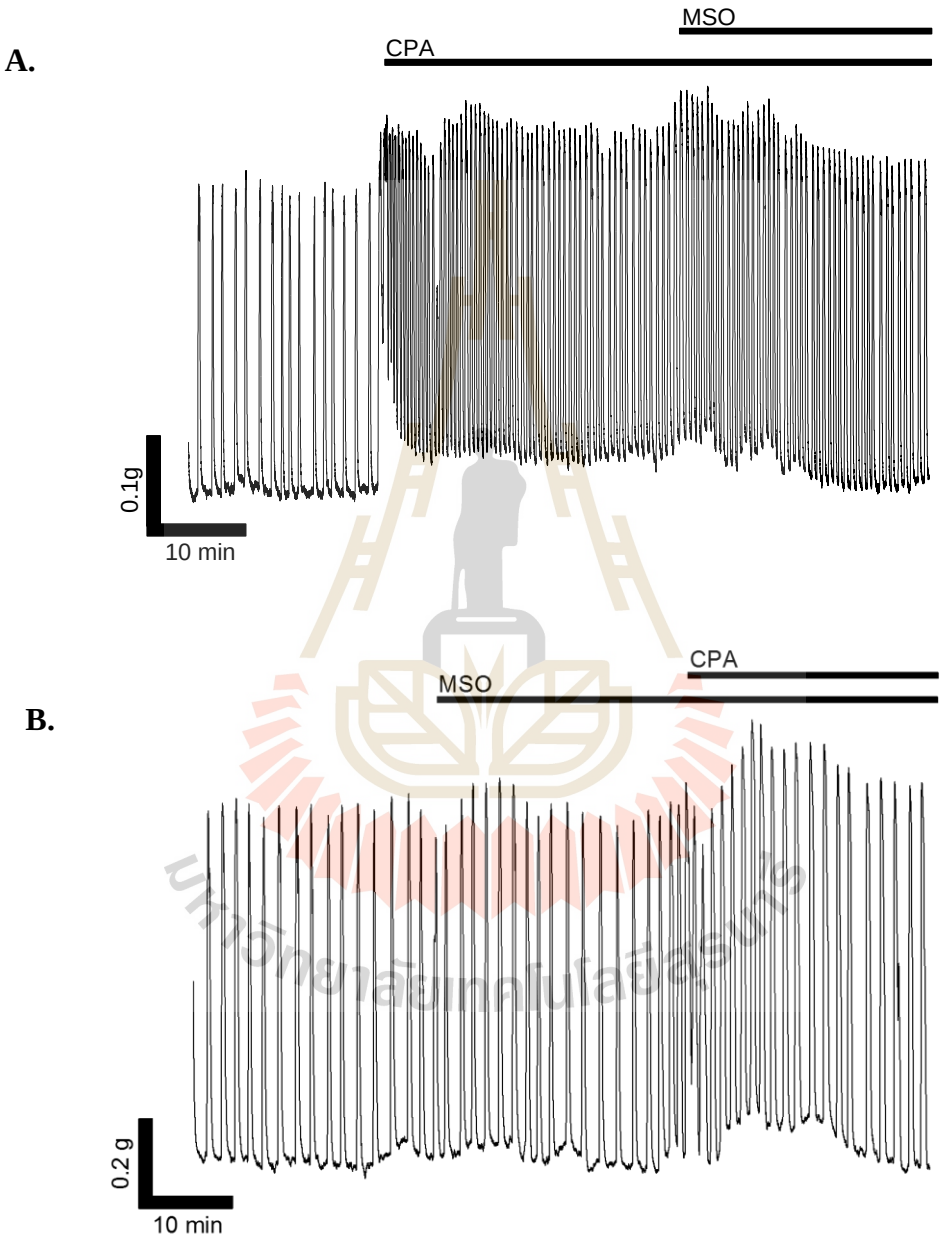

Figure 4.10 The effects of MSO on uterine contraction in the presence of SERCA pump inhibitor. CPA was added before (A) and after (B) MSO (n = 4).

Table 4.3 The effects of MSO on uterine contractions in the presence of SERCA pump inhibitor, CPA.

	Amplitude (% Mean ± S.E.M.)	**Frequency (% Mean ± S.E.M.)**	**AUC (% Mean ± S.E.M.)**	**n**
MSO (after)				
control	100	100	100	4
CPA	117.22 ± 6.14*	106.94 ± 13.28	109.59 ± 10.32	4
CPA + MSO	115.82 ± 7.42	118.12 ± 12.96	111.30 ± 3.75	4
MSO (before)				
control	100	100	100	4
MSO	115.26 ± 4.90*	91.44 ± 8.30	136.13 ± 8.27*	4
MSO + CPA	121.37 ± 5.97*	118.77 ± 3.60*	170.33 ± 12.11*	4

The *P*- value for amplitude, frequency and AUC of MSO treated are significantly different from the control ($P < 0.05$). Mean ± S.E.M. are given; n is number of animals.

4.4.8 Effects of MSO in the presence of estrogen receptor inhibitor

The effect of the MSO was also investigated in the presence of estrogen receptor inhibitor (fulvestrant). Application MSO in the presence of fulvestrant produced no significant changes in uterine contractions. The amplitude, frequency and AUC were summarized in Table 4.4. Similarly, if fulvestrant was added after an application of MSO, it produced no further increases in force. A typical effect is shown in Figure 4.11.

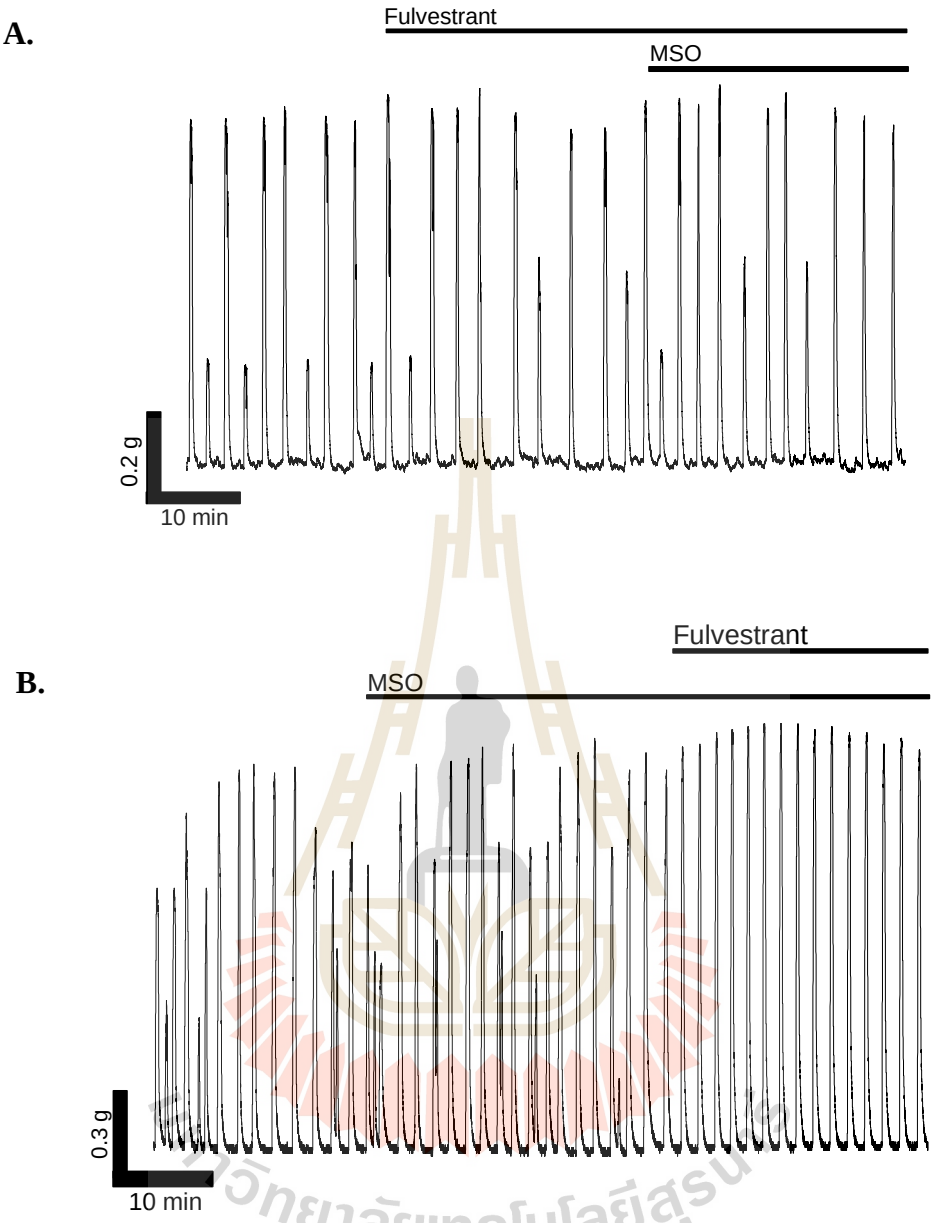

Figure 4.11 The effects of MSO in the presence of the estrogen receptor inhibitor. Fulvestrant was added before (A) and after (B) MSO (n = 4).

Table 4.4 The effects of MSO on uterine contractions in the presence of the estrogen receptor inhibitor.

	Amplitude (% Mean ± S.E.M.)	**Frequency (% Mean ± S.E.M.)**	**AUC (% Mean ± S.E.M.)**	**n**
MSO (after)				
control	100	100	100	4
Fulvestrant	104.58 ± 4.42	88.34 ± 8.62	90.52 ± 6.84	4
Fulvestrant + MSO	108.09 ± 4.38	80.32 ± 11.34	88.30 ± 4.89	4
MSO (before)				
control	100	100	100	4
MSO	109.10 ± 6.19	83.33 ± 11.34	90.46 ± 8.35	4
MSO + Fulvestrant	112.80 ± 12.37	62.27 ± 13.25	87.80 ± 14.51	4

The *P*- value for amplitude, frequency and AUC of MSO treated are significantly different from the control ($P < 0.05$). Mean ± S.E.M. are given; n is number of animals.

4.6 Discussion

It is well established that the mechanism of myometrium contraction is similar to other smooth muscles as both are caused by a rise in intracellular Ca^{2+} ($[Ca^{2+}]_i$). The rise of $[Ca^{2+}]_i$ for the myometrium contraction comes from two sources: the extracellular space with Ca^{2+} entering the cell through the plasma membrane voltage-gated calcium channels and an intracellular source from the sarcoplasmic reticulum (Vander et al., 2001; Kupittayanant et al., 2002). Ca^{2+}channel agonists have been used increasingly as uterotonic agents. These agents act to induce Ca^{2+} influx across the

cell membrane, thereby increasing the tone in the smooth muscle contraction. It is predictable, therefore, that uterine contractions can be stimulated by compounds which act to induce or enhance the entry of Ca^{2+} into the myocyte. Indeed, calcium agonists have been shown to stimulate uterine contractions in both the rat (Csapo et al., 1982) and human (Forman et al., 1982).

The aim of this experiment was to elucidate the effects and mechanisms of MSO on isolated rat uterine. The data provides direct evidence for the first time that MSO exhibits potent stimulation of phasic activity in rat uterus. Significant increase in the amplitude of spontaneous contractions may involve non-estrogenic effects and may relate to inhibition of K^{+}-channel and SERCA pump and/or stimulation intracellular $Ca^{2+}[Ca^{2+}]_i$ increase via L-type Ca^{2+} channel and myosin light chain kinase activation.

In many investigations, the data demonstrated that a lot of uterotonic constituents present in different plants usually mediate their effect through calcium. In order to test if MSO had a similar effect, the extract was assayed on various antagonist-induced contractions. As noted previously, nifedipine is a dihydropyridine calcium channel blocker that primarily blocks L-type calcium channels has been reported to reduce uterine contractions (Furberg et al., 1995; Hayashi et al., 2010). In the presence of MSO condition incubated with nifedipine also showed small force. In addition, contraction in smooth muscle is slower and longer lasting than instriated muscle. Regulation of actin and myosin does not work by way of troponin/tropomyosin but by phosphorylation of the regulatory myosin light chain. This is catalyzed by MLCK, which is calmodulin-dependent and, hence, again under the control of calcium (Squire and Morris, 1998; Michael, 2007). Longbottom

and colleagues (2000) published that wortmannin, selective inhibitor of MLCK reduced and abolished uterine force. The results from this experiment support a mechanism of action involving the Ca-calmodulin-MLCK pathway as the effects of wortmannin application in the presence of MSO, force was no longer produced.

On the other side, smooth muscle cells are known to express store-operated channels (SOCs) and these have been included as one of the possible channels for providing the inward current that is necessary to provide the depolarizing drive during the pacemaker depolarization (Berridge, 2012). The experiments in which SERCA was inhibited using antagonist; CPA that is specific inhibitor of Ca^{2+}-ATPase in the intracellular Ca^{2+} storage sites showed that application with CPA prevented any additional effects of MSO. However, CPA was still able to produce some stimulate of uterine contraction in the presence of MSO.

Furthermore, it is known that both Ca^{2+} channel activator and membrane depolarization caused by high K^+ can increase Ca^{2+} influx through the voltage-gated Ca^{2+} channel (Foster et al., 1983). In the present work, when the high K^+ solution was used to depolarize the myometrium and maintain intracellular Ca^{2+} at high levels, MSO was still able to alter force to tonic. Subsequently, the experiments with TEA, the blocker mainly of Ca^{2+}-activated K^+ channel was carried out. In previous studies, TEA increased the frequency of spontaneous myometrial contractions in myometrial strips from estrogen-primed rats, and also initiated spontaneous contractions in quiescent myometrial strips from term-pregnant women (Anwer et al., 1993). Moreover, TEA significantly increased mean integral tension in myometrium from non-pregnant (Aaronson et al., 2006). In this experiment the results represented partially modified the uterine contraction induced by MSO.

Oxytocin is one of many hormones that stimulate uterine contraction. Oxytocin binds to the oxytocin receptor (ORT), is a classical membrane with transmembrane domains linked through a G protein complex to a phospholipase-C protein kinase C signal transduction system. After oxytocin stimulation, there are markedly increase in intracellular concentration of inositol triphosphate and calcium ion (Ca^{2+}). The higher $Ca^{2+}$and calmodulin increase the myosin light chain kinase which catalyses the contraction response (Phaneuf et al., 1993). In addition, calcium activated chloride (Cl_{Ca}) channels have also been described in a rat myometrium stimulated with oxytocin. These channels therefore may be activated by Ca^{2+} from the sarcoplasmic reticulum and extracellular Ca^{2+} entry. Activation of Cl_{Ca} channels will lead to depolarization, as Cl^- leaves the cell. Thus, the channels may also play an important role in causing excitability of the myometrium and enhance Ca^{2+} entry through voltage-gated Ca^{2+}channels (Carl et al., 1996; Karaki et al., 1997; Wray et al., 2003). Similarly membrane depolarization causing Ca^{2+} entry may be caused by blocking of the delayed rectified potassium channels due to the increase in $[Ca^{2+}]_i$ by its release from the internal store and its entry from the extracellular site (Carl et al., 1996; Karaki et al., 1997). Moreover, Shmygol et al. (2006) have used mag-fluo-4 and confocal microscopy to obtain 3D reconstructions of the SR in uterine myocytes tonic contraction seen at the beginning of oxytocin application and showed that changes in frequency of contractions are mediated by the SR Ca^{2+}, and potentiation of contraction amplitude is achieved by sensitization of contractile machinery to Ca^{2+}.

The present study demonstrated the results when MSO were added in zero Ca^{2+} solution, little force was found as oxytocin-induced uterine contraction in zero Ca^{2+} solution. Thus, it is suggested that the uterotonic effect of MSO may also be

involved with the Ca from SR and would mimic that oxytocic activity, which activate Ca^{2+} influx through plasma membrane voltage-operated Ca^{2+} channels, resulting in stimulation of uterine contractions. Furthermore, the experiments with fulvestrant, a blocker of ERs which works by down-regulating the estrogen receptor (Kansra et al., 2005), when MSO was added in the presence of fulvestrant uterine contractility still increase. This demonstrated clearly the action of MSO is via a nongenomic ER pathway.

In conclusion, MSO has uterotonic effects on the isolated rat uterus. These findings support the traditional use of the plant for its abortificient and contraceptive properties. Moreover, MSO may be of potential value in the gynecological conditions linked with inducing labour and post-partum haemorrhage. Further study should becarried out to analysis which constituents responsible for its uterotonic effects.

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CHAPTER V

THE ESTROGENIC LIKE PROPERTIES OF MORINGA SEED OIL IN OVARIECTOMIZED RATS

The results from chapter III indicated that phytochemical screening of *Moringa oleifera* seed oil (MSO) composed of phytosterol as major constituents. It's established that phytoestrogens are popular to be used as an alternative choice for hormone replacement therapy in menopausal women. However, the estrogenic properties of MSO have never been reported. This aim of this chapter was to evaluate the estrogenic properties of this oil extract.

5.1 Abstract

Several herbal extracts have been used to prevent or relief menopausal symptoms. As with other herbal extracts, *Moringa oleifera* Lam. has been used in various gynecological disorders. However, its estrogenic properties have not yet been studied at least in the area of menopausal study. The aim of this study was therefore, to investigate the estrogen-like effects of *M. oleifera* seed oil (MSO) on prevention or relief menopausal symptoms using ovariectomized (OVX) rat model. The study was designed into two series including preventive and recovery study. In the preventive study, OVX rats were orally administered with MSO for 6 weeks starting from day 3 after ovariectomy. In the recovery study, OVX rats were administered with MSO for 6 weeks starting from day 60 after ovariectomy. Estrogenic properties were evaluated

using *in vivo* tests such as the degree of cornification of vaginal epithelium and uterotropic assays. Administration of MSO also significantly caused cornification of the vaginal epithelium. MSO could induce proliferation of the uterus. It increased the relative uterine weight as well as the luminal epithelium cell height. Its effects were analogous to those induced by a standard estrogen (Estradiol). These results suggest that MSO displayed estrogenic properties in OVX rats. The effects were as potent as a standard estrogen.

5.2 Introduction

Moringa oleifera Lam. (syn. *M. pterygosperma* Gaertn.) is a valued native plant of the western and sub-Himalayan tracts, India, Pakistan, Asia Minor, Africa and Arabia (Somali et al., 1984; Mughal et al., 1999). It is now distributed in Thailand, Philippines, Cambodia, Central America, North and South America and the Caribbean Islands (Morton, 1991). In Thailand, *M. oleifera* is locally known as "Ma-rum" and is grown and cultivated all over the country.

M. oleifera has been used in the traditional medicine passes down for centuries in many cultures around the world, for various gynecological purposes in both male and female such as semen deficiency, pregnancy and lactation (Anwar et al., 2007; Mahmood et al., 2010). With regard to the female reproductive system, the aqueous extract of *M. oleifera* roots and leaves have been reported to have unique estrogenic, antiestrogenic, progestational, and antiprogestational activities (Shukla et al., 1988a; 1988b, Nath et al., 1992). Oral administration of the extract progressively induced vaginal cornification, stimulated uterine histoarchitecture and increased the uterine wet weight of OVX rats (Shukla et al., 1988b). Biochemical

observations and histologic findings have been correlated with the anti-implantation action of the aqueous extract in light of its hormonal properties and various experiments can easily explain its use as an abortifacient (Nath et al., 1992). Subsequently, the phytochemical studies on *M. oleifera* root and leaves revealed major polyphenols and phytosterols such as quercetin, kaempferol, zeatin, caffeoylquinic acid, pterygospermin and β-sitosterol (Ghasi et al., 2000; Bennett et al., 2003; Siddhuraju et al., 2003; Bose et al., 2007).

Like moringa root and leaves phytochemical analyses have shown that moringa seed oil composed of several potential phytoestrogens such as campesterol, stigmasterol, and β-sitosterol (Lalas and Tsaknis, 2002; Anwar and Bhanger, 2003). However, to the best of our knowledge, the effects of MSO that related estogenic-like activities have never been studied.

In spite of the numerous literature reports on the nutritional and medicinal properties of moringa oil, no detailed scientific study has been carried out to validate or invalidate this claim on the reproductive functions, particularly the oil extracted from the seeds grown in Thailand. In order to obtain an explanation, its efficacy needs to be scientifically evaluated *in vivo* experiments. Thus, the current study was performed to evaluate the estrogenic efficacy of MSO administration on reproductive target tissues in ovariectomized rats.

5.3 Materials and methods

5.3.1 Chemicals

All of the reagents (analytical and HPLC grade) were obtained from Sigma Chemicals Company Co. (St. Louis, MO, U.S.A), and the standard solutions for the

determination of sterols were purchased from Larodan AB (Malmo, Sweden). The stock standard solution of 17β- estradiol (E8875, Sigma) was weighed and dissolved in a small volume of absolute ethanol (Merck, Darmstadt, Germany). After the powder was completely dissolved, the 1% tween 80 was added and the solution was allowed to stand at room temperature to evaporate the ethanol.

5.3.2 Experimental animals

A total of 50 female Wistar rats aged 3 months old (weighing 210-230 g), were used. Bilateral ovariectomy and sham-operation were performed under sodium pentobarbital anesthesia during diestrus period to keep the consistent lowest levels of sex hormones. All animals were maintained in an environmentally controlled animal care with constant temperature (25 ± 0.5°C), humidity (55-60%) and a 12 hr. light/dark cycle. They were fed with a standard laboratory rat diet (CP Co., Ltd, Thailand), and supplied *ad libitum* drinking water.

Care and use of animals and the experimental protocols were approved by the Institutional Animal Care and Use Committee, Suranaree University of Technology, Thailand.

5.3.3 Plant preparation and oil extraction

Plant preparation and oil extraction were as described in Chapter III.

5.3.4 Experimental procedures

The study was designed into two different series modified from Ferretti and colleagues (Ferretti et al., 2012), the first group was the preventive study and the second group was the recovery study. Each experiment series consisted of five sub-groups similarly; Group 1 (SHAM): Sham-operated controls received vehicle (1% Tween 80 in water); Group 2 (OVX): Ovariectomized controls received vehicle

(1% Tween 80 in water); Group 3 (LDMSO): Ovariectomized treated with MSO 0.25 mL/ 100g BW/day; Group 4 (HDMSO): Ovariectomized treated with MSO 0.50 mL/100g BW/day and Group 5 (E_2): Ovariectomized treated with 17β-estradiol 10 µg/kg BW/day. All animals were orally administered using gastric gavages feeding syringe. Treatments were performed at 09.00-12.00 AM and maintained in these conditions for 6 weeks. The administration process started on day 3 after ovariectomy for the preventive study and day 60 after ovariectomy for the recovery study (Figure 5.1).

At the end of the experiments, the animals were sacrificed under anesthesia and blood were collected from cardiac puncture for serum analysis. The uterus was removed immediately and weighed. Parts of the uterus were kept in neutral buffered formalin for histological analysis.

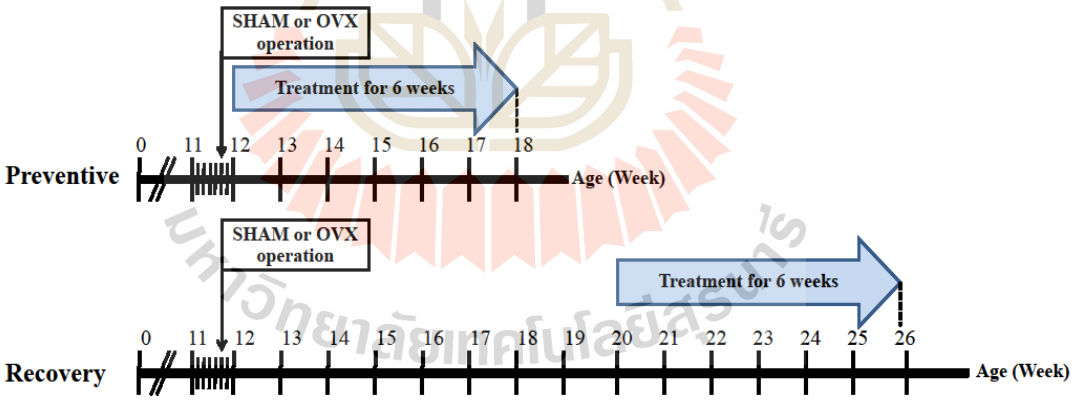

Figure 5.1 Schemes of experiment protocols.

5.3.5 Determination of body weight and relative uterine weight

Body weight of the animals was recorded weekly. At the end of the experiments rats were humanely sacrificed by CO_2 asphyxia. The uteri were rapidly

moved, trimmed of fat and connective tissue and their wet weights were measured. The relative weight (%RW) of uterine tissue was recorded and normalized with body weight (weight of uterus per 100 g of body weight)**.**

5.3.6 Vaginal cornification assay

Cornification of the vaginal epithelium cells in rats was checked daily between 8.00-10.00 AM by gently inserting the tip of dropper into the vagina, flushing 0.85% NaCl in and out. The vaginal lavage was stained with safranin O for 10 min and examined under a Nikon microscope (Hollywood International Ltd, Thailand). The vaginal epithelium cells observed were classified into the three cell types: leukocyte cells, nucleated cells and cornified cells. A total of 100 cells were counted, from which the percentage of cornified cells (%Co) was calculated (Thanamool et al., 2013).

5.3.7 Histological analysis

The uterus were carefully removed, cleaned, weighed and fixed in 10% formalin and then stained with Hematoxylin and Eosin (H&E). Histologic responses were quantified using a Nikon Eclipse 80i Upright microscope (Hollywood International Ltd., Thailand) and Cell^D imaging software (Olympus, EforL International Co., Ltd., Thailand). The thickness of luminal epithelium (micrometers) was measured at 5 different locations in three sections per animal to get an average value of epithelium cell height. All the measurements were made with the aid of the computer assisted program for histological quantification (Image J L.41 software).

5.3.8 Statistical analysis

The data were analyzed using Statistical Package for Social Sciences software version 17.0 (SPSS Inc., Chicago, IL, USA). One-way analysis of variance (ANOVA)

with Tukey's for *post-hoc* comparisons was performed between the means of control and individual treatment groups. Values of $P < 0.05$ indicate significant differences among groups.

5.4 Results

5.4.1 Effects of MSO on body weight and relative uterine weight

During the 6 weeks treatment period, no evidence of obvious toxicity and abnormal clinical signs was observed in any of the groups. As represented in Table 5.1, the animals from all five groups of two studies had similar initial mean body weights. At the end of the study, there was no significant difference in the body weight of all groups of preventive study. While, the results in the recovery study showed that OVX vehicle control group statically increased body weight compared to the SHAM group ($P < 0.05$). These weights were slightly altered in the MSO or E_2 treated animals compared with SHAM group, but lower than OVX vehicle controls.

Results of the uterotrophic assay showed that uterine wet weight from both OVX group tended to decrease when compared to SHAM group ($P < 0.05$). Figures 5A-B illustrates the values of %RW of treated and control animal groups in preventive and recovery studies, respectively. In both studies the mean values of uterine weight of OVX treated with HDMSO animals were similar to those of OVX treated E_2 and SHAM groups and showed increases in %RU ($P < 0.05$, n = 5) significantly higher compared to OVX vehicle control groups.

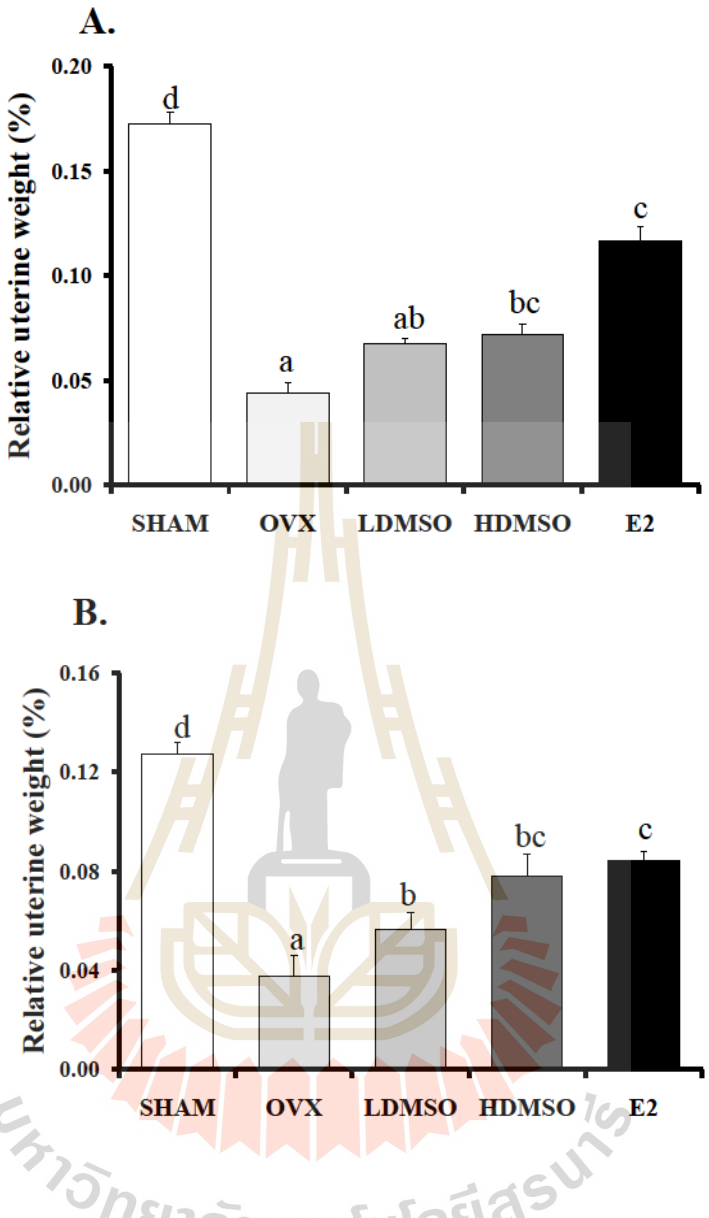

Figure 5.2 Mean values of relative uterine weight in the preventive study (A) and recovery study (B). Each treatment group are shown: SHAM received vehicle, OVX received vehicle, OVX received 0.25 mL/100g BW MSO (LDMSO), OVX received 0.50 mL/100g BW MSO (HDMSO) and OVX received E_2, respectively. Values are expressed as mean ± S.E.M. Data were analyzed by one-way ANOVA, followed by Tukey's *post hoc* test. Values that do not share the same superscript letters are significantly different ($P < 0.05$).

Table 5.1 Effects of MSO on body weight and the mass of uterus in ovariectomized rats.

Group	Treatment	Initial Body Weight (g)	Final Body Weight (g)	Uterine Wet Weight (g)
Preventive	(1) SHAM	225.00 ± 2.88^{a}	246.00 ± 4.00^{a}	0.41 ± 0.01^{d}
	(2) Vehicle control	226.25 ± 2.39^{a}	273.00 ± 5.77^{a}	0.13 ± 0.05^{a}
	(3) 0.25 mL/100g BW MSO	218.00 ± 1.66^{a}	265.00 ± 8.54^{a}	0.19 ± 0.01^{ab}
	(4) 0.50 mL/100g BW MSO	219.00 ± 1.00^{a}	253.00 ± 4.78^{a}	0.20 ± 0.01^{bc}
	(5) 17β-estradiol	222.00 ± 1.23^{a}	250.00 ± 3.16^{a}	0.30 ± 0.01^{c}
Recovery	(1) SHAM	225.00 ± 2.88^{a}	264.00 ± 8.12^{a}	0.32 ± 0.01^{d}
	(2) Vehicle control	222.00 ± 7.50^{a}	321.00 ± 6.63^{c}	0.09 ± 0.01^{a}
	(3) 0.25 mL/100g BW MSO	212.00 ± 4.78^{a}	282.00 ± 5.83^{ab}	0.18 ± 0.04^{b}
	(4) 0.50 mL/100g BW MSO	215.00 ± 3.41^{a}	292.00 ± 3.74^{bc}	0.17 ± 0.14^{b}
	(5) 17β-estradiol	213.00 ± 2.10^{a}	291.00 ± 4.01^{bc}	0.29 ± 0.01^{c}

All values represent as mean ± S.E.M. (n = 5 per group). Means with different superscripted letters in the same column indicate statistical significance ($P < 0.05$).

5.4.2 Determination of MSO on vaginal cornification

The estrous cycle of all rats in both preventive and recovery studies were monitored by daily checkup of vaginal epithelium cell smears comparing to standard E_2 administration. Smears of the vaginal epithelium cells from OVX rat control consisted of leukocytes and parabasal cells, which is representative of constant diestrus. In contrast, the vaginal cells from the OVX rats treated with E_2 or MSO (0.25 mL/100g BW MSO and 0.50 mL/100g BW MSO) demonstrated a large number of keratinized epithelial cells indicating that the status of estrus in the OVX rats was restored (Figure 5.3).

Figures 5.4A and B show the changes in vaginal cytology detected between 42 day treatment periods. Both groups received MSO illustrate the cornified cells with lesser than those of E_2 positive control group. The E_2 treated group revealed the cornified cells within 4 days in preventive group and 17 days in recovery group after E_2 administration and showed the persistent feature of estrus condition until the end of the experiment. While, in MSO treated groups, the cornified cells firstly presented on day 6 and 21 after treatment in the preventive study and recovery study, respectively. The high dosage of MSO, provoked a significant modification of the maximal vaginal smear in 48% of the preventive study (Figures 5.4A) and 38% in the recover study (Figure 5.4B). Moreover, treatment with MSO prolonged the estrus stage of the OVX rats supporting the potent estrogenic like activity.

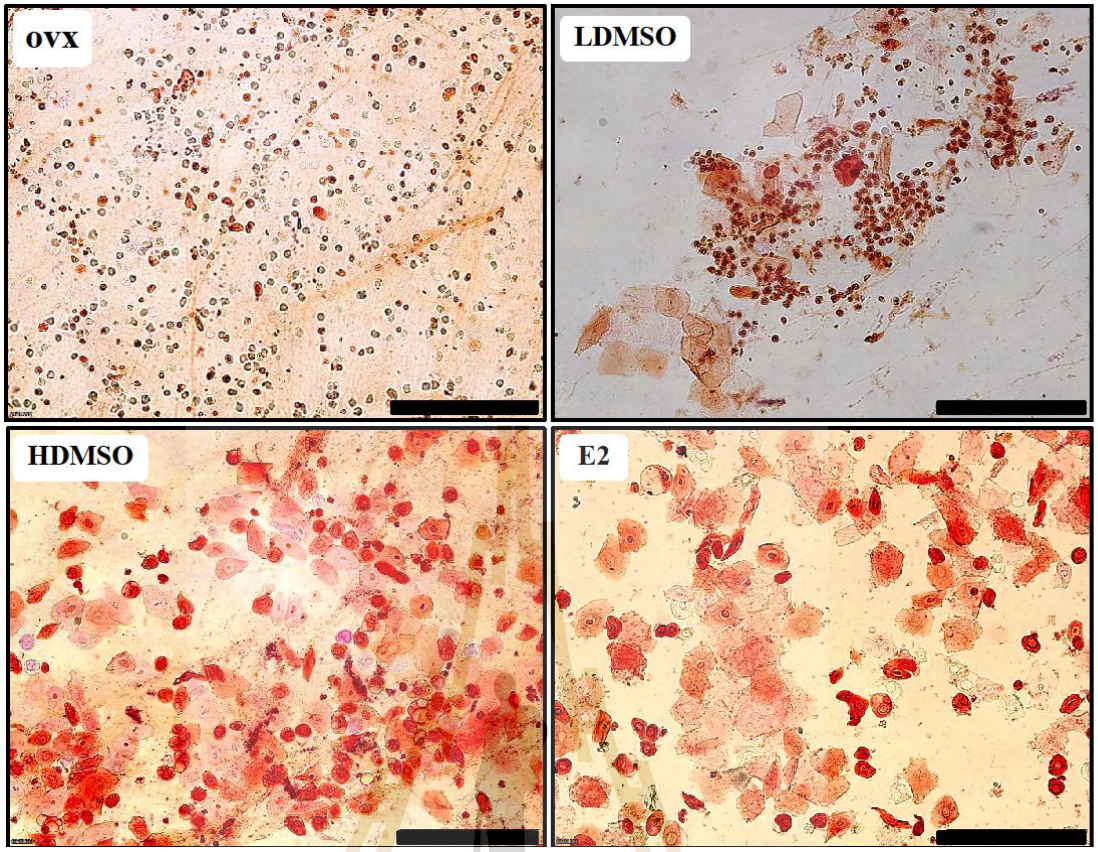

Figure 5.3 The effects of MSO on the estrous cycle on each treatment group; ovariectomized rats untreated (OVX); treated with 0.25 mL/100g BW MSO (LDMSO), treated with 0.50 m/100g BW MSO (HDMSO) and treated with 17β-estradiol (E_2). All vaginal epithelium cell smears were taken on day 28 of administration and stained with safranin O (Bars represent 50 μm, 200x).

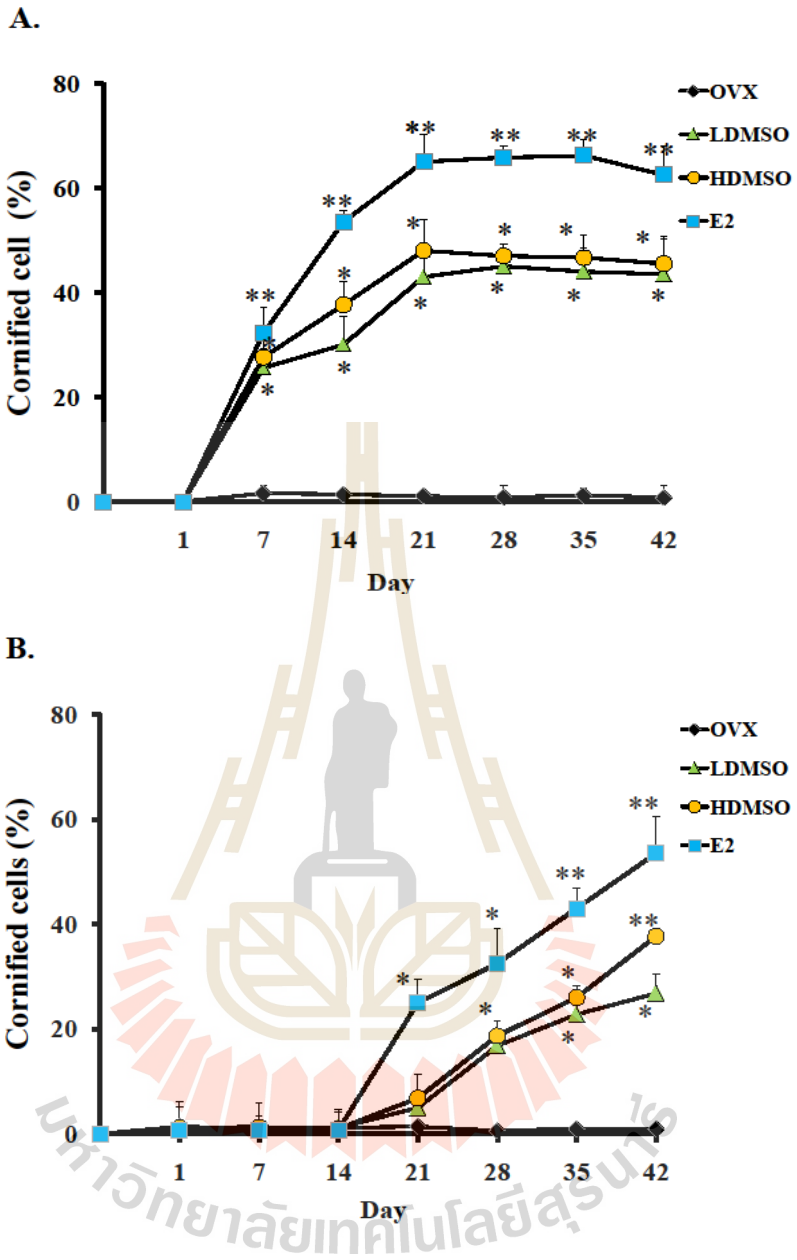

Figure 5.4 Effects of MSO on vaginal cornification in OVX rats between 42 day treatment period; (A) Preventive study group and (B) Recovery study group. Each treatment group are shown: OVX received vehicle, OVX received 0.25 mL/100g BW MSO (LDMSO), OVX received 0.50 mL/100g BW MSO (HDMSO) and OVX received E_2, respectively. Vertical bars represent the SEM (n = 5). $^{*}P < 0.05$, $^{**}P < 0.01$ vs. OVX control.

5.4.3 Effect of MSO on uterine histological changes

Under light microscopic determination, we observed pointed atrophy of the OVX rat uterus to about half of its former size in both study series when compared to SHAM vehicle group. The size increased to near normal in E_2 treated rats. The same results were obtained with MSO treatment indicating that after 6 weeks administration of MSO all doses caused a reverse of uterine atrophy (Figure 5.5A-B). Thus, MSO had a similar effect to E_2 on reversing the atrophy caused by ovariectomy.

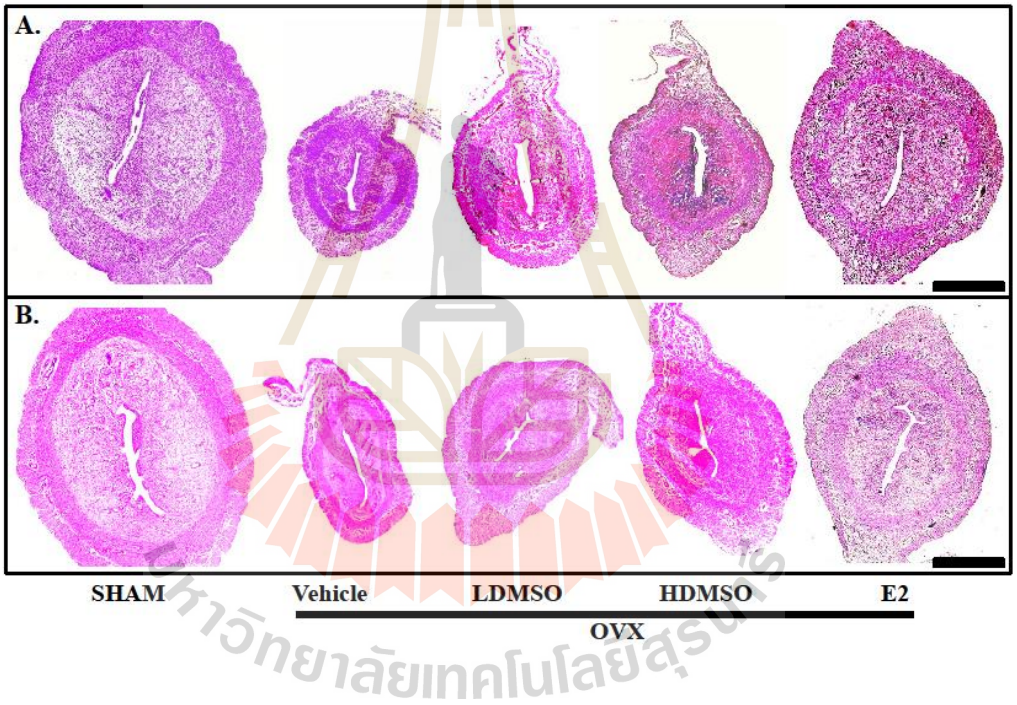

Figure 5.5 Representative photomicrographs of uterus cross sections in the OVX rats treated for 6 weeks from two study series; preventive study (A) and recovery study (B). Each treatment group are shown: SHAM received vehicle, OVX received vehicle, OVX received 0.25 mL/100g BW MSO (LDMSO), OVX received 0.50 mL/100g BW MSO (HDMSO) and OVX received E_2, respectively. Scale bars = 200 μm.

The uterus of SHAM-operated rat showed devious endometrium with tall columnar epithelium, large endometrial glands, and the stroma had a significant infiltration with polymorphonuclear cells. By contrast, the uterus of ovariectomized rats revealed a typical infantile condition with less cuboidal epithelium and the stroma was compacted. The glands were atrophy and vascularity was poor. As expected, administration of E_2 stimulated all the uterine structures as indicated by increases in the epithelial thickness, loose stroma and well developed uterine glands (Figures 5.6A-E, Figure 5.7A-E). Figures 5.6F and 5.7F showed the results of luminal epithelial cell height measurements. The uterus exhibited an increase in epithelial cell height in response to E_2 and MSO in both preventive and recovery studies. The increase in cell height was highest in rats treated with E_2, which induced a significant increase about 4 fold, whereas, OVX received MSO stimulated a significant increase about 2 fold respected to the OVX control group ($P < 0.05$). In both LDMSO and HDMSO, there were a shift from cuboidal to columnar epithelium. Endometrial glands again increased in size and contained a large amount of eosinophilic cytoplasm. However, no significance between each dose of MSO that induced the change of epithelium thickness was found.

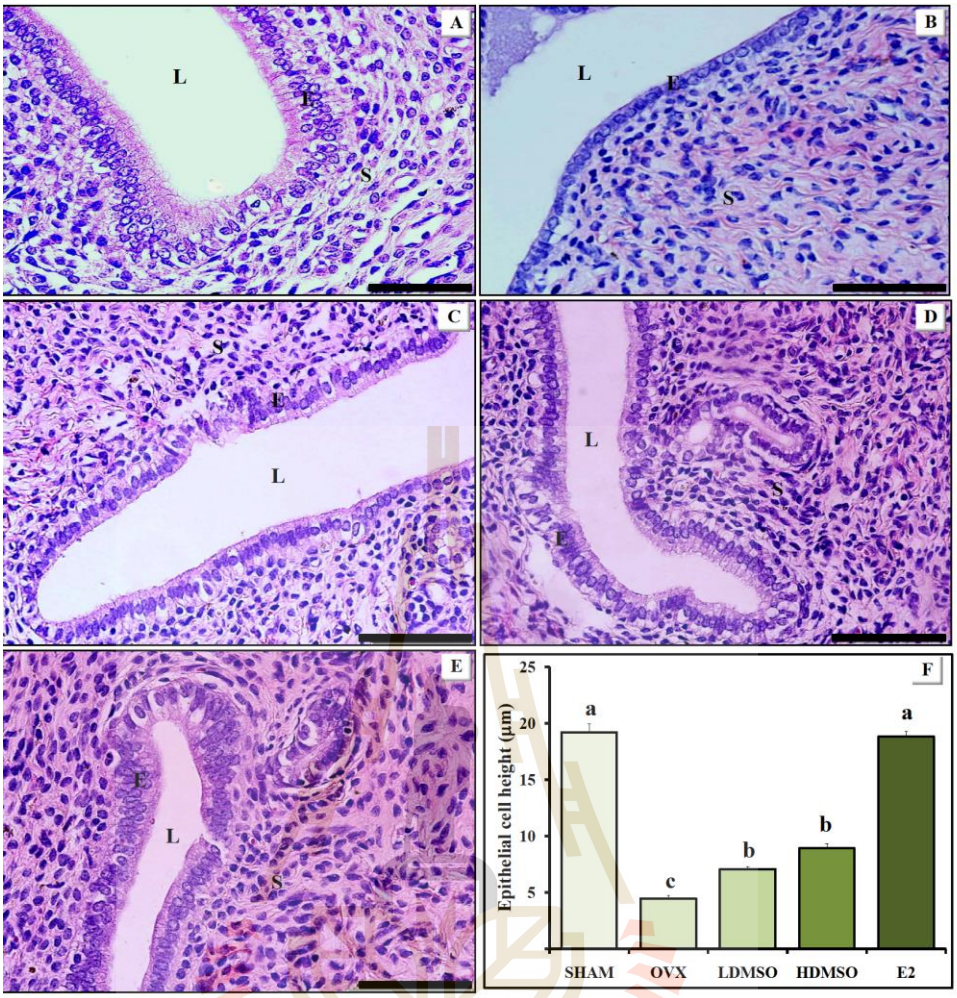

Figure 5.6 Hematoxylin and eosin staining of rats uterine cross sections of preventive study. All rats were administration 3 day after ovariectomy and treated for 42 days. Represent the height of the uterine epithelium (µm) after treated to SHAM received vehicle, OVX received vehicle, OVX received 0.25 mL/100g BW MSO (LDMSO), OVX received 0.50 mL/100g BW MSO (HDMSO) and OVX received E_2, respectively (A-E). S, stroma; L, lumen; E, epithelium. (Bars represent 50 µm). Photomicrographs indicate quantitative determination of the uterine epithelium cell height (F). Values are expressed as mean ± S.E.M. Data were analyzed by one-way ANOVA, followed by Tukey's *post hoc* test. Values that do not share the same superscript letters are significantly different ($P < 0.05$).

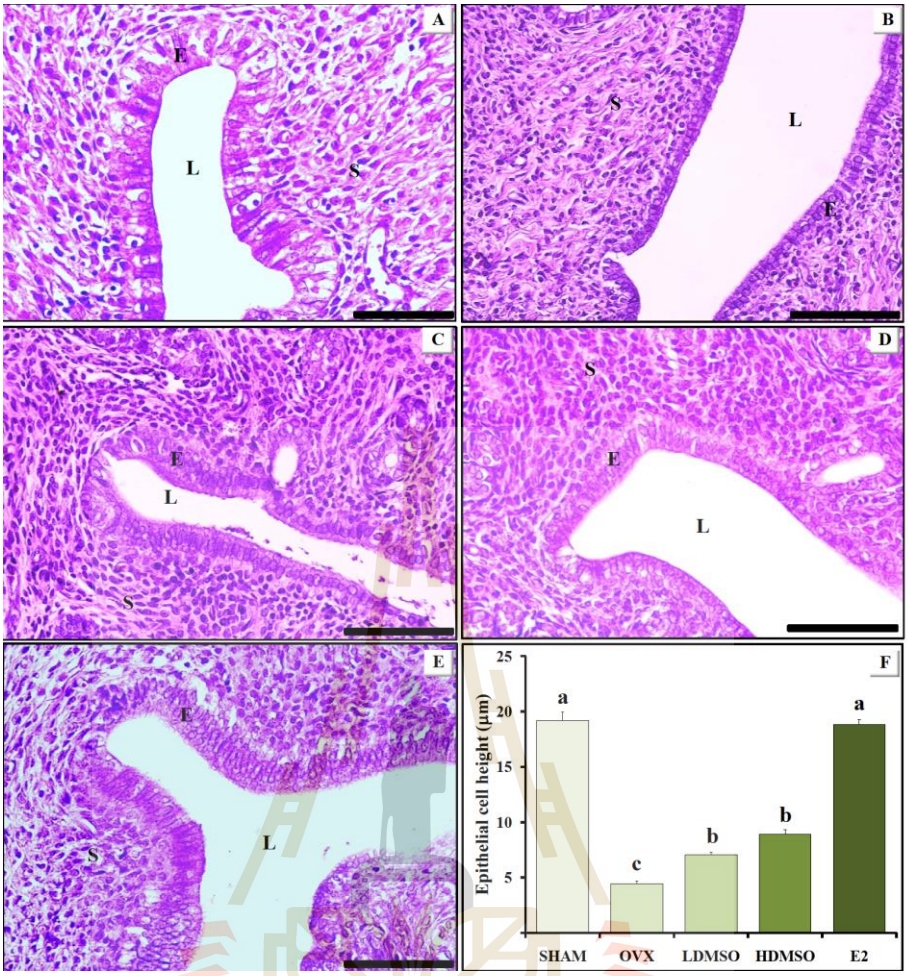

Figure 5.7 Hematoxylin and eosin staining of rats uterine cross sections of recovery study. All rats were administration 60 day after ovariectomy and treated for 42 days. Illustrating the height of the uterine epithelium (µm) after treated to SHAM received vehicle, OVX received vehicle, OVX received 0.25 mL/100g BW MSO (LDMSO), OVX received 0.50 mL/100g BW MSO (HDMSO) and OVX received E_2, respectively (A-E). S, stroma; L, lumen; E, epithelium. (Bars represent 50 µm)**.** Photomicrographs indicate quantitative determination of the uterine epithelium cell height (F). Values are expressed as mean ± S.E.M. Data were analyzed by one-way ANOVA, followed by Tukey's *post hoc* test. Values that do not share the same superscript letters are significantly different ($P < 0.05$).

5.5 Discussion

Phytoestrogens are a diverse class of non-steroidal compounds present in plants that have an affinity for estrogen receptor α and β for the peroxisome proliferator-activated receptor (PPAR) family and for the aryl hydrocarbon receptor (Mueller and Jungbauer, 2008; Mueller et al., 2010; Medjakovic et al., 2010). They are of biological interest because they exhibit estrogenic activity, both *in vitro* and *in vivo* (Adlercreutz, 1999). Moreover, phytoestrogens have long been recognized for their uterotropic activity in a variety of animal species, often showing dose-dependent effects (Jeffersona et al., 2002; Winuthayanon et al., 2009). In the present study, the effects of chronic treatments with MSO on the uterus of ovariectomized rats, particularly concerning its weight, size, morphology and structure, were investigated in comparison with those standard E_2 treatment, both in preventive and recovery studies.

Uterus is a primary target organ for estrogen; it shows enormous morphofunctional variations throughout the woman life span and in particular in reproductive cycle as well as in animal estrous cycle (Diel et al., 2002). It is generally accepted that the uterotrophic assay in ovariectomized rats is a sensitive parameter for determining the estrogenic activity of chemicals or natural compounds *in vivo*. This method is practical and can mimic human or animal consumption of such compounds because hepatic metabolism is involved with the bioactivity of estrogenic testing compounds (Reel et al., 1996; Sookvanichsilp et al., 2008). Estrogen is known to elicit uterine growth response in a non-genomic mechanism (Grunert et al., 1987; Sonnenschein et al., 1998) which involves inducing changes such as increase in vascular permeability, water imbibition, and cellular infiltration (Rockwell et al.,

2002). This sequence of events subsequently leads to an increase in uterine weight. In ovariectomized rats, circulating level of estrogen is very low because of the removal of ovary, which is the primary source of the steroid hormone (Cordial et al., 2006).

This study was investigated the estrogenic activity of MSO in the OVX rats model by applying the variety methods that are recognized. Uterotrophic assay revealed that oral administration of MSO had a significant effect on increasing the absolute uterine weight. The effect of MSO in the uterus of ovariectomized rats strongly indicates that some phytoestrogens of the plant led to an increase in uterine weight. Although, no significance was found between two dosages of MSO. However, the results indicated that MSO enhance stimulating uterotrophic even after ovariectomy up to 60 days and maintained the effect for longer, as seen at the end of treatment period (at day 42 of the experiment).

Nowadays, the vaginal cornification assay in ovariectomized rats has also been demonstrated as a practical means of evaluation of estrogenic activity in phytoestrogen-rich plants (Tsaknis et al., 1999). Estrous cycle is controlled by the synthesis of estrogen in the ovary (Lalas and Tsaknis, 2002). In the quantitative analysis of appearance of cornified cells at day 28 of MSO administration showed estrogen like activity in the ovariectomized rats in both studies. Estrogen also promotes cornification of the vaginal epithelial cells. The full cornification of vaginal epithelial cells requires a higher surge of estrogen level (Safranski et al., 1993). OVX rats treated with all dosages MSO showed an increase in this differentiation pattern. This finding suggests the presence of some components of MSO, which can stimulate estrogen-related changes. The mechanism which may be related E_2-induced vaginal epithelial proliferation is indirectly mediated by stromal $ER\alpha$ because both epithelial

and stromal ERα are required for E_2-induced vaginal epithelial proliferation, cornification, and normal stratification (Buchanan et al., 1999). Furthermore, the luminal epithelial height is well-established end points for evaluating estrogenicity of chemicals (Branham et al., 1993, Diel et al., 2000). Administration of MSO to ovariectomized rats also increased the height of the luminal epithelium. Moreover, it has the capacity to induce proliferative and stimulatory changes in estrogen target tissues that are analogous to those induced by estrogens. This is consistent with the phytochemical analysis which show phytosterols compounds as that might produce their effects through binding to estrogen receptors. Likewise, Alrefaie and colleagues (2010) suggested that E_2 initiates a series of responses in uterine cells in preparation for possibility of pregnancy, including cell hypertrophy and hyperplasia (Alrefaie et al., 2010).

To the best of our knowledge, this is the first report to demonstrate that MSO showed the estrogenic-like properties in postmenopausal rat model. These results clearly displayed the estrogenic responses to MSO by *in vivo* methods. The supporting data from GC-MS analysis of MSO showed the non-steroidal phytoestrogens such as β-sitosterol, stigmasterol, campesterol and stigmast-4-en-3-one similar to other studies that have been reported (Lalas and Tsaknis, 2002; Abdulkarim et al., 2005). The beneficial effects of MSO could be explained by the various bioactive of the many phytoestrogens found in the oil, particularly the non-steroidals (Kuiper et al., 1997; Björnström et al., 2005). It's effects could be due to its direct activity or combination with other mechanisms involving activate and/or not activate classical estrogen signaling classical estrogen signaling (Ahlbory-Dieker et al., 2009; O'Brien et al., 2006). The implications of this study could be appreciated

from both functional and therapeutic perspectives. Albeit the MSO shown in these study, further investigations are required to determine the extract mechanism on how MSO provide beneficial effects on uterine changes.

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CHAPTER VI

EFFECTS OF *MORINGA OLEIFERA* LAM. SEED OIL ON LIPID PROFILES, BONE BIOMARKERS AND REPRODUCTIVE HORMONES IN OVARIECTOMIZED RATS

As shown in the previous chapters, *Moringa oleifera* seed oil (MSO) revealed estrogenic like properties. It is therefore be used as an alternative medicine in prevention and treatment for menopause symptoms. It is also well known that the two most prevalent diseases associated with postmenopausal women are cardiovascular and osteoporosis diseases. Therefore, the examination of physiological changes focusing on lipid profiles, bone biomarkers, and reproductive hormones in ovariectomized (OVX) rats administered with MSO may be lead to more understanding about the roles and mechanisms of the oil in postmenopausal women using OVX rats as a model of study.

6.1 Abstract

Dietary fats and oils are known as macronutrients which provide concentrated source of energy for the metabolic processes. Many vegetable oil has been widely used for various medicinal approaches in hypolipidemic ailment. This chapter evaluated the metabolic effects of *M. oleifera* seed oil (MSO) administration in

ovariectomized (OVX) rats that were fed by MSO on day 3 after ovariectomy (Group 1) and fed MSO on day 60 after ovariectomy (Group 2). Each group was then divided into five subgroups; sham-operated control (SHAM); OVX rats treated with vehicle; OVX treated with MSO 0.25 mL/ 100g BW/day, OVX treated with MSO 0.50 mL/100g BW/day and OVX treated with 17β-estradiol 10 µg/kg BW/day. After 6 weeks of treatment; serum of rats in each treatment group was analyzed to determine lipid profiles [the total cholesterol (TC), triglyceride (TG), low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C)], bone biomarkers [alkaline phosphatase (ALP), serum calcium, serum phosphorus] and female reproductive hormone [Estradiol (E_2) and Luteinizing Hormone (LH)]. The urine samples were also collected for determined urine calcium, phosphorus and creatinine. OVX rats treated with MSO induced significant reductions in LDL-C, increased in HDL-C compared to the OVX-control group with elevating the serum levels of E_2. MSO treated rats restored the elevated serum levels of Ca, P and ALP. The oil also decreased urine Ca and P levels in all OVX groups compared with the SHAM group ($P < 0.05$). Furthermore, MSO could elevate serum E_2 but lowered serum LH concentration. Taken together, these findings suggest that MSO might has some beneficial in reducing plasma cholesterol, LDL-C, and bone biomarkers induced after ovariectomy in rats.

6.2 Introduction

Substantial increases in life expectancy have led to women expecting to live more than a third of their lives after menopause. Women's health and menopause is a rapidly expanding field of medical practice and scientific investigation. It is a field of

great social importance and impact, nationally and globally, in developed as well as developing countries. Depending on the health of the woman, the decline in estrogen that occurs during menopause may not only cause the transient various symptoms, it may also contribute to disease, particularly in women who already have an increased risk because of heredity, illness, or because of poor dietary and lifestyle choices (The National Heart, Lung, and Blood Institute, 2002).

After menopause, coronary heart disease (CHD) has become a major cause of morbidity and mortality in women (Van der Schouw et al., 1996). After the onset of menopause, the risk of CHD in women increases dramatically because of hormone deficiency especially in estrogens (Rosenberg et al., 1981). Decreased ovarian function involved in increased plasma concentrations of total and low-density lipoprotein cholesterol (LDL-C) and in an increased LDL/HDL ratio are among the important risk factors for the development of CHD (Assmann, 1993; Martin et al., 1986). Many research groups have reported that estrogens are important regulators of lipid homeostasis (Mendelsohn and Karas 1999; Basdevant, 1992). The beneficial effects of estrogen might be decreases LDL-C and increases HDL-C production of neurotrophic growth factors, which modulate neuronal growth, survival and aging (Srivastava et al., 2005).

To date, the use of herbal medicine has become a common practice due to the presence of various bioactive phytochemicals in herbal plant, which a medicinal herb with different uses both in traditional and modern medicine. Numerous studies revealed that consumption of some vegetable oil including soybean, rapeseed, flax seed, and walnut oil are affected plasma lipid levels. Corresponding to evidence from Navin and Rajmohan (2004), reported that virgin coconut oil has beneficial effect in

lowering lipid components. It reduced total cholesterol, triglycerides, phospholipids, LDL-C and very low-density lipoprotein (VLDL) cholesterol levels and increased HDL-C cholesterol in serum and tissues rats (Navin and Rajmohan, 2004). Also as flaxseed oil and sesame oil have a beneficial in reducing plasma cholesterol, LDL-C, and bone biomarkers induced by ovarian hormone deficiency in OVX rat (Boulbaroud et al., 2012). Furthermore, reported from pomegranate seed oil research showed some beneficial effects on the antioxidant status and LDL-C concentration after ovariectomy in rats (Tahmasbi et al., 2013). Considering the chemical composition of these oils revealed that they contain analogous phytochemicals that the main active components include vitamin E, phytosterols and unsaturated fatty acid.

The characteristics of MSO can be highly desirable especially with the current trend of replacing polyunsaturated vegetable oils with those containing high amounts of monounsaturated acids (Corbett, 2003). According to various studies, MSO is pleasant tasting, highly edible and resembles olive oil in its fatty acid composition (Ramachandran et al., 1980; Lowell, 1999). In addition it possesses behenic acid, lignoceric acid and traces of lauric n-pentadecanoic and pentadecenoid acids (Ferrao and Ferrao, 1970; Dahot and Memon, 1985). On the other hand, the extracted of MSO revealed various chemical constituents include α, γ, δ-tocopherols, sterol components (β-sitosterol, campesterol, stigmasteroland, Δ5-avenasterol, oleic acid, palmitic, stearic, behenic and arachidic acids (Tsaknis, 1999), which previous evidence indicated that these phytochemical associated with hypolipidemic activities. A variety of mechanisms may account for the effects of dietary fats and oils on bone and lipid

profiles, including alterations in calcium absorption, prostaglandin synthesis, formation of osteoblasts and lipid oxidation (Haag et al., 2003).

Although, the leaves extract and other parts of *M. oleifera* have been reported to hypocholesterolaemic action (Jain et al., 2010; Ghasi et al., 2000) their certain efficacies of MSO have not yet been proven and no scientific report exists about the usefulness of MSO in improving lipid profiles, biochemical parameters and reproductive hormones. Therefore, the main purpose of this chapter was to investigate the effects of administration of the MSO on lipid profiles, biochemical parameters and reproductive hormones in OVX rats.

6.3 Materials and methods

6.3.1 Animals and chemicals

Animals and chemicals were as described in Chapter V.

6.3.2 Experimental procedures

The experimental procedures were divided into two sets, preventive and recovery study detailed as below Table:

Table 6.1 The treatment regiment for lipid profiles, bone biomarkers and reproductive hormones evaluation.

Group	Administration procedure	Duration of treatment (Week)						N
		1	2	3	4	5	6	
Preventive study	3 day after ovariectomy	1. Sham operated rats (SHAM) received vehicle						5
		2. OVX received vehicle						5
		3. OVX received MSO (0.25 mL/100g BW/day)						5
		4. OVX received MSO (0.50 mL/100g BW/day)						5
		5. OVX received Estradiol (10μg/kg BW/day)						5
Recovery study	60 day after ovariectomy	1. Sham operated rats (SHAM) received vehicle						5
		2. OVX received vehicle						5
		3. OVX received MSO (0.25 mL/100g BW/day)						5
		4. OVX received MSO (0.50 mL/100g BW/day)						5
		5. OVX received Estradiol (10 μg/kg BW/day)						5

6.3.3 Collection and preparation of urine and blood samples

To collect urine samples, rats were transferred to individual metabolic cages for a 12 hr. fasting period. During that time, rats had free access to water. Urine was collected from 6.00 PM to 6.00 AM in acid-washed tubes and was frozen at -20°C until required for analysis.

At the end of the fasting period, rats were anesthetized with CO_2 asphyxia. Blood collections for the two studies were performed by cardiac puncture. All blood samples were collected in sample containers immediately and separated by centrifugation 3,000 × *g* for 15 min (4°C), sera were aliquoted into small volumes and stored at -20°C for biochemical determinations.

6.3.4 Determination for biochemistry parameters

Total serum cholesterol, high density lipoprotein cholesterol (HDL-C), low density lipoprotein cholesterol (LDL-C) and triglyceride concentration in serum were measured using diagnostic kits (Raichem). The measurements of lipid profiles were made using Reflotron; Roch Diagnostics GmbH.

The levels of serum calcium (S-Ca), serum phosphorus (S-P) and serum alkaline phosphatase (ALP) activity were measured on an automatic analyzer (Ciba-Corning 550, USA) using a diagnosticreagent kit for the in vitro determination. The urine calcium (U-Ca), phosphorus (U-P) and creatinine (Cr) concentrations were analyzed by the same method as the serum samples (Zhang et al., 2012).

The levels of 17β-estradiol (E_2) and luteinizing (LH) were analyzed by the Electrochemiluminescence rat immunoassay (ECLIA) on Elecsys and cobas e immunoassay analyzers (Roche Diagnostics, USA). Assay procedures were followed the instructions supplied by manufacturer.

6.3.5 Statistical analysis

Data were expressed as positive and negative mean standard deviation. ANOVA test with repeated measures was used to compare each parameter in each group after the treatment. Also, one-way ANOVA and Tukey's *post hoc* test were used to compare groups with each other. In addition, $P < 0.05$ was considered statistically significant.

6.4 Results

6.4.1 Effects of MSO on lipid profiles

Ovariectomy caused a considerable increase in the TC, TG, and LDL-C levels and decreased in HDL-C when compared to SHAM group ($P < 0.05$). OVX treated with E_2 illustrated remain the values of TC, TG, LDL-C and HDL-C resemble to SHAM group. Figure 6.1A-D shows the results of TC and TG, levels in preventive serum samples that were treated with MSO for 6 weeks. There was no significant difference in TC and TG levels between OVX control group and both MSO treated groups. The HDL-C level in serum samples of HDMSO and E_2 treated rats were increased significantly ($P < 0.05$), while, the LDL-C level in these groups were decreased significantly ($P < 0.05$) when compared to control.

In recovery study, the results of TC and TG levels in serum samples of E_2 and LDMSO treated rats were significantly decreased ($P < 0.05$) when respected to OVX control group. Additionally, the LDMSO and HDMSO treatment groups exhibited significant increases in the HDL-C and decreases in the LDL-C levels ($P < 0.05$) when compared to the OVX-control groups (Figure 6.2A-D).

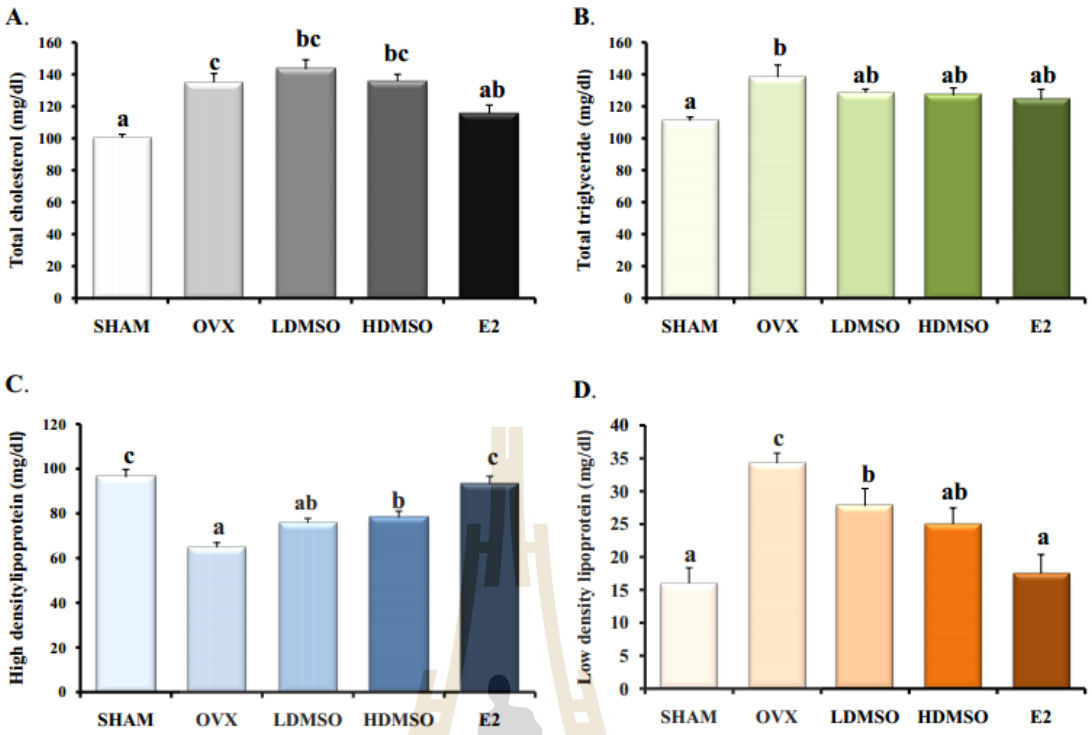

Figure 6.1 Effects of MSO on lipid profiles of preventive study. The total cholesterol level (A), triglyceride level (B), high density lipoprotein level; HDL-C (C) and low density lipoprotein level; LDL-C (D). All treatments are shown; SHAM received vehicle, OVX received vehicle, OVX received 0.25 mL/100g BW MSO (LDMSO), OVX received 0.50 mL/100g BW MSO (HDMSO) and OVX received E_2, respectively. Values are expressed as mean ± S.E.M. Data were analyzed by one-way ANOVA, followed by Tukey's *post hoc* test. Values that do not share the same superscript letters are significantly different ($P < 0.05$).

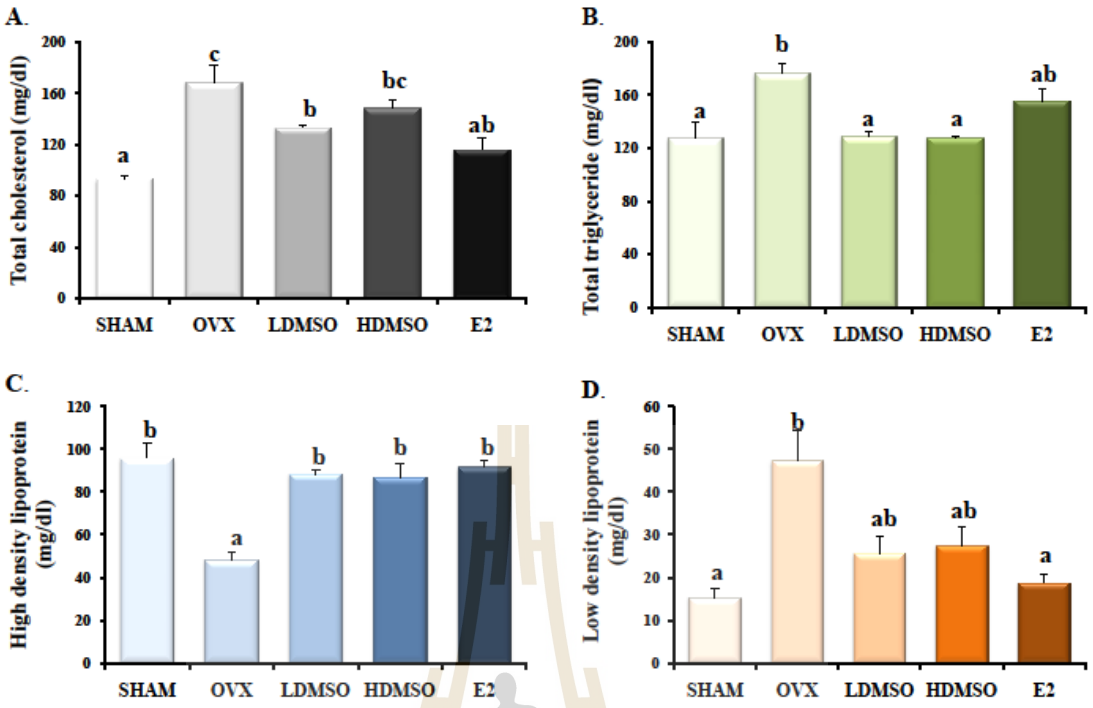

Figure 6.2 Effects of MSO on lipid profiles of recovery study. The total cholesterol level (A), triglyceride level (B), high density lipoprotein level; HDL-C (C) and low density lipoprotein level; LDL-C (D). All treatments are shown; SHAM received vehicle, OVX received vehicle, OVX received 0.25 mL/100g BW MSO (LDMSO), OVX received 0.50 mL/100g BW MSO (HDMSO) and OVX received E_2, respectively for 4 weeks. Values are expressed as mean ± S.E.M. Data were analyzed by one-way ANOVA, followed by Tukey's *post hoc* test. Values that do not share the same superscript letters are significantly different ($P < 0.05$).

6.4.2 Effects of MSO on serum-bone biomarkers

The measured serum ALP, calcium and phosphorus levels in OVX rats that treated with MSO are shown in Table 6.2. Both OVX rats groups from preventive and recovery study showed significant elevated levels of serum ALP compared with SHAM operated control rats ($P < 0.05$). Whereas, OVX rats received E_2 of two groups showed a significant decrease ($P < 0.05$) in ALP activity when compared to OVX control. The administration of all doses of MSO (LDMSO and HDMSO) decreased serum ALP ($P < 0.05$).

After 6 weeks treatment, OVX groups showed significant ($P < 0.05$) changes in decreased serum calcium level when compared to SHAM operated control. The mean calcium concentration in serum was significantly ($P < 0.05$) higher in OVX rats received E_2 and HDMSO treated from preventive study and E_2, LDMSO and HDMSO from recovery study as compared to OVX control animals. Phosphorus concentration in OVX animals was higher ($P < 0.05$) when compared to SHAM groups. OVX animals treated with MSO were slightly higher in serum phosphorus concentration, but there were no significant differences in the serum phosphorus levels among LDMSO and HDMSO groups. A significant ($P < 0.05$) change in serum phosphorous level was observed in OVX rats administered with E_2.

Table 6.2 Effects of MSO on serum-bone biomarkers of ovariectomized rats treated for 6 weeks.

Groups	Treatments	Serum alkaline phosphatase (IU/L)	Serum calcium (mg/dL)	Serum Inorganic phosphorus (mg/dL)
Preventive	SHAM	32.33 ± 11.40^{d}	10.97 ± 0.79^{a}	9.78 ± 0.49^{d}
	OVX	72.33 ± 10.34^{a}	9.15 ± 0.20^{c}	12.20 ± 0.91^{a}
	LDMSO	50.67 ± 8.09^{bc}	9.67 ± 0.16^{bc}	11.75 ± 0.71^{b}
	HDMSO	65.00 ± 10.58^{b}	10.33 ± 0.70^{b}	11.55 ± 0.79^{b}
	E_2	40.67 ± 5.81^{c}	10.42 ± 0.38^{b}	10.89 ± 0.39^{c}
Recovery	SHAM	56.00 ± 5.88^{d}	10.45 ± 0.26^{c}	11.93 ± 0.33^{c}
	OVX	96.00 ± 8.32^{a}	11.50 ± 0.45^{a}	13.62 ± 1.64^{a}
	LDMSO	80.67 ± 9.82^{b}	11.28 ± 0.22^{a}	13.32 ± 1.39^{a}
	HDMSO	94.66 ± 5.33^{b}	10.98 ± 0.22^{b}	11.74 ± 0.92^{c}
	E_2	76.50 ± 4.03^{c}	10.03 ± 0.41^{c}	12.66 ± 1.08^{b}

Values are expressed in mean ± S.E.M. of five animals. Data were analyzed by one-way ANOVA, followed by Tukey's *post hoc* test. Means with different superscripted letters in the same column indicate statistical significance ($P < 0.05$).

6.4.3 Effects of MSO on urine-bone biomarkers

Table 6.3 shows the level of urine calcium, phosphorus and creatinine. The analysis of urine samples of OVX control rats revealed significant ($P < 0.05$) increases in calcium, phosphorous and creatinine concentrations when compared with SHAM control rats. Feeding of OVX rats with E_2 had decreased all parameters to normal values similarly to the samples from SHAM group.

Urine calcium was slightly reduced as compared to OVX control group. In preventive study, there was a tiny alteration in urine calcium in MSO treated animals,

but not statistically significant. Unlike the recovery study that presented significantly increase ($P < 0.05$) in urine calcium level compared with OVX group.

Administration of MSO showed a significant reversal effect on urine phosphorus. In preventive study, the LDMSO, HDMSO and E_2 treated group showed significant decreases ($P < 0.05$), and all those treatments in recovery study represented significant decreases in urine phosphorus level ($P < 0.05$). Furthermore, the urine creatinine level of MSO treated from all treatments in both studies were significantly decreased ($P < 0.05$) when compared to OVX control group.

Table 6.3 Effects of MSO on urine-bone biomarkers of ovariectomized rats treated for 6 weeks.

Groups	**Treatment groups**	**Urinary Calcium (mg/dL)**	**Urinary phosphorus (mg/dL)**	**Urinary creatinine (μmol/L)**
Preventive	SHAM	8.12 ± 0.08^{c}	12.86 ± 2.10^{d}	117.05 ± 1.80^{c}
	OVX	11.57 ± 0.02^{a}	33.37 ± 7.17^{a}	154.83 ± 8.83^{a}
	LDMSO	10.35 ± 0.01^{b}	23.03 ± 3.35^{b}	111.00 ± 5.63^{bc}
	HDMSO	10.10 ± 0.05^{b}	18.76 ± 2.86^{c}	124.23 ± 7.67^{b}
	E_2	9.37 ± 0.01^{b}	15.72 ± 3.13^{d}	117.63 ± 15.13^{c}
Recovery	SHAM	13.07 ± 2.53^{d}	21.80 ± 1.01^{c}	129.40 ± 10.39^{c}
	OVX	28.30 ± 1.05^{a}	33.96 ± 1.48^{a}	168.83 ± 8.65^{a}
	LDMSO	24.60 ± 1.76^{b}	28.13 ± 2.67^{b}	131.00 ± 12.14^{b}
	HDMSO	24.76 ± 1.50^{b}	29.27 ± 2.54^{b}	124.67 ± 11.88^{c}
	E_2	19.05 ± 1.11^{c}	27.16 ± 0.99^{b}	115.17 ± 8.78^{d}

Values are expressed in mean ± S.E.M of five animals. Data were analyzed by one-way ANOVA, followed by Tukey's *post hoc* test. Means with different superscripted letters in the same column indicate statistical significance ($P < 0.05$).

6.4.4 Effects of MSO on female reproductive hormones

The results from all two studies (preventive and recovery) revealed that female reproductive hormones were similar. The E_2 levels of the OVX group were significantly decreased compared with those of the SHAM group ($P < 0.05$). Meanwhile, OVX that received MSO had significant increases in E_2 level as well as the OVX group that received E_2 standard ($P < 0.05$). All preventive treatments; LDMSO, HDMSO or E_2 the E_2 level was increased about 1.7, 2.24 and 2.61 fold compared to that of OVX rats. In recovery study, the E_2 level was increased about 2.24, 3.52 and 4.55 fold compared to that of OVX rats (Figure 6.3A-B).

The serum LH levels of the OVX group were significantly increased compared with those of the SHAM group ($P < 0.05$). Either, LDMSO or HDMSO group had a significant low level of serum LH compared to OVX control group ($P < 0.05$) both in preventive and recovery groups. The OVX group showed a significant low level of LH about 10.33 and 7.86 fold in preventive and recovery respectively when compared to SHAM group.

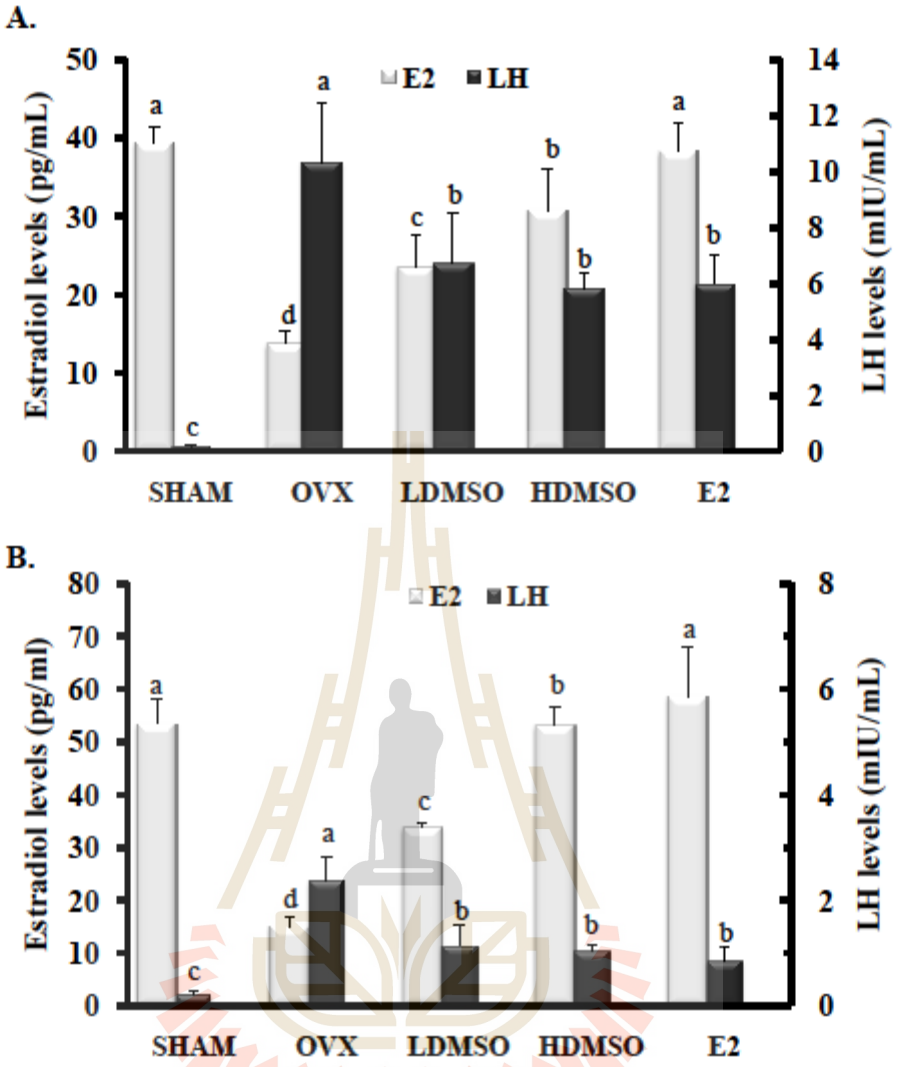

Figure 6.3 The effects of MSO on serum estradiol (E_2) and luteinizing hormone (LH) in preventive group (A) and recovery group (B). Serum levels of E_2 and LH were measured at the end of the treatment period. SHAM received vehicle, OVX received vehicle, OVX received 0.25 mL/100g BW MSO (LDMSO), OVX received 0.50 mL/100g BW MSO (HDMSO) and OVX received E_2, respectively. Bars indicate mean ± S.E.M. (n = 5). Data were analyzed by one-way ANOVA, followed by Tukey's *post hoc* test. Values that do not share the same superscript letters are significantly different ($P < 0.05$).

6.5 Discussion

Various pharmacological studies were carried out on the influence of the menopause on serum lipids and lipoproteins profiles. Estrogen declines precipitously after ovariectomy or menopause. This ovarian hormone deficiency increases the incidence of CVD due to hyperlipidemia, which is characterized by increases in TC, TG, and LDL-C and a reduction in HDL-C (Colditz et al., 1987; Jensen et al., 1990; Shlipak et al., 2000; Bonithon et al., 1990). Many experiments have been made to use herbal extracts to recover serum lipid levels in postmenopausal women suffering from hyperlipidemia associated with CVD (Lucus et al., 2001; Hidaka et al., 2004; Park et al., 2011).

It has been reported that ovariectomy caused an elevation of TC and LDL-C in serum, leading to the development of atherosclerosis and coronary heart disease (Park et al., 2014). As well as lipid and lipoprotein metabolism is markedly altered in postmenopausal women where elevated total cholesterol, LDL-C, VLDL-C and elevated lipoprotein a values have been observed (Signorelli et al., 2006). Estrogen has been shown to decrease LDL-C and increase high density lipoprotein HDL-C levels. Furthermore, during the menopause there is not only a reduction in the number of LDL-C liver receptors but also a decrease in the activity of the remaining receptors, and this may explain the increased lipoprotein and lipid concentrations in postmenopausal women (Walsh et al., 1991; Berg et al., 1996).

In the present study, MSO treatment restored LDL-C, HDL-C to control levels and no significant differences were observed between OVX treated with MSO and SHAM group clearly indicated in recovery study. The results from both groups indicated that oral administration of MSO for 6 weeks in OVX rats have

hypolipidemic effect and causes beneficial changes of cholesterol, HDL-C and LDL-C levels. Concerning addition of MSO to the OVX rats, these finding are different from some extents with previous reports showing that rats fed with corn oil and palm oil diet had significantly decreased in serum HDL-C (Shad et al., 2002; Scholtz et al., 2004). Nevertheless, our results resemble to the findings of Hansan and colleagues (2013) who elucidated the effect of soybean oil (SbO) and sesame oil (SO) supplemented on lipid profiles changes in OVX rats. They proposed that these two oils significantly increased the HDL-C level when compared to OVX rats. Additionally, Lim and coworkers (2014) indicated that feeding of *Citrus unshiu* peel extract to OVX rats have multiple metabolic benefits. It also inhibited the increase in lipoprotein levels and tended to increase HDL-C level compared to the OVX-control group.

These evidence may be possibly explains on the hypolipidemic effect of plant oils might be act through sterols composition. Phytosterols are naturally occurring compounds structurally related to cholesterol with different side chain configurations. Some phytosterols in the diet may have a role in modulating hormone related diseases based on their structural similarity to 17β-estradiol the main female estrogen in human (Barnes, 2004). Much information has shown that the main mechanism responsible for the cholesterol-lowering effect of free and esterified plant sterols is the inhibition of intestinal cholesterol absorption. Furthermore, different mechanisms such as competition with cholesterol for solubilisation in dietary mixed micelles, co-crystallisation with cholesterol to form insoluble mixed crystals, and interference with the hydrolysis process by lipases and cholesterol esterases are believed to contribute to the lowering of serum cholesterol concentrations by plant sterols (Brown, 2011).

There is also emerging evidence that plant sterols interfere with transport-mediated processes of cholesterol uptake. The consequence of all these actions, although their individual contribution on the overall effect remains to be established, is that intestinal cholesterol absorption is reduced, while more cholesterol is excreted in the feces (Trautwein et al., 2003).

Thus, the mechanism(s) of hypolipidemic activity of MSO could be possibly explained by presence of phytosterol that were according to many researches. Like Meguro et al. (2001) has explained several mechanisms about the cholesterol-lowering activity of plant sterols. It was reported that plant sterols which are structurally similar to cholesterol could displace cholesterol from mixed micelles, since they are more hydrophobic than cholesterol. This replacement causes a reduction of micellar cholesterol concentration and consequently lowers cholesterol absorption (Meguro et al., 2001). Furthermore, dietary oils and fats are composed different types of fatty acids (FA). These results may be possibly explains on the basis that MSO is rich in unsaturated fatty acids (UNSFAs), especially polyunsaturated fatty acids (PUSFAs) (Babatunde et al., 2011). PUSFAs stimulate the catabolic rate of LDL-C, thus resulting in the reduction of serum LDL-C (Choi et al., 1993). Therefore, it is possible that MSO could decrease the storage of cholesterol though this all mechanism mentioned above.

Further investigations regarding blood and urine biochemical parameters were also recorded in OVX rats. It is well known that calcium and phosphorus are widely accepted as phenotype markers for bone formation (Evans et al., 1990). Also, the present work indicated that the levels of serum and urinary Ca and P in OVX rats were significantly higher than the control group. These results are in agreement with

Elkomy et al. (2013), who recorded that the levels of serum Ca and P were increased in the ovariectomized rats. In the present study feeding of MSO to OVX rats significantly restored the decreased serum and urinary calcium and phosphorus levels induced by ovariectomy to normal levels, while it decreased urine calcium and phosphorous concentrations. These results suggest that oral supplementation with MSO were more effective in inhibiting bone resorption and in increasing bone formation. These findings were similar to those of Hansan et al. (2013) who proposed that dietary soybean, flaxseed and sesame oils induce hypocholesterolemic and anti-osteoporotic effects in ovariectomized rats. That those vegetable oils can effectively improve ovariectomy-induced osteoporosis in rats therefore they are considered promising natural dietary supplements for the treatment of postmenopausal osteoporosis in women. In a like manner with previous trials of Shuid et al. (2007), Boulbaroud et al. (2008) and Byun and Lee (2010) who reported that vegetable oils contain omega-3 and omega-6 polyunsaturated fatty acids which increased serum calcium and phosphorus concentrations and reduce urinary calcium and phosphorous excretion, thus enhanced bone formation.

Additionally, the biochemical parameters of bone formation (ALP) were also evaluated in this study. ALP is a non-collagenous protein secreted by osteoblast, which is essential for bone mineralization (Havill et al., 2006). In OVX group, the ALP activities were increased compared with the SHAM control. The increase in alkaline phosphatase can probably be related to abnormal bone formation and stimulated osteoblastic activity with increased ALP concentration in the serum (Farley et al., 1987). The administration of MSO improves these parameters, and might reduce bone resorption and increases the bone formation (Boulbaroud et al.,

2008). The beneficial effect of MSO on reduction of alkaline phosphatase activity might be due to the presence of high calcium content in the oil (Ishola et al., 1990; El-Siddig et al., 2006). The present study revealed that the raised bone ALP activity occurring with ovariectomy could contribute to high bone turnover rate, being characterized by an increase in both bone resorption and formation, but bone resorption excesses formation, leading to bone loss (Elwakf et al., 2014) thus, indicating an increase in the osteoblastic and osteoclastic activity, respectively, resulting in an overall net loss of bone with an increase in the excretion of urinary. The positive role of medical herbs supplemented diets was also achieved by the observed improvement of bone metabolic markers ALP and acid phosphatase (ACP) (Chiechi et al., 2002). Phytoestrogenic herbs exhibited comparable effects regarding all tested parameters, indicating almost similar ability of herbs to protect against bone loss (Boulbaroud et al., 2008). Furthermore, the creatinine results also showed significant differences increased in OVX groups compared with the SHAM control animals. The levels of creatinine in the urine may point to a kidney disease, certain muscular and neuromuscular disorders, or a blockage in the urinary tract. The MSO administration in OVX rats for six weeks exhibits the potential to improve urinary creatinine level such as E_2. However, there is need to evaluate in the long-term consumption of this oil extract for evaluated nephrotoxicity. Further studies are needed to evaluate the mechanism(s) by which supplemented by these oil can modulate bone tissue.

The current modulating effects of MSO supplemented on hormonal changes in OVX rats may be attributed to phytochemical constituents. In the view of female reproductive hormone change, another evidence reported that phytoestrogens have the

beneficial effect of restoring the profile of such sex hormones as E_2, FSH and LH in women. FSH and LH are key stimulators for follicular development. The preovulation secretion of FSH and LH is negatively regulated by circulating E_2 via the feedback control system of the hypothalamic-pituitary-ovarian axis (Arai et al., 1997; Zhang et al., 2014). The ovariectomy in female rats induced an abrupt increase in FSH level but a gradual increase in LH level. These increases are largely attributable to the removal of estradiol and inhibin, negative feedback regulators (Malaivijitnond et al., 2006). In the current study, MSO significant increased serum E_2 levels and moderated the illuminated serum LH levels resulting from the removal of E_2 in OVX rats, indicating that MSO has an efficacious role in regulating hypothalamic-pituitary function (Zhang et al., 2014).

Conclusively, these results demonstrated that MSO has multiple metabolic benefits in OVX rats. The oral administrationof MSO tended to reduced LDL-C levels and raised HDL-C levels and restored the elevated serum levels of Ca, P and ALP. These oils also decreased urinary Ca, P and creatinine. Furthermore, MSO could elevated serum estradiol but lowered serum LH concentration. Taken together, MSO could have protective effects against cardiovascular disease and anti-osteoporosis after menopause.

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CHAPTER VII

EFFECTS OF *MORINGA OLEIFERA* LAM. SEED OIL ON BONE QUALITY

Determination of bone biochemical markers in Chapter VI indicated that OVX rats treated by *M. oleifera* seed oil (MSO) could restore the levels of calcium (Ca), phosphorus (P) and alkaline phosphatase (ALP) and decrease urine Ca and P levels. It is interesting that oral administration of MSO may be related to osteoporosis prevention. Therefore, the effects of this oil on anti-osteoporosis action in osteoporosis model induced by ovariectomy will be focused in this Chapter.

7.1 Abstract

Several animal and clinical studies have shown that phytoestrogens, plant derived estrogenic compounds can be useful in treating postmenopausal osteoporosis. The purpose of this study was to determine the effects of MSO on ovariectomy-induced osteoporosis in rats. A total of twenty-five (3-month-old) female Wistar rats were used and randomly divided into sham-operated group and four ovariectomized (OVX) groups. Daily oral administration of 17β-estradiol (E_2) or MSO began 8 weeks after the surgery. At the end of the study, the changes of bone mineral density (BMD) and bone mineral content (BMC) were measured using peripheral Quantitative Computerized Tomography (pQCT). The results demonstrate that ovariectomy decreased BMD of trabecular tibial and femoral metaphysis and decreased BMC of

cortical tibial metaphysis, but had no effect on BMD and BMC in other compartments. Oral treatment with E_2 significantly increased BMD of trabecular tibial metaphysis, femoral metaphysis and BMC of trabecular tibial metaphysis. In MSO treated group BMD and BMC tended to increase, but the increase did not differ when compared to OVX rats. The proximal tibia histomorphometric in MSO treated OVX rats was similar to E_2-treated OVX rats, but less than SHAM controls. These suggest that administration of MSO in OVX rats for six weeks could prevent the increases in bone turnover and the decrease in bone mass.

7.2 Introduction

Osteoporosis is one of the most frequent diseases affecting one in ten people in the world and one in three women in their fifties. It is a systemic, chronic and metabolic bone disease characterized by a reduction and deterioration of the micro-architecture of bone tissue, with a consequent increase in bone frailty and susceptibility to fractures (Renno et al., 2006; Genant et al., 2007). Estrogen deficiency is responsible for bone loss in postmenopausal women. It is now accepted that postmenopausal osteoporosis should be regarded as a product of inflammatory disease triggered by estrogen deficiency (Chung et al., 2006). Hormone deficiency impair scancellous metaphyseal bone, which reduces of bone mineral density (BMD) and bone mineral content (BMC) in humans and animals (Riggs et al., 1982; Ohta et al., 2002). Hormone replacement therapy (HRT) can prevent the early phase of involutional bone loss and also restore the rate of bone resorption and formation to premenopausal levels in menopausal women (Thompson et al., 1995; Ahlborg et al., 2003; Curtis et al., 2005).

Todate, the pharmacological treatments including HRT, selective estrogen receptor modulators (SERMs) and bisphosphonate are the methods of choice for preventing osteoporosis after menopause. These methods have been shown to be effective either in increasing BMD and/or reducing fracture rates (Yoon et al., 2012). However, the side effects of these medications, including gastrointestinal tolerance problems in bisphosphonate and the potential malignancies in HRT (Kavancu et al., 2003; Tuner, 2001), may preclude their long-term use. Growing evidence of the benefits of natural foods for bone health provide an alternative option for prevention and/or treatment of osteoporosis. Among the therapeutic choice, phytoestrogens are estrogen agonists and can be effective especially when presence of estrogen is decreased (Mori- Okamoto et al., 2004).

Recently, various researches reported the estrogenic effects of plant oils on bone loss in ovariectomy rats (Saravani et al., 2014). The amount and the type of oils in the diet can have important effects on bone health. *M. oleifera* is a native plant tree that is native to the Himalayan Mountains of northern India and widely growing in Thailand. One of the most remarkable characteristics of moringa fruit is that its seeds are rich with estrogens. Moringa seeds are known to contain the estrogenic compounds that are chemically identical to those biosynthesized in human body. Since MSO contains phytoestrogens, this prediction may be true that MSO is able to prevent bone loss in postmenopausal women. In the present study, bone loss in female rats is induced by ovariectomy. The determination of bone loss therapy after MSO treatment has been performed in long bones, in trabecular and cortical compartments at metaphyseal and diaphyseal sites. Therefore, the main objective of this chapter was to investigate the effects of MSO on BMD and BMC in OVX rats.

7.3 Materials and methods

7.3.1 Experimental animals

This study was carried out with 25 female Wistar rats weighing 200-250 g. They were reared and maintained individually in an environmentally controlled animal laboratory (12 hr. light/dark schedule at 25 ± 2°C).The animals were either subjected to ovariectomy or to sham operation under ether anesthesia.

7.3.2 Experimental design

The rats were randomized into five groups. One group of rats was sham operated, while the rats of the other four groups were ovariectomized. The beginning of treatment started two months after OVX and sham operations. All treatments were as follows:

Group 1 (SHAM): Sham-operated controls received vehicle (1% Tween 80 in water).

Group 2 (OVX): OVX controls received vehicle (1% Tween 80 in water).

Group 3 (LDMSO): OVX treated with MSO 0.25 mL/100g BW/day.

Group 4 (HDMSO): OVX treated with MSO 0.50 mL/100g BW/day.

Group 5 (E_2): OVX treated with 17β-estradiol 10 µg/kg BW/day.

At the end of six weeks treatment period, all rats were sacrificed. Their right tibia and right femur were defleshed from adjacent tissues, wrapped in saline-soaked gauze bandages to prevent dehydration, and stored frozen at -20°C in small ziploc bags until the BMD and BMC were measured. The left tibiae and femoral were also used for histopathological study.

7.3.3 Bone measurement

Bone mineral density (BMD) and content were measured using peripheral quantitative computed tomography (pQCT) in the research M mode (XCT Research SA^+, Stratec Medizintechnik GmbH, Germany). The determination was performed in longbones (tibia and femur), in cortical and trabecular compartments, and in metaphyseal and diaphyseal sites. The trabecular BMD, trabecular BMC, cortical BMD, and cortical BMC for the tibial metaphysis and femoral metaphysis; and the cortical BMD and cortical BMC for the tibial diaphysis and femoral diaphysis were analyzed for each bone slice using XCT-5.50E software (Stratec Medizintechnik GmbH, Germany). The average of three scans made at all bone sites described above was analyzed.

7.3.4 Bone histology

After rats were sacrificed, the right proximal tibiae of sham rats and ovariectomized rats treated in all groups were selected for histopathological study. Bones were defleshed and placed in 10% phosphate-formalin buffer for at least 72 hr. Bones were cut to a small size and then decalcified in EDTA-G solution (EDTA disodium salt 14.50 g, NaOH 1.25 g, glycerol 15 mL and distilled water 100 mL) for 3 weeks by changing EDTA-G solution very week. After 3 weeks, the decalcified boneswere dehydrated in series of ethanol gradient and clearing in xylene. They were then embedded in paraffin, cut into section of 5 μm thickness, and stained with hematoxylin and eosin. The slides were analyzed under a Nikon Eclipse 80i Upright microscope (Hollywood International Ltd., Thailand) and Cell^D imaging software (Olympus, EforL International Co., Ltd., Thailand). The method of bone histopathological study was modified from Urasopon et al. (2008).

7.3.5 Statistical analysis

Results were reported as mean ± S.E.M. for each group. The significant differences between groups were examined by one-way analysis of variance followed by Tukey's protected least significant difference test. Differences were considered to be significance at the level of $P < 0.05$. The Statistical Packages for Social Science (SPSS) (version SPSS/PC 11.0, Chicago IL) was used.

7.4 Results

7.4.1 Bone mineral density (BMD)

The pQCT was used to evaluate the BMD of rats, which can separately examine cortical and trabecular bone changes. The effects of MSO on the BMD of all treatment groups are shown in Table 7.1. After ovariectomy for eight weeks, the trabecular BMDs in tibial metaphysis and femoral metaphysis of OVX rats were significantly decreased ($P < 0.05$, $P < 0.05$, respectively) when compared to SHAM rats. BMD were significantly increased in OVX rats treated with E_2 ($P < 0.05$) when compared to OVX rats. On the other hand, BMD of tibial metaphysis and femoral metaphysic were slightly increased in OVX that received MSO, but did not differ among groups (Table 7.1).

The cortical BMDs in all treatment groups are shown in Figure 7.2. The cortical BMDs of the tibial metaphysis, femoral metaphysis and femoral diaphysis in all treatment groups were slightly decreased, excepting for the tibial diaphysis, where the cortical BMD was decreased and significantly ($P < 0.05$) when compared to SHAM control rats. Effects of MSO treatment were not found in cortical BMDs of the tibial metaphysis, femoral metaphysis, tibial diaphysis and femoral diaphysis

compared with those of OVX rats. It is interesting to note that the effects of E_2 treatment on cortical BMDs were comparable to that of OVX treatment. It increased the cortical BMDs, but not significant (Figure 7.2).

Table 7.1 Effects of MSO on BMD.

Groups	BMD (10^3 mg/cm^3)					
	Trabecular bone		Cortical bone			
	Tibial metaphysis	Femoral metaphysis	Tibial metaphysis	Femoral metaphysis	Tibial diaphysis	Femoral diaphysis
SHAM	0.41 ± 0.01^{a}	0.54 ± 0.02^{a}	1.15 ± 0.02^{a}	0.97 ± 0.03^{a}	1.71 ± 0.05^{a}	1.38 ± 0.08^{a}
OVX	0.22 ± 0.02^{c}	0.35 ± 0.02^{c}	1.09 ± 0.05^{a}	0.94 ± 0.02^{a}	1.35 ± 0.08^{b}	1.11 ± 0.02^{b}
LDMSO	0.27 ± 0.06^{bc}	0.36 ± 0.04^{bc}	1.09 ± 0.02^{a}	0.95 ± 0.05^{a}	1.36 ± 0.09^{ab}	1.29 ± 0.09^{a}
HDMSO	0.26 ± 0.05^{bc}	0.38 ± 0.05^{bc}	1.12 ± 0.04^{a}	0.96 ± 0.04^{a}	1.38 ± 0.07^{ab}	1.31 ± 0.07^{a}
E_2	0.35 ± 0.05^{b}	0.43 ± 0.06^{b}	1.13 ± 0.01^{a}	0.96 ± 0.01^{a}	1.38 ± 0.09^{ab}	1.32 ± 0.08^{a}

All values are expressed as mean ± S.E.M. Data were analyzed by one-way ANOVA, followed by Tukey's *post hoc* test. Means with different superscripted letters in the same column indicate statistical significance ($P < 0.05$).

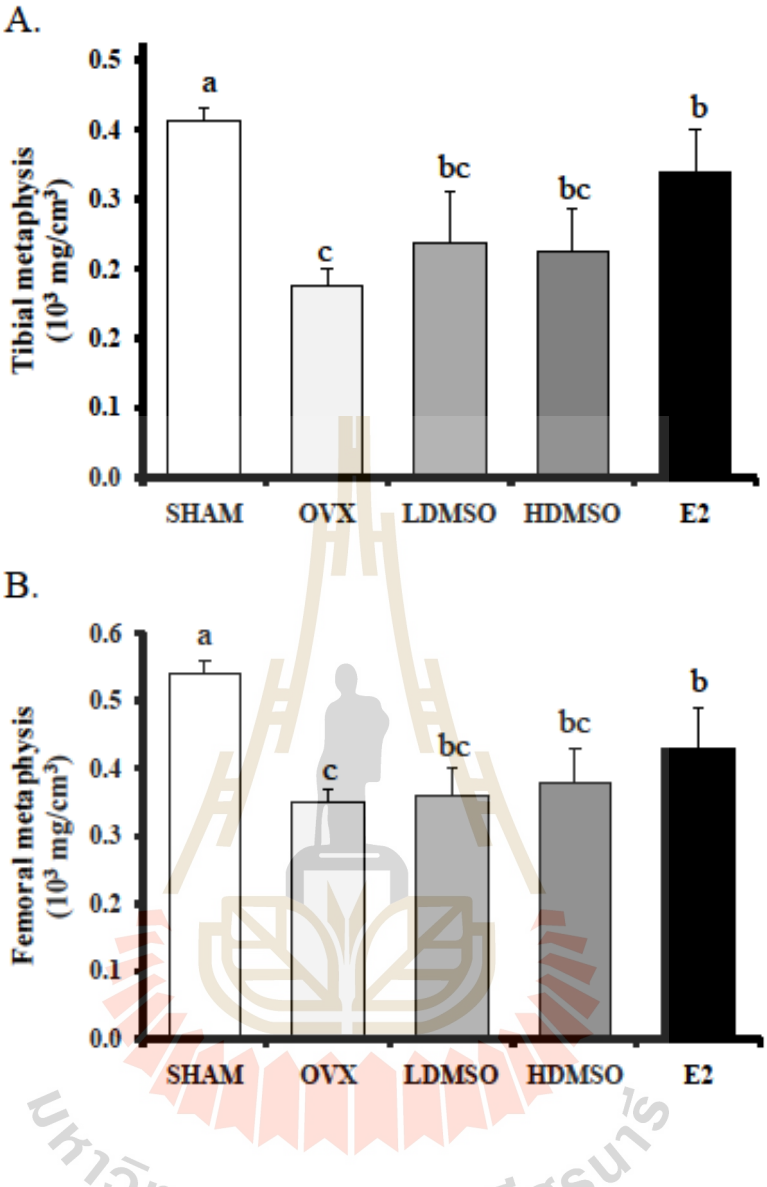

Figure 7.1 Trabecular BMDs of the tibial metaphysic and femoral metaphysis in sham operated control (SHAM), ovariectomized rats control (OVX), OVX treated with 0.25 mL/100g BW/day of MSO (LDMSO), OVX treated with 0.50 mL/100g BW/day of MSO (HDMSO) and OVX treated with 17β-estradiol (E_2), respectively. Bone mineral contents were determined at the end of the 6 weeks after treatments. Bars indicate mean ± S.E.M. (n = 5). Data were analyzed by one-way ANOVA, followed by Tukey's *post hoc* test. Values that do not share the same superscript letters are significantly different ($P < 0.05$).

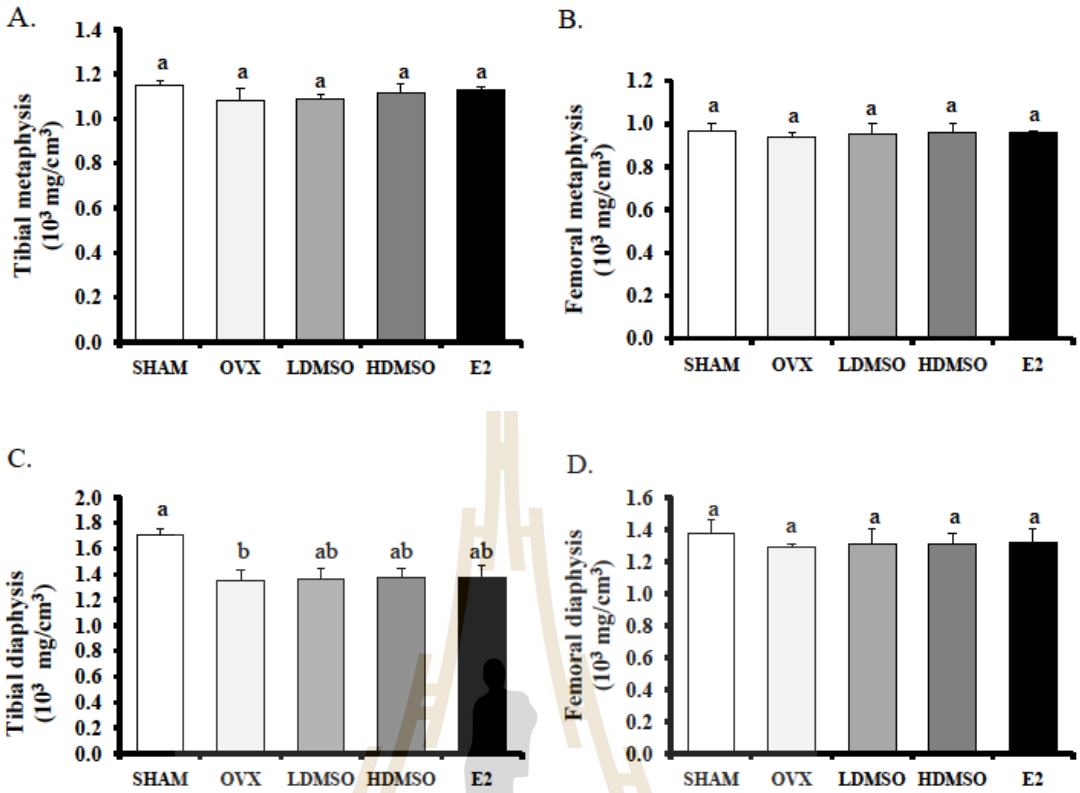

Figure 7.2 Cortical BMDs of the tibial metaphysis, femoral metaphysis, tibial diaphysis and femoral diaphysis in sham operated control (SHAM), ovariectomized rats control (OVX), OVX treated with 0.25 mL/100g BW/day of MSO (LDMSO), OVX treated with 0.50 mL/100g BW/day of MSO (HDMSO) and OVX treated with 17β-estradiol (E_2), respectively. Bone mineral contents were determined at the end of the 6 weeks after treatments. Bars indicate mean ± S.E.M. (n = 5). Data were analyzed by one-way ANOVA, followed by Tukey's *post hoc* test. Values that do not share the same superscript letters are significantly different ($P < 0.05$).

7.4.2 Bone mineral content (BMC)

The effects of MSO on BMC or bone mass in ovariectomized rats were determined. The results for BMCs in all compartments are shown in Table 7.2. The

trabecular BMCs of tibial metaphysis and femoral metaphysis were not changed, excepting for tibial metaphysis of OVX treatment that was significantly decreased ($P < 0.05$) when compared to SHAM rats. Trabecular BMCs of tibial metaphysis were significantly increased ($P < 0.05$) in OVX treated with E_2 when compared to OVX control rats (Figure 7.3).

The cortical BMCs in all treatment groups are shown in Figure 7.4. Six weeks of treatment period, rats in OVX group exhibited no changes of cortical BMCs in all bone sites in comparison with those of the SHAM group. The MSO and E_2 treatments showed no effects on cortical BMCs in all bone sites. However, in femoral diaphysis site, cortical BMCs was significantly increased in E_2 treated ($P < 0.05$) when compared to OVX rats.

Table 7.2 Effects of MSO on BMC.

Groups	BMC (mg/mm)					
	Trabecular bone		Cortical bone			
	Tibial metaphysis	Femoral metaphysis	Tibial metaphysis	Femoral metaphysis	Tibial diaphysis	Femoral diaphysis
SHAM	2.89 ± 0.18^{a}	1.54 ± 0.01^{a}	7.60 ± 0.55^{a}	10.09 ± 0.61^{a}	6.03 ± 0.03^{c}	10.78 ± 0.36^{b}
OVX	1.77 ± 0.43^{c}	1.27 ± 0.03^{b}	6.94 ± 0.27^{a}	6.68 ± 0.23^{b}	5.45 ± 0.05^{b}	9.66 ± 0.28^{c}
LDMSO	2.03 ± 0.36^{bc}	1.29 ± 0.02^{b}	6.72 ± 0.29^{a}	7.00 ± 0.54^{bc}	5.88 ± 0.07^{ac}	10.80 ± 0.16^{ab}
HDMSO	2.13 ± 0.22^{bc}	1.32 ± 0.02^{b}	7.32 ± 0.27^{a}	7.36 ± 0.31^{bc}	5.92 ± 0.06^{ac}	10.88 ± 0.16^{ab}
E_2	2.39 ± 0.25^{b}	1.35 ± 0.03^{b}	7.92 ± 0.19^{a}	7.89 ± 0.46^{bc}	6.32 ± 0.06^{a}	12.34 ± 0.18^{a}

All values are expressed as mean ± S.E.M. Data were analyzed by one-way ANOVA, followed by Tukey's *post hoc* test. Means with different superscripted letters in the same column indicate statistical significance ($P < 0.05$).

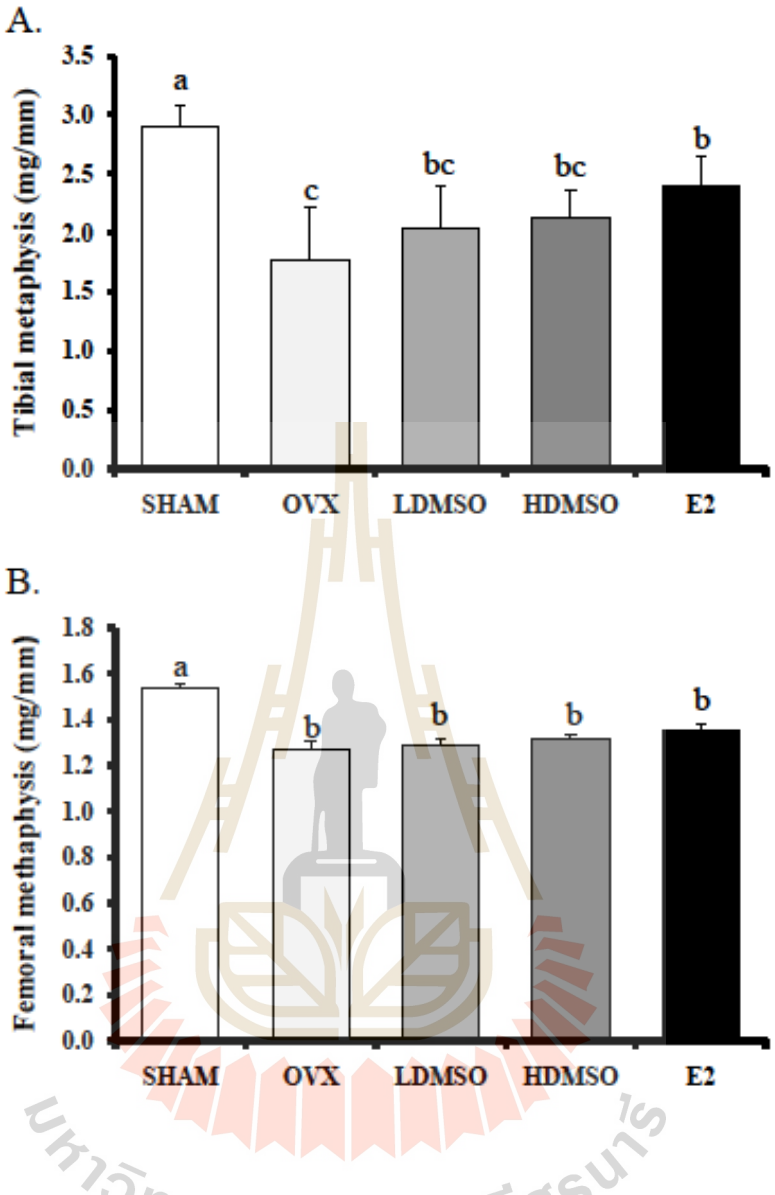

Figure 7.3 Trabecular BMCs of the tibial metaphysis and femoral metaphysis in sham operated control (SHAM), ovariectomized rats control (OVX), OVX treated with 0.25 mL/100g BW/day of MSO (LDMSO), OVX treated with 0.50 mL/100g BW/day of MSO (HDMSO) and OVX treated with 17β-estradiol (E_2), respectively. Bone mineral contents were determined at the end of the 6 weeks after treatments. Bars indicate mean ± S.E.M. (n = 5). Data were analyzed by one-way ANOVA, followed by Tukey's *post hoc* test. Values that do not share the same superscript letters are significantly different ($P < 0.05$).

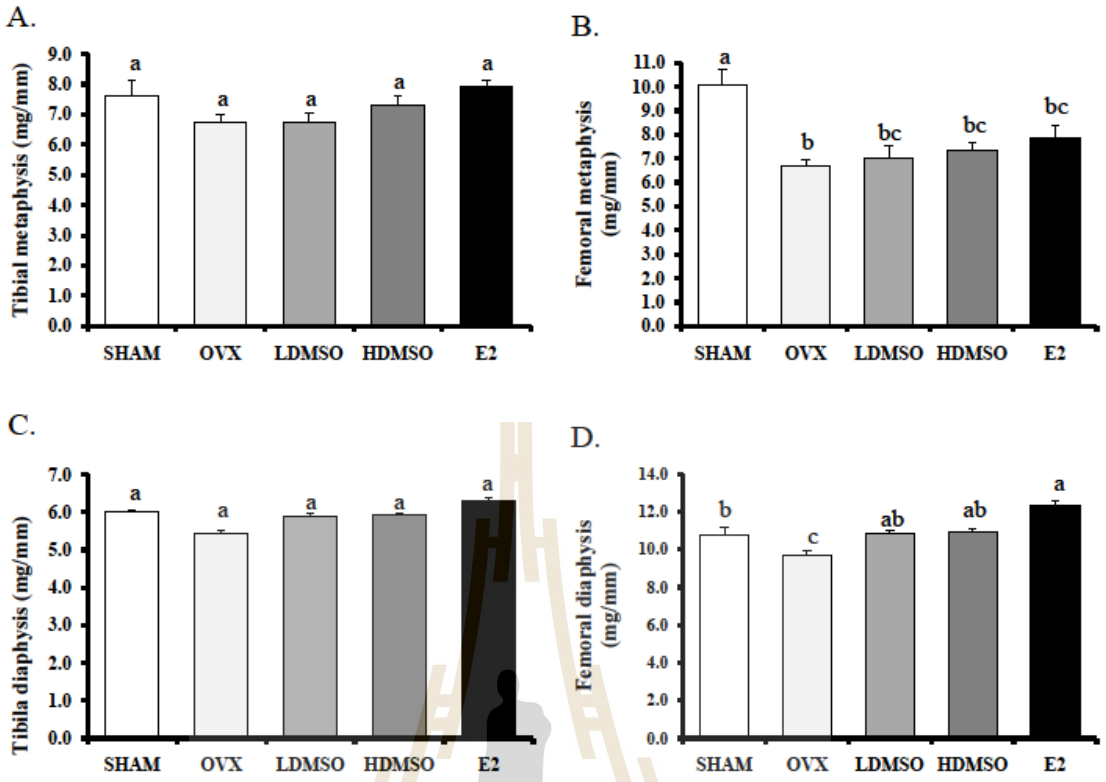

Figure 7.4 Cortical BMCs of the tibial metaphysis and femoral metaphysis in sham operated control (SHAM), ovariectomized rats control (OVX), OVX treated with 0.25 mL/100g BW/day of MSO (LDMSO), OVX treated with 0.50 mL/100g BW/day of MSO (HDMSO) and OVX treated with 17β-estradiol (E_2), respectively. Bone mineral contents were determined at the end of the 6 weeks after treatments. Bars indicate mean ± S.E.M. (n = 5). Data were analyzed by one-way ANOVA, followed by Tukey's *post hoc* test. Values that do not share the same superscript letters are significantly different ($P < 0.05$).

7.4.3 Bone histology

Histological sections revealed a normal trabecular conformation in SHAM rats. The area below epiphyseal plate, metaphyseal region was fully filled with

trabecular bones and its connectivity was intervened by small inter-trabecular spaces. Six weeks of the experimental period, OVX rats showed sparse and thinner trabeculae which were resulted in greater inter-trabecular spaces. Either HDMSO or E_2 treated rats were observed the thicker trabeculae more than OVX control. Six weeks of E_2 and MSO treatment, bone deterioration caused by ovariectomy was restored but less than SHAM control rats (Figure 7.5).

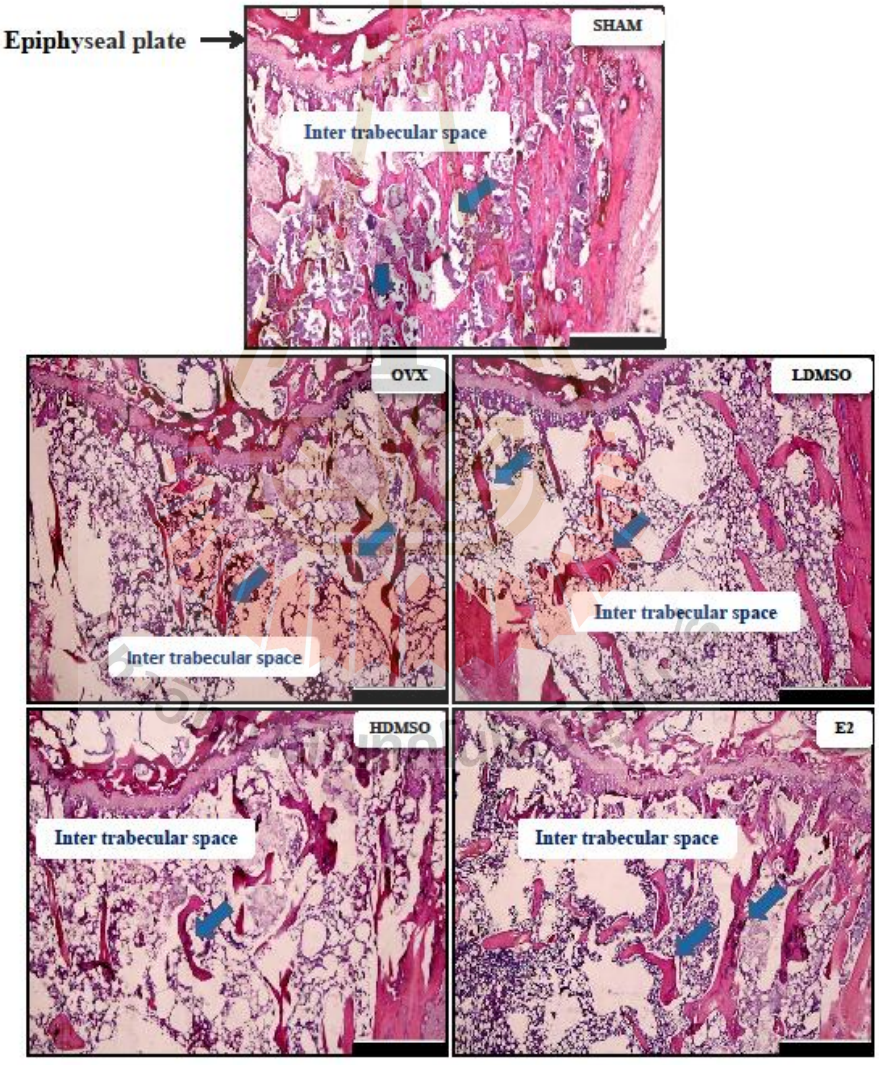

Figure 7.5 Histological section (H&E) in longitudinal and medio-lateral plane of the proximal tibia at the epiphyseal growth plate and metaphyseal area in sham operated

control (SHAM), ovariectomized rats control (OVX), OVX treated with 0.25 mL/100g BW/day of MSO (LDMSO), OVX treated with 0.50 mL/100g BW/day of MSO (HDMSO) and OVX treated with 17β-estradiol (E_2), respectively. Arrow indicated trabecular bone. Bar represents 200 μm.

7.5 Discussion

Osteoporosis represents a major public health problem among general populations particularly in postmenopausal women (Hertrampf et al., 2008). The OVX rat is an excellent preclinical animal model that correctly emulates the important clinical feature of the estrogen depleted human skeleton and the response of therapeutic agents (Kimmel, 1966). Rapid postmenopausal osteoporosis occurring in female rats following ovariectomy is characterized by a decrease intrabecular density and a deterioration of bone architecture, especially a decrease of the total number of trabeculae and an increase of the number of their perforations. The quantitative loss of bone and the changes of its internal structure are responsible for the increased fracture risk in postmenopausal osteopenia (Jee and Yao, 2001). In the present chapter, the antiosteoporotic effect of MSO was evaluated via determining BMD and BMC using pQCT. This instrument is the main method of BMD measurement in small animal research because of its ability to provide high-resolution *in vivo* measures of volumetric bone density and also has the ability to differentiate between cortical and cancellous bone compartments (Allen and Bloomfield, 2003).

Many evidences indicated that ovariectomy in the rat results in an increase in bone turnover rate and significant loss of cancellous bone such as the proximal femur, vertebral bodies and the metaphysis of long bones (Omi and Ezawa, 1995). The

micro-architectural alteration in cancellous bone is similar to those observed in postmenopausal and age dependent (Bonjour et al., 1999). It is well known that estrogen deficiency is an important risk factor in the pathogenesis of osteoporosis (Nian et al., 2009). Estrogen effects are exerted through ER_{α} and ER_{β}, which are present on both the cells of monocyte lineage and osteoblasts. Estrogen leads to the direct suppression of osteoclasts both by reducing the expression of receptor activator of nuclear factor kappa-B ligand (RANKL) on marrow cells and by increasing osteoprotegerin (OPG) secretion by osteoblasts that binds to and inactivates RANKL (Texel et al., 2008). Other indirect effects include suppression of interleukin-1 (IL-1), IL-6, IL-7 and tumornecrosis factor alpha (TNF-α) and increased production of insulin-like growth factor (IGF) and TGF-β by osteoblasts.

The use of dietary phytoestrogens as a possible option for the prevention of osteoporosis has raised considerable interest because of the increased concern about the risks associated with the use of hormone-replacement therapy (Branca, 2003). Previous chapters have reported that MSO has estrogen-like effects. Thus, this study inferred that the antiosteoporotic activity of MSO may be related with its estrogen-like activity. Following treatment for 6 weeks, density of tibial metaphysis and femoral metaphysis in the OVX groups are significantly lower than those of in the SHAM groups. These findings support the notion that this animal model successfully mimicked the effects of osteoporosis caused by the lack of ovarian hormone in the OVX model. Although, there were no significant alteration in the BMD and BMC of all compartments among MSO treated groups. However, feeding MSO in OVX rats tended to raise BMD and BMD more than OVX negative control. Furthermore, examination of histological sections of proximal tibia revealed that six weeks of the

experimental period, LDMSO and HDMSO treated rats showed the thicker trabeculae more than OVX rats while the OVX rats showed sparse and thinner trabeculae which are resulted in greater inter-trabecular spaces. Although, the results does not support the strong efficiency of MSO in prevention and treatment of post-menopausal osteoporosis, however, the results from chapter VI, MSO decreased the serum ALP levels and urine Ca, P and Cr after 6 weeks of treatment. Taken together, these data suggest that MSO can prevent OVX-induced increases in bone turnover in rats. The similar effects were observed with E_2.

Several studies demonstrated that phytoestrogens are widely reported for treatment and protection against various health problems, including bone diseases (Wilkinson et al., 2002; Hassan et al., 2013). Phytoestrogens are thought to protect from bone loss mainly through estrogen dependent mechanisms as phytoestrogens seemed to directly increase estrogen levels, as seen in the present study and in other investigations (Rice and Whitehead, 2008). Phytoestrogens associated estrogen raise may be related to their structural similarity to estrogens at the molecular level which may help to exert estrogen-like activities. It is well documented that estrogen has high affinity toward estrogen receptor ER_α and ER_β on osteoblasts (Beral et al., 2002) and activation of ER complex is vital in maintaining bone remodeling processes (Manolagas et al., 2002). Because phytoestrogens have a stable structure and low molecular weight, they can pass through cell membranes and interact with estrogen receptors (ERs) which allow them to act through the same intracellular pathways of estrogens (Toran-Allerand et al., 2002).

Furthermore, the long-term administration of some phytoestrogen as isoflavones was found to affect positively bone metabolism (Arjmandi et al., 1996;

Blair et al., 1996). Six-month genistein administration to postmenopausal women led to a significant increase in bone density and concurrent reduction in the concentration of biochemical markers of bone resorption (Turhan et al., 2008). After twelve-month genistein administration, the increase in bone density was comparable to the effects of estrogen hormonal replacement therapy (Potter et al., 1998; Morabito et al., 2002). Importantly, Polkowski and Mazurek (2000) suggested that the positive effect of isoflavones on bone metabolism may be mediated by at least two mechanisms. The first is the impact on osteoclasts via activation of apoptosis. The second is the inhibition of tyrosine-kinase activity via modulation of membrane ERs with consecutive changes in the activity of alkaline phosphatase. In several studies, long term period for administration at least 4-12 months was demonstrated (Haifeng et al., 2014). However, in this study the short-term administration of MSO (6 weeks) was conducted which may not long enough to observe pronounced changes in bone. Therefore, escalation in experimental period may be needed in further study.

When considering animal models of osteoporosis, it is important that bone sites should be taken into account. It is well known that, in ovariectomized animals, as in postmenopausal women, bone loss induced by ovarian deficiency mainly results from trabecular bone loss (Palumbo et al., 2009). In a study of ovariectomized rats, Ferreti et al. reported that bone mass started to decrease earlier and more extensively in trabecular than in cortical bone. Therefore trabecular bones are early detectors of bone loss in animal models of postmenopausal osteoporosis (Ferreti et al., 2010). Although BMD is an important determinant of bone strength, it does not take into account the architectural changes occurring in trabecular bone (Snyde et al., 1993; Kleerekope et al., 1995). The measurement of both trabecular bone microarchitecture

and BMD may improve the estimation of bone strength (Laib et al., 2001).There is strong evidence to support that the architecture of cancellous bone plays a significant role in bone strength and can be used to determine its biomechanical properties (Ulrich et al., 1999).

In conclusion, after ovariectomy at eight weeks, oral administered with MSO for six weeks has a tendency to increase BMD and BMC, but not significant when compared to OVX control group. The proximal tibia histomorphometric in MSO treated group shown the thicker trabeculae resemble to E_2 treated group. This information will aid the development of a better understanding of their biological roles on bone metabolism. Further studies are required to evaluate its mechanism for long term period.

7.6 References

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CHAPTER VIII

CONCLUSIONS

Biological actions of phytoestrogens are numerous and include several physiological systems. Phytoestrogens affect multiple signaling pathways through the activation of both intracellular and membrane estrogen receptors (ERs), as well as interaction with the metabolism of steroid hormones. Therefore, the impact of phytoestrogens on physiological processes in the organism seems to be very complex and may be related to large number of factors, which are not satisfactorily identified yet.

Moringa oleifera is one of the many plants used in herbal preparations in traditional medicine for treating numerous human ailments such as malnutrition, cardiovascular diseases and uterine conditions among others. Evidences from biochemical constituent research haves hown that this plant contained several of phytoestrogens (Tsaknis et al., 1999; Anwar et al., 2007). Despite its wide use by communities around the world, there is inadequate scientific information available on the actual pharmacological effects of *M. oleifera* seed oil (MSO) on estrogenic activities.

The principal aim of this thesis was to determine estrogenic activity with the intent of understanding the effects and their mechanisms for preventive and treatment for menopausal situation. The major findings can be concluded as follows.

8.1 Phytochemical compositions of MSO

In this study, moringa specimens were collected from Lopburi province, Thailand and then plant samples were confirmed and deposited by the botanists at the Royal Forest Department, Bangkok, Thailand. Clearly, this specimen is *M. oleifera* and classified to Moringaceae.

The data from qualitative phytochemical screening of MSO showed that alkaloids, flavonoids and phytosterols were observed. The GC-MS analysis of the MSO revealed the presence of major sterol groups which were β-sitosterol (3.46%), stigmasterol (1.91%), campesterol (1.90%), stigmast-4-en-3-one (0.32%) and cholest-5-en-3-ol (0.84%). In addition, α-tocopherols and oleic acid were detected to 0.25% and 0.30%, respectively.

8.2 Effects of MSO on isolated rat uterus

The oil of *M. oleifera* seed had uterotonic effects on isolated non-pregnant rat (Figure 8.1). MSO significantly increased the amplitude and AUC of contractions with EC_{50} value of 93.90 µL/100 mL. The uterotonic effects of the MSO were very similar to those of β-sitosterol which increase amplitude. In addition, the potentiation of force induced by MSO is produced by the Ca^{2+}-calmodulin-MLCK pathway, because force produced in the presence of the extract was abolished by the inhibition of L-type Ca^{2+} channels by nifedipine and inhibition of MLCK by wortmannin. The potentiation of spontaneous force induced by the MSO was dependent upon the Ca^{2+}-calmodulin-MLCK pathway, SR Ca^{2+} release and a Ca^{2+}-independent pathway. Other supporting comes from experiments in which Ca^{2+} was maintained at a high level via depolarization, when it was noted that the oil produced additional contraction.

Furthermore, although force transients are abolished by the inhibition of L-type Ca^{2+} channels and MLCK, some tonic force can still be produced by addition of MSO. It implies that, the mechanism of the uterotonic activity of MSO is due to acting via a non-estrogenic mechanism mediated β-sitosterol and/or other compositions in MSO, that may be further elucidated. Thus, this information give a better understanding of how MSO act on the uterus, which can be applied for the improvement of new agents with the potential to improve women's reproductive and gynaecological heath.

8.3 The estrogenic activity of MSO in ovariectomized rat

Two doses of MSO (0.25 mL/100g BW and 0.50 mL/100g BW) were chosen for all studies. The study was designed into two series including preventive and recovery study. In the preventive study, OVX rats were orally administered with MSO starting from day 3 after ovariectomy for 6 weeks. In the recovery study, OVX rats were administered with MSO starting from day 60 after ovariectomy for 6 weeks. The results from both series showed that MSO displayed estrogenic properties, confirmed by the increasing in percentage of vaginal cornification. Furthermore, MSO significantly increased serum E_2 levels and moderated the illuminated serum LH levels resulting from the removal of E_2 in OVX rats. This indicates that MSO has an efficacious role in regulating hypothalamic-pituitary function. The estrogenic activities of MSO on various parts are summarized as follows:

8.3.1 Effects of MSO on uterus

In this study MSO showed estrogen-like effects on uterus by reveal the increased in relative uterine weight and morphology changes of the luminal epithelia

compared to the OVX control group. These effects were lower in magnitude compared to the E_2 treated group, ensured that it would be safe to use in short term.

8.3.2 Effects of MSO on hypolipidemic

Oral administration of MSO for 6 weeks in OVX rats induced hypolipidemic effects and caused predominant increases in high density lipoprotein cholesterol (HDL-C) and decreases in low density lipoprotein cholesterol (LDL-C) levels in two studies. It is possible that MSO treatment could restore LDL-C to control levels as observed in SHAM group. This may be involved phytosterol constituents which act as estrogen and/ or antioxidant properties.

8.3.3 Effects of MSO on bone quality

The administration of MSO for 6 weeks from two month after ovariectomy restored the serum levels of calcium (Ca), Phosphorus (P) and alkaline phosphatase (ALP). Bone quantity as determined by peripheral Quantitative Computerized Tomography (pQCT) showed that MSO slightly effected bone mineral density (BMD) and bone mineral content (BMC) changes but tend to increase when compared to OVX control group and that histomorphometri of trabeculae in MSO treated OVX rats was similar to Estradiol-treated OVX rats. This suggests that MSO might have some benefits to decrease bone turnover.

As mentioned above, this is the first time to report the estrogenic activities of MSO. These finding revealed that MSO can stimulate the effects via both non-genomic and genomic pathways. These data clearly explain that MSO has affected on uterine contraction in non pregnant rats and possess positive property that mainly female reproductive functions, particularly restoring the circulating estradiol levels, improving lipid profiles and decreasing bone loss in the animal model of

postmenopausal condition. Summary mechanisms were represented in Figure 8.1. These observations imply that MSO may be useful in prevention and therapeutic in menopausal ailment of both cardiovascular disease and osteoporosis. However, to get better understanding how MSO affected to uterus, bone and other tissues, molecular mechanisms remains to be determined.

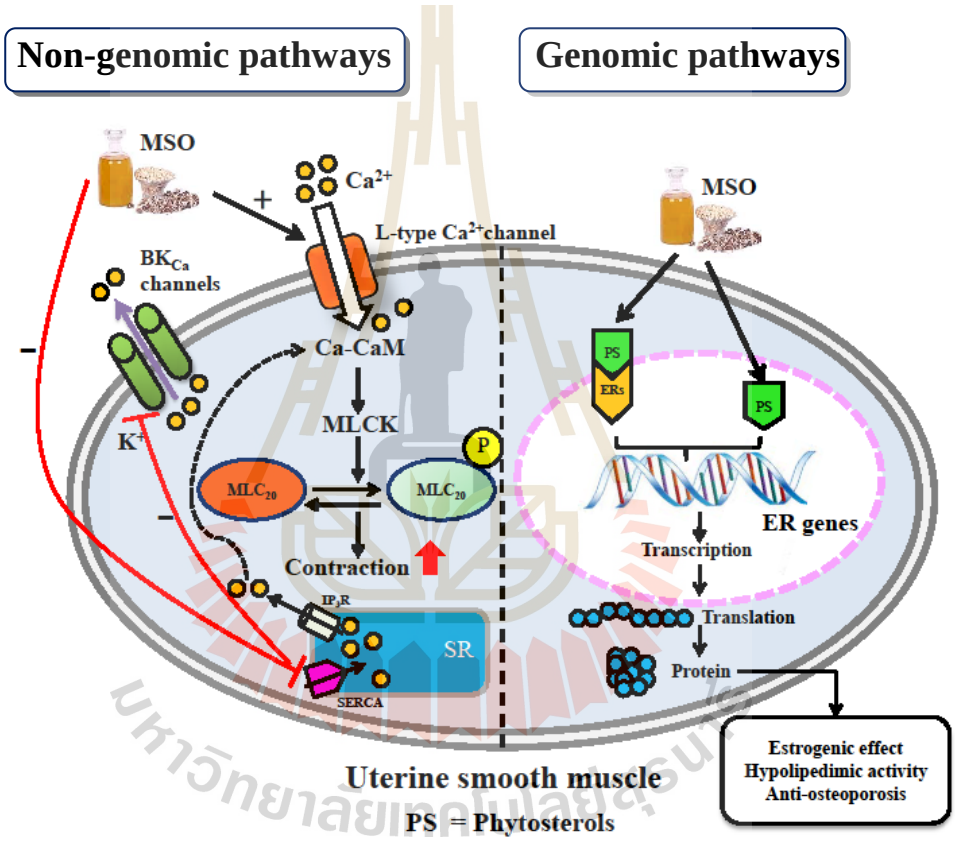

Figure 8.1 Schematic represents the mechanisms of the non genomic and genomic pathways modulated by MSO on their target organs. MSO also exhibit uterotonic activity via rapid non-genomic signaling pathways. MSO increased spontaneous contraction due to the Ca^{2+}-calmodulin-MLCK pathway, stimulate force via SR Ca^{2+} release. MSO may also acting to inhibit K^{+} channels and SR Ca^{2+} -ATPase. On the other hand, MSO may directly bind to estrogen receptors (ERs), resulting in

dimerization and activation of gene transcription that apparently regulate gene expression.

8.4 Future research

Future studies may include other molecular biology techniques such as polymerase chain reaction (PCR) and western blotting which can be implemented to examine signaling pathway show MSO are involved in restoring processes of menopausal symptoms. Moreover, these studies were demonstrated in an animal model; there should be a further study of this oil in a human model.

8.5 References

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CURRICULUM VITAE

FIRST NAME: PIYATHIDA **LAST NAME:** KUSOLRAT

GENDER: Female **NATIONALITY:** Thai

DATE OF BIRTH: January 11, 1974 **PLACE OF BIRTH:** Nakhon Ratchasima

EDUCATION BACKGROUND:

1995 B.Ed. (Chemistry), Nakhon Ratchasima Rajabhat University

2000 M.Sc. (Parasitology), Khon Kaen University, Thailand

GRANT:

A scholarship under the NRRU staff development fund, Nakhon Ratchasima Rajabhat University, Thailand

WORK EXPERIENCES:

2001-2016 Lecturer, Biology Program, Faculty of Science and Technology, Nakhon Ratchasima Rajabhat University, Nakhon Ratchasima, Thailand