THE EFFECTS OF *HELIOTROPIUM INDICUM* L. EXTRACT ON UTERINE INVOLUTION IN POSTPARTUM RATS

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ผลของสารสกัดหญ้างวงช้าง (*Heliotropium indicum* L.) ต่อการเข้าอู่ของมดลูกในหนูหลังคลอด

นางสาวสายาห์ องค์ศรีเจริญพร

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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THE EFFECTS OF *HELIOTROPIUM INDICUM* L. EXTRACT ON

UTERINE INVOLUTION IN POSTPARTUM RATS

Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for a Master's Degree.

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สายาห์ องค์ศรีเจริญพร : ผลของสารสกัดหญ้างวงช้าง (*Heliotropium indicum* L.) ต่อการเข้าอู่ของมดลูกในหนูหลังคลอด (THE EFFECTS OF *HELIOTROPIUM INDICUM* L. EXTRACT ON UTERINE INVOLUTION IN POSTPARTUM RATS). อาจารย์ที่ปรึกษา : รองศาสตราจารย์ สัตวแพทย์หญิง ดร.ศจีรา คุปพิทยานันท์, 241 หน้า.

จากปัญหาที่เกิดขึ้นทั่วโลกเกี่ยวกับการตายของมารดาซึ่งมีความสัมพันธ์กับการเข้าอู่ของมดลูกและการให้นมบุตร ทำให้เกิดการศึกษาผลของสมุนไพรเพื่อนำมาแก้ไขปัญหาเหล่านี้ อย่างไรก็ตามพืชสมุนไพรบางชนิดยังไม่มีข้อมูลทางวิทยาศาสตร์มายืนยันฤทธิ์ของสมุนไพรดังกล่าว การศึกษานี้ ทำการศึกษาในพืชสมุนไพร *Heliotropium indicum* L. (*H. indicum*) หรือ "หญ้างวงช้าง" วัตถุประสงค์ของการศึกษานี้คือ ศึกษาฤทธิ์การเป็นสมุนไพรของสารสกัดทุกส่วนของหญ้างวงช้าง โดยใช้เอทานอลในการสกัด (250 มก. ต่อ กก. น้ำหนักตัว ต่อวัน) ในหนูหลังคลอด โดยทำการศึกษา 1) องค์ประกอบทางเคมีของสารสกัดด้วยวิธีการทดสอบองค์ประกอบทางพฤกษเคมี 2) ศึกษาผลของสารสกัดต่อการเข้าอู่ของมดลูก โดยศึกษาผลต่อการหดตัวของมดลูก น้ำหนักของมดลูก ขนาดของมดลูกและเปอร์เซ็นต์คอลลาเจนในมดลูก 3) ศึกษาผลของสารสกัดต่อเต้านม โดยศึกษาผลต่อขนาดของต่อมน้ำนม เซลล์ที่มีความสามารถในการผลิตน้ำนม และองค์ประกอบทางเคมีของเต้านม 4) ศึกษาผลของสารสกัดต่อค่าบงชี้ในเลือด เช่น ตัวบ่งชี้ความเป็นพิษต่อตับ ฮอร์โมนเอสโตรเจนและโปรเจสเตอโรน นอกจากนี้ยังศึกษาพิษฉับพลันของสารสกัด โดยสังเกตจากการแสดงอาการเป็นพิษ ผลการศึกษาพบว่าสารสกัดหญ้างวงช้างมีส่วนประกอบของ อัลคาลอยด์ แทนนิน ฟลาโวนอยด์ สเตียรอยด์ ไกลโคไซด์ ฟีนอล ลิกนินและกรดคาร์บอกซิลิก นอกจากนี้ผลจากการวิเคราะห์สารสกัดหญ้างวงช้างแสดงสารประกอบหลายชนิด เช่น ไฟทอล กรดอะซิติก นีโอไฟติอาดีนและกรดเฮกซะเดคะโนอิก สารสกัดหญ้างวงช้างกระตุ้นการหดตัวของมดลูกและอาจออกฤทธิ์เสริมกันกับออกซิโตซินในการกระตุ้นการหดตัวของมดลูกหนูหลังคลอด นอกจากนี้สารสกัดหญ้างวงช้างยังช่วยเร่งให้มดลูกเข้าอู่ในหนูหลังคลอดโดยเพิ่มการหดตัวของมดลูกและช่วยลดน้ำหนัก ขนาด และเปอร์เซ็นต์คอลลาเจนในมดลูก นอกจากนี้สารสกัดหญ้างวงช้างยังช่วยเพิ่มขนาดของต่อมน้ำนมและเซลล์ที่มีความสามารถในการผลิตน้ำนมซึ่งทำให้การผลิตน้ำนมเพิ่มขึ้น ยิ่งไปกว่านั้นสารสกัดหญ้างวงช้างยัง ช่วยเพิ่มไขมันและโปรตีนในเต้านมซึ่งอาจเกี่ยวข้องกับการสังเคราะห์โปรตีนและไขมันในเซลล์เต้านม ผลการศึกษาแสดงให้เห็นว่าสารสกัดไม่มีผลทำให้ตัวบ่งชี้ความเป็นพิษต่อตับผิดปกติในสัตว์ทดลองที่ได้รับสารสกัดเป็นเวลา 5 วันหลังคลอด สารสกัดช่วยลดระดับของฮอร์โมนเอสโตรเจนและโปรเจสเตอโรนในวันที่ 1 และ 5 หลังคลอด ตามลำดับ ยิ่งไปกว่านั้นสารสกัดหญ้างวงช้าง ไม่ก่อให้เกิดการตายของสัตว์ทดลอง ผลจาก

การศึกษาในครั้งนี้เป็นข้อมูลทางวิทยาศาสตร์ที่ช่วยสนับสนุนผลของสารสกัดหญ้างวงช้างต่อ
มดลูกและเต้านมในช่วงหลังคลอด จากการศึกษาในสัตว์ทดลองหลังคลอด สารสกัดทุกส่วนของ
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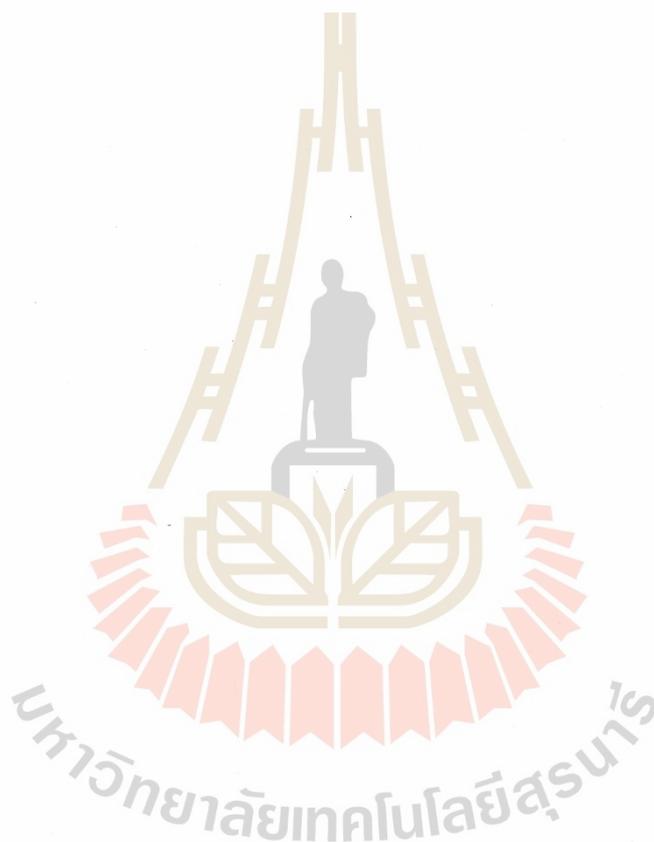
สาขาวิชาปรีคลินิก   ลายมือชื่อนักศึกษา _____

ปีการศึกษา 2563   ลายมือชื่ออาจารย์ที่ปรึกษา _____

SAYAH ONGSRICHAROENBHORN : THE EFFECTS OF
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HELIOTROPIUM INDICUM L./UTERUS/MAMMARY GLAND/RAT/
/CONTRACTION/INVOLUTION/POSTPARTUM

From the worldwide problems about maternal death related with uterine
involution and breastfeeding, the effects of medicinal plants for solving the problems
have been investigated. However, some traditional medicinal plants have no scientific
data to support their effects. In this study, the medicinal plant, *Heliotropium indicum*
L. (*H. indicum*) or "Ya Nguang Chang" was studied. The purposes of this study were
to explore the medicinal properties of the whole plant of *H. indicum* ethanoic extracts
(250 mg/ kg BW/ day) in postpartum rats. This study were aimed to 1) analyze
phytochemical components of the extract, 2) study the effects of the extract on uterine
involution by observing their effects on uterine contraction, uterine weight, uterine size,
and percent collagen of the uterus, 3) study the effects of the extract mammary glands
by observing their effects on alveoli size, parenchyma cells, and biochemical
components of the mammary glands, and 4) study the effects of the extract on blood
biochemical parameters such as parameters of hepatotoxicity, estradiol, and
progesterone. Moreover, the acute toxicity was observed for toxic symptoms. The
results showed various compounds in the whole plant extract of *H. indicum* such as
alkaloids, tannins, flavonoids, steroids, glycoside, phenols, lignins, and carboxylic

acids. Moreover, *H. indicum* showed many constituents such as phytol, acetic acid, neophytadiene, and hexadecanoic acid. The *H. indicum* extract can increase uterine contraction and may be synergistic with oxytocin- induced uterine contraction in postpartum rats. Moreover, the extract can help accelerate uterine involution in postpartum rats by enhancing uterine contraction and reducing the uterine weight, the uterine size, and % the collagen of the uterus. Moreover, *H. indicum* extract can help increase alveoli size and the parenchyma cells resulted in increasing milk production. Moreover, the extract helps increase lipid and protein in mammary gland tissue which could relate with lipid and protein synthesis in mammary epithelial cells. The results indicated that parameters of hepatotoxicity in this study did not show an abnormality when intake the extract within 5 days in the postpartum rats. The extract showed the decrease of estradiol and progesterone on day 1 and 5 postpartum, respectively. In addition, *H. indicum* had no lethal effects. The results from this study is the scientific data to support the effects of *H. indicum* extract on uterine tissue and mammary gland in postpartum period. Therefore, the whole plant of *H. indicum* extract can be intake during postpartum period to help accelerate uterine involution and to induce milk production in postpartum rats.

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LISTS OF ABBREVIATIONS

AA	arachidonic acid
AI	abdominal-inguinal
ALT	alanine aminotransferase
ANOVA	analysis of variance
AST	aspartate aminotransferase
AUC	area under the contraction
BaF_2	barium fluoride
BW	body weight
°C	degree celsius
Ca^{2+}	calcium ion
Ca-Cal-MLCK	calcium ions - calmodulin - myosin light chain kinase
$CaCl_2$	calcium chloride
CaM	calmodulin
Cl^-	chloride ion
cm	centimetre
cm^{-1}	typically centimeters
CO_2	carbon dioxide
COL	collagen
COX	cyclooxygenase
CT	cervicothoracic

LIST OF ABBREVIATIONS (Continued)

Cx-43	gap junction protein, Cx-43
C/S	cesarean section
Da	dalton
DAG	diacylglycerol
DNA	deoxyribonucleic acid
DP	prostaglandin D receptor
E_2	17β-estradiol
ECM	extracellular matrix
EP_1	prostaglandin E1 receptor
EP_2	prostaglandin E2 receptor
EP_3	prostaglandin E3 receptor
EP_4	prostaglandin E4 receptor
eV	electron volt
FP	prostaglandin F receptor
FTIR	Fourier Transform Infrared Spectroscopy
Etc	et cetera
FSH	follicle-stimulating hormone
g	gram
g/kg BW/day	gram per kilogram body weight per day
GC/MS	gas chromatography-mass spectrometry
HCl	hydrochloric acid

LIST OF ABBREVIATIONS (Continued)

H_2SO_4	sulphuric acid
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
hr	hour
KCl	potassium chloride
IP	prostaglandin I receptor
IP_3	inositol (1, 4, 5)-triphosphate
IP_3R	inositol triphosphate receptor
KMC	k-means cluster analysis
LH	luteinizing hormone
MECs	mammary epithelial cells
Mg	milligram
$MgSO_4$	magnesium sulfate
mg/kg BW/day	milligram per kilogram body weight per day
min	minute
mL	milliliter
mm	millimeter
mM	millimolar
MLCK	myosin light chain kinase
MLCP	myosin light chain phosphatase
mRNA	messenger ribonucleic acid
n	number of sample

LIST OF ABBREVIATIONS (Continued)

N	nitrogen
NaCl	sodium chloride
NaOH	sodium hydroxide
ng/ml	nanogram per milliliter
nmol/L	nanomoles per litre
O_2	oxygen
OCT	optimal cutting temperature compound
OT	oxytocin
OT1a	myometrial oxytocin receptors
OT1b	endometrial oxytocin receptors
P	progesterone
PAs	pyrrolizidine alkaloids
PDR	Lao People's Democratic Republic
PGs	prostaglandins
$PGF_{2\alpha}$	prostaglandin F2α
pg/mL	picogram per milliliter
pH	potential of hydrogen ion
PIP_2	phosphatidylinositol biphosphate
PP	postpartum period
PPH	postpartum bleeding or postpartum hemorrhage
pmol/L	picomoles per litre

LIST OF ABBREVIATIONS (Continued)

RNA	ribonucleic acid
RyR	ryanodine receptor
%RU	percentage of relative uterine weight
S.E.M	standard error of the mean
SERCA	SR Ca^{2+}-ATPase
SLRI	Synchrotron Light Research Institute
SPSS	Statistical Pagkage for the Social Sciences
SR	sarcoplasmic reticulum
μL	microliter
μm	micrometer
v/v	volume per volume
WHO	World Health Organization
5-HT2	serotonin receptors

CHAPTER I

INTRODUCTION

1.1 General uterine physiology

The uterus is the principal organ of breeding. It is divided into three parts by functionally and morphologically including the cervix, the uterine tube, and the main body of the uterus. Uterus has three layers, endometrium, myometrium, and perimetrium. The middle muscular layer is myometrium that compose of smooth muscle. Smooth muscle is prepared contractile forces. In an extracellular matrix found smooth muscle cells surround by collagen fibers, which help the transference of contractile forces originated by individual cells. Sheets of smooth muscle are composed of the "longitudinal layer" which a mesh of bundle of smooth muscle cells and the "circular layer", in which the fibers are prepared around the longitudinal axis of the organ (Lwiindi et al., 2015). The uterine smooth muscle contracts without nervous or hormonal stimulation (Wray, 1993). The uterus has pacemaker cells. It's not anatomically distinct, nor fixed in location. Pacemaker cells help involve by lowering threshold potential, so easy to produce action potential (Sergeant et al., 2000). Phasic myometrial contractions help parturition. It's managed by the progress of action potentials over the plasma membranes, effect from a transient rise in in the cytosolic free Ca^{2+} concentration (Young, 2007). Depolarization of the myometrial cell membrane is the most important event to an opening of voltage-sensitive Ca^{2+} channels (Wray, 1993). The inside Ca^{2+} current and the enlargement in $[Ca^{2+}]_i$, which follows

depolarization in the voltage-clamped cell. Moreover, this Ca^{2+} entry, at least in rat uterus, appears almost totally via L-type Ca^{2+} channels. Thus, upon excitation and depolarization of the myometrial cell membrane, external Ca^{2+} is important; without this Ca^{2+} there is no Ca^{2+} transient and no contraction (Wray et al., 2001).

1.2 Uterine contractility

Myosin and actin have significant collaboration in the uterus, light chains of myosin should be phosphorylated. Under physiological conditions, myosin light chain kinase (MLCK) is the selective and involved enzyme. Calcium ions-calmodulin complex activates MLCK and following cross-bridge cycling. Ca-Cal-MLCK pathway consist of spontaneous contraction and agonists-induced contraction. There are two ways for the enlargement in activator Ca^{2+}: entrance across the surface membrane by way of voltage-gated L-type Ca^{2+} channels and/or free from the sarcoplasmic reticulum (SR) (Wray, 2007). Spontaneous contraction, the resulting depolarization and result opening of L-type Ca^{2+} channels bring about this the major source of Ca^{2+} for contraction (Matthew et al., 2004). If L-type channels are blocked, the Ca^{2+} transients and contractions would abolish (Wray et al., 2003) such as nifedipine, a blocker of L-type Ca^{2+} channels, is present, no increase in intracellular Ca^{2+} or Ca^{2+} current appears with depolarization (Wray et al., 2001). In human myometrium, T-type Ca^{2+} channels may contribute to Ca^{2+} entry. Uterine contractions also fail, if Ca^{2+} is increased but MLCK is inhibited (Longbottom et al., 2000). Thus, the Ca^{2+}–calmodulin–MLCK pathway is important for uterine mechanical activity. Voltage- or ligand-gated plasma

membrane channels activate uterine contraction by increase Ca^{2+} influx into intracellular. Moreover, the way that works combine with these channels for activating uterine contraction is efflux from intracellular stores by way of the ryanodine receptors (RyR) and efflux from intracellular stores through the inositol triphosphate receptor (IP_3R) Ca^{2+} channels (Barrett et al., 2010). Calmodulin has four binding sites of Ca^{2+}, the reason a conformational change let the calmodulin complex to interact with inactive myosin light chain kinase (MLCK), so activating its enzymatic feature (Olson et al., 1990). The myosin light chain phosphorylates MLCK at on serine at position 19. In the cell, myosin is dephosphorylated by myosin light chain phosphatase. However, dephosphorylation of MLCK does not necessarily lead to uterine smooth muscle relaxation. Various mechanisms are complicated (Barrett et al., 2010).

Agonists-induced contraction, oxytocin is an agonist stimulation of IP_3 receptor. It's unable to generate force in the uterus if Ca^{2+} entry is restrained. Oxytocin can also stimulate uterine contraction by related to SR Ca^{2+} release. Both activities will enlarge and/or extend Ca^{2+} transients (Wray, 2007). The SR has both IP_3 and ryanodine (Ry) receptors. It now appears likely, however, that the Ca^{2+} released from these receptors has low effect on the activation of contraction. When the SR is disabled, this makes an increase in both Ca^{2+} transients and contractions. Cyclopiazonic acid is a drug which inhibits the SR Ca^{2+}-ATPase (SERCA) wanted to transport Ca^{2+} into the SR (Shmygol et al., 2007).
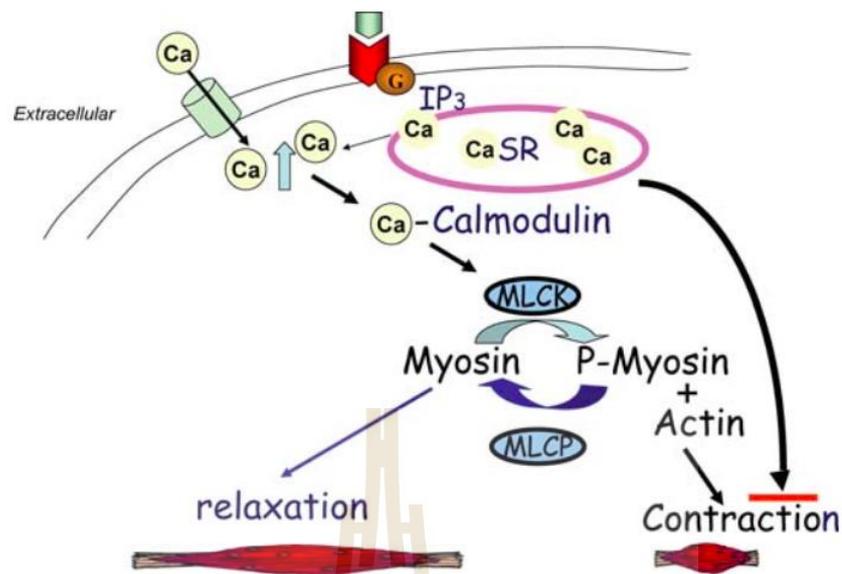
Figure 1.1 A diagram to show how Ca^{2+} entry activate smooth muscle contraction. The present of the SR to increase Ca^{2+} for contraction is not found for the uterus, but is referred for completeness, and the red bar represent its negative effect on contraction. Some Ca^{2+} entry into T-type channels may appear, but L-type Ca^{2+} entry predominates in the uterus. MLCK, myosin light chain kinase; MLCP, myosin light chain phosphatase; and SR, sarcoplasmic reticulum (Wray, 2007).

Oxytocin receptors are found in uterine tissue, mammary tissue and the ovary. It helps increases in intracellular Ca^{2+} levels. Oxytocin stimulates transmembrane receptors that stimulate the enzyme phospholipase C. The enzyme phospholipase C catalyzes the collapse of some phospholipids particularly phosphatidylinositol biphosphate (PIP_2). The two different second messenger products of PIP_2 are inositol triphosphate (IP_3) and diacylglycerol (DAG). The ionic basis for uterine smooth muscle contractility related to IP_3. It mobilizes calcium ions and second messenger effects in smooth muscle contraction. A secondary product of IP_3 synthesis, DAG might

encourage cell contraction along intracellular prostaglandin synthesis from arachidonic acid (AA) by cyclooxygenase (COX) enzymes (Morris and Malbon, 1999). Agonists such as acetylcholine help stimulate uterine smooth muscle contraction at myometrial muscarinic receptors (Lwiindi et al., 2015). Oxytocin bind to myometrial oxytocin receptors (OT1a) to directly motive uterine contraction. Stimulation prostaglandins and cholinergic releases act on endometrial oxytocin receptors (OT1b) important to uterine contraction (Dawood, 1995). Oxytocin and $PGF_{2\alpha}$ receptors were reported to be up- control by 17β-oestradiol (E_2) in late pregnancy especially at term (Kimura et al., 1996). Oxytocin might be activated chloride (Cl^-) channels, their uterotonic effect by depolarization of the smooth muscle cell membrane (Morrison et al., 1996). The effect of agonists on uterine contraction increase the power by a high dose of E_2. E_2 has been reported to up-regulation of oxytocin receptor in human, rat and mouse uterus (Lwiindi et al., 2015). Atropine is a non-specific muscarinic receptor antagonist. It induces relaxes smooth muscles and reduces the contractile effect of acetylcholine in the uterus (Kurtel et al., 1990). Salbutamol is a β_2-receptor stimulating agent. It decreases in uterine contractility even in dysmenorrhea women, so it can be used as an agent to relax the uterine smooth muscle to delay premature labor (Lwiindi et al., 2015)

1.3 Postpartum period

The postpartum period (PP) is a time after delivery. It's characterized by uterine involution and recovery of female reproductive organs. Postpartum involution of the uterus is imperative for the arrangement of the uterus for the next gestation. The non-involution of the after-delivery uterus will produce some of infertility. Several

researches had been studied about normal uterine involution in animals such as morphological, chemical changes, and histological (Ochiogu et al., 2013). The human uterine activity has been studied and has been reported in many papers. The uterine movement during initial postpartum along with the third stage of labor has potentially work on the basis of uterine contractions including, (1) spontaneous progression, (2) breast feeding, (3) cooling the uterus or (4) oxytocin administration. The basic physiology of postpartum hemostasis concerns with the uterine smooth muscle contractions was convinced by endogenous oxytocin and prostaglandins. The frequency of uterine contraction was increased by oxytocin management and baby suckling. The baby's suckling releases oxytocin cause nipple stimulation and leading to contraction of the myometrium, reduce blood flow. The cooling of the uterus can help blood vessels fasten and reduce the blood flow (Masuzawa and Yaeko, 2017). The other study of following real-time shows the noticeable enlargement in the intensity of contractions during early postpartum, indicate that greatly more activity in early postpartum than in late postpartum. The postpartum uterine contractions have the frequency lower than it was in late labor (Hendricks et al., 1962).

In animal study, the expressions of a variety of contraction-associated proteins, such as connexin 43, oxytocin receptor, and probably also the FP receptor were related with the decrease in progesterone and the increase in estrogen at term. Parturition stimulated the prostaglandins synthesis those specific to myometrial receptors as negotiators of uterine contractions. Prostaglandins (PGs) receptors have contractile (FP, EP_1, EP_3) and relaxant (EP_2, EP_4, IP, DP) properties. The study of FP and EP_2 expressions shows at day 16 EP_2 receptor mRNA expression was highest and decreased significantly to day 21 and one day postpartum. At day 16 of gestation, FP receptor

mRNA expression was low and increased significantly until delivery at day 22, then fell at one day postpartum. FP receptor maximal expression could supported two purposes, to convince contractions for parturition and to sustain contractions for a short time after labor to cause the involution of the uterus (Brodt-Eppley and Myatt, 1998). Prostaglandin $F_2\alpha$ is believed to present an important role in the control of myometrial contractility and introduction of parturition. FP receptor is obviously response for transducing the signal across the plasma membrane for many of physiological responses, including cell proliferation, cell death, smooth muscle contraction, and inhibition of steroid synthesis (Al-Matubsi et al., 2001). Hence, the labor can be convinced by various PGs or, conversely, deferred by PG synthesis inhibitors (Brodt-Eppley and Myatt, 1998).

In the myometrium, the uterine contractile activity was increased by gap junctions, that low resistant pathways in the middle of the smooth muscle cells. Cx-43 is the transcripts encoding of the myometrial gap junction protein. The study shows levels of Cx-43 transcripts, proteins, and gap junctions reduce rapidly in postpartum. Cx-43 proteins in the rat are significant enlargement before parturition and highest in delivery. The myometrial Cx-43 transcript levels being managed positively by estrogen and negatively by progesterone while pregnancy. Postpartum hemorrhage and the procedure of uterine involution were simplified by coordinated contractions (Lye et al., 1993). Gap junctions in the longitudinal muscle were increased numbers in rats killed 3 h after parturition. The studies show that a decline in progesterone levels were followed by extending in estradiol and $PGF_2\alpha$ are concurring with the formation of gap junctions. Gap junctions may correlate to the increased uterine activity needed for parturition. During day 21 of pregnancy found the frequency of gap junctions was

significantly higher in rat killed and the number of gap junctions was also significantly higher during delivery as compared day 21 of gestation. At day 21 the size of the gap junction was also significantly larger in the tissues from rats delivering and postpartum, as compared to pregnant rats between days 15 to 20 (Puri and Garfield, 1982).

In postpartum period, involution was related with baby sucking and oxytocin in the release of prolactin. The delay effect on mammary involution is a feature of oxytocin rather than vasopressin. The effects of oxytocin on mammary involution are due to a direction of the anterior pituitary and eliciting the release of prolactin (and perhaps other hormones) or a direction of oxytocin on the mammary gland. The mammary gland was contracted and ejected the milk from alveoli by oxytocin. Oxytocin can provoke the release of prolactin and increase milk yield (Benson and Folley, 1957). Moreover, some study shows the non-synaptic adrenoceptors play a considerable function in the regulation of the pregnant rat uterus and in delivery. This is supported by blockers (prazosine and urapidil) obstructed spontaneous postpartum rat uterus contractions in vivo (Gaspar et al., 1998). In postpartum period found some pathological disorders, the high risk of postpartum deaths is in the initially 24 h postpartum and the first postpartum week, and the disorders continues significantly until the second week after childbirth. Postpartum hemorrhage is commonplace causes of postpartum deaths that found in developing countries. Therefore, many researchers attend to study about protection and detection of postpartum deaths to reducing the maternal mortality (Li et al., 1996).

1.4 Postpartum hemorrhage (PPH)

Postpartum hemorrhage (PPH) is symptoms described as the loss of more than 500 ml or 1,000 ml of blood in the first 24 hours later parturition. The condition may primarily include a raised heart rate and an enlarged breathing rate. Blood is lost over cause of feeling freeze, blood pressure low, and the women may become nervous or senseless. The illness can appear up to six weeks following labor. PPH is the most reason of maternal mortality. All women who carry a pregnancy beyond 20 weeks' pregnancy are at risk for PPH. Even though maternal death rates have descent in the developed world, PPH can lead to maternal mortality anywhere (Smith, 2016). The healthy woman can death by PPH after delivery within hours if she is unattended. Injecting oxytocin instantly after parturition effectively decrease the risk of bleeding. Several countries in the world have maternal mortality rates in excess of 1,000 women per 100,000 live births, and 60% of maternal deaths in developing countries are relate with PPH evaluate by World Health Organization, counting for more than 100,000 maternal deaths per year (WHO, 2007). The rate of PPH increased from 1.5% in 1999 to 4.1% in 2009 (John R Smith, 2017).

1.5 Uterine involution

Uterine involution is the process occurs in postpartum period by which the uterus and the reproductive organs returning to its non-pregnant state (Bassam, 2009). Uterine involution shows decreases in size and weight of the uterus and returning the position to its pre-pregnant. The transition of uterine involution occurring during the first few days after childbirth (Medan, 2015). Uterine involution naturally uses autolysis or self-digestion and ischemia or localized anemia. Autophagy occurs in

postpartum uterine involution without an enhance of myocyte apoptosis. Autophagy cause decreases cytoplasm and collagen by collagenase (Keng-Fu Hsu, 2014).   In rat uterus during the postpartum period, involution is a wide remodeling procedure accompanied by the quick detachment of collagen. 85% of the total collagen during the end of pregnancy is erased within 4 days by the administration of oestradiol-17 at the time of childbirth (Ryan and Woessner, 1972). The study of the effect of hormones on collagen metabolism and collagenase activity in the guinea pig found progesterone was shown to prevent collagenase activity clearly while estrogen was less capability (Wahl et al., 1977). Ischemia or localized anemia is compensated from uterine contraction by the administration of oxytocin. Uterine contraction inhibited hemorrhage by constrict blood vessel between muscle, so the loss of blood in the endometrium. Lack of blood in uterus causes damage endometrium cell insight the uterus called amniotic fluid. The late process of uterine involution called the subinvolution.

In the animal study, day 3 postpartum showed that the wet weight of the uterus decreased to less than half of that on day 1 postpartum, going back to the weight of a non-pregnant uterus. In 3 days of postpartum, the collagen capacity of the uterus dropped to the levels of a non-pregnant uterus. Half of the collagen loss appeared on the first day after childbirth in rats, and that the losing of collagen continues quickly for around 2 days postpartum before slowing down. However, on 5 days postpartum showed the wet weight and collagen component of the rat uterus downfall by only 70% w. The degradation of tissue collagen causes the reduction in the size of the uterus (Elkhalil et al., 2005). The study by Grant in 1965 found that lactating and non-lactating rat was a similar rate of uterine involution at the 3rd day postpartum. At 24 hr., postpartum in both groups showed a detached of collagen removal. The number of

uterine muscle cells does not seem to change, although the size of the uterine muscle cells varies in postpartum period (Grant, 1965). Moreover, the levels of collagenase in rat uterine have been shown the highest levels within the first 72 h postpartum. Morphological studies have searched for the significance of the evident increase in macrophage activity postpartum and have associated this event to the quick loss of collagen. The electron microscopic study showed the involution of the rat on the day of parturition found the increased appearance of lysosomes containing cell rubble and collagen. In postpartum found the smooth muscle cells altered in some areas. The frequency of autophagic vacuoles cell organelles appeared to have declined at third day postpartum. The fifth and the seventh day postpartum found the ultrastructure of all cell types was substantially the same as the third day postpartum. The collagen component of the uterus was decreased 3~4 days following parturition and collagenase activity was increasing. Moreover, phagocytic intake of collagen increased during postpartum involution (Henell et al., 1983).

The other study shows an acid cathepsin and β-glucuronidase activities decreased during pregnancy and increased markedly during involution. Moreover, the levels of β-galactosidase and acid phosphatase were increases during involution. The enzyme was related to degrading the actomyosin complex of smooth muscle cells unchanged during gestation and involution. In addition, the alkaline proteinase inhibitor showed a rapid decline during postpartum (Henell et al., 1983). In addition to the study of the uterus there are also study about uterine cervix, the results show the changes in the circumference and mechanical properties, from the time of childbirth to 16 days after. The weight of cervix was decreased by loss of collagen, that obvious 24 hr after childbirth (Harkness and Harkness, 1961)

In human, the most intensive uterine involution period occurs in the first month after childbirth. Primiparous and multiparous women are the same trends of involution; however, in multiparous women have a period longer than 6–8 weeks (Paliulyte, 2017). The uterine sizes of multiparous woman are usually larger than in primiparous (Keng-Fu Hsu, 2014). When the size of the uterus returned to the pre-pregnant state size and found the normal estrous cycle were defined as the completion of uterine involution. Several factors like nutrition, care for offspring and season of parturition were important for completion of uterine involution and renewal of sexual after childbirth (Medan, 2015). Moreover, women delivered vaginally has the uterine involution faster than delivered by emergency C/S heedless to the weight of the infant. The women delivered newborns weighing more than 4 kg and the women who are their high vaginal swab showed deferrable the involution. During lactation, oxytocin released causes expanded uterine contraction, rush uterine involution, and so reduce postpartum blood loss (Anwar, 2009).

1.6 Mammary gland

The mammary gland is an exclusive organ accountable for milk formation, secretion, and involution to get ready the gland for the following lactation. The branching network of ducts in the mammary gland eliminates in alveoli (Jena et al., 2015). The mammary gland is a complicated and specialized tissue that has developed to prepare nourishments for the baby. The mammary epithelial cells are erased when they are no prolonged wanted lead to reduce the capacity of the mammary gland to produce milk. Apoptosis is a programmed cell death that appears in multicellular animals. It's the mechanism that responds to the annihilation of the secretory epithelium

(Watson, 2006). The rat mammary gland epithelium composed of branching ducts border to the nipple. The actions of systemic hormones and locally synthesized growth factors had effects on morphogenesis and differentiation of the mammary gland. The breast structural and functional change because of the epithelial extension during adolescence and cycles, secretory differentiation during pregnancy and lactation, and regression during involution. Rodents and humans can occur in these stages (Liska et al., 2018).

The mammary epithelial cells (MECs) are the complex that produce the milk and differentiate, regenerate, undergo apoptosis, and proliferate following a cyclic route of lactation – involution. Moreover, it was controlled by hormones, growth factors and other molecules. The major growth of the mammary gland occurs after puberty. The interesting physiological procedures of cells recover the structure of a virgin mammary gland occurs in the involution stage (Jenaa et al., 2019). Adipose connective tissue in mammary gland rats had rich with few glandular elements during the non-pregnant stage. The alveoli were increased during pregnancy became enlarge with milk and protuberance sac formation during lactation (Abunasef and El-Beshbishy, 2014). The invagination process of the mammary duct with mammary epithelial cells (MECs) into the fat pad before adolescence does not depend on the hormone. The expansion of mammary ducts was accelerated by the ovarian steroid hormones (Jena and Mohanty, 2017). During lactation, the ducts are transported milk by two layers of epithelial cells: include the inner luminal layer and the outer contractile myoepithelial layer. The alveoli made up of the lobules of the branching duct. The deepest layer of alveoli invented of epithelial cells that transform and exude milk after childbirth. The milk stasis and involution of the mammary epithelium are beginning after stop feeding (Jenaa et al.,

2019). Involution of the mammary gland is a necessary process when they become iterative at weaning. The collapse of the secretory epithelium and its restoration by adipocytes involve a two-step process of mammary gland involution. The remodeling is restrained and apoptotic cells can be appeared in the lumen of the alveoli at the first phase. In the second phase, apoptosis is along with reshaping of the enclosing stroma and re-differentiation of the adipocytes (Watson, 2006).

The basic physical area of milk creation is an alveolus, innerly with the excretory mammary cells and externally with a mesh of very tiny blood vessels and muscle cells. The milk production used the capillaries to deliver blood and precursors. The fresh milk was excreted by the alveoli through the ducts system. The mechanical of milk secretion is the electrical communications to the brain effect in the free of the oxytocin, which convinces contraction of the muscular surrounding of the alveoli causes milk ejection. Therefore, the arrangement of milk alters with time after delivery, the milk production cycle can be separated into a distinct phase of lactation. Instantly, after childbirth, the mammary gland excretes the colostrum. Colostrum composed of very excessive levels of alimentary demanded in the primary days of life and thus is valuable in fat and proteins for example immunoglobulins. In early lactation, the capacity of both total protein and casein is very high and then quickly reduces to attain their lowest level, after which both enhance slowly throughout the pause of lactation. In mid-lactation, the proportion of caseins and total protein degraded products, being the lowest. In late lactation, the milk yield becomes so low until milking is stopped. Many of continuous compositional alteration occurs, such as surging pH, changing salts balance, and decreasing the level of casein as a total protein, all of which adversely impact on the production of dairy outputs during late lactation (Kelly and Larsen,

2010).
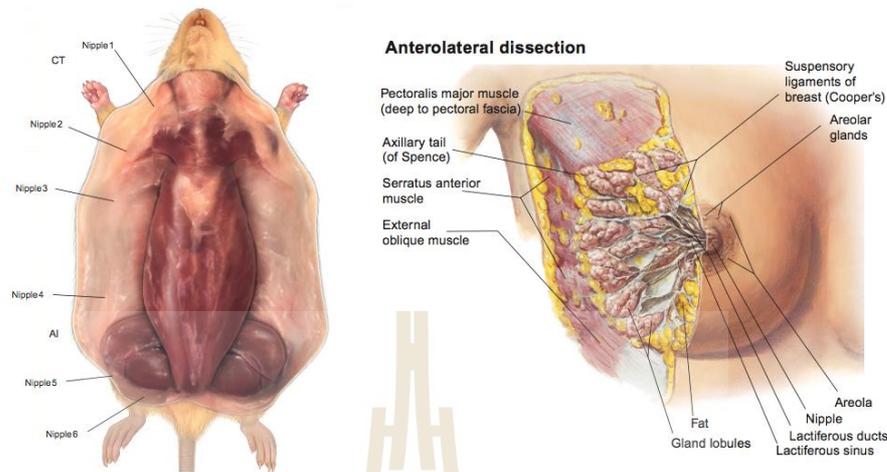
Figure 1.2 Rat regional mammary anatomy (left). Each gland terminates into a single collecting duct that deliver milk via a single nipple. The rat has six nipples. In humans (right), the single pair of mammary glands are detected over the pectoralis major muscle of the anterior chest (Treuting et al., 2017).

1.7 Previous study about traditional medicine on postpartum period

In many parts of the world, traditional medicinal plants have a significant function during pregnancy, delivery, and postpartum care. *Cinnamomum zeylanicum* is traditional medicinal plants used in uterine hemorrhage, stomachache, as an antiseptic and an astringent (Jayaprakasha et al., 2003). Moreover, *Symplocos racemosa* is used for treating various female disorders. It is astringent, cold and used in menorrhagia, blood disorders, for wound healing and to stop hemorrhage (Neelam et al., 2011). In Indian, cardamom used to ingredients to the maternal diet after 15 days of labor in the early morning, it is more efficient against strengthening the mother's body after labor, avoid more bleeding, etc (Rajith et al., 2010). The study in Nigeria found *Centaurea*

perrottetii is a medicinal plant that heals postpartum hemorrhage (Kankara et al., 2015). The studies of traditional medicinal plants for used in ante- and postpartum health care in December 2005 to Mars 2006 in Central and Northern Lao People's Democratic Republic (PDR) were collected. It showed 20 species to promote maternal health and postpartum resumption of strength, 16 species have a function of healing and contraction of the uterus and 8 species for promoting lactation. *Clerodendrum* species are used in traditional medicine to stoppage bleeding from wounds as well as stopping postpartum hemorrhage, the research does not found chemical composition these asserted effects. In the northern villages Lao Khao and Nam Vang, *Phlogacanthus* cf. *annamensis* used generally against postpartum hemorrhage and allay pain, chemical composition has been separated from this plant such as diterpene lactones, steroids, and triterpenes (Lundh, 2007).

Traditional Chinese maternal care is normally used to help in the restoration of women during postpartum. The previous study has reported the traditional medicine such as Sheng-Hua-Tang can increase myoelectric activities in the postpartum rabbit (Hong et al., 2003). Moreover, it showed significantly related with the anteverted uterus and was a predictor of the anteverted uterus. Sheng-Hua-Tang related to the step of uterine involution after childbirth, suggest that these therapies might possess the pharmacological potency of uterine contraction in postpartum women (Ho et al., 2011). Other studies found Si-Wu-Tang may be associated with health-related quality of women's life in Taiwan. Si-Wu-Tang has effects on uterine contraction by inhibiting uterine contraction so reduce uterine pain after delivery (Chang et al., 2013). The plant was used as the main ingredient of native American diets such as *Apios americana.* In Japan, Apios is used as a postpartum medication. The study of Apios showed Apios

can accelerate uterine involution, and proved the potential application of Apios in postpartum care. The parameter is the morphological changes, the hormone concentrations, and the collagen clearance process (Zheng et al., 2019).

Many studies were not clear about the mechanism of the extract on uterine involution and herbal medication care. These extracts may be related to hormones or other substances that affect reproductive organs in the postpartum period such as prostaglandin, estrogen, progesterone, and oxytocin. In cows study, $PGF_2\alpha$ was using during the early postpartum period can improve uterine involution and fertility (Nakao et al. , 1997) . The oral administration of herbal product with proven inducing contraction of the uterus leading to the expulsion of fetal and restorative actions, therefore, appears to be a secure and productive option both therapeutically and prophylactically. The scientists had reported the uses of medicinal plants were found efficient uterine restorative, which advantages the postpartum health and productivity of the animals when either used therapeutically or prophylactically (Ravi and Bhagwat, 2007).

1.8 Previous study about traditional medicine on mammary gland

Breastfeeding is widely knowing to have many health advantages for both infants and mothers. In developing countries, women believe in medicinal plants to increase breast milk production. Many researches have examined the effects of plant extracts on milk yield and milk ingredients to help increase milk production. The increase of milk production has a value on industry and individuals. The dietary supplementation of black seed or ginger fine powder were investigated their effects on hematological parameters, colostrum composition, milk yield and composition of

lactating ewes. The results indicated that dietary supplementation of medicinal plants to ewes' diet during the pregnancy period may enhance milk production on ewes (Hendawy et al., 2019). The study in cows receiving the green tea product showed an increased milk yield and had a significantly increased daily total of energy-corrected milk yield from week 2 to week 9 postpartum. Moreover, the green tea extract has no effect on milk composition such as fat, protein, and lactose. However, the milk protein and milk fat were higher in the group that receive the plant product when compare with the control group. Green tea composed of a polyphenol, major polyphenols in green tea are catechins, minor components in green tea are proteins, lipids, sterols, vitamins, caffeine, and theophylline. The phytochemical component of green tea extract is probably responsible for traditional use (Winkler et al., 2015). The blends of selected fermentation metabolites CitriStim yeast, Aspergillus niger, and plant extracts capsicum, cinnamaldehyde, and eugenol were studying in cow diets supplementation. The results showed that feeding supplemental plant extracts did not affect milk yield, but did affect milk components (Boyd et al., 2012).

In lactating rats, *Euphorbia hirta* (*E. hirta*) was investigated its effect on milk production. *E. hirta* is one of the curative plants used in Burkina Faso for the treatment of lactation deficiency. The preliminary phytochemical screening of the *E. hirta* aqueous extract exposed the presence of steroids and triterpenoids, tannins, flavonoids, coumarins, anthocyanosids, and reducing sugars. The results indicated that the extract-treated rats produced higher milk yield than blank and reference controls. *E. hirta* composed of several phytochemical compounds that may stimulate milk production in mammalian (Maya et al., 2018). *Musa paradisiaca L.* (*M. paradisiaca*) is a medicinal herb that used in traditional medicine to heal and control diarrhea, intestinal disorder,

blood disorders, venereal diseases, an anti-inflammatory, and analgesic. The *M. paradisiaca* flower show increase the milk manufacture of postpartum rats (Mahmood et al., 2012). Several phytochemicals have been discovered in *M. paradisiaca* such as serotonin, nor-epinephrine, tryptophan, indole compounds, and tannin (Dutta et al., 1983). In West Africa, *Gossypium herbaceum L.* (*Malvaceae*) (*G. herbaceum*) is used traditionally as a galactagogue, dysmenorrhea, spermatogenic, and for the removal of retained placenta. In humans, *G. Herbaceum* have the effective, secure and cost-effective for increase milk supply in lactation (Manjula et al., 2013). This plant contain with glycosides, linoleic acid, tannins, steroids, phenolics, oleic, palmitic, sitosterol, saponins, ergosterol, lipids, gossypol, and carbohydrates (Khaleequr et al., 2016). Moreover, condensed tannins (proanthocyanidins) can improve live weight gain, milk yield, ovulation rate, protein concentration (Guil-Guerrero et al., 2016). Many studies were not clear about mechanisms of the extract on milk production. These extracts may be related to hormones or other substances that affect mammary gland such as prolactin dopamine and oxytocin.

1.9 *Heliotropium indicum* L.

Heliotropium indicum (Family: Boraginaceae) (*H. indicum*) commonly known as the "Cock's comb". This plant is widely used in West Africa, India and the Philippines (Koffuor et al., 2012). The plant is informed to be very good in the traditional medicine and is believed to be useful in treating abdominal pain, insect bites, venereal diseases, fever, menstrual abnormal, and urticarial. The plant extract is carefully used as a diuretic for the treatment of kidney stone (Dash and Murthy, 2011). Some reports showed 24 h. after rats treated with *H. indicum* extract at dosage 1, 2, 4

and 5 g/kg bw there were no lethal effects. Acute toxicity study for 14 days showed no lethal effects in rats that were treated *H. indicum* extract at dosage 0.5, 1 and 2 g/kg bw but there were some changes in lung and kidney weight (Boye et al., 2012). Additional, pyrrolizidine alkaloids (PAs) presented in *H. indicum* have toxicity in human by destroying the liver (Dharmananda, 2001).

Figure 1.3 *Heliotropium indicum* Linn. (Family: Boraginaceae) (Roy, 2015).

Traditional Indian used *H. indicum* to heal disease of the dermis, poison bites, stomachache, abnormal nervous system, and used root juice of *H. indicum* to recover ophthalmic and fresh leaf extract is applied externally in fresh cuts and wounds. Whereas Tamil Nadu used leaf juice by boiling with coconut oil to kill dandruff. Eastern Nicaragua used the extracts (leaf and root together) for nursing whooping cough in children. The ethnopharmacological survey revealed that *H. indicum* is accepted to be useful in treating malaria, abdominal suffering, and dermatitis. In Jamaica, females used the infusion of the flower for the therapeutics of menorrhagia by taken orally (Roy, 2015). In Thailand, the dried flower and root of *H. indicum* is believed to expel the blood from the uterus during menstruation (Thiengburanathum, 1999). Moreover, *H. indicum* have effects that stimulated uterine contraction (Chaichanthipphayuth, 1980). Other folk cures include used of water extract of the

leaves for nursing of insect bites, skin disease, stings, diarrhea, fever, menstrual disorder, and urticarial. The leaves macerated with sugar cane juice is to be useful in curing insect stings and scorpion stings (Roy, 2015) . Effects of *H. indicum* on gastrointestinal had been studied but not clear, water extract of *H. indicum* do not have a clear effect on rat isolated small intestine specimen but showed relaxation effects on rabbit isolated small intestine specimens. Moreover, *H. indicum* alcohol extract has smooth muscle relaxant effects on rabbits. Water and alcohol extract does not have the effects on the smooth muscle of toad stomach (Chaichanthipphayuth, 1980).

The anti-fertility is one of the properties of *H. indicum* extract. The research examination of the ethanolic extract of the plant leaves showed the significant abortifacient property and moderate activity on implantation and sperm motility (Savadi et al., 2009). The whole plant *H. indicum* ethanolic extract showed stimulation of uterine contraction by the non-genomic active pathway. There are 2 ways in which the substance will active; 1) activated L-type Ca^{2+} channel for increased Ca^{2+} influx into the cell and 2) activated Ca^{2+} in sarcoplasmic reticulum by IP_3 receptors. The extract did not work through estrogen receptors (Kupittayanant and Kupittayanant, 2012) . Moreover, studying the site of action of an aqueous extract of the aerial part of *H. indicum* on smooth muscle reported the extract can induce uterine contraction. The extract has a chemical component that has stimulatory effects at adrenoceptors and possible enhance prostaglandin synthesis (Koffuor et al., 2012).

1.10 Aims

The effects of *Heliotropium indicum* L. extract on uterine involution in postpartum rats. There were five main aims to the program of this work, which were therefore to evaluate:

1) phytochemical components of ethanol extract of *Heliotropium indicum* L.

2) the effects of ethanol extract of *Heliotropium indicum* L. on uterine contractions in postpartum rats.

3) the effects of ethanol extract of *Heliotropium indicum* L. on uterine involution.

4) the effects of ethanol extract of *Heliotropium indicum* L. on histological structures by H&E staining and biochemical components of the uterus and mammary glands by Fourier Transform Infrared spectroscopy (FTIR).

5) the effects of ethanol extract of *Heliotropium indicum* L. on blood biochemical parameters in postpartum rats.

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CHAPTER II

GENERAL MATERIALS AND METHODS

The aims of this thesis were to investigate the effects of *H. indicum* extract on uterine contraction, uterine involution, mammary gland, blood biochemical parameters in postpartum rats, and to analyze phytochemical components of ethanol extract of *H. indicum.* Moreover, study the effects of *H. indicum* extract on histological structures by H&E staining and biochemical components of the uterus and mammary glands by Fourier Transform Infrared spectroscopy (FTIR). The study was conducted in both the Reproductive Physiology Laboratory, Suranaree University of Technology and Synchrotron Light Research Institute (SLRI). This chapter describes the general details of major materials and methods used in this research. More details relevant to each study are given in each chapter.

2.1 Chemicals

All the chemicals, reagents, and solvents used in the assay protocols were 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 company.

2.2 Preparation of plant materials

The whole plant of *H. indicum* were collected from Phra Nakhon Si Ayutthaya province, Thailand. The specimens were specified and authenticated by Royal Forest Department, Bangkok, Thailand. The plant sample were deposited at the herbarium with reference number BKF194426. The whole plant was extracted by ethanol (detail in Chapter III) and kept at -20 °C till used for experiment.

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 Care Unit, Suranaree University of Technology (SUT), Thailand. All animals were administered under environmentally controlled room provided with a 12:12 light and dark cycle at a temperature of approximately 25°C. They were fed with commercial food (C.P. Mice feed, Bangkok, Thailand) and allowed to access water *ad libitum*.

2.3.2 Vaginal cytology

Confirmation of mating and pregnancy was done by the vaginal plug method (Voss, 1979), as modified by Ochiogu et al. (2006), was used in discovering successful mating. The female rats were placed in a cage with a male rat of proven fertility. A vaginal smear of each female was taken on a glass slide with the aid of a wet cotton swab dipped in fresh normal saline and inserted into the vagina to a depth of approximately 1.5 cm. The wet smear was examined grossly for the presence of spermatozoa or protein coagulates as confirmation of successful mating. Thereafter, the females were separated from the males until delivery (Ochiogu et al., 2013)

2.3.3 Animal ethics

Female Wistar rats (200-250 g) were used in this study and maintained in accordance with the guidelines of the Committee on Care and Use of Laboratory Animal Resources, National Research Council, Thailand. The experiments performed on rats 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 experiment was carried out in both *in vitro* and *in vivo* which were divided into major eight parts as follows:

Part 1 was designed to examine phytochemical screening of *H. indicum* extract using standard chemical detection methods, GC-MS and FTIR (detailed in Chapter III).

Part 2 was designed to measure of isometric force of uterine contraction using organ bath system (detailed in Chapter IV).

Part 3 was designed to determine relative uterine weight (%RU) in postpartum rats (detailed in Chapter IV).

Part 4 was designed to measure uterine cross-section area and collagen of uterus in postpartum rats using image processing program (detailed in Chapter IV).

Part 5 was designed to measure biochemical components of uterus in postpartum rats using FTIR (detailed in Chapter IV).

Part 6 was designed to measure size and histological structures of mammary glands using image processing program (detailed in Chapter V).

Part 7 was designed to measure biochemical components of mammary glands using FTIR (detailed in Chapter V).

Part 8 was designed to measure blood parameters such as AST, ALT, estrogen, and progesterone (detailed in Chapter VI).

The rats in part 3-8 were divided into 2 main groups: 1) non-treated rats and 2) treated rats. There were 3 subgroups containing: 1) 1 day-postpartum rats 2) 3 day-postpartum rats 3) 5 day-postpartum rats. The extract was diluted to 250 mg/kg B.W. in the vehicle (Tween 80, 10% v/v) and was administered orally to the treated rats group at a volume of 0.8 mL/day for 1, 3, and 5 days, respectively. Only the vehicle was administered orally for the rests. The dose of the extract that we use came from previous study, it's shows effect on uterine contraction (Kupittayanant and Kupittayanant, 2012).

2.5 *In vitro* study

2.5.1 Phytochemical screening

H. indicum extracts were screened by different chemical tests for the identifying the basic phytochemical components present in the extract. The standard chemical tests for presence of alkaloids, flavonoids, saponins, steroid, glycoside, phenols, reducing sugar, carbohydrate, and protein were achieved to get a preparatory idea of the chemical component (Basak, 2016) (Dasarapu et al., 2015) (Ghosh et al., 2018). Moreover, *H. indicum* extract were analyzed for the component by GC-MS and FTIR (detailed in Chapter III).

2.5.2 Measurements of uterine contraction

The study was designed to measurement of isometric force of the uterus on day 1, 3, and 5 using organ bath system. The rats were euthanized by strangulation with CO_2 after the requirements of each group have been done. The uterus was collected. Longitudinal strips were dissected from each uterus. Whole thickness strips

were suspended in the direction of longitudinal smooth muscle fibers in organ baths of 25 mL each, filled with modified Krebs-Henseleit solution. The single chamber were linked to a digital signal and noted on a computer using Chart software (Kupittayanant, 2003). More details relevant to determine relative uterine contraction are given in Chapter IV.

2.6 *In vivo* study

2.6.1 Determine relative uterine weight

The uterine weight changed in postpartum period. Uterine weight was observed on day 1, 3, and 5 postpartum for measurement relative uterus (%RU). %RU were calculated using the following equation: (Uterine weight X 100) / Body weight. More details relevant to determine relative uterine weight are given in Chapter IV.

2.6.2 Uterine cross-section area

The uterus in postpartum period was returned size to its pre-pregnant stage. The uterine tissue on day 1, 3, and 5 postpartum in this study was cut and stained with Hematoxylin and Eosin. Histological investigation of the sample was performed using a light microscope. The uterine picture from light microscope was calculated uterine cross-section area by CellD program. More details relevant to uterine cross-section area are given in Chapter IV.

2.6.3 Relative collagen in the uterus determining by using k-means cluster analysis (KMC), MATLAB

The collagen in the uterus was changed in the postpartum period. The uterine tissue on days 1, 3, and 5 postpartum in this study was cut and stained with Masson's Trichrome. Histological investigation of the sample was performed using a light

microscope. The uterine picture from light microscope was relative calculated collagen in the uterus determining by using k-means cluster analysis (KMC), MATLAB. More details relevant to determine collagen in uterine are given in Chapter IV.

2.6.4 Biochemical components of the uterus determining by Fourier Transform Infrared spectroscopy (FTIR)

The uterine samples on days 1, 3, and 5 postpartum were analyzed. Fourier Transform Infrared spectroscopy (FTIR) is a technique use for the analysis of biological samples like tissue by detecting changes in macromolecular composition occurring during the uterine involution process. More details relevant to determine biochemical change in uterine are given in Chapter IV.

2.6.5 Mammary gland size

The mammary gland size in postpartum period was changed. The mammary glands tissue on days 1, 3, and 5 postpartum in this study was cut and stained with Hematoxylin and Eosin. Histological investigation of the sample was performed using a light microscope. The mammary glands picture from light microscope was calculated the mammary gland size by CellD program. More details relevant to mammary gland size are given in Chapter V.

2.6.6 Histological structures of the mammary glands by ImageJ

The histological structures of the mammary glands were changed in postpartum period. The mammary glands tissue on days 1, 3, and 5 postpartum in this study was cut and stained with Hematoxylin and Eosin. Histological investigation of the sample was performed using a light microscope. The mammary glands picture from light microscope was calculated the ratio of parenchyma cell and stroma cell in mammary glands determining by image processing using ImageJ. More details relevant to

histological structures of the mammary glands are given in Chapter V.

2.6.7 Biochemical components of the mammary glands by Fourier Transform Infrared spectroscopy (FTIR)

The mammary glands tissue on days 1, 3, and 5 postpartum was analyzed. Fourier Transform Infrared spectroscopy (FTIR) is a technique use for the analysis of biological samples like mammary glands tissue by detecting changes in macromolecular composition occurring during postpartum period. More details relevant to determine biochemical change in mammary glands are given in Chapter V.

2.6.8 Blood biochemical parameters

The blood samples were collected from the rats in treated-rats after treated the extract of *H. indicum* 250 mg/kg B.W. in the vehicle (Tween 80, 10% v/v) and the non-treated rats were treated vehicle a volume of 0.8 mL/day for 1, 3, and 5 days, respectively. Blood biochemical parameters including estradiol, progesterone, aspartate aminotransferase (AST), and alanine aminotransferase (ALT) were examined. More details relevant to determine blood biochemical parameters in postpartum rats are given in Chapter VI.

2.7 Statistical analyses

Registration of smooth muscle activity was expressed as changes in the strips' tension and calculated as AUC (area under the contraction). All data were expressed the mean $\pm$ S.E.M. and n refers to the number of strips from different animals. For paired two groups of data were analyzed by Paired-samples t-test. For three or more groups, data was analyzed by one-way ANOVA and post-hoc with Tukey's test. The parameters including uterine weight, uterine cross-section area, collagen determining, FTIR band area, alveoli size, ratio of parenchyma cells, and blood biochemical

parameters were expressed the mean $\pm$ S.E.M. and n refers to the number of animals. For paired two groups of data were analyzed by Independent-samples t-test. For three or more groups, data was analyzed by one-way ANOVA and post-hoc with Tukey's test. All statistical were analyzed by The Statistical Package for the Social Sciences (SPSS). The significance level at $P < 0.05$.
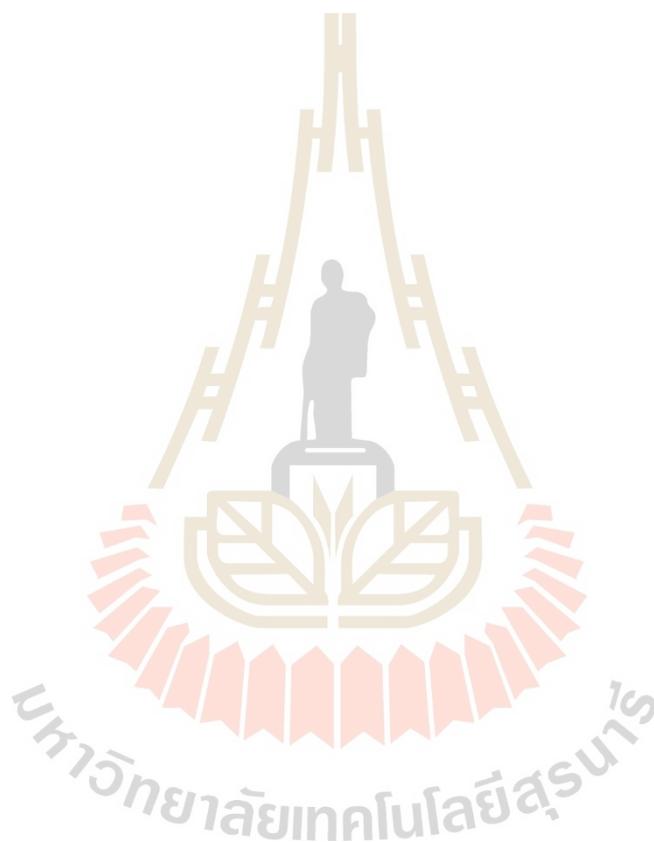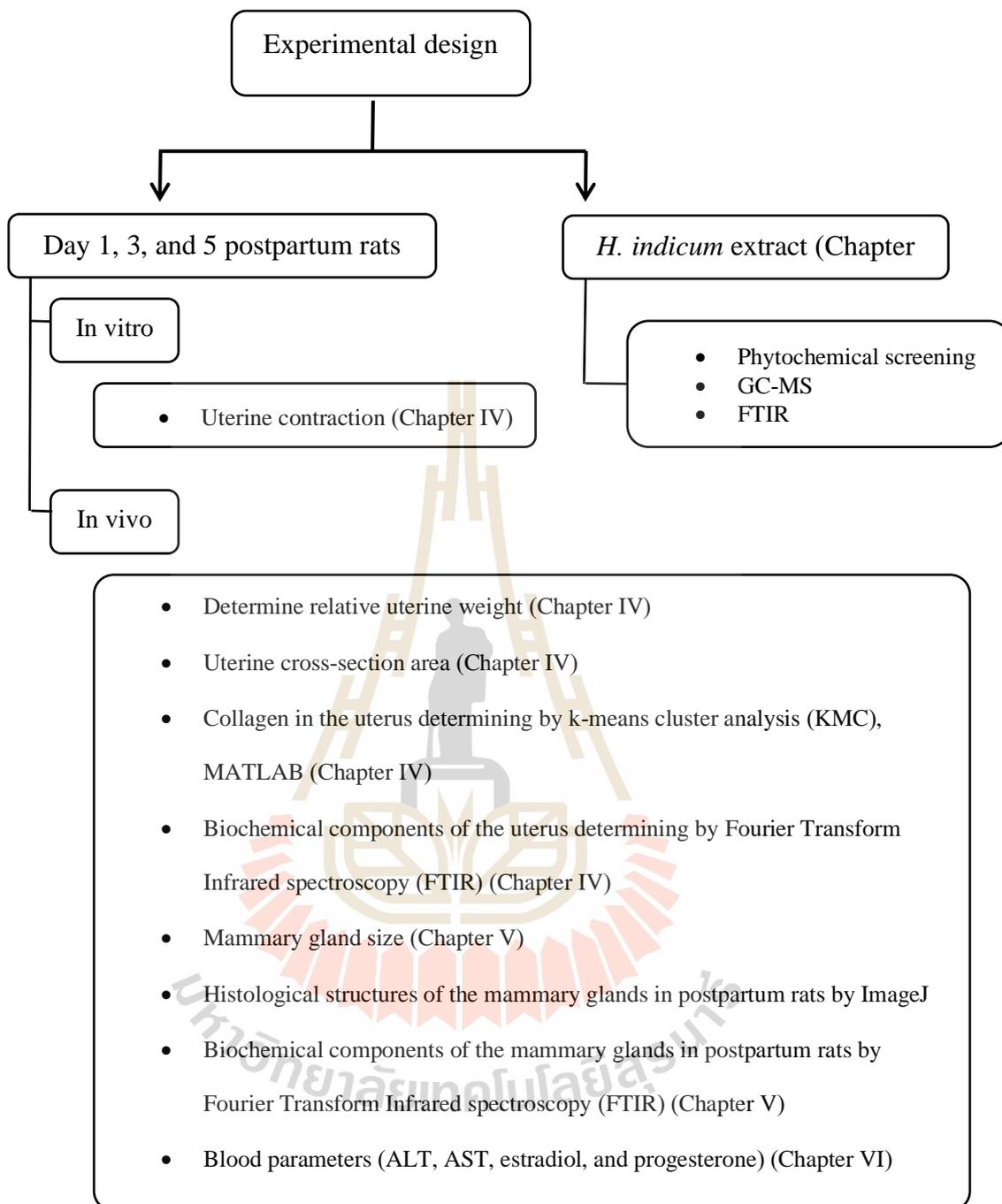
Figure 2.1 Experimental design.

2.8 References

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CHAPTER III

PHYTOCHEMICAL SCREENING OF *HELIOTROPIUM INDICUM* L. EXTRACT

3.1 Abstract

This study has investigated the effects of ethanol extracts of the whole plant of *Heliotropium indicum* Linn (*H. indicum*) on uterine involution and mammary gland in postpartum rats. The phytochemical constituent for supported their effects was studied. The whole plant of *H. indicum* was extracted by macerated with ethanol and shaken for 3 days. The mixture was filtered and evaporated by a rotary evaporator, dried by using lyophilizer and stored until use. In this work, phytochemical screening methods, Gas chromatography coupled with a mass spectrometry (GC-MS) analysis and Fourier transform infrared spectroscopy (FTIR) analysis were performed to identify phytochemical constituents of *H. indicum* extract. The phytochemical screening methods showed the presence of various active medical constituents in the *H. indicum* extract such as alkaloids, tannins, flavonoids, steroids, and glycoside. Moreover, *H. indicum* GC-MS analysis showed many constituents such as phytol, acetic acid, neophytadiene, hexadecanoic acid, hexadecanoic acid, ethyl ester, and 9,12-Octadecadienoic acid (Z,Z)- or linoleic acid ester, they have an effective anti-inflammatory, antimicrobial, antioxidant, and anticancer. Additional evidence created from the FTIR analysis of whole plant extract of *H. indicum* has shown the presence of

functional groups such as alkaloids, phenols, carboxylic acids, amines, flavonoids, phosphate compounds, glycoside, lignins and tannins, thus exposing the presence of vital phytoconstituents. Moreover, *H. indicum* had no acute toxicity and no lethal effects. on both mother and baby. Based on phytochemical screening, GC-MS analysis, FTIR analysis, and literature review, the presence of various compounds in whole plant extract of *H. indicum* is probably responsible for traditional uses. The *H. indicum* extract may have accelerated uterine involution, increased uterine contraction, changed blood biochemical parameters, and increased milk production.

3.2 Introduction

Heliotropium indicum Linn. (Family: Boraginaceae) (*H. indicum*) is a small annual or perennial herb distributed in Bangladesh, India, Srilanka, Nepal, Thailand, Tropical Asia, Africa, and Philippine (Ghani, 2003). The plant is traditionally used in many countries for wound healing, secretagogue stimulation, febrifuge, and cure eye infections. The roots powder is adapted to dermatitis and suppurating eczema in children in Senegal. Anti-inflammatory, gastroprotective, and anti-cancer properties were investigated for accomplishing to evaluate for confirm traditional used (Adelaja et al., 2008). The methanol extract of the dried roots of *H. indicum* was explored by the phytochemical screening test. The results showed the presence of tannins, saponins, flavonoids, steroids, and alkaloids (Rahman et al., 2011). In other studies, the sun-dried aerial part of *H. indicum* found alkaloids, cyanogenic glycosides, tannins, saponins, and steroids in aqueous and ethanol extract (Boye et al., 2012). The effects of crude aqueous extracts of the stem with leaves and roots of *H. indicum* on blood pressure in Wistar rats was investigated. The thin layer chromatography was used for the phytochemical

screening of extracts. The results revealed the presence of many secondary metabolites such as coumarins, flavonoids, anthocyanics, lignans, saponins, tannins, terpenes, and triterpenes in both extracts (Adjagba et al., 2017). The investigated of ethanolic leaf extract of *H. indicum* was for phytochemical screening. The presence of alkaloids, flavonoids, saponins, carbohydrate, glycoside, and phytosterol were found by standard procedure (Basak and Dey, 2016). Moreover, the ethanol extract of the whole plant of *H. indicum* was investigated phytochemical screening. They found flavonoids, phenol and tannins, steroids, alkaloids, and protein (Dasarapu et al., 2015).

Previous reports of phytochemicals from *H. indicum* have been showed the aerial parts contained pyrrolizidine alkaloid and the plant also contained rapone, lupeol, and an ester of retronecine. The roots of *H. indicim* contain a high total of estradiol and have a recent pyrrolizidine alkaloid, helindicine. The seeds show heliotrine, heliotridine-N-Oxide, heleurine-N- Oxide, europine-N-oxide, heleotrine-N-Oxide, and cynoglossine. Other alkaloids in seeds show spermidine, homo spermidine, and putrescine. Moreover, the spermine have been identified in the leaves of *H. indicum*. Alkaloids, several triterpenes, and steroids including lupeol, β- sitosterol, campesterol, and stigmasterol have been reported from the whole plant. The yields an essential oil which consists mainly of phytol (49.1%), 1-dodecanol (6.4%), and β-linalool (3.0%) (Roy, 2015). Additionally, pyrrolizidine alkaloids are expelled in milk. The pyrrolizidine alkaloids have different characterize, they were studied by many researchers in various investigations and most of them showed hepatoxic activity. The identified alkaloids are included heliotrine, lasiocarpine, indicine, 12- acetyl indicine, indicating, indicine- N- oxide, retronecine, tracheal- thamide, quinidine, echinate, heleurine, lasiocarpine- N- oxide, quinidine, putrescine, spermine, spermidine, and

lindelofidine (Ghosh et al., 2018).

Chromatography coupled with a mass spectrometry (GC-MS) technic found phytochemical constituent in *H. indicum* extract such as unknown (21%) and the remainder is fatty acids. Alkaloids that found in *H. indicum* extract can increase smooth muscle contraction from guinea pig (Pomeroy and Raper, 1971). In the present study, the alcoholic extract of *H. indicum* leaves were examined for possible larvicidal activity. GC-MS were chosen to identify and characterize the composition of curative value extracted from *H. indicum*. The results showed benzene acetaldehyde, 5H-1-Pyrindine, benzene acetic acid, phenol, ethyl ester, phytol, 9,12-Octadecadienoic acid (Z, Z)-, and many other components (Ramamurthy and Krishnaveni, 2014). The study of ethanolic *H. indicum* leaves extract found the four compounds were identified in by GC-MS analysis. The general compounds were squalene and phytol. The biological activities of common compounds are antibacterial, antioxidant, antitumor, cancer-preventive, chemopreventive, immunostimulant, and lipoxygenase-inhibitor for squalene and cancer-preventive for phytol (Meenatchi Ammal and Viji Stella Bai, 2013).

The Fourier transform infrared spectroscopy (FTIR) analysis of *H. indicum* has been few reported. The ethanolic *H. indicum* leaves extract were studied by FTIR. The results indicate the presence of some functional groups such as the C-I stretch at 490.14 cm^{-1}, the C-Br stretch at 516.62 cm^{-1}, the C-H bend at 824.83 cm^{-1}, 668.25 cm^{-1}, 2923.03 cm^{-1}, the C-Bl stretch at 824.83 cm^{-1}, the C-O stretch at 1057.84 cm^{-1}, the C=C stretch at 1615.72 cm^{-1} and 2360.41 cm^{-1}, C=O stretch at 1733.33 cm^{-1} and the OH stretch at 3383.83 cm^{-1}, 3734.83 cm^{-1} and 3820.39 cm^{-1}, respectively. The peak of these remarkable functional groups is environmentally friendly bioprocess with utilization in

pharmaceutical drug advancement (Malomo et al., 2016). *Heliotropium bacciferum* (*H. bacciferum*) is eminent in a medicinal perspective and belongs to Boraginaceae family, the same genus with *H. indicum.* The dominant IR peaks of various extracts of the plant were showed the different compounds such as aldehydes alcohols, amides, ketones, ethers, and carboxylic acids. The more strong bands taking at 2924 cm^{-1} , 2998 cm^{-1}, 2854cm^{-1}, 2853cm^{-1}, 1724cm^{-1}, 1489 cm^{-1}, and 1230 cm^{-1} corresponding to the stretching or bending vibrations of O–H or N–H or C–H, C=O and C–Cl or C–S, respectively, signify the being of amino acids, nitrates, alkenes, ethers, organic-halogen compounds, and carbohydrates (Ahmad et al., 2014).

From the previous study, *H. indicum* alcohol extract has been shown to induce uterine contraction in postpartum rats on 1, 3, and 5 days postpartum. However, the *H. indicum* was recognized for some of its components and biological properties, this plant also may hold some other considerable biological activities. The native *H. indicum* which grown in Thailand had never been identified and there is no research-based data to prove the conventional uses. The purpose of this study of this study was therefore to analyze phytochemical constituents of ethanol extract of *H. indicum*. In this study, preliminary phytochemical screening methods, Gas chromatography coupled with a mass spectrometry (GC-MS) analysis, and Fourier transform infrared spectroscopy (FTIR) analysis for identifying phytochemical constituents of *H. indicum* extract were used. The results from this study are valuable, not only for supporting their effects on uterine involution that help reduce the risk of postpartum hemorrhage but also for further research to develop drugs or products of maternal care.

3.3 Methodology

3.3.1 Collection and authentication of plant

The whole plant of *H. indicum* was collected from Phra Nakhon Si Ayutthaya province, Thailand in May 2017. The plants were specified and authenticated by Royal Forest Department, Bangkok, Thailand.

3.3.2 Ethanol extraction

The whole plants were dried and powdered. The powder of the whole plant was extracted by macerated with 70% ethanol and shaken for 3 days. The mixed was filtered through filter paper to remove particulates. The extract was evaporated by a rotary evaporator, dried by using lyophilizer and stored at -20 °C until use (Figure 3.1). The % yield of the extract was calculated using the following equation:

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

Where $W_{\text{crude extract}}$ is the mean weigh of crude extract and $W_{\text{dried plant}}$ is the mean weight of dried plant material.
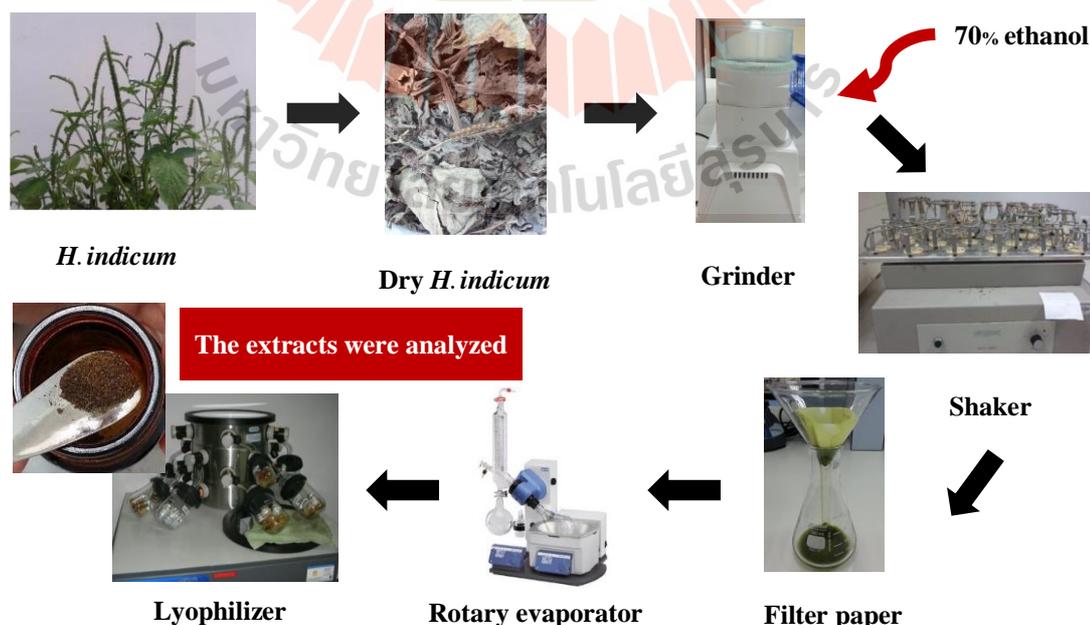
Figure 3.1 The method of *H. indicum* extraction.

3.3.3 Preliminary phytochemical screening

The lyophilized *H. indicum* extract concentration 20 mg/mL was tested for the presence of bioactive compounds, including protein, carbohydrate, reducing sugar, tannins, flavonoids, alkaloids, saponins, steroids, and glycosides.

Test for proteins: Ninhydrin test, the crude juice was tested by added to the boiled with 2 mL of 0. 2% solution of Ninhydrin. The violet color occurred indicating the presence of amino acids and proteins (Bansode and Chavan, 2013).

Test for carbohydrate: Iodine test, few drops of Iodine was added in other extract the dark blue color confirms the appearance of starch (Ram and Sinha, 2015).

Test for reducing sugar: Benedict's reagent test was performed by boiling 2 mL of Benedict's reagent with a crude extract. The reddish-brown color was showing indicated that the presence of reducing sugar (Jaradat et al., 2015).

Test for tannins: Extract was separately stirred with distilled water and then filtered. A few drops of 5% ferric chloride were then added. Black or blue-green coloration or precipitate was taken as positive result for the presence of tannins (Banso and Adeyemo, 2006).

Test for flavonoids: Twenty milligram of the extract was detained with acetone. The residue was extracted with warm water after evaporating the acetone on a water bath. The mixture was filtered while high temperature, the filtrate while hot, the filtrate could cool and used for the following test. Sodium hydroxide test: 2 mL of 10% NaOH solution was added, yellow solution indicates the presence of flavonoids which on adding dilute hydrochloric acid becomes colourless (Evans, 2002).

Test for alkaloids: Few mg (about 20 mg) of each extract was stirred

with 1% HCl (6 mL) on a water bath for 5 min and filtered. Wagner's test: Potassium iodide (2 g) and iodine (1.27 g) were dissolved in distilled water (5 mL) and the solution was diluted to 100 mL with distilled water. Few drops of this solution were added to the filtrate; a brown colored precipitate indicates the presence of alkaloids (Joshi et al., 2013)

Test for saponins: The foam test was performed. Shortly, a 1 mL aliquot of the *H. indicum* extract was shaken for 5 min. If saponins were present, they formed a stable foam in the test tube (Rauf et al., 2013).

Tests for steroids: Salkowski test, the crude extract (about 20 mg) was separately shaken with chloroform (2 mL) followed by the addition of concentrated H_2SO_4 (2 mL) along the side of the test tube, a reddish brown coloration of the interface indicates the presence of terpenoid (Ayoola et al., 2008).

Tests for glycosides: Cardiac glycoside (Keller-Killiani test): Extract (0.2 g) was shaken with distilled water (5 mL). To this, glacial acetic acid (2 mL) containing a few drops of ferric chloride was added, followed by H_2SO_4 (1 mL) along the side of the test tube. The formation of brown ring at the interface gives positive indication for cardiac glycoside and a violet ring may appear below the brown ring (Ayoola et al., 2008).

3.3.4 Gas chromatography coupled with a mass spectrometry (GC-MS) analysis

GC-MS analysis was carried out on a GC BRUKER model 450GC and gas chromatograph interfaced to a mass spectrometer instrument employing the following conditions: column: Rtx-5MS capillary column (30m x 0.25mm, fused silica 0.25 um) operating in electron impact mode at 70 eV; Helium gas (99.999%) was used

as carrier gas at a constant flow of 1 mL /min and an injection volume of 2 μL was employed (split ratio of 1:30) injector temperature 250 $^{\circ}$C; ion-source temperature 200 $^{\circ}$C. The oven temperature was programmed from 28 $^{\circ}$C, to 160 $^{\circ}$C ending with 230 $^{\circ}$C. Mass spectra were taken at 70 eV; a scan mode fragments from 35 to 400 Da. Total GC running time is 79.5 min. The relative percentage amount of each component was calculated by comparing its average peak area to the total areas. Software adopted to handle mass spectra and chromatograms was a NIST Mass Spectral Library 2008.

3.3.5 Fourier transform infrared spectroscopy (FTIR) analysis

Fourier transform infrared (FTIR) spectrum was analyzed to characterize the functional groups present in methanolic extract of *H. indicum*. Dried powder of *H. indicum* extract was used for FTIR analysis. Ten milligrams of the dried extract powder were loaded in ALPHA II FTIR spectrometer (Bruker Optics, Ettlingen, Germany) using the model discs. FTIR microspectroscopy analysis, spectra data were collected at an infrared microspectroscopy beamline (BL4.1 IR Spectroscopy and Imaging) at the Synchrotron Light Research Institute (SLRI). Spectra were acquired with an ALPHA II FTIR spectrometer (Bruker Optics, Ettlingen, Germany) , the measurement range from 4000 to 800 cm^{-1}. The microscope was connected to a software- controlled microscope stage and placed in a specially designed box that were purged by dry air. The measurements were performed in the scattering mode, using an aperture size of 10×10 μm with a spectral resolution of 4 cm^{-1}, with 64 scans co-added. Spectral acquisition and instrument control were performed using OPUS 7.2 (Bruker Optics Ltd, Ettlingen, Germany) software and analyzed by The Unscrambler X software (Thumanu et al., 2017). The spectra were smoothed with Savitsky–Golay smooth function to erase the noise. The spectra were baseline corrected and were

normalized with respect to specific bands for visual demonstration. Band areas were calculated from smoothed and baseline corrected spectra using OPUS software.

3.3.6 Acute Toxicity

60 pregnant rats were used. Rats were divided into 2 main groups: 1) non-treated rats and treated rats. They have 3 subgroups containing: 1) 1 day-postpartum rats 2) 3 day-postpartum rats 3) 5 day-postpartum rats. The extract of *H. indicum* were diluted to 250 mg/kg B.W. in the vehicle (Tween 80, 10% v/v) and were administered orally to the treated rats at a volume of 0.8 mL/day for 1, 3, and 5 days-postpartum. Only the vehicle was administered orally to the residue groups. Acute toxicity of extract was evaluated in healthy female Wistar rats, after treated extract of *H. indicum* respectively by gastric intubation. The animals were observed continuously for 24 hours for toxic symptoms such as behavioral changes, locomotion, convulsions, and mortality (Mohammad et al., 2014).

Methodology

Plant

Collection and authentication of plant

The whole plant of *H. indicum* was collected. The plants were specified by Royal Forest Department, Thailand.

Ethanol extraction

The whole plants were dried and powdered then extracted by macerated with 70% ethanol and shaken for 3 days. The mixed was filtered and evaporated by a rotary evaporator, dried by using lyophilizer and stored until use.

Preliminary phytochemical screening

The lyophilized *H. indicum* extract concentration 20 mg/mL was prepared and tested for the presence of protein, carbohydrate, reducing sugar, tannins, flavonoids, alkaloids, saponins, steroids, and glycosides.

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

GC-MS analysis was carried out on a GC BRUKER model 450GC and gas chromatograph interfaced to a mass spectrometer instrument. The relative percentage amount of each compound was analyzed by comparing its average peak area to the total areas, NIST Mass Spectral Library 2008.

Fourier transform infrared spectroscopy (FTIR) analysis

Ten milligrams of the dried extract powder were loaded in ALPHA II FTIR spectrometer using the model discs. Spectral were analyzed OPUS 7.2 software and The Unscrambler X software.

Acute Toxicity

Acute toxicity of extract was evaluated in healthy female Wistar rats, after treated extract of *H. indicum* .The animals were perceived continuously for 24 hours for toxic symptoms and mortality.

Figure 3.2 Summary of methodology.

3.4 Results

3.4.1 Plant identification

The voucher specimen (BKF194426) was identified and authenticated by Royal Forest Department, Bangkok, Thailand.

Taxonomic classification

Domain: Eukaryota Kingdom:

Plantae Phylum: Spermatophyta

Subphylum: Angiospermae

Class: Dicotyledonae

Order: Boraginales

Family: Boraginaceae

Genus: *Heliotropium*

Species: *Heliotropium indicum*

Plant description

Heliotropium indicum (Family: Boraginaceae) is a common smelly herb found throughout India in sunny localities, on wastelands, and widely determined as a tiny off the ground. The plant is an annual, upright; ascending hirsute branched about 20 to 60 cm tall and common smelly herb. The leaves are contrary or sub-opposite, alternate or straight forward or sub- alternate, ovate to obovate, hairy, and acute, 5 to 10 cm long. Leaf margins wavy and nerves on either side. The petiole is about 4 to 10 cm long. The flowers are green and proximately 5 mm in diameter. Flowers develop apically within the cymose, at maturity nutlets are present at the base of the inflorescence (cyme). Flowers are white or whitish violet, regular, sessile, axillary. Sepals-5, 3 mm long, diffused with hairs in outside, dark green, linear to lanceolate and

uneven or unequal. Distributed branched, hirsute with hairs in the stem and the root is tap root and branch. The fruits are dry 2 to 4 lobed, with or without united nutlets, 3 to 6 mm long (Ghosh et al., 2018).

Figure 3.3 *Heliotropium indicum* L.

3.4.2 The yield of *H. indicum*

The whole plant *H. indicum* extract was a dark brownish power. The yields of the whole plant extracts were 3.18%.

3.4.3 Phytochemical screening of *H. indicum* extract

Table 3.1 showed the qualitative estimation of elemental phytochemical components of the *H. indicum* extract. The results have been shown the presence of various active medical constituents in the *H. indicum* extract such as alkaloids, tannins, flavonoids, steroids, and glycoside. These compounds may be accountable for several medicinal activities of *H. indicum* (Table 3.2).

Table 3.1 Preliminary phytochemical screening of *H. Indicum.*

No.	Chemical constituents	*H. indicum* extract
1	Protein	-
2	Carbohydrates	-
3	Reducing sugar	-
4	Tannins	+
5	Flavonoids	+
6	Alkaloids	+
7	Saponins	-
8	Steroid	+
9	Glycoside	+

(+) detected, (-) absent

Table 3.2 The medicinal activities of component in *H. indicum* extract.

Phytochemical component	Medicinal activities
Tannins	Stimulate contraction of uterine smooth muscles and cardiac muscles (Calixto et al., 1986, Polya et al., 1995). Antimicrobial, anthelmintic, antidiarrhea (Liu et al., 2003).
Flavonoids	Antimicrobial, antidiarrhea, antioxidant, antiatherosclerotic, antiplatelet aggregation, antithrombogenic, antiviral, antiulcerative, antiinflammaroty, antiarthritis, antiosteoporotic, antileukemic activity (Cazarolli et al., 2008, Hodex et al., 2002)
Alkaloids	Some indole alkaloids are important medicinal activity. Alkaloid strychnine has strongly causing muscle contraction. The Ergot alkaloids such as ergometrine acts on the contraction of uterine muscle and ergotamine for migraine treatment (Kaushik et al., 2013). Antimicrobial (bacteria, fungi, protozoa), antihelmintic activity, antidiarrhea, antioxidant, antiinflammatory activity, antidepressant, renoprotective agent, anticancer, antimutagenic, hepatoprotetive activity, and cardioprotective activity (Singh et al., 2010, Tiwari et al., 2011).
Steroid	Antidiarrhea, cardioprotective, cholesterol lowering agents, antidiabetics, anti- inflammatory, anti- bacterial, antifungal, antiulcerative, antitumor/cancer, antiosteoporotic, reproductive enhancer, anti-fertility, aphrodisiac, and immunostimulant (Hörmann et al., 2012, Mbambo et al., 2012, Tiwari et al., 2011).
Glycoside	Cardiac glycosides reported to be present in *N. laevis* effect on the uterus of various species animals. Strophanthin show the activation of the non-pregnant guinea pig uterus (Sugimoto, 1913). and strophanthin induced the cat myometrium (Ransom, 1920). Digoxin and ouabain show increased both tone and the frequency of contractions of the human uterus *in vitro* (Norris, 1961).

3.4.4 Gas chromatography coupled with a mass spectrometry (GC-MS) of

H. indicum extract

The phytochemical components of the plant extract are present in Table 3.3. The mass spectrum of isolated compounds from methanolic extract of *H. indicum* were analyzed by GC/MS show in Figure 3.4. The GC/MS analysis of the *H. indicum* extract showed the presence of acetic acid (4.71%), 3-pentanone, 2-methyl (2.69%), propanoic acid, 2-oxo-, methyl ester (1.28%), 1,1-dimethoxyacetone (2.81%), hydroperoxide, 1-methylbutyl (3.69%), 3-pentanol, 2, 4-dimethyl (2.86%), 1H-indole (3.55%), propane, 2-methyl-2-[(1-methylethyl) thio] (1.01%), pyranone (1.27%), trachelanthamidine, 1, 2. beta.-epoxy- (0.63%), neophytadiene (1.07%), 2-Pentadecanone, 6, 10, 14-trimethyl- (0.41%), hexadecanoic acid (7.21%), hexadecanoic acid, ethyl ester (12.03%), heptadecanoic acid, ethyl ester (0.48%), phytol (20.42%), 9, 12-octadecadienoic acid (z,z)- (2.92%), 9, 12-octadecadienoic acid, ethyl ester (14.54%), (E)-9, 12-Octadecanoic acid, ethyl ester (13.93%), and octadecanoic acid, ethyl ester (2.43%). The biological activity of phytochemical components predicted in methanolic extract of *H. indicum* by GC-MS show in Table 3.4.
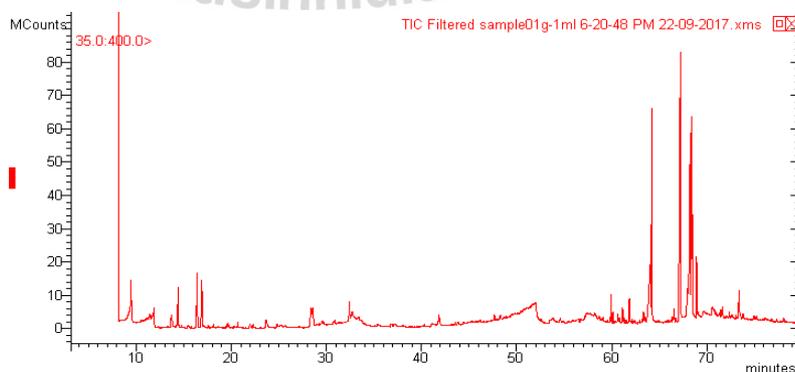
Figure 3.4 Mass spectrum of isolated compounds from ethanolic extract of *H. indicum.*

Table 3.3 Phytochemical components predicted in ethanolic extract of *H. indicum* by GC-MS.

No.	RT (min)	Peak Name	%Area
1	9.48	Acetic acid	4.71
2	11.87	3-Pentanone, 2-methyl	2.69
3	13.76	Propanoic acid, 2-oxo-, methyl ester	1.28
4	14.44	1,1-Dimethoxyacetone	2.81
5	16.38	Hydroperoxide, 1-methylbutyl	3.69
6	16.93	3-Pentanol, 2, 4-dimethyl	2.86
7	28.41	1H-indole	3.55
8	32.45	Propane, 2-methyl-2-[(1-methylethyl) thio]	1.01
9	32.71	Pyranone	1.27
10	41.88	Trachelanthamidine, 1, 2.beta.-epoxy-	0.63
11	59.92	Neophytadiene	1.07
12	60.12	2-Pentadecanone, 6, 10, 14-trimethyl-	0.41
13	64.05	Hexadecanoic acid	7.21
14	64.24	Hexadecanoic acid, ethyl ester	12.03
15	66.57	Heptadecanoic acid, ethyl ester	0.48
16	67.16	Phytol	20.42
17	68.00	9, 12-Octadecadienoic acid (z,z)-	2.92
18	68.20	9, 12-Octadecadienoic acid, ethyl ester	14.54
19	68.43	(E)-9, 12-Octadecanoic acid, ethyl ester	13.93
20	68.93	Octadecanoic acid, ethyl ester	2.43

Table 3.4 The biological activity of phytochemical components predicted in ethanolic extract of *H. indicum* by GC-MS.

Peak Name	Biological activity
Acetic acid	Anticancer, antiseptic, antibiotics, treatment for otitis externa (Ryssel et al., 2009)
Neophytadiene	Antimicrobial (Ahn et al., 2016)
Hexadecanoic acid (Palmitic acid)	Anti- inflammatory, antioxidant, hypocholesterolemic nematicide, pesticide, anti- androgenic flavor, hemolytic, 5-alpha reductase inhibitor, potent mosquito larvicide (Abubakar and Majinda, 2016)
Hexadecanoic acid, ethyl ester (Palmitic acid ethyl ester)	Antioxidant, hypocholesterolemic, nematicide, pesticide, antiandrogenic flavor, hemolytic, alphareductase inhibitor (Sudha et al., 2013)
Phytol	Cancer-preventive, antimicrobial, antimicrobial, anticancer, diuretic, anti-inflammatory (Sudha et al., 2013)
9,12- Octadecadienoic acid (Z,Z) - (Linoleic acid)	Antiinflammatory and antimicrobial activity (Huang et al., 2010)
9,12-Octadecadienoic acid, ethyl ester (Linolelaidic acid ethyl ester)	Anti-inflammatory and anti-diabetic (Kolar et al., 2019)
(E)-9-Octadecanoic acid ethyl ester (Oleic acid, ethyl ester)	Fatty acid steroids and primer pheromone (Kanimozhi and Bai, 2012)
Octadecanoic acid, ethyl ester (Stearic acid)	Play a role in the structure of cell membranes, modulate the behaviour of membrane-bound proteins and are precursors of prostaglandins, leukotrienes and thromboxanes (Capasso et al., 2003)

3.4.5 Fourier transform infrared spectroscopy and functional group analysis of *H. indicum* extract

The functional groups of the active compounds present in *H. indicum* extract were recognized by the FTIR spectroscopy, based on the peak values in the IR region. The results showed IR stretching frequency at 3334.94 cm^{-1}, 2972.71 cm^{-1}, 1727.87 cm^{-1}, 1607.76 cm^{-1}, 1375.38 cm^{-1}, 1236.24 cm^{-1}, 1079.03 cm^{-1}, 1028.79 cm^{-1}, 879.51 cm^{-1}, and 824.28 cm^{-1}. These were due to alkaloids, flavonoids, phenols, ketones, ester, carbonyl group, and glycoside. There was no absorbance in the middle of the regions of 1800–2200 cm^{-1} suggests that there was no cyanide group in this extract. The result shows that *H. indicum* extract does not composed any fatal toxic substances (Chandrashekar et al., 2018). The occurrence of functional groups in *H. indicum* extract was showed in Figure 3.5 and IR absorption frequencies are tabulated in Table 3.5. From FTIR analysis, lignins were found in *H. indicum* extract. Medicinal activities of lignins is applications in the treatment of obesity, diabetes, thrombosis, viral infections, cancer (Vinardell and Mitjans, 2017), anti-tumor, anti-virus, antioxidant, and antimicrobial activities (Spiridon, 2018). Moreover, some of lignins are the phytoestrogens. Diethylstilboestrol (lignins) is synthetic oestrogens, it has functionally similar to oestradiol (Burton and Wells, 2002).
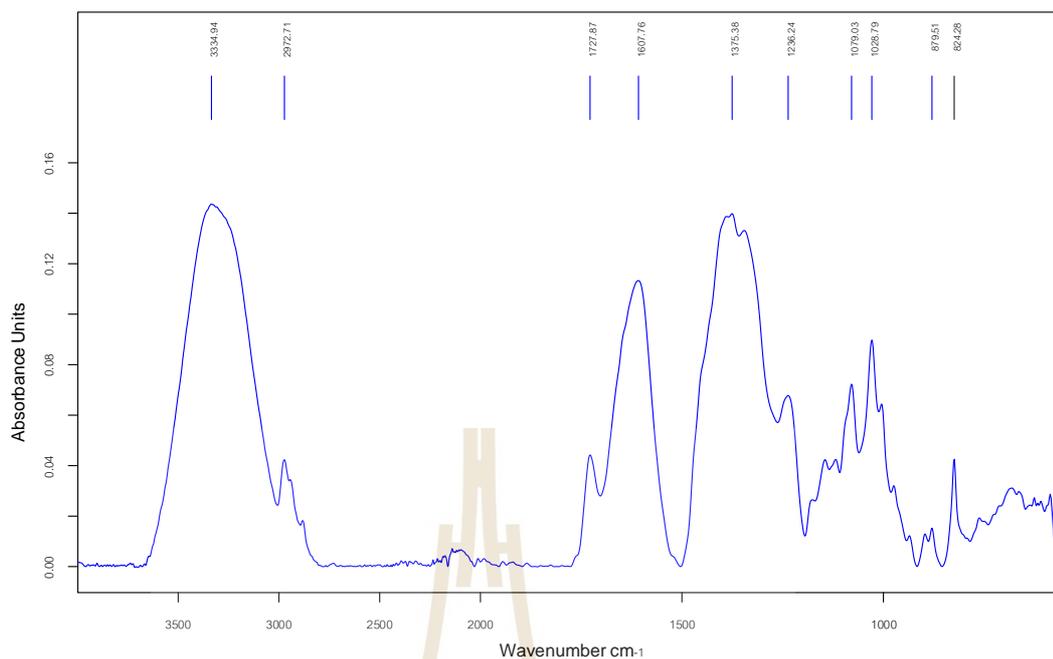
Figure 3.5 Fourier transform infrared spectrum of ethanolic extract of *H. indicum.*

Table 3.5 Major functional groups observed in the Fourier transform infrared spectra of *H. indicum* extract.

No.	IR spectral values(cm^{-1})	Description	Probable phytochemicals
1	3334.94	N–H stretching (Mitra and Syed, 2011)	Alkaloids and flavonoids (Chandrashekar et al., 2018)
2	2972.71	C-H stretching bends, polyatomic entitles with C bonded to two or three H, $C-CH_3$ (Saikia et al., 2009).	Alkaloids, flavonoids and polyphenols (Chandrashekar et al., 2018)
3	1727.87	C=O stretching, indicates the carbonyl groups (Gokulakumar and Narayanaswamy, 2008)	Ketones, ester carbonyl group, flavonoids and phenolic (Gokulakumar and Narayanaswamy, 2008)
4	1607.76	C=C stretching (Bykov, 2008)	Flavonoids, polyphenols and tannins (Kar, 2007)
5	1375.38	CH bending (Mitra and Syed, 2011)	Lignins (Mitra and Syed, 2011)
6	1236.24	PO^-_2 asymmetric stretching band (Wang et al., 2012)	Phosphate compound (Sahayaraj et al., 2015)
7	1079.03	Symmetric PO_2 ($vsPO_2-$) stretching (Mihoubi et al., 2017)	Phosphate compound (Sahayaraj et al., 2015)
8	1028.79	C-H deformation or C-O or C-C stretching, pertaining to carbohydrates (Ramamurthy and Kannan, 2007)	Phenols (O–H stretch, H–bonded), aromatics (C–C stretch (in–ring)), alcohols, carboxylic acids, esters, and ethers (C–O stretch) (Rajiv et al., 2017)
9	879.51, 824.28	Out of plane C-H bending (Kannan, 2014)	Glycopyrenose, glucose, galactose, glycoside (Prabu and Natarajan, 2012)

3.4.6 Acute Toxicity of *H. indicum* extract

After administered orally, the animals were recognized regularly for 24 hours for toxic conditions such as behavioral transformation, motion, seizures, and mortality. The results suggested that non-treated rats and treated rats did not show any data of acute toxicity of irregular clinical signs or death during experiment period. Moreover, the infant did not show death or disable.

Table 3.6 The summary of *H. indicum* phytochemical component found in this study.

No.	Chemical constituents	Phytochemical screening	FTIR
1	Protein	-	-
2	Carbohydrates	-	+
3	Reducing sugar	-	+
4	Tannins	+	+
5	Flavonoids	+	+
6	Alkaloids	+	+
7	Saponins	-	-
8	Steroid	+	+
9	Glycoside	+	+

(+) detected, (-) absent

3.5 Discussion

Therapeutic features of medicinal plants are due to the presence of diverse secondary metabolites such as flavonoids, alkaloids, saponins, glycosides, tannins, and sterols, their use as the active compound in medicinal preparations (Taylor et al., 2001).

The herbal medicines were used in many parts of the word for therapeutic and nutritional from different plants around the world. The drugs were synthesized from novel chemical entities, an excellent bio-resource of plants (Ncube et al., 2008). In this study, the primary phytochemical screening, GC-MS analysis, and FTIR analysis suggest that *H. indicum* extract are pharmaceutically significant due to the presence of the diverse medicinally phytochemical components and secondary plant metabolites. Even though their specific functions were not being examined in this study. The phytochemical screening was reported to obtain medicinal activity and physiological activity in Table 3.2., the GC-MS analysis of this plant showed the presence of bioactive compounds in Table 3.3, the biological activity of phytochemical components by GC-MS in Table 3.4, and FTIR analysis showed in Table 3.5.

The whole plant of *H. indicum* includes various phytochemicals. The pyrrolizidine alkaloids are a common component of a diverse genus of *Boraginaceae* family. They show high toxicity on the liver and lungs, cytotoxicity properties have also been reported in different studies. The young plant leaves, seedlings, and inflorescences exhibited high alkaloid levels. The concentration of alkaloids was decreased by 20% in the aged plant (Ghosh et al., 2018). In this study, two years old plants for extract and test their effects were used. Therefore, the study did not show any data of acute toxicity of abnormal clinical signs or death during the treatment period.

From the literature review, phytochemical screening of the whole plant of *H. indicum* exposed positive for flavonoids, phenols, tannins, saponins, alkaloids, and steroids. Flavonoids and tannins are phenolic compounds that act as primary antioxidants or free radical scavengers. Moreover, the extract absence of glycosides, reducing sugars and gum (Dasarapu et al., 2015). On the other hand, the results have

also shown the whole plant of *H. indicum* present of alkaloids, tannins, flavonoids, steroids, and glycoside. Their medicinal activity and physiological activity presented in this study (Table 3.2) and literature reviews that the potential use of this plant in various traditional medicines can be systematically evaluated. The phytochemicals such as alkaloids, tannins, and glycoside showed an effect on uterine contraction. The increased prolactin levels and expanding milk supply because of herbal galactagogues, most of them are believed to put their pharmacologic effects through interaction with dopamine receptors. Galactagogues are beneficial for women who are powerless to produce breast milk on their own due to baby prematurity, sickness of the mother or child, adoption, or representative motherhood (Gabay, 2002). Tannins are natural plant phenolic compounds that accelerate proteins and with a known capability to decrease proteolysis. Also, some of tannins have been shown to have antibacterial, antioxidant, and flavor-inducting effects (Singleton, 1981). Tannins showed a greater proportion of N absorbed in the lower gut, which leads to depressing urea synthesis and greater milk protein production (Jimenez, 2018). The presence of alkaloids, tannins, flavonoids, steroids, and glycoside in the plant extract is likely to be responsible for the effects on uterine contraction, uterine involution, and milk production.

Phytochemical components predicted in the ethanoic extract of *H. indicum* by GC-MS (Table 3.3) show many of components. Furthermore, phytols (both cis- and tran- forms) were abundant in *H. indicum* extract. It has a role as anti-itching agent, anticancer agent, immunostimulant (Chowdhury and Ghosh, 2012), insecticidal, and antihelmintic or antiseptic activity (Anand and Gokulakrishnan, 2012). Next, indole is an aromatic heterocyclic organic compound. It has a bicyclic structure, composed of a six- membered benzene ring combined to a five- membered nitrogen-

containing pyrrole ring. The normal properties of indole alkaloids that make them especially pharmacologically active were occurred by pyrrole ring with a nitrogen atom. Indole alkaloids are a class of alkaloids composed of a structural moiety of indole; many indole alkaloids also include isoprene groups and are thus called terpene indole or secologanin tryptamine alkaloids (El-Sayed and Verpoorte, 2007). Ergot alkaloids are a class of hemiterpenoid indole alkaloids. Narrows blood vessels and stimulates constriction of the uterus are the effects of ergotamine, a partial agonist of α-adrenergic and 5-HT$_2$ (serotonin receptors) receptors. Moreover, ergometrine is an agonist of α-adrenergic, 5-HT$_2$ and partly dopamine receptors mostly type D$_2$. Ergometrine has a greater selectivity in provoking the uterus compared with other ergot alkaloids (Katzung et al., 2009). Octadecanoic acid, ethyl ester has other names such as stearic acid. In evening primrose oil from the seeds of *Oenothera species* including *Oenothera biennis* composed linoleic acid, oleic acid, palmitic acid, and stearic acid. These necessary fatty acids play a function in the structure of cell membranes, adjust the behavior of membrane-bound proteins and are precursors of prostaglandins, leukotrienes, and thromboxanes that could lead to the healing effects. Nevertheless, the exact process of activity is not known. On the other hand, dong quai (Chinese angelica) is the root of *Angelica sinesis* (Oliv.) has a 2,000-year history as a therapeutic of dysmenorrhea, amenorrhea or excessive menstrual flow. The property of dong quai is said because of the presence of coumarins, phytosterols, flavonoids, and polysaccharides (Capasso et al., 2003).

Moreover, *H. indicum* GC-MS analysis show many constituents such as acetic acid (Ryssel et al., 2009), neophytadiene (Carretero et al., 2008), hexadecanoic acid (Abubakar and Majinda, 2016), hexadecanoic acid, ethyl ester (Sudha et al., 2013), and

9,12-Octadecadienoic acid (Z,Z)- or linoleic acid ester (Peyrat-Maillard et al., 2003), they have an effective anti-inflammatory, antimicrobial, antioxidant, and anticancer. The presence of phytochemical constituent by GC-MS of the *H. indicum* extract is probably responsible for traditional used. Additional evidence created from the FTIR analysis of whole plant extract of *H. indicum* has shown the presence of functional groups such as alkaloids, phenols, carboxylic acids, amines, flavonoids, phosphate compounds, glycoside, and tannins, thus exposing the presence of vital phytoconstituents (Table 3.5). From FTIR analysis, lignins were found in *H. indicum* extract. Medicinal activities of lignins is applications in the treatment of obesity, diabetes, thrombosis, viral infections, cancer (Vinardell and Mitjans, 2017), anti-tumor, anti-virus, antioxidant, and antimicrobial activities (Spiridon, 2018). Moreover, some of lignins are the phytoestrogens. The principle phytoestrogens are the isoflavones and lignins (for example, enterolactone and enterodiol). Diethylstilboestrol (lignins) is synthetic oestrogens, it has functionally similar to oestradiol (Burton and Wells, 2002).

Table 3.6 showed the summary of H. indicum phytochemical components by primary phytochemical screening and FTIR analysis. The primary phytochemical screening test can detect a chemical reaction of functional group and the FTIR technique can detect an infrared spectrum of absorption of functional group. From the table, each method shows difference results which may be related with the limitation of each method. Based on phytochemical screening, GC-MS, and FTIR analysis, the presence of various compounds in whole plant extract of *H. indicum* was found. The results suggest that of *H. indicum* extract may be have effects on uterine involution, uterine contraction, blood biochemical parameters, and milk production. Therefore, the extract on uterine contraction, uterine involution, uterine contraction, blood

biochemical parameters, and milk production were studied in the next chapter.

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CHAPTER IV

EFFECTS OF *HELIOTROPIUM INDICUM* L. EXTRACT ON UTERINE INVOLUTION IN POSTPARTUM RATS

4.1 Abstract

Involution is the process by which the uterus is transformed from pregnant to non-pregnant state. The uterus in the postpartum period returns size, position in the pelvic cavity, and regains its muscular tone to the pre-pregnant state. This process is primarily due to oxytocin, the hormone induced by lactation. Uterine involution needs a contraction of the uterus to prevent postpartum hemorrhage (PPH) that adversely affects the mother and baby. The healthy woman can death by PPH after delivery within hours if she is unattended. Modern and traditional medicine can be used to help accelerate uterine involution for reduces the risk of PPH. Traditional medicine indicates that *Heliotropium indicum* L. (*H. indicum*) can accelerate uterine involution. However, there is no scientific data to support this traditional claim. It is therefore worth studying the effects of *H. indicum* on uterine involution. The whole plants of *H. indicum* were dried and powdered. The powders of the whole plant were extracted by macerated with 70% ethanol and shaken for 3 days. The mixed was filtered through filter paper to remove particulates. The extract was evaporated under reduced pressure at a low temperature in a rotary evaporator, dried by using lyophilizer and stored at -20 °C until use. *In vitro* study, the 30 pregnant rats were used. Rats were divided into 3 groups: 1) 1 day-postpartum

rats 2) 3 day-postpartum rats and 3) 5 day-postpartum rats. The effect of concentration of *H. indicum* extract at 250 mg/100 mL on isolated uterine strips were measurement uterine contraction by tissue organ bath system. The experiments included: (i) uterine strips treated with spontaneous contraction followed by the application of *H. indicum* extract; (ii) uterine strips treated with spontaneous contraction followed by the application of *H. indicum* extract and then oxytocin in the continued presence of the extract and (iii) uterine strips treated with spontaneous contraction followed by the application of oxytocin and next *H. indicum* in the continued presence of oxytocin. *In vivo* study, the 60 pregnant rats were used. The rats were divided into 2 main groups: non-treated rats and treated rats. The main groups have 3 subgroups containing: 1) 1 day-postpartum rats 2) 3 day-postpartum rats 3) 5 day-postpartum rats. The extract of *H. indicum* were diluted to 250 mg/kg B.W. in the vehicle (Tween 80, 10% v/v) and were administered orally to the treated rats at a volume of 0.8 mL/day. Only the vehicle was administered orally to the residue groups. After administered orally, the uterine tissue was collected for analyses to determine the relative uterine weight, the relative uterine size, the relative collagen in the uterus determining by image processing using k-means cluster analysis (KMCA), MATLAB, and biochemical components of the uterus determining by Fourier Transform Infrared spectroscopy (FTIR). The results suggested that the *H. indicum* extract can induce uterine contraction and may be synergistic with oxytocin-induced uterine contraction in postpartum rats. Furthermore, the results showed the extracts can accelerate the reducing rate of uterine weight, uterine size, and the collagen of the uterus in postpartum period. In the part of FTIR study, the *H. indicum* extract have some effects on biochemical components of the uterine tissue on day 1, 3, and 5 postpartum. The *H. indicum* extract can interfere the pattern of lipid, protein, and carbohydrate and nucleic

acid. Especially, the collagen was significantly decreased on day 3 and 5 postpartum. The results from this study is the scientific data to support the effects of *H. indicum* extract on uterine tissue in postpartum period. Moreover, the extract can help accelerate uterine involution in postpartum rats by enhancing uterine contraction and reducing the uterine weight, the uterine size, and the collagen of the uterus. Therefore, the whole plant of *H. indicum* extract can be consumed during postpartum period to help reducing the risk of postpartum hemorrhage. The findings also confirm traditional uses that *H. indicum* could accelerate uterine involution.

4.2 Introduction

The death of a woman while pregnant or within 42 days of termination of pregnancy from any cause called maternal death. It associated with or worsen by the pregnancy or its administration but not from accidental or incidental causes. Data from World Health Organization (WHO) show in 2017 every day have 810 women die from preventable causes related to pregnancy and childbirth. Between 2000-2017, maternal mortality dropped by about 38% worldwide. Inequality in access to health services and the gap between rich and poor in some areas result in the high number of maternal deaths. Almost all maternal deaths (94%) occur in low and lower middle-income countries. Trends in estimates of maternal mortality ratio (maternal deaths per 100,000 live births, MMR) between 2000 and 2017, by country Thailand in 2017 had MMR at 37 (WHO et al., 2019). Women die because of complications during and following pregnancy and childbirth. Most of these problems develop during pregnancy and most are preventable or treatable. Other complications may exist before pregnancy but are

worsened during pregnancy, especially if not managed as part of the woman's care (WHO, 2016).

Postpartum bleeding or postpartum hemorrhage (PPH) is the symptoms characterized by the loss of more than 500 ml or 1,000 ml of blood within the first 24 hours later parturition. Signs and symptoms may primarily include: an enhanced heart rate and an enhanced breathing rate. Blood is lost which possibly cause of feeling cold, lowering blood pressure, and the women may become nervous or senseless. The illness can appear up to six weeks following labor. PPH is the most cause of maternal mortality. All women who carry a pregnancy beyond 20 weeks' pregnancy are at risk for PPH and its sequelae. Even though maternal mortality rates have descent greatly in the developed world, PPH remains a leading cause of maternal mortality elsewhere (Smith, 2016). The healthy woman can death by PPH after delivery within hours if she is unattended. Injecting oxytocin instantly after parturition effectively decrease the risk of bleeding (WHO, 2007).

Uterine involution is the process occur in postpartum period by which the uterus and the other genital organs returning to its pre-pregnant state (Bassam, 2009). The uterus in the postpartum period show returns weight and size and regains its muscular tone to the pre-pregnant state. Autolysis and phagocytosis are the main of involution. Autolysis or self-digestion cause the annihilation of a cell by the action of its own enzymes. During involution, autolysis reduces the size of muscle (Maibenco, 1960). The uterus during involution continuous decrease in size but not a decrease in the number of uterine muscles. This causes the uterine sizes of multiparous woman are usually larger than in nulligravidas (Hsu et al., 2014). The uterus has two main aims immediately after the delivery, the first is to regulate bleeding and the next is revert to

its pre-pregnant state. Bleeding is controlled by contraction of the muscles. Muscular tone can decrease the risk of PPH and constrict the blood vessels, causing less blood flow. This is the primary state of involution (Maibenco, 1960). At present, several countries used traditional therapeutically methods for treated severe bleeding and managed as part of the woman's care. In Thailand, the medicinal plant has also been used extensively in a variety of folk medicine and there have been used orally to treat uterine involution. However, some traditional medicinal plants do not have scientific data to support their effects on PPH and uterine involution. In this study, the *Heliotropium indicum* L was investigated.

Heliotropium indicum (Boraginaceae) (*H. indicum*), usually known as 'Indian heliotrope' is an annual, hairy plant that is widely distributed in waste places and settled areas. It is native to Asia. It is a coarse foetid herb, up to 2 feet high, hairy stem, white flowers with green calyx. *H. indicum* has been used in different traditional therapeutic and folklore systems of medicine for healing diverse diseases. Many reports showed effects of *H. indicum* to possess antibacterial activity, antitumor activity, antiinflammatory activity, antituberculosis activity, anti- proliferative activity, gastroprotective activity, antihyperglycemic, and immunostimulant activities (Mohammad et al., 2014). From previous study, *H. indicum* alcohol extract showed induce uterine contraction in postpartum rats on 1, 3 and 5 day of postpartum. *H. indicum* alcohol extract may reduce uterine size in involution period. Moreover, *H. indicum* may have a synergistic effect with oxytocin and it does not have acute toxicity.

The aims of present study were therefore to investigate the effects of ethanol extract of *H. indicum* on uterine involution in postpartum rats. The results from this study can be used as scientific data to support its effects on uterine involution for

reducing the risk of PPH. Moreover, the results from this study will provide the basic knowledge for further research to develop drugs or products for maternal care.

4.3 Methodology

4.3.1 Experimental animals

Female Wistar rats (200-250 g) were used in this study and maintained in accordance with the guidelines of the Committee on Care and Use of Laboratory Animal Resources, National Research Council, Thailand. The experiments performed on rats were conducted in accordance with the advice of the Institutional Animal Care and Use Committee, Suranaree University of Technology. Female Wistar rats were maintained under environmentally controlled room provided with a 12:12 light and dark cycle at a temperature of approximately 25°C. They were fed with commercial food (C.P. Mice feed, Bangkok, Thailand) and allowed to access water *ad libitum*. Used 100 female Wistar rats for this study.

Confirmation of mating and pregnancy were done by the vaginal plug method (Voss, 1979), as modified by Ochiogu et al. (2006). Briefly, two females were placed in a cage with a male of proven fertility. A vaginal smear of each female was taken on a labeled clean glass slide with the aid of a wet cotton swab dipped in fresh normal saline and inserted into the vagina to a depth of approximately 1.5 cm. The wet smear was examined grossly for the presence of protein coagulates (remnants of the copulatory plug) as evidence of successful mating. The light microscope was used to find the sperm and confirm the mating. This procedure was carried out at 12-hour intervals. The day remnants of the plug were regarded as day 1 of pregnancy. Thereafter, the females were separated from the males. Changes in body weight were

used in monitoring the progress of the pregnancy (Ochiogu et al., 2013).

4.3.2 Preparation of plant materials

The whole plants were dried and powdered. The powders of the whole plant were extracted by macerated with 70% ethanol and shaken for 3 days. The mixed was filtered through filter paper to remove particulates. The extract was evaporated by a rotary evaporator, dried by using lyophilizer and stored at -20 °C until use. More details relevant to preparation of plant materials are given in Chapter III.

4.3.3 Physiological solutions

All chemicals were obtained from Sigma- Aldrich Chemical Co. (St. Louis, MO, USA). The solvents and chemicals used were of analytical grade and obtained from Sigma® and Merck®. The physiological Krebs's solution (pH 7.4) was prepared in accordance with the following arrangement (mM): NaCl: 154.0; KCl: 5.4; $CaCl_2$: 2.0; $MgSO_4$: 1.2; glucose: 8.0; 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES): 10.0 (Kupittayanant et al., 2002). *H. indicum* extract (2.5 mg/mL) was used and directly dissolved in physiological solution.

4.3.4 Measurement of isometric contraction

30 pregnant rats were used. Rats were divided into 3 groups: 1) 1 day-postpartum rats 2) 3 day-postpartum rats 3) 5 day-postpartum rats, each group contains 10 rats.

The rats were euthanized by strangulation with CO_2 after the requirements of each group had been done. Uterus was collected. Longitudinal strips (1-2 mm X 0.5 mm X 10 mm) were dissected from each uterus. Whole thickness strips were suspended in the direction of longitudinal smooth muscle fibers in organ baths of 25 mL each, filled with modified Krebs-Henseleit solution. The single chamber was linked to a digital signal and noted on a computer using Chart software. The organ bath

contains Kreb-Henseleit solution was maintained at pH 7.4, and temperature of 37°C. The tissues were connected distally to hooks and proximally attached to the isometric force transducers. The electrical signal was recorded from the transducer and converted to the digital signal on a computer using Chart software (Kupittayanant, 2003). The representation of equipment used for tension measurement is showed in Figure 4.1.

Uterine strips were allowed by equilibrating for 30 min over which stable contractions occurred. Then, the applications of the extract or oxytocin were measurement. The measurement of tension was made while the tissue is continually perfused with physiological solution (control) or solution containing *H. indicum* extract 250 mg/ 100 mL. The measurement of tension was made while the tissue was continually perfused with physiological solution (control), oxytocin (10 nmol/L) or solution containing *H. indicum* extract 250 mg/100 mL. To study the mechanism of *H. indicum*, the experiments were included: (i) uterine strips treated with spontaneous contraction followed by the application of *H. indicum* extract; (ii) uterine strips treated with spontaneous contraction followed by the application of *H. indicum* extract and then oxytocin in the continued presence of the extract and (iii) uterine strips treated with spontaneous contraction followed by the application of oxytocin and next *H. indicum* in the continued presence of oxytocin (Kupittayanant et al., 2002). The experiment was studied on day 1, 3, and 5 postpartum.
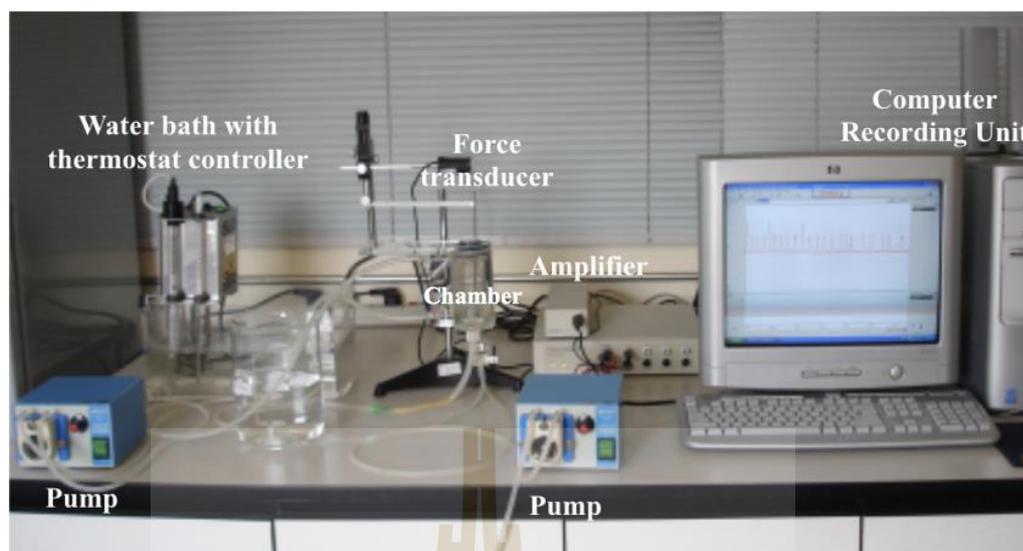
Figure 4.1 Representation of equipment used for tension measurement.

4.3.5 Uterine weight determining

60 pregnant rats were used. The rats were divided into 2 main groups: non-treated rats and treated rats. The main groups have 3 subgroups containing: 1) 1 day-postpartum rats 2) 3 day-postpartum rats 3) 5 day-postpartum rats. The extract of *H. indicum* were diluted to 250 mg/kg B.W. in the vehicle (Tween 80, 10% v/v) and were administered orally to the treated rats at a volume of 0.8 mL/day. Only the vehicle was administered orally to the residue group. After administered orally, the uterine tissues were collected. The uterus was weighed for uterine weight study. Moreover, the uterine horns were separated one for uterine size study, collagen estimation, and another one for FTIR study. Uterine weight was observed by weight scale for measurement relative uterus (%RU). %RU were calculated using the following equation: (Uterine weight X 100) / Body weight. Finally, the results were recorded and analyzed.

4.3.6 Uterine cross-section area determining

60 pregnant rats were used. The rats were divided into 2 main groups: non-treated rats and treated rat. The main groups have 3 subgroups containing: 1) 1 day-postpartum

rats 2) 3 day-postpartum rats 3) 5 day-postpartum rats. The extract of *H. indicum* were diluted to 250 mg/kg B.W. in the vehicle (Tween 80, 10% v/v) and were administered orally to the treated rats at a volume of 0.8 mL/day. Only the vehicle was administered orally to the residue group. After administered orally, the uterine tissues were collected. The uterine tissues were fixed in 10% buffered formalin then processed in a tissue processor and embedded in paraffin using routine methods. Representative transverse sections were cut and stained with Hematoxylin and Eosin. The uterine sectional thickness is 6 µm. Histological examination of the slides was performed using a light microscope. The uterine picture from light microscope was calculated for uterine cross-section area by CellD program (Dong et al., 2019). Assume, the uterus cross section is a disk shape with R and uterus size $A = \pi r^2$ and the uterine cross-section area represents to uterine size. Finally, the uterine cross-section area was relative to body weight. Moreover, the uterine picture from light microscope was calculated for myometrial layer per uterine cross-section area by CellD program.

4.3.7 Relative collagen in the uterus determining by using k-means cluster analysis (KMCA), MATLAB

60 pregnant rats were used. The rats were divided into 2 main groups: non-treated rats and treated rat. The main groups have 3 subgroups containing: 1) 1 day-postpartum rats 2) 3 day-postpartum rats 3) 5 day-postpartum rats. The extract of *H. indicum* were diluted to 250 mg/kg B.W. in the vehicle (Tween 80, 10% v/v) and were administered orally to the treated rats at a volume of 0.8 mL/day. Only the vehicle was administered orally to the residue group. After administered orally, the uterine tissues were collected. The uterine tissues were fixed in 10% buffered formalin then processed in a tissue processor and embedded in paraffin using routine methods. Representative transverse sections were cut and stained with Masson's Trichrome. Histological examination of the slides was performed using a light microscope. The

uterine sectional thickness is 6 µm. Then, the uterine histological image was analyzed by the k-means cluster analysis (KMCA), MATLAB. Finally, the collagen in the uterus was relative to body weight.
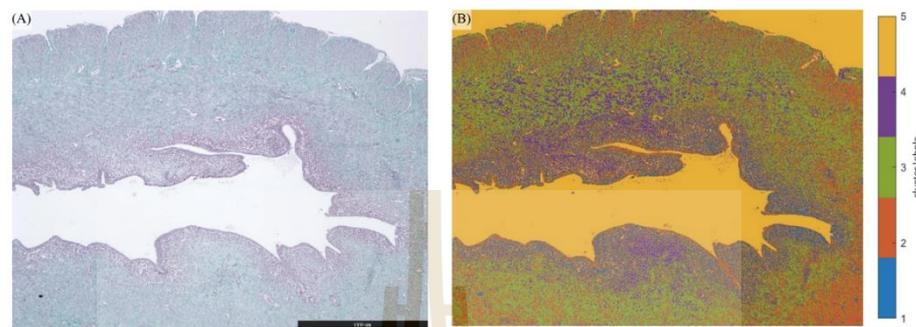
Figure 4.2 (A) uterine tissue with *Masson's trichrome stain*, (B) image segmented by k-means cluster analysis (KMCA).

To evaluate the collagen quantity, the uterine tissue was stained by Masson's trichrome, where the collagen area shows the green color under the optical microscope images as shown in Figure 4.2A From the optical microscope images, the collagen area is well visualized, however, the spatial distribution of the collagen area cannot be quantitatively determined, therefore the image segmentation rely on the k-mean clustering was performed. The k-means cluster analysis (KMCA) was used to create cluster groups based on RGB-implemented spectral color similarity (Andersen et al., 2011, Krafft et al., 2008, Lasch et al., 2004), creating a contrast image for identification of collagen location as shown in Figure 4.2B KMCA with k=4 was appropriate to classify the collagen per uterine cross-sectional area from the others. Figure 4.2B, the collagen area, and the background represent cluster number 3 (green) and cluster number 5 (yellow), respectively. To calculate the collagen per uterine cross-sectional area, first, the background was subtracted from the image, then the collagen cluster area was normalized by the summation area of cluster 1 to 4, which are the tissue

area, and, the collagen per uterine cross-sectional area was calculated as a percent ration respect to the tissue area. Finally, the collagen per uterine cross-sectional area was calculated relatively body weight. MATLAB code of k-means clustering analysis (KMCA) details are show in APPENDIX.

4.3.8 Biochemical components in the uterus determining by Fourier Transform Infrared spectroscopy (FTIR)

60 pregnant rats were used. The rats were divided into 2 main groups: non-treated rats and treated rats. Two main groups have 3 subgroups containing: 1) 1 day-postpartum rats 2) 3 day-postpartum rats 3) 5 day-postpartum rats. The extract of *H. indicum* were diluted to 250 mg/kg B.W. in the vehicle (Tween 80, 10% v/v) and were administered orally to the treated rats at a volume of 0.8 mL/day. Only the vehicle was administered orally to the residue group. After administered orally, the uterine tissues were collected. The sample preparation for FTIR microspectroscopy analysis, tissues were embedded in OCT compound (Tissue-Trek, Electron Microscopy Sciences, Hatfield, PA), and snap-frozen in liquid N_2. Then, the samples were transferred to a -80°C freezer and stored until cryo-sectioning. After that, the sample were cut using a cryostat (Leica 3050 S, Germany) and put on infrared transparent BaF_2 windows (13 × 2 mm) for infrared microspectroscopy. The uterine sectional thickness was 12 µm.

Fourier Transform Infrared spectroscopy (FTIR) is a technique use for the analysis of biological samples like tissue by detecting changes in macromolecular composition occurring during the uterine involution process such as collagen, protein, nucleic acid, and lipid. FTIR microspectroscopy analysis, spectra data were collected at an infrared microspectroscopy beamline (BL4.1 IR Spectroscopy and Imaging) at the Synchrotron Light Research Institute (SLRI). Spectra were acquired with a Vertex

70 FTIR spectrometer (Bruker Optics, Ettlingen, Germany) coupled with an IR microscope (Hyperion 2000, Bruker) with an MCT detector cooled with liquid nitrogen over the measurement range from 4000 to 800 cm^{-1}. The microscope was connected to a software- controlled microscope stage and placed in a specially designed box that were purged by dry air. The measurements were performed in the scattering mode, using an aperture size of 10×10 μm with a spectral resolution of 4 cm^{-1}, with 64 scans co-added. Spectral acquisition and instrument control were performed using OPUS 7.2 (Bruker Optics Ltd, Ettlingen, Germany) software and analyzed by The Unscrambler X software (Thumanu et al., 2017). The spectra were smoothed with Savitsky–Golay smooth function to erase the noise. The spectra were baseline corrected and were normalized with respect to specific bands for visual demonstration. Band areas were calculated from smoothed and baseline corrected spectra using OPUS software. Finally, the band areas in the uterus were relative to body weight.

4.3.9 Statistical analysis

Registration of smooth muscle activity were expressed as changes in the strips' tension. The effects of all the examined substances were based on changes in the smooth muscle strips' and calculated as AUC (area under the contraction). All data were expressed the mean $\pm$ S.E.M. and n refers to the number of strips from different animals. For paired two groups of data were analyzed by Paired-samples t-test. For three or more groups, data was analyzed by one- way ANOVA and post- hoc with Tukey's test. The parameters including uterine weight, uterine cross- section area, collagen determining, and FTIR band area were expressed the mean $\pm$ S.E.M. and n refers to the number of animals. For paired two groups of data were analyzed by Independent-samples t-test. For three or more groups, data were analyzed by one- way

ANOVA and post-hoc with Tukey's test. The Statistical Package for the Social Sciences (SPSS) were employed for all statistical analysis. The significance level was determined at $P < 0.05$.
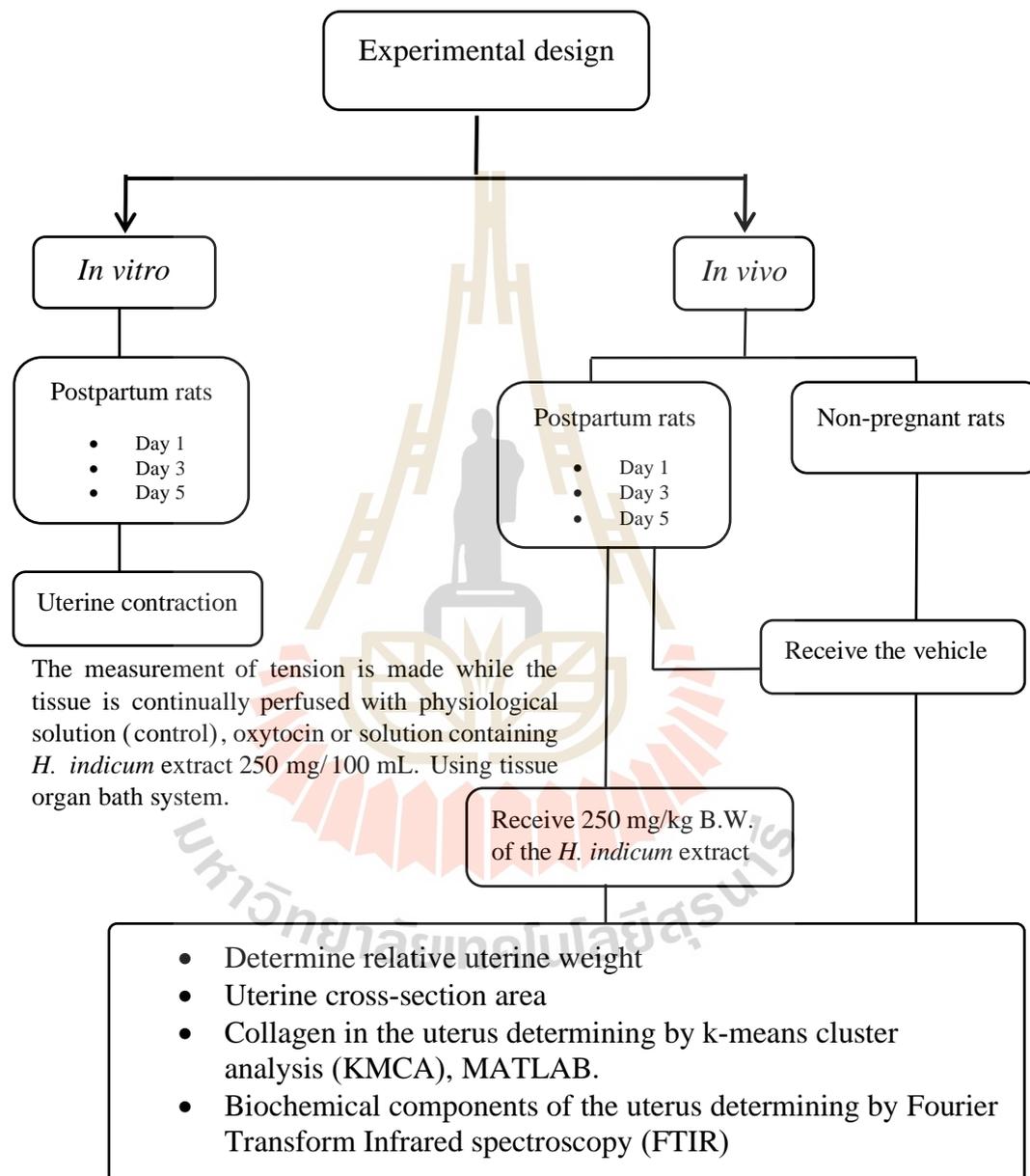
Figure 4.3 Summary of methodology.

4.4 Results

4.4.1 Effects of *H. indicum* extract on uterine contraction in postpartum rats

The concentration of *H. indicum* extract at 250 mg/100 mL were added into organ bath after the 30-minute equilibrium period and used as a control (100%). The application of *H. indicum* extract at the 250 mg/100 mL to isolated uterine strips significantly increased the AUC compared with control. In all samples, *H. indicum* extract had stimulating effect on spontaneous contractions. It produced a significant ($P < 0.05$) increase in AUC (Figure 4.4, Table 4.1). The effects of *H. indicum* extract on the day postpartum showed significant difference when compared another day. The results showed that the *H. indicum* extract had more potent effect at day 3 postpartum and dropped on day 5 postpartum (Figure 4.5).

Table 4.1 The effects of *H. indicum* extract on spontaneous contraction on day 1, 3, and 5 postpartum.

	AUC (%) (Mean ± S.E.M.)
Control	100
Day 1 postpartum	221.23 ± 8.94*
Day 3 postpartum	273.73 ± 20.53*
Day 5 postpartum	149.82 ± 3.44*

The *P*-value for AUC of *H. indicum* extract are significantly different from the control (*$P < 0.05$). Mean ± S.E.M. are given.
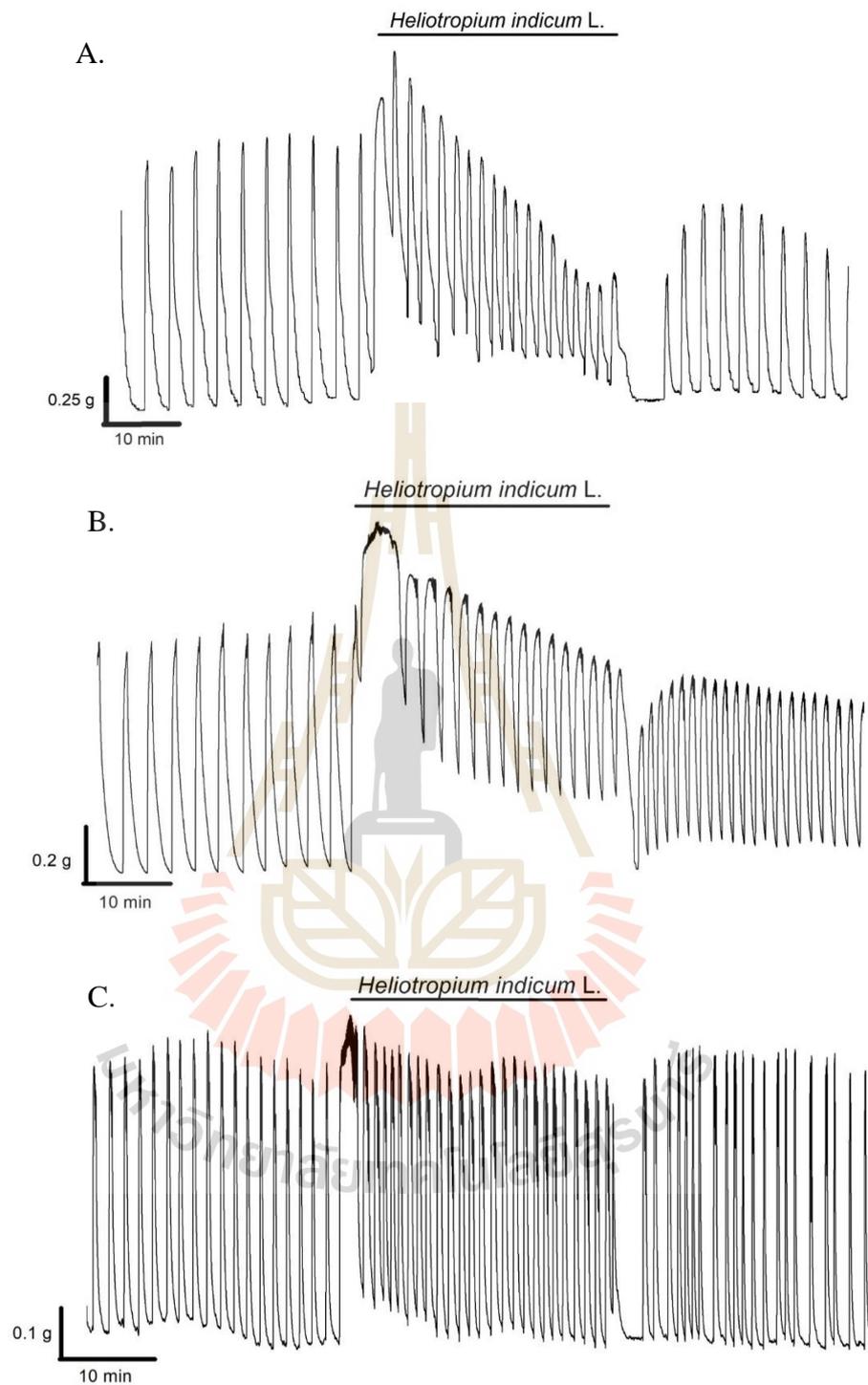
Figure 4. 4 The effects of *H. indicum* extract on spontaneous contraction. The increasing stimulation responses on day 1, 3, and 5 postpartum are shown in A, B and C, respectively.
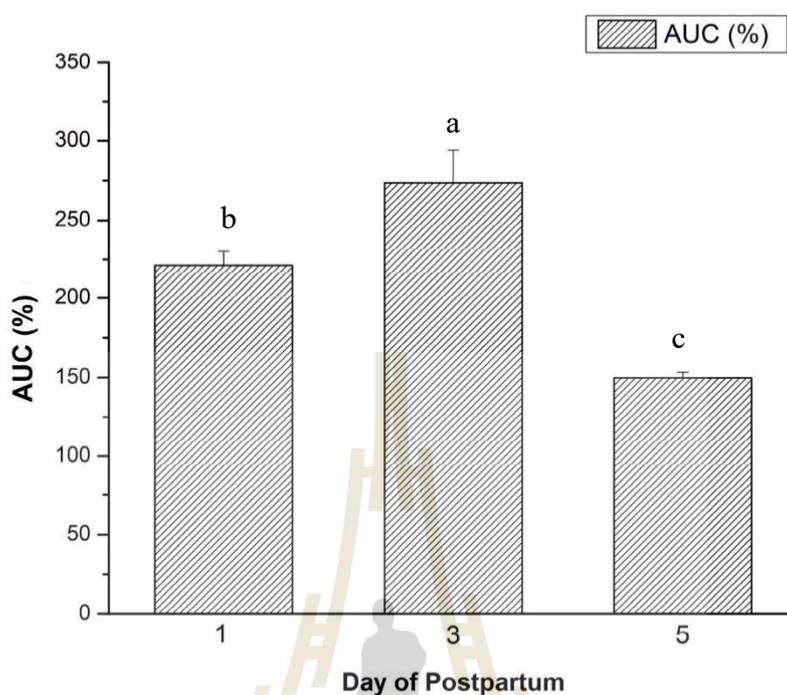
Figure 4.5 A graph showing the effects of *H. indicum* extract on spontaneous contraction. Comparison of the AUC among days of postpartum. The results show the *H. indicum* extract had more potent effect on day 3 postpartum and decreased on day 5 postpartum. Bars represent mean ± S.E.M. Values that do not share the same superscript letters are significantly different ($P < 0.05$).

4.4.2 Effects of *H. indicum* extract and oxytocin on uterine contraction in postpartum rats

Uterine strips were allowed by equilibrating with modified Krebs-Henseleit solution for 30 min over which stable contractions occur. The measurement of tension was made while the tissue is continually perfused with physiological solution (control), oxytocin (10 nmol/L) or solution containing *H. indicum* extract 250 mg/100 mL. To study the mechanism of *H. indicum*, the experiments were included: (i) uterine strips treated with spontaneous contraction followed by the application of *H. indicum*

extract; (ii) uterine strips treated with spontaneous contraction followed by the application of *H. indicum* extract and then oxytocin in the continued presence of the extract and (iii) uterine strips treated with spontaneous contraction followed by the application of oxytocin and next *H. indicum* in the continued presence of oxytocin (Kupittayanant et al., 2002). The experiments were studied on day 1, 3, and 5 postpartum.

Day 1 postpartum, the concentration of *H. indicum* extract at 250 mg/100 mL was added into organ bath after the 30-minute equilibrium period followed by the combination of *H. indicum* extract and oxytocin (Figure 4.6). The results showed *H. indicum* extract and the combination of *H. indicum* extract and oxytocin significantly increased uterine contraction when compared with spontaneous contraction. The percentage of area under the contraction (%AUC) of control, *H. indicum*, and the combination of *H. indicum* and oxytocin was 100, 201.24 ± 2.91, and 149.27 ± 11.11, respectively (Table 4.2).

Table 4.2 Effect of *H. indicum* extract and oxytocin (applied after) on uterine contraction on day 1 postpartum.

Day 1 postpartum	AUC (%) (Mean ± S.E.M.)
Control	100
H. indicum	201.24 ± 2.91*
H. indicum + OT	149.27 ± 11.11*

The *P*-value for AUC of *H. indicum* extract are significantly different from the control (*$P < 0.05$). Mean ± S.E.M. are given. OT is oxytocin.

Figure 4.6 Effect of *H. indicum* extract and oxytocin (applied after) on uterine contraction on day 1 postpartum rat.

Day 1 postpartum, the concentration of *H. indicum* extract at 250 mg/100 mL were added into organ bath after oxytocin-induced uterine contraction (Figure 4.7). The results showed oxytocin and the combination of *H. indicum* extract and oxytocin significantly increased uterine contraction when compared with spontaneous contraction. The percentage of area under the contraction (%AUC) of control, oxytocin, and the combination of *H. indicum* and oxytocin was 100, 331.09 ± 5.92, and 263.71 ± 11.28, respectively (Table 4.3).

Table 4.3 Effect of *H. indicum* extract on oxytocin-induced uterine contraction on day 1 postpartum.

Day 1 postpartum	AUC (%) (Mean ± S.E.M.)
Control	100
OT	331.09 ± 5.92*
H. indicum + OT	263.71 ± 11.28*

The *P*-value for AUC of *H. indicum* extract are significantly different from the control (*$P < 0.05$). Mean ± S.E.M. are given. OT is oxytocin.
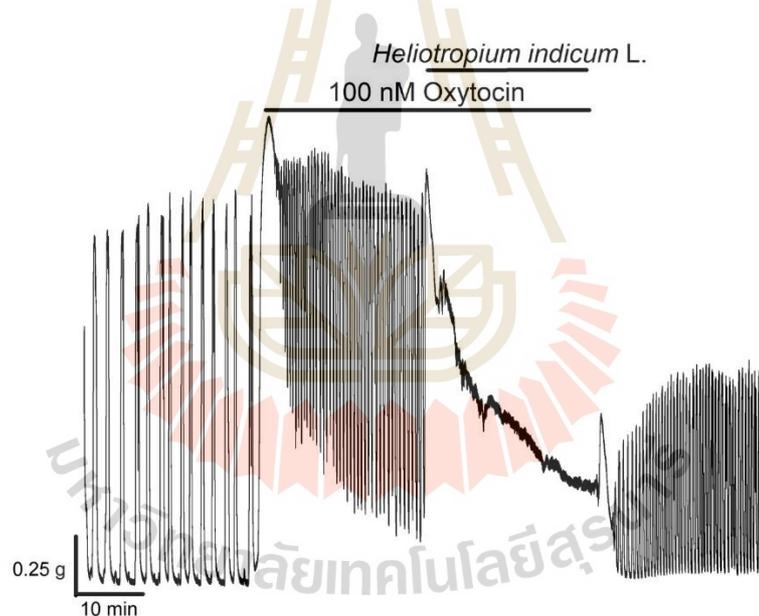
Figure 4.7 Effect of *H. indicum* extract on oxytocin-induced uterine contraction on day 1 postpartum rat.

Day 3 postpartum, the concentration of *H. indicum* extract at 250 mg/100 mL were added into organ bath after the 30-minute equilibrium period followed by the combination of *H. indicum* extract and oxytocin (Figure 4.8). The results showed *H. indicum* extract and the combination of *H. indicum* extract and oxytocin significantly

increased uterine contraction when compared with spontaneous contraction. The percentage of area under the contraction (%AUC) of control, *H. indicum*, and the combination of *H. indicum* and oxytocin was 100, 243.94 ± 2.28, and 312.52 ± 10.16, respectively (Table 4.4).

Table 4. 4 Effect of *H. indicum* extract and oxytocin (applied before) on uterine contraction on day 3 postpartum.

Day 3 postpartum	AUC (%) (Mean ± S.E.M.)
Control	100
H. indicum	243.94 ± 2.28*
H. indicum + OT	312.52 ± 10.16*

The *P*-value for AUC of *H. indicum* extract are significantly different from the control (*$P < 0.05$). Mean ± S.E.M. are given. OT is oxytocin.
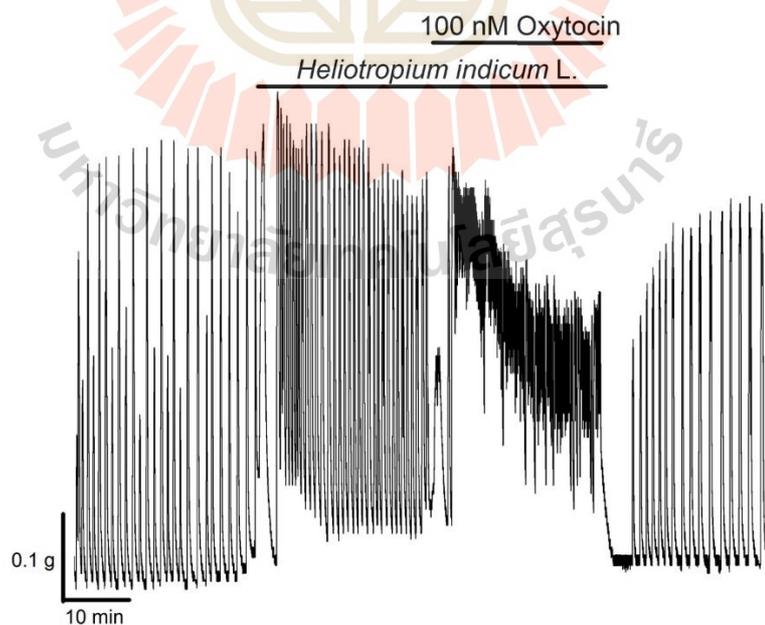
Figure 4. 8 Effect of *H. indicum* extract and oxytocin (applied before) on uterine contraction on day 3 postpartum rat.

Day 3 postpartum, the concentration of *H. indicum* extract at 250 mg/100 mL were added into organ bath after oxytocin-induced uterine contraction (Figure 4.9). The results showed oxytocin and the combination of *H. indicum* extract and oxytocin significantly increased uterine contraction when compared with spontaneous contraction. The percentage of area under the contraction (%AUC) of control, oxytocin, and the combination of *H. indicum* and oxytocin was 100, 304.11 ± 1.20, and 436.84 ± 7.95, respectively (Table 4.5).

Table 4.5 Effect of *H. indicum* extract on oxytocin-induced uterine contraction on day 3 postpartum.

Day 3 postpartum	AUC (%) (Mean ± S.E.M.)
Control	100
OT	304.11 ± 1.20*
H. indicum + OT	436.84 ± 7.95*

The *P*-value for AUC of *H. indicum* extract are significantly different from the control (*$P < 0.05$). Mean ± S.E.M. are given. OT is oxytocin.

Heliotropium indicum L.

100 nM Oxytocin

0.1 g

10 min
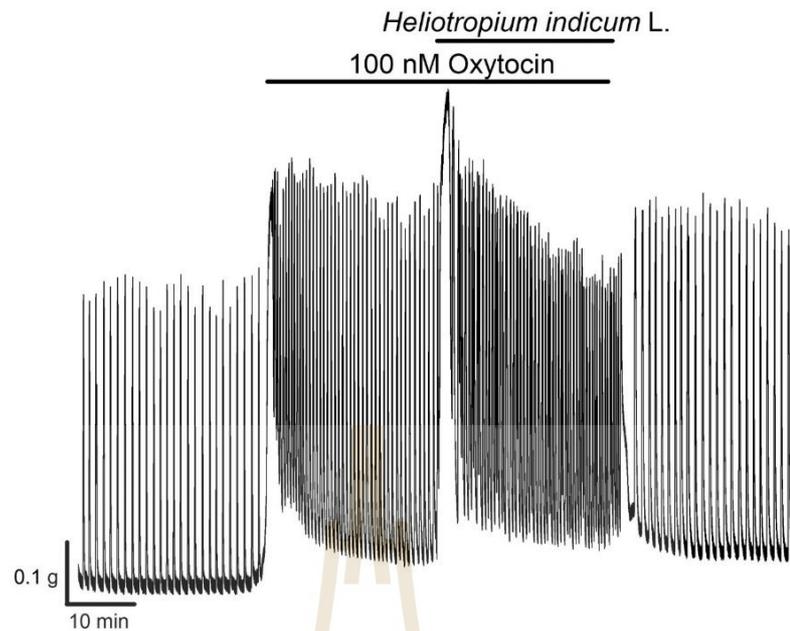
Figure 4.9 Effect of *H. indicum* extract on oxytocin-induced uterine contraction on day 3 postpartum rat.

Day 5 postpartum, the concentration of *H. indicum* extract at 250 mg/100 mL were added into organ bath after the 30-minute equilibrium period followed by the combination of *H. indicum* extract and oxytocin (Figure 4.10). The results showed *H. indicum* extract and the combination of *H. indicum* extract and oxytocin significantly increased uterine contraction when compared with spontaneous contraction. The percentage of area under the contraction (%AUC) of control, *H. indicum*, and the combination of *H. indicum* and oxytocin was 100, 146.62 ± 2.16, and 251.90 ± 6.80, respectively (Table 4.6).

Table 4.6 Effect of *H. indicum* extract and oxytocin (applied after) on uterine contraction on day 5 postpartum.

Day 5 postpartum	AUC (%) (Mean ± S.E.M.)
Control	100
H. indicum	146.62 ± 2.16*
H. indicum + OT	251.90 ± 6.80*

The *P*-value for AUC of *H. indicum* extract are significantly different from the control (*$P < 0.05$). Mean ± S.E.M. are given. OT is oxytocin.
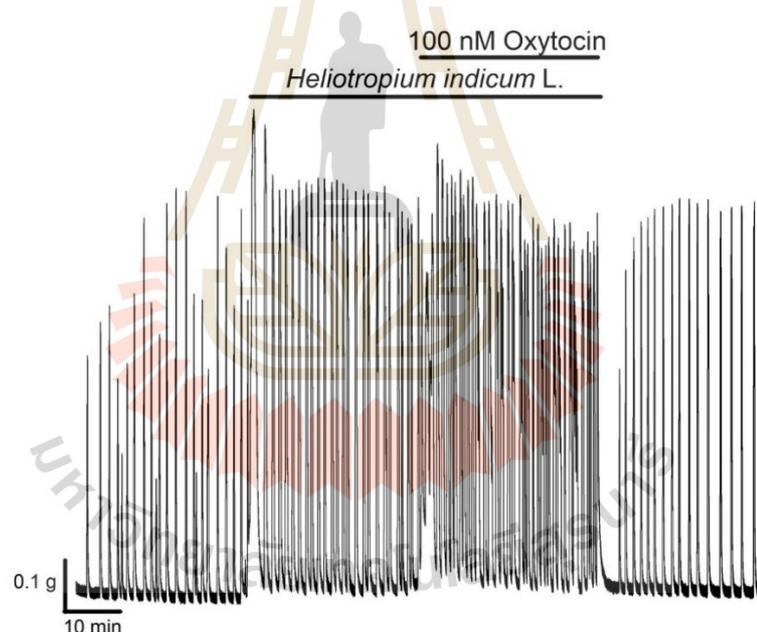
Figure 4. 10 Effect of *H. indicum* extract and oxytocin (applied after) on uterine contraction on day 5 postpartum rat.

Day 5 postpartum, the concentration of *H. indicum* extract at 250 mg/ 100 mL were added into organ bath after oxytocin- induced uterine contraction (Figure 4.11). The results showed oxytocin and the combination of *H. indicum* extract and oxytocin significantly increased uterine contraction when compared with

spontaneous contraction. The percentage of area under the contraction (%AUC) of control, oxytocin, and the combination of *H. indicum* and oxytocin was 100, 218.75 ± 2.75, and 297.56 ± 11.48 respectively (Table 4.7).

Table 4.7 Effect of *H. indicum* extract on oxytocin-induced uterine contraction on day 5 postpartum.

Day 5 postpartum	AUC (%) (Mean ± S.E.M.)
Control	100
OT	218.75 ± 2.75*
H. indicum + OT	297.56 ± 11.48*

The *P*-value for AUC of *H. indicum* extract are significantly different from the control (*$P < 0.05$). Mean ± S.E.M. are given. OT is oxytocin.
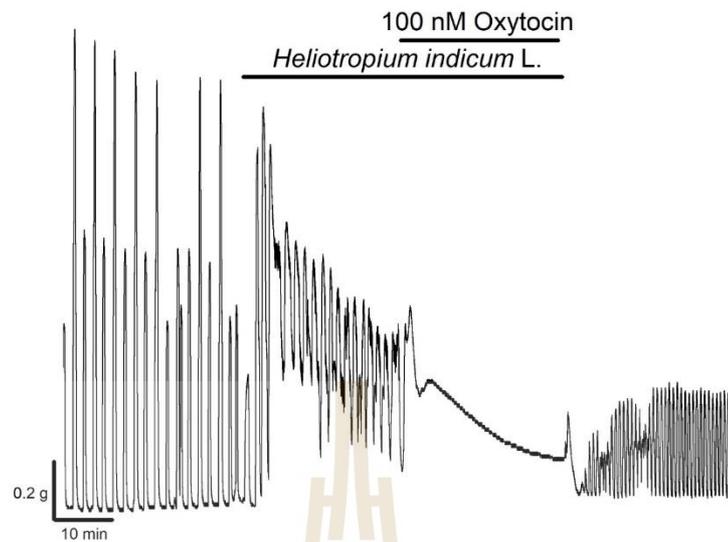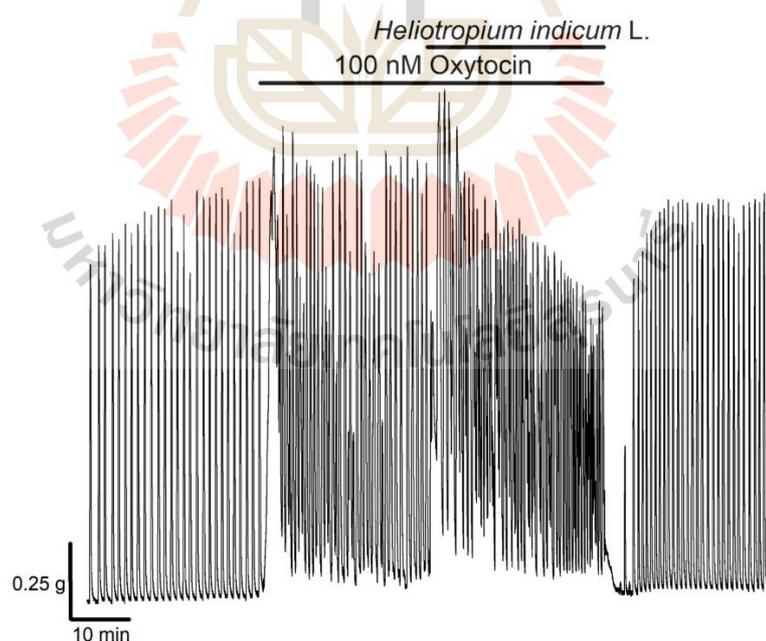

Figure 4.11 Effect of *H. indicum* extract on oxytocin-induced uterine contraction on day 5 postpartum rat.

The synergistic consider present if the effect of a combination is greater than that of each of the single compound such as $E(da,db) > E(da)$, and $E(da,db) > E(db)$. $E =$ the observed effect, and da and db are the doses of agents a and b (Williamson, 2001). In this study, the AUC of the combination of *H. indicum* and oxytocin was compared with *H. indicum* and oxytocin alone. The AUC of *H. indicum* extract alone divided by the combination between *H. indicum* and oxytocin on day 1, 3, and 5 postpartum, the ratio was 1.35, 0.78, and 0.58, respectively. Moreover, The AUC of oxytocin alone divided by the combination between *H. indicum* and oxytocin on day 1, 3, and 5 postpartum, the ratio was 1.26, 0.70, and 0.74, respectively.

When compare the effects of *H. indicum* extract which later-applied by oxytocin (*H. indicum* + Oxytocin) and after oxytocin-induced (Oxytocin + *H. indicum*) uterine contraction in same day of postpartum. The result showed the effects of *H. indicum* extract after oxytocin-induced uterine contraction (Oxytocin + *H. indicum*) significantly increased uterine contraction on day 1, 3, and 5 postpartum (Figure 4.12).
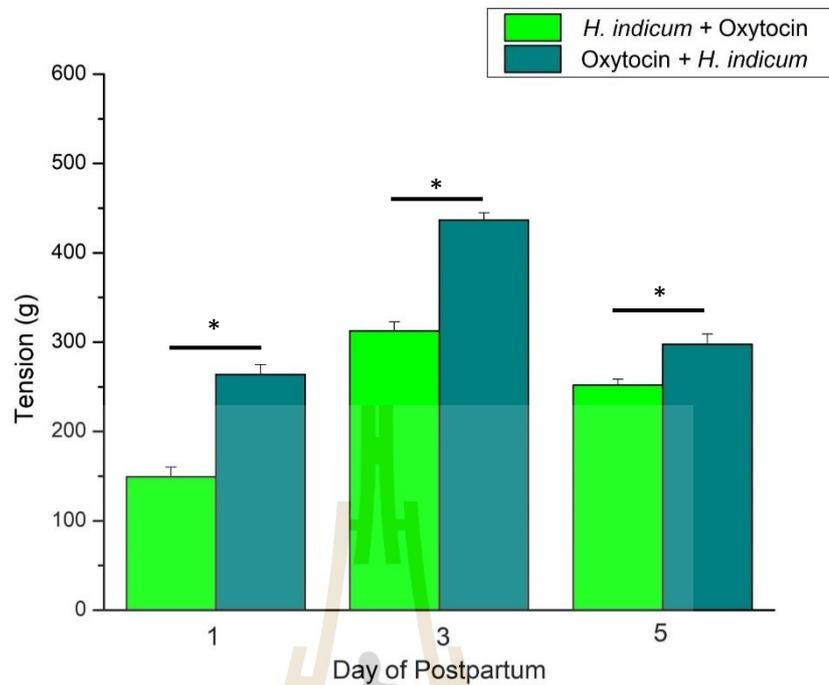
Figure 4.12 A graph showing the effects of *H. indicum* extract which later-applied by oxytocin on uterine contraction and on oxytocin- induced uterine contraction. Comparison of AUC (%) in same day of postpartum. The result shows the effects of *H. indicum* extract after oxytocin- induced uterine contraction (Oxytocin + *H. indicum*) significantly increases uterine contraction on day 1 and 3 postpartum. On day 5 postpartum, both groups are similar. Bars represent mean ± S.E.M. Values that do not share the same superscript letters are significantly different ($P < 0.05$). AUC: Area under the contraction.

4.4.3 Effects of *H. indicum* extract on uterine weight in postpartum rats

The uterine weight was observed on day 1, 3, and 5 postpartum. The results showed that the uterine weight was reduced both non- treated rats and treated rats. The uterine weight was decreased relatively compared to day 1 postpartum. In both groups, the uterine weight was significantly decreased from day 1 to day 5 postpartum and (Table 4. 8). When the relative uterine weight between groups at the

same day of postpartum was compared, the results showed the relative uterine weight of treated rats that receive the extract were significantly decreased on day 3 postpartum (Figure 4.13).

Table 4.8 Effect of *H. indicum* extract on uterine weight (%RU).

Group	Relative uterine weight (%) (Mean ± S.E.M.)
Non-treated rats	
Day 1 postpartum	100 [a]
Day 3 postpartum	51.06 ± 1.28 [b]
Day 5 postpartum	27.16 ± 0.88 [c]
Treated rats	
Day 1 postpartum	100 [a]
Day 3 postpartum	40.13 ± 0.50 [b]
Day 5 postpartum	25.06 ± 0.68 [c]

All values are expressed as mean + S.E.M. of 10 rats in each group ($n = 10$). Means with different superscripted letters indicate statistical significance ($P < 0.05$). Compare within group.
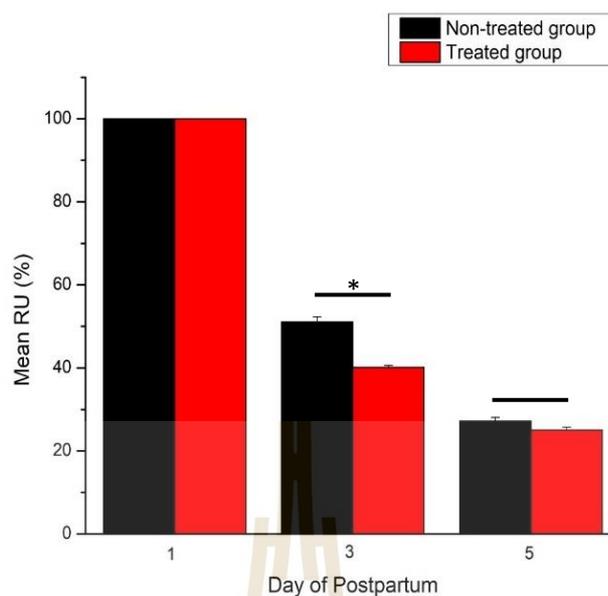
Figure 4.13 A graph showing the effects of *H. indicum* extract on uterine weight. Comparison of the relative uterine weight between groups at the same day of postpartum. The results show the relative uterine weight of treated rats that receive the extract were significantly decreased on day 3 postpartum. Bars represent mean ± S.E.M. Values that do not share the same superscript letters are significantly different (*P* < 0.05).

4.4.4 Effects of *H. indicum* extract on uterine cross-section area

The uterine cross-section area was observed on day 1, 3, and 5 postpartum. The uterine cross-section area represents to uterine size. The uterine cross-section area decreases relatively compared to day 1 postpartum. The relative uterine cross-section area in non-treated rats showed significant decrease on day 3 postpartum. Moreover, the relative uterine cross-section of treated rats that received the *H. indicum* extract was significantly decreased area on day 3 and 5 postpartum (Table 4.9). When the relative uterine cross-section area between groups at the same day of postpartum was compared, the results showed the relative uterine cross-section area of treated rats were significantly decreased on day 5 postpartum (Figure 4.15).

Table 4.9 Effect of *H. indicum* extract on uterine cross-section area.

Group	Relative uterine cross-section area (%) (Mean ± S.E.M.)
Non-treated rats	
Day 1 postpartum	100[a]
Day 3 postpartum	61.09 ± 6.46[b]
Day 5 postpartum	50.91 ± 1.08[b]
Treated rats	
Day 1 postpartum	100[a]
Day 3 postpartum	53.94 ± 2.19[b]
Day 5 postpartum	40.46 ± 2.45[c]

All values are expressed as mean $+$ S.E.M. of 10 rats in each group $(n = 10)$. When compare within group, means with different superscripted letters indicate statistical significance $(P < 0.05)$.
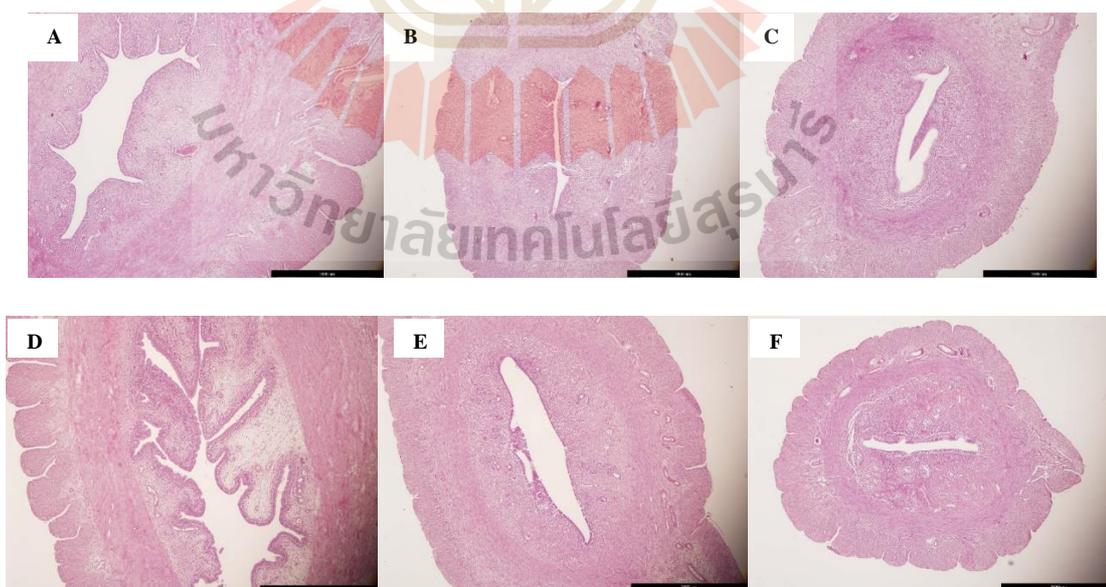
Figure 4.14 Representative images of hematoxylin and eosin staining on uterine histomorphology of the postpartum rats. A-C represent to non-treated rats on day 1, 3,

and 5 postpartum, respectively. D- F represent to treated rats on day 1, 3, and 5 postpartum, respectively. 4X magnification and bars represent 1000 μm.
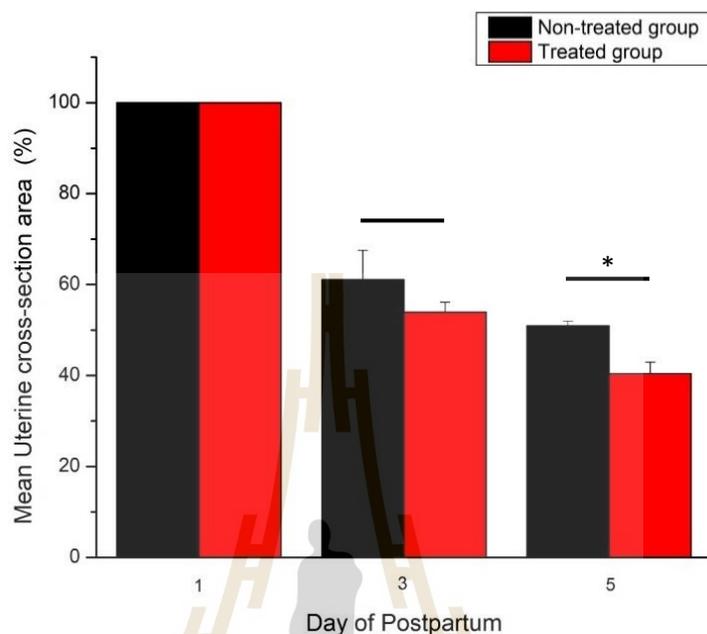
Figure 4.15 A graph showing the effects of *H. indicum* extract on uterine cross-section area. The relative uterine cross- section area between groups at the same day of postpartum is compared. The results show both groups are similar on day 3 postpartum. On day 5 postpartum, treated rats has the relative uterine cross-section area lower than non- treated rats. Bars represent mean ± S.E.M. Values that do not share the same superscript letters are significantly different (*P* < 0.05).

The myometrial layer per uterine cross- section area was observed on day 1, 3, and 5 postpartum. The myometrial layer per uterine cross-section area decreases relatively compared to day 1 postpartum. The relative myometrial layer per uterine cross- section area of both groups showed significant decrease on day 5 postpartum (Table 4.10). When the relative myometrial layer per uterine cross-section area between groups at the same day of postpartum was compared, the results showed

the relative myometrial layer per uterine cross-section area of treated rats were significantly decreased on day 5 postpartum (Figure 4.16).

Table 4.10 Effect of *H. indicum* extract on myometrial layer per uterine cross-section area.

Group	Relative myometrial layer per uterine cross-section area (%) (Mean ± S.E.M.)
Non-treated rats	
Day 1 postpartum	100[a]
Day 3 postpartum	92.24 ± 3.10[a]
Day 5 postpartum	70.62 ± 3.85[b]
Treated rats	
Day 1 postpartum	100[a]
Day 3 postpartum	82.76 ± 8.50[a]
Day 5 postpartum	58.98 ± 1.00[b]

All values are expressed as mean + S.E.M. of 10 rats in each group ($n = 10$). When compare within group, means with different superscripted letters indicate statistical significance ($P < 0.05$).
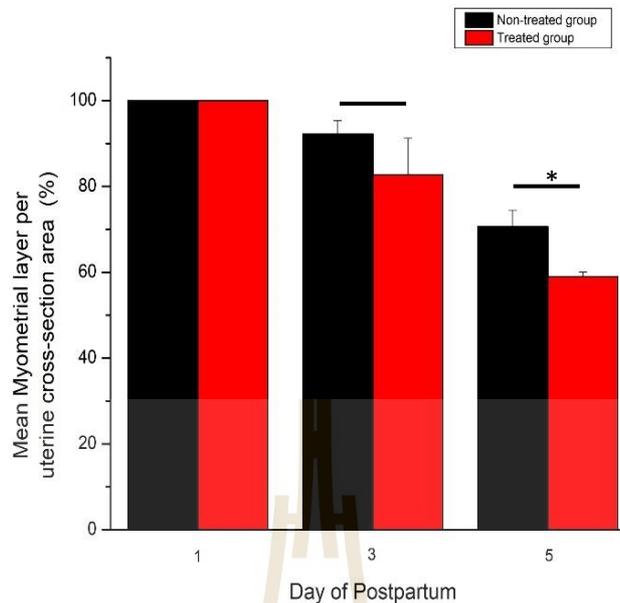
Figure 4.16 A graph showing the effects of *H. indicum* extract on myometrial layer per uterine cross-section area. The relative myometrial layer per uterine cross-section area between groups at the same day of postpartum is compared. The results show both groups are similar on day 3 postpartum. The relative myometrial layer per uterine cross-section area of treated rats are significantly decreased on day 5 postpartum. Bars represent mean ± S. E. M. Values that do not share the same superscript letters are significantly different ($P < 0.05$).

4.4.5 Effects of *H. indicum* extract on relative collagen in the uterus determining by using k-means cluster analysis (KMCA), MATLAB

The relative collagen in the uterus determining by using MATLAB build in function k-means clustering analysis (KMCA) was observed on day 1, 3, and 5 postpartum. The data was calculated by the program, to percent collagen in the uterus per tissue area. The collagen in the uterus decrease relatively compared to day 1 postpartum in both groups, non-treated rats showed significant decrease on day 5 postpartum and treated rats showed significant decrease from day 1 to day 5

postpartum (Table 4.11). When the relative collagen in the uterus between groups at the same day of postpartum was compared, the results showed the relative collagen in the uterus of treated rats were significantly decreased on day 3 and 5 postpartum (Figure 4.17).

Table 4.11 Effect of *H. indicum* extract on relative collagen in the uterus determining by using MATLAB build in function k-means clustering analysis (KMCA).

Group	Relative collagen in the uterus (%) (Mean ± S.E.M.)
Non-treated rats	
Day 1 postpartum	100[a]
Day 3 postpartum	96.71 ± 1.04[a]
Day 5 postpartum	90.85 ± 2.02[b]
Treated rats	
Day 1 postpartum	100[a]
Day 3 postpartum	86.48 ± 0.74[b]
Day 5 postpartum	78.68 ± 0.80[c]

All values are expressed as mean + S.E.M. of 10 rats in each group ($n = 10$). When compare within group, means with different superscripted letters indicate statistical significance ($P < 0.05$).
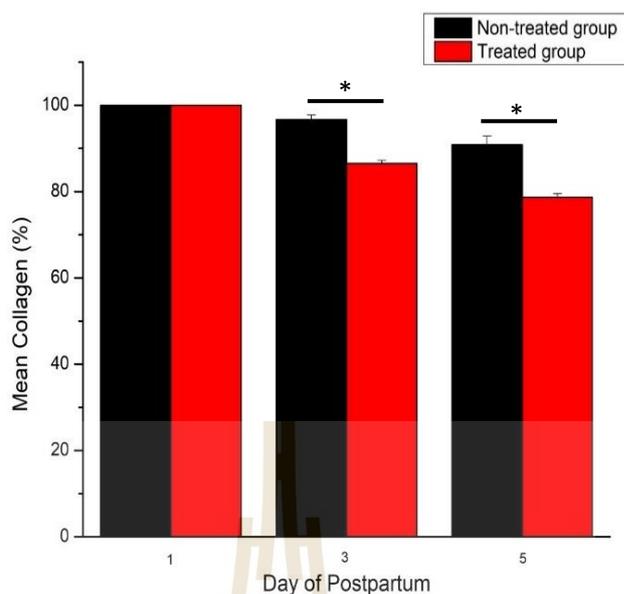
Figure 4.17 A graph showing the effects of *H. indicum* extract on the relative collagen of the uterus. The relative collagen in the uterus between groups at the same day of postpartum is compared. The results show the relative collagen in the uterus of treated rats are significantly decreased on day 3 and 5 postpartum. Bars represent mean ± S.E.M. Values that do not share the same superscript letters are significantly different ($P < 0.05$).

4.4.6 Effects of *H. indicum* extract on biochemical components in the uterus determining by Fourier Transform Infrared spectroscopy (FTIR)

Fourier Transform Infrared spectroscopy (FTIR) was used to identify and analysis the biological samples like tissue by detecting changes in macromolecular composition occurring during the uterine involution process based on the peak values in the IR region. The views of the uterine region were corrected by FTIR microscopic (Figure 4.18). The main bands were observed and detailed band assignments are given in Table 4.12. FTIR absorption spectrum of the sample shows distinct areas of the lipid region (3096-2811 cm^{-1}), the (C=O) triglycerides regions (1749-1732 cm^{-1}), the protein regions (1600-1276 cm^{-1}), carbohydrate, and nucleic acid regions (1257-950 cm^{-1}) were

investigated.
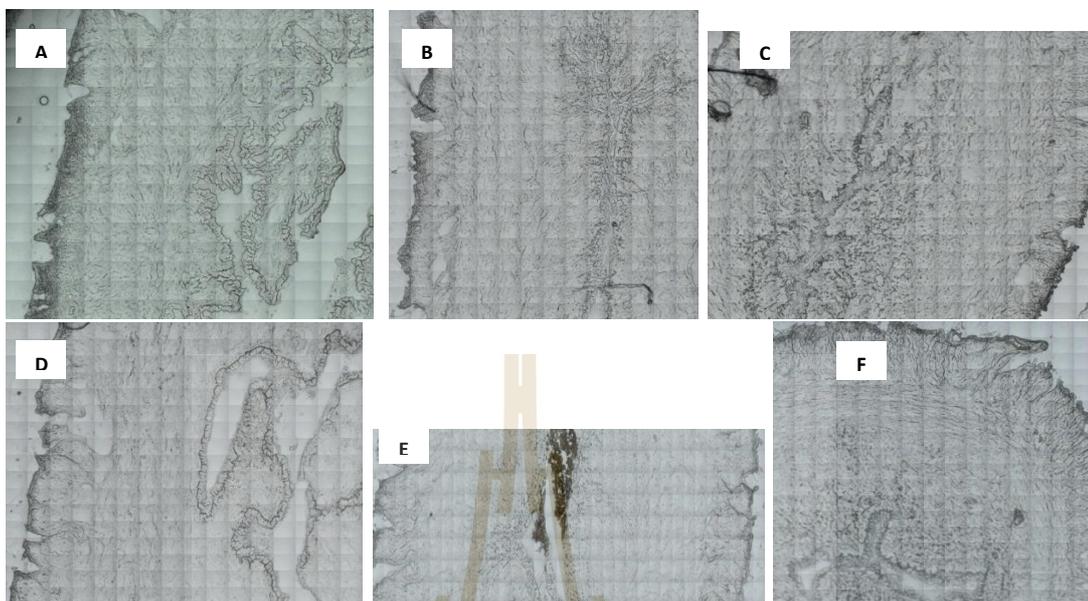
Figure 4.18 FTIR microscopic images of uterine tissue regions. A-C represent to non-treated rats on day 1, 3, and 5 postpartum, respectively. D-F represent to treat rats on day 1, 3, and 5 postpartum, respectively.

Table 4.12 General band assignment of FTIR spectrum of uterine tissue based on literature (Bozkurt et al., 2007, Movasaghi et al., 2008, Taylor et al., 2011).

Band No.	Spectrum	Description
1	3096-3033	C-H ring unsaturated lipid
2	3019-2811	Lipid, cholesterol esters
3	1749-1732	(C=O) triglycerides, cholesterol esters, phospholipids
4	1682-1633	Amide I protein C=O stretching
5	1350-1276	Amide III vibrations of collagen, N-H thymine
6	1257-1221, 1097-1072	PO^-2 asymmetric stretching, fully hydrogen-bonded: mainly nucleic acids with the little contribution from phospholipids, C-O-C, C-O dominated by the ring vibrations of polysaccharides C-O-P, P-O-P
7	1205-1100, 1062-949	PO^-2 symmetric stretching: nucleic acids and phospholipids, C–O stretch: glycogen, polysaccharides, glycolipids, C–N+–C stretch: nucleic acids, ribose-phosphate main chain vibrations of RNA

When compared within group of non-treated rats, the result has been shown in Table 4.13. The main bands area in non-treated rats were calculated relatively compared to day 1 postpartum in each band. Band No. 1 (unsaturated lipid) were significantly decreased on day 5 postpartum compared with day 1 postpartum. Band No. 2 (lipid, cholesterol esters) were significantly increased on day 1, 3, and 5 postpartum. Band No. 3 ((C=O) triglycerides) were significantly increased on day 5

postpartum. Band No. 4 (Amide I protein) were unchanged in postpartum periods. Moreover, Band No. 5 (Amide III vibrations of collagen) and Band No. 6 (PO−2 asymmetric stretching nucleic acids and carbohydrate) showed significant decreases on day 5 postpartum compared with day 1 postpartum. Finally, Band No. 7 (PO−2 symmetric stretching: nucleic acids and carbohydrate) were unchanged in postpartum periods.

Table 4.13 Changes in area values of the infrared bands for non-treated rats. The main bands area in non-treated rats were calculated relatively compared to day 1 postpartum in each band.

Non-treated	Postpartum day		
Band No.	**Day 1 (Mean ± SEM)**	**Day 3 (Mean ± SEM)**	**Day 5 (Mean ± SEM)**
1	100^a	94.40 ± 3.33^{ab}	87.35 ± 2.28^b
2	100^a	113.39 ± 2.27^b	138.60 ± 3.17^c
3	100^a	116.85 ± 9.48^{ab}	138.45 ± 6.72^b
4	100^a	100.31 ± 2.00^a	107.09 ± 4.00^a
5	100^a	93.84 ± 6.15^{ab}	82.74 ± 5.18^b
6	100^a	105.81 ± 4.14^{ab}	112.85 ± 4.09^b
7	100^a	107.79 ± 7.99^a	96.76 ± 5.43^a

All values are expressed as mean + S.E.M. of 10 rats in each group ($n = 10$). When compare within group, means with different superscripted letters indicate statistical significance ($P < 0.05$).

When compared within group of treated rats, the result shows in Table 4.14. The main bands area in treated rats were calculated relatively compared to day 1 postpartum in each band. In treated rats, they were received the extract showed Band No. 1 (unsaturated lipid) were significantly decreased on day 3 postpartum and stable

until day 5 postpartum. Band No. 2 (lipid, cholesterol esters), Band No. 3 ((C=O) triglycerides), and Band No. 4 (Amide I protein) were significantly increased on day1, 3, and 5 postpartum. Moreover, Band No. 5 (Amide III vibrations of collagen) were significantly decreased on day 3 and stable until 5 postpartum. Band No. 6 (PO−2 asymmetric stretching nucleic acids and carbohydrate) were significantly increased on day 1, 3, and 5 postpartum. Finally, Band No. 7 (PO−2 symmetric stretching: nucleic acids and carbohydrate) were unchanged in postpartum periods.

Table 4.14 Changes in area values of the infrared bands for treated rats. The main bands area in treated rats were calculated relatively compared to day 1 postpartum in each band.

Treated	Postpartum day		
Band No.	Day 1 (Mean ± SEM)	Day 3 (Mean ± SEM)	Day 5 (Mean ± SEM)
1	100[a]	85.81 ± 3.36[b]	85.66 ± 3.00[b]
2	100[a]	131.49 ± 3.07[b]	153.76 ± 3.53[c]
3	100[a]	138.06 ± 9.97[b]	188.33 ± 15.34[c]
4	100[a]	108.63 ± 2.58[b]	121.47 ± 1.88[c]
5	100[a]	75.75 ± 4.96[b]	66.66 ± 3.81[b]
6	100[a]	118.23 ± 3.67[b]	130.73 ± 4.44[c]
7	100[a]	98.03 ± 5.73[a]	95.44 ± 5.33[a]

All values are expressed as mean + S.E.M. of 10 rats in each group ($n = 10$). When compare within group, means with different superscripted letters indicate statistical significance ($P < 0.05$).

The main bands area in non-treated rats and treated rats were calculated relatively compared to day 1 postpartum in each band. When compare the relative band area of the biochemical components in the uterus between group at the same day of

postpartum the result showed in Figure 4.19. On day 3 postpartum, Band No. 1 (unsaturated lipid), Band No. 3 ((C=O) triglycerides), and Band No. 7 (PO−2 symmetric stretching: nucleic acids and carbohydrate) of non-treated rats no significantly when compared with treated rats. Band No. 2 (lipid, cholesterol esters), of both groups were similar. Moreover, Band No. 4 (Amide I protein), and Band No. 6 (PO−2 asymmetric stretching nucleic acids and carbohydrate) of treated rats were significantly increased when compared with non-treated rats. In the part of Band No. 5 (Amide III vibrations of collagen) of treated rats were significantly decreased when compared with non-treated rats. On day 5 postpartum, Band No. 1 (unsaturated lipid) and Band No. 7 (PO−2 symmetric stretching: nucleic acids and carbohydrate) of non-treated rats no significantly when compared with treated rats. Band No. 2 (lipid, cholesterol esters), Band No. 3 ((C=O) triglycerides), Band No. 4 (Amide I protein), and Band No. 6 (PO−2 asymmetric stretching nucleic acids and carbohydrate) of treated rats were significantly increased when compared with non-treated rats. In the part of Band No. 5 (Amide III vibrations of collagen) of treated rats were significantly decreased when compared with non-treated rats.
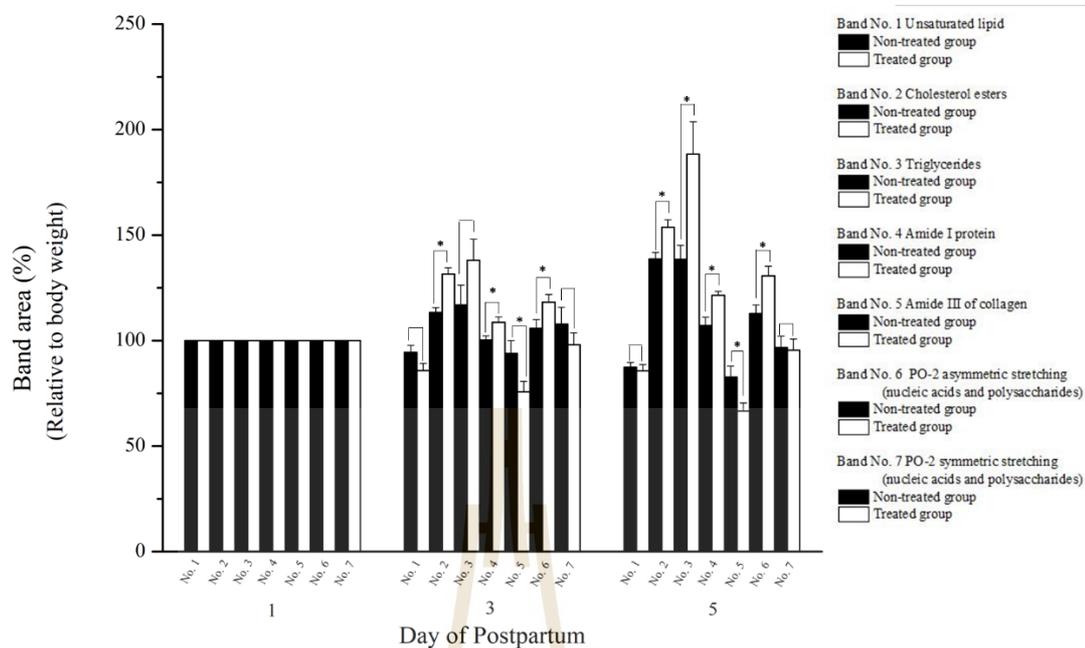
Figure 4.19 A graph showing the effects of *H. indicum* extract on FTIR band area of uterus in postpartum rat. The main bands area in both groups were calculated relatively compared to day 1 postpartum in each band. The relative band area of the biochemical components in the uterus between groups at the same day of postpartum are compared. Bars represent mean ± S.E.M. Asterisk are significantly different ($P < 0.05$).

4.5 Discussion

The study about the effects of *H. indicum* extract on uterine contraction in postpartum rats indicated that the extract can induce uterine contraction on day 1, 3, and 5 postpartum. The AUC of spontaneous contraction after administration of *H. indicum* extract at 250 mg/ 100 mL on isolated uterine strips were significantly increased compared with the spontaneous contraction without the extract. Moreover, the *H. indicum* extract had more potent effect at day 3 postpartum, but less on day 5 postpartum. The results of primary phytochemical screening in Chapter III showed that

H. indicum extract mainly contain alkaloids, tannins, flavonoids, steroids, and glycoside. The phytochemical such as alkaloids, tannins, and glycoside show some effects on uterine contraction (Calixto et al. , 1986, Polya et al. , 1995) . Indole alkaloids from *H. indicum* extract can also provoke contraction of the uterus such as ergotamine and ergometrine is a partial agonist of α-adrenergic and 5-HT2 (serotonin receptors) receptors, partly dopamine receptors (mostly type D_2) (Katzung et al., 2009). Both α-adrenergic receptors subclasses cause uterine contraction response of the uterus upon catecholamine stimulation (Bottari et al. , 1985) . Moreover, the stimulates contraction of human uterine smooth muscle (myometrium) were related with 5-hydroxytryptamine receptor. In pregnant human myometrium, the literature reviews were showed strong evidence for the expression of contractile 5-HT(2A) receptors, and this receptor is a possible target for new uterotonic therapies (Cordeaux et al. , 2009) . The other studies investigated if dopamine both stimulates and inhibits prolactin secretion through activation of the same dopamine D_2 receptor in GH4ZR7 cells. The results show that both the stimulatory and inhibitory actions of dopamine are plausible mediated by the same D_2 receptor subtype, the inhibitory action of dopamine is mediated through a G i protein; and the stimulatory action of dopamine is mediated through a PTX-insensitive pathway (Chang et al., 1997). Taken together, it is possible that the active constituents of are phytochemicals such as alkaloids, tannins, and glycoside and that the effect of the extract on uterine contraction stimulation may activate via a partial agonist of α-adrenergic and 5-HT2 (serotonin receptors) receptors, partly dopamine receptors (mostly type D_2). It would be of interest to investigate further this mechanism underlying.

Oxytocin is an agonist stimulation of IP_3 receptor. It's unable to generate force

in the uterus if Ca^{2+} entry is restrained. Oxytocin can also stimulate uterine contraction by related to SR Ca^{2+} release. Both activities will enlarge and/or extend Ca^{2+} transients (Wray, 2007). The SR has both IP_3 and ryanodine (Ry) receptors. The Ca^{2+} released from these receptors has low effect on the activation of contraction. When the SR is disabled, this makes an increase in both Ca^{2+} transients and contractions. Cyclopiazonic acid is a drug which inhibits the SR Ca^{2+}-ATPase (SERCA) wanted to transport Ca^{2+} into the SR (Shmygol et al., 2007). In this study, the measurement of tension was investigated from uterine strips treated with oxytocin added before and after the extract and then in the continued presence. The experiment was studied in postpartum uterus on day 1, 3, and 5 postpartum. The results showed the combination of *H. indicum* extract and oxytocin significantly increased uterine contraction when compared with spontaneous contraction alone. Moreover, the effects of *H. indicum* extract after oxytocin-induced uterine contraction (in the continued presence of oxytocin) were more powerful to increase uterine contraction on day 1, 3, and 5 postpartum. Thus, combination of *H. indicum* extract and oxytocin is useful, especially after received oxytocin. The study clearly showed that the *H. indicum* extract can be used with oxytocin to help accelerate uterine involution. Furthermore, if the mother did not response oxytocin, the *H. indicum* extract can be used to induce uterine contraction. This study on rat uterus gives the primary proofs that *H. indicum* ethanolic extract to produce contractility effects in postpartum period.

The method to evaluate drug synergistic effect was initially described by Gaddum in 1940 and Berenbaum 1989. The synergy is considered when the effect of a combination is greater than that of each of the single compound such as E(da,db) > E(da), and E(da,db) > E(db) whereby E is the observed effect and da and db are the

doses of agents a and b (Williamson, 2001). Thus, the ration less than 1 was considered as a synergistic effect. The results showed that the AUC ratio of *H. indicum* extract alone : the combination of *H. indicum* and oxytocin on day 1, 3, and 5 postpartum was 1. 35, 0. 78, and 0. 58, respectively and that the AUC ratio of oxytocin alone : the combination of oxytocin and *H. indicum* on day 1, 3, and 5 postpartum was 1.26, 0.70, and 0. 74, respectively. Taken together, the results suggested the synergistic effect of the combination of oxytocin and *H. indicum* on day 3 and day 5, but not day 1. From the results that mentioned earlier, the effect of *H. indicum* extract on uterine contraction may be synergistic with oxytocin-induced uterine contraction in postpartum rats. The possible mechanism(s) may be due to the stimulation of uterine contraction by the non-genomic active pathway. There are possibly 2 ways in which the substance will active; 1) activated L-type Ca^{2+} channel for increased Ca^{2+} influx into the cell and 2) activated Ca^{2+} in sarcoplasmic reticulum by IP_3 receptors. The extract did not work through estrogen receptors (Kupittayanant and Kupittayanant, 2012). The extract has chemical components that have stimulatory effects at adrenoceptors and possibly by enhancing prostaglandin synthesis (Koffuor et al., 2012). Finally, the potent stimulatory effects of the whole plant *H. indicum* extract on spontaneous contraction in postpartum period could confirm the medicinal use of *H. indicum* to help accelerate uterine involution and reduce the risk of postpartum hemorrhage.

The study of the effects of *H. indicum* extract on uterine weight in postpartum rats were observed on day 1, 3, and 5 postpartum. The results showed that the uterine weight was reduced in postpartum period. The uterine weight decreased relatively compared to day 1 postpartum in both non-treated rats and treated rats. When the relative uterine weight between groups was compared at the same day of postpartum.

The results showed significant decreases in the relative uterine weight on day 3 postpartum in treated rats that received the extract. The *H. indicum* extract enhanced the weight of the uterus to decrease faster than normal process on day 3 postpartum. In the part of uterine size, the effects of *H. indicum* extract on uterine cross-section area in postpartum rats were investigated on day 1, 3, and 5 postpartum. The uterine cross-section area represents to uterine size. The uterine cross-section area of non-treated rats and treated rats were decreased relatively compared to day 1 postpartum. The *H. indicum* extract showed the best size reducing rate. When the relative uterine cross-section area between groups at the same day of postpartum was compared, the results showed significant decreases in treated rats on day 5 postpartum. Thus, the results showed the extract can accelerate the reducing rate of uterine size. The study of enhanced myometrial autophagy in postpartum uterine involution shows postpartum autophagy developed suddenly in uterine myocytes without obvious apoptosis. Autophagy of myocytes may play a considerable role in uterine involution. Autophagy during postpartum period result in regress of size and weight of uterus (Hsu et al., 2014).

The myometrium is the muscular layer of the uterine wall that is complicated with contraction during delivery and postpartum. The principal part in the expansion of the uterus during pregnancy is muscular layer. The myometrium consists mainly of smooth muscle cells but also contains immune cells, blood and lymphatic vessels, fibroblasts, and connective tissue. The connective tissue is a framework that expands as the uterus distends during pregnancy (Lye, 1999). In pregnancy, the uterine smooth muscle layer or myometrium show hypertrophy and hyperplasia result in increased the weight of the uterus (Cunningham et al., 2010). Uterine involution show increase in

autophagy activity in the myometrium, resulting in decrease the uterine size during postpartum period (Hsu et al., 2014). This study showed the relative uterine cross-section area of treated rats significant decreases in on day 5 postpartum. The *H. indicum* extract had less effect on uterine contraction on day 5 postpartum when compared with another day. Moreover, the relative myometrial layer per uterine cross-section area of treated rats was significantly decreased on day 5 postpartum when compared non-treated rats. The results suggested that the reducing of myometrial layer related with uterine cross-section area and uterine contraction in postpartum period.

Collagen is the amplest the extracellular matrix protein in the human body, plays a vital role in equipping structure and tensile strength to tissues of the female pelvic floor and reproductive tract. The collagen type I and III are most abundant in the pelvic floor connective tissue, where the collagen type V has been found. Collagen type I fibers furnish most of tissue resistance to tension and collagen type III have greater flexibility and dilation to tissue, while collagen type V seems to be of minor prominence as it forms small fibers of very low tensile strength (Dhital et al., 2016). Rat uterus has four main uterine collagen types including fibril-forming collagen (COL) I and COL III, basement membrane COL IV, and microfibrillar COL VI. Uterine collagen content remodel during pregnancy and postpartum involution. The hormones and local factors such as 17ß-estradiol (E2) can restrain the loss of collagen from the involuting rat uterus *in vivo*, which reflects the inhibitory role of E_2 on collagenase activity. Moreover, progesterone (P_4) was also have an inhibitory effect on collagenase activity, preventing collagen degradation in postpartum period (Diao et al., 2011). The hypertrophy and hyperplasia of individual cells, especially smooth muscle cells, were involved with uterine size that increase on pregnancy. Postpartum blood loss or postpartum

hemorrhage were prevented by the uterus contraction after delivery. The rapid time course the myometrium contracts and the endometrium reforms. The literature review showed that estradiol (E_2), progesterone (P_4) or both could slow down uterine involution. It has been reported that the uterine smooth muscle cells synthesize collagenase, and this collagenase production were repressed by progesterone in vitro (Takamoto et al., 1998).

During pregnancy, the "transient" collagen were formed, apparently fated for degradation in the postpartum period. After parturition, the uterine collagen decrease by a means which is obvious even at 14 hours in human uterus. By the 8th day postpartum, the human uterus has lost proximately 72 percent of the total collagen which was showed at term. The remodeling in the collagen content of the uterus during pregnancy and involution follow fluctuations in total uterine weight. There is an augmentation in myometrial prolidase activity of approximately 75 percent, beginning 2 days postpartum and continue raised until the 8th day postpartum afterward falls to almost normal levels by the 5th postpartum week (Morrione and Seifter, 1962). In rat uterus, during the first four days postpartum, the collagen concentration was higher that found in nonpregnant controls. The concentration of collagen was lowest in early estrus, attained a peak in metestrus, and dropped again in diestrus (Smith and Kaltreider, 1963). The involuting uterus through a quick diminution in size primarily due to the degradation of the extracellular matrix, especially collagen for the uterus returns to its pre-pregnant state. The major proteinases that degrades collagen and is the most abundant in the uterus (Manase et al., 2006).

In this study, the effects of *H. indicum* extract on percent relative collagen in the uterus determining by using MATLAB build in function k-means clustering

analysis (KMCA) was observed on day 1, 3, and 5 postpartum. The collagen in the uterus were decreased relatively compared to day 1 postpartum in both groups. The relative collagen in the uterus between groups at the same day of postpartum was compared. On day 3 and 5 postpartum, collagen in the uterus of treated rats was significantly decreased compared with non-treated rats. The results suggested that the collagen in the uterus of treated rats that received the *H. indicum* extracts was reduced faster than non-treated rats. The metabolic breakdown of collagen and non-collagen protein was studied in the rat uterus exhibit postpartum involution. The collagen vanish from the uterus at a rate proportional to the surface of the fibre (Woessner, 1962). The disorganization of the uterine collagen in postpartum period were decreased progressively from day 3 to day 7 (Takamoto et al., 1998). From the results, it was therefore indicated that *H. indicum* extracts can stimulate the degradation of collagen involved with reducing weight and size of the uterus in postpartum period. Moreover, the extract can stimulate uterine contraction in postpartum period. Therefore, these results can support the folk medicinal use of *H. indicum* to accelerate uterine involution.

Blood biochemical parameters in rat were changed in the postpartum period such as reduced blood glucose levels. Moreover, the glycemic means were decreased during and after pregnancy and the triglyceride concentrations were expanded before and during pregnancy in association to after pregnancy. The total cholesterol levels showed no changes (Corvino et al., 2015). The literature review found that FTIR technique were identified proline and tyrosine at spectrum 1200 cm^{-1}, CH_2, and lipids at spectrum 1308 cm^{-1}, and the CH_3CH_2 bending modes found in protein side chains of multiple tissue types at spectrum 1450 cm^{-1}. Moreover, the spectrum at 1650 cm^{-1} has tentatively been assigned to amide I, lipids, and collagen (Miura and Thomas,

1995). Many of these features of uterus require alteration to the element of the extracellular matrix (ECM) of the tissues during pregnancy, which compose of fibrillar collagen, proteoglycans, hyaluronan, elastin and water (House et al., 2009). Moreover, the proteins approximately 60% to 68% were degraded by day 3 postpartum (Takamoto et al., 1998). The decline in uterine weight was related with a reduction in total protein, RNA, and DNA (Leathem et al., 1968).

The effects of *H. indicum* extract on biochemical components in the uterine tissue determining by Fourier Transform Infrared spectroscopy (FTIR) were observed on day 1, 3, and 5 postpartum. In this study, the change of biochemical components in the uterus during postpartum period were analyzed by FTIR and showed in Table 4.12 and Table 4.13. FTIR was used to identify and analysis the biological samples by detecting changes in macromolecular composition based on the peak values in the IR region. The main bands were observed and detailed band assignments are given in Table 4.11. FTIR absorption spectrum of the sample showed distinct areas of the lipid region (3096-2811 cm^{-1}), the (C=O) triglycerides regions (1749-1732 cm^{-1}), the protein regions (1600-1276 cm^{-1}), carbohydrate, and nucleic acid regions (1257-950 cm^{-1}).

The main bands area in non-treated rats and treated rats were calculated relatively compared to day 1 postpartum in each band. On the part of the lipid region (3096-1732 cm^{-1}) including Band No. 1 (unsaturated lipid), Band No. 2 (lipid, cholesterol esters), and Band No. 3 ((C=O) triglycerides). When compared within group, the result suggested that Band No. 1 (unsaturated lipid) of non-treated rats were significantly decreased on day 5 postpartum compared with day 1 postpartum. In treated rats, they were received the extract showed Band No. 1 (unsaturated lipid) were significantly decreased on day 3 postpartum and unchanged until day 5 postpartum.

Band No. 2 (lipid, cholesterol esters) of both groups were significantly increased on day 1, 3, and 5 postpartum. Band No. 3 ((C=O) triglycerides) of non-treated rats were significantly increased on day 5 postpartum. Band No. 3 ((C=O) triglycerides) of treated rats were significantly increased on day 3, and 5 postpartum. Thus, the *H. indicum* extract can interfere the pattern of lipid in the uterine tissue at spectrum 3096-1732 cm^{-1}.

On the part of the protein regions (1600-1276 cm^{-1}) including Band No. 4 (Amide I protein) and Band No. 5 (Amide III vibrations of collagen), the results suggested that Band No. 4 (Amide I protein) of non-treated showed were unchanged in postpartum periods. In treated rats, Band No. 4 (Amide I protein) were significantly increased on day 1, 3, and 5 postpartum. Moreover, Band No. 5 (Amide III vibrations of collagen) of non-treated rats were significantly decreased on day 5 postpartum but in treated rats were significantly decreased on day 3 postpartum. However, *H. indicum* extract showed slightly decreases in the total collagen (1350-1276 cm^{-1}) in the uterus better than normal group. Thus, the *H. indicum* extract can interfere the pattern of protein in the uterine tissue at spectrum 1600-1276 cm^{-1}.

The carbohydrate and nucleic acid regions (1257-950 cm^{-1}) includes Band No. 6 (PO^{-2} asymmetric stretching: nucleic acids and carbohydrate) and Band No. 7 (PO^{-2} symmetric stretching: nucleic acids and carbohydrate). The results showed Band No. 6 of non-treated rats were significantly increased on day 5 postpartum. In treated rats, Band No. 6 (PO-2 asymmetric stretching nucleic acids and carbohydrate) were significantly increased on day 1, 3, and 5 postpartum. In non-treated rats, Band No. 7 (PO-2 symmetric stretching: nucleic acids and carbohydrate) of both groups were unchanged in postpartum periods. Thus, the *H. indicum* extract can interfere the pattern

of the carbohydrate and nucleic acid in the uterine tissue at spectrum 1257-950 cm^{-1}.

The main bands area in non-treated rats and treated rats were calculated relatively compared to day 1 postpartum in each band. When the relative band area of the biochemical components in the uterus was compared between groups at the same day of postpartum the results showed in Figure 4.18. Band No. 2 (lipid, cholesterol esters), Band No. 3 ((C=O) triglycerides), Band No. 4 (Amide I protein), and Band No. 6 (PO−2 asymmetric stretching nucleic acids and carbohydrate) showed trend to increase in postpartum period. However, Band No. 1 (unsaturated lipid), Band No. 5 (Amide III vibrations of collagen), and Band No. 7 (PO−2 symmetric stretching: nucleic acids and carbohydrate) showed trend to decrease in postpartum period. After postpartum rats were treated by *H. indicum* extract, Band No. 2, Band No. 3, Band No. 4, and Band No. 6 seem to be increased more than non-treated group. Band No. 1 and Band No. 7 of treated rats showed no significant when compared with non-treated rats. Especially, Band No. 5 that represents to collagen were significantly decreased on day 3 and 5 postpartum.

In summary, the *H. indicum* extract can induce uterine contraction and may be synergistic with oxytocin-induced uterine contraction in postpartum rats. Furthermore, the results show the extract significantly decreased uterine weight on day 3 postpartum, uterine size on day 5 postpartum, and the collagen of the uterus on day 3 and 5 postpartum. The results of FTIR study indicated that the *H. indicum* extract has some effects on biochemical components of the uterine tissue on day 1, 3, and 5 postpartum. The *H. indicum* extract can interfere the pattern of the lipid region (3096-2811 cm^{-1}), the (C=O) triglycerides regions (1749-1732 cm^{-1}), the protein regions (1600-1276 cm^{-1}), carbohydrate, and nucleic acid regions (1257-950 cm^{-1}). Especially, band area of

collagen was significantly decreased on day 3 and 5 postpartum. In this study, the effects of the whole plant extract of *H. indicum* on the uterine contraction, the uterine weight, the uterine size, the collagen of the uterus, and the biochemical compounds of uterus were investigated. The results from this study is the scientific data to support the effects of *H. indicum* extract on uterine tissue in postpartum period. Moreover, the extract can help accelerate uterine involution in postpartum rats by enhancing uterine contraction and reducing the uterine weight, the uterine size, and the collagen of the uterus. Therefore, the whole plant of *H. indicum* extract can be consumed during postpartum period to help reduce the risk of postpartum hemorrhage. The findings therefore confirm traditional uses that *H. indicum* could accelerate uterine involution.
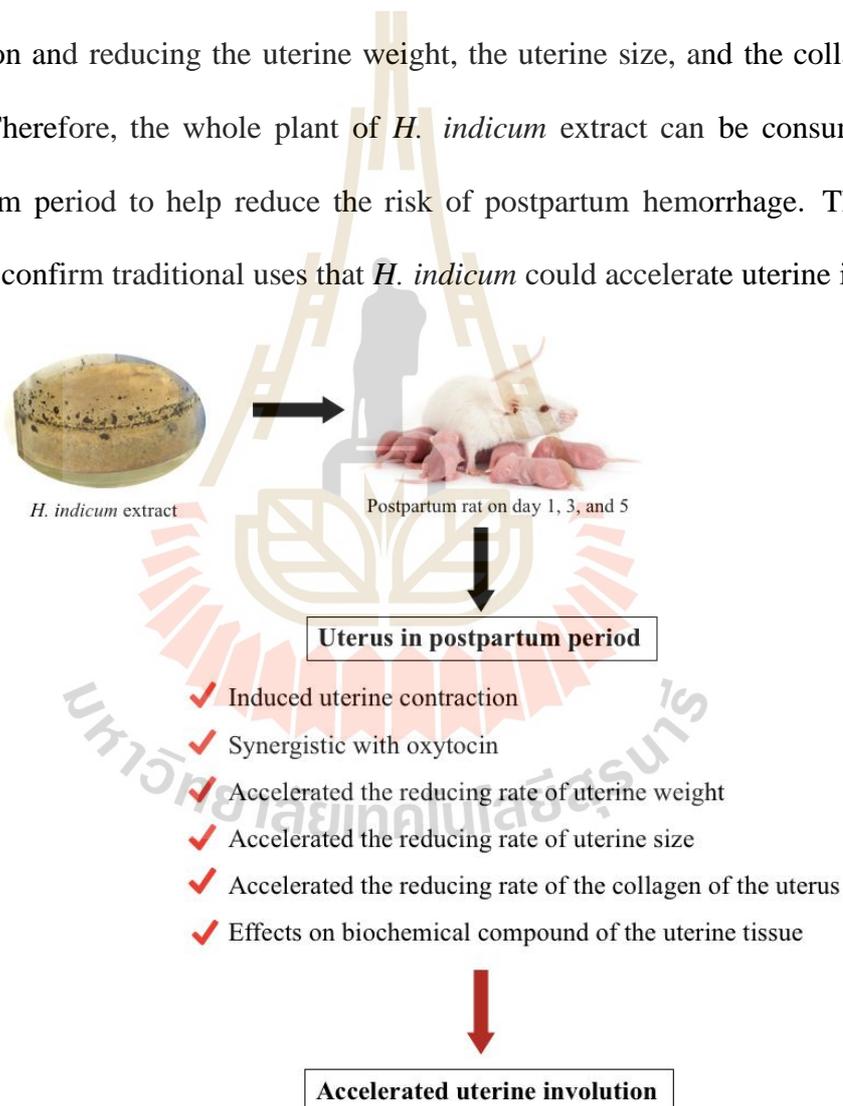
Figure 4.20 Diagram show the effects of *H. indicum* on uterine involution in postpartum rats.

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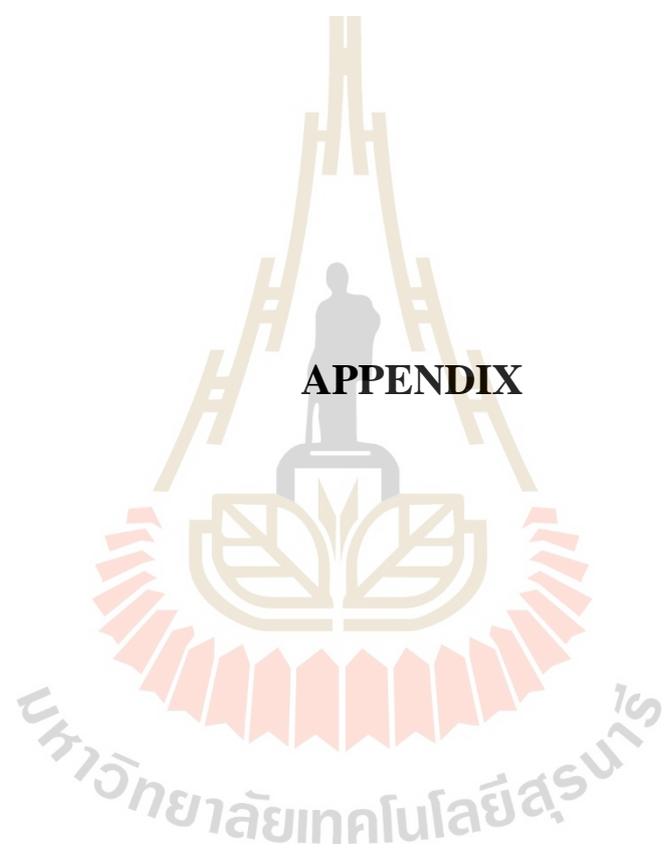
APPENDIX

APPENDIX

K-MEANS CLUSTER ANALYSIS (KMCA)

Methodology: k-means cluster analysis (KMCA)

The k-mean cluster analysis was performed to find groups of data that have comparable data characteristics. In the context of image segmentation, the one pixel of an image is a data point that has a character of color described as a component Red, Green, and Blue (RGB). All data points will pass to the data-partitioning algorithm based on Lloyd's algorithm (Lloyd, 1982), which allocates data points into k clusters defined by cluster centers, where k is chosen before the algorithm starts. The cluster centers were randomly picked for reference data characteristics, and the other data will be classified into the cluster member based on the similarity. The algorithm proceeds as follows: Randomly choose k initial cluster centers (in this study we use k=4), Compute data point-to-cluster centers similarity of all input data to each cluster centers. Then proceed with the batch update by assigning each data to the cluster with the closest cluster centers. Compute the average of the data in each cluster to obtain k (k=4) new representative data. Repeat until cluster assignments result does not change, or the maximum number of iterations is reached. Finally, the results of k-mean clustering will provide the similar color of the image assigned to the nearest similar color, therefore, the output is an image of clustered data as 4 clusters based on the similarity of color.

MATLAB code of k-means clustering analysis (KMCA)

```matlab
%%% K-means for Uterus's collagen

file_path = 'Here you input the image';

k_val =5; % kvalue for k-mean clustering

fullfile_name = dir(fullfile(file_path,'*.jpg'));% pattern to match filenames.
report_cluster_count = zeros(length(fullfile_name),k_val+1);

for file=1:length(fullfile_name)
    [file length(fullfile_name)]
    file_name = fullfile_name(file).name;
    report_cluster_count(file,1) = string(file_name);
    %raw_image = imread([ file_path '\' file_name]);
    image = double(imread([ file_path '\' file_name]));
    [num_rows,num_cols,rgb] = size(image);


    %figure(1000*file),imshow(raw_image);


    image2D = reshape(image,[num_rows*num_cols,rgb]);


    %weight = [1,1.5,1]; %Red Green Blue % fine-tune of color before clustering
```

```
target_image_2D = image2D;

dc_amp_vec_use = (1:1:rgb);


   % kmean-cultering code

   n=1;

   kn= k_val; % cluter number


   standardized_a = normalize(target_image_2D);


   for k = kn


       opts = statset('Display','final');


        [IDX_a, C_a, sumD_a, D_a] = kmeans(target_image_2D, k, 'Replicates', 2,

       'Options',opts);

       clustering_a = reshape(IDX_a, [num_rows,num_cols]);


       % because k-mean output in a random number of clustering

       % standardize cluster number (sort by low to related to green pigment)


       clus_map = clustering_a;

       C_green = sort(C_a(:,2));
```

```matlab
for newclus = 1:kn;

    oldclus = find(C_a(:,2)==C_green(newclus));

    clus_map(find(clus_map == oldclus))=newclus*10;

end


clus_map = clus_map./10;


figure(1000*file+k)

imagesc(clus_map)

if k == 5


    mymap = [0    0.4470    0.7410

        0.8500    0.3250    0.0980

        0.4660    0.6740    0.1880

        0.4940    0.1840    0.5560

        0.9290    0.6940    0.1250];

    c = colorbar ('Ticks',(1:1:5),'TickLabels',{'1','2','3','4','5'});

    c.Label.String = 'cluster labels';

    colormap(mymap)

    %file_name = erase(file_name,".jpg")

    print('-dtiff', '-r150', [ file_path '\Processed\' erase(file_name,".jpg")

'_clustered.jpg'])

    else

    colorbar ()
```

```
        colormap(lines(k)) %incease of

      end

      close all

    end

  for clus = 1:kn;

    report_cluster_count(file,clus+1)= string(numel(clus_map(find(clus_map ==
clus))));

  end

  imwrite(clus_map,colormap(mymap),[ file_path '\Processed\'
erase(file_name,".jpg") '_clustered_nobar.jpg']);

end
```
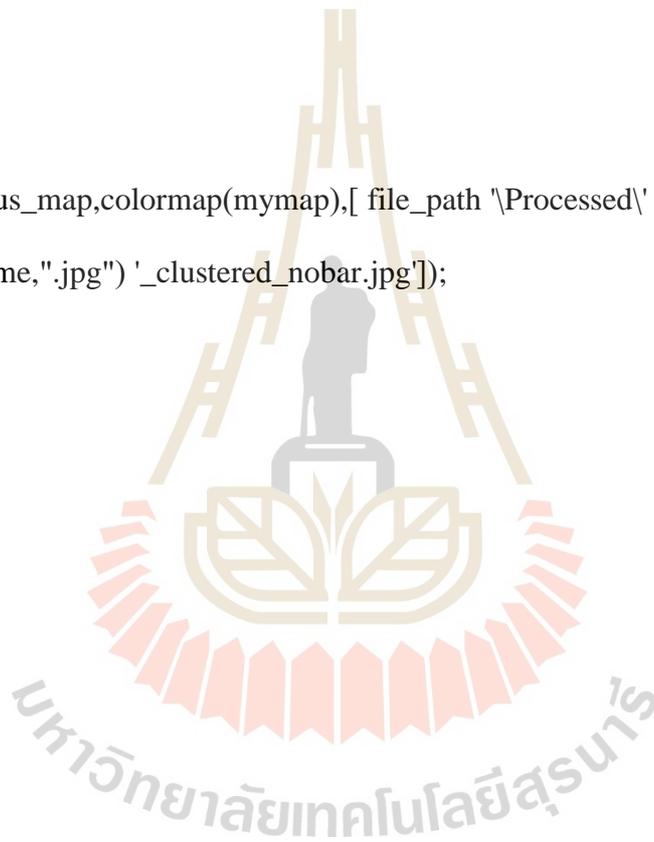
CHAPTER V

EFFECTS OF *HELIOTROPIUM INDICUM* L. EXTRACT ON MAMMARY GLAND IN POSTPARTUM RATS

5.1 Abstract

Breastfeeding is understood to move up growth in children, mainly abundant in terms of nutrients, protein, energy, water, and other. Many researches have exhibited the advantage of breastfeeding to both mother and baby. Worldwide problems about breastfeeding include quality and quantity that resulted in studying the effect of medicinal plant for solving problems. The whole plant of *Heliotropium indicum* L. (*H. indicum*) is used for traditional medicine in many parts of the world. Consequently, the use of natural products like the medicinal plants could be an option to relieve the lactation disturbances particularly lactation deficiency. Moreover, the effects of *H. indicum* extract on milk production have no reported. Therefore, the aim was to study the effects of ethanol extract of *H. indicum* on mammary gland in postpartum rats. The whole plant was dried and powdered. The powder of the whole plant was extracted by macerated with 70% ethanol and shaken for 3 days. The mixed were filtered through filter paper to remove particulates. The extract was evaporated under reduced pressure at a low temperature in a rotary evaporator, dried by using lyophilizer and stored at -20 °C until use. The 60 pregnant rats were used. The rats were divided into 2 main groups; non-treated and treated rats. Two main groups have 3 subgroups containing; 1) 1 day-postpartum

rats 2) 3 day-postpartum rats 3) 5 day-postpartum rats. The extract of *H. indicum* were diluted to 250 mg/kg B.W. in the vehicle (Tween 80, 10% v/v) and were administered orally to the treated rats at a volume of 0.8 mL/day for day 1, 3, and 5 postpartum. Only the vehicle was administered orally to the residue group. After administered orally, the mammary gland tissues were collected for analyses the alveoli size, histological structures of the mammary glands by ImageJ, and biochemical components of the mammary glands by Fourier Transform Infrared spectroscopy (FTIR). The results showed *H. indicum* extract can help increase alveoli size and the parenchyma cells involved for increases in milk production. In the part of FTIR analysis, the results showed the whole plant of *H. indicum* extract helps increase lipid and protein in mammary gland tissue which relates to lipid and protein synthesis in mammary epithelial cells. Therefore, the whole plant of *H. indicum* extract can be consumed during postpartum period to induce milk production in rats. This results also confirm the traditional use of the plant in the lactation insufficiency.

5.2 Introduction

The mammary gland is structurally and biologically created to deliver milk for descendants. The mammary gland consists of mammary epithelium, adipose tissue, and connective tissue stroma. (Kaye et al., 1995). The mammary gland histology shows the multiple cell types inside complex tissue. The mammary gland can be divided into two parts: the epithelial (or parenchymal) and the stromal (or mesenchymal). The parenchymal part consisted of distinct epithelial forms with clear morphologies and functional and proliferative activities, including the luminal epithelium of ducts, alveolar buds, and alveoli, as well as the underlying myoepithelial layer (Masso-Welch et al., 2000). The epithelium whose apical surface contacts the lumen of ducts, ductules,

and alveoli called the luminal epithelium. The form of the luminal epithelial cells scope from cuboidal or columnar in ducts to pyramidal (before secretion) or flattened (after secretion) in alveoli. The milk synthesis and secretion during lactation occurred in the alveolar luminal epithelium that functionally differentiated and secretory epithelium of the mammary gland. The myoepithelium compose of the basal epithelial cells that surround the luminal epithelium of ducts and alveoli. Moreover, the myoepithelial cells form a dense and continuous layer at the base of ducts. In pregnancy and lactation period, the myoepithelial cells enclosing alveoli and contract the alveolus during breastfeeding. During early involution, the regressing alveoli were surrounded by the myoepithelial cells form a more continuous thick sheet. The myoepithelium can be restrained at the place of completely regressed alveoli still at late time points after weaning, unrelated the luminal alveolar epithelium (Emerman and Vogl, 1986).

In humans, the mammary gland is a single pair located the superficial fascia of the anterior thoracic wall overhead the pectoralis major and spreading upwards into the axilla. The nipple was developed from milk buds and spread into the mammary lipid pad. In humans, the nipple was extended by many ducts that segmentally branching structure 15-20 separate lobes. Each lobe is emptied by its own lactiferous duct guiding to the nipple. Moreover, these lobes are isolated from one another by changeable number of adipose tissues. During human puberty, ductal is extended toward the mammary fat pad. The smooth muscle acts as contractile tissue that involved in delivery milk from the nipple. The humans have numerous major lactiferous ducts but the mouse nipple has a single major lactiferous duct. The ductal system of mouse was surrounded primarily by adipose tissue. Moreover, the ductal system of human was surrounded by fibrovascular and adipose tissue. In human pregnancy, the stroma becomes more

cellular and there is an associated decline in adipose tissue. In lactation, luminal alveolar cells generate large fat vacuoles and Golgi vesicles composed of lactose, protein, and water. Cessation of lactation causes involution with declined totals and size of glandular units and return to a non-secretory state. The decreased of glandular tissue and the expanded adipose tissue occur during menopause (Treuting and Dintzis, 2012). The rat mammary glands form extensive subcutaneous sheets of tissue that spread from the cervical to the inguinal regions as six gland pairs, each with its own nipple. The gland has a chain of lymph nodes and it's easy to dissect. The rat mammary gland epithelium includes the branching ducts come from the nipple and leading to smaller ducts, called ductules. The epithelium is planted in a sheath of stroma proximal to the nipple and surrounded by adipose tissue, depending on the developmental stage. The connective tissue capsule is spread around the fat pad (Masso-Welch et al., 2000).

The non-pregnant rats show the mammary gland had full adipose connective tissue with some glandular part. During pregnancy, the alveoli became enlarge. The milk was increased and the sac formed during lactation. The non-pregnant resting rat, the mammary gland H&E-stained sections revealed a group of folded ducts and full adipose connective tissue stroma (Abunasef and El-Beshbishy, 2014). The rat mammary gland changes during lactation and consists of a network of ducts are formed by epithelial cells that are the locate of milk secretion (McManaman and Neville, 2003). Lactating alveolar cells have synthetic ability for lipid and unique mechanism for lipid excretion (Mather and Keenan, 1998). The function to restrain paracellular relocate of substances between vascular and milk partitions during lactation were controlled by the epithelial cells that linked to each other via an apical junctional complex constituted of adherens- and tight-junctional portions (Nguyen et al., 2001). The luminal epithelium

is complete of basophilic secretions and casein micelles about lipid globules. The alveolar epithelium has a spongy feature due to intracellular lipid and osmotic swelling of lactose-containing secretory vesicles. The expulsion of the milk into the lumen of the excretory alveoli is constitutive. The apical face during secretion is the locate that milk fat globule becoming covered in plasma membrane. The milk fat globule can relate to a small sum of cytoplasm, which contain membrane-bound Golgi-derived secretory vesicles compose of lactose and milk proteins. The myoepithelial cells show the contraction of smooth muscle actin-rich dendritic processes for feedback to oxytocin causes blank of the alveolar lumina into the draining ductules and causes the apical face of the alveolar luminal epithelium to bulge into the alveolar lumen. The oxytocin receptor expression in myoepithelial cells effect on differences in the contractility state of near alveoli. The presentation of the gland continues similar during the remnant of the normal lactation cycle (21 days postpartum). The luminal epithelium has the duct function that believed to be as a channel for milk during lactation. Myoepithelial contraction at the base of ducts show shortening and widening of the ducts for decrease resistance to milk flow (Masso-Welch et al., 2000).

Involution is the loss of the excretory alveolar epithelium after weaning. It shows decreasing levels of circulating prolactin on the pause of suckling, parameters in milk that push the cell death and dilation of the luminal epithelium cells Moreover, involution of mammary gland shows increase activity of basement membrane-degrading enzymes. The weaning after 21 days of lactation is the involution time. At day 2 of involution, the histological mammary gland is still overshadowed by secretory epithelium. The apoptosis cause cell death occurs in involution period. Moreover, the

alveoli are also widened and packed with lipid and a basophilic substance, and infrequency cellular fragments (Masso-Welch et al., 2000).

The rat is a generally used example for various types of biochemical, physiological, and nutritional research. The component of rat milk, the values were constant during the 1st week of lactation, lead to expand during mid-lactation and start to lower during late lactation (Lonnerdal et al., 1976). The human milk fat constituent not significant change depend on the stage of lactation has been reported. The rat milk, the fat was influenced by the intake of the dam; low fat concentration in rat milk was reported with high zinc diets and relatively high fat concentration with low protein diets. The milk fat not depend on the constricted food intake (Mueller and Cox, 1946). Female mammals excreted milk fluid for give the rich nourishment to their children in the primary days of life. Milk is a mixture of adulterate salts, a common sugar and vitamins, in which fat is globules emulsifier, and which carry a protein. Moreover, the milk composed of the most of colloidal aggregates of thousands of molecules such as casein micelles, a greatness smaller than the fat globules (Kelly and Larsen, 2010).

The milk secretion, the milk protein gene expression and biosynthetic processes were occurred after the progesterone fall in parturition period, when removal of the placenta. The other hormones like cortisol and prolactin are present in suitable concentration if a decrease in progesterone is to encourage excretory stimulation (Nguyen et al., 2001). After the weaning, the proclaimed decrease in inorganic P and lactose were adversely related with an expand in all various milk components not include fat. The study reported the continuous transforms in the major milk components in rats during the first 20 days of feeding and for 3 days following weaning at 20 days postpartum (Nicholas and Hartmanna, 1991). Lactose is the major component in milk

(Kelly and Larsen, 2010). The concentration of lactose continued relatively unchanging at proximately 73 mM during the first 5 days of feeding and then expanded to a highest value of 126 mM at 15 days (Nicholas and Hartmanna, 1991). Casein is the major of milk protein. The thousands of molecules of each casein were carried by micelles (Kelly and Larsen, 2010). The research suggest that the total protein was slightly reduced from 8 to 6 g/ 100 ml during the first 2 days after birth, then a creeping enlarges to persistent levels of 7- 9 g/ 100ml for the remainder of lactation. The increasing of casein concentration was shown at birth to a highest at day 20 of lactation. Whereas, the serum was increased in the first 2 days after birth but unchanging for the continuing 18 days. The concentration of transferrin reduced at the day one of lactation, remained stable until day 10 and then rise to a highest concentration on day 20 (Nicholas and Hartmanna, 1991).

Milk fat is the amplest substance in milk. Triglycerides is the main component of milk fat. Moreover, it's few types of fatty acids in milk, varying in the form of the chain of carbon atoms and numbers of double bonds. Milk fat globules in the milk show diameters fluctuating from 0.1 to more than 10 mm (Kelly and Larsen, 2010). In milk, the concentration of fat (triacylglycerols) was decreased in the first 5 days postpartum and then unchanging for the continuing 15 days (Nicholas and Hartmanna, 1991). Other studies indicated that the concentration of milk fat increased in the first 10 days of breastfeeding (Chalk and Bailey, 1979). In milk, the concentration of casein was increased slowly from proximately 30% of the total protein at term to 50% at day 20 of breastfeeding (Nicholas and Hartmanna, 1991). Mineral salts are a component of milk, some of which are related with casein micelles (Kelly and Larsen, 2010).

Breastfeeding is understood to move up growth in children, mainly abundant in terms of power, nutrients, water, protein, and other. Many researches have exhibited the utilities of breastfeeding to both mother and baby. The exclusive breastfeeding up to six months of age were suggested by the World Health Organization (WHO) (WHO, 2009). The newborn and sixth month childhood have only 40% worldwide are breastfed exclusively (WHO, 2020). The baby who have been breastfed optimally have a decreased the probability of common childhood sickness for example gastrointestinal and respiratory infections (Story and Parish, 2008). Moreover, the breastfeeding is related with standard growth model in infants and protection averse to obesity. Therefore, infants breastfed exclusively for six months have lower the chance of progressing obesity compared with infants exclusively breastfed for one month (Kalies et al., 2005).

From the worldwide problems about breastfeeding include quality and quantity, many studies investigate the effect of medicinal plant for solving the problems. The study in a plant product compose of green tea and curcuma extract suggest that the cows receiving the plant product showed an inclination towards a raised milk yield. Moreover, the group of cows receiving the plant product have daily totals of milk proteins and milk fats higher than the group not receiving the plant product. The green tea or curcuma showed the same of polyphenols, it's remains unknown, the constituents accountable for their effects in this study remain mysterious (Winkler et al., 2015). Supplementation of black seed and ginger powder to ewes' diet may have an optimistic effect on milk fat, they can be used to enhance milk quality. The ingredients of herbs are not clear, they have the most effect either from the current study (Hendawy et al., 2019).

The whole plant of *Heliotropium indicum* L. (*H. indicum*) is used for healing herpes. Moreover, the juice of the bark is used orally by women for healing dysmenorrhea and the warm aqueous extract of the flower and buds is taken orally by the women as an emmenagogue in a small dose and abortive in an expanded treatment in West Indies (Ghosh et al., 2018). The dehydrated and powdered inflorescence of *H. indicum* were blended with milk or water and is used taken orally at the time of menses to get the desired result (Panthong et al., 1986). From literature reviews, they show that *H. indicum* is extensively used in treating diseases as a traditional medicine. The women with lactation failure or lactation deficiency always use the pharmaceutical galactogogues or lactogogues which are property that influence, maintain and increase breast milk production. Unfortunately, the greater of these drugs cause some side effects in mother and in infants (Sultana et al., 2013). Consequently, the use of natural products like the medicinal plants could be an option to relieve the lactation disturbances particularly lactation deficiency. The effects of *H. indicum* extract on milk production have no reported. Therefore, the aim of study was to examine the effects *H. indicum* extract on mammary glands in postpartum rats. The results from this study can be used as scientific data to support their effects on milk production for helping the worldwide problems about breastfeeding including quality and quantity of milk. Moreover, the results from this study are the basis knowledge for further research to develop drugs or products for maternal care.

5.3 Methodology

5.3.1 Experimental animals

Female Wistar rats (200-250 g) were used in this study and maintained in accordance with the guidelines of the Committee on Care and Use of Laboratory Animal Resources, National Research Council, Thailand. The experiments performed on rats were conducted in accordance with the advice of the Institutional Animal Care and Use Committee, Suranaree University of Technology. Female Wistar rats were maintained under environmentally controlled room provided with a 12:12 light and dark cycle at a temperature of approximately 25°C. They were fed with commercial food (C.P. Mice feed, Bangkok, Thailand) and allowed to access water *ad libitum*. Used 60 female Wistar rats for this study. More details relevant to experimental animals are given in Chapter IV. In this chapter, all of experiment used sample from the same rats in Chapter IV.

5.3.2 Preparation of plant materials

The whole plant was dried and powdered. The powder of the whole plant was extracted by macerated with 70% ethanol and shaken for 3 days. The mixed were filtered through filter paper to remove particulates. The extract was evaporated by a rotary evaporator, dried by using lyophilizer and stored at -20°C until use. More details relevant to preparation of plant materials are given in Chapter III.

5.3.3 Alveoli size determining

60 pregnant rats were used. The rats were divided into 2 main groups: non-treated rats and treated rats. Two main groups have 3 subgroups containing: 1) 1 day-postpartum rats 2) 3 day-postpartum rats 3) 5 day-postpartum rats. The extract of *H. indicum* were diluted to 250 mg/kg B.W. in the vehicle (Tween 80, 10% v/v) and were administered

orally to the treated rats at a volume of 0.8 mL/day for day 1, 3, and 5 postpartum. Only the vehicle was administered orally to the residue group. After administered orally, the mammary gland tissues were collected. The mammary gland tissues were fixed in 10% buffered formalin then processed in a tissue processor and embedded in paraffin using routine methods. Representative transverse sections were cut and stained with Hematoxylin and Eosin. The mammary gland sectional thickness is 6 µm. Histological examination of the slides was performed using a light microscope. The mammary gland picture from light microscope was calculated for alveoli size by CellD program.

5.3.4 Histological structures

60 pregnant rats were used. The rats were divided into 2 main groups: non-treated rats and treated rats. Two main groups have 3 subgroups containing: 1) 1 day-postpartum rats 2) 3 day-postpartum rats 3) 5 day-postpartum rats. The extract of *H. indicum* were diluted to 250 mg/kg B.W. in the vehicle (Tween 80, 10% v/v) and were administered orally to the treated rats at a volume of 0.8 mL/day for day 1, 3, and 5 postpartum. Only the vehicle was administered orally to the residue group. After administered orally, the mammary gland tissues were collected. The mammary gland tissues were fixed in 10% buffered formalin then processed in a tissue processor and embedded in paraffin using routine methods. Representative transverse sections were cut and stained with Hematoxylin and Eosin. The mammary gland sectional thickness is 6 µm. Histological examination of the slides was performed using a light microscope. The mammary gland picture from light microscope was calculated for the ratio of parenchyma cells and stroma cells in mammary gland determining by image processing using ImageJ (Figure 5.1).

Figure 5.1 Flow chart of the experimental procedure adapted from Chen et al., 2017.

5.3.5 Biochemical components in the mammary glands determining by Fourier Transform Infrared spectroscopy (FTIR)

60 pregnant rats were used. The rats were divided into 2 main groups: non-treated rats and treated rats. Two main groups have 3 subgroups containing: 1) 1 day-postpartum rats 2) 3 day-postpartum rats 3) 5 day-postpartum rats. The extract of *H. indicum* were diluted to 250 mg/kg B.W. in the vehicle (Tween 80, 10% v/v) and were administered orally to the treated rats at a volume of 0.8 mL/day for day 1, 3, and 5 postpartum. Only the vehicle was administered orally to the residue group. After administered orally, the mammary gland tissues were collected. The mammary gland tissue arrangement for FTIR microspectroscopy analysis. The mammary gland tissue was embedded in OCT compound (Tissue-Trek, Electron Microscopy Sciences, Hatfield, PA), and snap-

frozen in liquid N_2. Then, the samples were transferred to a $-80°C$ freezer and stored until cryo-sectioning. After that, a cryostat (Leica 3050 S, Germany) used to cut the sample and put the sample on infrared transparent BaF_2 windows (13×2 mm) for infrared micro-spectroscopy. The mammary gland sectional thickness was 7 µm.

Fourier Transform Infrared spectroscopy (FTIR) is a technique use for the analysis of biological samples like tissue by detecting changes in macromolecular composition occurring during the mammary gland in postpartum period such as protein, nucleic acid, and lipid. In mammary gland were used FTIR for check milk composition such as protein and lipid. Moreover, the effects of *H. indicum* extract on biochemical component of uterus and mammary gland in postpartum rat were analyzed by FTIR. The spectra data from FTIR microspectroscopy were collected at an infrared microspectroscopy beamline (BL4.1 IR Spectroscopy and Imaging) at the Synchrotron Light Research Institute (SLRI). Spectra were received with a Vertex 70 FTIR spectrometer (Bruker Optics, Ettlingen, Germany) coupled with an IR microscope (Hyperion 2000, Bruker) with an MCT detector cooled with liquid nitrogen over the measurement range from 4000 to 800 cm^{-1}. The microscope was linked to a software-controlled microscope stage and placed in a specially designed box that were purged by dry air. The measurements were performed in the mapping mode, using an aperture size of 10×10 µm with a spectral resolution of 4 cm^{-1}, with 64 scans co-added. Spectral acquisition and instrument control were performed using OPUS 7.2 (Bruker Optics Ltd, Ettlingen, Germany) software and analyzed by The Unscrambler X software (Thumanu et al., 2017). The spectra were smoothed for erase the noise by Savitsky-Golay smooth function. The spectra were baseline corrected and were normalized with respect to specific bands for visual demonstration. Band areas from mammary gland were

analyzed from smoothed and baseline corrected spectra using OPUS software.

5.3.6 Statistical analysis

All data were expressed the mean $\pm$ S.E.M. and n refers to the number of animals. For paired two groups of data were analyzed by Independent-samples t-test. For three or more groups, data were analyzed by one-way ANOVA and post-hoc with Tukey's test. The Statistical Package for the Social Sciences (SPSS) were employed for all statistical analysis. The significance level was determined at $P < 0.05$.

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┌─────────────────────────┐
│   Experimental design   │
└─────────────────────────┘
            │
      ┌───────────┐
      │  In vivo  │
      └───────────┘
            │
   ┌────────┴────────┐
   ▼                 ▼
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Postpartum rats	Postpartum rats
• Day 1	• Day 1
• Day 3	• Day 3
• Day 5	• Day 5

Receive 250 mg/kg B.W. of the *H. indicum* extract	Receive the vehicle

- **Alveoli size**

The mammary gland tissues were fixed in 10% buffered formalin then representative transverse sections were cut and stained with Hematoxylin and Eosin. Histological investigation of the samples was achieved using a light microscope. The mammary gland picture from light microscope was calculated for alveoli size by CellD program.

- **Histological structures of the mammary glands in postpartum rats by ImageJ**

The mammary gland tissues were fixed in 10% buffered formalin then representative transverse sections were cut and stained with Hematoxylin and Eosin. The mammary gland picture from light microscope was calculated for the ratio of parenchyma cell and stroma cell in mammary gland determining by image processing using ImageJ.
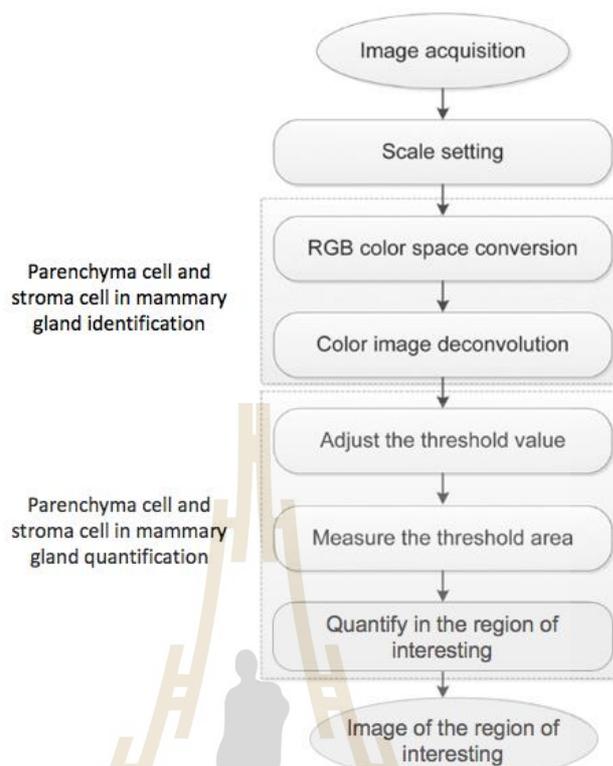
- **Biochemical components of the mammary glands in postpartum rats by Fourier Transform Infrared spectroscopy (FTIR)**

The sample preparation for FTIR microspectroscopy analysis, tissues were embedded in OCT compound and snap-frozen in liquid N_2. Then, the samples were cut by technique cryo-sectioning. After that, the sample were put on infrared transparent BaF_2 windows for FTIR analyzed in beamline 4.1 IR Spectroscopy and Imaging at the Synchrotron Light Research Institute (SLRI).

Figure 5.2 Summary of methodology.

5.4 Results

5.4.1 Effects of *H. indicum* extract on alveoli size

The alveoli size was observed on day 1, 3, and 5 postpartum. When compared within group, the results showed that the alveoli size was significantly increased non-treated rats on day 1 postpartum to day 5 postpartum (Table 5.1). The alveoli size of treated rats received the *H. indicum* extract was not significantly different from day 1 to day 5 postpartum (Table 5.1). The alveoli size between groups at the same day of postpartum was compared. On day 1 postpartum, the results showed the alveoli size of treated rats were significantly increased, compared non-treated rats. The alveoli size of both groups was similar on day 3 and 5 postpartum (Figure 5.3). The result suggested that *H. indicum* extract can help increase alveoli size on day 1 postpartum. Moreover, the extract can stabilize the alveoli size until day 5 postpartum.

Table 5.1 Effect of *H. indicum* extract on alveoli size.

Group	Alveoli size (µm) (Mean ± S.E.M.)
Non-treated rats	
Day 1 postpartum	73.00 ± 3.28^a
Day 3 postpartum	78.08 ± 3.47^{ab}
Day 5 postpartum	91.46 ± 3.63^b
Treated rats	
Day 1 postpartum	99.49 ± 3.70^a
Day 3 postpartum	85.25 ± 6.27^a
Day 5 postpartum	88.70 ± 4.63^a

All values are expressed as mean + S.E.M. of 10 rats in each group (n = 10). When compare within group, means with different superscripted letters indicate statistical significance (P < 0.05).

Figure 5.3 A graph showing the effects of *H. indicum* extract on alveoli size. The alveoli size between groups at the same day of postpartum are compared. The results show that the alveoli size of treated rats is significantly increased, compared non-treated rats on day 1 postpartum. Moreover, both groups are similar on day 3 and 5 postpartum. Bars represent mean ± S.E.M. Values that do not share the same superscript letters are significantly different (P < 0.05).

5.4.2 Effects of *H. indicum* extract on histological structures of the mammary glands by ImageJ

The mammary gland on day 1, 3, and 5 postpartum were calculated the ratio of parenchyma cells and stroma cells determining by image processing using ImageJ. When compared within group, the results showed that the ratio of parenchyma cells and stroma cells in mammary gland were similar in non-treated rats on day 1 postpartum to

day 5 postpartum (Table 5.2). In treated rats received the *H. indicum* extract, the result showed that the ratio of parenchyma cells and stroma cells in mammary gland was not significantly different from day 1 to day 3 postpartum then significantly increased on day 5 postpartum (Table 5. 2) . The ratio of parenchyma cells and stroma cells in mammary gland between groups at the same day of postpartum were compared. On day 1 postpartum, the results showed the ratio of parenchyma cells and stroma cells in mammary gland of both groups were not significantly different. .On day 3 postpartum, treated rats showed significant decreases in the ratio of parenchyma cell and stroma cell in mammary gland. On day 5 postpartum, treated rats were received the extract showed significant increases in the ratio of parenchyma cell and stroma cell in mammary gland (Figure 5.4). The result suggested that *H. indicum* extract can help increase the ratio of parenchyma cells and stroma cells in mammary gland on day 5 postpartum.
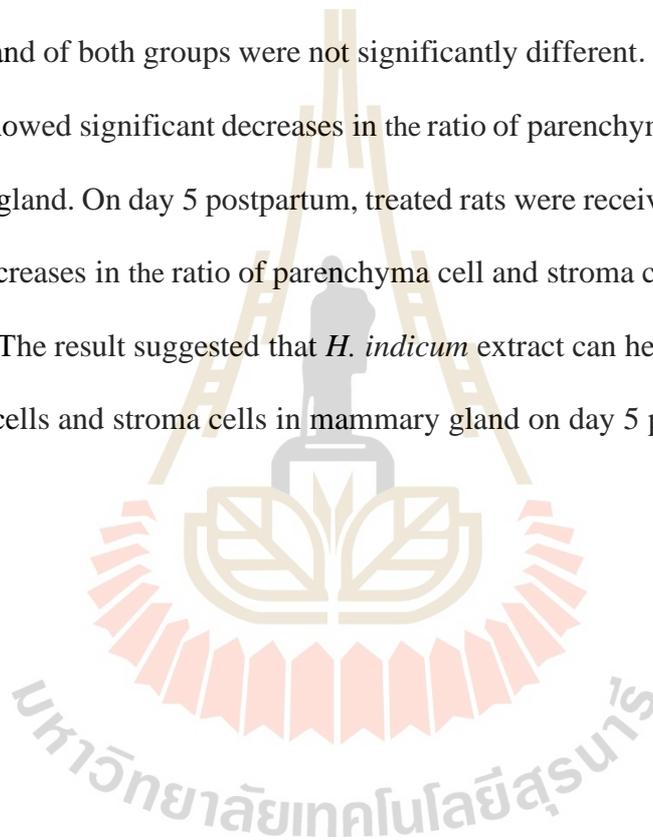
Table 5.2 Effect of *H. indicum* extract on the ratio of parenchyma cells and stroma cells in mammary gland.

Group	The ratio of parenchyma cells and stroma cells (Mean ± S.E.M.)
Non-treated rats	
Day 1 postpartum	1.02 ± 0.07^a
Day 3 postpartum	0.95 ± 0.04^a
Day 5 postpartum	1.10 ± 0.08^a
Treated rats	
Day 1 postpartum	0.88 ± 0.08^a
Day 3 postpartum	0.71 ± 0.04^a
Day 5 postpartum	1.78 ± 0.06^b

All values are expressed as mean + S.E.M. of 10 rats in each group ($n = 10$). When compare within group, means with different superscripted letters indicate statistical significance ($P < 0.05$).
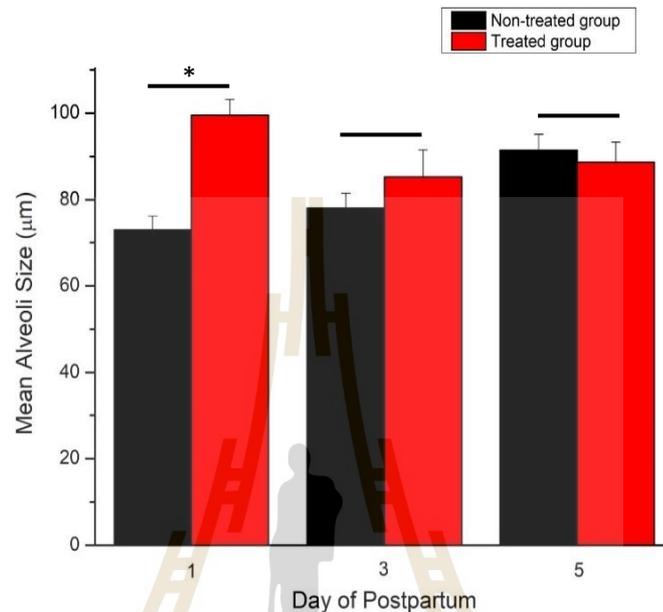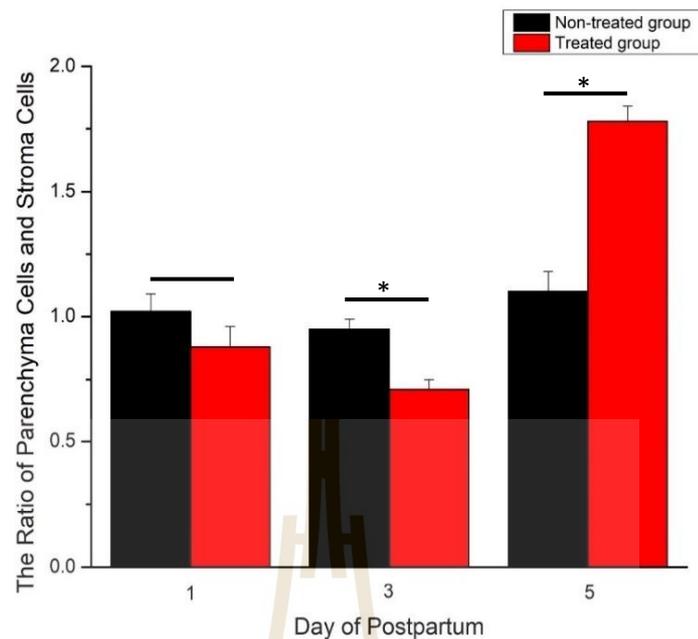
Figure 5.4 A graph showing the effects of *H. indicum* extract on the ratio of parenchyma cells and stroma cells in mammary gland. The ratio of parenchyma cells and stroma cells in mammary gland between groups at the same day of postpartum are compared. On day 1 postpartum, the results show the ratio of parenchyma cells and stroma cells in mammary gland of both groups are not significantly different. On day 3 postpartum, treated rats show significant decreases in the ratio of parenchyma cells and stroma cells in mammary gland. On day 5 postpartum, treated rats received the extract show significant increases in the ratio of parenchyma cells and stroma cells in mammary gland. Bars represent mean $\pm$ S.E.M. Values that do not share the same superscript letters are significantly different ($P < 0.05$).

5.4.3 Effects of *H. indicum* extract on biochemical components of the mammary glands determining by Fourier Transform Infrared spectroscopy (FTIR).

Fourier Transform Infrared spectroscopy (FTIR) was used to identify and analysis the biological samples like mammary gland tissue by detecting changes in macromolecular composition occurring during postpartum periods based on the peak values in the IR region. The main bands were observed and detailed band assignments are given in Table 5.3. FTIR absorption spectrum of the sample is showed distinct areas of the lipid region (3096-2811 cm^{-1}), the (C=O) triglycerides region (1749-1732 cm^{-1}), the protein region (1600-1276 cm^{-1}), carbohydrate, and nucleic acid region (1257-950 cm^{-1}) were investigated. The band area was changed when compared within group, showed the changing in macromolecular composition during postpartum period.
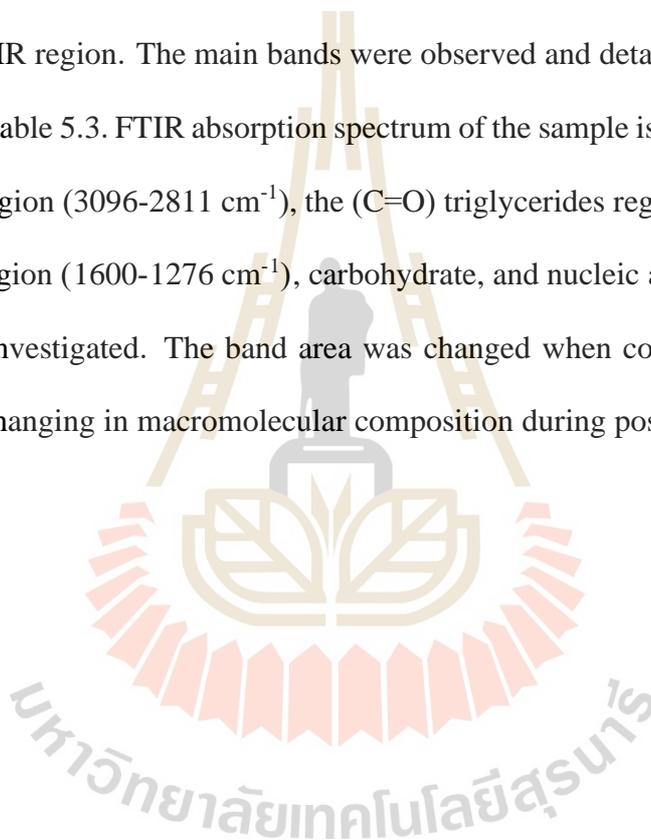
Table 5.3 General band assignment of FTIR spectrum of uterine tissue based on literature (Bozkurt et al., 2007, Movasaghi et al., 2008, Taylor et al., 2011).

Band No.	Spectrum (cm^{-1})	Description
1	3096-3033	C-H ring unsaturated lipid
2	3019-2811	Lipid, cholesterol esters
3	1749-1732	(C=O) triglycerides, cholesterol esters, phospholipids
4	1682-1633	Amide I protein C=O stretching
5	1559-1508	Amide II (protein N–H bend, C–N stretch)
6	1471-1370	CH$_2$ bending: mainly lipids, with the little contribution from proteins
7	1350-1276	Amide III vibrations of collagen, N-H thymine
8	1257-1221, 1097-1072	PO$^-$2 asymmetric stretching, fully hydrogen-bonded: mainly nucleic acids with the little contribution from phospholipids, C-O-C, C-O dominated by the ring vibrations of polysaccharides C-O-P, P-O-P
9	1177-1112	CO-O-C asymmetric stretching: glycogen and nucleic acids
10	1062-949	PO$^-$2 symmetric stretching: nucleic acids and phospholipids, C–O stretch: glycogen, polysaccharides, glycolipids, C–N+–C stretch: nucleic acids, ribose-phosphate main chain vibrations of RNA

When compared within group of non-treated rats, the result has been shown in Table 5.4. Band No. 1 (unsaturated lipid) were significantly increased on day 3 postpartum, compared day 1 postpartum and significantly decreased on day 5 postpartum, compared day 3 postpartum. Band No. 2 (lipid, cholesterol esters) and Band No. 3 ((C=O) triglycerides) were significantly decreased on day 1, 3, and 5 postpartum. Band No. 4 (Amide I protein) and Band No. 5 (Amide II protein) were significantly increased on day 3 postpartum, compared day 1 postpartum and significantly decreased on day 5 postpartum, compared day 3 postpartum. Band No. 6 (CH_2 bending: lipids and proteins) were significantly decreased on day 3 and 5 postpartum, compared day 1 postpartum. Band No. 7 (Amide III vibrations of collagen) were significantly decreased on day 5 postpartum, compared day 1 and 3 postpartum. Band No. 8 (PO^-2 asymmetric stretching nucleic acids and carbohydrate) and Band No. 10 (PO^-2 symmetric stretching: nucleic acids and carbohydrate) were unchanged in postpartum periods. Band No. 9 (CO-O-C asymmetric stretching: glycogen and nucleic acids) were significantly decreased on day 3 postpartum, compared day 1 and 5 postpartum.

Table 5.4 Changes in area values of the infrared bands for non-treated rats. Compared within group.

Non-treated	Postpartum day		
Band No.	Day 1 (Mean±SEM)	Day 3(Mean±SEM)	Day 5(Mean±SEM)
1	0.000172 ± 0.000012[a]	0.000440 ± 0.000042[c]	0.000321 ± 0.000019[b]
2	0.128888 ± 0.001640[a]	0.105155 ± 0.001753[b]	0.086119 ± 0.001005[c]
3	0.050728 ± 0.001832[a]	0.042423 ± 0.002045[b]	0.035131 ± 0.002913[c]
4	0.006948 ± 0.001740[a]	0.017556 ± 0.001108[c]	0.013758 ± 0.001159[b]
5	0.004608 ± 0.001579[a]	0.010214 ± 0.001422[c]	0.007595 ± 0.001258[b]
6	0.014819 ± 0.001315[a]	0.012268 ± 0.000473[b]	0.010412 ± 0.000472[b]
7	0.001239 ± 0.000082[a]	0.001154 ± 0.000118[a]	0.000459 ± 0.000045[b]
8	0.004637 ± 0.001005[a]	0.005176 ± 0.000888[a]	0.005581 ± 0.001090[a]
9	0.010319 ± 0.000571[a]	0.007488 ± 0.000322[b]	0.011149 ± 0.000707[a]
10	0.007394 ± 0.000284[a]	0.005205 ± 0.000630[a]	0.005718 ± 0.000558[a]

All values are expressed as mean + S.E.M. of 10 rats in each group ($n = 10$). When compare within group, means with different superscripted letters indicate statistical significance ($P < 0.05$).

When compared within group of treated rats, the result are shown in Table 5.5. Treated rats, they were received the extracts showed Band No. 1 (unsaturated lipid) that were significantly decreased on day 5 postpartum, compared day 1 and 3 postpartum. Band No. 2 (lipid, cholesterol esters) and Band No. 3 ((C=O) triglycerides) were significantly decreased on day 1, 3, and 5 postpartum. Band No. 4 (Amide I protein) and Band No. 5 (Amide II protein) were unchanged in postpartum periods. Band No. 6 (CH$_2$ bending: lipids and proteins) and Band No. 7 (Amide III vibrations

of collagen) were significantly decreased on day 1, 3, and 5 postpartum. Band No. 8 (PO−2 asymmetric stretching nucleic acids and carbohydrate) were significantly decreased on day 5 postpartum, compared day 3 postpartum. Band No. 9 (CO-O-C asymmetric stretching: glycogen and nucleic acids) were significantly decreased on day 5 postpartum, compared day 1 and 3 postpartum. Band No. 10 (PO−2 symmetric stretching: nucleic acids and carbohydrate) were significantly decreased on day 3 and 5 postpartum, compared day 1 postpartum.

Table 5.5 Changes in area values of the infrared bands for treated rats. Compared within group.

Treated	Postpartum day		
Band No.	Day 1 (Mean±SEM)	Day 3(Mean±SEM)	Day 5(Mean±SEM)
1	0.000431 ± 0.000022^a	0.000426 ± 0.000026^a	0.000266 ± 0.000030^b
2	0.124587 ± 0.003990^a	0.105685 ± 0.004700^b	0.086386 ± 0.004510^c
3	0.046000 ± 0.002198^a	0.040994 ± 0.002316^b	0.031945 ± 0.002512^c
4	0.016499 ± 0.000463^a	0.016490 ± 0.000418^a	0.015848 ± 0.001409^a
5	0.010740 ± 0.000504^a	0.011698 ± 0.000292^a	0.010438 ± 0.000547^a
6	0.013581 ± 0.000356^a	0.012708 ± 0.000930^b	0.010208 ± 0.000338^c
7	0.000929 ± 0.000049^a	0.000744 ± 0.000055^b	0.000545 ± 0.000040^c
8	$0.005096 \pm 0.000939^{ab}$	0.005602 ± 0.000327^a	0.004516 ± 0.000641^b
9	0.008462 ± 0.001092^a	0.007696 ± 0.000728^a	0.006085 ± 0.001008^b
10	0.003465 ± 0.000219^a	0.002534 ± 0.000216^b	0.002410 ± 0.000153^b

All values are expressed as mean + S.E.M. of 10 rats in each group ($n = 10$). When compare within group, means with different superscripted letters indicate statistical significance ($P < 0.05$).

When compared the band area of the biochemical components in the mammary gland between groups at the same day of postpartum the results are shown in Figure 5.5. Band No. 1 (unsaturated lipid) of treated rats were significantly increased on day 1 postpartum, compared non-treated rats. However, on day 3 and 5 postpartum they were similar. Band No. 2 (lipid, cholesterol esters) of both groups were similar in postpartum period. Treated rats, the Band No. 3 ((C= O) triglycerides) were significantly decreased on day 1 and 5 postpartum when compare with non-treated rats. Band No. 4 (Amide I protein) in treated rats were significantly increased on day 1 postpartum, compared non-treated rats. Then, day 3 and 5 postpartum were similar. Band No. 5 (Amide II protein) of treated rats were significantly increased on day 1, 3, and 5 postpartum, compared non-treated rats. Band No. 6 (CH$_2$ bending: lipids and proteins) in treated rats were significantly decreased on day 1 postpartum, compare non-treated rats. Then, day 3 and 5 postpartum showed the same area. On day 1 and 3 postpartum, Band No. 7 (Amide III vibrations of collagen) of treated rats were significantly decreased when compared non-treated rats. Both of groups were similar on day 5 postpartum. Moreover, Band No. 8 (PO−2 asymmetric stretching nucleic acids and carbohydrate) of both groups were similar in postpartum period. Band No. 9 (CO-O- C asymmetric stretching: glycogen and nucleic acids) of treated rats were significantly decreased compare non-treated rats on day 1 and 5 postpartum. Finally, Band No. 10 (PO−2 symmetric stretching: nucleic acids and carbohydrate) of treated rats were significantly decreased on day 1, 3, and 5 postpartum, compared non-treated rats.

A.

B.

C.
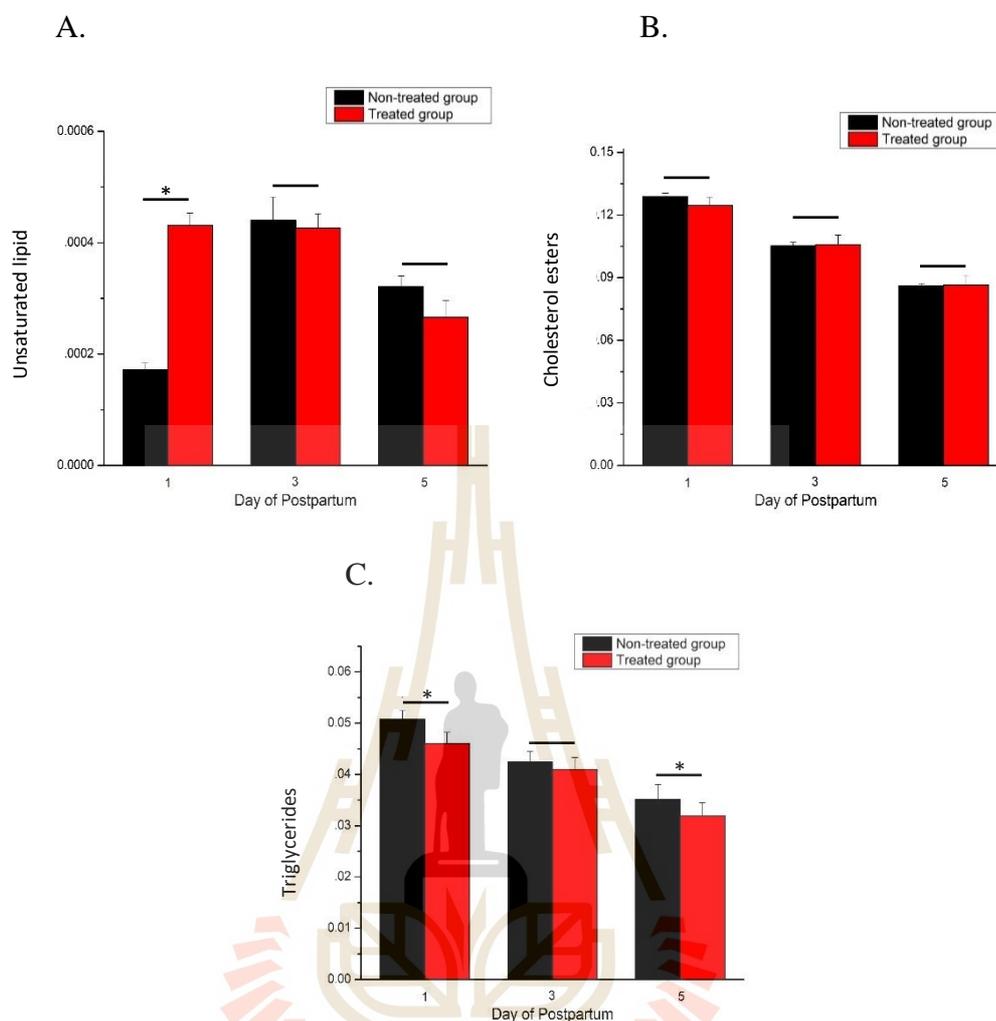
Figure 5.5 A graph showing the effects of *H. indicum* extract on band area of macromolecular composition changes in mammary gland during postpartum periods especially lipid region (3096-1732 cm^{-1}). The band area between groups at the same day of postpartum are compared. Alphabets include A, B, and C represent to Band No. 1 (unsaturated lipid), Band No. 2 (lipid, cholesterol esters), and Band No. 3 ((C=O) triglycerides), respectively. Bars represent mean ± S.E.M. Values that do not share the same superscript letters are significantly different ($P < 0.05$).
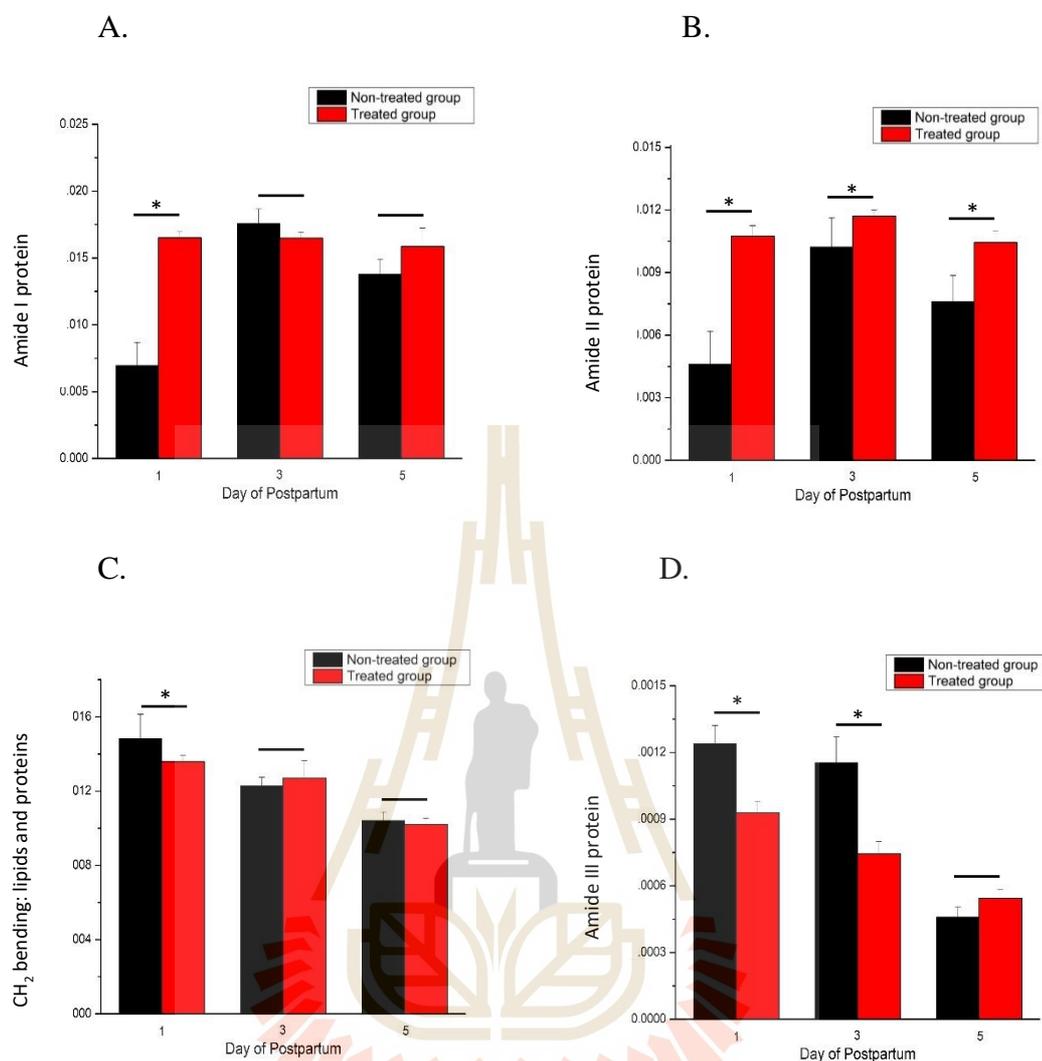
Figure 5. 6 A graph showing the effects of *H. indicum* extract on band area of macromolecular composition changes in mammary gland during postpartum periods especially protein regions (1600-1276 cm^{-1}). The band area between groups at the same day of postpartum are compared. Alphabets include A, B, C, and D represent to Band No. 4 (Amide I protein), Band No. 5 (Amide II protein), Band No. 6 (CH$_2$ bending: lipids and proteins), and Band No. 7 (Amide III vibrations of collagen), respectively. Bars represent mean ± S.E.M. Values that do not share the same superscript letters are significantly different (*P* < 0.05).
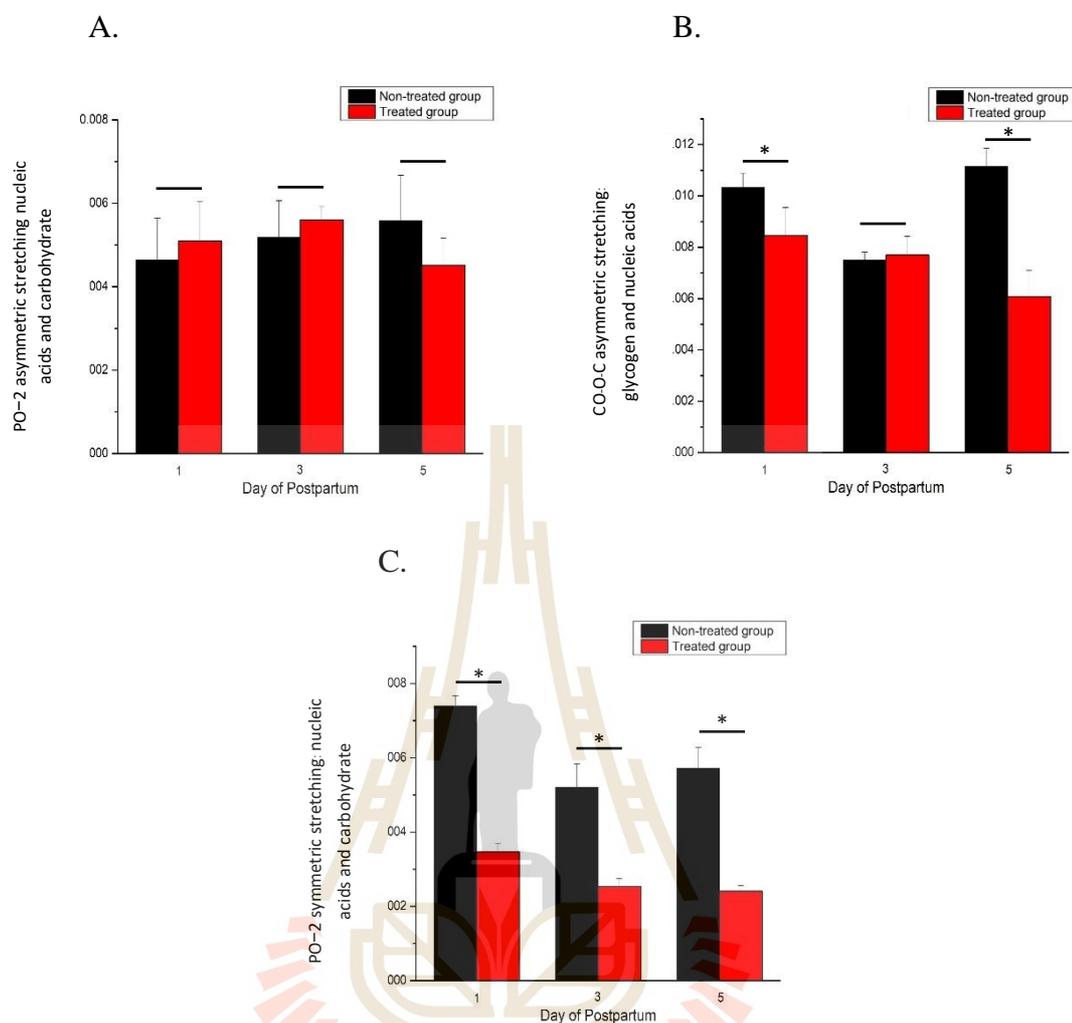
Figure 5. 7 A graph showing the effects of *H. indicum* extract on band area of macromolecular composition changes in mammary gland during postpartum periods especially carbohydrate and nucleic acid regions (1257- 950 cm^{-1}). The band area between groups at the same day of postpartum are compared. Alphabets include A, B, and C represent to Band No. 8 (PO− 2 asymmetric stretching nucleic acids and carbohydrate), Band No. 9 (CO-O-C asymmetric stretching: glycogen and nucleic acids), and Band No. 10 (PO−2 symmetric stretching: nucleic acids and carbohydrate), respectively. Bars represent mean ± S. E. M. Values that do not share the same superscript letters are significantly different ($P < 0.05$).

5.5 Discussion

Maternal care was applied by traditional medicinal plants such as involution and breastfeeding. Galactagogues is food or drug that promote the flow of mother's milk, it has benefits for mother who are powerless to create breast milk for baby (Gabay, 2002). The capability to produce milk progress during pregnancy when the mammary gland is converted from a simple ductal to an extremely potent exocrine organ. The conversion involves alteration in both of the cellular arrangement of the mammary gland and the structural. The features of alveolar cells are significant to the maturation of productive solute carry and secretory functions. Secretory differentiation of the mammary gland (lactogenesis) starts around mid-pregnancy in many species. Moreover, the lactogenesis separated into initiation and stimulation phases based on deviation in the configuration of mammary excretions, gene expression, and structural of alveolar cells (Nguyen et al., 2001).

Many studies reported traditional plants for the response of the traditional uses and galactagogues such as *Costus afer Ker-Gawl*. Alkaloids, terpenoids, flavonoids, tannins, cardiac glycosides, saponins, phenolic compounds, and anthraquinones are present in the plant (Ezejiofor et al., 2013). Moreover, *Euphorbia hirta* L. has been reported to contain alkanes, triterpenes, phytosterols, tannins, polyphenols, and flavonoids (Dadalto and Rosa, 2017). Other study shows *Euphorbia hirta* (L.) were studied on milk production in lactating rats. The study suggests that primary phytochemical screening shows the presence of steroids, triterpenoids, tannins, flavonoids, coumarins, anthocyanosids, and reducing sugars. Moreover, the literature showed that some lactogenic plants contain tannins, terpenoids and steroids, flavonoids, saponosids, anthocyans, alkaloids cardenolids, and quinone. Hence, the presence of

these phytochemical compounds in the plant could explain its medicinal use in lactation (Maya et al., 2018). Another galactagogues plant, *Desmodium adscendens (Sw.)* and *Gossypium herbaceum L.* (*Malvaceae*) contained indole tannins, alkaloids, carbohydrates, ergosterol, unsaturated fatty acids, tyramine, steroids, gossypol, oleic, palmitic, linoleic acid, and saponins (Addy, 1989, Khaleequr et al., 2016). Furthermore, tomato peels and seeds contain oxycarotenoids. In animal feeds, wet tomato byproducts can be blended with corn plants resulting in silage having of producing good milk in ruminants (Knoblich et al., 2005). Moreover, tannins are improving live weight gain, milk yield, ovulation rate, and protein concentration (Guil-Guerrero et al., 2016).

Heliotropium indicum L. (*H. indicum*) contains several phytochemical compounds including alkaloids, tannins, flavonoids, steroids, and glycoside which may explain its application in traditional medicine. The phytochemicals such as alkaloids, tannins, and glycoside show an effect on uterine contraction. The increased prolactin levels and expanding milk supply because of herbal galactagogues, most of them are believed to put their pharmacologic effects through interactions with dopamine receptors (Gabay, 2002). The effects of *H. indicum* extract on alveoli size in postpartum rats are shown in Table 5.1 and Figure 5.3. The alveoli size was observed on day 1, 3, and 5 postpartum and compared both within group and between groups. The results revealed that *H. indicum* extract can help increase alveoli size on day 1 postpartum and maintain the alveoli size till day 5 postpartum. The increasing size of alveoli may result in causing an increase in milk production.

Histological structures of the mammary gland are composed of two components, the parenchyma (the tissue achieve the special function of the organ) and the surrounding stroma (connective tissue framework of the organ). The parenchyma

composed of the alveoli, grape-like clusters where milk is stored, and branching ducts, which are tubular canals taking glandular secretions. The ducts and lobules include two main cell types: luminal epithelial cells and basal myoepithelial cells. The functional secretory cells called lobular luminal epithelial cells, it active during lactation. The myoepithelial cells have contractile features and are sensitive to oxytocin. Interlobular stroma consist of variable amounts of collagen (Treuting and Dintzis, 2012).

In this study, the effects of *H. indicum* extract on histological structures of the mammary glands in postpartum rats were analyzed by ImageJ program and the results are shown in Table 5.2 and Figure 5.4. The ratio of parenchyma cells and stroma cells in mammary gland was observed on day 1, 3, and 5 postpartum and compared both of within group and between groups. When compared within group, the results showed that the ratio of parenchyma cells and stroma cells in mammary gland were similar in non-treated rats on day 1 postpartum to day 5 postpartum. It means that the volume of the alveoli and the secretion was unchanged. Nevertheless, treated rats showed that the ratio of parenchyma cells and stroma cells in mammary gland was significantly increased on day 5 postpartum. However, the comparison of ratio between groups at the same day of postpartum, the results showed that the ratio were not significant different on day 1 postpartum. On day 3 postpartum, treated rats showed significant decreases in the ratio of parenchyma cell and stroma cell in mammary gland. Nevertheless, treated rats were received the extract showed significant increases in the ratio of parenchyma cells and stroma cells in mammary gland on day 5 postpartum. Therefore, the result indicated that the *H. indicum* extract increased the parenchyma cells which responsible for increases in milk production.

The rat is a generally used example for several types of nutritional,

physiological, and biochemical studies. The protein and carbohydrate enlarged somewhat in early lactation and later declined, but the developmental patterns of protein, carbohydrate, and fat were not very notable (Keen et al., 1981). The increasing expression of some milk protein genes, biosynthetic enzymes, and collection of neutral lipid droplets occurred in the initiation phase. The beginning of lactation shows three temporally distinct variation in milk composition take place. The earliest is a lower in sodium and chloride and an increase in the lactose concentrations of milk. These alterations appear immediately after childbirth and are largely complete by 72 h postpartum. The milk volume was increased at least 24 h and can be stored by end of the tight junctions that block the paracellular pathway. Hence, the decreased concentrations of sodium and chloride and increased concentrations of lactose in the mammary secretion is occur (Nguyen et al., 2001).

In this study, the change of biochemical component in mammary gland during postpartum period was found. The results were analyzed by FTIR and are shown in Table 5.4, Table 5.5, and Figure 5.5, Figure 5.6, and Figure 5.7. FTIR was used to identify and analysis the biological samples by detecting changes in macromolecular composition based on the peak values in the IR region. In this study, the FTIR absorption spectrum of the mammary gland showed distinct areas of the lipid region, the (C=O) triglycerides regions, the protein regions, carbohydrate, and nucleic acid regions. The band area was changed when compared within group, showing the changing in macromolecular composition during postpartum period. On the part of the lipid region ($3096\text{-}1732$ cm^{-1}) including Band No. 1 (unsaturated lipid), Band No. 2 (lipid, cholesterol esters), and Band No. 3 ((C=O) triglycerides) are shown in Figure 5.5. In non-treated rats, Band No. 1 (unsaturated lipid) were fell during day 1

postpartum, then increased on day 3 postpartum, and finally dropped on day 5 postpartum. However, in treated rats that received the extracts, the consistency of Band No. 1 (unsaturated lipid) was observed on day 1 and 3, then decreased on day 5 postpartum. When compared between groups, Band No. 1 of treated rats were significantly increased on day 1 postpartum. On day 3 and 5 postpartum both groups were similar. The results suggested that the *H. indicum* extract induced unsaturated lipid (3096-3033 cm^{-1}) higher than non-treated rats. Band No. 2 (lipid, cholesterol esters) of both groups were significantly decreased on day 1, 3, and 5 postpartum. When compared between groups, both groups were similar. The results suggested that the *H. indicum* extract have no effects on lipid and cholesterol esters at spectrum 3019-2811 cm^{-1} in mammary gland on postpartum period. Furthermore, Band No. 3 ((C=O) triglycerides) of both groups were significantly decreased on day 1, 3, and 5 postpartum. When compared between groups, Band No. 3 of treated rats were significantly reduced on day 1 and 5 postpartum when compared with non-treated rats. The results suggested that the *H. indicum* extract effectively reduced (C=O) triglycerides at spectrum 1749-1732 cm^{-1} on day 1 and 5 postpartum.

In human milk, the lipids are the major source of energy provided the baby (Delplanque et al., 2015) and gave the important portion of vital vitamins, polyunsaturated fatty acids, and bioactive constituent. Cholesterol supplying with breastfeeding regulate infant sterol metabolism and may influence long-term advantages. Some 98–99% of milk lipids are contained triacylglycerols, whose features depend on consolidated fatty acids. (Koletzko, 2016). The lipids in milk are composed of membrane-enclosed milk fat globules. The globule membrane is consisted mainly of phospholipids, cholesterols, and proteins (Pitkin et al., 1991). In this study, the *H.*

indicum extract has effects on mammary gland in postpartum period by increasing unsaturated lipid and no have effects on level of lipid and cholesterol esters. Moreover, the *H. indicum* extract effectively reduced triglycerides in mammary gland tissue on postpartum period. Many researches have shown considerable biological effects of the milk lipids on the recipient infant. Moreover, the human milk lipids provide the total energy intake in young children with a mean 44% of the power supply (Grote et al., 2016). The structural lipid incorporated in the improving central nervous system up to age 2 years of baby is myelin that main comprise by nervonic acid (Kinney et al., 1988).

On the part of the protein regions (1600-1276 cm^{-1}) include Band No. 4 (Amide I protein), Band No. 5 (Amide II protein), Band No. 6 (CH2 bending: lipids and proteins), and Band No. 7 (Amide III vibrations of collagen) shown in Figure 5.6. The results suggested that Band No. 4 (Amide I protein) of non-treated rats were fell during day 1 postpartum, then increased on day 3 postpartum, and finally dropped on day 5 postpartum. However, the amide I protein of treated rats were unchanged in postpartum periods. Compared between groups, the amide I protein of treated rats were significantly increased on day 1 postpartum, compared non-treated rats and the others day were similar. For this reason, the extracts in treated rats were induced the amide I protein at spectrum 1682-1633 cm^{-1} on day 1 postpartum and stable until day 5 postpartum. Band No. 5 (Amide II protein) of non-treated rats were fell during day 1 postpartum, then increased on day 3 postpartum, and finally dropped on day 5 postpartum. However, the amide II protein of treated rats were unchanged in postpartum periods. Compared between groups, the amide II protein of treated rats were higher than non-treated rats on day 1, 3, and 5 postpartum. For this reason, the extracts in treated rats increased the amide II protein at spectrum 1559-1508 cm^{-1} on day 1

postpartum and stable to day 5 postpartum. Band No. 6 (CH_2 bending: lipids and proteins) of non-treated rats were reduced on day 3 and 5 postpartum and in treated rats were declined on day 1 to 5 postpartum. When compared between groups, the CH_2 bending: lipids and proteins of treated rats were significantly decreased on day 1 postpartum but day 3 and 5 postpartum showing the same area with non-treated rats. Therefore, the extract in treated rats were decreased the CH_2 bending: lipids and proteins at spectrum 1471-1370 cm^{-1}, more effective on day 1 postpartum. Band No. 7 (Amide III vibrations of collagen), non-treated rats were significantly decreased on day 5 postpartum, compared day 1 and 3 postpartum. Conversely, Band No. 7 in treated rats were significantly decreased on day 1, 3, and 5 postpartum. On day 1 and 3 postpartum, Band No. 7 in treated rats were significantly decreased when compared non-treated rats and both of groups were similar in day 5 postpartum. Thus, the extracts in treated rats were decreased the amide III at spectrum 1350-1276 cm^{-1}, more effective on day 1 and 3 postpartum.

In human milk, the proteins are separated into the whey and casein fractions or complexes, with each contained by a noticeable array of specific proteins and peptides. Casein α-lactalbumin, lactoferrin, secretory immunoglobulin IgA, lysozyme, and serum albumin are the most generous proteins (Ballard and Morrow, 2013). In the mammary gland, the whey proteins for example α-lactalbumin and lactoferrin were synthesized; other proteins including serum albumin and some bioactive enzymes and protein hormones were transported to the milk from plasma. In addition, dimeric IgA is resulted by plasma cells in the mammary gland and is delivered into the milk by definite receptors. (Pitkin et al., 1991). The total protein in milk decline speedily during the first part of lactation, and thenceforth variation very little in humans (Lonnerdal et al., 1976).

The concentrations of lactoferrin and immunoglobulins (sIgA) remain high concentrations in colostrum at the first 48 h after birth then fall rapidly after day 2. About 36 h postpartum found the increasing in milk volume and the rates of formation and ∕ or excretion of almost all the elements of mature milk including, but not limited to, lactose, protein (mainly casein), lipid, glucose and free phosphate (Nguyen et al., 2001).

This study, the carbohydrate and nucleic acid regions $(1257\text{-}950\,cm^{-1})$ including Band No. 8 (PO−2 asymmetric stretching nucleic acids and carbohydrate), Band No. 9 (CO-O-C asymmetric stretching: glycogen and nucleic acids), and Band No. 10 (PO−2 symmetric stretching: nucleic acids and carbohydrate) as shown in Figure 5.7. Band No. 8 of non-treated rats were unchanged in postpartum periods and treated rats were significantly decreased on day 5 postpartum, compared day 3 postpartum. Moreover, Band No. 8 of both groups were similar in postpartum period when compared between day of postpartum. The result suggested that the extract was more efficient on day 5 postpartum. Band No. 9 (CO-O-C asymmetric stretching: glycogen and nucleic acids), in non-treated rats were low on day 3 postpartum and in treated rats were low on day 5 postpartum. Compared between groups, treated rats were significantly decreased on day 1 and 5 postpartum. This result indicated that the extract interferd the level of CO-O-C asymmetric stretching: glycogen and nucleic acids $(1177\text{-}1112\,cm^{-1})$ in mammary gland on postpartum period. Band No. 10 (PO−2 symmetric stretching: nucleic acids and carbohydrate) of non-treated rats were unchanged in postpartum periods but in treated rats were low on day 3 and 5 postpartum when compared day 1 postpartum. Compared between groups, treated rats were significantly decreased on day 1, 3, and 5 postpartum. The results suggested that the *H. indicum* extract was reduced the PO−2

symmetric stretching: nucleic acids and carbohydrate at spectrum 1062-949 cm^{-1} in mammary gland on postpartum period.

Human milk is the first dietary reveal in babyhood and the best nutritional selection for growth and healthy advancement of the newborn and infant (Brodribb, 2015). The concentrations of macronutrients, like proteins, carbohydrates, and lipids, variation during lactation stages (Lukacka et al., 2018). Human milk gives a persistence supply of carbohydrates to the newborn during early life, ensuring suitable nutrition, maturation, and growth of their comparatively immature physiological systems (Gridneva et al., 2019). Carbohydrates are also mention to as sugars or saccharides. The most considerable function is the storage of energy. In human milk, the principal carbohydrate is lactose, a disaccharide that compose of galactose joined by a β linkage to glucose (Pitkin et al., 1991). The lactose synthesis show in water being move into the milk, the rate of lactose formation of chemical compounds is a major controlling factor of milk production (Arthur et al., 1989). The lactose concentration and breastfeeding frequency of human milk are higher in 24 hours of lactation (Gridneva et al., 2019). The carbohydrate concentrations expand from day 1 to day 14, followed by slightly change to day 21 lactation. This reduction in carbohydrate concentration may be due to lowered activity of mammary lactose synthetase (Lonnerdal et al., 1976). From the result, treated rats received the *H. indicum* extract showed lower of carbohydrate band area than control group may be due to activity of mammary lactose synthetase.

The milk lipids and proteins were synthesized in the identical compartment, the endoplasmic reticulum in mammary epithelial cells (MEC) (Bousquet, 2002). One of the most enthusiastic lipid-synthesizing and secreting organs in the body is the

mammary gland (McManaman, 2015). The production of lipids to offer the newborn that are necessary for growth and survival are the one of the main functions of MEC. Milk fat globule (MFG) which buds from the MEC is the location for secreted the milk lipids (Cohen et al., 2018). Menin is the protein that abundant in the MEC. The cell cycle progression of MEC is controlled by menin. Moreover, lower of menin conducted increased epithelial cell apoptosis and resulted in extracellular matrix remodeling by down-regulating (Shi et al., 2017). The estimation of peptide transporters in the mammary gland may consequently provide novel insights into protein metabolism and secretion by the gland (Groneberg et al., 2002). From the result, the *H. indicum* extract has effectively increased the unsaturated lipid, the amide I protein, and amide II protein in the mammary gland on day 1 postpartum and stable until day 5 postpartum. Therefore, *H. indicum* extract can help increasing lipid and protein in mammary gland tissue which could relate with lipid and protein synthesis for milk secretion.

In summary, the effects of the whole plant extract of *H. indicum* on the mammary gland size, the rate of the parenchyma cells in mammary gland tissue, and the biochemical compound of mammary gland were investigated. The result showed *H. indicum* extract can help increase alveoli size and the parenchyma cells reason for increase in milk production. In the part of FTIR analysis, the result showed the whole plant of *H. indicum* extract helps to increase lipid and protein in mammary gland tissue which could relate with lipid and protein synthesis in mammary epithelial cells. Therefore, the whole plant of *H. indicum* extract can intake during postpartum period to induce milk production in rats. These results also confirm the traditional uses of the plant in the lactation insufficiency.
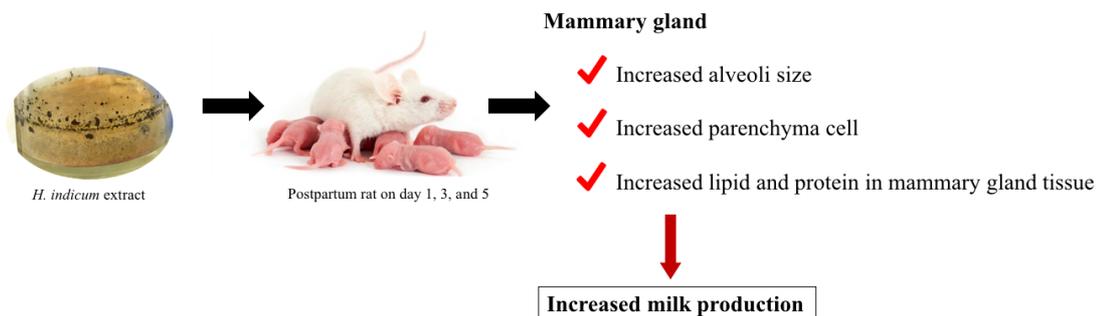
Figure 5.8 Diagram show the effects of *H. indicum* on milk production in postpartum rats.

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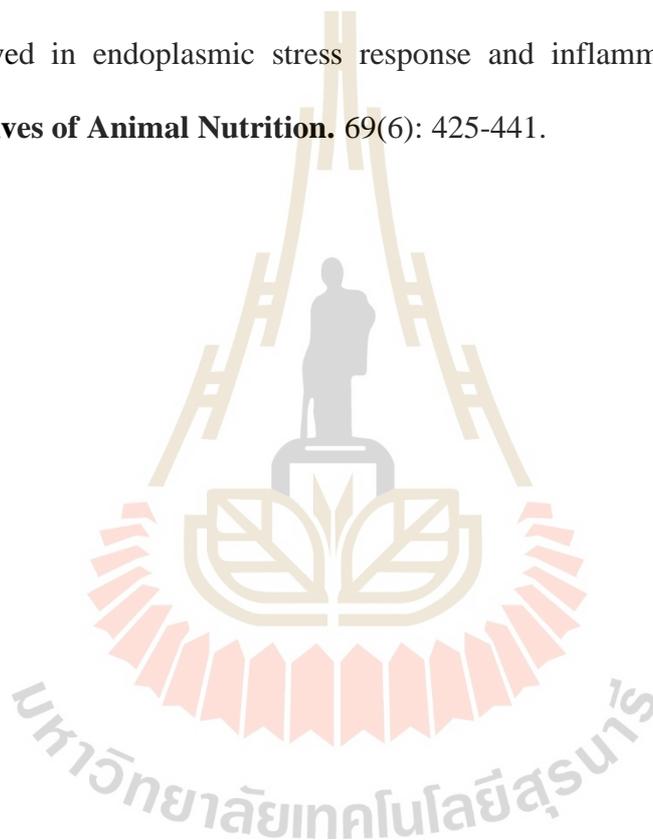
APPENDIX
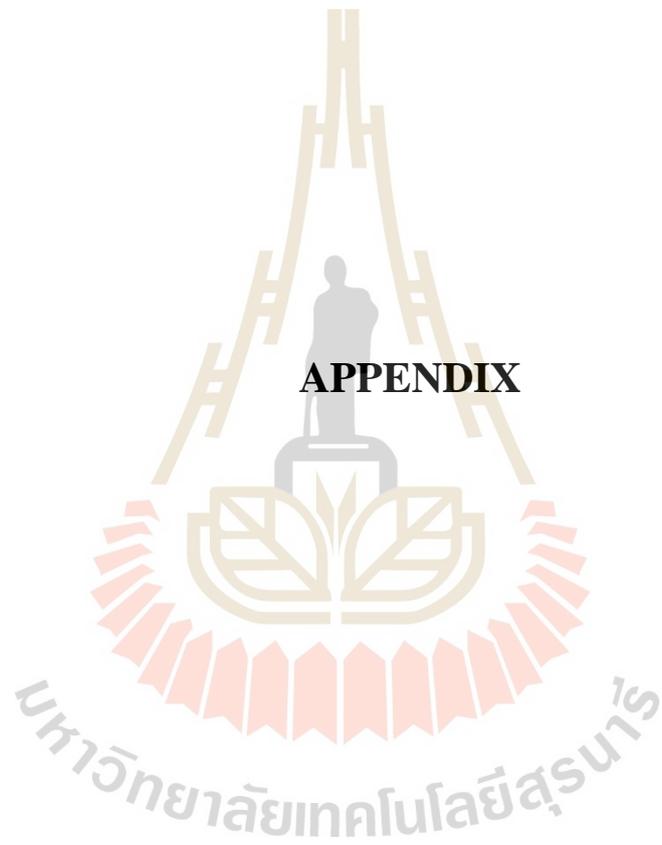
APPENDIX

IMAGE PROCESSING BY IMAGE-J

Image processing by ImageJ

Image acquisition is a prerequisite for further analysis, and the modified steps are required as previously reported from Chen et al., 2017. First, all images should be acquired under identical conditions. To avoid a short range on the grayscale (0-255), the automatic exposure and white balance should be turned off (Chen et al., 2017).
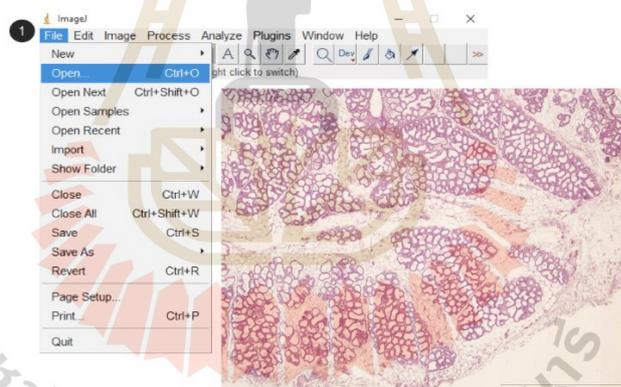
1) The quantitation of the region of interesting (hold, epithelium cells, lipid cells) using ImageJ software. First, started ImageJ software, then clicked "File" and clicked on the "Open" for loaded the picture.
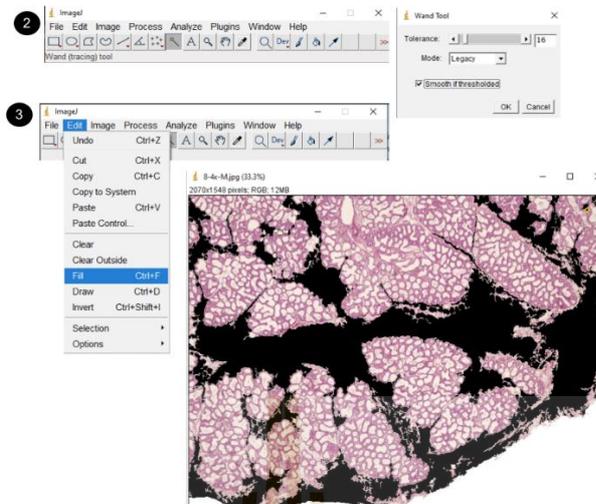
2-3) Used Wand Tool for selected area, after that clicked on the "Fill" for fill the color
on the lipid area. This tool help to separate area that easy to analyze.
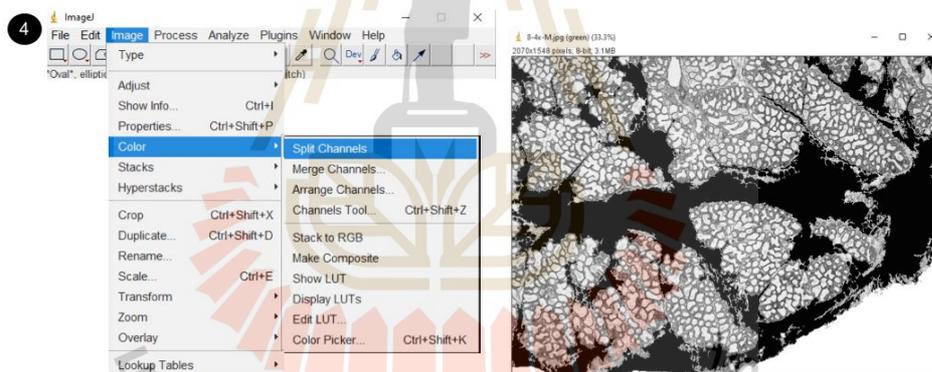
4) The area-based analysis was used to extract and quantify the regions of interest from
the image. This analysis allowed the performance of the algorithm to be evaluated. To
quantify the regions of interest (hold, epithelium cells, lipid cells), the manager was
chosen in the "Image" and clicked on the "Color" then "Spit Channels". The original
images were split into their red, blue and green components. Selected the channels that
show the most distinct area.
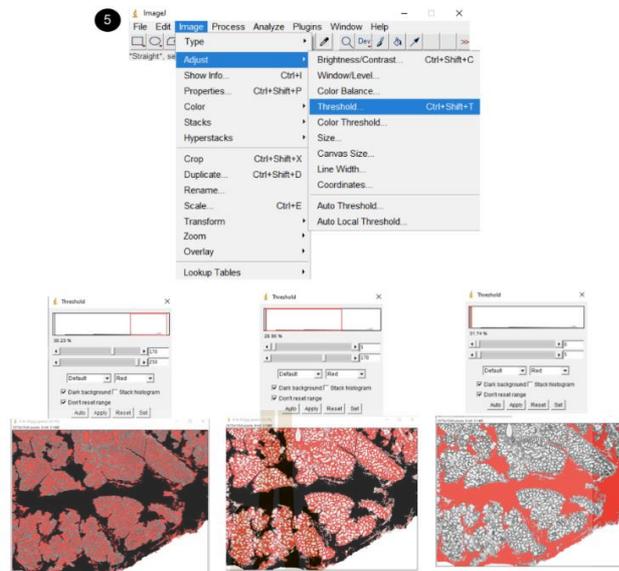
5) Use the channel from before step. The area of the region of interesting (hold, epithelium cells, lipid cells) was measured after we entered the "Image" menu, clicked on the "Adjust" box, and isolated the interesting area using the "Threshold" tool. The threshold was manually adjusted until the entire the interesting area was highlighted in red.
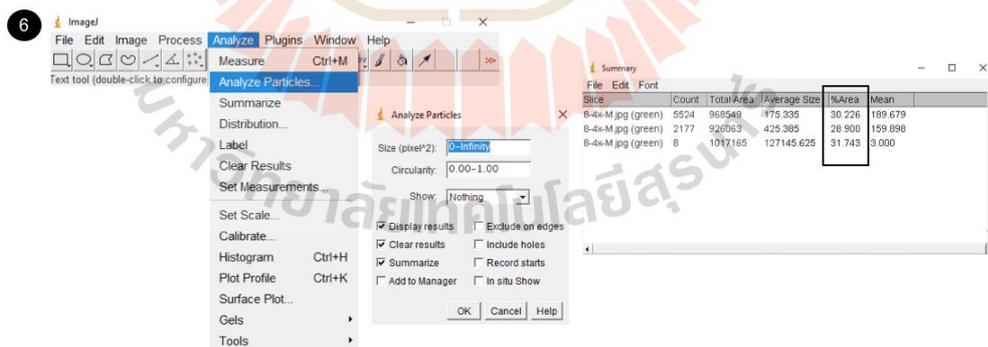
6) The measurement of the threshold area was performed as follows: we entered the set measurement dialog under the "Analyze" menu, and clicked on the "Analyze Particles", then checked in box that show in picture and clicked on the "OK". Next, the window of summary was show. We focus at %Area on the summary window, used that data for ratio of parenchyma per stroma cells analysis.

CHAPTER VI

EFFECTS OF *HELIOTROPIUM INDICUM* L. EXTRACT

ON BLOOD BIOCHEMICAL PARAMETERS IN

POSTPARTUM RATS

6.1 Abstract

The whole plant of *Heliotropium indicum* L. (*H. indicum*) is a traditional medicinal plant that used in many parts of the world. The study of blood biochemical parameters can explain the effects of *H. indicum* extract on the body and the safety of tradition uses. The typical parameters of hepatotoxicity were indicators of hepatocellular injury such as alanine aminotransferase (ALT) and aspartate aminotransferase (AST) . Other parameters are estradiol and progesterone, the 2 principal hormones implicated in the regulation of ovarian function and control of the reproductive cycle. The aim of this study was to investigate the effects of *H. indicum* on blood biochemical parameters in postpartum rats. The whole plant was dried and powdered. The powder was extracted by macerated with 70% ethanol and shaken for 3 days .The mixed were filtered through filter paper to remove particulates .Then, the extract was evaporated under reduced pressure at a low temperature in a rotary evaporator, dried by using lyophilizer and stored at - 20°C until use. Sixty postpartum rats were used .The rats were randomized into 2 main groups; non- treated rats and treated rats, which had 3 subgroups including; 1) 1 day-postpartum rats, 2) 3 day

-postpartum rats, and 3) 5 day-postpartum rats. The extract was diluted to 250 mg/kg B.W. in the vehicle (Tween 80, 10% v/v) and was administered orally to the treated rats at a volume of 0.8 mL/day for 1, 3, and 5 days, respectively. Only the vehicle was administered orally for the rests. After administered orally, the blood samples were collected and measured blood biochemical parameters from serum. Blood biochemical parameters included alanine aminotransferase (ALT), aspartate aminotransferase (AST), estradiol, and progesterone. The results indicated that the AST and ALT level in this study did not show an abnormality of the liver. The *H. indicum* extract has no hepatotoxicity when taken within 5 day in the postpartum rat. Moreover, the extract showed the decrease of estradiol and progesterone resulted in high collagenase activity that reduced the size and weight of the uterus on day 1 and 5 postpartum, respectively. In the uterus, the extract decreased progesterone resulted in high estradiol and PGF2α are concurring with the formation of gap junctions that may correlate to the increased uterine contraction on day 5 postpartum. Also, the extract decreases progesterone level on day 5 postpartum which can cause high prolactin that may relate to increases in milk production. Thus, the whole plant of *H. indicum* extract can intake during the postpartum period to accelerate uterine involution and induce milk production in rats. These results support the traditional uses of the plant.

6.2 Introduction

The typical blood biochemical parameters of hepatotoxicity were indicators of hepatocellular injury: alanine aminotransferase (ALT) and aspartate aminotransferase (AST) (Boone et al., 2005). Alanine amino transferase (ALT) has greater concentration in liver compared with other tissues of the body such as kidney, heart, and muscle.

Normal serum ALT is 7-56 U/L. The increase in ALT levels is the result of liver cell injury. The values up to 300 U/L are supposed nonspecific. The ALT levels higher than 500 U/L are occurred most often in patient with illness that affect originally hepatocytes such as viral hepatitis, ischemic liver injury, and toxin-induced liver damage (Kallei et al., 1964). Aspartate amino transferase (AST) has two disparate isoenzyme forms which are genetically detached, the mitochondrial and cytoplasmic form. Normal serum AST is 0 to 35 U/L. Most of the circulating AST activity in normal people is acquired from the cytosolic isoenzyme, whereas about 80% of AST activity of the liver is provided by the mitochondrial isoenzyme. AST levels with indicative pregnant patient in hyperemesis gravidarum is 73 U/L, in pre-eclampsia is 66 U/L, and 81 U/L is observed in hemolysis with low platelet count and raised liver enzymes (Thapa and Anuj, 2007).

The diagnostic importance in identifying the liver cell necrotic type condition and alcoholic hepatitis is the ratio of mitochondrial AST to total AST activity. An increased ALT was found in patients with cirrhosis and even in liver diseases. The mean ratio of 1.45 and 1.3 was found in alcoholic liver disease and post necrotic cirrhosis respectively (Giannini et al., 2003). However, the alcoholic hepatitis showed an AST/ALT ratio greater than 2, liver disease was powerfully suggestive of alcohol as an etiology. The ALT greater than AST in most chronic liver disease was not caused by alcohol (Al-Jameil et al., 2015). In pregnancy human, it has been accepted that AST, ALT, CGT and bilirubin concentration drops by 20% and that ALP rises by 300%. After delivery, the levels would be increasing by 25%. The AST and ALT 'overshoot' before return to nonpregnant state (David et al., 2000). Another study, the transaminase levels attained a peak following delivery, enlarged by a mean of 88% (AST) and 147%

(ALT) from pre-delivery concentration, but they had dropped by day 10 postpartum (Wilkinson, 1970).

The principal performance of the female reproductive system is to deliver the ova for sperm fertilization and to give a suitable limitation for embryo implantation, fetal growth and development, and delivery (Molina, 2013). The developing embryo appears after implant in the uterus, and after that, the placenta developed. The period of normal gestation is 40 weeks ends with parturition. The female breast shows differentiation during pregnancy to permit lactation and breast-feeding. The complicated occurrence in female reproductive physiology is controlled by the pituitary, the gonadal, and the placental hormones (Kibble and Halsey, 2015). Females have a short-lasting window of fertility. Moreover, the gametes were periodically produced. The hypothalamic-pituitary-ovarian axis is the main endocrine regulation of the reproductive system, both negative and positive feedback patterns (Haroun, 2016). The anterior pituitary gland releases follicle-stimulating hormone (FSH) in the first half of the cycle causes the ripening of the primary ovarian follicle to the mature Graafian follicle, after that the Graafian follicle excretes estrogen (Molina, 2013). Moreover, estrogens are produced by the placenta, liver, adrenal glands, fat and breast cells produce estrogens as well. During the fertile lifespan, the women is produced mainly estradiol, while estrone is the mighty estrogen during menopause and estriol during pregnancy (Grow, 2002). FSH was inhibited by estrogen and the luteinizing hormone (LH) was stimulated by estrogen, both hormones cause ovulation. The corpus hemorrhagicum was changed to a corpus luteum by LH. The production of LH was inhibited by progesterone make regression of the corpus luteum. The reduction of estrogen level provokes FSH secretion and a new cycle begins (Molina, 2013).

Pregnancy is a unique period that is accompanied by various biological and psychological changes. The levels of hormones such as progesterone and estrogen were changed in pregnant women, there is a lack of information about difference in levels of oxytocin, importance to parturition and lactation (Fuchs and Fuchs, 1984). The healthy women with a normal pregnancy were estimated estradiol and progesterone level by mass spectrometry. The women were obtained during gestation week 12 (first trimester), gestation week 22 (second trimester), gestation week 32 (third trimester), and approximately 1 year postpartum. The results show estradiol (pmol/L) at first trimester is $3,211.2 \pm 253.2$, second trimester is $15,546.1 \pm 961.5$, third trimester is $22,680.6 \pm 1493.7$, and postpartum is $<367.0 \pm 55.0$. The results show progesterone (nmol/L) at first trimester is 55.87 ± 2.78, second trimester is 134.51 ± 7.4, third trimester is 224.03 ± 10.56, and postpartum is 2.73 ± 0.62 (Soldin et al., 2005). Other study, Blood samples of the nineteen women, average age 33, were collected for a steroid hormone during their last 2 months of pregnancy and again within 2 months of delivery. The results show estradiol during pregnancy is 25.07 ng/ml and after delivery is 0.024 ng/ml. Progesterone during pregnancy is 174.11 ng/ml and after delivery 0.501 ng/ml (Buckwalter et al., 1999).

The uterus of rodents and the human endures cyclical alteration of development and regression. Estrogens made from the growing follicles provoke endometrial growth, in both species. In parts of progesterone is role in converting the estrogen-primed endometrium into a receptive state (Groothuis et al., 2007). In the pre-implantation stage, the progesterone affects tubal movement by contacting on specific receptors and performs on endometrial maturation and on uterine vascularization (Schneider et al., 1993). Moreover, the expansion of spiral arterioles in the late

secretary phase and at the time of implantation were controlled by progesterone (Al-Asmakh, 2007). During pregnancy and lactation of rats, the important reproductive process is the inhibition of gonadotropin release, and therefore the loss of ovulation. The suckling could be related with the low levels of estrogen, thus stopping the manifestation of the estrous cycle during lactation (Liu et al., 2013). The method employing gas-liquid chromatography with electron capture detection were used to analyze the plasma progesterone concentrations in rats. Progesterone concentration was found to decline before childbirth and to increase again during lactation. The progesterone concentration during postpartum period were high during the 4th day of lactation. Moreover, the progesterone concentration was decrease by removal of the litter or the placenta at delivery. In the 24 hr previous delivery, the concentration of progesterone decreased significantly from 114 ng/ml to 10 ng/ml of plasma, then 6 hr postpartum found the plasma progesterone concentration increased to 43 ng/ml and continued at this level until 2 days after labor. Day 4 of lactation, the concentration of progesterone increased to 123 ng/ml of plasma and remained at this level until at least the 8th day of lactation (Grota and Eik-nes, 1967). The postpartum estrus in Norway rats specifically, it is concerned with the appearance of ovulation, the vaginal cytology, and the plasma levels of estradiol-17 and progesterone were investigated. The plasma levels of estradiol-17beta and progesterone were analyzed throughout the 24 h postpartum estrus period by radioimmunoassay using kits from Diagnostic Product. The results show the estradiol levels ranged from 130.35 pg/ml at 3 h to a low of 5.35 pg/ml at 24 h after delivery. In the part of progesterone showed a peak level of 37.04 ng/ml at 12 h, and the intermediate levels ranged from 6.51 ng/ml to 23.01 ng/ml. In lactating rats on day 7, the estradiol levels are 15.83 ± 4.32 (pg/ml) and the progesterone

levels are 24.92 ± 2.04 (ng/ml). Day 10 of lactating, the estradiol levels are 6.76 ± 2.24 (pg/ml) and the progesterone levels are 32.73 ± 10.28 (ng/ml) (Ignacio et al., 2017).

The whole plant of *Heliotropium indicum* L. (*H. indicum*) is used for healing herpes, and the glue of plant parts is used for cleansing and dressing of wounds and ulcers in Rodrigues. Moreover, the juice of the bark is used orally by women for healing dysmenorrhea and the warm aqueous extract of the flower and buds is taken orally by the women as an emmenagogue in a small dose and abortive in an expanded treatment in West Indies (Ghosh et al., 2018). In Thailand, the dried inflorescence is chosen to do lasting sterilization when taken orally in the case of women. The dehydrated and powdered inflorescence blended with milk or water and used taken orally at the time of menses to get the desired result. (Panthong et al., 1986). In this study, the whole plant of *H. indicum* extract was used to study the effects of *H. indicum* extract on blood biochemical parameters in postpartum rats on days 1, 3, and 5 postpartum. Blood biochemical parameters include alanine aminotransferase (ALT), aspartate aminotransferase (AST), estradiol, and progesterone. The results of this study can be used as scientific data to support *H. indicum* extract effects on blood biochemical parameters to help support traditional uses. Moreover, the results from this study are the basis knowledge for further research to develop drugs or products for maternal care.

6.3 Methodology

6.3.1 Experimental animals

Female Wistar rats (200-250 g) were used in this study and maintained in accordance with the guidelines of the Committee on Care and Use of Laboratory Animal Resources, National Research Council, Thailand. The experiments performed

on rats were conducted in accordance with the advice of the Institutional Animal Care and Use Committee, Suranaree University of Technology. Female Wistar rats were maintained under environmentally controlled room provided with a 12:12 light and dark cycle at a temperature of approximately 25°C. They were fed with commercial food (C.P. Mice feed, Bangkok, Thailand) and allowed to access water *ad libitum*. Used 60 female Wistar rats for this study. More details relevant to experimental animals are given in Chapter IV. In this chapter, every experiment used sample from the same rats in Chapter IV.

6.3.2 Preparation of plant materials

The whole plant was dried and powdered. The powder of the whole plant was extracted by macerated with 70% ethanol and shaken for 3 days. The mixed were filtered through filter paper to remove particulates. The extract was evaporated by a rotary evaporator, dried by using lyophilizer and stored at -20°C until use. More details relevant to preparation of plant materials are given in Chapter III.

6.3.3 Blood biochemical parameters determining

60 pregnant rats were used. The rats were divided into 2 main groups: non-treated rats and treated rats. Two main groups have 3 subgroups containing: 1) 1 day-postpartum rats 2) 3 day-postpartum rats 3) 5 day-postpartum rats. The extract of *H. indicum* were diluted to 250 mg/kg B.W. in the vehicle (Tween 80, 10% v/v) and were administered orally to the treated rats at a volume of 0.8 mL/day for day 1, 3, and 5 postpartum. Only the vehicle was administered orally to the residue groups. After administered orally, the blood samples were collected at the end of the experiment by cardiac puncture. Blood samples were centrifuged (4°C) at 1500 g for 15 min to separating the serum. The serum samples were stored at -80°C until assay was

performed. The blood biochemical parameters were measure from serum consist of AST, ALT, estradiol, and progesterone.

Serum levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) concentrations were measured by using VITROS 5600 Integrated System Analyzer. Serum levels of estradiol concentrations were analyzed by the Competitive immunoassay using VITROS Estradiol Reagent Pack and VITROS Immunodiagnostic Products Estradiol Calibrator on VITROS 5600 Integrated System Analyzer. Serum levels of progesterone concentrations were analyzed by Competitive Chemilumunescence Immunoassay (CLIA) using VITROS Progesterone Reagent Pack and VITROS Immunodiagnostic Products Progesterone Calibrator on VITROS 5600 Integrated System Analyzer, Ortho Clinical Diagnostics, Raritan, New Jersey, USA (Oberkanins, 2017).

6.3.4 Statistical analysis

All data were expressed the mean $\pm$ S.E.M. and n refers to the number of animals. For paired two groups of data were analyzed by Independent-samples t-test. For three or more groups, data were analyzed by one-way ANOVA and post-hoc with Tukey's test. The Statistical Package for the Social Sciences (SPSS) were employed for all statistical analysis. The significance level was determined at $P < 0.05$.
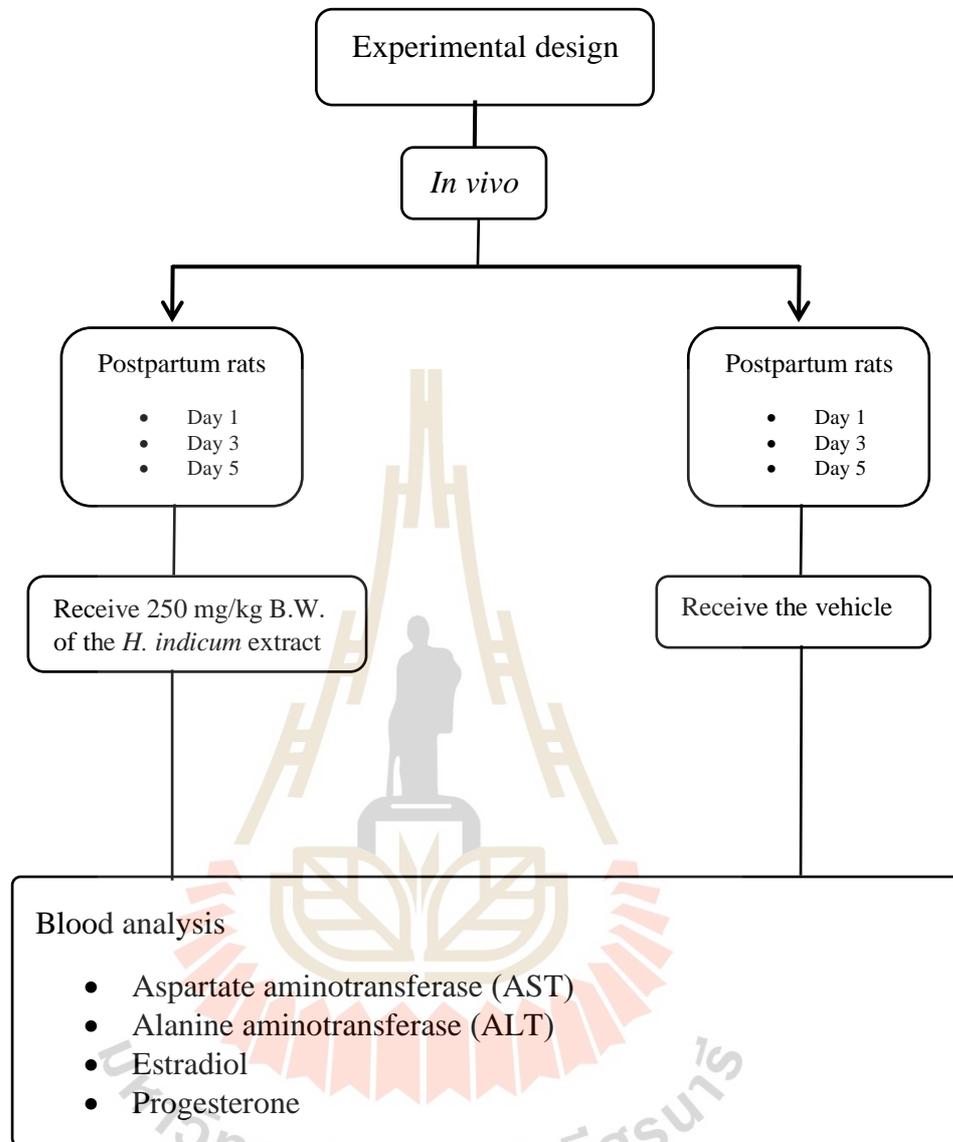
Figure 6.1 Summary of methodology.

6.4 Results

6.4.1 Effects of *H. indicum* extract on aspartate aminotransferase (AST) level

The AST levels were measured after delivery on day 1, 3, and 5 postpartum from non-treated rats and treated rats. The results showed AST levels on day 1 postpartum significantly increased when compared with day 3 postpartum in non-treated rats. The AST level of treated group showed no significant in postpartum period (Table 6.1). When compared between groups at the same day of postpartum, the AST level of both groups showed no significant on day 1, 3, and 5 postpartum (Figure 6.2).

Table 6.1 Effect of *H. indicum* extract on aspartate aminotransferase (AST) level in postpartum rat blood.

Group	AST level (U/L) (Mean ± S.E.M.)
Non-treated rats	
Day 1 postpartum	80.29 ± 7.48^a
Day 3 postpartum	55.38 ± 4.56^b
Day 5 postpartum	65.78 ± 4.00^{ab}
Treated rats	
Day 1 postpartum	83.86 ± 9.83^a
Day 3 postpartum	62.38 ± 5.02^a
Day 5 postpartum	69.40 ± 5.49^a

All values are expressed as mean + S.E.M. of 10 rats in each group ($n = 10$). When compare within group, means with different superscripted letters indicate statistical significance ($P < 0.05$).

Figure 6.2 A graph showing the effects of *H. indicum* extract on aspartate aminotransferase (AST) level in postpartum rat blood serum. Comparison between groups at the same day of postpartum. The AST level of both groups show no significant on day 1, 3, and 5 postpartum. Bars represent mean ± S.E.M. Values that do not share the same superscript letters are significantly different ($P < 0.05$).

6.4.2 Effects of *H. indicum* extract on alanine aminotransferase (ALT) level

The ALT level was measured after delivery on day 1, 3, and 5 postpartum from non-treated rats and treated rats. The results showed ALT level of non-treated rats on day 5 postpartum significantly increased when compared with day 3 postpartum. Moreover, the ALT level of treated rats was significantly increased on day 5 postpartum (Table 6.2). When compared between groups at the same day of postpartum, the ALT level of both groups showed no significant on day 1, 3, and 5 postpartum (Figure 6.3).

Table 6.2 Effect of *H. indicum* extract on alanine aminotransferase (ALT) level in postpartum rat blood.

Group	ALT level (U/L) (Mean ± S.E.M.)
Non-treated rats	
Day 1 postpartum	27.67 ± 1.40^{ab}
Day 3 postpartum	25.88 ± 3.35^{a}
Day 5 postpartum	33.70 ± 1.12^{b}
Treated rats	
Day 1 postpartum	26.50 ± 1.61^{a}
Day 3 postpartum	24.20 ± 0.88^{a}
Day 5 postpartum	32.50 ± 1.73^{b}

All values are expressed as mean + S.E.M. of 10 rats in each group ($n = 10$). When compare within group, means with different superscripted letters indicate statistical significance ($P < 0.05$).
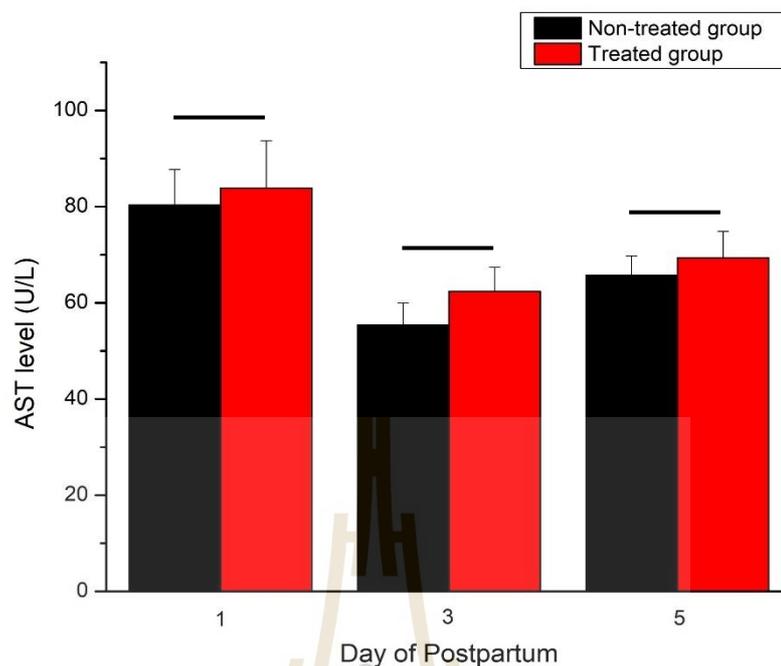
Figure 6.3 A graph showing the effects of *H. indicum* extract on alanine aminotransferase (ALT) level in postpartum rat blood. Comparison between groups at the same day of postpartum. The ALT level of both groups show no significant on day 1, 3, and 5 postpartum. Bars represent mean ± S.E.M. Values that do not share the same superscript letters are significantly different ($P < 0.05$).

6.4.3 Effects of *H. indicum* extract on estradiol level

The estradiol level was measured after delivery on day 1, 3, and 5 postpartum from non-treated rats and treated rats. The results showed estradiol level on day 1 postpartum significantly increased when compared with day 3 and 5 postpartum in both groups (Table 6.3). When the data between groups at the same day of postpartum were compared. Day 1 postpartum, estradiol level of treated rats was significantly decreased when compared with non-treated rats. The estradiol level was similar in both groups on day 3 and 5 postpartum (Figure 6.4).

Table 6.3 Effect of *H. indicum* extract on estradiol level in postpartum rat blood.

Group	Estradiol level (pmol/L) (Mean ± S.E.M.)
Non-treated rats	
Day 1 postpartum	128.04 ± 8.65^a
Day 3 postpartum	59.98 ± 15.61^b
Day 5 postpartum	57.85 ± 5.94^b
Treated rats	
Day 1 postpartum	79.90 ± 1.97^a
Day 3 postpartum	58.02 ± 5.54^b
Day 5 postpartum	55.52 ± 3.83^b

All values are expressed as mean + S.E.M. of 10 rats in each group ($n = 10$). When compare within group, means with different superscripted letters indicate statistical significance ($P < 0.05$).
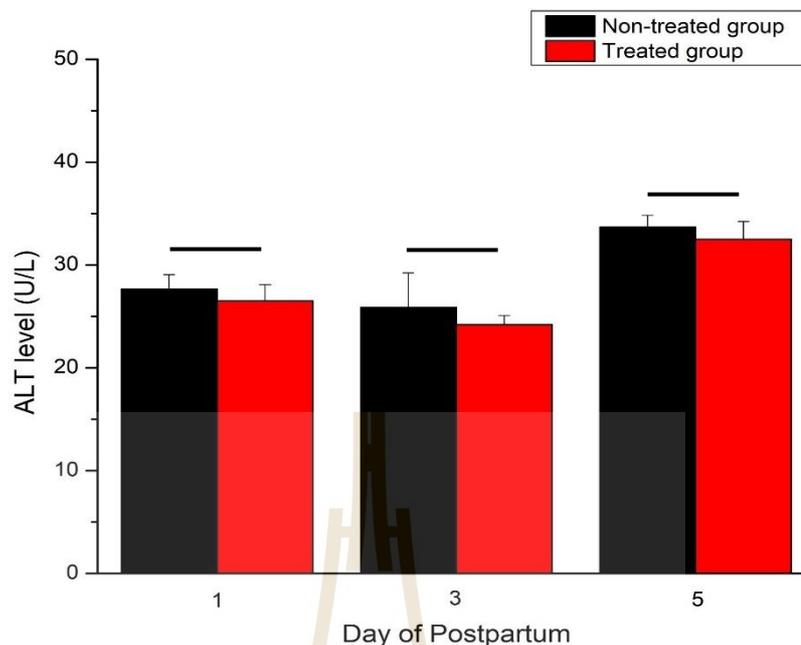
Figure 6.4 A graph showing the effects of *H. indicum* extract on estradiol level in postpartum rat blood. When the data between groups at the same day of postpartum are compared. Day 1 postpartum, estradiol level of treated rats was significantly decreased when compared with non-treated rats. The estradiol level was similar in both groups on day 3 and 5 postpartum. Bars represent mean ± S.E.M. Values that do not share the same superscript letters are significantly different ($P < 0.05$).

6.4.4 Effects of *H. indicum* extract on progesterone level

The progesterone level was measured after delivery on day 1, 3, and 5 postpartum from non-treated rats and treated rats. The results showed the progesterone level were significantly increased on day 1, 3, and 5 postpartum in both groups (Table 6.4). When the data between groups at the same day of postpartum were compared, the progesterone level of both groups on day 1 and 3 postpartum were similar. On day 5 postpartum, progesterone level of treated rats was significantly decreased when compared with non-treated rats (Figure 6.5).

Table 6.4 Effect of *H. indicum* extract on progesterone level in postpartum rat blood.

Group	Progesterone level (nmol/L) (Mean ± S.E.M.)
Non-treated rats	
Day 1 postpartum	38.20 ± 2.53^a
Day 3 postpartum	72.12 ± 0.64^b
Day 5 postpartum	151.20 ± 0.92^c
Treated rats	
Day 1 postpartum	37.74 ± 4.90^a
Day 3 postpartum	73.76 ± 0.67^b
Day 5 postpartum	129.60 ± 5.26^c

All values are expressed as mean + S.E.M. of 10 rats in each group ($n = 10$). When compare within group, means with different superscripted letters indicate statistical significance ($P < 0.05$).
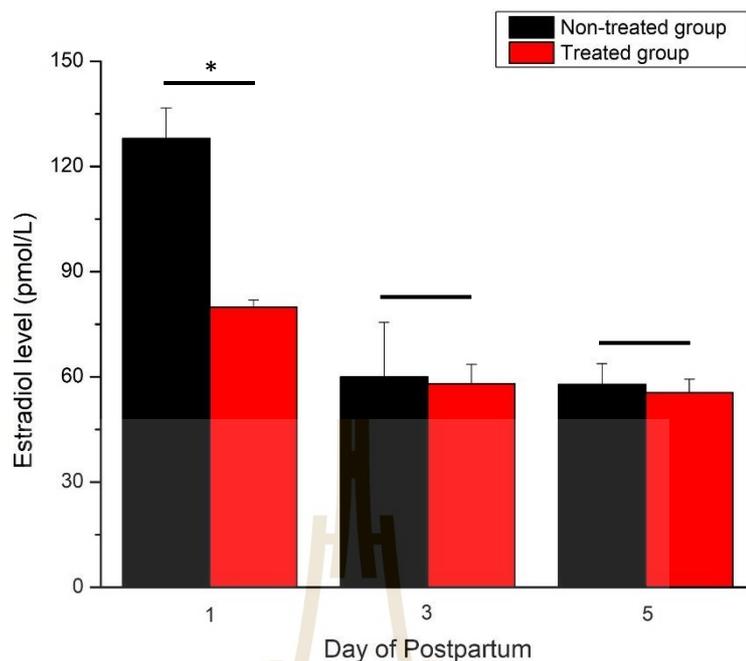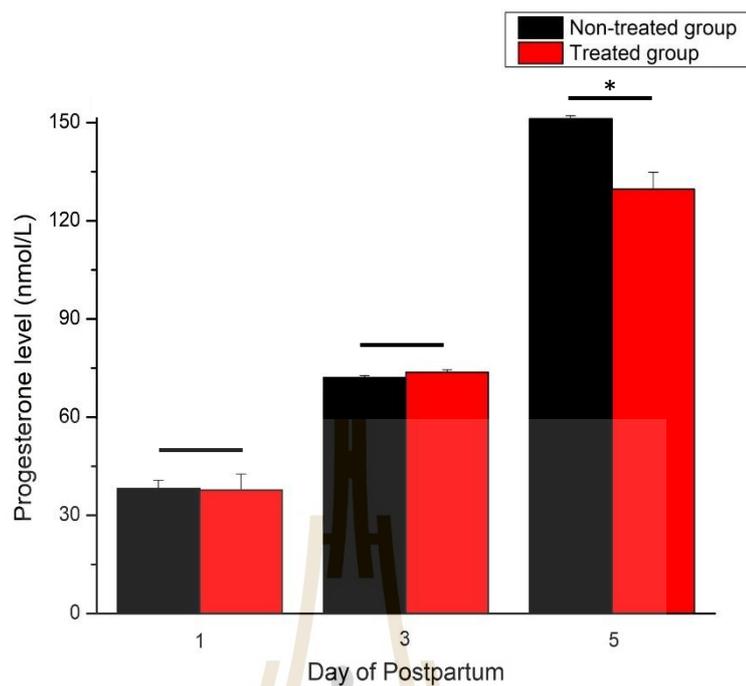
Figure 6.5 A graph showing the effects of *H. indicum* extract on progesterone level in postpartum rat blood. When the data between groups at the same day of postpartum are compared, the progesterone level of both groups on day 1 and 3 postpartum are similar. On day 5 postpartum, progesterone level of treated rats is significantly decreased when compared with non-treated rats. Bars represent mean ± S.E.M. Values that do not share the same superscript letters are significantly different ($P < 0.05$).

Table 6.5 Summary of blood biochemical parameter AST, ALT, AST/ALT, estrogen, and progesterone.

Group	AST level (U/L)	ALT level (U/L)	AST/ALT	Estradiol (pmol/L)	Progesterone (nmol/L)
Non-treated rats					
Day 1 postpartum	80.29 ± 7.48	27.67 ± 1.40	2.90	128.04 ± 8.65	38.20 ± 2.53
Day 3 postpartum	55.38 ± 4.56	25.88 ± 3.35	2.14	59.98 ± 15.61	72.12 ± 0.64
Day 5 postpartum	65.78 ± 4.00	33.70 ± 1.12	1.95	57.85 ± 5.94	151.20 ± 0.92
Treated rats					
Day 1 postpartum	83.86 ± 9.83	26.50 ± 1.61	3.16	79.90 ± 1.97	37.74 ± 4.90
Day 3 postpartum	62.38 ± 5.02	24.20 ± 0.88	2.58	58.02 ± 5.54	73.76 ± 0.67
Day 5 postpartum	69.40 ± 5.49	32.50 ± 1.73	2.14	55.52 ± 3.83	129.60 ± 5.26

6.5 Discussion

The whole plant of *Heliotropium indicum* L. (*H. indicum*) is a traditional medicinal plant that used in many parts of the world. The study of blood biochemical parameters can explain the effects of *H. indicum* extract on the body and the safety of tradition used. The typical parameters of hepatotoxicity were indicators of hepatocellular injury: alanine aminotransferase (ALT) and aspartate aminotransferase (AST) (Boone et al., 2005). In this study, the whole plant of *H. indicum* extract was used to study the effects of *H. indicum* extract on blood biochemical parameters in postpartum rats on days 1, 3, and 5 postpartum. The rats were divided into 2 main groups: non-treated rats and treated rats. The extract of *H. indicum* was diluted to 250 mg/kg B.W. in the vehicle (Tween 80, 10% v/v) and was administered orally to the treated rats at a volume of 0.8 mL/day for day 1, 3, and 5 postpartum. Only the vehicle was administered orally to the residue group. After administered orally, the blood samples were collected and measured blood biochemical parameters from serum. Blood biochemical parameters include alanine aminotransferase (ALT), aspartate aminotransferase (AST), estradiol, and progesterone.

From the literature reviews, the postpartum rats showed AST and ALT were 191.11 and 170.65 (U/L) respectively (Saidua et al., 2018). The study of haematology and serum chemistry parameters of the pregnant rat showed ALT level in non-pregnant rats was 58.9 U/l[4] and in postnatal days 1to 9 was 91.4 U/l[4] (LaBorde et al., 1999). Moreover, female Wistar rats were investigated about the activities of serum liver enzymes and the AST/ALT ratio. The AST, ALT, and AST/ALT ratio were 189.17 (U/L), 17.06 (U/L), and 11.09 respectively (Ndem and Ewere, 2016). Moreover, normal serum ALT is 7-56 U/L (Kallei et al., 1964) and normal serum AST is 0 to 35 U/L. The

AST levels with pregnant patient in hemolysis is 81 U/L (Thapa and Anuj, 2007). In this study, the results showed AST level of non-treated rats on day 1, 3, and 5 postpartum were 80.29 ± 7.48, 55.38 ± 4.56, and 65.78 ± 4.00 (U/L), respectively. The AST level of treated rats on day 1, 3, and 5 postpartum were 83.86 ± 9.83, 62.38 ± 5.02, and 69.40 ± 5.49 (U/L), respectively. When compared between groups at the same day of postpartum, the AST level of both groups showed no significant on day 1, 3, and 5 postpartum (Figure 6.2).

In the part of ALT level, the results showed ALT level of non-treated rats on day 1, 3, and 5 postpartum were 27.67 ± 1.40, 25.88 ± 3.35, and 33.70 ± 1.12 (U/L), respectively. The ALT level of treated rats on day 1, 3, and 5 postpartum were 26.50 ± 1.61, 24.20 ± 0.88, and 32.50 ± 1.73 (U/L), respectively. ALT level on day 5 postpartum showed a significant increase when compared with day 1 and 3 postpartum in both groups (Table 6.2). When compared between groups at the same day of postpartum, the ALT level of both groups showed no significant on day 1, 3, and 5 postpartum (Figure 6.3). Moreover, the AST/ALT ratio of non-treated rats on day 1, 3, and 5 postpartum were 2.90, 2.14, and 1.95, respectively. The AST/ALT ratio of treated rats on day 1, 3, and 5 postpartum were 3.16, 2.58, and 2.14, respectively (Table 6.5).

When the liver was injured or inflamed its reveals to various forms of toxic substances, the level of ALT and AST in the blood is normally raised. Therefore, tissue damage is directly related to the level of these enzymes in the blood (Ndem and Ewere, 2016). But in this case study in postpartum rats, there are some commentaries for the higher than normal in AST and ALT concentration. Firstly, both of AST and ALT are vastly distributed, the liver, kidney, heart, and muscle. The intracellular enzymes were released into the circulation by trauma to these tissues. The caesarean section and

perineal trauma implicate tissue damage, particularly skeletal muscle and myometrium, and this may explain the changes in these levels. The effect of exercise is a next reason for the upswing in the transaminase level. The labor, destruction of mitochondria in skeletal, cardiac and uterine muscle cells was stimulating the mitochondrial isoenzyme fraction of AST. The increase in AST and ALT after delivery may be partially described by enzyme release from contracting myometrium. The last reason is changes in blood volume and the blood concentration during the puerperium. After delivery, the haemoconcentration with loss of plasma volume is beginning and the blood volume slowly returns to nonpregnant levels. This period would be show increase transaminase levels (David et al., 2000). For these reasons, the AST and ALT level in this study did not show an abnormality of the liver. The *H. indicum* extract has no hepatotoxicity when taken within 5 day in the postpartum rats.

The next blood biochemical parameters in this study are estradiol and progesterone. Estradiol and progesterone is the 2 principal hormones implicated in the regulation of ovarian function and control of the reproductive cycle. Moreover, both of 2 hormones has effects on female reproductive organ (Molina, 2013). In this study, the results showed estradiol level of non‑treated rats on day 1, 3, and 5 postpartum were 128.04 ± 8.65, 59.98 ± 15.61, and 57.85 ± 5.94 (pmol/L), respectively. Estradiol level of treated rats on day 1, 3, and 5 postpartum were 79.90 ± 1.97, 58.02 ± 5.54, and 55.52 ± 3.83 (pmol/L), respectively. Day 1 postpartum, estradiol level of both groups was significantly increased when compared with day 3 and 5 postpartum (Table 6.3). When the data between groups at the same day of postpartum were compared. Day 1 postpartum, estradiol level of treated rats was significantly decreased when compared with non‑treated rats. The estradiol level was similar in both groups on day 3 and 5

postpartum (Figure 6.4). The results indicated that *H. indicum* extract decreased estradiol level on day 1 postpartum. The results showed progesterone level of non-treated rats on day 1, 3, and 5 postpartum were 38.20 ± 2.53, 72.12 ± 0.64, and 151.20 ± 0.92 (nmol/L), respectively. Progesterone level of treated rats on day 1, 3, and 5 postpartum were 37.74 ± 4.90, 73.76 ± 0.67, and 129.60 ± 5.26 (nmol/L), respectively. The progesterone level was significantly increased on day 1, 3, and 5 postpartum in both groups (Table 6.4). When compared between groups at the same day of postpartum, the progesterone level of both groups on day 1 and 3 postpartum were similar. On day 5 postpartum, the progesterone level of treated rats was significantly decreased when compared with non-treated rats (Figure 6.5).

Uterine involution is the process occurs in postpartum period by which the uterus and the reproductive organs returning to its non-pregnant state (Bassam, 2009). Uterine involution shows decreases size and weight of the uterus and returning the position to its pre-pregnant. The transition of uterine involution occurring during the first few days after childbirth (Medan, 2015). In rat uterus during the postpartum period, involution is a wide remodeling method controlled by the rapid removal of collagen. 85% of the total collagen during the end of pregnancy is erased within 4 days by the manipulation of oestradiol-17 at the time of childbirth (Ryan and Woessner, 1972). The study of the effect of hormones on collagen metabolism and collagenase activity in the guinea pig found progesterone inhibit collagenase activity clearly while estrogen was less effective (Wahl et al., 1977). The results from this study showed the *H. indicum* extract significant decreased estradiol level on day 1 postpartum. Moreover, the *H. indicum* extract was significantly decreased progesterone level on day 5 postpartum. From the literature review, the decrease of estradiol and progesterone can cause high

collagenase activity resulted in reduces the size and weight of the uterus on day 1 and 5 postpartum, respectively.

The role of progesterone is maintaining uterine quiescence during pregnancy, restraining prostaglandin synthesis and modulating the immune response to preserve pregnancy (Molina, 2013). In the myometrium, the uterine contractile activity was increased by gap junctions, that low resistant pathways between the smooth muscle cells, which increases electrical (Lye et al., 1993). Cx-43 is the transcripts encoding of the gap junction in the uterus. The levels of Cx-43 transcripts, protein, and gap junctions reduce rapidly in postpartum. Cx-43 proteins are significant enlargement before parturition and highest during delivery in the rodent. The myometrial Cx-43 transcript levels being managed positively by estrogen and negatively by progesterone during pregnancy. Postpartum hemostasis and the process of uterine involution were simplified by coordinated contractions (Lye et al., 1993). Gap junctions in the longitudinal muscle were increased numbers in rats killed 3 h after parturition. The studies show that a decline in progesterone levels were followed by extending in estradiol and $PGF_{2\alpha}$ are concurring with the formation of gap junctions. Gap junctions may correlate to the increased uterine activity needed for parturition. (Puri and Garfield, 1982). The results from this study showed the *H. indicum* extract significant decreased estradiol level on day 1 postpartum. Moreover, the *H. indicum* extract was significantly decreased progesterone level on day 5 postpartum. From the literature review, the decreased of progesterone resulted in high estradiol and $PGF_{2\alpha}$ are concurring with the formation of gap junctions may relate to the increased uterine contraction on day 5 postpartum.

The effect of estrogen on mammary gland is stimulates enlargement and

differentiation of the ductal epithelium, influences mitotic activity of duct cells, and provokes the development of connective tissue. Moreover, estrogen can also indirectly affect mammary gland development by promoting prolactin and progesterone levels and leading progesterone receptors in mammary epithelium. Progesterone preventing milk protein synthesis before parturition by antagonizes prolactin. In parturition, progesterone was dropping in circulating that connected with a simultaneous increase in prolactin secretion and the onset of lactation (Molina, 2013). The results from this study showed the *H. indicum* extract significant decreased estradiol level on day 1 postpartum. Moreover, the *H. indicum* extract significantly decreased progesterone level on day 5 postpartum. From the literature review, the decrease of progesterone also can cause high prolactin. Taken together, the *H. indicum* extract can increase in milk production on day 5 postpartum.

In this study, the effects of the whole plant extract of *H. indicum* on blood biochemical parameters including alanine aminotransferase (ALT), aspartate aminotransferase (AST), estradiol, and progesterone were investigated in postpartum rats. As discussion earlier, the AST and ALT level in this study did not show an abnormality of the liver. The *H. indicum* extract has no hepatotoxicity when intake within 5 day in the postpartum rat. Moreover, the extract showed the decrease of estradiol and progesterone which may cause high collagenase activity that reduces the size and weight of the uterus on day 1 and 5 postpartum, respectively. In the uterus, the extract decreased progesterone resulted in high estradiol and $PGF_{2\alpha}$ are concurring with the formation of gap junctions that may correlate to the increased uterine contraction on day 5 postpartum. Also, the extract decreased progesterone level on day 5 postpartum resulted in high prolactin that may relate to increase in milk production.

Therefore, the whole plant of *H. indicum* extract can be consumed during the postpartum period to accelerate uterine involution and induce milk production in rats. These results support the traditional use of the plant in the help uterine involution and the lactation insufficiency.
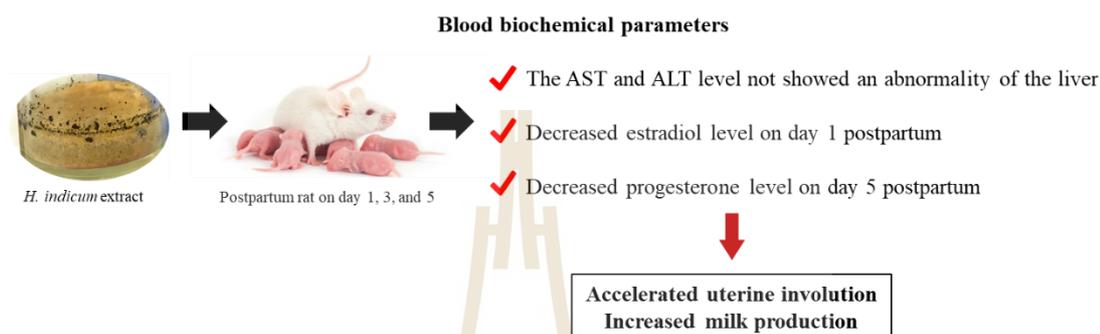
Figure 6.6 Diagram show the effects of *H. indicum* on blood biochemical parameters in postpartum rats.

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CHAPTER VII
CONCLUSIONS

Maternal death is the death of a woman while pregnant or in 42 days of ending of pregnancy from some reasons associated with or worsen by the pregnancy except from accidental or incidental causes (WHO et al., 2015). In some areas have inequity in access to health services and emphasizes the gap between rich and poor result in the high total of maternal deaths. The 94% of all maternal deaths happen in low and lower middle-income countries. Trends in estimates of maternal mortality ratio (maternal deaths per 100,000 live births, MMR) between 2000 and 2017, by country Thailand in 2017 had MMR at 37 (WHO et al., 2019). Most of these problems evolve while pregnancy and most are protectable or treatable (WHO, 2016). Postpartum hemorrhage (PPH) is the most reason of maternal death (Smith, 2016). Uterine involution is the process occur in postpartum period by which the uterus and the other reproductive organs returning to non-pregnant period (Bassam, 2009). The uterus in the postpartum period returns weight and size, and regains its muscular tone to the pre-pregnant state. Bleeding is controlled by contraction of the muscles. Muscular tone can decrease the risk of PPH and constrict the blood vessels, result in reduce blood flow. This is the initial state of involution (Maibenco, 1960). Breastfeeding is understood to move up growth in children, mainly sufficient in terms of energy, protein, nutrients, water, and other. Many studies have exhibited the benefits of breastfeeding to both mother and baby. The full breastfeeding up to six months of age were suggested by the World

Health Organization (WHO) with prolonged breastfeeding along with suitable supplementary meals up to two years of age or beyond (WHO, 2009). The children between birth and their sixth month have only 40% worldwide are breastfed exclusively (WHO, 2020). The baby who have been breastfed optimally have a decreased the risk of common childhood sickness for example gastrointestinal and respiratory infections. The literature review suggests that exclusive breastfeeding for six months (versus three to four months) decline rates of gastrointestinal infection, help appropriate development and can assist the mother lose weight (Story and Parish, 2008). Moreover, the infants breastfed exclusively for six months have lower the chance of progressing obesity compared with infants exclusively breastfed for one month (Kalies et al., 2005).

From the worldwide problem about maternal death and breastfeeding include quality and quantity that results many study the effect of medicinal plant for solving problems. Currently several countries used traditional therapeutically methods for severe bleeding and increase breast milk production. In Thailand, the medicinal plant has also been used extensively in a variety of folk medicine and there have been used orally to treat uterine involution and milk production. However, some traditional medicinal plants no have scientific data for supported their effects on PPH, uterine involution, and milk production. In this study, the medicinal plant, *Heliotropium indicum* L. (*H. indicum*) was investigated. The effects of *H. indicum* extract on uterine involution and mammary gland in postpartum rats using laboratory animal models were carefully examined. There were five main aims, which were: 1) to analyze phytochemical components of the extract, 2) to study the effects of the extract on uterine contractions, 3) to study the effects of the extract on uterine involution, 4) to study the effects of the extract on histological structures by H&E staining and to study

biochemical components of the uterus and mammary glands by Fourier Transform Infrared spectroscopy (FTIR), and 5) To study the effects of ethanol extract of *H. indicum* on blood biochemical parameters in postpartum rats. The results from this study can be used as scientific data to support their effects on uterine involution to help reduce the risk of PPH and increase breast milk production. Moreover, the results of this study are to provide basic knowledge for further research to develop drugs or products to maternal care. The major findings can be concluded as follows.

7.1 Phytochemical component of *H. indicum* extract

The herbal medicines were used in many parts of the word for therapeutic and nutritional from different plants around the word. The drugs were synthesized from novel chemical entities, an excellent bio-resource of plants (Ncube et al., 2008). Therapeutic features of medicinal plants are depending on the diverse secondary metabolites such as flavonoids, alkaloids, saponins, glycosides, tannins, and sterols, their use as active compound in medicinal preparations (Taylor et al., 2001). The ethnopharmacological project revealed that *H. indicum* is believed to be useful in abdominal injury, treating malaria, and skin disease. In Jamaica, people used the flower by taken orally for the nursing of menorrhagia in females (Roy, 2015). In Thailand, the dried flower and root of *H. indicum* is believed to expel the blood from the uterus during menstruation (Thiengburanathum, 1999). Moreover, *H. indicum* have effects that stimulated uterine contraction (Chaichanthipphayuth, 1980). Other folk cures include used of water extract of the leaves for healing of fever, urticaria, skin rashes, menstrual derangement, and diarrhea. The leaves of *H. indicum* were macerated with sugar cane juice is to be useful in curing insect stings and scorpion stings (Roy, 2015).

In this study, the primary phytochemical screening, GC-MS analysis, and FTIR analysis of whole plant *H. indicum* extracts were investigated. The results have shown the whole plant of *H. indicum* composed of alkaloids, tannins, flavonoids, steroids, and glycoside. Their medicinal activity and physiological activity were also summarized in this study (Table 3.2). Furthermore, *H. indicum* GC-MS analysis showed many constituents such as acetic acid (Ryssel et al., 2009), neophytadiene (Carretero et al., 2008), hexadecanoic acid (Abubakar and Majinda, 2016), hexadecanoic acid, ethyl ester (Sudha et al., 2013), and 9,12-Octadecadienoic acid (Z,Z)- or linoleic acid ester (Peyrat-Maillard et al., 2003), which have an effective anti-inflammatory, antimicrobial, antioxidant, and anticancer (Table 3.4). The presence of phytochemical constituent by GC-MS of the *H. indicum* extract is probably responsible for traditional uses. Additional evidence created from the FTIR analysis of whole plant extract of *H. indicum* has shown the presence of functional groups such as alcohols, phenols, carboxylic acids, amines, flavonoid, phosphate compound, glycoside, lignins, and tannins, thus exposing the presence of vital phytoconstituents (Table 3.5). The phytochemical constituents such as alkaloids, tannins, and glycoside showed effects on uterine contraction (Gabay, 2002). Alkaloids that found in *H. indicum* extract can increase smooth muscle contraction from guinea pig (Pomeroy and Raper, 1971). Tannins showed a greater proportion of N absorbed in the lower gut, which leads to depressing urea synthesis and greater milk protein production (Jimenez, 2018). The increased prolactin levels and expanding milk supply because of herbal galactagogues, most of them are believed to put their pharmacologic effects through interactions with dopamine receptors (Gabay, 2002).

In this study, the animals were recognized regularly for 24 hours for toxic

conditions such as behavioral transformation, motion, seizures, and mortality. The results suggested that non-treated rats and treated rats did not show any data of acute toxicity of irregular clinical signs or death during experiment period. Moreover, the infant did not show death or disable. From the results, the primary phytochemical screening, GC-MS analysis and FTIR analysis suggested that *H. indicum* extract are pharmaceutically significant due to the presence of the diverse medicinally phytochemical components and secondary plant metabolites. Even though their specific functions of constituents were not being examined in this study. The findings therefore confirmed traditional uses and revealed that *H. indicum* could accelerate uterine involution, induce uterine contraction, and increase milk production.

7.2 Effects of *H. indicum* extract on uterine involution in postpartum rats

Postpartum hemorrhage (PPH) is the most cause of maternal mortality. Postpartum bleeding or PPH is symptoms defined as the loss of more than 500 ml or 1,000 ml of blood within the first 24 hours later parturition (Smith, 2016). Uterine involution is the process occur in postpartum period by which the uterus and the other genital organs returning to its pre-pregnant state (Bassam, 2009). The uterus in the postpartum period returns size, position in the pelvic cavity, and regains its muscular tone to the pre-pregnant state. Moreover, the bleeding is controlled by contraction of the muscles. Muscular tone can decrease the risk of PPH and contraction of the muscles constricts the blood vessels, causing less blood flow. This is the early state of involution (Maibenco, 1960).

In this study, the effects of *H. indicum* extract on uterine contraction, uterine

weight, uterine size, percent collagen in the uterus, and biochemical component on uterine tissue of rats in postpartum period were investigated. From the results of this study, the *H. indicum* extract can induce uterine contraction and may be synergistic with oxytocin-induced uterine contraction in postpartum rats. Moreover, the effects of *H. indicum* extract after oxytocin-induced uterine contraction (in the continued presence of oxytocin) were more powerful to increase uterine contraction on day 1 and 3 postpartum. Thus, combination of *H. indicum* extract and oxytocin is useful, especially after received oxytocin. The study clearly showed that the *H. indicum* extract can be used with oxytocin to help accelerate uterine involution. Further, if the mother did not response oxytocin, the *H. indicum* extract can be used to induce uterine contraction. This study on rat uterus gives the primary proofs that *H. indicum* ethanolic extract to produce contractility effects on spontaneous contractions. According to the previous study, the possible mechanism(s) may be due to the stimulation of uterine contraction by the non-genomic active pathway. There are 2 ways in which the substance will active; 1) activated L-type Ca^{2+} channel for increased Ca^{2+} influx into the cell and 2) activated Ca^{2+} in sarcoplasmic reticulum by IP_3 receptors. The extract did not work through estrogen receptors as the extract did not further increased contraction in the presence of estrogen antagonist such as fulvestrant (Kupittayanant and Kupittayanant, 2012). Moreover, the studying the site of action of an aqueous extract of *H. indicum* on smooth muscle reported the extract can induce uterine contraction. The extract has chemical components that have stimulatory effects at adrenoceptors and possible enhance prostaglandin synthesis (Koffuor et al., 2012).

Furthermore, the results also showed that the extract can accelerate the reducing rate of uterine weight, uterine size and the collagen of the uterus in postpartum

period. In the part of FTIR study, the *H. indicum* extract have some effects on biochemical components of the uterine tissue on day 1, 3, and 5 postpartum. The *H. indicum* extract can interfere the pattern of lipid, protein, and carbohydrate and nucleic acid. Especially, Band No. 5 that represent to collagen were significantly decreased on day 3 postpartum. Postpartum blood loss or postpartum hemorrhage were prevented by the uterus contraction after delivery (Takamoto et al., 1998). The force of the uterine contractions and the spiral arteries contraction can temporarily cause ischemia of the endometrium (Choi et al., 2012). After the cells lack of nutritional and oxygen cause of autophagy (Flake et al., 2013). The physiological role of autophagy during uterine remodeling incidents appearing in the postpartum period reason of regress of size and weight of uterus (Hsu et al., 2014). Moreover, the involuting uterus through a quick diminution in size primarily due to the degradation of the extracellular matrix, especially collagen for the uterus returns to its pre-pregnant state. The major proteinases that degrades collagen and is the most abundant in the uterus (Manase et al., 2006). The uterine smooth muscle cells synthesize collagenase, and this collagenase production were repressed by progesterone *in vitro* (Takamoto et al., 1998). The results from this study is the scientific data to support the effects of *H. indicum* extract on uterine tissue in postpartum period. Moreover, the extract can help accelerate uterine involution in postpartum rats by enhance uterine contraction and reduce the uterine weight, the uterine size, and the collagen of the uterus. So, the whole plant of *H. indicum* extract can be consumed during postpartum period for help reduce the risk of postpartum hemorrhage. The findings therefore confirm traditional uses that *H. indicum* could accelerate uterine involution.
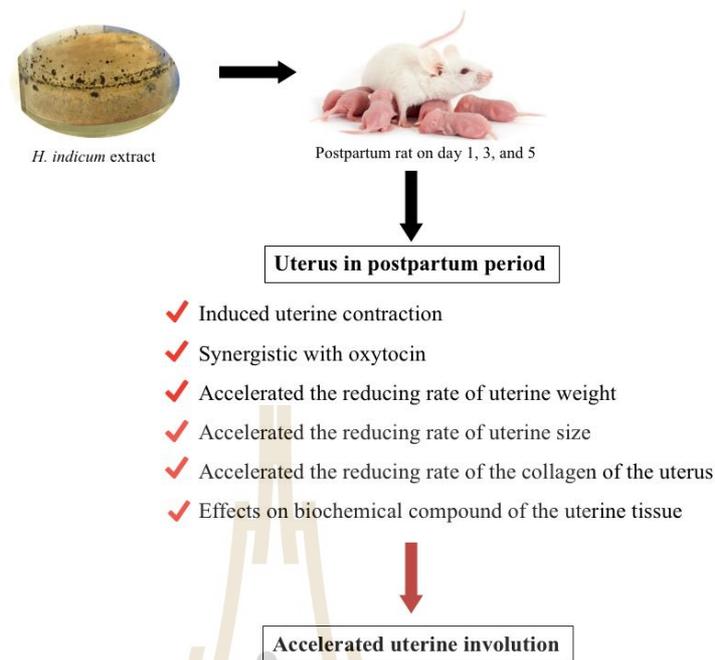
Figure 7.1 The summary diagram shows the effects of *H. indicum* on uterine involution in postpartum rats.

7.3 Effects of *H. indicum* extract on mammary glands in postpartum rats

The maternal care was applied by traditional medicinal plant such as involution and breastfeeding. Galactagogue is food or drug that promote the flow of mother's milk, it has benefit for women who are powerless to produce breast milk on their own due to baby prematurity, sickness of the mother or child, adoption, or representative motherhood (Gabay, 2002). *H. indicum* contains several phytochemical compounds including alkaloids, tannins, flavonoids, steroids, and glycoside which may explain its application in traditional medicine. The phytochemical compounds such as alkaloids, tannins, and glycoside show effect on uterine contraction. The increased prolactin

levels and expanding milk supply because of herbal galactagogues. Galactagogues are beneficial for women who are powerless to produce breast milk on their own due to baby prematurity, sickness of the mother or child, adoption, or representative motherhood (Gabay, 2002). Histological structures of the mammary gland are composed of two components, the parenchyma (the tissue achieve the special function of the organ) and the surrounding stroma (connective tissue framework of the organ). The parenchyma composed of the alveoli, grape-like clusters where milk is stored, and branching ducts, which are tubular canals taking glandular secretions. (Treuting and Dintzis, 2012).

In this study, the effects of *H. indicum* extract on alveoli size, the ratio of parenchyma cells and stroma cells, and biochemical component on mammary gland tissue of rats in postpartum period were investigated. The effects of *H. indicum* extract on mammary gland in postpartum rat (non-treated and treated rats) was observed on day 1, 3, and 5 postpartum and compared both of within group and between groups. The results revealed that *H. indicum* extract can help increase alveoli size on day 1 postpartum and maintain the alveoli size till day 5 postpartum. The increasing size of alveoli cause increase the milk production. Moreover, the ratio of parenchyma cells and stroma cells in mammary gland study suggested that when compared within group in treated rats the ratio was significantly increased on day 5 postpartum while non-treated rats unchanged. However, the comparison of ratio between groups at the same day of postpartum, treated rats received the extract showed significant increases in the ratio of parenchyma cells and stroma cells in mammary gland on day 5 postpartum. The results indicate that the *H. indicum* extract causes the parenchyma cells proliferation resulted in increasing milk production. In the part of FTIR analysis, the results showed the whole

plant of *H. indicum* extract helps increase lipid and protein in mammary gland tissue which relates to lipid and protein synthesis in mammary epithelial cells. Therefore, the whole plant of *H. indicum* extract can be consumed during postpartum period to induce milk production in rats. This results also confirm the traditional use of the plant in the lactation insufficiency.
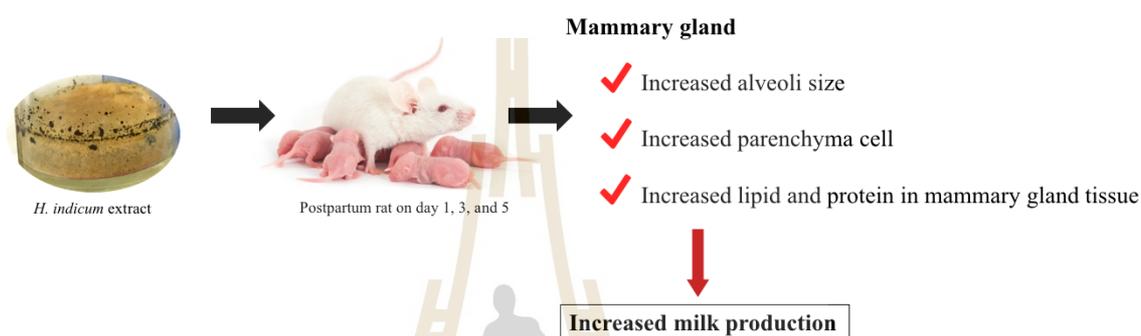

Figure 7.2 The summary diagram show the effects of *H. indicum* on milk production in postpartum rats.

7.4 Effects of *H. indicum* extract on blood biochemical parameters in postpartum rats

The whole plant of *Heliotropium indicum* L. (*H. indicum*) is a traditional medicinal plant that used in many parts of the world. The study of blood biochemical parameters can explain the effects of *H. indicum* extract on the body and the safety of tradition uses. The typical parameters of hepatotoxicity were indicators of hepatocellular injury: alanine aminotransferase (ALT) and aspartate aminotransferase (AST) (Boone et al., 2005). Other parameters are estradiol and progesterone, the 2 principal hormones implicated in the regulation of ovarian function and control of the reproductive cycle (Molina, 2013). The effects of *H. indicum* extract on blood

biochemical parameters in postpartum rats (non-treated and treated rats) was observed on day 1, 3, and 5 postpartum and compared both of within group and between groups. The extract was diluted to 250 mg/kg B.W. in the vehicle (Tween 80, 10% v/v) and was administered orally to treated rats at a volume of 0.8 mL/day for 1, 3, and 5 days, respectively. Only the vehicle was administered orally for the rests (non-treated rats). After administered orally, the blood samples were collected and measured blood biochemical parameters from serum. Blood biochemical parameters included alanine aminotransferase (ALT), aspartate aminotransferase (AST), estradiol, and progesterone. The results indicated that the AST and ALT level in this study did not show an abnormality of the liver. The *H. indicum* extract has no hepatotoxicity when taken within 5 days in the postpartum rats. Moreover, the extract showed the decrease of estradiol and progesterone which accelerated high collagenase activity that reduces the size and weight of the uterus on day 1 and 5 postpartum, respectively. In the uterus, the extract decreased progesterone resulted in high estradiol and $PGF_{2\alpha}$ which are concurring with the formation of gap junctions that may correlate to the increases in uterine contraction on day 5 postpartum. Also, the extract decreased progesterone level on day 5 postpartum which can cause high prolactin level that may relate to increases in milk production. Therefore, the whole plant of *H. indicum* extract can be consumed during the postpartum period to accelerate uterine involution and induce milk production in rats. These results support the traditional use of the plant in the help uterine involution and the lactation insufficiency.
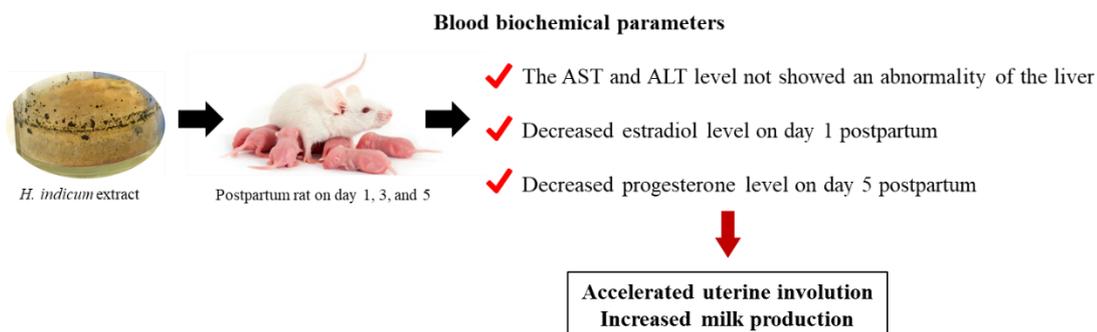
Figure 7.3 The summary diagram show the effects of *H. indicum* on blood parameters in postpartum rats.

7.5 Future research

The results clearly indicated that *H. indicum* is a medicinal plant with useful to accelerate uterine involution and reduce the chance of postpartum hemorrhage. Moreover, *H. indicum* can induce milk production for development and function in the newborn. However, this study was investigated in an animal model for physiological test. It would be interesting to discover the underlying mechanisms of this plant and studying the physiological interferences in a human model in the further.
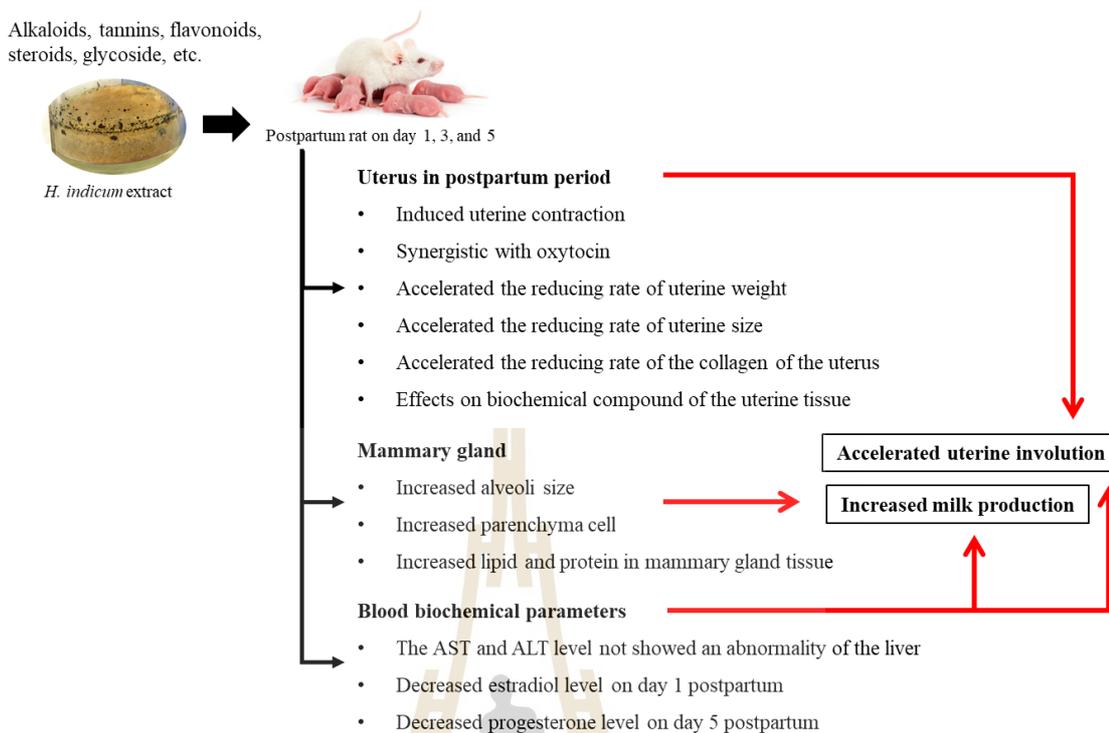
Figure 7.4 The summary diagram show the effects of *H. indicum* on uterine involution, milk production, and blood parameters in postpartum rats.

7.6 References

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