ESTROGENIC ACTIVITY OF *COSTUS SPECIOSUS* (Koen.) Sm. EXTRACT IN FEMALE RATS

Wanwisa Lijuan

A Thesis Submitted in Partial Fulfillment of the Requirements for the

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ฤทธิ์การเป็นเอสโตรเจนของสารสกัดจากเอื้องหมายนา (*Costus speciosus* (Koen.) Sm.)ในหนูแรทเพศเมีย

นางสาววันวิสาข์ ลิจ้วน

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

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วันวิสาข์ ลิจ้วน : ฤทธิ์การเป็นเอสโตรเจนของสารสกัดจากเอื้องหมายนา (*Costus speciosus* (Koen.) Sm.) ในหนูแรทเพศเมีย (ESTROGENIC ACTIVITY OF *COSTUS SPECIOSUS* (Koen.) Sm. EXTRACT IN FEMALE RATS) อาจารย์ที่ปรึกษา : รองศาสตราจารย์ สัตวแพทย์หญิง ดร.ศจีรา คุปพิทยานันท์, 240 หน้า.

เอื้องหมายนา (*Costus speciosus* (Koen.) Sm. เป็นพืชสมุนไพรพบทั่วไปในแถบเอเชียมีสรรพคุณเป็นยาแผนโบราณใช้ในการรักษาโรคหลายชนิด เอื้องหมายนาประกอบด้วยไฟโต-เอสโตรเจนหลายชนิดรวมทั้งไดออสจีนินและบีตาซิโตสเตอรอล วัตถุประสงค์หลักของการศึกษานี้คือศึกษาฤทธิ์การเป็นเอสโตรเจนของสารสกัดจากเหง้าและต้นเอื้องหมายนาในหนูแรทเพศเมียโดยศึกษา 1) ผลต่อมดลูก ช่องคลอด และเต้านม 2) ผลต่อระดับเอสตราไดออลและลิพิด โปรไฟล์ 3) ผลต่อการฝังตัวและ 4) ผลต่อการหดตัวของมดลูกและเปรียบเทียบผลกับสารมาตรฐาน พร้อมทั้งศึกษากลไกการออกฤทธิ์ทางสรีรวิทยา ผลการศึกษาพบว่าในสารสกัดจากเอื้องหมายนามีไดออสจีนินและบีตาซิโตสเตอรอลเช่นเดียวกับไฟโตเอสโตรเจนอื่นๆ การศึกษาฤทธิ์พบว่าการป้อนสารสกัดจากเอื้องหมายนาทั้งเหง้าและต้นขนาดต่ำและสูง (500 และ 1000 มก./กก.นน.) สามารถเพิ่มน้ำหนักมดลูก เพิ่มความหนาของเยื่อบุช่องคลอด เพิ่มจำนวนกระเปาะสร้างน้ำนมและท่อของเต้านมในหนูตัดรังไข่ สารสกัดจากเอื้องหมายนาไม่เพิ่มระดับเอสตราไดออลแต่ลดระดับคลอเรสเตอรอลและคลอเรสเตอรอลชนิดไม่ดี ที่เกิดจากการมีฤทธิ์คล้ายเอสโตรเจนของสารสกัดซึ่งไม่เกี่ยวข้องกับการออกฤทธิ์ผ่านโกนาโดโทรปินพิทูอิตารีย์โอวาเรียนแอกซิส นอกจากนี้พบว่าสารสกัดเอื้องหมายนาทั้งเหง้าและต้นมีฤทธิ์ต่อต้านการฝังตัวในหนูท้องระยะก่อนการฝังตัว โดยเอื้องหมายนามีผลไปรบกวนสมดุลระหว่างโปรเจสเตอโรนและเอสโตรเจนตลอดจนไปเพิ่มการหดตัวของมดลูก เมื่อศึกษาผลของสารสกัดเอื้องหมายนาต่อสรีรวิทยาการหดตัวของมดลูกพบว่าสารสกัดจากเหง้าและต้นเอื้องหมายนาสามารถเพิ่มการหดตัวของมดลูกในหนูปกติและหนูตัดรังไข่ได้ โดยมีฤทธิ์สูงสุดที่ 10 และ 30 มก./100 มล. ตามลำดับ โดยกลไกการออกฤทธิ์ไม่ได้เกิดจากการมีฤทธิ์เป็นเอสโตรเจนแต่เกิดจากการเพิ่มการหดตัวซึ่งเกิดจากการเข้าสู่เซลล์ของแคลเซียมผ่านแอลไทป์แคลเซียมชาแนลและการหลั่งแคลเซียมจากซาโคพลาสมิคเรติคูลัม สรุปได้ว่าเอื้องหมายนาทั้งเหง้าและต้นมีฤทธิ์การเป็นเอสโตรเจน

สาขาวิชาชีววิทยา ลายมือชื่อนักศึกษา_____

ปีการศึกษา 2553 ลายมือชื่ออาจารย์ที่ปรึกษา_____

ลายมือชื่ออาจารย์ที่ปรึกษาร่วม_____

WANWISA LIJUAN : ESTROGENIC ACTIVITY OF *COSTUS SPECIOSUS* (Koen.) Sm. EXTRACT IN FEMALE RATS. THESIS ADVISOR : ASSOC. PROF. SAJEERA KUPITTAYANANT, Ph.D. (DVM) 240 PP.

COSTUS SPECIOSUS (Koen.) Sm./ ESTROGEN/ RAT/ UTERUS/ VAGINA/ MAMMARY GLAND/ OVARIECTOMIZED RAT/ CONTRACTION/ CALCIUM

Costus speciosus (Koen.) Sm. is a medicinal plant widely distributed in Asia. The plant is traditionally used in the treatment of various ailments. *C. speciosus* contains phytoestrogens including diosgenin and β-sitosterol. The main aim of this study was to study estrogenic activities of *C. speciosus* rhizome and stem extracts in female rats by investigating 1) the effects on uterus, vagina and mammary gland, 2) the effects on serum estradiol level and lipid profile, 3) the effects on implantation, and 4) the effects on uterine contraction and compared the effects to known compounds. The underlying mechanisms of the extracts were also investigated. The results revealed that *C. speciosus* extracts contained diosgenin and β-sitosterol as well as other phytoestrogens. The data showed that administered orally of the extracts both low and high doses (500 and 1000 mg/kg B.W.) increased relative uterine weight, vaginal epithelium thickness and mammary gland alveoli and ducts. The extracts did not increase serum estradiol level but decreased total cholesterol and low-density lipoprotein cholesterol. These estrogenic effects were not involved with gonadotropin-pituitary-ovarian axis. In addition, the extracts had anti-implantation effects during pre-implantation periods. These occurred via an alteration of progesterone and

estrogen balance as well as an increase in uterotonic activity. The investigation of physiological effects of *C. speciosus* extracts on uterine contractility exhibited that *C. speciosus* rhizome and stem extracts are potent stimulators of the uterus in both non-pregnant and ovariectomized rats as they increased spontaneous contraction with a maximum effect of 10 and 30 mg/100 mL, respectively. The mechanisms of action were due to non-estrogen effect, but increasing contraction via Ca^{2+} entry on L-type calcium channel and sarcoplasmic reticulum Ca^{2+} release. In conclusion, the study clearly showed that both *C. speciosus* rhizome and stem have estrogenic activity.

School of Biology

Academic Year 2010

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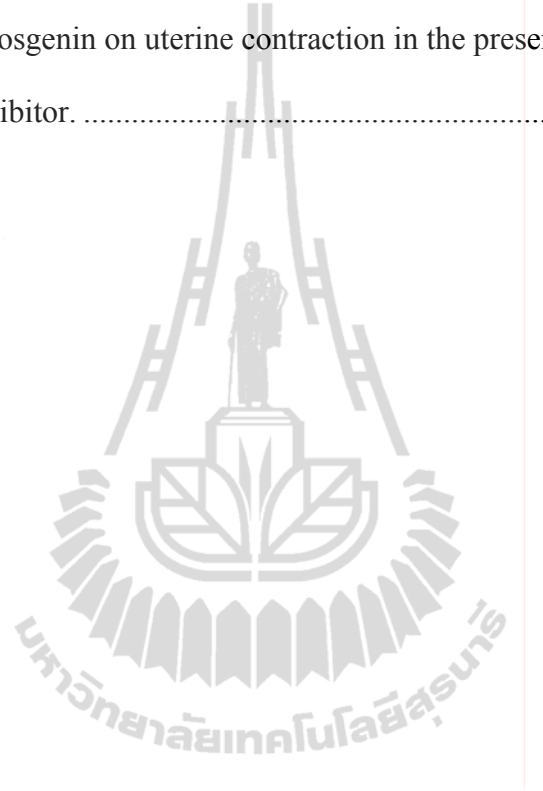

CHAPTER I

INTRDUCTION

1.1 Female Reproductive Cycle

A women's reproductive system produces sex hormones and functional gametes and also must be able to protect and support a developing embryo and nourish the newborn infant. The internal female reproductive organs are located in the lower abdomen and include the ovaries, fallopian tubes, uterus and vagina. The ovaries are located on both sides of and just superior to the medially located uterus. They produce ova and synthesis the female sex hormones that promote female sexual development. Uterus is a hollow and muscular organ, providing the space in which a fertilized ovum that mature into a child. Vagina is the muscular and fibrous tube that extends from the uterus to the outside. It provides a passage for uterine secretions, for the erect penis during intercourse and for the fetus at birth (Balusik, 2003). The maturity of reproductive organ begins at puberty, with the first menstruation and lasts until menopause, which is the cessation of reproductive function.

There are several important functional roles played by the female reproductive cycles. As shown in Figure 1.1, the changes associated with the different cycles are closely related. The primary role of ovarian cycle is to produce an ovum at regular intervals to make reproductive success. The secondary role of ovarian cycle is to regulate the endometrial (menstrual) cycle by means of the sex hormones, estrogen

and progesterone. The role of the endometrial cycle, in turn, is to ensure that the ling of the uterus is suitable for the implantation.

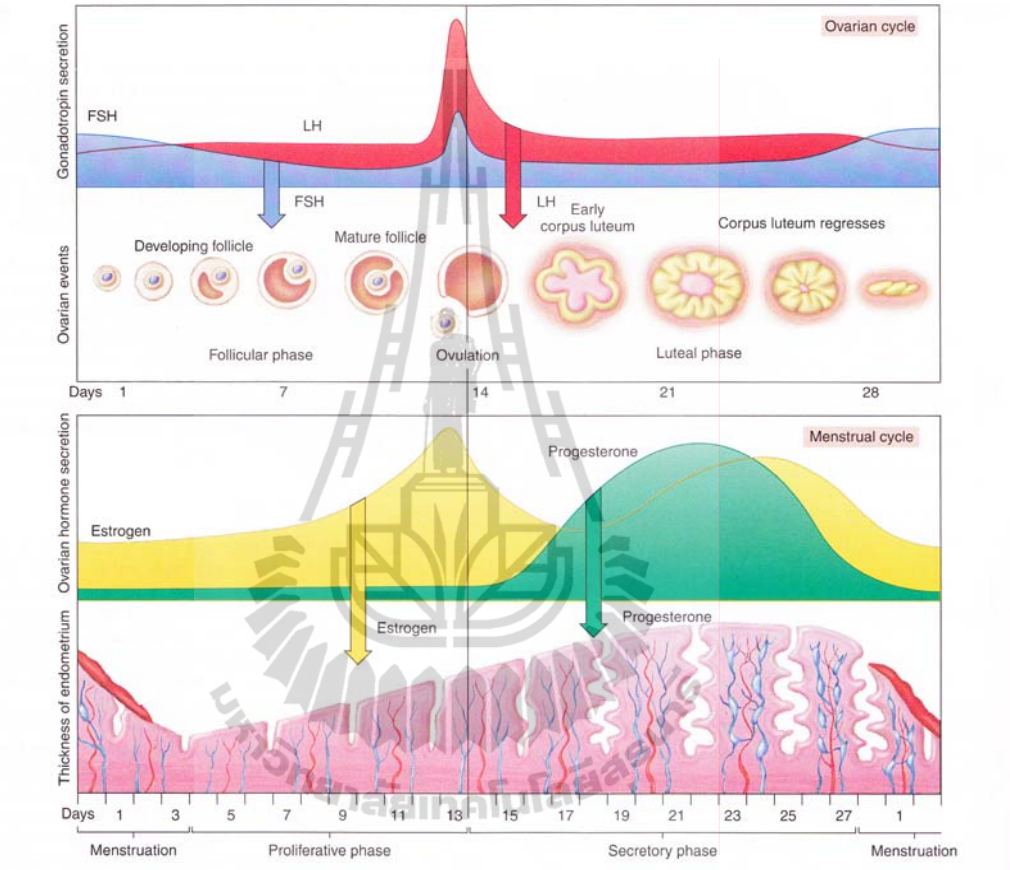

Figure 1.1 Diagrammatic represents female reproductive cycle (Fox, 2004).

1.1.1 Ovarian Cycle

The ovarian cycle is a regular pattern of growth, maturation and release of oocytes from the ovary. The ovarian cycle is divided into two phases, the follicular phase and the luteal phase. The follicular phase begins with the start of menstruation (day 1 of the menstrual cycle) and ends with ovulation; the luteal phase coincides with the remainder of the menstrual cycle (Stanfield and Germann, 2008).

During the follicular phase of the ovaries, some of the primary follicles grow, develop vesicle and become secondary follicles. Toward the end of the follicular phase, one follicle in one ovary reaches maturity and becomes a Graafian follicle. Follicle stimulating hormone (FSH) and luteinizing hormone (LH) stimulate follicle growth and maturation. FSH exerts its main effects on the follicle cells, whereas LH targets the thecal cells. As the follicles enlarge, LH stimulates the thecal cells to produce androgens. Follicular maturation is accompanied by rapid increase estrogen secretion, in turn, triggers the LH surge. Finally, the surge in LH secretion causes the wall of the Graafian follicle to rupture, resulting in ovulation.

The beginning of the luteal phase marks after ovulation. The ruptured follicle is then transformed into a gland, called the corpus luteum, which secretes estrogen and progesterone. If the oocyte is not fertilized, the corpus luteum reaches its maximum activity within 10 days of its formation and then begins to degenerate to scar tissue, called the corpus albicans. This degeneration causes a decline in plasma estrogen and progesterone levels that set the stage for menstruation and the beginning of the next follicular phase. If the oocyte is fertilized, the corpus luteum does not degenerate; instead it persists well into the gestation period (Stanfield and Germann, 2008).

1.1.2 Uterine Cycle

The uterine cycle is a series of structural and functional changes that occur in the endometrium of the uterus as it prepares each month for the possibility that a fertilized egg may arrive. It is divided into three phases: the menstrual phase, the proliferative phase and the secretory phase.

The menstrual phase, at first, blood vessels in the outermost layer of the endometrium begin to constrict, which reduces blood flow to the tissue. As a result, these tissues die and start to separate from the underlying endometrial tissue, which remain intact. The dead tissue gradually sheds from the endometrial surface, which causes rupture of blood vessels and bleeding.

The proliferative phase begins at the end of menstruation; the uterus renews itself in preparation for possible pregnancy, which might occur in the next ovulation. The endrometriul tissue that was spared from destruction in the menstrual phase begins to grow and the smooth muscle in the underlying myometrium thickens. The endometrial glands enlarge and blood vessels increase in abundance. Uterine changes in the proliferative phase are promoted by estrogens, whose plasma levels rise due to the growth an increasing secretory activity of the dominant follicle.

The secretory phase, the endometrium is transformed in such a way as to make it a favorable environment for implantation and subsequent housing and nourishment of the developing embryo. The blood supply of the endometrium becomes enriched as arteries branch. The endometrium glands enlarge further and begin to secrete fluids rich in glycogen, which the embryo uses as an energy source in its early stages of growth. These uterine changes are promoted by progesterone, whose plasma levels rise during the secretory phase. As the end of secretory phase approaches, the corpus luteum degenerates, causing plasma estrogen and progesterone levels fall. This decline causes a withdrawal of these hormones' growth and promoting influences on the endometrium trigger the previously described events of menstruation (Stanfield and Germann, 2008).

Vaginal cycle, in prepubertal and postmenopausal females, the vaginal epithelium is thin, which composed of a few layers of epithelial cells. In response to estrogens, this epithelium proliferates and subsequently consists of many more layers of epithelial cells. The histological of the vaginal epithelium changes in a characteristic manner during the menstrual cycle. Early in the cycle, the epithelium consists mainly of rounded basal cells that stain intensely with certain dyes. Maximal growth of the epithelium occurs during the periovulatory period. At this time, the basal cells are overlain with layers of more flattened cells; the outermost cells are very flat and keratinized (cornified). Toward the end of luteal phase, the vaginal epithelium becomes invaded with leukocytes and by the initiation of the next cycle (Hadley, 2007).

1.2 Implantation and Pregnancy

After a sperm enters an egg; its nucleus fuses with the egg's nucleus. The diploid cell that results from this fusion is called a zygote. Immediately following fertilization and while still in the fallopian tube, the zygote begins a series of mitotic divisions known as cleavage. Cleavage produces a ball of cells called a morula, which divide and release a fluid, resulting in a blastocyst. About six or seven after fertilization the blastocyst attaches to the uterine wall. After implantation the embryo continues to grow in size and complexity. At a certain stage, the embryo becomes a fetus which depends on the placenta for nourishment. After implantation, blastocyst slowly takes on the recognizable features of human infant. This nine months period of development is called pregnancy, which divided into three equal periods. Significant changes occur during each period (Prakash, 2007).

Hormone control of implantation: The uterus must be prepared for receiving the blastocyst in order to support implantation. Under estrogen domination, the uterus can not accommodate an implanting embryo and thus, premature entry of an embryo into the uterus results in its death. Generally, progesterone is comparatively high at the time of implantation. Under progesterone domination, the uterus is appropriately to engage in conversation with the blastocyst.

Hormone regulation of pregnancy: during the first three months of pregnancy, the corpus luteum is maintained by the actions of a hormone, human chorionic gonadotropin (hCG), which is secreted by placenta and exerts many effects as LH. The function of hCG is to maintain the corpus luteum of pregnancy beyond the period of menstrual cycle. It stimulates the corpus luteum to continue production of estrogen and progesterone, which are essential for the maintenances of the endometrium and also for the suppression of menstruation.

During pregnancy, plasma levels of estrogen and progesterone, which rise after formation of the corpus luteum, continue until parturition, at this time estrogen and progesterone levels fall. These hormones are important for maintaining the pregnancy and for preparing the mother's body for the delivery of the fetus. In pregnancy, progesterone exerts negative feedback on the hypothalamus and anterior pituitary that keeps rates of LH and FSH secretion low. Consequently, no new dominant follicles appear and no LH surges occur (Stanfield and Germann, 2008).

1.3 Menopause

Hormonal changes encounter fast in women, but proceed gradually in men (Jung, Jeon and Bai, 2008). Menopause is a natural process that occurs as part of

aging as a result of decreased estrogen and a disruption of menstrual periods for 12 months. It is defined by the World Health Organization (WHO) and the Stages of Reproductive Aging Workshop (STRAW) working group as the permanent cessation of menstruation resulting from loss of ovarian follicular activity (World Health Organization, 1996). Natural menopause determined retrospectively from the date of the last menstrual period after 12 months amenorrhea, with no other attributed causes. The age at which natural menopause occurs is between 45-55 years for women worldwide (Pathak and Parashar, 2010). Menopause is commonly divided into three phases (Figure 1.2); premenopause, perimenopause and postmenopause.

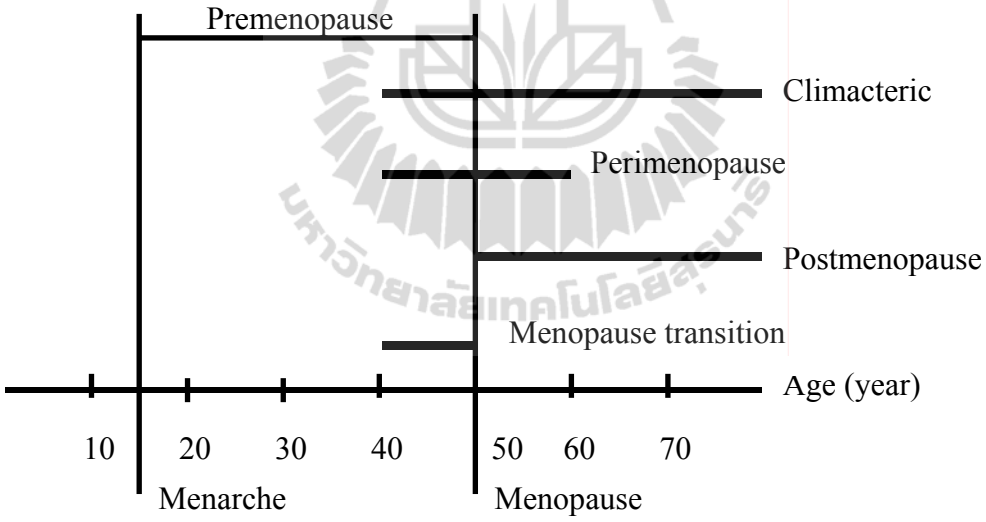

Figure 1.2 Diagrammatic represents changes in reproductive to non reproductive life in women (Chompootweep, Tankeyoon, Yamarat, Poomsuwan and Dusitsin, 1993).

Menopause is defined as the last menstrual period, which occurs retrospectively after a 12 months period of amenorrhea with no other pathological or physiological causes. Menopause transition is the time of menstrual irregularity before the last menstrual period. Premenopause is the entire reproductive time up to the last menstrual period. Perimenopause begins with the first clinical, endocrinological or

biological feature of menopause and ends 12 months after the last menstrual period. Postmenopause is defined as dating from the final menstrual period regardless of whether the menopause was induced or spontaneous. Climacteric is described as the transition from the reproductive to non reproductive state, in co-operating the perimenopause but extending for a longer and more variable time (Porter and Rees, 2002).

1.3.1 The Causes of Menopause

Reproductive function declines with aging in women. It stops at the time of menopause because of the depletion of ovarian follicles leading to the loss of ovarian hormones.

The normal functions of the hypothalamic-pituitary-ovarian axis are to control reproductive function. The hypothalamus secretes gonadotropin-releasing hormone (GnRH), which regulates the pituitary gland. In response to this, the pituitary produces gonadotrophins, including FSH and LH. These hormones are controlled by negative feedback from ovarian hormones, estrogen and progesterone.

The ovary contains of three main components, including granulosa cells, theca cells and ovarian stroma. Granulosa cells have FSH receptors, which FSH can convert androgens into estrogens. Theca cells have LH receptors, which LH produces primarily androgen and some estrogens. The ovarian stroma can produce all three classes of sex steroids; estrogen, androgen and progesterone. The number of oocytes produced is limited and maximal at 20 weeks of gestational age (7 million). From this time onwards, the number and quality of oocytes declines throughout life (Bruce and Rymer, 2009).

There are many hypotheses, but little is known about the exact mechanism involved. In the years leading up to menopause, there are irregular in length of the menstrual cycle, frequency of ovulation and concentrations of reproductive hormone (O' Connor, Holman and Wood, 2001). As a result of this, secretions of FSH and LH form the anterior pituitary gland increase due to the lack of negative feedback from estradiol. This gives rise to the postmenopausal state of hypogonadotrophic hypogonadism. The principal form of circulating estrogen in postmenopausal women is estrone, which is synthesized by peripheral conversion of androstenedione in the liver and adipose tissue (Longcope, Kato and Horton, 1969).

1.3.2 Symptoms of the Menopause

After reaching menopause, at least 85% of women have symptoms, the occurrence and intensity of which vary among individual (Jones and Lopez, 2006). Most menopausal symptoms can be classified into either physical or psychological in nature.

Physical symptom of the menopause is classically associated with the onset of vasomotor symptoms, which include hot flushes and night sweats. Hot flushes occur in 75% of postmenopausal women. It is attributed that they occur as a result of a central disorder of temperature regulation. The symptoms have characteristics of a heat dissipation response and consist of sweating on the face, neck and chest (Freedman, 2001). There is an acute rise in the skin temperature of several degrees centigrade (Sturdee and Reece, 1979), a transient increase in heart rate, a fluctuation of the electrocardiographic baseline, a pronounced decrease in skin resistance (Sturdee, Wilson and Pipili, 1978), and a slightly fall in the body's core temperature.

The symptoms usually last for 4-5 minutes. Other physical symptoms include urogenital tract changes (that occur to all tissues including epithelial thinning, reduced vascularity, decreased muscle bulk and increased fat deposition), irregular vaginal bleeding and menorrhagia, palpitations, headaches, bone and joint pain, asthenia, tiredness and breast tenderness. Most of the long-term symptoms associated with menopause are osteoporosis, cardiovascular disease and Alzheimer's disease.

Psychological symptoms are frequently reported around the time of the menopause. Symptoms include depression, loss of memory, irritability, poor concentration, tiredness and loss of confidence.

The determinants of experiencing menopausal symptoms are complex, representing biological and social factors. The prevalence of specific symptoms varies somewhat between national and ethnic groups but they are universal phenomena.

1.3.3 Hormone Replacement Therapy

Hormone replacement therapy (HRT) is indicated to manage problems caused, when the ovaries stop producing estrogen at the menopause. The resulting hypogonadal state may cause symptoms and detrimental changes in estrogen target tissues, including the brain, skeleton, skin and the cardiovascular and genitourinary systems. The concentration and function of hormone receptors varies in these organs and systems. Differences in genetics and general constitution may affect levels of circulating estrogen. Thus, there is variation between women in the development of menopausal symptoms, in the functional reaction of the target tissues to estrogen deficiency and in the response to HRT.

The broad aims of HRT are to reduce symptoms arising from estrogen depletion such as hot flushes, sleeplessness, lethargy, depression, vaginal dryness and to avoid causing disorders that may be more common with estrogen therapy such as endometrial and breast cancer (Collins, 2006).

Doses and routes of HRT vary by indication. Some commonly prescribed doses and regimens of postmenopausal systemic estrogens are associated with preservation of bone mineral density and in the case of combined hormone therapy, the added progestagen regimen prevents endometrial hyperplasia.

Estrogen has been used for many years as a hormonal supplement to treat menopausal symptoms. Systemic estrogens can be given by mouth, transdermally, intradermally (by patch, skin cream, or pellet), vaginally and by injection (Greendale, Lee and Arriola, 1999). Estrogen used for HRT includes 17β-eatradiol (E_2), conjugated equine estrogens or 17α-ethinyl estradiol (Imhof et al., 2006). Adverse effects of estrogen have been well studied. Breast tenderness and uterine bleeding are the most common side-effects from short-duration treatment trials. Others including, nausea and vomiting, headache, weight change, venous thomboembolic and cardiovascular event are also reported (Nelson, 2004).

Progestagens or progesterone are used to prevent endometrial hyperplasia in women with a uterus. Progestagens used for HRT can be divided into subgroups; the C-21 derivatives of progesterone (including, medroxyprogesterone acetate and dydrogesterone), C-19 derivatives of nortestosteron, and natural progesterone and similar compound. Investigational methods to deliver progestagens locally include an intrauterine device (Suhonen, Holmstrom and Lahteenmaki, 1997) and vaginal gel.

HRT provides short-term benefits and improves quality of life. However, short-term treatment has fewer risks. HRT generally should be used in the lowest effective dose and for the shortest time required. Long-term use is associated with increased risk for breast cancer and coronary artery disease (Timins, 2004).

1.4 Estrogen and Its Functions

The major sex hormones in the female are estrogen and progesterone. These hormones are steroids and derived from cholesterol. Only three estrogens are present in the plasma of human female; estradiol (E_2), estrone (E_1) and estriol (E_3). The principal estrogen secreted by the ovaries is 17β-estradiol, small amount of estrone is also secreted. Estriol is a weak estrogen. It is an oxidative product derived from both estradiol and estrone. The estrogen mainly promotes proliferation and growth of female reproductive tissues and also stimulates the development of the secondary sex characteristics in the female.

1.4.1 Effects of Estrogen on Female Reproductive System

Estrogen has numerous and diverse effects on the organs and tissues of the reproductive system. Estrogen stimulates cellular proliferation and growth of tissues of the reproductive tract. At puberty, estrogen causes an increase in size of the fallopian tubes, uterus, vagina and external genitalia. Conversely, estrogen depletion results in atrophy of these organs (Sperelakis and Banks, 1996).

The endometrium of the uterus is profoundly affected by estrogen, which cause thickening due to proliferation of the stromal cells, endometrial glands and blood vessels. Estrogen also increase the number of progesterone receptors in

endometrial cell. Endometrial sensitivity to progesterone requires previous exposure to estrogen.

In animal, it has been found that within the ovary, estrogen enhances its own production and amplifies the effects of FSH on granulosa cell function and follicular development. In the breasts, estrogen causes deposition of fat and growth of stromal tissue and ducts. Estrogen is responsible for breast enlargement but not for milk production.

There is a study reported the estrogenic effect on the basis of growth response of mammary epithelial structures and uterine weight in ovariectomized (OVX) rats. The percentage area of the mammary fat pad occupied by mammary epithelial structures is progressively increased by 17β-estradiol at a dose of 0.001 μg/day. The maximum effective dose of estradiol was 0.01 μg/day and a dose of 10 μg/day of estradiol decreased mammary size to control levels (Skarda, 2002).

1.4.2 Effects of Estrogen on Lipid Profile

As women enter menopausal years, the levels of estrogen begin to decline. The lipid profile changes observed after menopause are responsible from 20-25% (Kessler et al., 1997) and characterized by an increase in total cholesterol, low-density lipoprotein cholesterol (LDL) and triglyceride (TG), but a decrease in high-density lipoprotein (HDL) (Speroff, Rowan, Symons, Genant and Wilborn, 1996).

Oral estrogen therapy increases HDL-cholesterol, but decreases total cholesterol and LDL-cholesterol (Kim, Min, Ryu, Kwak and Ryoo, 1996; Mijatovic et al., 1997; Seed, Sands, Mclaren, Kirk and Darko, 2000; Godsland, 2001; Vehkavaara et al., 2001). Furthermore, it has an unfavorable increasing effect on triglyceride levels

(Godsland, 2001). When progestins were added to estrogen they can reduce the risk of endometrial cancer in women with intact uterus. However, the addition of progesterone can attenuate the beneficial effects of estrogens on the lipid profile by exerting variable effects on HDL-cholesterol levels.

1.4.3 Effects of Estrogen on Fertility Control

The most common uses of estrogens and progesterones are as oral contraceptives or hormone replacement therapy either postmenopausally or in cases of ovarian insufficiency. In addition, other uses for these hormones and drugs which affect their activities include fertility control and the treatment of certain hormone dependent cancer (Gard, 1998).

The hormonal formulations used include estrogens, combinations of estrogen plus progestin (Dao, Vanage, Marshall, Bardin and Koide, 1996). The most effective postcoital contraceptives include an administration of high doses of hormones to woman within 72 h after an unprotected coitus or an intrauterine device inserted within 10 days after the coitus, which alters the endometrial environment so that implantation can not occur.

Anordrin, an antiestrogen with estrogenic activity, has been used effectively in China with a failure rate of less than 0.5% (Chih et al., 1975). Since implantation of blastocysts in the uterus requires preparation of the endometrium by both estradiol and progesterone, blocking the action of these two hormones should be effective in preventing pregnancy.

1.5 Herbal Medication

Unanticipated increase in risk for breast cancer, stroke, heart attack and blood clots among women taking hormone replacement therapy has been reported (Rossouw et al., 2002). Herbal, phytochemical supplements or plant extracts with estrogenic properties are of increasing interest as potential alternatives to using HRT in treating menopausal symptoms (Wylie-Rosett, 2005). One such alternative includes the phytoestrogens, which are plant substances producing estrogenic effects (Lotke, 1998).

The first phytoestrogen discovered was in the 1940s (Bennetts, Underwood and Shier, 1946), which among sheep that were grazing on pastures containing red clover had multiple fertility problems. Moreover, immature animals were showing signs of estrus, ewes were unable to get pregnant and those that were pregnant often miscarried. The clover in these pastures had high amounts of isoflavones, formononetin and biochanin A (Rossiter and Beck, 1966).

Phytoestrogens are plant-based substances that bind to estrogen receptor and have weak estrogenic and anti-estrogenic activities. There are three main groups: isoflavones, lignans and cumestrans. Isoflavones are generally found in legumes such as soy and garbenzobeans and are consumed in the form of soy, miso and tofu. Genistein and daidzein are two plant isoflavones. Lignans are highly concentrated in seed oils such as flaxseed. Coumestans are the least important source of phytoestrogens for humans. They have steroid-like activity giving them estrogenic effects when digested. Red clover, sunflower seeds and bean sprouts contain high concentrations of coumestans.

Phytoestrogens are thought to be an alternative to estrogen replacement therapy during the menopause due to their positive estrogenic activity. This is supported by the observation that the menopause is less of problem in soy-consuming countries. Postmenopausal Japanese woman who eat large amounts of phytoestrogens reportedly have fewer hot flushes and night sweats. In Europe, 70-80% postmenopausal women suffer from hot flushes, compared with only 18% of women in China (Institute for Environment and Health, 1997).

Herbs have been used for centuries to treat illness and improve health, accounting for approximately 80% of medical treatment in the developing world (Bent and Ko, 2004). Several herbs have been reported as estrogenic agent for menopause. Their details are given below.

Red clover (*Trifolium pratense*) has gained increasing interest as a rich source of isoflavones that are metabolized to genistein and daidzein after consumption. The studies of red clover to treatment of vasomotor symptoms at a dose of 40-160 mg and duration of treatment about 12-16 weeks demonstrated a numerical reduction in the number of hot flushes compared with placebo. Moreover, there are no serious safety concerns associated with the short-term administration of red clover in any of these studies (Panay and Rees, 2005).

Black cohosh (*Cimicifugae racemosae*) is a native herb in Eastern North America, traditionally used for a variety of conditions, including vasomotor symptoms, menopausal anxiety and depression. A review of a number of clinical studies of a standardized extract of black cohosh has demonstrated efficacy for the alleviation of vasomotor symptoms, insomnia and low mood (Lieberman, 1998).

Evening primrose seed oil (*Oeothera biennis* L.), which is a rich source of γ-linolenic acid, an intermediate compound between *cis*-linolic acid and prostaglandin, is used to treat mood swing, irritability and breast tenderness associated with premenstrual syndrome and menopause (Rosett, 2005).

Ginseng (*Panax ginseng* C.A. Mey) is a perennial herb native to Korea and Chaina that has been extensively used in Eastern Asia. The result from clinical studies suggests that ginseng may be helpful with respect to quality of life outcomes, such as well-being, mood and sleep (Low Dog, 2005).

Among the phytoestrogen-rich plants, *Pueraria mirifica*, which a local name of white Kwao Krua or Kwao Krua Khao might be the most interesting one. This indigenous Thai herb has been used in traditional medicinal consumtion among menopausal women for purposes of rejuvenation and estrogen replacement (Suntara, 1931). The plant tubers were found to contain at least 13 known phytoestrogens, including miroestrol, deoxymiroestrol and isoflavonoids. The estrogenic effects of *P. mirifica* were exhibited in various reproduction organs. For example, there are induction of vaginal cornification and increased uterine weight in OVX rats (Malaivijitnod et al., 2004; 2006), prolongation of the menstrual cycle in mature female monkeys (Trisomboon, Malaivijitnod, Watanabe and Taya, 2004), suppression of serum LH and FSH levels in female monkeys (Trisomboon, Malaivijitnod, Watanabe and Taya, 2005; Trisomboon et al., 2006) and alleviation of menopausal symptoms in women (Muangman and Cherdshewasart, 2001).

Curcuma comosa Roxb. is an indigenous medicinal plant of Thailand, commonly known as Waan Chak Modluk. Rhizomes have long been used as an anti-inflammatory agent. It has been used widely for the treatment of postpartum uterine

bleeding, hemorrhoids, perimenopausal bleeding, uterine inflammation and promoting lactation (Perry, 1980). From previous phytochemical studies, the hexane extract of *C. comosa* exhibited estrogenic-like activities by causing an increase in uterine weight and cornification of vaginal epithelium (Piyachaturawat, Ercharuporn and Suksamrarn, 1995a, 1995b).

Pomegranates (*Punica granatum* L.) have been wildly used for health benefits. They are effective in reducing heart disease risk factors and may be effective against prostate cancer and osteoarthritis. The estrogenic effects of pomegranates seed and peel extracts in OVX rats have been demonstrated (Promprom et al., 2008). Oral administration of pomegranate seed extract at doses of 400 and 500 mg/kg B.W. induced vaginal corinfication on day 9 and day 7 after ovariectomy, respectively. With 400 and 500 mg/kg B.W., pomegranate peel extract, induced vaginal cornification on day 10 after ovariectomy. The effects of both seed and peel extracts lasted until day 14 after ovariectomy. The data suggest that pomegranates have estrogenic effects in OVX rats and they could be useful for health benefits in menopause.

1.6 *Costus speciosus* (Koen.) Sm.

Costus speciosus (Koen.) Sm. belongs to family Costaceae. It is commonly known as Ueang mai na (Smitinand, 2001). It is a medicinal plant abundantly found in various regions of Southeast and South Asia. *C. speciosus* is a succulent perennial herb, growing up to 1.8 meter high and having an erect stem. The plant possesses horizontal rhizomatous rootstock. It generally grows luxuriantly on clayey loam soil near inland forest under moderate shade. The plant propagated vegetatively through

rhizomes or via seeds dispersed by birds (Mandal, Thomas and Elanchezhian, 2007). The plant is traditionally used as anti-inflammatory, antihelminthic, astringent, depurative and stimulant. The rhizome is used in catarrhal, fevers, coughs, dyspepsia, skin diseases and worms (Chatterjee and Pakrashi, 1995). Moreover, the plant is used as anti-diabetic (Bavarva and Narasimhacharya, 2008). A number of compounds have been isolated from this plant, including diosgenin, tigogenin, saponin and β-sitosterol (Chandel, Shukla and Sharma, 1996).

1.7 Aims

There were four main aims of this study, which were interconnected; 1) to investigate the estrogenic effects of *C. speciosus* rhizome and stem extracts on reproductive organs, including uterus, vagina and mammary gland, 2) to investigate the effects of *C. speciosus* rhizome and stem extracts on serum estrogen level and lipid profile, 3) to test the anti-implantation effects of *C. speciosus* rhizome and stem extracts and 4) to examine the effects of *C. speciosus* rhizome and stem extracts on uterine contraction and compared their effects to the known compounds such as diosgenin and β-sitosterol. The underlying mechanisms of the extracts were also investigated.

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CHAPTER II

THE CHEMICAL COMPOSITIONS OF *COSTUS SPECIOSUS* (Koen.) Sm. RHIZOME AND STEM EXTRACTS

2.1 Abstract

Costus speciosus (Koen.) Sm. or Ueang mai na, belongs to the family Costaceae. It is a tropical to subtropical monocyledonous plant growing wild throughout Southeast and South Asia. The plant has many historical uses in Ayurveda for various purposes. The aim of this chapter was to analyze the chemical compositions of *C. speciosus* rhizome and stem extract. *C. speciosus* rhizome and stem were extracted by a soxhlet extractor and the chemical compositions were analyzed using gas chromatography and mass spectrometry (GC-MS). Twenty known and 8 unknown compounds were identified in *C. speciosus* rhizome extract whereas 26 known and 5 unknown compounds were identified in *C. speciosus* stem extract. Among these compounds diosgenin and β-sitosterol were the major compositions of the extract.

2.2 Introduction

Historically, natural products have provided an endless source of medicine. Plant-derived products have dominated the human pharmacopoeia for thousands of

years almost unchallenged (Raskin and Ripoll, 2004). About 70% of the world's population has incorporated traditional medicine into their primary health care (Farnsworth, Akenele, Bingel, Soejarto and Gue, 1995). These herbs are relatively cheap and available (Nwafor and Akah, 2005). There is growing interest in the use of medicinal plants because many plants and herbs belonging to various families and species are known to contain important phytoceuticals or nutraceuticals. The aim of this chapter was, therefore, to analyze the chemical compositions of *C. speciosus* rhizome and stem extracts.

2.2.1 Botanical Profile of *C. speciosus*

The scientific classification of *C. speciosus* belongs to the order Zingiberales, family Costaceae, genus *Costus*, and species *C. speciosus*. Its binomial name is *Costus speciosus* (Koen.) Sm. *C. speciosus* has a large number of common name in many languages including Keukand (Hindi), wild ginger, crape ginger (Peninsular) and Ueang mai na (Thailand).

2.2.2 Description of *C. speciosus*

C. speciosus (Figure 2.1A) is a tropical to subtropical monocyledonous plant (Kawano, Ichinose and Ebizuka, 2002) growing wild throughout Southeast and South Asia (Ebizuka and Inoue, 1996). It is a tall and dramatic landscape plant with large dark green leaves arranged on the stalk in a spiral form (Figure 2.1C). This plant can grow up to 3.1 meter tall in forest-free areas, but typically grow to about 1.8 meter tall in cooler regions where it roots hardy but dies back in winter. The flowers (Figure

2.1B) appear in the late summer or early fall and quite unusual looking. Flowers are white, arranged in terminal spikes. Bracts are bright red.

Figure 2.1 Morphology of *C. speciosus* (A), flower (B), leaves (C) and rhizome (D).

2.2.3 Uses in Traditional Medicine

The plant rhizome (Figure 2.1D) is a part used as medicine (Mandal, Thomas and Elanchezhian, 2007). The rhizome is used as tonic and antihelminthic. The sap from the crushed stem is used to treat diarrhea and eye infections. An infusion or a decoction from leaves is utilized in bath for relieving patients from high fever. The juice of fresh rhizome is considered to be a purgative. It is also applied on the head to have a cooling effect and to get relief from headache and ear pain. This plant is also used in India to control diabetes and it is known that diabetic people eat one leaf daily to keep their blood glucose low (Merina, 2004).

2.2.4 Pharmacological Studies

Previous studies reported that polyphenol content of methanolic extracts was high in roots when compared to leaves. Antioxidant activity indicates that the methanolic extracts showed more hydroxyl radical scavenging activity and free radical quenching ability (Vijayalakshmi and Sarada, 2008). It has been demonstrated that root of *C. speciosus* may serve as substituent for synthetic antioxidants. In addition, the screening for antibacterial activity of *C. speciosus* shows that plants commonly used for treating wounds cut and diarrhea posses significant antibacterial activity (Fasihuddin, Rahman and Hasman, 1993). The saponin obtained from *C. speciosus* seeds exhibited potent hypotensive and bradycardiac activities in dog (Banerji et al., 1981). In Chiangmai, Chiangrai and Lampang provinces, saponin was found in flowers of *C. speciosus* and used as pesticides (Promsttha, Chungyusuk and Sangwanish, 2003). Battacharya, Parik, Debnath, Pandey and Neogy (1972) reported that alkaloids from *C. speciosus* have been shown to process anticholinesterase

activity both *in vitro* and *in vivo*. In addition, Bavarva and Narasimhacharya (2008) reported that the ethanolic *C. speciosus* rhizome extract exhibited antihyperglycemic, antihyperlipidemic activities and is a potent antioxidant agent on diabetic rats. Moreover, an aqueous extract of *C. speciosus* showed significant hypoglycemic effect when it was administered orally with a simultaneous glucose load (Mosihuzzaman et al., 1994).

2.2.5 Previous Isolated Compositions

The chemical compositions were isolated from the rhizome of *C. speciosus* and elucidated as diosgenin, prosapogenin B of dioscin, diosgenone, cycloartano, 25-en-cycloartenol and octacosanoic acid (Qiao, Li, Dong, Xu and Wang, 2002). Gupta, Lal and Shukla (2001a) found two new compounds; B and C, isolated from the roots of *C. speciosus* that have been characterized as 24-hydroxyhentriacontan-27-one and 24-hydroxytriacontan-26-one by spectral data and chemical studies. Methy-triacontanoate, diosgenin and sitosterol have also been isolated and identified. A new sterol isolated from *C. speciosus* roots has been characterized as 5-stigmast-9(11)-en-3β-ol by spectroscopic data and chemical studies (Gupta, Lal and Shukla, 2001b). Tschesche and Pandey (2001) established the structures of the saponins B and C as gracillin and dioscin, respectively. In addition, diosgenin, sterols, fatty acid esters have been isolated from seed, rhizome, root, stem and triterpines from leaves (Vijayalakshmi and Sarada, 2008).

2.3 Materials and Methods

2.3.1 Plant Extraction and Chemical Analysis

The rhizome and stem of *C. speciosus* were collected from Nakhon Ratchasima province, Thailand, in August, 2009. The specimen was identified and authenticated by the Royal Forest Department, Bangkok, Thailand and a voucher specimen number BKF161284 deposited.

The rhizome and stem were dried and powdered. The powder of the plant was extracted in a soxhlet extractor with 95% ethanol (100 mL.) for 12 h, as shown in Figure 2.2A. The extract was evaporated under reduced pressure at low temperature in a rotary evaporator, dried by using lyophilizer (Figures 2.2B and 2.2C) and stored at -20°C until use. 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 weight of crude extract and $W_{\text{dried plant}}$ is the mean weight of dried plant material (Phrompittayarat et al., 2007).

Crude extracts of *C. speciosus* were analyzed for the compositions by GC-MS (Agilent Technologies 6850 gas chromatograph, coupled with an Agilent Technologies 5973 (EI) mass spectrum detector). The separation was performed on HP-5MS column, 30 m × 0.25 mm ID × 0.25 μm film thickness. The temperature of column was programmed from 50 to 200°C at 10°C/min, 200 to 260°C at 12°C/min, respectively. The injector temperature and the detector temperature were at 270°C. Helium was used as carrier gas with a constant flow rate of 1.0 ml/min. All separated compounds were identified from the recorded mass spectra by comparison with the

mass spectra from the National Institute of Standard and Technology (NIST) and Wiley libraries.

A

B

C

Figure 2.2 The apparatus used in the extraction process. (A) Soxhlet extractor, (B) rotary evaporator and (C) lyophilizer.

2.4 Results

2.4.1 The % Yield of the Extract

The rhizome extract was a brownish powder and the stem extract was a green sticky mass. The % yield of *C. speciosus* rhizome and stem extract was 7.21% and 8.68%, respectively.

2.4.2 Chemical Compositions of *C. speciosus*

The chemical compositions of the *C. speciosus* rhizome and stem extracts were analyzed using GC-MS technique. Twenty known and 8 unknown compounds were identified in *C. speciosus* rhizome extract whereas 26 known and 5 unknown compounds were identified in *C. speciosus* stem extract. The compositions of *C. speciosus* showed richness in fatty acids. The most compounds found in rhizome and stem extracts were diosgenin and β-sitosterol, respectively. In addition, phytosterol, such as stigmasterol and ergosterol were present. The chemical compositions of plant extracts are shown in Tables 2.1 and 2.2. The GC-MS chromatograms of *C. speciosus* rhizome and stem extracts are demonstrated in Figures 2.3 and 2.4.

Table 2.1 The identified compounds in *C. speciosus* rhizome extract.

Chemical Compounds	Retention time (min)
Phytosterol	
Diosgenin, spirost-5-en-ol	48.815
β-sitosterol	50.420
Diosgenin	52.698
Stigmasterol	47.346
Pennogenin	58.810
9,19-Cycloergost-24(28)-en-3-ol	54.696
Fatty acid	
Hexadecanoic acid	20.542
9,12-Octadecadienoic acid	22.837
Octadecanoic acid	23.241
Hexadecanoic acid, esthyl ester	20.893
Tetradecanoic acid	19.180
Other compounds	
9,12,15-Octadecatrien-1-ol	22.944
1, 3-Butanediol	3.929
2, 3-Butanediol	4.095

Table 2.1 (Continued).

Chemical Compounds	**Retention time (min)**
Other compounds	
2-Propanone, 1,3-dihydroxy	6.016
4-Isopropenyl-7-methyltricyclo-octan-6-one	28.432
Propanoic acid, 2-methyl, pentyl ester	9.114
2-Methoxy-4-vinylphenol	13.104
Di-n-octyl phthalate	28.593
Hexadecane	16.635

Table 2.2 The identified compounds in *C. speciosus* stem extract.

Chemical Compounds	Retention time (min)
Phytosterol	
β-sitosterol	50.397
Diosgenin	48.441
Ergost-5-en-3-ol	45.699
23-ethylcholesta-5	47.329
Fatty acid	
Hexadecanoic acid	20.524
Linoleic acid ethyl ester	23.277
9,12-octadecadienoic acid	22.796
Hexadecanoic acid, ethyl ester	20.893
Octadecadienoic acid	23.218
Other compounds	
Pyrolidine	4.393
9,12,15-octadecatrien-1-ol	22.914
Phenol, 2,6-dimethoxy	13.609
Propaonic acid, 2-oxo-,methyl ester	4.101
Anhydroyamogenin	39.070

Table 2.2 (Continued).

Chemical Compounds	Retention time (min)
Other compounds	
4-((1E)-3-Hydroxy-1-propenyl)-2- methoxyphenol	18.336
Pyridine-d5	12.081
7-amino-3-methylpyrimido (4,5-c) pyridazin-5 (6H)-one	17.266
4-vinylphenol	11.671
Phytol	22.492
Bis (2-ethylhexyl) phthalate	28.593
Neophytadiene	19.281
Xycaine	20.072
2,3-Dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one	10.553
1,2-Benzenedicarboxylic acid	19.565
Phenol,2-methoxy	13.104
2-Methoxy-4-vinylphenol	19.460

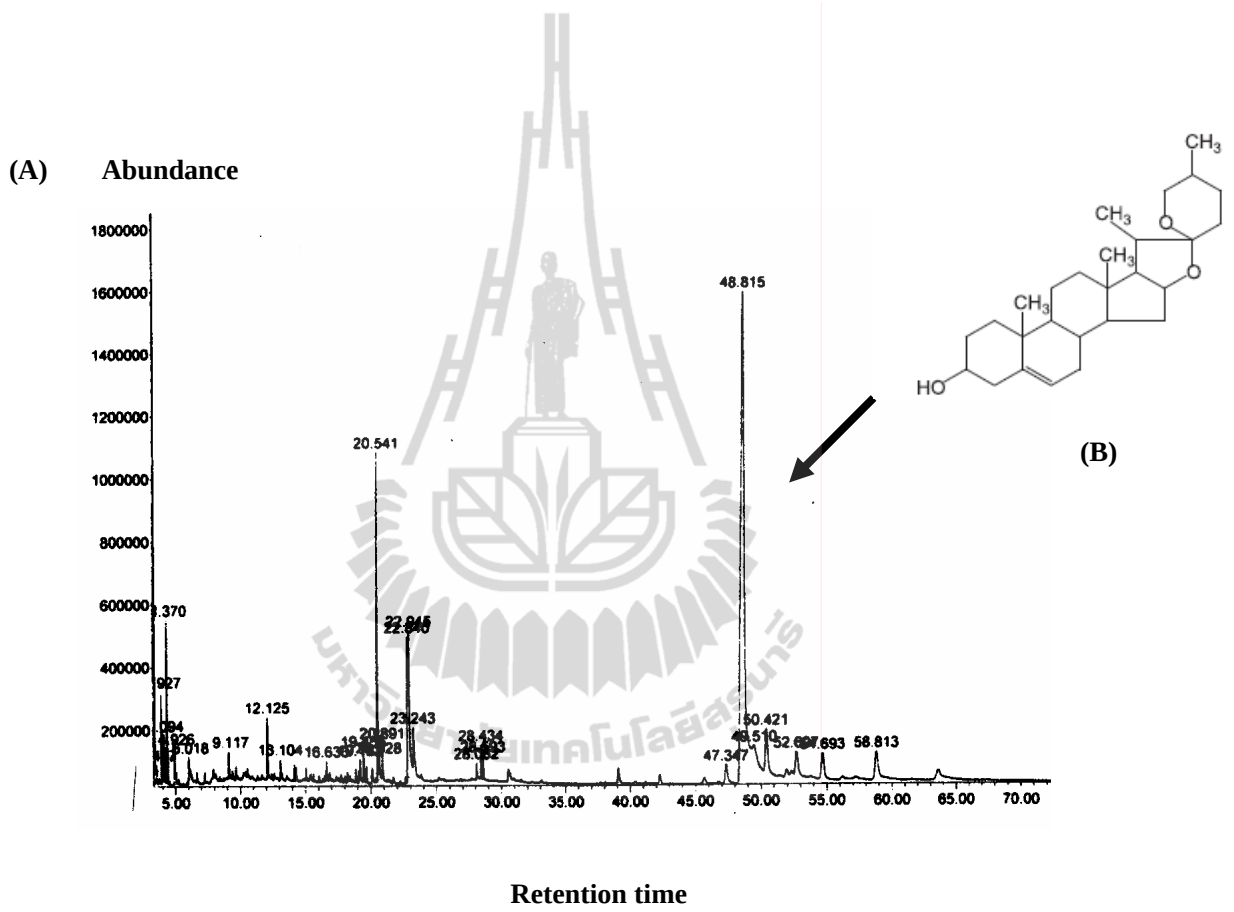

Figure 2.3 GC chromatogram of *C. speciosus* rhizome extract obtained by GC-MS (A) and chemical structure of diosgenin (B) (Au et al., 2004).

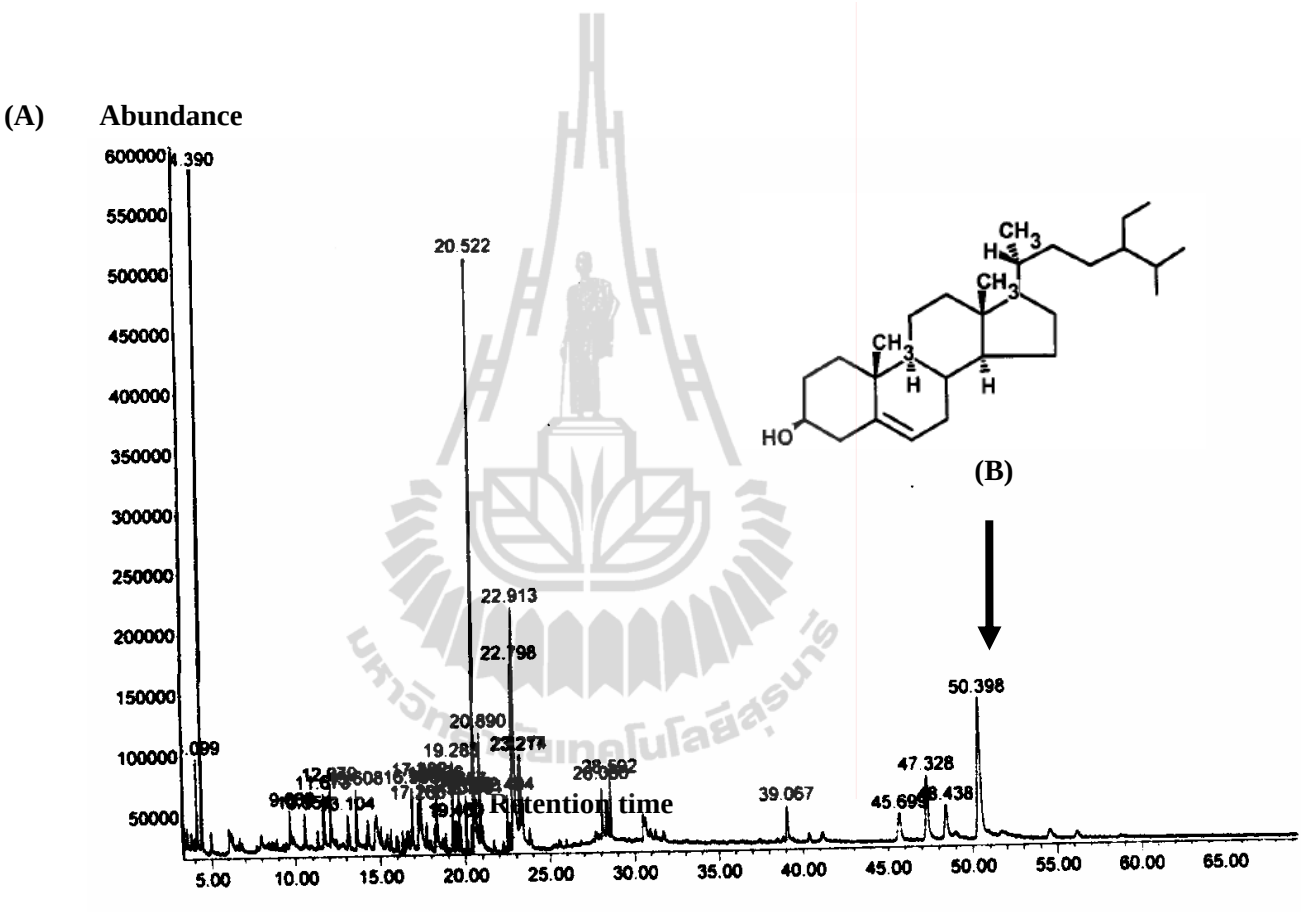

Figure 2.4 GC chromatogram of *C. speciosus* stem extract obtained by GC-MS (A) and chemical structure of β-sitosterol (B) (Turnbull, Frankos, Leeman and Jonker, 1999).

2.5 Discussion

C. speciosus has been reported for its medical usage. Compounds isolated from this plant were reported to have antioxidant (vijayalakshmi and Sarada, 2008), antibacterial (Fasihuddin, Rahman and Hasman, 1993), hypotensive and bradycardiac activities in dog (Banerji et al., 1981), anticholinesterase (Battachrya et al., 1972), antihyperglycemic activities in diabetic rats (Bavarva and Narasimhacharya, 2008) and pesticides (Promsttha, Chungyusuk and Sangwanish, 2003).

Similar to previous studies (Eliza, Daisy, Ignacimuthu and Duraipandiyan, 2009), the major compound found in *C. speciosus* rhizome is diosgenin. It is a steroid saponin that has been structurally similar to progesterone (Au et al., 2004). It has long been used as a raw material for the industrial production of steroid drugs including pregnanolone and progesterone which are used as the first birth control pills (Dweck, 2006). It is therefore believed that diosgenin can be converted into progesterone and other sex steroids *in vivo*. There are many plant sources of diosgenin, for example, the wild yam (*Dioscorea villosa*) which the root is used for numerous purposes, including the suppression of menopausal symptoms like hot flushes (Watson, 1993). It is also used for dysmenorrhea, ovarian and uterine pain (Burkill, 1985). In the market, there are over 50 formulations/creams containing diosgenin that may confer estrogen-like beneficial effects.

As with previous studies (Vijayalakshmi and Sarada, 2008), the major compound found in *C. speciosus* stem is β-sitosterol. It is one important kind of phytosterols that commonly occurs in raw material extracted from deodorizer distillations (Sheng and Chen, 2009). Among plant sterols, β-sitosterol has

demonstrated the greatest potential for the development of steroidal drugs. Both diosgenin and β-sitosterol belong to phytosterols.

Phytosterols are a group of steroid alcohols, which naturally found in vegetable product principally oil, but also pulses and dried fruits (Piironen, Toivo and Lampi, 2000). Phytosterols include a wide variety of molecules that are structurally similar to cholesterol. More than 100 types of phytosterols have been reported in plant species, but the most abundant are sitosterol, stigmasterol and campesterol (Moreau, Whitaker and Hicks, 2002; Berger, Jones and Abumweis, 2004; Kritchevsky and Shirley, 2005). The most research studies have focused on phytosterols, particularly, sterols which can reduce cholesterol in the blood. A substantial body of evidence indicated that the intake of 2 g/day of plant sterol could lower LDL-cholesterol levels by 5-15% in human including menopausal women (Katan et al., 2003,Rudkowska, 2008). Very few studies have evaluated the cholesterol-lowering effect of these plant sterols on women only. All studies agreed that plant sterols are effective in reducing LDL-cholesterol and total-cholesterol in postmenopausal women (Katan et al., 2003). However, it has also been shown that they can control other illnesses. For example, there is experimental and epidemiological evidence suggesting that they can protect agonist certain types of cancer such as colon, breast and prostate gland (Awad and Fink, 2000) and have positive effects on benign prostatic hyperplasia (Wilt, Macdonald and Ishani, 1999). Their actions as immune modulators and their anti-inflammatory properties have also been described (Bouic, 2001). A number of reports suggest that phytosterols may affect on the reproductive system in particular estrogenic activity (Elghamry and Hansel, 1969; Sammannoudy, Shareha, Ghannudi, Gillaly and Mougy, 1980; Malini and Vanithakumari, 1993; Rosenblum, Stauber, Van

Thiel, Campbell and Gavaler, 1993; Markaverich, Webb, Densmore and Gregory, 1995; Mellanen et al., 1996).

Diosgenin and β-sitosterol were found as the main composition of *C. speciosus* as shown in Table 2.1 and 2.2. It has been reported that the chemical compositions isolated from the plant have estrogenic activity as they increased the uterine weight of spayed albino rats (Tawari, Chaturvedi and Pandy, 1973) and disturbanced pregnancy in rats (Singh, Sanyal, Bhattachary and Pandy, 1972). It has also been reported that the fresh juice of *C. speciosus* rhizome increased the tone, amplitude and frequency of spontaneous contractions of the uterus isolated from rats, guinea-pig, rabbit, dog and human (Tewari, Prasad, Chaturvedi and Das, 1967). However, there are few studies reported about the mechanical action of estrogenic activity of *C. speciosus*. Thus, the understanding of such activity will help to improve health care related with estrogen deficiency, birth control or may be useful for uterine stimulation.

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CHAPTER III

EFFECTS OF *COSTUS SPECIOSUS* (Koen.) Sm. EXTRACTS ON FEMALE REPRODUCTIVE SYSTEM AND SERUM LIPID PROFILE IN OVARIECTOMIZED RATS

3.1 Abstract

Costus speciosus (Koen.) Sm. is a medicinal plant. It presents several phytoestrogens such as diosgenin and β-sitosterol. Thus, the purposes of this study were to investigate the effects of *C. speciosus* rhizome and stem extracts on physiological and morphological changes of uterus, vagina and mammary gland, serum estradiol level and serum lipid profile. Female Wistar rats were divided into 7 groups; sham-operated rats received vehicle (n = 9), bilaterally ovariectomized (OVX) rats received vehicle (n = 10), and OVX rats received a standard drug 17α-ethynylestradiol (n = 10), *C. speciosus* rhizome extract at a dose of 500 mg/kg B.W. (n = 8), *C. speciosus* rhizome extract at a dose of 1000 mg/kg B.W. (n = 9), *C. speciosus* stem extract at a dose of 500 mg/kg B.W. (n = 9) and *C. speciosus* stem extract at a dose of 1000 mg/kg B.W. (n = 9), respectively. Each was orally administered daily for 2 months. Administration of *C. speciosus* rhizome extract (500 mg/kg B.W.) to OVX rats produced significant increase in relative uterine weights compared with OVX rats received vehicle. In OVX rats received *C. speciosus*

rhizome and stem extracts, compared with control OVX rats received vehicle, there was a slightly increase in uterine cross section area and size, vaginal epithelium thickness in some area and alveolar and tubular structure proliferation of the mammary gland. *C. speciosus* rhizome and stem extracts produced no significant changes in serum estradiol levels compared with control OVX rats received only vehicle. However, *C. speciosus* rhizome and stem extracts decreased total cholesterol and LDL-cholesterol compared with control OVX rats received vehicle. In conclusion, *C. speciosus* rhizome and stem extracts exhibited weak estrogenic activity as shown by their effects on histological and morphological changes in the uterus, vagina and mammary gland ultra-structures. Moreover, the extracts produced some positive effects on lipid metabolism as shown by changes in lipid profile.

3.2 Introduction

The organs of the reproductive systems are concerned with the general process of reproduction and each is adapted for specialized tasks. Theses organs are unique in that their functions are not necessary for the survival of each individual. However, their functions are essential to the continuation of the human species. The function of the female reproductive system is to produce offspring. It produces germ cells for sexual reproduction, provides an environment for the transportation of the male germ cells, the spermatozoa to the fallopian tubes for fertilization and prepares a suitable environment for the development of the embryo. Moreover, it produces milk for the nourishment of the young offspring. Although the reproductive organs are established during the embryonic and fetal periods, they do not reach full maturity until puberty. The active reproductive period begins with menarche, the first menstruation, at

puberty and lasts until menopause, which is the cessation of reproductive function when the supply of ovarian follicle is depleted (LaBarbera, 1996).

The most important hormones of the female reproductive system are the estrogen and progesterone. Estrogen is a group of hormones originally defined by their ability to induce a period of sexual receptivity (estrus) in animals, the most important of which in humans are 17β-estradiol, estrone and estriol. The dominant form of estrogen in the body is 17β-estradiol. It is synthesized and secreted by the ovaries under the control of the pituitary gonadotropins (Gard, 1998).

Estrogen plays an important role in the growth, differentiation and development of primary sexual characteristics such as the uterus, ovaries and vagina. The estrogen is also responsible for development of the female secondary sex characteristics, such as the breasts and for regulation of reproductive cycle (Randall, Burggren and French, 1997). Moreover, estradiol also has a variety of pharmacological functions such as maintenance of bone mass, cardiovascular function and brain protection (Smith et al., 1994; Ciocca and Roig, 1995).

The menopause results in a sex hormone levels decline with age, and there is the possibility of some regression of the female secondary sexual characteristics. It is the period in a women's life during which the ovarian and uterine cycles cease. The ovaries are no longer responsive to gonadotrophic hormones and stop producing eggs and the female sex hormone (Mader, 2001). Menopausal symptoms vary greatly among women. The first sign of the menopause is the increasingly irregular nature of the menstrual cycle. The lack of estrogen during menopause can lead to many health problems, for example atrophy of the uterus, vagina, breasts, and external genitalia. Estrogen deficiency also promotes the changes on skin, skeleton and cardiovascular

system. The most likely cause for this difference is the deficiency of estrogen in menopause women. Hormone replacement therapy (HRT; estrogen and progestin) is often prescribed to suppress such estrogen-related diseases. However, the use of HRT may be associated with side-effects such as increases the risk of breast cancer and cardiovascular diseases. Therefore, diets rich in phytoestrogen-containing foods have been suggested to prevent or alleviate estrogen-related diseases. In addition, phytoestrogens are being increasingly promoted as the natural alternative to HRT.

Phytoestrogens are naturally occurring; plant based diphenolic compounds present in foods and are part of wider class of polyphenols found in all plants. They are structurally and functionally to estradiol. However, their estrogenic activities are generally much less than that of estradiol. The estrogenic activity ranges from 1/500 to 1/1000 of the activity of estradiol (The Institute of Food and Science and Technology, 2001). There are many different ways that phytoestrogens may work in the body. Also, the effects in various parts of the body may be different. They may act as mimics of estrogen or may act differently from estrogens. Which of these effects occur is unknown. The possible benefits of phytoestrogens have been focused on cancer (breast and prostate gland), menopause, osteoporosis, heart disease, diabetes and cognitive function. Phytoestrogens are thought to be an alternative to HRT (The Institute of Food and Science and Technology, 2001). These are supported by the observation that the menopause has few problems in soy-consuming countries. For example, postmenopausal Japanese women who eat large amounts of phytoestrogens reportedly have fewer hot flushes and night sweats. In Europe, 70-80% postmenopausal women suffer from hot-flushes, compared with only 18% of postmenopausal in China (Institute of environment and Health, 1997).

Several studies have been performed looking at phytoestrogens in the treatment of menopause. Lay literature recommends wild yam (*Dioscorea villosa*) containing diosgenin that has been used in treating hot flashes and excessive vaginal bleeding (Seidl and Stewart, 1998). In market, there are over 50 formulations/creams that contained extract of wild yam, which believed that diosgenin present in the cream may be estrogen-like beneficial effects (Au et al., 2004).

As shown in Chapter II, *C. speciosus* contains large amounts of diosgenin. Therefore, the aim of this chapter was to investigate the effects of *C. speciosus* rhizome and stem extracts on female reproductive system, including changes in physiology and morphology of the uterus, vagina and mammary gland. The effects on serum estradiol level and lipid profile were also investigated.

3.3 Materials and Methods

3.3.1 Preparation of Plant Extracts and Chemicals

The *C. speciosus* rhizome and stem extracts were diluted to 500 and 1000 mg/kg B.W. in the vehicle (Tween 80, 10% v/v) and were administered orally to the OVX rats at a volume of 1 mL/day. Only the vehicle (Tween 80, 10% v/v) was administered orally to the sham-operated rats and OVX rats (negative control group) at a volume of 1 mL/day

17α-ethnylestradiol obtained from Sigma chemical Co. (St. Louis, MO, USA) was diluted to 0.02 mg/kg B.W. in the vehicle (Tween 80, 10% v/v) and administered orally to the OVX rats at a volume of 1 mL/day.

3.3.2 Animals and Treatments

3.3.2.1 Ethics

Rats used in this study were 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 Institutional Animal Care and Use Committee, Suranaree University of Technology (SUT), Thailand.

3.3.2.2 Housing

Female Wistar rats at 9 weeks of age (180-200 g) 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*.

3.3.2.3 Surgical Procedure: Ovariectomy

Surgery of animals was done under pentobarbital sodium (30 mg/kg i.p.) anesthesia. Bilateral ovariectomies were performed from a dorsal approach with a small midline dorsal skin incision. The sham-operated rats were subjected to sham surgery exposing; the ovaries were not removed (Shih, Wu and Lin, 2001). After 14 days of endogenous hormonal decline (Tanee et al., 2007), the rats were divided into seven groups, each group contained 8-10 rats.

Group 1 (Sham-operated rats): Received the vehicle (served as a positive control group, n = 9).

Group 2 (Ovariectomized rats): Received the vehicle (served as a negative control group, n = 10).

Group 3 (Ovariectomized rats): Received 17α-ethynylestradiol at a dose of 0.02 mg/kg B.W. (served as a standard drug group, n = 10).

Group 4 (Ovariectomized rats): Received *C. speciosus* rhizome extract at a dose of 500 mg/kg B.W. (served as a test group, n = 8).

Group 5 (Ovariectomized rats): Received *C. speciosus* rhizome extract at a dose of 1000 mg/kg B.W. (served as a test group, n = 9).

Group 6 (Ovariectomized rats): Received *C. speciosus* stem extract at a dose of 500 mg/kg B.W. (served as a test group, n = 9).

Group 7 (Ovariectomized rats): Received *C. speciosus* stem extract at a dose of 1000 mg/kg B.W. (served as a test group, n = 9).

All the above treatments were given daily for two months. On the last day of the experiment, the animals were sacrificed by using an over dose of CO_2.

3.3.3 Body and Uterine Wet Weight Determination

Individual body weight was recorded twice, approximately 1 week prior to the administration of the extracts and at the end of the experiments.

The uteri were excised and all connective tissues were removed prior to wet weight recordings. To account for individual differences in body weight, an adjusted uterine wet weight was performed using the following formula:

Relative uterine wet weight = Uterine weight/ (Body weight × 100)

3.3.4 Hormone Determination

Estradiol (E_2) was analyzed by using the Electrochemiluminescence immunoassay (ECLIA) on Elecsys and cobas e immunoassay analyzer (Roche Diagnostics, USA). Assay procedures were according to the instructions supplied by manufacturer.

3.3.5 Serum Lipid Profile Analysis

Total-cholesterol, high density lipoprotein (HDL) cholesterol, low density lipoprotein (LDL) cholesterol and triglycerides (TG) were measured using Enzymatic color test and assayed by OLYMPUS analyzer (Olympus Life and Material Science, Germany).

3.3.6 Histological Examination

The uterus was measured in tissue section from the mid-region of each uterine horn. The vagina was prepared for longitudinal section. The mammary gland was cut to obtain sections from the abdominal inguinal region. These organs were fixed in 10% buffered formalin then processed in a tissue processor and embedded in paraffin using routine methods. Representative transverse sections (5-μm thick) were cut and

stained with Hematoxylin and Eosin. Histological examination of the slides was performed using a light microscope.

3.3.7 Statistical Analysis

All data were expressed the mean ± S.E.M.. The significant difference between control and treated groups was determined using one way analysis of variance (ANOVA). The Statistical Package for the Social Sciences (SPSS), version 11.5 was employed for all statistical analysis. The significance level was determined at $P < 0.05$.

3.4 Results

3.4.1 Effects of *C. speciosus* Extracts on Body and Relative Uterine Weights

The final body weights of OVX rats received vehicle were significantly higher than that of the sham-operated rats received vehicle, rats received the standard drug, and rats received *C. speciosus* extracts ($P < 0.05$). The results are summarized in Table 3.1 and Figure 3.1A.

The increscent of relative uterine weights at the end of treatment period is also shown in Table 3.1 and Figure 3.1B. The relative uterine weights in OVX rats received vehicle were significantly decreased compared with that of sham-operated rats received vehicle ($P < 0.05$). The relative uterine weights of rats treated with 17α-ethynylestradiol were significantly increased compared with OVX rats received vehicle ($P < 0.05$). The relative uterine weights of rats received *C. speciosus* rhizome

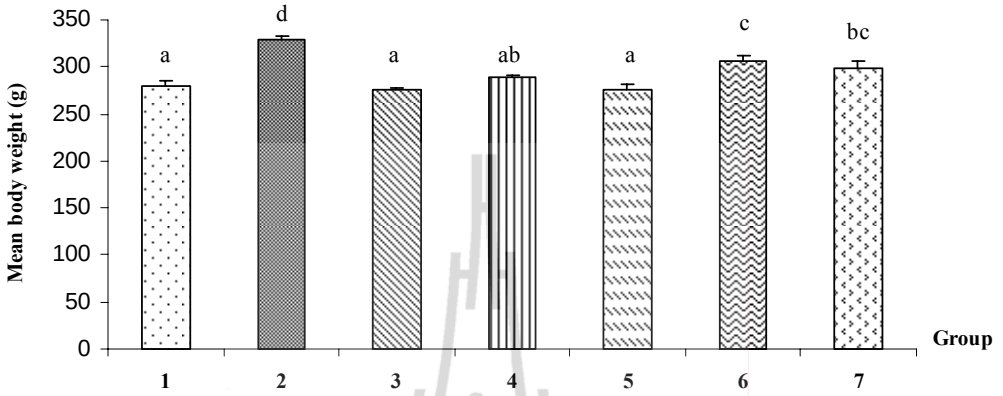
(500 mg/kg B.W.) were also significantly increased compared with OVX rats received vehicle ($P < 0.05$).

Table 3.1 Effects of *C. speciosus* extracts on body and relative uterine weights.

Group	Treatment	Body weight (g)	Relative uterine weight (mg/100 g B.W.)	n
1	Sham-operated rats received 10% v/v Tween 80	278.89 ± 6.96[a]	152.49 ± 7.40[d]	7
2	Ovariectomized rats received 10% v/v Tween 80	330.00 ± 2.98[d]	38.09 ± 2.41[a]	7
3	Ovariectomized rats received 17α-ethynylestradiol (0.02 mg/kg B.W.)	275.00 ± 2.69[a]	128.94 ± 10.33[c]	7
4	Ovariectomized rats received *C. speciosus* rhizome extract (500 mg/kg B.W.)	288.57 ± 3.40[ab]	56.13 ± 2.78[b]	7
5	Ovariectomized rats received *C. speciosus* rhizome extract (1000 mg/kg B.W.)	275.71 ± 6.49[a]	52.49 ± 3.60[ab]	7
6	Ovariectomized rats received *C. speciosus* stem extract (500 mg/kg B.W.)	307.14 ± 5.22[c]	51.47 ± 3.36[ab]	7
7	Ovariectomized rats received *C. speciosus* stem extract (1000 mg/kg B.W.)	298.57 ± 7.05[bc]	52.70 ± 2.57[ab]	7

[a, b, c, d] Significantly different ($P < 0.05$) when compared within a column. Mean ± S.E.M. are given; n is number of animals.

A



B

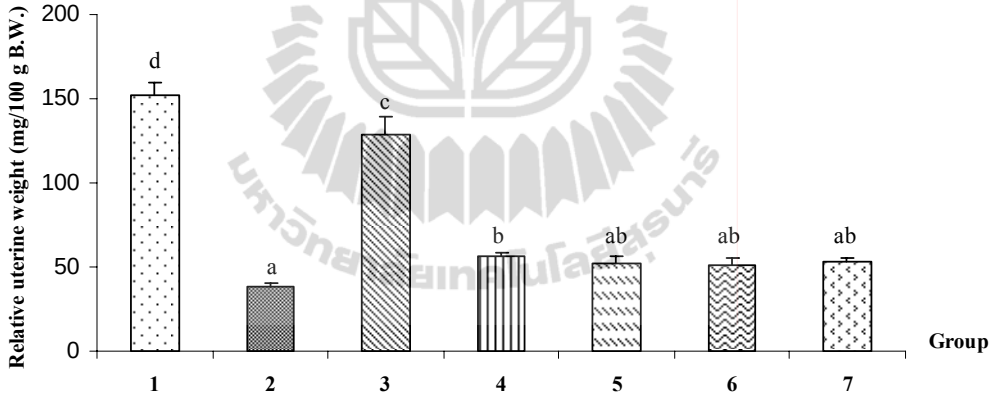


Figure 3.1 Effects of *C. speciosus* extracts on body weight (A) and relative uterine weights (B). 1, Sham-operated rats received 10% v/v Tween 80; 2, Ovariectomized (OVX) rats received 10% v/v Tween 80; 3, OVX rats received 17α-ethynylestradiol (0.02 mg/kg B.W.); 4, OVX rats received *C. speciosus* rhizome extract (500 mg/kg B.W.); 5, OVX rats received *C. speciosus* rhizome extract (1000 mg/kg B.W.); 6, OVX rats received *C. speciosus* stem extract (500 mg/kg B.W.); 7, OVX rats received *C. speciosus* stem extract (1000 mg/kg B.W.). [a, b, c, d] Significantly different ($P < 0.05$) when compared among groups.

3.4.2 Effects of *C. speciosus* Extracts on Serum Estradiol Levels

The effects of *C. speciosus* extracts on serum estradiol levels are summarized in Table 3.2 and Figure 3.2. A high serum estradiol levels was observed in the sham-operated rats received vehicle (22.00 ± 0.96 pg/ml, n = 5) and in the OVX rats received the standard drug (20.47 ± 0.96 pg/ml, n = 5) compared with OVX rats received vehicle (10.57 ± 0.08 pg/ml, n = 5). No significance was found among OVX rats received *C. speciosus* rhizome and stem extracts.

Table 3.2 Effects of *C. speciosus* extracts on serum estradiol levels.

Group	Treatment	Serum estradiol level (pg/ml)	n
1	Sham-operated rats received 10% v/v Tween 80	22.00 ± 0.96^{c}	5
2	Ovariectomized rats received 10% v/v Tween 80	10.57 ± 0.08^{a}	5
3	Ovariectomized rats received 17α-ethynylestradiol (0.02 mg/kg B.W.)	20.47 ± 0.96^{c}	5
4	Ovariectomized rats received *C. speciosus* rhizome extract (500 mg/kg B.W.)	12.43 ± 0.13^{ab}	5
5	Ovariectomized rats received *C. speciosus* rhizome extract (1000 mg/kg B.W.)	13.43 ± 1.55^{b}	5
6	Ovariectomized rats received *C. speciosus* stem extract (500 mg/kg B.W.)	10.63 ± 0.65^{a}	5
7	Ovariectomized rats received *C. speciosus* stem extract (1000 mg/kg B.W.)	10.57 ± 0.52^{a}	5

[a, b, c] Significantly different ($P < 0.05$) when compared within a column. Mean ± S.E.M. are given; n is number of animals.

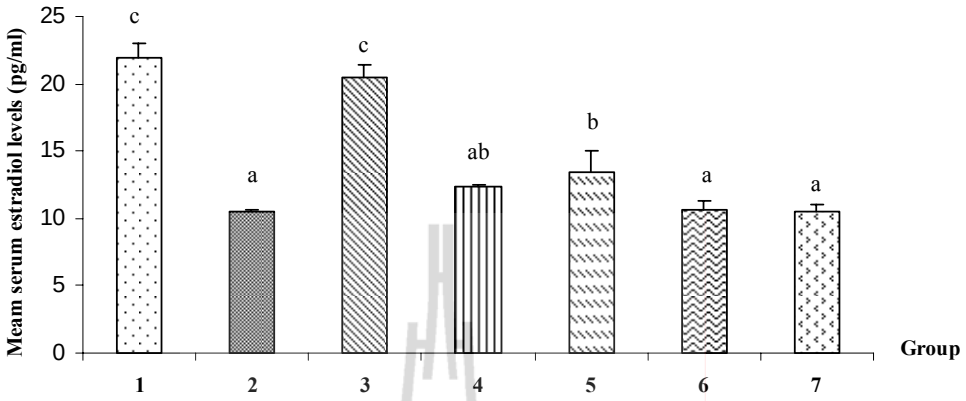


Figure 3.2 Effects of *C. speciosus* extracts on serum estradiol levels. 1, Sham-operated rats received 10% v/v Tween 80; 2, Ovariectomized (OVX) rats received 10% v/v Tween 80; 3, OVX rats received 17α-ethynylestradiol (0.02 mg/kg B.W.); 4, OVX rats received *C. speciosus* rhizome extract (500 mg/kg B.W.); 5, OVX rats received *C. speciosus* rhizome extract (1000 mg/kg B.W.); 6, OVX rats received *C. speciosus* stem extract (500 mg/kg B.W.); 7, OVX rats received *C. speciosus* stem extract (1000 mg/kg B.W.). [a, b, c] Significantly different ($P < 0.05$) when compared among groups.

3.4.3 Effects of *C. speciosus* Extracts on Serum Lipid Profile

Total cholesterol level was significantly increased in OVX rats received vehicle compared with sham-operated rats received vehicle ($P < 0.05$). In OVX rats treated with 17α-ethynylestradiol (0.02 mg/kg B.W.), but total cholesterol level was significantly decreased when compared with that of sham-operated and OVX rats received vehicle ($P < 0.05$). Moreover, the level of total cholesterol in the OVX rats treated with *C. speciosus* rhizome extract (500, 1000 mg/kg B.W.) and *C. speciosus* stem extract (500 mg/kg B.W.) tended to reduced when compared with OVX rats

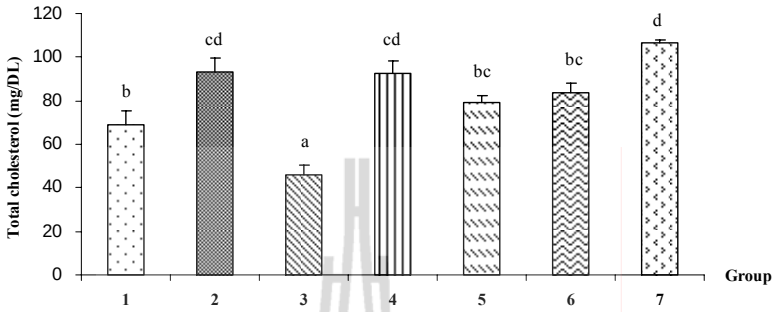
received only vehicle. Triglycerides level was significantly increased in OVX rats treated with the standard drug ($P < 0.05$) and there were no significant differences among sham-operated rats received vehicle, OVX rats received vehicle and OVX rats received *C. speciosus* extracts. HDL-cholesterol level was significantly reduced in OVX rats treated with 17α-ethynylestradiol ($P < 0.05$). *C. speciosus* rhizome (500, 1000 mg/kg B.W.) and *C. speciosus* stem extracts (500 mg/kg B.W.) tended to reduce HDL-cholesterol level in OVX rats when compared with OVX rats treated with vehicle. LDL-cholesterol level was significantly decreased in OVX rats treated with the standard drug when compared with OVX rats treated with vehicle ($P < 0.05$). *C. speciosus* rhizome and stem extracts, both doses, tended to reduce LDL-cholesterol level in OVX rats when compared with OVX rats received only vehicle. The data are shown in Table 3.3 and Figure 3.3.

Table 3.3 Effects of *C. speciosus* extracts on serum lipid profile.

Group	Treatment	Total cholesterol (mg/DL)	Triglyceride (mg/DL)	HDL-cholesterol (mg/DL)	LDL-cholesterol (mg/DL)	n
1	Sham-operated rats received 10% v/v Tween 80	68.67 ± 6.50^{b}	84.33 ± 8.61^{b}	44.83 ± 4.33^{b}	7.00 ± 2.00^{ab}	5
2	Ovariectomized rats received 10% v/v Tween 80	93.17 ± 6.69^{cd}	67.33 ± 4.60^{ab}	60.20 ± 2.98^{cd}	19.83 ± 3.78^{c}	5
3	Ovariectomized rats received 17α-ethynylestradiol (0.02 mg/kg B.W.)	45.83 ± 4.70^{a}	138.75 ± 8.63^{c}	22.33 ± 3.49^{a}	4.17 ± 0.60^{a}	5
4	Ovariectomized rats received *C. speciosus* rhizome extract (500 mg/kg B.W.)	92.67 ± 5.84^{cd}	58.33 ± 4.18^{a}	58.67 ± 3.33^{cd}	19.33 ± 3.38^{c}	5
5	Ovariectomized rats received *C. speciosus* rhizome extract (1000 mg/kg B.W.)	79.00 ± 3.21^{bc}	71.33 ± 4.63^{ab}	48.33 ± 2.67^{bc}	13.67 ± 0.33^{bc}	5
6	Ovariectomized rats received *C. speciosus* stem extract (500 mg/kg B.W.)	83.33 ± 4.91^{bc}	62.00 ± 8.50^{ab}	51.33 ± 2.96^{bc}	15.67 ± 5.33^{bc}	5
7	Ovariectomized rats received *C. speciosus* stem extract (1000 mg/kg B.W.)	106.67 ± 1.20^{d}	77.67 ± 2.40^{ab}	66.00 ± 1.15^{d}	18.33 ± 2.03^{c}	5

[a, b, c, d] Significantly different ($P < 0.05$) when compared within a column. Mean ± S.E.M. are given; n is number of animals.

A



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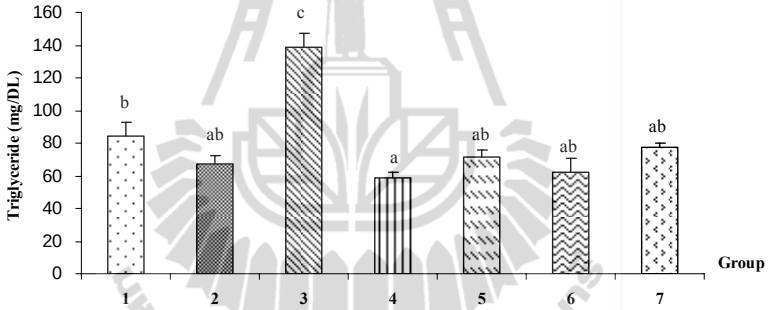
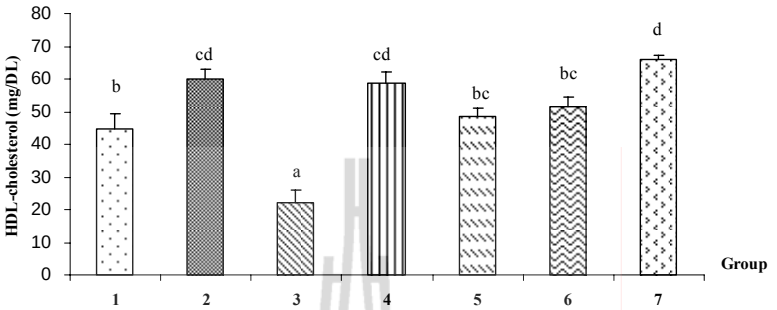


Figure 3.3 Effects of *C. speciosus* extracts on total cholesterol (A), triglyceride (B), HDL-cholesterol (C) and LDL-cholesterol (D). 1, Sham-operated rats received 10% v/v Tween 80; 2, Ovariectomized (OVX) rats received 10% v/v Tween 80; 3, OVX rats received 17α-ethynylestradiol (0.02 mg/kg B.W.); 4, OVX rats received *C. speciosus* rhizome extract (500 mg/kg B.W.); 5, OVX rats received *C. speciosus* rhizome extract (1000 mg/kg B.W.); 6, OVX rats received *C. speciosus* stem extract (500 mg/kg B.W.); 7, OVX rats received *C. speciosus* stem extract (1000 mg/kg B.W.). $^{a, b, c, d}$ Significantly different ($P < 0.05$) when compared among groups.

C



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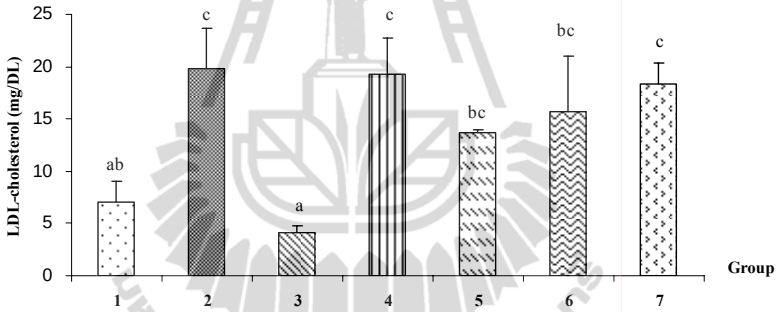

Figure 3.3 (Continued).

3.4.4 Effects of *C. speciosus* Extracts on Morphology of the Uterus, Vagina and Mammary Gland

The representative uterine histology is illustrated in Figure 3.4, showing atrophic uterus in OVX rats received vehicle. Conversely, in OVX rats received the standard drug; proliferative changes of the uterus were found. There was an increase in overall uterine cross-sectional area and size of all three uterine compartments; luminal and glandular epithelium, stroma and myometrium. Interestingly, in OVX rats received ethanolic extracts of *C. speciosus* rhizome (500 mg/kg B.W.) and stem (1000 mg/kg B.W.), proliferative changes in the uterus were also observed. There was an

increase in uterine glands and cross sectional area and size. However, the height of luminal epithelium was not different when compared with that of OVX rats received vehicle.

As shown in Figure 3.5, in the OVX rats received vehicle, the absence of ovarian stimulation led to an atrophy of the vaginal epithelium. The poorly stratified epithelium consisting of one to two atrophied cuboidal or flattened squamous cell layers were observed. In contrast, OVX rats treated with 17α-ethynylestradiol showed a keratinized stratified squamous multilayer epithelium cells which was similar to that of the sham-operated rats received vehicle. In the OVX rats received *C. speciosus* rhizome and stem extracts, epithelium thickness slightly increased in some areas. Whereas, the number of epithelium layer did not differ from OVX rats received vehicle.

In OVX rats received vehicle, the mammary gland lobules and ducts were atrophic. The bulk of the gland consists of fatty connective tissues. In contrast, OVX rats treated with 17α-ethynylestradiol showed more alveolar and tubular structure than in all other groups. The rats received *C. speciosus* rhizome and stem extract showed alveolar and tubular structures more than OVX rats received vehicle, but less than OVX rats received the standard drug. The representative mammary gland histology is illustrated in Figure 3.6.

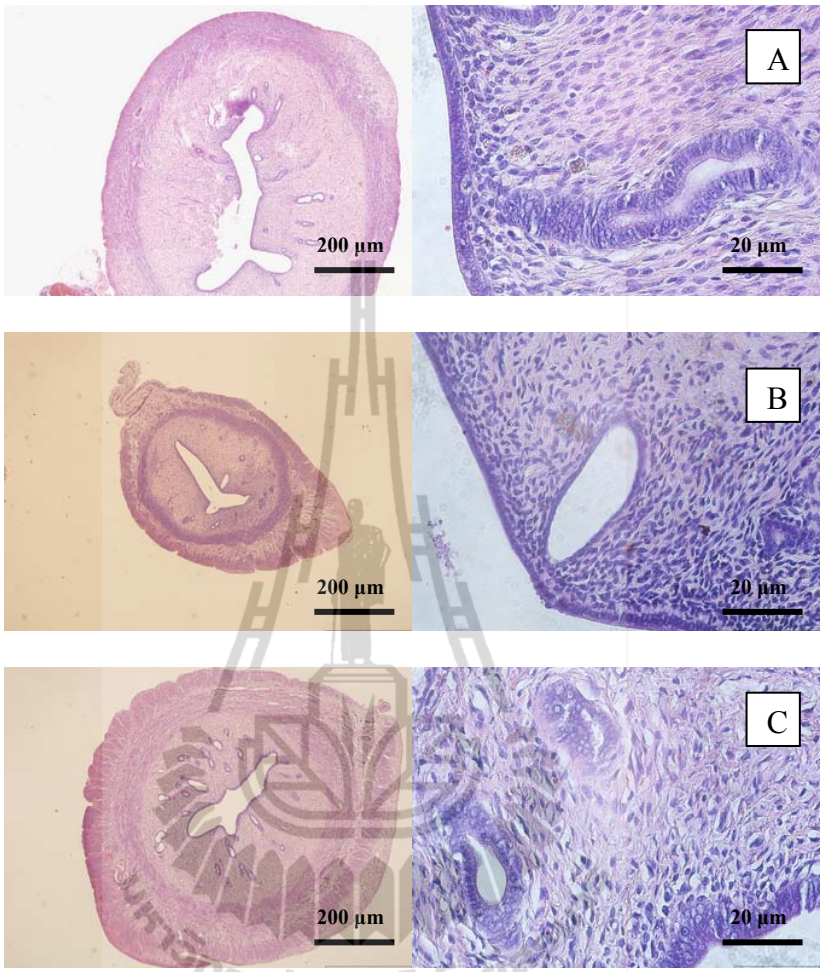

Figure 3.4 Histology of the uterus stained with Hematoxylin and Eosin. A, Sham-operated rats received 10% v/v Tween 80; B, Ovariectomized (OVX) rats received 10% v/v Tween 80; C, OVX rats received 17α-ethynylestradiol (0.02 mg/kg B.W.); D, OVX rats received *C. speciosus* rhizome extract (500 mg/kg B.W.); E, OVX rats received *C. speciosus* rhizome extract (1000 mg/kg B.W.); F, OVX rats received *C. speciosus* stem extract (500 mg/kg B.W.); G, OVX rats received *C. speciosus* stem extract (1000 mg/kg B.W.). The bar equals 200 µm in cross-section of uterine photographed (left) and 20 µm (right).

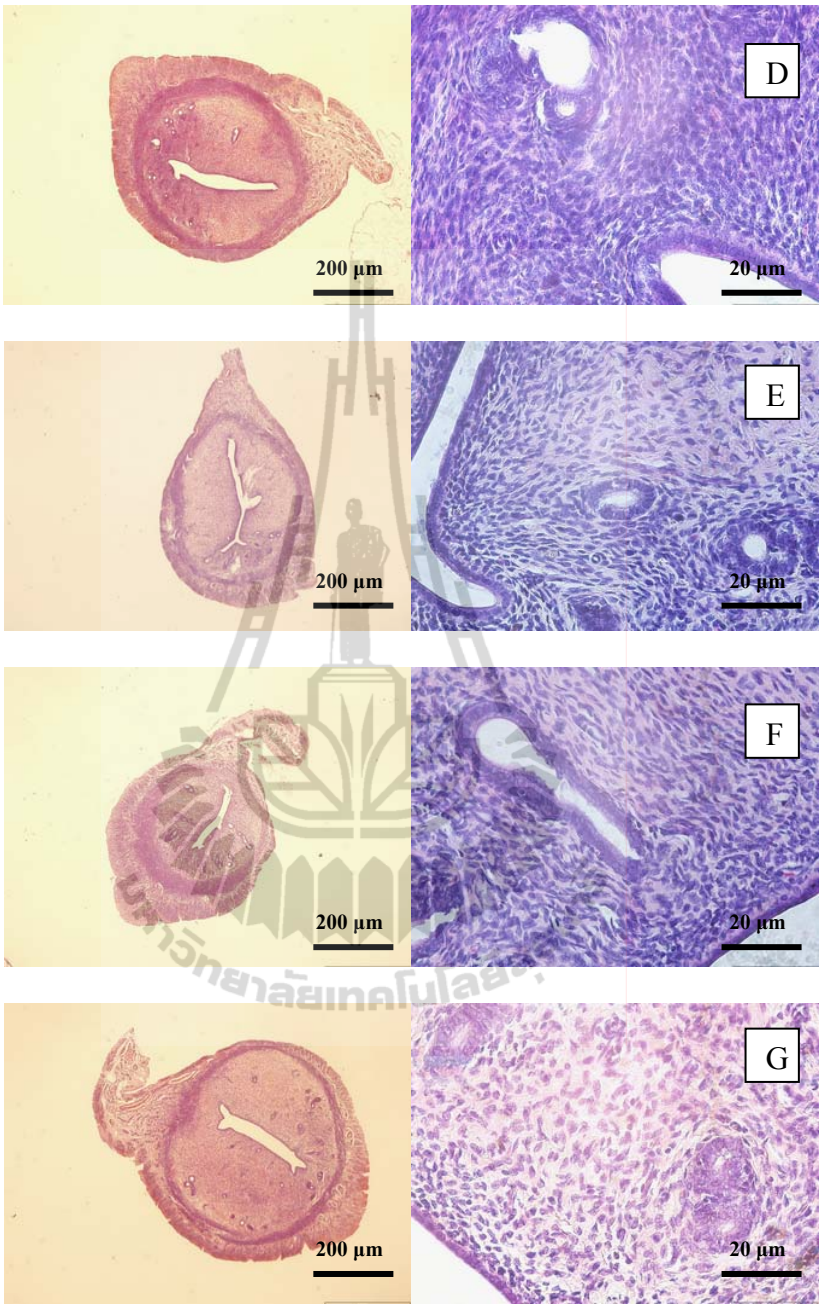

Figure 3.4 (Continued).

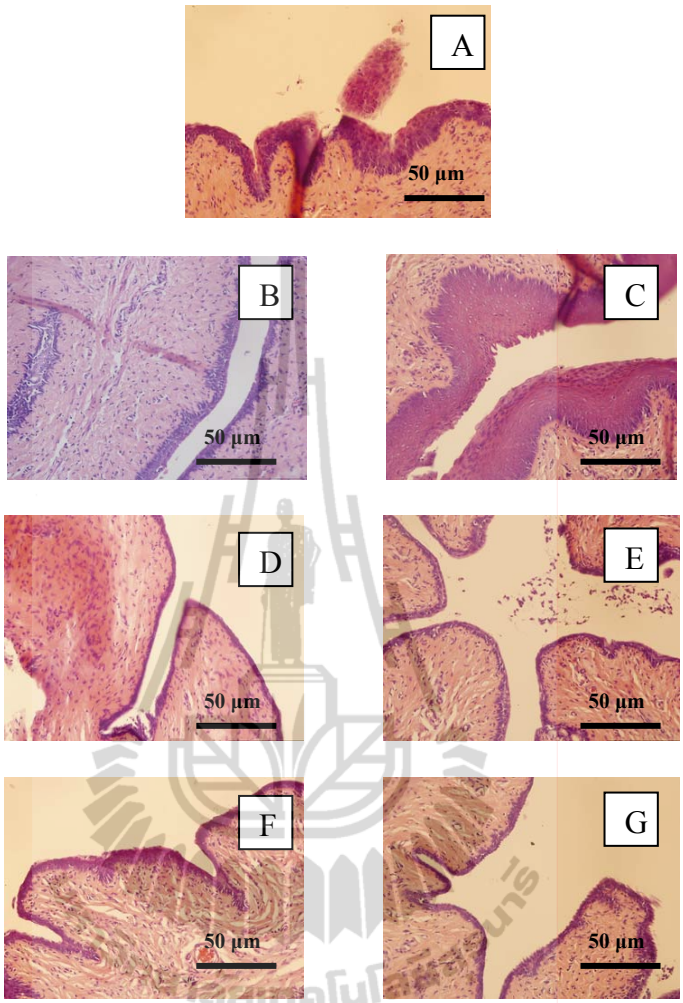

Figure 3.5 Histology of the vagina stained with Hematoxylin and Eosin. A, Sham-operated rats received 10% v/v Tween 80; B, Ovariectomized (OVX) rats received 10% v/v Tween 80; C, OVX rats received 17α-ethynylestradiol (0.02 mg/kg B.W.); D, OVX rats received *C. speciosus* rhizome extract (500 mg/kg B.W.); E, OVX rats received *C. speciosus* rhizome extract (1000 mg/kg B.W.); F, OVX rats received *C. speciosus* stem extract (500 mg/kg B.W.); G, OVX rats received *C. speciosus* stem extract (1000 mg/kg B.W.). The bar equals 50 µm in cross-section of vagina photographed.

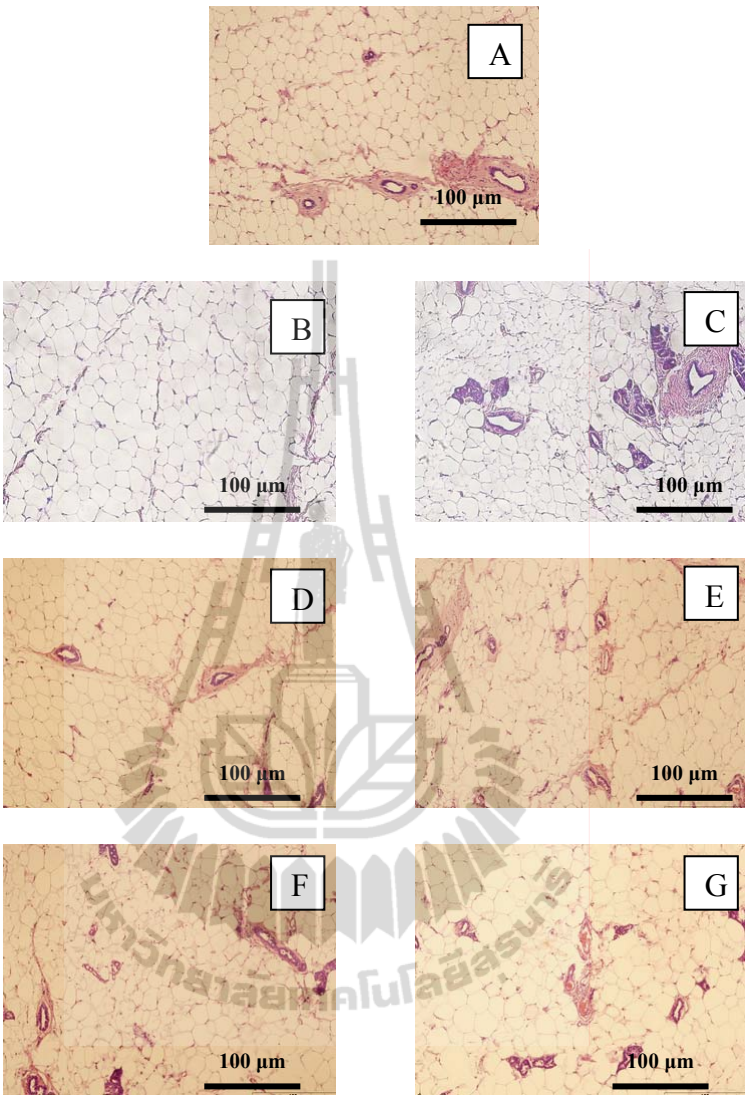

Figure 3.6 Histology of the mammary gland stained with Hematoxylin and Eosin. A, Sham-operated rats received 10% v/v Tween 80; B, Ovariectomized (OVX) rats received 10% v/v Tween 80; C, OVX rats received 17α-ethynylestradiol (0.02 mg/kg B.W.); D, OVX rats received *C. speciosus* rhizome extract (500 mg/kg B.W.); E, OVX rats received *C. speciosus* rhizome extract (1000 mg/kg B.W.); F, OVX rats received *C. speciosus* stem extract (500 mg/kg B.W.); G, OVX rats received *C. speciosus* stem extract (1000 mg/kg B.W.). The bar equals 100 µm in cross-section of the mammary gland.

3.5 Discussion

The female reproductive tract undergoes innumerable physiological and biochemical changes under the influence of ovarian hormones such as estrogen (Prakash and Mathur, 1979). If female rats are ovariectomized, estrogen deficiency can occur. Therefore, the female reproductive tract is atrophy. Administration of estrogenic substances to OVX rats leads to uterotropic changes. The aim of this chapter was designed to investigate the effects of *C. speciosus* rhizome and stem extracts on female reproductive organs in OVX rats and to compare their effects with 17α-ethynylestradiol.

The increased body weight of OVX rats compared to the sham-operated rats was perhaps due to ovariectomy-induced hyperphagia and treatment with estrogens reversed these effects. The results showed that administrations of *C. speciosus* rhizome and stem extracts (500, 1000 mg/kg B.W.) could prevent the OVX-induced increase in weight gain, similarly to 17α-ethynylestradiol (0.02 mg/kg B.W.).

It is well known that estrogen increases the weight of uterus. The results showed that the uterine weight of animals treated with 17α-ethynylestradiol could increase, but not more than sham-operated rats. Administration of *C. speciosus* rhizome (500 mg/kg B.W.) extract significantly increased relative uterine weight ($P < 0.05$). In Chapter II, GC-MS data showed that both *C. speciosus* rhizome and stem extracts contains β-sitosterol. In addition, there has been reported that the oral administration of β-sitosterol produced an estrogenic effect by increasing uterine weight in OVX rats (Baker et al., 1999). Taken together, the extracts clearly have uterotopic effects in OVX rats. This is via the effect of β-sitosterol.

Serum estradiol levels were not found to be significantly different between OVX rats and *C. speciosus* rhizome and stem extract treated rats. However, both doses of *C. speciosus* rhizome extracts tended to increase serum estradiol levels in OVX rats. In addition, it has been reported that no significant differences were found in plasma estradiol levels between intact and OVX rats as during most of the estrous cycle, estradiol concentrations are physiologically low (20-30 pg/mL).

A more reliable indicator of persistent estrogen action is from the relative uterine weight (Vongtau et al., 2000). However, the present results showed that in both the sham-operated rats and the rats received standard drug, serum estradiol levels were significantly increased compared with OVX rats ($P < 0.05$) and the levels were related to uterine weight.

Changes in lipid metabolism are often seen after menopause and are characterized by increasing LDL-cholesterol and triglyceride levels, decreasing HDL-cholesterol (Speroff, Rowan, Symons, Genant and Wilbron, 1996). Data from the experiments clearly showed that 17α-ethynylestradiol treatment decreased total, HDL and LDL-cholesterol, but increased triglyceride level. Moreover, *C. speciosus* rhizome extract (500, 1000 mg/kg B. W.) and *C. speciosus* stem extract (500 mg/kg B. W.) tended to decreased total cholesterol in OVX rats. In addition, *C. speciosus* rhizome and stem extracts both doses tended to reduced LDL-cholesterol. In contrast to human, the effects of estrogen administration on circulating lipid levels in rodents are well characterized (Lundeen, Carver, McKean and Winneker, 1997). In human and rodents, estrogen lowers by up regulating the hepatic LDL receptors, resulting in an increased removal of serum cholesterol from the circulation (Goss, Qi, Hu and Cheung, 2007). This effect results in a preferential reduction of LDL-cholesterol in

humans. However, in the rats, both HDL and LDL-cholesterol are reduced, because rat HDL contains apoprotein E (not found in human HDL), which also binds to the hepatic LDL receptor (Windler et al., 1980). Thus in the rats, as opposed to humans, HDL-cholesterol is a predominant from of circulating cholesterol and estrogen therapy lowers both HDL and LDL-cholesterol. Therefore, the rodent model for studying on lipid metabolism physiology may have some limitations, but still provides a good model to study on the effects of estrogenic substances on total cholesterol and LDL-cholesterol levels (Rachon, Vortherms, Seidlova-Wuttke and wuttke, 2008). As shown in Chapter II, diosgenin and β-sitosterol were found to be the major compound of *C. speciosus* extracts. Both of them belong to phytosterol that have been found to reduce cholesterol concentrations (Caswell, Denny and Lunn, 2008). Previous work showed that diosgenin could lower serum cholesterol in chickens and rabbits fed with cholesterol (Laguna, Gomez-Puyou, Pena and Guzman-Garcia, 1962) and it has a hypocholesterolemic effect by suppressing cholesterol absorption and increasing cholesterol secretion in rats (Son et al., 2007). Taken together, the present study confirmed that the hypocholesterolemic effects of the extract were due to diosgenin and β-sitosterol.

In the present study, a typical estrogenic effect reflected by a keratinized stratified squamous epithelium was observed in the rat vagina following a treatment with *C. speciosus* and 17α-ethynylestradiol. After treatments, the extracts represented estrogenic effect by inducing a typical keratinized stratified squamous epithelium which is similar to those findings in intact animals at estrus (Berger, El-Alfy, Martol and Labrie, 2005). Thus, these findings supported that in the absence of estrogen, the cell proliferation in the smooth muscle is inhibited in the female rat reproductive tract.

However, the results showed that the histological examination of uterus and vagina of *C. speciosus* extracts treated rats was not different when compared to OVX rats.

Mammogenesis is hormone dependent, and the morphology is dependent upon the hormonal status of the individual (Bergamn, Afifi and Heidger, 1996). At puberty, breast development is stimulated chiefly by estrogen and occurred after the establishment of menstrual cycles. Estrogen, progesterone and adrenal corticosteroids can induce breast enlargement. These hormones stimulate the intralobular ducts of the mammary gland to proliferate rapidly, branch and form numerous alveoli (Eroschenko, 1996). The results showed that histology of the mammary gland in OVX rats treated with *C. speciosus* demonstrated alveolar and tubular proliferation, while in OVX rats treated with vehicle; the mammary gland consisted of fatty connective tissues. In 17α-ethynylestradiol treated group, histology of the uterus and mammary gland showed cell proliferation. However, in OVX rats atrophic structures were observed. According to previous studies, diosgenin, when administered (s.c.) at a high dose (40 mg/kg B.W.) to OVX mouse for 15 days, it stimulated the growth of mammary epithelium by increasing number of ducts (Aradhana, Rao and Kale, 1992).

In conclusion, *C. speciosus* rhizome and stem extracts exhibited weak estrogenic activity as shown by slight effects on the reproductive organs and lipid profile. The estrogenic activity of *C. speciosus* rhizome and stem extracts shown in the present data could be due to the presence of diosgenin and β-sitosterol. Thus, they will be useful for health benefits in menopause women.

3.6 References

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CHAPTER IV

EFFECTS OF *COSTUS SPECIOSUS* (Koen.) Sm. EXTRACTS ON VAGINAL CYTOLOGY IN OVARIECTOMIZED RATS

4.1 Abstract

Costus speciosus (Koen.) Sm. is a medicinal plant. It is a source of bioactive compounds including diosgenin and β-sitosterol. The plant has been traditionally used in the treatment of various ailments. This chapter was aimed to assess the estrogenic effects of *C. speciosus* rhizome and stem extracts in ovariectomized (OVX) rats. The OVX rats were divided into six groups, each group consisted of 6 rats. They were the rats received vehicle (10% v/v Tween 80), 17α-ethynylestradiol (0.02 mg/kg B.W.), *C. speciosus* rhizome extract (500 mg/kg B.W.), *C. speciosus* rhizome extract (1000 mg/kg B.W.), *C. speciosus* stem extract (500 mg/kg B.W.), and *C. speciosus* stem extract (1000 mg/kg B.W.), respectively. These rats were administrated the extracts once daily and orally for 8 weeks. Estrogenic activity was determined by taking the percentage of vaginal cornification. Only leukocytes were found in the OVX rats received vehicle. Cornified cells were observed in the OVX rats treated with both *C. speciosus* rhizome and stem extracts, but these were less than that of OVX rats treated with the standard drug. In conclusion, *C. speciosus* extracts both rhizome and stem

exhibited estrogenic activity. This result will help to improve health care related with estrogen deficiency.

4.2 Introduction

The reproductive cycle or estrous cycle is a cyclic pattern as a result of the interaction of hormones from the hypothalamic-hypophyseal-ovarian axis. Generally, two types of reproductive cycles are found in mammals, based on the response of uterine epithelium to the stimulation of hormones (Prakash, 2007). These are called estrous cycle, which occurs in rodents, non primate mammals and menstrual cycle which occurs in primates. The duration of each reproductive cycle varies from species to species with significant fluctuation between cycle length and life expectancy of anyone species. For example, the reproductive cycle of female rats and mice repeats at intervals of four to five days whereas the monkey and human repeats every four weeks.

In rodents, the estrous cycle is approximate four to five days. There are four stages of estrous cycle including proestrus, estrus, metestrus (or diestrus I) and diestrus (or diestrus II). The four stages of this cycle can be determined according to the cell types in the vaginal smear (Marcondes, Bianchi and Tanno, 2002). During estrous cycle, the female physiological and psychological conditions change according to the different phases of the estrous cycle. Proestrus, typically lasts twelve to fourteen hours and the vaginal smear shows mainly nucleated epithelial cells. Estrus, typically lasts twenty-five to twenty seven hours and the vaginal smear shows mainly cornified cells. Metestrus is the shortest stage, lasting six to eight hours and shows many leukocytes and a few cornified cells. Diestrus, typically lasts fifty-five to

fifty seven hours and the vaginal smear consists of only leukocytes cells (Astwood, 1939; Mandal, 1951; Long and Evans, 1992; Hartman, 1994).

The estrous cycle is controlled by hypothalamic hormone. Gonadotropin releasing hormone (GnRH) secreted by the hypothalamus stimulates the release of follicle stimulating hormone (FSH) and luteinizing hormone (LH) from the anterior pituitary gland. FSH stimulates follicular growth and the secretion of estrogen from the growing follicles. LH stimulates the development of ovarian follicles and also secretion of estrogen to bring about ovulation and promote formation of the corpus luteum. Corpus luteum stimulates the production of estrogen and progesterone. During the estrous cycles, LH and FSH remain low but increase in the afternoon of the proestrus phase. Estradiol levels begin to increase at metestrus, reaching peak levels during proestrus but returning to baseline at estrus. Progesterone secretion also increases during metestrus and diestrus, but decreases afterwards. The progesterone value rise to reach its second peak towards the end of proestrus (Smith, Freman and Neil, 1975; Sportnitz, Socin and David, 1999).

Estrogen is produced by the ovaries. It plays a vital role in keeping vaginal tissues lubricated and healthy. When levels of estrogen are decreased, vaginal tissue becomes atrophic, thin, dry and shrunken (Branco, Cancelo, Villero, Nohales and Julia, 2005). At menopause, estrogen loss is believed to be the most common cause of vaginal symptoms. Ten to forty percent of women have vaginal dryness related urogenital disorders. The treatment of vaginal atrophy is estrogen replacement. Various forms of vaginal administration of estrogen, including vaginal creams, tablets or suppositories are available (Branco Cancelo, Villero, Nohales and Julia, 2005).

The Women's Health Initiative has published about concerning negative side effects of hormone replacement therapy (HRT) with estrogen and progestin (Writing Group for The Women's Health Initative Investigators, 2002), many women have been turning to herbal remedies. There are several medicinal plants that have been reported to have estrogenic activity on reproductive cycle including *Pueraria mirifica*, commonly known in Thai as "white kwao krua". Its tuberous root contains at least 13 known phytoestrogens. It has been reported that *P. mirifica* stimulated the proliferation of vaginal and uterine epithelium in female rats and women (Sukavattana, 1940; Pope, Grundy, Jone and Tait, 1958; Malaivijitnond, Kiatthaipipat, Cherdshewasart, Watanabe and Taya, 2004). Phytoestrogenic study of black tea (*Camellia sinensis*) extract in an oophorectomized rat has also been reported (Das, Das, Mukherjee, Mukherjee and Mitra, 2005). It was found that black tea extract supplementation for 21 days showed the revival of estrus stage from diestrus stage (Das, Das, Mukherjee, Mukherjee and Mitra, 2005). The ethanol extract of *Achyranthes aspera* Linn. root also exhibited estrogenic activity as shown by the significant increase in the vaginal epithelial cornification in immature rats (Vasudeva and Sharma, 2006). In addition, Gallo et al. (1998) found that vaginal opening occurred earlier in female receiving soy supplemented feed when compared with controls. This effect was significant at a high dose group. As shown in Chapter III, phytosterols such as diosgenin and β-sitosterol had been found in *C. speciosus* extracts. Thus, this chapter was aimed to evaluate the estrogenic activity of *C. speciosus* rhizome and stem extracts in bilaterally OVX rats using vaginal cornification assay.

4.3 Materials and Methods

4.3.1 Animals and Experimental Procedures

Female Wistar rats at 9 weeks of age were maintained under environmentally controlled room provided with a 12:12 light and dark cycle at a temperature of approximately 25°C, fed with commercial feed (C.P. Mice Feed, Bangkok, Thailand) and allowed to access water *ad libitum*. On day14 after ovariectomy, the animals were assigned into six groups, with 6 animals per group.

Group 1 (Ovariectomized rats): Received 1 mL of 10% Tween 80 in a distilled water (served as a control group, n = 6).

Group 2 (Ovariectomized rats): Received 1 mL of aqueous solution of 17α-ethynylestradiol at a dose of 0.02 mg/kg B.W. (served as a standard drug group, n = 6).

Group 3 (Ovariectomized rats): Received 1 mL of aqueous solution of *C. speciosus* rhizome extract at a dose of 500 mg/kg B.W. (served as a test group, n = 6).

Group 4 (Ovariectomized rats): Received 1 mL of aqueous solution of *C. speciosus* rhizome extract at a dose of 1000 mg/kg B.W. (served as a test group, n = 6).

Group 5 (Ovariectomized rats): Received 1 mL of aqueous solution of *C. speciosus* stem extract at a dose of 500 mg/kg B.W. (served as a test group, n = 6).

Group 6 (Ovariectomized rats): Received 1 mL of aqueous solution of *C. speciosus* stem extract at a dose of 1000 mg/kg B.W. (served as a test group, n = 6).

All these treatments were administrated once daily and orally for 8 weeks. Vaginal cornification was examined weekly.

4.3.2 Vaginal Cornification Assay

Vaginal samples were taken in the morning, during the photoperiod between 8.00 and 11.00 a.m. by inserting the tip of a medicine dropper into the vagina, flushing saline in and out and placing the fluid onto microscope slides and stained with methylene blue (Rhodes, Balestreire, Czambel and Rubin, 2002). The vaginal epithelium cells were observed and classified under a light microscope at a magnification x 20. The vaginal smears were classified by their cytology (Malaivijitnond et al., 2006). In this study, the appearance of cornified cells (or the majority of cornified cells) was used as an indicator of estrogenic activity.

4.4 Results

4.4.1 Estrogenic Activity of *C. speciosus* Extracts in Ovariectomized Rats

The estrogenic activity of *C. speciosus* extracts was evaluated through the vaginal cytology and the results were demonstrated in Figure 4.1. The results showed that the OVX rats received vehicle (control) exhibited only leukocytes. In contrast, the OVX rats administered with the standard drug showed cornified cells as early as on the fourth day of the treatments and lasted until the end of the treatments. As with the OVX rats administered with the standard drug, the OVX rats received *C. speciosus* rhizome and stem extracts at a difference dose showed vaginal cornification, the number of vaginal smear exhibited more nucleated and cornified cells when compared with that the OVX rats received vehicle (control).

4.4.2 Effects of the *C. speciosus* Extracts on Percentage of Ovariectomized Rats Having Vaginal Cornification

The percentage of rats having vaginal cornification summarized in Table 4.1 showed that the percentage of OVX rats having vaginal cornification in the control group was 0%, but was 100% in OVX rats with the standard drug. The administration of *C. speciosus* rhizome (1000 mg/kg B.W.) and stem (500 mg/kg B.W.) extracts showed an increased percentage of OVX rats having vaginal cornification.

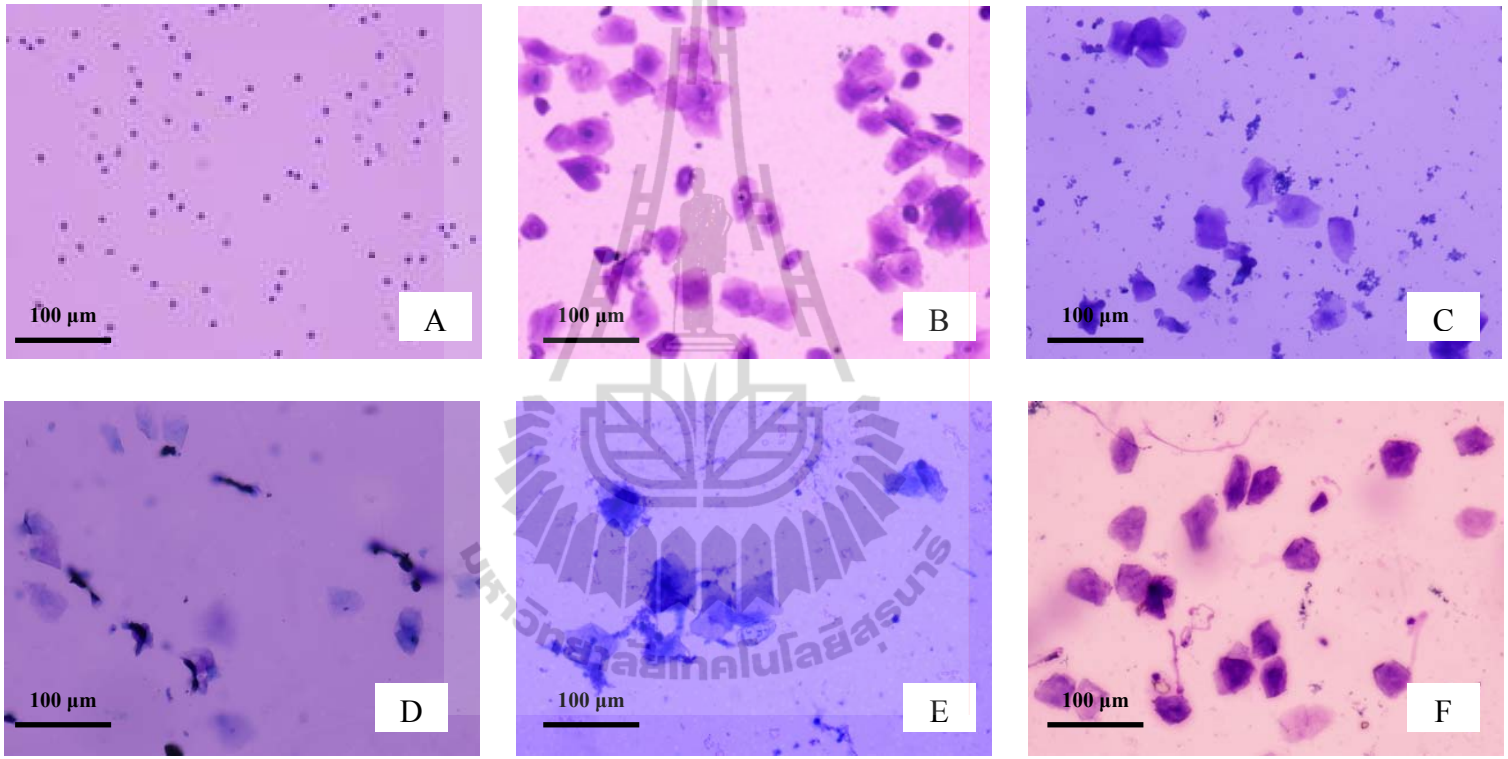

Figure 4.1 Representative photomicrographs of methylene blue stained vaginal smear (the bar equals 100 µm).

OVX rats received; vehicle (A), 17α-ethinylestradiol (0.02 mg/kg B.W.) (B), *C. speciosus* rhizome extract (500 mg/kg B.W.) (C), *C. speciosus* rhizome extract (1000 mg/kg B.W.) (D), *C. speciosus* stem extract (500 mg/kg B.W.) (E), *C. speciosus* stem extract (1000 mg/kg B.W.) (F).

Table 4.1 Percentage of ovariectomized rats having vaginal cornification.

Group	n	Ovariectomized rats having vaginal cornification (%)							
		Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7	Week 8
OVX rats received vehicle (10% v/v Tween 80)	6	0	0	0	0	0	0	0	0
OVX rats received 17α-ethinylestradiol (0.02 mg/kg B.W.)	6	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
OVX rats received *C. speciosus* rhizome extract (500 mg/kg B.W.)	6	16.67	16.67	16.67	50.00	33.33	33.33	50.00	33.33
OVX rats received *C. speciosus* rhizome extract (1000 mg/kg B.W.)	6	16.67	33.33	50.00	33.33	66.67	50.00	50.00	66.67
OVX rats received *C. speciosus* stem extract (500 mg/kg B.W.)	6	16.67	50.00	33.33	50.00	50.00	66.67	66.67	83.33
OVX rats received *C. speciosus* stem extract (1000 mg/kg B.W.)	6	0	50.00	16.67	66.67	66.67	33.33	66.67	16.67

4.5 Discussion

Changes in the vagina are closely correlated with the ovarian cycle. Understanding vaginal characteristic provides recognition of follicular activity during normal reproductive cycle or after estrogenic and other therapies. Vaginal cytology assay is a simple and inexpensive method for a determination of the estrogenic activity of the synthetic estrogen, xenoestrogens and phytoestrogen (Malaivijitnod, Chansri, Kijkuokul, Urosopon and Cherdshewasart, 2006). In normal animals, changes in the vagina were stimulated by the fluctuation of estrogen level, which acts directly on vaginal epithelium (Mandl, 1951). The vaginal smear during menopause was different from other phases. It was an atrophic typical smear; the predominant cells were the oval basal cells of various sizes. Cells from the intermediate layer were scarce. However, the neutrophilic cells were abundant. Menopausal smears vary according to the stage of menopause and estrogen levels (Erochenko, 1996). In this chapter, OVX rats were used as a model of cessation of estrogen that can lead to vaginal undergo atrophy. Administration of estrogenic substance such as 17α-ethynylestradiol clearly replaced the lack of natural estrogen. The 17α-ethynylestradiol induced opening of the vagina in 100% of rats and all cells in their vaginal smear were cornified. The pathogenesis of urogenital atrophy and its relationship to estrogen deficiency is directly correlated with the presence of estrogen receptors in the vaginal epithelium. In the vagina, 17β-estradiol exerts desirable effects in postmenopausal women (Santen et al., 2002). It stimulated a proliferation of epithelial cells thereby allowing acidification of the vaginal milieu with a lower pH prevents ascending infections. Furthermore, 17β-estradiol is necessary for lubrication of the vagina upon sexual arousal (Willhite and O' Connell, 2001).

The data also demonstrated that the ethanolic *C. speciosus* extracts exhibited estrogenic activity as shown by the vaginal epithelial cornification in OVX rats. The superficial cells of vagina could be shed into the lumen to form large squamous cells. The present data are consistent with the studies on OVX albino rats by injected *C. speciosus* plant extract on consecutive five days and compared with those receiving only estrogen for five days; showing the *C. speciosus* extract treated rats produced proliferation and cornification vaginal epithelium comparable with that found in the estrus phase (Panda, 2000).

The GC-MS data showed that *C. speciosus* rhizome and stem extracts contains diosgenin and β-sitosterol. Thus, plant extracts induced proliferation and cornification of vaginal epithelium may be due to the presence of β-sitosterol, which acts as an effective estrogen-like agonist in exerting vaginal cornification in adult OVX rats (Malini, 1987). Moreover, it has been reported that saponin-rich extracts from *Combretodendron africanum* injected into female rats stimulated uterine growth, lowered luteinizing hormone release and blocked the estrous cycle (Benie, El-Izzi, Tahiri, Duval and Thieulant, 1990). In contrast, the steroidal saponin isolated from *Ornithogalum saundersiae* injected into rats on the morning of proestrus at the level of 9 μg/kg inhibited estrogen production and prolonged the period of diestrus (Tamaru, Honda, Mimaki, Sashida and Kogo, 1997). Taken together, in this study, the estrogenic effects of *C. speciosus* rhizome and stem extracts on vaginal cytology are unlikely due to diosgenin, but probably due to β-sitosterol.

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CHAPTER V

ANTI-IMPLANTATION ACTIVITY OF *COSTUS SPECIOSUS* (Koen.) Sm. EXTRACTS IN PREGNANT RATS

5.1 Abstract

This study was aimed to investigate the anti-implantation activity of *Costus spesiosus* (Koen.) Sm. rhizome and stem extracts administered during pre-implantation and post-implantation periods by oral route in adult female Wistar rats. The pregnant rats were divided into 6 groups, each group contained 3-4 rats; received vehicle (10% v/v Tween 80), 17α-ethynylestradiol (0.02 mg/kg B.W.), *C. speciosus* rhizome extract (500 mg/kg B.W.), *C. speciosus* rhizome extract (1000 mg/kg B.W.), *C. speciosus* stem extract (500 mg/kg B.W.), *C. speciosus* stem extract (1000 mg/kg B.W.) from day 1 to 7 (pre-implantation) and day 8 to 14 (post-implantation) of pregnancy. Autopsy was performed on day 10 and day 20 of pregnancy. The results of pre-implantation studies showed that administration of *C. speciosus* rhizome and stem extracts (1000 mg/kg B.W., n = 4) and 17α-ethynylestradiol (0.02 mg/kg B.W., n = 4) significantly decreased the mean weight of the uterus ($P < 0.05$). Administration of *C. speciosus* stem extract (1000 mg/kg B.W., n = 4) significantly decreased a number of implantation site ($P < 0.05$). The results of post-implantation studies showed no significant difference in the number of implantation sites and the mean weight of the

fetuses when compared with the control group. In conclusion, the administration of *C. speciosus* stem extract at a dose of 1000 mg/kg B.W. produced anti-implantation effect during pre-implantation period.

5.2 Introduction

Pregnancy is defined as a period from fertilization to parturition. The duration of pregnancy is steady within a species and varies between species. In rats, gestation last to 21 days. Implantation is a process by which the conceptus comes into intimate contact with endometrium. Although the nature of implantation varies among mammalian species, it begins with the apposition of the blastocyst to the luminal epithelium of the endometrium and culminates in the formation of the definitive placenta (Bany and Cross, 2006). In rodents and human, the decidual reaction occurs during the first few days of pregnancy. Implantation in the rats is reported to occur around day 5 of gestation and decidulization has already started by this time which the fibroblast-like endometrial stromal cells transiently proliferate and then differentiate into large and polyploidy decidual cells.

Implantation and embryonic development are under hormonal control. Estrogen and progesterone are key regulators in all these processes. Estradiol stimulates the endometrial glandular epithelium to proliferate through estrogen receptor and growth factor-mediated mechanism (Brenner, West and McCellan, 1990). Progesterone counteracts the growth stimulatory effect of estrogen, induces differentiation in endometrium and is essential for normal implantation and the maintenance of the endometrium for pregnancy (Clarke and Sutherland, 1990; Ace and Okulicz, 1995).

Therefore, the appropriate in the estradiol/progesterone ratio is important for implantation.

The population explosion is one of the major problems present in the world. The increment of population raises so many sufferings, like lack of food, water, energy and raw material supply and a decline in mortality etc. In view of above discussion, scientists have started to talk this serious problem by developing the effective contraceptives. Among the three ways of controlling population including, abortion, sterilization or contraception, the contraceptive way of birth control is one of the most popularizing ways. The types of contraceptive method are being used such as mechanical method (diaphragm, cervical cap, intrauterine devices (IUD)), physiological method (oral pills), and surgical method (tubectomy/vasectomy) (Unny, Chauhan, Joshi, Dobhal and Gupta, 2003).

In female, the term contraceptive refers to those chemicals substances or devices act upon any of the reproductive organ/physiological aspect. They may be anti-ovulatory, affect the union of ova and sperm, be abortifacient, show anti-implantation activity, or have effect on the uterus. The most effective postcoital contraceptive includes high doses of hormones administered to women within 72 h after an unprotected coitus or an intrauterine device inserted within 10 days after the coitus (Fasoli, Parazzini, Cechetti and La Vecchi, 1989). The hormonal formulations used include estrogen, combinations of estrogen plus progestin, or androgen analogs such as testosterone esters and danazol. The adverse effects (Borell, 1970; Kannel, 1979; Takacs, 1979; Blashkova et al., 1981) caused by oral and injectable contraceptives are increased blood transaminase, cholesterol levels, indigestion, weight gain, headache, depression, fatigue, hypermenorrhea and intermenorrheal

bleeding. These also disturb the metabolism of lipid, protein, carbohydrates, enzymes and vitamins (Zaeslenin-Buthe, 1971; Bingel and Benoit, 1973a, b; Spellacy, 1974). Regarding to the importance of fertility control and side effects to the existing contraceptive methods, the usage of biologically active botanical substances or fertility-regulating agents of plant origin which are eco-friendly in approach and interfere with the natural patterns of reproduction becomes necessary (Dixit, 1992; Dehghan, Martin and Dehghanan, 2005).

For centuries, virtually every indigenous culture has been using plants and/or their various parts to control human population. The present study was set up with the objective to find new orally active non-steroidal contraceptive agents from authenticated plant samples using standard animal models for human use and welfare.

Many plant extracts have been reported to affect fertility in rodents. Gebri, Makonnen, Zerihum and Debella (2005) reported that methanolic extract of *Rumex steudelii* significantly decreased the number of implantation sites. Ravichandran, Suresh, Sathkumar, Elango and Srimivasan (2007) reported that hydroalcoholic extract of stem bark of *Ailanthus excelsa* Roxb. (Simaroubaceae) has anti-fertility activity. A strong anti-implantation (72%) and abortifacient activity (56%) was observed at the tested dose levels of 200 and 400 mg/kg. The studies of Montaserti, Pourheydar, Khazaei and Ghorbani (2007) showed that administration of *Physalis alkekengi* extract on day 1 to 5 of pregnancy significantly decreased the number of implantation sites, number and weight of neonates. These results suggest that the extract produced anti-fertility effect probably by inhibiting implantation. The ethanolic extract of *Allium cepa* showed significant anti-fertility activity. Pretreatment with ethanolic extract showed significant inhibition of number of implantation sites at

a dose of 300 mg/kg and there was no change in ovulation, hence the anti-fertility activity observed in the study with *Allium cepa* can be attributed largely to its anti-implantation activity (Thakare, Kthavade, Dhote and Deshpande, 2009).

C. speciosus is an herbaceous plant growing wild throughout Southeast and South Asia. The plant is traditionally used in the treatment of fevers, cough, worm infections, skin diseases and snake bite (Bavara and Narasimhacharya, 2008). The plant is reported to contain active constituents such as diosgenin, tigogenin, saponin, β-sitosterol (Chandel, Shukla and Sharma, 1996), sterol, fatty acid esters and triterpenes (Vijayalakshmi and Sarada, 2008) (see also in Chapter II) that have been known for their estrogenic properties. Thus, the aim of this chapter was performed to investigate the anti-implantation of the *C. speciosus* rhizome and stem extracts administered during pre- and post-implantation periods.

5.3 Materials and Methods

5.3.1 Animals

Female (200-300 g) and male (450-500 g) Wistar rats were obtained from SUT Animal House. The rats were housed in a temperature controlled (25°C), light-regulated (12h light and 12h dark) room and allowed to access standard food and water *ad libitum*.

5.3.2 Anti-implantation Determination

Anti-implantation activity was determined as described by Khanna and Choudhury (1968). Daily vaginal smears of the animals were taken and the stages of estrous cycle were determined. Proestrus animals were mated with males of proven

fertility (1 female: 1 male) and examined in the following morning for the evidence of copulation. Rats exhibiting the copulation plug or thick clumps of spermatozoa in their vaginal smears were separated and that day was designated as day one of pregnancy and these rats were divided into two experiments, including pre-and post-implantation studies. The pregnant rats were then randomly assigned into 6 groups, with 3-4 animals in each group. The experimental were designed as follows:

Group 1 (Pregnant rats): Received 1 mL of 10% Tween 80 in a distilled water (served as a negative control group).

Group 2 (Pregnant rats): Received 1 mL of aqueous solution of 17α-ethynylestradiol at a dose 0.02 mg/kg B.W. (served as a positive control group).

Group 3 (Pregnant rats): Received 1 mL of aqueous solution of *C. speciosus* rhizome extract at a dose 500 mg/kg B.W. (served as a test group).

Group 4 (Pregnant rats): Received 1 mL of aqueous solution of *C. speciosus* rhizome extract at a dose 1000 mg/kg B.W. (served as a test group).

Group 5 (Pregnant rats): Received 1 mL of aqueous solution of *C. speciosus* stem extract at a dose 500 mg/kg B.W. (served as a test group).

Group 6 (Pregnant rats): Received 1 mL of aqueous solution of *C. speciosus* stem extract at a dose 1000 mg/kg B.W. (served as a test group).

The anti-implantation studies were carried out in two sets of experiments; pre-implantation and post-implantation.

Pre-implantation effects: one set of experimental pregnant rats was treated with *C. speciosus* rhizome and stem extracts from day 1 of gestation and ending on treatment day 7. The rats were sacrificed on day 10 of pregnancy. Uteri were removed and weighed. A number of implantation sites were recorded. The percentage of pre-implantation losses was calculated by the following formula (Williamson, Okaka, and Evan, 1996): Anti-implantation activity =

$$\frac{\text{Number of implants in control group - Number of implants in test group}}{\text{Number of implants in control group}} \times 100$$

Post-implantation effects: in the second set of pregnant rats, the *C. speciosus* rhizome and stem extracts were given to the rats on day 8 through 14 of gestation. Rats in this group were sacrificed on day 20 of pregnancy. The uterus was excised and weighed. The number of implantation site was also investigated for the external malformations of fetuses (Steele and Copping, 1990).

5.3.3 Statistical Analysis

Data of anti-implantation activity study were analyzed for a statistical significance against their respective controls by using one way analysis of variance (ANOVA). *P* values less than 0.05 were considered to be significant. The values were expressed as mean ± S.E.M..

5.4 Results

5.4.1 Effects of *C. speciosus* Extracts on Pre-Implantation in Pregnant Rats

The results of pre-implantation are given in Table 5.1. These results obtained after administration of ethanolic extracts of *C. speciosus* in pregnant rats. An administration of *C. speciosus* stem extract at a dose of 1000 mg/kg B.W. significantly decreased ($P < 0.05$) a number of implantation site in pregnant rats. The mean weight of the uterus was significantly reduced ($P < 0.05$) in pregnant rats administered with *C. speciosus* rhizome and stem extracts at a dose of 1000 mg/kg B.W. Anti-implantation activity expressed as percentage in pre-implantation period were 17.24% and 19.52% with *C. speciosus* rhizome extract at a dose of 500 and 1000 mg/kg B.W., respectively while 24.14%, and 28.76%, with *C. speciosus* stem extract at a dose of 500 and 1000 mg/kg B.W., respectively.

An oral administration of 17α-ethynylestradiol at a dose of 0.02 mg/kg B.W. significantly decreased ($P < 0.05$) the mean weight of the uterus when compared with control. The percentage of anti-implantation activity in pre-implantation period was 26.41%. No side effects were observed in treated animals.

5.4.2 Effects of *C. speciosus* Extracts on Post-Implantation in Pregnant Rats

Table 5.2 showed anti-implantation activity in post-implantation period of *C. speciosus* rhizome and stem extracts. Administration of *C. speciosus* rhizome extract at a dose of 1000 mg/kg B.W. and *C. speciosus* stem extract at doses of 500 and 1000 mg/kg B.W. decreased the number of implantation site. The mean weight of fetuses

was decreased in pregnant rats administered with *C. speciosus* stem extract (500 mg/kg B.W.). The percentages of anti-implantation on post-implantation period of *C. speciosus* rhizome and stem extracts at a dose of 500 B.W. were 6.93% and 10.34% mg/kg, respectively.

An oral administration of 17α-ethynylestradiol at a dose of 0.02 mg/kg B.W. reduced the number of implantation site and mean weight of the fetuses. The percentage of anti-implantation activity in post-implantation period was 10.34%. No side effects were observed by gross examination of the fetuses. The animals exhibited normal pregnancy.

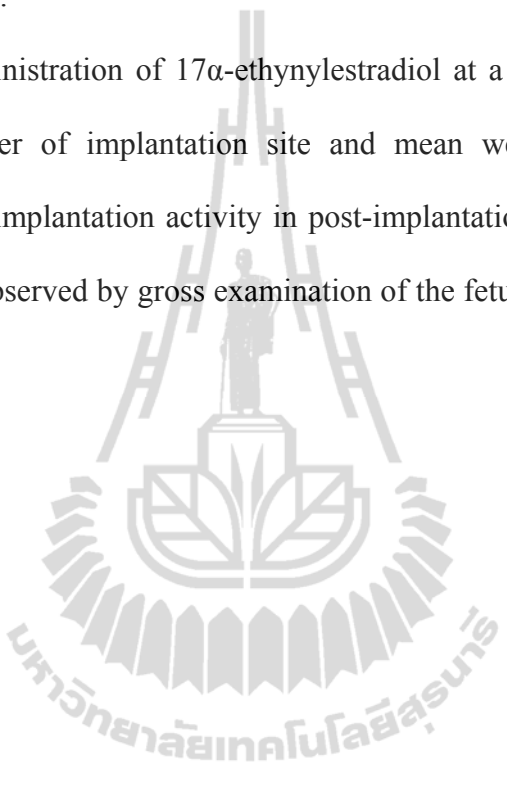

Table 5.1 Pre-implantation effect of *C. speciosus* extracts in pregnant rats treated on day 1 to 7 of pregnancy.

Group	Treatment	Number of implantation site	Mean weight of the uterus (mg)	Anti-implantation activity (%)
1	Pregnant rats received vehicle (10% v/v Tween 80)	14.50 ± 1.50^{b}	513.41 ± 42.53^{b}	0
2	Pregnant rats received 17α-ethynylestradiol (0.02 mg/kg B.W.)	10.67 ± 0.88^{ab}	370.82 ± 19.64^{a}	26.41
3	Pregnant rats received *C. speciosus* rhizome extract (500 mg/kg B.W.)	12.00 ± 0.00^{ab}	435.02 ± 22.47^{ab}	17.24
4	Pregnant rats received *C. speciosus* rhizome extract (1000 mg/kg B.W.)	11.67 ± 0.67^{ab}	365.15 ± 12.81^{a}	19.52
5	Pregnant rats received *C. speciosus* stem extract (500 mg/kg B.W.)	11.00 ± 1.68^{ab}	434.25 ± 45.33^{ab}	24.14
6	Pregnant rats received *C. speciosus* stem extract (1000 mg/kg B.W.)	10.33 ± 0.33^{a}	353.45 ± 22.41^{a}	28.76

[a, b]Significantly different ($P < 0.05$) when compared within a column. Mean ± S.E.M. are given.

Table 5.2 Post-implantation effect of *C. speciosus* extracts in pregnant rats treated on day 8 to 14 of pregnancy.

Group	Treatment	Number of implantation site	Mean weight of the fetuses (g)	Anti-implantation activity (%)
1	Pregnant rats received vehicle (10% v/v Tween 80)	9.67 ± 1.33	14.68 ± 1.46	0
2	Pregnant rats received 17α-ethynylestradiol (0.02 mg/kg B.W.)	8.67 ± 1.45	13.80 ± 1.53	10.34
3	Pregnant rats received *C. speciosus* rhizome extract (500 mg/kg B.W.)	10.67 ± 0.88	14.64 ± 0.00	6.93
4	Pregnant rats received *C. speciosus* rhizome extract (1000 mg/kg B.W.)	6.67 ± 3.53	15.85 ± 0.00	0
5	Pregnant rats received *C. speciosus* stem extract (500 mg/kg B.W.)	8.67 ± 1.86	13.96 ± 2.38	10.34
6	Pregnant rats received *C. speciosus* stem extract (1000 mg/kg B.W.)	9.67 ± 0.33	16.27 ± 0.00	0

Significantly did not different ($P > 0.05$) when compared within a column. Mean ± S.E.M. are given.

5.5 Discussion

In the study of anti-implantation activity, the *C. speciosus* extracts were administered for the first five days of pregnancy. This period corresponds to the period that begins after fertilization and involves in the stages before and after implantation (Elbetieha, Al-Hamood, Alkofahi and Bataineh, 1998). Normally, the implantation of conceptus occurs on gestation day 4-5 in rodents (Hodgen and Itskovits, 1988). Thus, chemical insults prior to completion of the implantation process should result in implantation loss.

The results obtained in Table 5.1 clearly indicated that the oral administration of *C. speciosus* rhizome and stem extracts on pre-implantation period showed anti-implantation activity in pregnant rats and was dose dependent. The result is consistent with previous studies showing that a mixture of saponin isolated from the rhizomes of *C. speciosus* effectively protected against pregnancy in rats by preventing implantation in 8 of 10 rats fed on day 1 to 7 of pregnancy (Tewari, Chaturvedi and Pandev, 1973). In the present study, *C. speciosus* rhizome and stem extracts were tested for their pre-and post-implantation properties. Among the tested groups, *C. speciosus* stem extract at a dose of 1000 mg/kg B.W. was the most potent in reducing the implantation sites and mean weight of uterus during pre-implantation period.

The anti-implantation effects of *C. speciosus* extracts might be attributed to one or more mechanisms. One possible mechanism could be an inhibition of implantation, which was shown by a decrease in the number of implantation site (pre-implantation period). Another mechanism, the anti-implantation effect of these extracts could be due to the disturbance of endocrine-endometrial synchrony which is dependent on

estrogen and progesterone balance. However, the exact mechanisms need further study.

Large numbers of anti-implantation plant extracts are known to exhibit estrogenic activity in rats (Dahanukar, kulkarni and Rege, 2000). Estrogenic substance may cause the expulsion of ova from the tube, disruption of luteotrophic activity of the blastocyst, disruption the functional equilibrium between the endogenous estrogen and progesterone which may result in failure in implantation. As shown in Chapter III, *C. speciosus* rhizome and stem extracts significantly increased relative uterine weight in OVX rats, indicating that the extracts had estrogenic properties.

Clinically, drugs that contract the uterine smooth muscle are used to induce labor or abortion. Such drugs include oxytocin, ergometrine and quinine (Olagbende-Dada, Ukpo, Coker and Adesina, 2009). The study of uterotonic responded by the *C. speciosus* extracts was characterized by an increase in the amplitude and frequency of uterine contractions (data shown in Chapter VI) indicating the anti-implantation effects of the *C. speciosus* extracts might be due to their uterotonic effects.

Evidences from animal studies suggested that ingestion of large quantities of phytoestrogens can adversely affect fertility. Previous studies reported that in sheep exposed to high levels of clover in their fodder resulted in high abortion rates and permanent sterility (Adams, 1995). Rats fed with a diet high in coumestrol maintained a state of anovulation, but the mechanism is still not well-known (Whitten, Lewis, Russellm and Naftolinm, 1995). These results suggested that phytoestrogens may affect the blastocyst implantation in animals by interfering with the endocrine system, since the implantation process is controlled and regulated by the axis. The negative

effects of saponins on animal reproduction have long been known and have been ascribed to their abortifacient, antizygotic and anti-implantation properties (Tewari, Chaturuedi and Pandey, 1973; Stolzenberg and parkhurst, 1976). In this study, diosgenin is a steroidal saponin belonging to the triterpene group that found in *C. speciosus* rhizome and stem extracts, which might be responsible for anti-implantation activity as well.

In conclusion, the results suggested that *C. speciosus* rhizome and stem extracts could be used as a contraceptive in early pregnancy. However, further experiments are required to elucidate their precise mechanisms of action.

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CHAPTER VI

EFFECTS OF *COSTUS SPECIOSUS* (Koen.) Sm. EXTRACTS ON UTERINE CONTRACTION IN NON-PREGNANT RATS

6.1 Abstract

Costus speciosus (Koen.) Sm. is an important medicinal plant species that has estrogen-like activity on rats. However, its possible uterotonic effects have not been clarified. The aims of the study were to investigate the effects of *C. speciosus* rhizome and stem extracts on rat uterine contraction and to study the mechanisms whereby the extracts exerted their effects. Longitudinal uterine smooth muscle strips were isolated. Isometric force was measured and the effects of *C. speciosus* rhizome and stem extracts were studied. *C. speciosus* rhizome and stem extracts produced an increase in spontaneous contraction. The maximum effect of *C. speciosus* rhizome and stem extracts was 10 mg/100 mL and 30 mg/100 mL, respectively. The amplitude, frequency and area under the contraction of the phasic contraction were significantly increased along with the basal tension. Force produced in the presence of the extracts was abolished by inhibition of L-type calcium channels or myosin light chain kinase (MLCK). The contractions were not potentiated by *C. speciosus* extracts following inhibition of K^+ channels or inhibition of the sarcoplasmic reticulum calcium-ATPase (SERCA). The actions of the extracts were not blocked by the estrogen receptor

blocker, fulvestrant. Interestingly, the extracts significantly induced amounts of force in the absence of extracellular calcium, which could be blocked by inhibition of SERCA, but not fulvestrant. Thus, *C. speciosus* rhizome and stem extracts stimulated phasic activity in rat uterus. The data suggested that the uterotonic effects were due to non-estrogenic effects acting to inhibit K^+ channels and SERCA, and thereby increasing contraction via calcium entry on L-type calcium channels and MLCK. Moreover, *C. speciosus* rhizome and stem extracts were able to increase contraction via calcium entry on L-type calcium channels and SR calcium release. In conclusion, *C. speciosus* rhizome and stem extracts may be useful uterine stimulants.

6.2 Introduction

The uterus is located in the pelvis anterior to the rectum and posterosuperior to the bladder. It is a hollow and muscular organ. Its dimension varies considerably, depending on both estrogenic stimulation and previous parturition. In a premenopausal woman who has never been pregnant, the uterus is about the size and shape of an inverted pear (Elaine, 2008). The function of the uterus is to house and nourish the embryo and fetus. In addition, contractions in the muscular wall of the uterus are important in parturition. There are anatomical differences of uterus between species. Primates have a simplex uterus, which a single uterus. In the duplex uterus, the horns are completely separated and may open into vagina and cervix, which can be found in rats and other rodents.

The wall of the uterus composed of three layers; the endometrium that lines the luminal surface, the smooth muscle layer or myometrium and the outer serosa. By characteristic of its predominant smooth muscle content the myometrium is

responsible for force development and contraction. The arrangement of the smooth muscle fibers, longitudinal and circular muscle layers, within the myometrium is species-dependent. Contractile activity of the myometrium is modulated by a broad array of agents often acting in a species-dependent manner.

Ion channels are macromolecular protein pores in the membrane that allow specific ion to pass in and out the cells. Elevation of intracellular Ca^{2+} generates contractions of myometrial cells. The entry of Ca^{2+} is carried through L-type Ca^{2+} channels leading to action potentials and they openings are induced by a depolarization. The membrane potential is regulated by other types of ion channels expressed in myometrium including T-type Ca^{2+}, Na^{+}, Cl^{-} and K^{+} channels. The activity of these channels can also be regulated by uterine contractants and relaxants, which affect intracellular free Ca^{2+} concentrations in the myometrium.

There are two sources of Ca^{2+} causing an increase in intracellular Ca^{2+}. These include an entry of Ca^{2+} across the surface membrane through voltage-gated L-type Ca^{2+} channels and/or a release of Ca^{2+} from the sacroplasmic reticulum (SR). In smooth muscle, such as the myometrium, when action potentials occur as a result of depolarization, they consequently open the L-type Ca^{2+} channels, which are the major sources of Ca^{2+} for contraction (Matthew, Shmygol and Wray, 2004). Therefore, the contractions are abolished if L-type channels are blocked (Wray et al., 2003). Release of Ca^{2+} from SR has been demonstrated in human and animal myometrial preparation (Taggart and Wray, 1998; Luckas, Taggart and Wray, 1999). This intracellular organelle has Ca-ATPase, known as SERCA, which enables it to take up Ca^{2+} (Shmygol and Wray, 2004). Both IP_3 and ryanodine receptors have been identified on the SR. Previous studies have clearly shown that Ca^{2+} signaling and contractility are

increased in myometrial preparation if SR Ca^{2+} release is inhibited (Taggart and Wray, 1998; Kupittayanant, Luckas and Wray, 2002).

As with other smooth muscles, changes in intracellular Ca^{2+} leads to interaction between myosin and actin crossbridges underlying uterine contraction. Intracellular Ca^{2+} concentrations increase as a result of Ca^{2+} entry through plasma membrane ion channels and/or release from the SR. The main activating pathway is taken to be calcium ions binding to calmodulin activation of myosin light chain kinase (MLCK) and therefore initiate the phosphorylation and subsequent cross-bridge cycling. The relaxation of the myometrium follows a reversal of Ca^{2+}-calmodulin-MLCK pathway. Thus, the myosin phosphatase and Ca^{2+} falls as L-type Ca^{2+} channels close and Ca^{2+} efflux mechanism are stimulated (Wray, 2007).

C. speciosus is an important medicinal plant species. The plant rhizome is used as medicine. In Ayurveda, the rhizomes were ascribed to be bitter, astringent, acrid, cooling, aphrodisiac, purgative, antihelminthic, depurative, febrifuga, and expectoral (Mandal, Thomas and Elanchezhian, 2007). In a previous report, it was demonstrated that fresh juice of *C. speciosus* rhizome increased tone, amplitude and frequency of rhythmic contractions of isolated uterus of rat, guinea pig, rabbit, dog and human (Tewari, Prasad, Chaturvedi and Das, 1967).

However, the effects of *C. speciosus* rhizome and stem extracts on uterine contraction as well as their mechanisms have never been elucidated. Therefore, the aims of the study were designed to investigate the effects of *C. speciosus* rhizome and stem extracts on rat uterine contraction and to study the mechanisms whereby the extracts exerts their effects.

6.3 Materials and Methods

6.3.1 Myometrium Tissue Preparation

Non-pregnant Wistar rats (200-300 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 advice of the Institutional Animal Care and Use Committee, Suranaree University of Technology (SUT), Thailand.

The rats were sacrificed by an over dose of CO_2. The uterus was removed and immediately immersed in buffered physiological Krebs' solution (pH 7.4). The uterus was then placed in a shallow dissecting dish containing Krebs' solution at 37°C, and under a microscope. The longitudinal muscle layer was separated from the endometrium and the circular layer. Five or six strips (1-2 mm × 0.5 mm × 10 mm) were dissected and either used immediately or stored for a maximum period of 12 h at 4°C (Buddhakala, 2007).

6.3.2 Measurement of Tension

The uterine strips were mounted vertically under a resting tension of 1 g in a single chamber (25 mL) connected to a digital signal and recorded on a computer using Chart software (Kupittayanant, 2003). The organ bath contained Krebs' solution maintained at pH 7.4, temperature of 37°C and gassed with O_2. The myometrial strip was attached at one end to a metal hook and another end was fixed to the force transducer. The electrical signal has been recorded from the transducer and converted to the digital signal on a computer using Chart software (Kupittayanant, 2003). The uterine strips were allowed to contract spontaneously. An equilibrium period for at

least 30 minutes was given before an application of any chemicals. The representation of equipments used for tension measurement is shown in Figure 6.1.

Figure 6.1 Representation of the equipments used for tension measurement. (A) Monitor, (B) CPU, (C) Keyboard, (D) Thermostat, (E) Transducer, (F) Organ Bath, (G) Bridge Amp, (H) Power Lab and (I) Peristaltic Pumps.

The strip was allowed to contract spontaneously and an equilibrium period of 30 minutes was given before an application of any chemical. The measurements were made whilst the tissue was continually perfused with physiological solution (control), solution containing *C. speciosus* rhizome or stem extract (10-70 mg/100 mL or 30-70 mg/100 mL), respectively. Wortmannin, nifedipine, TEA, CPA and fulvestrant were also used as indicated in the text.

6.3.3 Chemicals and Physiological Solutions

All chemicals were purchased from Sigma chemical Co. (St. Louis, MO, USA). Other drugs used for the investigation of physiological pathways were described below. Wortmannin, an inhibitor of MLCK, was dissolved in dimethyl sulfoxide

(DMSO) at a concentration of 4 μM (Longbottom et al., 2000). Nifedipine, an inhibitor of L-type Ca^{2+} entry was dissolved in ethanol at a concentration of 10 μM (Shmigol, Eisner and Wray, 1998). Tetraethlammonium (TEA), an inhibitor of calcium-activated potassium channels was dissolved in distilled water at a concentration of 5 mM. Cyclopiazonic acid (CPA), an inhibitor of the SERCA pump was dissolved in DMSO at a concentration of 20 μM (Kupittayanant, Luckas and Wray, 2002). Fulvestrant, an estrogen receptor antagonist was dissolved in DMSO at a concentration of 1 μM (Buzdar, 2008). These working solutions were diluted to the desired concentrations with Krebs' solution. The physiological Krebs' solution was composed as follows (mM): 154 NaCl; 5.4 KCl; 1.2 $MgSO_4$; 8 glucose; 2 $CaCl_2$ and 10 N-[2-hydroxyethyl] piperazine-N'-[2-ethanesulfonic acid] (HEPES).

6.3.4 Preparation of *C. speciosus* Rhizome and Stem Extracts

As described in 2.3.1, stocks of *C. speciosus* extracts were kept at -20°C. *C. speciosus* rhizome or stem extract was dissolved in Krebs' solution just before use.

6.3.5 Statistical Analysis

The data were analyzed using Microcal Origin Software. The following parameters of contraction were measured; force integral, frequency, amplitude, and duration. The phasic contractions in the extracts were measured over 30 minutes after their application. Results were expressed as percentages of control contractions (i.e. the control is 100%). To test the effects of applications of wortmannin, nifedipine, TEA, CPA, or fulvestrant following the extracts, contractions were compared for the 30 minutes in the extracts (i.e. 30-60 minutes after start of the extract exposure), to the

60-90 minutes in the extracts with an addition of wortmannin, nifedipine, TEA, CPA, or fulvestrant. Integrated force (area under the contraction) was measured over a 30 minutes period. In some experiments, changes in force amplitude were expressed with respect to basal (resting) force level (0%), and the peak force (100%) in control condition. Data were presented as mean ± S.E.M. and "n" represents the number of sample, each one from a different animal. Significance was tested using appropriate *t*-test and P values < 0.05 taken to be significant. Results were then expressed as percentages of control contractions (i.e. the control is 100%).

6.4 Results

6.4.1 Effects of *C. speciosus* Rhizome Extract on Spontaneous Contraction

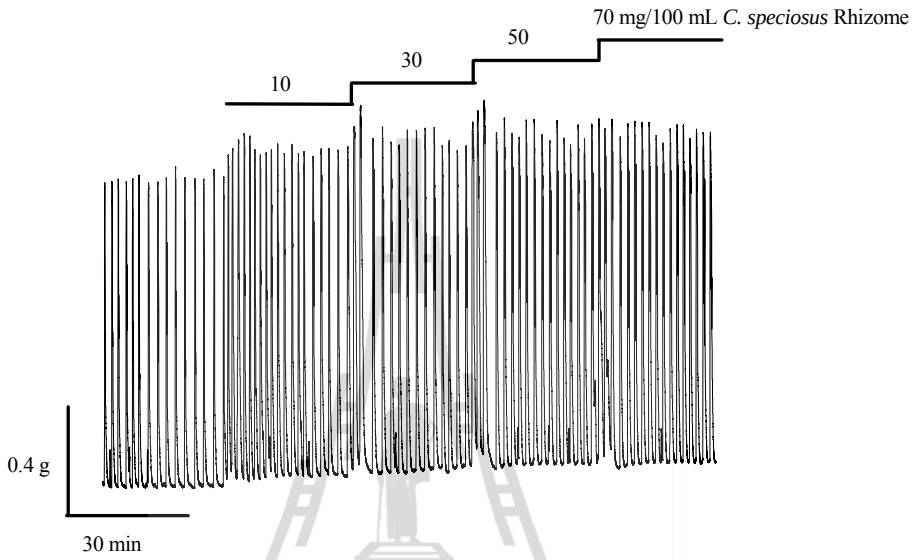
Under control conditions, spontaneous contraction of consistent amplitude, frequency and area under the contraction (AUC) was recorded for several hours, allowing the different concentrations of the extracts to be tested in the rat uterus (Figure 6.2 and Table 6.1). *C. speciosus* rhizome extract (10-70 mg/100 mL) was added to spontaneous active preparations for 30 minutes. At each contraction, it produced a significant increase in the amplitude, frequency, AUC and basal tension of the contractions (n = 5). The maximal stimulatory concentration on myometrium contractility occurred when its concentration was 10 mg/100 mL (n = 5). Thus, the concentration of 10 mg/100 mL was selected and used throughout the study.

6.4.2 Effects of *C. speciosus* Stem Extract on Spontaneous Contraction

As with *C. speciosus* rhizome extract, *C. speciosus* stem extract at concentrations of 30-70 mg/100 mL were applied to spontaneous active uterus for a

period of 30 minutes period (Figure 6.3 and Table 6.2). They produced significant increases in the amplitude, frequency, AUC, and basal tension of the contractions (n = 5). The maximal effect on the myometrium contractility was found with 30 mg/100 mL (n = 5). Thus, this concentration was used throughout the study.

A



B

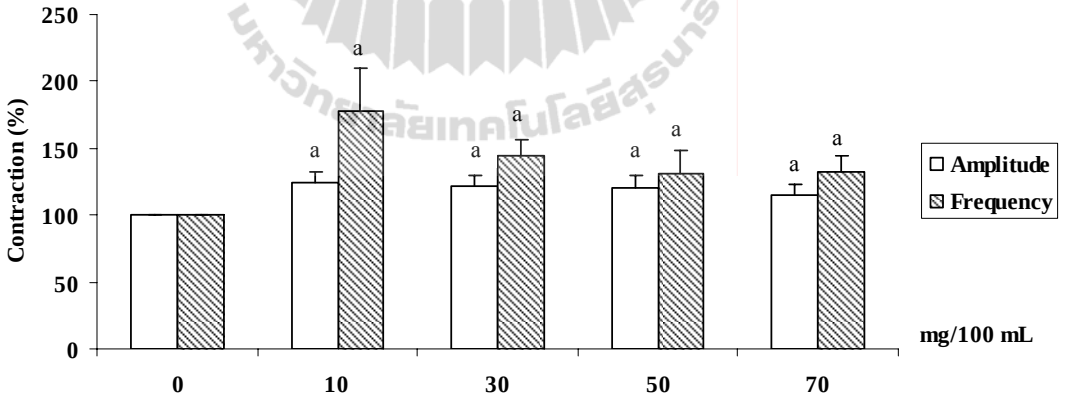
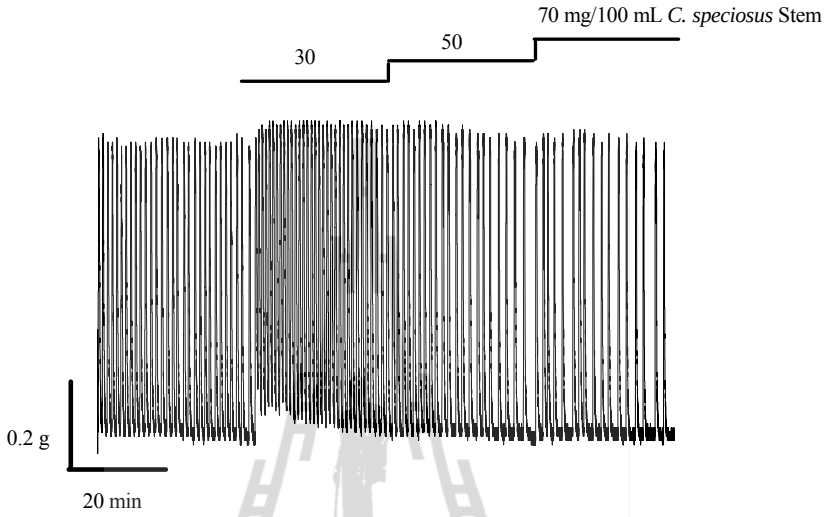


Figure 6.2 The effects of *C. speciosus* rhizome extract at various concentrations on spontaneous contraction (A) (typical of 5 other traces from different animals). Amplitude and frequency are presented as percentage of control responses [i.e. the control is 100%] (B). The *P*-values for amplitude and frequency of *C. speciosus* rhizome extract in ovariectomized rats are significantly different from the control ($^{a}P < 0.05$).

A



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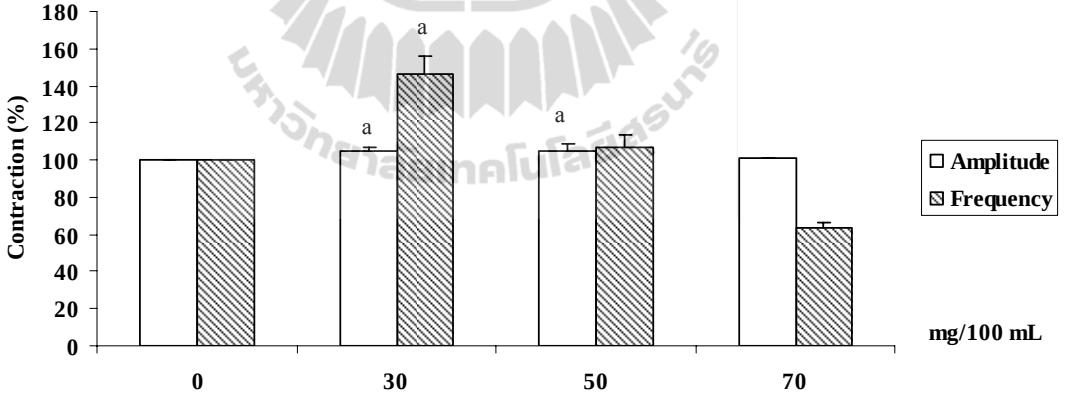


Figure 6.3 The effects of *C. speciosus* stem extract at various concentrations on spontaneous contraction (A) (typical of 5 other traces from different animals). Amplitude and frequency are presented as percentage of control responses [i.e. the control is 100%] (B). The *P*-values for amplitude and frequency of *C. speciosus* rhizome extract in ovariectomized rats are significantly different from the control ($^{a}P < 0.05$).

Table 6.1 The effects of *C. speciosus* rhizome extract at various concentrations on spontaneous contraction.

	Amplitude (% Mean ± S.E.M.)	Frequency (% Mean ± S.E.M.)	AUC (% Mean ± S.E.M.)	n
C. speciosus rhizome (mg/100 mL)				
0 (Control)	100	100	100	5
10	123.90 ± 8.44[a]	178.00 ± 32.12[a]	189.94 ± 22.63[a]	5
30	121.20 ± 9.01[a]	144.83 ± 12.07[a]	182.87 ± 7.69[a]	5
50	120.08 ± 9.22[a]	131.11 ± 17.36[a]	154.08 ± 19.24[a]	5
70	115.05 ± 7.65[a]	132.22 ± 12.22[a]	188.38 ± 20.75[a]	5

The *P*-values for amplitude, frequency and AUC of *C. speciosus* rhizome treated are significantly different from the control ([a]$P < 0.05$). Mean ± S.E.M. are given; n is number of animals.

Table 6.2 The effects of *C. speciosus* stem extract at various concentrations on spontaneous contraction.

	Amplitude (% Mean ± S.E.M.)	Frequency (% Mean ± S.E.M.)	AUC (% Mean ± S.E.M.)	n
C. speciosus stem (mg/100 mL)				
0 (Control)	100	100	100	5
30	105.09 ± 1.89[a]	146.67 ± 8.82[a]	241.49 ± 12.79[a]	5
50	105.07 ± 2.33[a]	106.67 ± 6.67	137.66 ± 20.50	5
70	100.75 ± 0.75	63.34 ± 3.34	84.39 ± 15.40	5

The *P*-values for amplitude, frequency and AUC of *C. speciosus* stem treated are significantly different from the control ([a]$P < 0.05$). Mean ± S.E.M. are given; n is number of animals.

6.4.3 Effects of *C. speciosus* Rhizome and Stem Extracts on Uterine Contractions in the Presence of the L-type Ca^{2+} Channel and MLCK Inhibitors

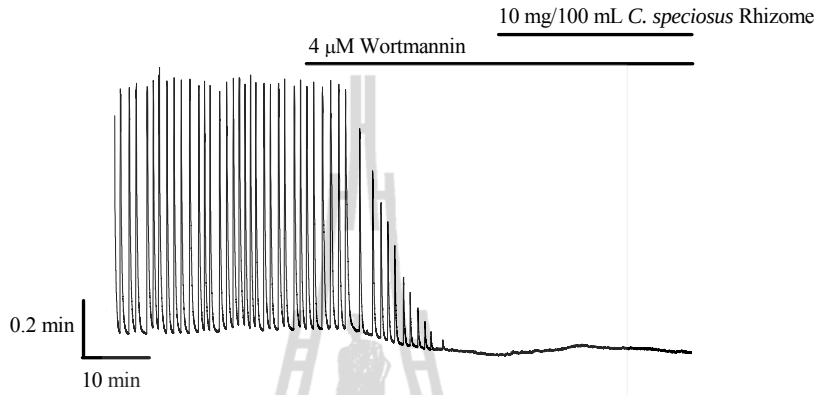
Uterine force can be produced by several pathways, but the main mechanism involves Ca^{2+}-calmodulin-MLCK (Longbottom et al., 2000). To investigate whether the increases in uterine force seen with the extracts were dependent on the calcium-calmodulin MLCK pathway, their effects in the presence of inhibitors of the L-type calcium channels and MLCK were examined (Figures 6.4 and 6.5).

As shown in Figures 6.4A and 6.5A, an application of wortmannin (4 μM) to spontaneous contracting uterus gradually reduced spontaneous force. The contraction was abolished after 30 minutes of the application. An addition of either *C. speciosus* rhizome (Figure 6.4A) or stem (Figure 6.5A) extract in the continued presence of wortmannin produced a small but consistent tonic force.

On the other hand, the effects of *C. speciosus* extracts in the presence of the potent inhibitor of MLCK, wortmannin, were studied (Figures 6.4B and 6.5B). Wortmannin (4 μM) in the continued presence of the extracts, gradually reduced active contraction and basal force in all studied preparations (n = 3).

The effects of *C. speciosus* rhizome and stem extracts on uterine contraction in the presence of wortmannin (4 μM), the MLCK inhibitor are summarized in Tables 6.3 and 6.4.

A



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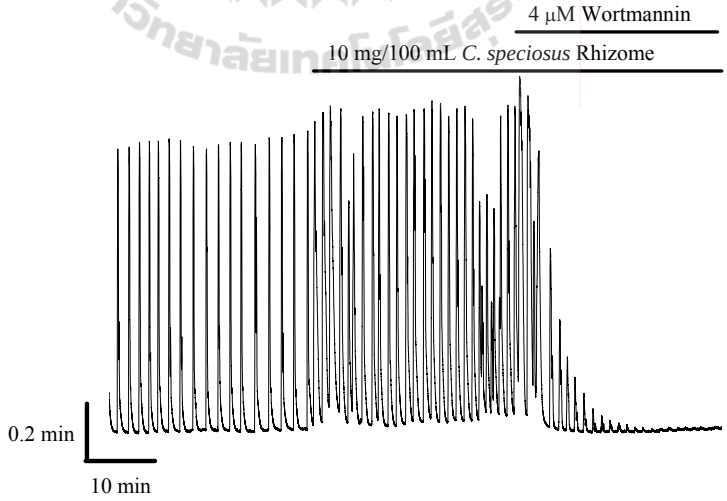
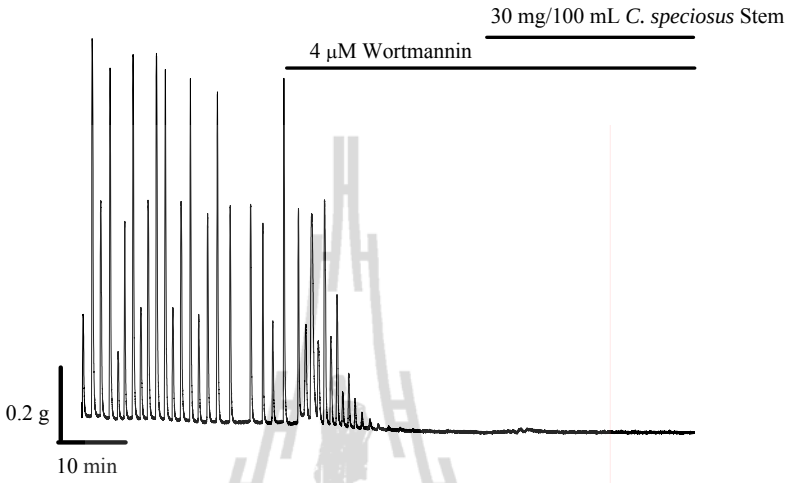


Figure 6.4 The effects of *C. speciosus* rhizome extract on uterine contraction in the presence of the MLCK inhibitor. Wortmannin was added before (A) and after (B) *C. speciosus* rhizome extract (typical of 3 other traces from different animals).

A



B

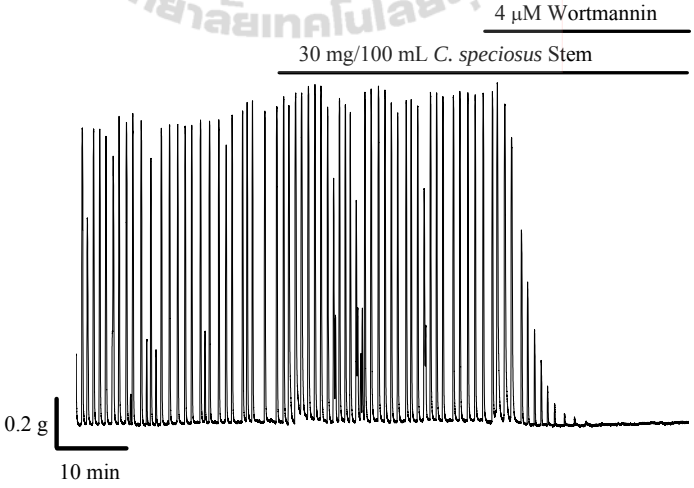


Figure 6.5 The effects of *C. speciosus* stem extract on uterine contraction in the presence of the MLCK inhibitor. Wortmannin was added before (A) and after (B) *C. speciosus* stem extract (typical of 3 other traces from different animals).

Table 6.3 The effects of *C. speciosus* rhizome extract on uterine contraction in the presence of the MLCK inhibitor.

	Amplitude (% Mean ± S.E.M.)	Frequency (% Mean ± S.E.M.)	AUC (% Mean ± S.E.M.)	n
Control	100	100	100	3
C. speciosus rhizome	108.35 ± 2.05[a]	165.00 ± 15.00[a]	182.96 ± 8.07[a]	3
C. speciosus rhizome + Wortmannin (10 min)	73.50 ± 9.60[a]	167.50 ± 7.50[a]	163.77 ± 1.65[a]	3
C. speciosus rhizome + Wortmannin (20 min)	17.35 ± 4.74[a]	80.00 ± 20.00	42.72 ± 2.14[a]	3
C. speciosus rhizome + Wortmannin (30 min)	0	0	0	3

The *P*-values for amplitude, frequency and AUC of wortmannin treated are significantly different from the control ([a]$P < 0.05$).

Mean ± S.E.M. are given; n is number of animals.

Table 6.4 The effects of *C. speciosus* stem extract on uterine contraction in the presence of the MLCK inhibitor.

	Amplitude (% Mean ± S.E.M.)	Frequency (% Mean ± S.E.M.)	AUC (% Mean ± S.E.M.)	n
Control	100	100	100	3
C. speciosus stem	100.41 ± 1.46	108.33 ± 8.33	113.32 ± 12.03	3
C. speciosus stem + Wortmannin (10 min)	69.42 ± 3.17[a]	126.25 ± 6.25	88.73 ± 2.13[a]	3
C. speciosus stem + Wortmannin (20 min)	18.03 ± 18.27[a]	0	0.80 ± 0.80[a]	3
C. speciosus stem + Wortmannin (30 min)	0	0	0	3

The *P*-values for amplitude, frequency and AUC of wortmannin treated are significantly different from the control ([a]$P < 0.05$).

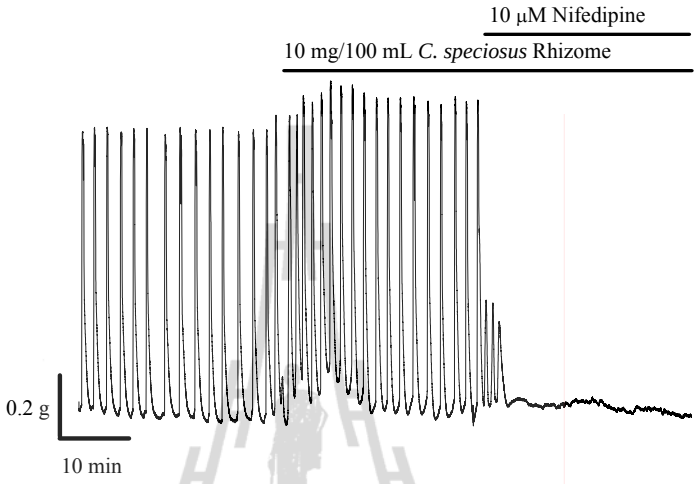
Mean ± S.E.M. are given; n is number of animals.

6.4.4 Effects of *C. speciosus* Rhizome and Stem Extracts on Uterine Contractions in the Presence of L-type Ca^{2+} channel inhibitor

The aim of this experiment was designed to investigate whether the increases in the uterine contraction induced by *C. speciosus* rhizome and stem extracts were dependent on an entry of extracellular Ca^{2+} via L-type Ca^{2+} channels. As shown in Figures 6.6A and 6.7A, an application of 10 μM nifidepine, L-type Ca^{2+} channel inhibitor, in the continued presence of the extracts rapidly inhibited and then abolished force (n = 5). However, basal force did not return to control levels, but remained somewhat elevated, and oscillatory.

In the uterus, some uterotonic agents can elicit a contraction when L-type Ca^{2+} channels are blocked (Kupittayanant, Luckas and Wray, 2002) and it has been suggested that this contraction occurs independently of the calcium-calmodulin-MLCK pathway (Kupittayanant, Luckas and Wray, 2002). To investigate this, the extracts were applied after application of nifedipine, the L-type Ca^{2+} channel inhibitor. As shown in Figures 6.6B and 6.7B, spontaneous force was abolished by applying of nifedipine. The extracts, however could elicit transient force with oscillatory in nature.

A



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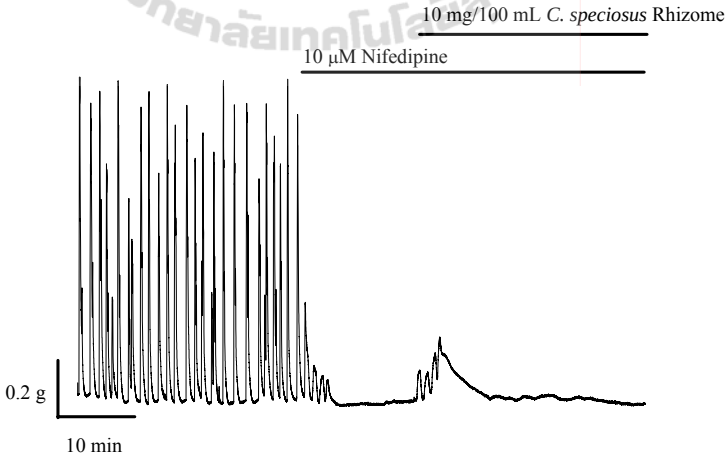
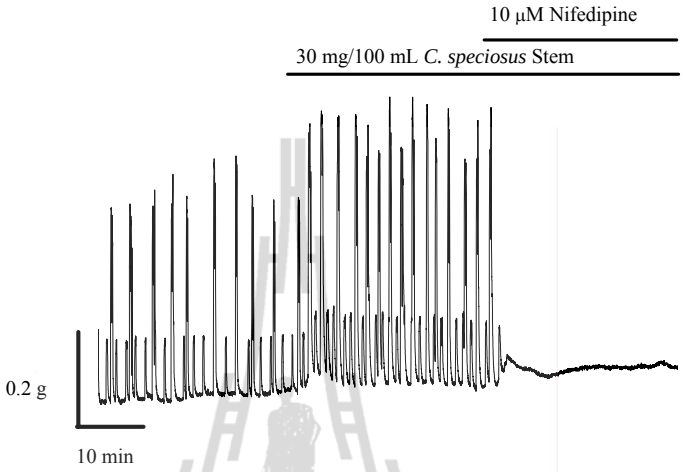


Figure 6.6 The effects of *C. speciosus* rhizome extract on uterine contraction in the presence of L-type Ca^{2+} channel inhibitor. Nifedipine was added before (A) and after (B) *C. speciosus* rhizome extract (typical of 5 other traces from different animals).

A



B

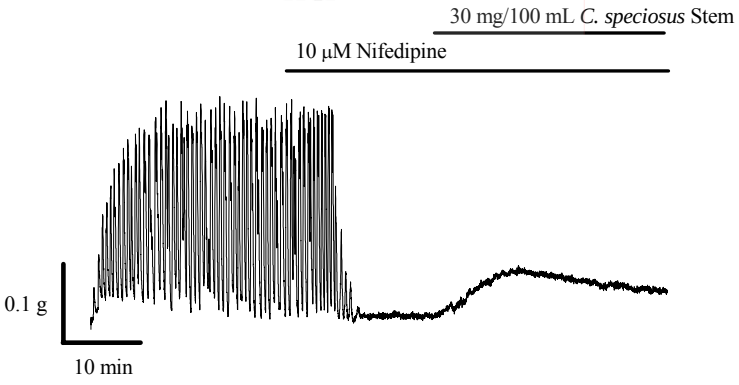


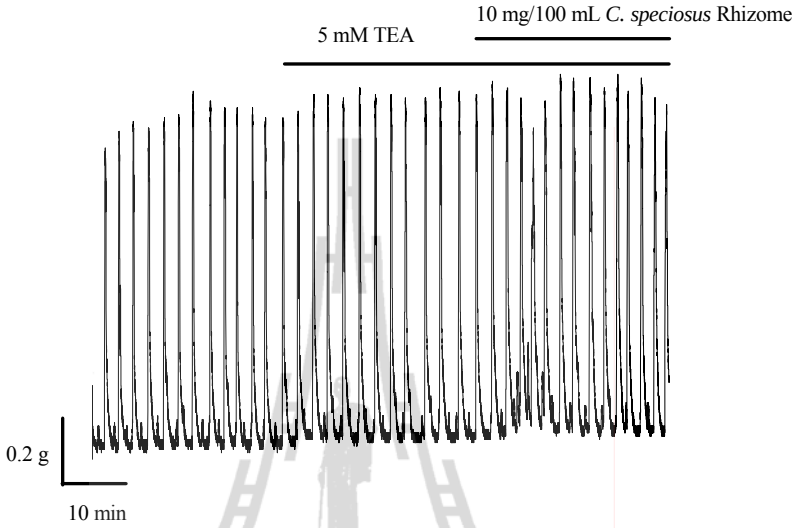
Figure 6.7 The effects of *C. speciosus* stem extract on uterine contraction in the presence of L-type Ca^{2+} channel inhibitor. Nifedipine was added before (A) and after (B) *C. speciosus* stem extract (typical of 5 other traces from different animals).

6.4.5 Effects of *C. speciosus* Rhizome and Stem Extracts on Uterine Contractions in the Presence of Calcium-Activated Potassium Channel Inhibitor

The effects of *C. speciosus* rhizome and stem extracts on force resembled those of calcium-activated potassium channel blockers, which potentiate force (Kupittayanant, Luckas and Wray, 2002). Thus, the question arose whether the extract effects were mediated by effects on those potassium channels. To do so, the potassium channels were blocked, with TEA (5 mM) and the effects of *C. speciosus* rhizome and stem extracts studied. Application of TEA produced a significant increase in the contraction amplitude (106.31 ± 1.35%) compared with the control (100%). Addition of *C. speciosus* rhizome extract in the continued presence of TEA, produced an increase in contraction amplitude, frequency and AUC to 107.11 ± 3.92%, 120.83 ± 4.16% and 115.85 ± 6.43%, respectively, compared with control (100%). When TEA was added after an addition of *C. speciosus* rhizome extract, it produced no further increases in force. The experimental traces are shown in Figure 6.8 and summarized in Table 6.5.

The effects of *C. speciosus* stem extract on uterine contractions in the presence of TEA were also studied. The effects were similar to those *C. speciosus* rhizome extract. Application of TEA produced a significant increase in amplitude of uterine contraction (n = 3). Addition of *C. speciosus* stem extract in the continued presence TEA produced an increase in amplitude, frequency and AUC of contraction to 118.76 ± 11.62%, 122.86 ± 2.86%, and 131.76 ± 3.14%, respectively, compared with control (100%). When TEA was added after an addition of *C. speciosus* stem extract, it reduced contraction. The experimental traces are shown in Figure 6.9 and summarized in Table 6.6.

A



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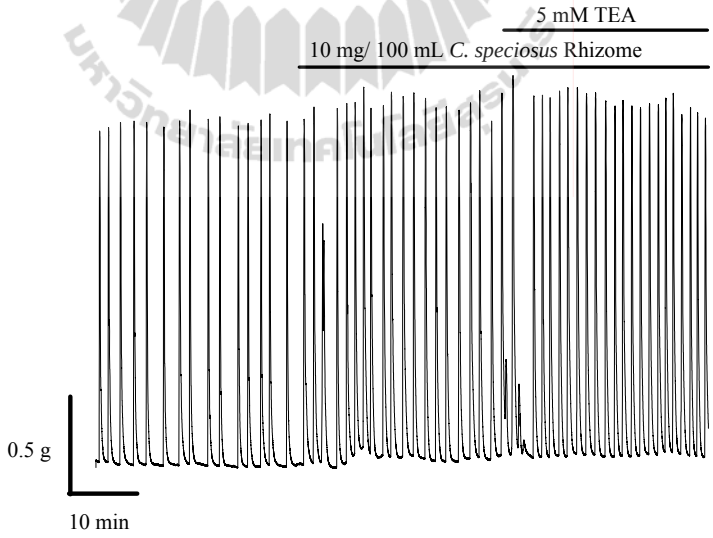
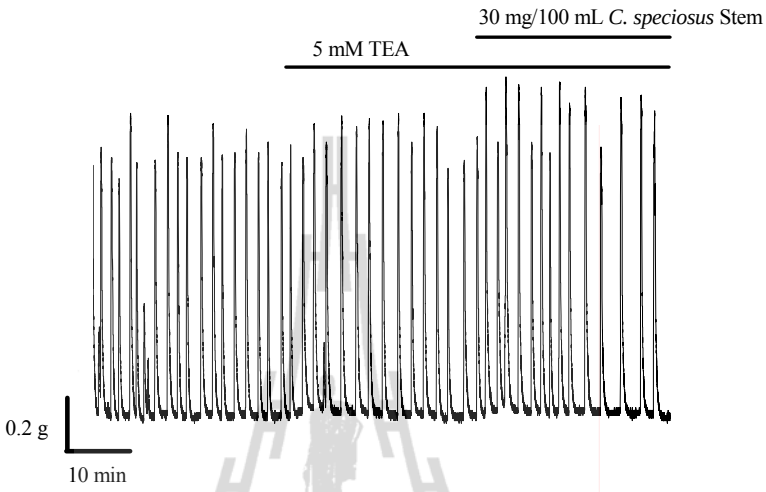


Figure 6.8 The effects of *C. speciosus* rhizome extract on uterine contraction in the presence of calcium-activated potassium channel inhibitor. TEA was added before (A) and after (B) *C. speciosus* rhizome extract (typical of 3 other traces from different animals).

A



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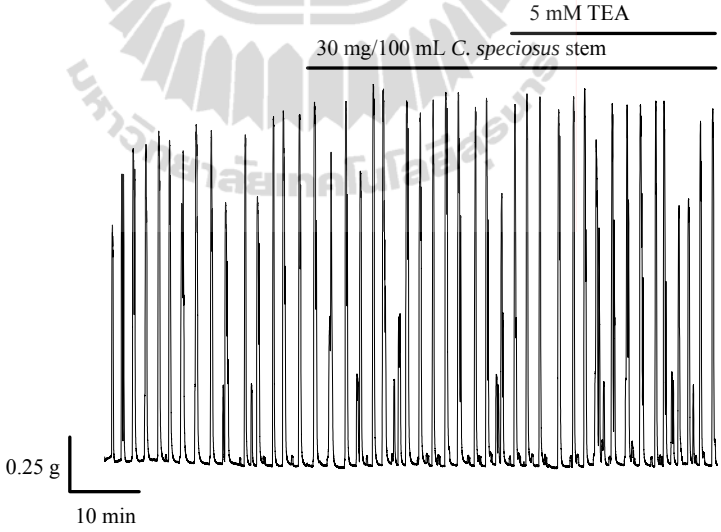


Figure 6.9 The effects of *C. speciosus* stem extract on uterine contraction in the presence of calcium-activated potassium channel inhibitor. TEA was added before (A) and after (B) *C. speciosus* stem extract (typical of 3 other traces from different animals).

Table 6.5 The effects of *C. speciosus* rhizome extract on uterine contraction in the presence of calcium-activated potassium channel inhibitor.

	Amplitude (% Mean ± S.E.M.)	Frequency (% Mean ± S.E.M.)	AUC (% Mean ± S.E.M.)	n
***C. speciosus* rhizome (after)**				
Control	100	100	100	3
TEA	106.31 ± 1.35[a]	100.00 ± 0.00	115.85 ± 6.43	3
TEA + *C. speciosus* rhizome	107.11 ± 3.92[a]	120.83 ± 4.16[a]	180.29 ± 4.59[a]	3
***C. speciosus* rhizome (before)**				
Control	100	100	100	3
C. speciosus rhizome	106.38 ± 0.91[a]	145.00 ± 5.00[a]	194.78 ± 3.31[a]	3
C. speciosus rhizome + TEA	101.94 ± 1.43	116.19 ± 1.90[a]	115.80 ± 1.15[a]	3

The *P*-values for amplitude, frequency and AUC of TEA treated are significantly different from the control ([a]$P < 0.05$).

Mean ± S.E.M. are given; n is number of animals.

Table 6.6 The effects of *C. speciosus* stem extract on uterine contraction in the presence of calcium-activated potassium channel inhibitor.

	Amplitude (% Mean ± S.E.M.)	Frequency (% Mean ± S.E.M.)	AUC (% Mean ± S.E.M.)	n
***C. speciosus* stem (after)**				
Control	100	100	100	3
TEA	109.96 ± 2.60[a]	100.00 ± 0.00	111.34 ± 6.53	3
TEA + *C. speciosus* stem	118.76 ± 11.62[a]	122.86 ± 2.86[a]	131.76 ± 3.14[a]	3
***C. speciosus* stem (before)**				
Control	100	100	100	3
C. speciosus stem	115.91 ± 2.35[a]	100.00 ± 0.00	134.92 ± 13.09	3
C. speciosus stem + TEA	101.54 ± 1.16	87.96 ± 7.23	102.55 ± 5.25	3

The *P*-values for amplitude, frequency and AUC of TEA treated are significantly different from the control ([a]$P < 0.05$).

Mean ± S.E.M. are given; n is number of animals.

6.4.6 Effects of *C. speciosus* Rhizome and Stem Extracts on Uterine Contractions in the Presence of SERCA Pump Inhibitor

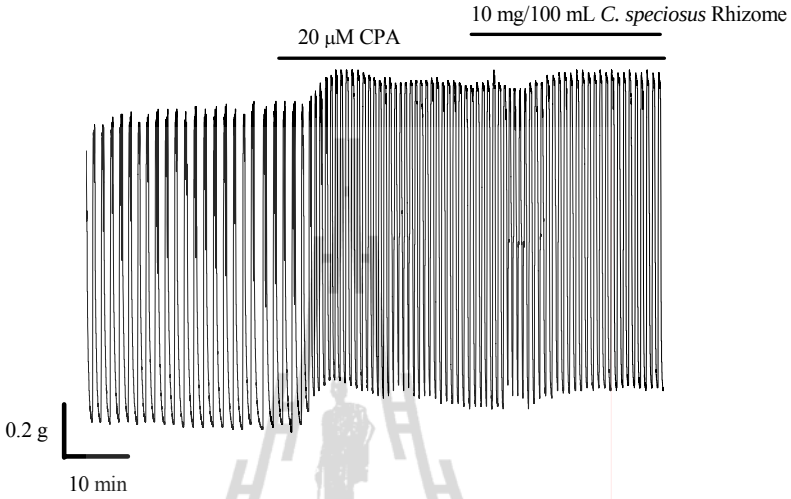
Release of Ca^{2+} from the SR can potentiate force in smooth muscles (Kupittayanant, Luckas and Wray, 2002) and thus the SR may be a target for *C. speciosus* extracts. In addition, the increase in basal tone found with the extracts could be due to releases of Ca^{2+} from the SR or inhibition of Ca^{2+} re-uptake by SERCA. The effects of *C. speciosus* extracts after inhibition of SERCA by CPA (20 μM) (Kupittayanant, Luckas and Wray, 2002; Taggart and Wray, 1998) were, therefore, elucidated.

When CPA was added to the contracting uterus, it significantly increased the frequency and AUC of contraction to 143.72 ± 14.54% and 188.23 ± 7.40%, respectively ($P < 0.05$, n = 3), compared with control (100%). Addition of *C. speciosus* rhizome extract in the continued presence CPA produced a decrease in frequency and AUC (94.19 ± 2.91% and 95.03 ± 7.40%), but the amplitude was increased (103.05 ± 1.82%). When CPA was added after *C. speciosus* rhizome extract (n = 3), it reduced the amplitude, frequency and AUC of contraction. The experimental traces are shown in Figure 6.10 and the data are summarized in Table 6.7.

The effects of *C. speciosus* stem extract on uterine contraction in the presence of CPA, the SERCA pump inhibitor are summarized in Table 6.8. Application of CPA produced a significant increase in amplitude, frequency and AUC of uterine contraction (n = 3). Addition of *C. speciosus* stem extract in the continued presence of CPA, decreased amplitude, frequency and AUC of contraction. When CPA was added after an addition of *C. speciosus* stem extract, it produced no significant change in

force. The experimental traces are shown in Figure 6.11 and the data are summarized in Table 6.8.

A



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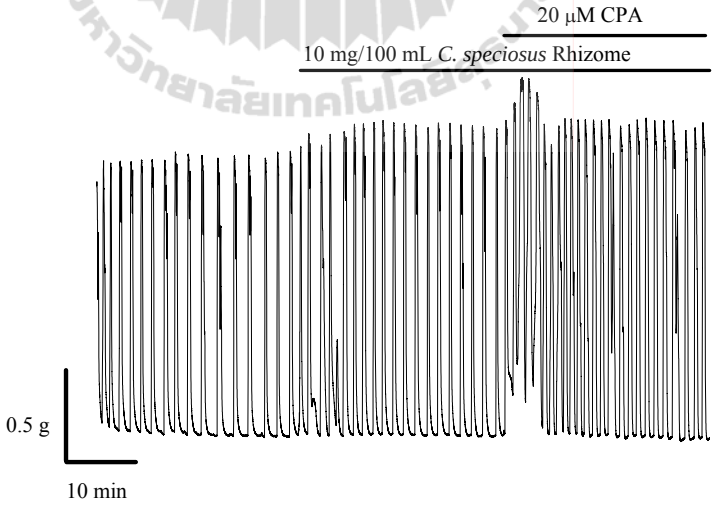
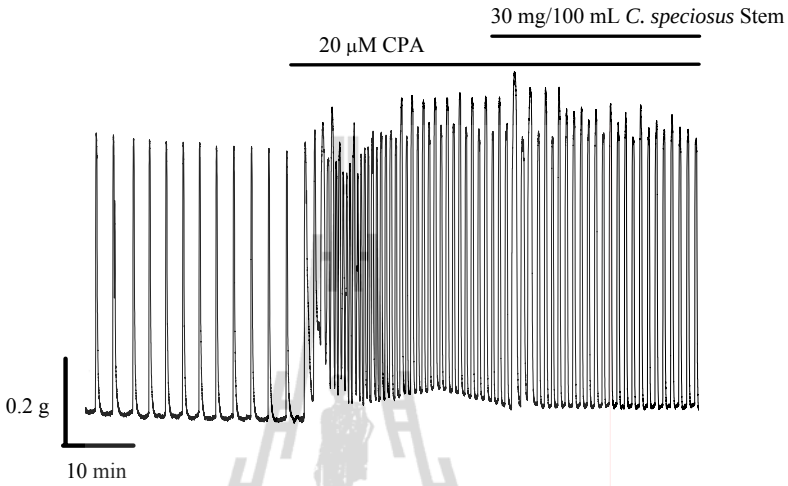


Figure 6.10 The effects of *C. speciosus* rhizome extract on uterine contraction in the presence of SERCA pump inhibitor. CPA was added before (A) and after (B) *C. speciosus* rhizome extract (typical of 3 other traces from different animals).

A



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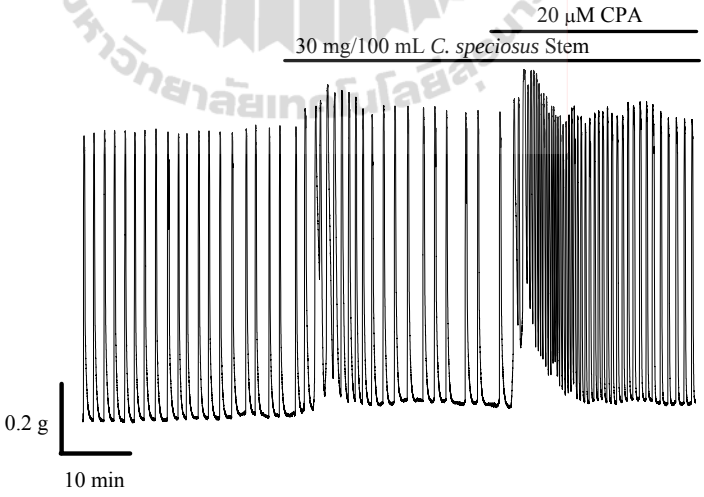


Figure 6.11 The effects of *C. speciosus* stem extract on uterine contraction in the presence of SERCA pump inhibitor. CPA was added before (A) and after (B) *C. speciosus* stem extract (typical of 3 other traces from different animals).

Table 6.7 The effects of *C. speciosus* rhizome extract on uterine contraction in the presence of SERCA pump inhibitor.

	Amplitude (% Mean ± S.E.M.)	Frequency (% Mean ± S.E.M.)	AUC (% Mean ± S.E.M.)	n
***C. speciosus* rhizome (after)**				
Control	100	100	100	3
CPA	101.09 ± 14.76	143.72 ± 14.54[a]	188.23 ± 7.40[a]	3
CPA + *C. speciosus* rhizome	103.05 ± 1.82	94.19 ± 2.91	95.03 ± 13.54	3
***C. speciosus* rhizome (before)**				
Control	100	100	100	3
C. speciosus rhizome	116.29 ± 5.11[a]	128.89 ± 24.43	132.09 ± 14.78	3
C. speciosus rhizome + CPA	102.23 ± 1.32	128.21 ± 25.68	143.19 ± 39.81	3

The *P*-values for amplitude, frequency and AUC of CPA treated are significantly different from the control ([a]$P < 0.05$).

Mean ± S.E.M. are given; n is number of animals.

Table 6.8 The effects of *C. speciosus* stem extract on uterine contraction in the presence of SERCA pump inhibitor.

	Amplitude (% Mean ± S.E.M.)	Frequency (% Mean ± S.E.M.)	AUC (% Mean ± S.E.M.)	n
***C. speciosus* stem (after)**				
Control	100	100	100	3
CPA	118.85 ± 5.74[a]	179.17 ± 4.17[a]	230.69 ± 30.32[a]	3
CPA + *C. speciosus* stem	104.89 ± 2.81	91.56 ± 5.29	94.05 ± 9.08	3
***C. speciosus* stem (before)**				
Control	100	100	100	3
C. speciosus stem	106.69 ± 3.80	111.11 ± 11.11	144.42 ± 3.29[a]	3
C. speciosus stem + CPA	101.27 ± 5.71	147.92 ± 14.86	122.37 ± 14.40	3

The *P*-values for amplitude, frequency and AUC of CPA treated are significantly different from the control ([a]$P < 0.05$).

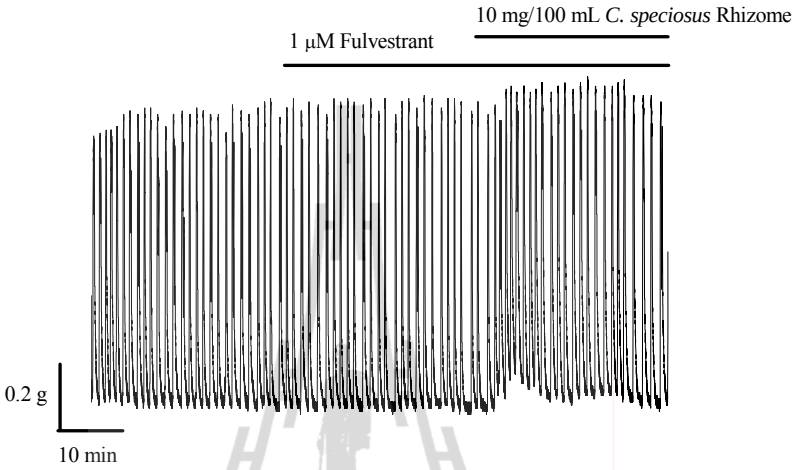
Mean ± S.E.M. are given; n is number of animals.

6.4.7 Effects of *C. speciosus* Rhizome and Stem Extracts on Uterine Contractions in the Presence of Estrogen Receptor Inhibitor

The effects of *C. speciosus* rhizome and stem extracts on uterine activity could be occurring through estrogen receptors. The effects of the extracts in the presence of fulvestrant (1 μM) (Buzdar, 2008) were studied. Application of fulvestrant to spontaneous contraction produced no significant changes in uterine contractions. The amplitude, frequency and AUC of contraction were 111.71 ± 7.48%, 108.93 ± 4.49% and 104.89 ± 10.13%, respectively, compared with control (100%). Addition of *C. speciosus* rhizome extract in presence of fulvestrant produced an increase in uterine contractility. When fulvestrant was added after *C. speciosus* rhizome extract, there was no significant change in force. The experimental traces are shown in Figure 6.12 and the data are summarized in Table 6.9.

The effect of *C. speciosus* stem extract on uterine contraction in the presence of fulvestrant was also investigated. Similar to *C. speciosus* rhizome extract, significant change in uterine contractions was not found upon an application of fulvestrant to spontaneous contraction. The amplitude, frequency and AUC of contraction were 106.51 ± 4.64%, 106.76 ± 4.37% and 105.38 ± 10.41%, respectively, compared with control (100%). An addition of *C. speciosus* stem extract in the presence of fulvestrant produced an increase in uterine contractility. When fulvestrant was added after an addition of *C. speciosus* stem extract, significant change in force did not occur. The experimental traces are shown in Figure 6.13 and the data are summarized in Table 6.10.

A



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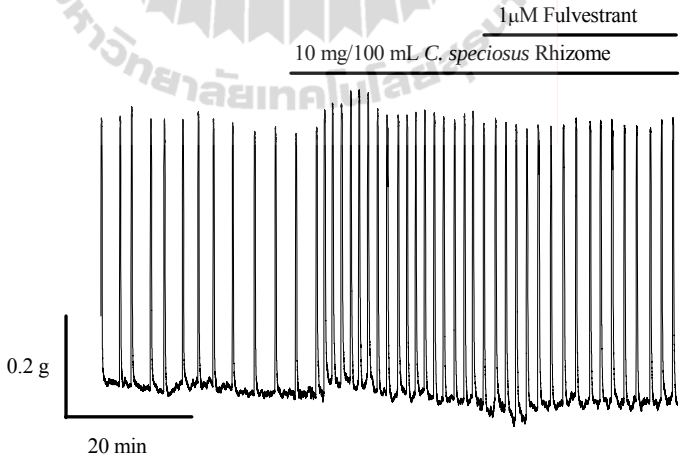
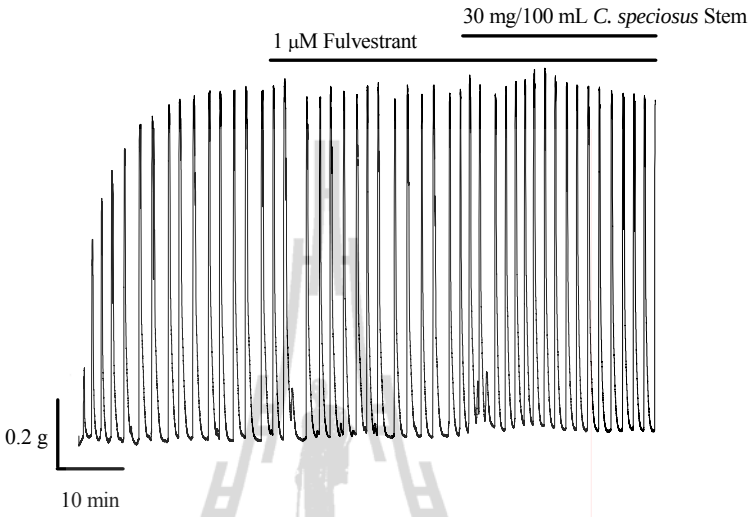


Figure 6.12 The effects of *C. speciosus* rhizome extract on uterine contraction in the presence of estrogen receptor inhibitor. Fulvestrant was added before (A) and after (B) *C. speciosus* rhizome extract (typical of 3 other traces from different animals).

A



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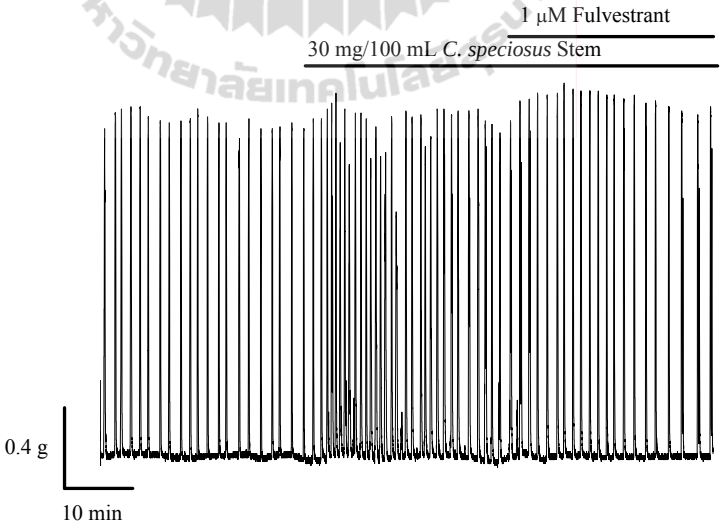

Figure 6.13 The effects of *C. speciosus* stem extract on uterine contraction in the presence of estrogen receptor inhibitor. Fulvestrant was added before (A) and after (B) *C. speciosus* stem extract (typical of 3 other traces from different animals).

Table 6.9 The effects of *C. speciosus* rhizome extract on uterine contraction in the presence of estrogen receptor inhibitor.

	Amplitude (% Mean ± S.E.M.)	Frequency (% Mean ± S.E.M.)	AUC (% Mean ± S.E.M.)	n
***C. speciosus* rhizome (after)**				
Control	100	100	100	3
Fulvestrant	102.15 ± 2.15	108.93 ± 4.49	104.89 ± 10.13	3
Fulvestrant + *C. speciosus* rhizome	104.13 ± 0.01[a]	126.67 ± 26.67	232.78 ± 18.97[a]	3
***C. speciosus* rhizome (before)**				
Control	100	100	100	3
C. speciosus rhizome	112.79 ± 0.84[a]	158.34 ± 8.34[a]	176.95 ± 4.12[a]	3
C. speciosus rhizome + Fulvestrant	100.54 ± 2.54	108.47 ±10.41	153.28 ± 9.11	3

The *P*-values for amplitude, frequency and AUC of fulvestrant treated are significantly different from the control ([a]$P < 0.05$).

Mean ± S.E.M. are given; n is number of animals.

Table 6.10 The effects of *C. speciosus* stem extract on uterine contraction in the presence of estrogen receptor inhibitor.

	Amplitude (% Mean ± S.E.M.)	Frequency (% Mean ± S.E.M.)	AUC (% Mean ± S.E.M.)	n
***C. speciosus* stem (after)**				
Control	100	100	100	3
Fulvestrant	106.51 ± 4.64	106.76 ± 4.37	105.38 ± 10.41	3
Fulvestrant + *C. speciosus* stem	108.13 ± 2.24[a]	118.34 ± 1.67[a]	122.37 ± 10.88	3
***C. speciosus* stem (before)**				
Control	100	100	100	3
C. speciosus stem	117.75 ± 8.58	139.40 ± 23.54	178.60 ± 63.79	3
C. speciosus stem + Fulvestrant	119.91 ± 17.57	108.34 ± 8.34	85.51 ± 18.27	3

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

6.5 Discussion

The aims of study were to investigate, in non-pregnant rats, the effects of *C. speciosus* rhizome and stem extracts on uterine contraction and to examine the mechanisms whereby the extracts exerted their effects. The results revealed that *C. speciosus* rhizome and stem extracts potently potentiated spontaneous contractions. Both *C. speciosus* rhizome and stem extracts significantly increased the amplitude, frequency and AUC of the phasic contraction as well as the basal tension. The potentiation of a spontaneous force induced by the extracts was dependent upon the Ca^{2+}-calmodulin-MLCK pathway, but the extracts were also able to stimulate force via SR Ca^{2+} release. When the effects of *C. speciosus* and stem extracts were studied after fulvestrant treatment, they were still effective. The conclusion is that *C. speciosus* rhizome and stem extracts are uterine stimulants acting via a non-estrogen mechanism on both Ca^{2+} entry and SR Ca^{2+} release. Moreover, the effects of the extracts on spontaneous contraction resembled those of inhibiting K^+ channels or SERCA and pre-incubation with TEA or CPA prevented the extracts exerting their effects. This suggests that the potentiation of force is due to the extracts acting to inhibit K^+ channels, but may also involve in an inhibition of the SR Ca^{2+}-ATPase.

Uterine force can be produced by several pathways, but the main mechanism involves in Ca^{2+}-calmodulin-MLCK (Wray, 2007). To investigate whether the increases in uterine force were dependent on the Ca^{2+}-calmodulin-MLCK pathway, the effects of *C. speciosus* extracts in the presence of nifedipine and MLCK inhibitors, wortmannin were investigated. Nifedipine is classified as a dihydropyridine and exerts its effects by binding to the inside of voltage-gated L-type channels, inhibiting the action potential as well as the contractility (Nayler and Poole-

Wilson, 1981). Wortmannin is the most selective inhibitor of MLC phosphorylation and a tonic force in smooth muscle (Burdyga and Wray, 1998).

It was found that the pathway to increase uterine contraction by *C. speciosus* extracts is occurred via a calcium dependent pathway. Supporting this conclusion comes from the experiments with MLCK inhibition; force transients were no longer produced in the presence of the extract. Furthermore, force produced in the presence of the extract was abolished when Ca^{2+} entry was inhibited. The effects of inhibition of L-type calcium channels, removal of external Ca^{2+} or inhibition of MLCK, suggesting that they are produced by the Ca^{2+}-calmodulin-MLCK pathway.

In the uterus, some uterotonic agents can elicit a contraction when L-type Ca^{2+} channels are blocked (Oishi, Takano, Ohmuro and Minakava-Mutsun, 1991; Kupittayanant, Luckas and Wray, 2002). Although force transients are abolished in nifedipine, but some force can still be produced via SR Ca^{2+} release. It is also known that there is an internal Ca^{2+} store (SR). These results indicated that *C. speciosus* extracts stimulated uterine contraction via SR calcium release. The evidence for this comes from the effects of *C. speciosus* extracts after nifedipine, which had clearly blocked L-type Ca^{2+} channels that clear increases of force were seen when the extract was added. This is one of the most interesting findings as few agents including oxytocin have previously been found to be so active. Many factors including Ca^{2+} itself and pH can influence SR Ca^{2+} release (Shabir, Borisova, Wray and Burdyga, 2004; Wray and Burdyga, 2010). Future studies investigating the mechanisms underlying the effects of *C. speciosus* extracts on SR Ca releases would be of interest.

TEA, a nonselective K^+ blocker also stimulates the mechanical activity of rat myometrial (Sanborn, 2000). These results exhibited that TEA increased the

amplitude of spontaneous uterine contraction. After exposuring to TEA, the *C. speciosus* extracts were without effect. There are the data from other sterols, especially cholesterol. These compounds can modulate K^+ channel activity. Specifically, in the uterus, cholesterol manipulation can have marked effects on Ca^{2+} signaling and contracting via effects on Ca^{2+}-activated K^+ channels (Shmygol, Noble and Wray, 2007; Zhang, Kendric, Quenby and Wray, 2007).

CPA is a very useful pharmacological tool for evaluation of the role of SERCA in smooth muscle contraction. It acts as a highly specific inhibitor of the SERCA without affecting plasma membrane Ca^{2+}-ATP (Seidler, Jona, Vegh and Martonosi, 1989). Release of Ca^{2+} from the SR can potentiate force in smooth muscle (Wray, Burdyga and Noble, 2005) and the *C. speciosus* extracts were also stimulated force via SR Ca^{2+} release. In addition, an increase in basal tone found with the *C. speciosus* extracts could be due to the release of Ca^{2+} from the SR or inhibition of Ca^{2+} reuptake by SERCA. Inhibition of SERCA may explain the increase in basal tone found with extract application. Previous studies showed that when SERCA is inhibited; there is an increase in intracellular Ca^{2+} (Kupittayanant, Luckas and Wray, 2002), which may lead to change of basal tone.

Inhibiting estrogen production or reducing the binding of estrogen to the estrogen receptor (ER) is established a rational for the design of therapeutic agents to treat hormone-sensitive breast cancer (Fuqua et al., 1992). Fulvestrant is an ER antagonist with no agonist effect (Wakeling, Dukes and Bowler, 1991; Fisher et al., 2001), which binds, blocks and degrades the ER. The data suggest that the action of *C. speciosus* extracts is not via estrogen receptor. Support for this conclusion comes

from the experiments with fulvestrant, a blocker of estrogen receptors. When fulvestrant was added after *C. speciosus* extracts no change in force occurred.

In conclusion, the data demonstrated a significant stimulation of uterine activity by *C. speciosus* rhizome and stem extracts. The stimulation of uterine activity of this plant extracts may be a useful source of uterine stimulant for slowly progressing labors (Quenby, Pierce, Brigham and Wray, 2004). However, further studies in human myometrium are required to develop these suggestions.

6.6 References

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CHAPTER VII

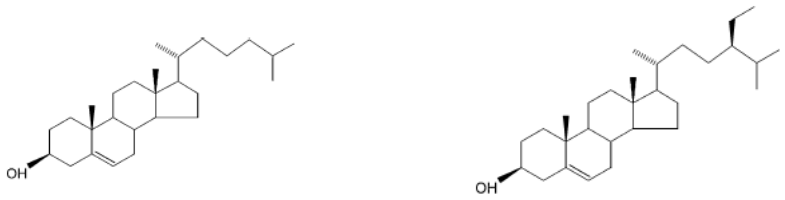
EFFECTS OF β-SITOSTEROL AND DIOSGENIN ON UTERINE CONTRACTION IN NON-PREGNANT RATS

7.1 Abstract

Costus speciosus (Koen.) Sm. is an important medicinal plant that has been used in human and animal medicines. β-sitosterol and diosgenin have been found to be major components of this plant. Thus, the aim of this chapter was to clarify whether the potentiation of uterine force produced by *C. speciosus* rhizome and stem extracts was due to β-sitosterol and diosgenin. Rats were sacrificed by an over dose of CO_2 and the longitudinal uterine smooth muscle strips were isolated. The effects of β-sitosterol and diosgenin with or without *C. speciosus* extracts on uterine contraction were measured. The results showed that β-sitosterol (0.02, 0.2, 2 mg/100 mL) increased uterine spontaneous contraction. The amplitude of the phasic contraction was significantly increased along with the basal tension. The effects of β-sitosterol were partially similar to those of *C. speciosus* extracts. Diosgenin, depending upon its concentration (0.02, 0.2, 2 mg/100 mL), had either no effect or was inhibitory on force. Interestingly, neither β-sitosterol nor diosgenin induced force in the absence of extracellular calcium. The data suggested that a significant stimulation of uterine activity by *C. speciosus* can largely but not entirely be accounted for by its constituent, β-sitosterol.

7.2 Introduction

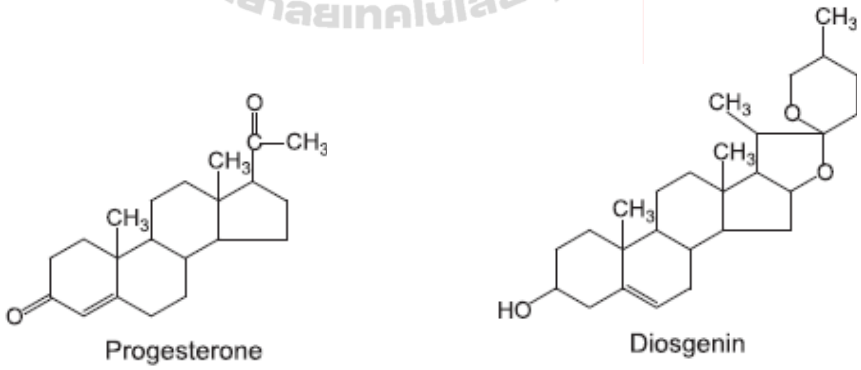
β-sitosterol (24-ethyl-5-cholestene-3-ol) is a weakly estrogenic phytosterol found in oil, seeds, nut, pulses, cereals and vegetables. It has been reported to reduce serum cholesterol and to prevent cardiovascular disease by inhibition of cholesterol absorption in intestine (Miettinen, Puska, Gylling, Vaniianen and Vartiainen, 1995; ostlund, 2002; Pouteau et al., 2003). Moreover, β-sitosterol is known to regulate key molecules involved in inflammation, the immune response, anti-cancer defenses and apoptosis and it was found to be significantly reduced in type 2 diabetes patients by a possible role to lower the blood glucose level (Sutherland et al., 1992; Awad and Fink, 2000; Bouic, 2002). β-sitosterol is structurally related to animal cholesterol (Figure 7.1) and can possibly act as a precursor of sex steroids (Monghadasian, 2000). There are several evidences suggest that β-sitosterol's effects on steroidogenesis may be mediated by its effects on cholesterol status. However, the physiological mechanisms underlying these beneficial effects of β-sitosterol are not well understood.



A B

Figure 7.1 Chemical structures of cholesterol (A) and β-sitosterol (B) (Christiansen, Karjalainen, Seppänen-Laakso, Hiltunen and Yliruusi, 2003).

Diosgenin (3β-hydroxy-5-spirostene) is a steroidal sapogenin belonging to the triterpene group that has been suggested structurally similar to progesterone (Figure 7.2). It is found in several plants including Dioscorea species, fenugreek and *Costus speciosus* (Liu, Wange, Ju, Wong and Wu, 2005). Many literatures reported that diosgenin can be absorbed through the gut and plays an important role in the control of cholesterol metabolism (Roman, Thewles and Coleman, 1995), produces changes in the lipoxynase activity of human erythroleukaemia (Nappez, Liagre and Beneytout, 1995) and is responsible for morphological and biochemical changes in megakaryocyte cells (Beneytout, Nappez, Leboutet and Malinvaud, 1995). It also has estrogenic effects by stimulated the growth of mammary gland of ovariectomized mice (Aradhana and Kale, 1992) and antitumor activity (Moalic et al., 2001; Corbiere et al., 2003). Furthermore, diosgenin is mainly used as starting material for partial synthesis of oral contraceptives, sex hormones and other steroids (Zenk, 1978).



A B

Figure 7.2 Chemical structures of progesterone (A) and diosgenin (B) (Au et al., 2004).

C. speciosus is an important medicinal plant that has been used in many human and animal medicines. The rhizome and stem of this plant have been found to be the rich sources of β-sitosterol and diosgenin. They also contain sterols and fatty acids in their seed and stem and triterpines in leaves (Vijayalakshmi and Sarada, 2008). It has been shown that the extracts from all its parts have therapeutic properties. The experimental evidences have shown its antioxidant activity, anti-inflammatory and anti-diabetic (Vijayalakshmi and Sarada, 2008; Verma and Khosa, 2009). β-sitosterol and diosgenin have been found to be the major components of this plant. Thus, this chapter was aimed to clarify whether the potentiation of uterine force produced by *C. speciosus* rhizome and stem extracts was due to β-sitosterol and diosgenin.

7.3 Materials and Methods

7.3.1 Chemicals and Physiological Solutions

All chemicals were purchased from Sigma chemical Co. (St. Louis, MO, USA). Drugs for investigation of physiological pathways used in this chapter were β-sitosterol and diosgenin. The purity of β-sitosterol and diosgenin was 75% and 95%, respectively. Both the β-sitosterol and diosgenin were dissolved in ethanol. In this study, their final concentration used had never exceeded 0.01% (v/v).

7.3.2 Preparation of *C. speciosus* Rhizome and Stem Extracts

As described in 2.3.1 (plant extraction), the *C. speciosus* rhizome or stem extract was dissolved in Krebs' solution just before use.

7.3.3 Myometrial Tissue Preparation

Non-pregnant Wistar rats (200-300 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 advice of the Institutional Animal Care and Use Committee, Suranaree University of Technology (SUT), Thailand. Preparations of myometrium tissues were prepared as described in 6.3.1.

7.3.4 Measurements of Tension

Measurements of tension were performed as described in 6.3.2. Briefly, the uterine strip was mounted vertically under resting tension of 1 g in a single chamber (25 mL) tissue bath connected to a force transducer. The strip was allowed to contract spontaneously and an equilibrium period of 30 minutes was given before an application of any chemical. The measurements were made whilst the tissue was continually perfused with physiological solution (control) or solution containing *C. speciosus* rhizome and stem extracts at a concentration of 10 mg/100 mL or 30 mg/100 mL, respectively. Nifedipine, an inhibitor of L-type channels (Shmigol, Eisner and Wray, 1998), β-sitosterol and diosgenin were also used as indicated in the text.

7.3.5 Statistical Analysis

All data were analyzed and statistical significance was tested using the student'*t*-test, and P values < 0.05 taken to be significant. All values represented mean

± S.E.M., n is the number of sample. The results were then expressed as percentages of control contraction (i.e. the control is 100% as described in 6.3.5).

7.4 Results

7.4.1 Effects of β-sitosterol on Spontaneous Contraction

The effects of β-sitosterol (0.02, 0.2, 2 mg/100 mL) on spontaneous contraction were investigated. As shown in Figure 7.3 and Table 7.1, β-sitosterol produced a significant increase in the amplitude, frequency and AUC of the contraction (n = 5). Initial dose response curves over the range from 0.02 to 2 mg/100 mL showed that maximal effects were achieved at around 0.02 mg/100 mL, and thus, a dose of 0.02 mg/100 mL of β-sitosterol was used (n = 5). It has been shown that the estrogenic activity of phytoestrogens ranges from 1/500 to 1/1000 of the activity of estrogen (Cassidy, 1999). Thus, the selected concentration was in the ranges.

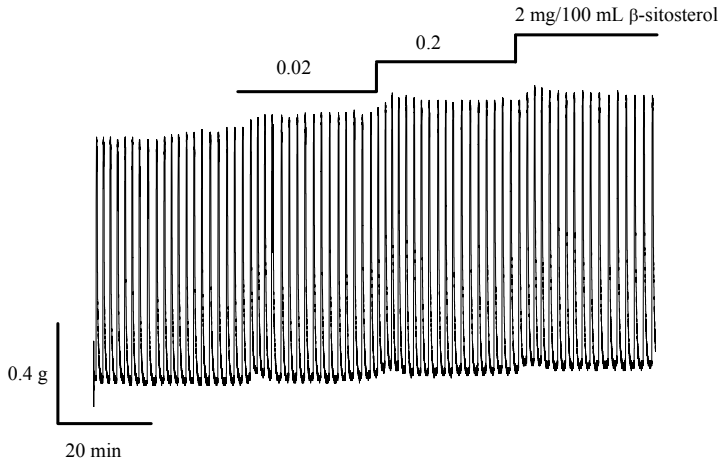


Figure 7.3 The effects of β-sitosterol at various concentrations on spontaneous contraction (typical of 5 other traces from different animals).

Table 7.1 Effects of β-sitosterol at various concentrations on spontaneous contraction in non-pregnant rats.

	Amplitude (% Mean ± S.E.M.)	Frequency (% Mean ± S.E.M.)	AUC (% Mean ± S.E.M.)	n
β-sitostero (mg/100 mL)				
0 (Control)	100	100	100	5
0.02	108.10 ± 1.77[a]	104.17 ± 4.17	112.77 ±0 .72[a]	5
0.20	110.43 ± 3.12[a]	116.67 ± 16.67	120.34 ± 7.17[a]	5
2.00	112.63 ± 4.78[a]	100.00 ± 0.00	96.18 ± 20.79	5

The *P*-values for amplitude, frequency and AUC of β-sitosterol treated are significantly different from the control ([a]$P < 0.05$).

Mean value ± S.E.M. are given; n is number of animals.

7.4.2 Effects of β-sitosterol in the Presence of *C. speciosus* Extracts

As shown in Chapter VI, it is clear that *C. speciosus* potentiate uterine contraction. As β-sitosterol is one of the major components found in the extracts, and has previously been found to be a phytoestrogen, it was of interest to determine whether the effects of *C. speciosus* extracts were due to β-sitosterol.

Effects of β-sitosterol in the Presence of *C. speciosus* Rhizome Extract

As shown in Figure 7.4A and Table 7.2, application of β-sitosterol produced significant increases in force. The amplitude of force was significantly increased; 107.55 ± 0.91%, compared with control, 100%. However the frequency of the contractions was not significantly increased (104.76 ± 4.76%; compared with control, 100%). As with *C. speciosus* extracts, β-sitosterol increased the basal force.

An application of *C. speciosus* rhizome extract in the continued presence of β-sitosterol produced further increases in force, although its effect could be transient, (Figure 7.4A.) The area under the contraction was increased to 137.60 ± 9.58%, compared with β-sitosterol, 104.15 ± 12.52% (n = 3). The amplitude and the frequency of the contractions increased significantly to 117.86 ± 15.56% and 113.24 ± 7.97%, respectively (compared with β-sitosterol, 107.55 ± 0.91% and 104.76 ± 4.76%, respectively). However, when β-sitosterol was added after *C. speciosus* rhizome extract, no further increases in force were observed (Figure 7.4B, n = 3).

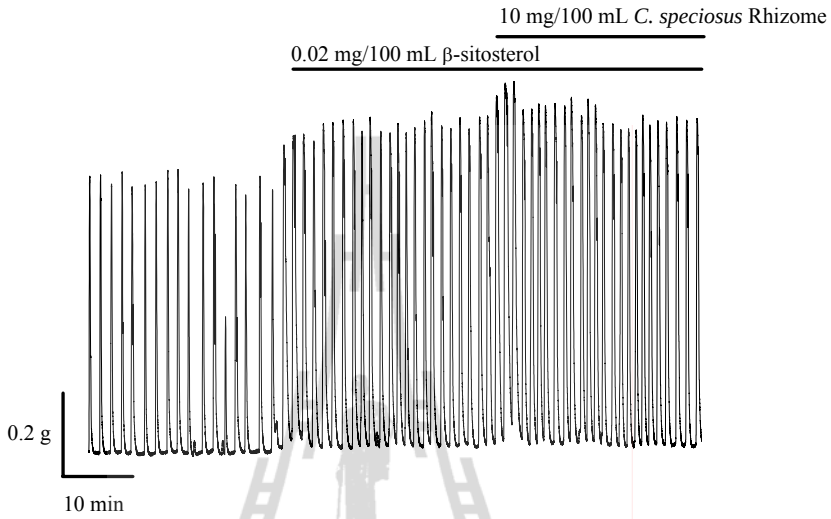
Effects of β-sitosterol in the Presence of *C. speciosus* Stem Extract

The effects of β-sitosterol in the presence of *C. speciosus* stem extract as shown in Table 7.3 were similar to those with *C. speciosus* rhizome extract. As shown

in Figure 7.5A, application of β-sitosterol produced a significant increase in force. The amplitude of force was increased, 112.14 ± 7.30%, compared with control, 100%. However, the frequency of the contractions did not significantly increase (102.50 ± 9.46%; compared with control, 100%). As with *C. speciosus* extracts, β-sitosterol increased the basal force, Figure 7.4B.

As with the *C. speciosus* rhizome extracts, an application of *C. speciosus* stem extract in the continued presence of β-sitosterol produced further increases in force, although its effect could be transient, Figure 7.5A. The area under the contraction was increased to 124.31 ± 3.34%, compared with β-sitosterol alone, 113.17 ± 4.07% (n = 3). The frequency of the contractions increased significantly to 121.75 ± 3.54% (compared with β-sitosterol, 102.50 ± 9.46%). However, when β-sitosterol was added after *C. speciosus* stem extract, no further increases in force were observed (Figure 7.5B, n = 3).

A



B

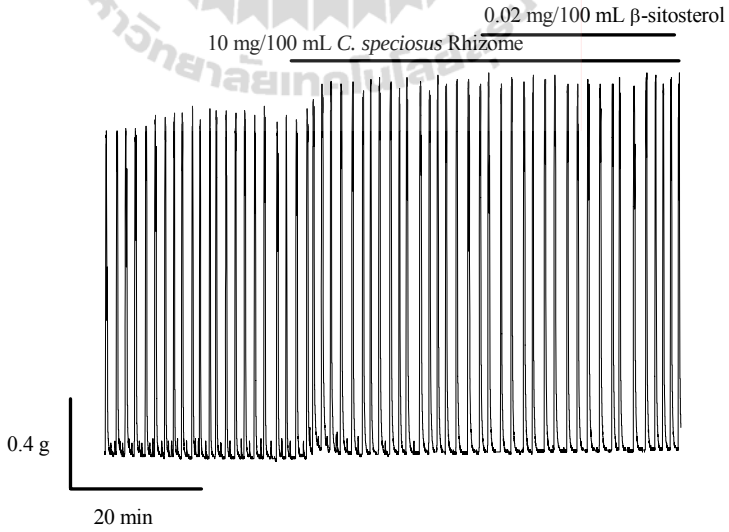
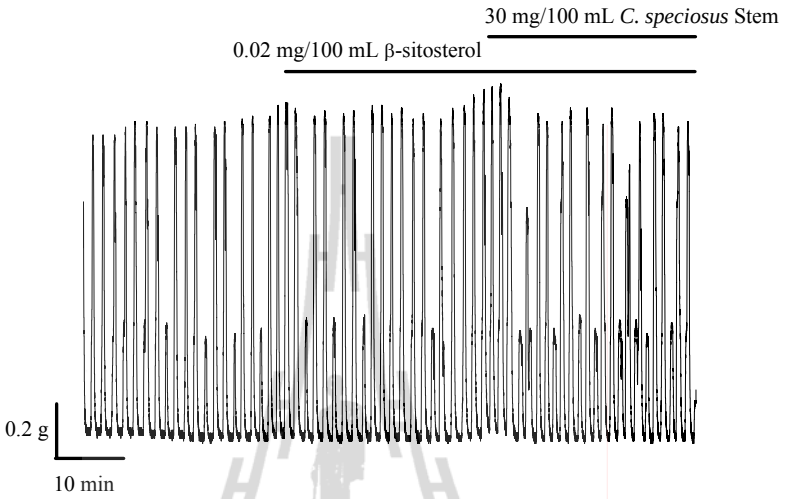


Figure 7.4 The effects of β-sitosterol in the presence of *C. speciosus* rhizome extract. β-sitosterol was added before (A) and after (B) *C. speciosus* rhizome extract (typical of 3 other traces from different animals).

A



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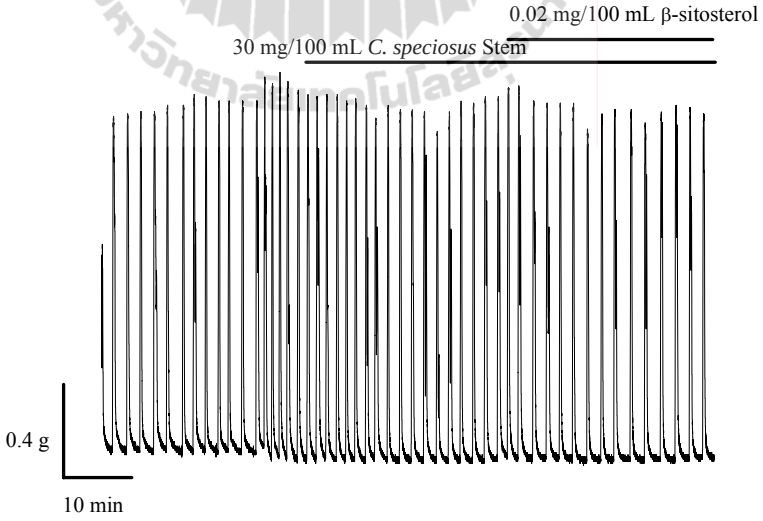


Figure 7.5 The effects of β-sitosterol in the presence of *C. speciosus* stem extract. β-sitosterol was added before (A) and after (B) *C. speciosus* rhizome extract (typical of 3 other traces from different animals).

Table 7.2 Effects of β-sitosterol in the presence of *C. speciosus* rhizome extract.

	Amplitude (% Mean ± S.E.M.)	Frequency (% Mean ± S.E.M.)	AUC (% Mean ± S.E.M.)	n
***C. speciosus* rhizome (after)**				
Control	100	100	100	3
β-sitosterol	107.55 ± 0.91[a]	104.76 ± 4.76	104. 15 ± 12.52	3
β-sitosterol + *C. speciosus* rhizome	117.86 ±15.56[a]	113.24 ± 7.97	137.60 ± 9.58[a]	3
***C. speciosus* rhizome (before)**				
Control	100	100	100	3
C. speciosus rhizome	121.71 ± 7.95[a]	106.21 ± 2.76[a]	171.57 ± 15.39[a]	3
C. speciosus rhizome + β-sitosterol	103.22 ± 1.22[a]	103.33 ± 3.33	91.98 ± 3.36[a]	3

The *P*-values for amplitude, frequency and AUC of *C. speciosus* rhizome are significantly different from the control ([a]$P < 0.05$).

Mean value ± S.E.M. are given; n is number of animals.

Table 7.3 Effects of β-sitosterol in the presence of *C. speciosus* stem extract.

	Amplitude (% Mean ± S.E.M.)	Frequency (% Mean ± S.E.M.)	AUC (% Mean ± S.E.M.)	n
***C. speciosus* stem (after)**				
Control	100	100	100	3
β-sitosterol	112.14 ± 7.30	102.50 ± 9.46	113.17 ± 4.07[a]	3
β-sitosterol + *C. speciosus* stem	100.67 ± 3.58	121.75 ± 3.54[a]	124.31 ± 3.34[a]	3
***C. speciosus* stem (before)**				
Control	100	100	100	3
C. speciosus stem	101.16 ± 2.46	157.50 ± 7.50[a]	148.28 ± 0.16[a]	3
C. speciosus stem + β-sitosterol	102.35 ± 3.67	111.25 ± 1.25	101.73 ± 1.73	3

The *P*-values for amplitude, frequency and AUC of *C. speciosus* stem are significantly different from the control ([a]$P < 0.05$).

Mean value ± S.E.M. are given; n is number of animals.

7.4.3 Effects of β-sitosterol on Uterine Contraction in the Presence of L-type Ca^{2+} Channel Inhibitor

As shown in Chapter VI, it is clear that *C. speciosus* potentiate uterine contraction in the absence of extracellular calcium. As β-sitosterol is one of the major components found in the extracts, it was of interest to determine whether the force elicited in the absence of external calcium were due to β-sitosterol.

As shown in Figure 7.6, the effect of β-sitosterol (0.02 mg/100 mL) was applied in the continued presence of 10 μM nifedipine, no force transients were observed.

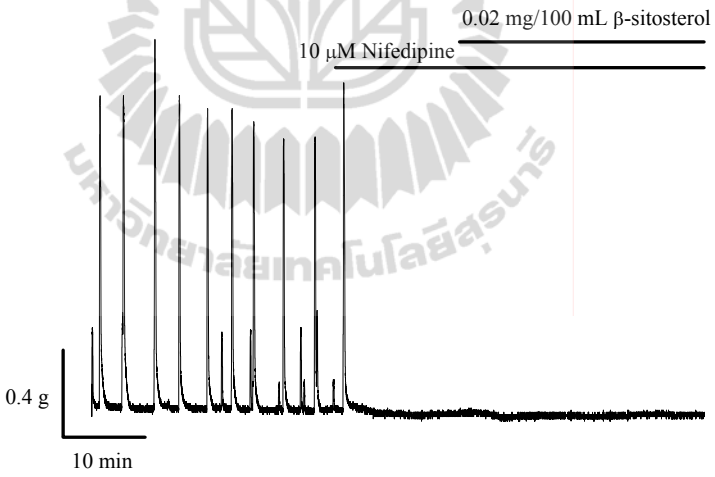


Figure 7.6 The effect of β-sitosterol on uterine contraction in the presence of L-type Ca^{2+} channel inhibitor, nifedipine (typical of 3 other traces from different animals).

7.4.4 Effects of Diosgenin on Spontaneous Contraction

Diosgenin, a steroidal saponin is one the major compositions found in *C. speciosus* extracts, it was worth determining whether the effects of the extracts on uterine contraction were due to this compound.

Under control condition, spontaneous contractions of the consistent amplitude, frequency and AUC were recorded. Diosgenin at the concentrations of 0.02, 0.2 and 2 mg/100 mL were applied to the contracting myometrium strips. As shown in Figure 7.7 and Table 7.4, each concentration of diosgenin reduced the frequency and AUC of uterine contraction. Diosgenin up to 2 mg/100 mL produced no significant effects on the amplitude of uterine contraction. Thus, a concentration of 0.02 mg/100 mL of diosgenin was used in this study.

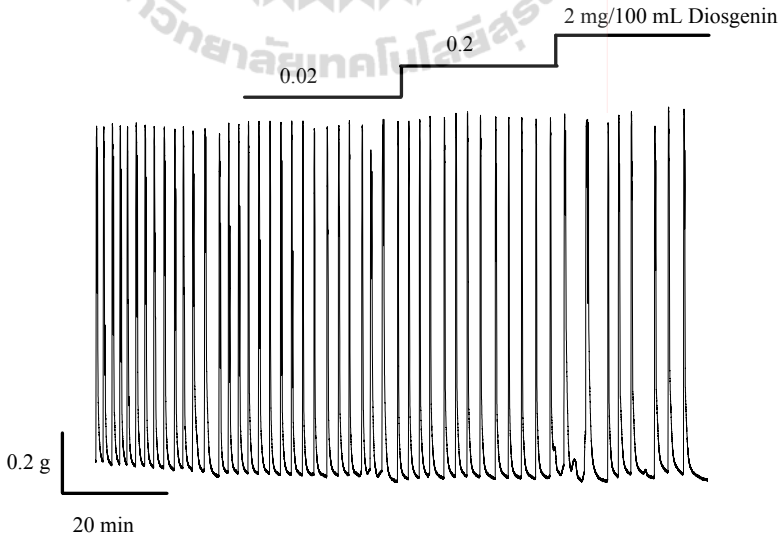


Figure 7.7 The effect of diosgenin at various concentrations on spontaneous contraction (typical of 3 other traces from different animals).

Table 7.4 Effects of diosgenin at various concentrations on spontaneous contraction in non-pregnant rats.

	Amplitude (% Mean ± S.E.M.)	Frequency (% Mean ± S.E.M.)	AUC (% Mean ± S.E.M.)	n
Diosgenin (mg/100 mL)				
0 (Control)	100	100	100	3
0.02	102.85 ± 2.43	90.48 ± 9.52	107.41 ± 15.06	3
0.20	104.54 ± 2.78	85.71 ± 14.29	98.45 ± 14.98	3
2.00	102.52 ± 2.37	80.95 ± 19.05	66.82 ± 12.46	3

The *P*-values for amplitude, frequency and AUC of diosgenin treated are not significantly different from the control ($P > 0.05$).

Mean value ± S.E.M. are given; n is number of animals.

7.4.5 Effects of Diosgenin in the Presence of *C. speciosus* Extracts

As shown in Chapter VI, it is clear that *C. speciosus* potentiate uterine contraction. As with β-sitosterol, diosgenin is one of the major compositions found in the extracts, it was worth determining whether the effects of *C. speciosus* extracts on uterine contraction were due to diosgenin.

Effects of Diosgenin in the Presence of *C. speciosus* Rhizome Extract

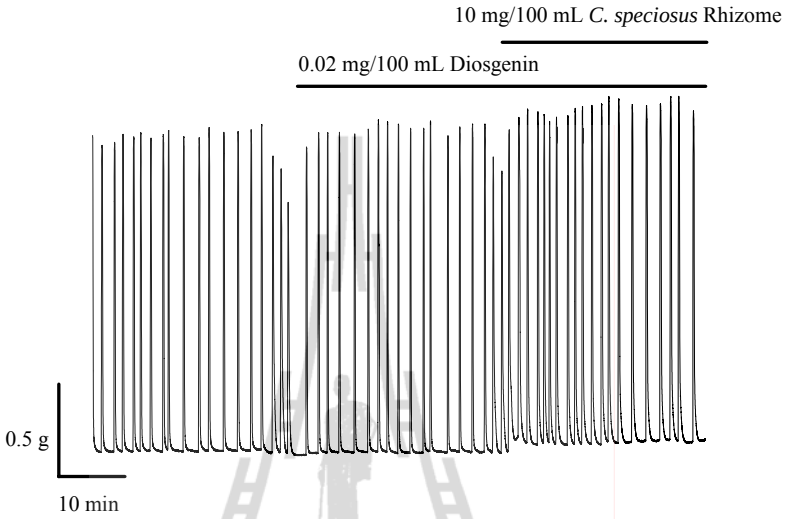
The effects of diosgenin on uterine contraction in the presence of *C. speciosus* rhizome extract are summarized in Table 7.5. Application of diosgenin reduced the frequency and AUC of contraction to 80.55 ± 10.02% and 99.19 ± 3.30%, respectively, compared with control (100%). When *C. speciosus* rhizome extract was added in the continued presence of diosgenin, it produced an increase in the amplitude (113.45 ± 4.43%), frequency (150.00 ± 16.67%) and AUC (227.72 ± 11.67%) of contraction, as can be seen in Figure 7.8A. As shown in Figure 7.8B, when diosgenin was added after an addition of *C. speciosus* rhizome extract, it reduced amplitude, frequency and AUC of contraction to 103.88 ± 12.78%, 103.11 ± 7.76% and 91.87 ± 6.45%, respectively, compared with control (100%).

Effects of Diosgenin in the Presence of *C. speciosus* Stem Extract

As shown in Figure 7.9, diosgenin produced a decreases in amplitude and AUC to 86.26 ± 9.78% and 79.51 ± 9.63%, respectively, compared with control (100%). When *C. speciosus* stem extract was applied in the continued presence of diosgenin, it produced an increase in amplitude (108.60 ± 8.89%), frequency (105.17 ± 5.17%) and AUC (100.44 ± 11.55%) of contraction, compared with control (100%). When

diosgenin was applied after addition of *C. speciosus* stem extract, amplitude, frequency and AUC were decreased to 92.49 ± 10.18%, 117.26 ± 3.90% and 88.61 ± 2.52%, respectively, compared with control (100%). The experiment traces are demonstrated in Figure 7.9 and the data were summarized in Table 7.6.

A



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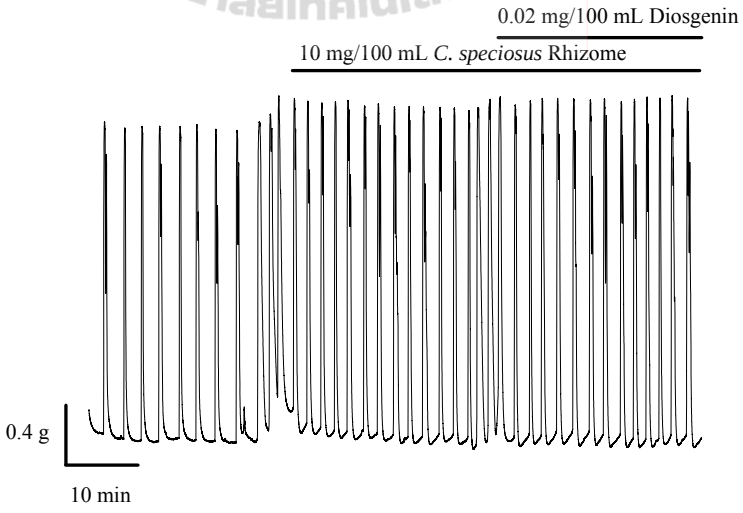
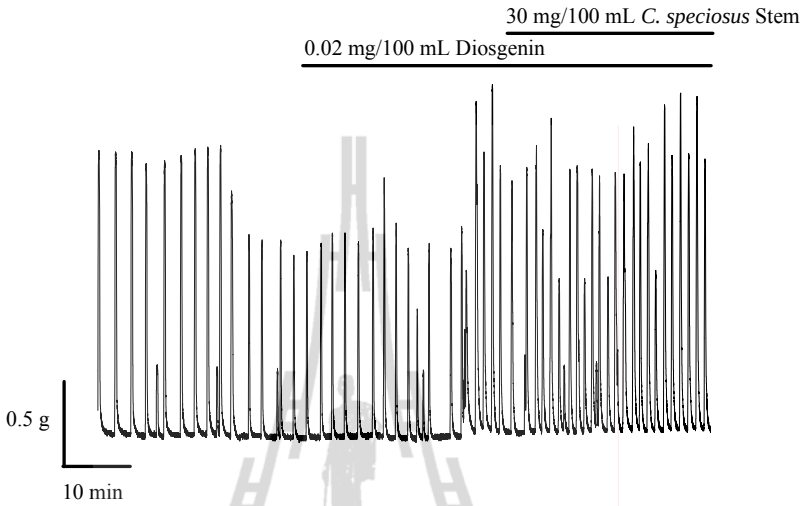


Figure 7.8 The effects of diosgenin in the presence of *C. speciosus* rhizome extract. Diosgenin was added before (A) and after (B) *C. speciosus* rhizome extract (typical of 3 other traces from different animals).

A



B

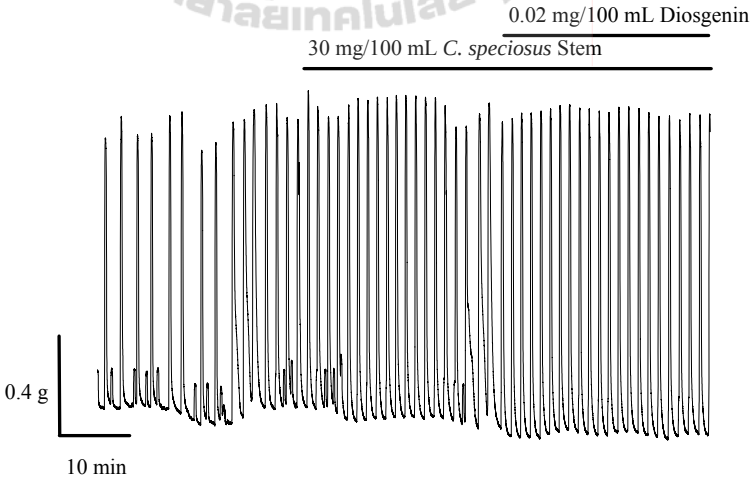


Figure 7.9 The effects of diosgenin in the presence of *C. speciosus* stem extract. Diosgenin was added before (A) and after (B) *C. speciosus* stem extract (typical of 3 other traces from different animals).

Table 7.5 Effects of diosgenin in the presence of *C. speciosus* rhizome extract.

	Amplitude (% Mean ± S.E.M.)	Frequency (% Mean ± S.E.M.)	AUC (% Mean ± S.E.M.)	n
***C. speciosus* rhizome (after)**				
Control	100	100	100	
Diosgenin	105.83 ± 2.23	80.55 ± 10.02	99.19 ± 3.30	3
Diosgenin + *C. speciosus* rhizome	113.45 ± 4.43[a]	150.00 ± 16.67	227.72 ± 11.67[a]	3
C. speciosus **rhizome (before)**				
Control	100	100	100	3
C. speciosus rhizome	117.08 ± 4.86[a]	131.67 ± 9.28[a]	139.40 ± 12.29	3
C. speciosus rhizome + Diosgenin	103.88 ± 2.78	103.11 ± 7.76	91.87 ± 6.45	3

The *P*-values for amplitude, frequency and AUC of *C. speciosus* rhizome are significantly different from the control ([a]$P < 0.05$).

Mean value ± S.E.M. are given; n is number of animals.

Table 7.6 Effects of diosgenin in the presence of *C. speciosus* stem extract.

	Amplitude (% Mean ± S.E.M.)	Frequency (% Mean ± S.E.M.)	AUC (% Mean ± S.E.M.)	n
***C. speciosus* stem (after)**				
Control	100	100	100	3
Diosgenin	86.26 ± 9.78	100.00 ± 0.00	79.51 ± 9.63	3
Diosgenin + *C. speciosus* stem	108.60 ± 8.89	105.17 ± 5.17	100.44 ± 11.55	3
***C. speciosus* stem (before)**				
Control	100	100	100	3
C. speciosus stem	113.98 ± 4.48[a]	133.33 ± 0.00[a]	137.65 ± 8.70[a]	3
C. speciosus stem + Diosgenin	92.49 ± 10.18	117.26 ± 3.90[a]	88.61 ± 2.52	3

The *P*-values for amplitude, frequency and AUC of *C. speciosus* stem are significantly different from the control ([a]$P < 0.05$).

Mean value ± S.E.M. are given; n is number of animals.

7.4.6 Effects of Diosgenin on Uterine Contraction in the Presence of L-type Ca^{2+} Channel Inhibitor

As shown in Chapter VI, it is clear that *C. speciosus* potentiate uterine contraction in the absence of extracellular calcium. As diosgenin is one of the major compositions found in the extracts, it was worth determining whether the force elicited in the absence of external calcium were due to diosgenin.

As shown in Figure 7.10, the effect of diosgenin (0.02 mg/100 mL) was applied in the continued presence of 10 μM nifedipine, no force transients were observed.

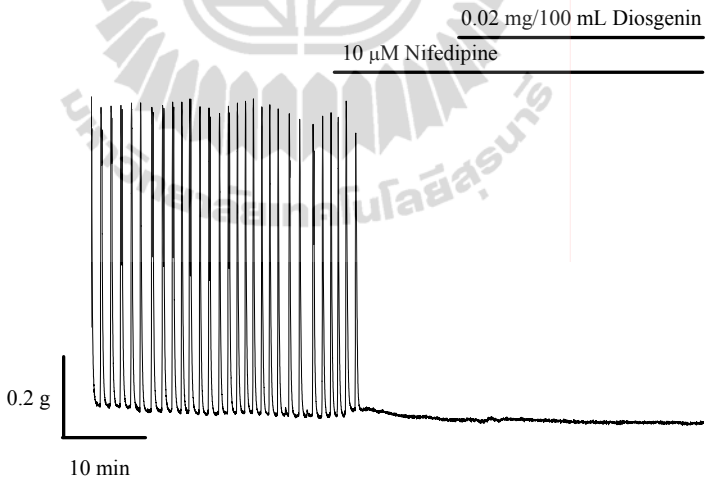


Figure 7.10 The effect of diosgenin on uterine contraction in the presence of L-type Ca^{2+} channel inhibitor, nifedipine (typical of 3 other traces from different animals).

7.5 Discussion

In Chapter II, the chemical compositions of the *C. speciosus* rhizome and stem extracts analyzed using GC-MS technique showed that the most compounds found in the plant extracts were β-sitosterol and diosgenin. Thus, the aim of this chapter was established to clarify whether the effects of the extracts on uterine contraction were due to β-sitosterol and diosgenin.

In non-pregnant rats, the results showed that β-sitosterol and diosgenin exhibited the differential effects on uterine contraction. β-sitosterol increased the amplitude, frequency and AUC of the contraction. In contrast, diosgenin decreased all of those parameters of spontaneous contraction.

β-sitosterol is a common phytoestrogen and appears to be a major uterotonic agent in pomegranate seeds (Promprom, Kupittayanant, Indrapichate, Wray and Kupittayanant, 2010). In other cell types, an inhibitory action of β-sitosterol on the SR Ca^{2+}-ATPase (SERCA) has been reported (Bao, Li, Deng, Landry and tabas, 2006). It has been reported that β-sitosterol increased uterine contraction via calcium entry on L-type calcium channels and MLCK (Promprom, Kupittayanant, Indrapichate, Wray and Kupittayanant, 2010). In addition, when the β-sitosterol was applied after an application of nifedipine, no force was observed during the application of β-sitosterol, which was different from *C. speciosus* extracts. Thus, β-sitosterol is probably not the only agent in the extracts responsible for potentiation of uterine force, is a key mediator of its actions. In *C. speciosus* extracts, it remains to be established which additional component of the extract contributes to potentiating spontaneous contraction. Significant amounts of the erogostane steroid, 9, 19-cycloergost-24(28)-en-3-ol, were also found in the extracts and this may be contributing.

Diosgenin is found in a variety of plants, particularly yams and fenugreek as well as *C. speciosus* (Puri, Jefferies and Hardman, 1976). It has a variety of medicinal uses such as in a treatment of diabetes (Gupta, Gupta and Lal, 2001) and hypercholesterolemia (Sauvaire, Ribes, Baccou and Loubatieeres-Mariani, 1991). It has not been studied in the uterus. However, in vascular smooth muscle it has recently been found to reduce tone, partly through activating calcium-activated potassium channels and reducing Ca^{2+} entry (Au et al., 2004; Dias et al., 2007). At the high concentrations, it was found that diosgenin relaxed the rat uterus, suggesting a common action on contractility in the smooth muscle. The relaxant effect of diosgenin was overcome by the extracts suggesting a more potent effect of other components, such as β-sitosterol.

Taken together, the results indicated that one or more active compounds in the extracts possessed an activity on uterine stimulation. Significant amounts of the ergosterol, a sterol which is found in fungi, bacteria, algae and higher plants, were also found in the extracts. Ergosterol is a biological precursor to vitamin D_2 by the action of ultraviolet light (Rajakumar, Greenspan, Thomas and Holick, 2007). It is one of the pharmaceutical constituents found in ergort alkaloids.

The ergort alkaloids are complex family of mycotoxins derived from prenylated tryptophane in several species of fungi. They are well known from their historical role in human toxicoses. In mammals, ergort alkaloids affect the central and sympathetic nervous systems. This results in many symptoms such as lowered immune response, reduced lactation and reproductive capability, disturbance in sleep/wake cycle (Panaccione, 2005). In 1582, ergort was used for the first time as an oxytocic drug by administering 0.5 mg ergort to stimulate uterine contractions of labor (Pieter and

Akosva, 1995). Moreover, it has been reported that ergort directly stimulates smooth muscle in many organs such as stimulates and increases blood pressure (Dale, 1906). Therefore, the potentiation of uterine force produced by the extracts may be partly due to this compound.

There is a recent report indicated that *Costus Lucanusianus* showed oxytocic effects on isolated non-pregnant rat uterus. It was suggested that both oxytocin and the extract both produced a dose dependent increase in the contraction of the isolated uterus. The contractile effect produced by 200 mg/mL of the extract was noted to be similar to that of 0.16 IU oxytocin (Owolabi, Omogbai and Falodun, 2010). Earlier researchers have reported that estrogens possess some oxytocic effect and hence may be involved in increasing uterine contractility (Tafesse, Mekonnen and Makonnen, 2005). Oxytocin was reported to biologically act via binding to a uterine membrane receptor, with seven transmembrane domains and belongs to the class I G protein-coupled receptor family and thereby stimulates a cascade of events leading to the contraction of uterine contractile protein; actin and myosin (McKillen, Thomas and Taylor, 1999; Gimpl and Fahrenholz, 2001; Muller et al., 2006). Similarly, the increase in the uterine stimulated contractile activity of the extracts observed in the present results could be due to the presence of oxytocic-like compounds. When considering the phytochemical compositions of the extracts, both rhizome and stem composed of ergosterol which has an oxytocic property. Therefore, the biological active substances of *C. speciosus* rhizome and stem extracts could be partially due to this compound.

In conclusion, the effects of *C. speciosus* rhizome and stem extracts on uterine stimulated contraction were not found to be due to diosgenin, but β-sitosterol and other compounds such as ergosterol may be contributing.

7.6 References

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CHAPTER VIII

EFFECTS OF *COSTUS SPECIOSUS* (Koen.) Sm. EXTRACTS ON UTERINE CONTRACTION IN OVARIECTOMIZED RATS

8.1 Abstract

Costus speciosus (Koen.) Sm. is one of many plants that contain estrogen-like action substances. However, the estrogenic activities of *C. speciosus* rhizome and stem extracts on uterine contractility in ovariectomized rats are not well understood. Thus, the purpose of this chapter was to investigate the effects of *C. speciosus* rhizome and stem extracts on uterine contractility in ovariectomized (OVX) rats. The uterine strips were taken from the uterus of OVX rats (n = 3). The results showed that both *C. speciosus* rhizome (10 mg/100 mL) and stem (30 mg/100 mL) extracts increased the amplitude, frequency and AUC of contraction. The mechanisms whereby the extracts exerted their effects on uterine force were necessarily resembled to those of non-pregnant rats. However, the potentiation of uterine force was more transient compared with those of non-pregnant rats. In conclusion, *C. speciosus* rhizome and stem extracts increased phasic contraction in uterine strips isolated from OVX rats.

8.2 Introduction

By the year 2030, the World Health Organization estimates 1.2 billion women will be age of 50 or over, which closely related to the age of menopause. After menopause, the loss of estrogen often causes morphological changes of reproductive organs such as the uterus is atrophy and becomes smaller. Generally, ovarian steroids are the most important factors affecting uterine morphology and also have an important role on regulating uterine smooth muscle contractile activity. Several studies reported that the administration of estrogen to women deprived of ovarian function as a result of surgical menopause (Krohn, Lackner and Soskin, 1937) or primary amenorrhea (Wilson and Kurzrok, 1938; Henry, Browne and Venning, 1950) induces a normal proliferative pattern of uterine contractions. The characteristics of spontaneous myometrial contractility change are due to the direct actions of ovarian hormones and depend on the stage of the menstrual cycle or stage of pregnancy (Bulbul et al., 2007).

Observational studies from Wray and Noble (2008) reported that the electrical and mechanical activities in the rat myometrium showed a relative quiescence during proestrus, with little propagation of any electrical event. In addition, they showed that Ca^{2+} signaling and spontaneous myometrial contractions are greater in metestrus and diestrus compared to proestrus and estrus. Their observations are in agreement with Ishikawa and Fuchs (1978), who found that spontaneous action potentials were often not propagated in these tissues *in vivo*.

Since ancient times, women throughout the world have been using plant extract to treat uterine disorders, menstrual complaints, pregnancy and childbirth. Many plants are commonly used for menopause and menopause-related symptoms including

Black cohosh (*Cimicifuga recemosa*), dong quai (*Angelica sinensis*), wild yam (*Dioscorea villosa*), evening primrose (*Oenothera biennis*), ginkgo (*Ginkgo biloba*), ginseng (*Panax ginseng*), kava (*Piper methyaticum*), motherwort (*Leonurus cardiaca*) and St John's wort (*Hypericum perforatum*). Wild yam and evening primrose are more commonly used for premenstrual syndrome and early menopausal symptoms. Other plants such as ginkgo, ginseng and St John's wort are used primarily for sleep disturbances, nervousness, depression, mood swings and memory loss (Geller and Studee, 2006).

As shown in Chapter III-V, the data demonstrated that *C. speciosus* extracts clearly has estrogenic properties. Moreover, as demonstrated in Chapter II, phytochemical compositions of plant extracts such as β-sitosterol and diosgenin have been found. As demonstrated in Chapter VI and VII, plant extracts were potent uterine stimulants in non-pregnant rats. In animal studies, it has long been known that ovariectomy can lead to uterine dysfunction as a result of ovarian hormone depletion. Thus, changes in sex hormones may play a role in regulation of smooth muscle contractility. However, it has never been investigated. Therefore, the purposes of this chapter were designed to investigate the behavior of uterine contractility isolated from ovariectomized (OVX) rats and to examine the effects of *C. speciosus* rhizome and stem extracts on uterine contraction. The underlying mechanisms of plant extracts were also investigated and compared with those of non-pregnant rats.

8.3 Materials and Methods

8.3.1 Myometrial Tissue Preparation

The rats used in this study were 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 performed in accordance with advice of the Institutional Animal Care and Use Committee, SUT. Rats were bilaterally ovariectomized under pentobarbital sodium anesthesia as described in 3.3.2.3. Fourteen days after surgery, they were sacrificed by given an over dose of CO_2. The uteri were taken and the myometrium preparations were prepared as described in 6.3.1.

8.3.2 Measurements of Tension

Measurements of tension were necessary resemble to those described in 6.3.2. Briefly, the uterine strip was mounted vertically under resting tension of 1 g in a single chamber (25 mL) tissue bath connected to a force transducer. The strip was allowed to contract spontaneously and an equilibrium period of 30 minutes was given before an application of any chemical. The measurements were made whilst the tissue was continually perfused with physiological solution (control) or solution containing *C. speciosus* rhizome or stem extracts at the concentrations of 10-50 mg/100 mL and 30-70 mg/100 mL, respectively. In some experiments, nifedipine, diosgenin or β-sitosterol was used for investigating the mechanisms whereby the extracts exerted their effects.

8.3.3 Statistical Analysis

Parameters that were measured include frequency, amplitude and AUC. The data were given as mean ± S.E.M. and "n" represents the number of sample, each one from a different animal. Significance was tested using appropriate *t*-test and *P* values < 0.05 taken to be significant. The results were then expressed as percentages of control contractions (i.e. the control is 100%).

8.4 Results

8.4.1 The Spontaneous Contraction in Ovariectomized Rats

Under control conditions, the uterine strips taken from OVX rats were able to produce spontaneous contraction (n = 6). However, spontaneous uterine contraction of OVX rats was not different from those of non-pregnant rats. In OVX rats, the mean amplitude was 1.14 ± 0.19 g and the frequency of the contraction was 1.00 ± 0.00 contractions per minute. In non-pregnant rats, the mean amplitude and frequency of the contraction was 1.01 ± 0.15 g and 1.00 ± 0.00 contractions per minute, respectively. As can be seen in Figure 8.1, where the spontaneous contraction of OVX rats and non-pregnant rat records have been expanded and overlapped, the force transient taken from OVX rats is slightly higher ($P > 0.05$) than that of taken from non-pregnant rats.

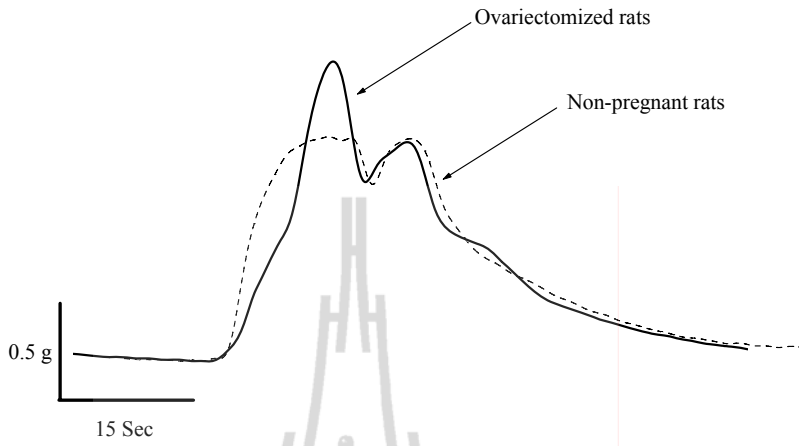


Figure 8.1 Representative superimposed spontaneous force records taken from ovariectomized and non-pregnant rats (typical of 6 other traces from different animals).

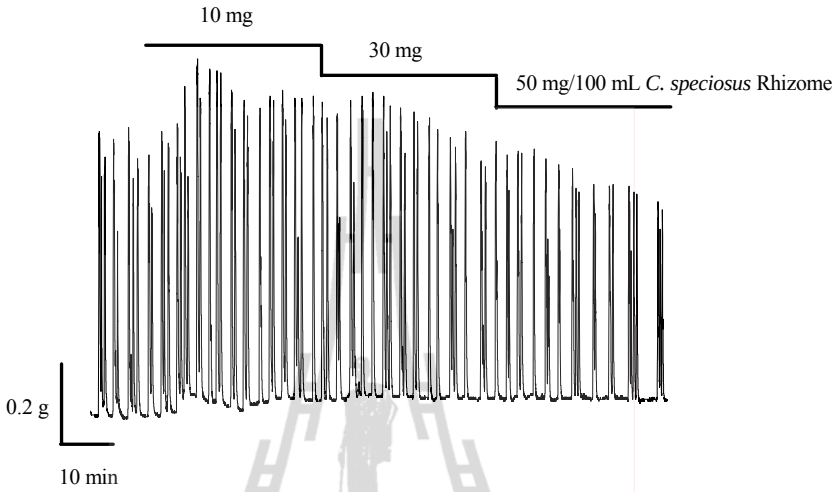
8.4.2 Effects of *C. speciosus* Rhizome and Stem Extracts on Spontaneous Contraction in Ovariectomized Rats

The effects of *C. speciosus* rhizome extract (10, 30, 50 mg/100 mL) were examined (n = 3). Each concentration was applied for 30 minutes. At a dose of 10 mg/100 mL, the extract produced an increase in amplitude, frequency and AUC of the uterine contraction, Figure 8.2A. The mean value of contraction was 140.82 ± 8.82%, 103.03 ± 3.03%, and 200.84 ± 35.28%, respectively. At high concentrations, the extract produced a decrease in amplitude, frequency and AUC of the uterine contraction, Figure 8.2A. The data are summarized in Figure 8.2B and Table 8.1.

C. speciosus stem extract (30, 50, 70 mg/100 mL) was added to spontaneous active preparations for 30 minutes (n = 3). As shown in Figure 8.3A, at a dose of 30 mg/100 mL, the extract produced an increase in the amplitude, frequency and AUC of

contraction to 109.91 ± 4.22%, 106.70 ± 3.88%, and 122.71 ± 4.61%, respectively. At high concentrations, a decrease in amplitude, frequency and AUC of the uterine contraction was observed, Figure 8.3A. The data are summarized in Figure 8.3B and Table 8.2.

A



B

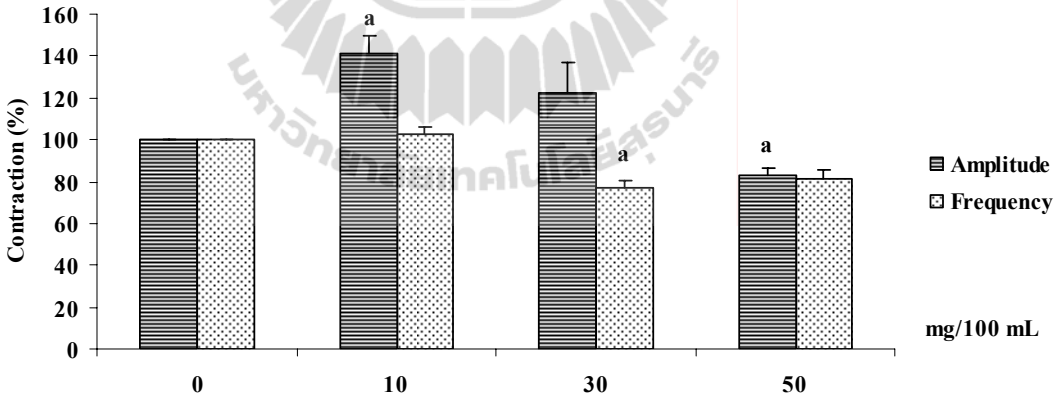
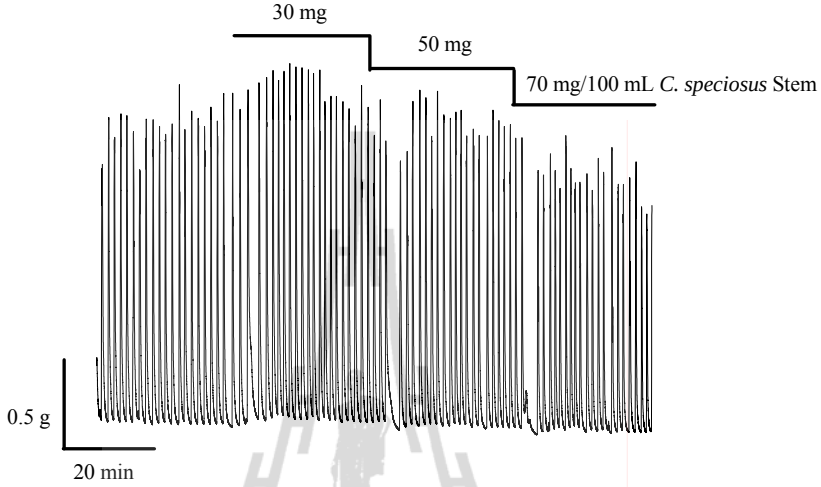


Figure 8.2 The effect of *C. speciosus* rhizome extract at various concentrations on spontaneous contraction in ovariectomized rats (A) (typical of 3 other traces from different animals). Amplitude and frequency are presented as percentage of control responses [i.e. the control is 100%] (B). The *P*-values for amplitude and frequency of *C. speciosus* rhizome extract in ovariectomized rats are significantly different from the control ($^{a}P < 0.05$).

A



B

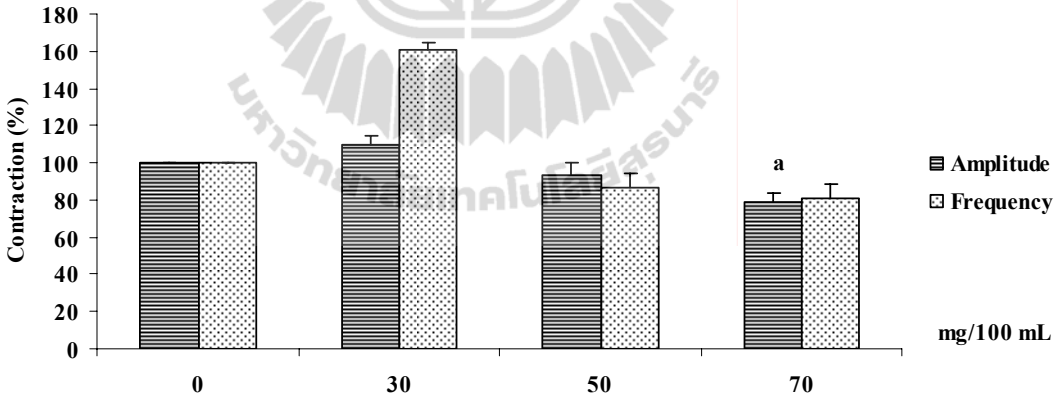


Figure 8.3 The effect of *C. speciosus* stem extract at various concentrations on spontaneous contraction in ovariectomized rats (A) (typical of 3 other traces from different animals). Amplitude and frequency are presented as percentage of control responses [i.e. the control is 100%] (B). The *P*-values for amplitude and frequency of *C. speciosus* rhizome extract in ovariectomized rats are significantly different from the control ($^{a}P < 0.05$).

Table 8.1 The effects of *C. speciosus* rhizome extract at various concentrations in ovariectomized rats.

	Amplitude (% Mean ± S.E.M.)	Frequency (% Mean ± S.E.M.)	AUC (% Mean ± S.E.M.)	n
C. speciosus rhizome extract (mg/100 mL)				
0 (Control)	100	100	100	3
10	140.82 ± 8.82[a]	103.03 ± 3.03	200.84 ± 35.28[a]	3
30	122.61 ±14.06	77.02 ± 3.22[a]	139.85 ±33.19	3
50	83.23 ± 2.77[a]	81.18 ± 4.40	180.47 ± 17.62	3

The *P*-values for amplitude, frequency and AUC of *C. speciosus* rhizome extract in ovariectomized rats are significantly different from the control ([a]$P < 0.05$). Mean ± S.E.M. are given, n is number of animals.

Table 8.2 The effects of *C. speciosus* stem extract at various concentrations in ovariectomized rats.

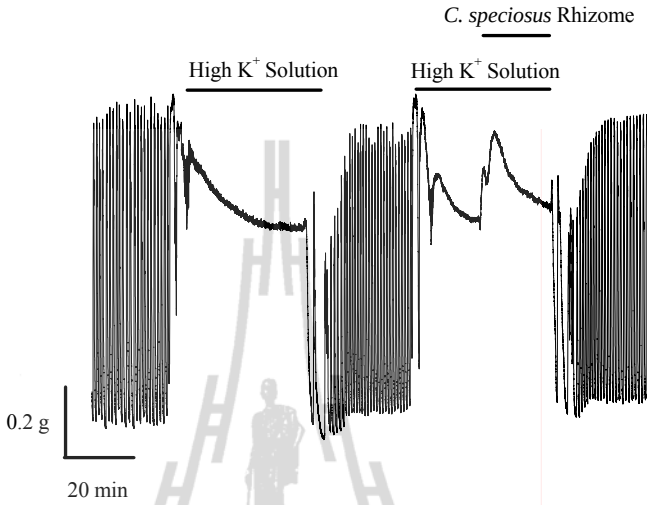
	Amplitude (% Mean ± S.E.M.)	Frequency (% Mean ± S.E.M.)	AUC (% Mean ± S.E.M.)	n
C. speciosus stem extract (mg/100 mL)				
0 (Control)	100	100	100	3
30	109.91 ± 4.22	106.70 ± 3.88	122.71 ± 4.61[a]	3
50	93.69 ± 6.32	86.62 ± 7.31	89.00 ± 4.24	3
70	79.07 ± 4.32[a]	80.85 ± 7.89	46.78 ± 8.29[a]	3

The *P*-values for amplitude, frequency and AUC of *C. speciosus* stem extract in ovariectomized rats are significantly different from the control ([a]$P < 0.05$). Mean ± S.E.M. are given, n is number of animals.

8.4.3 Effects of *C. speciosus* Rhizome and Stem Extracts in the Presence of High K^+ Solution

The aim of these experiments was to investigate the effects of *C. speciosus* extracts when intracellular Ca^{2+} was maintained at a high level by exposing the uterus to high K^+ solution. As can be seen in Figure 8.4, when *C. speciosus* rhizome or stem extract was added in the continued presence of 40 mM K^+ solution (n = 3), a tonic force was produced upon the addition of the extracts, compared to high K^+ alone.

A



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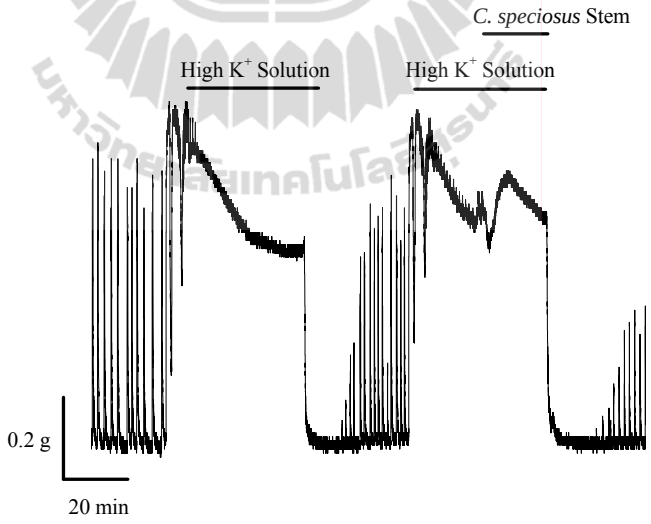


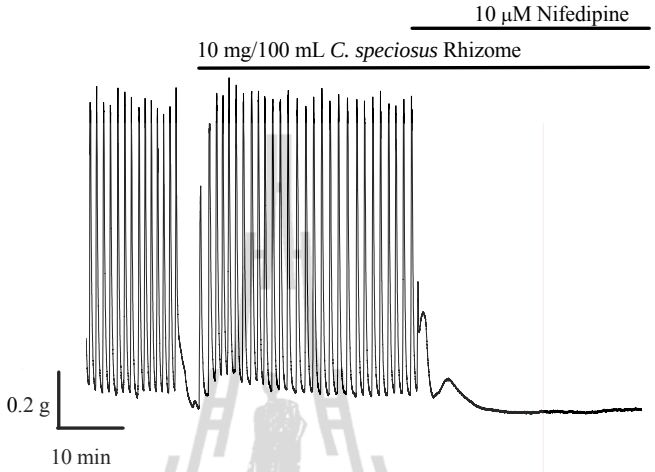
Figure 8.4 Effects of *C. speciosus* rhizome (A) and stem (B) extracts in the presence of high K^+ solution (40 mM) in ovariectomized rats (typical of 3 other traces from different animals).

8.4.4 Effects of *C. speciosus* Rhizome and Stem Extracts on Uterine Contraction in the Presence of L-type Ca^{2+} Channel Inhibitor

The experiments were performed to see whether an increase in the contraction induced by *C. speciosus* rhizome (10 mg/100 mL) and stem (30 mg/100 mL) extracts was dependent on an entry of extracellular Ca^{2+} via L-type Ca^{2+} channels. As shown in Figures 8.5A and 8.6A, either *C. speciosus* rhizome (10 mg/100 mL) or stem (30 mg/100 mL) extract was applied in the continued presence of 10 μM nifedipine and the contraction was observed. The application of 10 μM nifedipine rapidly inhibited the increased force induced by the extract (n = 3).

As shown in Figures 8.5B and 8.6B, when *C. speciosus* rhizome or stem extracts was applied after an application of 10 μM nifedipine, force transients could be observed upon the application of the extracts.

A



B

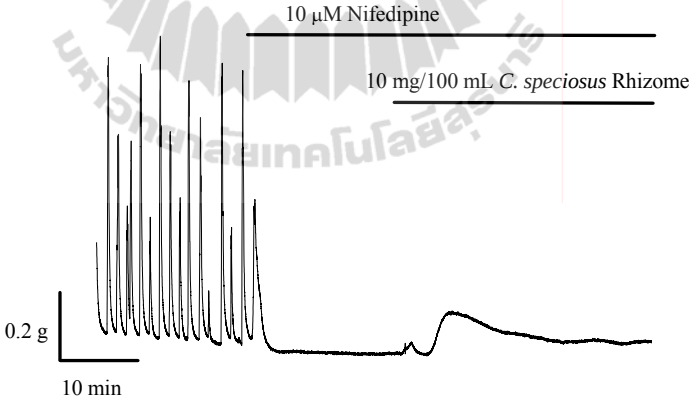
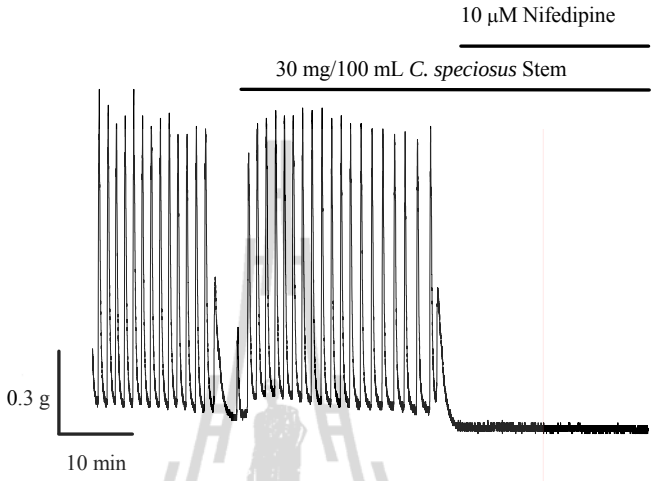


Figure 8.5 Effects of *C. speciosus* rhizome extract on uterine contraction in the presence of L-type Ca^{2+} channel inhibitor. Nifedipine was added before (A) and after (B) *C. speciosus* rhizome extract (typical of 3 other traces from different animals).

A



B

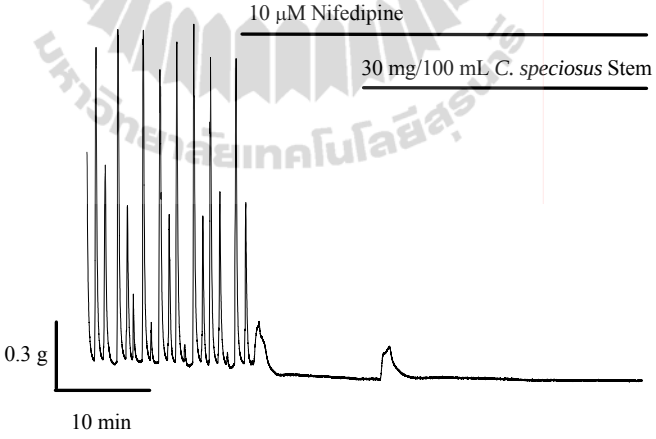


Figure 8.6 Effects of *C. speciosus* stem extract on uterine contraction in the presence of L-type Ca^{2+} channel inhibitor. Nifedipine was added before (A) and after (B) *C. speciosus* stem extract (typical of 3 other traces from different animals).

8.4.5 Effects of β-sitosterol in the Presence of *C. speciosus* Rhizome and Stem Extracts

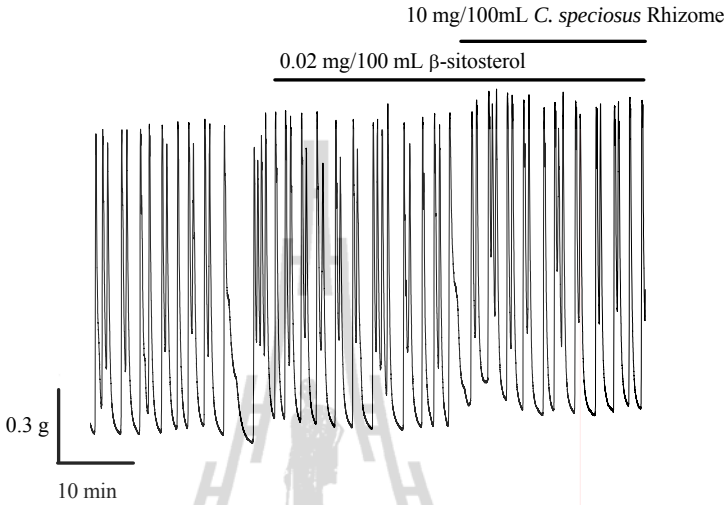
Effects of β-sitosterol in the Presence of *C. speciosus* Rhizome Extract

The effects of β-sitosterol (0.02 mg/100 mL) on spontaneous contraction in OVX rats were investigated (n = 3). As shown in Figure 8.7A, the mean value of the amplitude, frequency and AUC of contraction was increased to 103.44 ± 4.50%, 112.50 ± 12.50% and 112.93 ± 28.60%, respectively, compared with spontaneous control (100%). When *C. speciosus* rhizome extract was added in the continued presence of β-sitosterol, it produced an increase in amplitude, frequency and AUC of contraction to 109.86 ± 6.18%, 108.34 ± 8.34% and 130.66 ± 19.44%, respectively, compared with β-sitosterol alone (100%). However, when β-sitosterol was added after an addition of *C. speciosus* rhizome extract, it reduced uterine contraction in OVX rats, as shown in Figure 8.7B. The data are shown in Table 8.3.

Effects of β-sitosterol in the Presence of *C. speciosus* Stem Extract

The effects of *C. speciosus* stem extract on uterine contraction in OVX rats in the presence of β-sitosterol are shown in Table 8.4. As shown in Figure 8.8A, an application of β-sitosterol (0.02 mg/100 mL) produced an increase in the amplitude (104.34 ± 2.71%), compared with spontaneous control (100%). When *C. speciosus* stem extract was applied in the continued presence of β-sitosterol, it increased the frequency of contraction to 120.00± 11.55%, compared with β-sitosterol control (100%). When β-sitosterol was applied after an addition of *C. speciosus* stem extract, the amplitude, frequency and AUC of contraction was reduced to 101.98 ± 6.27%, 70.84 ± 4.17% and 76.79 ± 4.62%, respectively, as can be seen in Figure 8.8B.

A



B

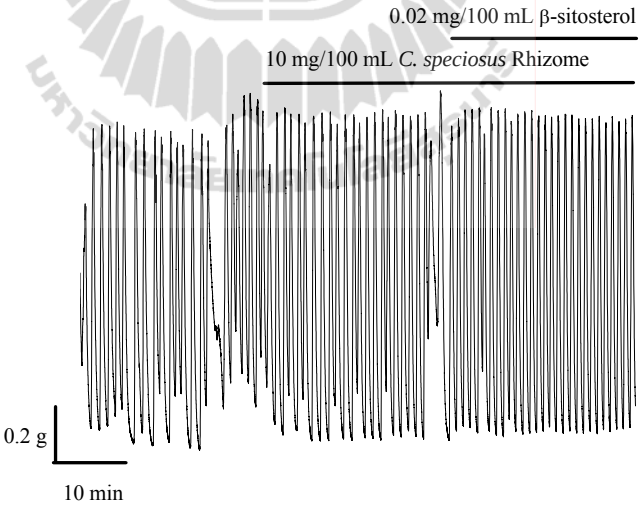
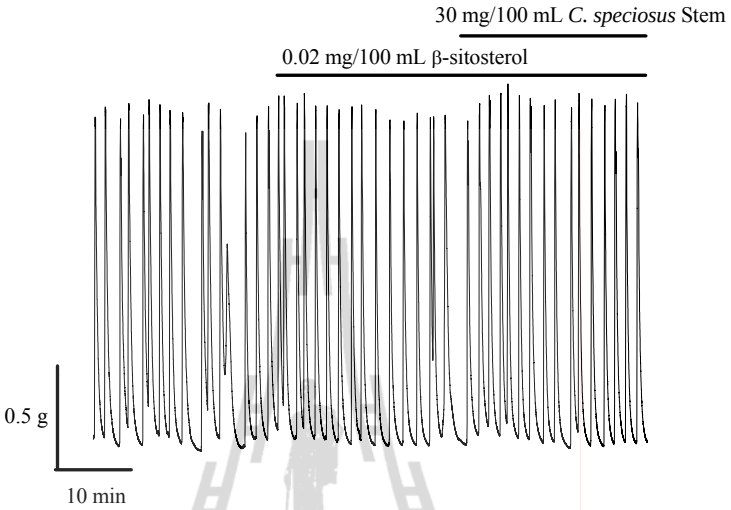


Figure 8.7 Effects of β-sitosterol in the presence of *C. speciosus* rhizome extract. β-sitosterol was added before (A) and after (B) *C. speciosus* rhizome extract (typical of 3 other traces from different animals).

A



B

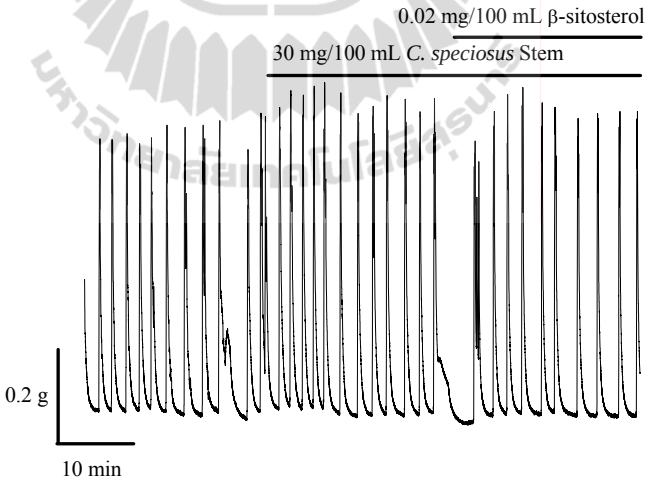


Figure 8.8 Effects of β-sitosterol in the presence of *C. speciosus* stem extract. β-sitosterol was added before (A) and after (B) *C. speciosus* stem extract (typical of 3 other traces from different animals).

Table 8.3 Effects of β-sitosterol in the presence of *C. speciosus* rhizome extract.

	Amplitude (% Mean ± S.E.M.)	Frequency (% Mean ± S.E.M.)	AUC (% Mean ± S.E.M.)	n
***C. speciosus* stem (after)**				
Control	100	100	100	3
β-sitosterol	103.44 ± 4.50	112.50 ± 12.50	112.93 ± 28.60	3
β-sitosterol + *C. speciosus* rhizome	109.86 ± 6.18	108.34 ± 8.34	130.66 ± 19.44	3
***C. speciosus* stem (before)**				
Control	100	100	100	3
C. speciosus rhizome	113.13 ± 3.77[a]	137.04 ± 14.19	139.14 ±10.20	3
C. speciosus rhizome + β-sitosterol	97.39 ± 2.78	88.75 ± 1.25[a]	85.92 ± 5.10	3

The *P*-values for amplitude, frequency and AUC of *C. speciosus* rhizome are significantly different from the control ([a]$P < 0.05$).

Mean value ± S.E.M. are given; n is number of animals.

Table 8.4 Effects of β-sitosterol in the presence of *C. speciosus* stem extract.

	Amplitude (% Mean ± S.E.M.)	Frequency (% Mean ± S.E.M.)	AUC (% Mean ± S.E.M.)	n
***C. speciosus* stem (after)**				
Control	100	100	100	3
β-sitosterol	104.34 ± 0.24	103.33 ± 3.33	98.96 ± 12.66	3
β-sitosterol + *C. speciosus* stem	102.24 ± 4.65	120.00 ±11.55	97.73 ± 1.90	3
***C. speciosus* stem (before)**				
Control	100	100	100	3
C. speciosus stem	102.70 ± 12.05	117.15 ± 2.86[a]	99.45 ± 3.93	3
C. speciosus stem + β-sitosterol	101.98 ± 6.27	70.84 ± 4.17[a]	76.79 ± 4.62[a]	3

The *P*-values for amplitude, frequency and AUC of *C. speciosus* stem are significantly different from the control ([a]$P < 0.05$).

Mean value ± S.E.M. are given; n is number of animals.

8.4.6 Effects of β-sitosterol on Uterine Contraction in the Presence of L-type Ca^{2+} Channel Inhibitor

The following experiments were to investigate the effect of β-sitosterol (0.02 mg/100 mL) in the presence of the L-type Ca^{2+} channel inhibitor, nifedipine. Figure 8.9, no force transient was observed during the application of β-sitosterol.

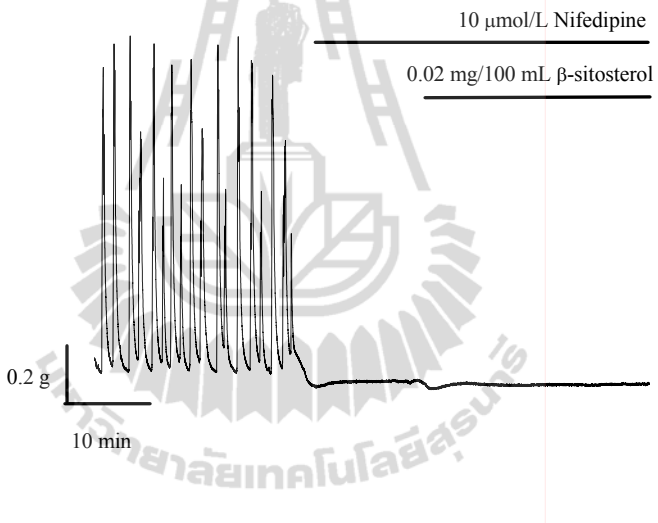


Figure 8.9 Effects of β-sitosterol on uterine contraction in the presence of L-type Ca^{2+} channel inhibitor (typical of 3 other traces from different animals).

8.4.7 Effects of Diosgenin in the Presence of *C. speciosus* Rhizome and Stem Extracts

Effects of Diosgenin in the Presence of *C. speciosus* Rhizome Extract

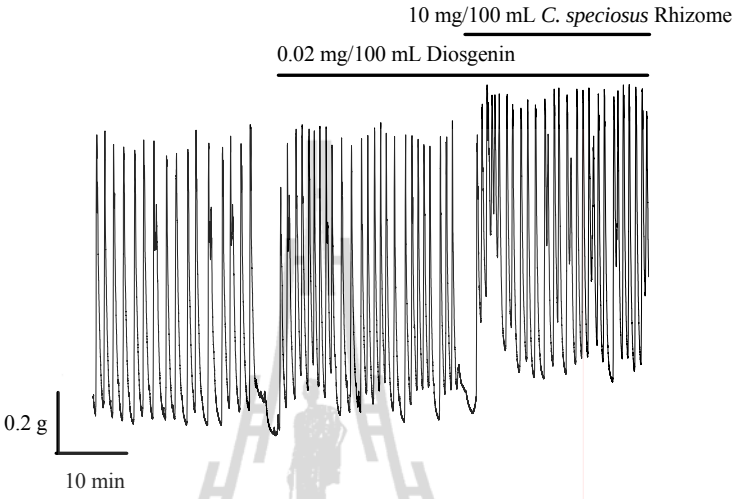
As shown in Figure 8.10A, an application of diosgenin (0.02 mg/100 mL) decreased the mean value amplitude of contraction to 87.71 ± 10.30%, compared with spontaneous control (100%). When *C. speciosus* rhizome extract was applied in the continued presence of diosgenin, it increased the amplitude and AUC of contraction

to 119.69 ± 0.94% and 165.34 ± 24.11%, respectively, compared with diosgenin control (100%). When diosgenin was applied after an addition of *C. speciosus* rhizome extract, it reduced the amplitude (101.97 ± 0.29%), frequency (87.50 ± 12.50%) and AUC (92.15 ± 0.29%) of uterine contraction, as shown in Figure 8.10B. The data are summarized in Table 8.5.

Effects of Diosgenin in the Presence of *C. speciosus* Stem Extract

As shown in Figure 8.11A, diosgenin decreased amplitude (87.75 ± 6.74%) and AUC (67.89 ± 11.77%) of uterine contraction in OVX rats. An application of *C. speciosus* stem extract in the continued presence of diosgenin increased the amplitude (112.02 ± 2.02%) and AUC (127.34 ± 7.81%) of uterine contraction, compared with spontaneous control (100%). As shown in Figure 8.11B, an application of diosgenin after addition of *C. speciosus* stem extract reduced the amplitude (90.25 ± 6.25%), frequency (91.67 ± 8.34%) and AUC (62.02 ± 17.29%) of uterine contraction, compared with diosgenin control (100%) in OVX rats. The data are summarized in Table 8.6.

A



B

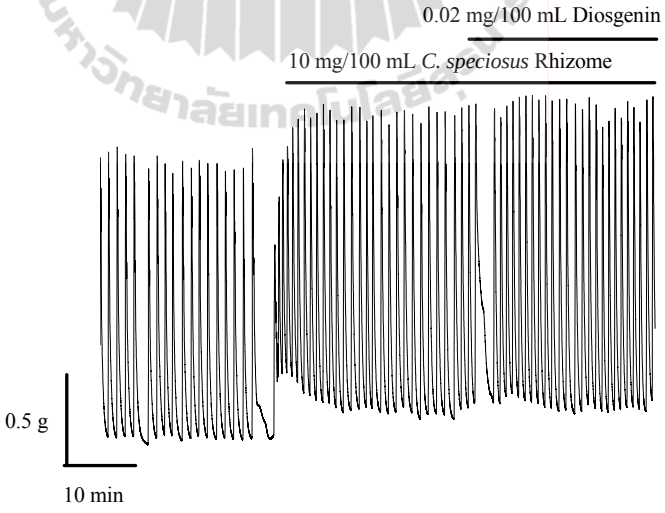
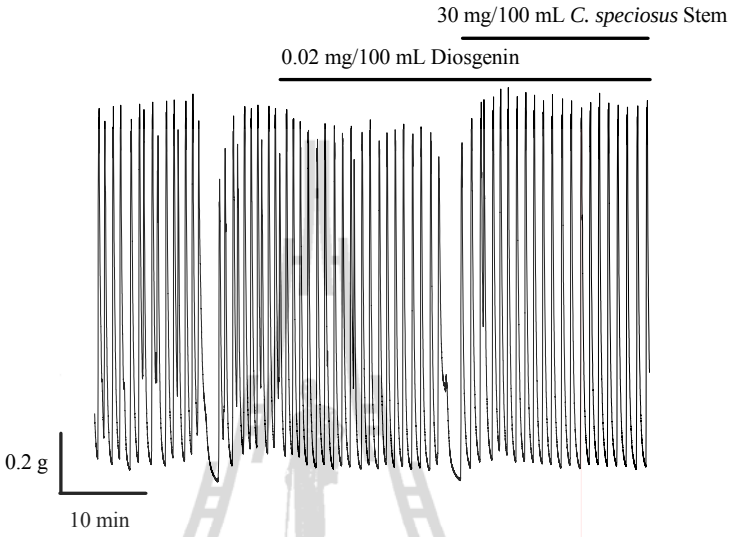


Figure 8.10 Effects of diosgenin in the presence of *C. speciosus* rhizome extract. Diosgenin was added before (A) and after (B) *C. speciosus* rhizome extract (typical of 3 other traces from different animals).

A



B

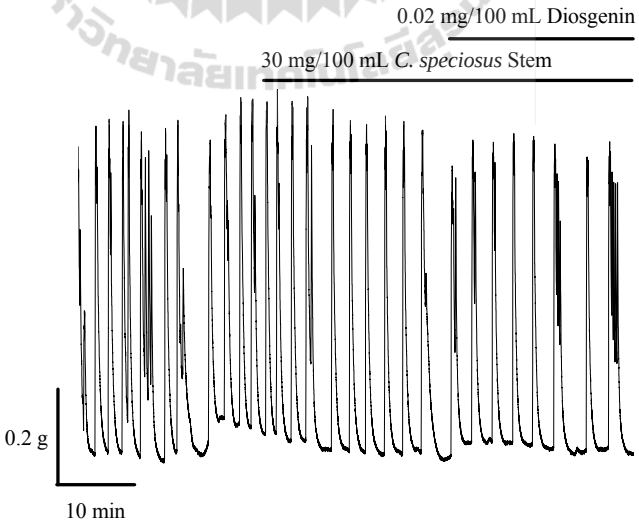


Figure 8.11 Effects of diosgenin in the presence of *C. speciosus* stem extract. Diosgenin was added before (A) and after (B) *C. speciosus* stem extract (typical of 3 other traces from different animals).

Table 8.5 Effects of diosgenin in the presence of *C. speciosus* rhizome extract.

	Amplitude (% Mean ± S.E.M.)	Frequency (% Mean ± S.E.M.)	AUC (% Mean ± S.E.M.)	n
***C. speciosus* rhizome (after)**				
Control	100	100	100	3
Diosgenin	87.71 ± 10.32	110.00 ± 10.00	125.28 ± 24.64	3
Diosgenin + *C. speciosus* rhizome	119.69 ± 094	100.00 ± 0.00	165.34 ± 24.11	3
***C. speciosus* rhizome (before)**				
Control	100	100	100	3
C. speciosus rhizome	110.65 ± 8.95	119.65 ± 5.36	140.46 ± 22.56	3
C. speciosus rhizome + Diosgenin	101.97 ± 0.29	87.50 ± 12.50	92.15 ± 9.28	3

The *P*-values for amplitude, frequency and AUC of *C. speciosus* rhizome are not significantly different from the control ($P > 0.05$). Mean value ± S.E.M. are given; n is number of animals.

Table 8.6 Effects of diosgenin in the presence of *C. speciosus* stem extract.

	Amplitude (% Mean ± S.E.M.)	Frequency (% Mean ± S.E.M.)	AUC (% Mean ± S.E.M.)	n
***C. speciosus* stem (after)**				
Control	100	100	100	3
Diosgenin	87.75 ± 6.74	100.00 ± 0.00	67.89 ± 11.77	3
Diosgenin + *C. speciosus* stem	112.02 ± 2.02[a]	100.00 ± 0.00	127.34 ± 7.81	3
***C. speciosus* stem (before)**				
Control	100	100	100	3
C. speciosus stem	105.15 ± 5.15	122.22 ± 11.11	123.39 ± 3.08[a]	3
C. speciosus stem + Diosgenin	90.25 ± 6.25	91.67± 8.34	62.02 ± 17.29	3

The *P*-values for amplitude, frequency and AUC of *C. speciosus* stem are significantly different from the control ([a]$P < 0.05$).

Mean value ± S.E.M. are given; n is number of animals.

8.4.8 Effects of Diosgenin on Uterine Contraction in the Presence of L-type Ca^{2+} Channel Inhibitor

The effects of diosgenin (0.02 mg/100 mL) in the presence of 10 μM nifedipine were investigated (n = 3). As shown in Figure 8.12, a spontaneous force was abolished by applying 10 μM nifedipine. When diosgenin was applied in the continued presence of nifedipine, no force transient was observed.

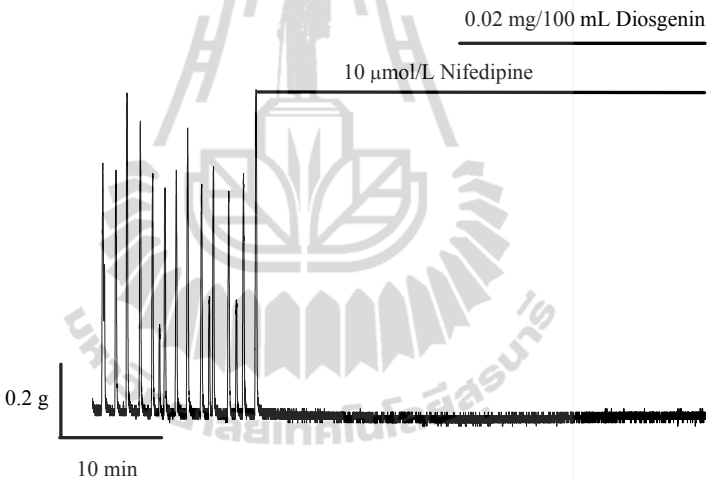


Figure 8.12 Effects of diosgenin on uterine contraction in the presence of L-type Ca^{2+} channel inhibitor (typical of 3 other traces from different animals).

8.5 Discussion

The loss of follicles results in estradiol depletion in OVX rats similar to those seen in the final stage of female reproduction, menopause. This is termed persistent anestrus, or persistent diestrus. After the cessation of ovulation and entry into persistent estrus, there is still some maturation of follicles, followed by without ovulation and culminating in complete follicular depletion. Morphology of the

reproductive tract is characterized by a low, somewhat mucified vaginal epithelium and a low inactive endometrial epithelium (Westwood, 2008). Whether, the myometrium is active, has never been clarified.

The results of the present study indicated that the OVX myometrium is still active. Under control conditions, the spontaneous contraction of consistent amplitude, frequency and area under the contraction (AUC) could be recorded for several hours. Though the uterus of the OVX rats behaved similarly to those of non-pregnant rats in several aspects, however, the amplitude of spontaneous contraction was slightly increased in ovariectomized rats compared with normal rats. This observation is consistent with Vedernikov, Hartke, de Long, Saade and Garfield (2003) who found a higher spontaneous contractile activity in uterine rings isolated from OVX rats. The increased spontaneous contractile activity in OVX rats may result from the removal of the genomic and non-genomic suppressive effects of 17β-estradiol, causing electrical instability of the myometrial cells of sarcolemmal membrane (Vedernikov, Hartke, de Long, Saade and Garfield, 2003).

The present data also showed that *C. speciosus* rhizome and stem extracts stimulated uterine strips taken from OVX rats at the same concentrations as observed in non-pregnant rats. However, at high concentrations, the effects of the extracts were transient. This may be due to the genomic and non-genomic suppressive effects of 17β-estradiol as previously described (Vedernikov, Hartke, de Long, Saade and Garfield, 2003). As with non-pregnant rats, the results indicated that in OVX rats the pathways lead to increase uterine contraction by the extracts occurred via calcium entry on L-type Ca^{2+} channels and SR calcium release. Moreover, it was found that force transients stimulated by plant extracts were abolished by nifedipine, the

inhibitor of L-type Ca^{2+} channels. When extracellular Ca^{2+} was absent, the extracts of both rhizome and stem caused a small force presumably by releasing Ca^{2+} from the SR. This was also the case observed in non-pregnant rats.

Uterine smooth muscle contraction is mediated mainly via an increase in intracellular Ca^{2+} and is accomplished by excitation-contraction coupling mechanisms (Zhang et al., 2005). A high K^+ solution could depolarize the cellular membrane of uterine smooth muscle (Bolton, 1979; Zhang et al., 2005). In the present study, when the high K^+ solution was used to depolarize the uterus and maintain intracellular Ca^{2+} at high levels, *C. speciosus* rhizome and stem extracts were still able to alter force to tonic. Thus, the data supported that, in OVX rats, a mechanism of action on elevating tone involving the non- Ca^{2+}-calmodulin-MLCK pathway.

As with non-pregnant rats, β-sitosterol stimulated uterine contraction in OVX rats. It has also been reported that β-sitosterol at a dose of 1 mg/100 mL increased amplitude and AUC of uterine contraction in OVX rats (Promprom, 2009). In OVX rats, diosgenin decreased uterine contraction which could be observed in non-pregnant rats as well. In addition, several studies suggested that, the binding affinity of different phytoestrogens appears to depend on the model system or cell type (Giammarino et al., 2008) and that estrogenic compounds may exhibit differential binding preference and relative binding affinity for both ER subtypes and for ER from different species (Matthews, Celius, Halgren and Zacharewski, 2000). Taken together, it is of interest to investigate such the effects in the future.

There is evidence reported that estrogen inhibits spontaneous myometrial activity in the rat (Fuchs, 1974, 1976; Downing, Lye, Bradshaw and Porter, 1978) as well as in other species such as rabbit (Coutinho and DeMattos, 1968; Boling and

Blandau, 1971) and ewe (Lye and Porter 1978; Lye, 1980). Results from several studies suggested that exogenous 17β-estradiol and progesterone given to OVX rats are decreased uterine contractility both *in vivo* (Downing, Poster and Redstone, 1981) and in freshly isolated uterine ring (Vedernikov et al., 2003). Decreasing uterine contraction may be explained by the effects of 17β-estradiol. Estrogens have been shown to hyperpolarize smooth muscle by an activation of outward K^+ current (Harder and Coulson, 1979), thereby suppressing spontaneous muscle contraction, inhibiting Ca^{2+} entry (Osa and Ogasawara, 1984; Han, Karaki, Ouchi, Akishita and Oroimo 1995), suppressing the voltage-dependent calcium (Ca^{2+}) current (Yamamoto, 1995) and may be also result from inhibition of smooth muscle cell pacemaker activity. Thus, to further investigate the effect of ovarian steroid hormones on the uterine taken from ovariectomized rats is a prerequisite for the understanding of physiological processes.

In conclusion, the present study demonstrated that *C. speciosus* rhizome and stem extracts stimulated a contraction in uterine strips taken from OVX rats. The mechanisms whereby the extracts exerted their effects were similar to those observed in non-pregnant rats.

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CHAPTER IX

CONCLUSION

Costus speciosus (Koen.) Sm. belongs to family Costaceae. It is commonly known as Ueang maina (Smitinand, 2001). The plant is traditionally used in the treatment of fevers, cough, worm infections, skin diseases and snake bite (Bavara and Narasimhacharya, 2008). It is reported to contain active compounds including diosgenin, tigogenin, β-sitosterol, sterol and fatty acid ester that have been known for their estrogenic properties (Vijayalakshmi and Sarada, 2008). The estrogenic effects of this plant have never been investigated and the physiological mechanisms whereby the plant exerts its effects have never been studied. Therefore, this study focused on investigating estrogenic effects of *C. speciosus* in female rats. There were four main aims which were interconnected; 1) to investigate the effects of *C. speciosus* extracts on reproductive organs, including the uterus, vagina and mammary gland, 2) to investigate the effects of *C. speciosus* extracts on serum estradiol level and lipid profile, 3) to test the anti-implantation effects of *C. speciosus* extracts and 4) to examine the effects of *C. speciosus* extracts on uterine contraction and compared their effects to the known compounds such as diosgenin and β-sitosterol. The underlying mechanisms of the extracts were also investigated. The major findings can be summarized as follows.

9.1 The Chemical Compositions of *C. speciosus* Rhizome and Stem Extracts

Ethanolic extracts of *C. speciosus* rhizome and stem were identified for their chemical compositions using GC-MS technique. It was found that *C. speciosus* rhizome extract contained 20 known and 8 unknown compounds and that diosgenin was the major compound found in the extract. *C. speciosus* stem extract contained 26 known and 5 unknown compounds. Among these compounds, β-sitosterol was the major compound. It is interesting to note that, other phytoestrogens such as ergosterols as well as fatty acid were observed in both rhizome and stem extracts as demonstrated in previous studies (Vijayalakshmi and Sarada, 2008).

9.2 Effects of *C. speciosus* Extracts on Female Reproductive System and Serum Lipid Profile in Ovariectomized Rats

Menopause can lead to a decline in reproductive function and a change in lipid metabolism as a result of estrogen depletion. Physiologically, ovariectomized (OVX) rats have long been used as a model of study for menopausal women (Speroff, Rowan, Symons, Genant and Wilbron, 1996). Since *C. speciosus* extracts contained β-sitosterol and diosgenin that have estrogen-like activity; it was worth investigating the effects of *C. speciosus* extracts on female reproductive organs, serum estradiol level, and serum lipid profile in OVX rats. To do so, the extracts were orally administered to OVX rats and the effects were observed. The results showed that *C. speciosus* rhizome and stem extracts produced increases in relative uterine weights of OVX rats. Histological examination revealed that, in OVX rats, *C. speciosus* rhizome and stem extracts increased the uterine cross section area and size, vaginal epithelium

thickness, and mammary gland alveoli and ducts. *C. speciosus* rhizome and stem extracts also had some positive effects on lipid metabolism. They tended to decrease total cholesterol and LDL-cholesterol. However, the extracts produced no significant changes in serum estradiol level in ovariectomized rats. Thus, ingestion of *C. speciosus* rhizome and stem extracts can adverse the decline of reproductive function and the change in lipid metabolism when estrogen was depleted. Though the effects remained unclear, however, the lack of effects on increasing serum estradiol level indicated that such the effects do not involve in the hypothalamic-pituitary-ovarian axis.

9.3 Effects of *C. speciosus* Extracts on Vaginal Cytology in Ovariectomized Rats

Estrogen plays an important role in keeping vaginal tissue healthy; decreasing in estrogen levels vaginal tissue becomes atrophic-thin, dry and shrunken (Branco, Cancelo, Villero, Nohales and Julia, 2005). As *C. speciosus* extracts contained substances with estrogen-like activity, namely, β-sitosterol and diosgenin. It was of interest to investigate the effects of *C. speciosus* extracts on vaginal cytology in OVX rats. To test this, the extracts were orally administered to OVX rats and the effects were examined. The results showed that oral administration of ethanolic extract of *C. speciosus* rhizome extract at both doses (500 and 1000 mg/kg B.W.) induced vaginal corinfication in OVX rats as early as on day 14 after ovariectomy. This was also the case for *C. speciosus* stem extract (500 mg/kg B.W.). However, *C. speciosus* stem extract with a dose of 1000 mg/kg B.W. started to induce vaginal cornification on day 21 after ovariectomy. The effects of both extracts lasted until 8 weeks after

ovariectomy. The data suggested that *C. speciosus* has estrogenic effects in OVX rats and that ingestion of the plant may be useful for vaginal health benefit in menopause.

9.4 Anti-implantation Activity of *C. speciosus* Extracts in Pregnant Rats

Evidence from animal studies suggested that ingestion of large quantities of phytoestrogens can adversely affect fertility (Adams, 1995). The results of pre-implantation studies showed that *C. speciosus* rhizome and stem extracts at a dose of 1000 mg/kg B. W. significantly decreased the mean weight of the uterus ($P < 0.05$). However, no significant difference in the number of implantation sites and mean weight of the fetuses was found during post-implantation period. The results suggested that estrogenic substances such as β-sitosterol may affect the blastocyst implantation by disturbing the functional equilibrium between estrogen and progesterone as well as by increasing uterine contractility which may result in anti-implantation.

9.5 Effects of *C. speciosus* Extracts on Uterine Contraction in Non-Pregnant Rats

C. speciosus rhizome and stem extracts are potent stimulators of phasic activity in non-pregnant rat uterus as they increased spontaneous contraction with a maximum effect at 10 mg/100 mL and 30 mg/100 mL, respectively. The amplitude, frequency and AUC of the phasic contraction were significantly increased along with the basal tension. The pathways in which the extracts increased spontaneous contraction were due to the Ca^{2+}-calmodulin-MLCK pathway, but the extracts were also able to

stimulate force via SR Ca^{2+} release. Though *C. speciosus* extracts contained estrogenic-like substances such as β-sitosterol and diosgenin, they were acting via a non-estrogen mechanism on both Ca^{2+} entry and SR Ca^{2+} release. Moreover, the results suggested that the potentiation of force is not only due to the extracts acting to inhibit K^+ channels, but may also involve an inhibition of the SR Ca^{2+}-ATPase.

9.6 Effects of β-sitosterol and Diosgenin on Uterine Contraction in Non-Pregnant Rats

The most compounds found in the plant extracts were β-sitosterol and diosgenin. Thus, it was interesting to investigate whether the potentiation of force of the extracts was due to β-sitosterol and diosgenin. The results showed that β-sitosterol and diosgenin exhibited different effects on uterine contraction. β-sitosterol increased, but diosgenin decreased the contraction. Though, β-sitosterol is a key mediator of actions, it was probably not the only agent in the extracts responsible for potentiation of uterine force. Significant amounts of other phytoestrogens were also found in the extracts, and these may be contributing.

9.7 Effects of *C. speciosus* Extracts on Spontaneous Contraction in Ovariectomized Rats

Without ovarian hormones, myometrium strips taken from OVX rats were able to produce spontaneous contractions. As with non-pregnant rats, the uterine contraction of OVX rats can be stimulated by *C. speciosus* extracts. Surprisingly, the pathways in which the extracts potentiated force were similar to those observed in

non-pregnant rat uterus. These confirmed that the ovarian hormones do not regulate, but modulate uterine contraction.

In summary, the present data clearly showed that *C. speciosus* has estrogenic activity. However, the experiments were undertaken in an animal model. It would be, therefore, interesting to further investigate such the effects in human model.

9.8 References

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