ESTROGENIC ACTIVITIES OF POMEGRANATE (*PUNICA GRANATUM* L.) EXTRACT IN OVARIECTOMIZED RATS

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ฤทธิ์การเป็นเอสโตรเจนของสารสกัดจากทับทิม (*Punica granatum* L.) ในหนูตัดรังไข่

นางสาววิลาวัณย์ พร้อมพรม

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
สาขาวิชาชีววิทยาสิ่งแวดล้อม
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วิลาวัณย์ พร้อมพรม : ฤทธิ์การเป็นเอสโตรเจนของสารสกัดจากทับทิม (*Punica granatum* L.) ในหนูตัดรังไข่ (ESTROGENIC ACTIVITIES OF POMEGRANATE (*PUNICA GRANATUM* L.) EXTRACT IN OVARIECTOMIZED RATS) อาจารย์ที่ปรึกษา : ผู้ช่วยศาสตราจารย์ สัตวแพทย์หญิง ดร. ศจีรา คุปพิทยานันท์, 211 หน้า

เป็นที่ทราบกันดีว่าทับทิม (Pomegranate, *Punica granatum* L., Punicaceae) มีเอสโตรเจน (estradiol estrone และ estriol) เป็นองค์ประกอบและแสดงฤทธิ์การเป็นเอสโตรเจนในหนูถีบจักร ยิ่งไปกว่านั้นในเมล็ดทับทิมยังเป็นแหล่งของไฟโตเอสโตรเจน วัตถุประสงค์ของการวิจัยมีดังนี้ 1) เพื่อศึกษาผลของสารสกัดจากทับทิมต่อระดับซีรั่มเอสโตรเจน การปกป้องกระดูกรวมถึงความหนาแน่นของกระดูก ระบบสืบพันธุ์ (น้ำหนักมดลูก เซลล์ของช่องคลอด และการพัฒนาของเต้านม) ระดับไขมัน (ไลโปโปรตีนความหนาแน่นต่ำ ไลโปโปรตีนความหนาแน่นสูง และไตรกลีเซอร์ไรด์) 2) เพื่อทดสอบผลของสารสกัดในการต่อต้านการฝังตัวของตัวอ่อน และ 3) เพื่อทดสอบผลของสารสกัดจากทับทิม ต่อการหดตัวของกล้ามเนื้อเรียบมดลูก และเปรียบเทียบผลของสารสกัดกับ β-sitosterol พร้อมทั้งศึกษากลไกการออกฤทธิ์ โดยใช้หนูทดลอง 8 กลุ่ม (กลุ่มละ 6-10 ตัว) ดังนี้ หนูที่ไม่ตัดรังไข่ หนูตัดรังไข่ หนูตัดรังไข่ที่ได้รับฮอร์โมนเอสโตรเจน 2 ขนาดในปริมาณที่แตกต่างกัน (0.17 และ 0.7 มก./กก. ต่อน้ำหนักตัว) หนูตัดรังไข่ที่ได้รับสารสกัดจากเมล็ดและเปลือกทับทิม 2 ขนาดในปริมาณที่แตกต่างกัน (100 และ 1000 มก./กก. ต่อน้ำหนักตัว) ทำการทดลองเป็นระยะเวลา 2 เดือน สารสกัดจากทั้งเมล็ดและเปลือกทับทิม ทำให้มดลูกมีน้ำหนักเพิ่มขึ้น เหนี่ยวนำให้เยื่อบุช่องคลอดหนาขึ้น และทำให้ผนังเยื่อบุมดลูกแบ่งตัว ส่วนในเต้านมนั้นพบว่าสารสกัดจากเมล็ดและเปลือกทับทิม สามารถเพิ่มจำนวนท่อของเต้านม สารสกัดจากเมล็ดและเปลือก ทับทิมในขนาด 1000 มก./กก. ต่อน้ำหนักตัว มีแนวโน้มที่จะเพิ่มความหนาแน่นของกระดูก ในการศึกษาครั้งนี้ ไม่สามารถเปรียบเทียบผลของสารสกัดจากเมล็ดและเปลือกทับทิมต่อระดับไขมันในเลือดได้ เพราะข้อมูลที่ได้ให้ผลตรงข้ามกับทฤษฎีที่เคยระบุไว้ นอกจากนี้ยังพบว่า สารสกัดจากเมล็ดและเปลือกทับทิมมีฤทธิ์ต้านการฝังตัวของตัวอ่อน

เมื่อศึกษาผลของสารสกัดจากเมล็ดและเปลือกทับทิมต่อการหดตัวของมดลูก พบว่า สารสกัดจากเมล็ดและเปลือกทับทิมสามารถเพิ่มการหดตัวของมดลูกตามลำดับความเข้มข้น โดยมีฤทธิ์สูงสุดที่ความเข้มข้น 250 มก. /100 มล. และ 70 มก. /100 มล. ตามลำดับ

สาขาวิชาชีววิทยา ลายมือชื่อนักศึกษา _____

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ลายมือชื่ออาจารย์ที่ปรึกษ ารว่ม _____

WILAWAN PROMPROM : ESTROGENIC ACTIVITIES OF POMEGRANATE (*PUNICA GRANATUM* L.) EXTRACT IN OVARIECTOMIZED RATS. THESIS ADVISOR : ASST. PROF. SAJEERA KUPITTAYANANT, Ph.D. (DVM), 211 PP.

POMEGRANATE/*PUNICA GRANATUM* L./ESTROGENIC ACTIVITY/ UTERUS/ MAMMARY GLAND/VAGINA/OVARIECTOMIZED RATS

Pomegranate (*Punica granatum* L., Punicacease) is known to contain estrogens (estradiol, estrone, and estriol) and show estrogenic activities in mice. In addition, pomegranate seed is a rich source of phytoestrogens. The aims of the study were therefore; 1) to investigate the effects of the pomegranate extract on serum estrogen level, bone protection including bone mineral densitometry, reproductive actions, (uterine weight, vaginal cytology, mammary gland development), lipid profile (low-density lipoprotein, high-density lipoprotein, and triglycerides), 2) to test the effects of the pomegranate extract on anti-implantation, and 3) to examine the effects of the pomegranate extract on contraction and compare its effect to a known compound such as β-sitosterol. The underlying mechanism of the extract was investigated. Rats were divided into eight groups (n=6-10); sham operated rats received vehicle (10% v/v Tween 80, p.o.), ovariectomized rats received vehicle (10% v/v Tween 80, p.o.), ovariectomized rats received 17β-estradiol at the different doses (0.17 or 0.7 mg/kg B.W. (s.c.)), and ovariectomized rats received methanolic extracts of the pomegranate seed or peel at the different doses (100 and 1000 mg/kg B.W. (p.o.)). These rats were administrated daily for 2 months. In ovariectomized rats, the

pomegranate seed and peel extracts produced an increase in uterine wet weight and induced cornification of the vagina and proliferation of the uterine endometrium. Increases in interlobular ducts were found in the mammary glands of pomegranate seed and peel extracts administrated rats. The effects of pomegranate seed and peel extracts on bone mineral densities measured using dual energy x-ray absorptiometry showed that pomegranate seed and peel extracts (1000 mg/kg B.W.) had a tendency to increase bone mineral densities. In this study, the effects pomegranate seed and peel extracts on lipid profile were difficult to explain because the data suggest the opposite theory. In addition, the pomegranate seed and peel extracts exerted anti-implantation activity. The effect of pomegranate seed and peel extracts on uterine contractility revealed increases in spontaneous contractions in concentration-dependent manner with the maximum effect of 250/100 mL and 70 mg/100 mL, respectively.

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LIST OF ABBREVIATIONS

˚C	=	degree Celsius
BMD	=	bone mineral densitometry
LDL	=	low density lipoprotein
HDL	=	high density lipoprotein
FSH	=	follicle stimulating hormone
LH	=	luteinizing hormone
E_1	=	estrone
E_2	=	estradiol
E_3	=	estriol
P_4	=	progesterone
GC/MS	=	gas chromatography/mass spectrometry
OVX	=	ovariectomized
L	=	leukocyte cells
O	=	nucleated cell
Co	=	cornified cell
B.W.	=	body weight
HRT	=	hormone replacement therapy
DXA	=	dual energy x-ray absorptiometry
ERT	=	estrogen replacement therapy
CHD	=	coronary heart disease

LIST OF ABBREVIATIONS (Continued)

TC	=	total cholesterol
mg/Kg	=	milligram/kilogram
g	=	gram
mg	=	milligram
MLCK	=	myosin light chain kinase
MLCP	=	myosin light chain phosphatase
MLC_{20}	=	regulatory 20-kDa of myosin light chain
Ca^{2+}	=	calcium ion
Ca-ATPase	=	calcium adenosine triphosphatase
$[Ca^{2+}]$	=	calcium concentration
$[Ca^{2+}]_i$	=	cytosolic calcium concentration
$CaCl_2$	=	calcium chloride
CaM	=	calmodulin
CICR	=	calcium-induced calcium release
Cl	=	chloride
CO_2	=	carbon dioxide
DMSO	=	dimethy sulfoxide
HEPES	=	N-2-hydroxyethylpipeperazine-N-2-
s.c.	=	subcutaneous injection
p.o.	=	per os (via oral)
hr	=	hour

LIST OF ABBREVIATIONS (Continued)

IP_3	=	inositol (1, 4, 5)-tris-phosphate
L	=	liter, volume
L-Type	=	L-Type calcium channel
T-type	=	T-Type calcium channel
min	=	minute, time
mL	=	milliter,volume
mM	=	millimolar, concentration
mol	=	moles
n	=	sample size
Na	=	sodium
%	=	percent
O_2	=	oxygen
pH	=	-log of hydrogen concentration
S.E.M	=	standard error of the mean
SR	=	sarcoplasmic reticulum
Ry	=	ryanodine
µl	=	microliter
µm	=	micrometer
µM	=	micromolar
wt	=	weight
v/v	=	volume : volume
AUC	=	area under the curve

LIST OF ABBREVIATIONS (Continued)

TEA	=	tetraethylammonium
Y-27632	=	ROK inhibitor

CHAPTER I

INTRODUCTION

1.1 Biology of Menopause

1.1.1 Causes of Menopause

Menopause is defined as the permanent cessation of menstruation that results from loss of ovarian follicular activity, after at least 12 month of amenorrhea. The average age of menopause is between 51 and 52 years, with a range of 40-60 years. (Mishell et al., 1997). Median age at the menopause is currently around 50 in Western industrialized societies (in Britain it is 50.78 years, in the United States 49.8 years and in White South Africans 48.7 year). It occurs earlier in Black women (Ginsburg, 1991). Thai women reach menopause at 47.3 years (Chirawatkul, 1993; Chompootweep et al., 1993; Department of Health, 1996). In addition to race, nutrition and smoking influence the age at menopause. It has been suggested that age at menopause may be a biological marker of ageing, later menopausal age being associated with longevity (Snowden, 1990).

The biological bases of the menopause are changes that occur in the structure and function of the ovaries. The ovaries also produce the hormones estrogen and progesterone, which regulate menstruation and ovulation. The number of ovarian granulose cells available for hormone secretion appears to be a critical determinant of age at menopause. The supply of oocytes is finite: about 7 million germ cells can be found in the ovaries of the human fetus at the fifth month of intrauterine life but these

cells do not thereafter divide. The rate of follicle decline is approximately linear on a semi logarithmic scale until an age of about 35-40 years. It accelerates thereafter until after the menopause, when essentially no follicles remain (Richardson et al., 1987).

As women approach the menopause, menstrual cycles become irregular. The progressive shortening of the cycle is caused by shortening of the follicular rather than the luteal phase (Lenton et al., 1984). About 25% of women aged 40-45 years and 40% in the 45-50 age group have anovulatory cycles. An important consequence of the increasing number of anovulatory cycles is unopposed oestrogenic stimulation of the uterus which underlies increased incidence of endometrial hyperplasia that occurs at this time.

During the menopausal transition, hormonal levels are variable and unpredictable, and endocrine assessment of ovarian function of poor predictive value with respect to timing of the menopause (Burger, 1994). All possible combinations of hormonal patterns can be observed during this phase. The menopausal transition refers to the time of the perimenopause prior to the last menstrual period, and is a transition phase from fertile ovulatory cycles with well-characterized hormonal profiles to the postmenopause with low estrogen, progesterone and high gonadotrophin follicle-stimulating hormone (FSH), luteinizing hormone (LH) levels. In order to explain the pathophysiological changes seen at the time of the menopause, it is necessary to explain the function of the hypothalamic-pituitary-ovarian axis in premenopausal women. The hypothalamus secretes gonadotrophin-releasing hormone in a pulsatile fashion, which regulates the pituitary gland. In response to this, the pituitary produces gonadotrophins, namely FSH and LH. These hormones are responsible for the regulation of ovarian function.

A number of terms including “climacteric”, “perimenopause”, “menopausal transition”, “postmenopause” and “menopause” have been used to refer to the stages of reproductive ageing. The terms “menopausal transition” and “perimenopause” were recommended for use in place of the term “climacteric” in 1996, with release of the definition by the World Health Organization (WHO). The menopausal transition begins with variations in menstrual cycle length and a monotropic rise in FSH; no associated increase in LH, and ends with the final menstrual period, classically confirmed only when followed by 12 months of amenorrhea. The perimenopause, which literally means “about or around the menopause,” begins at the same time as the menopausal transition and ends 1 year after the final menstrual period. The median age at the final menstrual period is 51.4 year (Mckinlay, 1992). Although, these two terms were initially used interchangeably, there is a slight difference in their definition according to the Stages of Reproductive Ageing Workshop (STRAW) proposed a system that divides female reproductive ageing into five stages before the final menstrual period, and two afterwards (Soules, 2001). Three stages (early (-5), peak (-4) and late (-3) reproductive describe the years before the perimenopause. Stage-3 is characterized by regular cycles but elevated levels of FSH in the follicular phase. Stage -2 (the early menopause transition) is characterized by variable cycle length. Stage -1 (late transition) is characterized by two or more stopped cycles and 60 or more days of amenorrhoea. Stage +1 is the first 5 years after the final menstrual period. Stage +2 is the late postmenopause.

The normal pituitary of hypothalamic gonadotrophin-releasing hormone, the normal pituitary secretes FSH and LH in a cyclical pattern, characterized particularly by an early follicular phase rise in FSH, a mid-cycle peak of both

gonadotrophins, and relatively low levels of both during the luteal phase. Follicular-phase FSH drives ovarian production of estradiol, increasing levels of which in the late follicular phase trigger the mid-cycle LH surge. After ovulation, the granulose cells of the dominant follicle are luteinized to form the corpus luteum, which is the source of progesterone. The granulosa cells are also the source of the ovarian inhibins A and B. Inhibin A is a product of the dominant follicle. Its levels are high mid-cycle, and concentrations are also high during the luteal phase, when it is produced by the corpus luteum. Inhibin B is a product of the granulose cells of the cohort of antral follicles from which the dominant follicle is derived. Its levels are high in the early follicular phase; after a mid-cycle peak, it decreases throughout the luteal phase (Groome, 1996).

Levels of serum FSH during the follicular phase increase progressively with age in women who continue to have regular cycle, particularly in those over the age of 40-50 years. The concentration of inhibin B changes little until the age of 40 years, when there is a decline that is inversely correlated with the increasing FSH. The menopausal transition is a time of marked hormonal instability. Repetitive sampling in an individual woman may show various patterns, with high or low FSH, estradiol and inhibin. In a population of women, the major changes around the final menses are progressive increase in FSH and a progressive decrease in estradiol. The postmenopausal state is characterized by elevated FSH and LH, low estradiol and progesterone, and well-preserved levels of testosterone.

1.1.2 Menopause Symptoms

Menopausal symptoms affect about 70% of women approaching menopause. Typical menopause symptoms, such as hot flashes or night sweats are caused by changing hormonal levels in the female reproductive system. The symptoms of menopause can be divided into early and late onset symptoms. Early symptoms include abnormal vaginal bleeding, hot flashes, and mood changes. Late symptoms include vaginal dryness and irritation, osteoporosis, coronary heart disease, and Alzheimer's disease. Many symptoms are associated with the menopause, but the two that are usually the most significant and therefore most distressing to women are the hot flush, which often leads to insomnia, and vaginal dryness. These symptoms are directly related to a decrease in estrogen levels and experienced by over 70% of women. Most menopausal symptoms can be classified into either physical or psychological symptom in nature. The menopause is classically associated with the onset of vasomotor symptoms, which include hot flushes and night sweats. Other physical symptoms include palpitations, headaches, bone and joint pain, asthenia, tiredness, and breast tenderness. The determinants of experiencing menopausal symptoms are complex, representing biological, psychological and social factors.

Hot flushes are the most common symptom of the climacteric and occur in 75% of postmenopausal women, although only 30% of women seek medical help (Belchetz, 1994). Hot flushes tend to last longer and be more severe in women who have had a surgically induced menopause (Bachmann, 1999). They tend to occur most often in the first year after the final period and can occur at any time of day or night. Despite multiple theories, the exact pathophysiology of the hot flush is not yet known. It is postulated that they are a result of a central disorder of temperature

regulation and that the hypothalamus is pivotal as hot flushes have been recorded in patients with pituitary insufficiency. It is recognized that hot flushes occur with the pulsatile release of LH (Rebar, 1987). The symptoms are characteristic of a heat dissipation response and consist of sweating on the face, neck and chest as well as peripheral vasodilatation (Freedman, 2001).

Urogenital symptomatology is the most prevalent consequence of the menopause, and affects at least 50% of postmenopausal women (Milsom, 1998). Estrogen receptors have been located in the vaginal walls of both pre and postmenopausal women (Chen, 1999). At the time of the menopause, there are many changes in the vaginal area. These include decreased blood flow and a reduction in the elasticity and distensibility of the vaginal walls that can in turn, cause dyspareunia (Pandit, 1997). The epithelium becomes less cellular and more easily traumatized which can result in postmenopausal bleeding. There is a loss of cellular glycogen and decreasing lactic acid, which results in the vaginal pH changing from a more acidic environment (pH 4-5) in the premenopausal state to a more alkaline environment (pH 6-8) in the postmenopausal state. This can increase susceptibility to pathogenic invasion (Pandit, 1997; Melis, 2000). All of these changes can cause vaginal irritation, dryness, burning, and itching (Willhite, 2001).

Irregular vaginal bleeding and menorrhagia are often pathopneumonic of the menopause. These symptoms are due to the depletion of the ovarian follicle pool leading to an increase in the number of anovulatory cycles.

Palpitation is an unpleasant awareness of an abnormal beating of heart. This symptom may be brought on by a variety of cardiac disorders, such as cardiomyopathy, valvular heart disease and coronary artery disease. Several non-

cardiac disorders may also cause palpitations, and in this case are an effect of the disease upon cardiac rhythm. Palpitations occur frequently in perimenopausal woman, are usually benign and seem to be related to the increased sympathetic activity caused by the menopause (Rosano, 2000).

Women have more migraines than men. Menses, pregnancy and menopause affect the frequency and treatment of headaches. The mechanisms that underlie sex-related differences in the prevalence of these conditions remain obscure and are likely to involve both physiological and psychosocial factors. In terms of physiological factors relevant to the development of headaches, direct evidence of sex-related differences in the properties of dural afferent fibres or durally activated second-order trigeminal sensory neurons has yet to be provided.

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. There is a higher incidence of depressive illness in women than in men, and this is exacerbated during the perimenopause (Montgomery, 1987). There is a lack of evidence that these symptoms are directly due to estrogen deficiency. However, there are estrogen, progesterone and testosterone receptors in a number of centres of the brain, so it is possible that hormone deficiency at the time of the menopause could induce psychological symptoms.

1.1.3 Hormone Replacement Therapy

Hormone Replacement Therapy (HRT) is the use of synthetic or natural female hormone to make up for the decline or lack of natural hormones produced in

woman's body. HRT is sometime referred to as estrogen replacement therapy (ERT), because the first medications that were used in the 1960s for female hormone replacements were estrogen. Furthermore, HRT has a proven track record of preventing osteoporosis (Recker, 1999). Estrogen replacement therapy may also bestow some cardiovascular benefits for some women. However, recent studies have shown an increase in coronary events during the initial year of HRT in women with history myocardial infarction or atherosclerosis (Alexander et al., 2001; Grodstein, 2001).

Hormone replacement therapy is used to supplement the body with either estrogen alone or estrogen and progesterone in combination during and after menopause. Estrogen and progesterone are hormones that are produced by a woman's ovaries. When the ovaries no longer produce adequate amounts of these hormones (as in menopause); HRT can be given to supplement the body with adequate levels of estrogen and progesterone.

The hormones used in HRT are estrogen and progestogen (synthetic progesterone). Prolonged use of unopposed estrogen can cause endometrial hyperplasia and carcinoma; hence, women with a uterus should be given estrogen in combination with some form of progestogen for endometrial protection. Women who have undergone hysterectomy can be prescribed estrogen therapy alone.

The estrogen contained in most HRT preparations (estradiol, estrone, estriol) are "natural estrogens", so-called because they give rise to plasma estrogen identical to those produced by the premenopausal ovary.

Only a very few HRT preparations contain the more potent but less expensive synthetic estrogens ethinylestradiol and mestranol, as used in the combined contraceptive pill.

Most HRT users in the UK take oral estrogen therapy, which is convenient to use, is inexpensive, and can be changed easily if problems arise. Oral estrogens can be associated with mild gastrointestinal side-effects (nausea, abdominal cramps), however, and, because of the first-pass effect through the liver, a high percentage is metabolized rapidly and inactivated before achieving any systemic effect.

Subcutaneous estrogen implants are often prescribed at the time of hysterectomy and oophorectomy. They comprise biodegradable crystalline pellets of estradiol that release the hormone slowly over several months. Several doses are available, and a testosterone implant may be inserted simultaneously to improve libido. Insertion is performed under local anaesthesia, usually into the anterior abdominal wall and repeated after 6-8 months when symptoms have returned.

Most vaginal estrogen preparations provide low-dose therapy to improve urogenital symptoms with minimal systemic absorption. If a high dose is given, vaginal estrogens are absorbed systemically. Low-dose topical vaginal estrogens can be used safely in women with contraindications to systemic HRT. An intravaginal ring that delivers systemic estradiol is also marketed for treatment of menopausal symptoms in hysterectomized women.

Progestogens

Progestogens can be delivered via a hormone-releasing intra-uterine system with systemic estrogen as part of an HRT regimen. The intrauterine progestogen causes atrophy of the endometrium, induces amenorrhoea and reduces systemic

progestogenic side effects (Raudoskoski, 1995). Progestogens-releasing intrauterine systems are widely used for contraceptive purposes and are now licensed in the UK.

Side effects of estrogen

Low-dose estrogen replacement therapy has relatively few side-effects. Breast tenderness, leg cramps at night, nausea and mild fluid retention are the most common. Women starting HRT many years after their natural menopause are likely to experience more hormonal side-effects than those starting HRT while still menstruating. Estrogenic side-effects can often be improved by reducing the estrogen dose or using an alternative route of administration.

Women usually attribute weight gain to HRT, though there is little scientific evidence to support this. Most women tend to gain weight as they take HRT or not. With improved general well-being when taking HRT, many women are motivated to lose weight.

Side effects of progestogens

Many women experience a return of unpleasant side-effects similar to premenstrual syndrome while taking the progestogen phase of sequenstrual syndrome while taking the progestogen phase of sequential HRT preparations. This manifests particularly as mood swings, irritability and depression; physical symptoms include breast tenderness, fluid retention and abdominal bloating. These symptoms can be a major disincentive to taking HRT, but can often be helped by changing the type of progestogen and prescribing it at the lowest possible dose for the fewest number of day (10-12 days). There is wide individual variation in tolerance to progestogens and if side-effects are problematic, several changes of preparation may be necessary to find one that is suitable. Women who take continuous combined estrogen and

progestogen combinations often tolerate the progestogen better, despite, taking a greater overall dose than with sequential preparation.

1.2 Estrogen and Hormone Replacement Therapy

1.2.1 Estrogen and Its Function

Estrogen is not one hormone; it is the name of a group of hormones. There are three principle forms of estrogen found in the human body estrone, estradiol and estriol, also known as E_1, E_2 and E_3, respectively. There is also a group of compounds called phytoestrogens, generally found in food, which can "estrogen like" effects in the body. Estradiol (E_2) is the primary estrogen produced by the ovaries. Estrone (E_1) is from estradiol. It is a weak estrogen and is the most abundant estrogen found in the body after menopause. Estriol (E_3) is produced in large amounts during pregnancy and is a breakdown product of estradiol. Estriol is also a weak estrogen and may have anti-cancer effects. Before menopause estradiol is the predominant estrogen. After menopause estradiol levels drop more than estrone so that now estrone is the predominant estrogen. The most important of which in humans are 17β-estradiol, estrone and estriol. The dominant form of estrogen in body is 17β-estradiol. They are synthesized and secreted by the ovaries under the control of the pituitary gonadotropin and follicle stimulating hormone (Gard, 1998).

Estrogen plays an important role in the growth, differentiation and development of primary sexual characteristic such as the uterus, ovaries and vagina. The estrogens are also responsible for development of the female secondary sex character, such as the breasts and for regulation of reproductive cycle (Randall, 1997). Moreover, estradiol also has a variety of pharmacological functions such as

maintenance of bone mass, cardiovascular function and brain protection (Smith, 1994; Ciocca and Roig, 1995).

Estrogen replacement is frequently the treatment of choice for maintaining reproductive function and bone mineral density in post-menopausal women.

1.2.2 Effect of Estrogen on Bone Mass

Estrogen plays an important role in the growth and maturation of bone as well as in the regulation of bone turnover in adult bone. During bone growth estrogen is needed for proper closure of epiphyseal growth plates both in females and in males. Also in young skeleton estrogen deficiency leads to an increased osteoclast formation and an enhanced bone resorption. In menopause, estrogen deficiency induces cancellous as well as cortical bone loss. Highly increased bone resorption in cancellous bone leads to general bone loss and destruction of local architecture because of penetrative resorption and microfractures. In cortical bone the first response of estrogen withdrawal is enhanced endocortical resorption. Later, also intracortical porosity increases. These lead to decrease bone mass, disturbed architecture and reduced bone strength. At cellular level in bone, estrogen inhibits differentiation of osteoclasts thus decreasing their number and reducing the amount of active remodeling units. This effect is probably mediated through some cytokines, IL-1 and IL-6 being strongest candidates. Estrogen regulates the expression of IL-6 in bone marrow cells by a so far unknown mechanism. It is still uncertain if the effect of estrogen on osteoblasts is director is due to coupling phenomenon between bone formations to resorption (Kalervo, 1996).

Estrogen replacement therapy is effective in reducing postmenopausal bone loss, and decreases fracture risk (Hutchinson et al., 1979; Grady et al., 1992; Mack et al., 1989). Recently, estrogen has been reported to decrease the synthesis of cytokines such as interleukin-1 and-6 both of which stimulate bone resorption (Jilka et al., 1992; Horowitz, 1993). In addition, estrogen has a capability to inhibit osteoclastogenesis and increase the rate of apoptotic osteoclast death as well (Hughes et al., 1995). In osteoblasts, estrogen can stimulate the release of TGF-β which is a cytokine for osteoblastic inhibition (Oursler et al., 1991; Turner et al., 1994). Estrogen also increases the expression of the receptors for 1, 25 $(OH)_2D_3$ growth hormone, progesterone, and modulates PTH responsiveness in osteblastic cells (Compston et al., 2001). Bone consists of a matrix with embedded cell such as osteoclasts and osteoblasts. Osteoclast cells break down bone, whilst osteoblasts cells form new bone tissue. When the activity of both osteoblasts and osteoclasts is equal (or coupled), the amount of bone, or bone mass, remains constant. If bone density falls below a certain threshold, the risk of fracture is high. Bone loss is particularly notable in women at the menopause. When oestrogen production ceases, and excess loss is associated with increased risk of osteoporosis. There are many factors that influence the rate of age-related bone loss (Goulding, 2000).

Interestingly, phytoestrogens are non-steroidal compound naturally occurring in a wide range of foods of plant origin. They are able to ‘compete’ with or mimic the main circulating oestrogens of most mammals. The phytoestrogens which are known to bind to the estrogenic receptor sites of the cell and trigger the components and processes of estrogenic activity have a promising role in the treatment of osteoporosis (Adams, 1998). The isoflavones found in soybeans, such as

genistein, were found to prevent bone loss in the ovariectomized rat model of osteoporosis (Bahram et al., 1996). Furthermore, phytoestrogens might have beneficial effects on bone metabolism and osteoporosis but the evidence from experimental and observational studies are very limited (Knight, 1996).

Potter et al. (1988) reported the effects of soy protein and phytoestrogen on BMD in postmenopausal women. In this study the group taking soy protein, with associated high concentrations of isoflavone, significantly increased both bone mineral density and content in the lumbar spine, but not in other skeletal areas, as compared to group consuming casein dry milk. In addition, pomegranate extract can improves a depressive state and protect bone loss in menopausal syndrome model ovariectomized mice (Junko, 2004).

1.2.3 Effect of Estrogen on Lipid Metabolism

Menopause is a normal biological event associated with depletion of functional ovarian follicles that are the source of oestradiol production. Accodingly there is a marked decrease in oestradiol levels. So, that the major anti-atherosclerotic effect of estrogen is associated with its beneficial influence on lipid metabolism, including increased high-density lipoprotein (HLD), decreased low-density lipoprotein (LDL), lipoprotein and total cholesterol concentrations (Seed and Crook, 1994). Estrogen is associated with elevations in HDL cholesterol, especially HDL, by up to 20% (Pasty et al., 1993) and reduction in LDL cholesterol by up to 19% and apolipoprotein A-I increases by 13% to 22% (Nabulsi et al., 1993). In addition, serum triglyceride levels are elevated by 16% to 42%. Although a mild reduction in total apolipoprotein B levels has been reported, large very low density (VLDL)

apolipoprotein B levels increase by ≈ 30%. The mechanism appears to be increased production rather than decreased clearance. Oral estrogens appear to have effects on VLDL and LDL cholesterol, but transdermal estrogen do not; and although HDL levels increase with transdermal estrogen. This effect has been postulated to be due to supraphysiologic concentration of estrogen in the portal circulation after intestinal absorption, leading to alterations in hepatic metabolism of lipids (Walsh et al., 1991).

Soma et al. (1993) found a 50% reduction in lipoprotein with the combination therapy, a 30% reduction in LDL and a 19% increase in HDL. Similar findings were reported in a recent study by Nabulsi et al. (1993). The elevations in HDL, HDL_2, HDL_3, and apolipoprotein A-I and the reduction in LDL, apolipoprotein B and lipoprotein were similar between estrogen, combined estrogen and progesterone. To address these issues objectively, the postmenopausal estrogen/progestin intervention trial, a randomized, controlled trial of conjugated estrogen with and without the addition of progesterone (medroxyprogesterone or micronized progesterone) was undertaken. This 3-year study of 875 postmenopausal women found significant elevations in total HDL cholesterol with all regimens, although the combination therapy significantly attenuated this increase. The average increase in HDL levels was 5.6 mg/dl in the estrogen group and 1.2 to 4.1 mg/dl in the progesterone group. The HDL subtypes were not evaluated. The LDL cholesterol was lowered to the same degree in all active treatment groups (14.5 to 17.7 mg/dl). The increase in triglyceride levels (11.4 to 13.7 mg/dl) did not differ significantly between estrogen and combination therapy.

In addition to lowering lipid levels, estrogen (but not progesterone or testosterone) also demonstrates marked antioxidant properties. At high local

concentrations, 17-beta-estrdiol has been found to inhibit LDL oxidation and reduce cholesterol ester formation in vitro preparation (Negre et al., 1993).

However, effects of phytostrogens on lipid profiles, vascular reactivity, thrombosis and cellular proliferation have been reported. When patients with type II hyperlipoproteinemia (mean TC 409 mg/dL) were placed on high soy diets for four week, the total cholesterol and LDL decreased by 16% and soy protein consumption in humans revealed an improvement in total cholesterol by 9% and LDL by 13%, as well as a decrease in triglyceride levels of 10% (Anderson and Johnstone, 1995). In fact, soy bean isoflavones have reported to prevent cardiovascular disease (Potter, 1998).

1.2.4 Effect of Estrogen on Female Reproductive Organ

Estrogen is known as a "female hormone" because it plays a key role in shaping and preparing it for uniquely female function such as pregnancy. For example, estrogen is for the development of breast and hips. In addition, the vagina, uterus and mammary glands depend on the presence of estrogen in the body to mature. The most potent naturally occurring estrogen in humans is E_2, E_1 and E_3. Estradiol is the predominant estrogen during the premenopausal period, and is mainly secreted by the ovaries. After menopause, the main estrogen is estrone. Estrone is synthesized in adipose tissues from adrenal dehydroepiandrosterone. During pregnancy, estriol is produced in large quantities by the placenta. Estradiol occurs in all mammals, regardless of sex or age; its function, however, is related to control of female reproduction through cyclic release of anterior pituitary hormones and to cyclic changes of the female reproductive tract. During pregnancy, estradiol

contributes to uterine growth, placental development, parturition, and the development of the mammary gland. In particular, the ovaries stop producing oestrogen and progenterone and there is an increased production of gonadotropin hormones such as LH and FSH. Alteration of these hormones can cause a wide variety of vasomotor, vaginal and psychological symptoms and diseases including tissue atrophy, sexual dysfunction, impaired sleep and emotion disturbances. Currently, hormone replacement therapy is commonly used to combat the symptoms and diseases associated with decreasing estrogen and progesterone levels. However, HRT is associated with adverse effects and an increased risk of endometrial or breast cancer. Many women are therefore against HRT and choose to phytoestrogen for female body with symptoms arising primarily from the loss of estrogen.

Chansakaow et al. (2000); Ingham et al. (2002); and Malaivjitnond et al. (2004) reported the effects of *Pueraria mirifica*, a Thai herb, belongs to the same family of soybean and *Pueraria lobata.* Its tuberous root was found to contain at least 13 known phytoestrogen. The estrogenic effect of *P. mirifica* were exhibited in various reproductive organs, that is; induced vaginal cornification and increased uterine weight in ovariectomized rats, prolongation of the menstrual cycle in mature female monkeys and alleviation of menopausal symptoms in women (Trisomboon, 2004).

Einer et al. (1998) investigated the oestrogenic effects of *Cimicifuga racemosa* on uterine growth in immature mice and on vaginal cornification in ovariectomized rats. Vehicle (negative control), estradiol-benzoate (positive control, 0.4 mg/kg) and a commercially available extract of *C. racemosa* (rhizome of *C. racemosa* extracted with a 50% water/ethanol mixture, 6-600 mg/kg) were

administered for three days. Estradiol-benzoate increased significantly the average wet weight of the mouse uteri. In contrast, no signs of uterotrophic or vaginotrophic effects were found in the mice and rats treated with the extra. Eagon et al. (1997, 1999) found that an extract of *C. racemosa* obtained from the root (not rhizome) of the plant administered to ovariectomized rats for three weeks, increased the uterine growth. Also, pomegranate seed oil and extracts might be employed in menopausal women as external and internal phytoestrogen medicaments, as a possible alternative or supplement to conventional hormone replacement therapy (Lansky, 1999). In addition, mixtures of pomegranate seed, juice and peel products paradoxically have been reported not only prevent abortion (Ramirez et al., 1988), but also conception (Gujral et al., 1960). However, such studied were performed on pomegranates grown India.

1.3 Alternative Therapies in Menopause

Hormone replacement therapy has traditionally been used for treatment of menopausal disorders. However, not all women can, or prefer to, take HRT. Despite the potential health benefits of estrogens, commonly conjugated equine estrogens in the United States, and 17β-estradiol in Europe, estrogens have the disadvantage of being tissue agonists for breasts and endometrial tissues. Adding progestin to estrogen unwanted side-effects, i.e. vaginal bleeding, and bloating and depression. Side effects such as cholelethiasis, breast tenderness, mood changes and venous thromboembolism may result from conventional HRT. Thus, alternative therapies, which include natural products such as phytoestrogens and herbs, as well as raloxifene, aselective estrogen receptor modulator (SERM), offer attractive options. Alternative therapies may

protect against breast and endometrial cancer, obviate the need for progestin, have fewer side-effects and still provide health benefits.

1.3.1 Phytoestrogen-rich Herbs

Phytoestrogens are compounds found in plants and foods, with estrogen-like biological activity. They are generally considered safe for long-term daily use for all people, including pregnant and lactating women. Since phytoestrogens are soluble in water, vinegar, and alcohol, they are easily extracted from herbs. Their main classes are: isoflavones, coumestans and lignans. Soybeans and soy foods are the most significant dietary sources of isoflavones, as sprouts of clover and alfalfa are of coumestans, and oilseed, such as flaxseed, are of lignans.

Plants contain a wide variety of naturally occurring chemicals. Many of these chemicals were named “phytoestrogen” because “phyto” which means plant was combined with “estrogen” due to their estrogenic activity. They are non-steroidal naturally occurring phenolic compounds that can be divided into two groups: firstly, the flavonoids that are further subdivided into isoflavones, coumestans and prenyl flavonoids; and secondly the non-flavonoids, comprising the lignans (Figure 1.1).

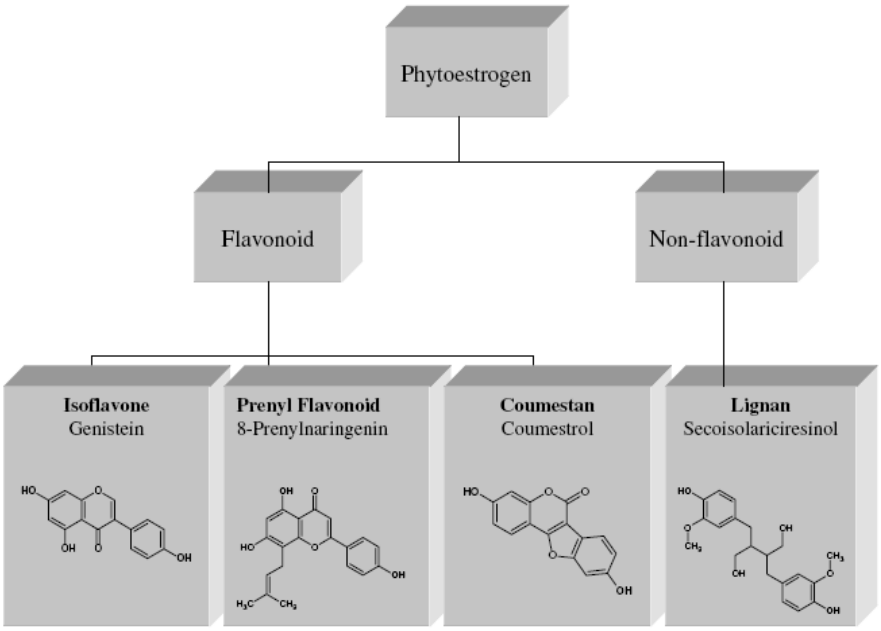

Figure 1.1 The different classes of phytoestrogens (Martin, 2007).

All are polyphenols that have a structural similarity to estradiol (Figure 1.2) and possess estrogenic activity due to having a similar "A" ring to that of estradiol and possessing two hydroxyl groups (shown in bold in Figure 1.1) at positions that afford the correct distance between facilitate binding to the estrogen receptor (Zand et al., 2000). The isoflavone phytoestrogen share a common structure (Figure 1.2), with genistein having the important-OH groups at positions 7 and 4. Biochanin A has a methoxy group at position 4 and prunetin has a methoxy group at position 7 resulting in less estrogenic activity as the methoxy groups hinder binding to the estrogen receptor. In quercetin the "B" ring is attached to position 2 and there is an OH group at position 3. Virtually all fruits and vegetables are known to be rich in phytoestrogen.

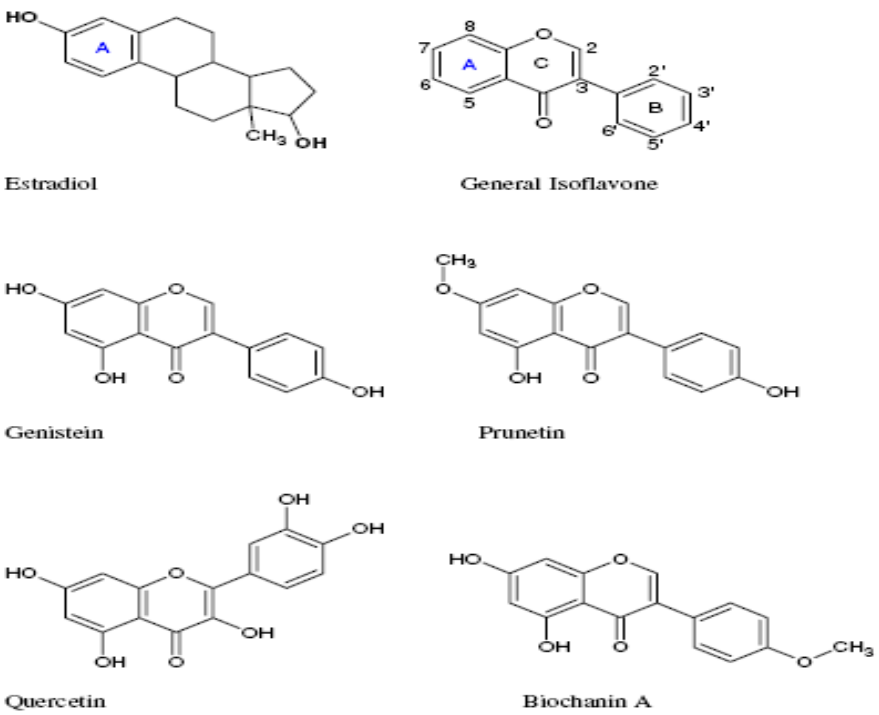

Figure 1.2 The structure of estradiol and selected phytoestrogen (Martin, 2007).

Some phytoestrogen-rich herbs and food, however, contain other constituents that may make them unsafe for daily or long-term use. Soybeans, for instance, contain phytoestrogens that can influence menstrual cycles (Cassidy, 1994).

Black cohosh (*Cimicifuga racemosa*) has been studied intensively, but no specific active ingredient has been isolated. The beneficial effects of taking a daily dose of 1-2 ml of black cohosh root tincture appear slowly, reaching a maximum in 4 weeks. Black cohosh tincture seems to reduce secretion of LH (Tyler, 1994). Side effects from capsules and teas are common and may include dizziness, headache, nausea, and visual disturbances. Black cohosh may increase menstrual; it is considered unsafe for pregnant women and is not recommended for daily use.

In the general population, black cohosh has been shown to be an effective treatment for hot flashes. There is some evidence it can be as estrogen replacement therapy for some patients. Often significant improvements in symptoms appear within 4 weeks (Lieberman, 1998; Lehmann, 1998). Laboratory evidence of estrogenic activity has been conflicting. Whether black cohosh has an estrogen-like beneficial effect on osteoporosis is uncertain (Einer, 1996; Kruse, 1999).

However, it appears that black cohosh is not a phytoestrogen. Some authors have suggested that black cohosh can be safely used in women with a history of breast cancer, citing laboratory evidence that black cohosh does not stimulate the proliferation of estrogen receptor (ER) positive breast cancer cell . Black cohosh was not better than placebo for breast cancer, though a reduction in night sweats was observed (Jacobson, 2001).

Chaste tree (*Vitex agnus-castus*), or chaste berry is used to treat premenstrual breast pain and fibrocystic breast (Mills and Bone, 2000). It is also used for different types of menstrual irregularities and for sexual dysfunction, reducing sexual desire, as well as historically for decreased libido. Chastetree stimulates LH and may decrease FSH (Weiss, 1988).

Motherwort (*Leonurus cardiac*) is Everywoman's herb. Tincture of the flowering plant ease menstrual cramping, relieves cools hot flashes. As a calmative, it has no equal; but its strongest ability is in providing dependable relief for women experiencing tachycardia, palpitations, or hyperthyroidism (Weiss, 1988). Daily use is considered safe for all women.

Red clover (*Trifolium pretense*) blossoms and leaves unusually rich in amount and variety of phytoestrogens, containing ten times more phytoestrogens than

soy (Reinli, 1996) and twice as many kinds (He, 1996). Red clover has a soild anti-cancer reputation (Fotsis, 1995; Pagliacc, 1994).

Dong Quai (*Angelica sinsensis*) is used for dysmenorrheal, amenorrhea, and menopausal symptoms. Dong Quai has not been shown to be effective for hot flashes (Hirata, 1997). It is safe for patients with estrogen-dependent cancers. Donguai constituents can be carcinogenic, mutagenic and photocarcinogenic even without excessive light exposure. Dong quai is widely used in Chinese medicine, a Chinese herb traditionally prescribed as folksy medicine for women.

Soy contains special chemicals that seem to fight illness and disease. These chemicals are known as phytochemicals. Phytoestrogens, a special kind of phytochemical, appear in high quantities in soy products. These phytoestrogens are a weaker form of our own natural estrogen, and seem to help combat the symptoms of menopause. A particularly beneficial type of soy estrogen is the isoflavone. Isoflavones are linked to the reduction of serious illnesses that plague menopausal women, including osteoporosis and heart disease (Setchell, 1998).

Ginseng root (*Panax ginseng*) is an ancient and revered East-Asian aphrodisiac with estrogenic activity that is claimed to improve menopausal symptoms. A single randomized, double-blind, placebo-controlled study of 384 post-menopausal women found no benefit of ginseng over placebo for hot flashes and overall quality of life, although there were positive effects on mood (Wiklund, 1999). Follicular stimulating hormone and estrogen blood levels and endometrial thickness were also the same.

Pomegranates (*Punica granatum* L.)

A pomegranate is a shrub or small tree. It is found growing wild in the warm valleys and outer hill of the Himalayas. It has been extensively used as a folk medicine in many cultures (Langley, 2000). Pomegranate is a rich source of crude fibers, pectin, sugars, and several tannins (Gil et al., 2000). In addition, it has recently been reported that pomegranate contains some species of flavonoids and anthocyanidins in their seed oil and juice. Moreover, pomegranate juice is rich in antioxidants which general possess numerous important biological properties against cholesterol oxidation, protection against atherogenesis, anti-inflammatory, anti-aging, and protection against Alzheimer's disease and diabetes. The rind of the fruit is antihelminthic, useful in diarrhea and dysentery. Furthermore, the chemopreventive and adjuvant therapeutic applications of pomegranate to human breast cancer have been warranted recently (Kim et al., 2002).

Furthermore, the effects of pomegranate compound on low-density lipoproteins and aggregation of platelets are beneficial because they reduce some of the major risk factors for coronary heart disease (Sudheesh, 1999) and pomegranate juice was found to slow down cholesterol oxidation by almost half, and reduce the retention of disproportionate LDL cholesterol (Sumner et al., 2005). The rinds of fruits are valued as astringents in diarrhea and dysentery. In folk medicine pomegranate preparations from the dried pericarp and the juice of the fruits are employed as orally medication in the treatment of colic, colitis, leucorrhea, menorrhagia, oxyuriasis, paralysis, and external application to caked breast and to the nape of the neck in mumps and headache. Australian researchers found that their scientific investigation of pomegranate flower extract improved hyperglycemia in

Type II diabetes and obesity at least partially (Li et al., 2005). The seed and juice extracts were diluted to 20 and 10% have been used to treat menopausal symptoms and bone loss (Junko et al., 2004). Part of toxicity of pomegranate has not been intensively studied. Amorin (1995) observed no toxic effects in mice treated with aqueous extracts of pomegranate similar to those used in folk medicine. Pomegranate fruits also (excluding the peel) are not toxic but roots and bark are alkaloid content (Fuentes et al., 1985).

However, one of the most remarkable characteristic of pomegranate fruit is that its seeds are the richest plant source of estrogens. Pomegranate seeds are known to contain the estrogenic compounds, estrone and estradiol that are chemically identical to those biosynthesized in human body (Heftmann et al., 1996). Pomegranate seeds contain not only estrogen (estradiol, estrone and estriol) but also other steroids such as testosterone and β-sitosterol and coumesterol. In our preliminary High Performance Liquid Chromatography (HPLC) assay, isoflavone phytoestrogens such as genistein and daidzein are identified in both seed and peel extracts.

1.4 Aims

There were three main aims to the program of this work, which were interconnected: 1) to investigate the effects of the pomegranate extract on serum estrogen level, bone protection, including BMD, reproductive actions, including uterine weight, vaginal cytology, mammary gland development, and lipid profile; including LDL, HDL and triglycerides; 2) to test the effects of the pomegranate extract on anti-implantation; and 3) to examine the effects of the pomegranate extracts

on contraction and compared its effect to the known compounds such as β-sitosterol. The underlying mechanism of the extracts was also investigated.

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CHAPTER II

GENERAL MATERIALS AND METHODS

This Chapter will give a general description of major mater materials and methods used in the work presented in this thesis. More details pertinent to each study are given in each Chapter.

In Chapters VII, VIII, and IX, experiments were performed on animal tissues to establish valid models for physiological mechanisms in human.

2.1 Plant Preparation

2.1.1 Plant Collection and Preparation of the Extracts

Fresh Thai pomegranate fruits were collected from the field in the areas of Nakhon Ratchasima, Thailand, during April to May. The plant and its fruit was identified and confirmed by the Royal Forest Department of Thailand and a voucher specimen (Herbarium No.080252) has been deposited in the laboratory for future references.

The pomegranate seed and peel were manually isolated. The powder of seed and peel (250 g) were extracted in 1 L methanol to Soxhlet extraction (Figure 2.1A). The methanol extract was concentrated under vacuum in a rotary evaporator (Figure 2.1B) to yield semi-solid mass, which was further dried by lyophilized and kept at -20^{o}C. Pomegranate seed was prepared in Tween-80 10% (v/v) and

pomegranate peel was prepared in distilled water. They were administered to the rats orally by means of an intragastric catheter.

A

B

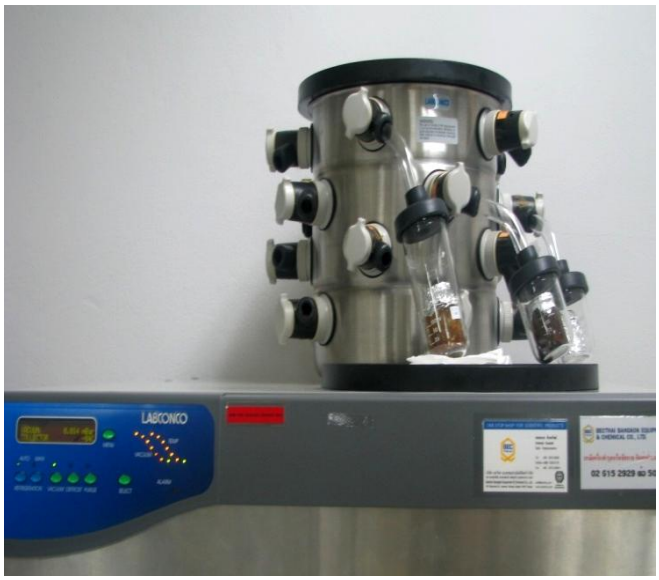


Figure 2.1 Soxhlet extraction (A) and lyophilizer apparatus used in the extraction process (B).

2.1.2 Composition and Identification of the Extract

Composition of the pomegranate seed and peel extracts was investigated by Gas chromatography/mass spectrometry (GC/MS). The identification of the extract composition was based on comparisons with mass spectra and retention indices of authentic reference compounds where possible. GC/MS analysis was performed on a Hewlett-Packard 5973 (IE) MS selective detector coupled with a Hewlett Packard 6890 gas chromatograph equipped with a cross-linked 5% PHME siloxane HP-5MS capillary columm (30 m x 0.25 mm; film thickness, 0.25 µm). The gas chromatographic conditions were as follows: carrier gas, helium with a flow rate of 1.0 mL/min; column temperature, 50^{o}C at 6^{o}C/min; injector temperature, 250^{o}C; volume injected 0.1 µL of oil; split ratio, 250: 1. Compound identification was based on comparisons with mass spectra and retention indices of authentic reference compounds where possible. The rest of the extract obtained was stored at 4^{o}C until use in the physiological experiments. A working solution was obtained by dissolving the extract in physiological solution.

2.2 Animal Preparations

2.2.1 Housing

Rats 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 the Institutional Animal Care and Use Committee, Suranaree University of Technology (SUT), Thailand.

Wistar rats (200-250g) were housed in the animal house of the SUT under a controlled environment (23-25^{o}C) and illumination (12 hr light, 12 hr dark) room. Rats were given tap water and a standard diet *ad libitum*.

2.2.2 Bilateral Ovariectomized Procedure

Ovariectomized (OVX) procedure was performed by anesthetizing rats with isoflurane. After placing the animal in right lateral recumbency on a water-jacketed heating pad to maintain body temperature, the flank region of the anesthetized rat was clipped and aseptically prepared. An incision was made on the animal's right flank. The right ovary was located and a circumferential suture was placed around the ovarian artery and vein. The right ovary was removed just distal to the suture. The incision was sutured closed. The same procedure was repeated to remove the left ovary. This procedure was also preformed on the sham-operated groups except that both ovaries were exteriorized and then replaced into the abdominal cavity.

2.2.3 Animal and Administration Procedure

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 the Institutional Animal Care and Use Committee, , (SUT).

The rats were divided into eight groups. Each group contained 6-10 rats. It has been reported that the estrogenic activity of phytoestrogens ranges from 1/500 to

1/1000 mg/kg B.W. (Cassidy, 1999), the dose 100 and 1000 mg/kg B.W. of pomegranate seed and peel extracts were used. Therefore, the experiments were designed as follows:

Group 1 (sham operated control): received 1 ml of 10% (v/v) Tween 80 suspension (p.o.).

Group 2 (ovariectomized rats): received 1 ml of 10% (v/v) Tween 80 suspension (p.o.).

Group 3 (standard): received 17β-estradiol at a dose of 0.17 mg/kg B.W. (s.c.).

Group 4 (standard): received 17β-estradiol at a dose of 0.7 mg/kg B.W. (s.c.).

Group 5 (test): received 1 ml aqueous solution of pomegranate seed extract in 10% (v/v) Tween 80 at a dose of 100 mg/kg B.W. (p.o).

Group 6 (test): received 1 ml aqueous solution of pomegranate seed extract in 10% (v/v) Tween 80 at a dose of 1000 mg/kg B.W. (p.o).

Group 7 (test): received 1 ml aqueous solution of pomegranate peel extract in distilled water at a dose of 100 mg/kg B.W. (p.o).

Group 8 (test): received 1 ml aqueous solution of pomegranate peel extract in distilled water at a dose of 1000 mg/kg B.W. (p.o).

All these were administrated daily for 2 months. At the end of the experiments, the rats were sacrificed under CO_2 anesthesia.

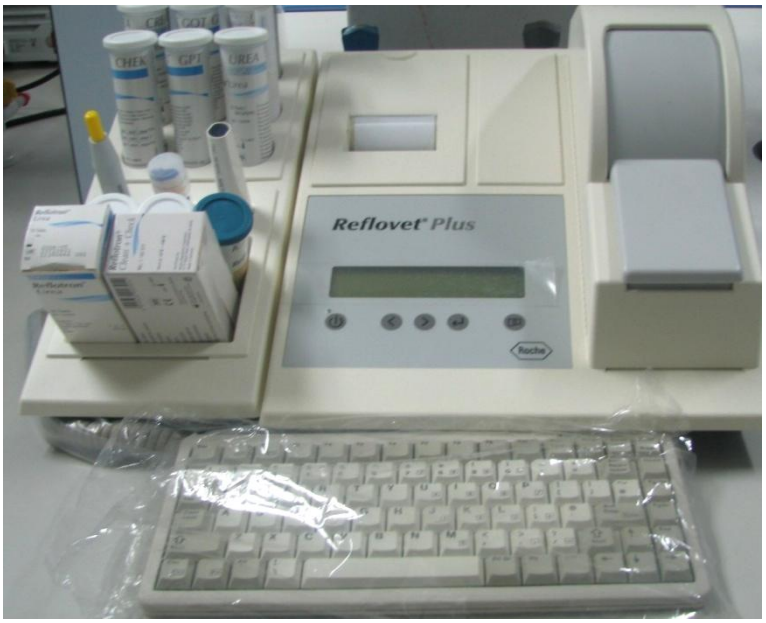
2.3 Lipid Profile Analysis

Blood samples were collected from cardiac puncture. Blood samples were centrifuged at 3000 x g for 10 min at 4^oC to obtain serum. All biochemical determinations are performed in serum. Total serum cholesterol, high density lipoprotein (HDL), low density lipoprotein (LDL), triglyceride concentration in serum was measured by diagnostic kits (Raichem). The measurements of lipid fraction were made using Reflotron; Roch Dianostics GmbH (Figure 2.2A).

2.4 Histological Analysis

The uterus, vagina and mammary glands were fixed in 10% buffered formalin for 48 hr. Uterine were cut for three cross section per area. Mammary glands were cut to obtain sections from the nipple through the fat pad toward the abdominal muscles. Vagina was prepared for longitudinal sections. All samples were embedded in paraffin and 3-µm thick sections were cut, mounted, and stained with hematoxylin and eosin (H&E) for microscopic analysis (Rimoldi, 2007).

A



B

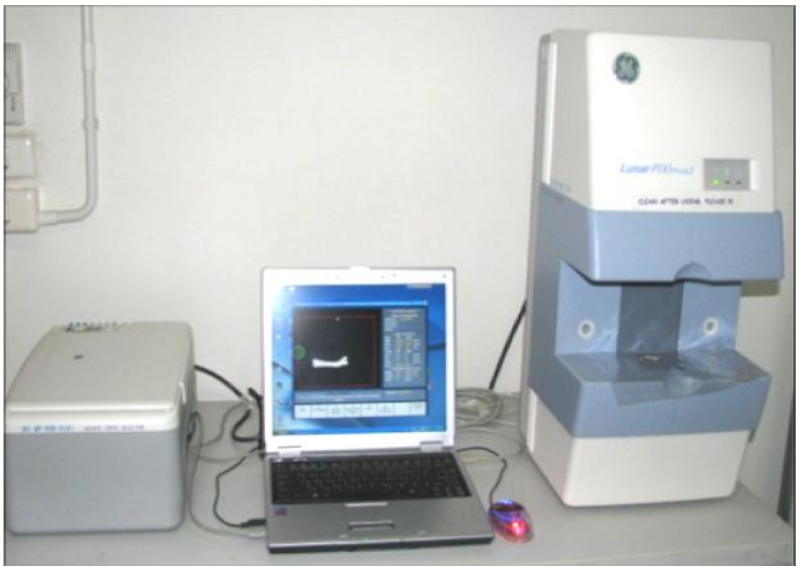


Figure 2.2 Picture shows the set up of Reflotron (Roch Dianostics GmbH) (A). Dual energy-x-ray absorptioneter (PIXImus) in the present study, computer with Software version 4.1 (B).

2.5 Study of Reproductive Hormones

Blood samples from all animals were collected by cardiac puncture and centrifuged at 3000 x g, 4^{o}C for 10 min. Serum was stored frozen at frozen at -20^{o}C. Four hundred microliters of blood samples were collected from each animal for hormonal assay. 75 µl of serum was used for each hormonal assay. The absorbance was measured at 450 nm using a micro plate reader and generated a standard curve by 4 parameter logistic fitting. The estrogenic activity of the sample was calculated from the standard curve. Serum 17β-estradiol and LH concentrations were measured by ELISA microwell kits (Ganguly, 2007).

2.6 Bone Mineral Density

The right femurs were freed from soft tissue using small scissors, tweezers and cotton gauze. Bone mineral density (BMD) was performed on the right femur by a dual energy x-ray absorptiometer (PIXImus) lunar Corp., USA, version 1.4 (Thongchote, 2007 ; Figure 2.2B).

2.7 Vaginal Cytology

Vaginal epithelium was checked weekly between 09.00-10.00 A.M. The vaginal cells were smeared onto a slide with a drop of 0.9% normal saline solution, observed under a light microscope, and identified and then their cell types were recorded. The Vaginal cells were categorized into the following 3 types: leukocyte cells (L), nucleated cell (O), and cornified cell (Co). The representative cell-type was determined by selecting the type of cells that comprised the majority of cells. The results of examination of vaginal smear cells from 5 rats in each treatment group were

expressed as a mode value (most frequently occurring cell type in 5 rats). The appearance of cornified cells was used as an indicator of estrogenic activity (Malaivijitnond et al., 2006).

2.8 Anti-implantation Studies

Vaginal smears from each rat were monitored daily. Only rats with normal estrous cycle were selected. Anti-implantation activity was determined as described by Khanna and Chaudhary (1968). The female rats were housed with the male rats in the ratio of 2: 1 and examined the following morning for evidence of copulation. The rats exhibiting thick clumps of spermatozoa in their vaginal smears were separated and that day was designated as day 1 of pregnancy.

2.9 The Investigation of Physiological Effects of Pomegranate on Uterine Activities

2.9.1 Myometrial Tissue Preparation

In Chapters VII, VIII and IX, the rats were sacrificed under CO_2 anesthesia. Myometrial were obtained from uterus. The uterus was removed and immediately immersed in buffered physiological Krebs' solution (pH 7.40) containing (mM): 154 NaCl; 5.4 KCL; 1.2 $MgSO_4$; 12 glucose; 2 $CaCl_2$, and 10 N-[2-hydroxyethyl] piperazine-N'-[2-ethanesulphonic acid] [HEPES]. The uterus was then placed in a shallow dissecting dish containing Krebs' solution at room temperature under a light microscope. The longitudinal layer was separated from the endometrium and circular layers. Five or six strips (1-2 mm x 0.5 x 10 mm) of longitudinal muscles were then

dissected. The strips were either used immediately or stored for a maximum of 12 hr at 4^{o}C.

2.9.2 Measurements of Tension

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 (Figure 2.3).

The organ bath contained Krebs' solution maintained at pH 7.4, temperature of 37^{o}C, and gassed with O_2. The myometrial strip was attached at one end to metal hooks and another hook was fixed to a transducer. The electrical signal was recorded from the transducer and converted to the digital signal on a computer using Chart software (Kupittayanant et al., 2002). The strips were allowed to contract spontaneously. A equilibrium period of 30 min was given before the administration of any chemical.

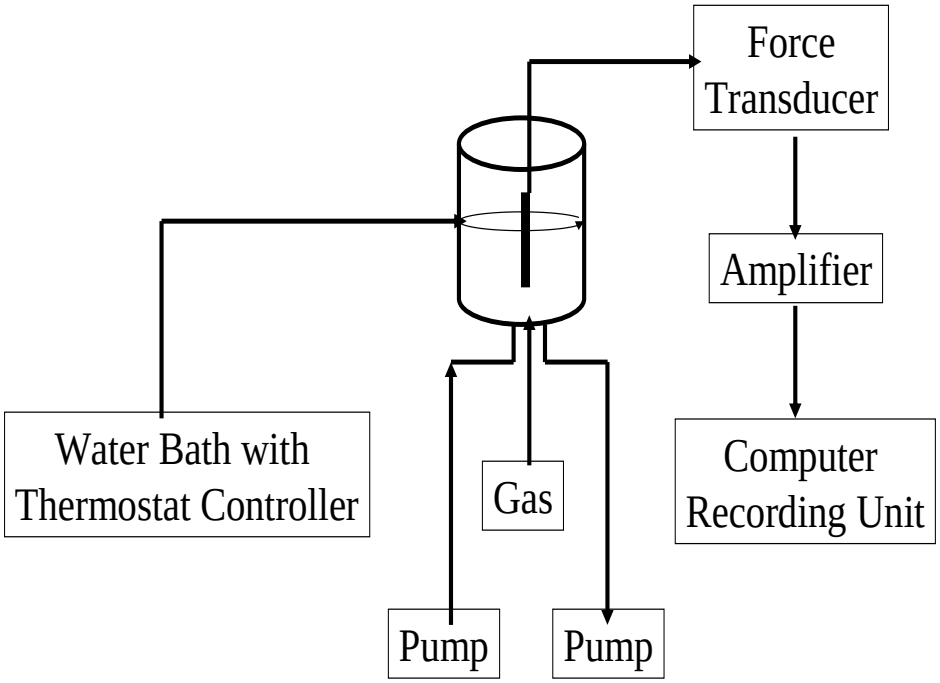


Figure 2.3 Schematic representation of the set up used for tension measurements (Buddhakala, 2007).

2.10 Chemicals

All chemicals were purchased from Sigma® unless stated otherwise. Information of pomegranate seed and peel extracts were given in the individual chapter concerned. Stocks of pomegranate seed and peel extracts were prepared and kept as recommended by Promprom et al. (2008). Dissolved vehicles used (e.g. DMSO, ethanol) did not alter the myometrial contraction integral. The dilutions were made on the day of the experiment.

2.11 Statistical Analysis

All data in Chapter I-VI were expressed as the mean ± S.E.M. The difference between the two data sets obtained from the intact, control, and pomegranate groups was analyzed by using Student's *t*-test (SPSS Statistical Methods, Version 13) taking $P<0.05$ as statistically significant.

Statistical Analysis of Tension Measurements: The result data were analyzed using Microcal Origin Software (Massachusetts, USA). Parameters that were measured include maximum tension development of each contraction, the contraction integral (total tension developed in each contraction), contraction duration, and contraction frequency. Data were then presented as mean ± S.E.M and "*n*" represents the number of samples, each one from a different animal. Significance was tested using appropriate *t* tests or ANOVA and *P* values <0.05 taken to be significant. Results were then expressed as percentages of control contractions (i.e. the control is 100%).

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CHAPTER III

EFFECTS OF POMEGRANATE (*PUNICA GRANATUM* L.) EXTRACT ON UTERUS, MAMMARY GLAND AND VAGINA

3.1 Abstract

Pomegranate (*Punica granatum* L.) is weakly estrogenic and heuristically of interest for the treatment of menopausal symptoms and sequela. The purpose of this study was designed to compare the effects of 17β-estradiol and a phytoestrogen containing pomegranate extract on proliferative and morphogenetic relations in the uterus, mammary glands and vagina in ovariectomized rats. The study was performed in Wistar rats which divided into 8 subgroups; sham operated rats received vehicle (10% v/v Tween 80, p.o.), ovariectomized rats received vehicle (10% v/v Tween 80, p.o.), ovariectomized rats received 17β-estradiol at the different doses (0.17 or 0.7 mg/kg B.W. (s.c.)) and ovariectomized rats received methanolic extracts of the pomegranate seed or peel extracts at the different doses (100 and 1000 mg/kg B.W., (p.o.)). Uterus, mammary glands and vagina were morphologically investigated. Serum E_2 and LH levels were also measured as indicators of a hypothalamic/pituitary effect of the test substances. The pomegranate seed and peel extracts produced a significant increase in uterine weight compared with ovariectomized rats. Histologic analysis showed that pomegranate seed and peel extracts produced slightly increases in uterine weight and endometrial thickness. Pomegranate seed extract

(1000 mg/kg B.W.) induced hyperplastic epithelium in vagina endometrial thickness compared with ovariectomized rats. Interlobular ducts were found in the mammary gland of pomegranate seed and peel extracts compared with ovariectomized rats. In conclusion, the pomegranate seed and peel extracts exhibits estrogenic activity on uterus, vagina and mammary glands. Serum LH levels in ovariectomized rats were inhibited by E_2 (both doses) but not by pomegranate extract.

3.2 Introduction

During the period of menopause and postmenopause, many women experience one or more symptoms such as hot flashes, depression, urinary problems, sleeping disorders, vaginal dryness, and joint pain, largely due to a lack of estrogens. Hormone replacement therapy helped to relieve menopausal symptoms, in addition, a risk of osteoporosis, cardiovascular disease, and dementia from Alzheimer's disease. The sex hormone estrogens play various roles in both the male and female body. Estrogens are used in hormone replacement therapy (HRT) to prevent hot flashes and osteoporosis in postmenopausal women (Kenny and Prestwood, 2000). 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 fallopian tubes, uterus, vagina, and external genitalia. Conversely, estrogen deprivation result in atrophy of these organs (Sperelakis and Banks, 1996). Postmenopausal estrogen replacement therapy is a commonly used treatment for climacteric symptoms in a short term and long term. Low serum levels of 17β-estradiol (E_2) often result in symptoms and degenerative

processes. This has prompted women to receive hormone replacement therapy (HRT) to prevent these ageing associated symptoms or diseases (Burger, 2003). Because estrogens alone stimulate endometrial proliferation, which may result in cancer, they have to be given in combination with progestins (Albertazzi and Sharma, 2005). In addition, estrogen replacement therapy is associated with increased breast and endometrial cancer risk (Beral, 2003). Epidemiological studies suggest that eating a diet rich in phytoestrogens can help to relieve hot flushes and the incidence of cancer in oriental women (Kurzer, 1997). Since side-effects of traditional estrogen replacement therapy include a slight but significant increase in the risk of developing breast and endometrial cancer, women are increasingly using herbal remedies as alternative therapy (Setchell, 1998). This has resulted in a search for HRT alternatives and plant-derived, so-called phytoestrogens are vigorously promoted.

Phytoestrogens (also called plant estrogens) are a group of substances found in plant foods. They are structurally similar to estrogen, (Mackey and Eden, 1998). They are non-steroid compounds and have weaker hormonal effects: binding weakly to the ∞-estrogen receptors of the uterus, ovaries and breast and more strongly to the beta-estrogen receptors found in the brain, arteries and bone (Setchell, 1988). The phytoestrogens with the most powerful estrogenic action are genistein, daidzein and glycitein that belong to the class of isoflavones.

Pomegranate (*Punica granatum* L.) is one of the plants received alternative for HRT. It has several advantages for human health. Pomegranate juice, seed oil, peel or flower extracts, and their derivatives have been reported to kill bacteria and viruses, or to fight vascular disease, diabetes and cancer (Ephraim and Robert, 2007). Furthermore, the chemo preventive and adjuvant therapeutic applications of

pomegranate to human breast cancer have been warranted recently (Kim et al. 2002). Owing to these significant biological activities, pomegranate juice is being increasingly popularized in Japan.

However, one of the most remarkable characteristics of pomegranate seed are known to contain the estrogenic compounds, estrone and estradiol, that are chemically identical to those biosynthesized in human body (Heftmann et al., 1966), and coumesterol as well (Moneam et al., 1988). According to Kim et al., (2002), pomegranate seed contain not only estogens (estradiol, estrone, and estriol) but also other steroids, such as testosterone and β-sitosterol, and coumesterol. Using preliminary HPLC assay, isoflavone phytoestrogens such as genistein and daidzein were also detected in the pomegranate extract containing seed used in this study. The OVX rat is a widely used model to study estrogen withdrawal and replacement because many phenomena in rat model are similar to those occurring in postmenopausal women (Guillermo, 2007).

Therefore, the aims of this chapter were to 1) examine a number of physiological and morphological effects induced in the mammary glands, uterus, and vagina after oral administration of two dosed of pomegranate seed and peel extracts in ovariectomized rats and to 2) compare with those induced by E_2 treatment. In the present study, tissue samples were histologically examined.

3.3 Materials and Methods

3.3.1 Identification of the Pomegranate Constituents

Composition of the pomegranate extract was investigated by GC/MS. The identification of the extract composition was based on comparisons with mass spectra

and retention indices of authentic reference compounds where possible. GC/MS of pomegranate seed and peel analysis's were performed on a Hewlett-Packard 5973 (IE) MS selective detector coupled with a Hewlett Packard 6890 gas chromatograph equipped with a cross-linked 5% PHME siloxane HP-5MS capillary column (30 m x 0.25 mm; film thickness, 0.25 μm). The gas chromatographic conditions were as follows: carrier gas, helium with a flow rate of 1.0 mL/min; column temperature, 70^{o}C at 10^{o}C/min; injector temperature, 270^{o}C; volume injected 1.0 μL of oil; split ratio, 20: 1.

3.3.2 Animals and Chemical Exposures

β-Estradiol 3-benzoate was obtained from Sigma Chemical Co. (St.Louis, MO, USA). All solvents/chemicals used were of analytical grade and obtained from Sigma® and Merck®.

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 the Institutional Animal Care and Use Committee, SUT.

The rats were divided into eight groups as follows. Each group contained 6-9 rats. It has been reported that the estrogenic activity of phytoestrogens ranges from 1/500 to 1/1000 mg/kg B.W.; thus, these doses were used.

Group 1 (sham operated control): received 1 ml of 10% (v/v) Tween 80 suspension (p.o.).

Group 2 (ovariectomized rats):	received 1 ml of 10% (v/v) Tween 80 suspension (p.o.).
Group 3 (standard):	received 17β-estradiol at a dose of 0.17 mg/kg B.W. (s.c.).
Group 4 (standard):	received 17β-estradiol at a dose of 0.7 mg/kg B.W. (s.c.).
Group 5 (test):	received 1 ml aqueous solution of pomegranate seed extract in 10% (v/v) Tween 80 at a dose of 100 mg/kg B.W. (p.o).
Group 6 (test):	received 1 ml aqueous solution of pomegranate seed extract in 10% (v/v) Tween 80 at a dose of 1000 mg/kg B.W. (p.o).
Group 7 (test):	received 1 ml aqueous solution of pomegranate peel extract in distilled water at a dose of 100 mg/kg B.W. (p.o).
Group 8 (test):	received 1 ml aqueous solution of pomegranate peel extract in distilled water at a dose of 1000 mg/kg B.W. (p.o).

All these rats were administrated daily for 2 months. At the end of the experiments, the rats were sacrificed under CO_2 anesthesia. The uterus were removed and weighed, and mammary glands and vagina were dissected. The six excised uterus, mammary glands and vagina were fixed in 10% buffered formalin. Uterus was cut three cross-sections per area. Mammary glands were cut to obtain sections from the

nipple through the fat pad toward the abdominal muscles. Vagina was prepared for longitudinal sections. All samples were embedded in paraffin, and 3 µM thick sections were cut, mounted, and stained with hematoxylin and eosin (H&E) for microscopic determination.

3.3.3 Reproductive Organ Measurement

Uterus, mammary glands, and abdominal fat were calculated for each animal. All organ wet weight analysis was performed using digital weight scales and organ weight was reported as absolute and relative wet weight (organ weight/body weight x 100).

For mammary gland measurement, the proximal fourth or fifth of left or right abdomino-inguinal mammary glands were examined, after exfoliating the skin and fat pad from the body.

3.3.4 Hormone Analysis

Blood samples were collected from the cardiac puncture of animals. Serum LH and 17β-estradiol concentrations were measured by using ELISA micro well kit as described in 2.5.

3.3.5 Data Analysis

Data were expressed as mean ± S.E.M. The differences of parameters were statistically evaluated using ANOVA and two-paired Student's *t*-test, $P<0.05$ was considered as statistically significant. Morphological features were statistically analyzed with contingency tables compared with controls.

3.4 Results

3.4.1 Chemical Constituents of Pomegranate

The fingerprint of pomegranate seed and peel was obtained by GC-MS. The compounds were identified as shown in Table 3.1 and 3.2.

Table 3.1 The compounds identified in pomegranate seed using GC-MS.

No.	Compound identified	Relative constituents (%)	Retention time (min)
1	Beta-Tocopherol	18.30	30.16
2	Tricyclo	15.72	18.23
3	β-Sitosterol	14.93	40.55
4	9,12-Octadecadienoic acid	11.04	17.01
5	9,12-Octadecadienoic acid	5.40	22.41
6	Octadec-9-enoic acid	5.05	17.05
7	Palmitinic acid	3.84	15.30
8	Unknown	3.15	10.37
9	Taraxasterol	2.19	44.06
10	2,6,10,14,18,22-Tetracosahexaene	2.16	24.53
11	Cyclohexene,5-methyl-3-(1-methylethenyl)	2.04	18.33
12	Fucosterol	1.82	41.23
13	6-Butyl-1,4-cycloheptadiene	1.68	24.67

Table 3.1 (Continued).

No.	Compound identified	Relative constituents (%)	Retention time (min)
14	Nonanoic acid	1.683	24.67
15	Octadecanoic acid (CAS)	1.47	17.18
16	Hexadecanoic acid	1.353	20.21
17	Methylcholesterol	1.351	35.96
18	Alpha-Fenchene	1.14	18.55
19	β-Thujaplicinol	0.952	13.06
20	Eicosanoic acid	0.791	18.87
21	2-Furancarboxaldehyde	0.74	6.78
22	Isoflavone	0.671	27.06
23	Unknown	0.579	22.70
24	3-Fluoroanisole	0.475	4.84
25	Unknown	0.347	19.67
26	9,12-Octadecadienoic acid	0.254	16.52
27	4H-pyran-4-one	0.23	5.68
28	8-Octadecenoic acid	0.213	7.94
29	Phenol,2-methoxy-4-vinyl	0.21	16.57
30	Unknown	0.26	15.65

Table 3.2 The compounds indentified in pomegranate peel using GC-MS.

No.	Compound identified	Relative constituents (%)	Retention time (min)
1	2-Furancarboxaldehyde	61.031	6.87
2	β-Sitosterol	7.207	40.13
3	4H-Pyran-4-one	5.117	5.69
4	4-Methyleneproline	3.378	8.76
5	1,6-Anhydro-beta-D-glucopyransoe	3.342	10.21
6	1,3-Benzenetriol-5-methyl	3.163	4.80
7	1,2,3-Benzenetriol	2.906	8.87
8	Unknown	2.825	3.16
9	9-Octadecenoic	2.176	16.91
10	2-Furancarboxaldehyde	1.969	3.51
11	Hexadecanoic acid	1.484	15.21
12	Methy 2-furoate	1.457	4.90
13	Unknown	1.374	8.82
14	Cis-linoleic acid	1.028	16.87
15	4H-Pyran-4-one	0.855	3.66
16	Barbituric acid	0.69	7.78

3.4.2 Effects of Pomegranate on Body, Relative Uterine, and Mammary Gland Weight

The effects of pomegranate on body weight during the study are shown in Figure 3.1A and Table 3.3. At the end of the study, the body weight of ovariectomized rats was significantly increased when compared with sham operated control. The pomegranate seed and peel extracts caused a significant increase in mean body weight than that of the sham operated control ($P<0.05$). 17β-estradiol at the different doses (0.17 and 0.7 mg/kg B.W.) did not cause any difference in the body weight compared to sham operated control.

The uterine weight, as expected, of ovariectomy was significantly reduced when compared with sham operated control ($P<0.05$) (Figure 3.1B, Table 3.3). Pomegranate seed and peel extracts completely increased uterine weight as observed in ovariectomized rats. But both increases were less than that of sham operated control. Conversely, 17β-estradiol (0.17 and 0.7 mg/kg B.W.) significantly increased the uterine weight compared with ovariectomized rats ($P<0.05$).

The mammary gland weight of ovariectomized control was significantly increased compared with the sham operate control. The mammary gland weight of rats received pomegranate seed extract at a dose of 1000 mg/kg B.W. was also significantly increased compared with that of the sham operated control and ovariectomized rats. 17β-estradiol (0.17 and 0.7 mg/kg B.W.) did not cause any difference in the mammary gland weight compared to sham operated control. (Table 3.3 and Figure 3.1C).

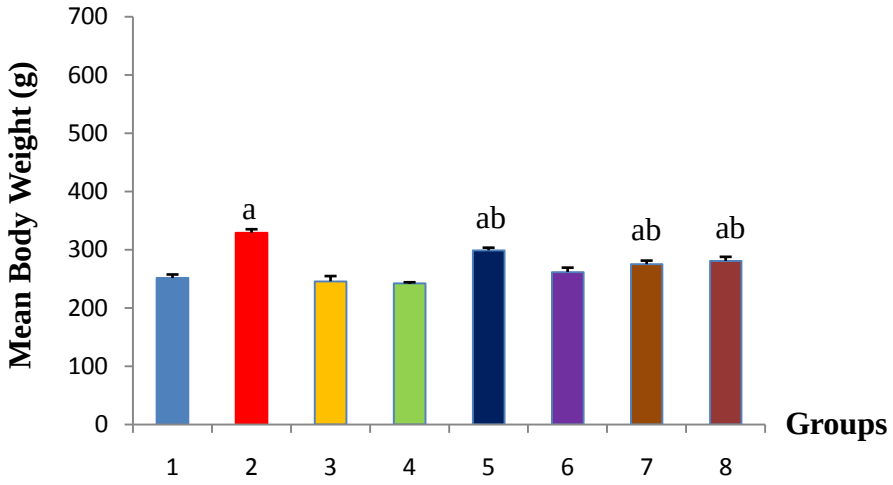
Table 3.3 Effects of pomegranate seed and peel extracts on body, relative uterine, and mammary gland weight.

Group	Body weight (g)	Uterus (mg)	Mammary glands (g)
Sham operated control	251.25 ± 6.10	138.94 ± 8.61	4.51 ± 0.30
Ovariectomized rats	329.00 ± 6.04[a]	25.23 ± 1.12[a]	7.52 ± 0.53[a]
Standard 17β-estradiol [0.17 mg/kg B.W. (s.c.).]	245.71 ± 8.95	294.70 ± 21.95[ab]	3.92 ± 0.40
Standard 17β-estradiol [0.7 mg/kg B.W. (s.c.).]	242.00 ± 2.00	532.94 ± 120.01[ab]	4.14 ± 0.36
Pomegranate seed extract [100 mg/kg B.W. (p.o.).]	298.88 ± 4.48[ab]	78.32 ± 7.26[ab]	6.10 ± 0.53[a]
Pomegranate seed extract [1000 mg/kg B.W. (p.o.).]	261.50 ± 7.60	70.64 ± 2.32[ab]	6.05 ± 0.41[ab]
Pomegranate peel extract [100 mg/kg B.W. (p.o.).]	275.00 ± 6.19[ab]	50.88 ± 3.62[ab]	7.18 ± 0.47[a]
Pomegranate peel extract [1000 mg/kg B.W. (p.o.).]	280.50 ± 7.16[ab]	45.29 ± 3.54[ab]	6.95 ± 0.47[a]

[a] $P<0.05$ vs sham operated control

[b] $P<0.05$ vs ovariectomized rats

A



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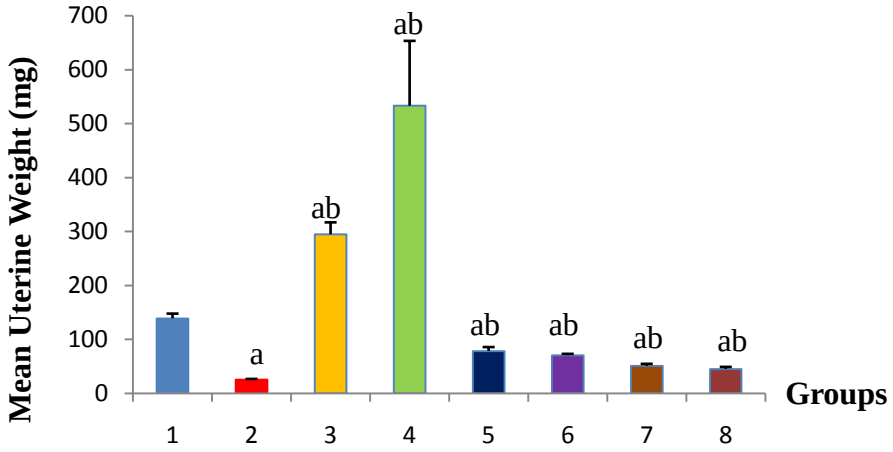


Figure 3.1 Effects of pomegranate seed and peel extracts on body (A), uterine (B) and mammary gland (C) weight. 1 = Sham operated control; 2 = Ovariectomized rats; 3 = 17β-estradiol (0.17 mg/kg B.W.); 4 = 17β-estradiol (0.7 mg/kg B.W.); 5 = Pomegranate seed (100 mg/kg B.W.); 6 = Pomegranate seed (1000 mg/kg B.W.); 7 = Pomegranate peel (100 mg/kg B.W.); 8 = Pomegranate peel (1000 mg/kg BW). (Mean ± S.E.M, n= 6-10).

C

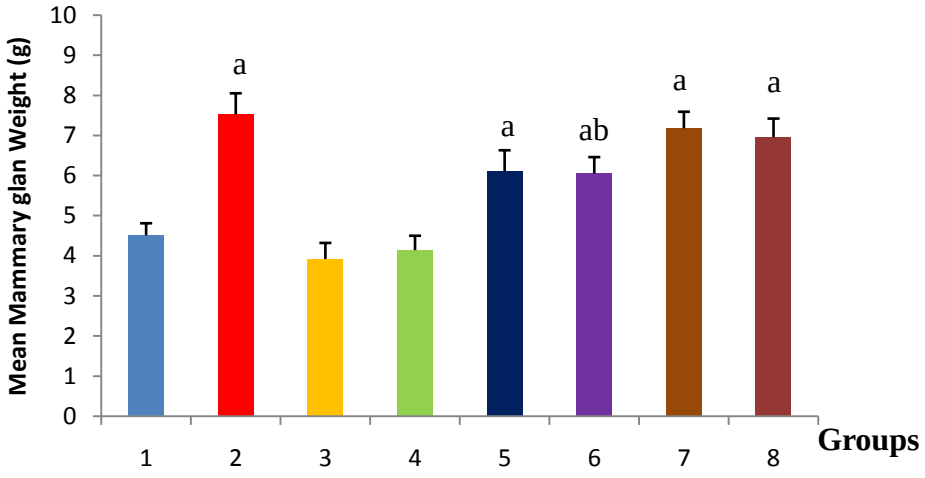


Figure 3.1 (Continued) Effects of pomegranate seed and peel extracts on body (A), uterine (B) and mammary gland (C) weight. 1 = Sham operated control; 2 = Ovariectomized rats; 3 = 17β-estradiol (0.17 mg/kg B.W.); 4 = 17β- estradiol (0.7 mg/kg B.W.); 5 = Pomegranate seed (100 mg/kg B.W.); 6 = Pomegranate seed (1000 mg/kg B.W.); 7 = Pomegranate peel (100 mg/kg B.W.); 8 = Pomegranate peel (1000 mg/kg BW). (Mean ± S.E.M, n=6-10).

3.4.3 Effects of Pomegranate Seed and Peel on Serum Level of E_2 and LH

The effects of pomegranate on serum E_2 levels in sham operated control, ovariectomized rats, ovariectomized rats received pomegranate seed and peel extracts and 17β-estradiol are shown in Figure 3.2A and Table 3.4. Serum E_2 was significantly increased in both doses of 17β-estradiol (0.17 and 0.7 mg/kg B.W.) (245 ± 69.07 and 262.31 ± 72.57 pg/mL) compared with sham-operated control and ovariectomized rats (73.62 ± 13.58 and 59.88 ± 10.42 pg/mL). Pomegranate seed extract and pomegranate peel extract (1000 mg/kg B.W.) caused significant increases in serum E_2 compared with sham operated control. Serum LH were significantly increased in ovariectomized rats ($P<0.05$) (Figure 3.2B and Table 3.4), compared with sham operated control (0.69 ± .33) and 0.07 ± .00 mIU/mL). The administration of pomegranate seed and peel extracts increased serum LH compared to sham operated control, but these increases did not differ when compared to ovariectomized rats.

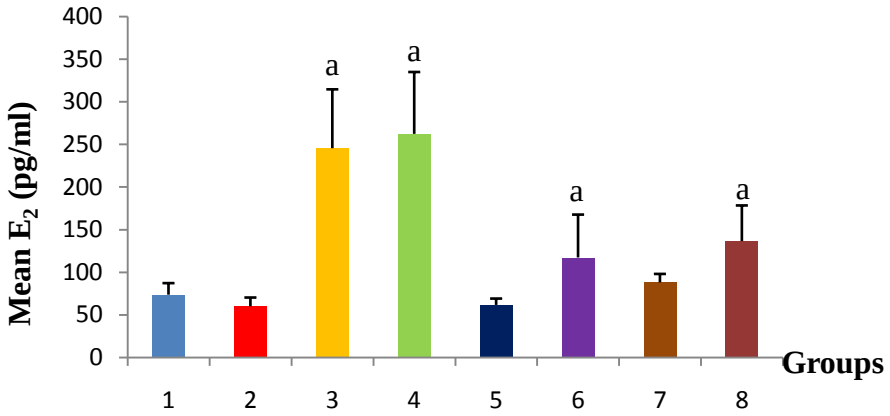
Table 3.4 Effects of pomegranate seed and peel extracts on E_2 and LH.

Group	Estradiol (pg/ml)	Luteinizing hormone (LH) (mIU/ml)
Sham operated control	73.62 ± 13.58	0.07 ± 0.00
Ovariectomized rats	59.88 ± 10.42	0.69 ± 0.33[a]
Standard 17β-estradiol [0.17 mg/kg B.W. (s.c.).]	245.45 ± 69.07[a]	0.07 ± 0.00
Standard 17β-estradiol [0.7 mg/kg B.W. (s.c.).]	262.31 ± 72.57[a]	0.07 ± 0.00
Pomegranate seed extract [100 mg/kg B.W. (p.o.).]	61.65 ± 7.45	0.84 ± 0.87[a]
Pomegranate seed extract [1000 mg/kg B.W. (p.o.).]	117.28 ± 50.33[a]	1.58 ± 1.69[a]
Pomegranate peel extract [100 mg/kg B.W. (p.o.).]	88.05 ± 9.94	0.28 ± 0.25[a]
Pomegranate peel extract [1000 mg/kg B.W. (p.o.).]	136.48 ± 41.87[a]	0.44 ± 0.35[a]

[a] $P<0.05$ vs sham operated control

[b] $P<0.05$ vs ovariectomized rats

A



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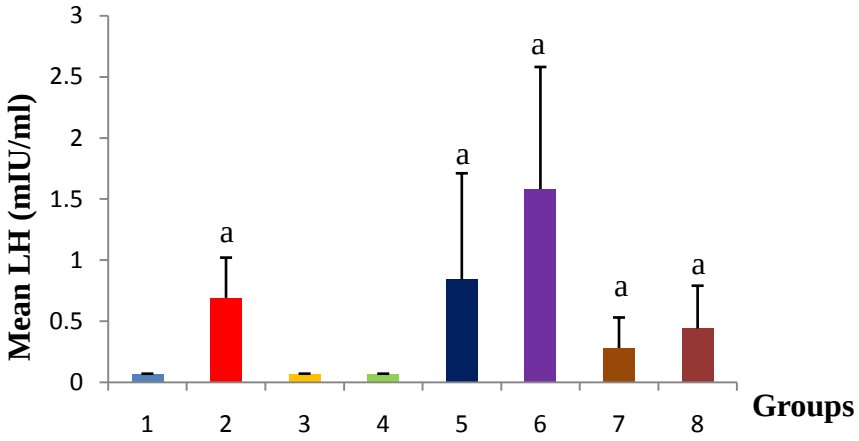

Figure 3.2 Effects of pomegranate seed and peel extracts on serum E_2 levels (A), and LH (B); 1 = Sham operated control; 2 = Ovariectomized rats; 3 = 17β-estradiol (0.17 mg/kg B.W.); 4 = 17β-estradiol (0.7 mg/kg B.W.); 5 = Pomegranate seed (100 mg/kg B.W.); 6 = Pomegranate seed (1000 mg/kg B.W.); 7 = Pomegranate peel (100 mg/kg B.W.); 8 = Pomegranate peel (1000 mg/kg B.W.). (Mean ± S.E.M, n=6-10).

3.4.4 Effects of Pomegranate Seed and Peel Extracts on Morphology of Mammary Gland, Uterine and Vagina

Figure 3.3 shows microscopic preparations of representative uterus taken from one animal per treatment group. In ovariectomized rats, (Figure 3.3 (2)) all epithelium structures revealed atrophic. The endometrium was composed of cuboidal inactive cells. The connective tissue was an unorganized lax syncytium of round nuclei. All uterine structures were hypertrophic and hyperplastic with both doses of E_2 treatments (Figure 3.3 (3 and 4)). Pomegranate seed and peel extracts increased uterine weight and uterine glands (Figure 3.3 (5, 6, 7 and 8)). There was a slight increase of endometrial proliferation. With the pomegranate seed and peel extracts, endometrial cells were stimulated, but no pathological signs were detected.

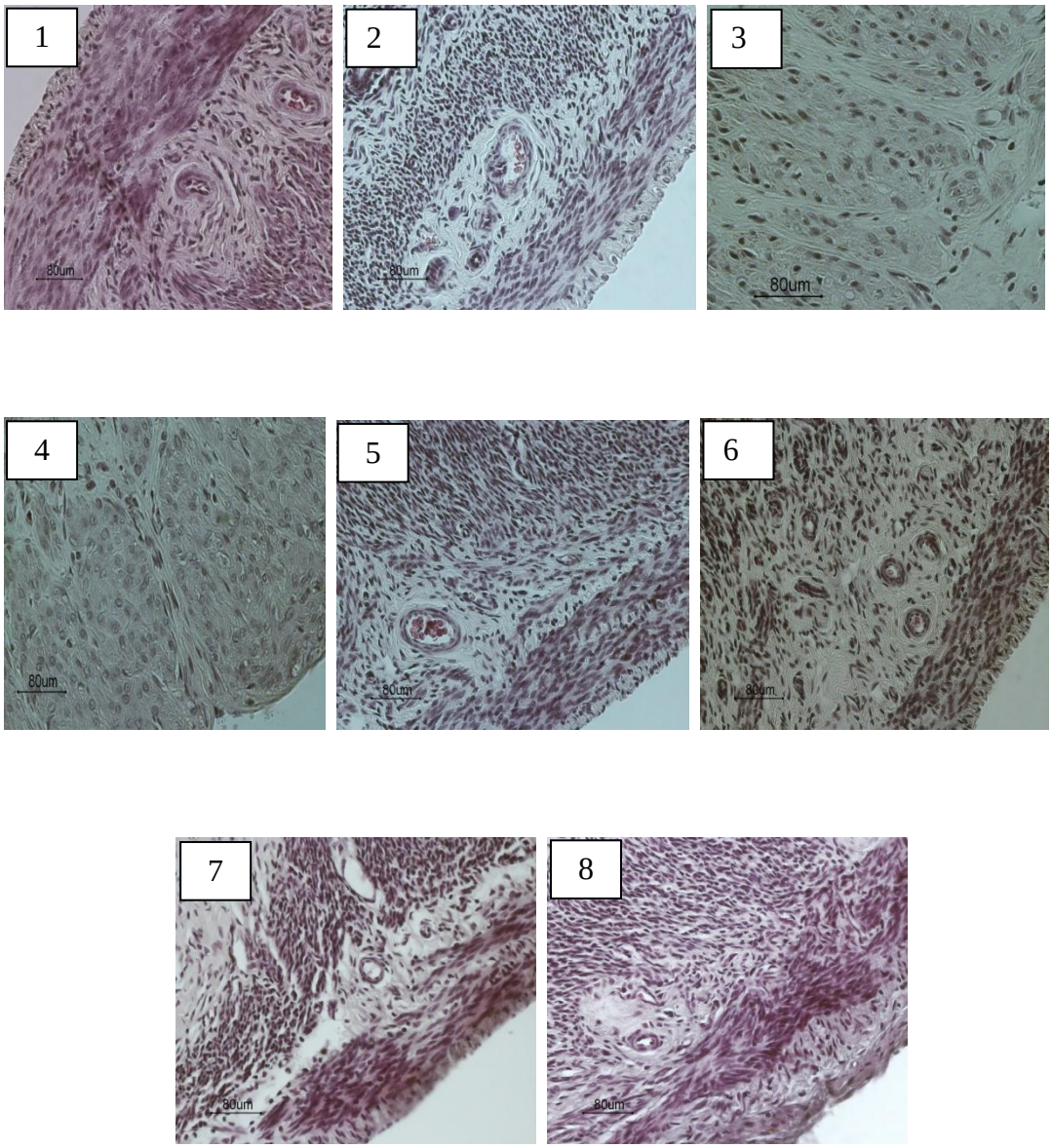

Figure 3.3 Representative photomicrographs of uterine histology.

1 = Sham operated control; 2 = Ovariectomized rats ; 3 = 17β-estradiol (0.17 mg/kg B.W.); 4 = 17β-estradiol (0.7 mg/kg B.W.); 5 = Pomegranate seed (100 mg/kg B.W.); 6 = Pomegranate seed (1000 mg/kg B.W.) ; 7 = Pomegranate peel (100 mg/kg B.W.); 8 = Pomegranate peel (1000 mg/kg B.W.). (Mean ± S.E.M, n= 6-10), (magnification of all pictures is 400x).

Figure 3.4 shows microscopic preparations of representative vaginae taken from one animal per pretreatment group. Atrophic vaginal epithelium was observed in ovariectomized control animals (Figure 3.4). These were composed of flattened cells with no cornification.

The standard drug, 17β-estradiol (0.17 and 0.7 mg/kg B.W.) displayed a typical squamous mutilayered epithelium. With pomegranate seed and peel extracts, epithelium thickness seemed slightly augmented in some areas. The number of layers did not differ from ovariectomized rats (two to three on average) and cornification could not be found.

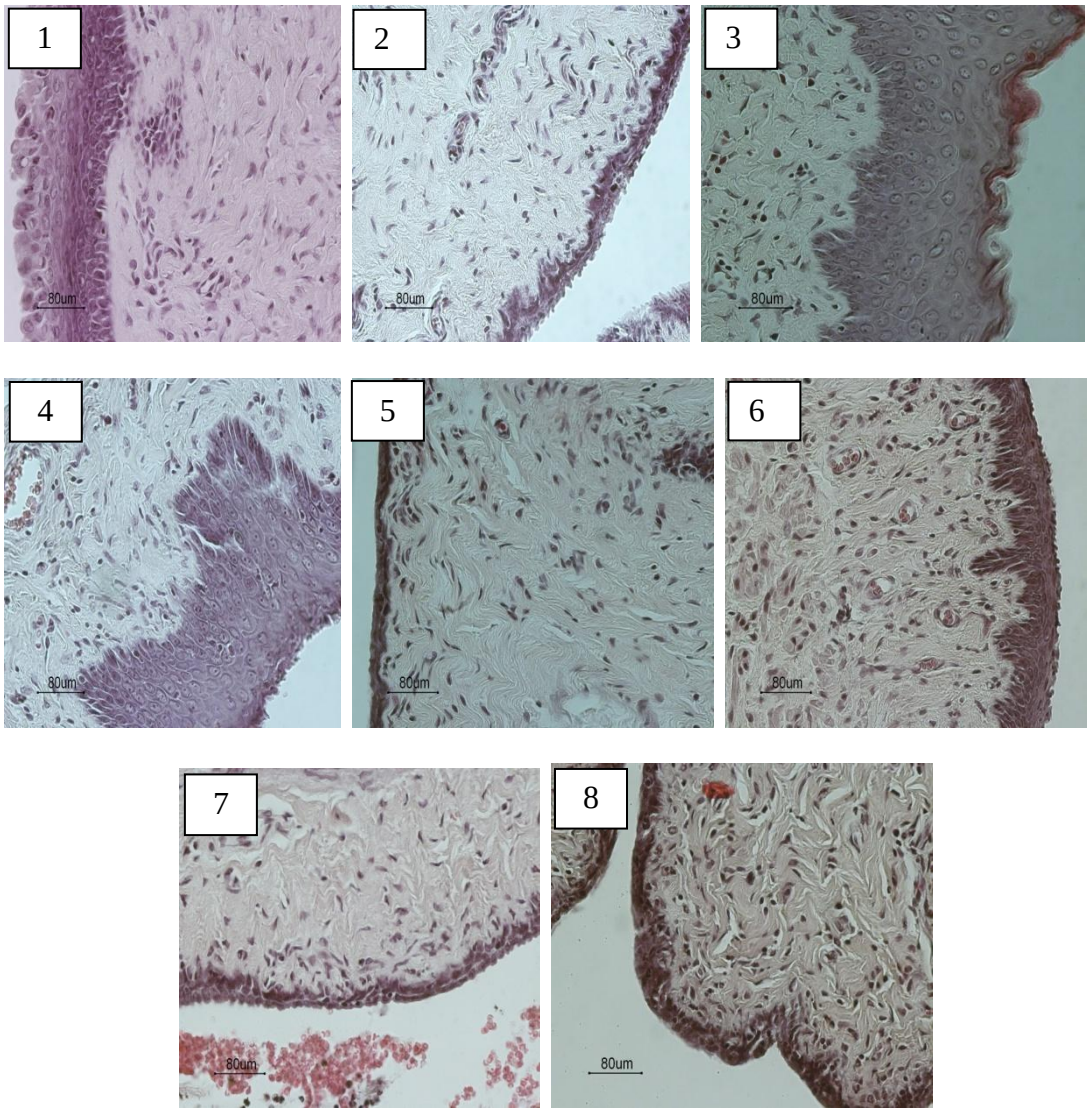

Figure 3.4 Representative photomicrographs of vagina histology.

1 = Sham operated control; 2 = Ovariectomized rats; 3 = 17β-estradiol (0.17 mg/kg B.W.); 4 = 17β-estradiol (0.7 mg/kg B.W.); 5 = Pomegranate seed (100 mg/kg B.W.); 6 = Pomegranate seed (1000 mg/kg B.W.); 7 = Pomegranate peel (100 mg/kg B.W.); 8 = Pomegranate peel (1000 mg/kg B.W.). (Mean ± S.E.M, n= 6-10), (magnification of all pictures is 400x).

Figure 3.5 shows microscopic preparations of representative mammary glands taken from one animal per treatment group. In ovariectomized rats, (Figure 3.5 (2)) all epithelial structures revealed atrophic. Deep in the fat pad, scarce clusters of densely packed terminal structures were found. Many did not show luminal formation. In ovariectomized rats, tubular ducts were not found. In comparison to the scarcity in controls, there were more luminal and alveolar structures in the glands of group treated with the standard drug 17β-estradiol (0.17 and 0.7 mg/kg B.W.). In pomegranate seed extracts-treated group, there was a slight increase of tubular ducts of the mammary glands. In pomegranate peel extract-treated mammary glands, there was luminal formation. In 17β-estradiol (0.17 and 0.7 mg/kg B.W.) treated groups, the secretory materials were found whereas pomegranate seed and peel extracts stimulated lobulo-alveolar and tubular growth without secretory activity.

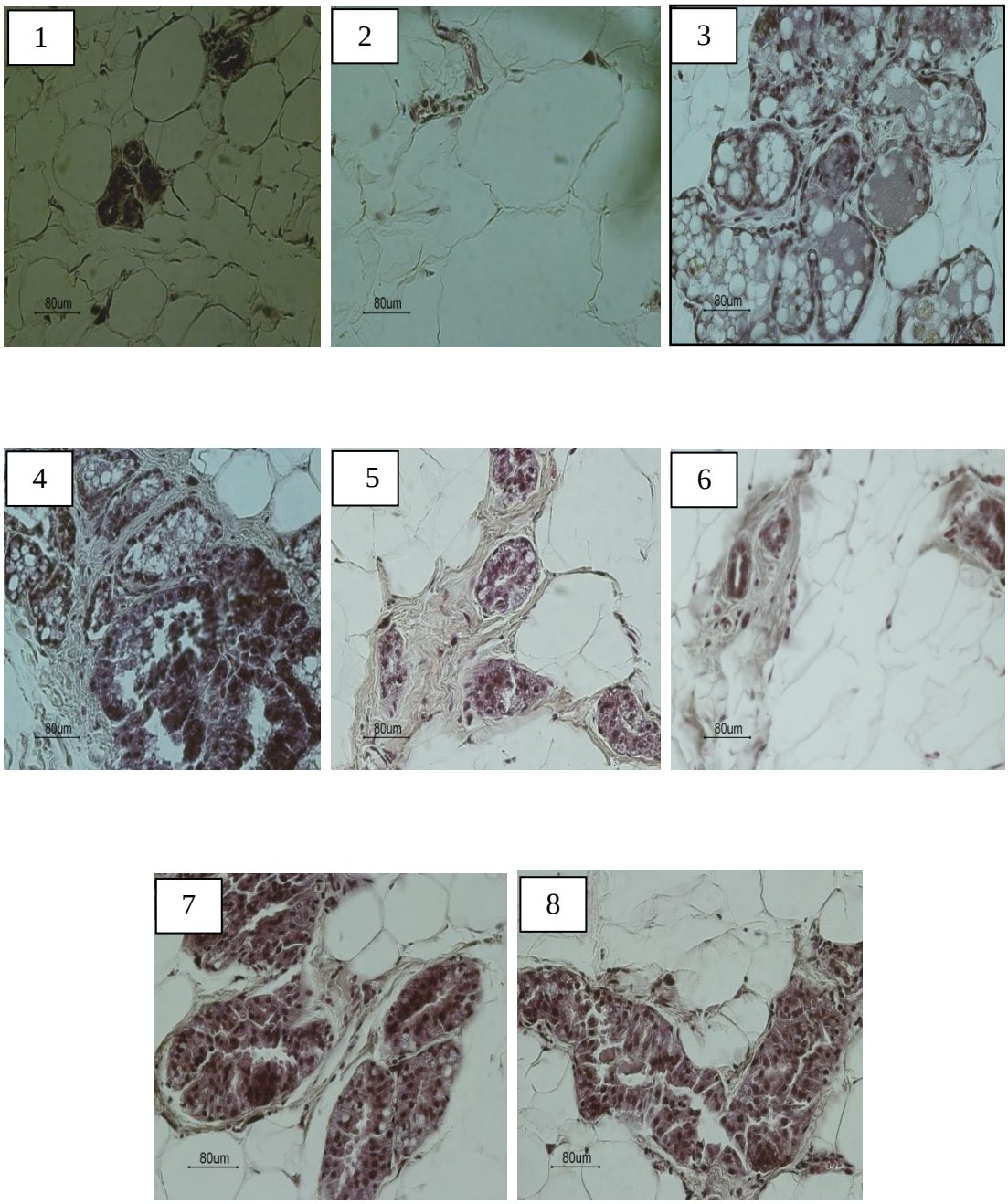

Figure 3.5 Representative photomicrographs of mammary gland histology.

1 = Sham operated control; 2 = Ovariectomized rats; 3 = 17β-estradiol (0.17 mg/kg B.W.); 4 = 17β-estradiol (0.7 mg/kg B.W.); 5 = Pomegranate seed (100 mg/kg B.W.); 6 = Pomegranate seed (1000 mg/kg B.W.); 7 = Pomegranate peel (100 mg/kg B.W.); 8 = Pomegranate peel (1000 mg/kg B.W.). (Mean ± S.E.M, n=6-10), (magnification of all pictures is 400x).

3.5 Discussion

This study was specifically designed to compare the effect of pomegranate seed and peel extracts, and 17β-estradiol treatments on uterine, vagina and mammary glands in the ovariectomized rats. The administration of estrogenic substance such as 17β-estradiol clearly replaced the lack of natural estrogen as a result from ovariectomy. Chronic 17β-estradiol treatment leads to endometrial hyperplasia formation, and the numbers of perpendicular oriented mitoses become much more elevated than that after 17β-estradiol injection. Parameters, such as uterine weight, proliferation, and morphogenetic changes, were used to estimate uterine sensitivity to estradiol. Uterine weight and proliferation begin to be affected by estrogen within several hours after administration of estrogens (Couse and Korach, 1999). Therefore, these parameters can estimate both short and long term estrogen actions. However, changes in types, shapes, and architecture of tissue structures appear only under long-term estrogen exposure.

The effects of two doses of pomegranate seed and peel extracts with those of two doses of 17β-estradiol were compared in ovriectomized rats. Pomegranate seed and peel extracts significantly produced increases in uterine weight and endometrial thickness. The observations in this study were similar in degree to changes in uterine weights that have been observed in long term studies of lasofoxifene in ovaiectomized rats (Mark, 2008). Increased endometrial thickness was also observed clinically in women (McClung, 2006). Therefore, the uterine changes that were seen in this study appear to represent the biologic activity of pomegranate seed and peel extracts on the uterus.

Vaginal epithelium also subject to estrogenic action in that 17β-estradiol causes proliferation and cornification were observed. In the present study, there were increases in vagina epithelial height, number of layers, and cornification in all samples from both 17β-estradiol (0.17 and 0.7 mg/kg B.W.). Pomegranate seed extract (1000 mg/kg B.W.) was to increase epithelial height only slightly. Hence, the morphological changes induced by pomegranate in vaginal epithelium are not identical to those exerted by 17β-estradiol, suggesting that the mechanism by which this phytoestrogen affect vaginal epithelium are different from those mediating the effects of 17β-estradiol.

Mammary gland tissues were scarce in the ovariectomized rats, and a few detectable terminal structures were collapsed. 17β-estradiol (0.17mg/kg B.W.) induced luminar formation but no secretion, whereas in the mammary glands of 17β-estradiol treated (0.17 mg/kg B.W.) animals, ample tissue and signs of secretion were observed. Pomegranate seed and peel extracts have marked effects on mammary gland morphology. In deep terminal mammary structures, epithelial proliferation was increased by both17β-estradiol doses. Stimulation of mammary cell proliferation is an increased risk factor for the development of cancer (Gadducci, 2005).

In summary, pomegranate seed and peel extracts produced slight increases in uterine weight and endometrial thickness. The pomegranate seed and peel extracts exhibits estrogenic activity on uterus, vagina and mammary glands. Serum LH levels were inhibited by E_2 (both doses) but not by pomegranate extract.

3.6 References

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CHAPTER IV

THE ESTROGENIC ACTIVITY OF POMEGRANATE (*PUNICA GRANATUM* L.) EXTRACT ON INDUCING VAGINAL CORNIFICATION

4.1 Abstract

Pomegranate (*Punica granatum* L., Punicaceae) is used for the treatment of various ailments. In chapter III, GC/MS data revealed that the methanolic extracts of pomegranate seed and peel contains β-sitosterol. This compound is known to have estrogenic activity. However, their estrogenic activity is not well understood. Thus, the aim of the present study was to evaluate the estrogenic activity of methanolic extracts of pomegranate seed and peel in bilaterally ovariectomized mature rats and compared to the estrogen standard drug, 17β-estradiol. The rats were divided into eight groups (n=6-9); sham operated rats received vehicle (10% v/v Tween 80, p.o.), ovariectomized rats received vehicle (10% v/v Tween 80, p.o.), ovariectomized rats received 17β-estradiol at the different doses (0.17 or 0.7 mg/kg B.W. (s.c.)), and ovariectomized rats received methanolic extracts of the pomegranate seed or peel at the different doses (100 and 1000 mg/kg B.W. (p.o.)). These rats were administrated weekly for 7 weeks. Estrogenic activity was assessed by taking percentage vaginal cornification. The methanolic extracts of pomegranate showed increase in percentage vaginal cornification of pomegranate seed (1000 mg/kg B.W.). In conclusion, the pomegranate seed and peel extracts exhibits estrogenic activity and that will be useful for health benefits in menopause.

4.2 Introduction

Estrogen plays an important role in the growth, differentiation and function of many bodily targets, including the female and male reproductive system. Estrogen also has a variety of pharmacological functions, such as maintenance of bone mass, cardiovascular protection, and brain protection (Ciocca and Roig, 1995; Smith, et al., 1996). Estrogen deficiency during menopause can lead to risk for many health problems, such as hot flashes, sleeping disorders, vaginal dryness, reduced bone density, mood swings, and cardiovascular diseases, etc. (Palacios, 1999; Rymer, 2003). Hormone replacement therapy is suspected to increase the risk of breast cancer and to cause other undesirable side effects, such as breast tenderness and uterine bleeding (Fernandez, 2003; Humphries, 2003). Because of these undesirable side effects, women are increasingly using herbal remedies as alternative therapy. In addition, phytoestrogen is an alternative choice for estrogen replacement therapy. Phytoestrogens can be found in various species of plants especially from soy and leguminous herb. Phytoestrogens can prevente and treatment of diseases such as osteoporosis and breast cancer (Jordan, 2004). It has been found that natural compounds known as phytoestrogens, obtained from certain plants and possessing estrogen-like activity (Paola and David, 2002), can be used for the management of menopausal symptoms with few side effects (Thompson, 1993; Glazier and Bowman, 2001). Moreover, these phytoestrogens are structurally and functionally similar to estrogen (Knight, 1996).

Pomegranate (*punica granatum* L.) is an important tree of the tropical and subtropical regions of the word which is valued for its delicious edible fruit (Kader et al., 1984). The edible part of the fruit is called arils which are eaten fresh and can be

preserved as syrup or used for making jam. In addition, the tree is also valued for its pharmaceutical properties. The fruit peel, stem root bark and leaves are a good source of secondary products such as tannins, dyes and alkaloids. Pomegranate is known to contain estrogens (estradiol, estrone and estriol) and show estrogenic activities in mice (Jonko, 2004). Furthermore, pomegranate seed contain not only estrogens but also other steroids such as testosterone and β-sitosterol and coumesterol whereas anthocyanins and phenolic acids are the main ingredients of pomegranate juice. The extract of pomegranate seed and peel as investigated in this thesis (Chapter III) contains β-sitosterol. Thus, the aim of the present study was to evaluate the estrogenic activity of methanolic extracts of pomegranate seed and peel in bilaterally ovariectomized mature rats and to compare the effect with estrogen standard drug, 17β-estradiol, using vaginal cornification assay.

4.3 Materials and Methods

4.3.1 Plant Collections and Preparation of the Extracts

As described in 2.1.1, a stock of pomegranate seed and peel was kept at -20^{o}C. Pomegranate seed was prepared in Tween-80 10% (v/v) and pomegranate peel was prepared in distilled water. They were administered to the rats orally by means of an intragastric catheter.

4.3.2 Animal and Experimental Procedures

Estrogenic activity of the methanolic extract was assessed in bilaterally ovariectomized mature female Wistar rats weighing 200-250 g. The parameters of assessment were percentage of rats having vaginal cornification. The ovariectomized rats were divided into seven groups (6-9 rats). It has been reported that the estrogenic

activity of phytoestrogens ranges from 1/500 to 1/1000 to the activity of the estrogen standard drug (Cassidy, 1999). Thus, the doses of 100 or 1000 mg/kg B.W. of pomegranate seed and peel extracts were used:

Group 1 (ovarietomized rats control): received 1 ml of 10% (v/v) Tween 80 suspension (p.o.).

Group 2 (standard): received 17β-estradiol at a dose of 0.17 mg/kg B.W. (s.c.).

Group 3 (standard): received 17β-estradiol at a dose of 0.7 mg/kg B.W. (s.c.).

Group 4 (test): received 1 ml aqueous solution of pomegranate seed extract in 10% (v/v) Tween 80 at a dose of 100 mg/kg B.W. (p.o).

Group 5 (test): received 1 ml aqueous solution of pomegranate seed extract in 10% (v/v) Tween 80 at a dose of 1000 mg/kg B.W. (p.o).

Group 6 (test): received 1 ml aqueous solution of pomegranate peel extract in distilled water at a dose of 100 mg/kg B.W. (p.o).

Group 7 (test): received 1 ml aqueous solution of pomegranate peel extract in distilled water at a dose of 1000 mg/kg B.W. (p.o).

All these were administrated weekly for 7 weeks. Vaginal cornification was examined weekly.

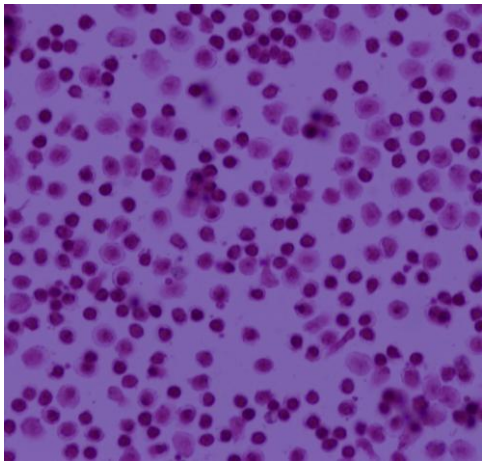
4.3.3 Vaginal Cornification Assay

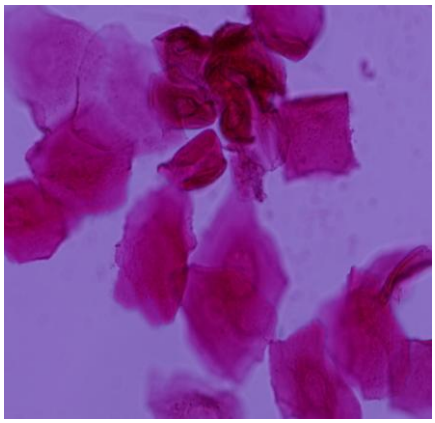
Vagina smear was performed as described in 2.7. Vaginal cells were categorized into the following 3 types: leukocyte cell, nucleated cell and cornified cell.

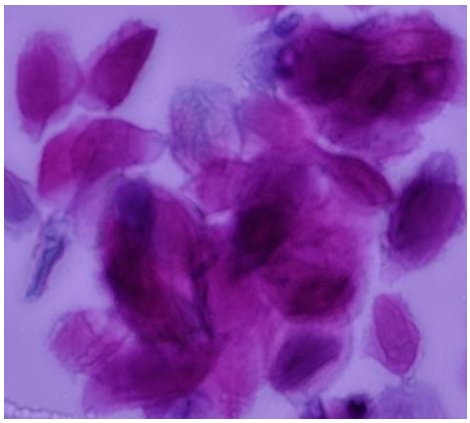
4.4 Results

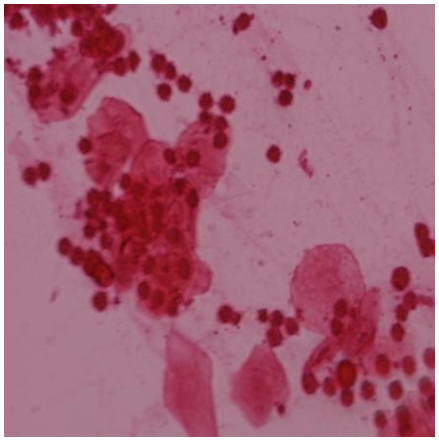
Assessments of estrogenic activity of methanolic extracts from pomegranate seed and peel were done by taking percentage vaginal cornification. The vaginal smear of ovariectomized control did not show any vaginal cornification. Only leukocytes were found (Figure 4.1A). The percentage of rats having vaginal cornification (Table 4.2) obtained 1000 mg/kg B.W. of the methanolic extract of pomegranate seeds was less than that of the standard drug 17β-estradiol at a dose of 0.17 and 0.7 mg/kg B.W.

Both pomegranate seed and peel extracts, at the different doses (100 and 1000 mg/kg B.W., induced vaginal opening. The smear showed proestrous or estrous conditions. The number of cornified cells in vaginal smears was considerably higher (+ to + +) than that of the controls (0 to +), but notably less than that of the standard drug17β-estradiol at a dose of 0.17 and 0.7 mg/kg B.W. (+ + +). The results are summarized in Table 4.1.

A 

B 

C 

D 

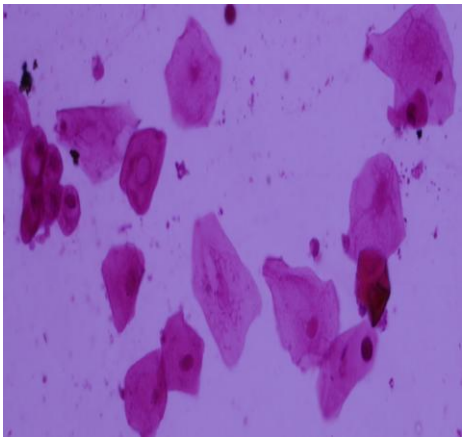
E 

Figure 4.1 Photomicrograph (20x) of methylene blue vaginal smear of control rat showing only leukocytes (A). 17β-estradiol [0.17mg/kg B.W.] (B) and [0.7 mg/kg B.W.] (C). Pomegranate seed at the different doses [100 and 1000 mg/kg B.W.] (D and E). Pomegranate peel at the different doses [100 and 1000 mg/kg B.W.] (F and G).

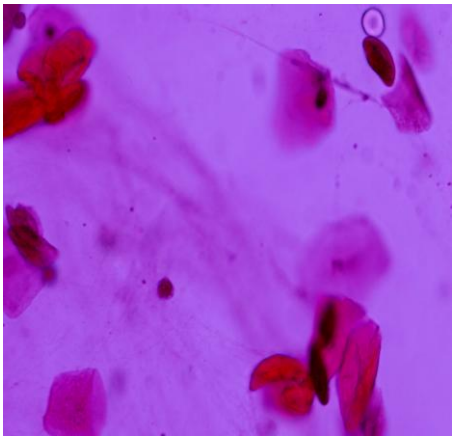
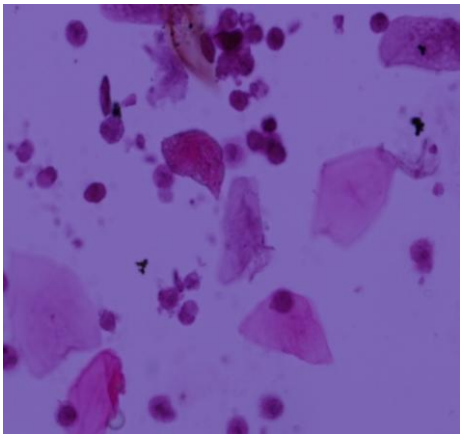
F  G 

Figure 4.1 (Continued) Photomicrograph (20x) of methylene blue vaginal smear of control rat showing only leukocytes (A). 17β-estradiol [0.17mg/kg B.W.] (B) and [0.7 mg/kg B.W.] (C). Pomegranate seed at the different doses [100 and 1000 mg/kg B.W.] (D and E). Pomegranate peel at the different doses [100 and 1000 mg/kg B.W.] (F and G).

Table 4.1 Estrogenic activity of the methanolic extract of pomegranate.

Group	N	Treatment (dose)	Vaginal cornification
1	5	Control 10% v/v Tween 80 (p.o.)	Vagina not open
2	5	Standard 17β-estradiol [0.17 mg/kg B.W. (s.c.).]	+ + +
3	5	Standard 17β-estradiol [0.7 mg/kg B.W. (s.c.).]	+ + +
4	5	Pomegranate seed extract [100 mg/kg B.W. (p.o).]	+ to + +
5	5	Pomegranate seed extract [1000 mg/kg B.W. (p.o).]	+ to + +
6	5	Pomegranate peel extract [100 mg/kg B.W. (p.o).]	+ to + +
7	5	Pomegranate peel extract [1000 mg/kg B.W. (p.o).]	+ to + +

+ = nucleated epithelial cells, ++ = nucleated and cornified cells, +++ = cornified cells only

Table 4.2 Effect of methanolic extracts of pomegranate seed and peel on vaginal cornification in bilaterally ovariectomized Wistar rats.

Group	N	Treatment (dose)	% of rats having vaginal cornification					
			Week 2	Week 3	Week 4	Week 5	Week 6	Week 7
1	5	Control 10% v/v Tween 80 (p.o.)	0	0	0	0	0	0
2	5	Standard 17β-estradiol [0.17 mg/kg B.W. (s.c.)]	100%	100%	100%	100%	100%	100%
3	5	Standard 17β-estradiol [0.7 mg/kg B.W. (s.c.)]	100%	100%	100%	100%	100%	100%
4	5	Pomegranate seed extract [100 mg/kg B.W. (p.o)]	80%	40%	40%	40%	40%	20%
5	5	Pomegranate seed extract [1000 mg/kg B.W. (p.o)]	100%	80%	80%	80%	80%	60%
6	5	Pomegranate peel extract [100 mg/kg B.W. (p.o)]	40%	20%	20%	40%	20%	20%
7	5	Pomegranate peel extract [1000 mg/kg B.W. (p.o)]	80%	80%	40%	40%	40%	20%

4.5 Discussion

During the reproductive cycle, vagina and the female reproductive tract undergo innumerable physiologic and biochemical changes under the influence of ovarian hormones such as estrogen (Prakash and Mathur, 1979). If female rats are ovariectomized, this can lead to vagina undergo atrophy and the process of vaginal keratinization and cornification can no longer be observed. However, administration of estrogenic substances to ovaricetomized females helps prevent atrophic changes of the organs and also stimulate the process of ovaginal keratinization and cornification (Vijayanarayana et al., 2007). In the present study, administration of estrogenic substance such as 17β-estradiol clearly replaced the lack of natural estrogen as a result of ovariectomy. Interestingly, it was found that the methanolic extract of pomegranate seed and peel extracts may have some estrogenic effects in ovariectomized rats as found in previously report in the same plant assessing the same parameters, despite different animal models (Sharaf, 1964; Heftmann, 1966).

The data also demonstrated that the methanolic extracts of pomegranate seed and peel caused keratinization and cornification of the vaginal epithelium. Upon the administration of the extracts, the superficial cells of vagina could be shed into the lumen to form large squamous cells. The effect on the vagina seemed to be a dose dependent as the higher dose of the extracts the higher percentage of rats having vaginal cornification were found. Thus, the increase in percentage vaginal cornification shown by the methanolic extract of pomegranate seed and peel extracts can be attributed to its estrogenic activity.

GC/MS data revealed that the methanolic extract of pomegranate seed and peel contains β-sitosterol. This compound is known to have estrogenic activity

(Kuiper, 1998). Thus, the estrogenic activity of pomegranate seed and peel extracts shown in the present study could be due to the presence of β-sitosterol.

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CHAPTER V

EFFECTS OF POMEGRANATE (*PUNICA GRANATUM* L.) EXTRACT ON BONE LOSS AND SERUM LIPID PROFILE IN OVARIECTOMIZED RATS

5.1 Abstract

Pomegranate (*Punica granatum* L.) is an important source of bioactive compounds and has been used for folk medicine for many centuries. The aim of this study was to investigate whether pomegranate seed and peel extracts were effective on experimental menopausal syndromes in ovariectomized rats. The rats were divided into eight groups, (n=6); sham operated rats received vehicle (10% v/v Tween 80, p.o.), ovariectomized rats received vehicle (10% v/v Tween 80, p.o.), ovariectomized rats received 17β-estradiol at the different doses (0.17 or 0.7 mg/kg B.W. (s.c.)), and ovariectomized rats received methanolic extracts of the pomegranate seed or peel at the different doses (100 and 1000 mg/kg B.W. (p.o.)). These rats were administrated daily for 2 months. The effects of pomegranate seed and peel extracts on bone mineral densities were measured using dual energy x-ray absorptiometry (DXA). The results showed that pomegranate seed and peel extracts (1000 mg/kg B.W.) have a tendency to increase bone mineral densities. In this study, the effects of pomegranate seed and peel extracts on lipid profile were difficult to explain because the data suggest the opposite theory. Moreover, the estrogen replacement therapy has shown no

potentiation of lipid profile. In conclusion, pomegranate seed and peel extracts can help to protect bone loss induced by estrogen deficiency.

5.2 Introduction

Menopause is a normal biological event associated with depletion of functional ovarian follicles that are the source of estradiol production. Accordingly, there is a marked decrease in estradiol levels. The fall in estrogen levels results in an unfavourable lipid profile which is believed to have deleterious effect on the cardiovascular system and the rate of calcium loss from bones in postmenopausal women (Tikkanen, 1996). It is well known that lipid metabolism is influenced by sex hormones in animals and humans (Gevers, 1994). Sex hormones such as estrogen have a major impact on atherosclerotic processes. Studies in animal models have shown that estrogen inhibited the development of atherosclerotic lesions (Sullivan, 1995). Estrogen deficiency is associated with changes in blood lipid levels. It is now clear that estrogen deficiency plays a key pathogenetic role in the development of coronary heart disease (CHD) in women, as supported by several epidemiological findings (Rosano, 2002). The risk of CHD in women increases dramatically at the onset of menopause.

Women with an early menopause are at an increased risk of cardiovascular disease (Schouw, 1996; Hu, 1999). Cardiovascular risk factor changes occurring with menopause have been considered the biological mechanism (Matthews, 1989). There is an evidence to show that menopause is accompanied by unfavorable levels of several cardiovascular risk factors (Akahoshi, 1996). The risk of coronary heart

disease drastically increases after menopause. Arteriolosclerosis induced by lipid metabolism abnormality after menopause has been recognized as a major risk factor for cardiovascular disease. The mechanism through which menopause exerts its effect on the cardiovascular system is still unknown.

Increased levels of serum total cholesterol after cessation of menses have been found in most studies on menopause and risk factors (Dallongeville, 1995). The major anti-atherosclerotic effect of estrogen is associated with its beneficial influence on lipid metabolism, including increased high-density lipoprotein (HDL), decreased low-density lipoprotein (LDL), lipoprotein and cholesterol concentrations (Seed, 1994).

Osteoporosis is a skeletal disorder characterized by bone mineral loss and impaired strength that places the bone at increased risk of fracture. The individuals at the greatest risk are women because of the accelerated bone loss associates with reduced estrogen production that results from loss of ovarian function at menopause. Individuals aged 50 years and older also represent the most sedentary segment of the adult population (King, 1998). This fact has important implications because physical activity induces the mechanical stress that maintains skeletal integrity. Bone mineral density (BMD), bone mineral content (BMC) and bone size are important risk determinants for osteoporotic fractures (Yega, 1998).

Postmenopausal estrogen replacement therapy (ERT) has shown potential for reduction or prevention of coronary heart disease (Stampfer et al., 1991), and is considered to be the most effective method to reduce the rate of osteoporosis in postmenopausal women. However, ERT may be accompanied by unacceptable side

effects such as endometrial and breast cancer in some women. Therefore, ERT is recommended only for women who have no contraindications. Thus, it would be most helpful to discover a natural and safe dietary substance that minimizes bone loss and or improves lipid metabolism in postmenopausal women. Recently, phytoestrogens received much attention for the prevention of menopause and have been looked on as an alternative to estrogen (Wuttke et al., 2002). Phytoestrogens are a group of plant-derived, biologically active substances with a chemical structure similarity accounts for the ability of these to substances to interact with the ERs in various cells and act as agonists or antagonists via ER-dependent signaling pathways (Kuiper, 1998; Miksicek, 1994).

It is well known that pomegranate seed contain the estrogenic compounds, estrone and estradiol, that are chemically identical to those biosynthesized in human body (Heftman, 1996), and coumesterol as well (Moneam et al., 1988). According to Kim et al. (2002), pomegranate seed contains not only estrogens (estradiol, estrone, and estriol) but also other steroids such as testosterone and β-sitosterol, and coumesterol, whereas, anthocyanins and phenolic acids are the main ingredients of pomegranate juice. Then, the question raised is whether pomegranate can, be as the richest plant source of estrogen, exhibit additional important biological actions on menopausal syndrome in women?

Animal models are routinely used to assess bone health and lipid metabolism because of the time and difficulties associated with performing these studies in human participants. Ovariectomize induces bone loss and lipid profile characteristics similar to some of the bone and lipid changes observed after

oophorectomy or menopause in humans (Jee, 2001). This study was therefore undertaken to compare the effect of estradiol-3benzoate and pomegranate seed and peel extracts on the serum lipid profile and mineral bone density in ovariectomized rats.

5.3 Materials and Methods

5.3.1 Measurements of Bone Mineral Density (BMD)

The right femurs of the rats were cleaned of adhering soft tissues using small scissors, tweezers and cotton gauze. BMD of the right femurs was measured by a dual energy x-ray absorptiometer (PIXImus Lunar Corp., USA version 1.4). BMD values were presented as g/cm^2.

5.3.2 Measurements of Serum Lipid

Blood was collected by cardiac puncture technique. Whole blood was allowed to clot and then centrifuged at 3000 g for 15 minutes to obtain serum, and kept at -80 ^{o}C until the determination of serum lipid. Serum levels of total cholesterol (TC), high-density lipoproteine cholesterol, low-density lipoprotein cholesterol, and triglyceride were determined using Reflotron (Roch Dianostics GmbH).

5.3.3 Statistical Analysis

All data are expressed as mean ± S.E.M. Statistical analysis was performed using the Student's t test, and values of $P<0.05$ were regarded as significant.

5.4 Results

5.4.1 Effects of Pomegranate Extracts on Bone Mineral Density

The right femur was determined by a dual energy x-ray absorptiometer (PIXImus Lunar Corp., USA version 1.4) and analysis were respectively shown in Figure 5.1 and Table 5.1. The femur bone mineral densities in the vehicle treated ovariectomized control was significantly decreased compared with the sham operated control (0.202 ± 0.001 and 0.199 ± 0.003 g/cm^2, respectively). This indicated that ovariectomy caused a decrease in BMD and that estrogen deficiency caused by ovariectomized controls stimulated marked osteoclast differentiation resulting in severe bone loss. There were significant increases in the BMD of the femurs after 8 weeks of the treatment with 17β-estradiol (0.17 and 0.7 mg/kg B.W.). The administration of pomegranate seed extract (1000 mg/kg B.W.) was not statistically different when compared between the sham operated controls and ovariectomized rats. However, it showed a tendency to increase BMD (0.200 ± 0.005 g/cm^2).

5.4.2 Effects of Pomegranate Extracts on Serum Lipid Profile in Rats

The levels of total serum cholesterol in vehicle treated ovariectomized control did not differ when compared to sham operated control (Figure 5.2A and Table 5.2). In ovariectomized rats treated with 17β-estradiol (0.17 and 0.7 mg/kg B.W.) the levels were significantly increased when compared to that of sham operated controls and ovariectomized rats.

However, the levels of total serum cholesterol in the groups treated with pomegranate seed extract (100 mg/kg B.W.) and pomegranate peel (100 and 1000 mg/kg B.W.) extract did not differ when compared to sham operated control.

Clinical studies have shown that estrogen therapy decreases total cholesterol and LDL cholesterol but increases HDL cholesterol (Selzman, 1997). The serum HDL in the vehicle treated ovariectomized rats was increased compared with the sham operated controls ($P<0.05$) (Figure 5.2 B and Table 5.2). The serum HDL in ovariectomized rats treated with 17β-estradiol (0.17 and 0.7 mg/kg B.W.) were significantly increased compared with the sham operated controls and ovariectomized controls. As can be seen, the methanolic extract of pomegranate seed and peel extracts caused increases in serum HDL but this did not differ when compared to sham operated control and ovariectomized rats.

However, the serum LDL in the treated ovariectomized rats was significantly increased compared with the sham operated control (Figure 5.2 C and Table 5.2). The serum LDL in ovariectomized control treated with 17β-estradiol (0.17 and 0.7 mg/kg B.W.) were significantly increased compared with the sham operated controls. The serum LDL in the rats treated with pomegranate seed and peel extracts (100 and 1000 mg/kg B.W.) were decreased serum LDL compared to ovariectomized rats but this did not differ when compared to sham operated control.

In addition, serum triglyceride in the vehicle treated ovariectomized control was significantly increased compared with the sham operated control (Figure 5.2 D and Table 5.2). The serum triglyceride in ovariectomized control treated with 17β-estradiol (0.17 and 0.7 mg/kg B.W.) were significantly decreased when compared

with the sham operated controls and ovariectomized rats. The serum triglyceride in the rats treated with pomegranate seed extracts (100 and 1000 mg/kg B.W.) was significantly decreased when compared with the ovariectomized rats.

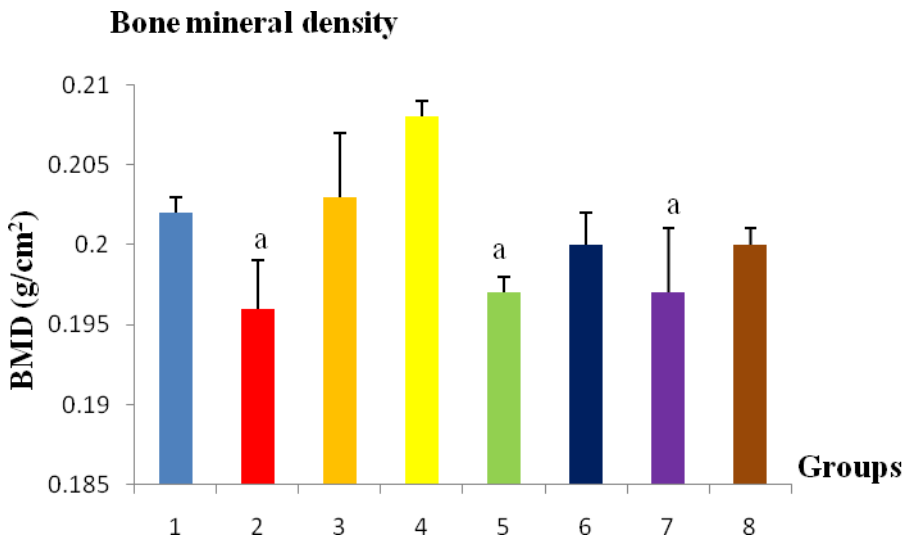
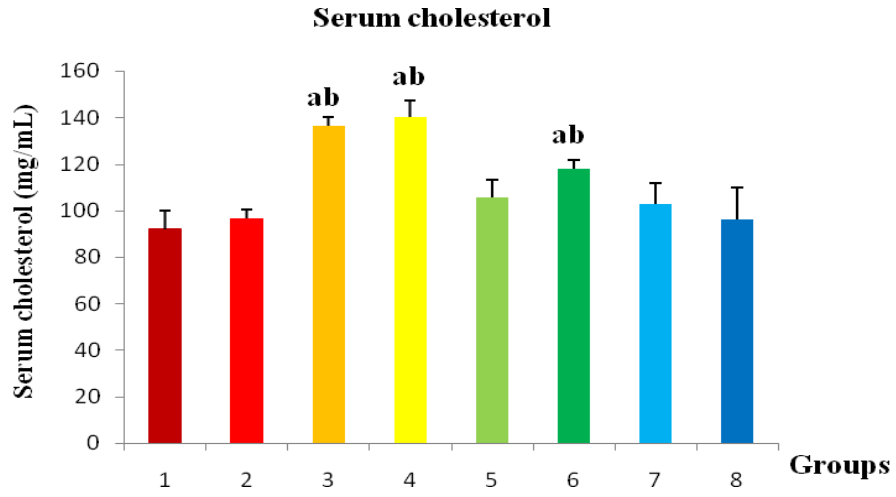


Figure 5.1 Effects of pomegranate on bone mineral densities of the right femur.

1 = Sham operated control; 2 = Ovariectomized rats; 3 = 17β-estradiol (0.17 mg/kg B.W.); 4 = 17β-estradiol (0.7 mg/kg B.W.); 5 = Pomegranate seeds extract (100 mg/kg B.W.); 6 = Pomegranate seeds extract (1000 mg/kg B.W.); 7 = Pomegranate peel extract (100 mg/kg B.W.); 8 = Pomegranate peel extract (1000 mg/kg B.W.); (mean ± S.E.M, n=6).

A



B

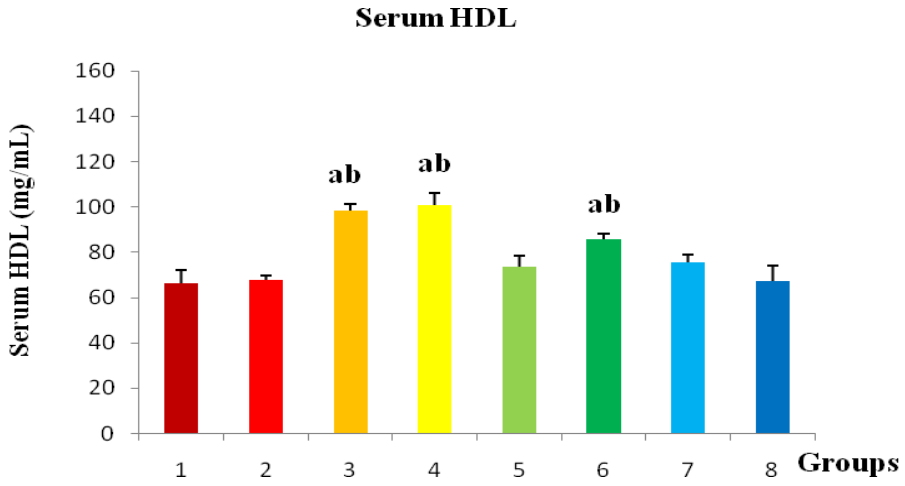
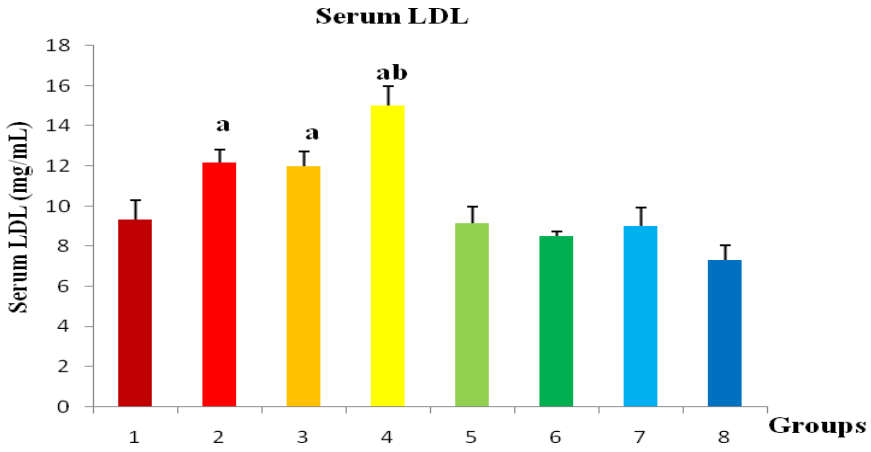


Figure 5.2 Effects of pomegranate extract on serum cholesterol (A), serum HDL (B), serum LDL (C) and serum triglyceride (D). 1 = Sham operated control; 2 = Ovariectomized rats; 3 = 17β-estradiol (0.17 mg/kg B.W.); 4 = 17β-estradiol (0.7 mg/kg B.W.); 5 = Pomegranate seeds extract (100 mg/kg B.W.); 6 = Pomegranate seeds extract (1000 mg/kg B.W.); 7 = Pomegranate peel extract (100 mg/kg B.W.); 8 = Pomegranate peel extract (1000 mg/kg B.W.); (mean ± S.E.M, n=6).

C



D

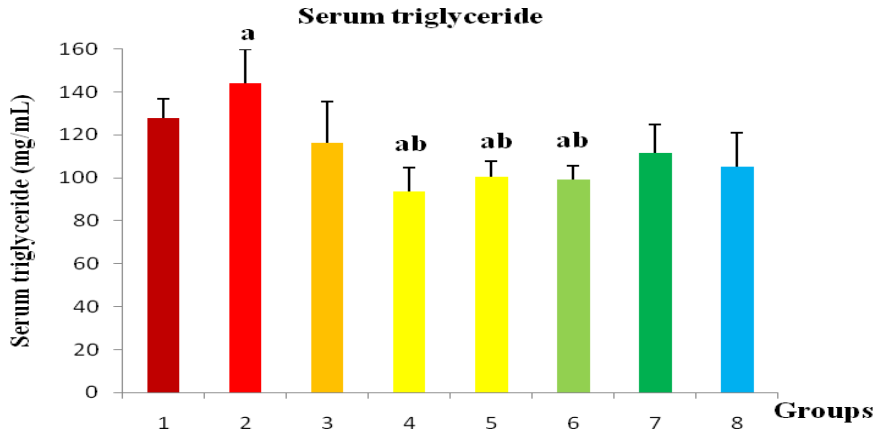


Figure 5.2 (Continued) Effects of pomegranate extract on serum cholesterol (A), serum HDL (B), serum LDL (C) and serum triglyceride (D). 1 = Sham operated control; 2 = Ovariectomized rats; 3 = 17β-estradiol (0.17 mg/kg B.W.); 4 = 17β-estradiol (0.7 mg/kg B.W.); 5 = Pomegranate seeds extract (100 mg/kg B.W.); 6 = Pomegranate seeds extract (1000 mg/kg B.W.); 7 = Pomegranate peel extract (100 mg/kg B.W.); 8 = Pomegranate peel extract (1000 mg/kg B.W.); (mean ± S.E.M, n=6).

Table 5.1 Effect of pomegranate extract on bone mineral density.

Group	BMD (g/cm^2)
Sham operated control	0.202 ±0.001
Ovariectomized rats	0.195 ±0.003[a]
Standard 17β-estradiol [0.17 mg/kg B.W. (s.c.).]	0.203 ±0.005
Standard 17β-estradiol [0.7 mg/kg B.W. (s.c.).]	0.208 ±0.001[a,b]
Pomegranate seed extract [100 mg/kg B.W. (p.o.).]	0.197 ± 0.001[a]
Pomegranate seed extract [1000 mg/kg B.W. (p.o.).]	0.200 ± 0.002
Pomegranate peel extract [100 mg/kg B.W. (p.o.).]	0.198 ± 0.005[a]
Pomegranate peel extract [1000 mg/kg B.W. (p.o.).]	0.200 ± 0.001

Results are mean ± S.E.M; n=6

[a] $P<0.05$ vs sham operated control

[b] $P<0.05$ vs ovariectomized control

Table 5.2 Effect of pomegranate extract on serum lipid profile.

Group	Lipid parameters (mg/dL)			
	Total serum cholesterol	HDL-cholesterol	LDL-cholesterol	Triglycerides
Sham operated control	92 ± 8.09	66.17 ± 5.89	9.33 ± 0.95	127.67 ± 8.90
Ovariectomized rats	96.83 ± 3.47	67.67 ± 2.21	12.17 ± 0.61[a]	144.00 ± 17.08[a]
Standard 17β-estradiol [0.17 mg/kg B.W. (s.c.).]	136.50 ± 3.83[ab]	98.50 ± 2.87[ab]	12.00 ± 0.73[a]	116.33 ± 19.12
Standard 17β-estradiol [0.7 mg/kg B.W. (s.c.).]	140.33 ± 6.86[ab]	100.83 ± 5.43[ab]	15.00 ± 0.96[ab]	93.83 ± 10.78[ab]
Pomegranate seed extract [100 mg/kg B.W. (p.o.).]	105.69 ± 7.74	73.67 ± 4.57	9.17 ± 0.79	100.50 ± 7.35[ab]
Pomegranate seed extract [1000 mg/kg B.W. (p.o.).]	118.17 ± 3.58[ab]	85.50 ± 2.66[ab]	8.50 ± 0.24	99.00 ± 6.52[ab]
Pomegranate peel extract [100 mg/kg B.W. (p.o.).]	103.00 ± 4.52	75.33 ± 3.63	9.00 ± 0.93	111.67 ± 12.98
Pomegranate peel extract [1000 mg/kg B.W. (p.o.).]	96.33 ± 8.74	67.33 ± 2.41	7.33 ± 0.71	105.3 ± 15.57

Results are mean ± S.E.M; n=6 [a]$P<0.05$ vs. sham operated control, [b]$P<0.05$ vs ovariectomized control.

5.5 Discussion

It is well known that bone loss is a characteristic of menopausal syndrome and is attributable to the cessation of ovarian function and tapering off of estrogen secretion. Furthermore, estrogen replacement therapy can prevent the early phase of involutional bone loss and also restore the rate of bone resorption and formation to pre-menopausal levels in menopause women (Barzel, 1988; Heshmati et al., 2002).

The rat model is useful in studying of osteoporosis. The ovariectomized rat was judged to be the standard animal for the study of bone loss caused by estrogen deficiency (Thompson, 1995).

The main purpose of this study was to evaluate whether pomegranate extracts is effective in preventing bone loss due to ovariectomy and if so, whether its functions in a manner similar to estrogen. One of the treatment groups in this study received estrogen. This group served as a positive control group, because the bone conserving effects of estrogen are well established in an ovariectomized rat model of osteoporosis. Data on bone mineral density the observations of other investigators that bone loss due to ovarian hormone deficiency is prevented by estrogen administration (Kalu, 1991).

In the aforementioned results, it is shown that these pomegranate seed and peel extracts (1000 mg/kg B.W.) exert significant protective effect on the bone density of the femur. The data demonstrated that the methanolic extracts of pomegranate seed and peel similarly characterized phytoestrogens. In addition, phytoestrogens have shown a weak estrogenic effect on bone in human and animal studies (Messina, 2000).

Phytoestrogens might have beneficial effects on bone metabolism and osteoporosis but the evidence from experimental and observation studies is very limited (kning, 1996; Murkies et al., 1998; Tham et al., 1998). In oophorectomized rats, phytoestrogens were reported to prevent or reduce bone loss (Draper et al., 1997; Fanti et al., 1998). In a human clinical study, Potter et al. (1998) reported the effects of soy protein and phytoestrogens on BMD in postmenopausal women. In this study the group taking soy protein, with associated high concentrations of isoflavone, significantly increased both bone mineral density and content in the lumbar spine, but not in other skeletal area, as compared to group consuming casein dry milk.

Regarding the role of estrogens in lipid metabolism, estrogen insufficiency is thought to be largely responsible for an increase in adiposity during menopause because postmenopausal women under estrogen replacement therapy do not display the characteristic pattern of abdominal weight gain usually associating with menopause (Gambacciani et al., 1997). When female enters menopause, the final stage of female reproductive age, the ovary and uterus begins to shrink and degenerate. Thus, estradiol and progesterone production sharpy declines, which disrupt the feedback, control mechanisms of the hypothalamic pituitary ovarian axis. The increase in follicle stimulating and luteinizing hormone levels, together with the decrease in estrogen level, lead to other menopausal disorders, such as heart disease, osteoporosis, etc. (Tsavachidou, 2002). It is well known that abdominal adiposity leads to dyslipidemia. Changes in lipid metabolism seen after menopause are characterized by an overall shift toward a more atherogenic lipid profile, which is also seen in metabolic syndrome (increased LDL cholesterol and triglyceride levels but

decreased HDL cholesterol). These adverse changes in lipid metabolism seen after menopause may also contribute to the increased risk of coronary heart disease.

Clinical studies have showed that estrogen therapy decreases total and LDL cholesterol but increases HDL cholesterol (Selzman et al., 1997). Data from experiment did not clearly show that estrogen replacement therapy subcutaneously of 17β-estradiol 3-bebzoate; E_2B (0.17 and 0.7 mg/kg B.W.) administration in ovariectomized had significantly increased cholesterol, LDL cholesterol and HDL cholesterol when compared to ovariectomized control. However, serum triglycerides were unaffected. This is in contrast to the report of Dominik (2007) showing that E_2B (10 mg/kg B.W.) treated animal, in which plasma HDL cholesterol and LDL cholesterol were significantly decreased but triglycerides were slightly increased compared to control animals. Estrogen administration in rats did not only decrease LDL cholesterol but also HDL cholesterol levels. This is due to the fact that the predominant plasma cholesterol in rat is HDL but not LDL, comprising approximately 60% to 70% of the total cholesterol pool. Thus, the extracts did not have clearly effects on lipid profile in ovariectomized rats. The increase in serum cholesterol level following hormone therapy is, presumably, due to an increase in hepatic secretion of cholesterol. Administration of methanolic extracts of pomegranate seed and peel were not able to compare with β-estradiol 3-bebzoate and ovariectomized control. The present study suggested that feeding pomegranate extracts orally was not beneficial on improving serum lipid profile in ovariectomized rats. The chemical constituents of both pomegranate seed and peel were established and analyzed by GC-MS. According

to the fingerprint, there was considerable partly of β-sitosterol. This compound is known to have estrogenic activity (Kuiper, 1998).

In conclusion, the results show that pomegranate seed and pomegranate peel (1000 mg/kg B.W.), have a tendency to increase bone mineral densities. In this study, the effects of pomegranate seed and peel extracts on serum lipid profile were not as effective as in ovariectomized rats as estrogen replacement therapy. This information will aid the development of a better understanding of their biological roles in human bone metabolism and cardiovascular disease.

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CHAPTER VI

ANTI-IMPLANTATION ACTIVITY OF POMEGRANATE EXTRACT IN PREGNANT RATS

6.1 Abstract

Many plants are known to possess anti-fertility activity. However, limited attempts have been made to scientifically evaluate these postulates. Pomegranate (*Punica granatum* L., Punicaceae) seed and peel have been shown to possess abortifacient activity. The aim of the present study was to evaluate the anti-implantation activity of methanolic extracts of pomegranate seed and peel in pregnancy rats. The rats were divided into five groups, (each group contained 6 rats); pregnant rats control received distilled water; pregnant rats received methanolic extracts of pomegranate seed and peel at the different doses (100 and 1000 mg/kg B.W. (p.o.)) from day 1 to day 7 of pregnancy. Treatment of dams during the implantation period showed no signs of toxicity, and no significant difference was observed with pregnant rats control groups. The percentage of implantation in the group treated with pomegranate seed and peel extracts (100 mg/kg B.W.) did not differ, but the percentage of implantation in the groups fed with pomegranate seed and peel extracts (1000 mg/kg B.W.) differed from the groups that fed with a dose of pomegranate seed or peel extracts (100 mg/kg B.W.). In conclusion, the pomegranate seed and peel extracts (100 and 1000 mg/kg B.W.) exerted anti-implantation activity. Thus, the understanding of such activity will help to control population.

6.2 Introduction

Population control is of great importance for a country's development and progress. For centuries, efforts have been targeted to develop safe and effective contraceptives from natural sources. The use of natural products as alternative therapy is common in countries with populations that do not have access to scientific medical assistance. In Central and Latin America, a decoction or infusion ingestion prepared with plant extracts is used popularly for fertility control, and for producing temporary sterility, possibly by interfering with embryonic implantation. Several plants have confirmed as antifertility, abortive, uterine stimulant, estrogenic or cytotoxic agents in animals and humans (Farnsworth et al., 1975). The importance of plants as a source of antifertility drugs has been emphasized by many researchers (Chaudhury, 1986). Anti-fertility agents obtained from indigenous plants would be of immense benefit especially to inhabitants of developing countries, since the cost of these drugs would be within their means. Moreover, a drug prescribed by the traditional medical practitioner, in the form of a crude extract or a semi purified isolate would be more acceptable to rural people in addition to being freely available.

Implantation is a very crucial event in reproductive physiology. Several biochemical, biophysical and hormonal changes take place prior to this event (Laloraya, 1990). Embryo implantation in the human is still a poorly understood process. The ovum is fertilized in the ampulla, near the ampullary-isthmic junction, where it resides for some 72 hours (Croxatto et al., 1978). During this time period cell division and compaction occur to form a morula. Under the influence of ovarian steroids, the autonomic nervous system, and the developing embryo itself, the morula is transported through the isthmus to the uterus (Croxatto, 2002). Following entry of

the morula into the uterine cavity, cell polarity is established and lineage differentiation occurs, forming a blastocyst. The blastocyst begins to express and transcribe over 500 previously dormant genes. This "activated" blastocyst hatches from the zona pellucid approximately 72 hours after entry into the uterine cavity.

Hatching, as it is understood from animal models, is a consequence of hydrostatic pressure exerted by the expanding blastocyst and proteolytic enzymes released by the blastocyst (e.g., strypin) and the endometrium (e.g., tryptase) that lyses the zona pellucid (Perona and Wassarman, 1986; Lee et al., 1997; Sullivan, 2004). The blastocyst then begins to invade the endometrium, a process called implantation. Implantation occurs 7 to 10 days after fertilization and usually occurs on the posterior wall of the fundus or corpus of the uterus.

Biochemical communication between the preimplantation blastocyst and the endometrium occurs prior, during, and after hatching. Chorionic gonadotropin released from the blastocyst and cytokines from both the blastocyst and endometrium begin the process of blastocyst endometrium signaling essential for implantation. Concurrently, ovarian steroids prepare the uterus for implantation. The preovulatory increase in the secretion of 17β-estradiol stimulates proliferation and differentiation of the endometrial epithelial cells. The marked increase in progesterone production after ovulation causes endometrial stromal edema, leading to effective closure of the uterine lumen whereby the blastocyst comes into intimate contact with the endometrial epithelium. Implantation begins 6 to 7 days after fertilization (Vigano et al., 2003). Once adhesion of the blastocyst to endometrium is complete, invasion begins and the trophoblast penetrates the uterine epithelium. By day 10, post fertilization, the

blastocyst is completely embedded in subepithelial stromal tissue and the uterine epithelium grows to cover the implantation site (Benirschke, 1991).

In the rat, zona pellucid lysis occurs on day 5 of pregnancy and the blastocysts then attach to the uterus (Surani, 1975). Giavini et al. (1984) revealed that drugs and other common chemicals pass from general circulation into uterine fluid and penetrate readily into the embryo before its implantation. But there is little information about the toxic effects of chemicals or physical agents on mammalian embryos during this period (Persaud, 2000). The preimplantation period of pregnancy is considered to be an "all-or-none" period, i.e., the period during which maternal exposure to exogenous agents may cause either embryolethality or a normal fetus at delivery (Giavini et al., 1990).

In order for blastocyst implantation to occur in the rat, the endometrium must be adequately differentiated, a state which results from the sequential action of progesterone (P_4) and 17β-estradiol (E_2) (Abrahamson, 1993). Implantation has long been known to be a steroid hormone dependent process. Coordinated action of both estrogen (E_2) and progesterone (P_4) are necessary for the preparation and process of implantation into the mouse endometrium. During normal mouse pregnancy, an E_2 surge on day (d) 1 (d1 = vaginal plug) stimulates uterine epithelial cell proliferation. A decrease in E_2 levels on d2 leads to apoptosis of a large number of epithelial cells. P_4, from the newly formed corpra lutea on day 3, initiates uterine stromal cell proliferation. In conjunction with the high P_4, an acute E_2 spike on day 4 further stimulates uterine stromal proliferation and renders the uterus receptive for the blastocyst to implant (Carson, 2000). In mice, the process of implantation consists apposition between the trophectoderm layer of the blastocyst with the luminal

epithelium, attachment of these layers and finally, invasion of the uterine luminal epithelium by the embryo. Upon invasion, the uterine stromal cell is rapidly remodeled in the process of decidualization (Lee and Demayo, 2004).

There are many different ways in which herbs can impair fertility. Some herbs may affect the ovary, while others act upon the uterus, affect normal hormone production or block certain hormones. Some herbs have the ability to interfere with implantation. A large number of plants has been used in folk medicine for centuries, including *Punica granatum;* a fruit used by ancient women to prevent conception. This is more for historical interest. Rudolf Fritz Weiss (2001) notes the seeds contain an oestrone identical to the genuine hormone (estrogen) and states *Punica granatum* seeds are the best source of plant oestrone to date.

Punica granatum L. has acquired various regional names pomegranate. This plant is reputed to possess varied medicinal properties, such as pomegranate juice, seed oil, peel or flower extracts, and their derivatives to kill bacteria and viruses, or to fight vascular disease, diabetes and cancer. The pomegranate is a symbol of life, longevity, health, femininity, fecundity, knowledge, morality, immortality and spirituality. The bark and roots believed to have anthelimintic and vermifuge properties (Naovi et al., 1991). The peels a powerful astringent and cure for diarrhea and oral aphthae, and the juice a “refrigerant” (Arseculeratne et al., 1985) and blood tonic (Lad and Frawley, 1986). In India (Nagaraju and Rao, 1990), dried pomegranate peel are decocted in water and employed both internally and externally for numerous problem demanding astringents and/or germicides, especially for aphthae, diarrhea and ulcers. Mixtures of pomegranate seed, juice and peel products paradoxically have

been reported to not only prevent abortion (Ramirez et al., 1988) but also conception (Gujral et al., 1960; Jochle, 1971).

Therefore, the aims of the present study were to investigate 1) if pomegranate seed and peel extracts, in doses higher than that used popularly to contraception, interfere the reproductive performance of rats and 2) the correlation of the ingestion of this extract with possible alterations of embryonic implantation of the rats.

6.3 Materials and Methods

6.3.1 Animals

After a ten days of acclimatization under standard conditions 22 ± 4^{o}C, 12-h light/dark, virgin female Wistar rats, weighing 200-230 g each, were maintained in a temperature and light-controlled room, with access to water and food *ad libitum*.

6.3.2 Experimental Procedure

Vaginal smears from each rat were monitored daily. Only rats with normal oestrous cycle were selected. The selection of animals for using in the study was determined by the presence of at least two consecutive 4-day oestrous cycles. Anti-implantation activity was determined as described by Khanna and Chaudhary (1968). Rats found in proestrus phase of cycle were caged with males of proven fertility. Mating was monogamous (one male to one female). Every morning during the mating period, each female was examined for the presence of sperm plugs or sperm in vaginal smears. The day when spermatozoa were noted in the vaginal smear was designated as day 1 of pregnancy and those rats were divided into five groups (n=6). The mated

females were randomly divided into five groups; one control and four experimental groups which received daily treatment by gavages during the implantation period (day 1-7 of pregnancy). The doses used in this experiment were higher than those used popularly, because previous studies carried out in our laboratory showed that the popular dose presented no alteration in reproductive performance [unpublished data].

The experimental groups were designed as follows:

Group1 (pregnant rat control): received 1 mL of distilled water.

Group 2 (test): received 1 mL of aqueous solution of pomegranate seed extract in distilled water at a dose of 100 mg/kg B.W. (p.o.).

Group 3 (test): received 1 mL of aqueous solution of pomegranate seed extract in distilled water at a dose of 1000 mg/kg B.W. (p.o.).

Group 4 (test): received 1 mL of aqueous solution of pomegranate peel extract in distilled water at a dose of 100 mg/kg B.W. (p.o.).

Group 5 (test): received 1 mL of aqueous solution of pomegranate peel extract in distilled water at a dose of 1000 mg/kg B.W. (p.o.).

On day 10, pregnant rats were euthanized under CO_2. Uterine horns were removed. The numbers of implantation sites in both horns of uterus were recorded. Distribution of implantation and embryos in development were recorded. Anti-implantation was expressed as percentages using the following formula (Williamson, 1996). ; Anti-implantation activity =

$$\frac{\text{No. of implants in control (Group 1)-No. of implants in test group (Group 2-5) x 100}}{\text{No. of implants in control group (Group 1)}}$$

6.3.3 Statistical Analysis

The data were statistically analyzed and expressed as mean ± S.E.M. Statistical analysis of the variance between control and experimental values was done using Student's *t*-test ($P<0.05$) (Gupta, 1978).

6.4 Results

6.4.1 Weight Gain

The results obtained from the methanolic extract of pomegranate seed and peel were given in Table 1 and 2. No significant differences were found between body weight gain in Group 2-5 and Group 1 at day 10 of pregnancy (Table 1).

6.4.2 Anti-implantation Activity

An anti-implantation activity is expressed as percentage of implantation in uteri laparotomised on day 10 of pregnancy. These were 10.83 ± 0.7% in the control group, and 10.17 ± 0.4%, 10.17 ± 0.5%, 9.83 ± 0.5% and 9.67 ± 1.4% in group 2, 3, 4 and 5 respectively. Table 2 shows number of implantations in control and treated animals. Treated animals showed 6.09 %, 6.09 %, 9.23% and 10.71% inhibition of blastocyst implantation as compared control animals. No significant difference was observed in respect to the implantation index among groups. The methanolic extract of pomegranate seed and peel showed statistically significant ($P<0.05$) increase in visual examination. There was also no change in the body weight of animals.

Table 6.1 Effects of pomegranate seed and peel extracts on body weight of all groups.

Group	Body weight (g)	
	1st day of pregnancy	10th day of pregnancy
Control	218.33 ± 1.66	238.33 ± 3.07
Pomegranate seed (100 mg/kg B.W.)	218.33 ± 1.66	236.67 ± 4.21
Pomegranate seed (1000 mg/kg B.W.)	220.00 ± 0.00	245.00 ± 2.23
Pomegranate peel (100 mg/kg B.W.)	218.33 ± 1.66	241.67 ± 3.07
Pomegranate peel (1000 mg/kg B.W.)	221.67 ± 1.66	231.67 ± 4.01

*$P<0.05$, when compared with control

Table 6.2 Maternal organ weight of Wistar rats treated with pomegranate seed and peel extracts.

Group	Maternal Organs (g)	
	Ovary	Liver
Control	0.105 ± .00	11.10 ± .00
Pomegranate seed (100 mg/kg B.W.)	0.095 ± .00	10.60 ± .00
Pomegranate seed (1000 mg/kg B.W.)	0.096 ± .00	10.39 ± .00
Pomegranate peel (100 mg/kg B.W.)	0.075 ± .00	10.04 ± .01
Pomegranate peel (1000 mg/kg B.W.)	0.098 ± .00	10.24 ± .00

*$P<0.05$, when compared with control

Table 6.3 Effects of pomegranate seed and peel extracts on implantation in rats when fed orally from days 1 to 7 of pregnancy.

Group	Numbers rats without implantation sites	Uterine weight (mg)	Numbers of implantation mean ± S.E.M.	% anti-implantation
Control	Nil	388.70 ± 29.97	10.83 ± 0.7	Nil
Pomegranate seed (100 mg/kg B.W)	Nil	397.94 ± 39.39	10.17 ± 0.4	6.09
Pomegranate seed (1000 mg/kg B.W)	Nil	527.83 ± 39.06*	9.83 ± 0.5	9.23
Pomegranate peel (100 mg/kg B.W)	Nil	424.71± 20.38	10.17 ± 0.5	6.09
Pomegranate peel (1000 mg/kg B.W)	Nil	411.56 ± 26.49	9.67 ± 1.4	10.71

*$P<0.05$, when compared with control

6.5 Discussion

In the present study, the seed and peel of pomegranate were tested for their anti-implantation activity. The loss of implantation caused by methanol extract may be due to antizygotic, blastocytotoxic or anti-implantation (Hafez, 1970). Pre-implantation losses can also arise due to disruption of events that are prerequisite for fertilization or impairment in the production of cytokines, growth factors and various types of adhesion molecules, either by the developing blastocyst or by uterine epithelium around the site of implantation (Denker and Haimovici, 1993). Hence, the anti-implantation activity of these compounds may be due to an imbalance in endogenous estrogen and progesterone levels. Furthermore, sitosterol has been shown to affect reproductive tissues in that β-sitosterol may act as an abortifacient in animals (Burck, 1982). Therefore, β-sitosterol, the most constituents found in pomegranate seed and peel extracts will help to antiimplantion. In the present study, the decrease in the wet weight of the ovaries in the extract treated animals compared to the control animals may indicate inhibition of ovulation through suppression of follicular stimulating hormone.

In conclusion, the present study indicates that the methanolic extracts of pomegranate seed and peel exert significant anti-implantation activity. In the future, it is worth investigating the mechanism whereby the methanolic extracts of pomegranate seed and peel exert its anti-implantation activity.

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CHAPTER VII

EFFECTS OF POMEGRANATE (*PUNICA GRANATUM* L.) EXTRACT ON UTERINE CONTRACTILITY

7.1 Abstract

The pomegranate (*Punica granatum* L., Punicaceae) is known to contain estrogens (estradiol, estrone, and estriol) and shows estrogenic activities in rats, but its possible uterotonic effects have not yet been demonstrated. The aims of the study were to 1) investigate the effects of pomegranate seed and peel extracts on rat uterine contraction, 2) examine the effects on spontaneous phasic contraction, and 3) study the mechanisms whereby they exert their effects. Pomegranate seeds and peels were methanolic extracted and structural constituents were then analyzed using GC/MS. Rats were humanely killed by asphyxiation with CO_2, and longitudinal uterine smooth muscle strips were isolated. Isometric force was measured and the effects of pomegranate seed and peel extracts were studied. Pomegranate seed and peel extracts increased spontaneous contraction in a concentration dependent manner with a maximum effect at of 250 mg/100 mL and 70 mg/100 mL, respectively. The amplitude and the frequency of the phasic contraction were significantly increased as well as the basal tension. Force produced in the presence of pomegranate seed and peel extracts were abolished by inhibition of L-type calcium channels or myosin light chain kinases (MLCK). Contractions were not potentiated by pomegranate extract

following inhibition of K^+ channels. In summary, pomegranate extract may be a useful uterine stimulant.

7.2 Introduction

Under physiological conditions there appears little doubt that myosin light chain kinase (MLCK) is the selective and dedicated enzyme that brings this about (Moore and Bernal, 2001). Calcium ions binding to calmodulin activate MLCK and therefore initiate the phosphorylation and subsequent cross-bridge cycling. There are two sources for the increase in activator Ca^{2+}: entry across the surface membrane through voltage-gated L-type Ca^{2+} channels and/or release from the sarcoplasmic reticulum (SR). This Ca^{2+} is from the extracellular space via VOCCs or ROCCs or from the SR via IP_3 or RyR receptor/Ca release channels. Calcium ions binding to calmodulin activate MLCK and therefore initiate the phosphorylation and subsequent cross-bridge cycling, which force development and shortening of muscle (Walsh, 1994). Relaxation of the smooth muscle follows the closing of the Ca^{2+} channels and lowering of the cytoplasmic Ca^{2+} concentration by the action of Ca^{2+}-ATP ase active transport pumps (Fox, 2004). Under those conditions, calmodulin dissociates from the myosin light-chain kinase, thereby inactivating this enzyme. The phosphate groups that were added to the myosin are then removed by a different enzyme, a myosin phosphatase. Dephosphorylation inhibits the cross bridge from binding to actin and producing another power stroke.

Ion channel currents have been very well characterized in rat and human myometrium. For instance, rat myometrium is reported to have both slow L-type Ca^{2+} channels and fast Na^+ channels, but not T-type Ca^{2+} channels (Ohya and Sperelakis,

1989), while pregnant human myometrium is known to express both T-type and L-type Ca^{2+} channels (Young et al., 1993).

The sarcoplasmic reticulum (SR) is able to take up Ca^{2+} agonist the electrochemical gradient due to ATP-dependent Ca^{2+} pump in the SR membrane (Shmygol and Wray, 2004). The role of the SR is to feedback and limit contractility by contribution of Ca^{2+} induced Ca^{2+} release (CICR) through ryanodine (RyR) gated calcium channels producing force (Taggart and Wray, 1998).

Release of Ca^{2+} from uterine SR has been demonstrated in human and animal myometrial preparations (Taggart and Wray, 1998; Luckas et al. 1999). Both IP_3 and ryanodine (Ry) receptors have been identified on the SR. It now seems likely, however, that the Ca^{2+} released from these receptors contributes little to the activation of contraction. The evidence for this comes from experiments in a variety of species that show an increase in both Ca^{2+} transients and contraction when the SR is disabled (Taggart and Wray, 1998a; Kupittayanant et al., 2002; Noble and Wray, 2002). This can be done using drugs such as cyclopiazonic acid which inhibit the SR Ca^{2+} -ATPase (SERCA) required to transport Ca^{2+} into the SR.

Punica granatum L. (Punicaceae), commonly called pomegranate, is a large deciduous shrub or small tree used medicinally in Europe, Indo-China, the Philippine Islands and South Africa. The plant is used in folklore medicine for the treatment of various diseases, such as ulcer, hepatic damage, snakebite, etc (Ajaikumar, 2004). The rind is valued as an astringent in diarrhea and dysentery. The pomegranate seed are considered to be stomachic and the pulp cardiac and stomachic (Satyavati et al., 1978). In addition, it has been reported that pomegranate treatment of treatment of colic, colitis, diarrhea, dysentery, leucorrhea, menorrhagia, oxyuriasis,

paralysis and rectocele, and as external applications to caked breast (Duke and Ayensu, 1985) and to the nape of the neck in mumps (Boulos, 1983) and headace (Ayensu, 1981). Furthermore, a number of therapeutic actions of these materials have been described including vermifugal, taenicidal, astringent, antispasmodic, antihysteric, and diuretic, carminative. Sudorific, galactogogue, and emmennagogue (Bianchini, 1979). Pomegranate flowers serve as a remedy for diabetes mellitus (Saxena and Vikram, 2004). Modern uses of pomegranate derived products now include treatment of acquired immune deficiency syndrome (AIDS) (Lee and Watson, 1998). In addition, mixtures of pomegranate seed, juice and peel products paradoxically have been reported to not only prevent abortion (Ramirez et al., 1988) but also conception (Gujral et al., 1960; Jochle, 1971). However, the use of pomegranate as uterotonic agents is not well understood and effects of pomegranate on uterine contraction have not yet been demonstrated.

Therefore, the aims of this chapter were to 1) investigate the effects of pomegranate seed and peel extracts on rat uterine contraction, 2) examine the effects on spontaneous phasic contraction, and 3) study the mechanisms whereby they exert their effects.

7.3 Materials and Methods

7.3.1 Chemicals and Physiological Solution

All chemicals were purchased from Sigma® unless state otherwise. Antagonists for investigation of physiological pathways used were as follows. Wortmannin was dissolved in DMSO at a concentration of 4 μM. Nifedipine was

dissolved in DMSO at a concentration of 10 μM. Tetraethylammonium (5 mM) was dissolved in distilled water.

7.3.2 Preparations of Pomegranate Seed and Peel Extracts

Fresh pomegranate fruits were collected from fields in the area of Nakhon Ratchasima Province, Thailand, during April to May. As described in 2.1.1 (Plant collections and preparation of extract), the yield of pomegranate seed and peel was 25.06% and 34.70% respectively. Both extracts were dissolved in Krebs's solution just before use.

7.3.3 Myometrial Tissue Preparations

Tissue preparations were essentially the same as those described in Chapter 2. Non-pregnant Wistar rats (200-250 g) were used in this study and maintained in accordance with the guidelines of the committee on Care and Use of Laboratory Animal Resources, National Research Council, Thailand. The experiments performed on rats were conducted in accordance with the advice of the Institutional Animal Care and Use Committee, (SUT), Thailand. Myometial tissue preparations were dissected and provided for tension measurements as those described in 2.9.1.

7.3.4 Measurements of Tension

The uterine strip was mounted vertically under resting tension of 1 g in a single chamber (25 mL) tissue bath connected to force transducer (as described in 2.8). The organ bath contained Krebs' solution maintained at pH 7.4, temperature of 37 ^{o}C, and gassed with O_2. The myometrial strip was attached at each end to metal

hooks and another hook was fixed to a transducer. The electrical signal was recorded from the transducer and converted to the digital signal on a computer using Chart software (Kupittayanant, 2003). The strips were allowed to contract spontaneously and an equilibrium period of 30 min was given before the application of any chemical. The measurements were made whilst the tissue was continually perfused with physiological solution (control) or solution containing pomegranate seed and peel extracts 200-260 mg/100 mL, and 30-90 mg/100 mL respectively. Wortmannin, an inhibitor of myosin light chain kinase (MLCK), (Longbottom, 2000); nifedipine, an inbitor of voltage-gated L-type channels, (Nayler, 1981); tetraethylammonium (TEA), an inhibitor of calcium-activate potassium channels (Kupittayanant et.al., 2002), were used, as indicated in the text.

7.3.5 Statistical Analysis

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 tests or ANOVA and P values < 0.05 taken to be significant. Results were then expressed as percentages of control contractions (i.e. the control is 100% as described in 2.12).

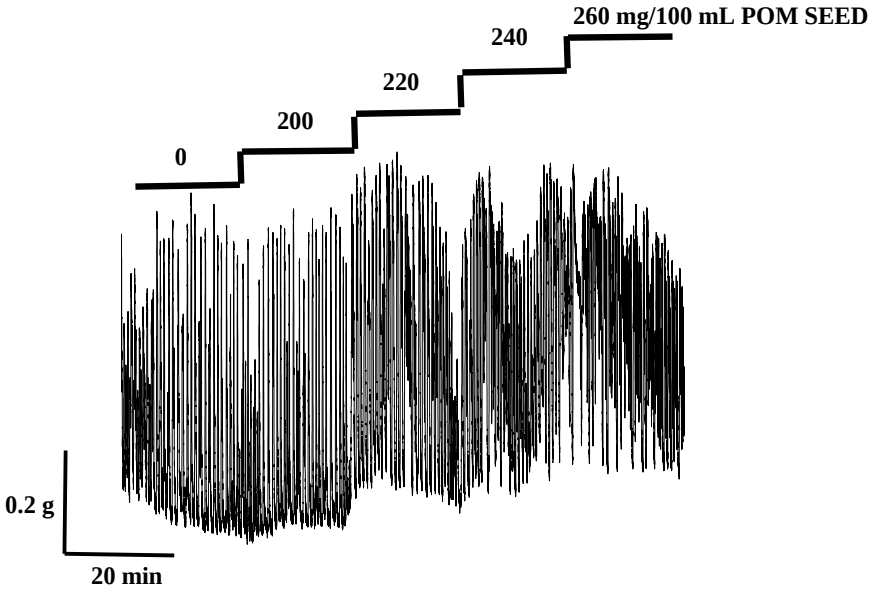
7.4 Results

7.4.1 Effects of Pomegranate Seed Extract on Spontaneous Contractions

Spontaneous contractions of consistent amplitude, frequency and area under the curve could be recorded for several hours allowing the effects of the different concentrations of the extract to be examined (Figure 7.1 and Table 7.1).

Pomegranate seed extract (200-260 mg/100 mL) was added to spontaneous active preparations for 30 min. At each contraction, it produced a significant increase in the amplitude and the frequency of the contractions, and the basal tension. An initial significant induction was observed with 220 mg/100 mL of pomegranate seed extract whereas the maximal stimulatory concentration on myometrium contractility occurred when its concentration was in between 240-260 mg/100 mL, n=5. Thus, the concentration of 250 mg/100 mL was used throughout the study. A significant increase of frequency was found with a concentration of 240 and 260 mg/100 mL (118.2 ± 6.12% and 114.72 ± 4.97%, respectively). The amplitude of the contraction was significantly increased with a concentration of 220 and 240 mg/100 mL (108.02 ± 3.10% and 108.79 ± 3.18%, respectively). The AUC of the contraction was significantly increased with a concentration of 220, 240 and 260 mg/100 mL (132.10 ± 11.75%, 152.65 ± 19.06% and 134.87 ± 17.24%, respectively).

A



B

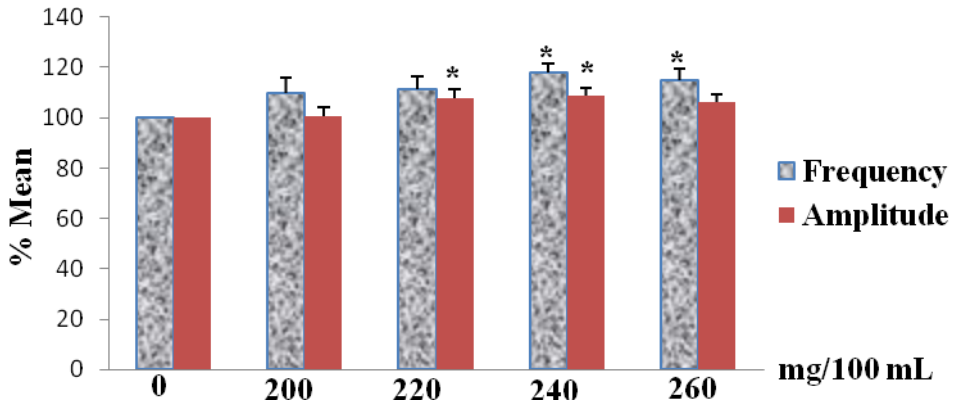
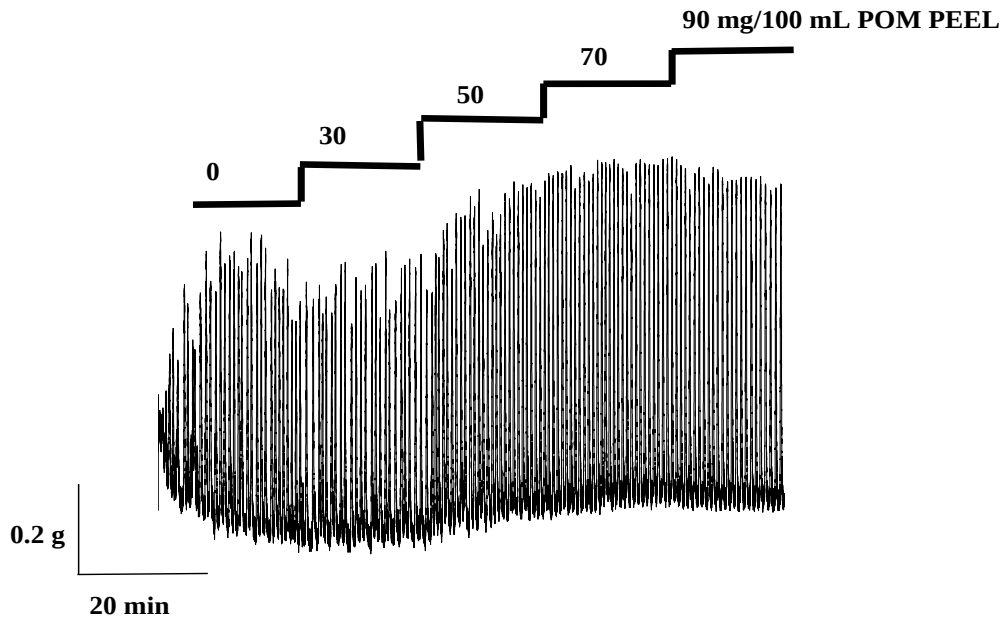


Figure 7.1 The effects of pomegranate seed extract at various concentrations on spontaneous contractions. Amplitude and frequency are presented as percentage of control responses, [i.e. the control is 100% (B)].

7.4.2 Effects of Pomegranate Peel Extract on Spontaneous Contraction

Pomegranate peel extract (30-60 mg/100 mL) was added to spontaneous active preparations for 30 min. It produced a significant increase in the amplitude of rat uterine contraction (Figure 7.2 and Table 7.2). An initial significant induction was observed whit 50 mg/100 mL of pomegranate peel extract whereas the maximal stimulatory concentration on myometrium contractility occurred when its concentration was in between 70-90 mg/100 mL, n=4. Thus, the concentration of 70 mg/100 mL was used throughout the study. Pomegranate peel extract (50-90 mg/100 mL) significantly increased the amplitude of spontaneous contractions in rat uterus (116.90 ± 6.80%, 131.58 ± 12.81%, and 121.61 ± 8.64%, respectively). The frequency of the contraction was increased to 114.82 ± 1.77%, 119.82 ± 6.73% and 121.96 ± 7.18% respectively and the area under the contraction (AUC) of the contraction was increased to 111.86 ± 4.59%, 123.59 ± 6.97%, 149.23 ± 17.85%, and 125.62 ± 5.89%. All were compared to 100% of the control (Table 7.2 and Figure 7.2) A, B.

A



B

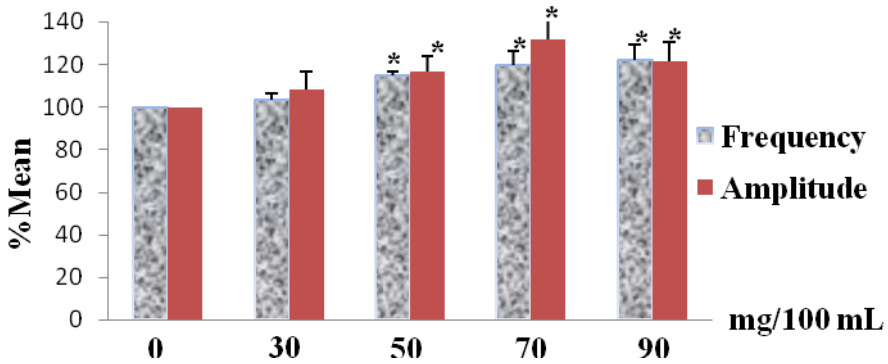


Figure 7.2 The effects of pomegranate peel extract at various concentrations on spontaneous contraction. Amplitude and frequency are presented as percentage of control responses, [i.e. the control is 100% (B)].

Table 7.1 The effects of pomegranate seed extract at various concentrations on spontaneous contractions.

	Amplitude (% Mean ± S.E.M)	Frequency (% Mean ± S.E.M)	AUC (% Mean ± S.E.M)	n
Pomegranate seed (mg/100 mL)				
0 (control)	100	100	100	5
200	100.54 ± 3.44	110 ± 6.12	107.99 ± 11.75	5
220	108.02 ± 3.10*	111.5 ± 5.09	132.10 ± 12.54*	5
240	108.79 ± 3.18*	118.22 ± 3.26*	152.65 ± 19.06*	5
260	106.35 ± 2.83	114.72 ± 4.97*	134.87 ± 17.24*	5

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

Table 7.2 The effects of pomegranate peel extract at various concentrations on spontaneous contractions.

	Amplitude (% Mean ± S.E.M)	Frequency (% Mean ± S.E.M)	AUC (% Mean ± S.E.M)	n
Pomegranate peel (mg/100 mL)				
0 (control)	100	100	100	4
30	108.24 ± 8.68	103.12 ± 3.12	111.86 ± 4.59*	4
50	116.90 ± 6.80*	114.82 ± 1.77*	123.59 ± 6.97*	4
70	131.58 ± 12.81*	119.82 ± 6.73*	149.23 ± 17.85*	4
90	121.61 ± 8.64*	121.96 ± 7.18*	125.62 ± 5.89*	4

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

7.4.3 Effects of Pomegranate Seed Extract on Uterine Contractions in the Presence of the MLCK Inhibitor

Having demonstrated the effects of pomegranate seed on spontaneous contractions, the effects of pomegranate seed extracts on uterine contraction in the presence of wortmannin, the MLCK inhibitor, were investigated. The effects are summarized in Table 7.3. When pomegranate seed was applied in the continued presence of wortmannin, it gradually reduced force. As can be seen in Figure 7.3A; a significant reduction occurred after 10 min (mean amplitude of contractions 84.76 ± 1.58% compared to 100% control), and by 40 min contraction were abolished. In addition, pomegranate seed extract consistently increased basal force, even in the presence of wortmannin.

Table 7.3 The effects of pomegranate seed on uterine contractions 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
Pomegranate seed	118.47 ± 4.32*	133.33 ± 9.62*	256.6 ± 46.91*	3
Pomegranate seed + Wortmannin (10 min)	84.76 ± 1.58*	143.64 ± 35.64*	193.58 ± 47.06*	3
Pomegranate seed + Wortmannin (20 min)	67.80 ± 4.9*	140.47 ± 21.16*	137.15 ± 21.75*	3
Pomegranate seed + Wortmannin (30 min)	33.06 ± 7.74*	118.24 ± 30.25*	94.73 ± 36.24*	3
Pomegranate seed + Wortmannin (40 min)	18.43 ± 6.64*	62.69 ± 33.95*	74.25 ± 37.40*	3

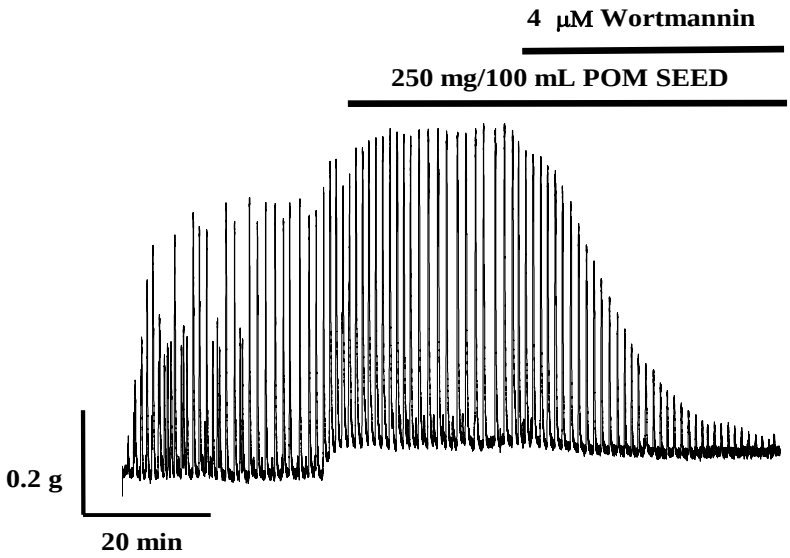
The *P*-values for amplitude, frequency, and area under the curve of pomegranate seed on wortmannin treated are significantly different from the control (*$P<0.05$). Mean value ± S.E.M are given; n is number of animals.

Table 7.4 The effects of pomegranate peel on uterine contractions 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
Pomegranate peel	119.60 ± 6.05*	132.53 ± 10.31*	163.19 ± 7.17*	3
Pomegranate peel + Wortmannin (10 min)	93.64 ± 1.55*	111.10 ± 5.53	114.22 ± 15.96	3
Pomegranate peel + Wortmannin (20 min)	85.02 ± 2.06*	101.58 ± 15.00	94.15 ±5.45*	3
Pomegranate peel + Wortmannin (30 min)	59.19 ± 12.91*	96.02 ± 13.20*	67.35 ± 1.50*	3
Pomegranate peel + Wortmannin (40 min)	40.38 ± 20.23*	57.14 ± 2.97*	50.04 ± 2.52*	3

The *P*-values for amplitude, frequency, and area under the curve of pomegranate peel on wortmannin treated are significantly different from the control (*P<0.05). Mean value ± S.E.M are given; n is number of animals.

A



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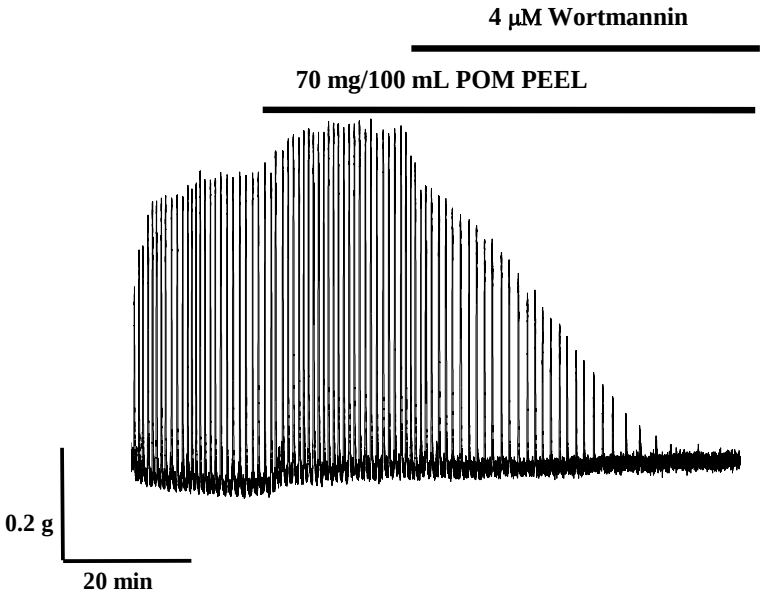


Figure 7.3 The effects of pomegranate extraction uterine contractions in the presence of the MLCK inhibitor, pomegranate seed (A), pomegranate peel (B).

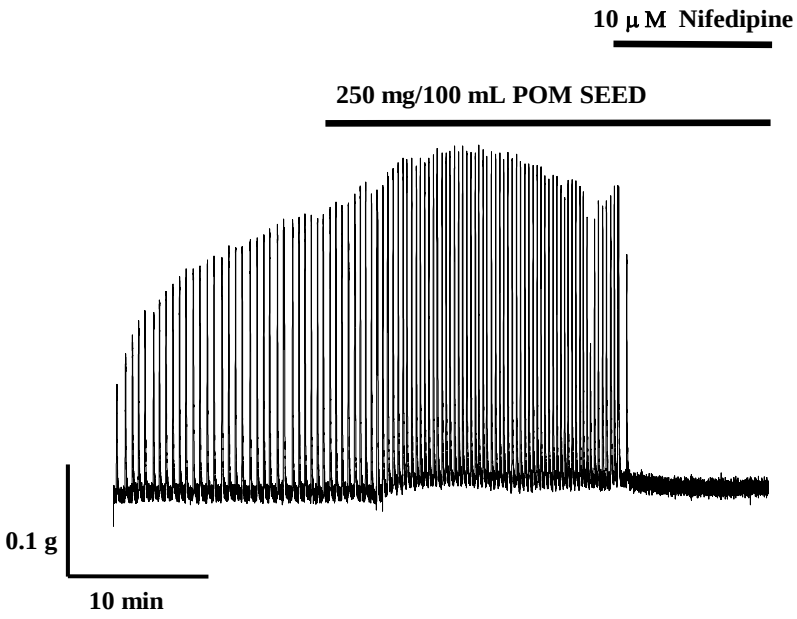
7.4.4 Effects of Pomegranate Peel Extract on Uterine Contractions in the Presence of the MLCK Inhibitor

The effects are summarized in Table 7.4. When 4 µM was added to spontaneously contracting uterus, it gradually reduced spontaneous force. As can be seen in Figure 7.3B; a significant reduction occurred after 10 min (mean amplitude of contractions 93.64 ± 1.55%, compared to 100% control) and by 40 min contraction were slightly abolished. In addition, pomegranate seed extract consistently increased basal force, even in the presence of the MLCK inhibitor.

7.4.5 Effects of Pomegranate Seed and Peel Extracts on Uterine Contractions in the Absence of External Ca^{2+}

The following experiments were investigated to see whether increases in the contraction induced by pomegranate seed and peel extracts were dependent on an increase in extracellular Ca^{2+} via L-type Ca^{2+} channels. As can be seen in Figure 7.4A and B, either pomegranate seed (250 mg/100 mL) or pomegranate peel (70 mg/100 mL) was applied in the continued presence of 1µM nifedipine and the contraction observed. The application of 1µM nifedipine rapidly inhibited force induced in the continued presence of the extract and no force transients were produced as long as nifedipine was present (n=3). Basal force however, did not return to control levels but remained elevated.

A



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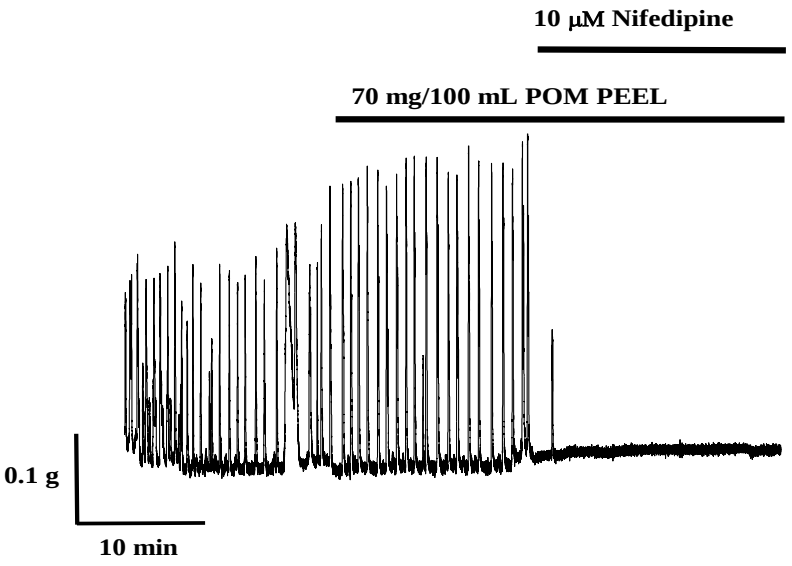


Figure 7.4 Effects of pomegranate seed and peel extracts on uterine contractions in the absence of external Ca^{2+} (A and B).

7.4.6 Effects of Pomegranate Seed Extract in the Presence of K^+ Channel Inhibitor

The effects of pomegranate seed extract on force were resembled to those of potassium channel blockers, which prolong the action potential and thereby potentiate fore (Kupittayanant et al., 2002; Heaton et al., 1993). Thus, the question arose whether the extract effects were mediated by effecting on potassium channels. Therefore potassium channels were blocked with tetraethylammonium (TEA, 5mM ; a concentration known to block all potassium channels) and the effects of pomegranate seed extract (n=3) studied. Application of TEA produced a significant increase in the contraction amplitude, frequency and AUC to 120.02 ± 3.35%, 115.55 ± 2.93% and 118.74 ± 3.57%, respectively (P<0.05, n=3); compared with the control (100%), but no further significant increase occurred upon addition of pomegranate seed extract in the continued presence of K^+ channel. A significant increase of contraction amplitude, frequency and AUC to 122.49 ± 2.28%, 127.77 ± 9.09% and 133.15 ± 8.83% compared with 100% of the control was also found. The effects are summarized in Table 7.5 and a typical effect is shown in Figure 7.5A.

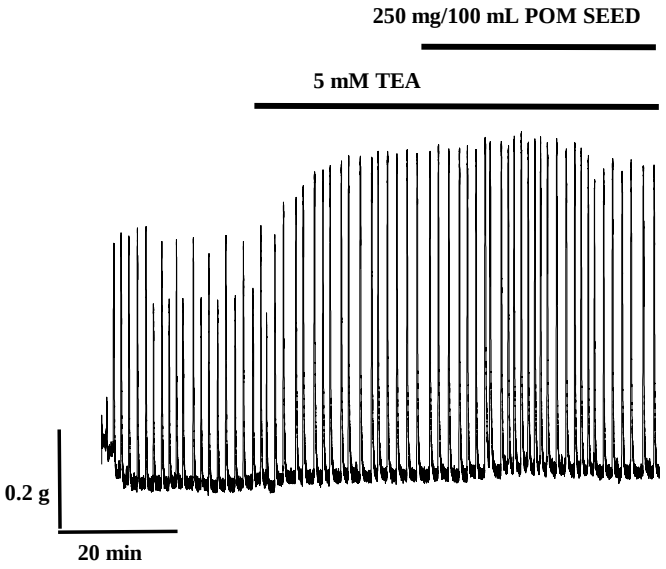
Similarly if TEA was added after an addition of pomegranate seed extract, it produced no further increases in force (Figure 7.5B).

7.4.7 Effects of Pomegranate Peel Extract in the Presence of K^+ Channel Inhibitor

As with the pomegranate seed extract, the pomegranate peel extract produced the same effects. The effects of pomegranate peel extract on force were resembled to those of potassium channel blockers, which prolong the action potential and thereby potentiate fore (Kupittayanant et al., 2002; Heaton et al., 1993). Thus, the question arose whether the extract effects were mediated by effects on potassium channels. Therefore, potassium channels were blocked, whit tetraethylammonium (TEA, 5mM; a concentration known to block all potassium channels) and the effects of pomegranate seed extract (n=3) studied. Application of TEA produced a significant increase in the contraction amplitude, frequency and AUC to 137.87 ± 13.48%, 116.66 ± 3.33% and 195.33 ± 30.45%, respectively (P<0.05, n=3); compared with the control (100%), but no further significant increase occurred upon addition of pomegranate seed extract in the continued presence of K channel. A significant increase in contraction amplitude, frequency and AUC to 139.82 ± 12.59%, 113.33 ± 3.33% and 183.48 ± 29.94% compared with 100% of the control was also found. The effects are summarized in Table 7.6 and a typical effect is shown in Figure 7.6A.

Similarly if TEA was added after an administration of pomegranate seed extract, it produced no further increases in force (Figure 7.6B).

A



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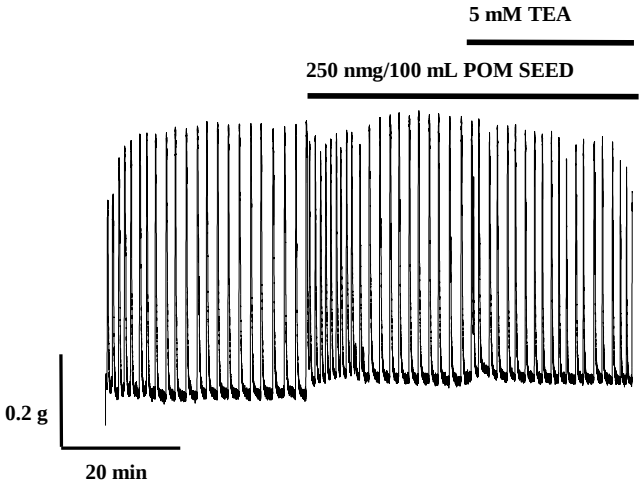
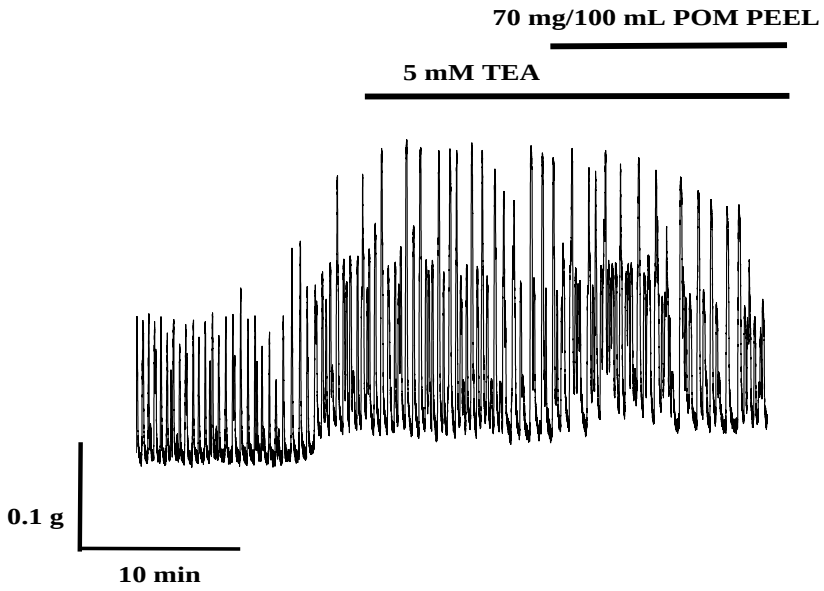


Figure 7.5 Effects of pomegranate seed extract in the presence of K^+ channel inhibitor.

A



B

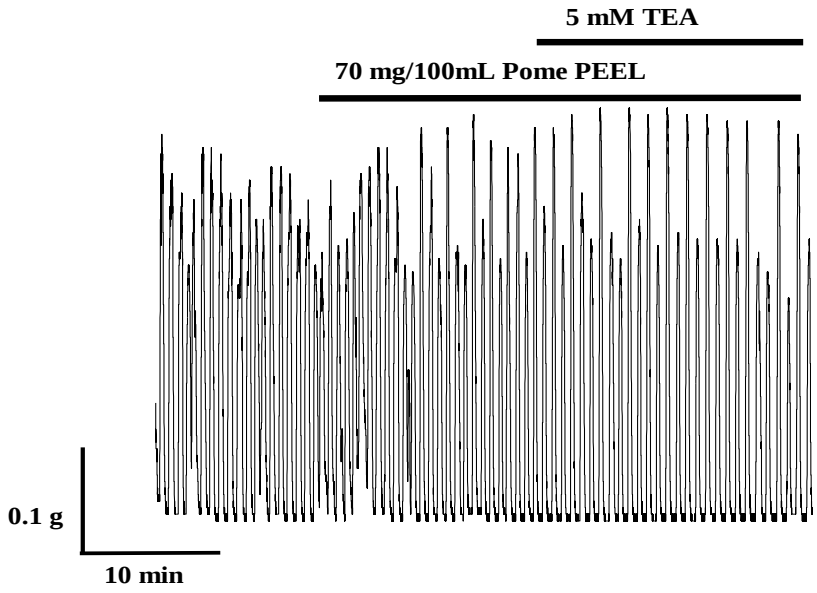


Figure 7.6 Effects of pomegranate peel extract in the presence of K^+ channel inhibitor.

Table 7.5 Effects of pomegranate seed extract in the presence of K channel inhibitor.

	Amplitude (% Mean ± S.E.M)	Frequency (% Mean ± S.E.M)	AUC (% Mean ± S.E.M)	n
Pomegranate seed (after)	100	100	100	3
TEA	120.02 ± 3.35*	115.55 ± 2.93*	118.74 ± 3.57*	3
TEA + pomegranate seed	122.49 ± 2.28*	127.77 ± 9.09*	133.15 ± 8.83*	3
Pomegranate seed (before)	100	100	100	3
Pomegranate seed	109.04 ± 3.13*	151.19 ± 13.41*	168.11 ± 16.99*	3
Pomegranate seed +TEA	118.25 ± 3.58*	118.64 ± 3.25*	129.22 ± 8.69*	3

The *P*-values for amplitude, frequency, and area under the curve of pomegranate seed on tetraethylammonium (TEA) treated are significantly different from the control (*$P<0.05$). Mean value ± S.E.M are given; n is number of animals.

Table 7.6 Effects of pomegranate peel extract in the presence of K channel inhibitor.

	Amplitude (% Mean ± S.E.M)	Frequency (% Mean ± S.E.M)	AUC (% Mean ± S.E.M)	n
Pomegranate peel (after)	100	100	100	3
TEA	137.87 ± 13.48*	116.66 ± 3.33*	195.33 ± 30.45*	3
TEA + pomegranate peel	139.82 ± 12.59*	113.33 ± 3.33*	183.48 ± 29.94*	3
Pomegranate peel (before)	100	100	100	3
Pomegranate peel	122.75 ± 7.59*	115.53 ± 4.83*	112.90 ± 11.24	3
Pomegranate peel + TEA	122.03 ± 7.13*	118.56 ± 3.61*	100.24 ± 9.98	3

The *P*-values for amplitude, frequency, and area under the curve of pomegranate peel on tetraethylammonium (TEA) treated are significantly different from the control (*$P<0.05$). Mean value ± S.E.M are given; n is number of animals.

7.5 Discussion

Pomegranate seed and peel extracts potently potentiate spontaneous contractions. Both pomegranate seed and peel extracts caused significant increases in the amplitude, frequency and AUC of phasic contraction and also increased basal tension. The effects of pomegranate seed and peel extracts were indistinguishable from those of β-sitosterol. The potentiation of force induced by pomegranate seed and peel extracts was however insufficient to overcome the effects of inhibition of L-type calcium channels, removal of external Ca^{+} or inhibition of MLCK, suggesting that they are produced by the Ca^{2+} -calmodulin-MLCK pathway. The effects of the extract on spontaneous contraction were resembled to those of inhibiting K^{+} channels.

As can be seen in figure 7.1 and 7.2 the effects of pomegranate extract particularly on the amplitude and frequency of contractions were significant. These data demonstrate that pomegranate extract is a powerful uterine stimulant. An increase in basal tone was also consistently found. The putative mechanisms for the effects on phasic contractions and basal tone will be discussed.

It was found that the increases in contraction produced by the extracts were dependent on external calcium and myosin light chain kinase. In addition, the extract could not produce force in the absence of external calcium entry. Force produced in the presence of the extract was abolished when Ca^{2+} entry was inhibited. Furthermore, support for this conclusion comes from the experiments with MLCK inhibition; force was no longer produced by the extract. Thus the data support a mechanism of action involving the Ca^{2+} -calmodulin-MLCK pathway rather than that of Ca^{2+} sensitization. Nifedipine application however, did not reverse the increase in basal tone, caused by

the extract, although it did abolish the spontaneous contractions. This suggests that the mechanism causing the elevation of basal tone is not dependent upon Ca^{2+} entry and may therefore involve the internal Ca^{2+} store i.e. the SR.

The data suggest that the pomegranate seed and peel extracts are potentiating force by an inhibition of K^+ channels and an effect on the SR. After exposure to TEA, the pomegranate seed and peel extracts had no effect, pointing to K^+ channels being a target. There are data from other sterols, especially cholesterol, that these compounds can modulate K^+ channel activity. Specifically in the uterus cholesterol manipulation can have marked effects on Ca^{2+} signaling and contractility (Zhang, 2007) via effects on Ca^{2+} -activated K^+ channels (Shmygol, 2007). Cholecalciferol, a vitamin D_3 precursor has also been shown to affect K^+ channel activity in vascular smooth muscle (Borges, 1999). The effects of pomegranate seed and peel extracts closely resembled to that of 5 mM TEA, a concentration known to inhibit most K^+ channels. The application of TEA inhibits the repolarizing drive in the uterine myocytes leading to increase excitability and prolonge force and Ca transient (Kupittayanant et al., 2002; Taggart and Wray, 1998). Further studies are suggested to determine which type of K^+ channels are the main targets of the extract, along with measurements of electrical activity. Both pomegranate seed and peel extracts increased basal tension. As a result of the same concentration of the effect of inhibiting the sarcoplasmic reticulum on contraction of cyclopiazonic acid ; CPA, 20 µM (Kupittayanant et al., 2002).

In conclusion, the data demonstrating a significant stimulation of uterine activity by pomegranate seed and peel extracts, which can largely be accounted for by its constituent, β-sitosterol. No evidence for Ca^{2+} -senstization mechanisms could be

found but the data support the Ca^{2+}-calmodulin-MLCK pathway being directly involved in underlying the effects of the pomegranate seed and peel extracts on contractions. This potentiation of force as suggested is due to the extract acting to inhibit K channels, and may also involve an inhibition of the SR Ca^{2+} -ATPase. The pomegranate extracts may be a useful uterine stimulant for dystocia, preterm labor and post term labor (Adam, 2007).

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CHAPTER VIII

EFFECTS OF POMEGRANATE EXTRACT ON SPONTANEOUS CONTRACTILITY IN OVARIECTOMIZED RATS

8.1 Abstract

As shown in Chapter III, the pomegranate (*Punica granatum* L.) seed and peel extracts exhibited estrogenic activity. Ovariectomized rats are routinely used to assess for estrogen deficiency. However, the estrogenic activities of pomegranate seed and peel extracts on uterus contractility in ovariectomized rats are not well understood. Therefore, the aims of this chapter were to investigate effects of pomegranate seed and peel extracts on the spontaneous contractions in isolated uterine strips from ovariectomized rats. Uterine activity was evaluated in tissues obtained from bilaterally ovariectomized rats (n=4-5). All concentration (200-260 mg/100 mL) of pomegranate seed extract significantly decreased the amplitude. Significant increases in the frequency of the spontaneous contractions were found with 200-260 mg/100 mL. All concentration (30-90 mg/100 mL) of pomegranate peel extract significantly increased amplitude. Significant increases in frequency were found at concentrations of 30-70 mg/100 mL. In summary, pomegranate seed and peel extracts are potent stimulators of phasic activity of the uterus taken from ovariectomized rats.

8.2 Introduction

The biology and physiology of mammal reproductive system are hormonally regulated. An abnormality in the dynamics of hormone production, metabolism and elimination has been associated with pathophysiological conditions of the reproductive system (Pedram et al., 2002). In fact, estrogens influence cell growth and differentiation of many tissues and also play an important role on the contractile activity of uterine smooth muscles during the follicular phase (Suarez and Pacey, 2005).

Gross morphology, behavior, and responsiveness of the uterus vary with the stage of the menstrual or estrus cycle in a manner that suggests they are regulated by the relative concentrations of estrogens and progesterone. While the type and extent of changes are to some degree species dependent, some generalizations hold across species. Uterine taken during estrus, the estrogen-dominated state, are hyperemia, hyperplastic, and hypertrophied in comparison to progesterone-dominated uterine. Estrogen-dominated uterine show increased spontaneous activity and increased excitability. Ovarian steroids are one of the most important factors affecting uterine morphology and motility. The characteristics of spontaneous myometrial contractility change due to direct actions of these hormones. Uterine function and activity changes the stage of the estrus cycle or stage of pregnancy (Bulbul et al., 2007). During the follicular period, when estrogen levels are high, the amplitude of uterine contraction increases in humans (Oike, 1990). Moreover, increased contractile tension following 17β-estraiol treatment has been demonstrated in vitro in ovariectomized rats (Vedernikov, 2003). Animal studies have shown that ovariectomy results in bladder mucosa atrophy, smooth muscle (SM) atrophy, decreased

compliance, decreased depressor contractility, decreased blood flow, and tissue hypoxia (Lin, 2006).

Therefore, changes in sex hormone in female reproductive system may play a role in the regulation of smooth muscle contractility. The aims of this chapter were to investigate the profile of spontaneous contractions in ovariectomized rats and the response to pomegranate seed and peel extracts.

8.3 Materials and Methods

8.3.1 Preparations of Pomegranate Seed and Peel Extracts

As described in 2.1 (Plant collection and preparation of extract), pomegranate seed and peel extracts were dissolved in Krebs's solution just before use.

8.3.2 Myometrial Tissue Preparations

Tissue preparations were essentially the same as those described in Chapter II. Non-pregnant Wistar rats (200-250 g) were used in this study and maintained in accordance with the guidelines of the committee on Care and Use of Laboratory Animal Resources, National Research Council, Thailand**.** The experiments performed on rats were conducted in accordance with the advice of the Institutional Animal Care and Use Committee, (SUT), Thailand. Rats were bilaterally ovariectomized under isoflurane anesthesia as those described in 2.2.2. On day 14 after ovariectomy, rats were sacrificed. Myometial tissue preparations were dissected as those described in 2.9.1. Briefly, an incision was made on the animal abdomen. Both uterine horns were exposed and excised. The horns were placed in a peteri dish containing Krebs' solution.

8.3.3 Measurements of Tension

The uterine strip was mounted vertically under resting tension of 1 g in a single chamber (25 mL) tissue bath connected to force transducer (as described in 2.8). The organ bath contained Krebs' solution maintained at pH 7.4, temperature of 37^{o}C, and gassed with O_2. The myometrial strip was attached at each end to metal hooks and another hook was fixed to a transducer. The electrical signal has been recorded from the transducer and converted to the digital signal on a computer using Chart software (Kupittayanant, 2003).

8.3.4 Statistical Analysis

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* tests or ANOVA and $P<0.05$ taken to be significant. Results were then expressed as percentages of control contractions (i.e. the control is 100% as described in 2.12).

8.4 Results

8.4.1 The Profile of Spontaneous Contractions in Ovariectomized Rats

The uterine strips taken from the uterus of ovariectomized rats when placed in the appropriate in vitro condition, e.g. buffered physiological Krebs' solution were able to produce regular spontaneous contractions for many hours. The mean time for spontaneous contraction commence was 2.67 ± 0.54 min (n=10). The amplitude, frequency and AUC of the contraction were 0.23 ± 0.02, 0.93 ± 0.06 and 55.33 ± 5.23 contractions per minute, respectively. An example of typical control of uterine spontaneous contraction in ovariectomized rats is shown in Figure 8.1.

In normal rats, the mean time for spontaneous contraction to commence was 1.62 ± 0.66 min (n=10). The amplitude, frequency and AUC of the contraction were 0.57 ± 0.05, 0.95 ± 0.05 and 84.94 ± 9.16 contractions per minute respectively. Thus, the amplitude and AUC of the normal rats was significantly different from that of the ovariectomized rats, but the frequency did not differ when compared to that of the ovariectomized rats.

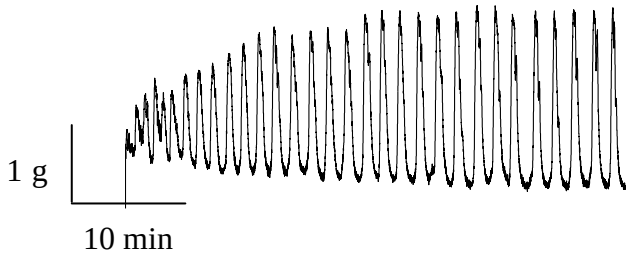


Figure 8.1 A typical recording of uterine spontaneous contraction in ovariectomized rats.

8.4.2 Effects of Pomegranate Seed Extract on Spontaneous Contraction in Ovariectomized Rats

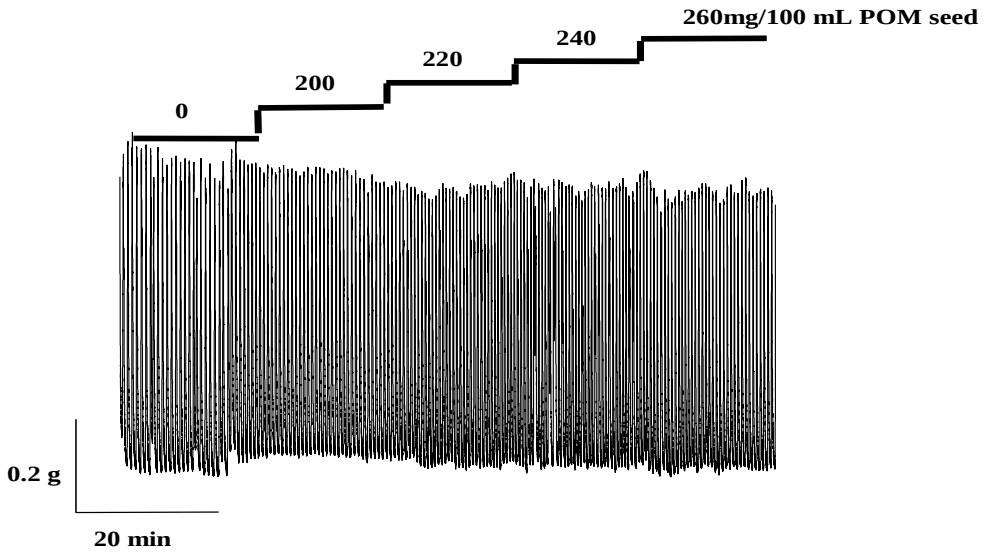
Pomegranate seed extract (200, 220, 240, 260 mg/100 mL) was added to spontaneously active preparation for 30 min (n=5). It produced a significant increase in the frequency of rat uterine contractions as shown in Figure 8.2 and Table 8.1. The value of contraction frequency was 115.88 ± 5.90% 126.55 ± 9.30% and 137.96 ± 11.01% and 119.55 ± 6.97%, respectively. The amplitude was 91.46 ± 2.29%, 87.80 ± 5.28% and 82.28 ± 7.67% compared with 100% of the control. However, the area

under the curve (AUC) was increased to 108.97 ± 10.29%, 106.07 ± 15.79%, 103.27 ± 16.13% and 89.15 ± 14.66%, respectively (P<0.05).

8.4.3 Effects of Pomegranate Peel Extract on Spontaneous Contraction in Ovariectomized Rats

Pomegranate peel extract (30, 50, 70, 90 mg/100 mL) was added to spontaneously active preparation for 30 min (n=4). It produced a significant increase in the amplitude of rat uterine contractions as shown in Figure 8.3 and Table 8.2. The value of contraction amplitude was 112.35 ± 3.43%, 112.77 ± 0.75%, 118.03 ± 2.04% and 106 ± 2.38%, respectively. The area under the curve (AUC) was increased to 137.48 ± 7.37%, 146.49 ± 5.88% and 121.83 ± 8.27 % compared with 100% of the control and at various concentrations (50-90 mg/ 100 mL). However, in response to the concentration of 50 and 70 mg/100 mL, the frequency was significant increase to 133.92 ± 10.91% and 137.01 ± 6.12%, respectively.

A



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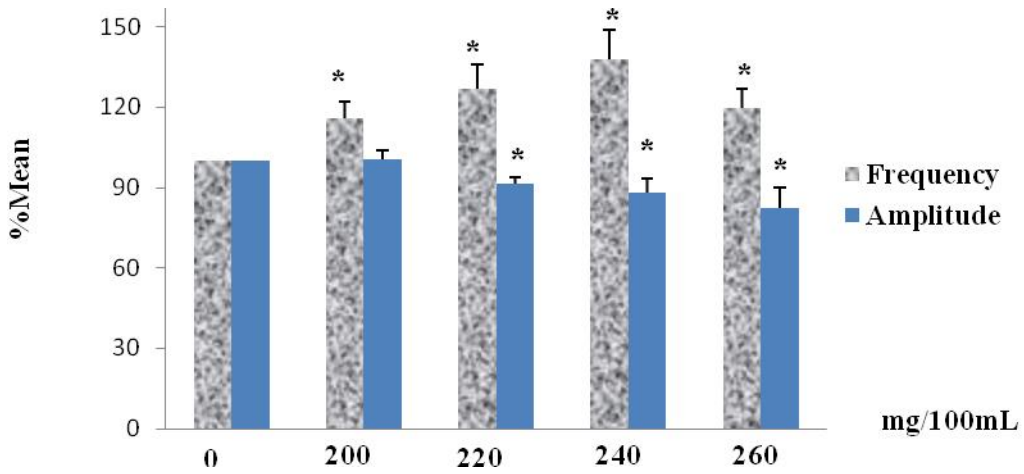
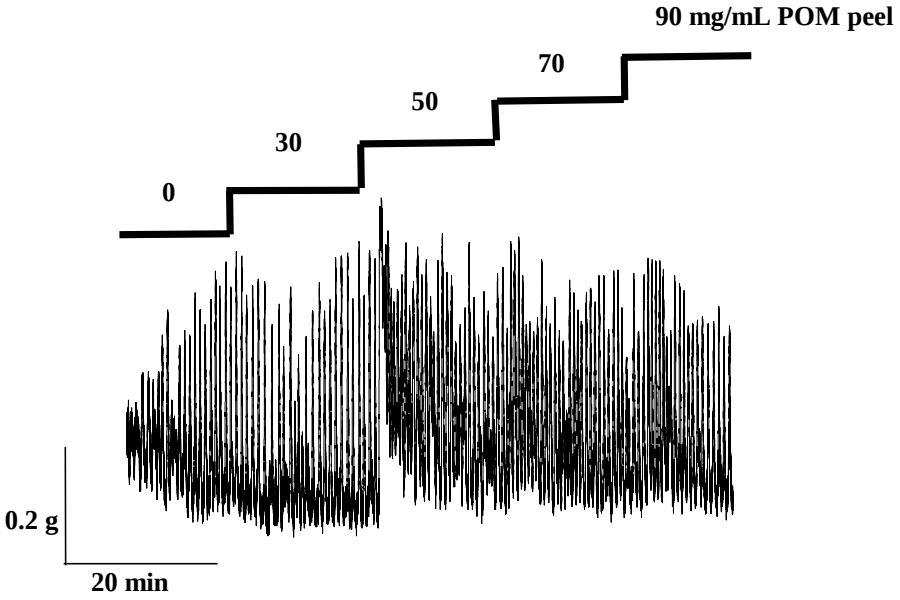


Figure 8.2 The effect of pomegranate seed (A) at various concentrations in ovariectomized rats. Frequency and amplitude are presented as percentage of control responses, [i.e. the control is 100% (B)].

A



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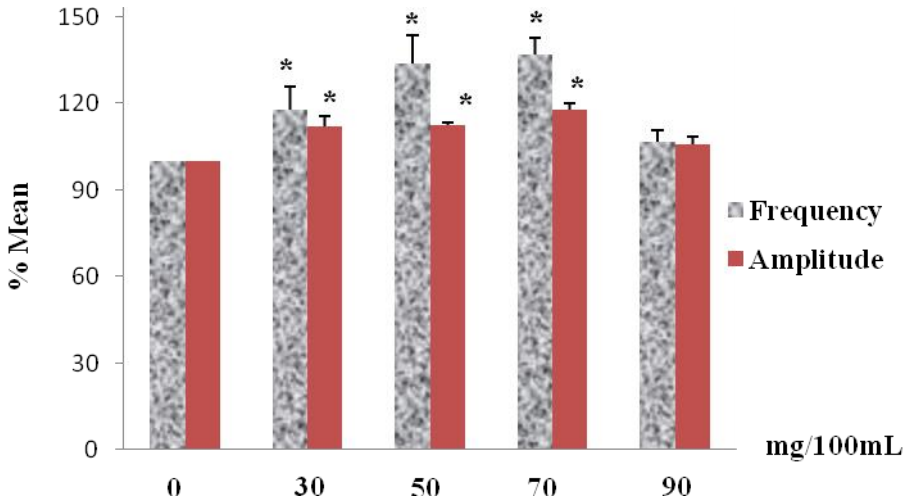

Figure 8.3 The effect of pomegranate peel (A) at various concentrations in ovariectomized rats. Frequency and amplitude are presented as percentage of control responses, [i.e. the control is 100% (B)].

Table 8.1 The effect of pomegranate seed extract at various concentrations in ovariectomized rats.

	Amplitude (% Mean ± S.E.M.)	Frequency (% Mean ± S.E.M.)	AUC (% Mean ± S.E.M.)	n
Pomegranate seed (mg/100 mL)				
0 (Control)	100	100	100	5
200	100.31 ± 3.38	115.88 ± 5.90*	108.97 ± 10.29	5
220	91.46 ± 2.29*	126.55 ± 9.30*	106.07 ± 15.79	5
240	87.80 ± 5.28*	137.96 ± 11.01*	103.27 ± 16.13	5
260	82.28 ± 7.67*	119.55 ± 6.97*	89.15 ± 14.66	5

The *P*-values for amplitude, frequency, and area under the curve of pomegranate seed are significantly different from the control (*P<0.05). Mean value ± S.E.M are given; n is number of animal.

Table 8.2 The effect of pomegranate peel extract at various concentrations on ovariectomized rat.

	Amplitude (% Mean ± S.E.M.)	Frequency (% Mean ± S.E.M.)	AUC (% Mean ± S.E.M.)	n
Pomegranate peel (mg/100 mL)				
0 (Control)	100	100	100	4
30	112.35 ± 3.43*	117.96 ± 8.48*	113.14 ± 12.98	4
50	112.75 ± 0.75*	133.92 ± 10.91*	137.48 ± 7.37*	4
70	118.03 ± 2.04*	137.01 ± 6.12*	146.49 ± 5.88*	4
90	106.03 ± 2.38*	106.81 ± 4.35	121.83 ± 8.27*	4

The *P*-values for amplitude, frequency, and area under the curve of pomegranate peel are significantly different from the control (*P<0.05). Mean value ± S.E.M are given; n is number of animal.

8.5 Discussion

It is well known that ovariectomy induces some physiological of uterus such as uterus size, stretch and the lack of ovarian hormones. Regarding the role of ovarian steroid hormones on uterus, exogenous 17β-estradiol depresses uterine contractility both in vivo (Downing, 1981), and in freshly isolated uterine rings (Vedernikov, 2003). Progesterone had no effect of its own but suppressed the inhibitory action of 17β-estradiol. In these experiments, 17β-estradiol was administered for a prolonged period of time (days). Osa and Ogasawara (1984) compared the potency of progesterone, oestradiol and stilbestrol on rat myometrium at dioestrous. Spontaneous activity was decreased mostly by stilbesterol, then oestradiol and then progesterone. Such a rapid action suggests that 17β-estradiol is acting via plasma membrane receptors but there is also evidence of action via a longer term genomic pathway, as the effects of 17β-estradiol could be reduced by prior treatment of myometrium with inhibitors of protein synthesis and transcription (Gutierrez, 1998). Thus, it is conceivable that the pomegranate extracts administered as a rich plant source of estrogens is able to regulate uterine contractility instead of insufficient endogenous estrogens.

The present experiments revealed that pomegranate seed and peel extracts exhibited differential effects on the amplitude and the frequency of contraction in ovariectomized rats. Pomegranate seed extract increased the frequency of spontaneous contraction in ovariectomized rats. However, this agent did not affect the amplitude and the area under the contraction (AUC) of these contractions. In contrast, pomegranate peel extract increased the amplitude of spontaneous contraction in

ovariectomized rats. However, both extracts increased the frequency of ovariectomized rats.

There is an evidence reported that estrogen decreased the contractile activity of the rat vagina and the rabbit uterus and oviduct (Boling, 1965; Coutinho, 1968; Boling, 1971). However, there appears to be no evidence from the present that endogenous estrogen in the ewe reduces uterine activity (Hawk, 1975). Exogenous estradiol generally stimulated the frequency of uterine contraction. Pomegranate seed extract (220-260 mg/100 mL) decreased amplitude of spontaneous contraction in ovariectomized rats. Thus, an excessive amount of exogenous estrogen may exert an adverse effect on uterine activity. These factors can affect the pomegranate seed and peel extracts responses in these tissues and may cause this controversy effect between ovariectomized and normal rats. In the future the experimental will be designed to find out whether the different between pomegranate seed and peel were related to the physiological and hormonal states of the rats. Also, it can be tested the effect of pomegranate seed and peel extracts in ovariectomized rat uterus treated with different hormones such as estraiol or in normal rat at known stage of oestrus cycle (Wray and Noble, 2008). An increase in the external Ca^{2+}concenrtration can be reversed the effect of pomegranate on spontaneous contraction compared with control.

In conclusion, the present study demonstrated that pomegranate seed and peel extracts are potent stimulators of phasic activity in the uterus of ovariectomized rats. However, further studies are needed to determine the mechanism that underlies the interactions between pomegranate seed and peel extracts and 17β-estradiol responses related to hormonal regulation of strips in the ovariectomized and normal uterus.

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CHAPTER IX

EFFECTS OF β-SITOSTEROL ON UTERINE CONTRACTILITY

9.1 Abstract

Pomegranate (*Punica granatum* L.) contains not only estrogens (estradiol, estrone and estradiol) but also other steroids such as testosterone and β-sitosterol. The aims of this chapter were to investigate the effects of β-sitosterol on spontaneous contractions in normal and ovariectomized rats and to study mechanisms whereby they exerted their effects. The results showed that the effect of β-sitosterol was similar indistinguishable to those of pomegranate extracts. Thus, it increased uterine contractions, irrespectively of how they were produced, via the inhibition of L-type calcium channels or myosin light chain kinases (MLCK). Contractions were not potentiated by β-sitosterol following inhibition of K^+ channels.

9.2 Introduction

β-sitosterol is one of several phytosterols with chemical structures similar to that of cholesterol. It is white colour and waxy in nature. High levels of β-sitosterol are found in rice bran, wheat germ, corn oils, and soybeans. β-sitosterol differs from cholesterol by the presence of an extra ethyl group. β-sitosterol (24-ethyl-5-cholestene-3-ol), a well-known plant sterol, has been reported to reduce serumcholesterol levels and to prevent cardiovascular events mainly by inhibition of

cholesterol absorption in the intestines (Miettinen, 1995; Ostund, 2002; Pouteau, 2003). β-Sitosterol is also known to regulate key molecules involved in inflammation, anti-cancer, and apoptosis (Bouic, 2002; Awad, 2000). In addition, the plasma β-sitosterol concentration was found to be significantly reduced in type 2 diabetes patients (Sutherland, 2000). However, the molecular mechanisms underlying these beneficial effects of β-sitosterol are largely unknown.

The characteristics of pomegranate fruit are that its seeds are the richest plant source of estrogens. Pomegranate seeds are known to contain the estrogenic compounds, estrone and estradiol, that are chemically identical to those biosynthesized in human body (Heftmann et al., 1966), and coumesterol as well (Moneam et al., 1988). According to Kim et al. (2000), pomegranate seed contain not only estrogen (estradiol, estrone and estriol) but also other steroids such as testosterone and β-sitosterol, and coumesterol, whereas, anthocyanins and phenolic acids are the main ingredients of pomegranate juice. It has been recently reported that the pomegranate peel and seed oils contains a substantial amount of polyphenols such as sugar-bound flavonoids quercetin and kaempferol (Chauhan, 2001), flavonoid diglycoside, ellagic acid and ellagic tannin (Poyrazoglu, 2002) and organic acids. On the other hand, there were some reports that the pomegranate seed and peel extracts contain steroid hormones including estrone (Dean, 1971; Moneam, 1988), estradiol (Abd, 1998) and testosterone (Lau, 2003). However, the use of pomegranate compared with β-sitosterol as uterotonic agents is not well understood and effects of pomegranate compared with β-sitosterol on uterine contraction have not yet been demonstrated.

Therefore, the aims of this chapter were to investigate the effects of β-sitosterol on spontaneous contractions in normal and ovariectomized rats and to study mechanisms whereby it exerted the effects.

9.3 Materials and Methods

9.3.1 Chemicals and Physiological Solution

All chemicals were purchased from Sigma® unless state otherwise. Antagonists for investigation of physiological pathways used were as follows; β-sitosterol was dissolved in ethanol. Nifedipine was dissolved in DMSO at a concentration of 10 μM. Tetraethylammonium (5 mM) was dissolved in distilled water.

9.3.2 Preparations of Pomegranate Seed and Peel Extracts

Fresh pomegranate fruits were collected from fields in the area of Nakhon Ratchasima, Thailand, during April to May. As described in 2.1.1 (Plant collections and preparation of extract), the yield of pomegranate seed and pomegranate peel was 25.06% and 34.70% respectively. The extract was dissolved in Krebs's solution just before use.

9.3.3 Myometrial Tissue Preparations

Tissue preparations were essentially the same as those described in Chapter II. Non-pregnant Wistar rats (200-250 g) and ovariectomy rats were used in this study and maintained in accordance with the guidelines of the committee on Care and Use of Laboratory Animal Resources, National Research Council, Thailand. The

experiments performed on rats were conducted in accordance with the advice of the Institutional Animal Care and Use Committee, Surannaree University of Technology (SUT), Thailand. Myometrial tissue preparations were dissected and provided for tension measurements as those described in 2.9.1.

9.3.4 Measurements of Tension

The uterine strip was mounted vertically under resting tension of 1 g in a single chamber (25 mL) tissue bath connected to force transducer (as described in 2.8). The organ bath contained Krebs' solution maintained at pH 7.4, temperature of $37^{o}C$, and gassed with O_2. The myometrial strip was attached at each end to metal hooks and another hook was fixed to a transducer. The electrical signal was recorded from the transducer and converted to the digital signal on a computer using Chart software (Kupittayanant, 2003). The strips were allowed to contract spontaneously and an equilibrium period of 30 min was given before the application of any chemical. The measurements were made whilst the tissue was continually perfused with physiological solution (control) or solution containing pomegranate seed and peel extracts 200-260 mg/100 mL, and 30-90 mg/100 mL respectively. Nifedipine, an inhibitor of voltage-gated L-type channels, (Nayler, 1981); tetraethylammonium (TEA), an inhibitor of calcium-activate potassium channels (Kupittayanant et al., 2002), were also used, as indicated in the text.

9.4 Statistical Analysis

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*-

tests or ANOVA and $P<0.05$ taken to be significant. Results were then expressed as percentages of control contractions (i.e. the control is 100% as described in 2.12).

9.5 Results

9.5.1 Effects of β-sitosterol on Spontaneous Contraction in Normal and Ovariectomized Rats

In normal rats, spontaneous contractions of consistent amplitude, frequency and under the curve were recorded. The estrogenic activity of the estrogen standard drug was studied. Doses of 1/500 and 1/1000 were recommended in vivo (Cassidy, 1999). Thus, a dose of 1mg/100 mL of β-sitosterol was used in this study (n=3). As shown in Figure 9.1A and Table 9.1, the mean values of contraction amplitude was significantly increased to 110.43 ± 0.02% and the AUC was significantly increased to 130.99 ± 9.9% (all were compared with the control (100%)). However, the increase frequency of the contractions was not significantly different (102.77 ± 2.77%; compared with the control, 100%).

The effects of β-sitosterol (1 mg/100 mL) on spontaneous contraction in ovariectomized rats were investigated (n=3). As shown in Figure 9.1B and Table 9.1, the mean values of contraction amplitude was significantly increased to 112.03 ± 0.87%, contraction AUC to 106.07 ± 0.03%; compared with the control (100%). However, the decreased frequency of the contractions was not significantly different (94.43 ± 2.56%; compared with the control, 100%).

9.5.2 Effects of Pomegranate Seed and Peel on β-sitosterol

The effects of pomegranate seed on β-sitosterol-induced contraction were investigated Figure 9.2 (A and B). When 250 mg/100 mL pomegranate seed was applied in the continued presence of β-sitosterol, it significantly increased the contraction. With β-sitosterol, the amplitude, frequency, and AUC of the contraction was increased to 106.61 ± 0.40%, 120.54 ± 5.50% and 148.6 ± 11.53%, respectively (compared to 100% of the control (Table 9.2)).

The effects of pomegranate peel on β-sitosterol can be seen in Figure 9.3 (A and B). With β-sitosterol, the amplitude, frequency, and AUC of the contraction was reduced to 95.39 ± 2.69%, 108.54 ± 12.59% and 103.21 ± 9.67%, respectively (compared to 100% of the control (Table 9.2)).

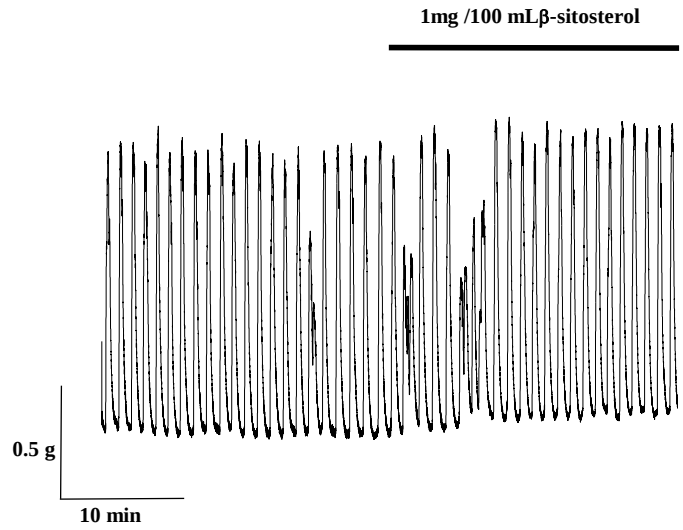
9.5.3 Effects of Tetraethylammonium on β-sitosterol

As shown in Figure 9.4 (A and B), TEA, (5mM) produced a significant increase in the contraction amplitude, frequency and AUC to 127.7 ± 0.80%, 120.35 ± 4.64%, 125.80 ± 2.78%, respectively ($P<0.05$, n=3); compared with the control (100%). The effect of TEA on β-sitosterol can be seen in Figure 9.4. With β-sitosterol, the amplitude, frequency, and AUC of the contraction were reduced to 121.00 ± 1.25%, 106.66 ± 6.66% and 116.14 ± 4.32%, compared to 100% of the control (Table 9.3).

9.5.4 Effects of β-sitosterol on Uterine Contractions in the Absence of External Ca^{2+}

The following experiments were to investigate whether increases in the contraction induced by β-sitosterol were dependent on an increase in extracellular Ca via L-type Ca channels. As can be seen in Figure 9.5, the effect of β-sitosterol (1 mg/100 mL) was applied in the continued presence of 1μM nifedipine and the contraction observed. The application of 1 μM nifedipine rapidly inhibited force induced in the continued presence of the extract and no force transients were produced as nifedipine was present (n=3). Basal force however did not return to control levels but remained elevated.

A



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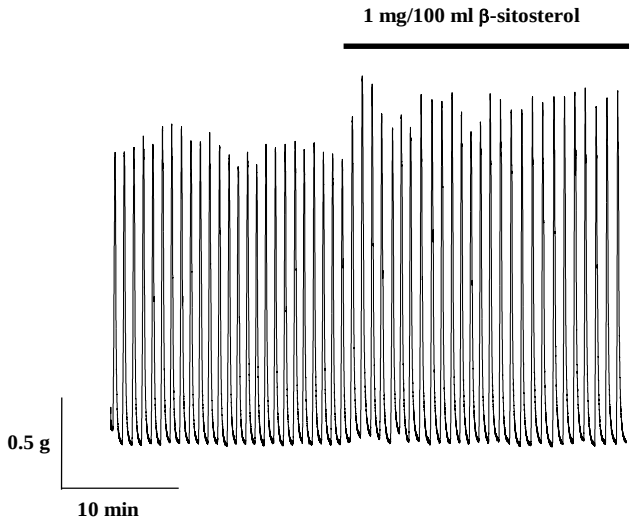


Figure 9.1 The effect of β-sitosterol on spontaneous contraction in normal rats (A); ovariectomized rats (B).

Table 9.1 The effects of β-sitosterol on spontaneous contraction in normal and ovariectomized rats.

	Amplitude (% Mean ± S.E.M.)	Frequency (% Mean ± S.E.M.)	AUC (% Mean ± S.E.M.)	n
Spontaneous normal rats				
Control	100	100	100	3
β-sitosterol	110.43 ± 0.02*	102.77 ± 2.77	130.99 ± 9.9*	3
Spontaneous ovx rats				
Control	100	100	100	3
β-sitosterol	112.03 ± 0.87*	97.43 ± 2.56	106.07 ± 0.03*	3

The *P*-values for amplitude, frequency, and area under the curve of spontaneous are significantly different from the control (*$P<0.05$). Mean value ± S.E.M. are given; n is number of animal.

Table 9.2 The effects of pomegranate seed and peel on β-sitosterol.

	Amplitude (% Mean ± S.E.M.)	Frequency (% Mean ± S.E.M.)	AUC (% Mean ± S.E.M.)	n
Spontaneous contraction				
Control	100	100	100	3
POM seed	107.43 ± 1.52*	117.98 ± 5.51*	143.91 ± 13.98*	3
Pomegranate seed + β-sitosterol	106.61 ± 0.40*	120.54 ± 5.50*	148.6 ± 11.53*	3
Control	100	100	100	3
POM peel	104.76 ± 1.18*	124.78 ± 8.54*	123.75 ± 12.68*	3
Pomegranate peel + β-sitosterol	95.39 ± 2.69	108.54 ± 12.59	103.21 ± 9.67	3

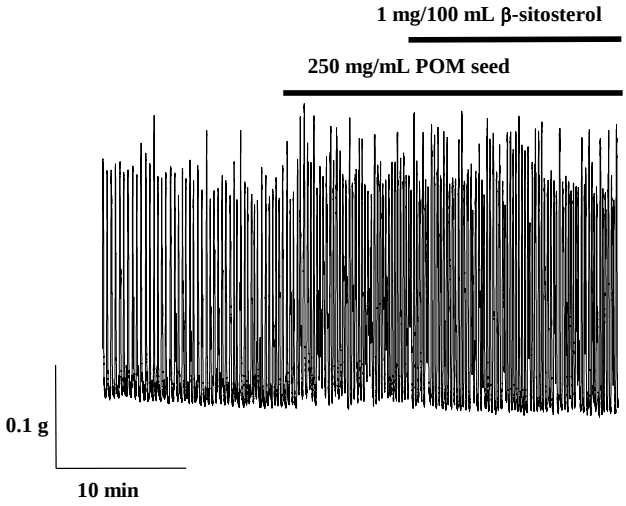
The*P* -values for amplitude, frequency, and area under the curve of pomegranate seed and peel are significantly different from the control (*P<0.05). Mean value ± S.E.M. are given; n is number of animal.

Table 9.3 Effects of tetraethylammonium on β-sitosterol.

	Amplitude (% Mean ± S.E.M.)	Frequency (% Mean ± S.E.M.)	AUC (% Mean ± S.E.M.)	n
Spontaneous normal rats				
Control	100	100	100	3
TEA	127.7 ± 0.80*	120.35 ± 4.64*	125.80 ± 2.78*	3
TEA+β-sitosterol	121.00 ± 1.25*	106.66 ± 6.66*	116.143 ± 4.32*	3
TEA	100	100	100	3
TEA+β-sitosterol	96.61 ± 0.76	88.83 ± 6.41	92.35 ± 3.46	3

The *p*-values for amplitude, frequency, and area under the curve of tetraethylammonium are significantly different from the control (*$P<0.05$). Mean value ± S.E.M. are given; n is number of animal.

A



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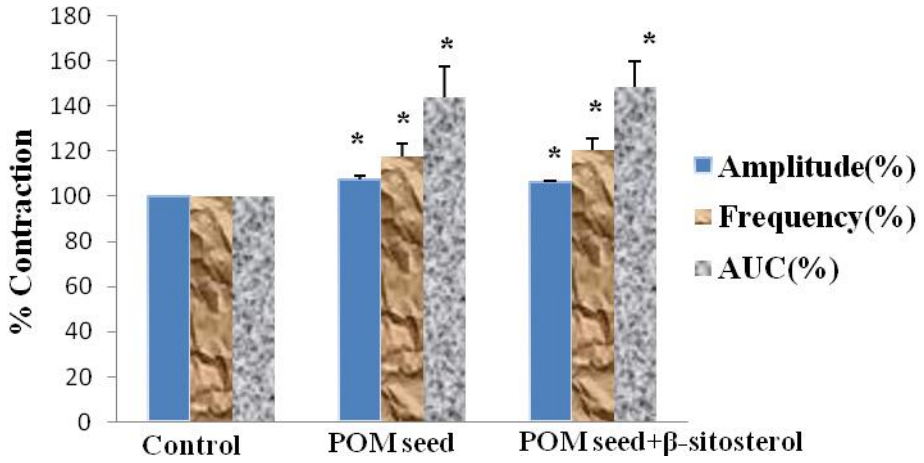
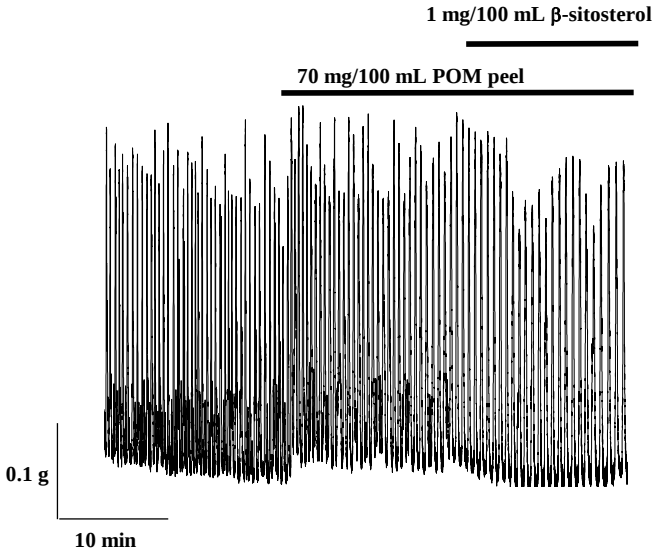


Figure 9.2 The effect of pomegranate seed on β-sitosterol.

A



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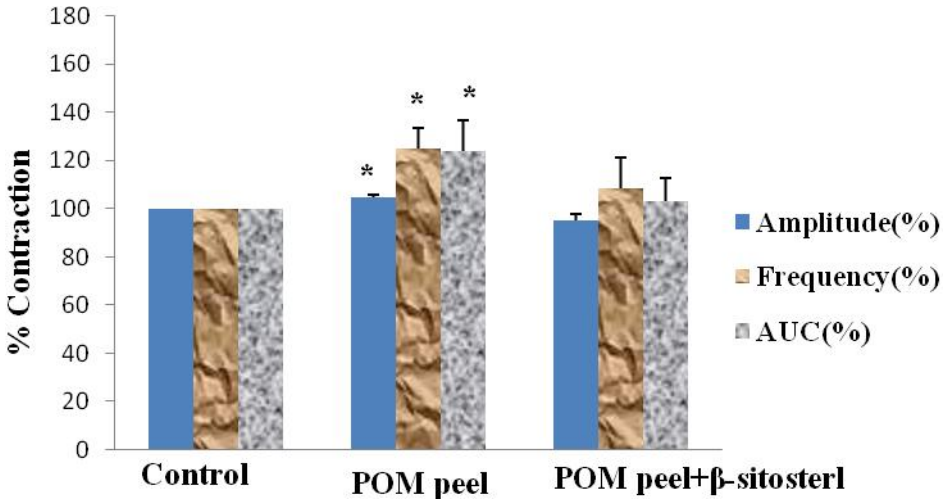
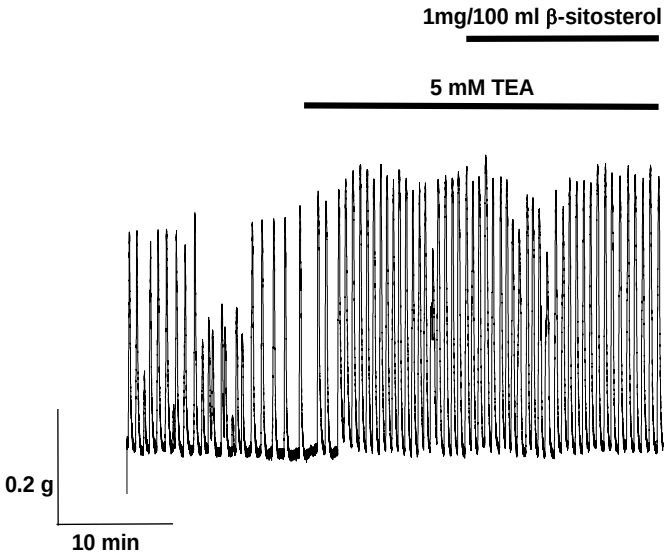


Figure 9.3 The effect of pomegranate peel on β-sitosterol.

A



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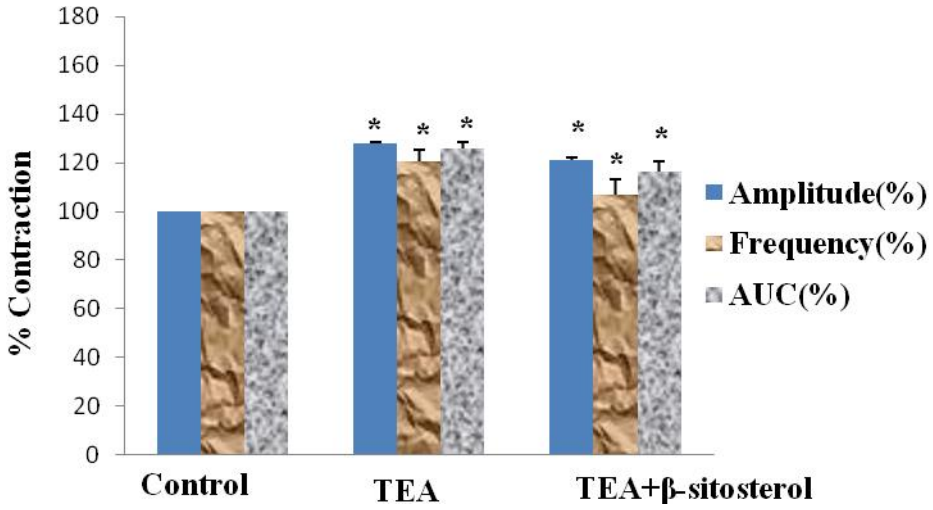

Figure 9.4 The effect of tetraethylammonium on β-sitosterol.

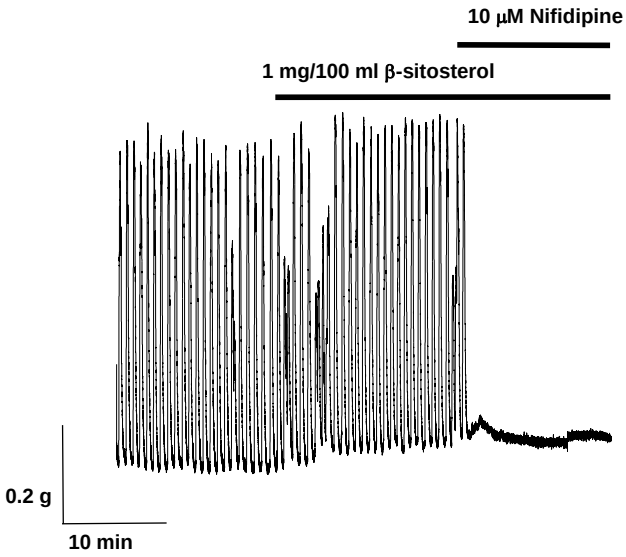

Figure 9.5 Effect of β-sitosterol on uterine contractions in the absence of external Ca^{2+}.

9.5 Discussion

β-sitosterol was found as the main constituents of pomegranate seed and peel extracts as shown in Table 3.1. The effects of β-sitosterol on myometrial contraction have not been investigated. Therefore, the aim of this Chapter was to evaluate the effects of β-sitosterol on myometrial contractile activities. The results showed that the effects of β-sitosterol was similarly indistinguishable to those of pomegranate extracts; suggesting the effects of pomegranate extracts found could be due to the effects of β-sitosterol.

It has revealed that the β-sitosterol potently potentiates spontaneous contractions. Both the amplitude and AUC of the phasic contraction were

significantly increased as well as the basal tension. The increased frequency of the contraction was not significantly different.

These results reported here show effect of β-sitosterol on spontaneous in normal and ovariectomized rats. The amplitude and the frequency were not statistically different between the normal and ovariectomized rats. However, AUC of the normal rats was significantly increased in the contractions arising spontaneously. The potentiation of force induced by β-sitosterol was however insufficient to overcome the effects of inhibition of L-type calcium channels. The effects of the β-sitosterol on spontaneous contractions resembled to those of inhibiting K^+ channels with TEA prevented the β-sitosterol exerting its effects.

In summary, the effects found were the same as those observed in β-sitosterol on spontaneous contractions. Interestingly, its effects were likely to increase amplitude of the phasic contraction and to significantly increase the basal tension. Thus, the effects of pomegranate seed and peel extracts were indistinguishable from those of β-sitosterol.

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CHAPTER X

CONCLUSION

The main aims of this thesis were to investigate 1) the effects of the pomegranate (*Punica granatum* L.) extracts on serum estrogen level, bone protection (bone mineral densitometry), reproductive actions (uterine weight, vaginal cytology, mammary gland development), lipid profile (low-density lipoprotein, high-density lipoprotein, and triglycerides); 2) the effects of the pomegranate extracts on anti-implantation; and 3) the effects of the pomegranate extracts on contraction and compared its effect to the known compounds such as β-sitosterol. The underlying mechanism of the extracts was also investigated.

10.1 Identification of Pomegranate Seed and Peel Extracts

Pomegranate seed and peel extracts were analyzed using GC/MS. Pomegranate seed extract contains twenty-five compounds and four unknown. The retention time was 40.55 min, indentified as β-sitosterol (14.93%). Pomegranate peel extract contains fourteen compounds and two unknown. The retention time was 40.13 min, indentified as β-sitosterol (7.02%).

10.2 Effects of Pomegranate (*Punica granatum* L.) Extract on Uterus, Mammary Gland and Vagina

Pomegranate expressed weakly estrogenic and heuristically of interest for the treatment of menopausal symptoms. The ovariectomized rat is a widely used model to study estrogen withdrawal and replacement because many phenomena in this rat model are similar to those occurring in postmenopausal women (Guillermo, 2007). The histological analysis showed that pomegranate seed and peel extracts produced slight increases in uterine weight and endometrial thickness. Pomegranate seed extract (1000 mg/kg B.W.) induced vaginal hyperplastic epithelium and endometrial thickness compared with ovariectomized rats. However, both pomegranate seed and peel extracts (1000 mg/kg B.W.) stimulated interlobular duct growth without resulting in secretory activity. Serum LH levels were inhibited by E_2 (both doses) but not by pomegranate extracts.

10.3 The Estrogenic Activity of Pomegranate (*Punica granatum* L.) Extract on Inducing Vaginal Cornification

Pomegranate seed extract exhibits estrogenic activity in mice (Junko, 2004). Pomegranate seed and peel extracts at the different doses (100 and 1000 mg/kg B.W., (p.o.)) induced vaginal opening and the smear showed proestrous or estrous conditions. Increase in percentage vaginal cornification was found with the application of the methanolic extracts of pomegranate (1000 mg/kg B.W.).

10.4 Effects of Pomegranate (*Punica granatum* L.) Extract on Bone Loss and Serum Lipid Profile in Ovariectomized Rats

The rat model is useful in studying osteoporosis and the ovariectomized rat was judged to be the standard animal for the study of bone loss caused by estrogen deficiency (Thompson, 1995). The results showed that pomegranate seed and peel extracts (1000 mg/kg B.W.) have a tendency to increase bone mineral densities. However, in view of the effects of pomegranate seed and peel extracts on lipid profile, it was difficult to explain because the data suggest the opposite theory. The estrogen replacement therapy, using pomegranate extract, has shown no potency for lipid profile.

10.5 Anti-implantation Activity of Pomegranate Extract in Pregnant Rats

Pomegranate seed, juice and peel products paradoxically have been reported to not only prevent abortion (Ramirez et al., 1988) but also conception (Gujral et al., 1960; Jochle, 1971; Zhan, 1995). The methanolic extracts of pomegranate seed and peel at the different doses (100 and 1000 mg/kg B.W., (p.o)) exerted significant anti-implantation activities.

10.6 Effects of Pomegranate (*Punica granatum* L.) Extract on Uterine Contractility

Under control conditions, the spontaneous contraction of rat myometrial smooth muscles can be increased contraction by the extracts of pomegranate seed and

peel (200-260 mg/100 mL and 30-90 mg/100 mL). Pomegranate seed and peel extracts increased spontaneous contraction in a concentration dependent manner with a maximum effect at of 250 mg/100 mL and 70 mg/100 mL, respectively. The amplitude and the frequency of the phasic contraction were significantly increased as well as the basal tension. Force produced in the presence of pomegranate seed and peel extracts were abolished by inhibition of L-type calcium channels or myosin light chain kinases (MLCK). Contractions were not potentiated by pomegranate extract following inhibition of K^+ channels.

10.7 Effects of Pomegranate (*Punica granatum* L.) Extract on Spontaneous Contractility in Ovariectomized Rats

All concentration (200-260 mg/100 mL) of pomegranate seed extract significantly decreased the amplitude. Significant increases in the frequency of the spontaneous contractions were found with 200-260 mg/100 mL. All concentration (30-90 mg/100 mL) of pomegranate peel extract significantly increased amplitude. Significant increases in frequency were found at concentrations of 30-70 mg/100 mL. Thus, pomegranate seed and peel extracts are potent stimulators of phasic activity of the uterus taken from ovariectomized rats.

10.8 Effects of β-sitosterol on Uterine Contractility

The results showed that the effect of β-sitosterol was similar indistinguishable to those of pomegranate extracts. Interestingly, its effects were likely to increase amplitude of the phasic contraction and to significantly increase the basal tension. The potentiation of force induced by β-sitosterol was however

insufficient to overcome the effects of inhibition of L-type calcium channels. The effects of the β-sitosterol on spontaneous contractions resembled to those of inhibiting K^+ channels with TEA prevented the β-sitosterol exerting its effects.

Thus, it increased uterine contractions via the inhibition of L-type calcium channels or myosin light chain kinases (MLCK). Contractions were not potentiated by β-sitosterol following inhibition of K^+ channels.

10.9 Future Work

The results clearly show that pomegranate, grown in Thailand, has estrogenic activity. However, the experiments were undertaken in animal models. Thus, in the future, it would be interesting to investigate such the effects in human model.

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APPENDICES

APPENDIX A

THE EFFECTS OF POMEGRANATE SEED EXTRACT AND β-SITOSTEROL ON RAT UTERINE CONTRACTIONS

APPENDIX B

EFFECTS OF POMEGRANATE EXTRACTS ON RAT UTERINE CONTRACTION

ESTROGENIC EFFECTS OF POMEGRANATE EXTRACTS IN OVARIECTOMIZED RATS

EFFECTS OF THAI POMEGRANATE TREATMENT IN MAMMARY GLAND, UTERUS, AND VAGINA

The Effects of Pomegranate Seed Extract and β-Sitosterol on Rat Uterine Contractions

Wilawan Promprom, MSc, Pakanit Kupittayanant, PhD, Korakod Indrapichate, PhD, Susan Wray, PhD, and Sajeera Kupittayanant, PhD

*The aim of this study was to investigate the effects of pomegranate (*Punica granatum *L., Punicaceae) seed extract on uterine contractility. Pomegranate seeds were methanolic extracted and their constituents analyzed using gas chromatography and mass spectrometry. Isometric force was measured in strips of longitudinal rat myometrium and the effects of pomegranate seed extract studied. We found β-sitosterol to be the main constituent of the extract (16%) and its effects were also investigated. Pomegranate seed extract and β-sitosterol increased spontaneous contractions* in a concentration-*dependent* manner *with a maximum effect at 250 mg/100 mL and 1 mg/100 mL, respectively. The amplitude and frequency of the phasic contraction were significantly increased along with basal tension. The effects of pomegranate seed extract were very similar to those of β-sitosterol. Force produced in the presence of pomegranate seed extract was abolished by the inhibition of L-type calcium channels or myosin light chain kinase (MLCK). Contractions were not potentiated by pomegranate extract following the inhibition of K channels or inhibition of the sarcoplasmic reticulum calcium ATPase (SERCA). The actions of β-sitosterol and the extract were not blocked by the estrogen receptor blocker, fulvestrant. We conclude that pomegranate seed extract is a potent stimulator of phasic activity in rat uterus. Our data suggest that the uterotonic effect is due to nonestrogenic effects of β-sitosterol acting to inhibit K channels and SERCA and thereby increasing contraction via calcium entry on L-type calcium channels and MLCK. We suggest that pomegranate extract and β-sitosterol may be a useful uterine stimulant.*

KEY WORDS: *Punica granatum* L, Punicaceae, pomegranate, smooth muscle, potassium channel, calcium, sarcoplasmic reticulum calcium ATPase, SR.

The pomegranate (*Punica granatum* L., Punicaceae) is an ancient, mystical, and highly distinctive fruit that has been used in folk medicine in many cultures.[1] Pomegranates are a rich source of crude fibers, pectin, sugars, and several tannins.[2] They also contain species of flavonoids and anthocyanidins in their seed oil and juice.[3] The seeds have been shown to contain a variety of estrogenic compounds,[4,5] as well as other steroids such as testosterone, β-sitosterol, and coumesterol.[5,6]

It has been shown that the extracts of all parts of the fruit appear to have therapeutic properties.[7] Most research has focused on its antioxidant, anticarcinogenic, and anti-inflammatory properties.[7] In addition, pomegranate seed extract has a uterotropic effect as it increased uterine weight and induced vaginal cornification in ovariectomized animals.[8]

To the best of our knowledge, the uterotonic effect of pomegranate seed has not been studied. As there is a clinical need to find better drugs to help control uterine activity,[9] and novel compounds are sought, the aim of the

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study was therefore to investigate the effects of pomegranate seed extract on uterine contractions. We particularly examined the effects on spontaneous phasic contractions and the mechanisms whereby pomegranate exerts its effects. The effects of the extract were then compared to β-sitosterol, one of the common plant estrogens isolated from pomegranate seeds.[5,6] Recently, it has been reported that plant sterols such as β-sitosterol may act to inhibit the sarcoplasmic reticulum calcium ATPase or SERCA[10] and that sterols can affect calcium-activated K channels[11]; we therefore investigated these possibilities. We find that pomegranate extract significantly stimulates uterine activity. Some of these data have been reported in abstract form.[12]

MATERIALS AND METHODS

Plant Material

Fresh pomegranate fruits were collected from fields in the area of Nakhon Ratchasima, Thailand, during April to May. The plant and its fruit was identified and confirmed by the Royal Forest Department of Thailand and a voucher specimen (Herbarium No 080252) deposited in the laboratory for future reference.

Extraction and Isolation

The pomegranate seeds were manually isolated. They were cleaned, air-dried, powdered, and subjected to Soxhlet extraction with methanol. The extract was filtered through a filter paper, evaporated in a rotary evaporator, and dried by a lyophilizer. The yield was 25.06%.

Gas chromatography/mass spectrometry (GC/MS) analyses were performed on a Hewlett-Packard 5973(IE) MS selective detector coupled with a Hewlett Packard 6890 gas chromatograph equipped with a cross-linked 5% PHME siloxane HP-5MS capillary column (30 m × 0.25 mm; film thickness, 0.25 μm). The gas chromatographic conditions were as follows: carrier gas, helium with a flow rate of 1.0 mL/min; column temperature, 50°C at 6°C/min; injector temperature, 250°C; volume injected, 0.1 μL of the oil; split ratio, 250:1. Compound identification was based on comparisons with mass spectra and retention indices of authentic reference compounds where possible. The rest of the extract obtained was stored at 4°C until use in the physiological experiments. A working solution was obtained by dissolving the extract in physiological solution. We also purchased β-sitosterol, a major component of pomegranate seeds.

Animals

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

The rats were humanely killed by cervical dislocation under carbon dioxide (CO_2) anesthesia. The uterus was removed and immediately immersed in buffered physiological solution (pH 7.40) containing (mmol/L) 154.0 NaCl; 5.4 KCl; 1.2 $MgSO_4$; 12.0 glucose; 2.0 $CaCl_2$, and 10.0 *N*-[2-hydroxyethyl]piperazine-*N*-[2-ethanesulfonic acid] (HEPES). The uterus was placed in a shallow dissecting dish containing physiological solution at 37°C, and under a microscope, the longitudinal muscle layer was separated from the endometrium and circular muscle layer. Five or six strips (1-2 mm × 0.5 mm × 10 mm) were dissected and either used immediately or stored for a maximum of 12 hours at 4°C.

Tension Measurement

The uterine strips were mounted vertically under a resting tension of 1 g in a tissue bath (25 mL Panlab s.l. for AD-Instruments Pty Ltd., Spain) connected to a force transducer (AD Instruments Pty Ltd., Spain) using silk threads. The electrical signal from the transducer was amplified and converted to a digital signal and recorded on a computer using Chart software (AD Instruments Pty Ltd., Australia). The tissue-bathing medium used was physiological saline solution maintained at pH of 7.40, temperature of 37°C, and gassed with 100% O_2. The strips were allowed to contract spontaneously and an equilibrium period of at least 30 min was given before the application of any chemical. The measurements were made while the tissue was continually perfused with physiological solution (control) or solution containing pomegranate seed extract between 200 and 260 mg/100 mL. In some experiments, the known component of the extract, β-sitosterol (0.5-1.5 mg/100 mL, dissolved in physiological solution) was used. Wortmannin, an inhibitor of myosin light chain kinase (MLCK)[13]; nifedipine an inhibitor of L-type Ca

entry[14]; tetraethylammonium (TEA), an inhibitor of calcium-activated potassium channels; and cyclopiazonic acid (CPA), an inhibitor of the SERCA pump[15]; and fulvestrant, an estrogen receptor antagonist,[16,17] were also used, as indicated in the text.

Chemicals

All chemicals were purchased from Sigma unless stated otherwise. The purity of β-sitosterol, which was used as a positive control, was 75%.

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 pomegranate extract or β-sitosterol were measured over 20 minutes from the start of their application. Results were expressed as percentages of control contractions (ie, the control is 100%). To test the effects of stimulation with CPA, TEA, or fulvestrant following pomegranate extract, contractions were compared for 10 minutes in CPA and pomegranate extract (ie, 11-20 minutes after start of pomegranate extract exposure), to the 11 to 20 minutes in pomegranate extract without the addition of CPA, TEA, or fulvestrant. Integrated force (area under the curve) was measured over a 10- or 20-minute period as appropriate. In some experiments, changes in force amplitude are expressed with respect to basal (resting) force level (0%) and the peak force (100%) in control condition. Throughout, data are presented as mean ± SEM and "n" represents the number of samples, each one from a different animal. Significance was tested using appropriate *t* tests and *P* values <.05 taken to be significant.

RESULTS

Gas Chromatography/Mass Spectrometry Analysis

The GC/MS analysis showed 4 main compounds that had retention times (minutes) of 40.55 (18.30%), 18.23 (15.72%), 30.16 (14.93%), and 17.01 (11.04%). These corresponded to tocopherol, 6-butyl-1,4-cycloheptadiene, β-sitosterol, and octadecadienoic acid, respectively. Traces of 22 other known compounds, mainly essential oil (0.1%-5%), and 4 unknown compounds were detected (data not shown).

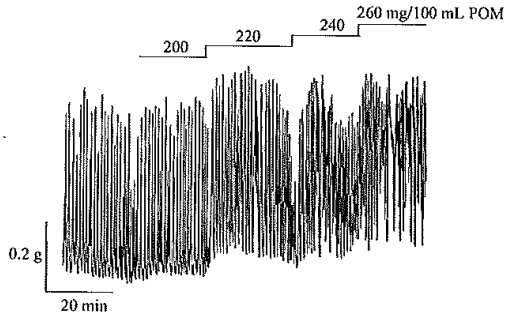

Figure 1. The effects of pomegranate seed extract (POM) on spontaneous contraction. The presence of increasing cumulative concentrations of POM (200-260 mg/100 mL) is shown.

Spontaneous Uterine Activity—Dose Dependency of Pomegranate Extract

Under control conditions, spontaneous contractions of consistent amplitude and frequency could be recorded for several hours, allowing the effects of the different concentrations of the extract to be examined (see Figure 1). The effects of increasing cumulative concentrations of pomegranate seed extract (200-260 mg/100 mL) were examined; each concentration was applied for 30 minutes. Pomegranate seed extract, in a concentration-dependent manner, increased uterine contractility arising spontaneously (n = 5). An example of this is shown in Figure 1. At each concentration, the extract increased the amplitude and the frequency of the contractions and increased basal tension. The stimulatory effects of pomegranate seed extract could be seen within 5 minutes of application and were maintained as long as it was present in the bath. These effects were irreversible over the timescale of the experiments. The threshold concentration at which an effect was consistently observed with 220 mg/100 mL of pomegranate seed extract, and the maximal stimulatory concentration on myometrium contractility occurred between 240 and 260 mg/100 mL (n = 5). Thus, the concentration of 250 mg/100 mL was used throughout the remainder of the study.

Effects on Parameters of Contraction

The application of pomegranate seed extract (250 mg/100 mL) to the rat myometrial preparations produced significant potentiating effects on spontaneous force (n = 5). The frequency of the contractions increased significantly

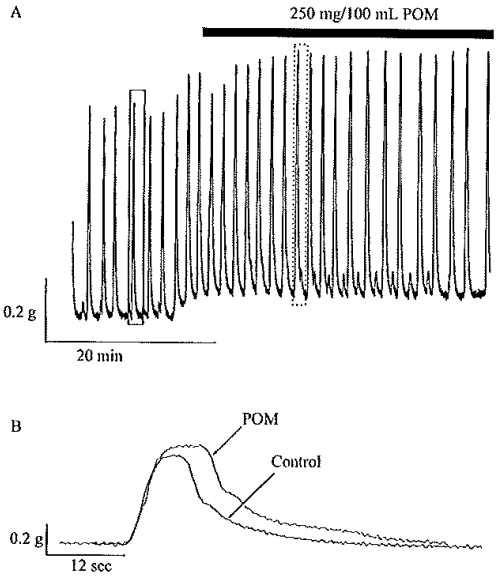

Figure 2. The effects of pomegranate seed extract (POM) on spontaneous contraction. Pomegranate seed extract (250 mg/100 mL) is added to spontaneously contracting uterus (A). Superimposed force records taken from (A), under control conditions and in the presence of POM (dotted trace; B).

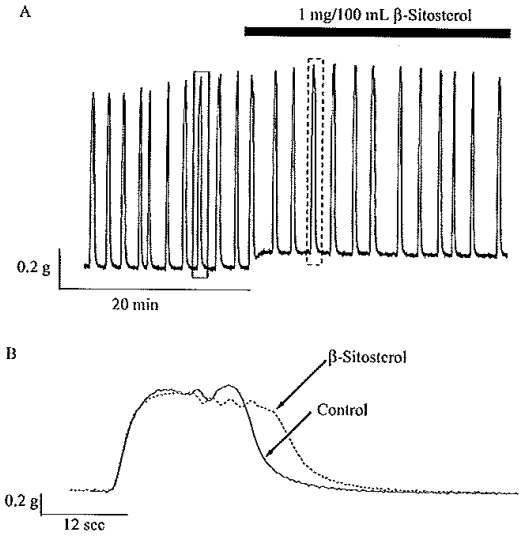

Figure 3. The effects of β-sitosterol on spontaneous contraction. β-sitosterol (1 mg/100 mL) is added to spontaneously contracting uterus (A). Superimposed force records taken from (A), under control conditions and in the presence of β-sitosterol (dotted trace; B).

to 134% ± 11%. The amplitude of force was also significantly increased; 130% ± 12%, as was its duration; 163% ± 3% (all compared with control, 100%). The mean increase in integrated force over the last 20 minutes in extract was 146% ± 12%. A typical example is shown in Figure 2A. It can be seen in Figure 2B, where control and pomegranate seed extract records have been expanded and overlapped, that there is a clear effect of pomegranate seed extract to prolong the force transient, due to an effect of prolonging the plateau phase and also significantly slowing the relaxation rate (172% ± 23%). In addition, pomegranate seed extract consistently increased basal force by 6% ± 1% (see Figure 2A).

Effects of β-sitosterol

As shown above, it is clear that pomegranate seeds potentiate uterine contraction. As β-sitosterol is one of the major components found in the extract and has previously been found to be a phytoestrogen, it was of interest to determine whether the effects of pomegranate seed extract were due to this compound. The estrogenic activity of phytoestrogens range from 1/500 to 1/1000 of the activity of estrogen.[18] Initial dose response curves over the range 0.5 to 1.5 mg/100 mL showed that maximal effects were achieved at around 1 mg/100 mL, and thus, a dose of 1 mg/100 mL of β-sitosterol was used (n = 5). As shown in Figure 3A, application of β-sitosterol produced significant increases in force (125% ± 3% compared with control integrated force). The amplitude of force was significantly increased; 111% ± 3%, as was its duration; 138% ± 8% (all compared with control, 100%). However, the frequency of the contractions was not significantly increased (107% ± 3%; compared with control, 100%). As with the pomegranate seed extract, β-sitosterol increased the basal force to 7% ± 1% (see Figure 3A). It is interesting to note that β-sitosterol also prolonged the force transient (Figure 3B).

Effects of Ca-dependent force pathway modulation

Uterine force can be produced by several pathways, but the main mechanism involves Ca-calmodulin-MLCK.[19] To investigate whether the increases in uterine force were

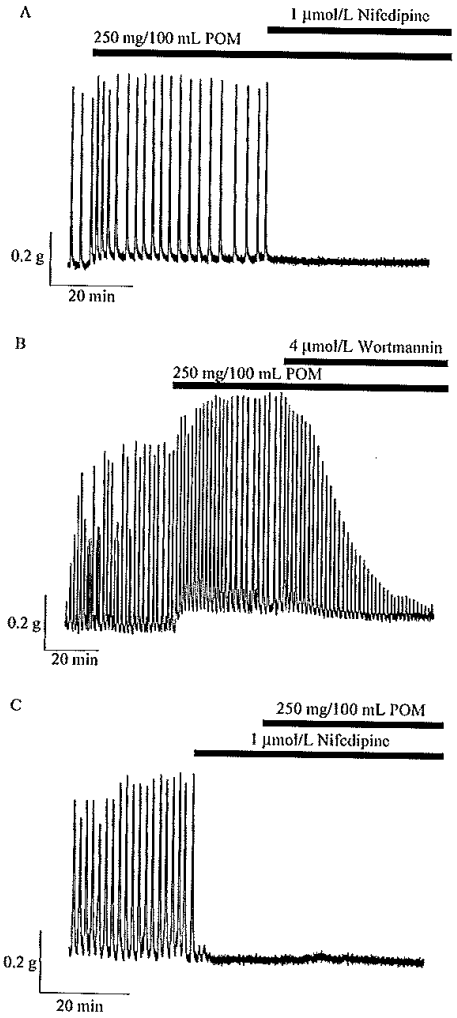

Figure 4. The effects of nifedipine and wortmannin on rat uterine contractions induced by pomegranate seed extract (POM). Control POM application followed by POM and nifedipine (1 μmol/L; A). Control POM application followed by POM and wortmannin (4 μmol/L; B). Nifedipine (1 μmol/L) application followed by POM (250 mg/100 mL; C).

dependent on the calcium-calmodulin MLCK pathway, we studied the effects of the extract in the presence of the L-type calcium channels and MLCK inhibitors (Figure 4). As can be seen in Figure 4A, the application of 1 μmol/L nifedipine in the continued presence of the extract rapidly inhibited and then abolished force ($n = 5$). Basal force, however, did not return to control levels but remained elevated.

We next investigated the effects of pomegranate seed extract in the presence of a potent inhibitor of MLCK, wortmannin (Figure 4B). As shown in Figure 4B, wortmannin (4 μmol/L) in the continued presence of the extract gradually reduced force in all preparations studied (n = 5); a significant reduction occurred after 10 minutes (mean amplitude of contractions 86% ± 1%) and by 45 ± 9 minute contractions were abolished.

In the uterus, some uterotonic agents can elicit a contraction in a 0-Ca solution or when L-type Ca channels are blocked,[15,20] and it has been suggested that this contraction occurs independently of the calcium-calmodulin-MLCK pathway.[15,20] To investigate this, the extract was applied after application of nifedipine. As can be seen in Figure 4C, no force is observed during the application of the extract (n = 5).

Role of K Channels

The effects of pomegranate extract on force resembled those of potassium channel blockers, which prolong the action potential and thereby potentiate force.[15,21] Thus, the question arose whether the extract effects were mediated by effects on potassium channels. We therefore blocked potassium channels, with TEA (5 mmol/L) and studied the effects of pomegranate seed extract (n = 6). Application of TEA produced increases in force (137% ± 4% compared with control integrated force), but no further significant increase occurred upon addition of pomegranate seed extract in the continued presence of K channel inhibition (110% ± 12%; Figure 5A). Similarly, if TEA was added after pomegranate seed extract, it produced no further increases in force (Figure 5B). These data suggest that the potentiating effect of TEA was prevented by pomegranate seed extract.

Role of Sarcoplasmic Reticulum

Release of Ca from the sarcoplasmic reticulum (SR) can potentiate force in smooth muscles,[22] and thus the SR may be a target for pomegranate extract. In addition, the increase in basal tone found with the extract could be due to the release of Ca from the SR or inhibition of Ca reuptake by SERCA. We therefore elucidated the effect of pomegranate extract after the inhibition of SERCA by cyclopiazonic acid (20 μmol/L).[15,23] As expected, inhibiting SERCA increased uterine force. As can be seen in Figure 6A, the extract was unable to potentiate force after

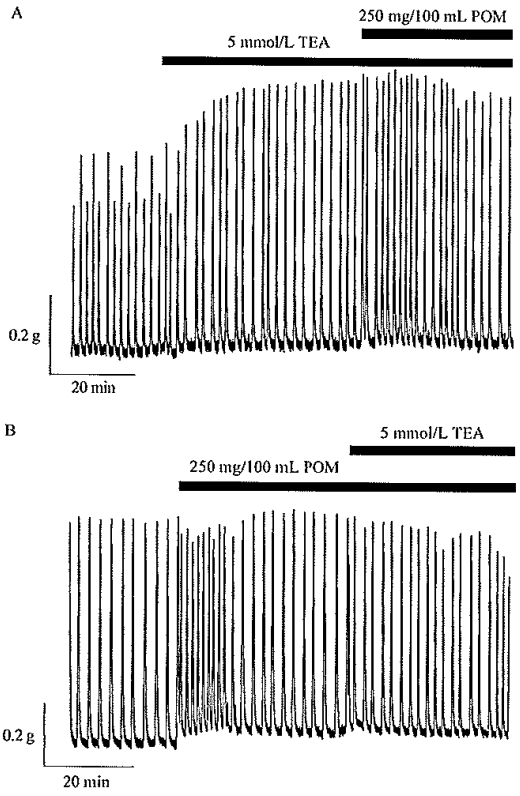

Figure 5. The effects of pomegranate seed extract (POM) on contraction in the presence of the calcium-activated potassium channel inhibitor. Tetraethylammonium (TEA, 5 mmol/L) is added before (A) and after (B) POM (250 mg/100 mL).

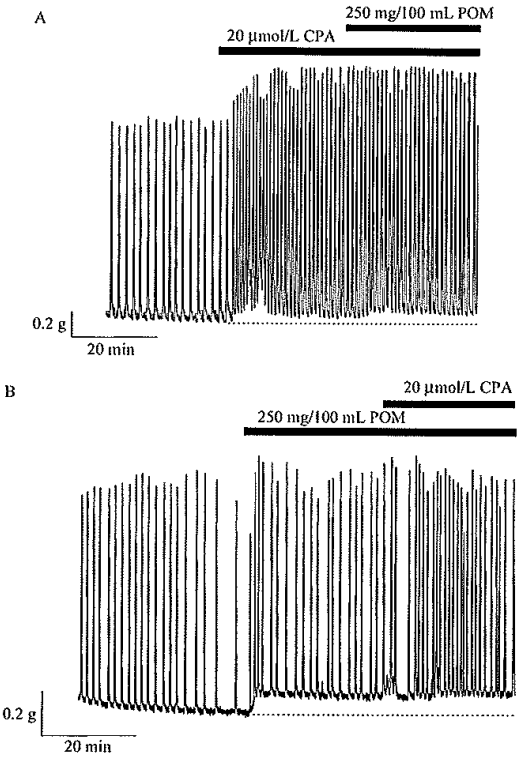

Figure 6. The effects of pomegranate seed extract (POM) on contraction in the presence of the sarcoplasmic reticulum calcium-ATPase inhibitor. Cyclopiazonic acid (CPA, 20 μmol/L) application is added before (A) and after (B) POM (250 mg/100 mL).

CPA treatment (n = 5). The application of CPA produced increases in force (227% ± 18% compared with control integrated force). No further increase occurred upon addition of pomegranate seed extract in the continued presence of CPA (103% ± 4%; Figure 6A). As can be seen in Figure 5A, CPA caused an increase in basal tone (≅5%); no further increase occurred in the presence of pomegranate. If CPA was added after pomegranate seed extract (n = 5), it produced no further increases in force amplitude (Figure 6B), or basal tone, but the contraction frequency was increased.

Effects of Fulvestrant

The above results clearly show the effects of pomegranate seed extract, which could be occurring through either the nonreceptor estrogen-like actions of β-sitosterol or via its estrogen receptor-mediated actions. We therefore blocked the estrogen receptors, with fulvestrant (1 μmol/L),[16,17] and studied the effects of pomegranate seed extract (n = 5) and β-sitosterol (n = 6). Application of fulvestrant to spontaneous active uterus produced no significant changes in uterine contractions. The amplitude, frequency, and area under the curve of spontaneous contraction after the application of fulvestrant were 105% ± 2%, 104% ± 10%, and 97% ± 10% (all compared with control, 100%). Application of pomegranate seed extract in the continued presence of fulvestrant produced significant increases in the amplitude of the contraction, frequency, and area under the curve (Figure 7). The increases in the frequency and area under the curve were 105 ± 3, 142% ± 11% and 131% ± 4% (all compared

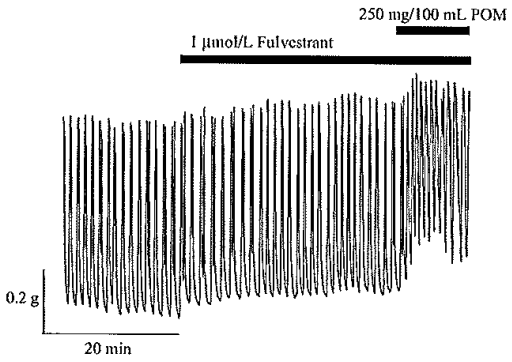

Figure 7. The effects of pomegranate seed extract (POM) on contraction in the presence of the estrogen receptor antagonist. Fulvestrant (1 μmol/L) application is added before POM (250 mg/100 mL).

with fulvestrant control, 100%). β-sitosterol was also able to significantly increase force in the presence of fulvestrant (data not shown).

DISCUSSION

This study is the first to show the effects of pomegranate seed extract on uterine contraction and demonstrates that it potently potentiates spontaneous contractions. Both the amplitude and the frequency of the phasic contraction were significantly increased as well as the basal tension. The effects of pomegranate seed extract were very closely matched to those of β-sitosterol. The potentiation of force induced by the extract was dependent upon the Ca-calmodulin-MLCK pathway. Pre-incubation with TEA or CPA prevented the extract exerting its effects, suggesting that K channels or SERCA are the targets for its actions. When the effects of pomegranate seed extract and β-sitosterol were compared after fulvestrant treatment, they were still effective. Thus, our conclusion is that pomegranate seed extract is a powerful uterine stimulant acting via a nonestrogen mechanism.

In agreement with previous studies,[5,6] we showed that one of the major constituents isolated in pomegranate seed extract is β-sitosterol. Other constituents, mainly essential oils, have been demonstrated to relax rather than contract uterine smooth muscle.[24] Thus, the predominant effect on uterine contractions of an extract of pomegranate is potentiating, and we show that β-sitosterol is the active agent responsible for this effect. It also increased basal tone as did the extract.

We found that the pathway to increase uterine contraction by pomegranate seed extract occurred via calcium-dependent pathway as: (a) addition of the extract could not produce force in the absence of external calcium entry and, (b) force produced in the presence of the extract was abolished when Ca entry through L-type Ca channels was inhibited. Further support for this conclusion comes from the experiments with MLCK inhibition; force was no longer produced by the extract. Thus, our data support a mechanism of action involving the Ca-calmodulin-MLCK pathway rather than that of Ca-sensitization. Nifedipine application, however, did not reverse the increase in basal tone caused by the extract, although it did abolish the spontaneous contractions. This suggests that the mechanism causing the elevation of basal tone is not dependent upon Ca entry and may therefore involve the internal Ca store, that is, the SR. This is discussed further later.

Our data suggest that the extract is potentiating force by an inhibition of K channels and an effect on the SR. The effects of pomegranate seed extract on the uterus resembled those of the K channel inhibitor 5 mmol/L TEA,[15,23] and after exposure to TEA, the extract was without effect. There are data from other sterols, especially cholesterol, that these compounds can modulate K channel activity. Specifically in the uterus, cholesterol manipulation can have marked effects on Ca signaling and contractility[25] via effects on Ca-activated K channels.[11] Cholecalciferol, a vitamin D_3 precursor, has also been shown to affect K channel activity in vascular smooth muscle.[26] Further studies are suggested to determine which type of K channels are the main targets of the extract, along with measurements of electrical activity.

The experiments in which SERCA was inhibited using CPA point to an involvement of the SR in mediating the effects of pomegranate extract. Pre-incubation with CPA prevented any additional effects of the extract occurring. However, CPA was still able to produce some stimulation of the uterus following pomegranate application. The role of the SR in spontaneous activity of the uterus remains enigmatic, as its inhibition promotes contractility, suggesting it is not acting as a source of Ca for spontaneous contractions.[27] Nor is it likely that it is acting via the release of Ca sparks from the SR that activate Ca-activated K channels, as such a mechanism is absent in the uterus.[28] Thus, it is difficult to interpret our findings in further detail. We can find no evidence in smooth muscle that β-sitosterol potentiates force by inhibiting SERCA. There is evidence however that phytoestrogen can affect SERCA in cardiac muscle.[29] In addition, there

is clear evidence in macrophages that plant sterols such as sitosterol can inhibit SERCA.[9]

Inhibition of SERCA may explain the increase in basal tone found with extract application. Our previous studies have shown that when SERCA is inhibited; there is an increase in intracellular [Ca],[15] which may lead to change of basal tone. Our data with nifedipine support the source of Ca being intracellular, as nifedipine abolished phasic contractions but did not restore basal force to resting condition.

CONCLUSION

In conclusion, we have presented novel data demonstrating a significant stimulation of uterine activity by pomegranate seed extract, which can largely be accounted for by its constituent, β-sitosterol, acting as a nonestrogen receptor-mediated mechanism. The stimulation of uterine activity may contribute to the antifertility effects of pomegranates, and they may be a useful source of uterine stimulant for slowly progressing labours,[30] although further studies in human myometrium are required to develop these suggestions. Although not studied, it is speculated that, as the pathways for calcium signaling and contractility are similar in the pregnant to nonpregnant myometrium, and in human and rat myometrium, that similar effects of pomegranate seed extract will be seen in the pregnant human uterus.

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