ผลของกวาวเครือขาว *Pueraria mirifica* Airy Shaw & Suvatabandhu ต่อการรักษาภาวะกระดูกพรุนที่เหนี่ยวนำโดยการตัดรังไข่ในหนูแรท

นางสมฤดี หาญมานพ

วิทยานิพนธ์นี้ เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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THE THERAPEUTIC EFFECTS OF WHITE KWAO KRUA *Pueraria mirifica* Airy Shaw & Suvatabandhu ON OVARIECTOMY - INDUCED OSTEOPOROTIC RATS

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สมฤดี หาญมานพ : ผลของกวาวเครือขาว *Pueraria mirifica* Airy Shaw & Suvatabandhu ต่อการรักษาภาวะกระดูกพรุนที่เหนี่ยวนำโดยการตัดรังไข่ในหนูแรท (THE THERAPEUTIC EFFECTS OF WHITE KWAO KRUA *Pueraria mirifica* Airy Shaw & Suvatabandhu ON OVARIECTOMY - INDUCED OSTEOPOROTIC RATS) อ. ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. ดร. สุจินดา มาลัยวิจิตรนนท์; 91 หน้า.

กวาวเครือขาวเป็นพืชสมุนไพรไทย ที่มีสารไฟโตเอสโตรเจนในปริมาณสูง มีรายงานว่ากวาวเครือขาว สามารถป้องกันการสูญเสียมวลกระดูกในหนูแรทเพศเมียและเพศผู้ที่ตัดต่อมบ่งเพศออกได้ การศึกษาครั้งนี้จึงมีวัตถุประสงค์เพื่อศึกษาผลของกวาวเครือขาวต่อการรักษาภาวะกระดูกพรุนที่ถูกเหนี่ยวนำโดยการตัดรังไข่ในหนูแรทเพศเมีย ทำการทดลองโดยนำหนูแรทเพศเมียอายุ 6 เดือน มาแบ่งออกเป็น 2 กลุ่ม คือ กลุ่มที่ไม่ตัดรังไข่ (SH) จำนวน 13 ตัว และกลุ่มที่ตัดรังไข่ (OVX) จำนวน 45 ตัว เลี้ยงหนูไว้เป็นเวลา 90 วัน เพื่อเหนี่ยวนำให้เกิดภาวะกระดูกพรุน เจาะเลือดทุกๆ 30 วัน เพื่อนำมาวัดระดับของ alkaline phosphatase (ALP) และ tartrate resistant acid phosphatase 5b (TRAP 5b) ในซีรั่ม สุ่มหนูแต่ละกลุ่มออกมา 5 ตัว เพื่อทำการุณยฆาตและเก็บกระดูกมาวัดความหนาแน่นกระดูก (BMD) มวลกระดูก (BMC) และศึกษาโครงสร้างระดับจุลวิทยาและวัดพื้นที่หน้าตัดเนื้อกระดูกโปร่ง (%BA) และ หลังจากนั้นนำหนูกลุ่ม SH ที่เหลือ มาป้อนน้ำกลั่นเป็นเวลา 90 วัน และแบ่งหนูกลุ่ม OVX ที่เหลือออกเป็น 5 กลุ่ม กลุ่มละ 8 ตัว และป้อนกวาวเครือขาวขนาด 0, 10, 100 และ 1,000 มก. / กก. น้ำหนักตัว/ วัน (กลุ่ม PM0, PM10, PM100 และPM1000 ตามลำดับ) และ ฮอร์โมนเอสโตรเจนสังเคราะห์ (17-α ethinylestradiol: EE) ขนาด 0.1 มก. / กก. น้ำหนักตัว/ วัน เป็นเวลา 90 วัน เจาะเลือดทุกๆ 30 วัน เพื่อนำมาวัดระดับของ ALP และ TRAP 5b ในซีรั่ม ภายหลังจากการให้สารนาน 90 วัน นำหนูมาการุณยฆาต และเก็บกระดูกมาวัด BMD BMC และ %BA

จากการศึกษาพบว่าภายหลังการตัดรังไข่ในหนูแรทเป็นเวลา 90 วัน ค่า BMD, BMC และ %BA ลดลงอย่างมีนัยสำคัญทางสถิติ ($p<0.01$) เมื่อเปรียบเทียบกับกลุ่มที่ไม่ได้ตัดรังไข่ ระดับ ALP สูงขึ้นตลอด 90 วัน แต่ระดับ TRAP 5b เพิ่มขึ้นเฉพาะในวันที่ 30 ($p<0.05$) เมื่อให้กวาวเครือขาวในขนาดต่าง ๆ หรือ EE เป็นเวลา 90 วัน พบว่าค่า BMD BMC และ %BA ของหนูเหล่านี้มีค่าสูงกว่ากลุ่ม PM0 อย่างมีนัยสำคัญทางสถิติ ($p<0.05$) ระดับ ALP ในกลุ่มที่ได้รับกวาวเครือขาวไม่แตกต่างจากกลุ่ม PM0 แต่ระดับ TRAP 5b ลดลงอย่างมีนัยสำคัญทางสถิติ ($p<0.01$) และไม่สัมพันธ์กับขนาดของกวาวเครือขาวที่ให้ ในขณะที่เมื่อให้ EE ไม่พบความแตกต่างของทั้งระดับ ALP และ TRAP 5b จากกลุ่ม PM0

จากผลการศึกษาครั้งนี้สรุปได้ว่า กวาวเครือขาวมีแนวโน้มที่จะรักษาภาวะกระดูกพรุน ในหนูแรทเพศเมียที่ถูกตัดรังไข่ได้ โดยไปยับยั้งการสลายกระดูก โดยกลไกการออกฤทธิ์ของกวาวเครือขาวแตกต่างจากของฮอร์โมนเอสโตรเจนสังเคราะห์

สาขาวิชา สรีรวิทยา ..... ลายมือชื่อนิสิต .....

ปีการศึกษา 2553 ..... ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก.....

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KEYWORDS : Osteoporosis/ Ovariectomized rats/ *Pueraria mirifica*/ ALP, TRAP

SOMRUDEE HANMANOP: THE THERAPEUTIC EFFECTS OF WHITE KWAO KRUA *Pueraria mirifica* Airy Shaw & Suvatabandhu ON OVARIECTOMY-INDUCED OSTEOPOROTIC RATS. ADVISOR: ASSOC. PROF. SUCHINDA MALAIVIJITNOND, Ph.D., 91 pp.

Pueraria mirifica (PM) is a Thai herb which contains high amount of phytoestrogens. It was reported to prevent bone loss in gonadectomized rats. Thus, this study aimed to investigate the therapeutic effects of PM on bone loss in ovariectomized rats. Six months old female rats were divided into 2 groups; sham operation (SH group, n=13) and ovariectomy (OVX group, n=45). After operation, they were kept for 90 days to induce bone loss. Blood samples were collected every 30 days for serum alkaline phosphatase (ALP) and tartrate resistant acid phosphatase 5b (TRAP 5b) assays. On day 90, 5 rats in each group (OVX_{90} and SH_{90} groups) were randomly selected and euthanized. Bone was collected and measured for bone mineral density (BMD), bone mineral content (BMC) and %trabecular bone area (%BA). The remaining SH rats (n=8) were gavaged daily with distilled water for 90 days. The remaining OVX rats were subdivided into 5 groups (n=8/group) and gavaged daily with 0, 10, 100 and 1,000 mg/ kg BW/ day of PM (PM0, PM10, PM100 and PM1000 groups, respectively) and 0.1 mg/ kg BW/ day of 17-α ethinylestradiol (EE group) for 90 days. Blood samples were collected every 30 days for serum ALP and TRAP 5b assays. After 90 days of treatment, rats were euthanized and collected bone for BMD, BMC and %BA determinations.

The results showed that BMD, BMC and %BA were significantly lower in the OVX_{90} rats than the SH_{90} rats ($p<0.01$), serum ALP levels were higher ($p<0.05$) throughout the 90 days of bone loss induction period, while the serum TRAP 5b levels was significantly higher only on D_{30} ($p<0.05$). After 90 days of the PM or EE treatment, BMD, BMC and %BA of rats were significantly higher than those of the PM0 group ($p<0.05$). Although serum ALP levels of PM or EE treated groups were not different from those of the PM 0 group, serum TRAP 5b levels were significantly lower ($p<0.01$), but not depended on doses. Treatment of EE had no effects on serum ALP and TRAP 5b levels, compared to the PM0 group.

These results indicate that PM consumption tends to restore the established osteoporosis in OVX rats by reducing bone loss. However, the mechanism of the effects of PM is different from those of synthetic estrogens.

Field of Study : Physiology ..... Student's Signature Somrudee Hanmanop

Academic Year : 2010 ..... Advisor's Signature S. Malaivijitnond

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ALP	=	alkaline phosphatase
BA	=	trabecular bone area
BMC	=	bone mineral content
BMD	=	bone mineral density
BMUs	=	bone multicellular units
BW	=	body weight
cm	=	cubic centimeter
CtBMC	=	cortical bone mineral content
CtBMD	=	cortical bone mineral density
DXA	=	dual-energy X-ray absorptiometry
E_2	=	17 β-estradiol
EE	=	17 α-ethinylestradiol
ER	=	estrogen receptor
ERα	=	estrogen receptor α
ERβ	=	estrogen receptor β
ERE	=	estrogen responsive element
ERT	=	estrogen replacement therapy
FD	=	femoral diaphysis
FM	=	femoral metaphysis
g	=	gram
IL 6	=	interleukin 6
IL I	=	interleukin I
M-CSF	=	granulocyte-macrophage colony-stimulating factor
ml	=	milliliter
mm	=	millimeter
OPG	=	osteoprotegerin
ORX	=	orchidectomy
OVX	=	ovariectomy

P. mirifica, PM	=	*Pueraria mirifica*
PGE	=	prostaglandin-E_2
pQCT	=	peripheral Quantitativ Computed Tomography
PTH	=	parathyroid hormone
RANK	=	receptor activator of nuclear factor-kappa B
RANKL	=	receptor activator of nuclear factor-kappa B ligand
SERM	=	selective estrogen receptor modulator
TbBMC	=	trabecular bone mineral content
TbBMD	=	trabecular bone mineral density
TD	=	tibia diaphysis
TM	=	tibia metaphysis
TRAP	=	tartrate resistant acid phosphatase
WHO	=	World Health Organization
wk	=	week

CHAPTER I

INTRODUCTION

Osteoporosis is a condition characterized by low bone density and micro architectural deterioration of bone tissue, resulting in an enhance bone fragility and an increased susceptibility to fracture, especially of the hip, spine and wrist (Kanis *et al*., 1994). Osteoporosis has recently emerged as a significant public health problem, which becoming increasingly prevalent with aging of the world's population, and the incidence was higher in women than in men. Osteoporosis is a silent problem as the bone loss typically occurs without symptoms. Many patients are unaware of this problem until they have experienced a fracture. Nearly all fractures make patients being hospitalized and cause considerable morbidity, disability and mortality. The National Osteoporosis Foundation (NOF) of the United States indicates that about 44 million Americans are on the risk of this disease by virtue of having low bone mineral density. Presently, 10 million adults are osteoporosis, and the majority of them are women (Setchell and Lydeking-Olsen, 2003). In Thailand, an increase in the life span of Thai people has proceeded to an increased incidence of osteoporosis and bone fracture. The prevalence of osteoporosis in Thai women occurs rapidly after the age of 55 years and reaches a level of more than 50% after the age of 70 years (Limpaphayom *et al*., 2001). The worst consequence of the bone fracture might not be death. It is that the post-fracture patients need to live dependently, with an impaired quality of life, for many years. The primary goal of osteoporosis treatment is to prevent a fracture, by slowing or decreasing bone loss, maintaining bone strength and minimizing or eliminating factors that induce fractures.

Estrogen plays an important role in bone homeostasis (Compston, 2001). It regulates the bone homeostasis directly to bone cells, and indirectly on the immune system and oxidative stress (Weitzman and Pacific, 2006). It has been reported that the loss of estrogen during menopause could cause the decrease in bone mineral density

(BMD) and bone mineral content (BMC) (Riggs *et al*., 1982; Ohta *et al*., 2002). Thus, estrogen deficiency is a major pathogenic factor in the development of osteoporosis in postmenopausal women. Though estrogen replacement therapy has potential benefits on prevention or cure for osteoporosis, the higher and longer exposure to estrogen is considered to be a main risk factor for the development of breast cancer (Fontages *et al*., 2004), endometrial cancer (Sulak, 1997; Canavan and Doshi, 1999), hypertriglyceridemia and angiogenesis (Lissin and Cooke, 2000). Considering these problems, many researchers/scientists have tried to develop the alternative drugs for bone loss therapy which have no side effects on the other organs.

Over the past several years, there are many agents and chemicals that have the potential for osteoporosis treatment which can be categorized into 2 groups; 1) antiresorptive agents and 2) anabolic agents. **Antiresorptive agents** include selective estrogen receptor modulator (SERMs), bisphosphonates, calcitonin and estrogen. The antiresorptive agents can suppress bone resorption by reducing bone turnover. SERMs, such as tamoxifen and raloxifene, exhibit both estrogenic and antiestrogenic actions. The effect of tamoxifen on breast tumor has been researched for decades and found that it could prevent the recurrences of ER-positive breast tumor (Fisher *et al*., 1996). The use of tamoxifen, at the same time, elicits a beneficial estrogenic effect on bone loss prevention (Love *et al*., 1992). On the negative outcome, however, tamoxifen can lead to the endometrial cancer (Bernstein *et al*., 1999). Similar to the tamoxifen, raloxifene can function as an estrogen agonist on bone cells and prevent bone loss (Delmas *et al*., 1997; Ettinger *et al*., 1999) and behaves as an estrogen antagonist on breast cells and cures the breast cancer (Cummings *et al*., 1999). Oral bisphosphonates are currently the most potent antiresorptive agents for prevention or treatment of osteoporosis. It can reduce the vertebral and hip fracture by 50 % to 60 % in postmenopausal women (Liberman *et al*., 1995; Bauer et al., 2004 and Cheshut *et al*., 2004). However, bisphosphonates have adverse side effects on gastrointestinal tract, such as induction of esophageal ulceration, acid reflux and vomiting, and heart burn, which make the hesitation to use this drug in some women. The weak points for antiresorptive agents are that they do not be able to build a new bone, or bone

formation, thus, it might not be justified to use in osteoporotic patients. **Anabolic agents**, such as recombinant human parathyroid hormone (hPTH) analogs, can increase bone formation by stimulating the osteoblast activity. The hPTH significantly increased BMD at all skeletal sites except at the radius, and subsequently significantly reduced the risk of bone fracture in postmenopausal women (Cranney *et al*., 2006). Though hPTH shows the beneficial effects on bone formation, the application of this drug to humans is still equivocal, because hPTH can also induce a malignant bone cancer (osteosarcoma) (Vahle *et al*., 2004).

In Thailand, the diagnosis of the onset of osteoporosis is usually ignored. Most of the people are unaware of it until the disease is well advanced. The patients come to realize about this when it is too late to return. The patients with a low BMD or used to be an osteoporotic fracture should particularly consider for the treatment. The antiresorptive agents could reduce bone resorption, but it does not increase a bone formation. The anabolic agents can induce a new bone formation, but their side effects and cost are the main issue to be concerned. Therefore, the agent, which can induce bone formation, has fewer side effects and is cheap, should be sought out. The indigenous herb should be one of the choices.

Phytoestrogens, plant derived substances, have structures related to estrogens and can bind to estrogen receptors. They are currently used by many women as an alternative drug for estrogen replacement therapy. Phytoestrogens are classified into 4 groups; isoflavones, lignans, coumestans and stilbenes, which can be found, for example, in soy, red clover, flaxseed and black cohosh (Dang *et al*., 2005). Soy isoflavones have been reported their estrogenic effects on reproductive organs and their anabolic effects on bone (Ishimi *et al*., 2000). In 2001, Picherit *et al*. assessed the effects of daily consumption of soy isoflavone on bone loss in adult ovariectomized (OVX) rats. After ovariectomy for 80 days, rats were fed a powder of soy isoflavone in various doses, 0 (or control), 40 and 80 mg/kg/day. On day 164 or 84 days after treatments, the OVX rats fed with isoflavone at doses of 40 and 80 mg/ kg/ day showed a lower concentration of serum osteocalcin, a marker of bone formation, and deoxypyrinidinoline, a marker of bone resorption, than those in the OVX control rats.

However, the BMD as well as the trabecular bone area in isoflavone fed rats were not different from the OVX control rats. In 2006, Devareddy *et al*., examined the effects of isoflavone in the form of soy protein on the improvement of microstructural architecture of trabecular bone which was induced by ovariectomy. After ovariectomy for 90 days, the OVX rats were treated with soy protein enriched with soy isoflavone. After 125 days of treatment, none of the treatment groups showed the improvement of BMD or micro-architectural properties of the lumbar vertebra, but the BMC and BMD of tibia were increased, though it was lower than that of the sham group. These results indicated that the soy isoflavone dose-dependently reduced the initiation of bone loss but it could not recover the established bone loss.

Pueraria mirifica Air Shaw & Suvatabandhu, an indigenous Thai herb, known in Thai as "white Kwao Krua" has been used in a traditional medicine for a long time as a rejuvenating agent and a hormone replacement therapy for elderly. It belongs to the family Leguminosae, subfamily Papilinoidea as soy does. The tuberous roots of *P. mirifica* contain many isoflavonoid compounds, such as daidzin, daidzein, genistin, genistein, puerarin and mirificin, and other phytoestrogen compounds, such as miricoumestan, miroestrol and deoxymiroestrol (Chanakaow *et al*., 2000; Ingham *et al*., 2002; Cherdshewasart *et al*., 2007; 2008). The compounds that make *P. mirifica* different from other phytoestrogens containing plants in the family Leguminosae are miroestrol and deoxymiroestrol. The estrogenic activity of miroestrol was previously estimated to be about 0.25 times of 17β-estradiol, tested by vaginal cornification assay in rats (Jones *et al*., 1961). Miroestrol is considered to be the compound having the highest estrogenic potency among other known phytoestrogens.

The estrogenic potency of *P. mirifica* has been tested in reproductive organs of rodents, monkeys and humans. The OVX rats fed with *P. mirifica* exhibited a vaginal cornification and an increased uterine weight (Malaivijitnond *et al*., 2006; 2010). Feeding of *P. mirifica* at a dose of 1,000 mg/kg/day for 90 days could ameliorate an increase in serum luteinizing hormone (LH) and follicle stimulating hormone (FSH) levels in orchidectomized (ORX) and OVX rats (Malaivijitnond *et al*., 2004). Oral administrations of *P. mirifica* at doses of 10, 100 and 1,000 mg/day in adult cycling and aged

menopausal cynomolgus monkeys indicated a reduction of serum LH and FSH levels (Trisomboon *et al*., 2005; 2006). A daily consumption of the crude *P. mirifica* at a dose of 200 mg for 3 weeks per month improved the menopausal symptoms, such as hot flushes, frustration, sleep disorder, skin dryness, high blood cholesterol, oligomenorrhea and amenorrhea in postmenopausal women (Muangman and Cherdshewasart, 2001). Other than the estrogenic activity on reproductive organs, *P. mirifica* also exhibited the effects on bone loss prevention in rats and monkeys. The menopausal cynomolgus monkeys treated with *P. mirifica* at a dose of 1,000 mg/day for 90 days had a reduction in serum PTH levels and followed by a decline in serum calcium levels (Trisomboon *et al*., 2004). Treatment of *P. mirifica* at doses of 10, 100 and 1,000 mg/kg BW/day for 90 days in ORX and OVX rats significantly prevented bone loss in trabecular and cortical bone of the 4th lumbar vertebra, tibia and femur, dose-dependently (Urasopon *et al*., 2007; Urasopon *et al*., 2008). Based on these results, *P. mirificain* should be potentially useful for prevention of osteoporosis caused by sex hormone deficiency. In addition, *P. mirifica* showed the anti–proliferative effects on the growth of MCF – 7 cells, breast cancer cell-lines (Cherdshewasart *et al*., 2004; 2007).

As mentioned above, osteoporosis is a silent disease. Many patients are unaware of the onset of the disease. They always come to the hospital when bone loss is on the serious stage or when bone fracture occurs. At this point, they asked for the therapeutic agents. However, the cost of therapeutic drugs for bone loss is very expensive and the side effects are in concern. Therefore, the therapeutic effects of *P. mirifica* on bone loss in osteoporotic rats are carried out in this study.

The aim of this study is to investigate the therapeutic effects of *P. mirifica* on ovariectomy-induced osteoporotic rats. The knowledge gained from this study is hopefully to be applied for osteoporosis treatment in humans.

Research Questions

Could *P. mirifica* at the doses of 10, 100 and 1,000 mg/ kg BW/ day prevent a further bone loss and cure the established osteoporosis in ovariectomy - induced osteoporotic rats?

Hypothesis

P. mirifica at the doses of 10, 100 and 1,000 mg/ kg BW/ day could prevent a further bone loss and reverse the established bone loss in ovariectomy - induced osteoporotic rats.

Objectives

1. To investigate if a 90-day ovariectomy in female rats can induce bone loss

2. To study the effects of *P. mirifica* on BMD and BMC of osteoporotic rats.

3. To study the effects of *P. mirifica* on trabecular bone tissue area of osteoporotic rats.

4. To study the effects of *P. mirifica* on serum ALP and TRAP levels of osteoporotic rats.

CHAPTER II

REVIEW OF LITERATURE

Osteoporosis is a major public health problem and becoming increasingly prevalence with aging of the world population. Estrogen deficiency has been recognized as a key factor of osteoporosis development. There are many pharmacological agents for osteoporosis treatment but the cost of drugs is high and the safety is in concern. Therefore, the alternative drugs for osteoporosis treatment without side effects have been a focal of interest.

This chapter first summarizes about the bone, such as bone structure, bone cell and lineage, bone remodeling, bone markers, estrogen and bone homeostasis, osteoporosis, antiresorptive agents, and anabolic agents. Later, the content focuses on phytoestrogens, estrogenic and anti-estrogenic activities of isoflavones, *P. mirifica* and animal model for bone loss.

1. Bone

1.1. Bone structure

Bone is a specialized connective tissue that forms, together with cartilage, the skeletal system to serve 3 major functions; 1) mechanical support and sites for attachment of muscles and tendons which are essential for locomotion 2) as a protector of internal organs, such as those contained in the cranial and thoracic cavities and bone marrow, and 3) metabolic function as a reservoir of ions, especially calcium and phosphate, for maintaining calcium homeostasis and acid-base regulation.

Bone consists of extracellular matrix and bone cells. The extracellular matrix includes organic matrix and phosphate and calcium salts in the form of hydroxyapatite crystals ($Ca_{10}(PO_4)_6(OH)_2$) (Nakamura, 2007). The constituent of organic matrix of bone

is type I collagen (95%) and non-collagenous proteins (5%), The non-collagenous proteins include osteonectin, vitamin K-dependent proteins (osteocalcin and osteopontin), fibronectin (Lemann *et al*., 1972) and ground substances which are primarily composed of glycoproteins and proteoglycans. The collagen as well as ground substance can be mineralized.

Generally, there are 2 structures of the bone, cortical and trabecular (Figure 2.1). Cortical and trabecular bones are differences in both structure and function. The cortical bone appears as a compact, dense layer that forms the outermost layer along the length of long bone and is more calcified than trabecular bone. Trabecular (or spongy) bone has the appearance of a sponge or a meshwork locating in the interior part of the bone. Trabecular bone has lower calcium content than the cortical bone. Functionally, cortical bone provides mechanical and protective functions while trabecular bone provides metabolic functions with higher turnover rate than that of the cortical bone (Gustavo and Bruce, 2008).

Bone is a dynamic metabolized connective tissue working throughout our life. The homeostasis of bone maintains by balancing between the process of bone formation and bone resorption, which will keep the bone volume and bone strength. Three types of bone cells, that is, osteoclast, osteoblast and osteocyte, involve in bone formation and bone resorption.

1.2 Bone cells and lineage

In actively growing bone, 4 types of cells are found in the bone tissue; osteoprogenitor cell, osteoblast, osteocyte and osteoclast. All of these cells are differences in embryological origin, microscopic appearance and function.

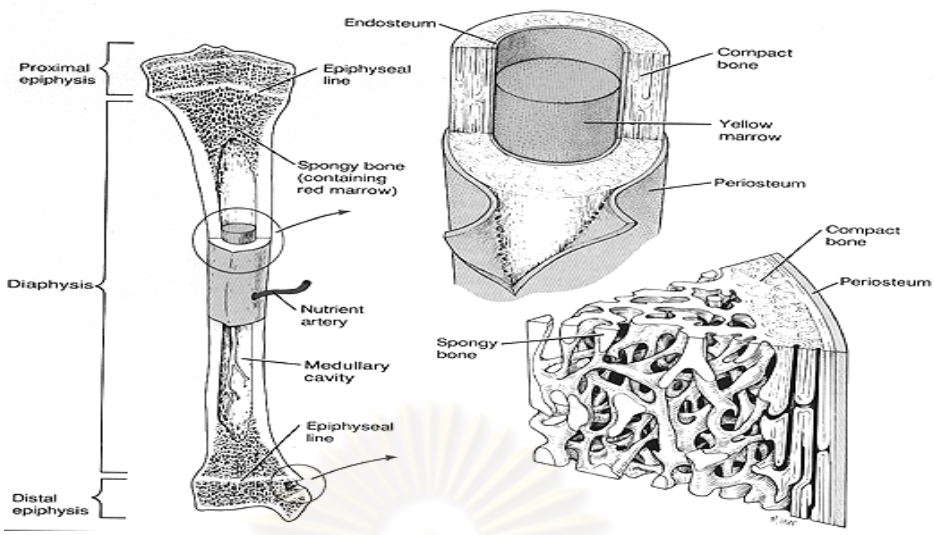


Figure 2.1 Bone structure. It consists of cortical and trabecular (or spongy) bone. The cortical bone appears as a compact dense layer that forms the outermost layer along the length of bone. Trabecular bone forms a meshwork in the interior part of the bone.

(Available from: http://homepage.mac.com/myers/misc/bonefiles/bonestruct.html)

1. Osteoprogenitor cells

The osteoprogenitor cells are originated from mesenchymal stem cells. In resting phase, they are referred to as bone-lining cells which are found on the surface of bone or the endosteum and at the trabeculae of the cartilage at the metaphyses (Fawcett *et al*., 1994). The osteoprogenitor cells appear as flattened cells with lightly stained, elongated nuclei and attenuated cytoplasm (Lynn *et al*., 1995). They are most active during bone remodeling process and also function in maintaining and nutritionally supporting the osteocytes which are embedded in the bone matrix.

2. Osteoblasts

The osteoblasts are differentiated bone-forming cells that descend from mesenchymal stem cells. Osteoblasts are located at the surface of the bone tissue and lined as a single layer. The osteoblasts appear as cuboidal or columnar cells with basophilic cytoplasm, abundant rough endoplasmic reticulum, and prominent Golgi

apparatus. They are responsible for the synthesis of several bone matrix components, including type I collagen, proteoglycans, glycoproteins, osteocalcin, osteonectin and osteopontin (Junqueiva *et al*., 1986). Oteoblasts also produce growth factors that have important autocrine and paracrine effects on bone growth (Fawcett *et al*., 1994). They have plasma membrane receptors for several hormones, such as PTH. In the nuclei, the receptors for estrogen and 1, 25 $(OH)_2D_3$ (Baron, 1993) are also found. Osteoblasts appear to initiate the calcification process. They secrete matrix vesicles which are rich in **alkaline phosphatase (ALP), one of the markers of bone formation** (Fawcett *et al*., 1994).

3. Osteocytes

The osteocytes are originated from differentiated osteoblasts. They have embedded themselves within the calcified bone matrix and resided in the lacunae between the lamellae of matrix. That makes the cells being a lenticular shape. Compared to osteoblast, the flat, almond-shaped osteocytes usually exhibit less cytoplasmic basophilia, reduced rough endoplasmic reticulum and Golgi complex, and more condensed nuclear chromatin. The nucleus may be the only prominent feature. The processes of adjacent cells form a communicating network connected together with gap junctions that provide for the intercellular flow of several ions and small molecules. In addition, osteocytes are also responsible for bone turnover process and acting as mechano-sensory cells that play an important role in functional adaptation of bone (Aarden *et al*., 1994)

4. Osteoclasts

The osteoclasts are the bone resorbing cells. They are derived from hematopoietic stem cells. They are giant multinucleated cells. Osteoclasts are usually found in contact with the calcified bone surface as well as within the lacuna. The most prominent features of osteoclasts are the regions containing numerous plasma membranes folding that form microvillous structures which are called the ruffled border. It is surrounded by the ring of actins that serves to attach the osteoclast cells to the bone surface. This is the site for secretion of hydrogen and chloride ions, proteolytic

enzymes, and collagenase. In addition, osteoclast has a great number of lysosomes which contain lysosomal enzymes that makes the osteoclast cell can be responsible for the degradation of bone matrix. The organelles inside the cells are acidophilic cytoplasm with many vacuoles, abundant Golgi complexes, mitochondria and free polysomes, and some rough endoplasmic reticulum.

Osteoblast and osteoclast coupling is mediated through a membrane-bound receptor activator of nuclear factor-kappa B ligand (RANKL), which is the factor necessary for osteoclastogenesis. RANKL interacts with its receptor (RANK) expressed on the osteoclasts and their precursors. The binding between RANK and its ligand stimulates osteoclast differentiation and prevents osteoclast cells death (Roodman, 2006). Concurrently, a decoy receptor knows as osteoprotegerin (OPG), which is produced by osteoblasts, can inhibit RANK – RANKL signaling (Yeung, 2004). There are many factors stimulating RANKL expression, such as PTH, vitamin D and cytokines. Conversely, estrogen, transforming growth factor beta (TGF - β), and mechanical force inhibit RANKL expression (Figure 2.2).

1.3 Bone remodeling

Bone is a dynamic connective tissue which is suited for its supportive and protective functions of the skeletal. To maintain the mechanical integrity, bone tissue undergoes a remodeling, an active process that is mediated through the coupled processes of which there are a removal of old bones and a replacement with newly formed bone. This process is complicated and requires an interaction between all types of bone cells, which is regulated by a variety of biochemical and mechanical factors. A bone remodeling process most frequently occurs at the surface of trabecular bone. It also takes place on both periosteum and endosteum surface of compact bone. In adult, the rate of bone resorption and formation are equal, but in aging and postmenopausal women, the resorption exceeds the formation process. This can lead to the alteration in bone architecture and decreases bone mass which finally causes an increased high risk of skeletal fracture.

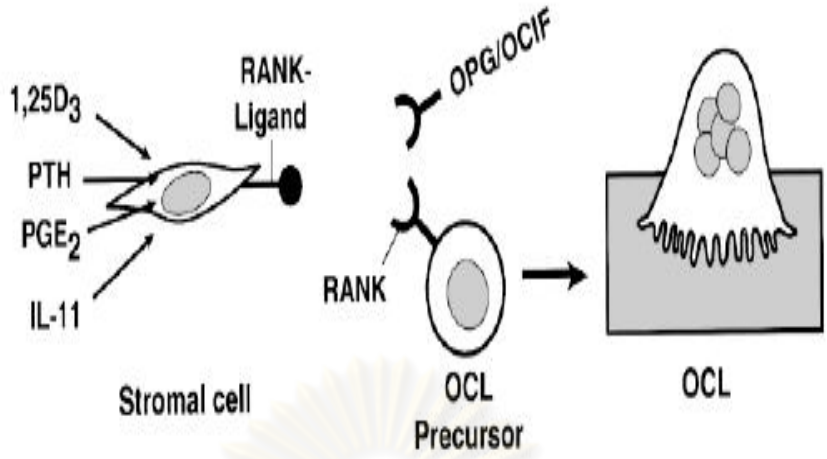


Figure 2.2 Role of RANKL in osteoclastogenesis. Osteotropic factors induce upregulation of RANKL on marrow stromal cells and osteoblasts. RANKL binds to the RANK receptor on osteoclast precursors and induces osteoclast formation.

(Available from: http://elsevierhealth.com/pdfs/journals/PIIS0301472X99000612.pdf)

Bone remodeling maintains the renewal of the skeletal. Groups of cells incorporating in bone remodeling process are named “bone multicellular units” (BMUs) (Eriksen *et al*., 1994). Each remodeling cycle initiates with 1) the resting (or quiescent) phase, in which bone lining cells are inactivated, and 2) the resorbing phase, whereby certain factors, such as osteoclastic precursors are activated by transcription factors or growth factors to become mature multinucleated osteoclasts and start the osteoclastic bone resorption. After the resorption is terminated the area is invaded by preosteoblast, which is then differentiated into an active osteoblast and this step is called 3) reversal phase. After the osteoblasts are activated, they synthesize a new matrix which is subsequently mineralized, and this step is called 4) bone formation (Figure 2.3). In conclusion, bone remodeling process is composed of an activation, resorption and formation sequence. The complete remodeling cycle takes about 3-6 months. Generally, the bone formation phase takes at least 4 times longer than the bone resorption phase (Baron, 2003).

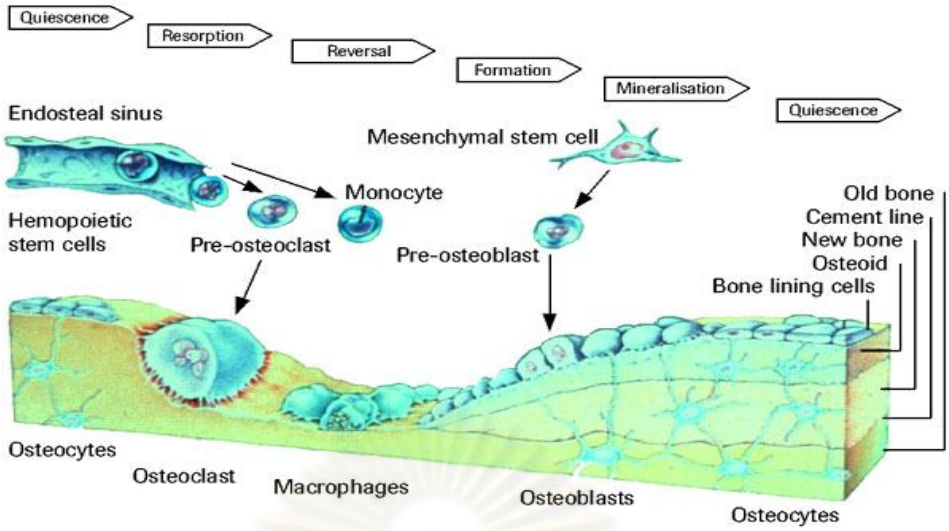


Figure 2.3 Bone remodeling process. During this process, old bone is resorbed by osteoclasts and replaced with new osteoid, secreted by osteoblast. The matrix is then mineralized to generate a new bone.

(Available from: http://www.roche.com/pages/facets/11/ostedefe.htm)

1.4 Bone markers

During bone remodeling, osteoclast and osteoblast synthesized numerous molecules, such as ALP and osteocalcin. The rate of synthesis and release of these molecules indicate abnormality of bone and mineral metabolism and it could be used as biochemical markers for diagnosis and monitoring of what anabolic bone formation drugs should be used for osteoporosis treatment (Yves *et al.*, 2008). Bone marker measurements are noninvasive, inexpensive, and can be repeated often. Unfortunately, most of the studies that provided insight on clinical situations did not focus on markers as a primary endpoint. Bone markers have been useful in clinical practice and have been helpful in understanding the pathogenesis of osteoporosis and the mechanism of actions of therapies (Watts, 1999). Biochemical markers that reflect the remodeling process and can be measured in blood or urine fall into three categories: 1) enzymes or proteins that secreted by cells involved in the remodeling process, 2) breakdown products generated in the resorption of old bone, and, 3) by products produced during

the synthesis of new bone (Watts, 1999; Swaminathan, 2001) (Table 2.1). Followings are example of bone markers used in clinical diagnosis and researches.

Alkaline phosphatase (ALP) is an enzyme that is synthesized and released by osteoblasts and then can indicate the osteoblast activity. ALP is a membrane-bound protein with enzymatic activity that originates from various tissues, such as liver, bone, intestine, and placenta. Two major circulating forms of ALP, the liver and the bone isoenzyme, are posttranslationally different in their glycosylation patterns. In adults, only about 50% of ALP is derived from bone, wile the remainder mostly emanates from liver tissue. In the bone, ALP anchored to the outer plasma membrane of osteoblast by a glycan linkage to phosphatidyl-inositol (Low and Finean, 1997). Several possible roles in bone formation of bone ALP have been proposed. The enzyme hydrolyzes phosphate-ester and provides supplemental phosphates for deposition in the hydroxyapatite, destroys local inhibitors of the mineral crystal growth, or acts as a calcium-binding protein or Ca^{2+} ATPase. Bone ALP can be released to the circulation by action of glycan-inositol phosphate specific hydrolase which cleaves the molecule at the inositol-PO_4 linkage site. The levels of serum ALP can be increased as a consequence of an increased release from bone cells during the process of bone formation. Recently developed immunoradiometric or enzyme immunoassay has facilitated the determination of serum levels of bone specific ALP, despite a residual and significant cross reactivity with the liver isoenzyme. Bone specific ALP provides a higher specificity for bone than the other bone formation markers. It has been shown that the bone turnover is increased by increase in levels of serum total ALP and its isoenzyme, bone specific ALP in OVX rats. The replacement of E_2 in OVX rats decreased the levels of serum total ALP and bone specific ALP to the basal levels (Sozer *et al*., 2006).

Tartrate resistant acid phosphatase (TRAP) is a lysosomal enzyme found in bone, prostate, platelets, erythrocytes, and spleen. TRAP can be measured in serum or plasma by electrophoresis or by enzyme linked immunoassay (EIA). Two forms of TRAP circulate in blood as TRAP 5a and TRAP 5b. TRAP 5b is derived from osteoclasts. Osteoclasts secrete TRAP 5b into the blood circulation as an active enzyme, which is inactivated and degraded to fragments before it is removed from the circulation. The

levels of serum or plasma TRAP 5b reflects the number of osteoclasts and can predict the future bone loss (Alatalo *et al*., 2003; Chu *et al*., 2003). The number and activity of osteoclasts are tightly coupled, suggesting that circulating TRAP 5b is a useful marker of bone resorpton. Serum TRAP 5b levels of male rats were increased as early as 5 day after ORX, whereas the significant bone loss, quantified by pQCT, was observed at 11 days after operation. This suggests that elevated serum TRAP 5b levels could use to predict a future bone loss. Interestingly, after the rapid elevation of serum TRAP 5b, it started to decline and returned to the control level within 17 days after operation (Alatalo *et al*., 2003). Similar results were also obtained in the OVX rats. They showed an even faster elevation of serum TRAP 5b after operation and it reduced to the control level within the first week (Surve *et al.,* 2001).

Table 2.1 Summary of biochemical markers of bone turnover

Bone markers	Sample	Major source
Bone formation		
Total alkaline phosphatase (ALP)	Serum	Liver/bone
Bone alkaline phosphatase (BALP)	Serum	Bone (osteoblasts)
Osteocalcin	Serum/ plasma	Bone (osteoblasts)
Carboxy terminal propeptide of type I procollagen (PICP)	Serum	Bone (osteoblasts) and skin (fibroblasts)
Bone resorption		
TRAP 5b	Serum/ plasma	Bone (osteoclast)
Pyridinoline cross-linkis (PYD)	Urine	Bone and cartilage
Deoxypyridinoline cross-link (DPD)	Serum/ urine	Bone and dentine
N-terminal telepeptide (NTx)	Serum/ urine	Bone and dentine
C- terminal telepeptide (Cross-laps)	Serum/ urine	Bone and dentine

Bone markers are useful in clinical diagnosis and are helpful in understanding the pathogenesis of osteoporosis and mechanism of action of therapies. In clinical trials, bone markers help to select the optimal dose and to understand the time course of the onset of treatment of osteoporosis. Other than the diagnosis and treatment of osteoporosis, the determination of bone markers can help to detect the symptom of bone loss earlier and can help to select the most suitable treatment for the patients.

Bone remodeling is regulated by various hormones and many local factors which affect the functions of bone cells. Estrogen is one of major functional hormones in bone remodeling found in females. This hormone interacts with various organ systems, for example, reproductive system, cardiovascular system and skeletal system.

1.5 Estrogen and bone homeostasis

Estrogen plays an important role in maintaining the skeletal homeostasis in adult men as well as in adult women. At present, it is certain that osteoblast, osteoclast, osteocytes and their progenitor cells have estrogen receptors (ERs; ERα and ERβ), although the concentration is lower than those found in the reproductive tissues. (Nakamura, 2007). Estrogen regulates bone homeostasis by binding to the ERs, and modulates the biosynthetic activity of osteoblast, osteoclast and their progenitor cells.

The osteoclast develops a network of autocrine and paracrine communication with stromal and osteoblast cells (Figure 2.4). RANKL is produced by osteoblast and activates the osteoclast differentiation. RANKL is found on the surface of stromal-osteoblastic lineage cells. Cell-to-cell communication between these two cells allowed RANKL to bind to its membrane receptor (RANK receptor) which is expressed on the surface of osteoclast cells. In response to RANKL signaling, osteoclast is differentiated, increases in activity, and decreases in apoptosis. The stromal-osteoblast lineage cells (mature osteoclast) also secrete an osteoprotegerin (OPG), a soluble decoy receptor that neutralizes a RANKL. OPG acts as a decoy by blocking the binding of the receptor activator of NF-$\kappa\beta$ (RANK) to RANKL on mature osteoblast/ stromal cells (Simonet,

1997). This interaction inhibits the differentiation of osteoclast precursor into a mature osteoclast.

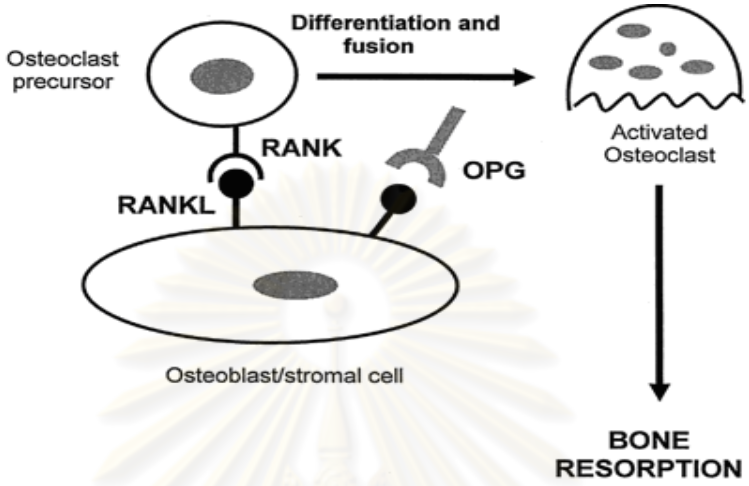


Figure 2.4 A schematic overview of RANKL/ RANK/ OPG system. RANKL mediates a signal for osteoclast formation through RANK expressed on osteoclast progenitor.

(Available from: http://www. Medscape. Com/ viewarticle/ 479893_2)

Estrogen stimulates the production of OPG, an anti-osteoclastogenic factor, and suppresses bone resorption. In addition, estrogen regulates the production of cytokines by bone marrow stromal cells and osteoblast. Estrogen decreases many cytokines production, such as IL1, IL6 and TNF-α. These cytokines have been shown to inhibit apoptosis of osteoclast and stimulate osteoclast proliferation and differentiation from haemopoietic stem cells (Hughes *et al*., 1997; Jimi *et al*., 1996). Estrogen has also stimulated the bone formation by decreasing an osteoblast apoptosis, increasing a lifespan of osteoblast, and stimulating an osteoblast activity via the increase in type I collagen production (Nakamura, 2007).

Estrogen deficiency affects bone remodeling in several ways. Firstly, it increases the activation frequency of BMUs which leads to a higher bone turnover. Secondly, it induces a remodeling imbalance by prolonging the resorption phase and shortening the formation phase (Compston, 2000). As a consequence of these changes, the volume of the resorption cavity is increased beyond the capacity of the osteoblasts to refill it. As

written above that estrogen decreases secretions of many bone related cytokines, estrogen deficiency results in an increase in the production of IL-1, IL-2, TNF-α, granulocyte-macrophage colony-stimulating factor (M-CSF), and prostaglandin-E_2 (PGE_2).

Estrogen deficiency is one of the most important factors in the pathogenesis of osteoporosis in post-menopausal women. Estrogen plays an important role in maintaining bone mass in adult women by maintaining remodeling balance. In post-menopausal women, the lack of estrogen leading to decrease the bone mass and increase the risk for osteoporosis.

1.6 Osteoporosis

Osteoporosis is a condition of bone disorder characterized by a reduced BMD and micro-architectural deterioration of bone tissue, resulting in increased bone fragility and bone fracture (Figure 2.5). Osteoporosis is mainly seen in elderly people. The decrease in sex steroid hormones, bone fracture and their complications are the relevant clinical squeal of osteoporosis. The most common fractures are the hip, spine and wrist. However, most fractures in older adults are due in part to a low bone mass. After the bone fracture, some patients may be fully recovered or some may have a chronic pain, disability or death. Other than the physiological symptoms, the fractures can also cause psychological symptoms, e. g. depression and lost of self – esteem, because the patients are dependent to other persons.

The diagnosis of osteoporosis is done by the measurement of BMD. An areal BMD is expressed in the absolute term of grams of mineral per square centimeter scanned (g/cm^2). The BMD diagnosis of normal, low bone mass, osteoporosis and severe or established osteoporosis is based on WHO classification as follows;

Normal: BMD is within -1 SD of a young normal adults (t – score at -1 and above)

Low bone mass (osteopenia): BMD is between -1.0 and -2.5 SD of a young normal adult (T – score between -1.0 and -2.5)

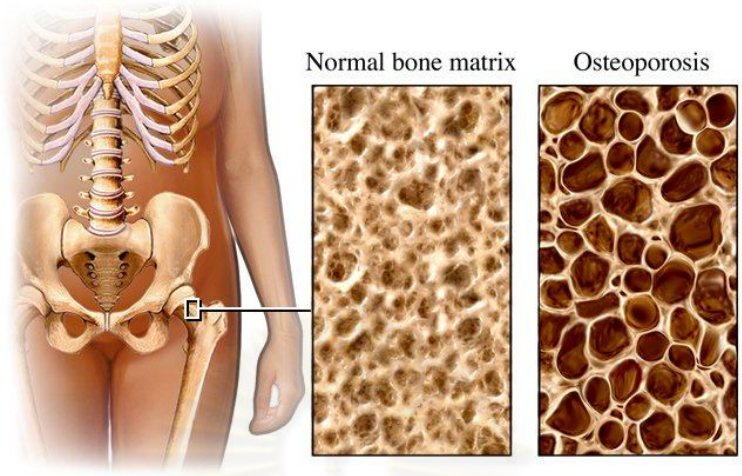


Figure 2.5 Normal bone matrix and osteoporosis. Osteoporosis is characterized by a decrease in bone density and a loss of bone connection.

(Available from: www.tongkatsu.com/2010/03/ostoporosis-in-menopause/)

Osteoporosis: BMD is -2.5 SD or more below that of a young normal adult (T – score at or below -2.5)

In post-menopausal women, the WHO diagnostic T – score criteria are applied to the BMD measurement done by a central dual - energy X - ray absorptiometry (DXA) at the lumbar spine and femoral neck.

Most patients suffered from osteoporosis are post-menopausal women. Women exhibit two phases of age related bone loss; early and late. **The early phase** begins at the menopause, this phase accounts for losses of 20-30% in trabecular bone, but for only 5-10% in cortical bone. Bone loss in this phase associates with a decline in serum estrogen levels which can be prevented by estrogen treatment. Although the menopause induces a rapid bone loss, a part of the decrease in the BMD relates to the increase in remodeling rate induced by the large increase in BMU numbers. A rapid bone loss in this phase produces an increased outflow of calcium from bone into the

extracellular pool. However, the hypercalcemia is prevented by a compensatory increase in urinary calcium excretion, a decrease in intestinal calcium absorption, and partial suppression of PTH secretion. As assessed the biochemical markers, the bone resorption markers increase by 90% at the menopause, whereas the bone formation markers increase by only 40%. The increase in bone turnover and remodeling imbalance leads to an accelerated bone loss. **The late phase** of bone loss is associated with a progressive increase in levels of serum PTH and in biochemical markers of bone turnover. The PTH secretion increases and maintains normal levels of serum ionic calcium by resorption of bone, a main body calcium stores (about 99% of calcium in the body) (Riggs *et al*., 2002).

Osteoporosis is a worldwide health problem. The prevalence is currently high and the therapies are aimed to adjust the imbalance of the bone remodeling. The therapeutic agents for osteoporosis are classified as antiresorptive agents and anabolic agents. **Antiresorptive agents** are agents acting on inhibiting the activity of osteoclasts and, therefore, reducing bone resorption. Currently, available antiresorptive agents include bisphonates, selective estrogen-receptor modulators (SERMs), calcitonin and estrogen. **Anabolic agents** act by stimulating a formation of the new bone. The only anabolic agent currently available is the teriparatide; a recombinant human PTH (hPTH).

1.7 Antiresorptive agents

1. Bisphosphonates

Bisphosphonates are structural analogues of pyrophosphate, a naturally occurring bone resorption inhibitor. Bisphosphonates have a strong affinity for the hydroxyapatite crystals in bone, where they act as potent inhibitors of bone resorption by decreasing osteoclast recruitment, activity, and life span (Van Beek, *et al*., 2002). Once absorbed into the systemic circulation, approximately 50% of a bisphosphonate distributes into bone resorption sites, while the remaining 50% is excreted renally. Bisphosphonates have very long half-lives in bone. Once bone resorption at a remodeling site is inhibited, bisphosphonates are either released back into the

circulation and eliminated or are incorporated into the new bone matrix, where they remain inactive until they are released at a new remodeling cycle. Both alendronate and risedronate are popularly used bisphosphonates. They reduce the incidence of vertebral fracture by 40-50% in osteoporotic women, and they can improve BMD and reduced vertebral fracture risk (Orcel and Beaudreuil, 2002). Bisphosphonates suppress bone resorption within a few weeks after treatment, and the risk of radiological or vertebral fracture can be reduced within 6-12 months (Eastell *et al*., 2011). Randomized clinical studies have shown a similar frequency of GI adverse effects with alendronate and risedronate compared with the placebo, although patients have an upper-GI-tract disease or a nonsteroidal anti-inflammatory drug use (Diel *et al*., 2007). Thus, use of bisphosphonates on osteoporotic treatment could cause some adverse effects.

2. Selective Estrogen Receptor Modulators (SERMs)

SERMs are the substances that have mixed agonistic and antagonistic effects after binding on estrogen receptors. Raloxifene is the only SERM that approved to be used in prevention and treatment of osteoporosis. Raloxifene has estrogen-like effects on lipid metabolism and bone, and has estrogen antagonistic effects on endometrial and breast tissues (Michael *et al*., 2004). Based on its antagonistic effects on the endometrium, raloxifene does not cause a vaginal bleeding or a risk of endometrial cancer. Raloxifene significantly increases BMD, but to a lesser extent than those of estrogen and bisphosphonates. Treatment of 60 mg/ day of raloxifene hydrochloride in comparison with placebo in healthy postmenopausal women, with normal BMD or osteopenia showed a 1-2% of increase in BMD from the baseline at all body sites. In addition to the effects of raloxifene on BMD and fractures, it did not alter performance on cognitive tests in post-menopausal women, but it increases the risk of venous thromboembolism (VTE) up to threefold (Roger *et al*., 2004). The most common adverse effects associated with raloxifene are leg cramps and hot flushes. Raloxifene should not be used in postmenopausal women with hot flushes, since it can make the symptoms worse.

3. Calcitonin

Calcitonin is a polypeptide hormone secreted by C-cells of the thyroid gland. A salmon calcitonin has been approved by FDA for the treatment of osteoporosis in women who experienced menopause for more than 5 years (Chesnut *et al*., 2008). Calcitonin is available as an injection or a nasal spray form. Adverse effects associated with the injection include local reactions, such as flushing, and rash, and systemic reactions of a rare allergic type. Thus, calcitonin has a limitation of use. Adverse effects are rare with the intranasal formulation. Mostly the patients complain about the nasal dryness, soreness, irritation, itching, and epistaxis. In postmenopausal women with a low BMD, nasal spray of 200 IU of calcitonin increased a spine BMD of 1-3% (Adami *et al*., 1995). Thus, calcitonin is considered as a third-line therapy for osteoporosis in postmenopausal women.

4. Estrogen

Estrogen replacement therapy (ERT) has been approved by FDA only for use in the prevention of osteoporosis. Several controlled trials and observational studies have demonstrated that estrogen significantly increased BMD in both early and late menopausal women. In addition, the ERT could be the most beneficial if it has been started soon after the onset of menopause and continued to use longer than 7 years. It has long been thought that higher doses of estrogen were needed for bone loss protection, however, recent studies showed that use of lower doses of estrogen (doses equivalent to 0.3 mg of conjugated equine estrogen) in combination with adequate calcium intake are high efficiency. Although ERT has been proven to increase BMD, the anti-fracture efficacy has been consistently shown only in observational studies. In addition, ERT has adverse effects on increasing the risk of breast cancer, endometrial cancer, colon cancer and stroke. Considering these problems, higher and longer use of ERT should be aware.

1.8 Anabolic agents

Anabolic agents are the chemicals that have the capacity to increase bone mass and also to improve bone quality and bone strength. The recombinant hPTH is the only anabolic agent for the treatment of low bone mass. Two forms of recombinant hPTH have been evaluated in clinical trials, hPTH (I-34) and the intact 84-amino acid form, hPTH (I-84). The hPTH exerted a potent anabolic effect on the skeletal by increasing the rate of bone remodeling, resulting in a positive remodeling balance. New bone formation occurs on quiescent surfaces and, as a result, trabecular architecture becomes closely resemble to the normal bone. The mechanism of action of hPTH is still equivocal. However, the multiple pathways on alterations of the activity of osteoblasts, bone lining cells, osteoclasts and osteocytes were proposed. PTH stimulates bone formation by increasing the number of osteoblasts, partly by delaying osteoblasts apoptosis. PTH is able to increase the bone size. To date, clinical trials of PTH have been carried out in postmenopausal women with osteoporosis. In 2001, Robert and co-workers studied the effects of PTH in postmenopausal women, who also had a prevalent of vertebral fracture and found that PTH decreased the risk of vertebral and non-vertebral fractures and increased vertebral, femoral, and total-body BMD.

A negative issue of using of hPTH is the increase in risk of osteosarcoma, tested in Fischer rats (Vahle *et al.*, 2004). Because of this unexpected finding, treatment of hPTH in severe osteoporotic postmenopausal women was determined. Osteosarcoma was found with all doses, and in the lower-dose ranges it was first detected after 20 months of therapy. Because of the incidence of osteosarcoma in rats, patients who might be an increased risk of osteosarcoma, such as Paget' s disease and those having prior radiation therapy of the skeletal, bone metastasis, hypercalcemia should not receive hPTH therapy. The safety and efficacy of PTH treatment, longer than 2 years, has not been evaluated, use of this drug for more than 2 years is not recommended.

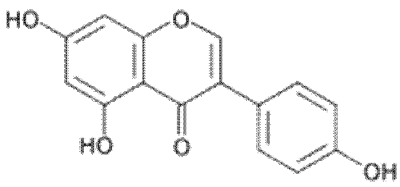
As mentioned above, the antiresorptive agent can reduce the bone resorption, but it does not increase the bone formation. The anabolic agents can induce a new bone formation, but their side effects and cost are the main issue to be concerned.

Therefore, the agents which can induce bone formation, no side effects, and cheap should be sought. The indigenous herb is becoming one of the choices.

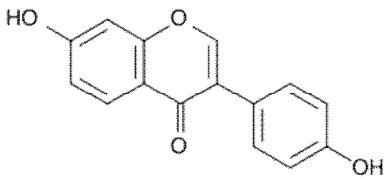
2. Phytoestrogens

Phytoestrogens are natural compounds found in plants. They are non-steroidal compounds and can exert estrogenic and anti-estrogenic effects on the target organs after binding to the ERs (Terraeaux, 2003). The structure of phytoestrogens is similar to those of endogenous estrogens because they have aromatic ring with hydroxyl group, and nearly identical distant exist between two hydroxyl groups (Murkies *et al*., 1998; Chen and Anderson, 2002). (Figure 2.6)

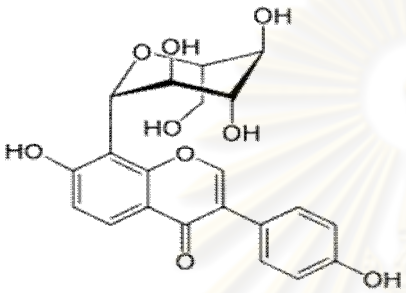
Phytoestrogens can be classified into 4 classes; isoflavones, coumestans, lignans and stilbenes. Isoflavones are considerably the most important phytoestrogens, especially genistein and daidzein. The major sources of isoflavones are present in soy and soy products, such as tofu, soy yoghurt and soy noodle. Isoflavones found in food are in glycoside form, such as genistin and daidzin, and they were metabolized by intestinal glucosidase enzyme to aglycoside form, genistein and daidzein. Following absorption, isoflavones undergo the hepatic conjugation to glucuronic acid or sulphate and produce forms that excreted in urine and feces. The serum and urinary concentrations of isoflavones increase in time after consumption, indicating that absorption occurs in a dose dependent manner (Ebeling and Akesson, 2001). Isoflavones have beneficial effects for health when consumed at optimal doses. They can prevent a cardiovascular disease, breast cancer and osteoporosis and decrease menopausal symptoms, such as hot flush (Yamaguchi, 2002; Benassayag *et al*., 2002; Cornwell *et al*., 2003).



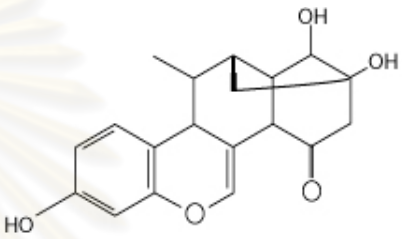
genistein



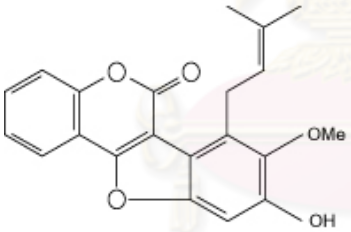
daidzein



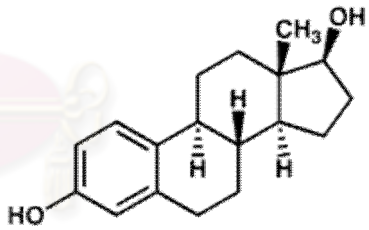
puerarin



deoxymiroestrol



mirificoumestan



17β- estradiol

Figure 2.6 Chemical structures of phytoestrogens (genistein, daidzein and puerarin, deoxymiroestrol, mirificoumestan) and 17β- estradiol.

3. <u>Estrogenic and anti-estrogenic activities of isoflavones</u>

The estrogenic and anti-estrogenic effects of isoflavones are achieved via ERs. They have a higher affinity for ERβ than the ERα. Isoflavones bind to ERs in the cytosol

and form an isoflavone-ER complex, translocate into nucleus, activate the estrogen responsive element (ERE), which involved in the regulation of mRNA synthesis and the production of new proteins. In the culture of human endometrial cells, high concentrations (10^{-8} – 10^{-6} M) of isoflavones showed the estrogenic activity by increase in cell proliferation and ALP activity. However, they showed an anti-estrogenic activity when co-incubated with estradiol (Kayisli *et al*., 2002). Genistein induced the proliferation of human MCF-7 breast cancer cells. In the animal study, genistein, daidzein, and equol significantly increased uterine weight in OVX rats (Ishimi *et al*., 2000; Picherit *et al*., 2000). Genistein and daidzein increased uterine epithelial cell height and uterine gland numbers in immature mice (Jefferson, 2002).

The estrogenic effects of phytoestrogens on bone have been reported, as an anabolic effect on bone metabolism (Yamaguchi and Gao, 1998; Gao, 1999). In bone cell culture, genistein (10^{-6}– 10^{-5} M) induced a significant increase in calcium content and ALP activity, a bone formation marker (Yamaguchi and Gao, 1998). In MC3T3-E1 cell culture, genistein (10^{-6} – 10^{-5} M) and daidzein (10^{-6} – 10^{-5} M) caused a significant increase in cell proliferation, ALP activity and DNA content (Sugimoto and Yamaguchi, 2000). Isoflavones could also inhibit the osteoclast cell formation from bone marrow cells and stimulated apoptosis of these cells (Gao, 1999). In OVX rat and mice, administration of genistein and daidzein could prevent bone loss caused by estrogen deficiency, in the dose-dependent manner (Ishimi *et al*., 1999; 2000; Picherit, 2000).

The therapeutic effects of isoflavones on established bone loss have also been investigated. In 2000, Picherit and co–workers studied the effects of soybean isoflavones on osteoporosis in OVX rats. After ovariectomy for 80 days, the OVX rats were treated with soybean isoflavone for 84 days. The results indicated that the BMD and trabecular bone area were greater in isoflavone fed rats than in the OVX rats. In 2006, Devareddy *et al*. examined the effects of isoflavones in the form of soy protein on the improvement of micro-structural architecture of trabecular bone which was induced by ovariectomy for 90 days. After 125 days of treatment with soy protein mixed with isoflavones, none of the treatment groups showed the improvement of BMD or micro-architectural properties of the lumbar vertebra. However, the BMC and BMD of tibia

were increased, though it was lower than that of the sham group. These results indicated that the soy isoflavone consumption could prevent bone loss but did not reverse an established osteoporosis.

4. *Pueraria mirifica* Airy Shaw & Suvatabandhu

P. mirifica, an indigenous Thai herb, is known in Thai as "white Kwao Krua" (Figure 2.7). It has been used in a traditional medicine for a long time as a rejuvenating agent and hormone replacement therapy for elderly. It belongs to the family Leguminosae, subfamily Papilinoideae as soy does. Phytochemical studies have shown that the tuberous root of *P. mirifica* contains various kinds of phytoestrogens, such as miroestrol, puerarin, coumestrol, daidzin, daidzein, mirificin, mirificoumestan, genistin, genistein and kwakurin (Pope and John, 1960; Chansakaow *et al*., 2000; Ingham *et al*., 2002; Cherdshewasart *et al*., 2007; 2008). The compounds that make *P. mirifica* different from other phytoestrogen containing plats in the family Leguminosae are miroestrol and deoxymiroestrol. The estrogenic activity of miroestrol was previously estimated to be about 0.25 times of 17β –estradiol, tested by vaginal cornification assay in rats (Jones *et al*., 1961). Deoxymiroestrol possessed stronger estrogenic effects on MCF – 7 human breast cancer cells than the miroestrol (Matsomura *et al*., 2005).

Several pharmacological studies of *P. mirifica* mostly concerned about its estrogenic activities in animals. Feeding *P. mirifica* powder suspended in water to OVX rats stimulated the proliferation of vaginal epithelium and uterus endometrium and reduced serum LH and FSH levels (Malaivijitnond *et al*., 2004; 2006). The reduction of serum FSH and LH levels has also been investigated in cyclic and aged menopausal monkeys. *P. mirifica* suppressed folliculogenesis and ovulation in adult female monkeys after a single or long–term feeding (Trisomboon *et al*., 2004; 2005). In menopausal women, daily consumption of *P. mirifica* powder at a dose of 200 mg/day can relieve climacteric symptoms, such as hot flushes, frustration, sleep disorder and skin dryness (Muangman and Cherdshewasart, 2001). In addition, *P. mirifica* showed the anti–

proliferative effects on the growth of MCF–7 cells, breast cancer cell lines (Cherdshewasart *et al*., 2004; 2007).

Figure 2.7 The Pueraria *mirifica*: woody climbers and tuberous roots in which estrogenic compounds contain.

The beneficial effect of *P. mirifica* on bone loss has also been investigated. In 2004, Trisomboon, *et al*. demonstrated that feeding of *P. mirifica* at a dose of 1,000 mg/day for 90 days in menopausal cynomolgus monkeys had a reduction in serum PTH levels and followed by a decline in serum calcium levels. This result indicated that *P. mirifica* ameliorated bone loss in age menopausal monkeys. In 2007 and 2008a, Urasopon *et al*. reported that *P. mirifica* could prevent bone loss in ORX and OVX rats. They reported that treatment of *P. mirifica* at doses of 10, 100 and 1,000 mg/kg BW/day for 90 days in ORX and OVX rats significant prevents bone loss in trabecular and cortical bone of the 4th lumbar vertebra, tibia and femur, dose-dependently. Based on these results, *P. mirifica* should be potentially useful for prevention of osteoporosis caused by sex hormone deficiency. As mentioned previously, osteoporosis is a silent disease and many patients are unaware of the onset of this disease. They always come to the hospital when bone loss is on the serious stage or bone fracture occurs. At this point, the therapeutic agents are necessary. However, the cost of the drugs for osteoporosis is very expensive and the side effects are concerned. Therefore, the therapeutic effects of *P. mirifica* on ovariectomy–induced osteoporotic rats were investigated in this study.

5. **Animal model for the study of bone loss**

There is a great need to characterize the available animal models for the study of postmenopausal osteoporosis, to understand the pathogenesis of the disease, and to investigate the new therapies. The animal model to study the estrogen deficiency-induced osteoporosis should be able to reflect changes in human bone. The Food and Drug Administration (FDA) guideline has appropriately designed the need for rat experimentation in the preclinical evaluation of agents used in the prevention or treatment of postmenopaual osteoporosis. The OVX rat is an excellent preclinical animal model that correctly emulates the important clinical feature of the estrogen depleted human skeleton and the response of therapeutic agents. They have trabecular bone remodeling at the site which is similar to those seen in human trabecular bone and the anatomy of their skeletal has many similarities with the human skeletal (Mosekide, 1995).

The site-specific development of trabecular osteoporosis is one of the most reproducible biologic responses in skeletal research. The predominant cellular activity on endoosteal (trabecular) bone surface is remodeling. Though the cortical bone displays a low level of intracortical remodeling in rats (Jee and Yao, 2001), because of the lack of Haversian systems, another limitation is the absence of impaired osteoblast function during the late stages of estrogen deficiency (Wronski and Yen, 1991). The major drawback of the rat skeleton is that some bones retain lifelong growth and do not fuse epiphyses. Longitudinal bone growth increases transiently after OVX in rats, but this can be minimized by the use of 6 to 9 month olds rats. At the 6 to 9 months of age, rats bone mass in cortical and trabecular compartment of the longitudinal bone reach a plateau or show slow increase in growth. The ovariectomy in 6 months old rats showed a similar phenomenon of bone loss as those found in menopausal women (Urasopon *et al*., 2008). Therefore, this study used rats at the age of 6 months old as an animal model for the study of the therapeutic effects of *P. mirifica* on osteoporosis induced by estrogen deficiency.

CHAPTER III

MATERIALS AND METHODS

The experiment was divided into 2 periods as follows (Figure 3.1);

1. **Bone loss induction;** to examine the effects of ovariectomy on the induction of bone loss in female rats
2. **Treatment;** to examine the therapeutic effects of *P. mirifica* on ovariectomy-induced osteoporotic rats

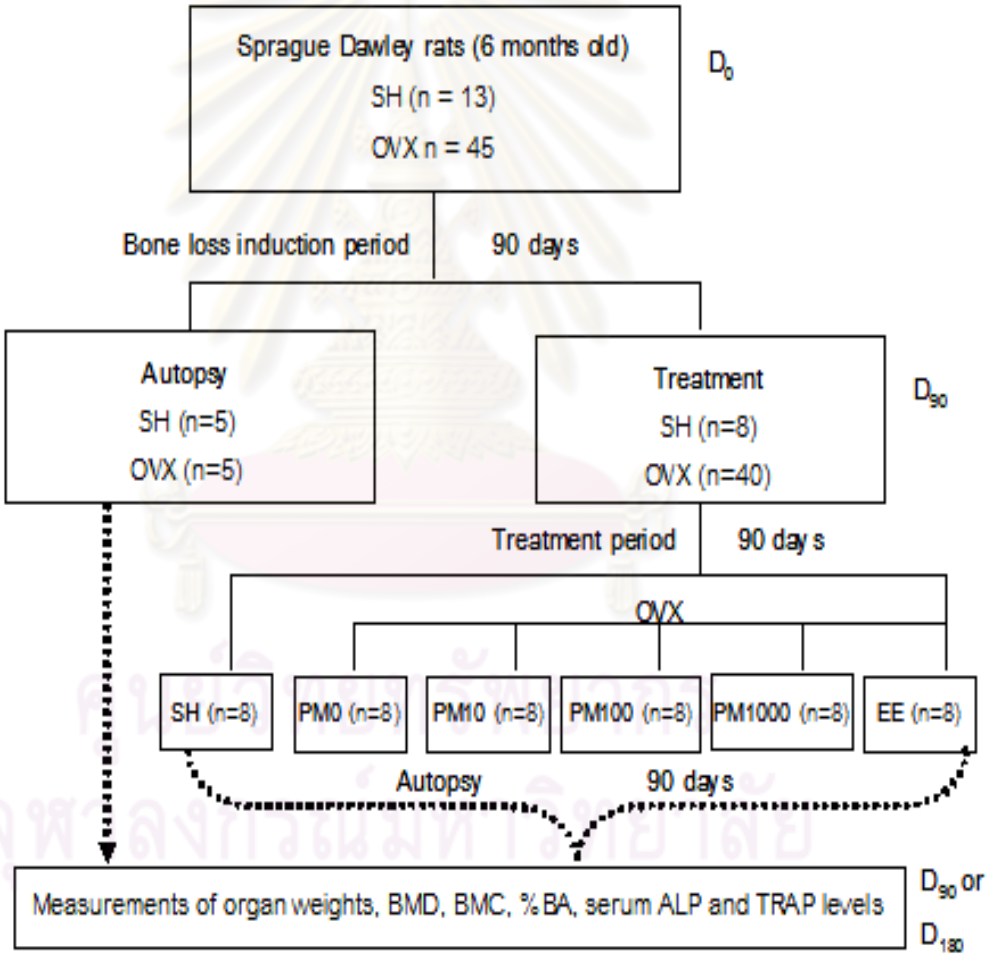


Figure 3.1 The diagram shows the experimental design of the study. SH = sham operated rats, OVX = ovariectomized rats, PM = *P. mirifica*, D = day of the experiment, %BA = %trabecular bone area, BMD = bone mineral density, BMC = bone mineral content, ALP = alkaline phosphatase, TRAP = tartrate resistant acid phosphatase.

1. Animals

Two months old female Sprague–Dawley rats were obtained from the National Laboratory Animal Center, Mahidol University, Nakhon Pathom, Thailand. They were housed in stainless steel cages with sawdust bedding, 5 animals/ cage, in a room with 12-h light/ 12-h dark cycle and room temperature about 25 ± 2 ºC at the Primate Research Unit, Department of Biology, Faculty of Science, Chulalongkorn University. The animals were fed with a standard rodent diet (C.P. 082, Lot No. 17 S. W. T. Co., Ltd, Thailand) for 3.5 months or until they were 5.5 months old. Two weeks in advance and during the experimental period, rats were fed a soy-bean free rat diet (C. P. 082G/SBF, Lot No. 080101, S. W. T. Co., Ltd, Thailand) to minimize the phytoestrogens content in the diet. The isoflavone contents and ingredients of the standard rodent diet and soybean-free diet are shown in Table 3.1 and 3.2.

Table 3.1 Isoflavone contents of the standard rodent diet and soybean-free diet (Usasopon *et al*., 2008).

Sample	Isoflavones (mg/ 100 g sample)				
	Daidzin	Daidzein	Genistin	Genistein	Total
Rodent diet (C.P. 082)					
Lot no. 2	20.7±0.6	10.2±3.4	38.6±2.6	1.4±0.7	70.9±3.0
Lot no. 10	12.2± 0.2	4.5±0.6	20.7±0.9	1.2±0.3	38.6±2.9
Lot no. 18	26.2±0.9	9.1±1.0	36.2±1.1	0.9±0.6	72.4±3.7
Lot no. 21	13.7±0.8	5.4±0.3	22.7±0.8	0.8±0.5	42.5±0.8
Lot no. 24	18.8±1.8	10.0±0.2	28.8±3.5	0.4±0.1	58.0±5.6
Soybean-free diet (C.P. 082/SBF) Lot no. 050119	0.9±0.1	3.0±0.1	2.2±0.1	nd	6.1±0.2

nd = not detected

Table 3.2 Ingredients of the standard rodent diet and soybean-free diet.

Ingredients	Percentage of diet	
	Standard rodent diet	Soybean-free diet
Moisture	6.69	4.64
Protein	27.6	28.9
Fat (Ether extraction)	2.45	2.94
Fat (Acid hydrolysis)	8.36	10.8
Fiber	3.69	2.98
Ash	5.98	4.52
Calcium	1.17	0.91
Phosphorus	0.93	0.792
Sodium Chloride	0.51	0.44
Vitamin D	4,000 i.u. / kg feed	4,000 i.u. / kg feed

To minimize the increase in body weight caused by ovariectomy (Mcelroy and Wade, 1987), the food consumption of OVX rats was adjusted weekly to the level of the sham-operated rat's consumption. Water and rat diet were supplied *ad libitum.* The experimental protocol was approved by the Institutional Animal Care and Use Committee of Faculty of Science, Chulalongkorn University (Protocol Review no. 0823003).

2. Experimental designs

At 6 months of age, 58 of rats were collected the blood samples by cardiac puncture and this day was designed as day 0 (D_0). Rats were divided into 2 groups; sham control (n=13) and ovariectomy (n=45). Before starting the operation, food was withdrawn from the animals for 12 hours. In the ovariectomy or OVX group, rats were bilaterally ovariectomized from a dorsal approach. In the sham control or SH group, the procedures were repeated except that ovaries were not cut out. They were kept for 90

days after the surgery to induce bone loss (the bone loss induction period) before submitting to the next step of the study (Urasopon *et al*., 2007, 2008a).

After 90 days of the bone loss induction period, 5 rats in each group (SH and OVX groups) were randomly selected and euthanized. Their right tibia, femur and lumbar vertebra were defleshed from the adjacent tissues, wrapped in saline-soaked guaze to prevent dehydration, and stored frozen at -20ºC in ziplock until BMD and BMC were measured to confirm the ovariectomy-induced bone loss (Urasopon *et al*., 2007, 2008a). Their left tibia was defleshed from the adjacent tissues, fixed in 10% phosphate buffer formalin and manipulated for histological examination. The uterus was removed and weighed. The remaining rats in the SH group (8 rats) and OVX group (40 rats) were subdivided into 6 groups (8 rats/group) as follows;

1) Sham control rats fed with distilled water (SH)
2) Ovariectomized rats fed with distilled water (PM0)
3) Ovariectomized rats fed with 10 mg/kg BW/day of *P. mirifica* (PM10)
4) Ovariectomized rats fed with 100 mg/kg BW/day of *P. mirifica* (PM100)
5) Ovariectomized rats fed with 1,000 mg/kg BW/day of *P. mirifica* (PM1000)
6) Ovariectomized rats fed with 0.1 mg/kg BW/day of 17α-ethinylestradiol (EE)

The treatment period was 90 days. In SH and PM0 groups, rat were gavaged daily with 1 ml of distilled water. In EE group, rats were gavaged daily with 0.1 mg/kg BW of 17 α- ethinylestradiol. In the PM10, PM100 and PM1000 groups, rats were gavaged daily with 10, 100 and 1,000 mg/kg BW of *P. mirifica* in 1 ml distilled water, respectively. The rats were gavaged at 08.00-10.00 AM using a gastric feeding needle.

During the experimental period, all rats were weighed once a week and these body weights were used to adjust the quantity of *P. mirifica* or 17 α- ethinylestradiol treatment. At the end of 90 days of experimental period, all rats were sacrificed. Their right tibia, femur and lumbar vertebra were defleshed from the adjacent tissues, wrapped in saline-soaked guaze to prevent dehydration, and stored frozen at -20ºC in ziplock until BMD and BMC were measured. Their left tibia were defleshed from the

adjacent tissues, fixed in 10% phosphate buffer formalin and manipulated for histological examination. The uterus was removed and weighed.

3. Blood collection

Blood samples were collected on day 0 and every 30 days throughout the bone loss induction period (D_0, D_{30}, D_{60}, and D_{90}) and treatment period (D_{120}, D_{150}, and D_{180}). Blood samples were collected by cardiac puncture under ether anesthesia. After sampling, the blood was chilled on ice immediately and centrifugued at 2,500 rpm for 15 min at 4 ^{o}C. The serum was removed and kept at -20 ^{o}C for the determination of ALP and TRAP 5b levels.

4. The preparation *of P. mirifica* suspension

The tuberous roots of *P. mirifica* used in this study were the *P. mirifica* cultivar Wichai III, which were collected from the field in Chiang Mai Province, Thailand. The voucher specimen of *P. mirifica* was deposited at the Herbarium of the Department of Botany, Faculty of Science, Chulalongkorn University, Bangkok, Thailand. The same lot of tuberous roots of *P. mirifica* was washed, sliced and completely dried in a hot-air oven at 70 ^{o}C. The dried tuberous roots of *P. mirifica* were ground into a powder at a size of 100 Mesh. The suspension of *P. mirifica* was freshly prepared by suspending the *P. mirifica* powder in distilled water. The rats were gavaged daily with the suspension of *P. mirifica* at a dose of 10, 100 or 1,000 mg/kg BW in 1ml of distilled water. The dose range used in this study was followed the previous study, in which the low dose (10 mg/kg BW/day) significantly prevented bone loss in the OVX and ORX rats and the doses of 100 and 1,000 mg/kg BW/day completely prevented bone loss (Urasopon *et al*., 2007, 2008a).

5. The preparation of 17 α–ethinylestradiol solution

The 17 α–ethinylestradiol powder (98 % purity, HPLC grade, Sigma, St. Loius, MO) was weighed and dissolved in a small volume of absolute ethanol. After the powder was completely dissolved, the distilled water was added and the solution was allowed to stand at room temperature to evaporate the ethanol. The stock solution was then diluted with distilled water to give a final dose of 0.1 mg/kg BW/day/ 1 ml of distilled water. The stock 17 α – ethinylestradiol solution was kept in the dark bottles at 4 ^{o}C until the feeding time. The oral dose of 0.1 mg/kg BW/day of 17α–ethinylestradiol used in this study was previously reported to prevent bone loss in OVX and ORX rats and the effect of this dose was equivalent to the dose of 100 mg/kg BW/day of *P. mirifica* (Urasopon *et al*., 2007, 2008a).

6. Uterus weights

After euthanized the rats, the uterus was dissected, the connective tissue was removed, and the organs were weighed. The relative uterus weight (%) was obtained by the division of the uterus weight by the body weight × 100.

7. Serum total alkaline phosphatase (ALP) assay

Serum ALP is an index of bone formation. Serum samples were sent to the Faculty of Veterinary Technology, Kasetsart University for the determination. In this assay, ALP functions as an enzyme in the enzyme-linked immunoassay (EIA) system. ALP catalyzes the hydrolysis of orthophosphoric monoester, and delivers phosphate and corresponding alcohols. If the phosphoric ester is electro-inactive while the enzymatic product is electro-active in the working potential range, electrochemical methods can be used for detection. In this experiment the determination of ALP activity is conducted with *p*-nitrophenylphosphate (*p*NPP) as the substrate. The ALP catalyzed reaction process is shown below;

In the test, the well plate was coated with an antibody specific to rat ALP and incubated for 60 min. Then the plate was washed by washing buffer. After washing, standard, control, and serum samples were pipette and incubated in the wells for 2 hours at 37 ºC. The liquid of each well was removed. The substrate solution was added to all wells and incubated for 1 hour at 37 ºC. The stop solution was added to each well. The ALP activity was determined with the color developed by a chromogenic substrate. The enzyme activity was determined as the change of absorbance at 450 nm. The coefficient variations (%CVs) of the intra and inter-assay of ALP were 2.8% and 6.3%, respectively.

8. Serum tartrate-resistant acid phosphatase 5b (TRAP 5b) assay

TRAP-5b is typically expressed in osteoclasts and then secreted into blood circulation. Therefore, serum TRAP 5b has been regarded as a useful marker for bone resorption. Rat TRAP 5b EIA features high specificity for the 5b isoform of TRAP in serum. By utilizing specific monoclonal antibodies to capture the 5b isoform, it avoids interference from TRAP 5a, which is not specific for bone resorbing osteoclasts. Rat TRAP 5b detects the enzyme activity of TRAP 5b based on the enzymatic immunocapture assay method (EIA). A monoclonal antibody to rat TRAP 5b with high specificity for active TRAP 5b is allowed to react with the sample. After the immunoreaction, bound TRAP 5b activity is measured by addition of *p*-nitrophenyl phosphate (*p*NPP) as a substrate.

Rat serum samples were analyzed by RatTRAP ™ Assay Kit (IDS Ltd. UK; Lot No. 6482, UK). The protocol was followed the instruction provided by the Immunodiagnostic System Limited, UK. In the test, the monoclonal antibody of rat TRAP is incubated in anti-mouse IgG-coated microtiter wells for 60 min. Then the plate was washed by washing buffer. After washing, standard, control, and serum samples were incubated in the wells for 60 min. The bound TRAP 5b activity was determined with a chromogenic substrate to develop color. The reaction was stopped and the absorbance of the

reaction mixture was read by a microplate reader at 405 nm (Songlin *et al*., 2009). The coefficient variations (%CVs) of intra and inter-assay of TRAP 5b were 3.64% and 4.18%, respectively.

9. Bone histomorphometry

After rats were sacrificed, the left proximal tibia was defreshed and placed in 10% phosphate buffer formalin for at least 72 hours. Bones were cut into a small size and then decalcificed in EDTA – G solution (EDTA disodium salt 14.50 g, NaOH 1.25 g, glycerol 15 ml and distilled water 100 ml) for 4 weeks by changing EDTA – G solution every week. After 4 weeks, the decalcified bones were dehydrated in series of ethanol gradient and clearing in xylene. They were then embedded in paraffin, cut into section of 5 μm thickness, and stained with Hematoxylin and Eosin (H&E) (Urasopon *et al*., 2007, 2008a). The slides were analyzed under the light microscope (ZEISS: Axiostar plus) and photographed using a digital camera.

The Digital Image Processing Software Image Pro (Plus Software Media Cybernatics, Inc., USA) was used for quantitative bone histomorphometric measurements. The studied region of proximal tibia metaphysis was trabecular bone between 2 and 4 mm distal to the growth plate-epiphyseal junction (Figure 3.2). In each section, 4 non-overlapped windows of 1×1 mm^2 were determined, and 5 sections in each rat were selected for the study (Figure 3.3). Then the total trabecular bone areas were summed up (modified from Cui *et al*., 2004). The percentage of trabecular bone area (%BA) was expressed by the sum of trabecular bone area/ sum of bone area (or total area), and then multiplied by 100 as shown in the following equation;

$$\text{\% Trabecular bone area (\%BA) (in each section)} = \frac{\text{(Sum of trabecular bone area} \times 100)}{\text{Sum of the bone area}}$$

Trabecular bone area of each rat is an average value of %BA of 5 sections as shown in the following equation;

$$\text{\%BA of each rat} = (\%BA_1 + \%BA_2 + \%BA_3 + \%BA_4 + \%BA_5)/\ 5$$

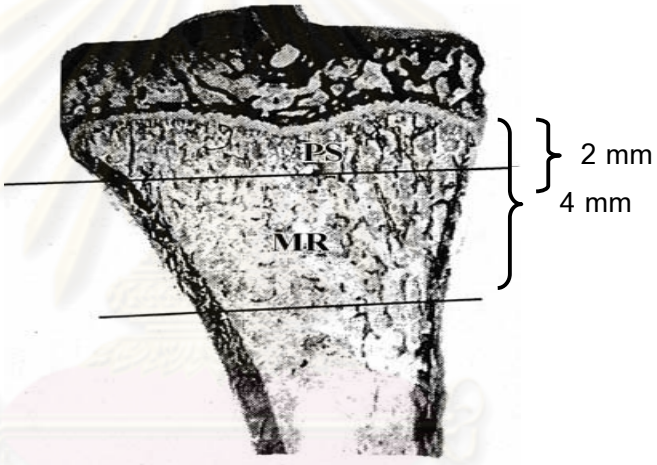
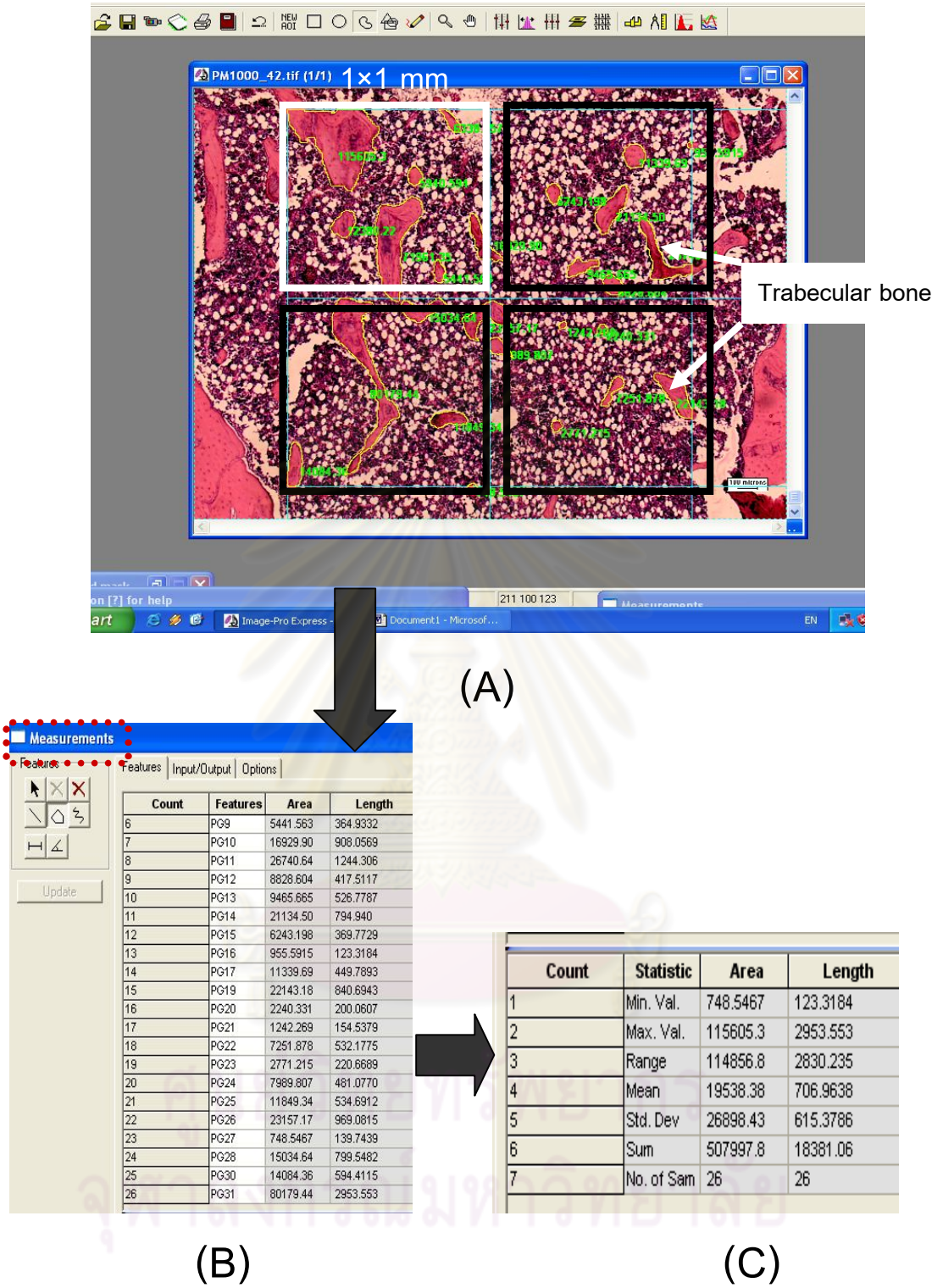


Figure 3.2 The studied region of proximal tibia metaphysis was trabecular bone between 2 and 4 mm distal to the growth plate-epiphyseal junction.
(Available from: http://www.chinaphar.com/1671-4083/25/678.pdf)



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7	PG10	16929.90	908.0569
8	PG11	26740.64	1244.306
9	PG12	8828.604	417.5117
10	PG13	9465.665	526.7787
11	PG14	21134.50	794.940
12	PG15	6243.198	369.7729
13	PG16	955.5915	123.3184
14	PG17	11339.69	449.7893
15	PG19	22143.18	840.6943
16	PG20	2240.331	200.0607
17	PG21	1242.269	154.5379
18	PG22	7251.878	532.1775
19	PG23	2771.215	220.6689
20	PG24	7989.807	481.0770
21	PG25	11849.34	534.6912
22	PG26	23157.17	969.0815
23	PG27	748.5467	139.7439
24	PG28	15034.64	799.5482
25	PG30	14084.36	594.4115
26	PG31	80179.44	2953.553

Count	Statistic	Area	Length
1	Min. Val.	748.5467	123.3184
2	Max. Val.	115605.3	2953.553
3	Range	114856.8	2830.235
4	Mean	19538.38	706.9638
5	Std. Dev	26898.43	615.3786
6	Sum	507997.8	18381.06
7	No. of Sam	26	26

Figure 3.3 The image of trabecular bone of tibia metaphysis (A) and the measurement functional tool was used to calculate the trabecular bone area (Plus Software Media Cybernatics, Inc., USA) (B, C).

10. Bone measurement

BMD and BMC were measured using a peripheral Quantitative Computed Tomography (pQCT) (Figure 3.4). The pQCT can measure a 3-dimension image of bone and can provide a real volumetric density. The determination has been performed both in axial and long bones, in cortical and trabecular compartments, at metaphyseal and diaphyseal sites as follows (Figure 3.5);

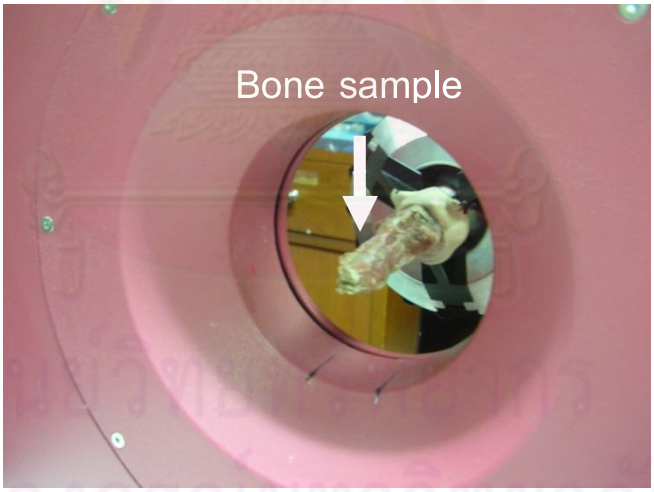
Tibia: Proximal tibia metaphysis (TM) was cross-sectionally scanned at 2, 2.5 and 3 mm below the growth plate of the tibia. Tibia diaphysis (TD) was scanned at the midpoint (50% of the length of tibia) and at both sides of the midpoint, 1 mm apart.

Femur: Distal femur metaphysis (FM) was cross-sectionally scanned at 2, 2.5 and 3 mm above the growth plate and femoral diaphysis (FD) was scanned at the midpoint (50% of the length of the femur) and at both sides of the midpoint, 1 mm apart.

The fourth lumbar vertebra (L4): Fourth lumbar vertebra was cross-sectionally scanned at the midpoint of cranio-caudal longitudinal axis and both sides of the midpoint, 1 mm apart from the midpoint along the longitudinal axis of the vertebra.

The trabecular compartment was determined by the contour mode 2 and peel mode 2 with the threshold value of 750 mg/cm^3, and the cortical compartment was determined by the separation mode 2 with the threshold value of 900 mg/cm^3. After completion of the scanning, the parameters were analyzed for each bone slice using XCT- 5.50E software (Stratec Medizintechnik GmbH., Germany): trabecular bone mineral density (TbBMD), trabecular bone mineral content (TbBMC), cortical bone mineral density (CtBMD), and cortical bone mineral content (CtBMC) for the TM, FM and L4; and CtBMD and CtBMC for the TD and FD. The average of three scans made at all bone sites described above was analyzed (Urasopon *et al*., 2007, 2008a).

(A)



(B)

Figure 3.4 Peripheral Quantitative Computed Tomography (pQCT) (A) and bone scanning by pQCT (B).

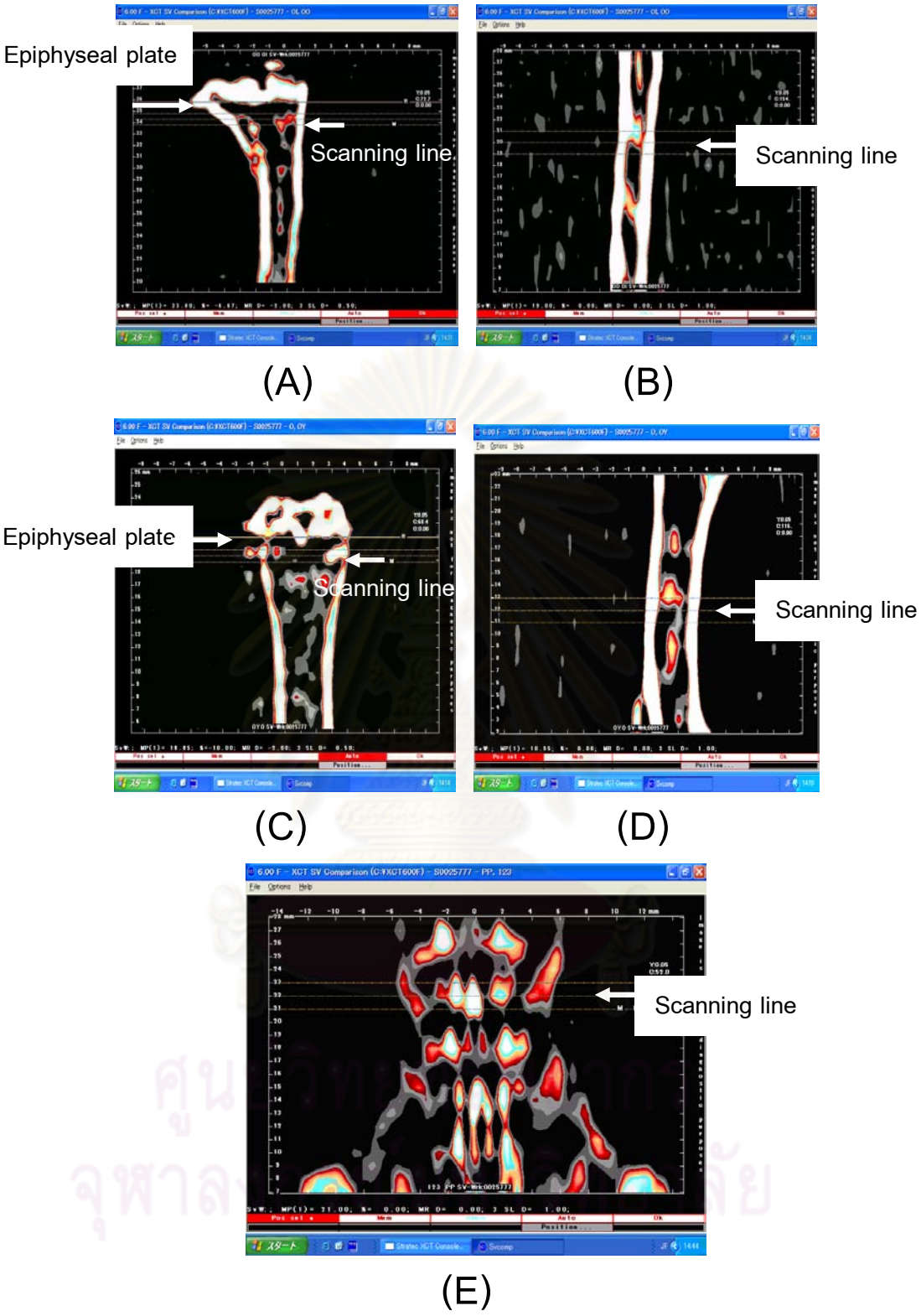


Figure 3.5 The longitudinal preview of bone scanning at proximal tibia metaphysis (TM) (A), middle length of tibia or tibia diaphysis (TD) (B), metaphysis of femur (FM) (C), middle length of femur or femoral diaphysis (FD) (D), middle length of fourth lumbar vertebra (L4) (E), using pQCT.

11. Data analysis

The result was reported as mean ± SEM. The significant differences between groups were examined by one-way analysis of variance (ANOVA). LSD Post-Hoc test was used for multiple comparisons among groups. The statistical differences were considered at the probability level *(p*-value) of lower than 0.05 ($p < 0.05$). The Statistical Packages for Social Science (SPSS) version 15.0 was used.

CHAPTER IV

RESULTS

1. Changes in body weights and relative uterus weights

1.1 Body weights

The body weights of the rats during the experimental periods are shown in **Figure 4.1.** There were no significant difference in mean initial body weights (Day_0) between SH and OVX (PM0) rats ($p>0.05$). Relative to Day_0, the mean body weights highly increased over the 90 days after ovariectomy in the OVX (PM0) rats and showed a highly significant difference to the SH rats at each time point ($p<0.05$ and $p<0.001$), starting from 21 days or 3 weeks of the pre-treatment period. After 90 days of *P. mirifica* treatment, the body weights of OVX rats were decreased, in a dose dependent manner. The body weights of PM10 group were not significantly different from those of the OVX (PM0) group throughout the treatment period, whereas it was lower in the PM100 group, starting from 28 days of the treatment period. The body weights of EE and PM1000 groups were significantly decreased to those of the SH rat, starting from 2 weeks of the treatment period. The body weights of SH rats were slightly increased in the last 90 days of the study period, but showed no significant difference from D_0 ($p>0.05$).

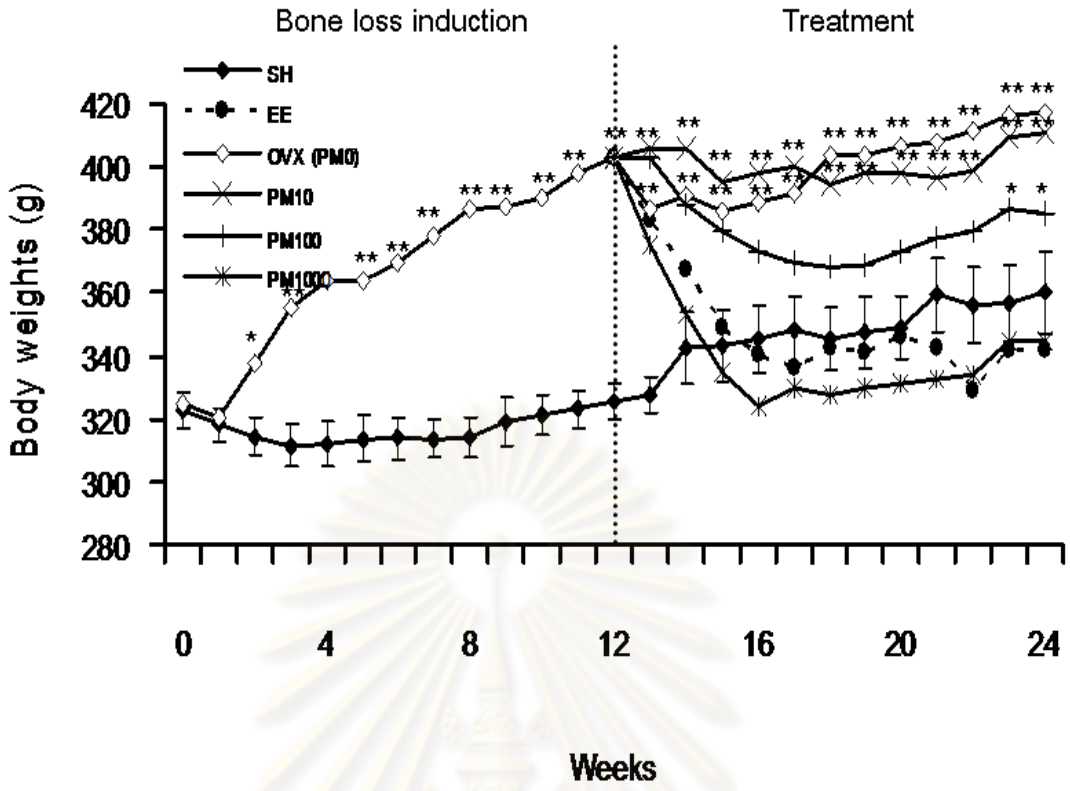


Figure 4.1 Body weights in sham operated rats (SH) and ovariectomized rats (OVX) treated with 0.1 mg/kg BW/day of esthinylestradiol (EE), or *P. mirifica* powder suspension at 0, 10, 100 and 1,000 mg/kg BW/day (PM0, PM10, PM100 and PM1000, respectively) for 90 days. The data are expressed as only the mean because the SE values were overlapped and difficult to understand, except for the SH group. *, ** = $p < 0.05$ and 0.001 compared to the SH group.

1.2 Relative uterus weights

The relative uterus weights are shown in **Figure 4.2.** Ninety days after ovariectomy, the relative uterus weight was decreased by 81.46% in comparison with the SH_{90} rats ($p<0.001$). After the rats were kept for the further 90 days, the relative uterus weight in the OVX (PM0) group was significantly lowered than that of the SH_{180} group ($p<0.001$). Ninety days after treatment with PM10, PM100 and PM1000, relative uterine weights were significantly dose-dependently increased by 30.30%, 62.64% and 77.38% over that of the PM0 rats ($p<0.001$). The EE treatment significantly increased a relative uterine weight by 73.86% over the PM0 rats ($p<0.001$). In addition, the relative uterine weights of the PM1000 and EE rats were higher than that of the SH_{180} rats by 42.06% and 21.37%, respectively.

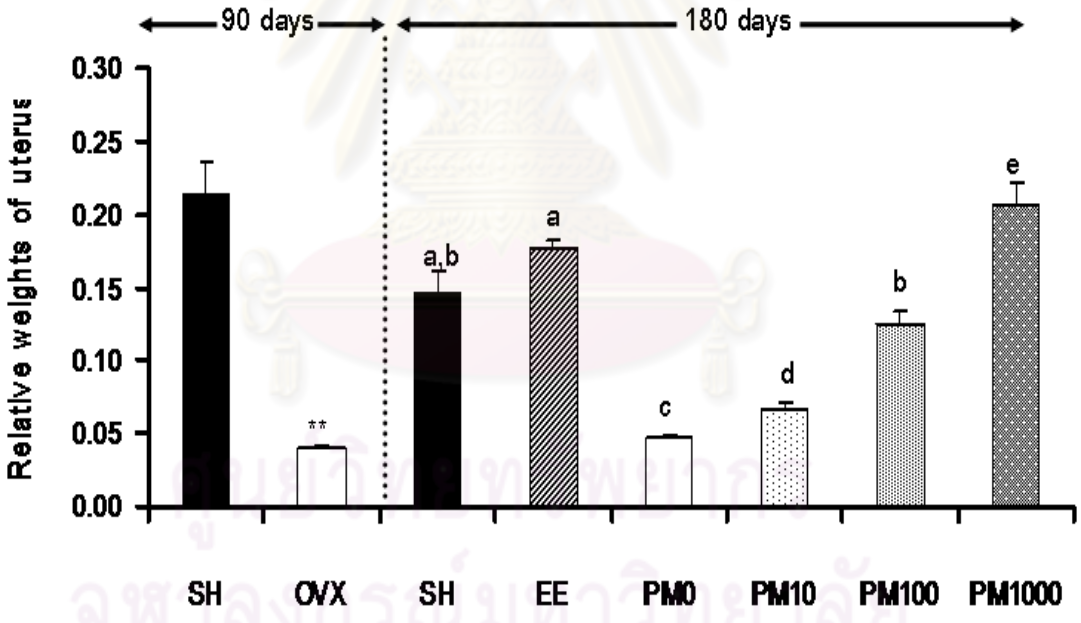


Figure 4.2 Relative weights of uterus in sham operated rats (SH) and ovariectomized rats (OVX) at 90 and 180 days after surgery, and the 90-day OVX rats treated with 0.1 mg/kg BW/day of esthinylestradiol (EE), or *P. mirifica* powder suspension at 0, 10, 100 and 1,000 mg/ kg BW/day (PM0, PM10, PM100 and PM1000, respectively) for 90 days. Data are means ± SEM. ** = $p < 0.001$ compared to the SH group. Means that do not share the same super script letters are significantly different from each other.

2. Changes in bone mineral density, bone mineral content and bone histology

2.1 Bone mineral density after ovariectomy

The trabecular bone mineral densities (TbBMDs) in proximal tibia metaphysis (TM), distal femoral metaphysis (FM) and the fourth lumbar vertebra (L4) for SH and OVX groups at 90 and 180 days after surgery are shown in **Figure 4.3**. The influence of ovariectomy on BMD was expressed by comparison between the SH and OVX groups after ovariectomy for 90 and 180 days. After ovariectomy for 90 days TbBMDs in TM, FM and L4 were significantly decreased ($p<0.01$) by 36.86%, 35.20%, and 19.55%, respectively, when compared to the SH_{90} rats. After ovariectomy for 180 days TbBMDs in TM, FM and L4 were significantly decreased ($p<0.01$) by 38.24%, 42.24%, and 12.58%, respectively, when compared to the SH_{180} rats. Increasing in age of rats from 9 months (SH_{90}) to 12 months (SH_{180}) old, TbBMDs in TM, FM and L4 tended to decrease by 4.78%, 3.45% and 2.75%, respectively, with no significant differences ($p>0.05$).

The cortical bone mineral densities (CtBMDs) in proximal tibia metaphysis (TM), distal femoral metaphysis (FM), the fourth lumbar vertebra (L4), tibia diaphysis (TD) and femoral diaphysis (FD) for SH and OVX groups at 90 and 180 days after surgery are shown in **Figure 4.4**. The ovariectomy for 90 days did not obviously affect the CtBMDs of L4, TD and FD, except for TM and FM. The CtBMDs of TM and FM were significant decreased by 2.96% and 3.82% ($p<0.05$), respectively, when compared to the SH_{90} rats. After OVX for 180 days, the CtBMDs of TM, FM, L4, TD and FD were significantly decreased by 5.67%, 4.96%, 2.60%, 2.64% and 4.69% ($p<0.001$) when compared to the SH_{180} rats. Increasing in age of rats from 9 months (SH_{90} group) to 12 months (SH_{180}) old increased the CtBMD of TM by 5.07% and increased in the CtBMD of FD by 2.03% ($p>0.05$).

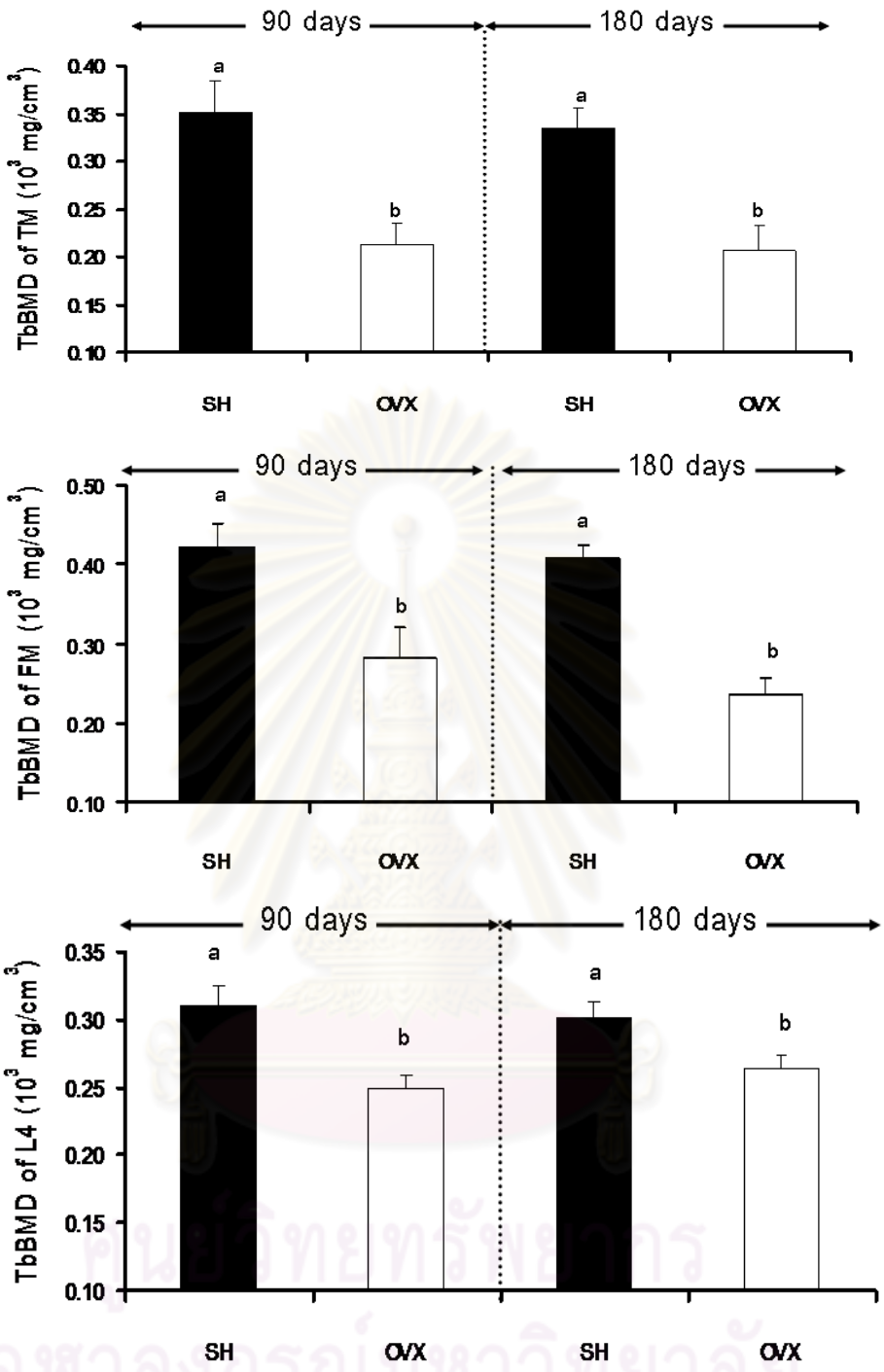


Figure 4.3 Trabecular bone mineral densities (TbBMDs) of tibia metaphysis (TM), distal femoral metaphysis (FM) and the fourth lumbar vertebra (L4) in sham operated rats (SH) and ovariectomized rats (OVX) at 90 and 180 days after surgery. Data are means ± SEM. Means that do not share the same super script letters are significantly different from each other ($p<0.01$).

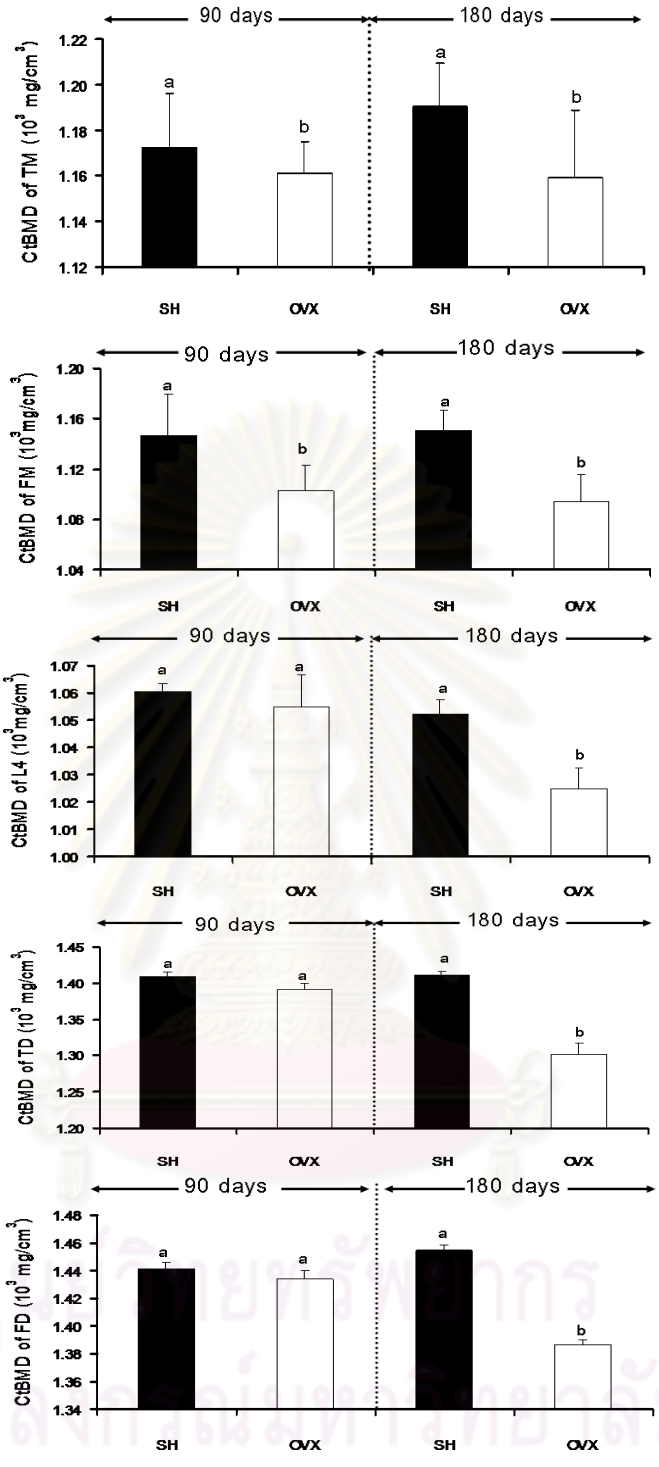


Figure 4.4 Cortical bone mineral densities (CtBMDs) of TM, FM, L4, TD and FD in sham operated rats (SH) and ovariectomized rats (OVX) at 90 and 180 days after surgery. Data are means ± SEM. Means that do not share the same super script letters are significantly different from each other.

2.2 Bone mineral density after EE or *P. mirifica* treatment

The trabecular bone mineral densities (TbBMDs) in proximal tibia metaphysis (TM), distal femoral metaphysis (FM) and the fourth lumbar vertebra (L4) in SH and OVX rats at 90 days after surgery and treated with EE or PM0, PM10, PM100 and PM1000 for 90 days are shown in **Figure 4.5.** Treatments of PM100 and PM1000 significantly prevented the decrease in TbBMDs of FM and L4 ($p<0.05$), but did not in TM ($p>0.05$). The preventive effect of EE was significant and comparable to that of the PM100 and PM1000 in FM and L4. EE did not exhibit the preventive effect on TM as well.

The cortical bone mineral densities (CtBMDs) in proximal tibia metaphysis (TM), distal femoral metaphysis (FM), the fourth lumbar vertebra (L4), tibia diaphysis (TD) and femur diaphysis (FD) in SH and OVX rats at 90 days after surgery and treated with EE or PM0, PM10, PM100 and PM1000 for 90 days are shown in **Figure 4.6.** Effects of PM10, PM100 and PM1000 as well as EE significantly prevented the decrease in CtBMDs of TM, FM, L4, TD and FD ($p<0.05$). Interestingly, the CtBMDs of FM in all *P. mirifica* treatment groups were significantly higher than those of the SH and EE groups.

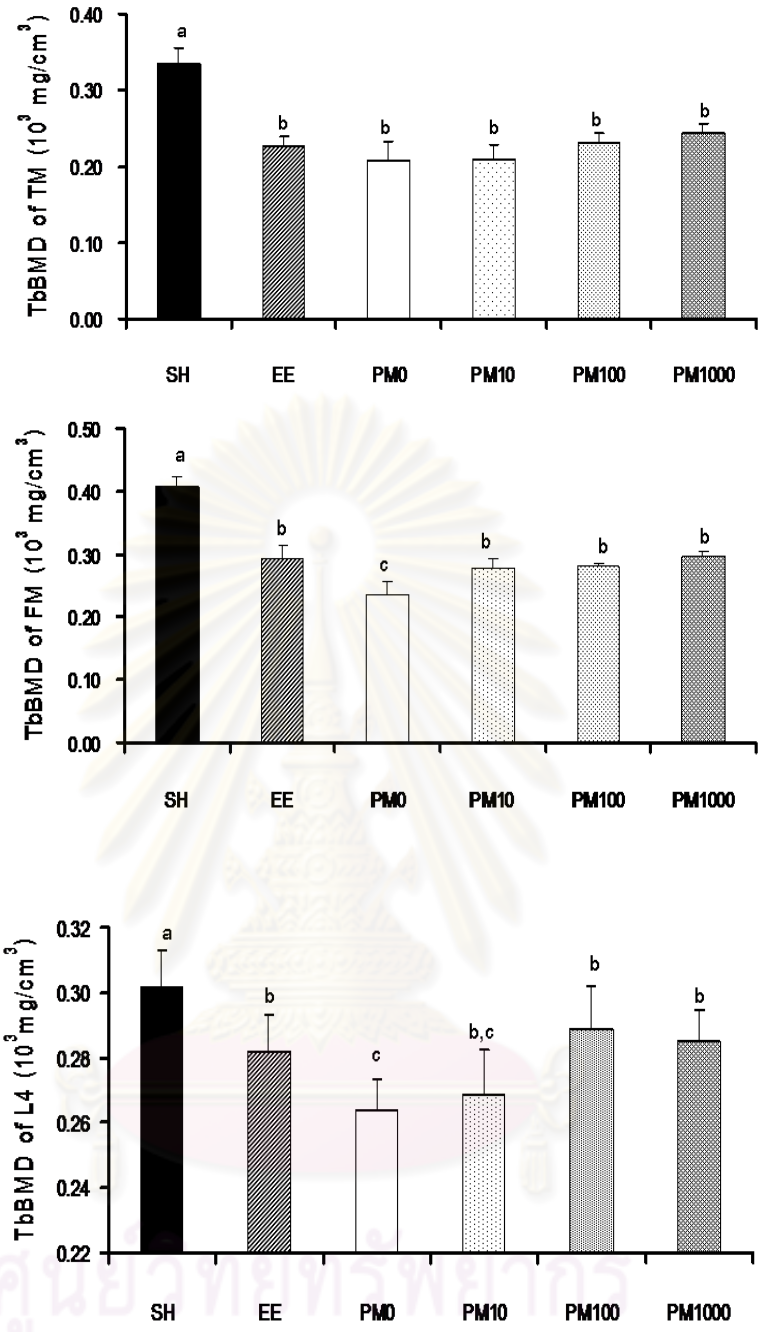


Figure 4.5 Trabecular bone mineral densities (TbBMDs) of tibia metaphysis (TM), distal femoral metaphysis (FM) and the fourth lumbar vertebra (L4) in sham operated rats (SH) and ovariectomized rats (OVX) at 90 days after surgery and treated with 0.1 mg/kg BW/day of esthinylestradiol (EE) or *P. mirifica* powder suspension at 0, 10, 100 and 1,000 mg/kg BW/day (PM0, PM10, PM100 and PM1000, respectively) for 90 days. Data are means ± SEM. Means that do not share the same super script letters are significantly different from each other.

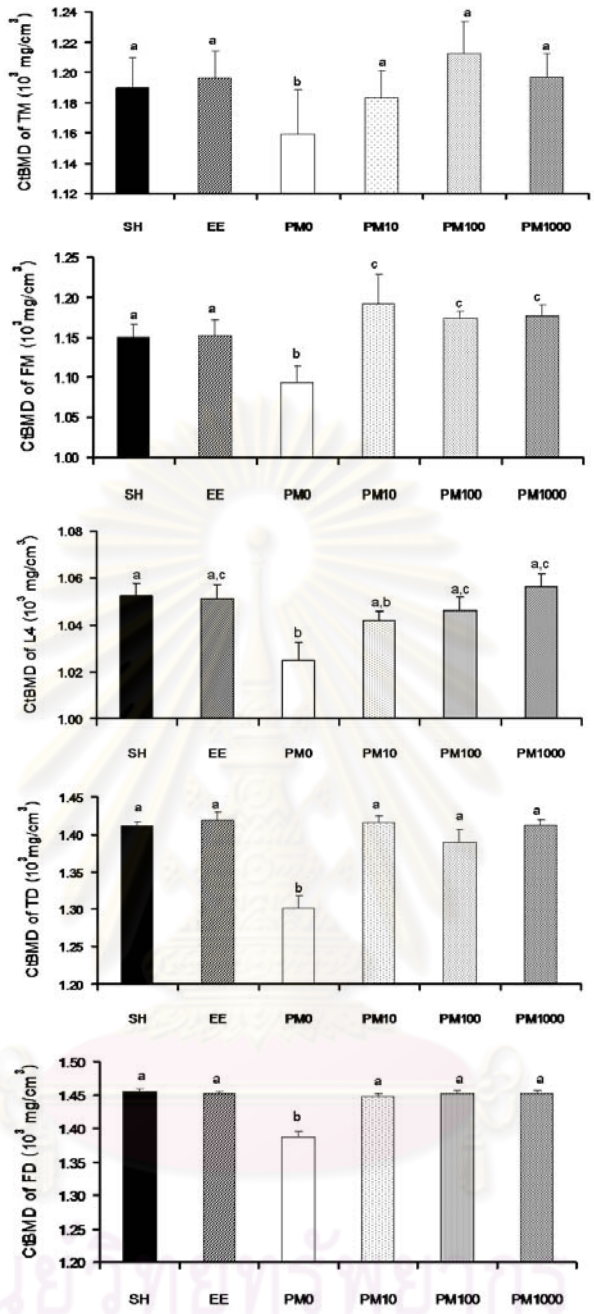


Figure 4.6 Cortical bone mineral densities (CtBMDs) of TM, FM, L4, TD and FD in sham operated rats (SH) and ovariectomized rats (OVX) at 90 days after surgery and treated with 0.1 mg/kg BW/day of esthinylestradiol (EE) or *P. mirifica* powder suspension at 0, 10, 100 and 1,000 mg/kg BW/day (PM0, PM10, PM100 and PM1000, respectively) for 90 days. Data are means ± SEM. Means that do not share the same super script letters are significantly different from each other.

2.3 Bone mineral content after ovariectomy.

The trabecular bone mineral contents (TbBMCs) in proximal tibia metaphysis (TM), distal femoral metaphysis (FM) and the fourth lumbar vertebra (L4) in SH and OVX rats at 90 and 180 days after surgery are shown in **Figure 4.7**. After ovariectomy for 90 days the TbBMCs of TM and FM significantly decreased ($p<0.05$) by 29.56% and 16.96%, respectively, when compared to the SH_{90} rats, but not in the L4. Changes of TbBMCs after ovariectomy for 180 days were similar to those of the 90-day ovariectomy, that is, only TbBMCs of TM and FM were significantly decreased ($p<0.001$) by 31.69% and 36.71%, respectively, when compared to the SH_{180} rats. Increasing in age of rats from 9 months (SH_{90}) to 12 months (SH_{180}) old had no effects on TbBMCs of TM, FM and L4.

The cortical bone mineral contents (CtBMCs) in proximal tibia metaphysis (TM), distal femoral metaphysis (FM), the fourth lumbar vertebra (L4), tibia diaphysis (TD) and femoral diaphysis (FD) in SH and OVX rats at 90 and 180 days after surgery are shown in **Figure 4.8**. After ovariectomy for 90 days only the CtBMC of L4 was significantly decreased ($p<0.05$) by 17.88% when compared to the SH_{90} rats. However, after ovariectomy for 180 days the CtBMCs of L4, TD and FD were significantly decreased ($p<0.05$) by 27.07%, 36.86%, and 7.30% when compared to the SH_{180} rats. After ovariectomy for 90 and 180 days, significant changes in CtBMCs of TM and FM were not found ($p>0.05$). Increasing in age of rats from 9 months to 12 months old significantly increased ($p<0.01$) the CtBMCs of TD and FD by 38.66% and 4.50%, respectively.

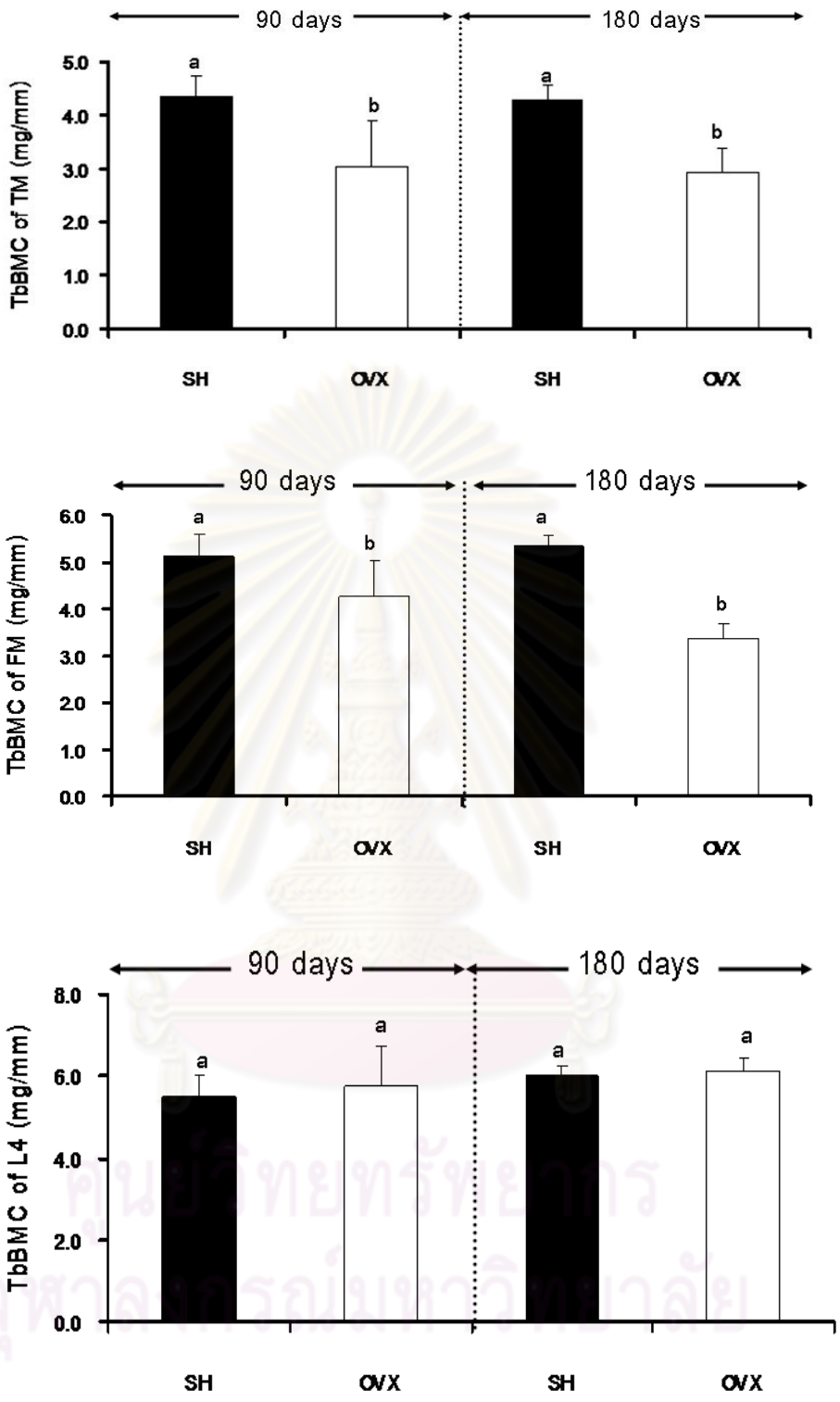


Figure 4.7 Trabecular bone mineral contents (TbBMCs) of tibia metaphysis (TM), distal femoral metaphysis (FM) and the fourth lumbar vertebra (L4) in sham operated rats (SH) and ovariectomized rats (OVX) at 90 and 180 days after surgery. Data are means ± SEM. Means that do not share the same super script letters are significantly different from each other.

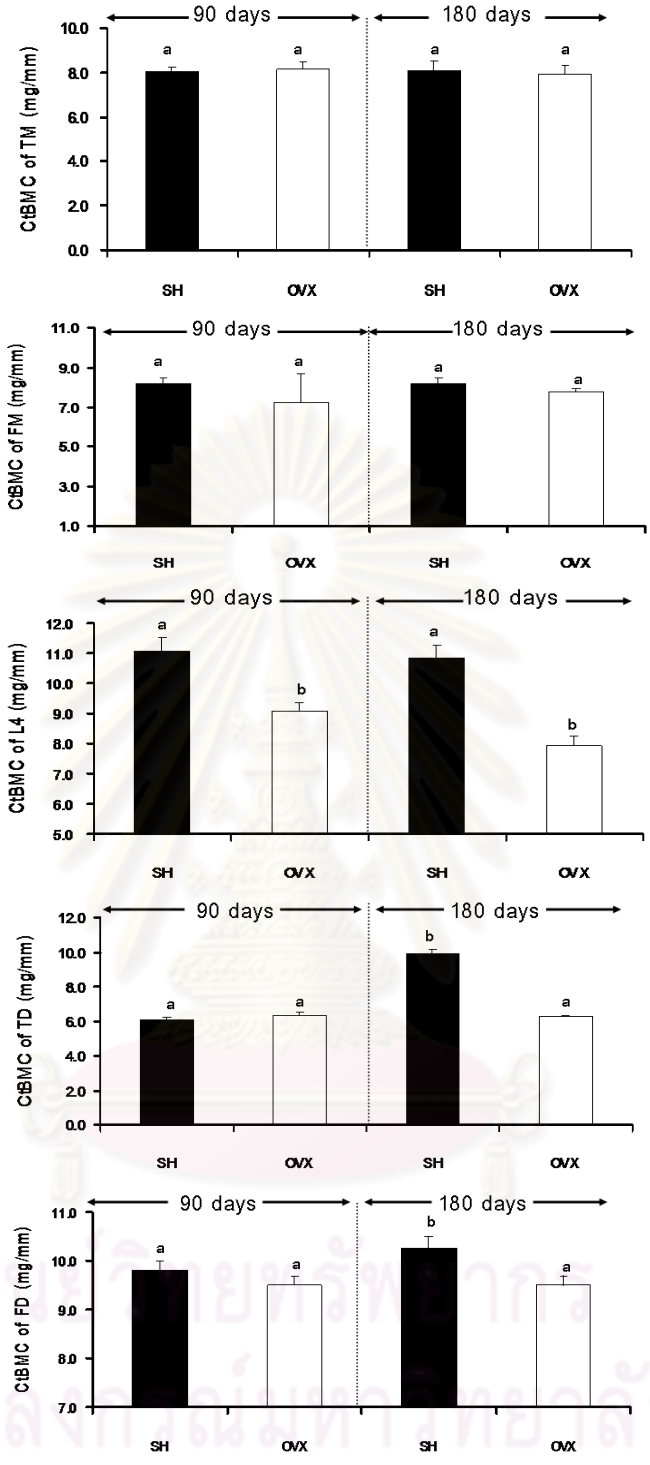


Figure 4.8 Cortical bone mineral contents (CtBMCs) of TM, FM, L4, TD and FD in sham operated rats (SH) and ovariectomized rats (OVX) at 90 and 180 days after surgery. Data are means ± SEM. Means that do not share the same super script letters are significantly different from each other.

2.4 Bone mineral content after EE or *P. mirifica* treatment

The trabecular bone mineral contents (TbBMCs) in proximal tibia metaphysis (TM), distal femoral metaphysis (FM) and the fourth lumbar vertebra (L4) in SH and OVX rats at 90 days after surgery and treated with EE or PM0, PM10, PM100 and PM1000 for 90 days are shown in **Figure 4.9.** Treatment of PM100 and EE significantly prevented the decrease ($p<0.05$) in TbBMCs of FM by 32.84% and 36.05% when compared to the PM0 rats. However, changes in other bone regions in other doses of *P. mirifica* treatment were not found.

The cortical bone mineral contents (CtBMCs) in proximal tibia metaphysis (TM), distal femoral metaphysis (FM), the fourth lumbar vertebra (L4), tibia diaphysis (TD) and femoral diaphysis (FD) in SH and OVX rats at 90 days after surgery and treated with EE or PM0, PM10, PM100 and PM1000 for 90 days are shown in **Figure 4.10**. Treatment of PM100 significantly prevented the decrease ($p<0.05$) in CtBMCs of L4, TD and FD by 26.44%, 10.62% and 3.80%, respectively when compared to the PM0 rats. In contrast, treatment of EE prevented the decrease in CtBMCs of TM and FM by 7.57% and 26.57%, respectively, which were not found in *P. mirifica* treatments, and also in CtBMC of TD by 3.27%.

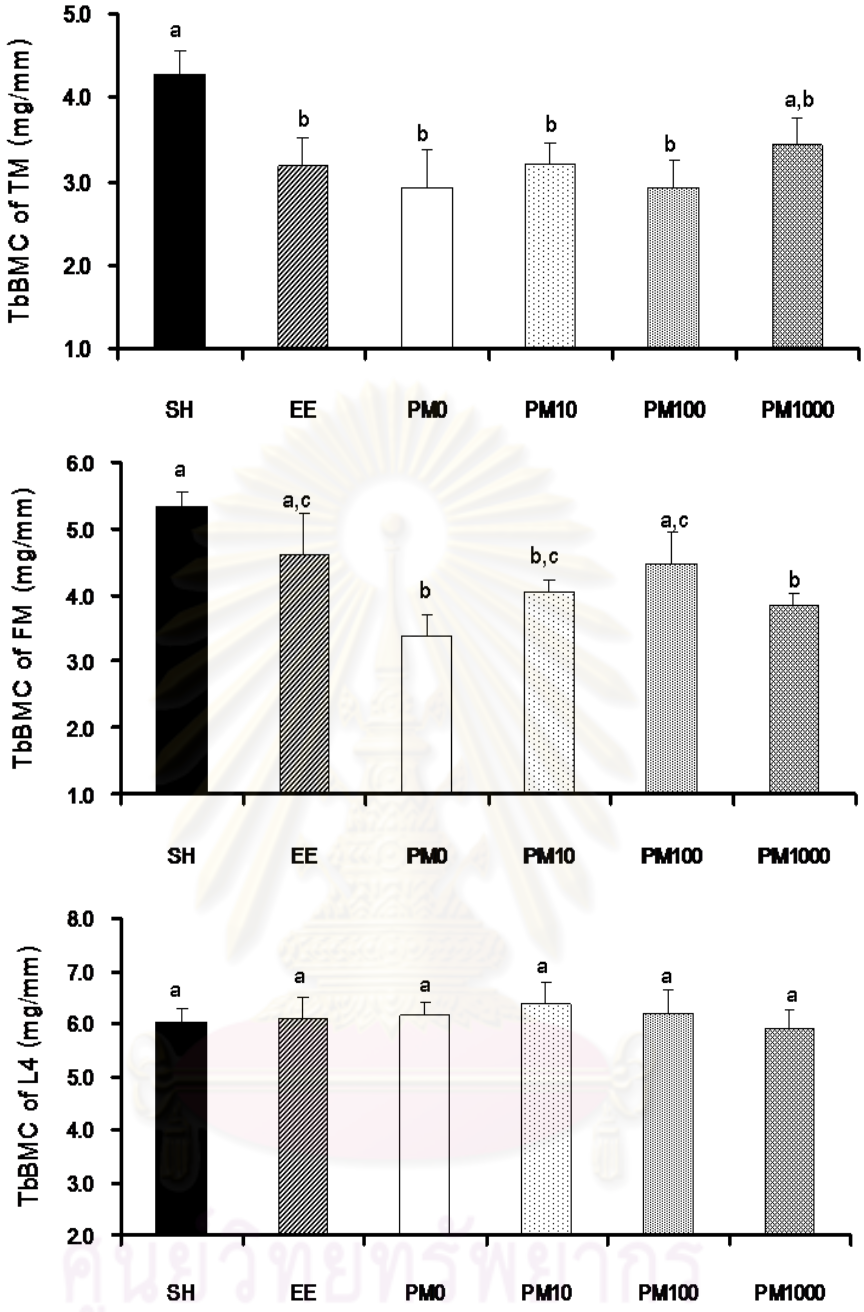


Figure 4.9 Trabecular bone mineral contents (TbBMCs) of tibia metaphysis (TM), distal femoral metaphysis (FM) and the fourth lumbar vertebra (L4) in sham operated rats (SH) and ovariectomized rats (OVX) at 90 days after surgery and treated with 0.1 mg/kg BW/day of esthinylestradiol (EE) or *P. mirifica* powder suspension at 0, 10, 100 and 1,000 mg/kg BW/day (PM0, PM10, PM100 and PM1000, respectively) for 90 days. Data are means ± SEM. Means that do not share the same super script letters are significantly different from each other.

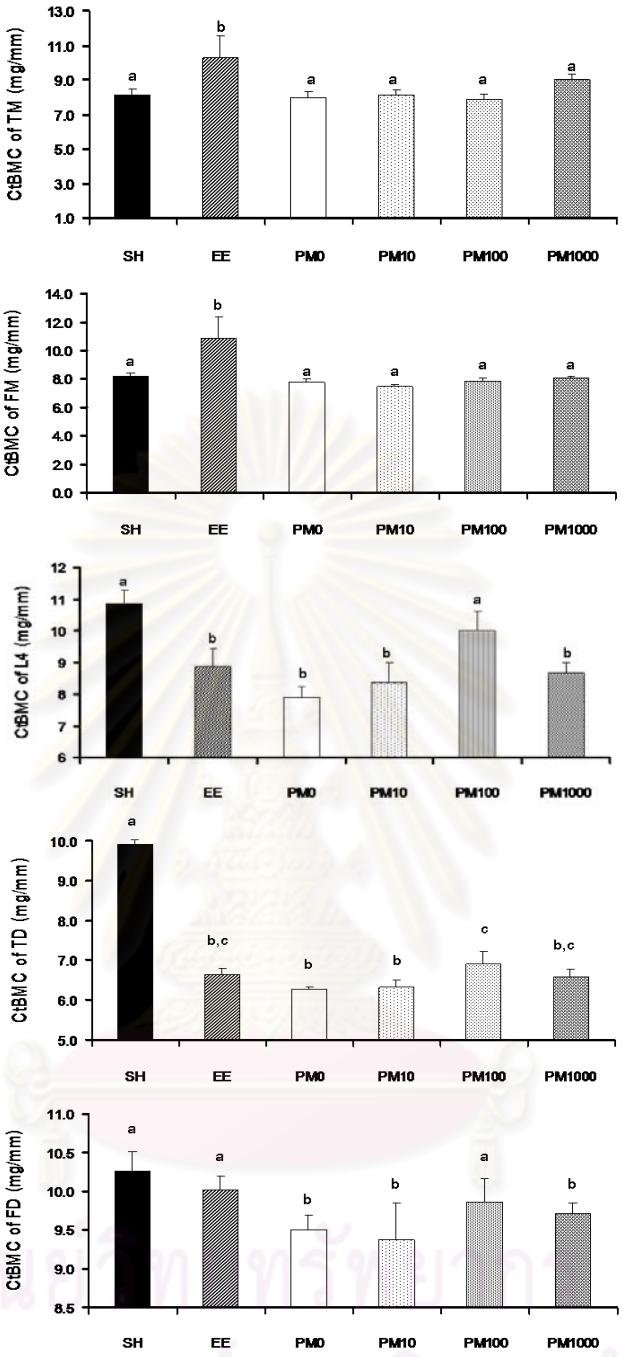


Figure 4.10 Cortical bone mineral contents (CtBMCs) of TM, FM, L4, TD and FD in sham operated rats (SH) and ovariectomized rats (OVX) at 90 days after surgery and treated with 0.1 mg/kg BW/day of esthinylestradiol (EE) or *P. mirifica* powder suspension at 0, 10, 100 and 1,000 mg/kg BW/day (PM0, PM10, PM100 and PM1000, respectively) for 90 days. Data are means ± SEM. Means that do not share the same super script letters are significantly different from each other.

1.3 The therapeutic effects of EE and PM on trabecular BMD (TbBMDs)

From the results presented previously, bone loss could be observed earlier and greater on a trabecular compartment (Tb) than a cortical compartment (Ct), and on BMD than BMC. Thus, changes on TbBMD should be a better marker to detect the therapeutic effects of EE and PM in ovariectomy-induced osteoporotic rats. Regarding the significant difference of TbBMDs between the *P. mirifica* treated groups and the PM0 (OVX_{180}) group was mainly found in the PM1000 group, thus only the TbBMDs of PM1000 were selected for the determination of the therapeutic effects of *P. mirifica* by comparison with the PM0 (OVX_{90}). The determination of the therapeutic effects of EE and PM1000 on bone loss, based on TbBMDs, was done only for the distal femur metaphysis (FM) and the fourth lumbar vertebra (L4) where the significant increase in comparison with PM0 (OVX_{180}) was observed.

The therapeutic effects of PM1000 and EE in FM and L4 of OVX rats at 90 days after ovariectomy are shown in **Figure 4.11**. Treatments of PM1000 and EE were not significant increase in TbBMD of FM ($p>0.05$) when compared to the PM0 (OVX_{90}), however, TbBMDs of L4 were significantly increased ($p<0.05$) by 4.61% and 5.043%, respectively.

1.4 The therapeutic effects of EE and PM on cortical BMD (CtBMDs)

The therapeutic effects of PM1000 and EE on CtBMDs were determined at proximal tibia metaphysis (TM), distal femur metaphysic (FM), tibia diaphysis (TD) and femur diaphysis (FD) as shown in **Figure 4.12**. Treatment of PM1000 significantly increased ($p<0.05$) in CtBMD of TM, FM, TD and FD by 3.05%, 6.78%, 1.50% and 13.46%, respectively, when compared to the OVX_{90} groups. Treatment of EE significantly increased ($p<0.05$) in CtBMD of TM, FM, TD and FD by 3.08%, 4.58%, 1.93% and 11.11%, respectively, when compared to the OVX_{90} groups.

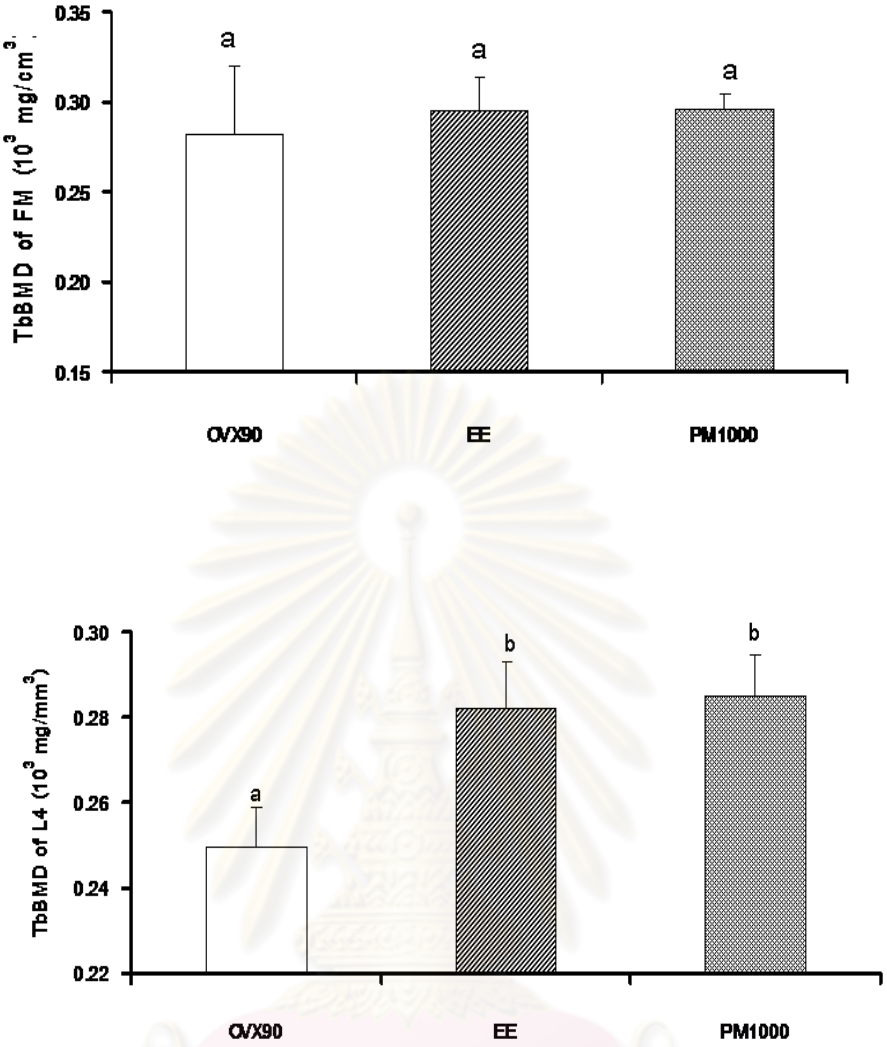


Figure 4.11 The therapeutic effects of 1,000 mg/kg BW/day of *P. mirifica* treatment (PM1000) and 0.1 mg/kg BW/day of esthinylestradiol (EE) on TbBMDs of distal femoral metaphysis (FM) and the fourth lumbar vertebra (L4) in OVX rats after 90 days of ovariectomy. Data are means ± SEM. Means that do not share the same super script letters are significantly different from each other.

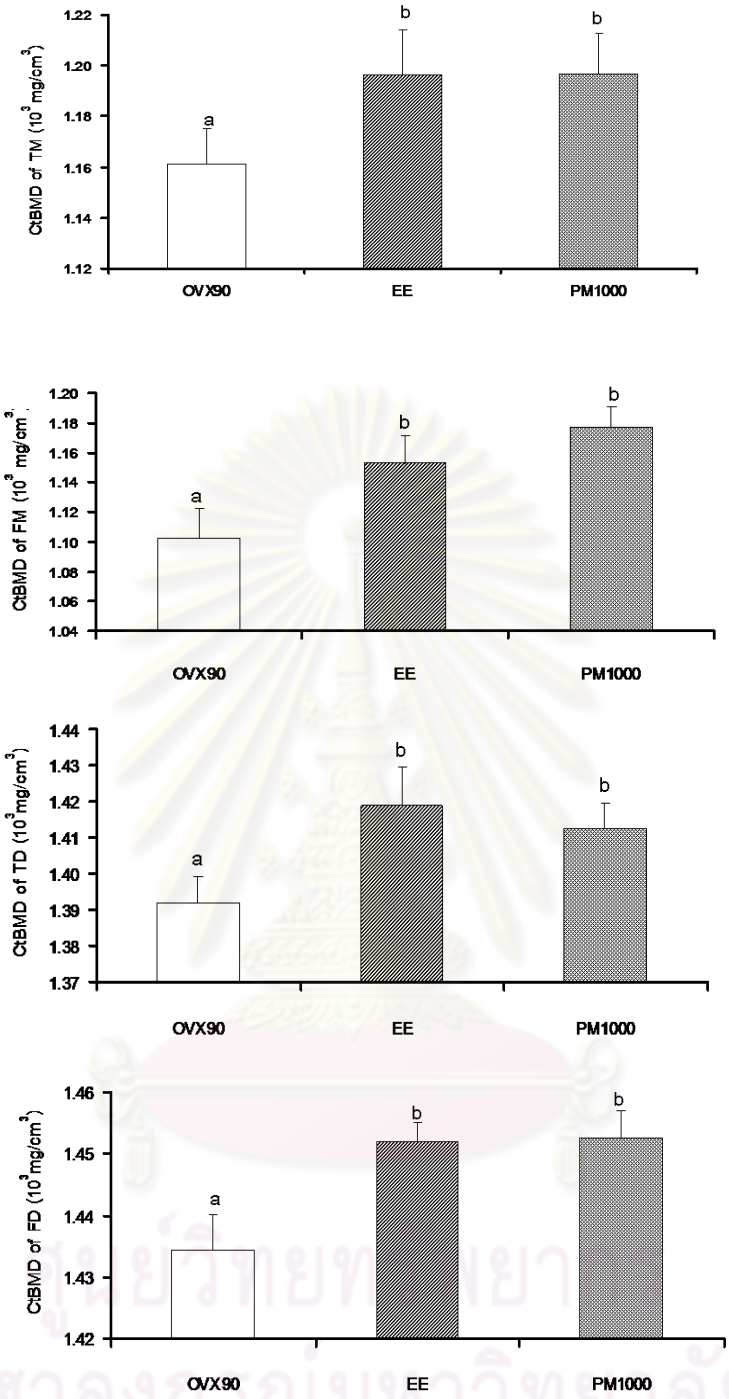


Figure 4.12 The therapeutic effects of 1,000 mg/kg BW/day of *P. mirifica* treatment (PM1000) and 0.1 mg/kg BW/day of esthinylestradiol (EE) on CtBMDs of proximal tibia metaphysis (TM), distal femur metaphysic (FM), tibia diaphysis (TD) and femur diaphysis (FD) in OVX rats after 90 days of ovariectomy. Data are means ± SEM. Means that do not share the same super script letters are significantly different from each other.

2.5. Bone histology after ovariectomy and after EE or *P. mirifica* treatment

Bone histology in SH and OVX rats at 90 and 180 days after surgery are shown in **Figure 4.13**. Histological sections revealed a normal trabecular conformation in SH rats. The metaphyseal region was fully filled with trabecular bones and its connectivity was intervened by small inter-trabecular spaces. After ovariectomy for 90 days, the trabecular bone in OVX rats showed sparse and thinner trabeculare which was resulted in greater inter-trabecular spaces. This confirms the success in the induction of bone loss by ovariectomy in this study. The trabecular bone area in rats after OVX for 180 days was kept being reduced. Treatment of *P. mirifica* and EE could restore the trabecular bone loss. The thicker trabecular was observed in PM100 and PM1000 rats. Bone deterioration caused by OVX was also restored by a 90-day EE treatment.

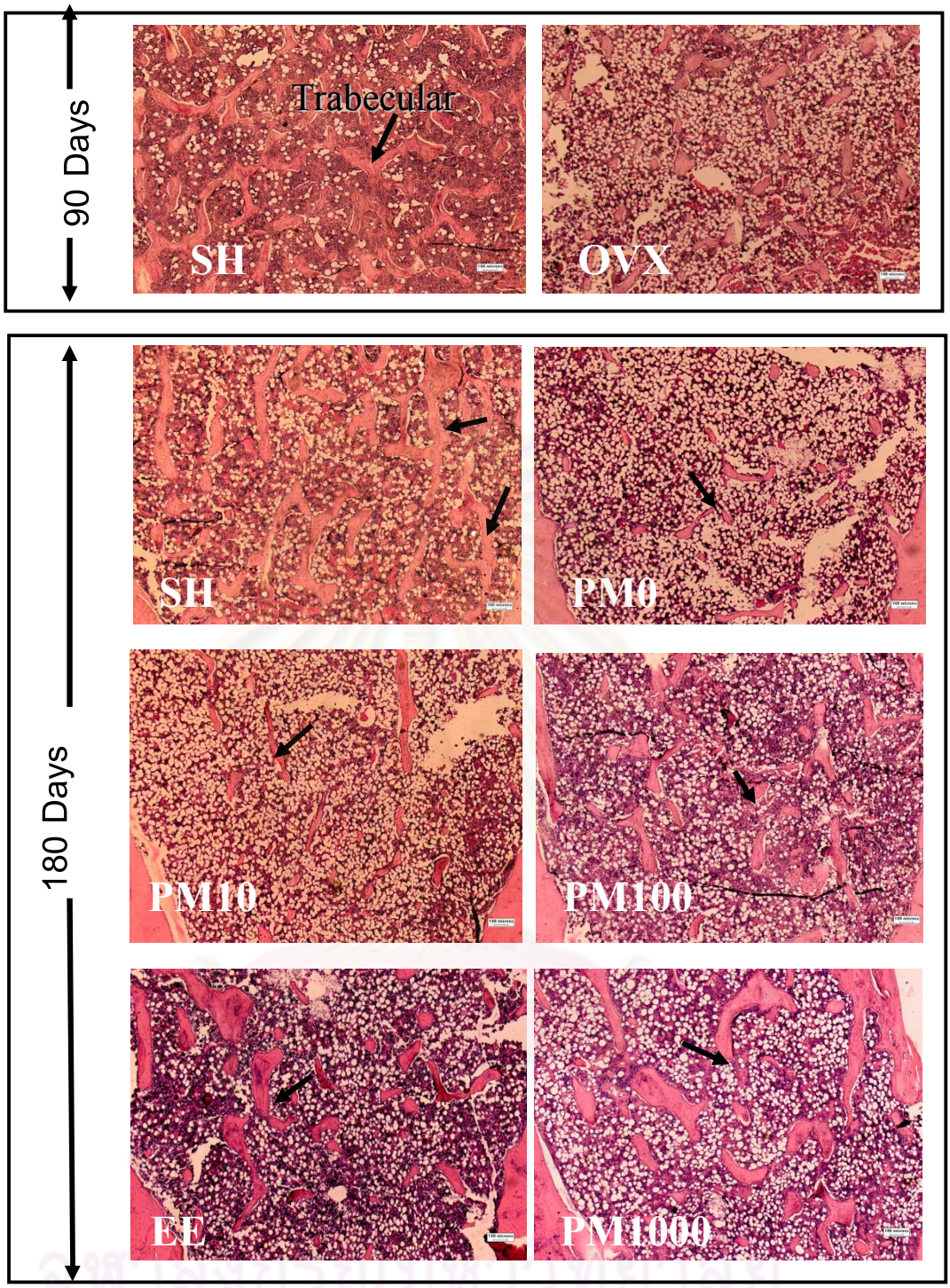
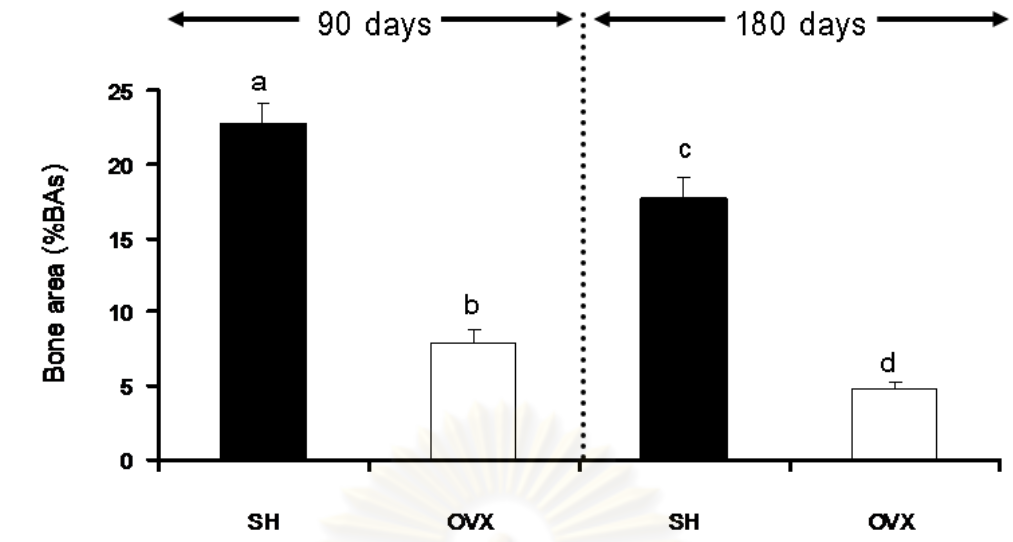


Figure 4.13 Histological section (stained with H&E (50×)) in sham operated rats (SH) and ovariectomized rats (OVX) at 90 and 180 days after surgery, and the 90- day OVX rats treated with 0.1 mg/kg BW/day of esthinylestradiol (EE) or, and *P. mirifica* powder suspension at 0, 10, 100 and 1,000 mg/kg BW/day (PM0, PM10, PM100 and PM1000, respectively) for 90 days.

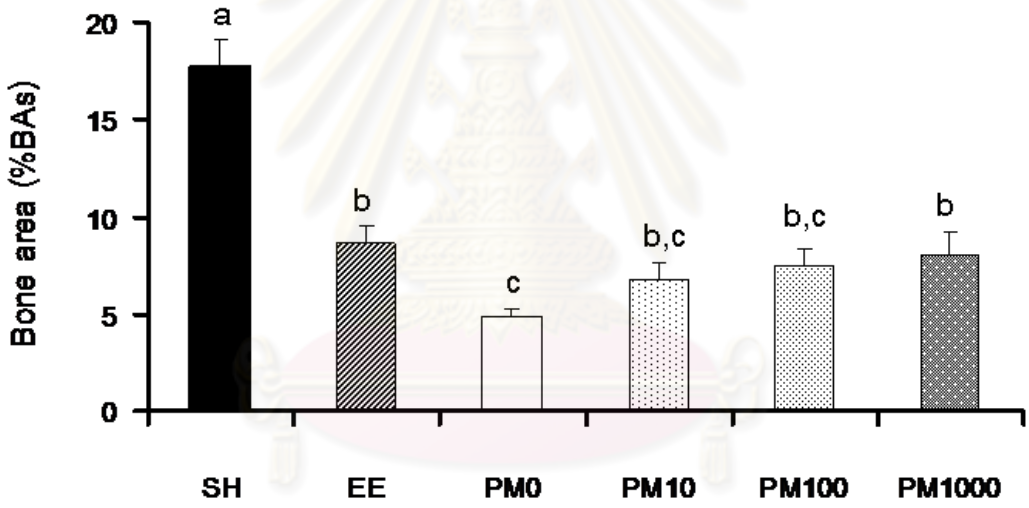
2.6. Changes in trabecular bone area

The % trabecular bone areas (%BAs) in SH and OVX rats at 90 and 180 days after surgery are shown in **Figure 4.15 A.** Comparison between the SH and OVX groups after ovariectomy for 90 and 180 days, the %BA in both OVX groups were highly significantly lower than those of the SH groups (SH_{90} group = 22.79 ± 1.34 and OVX_{90} group = 7.88 ± 0.99 ($p<0.001$), and SH_{180} group 17.71 ± 1.42 and OVX_{180} = 4.83 ± 0.42 ($p<0.001$)) (**Figure 4.14 A**). Increasing in age of rats from 9 months (SH_{90} group) to 12 months (SH_{180} group) old also decreased the %BA by 22.29% ($p<0.01$). Keeping rats in the stage of ovariectomy from 90 days (OVX_{90} group) to 180 days (OVX_{180} group), the %BA also showed a significant decrease by 38.70% ($p<0.05$).

The % trabecular bone areas (%BAs) of the 90-day OVX rats treated EE or PM10, PM100 and PM1000 for 90 days are shown in **Figure 4.14 A**. Treatment of *P. mirifica* to the OVX rats could prevent the progressive reduction of %BA from that of the OVX_{90} group (%BA = 7.88 ± 0.99), especially at the higher doses (%BA = 4.83 ± 0.42, 6.73 ± 0.92, 7.45 ± 0.93 and 8.01 ± 1.27 for PM0, PM10, PM100 and PM1000, respectively). The %BA in the PM1000 group was nearly similar to that of the EE group ($p>0.05$). Moreover, the %BA in PM1000 and EE groups was significantly higher than the PM0 group by 65.8% ($p<0.05$) and 79.46% ($p<0.05$), respectively, and rather higher than that of the OVX_{90} group, by 1.64% and 10.91%, respectively. Thus, feeding of PM1000 and EE to the rats seems to recurrence the %BA. However, the increases in %BA in the PM1000 and EE groups were still lower than that of the SH group by -54.77 % and -51.04 %, respectively ($p<0.001$).



(A)



(B)

Figure 4.14 Percent trabecular bone areas (%BAs) in sham operated rats (SH) and ovariectomized rats (OVX) at 90 and 180 days after surgery (A), and the 90-day OVX rats treated with 0.1 mg/kg BW/day of esthinylestradiol (EE) or *P. mirifica* powder suspension at 0, 10, 100 and 1,000 mg/kg BW/day (PM0, PM10, PM100 and PM1000, respectively) for 90 days (B). Data are means ± SEM. Means that do not share the same super script letters are significantly different from each other.

3. Changes in serum bone markers

3.1. Serum alkaline phosphatase (ALP) levels

On D_0, serum ALP levels were not significant differences between the SH and OVX rats ($p>0.05$) (**Figure 4.15**). Serum ALP levels were significantly increased after ovariectomy over 90 days ($p<0.05$). However, treatment of *P. mirifica* gradually and significantly reduced the serum ALP levels to the SH levels at D_{180}, though it was not depended on doses. At D_{180}, serum ALP levels in PM10 and PM1000 were not different from the SH level ($p>0.05$). The EE treatment could not reduce the increased serum ALP levels, and the levels were kept higher than the SH levels until day 180.

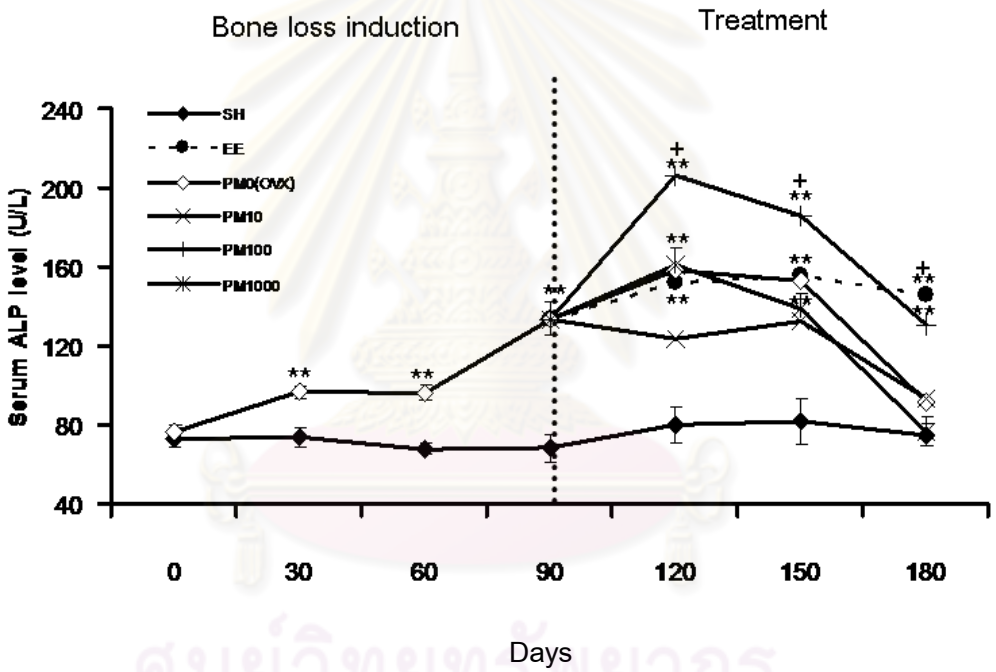


Figure 4.15 Serum alkaline phosphatase (ALP) levels in sham operated rats (SH) and ovariectomized rats (OVX) treated with 0.1 mg/kg BW/day of esthinylestradiol (EE) or *P. mirifica* powder suspension at 0, 10, 100 and 1,000 mg/kg BW/day (PM0, PM10, PM100 and PM1000, respectively) for 90 days. The data are expressed as only the mean because the SE values were overlapped and difficult to understand, except for the SH group. Data are mean ± SEM. ** = $p < 0.01$ compared to the SH group. $^{+}$ = $p < 0.01$ compared to PM0 group.

3.2 Serum tartrate resistant acid phosphatase 5b (TRAP 5b) levels

On D_0, serum TRAP 5b levels were not significant differences between the SH and OVX rats ($p>0.05$). (**Figure 4.16).** After ovariectomy, serum TRAP levels were significantly increased only at D_{30} ($p<0.05$) when compared to the SH group). After *P. mirifica* treatment, serum TRAP levels in PM1000 group significant increased throughout the treatment period ($p< 0.05$), whereas it was significantly increased only on D_{120} and D_{180} for PM10 and PM100 groups when compared to the SH group. Serum TRAP levels in PM0 group were kept consistency throughout the treatment period and not difference from those of the SH group ($p>0.05$)

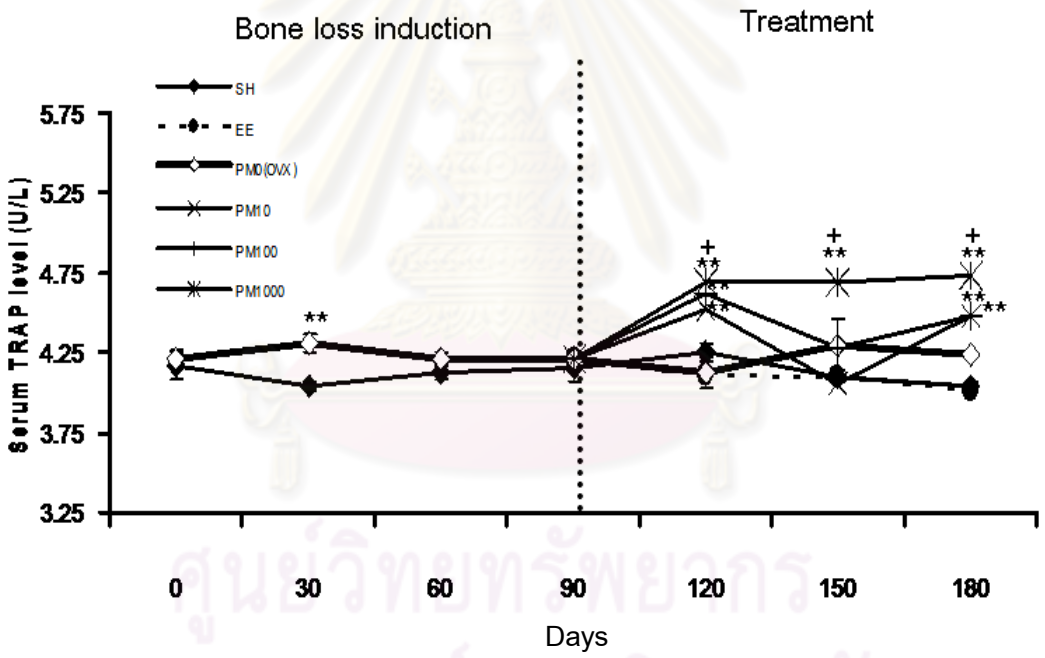


Figure 4.16 Serum tartrate resistant acid phosphatase (TRAP) levels in sham operated rats (SH) and ovariectomized rats (OVX) treated with 0.1 mg/kg BW/day of esthinylestradiol (EE) or *P. mirifica* powder suspension at 0, 10, 100 and 1,000 mg/kg BW/day (PM0, PM10, PM100 and PM1000, respectively) for 90 days. The data are expressed as only the mean, because the SE values were overlapped and difficult to understand, except for the SH group. Data are mean ± SEM. * = $p<0.05$, ** = $p < 0.01$ compared to the SH group. $^{+}$ = $p < 0.01$ compared to PM0 group.

CHAPTER V

DISCUSSION

Recently it was reported that *P. mirifica* could exhibit preventive effects on bone loss in rats (Urasopon *et al*., 2007; 2008a), monkeys (Trisomboon *et al*., 2004) and humans (Manonai *et al*., 2008). *P. mirifica* feeding for 90 days in OVX and ORX rats effectively prevented bone loss in trabecular and cortical compartments at the various sites in axial bone (4^{th} lumbar vertebra) and long bones (tibia and femur) by the determination of BMD and BMC (Urasopon *et al*., 2007; 2008a). *P. mirifica* decreased the serum PTH and calcium levels in postmenopausal cynomolgus monkeys (Trisomboon *et al*., 2004). After 24 weeks of treatment, *P. mirifica* at doses 20, 30 and 50 mg/day significantly decreased serum bone-specific ALP levels in menopausal women compared with the placebo group (Manonai *et al*., 2008). Regarding that the osteoporosis is a silent disease and gradually progressive, most of patients thus are not aware of it and rarely search for the preventive methods. The therapeutics is used when the bone fracture occurs. Therefore, the therapeutic effects of *P. mirifica* on bone loss in osteoporotic rats are carried out in this study. However, prior to the determination of the effects of *P. mirifica* on bone loss in osteoporotic rats, the effects of ovariectomy (90 and 180 days) on the bone loss induction needed to be confirmed.

1. The effects of ovariectomy and *P. mirifica* on body weight

Adipose tissue is highly responsive to estrogen and expresses both ER types (ERα and ERβ) (Pedersen *et al*., 1996; Anwar *et al*., 2001). The phytoestrogens and estrogens have direct effects in energy metabolism (anti-lipogenic effects) on adipose tissue (Naaz *et al*., 2003; Joyner *et al*., 2001). Loss of estrogen after ovariectomy leads to increase in body weight and adipose weight (Butera, 2010; Naaz *et al*., 2003: Picherit *et al*., 2000), and this is prevented or reversed by estrogen replacement (Mohamed *et al*., 2000). Treatment of isoflavone or synthetic estrogen could decrease the weight gain

(Picherit *et al*., 2000; Lin *et al*., 2008). In the present study, the body weight of rats after 90 days of ovariectomy was significantly higher than that of the SH rats, confirming that rats were in the stage of endogenous estrogen deficiency. After 90 days of *P. mirifica* treatment, the body weight gains of the OVX rats could be dose-dependently reversed as also observed in the EE group. The results indicate that phytoestrogens containing in *P. mirifica* exhibited estrogenic effect on rat body weights. In addition, the effects of PM1000 were equal with those of the EE.

2. The effects of ovariectomy and *P. mirifica* on relative uterus weight

Almost every organs of the body could be found both types of ERs (Kuiper *et al*., 1997). However, different tissues have different proportions of the 2 receptor types. ERα is the receptor subtype predominantly expressed in the uterus. It has previously reported that the estradiol mediated increase in uterine weight is solely mediated via ERα (Linberg *et al*., 2002). The uterine weight changes reflect the serum level of endogenous estrogens or estrogens-like substances. In the present study, the body weights of rats were altered after *P. mirifica* and EE treatment, thus the weights of uterus were presented as relative uterus weights.

In this study, 90 days after ovariectomy in OVX rats, the relative uterine weights were significantly decreased in comparison with the SH rats. It indicates that rats were in the stage of endogenous estrogen deficiency (Duncan *et al*., 1999; Kippo *et al*., 1995; Malaivijitnond *et al*., 2004). The administration of EE increased the uterine weight in OVX rats as reported in the previous studies (Narayama *et al*., 2006; Picherit *et al*., 2000). Treatment with PM10, PM100, and PM1000 for 90 days significantly increased relative uterine weights in a dose-dependent manner. In addition, the relative uterine weight in PM1000 group were significantly higher than the SH groups (SH_{90} and SH_{180}). From this result, it can be concluded that use of *P. mirifica* when the effects on bone are needed the undesirable side effects on the reproductive organ should be aware, especially when high doses and long-term consumption are used. Thus, further study is necessary to be conducted to determine the appropriate dose of *P. mirifica* for bone loss therapy in humans.

3. The effects of ovariectomy and *P. mirifica* on bone mineral density, bone mineral content and bone area

3.1 The effects of ovariectomy on BMD and BMC

The OVX rats are determined as a standard model of human menopausal osteoporosis caused by sex hormone deficiency (Ren *et al*., 2007). Most indices of bone mass in cortical and trabecular compartments of longitudinal bone growth of rats are reaching a plateau at 6 to 9 months old. Additionally, age-related decrease in bone mass starts to occur in 12 months old rats (Ke et al., 1996). Thus, rats at 6 months old were selected for ovariectomy and used in the present study. This model is supposed to have no confounding effects by rapid bone growth as found in younger rats or have lesser effect of bone loss caused by aging. The measurement of BMD and BMC in this study was using peripheral Quantitative Computed Tomography (pQCT). The pQCT separates cortical and trabecular bone compartments and thus can monitor metabolic changes very quickly and precisely. In the present study, effects of ovariectomy on bones were found to depend on bone types (axial bone or long bone), bone sites (metaphysis or diaphysis), and bone compartments (trabecular or cortical) and agreed with the previous report (Urasopon *et al*., 2007; 2008a). The decreases in trabecular compartments (BMDs and BMCs) in many bone types were presented clearly within 90 days after ovariectomy but the effects were found to be much smaller in cortical compartments. The decreases in BMDs and BMCs were greater in cortical compartments after ovariectomy for 180 days. This result is in agreement with the previous published reports that the effects of 3 month ovariectomy on BMDs and BMCs were found to be much lesser in cortical compartments than in trabecular compartments (Urasopon *et al*., 2007; 2008a). Thus, it can be concluded that bone loss can occur in the trabecular compartment faster than the cortical compartment. This also confirms that the response of trabecular bones to estrogen deficiency (or ovariectomy) is great in the early phase, while the response of cortical bones is great in the late phase (Thompson

et al., 1995). Following ovariectomy, rapid loss of trabecular bone mass and strength occurs, which then proceed in a less rapid rate in a site-specific fashion to reach steady state of bone mass with an increase in the rate of bone turnover.

Estrogen deficiency affects remodeling in several ways. It increases the activation frequency of BRUs, which leads to a higher bone turnover and induces a remodeling imbalance by prolonging the resorption phase and shortening the formation phase. As a consequence of these changes, the volume of the resorption cavity is increased beyond the capacity of the osteoblasts to refill. In trabecular bone, the extended osteoclast lifespan increases resorption depth, leading to trabecular plate perforation and loss of trabeuular connectivity (Parfit, 2000; Eriksen *et al*., 1999). In cortical bone of the diaphysis of long bones, ovariectomy stimulates periosteal bone growth. On the other hand, the mid-diaphyseal endoosteum in the OVX rat exhibits increased bone resorption, leading to an enlargement in this size of the medullary cavity (Riggs *et al*., 2002). As a results of these changes, cortical bone changes slowly only in the early phase. Nevertheless, there have been several studies indicating that the earliest changes in cortical bone width and medullary cavity size to be between 90-120 days after ovariectomy and reached a steady state at 180 days after ovariectomy or more (Jee and Yao, 2001). Thus, the rat is not a suitable animal model for the study of the effect of ovariectomy on cortical bone because of the lack of Haversian systems, while another limitation is the absence of impaired osteoblast function during the late stage of estrogen deficiency (Wronski and Yen, 1991).

In the present study, developing of the staus of estrogen deficiency in female rats by ovariectomy and kept for 90 days could clearly induce bone loss, especially for the TbBMD. However, when the rats were kept on that status exceeded 90 days, e.g. 180 days, bone loss was gradually progressively occurred and had no significance from that of the 90 days. Thus, the 90-day OVX rats were suitable models for osteoporosis study, and changes of TbBMD should be a good marker.

3.2. The preventive effects of *P. mirifica* on BMD and BMC

The 90 days after the *P. mirifica* treatment, TbBMDs of FM and L4 were higher than those of the PM0 group, but it was lower than those of the SH group. In 2000, Picherit and co–workers studied the effects of soybean isoflavones on osteoporosis in OVX rats. After ovariectomy for 80 days, the OVX rats were treated with soybean isoflavone for 84 days. The results were shown that the BMD and trabecular bone area were greater in isoflavone fed rats than in OVX rats, but it was lower than those of the SH group. In 2006, Devareddy *et al*., examined the effects of isoflavones in the form of soy protein on the improvement of microstructural architecture of trabecular bone which was induced by ovariectomy. After ovariectomy for 90 days, the OVX rats treated with soy protein mixed with isoflavones. After 125 days of treatment, none of the treatment groups showed the improvement of BMD or microarchitectural properties of the lumbar vertebra. However, the BMC and BMD of tibia were increased, though it was lower than of the sham group.

In cortical bone, after treatment of *P. mirifica* for 90 days, the BMDs of TM, FM, L4, TD and FD were higher than those of the PM0 group. Interestingly, the CtBMD of FM in all *P. mirifica* treatment groups were significantly higher than those of the SH and EE groups. This is different from those reported by Urasopon *et al*. (2008a) that feeding of 10-1,000 mg/kg BW/day of *P. mirifica* to the OVX rats could prevent the decrease in CtBMDs of L4 dose-dependently. However, in the ORX rats fed with 10-1,000 mg/kg BW/day of *P. mirifica* could prevent the decrease in CtBMDs of both FM and L4. Similar to this study, treatment of PM1000 to the OVX rats was also higher than the SH rats.

Treatment of *P. mirifica* in OVX rats could rarely obtain the increase in BMCs (both TbBMC and CtBMC), compared to the PM0 group. Surprisingly, increase in TbBMC of FM, and CtBMCs of L4, TD and FD could be observed only in the PM100 group. In contrast, treatment of EE prevented the decrease in CtBMCs of TM and FM, which were not found in the PM100 treatment. In agreement with Urasopon *et al*. (2007; 2008a) reported that *P. mirifica* significantly prevented bone loss in cortical bone of L4, tibia and femur, dose-dependently, in the ORX and OVX rats. The dose of *P. mirifica* on

the prevention of bone loss, which was comparable to the preventive effect of 0.1 mg/kg BW/ day of EE was 100 mg/kg BW/day.

3.3. The therapeutic effects of *P. mirifica* on BMD

From the results gained, bone loss could be observed earlier and greater on BMD than BMC. Thus, changes on BMDs were selected and determined for the therapeutic effects of *P. mirifica* on bone loss. The therapeutic effect of *P. mirifica* on bone loss is considered from a comparison between PM0 (OVX_{90}) and PM1000 groups. In the present study, PM1000 could restore established bone loss in TbBMD of L4 and CtBMD of TM, FM, TD and FD, as seen in the EE group. However, these results disagreed with those of Picherit *et al*. (2000), they reported that consumption of 20-80 mg/kg BW/day of isoflavone for 84 day did not restored BMD in total femur, diaphysis and metaphysis of femur. In 2006, Devareddy *et al*. examined the effects of isoflavones in the form of soy protein on BMC and BMD of 90-day OVX rats and found that the BMC and BMD of tibia were increased, though it was lower than of the sham group. They summarized that soy isoflavone could prevent the further bone loss but did not reverse the established bone loss in osteoporotic rats.

Urasopon *et al*., 2008 analyzed the amount of the major five isoflavones, puerarin, daidzin, daidzein, genistin and genistein, in *P. mirifica*. They found that puerarin accounted for about half of the total isoflavone content in *P. mirifica*. Isoflavones exhibit estrogenic activity by binding at ERs, ERα and ERβ, with a higher binding affinity and expression of ERβ. ERβ is expressed in many tissues including bone, especially at the trabecular bone. Osteoblast, osteoclast and their progenitor cells have ERs. Genistein and daidzein could exhibit estrogenic effects on bone, as an anabolic effect on bone metabolism. Puerarin (10 and 25 μmol/L) significant increased cell growth, increased ALP activity and mineral nodules formation in osteoblast cultures. It can be suggested that puerarin had a stimulatory effect on osteoblast bone formation (Zhang *et al*., 2007). As known that the tuberous root of *P. mirifica* contains at least 22 phytoestrogens (Pope and John, 1960; Chansakaow *et al*., 2000; Ingham *et al*., 2002; Cherdshewasart *et al*., 2007; 2008), the preventive and therapeutic effects of *P. mirifica*

on bone loss may be caused by other phytoestrogens that were not mentioned previously, such as miroestrol and deoxymiroestrol.

3.4. Bone area

The response of bone loss to ovariectomy of female rats was site and compartment specifics. The regions of trabecular compartment responded rapidly to the interventions, while cortical compartment was, as it lacked remodeling, relatively non-responsive (Thompson *et al*., 1995; Bloomfield *et al*., 2002). Thus, the bone sites which have been preferably used as an indicator of bone loss was trabecular area at the proximal tibia metaphysis (Zhang *et al*., 2007; Filipovic *et al*., 2009).

In agreement with previous published reports that the ovariectomy reduced % trabecular bone areas (%BAs) and increased bone marrow cavities of the proximal tibia metaphysis, whilst the estrogen reversed the effects (Zhang *et al*., 2007; Fillipovic *et al*., 2009). The trabecular bone area could also be increased, in a dose-dependent manner, by *P. mirifica* treatment. *P. mirifica* and EE do not only prevent the bone loss (anti-osteoporosis effect), but they also restored the established osteoporosis (anabolic effect) in female rats as seen that the trabecular bone area in PM1000 and EE groups were higher than the OVX_{90} group by 1.64% and 10.91%, respectively. Thus, the use of *P. mirifica* should promise major advances to bone health in postmenopausal women.

4. The effects of ovariectomy and *P. mirifica* on serum bone marker

4.1 Bone formation marker

Alkaline phosphatase (ALP) has been clinically available for several years as a marker for bone formation. Serum ALP consists of several dimeric isoforms that originate from various tissues, such as liver, bone, intestine, spleen, kidney and placenta. In adults with normal liver function, approximately 50% of total ALP activity arises from the liver and 50% arises from the bone. In agreement with the previous published reports, the ovariectomy increased serum ALP levels in the present study starting from D_{30}

whereas the levels were stable in the SH rats throughout the study period (Li and YU, 2003; Lee *et al*., 2004; Shirke *et al*., 2009). It suggests then that the OVX rats have increased bone turnover (Shirke *et al*., 2009). Controversial results of the effect of estrogen and isoflavone on serum ALP in OVX rats have also been reported, where they either cause an increase or decrease in this enzyme serum level. Although the effects of *P. mirifica* on serum ALP levels in OVX rats in this study were inconsistent, and did not display a linear or simple, if any, dose-dependency, the serum ALP levels in PM100 and PM1000 rats were higher than those in the PM0 and SH rats at D_{120} and D_{150}. In agreement with previous reported, rats fed on the soy diet were reported to show a further elevation in serum ALP activity (Arjanmandi *et al*.,1998). Genistein, coumestrol and daidzein are known to stimulate ALP activity of an osteoblast –like cell line, MC3T3-E1 cells (Kanno *et al*., 2004).

4.2 Bone resorption marker

High amount of tartrate resistant acid phosphatase 5 b (TRAP 5b) is expressed by bone - resorbing osteoclasts. Osteoclast secreted TRAP 5b into the blood circulation. Serum TRAP 5b was elevated in patients with bone diseases (Halleen *et al*., 2000; Chu et al., 2003; Mose *et al* 2003), and decrease after antiresorptive treatment (Koizumi *et al*., 2003; Voskaridou *et al*., 2003). In rats, after ovariectomy for 3 months serum TRAP levels were significantly increased (Songlin *et al*., 2009). In the present study, serum TRAP levels were significantly increased after ovariectomy for 30 days. The ovariectomy causes a rapid bone loss and develops an osteoporosis due to estrogen withdrawal (Urasopon *et al*., 2008b). Changes (or increases) in serum TRAP levels in the OVX model could be detected within only the first week after ovariectomy, because it reduced to the control level thereafter (Surve *et al*., 2001). The elevated serum TRAP levels can used to predict a future bone loss (Alatalo *et al*., 2003).

After *P. mirifica* treatment, serum TRAP levels were significant and consistent increased in PM1000 group. However, in the EE group, serum TRAP levels were kept low throughout the treatment period. Thus, it seems to be that *P. mirifica* and EE exert on different pathways on bone loss prevention and therapy. Based upon the results gained

in the present study, it is still difficult to explain how EE and *P. mirifica* affect on bone cells. Thus, the further determination of the other biochemical markers of bone resorption, e.g. sialoprotein or pyridinoline cross-linking telopeptides, should be done.

Based on these results it is possible, however, to conclude that *P. mirifica* consumption could prevent bone loss (anti-osteoporotic effect) and restore the established osteoporosis (anabolic effect) in female rats. At present, the only available anabolic agent that stimulates osteoblasts to form bone that is sold in the pharmaceutical market is PTH (Ng, 2009). However, the PTH has undesirable side effects, including nausea, vomiting, headache, leg cramps and dizziness, and, most importantly, it is very expensive. Thus, PTH treatment is reserved for patients with severe osteoporosis who are unable to take other medications or for whom other medications are not effective (Ng, 2009). Regarding the results presented here, *P. mirifica* might be a candidate of choice, because *P. mirifica* also confers beneficial effects in terms of the reduction of tumorigenesis and tumor growth (Cherdshewasart *et al*., 2007). The further study on the appropriate doses of *P. mirifica* for bone loss therapy and did not have the side effects on the other organs should be investigated.

CHAPTER VI

CONCLUSION

From this study, it can be concluded the findings as follows;

1. Developing of estrogen deficiency status for 90 days by ovariectomy in female rats could remarkably induce bone loss, especially for the TbBMD, bone loss occurred by 20-37%. However, when the rats were kept on that status exceeded 90 days, that is 180 days in this study, bone loss was gradually progressively and had no significant difference from D_{90}, bone loss occurred by 13-42%. Thus, the 90-day OVX rats were the most suitable models for osteoporosis study.

2. Bone loss could be observed earlier and greater on trabecular compartment (Tb) than on cortical compartment (Ct), and on bone mineral density (BMD) than bone mineral content (BMC) as follows; Tb**BMD>** Ct**BMD**> Tb**BMC**> Ct**BMC**, respectively. Thus, changes on TbBMD should be a good marker on osteoporosis study.

3. Using changes of body weight gain after ovariectomy or *P. mirifica* treatment, in addition to changes of uterus weights, should be an alternative indicator of the estrogen deficiency follow-up in rats.

4. *P. mirifica* consumption could improve a micro-architectural deterioration of bone tissue in rats.

5. *P. mirifica* could prevent a further bone loss (anti-osteoporotic effect) and restored the established osteoporosis (anabolic effect) in ovariectomy-induced osteoporotic rats.

6. The mechanisms of actions of *P. mirifica* on bone cells should be different from those of synthetic estrogens, especially for bone loss prevention.

7. *P. mirifica* should be one of alternative choices for osteoporosis treatment in humans, because it distributes throughout Thailand, is cheap and has no side effects on cancers. This should be a value-added to *P. mirifica* on the commercial scale which is popularly used for cosmetics and food supplements nowadays.

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APPENDIX

Chemicals

1. The list of chemical substance used in this study were given below;

Chemical	Company
17α-ethinylestradiol	Sigma, USA
EDTA, disodium salt	Merck, Germany
Diethyl ether	Sigma, USA
Formaldehyde	Merck, Germany
Normal saline	Thainakornpatana Co., Ltd., Thailand
Sodium Hydroxide (NaOH)	Merck, Germany

2. Composition of 10% phosphate buffered formalin

1. Formaldehyde	100	ml
2. Distilled water	900	ml
3. Sodium phosphate monobasic (NaH_2PO_4)	4.0	g
4. Sodium phosphate dibasic (Na_2HPO_4)	6.5	g

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