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ของหนูเมาส์

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ESTROGENIC ACTIVITY AND MECHANISM OF ACTION OF PUERARIN PHYTOESTROGEN ON MOUSE OSTEOCLAST

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A Thesis Submitted in Partial Fulfillment of the Requirements
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Thesis Title	ESTROGENIC ACTIVITY AND MECHANISM OF ACTION OF PUERARIN PHYTOESTROGEN ON MOUSE OSTEOCLAST
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Field of Study	Zoology
Thesis Advisor	Professor Suchinda Malaivijitnond, Ph.D.
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งานวิจัยนี้มีวัตถุประสงค์เพื่อศึกษาผลและกลไกการออกฤทธิ์ของสารสกัดกวาวเครือขาว *Pueraria mirifica* (PME) และพูราริน (PU) ต่อเซลล์สลายกระดูกของหนูเมาส์และปฏิสัมพันธ์ระหว่างเซลล์สร้างกระดูกและเซลล์สลายกระดูก แบ่งการทดลองออกเป็น 3 ชุด คือ การทดลองที่ 1 ศึกษาผลของ PME และ PU ต่อการต้านการสร้างเซลล์สลายกระดูก บ่มเซลล์ต้นกำเนิดเซลล์สลายกระดูก (BMMs) ของหนูเมาส์ ร่วมกับ 0.3% DMSO, PME ที่ความเข้มข้น 1, 5 และ 10 มก./มล., PU ที่ความเข้มข้น 0.1, 10 และ 1000 นาโนโมลาร์, และ E_2 ที่ความเข้มข้น 10 นาโนโมลาร์ เป็นเวลา 24 ชม. วัดการเพิ่มจำนวนเซลล์ การเจริญของเซลล์ การรวมตัวของเซลล์ และการกัดกร่อนกระดูก พบว่า PME และ PU ยับยั้งการเจริญของ BMMs ไปเป็นเซลล์สลายกระดูกวัยอ่อนและการรวมตัวของเซลล์สลายกระดูกวัยอ่อนไปเป็นเซลล์สลายกระดูกเจริญวัยจากการชักนำของแรงค์ไลแก้น (RANKL) โดย PME ที่ความเข้มข้นต่างกันออกฤทธิ์ต่างกัน นั่นคือ PME ที่ความเข้มข้นต่ำ (1 มก./มล.) ยับยั้งการรวมตัวของเซลล์สลายกระดูกวัยอ่อน ในขณะที่ PME ที่ความเข้มข้นสูง (10 มก./มล.) ยับยั้งการเจริญของ BMMs ไปเป็นเซลล์สลายกระดูกวัยอ่อน และลดระดับเอ็มอาร์เอ็นเอของยีน *Nfatc1* และ *Tm7sf4* นอกจากนี้ยังพบว่า PME ที่ความเข้มข้นต่ำและ PU ออกฤทธิ์ผ่านตัวรับเอสโทรเจน (ERs) ในขณะที่ PME ที่ความเข้มข้นสูงออกฤทธิ์ผ่านวิถีอื่นด้วยเช่นกัน การทดลองที่ 2 ศึกษาผลของ PME และ PU ต่อการชักนำให้เกิดการตายของเซลล์สลายกระดูก บ่ม BMMs ร่วมกับ 0.3% DMSO, E_2 ที่ความเข้มข้น 10 นาโนโมลาร์, PME ที่ความเข้มข้น 1 และ 10 มก./มล., และ PU ที่ความเข้มข้น 1000 นาโนโมลาร์ เป็นเวลา 72 ชม. จากนั้นตรวจสอบการตายของเซลล์ พบว่าเฉพาะ PME เท่านั้นที่ชักนำให้เกิดการตายของ BMMs ในขณะที่ทั้งสามกลุ่มการทดลองมีการลดลงของระดับเอ็มอาร์เอ็นเอของยีน *FasL* เมื่อให้สารนาน 24 ชม. การทดลองที่ 3 ศึกษาผลของ PME และ PU ต่อการสื่อสารระหว่างเซลล์สร้างและสลายกระดูก ในสภาวะที่มีการเลี้ยงเซลล์แบบเดี่ยว (เซลล์ต้นกำเนิดเซลล์สร้างกระดูก (ST2) หรือ BMMs อย่างใดอย่างหนึ่ง) และในสภาวะที่มีการเลี้ยงเซลล์สองชนิดร่วมกัน บ่มเซลล์ร่วมกับ 0.025% DMSO, E_2 ที่ความเข้มข้น 10 นาโนโมลาร์, PME ที่ความเข้มข้น 1 มก./มล., และ PU ที่ความเข้มข้น 1000 นาโนโมลาร์ พบว่าเมื่อเลี้ยงเซลล์ ST2 เพียงลำพัง PME ลดการแสดงออกของยีน *Rankl* ส่งผลให้อัตราการแสดงออกระหว่างยีนแรงค์ไลแก้น/ออสทีโอโปรทีจีริน (*Rankl/Opg*) ลดลง อย่างไรก็ตาม PME, PU และ E_2 ไม่มีผลต่อการแสดงออกของยีน *Rankl, Opg, Rank*, *Opg*, *EphB4* และ *EfnB2* เมื่อบ่มเซลล์ BMMs เพียงลำพังหรือเมื่อบ่มเซลล์ทั้งสองชนิดร่วมกัน จากงานวิจัยนี้สรุปได้ว่า PME และ PU สามารถออกฤทธิ์โดยตรงต่อเซลล์สลายกระดูกในการต้านการเจริญและการทำงานของเซลล์สลายกระดูกและออกฤทธิ์ทางอ้อมผ่านเซลล์สร้างกระดูกต่อการส่งสัญญาณ RANKL/OPG/RANK ผลการวิจัยในครั้งนี้นอกจากจะเป็นการเพิ่มองค์ความรู้และความเข้าใจถึงการออกฤทธิ์ของ PME และ PU ต่อเซลล์กระดูกแล้ว ยังตอกย้ำถึงศักยภาพของ PME และ PU ในการพัฒนาไปเป็นยาเพื่อใช้ในการรักษาผู้ป่วยโรคกระดูกพรุนอีกด้วย

ภาควิชา	ชีววิทยา	ลายมือชื่อนิสิต .....
สาขาวิชา	สัตววิทยา	ลายมือชื่อ อ.ที่ปรึกษาหลัก .....
ปีการศึกษา	2558	ลายมือชื่อ อ.ที่ปรึกษาร่วม .....

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KEYWORDS: PUERARIA MIRIFICA, OSTEOPOROSIS, OSTEOCLAST, OSTEOBLAST, CROSS-TALK

SAROCHA SUTHON: ESTROGENIC ACTIVITY AND MECHANISM OF ACTION OF PUERARIN PHYTOESTROGEN ON MOUSE OSTEOCLAST. ADVISOR: PROF. SUCHINDA MALAIVIJITNOND, Ph.D., CO-ADVISOR: PROF. KOICHI MATSUO, M.D., Ph.D., 86 pp.

The effects and mechanisms of action of *Pueraria mirifica* extract (PME) and puerarin (PU) on primary mouse osteoclasts and cross-talk between osteoblasts and osteoclasts were investigated. Three experimental series were set up. Experiment I: to study the effects of PME and PU on anti-osteoclastogenesis, mouse bone marrow macrophages (BMMs) were incubated with 0.3% DMSO, 1, 5 and 10 mg/ml PME, 0.1, 10 and 1000 nM PU, and 10 nM E_2 for 24 h, and cell proliferation, differentiation, fusion and resorptive function were determined. PME and PU suppressed the RANKL-induced differentiation of BMMs into pre-osteoclasts and the fusion of pre-osteoclasts to multi-nucleated mature osteoclasts. The PME showed a differential dose-response effect, where a low PME dose (1 mg/ml) suppressed the fusion stage of mononuclear pre-osteoclast and a high PME dose (10 mg/ml) suppressed the differentiation from BMMs to pre-osteoclasts and decreased mRNA expression levels of *Nfatc1* and *Tm7sf4*. The mechanisms of the low PME dose and PU were passed through estrogen receptors (ERs). Nevertheless, the anti-osteoclastogenic activity of a high PME dose was not significantly suppressed by ER antagonists, suggesting that PME might act via one or more alternative pathway(s). Experiment II: to study the effects of PME and PU on induction of osteoclast apoptosis, BMMs were treated with 0.3% DMSO, 10 nM E_2, 1 and 10 mg/ml PME and 1000 nM PU for 72h, and cell apoptosis was determined. PME, but not PU and E_2, induced apoptosis in mouse BMMs-preosteoclasts, while *FasL* mRNA expression levels were decreased after 24-h exposure to all three treatments. Experiment III: to study the effects of PME and PU on the cross-talk between osteoblasts and osteoclasts, each single-cell type culture (either mouse osteoblast ST2 precursors or BMMs) and a two-cell type coculture condition were conducted. Cells were treated with 0.025% DMSO, 10 nM E_2, 1 mg/ml PME and 1000 nM PU. Exposure of ST2 cells alone with the PME reduced *Rankl* expression, which led to a significant decrease in *Rankl/Opg* ratio. However, exposure of BMMs alone or two-cell type coculture with PME, PU and E_2 had no effects on expression of *Rankl, Opg*, *Rankl/Opg* ratio, *Rank*, *EphB4* and *EfnB2*. In conclusion, PME and PU elicit the anti-osteoclastogenic activity of both directly on osteoclasts and indirectly via the regulation of osteoblasts through RANKL/OPG/RANK signal This study increases the knowledge and understanding of the mode of action of PME and PU on bone cells which corroborates the high potential of PME and PU to be developed as anti-resorptive agents for osteoporotic patients.

Department:	Biology	Student's Signature	.....
Field of Study:	Zoology	Advisor's Signature	.....
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CHAPTER I
GENERAL INTRODUCTION

Osteoporosis is the most common metabolic bone disease in human of which bone mass depletion and bone microarchitecture deterioration enhance risk of bone fractures. World Health Organization (WHO) states the definition of osteoporosis as their bone mineral density (BMD) is less than or equal to -2.5 standard deviation (SD) of the average of those of the young adults. As osteoporosis associates with age, the number of osteoporotic patients aged over 50 years is expected to increase to nearly 14 million by 2020 while the other 47 million people will be the low bone mass. Comparing between elderly men and women, women have a higher possibility to be osteoporosis and osteoporotic-induced bone fracture than the men. About 40% of over 50 years old women have a fracture risk of spine, wrist or hip and the number of hip fracture patients have been extrapolated to be 6.3 million by 2050 (Cole et al., 2008). Thus, osteoporosis has been concerned as a major public health problem and the prevalence is continuously increased all over the world as the expansion of life expectancy of world population (Gass and Dawson-Hughes, 2006; Shuler et al., 2012).

Since women are in the higher risk of osteoporosis than men, especailly when the women enter into the post-menopausal peroid, thus estrogens might be a major player of the osteoporosis induction. Estrogen is defined as the regulator hormone to control bone remodeling in both women and men (Khosla et al., 2012). Bone is a dynamic organ where the bone remodeling happens throughout the animals' life. This self-generation process begins with the elimination of bone microarchitecture damage then the replacement of old bone with new bone at the same site which is continuously modulated by the functions of osteoclasts (or bone resorbing cells) and osteoblasts (or bone forming cells), respectively (Raggatt and Partridge, 2010). Estrogen deficiency causes an impaired function of bone resorption and bone formation and then leads to the pathology of osteoporosis. There are two classes of

the osteoporotic therapeutic strategies; anti-resorptive agents which suppress osteoclast function and anabolic agents which activate osteoblast function. Anti-resorptive therapies are widespread used and divided to 5 groups which are estrogens, selective estrogen receptor modulators (SERMs), bisphosphonates, calcitonin, and monoclonal antibodies (Chen and Sambrook, 2012). Since 85% of the osteoporotic patients are post-menopausal women, estrogen replacement therapy is effective to prevent or remedy the symptom (Ettinger et al., 1985). However, since the long-term use of synthetic estrogen activates the developing of breast and endothelium canners as well as other adverse effects such as cardiovascular and metabolic diseases (Rachner et al., 2011), the natural products which show the least side effects and have similar activity with that of estrogens have attracted considerable attention (Glazier and Bowman, 2001).

Pueraria mirifica or white Kwao Krua is an endemic Thai herbal plant. Its tuberous roots have been used for folklore medicine for several decades. The result of high performance liquid chromatography (HPLC) shows that the extract of *P. mirifica*'s roots contain at least 17 phytoestrogenic substances and puerarin which can only be isolated from *Pueraria* plant is the highest phytoestrogen (Malaivijitnond, 2012). Previous studies denoted that *P. mirifica* could exhibit estrogenic activity on reproductive organs of mice (Jaroenporn et al., 2006), rats (Malaivijitnond, 2004) and monkeys (Trisomboon et al., 2007). The estrogenic effects of *P. mirifica* and puerarin on bone have also been assessed both *in vivo* and *in vitro*. The study in gonadectomy-induced osteoporotic rats shows that *P. mirifica* could prevent as well as restrain BMD and bone mineral content (BMC) of rats (Urasopon et al., 2007, 2008; Suthon et al., 2016). The underlying mechanisms of action were that *P. mirifica* and puerarin stimulated osteoblast function as indicated by an increased alkaline phosphatase (Alp) mRNA expression, and suppressed osteoclast differentiation through decrease in the ratio of receptor activator of nuclear factor κB ligand/osteoprotegerin (RANKL/OPG) in rat osteoblast-like UMR106 and primary baboon osteoblasts (Tiyasatkulkovit et al., 2012, 2014).

The anti-osteoporotic effects of *P. mirifica* were evident in estrogen deficient-induced osteoporotic rats (Urasopon et al., 2007, 2008; Suthon et al., 2016) as well as rat UMP106 osteosarcoma cells and baboon primary osteoblasts (Tiyasatkulkovit et al., 2012, 2014). However, during bone remodeling process, osteoblasts and osteoclasts work together in order to keep the balance of the remodeling, the understanding of the estrogenic effect of *P. mirifica* extract and its phytoestrogens should also be performed in osteoclasts. Generally, osteoclasts, osteoblasts and also osteocytes coordinately communicated together all the time. While osteoclasts resorb the damaged bone, they produce the coupling factors, especially membrane-bound molecule ephrinB2 ligand which binds with EphB4 tyrosine kinase receptor (EphB4) on osteoblast surface then the signal induces osteoblast differentiation but suppresses osteoclast function. Likewise, osteoblasts and osteocytes regulate osteoclasts by producing RANKL and OPG, the decoy protein for RANKL, to activate and inhibit osteoclast differentiation, respectively (Matsuo, 2009). Furthermore, osteoblasts also secrete Fas ligand (FasL), a pro-apoptotic molecule, to control osteoclast lifespan (Krum et al., 2008a; Wang et al., 2015). The balance between bone resorption and bone formation sustains the constancy of bone mass and mineral homeostasis. Thus, to study the effects of *P. mirifica* extract on each osteoblast or osteoclast cell type in the cell culture condition is not truly mimic the real situation within the bone and the bone remodeling process. This study investigates the estrogenic efficacy and mechanisms of actions of *P. mirifica* extract and puerarin on primary mouse osteoclasts and on the interaction between osteoclasts and osteoblasts in the coculture condition. The 17β-estradiol is used as a positive control to compare the mechanisms of actions with those of the *P. mirifica* extract and puerarin.

OBJECTIVES

1. To determine if and how *P. mirifica* and puerarin phytoestrogen exhibit estrogenic effect on primary mouse osteoclastogenesis *in vitro*
2. To determine if *P. mirifica* and puerarin phytoestrogen induce apoptosis in primary mouse osteoclast *in vitro*
3. To determine if *P. mirifica* and puerarin phytoestrogen mediate the communication between primary mouse osteoclast and osteoblast-precursor ST2 mouse cell *in vitro*

CHAPTER II
LITERATURE REVIEWS

This chapter firstly summarizes the basic knowledge of bone biology including bone structure, bone cells, bone remodeling, its mechanism of action and osteoporosis. Then, estrogens are reviewed in part of the structures, receptors and mechanisms of actions as well as the function on differentiation of bone forming cells (osteoblastogenesis) and bone resorbing cells (osteoclastogenesis). Finally, the information of *P. mirifica*, and its phytoestrogen substances, estrogenic activity and effects on bone are described.

1. Bone biology

Bone is endoskeleton of vertebrates required for multifunction such as mechanical support and protection, locomotion, hematopoiesis, endocrine system and mineral homeostasis. Its hierarchical structure manages from nanometer to macroscopic scale which shows a unique morphology. The synchronized action of bone cells, calling basic multicellular unit (BMU), is to maintain the bone function, skeleton mass and mineral homeostasis.

1.1. Bone structure

Bone structure organizes in hierarchy (Figure 2.1); the macroscopic structure consists of 2 parts, cortical and trabecular (spongy or cancellous) bone which are distinguished by the porosity, location and function. Cortical bone is the dense bone which forms the outside shell of the bone in order to encircle the bone marrow cavity and to carry the mechanical loading. Harversian system or osteon is the functional unit of cortical bone. It is composed of lamellae that surround Haversian canal at the center. Between each layer of lamellae is the empty space calling lacunae which fill up with bone cells and bone matrix. Lacunae are connected by canaliculi, a microscopic canal. Each osteon connects by perforating or Volkmann canals that lead to the establishment of the network of blood vessel throughout this bone composite. For trabecular bone, it is a plate-like loose bone dividing into broader plates and thinner rods. Both structures connect together within the marrow cavity (Figure 2.2). The trabecular architecture is important on supporting the major stresses within bone even if it comprises only 25-30% of the total bone volume (Burr and Akkus, 2014).

At nanostructure, bone is composed of 3 major fundamental components; minerals, organics and water. Minerals are the main components which take about 65% of the total bone. Calcium phosphate and calcium carbonate are the major minerals in bone which form the mineral plates, called hydroxyapatite crystal in the formula of $Ca_{10}(PO_4)_6(OH)_2$. Hydroxyapatite plays a role for bone strength to resist with numerous pressures. Moreover, hydroxyapatite can release soluble form of bicarbonate (HCO^-_3), calcium ion (Ca^{2+}) and phosphate ion (P_i) into the blood circulation and maintain mineral homeostasis. Organics are about 25% of the total bone dividing into collagens and noncollagenous proteins. Type I collagen is the highest proportion of organic protein of bone (almost 90% of organics). Type I collagen fiber is built from the 2 α1-chains and 1 α2-chain which form triple helix structures and then are assembled to the fibers. The fiber orientations are identical in the sense that they have holes between the ends of each fiber, so-call D-spacing patter. This cavity is for hydroxyapatite deposition which then produces bone

elasticity together with strength. Noncollagenous proteins are only 10% of the organic phase of bone. It consists of 15% of the cell proteins (proteoglycans, glycoproteins, hyarulonan and serum proteins) and 85% of extracellular matrix (ECM) which plays a major role on cell proliferation, size of collagen fibers, mineralization and cellular signaling. Water is important for bone in the sense of the link between hydroxyapatite and collagen which helps the bone not too stiffness and easily fracture although water is only 10% of the total bone volume. The hierarchical structure of bone is generally organized by bone cells including osteoclasts, osteoblasts and osteocytes (Burr and Akkus, 2014).

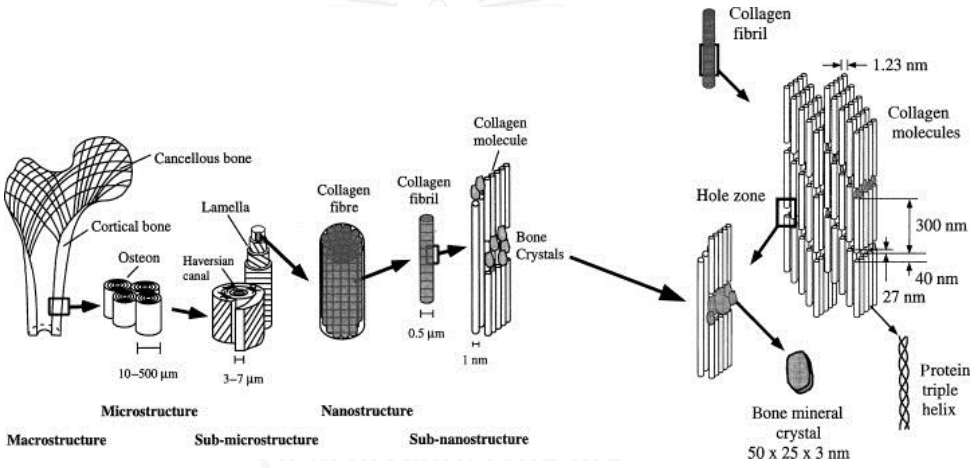

Figure 2.1 Hierarchical structural organization of bone. Bone comprises of several levels from the biggest to the smallest scale ordering as macrostructure, microstructure, sub-microstructure, nanostructure and sub-nanostructure, repectively. The fundamental components of bone are collagen molecules and hydroxyapatite crystal. (Ritchie et al., 2005)

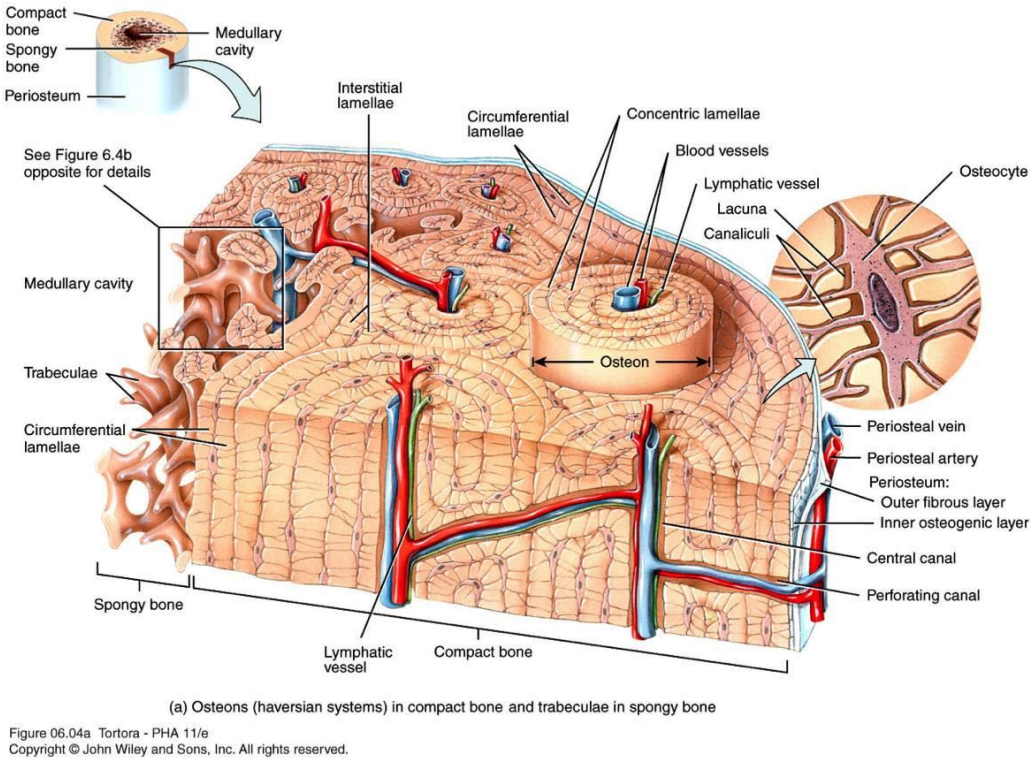

Figure 2.2 The macroscropic structure of bone. Cortical (compact) bone consists of many osteons which are linked together by Volkmann (perforating) canals. Osteocytes embed in lacuna and penetrate their processes along canaliculi. Trabecular (spongy) bone connects within the marrow cavity. (Available in: https://classconnection.s3.amazonaws.com/249/flashcards/186249/png/compacbone-1439695648A33A4B727.png)

1.2. Bone cells: the developments and functions

1.2.1. Osteoclasts

Osteoclasts are giant multinucleated cells functioning to remove mineralized bone matrix. Osteoclastogenesis undergoes from the hematopoietic stem cells expressing c-Fms receptor, which binds with macrophage colony stimulating factor (M-CSF), and commit to osteoclast precursors. M-CSF supports proliferation and survival of the precursor cells and also prepares the cells to be the mature osteoclasts by stimulating the expression of receptor activator of nuclear factor κB (RANK). RANK can bind with RANKL secreted by osteoblasts and be activated. However, osteoblasts also secrete OPG which acts as a soluble decoy receptor for RANKL and competes RANK to bind with RANKL which then suppresses osteoclastogenesis. Thus, RANKL/OPG expression ratio is indicator for osteoclast differentiation and function (Raggatt and Partridge, 2010)

Since osteoclasts characterized as multinucleated tartrate-resistance acid phosphatase (TRAP) and calcitonin receptor expressed cells, osteoclast differentiation consists of 2 subsequential steps; differentiation of monocyte/macrophage precursor cells to preosteoclasts and forming of multinucleated cells to mature osteoclasts, respectively (Figure 2.3B). The mechanism of RANKL-induced osteoclastogenesis initiates with the activation of mitogen-activated protein kinases (MAPKs), including Jun N-terminal kinase (JNK) and p38, which leads to the translocation of the transcription factors nuclear factor κB (NF-κB) to the nucleus. Then, the nuclear factor of activated T-cells cytoplasmic 1 (NFATc1), an osteoclast mater transcription protein, starts the autoamplification and induces the expression of osteoclast-specific genes such as *TRAP*, *Tm7sf4*, *calcitonin receptor*, *cathepsin K*, and *β3-integrin* genes (Asagiri and Takanayagi, 2007). As mature osteoclasts are created by the fusion of preosteoclasts, dendritic cell-specific transmembrane protein (DC-STAMP) encoded by *Tm7sf4* is necessary for multinucleation process. As seen in *Tm7sf4*$^{-/-}$ mice, they showed a complete abrogation of osteoclast fusion and an osteopetrosis (Yagi et al., 2005).

At the terminal stage of the differentiation, osteoclasts adhere on bone surface and form two unique membrane structures, including sealing zone and raffled border, then become polarization. Sealing zone is an actin ring-like structure creating to provide resorbed microenvironment and separating the area from general extracellular matrix. Bone resorption occurs at raffled border, a villous-like membrane locating around the middle bottom of cells, by acidification and proteolysis the hydroxyapatite and organic composition of bone, respectively (Oikawa et al., 2012).

1.2.2. Osteoblasts

Osteoblasts are mononuclear cells functioning for new bone formation and also regulating osteoclasts. As osteoblastic linage consists of pluripotent mesenchymal stem cells (MSCs), osteoprogenitor cells, preosteoblasts, osteoblasts, bone lining cells and osteocytes, the regulation of expression of osteoblastogenic genes is different in each stage (Figure 2.3A). Runt-related transcription factor 2 (RUNX2) or core binding factor-1 (Cbfa1) is an osteoblast master transcription factor controlling the commitment of MSCs to ostoprogenitor cells, preosteoblasts and subsequently osteoblasts (Marie, 2008). RUNX2 binds at the promoter of the osteoblastogenic genes that are *Alp*, *type I collagen* (*Col I*), *osteopontin* (*Opn*) and *osteocalcin* (*Ocn*) which indicate the osteoblast characteristics. *Runx2*$^{-/-}$ mice were reportedly completely absent of mineralization at the embryonic of day 15.5. This suggests that RUNX2 is necessary for ossification and skeletogenesis (Takarada et al., 2013). The osteogenic factors such as bone morphogenetic proteins (BMPs), parathyroid hormone (PTH), 1,25-dihydroxyvitamin-D_3 (Vit D_3), insulin-like growth factors (IGFs) and loading stress activate RUNX2 through stimulating the wingless-type MMTV integration site family (Wnt) signaling pathway. Osterix (Osx) is one of the RUNX2 downstream transcriptional factors which supports RUNX2 to activate osteoblast differentiation. *Osx*-deficient mice are lack of a mineralized matrix and no bone formation. This indicates that Osx is vital for new bone formation (Nakashima et al., 2002). Furthermore, osteoblasts also control

osteoclast differentiation as a consequence of the function of activating transcription factor 4 (ATF4) which binds with the promoter of *Rankl* gene (Karsenty, 2008). On contrarily, expression of *Opg* gene is controlled by synergistic activity of β-catenin, T-cell factor/lymphocyte enhancer factor protein and early B-cell factor 2 on *Opg* promoter (Kieslinger et al., 2005).

At the terminal stage of differentiation, mature osteoblasts are characterized by a cuboidal shape, large Golgi body and several rough endoplasmic reticulums. These cells undergo three possible fates; undergoing apoptosis, covering bone surface and becoming bone lining cells or mineralizing and changing to osteocytes. Most of mature osteoblasts (about 50-70%) are apoptosis, while 5-20% of the population start to produce the osteoid, an unmineralized protein matrix, by producing organic phase and mineralizing hydroxyapatite crystals before entombing themselves within the bone and become the osteocytes (Bellido, 2014).

1.2.3. Osteocytes

Osteocytes are subpopulation of osteoblasts which account for 90-95% of all bone cells. These cells cocoon in fluid-filled lacunae and penetrate their long dendrite-like processes throughout canalicular system which connect to other osteocytes, osteoblasts and osteoclasts. The processes contact other neighboring cells via gap junction which forms the connected cellular networks and maintains integrity of bone as tissue. Then, osteocytes are suggested to play an important role for sensing mechanical stress within bone and detecting microarchitecture damage. Osteocytes also inhibit osteoclastogenesis by secreting RANKL/OPG and enhance osteoblastogenesis by producing sclerostin, the molecule inhibiting Wnt-signaling pathway (Raggatt and Partridge, 2010; Bellido, 2014).

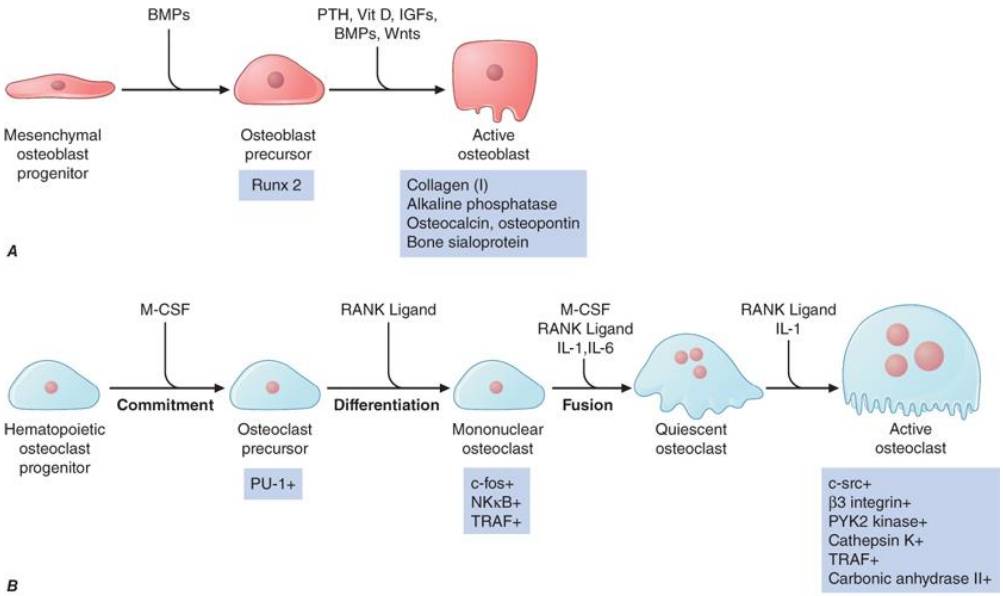

Figure 2.3 The development pathways of osteoblasts (A) and osteoclasts (B). Osteoblasts derive form mesenchymal stem cells and osteoclasts derive from hematopoeitic stem cells under the percisely consequential steps (Available in: http://accessmedicine.mhmedical.com/data/Books/1130/kas_ch423_ f001.png)

1.3. Bone remodeling and its mechanisms

Bone remodeling is the bone rebuilt process which is initiated by the bone resorption and then followed by the bone formation at the site of the resorption in order to maintain bone mass and repair microarchitecture damage. As this process is important to sustain the mineral homeostasis and the integrity of skeleton, the synchronized activities of multiple cellular participants or BMUs are required. These temporarily anatomical structures consist of osteoclasts, osteoblasts, osteocytes and bone lining cells which multitude signals among BMUs and control each other. The process of bone remodeling coordinates with three sequential phases of resorption, reversal and formation phase (Figure 2.4; Matsuo and Irie, 2008; Raggatt and Partridge, 2010; Sims and Martin, 2015).

At the resorption phase, osteocytes and osteoblasts detect the signaling such as mechanical stress or hormones then initiate remodeling by activating osteoclastogenesis. Normally, osteocytes within bone matrix secrete the transforming growth factor β (TGF-β) to supress osteoclastogenesis; however, mechanical stress from daily skeleton movement causes osteocyte apoptosis leading to the low level of TGF-β. Osteoblasts are also activated by mechanical signal to secrete the matrix metalloproteinase 13 (MMP-13) which degrades unmineralized osteoid and opens the surface for osteoclast attachment. As both osteoblasts and osteocytes express PTH receptors and estrogen receptors (ERs), PTH and estrogens can regulate proliferation and differentiation of osteoclasts by controlling expression level of RANKL/OPG ratio. Then mature osteoclasts are created and start to resorp the damaged bone by making Howship's resorption lacunae of where the formation is filled up (Matsuo and Irie, 2008; Raggatt and Partridge, 2010; Sims and Martin, 2015).

During the reversal phase from resorption to formation, osteoclasts produce coupling factors, the osteoclast-derived molecules, to induce new bone formation by osteoblasts in the BMUs. Then, the osteoprogenitor cells are recruited to the site and osteoblast differentiation and function are activated. Coupling signals secreted by osteoclasts are the necessary message to connect resorption with formation which can be divided into three main classes; matrix-derived factors, osteoclast-secreting

factors, and osteoclast membrane-bound factors. Matrix-embed factors such as TGF-β, IGF-1, BMP-2 and IGFs are released while osteoclasts resorp bone, then activate osteoprogenitor cells for recruitment, migration and differentiation. Osteoclasts also secrete soluble coupling factors such as cardiotrophin-1, sphingosine-1-phosphate, Wnt 10b, BMP-6 and complement factor 3a (C3a). Membrane-bound ephrinB2 ligand (EphB2) is well-known membrane-bound coupling factors which is expressed and embedded onto differentiating osteoclast surface and binds with EphB4 tyrosine kinase receptor (EphB4) expressed on osteoblasts. EphrinB2/EphB4 complex provides bidirectional signaling of which forward signaling enhances osteoblast differentiation and reverse signaling inhibits osteoclast function. Indeed, several mechanisms are required for coupling signals between osteoclasts and osteoblasts (Matsuo and Irie, 2008; Raggatt and Partridge, 2010; Sims and Martin, 2015).

Formation phase starts when MSCs or osteoprogenitor cells return to Howship's resorption lacunae and differentiate to mature osteoblasts. These cells are responsible for fulfilling the cavity by secreting organic matrix such as type I collagen and noncollagenous proteins, and depositing hydroxyapatite into osteoid. Osteocytes support osteoblast differentiation and function by reducing the level of sclerostin which suppress osteoblastic bone formation in resting condition. Additionally, osteoclastogenesis is repressed owning to high expression of OPG from osteoblasts. Then, Howship's lacunae are filled up leading to the termination of the bone remodeling. Osteoblasts undergo apoptosis, embed into bone as osteocytes or cover bone surface as bone lining cells. Bone gets into resting stage until the new signaling triggers next bone remodeling process (Matsuo and Irie, 2008; Raggatt and Partridge, 2010; Sims and Martin, 2015).

Bone remodeling happens throughout the human's life and relates to bone metabolic diseases. The resorption phase occurs within 3 weeks, whereas the formation phase takes approximately 3-4 months. The balance of these two processes maintains BMD. During puberty, the bone formation occurs greater than the bone resorption and reaching of the peak bone mass at 20s before the BMD continuously losses when the human become older (Manolagas, 2000).

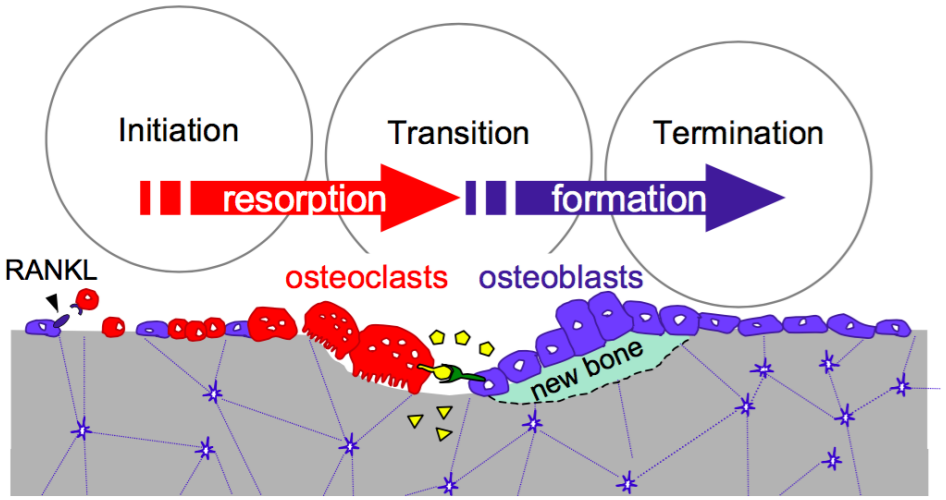

Figure 2.4 Bone remodeling process. There are three major phases of the process starting from resorption phase, reversal phase and then formation phase. (Matsuo and Irie, 2008)

1.4. Bone metabolic disorder: osteoporosis

Osteoporosis is the bone metabolic disorder that bone mass depletion and bone microarchitecture deterioration enhance the risk of bone fracture. The definition of osteoporosis is that their BMD is less than or equal to -2.5 SD of that average of the young adults. The dual x-ray absorptiometry (DXA; the standard tool to measure the BMD) and the fracture risk assessment tool (FRAX) are used to diagnose and predict osteoporosis (Rachner et al., 2011). According to two phases of bone remodeling, bone formation and bone resorption, either fail to produce new bone by osteoblasts or accelerate to resorp bone by osteoclasts causes bone fragility in osteoporotic patients (Raisz, 2005). Therefore, the therapeutic strategies distinguish into 2 classes; anabolic agents which activate osteoblast function and anti-resorptive agents which suppress osteoclast function. Currently, there are only the three forms of PTH; N-terminal (1-34) fragment, teriparatide and the full-length (1-84) which are used as anabolic agents (Raisz, 2005). Whereas, the anti-resorptive therapies are widespread used and divided to 5 groups; bisphosphonates, estrogens, selective estrogen receptor modulators (SERMs), calcitonin, and monoclonal antibodies (Chen and Sambrook, 2012).

Estrogen deficiency is critical to the pathogenesis of osteoporosis as 85% of all osteoporotic patients are post-menopausal women. At menopausal period, both markers of bone formation and resorption increase indicating that loss of estrogen trigs the acceleration of bone remodeling process. Then, synthetic estrogen has been used as anti-osteoporotic agent and shows the efficiency to prevent or remedy the osteoporotic symptoms (Raisz, 2005).

2. Estrogens and skeleton

2.1. Estrogen structure and mechanisms of action

Estrogens are important sex hormones secreted from the ovary to regulate the development and maintenance of female reproductive system. Its chemical structures are derived from cholesterol precursor which contains 18 carbon atoms and has phenol properties. Estrogens are a group of sex hormones and consisted of three commons structures of estrone (E_1), 17β-estradiol (E_2) and estriol (E_3; Figure 2.5). The biological active estrogen in premenopausal women is E_2 whereas E_1 and E_3 play a role in postmenopausal and pregnant women, respectively. Estrogens are converted from testosterone by aromatase enzyme and E_2 is the strongest and most influence to humans' body compared with the other structures (Jordan et al., 1985).

Estrogen functions by binding with estrogen receptors (ERs), the member of nuclear transcription factors, which can be divided into ERα and ERβ. The structure of ERs subdivides into A-F domains. The N-terminal A/B domain encodes the transcriptional activation function-1 (AF-1). C domain or the DNA-binding domain (DBD) is essential for ERs to bind with specific DNA sequence then activates or represses the transcription of specific genes. D domain is the flexible hinge region connecting B domain with C domain. The C-terminal E/F domain consists of two parts; the ligand-binding domain (LBD) where the hormones bind and the ligand-dependent transcription activation function-2 (AF-2). ERα and ERβ are highly conserved in the DBD (90%) and LBD (65%) region. As similar to other genomic pathways, once estrogens bind at LBDs, ERs form homodimers (ERα:ERα or ERβ:ERβ) or heterodimers (ERα:ERβ), then bind at estrogen response elements (EREs) on specific DNA sequences and modulate the gene expression. Estrogen also acts through non-genomic pathway by binding with membrane-localized ERs. This interaction activates intracellular signaling cascades such as MAPKs or phosphatidylinositol 3-kinase/protein kinase B (PI3K/AKT) which subsequently stimulates target proteins in cytosol or transcription factors for gene regulation depending on cell types. The ERs express in various organs including urogenital tract,

central nervous system, cardiovascular system and bone (Nilsson and Gustafsson 2011).

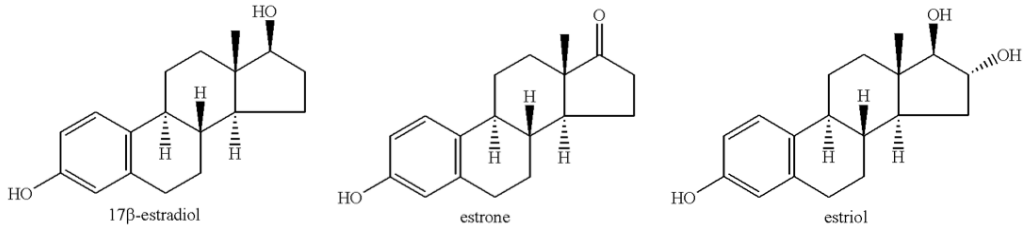

Figure 2.5 Estrogen structures .The common structures of physiological estrogens; estrone (E_1), 17β-estradiol (E_2) and estriol (E_3). (Available in: http://patentimages storage.googleapis.com/US6265147B1/ US06265147-20010724-C00001. png)

2.2. Estrogen and bone

Estrogen is an essential regulator for bone metabolic homeostasis in women and men by maintaining the balance of bone remodeling through the anti-resorptive and anabolic effects. Loss of estrogen in postmenopausal women or in old men at the age of greater than or equal to 50 years decreases BMD and then induces the risk of osteoporosis (Rachner et al., 2011). In agreement with the postmenopausal women, the ovariectomy-induced estrogen deficiency in monkeys, rats and mice also caused bone loss (Trisomboon et al., 2007; Malaivijitnond, 2004; Jaroenporn et al., 2006). Then, the effect of estrogen through ERs was confirmed in the ERαβKO male and female mice which had the decreased cortical and trabecular BMDs. Since osteoclasts, osteoblasts and osteocytes express both ERα and ERβ, the efficacy of estrogens on bone becomes a focal point of interest (Khalid and Krum, 2016).

2.2.1. Estrogen and osteoclast

In vivo study showed that specific deletion of ERα in mice osteoclasts increased bone remodeling and then induced the loss of trabecular bone mass (Nakamura et al., 2007). This suggests that estrogen limited either number or lifespan of mature osteoclasts. During RANKL-induced osteoclastogenesis, estrogen directly reduced the number of osteoclasts through non-genomic pathway by decreasing the activity of JNK1 and c-Jun which suppressed the osteoclast differentiation (Shevde et al., 2000; Srivastava et al., 2001). When estrogen bound with ERα, it interacted with BCAR1 scaffolding protein and formed ERα/BCAR1 complex which abolished the activity of TNF receptor-associated factor 6 (TRAF6), a mediator of RANKL signaling, and led to a decrease in activation of NF-κB of osteoclasts (Robinson et al., 2009). Moreover, estrogen downregulated the level of osteoclast stimulatory transmembrane protein (OC-STAMP) mRNA expression and then the formation of the multinucleated osteoclasts from mononucleated preosteoclasts were reduced (Kim et al., 2011).

Estrogen also regulates osteoclasts indirectly via osteoblasts by repressing the secretion of pre-osteoclastic cytokines and inducing the apoptosis. As RANKL is an important protein for osteoclastogenesis, estrogen suppressed RANKL production through ERα binding then interacted and inhibited Runx2 activity (Khalid et al., 2008). Estrogen also stimulated transcription of *Opg* (Hofbauer et al., 1999), and decreased the proliferation and differentiation of osteoclasts. Furthermore, estrogen activates osteoblast-induced osteoclast apoptosis by enhancing the *Fasl* expression of osteoblasts which can bind with pro-apoptotic Fas receptor embedded on osteoclasts (Krum et al., 2008a; Wang et al., 2015). Thus, treatment of osteoblasts with estrogen enhances the expression of *FasL* after estrogen binds with ERα and directly upregulates the enhancer on the downstream of the *FasL* transcription starting site (Krum et al., 2008a).

2.2.2. Estrogen and osteoblast

Estrogen stimulates osteoblastogenesis in several manners such as reduction of apoptosis, suppression of oxidative stress and activation of osteoblast function which lead to an increase in bone formation. Anti-apoptotic activity of estrogen on osteoblasts occurred through the activation of mRNA expression of B-cell lymphoma protein 2 (Bcl2), an anti-apoptotic protein, which then enhanced their lifespan (Pantschenko et al., 2005). The products of oxidative stress including reactive oxygen species (ROS) which were accumulated during estrogen deficient condition inhibited Wnt signaling and suppressed osteoblastogenesis (Almeida et al., 2007). Estrogen activated osteoblast formation by stimulating the transcription of *Alp* (Krum et al., 2008b) and reducing NF-κB activity which led to upregulation of Fos-related antigen 1 (Fra-1; the essential transcriptional factor for bone matrix embed) expression (Chang et al., 2009).

3. *Pueraria mirifica* plant

3.1. *P. mirifica* and its phytoestrogens

P. candollei var. *mirifica* (Airy Shaw et. Suvatabandhu) Niyomdham (1992) (syn. *P. mirifica*) or white Kwao Krua is an endemic Thai herbal plant distributing in the deciduous forest throughout Thailand. *P. mirifica* is taxonomically categorized in family Leguminosae, subfamily Papilionoidae or the soy, bean and pea subfamily. It is a woody prenial climber plant with purple-blue flowers and white tuberous roots. The tuberous roots of *P. mirifica* contain high amount of phytoestrogens, plant-derived non-steroidal compounds which can bind with ERs and elicit estrogenic activity (Malaivijiitnond et al., 2012). The high performance liquid chromatography analysis indicates that this plant contains at least 17 phytoestrogen substances which are divided into three main classes; chromenes (miroestrol, deoxymiroestrol and isomiroestrol), isoflavonoids (daidzein, daidzin, genistin, genistein, kwakhurin, kwakhurin hydrate, tuberosin, puerarin, mirificin and puermiricarpene) and coumestrans (coumestrol, mirificoumestan, mirificoumestan hydrate and mirificoumestan glycol) (Cherdshewasart and Sriwatcharakul, 2007).

Cheomenes and isoflavonoids are the major active substances in *P. mirifica* (Figure 2.6). Miroestrol is the first phytoestrogen isolated from *P. mirifica* extract and is the highly potent estrogenic activity (Cain, 1960). Deoxymiroestrol is mentioned to be an original phytoestrogen substance in *P. mirifica*, however, it is oxidized to miroestrol and isomiroestrol during the isolation and extraction (Chansakaow et al., 2000). Puerarin, a specific-*Pueraria* genus substance, is the highest amount among other phytoestrogens isolated from *P. mirifica.* Even if puerarin exhibits weak estrogenic potency (Malaivijiitnond et al., 2010), it has high antioxidant capacity for ROS scavenging (Zhou et al., 2013).

The basic structure of phytoestrogens in *P. mirifica,* such as miroestrol, puerarin, genistein and daidzein, is the two benzene rings which are linked by a heterocyclic pyrane ring and one hydroxyl group (-OH) joining to each benzene ring. These hydroxyl groups are important for *P. mirifica* phytoestrogens when they bind

with ERα and ERβ and exhibit estrogenic activity (Chen and Anderson, 2002). Generally, phytoestrogens are considered as alternative agents for ERT.

The tuberous roots of *P. mirifica* have been used as a rejuvenating agent for women and men in Thai folklore medicine for several decades. Taking of peppercorn-size pill of *P. mirifica* (about 250 mg) once at night for 3-6 months could attenuate the aging process such as sagging breast, wrinkle skin, grey hair and bone loss which is the consequence of estrogen deficiency in post-menopausal women (Malaivijiitnond et al., 2012).

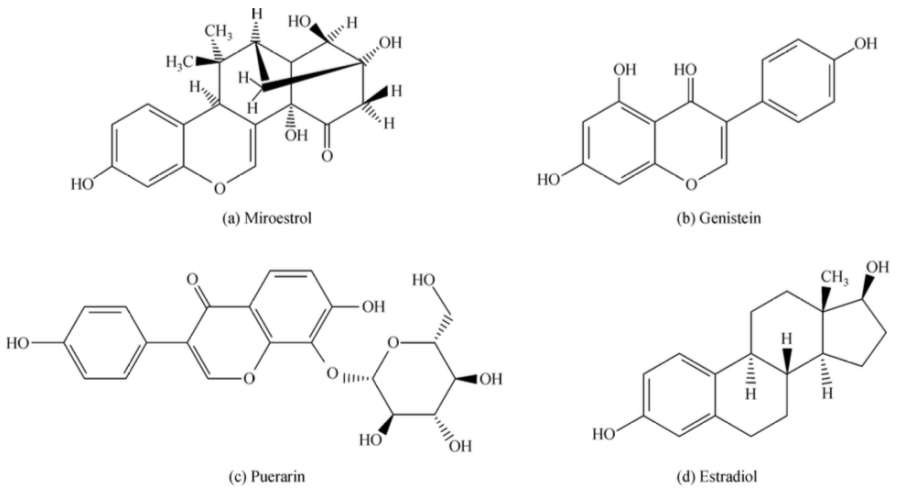

Figure 2.6 Major phytoestrogen substrances in *P. mirifica* and 17β-estradiol. (Malaivijitnond, 2012)

3.2. *P. mirifica* and its phytoestrogens on bone

As loss of estrogen in postmenopausal women is a major causation of osteoporosis. The estrogenic effects of *P. mirifica* on bone have been tested both *in vivo* and *in vitro*. Treatment with crude powder of *P. mirifica* at concentrations of 10, 100 and 1,000 mg/kg BW/day in ovariectomized and orchidectomized rats demonstrated that *P. mirifica* protected bone loss by increasing trabecular and corticel BMD of 4^{th} lumbar vertebra, tibia and femur in a dose-dependent manner (Urasopon et al., 2007, 2008). *P. mirifica* also retained the tibia BMD in estrogen deficient-induced osteoporotic rats (Suthon et al., 2016). This confirms that *P. mirifica* could attenuate bone loss caused by estrogen deficiency. Later, the effects of *P. mirifica* and its phytoestrogens on bone cells have been evaluated.

In osteoblast linage, *P. mirifica* and its phytoestrogens stimulated osteoblastogenesis in multiple levels. Puerarin increased the proliferation, differentiation and matrix mineralization in primary rat osteoblasts (Wang et al., 2014). Genistein and daidzein significantly enhanced proliferation, ALP activity and osteoblastogenic gene expression in mouse osteoblast MC3T3-E1 (Yamaguchi and Sugimoto, 2000) and human osteoblast MG63 (Morris et al., 2006). *P. mirifica* and puerarin activated ostoblastic bone formation by increasing *Alp* mRNA expression while suppressed osteoclastic bone resorption by increasing the ratio of RANKL/OPG in rat osteoblast UMR106 and primary baboon osteoblasts through the ER-dependent manner (Tiyasatkulkovit et al., 2012, 2014).

P. mirifica directly regulated osteoclast linage cells via suppression of RANKL-induced osteoclastogenesis. The study in osteoclast-like RAW264.7 cells showed that the treatment of *P. mirifica* extract and coumestrol decreased number of mature osteoclasts (Kanno et al., 2004; Sasivoey et al., 2014) through interrupting ERK1/2 pathway (Kanno et al., 2004). Additionally, genistein and daidzein inhibited the translocation of NF-κB leading to the suppression of osteoclastogenic gene transcription in osteoclast-like RAW264.7 cells (Garcia Palacios et al., 2005). However, the direct effects and mechanisms of action of *P. mirifica* extract and puerarin in

primary osteoclasts and coculture condition have not been elucidated and awaited for the investigation.

CHAPTER III
ANTI-OSTEOCLASTOGENIC ACTION OF *Pueraria mirifica* EXTRACT ON MOUSE OSTEOCLASTS

Introduction

Estrogen deficiency during the postmenopausal period can cause bone mass depletion and bone microarchitecture deterioration, which subsequently lead to osteoporosis (Lane, 2006). Synthetic E_2 can be effective in preventing or curing osteoporosis symptoms (Ettinger et al., 1985), but there is concern about such treatment with the risk of developing estrogen-dependent cancers (Beral, 2007; Narod, 2011). Thus, alternative potential reagents instead of synthetic estrogen, such as the use of natural based phytoestrogens, have attracted considerable attention (Glazier and Bowman, 2001). Tuberous roots of *P. mirifica* contain many phytoestrogen substances with puerarin (PU) as the major constituent and miroestrol as the high potency in estrogenic activity (Chansakaow et al., 2000; Cherdshewasart et al., 2007; Malaivijitnond, 2012). Previous studies found that *P. mirifica* extract (PME) could prevent bone loss in estrogen deficient-induced osteoporosis in both sexes of rats (Suthon et al., 2016; Urasopon et al., 2007, 2008). The mechanisms of action of PME and PU were evaluated in the rat osteoblast-like UMR-106 cell line and in primary baboon osteoblasts (Tiyasatkulkovit et al., 2012, 2014), where PME and PU enhanced osteoblast proliferation, differentiation and mineralization in these cells via interaction with ERs (Tiyasatkulkovit et al., 2012, 2014). In addition, the effects of PU and other phytoestrogen components isolated from *P. mirifica* on bone have mainly been examined on osteoblasts (Kanno et al., 2004; Wang et al., 2014). In particular, the action of PME and PU on osteoclastogenesis has not yet been investigated.

Generally, to maintain the bone structure and function, bone remodeling occurs via cross-talk among osteoclasts, osteoblasts and osteocytes (Matsuo, 2009). Osteoclasts initiate the process by clearing damaged sites on the bone to produce a

free space before allowing osteoblasts to fill the cavity (Zaidi, 2007). However, the accelerated function of osteoclasts after estrogen depletion causes an inequality in the bone homeostasis resulting in bone loss and subsequent osteoporosis (Raisz, 2005). Estrogens exert their activity on osteoclasts through the ERα and ERβ subtypes that are present during osteoclastogenesis (Denger et al., 2008) and control the formation as well as the function and lifespan of osteoclasts (Oursler et al., 1991). With respect to the similarity of chemical structures between phytoestrogens and estrogens, PME, which contains a high amount of phytoestrogens (Chansakaow et al., 2000; Cherdshewasart et al., 2007; Malaivijitnond et al., 2012), and PU would be expected to affect osteoclastogenesis.

Therefore, this study aimed to investigate the effects of PME and PU on the entire process of osteoclastogenesis, including (i) osteoclast precursor viability and proliferation, (ii) osteoclast differentiation and multi-nucleated cell formation (or fusion) and (iii) osteoclast resorptive function. The estrogenic activity of PME and PU via ERs on osteoclast differentiation was also determined, where E_2 was used as a positive control to compare the mechanisms of action with those of PME and PU.

Materials and Methods

General reagents and the PME

Extract of *P. candollei* var. *mirifica* (Airy Shaw et. Suvatabandhu) Niyomdham (1992) (syn. *P. mirifica*) (Lot: 112747) was provided by Dr. I. Sandford Schwartz (Smith Naturals Co., Ltd., Thailand). The *P. mirifica* tuberous roots were collected from Chiang Mai province, Thailand, dried and sliced before shipping to the USA in order to conduct the extraction with ethanol at Bio-Botanica Inc., New York. The obtained PME was analyzed by liquid chromatography-mass spectrometry (LC/MS). PU was the major phytoestrogen at 16.5 mg/100 g of PME, and only 2.3, 0.925, 0.695, 0.201 and 0.173 mg/100 g of PME for daidzin, genistin, daidzein, miroestrol, and genistein. It is undetectable for deoxymiroestrol.

Dimethyl sulfoxide (DMSO; 99.5% purity) and E_2 were from Sigma (USA) and synthetic PU (99% purity, HPLC) was from Pure Chemistry Scientific Inc., USA.

Mouse bone marrow cells

To subtle the variation between the primary osteoclast cells, the inbred strain of C57BL/6Mlac mouse was used in this study. Female C57BL/6Mlac mice of 8–10 weeks old were purchased from the National Laboratory Animal Center, Mahidol University, Thailand. The experimental protocol for laboratory animals was approved by the Ethical Animal Care and Use Committee, Faculty of Science, Chulalongkorn University (Protocol review no. 1423001). Mice were housed in a room with controlled light, temperature and humidity at the Laboratory Animal Unit, Department of Biology, Faculty of Science, Chulalongkorn University, with a light-dark cycle of 12:12 h (lights on 06.00 h), 22–25 °C and 50–60% relative humidity. Animals were fed with standard rodent diet (Perfect Companion Group, Bangkok, Thailand) and water *ad libitum*. For euthanasia, mice were intraperitoneally injected with 100 mg/kg pentobarbital sodium. Bone marrow cells (BMCs) were isolated from the tibia and femur under sterile conditions. In brief, the extraneous soft connective tissues around the bones were removed using a scalpel blade. The bones were then rinsed with phosphate-buffer saline (PBS) pH 7.4 without calcium and magnesium (Gibco, USA) and kept in phenol red-free α-MEM media (Gibco, USA) with 1% penicillin streptomycin (Gibco, USA). Both sides of the bones were cut using bone scissors, and BMCs were flushed using 22G and 25G needles for the femur and tibia, respectively, and then incubated in phenol red-free α-MEM media (Gibco, USA) with 10% (v/v) fetal bovine serum (FBS; Gibco, USA) and 1% penicillin streptomycin at 37 °C with 5% (v/v) CO_2 for 4–6 h. Non-adherent BMCs were collected as osteoclast progenitor cells.

Cell viability (MTT) assay

Osteoclast precursor viability was investigated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; catalog no. 0793; Ultra Pure Grade, Ameresco, USA) assay (Tiyasatkulkovit et al., 2012). Non-adherent BMCs at a density of 1 x 10^6 cells/ml were cultured in 96-well plates with 10 ng/ml M-CSF (R&D system, USA) for 3 days. BMCs transformed into osteoclast precursors or bone marrow derived-macrophages (BMMs) and then 0.3% (v/v) DMSO, 10 nM E_2, PME (1, 5, 10, 50 and 100 mg/ml) and PU (0.1, 10 and 1000 nM) were added. After incubation for 24 h the cell viability was determined following the manufacturer's instructions. The absorbance was measured using a microplate reader (model Multiskan EX; Thermo Scientific, USA) at 540 nm with a reference wavelength of 595 nm. The assay was performed in triplicate upon each of six independent samples (n = 6).

Cell proliferation assay

Osteoclast precursor proliferation was investigated using the 5-bromo-2'-deoxyuridine (BrdU) assay (Tiyasatkulkovit et al., 2012). Non-adherent BMCs at a density of 1 x 10^6 cells/ml were cultured in 96-well plates together with 10 ng/ml M-CSF (R&D system, USA) for 3 days before transforming into BMMs and then 0.3% (v/v) DMSO, 10 nM E_2, PME (1, 5 and 10 mg/ml) and PU (0.1, 10 and 1000 nM) were added and incubated for 24 h. Cell proliferation was then determined using the BrdU enzyme-linked immunosorbent assay kit (catalog no. 11647229001; Roche, Germany) following the manufacturer's instruction. The absorbance was measured using a microplate reader (model Multiskan EX; Thermo Scientific, USA) at 450 nm with the reference wavelength of 690 nm. The BrdU assay was performed in triplicate with six independent samples in each replicate (n = 6).

Osteoclast differentiation assay

Non-adherent BMCs at a density of 1 x 10^6 cells/ml were cultured with phenol red-free α-MEM media (Gibco, USA) with 10% (v/v) FBS (Gibco, USA) including 10 ng/ml M-CSF for 3 days whereupon the cells were differentiated into BMMs. The BMMs were then activated with the M-CSF/RANKL (R&D system, USA) together with 0.3% (v/v) DMSO, 10 nM E_2, PME (1, 5 and 10 mg/ml) and PU (0.1, 10 and 1000 nM) for 4 days. The cells were then stained using a TRAP staining kit (catalog no. PMC-AK04F; Cosmo Bio, Japan), following the manufacturer's instruction with slight modification (Kuroda et al., 2008). In brief, cells were washed with PBS before fixing with 100 μl of 10% (v/v) formalin neutral buffer at room temperature for 10 min and then washed with 100 μl of distilled water twice. Then 50 μl of TRAP staining substrate was added and incubated at 37 °C for 30 min. Cells were washed twice with 100 μl of distilled water and kept in distilled water afterwards. As TRAP staining typifies differentiated osteoclasts, mononuclear TRAP-positive cells were defined as pre-osteoclasts and multi-nucleated TRAP-positive cells with at least three nuclei were defined as mature osteoclasts.

Cells were examined under a compound light microscope (model Axio Vert.A1; Carl Zeiss Micro-Imaging GmbH, Germany), and the mononuclear pre-osteoclasts and multi-nucleated mature osteoclasts were separately counted using the ImageJ 1.49 program. The TRAP staining was performed in triplicate with six independent samples in each replicate (n = 6).

Genes expression analysis

Cells were collected in a Trizol reagent (Applied Biosystems, USA), and the total RNA was extracted using the phenol-chloroform method. The quantity and purity of the RNA samples were checked by measuring the absorbance at a wavelength of 260 and 280 nm using a NanoDrop 2000 spectrometer (Thermo Fisher Scientific, USA), where a A_{260}/A_{280} ratio of 1.8–2.0 was accepted. One μg of the total

RNA was reversed transcribed to cDNA using a High Capacity cDNA Reverse Transcription Kit (catalog no. 4374966; Applied Biosystems, USA).

The expression levels of the genes associated with osteoclast differentiation, *Nfatc1*, and osteoclast fusion, *Tm7sf4* encoded for DC-STAMP, were examined using quantitative real time reverse transcriptase (qRT)-PCR with the previously reported primers (Kasahara et al., 2010; Lange and Yutzey, 2006) as listed in Table 3.1. *Gapdh* was used as a housekeeping gene. The specificity of the *Nfatc1* and *Tm7sf4* primers was tested by conventional PCR using the PCR Super Mix (catalog no. 10572014; Applied Biosystems, USA). The PCR amplicons were mixed with Novel Juice (catalog no. LD001; GeneDirex, Taiwan) and visualized after resolution through a 1.2% (w/v) agarose gel using a GelDoc-lt2 310 Imager (UVP, USA). The qRT-PCR was performed using the StepOne^{TM} Plus Real-Time PCR System (Applied Biosystems, USA) in a 20 μl reaction mixture containing 1x POWER SYBR Green PCR Master Mix (catalog no. 4367659; Applied Biosystems, USA), 200 nM each of the respective forward and reverse primers and 50 ng of the cDNA sample. The reaction was performed at 95°C for 10 min, followed by 40 cycles of 95°C for 5 sec and 61 °C for 1 min, and then a dissociation curve step. The relative expression levels of the target genes were calculated by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Each qRT-PCR was performed from 5–7 independent samples.

Table 3.1 *Mus musculus* primers used in the qRT-PCR analyses

Genes	Accession no.	Product size (bp)	Primer sequences (5' to 3')
Nfatc1	NM016791	190	CGGGAAGAAGATGGTGCTGT
			TTGGACGGGGCTGGTTAT
Tm7sf4	NM029422.4	370	CCGCTGTGGACTATCTGCTG
			CTCAATGGCTGCTTTGATCG
Gapdh	NM008084	191	AACGACCCCTTCATTGAC
			TCCACGACATACTCAGCAC

Nfatc1, nuclear factor of activated T-cells cytoplasmic 1; *Tm7sf4*, gene encoding for dendritic cell-specific transmembrane protein; *Gapdh*, glyceraldehyde 3-phosphate dehydrogenase

Bone resorption assay

Bone slices were prepared as reported (Zhao et al., 2006). Bones were incubated overnight in 96-well plates in phenol red-free α-MEM media (Gibco, USA) with 10% (v/v) FBS (Gibco, USA) and 1% penicillin streptomycin before the non-adherent BMCs (1 x 10^6 cells/ml) were seeded onto the bone and activated with 10 ng/ml M-CSF for 3 days. Then, 0.3% (v/v) DMSO, 10 nM E_2, PME (1 and 10 mg/ml) and 1000 nM PU were added to the BMMs together with the M-CSF/RANKL system and cultured for 6 days. Thereafter, bone slices were analyzed for the amount of resorbed area using wheat germ agglutinin (WGA) (Selander et al, 1994). Briefly, the bone slices were sunk into 50 mM NH_4OH and the osteoclasts were then removed by sonication for 60 sec. After that, bone slices were incubated with peroxidase-conjugated WGA-lectin (Sigma, USA) for 1 h before incubating with the 3,3'-diaminobenzidine (DAB) peroxidase substrate kit (Vector Laboratory, USA) for 1–3 min. The resorbed area, revealed as a brown color, was examined under a stereomicroscope (model Stemi 508; Carl Zeiss Micro-Imaging GmbH, Germany) and the percent of resorption area was analyzed by ImageJ 1.49 program. Each pit assay was performed in triplicate from each of six independent samples (n = 6).

ER antagonists

BMMs were each pre-treated with 1 μM of the ERα antagonist 1,3-*Bis*(4-hydroxyphenyl)-4-methyl-5-[4-(2-piperidinylethoxy)phenol]-1*H*-pyrazoledihydro chloride (MPP; Tocris Bioscience, UK), the ERβ antagonist 4-[2-Phenyl-5,7*bis* (trifluoromethyl) pyrazolo [1,5-*a*] pyrimidin-3-yl] phenol (PHTPP; Tocris Bioscience, UK) and both ERα and ERβ antagonists (MPP/PHTPP) at 37 °C with 5% (v/v) CO_2 for 2.5 h. Then, 3% (v/v) DMSO, 10 nM E_2, PME (1 and 10 mg/ml) and 1000 nM PU were added to the culture system together with M-CSF/RANKL and incubated for another 4 days. The TRAP staining was performed following the protocol mentioned above in triplicate with six independent samples in each replicate (n = 6).

Statistical analysis

The data are expressed as the mean ± one standard error (1 S.E.) Two-group comparisons were analyzed by an independent sample t-test. Comparisons of more than two groups were performed by a one-way analysis of variance (ANOVA) followed by Least Significant Difference (LSD) or Duncan post-test. Significance was accepted at the $p < 0.05$ level. All data were analyzed using the IBM SPSS Statistics 22 Program.

Results

Effects of PME and PU on viability and proliferation of osteoclast precursors

The cytotoxicity of PME and PU on BMMs was investigated first. After incubation for 24 h, the incubation with PME at 50 or 100 mg/ml significantly ($p < 0.001$) decreased the viability of BMMs to 50–70% of that of the vehicle-treated control, while the lower PME doses (1, 5 and 10 mg/ml) and the entire tested range of PU (0.1–1000 nM) as well as 10 nM E_2 treatment had no detrimental effect (Figure 3.1A). This indicates that PME at 50 mg/ml or higher was toxic to osteoclast precursor cells, and so PME was evaluated at 1, 5 and 10 mg/ml in the subsequent assays.

Exposure of the BMMs to 10 nM E_2, PME (1, 5 and 10 mg/ml) or PU (0.1, 10 and 1000 nM) in the presence of M-CSF for 24 h resulted in a significantly reduced cell proliferation only in the case of 10 mg/ml PME, which showed a 20% reduced cell proliferation level compared to the control ($p = 0.021$; Figure 3.1B).

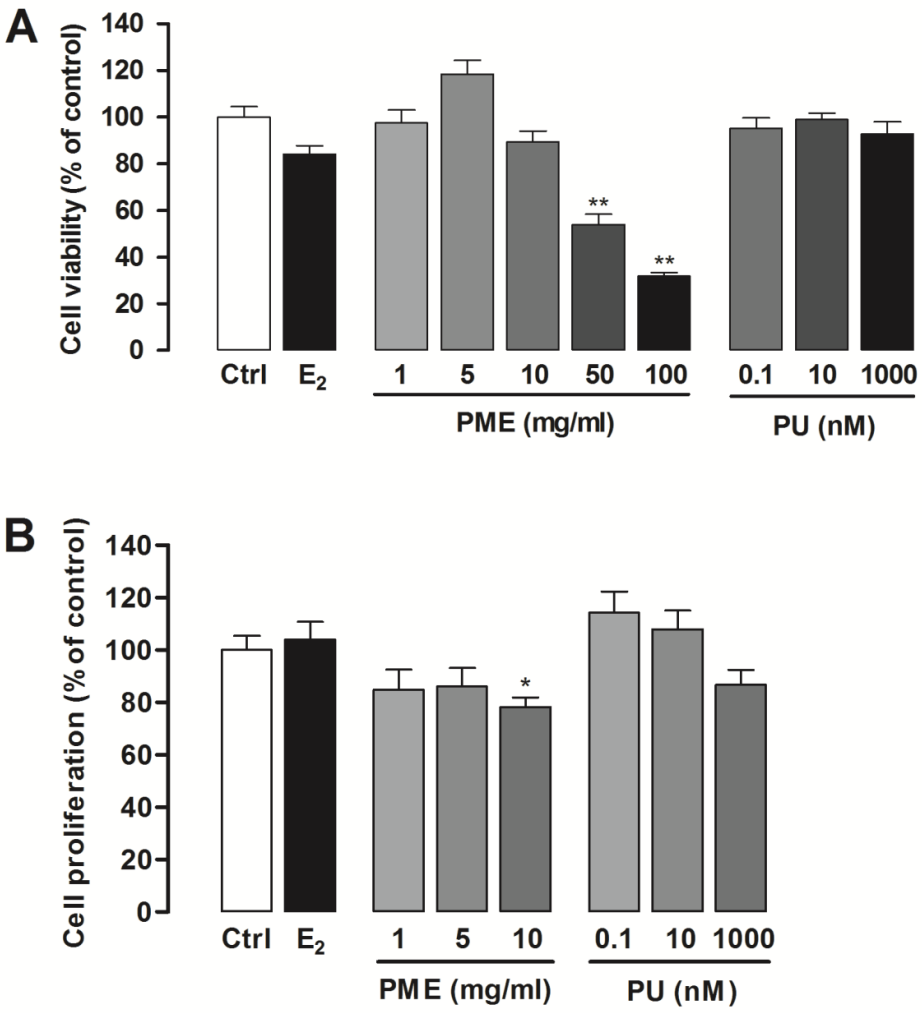

Figure 3.1 Relative cell (A) viability and (B) proliferation of BMMs after exposure to 0.3% (v/v) DMSO (Ctrl), 10 nM E_2, 1, 5, 10 and 100 mg/ml *P. mirifica* extract (PME), or 0.1, 1 and 1000 nM puerarin (PU). Values are relative to that of the control (set to 100%). *$p < 0.05$ and **$p < 0.001$, compared with the control.

Effects of PME and PU on osteoclast differentiation

The effects of PME and PU on the RANKL-induced osteoclast differentiation (from BMMs to mononuclear pre-osteoclasts) and multi-nucleation (from mononuclear pre-osteoclasts to multi-nucleated mature osteoclasts) were examined. After 4 days of exposure, all doses of PME and PU significantly lowered the number of multi-nucleated osteoclasts compared to the control group, with PME showing the strongest effect and PU having a dose-dependent effect (Figure 3.2A). As mature osteoclasts were not detected in the 5 and 10 mg/ml PME treatments, the numbers of mononuclear pre-osteoclasts were counted. As shown in Figures 3.2B and D, PME at doses of 5 and 10 mg/ml highly significantly decreased the number of pre-osteoclasts ($p < 0.001$), whereas 1 mg/ml PME, 10 nM E_2, and 0.1–1000 nM PU treatments significantly increased the number of mononuclear pre-osteoclasts compared to the control group. To determine the differential response between high and low doses of PME on pre-osteoclast differentiation and multi-nucleated cell formation, the ratio of multi-nucleated cells to mononuclear cells was calculated (Figure 3.2C). The high doses of PME suppressed the differentiation of BMMs to pre-osteoclasts (Figure 3.2B), while the low dose of PME suppressed the formation of multi-nucleated mature osteoclasts, but not the differentiation of BMMs to pre-osteoclasts (Figure 3.2D).

Nonetheless, significantly decreased mRNA levels of *Nfatc1*, an essential gene for osteoclast differentiation, were observed only in the 10 mg/ml PME treatment, while mRNA levels in the other groups were comparable to the control group (Figure 3.3A). This confirms the suppression of BMM differentiation by the high dose of PME. On the other hand, *Tm7sf4* encoding DC-STAMP, which is essential for osteoclast fusion, was not suppressed by 1 mg/ml PME, but it was in the 10 mg/ml PME treatment (Figure 3.3B). Therefore, observed inhibitory effects of 1 mg/ml PME on osteoclast fusion is unlikely due to downregulation of *Tm7sf4* expression.

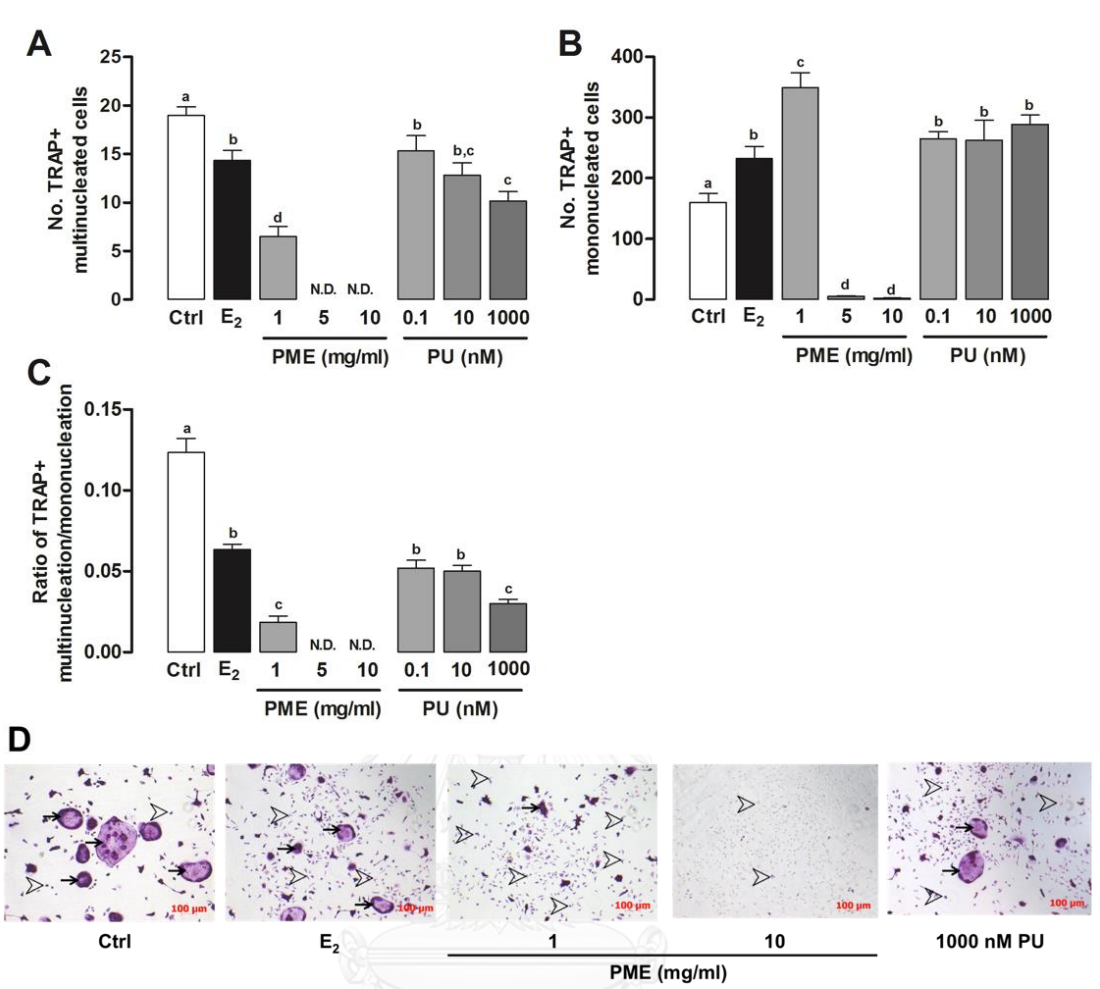

Figure 3.2 The number of TRAP staining-positive (A) multi-nucleated and (B) mono-nucleated osteoclasts. Cells were exposed to 0.3% (v/v) DMSO (Ctrl), 10 nM E_2, 1, 5 and 10 mg/ml PME, or 0.1, 1 and 1000 nM PU for 4 days. (C) The ratio of multi/mono-osteoclasts. The different letters on each column indicate significant differences ($p < 0.05$). (D) The multi-nucleated osteoclasts are indicated by arrows, and the mononuclear pre-osteoclasts are indicated by arrowheads.

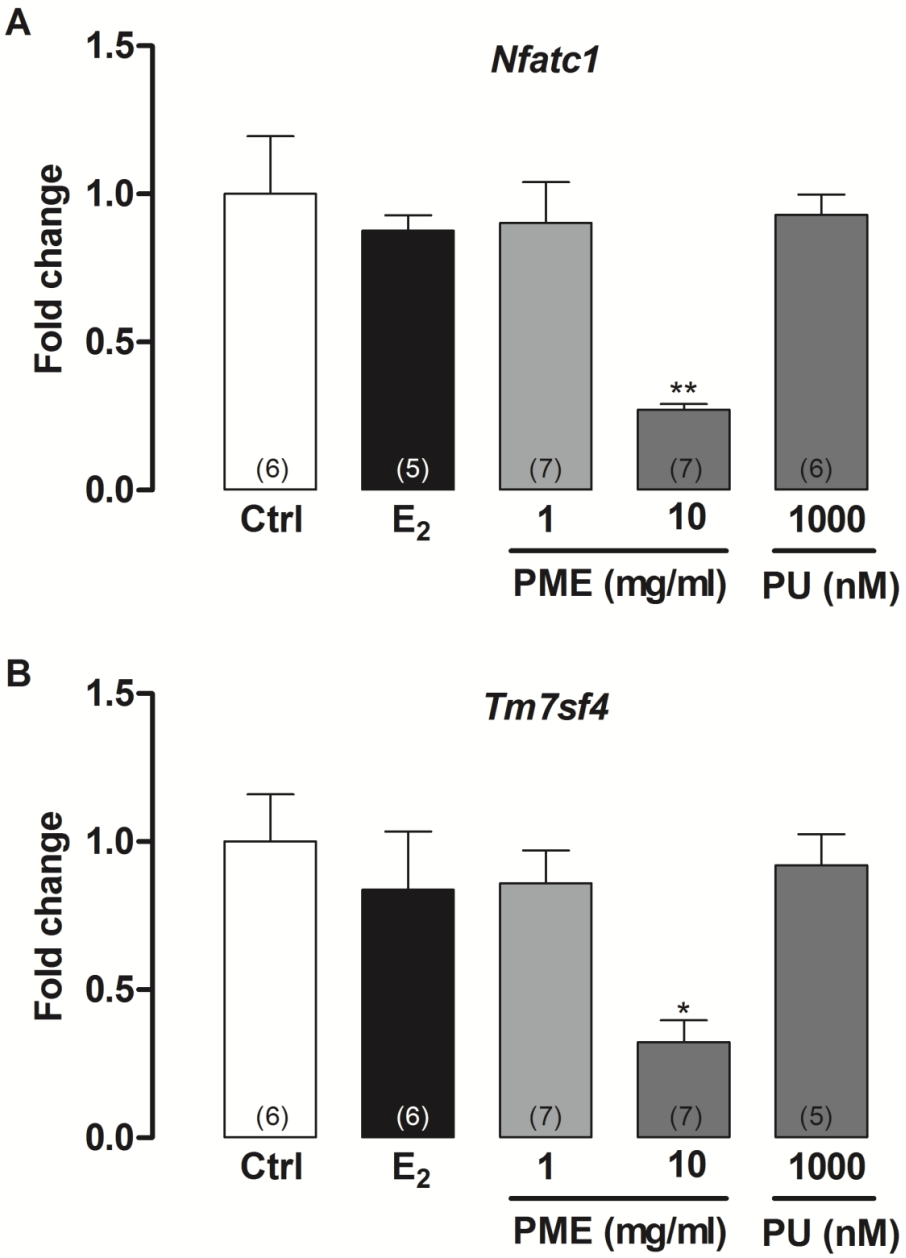

Figure 3.3 The mRNA expression of (A) *Nfatc1* and (B) *Tm7sf4* after a 24-h exposure to the treatments, as quantified by qRT-PCR. * and ** represent $p < 0.05$ and 0.001, respectively, compared with the control group.

Effects of PME and PU on osteoclast activity

After exposing BMMs to M-CSF/RANKL and estrogen/phytoestrogens for 6 days (Figure 3.4A) the percent resorption area had decreased, especially in the 10 mg/ml PME treatment that had decreased by up to 85% compared with the control group. Thus, it is interesting to verify if the decreased resorption area was caused by a direct suppressive effect of PME and PU on mature osteoclast activity or an indirect effect through lowering osteoclast differentiation and formation. To test this, the BMMs were first incubated with M-CSF/RANKL for 4 days, differentiated to mature osteoclasts, and then the estrogen/phytoestrogens were added and incubated for another 6 days, totally 10 days of incubation, before the bone slices were analyzed for the resorbed area. The additional 6-day activation of the mature osteoclasts produced a continuously resorbed bone surface in the control group (Figure 3.4B) by 25% compared with the initial control (IC) of 4-day incubation (after only M-CSF/RANKL incubation). However, no significant differences were detected between the all treatments and the control group (Figure 3.4B). Thus, once the osteoclasts become mature, estrogens and phytoestrogens did not affect the osteoclast activity.

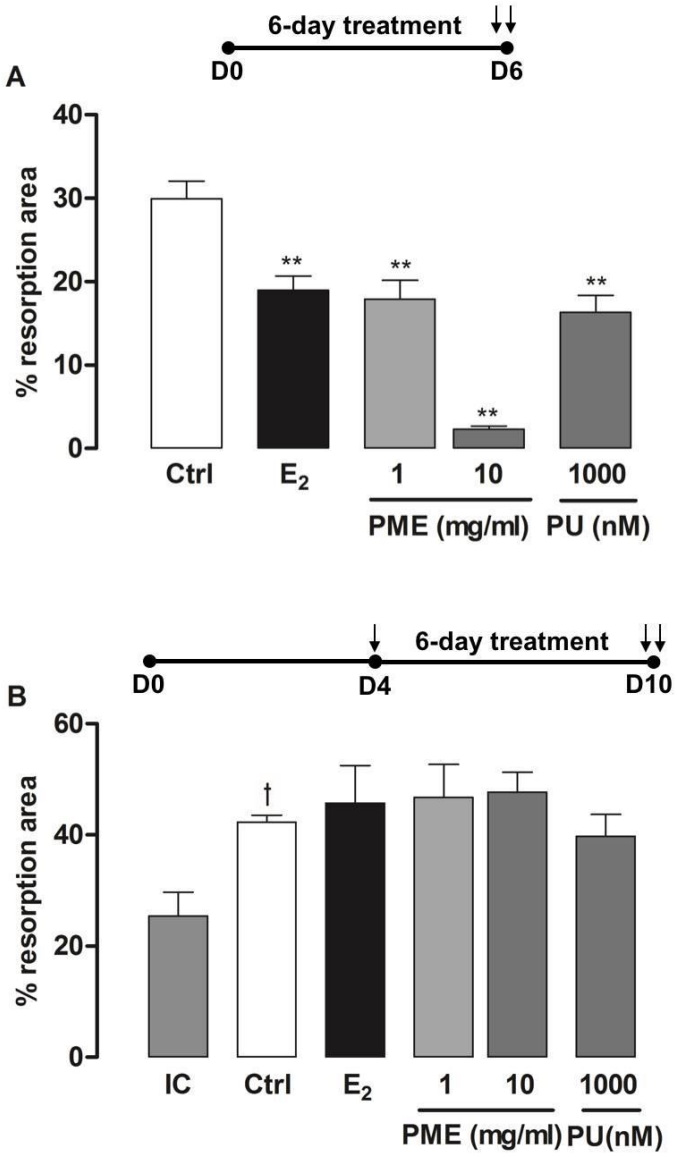

Figure 3.4 Percent resorption area of mature osteoclasts after BMMs was activated with M-CSF/RANKL system together with the exposure to the 0.3% (v/v) DMSO (Ctrl), 10 nM E_2, 1 and 10 mg/ml *P. mirifica* extract (PME) and 1000 nM puerarin (PU) after a (A) 6-day incubation or (B) the treatments were performed after the osteoclasts became mature over 4 days and were then incubated for another 6 days. The timeline indicates the duration of the experiment. ↓ and ↓↓ indicate the day that the bone resorption was determined in the IC group, and the Ctrl, E_2, PME and PU group, respectively. ** $p < 0.001$ compared with the control group. † $p < 0.05$ compared with the initiation control (IC).

Suppressive effects of PME and PU on osteoclast multi-nucleation are mediated via ERs

As mentioned above, PME, PU and E_2 could suppress osteoclast multi-nucleation. To determine if the effects of PME and PU were mediated through an ER dependent mechanism, BMMs were pre-incubated with the M-CSF/RANKL system with or without ERα, ERβ or ERα/ERβ antagonists before adding E_2, PME and PU and then incubating for 4 days. The ERα, ERβ or ERα/ERβ antagonists alone had no effect on the differentiation and multi-nucleation of osteoclasts (Figure 3.5). Corresponding to the above results for osteoclast differentiation and formation, exposure to 10 nM E_2, 1000 nM PU, and 1 or 10 mg/ml PME decreased the number of mature osteoclasts compared to the control group. Furthermore, the suppressive effects were prevented by both subtypes of ER antagonists (Figures 3.5A–C), except for in the case of 10 mg/ml PME, where mature osteoclasts were not detected whether the cells were cultured in the presence or absence of ER antagonists (Figure 3.5D).

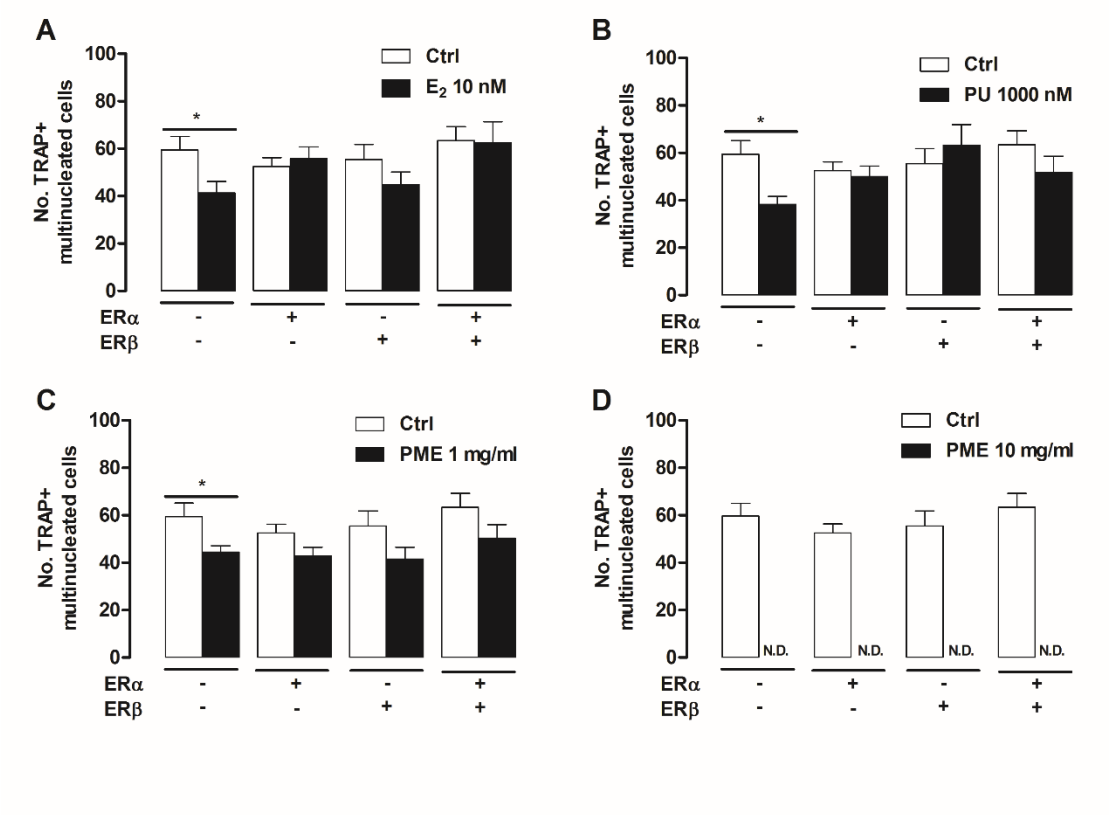

Figure 3.5 The number of mature osteoclasts determined by TRAP staining after treatment with (A) 10 nM E_2, (B) 1000 nM puerarin (PU) and (C, D) *P. mirifica* extract (PME) at (C) 1 mg/ml and (D) 10 mg/ml in the absence or presence of ERα, ERβ or ERα/ERβ antagonists. * represents $p < 0.05$ compared with the control group.

Discussion

Phytoestrogens have been considered for decades for their potential to prevent bone loss. *P. mirifica* is a remarkable, phytoestrogen-rich herb that has been accessed for its efficacy to prevent bone loss *in vivo* (Suthon et al., 2016; Urasopon et al., 2007, 2008; Udomsuk et al., 2012) and *in vitro* (Tiyasatkulkovit et al., 2012, 2014). Although it has been reported that PME contained at least 17 phytoestrogens (Malaivijitnond et al., 2012) of which PU is the major constituent and miroestrol has the highly potent estrogenic activity, in this study only PU was selected to determine if the anti-osteoclastogenic action was comparable with PME and E_2, because miroestrol is unstable, and no synthesized miroestrol has been commercialized. Treatment with PME prevented bone loss by increasing the BMC and BMD in orchidectomized and ovariectomized rats (Suthon et al., 2016; Urasopon et al., 2007, 2008). The underlying mechanism for PME and PU has been reported to be the stimulated differentiation and enhanced mineralization of rat and baboon osteoblasts (Tiyasatkulkovit et al., 2012, 2014). Many studies on the effects of PME and PU on osteoblasts have been conducted, but not on osteoclasts. Osteoblast bone formation is tightly coupled to osteoclast bone resorption, and as PME and PU could increase the ratio of OPG/RANKL (Tiyasatkulkovit et al., 2012, 2014; Udomsuk et al., 2012), PME and PU should affect osteoclast differentiation and fusion.

Osteoclastogenesis has several consecutive steps to create mature osteoclasts. Hematopoietic stem cells commit to M-CSF and undergo differentiation to osteoclast precursors (or BMMs), and then pre-osteoclasts which are activated to fuse and form multi-nucleated osteoclasts under the influence of M-CSF and RANKL. Development towards mature osteoclasts is characterized by dynamic osteoclast-specific gene expression, including *Nfatc1*, *Tm7sf4* (DC-STAMP) and *Acp5* (TRAP) (Boyle et al., 2003). The DC-STAMP-expressing mononuclear osteoclast becomes the fusion competent cell, which then can fuse with other cells. Mature multi-nucleated osteoclasts have the unique characters of a ruffled membrane in a sealing zone that bind to the bone. Once multi-nucleated osteoclasts attach to the bone surface they secrete TRAP enzyme to digest the collagen and HCl to resorb the hydroxyapatite

(Yavropoulou and Yovos, 2008). While osteoclast precursors are important to create mature osteoclasts, this study showed that a high dose of PME (10 mg/ml) could decrease the osteoclast precursor proliferation. This might be because the phytoestrogen substances in PME, such as genistein, arrest the cell cycle by activating phosphorylation of mitogen-activated kinase (MAPK) p38, as reported previously in the murine macrophage cell line RAW246.7 (Cui et al., 2014).

After proliferation, the differentiation and multi-nucleated formation were initiated with activation of MAPKs, including Jun N-terminal kinase (JNK) and p38, resulting in translocation of the transcription factor nuclear factor κB (NF-κB) to nucleus. Then, *Nfatc1* induces osteoclast precursor differentiation and pre-osteoclast multi-nucleation, respectively (Asagiri and Takayanagi, 2007). Since only high doses of PME (5 and 10 mg/ml) decreased the number of mononuclear pre-osteoclasts, together with a low level of *Nfatc1* and *Tm7sf4* mRNA expression, in the case of treatment with 10 mg/ml PME, this suggests that PME suppressed at least the differentiation of osteoclasts via the NFATc1 pathway resulting in the down-regulation of subsequent genes, i.e. *Tm7sf4* in this study. However, since a low dose of PME (1 mg/ml) and PU both suppressed the fusion stage of osteoclasts, indicated by the high numbers of TRAP-positive pre-osteoclasts and lower numbers of multi-nucleated mature osteoclasts in comparison with the control group, PME would have a differential dose-response effect on osteoclastogenesis. Previously, it was reported that PME (Saisavoey et al., 2014) and its phytoestrogens, such as genistein and daidzein (Garcia Palacios et al., 2005), suppressed multi-nucleation in the osteoclast-like RAW264.7 cells. Possibly, phytoestrogen substances in PME and PU interfered with c-Jun activity (Shevde et al., 2000) and JNK1 activity (Srivastava et al., 2001), as seen in the E_2 treatment. Although DC-STAMP is a key regulator of osteoclast multi-nucleation (Yagi et al., 2005), changes in its mRNA expression level were not detected after PME (1 mg/ml) or PU treatment. As the pathway controlling cell-cell fusion downstream of DC-STAMP requires several steps to produce membrane-bound fusion molecules (Oikawa et al., 2012), the effect of PME (1 mg/ml) and PU might affect other steps of signal transduction such as osteoclast stimulatory

transmembrane protein (OC-STAMP), another osteoclast formation regulator gene. Previously, it was reported that E_2 repressed the expression of OC-STAMP in RAW264.7 cells (Kim et al., 2011).

Both ERα and ERβ antagonists completely abolished the anti-osteoclastogenic effect of a low PME dose (1 mg/ml), but not the high PME dose (10 mg/ml), and also all the doses of PU tested in comparison with that of 10 nM E_2. This suggests that PME mediates osteoclast differentiation and fusion also through one or more estrogen-independent pathways. As reactive oxygen species (ROS) are essential for osteoclast formation, inhibition of ROS production resulted in abrogation of multi-nucleated osteoclast formation (Lee et al., 2005). Thus, phytoestrogens may directly reduce ROS or indirectly up-regulate antioxidant-related genes in osteoclasts (Lee et al., 2014). It was reported that the phytoestrogens in PME, especially PU, could exhibit antioxidative activity (Cherdshewasart and Sutjit, 2008). As such, a high PME dose (10 mg/ml) might exhibit an antioxidative activity and then induce apoptosis of osteoclasts. Alternatively, PME may decrease the pro-inflammatory cytokine TNF-α pathway leading to a decreased level of the TNF-α-induced *c-fos* and *Nfatc1* expression in osteoclasts as shown with genistein and daidzein treatment (Karieb and Fox, 2011). Moreover, genistein-induced osteoclast apoptosis was found to be mediated through the intracellular Ca^{2+} signaling pathway (Gao and Yamaguchi, 1999). Taken together, the suppressive effects of E_2, all the doses of PU, and a low PME dose (1 mg/ml) on multi-nucleation, are possibly via estrogen-dependent pathway, which did not affect *Tm7sf4* expression. It is possible that PU, a low PME dose (1 mg/ml), and E_2 (in this study) might suppress cell-cell fusion through other genes such as *Oc-stamp*, since treatment of E_2 in the RAW264.7 cells downregulated the *Oc-stamp* expression (Kim et al., 2011). However, a high PME dose (10 mg/ml) likely exerts its effect on osteoclast precursor differentiation through estrogen-independent pathways, which decreases expression of *Tm7sf4* and other genes as a result of the suppression of *Nfatc1* expression at the early state of osteoclastogenesis.

Consistent with this study, a previous study in human osteoclasts (Sorensen et al., 2006) revealed that after osteoclasts reached a mature stage the cells remain actively resorbing bone regardless of subsequent PME, PU or E_2 treatment. The resorbing activity could be suppressed indirectly by decreasing the number of mature osteoclasts or decreasing osteoclast differentiation and fusion, as seen in the genistein treatment (Li and Yu, 2003). Since high doses of PME did not act typically on osteoclasts by estrogenic activity, further investigation is required to find out whether PME elicits any antioxidative activity on osteoclastogenesis.

In conclusion, the present study showed the anti-osteoclastogenic action of PME and PU, which should result in a decreased bone resorption. Considering the previous studies reporting that PME and PU stimulated osteoblastogenesis and function (Tiyasatkulkovit et al., 2012, 2014), PME and PU should be candidates as anti-osteoporotic agents to be developed for human use in the future. It is worth noting that PME showed a differential dose-response effect, where a low dose (1 mg/ml) suppressed the fusion stage of osteoclast formation and a high dose (10 mg/ml) suppressed the differentiation of osteoclast precursors. Furthermore, only the low PME dose affected the osteoclasts in a comparable manner to that of E_2, but the higher PME dose also acted through ER-independent pathways. The effect of PME on osteoclasts might be depended on dose of treatment and developmental stage of cells, which needs to be investigated further to help understand the underlying mechanisms.

CHAPTER IV

Pueraria mirifica EXTRACT INDUCES MOUSE OSTEOCLAST APOPTOSIS

Introduction

Osteoclasts play an important role in resorption phase in bone remodeling process. The acceleration of resorbing activity causes the impairment of bone homeostasis and leads to the bone metabolic disorders such as rheumatoid arthritis and osteoporosis. The strategies to keep the rate of bone resorption are not only the regulation of osteoclastogenesis but it is also the management of the death rate of osteoclasts by programed cell death (Manolagas, 2000). Apoptosis is the process of programed cell death to manage cell population in tissues during development and aging of animals. The fatty acid synthase ligand/Fas receptor (FasL/Fas) system is the major apoptotic mechanism of osteoclasts. *FasL* and *Fas* express during osteoclastogenesis including in osteoclast precursors and mature osteoclasts (Park et al., 2005). It was also reported that the impairment of FasL/Fas interaction in conditional knockout mice led to severe dysfunction in immune system (Roths et al., 1984; Watanabe-Fukunaga et al., 1992).

Synthetic E_2 has been reported to induce apoptosis in osteoclasts by stimulating the expression of *FasL* but not *Fas* in mature osteoclasts and initiating autocrine apoptotic mechanism (Imai et al., 2009). As the study in the Chapter III showed that the high dose of PME (10 mg/ml) decreased osteoclast precursor proliferation, thus this high PME dose was expected to induce the apoptosis in osteoclast linage cells. Therefore, this study aimed to investigate the apoptotic effects of PME and PU during osteoclast differentiation (BMMs-preosteoclasts) in comparison with E_2 and the mRNA expression of *FasL*.

Materials and Methods

PME and reagents

The PME was provided from the Smith Naturals Co., Ltd., Thailand. The extraction process and the contents of major phytoestrogens in the extract were reported in Chapter III. DMSO 99.5% purity (Sigma, MO, USA), E_2 (Sigma, MO, USA) and synthetic PU (99% purity, HPLC, Pure Chemistry Scientific Inc., MA, USA) were purchased.

Animals and mouse osteoclast culture

Female C57BL/6Mlac mice at 8-week old were purchased from the National Laboratory Animal Center, Mahidol University, Thailand and housed at the Laboratory Animal Unit, Department of Biology, Faculty of Science, Chulalongkorn University as described in Chapter III. Mice were euthanatized by intraperitoneal injection with 100 mg/kg pentobarbital sodium before collecting tibia and femur of under sterile condition in order to isolate bone marrow cells (BMCs) for osteoclast culture. BMCs were incubated in phenol red-free α-MEM media (Gibco, CA, USA) with 10% FBS (Gibco, CA, USA) and 1% penicillin streptomycin (Gibco, CA, USA) at 37 °C and 5% CO_2 for 5h and then non-adherent BMCs were collected and used as osteoclasts progenitor cells. The laboratory animal use was approved by the Ethical Animal Care and Use Committee, Faculty of Science, Chulalongkorn University (Protocol Review no. 1423001).

Cell apoptosis assay

To investigate the effects of E_2, PME and PU on osteoclast apoptosis, the DNA fragmentation was determined by the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay (Szymczyk et al., 2006). Non-adherent BMCs were seeded at the density of $2x10^5$ cell/ml in 8-chamber glass slides and cultured in phenol red-free α-MEM media with 10% FBS and 1% penicillin streptomycin at 37 °C

and 5% CO_2 together with 10 ng/ml M-CSF (R&D system, MN, USA). After 3 days of incubation, the non-adherent BMCs transformed into BMMs.

From the results of PME and PU on osteoclast differentiation in the Chapter III, only PME at the concentrations of 1 and 10 mg/ml and PU at the concentration of 1000 nM were selected for the apoptosis study. BMMs were treated with 0.3% DMSO, 10 nM E_2, 1 and 10 mg/ml PME and 1000 nM PU in the presence of M-CSF and RANKL (R&D system, MN, USA) for 72 h. The incubation time at 72 h was selected for this study, instead of 96 h, in an attempt to detect the osteoclast apoptosis before the cells become mature. Following the manufacture's instruction, cells were fixed with 4% paraformaldehyde and stained with Fluorescein FragEL^{TM} DNA fragmentation kit (catalog number QIA39, Calbiochem, Darmstadt, Germany). The images were taken under the fluorescent microscope (model BZ-9000, Keyence, Osaka, Japan) using a fluorescein filter (ex 465-495 nm) to indicate apoptotic cells and using a DAPI filter (ex 330-380) to visualize total cell population. Cells was counted by ImageJ 1.49c program and then apoptotic rate (%) was calculated by (number of apoptotic cells/total cells) x 100. The assay was performed in duplicates in each treatment.

Apoptotic gene expression

To determine whether the osteoclast apoptosis was associated with *FasL* expression, qRT-PCR was performed. In brief, BMCs were seeded with the density of $8x10^5$ cell/ml in 6-well plates and then cultured in phenol red-free α-MEM media with 10% FBS and 1% penicillin streptomycin at 37 ^{o}C and 5% CO_2 together with 10 ng/ml M-CSF for 3 days, and the BMCs were differentiated into BMMs. Then, BMMs were exposed to 0.3% DMSO, 10 nM E_2, 1 and 10 mg/ml PME and 1000 nM PU in the presence of M-CSF/RANKL. After 4h and 24 h of incubation, cells were collected in trizol reagent (Thermo Fisher Scientific, MA, USA) before total RNA was extracted using phenol-chloroform technique. cDNA synthesis was done by High Capacity cDNA Reverse Transcription kits (catalog number 4374966, Applied Biosystems, CA, USA) and qRT-PCR was performed by 1X POWER SYBR Green PCR Master Mix (catalog

number 4367659, Applied Biosystems, CA, USA). The primers for *FasL* and *Gapdh* were followed the previous reports as denoted in Table 4.1. qRT-PCR was carried out at 95 $^{\circ}$C for 5 sec. and 61°C for 1 min for 40 cycles, followed by a dissociation curve step. The $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen et al., 2001) was used to calculate relative cexpression levels of the target gene. qRT-PCR was performed 6-8 independent samples for each treatment.

Table 4.1 *Mus musculus* primers used in qRT-PCR experiment

Genes	Accession no.	Product size (bp)	Primer sequences (5' to 3')	References
FasL	NM010177.4	200	TCCAGGGTGGGTCTACTTACTAC CCCTCTTACTTCTCCGTTAGGA	Komatsu et al., 2003
Gapdh	NM008084	191	AACGACCCCTTCATTGAC TCCACGACATACTCAGCAC	Kasahara et al., 2010

FasL, fatty acid synthase ligand; *Gapdh,* glyceraldehyde 3-phosphate dehydrogenase

Statistical analysis

The data are expressed as mean ± SE. Comparison between groups was performed by one-way analysis of variance (ANOVA) followed by LSD post-test. The significant level was accepted at $p < 0.05$. All data were analyzed using IBM SPSS Statistic 22 Program.

Results

Apoptotic effect of PME on mouse osteoclasts

PME at either low (1 mg/ml) or high (10 mg/ml) dose significantly increased cell apoptotic rate for 2.4 ($p = 0.014$) and 3.7 times ($p = 0.001$), respectively, compared to the control group (Figure 4.1). However, 1000 nM PU had no apoptotic effect on mouse osteoclasts. Treatment with 10 nM E_2 marginally increased, but not significantly different, the apoptotic rate ($p = 0.163$).

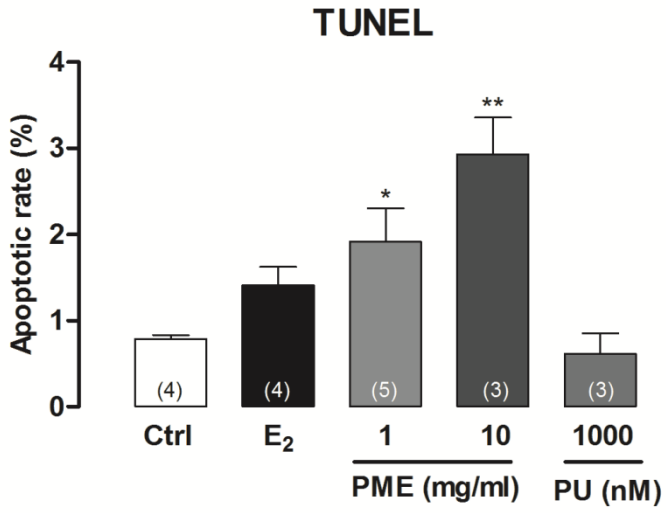

Figure 4.1 Cell apoptotic rate (%) of BMMs after exposure to 0.3% DMSO (Ctrl), 10 nM 17β-estradiol (E_2), 1 and 10 mg/ml *P. mirifica* extract (PME) and 1000 nM puerarin (PU) together with M-CSF/RANKL for 72h. * and ** represent $p < 0.05$ and 0.001, respectively, compared with the control group.

The expression of *FasL* in osteoclast apoptosis

After 4h of exposure, treatments of 1 and 10 mg/ml PME and 1000 nM PU, except the 10 nM E_2, significantly decreased the levels of *FasL* mRNA expression (p = 0.005, 0.001 and 0.011, respectively; Figure 4.2A). However, all treatments of 10 nM E_2, 1 and 10 mg/ml PME and 1000 nM PU significantly reduced the levels of *FasL* mRNA expression (p = 0.038, 0.018, 0.008 and 0.012, respectively) compared with the control group after 24h of incubation (Figure 4.2B). It is worthily noted that the reduction of *FasL* mRNA expression was greater after the longer time of incubation.

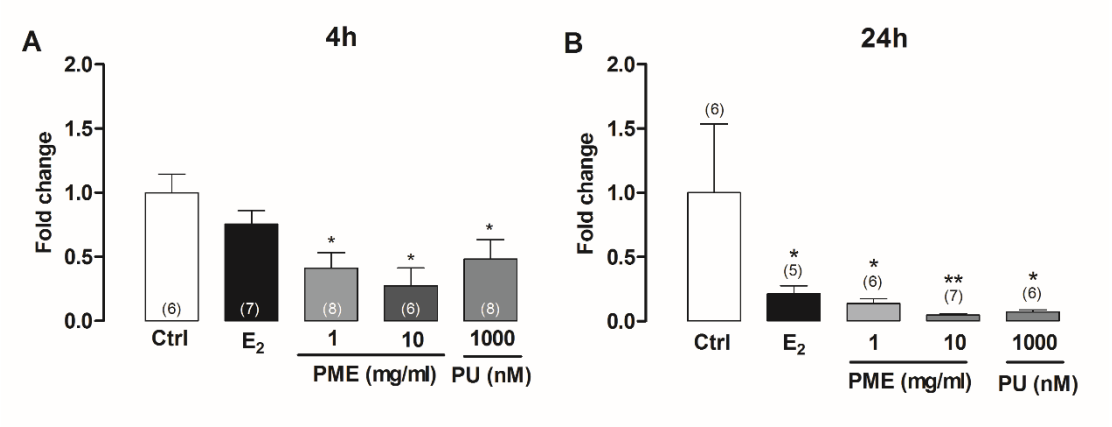

Figure 4.2 The mRNA expression of *FasL* at (A) 4h and (B) 24h of BMMs after exposure to the 0.3% DMSO (Ctrl), 10 nM 17β-estradiol (E_2), 1 and 10 mg/ml *P. mirifica* extract (PME) and 1000 nM puerarin (PU) together with M-CSF/RANKL as quantified by qRT-PCR. * and ** represent $p < 0.05$ and 0.01 compared with the control group, respectively.

Discussion

The apoptotic effect of phytoestrogen substances including PU, daidzein and genistein have been previously tested in breast cancer cells (Rice and Whitehead, 2006). Although *P. mirifica* has been locally used for treatment of several diseases (Malaivijitnond, 2012), no research focused on apoptotic effect of PME and PU on osteoclast lineages. This is the first study showing that PME could induce apoptosis in mouse BMMs-preosteoclasts.

Apoptosis is the major process to control cells' lifespan and population in various tissues. FasL and Fas are the best characterized death ligand and corresponding death receptor of apoptosis, respectively. When FasL bound with Fas at the cell membrane, the transduction signal was produced of which MAP kinase p38 started to activate caspase-8 and then subsequently stimulated executioner caspase-3 which finally led to the formation of apoptotic bodies (Bhattacharyya et al., 2003; Elmore, 2007). This study showed that PME, but not PU, induced apoptosis indicating by the TUNEL assay. Thus, the increased *FasL* mRNA levels in the PME treated groups were expected. However, the results were not followed the expectation, PME, PU as well as E_2 reduced the levels of *FasL* mRNA expression during RANKL-induced mouse osteoclast differentiation. Thus, the apoptosis of mouse BMMs-preosteoclasts was assumed to happen via other pathways which were not determined here. Recently, it was reported that genistein (≥ 50 μM), one of phytoestrogens isolated from PME, directly induced phosphorylation of MAP kinase p38, decreased viability and arrested G2/M phase of cell cycle resulting in apoptosis of mouse RAW264.7 macrophage-like cells (Cui et al., 2014). It might also be possible that *P. mirifica* phytoestrogens bound with peroxisome proliferator-activated receptor-γ (PPAR-γ) and induced BMMs-preosteoclasts apoptosis since PPAR-γ expressed during osteoclast differentiation and played an important role for osteoclastogenesis (Wan et al., 2007). The PPAR-γ ligand induced apoptosis after binding with PPAR-γ (Elrod and Shun, 2008) and stimulating the p53-dependent mechanism in cancer cells (Nagamine et al., 2003). It was previously reported that E_2-induced osteoclast apoptosis was regulated by FasL level which was secreted by

osteoblasts (Wang et al., 2008a; Wang et al., 2015). Thus, it was observed that E_2 induced apoptosis in mature osteoclasts by activating *FasL* mRNA expression in RAW264.7 (Imai et al., 2009). On contrarily, 10 nM E_2 marginally downregulated *FasL* mRNA expression, but showed the induction of apoptosis in this study. Thus, treatments of estrogen/phytoestrogen in the coculture between osteoclast and osteoblast should help to clarify this doubt as it mimics the real situation of cell interaction within bone multicellular unit. Besides, the determination of changes of expression of other genes or proteins in the down-stream of FasL such as caspase-3 should also be considered.

Other than the apoptosis, the expression of *FasL* and *Fas* were detected during differentiation of osteoclast linage cells, from osteoclast precursors to mature osteoclasts, and FasL was a major molecule to stimulate this process (Park et al., 2005). In the same line with the results of Chapter III showing that PME and PU suppressed osteoclastogenesis, this study showed that PME and PU decreased levels of *FasL* mRNA expression. Thus, reduced *FasL* expression might not result in reduction of mouse osteoclast apoptosis, but it might reduce mouse osteoclast differentiation instead.

Taken together between this study that PME and PU induced mouse osteoclast apoptosis and the study in Chapter III that PME and PU suppressed osteoclastogenesis, it was clearly shown that PME and PU had negative effects on osteoclast. Thus, *P. mirifica* might be outstanding candidate to develop as anti-osteoporotic agent due to its activation on osteoblast function (Tiyasatkulkovit et al., 2012, 2014) and osteoclast apoptosis and suppression of osteoclast proliferation, differentiation and resorption function

CHAPTER V

Pueraria mirifica PHYTOESTROGEN INTERUPT RANKL/OPG/RANK SIGNALING IN OSTEOBLAST PRECURSOR ST2 CELLS, BUT NOT IN OSTEOBLAST-OSTEOCLAST COCULTURE

Introduction

Pueraria mirifica is an endemic Thai plant belonging to family Leguminosae. Its tuberous root contains high amount of phytoestrogens, and PU is a major constituent (Malaivijitnind, 2012). Since phytoestrogens in *P. mirifica* can bind with ERs (Morito et al., 2001) and ERs expressed in both osteoblasts (Harada and Rodan, 2003) and osteoclasts (Denger et al., 2008), *P. mirifica* becomes one of the candidate herbal medicines for pharmaceutical company to be developed as anti-osteoporotic agent for human use. At present, many researchers conducted research to test the efficacy and to understand the mechanism of action of the PME and PU on osteoblasts and osteoclasts, and on bone mass in ovariectomized rodents (Urasopon et al., 2008; Udomsuk et al., 2012; Wang et al., 2014; Suthon et al., 2016). *P. mirifica* exhibited anti-osteoporosis effects in estrogen deficient-induced osteoporotic rats (Suthon et al., 2016; Urasopon et al., 2008). The cellular mechanisms of actions have been investigated; PME and PU activated differentiation of rat osteoblast-like UMR-106 cells and primary baboon osteoblasts (Tiyasatkulkovit et al., 2012, 2014), whereas it suppressed osteoclasts multinucleation differentiation and formation (as seen in Chapter III) and induced osteoclast apoptosis (as seen in Chapter IV). Moreover, miroestrol, a highly potent phytoestrogenic chemical of *P. mirifica*, upregulated the *Opg* mRNA expression while decreased *Rankl* mRNA levels in ovariectomized mice (Udomsuk et al., 2012).

Bone is a dynamic organ of which the bone remodeling process happens throughout the animals' life by the co-action of, at least, three types of bone cells; osteoblast, osteoclast and osteocyte. During bone remodeling, multiple signals were

secreted from osteoblasts and osteoclasts and affected differentiation and function of other cells (Matsuo and Irie, 2008). At the initiation phase, osteoblasts secreted RANKL which bound to RANK on membrane of osteoclast precursors and then stimulated osteoclastogenesis (Matsuo, 2009). At the same time, osteoblasts also secreted OPG, the decoy receptor of RANKL, which ceased the osteoclastogenesis and balanced the number of mature osteoclasts (Raggatt and Partridge, 2010). At the transition phase from bone resorption to bone formation, osteoclasts produce coupling factors such as ephrinB2 which specifically binds to EphB4 expressed and embedded on the plasma membrane of osteoblasts. Later, EphB4 shows a forward signaling to induce osteoblast differentiation and a reverse signaling to inhibit osteoclast differentiation (Matsuo and Otaki, 2012). As such, it is clearly seen that to study the effects of PME on either type of bone cells alone do not completely represent the real condition in bone remodeling. Therefore, this study aims to investigate whether PME and PU in comparison with E_2 mediate the osteoblast-osteoclast communication.

Materials and Methods

PME and chemicals

The process of the ethanol extraction of PME, and the quantities of phytoestrogens in the PME determined by LC/MS could be seen in Chapter III. DMSO (99.5% purity) and E_2 were purchased from Sigma (MO, USA) and synthetic PU (99% purity, HPLC grade) was purchased from Pure Chemistry Scientific Inc., MA, USA.

Osteoblast culture

Following the standardized protocol reported previously (Zhao et al., 2006), mouse bone marrow-derived ST2 stroma cells (Riken Cell Bank, Tsukuba, Japan) were cultured in phenol red-free α-MEM media (Gibco, CA, USA) with 10% FBS (Gibco, CA, USA) and 1% penicillin streptomycin (Gibco, CA, USA) at 37 $^{\circ}$C and 5% CO_2. Only

early passaged cells (< 12 passages) were used in the experiment. In order to induce the differentiation of ST2, cells were seeded into 6-well plate at the density of 2×10^5 cell/ml for 1 day. After the cells attached to the plate, they were cultured with 10^{-8} M 1,25-dihydroxyvitamin D_3 (Vit D_3; Tocris, Bristol, UK) and 10^{-7} M dexamethasone (Dex; Sigma, MO, USA) for 3 days. Then, ST2-derived osteoblast cells were exposed to 0.3% DMSO, 10 nM E_2, 1000 nM PU, and 1 mg/ml PME in the presence of Vit D_3 and Dex for another 1 day before the cells were collected and measured the mRNA levels of *Rankl*, *Opg*, and *EphB4* genes by qRT-PCR technique. In this study, only a single dose of PME and PU that showed the effects on osteoclastogenesis (Chapter III) and apoptosis (Chapter IV) was selected. Although it was well-known that many forms of Eph-ephrin were reported, only EphB4 and ephrinB2 were selected for this study because they denote the control of bone homeostasis by gain- and loss- function experiments (Zhao et al., 2006).

Osteoclast culture

BMCs were isolated from tibia and femur of 8-12 weeks old C57BL/6 mice which purchased from the National Laboratory Animal Center, Mahidol University, Thailand. Cells were culture in phenol red-free α-MEM media with 10% FBS and 1% penicillin streptomycin at 37 °C and 5% CO_2. Bone marrow cells were seeded into 6-well plate at the density of 8×10^5 cell/ml and cultured in the presence of 10 ng/μl M-CSF (R&D, MN, USA). After 3 days of incubation, BMCs differentiated into BMMs, the osteoclast precursors, then 10 ng/μl M-CSF and 10 ng/μl RANKL (R&D, MN, USA) were added to induce osteoclastogenesis. Together with the addition of M-CSF and RANKL, BMMs were also incubated with 0.3% DMSO, 10 nM E_2, 1000 nM PU, and 1 mg/ml PME. After 1 day of incubation, cells were collected and measured mRNA levels of *Rank* and *EfnB2*, encoded ephrinB2, by qRT-PCR.

Osteoblast-osteoclast coculture

For osteoblast-osteoclast coculture, ST2 were seeded at the density of 2×10^5 cell/ml into 6-well plate for 1 day. The next day, spleen was collected from 8-12 weeks old C57BL/6 mice. Spleen cells were flushed out using 25G needle and filtrated through cell strainer before they were incubated in phenol red-free α-MEM media with 10% FBS and 1% penicillin streptomycin at 37 °C and 5% CO_2 for 5-6 h. Then, non-adhered spleen cells were collected and used as osteoclast precursors in this study following (Zhao et al., 2006). Cells were seeded onto 6-well plate which contained ST2 cells at the density of 1×10^6 cell/ml. Later, 10^{-8} M Vit D_3 and 10^{-7} M Dex were added into the coculture condition and incubated for 3 days. Then, 0.025% DMSO, 10 nM E_2, 1000 nM PU, and 1 mg/ml PME were added into the coculture condition together with Vit D_3 and Dex, and incubated for another 1 day. Cells were collected and determined mRNA levels of *Rankl*, *Opg*, *Rank*, *EphB4* and *EfnB2* by qRT-PCR.

Expression of genes associated with osteoblast and osteoclast functions

Osteoblasts and osteoclasts were collected in Trizol reagent (Thermo Fisher Scientific, MA, USA), then total RNAs were extracted by phenol-chloroform technique. The quantity and purity of total RNAs were measured by Nanodrop2000 (Thermo Fisher Scientific, MA, USA) with the ratio of 2.0 ±0.2 was accepted. The cDNA was synthesized from 1 μg of total RNA using Tetro cDNA synthesis kit (Bio Line, London, UK).

The expression of mRNA levels of *Rankl*, *Opg* and *EphB4* of osteoblasts, and *Rank* and *EfnB2* of osteoclasts were detected by qRT-PCR technique. Primers used in this study were followed the previous reports and shown in Table 5.1. Briefly, each reaction mixture contains of 1X SensiFAST SYBR Hi-ROX Mix (Bio Line, London, UK), 400 nM each of forward and reward primers and 100 ng of cDNA in the final volume of 20 μl. The reaction was carried out at 95°C for 2 min, and 40 cycles of 95°C 5 sec and 60°C for 30 sec, following with the melting curve determination. *Gapdh* (for

osteoclast) and *β-actin* (for osteoblast) were used as the house keeping genes. The $2^{-\Delta\Delta Ct}$ method was used to analyze relative expression level of target genes. qRT-PCR was performed with 6 independent samples in each replication.

Table 5.1 Mus musculus primers used in qRT-PCR experiment

Genes	Accession no.	Product size (bp)	Primer sequences (5' to 3')	References
Rankl	NM011613.3	190	TCCTGAGACTCCATGAAAACGCAG GCCACATCCAACCATGAGCCTTC	Ren et al., 2015
Opg	NM008764.3	251	TGAGAGAACGAGAAAGACCTGC CGGATTGAACCTGATTCCCTAT	Ren et al., 2015
Rank	NM009399.3	351	AAGATGGTTCCAGAAGACGGT CATAGAGTCAGTTCTGCTCGGA	Yan et al., 2001
EphB4	NM001159571.1	388	CAGGTGGTCAGCGCTCTGGAC ATCTGCCACGGTGGTGAGTCC	Yang et al., 2013
EfnB2	NM010111.5	152	AGGAATCACGGTCCAACAAG ACTTCGGAACCCAGGAGATT	Sohl et al., 2009
β-actin	NM007393	154	GGCTGTATTCCCCTCCATCG CCAGTTGGTAACAATGCCATGT	Wang and Seed, 2013
Gapdh	NM008084	191	AACGACCCCTTCATTGAC TCCACGACATACTCAGCAC	Kasahara et al., 2010

Rankl, receptor activator of nuclear factor κB ligand; *Opg*, osteoprotegerin; *Rank*, receptor activator of nuclear factor kappa-B; *EphB4*, ephrin type-B receptor 4; *EfnB2*, ephinB2; *Gapdh,* glyceraldehyde 3-phosphate dehydrogenase

TRAP staining

To ensure that the spleen cells could be completely differentiated into multinucleated mature osteoclast in the coculture condition, the cells were stained with the TRAP staining kit (Cat no. PMC-AK04F, Cosmo Bio, Tokyo, Japan). ST2 cells were seeded at the density of 5 x 10^5 cell/ml into 24-well plate for 1 day. The non-adhered mouse spleen cells were prepared as previously described and added onto 24-well plate which contains 2 x 10^6 cell/ml of ST2 cells. Later, 10^{-8} M Vit D_3 and 10^{-7} M Dex were added into the coculture condition and incubated for 3 days. Then, 0.025% DMSO, 10 nM E_2, 1000 nM PU, and 1 mg/ml PME were added into the coculture condition together with Vit D_3 and Dex, and incubated for another 4 day. Cells were washed with 1xPBS pH 7.4 without calcium and magnesium (Gibco, CA, USA) before the cells were fixed with 100 μl of 10% formalin neutral buffer at room temperature for 10 min and then washed with 100 μl of distilled water twice. Fifty microliters of TRAP staining substrate were added and cells were incubated for 20-30 min at 37°C. After that, cells were washed with 100 μl of distilled water twice, and number of TRAP positive (greater than or equal 3 nuclei) osteoclasts was observed.

Statistical analysis

Data are expressed as mean ± S.E. from 2 independent replications. Normal distribution of data was checked by Kolmogorov-Smirnov. Comparisons among treatments were analyzed using one-way analysis of variance (ANOVA) with LSD post-hoc test. The level of statistical significance was set at $p < 0.05$. All data were analyzed using IBM SPSS statistics 22 Program.

Results

mRNA gene expression in osteoblasts

No significant differences of *Opg* and *EphB4* mRNA levels (Figures 5.1B and D) in all treatment groups compared with the control (0.3% DMSO) group were detected. E_2 and PME treatments could significantly downregulate the *Rankl* expression for 41% (p = 0.005) and 40% (p = 0.009), respectively (Figure 5.1A). Therefore, the *Rankl/Opg* ratio were significantly lowered in the E_2 and PME groups for 43.3% (p = 0.016) and 38.5% (p = 0.017), respectively (Figure 5.1C) compared with the control group.

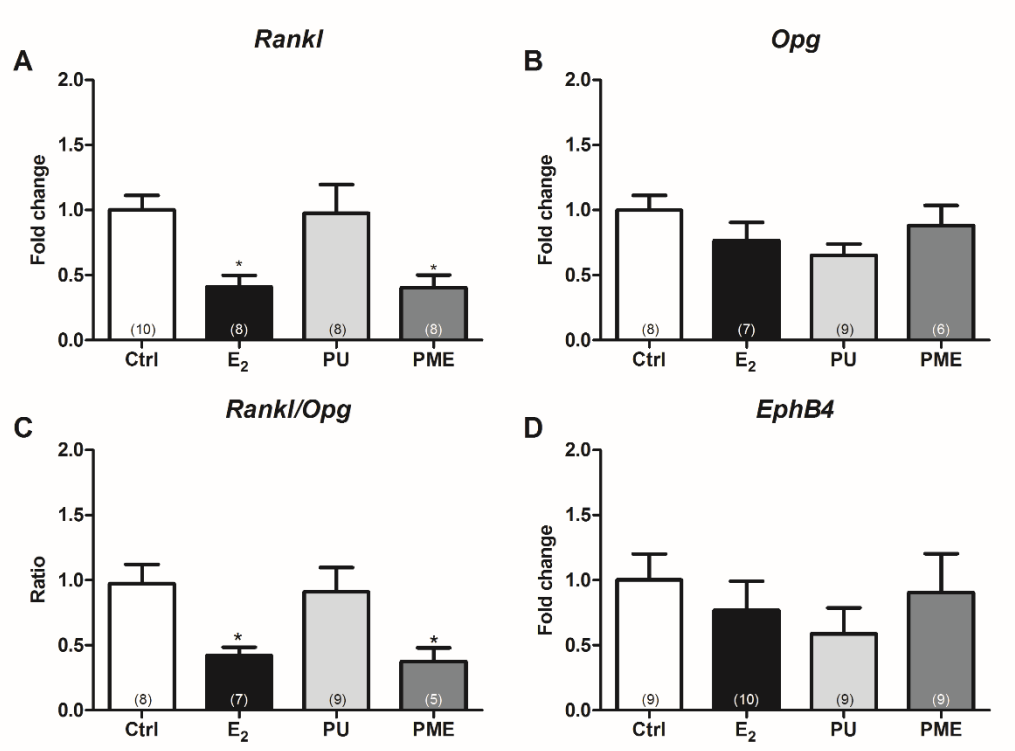

Figure 5.1 The mRNA expression of (A) *Rankl*, (B) *Opg*, (C) Rankl/Opg ratio and (D) *EphB4* of osteoblast precursor ST2 cells after a 24-h exposure to the 0.3% DMSO (Ctrl), 10 nM 17β-estradiol (E_2), 1000 nM puerarin (PU), and 1 mg/ml *P. mirifica* extract (PME) as quantified by qRT-PCR. *p < 0.05 compared with the control group.

mRNA gene expression in osteoclasts

After 1-day exposure of BMMs to E_2, PU and PME, no significant differences of *Rank* and *EfnB2* expression in comparison with the control group were detected (Figure 5.2).

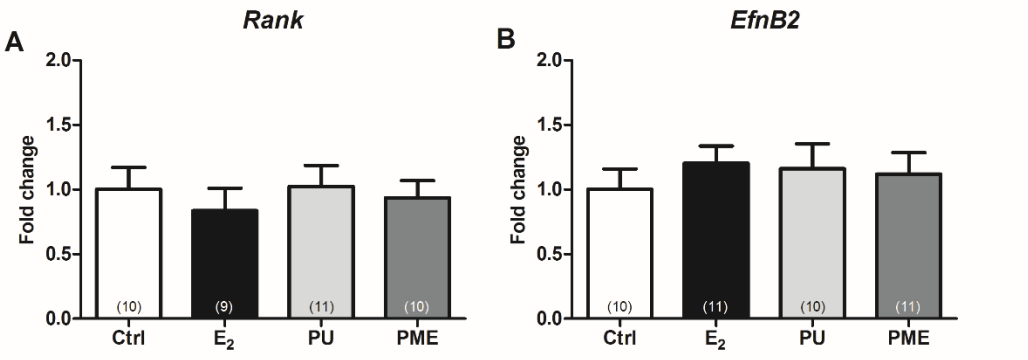

Figure 5.2 The mRNA expression of (A) *Rank* and (B) *EfnB2* from BMMs-preosteoclasts after 24-h exposure to the 0.3% DMSO (Ctrl), 10 nM 17β-estradiol (E_2), 1000 nM puerarin (PU), and 1 mg/ml *P. mirifica* extract (PME).

mRNA gene expression in osteoblasts and osteoclasts coculture

After exposure the osteoblast-osteoclast cells with E_2, PU and PME for 1 day, the mRNA levels of *Rankl, Opg*, *Rankl/Opg* ratio, *Rank*, *EphB4* and *EfnB2* were not significantly different in all three treatment groups compared with the control group (Figure 5.3).

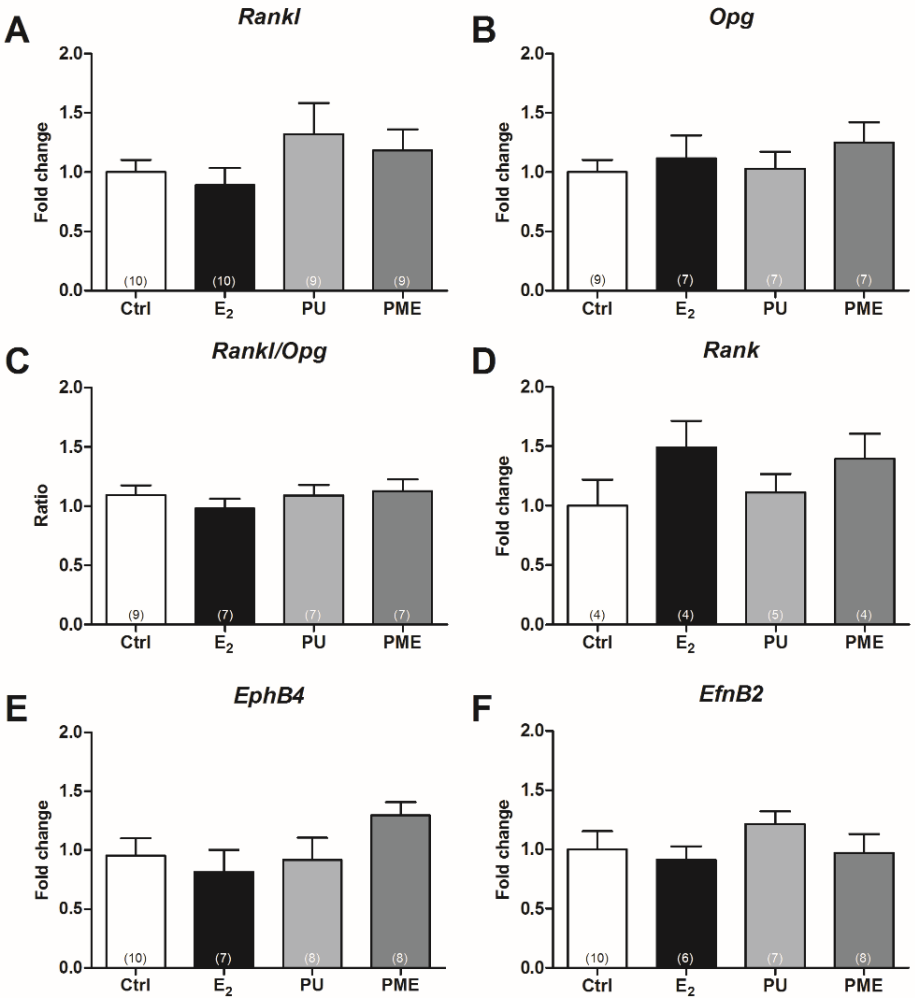

Figure 5.3 The mRNA expression of (A) *Rankl*, (B) *Opg*, (C) Rankl/Opg ratio, (D) *Rank*, (E) *EphB4*, and (F) *EfnB2* from osteoblast-osteoclast coculture after a 24-h exposure to 0.025% DMSO (Ctrl), 10 nM 17β-estradiol (E_2), 1000 nM puerarin (PU), and 1 mg/ml *P. mirifica* extract (PME) as quantified by qRT-PCR.

TRAP staining

Consistent with the results of gene expression, M-CSF and RANKL secreted from ST2-precursor osteoblast could stimulate the differentiation and fusion of BMMs to multinucleated mature osteoclasts under the coculture condition as indicated by TRAP staining. Visually detected, but not counted, treatments of 10 nM E_2, 1000 nM PU and 1 mg/ml PME had no effect on differentiation and formation of multinucleated osteoclasts in the coculture condition (Figure 5.4).

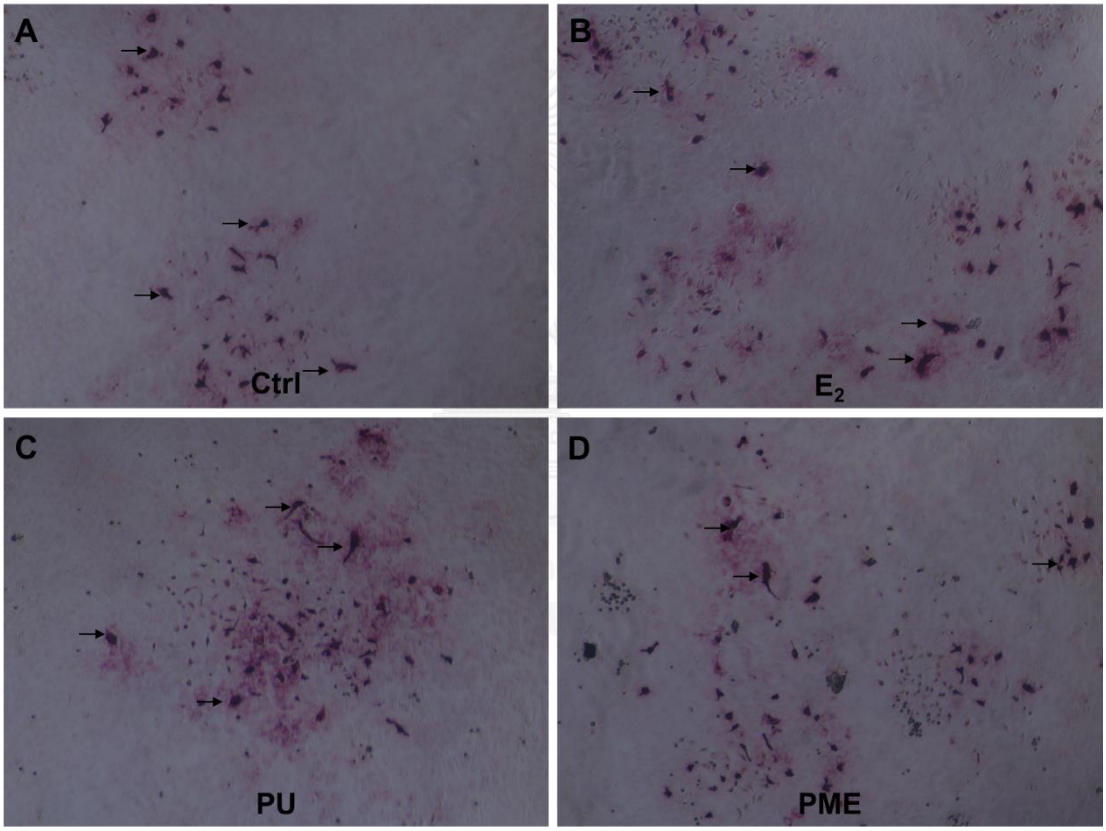

Figure 5.4 The characteristics of multinucleated mature osteoclasts in coculture condition after 24-h exposure to (A) 0.025% (v/v) DMSO (Ctrl), (B) 10 nM E_2, (C) 1000 nM puerarin (PU), and (D) 1 mg/ml *P. mirifica* extract (PME). Arrows indicated TRAP-positive (greater than or equal 3) multinucleated cells.

Discussion

The osteoprotective effects of *P. mirifica* and PU on bone have been studied for a decade (Urasopon et al., 2008). The mechanisms of action revealed that PME and PU directly activated osteoblastogenesis (Tiyasatkulkovit et al., 2012, 2014) and suppressed osteoclastogenesis (see Chapter III) when either osteoblasts or osteoclasts were exposed alone to the treatments. This is the first study to investigate the effects of PME and PU on cross-talk signaling between osteoblasts and osteoclasts. In agreement with the previous study in primary baboon osteoblasts (Tiyasatkulkovit et al., 2014), our study showed that 1 mg/ml of PME did not alter *Opg* mRNA level, but decreased *Rankl* mRNA level, leading to decreased ratio of Rankl/Opg in mouse osteoblast precursor ST2 cells. As this effect was not seen in the PU treatment, it might be possible that other phytoestrogen substances in the PME elicited the suppressive effect on *Rankl* expression. The underlying mechanism of PME on Rankl expression might pass through ERα which directly interacts and inhibits RUNX2, the master regulator of osteoblastogenesis. This is because the treatment of E_2 in osteoblast-like MC3T3-E1 cells (Khalid et al., 2008) suppresses Rankl expression (Frenkel et al., 2010). On the other hand, both estrogen (E_2) and phytoestrogens (PME and PU) did not affect *Rank* mRNA expression in mouse BMMs-preosteoclasts which is similar to that found in murine monocytic cell line RAW264.7 after E_2 treatment (Shevde et al., 2000). It was assumed that phytoestrogens and estrogens controlled the osteoclast differentiation through non-genomic ER pathways and might act after RANKL-induced osteoclast differentiation was completed by suppressing c-JUN (Shevde et al., 2000) and JNK activity (Srivastava et al., 2001). Since RANK was an upstream gene target of RANKL signaling, it was not affected by estrogenic activity of E_2, PME and PU. This suggested that phytoestrogens/estrogens regulated osteoclastogenesis indirectly by limiting the level of RANKL secreted from osteoblasts without directly altering *RANK* expression in BMMs-preosteoclasts.

Even though it was reported that ovariectomy-induced estrogen deficient rats showed the decrease in *EphB4* and *EfnB2* mRNA and protein expression (Yan and Ye, 2015), changes of *EphB4* and *EfnB2* mRNA expression in osteoblast precursor ST2

cells and mouse BMMs-preosteoclasts, respectively, were not observe here. Similarly, E_2 did not change the level of *EphB4* expression in *in vitro* HOC-7 ovarian carcinoma cells (Kumar et al., 2007) and primary macaque neurons (Bethea and Reddy, 2012), but it activated level of *EfnB2* expression in in vivo mouse mammary gland (Nikolova et al., 1998). As EphB4/ephrinB2 complex signaling recently revealed in osteoblast-osteoclast communication (Zhao et al., 2006), the mechanisms of E_2 on the regulation of *EphB4* and *EfnB2* expression in bone cells have been not clearly understood. From the basic knowledge that EphB4 and ephrinB2 regulated cascade of Wnt signaling in both osteoblasts and osteoclasts (Matsuo and Otaki, 2012), it might be possible that E_2, PME and PU did not directly affect the expression of EphB4/EfnB2 but interact with the downstream signaling of this complex such as Wnt pathway.

Interestingly, in the coculture condition when ST2 cells and BMMs differentiated to osteoblast-like cells and osteoclasts, E_2, PME and PU treatments did not alter the mRNA level of *Rankl, Opg, Rank, EphB4* and *EfnB2.* This might be because there are multiple signaling pathways among these two cell types other than RANKL/OPG/RANK and EphB4/ephrinB2 systems. For example, the osteoclastogenesis was also activated through the binding of ligands form osteoblasts with Ig-like receptor (Takayanagi, 2007) or immunoreceptor tyrosine-based activation motive (ITAM; Kuroda et al., 2008) presenting on osteoclast surface Osteoclasts also secreted a chemokine sphingosine-1-phosphate (S1P) which could stimulate osteoblastogenesis and mineralization (Pederson et al., 2009). Similarly, the E_2 suppressed number of osteoclasts by decreasing RANKL trafficking but did not change the levels of *Rankl* and *Opg* mRNA expression in osteoblast-osteoclast coculture (Martin et al., 2015). This suggests that the effects of E_2, PME and PU on the cross-talk signals between cells in coculture condition were different from those in the single cell culture condition.

In conclusion, the present study showed that PME phytoestrogens and E_2 mediates RANKL/OPG/RANK signaling in osteoblast precursor ST2 cells and suppresses osteoclastogenesis, but not in osteoblast–osteoclast coculture and

EphB4-ephrinB2 signaling. These results increase our knowledge and understanding on the mode of action of PME on bone cells, and these data support the development of *P. mirifica* as anti-osteoporotic agent for human use.

Chapter VI
GENERAL DISSCUSSION AND CONCLUSIONS

The anti-osteoporotic effects of *Pueraria mirifica* or white Kwao Krua have been mentioned for a decade (Urasopon et al., 2007; 2008). The mechanism of action of PME and PU on osteoblasts, including osteoblast-like UMR 106 rat cells and primary baboon osteoblasts, has been thoroughly verified (Tiyasatkulkovit et al., 2012, 2014). In the present study, the estrogenic effects of PME and PU in comparison with E_2 on osteoclast and osteoblast-osteoclast coculture were elucidated.

Osteoclasts play an important role for bone remodeling as these cells are responsible for removing damaged bone before osteoblasts migrate to the site and form a new bone. This study investigated the estrogenic effects and mechanisms of action of PME and PU on osteoclasts in three consequential stages; (i) anti-osteoclastogenesis (proliferation, differentiation, multi-nucleated cell formation and function), (ii) induction of apoptosis and (iii) interfering cell-cell communication, respectively. In conclusion, the anti-osteoclastogenic effects of PME and PU are dose deferential effects which acted either directly on osteoclasts or indirectly via the regulation of osteoblasts through RANKL/OPG/RANK signal. The PME and PU decreased BMMs differentiation (of BMMs to mono-nucleated preosteoclasts), multi-nucleated formation (of mono-nucleated cells to multi-nucleated mature osteoclasts) and function, but induced BMMs apoptosis.. Only a high PME dose, but not all 0.1 – 1000 nM dose ranges of PU, decreased BMMs proliferation. After the calculation, it was found that a high (10 mg/ml) PME dose contains 4000 nM PU which much exceeds the highest PU dose used in this study. Thus, it is possible that if the pure PU dose is as high as or greater than 4000 nM, the suppression on proliferation might be seen. The mode of action of PME and PU on osteoclasts occurs through ER binding via the RANKL/OPG/RANK signaling pathway, but not

through the EphB2-EphB4 signaling. It should be noted that PME and PU could not interfere the cross-talk between the osteoblast-osteoclast cells in coculture condition once the cells have been differentiated and started to secrete the cytokines. Generally, osteoblast and osteoclasts have several alternative pathways to commute. Since expression of genes such as *Nfatc1*, *Tm7sf4* and *FasL* was not corresponded with the differentiation and apoptosis of osteoclasts determined, and the low and high doses of PME show the different actions through the ER, the further study needs to be conducted to clarify these several ambiguous points.

Although antiresorptive agents such as bisphosphonate are currently popularly used to remedy bone metabolic disorders, they fully destroy osteoclast function resulting in the impairment of bone remodeling and bone microarchitecture (Suresh et al., 2014). At this point, the effect of PME and PU on bone metabolism was completely investigated in osteoblasts, osteoclasts and coculture of two-cell type. PME and PU activated osteoblastogenesis and enhanced bone formation (Tiyasatkulkovit et al., 2012, 2014), meanwhile it suppressed osteoclastogenesis resulting in the increase in BMD and BMC in gonadectomy-induced osteoporotic rats (Urasopon et al., 2007, 2008; Suthon et al., 2015) (Figure 6.1). This present study corroborates the high potential of PME and PU to be developed as alternative anabolic agents for bone metabolic diseases such as osteoporosis and rheumatoid arthritis for human use.

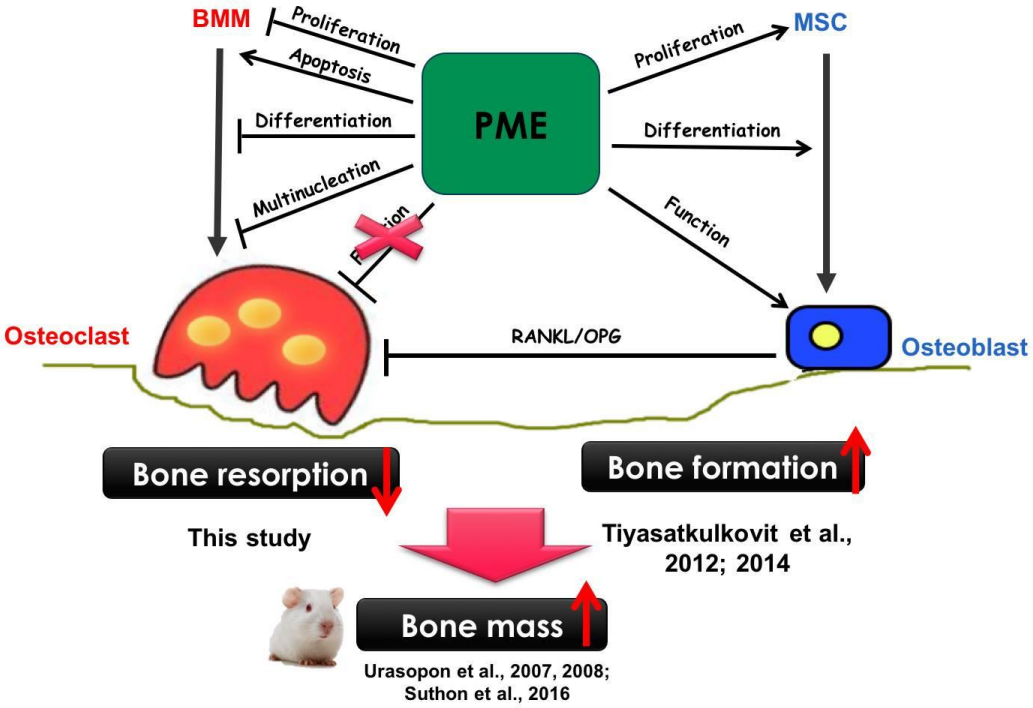

Figure 6.1 Schematic of *P. mirifica* extract (PME) on bone metabolism.

Since the effect on osteoclastogenesis of a high PME dose differs from those of a low PME dose and pure PU, the mechanism of action of a high (10 mg/ml) PME dose on osteoclast differentiation and BMMs apoptosis should be investigated further. Moreover, if PME and PU could disturb the cross-talk between osteoclasts and osteoblasts when the onset of the treatment is set earlier should be tested. Besides, the other possible pathways of communication between these two cells should also be determined.

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APPENDIX

VITA

Miss Sarocha Suthon was born on April 25th, 1991 at Chumphon Province, Thailand. She has graduated a Bachelor of Science in Biology (B.Sc. in Biology with First Class Honor) from the Department of Biology, Faculty of Science, Chulalongkorn University since 2012. She has then continued her study as a graduate student in Master of Science in Zoology, at the Department of Biology, Faculty of Science, Chulalongkorn University. She has been awarded the Royal Government of Thailand Scholarship for her graduate study by the Development and Promotion of Science and Technology Talent Project (DPST) under The Institute for the Promotion of Teaching Science and Technology (IPST) of Thailand since 2009. She has carried on her Master research under the support of the 90th Anniversary of Chulalongkorn University Fund (Ratchadaphiseksomphot Endowment Fund).

Sarocha has published her scientific works in an international ISI journal and presented a part of her Master's thesis in both national and international meetings. She published her research article entitled "Anti-osteoporotic effects of Pueraria candollei var. mirifica on bone mineral density and histomorphometry in estrogen-deficient rats" in the Journal of Natural Medicines (IF2015 = 1.670) in 2016. She also reported a part of her Master's thesis and published as proceedings in the Eleventh Conference on Science and Technology for Youths which was held in Bangkok, Thailand on June 11, 2016. During her graduate practice, she also orally presented a part of her work at the Eighth Congress of the Asia and Oceania Society of Comparative Endocrinology (AOSCE) which was held in Korea University College of Medicine, Seoul, South Korea on June 24, 2016.