การศึกษาผลและกลไกการออกฤทธิ์ของสารไฟโทเอสโทรเจนที่สกัดได้จากกวาวเครือขาว *Pueraria mirifica* และสารไฟโทเอสโทรเจนสังเคราะห์ต่อการสร้างและสูญสลายกระดูกในหลอดทดลอง

นางวัชราภรณ์ ติยะสัตย์กุลโกวิท

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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IN VITRO STUDY OF EFFECTS AND MECHANISMS OF WHITE KWAO KRUA *Pueraria mirifica* EXTRACTED PHYTOESTROGENS AND SYNTHETIC PHYTOESTROGENS ON BONE FORMATION AND RESORPTION

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วัชราภรณ์ ติยะสัตย์กุลโกวิท : การศึกษาผลและกลไกการออกฤทธิ์ของสารไฟโทเอสโทรเจนที่สกัดได้จากกวาวเครือขาว *Pueraria mirifica* และสารไฟโทเอสโทรเจนสังเคราะห์ต่อการสร้างและสูญสลายกระดูกในหลอดทดลอง (IN VITRO STUDY OF EFFECTS AND MECHANISMS OF WHITE KWAO KRUA *Pueraria mirifica* EXTRACTED PHYTOESTROGENS AND SYNTHETIC PHYTOESTROGENS ON BONE FORMATION AND RESORPTION) อ.ที่ปรึกษาวิทยานิพนธ์หลัก : ศ. ดร. สุจินดา มาลัยวิจิตรนนท์, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม Prof. John L. VandeBerg, รศ. นพ. ดร. นรัตถพล เจริญพันธุ์, 141 หน้า.

การศึกษาครั้งนี้มีวัตถุประสงค์เพื่อศึกษาผลและกลไกการออกฤทธิ์ของสารไฟโทเอสโทรเจนที่สกัดได้จากกวาวเครือขาว *Pueraria mirifica* (PM) ซึ่งเป็นพืชสมุนไพรไทยที่มีสารไฟโทเอสโทรเจนหลายชนิด รวมทั้งจีนีสทีอีน (GEN) และพูรารินิ (PU) ต่อการสร้างและการสลายกระดูกในเซลล์ออสทีโอบลาสท์ของหนูแรทสองชนิด คือ เซลล์มะเร็ง (osteosarcoma UMR106) และเซลล์ปฐมภูมิ (primary cell) และเพื่อพัฒนากวาวเครือขาวไปเป็นยารักษาโรคกระดูกพรุนในคน จึงได้ทำการเลี้ยงเซลล์ออสทีโอบลาสท์ปฐมภูมิของลิงบาบูนในหลอดทดลองเป็นครั้งแรก ทำการทดลองบ่มสารสกัด PM, GEN และ PU กับเซลล์ออสทีโอบลาสท์ ทั้งเซลล์มะเร็งและเซลล์ปฐมภูมิของหนูแรท และเซลล์ปฐมภูมิของลิงบาบูน ที่ความเข้มข้นและที่เวลาต่าง ๆ จากนั้นวัดการเจริญของเซลล์ การแสดงออกของยีนที่เกี่ยวกับกระบวนการสร้างและสลายกระดูก และการสะสมแคลเซียมในหลอดทดลอง โดยวิธีบีอาร์ดียู คิวอาร์ที-พีซีอาร์ และย้อมสีอะลิซารินเรด ตามลำดับ พบว่าสารสกัด PM, GEN และ PU ลดการเจริญของเซลล์มะเร็งออสทีโอบลาสท์ของหนูแรท เพิ่มปริมาณการแสดงออกของยีนที่เกี่ยวกับการสร้างกระดูก คือ อัลคาไล ฟอสฟาเทส (ALP) และยีนที่เกี่ยวกับการสลายกระดูก คือ ออสทีโอโปรทีจีริน (OPG) ในขณะที่ปริมาณแรงค์ไลแก้น (RANKL) มีการแกว่งไกว แต่โดยรวมทำให้อัตราส่วนของ RANKL ต่อ OPG ซึ่งเป็นดัชนีบ่งชี้การพัฒนาของเซลล์ออสทีโอคลาสท์ มีค่าลดลง มีการสะสมของแคลเซียมเพิ่มขึ้น พบว่าสารทั้งสามชนิดออกฤทธิ์ผ่านตัวรับเอสโทรเจน (ERs) เนื่องจากเมื่อให้สารที่ไปขัดขวางการจับ ERs (ICI182780) ทำให้ปริมาณการแสดงออกของยีน ALP ลดลง เมื่อทำการศึกษาลำดับถัดไปในเซลล์ออสทีโอบลาสท์ปฐมภูมิของหนูแรทและลิงบาบูน พบว่าเซลล์ทั้งสองชนิดมีการตอบสนองต่อสารสกัด PM, GEN และ PU ที่คล้ายกัน โดยสารทั้งสามชนิดมีผลกระตุ้นการเจริญของเซลล์และการแสดงออกของยีนที่เกี่ยวกับการสร้างกระดูก (ALP ในหนูแรท และ ALP และ type I collagen ในลิงบาบูน) อย่างไรก็ตามพบว่าปริมาณการแสดงออกของ RANKL และอัตราส่วนของ RANKL ต่อ OPG ในเซลล์ออสทีโอบลาสท์ของลิงบาบูนลดลง ในขณะที่ไม่มีการเปลี่ยนแปลงของยีนดังกล่าวในเซลล์ออสทีโอบลาสท์ของหนูแรท โดยสรุป สารสกัด PM กระตุ้นการสร้างกระดูก โดยไปเพิ่มการแสดงออกของยีนที่เกี่ยวกับการพัฒนาของเซลล์ออสทีโอบลาสท์ และยับยั้งการแสดงออกของยีนที่เกี่ยวกับการพัฒนาของเซลล์ออสทีโอคลาสท์ ที่ผ่าน ERs ซึ่งเป็นการยืนยันว่า PM เป็นพืชสมุนไพรที่มีศักยภาพสูง น่าที่จะพัฒนาไปเป็นยารักษาโรคกระดูกพรุนเพื่อใช้ในคน ต่อไป และจากผลการตอบสนองที่แตกต่างกันของเซลล์กระดูกทั้งสามชนิดต่อสารสกัด PM จึงกล่าวได้ว่าในการศึกษาผลและกลไกการออกฤทธิ์ของสารสกัด PM ในหลอดทดลองควรใช้เซลล์ออสทีโอบลาสท์มากกว่าหนึ่งชนิด

สาขาวิชา วิทยาศาสตร์ชีวภาพ ลายมือชื่อนิสิต .....

ปีการศึกษา 2555 ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก .....

ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม .....

ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม .....

5173855823 : MAJOR BIOLOGICAL SCIENCES
KEYWORDS: *Pueraria mirifica* / OSTEOPOROSIS / UMR106 / RATS / BABOON
WACHARAPORN TIYASATKULKOVIT: IN VITRO STUDY OF EFFECTS AND MECHANISMS OF WHITE KWAO KRUA *Pueraria mirifica* EXTRACTED PHYTOESTROGENS AND SYNTHETIC PHYTOESTROGENS ON BONE FORMATION AND RESORPTION. ADVISOR: PROF. SUCHINDA MALAIVIJITNOND, Ph.D., CO-ADVISOR: PROF. JOHN L. VANDEBERG, Ph.D., ASSOC. PROF. NARATTAPHOL CHAROENPHANDHU, M.D., Ph.D., 141 pp.

This study aimed to investigate the effects and mechanisms of actions of *Pueraria mirifica* (PM) extract, a Thai herb which contained various types of phytoestrogens, including genistein (GEN) and puerarin (PU), on bone formation and resorption process in two types of rat osteoblast cells, osteosarcoma UMR106 and primary cells. To develop the PM as an alternative drug for osteoporosis treatment in humans, the culture system of baboon primary osteoblast cells was first established. UMR106 rat osteosarcoma cells, rat and baboon primary osteoblast cells were incubated with PM extract, GEN and PU in different doses and time-courses. Cell proliferation, mRNA expression of genes associated with bone formation and resorption, and mineralization processes of osteoblast cells were determined by BrdU assay, qRT-PCR and Alizarin Red S staining, respectively. In the UMR106 cells, PM extract, GEN and PU decreased cell proliferation, increased mRNA expression of the gene associated with bone formation, i.e., alkaline phosphatase (ALP), and the gene associated with bone resorption, i.e., osteoprotegerin (OPG), while the mRNA levels of receptor activator of nuclear factor-κB ligand (RANKL) were varied, however, the RANKL/OPG ratios, an indicator of bone resorption process, were decreased. The *in vitro* calcium deposition was also increased. The effects of the PM extract and phytoestrogens were passed through the estrogen receptors (ERs) by observing the abolishment of ER antagonist (ICI182780) on expression of ALP mRNA. Further study in rat and baboon primary osteoblast cells indicated that the responses of these two cell types to the PM extract, GEN and PU were nearly in the same line. All three treatments stimulated cell proliferation and expression of genes associated with bone formation (ALP in rat, and ALP and type I collagen in baboon osteoblast cells). However, the expression of RANKL mRNA levels and the RANKL/OPG ratios in baboon osteoblasts were decreased, whilst no alterations of those genes were observed in primary rat osteoblasts. In conclusion, the PM extract induced bone formation by enhancing expression of genes associated with osteoblast differentiation and suppressing expression of genes associated with osteoclast differentiation, in an ER-dependent manner. This study corroborates the high potential of the PM herb to be developed as anti-osteoporotic drug for human use. In regard to the different responses of these three cell types to the PM extract, it suggests that research on effects and mechanisms of actions of PM extract *in vitro*, at least more than one cell types of osteoblasts should be used.

Field of Study: Biological Sciences Student's Signature ……..……....

Academic Year: 2012 Advisor's Signature …….....

Co-advisor's Signature …….....

Co-advisor's Signature …….....

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LIST OF ABBREVIATIONS

ALP	=	Alkaline phosphatase
BCA	=	Biconchoninic acid
BMC	=	Bone mineral content
BMD	=	Bone mineral density
BrdU	=	5-Bromo-2´- deoxyuridine
cDNA	=	Complementary deoxyribonucleic acid
cm^2	=	Square centimeter
DMEM	=	Dulbecco's modified Eagle's medium
DMSO	=	Dimethyl sulfoxide
E_2	=	17β-estradiol
ECM	=	Extracellular matrix
ER	=	Estrogen receptor
ERα	=	Estrogen receptor alpha
ERβ	=	Estrogen receptor beta
ERT	=	Estrogen replacement therapy
GH	=	Growth hormone
HPLC	=	High performance liquid chromatography
LC/MS	=	Liquid chromatography/ mass spectophotometry
IL	=	Interleukin
μg	=	Microgram

µl	=	Microliter
µM	=	Micromolar
mg	=	Milligram
min	=	Minute
ml	=	Milliliter
mm	=	Millimeter
mM	=	Millimolar
mRNA	=	Messenger ribonucleic acid
MTT	=	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
nM	=	Nanomolar
nm	=	Nanometer
OPG	=	Osteoprotegerin
Osx	=	Osterix
PBS	=	Phosphate-buffered saline
PCR	=	Polymerase chain reaction
PM	=	*Pueraria mirifica*
PTH	=	Parathyroid hormone
RNA	=	Ribonucleic acid
qRT-PCR	=	Quantitative real-time polymerase chain reaction
RANKL	=	Receptor activator of nuclear factor kappa-β ligand
Runx2	=	Runt-related transcription factor 2
SERMs	=	Selective estrogen receptor modulators
TGF	=	Transforming growth factor
TNF	=	Tumor necrosis factor
Vol	=	Volume

CHAPTER I

GENERAL INTRODUCTION

Osteoporosis is a disease in which the density and quality of bone are reduced which leads to the weakness and increased risk of fracture of the skeleton, rate of morbidity and mortality (Lane, 2006; Cole et al., 2008). Osteoporosis and associated fractures are a major public health problem in aging population worldwide (Lane, 2006). The risk for osteoporotic fracture in women is greater than in men (30-50% in women and 15-30% in men; Randell et al., 1995; Wright, 2006). Additionally, about half of women over the age of 50 have a fracture of the spine, wrist or hip. The annual incidence rate of osteoporotic fractures in women is also greater than the combined incidence rates of heart attack, stroke and breast cancer. Approximately, 1.6 million hip fractures occur worldwide in each year and the incidence could increase up to 6.3 million by the year 2050 (Cole et al., 2008). There is an increase in incidence of hip fractures in the developed cities in Asia. In Thailand, the number of the cases of hip fracture for men and women is 114 and 289 per 100,000 populations (Damodaran et al., 2000). Together with the increasing in number of hip fracture, the prevalence of osteoporosis in Thai women progressively increases in relation to the advancing age, more than 50% of the prevalence is occurred in women aged over 70 years (Limpaphayom et al., 2001). Since the medical cost for osteoporosis is expensive, this can cause the financial burden to the elderly. Over the next 20 years, the costs of medical care of osteoporosis-related fractures in the non-White population could raise from 1.9 billion (12% of total costs) in 2005 up to over 4.7 billion (19% of total) by 2025 (Berge et al., 2007).

Bone is a dynamic tissue that serves in mechanical as well as homeostatic functions. It undergoes a continual self-regeneration process that called remodeling. Remodeling is continuous and coordinates a cycle of removal of old bone by osteoclast (or bone resorbing) cells which is followed by the deposition of new bone by osteoblast (or bone forming) cells in response to micro damage and variable mechanical loadings throughout life (Sommerfeldt and Rubin, 2001). The underlying

mechanism in all cases of osteoporosis is an imbalance between bone resorption and bone formation. The three main mechanisms by which osteoporosis developed are an inadequate peak bone mass (the skeleton develops insufficient mass and strength during growth), excessive bone resorption, and inadequate formation of new bone during remodeling (Raisz, 2005). Osteoporosis develops less often in men than in women because men have larger skeletons, their bone loss starts later and progresses more slowly, and they have no period of rapid hormonal change (i.e., menopause as it occurs in women) (Wright, 2006).

In women, estrogen deficiency during the postmenopausal period has been recognized as a critical factor in osteoporosis development. Estrogen deficiency strongly determines the increasing rate of bone resorption as well as decreasing the deposition (Erben et al., 2000). Therefore, entering the menopause with a sudden loss of estrogen could result in decrease in bone mineral density (BMD) and bone mineral content (BMC) (Riggs et al., 1982; Ohta et al., 2002). In men, it was traditionally assumed that decreased serum testosterone level was responsible for age-related bone loss**.** However, in these recent years, research results have provided evidences that estrogen also played a role in bone homeostasis in men as well as women (Smith et al., 1994; Khosla et al., 2001). The sources of estrogen in men are both intratesticular and extragonadal, which play a physiological significance throughout adult life. For example, men with a mutation of the gene encoding the aromatase enzyme or a mutation of the estrogen receptor (ER) exhibited a failure of epiphysial fusion, osteopenia and delayed bone age (Smith et al., 1994). During aging in men, not only serum testosterone level was decreased, but bioavailable estradiol level was also decreased (Janssens and Vanderchueren, 2000). In addition, a combination of cross-sectional and observational studies of aging men showed a better correlation between serum estradiol and BMD than a testosterone and BMD at various skeletal sites (Slemenda et al., 1997; Khosla, 2008; Mellstrom et al., 2008)

Estrogen has long been known to influence growth, differentiation and function of bone during the prepubertal, adulthood and old age. The main function of estrogen is to preserve bone and to increase endocortical bone formation during puberty. After menopause, estrogen level dramatically decreases in women and bone loss occurs (Ohta et al., 2002; Jarvinen et al., 2003). Estrogen exerts its effects on target cells by

interacting with specific ERs. Two types of ERs are present in bone, ERα and ERβ (Mosselman et al., 1996; Kuiper, 1998; Centrella and McCarthy, 2012). *In vitro* studies indicated the presence of ERα in osteoblast cells of mouse (Bellido et al., 1993), rat (Damien et al., 2000) and human osteosarcoma cell line or osteoblast-like cell (Ikegami et al., 1994).The relative abundances of ERα and ERβ vary throughout the skeleton. ERα is predominantly expressed by cortical bone cells, whereas both isoforms are expressed by trabecular bone content in female mice. ERβ has been detected in rat osteoblast and rat osteosarcoma cell line (UMR-106) (Onoe et al., 1997) and human osteoblast cell line SV-HFO (Arts et al., 1997).

Although hormone replacement therapy (HRT) is effective in reducing bone loss, it is recently reported that HRT is associated with a high risk of breast, endometrial and ovarian cancers, venous thromboembolism and stroke. Several agents, such as bisphosphonates, calcitonin and raloxifene are available for osteoporosis treatment, they however exhibit various severity in adverse side effects and very expensive. For example, bisphosphonates are associated with gastrointestinal side effects, osteonecrosis of the jaw and subtrochonteric fracture (Manolagas et al., 2002). Regarding these problems, alternative drugs for bone loss therapy with less adverse effects and inexpensive are being sought by researcher throughout the globe.

Phytoestrogens are plant-derived non-steroidal compounds that bind to ERs and have estrogen-like biological activities (Tham et al., 1998). Common phytoestrogens can be found in various natural dietary sources, such as, soybean, soy product, whole grain breads, vegetables and tea. Phytoestrogens are generally categorized into three major classes according to the chemical structure: isoflavones, lignans, and coumestans. Soy food and soybeans supply the most significant dietary source of isoflavones (Setchell, 1998). The main soy isoflavones, genistein, daidzein and glycitein, are structurally similar to 17β-estradiol (E_2) and are considered as a potential alternative to estrogen replacement therapy (Cornwell et al., 2004).

Pueraria mirifica, is a Thai phytoestrogen-rich herb which distributes throughout only Thailand (Cherdshewarard et al., 2007b) and known in Thai as white Kwao Krua. Its tuberous root is composed mainly of isoflavones, such as, puerarin, daidzin, genistin, daidzein, genistein (Cherdshewasart et al., 2007b; Cherdshewasart and Sriwatcharakul, 2007) and miriestrol (Chansakaow et al., 2000)

which exhibit estrogenic activity as that of estrogen. The estrogenic effects of *P. mirifica* on reproductive organs have been thoroughly examined in female as well as in male laboratory animals (Malaivijitnond et al., 2004, 2006; Trisomboon et al., 2004, 2005, 2006; Cherdshewasart et al., 2007a). *P. mirifica* could also reduce serum parathyroid hormone (PTH) and calcium levels in female cynomolgus monkeys (Trisomboon et al., 2004). In addition, *P. mirifica* was also reported to increase the BMD and BMC in gonadectomy-induced osteoporotic rats (Urasopon et al., 2007, 2008).

Although estrogenic effects of *P. mirifica* on bone loss prevention in osteoporotic rats were clearly shown (Urasopon et al., 2007, 2008), no reports explain how *P. mirifica* affect the bone and what the mechanisms of actions are. Based on these reasons, the mechanisms of action of *P. mirifica* extract, in comparison with the synthetic phytoestrogens (genistein and puerarin) and synthetic estrogen (E_2), on bone formation and resorption in rat osteosarcoma cell line (UMR 106) and primary osteoblast cells derived from tibia and fibular of adult female rats and baboon were determined in the present study.

This thesis could establish for the first time baboon primary osteoblast cell culture. Although a lot of baseline data of bone biology and physiology in rodents have been accumulated, the bone biology of rodents is different from those of humans. Rodents are lacking of the Harversian system in bone and their growth plate remained open until the end of their life, resembling immature skeleton (Jerome and Peterson, 2001; Bagi et al., 2011). Baboons are the closes living animals to humans which have a lot of similarity in their anatomy and physiology to humans (Turner, 2001), including having a osteon (or Haversian) remodeling in cortical bone as observed in humans (Aufdemort et al., 1993; Mahaney et al., 1995; Wang et al., 1998; Phelps et al., 2000). In that regard, the baboon model should fill up the gap between rodent and human bone research.

OBJECTIVES

1. To determine if and how *P. mirifica*-extracted phytoestrogens and synthetic phytoestrogens affect the formation and resorption of rat osteosarcoma cell line (UMR 106).

2. To determine if and how *P. mirifica-extracted* phytoestrogens and synthetic phytoestrogens affect the formation and resorption of rat primary osteoblast cells.

3. To establish the baboon primary osteoblast culture system of which the translation of the results to human application is needed.

4. To determine if and how *P. mirifica*-extracted phytoestrogens affect the formation and resorption of baboon primary osteoblast cells.

5. To compare the mechanisms of actions of *P. mirifica*-extracted phytoestrogens and synthetic phytoestrogens on the expression of bone formation and resorption gene markers between rat osteosarcoma cell line, rat and baboon primary osteoblast cells.

ANTICIPATED BENEFITS

1. To understand the mechanisms of actions how *P. mirifica* herb–extracted phytoestrogens and synthetic phytoestrogens acts on bone remodeling of rat and baboon osteoblast cells.

2. To apply the knowledge gained from this study for the next step of the preclinical trial of *P. mirifica* in nonhuman primates *in vivo*.

3. To provide basic information for the development of anti-osteoporotic drugs, based on *P. mirifica*-extracted phytoestrogens, for the human use.

CHAPTER II

LITERATURE REVIEW

Phytoestrogens have recently attracted attention for their potential in the prevention and therapeutics of postmenopausal osteoporosis, in regard to their binding affinities to ERs and estrogen-like biological activities (Tham et al., 1998). Many scientific reports indicated that soy phytoestrogens, such as genistein, daidzein and glycitein, are structurally similar to E_2 and are considered as a potential alternative to estrogen replacement therapy in osteoporosis treatment (Cornwell et al., 2004). *P. mirifica*, a Thai phytoestrogen- rich herb, were reported to comprise of mostly isoflavones, such aspuerarin, daidzin, genistin, daidzein and genistein (Cherdshewasart et al., 2007b; Cherdshewasart and Sriwatcharakul, 2007). Recently, it has been demonstrated to possess an estrogenic activity on bone by increase in BMD and BMC in osteoporotic rats induced by gonadectomy (Urasopon et al., 2007, 2008). However, the underlying cellular mechanisms of *P. mirifica* on bone were not known.

This chapter is first introducing the readers to bone physiology which includes bone structure, bone cells and functions, bone remodeling and its regulation. Secondly, the phytoestrogens are reviewed in the relation to their source, metabolism, estrogenic activity and their effects on bone metabolism, such as bone formation and bone resorption. Lastly, the source, chemical components, and estrogenic activity of *P. mirifica* on reproductive organs and bones are focused.

1. Bone physiology

1.1 Bone structure

Bone, the major component of the skeleton, is a special connective tissue hardened by mineralization with calcium and phosphate in the form of hydroxyapatite [3Ca3(PO4)2·(OH)2]. It has a well-recognized mechanical function by providing protection and supporting for the body structures, aiding locomotion and being a

major source of calcium ion in the body. Bone is a highly dynamic structure which is constantly remodeled throughout life (Sommerfeldt and Rubin, 2001). Morphologically, there are two forms of bone; a hard outer shell, called cortical (or compact) bone, and an inner network of fiber, called trabecular (or cancellous) bone (Figure 2.1). Cortical bone, which comprises 80% of the skeleton mainly on the outer part, is dense and compact. It has a slow turnover rate and high resistant to bending and torsion. The major part of the cortical bone is calcified, which functions to provide mechanical strength and protection, but it can also participate in metabolic responses, particularly when there is severe or prolonged mineral deficit. Trabecular bone has a loosely organized, porous matrix and represents 20% of the skeleton mass. It is found inside the long bone throughout the bodies of the vertebrae and in the inner portion of pelvis and other large flat bones. Trabecular bone is less dense, more elastic, and has a higher turnover rate than cortical bone, and thus exhibits a major metabolic function. Trabecular bone contributes to mechanical support and provides the initial supplies of mineral in acute deficiency states (Sommerfeldt and Rubin, 2001; Marks et al., 2002; Sambrook and Cooper, 2006)

All bones are composed of an organic matrix, bone cells and mineral elements. The organic bone matrix comprises 95% of collagen fiber, mainly type I collagen, and 5% of non-collagenous protein, such as osteoponin, osteocalcin, sialoprotein, thrombospondin and fibronectin (Marks et al., 2002). The major non-collagenous protein in bone matrix is osteocalcin (bone Gla protein), which plays a role in calcium binding, stabilization of hydroxyapatite in the matrix, and regulation of bone formation. Osteocalcin is a negative regulator of bone formation, which appears to inhibit premature or inappropriate mineralization.

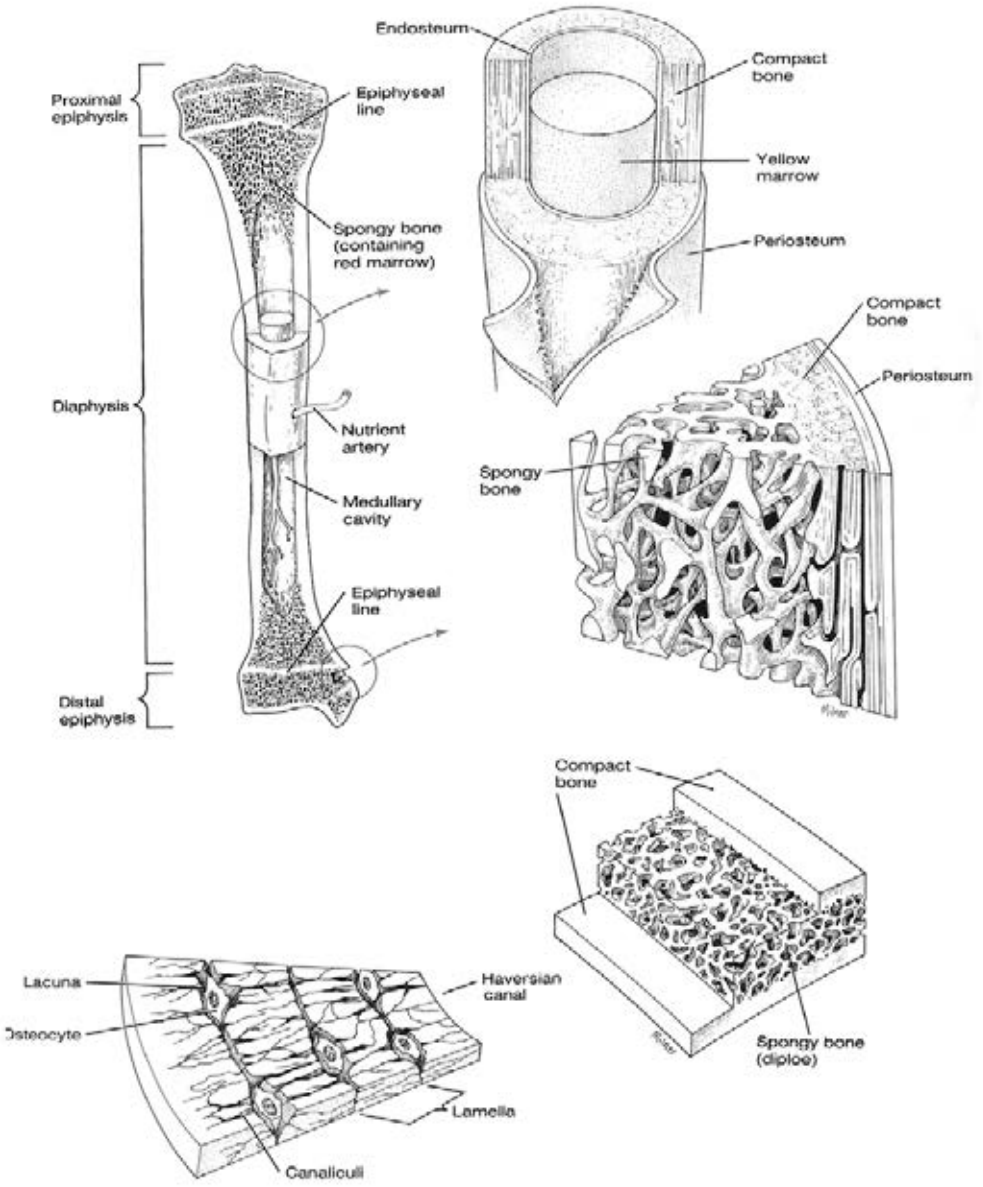

Figure 2.1 Bone structure. Bone is composing of cortical and trabecular compartments. Cortical bone is mainly in diaphysis sites, and trabecular bone is mainly in metaphysis and epiphysis sites.

(Available in: http://www.bathead.com/ANATOMY/bone1.jpg)

1.2 Bone cells and functions

Bone is composed of four different cell types, osteoblast, osteocyte, bone lining cells and osteoclast. These bone cells are necessary for bone growth and bone development. All bone cells found in our body have specific location and functions. Osteoblast, osteoclast and bone lining cells are present on bone surface, whereas osteocytes permeate the mineralized interior (Figure 2.2). Osteoblasts, osteocytes and bone lining cells originate from mesenchymal stem cells, whereas osteoclasts arise from the fusion of mononuclear precursors, which originate in the various hemopoietic tissues (Sambrook and Cooper, 2006) .

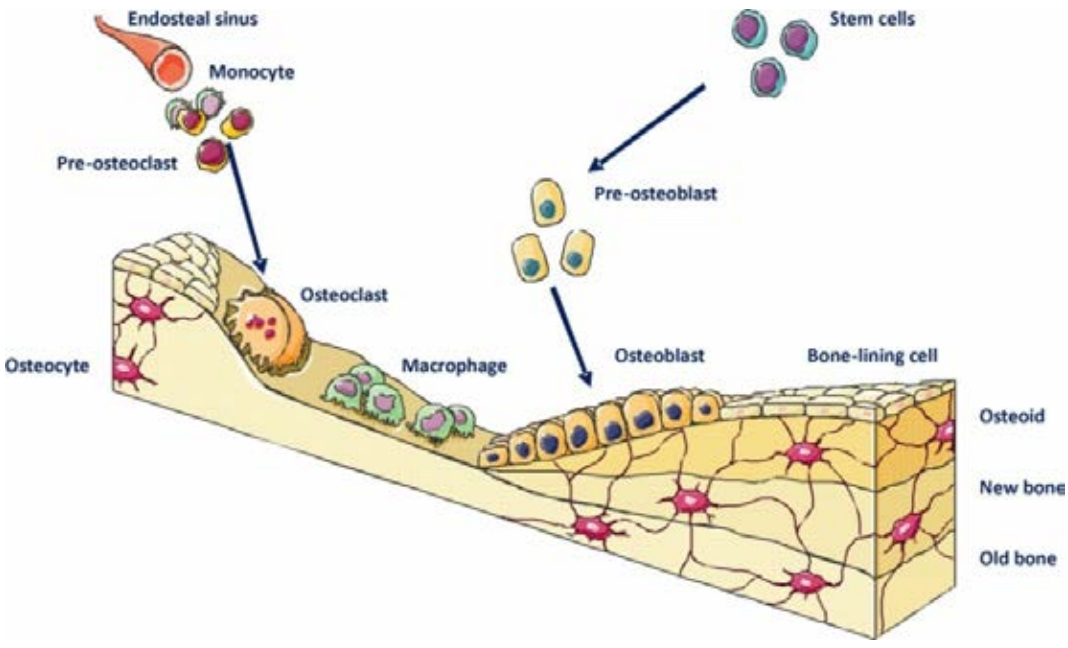

Figure 2.2 Bone cells. Bone is composed of four different cell types, osteoblast, osteoclast, osteocyte and bone lining cells.

(Available from: http://www.intechopen.com)

1.2.1 Osteoblasts

Osteoblasts have a very important role in creating and maintaining skeletal architecture. These cells are responsible for the production of bone matrix, mineralization, and regulation of osteoclasts. Osteoblasts are mononuclear and when they are active, a large Golgi apparatus and an abundant rough endoplasmic reticulum are visible. It secrets type I collagen and the non-collagenous proteins of the bone matrix. Generally, bone formation occurs in three successive phases: the production and the maturation of osteoid matrix following by mineralization of the matrix. Osteoblasts produce osteoid by rapidly depositing collagen and follow by an increase in the mineralization rate to equal that of collagen synthesis. In the final stage the rate of collagen synthesis decreases and mineralization continues until the osteoid become fully mineralized. Osteoblasts form tight junctions with adjacent osteoblasts and have regions of the plasma membrane specialized in vesicular trafficking and secretion. As they differentiate, osteoblasts acquire the ability to secrete bone matrix. Ultimately, some osteoblasts become trapped in their own bone matrix, changing their phenotype and developing into osteocytes, which gradually stop secreting osteoid (Sommerfeldt and Rubin, 2001).

1.2.2 Osteocytes

Osteocytes are mature osteoblast that have been trapped in the bone matrix and are the most abundant cells in bone. Osteocyte functional activity and morphology vary according to cell age. A young osteocyte has most of structural characteristic of the osteoblast but decreases in cell volume and capacity of protein synthesis. An older osteocyte, locating deeper within the calcified bone, presents with a further decrease in cell volume and an accumulation of glycogen in the cytoplasm. Each osteocyte occupies a space or lacunae within the matrix and extends filopodial processes through the canaliculi in the matrix to contact processes of adjacent cells. These cells have the capacity not only to synthesize, but also reabsorb matrix to a limited extent. Osteocyte cells communicate with each other and with the surrounding medium through extensions of their plasma membrane. Because the diffusion of nutrient and metabolites through the mineralized matrix is limited, filopodial connection permits the communication between the neighboring osteocytes and internal and external bone

to the blood vessels transversed the matrix. Therefore, osteocytes are thought to act as mechanosensors, instructing osteoclasts to resorb bone and osteoblasts to form the new osteocyte (Lozupone et al., 1996).

1.2.3 Bone lining cells

Bone lining cells are flat, elongated, inactive cells that cover bone surface that are undergoing neither bone formation nor bone resorption. An important aspect concerning bone lining cells is that the retraction or removing of these cells is a mandatory step in starting osteoclastic bone resorption. Retraction exposes the underlining osteoid to proteolytic enzymes of osteoclasts, and further to osteoclastic resorption (Lozupone et al., 1996).

1.2.4 Osteoclasts

Osteoclasst are large, multinucleated cells which resorb bone. When active, osteoclasts directly approach on the bone surface and have two plasma membrane specializations: a ruffled border and a clear zone. The ruffle border is the central, highly infolded area of the plasma membrane where the bone resorbtion is taken place. The clear zone is a multifilament-rich, organelle-free area of the plasma membrane that surrounding the ruffled border and serves as points of attachment of osteoclast to the underlying bone matrix. Active osteoclasts exhibit a characteristic polarity. Nuclei are typically located in the part of cell most removed from bone surface and are interconnected by cytoskeleton proteins. Osteoclasts contain multiple circular nuclear Golgi stacks, a high density of mitochondria, and abundant lysosomal vesicles that arise from the Golgi and cluster near ruffled border. Osteoclasts resorb bone by acidification and proteolysis of the bone matrix and of the hydroxyapatite crystals encapsulated within the sealing zone. The first process occurring during bone matrix resorption is mobilization of the hydroxyapatite crystal by digestion of their link to collagen. Then the residual collagen fibers are digested by either cathepsins or activated collagenases, and the digested residues are internalized and transport across the cells and released at the basolateral domain (Sommerfeldt and Rubin, 2001).

1.3 Bone remodeling and its regulation

Bone remodeling is a lifelong process and composed of two sub-processes, bone formation from osteoblast cells and bone resorption from osteoclast cells. During childhood and the beginning of adulthood, bone becomes larger, heavier and denser by the process of bone modeling, of which formation exhibits a higher rate than resorption. Even after bones have reached the adult shape and size in a fully growth body, renewal of bone is essential for maintenance of bone strength throughout life. In homeostatic equilibrium, resorption and formation are balanced so that old bone is continuously replaced by new tissue which adapts to mechanical load (Frost, 1990). An imbalance in bone remodeling can lead to osteoporosis. Osteoclast and osteoblast closely collaborate in remodeling process in what is called a basic multicellular unit (BMU). The organization of the BMUs in cortical and trabecular bone differs, mainly on morphological rather than biological aspects. In cortical bone, the BMU forms a cylindrical canal about 2,000 μm long and 150-200 μm wide and gradually burrows through the bone with a speed of 20-40 μm /day. During a cycle, 10 osteoclasts dig a circular tunnel in the dominant loading direction and they are followed by several thousand of osteoblasts that fill the tunnel. In this manner, between 2% and 5% of cortical bone is being remodeled each year. The trabecular bone is more actively remodeled than cortical bone due to the larger surface to volume ratio. Osteoclasts travel across the trabecular surface with a speed of 25 μm/day, and dig a stretch with a depth of 40-60 μ (Martin et al, 1998; Hadjidakis and Androulakis, 2006)

Remodeling sequence (Figure 2.3) is initiated by the activation of the bone surface prior to resorption, through the retraction of bone lining cells and the digestion of endosteal membrane by collagenase action. Once exposed, the mineralized surface attracts the circulating osteoclasts coming from the nearby vessels. This activation takes about three days after this period of time, newly formed osteoclasts begin to resorb bone throughout the process of tunneling in cortical bone and surface erosion in trabecular bone. The transition from osteoclastic to osteoblastic activity (reversal phase) takes about four weeks. The formation is initiated with a single layer of mineralized tissue (cement line) and followed by deposition of consecutive layers of osteoid. The formation phase in adult humans averages about three months. During formation, osteoid mineralization begins after a period of about ten days (lag

mineralization time). Once begun, approximately 60% of the mineralization occurs within a few days, but it is not completed until about six months. When the refilling is completed (resting phase), the osteoblasts become osteocytes or bone lining cells (Sambrook and Cooper, 2006).

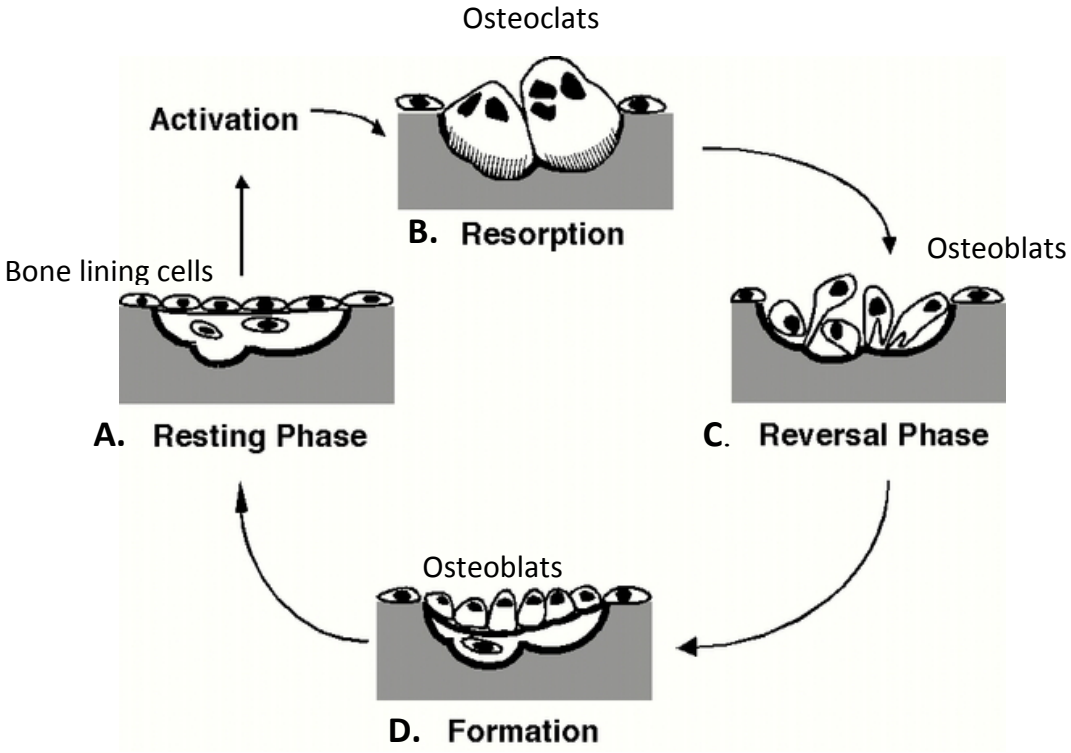

Figure 2.3 Bone remodeling sequence starts when the lining cells reveal the bone surface upon activation (A). Osteoclast precursors arrive at the site and become active osteoclasts as they start to dig out a resorption pit (B). When the osteoclasts have finished the process of resorption, osteoblasts are attracted to the site (C). These osteoblasts lay down the organic matrix (mainly type I collagen), which is subsequently mineralized (D). At the end of this process, old bone has been replaced by new bone.

(Available from:http://www.endotext.org/parathyroid/parathyroid1/parathyroid1.html)

The balance bone formation and resorption is influenced by hormones and many other proteins secreted by both hemopoietic bone marrow cells and bone cells. There are both systemic and local regulations of bone cell function (Sambrook and Cooper, 2006).

1.3.1 Endocrine regulation of bone remodeling

Parathyroid hormone

Parathyroid hormone or parathormone (PTH) is a hyperglycemic hormone which is produced in parathyroid glands in response to hypocalcemia and functions in stimulating bone resorption. PTH controls the homeostasis of calcium by showing a direct action on the bone and kidney, and indirectly on the intestine. PTH could exhibit a dual effect of formation and resorption when it is continual and intermittent supply. PTH would stimulate bone resorption through the synthesis of a factor favoring osteoclastogenesis on the part of osteoblastic cells, that is, the receptor activator of nuclear factor κB ligand (RANKL), and stimulate the formation by increase in growth factors (Swarthout et al., 2002).

Calcitonin

Calcitonin is produced in the C or parafollicular cell of thyroid, it is the inhibitor of bone resorption by reducing the number and activity of osteoclast. In addition, calcitonin enhances osteoblastic bone formation (Wallach et al., 1993)

1, 25$(OH)_2$ vitamin D_3 or calcitriol

Vitamin D is a steroid hormone which favors the intestinal absorption of calcium and phosphate, and therefore bone mineralization. It is necessary for normal growth of the skeleton. Numerous effects of vitamin D on bone have been demonstrated. As a transcriptional regulator of bone matrix proteins, it induces the expression of osteocalcin and suppresses synthesis of type I collagen. In cell cultures, vitamin D stimulates differentiation of osteoblasts and provides the proper balance of calcium and phosphorus to support mineralization (Deluca, 2004).

Estrogens

Estrogens play a major role in the development of the skeleton in both male and female, during adolescence. Estrogens have dual effect on bone metabolism. On the one hand they favor bone formation, increasing the number and function of the osteoblast, and on the other hand they reduce resorption. ERs have been described in human osteoblasts, osteocytes, and osteoclasts (Centrella and McCarthy, 2012). Estrogens can increase the level of osteoprotegerin (OPG), a protein produced from osteoblast that inhibits bone resorption, therefore they play an important role in the regulation of osteoclastogenesis. For this reason, estrogen deficiency during menopause constitutes the most important pathogenic factor in bone loss (Anandarajah, 2008).

Androgens

Androgens have an anabolic effect on bone through the stimulation of the osteoblast receptors, increase in cortical bone size and maintain trabecular bone mass. They act as mediators of the peak growth hormone (GH) in puberty. Androgen deficiency is associated with lower bone density (Vanderschueren et al., 2004).

Growth hormone

Growth hormone (GH) acts both directly and indirectly on bone. GH acts directly on osteoblast cell via its receptors binding, thus increases the synthesis of collagen, osteocalcin and alkaline phosphatase. The indirect action is produced through an increase is synthesis of insulin- like growth factor (IGF) I and II by osteoblast. These factors stimulate the proliferation and differentiation of osteoblast or increasing number and function. GH has been considered as a local growth factor, since it is not only synthesized in the adenohypophysis, but also in almost all the cells of organism, including osteoblast, having both autocrine and paracrine effect in addition to the endocrine (Ohlsson et al., 1998).

1.3.2 Local regulation of bone remodeling

Bone remodeling also regulated by local factors. These are principally growth factors and cytokines, and recently the bone matrix proteins have been implicated as modulators of other local factors.

Growth factors

These are polypeptide produced by the bone cells themselves or intra-osseous tissue, and act as modulators of the cellular functions, fundamentally growth, proliferation and differentiation. IGF- I and II, synthesized by liver and osteoblast, increase the number and function of osteoblast and stimulate collagen synthesis. Transforming growth factor (TGF)-β is a potent stimulator of bone formation by promoting osteoblastic differentiation and synthesis of osteoid matrix. Likewise, TGF-β inhibits resorption by decreasing in differentiation of osteoclast and stimulating their apoptosis. Bone morphogenic protein (BMP), which is included in TGF-β family, is abundant in bone tissue and considered to be the most powerful factor in osteoblast differentiation (Baylink et al., 1993).

Cytokines

Cytokines are polypeptide synthesized in the lymphocyte and monocyte cells and play an important role in multiple cellular functions, such as the immunological response, inflammation and hematopoiesis. Cytokines which are importance in bone are interluekin1 (IL-1), interleukin 6 (IL-6), interleukin 11 (IL-11) and prostaglandin (PG). IL-1 directly stimulates osteoclastic resorption and inhibiting the apoptosis of osteoclasts. IL-6 is also stimulates bone resorption and is produced in response to PTH, IL-1 and 1, 25(OH) $_2$ vitamin D_3 stimulation (Magnolas and Jilka, 1995).

Matrix proteins

Matrix proteins from osteoblast cells act as growth factor modulator and play a major role in regulation of the differentiation of osteoblast and osteoclast cells. Collagen I is one of the earliest markers which regulates the osteoprogenitor cell, and alkaline phosphatase protein could participate in the regulation of proliferation,

migration and differentiation of osteoblastic cells. Moreover osteoblasts express several key proteins, such as osteocalcin, osteoponin, RANKLand OPG. The balances between RANKL and OPG are involved in the regulation of osteoclast function. RANKL binds to RANK receptor on the osteoclast precursors and stimulates osteoclast proliferation, whereas OPG is decoy receptors of RANKL, blocks RANKL binding to its receptor, decreases osteoclast activity and thus blocks bone resorption (Jane and Gary, 2001).

1.3.3 Molecular regulation of osteoblast development

Osteoblasts derive from pluripotent mesenchymal stem cells which also transform to chondrocytes, myoblasts, adipocytes and tendon cells, depending on the transcription factors that regulate the pathway. Three of these transcription factors are core binding factor A1 (Cbfa1), osterix (Osx) and activating transcription factor 4 (ATF4), which have been identified as controllers of the osteoblast lineage. In the absence of Cbfa1 and Osx, no osteoblasts are formed (Stains et al., 2003; Krane, 2005).

Cbfa1 or Runt-related transcription factor (Runx2) is a transcription factor expressed specifically in osteoblast progenitors as well as in mature osteoblasts (Ducy et al., 1997). The ultimate demonstration that Runx2 acts as a pivotal activator of osteoblast differentiation came from genetic studies in mice and humans. In Runx2-deficit mice, osteoblasts fail to differentiate, leading to a complete absence of endochondral and intramembranous bone formation (Komori et al., 1997; Otto et al., 1997). The critical importance of Runx2 for osteoblast differentiation was reported in mice that lacking one allele of Runx2. They displayed hypoplastic clavicle and delayed closure of the frontal bone. Indeed, it is essential for osteoblast differentiation and maturation by regulating of the genes encoding bone extracellular matrix proteins including osteoponin, osteocalcin, bone sialoprotein and type I collagen. The binding cites of Runx2 are also present in the regulatory sequences of most genes that are required for the synthesis of extracellular matrix (Kern et al., 2001).

Osx is another transcription factor involved on osteoblast differentiation and acts downstream of Runx2 to induce osteoblast differentiation. Inactivation of Osx in

mice results in perinatal lethalithy due to arrested osteoblast differentiation and complete absent of bone formation. While Runx2 is normally expressed in Osx-deficient embryos, Osx is not expressed in Runx2-deficient embryos. This indicates that Osx acts downstream of Runx2 in the transcriptional cascade of osteoblast differentiation (Nakashima et al., 2002).

During osteoblast differentiation, the osteoblast goes through three major phenotypically stages, including proliferation, maturation and extra-cellular matrix synthesis, and matrix mineralization. Cells in each of these phases have been described as early pre-osteoblasts, proliferating osteoblasts, mature osteoblasts, preosteocytes (osteoid osteocytes) and finally mature osteocytes within the mineralized matrix. Several studies support the hypothesis that proliferation is strictly dependent upon the synthesis of bone-specific extracellular matrix, whose maturation contributes to up-regulate the proliferation stage. During the active proliferation phase, osteoblast-committed progenitor cells (pre-osteoblasts) express genes that support proliferation and several genes encoding for extracellular matrix proteins, such as type I collagen and fibronectin, and type I collagen is the main structural component of bone matrix. The precursors that undergo proliferation and differentiate into pre-osteoblasts are elliptical cells that are unable to deposit bone matrix but are still capable to proliferate. In this phase, BMP-2 and BMP-5 play a significant role in increasing ALP activity and osteocalcin synthesis (Yamaguchi et al. 1991; Neve et al., 2011). Immediately after the growth arrested, a developmental sequence involving the selective expression of specific genes that characterize the differentiated osteoblast phenotype (e.g. ALP and osteocalcin gene expression) occurs (Stein et al., 1990). The accumulation of matrix proteins contributes, in part, to the cessation of cell proliferation. The active bone-matrix secreting osteoblasts are cuboidal cells, with a large Golgi apparatus and an abundant rough endoplasmic reticulum, and are provided with regions of plasma membrane specialized in the trafficking and secretion of vesicles that facilitate the deposition of bone matrix; these cells communicate with each other through tight junctions (Anderson, 2003). During the post proliferative phase, which is characterized by the high synthesis of ALP, the extra-cellular matrix progresses into the mineralization phase in which osteoblasts synthesize several proteins that are associated with the mineralized matrix, including sialoprotein,

osteopontin and osteocalcin (Gerstenfeld et al. 1987; Owen et al. 1990; Neve et al., 2011). Osteopontin is expressed during the stage of active proliferation and decreases immediately after the post-proliferative stage and increases again at the onset of mineralization, achieving the greatest level of expression during mineralization (Lian and Stein, 1995). Unlike osteopontin, osteocalcin is expressed by osteoblasts only in the post-proliferative phase. Osteocalcin is maximally expressed during mineralization (Owen et al., 1990). Several studies suggest that osteocalcin is involved in the regulation of mineral deposition and that it acts as a bone matrix signal that promotes osteoblast differentiation and activation (Chenu et al. 1994; DeFranco et al. 1991; Liggett et al. 1994). This confirms that osteocalcin is a marker of mature osteoblasts (Neve et al., 2011). Osteocalcin synthesis is regulated by various hormones, such as 1, 25$(OH)_2$ vitamin D_3, and growth factors (e.g. TGF-β).

Several cell-derived structures and organelles are responsible for the initiation and control of the mineral nucleation event. The extracellular matrix surrounding the osteoblast cells provides an oriented surface for mineral deposition and defines both the sites where mineralization will commence and the sizes to which the crystals will grow. Calcium and phosphate ions are the main constituents of hydroxyapatite crystal in the bone mineralization (Henrikzen et al., 2009). Collagen provides the template for mineral deposition in bone, and the size and organization of the collagen fibrils limit the dimensions that mineral crystals can attain. However, without the non-collagenous proteins, the mineralization process does not occur in a measurable time period. ALP is an important component in hard tissue formation, highly expressed in mineralized cells. ALP acts both to increase the local concentration of inorganic phosphate, a mineralization promoter, and to decrease the concentration of extracellular pyrophosphate, an inhibitor of mineral formation (Golub and Boesze, 2007). Osteocalcin also plays an important role in mineralization stage, because it is a promotor for the nucleation of calcium and phosphate crystallization. Indeed, without osteocalcin the forming cluster of calcium and phosphate are loosely bound to the collagen fibrils (Hempel et al, 2004). The onset and progression of matrix mineralization processes might be responsible for the down-regulation of genes expressed by mature osteoblasts during the same processes of extra-cellular matrix maturation and organization. At the end of the synthesis and mineralization of the

extracellular matrix, cellular levels of ALP mRNA decline (Lian and Stein 1995) and 50%–70% of mature osteoblasts undergo apoptosis, whereas the remainder can differentiate into lining cells or osteocytes (Franz-Odendaal et al, 2006). Bone lining cells remain on the bone surface, regulate the influx and efflux of mineral ions and retain the ability to re-differentiate into secreting osteoblasts upon exposure to various stimuli (hormones, or mechanical forces; Clarke, 2008). Osteocytes are metabolically quiescent osteoblasts embedded in bone matrix. They communicate with other bone cells through cell processes and function as strain and bone formation stress sensors (Lozupone et al. 1996).

1.3.4 Control of bone remodeling by osteoblasts

Osteoblasts play a major role in the process of bone formation, in the induction and regulation of extra cellular matrix mineralization and in the control of bone remodeling. During bone remodeling, bone formation is tightly coupled to bone resorption, and direct contacts between osteoclasts and osteoblasts have been proposed to maintain this relationship. Numerous other growth-regulatory factors affect osteoblast and osteoclast function, including TGFs, fibroblast growth factors (FGFs) and cytokines, such as IL 1, 6 and 11 and tumor necrosis factor (TNF)-α. Hormones, such as estrogens, PTH and 1, 25$(OH)_2$ vitamin D_3 activated receptors expressed by osteoblast to maintain mineral homeostasis (Sambrook and Cooper, 2006) and to influence a variety of osteoclast function, mostly via the cellular pathway called RANKL/OPG pathway.

The concept that the activation and regulation of osteoclasts in bone resorption require an interaction between osteoblast and osteoclast was proposed many years ago on the basis of the *in vitro* study, that in order to obtain mature osteoclasts, the presence of osteoblast is necessary. The molecular mechanism underlying this relationship was understood with the identification of RANK/ RANKL/ OPG system (Simonet et al., 1997; Jane and Gary, 2001; Neve et al., 2011)

The RANK/RANKL/OPG system

RANKL and its decoy receptor OPG are cytokines of TNFα superfamily and play a key role in the regulation of bone turnover. RANKL or osteoclast differentiation factor is expressed by osteoblasts both in a membrane-bound form and as a secreted protein. RANKL is essential factor for the recruitment, differentiation, activation and survival of osteoclastic cells through binding to its specific receptor RANK, which presents on the surface of osteoclast precursors and mature osteoclasts (Glantschnig et al., 2003). OPG is a soluble protein which is also secreted by osteoblasts and is the natural decoy receptor for RANKL. OPG prevents the interaction of RANKL with the RANK receptor and potently inhibits bone resorption. OPG-deficit mice exhibit an osteoporotic phenotype and present an increase number of osteoclasts (Lacey et al., 1998), whereas the over-expression of OPG reduces osteoclast formation and leads to osteopetrosis (Simonet et al., 1997). Through the modulation of RANKL and OPG, the ratio of RANKL and OPG is directly responsible for controlling the maturation and activation of osteoclasts and thus bone resorption. The majority of factors affecting the resorption rate mediate their effects by causing an alteration in one or both of these cytokines (Hofbauer and Schoppet, 2004; Kearns et al., 2008) (Figure 2.4).

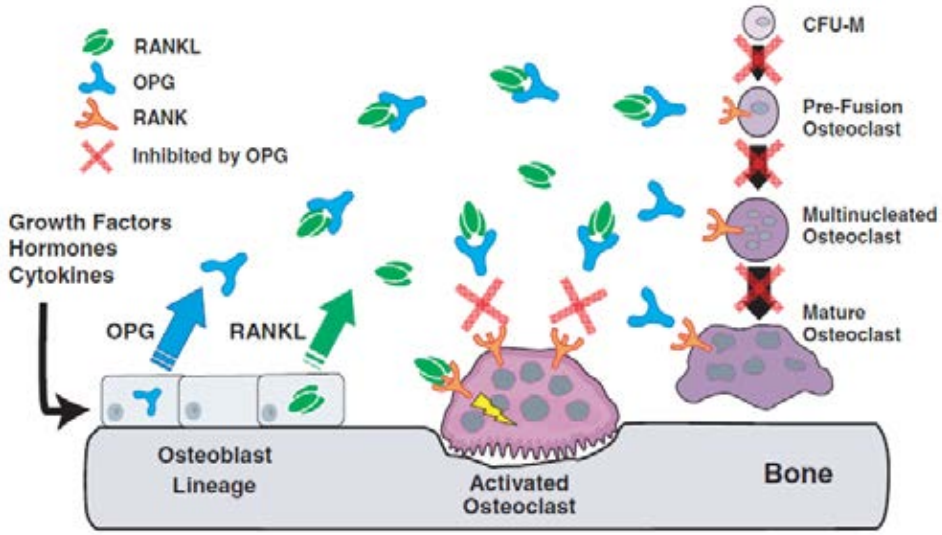

Figure 2.4 The RANK/RANKL/OPG system. RANKL binds to RANK receptor on the surface of osteoclast precursors leading to the activation of Nuclear Factor κB and the transcription of genes involved in osteoclastogenesis. OPG, by interacting with RANKL, prevents RANKL/RANK binding and subsequently inhibits osteoclastogenesis (Kearns et al., 2008).

1.3.5 Effects of estrogens on bone

Estrogens, a sex steroid hormone, are important regulators of bone development and homeostasis. Estrogens are represented by a large number of molecules, both steroidal and non-steroidal in nature. The most abundant estrogenic steroids in humans include estrone (E_1), E_2 and estriol (E_3) (Figure 2. 5). In premenopausal women E_2 produced in the ovaries is the main circulating estrogen whereas in postmenopausal women E_1, synthesized in several extraovarian sites, including bone, is the most abundant circulating estrogen in postmenopausal women. E_2 can be formed by aromatization of testosterone (Almeda, 2010).

Estrogens act by binding to either ERα or ERβ that belongs to the large family of nuclear receptors. ERs are ligand-activated transcription factors that homo- or heterodimerize upon ligand binding and directly bind to specific DNA sequences called estrogen response elements (EREs) in regulatory regions of target genes (Klock et al., 1987; Pacifici, 2006). DNA-bound ERs interact with several regulator proteins

to form a multiprotein complex that activates or responses the general transcription machinery. Additionally, ERs also have the ability to associate indirectly with gene promoters through protein-protein interactions with other transcription factors. Within minutes of ligand binding, estrogens are able to evoke rapid cellular responses that are incompatible with the classical mode of receptor action. These are called nongenomic action of estrogens which activates intracellular signaling cascade, like the mitogen-activated protein kinase (MAPK) or the phosphatidylinositol 3-kinase (P13K). Such actions are thought to result from ligand binding to ERs localized in the cytoplasm or the plasma membrane (Manolagas et al., 2004).

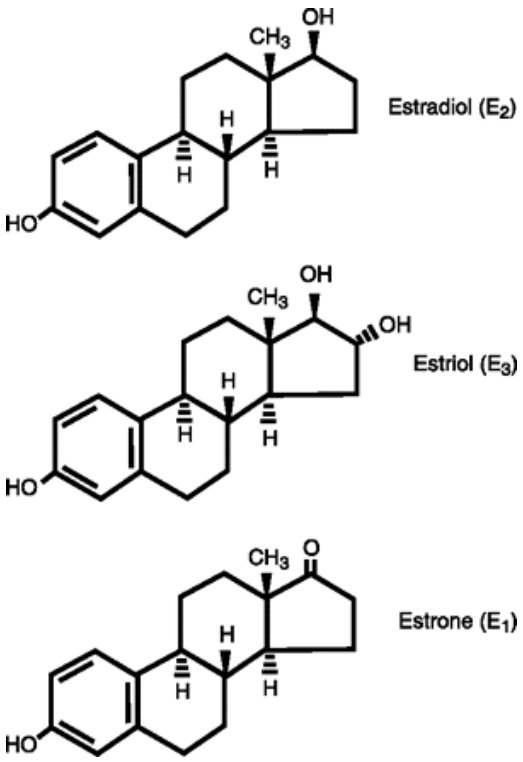

Figure 2.5 Estrogen structure. The structure of the predominant physiological estrogens including estrone (E_1), 17β-estradiol (E_2) and estriol (E_3) (Watson et al., 2008)

Estrogens are important regulators of skeletal growth and homeostasis. During growth, the physiologic actions of these steroids contribute the sexual dimorphism of the skeleton, the timing of epiphyseal closure, and the determination of peak bone mass. In the adult, estrogens play an important role in bone remodeling and attenuate the development of the osteoblasts and osteoclasts. Estrogens influence skeletal physiology during postnatal life in women and in men as well. This conclusion comes from the positive correlation between BMD and plasma E_2 concentration rather than to testosterone level in aging men. Confirming this conclusion, decreased BMD has been observed in men that have aromatase deficiency (Slemenda et al., 1997; Khosla et al., 2001, 2008; Mellstrom et al., 2008). Many cell types, including osteoblast and osteoclast lineages in the bones, express ERs. Therefore, bone cells are direct targets of estrogen action. ERα is predominantly expressed by cortical bone cells, whereas both forms of ERs are expressed by trabecular bone content in female mice (Centrella and McCarthy, 2012). ERα has been reported to be expressed in mouse (Bellido et al., 1993), rat (Davis et al., 1994) and human osteosarcoma cell line (Ikegami et al., 1994), as well as in human osteoblast-like cells (Chen et al., 2004). ERβ has been detected in rat osteoblast, rat osteosarcoma cell line (UMR-106) (Onoe et al., 1997) and in human osteoblast cell line SV-HFO (Arts et al., 1997: Chen et al., 2004).

Estrogens are thought to play a role in bone remodeling process. Estrogens inhibit bone remodeling and bone resorption, and enhance bone formation. Conversely, loss of estrogens in postmenopausal women increases osteoclastogenesis and bone resorption. These are the most important factor for the development of osteoporosis (Kato, 2009)

In osteoblast lineage, estrogens regulate osteoblastogenesis in multiple levels, including proliferation, differentiation and apoptosis. Several studies using human or rat osteoblastic cell lines have found a consistent inhibitory effect of estrogen on cell proliferation. In contrast, other investigators have reported that estrogens increase osteoblastic cell proliferation *in vitro*. This inconsistence might be due to different stage of cellular differentiation, cell system heterogeneity and expression level of ERs In differentiation stage, effects of estrogens have been variable, depending on the model system used. In some studies, estrogens have been shown to increase some markers of osteoblast differentiation, such as type I collagen, ALP, osteocalcin and

formation of mineralization nodules. In rat osteosarcoma cell lines, primary cultures of ROB or hOB cells and immortalized mouse MC-3T3-E1 cells increased ALP expression after exposed to estrogens. However, in some other studies estrogens exert suppressive effects on these same markers of osteoblast cell differentiation (Almeida, 2010).

The effects of estrogens on osteoclasts are mainly indirect and mediated by proteins secreted from osteoblasts. These proteins include RANKL and OPG. They regulate the differentiation of osteoclast precursors to mature osteoclasts, and then modulate the activity of the mature osteoclast and regulate its rate of apoptosis. There is also evidence indicating that estrogens have direct effect on osteoclast lineage cells. Estrogens induced apoptosis in osteoclast through activation of the Fas/FasL system via ERα and that leads to suppression of bone resorption through truncating the short life span of differentiated osteoclasts (Anandarajah, 2008).

2. Phytoestrogens

Phytoestrogens are plant-derived non-steroidal compounds that bind to ERs and have estrogen-like biological activities. Common phytoestrogens found in natural dietary sources are included soybean, soy product, whole grain breads, vegetables and tea. Phytoestrogens are generally categorized into three major classes according to the chemical structure: isoflavonoids (i.e., genistein, genistin, daidzein, daidzin, biochanin A, formononetin, puerarin and pratesenin), lignans and coumestans (i.e., coumestrol, 4'-o-methylcumestrol, miroestrol and deoxymiriestrol). Isoflavonoids are a large chemical class of flavanoids and there are the most extensively studied phytoestrogens (Malaivijitnond, 2012).

The basic structures of isoflavonoids consist of two benzene rings, which are linked by a heterocyclic pyrane ring, and one hydroxyl group (-OH) is found attached to each benzene ring (Figure2.6). Structurally, isoflavonoids exhibit a similarity to E_2 . They have an aromatic ring with hydroxyl groups and nearly identical distance exist between two hydroxyl groups. Genistein and daidzein differ by only one hydroxyl group on the A ring of the isoflavone structure, and they bind to both ERα and ERβ.

Therefore, isoflavonoids are considered as a potential alternative to ERT (Setchell, 1998; Chen et al., 2002; Cornwell et al., 2004).

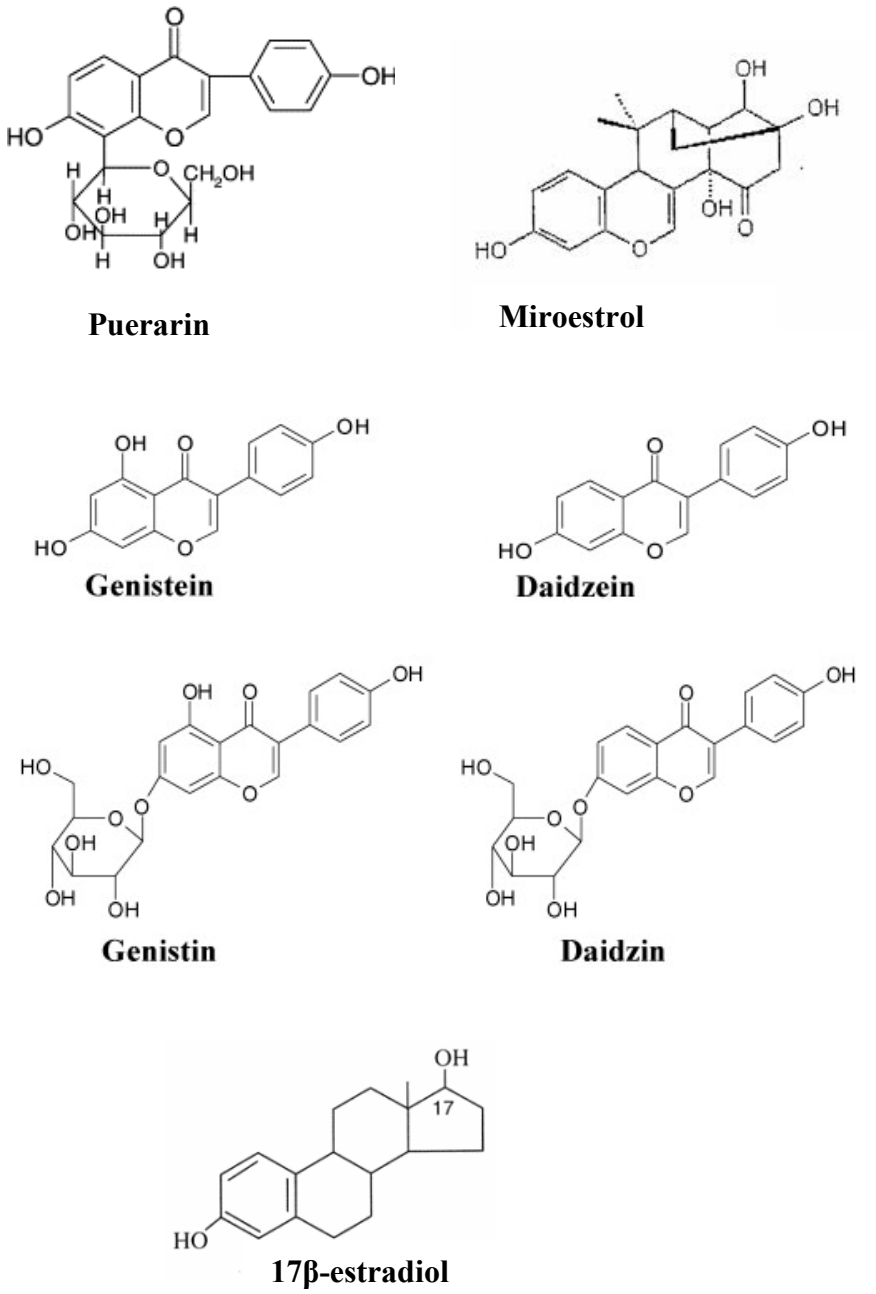

Figure 2.6 Chemical structures of major isoflavonoids (daidzin, daidzein, puerarin, genistin and genistein), miroestrol, and 17β-estradiol.

2.1 Source of phytoestrogens

The isoflavonoids are found almost exclusively in legumes and beans, and in products that contain most or all of beans. Second generations of soy foods are made by adding soy ingredients to a wide variety of manufactured foods, hence the isoflavonoid content is diminished, for example tofu, yoghurt and soy noodle. Lignans occur in high concentration in flaxseed, also known as linseed, and in lesser concentration in whole grain cereals, vegetables, fruits, and seeds. Coumestans are found predominantly with germination, for example, beans sprouting and also in fodder crops. Mostly, people consume phytoestrogen-rich foods and dietary supplements for menopausal symptom relief, such as bone health, cardiovascular risk reduction and breast and prostate preventive health (Murkies et al., 1998; Button and Patel, 2004).

2.2 Metabolism of isoflavonoid phytoestrogens

Most isoflavonoids exist in plant as glycosidic conjugates, generally locate in the cell vacuoles. The major isoflavonoids, genistein and daidzein, commonly exist as inactive glycosides. They are also derived from precursors, biochanin A and formononectin, which are converted to genistein and daidzein, respectively, after breaking down by intestinal bacteria or intestinal glycosidases enzymes. Daidzein is further partially metabolized to equol and O-desmethyl angiolensin (O-DMA). In humans, after consumption of plant isoflavones, complex enzymatic metabolic conversions occur in the gastrointestinal tract, resulting in the formation of heterocyclic phenols with a close similarity in structure to estrogens. Absorbed phytoestrogen metabolites undergo enterohepatic circulation and may be excreted in the bile deconjugated by intestinal flora, reabsorbed, reconjugated by the liver, and excreted in the urine. Isoflavonoids can be measured in urine, plasma, faeces, semen, bile, saliva, and breast milk. Concentrations of the different phytoestrogen metabolites vary widely between individuals even when a controlled quantity of isoflavonoids supplement is administered. As dietary phytoestrogen metabolism is predominantly determined by the gastrointestinal flora, the antibiotic use, or bowel disease and gender will modify metabolism (Murkies et al., 1998; Wong, 2002).

2.3 Phytoestrogens and its estrogenic activities

Phytoestrogens are of biological interest, because they exhibit both *in vitro* and *in vivo* estrogenic and antiestrogenic actions. Estrogenic property of phytoestrogens varies widely depending on the type of phytoestrogens and target tissue (Gao and yamagichi, 1999; Jefferson et al., 2002). Phytoestrogens may elicit weak estrogenic response or block estrogenic actions in estrogen responsive tissues. Whether they act as agonists or antagonists depends on many factors, including receptor numbers and concentrations and binding affinities of competing estrogen. They exert an antagonistic effect when estrogen concentration is high (Messina et al., 1994; Hwang et al., 2006).

Phytoestrogens have a higher affinity for ERβ than ERα (Kuiper et al., 1998). Study on the interaction of phytoestrogens with ERs in human breast cancer cell line, MCF-7, showed that both genistein and coumestrol can bind ERs in the cytoplasm and be translocated to the nucleus. Genistein has one-third of binding potency of E_2 with ERβ, and the one-thousandth with ERα as determined by expression of luciferase reporter gene constructed in kidney cells that have been co-transfected with ERβ and ERα (Kuiper et al., 1998). Thus, ERβ is believed to be important for the action of phytoestrogens. The ERβ mostly found in bone and prostate gland, and ERα is found mainly in breast, uterus and testes (Wong, 2002). The estrogenic activity of phytoestrogens is significant, especially for ERβ, and they may trigger many biological responses as those of physiological estrogens have done.

Phytoestrogens have been shown to influence many aspect of mammalian reproductive process. In female, the increase in uterine weight in ovariectomized animals after phytoestrogen treatment was reported. Genistein, cumestrol, miroestrol and equol significantly increased uterine weight in ovariectomized rats and mice (Ishimi et al., 2000; Jefferson et al., 2002; Deil et al., 2006). In women, oral administration of genistein could modulate the consequences of the postmenopausal estrogen deficiency state (Ishimi et al, 2000).

Moreover, isoflavonoids can exhibit anticarcinogenic activity *in vivo*. Feeding rat and mice with soy-fortified diets produced predominantly less breast tumor proliferation (Messina and Wood, 2008). Genistein has exerted both proliferative

(estrogenic) and antiproliferative (antiestrogenic) effects in human cell lines (Wang et al., 1996). In the human ER-positive MCF-7 breast cancer cell line, these effects are biphasic and depend on concentration used, with stimulation of cell growth occurring at low concentrations of genistein (0.01 - 10 μM) and inhibition at higher concentrations (10 - 100 μM). At low concentration (0.5 μM), genistein competes with E_2 for binding to the ER with a 50% inhibition and stimulates the expression of pS2 mRNA, a specific marker of ER-mediated estrogen like activity (Wang et al., 1996). It has been proposed that genistein, and perhaps other phytoestrogens, inhibit tumor cell growth by interfering with the tyrosine kinase activity of activated growth factor receptors and cytoplasmic tyrosine kinases, which are essential for the transduction of mitogenic signals (Peterson, 1995).

Miroestrol has known as the most potent phytoestrogen that exhibits its estrogenic activity in immature mouse uterine weight and rat vaginal cornification assay (Pop et al., 1958). Subcultaneous injection of miroestrol to ovariectomized rat has one-quarter activity of E_2 and double activity of E_1 on vaginal cornification assay.

Puerarin is a phytoestrogen with weak estrogenic activity, significantly induced a vaginal cornification in mature female rat after a long term subcutaneous injection of 140 days (Malaivijitnond et al., 2010). On the other hand, the very high dose of the dietary puerarin at 3,000 mg/kg of feed significantly increased uterine weight in ovariectomized rats (Rachon et al., 2007). Puerarin upregulated the expression of three estrogens responsive genes, that are insulin-like growth factor1 (IGF-1), progesterone receptor (PR) and complement protein 3 (C3), in the rat uterus (Rachon et al., 2007). Besides, puerarin suppressed the invasion and vasculaization of estrogen-stimulated endometriotic tissue in endometriotic stromal cells (ESCs) (Wang et al., 2011)

2.4 Phytoestrogens and bone

2.4.1 Effects of isoflavonoids on osteoblastic bone formation

Numerous *in vitro* studies with human and animal osteoblasts or osteoblast-like cell lines have explored the action of soy isoflavones on bone formation. Genistein (1 to 10 μM) induced a significant increase in calcium content, ALP activity and DNA content in bone tissues. The stimulatory effect of genistein (10 μM) was equal to that

of E_2 (0.1 to 1 nM) (Yamaguchi and Gao, 1997). This anabolic effect of genistein on bone components was also inhibited by anti-estrogen tamoxifen. Genistein and daidzein at concentrations of 1 and 10 μM stimulated increase in DNA content, protein synthesis and ALP in mouse osteoblast-like cells MC3T3-E1 (Sugimoto and Yamaguchi, 2000). In addition, soy isoflavonoids at concentrations of 0.1 to 10 μM have improved the differentiation and mineralization in MC3T3-E1 cells (Kanno et al., 2004; Ge et al., 2006). Similarly, it was found that genistein and daidzein at concentration 1 μM could stimulate bone nodule formation in primary osteoblasts obtained from newborn Sprague–dawley rat calvarias (Chang et al., 2003). Furthermore, it has been proposed that isoflavones may play an important role in bone remodeling because genistein and daidzein (10 μM) were able to inhibit TNF-α induced apoptosis and modulate the production of IL-6 and prostaglandin in MC3T3-E1 osteoblastic cells (Suh et al., 2003). Genistein, 0.1 μM for 72 hours, has been found to stimulate the production of OPG by human trabecular osteoblasts, providing a new mechanism for the bone-sparing effect of soy isoflavones (Viereck et al., 2002). Daidzein, at a concentration of 1 μM, exerted its anti-resorptive action by regulating Runx2 production and by stimulating the secretion of OPG and RANKL (De Wilde et al., 2004). Moreover, the anabolic effects of daidzein in primary cultures of osteoblasts could be mediated by an increased production of BMPs, in particular BMP2 (Jia et al., 2003). To conclude this section, it is important to specify that the effective doses of isoflavonoids used in nearly all these *in vitro* studies were closed to 1 μM.

2.4.2 Effects of isoflavonoids on osteoclastic bone resorption

Genistein (0.1 to 10 μM) completely inhibited the effects of bone resorbing factors (PTH, prostaglandin and lipopolysacharide) in a tissue culture system (Yamaguchi and Gao, 1998). Genistein and daidzein suppressed osteoclastic bone resorption by numerous possible mechanisms, that are, induction apoptosis (Gao and Yamaguchi, 1999; Rassi et al., 2002), inhibition of protein tyrosine kinases (Blair et al.,1996; Williams et al., 1998), activation of protein tyrosine phosphatases (Gao and Yamaguchi, 2000), changes in intracellular concentrations of Ca^{2+} and membrane depolarization (Okamoto et al., 2001).

2.4.3 Action of dietary isoflavonoids on animal bone

Most of the studies of the effects of soy isoflavones on bone have been performed in female ovariectomized rodents. This model was used to mimic postmenopausal osteoporosis, and has provided convincing data on the significant improvement of trabecular and/or cortical BMD or other end points (i.e., femur ash weight, mechanical strength, markers of bone turnover) (Omi et al., 1994; Harrison et al., 1998; Ishimi et al., 1999). A beneficial effect of genistein on bone tissue, that is, increase in femur ash weights and calcium contents in the femoral metaphyseal and diaphyseal tissue has also been described in lactating ovariectomized rats (Anderson et al., 1998) and in elderly female rats (Yamaguchi and Gao 1998). However, many other experiments found minimal or no effects of purified isoflavones on BMD and bone biomarkers of remodelling activity of ovariectomized rats (Picherit et al., 2001; Breitman et al., 2003; Bahr et al., 2005). Other studies, one with ovariectomized monkeys (Jayo et al., 1994) and the other with growing pigs (De Wilde et al., 2004), have also found no effects of soy isoflavones on bone mineral loss. This suggests that modulation in responsiveness to soy isoflavones could be due to species and also life-stage differences.

3. *Pueraria mirifica*

Pueraria mirifica (Airy Shaw and Suvatabandhu) is an endemic Thai plant which contains high amount of phytoestrogen and known in Thai as white Kwao Krua. It belongs to the family Leguminosae, subfamily Papilionoideae or the soy, bean and pea subfamily. The plant is commonly found in the forest throughout Thailand and abundantly found in the north, west and north-east of Thailand. *P. mirifica* is a woody prenial climber with tuberouse roots that look like a chain of round-shape bulbs of various sizes and connect to the next one via small root. Its tuberous root has a round or ellipse-shape and contains whitish starch granules. The flower color is purple-blue (Figure 2.7) (Ingham et al., 2002; va der Maesan, 2002; Malaivijitnond, 2012).

The globular or pear-shape tuberous roots of *P. mirifica* have been used by the local community in Thailand for traditional Thai medicine as a source of rejuvenating quality in aged women and men for nearly one hundred years. The usage of tuberouse

root of *P. mirifica* is by being sliced, sun-dried, pulverized and mixed with honey to give a peppercorn-size pill, which is equivalent to about 250 mg/50 kg BW or 5 mg/kg BW dosage, and taken once a day at night time for 3-6 months (Ingham et al., 2002; Malaivijitnond, 2012). Recently, *P. mirifica* has been popularly used to mitigate the symptons of ageing process and estrogen deficiency, such as sagging breasts, wrinkled skin, grey hair and bone loss. Nowadays, *P. mirifica* has been included into the cosmetic, dietary supplement and pharmaceutical products, and various forms are sold on the market (Malaivijitnind, 2012). In addition, *P. mirifica* has been extensively studied for its contents and estrogenic activities on reproductive organs and also recently on bones.

Figure 2.7 The *Pueraria mirifica* : woody climbers (a), its leaves and flowers (b) and tuberous roots (c) (Malaivijitnond, 2012).

3.1 Chemical constituents of *P. mirifica*

Many chemicals possessing estrogenic activities contained in *P. mirifica* are determined by high performance liquid chromatography (HPLC) technique. The first phytoestrogens isolated from *P. mirifica* was miroestrol, which has the highest estrogenic potency among all the subsequentially isolated phytoestrogens from *P. mirifica* (Pope et al., 1958; Malivijitnond, 2012). The estrogenic activity is found in the roots, leaves, and stem of the plant, and the root is the mostly comprise of estrogenic activity when compared to those of leaves and stems. The tuberous root of *P. mirifica* contained at least 17 phytoestrogens, daidzein, daidzin, genistin, genistein, kwakhurin, kwakhurin hydrate, tuberosin, puerarin mirificin, puemiricarpene, coumestrol, mirificoumestan, mirificoumestan glycol, miricoumestan hydrate, miroestrol, deoxymiroestrol, and isomiroestrol (Malaivijitnond, 2012). Because puerarin and miroestrol are only found in *P. mirifica*, and so they can be used as index of species identification and qualification.

Daidzin, puerarin and puemiricarpene are the main compounds, and other remaining compounds are at low concentration (Ingham et al., 2002). Generally, puerarin accounted for about haft of the total isoflavonoid content with a slightly comparable amount of genistein and daidzein (Cherdshewasart et al, 2007; Urasopon et al., 2008). *P. mirifica* dry powder of 100g contains 46.1 mg of daidzein and 2-3 mg of miroestrol and deoxymiroestrol (Chansakaow et al., 2002). The content of puerarin in *P. mirifica* as analyzed by HPLC ranged from 53.3-870.5 μg/g of *P. mirifica* ground tuber powder (Cherdshewasart et al, 2007; Urasopon et al., 2008). By HPLC analysis of *P. mirifica* cultivar Wichai III, 100 g of dry powder is composed of 169.1 mg of isoflavonoids (Muangman and Cherdshewasart, 2001). Phytoestrogens content analyzed in *P. mirifica* roots collectected from 76 provinces of Thailand by HPLC technique contained the total five isoflavonoids (puerarin, daidzin, daidzein, genistin and genistein) ranging over 18.1-198.3 mg/100g of dry powder. The high variability of isoflavonoid contents in *P. mirifica* roots is probably influenced by genetic and environment factors.

3.2 Estrogenic activity of *P. mirifica*

The estrogenic activity of *P. mirifica* was reported many years before the identification of the plant species was certain in 1952 (Malaivijitnond, 2012). The key chemicals, as well as crude extract, were tested in cells, animals and humans, and exhibited estrogenic effects. The *in vitro* assay for *P. mirifica* cultivar Wichai-III tuberous extract showed a biphasic response, with proliferation of MCF-7 at low concentration (1μg/ml) and antiproliferation at high concentration (1,000 μg/ml) (Cherdshewasart et al., 2004a). *P. mirifica* exhibited a higher binding affinity to hERβ than to hERα (Boonchird et al., 2010). At high concentration, *P. mirifica* tuberous extract also induced antiproliferation of HeLa cells (Cherdshewasard et al., 2004b). In addition, when high concentration of *P. mirifica* extract (1,000 μg/ml) was coincubated in the same assay with E_2 in MCF-7 cells, a significant agonistic effect was observed, by competing to bind the ERα of MCF-7 Cells (Cherdshewasart et al., 2004a). Puerarin, produced a significant proliferative activity in MCF-7 cells at high concentration (1 μM), whereas E_2 showed a similar effect at much lower dose (0.01 nM). In comparison, puerarin exhibited a proliferative effect that was 10^2-10^5 times lower than that of E_2. However, puerarin did not show any cytotoxicity at high concentration (1 μM) (Cherdshewasard et al., 2008). Miroestrol exhibited the strong estrogenic activity equivalent to that of E_2 , in terms of the induction of expression of the aryl hydrocarbon receptor (AhR) and ER related genes (Udomsuk et al., 2011a) and the regulation of testicular gene related sex hormone synthesis pathway (Udomsuk et al., 2011b).

The estrogenic effects of *P. mirifica* on male and female reproductive organs have been thoroughly examined in mice, rats and monkeys. Daily feeding of *P. mirifica* root powder induced a vaginal cornification and an increased uterine weight, decreased serum follicle stimulating hormone (FSH) and luteinizing hormone (LH) levels in ovariectomized rats (Malaivijitnond et al., 2004, 2006). In adult female monkeys, long-term administration of *P. mirifica* prolonged the menstrual cycle length and suppressesed the folliculogenesis and ovulation together with the reduction of serum FSH, LH, E_2 and progesterone levels (Trisomboon et al., 2004, 2005).

Although *P. mirifica* can influence the reproductive function in both sexes, the response in females is greater and more sensitive than in males (Malaivijitnond et al., 2004; Jaroenporn et al., 2006, 2007). The reduction in FSH levels was observed only in female rats, along with the increase in the uterus weight and vaginal cornification, while the increase in epididymis weight only occurred in male rats after treatment at the high dose of 1,000 mg/kg/BW) of *P. mirifica* for 14 days (Malaivijitnond et al., 2004). In addition, administration of *P. mirifica* at doses of 10-1000 (mg/kg/BW) for 90 days increased the uterus weights of female rats (Urasopon et al., 2008), but no alteration of the seminal vesicle and prostate gland of male rats were found(Urasopon et al., 2007).

3.3 *P. mirifica* and its phytoestrogen on bone

Since the estrogenic activity of *P. mirifica* extract and tuber powder in the reproductive organs has been found as mentioned above, the effects of such treatment on bone tissue were evaluated to its potential use for osteoporosis treatment. The effect of *Pueraria* plant extract on the prevention of bone loss was first report in *P. lobata*, which locally found in China and Korea (Wang et al., 2003, 2005). Subsequent research in *P. mirifica* revealed that crude powder of *P. mirifica* could increase BMD and BMC in osteoporotic rats in trabecular and cortical bones of the 4th lumbar vertebra, tibia and femur in a dose-dependent manner (Urasopon et al., 2007, 2008). Furthermore, *P. mirifica* decreased serum PTH and calcium levels on aged menopausal monkeys (*Macaca fascicularis*) after long-term treatment with 1,000 mg/day, indicating that *P. mirifica* ameliorates bone loss caused by estrogen deficiency (Trisomboon et al., 2004).

The effects of isoflavonoid compounds, such as puerarin, genistein and daidzein, have been reported in various types of bone cell culture. Puerarin at concentration of 0.01 μM increases the proliferation and matrix mineralization, promotes nitric oxide synthesis and increased mRNA expression of BMP-2, SMAD4, Runx2, and OPG in mouse primary osteoblast cells (Sheu et al., 2012). Genistein and daidzein significantly increased in the proliferation, alkaline phosphatase activity, and DNA content of the mouse osteoblastic cell line MC3T3-E1 (Yamaguchi et al.,

2000) and human osteoblastic cell line MG63 (Morris et al., 2006). However, the mechanism of actions of *P. mirifica* on bone cells is not been clearly understood and need to be undertaken.

4. Comparison of bone physiology between rats, baboons and humans

To maintain the bone mass throughout life in humans, the bone undergoes remodeling. The cortical bone remodeling is continuously broken down by osteoclasts and rebuilt by osteoblasts. These two types of bone cells are collaborating within BMU. Osteoclasts create a resorption cavity that is filled with new bone by osteoblasts. In remodeling of cortical bone, a cutting cone of osteoclasts excavates a tunnel, closely follows by a closing cone of osteoblasts that fills the tunnel with new bone. In this way, the BMU creates a new osteon or Harversian system (Figure 2.8). In trabecular bone, the BMU is similar, but moves across the trabecular surface (Van Oers et al., 2008).

Currently, postmenopausal osteoporosis is a major health problem in women worldwide. There is extensively literature studied indicating that the ovariectomized rats exhibit most of the characteristic of human postmenopausal osteoporosis (Frost, 1992; Urasopon 2007, 2008). Thus, rodents (rats or mice) are the most commonly used animal model for osteoporosis. One of advantages is that rodent have shorter life spans which enables studies the effects of ageing on bone (Barlet et al., 1994). With the fast generation time, rodents are often a starting point for preliminary screening, efficacy and toxicity of new pharmacological or therapeutic agents, following by verification in other species, before undertaking clinical trials in human patient (Turner, 2001).

Although information of rat bone is valuable, rat model has several limitations to its similarity to the human condition in both reproductive and bone physiology aspects. Rats have four to five day estrous cycle that does not involve a true luteal phase. In addition, they do not experience a natural menopause (Frost, 1992; Lelovas et al., 2008). Furthermore, for bone physiology, rats are lack of Harversian system, the cortical bone does not undergo osteonal (or Harversian) remodeling and growth plate does not close in less than 30 months (Lelovas et al., 2008). Therefore, rats are

unsuitable model for human osteoporosis (Turner, 2001). To mimic the human reproductive and bone physiology, the animals which have osteonal remodeling are focused. Most recent investigations of bone metabolism have examined in nonhuman primates.

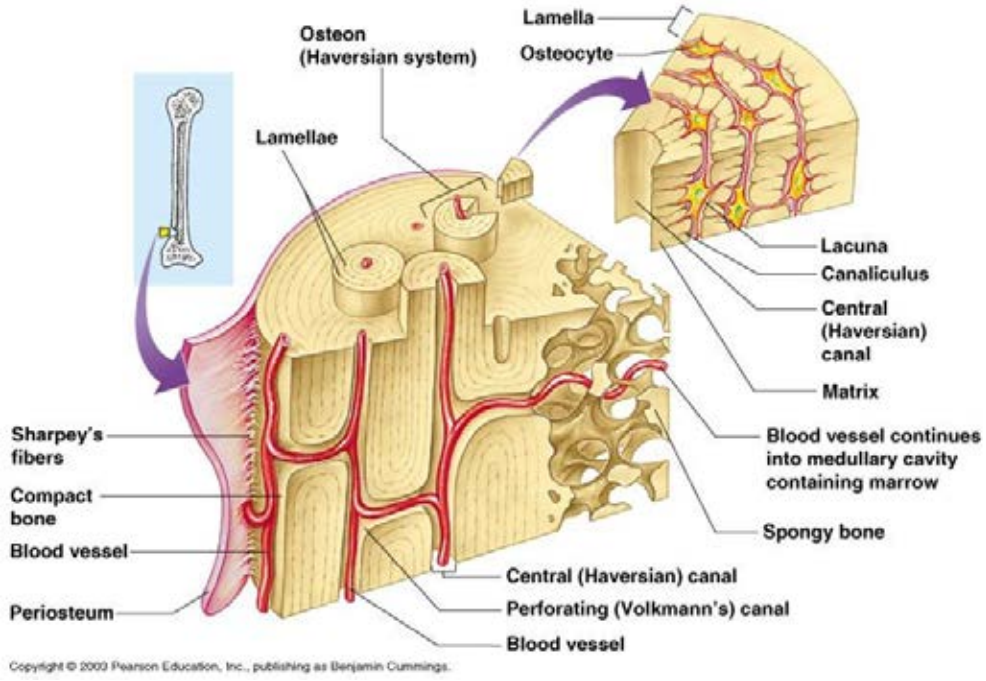

Figure 2.8 Structural unit of cortical (or compact) bone also called a Haversian system. Osteons are essentially long cylinders of bone; the hollow center is called the central canal, and is where blood vessels, nerves, and lymphatic vessels are found. Cortical bone is laid down around the central canal in rings (lamella)

(Available
from: http://www.usi.edu/science/biology/mkhopper/hopper/BIOL2401/LABUNIT1/01Ex8OverSkeleton/LabEx9Images/osteonlabeled.jpg)

Nonhuman primates, especially the old world monkeys, such as baboons and macaques, second only to apes in genetic proximity to humans, exhibit many biological and physiological similarities to those of humans. Age-related changes in bone mass and bone structure, particularly in older females, have been demonstrated in several old World monkey species (Aufdemort et al., 1993; Mahaney et al., 1995; Wang et al., 1998; Phelps et al., 2000). An important feature of nonhuman primate bone is the presence of Harversian osteonal remodeling in cortical bone, as observed in humans (Jerome and Peterson, 2001). This feature is not normally present in rodent bone and is one of the major reasons that nonhuman primates are suitable models for studies of bone biology in humans.

Baboon is one of the old world monkey species that shows various similarities to humans regarding skeletal biology. The life span of baboon is approximately 30 years and both male and female baboons showed bone loss with age (Aufdemorte et al., 1993; Harvill et al., 2008), and females showed increased bone turnover after ovariectomy (Jerome et al., 1986), similar to that seen in postmenopausal women. Furthermore, in a study of material properties of bone, relative to dogs, cows, and rabbits, baboon bone was more similar to the human bone regarding fracture, microstructural, and compositional properties. Specifically, baboon bone was not significantly different from the human bone in terms of mineral density, organic density, volume fraction, fracture surface pattern and length of collagen-mineral bundles (Wang et al., 1998). Baboons also resembles to humans in reproductive endocrinology, especially for the estrogen profiles which are known to be important in bone metabolism. They also had menstrual cycles involving timing and phases similar to humans and underwent a natural menopause (Brommage, 2001). These aspects of baboon skeletal biology and sex steroid hormone secretion emphasized their value as a model for bone research in humans.

CHAPTER III

PHYTOESTROGENS FROM *Pueraria mirifica* INCREASED BONE FORMATION THROUGH THE DIFFERENTIATION AND MINERALIZATION OF RAT OSTEOBLAST-LIKE UMR106 CELLS

Introduction

Osteoporosis, a metabolic bone disease characterized by low bone density and microarchitectural deterioration, progressively reduces the quality of bone strength, predisposing patients to the vertebral fractures, fragility fractures of the neck of the femur, and fracture of the wrist (Lane, 2006; Cole et al., 2008). The economic burden of osteoporosis is markedly increased paralleling the expansion of aging world population (Lane, 2006). Approximately 1.6 million patients with osteoporotic hip fractures were reported worldwide in 2000, and the incidence has been predicted to reach 6.3 million by 2050 (Cole et al., 2008). Therefore, development of effective preventions, early interventions and treatments is needed to mitigate clinical complications of osteoporosis and the accompanying economic impact. Since E_2 is an important hormone for maintaining bone mass, postmenopausal women with estrogen deficiency thus are a prone to develop osteoporosis (Lane, 2006). Although ERT is an effective therapy for postmenopausal bone loss, long-term exposure to synthetic estrogens markedly increases the prevalence of malignant neoplasia in several tissues, such as mammary gland, endometrium, and ovary (Manolagas et al., 2002).

Phytoestrogens, an estrogen-like compound of plant origin, are capable of binding to ER with minimal cancer risk (Tham et al., 1998), and have attracted attention for their potential in the prevention and therapeutic of postmenopausal osteoporosis (Tham et al., 1998; Setchell and Lydeking-Olsen, 2003). Recently, the tuberous root extract of phytoestrogen-rich herb *Pueraria mirifica* has been shown to effectively prevent osteoporosis in ovariectomized rats by increasing BMD and BMC (Urasopon et al., 2007, 2008). However, the exact anti-osteoporotic mechanism of *P. mirifica* extract and its major constituent, puerarin, in osteoblasts (bone-forming cells) remained elusive.

P. mirifica belongs to the family Leguminosae endemic to Thailand (Chansakaow et al., 2000; Malaivijitnond, 2012). Its tuberous root contains a number of isoflavones, such as puerarin, daidzin, genistin, daidzein and genistein (Cherdshewasart et al., 2007a, b; Cherdshewasart and Sriwatcharakul, 2007) and exert estrogenic activity on bone like estrogen (Morabito et al., 2002; Marini et al., 2007; Urasopon et al., 2008). For instance, a randomized double blind placebo-controlled study in postmenopausal women showed that genistein administration significantly increased BMD and the circulating levels of bone formation markers, e.g., ALP, osteocalcin and IGF-1 (Morabito et al., 2002; Marini et al., 2007). It was shown that genistein exerted its action by binding to ERα and ERβ (Kuiper et al., 1998). Since the chemical structures and actions of isoflavones resemble those of E_2, it is possible that *P. mirifica* extract and its major isoflavone compound, puerarin, may also induce positive estrogenic effect on osteoblasts.

Although it was possible that *P. mirifica* extract prevented bone loss by promoting bone formation, whether it affected proliferation, differentiation and mineralization stage of osteoblasts was not known. Under normal conditions, to induce bone formation, stromal cells or osteoprogenitor cells undergo proliferation, followed by differentiation with a slowdown of proliferation (Owen et al., 1990; Zaidi, 2007). When osteoblasts differentiate, they sequentially express different markers specific to each stage of maturation, i.e. Runx2, osterix, ALP, and osteocalcin in this order (Stein et al., 2004; Komori, 2006). Moreover, differentiated osteoblasts also express RANKL and OPG, both of which are commonly used as markers for the assessment of osteoblast-regulated osteoclast function and bone resorption. In addition, during the post proliferative phase, the mature osteoblast produced extracellular matrix and mineralization of the extracellular matrix would be occurred (Abdallah et al., 2005; Lin et al., 2007). Therefore, the objectives of the present study were (i) to investigated the effect of *P. mirifica* extract and its isoflavones, puerarin and genistein, on proliferation and viability of osteoblasts, (ii) to determine the effect of *P. mirifica* extract, puerarin and genistein on the expressions of osteoblastic differentiation markers and osteoblast-regulated osteoclast function, and mineralization of osteoblasts, and (iii) to investigate whether ERs mediated the estrogenic actions of *P. mirifica* extract, puerarin and genistein in osteoblasts.

Materials and Methods

Preparation of *P. mirifica* extract and high-performance liquid chromatography (HPLC)

The tuberous roots of *P. mirifica* cultivar SARDI 190 (lot no.0070317) were purchased from Dr. Sompoch Tubcharoen, Kasetsart University, Kamphaeng Saen Campus, Thailand, and was authenticated as *P. mirifica* by comparing with the voucher specimens (nos.BCU010250 and BCU011045) kept at the Professor Kasin Suvatabhandhu Herbarium, Department of Botany, Faculty of Science, University, Thailand. The specimens were washed, sliced, dried in a hot-air oven at 70 ˚C, and ground. One gram of tuberous powder was extracted twice by mixing with 4 ml of 70% ethanol (Urasopon et al., 2008). The supernatants collected from two extractions were mixed together and dried for 4 h in a centrifugal concentrator at room temperature. Dry sample was kept at 4 ˚C until used in cell culture, and some portion was used for HPLC analysis.

The concentrations of isoflavones in *P. mirifica* extract (i.e., puerarin, daidzin, genistin, daidzein and genistein) were determined by HPLC, as described previously (Malaivijitnond et al. 2004). Briefly, 10 µl of extracted solution was injected through a sensory guard column [model Hyperclone ODS(C18); Phenomenex, Torrance, CA, USA] into a HPLC system (model Agilent 1000; Agilent, Waldbronn, Germany) equipped with a reverse phase Symmetry C18 column (250 mm×4.6 mm, 5 µm; Phenomenex). Mobile phase consisted of 0.1% vol/vol phosphoric acid and acetonitrile with gradient elution (flow rate 1 ml/min). Ultraviolet detection was performed at a wavelength corresponding to the most intense absorption maximum at 255 nm. The isoflavone concentrations in each sample were analyzed in duplicate by comparing the retention times, and the amounts were quantified using the peak area of the standard curves. The standard compounds of daidzin, genistin, daidzein, and genistein (catalog nos. 30408, 48756, D7802 and G6649, respectively) were purchased from Sigma (St. Louis, MO, USA), and puerarin (catalog no. P5555) was purchased from Fluka (Buchs, Switzerland).

Cell culture

Rat osteoblast-like UMR106 cells (American Type Culture Collection (ATCC) no. CRL-1661) were cultured in 100 mm petri-dish with Dulbecco's modified Eagle's medium (DMEM; Sigma) supplemented with 10% fetal bovine serum (FBS; PAA, Pasching, Austria), and 100 U/ml penicillin-streptomycin (Gibco, Grand Island, NY, USA). Cells were maintained at 37 ˚C in 5% CO_2 incubator, and subcultured according to the ATCC's protocol.

Experimental design

Prior to determination of the effects of *P. mirifica* and its phytoestrogens on proliferation, expressions of markers of osteoblast differentiation and osteoblast-regulated osteoclast function, and mineralization, rat osteoblast-like UMR106 cells were first investigated for the osteoblast characteristics. Cell morphology was monitored under inverted-light microscope, mRNA expression of ERs (ER-α and ER-β), osteoblast differentiation marker genes i.e.; Runx2, osterix, ALP and osteocalcin, osteoblast-regulated osteoclast function i.e.; RANKL and OPG were investigated by quantitative real time PCR (qRT-PCR), and the *in vitro* mineralization was determined by Alizarin Red S staining.

In addition, the response of UMR106 to estrogens was investigated by incubating osteoblast cells with 10 nM E_2 with the incubation time varied, 24, 48 and 72 h. Cell proliferation and cell viability were determined by 5-bromo-2´-deoxyuridine (BrdU) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays, respectively, while the mRNA expressions of osteoblast differentiation marker gene, ALP mRNA expression, was determined by qRT-PCR

To investigate the effects of phytoestrogens and *P. mirifica* extract, on rat osteoblast cells, UMR106 cells were incubated for 24, 48 or 72 h with vehicle (0.3% vol/vol dimethyl sulfoxide (DMSO); control group), or various concentrations of genistein (0.1, 10 and 1000 nM), puerarin (0.1, 10 and 1000 nM) or *P. mirifica* extract (1, 10 and 100 μg/ml). Cell proliferation and cell viability were determined by BrdU and MTT assays, respectively, while the mRNA expressions of osteoblast differentiation markers (i.e., runx2, osterix, ALP, osteocalcin, RANKL and OPG)

were quantified by qRT-PCR. In addition, ALP level and activity were also determined. The *in vitro* mineralization was determined by Alizarin Red S staining and quantified by semi-quantitative fluorescence analysis of calcein binding. To describe if genistein, puerarin and *P. mirifica* extract exerted its estrogenic effect through ER as those of E_2, UMR106 cells were pre-incubated for 2 h with ER antagonist (10 nM ICI182780; half maximal inhibitory concentration of ~0.3 nM, Sigma), and then incubated for 48 h with 10 nM ICI182780 plus 10 nM E_2, 1000 nM genistein, 1000 nM puerarin, or 100 μg/ml *P. mirifica* extract and the ALP expression was determined.

Total RNA preparation

Total RNA samples were extracted from UMR106 cells by using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Purity of the total RNA was determined by the ratio of absorbance reading at 260 and 280 nm, the ratio of which was between 1.8 and 2.0. One microgram of total RNA was then reverse-transcribed with iScript cDNA synthesis kit and oligo-dT20 (Bio-Rad, Hercules, CA, USA) to cDNA by Bio-Rad MyCycler. Rat β-actin served as a control gene to check the consistency of the reverse transcription (% coefficient of variation <5%, n = 8).

Quantitative real-time PCR (qRT-PCR)

Primers used in the present study are listed in Table 3.1. All primers were first verified by conventional PCR followed by amplicon sequencing. Conventional PCR was performed by Bio-Rad MyCycler with GoTaq Green Master Mix (Promega, Madison, WI, USA). Thereafter, PCR products were visualized on 1.5% agarose gel stained with 1 μg/ml ethidium bromide (Sigma) under UV transilluminator (Alpha Innotech, San Leandro, USA). qRT-PCR and melting curve analysis were performed in triplicate by Bio-Rad MiniOpticon real-time PCR system with iQ SYBR Green SuperMix (Bio-Rad) for 40 cycles at 95˚C for 60 s, 53–60 ˚C annealing temperature for 30 s, and 72˚C for 30 s.

Table 3.1 Rat (*Rattus norvegicus*) oligonucleotide sequences used in the PCR experiments

Gene	Accession no.	Primer (forward/reverse)	Product length (bp)	Anealing Temp.(°C)
Estrogen receptors (ER)				
Estrogen receptors-α	NM_012689.1	5´-CCAAGTCCACTTGTGATCAAGC-3´ 5´-TTGAGGCTTCACTGAAGGGTC-3´	148	58
Estrogen receptors-β	NM_012754	5´-AAAGCCAAGAGAAACGGTGGGCAT-3´ 5´-GCCAATCATGTGCACCAGTTCCTT-3´	204	60
Osteoblast-related genes				
Osterix	AY177399	5´-GCCTACTTACCCGTCTGA-3´ 5 -CTCCAGTTGCCCACTATT-3´	139	55
Runx2	XM_001066909	5´-TAACGGTCTTCACAAATCCTC-3´ 5´-GGCGGTCAGAGAACAAACTA-3´	135	55
Alkaline phosphatase	NM_013059	5´-GCAGGATCGGAACGTCAAT-3´ 5´-ATGAGTTGGTAAGGCAGGGTC-3´	144	56
Osteocalcin	J04500	5´-CACAGGGAGGTGTGTGAG-3´ 5´-TGTGCCGTCCATACTTTC-3´	203	57
OPG	NM_012870	5´-ATTGGCTGAGTGTTCTGGT-3´ 5´-CTGGTCTCTGTTTTGATGC-3´	140	53
RANKL	NM_057149	5´-TCGCTCTGTTCCTGTACT-3´ 5´-AGTGCTTCTGTGTCTTCG-3´	145	53
Housekeeping gene				
β-actin	NM_031144	5´-CAGAGCAAGAGAGGCATCCT-3´ 5´-GTCATCTTTTCACGGTTGGC-3´	185	56

Cell proliferation assay

UMR106 cells were seeded in 96-well culture plate (1000 cells/well). After a 48-h incubation with 10 nM E_2, 0.1, 10, and 1000 nM genistein, 0.1, 10, and 1000 nM puerarin, or 1, 10, and 100 μg/ml *P. mirifica* extract, cell proliferation was determined by BrdU enzymelinked immunosorbent assay kit (catalog no. 11647229001; Roche, Mannheim, Germany), according to the manufacturer's instruction. Briefly, UMR 106 cells were seeded in 96-well culture plate at 1000 cells/well. After 48-h incubation with E_2, phytoestrogens and *P. mirifica* extract, cells were fixed and later incubated with anti-BrdU-peroxidase antibody to detect BrdU-incorporated cells. Substrate solution containing tetramethylbenzidine was added for color development. Since BrdU incorporated into the newly synthesized DNA of proliferating cells, the amount of BrdU in each well represented cell proliferation. The absorbance of each well was measured at 370 nm with the reference wavelength of 490 nm by a microplate reader (Wallac; model 1420, Turku, Finland). The BrdU assay was performed in triplicate with 6 independent samples (n = 6).

Cell viability assay

UMR106 cells were seeded in 96-well culture plate (1000 cells/well). After a 48-h incubation with 10 nM E_2, 0.1, 10, and 1000 nM genistein, 0.1, 10, and 1000 nM puerarin, or 1, 10, and 100 μg/ml *P. mirifica* extract, cell viability was determined by MTT assay. In viable cells, MTT was converted by a mitochondrial reductase to purple formazan crystal. Briefly, 40 μl of MTT (catalog no. M2128; Sigma) was pipetted into each well to obtain a final concentration of 1 mg/ml, and incubated at 37 ˚C for 3.5 h prior to removing culture medium. Thereafter, 150 μl of solvent containing 4 mM HCl and 0.1% vol/vol Nonidet P-40 in isopropanol was added to dissolve the formazan crystal. The absorbance of each well was determined at 590 nm with the reference wavelength of 620 nm. The MTT assay was performed in triplicate with 6 independent samples (n = 6).

Alkaline phosphatase (ALP) assay

ALP level was histochemically examined by ALP staining. The stained cells were seen in blue-violet when the ALP presented. Briefly, UMR 106 cells were seeded in 24-well plate at density $5x10^4$ cells/well for 4 days. Cells were incubated with 10 nM of E_2, and high dose of each phytoestrogens and *P. mirifica* extract (1000 nM genistein, 1000 nM puerarin, and 100 µg/ml of *P. mirifica* extract) for 48 h. Then UMR 106 cells were washed with PBS and fixed with 70% ethanol. The cells were incubated with diazonium salt solution (0.05% of Fast Violet B salt (Sigma) and 4% of Naphthol AS-MX (Sigma)) for 30 minutes. After being rinsed with deionized water and air-dried, cells were observed and photographed (Bidarra *et al*., 2011).

For quantitative ALP analyses, ALP activity was also examined. Briefly, UMR 106 cells were seeded in 24-well plate at density $5x10^4$ cells/well for 4 days. Cells were incubated with 10 nM E_2, and high dose of each phytoestrogen and *P. mirifica* extract (1000 nM genistein, 1000 nM puerarin, and 100 µg/ml *P. mirifica* extract) for 48 hours. The cells were lysated with 1% v/v Triton X-100 under brief sonication in ice, and then incubated with the substrate, 2 mM ρ–nitrophenol phosphate in 0.2 M bicarbonate buffer (pH 10), 0.05% v/v Triton X-100, and 4 mM $MgCl_2$ (Sigma) for 1 h at 37 °C. The reaction was stopped by adding 1 M NaOH and absorbance was read at 405 nm in a microplate reader (model 1420; Wallac, Turku, Finland). The amount of product was obtained from a ρ-nitrophenol standard curve. The enzymatic activity was normalized to total protein concentration, obtained using the bicinchoninic acid protein assay (BCA, Pierce), and expressed as nmol/min/mg protein (Bidarra et al., 2011).

Protein concentration determination

The bicinchoninic acid protein assay (BCA, Pierce) was used to detect and quantify total protein content for ALP assay. Briefly, UMR 106 cells were lysated for each condition by brief sonication in ice with 1% v/v Trition X-100. Then cell lysates were incubated with the bicinchoninic acid (BCA) working reagent for 1 h at 37 ˚C. The absorbance was read at 540 nm in a microplate reader (Wallac; model 1420, Turku, Finland) (Bidarra et al., 2011).

Mineralization assay

The osteoblast-like UMR 106 cells were seeded in 24-well plate at density $2x10^4$ cells/well the medium containing 50 mM β-glycerophosphate and 50 μg/ml L-ascorbate 2-phospate (Sigma) and treated with 10 nM of E_2, and high dose of each phytoestrogens and *P. mirifica* extract (1000 nM genistein, 1000 nM, puerarin, and 100 μg/ml *P. mirifica* extract) until day 6. The culture medium was replaced with fresh treatment medium every 3 days. The presence of mineralized nodules was determined by staining with Alizarin Red S (Sigma). Briefly, after removal of the culture medium, the wells were washed twice with PBS and the cells were fixed by adding 70% cold ethanol to each well for 1 hour. The cells were washed extensively with deionized water and stained with 40 mM Alizarin Red S at pH 4.2 for 10 minutes at room temperature. The cells were then washed 5 times with distilled water to wash the nonspecifically bound stain. The red mineralized nodule staining was photographed with Canon EOS 500D (Canon USA Inc., New York, USA).

For quantitative analysis of mineralized calcium, UMR 106 cells were exposed to the complete medium containing 1 μg/ml calcein (Sigma) for 4 hours at 5%CO_2 and 37 $^{\circ}$C prior to quantitation. The osteoblast cells were washed 3 times with PBS and overlaid with 1 mL PBS. Then the bound calcien fluorescence was read in a fluorescence multiwell plate reader (model 1420; Wallac, Turku, Finland) at 485 nm excitation and 530 nm emissions (Hale et al., 2000).

Statistical analysis

The results are expressed as means ± SE. Data were tested for homogeneity of variance. Two-group comparisons were analyzed by Mann–Whitney test. Multiple comparisons were performed by Kruskal-Wallis One-Way Analysis of Variance (ANOVA) followed by Dunnett's post-test. The level of significance was $p < 0.05$. All data were analyzed by GraphPad Prism 5 (Graphpad, San Diego, CA, USA).

Results

Phytoestrogen contents in *P. mirifica* extract

As shown in Figure 3.1, five isoflavone phytoestrogens, i.e., puerarin, daidzin, genistin, daidzein and genistein were identified in *P. mirifica* extract by HPLC technique. The retention times were 13.702, 17.976, 23.931, 34.359 and 43.308 minutes, respectively. Puerarin with a concentration of 35.3 mg/100 g was the major component of the extract. The concentrations of daidzin, genistin, daidzein and genistein were 2.3, 0.4, 11.2 and 0.9 mg/100 g, which were lower than that of puerarin by 15-, 90-, 3-, and 40-fold, respectively.

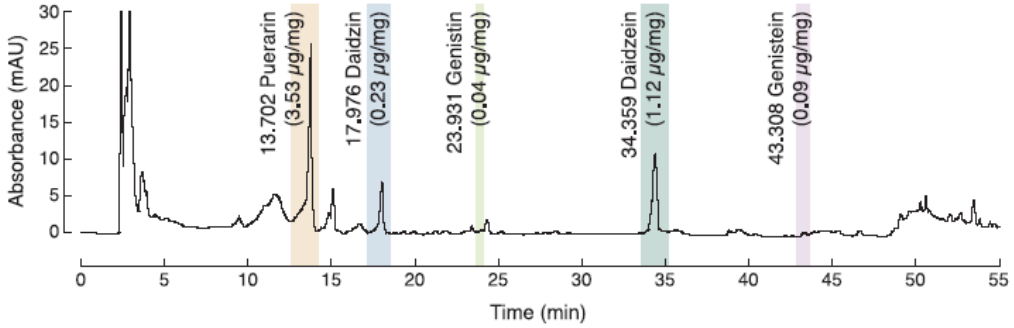

Figure 3.1 A chromatogram of *P. mirifica* extract reveals its components, i.e., puerarin, daidzin, genistin, daidzein and genistein. The total retetion time and concentration of each component as determined by HPLC are also presented.

The characteristics of osteoblast-like UMR106 cells

To determine the characteristics of osteoblast-like UMR106, the cells were photographed under a inverted-light microscope. UMR106 cells rapidly proliferation as shown in Figure 3.2 A-C. From the day of seeding with concentration of $5x10^5$ cells in 100 mm culture plate (Day 0; Figure 3.2A), the round shape of osteoblast cells were attached and adhered onto the culture plate within 2 days (Figure 3.2B). UMR106 cells were greater than 80% of cell confluent in day 4 and the morphology of these cells display as spindle shape (Figure 3.2C).

UMR106 also showed an *in vivo* mineralization when supplement the culture medium with 50 nM β-glycerophosphate. The extracellular calcium was deposited and formed mineralized nodules with the red staining of Alizarin Red S on day 4 thereafter (Figure 3.3A and D) and these mineralized nodules increased in number with progressive day as shown in day 7 and day 9 (Figure 3.3 B,C and E,F).

Osteoblast-like UMR106 cells expressed ERs and responded to E_2

Prior to investigating the effects of isoflavones on osteoblasts, we first determined whether osteoblast-like UMR106 cells expressed ERs and were responsive to estrogens. qRT-PCR revealed that UMR106 cells strongly expressed both ERα and ERβ mRNAs (Figure 3.4A), suggesting that UMR106 cells could respond to estrogens and phytoestrogens through ERs.

After exposure to 10 nM E_2, cell viability as determined by MTT assay was not changed (Figure 3.4B), but the proliferation rate as determined by BrdU assay was significantly decreased ($p< 0.05$) (Figure 3.4C). Further time-dependent study showed that UMR106 cells upregulated the mRNA expression of an osteoblast differentiation marker ALP after 48- and 72- h exposure to E_2 as compared to the corresponding vehicle treated groups (Figure 3.4D).

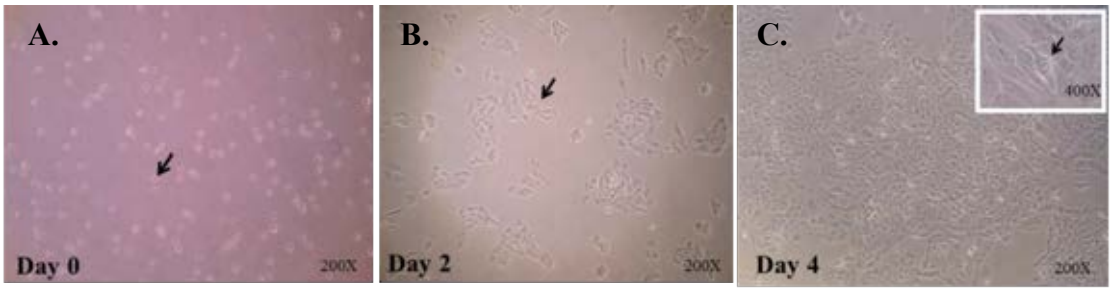

Figure 3.2 The UMR-106 osteoblast-like cells from day 0 (A), the round shape of osteoblast cells were seeded to 100 mm culture plate at concentration of $5x10^5$ cells. At day 2 (B), the osteoblast cells attached to the culture plate, rapidly proliferated and (C) had 80% of cell confluent at day 4 of culture. The arrows indicate osteoblast cells.

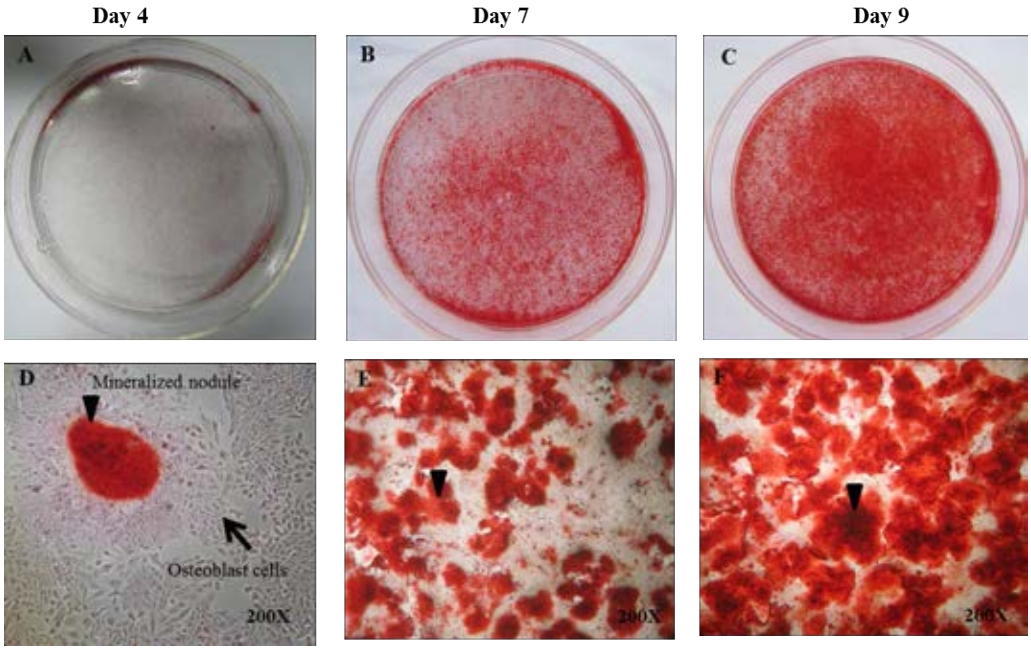

Figure 3.3 The *in vitro* mineralization of UMR-106 osteoblast-like cells. UMR-106 cells were cultured in 100 mm culture plate at concentration of $5x10^5$cells in DMEM supplement with 50 nM β-glycerophosphate. The extracellular calcium deposition was determined by Alizarin Red S staining. The extracellular calcium was formed a mineralized nodule in red color from day 4 (A) of culturing and these nodules increased in number by day 7 (B) and 9 (C). The mineralized nodules of day 4 (D), day 7 (E) and day 9 (F) were magnified for 200x times. Arrow heads indicates mineralized nodule.

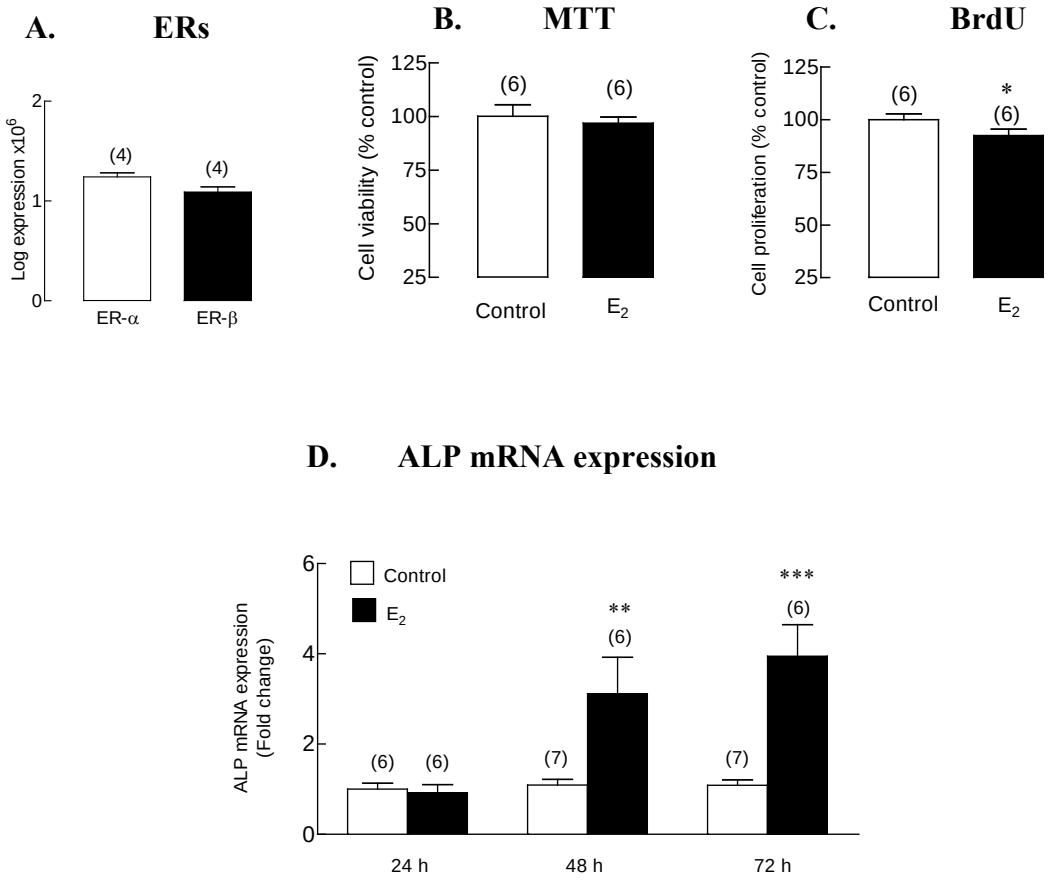

Figure 3.4 The mRNA expression of ER-α and ER-β in UMR106 cells, as determined by qRT-PCR (A). Cell viability (B) and cell proliferation (C) of UMR 106 after exposure to 10 nM E_2, as determined by MTT and BrdU assays, respectively. The mRNA expression of ALP in UMR106 treated for 24, 48, or 72 h with 10 nM E_2. The numbers in parentheses represent the number of independent samples. *p< 0.05, **p<0.01, *** p<0.001 compared with the control group.

Effects of phytoestrogens and *P. mirifica* extract on cell viability and proliferation

Consistent with the results of E_2, after treated UMR106 cells for 48 h with 0.1, 10, or 1000 nM genistein, 0.1, 10, or 1000 nM puerarin, and 1, 10, or 100 µg/ml *P. mirifica* extract, cell proliferation was significantly decreased (p<0.05 and 0.01) (Figure 3.5 D,E,F) with no change on cell viability (Figure 3.5 A,B,C)

Effects of phytoestrogens and *P. mirifica* extract on the expression of genes associated with osteoblast differentiation

Since this study found that UMR106 cells responded to E_2 by upregulating ALP expression, the osteoblast differentiation marker expression. i.e., Runx2, osterix, ALP and osteocalcin were investigated in UMR106 cells after incubated the cells for 24, 48 or 72 h with 0.1, 10 or1000 nM genistein, 0.1, 10, or 1000 nM puerarin, or 1, 10, or 100 µg/ml *P. mirifica* extract. After 24 h of incubation, there were no significant changed of mRNA levels in any doses of all three treatments as shown in Figure 3.6, 3.7 and 3.8. At 48 and 72 h of incubation, genistein significantly increased the mRNA expression of osterix (only at high dose; 1000 nM) and ALP (all three does at 48 h and only the dose of 10 nM at 72 h), however, Runx2 and osteocalcin were not changed (Figure 3.6 A-D). Puerarin significantly increased mRNA expression of only ALP, but not Runx2, osterix or osteocalcin at either 48 or 72 h of incubation (Figure 3.7 A-D). *P. mirifica* extract at all doses significantly increased the mRNA expression of ALP at only 48 h of incubation (Figure 3.8C). On contrary, only at 72 h of incubation, a high dose of *P. mirifica* extract (100 µg/ml) significantly increased the mRNA expression of osterix and osteocalcin (Figure 3.8B and D). Taken together, *P. mirifica* extract, genistein and puerarin all upregulated the ALP mRNA expression, both of genistein and *P. mirifica* extract upregulated osterix mRNA expression, and only *P. mirifica* extract upregulated osteocalcin mRNA expression.

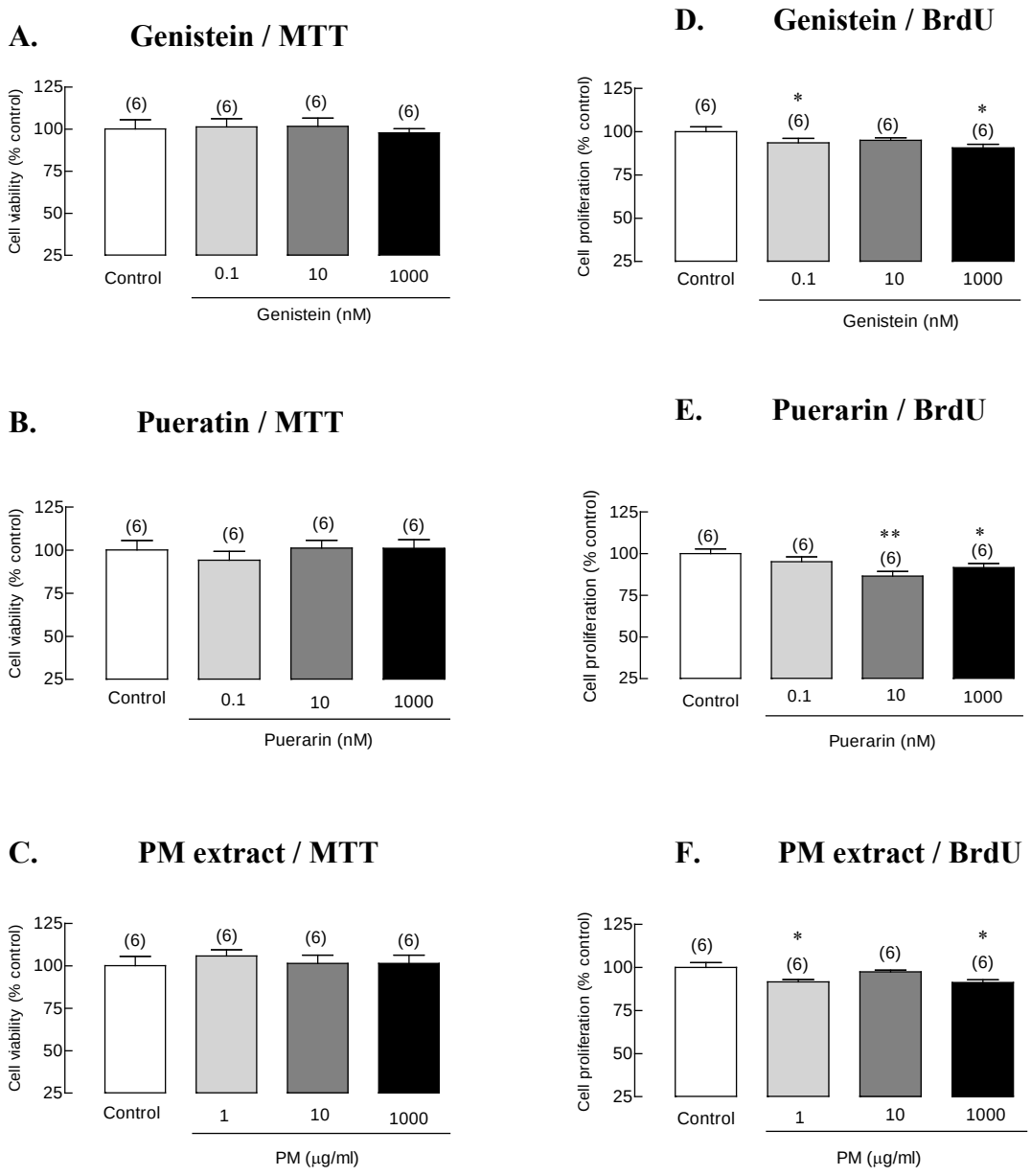

Figure 3.5 Cell viability (A-C) and cell proliferation (D-F) of UMR106 cells after 48-h exposure to vehicle (control), 0.1, 10, or 1000 nM genistein, 0.1, 10, or 1000 nM puerarin, and 1, 10, or 100 μg/ml PM extract as determined by MTT assay and BrdU assay, respectively. The numbers in parentheses represent the number of independent samples. *p< 0.05, **p<0.01 compared with the control group.

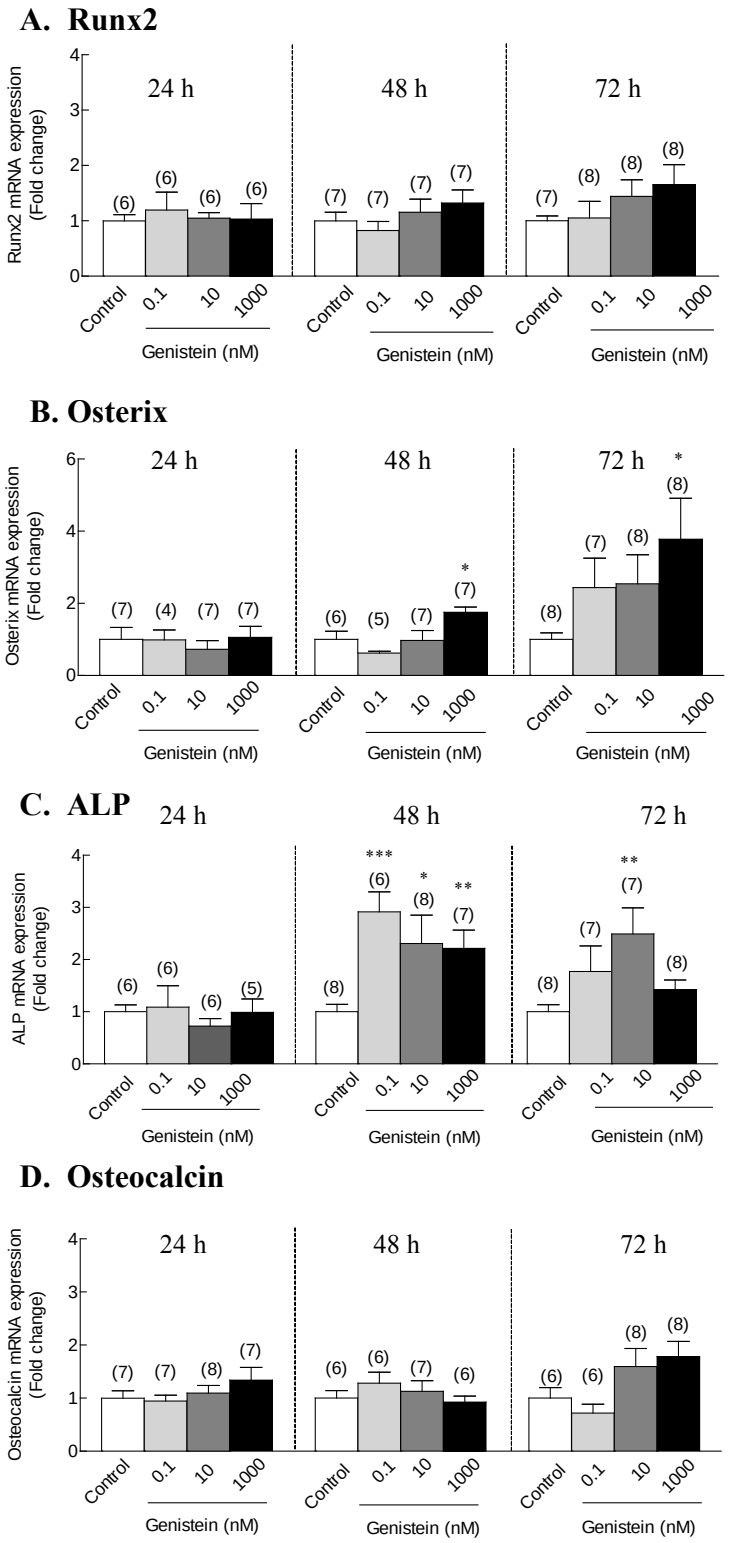

Figure 3.6 The mRNA expression of Runx2 (A), osterix (B), ALP (C) and osteocalcin (D) in UMR 106 cells treated for 24h, 48h or 72h with vehicle (control), and 0.1, 10, or 1000 nM genistein as determined by qRT-PCR. The numbers in parentheses represent the number of independent samples. *p< 0.05, **p<0.01, ***p<0.01 compared with the control group.

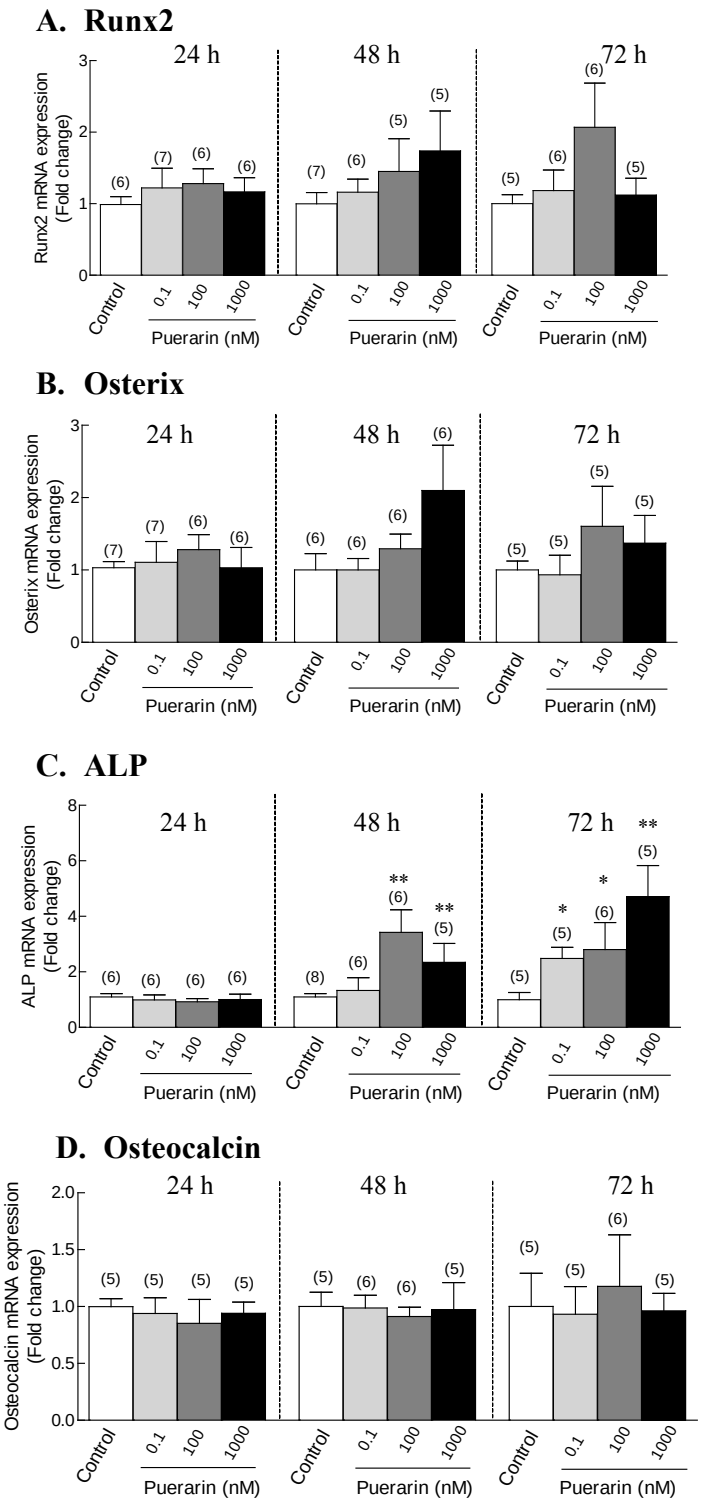

Figure 3.7 The mRNA expression of Runx2 (A), osterix (B), ALP (C) and osteocalcin (D) in UMR 106 cells treated for 24h, 48h or 72h with vehicle (control), and 0.1, 10, or 1000 nM puerarin as determined by qRT-PCR. The numbers in parentheses represent the number of independent samples. *p< 0.05, **p<0.01 compared with the control group.

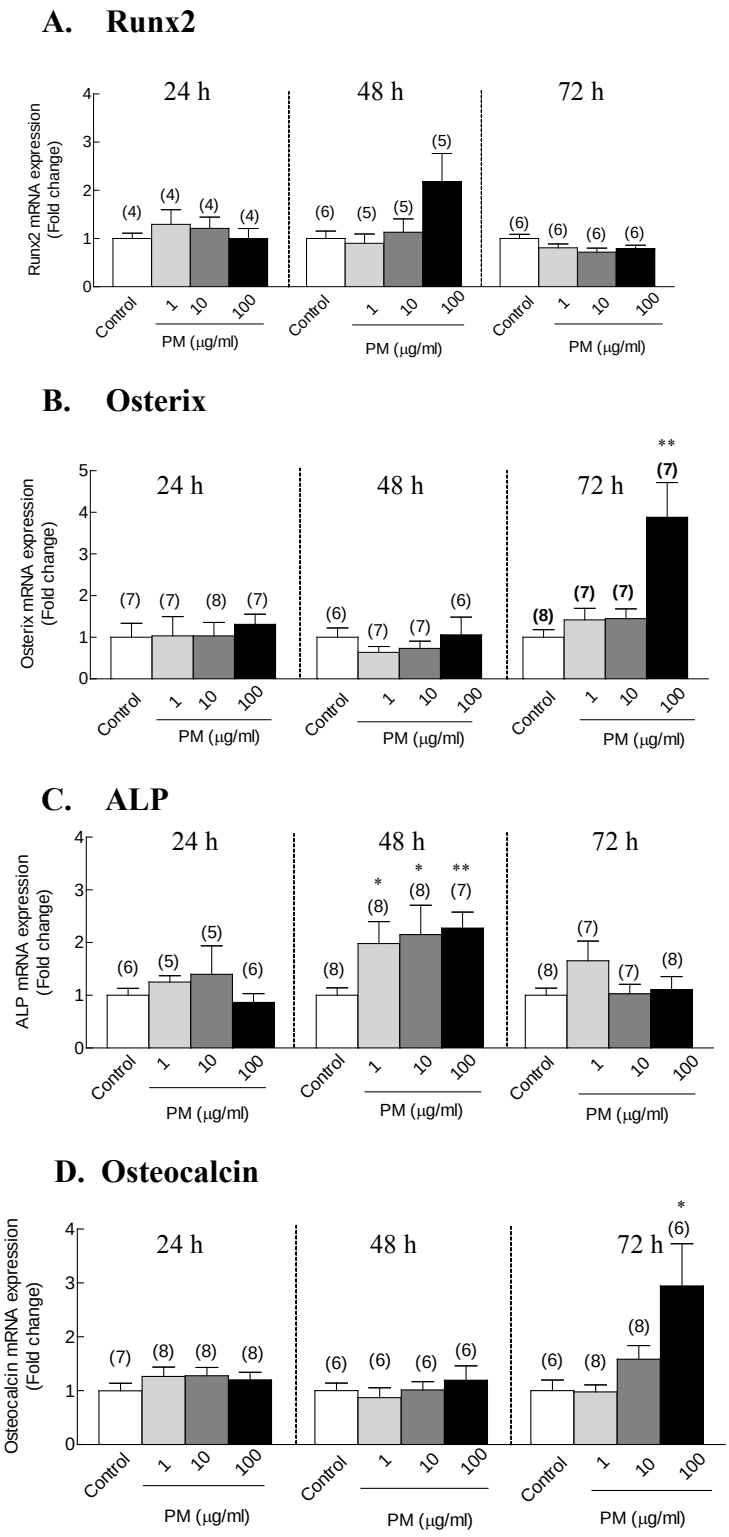

Figure 3.8 The mRNA expression of Runx2 (A), osterix (B), ALP (C) and osteocalcin (D) in UMR 106 cells treated for 24h, 48h or 72h with vehicle (control), and 1, 10, or 100 μg/ml *P. mirifica* extract (PM) as determined by qRT-PCR. The numbers in parentheses represent the number of independent samples.*p< 0.05, **p<0.01 compared with the control group.

Effects of phytoestrogens and *P. mirifica* extract on ALP activity

ALP activity is considered as an early osteogenic marker, since ALP expression increases from the beginning of cell differentiation and increases throughout extracellular matrix maturation (Stein et al., 1990**).** As shown in Figure 3.9, after incubated UMR106 cells for 48 h with 10 nM E_2, 1000 nM genistein, 1000 nM puerarin, and 100 µg/ml *P. mirifica* extract, the cytochemical analysis of ALP (Figure 3.9A) and the levels of ALP activity (Figure 3.9B) normalized to a total protein concentration were not changed.

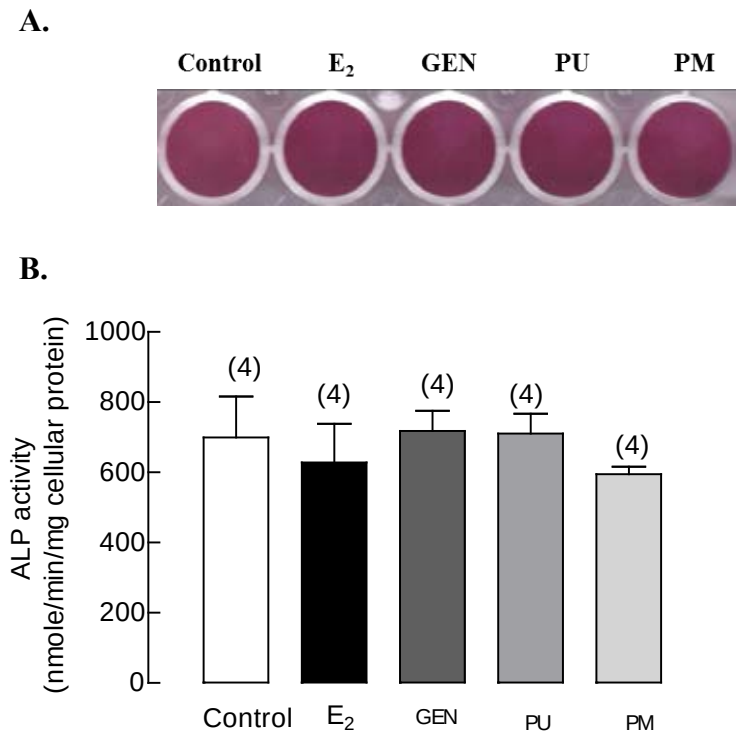

Figure 3.9 The ALP levels (A) and ALP activity (B) in UMR106 cells treated for 48 with 10 nM E_2, 1000 nM genistein (GEN), 1000 nM puerarin (PU), or 100 µg/ml *P. mirifica* extract (PM) as determined by ALP staining and ALP activity, respectively. The numbers in parentheses represent the number of independent samples.

Effects of phytoestrogens and *P. mirifica* extract on the extracellular calcium deposition

Calcium deposition in mineralization phase of osteoblast cell culture system is considered as a functional *in vitro* endpoint reflecting advanced osteoblast cell differentiation and bone formation. As shown in Figure 3.10A, after incubated UMR106 cells for 6 days with 10 nM E_2, 1000 nM genistein, 1000 nM puerarin and 100 µg/ml of *P. mirifica* extract, calcium depositions were clearly increased as indicated by red mineralized nodules. The amount of calcium deposition quantified by calcein fluorescent staining was significantly increased ($p< 0.05$) after all four treatments.

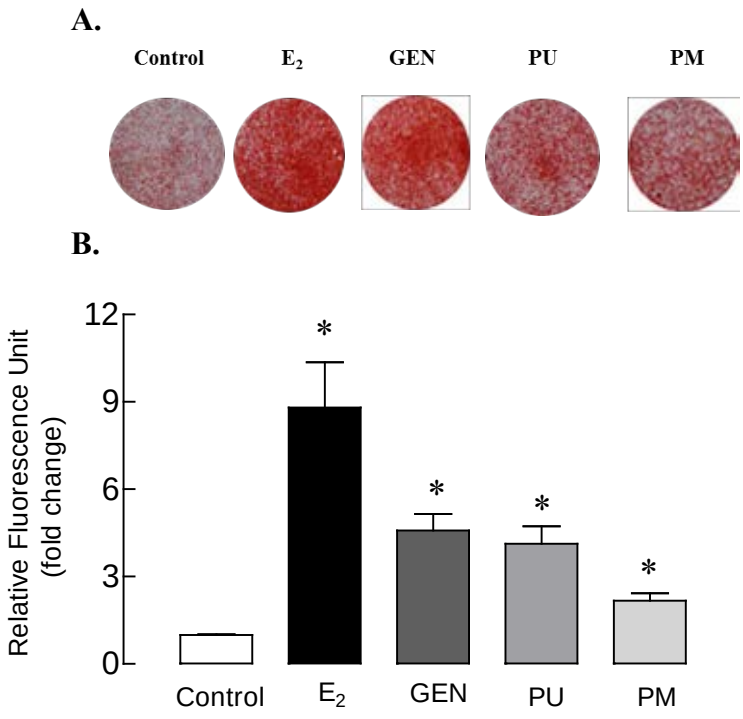

Figure 3.10 The extracellular calcium deposition in UMR 106 cells treated for 6 days with 10 nM E_2, 1000 nM genistein (GEN), 1000 nM of puerarin (PU), or 100 µg/ml of PM extract (PM) as determined by Alizarin Red S staining (A) and quantified by calcien fluorescent staining (B). The numbers in parentheses represent the number of independent samples.*$p< 0.05$ compared with the control group.

Effects of phytoestrogens and *P. mirifica* extract on the expression of genes associated with osteoblast-regulated osteoclast function

Differentiated osteoblast cells also expressed RANKL and OPG, both of which are commonly used as markers for the assessment of osteoblast-regulated osteoclast function and bone resorption. As shown in Figure 3.11, 3.12 and 3.13A and B, after incubated UMR106 cells for 24, 48 or 72 h with 0.1, 10 or 1000 nM genistein, 0.1, 10, or 1000 nM puerarin, and 1, 10, or 100 µg/ml *P. mirifica* extract, the mRNA expression levels of RANKL and OPG were sporadically increased or decreased. Consistent with the genistein and puerarin, *P. mirifica* extract, in sum, significantly decreased the mRNA expression of RANKL after 48 h of incubation. On contrary, the expression of OPG mRNA was mostly increased after 72 h incubation with genistein, puerarin and *P. mirifica* extract. The RANKL/OPG ratios, an index of osteoclastogenic stimulus, as shown in Figure 3.11, 3.12 and 3.13, were significantly decreased after 48 h incubation with genistein, puerarin, and *P. mirifica* extract.

ER mediated the estrogenic effect of *P. mirifica* extract on osteoblast-like UMR106 cells

To confirm that ER mediated the estrogenic actions of E_2, genistein, puerarin, and *P. mirifica* extract, UMR106 cells were exposed to high affinity ER antagonist (10 nM of ICI182780). As shown in Figure 3.6 A-D, left panel, UMR106 cells treated with E_2, genistein, puerarin, or *P. mirifica* extract significantly increased the mRNA expression of ALP by 3-, 2-, 2- and 2-fold, respectively, as compared to the respective vehicle-treated groups. On the other hand, in the presence of ICI182780, none of the four treatments showed the stimulatory effect on ALP expression (Figure 3.14 A-D, right panel), suggesting that ER mediated the upregulation of ALP mRNA expression.

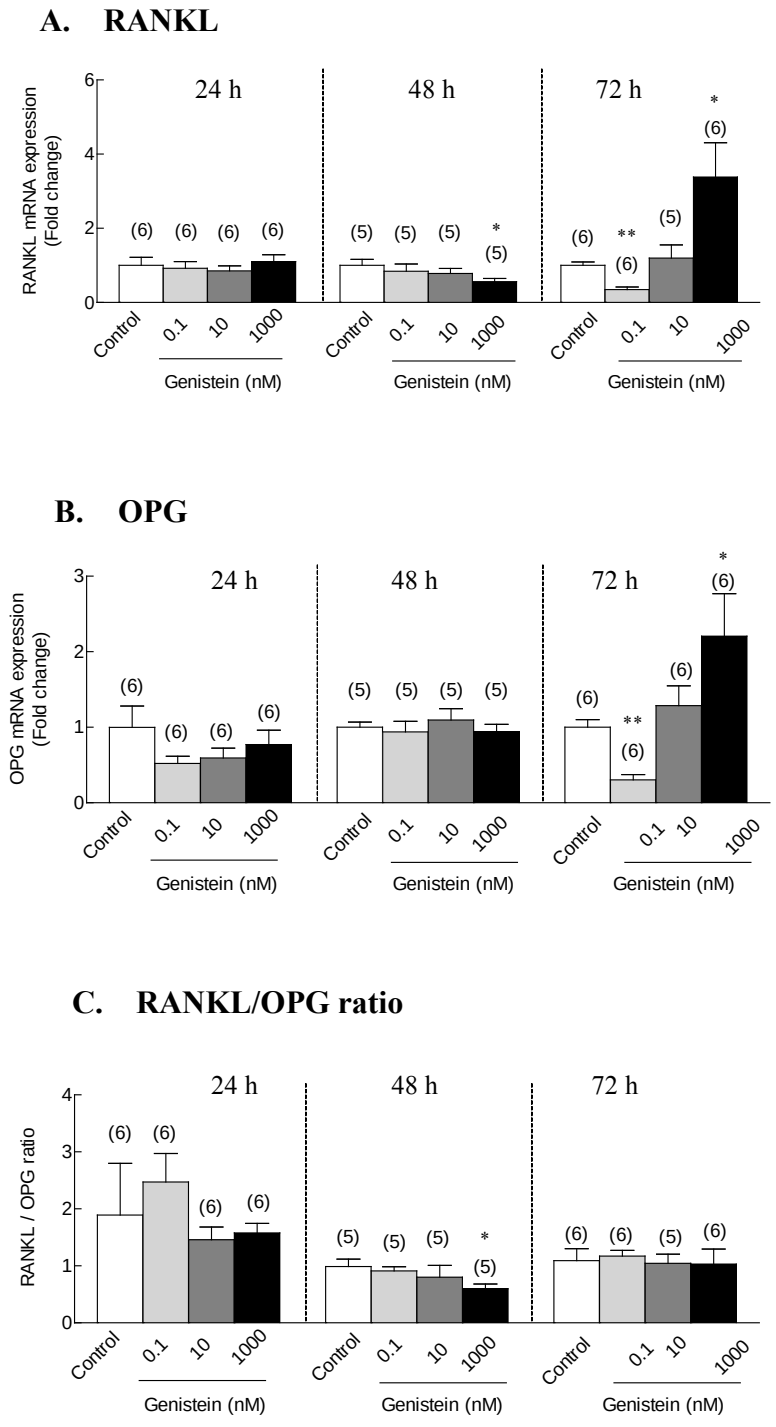

Figure 3.11 The mRNA expression of RANKL(A), OPG (B), and RANKL/OPG ratios (C) in UMR 106 cells treated for 24h, 48h or 72h with vehicle (control), or 0.1, 10, or 1000 nM genistein as determined by qRT-PCR. The numbers in parentheses represent the number of independent samples.*p< 0.05, **p<0.01 compared with the control group.

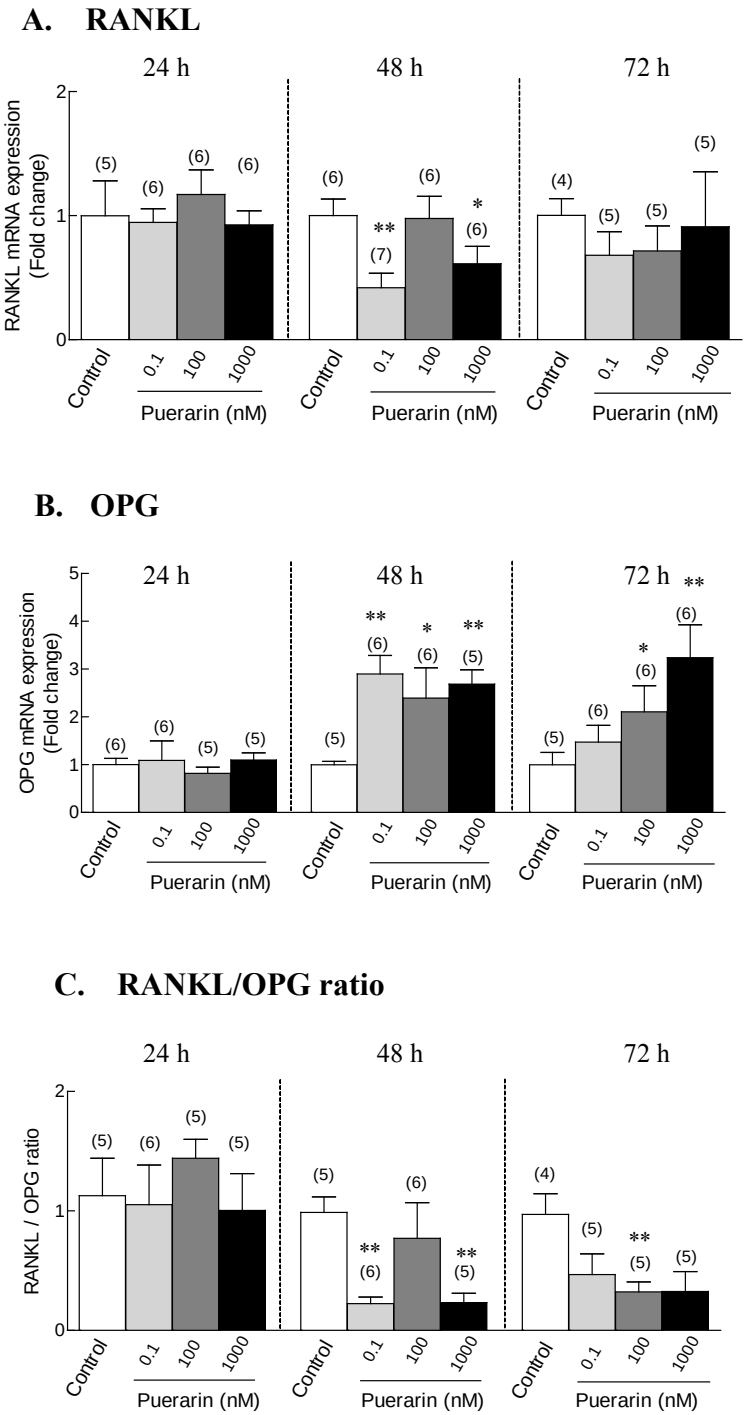

Figure 3.12 The mRNA expression of RANKL(A), OPG (B), and RANKL/OPG ratios (C) in UMR 106 cells treated for 24h, 48h or 72h with vehicle (control), or 0.1, 10, or 1000 nM puerarin as determined by qRT-PCR. The numbers in parentheses represent the number of independent samples.*p< 0.05, **p<0.01 compared with the control group.

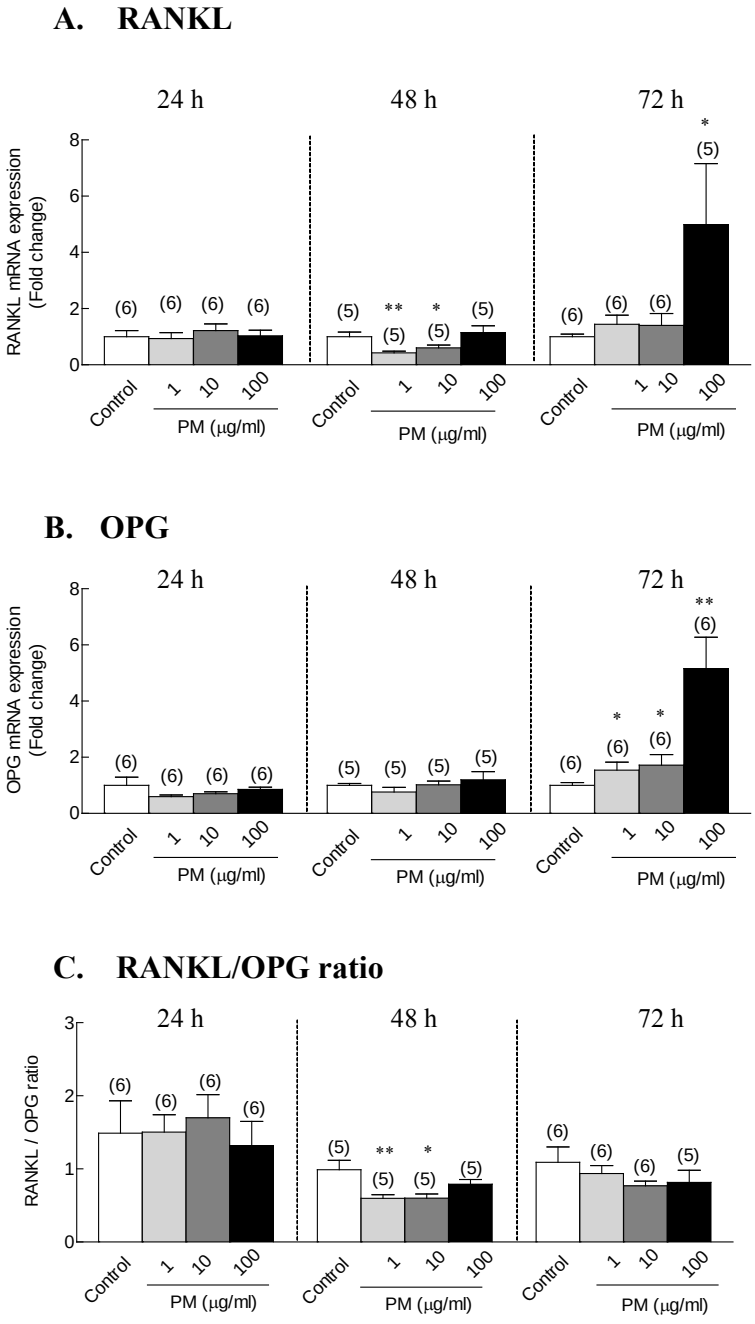

Figure 3.13 The mRNA expression of RANKL(A), OPG (B), and RANKL/OPG ratios (C) in UMR 106 cells treated for 24h, 48h or 72h with vehicle (control), or 1, 10, or 100 μg/ml of *P. mirifica* extract as determined by qRT-PCR. The numbers in parentheses represent the number of independent samples.*p< 0.05, **p<0.01 compared with the control group.

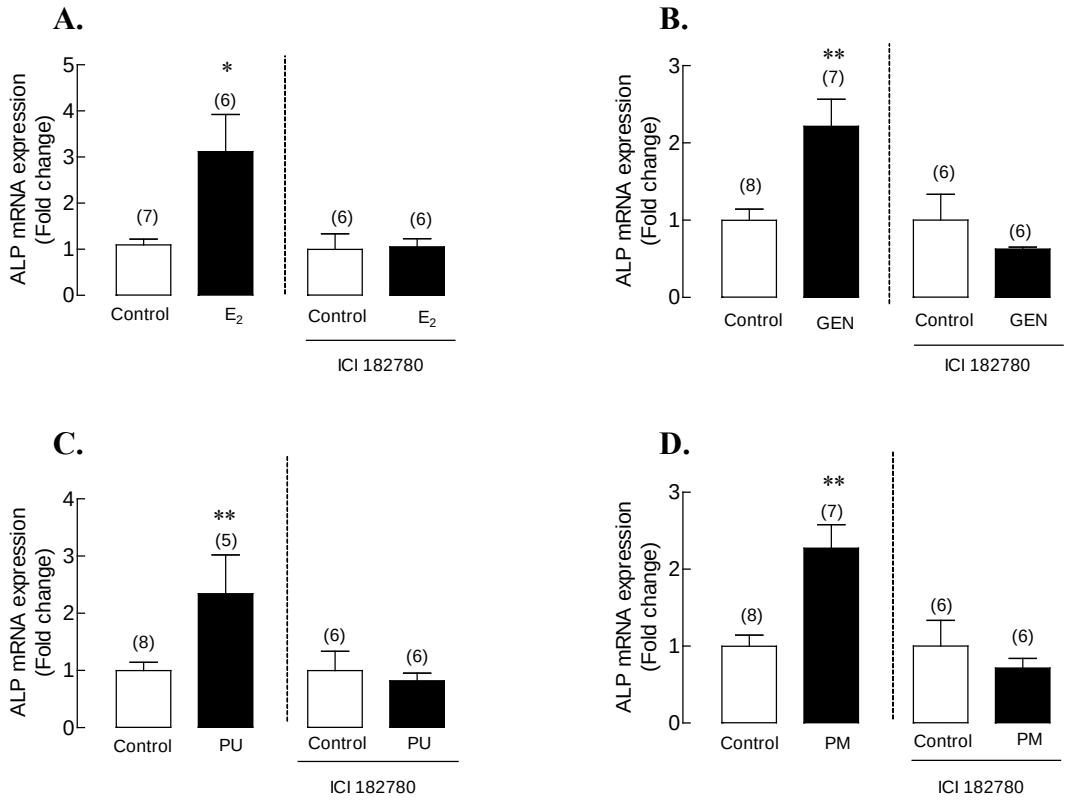

Figure 3.14 The ALP mRNA expression in UMR106 cells treated for 48 h with 10 nM E_2 (A), 1000 nM genistein (GEN; B), 1000 nM puerarin (PU; C), or 100 μg/ml *P. mirifica* extract (PM; D) in the absence (left panel) or presence (right panel) of 10 nM ICI 182780 (ER antagonist). The numbers in parentheses represent the number of independent samples.*p< 0.05, **p<0.01 compared with the control group.

Discussion

Postmenopausal osteoporosis is caused by a decrease in estrogen level that leads to an increased rate of bone remodeling Currently available treatments for postmenopausal osteoporosis are including hormone replacement therapy, calcitonin, bisphosphonates, and selective estrogen receptor modulators (SERMs), such as raloxifene. Although hormone replacement therapy is effective in reducing postmenopausal bone loss, it is associated with a higher risk for breast, endometrial, and ovarian cancer, cardiovascular disease, venous thromboembolism, and stroke (Manolagas et al., 2002). In addition, several agents, such as bisphosphonates, calcitonin and raloxifene, exhibit various severity in adverse side effects and very expensive. For example, bisphosphonates are associated with gastrointestinal side effects; osteonecrosis of the jaw and subtrochonteric fracture (Manolagas et al., 2002). Recently, the demands for alternative and natural strategies for osteoporosis prevention and treatment are increased. The natural alternatives currently under investigation, phytoestrogens appear to offer the most potential for the prevention of bone loss (Button and Patel, 2004).

Phytoestrogen has attracted attention for its potential in the prevention of bone loss (Tham et al. 1998; Setchell and Lydeking-Olsen 2003). Genistein, a major phytoestrogen component in soybean, has been most investigated its effects on the prevention of bone loss in animal models both *in vivo* and *in vitro*. It increased bone mass by stimulating osteoblastic bone formation and inhibiting osteoclastic bone resorption (Gao & Yamaguchi, 1999a; Sugimoto & Yamaguchi, 2000; Chen et al., 2003; Heim et al., 2004). Thai phytoestrogen-rich herb *P. mirifica* has also shown estrogenic effect on bone by increasing BMD and BMC in both ovariectomized and orchidectomized rats (Urasopon et al. 2007, 2008. In the present study, the mechanisms of *P. mirifica* on osteoblast cells including proliferation, differentiation, and mineralization process during osteoblast development were investigated. Osteoblast-like UMR106 cells used in the present study constitutively expressed ER-α and ER-β transcripts, and were responsive to E_2 and phytoestrogens by upregulating ALP expression (Li et al. 2005). Puerarin was identified as the major component of *P. mirifica* extract, which upregulated the expression of osteoblast differentiation markers, especially ALP, in an ER-dependent manner. However, *P. mirifica* extract

and two phytoestrogens (genistein and puerarin) were found to modestly decrease cell proliferation rate, suggesting that they probably induced bone formation by enhancing osteoblast differentiation rather than proliferation. Since cell viability as determined by MTT assay was not altered, consistent with that observed in *P. lobata* extract-treated osteoblast-like SaOS-2 cells (Huh et al. 2006), *P. mirifica* extract and puerarin might be protective against apoptosis. In diabetic rats, it was evident that the expression of caspase-3, a marker of apoptosis, was decreased after administration of puerarin (Liang et al. 2012). Further investigation is, therefore, required to demonstrate whether puerarin induces bone formation, in part, by inhibiting the caspase-3-mediated osteoblast apoptosis.

After the proliferation stage, osteoblasts normally undergo differentiation through a two-step sequential event, i.e., extracellular matrix maturation (early differentiation) and mineralization (late differentiation) (Owen et al., 1990; Ducy et al., 2000), which are controlled by different regulators. For example, early development from mesenchymal cells to osteoblastic lineage is determined by the key transcription factors, Runx2 and osterix (Komori, 2006). Runx2 induces mesenchymal stem cell proliferation and initiates their differentiation into pre-osteoblasts (Komori, 2006; Ducy et al., 2000). Osterix is downstream from Runx2, and is also essential for osteoblast differentiation (Komori, 2006). Pre-osteoblasts also proliferate under the stimulation of various humoral factors, e.g., IGF-1, fibroblast growth factor-2, and bone morphogenetic proteins (Zaidi, 2007; Ou et al., 2010). Once osteoblast proliferation declines, expression of a marker of matrix maturation phase, ALP, is gradually increased (Owen et al., 1990; Stein et al., 2004; Zaidi, 2007). Finally, bone matrix is mineralized, concurrently with an increase in osteocalcin expression (Owen et al., 1990; Stein et al., 2004). Herein, after a 48-h exposure to *P. mirifica* extract, genistein or puerarin, ALP transcript, but not Runx2, osterix and osteocalcin transcripts, was markedly increased, suggesting that *P. mirifica* extract and its phytoestrogens promoted bone formation by enhancing early osteoblast differentiation into matrix maturation stage. After 72-h exposure, *P. mirifica* extract and genistein also increased osterix transcript. Furthermore, high dose of *P.mirifica* extract markedly increased osteocalcin transcript. The results suggesting that the different isoflavone phytoestrogens derivatives, puerarin and genistein, which found

in *P. mirifica* extract might induced the different in mRNA transcript since the binding affinity to ER-α and ER-β was different (Morito et al., 2001). However, in the present study, ALP activity was not changed after 48-h exposure to *P. mirifica* extract, genistein and puerarin. The results obtained might indicate that the induction of mRNA synthesis at the transcription is not preceded by an effect on the translation of protein. As UMR 106 cells exhibit high proliferation, well differentiated, express high level of ALP activity and enhanced the rapid rate of mineralization (Standford et al., 1995), therefore, translational changed of protein possibly occurred and saturated before day 4 that these cells in this study reached confluent. In addition, the previous study by Morris et al.(2000) performed in human osteosarcoma osteoblast MG-63 cells shown that genistein at concentration rage from 2.5 to 30 μM reduced the ALP activity. Conversely, genistein at concentration ranging from 1 to10 μM induced ALP activity in mouse osteoblast-like cells MC3T3-E1 (Sugimoto and Yamaguchi, 2000). Therefore, the effect of phytoestrogens on osteoblast ALP activity *in vitro* seems to depend on the cell type, proliferative rate and confluent of each cell.

At the late differentiation, after osteoblasts have produced a complete and maturated extracellular matrix (ECM), the mineralization is occurred by the deposition of hydroxyapatite mineral (calcium and phosphate) in the ECM. The mineralization is controlled by many factors including calcium and phosphate concentrations, enzyme activity, and the composition of the ECM (Eijken, 2007). Initiation of mineralization requires the precipitation and attachment of hydroxyapatite crystals to the ECM. There are at least two possible processes which could be involved in the control of mineralization. The first, directly from cellular activity via the production of vesicles from outer plasma membranes of osteoblast, It has been demonstrated that after high in calcium content vesicles are released into the ECM, they initiate formation of the first mineral crystal (Kirsch et al., 1997). The second depends upon the presence of organic components including collagen and ALP. Collagen plays a role as a nucleation template for the mineralized take place. The osteoblast differentiation marker ALP is suggested to play a role in the mineralization process, where it hydrolyses organic phosphate substrates to release free inorganic phosphate. This enzyme is highly expressed in vesicles together with several phosphate and calcium transporters. These transporters increase local calcium

and phosphate concentrations and thereby initiate hydroxyapatite crystal formation (Boskey et al., 1996; Eijken, 2007)

In the present study, by supplemented the osteoblast culture with β-glycerophosphate, osteoblasts can be induced to produce vast extracellular calcium deposits *in vitro*. After exposure to high dose of *P. mirifica* extracts (100 μg/ml) genistein (1000 nM) or puerarin (1000 nM), the calcium deposition and the formation of mineralized nodules were increased. Consistent with observed in MG63 (Morris et al., 2006) and mouse bone marrow-derived mesenchymal stem cells (BMSC) (Liao et al., 2007), genistein at concentrations of 2500 nM and 1000 nM increased calcium deposition. In addition, Zhang et al. (2007) also demonstrated that 10-100 μM puerarin increased mineralizing nodules in rat primary osteoblasts via the phosphoinositide-3-kinase/Akt pathway.

Since ICI182780 completely abolished the *P. mirifica* extract-, genistein- and puerarin-induced upregulation of ALP transcript, their estrogenic actions were mediated by ERs, similar to the actions of E_2. Indeed, the observed increase in ALP expression after exposure to E_2 and phytoestrogens was consistent with the previous reports of *in vitro* studies in primary osteoblasts and osteoblast cell lines (Zhang et al., 2007; Li et al., 2005). In addition, differentiated osteoblasts, in turn, control bone remodeling process by producing RANKL and OPG (Teitelbaum, 2000). After being released from osteoblasts, RANKL binds to its receptor, RANK, on osteoclast precursors, thereby inducing differentiation of these cells into osteoclasts. The osteoclastogenesis is inhibited by OPG, a soluble decoy receptor of RANKL. Thus, the balance between the expression of osteoclastogenic stimulator (RANKL) and inhibitor (OPG) maintains equal bone formation and resorption in bone remodeling (Teitelbaum, 2000). However, osteoblasts are targets of several osteoclastogenic agents, such as parathyroid hormone (PTH) and prolactin, that shift the RANKL/OPG ratio toward bone resorption, which eventually leads to bone loss and osteoporosis (Charoenphandhu et al., 2010; Zaidi, 2007; Teitelbaum, 2000). In the present study, *P. mirifica* extract, genistein and puerarin significantly upregulated the OPG mRNA expression although RANKL expression was upregulated by the high-dose *P. mirifica* extract (100 ug/ml) and genistein (1000 nM) at 72 h exposure. Puerarin might have greater anti-osteoporotic potency than *P. mirifica* extract and genistein since it could

reduce the RANKL mRNA expression, thereby decreasing the RANKL/OPG ratio by ~80%. *P. mirifica* extract and puerarin thus exerted a positive effect on bone not only by increasing osteoblast differentiation, but also by suppressing the osteoclast-mediated bone resorption. Consistent with this finding, Li et al. (2004) reported that 10 and 1000 nM puerarin could suppress osteoclast activity in osteoblast/osteoclast co-culture with bovine bone slices by reducing the number and surface area of osteoclast absorption lacunae. The puerarin-induced decrease in RANKL/OPG ratio could explain the suppressed osteoclast activity in this co-culture system.

In conclusion, the present study provided corroborative evidence for the first time that *P. mirifica* extract and its major isoflavone, puerarin, were likely to enhance bone formation by promoting osteoblast differentiation and mineralization, as indicated by the upregulation the mRNA expression of osteoblast differentiation marker gens and increased in calcium deposition. Moreover, both *P. mirifica* extract and puerarin may suppress osteoclast activity since they predominantly upregulated OPG expression, thereby decreasing the RANKL/OPG ratio. Therefore, it would be the worth exploring to develop a Thai phytoestrogen rich herb *P. mirifica* for alternative drugs for bone loss therapy with less adverse effects for human.

CHAPTER IV

PHYTOESTROGEN FROM *Pueraria mirifica* INCREASED PROLIFERATION AND UPREGULATED mRNA EXPRESSION OF OSTEOBLASTIC DIFFERENTIATION MARKERS IN RAT PRIMARY OSTEOBLAST CELLS

Introduction

Osteoporosis, a disease of bone mass depletion and bone micro-architectural deterioration, is a major public health problem in aging population. In women, estrogen deficiency in the postmenopausal period has been recognized as a critical factor in osteoporosis development (Erben et al., 2000; Cole et al., 2008). Although ERT is effective in reducing bone loss, it is recently reported that HRT is associated with a high risk for breast, endometrial and ovarian cancers (Manolagas et al., 2002). Regarding these problems, alternative drugs for bone loss therapy with less adverse effects are being sought.

P. mirifica, a Thai phytoestrogen- rich herb and known in Thai as white Kwao Krua, belongs to the family Leguminosae subfamily Papilinoideae. Its tuberous root is composed of mostly isoflavonoid, such aspuerarin, daidzin, genistin, daidzein and genistein (Cherdshewasart et al., 2007a; Cherdshewasart and Sriwatcharakul, 2007). It clearly exhibits estrogenic activity in rat and monkey reproductive system as estrogen does (Malaivijitnond et al., 2004, 2006; Trisomboon et al., 2004, 2005, 2006; Cherdshewasart et al., 2007). Other than the effects on reproductive organs, *P. mirifica* has been demonstrated to prevent bone loss in osteoporotic rats by increasing in BMD and BMC (Urasopon, 2007, 2008). In addition, the mechanisms of the plant extract and its phytoestrogens was determined in osteoblast-like UMR106 cells (Tiyasatkulkovit et al., 2012). UMR106 cells are a rat clonal osteosarcoma cell line which has the osteoblast-like phenotypic properties. The results showed that *P. mirifica* extract, puerarin, and genistein promoted bone formation by a significantly

increased osteoblast differentiation through the upregulation of ALP mRNA expression (Tiyasatkulkovit et al., 2012) and increase in extracellular calcium deposition and *in vitro* mineralization in rat osteoblast-like UMR106 cells.

Although UMR-106 cells are the powerful research tool to study bone biology because it has an unlimited proliferation capacity, easily to manipulate, and, because of the properties of cell line, the results gained are consistency (Midura et al., 1990; Celic et al., 1998), these cells indeed do not represent the rat *in vivo* situation. To determine the molecular mechanisms on bone cells, it needs to duplicate the experiment from UMR106 cells in the rat primary cells, which are supposed to be a superior *in vivo* model. Therefore, the aim of the present study was to investigate the effects of phytoestrogens from *P. mirifica* on cell proliferation and mRNA expression of bone formation and resorption markers of primary rat osteoblast cells.

Materials and Methods

Preparation of *P. mirifica* extract and identification of the phytoestrogen contents

The tuberous roots of *P. mirifica* cultivar SARDI 190 (lot no.0070317) were purchased from Dr. Sompoch Tubcharoen, Kasetsart University, Kamphaeng Saen Campus, Thailand, and were authenticated as *P. mirifica* by comparing with the voucher specimens (nos.BCU010250 and BCU011045) kept at the Professor Kasin Suvatabhandhu Herbarium, Department of Botany, Faculty of Science, University, Thailand. The specimens were washed, sliced, dried in a hot-air oven at 70℃, and ground. One gram of tuberous powder was extracted twice by mixing with 4 ml of 70% ethanol (Urasopon et al., 2008). The supernatants collected from two extractions were mixed together and dried for 4 h in a centrifugal concentrator at room temperature. Dry sample was kept at 4 ˚C until used in cell culture, and some portion was used for HPLC analysis. Five isoflavone phytoestrogens, i.e., puerarin, daidzin, genistin, daidzein and genistein were identified in *P. mirifica* extract by HPLC technique. Puerarin with a concentration of 35.3 mg/100 g was the major component of the extract. The concentrations of daidzin, genistin, daidzein and genistein were

2.3, 0.4, 11.2 and 0.9 mg/100 g, respectively, as described in the previous report (Tiyasatkulkovit et al., 2012).

Isolation and culture of primary rat osteoblast cells

The tibia and fibular bone of eight-week-old female Sprague-Dawley rats were used in this study. The bone specimens were obtained from the National Laboratory Animal Centre, Mahidol University, Thailand. To earn high value of animal use, the bones were collected from animals which were also subjects of other project. The animal use protocol was approved by the Institutional Animal Care and Use Committee of the National Laboratory Animal Centre, Mahidol University. The animals were housed in the husbandry unit with 12/12 h dark/light cycle and were fed regular rat chow (Perfect Companion, Bangkok, Thailand), and reverse osmosis water *ad libitum*. The room temperature was 25 ˚C and the humidity was 55%.

The bones were removed extraneous soft connective tissue from the outer surface of the bone by scraping with a sterile scalpel blade and rinsed with sterile phosphate-buffered saline (PBS). The bone was transferred to a sterile Petri dish containing Dulbecco's modified Eagle's medium (DMEM) (Sigma Chemical, St. Louis, MO, USA) with antibiotics (100 IU/ml penicillin and 100 µg/ml streptomycin (Gibco)). Bone was cut into small fragments of 1-3 mm^2 and extensively washed in DMEM for 5 times or until no hematopoietic marrow was remained or the white color of bone fragment was visible. Bone chips were transferred to culture flask containing 10 ml of DMEM and digested with 0.25% collagenase (Sigma Chemical, St. Louis, MO, USA) at 37 ˚C for 2h. The digestion was stopped by removing the DMEM and collagenase and adding 10 ml of DMEM with 10% fetal bovine serum (FBS; Sigma Chemical, St. Louis, MO, USA). Bone chips were transferred to culture flask with complete medium containing 10 ml of DMEM supplemented with 30% FBS, 100 IU/ml of penicillin and 100 µg/ml of streptomycin, 50 µg/ml of L-ascorbate 2-phosphate and 100 µM of sodium pyruvate (Gibco, Grand Island, NY, USA). Bone cell cultures were incubated at 5% CO_2, 37℃ for 3 days with undisturbed. Cells growth was maintained in the same complete medium but containing only 15% FBS and the medium was changed every 3 days. The osteoblast confluence cells were

subcultured with 5% trypsin (Gibco, Grand Island, NY, USA) and cells were passaged to new culture plate for the consequence experiment. All above procedures were carried on under sterile condition.

Experimental design

To ensure that the isolated and cultured cells were rat primary osteoblast, characteristics of the osteoblast cells were monitored. Cell morphology was observed under an inverted-light microscope, and the *in vitro* mineralization was determined by Alizarin Red S staining. In addition, the mRNA expression of ERα and ERβ were determined by qRT-PCR. Besides, the responses of primary osteoblast cells to E_2 (Sigma Chemical, St. Louis, MO, USA) were monitored by determining (i) cell proliferation using BrdU assay, (ii) mRNA expressions of osteoblast differentiation associated genes, Runx2, osterix, ALP, and osteocalcin, using qRT-PCR.

To investigate the effects of phytoestrogens and *P. mirifica* extract on rat primary osteoblast cells, the confluent of rat primary osteoblast cells were incubated for 48 with 0.3% vol/vol DMSO (vehicle or control group), various concentrations of genistein (0.1, 10 and 1000 nM), puerarin (0.1, 10 and 1000 nM), or *P. mirifica* extract (1, 10 and 100 µg/ml). The concentrations of genistein, puerarin and *P. mirifica,* and the incubation time used in this study were followed our previous study in the rat osteosarcoma cell line (Tiyasatkulkovit et al., 2012). Cell proliferation was determined by BrdU assay. The mRNA expressions of bone formation markers, Runx2, osterix, ALP and osteocalcin, and bone resorption markers, RANKL and OPG, were quantified by qRT-PCR.

Cell proliferation

Cell proliferation was determined by BrdU enzyme-linked immunosorbent assay kit (catalog no. 11647229001; Roche, Mannheim, Germany), according to the manufacturer's instruction. Briefly, the second passages of rat primary osteoblast cells were seeded in 96-well culture plate at 1000 cells/well. After 48-h incubation with E_2, phytoestrogens and *P. mirifica* extract, cells were fixed and later incubated with anti-BrdU-peroxidase antibody to detect BrdU-incorporated cells. Substrate solution containing tetramethylbenzidine was added for color development. Since BrdU incorporated into the newly synthesized DNA of proliferating cells, the amount of BrdU in each well represented cell proliferation. The absorbance of each well was measured at 370 nm with the reference wavelength of 490 nm by a microplate reader (model 1420; Wallac, Turku, Finland). The BrdU assay was performed in triplicate with 7 independent samples (n = 7).

Total RNA preparation and qRT-PCR

Total RNA samples were extracted from rat primary osteoblast cells by using TRIzol reagent (invitrogen, Carlsbad, CA, USA). Purity of the total RNA was determined by the ratio of absorbance reading at 260 and 280 nm, the ratio of which was between 1.8 and 2.0. One microgram of total RNA was then reverse-transcribed with iScript cDNA synthesis kit and oligo-dT20 (Bio-Rad, Hercules, CA, USA) to cDNA by Bio-Rad MyCycler. Rat β-actin served as a control gene to check the consistency of the reverse transcription (% coefficient of variation <5%, n = 6).

Primers used in the present study are listed in Table 3.1. All primers were first verified by conventional PCR followed by amplicon sequencing. Conventional PCR was performed by Bio-Rad MyCycler with GoTaq Green Master Mix (Promega, Madison, WI, USA). Thereafter, PCR products were visualized on 1.5% agarose gel stained with 1 μg/ml ethidium bromide (Sigma) under UV transilluminator (Alpha Innotech, San Leandro, USA). qRT-PCR and melting curve analysis were performed in triplicate by Bio-Rad MiniOpticon real-time PCR system with iQ SYBR Green SuperMix (Bio-Rad) for 40 cycles at 95 ˚C for 60 s, 53–60 ˚C annealing temperature for 30 s, and 72 ˚C for 30 s.

Evaluation of calcium nodules formation and mineralization

The second passage of rat primary osteoblast cells were seeded in 100 mm cell culture plate at density $5x10^5$ cells and culture for 21 days in complete medium containing 50 mM β-glycerophosphate and 50 µg/ml L-ascorbate 2-phospate (Sigma). The culture medium was replaced with fresh medium every 3 days. The presence of mineralized nodules was determined by staining with Alizarin Red S (Sigma). Briefly, after removal of the culture medium, the wells were washed twice with PBS and the cells were fixed by adding 70% cold ethanol to each well for 1 hour. The cells were washed extensively with deionized water and stained with 40 mM Alizarin Red S at pH 4.2 for 10 minutes at room temperature. The cells were then washed 5 times with distilled water nonspecifically bound stain. The red mineralized nodules staining were taken an image under inverted light microscope.

Statistical analysis

The results are expressed as means ± SE. Data were tested for homogeneity of variance. Two-group comparisons were analyzed by Mann–Whitney test. Multiple comparisons were performed by Kruskal-Wallis One-Way Analysis of Variance (ANOVA) followed by Dunnett's post-test. The level of significance was $p < 0.05$. All data were analyzed by GraphPad Prism 5 (Graphpad, San Diego, CA, USA).

Results

The characteristic of primary rat osteoblast cells

To determine the characteristics of rat primary osteoblast cells, the cells were photographed under inverted-light microscope. During primary culture, round or polygonal cells were observed migrating from a few of bone pieces and attached to culture dish on the thrid day of culture (Figure 4.1A). As the culture time increased, the cells become more triangular and spindle-shaped. The cells form nearly confluent cell layer around the bone tissue (covering 80% of the cell culture dish) after day 7 (Figure 4.1B). In addition, rat primary osteoblast cells showed an *in vivo* mineralization when culture the second passage of these cells and supplement the culture medium with 50 nM β-glycerophosphate. The extracellular calcium deposition showed as mineralized nodule with red crystalized stain after determined by Alizarin Red S staining from day 14 (Figure 4.2A, C) and these mineralized nodule increased in number with progressive day as shown in day 21 (Figure 4.2B, D).

Rat primary osteoblast cells expressed ERs, and responded to E_2

Prior to study the estrogenic effects of phytoestrogens on rat primary osteoblast cells, the expression of both ERα and ERβ mRNAs and the response of these primary cells to E_2 were determined. Rat primary osteoblast cells express both ERα and ERβ (Figure 4.3 A). After exposure to 10 nM E_2 for 48h, rat primary osteoblast cells increased in cell proliferation (Figure 4.3 B) and increased the osteoblast differentiation marker gene, ALP mRNA expression (Figure 4.3 C) but not Runx2, osterix and osteocalcin mRNA expression (data not shown).

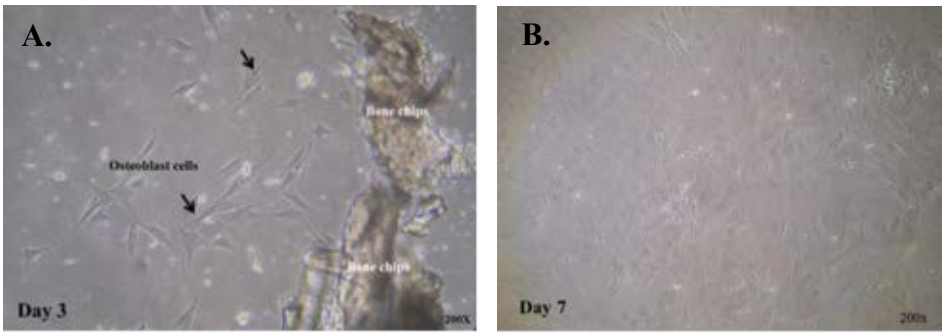

Figure 4.1 The rat primary osteoblast cells were isolated and cultured from rat tibia and fibular bone. Cells migrated from the bone chips after day 3 of culture (A). The osteoblast cells attached to the culture plate, proliferated and had 80% of cell confluent at day 7 of culture. The arrows indicate osteoblast cells.

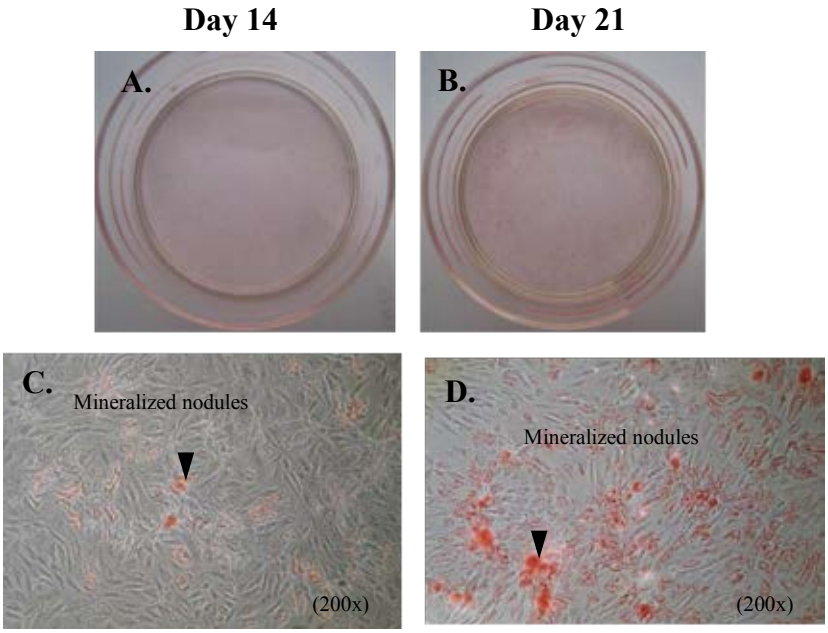

Figure 4.2 The *in vitro* mineralization of rat primary osteoblast cells. Cells were cultured in 100 mm culture plate at concentration of $5x10^5$cells in DMEM supplemented with 50 nM β-glycerophosphate. The extracellular calcium deposition was determined by Alizarin red staining. The extracellular calcium was formed a mineralized nodule in red color from day 14 (A) of culturing and these nodules increased in number by day 21 (B). The mineralized nodules of day 14 (C) and day 21 (D) were magnified for 200x times. Arrow heads indicates mineralized nodule.

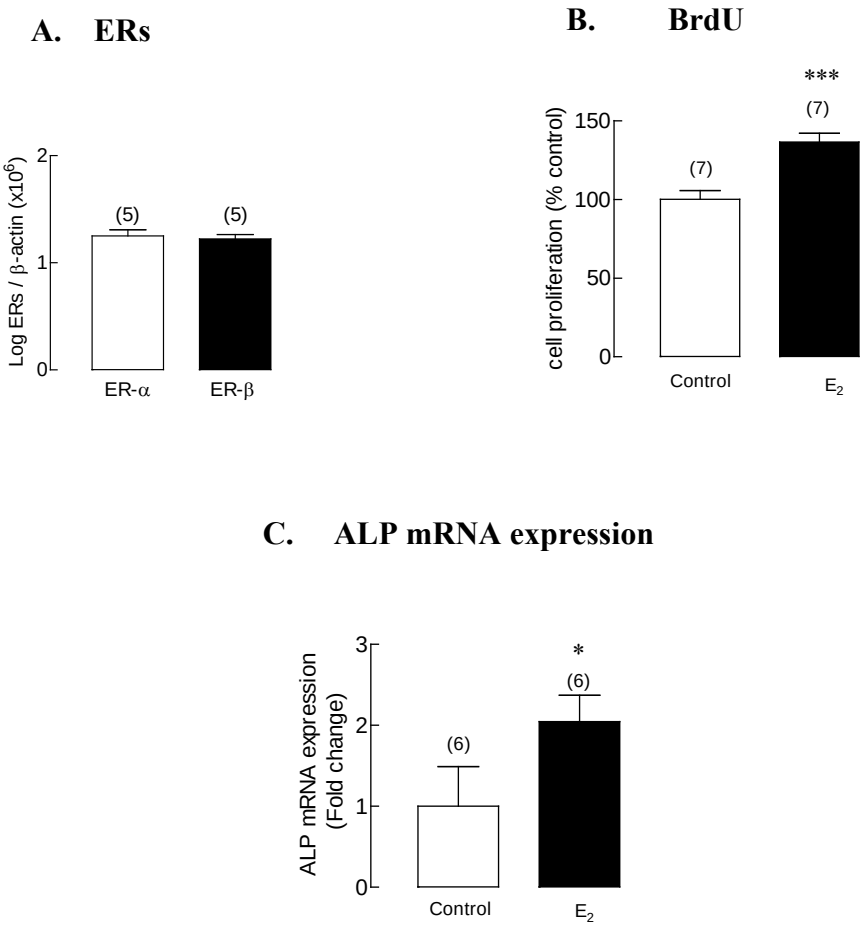

Figure 4.3 The mRNA expression of ERα and ERβ in rat primary osteoblast cells, as determined by qRT-PCR (A). Cell proliferation (B) of rat primary osteoblast cells after exposure to 10 nM E_2, as determined by BrdU assay. The mRNA expression of ALP in rat primary osteoblast cells treated for 48 h with 10 nM E_2. The numbers in parentheses represent the number of independent samples.*p< 0.05, *** p<0.001 compared with the control group.

Effects of phytoestrogens and *P. mirifica* extract on cell proliferation

In consistent with the response to $E_{2,}$ exposure of rat primary osteoblast cells with 1000 nM genistein, 1000 nM puerarin, and 100 µg/ml *P. mirifica* extract for 48 h significantly increased cell proliferation ($p< 0.05$ and $p<0.001$), indicating by a significant increased in BrdU incorporation cells (Figure 4.4).

Effects of phytoestrogens and *P. mirifica* extract on the expression of genes associated with osteoblast differentiation

After 48 h of incubation, genistein (10 and 1000 nM), puerarin (1000 nM) and *P. mirifica* extract (10 and 100 µg/ml) significantly increased the expression of ALP mRNA (Figure 4.5, 4.6 and 4.7). However, puerarin and *P. mirifica* extract did not change the expression of Runx2, osterix and osteocalcin (Figure 4.6 and 4.7) whereas the genistein significantly increased osteocalcin mRNA expression at concentration of 0.1 and 10 nM (Figure 4.5).

Effects of phytoestrogens and *P. mirifica* extract on the expression of genes associated with osteoblast-regulated osteoclast function

After 48h of incubation and at any dose of treatments, genistein, puerarin, and *P. mirifica* extract did not significantly affect on RANKL and OPG mRNA expression, authough the mRNA OPG levels tended to increase (Figure 4.8). Interestingly, no alterations on RANKL/OPG ratios were found in any treatment groups (Figure 4.9)

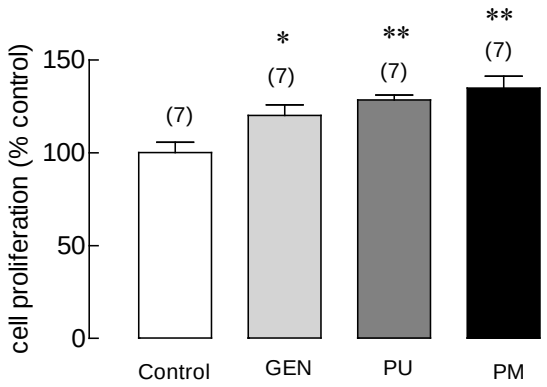

Figure 4.4 Cell proliferation of rat primary osteoblast cells after 48-h exposure to vehicle (control), 1000 nM genistein (GEN), 1000 nM puerarin (PU), or 100 µg/ml *P. mirifica* extract (PM) as determined by BrdU assay. The numbers in parentheses represent the number of independent samples. *$p < 0.05$, **$p<0.01$ compared with the control group.

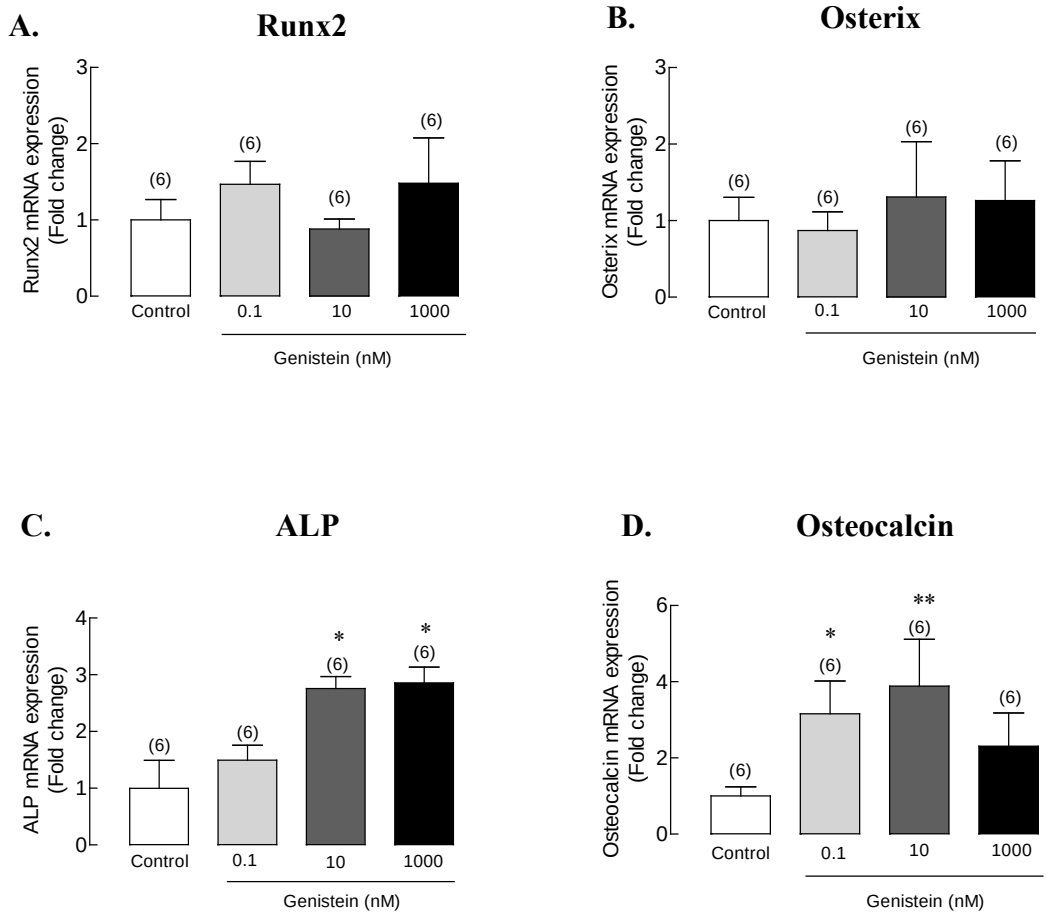

Figure 4.5 The mRNA expression of Runx2 (A), osterix (B), ALP (C) and osteocalcin (D) in rat primary osteoblast cells treated for 48h with vehicle (control), and 0.1, 10, or 1000 nM genistein as determined by qRT-PCR. The numbers in parentheses represent the number of independent samples.*p< 0.05, **p<0.01 compared with the control group.

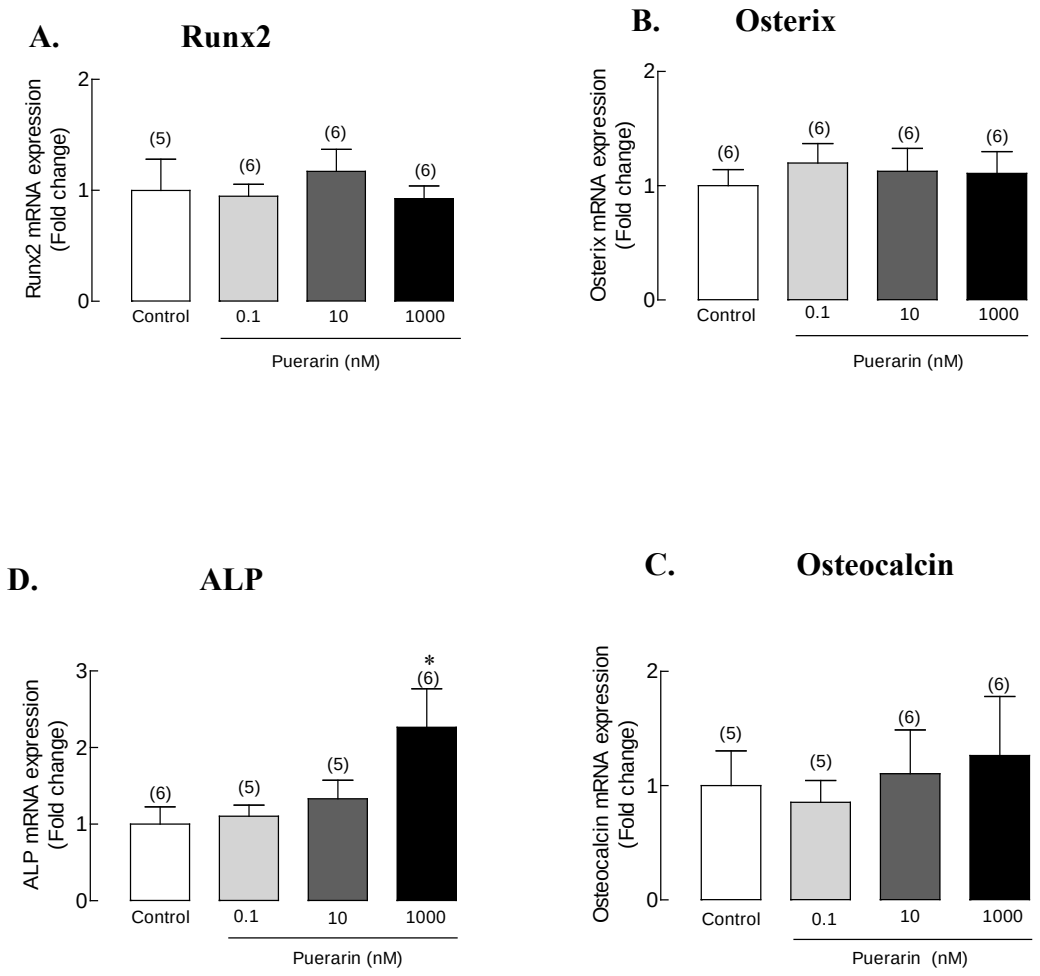

Figure 4.6 The mRNA expression of Runx2 (A), osterix (B), ALP (C) and osteocalcin (D) in rat primary osteoblast cells treated for 48h with vehicle (control), and 0.1, 10, or 1000 nM puerarin as determined by qRT-PCR. The numbers in parentheses represent the number of independent samples.*p< 0.05 compared with the control group.

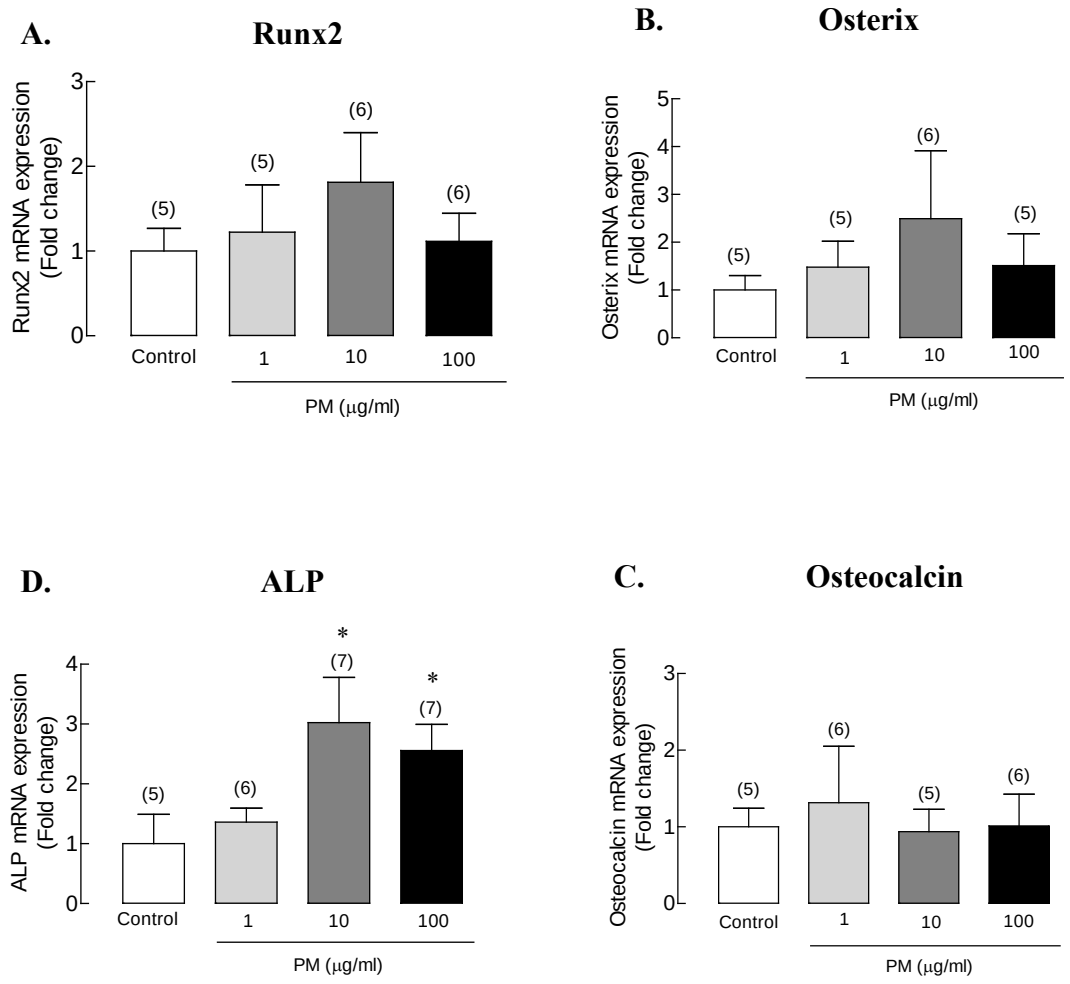

Figure 4.7 The mRNA expression of Runx2 (A), osterix (B), ALP (C) and osteocalcin (D) in rat primary osteoblast cells treated for 48h with vehicle (control), and 1, 10, or 100μg/ml *P. mirifica* extract (PM) as determined by qRT-PCR. The numbers in parentheses represent the number of independent samples.*p< 0.05 compared with the control group.

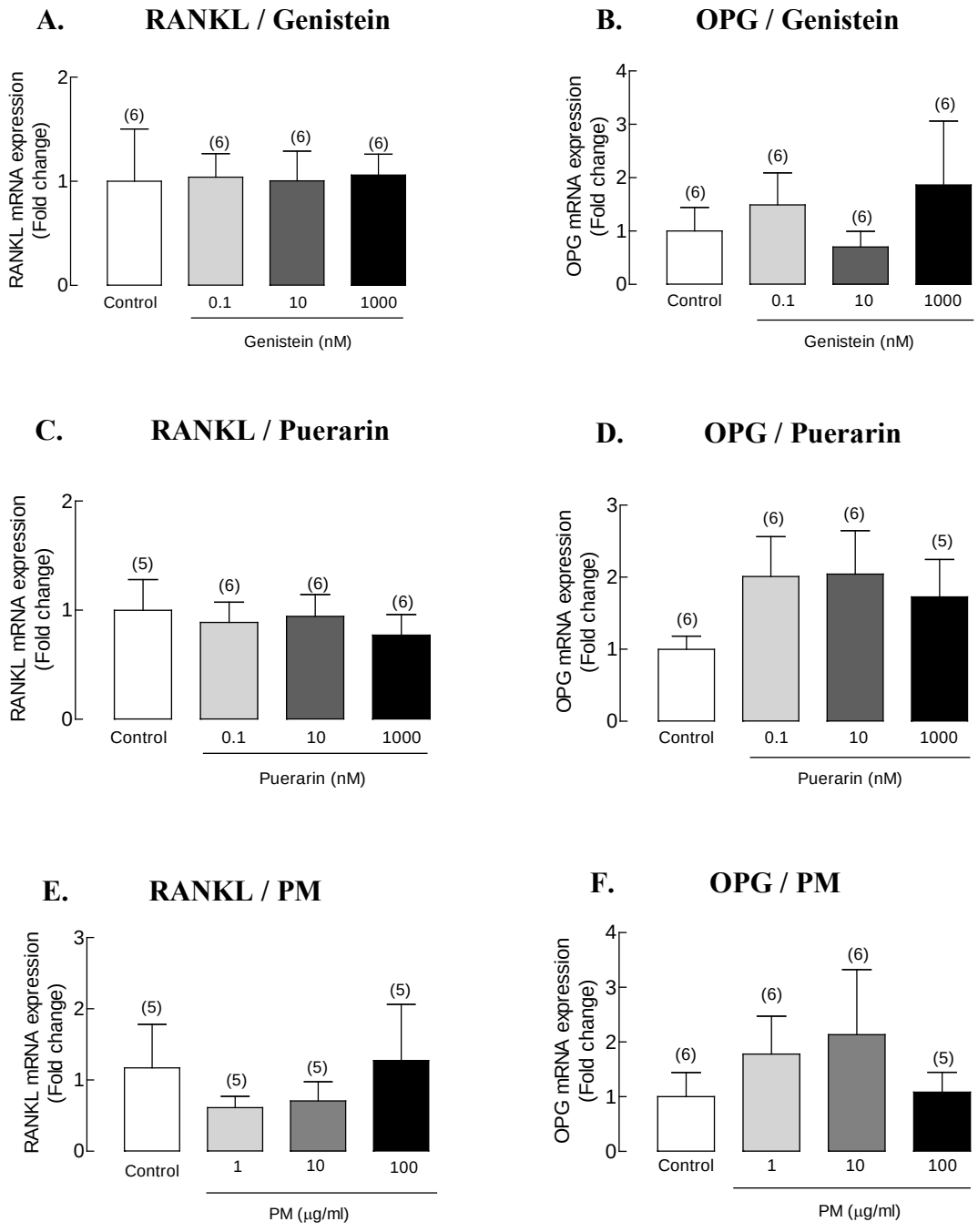

Figure 4.8 The mRNA expression of RANKL (left panel) and OPG (right panel) in rat primary osteoblast cells treated for 48h with vehicle (control), 0.1, 10, or 1000 nM genistein (A,B) , 0.1, 10, or 1000 nM puerarin (C,D), and 1, 10, or 100μg/ml *P. mirifica* extract (PM; E,F) as determined by qRT-PCR. The numbers in parentheses represent the number of independent samples.

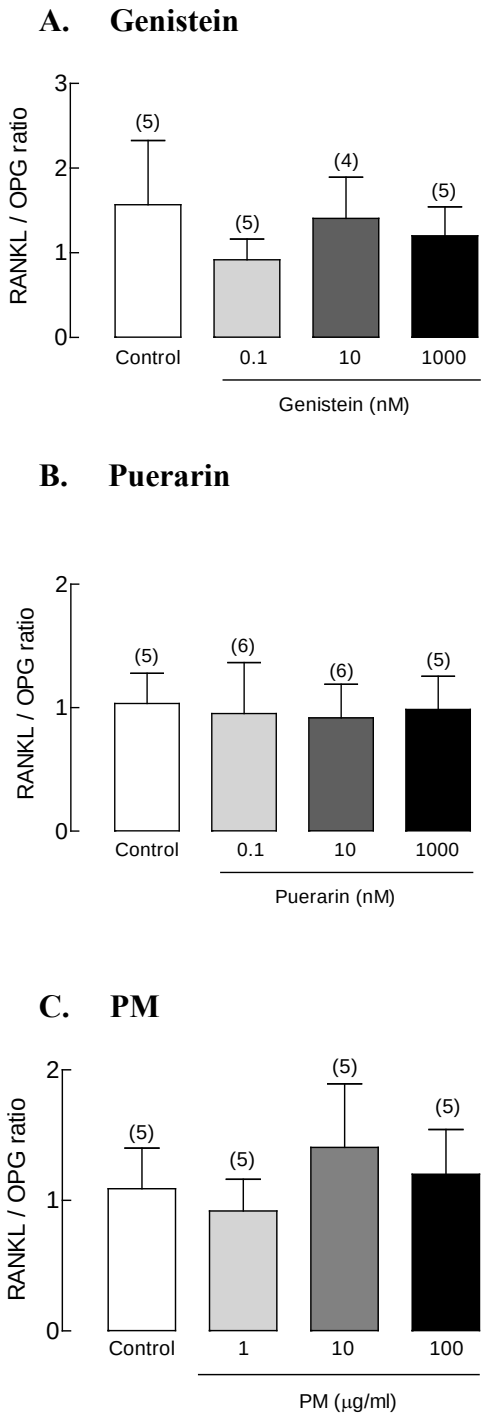

Figure 4.9 The RANKL/OPG ratios in rat primary osteoblast cells treated for 48h with vehicle (control), 0.1, 10, or 1000 nM genistein (A) , 0.1, 10, or 1000 nM puerarin (B), and 1, 10, or 100μg/ml *P. mirifica* extract (PM; C) as determined by qRT-PCR. The numbers in parentheses represent the number of independent samples.

Discussion

The osteoblast immortalized cell lines are worldwide selected modelfor *in vitro* study in bone research because they exhibited the feature of osteoblast phenotype, stability, homogeneity, reproducibility and were rapidly proliferated *in vitro* (Midura et al., 1990; Celic et al., 1998). However, most of osteoblast cell lines are cancer cells, commonly osteosarcoma. These cells frequently have undergone transformation, gene expression and signaling pathway have been modified or altered, and chromosome or receptor expression were changed (Karin and Farach-Carson, 2004). Primary cultures of osteoblast that underwent differentiation in the culture condition could provide choice of *in vitro* alternative. Primary cultured cells often behave in a manner thought to be more similar to the *in vivo* situation, especially for a type of cellular memory (Kasperk et al., 1995).

In the present study, the effects of *P. mirifica* extract and its phytoestrogen was determined in rat primary osteoblast cells derived from the long bone (tibia and fibular) of the female Sprague-Dawley rats. These osteoblast cells directly migrated from the small pieces of bone which shown the osteoblast characteristics. They underwent proliferation, differentiation and mineralization in culture condition. *P. mirifica* extract and its phytoestrogen, puerarin and genistein, increased cell proliferation by increasing BrdU incorporating cells during S phase of cell cycle which represented an increase in new DNA synthesis in rat primary osteoblast cells. This finding was consistent with the study in mouse primary osteoblast cells indicating that 1000 nM genistein increased cell proliferation (Pan et al., 2005). In contrast, the previous report (Tiyasatkulkovit et al., 2012) performed in UMR106, rat osteosarcoma cell lines, reported that *P. mirifica* extract and its phytoestrogens decreased cell proliferation but not the cell viability. However, the other investigation found that genistein increased cell proliferation in primary rat cavarial osteoblast cells and it has no effect in UMR106 cells (Li et al., 2005). In human osteosarcoma cell line (hFOB), high concentration of genistein (1000 nM) decreased cell proliferation by 20% (Rickard et al., 2003). These results suggesting that the osteoblastic cells displayed a unique growth characteristic and different response to the growth stimuli which were depend on the cell types (Karin and Farach-Carson, 2004).

P. mirifica extractand its phytoestrogens increased osteoblast differentiation as indicated by the increase in the mRNA expression of ALP, the extracellular matrix protein that plays a major role in the regulation of differentiation and mineralization of osteoblastic cells to form a new bone. Moreover, genistein seemed to be higher potential than the P. mirifica and puerarin because it also increased the expresstion of osteocalcin. Contrary to the results from rat osteoblast-like UMR106 cells, *P. mirifica* extract and its phytoestrogen increased RANKL and OPG mRNA expression and decreased RANKL/OPG ratios (Tiyasatkulkovit et al., 2012). *P. mirifica* extract and its phytoestrogens did not alter the expression of RANKL and OPG genes, and RANKL/OPG ratios. This indicates that *P. mirifica* extract, genistein and puerarin did not affect bone resorption process in rat primary osteoblast.

To describe the different of transcriptional changes in osteoblast specific genes after phytoestrogens treatment, the affinity binding of phytoestrogen to ER isoforms in osteoblast cells were focused. The two isoforms have been shown to exhibit different ligand binding affinities in osteoblasts (Kuiper et al., 1997), and acted as homodimers, exert differential transcriptional regulation at estrogen-responsive DNA elements (ERE) and promoters (Paech et al., 1997; Saville et al., 2000). In addition, one report has shown that ERβ inhibits the transcriptional activity of ERα, possibly through ERα/β heterodimer formation (Hall and McDonnell, 1999). Kuiper et al. (1998) reported that the relative binding affinity of phytoestrogen genistein for ERβ is greater than for ERα. Taken together, these findings suggested that the divergent responses of any specific gene to phytoestrogens, including their unique regulation of osteoblast and osteoclast functions, could be caused by the particular ligand bound to the receptor isoforms in osteoblast cells (Waters et al., 2001)

UMR 106 cells prolifated than the primary osteoblast cells. Furthermore, UMR106 cells were well differentiated, expressed high level of ALP and enhanced the rapid rate of mineralization *in vitro* when compared to the rat primary osteoblast cells (Standford et al., 1995). However, to mimic the rat bone physiology, it still needs to duplicate the experimental procedure in the rat primary bone cells because, from the present study, cell lines were responded to *P. mirifica* extract and phytoestrogen for the expression of osteoblast-regulated osteoclast function genes in the different

way from those of the primary rat osteoblast cells. This might be due to the difference of heterogeneity of cells and the different stage of cellular differentiation (Almeida, 2010).

In conclusion, the present study verified the molecular mechanisms of action of *P. mirifica* and its phytoestrogens of rat primary osteoblast cells on bone formation. Furthermore, the mechanisms of actions of rat primary osteoblast cells were mostly congruent with those of the rat osteosarcoma cell line UMR106 (Tiyasatkulkovit et al., 2012) except for the bone resorption process. Thus, to conduct the experiment to answer about the mechanisms of actions of phytoestrogens and phytoestrogen-containing plant on bone cells, at least two cell types are recommended to use.

CHAPTER V

A NOVEL BABOON PRIMARY OSTEOBLAST CELL CULTURE AND EFFECS OF PHYTOESTROGENS ON BONE FORMATION AND RESORPTION

Introduction

Nonhuman primates (NHPs), especially macaques and baboons which are second only to apes in genetic proximity to humans, exhibit many biological, physiological and anatomical similarities to those of humans. Thus, much research aimed at translation to the human condition, such as development of drugs and vaccines for cardiovascular disease (Shen, 2010), neurodegenerative diseases (Schneider et al., 2012), infectious diseases (Zompi and Harris, 2012), and bone diseases (Smith et al., 2009) have been conducted with NHPs. As observed in humans, an important feature of NHP bone is the presence of osteonal or Harversian remodeling in cortical bone (Jerome and Peterson, 2001), which is not normally present in rodent bone. In addition to resembling humans in regard to Harversian remodeling, NHPs, especially baboons and especially older females, resemble humans in age-related changes in bone mass and bone structure (Aufdemorte et al., 1993; Wang et al., 1998).

Baboons show many similarities to humans regarding skeletal biology (Smith et al., 2009; Baek et al., 2008). Moreover, they resemble humans in reproductive endocrinology, especially in regard to estrogen profile which is important in bone metabolism, and they undergo natural menopause (Chen et al., 1998; Martin et al., 2003). Both male and female baboons exhibit bone loss with age (Aufdemorte et al., 1993; Havill et al., 2008), and females exhibit increased bone turnover after ovariectomy (Jerome et al., 1986), similarly to postmenopausal women. Compared to dogs, cows, and rabbits in relation to material properties of bone, baboons are more similar to humans in regard to fracture and microstructural and compositional

properties, particularly bone mineral density, organic density, volume fraction, fracture surface pattern and length of collagen-mineral bundles (Wang et al., 1998). These aspects of baboon skeletal biology and estrogen profile have highlighted the value of baboons as a model for postmenopausal osteoporosis.

Postmenopausal osteoporosis and associated fractures are a major public health problem in the aging population worldwide and contribute significantly to the increasing economic healthcare burden (Cole et al., 2008; Lane, 2006). Although estrogen replacement therapy is effective, there are many side effects which are of considerable concern (Manolagas et al, 2002). Phytoestrogens, i.e., plant estrogen-like compounds, have attracted attention as potential inexpensive and effective alternatives to estrogen replacement therapy in the treatment of osteoporosis. *Pueraria mirifica* is a phytoestrogen-rich herb which is widely used as a dietary supplement and other products in USA, China, Japan, Korea and Thailand. Its estrogenic activity has been tested in various animal species, including humans (Malaivijitnond et al., 2004, 2006; Trisomboon et al., 2004, 2005, 2006; Cherdshewasart et al., 2007a; Malaivijitnod, 2012). Recently, *P. mirifica* has also been reported to prevent bone loss in both male and female osteoporotic rats (Urasopon et al., 2007, 2008). The mechanisms identified in research with rat bone cells *in vitro* were clearly shown to be induction of bone formation and suppression of bone resorption by upregulated mRNA expression of alkaline phosphatase (ALP) and osteoprotegerin (OPG), respectively (Tiyasatkulkovit et al., 2012). However, based on the regulatory guidelines of the U.S. Food and Drug Administration (2004), results from two species of animals are required to assess safety of any new therapeutic agent for treating osteoporosis in humans(Smith et al., 2009. One of the two suggested species is rat because its bone is well characterized, and the second species should have the intracortical bone remodeling. While no single animal model exactly mimics the human condition, NHPs, especially baboons, are considered to be the most closely related species to humans in relation to bone physiology (Jerome and Peterson 2001; Black and Lane, 2002; Havill et al., 2004; Smith et al., 2009). Research on *P. mirifica* for treatment of osteoporosis has been thoroughly conducted with rats (Urasopon et al., 2007, 2008), and we now extend those studies to the baboon model. Although the baboon clearly serves as a good model for humans in regard to skeletal maintenance and turnover, the regulatory

processes involved in bone turnover at the cellular level, have not previously been established in baboons, or in an *in vitro* baboon model.

Therefore, the goal of this study was to establish baboon osteoblast cell cultures and to determine the effects and mechanisms of action of *P. mirifica* extract and some major phytoestrogens contained in *P. mirifica* on baboon primary osteoblast cells.

Materials and Methods

Animals

Female baboons (*Papio hamadryas*), ranging in age from 10 to 15 years (roughly equivalent to 30 to 45 years of age in humans), at the Southwest National Primate Research Center (SNPRC), Texas Biomedical Research Institute, Texas, USA, were selected for this study. They were housed outdoors in social groups and fed commercial monkey chow to which they had *ad libitum* access. Animal care personnel and staff veterinarians provided daily maintenance and health care to all animals in accordance with the Guide for the Care and Use of Laboratory Animals. The animals were euthanized for purposes not related to this study, and fibular bone was collected under sterile conditions. Clinical records for each animal were examined to be certain that animals with medical conditions known to affect bone metabolism (e.g. diabetes and chronic renal disease) were excluded from this study.

Bone cell isolation and culture

Immediately after the bone was collected and while maintaining sterile conditions, the extraneous soft connective tissue from the outer surface were removed by a scalpel blade, and the bone was rinsed with phosphate-buffered saline pH 7.4 without calcium and magnesium (Gibco, Grand Island, NY, USA), and transferred to a Petri dish containing Dulbecco's modified Eagle's medium (DMEM) (Sigma Chemical, St. Louis, MO, USA) with 100 IU/ml of penicillin and 100 μg/ml of streptomycin (Gibco, Grand Island, NY, USA). The bone sample was cut into small fragments of 1-3 mm^2 and extensively washed in 10 ml of DMEM five times or until no hematopoietic marrow remained, i.e., until the white color of the bone fragment was visible. Bone chips were transferred to a culture flask containing 30 ml of DMEM

and digested with 0.25% collagenase (Sigma Chemical, St. Louis, MO, USA) at 37 °C for 4h. The digestion was stopped by removing the DMEM and collagenase and adding 30 ml of DMEM with 10% fetal bovine serum (FBS; Sigma Chemical, St. Louis, MO, USA). Bone chips were transferred to a culture flask with complete medium containing 30 ml of DMEM supplemented with 30% FBS, 100 IU/ml of penicillin and 100 µg/ml of streptomycin, 50 µg/ml of L-ascorbate 2-phosphate and 100 µM of sodium pyruvate (Gibco, Grand Island, NY, USA). Bone cell cultures were incubated at 5% CO_2, 37 °C for 6 days. Cell growth was maintained in the same complete medium but containing only 15% FBS, and the medium was changed every 3 days. All of the above procedures were conducted under sterile conditions.

To ensure that the bone cells collected were osteoblast cells, cell morphology was observed by inverted-light microscopy and ALP activity, a marker of osteoblast differentiation, was assessed by ALP staining; mRNA expression levels of estrogen receptor (ER)-α and ER-β were determined by quantitative real-time PCR (qRT-PCR); and mineralization was confirmed by Alizarin Red S staining. The responses of baboon primary osteoblast cells to 17β-estradiol (E_2; Sigma Chemical, St. Louis, MO, USA) were monitored by determining (i) cell proliferation using 5-bromo-2′-deoxyuridine (BrdU) assay, (ii) mRNA expression of three osteoblast differentiation associated genes, ALP, type I collagen and osteocalcin; and two genes associated with osteoblast-regulated osteoclast function, receptor activator of nuclear factor- κB ligand (RANKL) and OPG, using qRT-PCR, and (iii) mineralization using Alizarin Red S staining and quantification by semi-quantitative fluorescence analysis of calcein binding.

Effects of *P. mirifica* and phytoestrogens on primary baboon osteoblast cells

P. mirifica extract (lot no. 121408) used in this study was provided by Dr. I. Sandford Schwartz, Smith Naturals Co., Ltd, Thailand. *P. mirifica* was cultivated and the tuberous roots were collected from Chiang Mai province, northern Thailand. The tuberous roots were cut, dried and shipped to Bio-Botanica Inc., New York, USA for extraction and standardization. The *P. mirifica* powder was exhaustively extracted with concentrated ethanol. The pooled extracts were distilled under reduced pressure

at a temperature not exceeding 45°C to remove all of the alcohol to obtain a concentrated extract. The liquid chromatography–mass spectrometry (LC/MS) profile of the extract indicating the three major phytoestrogens is shown in Figure 5. 1. Each 100 g of *P. mirifica* extract contained 29.90, 0.47 and 34.17 mg of miroestrol, genistein and puerarin, respectively. Only synthetic puerarin (LKT Laboratories Inc., St. Paul, MN, USA) and genistein (Sigma, St. Louis, MO, USA) were selected and tested in baboon primary osteoblast cells, because miroestrol is unstable when it is isolated or synthesized as a pure compound, although it exhibited very high estrogenic activity (Malaivijitnond, 2012).

To investigate the effects of *P. mirifica* extract, puerarin and genistein phytoestrogens on baboon primary osteoblast cells, cells were incubated with 100 μg/ml of *P. mirifica* extract (PM group), 1000 nM of genistein (GEN group), 1000 nM of puerarin (PU group) or 0.3% vol/vol dimethyl sulfoxide (vehicle or control group). The concentrations of *P. mirifica*, puerarin and genistein used in this study were established in our previous study with the rat osteosarcoma cell line (Tiyasatkulkovit et al., 2012). Cell proliferation was determined by BrdU assay. The levels of mRNA expression of bone formation markers, runt-related transcription factor2 (Runx2), osterix, ALP, type I collagen and osteocalcin; and bone resorption markers, RANKL and OPG, were quantified by qRT-PCR. *In vitro* mineralization was determined by Alizarin Red S staining and quantified by semi-quantitative fluorescence analysis of calcein binding as was done for the E_2 treatment group.

ALP activity

The ALP activity in baboon primary osteoblast cells was assessed by using SigmaFast™ BCIP/NBT (Sigma Chemical, St. Louis, MO, USA) as the substrate. According to the manufacturer's instructions, second passages of baboon primary osteoblast cells were seeded in 24-well plates at a density of $5x10^4$ cells/well for 6 days. Cells were fixed with 70% ethanol and later incubated with BCIP/NBT solution for 10 minutes. The cells were washed, and the ALP positive cells were stained and visualized as a dark blue-violet color.

Cell proliferation

Cell proliferation was determined by a BrdU enzyme-linked immunosorbent assay kit (catalog no. 11647229001; Roche, Mannheim, Germany), according to the manufacturer's instructions. Briefly, second passages of baboon primary osteoblast cells were seeded in 96-well culture plates at 1000 cells/well. After 48-h incubation with E_2, *P. mirifica* extract or phytoestrogens, cells were fixed and later incubated with anti-BrdU-peroxidase antibody to detect BrdU-incorporated cells. Substrate solution containing tetramethylbenzidine was added for color development. Since BrdU is incorporated into the newly synthesized DNA of proliferating cells, the amount of BrdU in each well represented cell proliferation. Optical density was quantified by a microplate reader at 370 nm with the reference wavelength of 490 nm (Mutiskan® spectrum; Thermo Scientific, Waltham, MA, USA). The absorbance of control cells was normalized to 1, and relative proliferation of E_2, *P. mirifica* extract or phytoestrogen-exposed cells was presented as fold change compared to the control group. The BrdU assay was performed in triplicate with five independent samples (n = 5).

Total RNA preparation and qRT-PCR

The baboon primary osteoblast cells were cultured in 6-well plates at a concentration of $2x10^5$ cells/well. After 48-h incubation with E_2, *P. mirifica* extract or phytoestrogens, total RNA samples were extracted by the QIAshredder™ (catalog no. 79654; Qaigen, Texas, USA) and purified by RNeasy® Mini Plus (catalog no. 74134; Qaigen). Purity of the total RNA was determined by the ratio of absorbance at 260 and 280 nm, the ratio of which was between 1.8 and 2.0. One microgram of total RNA was then reverse-transcribed with iScript Select cDNA synthesis kit (catalog no. 1708896; Bio-Rad, Hercules, CA, USA) to cDNA by the Applied Biosystems GeneAmp® PCR System 9700 (Applied Biosystems, CA, USA). Baboon β-actin served as a control gene to check the consistency of the reverse transcription (coefficient of variation <5%, n = 6).

Primers used in the present study are listed in Table 5.1. The baboon primers were designed by Primer3 program based on the primers for rat and human genes, and

the baboon sequences which are located at the NCBI site. All primers were first verified by conventional PCR and the sizes of amplicons were confirmed. The sequence identity between baboon and rat genes was 82.6-96.1%, and between baboon and human genes was 97.2-99.5%. Conventional PCR was performed by Bio-Rad MyCycler with GoTaq Green Master Mix (Promega, Madison, WI, USA). Thereafter, PCR products were visualized on 1.5% agarose gel stained with 1 μg/ml ethidium bromide (Sigma Chemical, St. Louis, MO, USA) under a UV transilluminator (Eastman Kodak, Rochester, NY, USA). qRT-PCR and melting curve analysis were performed in triplicate by Bio-Rad CFX96™ Real-Time PCR Detection system with SsoFast™ EvaGreen® Supermix (catalog no. 1725203; Bio-Rad) for 40 cycles at 95 °C for 60 s, 56–60 °C annealing temperature for 30 s, and 72 °C for 30 s.

Calcium nodules formation and mineralization

The presence of mineralized nodules of baboon bone cells was determined by staining with Alizarin Red S (Sigma Chemical, St. Louis, MO, USA). The baboon primary osteoblast cells were seeded in 24-well plates at a density of $2x10^4$ cells/well and cultured for 14 days in complete medium containing 50 mM β-glycerophosphate and 50 μg/ml of L-ascorbate 2-phospate (Sigma Chemical, St. Louis, MO, USA). The culture medium was replaced with fresh medium every 3 days. When the mineralized nodules were determined, the culture medium was removed, the wells were washed twice with PBS, and the cells were fixed by adding cold 70% ethanol to each well and incubating for 1 hour. Cells were washed extensively with deionized water and stained with 40 mM Alizarin Red S at pH 4.2 for 10 minutes at room temperature and then washed five times with distilled water. The red mineralized nodule staining was observed under a Leica inverted light microscope and the images were taken with the Leica Application Suite software (Leica Microsystem, Wechsler, Germany). For quantitative analysis of mineralized calcium, baboon primary osteoblast cells were exposed to the complete medium containing 1 μg/ml of calcein (Sigma Chemical, St. Louis, MO, USA) for 4 hours at 5% CO_2 and 37 °C prior to quantitation. The osteoblast cells were washed three times with PBS and overlaid with 1 mL PBS. Then

the bound calcein fluorescence was read in a fluorescence multiwell plate reader (model 1420; Wallac, Turku, Finland) at 485 nm excitation and 530 nm emissions (Hale et al., 2000).

Statistical analysis

The results are expressed as means ± SE. Data were tested for homogeneity of variance. Two-group comparisons were analyzed by Mann–Whitney test. Multiple comparisons were performed by Kruskal-Wallis One-Way Analysis of Variance (ANOVA) followed by Dunnett's post-test. The level of significance was $p < 0.05$. All data were analyzed by GraphPad Prism 5 (Graphpad, San Diego, CA, USA).

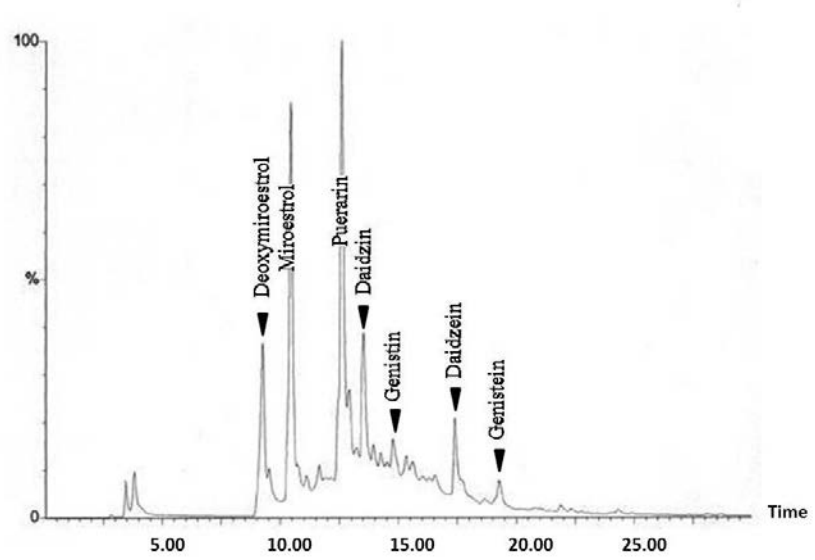

Figure 5.1 A chromatogram of *P. mirifica* extract reveals its miroestrol and isoflavonoid components, i.e., puerarin, daidzin, genistin, daidzein and genistein. The total retetion time and percent of concentration of each component as determined by LC/MS are also presented.

Table 5. 1 Baboon (*Papio hamadryas*) primers used in the qRT-PCR experiment

Genes	Primers (Forward/Reverse)	Product length (bp)	Anealing Temp.(°C)
β-actin	5′-CACACGCAGCTCATTGTAGA-3′	153	56
	5′-GGCATGGGTCAGAAGGATT-3′		
ER-alpha	5′-AGGGTGGCAGAGAGAGATTG-3′	156	60
	5′-TCTTGAAGAAGGCCTTGCAG-3′		
ER-beta	5′-ATCAGCCCCACCATTAACAC-3′	206	60
	5′-GAGCCACCCCATGTACTGAT-3′		
Runx2	5′-CAAAATGAGCGACGTGAGC-3′	214	58
	5′-GGCGATGATCTCCACCAT-3′		
Osterix	5′-TGCATCTCTTCCACACTTGC-3′	166	56
	5′-TGACGGGCAGTAGCTATGAG-3′		
Type I collagen	5′-CAGAGTGGCACATCTTGAGG-3′	283	56
	5′-TGGTTTCGACTTCAGCTTCC-3′		
ALP	5′-AACCACCACGAGAGTGAACC-3′	150	56
	5′-TCCCTGATGTTATGCACGAG-3′		
Osteocalcin	5′-TGGACTTTAGCTCTCCATCTCTG-3′	102	58
	5′-ATCGCATGAAAGCATGGAA-3′		
RANKL	5′-TCAGAAGATGGCACTCACTG-3′	215	58
	5′-AGCAAAAGGCTGAGCTTCAA-3′		
OPG	5′-TGTATTTCGCTCTGGGGTTC-3′	153	56
	5′-CTGCAGTACGTCAAGCAGGA-3′		

Results

Characterization of osteoblast phenotype

At day 0 after 4-hour collagenase digestion of bone pieces, most of the soft tissue was removed from the bone surface. During primary culture of the first passage, round or polygonal cells were observed migrating from bone chips; these cells became attached to the culture dish surface on day 6 of culture (Figure 5.2 A). As the culture time increased, the cells proliferated and became more triangular, short, spindle-shape or polygonal. These cells formed a nearly confluent cell layer around the bone tissue covering 80% of the culture dish by day 13 of culture (Figure 5.2 B). During osteoblast cell differentiation, these cells secreted matrix protein, and ALP was visualized as dark blue-violet staining (Figure 5.3 A, B). In addition, primary baboon osteoblast cells became multilayered, and a thick extracellular matrix was formed. At day 14 of culture, these cells exhibited mineralization capacity as shown by Alizarin Red S staining of extracellular calcium deposition and of the mineralized nodules (Figure 5.3 C, D).

ER expression in baboon primary osteoblast cells and response to 17β-estradiol

Baboon primary osteoblast cells expressed both ERα and ERβ (Figure 5.4 A). After exposure to 10 nM of E_2 for 48h, proliferation of baboon primary osteoblast cells was increased by 2 fold (Figure 5.4 B). The mRNA expression of osteoblast differentiation associated genes, ALP, type I collagen and osteocalcin (Figure 5.4 C), were upregulated approximately 1.5-2 fold, while the mRNA expression of osteoclast maturation associated genes, RANKL and RANKL/OPG ratio, were suppressed by 50% (Figure 5.4 D). No significant alterations were found in OPG mRNA expression (Figure 5.4 D) or calcium deposition (Figure 5.4 E) after exposure to 10 nM E_2.

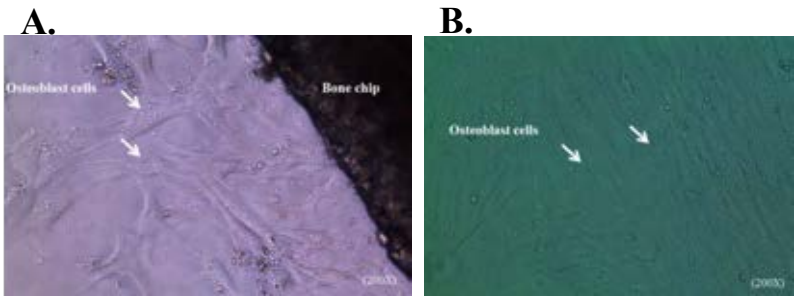

Figure 5.2 The baboon primary osteoblast cells were isolated and cultured from fibular bone. Osteoblast cells migrated from the bone pieces after day 6 of culture. The osteoblast cells attached to the culture plate, proliferated and had 80% of cell confluent after day 13 of culture (B). The arrows indicate osteoblast cells.

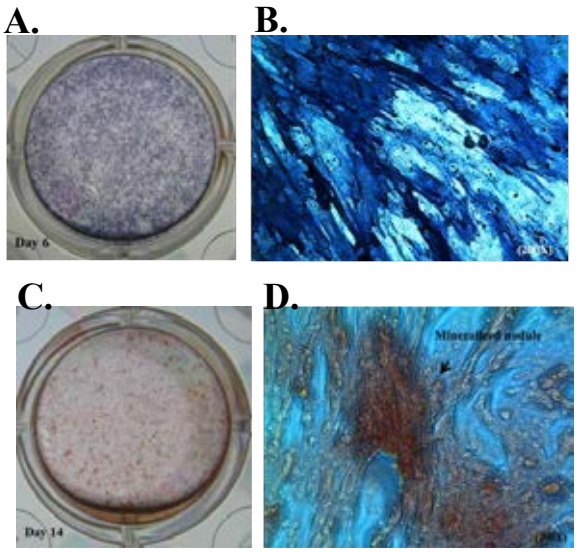

Figure 5.3 The phenotype of baboon primary osteoblast cells. ALP activity (A, B) and calcium deposition (C, D) were determined by ALP staining and Alizarin Red S staining, respectively. At day 6 of culture, the cells secreted ALP, as visualized a positive dark blue-violet staining (A). At day 14 of culture, cells exhibited the mineralization capacity as shown a red staining of extracellular calcium (B). The ALP activity (B) and the mineralized nodules (D) were magnified for 200x times.

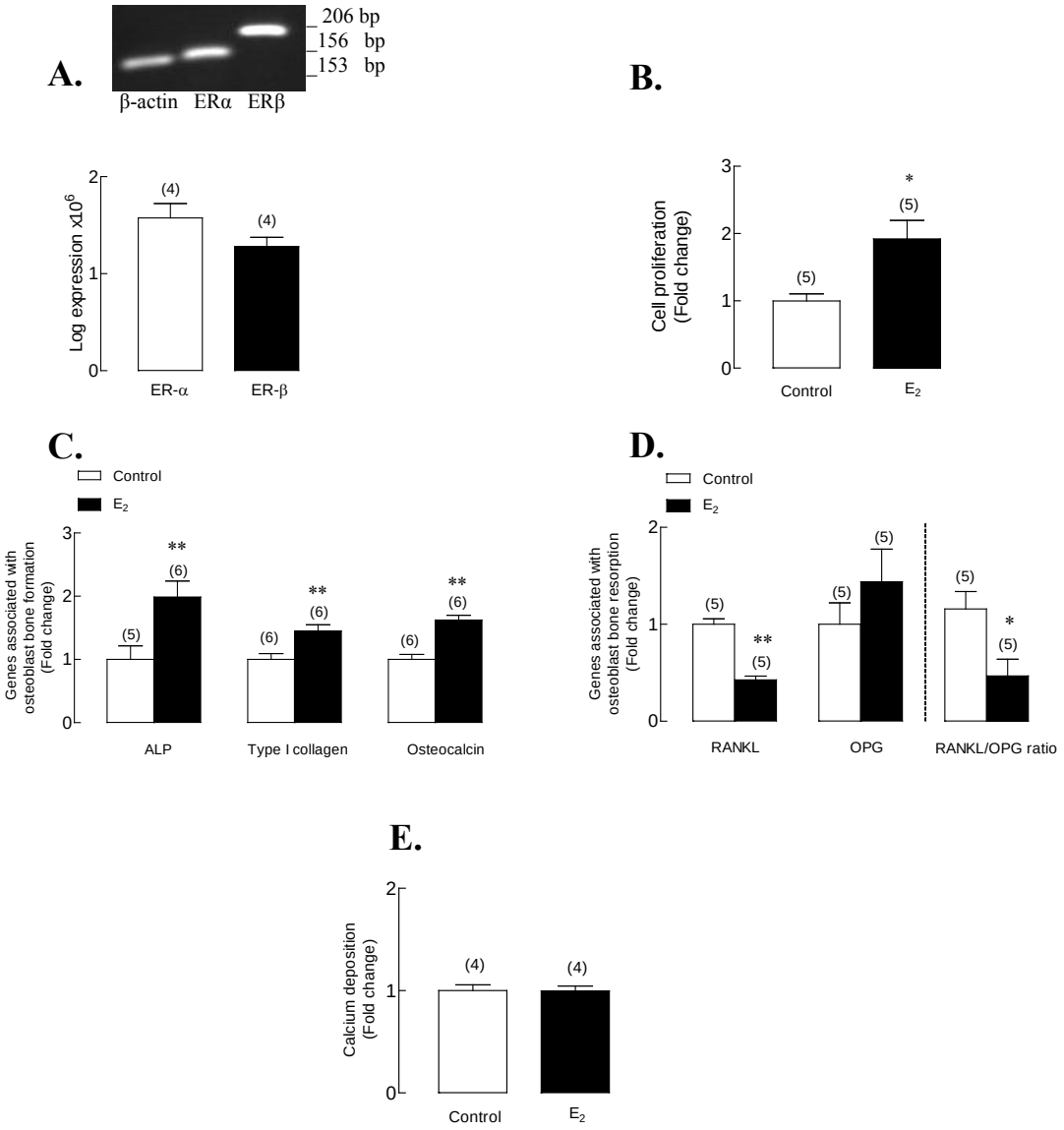

Figure 5.4 The mRNA expression of ERα and ERβ in baboon primary osteoblast cells, as determined by qRT-PCR (A). Cell proliferation (B) of baboon primary osteoblast cells after exposure to 10 nM E_2, as determined BrdU assay. The mRNA expression of ALP, type I collagen, and osteocalcin (C), RANKL, OPG and the RANKL/OPG ratio (D), and calcium deposition (E), after treated cells for 48 h with 10 nM E_2. The numbers in parentheses represent the number of independent samples.*p< 0.05, ** p<0.01 compared with the control group.

Effects of *P. mirifica* extract and phytoestrogens on baboon primary osteoblast cells

Consistent with the response to E_2, exposure of baboon primary osteoblast cells to 100 µg/ml of *P. mirifica* extract, 1000 nM of genistein or 1000 nM of puerarin for 48 h highly significantly increased cell proliferation ($p<0.001$), indicated by BrdU incorporation (Figure 5.5).

As shown in Figure 5.6 C and D, after incubation with *P. mirifica* extract, genistein or puerarin for 48 h, the expression levels of osteoblast differentiation associated genes, ALP and type I collagen, were highly significantly increased ($p<0.001$). However, none of these treatments affected the expression of Runx2, osterix or osteocalcin (Figure 5.6 A, B and E).

Differentiated osteoblast cells also expressed RANKL and OPG, both of which are commonly used as markers for assessment of osteoblast-regulated osteoclast function and bone resorption. As shown in Figure 5.7A, after incubation with *P. mirifica* extract, genistein and puerarin for 48 h, the levels of RANKL mRNA expression were significantly decreased ($p<0.05$ and 0.001) while the expression of OPG mRNA was not altered (Figure 5.7 B). However, the RANKL/OPG ratios were significantly decreased only in the *P. mirifica* and genistein groups ($p<0.001$ and $p<0.05$, respectively).

After the baboon primary osteoblast cells were exposed to *P. mirifica* extract, genistein, or puerarin for 14 days, the calcium deposition was quantified by semi-quantitative fluorescence analysis of calcein binding. There were no significant differences of calcium deposition among treatment groups, as shown in Figure5.8.

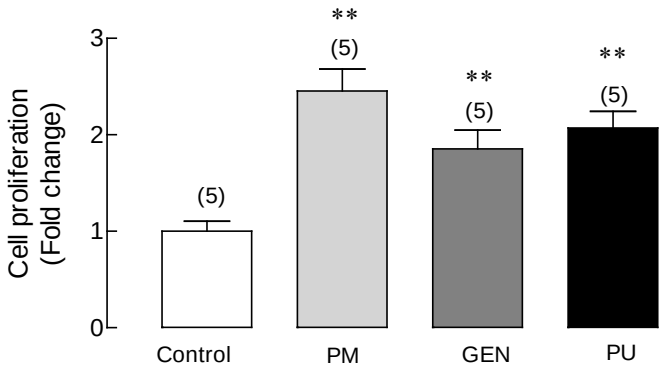

Figure 5.5 Cell proliferation of baboon primary osteoblast cells after 48-h exposure to vehicle (control), and high dose of *P. mirifica* extract (100 µg/ml), genistein (1000 nM),and puerarin (1000 nM), as determined by BrdU assay. The numbers in parentheses represent the number of independent samples. ** $p<0.01$ compared with the control group.

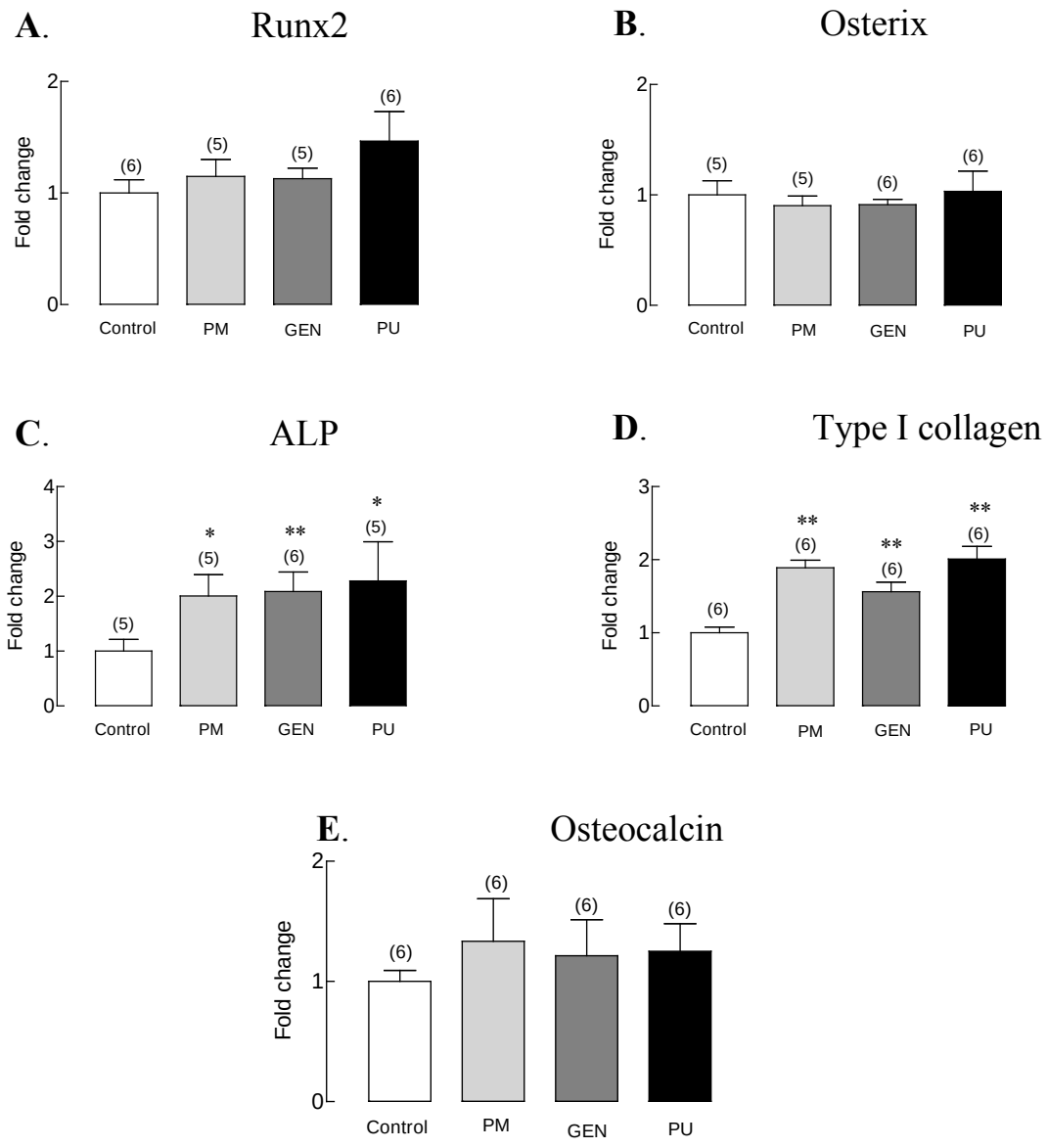

Figure 5.6 The mRNA expression of Runx2 (A), osterix (B), ALP (C), type I collagen (D), and osteocalcin (E) in baboon primary osteoblast cells treated for 48h with vehicle (control), and high dose of *P. mirifica* extract (100 µg/ml), genistein (1000 nM), and puerarin (1000 nM) as determined by qRT-PCR. The numbers in parentheses represent the number of independent samples.*p< 0.05, **p<0.01 compared with the control group.

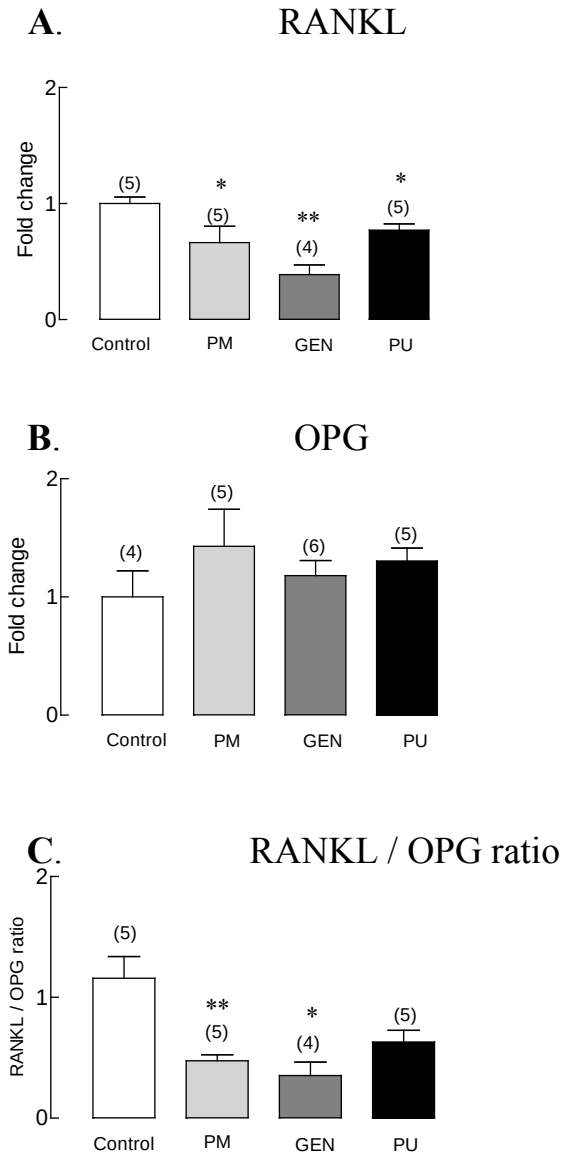

Figure 5.7 The mRNA expression of RANKL (A), OPG (B), and the RANKL/OPG ratio (C) in baboon primary osteoblast cells treated for 48h with vehicle (control), and high dose of *P. mirifica* extract (100 µg/ml), genistein (1000 nM), and puerarin (1000 nM) as determined by qRT-PCR. The numbers in parentheses represent the number of independent samples.*p< 0.05, **p<0.01 compared with the control group.

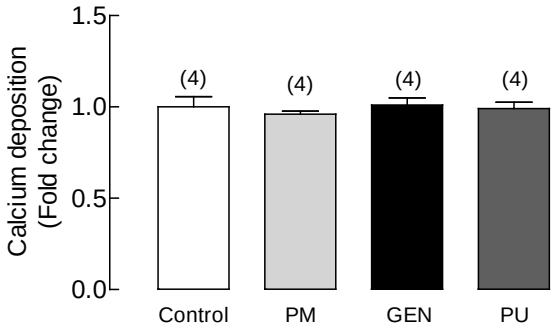

Figure 5.8 The extracellular calcium deoisition in baboon primary osteoblast cells after exposure vehicle (control), and high dose of *P. mirifica* extract (100 μg/ml), genistein (1000 nM), and puerarin (1000 nM) for 14 days as determined by calcein fluorescent staining. The numbers in parentheses represent the numbers of independent samples.

Discussion

Since the use of human primary osteoblast cells is impractical, it is necessary to establish an alternative primate model for human bone diseases and preclinical tests of new candidate drugs for treating bone conditions. Baboon is one of the closest NHPs, in terms of evolution and physiology, to humans, and their whole genome sequences and basic information on bone biology have been established (Havill et al., 2004, 2008). The first accomplishment of our study was the isolation and culture of primary osteoblast cells derived from fibular bones of baboons. These cells exhibited the same osteoblast characteristics, including proliferation, differentiation and mineralization during development, as those reported for rat osteosarcoma UMR 106 cell lines (Forrest et al., 1985; Tiyasatkulkovit et al., 2012), rat primary osteoblast cells (Heim et al., 2004), human MG-63, Saos-2 and U-2 OS cell lines (Pautke et al., 2004), and human primary osteoblast cells (Viereck et al., 2002; Heim et al., 2004). Baboon primary osteoblast cells expressed the genes associated with osteoblast bone formation, i.e., Runx2, osterix, ALP, type I collagen and osteocalcin, during cell differentiation. Furthermore, calcium deposition during the mineralization phase in

cell culture system was also observed, reflecting a functional *in vitro* endpoint of advanced osteoblast cell differentiation. The calcium deposition in baboon bone cells is amorphous hydroxyapatite similar to that seen in human bone (Neve et al., 2011). Likewise, these baboon primary osteoblast cells expressed both ERα and ERβ mRNA and could respond to 17β-estradiol by increased proliferation and expression of ALP, type I collagen and osteocalcin genes similar to those of human primary osteoblast cells (O'Shaughnessy et al., 2000; Viereck et al., 2002; Heim et al., 2004). Considering all of the observations mentioned above, baboon primary osteoblast cell cultures appear to be an excellent *in vitro* model for studying cellular and molecular mechanisms of bone metabolism and translating the results to human applications. In that regard, the baboon model can fill up the gap between rodent and human bone research.

In many women, osteoporosis develops rapidly during the postmenopausal period after ovarian function has ceased and plasma estrogen levels become extremely low (Erben et al., 2000). Although estrogen replacement therapy (ERT) is effective in reducing bone loss, ERT is associated with a high risk of breast, endometrial and ovarian cancers (Manolagas et al., 2002). Recently, phytoestrogens and phytoestrogen-containing plants, i.e., *P. mirifica*, have attracted attention as alternative to ERT (Malaivijitnond, 2012). *P. mirifica* has been widely used and is commercially sold in USA, China, Japan, Korea and Thailand as dietary supplements, cosmetics, and pharmaceutical products. *P. mirifica* is claimed to prevent and/or to cure many diseases, and one of them is osteoporosis. *P. mirifica* has been demonstrated to prevent bone loss in both sexes of gonadectomized rats by increasing BMD and BMC (Urasopon et al., 2007, 2008). Recently, the mechanisms of actions were clarified in the rat osteosarcoma UMR 106 cell line; *P. mirifica* extract and its major phytoestrogens, puerarin and genistein, significantly increased the expression of genes associated with osteogenic differentiation (bone formation), ALP, and the gene associated with osteoclastogenic inhibitor (bone anti-resorption), OPG (Tiyasatkulkovit et al., 2012). However, as mentioned previously, rat bone is not a good representative of human bone because it does not exhibit Harversian remodeling in cortical bone which is an important feature of human bone remodeling (Jerome and Peterson, 2001). Moreover, the results from rat osteosarcoma cell lines may not reflect

biological activity of animal or human osteoblasts in *vivo*. Therefore, in developing *P. mirifica* as an alternative drug for osteoporosis treatment, the investigation of its effects in an animal model which is closely related and highly similar to humans in regard to bone physiology is necessary. The establishment of baboon primary osteoblast cultures was the first step required for developing this model.

During the bone formation process, as osteoblast cells differentiate, they sequentially express different markers specific to each stage of maturation, i.e., Runx2, osterix, ALP, type I collagen, and osteocalcin (Komori, 2006). Runx 2 is required for mesenchymal cell differentiation to preosteoblasts (Zhang, 2010); and osterix, the only osteoblast-specific transcription factor, is essential for the differentiation of pre-osteoblasts into functional osteoblasts (Baek et al., 2008). Thus, Runx2, osterix and ALP genes are expressed during early differentiation and the osteocalcin and type I collagen genes are expressed during maturation. Osteoblasts express ALP, type I collagen and osteocalcin when they commenced to produce extracelluar matrix (Zhang, 2010). Based on the increase in proliferation, lack of alteration of Runx 2 and osterix mRNA expression, and increase in ALP and type I collagen mRNA expression in baboon primary osteoblasts, we conclude that *P. mirifica* extract and its phytoestrogens stimulate mitosis of osteoprogenitor cells and mineralization, while they have no effect on the differentiation of osteoprogenitor cells to preosteoblasts and subsequent mature osteoblasts (Zhang, 2010). These results were similar to those previously reported for rat UMR 106 osteoblast-like cells (Tiyasatkulkovit et al., 2012) and help to support the premise that *P. mirifica* stimulates bone formation.

Moreover, differentiated osteoblasts also expressed RANKL and OPG, both of which are commonly used as markers for the assessment of osteoblast-regulated osteoclast function and bone resorption. RANKL is an essential factor for the recruitment, differentiation, activation and survival of osteoclastic cells through binding to its specific receptor RANK, which present on the surface of osteoclast precursors and mature osteoclasts. OPG is the natural decoy receptor for RANKL. OPG prevents the interaction of RANKL with the RANK receptor and potently inhibits bone resorption (Simonet et al., 1997; Glantsching et al., 2003). Contrary to results from rat UMR 106 osteoblast-like cells indicating that *P. mirifica* increased

RANKL as well as OPG gene expression (Tiyasatkulkovit et al., 2012); there were no alterations in OPG mRNA levels, and RANKL mRNA levels were decreased in the baboon primary osteoblast cells after exposure to 100 µg/ml of *P. mirifica* for 48 h. However, in general, *P. mirifica* decreased the RANKL/OPG ratio in both rat and baboon cells. This observation leads to the conclusion that *P. mirifica* suppresses bone resorption through an anti-osteoclastogenic effect in baboon bone cells.

Theoretically, after the actions we observed in osteoblast and osteoclast cells, mineralization of the extracellular matrix would be occur in order to form a new bone (Abdallah et al, 2005; Lin et al., 2007). However, aside from the effects on bone formation and resorption, E_2, *P. mirifica*, genistein and puerarin had no effect on calcium deposition in baboon primary osteoblast cells. In rat primary osteoblast cells, calcium deposition could be first observed after 21 days of exposure to E_2 (O'Shaughnessy et al., 2000). Thus, the duration of the 14-day culture of primary baboon bone cells might be too short for estrogens and phytoestrogens to enhance the formation of calcium nodules. Another possible explanation is that osteoblast cells used in this study were isolated from fibular bones of fully mature baboons, while the rat osteoblast cells were collected from neonatal rats (O'Shaughnessy et al., 2000). Supporting this explanation is the observation that exposure of human fetal bone cells to dexamethasone for 14 days resulted in strongly visible nodule formation by comparison with the untreated control group, whereas no obvious difference between treated and untreated human adult bone cells was observed at the 14-day time point (Lin et al., 2007).

In the baboon primary bone culture system, E_2, *P. mirifica* extract and its phytoestrogens enhanced bone formation by similar mechanisms to those found in the rat osteosarcoma UMR 106 cell line (Forrest et al., 1985; Tiyasatkulkovit et al., 2012), and also to those in human osteosarcoma Saos-2 osteoblast-like cells exposed to genistein (Karieb and Fox, 2012). However, different mechanisms involved in the inhibition of osteoclast differentiation and subsequent bone resorption after estrogen and phytoestrogen treatments were observed between rat osteoblast cells (Tiyasatkulkovit et al., 2012) and baboon osteoblast cells. In addition, E_2, *P. mirifica* extract and its phytoestrogens increased cell proliferation of baboon primary osteoblast cells as indicated by increase in incorporation of the thymidine analog

BrdU to the new DNA of baboon osteoblasts, as occurs in human ostosarcoma Saos-2 osteoblast-like cells, while proliferation was decreased in rat osteoblast-like cells (Tiyasatkulkovit et al., 2012) and rat primary osteoblasts (Ma et al., 2011). Baboon gene sequences are much more similar to human gene sequences (97.2-99.5%) than are rat gene sequences (82.6-96.1%), and baboons are much more similar to human physiologically than are rats; consequently, the results from baboon osteoblast cells more closely represent the human condition than do results from rat osteoblast cells.

In conclusion, establishment of baboon primary osteoblast cell cultures has provided a new tool for studying osteoblast biology. This tool can be extremely useful in the evaluation of candidate therapeutic agents intended for treatment of degenerative bone disease in humans. In addition, the definition of mechanisms by which *P. mirifica* affects baboon osteoblast cells strengthens the high potential of this herb to be developed as anti-osteoporotic drug for human use.

CHAPTER VI

GENERAL DISCUSSION AND CONCLUSIONS

P. mirifica, a Thai traditional herb, which has been used to remedy menopausal symptoms. Because its tuberous root is rich in phytoestrogens, mainly isoflavonoids, such as,puerarin, daidzin, genistin, daidzein, genistein and miroestrol (Chansakaow et al., 2000; Cherdshewasart et al., 2007b; Cherdshewasart and Sriwatcharakul, 2007). *P. mirifica* exhibited estrogenic activity on reproductive organs in female as well as in male laboratory animals as that of estrogen (Malaivijitnond et al., 2004, 2006; Trisomboon et al., 2004, 2005, 2006; Cherdshewasart et al., 2007a). Besides, *P. mirifica* could also reduce serum PTH and calcium levels in female cynomolgus monkeys (Trisomboon et al., 2004), and increase the BMD and BMC in gonadectomy-induced osteoporotic rats (Urasopon et al., 2007, 2008).

In the present study, the mechanisms of action of *P. mirifica* extract and its phytoestrogens, genistein and puerarin, on bone metabolism were investigated. *P. mirifica* extract which used in this study were collected from two different locations; Nakornpathom and Chiangmai Province. As shown in the chromatogram analysis, the major isoflavonoid isolated from the tuber powder of both two *P. mirifica* roots, was puerarin (35.3 mg/100 g in Nakornpathom and 34.17 mg/100g in Chiangmai, respectively). It is clearly shown that the concentration of each phytoestrogen was found in *P. mirifica* tuberous roots varied and depended on the plants genetic and environment where the plant cultivated (Cherdshewasart et al., 2007b). Using LC/MS technique, miroestrol was able to be isolated from *P. mirifica* roots collected from Chiangmai Province (29.9 mg/100g). Although miroestrol had high estrogenic potency than other phytoestrogens (Benson et al., 1961; Mebe et al., 1992; Mutsumura et al., 2005), it was not selected to test in either rat or baboon bone cells in this study, because it is unstable in the purified form. Therefore, this study has been focused only on the mechanisms of *P. mirifica* extract, genistein and puerarin on the bone metabolism.

Estrogenic activity of phytoestrogens varies widely depending on their structures and target tissues (Gao and Yamaguchi, 1999b; Jefferson et al., 2002). Phytoestrogens have higher affinity for ERβ than the ERα. For example, genistein has the one-third of binding potency with ERβ, and the one-thousandth with ERα compare to E_2 as determined by expression of luciferase reporter gene which was co-transfected with ERβ and ERα in the kidney cells (Kuiper et al., 1998). Thus, ERβ is believed to be important for the action of phytoestrogens.

Osteoblasts have a very important role in creating and maintaining the bone mass. These cells are responsible for the production of bone matrix, mineralization and regulation of osteoclasts (Sommerfeldt and Rubin, 2001). Osteoblasts have been reported to express both isoforms of the ERs (ERα and ERβ), and the ratio of these two ERs were changed during osteoblast differentiation in different pattern in different cell types (Monroe et al., 2003). ERβ levels increased almost 10-fold with the ERα increasing only slightly during differentiation to mature osteoblast in human osteoblast cells (Arts et al., 1997). On the other hand, in the rat osteoblast cells, ERα was very low in undifferentiated osteoblast but significantly increased as the cells differentiated and ERβ remained high at all stages of differentiation (Onoe et al., 1997). Currently, there remained many unknowns in the ER isoform actions, including the relationship between changes of the ratios of these two receptors and the pattern of gene expressions. The present study demonstrated that rat osteosarcoma UMR106 osteoblast-like cells, rat primary osteoblast cells and baboon primary osteoblast cells express both ERα and ERβ in the same ratio at the day that cells reached confluent. In addition, this study first reported that *P. mirifica* extract and its phytoestrogens, puerarin and genistein, modulated its estrogenic activity on osteoblast cells via estrogen receptors. However, the specific affinity and activity for each ER isoform (ERα and ERβ) need a further investigation.

To investigate the effects of *P. mirifica* on bone formation and resorption process via the regulation of osteoblast cells, three stages of osteoblast differentiation, including proliferation, matrix maturation and mineralization were performed in this study. *P. mirifica* extract decreased cell proliferation in rat osteosarcoma UMR106 osteoblast-like cell, whereas increased cell proliferation in rat primary osteoblast cells and baboon primary osteoblast cells. The differences in cell response might be due to

the different heterogeneity of cells and the different stage of cellular differentiation (Almeida, 2010). The decrease in proliferation in rat osteosarcoma UMR106 osteoblast-like cell is normally observed when osteoblasts underwent well differentiated (Owen et al. 1990; Stein et al. 2004). Comparing between two rat cell culture systems, osteosarcoma and primary osteoblasts during, differentiation stage, the cellular changes were observed after 48 h of exposure to *P. mirifica* extract and its phytoestrogens. *P. mirifica* extract and phytoestrogens significantly increased mRNA expression of ALP in both rat cell types. ALP is the matrix protein synthesized from osteoblasts during post-proliferative phase. It plays a major role for mineralization stage. ALP acts both to increase the local concentration of inorganic phosphate, a mineralization promoter, and to decrease the concentration of extracellular pyrophosphate, an inhibitor of mineral formation (Golub and Boesze-Battaglia, 2007). Consistent to the differentiation, *P. mirifica* extract, puerarin and genistein also increased calcium deposition in mineralization phase of rat osteosarcoma UMR106 osteoblast-like cell. Furthermore, *P. mirifica* extract, puerarin and genistein increased mRNA expression of OPG after 48 h of incubation, while the mRNA expression of RANKL was varied in both cell types of rat osteoblasts. Lastly, the RANKL/OPG ratios were decreased only in rat osteosarcoma cells. These results suggested that *P. mirifica* and its phytoestrogens inhibited the differentiation of osteoclast cells, and subsequently decreased bone resorption process in rat osteosarcoma cell lines. The mechanisms in both rat osteoblast cell models have been clearly shown that *P. mirifica* and it major phytoestrogen, puerarin, promote bone formation by increasing in osteoblast differentiation in both cell type of rats and inhibiting osteoclast differentiation only in rat osteosarcoma.

To develop *P. mirifica* as a new therapeutic agent for osteoporotic treatments in humans, researching only in rat model is not adequate, because the bone biology of humans and rats is not alike. Since the use of human primary osteoblast cells are imposed ethical restriction, it therefore necessary to establish an alternative model for human bone biology and preclinical studies of the new drug discovery. Baboon, one of the NHPs which has genetics, reproductive endocrinology and also bone physiology comparable to those of humans (Smith et al., 2009), is attracted attention. This study was first established the culture system of baboon primary osteoblast cell

derived from the fibular bone. The effects and mechanisms of actions of phytoestrogens in *P. mirifica* extract on baboon primary osteoblast cells were later investigated. Consistent with those of rat primary osteoblast cells, *P. mirifica* extract and its phytoestrogens increased cell proliferation in baboon primary osteoblast cells. *P. mirifica* extract and its phytoestrogens also promoted osteoblast differentiation by upregulating type I collagen and ALP mRNA expression. On contrary to those in both cell type of rat bone cells, *P. mirifica* extract inhibited osteoclast differentiation by suppressing RANKL mRNA expression while there was no changes in OPG mRNA levels. Although the cellular response of rat and baboon osteoblast cells after treatment with *P. mirifica* extract were not exactly similar, the final results were the same that is *P. mirifica* extract and its phytoestrogens promote bone formation and inhibit bone resorption.

Currently, there are two primary types of drugs used in osteoporotic treatment in humans. One is antiresorptive agents which mainly inhibit bone resorption and another is anabolic agents which mainly build the bone mass. Most drugs act as antiresorptive agents against bone resorption, such as bisphosphonates, estrogens, selective estrogen receptor modulators (SERMs), and calcitonin. These could reduce bone loss, stabilize the microarchitecture of the bone, and decrease bone turnover. However, it is relatively rare for anabolic drug (Waalen, 2010). Since it indicates that *P. mirifica* does not only inhibit bone resorption, but it also increases new bone formation, *P. mirifica* should be developed as antiosteoporosis and anabolic drug for bone in the future.

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APPENDICES

APPENDIX A

Comparison of the homology of genes sequence of baboon to humans and rats

No.	Baboon genes	Identity of gene (%) compare to	
		Humans	**Rats**
1	Beta actin	**98.1%** (Beta actin : chr7:5568792-5569288)	**92.6 %** (Beta actin :chr12:12048088-12048537)
2	ER alpha	**99.5%** (ESR1: chr6:152163732-152163922)	**90.7%** (ESR1 :chr1:35559657-35559774)
3	ER beta	**97.1%** (ESR2: chr14:64723944-64727466)	**88.5%** (ESR2 : chr6:98707009-98745364)
4	Type I collagen	**97.6%** (COL1A1: chr17:48264001-48264283)	**93.1%** (COL1A1: chr10:83636679-83636961)
5	ALP	**97.5%** (ALP : chr1:21887119-21890709)	**88.4%** (ALP: chr5:156513108-156516180)
6	osteocalcin	**97.3%** (MGP/Gla protein :chr12:15035906-15037179)	**86.2%** (MGP/Gla proterin: chr4:173911351-173911415)
7	Runx2	**97.2%** (Runx2: chr6:45390314-45390694)	**96.1%** (Runx2 :chr9:11870198-11870602)
8	Osterix	**98.5%** (Osterix: chr12:53714349-53715249)	**91.5%** (Osterix:chr7:141100420-141101067)
9	RANKL	**99.3%** (RANKL :chr13:43155262-43175117)	**82.6%** (RANKL: chr15:59419425-59419590)
10	OPG	**97.7%** (OPG: chr8:119940977-119945539)	**82.6%** (OPG: chr15:59419425-59419590)

APPENDIX B

List of publications

1. Research papers

   1.1 **Tiyasatkulkovit, W.**, Charoenphandhu, N., Wongdee, K., Thongbunchoo, J., Krishnamra, N., and Malaivijitnond, S. 2012. Upregulation of osteoblastic differentiation marker mRNA expression in osteoblast-like UMR106 cells by puerarin and phytoestrogens from *Pueraria mirifica*. Phytomedicine 19(13):1147-55. (IF 2011 = 3.268)

2. Proceedings

   2.1 **Tiyasatkulkovit, W.**, Charoenphandhu, N., Thongbunchoo, J., and Malaivijitnond, S. 2010. Effects of phytoestrogens found in Thai herb *Pueraria mirifica* on bone formation. 15th Biological Sciences Graduate Congress. 15-17 December 2010, University of Malaya, Malaysia.

   2.2 **Tiyasatkulkovit, W.**, Charoenphandhu, N., VandeBerg, J. L., and Malaivijitnond, S. 2013. Mechanisms and effects of Pueraria mirifica extracted phytoestrogens and synthetic phytoestrogens on bone formation and resorption. The RGJ-Ph.D. Congress XIV. 5-7 April 2013, Chonburi, Thailand.

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