The Hong Kong Polytechnic University

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Modern approach to study the osteoprotective effects of Herba *Epimedii*

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A thesis submitted in partial fulfillment of the requirements for the degree of Master of Philosophy

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CERTIFICATE OF ORIGINALITY

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Abstract

Herba *Epimedii* is one of the most frequently herbs prescribed for treatment of osteoporosis in China over centuries. In this study we aimed to develop both *in vivo* and *in vitro* screening platforms to evaluate the osteoprotective effects of total extract (HEP), total flavonoids (TF), non-flavonoids (NF) and five flavonoid compounds from Herba *Epimedii* for identification of active ingredients using modern approach, and hence to provide scientific evidence at tissue, cellular and molecular levels for evaluating the use of Herba *Epimedii*.

To verify the efficacy of HEP, TF and NF on BMD maintenance, ovariectomized C57BL/6J mice were orally administered with HEP (1.4 mg/g/day), TF (0.2 mg/g/day), NF (1.05 mg/g/day), 17β-estradiol (4 μg/g/day) or vehicle for four weeks. All treatments except vehicle prevented OVX-induced reductions in total and trabecular BMD of distal femur. Absence of uterotrophic effects of HEP, TF and NF suggested these treatments might be safer alternatives for potential prevention of osteoporosis. To determine the optimal dose of TF, ovariectomized mice were treated with four doses of TF (50, 100, 200 and 400 μg/g/day), 17β-estradiol (4 μg/g/day) or vehicle for six weeks. TF exerted

osteoprotective effects by increasing bone mass and bone strength while decreasing bone turnover and calcium loss through urinary excretion. TF treatment at 100μg/g/day was the optimal dose for osteoprotection.

To study *in vitro* effects, rat osteoblast-like UMR-106 cells were treated with HEP (0.0625-1 μg/ml), TF (0.0625-2 μg/ml), NF (0.0625-2 μg/ml) or five flavonoids compounds (i.e. baohuoside I, sagittatoside B, korepimedoside C, epimedin B and sagittatoside A) (10^{-12}-10^{-6} M) for 48hr. Their effects on cell proliferation, differentiation and modulation of osteoclastogenesis were evaluated by MTS assay, ALP activity assay and OPG/RANKL mRNA expression respectively. HEP, TF and NF exerted osteoprotective effects by increasing cell proliferation, differentiation and suppressing osteoclastogenesis. All five flavonoid compounds stimulated osteoblastic proliferation and differentiation. TF and sagittatoside A were more potent than the remaining fractions or compounds.

In vivo and *in vitro* effects as well as the molecular actions of icariin, a marker flavonoid compound in Herba *Epimedii*, were studied. Icariin (0.3 mg/g/day) significantly increased BMD and bone strength and decreased urinary calcium loss in ovariectomized mice. UMR-106 cells treated with icariin ($10^{-12}10^{-6}$ M) increased cell proliferation and differentiation and suppressed

osteoclastogenesis. Since the effects of icariin and HEP were similar to that of 17β-estradiol, their molecular actions might be mediated through activation of ER. The mechanistic study showed that the effects of icariin on the osteoblastic functions involved the participation of ER and its actions were mediated through ligand-independent phosphorylation of ER instead of direct ERE binding. Owing to the complex ingredients in HEP, the molecular action of HEP was less clearly defined that cell proliferation and upregulation of OPG mRNA expression but not differentiation, was ER-dependent and its action was not mediated by ERE binding.

In conclusion, Herba *Epimedii* and its derived fractions or compounds demonstrated osteoprotective effects *in vivo* and *in vitro*, which might be regarded as a potential candidate for management of osteoporosis.

List of Publications

1. Lai WP, Mok SK, Pang WY, Wang XL, Yao XS, Favus MJ, Wong MS. Icariin, an active ingredient in *Herba Epimedii*, exerts bone protective effects *in vivo* and *in vitro*. *Proceedings of the 29th American Society of Bone and Mineral Research*, Hawaii, USA, September 2007
2. S. K. Mok, K. H. Lai, M. S. Wong. The anti-osteoporotic effect of total flavonoid extract of *Herba Epimedii* in ovariectomized mice. *Proceedings of 2007 Hong Kong-Macau Postgraduate Symposium on Chinese Medicine*, HK; August 2007
3. S. K. Mok, C. Y. Fung, M. S. Wong. The effect of four flavonoid compounds isolated from *Herba Epimedii* in UMR-106 cells. *Proceedings of 2007 Hong Kong-Macau Postgraduate Symposium on Chinese Medicine*, HK; August 2007
4. S. K. Mok, W. P. Lai, P.C. Leung, Y. Zhang, Y. Cheng, X.S. Yao, M.S. Wong. (2006) In vivo and in vitro osteoprotective effects of *Herba Epimedii* extract and its active ingredient, icariin. *Proceedings of International Conference on Progress in Bone and Mineral Research 2006*, Vienna, Austria.

5. M. S. Wong, S. K. Mok, W. P. Lai, P.C. Leung. (2006) Icariin, an active ingredient in *Herba Epimedii*, exerts estrogen-like effects in rat osteoblastic (UMR-106) cells. *Proceedings of the Endocrine Society's 88th Annual meeting 2006*, Boston, U. S. A.

6. Pang Wai-Yin, Mok Sao-Keng, Lai Wan-Ping, Wong Man-Sau. (2006) Proteomic approach to study the molecular mechanism involved in achieving optimal peak bone mass. *Proceedings of Guangdong-Hong Kong Joint Biophysics Symposium*, Hong Kong, P. R. C.

7. Mok Sao-Keng, Pang Wai-Yin, Lai Wai-Ping, Zhang Yan, Leung Ping-Chung, Yao Xin-Sheng, Wong Man-Sau. (2006) In vivo effects of *Herba Epimedii* (Yinyanghuo) and *Rhizoma Drynariae* (Gusuibu) on bone and mineral metabolism. *Proceedings of 2006 Hong Kong-Macau Postgraduate Symposium on Chinese Medicine*, Hong Kong, P. R. C.

8. Mok Sao-Keng, Lai Wai-Ping, Wong Man-Sau. (2005) Development of screening platform for identification of potential therapeutic targets for prevention and treatment of osteoporosis by studying *Herab Epimedii* (Yinyanghuo). *Proceedings of 2005 Hong Kong-Macau Postgraduate Symposium on Chinese Medicine*, Hong Kong, P. R. C.

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Table of contents

Title page

Certificate of originality ……i

Abstract ……..……..……...ii

List of publications .....…...v

Acknowledgements ……..……vi

Table of contents ……..ix

List of figures and tables ...…….xvi

Abbreviations ……..….xxiii

Chapter 1: Introduction ……1

1.1 Osteoporosis ……..…….……..2

1.1.1 Definition of osteoporosis …….2

1.1.2 Types of osteoporosis ……...…4

1.1.3 Prevalence of osteoporosis …….……..4

1.1.4 Diagnosis of osteoporosis ……5

1.1.4.1 BMD measurements ……...……5

1.1.4.2 Biochemical tests ……...….7

1.1.5 Current therapies and drawbacks ……10

1.2 Bone physiology ……12

1.2.1 Bone structure ……..12

1.2.2 Bone compositions ……..……14

1.2.3 Bone cells ……..…….……..14

1.2.4 Bone remodeling ……17

1.2.5 OPG/RANKL/RANK system & bone ……20

1.3 Estrogen and bone ……..23

1.3.1 Estrogen ……..23

1.3.2 Estrogen receptors ……..25

1.3.3 ER signaling ……...27

1.3.4 Estrogen's actions on bone ……31

1.3.5 Hormone replacement therapy ……...33

1.3.6 Phytoestrogens ……...33

1.4 Alternative approach for osteoporosis management ……..36

1.4.1 Traditional Chinese Medicine ……36

1.4.2 Problems of TCM ……..36

1.4.3 Herba *Epimedii* …….……38

1.4.4 Modern approaches ……...43

1.4.4.1 Bioactivity-guided fractionation ……..……..43

1.4.4.2 Efficacy-based approach to develop *in vivo* and *in vitro* screening platform …….……44

1.4.4.3 Investigation on molecular actions ……...…45

Chapter 2: Objectives and significance ……..……48

2.1 Objectives ……49

2.2 Significance ……...……..51

Chapter 3: *In vivo* and *in vitro* screening of biologically active fractions and compounds in Herba *Epimedii* for potential treatment of osteoporosis …...52

3.1 Introduction ……..…..53

3.2 Materials and Methods …….……..56

3.2.1 Preparations of Herba *Epimedii* extracts and flavonoids compounds 56

3.2.2 Animals ……60

3.2.3 Sample collection ……64

3.2.4 Biochemical assays of serum samples ……64

3.2.5 Biochemical analysis of urine samples ……..…….66

3.2.6 Bone mineral density analysis by peripheral computed tomography (pQCT) ……67

3.2.7 Culture rat osteoblast-like cell line (UMR-106) …….……68

3.2.8 Cell proliferation assay …….……..69

3.2.9 Alkaline phosphatase (ALP) activity assay ……70

3.2.10 Reverse transcription-Real time-PCR …….70

3.2.11 Statistical analysis ……...72

3.3 Results ……74

3.3.1 Effect of total extract, total flavonoids and non-flavonoids of Herba *Epimedii* on body weight ……75

3.3.2 Effect of total extract, total flavonoids and non-flavonoids of Herba *Epimedii* on uterus weight ……..77

3.3.3 Effect of total extract, total flavonoids and non-flavonoids of Herba *Epimedii* on serum calcium and phosphorus levels ……79

3.3.4 Effect of total extract, total flavonoids and non-flavonoids of Herba *Epimedii* on bone mineral density analyzed by pQCT …….……...81

3.3.5 Effect of total flavonoids on body weight ……..83

3.3.6 Effect of total flavonoids on uterus weight ……85

3.3.7 Effect of total flavonoids on serum and urinary calcium and phosphorus ……..……87

3.3.8 Effect of total flavonoids on serum alkaline phosphatase ……..90

3.3.9 Effect of total flavonoids on bone mineral density of femur analyzed by pQCT ……..……..92

3.3.10 Effect of total flavonoids on polar stress-strain index (polar-SSI) analyzed by pQCT ……95

3.3.11 Effect of total extract of Herba *Epimedii* on cell proliferation and alkaline phosphatase activity ……..…….98

3.3.12 Effects of total extract of Herba *Epimedii* on mRNA expressions of OPG and RANKL ……..…100

3.3.13 Effect of total flavonoids on cell proliferation and alkaline phosphatase activity ……...…103

3.3.14 Effect of total flavonoids on mRNA expressions of OPG and RANKL ……105

3.3.15 Effect of non-flavonoids on cell proliferation and alkaline phosphatase activity …….107

3.3.16 Effect of non-flavonoids on mRNA expressions of OPG and RANKL ……109

3.3.17 Effect of flavonoids compounds on cell proliferation …….111

3.3.18 Effect of flavonoids compounds on alkaline phosphatase activity …….114

3.4 Discussion ……..117

3.5 Conclusion …….131

Chapter 4: Characterization of *in vivo* and *in vitro* effects and mechanism of actions of icariin, a marker flavonoids compound in Herba *Epimedi* ……132

4.1 Introduction ……...……133

4.2 Materials and Methods ……..……136

4.2.1 Animal study ……..136

4.2.2 Culture of rat osteoblast-like cell line (UMR-106) ……...…138

4.2.3 Cell proliferation assay ……..138

4.2.4 Alkaline phosphatase activity assay ……..139

4.2.5 Reverse transcription-Real time-PCR ……...…140

4.2.6 Transient transfection and Estrogen receptors (ER) mediated luciferase activity assay ……..……141

4.2.7 Immunobloting …….…….142

4.2.8 Statistical analysis ……143

4.3 Results ……...146

4.3.1 Effect of icariin on body weight ……147

4.3.2 Effect of icariin on uterus index ……149

4.3.3 Effect of icariin on serum and urinary calcium and phosphorus

concentrations …….149

4.3.4 Effect of icariin on serum alkaline phosphatase ……154

4.3.5 Effect of icariin on total and trabecular BMD of distal femur analyzed by pQCT ……..156

4.3.6 Effect of icariin on polar-SSI of distal femur analyzed by pQCT ….158

4.3.7 *In vitro* effect of icariin on cell proliferation and ALP activity …….160

4.3.8 *In vitro* effect of icariin on modulation of osteoclastogenesis ……...162

4.3.9 Effect of ER antagonist on stimulatory effect of icariin and total extract of Herba *Epimedii* on cell proliferation and ALP activity …….165

4.3.10 Effect of ER antagonist on modulating effect of icariin and total extract of Herba *Epimedii* on osteoclastogenesis ……..169

4.3.11 Effect of icariin and total extract of Herba *Epimedii* on ER-mediated luciferase activity ……...172

4.3.12 Effect of icariin and total extract of Herba *Epimedii* on phosphorylation of ERα at serine 118 residue ……...…..174

4.4 Discussion ……176

4.5 Conclusion ……182

Chapter 5: Discussion and Conclusion …….183

5.1 Discussion …….184

5.2 Conclusion ……191

References ……..……..193

List of figures and tables

Figure 1-1: A photograph showing a normal bone (left) and an osteoporotic bone (right). …….....3

Figure 1-2: Biochemical markers of bone turnover. …….9

Figure 1-3: A schematic diagram showing the structure of a femur. ……..…13

Figure 1-4: A schematic diagram showing the four types of bone cells, which are osteoclast, osteoblast, osteocytes and lining cell. ……..……16

Figure 1-5: A schematic diagram illustrating the process of bone remodeling. ……..……18

Figure 1-6: Mechanism of osteoclastic bone resorption. ……19

Figure 1-7: Relationship among OPG, RANKL and RANK in regulating osteoclastogenesis and bone resorption. ……..22

Figure 1-8: Chemical structures of estrogen known as (A) 17 β -estradiol, (B) estrone and (C) estriol. …….24

Figure 1-9: Structural domains of a generic member of the nuclear receptor family. ……..26

Figure 1-10: Schematic illustration of ER signaling mechanisms. ……..... 29

Figure 1-11: Three main signaling pathways activated by membrane ER: the

Ras-Raf-MEK-MAPK, Sct-PI3K-Akt-eNOS and PLC-PKC-cAMP-PKA. …….. ……...30

Figure 1-12: Summary of stimulatory and inhibitory effects of estrogen on osteoclast formation and function by cytokines in bone marrow microenvironment. ……..32

Figure 1-13: Classification of phytoestrogens and members of each group. ...35

Figure 1-14: Photographs showing the fresh (upper) and dried (lower) leaves of *E. koreanum* Nakai. …….....40

Figure 1-15: A flow chart showing the extraction of Herba *Epimedii* ……..…47

Figure 3-1: The HPLC profile of flavonoids compounds (1, Icariin; 2, Baohuoside I; 3, Sagittatoside B; 4, Korepimedoside C; 8, Epimedin B; 9, Sagittatoside A). …….57

Figure 3-2: Chemical structures of (A) a general flavonoids, (B) Baohuoside I, (C) Sagittatoside B, (D) Korepimedoside C, (E) Epimedin B and (F) Sagittatoside A. …….58

Figure 3-3: Systematic diagrams showing the experimental designs of (A) the first and (B) the second animal studies. ……...62

Figure 3-4: Effect of total extract, total flavonoids and non-flavonoids of Herba *Epimedii* on body weight. ……....76

Figure 3-5: Effects of total extract, total flavonoids and non-flavonoids of Herba *Epimedii* on uterus index. ……78

Figure 3-6: Effect of total extract, total flavonoids and non-flavonoids of Herba *Epimedii* on serum (A) calcium and (B) phosphorus concentrations. ……80

Figure 3-7: Effect of total extract, total flavonoids and non-flavonoids of Herba *Epimedii* on bone mineral density analyzed by pQCT. 82

Figure 3-8: Effect of total flavonoids on body weight. ……84

Figure 3-9: Effect of total flavonoids on uterus index. …….....86

Figure 3-10: Effect of total flavonoids on serum and urine chemistry. ……....88

Figure 3-11: Effect of total flavonoids on serum ALP. …….....91

Figure 3-12: Effect of total flavonoids on BMD of midshaft, distal and proximal end of femur analyzed by pQCT. …….....93

Figure 3-13: Effect of total flavonoids on polar stress-strain index (polar-SSI) analyzed by pQCT. ……..96

Figure 3-14: Effect of total extract on (A) cell proliferation and (B) alkaline phosphatase activity. ……...99

Figure 3-15: Effect of total extract of Herba *Epimedii* on mRNA expressions of

(A) OPG/GAPDH, (B) RANKL/GAPDH and (C) OPG/RANKL. ……...101

Figure 3-16: Effect of total flavonoids on (A) cell proliferation and (B) alkaline phosphatase activity. ……..……..……104

Figure 3-17: Effect of total flavonoids on mRNA expressions of (A) OPG/GAPDH, (B) RANKL/GAPDH and (C) OPG/RANKL...106

Figure 3-18: Effect of non-flavonoids on (A) cell proliferation and (B) alkaline phosphatase activity. ……..…….108

Figure 3-19: Effect of total flavonoids on mRNA expressions of (A) OPG/GAPDH, (B) RANKL/GAPDH and (C) OPG/RANKL. ……..……..110

Figure 3-20: Effect of flavonoids compounds (A) Baohuoside I, (B) Sagittaoside B, (C) Korepimedoside C, (D) Epimedin B, (E) Sagittatoside A on cell proliferation. …….112

Figure 3-21: Effect of flavonoids compounds (A) Baohuoside I, (B) Sagittaoside B, (C) Korepimedoside C, (D) Epimediin, (E) Sagittatoside A on ALP activity. ……..…...115

Figure 4-1: A schematic diagram of experimental design. ……...135

Figure 4-2: Chemical structures of (A) 17β-estradiol, (B) ICI 182780, (C)

flavonoid backbone and (D) icariin. …….……142

Figure 4-3: The circle maps of (A) ERα plasmid, (B) ERβ plasmid, (C) ERE-containing luciferase reporter vector and (D) pRL-TK vector. …….…...…143

Figure 4-4: Effect of icariin on body weight. ……...…146

Figure 4-5: Effect of icariin on uterus index calculated from (A) wet and (dry) uterus weight. …….……148

Figure 4-6: Effect of icariin on (A) serum calcium, (B) serum phosphorus, (C) urinary calcium and (D) urinary phosphorus levels. …….....150

Figure 4-7: Effect of icariin on serum ALP. ……...……..155

Figure 4-8: Effect of icariin on (A) total and (B) trabecular BMD distal end of femur analyzed by pQCT. …….....153

Figure 4-9: Effect of icariin on polar-SSI of distal femur analyzed by pQCT. ……..……..155

Figure 4-10: Effect of icariin on (A) cell proliferation and (B) alkaline phosphatase activity. …….…….157

Figure 4-11: Effect of icariin on mRNA expressions of (A) OPG/GAPDH, (B) RANKL/GAPDH and (C) OPG/RANKL. ……..…..….159

Figure 4-12: Effect of ICI 182,780 on the stimulatory effect of 17β-estradiol,

HEP extract and icariin on UMR-106 (A) cell proliferation and (B) ALP activity. ……..……..…..162

Figure 4-13: Effect of ICI 182,780 on mRNA expressions of (A) OPG, (B) RANKL and (C) OPG/RANKL. ……..…….....164

Figure 4-14: Effect of icariin and HEP extract on (A) ERα- and (B) ERβ-mediated luciferase activity in UMR-106 cells. ……167

Figure 4-15: Effect of icariin and total extract of Herba *Epimedii* on phosphorylation of ERα at serine 118 residue. ……..169

Table 1-1: World Health Organization definition of osteoporosis. …….3

Table 1-2: A summary table showing the frequency of usage of different Chinese herbs in treating osteoporosis in China. …..……...42

Table 3-1: Compositions of normal calcium diet (0.6% Ca, 0.65% P) from Harlan Teklad (No. 98005).……..63

Table 3-2: Details of the primers used for probing OPG, RANKL and GAPDH in real time-PCR. …….…….73

Table 3-3: A summary table showing the effects of total extract, total flavonoids and non-flavonoids on cell proliferation, ALP activity and OPG/RANKL mRNA expression. ……..129

Table 3-4: A summary table showing the effects of five flavonoid compounds

on cell proliferation and ALP activity. ……..130

Abbreviations

AF	Transcription activation functions
ALP	Alkaline phosphatase
AP-1	Activator protein-1
BALP	Bone-specific alkaline phosphatase
BGP	Bone gal protein
BMD	Bone mineral density
Ca/Cr	Calcium to creatinine ratio
cDNA	Complementary DNA
DBD	DNA binding domain
DEXA	Dual-energy X-ray absorptiometry
DMEM	Dulbecco’s Modified Eagle Medium
DNA	Deoxyribonucleic acid
DPD	Deoxypyridinoline
E2	17β-estradiol
ECL	Enhanced Chemiluminescence
EGF	Epidermal growth factor
ER	Estrogen receptors (α and β)

ERE	Estrogen response elements
FBS	Fetal bovine serum
FDA	Food and Drug Administration
GAPDH	Glyceralehyde-3-phosphate dehydrogenase
HEP	Total extract of Herba Epimedii
HPLC	High performance liquid chromatography
HRT	Hormone replacement therapy
ICI	ICI 182,780
IGF	Insulin-like growth factor
IL-6	Interleukin-6
LBD	Ligand binding domain
MAPK	Mitogen-activated protein kinase
mRNA	Messenger RNA
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
NF	Non-flavonoids of Herba Epimedii
OCPC	O-cresol-phthalein complexone
OPG	Osteoprotegerin
OVX	Ovariectomy

P/Cr	Phosphorus to creatinine ratio
PBS	Phosphate-buffered saline
pERα	Phospho-ERα
PMS	Phenazine methosulfate
pQCT	Pheripheral quantitative computed tomography
PTH	Parathyroid hormone
PVDF	Polyvinylidene difluoride
QCT	Quantitative computed tomography
QUS	Quantitative ultrasound
RANK	Receptor activator nuclear factor-kappa B
RANKL	Receptor activator nuclear factor-kappa B ligand
RNA	Ribonucleic acid
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
TCM	Traditional Chinese Medicine
TF	Total flavonoids of Herba Epimedii
TGF-β1	Transforming growth factor beta 1
TNF-α	Tumor necrosis factor –alpha
TRAP	Tartrate-resistant acid phosphatase
TTBS	Tween 20 Tris-buffered saline

Chapter 1

Introduction

1.1 Osteoporosis

1.1.1 Definition of osteoporosis

Osteoporosis literally means porous bone (Figure 1-1). According to World Health Organization (WHO), osteoporosis is a progressive systemic skeletal disease characterized by low bone mass and microarchitectural deterioration of bone tissue, with a consequent increase in bone fragility and susceptibility to fracture. It is usually described as a “silent disease” because there are no symptoms until the first fracture occurs. Hip and vertebral fractures are the most important types of fracture as they increase the risk of morbidity and mortality. The patients will suffer from a different extent of chronic pain, bone deformity, functional limitations and emotional discomfort, which subsequently decrease their quality of life. In 1994, the WHO Study Group established a diagnostic scale using the bone mineral density (BMD) of young healthy adults as a reference (Table 1-1). The scale is divided into four categories based on the number of standard deviation units or T-score varied from the reference and the presence/absence of a fracture (Ferguson, 2004). People having T-score less than 2.5 are considered as patients with osteoporosis.

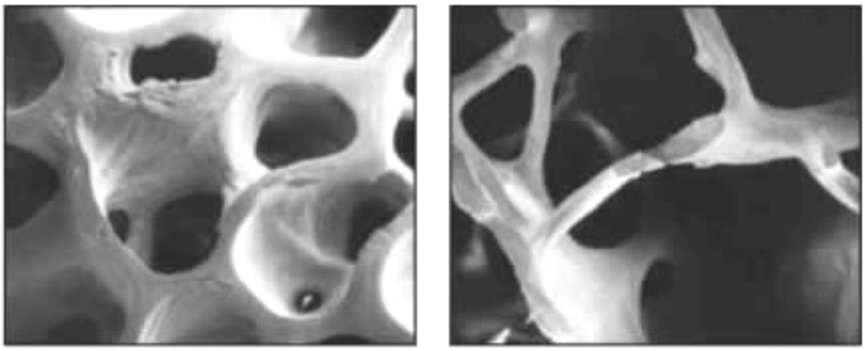


Figure 1-1: A photograph showing a normal bone (left) and an osteoporotic bone (right).

The normal bone shows a pattern of strong interconnected plates of bone. Much of this bone is lost in osteoporosis and the remaining bone has a weaker and disconnected rod-like structure. (Adapted from the website: http://www.surgeongeneral.gov/library/bonehealth/images/Figure2-5.jpg)

Diagnostic Category	T-score	Bone Mineral Density
Normal	>−1	Within 1 SD of a young normal adult
Low bone mass	−1 to −2.5	Between 1 and 2.5 SD below that of a young normal adult
Osteoporosis	<−2.5	>2.5 SD below that of a young normal adult
Severe osteoporosis	<−2.5 and ≥1 fragility fracture	>2.5 SD below that of a young normal adult

Adapted from WHO Technical Report Series.[10]

Table : World Health Organization definition of osteoporosis.

1.1.2 Types of osteoporosis

Osteoporosis can be classified into two different categories, primary (or idiopathic) and secondary osteoporosis. Primary osteoporosis occurs with no apparent causal factors, while secondary osteoporosis is caused by different diseases or medications such as glucocorticoids. There are two kinds of primary osteoporosis, type I (postmenopausal) and type II (age-related or senile). Type I osteoporosis generally develops in women after menopause when estrogen level is greatly decreased that leads to an increase in bone resorption. Type II osteoporosis usually occurs in the elderly over 70, and is resulted from age-related reduction in calcium absorption, vitamin D synthesis and physical activity that decreases bone formation.

1.1.3 Prevalence of osteoporosis

As the population grows and ages, osteoporosis is becoming a major global healthcare problem. It affects one in three women and one in five men over 50 worldwide which are more prevalent than breast cancer and prostate cancer respectively (Keen R, 2007). Approximately 1.6 million hip fractures occur each year worldwide, and the incidence is expected to increase to 6.3 million by 2050 (Cooper C et al., 1992). According to International

Osteoporosis Foundation (IOF), the annual direct costs for osteoporotic fractures of people in the workplace in the USA, Canada and Europe is about USD48 billions (IOF, 2002). Indirect costs which are at least 20% of the direct costs will substantially add the total cost of care for fractures (Lindsay R., 2001). These costs are expected to double or triple over the next few decades, and to increase to USD131.5 billion by 2050 (Gass M et al., 2006). Therefore effective approaches for osteoporosis management are important to reduce the rate and the socio-economic burden of osteoporosis-related fractures.

1.1.4 Diagnosis of osteoporosis

1.1.4.1 BMD measurements

The World Health Organization has used BMD to define normal, osteopenia and osteoporosis. BMD measurement is the primary test involved in the diagnosis of osteoporosis and the clinical evaluation of anti-osteoprosis drugs. BMD is considered as a surrogate marker of bone strength and a standard predictor of fracture risks (Ammann P et al., 2003). It can predict approximately 60-70% of a patient’s total fracture risk and fracture risk approximately doubles for every decrease in SD score (Moyad, 2003).

The most widely used methods of BMD determination include

dual-energy X-ray absorptiometry (DEXA or DXA), quantitative computed tomography (QCT) and quantitative ultrasound (QUS) (Faulkner KG, 2001). Originally, BMD measurement is obtained by photon absorptiometry which depends on the relationship between bone mineral content and the resistance for photons of radioisotopes to pass through the bone tissue. The denser the bone, the fewer photons can be detected and so the BMD calculated is higher. In 1987, this technique has developed into DEXA using X-ray as a photon source, which is the WHO-accepted gold standard for diagnosis of osteoporosis. QCT is a more sensitive method that produces a three-dimensional image of the bone being measured. It has advantages over DEXA because it is capable of measuring volumetric density, and differentiating between the cortical bone and trabecular bone, which makes it more specific in identifying early bone loss. Although it is precise enough to detect skeletal changes over time or in response to certain treatment, frequent use is not recommended as it employs a greater dose of radiation than DEXA. QUS uses a mechanical wave (i.e. sound) instead of X-ray to assess bone tissue. This technique is becoming more popular due to the absence of radiation exposure and the ease of application. However, it cannot replace DEXA measurements of the spine and hip.

1.1.4.2 Biochemical tests

In addition to BMD, biochemical tests of serum and urine may provide more information for diagnosing osteoporosis and predicting fracture risk than BMD measurement alone. The biochemical markers of bone turnover can be divided into two types, which are related to bone formation and bone resorption (Figure 1-2). For bone formation markers, osteocalcin or bone gal protein (BGP) and bone-specific alkaline phosphatase (BAP or bone ALP) are more useful than C- and N-terminal of propeptides of type I collagen due to tissue specificity and assay sensitivity. In addition, current assays for quantifying osteocalcin and bone ALP are more effective at differentiating between normal and disease states compared with those for propeptides of type I collagen (Land, 2006). Bone resorption markers are secreted during osteoclastic activity, which include the tartrate-resistant acid phosphatase (TRAP) and the collagen breakdown products such as pyridinoline, deoxypyridinoline, C- and N-terminal cross-linked telopeptides. The tests of collagen derivatives and the telopeptides are considered appropriate as the results closely reflect those of bone histology and calcium kinetic studies. A new assay for TRAP 5b, the osteoclasts-specific isoform, is now considered as a promising marker for vertebral fracture prediction.

Although biochemical tests have not been widely used for clinical diagnosis of osteoporosis, it is useful in monitoring therapy. Upon treatment for osteoporosis, any changes in bone formation and resorption can be seen after a period of 3-6 months using biochemical methods, while any changes in BMD may take at least a year using DEXA. It is also important to distinguish between the fast bone losers and slow bone losers, which helps to identify or solve the problem in an earlier stage (Watts NB, 1999). Absence of radiation exposure is another advantage over DEXA and QCT. However, there are several limitations for these tests. First, the biochemical markers can only give information on the whole skeleton instead of a specific site like the radiological techniques do. Second, there are no internationally agreed standards for the markers of bone turnover due to the difficulty in obtaining reproducible results. Third, high biological variability and diurnal variations limit the use of biochemical markers in researches and clinical trials rather than treatment decisions in patients (Felsenberg D, 2005).

Bone formation	Bone resorption
Serum Osteocalcin Bone specific alkaline phosphatase Procollagen type I C-/N-extension peptide (PICP, PINP)	Serum Pyridinoline cross-linking telopeptides (C- and N-telopeptides, CTx, NTx, ICTP) Free pyridinoline and deoxypyridinoline Tartrate-resistant acid phosphatase Bone sialoprotein Urine Pyridinoline cross-links: Pyridinoline Deoxypyridinoline Pyridinoline cross-linking telopeptides (C- and N-telopeptides, CTx, NTx)

Figure 1-2: Biochemical markers of turnover. (Adapted from Ebeling PR, 2001)

1.1.5 Current therapies & drawbacks

Under the Food and Drug Administration (FDA) guidelines, drugs to treat osteoporosis must be shown to preserve or increase bone mass and maintain bone quality in order to reduce the risk of fractures. Drugs currently approved for prevention and/or treatment of osteoporosis include estrogens, raloxifene (a selective estrogen receptor modulators), bisphosphonates, calcitonin and teriparatide (a bioactive parathyroid hormone fragment), each having their own advantages and disadvantages. Estrogen or hormone replacement therapy (HRT) was regarded as a golden standard for prevention of osteoporosis for many decades as numerous clinical studies have shown positive effects on bone health. However, emerging evidence showing increases in risk for breast cancer, stroke, thrombosis and cardiovascular disease after HRT that may outweigh its benefits on bones (Compston JE, 2004; Levine JP, 2003). Due to low solubility and high affinity to calcium, bisphosphonates need to be taken on an empty stomach with plenty of water and the patient has to remain in an upright position for at least 30 minutes afterwards. Several side effects such as gastrointestinal discomfort (e.g. nausea, stomach aches, and diarrhea) and acute phase reaction (e.g. temperature rise, bone pains, myalgias, increase in IL-6 and C reactive proteins, etc.) have been reported after oral and intravenous

administration respectively (Fleisch H, 2000). Also the short half-life in blood but long half-life in bones has made prescription of a precise dose of bisphosphonates for optimal therapeutic effect difficult. Although the clinical studies have not demonstrated an increased incidence of tumors in bone or other tissues in human, teriparatide have shown to increase the number of rats developing osteosarcoma, which implying a potential risk of getting osteosarcoma with teriparatide treatment (Delmas PD et al, 2006). Calcitonin has been reported to lose its effectiveness on inhibition of bone resorption over a continuous treatment, which limiting its regimen to an intermittent administration (Wada S et al., 2001). Besides the pharmacological side effects, expensive cost and inconvenience of administration are the common drawbacks of these drugs.

1.2 Bone physiology

1.2.1 Bone structure

Bone is a dynamic living tissue that contains bone cells, vessels, extracellular bone matrix proteins and crystals of calcium hydroxyapatite. There are two kinds of structure in a long bone (e.g. femur and tibia), namely the cortical (or compact) and trabecular (or cancellous or spongy) bone. The structure of a femur is shown in Figure 1-3. Cortical bone represents nearly 80% of the skeletal mass, which has a high density and low surface/volume ratio and thus a slow turnover rate. It forms the outer layer of the long bones and provides the strength against bending and torsion. Trabecular bone represents about 20% of the skeletal mass, but 80% of the bone surface and has a high turnover rate. It forms the interior scaffold that maintains the elasticity of bone against compression. It is reported that about 25% of trabecular bone is remodeled annually compared to only 2.5% of cortical bone (Bartl et al., 2004). About 90% of the adult bone is formed by the end of adolescence and further bone growth during the adulthood is very small.

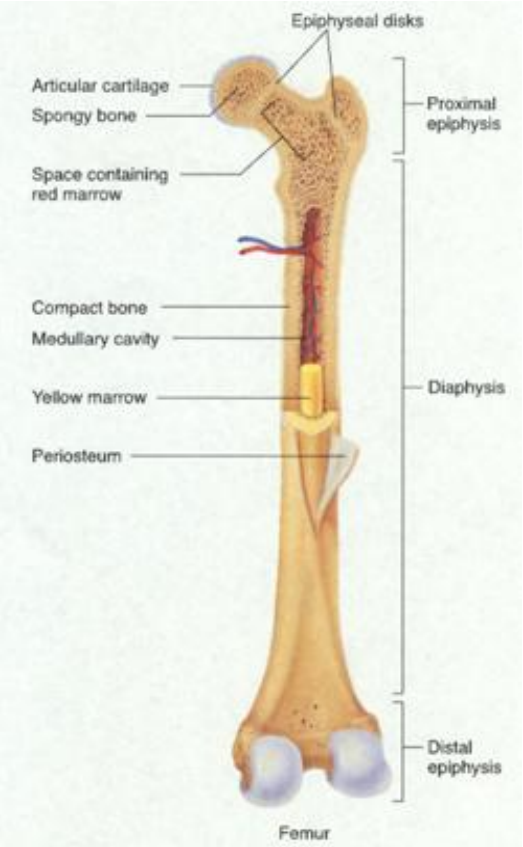


Figure 1-3: A schematic diagram showing the structure of a femur.

1.2.2 Bone compositions

The chemical composition of bone consists of both organic and inorganic phase. The organic phase refers to the extracellular bone matrix or osteoid, in which about 90% is type I collagen and 10% is non-collagenous proteins (e.g. osteocalcin, osteonectin, proteoglycans, bone morphogenetic proteins and bone-derived growth factors. The organic matrix contributes to the structure and tensile strength of bone. The inorganic phase refers to the mineral salts known as hydroxyapatites, which are mainly made up of calcium and phosphates. The process describes the deposition of hydroxyapatites in the bone matrix is known as mineralization, which provides stiffness and compressive strength to bone.

1.2.3 Bone cells

Bone tissue contains four types of bone cells, which are osteoblasts (bone forming cells), osteoclasts (bone resorbing cells), osteocytes and bone lining cells (Figure 1-4). Osteoblasts are originated from mesenchymal stromal cells and are responsible for bone formation by synthesizing an extracellular matrix (mostly type I collagen and several non-collagenous proteins) within which mineral ions crystallize. Osteocytes are the most abundant bone cells that are

derived from osteoblasts entrapped within their mineralized extracellular matrix or osteoids. Bone lining cells refer to the inactive osteoblasts that cover 80-95% of the internal surface of bone. Osteoclasts are originated from hematopoietic monocyte/macrophage precursor cells and are responsible for bone resorption by dissolving bone minerals and organic matrix.

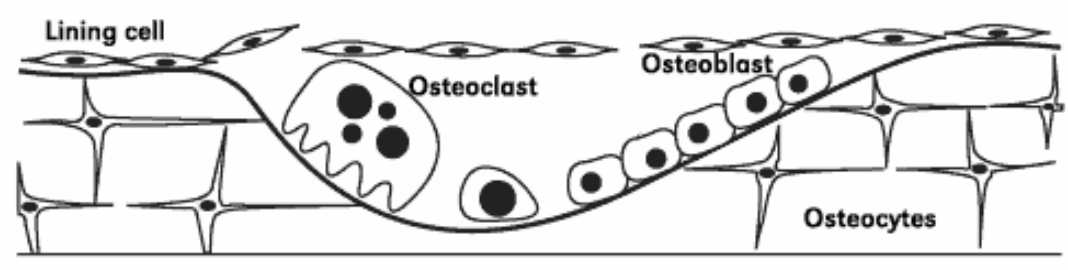


Figure 1-4: A schematic diagram showing the four types of bone cells, which are osteoclast, osteoblasts, osteocytes and lining cells. (Adapted from the website: http://www.surgeongeneral.gov/library/bonehealth/chapter_2.html)

1.2.4 Bone remodeling

Osteoclasts and osteoblasts play critical roles in bone remodeling in which bone formation and resorption are closely regulated to maintain constant bone mass. Under normal circumstances the amount of bone resorbed and formed within individual remodeling unit is closely balanced. Osteoporosis occurs when the rate of bone resorption exceeds that of bone formation. One remodeling cycle takes about 120 days, and is divided into four stages including quiescence, resorption, reversal and formation (Figure 1-5). The mechanism of bone resorption by osteoclasts was illustrated in Figure 1-6. The control of bone remodeling is complex, which depends on both systemic and local factors and results from the interaction of mechanical stresses, systemic hormones and locally produced cytokines, prostaglandins and growth factors (Ferguson N, 2004; Bartl R et al., 2004).

1. Quiescence:
A bone surface is covered by a protective layer of dormant bone cells (lining cells).

2. Resorption:
Osteoclasts invade the bone surface and erode it by dissolving the mineral and the matrix.

3. Reversal
When resorption is completed, osteoclasts retrieved while osteoblasts migrate to the resorption site.

4. Formation
Osteoblasts fill in the cavities with bone matrix proteins in which minerals crystallize to form new bone.

5. Quiescence:
The bone remains resting before the next remodeling cycle comes.

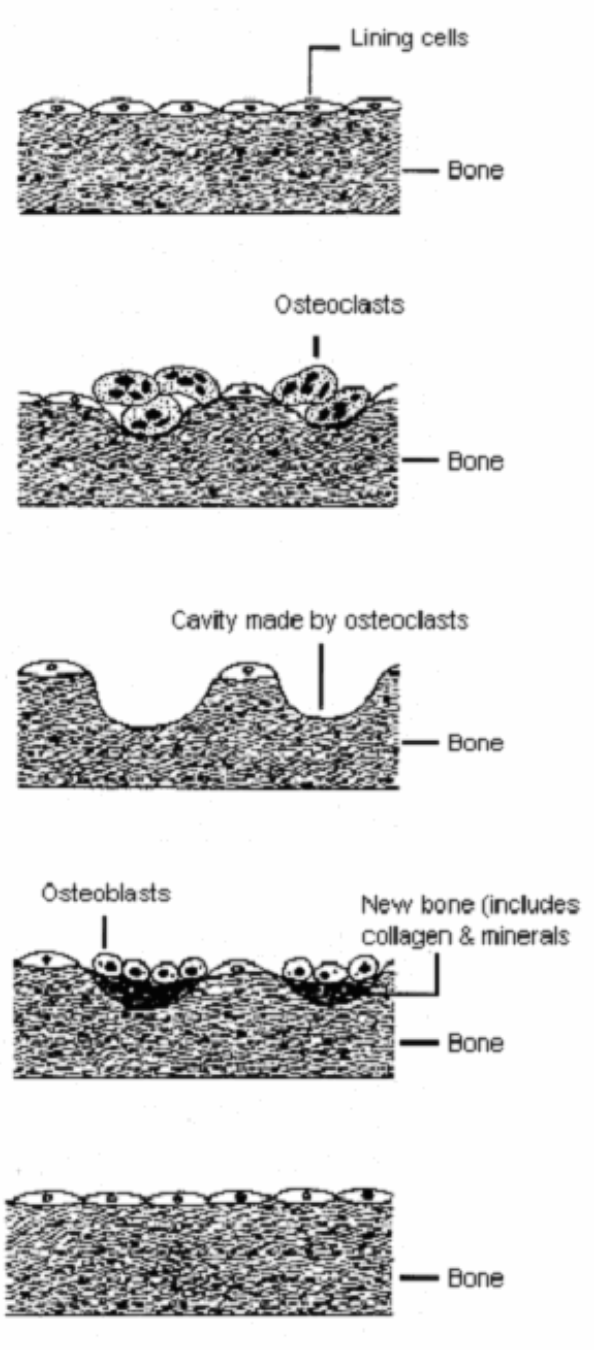


Figure 1-5: A schematic diagram illustrating the process of bone remodeling.

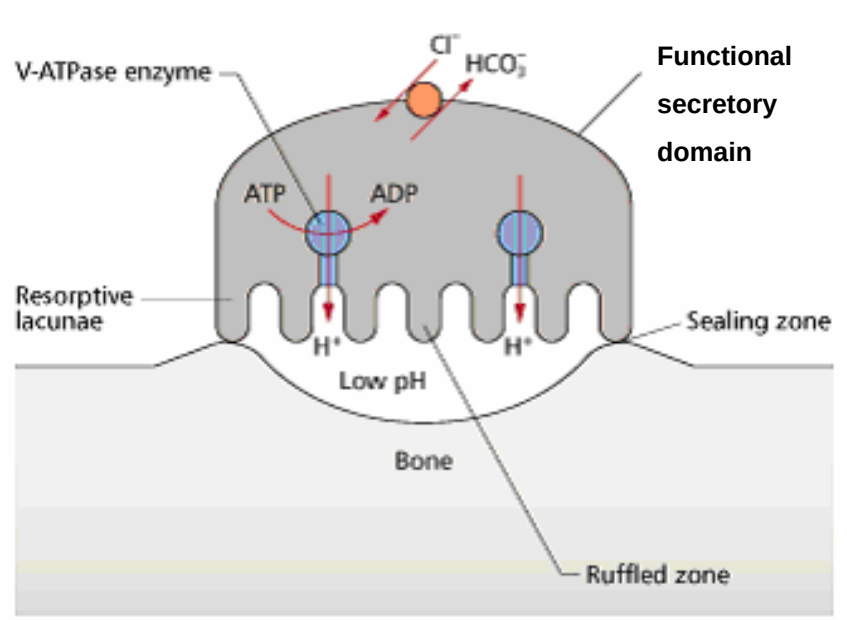


Figure 1-6: Mechanism of osteoclastic bone resorption.

When initiating bone resorption, osteoclasts undergo cytoskeleton reorganization to form an attachment ring at the sealing zone for anchoring to bone matrix. Osteoclasts become polarized and three distinct membrane domains appear: a ruffled zone, a sealing zone and a functional secretory domain. The proton pump and HCO_3^-/Cl^- exchanger produce an acidic (~ pH 4.5) microenvironment for dissolution of hydroxyapatite crystals and degradation of matrix proteins in the resorption lacunae. The resorption products are endocytosed and then excytosed through the functional secretory domain into circulation. After that, osteoclasts depolarize and relocate to a new resorption site or undergo apoptosis. (Adapted from the website: http://www.chemsoc.org/chembytes/ezine/images/2001/kee_oct01_fig1.gif)

1.2.5 OPG/RANKL/RANK system and bone

The recent identification of osteoprotegerin (OPG), receptor activator of nuclear factor kappa-B ligand (RANKL) and its cognate receptor RANK has led a new molecular perspective on osteoclast biology and bone homeostasis (Theoleyre A et al., 2004). OPG was discovered by three laboratories in 1997 and was identified as a novel member of the TNF receptor superfamily in a sequencing project of fetal rat intestinal cDNA in 1998 (Kostenuil PJ et al., 2001; Aubin JE et al., 2000). It was named according to its ability to protect bone by scavenging bone mass in ovariectomized rat model of postmenopausal women (Aubin JE et al., 2000). OPG is secreted by osteoblasts and acts as a soluble decoy receptor for RANKL, which blocks the interaction between RANK and RANKL and thereby inhibiting osteoclastogenesis for bone resorption (Figure 1-7).

OPG and RANKL are critical regulators of bone resorption and their importance were well demonstrated in OPG or RANKL transgenic and knockout mice. OPG -/- mice dramatically developed osteoporosis, in which BMD and bone strength were decreased while fracture incidence, bone turnover, osteoclast number, cortical and trabecular bone porosity were increased. Contrarily, OPG +/+ mice suffered from osteopetrosis in which bone mass and

bone volume were increased. RANKL -/- mice were osteopetrotic due to an absence of osteoclasts while RANKL +/+ mice were severely osteoporotic (Blair JM et al., 2006; Aubin JE et al., 2000; Kostenuil PJ et al., 2001; Theoleyre A et al., 2004). Bone resorption induced by soluble RANKL injection in mice could be prevented by OPG, which demonstrated the opposing actions of OPG and RANKL (Kostenuil PJ, 2005). The ratio of OPG/RANKL may therefore be regarded as the ultimate determinant of bone resorption.

OPG therapy is one of the potential therapeutic approaches for osteoporosis. The first randomized, double-masked, placebo-controlled clinical trial that involved 52 postmenopausal women showed single subcutaneous injection of OPG (human recombinant OPG) induced rapid and sustained decrease in bone turnover marker (i.e. N-telopeptide/creatine) dose-dependently (Bekker PJ et al., 1999). Gene therapy with OPG increased BMD and decreased osteoclast surfaces significantly in both Sham and OVX mice compared with untreated controls (Kostenuik PJ et al., 2004). BMD increase by OPG treatment was shown to be similar to that by bisphosphonate treatment (Kostenuik PJ et al., 2005). The efficacy, safety and convenience of OPG therapy may receive extensive study in the future and open up a new possible way for osteoporosis treatment.

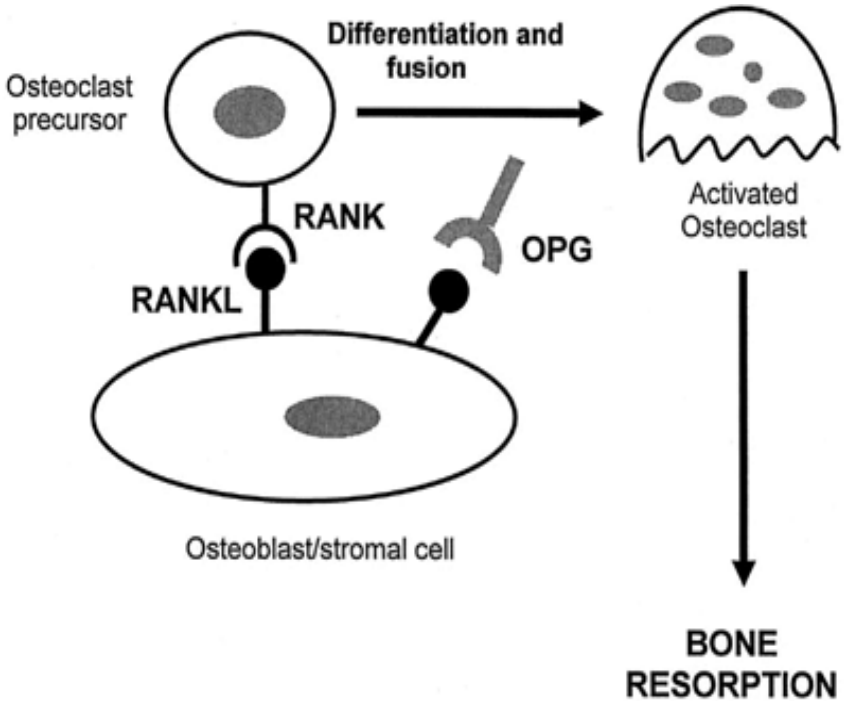


Figure 1-7: Relationship among OPG, RANKL and RANK in regulating osteoclastogenesis and bone resorption.

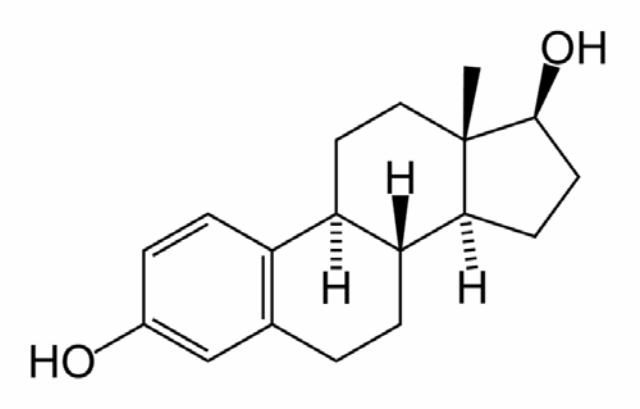
Interaction between RANK and RANKL induces differentiation and maturation in osteoclast precursor to become active osteoclast, which is capable of bone resorption. OPG, secreted by osteoblast, inhibits osteoclastogenesis and bone resorption by competing RANKL with RANK. (Adapted from the website: http://www.medscape.com/content/2004/00/47/98/479893/art-smj479893.fig1.gif)

1.3 Estrogen and bone

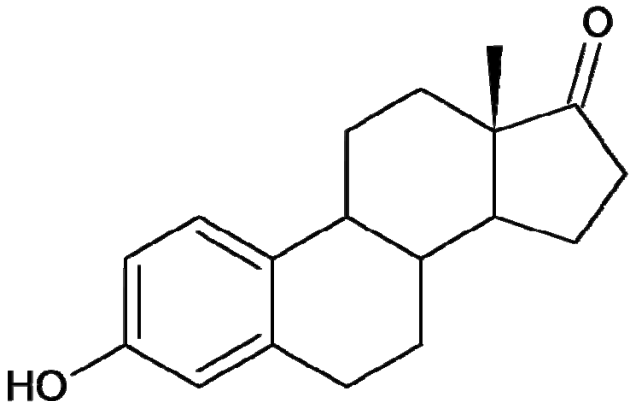
1.3.1 Estrogen

Estrogen is a group of steroid hormones that is mainly synthesized in the ovary (in female), testis (in male) and multiple peripheral tissues. The most potent and dominant estrogen in human is 17β-estradiol, but lower levels of estrone and estriol are also present (Figure 1-8). Estrogen regulates a plethora of physiological processes in mammals, including reproduction, cardiovascular protection, bone integrity, cellular homeostasis and behavior (Manavathi B et al., 2006). Its actions are initiated by direct or indirect binding to the estrogen receptors.

(A)



(B)



(C)

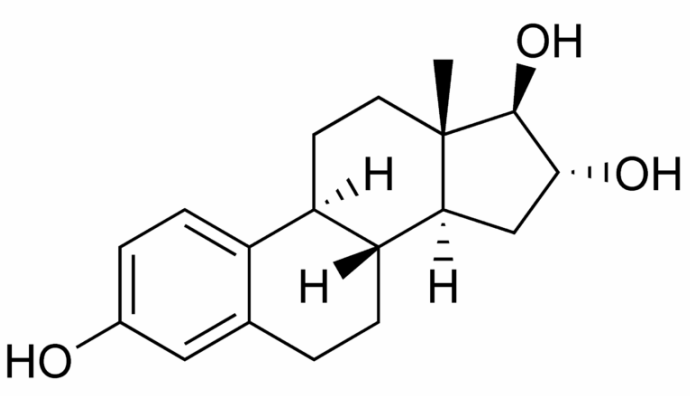


Figure 1-8: Chemical structures of estrogen known as (A) 17 β -estradiol, (B) estrone and (C) estriol. (Adapted from the website: http://en.wikipedia.org/wiki/Estradiol)

1.3.2 Estrogen receptors

The biological effects of estrogen are mediated by the two known isoforms of the estrogen receptor α and β, which are members of a large superfamily of nuclear receptors. Human ERα and ERβ are encoded by different genes located on chromosome 6 and 14 respectively. The structure of ERs can be divided into several functional domains: the N-terminal domain (A/B domain) is involved in the activation of gene transcription; the DNA binding domain (DBD or C domain) contains two zinc fingers involved in specific DNA-binding and receptor dimerisation: the hinge domain (D domain) contributes flexibility to the DNA- and ligand-binding domain and may influence DNA-binding of individual receptors; the ligand-binding domain (LBD or E domain) contains regions that are important for ligand binding, receptor dimerisation, nuclear localization and interactions with transcriptional coactivators and corepressors; the C-terminal domain (F domain) contributes to the transactivation capacity of the receptors but may have other functions (Figure 1-9) (Acconcia F et al., 2006; Hughes I et al., 2003). ERs contain two transcription activation functions (AFs) in the A/B and E domain which allows ligand-independent and ligand-dependent transactivation activities respectively.

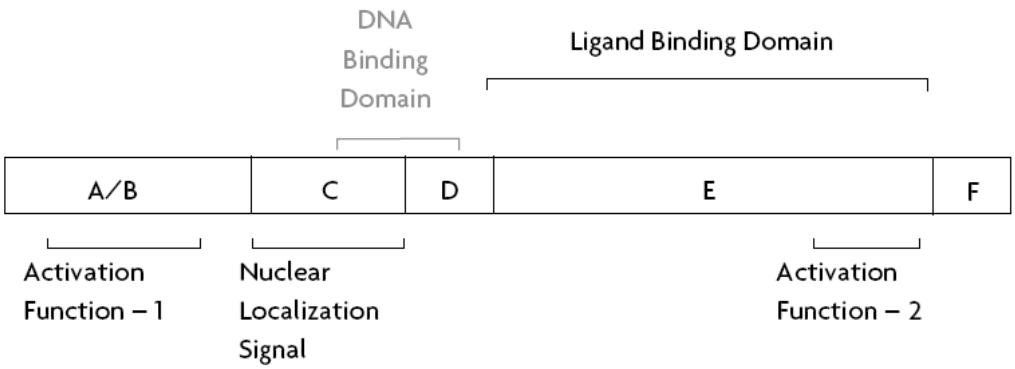


Figure 1-9: Structural domains of a generic member of the nuclear receptor family. (Adapted from Hughes I et al., 2003)

1.3.3 ER signaling

ER act as transcription factors in which their actions can be divided into genomic (classical) and nongenomic (nonclassical) (Figure 1-10). Genomic mechanism of ER involves estrogen or high-affinity ligand binding to ER which induces the dissociation of receptors from the heat shock proteins (Hsps), dimerisation, and translocation to the nucleus, where the ligand-ER complexes bind to the specific response elements known as estrogen response elements (ERE) located in the promoters of estrogen sensitive genes (Acconcia F et al., 2006). The subsequent activation of gene transcription relies on the recruitment of coactivators and coreppressors and the basal transcription machinery. Around one third of the genes in human that are regulated by ER do not contain ERE-like sequences (O'Lone R et al., 2004). In ERE-independent genomic actions, ER can regulate gene expression without directly binding to DNA by modulating the function of other classes of transcription factors through protein-protein interaction (Bjornstern L et al., 2005; Zhang D et al., 2006). Some examples of such action include the interaction of ER with the activator protein 1 (AP-1) transcription factor complex, the Sp1 transcription factor, the GATA-1 transcription factor and STAT5 transcription factor. Apart from traditional binding of estrogen to ER, estrogenic effects can be mediated

through ligand-independent activation of ER pathway. For example, binding of epidermal growth factor (EGF) and insulin-like growth factor (IGF) to its cognate receptors are capable of activating ER through mitogen-activated protein kinase (MAPK) pathway that leads to phosphorylation Serine 118 of ERα (Lannigan DA, 2003). Since 1977, evidence has been accumulating that estrogen exerts extranuclear or nongenomic actions that is too rapid (i.e. a few seconds or minutes) to be explained by the activation of RNA and protein synthesis (Manavathi B et al., 2006). The nongenomic action mediated by binding of estrogen and membrane ER involves activation of various signaling pathways such as Ras-Raf-MEK-MAPK, Sct-PI3K-Akt-eNOS and PLC-PKC-cAMP-PKA (Figure 1-11). It is important to note that the regulation of gene expression by ER is a multifactorial process, involving both genomic and nongenomic actions that often converge at certain response elements located in the promoters of target genes (Bjornstrom L et al., 2005). Therefore the response of estrogens may be different depending on the cellular context.

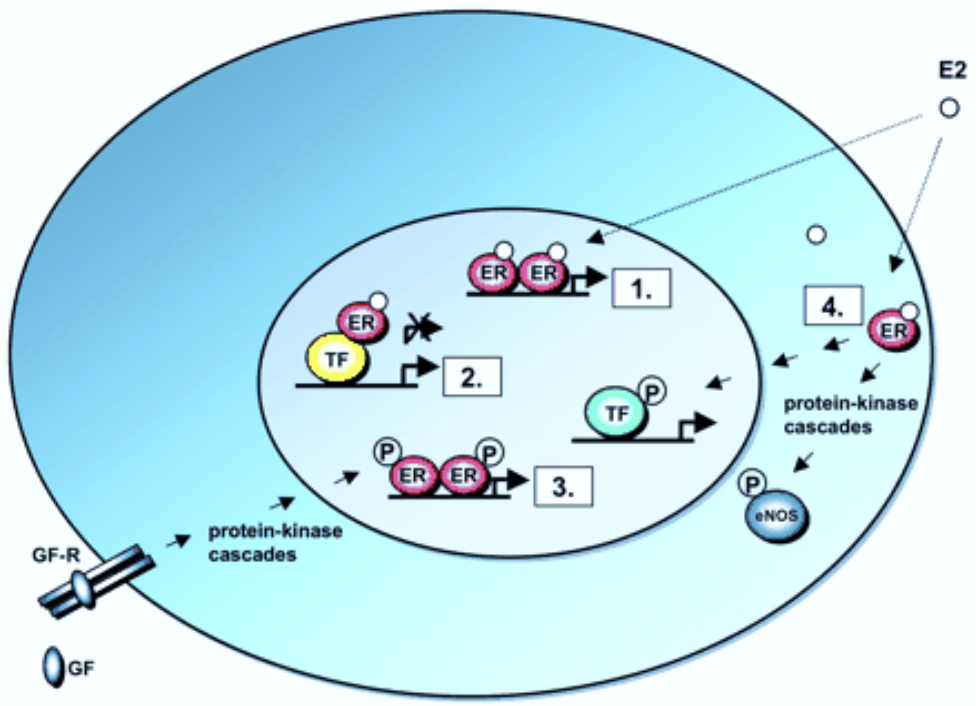


Figure 1-10: Schematic illustration of ER signaling mechanisms.

1. Classical mechanism of ER action: Nuclear E2-ER complexes bind directly to EREs in the target gene promoters. 2. ERE-independent genomic actions: Nuclear E2-ER complexes are tethered through protein-protein interactions to a transcription factor complex (TF) that contacts the target gene promoters. 3. Ligand-independent genomic actions: Growth factors (GF) activate protein kinase cascades, leading to phosphorylation (P) and activation of nuclear ERs at EREs. 4. Nongenomic actions: Membrane E2-ER complexes activate protein kinase cascades, leading to altered functions of proteins in the cytoplasm, e.g. activation of eNOS, or to regulation of gene expression through phosphorylation and activation of a TF. (Adapted from Bjornstrom L et al., 2005)

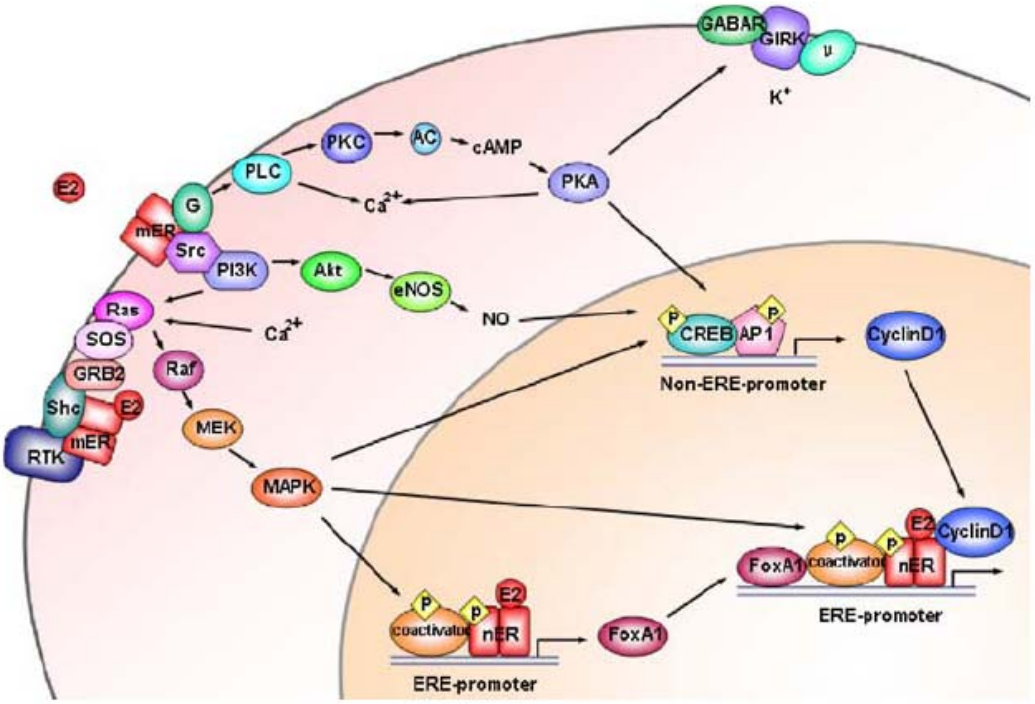


Figure 1-11: Three main signaling pathways activated by membrane ER: the Ras-Raf-MEK-MAPK, Sct-PI3K-Akt-eNOS and PLC-PKC-cAMP-PKA.

(Adapted from Zhang D et al., 2006)

1.3.4 Estrogen's actions on bone

In 1940s, Albright et al. related the causation of postmenopausal osteoporosis to estrogen deficiency and found that estrogen treatment improved calcium balance in postmenopausal women. Their studies were validated by densitometric studies demonstrating that accelerated bone loss induced by ovariectomy could be prevented by estrogen therapy (Riggs BL et al., 2002). The mechanisms of action of estrogen on bone include reduction in bone turnover, inhibition of osteoclastic activity, stimulation of osteoblastic activity, calcitonin secretion, gastrointestinal absorption of calcium, and blood flow through the bone, modulation of PTH secretion, and improvement of central nervous functions that decrease tendency to fall (Ferguson N, 2004; Bartl R et al., 2004; Avioli LV, 2000; Gass M et al., 2006). As shown in Figure 1-12, estrogen inhibits bone resorption by inducing small but cumulative changes in multiple estrogen-dependent regulatory factors. Estrogen deficiency increases bone turnover, prolongs bone resorption phase and shortens bone formation phase, which results in bone loss and various fracture risks (Riggs BL et al., 2000).

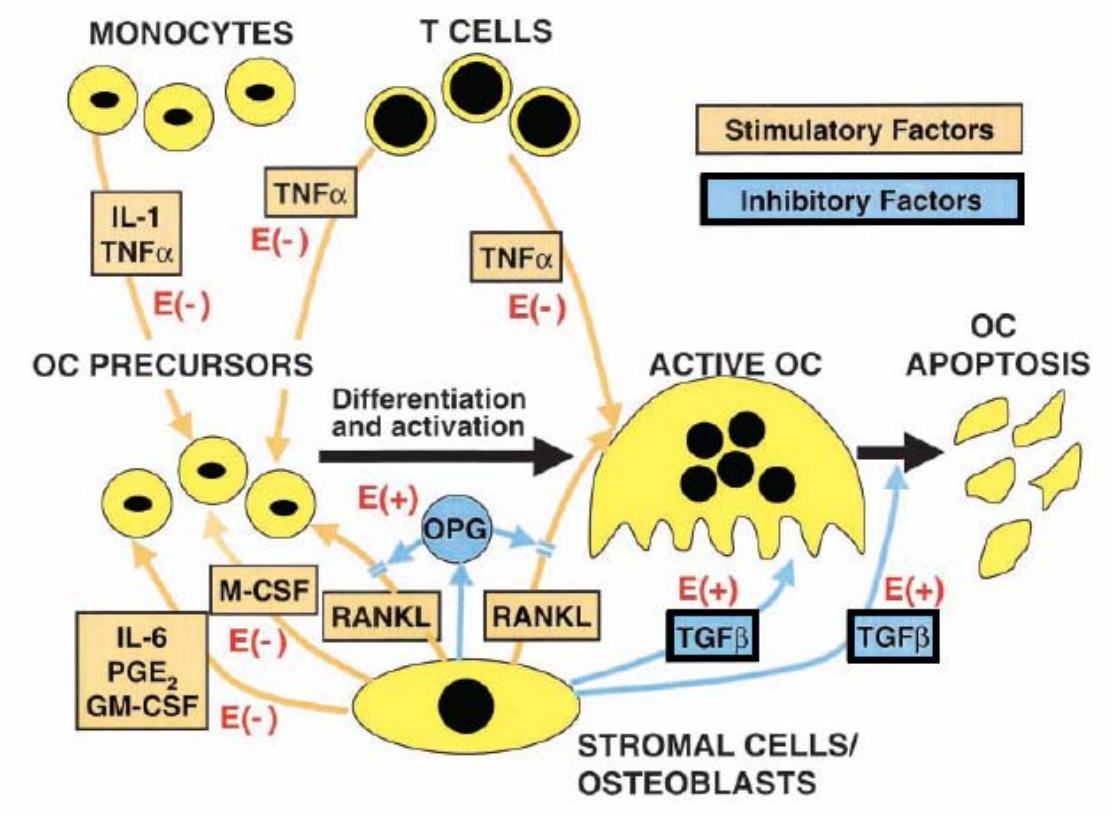


Figure 1-12: Summary of stimulatory and inhibitory effects of estrogen on osteoclast formation and function by cytokines in bone marrow microenvironment.

E(+) represents positive effects of estrogen on the regulatory factors while E(-) represents negative effects. Stimulatory and inhibitory factors are shown in thin and thick rectangles respectively. (Adapted from Riggs BL et al., 2000)

1.3.5 Hormone replacement therapy

As estrogen is recognized as a causative agent for postmenopausal osteoporosis, estrogen or hormone replacement therapy was thought to be a direct solution for restoring bone mass. For many decades HRT was regarded as a golden standard for treatment of osteoporosis as many clinical studies have shown positive effects on bone health. However, emerging evidence showing increases in risk for breast cancer, ovarian cancer, stroke, thrombosis and cardiovascular disease after HRT, and the risks increase with duration of treatment, which may outweigh its benefits on the bones (Balasch J, 2003). It should be noted that estrogen therapy (in various formulations) is FDA-indicated for prevention of osteoporosis, but not FDA-approved for treatment of osteoporosis as the fracture data required by FDA have not been submitted (Levine, 2003). The current consensus is that HRT is no longer considered as a front-line option for prevention of osteoporotic fractures, and it should be applied to osteoporotic women who have menopausal symptoms and to older women who are intolerant of other therapies (Compston, 2004). A minimal dosage and intervention period for desirable effects on bone was recommended before the potential risks of HRT are thoroughly assessed.

1.3.6 Phytoestrogens

Phytoestrogens is a group of biologically active plant substances with a chemical structure that is similar to estrogen, which can mimic or modulate estrogenic or antiestrogenic effects usually by binding to estrogen receptors (Usui T, 2006; Hughes I et al., 2003). The majority of phytoestrogens belong to a large group of substituted phenolic compounds known as flavonoids. Phytoestrogens can be structurally subdivided into three main classes: isoflavones, coumestans and lignans (Whelan AM et al., 2006) (Figure 1-13).

Several animal studies have provided convincing data on the significant improvement of bone mass or other endpoints in ovariectomized animals after phytoestrogens (e.g. genistein, daidzein and icariin) treatments (Ishimi Y et al., 2000; Picherit C et al., 2000; Bao JR et al., 2005). Observational epidemiologic studies have also examined the link between dietary intake of phytoestrogens and bone mass in humans and reported that soy phytoestrogens are beneficial in maintaining or modestly improving bone mass in postmenopausal women (Usui T, 2006). Many clinical studies have supported the use of phytoestrogens (e.g. isoflavones and icariin) in increasing BMD and decreasing bone turnover in postmenopausal women, and the results are somewhat comparable to HRT (Hughes I et al., 2003; Zhang G et al., 2007). The beneficial effects of phytoestrogens are of particular interest since HRT is known to associate with

increased risk of cancers, and may therefore be considered as an alternative for management of osteoporosis.

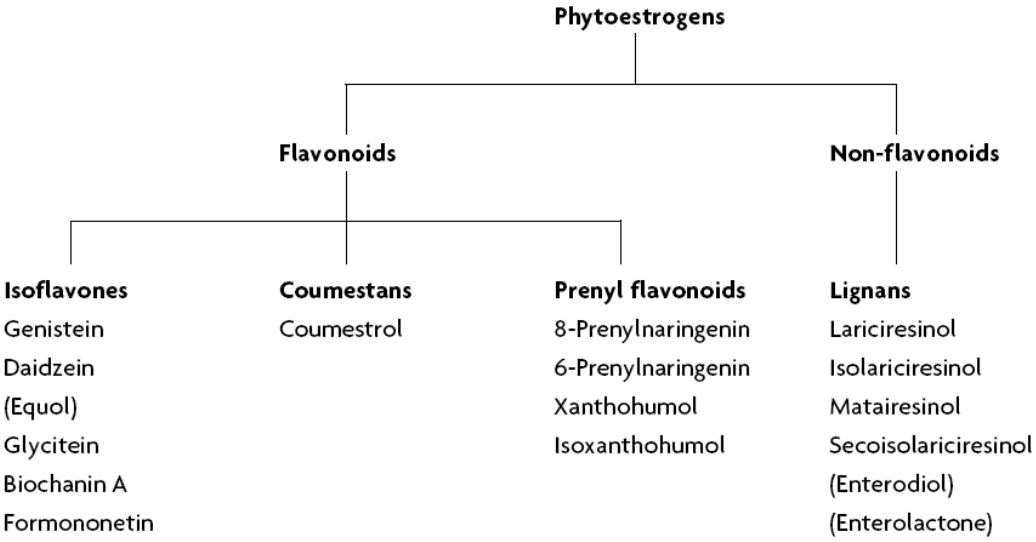


Figure 1-13: Classification of phytoestrogens and members of each group.

(Adapted from Hughes I et al., 2003)

1.4 Alternative approach for osteoporosis management

1.4.1 Traditional Chinese Medicine

Traditional Chinese Medicine (TCM) is one of the oldest forms of medicine in the world, which has been formed for more than two thousand years (Lu AP et al., 2004). TCM is composed of a wide range of therapies including herbal medicines, acupuncture, qigong, taiji, moxibustion and Chinese massage. There is a rapidly growing and sustained interest in TCM as an alternative or a complement to Western medical treatment internationally. This interest is fueled by a combination of factors including (1) recognition of potential benefits of TCM; (2) dissatisfaction with the traditional Western medical model; (3) an increasing commitment to holistic care; (4) skepticism regarding adverse drug effects and (5) increasing evidence for the interaction of psychological factors and outcomes of disease and treatment and consumer demand (Davidson P et al., 2003).

1.4.2 Problems of TCM

Although TCM has a very long history of usage, it is still not widely accepted by many science researchers and medical professionals due to three main reasons. Firstly the active ingredients in TCM are neither identified nor characterized. TCM is usually prescribed or taken in formulae containing

several herbs. The more the number of herbs used, the more complex the mixture is and therefore more complicated to analyze. Also interaction among a vast number of compounds may result in synergistic, additional or antagonistic effects that give the false portrait of individual herb in the formula. Secondly the mechanisms of actions are not well elucidated. Diagnosis and treatment with reference to TCM theory that based on several philosophical frameworks (e.g. the theory of Yin-yang, the Five Elements, Zang Fu and Meridian or Jingluo) are not operated within a scientific paradigm. TCM emphasizes on acting on multiple targets to achieve balance or harmony in the body that keeps people healthy. These vague concepts give little evidence and direction for investigation of molecular actions. Both complexity and limited information may be suggested for the reason why many TCM studies mainly focus on the effects rather than the mechanisms. Furthermore the majority of the trials examined by the researchers were of poor methodological quality that complicated the interpretation of trial results. Thirdly the herb or the derived compounds used are not standardized with quality control system. This may result in varying profiles of chemical compositions that make comparison or analysis of different research findings difficult, which subsequently hinder the progress to modernize TCM for potential prevention and treatment of various

diseases.

1.4.3 Herba *Epimedii*

Some TCM have been used to treat bone diseases or relieve the relevant symptoms over centuries. Herba *Epimedii* is one of the most frequently used kidney-tonifying herbs for treatment of bone diseases including osteoporosis in China (Figure 1-14). According to Cheng Dong, after reviewing 100 recent articles of research and clinical aspects, 16 single herbs including Herba *Epimedii* were found to be used over 50% for treatment of osteoporosis (Cheng D et al., 2003). Among twenty TCM formulae or commercially available anti-osteoporotic products that of different degrees of efficacy, Herba *Epimedii* remains to be the most popular or important single herb used (Table 1-2).

The Chinese refer Herba *Epimedii* as Yinyanghuo (淫洋藿), which has been loosely translated as “licentious goat plant” and then adopted as “horny goat weed” by the Western countries. It is a genus of around forty related plant species, which are grown in Asia and the Mediterranean region. Several species such as *E. sagittatum* Maxim. (箭葉淫洋藿), *E. koreanum* Nakai (朝鮮淫洋藿), *E. wushanense* T. S. Ying (巫山淫洋藿),, *E. pubescens* Maxim. (柔毛淫洋藿), *E. brevicornum* Maxim. (心葉淫洋藿) are used for medicinal purposes. *In vitro*

studies have demonstrated an increase in osteoblastic cell proliferation, differentiation, mineralization and osteocalcin secretion with icariin treatment (Chen KM, et al., 2005; Meng FH, et al., 2005). It has been reported that flavonoids from Herba *Epimedii* reduced bone resorption, serum interleukin-6 concentration and increased serum estrogen level without hypertrophic effect on uterus in ovariectomized rats (Zhang G, et al., 2006; Jiang YN, et al., 2002). It has also been shown to increase bone mineral density, trabecular thickness, femoral breaking strength and decrease trabecular separation. A recent clinical study conducted in 110 Chinese postmenopausal women has shown that administration of Herba *Epimedii* for 1 year led to a 2 % increase in BMD and no side effects were observed (Qin L, et al., 2005). The first clinical trial on HEP-derived compounds icariin, daidzein and genistein in a ratio of 20:5:1 has demonstrated to increase BMD (lumbar and femoral neck) and decrease bone resorption marker (urinary DPD) significantly in 100 healthy postmenopausal women (Zhang G et al., 2007).These results suggested a potential value of Herba *Epimedii* and its derived compounds on prevention and treatment of osteoporosis.

Figure 1-14: Photographs showing the fresh (upper) and dried (lower) leaves of *E. koreanum Nakai.*

藥名 (Name)	使用率 (Frequency)	藥名 (Name)	使用率 (Frequency)
淫羊藿 (仙靈脾) Herba Epimedii	18	女貞子 Fructus Ligustri Lucidi	2
杜仲 Eucomm iaulm oides Oliv.	8	巴戟天 Radix Morindae Officinalis	2
熟地 Radix Rehmanniae Preparata	8	紫河車 Dried Human Placenta	2
補骨脂 Psoralea corylifolia L.	7	山藥 Rhizoma Dioscoreae	2
丹參 Radix Salviae Miltiorrhiae	6	知母 Rhizoma Anemarrhenae	2
骨碎補 Rhizoma Drynariae	5	龍骨 Euphorbia trigona	2
當歸 Radix Angelicae Sinensis	5	黃精 Rhizoma Polygonati	1
黃芪 Radix Astragali	5	黃柏 Cortex Phellodendri	1
牡蠣 Concha Ostreae	5	澤瀉 Rhizoma Alismatis	1
生地 Rehmannia glutinosa	4	功勞葉 Mahonia leaf	1
續斷 Radix Dipsaci	4	川芎 Rhizoma Chuanxiong	1
蛇床子 Cnidi Fructus	3	白朮 Rhizoma Atractylodis Macrocephalae	1
枸杞 Lycium chinense	3	白芍 Radix Paeoniae Alba	1
胡桃肉 Semen Persicae	3	覆盆子 Palmleaf Raspberry Pruit	1
山茱萸 Cornus officinalis	3	茯苓 Rhizoma Smilacis Glabra	1
龜板 Plastrum Testudinis	3	旱蓮草 Eclipta prostrata L	1
鹿角 Cornu Cervi	3	紅花 Flos Carthami	1
仙茅 Curculigo orchioides	2	首烏 Radix Polygoni Multiflori	1
牛膝 Radix Achyranthis Bidentatae	2	菟絲子 Cuscuta europaea L.	1

Table 1-2: A summary table showing the frequency of usage of different Chinese herbs in treating osteoporosis in China.

Twenty TCM formulae or commercially available anti-osteoporotic products (e.g. " Migupian密骨片", " Qiangshen migu ye強腎密骨液"," Bushen jiangu tang補腎健骨湯"," Bushen zhuanggu jiaonang補腎壯骨膠囊", " Qingewan 青蛾丸", " Bugu erhao補骨二號", " Xianling gubao jiaonang 仙靈骨葆膠囊", "Gushukang 骨疏康", "Huiguwan 回骨丸", "Hugu heji 護骨合劑", "Erxiantang 二仙湯", "Yishen tiansui tang 益腎填髓湯", "Gusongbao 骨松寶", "Qianlinghuo heji 黔嶺藿合劑", "Guhuo jiaonang 骨活膠囊", "Zhipi bugu dan 治痺補骨丹", "Xianzhengubao 仙珍骨寶", "Xianlingzhenbao 仙靈真寶", "Xianguning 仙骨寧", "Kanggusong 抗骨鬆") were surveyed and the major ingredients were counted.

1.4.4 Modern approaches

Herba *Epimedii* is chosen for the present study based on its frequency of usage and previous studies. As mentioned above, inability to identify for the active ingredients and to characterize the molecular actions of Herba *Epimedii* makes this herb a minor alternative for management of osteoporosis in places other than China. In order to gain a higher acceptance, *in vivo* and *in vitro* effects of Herba *Epimedii* as well as the molecular actions are studied.

1.4.4.1 Bioactivity-guided fractionation

Bioactivity-guided fractionation is one of the modern techniques for isolation and identification of the active ingredients in a medicinal herb. In this study, the three extracts of Herba *Epimedii* used were obtained from Prof. Yao's research team, which were the total extract, the total flavonoids and the non-flavonoids (Figure 1-15). Briefly the extract of Herba *Epimedii* was fractionated on the basis of polarity. The fractions were subjected to different bioassays such as MTS and ALP assays to evaluate their relative bioactivity. When the desirable effects were observed in either one fraction, further fractionation was carried out to produce different smaller fractions of higher purity. Different single compounds were finally isolated from these smaller

fractions. This technique provides a systematic approach to fractionate the extracts for isolation of single compounds, and to assess the bioactivity of these substances for targeting the active ingredients. This method helps to keep the researchers on the right track and to allocate resources to the ingredients of high potential value, which in turn maximize the use of resources and time. It is a commonly adopted method for studying TCM other than Herba *Epimedii*.

1.4.4.2 Efficacy-based approach to develop *in vivo* and *in vitro* screening platform

Development of many conventional medicines relies on the mechanism-based approach that requires the understanding of the mechanisms before lounging evaluation of efficacy in clinical trials. Unlike the Western medicines, TCM is usually studied or developed using the efficacy-based approach, in which investigation is started with evaluation of efficacy. As the ultimate requirements of drugs should be efficacy and safety while the elucidation of mechanisms is not a must, this approach helps to save resources by avoiding unnecessary research into ineffective candidates. Since many TCM researches were criticized by low methodological quality, some well-defined endpoints and models were chosen for this study. We used ovariectomized mice

and rat osteoblast-like cells as *in vivo* and *in vitro* models respectively, which are clinical relevant and suitable models for fast screening of active ingredients. The osteoprotective effects were evaluated using different endpoints such as BMD, bone strength marker, serum ALP and urinary Ca/Cr for *in vivo* study, and osteoblastic cell proliferation, differentiation and suppression of osteoclastogenesis for *in vitro* study. Further information on the models and the endpoints will be discussed in the following chapter.

1.4.4.3 Investigation on molecular actions

Although lacking of knowledge about the mechanisms involved does not have to prevent the use of clinically efficacious therapies, characterization of molecular actions is useful in gaining further improvement of efficacy and universal acceptance of the herb or its compounds. Therefore after confirming the efficacy of Herba *Epimedii*, the last part of this study focused on the investigation on molecular actions of Herba *Epimedii* and its marker compound icariin. As Herba *Epimedii* was claimed to be phytoestrogenic and icariin was a potent phytoestrogen, their actions were thought to be mediated through activation of estrogen receptors. Their biological actions at molecular level were demonstrated using ER antagonist, ERE-mediated transactivation assays

and immunobloting of phospho-ERα/ERα.

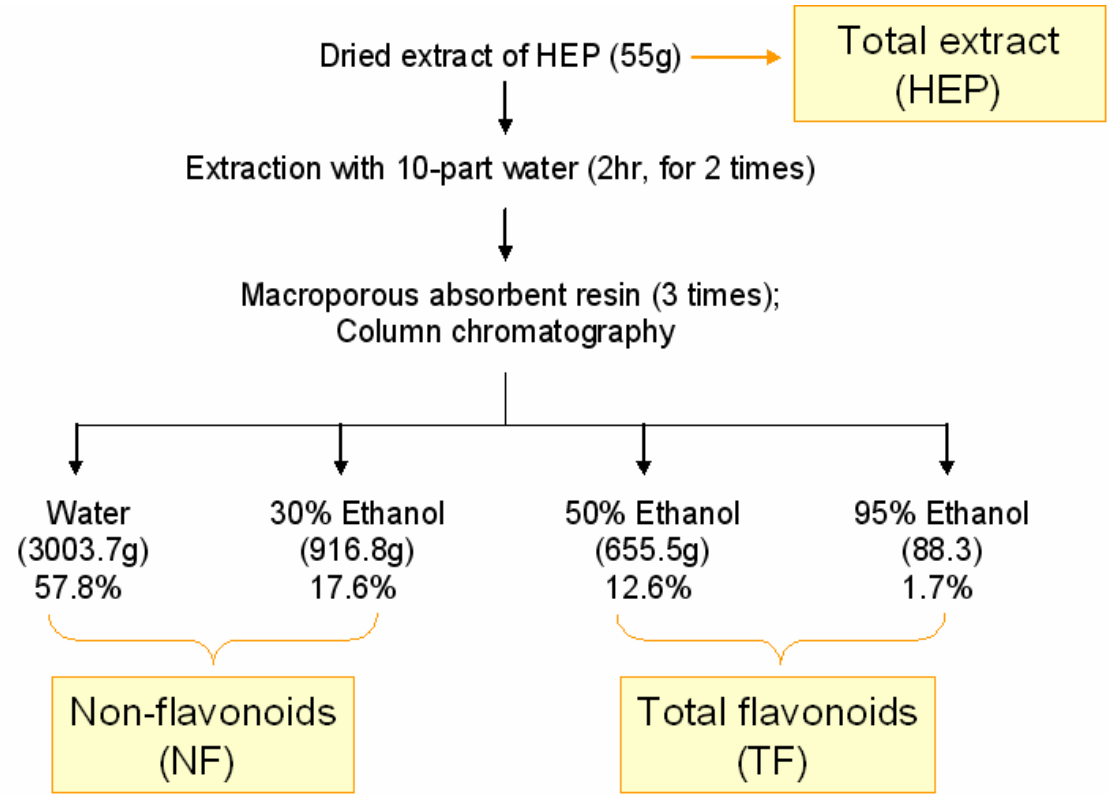


Figure 1-15: A flow chart showing the extraction of Herba *Epimedii* .

Chapter 2

Objectives and Significance

2.1 Objectives

In this study we aimed to develop both *in vivo* and *in vitro* screening platforms to identify the active ingredients of Herba *Epimedii* and to provide scientific evidence at tissue, cellular and molecular levels for evaluating the osteoprotective effects of Herba *Epimedii*. This study was generally divided into two parts, which was the efficacy study (*in vivo* and *in vitro*) of Herba *Epimedii*-derived fractions or compounds in Chapter 3 and the efficacy and mechanism study of icariin in Chapter 4. *In vivo* effects of total extract, total flavonoids, non-flavonoids and icariin on bone and mineral metabolism was studied in ovariectomized C57BL/6J mice, while *in vitro* effects of total extract, total flavonoids, non-flavonoids and flavonoids compounds (including icariin) on osteoblastic functions were studied in rat osteoblast-like UMR-106 cells. The specific aims were:

1. To determine if total extract, total flavonoids, non-flavonoids and icariin exerted trophic effect on uterus
2. To study the effects of total extract, total flavonoids, non-flavonoids and icariin on serum and urinary calcium and phosphorus levels
3. To study the effects of total extract, total flavonoids, non-flavonoids and icariin on bone mineral density and polar stress-strain index

4. To study the effects of total extract, total flavonoids, non-flavonoids and flavonoids compounds on cell proliferation using MTS assay
5. To study the effects of total extract, total flavonoids, non-flavonoids and flavonoids compounds on cell differentiation using ALP assay
6. To determine the effects of total extract, total flavonoids, non-flavonoids and icariin on modulation of osteoclastogenesis by evaluating OPG/RANKL mRNA expression
7. To compare the efficacy and potency of different Herba *Epimedii* fractions or compounds on enhancement of osteoblastic functions
8. To investigate the molecular actions of total extract and icariin using ER antagonist and ERE-mediated transactivation assay

2.2 Significance

Ability to establish both *in vivo* and *in vitro* screening platforms for identification of the active ingredients of Herba *Epimedii* for potential prevention and treatment of osteoporosis is the main significance of this study. Determination of active ingredients helps to provide systemic scientific evidence supporting the use of Herba *Epimedii* for alternative management of osteoporosis. It may also help to provide useful information for culturing or selecting the best species that contains a higher proportion of active ingredients among different species of *Epimedium* for high quality herbs or products. When the chemical composition profile is established, it may be used as a reference for evaluating the quality of Herba *Epimedii*-derived healthcare products, which makes standardization more reliable. Furthermore, investigation on the molecular actions of Herba *Epimedii* and icariin provided more evidence to widen the acceptance of Herba *Epimedii*.

Chapter 3

In vivo and *in vitro* screening of biologically active fractions and compounds in Herba *Epimedii* for potential treatment of osteoporosis

3.1 Introduction

In this chapter, both *in vivo* and *in vitro* effects of Herba *Epimedii* and its derived fractions were studied. Proper selection of *in vivo* and *in vitro* models was a basic criterion for developing a valid screening platform for studying Herba *Epimedii*. Generally, the models chosen should be stable, responsive (high response potential and short duration), easy to manipulate and clinically relevant.

Mice and rats were two commonly used animal models to study skeletal remodeling mechanisms relevant to human (Srivastava AK et al., 2000). C57BL/6J mice were used in this study, which required a shorter time for feeding and responses and a smaller amount of herbal extracts when compared to Wistar or Sprague-Dawley rats. C57BL/6J is one of the most widely used inbred strains, which results from a crossing of no more than 20 consecutive generations of brother-sister mating. Inbred mice provide both genetic and phenotypic uniformity, and therefore are regarded as a valuable tool for studying the genetic regulation of skeletal phenotypes (Bouxsein ML et al., 2005). For this study, C57BL/6J mice are particularly useful as they are generally classified as low BMD individuals when compared with other inbred strains such as BALB/cByJ and C3H/HeJ (Judex S et al., 2004). Previous studies have demonstrated that ovariectomy in C57BL/6J mice resulted in significant increase in urinary and

fecal calcium, marked decrease in percentage of calcium absorbed, and trabecular bone loss in tibias and lumbar vertebrae (Kalu DN et al., 1999; Li CY et al., 2005). These results suggest that ovariectomized C57BL/6J mice are suitable model for investigating variations in bone properties related to osteoporotic conditions in postmenopausal women.

Rat osteoblast-like UMR-106 cells were used as an *in vitro* model in this project. UMR-106 cells have been considered as preosteoblasts that explicit osteoblastic characteristics and morphological features similar to osteoblast lineage cells *in vivo*, which is a suitable model for studying the hormonal regulation of the osteoblasts (Partridge NC et al., 1983; Shinji Imai et al., 1998; Gray TK et al., 1987). Due to instability, batch variations and handling difficulties, the use of primary osteoblasts (e.g. calvarial, femoral cells) was abandoned. Other cell lines such as UMR-108 (rat), MC3T3-E1 (mouse), Saos-2, MG-63 and U-2 OS (human) are commercially available in the American Type Culture Collection. Among these cell lines, UMR-106 cells are one of the most frequently used cell line. Higher ALP activity or responsiveness than UMR-108, easier manipulation than MC3T3-E1 and Saos-2, and more extensively used or studied than MG-63 and U-2 OS urged for the use of UMR-106. Ability to undergo prolonged growth and subculture through many passages without losing

osteoblastic properties made UMR-106 cells a convenient model for screening the active ingredients.

In addition to the experimental models, endpoint measurements were important to determine the reliability and validity of the study. *In vivo* effects were evaluated by bone mineral density, a surrogate marker of bone strength (polar stress-strain index), serum and urinary calcium and phosphorus, and a bone turnover marker (serum ALP activity), whereas *in vitro* effects were assessed by relative rate of cell proliferation, differentiation (ALP activity) and suppression on osteoclastogenesis (OPG/RANKL mRNA expression). These endpoints were chosen because they were well-defined representatives of bone and mineral metabolism or osteoblastic functions, and were relatively easy and inexpensive to perform, which offered a cost-effective approach to studying Herba *Epimedii*.

3.2 Materials and Methods

3.2.1 Preparations of Herba *Epimedii* extracts and flavonoid compounds

In this study three fractions derived from *Epimedium koreanum* Nakai were used. They were classified as total extract (HEP), total flavonoids (TF) and non-flavonoids (NF). The total extract was obtained from the dried aerial part of Herba *Epimedii* and was prepared by Liaoning Xintai Pharmacy Company Limited. The total extract was subjected to water extraction and column chromatography to yield four fractions with different polarities, namely water, 30% ethanol, 50% ethanol and 95% ethanol. The fractions of water and 30% ethanol were pooled and regarded as the non-flavonoids fraction while the fractions of 50% and 95% ethanol were pooled to give total flavonoids fraction (Figure 1-15). In addition to these fractions, five flavonoid compounds were isolated from total flavonoids fraction by Prof. Yao's research team and were studied. They were known as Baohuoside I ($C_{27}H_{30}O_{10}$; MW: 514), Sagittatoside B ($C_{32}H_{38}O_{14}$; MW: 646), Korepimedoside C ($C_{41}H_{52}O_{21}$; MW: 880), Epimedin B ($C_{33}H_{40}O_{14}$; MW: 660) and Sagittatoside A ($C_{33}H_{40}O_{15}$; MW: 676). The HPLC profiles and the chemical structures were shown in Figure 3-1 and Figure 3-2 respectively.

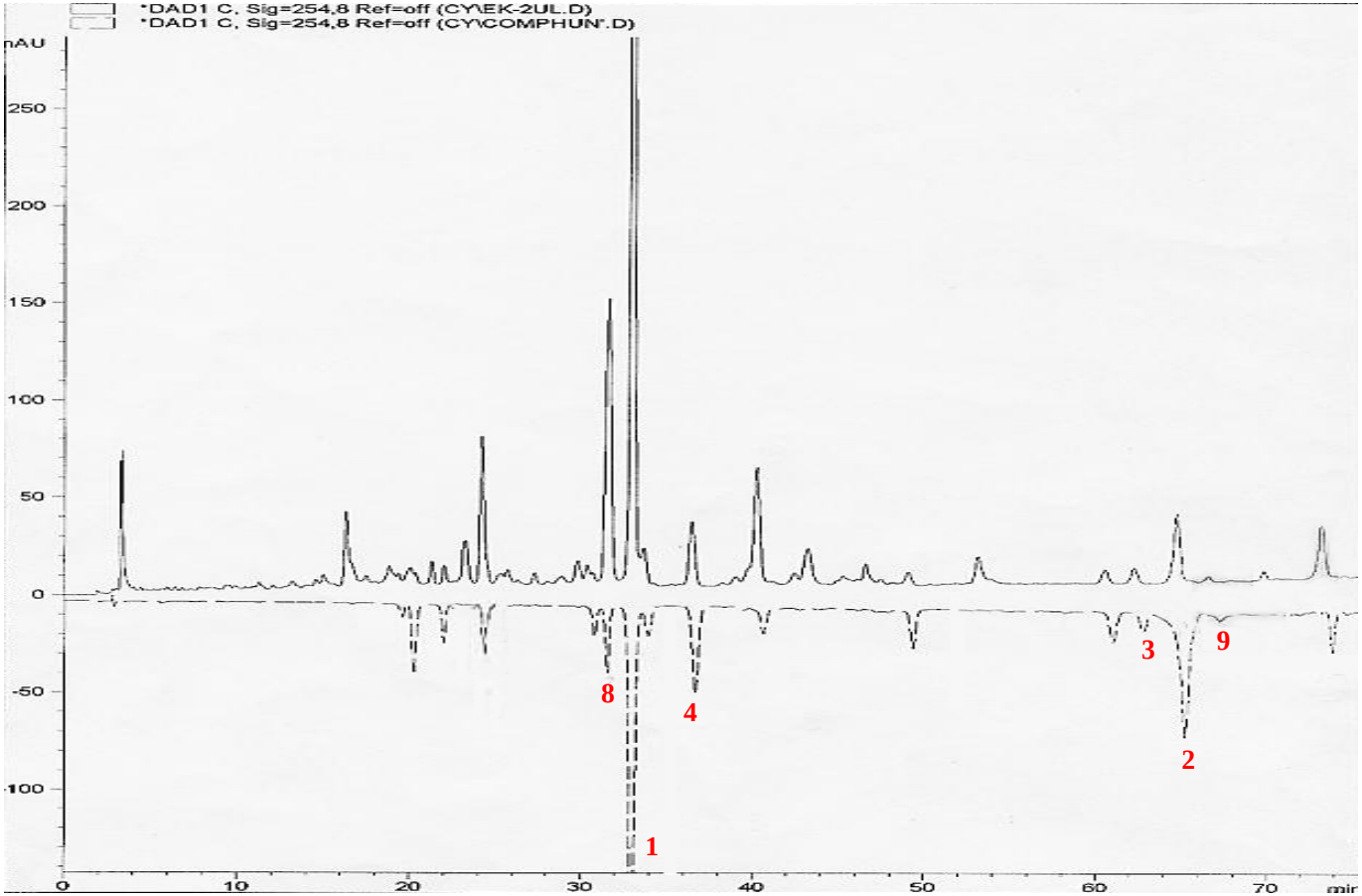


Figure 3-1: The HPLC profile of flavonoids compounds (1, Icariin; 2, Baohuoside I; 3, Sagittatoside B; 4, Korepimedoside C; 8, Epimedin B; 9, Sagittatoside A). (Adapted from Prof. Yao's research team)

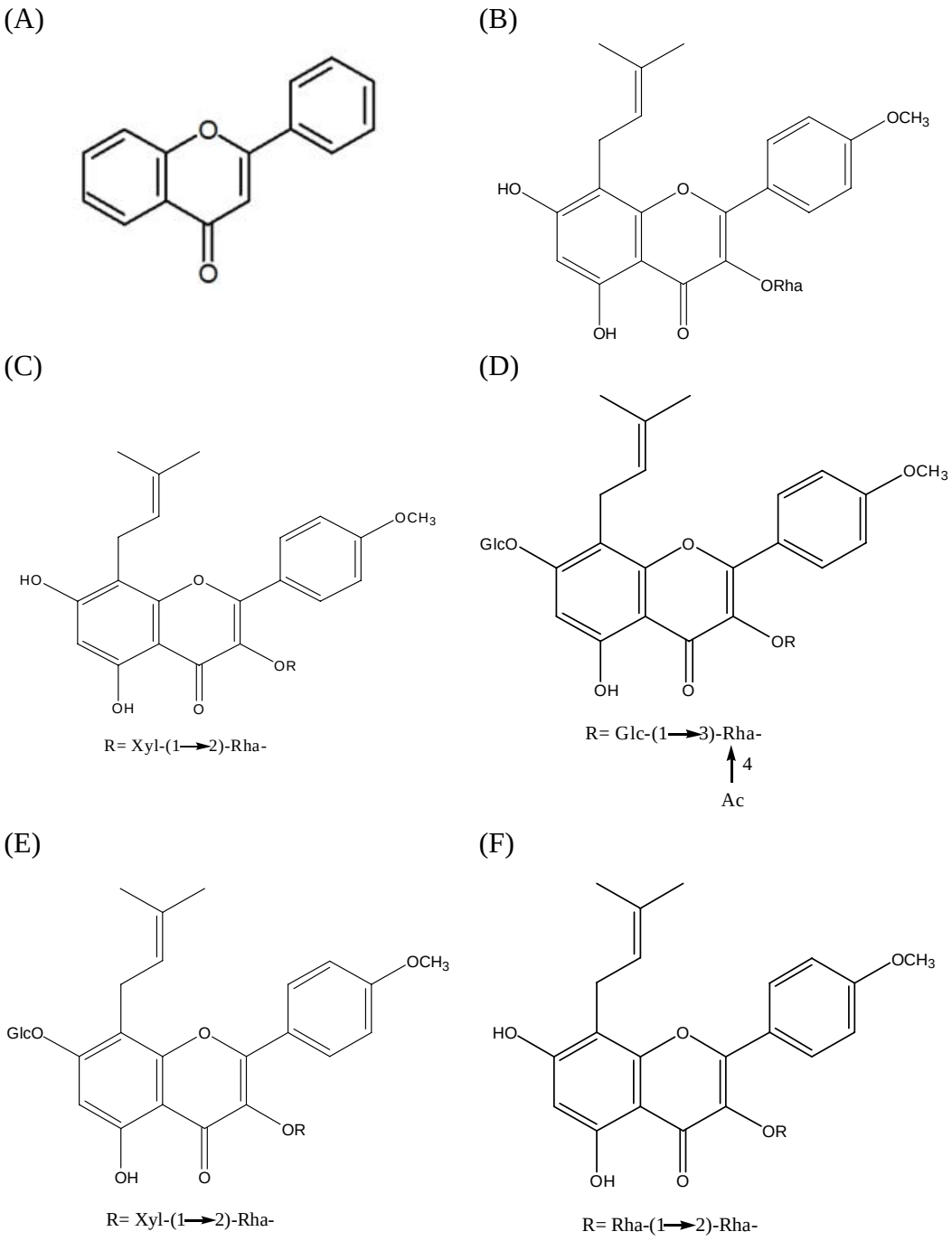


Figure 3-2: Chemical structures of (A) a general flavonoid, (B) Baohuoside I, (C) Sagittatoside B, (D) Korepimedoside C, (E) Epimedin B and (F) Sagittatoside A.

The side chains Rha, Xyl, Glc and Ac refer to rhamnose ($C_6H_{12}O_5$), xylose

($C_5H_{10}O_5$), glucose ($C_6H_{12}O_6$) and acetyl group (CH_3O) respectively.

3.2.2 Animals

There are two animal studies in this project and the experimental designs were shown in Figure 3-3. In the first study, all three fractions from Herba *Epimedii* were used to verify the *in vivo* beneficial effects of Herba *Epimedii* on bone and mineral metabolism. A total of 40 female C57BL/6J mice were purchased from Laboratory Animal Services Centre (the Chinese University of Hong Kong, HK). They were placed in cages in a room providing alternating 12 hr light/dark period with temperature of 22±2 ˚C and humidity of 55±10 %, and were allowed to consume distilled water and normal diet (0.6 % Ca, 0.35 % P) *ad libitum*. The diet was purchased from Harlan Teklad (Madison, WI) and the composition was shown in Table 3-1. They were randomly separated into 6 groups including Sham+vehicle (Sham, N=6), OVX+vehicle (OVX, N=7), OVX+17β-estradiol (E2, N=6), OVX+total extract (HEP, N=7), OVX+total flavonoids (TF, N=7) and OVX+non-flavonoids (NF, N=7). When they were 2 month-old, all of them were ovariectomized except the sham-operated group. After 18-day recovery from surgery, they were orally administered with vehicle, 17β-estradiol (4 μg/g/day), total extract (1.4 mg/g/day), total flavonoids (200 μg/g/day) or non-flavonoids (1.05 mg/g/day) for four weeks. The dosages were determined on the basis of other animal and clinical studies (Xu B et. al., 2004;

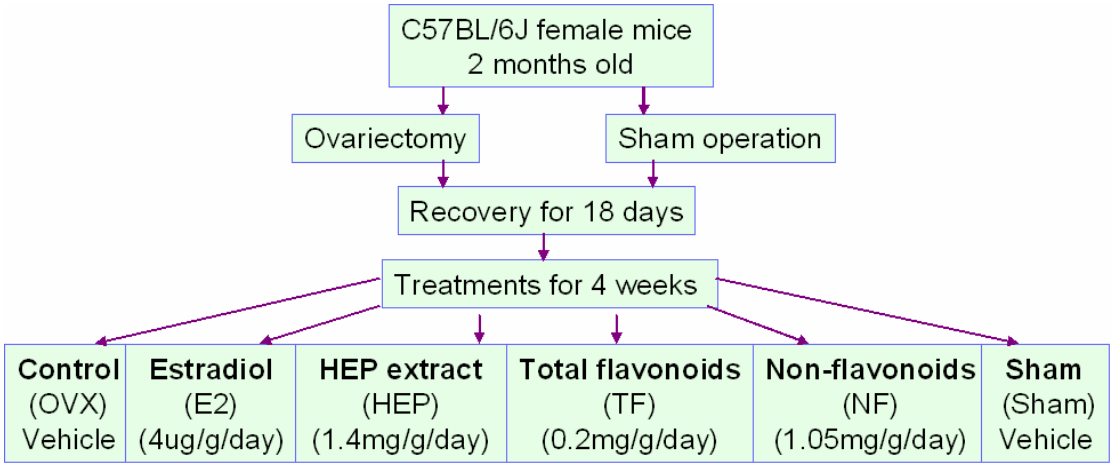
Zhang YF et. al., 1999; Ma HP, et. al., 2002; Ma HP, et. al., 2003; Jiang YN, et. al., 2002). The feeding part was done by Dr. Zhang Yan.

For the second study, four different dosages of total flavonoids were used to investigate the dose response of total flavonoids on bone and mineral metabolism. A total of 56 female C57BL/6J mice were handled in conditions similar to that in the first study. When they were 1 month-old, they were randomly separated into 7 groups including Sham+vehicle (Sham, N=8), OVX+vehicle (OVX, N=8), OVX+17β-estradiol (E2, N=8), OVX+total flavonoids of different dosages (TF50, TF100, TF200, TF400, N=8). After recovery from surgery, they were orally administered with vehicle, 17β-estradiol (4 μg/g/day) or total flavonoids (TF50, 50 μg/g/day; TF100, 100 μg/g/day; TF200, 200 μg/g/day and TF400, 400 μg/g/day) for six weeks. Before sacrifice mice were placed in the metabolic cage individually for 24 hr for collection of urine samples.

For both studies, body weight was recorded every week. At sacrifice uterus weight was measured. Serum, femurs and tibia were collected for further analysis. Urine samples were collected from mice in the second study.

Before operation or sacrifice, mice were anesthetized by peritoneal injection of ketamine (100 mg/kg mouse) and xylazine (10 mg/kg mouse).

(A)



(B)

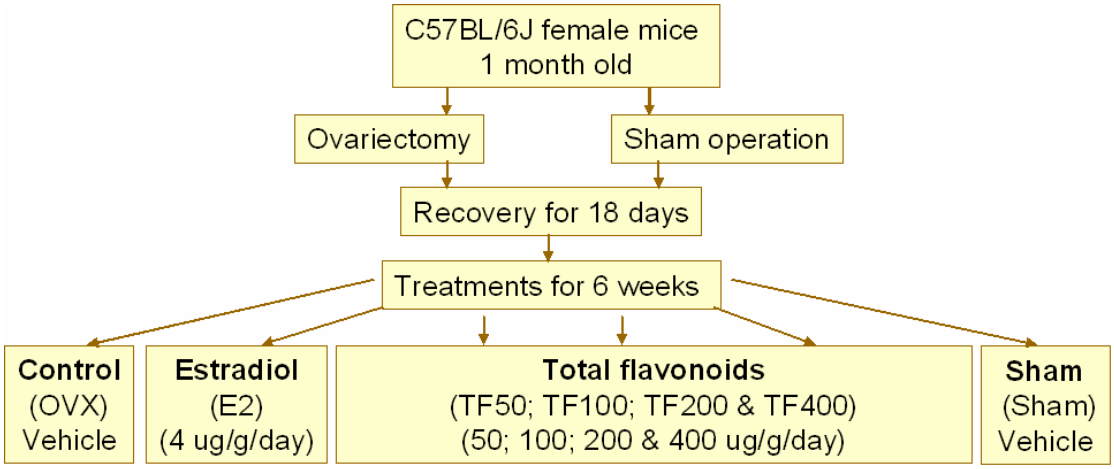


Figure 3-3: Systematic diagrams showing the experimental designs of (A) the first and (B) the second animal studies.

Table 3-1: Compositions of normal calcium diet (0.6% Ca, 0.65% P) from Harlan Teklad (No. 98005)

Components	Normal calcium diet (No. 98005) g/kg
Casein	110.0
Egg white solids, sprayed dried	97.9
DL-Methionine	3.0
Sucrose	551.1
Corn starch	100.0
Corn oil	50.0
Cellulose	20.0
Vitamin mixture[a]	10.0
Mineral mixture[b]	0.042

[a] Vitamin mixture from Harlan Teklad (No. 40060) included (mg/kg diet): p-aminobenzoic acid, 110.1; ascorbic acid, 1,016.6; biotin, 0.44; vitamin B12, 29.7; calcium panthothenate, 66.1; choline dihydrogen citrate, 3,496.9; folic acid, 1.98; inositol, 110.1; menadione, 49.5; niacin, 99.1; pyridoxine-HCL, 22.0; riboflavin, 22.0; thiamin-HCL, 22.0; dry vitamin A retinyl palmitate (500,000 U/g), 39.7; dry cholecalciferol (500,00 U/g), 4.4; dry vitamin E DL-a-tochopherol acetate (500 U/g), 242.3 and cornstarch (diluent), 4,666.9.

[b] Mineral mixture from Harlan Teklad included (mg/kg diet): potassium phosphate, monobasic, 24.6; calcium carbonate, 14.74; potassium chloride, 5.6; sodium bicarbonate, 4.62; magnesium oxide, 3.83; sodium chloride, 3.7; sodium selenite, 0.5; ferric citrate, 0.21; manganous carbonate, 0.123; zinc carbonate, 0.056; chromium potassium sulfate, 0.0193; cupric carbonate, 0.011 and potassium iodate, 0.0004.

3.2.3 Sample collection

Before sacrifice each mouse was placed in the metabolic cage for 24 hr and free access of distilled water and normal diet was allowed. The urine samples were collected and stored at -20°C before biochemical analysis.

At sacrifice blood was collected from the orbital venous sinus of mice. The blood samples were incubated at room temperature for at least 30 min before centrifugation at 4°C at 2,000 rpm for 20 min. The supernatant collected was serum samples and were stored at -80°C before biochemical analysis.

After removal of the surrounding adipose tissues, the wet weight of uterus was recorded. The dry weight of uterus was obtained by placing the uterus in an 80°C oven for 24 hr. The uterus index or uterus/body weight ratio was calculated by normalizing the wet or the dry weight of uterus with the final body weight of mice.

Femurs and tibias were collected and wrapped in gauzes saturated with physiological saline. The bones were stored at -20°C before analysis.

3.2.4 Biochemical assays of serum samples

Serum calcium concentration was determined by a o-cresolphthalein

complexion (OCPC) method using a commercial kit (Wako Pure Chemical Industries Ltd., Japan). This method makes use of the ability of calcium to produce a purplish red color when combine with OCPC under an alkaline condition. Briefly, 200 μl alkaline buffer and 20 μl OCPC solution were added into a 96-well microplate containing 2 μl serum samples. The microplate was incubated at room temperature for 5 min and absorbance at 570 nm was measured. The intensity of purplish red color produced by OCPC was directly proportional to the calcium content.

The inorganic phosphorus concentration in serum was determined by a p-methylaminophenol reduction method using a commercial kit (Wako Pure Chemical Industries Ltd., Japan). The principle of this method relies on the formation of a blue substance called molybdenum blue by reducing phosphomolybdic acid, which is a product formed from inorganic phosphorus and molybdate in the presence of p-methylaminophenol (a reducing agent). Briefly, 12.5 μl serum and 1 ml p-methylaminophenol/molbdate solution were mixed and incubated at 37°C for 20 min. Absorbance at 750 nm was measured. The intensity of blue color developed was directly proportional to the inorganic phosphorus level.

Serum alkaline phosphatase (ALP) was determined using the ALP substrate

solution purchased from Stanbio Laboratory, USA. This enzymatic assay bases on the ability of ALP to hydrolyzes the substrate called p-nitrophenyl phosphate to form p-nitrophenol and phosphates at pH 10.4 and 37°C, in which the color changes from colorless to yellow. After mixing 2.5µl serum and 100µl ALP substrate solution, the absorbance change per minute was measured at 405 nm. The concentration of ALP was expressed in unit per liter (U/L), which refers to the amount of enzyme used to produce one mmol/L of p-nitrophenol per minute. It was calculated using an equation shown below.

$$\text{ALP (U/L)} = \frac{\text{Absorbance change per minute}}{0.01875} \times \frac{\text{Total volume}}{\text{Sample volume}}$$

3.2.5 Biochemical analysis of urine samples

Urinary calcium and phosphorus concentrations were determined using the methods (i.e. OCPC method and p-methylaminophenol reduction method for quantification of serum calcium and phosphorus respectively) described above. Concentration of creatinine in urine was determined by a colorimetric method using a commercial kit (Wako Pure Chemical Industries Ltd., Japan). This method is based on the principle of Jaffe reaction, in which creatinine reacts with picric acid under an alkaline condition to produce a reddish orange color. Briefly,

urine samples were 10-fold diluted with distilled water and deproteinized with sulfuric acid to remove any interfering proteins before mixing with alkaline picrate solution in a ratio of 1:1. The reaction mixture was incubated at 25-30°C for 20 min and absorbance was measured at 570 nm. The intensity of reddish orange color was directly proportional to the creatinine content. Urinary calcium and phosphorus concentrations were normalized with creatinine concentration and were expressed as urinary calcium to creatinine ratio (Ca/Cr) and phosphorus to creatinine ratio (P/Cr) respectively.

3.2.6 Bone mineral density analysis by peripheral computed tomography (pQCT)

The pQCT scanning was performed using XCT-2000 (StraTec Medizinetecnik, Germany). Femurs or tibias were placed on a plastic holder and oriented at the center of the scanning area. Long axis of diaphysis was adjusted parallel to the scanning direction. The midshaft, 1.5 mm away from the apex of femoral proximal and distal end were scanned at a voxel size of 0.3 mm^2. BMD (in mg/ccm) and polar stress-strain index (polar-SSI) (in mm^3) of cortical and trabecular bone were measured. BMD is a surrogate marker of bone strength and a standard predictor of fracture risks (Ammann P et al., 2003). SSI is a newly

introduced index calculated by the pQCT to reflect torsional bone strength (Hasegawa Y et al., 2001). It was calculated as:

$$SSI = \sum_{i-1,n} r_i^2 * a\mathrm{CD/ND}/r_{\max}$$

where *r* is the distance of a voxel to the center of gravity, *r*max is the maximum distance of a voxel to the center of gravity (mm), *a* is the area of a voxel (mm^2), CD is the measured volumetric cortical density (mg/cm^2), and ND is the normal physiological density (1200 mg/cm^2).

3.2.7 Culture of rat osteoblast-like cell line (UMR-106)

UMR-106 cells (ATCC no. CRL-1661) were routinely cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10 % fetal bovine serum (FBS), penicillin 100 U/ml and streptomycin 100 μg/ml under the conditions of 37 C, 95 % air and 5 % CO_2. Fresh medium replenishment was carried out every three days. At 80-90 % confluence, cells were seeded in 96-well, 12-well and 6-well plate at a density of 2,500, 65,000 and 125,000 cells per well respectively for different assays. After 48 hr, the medium was switched to phenol red-free DMEM supplemented with 1 % dextran-charcoal-stripped serum (sFBS) for 24 hr. Cells were then treated with vehicle, 17β-estradial (10^{-8}M), HEP extracts or flavonoids compounds for 48 hr. DMEM, FBS and

penicillin-streptomycin-glutamine were purchased from Life Technologies Inc. (Carlsbad, CA, USA), and 17β-estradial was purchased from Sigma-Aldrich (St. Louis, MO, USA).

3.2.8 Cell proliferation assay

UMR-106 cells were seeded in a 96-well plate at a density of 2,500 cells per well. After 48 hr, the medium was switched to phenol red-free DMEM supplemented with 1 % dextran-charcoal-stripped serum (sFBS) for 24 hr. Cells were then treated with vehicle, 17β-estradial (10^{-8}M) or compounds to be investigated for 48 hr. The MTS assay was used as an indirect colorimetric measurement of cell proliferation. Briefly the medium was discarded and replaced with 100 μl of MTS/PMS solution (Promega, Madison, WI, USA). The plate was incubated at 37 ˚C for 1 hr followed by measurement at a wavelength of 490 nm using a microplate reader (Bio-Rad Laboratories Inc., CA). Dehydrogenases found in metabolically active cells reduce MTS into aqueous soluble formazan, and production of formazan is directly proportional to the number of viable cells.

3.2.9 Alkaline phosphatase (ALP) activity assay

UMR-106 cells were seeded in a 96-well plate at a density of 2,500 cells per well. After 48 hr, the medium was switched to phenol red-free DMEM supplemented with 1 % dextran-charcoal-stripped serum (sFBS) for 24 hr. Cells were then treated with vehicle, 17β-estradial (10^{-8}M) or compounds to be investigated for 48 hr. After treatment, cells were rinsed twice with ice-cold phosphate buffered saline (PBS) and lysed by placing the microplate in -80˚C refrigerator for 15 min. After removing from refrigerator, 100μl 10mM ALP reagent (Sigma-Aldrich, St. Louis, MO, USA) was added to each well followed by incubation at 37˚C for 30 min. Absorbance at 405nm was measured using a microplate reader (Bio-Rad Laboratories Inc., CA). The ALP in the sample hydrolysed p-nitrophenylphosphate in the reagent to p-nitrophenol, changing the color from colorless to yellow. To normalize the result, Bradford protein assay was carried out.

3.2.10 Reverse transcription-Real time-PCR

After treatment, cells were rinsed twice with ice-cold PBS and lysed with 1 ml Trizol (Life Technologies Inc., Carlsbad, CA, USA). Total RNA was collected according to the manufacturer's instructions. Before reverse transcription, agarose gel electrophoresis was conducted to check for the integrity of RNA and

two distinct (28S and 18S) bands should be observed. 2 μg total RNA, 0.5 μg oligo-dT(12-18) primers, 10 mM dNTPs, PCR buffer and Superscript II reverse transcriptase (Life Technologies Inc., Carlsbad, CA, USA) were used to generate the complementary DNA. The cDNA was diluted to 1/10 with DEPC-treated water and 5 μl was used for the quantitative real time-PCR. The mRNA expressions of OPG, RANKL and GAPDH were investigated. The primers targeting genes of OPG, RANKL and GAPDH were shown in Table 3-2. The reaction mixture containing diluted cDNA, primers, and iQ SYBR Green Supermix (Bio-Rad Laboratories, CA) were subjected to PCR consisting initial denaturation at 95 ˚C for 180 sec and 40 cycles of denaturation at 95 ˚C for 12 sec, primer annealing for 12 sec, DNA polymerization at 72 ˚C for 18 sec. Agarose gel electrophoresis was performed to check for the sizes of the PCR products and the expected sizes were shown in Table 3-2. A relative standard curve was prepared by using a series of dilution of vehicle-treated cDNA to quantify the concentration of PCR products. GAPDH is a house-keeping gene that serves as a reference for result normalization. The result was expressed as a ratio of the target/reference mRNA expression.

3.2.11 Statistical analysis

The data were analyzed by the non-paired student's t-test between Sham or OVX or C group and each treatment group instead of multiple comparisons. This method offered a greater chance of detecting subtle but important biological differences between groups when the sample size was modest. The GraphPad Prism version 4.4 software was used. Results were reported as Mean ± SEM. A *p*-value < 0.05 was considered statistically significant.

Primer	Orientation	Sequence	Annealing temperature (˚C)	Product size (bp)
OPG	Sense	GACGAGATTGAGAGAACGAG	56	502
	Anti-sense	GGTGCTTGACTTTCTAGGTG		
RANKL	Sense	TCAGGAGTTCCAGCTATGAT	58	298
	Anti-sense	CCATCAGCTGAAGATAGTCC		
GAPDH	Sense	TACATTTTGCTGATGACTGG	55	202
	Anti-sense	TGAATGGTAGGAGCTTGACT		

Table 3-2: Details of the primers used for probing OPG, RANKL and GAPDH in real time-PCR.

3.3 Results

In this section, *in vivo* study followed by *in vitro* study was introduced. Two animal studies were conducted to confirm the efficacy of three extracts from Herba *Epimedii* (i.e. HEP, TF and NF), to screen for the active fractions and to study the optimal dose of the fractions for bone health protection. As total flavonoids was generally considered as a major source of active ingredients, it was of greater potential and research interest that deserved further investigations. Therefore total flavonoids instead of non-flavonoids was studied in the second animal study due to time and resources limitations. For *in vitro* study, five flavonoids compounds (i.e. Baohuoside I, Sagittatoside B, Korepimedoside C, Epimedin B, Sagittatoside A) were studied in addition to three extracts from Herba *Epimedii*.

3.3.1 Effect of total extract, total flavonoids and non-flavonoids of Herba *Epimedii* on body weight

During the study, mice were paired-fed and body weight was recorded weekly. Percentage change in body weight was calculated using the equation: (final body weight - initial body weight) / initial body weight x 100%. In Figure 3-4, all ovariectomized mice had higher body weight change than the sham-operated mice as a result of ovary removal. As oral estradiol treatment compensated for the effect of ovariectomy, body weight change in estradiol-treated mice was not significantly different from that in sham mice. Total extract, total flavonoids and non-flavonoids of Herba *Epimedii* did not prevent the OVX-induced body weight gain.

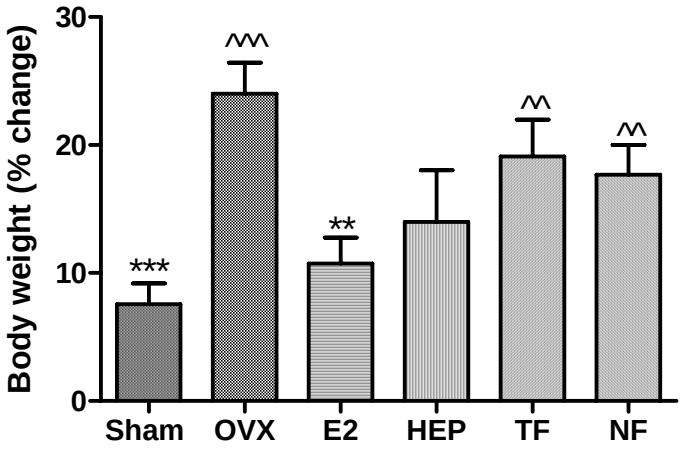


Figure 3-4: Effect of total extract, total flavonoids and non-flavonoids of Herba *Epimedii* on body weight change.

Mice of 2 months old were ovariectomized or sham-operated. After recovery from surgery, vehicle (Sham or OVX), 17β-estradiol (E2 4 μg/g/day), HEP extract (HEP 1.4 mg/g/day), total flavonoids (TF 0.2 mg/g/day) or non-flavonoids (NF 1.05 mg/g/day) were orally administered for four weeks. During the study, body weight was measured weekly and percentage change in body weight was calculated. Results were expressed as mean ± SEM (n = 6-8). (**p<0.01; ***p<0.001 vs OVX; ^^p<0.01; ^^^p<0.001 vs Sham)

3.3.2 Effect of total extract, total flavonoids and non-flavonoids of Herba *Epimedii* on uterus weight

To investigate if total extract, total flavonoids and non-flavonoids of Herba *Epimedii* exerted trophic effect on uterus, the uterus to body weight ratio was calculated. As shown in Figure 3-5, uterus to body weight ratio was significantly lowered in all groups except E2 when compared with Sham. A high uterus index in E2 group indicated a presence of uterotrophic effect, while low uterus index in HEP, TF and NF groups suggested the opposite.

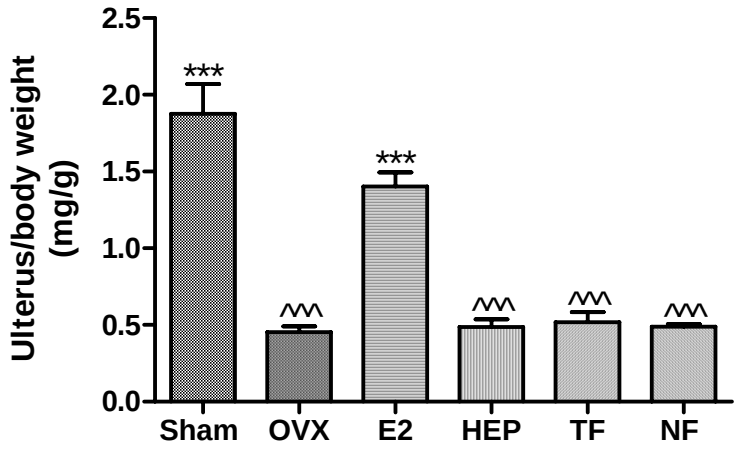


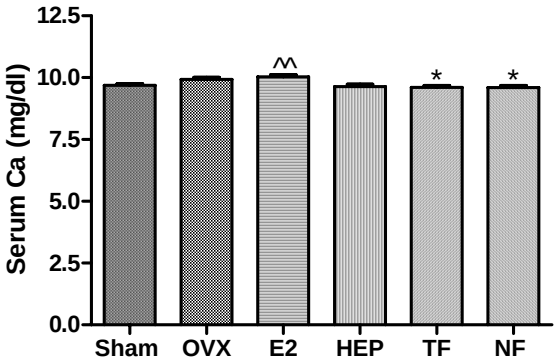
Figure 3-5: Effects of total extract, total flavonoids and non-flavonoids of Herba *Epimedii* on uterus index.

After administration of vehicle (Sham or OVX), 17β-estradiol (E2 4 ug/g/day), HEP extract (HEP 1.4 mg/g/day), total flavonoids (TF 0.2 mg/g/day) or non-flavonoids (NF 1.05 mg/g/day) for four weeks, body weight and uterus weight were measured. The uterus index was calculated as the ratio of uterus to final body weight. Results were expressed as mean ± SEM (n = 6-8). ***$p<0.001$ vs OVX; ^^^$p<0.001$ vs Sham)

3.3.3 Effect of total extract, total flavonoids and non-flavonoids of Herba *Epimedii* on serum calcium and phosphorus levels

To determine if ovariectomy and Herba *Epimedii* extracts altered calcium homeostasis, serum calcium and phosphorous concentrations were measured. In Figure 3-6A, serum calcium concentration remained more or less unchanged in all groups except E2 group when compared with Sham group. When compared with OVX group, TF and NF treatments caused slight and significant decrease in serum calcium level. These results suggested that serum calcium level was tightly regulated that it was not largely affected by ovariectomy and different treatments. Serum phosphorus concentrations were significantly higher in all groups except HEP group when compared with Sham group (Figure 3-6B). As PTH increases renal phosphorus excretion and decreases serum phosphorus level, high serum phosphorus concentration suggested that PTH levels might be low in the corresponding mice.

(A)



(B)

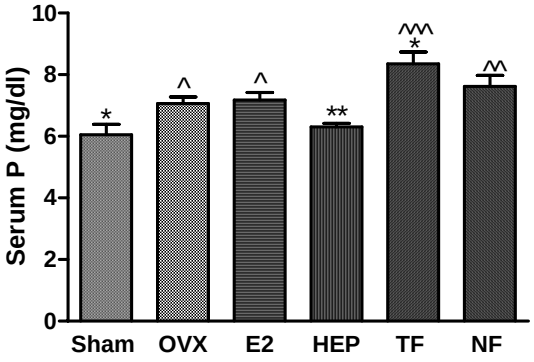


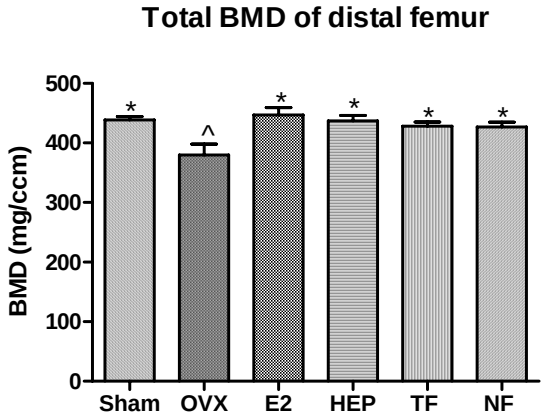
Figure 3-6: Effect of total extract, total flavonoids and non-flavonoids of Herba *Epimedii* on serum (A) calcium and (B) phosphorus concentrations.

Mice were treated with vehicle (Sham or OVX), 17β-estradiol (E2 4 μg/g/day), HEP extract (HEP 1.4 mg/g/day), total flavonoids (TF 0.2 mg/g/day) or non-flavonoids (NF 1.05 mg/g/day) for four weeks. Before sacrifice serum was collected from orbital venous sinus of mice. Concentrations of calcium and phosphorus were determined by colorimetric methods described previously. Serum calcium and phosphorus concentrations were shown in (A) and (B) respectively. Results were expressed as mean ± SEM (n = 6-8). (*$p<0.05$; **$p<0.01$ vs OVX; ^$p<0.05$; ^^$p<0.01$; ^^^$p<0.001$ vs Sham)

3.3.4 Effect of total extract, total flavonoids and non-flavonoids of Herba *Epimedii* on bone mineral density analyzed by pQCT

As trabecular bone has a higher bone turnover rate and responsiveness than cortical bone in response to treatments, total and trabecular bone mineral density of femur distal end were determined. As shown in Figure 3-7A and B, total and trabecular BMD was significantly decreased in OVX group when compared to Sham group. Both total and trabecular BMD were restored to a level similar to Sham group in all groups except OVX group. These results suggested that Herba *Epimedii* extracts exerted a beneficial effect on suppressing ovariectomy-induced bone loss.

(A)



(B)

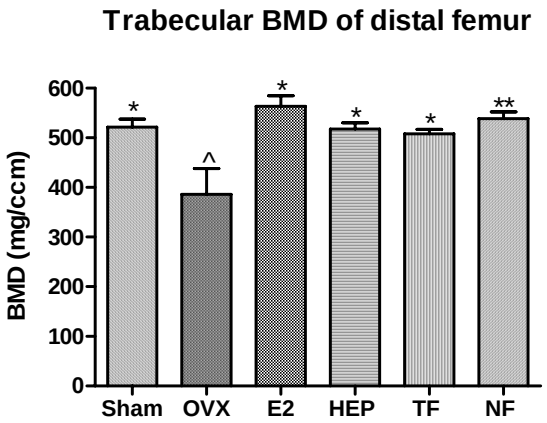


Figure 3-7: Effect of total extract, total flavonoids and non-flavonoids of Herba *Epimedii* on bone mineral density analyzed by pQCT.

After 4-week treatment with vehicle (Sham or OVX), 17β-estradiol (E2 4 μg/g/day), HEP extract (HEP 1.4 mg/g/day), total flavonoids (TF 0.2 mg/g/day) or non-flavonoids (NF 1.05 mg/g/day), femurs were collected and subjected to pQCT analysis. Bone tissue at 1.5mm away from the apex of femoral distal end was scanned. (A) Total BMD and (B) trabecular BMD were measured. Results were expressed as mean ± SEM (n = 6-8). (*$p<0.05$; **$p<0.01$ vs OVX; ^$p<0.05$ vs Sham)

3.3.5 Effect of total flavonoids on body weight

In the second animal study, *in vivo* effects of total flavonoids were studied. Mice were paired-fed and body weight was recorded weekly throughout the study. In Figure 3-8, body weight change was significantly higher in all ovariectomized mice except those treated with estradiol when compared to sham mice. This result demonstrated that ovariectomy-induced body weight gain might be prevented by administration of 17β-estradiol but total flavonoids.

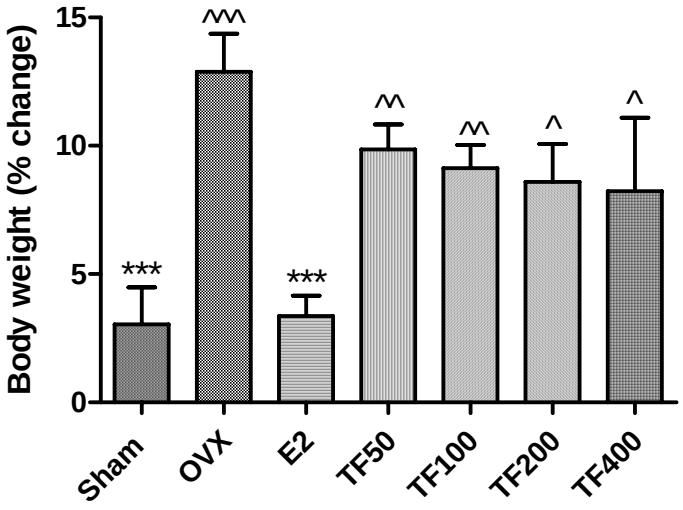


Figure 3-8: Effect of total flavonoids on body weight change.

Mice of 1 month old were sham-operated or ovariectomized. After 18-day recovery, mice were treated with vehicle (Sham or OVX), 17β-estradiol (E2; 4μg/g/day) or four doses of total flavonoids (TF50, 50 μg/g/day; TF100, 100 μg/g/day; TF200, 200 μg/g/day and TF400, 400 μg/g/day) for six weeks. Body weight measurement was taken weekly and percentage change in body weight was calculated. Results were expressed as mean ± SEM (n =6-8). (***$p<0.001$ vs OVX; ^$p<0.05$; ^^$p<0.01$; ^^^$p<0.001$ vs Sham)

3.3.6 Effect of total flavonoids on uterus weight

As observed in the first animal study, treatment with total flavonoids at 200 μg/g/day did not increase uterus index. To confirm if total flavonoids at other doses did not exerted trophic effect on uterus, the uterus to body weight ratio was determined. Uterus index calculated from wet and dry uterus weight were shown in Figure 3-9A and B respectively. In both figures, E2 treatment caused a significant increase in uterus index while the remaining groups showed significant decrease in uterus index when compared with Sham group. Besides Sham and E2 group, wet uterus to body weight ratio was significantly higher in TF50 and TF100 group when compared with OVX, while dry uterus to body weight remained unchanged in all total flavonoids groups. These results indicated that total flavonoids did not exert trophic effect on uterus.

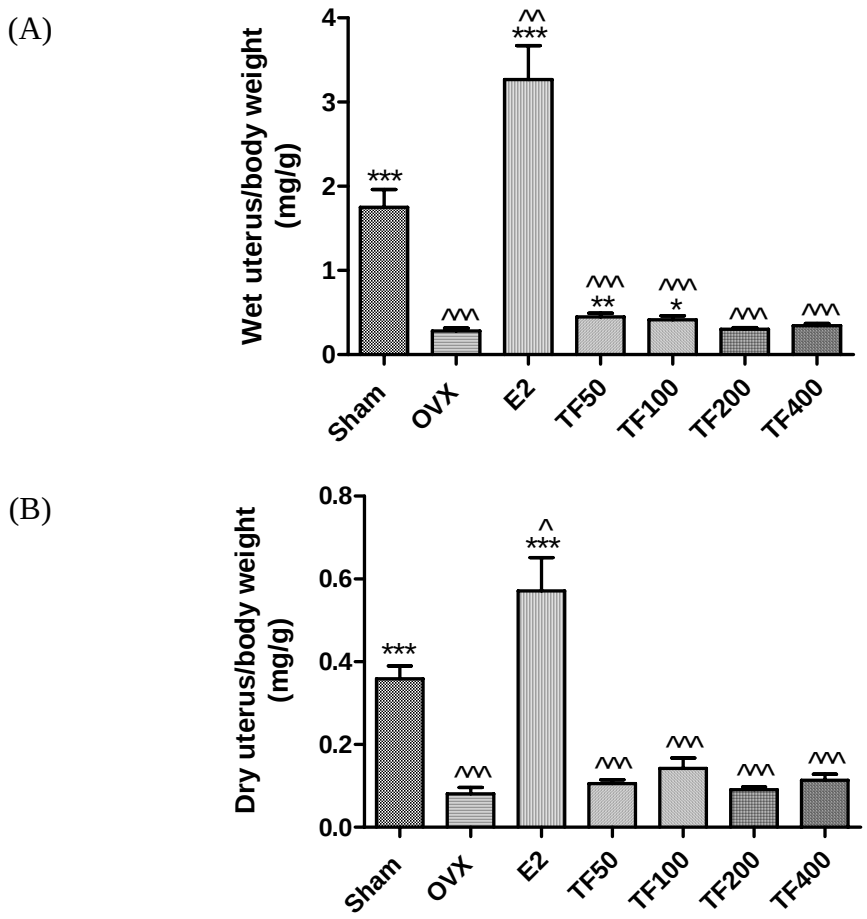


Figure 3-9: Effect of total flavonoids on uterus index.

After administration of vehicle (Sham or OVX), 17β-estradiol (E2; 4 μg/g/day), or four doses of total flavonoids (TF50, 50 μg/g/day; TF100, 100 μg/g/day; TF200, 200 μg/g/day and TF400, 400 μg/g/day) for six weeks, body weight and uterus weight were measured. The uterus index was calculated as the ratio of uterus to final body weight. Wet uterus/body weight and dry uterus/body weight were shown in (A) and (B) respectively. Results were expressed as mean ± SEM (n = 6-8). (*$p<0.05$; **$p<0.01$; ***$p< 0.001$ vs OVX; ^$p<0.05$; ^^$p<0.01$; ^^^$p<0.001$ vs Sham)

3.3.7 Effect of total flavonoids on serum and urinary calcium and phosphorus

To study if total flavonoids altered calcium and phosphorus homeostasis, serum calcium and phosphorus concentrations were measured. As shown in Figure 3-10A, all treatments except TF400 group caused significant decrease in serum calcium concentration when compared with Sham group. TF200 and TF400 group had significantly lower serum calcium concentration than OVX group. All treatments except TF200 and TF400 showed higher serum phosphorus levels when compared with Sham group, and the highest serum phosphorus level was found in OVX group (Figure 3-10B).

To study if total flavonoids prevented ovariectomy-induced increase in calcium excretion, 24 hr-urine samples were collected. Urinary calcium and phosphorus to creatinine ratios were determined. As shown in Figure 3-10C, OVX and TF400 group showed significant increase in urinary Ca/Cr when compared with Sham group. All treatments except TF400 showed significant decrease in urinary Ca/Cr when compared with OVX group. Urinary phosphorus to creatinine ratio was not significantly different among all groups (Figure 3-10D). This result suggested that total flavonoids at lower doses prevented urinary calcium loss through excretion in ovariectomized mice.

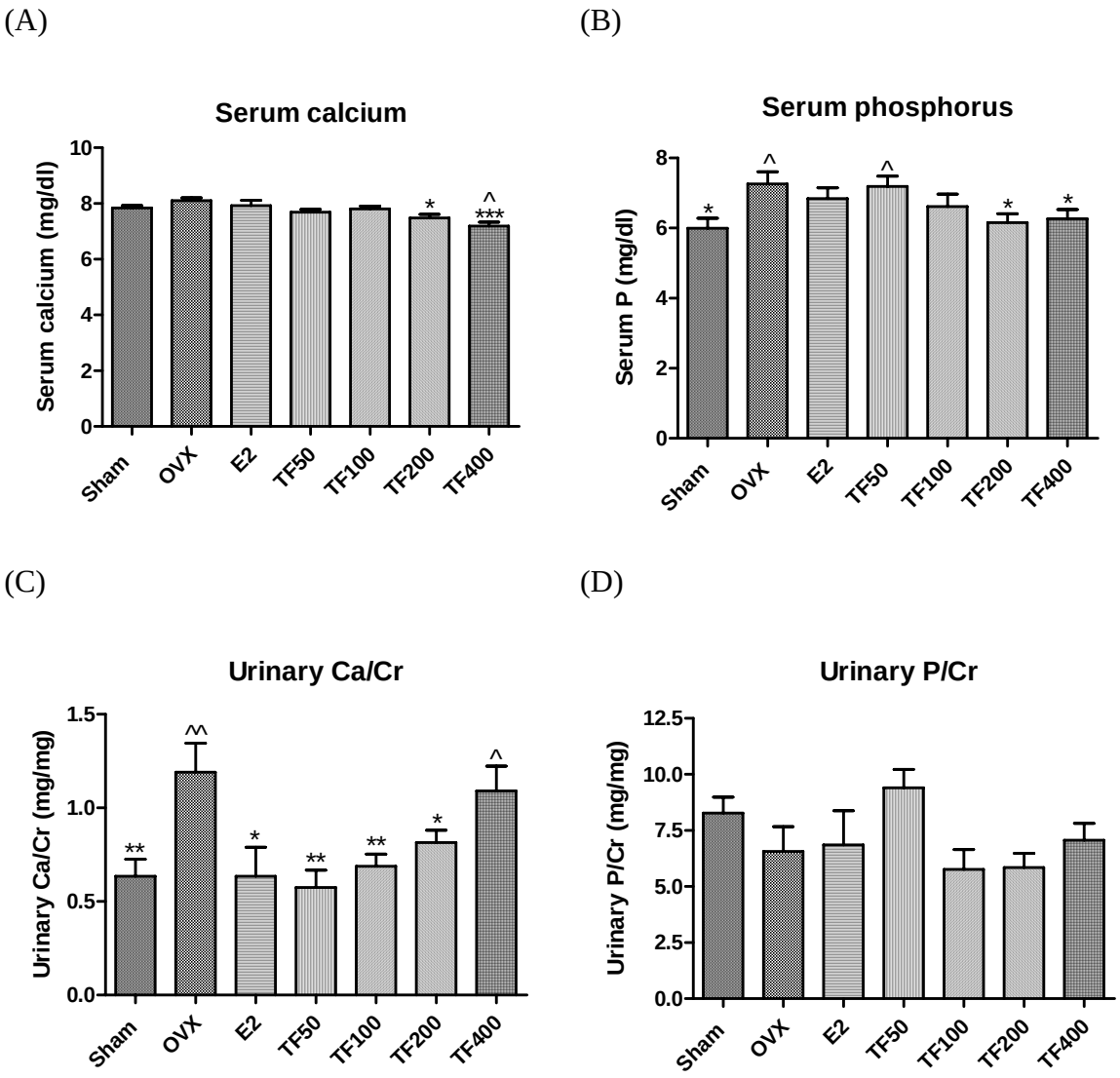


Figure 3-10: Effect of total flavonoids on serum and urine chemistry.

Mice were treated with vehicle (Sham or OVX), 17β-estradiol (E2; 4 μg/g/day), or four doses of total flavonoids (TF50, 50 μg/g/day; TF100, 100 μg/g/day; TF200, 200 μg/g/day and TF400, 400 μg/g/day) for six weeks. The day before sacrifice, mice were placed in metabolic cages for 24 hr and urine was collected. Urinary calcium and phosphorus

concentrations were normalized with creatinine and expressed as (C) urinary Ca/Cr and (D) urinary P/Cr respectively. At sacrifice serum was collected from orbital venous sinus of mice. (A) Serum calcium and (B) phosphorus concentrations were measured. Results were expressed as mean ± SEM (n = 6-8). (*p<0.05; **<0.01; ***p<0.001 vs OVX; ^p<0.05; ^^p<0.01; ^^^p<0.001 vs Sham)

3.3.8 Effect of total flavonoids on serum alkaline phosphatase

To determine if total flavonoids could suppress ovariectomy-induced increase in serum ALP concentrations, serum ALP activity was calculated. As shown in Figure 3-11, OVX group had a higher serum ALP activity than Sham group. Mice treated with TF100 and TF400 showed significant decrease in serum ALP activity when compared with OVX group. It was unexpected that serum ALP activity was the highest in E2 group. These results suggested that total flavonoids prevented OVX-induced high bone turnover.

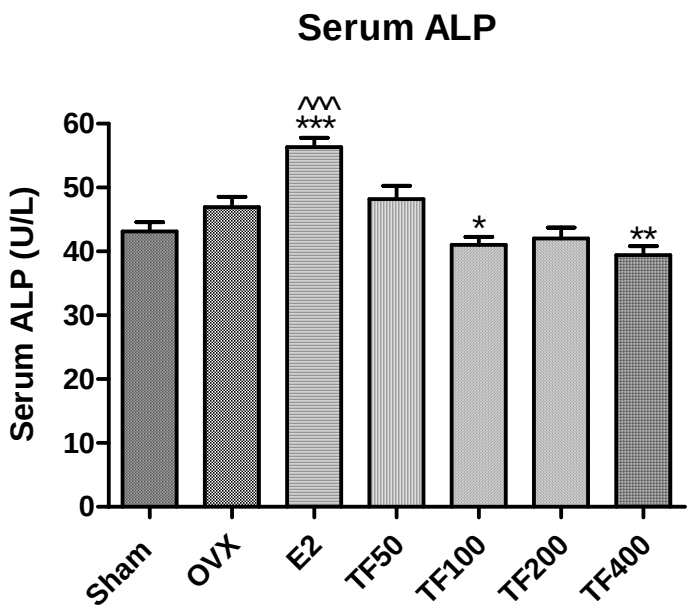


Figure 3-11: Effect of total flavonoids on serum ALP.

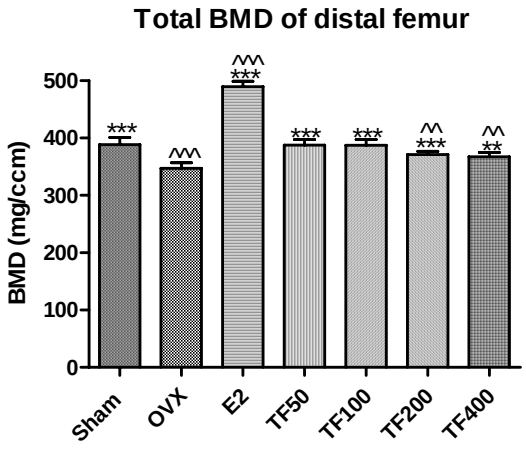
Mice were treated with vehicle (Sham or OVX), 17β-estradiol (E2; 4 μg/g/day), or four doses of total flavonoids (TF50, 50 μg/g/day; TF100, 100 μg/g/day; TF200, 200 μg/g/day and TF400, 400 μg/g/day) for six weeks. At sacrifice serum was collected from orbital venous sinus of mice, and serum ALP was measured. Results were expressed as mean ± SEM (n = 6-8). (*p<0.05; **<0.01; ***p<0.001 vs OVX; ^^^p<0.001 vs Sham)

3.3.9 Effect of total flavonoids on bone mineral density of femur analyzed by pQCT

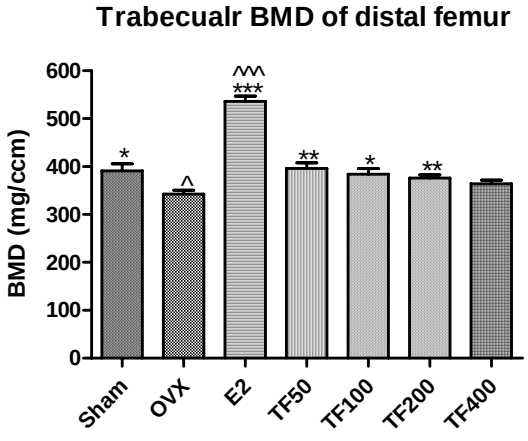
Different from the first animal study, BMD of three sites (i.e. proximal end, distal end and midshaft) of femur were analyzed instead of one (i.e. distal end) to allow deeper understanding of effect of total flavonoids on BMD restoration. Trabecular bone is highly present in proximal and distal end, and therefore both total and trabecular BMD were measured. Due to the limited amount of trabecular bone, only cortical BMD was measured at midshaft.

As shown in Figure 3-12A, all treatments significantly increased total BMD of distal femur when compared with OVX group. Trabecular BMD was significantly higher in all treatments except TF400 when compared with OVX group (Figure 3-12B). Total BMD of proximal femur was higher in all treatments and significance was observed in Sham, E2 and TF200 group when compared with OVX group (Figure 3-12C). Mice treated with total flavonoids showed higher trabecular BMD of proximal femur than OVX group (Figure 3-12D). In Figure 3-12E, all treatments showed higher cortical BMD of femur midshaft when compared with OVX group and significance was observed in E2 and TF50 group.

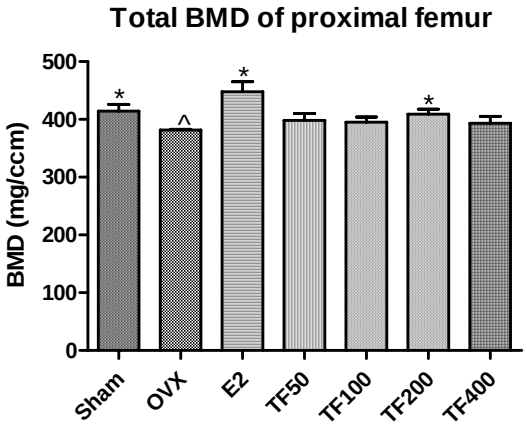
(A)


Total BMD of distal femur
BMD (mg/ccm)
0
100
200
300
400
500
Sham
OVX
E2
TF50
TF100
TF200
TF400

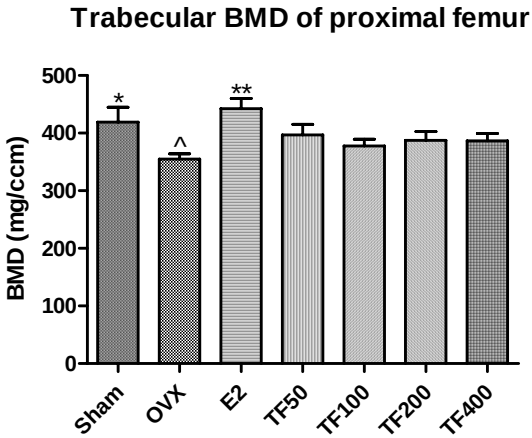
(B)


Trabecualr BMD of distal femur
BMD (mg/ccm)
0
100
200
300
400
500
600
Sham
OVX
E2
TF50
TF100
TF200
TF400

(C)


Total BMD of proximal femur
BMD (mg/ccm)
0
100
200
300
400
500
Sham
OVX
E2
TF50
TF100
TF200
TF400

(D)


Trabecular BMD of proximal femur
BMD (mg/ccm)
0
100
200
300
400
500
Sham
OVX
E2
TF50
TF100
TF200
TF400

(E)

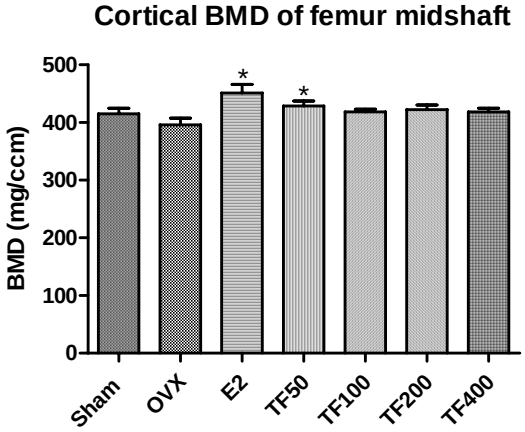

Cortical BMD of femur midshaft
BMD (mg/ccm)
0
100
200
300
400
500
Sham
OVX
E2
TF50
TF100
TF200
TF400

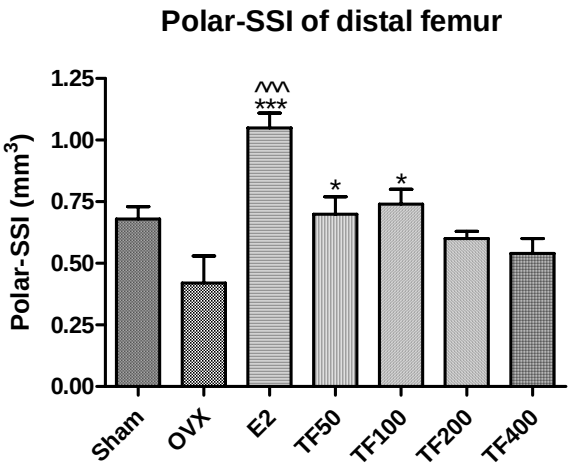
Figure 3-12: Effect of total flavonoids on BMD of midshaft, distal and proximal end of femur analyzed by pQCT.

After treatment with vehicle (Sham or OVX), 17β-estradiol (E2; 4 μg/g/day), or four doses of total flavonoids (TF50, 50 μg/g/day; TF100, 100 μg/g/day; TF200, 200 μg/g/day and TF400, 400 μg/g/day) for six weeks, femurs were collected at sacrifice. Femurs were subjected to pQCT analysis. (A) Total BMD and (B) trabecular BMD of distal femur. (C) Total BMD and (D) trabecular BMD of proximal femur. (E) Cortical BMD of femur midshaft. Results were expressed as mean ± SEM (n = 6-8). (*$p<0.05$; **$p<0.01$; ***$p<0.001$ vs OVX; ^$p<0.05$; ^^$p<0.01$; ^^^$p<0.001$ vs Sham)

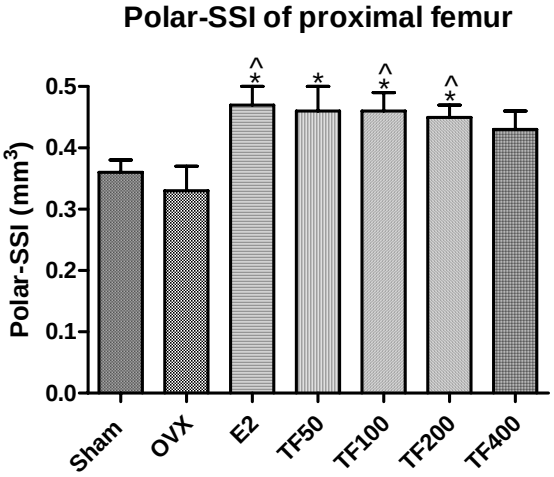
3.3.10 Effect of total flavonoids on polar stress-strain index (polar-SSI) analyzed by pQCT

The SSI is calculated from the BMD value based on the geometric values for the bone and is used as an indicator to assess bone fragility as well as BMD (Fukuda S et al., 2004). As shown in Figure 3-13A, polar-SSI of distal femur was lowest in OVX group and highest in E2 group. TF50 and TF100 treatment significantly increased polar-SSI when compared with OVX group. Polar-SSI of proximal femur was slightly lower in OVX group than Sham group (Figure 3-13B). Treatment with E2 and total flavonoids except TF400 significantly increased polar-SSI of proximal femur when compared to OVX group. Polar-SSI decreased with increasing concentration of total flavonoids. In Figure 3-13C, all treatments increased polar-SSI but significant difference was only observed in E2 group when compared to OVX group.

(A)



(B)



(C)

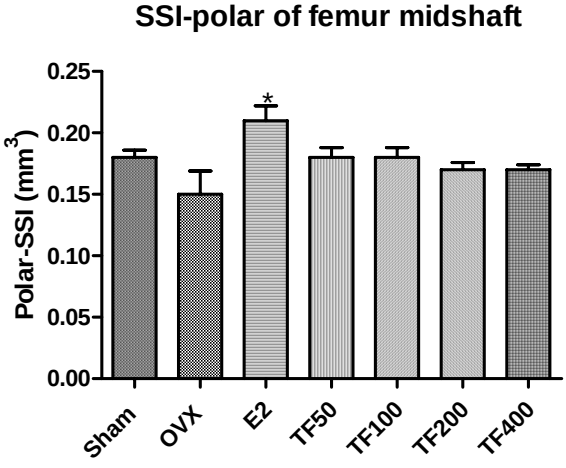


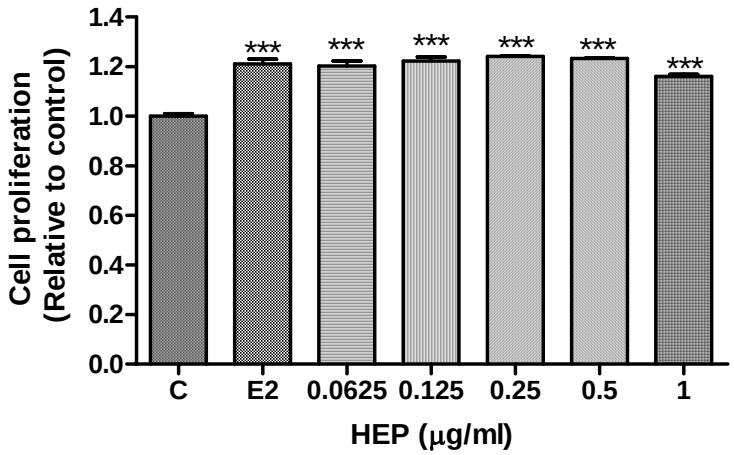
Figure 3-13: Effect of total flavonoids on stress-strain index-polar (SSI-polar) analyzed by pQCT.

After treatment with vehicle (Sham or OVX), 17β-estradiol (E2; 4 μg/g/day), or four doses of total flavonoids (TF50, 50 μg/g/day; TF100, 100 μg/g/day; TF200, 200 μg/g/day and TF400, 400 μg/g/day) for six weeks, femurs were collected at sacrifice. Femurs were subjected to pQCT analysis. Femoral polar-SSI of distal end, proximal end and midshaft were shown in (A), (B) and (C) respectively. Results were expressed as mean ± SEM (n = 6-8). (*$p<0.05$; ***$p<0.01$ vs OVX; ^$p<0.05$; ^^^$p<0.001$ vs Sham)

3.3.11 Effect of total extract of Herba *Epimedii* on cell proliferation and alkaline phosphatase activity

To study *in vitro* effect of total extract on cell proliferation and differentiation, MTS and ALP assays were conducted respectively. As shown in Figure 3-14A, UMR-106 cells treated with total extract at all concentrations increased cell proliferation significantly to a similar extent as E2. ALP activity was significantly increased in cells with all treatments except HEP at 0.0625 μg/ml (Figure 3-14B).

(A)



(B)

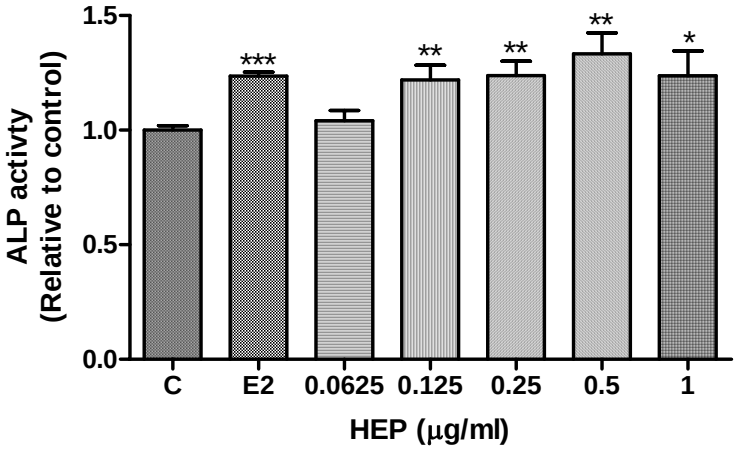


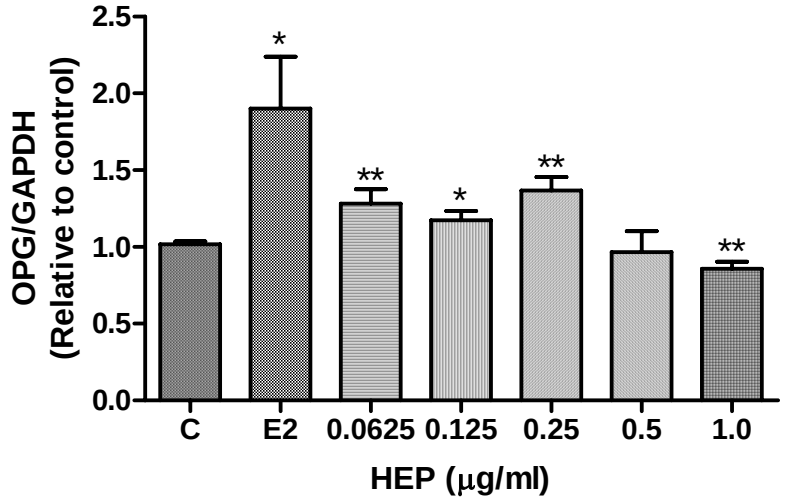
Figure 3-14: Effect of total extract on (A) cell proliferation and (B) alkaline phosphatase activity.

UMR-106 cells were treated with vehicle (C) as negative control, 17β-estradiol (E2; 10^{-8}M) as positive control or HEP (0.0625, 0.125, 0.25, 0.5 and 1 μg/ml) for 48 hr. The relative proliferation rate and ALP activity were determined using MTS and ALP assays previously described, and were shown in (A) and (B) respectively. Results were expressed as mean ± SEM (n = 18-24). (*$p<0.05$; **$p<0.01$; ***$p<0.001$ vs C)

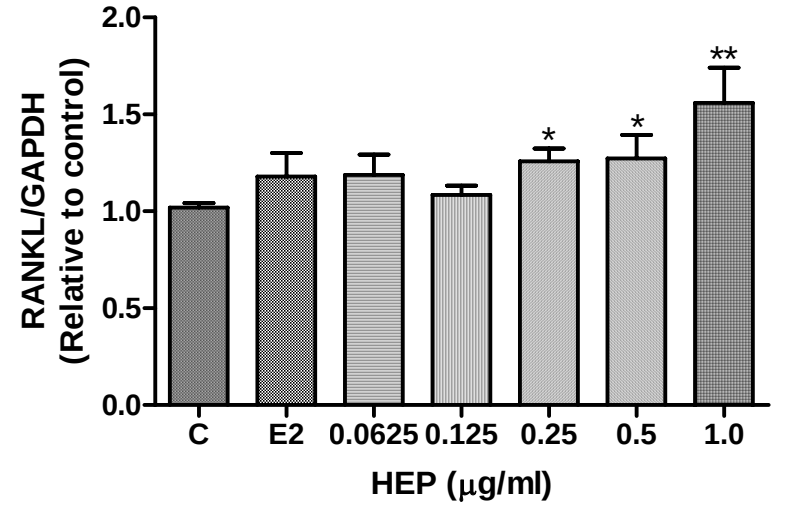
3.3.12 Effects of total extract of Herba *Epimedii* on mRNA expressions of OPG and RANKL

To study the effect of total extract on modulation of osteoclastogenesis, OPG/RANKL mRNA expressions were determined. OPG/GAPDH mRNA expression was significantly upregulated in cells treated with E2 and HEP at 0.0625 to 0.25 μg/ml while opposite result occurred in cells treated with HEP at 0.5 and 1μg/ml (Figure 3-15A). RANKL/GAPDH mRNA expression increased with increasing concentrations of HEP and significance was observed in cells treated with HEP at 0.25 to 1μg/ml (Figure 3-15B). As shown in Figure 3-15C, low concentrations of HEP (0.0625 to 0.25μg/ml) caused a significant increase in OPG/RANKL mRNA expression, while high concentrations (0.5 and 1μg/ml) resulted in a significant decrease.

(A)



(B)



(C)

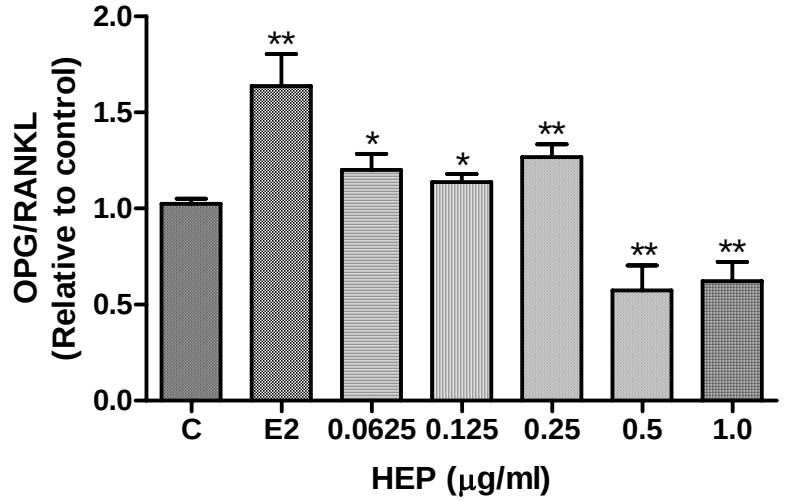


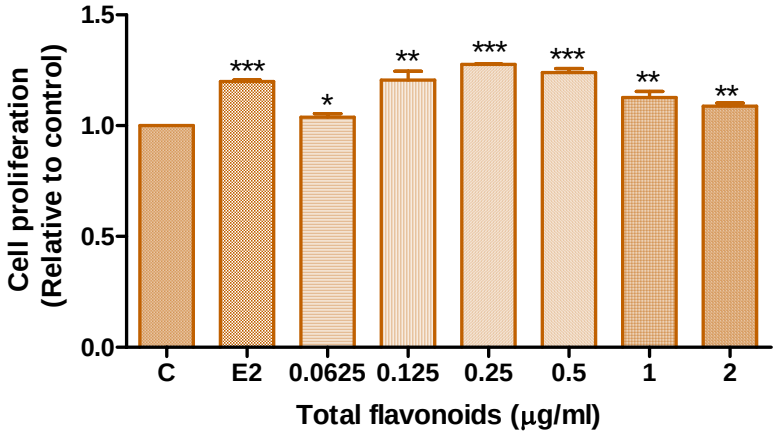
Figure 3-15: Effect of total extract of Herba *Epimedii* on mRNA expressions of (A) OPG/GAPDH, (B) RANKL/GAPDH and (C) OPG/RANKL.

UMR-106 cells were treated with vehicle (C) as negative control, 17β-estradiol (E2; 10^{-8}M) as positive control or HEP (0.0625, 0.125, 0.25, 0.5 and 1 μg/ml) for 48 hr. Total RNA was isolated and subjected to reverse transcription real time-PCR. OPG and RANKL mRNA expressions were normalized with that of GAPDH, which is a house-keeping gene for internal control. mRNA expressions of OPG/GAPDH, RANKL/GAPDH and OPG/RANKL were shown in (A), (B) and (C) respectively. Results were expressed as mean ± SEM (n = 4-6). (*$p<0.05$; **$p<0.01$; ***$p<0.001$ vs C)

3.3.13 Effect of total flavonoids on cell proliferation and alkaline phosphatase activity

To study the effect of total flavonoids on cell proliferation and differentiation, MTS and ALP assays were conducted respectively. Treatment of total flavonoids at all concentrations (0.0625 to 2μg/ml) resulted in significant increase in cell proliferation with a maximum at 0.25μg/ml (Figure 3-16A). ALP activity was significantly increased in cells treated with total flavonoids at 0.125 to 0.5μg/ml (Figure 3-16B).

(A)



(B)

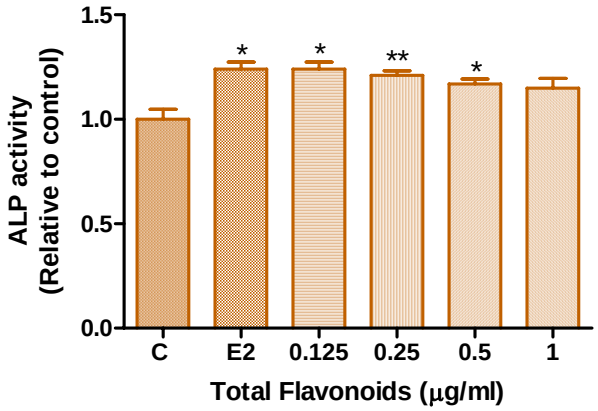


Figure 3-16: Effect of total flavonoids on (A) cell proliferation and (B) alkaline phosphatase activity.

UMR-106 cells were treated with vehicle (C) as negative control, 17β-estradiol (E2; 10^{-8}M) as positive control or total flavonoids (0.0625, 0.125, 0.25, 0.5, 1 and 2 μg/ml for MTS assay; 0.125, 0.25, 0.5 and 1 μg/ml for ALP assay) for 48 hr. The relative proliferation rate and ALP activity were determined using MTS and ALP assays previously described, and were shown in (A) and (B) respectively. Results were expressed as mean ± SEM (n = 18-24). (*$p<0.05$; **$p<0.01$; ***$p<0.001$ vs C)

3.3.14 Effect of total flavonoids on mRNA expressions of OPG and RANKL

To study the effect of total flavonoids on modulation of osteoclastogenesis, OPG/RANKL mRNA expressions were determined. Treatment with total flavonoids (0.0625 and 0.25μg/ml) significantly increased OPG/GAPDH mRNA expression to an extent higher than E2 (Figure 3-17A). RANKL/GAPDH mRNA expression was not largely altered by treatment of total flavonoids and E2 (Figure 3-17B). OPG/RANKL mRNA expression was significantly upregulated in cells treated with E2 and total flavonoids (Figure 3-17C).

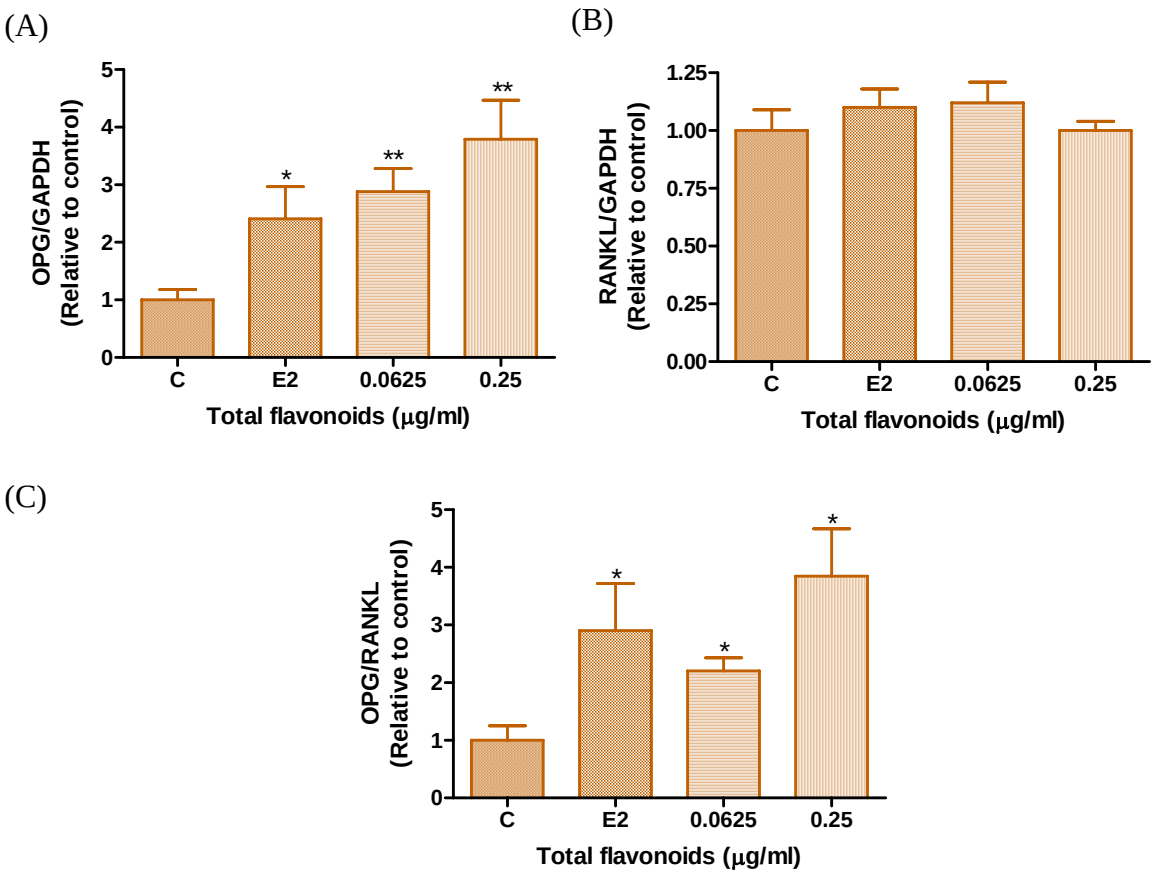


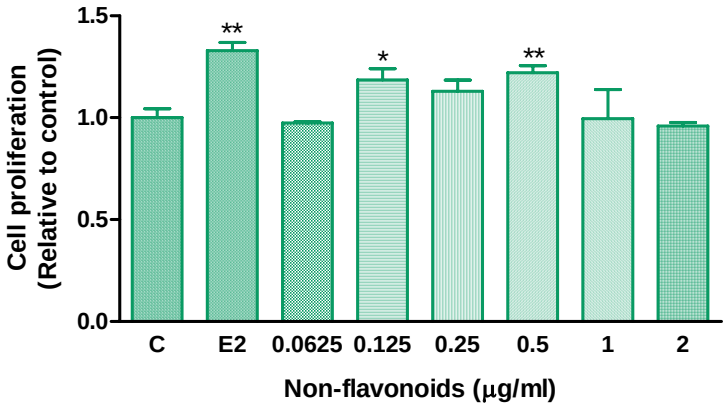
Figure 3-17: Effect of total flavonoids on mRNA expressions of (A) OPG/GAPDH, (B) RANKL/GAPDH and (C) OPG/RANKL.

UMR-106 cells were treated with vehicle (C) as negative control, 17β-estradiol (E2; 10^{-8}M) as positive control or total flavonoids (0.0625 and 0.25 μg/ml) for 48 hr. Total RNA was isolated and subjected to reverse transcription-real time-PCR. OPG and RANKL mRNA expressions were normalized with that of GAPDH, which is a house-keeping gene for internal control. mRNA expressions of OPG/GAPDH, RANKL/GAPDH and OPG/RANKL were shown in (A), (B) and (C) respectively. Results were expressed as mean ± SEM (n = 4-6). (*$p<0.05$; **$p<0.01$; ***$p<0.001$ vs C)

3.3.15 Effect of non-flavonoids on cell proliferation and alkaline phosphatase activity

To determine the effect of non-flavonoids on cell proliferation and differentiation, MTS and ALP assays were conducted respectively. As shown in Figure 3-18A, treatment with non-flavonoids at 0.125 and 0.5μg/ml caused significant increase in cell proliferation. ALP activity was significantly stimulated in cells except those treated with non-flavonoids at 0.5μg/ml (Figure 3-18B).

(A)



(B)

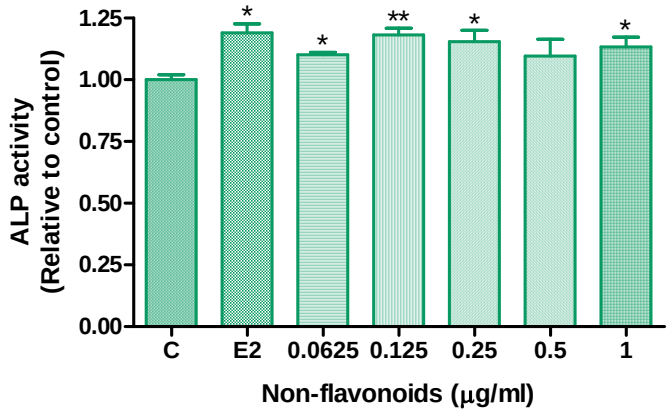


Figure 3-18: Effect of non-flavonoids on (A) cell proliferation and (B) alkaline phosphatase activity.

UMR-106 cells were treated with vehicle (C) as negative control, 17β-estradiol (E2; 10^{-8}M) as positive control or non-flavonoids (0.0625, 0.125, 0.25, 0.5, 1 and 2 μg/ml for MTS assay; 0.0625, 0.125, 0.25, 0.5 and 1 μg/ml for ALP assay) for 48 hr. Cell proliferation and ALP activity were determined using MTS and ALP assays previously described, and were shown in (A) and (B) respectively. Results were expressed as mean ± SEM (n = 18-24). (*p<0.05; **p<0.01; ***p<0.001 vs C)

3.3.16 Effect of non-flavonoids on mRNA expressions of OPG and RANKL

To investigate the effect of non-flavonoids on modulation of osteoclastogenesis, OPG/RANKL mRNA expression was determined. Cells treated with E2 and non-flavonoids at 0.25μg/ml showed significant increase in OPG/GAPDH mRNA expression (Figure 3-19A). RANKL/GAPDH mRNA expression was slightly increased in cells with E2 and non-flavonoids treatment (Figure 3-19B). OPG/RANKL mRNA expression was significantly upregulated in cells treated with non-flavonoids at 0.25μg/ml to a level lower than that of E2 (Figure 3-19C).

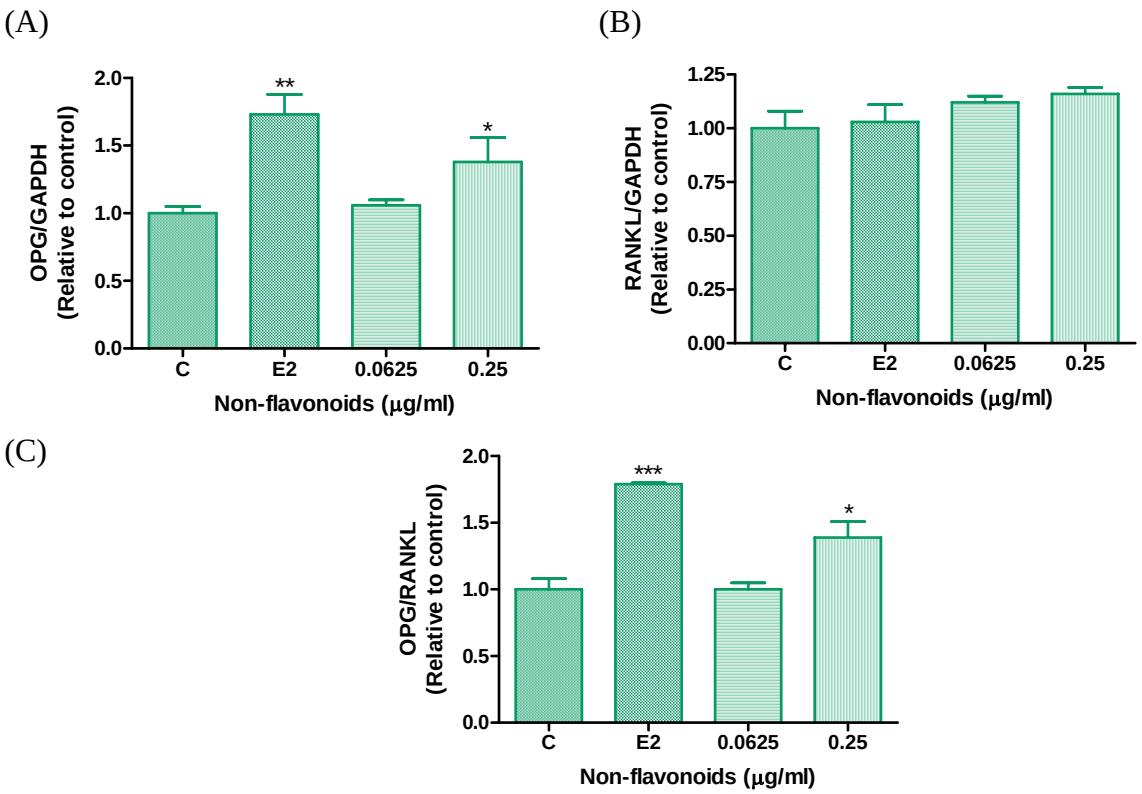


Figure 3-19: Effect of non-flavonoids on mRNA expressions of (A) OPG/GAPDH, (B) RANKL/GAPDH and (C) OPG/RANKL.

UMR-106 cells were treated with vehicle (C), 17β-estradiol (E2; 10^{-8}M) or non-flavonoids (0.0625 and 0.25 μg/ml) for 48 hr. Total RNA was isolated and subjected to reverse transcription-real time-PCR. OPG and RANKL mRNA expressions were normalized with that of GAPDH, which is a house-keeping gene for internal control. mRNA expressions of OPG/GAPDH, RANKL/GAPDH and OPG/RANKL were shown in (A), (B) and (C) respectively. Results were expressed as mean ± SEM (n = 4-6). (*$p<0.05$; **$p<0.01$; ***$p<0.001$ vs C)

3.3.17 Effect of five flavonoid compounds on cell proliferation

Flavonoid compounds are generally considered as the active ingredients in many Chinese herbs and received more attention from the researchers. From the results shown above, total flavonoids demonstrated a stronger effect on promoting cell proliferation, ALP activity and OPG/RANKL mRNA expression. Therefore five flavonoid compounds with relatively higher abundance in the total flavonoids fraction of Herba *Epimedii* were studied. To determine the effect of baohuoside I, sagittaoside B, korepimedoside C, epimedin B and sagittatoside A on cell proliferation, MTS assays were carried out. Treatment with baohuoside I (10^{-12}M) increased cell proliferation significantly (Figure 3-20A). Cells treated with sagittatoside B and korepimedoside C at all concentrations except 10^{-6}M showed significant increase in cell proliferation (Figure 3-20B and 3-20C). Treatment with epimedin B and sagittatoside A at 10^{-10}M and 10^{-8}M resulted in significant increase in cell proliferation (Figure 3-20D and 3-20E).

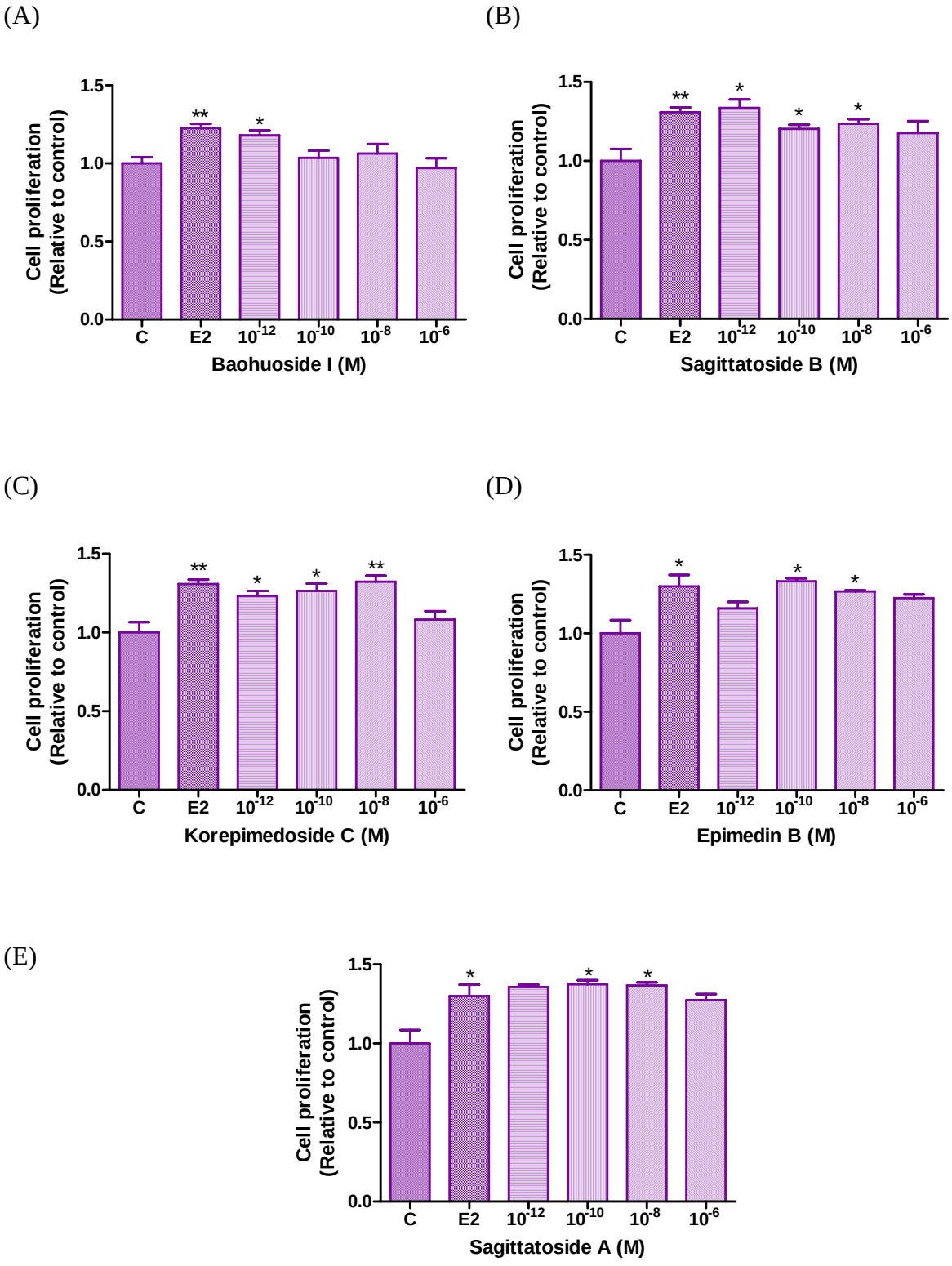


Figure 3-20: Effect of flavonoids compounds (A) Baohuoside I, (B) Sagittaoside B, (C) Korepimedoside C, (D) Epimedin B, (E) Sagittatoside A on cell proliferation.

UMR-106 cells were treated with vehicle (C), 17β-estradiol (E2; 10^{-8}M) or flavonoid compounds (10^{-12} to 10^{-6}M) for 48 hr. Cell proliferation rate of (A) Baohuoside I, (B) Sagittaoside B, (C) Korepimedoside C, (D) Epimedin B, (E) Sagittatoside A were determined by MTS assay previously described. Results were expressed as mean ± SEM (n = 18-24). (*p<0.05; **p<0.01; ***p<0.001 vs C)

3.3.18 Effect of flavonoids compounds on alkaline phosphatase activity

To determine the effect of baohuoside I, sagittaoside B, korepimedoside C, epimedin B and sagittatoside A on cell differentiation, ALP assays were carried out. Treatment with baohuoside I at 10^{-8} and 10^{-6}M increased ALP activity significantly (Figure 3-21A). ALP activity was significantly higher in cells treated with korepimedoside C at 10^{-10} and 10^{-6}M (Figure 3-21C). All treatments with sagittatoside B, epimedin B and sagittatoside A (10^{-10} to 10^{-6}M) increased ALP activity significantly (Figure 3-21B, 3-21D and 3-21E).

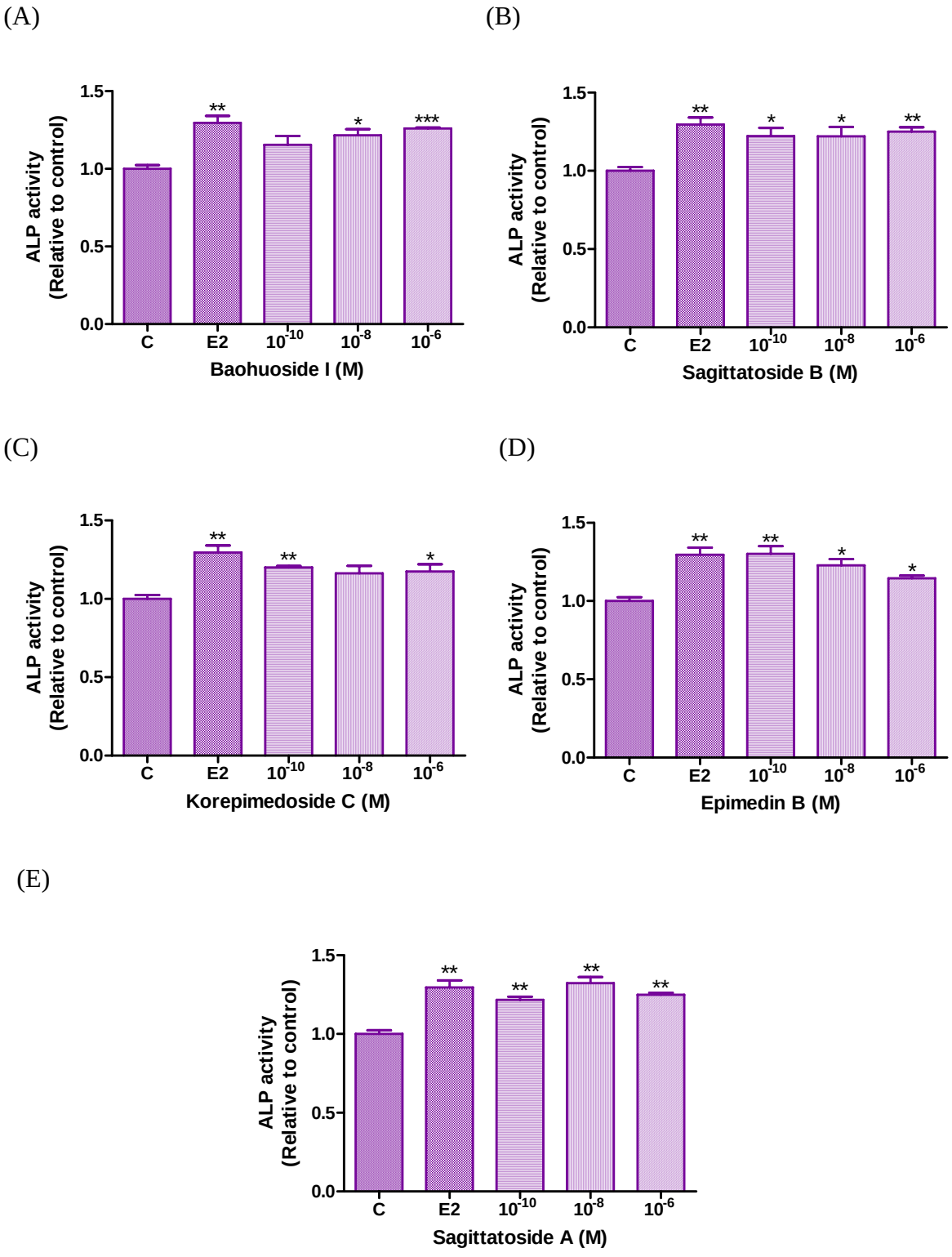


Figure 3-21: Effect of flavonoids compounds (A) Baohuoside I, (B) Sagittaoside B, (C) Korepimedoside C, (D) Epimediin, (E) Sagittatoside A on ALP activity.

UMR-106 cells were treated with vehicle (C), 17β-estradiol (E2; 10^{-8}M) or flavonoids compounds (10^{-10} to 10^{-6}M) for 48 hr. ALP activity of (A) Baohuoside I, (B) Sagittaoside B, (C) Korepimedoside C, (D) Epimedin B and (E) Sagittatoside A were determined by ALP assay previously described. Results were expressed as mean ± SEM (n = 18-24). (*p<0.05; **p<0.01; ***p<0.001 vs C)

3.4 Discussion

Estrogen deficiency is the main cause for postmenopausal osteoporosis. Characteristics associated with estrogen deficiency in ovariectomized animals include body weight gain, uterus atrophy, negative calcium balance, high bone turnover and bone loss with increased fragility. To study *in vivo* effects of three extracts of Herba *Epimedii*, these characteristics were examined accordingly.

The OVX-induced body weight gain is analogous to that in postmenopausal women, and was regarded as one of the observations for successful operations as well as osteoporotic conditions. Ovariectomy increased body weight significantly and estrogen treatment suppressed such increase in both animal studies. These results were in agreement with some previous reports (Wu J et al., 2004). It is well recognized that estrogen regulates the energy metabolism and estrogen deficiency was shown to increase appetite, food intake but decrease serum leptin and spontaneous physical activity (Ainslie DA et al., 2001). As the mice were paired-fed that the amount of food available was the same in each group, the OVX-induced weight gain might due to a decrease in daily activity instead of food intake. Treatment with total extract, total flavonoids and non-flavonoids suppressed OVX-induced weight gain slightly, which indicated that these extracts were weaker suppressors on weight gain than estrogen.

The uterus weight was significantly lowered by ovariectomy and estrogen treatment could restore it (Lee YB et al., 2004; Xie F et al., 200). All OVX mice except those treated with estradiol encountered uterus atrophy while E2 group had a similar uterus index to Sham group in both animal studies. The OVX-induced uterus atrophy was resulted from estrogen deficiency in which the thickness and weight of uterus was no longer maintained. Unlike estradiol treatment, total extract, total flavonoids and non-flavonoids could not compensate for the effect of ovariectomy, which suggesting that these extracts did not exhibit uterotrophic effect as estradiol did.

Despite statistical significance observed in some groups, serum calcium concentrations remained stable in a narrow range irrespective of ovariectomy. This might be due to the fact that calcium metabolism is tightly regulated. Increase in serum phosphorus concentrations in OVX mice treated with total flavonoids and non-flavonoids might imply a low PTH status as PTH increases phosphorus excretion and increases serum phosphorus concentration. The indirect action of estrogen on bone that is mediated by changes in PTH secretion in both experimental animals and human includes stimulation of intestinal calcium absorption and renal calcium reabsorption (Riggs BL et al., 2002). Urinary calcium was considered as an indicator of calcium absorption and

reabsorption (Zhang G et al., 2006). Ovariectomy increased urinary calcium concentration while estrogen treatment suppressed the increase. Total flavonoids treatment suppressed the OVX-induced urinary calcium elevation dose-dependently, in which lower concentration was more effective. These results suggested that total flavonoids at lower concentrations could prevent calcium loss through urinary excretion as estradiol did, which might contribute to conservation of bone mass.

It is well known that ALP facilitates mineralization by splitting inorganic phosphate from organic phosphate causing an increase in the calcium-phosphate product as well as splitting a potent mineralization inhibitor called inorganic pyrophosphate (Rudberg A et al., 1999). However the more precise function of this enzyme is still unclear. Previous reports had demonstrated that OVX led to serum ALP elevation and the increase could be prevented by estrogen treatment (Nian H et al., 2006; Lee YB et al., 2004). In the second animal study, serum ALP was increased in OVX group and significant decrease was observed in TF100 and TF400 group when compared with OVX group. This result suggested that OVX increased bone turnover due to estrogen deficiency while some total flavonoids treatments suppressed it. It was surprising to find that estradiol treatment resulted in serum ALP elevation, which was in contrary to the previous

studies. It was reported that high-dose estradiol (i.e. 71.4 μg/mouse/day) that was similar to the dosage used in our study (i.e. 4 μg/g/day) induced de novo bone formation or osteogenesis, trabecular bone gain and ALP activity elevation in female mice (Samuels A et al., 1999). Together with higher BMD, polar-SSI and uterus index in estradiol-treated mice than in sham-operated mice, it was suggested that high serum ALP might be resulted from higher bone formation rate than resorption rate.

In the first animal study, treatment with estradiol, total extract, total flavonoids and non-flavonoids increased both total and trabecular BMD significantly when compared to OVX group. Ability to increase total and trabecular BMD decreased in an order: E2 > HEP > TF > NF and E2 > NF > HEP > TF respectively. These results confirmed the beneficial effects of estradiol and three extracts of Herba *Epimedii* on restoring normal bone mass in ovariectomized mice. To screen for the active ingredient, total flavonoids rather than non-flavonoids was studied within a limited timeframe as flavonoids was of greater osteoprotective potentials basing on the literature and our *in vitro* study. In the second study, total, trabecular and cortical BMD were increased by total flavonoids treatment in distal and proximal femur and its midshaft. Change in total and trabecular BMD at the three sites investigated were more or less the

same, in which the BMD decreased with increasing concentration of total flavonoids (i.e. E2 > TF50 > TF100 > TF200 > TF400). Polar-SSI, a recently introduced indicator that reflects torsional bone strength, was shown to be directly proportional to the breaking force of long bones in cadaveric or animal studies (Horikoshi T et al., 1999). Similar to BMD, OVX group had the lowest polar-SSI while E2 group had the highest index in all three sites investigated. Polar-SSI decreased with increasing concentration of total flavonoids. Taken together, total flavonoids exerted osteoprotective effect by increasing total and trabecular BMD and polar-SSI, which reflecting higher bone strength and lower fracture risk. Lower concentrations of total flavonoids appeared to be more effective in osteoprotection.

It was observed that BMD reduction in response to ovariectomy was highest in distal end (10.6% in total BMD; 12.4% in trabecular BMD), followed by proximal end (7.9% in total BMD; 15.3% in trabecular BMD) and midshaft (4.6% in cortical BMD). The BMD increase in OVX mice in response to estradiol treatment was higher in distal end (41% in total BMD; 56% in trabecular BMD) than proximal end (17% in total BMD; 25% in trabecular BMD) and midshaft (14% in cortical BMD). These observations suggested that distal femur was more responsive to ovariectomy and treatment, which served as an

ideal site to monitor BMD for therapy evaluation. As cortical bone has a much lower remodeling rate than trabecular bone, it was reasonable to see a small BMD change in cortical bone and longer period was required for a marked difference. Unlike BMD, polar-SSI was increased to a greater extent in proximal femur instead of distal femur. As bone strength does not solely depend on BMD, other determinants like bone geometry (shape; size), microarchitecture (trabecular architecture; cortical thickness and porosity) and bone tissue quality (mineral to matrix ratio; crystal size; microdamage; type and cross-link of collagen) have to be taken into account (Felsenberg D et al., 2005). This result might be due to greater microarchitectural changes that improved bone strength in proximal femur and reduced the risk of fracture in such an important loading site.

Although total flavonoids was not as potent as estradiol in restoring normal BMD and bone strength after ovariectomy, its beneficial effects on bone could be clearly observed. A weaker potency of total flavonoids might due to the presence of some inactive compounds that counteracted the positive effects of other active compounds in the fraction. Total flavonoids might conserve bone mass through suppressing OVX-induced increase in bone turnover and urinary calcium excretion. On the whole, the optimal dose of total flavonoids was found to be

TF100. An advantage of total flavonoids over estradiol lied in the fact that total flavonoids did not induce uterotrophy while estradiol did, which implying total flavonoids was a safer and potential candidate for alternative management of osteoporosis.

Previous studies had demonstrated *Epimedium* possessed estrogenic properties, which was likely accounted for its beneficial effects against menopausal symptoms, osteoporosis and breast cancer on animal models (Zhang G et al., 2006; Naeyer AD et al., 2005; Yap SP et al., 2005; Zhang CZ et al., 2005; Zhang G et al., 2007). In order to evaluate the effects of Herba *Epimedii* and its derived compounds accurately, UMR-106 cells were incubated in phenol red-free DMEM supplemented with charcoal-stripped serum during the investigation except for routine culture and plating procedure. Phenol red, a pH indicator with similar structure to nonsteroidal estrogens, was shown to stimulate cell proliferation and protein synthesis in estrogen-responsive cells including bone cells (Berthois Y et al., 1986; Ernst M et al., 1989). Its estrogenic effects reduced the sensitivity of the assay system and partially masked the effects of the tested compounds, which led to an underestimation of the response potential and therefore should be eliminated in our experiments. Similarly, charcoal-stripped serum was used to minimize the estrogenic activity exerted by steroid hormones

present in the untreated serum.

Estrogen promotes osteoblast proliferation, differentiation and functions while it inhibits osteoclasts formation and activity at cellular level (Riggs BL et al., 2002). Basing on the evidence that 17β-estradiol acted directly on UMR-106 cells, 17β-estradiol was used as the positive control paralleled to that in the mice study. Estradiol caused a 1.2 to 1.3-fold increase in cell proliferation, which was similar to the maximum response of Herba *Epimedii* extracts. The proliferative effect of total extract and total flavonoids were stronger than that of non-flavonoids. Treatment at the highest concentration (i.e. 1μg/ml) offered the smallest promotion on cell proliferation, which suggested that high doses might induce cytotoxicity. These results suggested that total extract, total flavonoids and non-flavonoids exerted stimulatory effects on cell proliferation, which contributed to the protection against osteoporosis.

ALP activity is a widely accepted marker of osteoblast differentiation both *in vitro* and *in vivo* (Hua NA et al., 2006; Gray TK et al., 1987). Total extract, total flavonoids and non-flavonoids were able to stimulate ALP activity significantly. The effectiveness in stimulation of ALP decreased in an order: total extract > total flavonoids and estradiol > non-flavonoids. These results suggested that all three extracts offered osteoprotective effects by enhancing cell

differentiation.

As the OPG/RANKL/RANK system has shown to play a critical role in the regulation of osteoclasts and bone resorption, the ratio of OPG/RANKL represents a determinant of bone resorption. Previous studies had demonstrated the ability of estrogen to increase OPG production while its effect on RANKL expression remained either undetermined or inconsistent (Rogers A et al., 2005; Aubin JE et al., 2000; Kostenuik PJ et al., 2001; Theoleyre S et al., 2004). Total flavonoids was more effective in upregulating OPG mRNA expression than estradiol followed by total extract and non-flavonoids. It was unexpected to observe a slight increase in RANKL mRNA expression in estradiol-treated cells as estrogen decreased RANKL expression theoretically. RANKL mRNA expression increased with increasing concentrations of total extract and non-flavonoids. Similar to OPG mRNA expression, OPG/RANKL mRNA expression was upregulated in an order: total flavonoids > estradiol > non-flavonoids and total extract. These results suggested that total extract, total flavonoids and non-flavonoids exerted osteoprotective effects through suppression on osteoclastogenesis. Moreover, total extract at high concentrations downregulated OPG/RANKL mRNA expression, which suggested that desirable modulation of osteoclastogenesis occurred at lower concentrations.

On the whole, the osteoprotective effect offered by total flavonoids was stronger than total extract while that by non-flavonoids was the weakest (Table 3-3). As non-flavonoids contained less active ingredients (such as phytoestrogenic or phenolic compounds) or more compounds that arrested the osteoprotective actions of some active compounds than total flavonoids, its presence reduced the efficacy of total extract and resulted in a moderate effect between total flavonoids and non-flavonoids.

Based on the literature and the results gathered, flavonoids were believed to be the active fraction that contained more potent ingredients than other fractions. Five flavonoid compounds of higher abundance (i.e. baohuoside I, sagittatoside B, korepimedoside C, epimedin B and sagittatoside A) were isolated and studied using MTS and ALP activity assays. A summary table showing the biological effects of five flavonoid compounds was shown in Table 3-4. All treatments except baohuoside I increased cell proliferation with a similar extent to estradiol. The proliferative effect decreased with increasing concentration of baohuoside I, which suggested that baohuoside I was more effective at low concentration (e.g. 10^{-12}M). The effective range of all treatments except baohuoside I lied between the physiological concentrations (e.g. 10^{-12}M to 10^{-8}M), which suggested that these compounds were potential potent agents for promoting osteoblastic

proliferation *in vivo*. All treatments caused a 1.2 to 1.3-fold increase in ALP activity, which was slightly lower than estradiol treatment. Among all, sagittatoside A was most effective in promoting cell proliferation and differentiation, and might have a higher value for further investigations. These results suggested that all five flavonoid compounds offered beneficial effects on bone by stimulating osteoblastic proliferation and differentiation.

These flavonoid compounds were conjugated with different number and types of sugar moieties (one sugar: baohuoside I; two sugars: sagittatoside A and B; three sugars: korepimedoside C and epimedin B) but contained a common isopentenyl group at C-8 position. The presence of isopentenyl group at C-8 was demonstrated to play a crucial role in binding at estrogen receptors (Wang Z et al., 2004; Kitaoka M et al., 1998). As these compounds had a similar stimulatory effect, it was suggested that C-8 isopentenyl group might be more important than the number of sugar moieties for their activities. The steric hindrance imposed by the sugar moieties might prevent the receptor-ligand interaction, in which the location of the sugar moieties was said to be more important than the number.

Osteoblastic functions were evaluated in terms of cell proliferation, differentiation and modulation of osteoclastogenesis. It was observed that OPG/RANKL mRNA expression was a more sensitive endpoint measurement in

determining response potential of different treatments than MTS and ALP activity assays. Therefore the effectiveness of the flavonoid compounds could be more accurately compared by measuring OPG/RANKL mRNA expression.

Taken both *in vivo* and *in vitro* study together, Herba *Epimedii* was demonstrated to exert osteoprotective effects by preventing OVX-induced bone loss and promoting osteoblastic functions without observable side effects, which supported its usage as an alternative therapy for osteoporosis.

Treatment		Dose (μg/ml)				
		0.0625	0.125	0.25	0.5	1.0
Cell proliferation	HEP	1.20±0.02***	1.22±0.02***	1.24±0.01***	1.23±0.01***	1.16±0.01***
	TF	1.04±0.02*	1.21±0.04**	1.28±0.01***	1.24±0.02***	1.13±0.03**
	NF	0.97±0.01	1.19±0.06*	1.13±0.06	1.22±0.04**	1.00±0.14
ALP activity	HEP	1.04±0.04	1.22±0.06**	1.24±0.06**	1.33±0.09**	1.24±0.11*
	TF	NA	1.24±0.04*	1.21±0.02**	1.17±0.02*	1.15±0.05
	NF	1.10±0.01*	1.18±0.03**	1.16±0.05*	1.10±0.07	1.13±0.04*
OPG/RANKL mRNA expression	HEP	1.20±0.08*	1.14±0.04*	1.27±0.07**	0.58±0.13**	0.62±0.10**
	TF	2.20±0.23*	NA	3.85±0.82*	NA	NA
	NF	1.00±0.05	NA	1.39±0.12*	NA	NA

Table 3-3: A summary table showing the effects of total extract, total flavonoids and non-flavonoids on cell proliferation, ALP activity and OPG/RANKL mRNA expression.

UMR-106 cells were treated with vehicle (C), total extract (HEP), total flavonoids (TF) or non-flavonoids (NF) at designated concentrations for 48 hr. The relative rate of cell proliferation, ALP activity and OPG/RANKL mRNA expression were determined. Results were expressed in mean ± SEM. (NA: Not available; *p<0.05; **p<0.01; ***p<0.001 vs C)

Treatment		Dose (M)			
		10^{-12}	10^{-10}	10^{-8}	10^{-6}
Cell proliferation	Baohuoside I	1.18±0.03*	1.04±0.05	1.06±0.06	0.97±0.06
	Saigittatoside B	1.34±0.05*	1.20±0.03*	1.24±0.03*	1.18±0.08
	Korepimedoside C	1.23±0.03*	1.26±0.05*	1.32±0.04**	1.08±0.05
	Epimedin B	1.16±0.04	1.33±0.02*	1.27±0.01*	1.22±0.02
	Sagittatoside A	1.36±0.02	1.37±0.03*	1.37±0.02*	1.27±0.04
ALP activity	Baohuoside I	NA	1.15±0.06	1.22±0.04*	1.26±0.01***
	Saigittatoside B	NA	1.22±0.05*	1.22±0.06*	1.25±0.03**
	Korepimedoside C	NA	1.20±0.01**	1.16±0.05	1.18±0.05*
	Epimedin B	NA	1.30±0.05**	1.23±0.04*	1.15±0.02*
	Sagittatoside A	NA	1.22±0.02**	1.32±0.04**	1.25±0.01**

Table 3-4: A summary table showing the effects of five flavonoid compounds on cell proliferation and ALP activity.

UMR-106 cells were treated with vehicle (C), baohuoside I, sagittatoside B, korepimedoside C, epimedin B or sagittatoside A at designated concentrations for 48 hr. The relative rate of cell proliferation and ALP activity were determined. Results were expressed in mean ± SEM. (NA: Not available; *p<0.05; **p<0.01; ***p<0.001 vs C)

3.5 Conclusion

In the first animal study, total extract, total flavonoids and non-flavonoids of Herba *Epimedii* were shown to exert anti-osteoporotic effect in ovariectomized mice by increasing BMD. In the second study, the beneficial effects of total flavonoids demonstrated on bone included increase in BMD and bone strength as well as decrease in bone turnover and calcium loss through urine. Optimal dose of total flavonoids was found to be TF100. Absence of trophic effect on uterus made all extracts of Herba *Epimedii* advantageous to be a potential safer alternative for prevention and treatment of osteoporosis.

In vitro study suggested that total extract, total flavonoids and non-flavonoids and five flavonoid compounds (i.e. baohuoside I, sagittatoside B, korepimedoside C, epimedin B and sagittatoside A) from Herba *Epimedii* exerted osteoprotective effects by stimulating cell proliferation, differentiation and suppressing osteoclastogenesis in rat osteoblast-like UMR-106 cells. Total flavonoids and sagittatoside A had a higher efficacy than the remaining extracts and single compounds respectively.

Both *in vivo* and *in vitro* screening platforms were properly established, and evidence at bone tissue and cellular levels had supported the use of Herba *Epimedii* for management of osteoporosis.

Chapter 4

Characterization of *in vivo* and *in vitro* effects and mechanism of actions of icariin, a marker flavonoid compound in Herba *Epimedii*

4.1 Introduction

Herba *Epimedii* is one of the most effective and frequently used herbs prescribed in formulae for treatment of osteoporosis in China. Being a marker flavonoid compound of high abundance, icariin was the most popular single compound being studied in Herba *Epimedii*. Previous studies had demonstrated that icariin increased cell proliferation, ALP activity, calcium of matrix and mineral nodes, TGF-β1 mRNA expression and decreased mRNA expressions of IL-6 and TNF-α in calvaria-derived osteoblasts (Wang JQ et al., 2002; Cai ML et al., 2004; Chen H et al., 2005). Stimulatory effects of icariin on osteoblast-like cells (e.g. UMR-106 and MC3T3-E1) on cell proliferation, ALP activity and type I collagen expression had been reported (Yin XX et al., 2007; Meng FH et al., 2005; Xue Y et al., 2005). Comparatively, *in vivo* or clinical data concerning icariin were limited that only two studies were carried out in the past few decades. Bao JR et al. had demonstrated that icariin treatment (75, 150 or 225 mg/kg/day) increased BMD (femur, lumbar spine and whole body), maximum load and flexural rigidity of femur, and decreased bone turnover markers (TRAP and bone ALP) in ovariectomized rats (Bao JR et al., 2005). One 24-month clinical trial had showed that treatment with icariin in combination with daidzein and genistein (60mg: 15mg: 3mg supplemented

with 300 mg calcium per day) significantly increased BMD (femoral neck and lumbar spine) and decreased bone resorption marker (urinary DPD) in 100 late postmenopausal women (Zhang G et al., 2007). These studies suggested that icariin exerted osteoprotective effects and it was worthy to carry out further investigations.

In this chapter, *in vivo* and *in vitro* effects as well as the molecular actions of icariin were studied. Ovariectomized C57BL/6J mice and rat osteoblast-like UMR-106 cells were used as *in vivo* and *in vitro* models respectively, which were suitable models for studying osteoprotective effects of icariin. *In vivo* effects were evaluated by BMD, a surrogate marker of bone strength (polar-SSI), serum and urinary calcium and phosphorus, whereas *in vitro* effects were assessed by relative rate of cell proliferation, differentiation (ALP activity) and suppression on osteoclastogenesis (OPG/RANKL mRNA expression). These endpoints were good parameters for outcome evaluation. As animal study was more time, resources and labor-demanding than *in vitro* study, a single dose instead of several doses was used to verify the efficacy of icariin. In this way, however, the dose-dependent effects of icariin on bone loss prevention could not be observed.

Since there was scarce evidence on the molecular actions of icariin, it was

of particular interest to characterize its actions in UMR-106 cells. Herba *Epimedii* was described to be estrogenic and exerted osteoprotective effects comparable to estradiol, icariin was thereby suspected as a phytoestrogen. To investigate the molecular actions of icariin, we first determine the importance of ER for any beneficial effects on osteoblastic functions exerted by icariin, and then determine if its actions were mediated by activation of ER. This study was probably one of the pioneering studies on icariin, which demonstrating both efficacy and mechanism of action.

4.2 Materials and Methods

4.2.1 Animal study

A total of 32 female C57BL/6J mice were purchased from Laboratory Animal Services Centre (the Chinese University of Hong Kong, HK). They were placed in cages in a room providing alternating 12 hr light/dark period with temperature of 22±2 ˚C and humidity of 55±10 %, and were allowed to consume distilled water and normal diet (0.6 % Ca, 0.35 % P) *ad libitum*. The diet was purchased from Harlan Teklad (Madison, WI) and the composition was shown in Table 3-1. They were randomly separated into 4 groups including Sham+vehicle (Sham, N=8), OVX+vehicle (OVX, N=8), OVX+17β-estradiol (E2, N=8) and OVX+icariin (Icariin, N=8). When they were 1 month-old, they were either ovariectomized or sham-operated. After 18-day recovery from surgery, they were orally administered with vehicle, 17β-estradiol (4 μg/g/day) and icariin (0.3 mg/g/day) for six weeks (Figure 4-1). The dosages were determined on the basis of other animal studies (Bao JR et al., 2005). Icariin was obtained from Prof. Yao of Shenyang Pharmaceutical University.

After treatment, collection of serum and urine samples, biochemical assays and pQCT analysis were conducted in the same way as described in Chapter 3.

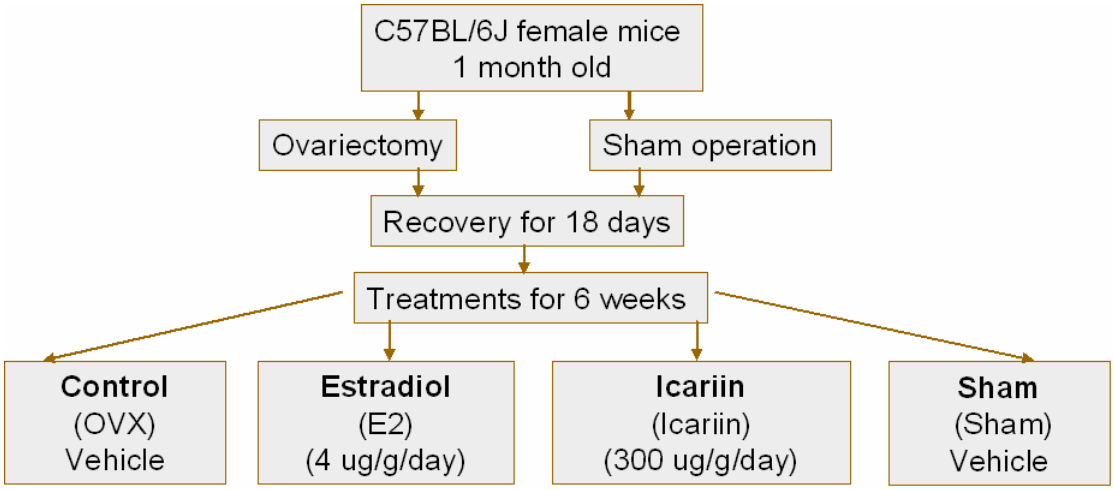


Figure 4-1: A schematic diagram of experimental design.

4.2.2 Culture of rat osteoblast-like cell line (UMR-106)

UMR-106 cells (ATCC no. CRL-1661) were routinely cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10 % fetal bovine serum (FBS), penicillin 100 U/ml and streptomycin 100 μg/ml under the conditions of 37 C, 95 % air and 5 % CO_2. Fresh medium replenishment was carried out every three days. At 80-90 % confluence, cells were seeded in 96-well and 12-well plate at a density of 2,500 and 65,000 cells per well respectively for different assays. After 48 hr, the medium was switched to phenol red-free DMEM supplemented with 1 % dextran-charcoal-stripped serum (sFBS) for 24 hr. Cells were then treated with vehicle, 17β-estradial (10^{-8}M), HEP extract or icariin (10^{-8}M) for 48 hr. DMEM, FBS and penicillin-streptomycin-glutamine were purchased from Life Technologies Inc. (Carlsbad, CA, USA). 17β-estradial and icariin was purchased from Sigma-Aldrich (St. Louis, MO, USA) and LKT Laboratory (USA) respectively.

4.2.3 Cell proliferation assay

UMR-106 cells were seeded in a 96-well plate at a density of 2,500 cells per well. After 48 hr, the medium was switched to phenol red-free DMEM supplemented with 1 % dextran-charcoal-stripped serum (sFBS) for 24 hr. Cells

were then treated with vehicle, 17β-estradial (10^{-8}M) or icariin (10^{-12} to 10^{-6}M) in the presence or absence of ICI 182,780 (10^{-8}M) for 48 hr (Figure 4-2). The MTS assay was used as an indirect colorimetric measurement of cell proliferation. Briefly the medium was discarded and replaced with 100 μl of MTS/PMS solution (Promega, Madison, WI, USA). The plate was incubated at 37 ˚C for 1 hr followed by measurement at a wavelength of 490 nm using a microplate reader (Bio-Rad Laboratories Inc., CA). Dehydrogenases found in metabolically active cells reduce MTS into aqueous soluble formazan, and production of formazan is directly proportional to the number of viable cells.

4.2.4 Alkaline phosphatase activity assay

UMR-106 cells were seeded in a 96-well plate at a density of 2,500 cells per well. After 48 hr, the medium was switched to phenol red-free DMEM supplemented with 1 % dextran-charcoal-stripped serum (sFBS) for 24 hr. Cells were then treated with vehicle, 17β-estradial (10^{-8}M) or icariin (10^{-12} to 10^{-6}M) in the presence or absence of ICI 182,780 (10^{-8}M) for 48 hr (Figure 4-2). After treatment, cells were rinsed twice with ice-cold phosphate buffered saline (PBS) and lysed by placing the microplate in -80˚C refrigerator for 15 min. After removing from refrigerator, 100μl 10mM ALP reagent (Sigma-Aldrich, St.

Louis, MO, USA) was added to each well followed by incubation at 37˚C for 30 min. Absorbance at 405nm was measured using a microplate reader (Bio-Rad Laboratories Inc., CA). The ALP in the sample hydrolysed p-nitrophenylphosphate in the reagent to p-nitrophenol, changing the color from colorless to yellow. To normalize the result, Bradford protein assay was carried out.

4.2.5 Reverse transcription-Real time-PCR

After treatment, cells were rinsed twice with ice-cold PBS and lysed with 1 ml Trizol (Life Technologies Inc., Carlsbad, CA, USA). Total RNA was collected according to the manufacturer's instructions. Before reverse transcription, agarose gel electrophoresis was conducted to check for the integrity of RNA and two distinct (28S and 18S) bands should be observed. 2 μg total RNA, 0.5 μg oligo-dT(12-18) primers, 10 mM dNTPs, PCR buffer and Superscript II reverse transcriptase (Life Technologies Inc., Carlsbad, CA, USA) were used to generate the complementary DNA. The cDNA was diluted to 1/10 with DEPC-treated water and 5 μl was used for the quantitative real time-PCR. The mRNA expressions of OPG, RANKL and GAPDH were investigated. The primers targeting genes of OPG, RANKL and GAPDH were shown in Table 3-2.

The reaction mixture containing diluted cDNA, primers, and iQ SYBR Green Supermix (Bio-Rad Laboratories, CA) were subjected to PCR consisting initial denaturation at 95 ˚C for 180 sec and 40 cycles of denaturation at 95 ˚C for 12 sec, primer annealing for 12 sec, DNA polymerization at 72 ˚C for 18 sec. Agarose gel electrophoresis was performed to check for the sizes of the PCR products and the expected sizes were shown in Table 3-2. A relative standard curve was prepared by using a series of dilution of vehicle-treated cDNA to quantify the concentration of PCR products. GAPDH is a house-keeping gene that serves as a reference for result normalization. The result was expressed as a ratio of the target/reference mRNA expression.

4.2.6 Transient transfection and Estrogen receptors (ER) mediated luciferase activity assay

Cells were seeded in a 12-well plate at a density of 65,000 cells per well. The medium was changed to phenol red-free DMEM supplemented with 1 % dextran-charcoal-stripped serum for 48 hr before transient transfection. During transfection, cells were incubated with 0.4 μg ER-α or ER-β plasmid, 0.4 μg ERE-containing luciferase reporter vector (obtained from DR. Vincent Giguere of the McGill University), 0.1 μg pRL-TK internal control reporter vector

(Promega, Madison, WI, USA) which had complexed with lipofectamine (Promega, Madison, WI, USA) in phenol red-free DMEM without antibiotics and serum for 5 hr. The constructs of ER-α, ER-β, ERE-luciferase reporter and pRL-TK vectors were shown in Figure 4-3. Cells were then treated with vehicle, ICI 182,780 (10^{-8}M), 17β-estradial (10^{-8}M), icariin (10^{-8}M) or HEP at 0.25 μg/ml for 24 hr. After treatment, luciferase activity was measured using the Dual Luciferase Reporter assay System (Promega, Madison, WI, USA) and the signal was detected by TD-20/20 Luminometer (Turner Design, Sunnyvale, CA, USA). The estrogen promoter activity was expressed as firefly luminescence normalized with Renilla luminescence.

4.2.7 Immunoblotting

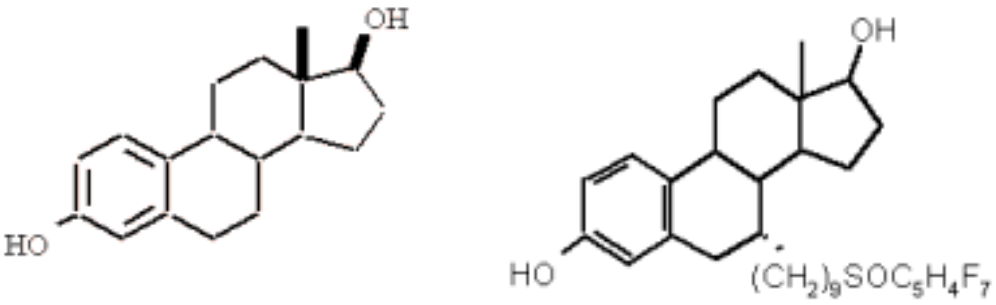
After treatment, cells were rinsed twice with ice-cold PBS and lysed with Nonidet P-40 lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM CaCl2, 1 mM MgCl2, 10 % glycerol, 1 % Nonidet P-40) supplemented with protease inhibitors (2 ug/ml aprotinin, 2 ug/ml leupeptin and 1 mM PMSF) and phosphatase inhibitors (1 mM sodium orthovanadate, 10 mM NaF). Protein concentrations of the cell lysates were determined using Bradford assay. Equal amount of proteins were separated by SDS-PAGE on a 10% reducing gel at a

constant voltage (i.e. 200V) for about 1 hr, and were transblotted onto a PVDF membrane (Immobilin-P, Millipore Corp., Ma, U.S.A.) at 100V for 1.5 hr at 4˚C. Immuno-detection was performed after blocking the non-specific binding sites on the membrane with 5% skimmed milk TTBS solution. The membrane was probed with the primary antibodies such as monoclonal rabbit anti-human phospho-ERα at serine 118 residue (1:2000; Upstate) or anti-human ERα (1:3000; Sigma, St. Louis, MO, U.S.A.), and was then probed with the corresponding secondary antibodies such as horseradish peroxidase-conjugated anti-rabbit antibodies (1:2000; Santa Cruz Biotechnology, Inc., CA) after TTBS washing. The antigen-antibody complexes were detected with enhanced chemiluminescence (ECL) reagent and visualized by the Lumi-Imager using Lumi Analyst version 3.10 software (Roche, Mannheim, Germany).

4.2.8 Statistical analysis

Results were reported as Mean ± SEM. The non-paired student's t test was used to calculate statistical significance between the control group and each treatment group. A p-value < 0.05 was considered statistically significant.

(A) (B)



(C) (D)

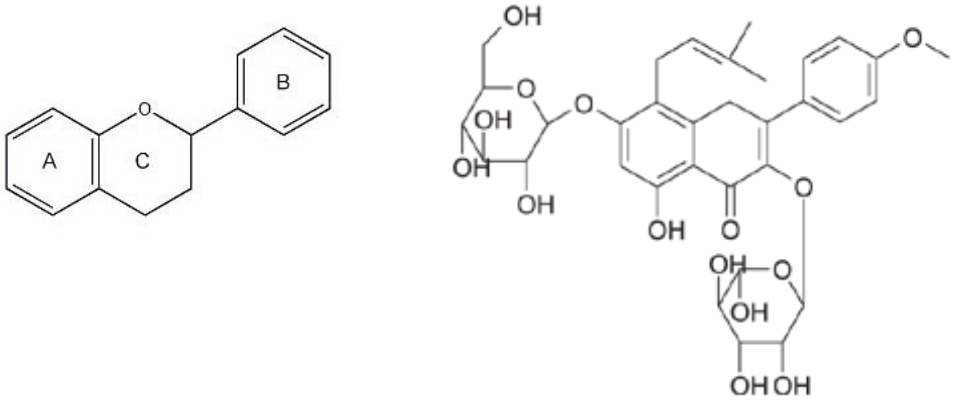
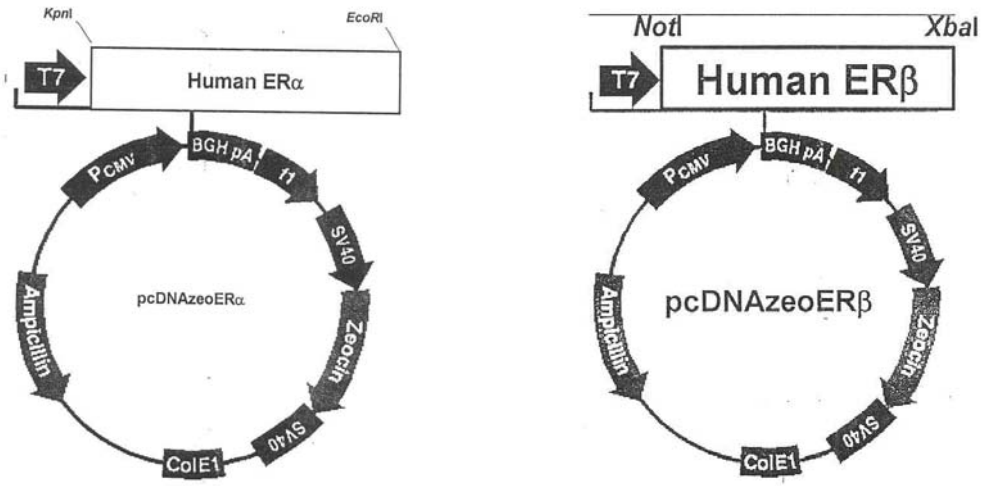


Figure 4-2: Chemical structures of (A) 17β-estradiol, (B) ICI 182,780, (C) flavonoid backbone and (D) icariin.

(A) (B)



(C) (D)

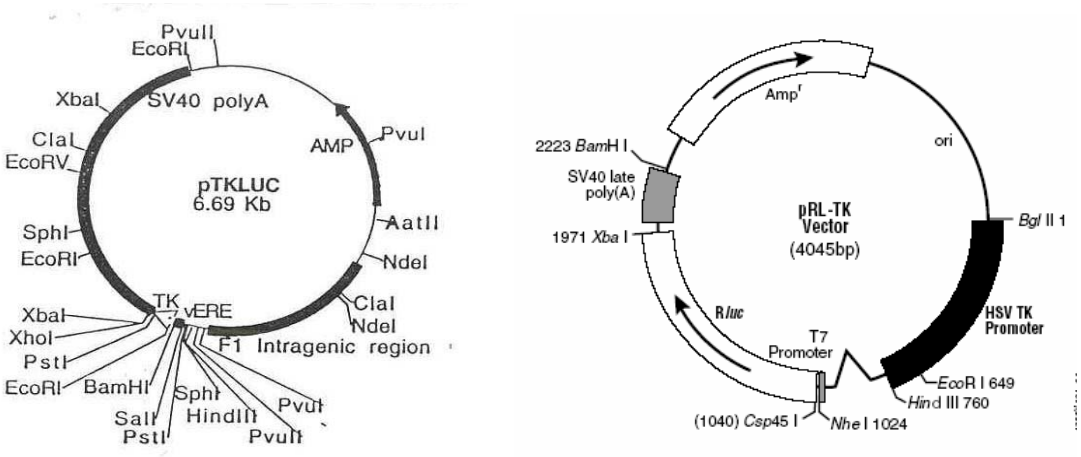


Figure 4-3: The circle maps of (A) ERα plasmid, (B) ERβ plasmid, (C) ERE-containing luciferase reporter vector and (D) pRL-TK vector.

4.3 Results

In this section, *in vivo* study was introduced first and was followed by *in vitro* and mechanistic study. The animal study was conducted to confirm the efficacy of icariin and a single dose was used due to time and resources limitations. For *in vitro* study, effects of icariin at different physiological concentrations were examined. Based on the *in vitro* results, an optimal dose of icariin (i.e. 10^{-8}M) and total extract (i.e. 0.25 μg/ml) of Herba *Epimedii* was determined and used in the mechanistic study.

4.3.1 Effect of icariin on body weight

During the study, mice were paired-fed and body weight was recorded weekly. Percentage change in body weight was calculated using the equation: (final body weight - initial body weight) / initial body weight x 100%. In Figure 4-4, ovariectomy resulted in significant body weight gain in mice when compared with sham operation. Estradiol treatment prevented OVX-induced weight gain significantly while icariin caused a slight decrease in body weight change.

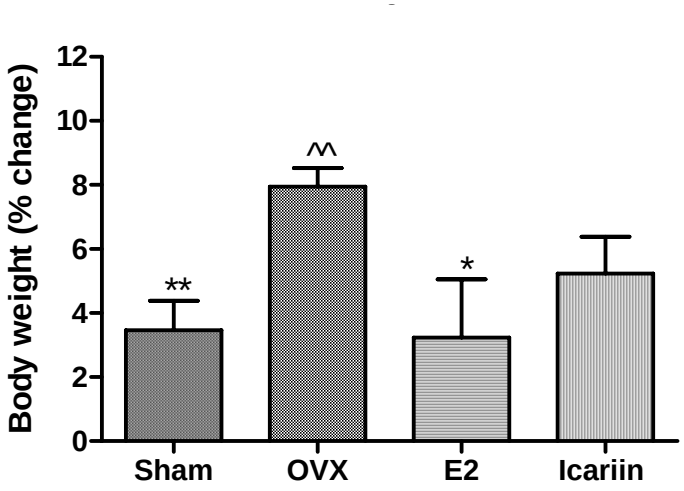


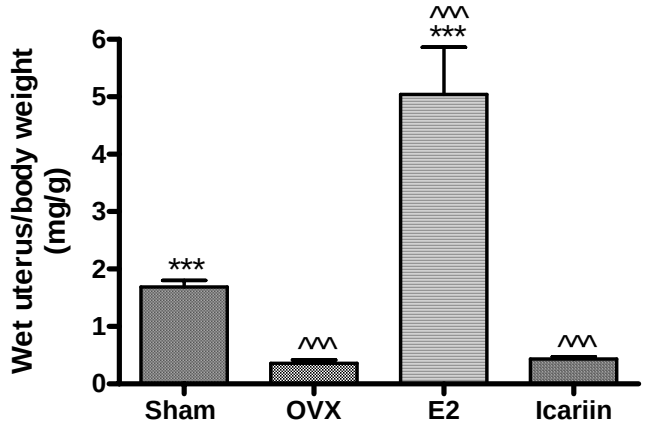
Figure 4-4: Effect of icariin on body weight.

Mice of 1 month old were ovariectomized or sham-operated. After 18-day recovery, vehicle (Sham or OVX), 17β-estradiol (E2 4 μg/g/day) or icariin (Icariin 0.3 mg/g/day) were orally administered for six weeks. During the study, body weight was measured weekly and percentage change in body weight was calculated. Results were expressed as mean ± SEM (n = 6-9). (*$p<0.05$; **$p<0.01$ vs OVX; ^^$p<0.01$ vs Sham)

4.3.2 Effect of icariin on uterus index

To study if icariin exerted trophic effect on uterus, the uterus to body weight ratio was determined. Uterus index calculated from wet and dry uterus weight were shown in Figure 4-5A and 4-5B respectively. In both figures, uterus indices of OVX and icariin group were significantly lower than Sham group. E2 treatment caused a significant increase in uterus index when compared with Sham and OVX group. These results indicated that icariin did not exert trophic effect on uterus.

(A)



(B)

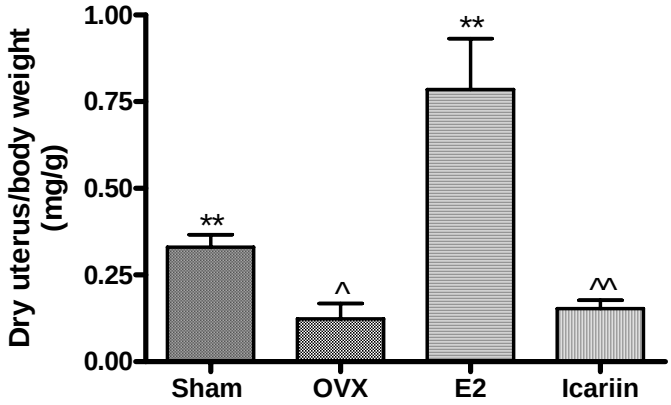


Figure 4-5: Effect of icariin on uterus index calculated from (A) wet and (B) dry uterus weight.

After administration of vehicle (Sham or OVX), 17β-estradiol (E2; 4 μg/g/day) or icariin (Icariin; 0.3 mg/g/day) for six weeks, body weight and uterus weight (wet and dry) were measured. The uterus index was calculated as the ratio of uterus to final body weight. Wet uterus/body weight and dry uterus/body weight were shown in (A) and (B) respectively. Results were expressed as mean ± SEM (n = 6-9). (**p<0.01; ***p<0.001 vs OVX; ^p<0.05; ^^p<0.01; ^^^p<0.001 vs Sham)

4.3.3 Effect of icariin on serum and urinary calcium and phosphorus concentrations

To study if icariin altered calcium and phosphorus homeostasis, serum calcium and phosphorus concentrations were measured. As shown in Figure 4-6A, icariin treatment caused a slight but significant decrease in serum calcium concentration when compared with OVX group. Serum phosphorus concentrations were not significantly changed among all groups (Figure 4-6B).

To study if icariin prevented ovariectomy-induced increase in calcium excretion, 24 hr-urine samples were collected. Urinary calcium and phosphorus to creatinine ratios were determined. As shown in Figure 4-6C, urinary Ca/Cr was higher in OVX group than Sham group. Treatment with estradiol and icariin resulted in significant decrease in urinary Ca/Cr when compared with OVX group. Urinary phosphorus to creatinine ratio was significantly lowered in icariin-treated mice when compared to sham-operated mice, while the ratio was not significantly altered in the remaining groups (Figure 4-6D). This result suggested that icariin prevented calcium loss through excretion in ovariectomized mice.

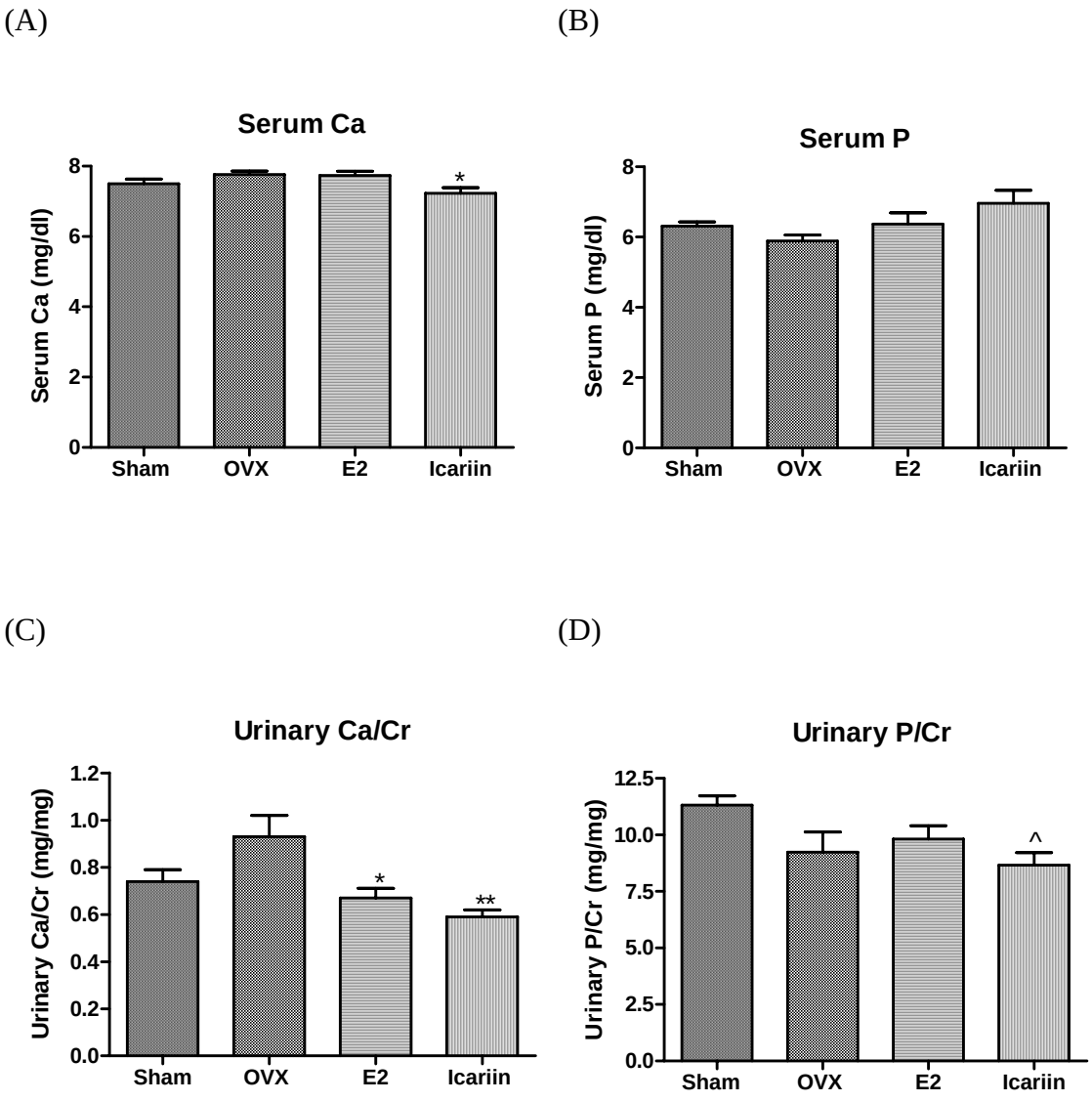


Figure 4-6: Effect of icariin on (A) serum calcium, (B) serum phosphorus, (C) urinary calcium and (D) urinary phosphorus levels.

Mice were treated with vehicle (Sham or OVX), 17β-estradiol (E2; 4 μg/g/day) or icariin (Icariin; 0.3 mg/g/day) for six weeks. Before sacrifice serum was collected from orbital venous sinus of mice. Calcium and phosphorus concentrations were

determined by colorimetric methods described previously. Serum calcium and phosphorus concentrations were shown in (A) and (B) respectively. The day before sacrifice, mice were placed in metabolic cages for 24 hr and urine was collected. Urinary calcium and phosphorus concentrations were normalized with creatinine and expressed as urinary Ca/Cr and P/Cr that were shown in (C) and (D) respectively. Results were expressed as mean ± SEM (n = 6-9). (*$p<0.05$; **$p<0.01$ vs OVX; ^$p<0.05$ vs Sham)

4.3.4 Effect of icariin on serum alkaline phosphatase

To determine if icariin could suppress ovariectomy-induced increase in serum ALP concentrations, serum ALP activity was calculated. As shown in Figure 4-7, OVX group had a significantly higher serum ALP activity than Sham group. Mice treated with icariin had significantly lower serum ALP activity when compared with OVX group. It was unexpected that serum ALP activity was the highest in E2 group. These results suggested that icariin prevented OVX-induced increase in bone turnover.

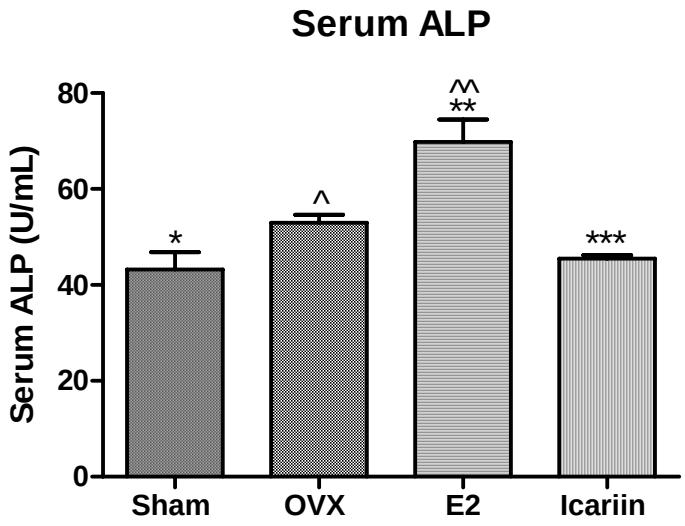


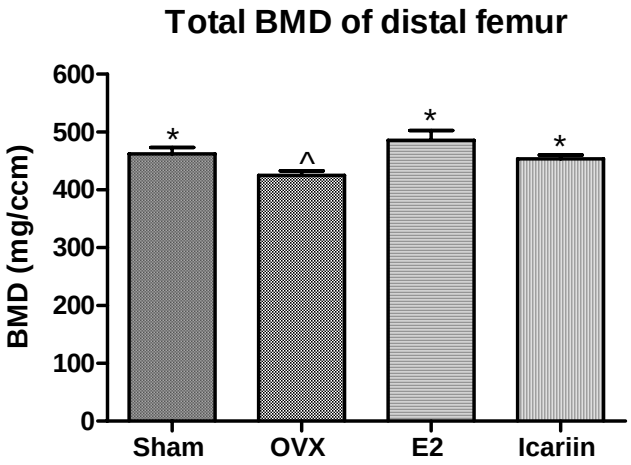
Figure 4-7: Effect of icariin on serum ALP.

After 6-week treatment with vehicle (Sham or OVX), 17β-estradiol (E2; 4 μg/g/day), or icariin (Icariin; 300 μg/g/day), mice were sacrificed. Serum was collected from orbital venous sinus of mice and serum ALP was measured. Results were expressed as mean ± SEM (n = 6-9). (*p<0.05; **<0.01; ***p<0.001 vs OVX; ^p<0.05; ^^p<0.01 vs Sham)

4.3.5 Effect of icariin on total and trabecular BMD of distal femur analyzed by pQCT

As trabecular bone has a higher bone turnover rate and responsiveness than cortical bone in response to treatments, total and trabecular bone mineral density of femur distal end were determined. As shown in Figure 4-8A and 4-8B, total and trabecular BMD was significantly decreased in OVX group when compared to Sham group. Both total and trabecular BMD were restored to a level similar to Sham group in Icariin group. These results suggested that icariin exerted a beneficial effect on suppressing ovariectomy-induced bone loss.

(A)



(B)

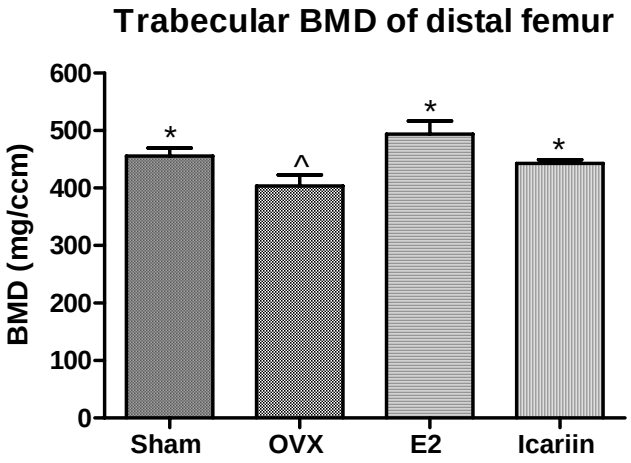


Figure 4-8: Effect of icariin on (A) total and (B) trabecular BMD distal end of femur analyzed by pQCT.

After treatment with vehicle (Sham or OVX), 17β-estradiol (E2; 4 μg/g/day) or icariin (Icariin; 0.3 mg/g/day) for six weeks, femurs were collected at sacrifice. Distal femurs were subjected to pQCT analysis and (A) total and (B) trabecular BMD were determined. Results were expressed as mean ± SEM (n = 6-9). (*$p<0.05$ vs OVX; ^$p<0.05$ vs Sham)

4.3.6 Effect of icariin on polar-SSI of distal femur analyzed by pQCT

The SSI is calculated from the BMD value based on the geometric values for the bone and is used as an indicator to assess bone fragility as well as BMD (Fukuda S et al., 2004). As shown in Figure 4-9, polar-SSI of distal femur was lowest in OVX group and highest in Sham group. Estradiol and icariin treatment significantly increased polar-SSI when compared with OVX group. This result suggested that icariin treatment could prevent the OVX-induced decrease in bone strength.

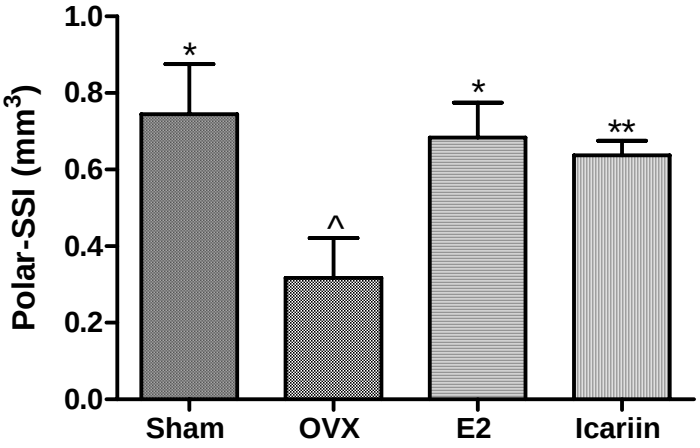


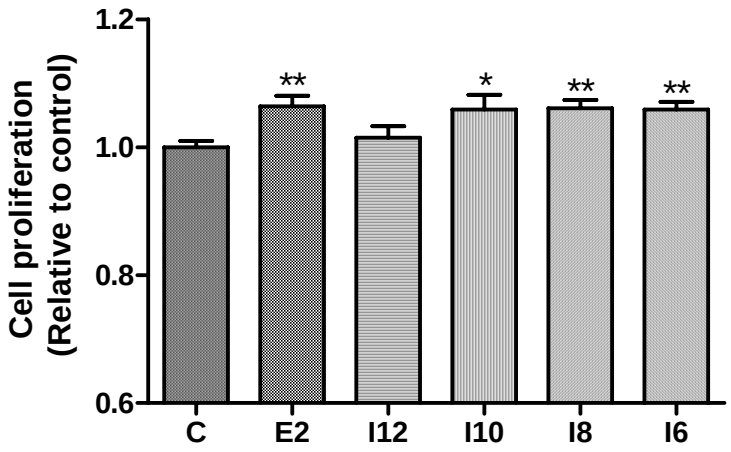
Figure 4-9: Effect of icariin on polar-SSI of distal femur analyzed by pQCT.

After treatment with vehicle (Sham or OVX), 17β-estradiol (E2; 4 μg/g/day) or icariin (Icariin; 0.3 mg/g/day) for six weeks, femurs were collected at sacrifice. Distal femurs were subjected to pQCT analysis and polar-SSI was determined. Results were expressed as mean ± SEM (n = 6-9). (*$p<0.05$; **$p<0.01$ vs OVX; ^$p<0.05$ vs Sham)

4.3.7 *In vitro* effect of icariin on cell proliferation and ALP activity

After confirming *in vivo* effect of icariin on bone health, *in vitro* effect on cell proliferation and differentiation in UMR-106 cells were determined by MTS and ALP activity assays. As shown in Figure 4-10A, cell proliferation was significantly stimulated in cells treated with E2 and icariin (10^{-10} to 10^{-6}M). All treatments except icariin at 10^{-6}M showed significant increase in ALP activity (Figure 4-10B). These results suggested that icariin exerted promoting effect on cell proliferation and differentiation.

(A)



(B)

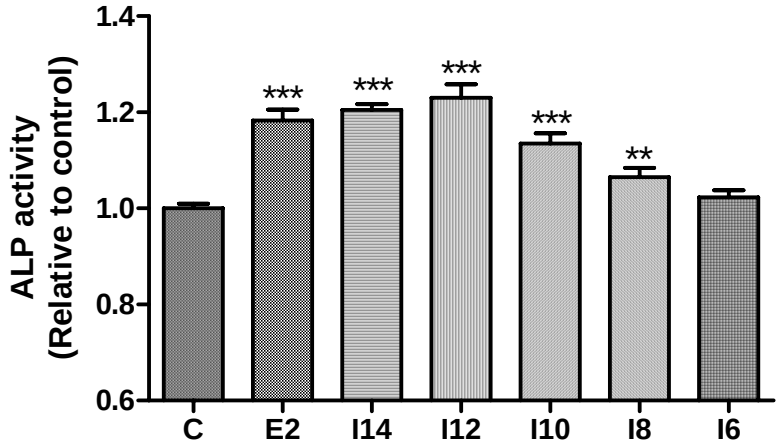


Figure 4-10: Effect of icariin on (A) cell proliferation and (B) alkaline phosphatase activity.

UMR-106 cells were then treated with vehicle (C) as negative control, 17β-estradiol (E2; 10^{-8}M) as positive control or icariin (I12 to I6; 10^{-12} to 10^{-6}M for MTS assay; I14 to I6; 10^{-14} to 10^{-6}M for ALP assay) for 48 hr. (A) Cell proliferation and (B) ALP activity were determined using MTS and ALP assays respectively. Results were expressed as mean ± SEM (n = 18-24). (*$p<0.05$; **$p<0.01$; ***$p<0.001$ vs C)

4.3.8 *In vitro* effect of icariin on modulation of osteoclastogenesis

To study the effect of icariin on modulation of osteoclastogenesis, OPG/RANKL mRNA expression was determined. OPG/GAPDH mRNA expression was significantly upregulated in E2 and icariin-treated cells (Figure 4-11A). RANKL/GAPDH mRNA expression increased with increasing concentration of icariin and statistical significance was observed cells treated with 10^{-8} and 10^{-6}M (Figure 4-11B). E2 and icariin treatment at all concentrations showed significant upregulation of about 2-fold in OPG/RANKL mRNA expression (Figure 4-11C). These results suggested that icariin suppressed osteoclastogenesis.

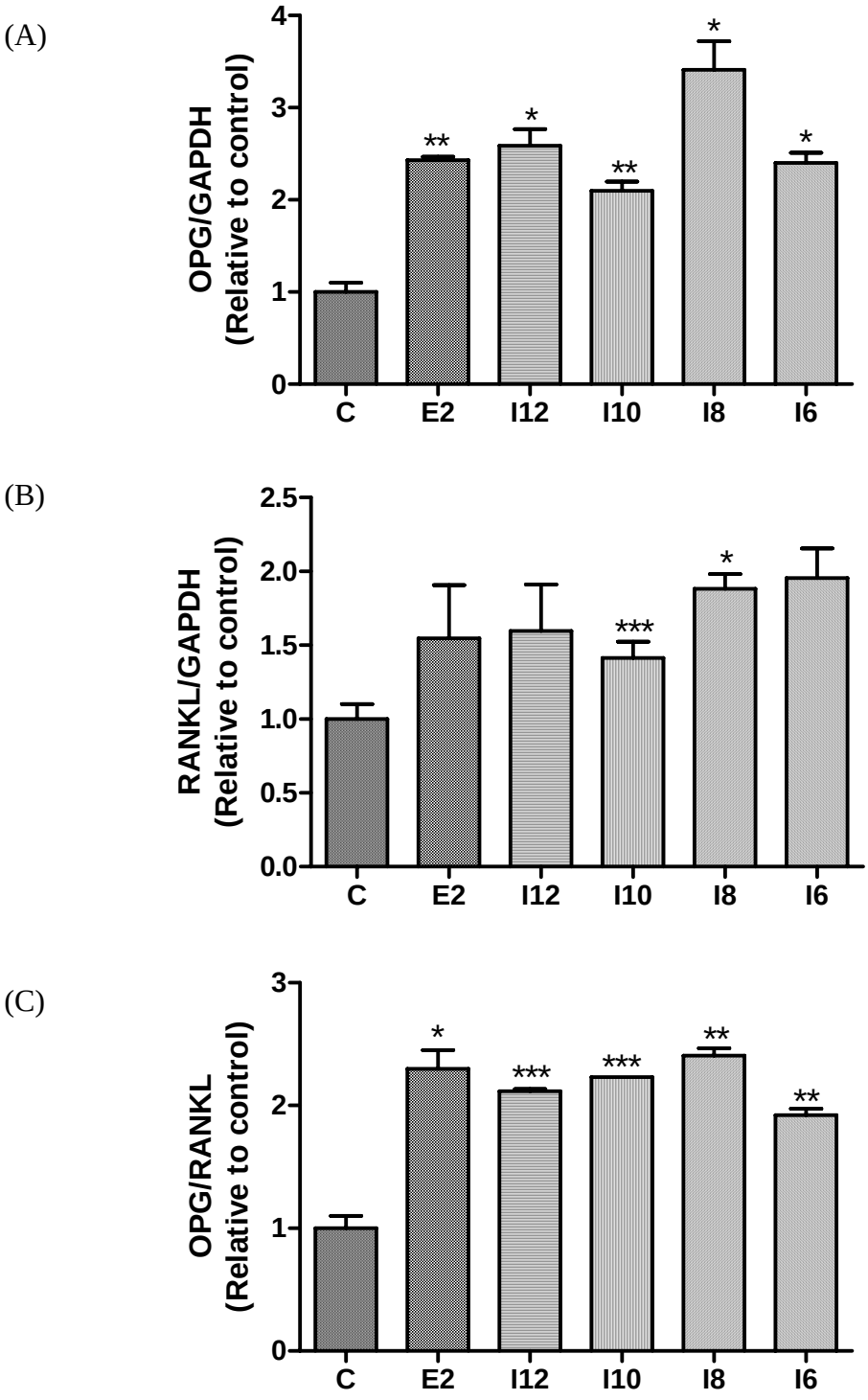


Figure 4-11: Effect of icariin on mRNA expressions of (A) OPG/GAPDH, (B) RANKL/GAPDH and (C) OPG/RANKL.

UMR-106 cells were treated with vehicle (C) as negative control, 17β-estradiol

(E2; 10^{-8}M) as positive control or icariin (I12 to I6; 10^{-12} to 10^{-6}M) for 48 hr. Total RNA was isolated and subjected to reverse transcription real time-PCR. OPG and RANKL mRNA expressions were normalized with that of GAPDH, which is a house-keeping gene for internal control. mRNA expressions of OPG/GAPDH, RANKL/GAPDH and OPG/RANKL were shown in (A), (B) and (C) respectively. Results were expressed as mean ± SEM (n = 4-6). (*$p<0.05$; **$p<0.01$; ***$p<0.001$ vs C)

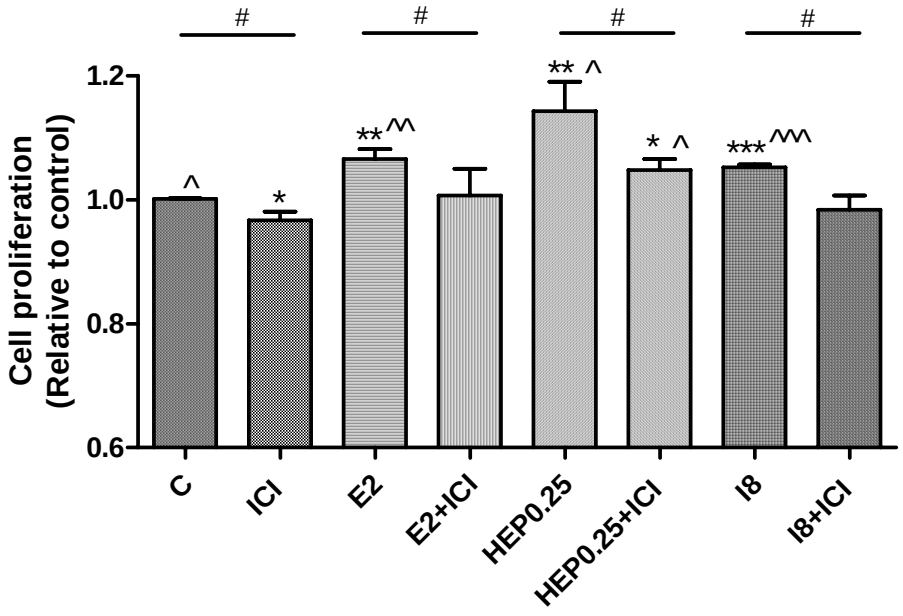
4.3.9 Effect of ER antagonist on stimulatory effect of icariin and total extract of Herba *Epimedii* on cell proliferation and ALP activity

From results shown above, effect of HEP and icariin were similar to that of 17β-estradiol. As 17β-estradiol exerted its effect through activation of estrogen receptors (ER), it was hypothesized that actions of HEP and icariin involved ER. Based on the experimental results, the optimal dose of icariin and HEP were found to be 10^{-8}M and 0.25 μg/ml respectively, which were used in the mechanistic study. To test this hypothesis, an ER antagonist called ICI 182,780 (ICI) was co-treated with HEP or icariin to see if their actions were inhibited.

Cell proliferation was significantly decreased in cells treated with ICI 182,780. In the presence of ICI 182,780, stimulatory effects of E2, icariin and HEP on cell proliferation were inhibited as statistical difference was observed between each treatment +/- ICI 182,780 (Figure 4-12A). In Figure 4-12B, ICI 182,780 treatment alone caused a slight decrease in ALP activity. No statistical difference in ALP activity was observed between each treatment in the presence and absence of ICI 182,780. These results suggested that ER was required for the actions of E2, icariin and HEP for promoting cell proliferation but not ALP activity. The actions of E2, icariin and HEP might involve ER-independent

mechanism as their stimulatory effects on ALP activity were not abolished by ICI 182,780 treatment.

(A)



(B)

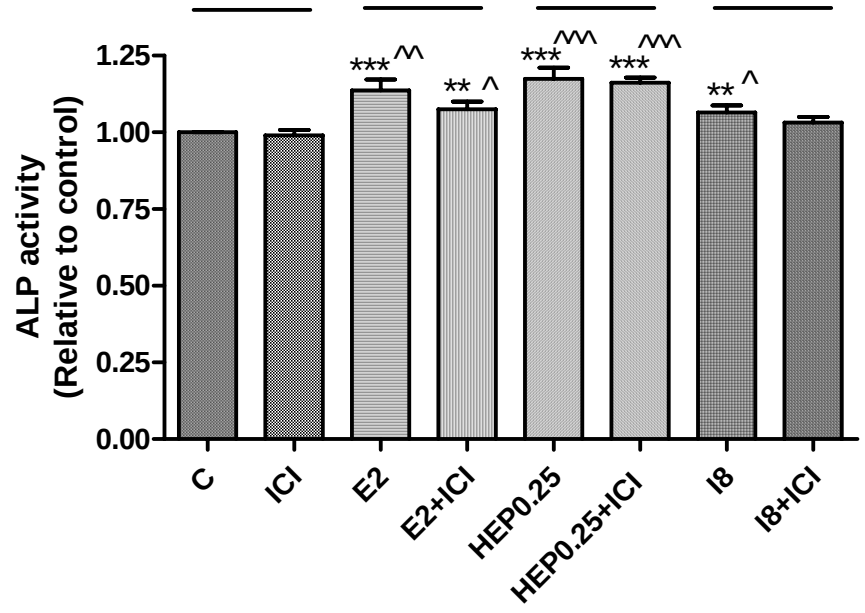


Figure 4-12: Effect of ICI 182,780 on the stimulatory effect of 17β-estradiol, HEP extract and icariin on UMR-106 (A) cell proliferation and (B) ALP activity.

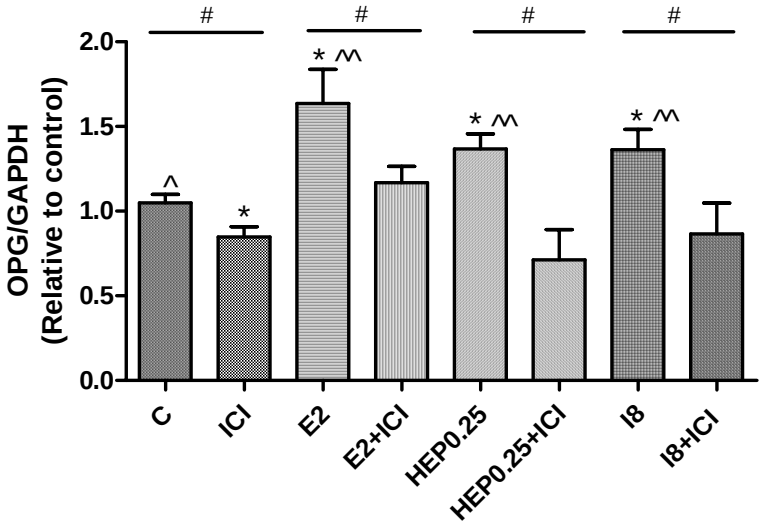
Cells were treated with vehicle (C), 17β-estradiol (E8; 10^{-8}M), HEP (0.25 μg/ml)

or icariin (I8; 10^{-8}M) in the presence or absence of ICI 182,780 (ICI; 10^{-6}M) for 48 hr. ICI 182,780 is a pure non-selective ER antagonist. Effect of E2, HEP and icariin with or without ICI 182,780 co-treatment on cell proliferation and ALP activity were shown in (A) and (B) respectively. Results were expressed as mean ± SEM (n = 18-24). (*p<0.05; **p<0.01; ***p<0.001 vs C; ^p<0.05; ^^p<0.01; ^^^p<0.001 vs ICI; # indicated a difference between each treatment with or without ICI)

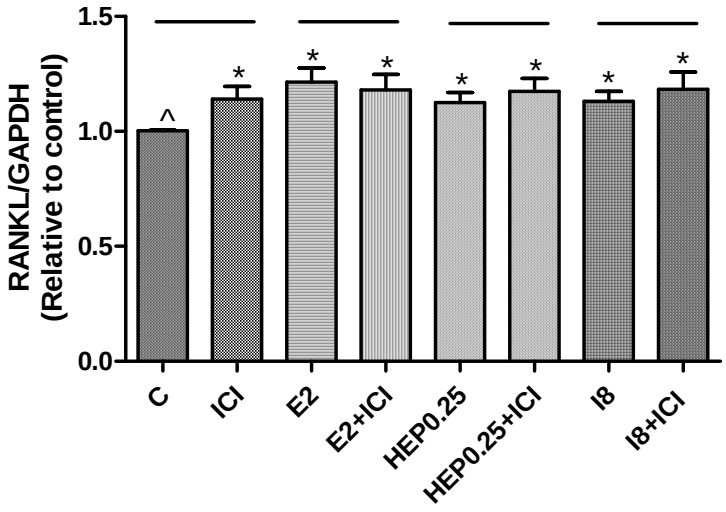
4.3.10 Effect of ER antagonist on modulating effect of icariin and total extract of Herba *Epimedii* on osteoclastogenesis

To investigate if ER was required for the modulating effect of icariin on osteoclastogenesis, OPG/RANKL mRNA expression in cells treated with icariin in the presence or absence of ICI 182,780 was determined. As shown in Figure 4-13A, OPG/GAPDH mRNA expression was significantly decreased in cells treated ICI 182,780 alone. In the presence of ICI 182,780, OPG/GAPDH mRNA expression was not upregulated by E2, HEP and icariin. RANKL/GAPDH mRNA expression was significantly increased in all treatment when compared with control (Figure 4-13B). OPG/RANKL mRNA expressions were significantly decreased in cells treated with ICI 182,780 and its co-treatment with HEP and icariin (Figure 4-13C). These results suggested that ER was required for the actions of HEP and icariin for upregulating OPG/GAPDH and OPG/RANKL mRNA expressions.

(A)


OPG/GAPDH
(Relative to control)
2.0
1.5
1.0
0.5
0.0
#
#
#
#
* ^^
* ^^
* ^^
^
*
C
ICI
E2
E2+ICI
HEP0.25
HEP0.25+ICI
I8
I8+ICI

(B)


RANKL/GAPDH
(Relative to control)
1.5
1.0
0.5
0.0
^
*
*
*
*
*
*
*
C
ICI
E2
E2+ICI
HEP0.25
HEP0.25+ICI
I8
I8+ICI

(C)

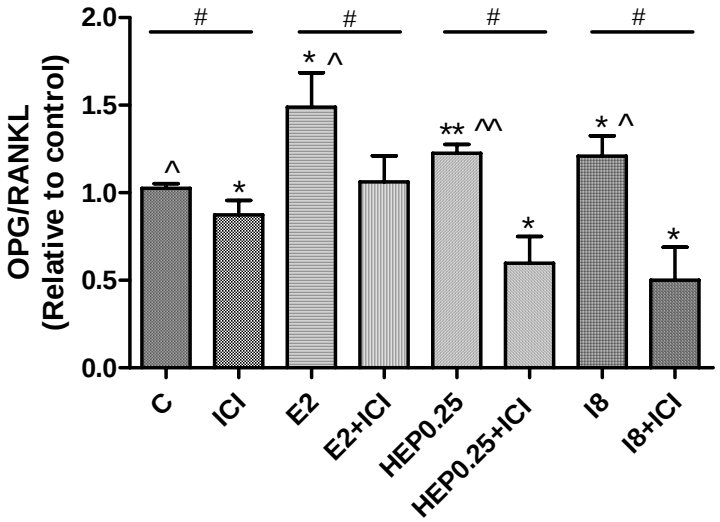

OPG/RANKL
(Relative to control)
2.0
1.5
1.0
0.5
0.0
#
#
#
#
* ^
** ^^
* ^
^
*
*
*
C
ICI
E2
E2+ICI
HEP0.25
HEP0.25+ICI
I8
I8+ICI

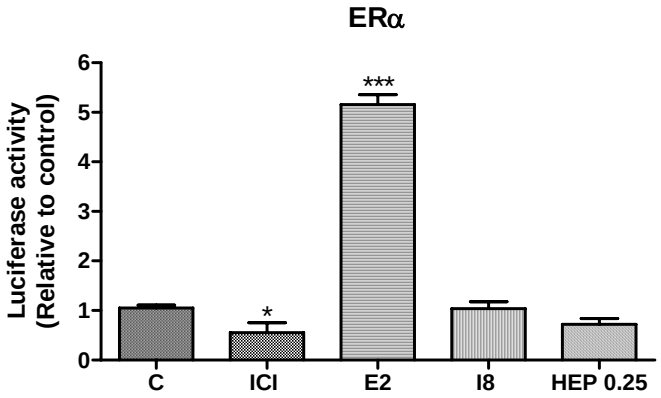
Figure 4-13: Effect of ICI 182,780 on mRNA expressions of (A) OPG/GAPDH, (B) RANKL/GAPDH and (C) OPG/RANKL.

Cells were treated with vehicle (C), 17β-estradiol (E8; 10^{-8}M), HEP (0.25 μg/ml) or icariin (I8; 10^{-8}M) in the presence or absence of ICI 182,780 (ICI; 10^{-6}M) for 48 hr. ICI 182,780 is a pure non-selective ER antagonist. Effect of E2, HEP and icariin with or without ICI 182,780 co-treatment on OPG/GAPDH, RANKL/GAPDH and OPG/RANKL mRNA expression were shown in (A),(B) and (C) respectively. Results were expressed as mean ± SEM (n = 4-6). (*p<0.05; **p<0.01 vs C; ^p<0.05; ^^p<0.01 vs ICI; # indicated a difference between each treatment with or without ICI)

4.3.11 Effect of icariin and total extract of Herba *Epimedii* on ER-mediated luciferase activity

As inhibition on cell proliferation, ALP activity and OPG/RANKL mRNA expressions were observed in cells co-treated with ICI and icariin, it was hypothesized that the actions of icariin were mediated through activation of ER. To test this hypothesis, ER-mediated luciferase activity assays were conducted. In Figure 4-14A and 4-14B, ICI 182,780 treatment resulted in significant decrease in ERα or ERβ-mediated luciferase activity, while E2 treatment significantly increased the activity. Icariin and HEP treatment remained more or less unchanged when compared with control. Inability of icariin and HEP to stimulate ER-mediated luciferase activity suggested that their actions were not mediated by direct binding of ERE.

(A)



(B)

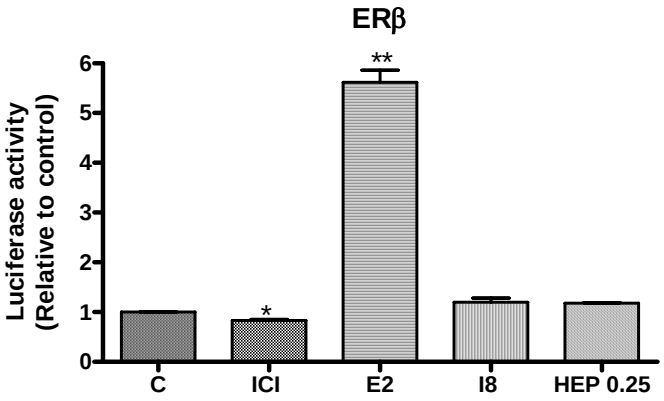


Figure 4-13: Effect of icariin and HEP extract on (A) ERα- and (B) ERβ-mediated luciferase activity in UMR-106 cells.

Cells were transiently transfected with 0.4 μg ERα or ERβ plasmid, 0.4 μg ERE-containing luciferase reporter vector and 0.1 μg pRL-TK luciferase internal reporter vector for 5 hr. After transfection, cells were treated with vehicle (C), ICI 182,780 (ICI; 10^{-6}M), 17β-estradiol (E8; 10^{-8}M), icariin (I8; 10^{-8}M) or HEP (0.125, 0.25, 1 μg/ml) for 24 hr. After treatment, the relative ERα-mediated and ERβ-mediated luciferase activity was determined and shown in (A) and (B) respectively. Results were expressed as mean ± SEM (n = 3-6). (*$p<0.05$;

***p<0.001 vs C)

4.3.12 Effect of icariin and total extract of Herba *Epimedii* on phosphorylation of ERα at serine 118 residue

From the above results, the actions of total extract was independent of the presence and direct binding of ER as they were not abolished by ER antagonist and total extract did not induce ERE-mediated transactivation. Therefore total extract was not subjected to the immunodetection of phospho-ERα and ERα proteins. To determine if icariin activated ERα indirectly, the relative degree of phosphorylation at serine 118 residue was measured in cells treated with icariin for 24 hr. The ratio of phospho-ERα to ERα (pERα/ERα) represents the relative amount of activated ERα. As shown in Figure 4-15, both estradiol and icariin significantly increased pERα/ERα by 74% and 53% respectively when compared with vehicle control. This result suggested that icariin could activate ERα by phosphorylation.

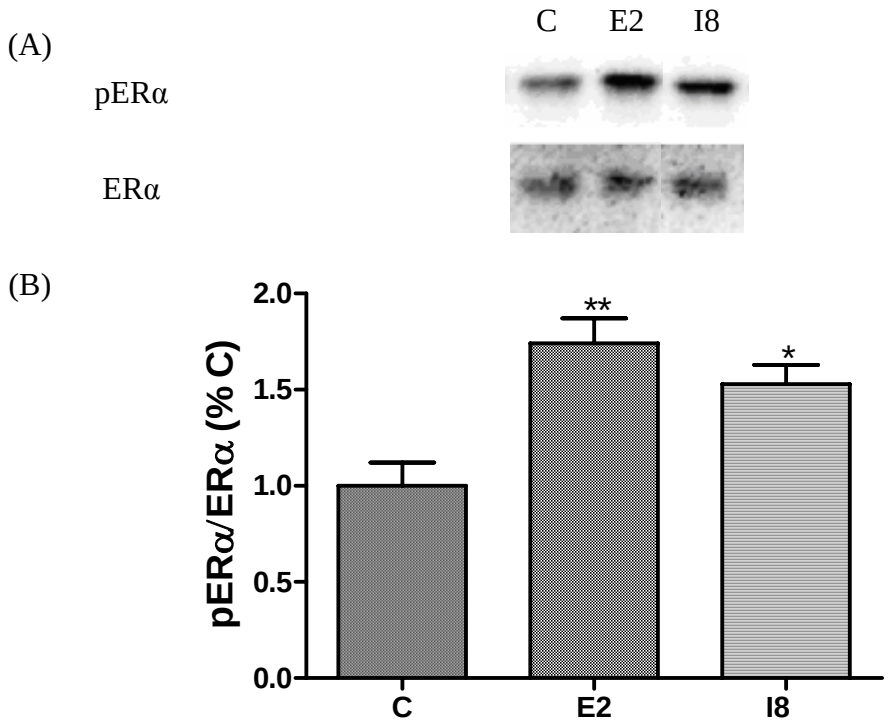


Figure 4-15: Effect of icariin and total extract of Herba *Epimedii* on phosphorylation of ERα at serine 118 residue.

Cells were treated with vehicle (C), 17β-estradiol (E8; 10^{-8}M) or icariin (I8; 10^{-8}M) for 24 hr and cell lysates were collected. The proteins were transblotted onto a membrane and probed with anti-phospho-ERα at serine 118 residue (pERα) and anti-ERα (ERα) primary antibodies followed by their corresponding secondary antibodies. Relative intensity of chemiluminescence was measured and anti-phospho-ERα to anti-ERα ratio was calculated. Protein bands of pERα and ERα and a graphical representation of pERα/ERα were shown in (A) and (B) respectively. Results were expressed as mean ± SEM (n = 3). (*$p<0.05$; **$p<0.01$ vs C)

4.4 Discussion

Herba *Epimedii* has long been used in TCM to treat osteoporosis and prevent bone fracture. Researchers had demonstrated the flavonoids fraction exhibited anti-osteoporotic effects, which might mainly be contributed by icariin, the most abundant flavonoid compound (i.e. > 70%) in the fraction. In the present study *in vivo* and *in vitro* effects as well as molecular actions of icariin were evaluated systematically.

Body weight gain, uterus atrophy, high bone turnover and bone loss with subsequent decrease in bone strength were commonly observed in ovariectomized animals due to estrogen deficiency. Our results showed that icariin did not prevent OVX-induced weight gain and uterus atrophy as estradiol did. Despite statistical significance in icariin group, serum calcium concentrations remained stable in a narrow range because of the tightly regulated mechanism of calcium homeostasis. Increase in serum phosphorus concentration in icariin group might indicate a low PTH status as PTH decreases serum phosphorus level. The indirect action of estrogen on bone that is mediated by changes in PTH secretion in both experimental animals and human includes stimulation of intestinal calcium absorption and renal calcium reabsorption (Riggs BL et al., 2002). Urinary calcium was considered as an

indicator of calcium absorption and reabsorption (Zhang G et al., 2006). Icariin treatment decreased OVX-induced increase in urinary calcium level to a greater extent than estradiol, which suggested that icariin was effective in preventing calcium loss through urinary excretion and might contribute to conservation of bone mass. Icariin resulted in smaller significant increase in total BMD, trabecular BMD and polar-SSI than estradiol. Taken together, icariin exerted osteoprotective effect by suppressing calcium loss through urine and increasing total and trabecular BMD and polar-SSI, which reflecting higher bone strength and lower fracture risk. Lack of uterotrophic effect of icariin was more advantageous than estradiol as it represented a safer tissue specific candidate for treatment of osteoporosis.

Estrogen promotes osteoblast proliferation, differentiation and functions while it inhibits osteoclasts formation and activity at cellular level (Riggs BL et al., 2002). Basing on the evidence that 17β-estradiol acted directly on UMR-106 cells, 17β-estradiol was used as the positive control paralleled to that in the mice study. The osteoblastic functions in terms of cell proliferation, differentiation and relative determinant of osteoclastogenesis were evaluated by cell proliferation rate, ALP activity and OPG/RANKL mRNA expression respectively. Icariin produced a similar stimulatory effect on cell proliferation

and ALP activity as estradiol. Previous studies had demonstrated the ability of estrogen to increase OPG production while its effect on RANKL expression remained either undetermined or inconsistent (Rogers A et al., 2005; Aubin JE et al., 2000; Kostenuik PJ et al., 2001; Theoleyre S et al., 2004). Both estradiol and icariin treatment led to a 2 to 3-fold increase in OPG mRNA expression. Upregulation of RANKL mRNA expression in estradiol-treated cells was unexpected as estrogen decreased RANKL expression theoretically. RANKL mRNA expression increased with increasing concentrations of icariin. Similar to OPG mRNA expression, about 2-fold increase in OPG/RANKL mRNA expression was observed in estradiol and icariin group. These results suggested that icariin exerted osteoprotective effects in a similar manner with estradiol, in which cell proliferation and differentiation were promoted while osteoclastogenesis was suppressed.

As the effects of icariin as well as total extract of Herba *Epimedii* were similar to that of estradiol, it was believed that their actions were mediated through activation of ER. Stimulatory effects of icariin and HEP on cell proliferation were inhibited by the presence of ICI 182,780, which suggested that ER was required for their actions for promoting cell proliferation. Inability of ICI 182,780 alone or its cotreatments to suppress ALP activity was

unexpected as it was reported that ALP expression was estrogen-dependent, which suggested that regulation of ALP activity by icariin and HEP might not involve direct participation of ER. Upregulation of OPG and OPG/RANKL mRNA expression was prevented in cotreatment with icariin, HEP or estradiol and ICI 182,780, while RANKL mRNA expression remained unchanged with or without ICI 182,780. These results suggested that upregulation of OPG mRNA expression involved the participation of ER. Although the importance of ER for the actions of icariin and HEP was demonstrated, it was still unclear if they induce direct activation of ER. To answer this question, the ER-mediated transactivation assay using ERE-promoter was conducted. Failure to stimulate both ERα- and β-mediated luciferase activity suggested that the actions of icariin and HEP were not mediated by direct ERE-binding and was said to be ERE-independent. Since there are different mechanisms of ER signaling, only about one-third of estrogen responsive genes contain ERE sequence and therefore the remaining genes may induce their biological responses by indirect interaction of ER and DNA through transcription factors or ligand-independent phosphorylation of ER. ERα phosphorylation can take place at its serine residue 118 (Ser118), 104 (Ser104) and 167 (Ser167) within AF-1, which in turns modulates the transcriptional activity (Chen DS et al.,

2002). To determine if icariin exhibit ligand-independent actions, the ratio of phosphorylated ERα (Ser118) to total ERα expression was measured. ER phosphorylation at Ser118 was preferentially chosen as Ser118 is highly conserved residue and represents the major site of phosphorylation in response to estradiol (Lannigan DA et al., 2003). Ability of icariin to increase phosphorylated ERα to ERα ratio indicated that icariin was able to activate ER through ligand-independent mechanism. Previous reports had shown that presence of isopentenyl group at C-8 position facilitates the docking and binding at ER (Wang Z et al., 2004; Kitaoka M et al., 1998). Icariin also had an isopentenyl group at C-8 position in which the effects might be strengthened. Collectively, the actions of icariin on cell proliferation, differentiation and OPG mRNA expression required the participation of ER and might be mediated through the ligand-independent pathway without direct ERE binding. The actions of icariin was somewhat similar to estren (4-estren-3α,17β-diol), a synthetic steroid or xenoestrogen that prevents bone loss and maintains bone strength, in which transcriptional activity was not mediated by ERE binding. If the actions of icariin targeted on bone tissue rather than reproductive system like estren does, it would be particularly beneficial to management of osteoporosis. Further investigations are necessary to clarify the molecular

mechanisms of icariin.

4.5 Conclusion

Icariin was confirmed to be effective in increasing total BMD, trabecular BMD and bone strength while decreasing calcium loss through urinary excretion in ovariectomized mice. It was regarded as a potential safer alternative for prevention and treatment of osteoporosis due to the absence of uterotrophic effect. *In vitro* study demonstrated that icariin exerted osteoprotective effects by stimulating cell proliferation, differentiation and suppressing osteoclastogenesis in rat osteoblast-like UMR-106 cells. The mechanistic study showed that the effects of icariin on the osteoblastic functions involved the participation of ER and its actions were mediated through ligand-independent phosphorylation of ER instead of direct ERE binding. Owing to the complex ingredients in total extract of Herba *Epimedii*, the molecular actions of total extract was less clearly defined, in which cell proliferation and upregulation of OPG mRNA expression but not ALP activity, was ER-dependent and its actions were not mediated by ERE binding. To conclude both *in vivo* and *in vitro* results suggested icariin as a potential and effective candidate for management of osteoporosis.

Chapter 5

Discussion and Conclusion

5.1 Discussion

Osteoporosis is one of the major serious health problems in the world, which affects over millions of people and accounts for billions of cost for osteoporosis-related fractures. The current therapies such as hormone replacement therapy, selective estrogen receptor modulators, bisphosphonates, calcitonin and parathyroid hormone are effective agents but long-term compliance led to economic burden and various side effects (e.g. breast and endometrial cancers, cardiovascular diseases and gastrointestinal discomforts) that may outweigh their benefits. TCM has been considered as important complementary and alternative medicines for treatment of different diseases including osteoporosis in the Asian community, which represents a safer and inexpensive medication for long-term usage. However TCM has not been universally accepted since the active ingredients and the mechanism of actions of the herbal medicines were neither identified nor characterized, which hinders the progress to modernize TCM for more extensive use.

Herba *Epimedii* is one of the most popular Chinese herbs being used for prevention and treatment of osteoporosis in China. Previous studies had demonstrated that Herba *Epimedii* and its flavonoid ingredients exerted beneficial effects on bone protection through improving BMD, bone strength,

bone microarchitecture, osteoblast proliferation, differentiation, mineralization and matrix protein synthesis while suppressing bone resorption rate. Due to the problems associated with TCM, Herba *Epimedii* remains to be a minor alternative for management of osteoporosis in places other than China. In order to raise the public acceptance of this herb, we conducted a systematic study on evaluating both *in vivo* and *in vitro* effects as well as identifying the active ingredients and molecular actions of Herba *Epimedii* using modern approaches such as bioactivity-guided fractionation and effective screening platforms.

In chapter 3, we confirmed that total extract, total flavonoids and non-flavonoids of Herba *Epimedii* exerted osteoprotective effects in ovariectomized mice by increasing femoral total and trabecular BMD significantly. The increase in total and trabecular BMD decreased in an order: E2 > HEP > TF > NF and E2 > NF > HEP > TF respectively. As total flavonoids is of greater osteoprotective potentials basing on the literature and our *in vitro* study, total flavonoids rather than non-flavonoids was studied in the second mice study within a limited timeframe. It was observed that total flavonoids increased total BMD, trabecular BMD and polar-SSI (a surrogate marker for bone strength) significantly. The increase in total BMD, trabecular BMD and polar-SSI decreased with increasing concentration of total flavonoids

(i.e. E2 > TF50 > TF100 > TF200 > TF400) in distal, proximal and midshaft of femur. Serum ALP and urinary calcium levels were significantly decreased in response to total flavonoids. Taken together, total flavonoids exerted osteoprotective effect by increasing BMD and bone strength, while decreasing bone turnover and calcium loss through urine excretion. Optimal dose of total flavonoids was found to be TF100 (i.e. 100 μg/g/day). On the whole, the osteoprotective effect offered by total flavonoids was stronger than total extract while that by non-flavonoids was the weakest. As non-flavonoids contained less active ingredients (such as phytoestrogenic or phenolic compounds) or more compounds that arrested the osteoprotective actions of some active compounds than total flavonoids, its presence reduced the efficacy of total extract and resulted in a moderate effect between total flavonoids and non-flavonoids. Inability of total extract, total flavonoids and non-flavonoids to induce trophic effects on uterus makes them advantageous to be a potential safer alternative for prevention and treatment of osteoporosis.

For *in vitro* study, effects of total extract, total flavonoids and non-flavonoids were assessed by evaluating cell proliferation rate, ALP activity and OPG/RANKL mRNA expression. All three extracts exhibited osteoprotective effects by stimulating osteoblast-like UMR-106 cell

proliferation, differentiation and suppressing osteoclastogenesis. Lower concentrations were more desirable as high concentrations (i.e. 1 μg/ml) induced cytotoxicity and downregulated OPG mRNA expression. Additional five flavonoid compounds of higher abundance in total flavonoids (i.e. baohuoside I, sagittatoside B, korepimedoside C, epimedin B and sagittatoside A) were studied. All these compounds increased cell proliferation and differentiation significantly. The effective range of these compounds except baohuoside I lied between the physiological concentrations (e.g. 10^{-12}M to 10^{-8}M), which suggested they were potential potent agents for promoting osteoblastic proliferation *in vivo*. Total flavonoids and sagittatoside A had a higher efficacy than the remaining extracts and single compounds respectively. It was suggested that the commonly found C-8 isopentenyl group might be more important than the different number of sugar moieties in the five compounds for their activities.

Icariin, the most abundant flavonoid compound (i.e. > 70%) in total flavonoids, may mainly account for the beneficial effects of total flavonoids. In chapter 4, we confirmed that icariin exerted osteoprotective effects by increasing total BMD, trabecular BMD and bone strength and decreasing calcium loss through urinary excretion in ovariectomized mice, while

stimulating cell proliferation, differentiation and suppressing osteoclastogenesis in UMR-106 cells. Absence of uterotrophic effect of icariin suggested icariin as a potential and effective candidate for management of osteoporosis. In addition to the efficacy, the mechanism of actions of icariin is worthy of studying. As the effects of icariin as well as total extract of Herba *Epimedii* were similar to that of estradiol, it was believed that their actions were mediated through activation of ER. The mechanistic study showed that the effects of icariin on the osteoblastic functions involved the participation of ER and its actions were mediated through ligand-independent phosphorylation of ER instead of direct ERE binding. Owing to the complex ingredients in total extract of Herba *Epimedii*, the molecular actions of total extract was less clearly defined, in which cell proliferation and upregulation of OPG mRNA expression but not differentiation, was ER-dependent and its actions were not mediated by ERE binding.

In this study we successfully established both *in vivo* and *in vitro* screening platforms to demonstrate the osteoprotective effects of Herba *Epimedii* and its fractions and flavonoid compounds. Selection of models and endpoints are two important criteria for valid results with methodological quality. Although ovariectomized C57BL/6J mice and rat osteoblast-like

UMR-106 cells were suitable models for this study, there are still some limitations in these models. First the mice used were of young or adolescent ages that their peak bone mass were not achieved when the experiment commenced. This situation is different from those postmenopausal osteoporosis patients whose ages are usually over 50 years old. As these mice still undergo active growing, they are more responsive upon treatment and this makes fast *in vivo* screening possible. Second the effects observed in UMR-106 cells may not completely represent or predict the real situation in the osteoblasts since UMR-106 cells are not normal or primary cells. On the whole, responsiveness, stability, ease of manipulation and relevance of these models make them more preferable to other available candidates for cost-effective screening of active ingredients in Herba *Epimedii*. To evaluate the bone quality in response to different treatments, BMD and polar-SSI of femurs were measured by pQCT. As bone strength does not solely depend on BMD, other determinants like bone geometry, microarchitecture and bone tissue quality have to be taken into account. Therefore examination on bone micrarchitecture by micro-computed tomography and breaking force by biomechanical testings provide more evidence for evaluating the efficacy of Herba *Epimedii*. Bone tissue other than long bones like lumber spine may also be measured because it is highly

important in the skeletal system and its fracture results in serious conditions. In addition some promising bone formation and resorption markers such as serum osteocalcin and urinary DPD may be measured in mice treated with more potent fraction of Herba *Epimedii*. Generally the endpoints measured in this study were representative and adequate for substantiating the beneficial effects of Herba *Epimedii* and its fractions. However further measurements or resources should be taken to the fraction with high potency.

5.2 Conclusion

In the present study, both *in vivo* and *in vitro* screening platforms for identifying the active ingredients of Herba *Epimedii* were developed. The first animal study demonstrated that total extract, total flavonoids and non-flavonoids of Herba *Epimedii* exerted ant-osteoporotic effects by increasing total and trabecular BMD in ovariectomized C57BL/6J mice. In the second *in vivo* study, the beneficial effects of total flavonoids demonstrated included increase in BMD and bone strength as well as decrease in bone turnover and calcium loss through urinary excretion. Absence of trophic effect on uterus made all extracts of Herba *Epimedii* advantageous to be a potential safer alternative for prevention and treatment of osteoporosis.

In vitro study suggested that total extract, total flavonoids and non-flavonoids and five flavonoid compounds (i.e. baohuoside I, sagittatoside B, korepimedoside C, epimedin B and sagittatoside A) from Herba *Epimedii* exerted osteoprotective effects by stimulating cell proliferation, differentiation and suppressing osteoclastogenesis in rat osteoblast-like UMR-106 cells. Total flavonoids and sagittatoside A had a higher efficacy than the remaining extracts and single compounds respectively.

Icariin exerted osteoprotective effects by increasing total BMD, trabecular

BMD and bone strength while decreasing calcium loss through urinary excretion in ovariectomized C57BL/6J mice. Absence of uterotrophic effect was observed. Icariin exhibited beneficial effects by stimulating cell proliferation, differentiation and suppressing osteoclastogenesis in rat osteoblast-like UMR-106 cells. The effects of icariin on the osteoblastic functions involved the participation of ER and its actions were mediated through ligand-independent phosphorylation of ER instead of direct ERE binding.

To conclude both *in vivo* and *in vitro* results suggested total extract, total flavonoids, non-flavonoids and icariin from Herba *Epimedii* as potential and effective candidates for management of osteoporosis. Total flavonoids and sagittatoside A had a higher efficacy than the remaining extracts and single compounds respectively.

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