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Chemical Technology

Modern approach to study the osteoprotective effect of Rhizoma Drynariae

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CERTIFICATE OF ORIGINALITY

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(Signed)

Pang Wai Yin

(Name of Student)

Abstract

Rhizoma Drynariae (RD, Gu Sui Bu) has been one of the most frequently used herbs prescribed for bone fracture healing in China for thousands of years. It is postulated that total flavonoids fraction of Rhizoma Drynariae and its active ingredients benefits the prevention and treatment of osteoporosis.

In the present study, the dose response effects of RD total flavonoids extract and its active ingredient, Naringin, on bone metabolism were evaluated using 4-week old ovariectomized (OVX) female C57BL/6J mice orally administered with corresponding agents for 6 weeks. We found that 0.173 mg/g/day RD total flavonoids and 0.4 mg/g/day Naringin significantly restored bone mineral densities (BMD) and bone strengths at mice distal femur, proximal tibia, and lumbar spine from OVX-induced osteopenia without exhibiting uterotrophic side-effect ($p<0.05$ versus vehicle-treated OVX mice). Decreased levels of urinary calcium and urinary deoxypyridinolines (Dpd) suggested that RD total flavonoids and Naringin might exert bone protective effect via its inhibiting effect on bone resorption ($p<0.05$ versus vehicle-treated OVX mice).

The osteoprotective effects of RD total flavonoids, Naringin and other RD isolated single compounds were also investigated using rat osteoblastic-like UMR-106 cells. RD total flavonoids, Naringin and some RD single compounds significantly stimulated cell proliferation and differentiation in a dose-dependent manner ($p<0.05$ versus vehicle-treated). The optimal doses of each agent were deduced from the corresponding cell proliferative effects. The involvement of estrogen receptor (ER) in

their activities was determined. We found that RD total flavonoids and Naringin modulated osteoclastogenesis by increasing osteoprotegrin (OPG) and decreasing receptor activator of NF-κB ligand (RANKL) mRNA expression simultaneously (p<0.05 versus vehicle-treated). The modulation of osteoclastogenesis by Rhizoma Drynariae and Naringin were abolished by co-treating with estrogen antagonist, ICI 182,780, suggesting that the activation of osteoblastic functions were ER-mediated. From the detection of ER-α / ER-β-mediated estrogen receptor element (ERE)-luciferase activity and the protein expression of phospho-ER-α, RD total flavonoids and Naringin were found to activate ER-α phosphorylation as well as direct ERE binding. The results suggest that RD total flavonoids and Naringin exert stimulatory effects on osteoblastic functions through mechanisms that are similar to those of estrogen or growth factors which promote cell activities.

Taken together, Rhizoma Drynariae total flavonoids and Naringin treatment can effectively suppress the OVX-induced uncoupled bone remodeling possibly by both an increase in osteoblastic activities and a decrease in osteoclastogenesis in an ER dependent manner. The present study provides the evidence that Rhizoma Drynariae and its active ingredients can be developed as alternative therapeutic agents for the prevention and treatment of osteoporosis.

List of Publications

1. MOK, S.K., PANG, W.Y., LAI, W.P., ZHANG, Y., LEUNG, P.C., YAO, X.S., Wong, M.S. *In vivo* effects of Herba Epimedii (Yinyanghuo) and Rhizoma Drynariae (Gusuibu) on bone and mineral metabolism. Proceedings, 2006 Hong Kong-Macau Postgraduate Symposium on Chinese Medicine, Hong Kong, PRC, Abstract C-17, pp.114-115.
2. MOK, S.K., LAI, K.H., PANG, W.Y., LAI, W.P., ZHANG, Y., LEUNG, P.C., YAO, X.S., WONG, M.S. The anti-osteoporotic effect of total flavonoid extract of *Herba Epimedii* in ovariectomized mice. Proceedings, 2007 Hong Kong-Macau Postgraduate Symposium on Chinese Medicine, Hong Kong, PRC, Abstract C-11, pp. 71-72.
3. MOK, S.K., FUNG, C.Y., PANG, W.Y., LAI, W.P., ZHANG, Y., WANG, X.L., CHENG, Y., LEUNG, P.C., YAO, X.S., WONG, M.S. The effect of four flavonoid compounds isolated from Herba Epimedii in UMR-106 cells. Proceedings, 2007 Hong Kong-Macau Postgraduate Symposium on Chinese Medicine, Hong Kong, PRC, Abstract C-12, pp. 73-74.
4. PANG, W.Y., WONG, C.Y., WONG, M.S. The osteoprotective effects of flavonoid fractions of *Rhizoma Drynariae in vivo*. Proceedings, 2007 Hong Kong-Macau Postgraduate Symposium on Chinese Medicine, Hong Kong, PRC, Abstract C-13, pp. 75-76.
5. CHAN, C.Y., PANG, W.Y., WONG, M.S. The *in vitro* osteoprotective effects of three flavonoid compounds isolated from *Rhizoma Drynariae*. Proceedings, 2007 Hong Kong-Macau Postgraduate Symposium on Chinese Medicine, Hong Kong, PRC, Abstract C-14, pp. 77-78.
6. PANG, W.Y., WANG, X.L., MOK, S.K., LAI, W.P., YAO, X.S., WONG, M.S. Total flavonoid fraction of *Rhizoma Drynariae* and its active ingredient, Naringin, exert bone protective effect *in vivo* and *in vitro*. J Bone Miner Res 22 (suppl), 29th Annual Meeting

of the American Society for Bone and Mineral Research 2007, Hawaii, USA, Abstract W342, pp. S448.

7. LAI, W.P., MOK, S.K., PANG, W.Y., WANG, X.L., YAO, X.S., FAVUS, M.J., WONG, M.S. Icariin, an active ingredient in *Herba Epimedii*, exerts bone protective effects in vivo and in vitro. J Bone Miner Res 22 (suppl), 29th Annual Meeting of the American Society for Bone and Mineral Research 2007, Hawaii, USA, Abstract W337, pp. S446.
8. PANG, W.Y., WANG, X.L., MOK, S.K., LAI, W.P., YAO, X.S., WONG, M.S. Naringin, a citrus flavonoid, exerts estrogen-like activity in rat osteoblastic-like UMR-106 cells. Proceedings, 2008 International Symposium on Functional Foods, Hong Kong, PRC, Abstract P-4, pp. 5.
9. PANG, W.Y., WANG, X.L., MOK, S.K., LAI, W.P., YAO, X.S., WONG, M.S. Naringin, a citrus flavonoid, exerts bone protective effects in ovariectomized C57BL/6J female mice. Proceedings, 2008 International Symposium on Functional Foods, Hong Kong, PRC, Abstract P-5, pp. 6.

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Although it is not a high time to confirm my career, I believe the spirit and attitude that I have learnt from this postgraduate study would lead me to a brighter path in the future. I am obliged to all those who had given me a hand in this study.

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Abbreviations and Symbols

ALP	Alkaline phosphatase
ANOVA	Analysis of variance
BMC	Bone mineral content
BMD	Bone mineral density
BSA	Bovine serum albumin
BASP	Bone-specific alkaline phosphatase
BMP-2	Bone morphogenic protein 2
Ca	Calcium
cDNA	Complementary DNA
Cr	Creatinine
DEXA	Dual-energy X-ray absorptiometry
DLR	Dual Luciferase Reporter
DMEM	Dulbecco’s modified Eagle medium
Dpd	Deoxypyridinolines
E2	17β-estradiol
ECL	Enhanced chemiluminescence
EIA	Enzyme immunoassay
ER	Estrogen receptor
ER α	Estrogen receptor alpha
ERβ	Estrogen receptor beta
ERE	Estrogen receptor element
ERT	Estrogen replacement therapy
FBS	Fetal bovine serum
FDA	Food and Drug Administration

GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
HPLC	High performance liquid chromatography
HRT	Hormone replacement therapy
IGF-I	Insulin growth factor I
mRNA	Messenger RNA
NCPs	Non-collagenous proteins
OPG	Osteoprotegerin
OVX	Ovariectomy
P	Phosphate
PBS	Phosphate-buffered saline
pER	Phosphorylated estrogen receptor
PMS	Phenazine methosulfate
PNP	*p*-nitrophenylphosphate
pQCT	Peripheral quantitative computed tomography
PVDF	Polyvinylidene fluoride
RANKL	Nκ-B ligand (RANK ligand)
RD	Rhizoma Drynariae
RD TF	Rhizoma Drynariae total flavonoids
PTH	Parathyroid hormone
sCa / sP	Serum Calcium / serum Phosphorus
SDS-PAGE	Sodium dodecyl sulfate-polyacrlamide gel electrophoresis
SEM	Standard error mean
SERMs	Selective estrogen receptor modulators
SSI	Stress-strain index
TCM	Traditional Chinese Medicine
TGF-β	Transforming growth factor β

TK	Tyrosine kinase
TRACP 5b	Tartrate-resistant acid phosphatase 5b
uCa/Cr // uP/Cr	Urinary Calcium/Creatinine // urinary Phosphorus/Creatinine
µCT	Micro-computed tomography
WHO	World Health Organization

Chapter 1

Introduction

1.1 Postmenopausal Osteoporosis

Osteoporosis is a skeletal disorder characterized by compromised bone strength and impaired microarchitecture which afflict a person to an increased of risk of fracture (Gass and Dawson-Hughes 2006). Postmenopausal osteoporosis is due to estrogen deprivation during menopause. Several evidences confirmed that a higher incidence of osteoporosis appears during the first four to five years after the last menstruation (Pansini 2006). This disorder leads to increase bone resorption which is not compensated by bone formation.

Hip and vertebral fractures are the most common fractures in patients with osteoporosis. Hip fractures are associated with a substantially increased risk of death, whereas vertebral fractures are associated with chronic back pain, functional limitations and increased risks of mortality. In the United States alone, there are over 1.5 million fractures attributed to this disease every year, and approximately 40% of the patients would experience hip or spine fractures. The direct healthcare expenditures have reached from US$12 billion to $18 billion per year (Gass and Dawson-Hughes 2006). In China, the year 2000 census revealed that 6.97% of the total population had primary osteoporosis (Liu, Piao et al. 2002). Due to its increasing prevalence in the world, the medical costs associated with the treatment and management of osteoporosis have become governments' major social and economic

burdens. Estrogen replacement therapy (ERT), the major regime for the prevention and treatment of osteoporosis, is recently found to increase the risk of female patients developing breast and ovarian cancers. Therefore, alternative medications of non-estrogenic therapeutic agents with a lower cost and fewer side effects, such as the applications of traditional Chinese medicine (TCM), are worth exploring.

1.1.1 Diagnosis of Postmenopausal Osteoporosis

The assessment of existing bone mass is the ultimate parameter for evaluating patients for osteoporosis. The WHO (World Health Organization) has established diagnostic criteria for osteoporosis on the basis of BMD T-scores. This score could only give a general picture for the estimation of the prevalence of osteoporosis at different ages but not a reliable assessment for specific patients. Besides BMD measurement, biochemical markers of bone turnover have become widely used in clinical research as they directly reflect the bone turnover rate and bone loss that occurs with estrogen deficiency. Markers of bone formation are typically measured in serum. Currently available bone formation markers include bone-specific alkaline phosphatase (BSAP), osteocalcin and type I collagen. Bone resorption markers are secreted during osteoclastic activity and collagen breakdown. Several assays are known to be available in serum and urine such as urinary Dpd (Deoxypyridinolines) (Lane 2006).

1.1.2 Current Available Therapies for Postmenopausal Osteoporosis

From the 1980s to 1990s, hormonal replacement therapies, particularly estrogen and an injectable synthetic salmon calcitonin, were the only agents available in the market. In late 1990s, the US FDA (Food and Drug Administration) approved the first bisphosphonate, alendronate, as an effective agent for the treatment of osteoporosis. Raloxifene, which is regarded as the first selective estrogen receptor modulator (SERM), was approved for the prevention of this disease. In early 2000, risedronate became the second bisphosphonate which regarded as an antiresorptive drug (Pansini 2006).

HRT (Hormone replacement therapy)

HRT is the major available treatment. It is a method to replace the lost endogenous estrogen by delivering 17β-estradiol orally. Although HRT increases the bone mass and reduces fracture risks in postmenopausal women, the increase of risk of breast cancer and cardiovascular disease associated with the combined use of conjugated equine estrogens outweighs its benefit (Gass and Dawson-Hughes 2006).

SERMS (Selective Estrogen Receptor Modulators)

Raloxifene is the only SERM currently approved for the prevention and treatment of osteoporosis (Gass and Dawson-Hughes 2006). It acts as an estrogen agonist on bone metabolism and has partial anti-estrogenicity in breast and endometrium tissues. It is

effective in reducing vertebral fractures; however, several findings demonstrated that raloxifene also increases the risks of deep vein thrombosis and pulmonary embolism to an extent similar as HRT (Gass and Dawson-Hughes 2006).

1.1.2.1 Limitations and the Adverse Effects

While most of the osteoporotic patients in worldwide are receiving the treatment mentioned above, several findings were indicated that long-term treatment associated with some adverse effects. Current findings demonstrated that estrogen replacement therapy and bisphosphonate supplement were associated with increased risks of breast, ovarian and endometrial cancers (Pansini 2006). Apart from cancers, most of the agents such as bisphosphonates used in treating osteoporosis would seriously and adversely affect the gastrointestinal tract and cause gastrointestinal irritation and nausea. The economic burden for post-caring increases over time (Tuck and Francis 2002).

Owing to the rising prevalence of cancers and high expenditures of post-caring, a growing interest is seen in investigating an alternative strategy to alleviate osteoporosis in postmenopausal women (Wang, Wu et al. 2003; Zhang, Qin et al. 2007).

1.1.3 Alternative Treatment

The development of complementary medicine has become one of the concerns in treating osteoporosis. Most of the patients now believe that herbal or other traditional therapies associated with conventional treatment could decrease the risk of having side effects. Chiechi LM reviewed that it is possible to obtain the same preventive effects of HRT with alimentary interventions with phytoestrogens (Chiechi 1999). The mechanisms of the actions of phytoestrogens in preventing and treating osteoporosis are worth studying.

1.1.3.1 Phytoestrogens

Phytoestrogens are secondary metabolites produced in a variety of plants. It is postulated that they can induce biological activities and can mimic or modulate the actions of endogenous estrogens. The broad classes of phytoestrogenic compounds include isoflavonoids, coumestans and lignans. All have biphenolic structures which are regarded as estrogen-resemble. Due to this specificity, phytoestrogens have the ability to bind to the estrogen receptor (ER) which is required for ligand-association. They can act like partial ER agonists or antagonists. Recent evidence supports the fact that a protective effect of high phytoestrogen diets to reduce the incidence of certain hormone-responsive cancers, such as breast and prostate cancers. A study by Harris et

al. has revealed that the incidence of mortality from breast cancer is much lower in the Asian countries. Although the actual factors that contribute this difference are still unclear, it is believed that the health consequences of preventing cancers are due to the protective effects of phytoestrogens diets (Harris, Besselink et al. 2005). Recent studies have shown that potent phytoestrogens flavonoids extracted from traditional Chinese medicinal herbs have significant effects in preventing bone loss in late postmenopausal women (Zhang, Qin et al. 2007).

1.1.3.2 Traditional Chinese Medicine (TCM)

The theories and literatures of traditional Chinese medicines on menopause and bone loss have been developed for thousands of years. This is based on harmonizing the body dynamic balance to prevent or treat osteoporosis with minimal side effects. In terms of TCM, osteoporosis occurs due to an insufficiency of *kidney yin* or *kidney yang*. In Chinese medicine theory, *kidney* is responsible for the congenital functions. Ancient Chinese believed that the occurrence of menopause is a result of depleting *kidney yin* or *yang*, which equals the decline of congenital functions. One of the theories in TCM is that *kidney* controls the bones. The *kidney* regulates the vital *essence* which supplies nutrients to bones and maintains them in normal conditions. Based on this relationship, some of the osteoporosis-treating herbal therapies consist

the so-called *kidney*-tonifying herbs (Xu, Lawson et al. 2005).

Several *kidney*-tonifying herbs, such as Herba Epimedii and Drynariae Fortunei, have been used in the Asian countries for the treatment of bone diseases and promotion of bone healing for thousands of years. However, such knowledge was just acquired by clinical experience but without scientific proof. This is the reason why TCM has not been widely accepted in Western societies (Wong and Rabie 2006).

As Asian cultures are developing rapidly in recent decades, there is an increasing number of people concern about the use of TCM in treating metabolic diseases. In addition, there is a growing trend of scientists worldwide to explore TCM by using a Western approach. Several *kidney*-tonifying herbs have been proven as effective in promoting bone formation but the mechanisms of their actions have not been fully elucidated (Wong and Rabie 2006). There are still many technical problems that need to be tackled for developing TCM into modern therapeutic agents. The most important issue is to identify the active ingredients of each herbal medicine and to study their mechanisms of actions with state-of-art technology so that they can be accepted internationally. Since last few decades, TCM has become one of the popular alternative medicines since it has fewer side effects and is cost-effective. It has been proven that phytoestrogens extracted from herbal medicines, sustain estrogenic-like activities which are potently act as alternative therapeutic agents in dietary

supplement, as well as the treatment of menopausal symptoms (Zhang, Wang et al. 2005).

Rhizoma Drynariae (Gu Sui Bu) is one of the common Chinese *kidney*-tonifying herbs used to manage bone diseases. It is hypothesized that Rhizoma Drynariae would give benefits to osteoporotic patients worldwide.

1.2 Rhizoma Drynariae (Gu Sui Bu)

Rhizoma Drynriae (RD) is characterized as the dried rhizome of perennial pteridophyte *Drynariae Fortunei* (Kunze) J. Smith (Polypodiaceae) (Figure 1.1). In Chinese, “Gu Sui Bu” means bone fractures healer. It was already in the prescriptions to treat osteoporosis in ancient China for treating bone fractures and osteoporosis (Table 1.1). It is not surprising that RD has been reported as a good enhancer for bone fracture healing (Wong and Rabie 2006c). Similar to HRT, receiving RD is postulated to restore the estrogen-deficiency-induced bone loss and associate the decrease of bone fractures risk.

Table 1.1 TCM formulae, which include Rhizoma Drynariae, in treating bone-related diseases. Rhizoma Drynariae (in Chinese: 骨碎補) is widely prescribed in TCM formulae treated for bone fractures (left hand column) and osteoporosis (right hand column)

Bone fractures	**Osteoporosis**
強筋健骨外敷散 續斷, **骨碎補**, 土鱉蟲, 刺五加, 當歸, 赤芍, 乳香, 没藥, 地龍, 白及, 五倍子 **藥酒(relieve pain from bone fractures)** ***天麻酒*** 茄子根, 大麻仁, **骨碎補**, 當歸,牛膝(酒浸切焙),熟地, 白花蛇(酒浸去皮骨炙), 天麻,酒 等 ***地仙酒*** 肉蓯蓉, 炮附子, 木鱉子, 天南星, 白附子, 菟絲子, 赤小豆, **骨碎補**, 何首烏, 地龍, 羊膝, 人參, 黃芪, 白术, 茯苓, 白酒	***強腎密骨液*** 威靈仙, 杜仲, 熟地, **骨碎補**, 鹿含草, 伸筋草, 透骨草, 生南星, 生草烏, 藏紅花 ***骨疏康顆粒*** 淫羊藿, 熟地黃, **骨碎補**, 黃耆, 丹參, 木耳, 黃瓜子, 糊精 ***健脾補腎方*** 人參, 黃芪, 白朮, 灸甘, 淫羊藿, **骨碎補**, 續斷, 杜仲, 熟地, 首烏

1.2.1 Literature Review

Lin et al. investigated the biochemical effects of ten different traditional Chinese medicines *in vitro*, among them only the aqueous crude extracts of Rhizoma Drynariae had beneficial effects on bone cell metabolism. The stimulatory effects on bone cell activites are probably mediated by the induction of apoptosis of the osteoclast cell population (Lin, Sun et al. 2002). Moreover, RD promotes osteoblasts differentiation and mineralization through the regulation of bone morphogenetic protein-2, alkaline phosphatase, type I collagen and collagenase-1 (Jeong, Lee et al. 2004). In addition, Jeong et al. demonstrated that RD total extracts promote osteoblasts proliferation and differentiation *in vitro* (Jeong, Lee et al. 2005) and demonstrated the dose-dependent inhibitory effect on bone resorption *in vitro.* The effect was mediated by its inhibition of osteoclast-mediated intracellular processing of cathepsin K (Jeong, Kang et al. 2003). Recently, Jia et al. demonstrated that RD mimicked the effect of estrogen in maintaining ovariectomized rats' normal trabecular structure and connection by inhibiting bone turnover of postmenopausal osteoporosis (Jia, Wang et al. 2006). Another investigation showed that the total flavones of Rhizoma Drynariae promoted the functions of elevating blood calcium, increasing bone mineral density *in vivo* (JG 2004). Apart from the RD total extracts, studies about single compounds extracted from this herb are rare.

1.2.2 Preparation and Isolation of RD Crude Extracts and Flavonoids Fractions

(work done by Xin-luan Wang, ShenYang Pharmaceutical University)

50 kg dried Rhizoma Drynariae was carefully selected for the preparation of the herbal extracts. 8X volume of 60% ethanol were administered and the solvent was collected as the RD crude extracts (Yield: 3.4kg). The crude extracts were then differentiated into four different fractions by passing the following solvents (A) water; (B) 30% Ethanol; (C) 50% Ethanol; (D) 95% Ethanol into a macroporous resin column (Ø 25 x 150 cm). The four fractions with varied polarities were then ready for use after freeze-drying. Figure 1.2 shows a simplified flow chart of the preparation and isolation of RD fractions. According to chemical analysis done by ShenYang Pharmaceutical University, RDA was regarded as non-flavonoid fraction, while RDB, RDC and RDD were combined as the total flavonoid fraction.

<u>Total flavonoids fractions</u>

Flavonoids are water soluble polyphenolic molecules found in small quantities in numerous plants, including fruit, vegetables and wine (Mink, Scrafford et al. 2007). The backbone structure of flavonoid compounds is very similar to estrogen. Among the various kinds of flavonoids, hesperidin from citrus fruits promotes an inhibitory effect in bone loss by reducing the number of osteoclasts in ovarietomized mice

(Chiba, Uehara et al. 2003). Soy flavonoids (isoflavones) such as genistein and daidzein are the most popular natural isolated compounds in preventing osteoporosis. They give significant positive effects in alleviating postmenopausal symptoms (Mathey, Mardon et al. 2007). It is believed that flavonoid compounds might participate in the mechanisms of maintaining bone health. In the study, the crude extracts were further separated into four fractions according to their flavonoids-nature. Total flavonoids fraction is a mixture of all the flavonoids compounds of the herb.

Non-flavonoids fraction

This is the fraction which depleted all the flavonoids compounds.

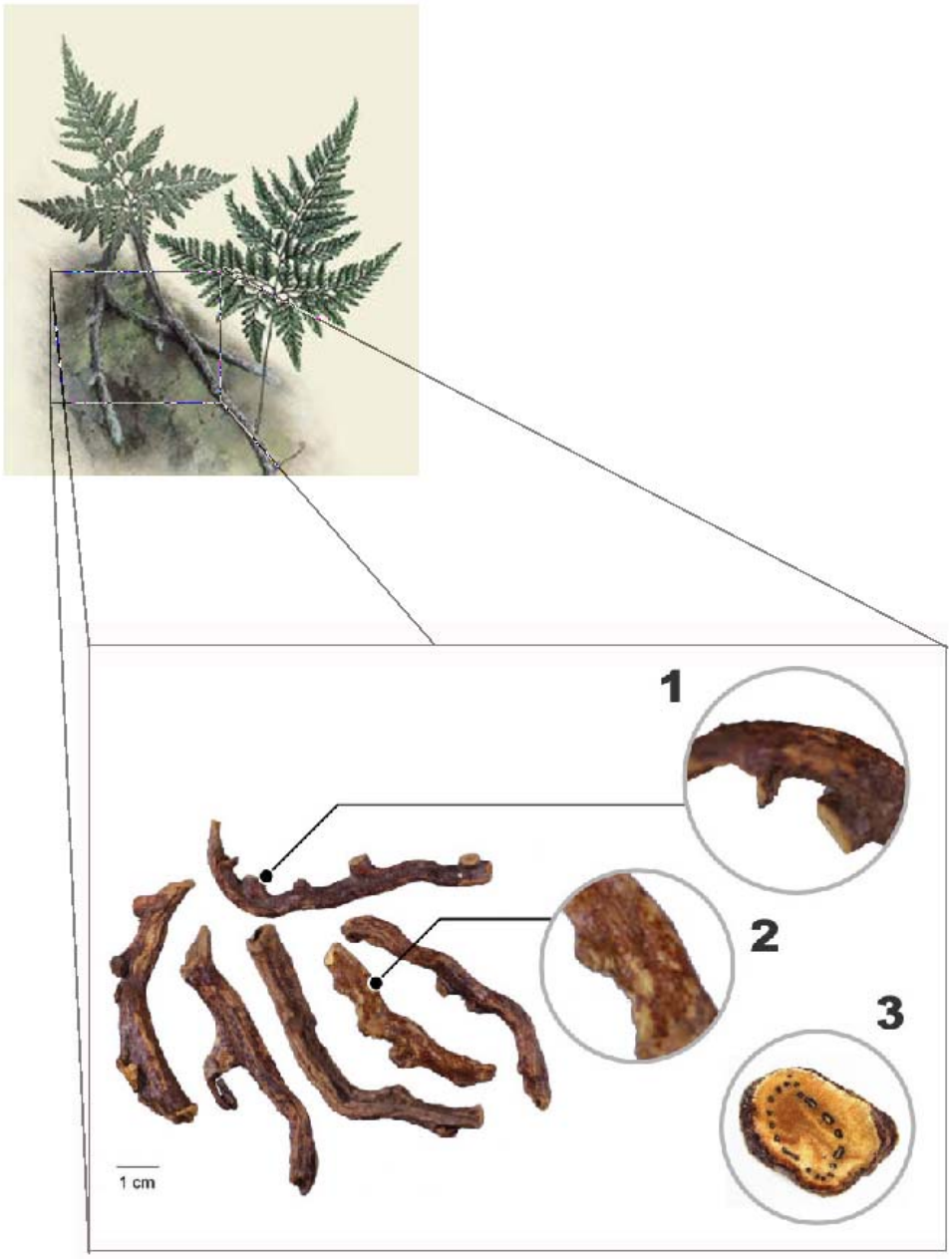


Figure 1.1 The dried form of Rhizoma Drynariae. *(Adopted from Easily Confused Chinese Medicines in Hong Kong : www.hkcccm.com/photos/43_1_s.jpg)*

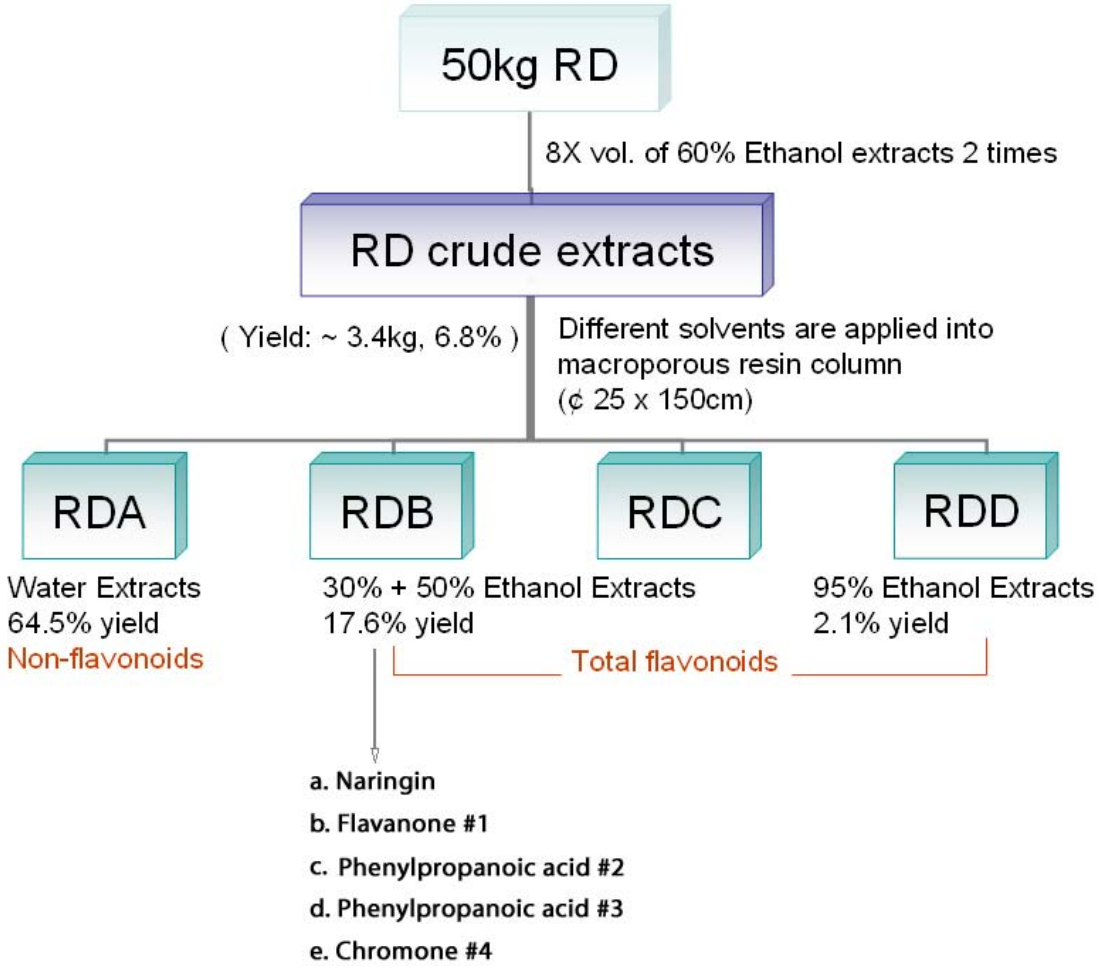


Figure 1.2 A simplified flow chart of the extraction of Rhizoma Drynariae. RD crude extracts were obtained from dried RD washed with 8X volume of 60% ethanol twice. Further differentiation of flavonoids compounds were done by dissolving them in four different solvents: water, 30%/50% and 95% ethanol. The mixture of RDB, RDC and RDD are regard as total flavonoids fraction. 5 single compounds, which were selected for the molecular studies, were all derived from the RDB fraction.

1.2.3 Preparation and Isolation of RD Single Compounds *(work done by Xin-luan Wang, ShenYang Pharmaceutical University)*

The major compositions of the RD crude extracts were obtained by using HPLC (High Performance Liquid Chromatography) techniques. Figure 1.3 shows that Naringin is the most abundant single compound of the RD crude extract. Other single compounds were derived from 30% Ethanol (RDB) extracts. They were named Flavonone #1, Phenylpropanoic acid #2, Phenylpropanoic acid #3 and Chromone #4 (Table 1.1). Figure 1.4 shows the abundance of Naringin, Flavonone #1 and Chromone #4 in 30% Ethanol RD extract.

Naringin

Naringin is the most abundant isolated flavonoid compound of Rhizoma Drynariae. Table 1.1 shows its chemical structure. It consists of more than 30% but less than 100% in total flavonoids fraction (Xie 2004). In this study, the RD crude extracts we used contained 53.3% Naringin (Fig. 1.3). The results display that Naringin is the major component of RD. Research on Naringin has focused on its anti-oxidant and anti-cholesterol effects. Naringin was also shown to have an inhibitory effect on HMG-CoA reductase inhibitory effect. Based on its ability, it is suggested that Naringin can trigger bone mass production through its ability to activate the BMP-2

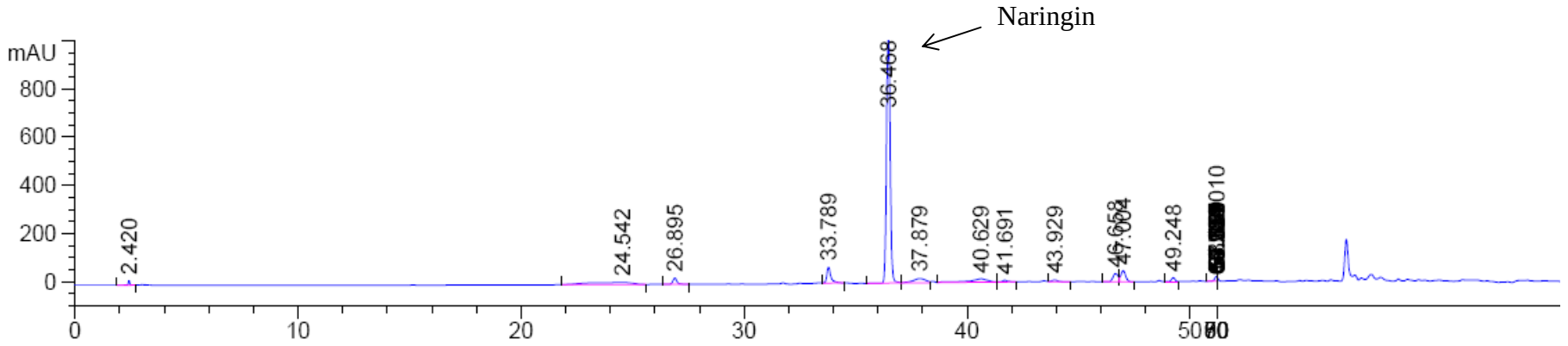


Figure 1.3 The HPLC profile of Rhizoma Drynariae total extracts. There are eleven isolated peaks which could be identified in HPLC profile. According to the chemical analysis, about 53.3% of the content is regarded as Naringin. It is believed that Naringin is the most bio-active ingredient in Rhizoma Drynariae crude extracts. *(Adapted from the chemists of Shenyang Pharmaceutical University)*

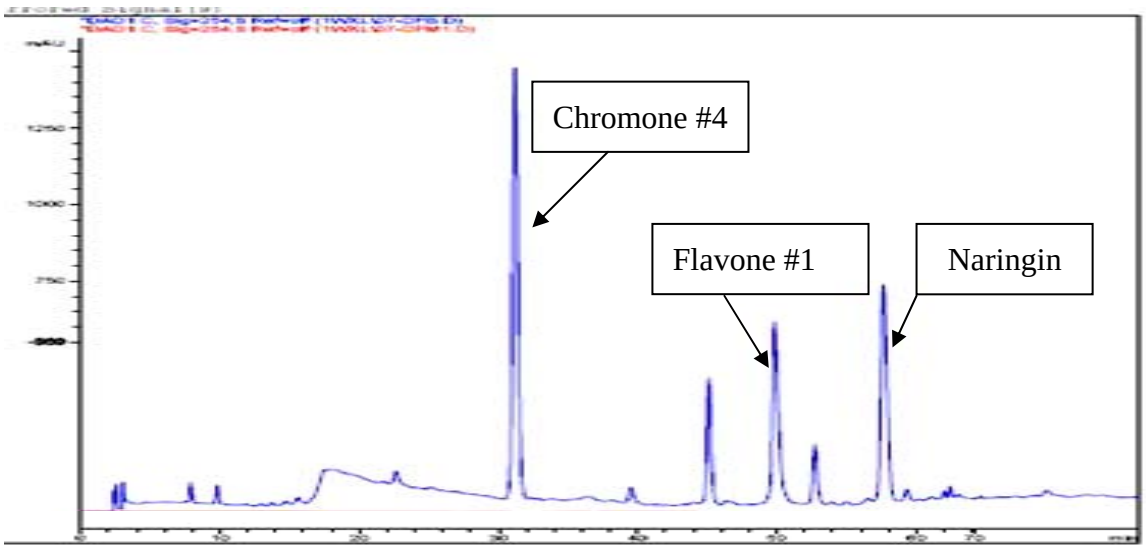
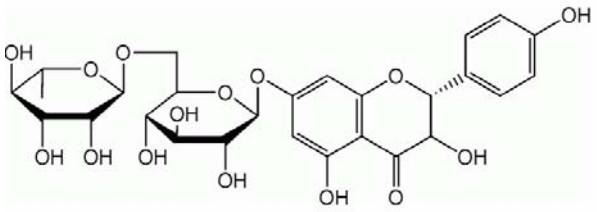
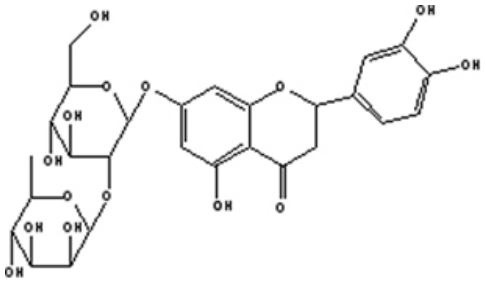
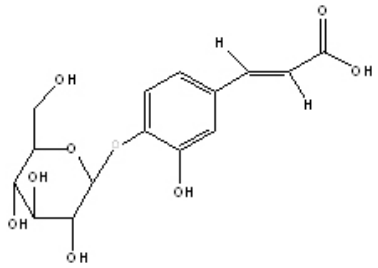
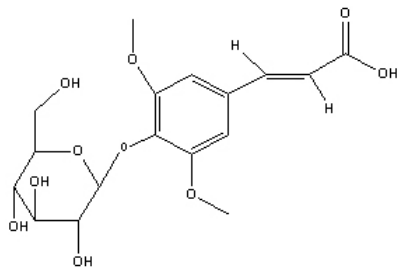
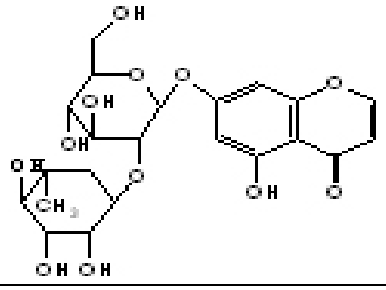


Figure 1.4 The HPLC profile of 30% ethanol Rhizoma Drynariae extract (RDB)

There are about five major peaks in the profile. The most abundant one is Chromone #4. Naringin and Flavone #1 had similar quantities to Naringin.

Table 1.2 The molecular sizes and the chemical structures of 4 single compounds isolated from RD

Single compounds	Molecular size	Chemical Structure
Naringin	580	
Flavone #1	596	
Phenylpropanoic acid #2	342	
Phenylpropanoic acid #3	386	
Chromone #4	486	

promoter (Wong and Rabie 2006). Wong and Rabie demonstrated that Naringin promotes osteoblastic cells proliferation (Wong and Rabie 2006b), as well as improves bone healing in a rabbit model (Wong and Rabie 2006a). Although the efficacy of Naringin has been confirmed, the mechanisms of actions are still unclear. The estrogenic effect of Naringin on bone is worth studying.

Other bio-active single compounds

Apart from Naringin, other active single compounds extracted from Rhizoma Drynariae are believed to be effective in treating osteoporosis. In this study, four more single compounds were selected for further molecular investigations (Table 1.1). Flavone #1 and Chromone #4 were the most abundant isolated compounds found in 30% Ethanol (RDB) extract (Fig. 1.4). Phenylpropanoic acid #2 and Phenylpropanoic acid #3 were classified as non-flavonoid compounds. They were also extracted from the RDB fraction (30% Ethanol extract) which contained about 1.6% and 0.5% in RD crude extracts respectively. It is hypothesized that these compounds would promote an osteoprotective effect *in vitro*.

1.3 Bone Biology

1.3.1 Composition of Bone

Bone consists of 20 to 40% organic matrix, mostly are Type I collagen, small quantities of type III, V and X, and other non-collagenous proteins (NCPs). Apart from the organic matrix, 50-70% of bone is inorganic mineral, such as hydroxyapatite that houses many impurities like carbonate, citrate, magnesium, etc. These impurities make the bone crystal imperfect, thus provide an advantage of enabling the release of calcium, phosphorus and magnesium (Hong-wen Deng 2005).

1.3.2 Bone Anatomy and Physiology

A typical long bone consists of a central cylindrical diaphysis and an epiphysis at each end. The metaphysis is the conical region connecting the diaphysis with the epiphysis (Figure 1.5). The joint is the connecting point between femur and tibia. The metaphysis is composed of a sponge-like network of interconnected trabecular plates and spicules generated by the growth plate complex. It is the region for BMD measurements. The total cross sectional area of the growth plate is also measured in metaphysis. The cortical bone is a semi-solid shell that covers the entire bone. It is thin in the epiphyseal and metaphyseal regions, but thick in the diaphyseal region. The cortical BMD could be detected in the diaphysis (Hong-wen Deng 2005).

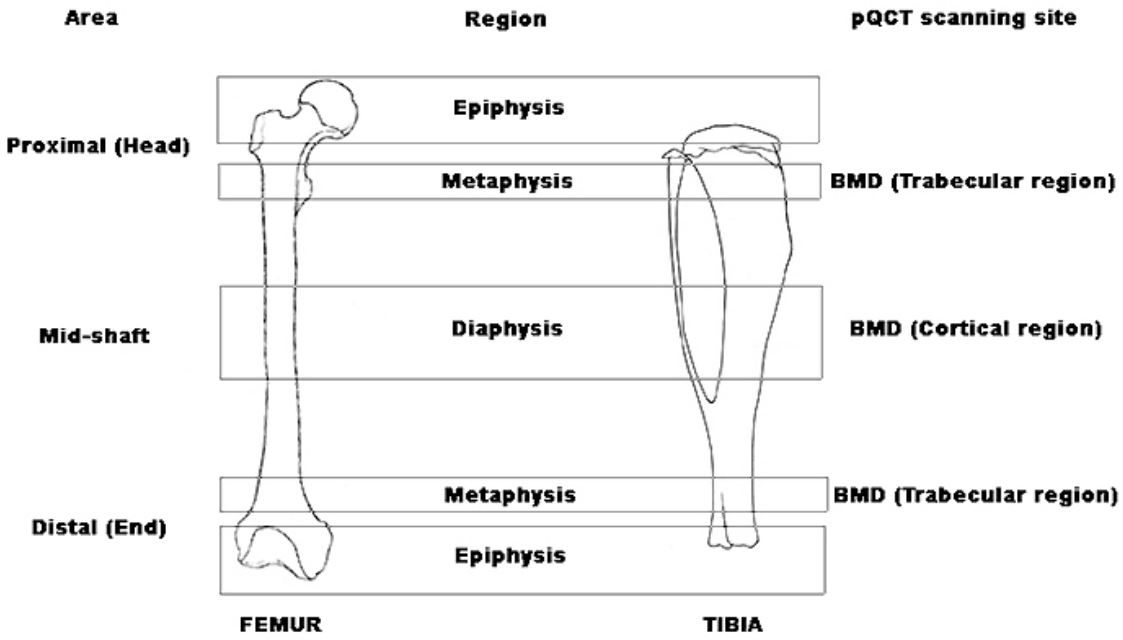


Figure 1.5 The typical mouse femur and tibia. The proximal area consists of the epiphyseal and metaphyseal regions. Trabecular BMD is measured in the metaphyseal growth plate. The cortical BMD is scanned in the diaphysis region in the mid-shaft area.

1.3.3 Bone Remodeling

Bone remodeling is a dynamic, lifelong process in which an old bone is removed from the skeleton to make room for new bone formation. This is a coupled process involving bone resorption by osteoclasts and new bone formation by osteoblasts. Usually, the bone resorption and formation rates are in balance and maintain skeletal strength and integrity can be maintained. However, for women over 30 years old, bone resorption may begin to outpace bone formation. The failure to reach peak bone mass or the uncoupling of remodeling may result in bone fragility (McCormick 2007).

1.3.3.1 Osteoblasts

Osteoblasts are bone cells responsible for bone formation. They synthesize almost all of the constituents of the bone matrix and direct its subsequent mineralization. Bone morphogenetic proteins (BMPs), ALP and osteocalcin are the common bone markers in osteoblastogenesis. Other growth factors such as TGF-β are also responsible for the actions of osteoblasts proliferation. Osteoblasts regulate the production of osteoclasts and the bone resorption cells by secreting the receptor activator of NF-κB ligand (RANKL). The stromal-osteoblast lineage cells also secrete osteoprotegrin (OPG). It is a soluble decoy receptor that neutralizes RANKL (Riggs 2000). Although estrogen is known to be the key sex hormone governing bone homeostasis, the RANK /

RANKL / OPG system is now being recognized as the primary regulator of bone remodeling. Studying this system may help us understand the physiology of bone loss and bone fragility (McCormick 2007). Osteoblasts play an important role in regulating bone remodeling (Figure 1.7).

The UMR-106 cell line (**CRL-1661™)** is a clonal derivative of a transplantable rat osteosarcoma induced by the injection of radiophosphorous (32P) (ATCC 2007). In this study, this cell line was utilized to investigate the mechanism of the actions of Rhizoma Drynariae or its isolated compounds that are involved in osteoblastic cell activities. This fast growing property shortens the screening process and the cell is easy to handle.

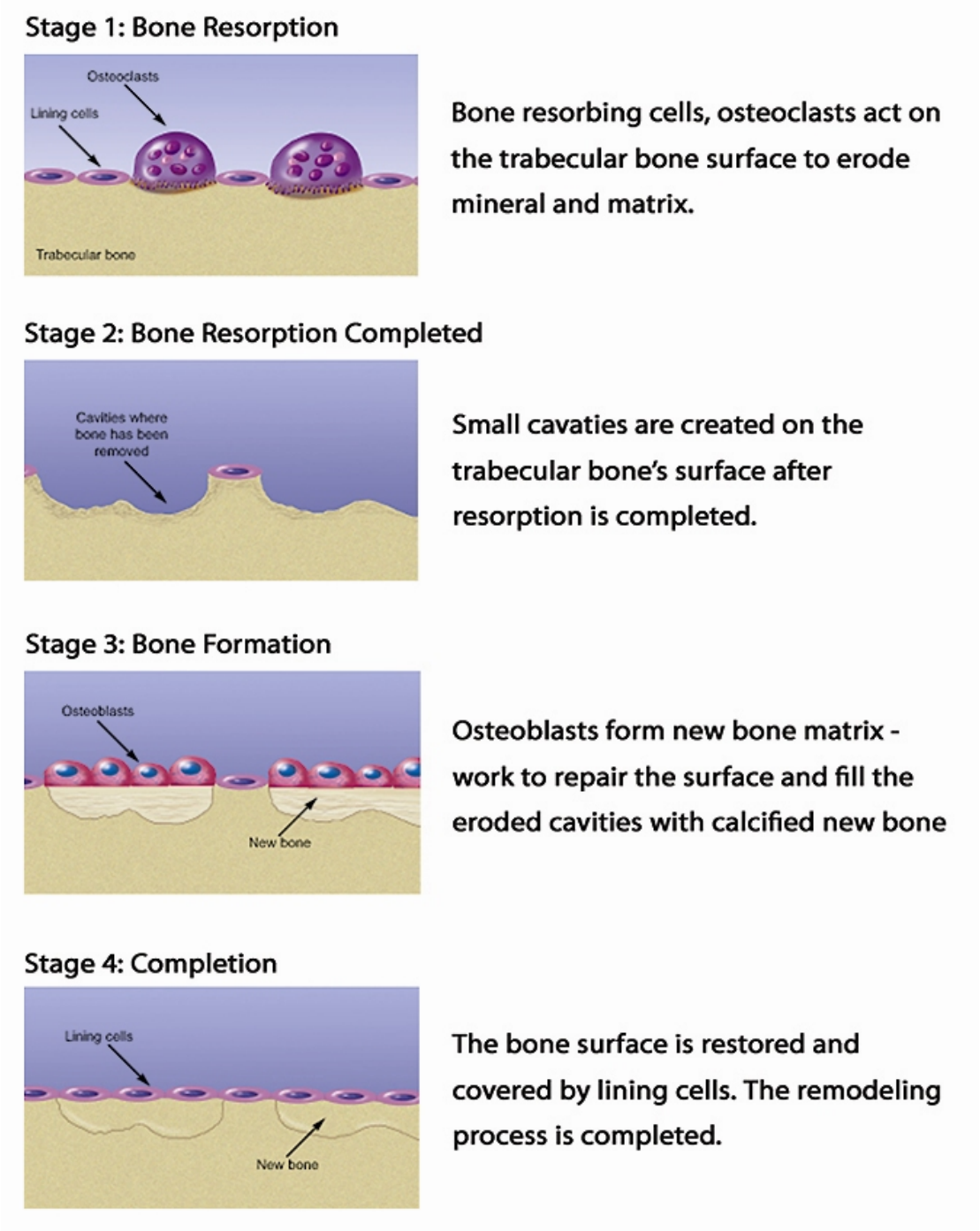


Figure 1.6 The bone remodeling process. *(Adapted from (Lilly 2001)*

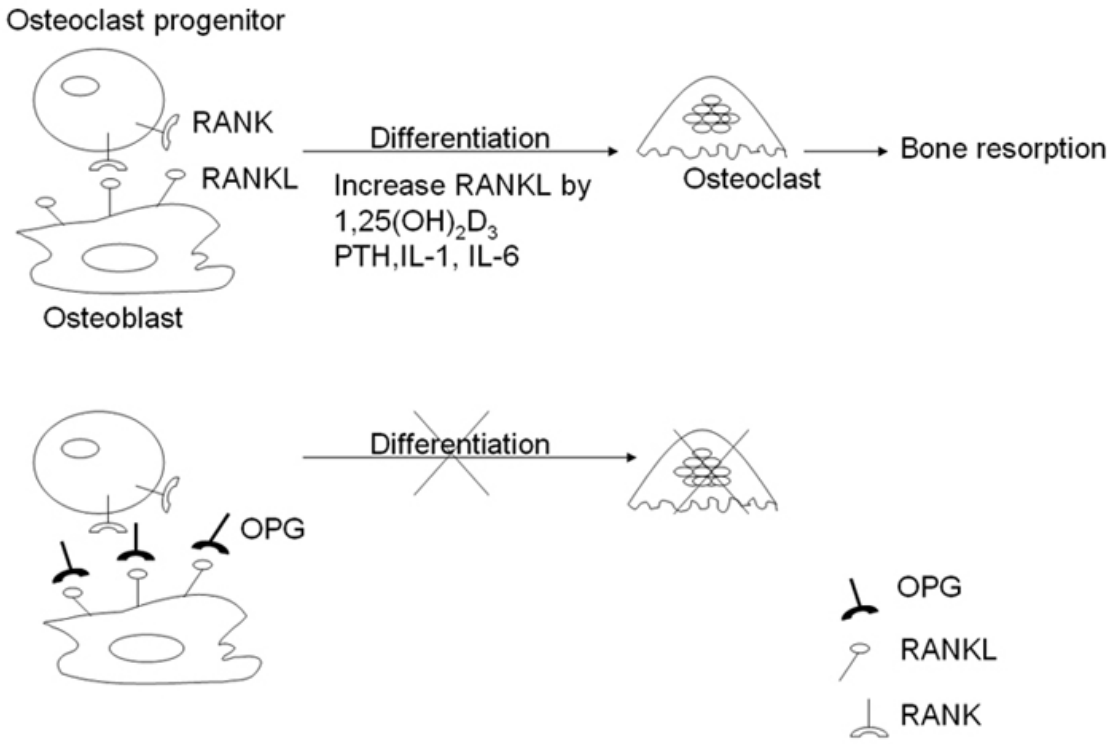


Figure 1.7 The roles of OPG and RANKL in osteoclastogenesis RANKL mRNA expression in the presence of OPG mRNA production accounts for a net reduction in osteoclast differentiating signals from osteoblasts as the relatively abundant OPG saturates RANKL. RANKL binding to the receptor on osteoclast progenitor cell is thus inhibited. The differentiation of osteoclasts is then directly suppressed *(Adopted from (Mackie, Fisher et al. 2001)*

1.3.3.2 Osteoclasts

Osteoclasts are specialized bone cells whose primary function is to resorb bone and provide sites for new bone formation. The resorptive phase of bone remodeling is initiated by the attachment of osteoclasts to the bone surface (Jeong, Kang et al. 2003). Osteoclastogenesis, which defined as the formation of activated ostoeclasts, would be happened when stromal-osteoblast lineage cells contact the osteoclast lineage cells, the RANKL which is secreted from osteoblasts is allowed to bind to its physiologic receptor, RANK, and potently stimulate all aspects of osteoclastic functions (Riggs 2000). As mentioned in 1.3.3.1, OPG, a soluble decoy receptor that blocks RANKL, can maintain the control of the remodeling process.

1.3.3.3 Hormonal Regulation - Estrogen

17β-estradiol (E2) is the most potent and dominant estrogen in humans (Lanyon, Armstrong et al. 2004). Estradiol plays an important role in regulating the growth, differentiation and physiology of the reproductive process through the estrogen receptors (ER). It also affects other tissues, such as bone, liver, brain and cardiovascular system (Pearce and Jordan 2004). Estrogens circulate in men and women, and play an important role in the maintenance of bone homeostasis and bone metabolism. Estrogens have been identified as the major inhibitor of bone resorption

in both men and women and estrogens slow the rate of bone remodeling and protect against bone loss by attenuating the rate of differentiation of osteoblasts and osteoclasts from the precursor cells as well as affecting the apoptosis of osteoblasts and osteoclasts. With reduced estrogen levels, the body natural ability is unable to control the production of RANKL, and increased osteoclastogenesis (McCormick 2007).

The classical recognized pathway of estrogen in osteoblasts involves the binding of estrogen to the receptors (ERs) in the nucleus, after which the ERs dimerize and bind to specific response elements known as estrogen response elements (EREs) located in the promoters of target genes. The responsive genes are responsible for increasing the number of osteoblasts. Figure 1.8 shows the classical pathway of estrogen regulation in osteoblast. 17β-estradiol has been used as a positive control in the study of the estrogenicity of Rhizoma Drynariae or its isolated compounds. Besides the biological effects of estrogens mediated through estrogen receptor (ER) α and β which act as ligand-activated transcription factors, there is evidence for signaling pathways which is deviated from this classical model. It has been found that one third of the human genomes that are regulated by ERs do not contain any ERE-like sequences. Several hypothetical actions have been confirmed that ERs may involve in the regulation of gene expression without directly bind to DNA. The actions are postulated to modulate

the function of other classes of transcription factors through protein-protein interactions in the nucleus. Figure 1.9 illustrates the regulation of transcription by ERs without any ERE binding (Bjornstrom and Sjoberg 2005).

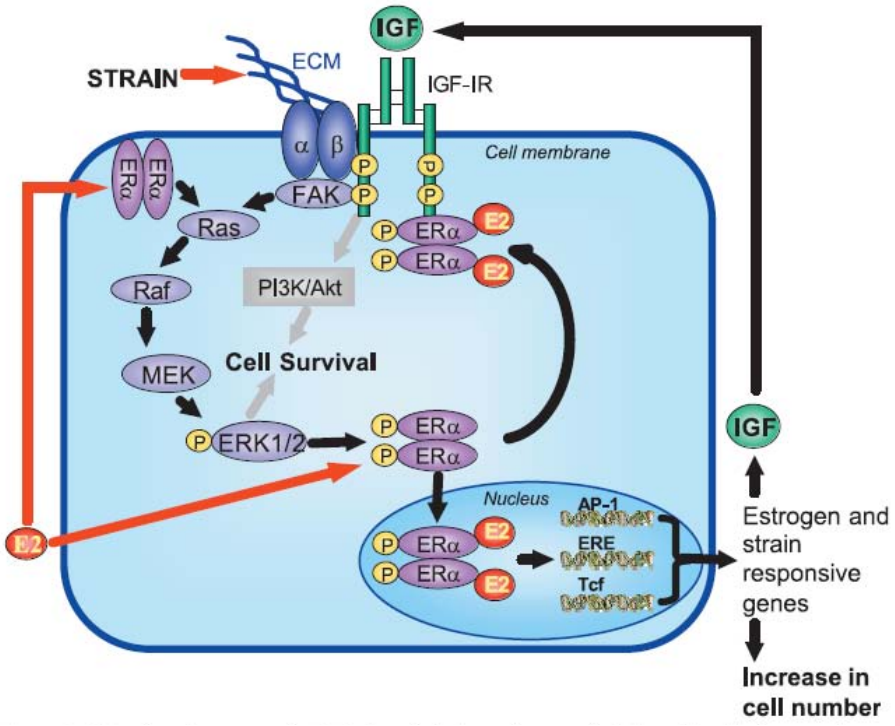


Figure 1.8 Possible pathways for estrogen and strain signal transduction in osteoblasts. In order for bone cells to adjust bone mass in response to changes in mechanical loading, resident bone cells respond to the local strains that such loading engenders. Integrins, in conjunction with the IGF-IR, play an important role in the initial transduction process, which initiates a cascade of events, including activation of the MAPK/ERK pathway and subsequently ER-α. As with estrogen (E2), strain causes ERK1/2 to phosphorylate ER-α resulting in both classical activation of gene transcription within the nucleus and ER participation in extranuclear signaling events. IGF-I and –II genes regulated by E2 and strain interact in an autocrine or paracrine fashion at the IGF-IR, which in turn requires ER-α to be present for signaling to occur. *(Adapted from (Lanyon, Armstrong et al. 2004)*

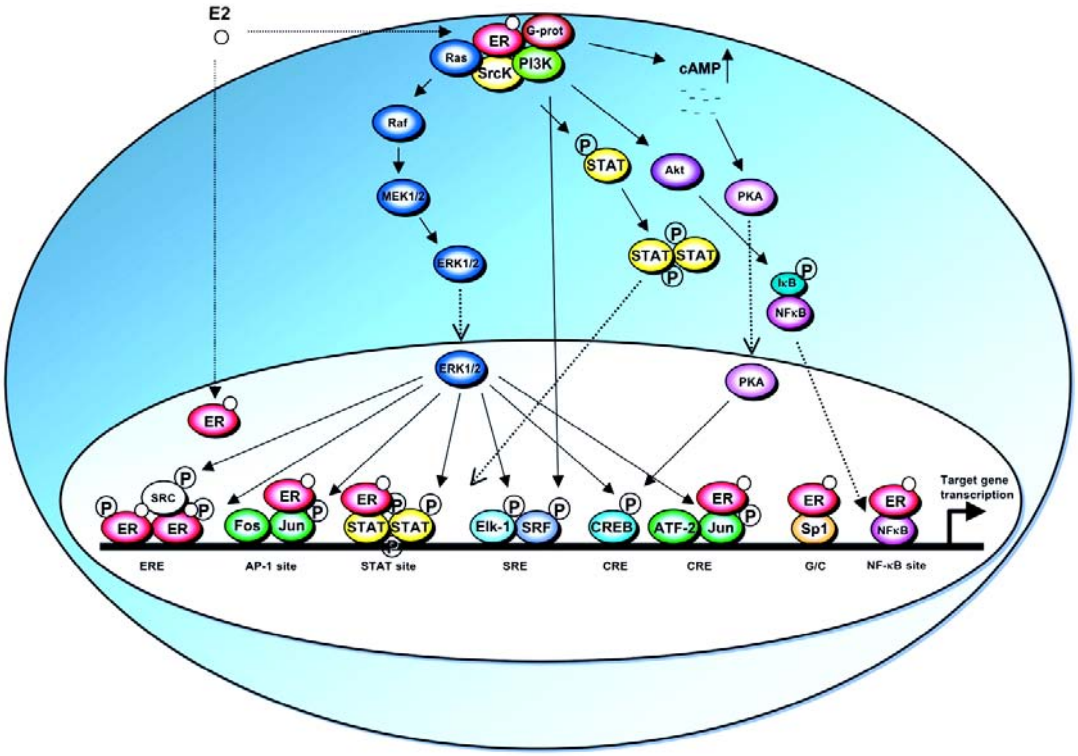


Figure 1.9 Schematic illustration of how genomic and nongenomic actions of ERs on a target gene promoter may converge. Nuclear E2-ER complexes bind to EREs and transcription factor complexes, *e.g.* AP-1, STATs, ATF-2 (activation transcription factor 2)/c-Jun, Sp1, and NF-κB, that are bound to their cognate DNA binding sites. Membrane E2-ER complexes activate protein-kinase cascades, leading to phosphorylation (P) of target transcription factors, *e.g.* AP-1, STATs, Elk-1, SRF (serum response factor), CREB and NF-κB. The phosphorylation results in their transcriptional activation and/or in modulation of the transcriptional activities of ER-AP-1, ER-STAT, ER-Sp1 and ER-NF-κB complexes at the promoter. Protein-kinase cascades also target ERs themselves and steroid receptor coactivator coactivators, resulting in an enhanced transcriptional activity of ERs at EREs. The distinct actions of ERs at multiple response elements provide an extremely fine degree of control for the regulation of target gene transcription. *(Adapted from (Bjornstrom and Sjoberg 2005)*

1.3.3.4 Biochemical Markers of Bone Turnover

Biochemical markers of bone turnover are commonly divided into bone formation and resorption markers. Quantitative changes in the markers reflect the dynamic process of bone metabolism. If a patient has significant higher than normal bone formation and resorption markers, it reflects that high bone turnover rate has happened and an associated bone loss that might occur with estrogen deficiency (Lane 2006).

Markers of bone formation are usually released from osteoblasts and it is possible to detect them in serum. Common useful markers, such as bone-specific alkaline phosphatase (BSAP) and osteocalcin mentioned in section 1.1.1, are widely used in clinical research. Type I collagen is another common bone formation marker. However, a currently available assay could not differentiate bone derived type I collagen from serum, BSAP and osteocalcin are more reliable than type I collagen marker. Bone resorption markers, including the collagen breakdown products like urinary pyridinoline (Pyd), deocypyridinoline (Dpd), and cross-linked and N-telopeptides (NTx), are secreted during osteoclastic activity (Lane 2006). Elevated levels of resorption markers indicate increased osteoclastic activity and a higher risk for osteoporortic hip fracture, independent of BMD (McCormick 2007). Tartrate-resistant acid phosphatase 5b (TRACP 5b), an osteoclast-specific isoform of lysosomal enzyme, is considered to be a promising marker for predicting vertebral

fractures. It is more stable and specific than TRACP (Lane 2006).

1.4 Evaluation of Efficacy and Molecular Mechanisms

Osteoporotic fractures are the major cause of morbidity and disability in postmenopaual women. World-wide monitoring of osteoporosis in patients aims to prevent them from fractures. Fracture risks, however, could not be directly measured *in vivo*. The WHO has determined the measurement of bone mineral content (BMC) or bone mineral density (BMD) as the major diagnosis of osteoporosis. BMD typically represented about 60-80% of bone strength when bone samples are compared in a laboratory setting under control loading conditions. According to the statement, it is assumed that there is a direct relationship between BMD and bone strength. In terms of pre-clinical studies, we can examine the bone strengths *ex vivo* by applying bone specimens on force/deformation engine under compression. DEXA (Dual-energy X-ray absorptiometry) and pQCT can be used for the BMD assessment (Prentice 2004). Apart from mechanical end points assessment, serum and urinary biochemical markers can demonstrate metabolic changes *in vivo* (McCormick 2007).

1.4.1 Serum and Urine Biochemical Markers

Calcium (Ca) and phosphorus (P) are the most abundant minerals in body and

constitute major portions of the hydroxyapatite crystal in bone mineralization. Phosphorus and calcium levels are maintained through PTH, vitamin D and changes in renal tubular reasborption. Serum and urine Ca and P levels are therefore combined to investigate the mineral regulations in bone homeostasis. For example, excessive urine calcium loss is commonly found in osteoporotic patients. Since urinary calcium could be affected by its volume, urinary calcium per creatinine ratio (uCa/Cr) can be used as a general screening tool for urine calcium levels (McCormick 2007)

1.4.2 Biomechanical Bone Strength

In this study, mechanical bone strength was determined based on the "force and deformation" and "stress and strain" concepts.

Force and deformation

The biomechanical bone strength of tibia could be directly measured *ex vivo*. Special applications, such as materials testing machines were accommodated to test the tibia bone strength based on its flexural loading (bending), stiffness, flexural modulus, etc (Olsen 2007). A three-point bending test was applied to the measurement of flexural loading (Figure 1.10). In this test, the uniform stress is concentrated under a center loading point. A force/deformation curve is driven for further calculation. Maximum load is calculated for the increments of load while flexural modulus is calculated from

the slope of the stress versus the force/deformation curve (Instron 2007).

Stress and Strain

As the data generated from the force-deformation curve could be affected by the size of specimen and the load mode applied, the material properties of the bone could not be truly reflected. It is necessary to transfer it into a stress-strain relationship for the understanding of the material properties (Hong-wen Deng 2005).

The stress-strain index could be simulated in pQCT. Computed tomography techniques could generate the polar stress-strain index (polar SSI) for references. In this study, both of the measurements were applied to interpret the therapeutic effects of Rhizoma Drynariae on biomechanical bone strengths (Hong-wen Deng 2005).

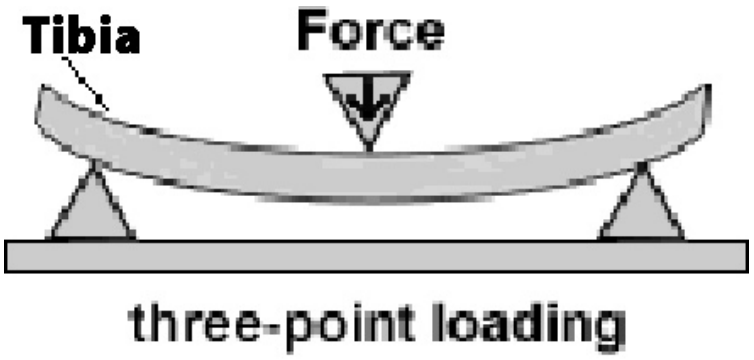


Figure 1.10 The principle of 3-point loading. Extensive force is applied on the centre of the intact long bone. The loaded stress and the change of extension were recorded simultaneously. The force/deformation curve was deduced for further calculations of mechanical properties. *(Adapted from Instron 2007)*

1.4.3 Bone Mineral Densities (pQCT)

Quantitative computed tomography (QCT) is an established technique for the determination of bone mineral density (BMD) in the axial and appendicular skeleton. Peripheral QCT (pQCT) is a special type of computed tomography in which scans of the appendicular skeleton are performed at a low radiation dosage. Bone development can be assessed by pQCT at peripheral sites in studies of the bone loss in experimental models, in monitoring the effectiveness of therapeutic interventions, etc. For monitoring of bone densities, proximal tibia metaphysic has been the preferred site for the pQCT measurements. This site is rich in cancellous bone and reacts with the greatest magnitude of physical changes, such as OVX. Another ideal site to study the cancelleous and cortical bone is the distal femur metaphysic. Since very few scanner algorithms can separate cancelleous and cortical bone in a perfect way, a site of “absolute” cortical bone should also be measured. One of the recommended sites with high precision in longitudinal studies is the mid-diaphysis of tibia (Gasser 2002).

Chapter 2

Objectives and Hypothesis

2.1 Objectives of the Study

Osteoporosis is a metabolic bone disease associated with the increase of the risk of fractures incidence (Tuck and Francis 2002). It arouses public concerns to assess patients at risk to allow for the prevention and early intervention.

TCM has been widely used in orthopaedic clinical practice for thousands years for the treatment of fractures and joint diseases (L, G et al. 2003). Some medicinal herbs are demonstrated to be "*kidney*-tonifying" are used in traditional Chinese medicine formulae for the prevention and treatment of osteoporosis. In 2006, Wang *et al.* reviewed that Rhizoma Drynariae is one of the herbal medicine that has significant positive effects in preventing osteoporosis (Wang, Zhang et al. 2006). However, the active ingredients of RD have not been completely identified.

The objective of this study is to develop Rhizoma Drynariae as an alternative treatment of postemenopausal osteoprorsis, by studying the optimal osteoprotective effects of RD total flavonoids on bone and mineral metabolism using osteoporotic female mice. Another aim is to use UMR-106 cells to screen the bioactive osteoprotective ingredients in RD. Based on the information provided by the screening, we can characterize the mechanisms of actions of RD followed by detailed investigation.

2.2 Significance of the study

By demonstrating the efficacy, elucidating the mechanisms of actions and identifying active ingredients of Rhizoma Drynariae, it is hoped to improve the international acceptance of RD.

Chapter 3

Methodology

In this thesis, the *in vivo* and *in vitro* osteoporotic effects of total flavonoids fraction of Rhizoma Drynariae (RD TF) and the single compounds extracted from the herb such as Naringin are demonstrated. The results on RD TF and other single compounds could be found in Chapter 4, 5 and 6. The integrated methodologies of the experimental set-ups are shown in this chapter.

3.1 Materials and Methods: *In Vivo* Study

3.1.1 Animals Care and Diet

Eighty one-month old female C57B/L6J mice (Jackson Laboratory) were housed in environmentally controlled central animal facilities. The animals were acclimated in 22 ^{o}C, light:dark (12:12) conditions, and fed with a normal calcium level control diet (Appendix 1) for 2 days before the treatment. The mice were either sham-operated or ovariectomized (OVX) at one-month old. After recovery for two weeks, the mice were randomly selected and divided into 8 groups. One group was sham vehicle (2% Ethanol), the others were OVX vehicle; OVX 17β-estradiol (2μg/g/day); 3 groups of OVX Rhizoma Drynariae total flavonoids herbal extracts which were divided in 3 dosages: *Low dose:* 0.087; *Medium dose:* 0.173 and; *High dose:* 0.347 mg/g/day; lastly, 2 groups of OVX Naringin. The dosages used in the OVX Naringin groups were 0.2 and 0.4 mg/g/day. The total flavonoids fraction of RD was obtained from

Shenyang Pharmaceutical University, while Naringin was obtained from Sigma-aldrich (#N1376). Tables 3.1 and 3.2 show the abbreviation of the mice groups and the dosages used in treatment. All the mice were weighed on a weekly basis, and they had free access to distilled water and diet for six consecutive weeks. One day before the end of treatment, the mice were placed in metabolic cages for 24 hours. Their urine was collected and centrifuged (4000rpm for 20 min) immediately to remove impurities. The urine samples were then stored in -20 ^{0}C until use. At the end of the treatment, mice serum was collected, followed by the collection of uteri and bone specimens, including femur, tibia and lumbar spine. The mice serum was stored in -80 ^{o}C after centrifuging the blood sample at 1200 rpm for 15 minutes. Uteri were collected for weight measurement. Lumbar spine, left femur and tibia were cleaned and wrapped with saline cotton before storing in -20 ^{0}C for bone scans, while right femur and tibia were frozen immediately in liquid nitrogen for molecular assays, such as mRNA expression. Figure 3.1 shows the experimental set-up of the *in vivo* study.

Table 3.1. Abbreviations of treatment groups used in Chapter 4. This is aimed to study the *in vivo* osteoporotic effects by administrating different dosages of Rhizoma Drynariae total flavonoids.

Abbreviation	Operation	Drug
V	OVX	Veh (2% Ethanol)
E	OVX	2 μg/g 17β-estradiol
L	OVX	0.087 mg/g RD TF
M	OVX	0.173 mg/g RD TF
H	OVX	0.347 mg/g RD TF
S	Sham	Veh (2% Ethanol)

Table 3.2. Abbreviations of treatment groups used in Chapter 5. This is aimed to study the *in vivo* osteoporotic effects by administrating different dosages of Naringin.

Abbreviation	**Operation**	**Drug**
V	OVX	Veh (2% Ethanol)
E	OVX	2 ug/g 17β-estradiol
N 0.2	OVX	0.2 mg/g/day naringin
N 0.4	OVX	0.4 mg/g/day naringin
S	Sham	Veh (2% Ethanol)

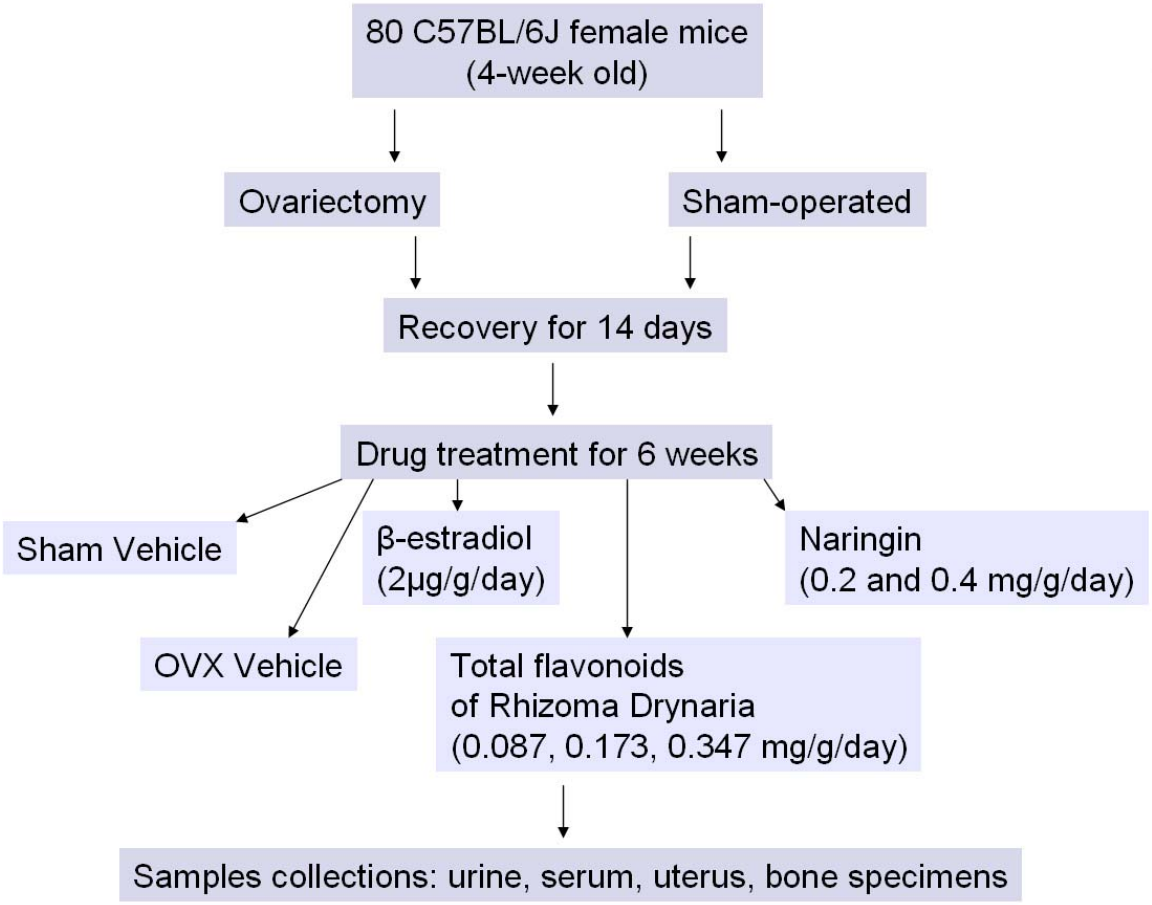


Figure 3.1 Experimental set-up of the animal study. Eighty 4-week old C57BL/6J female mice were ovariectomized or sham-operated. After 2 weeks of recovery, total flavonoids fraction of RD (RD TF) or Naringin was administered daily. Vehicle and 17β-estradiol were prepared as negative and positive control groups respectively. After 6 weeks of drug treatment, urine samples were collected to study the metabolic effects of collagen degradation and mineral reservation. Serum samples were collected for biochemical markers assays. The uterotrophic effect of drugs could be observed by uteri weight. The bone specimens including femur, tibia and lumbar spine were obtained for bone scanning and bone-specific mRNA expression.

3.1.2 Systemic Serum and Urinary Biomarkers of Bone Metabolism

Serum and urinary calcium (sCa, uCa) and phosphorus (sP, uP) were assessed in this study. sCa and uCa were measured by using the OCPC (O-cresolphthalein complexon color development) method, while sP and uP were determined by the p-Methylaminophenol method. Calcium and phosphorus levels were determined by using commercial kits, Wako, Japan; sCa/uCa: 3272-21801; sP/uP:270-49801, repectively.

In addition, urinary excretion of deoxy-pyridinoline (Dpd), a collagen degradation product, was measured for the determination of the effects of bone resorption. The urinary samples were assayed in Quidel Metra® DPD EIA kit with a microplate reader. Urinary creatinine concentration, which was used as internal control, was also assessed with the Jaffe method by using commercial kit (Wako, Japan #277-10501). Dpd concentrations were expressed as DPD excretion per unit of creatinine.

3.1.3 Assessment of Bone Density Using pQCT

The left femur, tibia and lumbar spine region L1 were scanned using pQCT (peripheral Quantitative Computed Tomography). Trabecular and cortical bone densities were measured using StraTec XCT 2000 machine (Norland Stratec Medizintechnik GmbH, Birkenfeld, Germany). The proximal, mid-shaft and distal

regions of the femur and tibia were scanned. The proximal site was defined as 2.5mm away from the femur/tibia head. The mid-shaft was the middle region of each bone. The distal site was 2.5mm away from the end of the femur or tibia. All scans were performed using the developed protocol designed for studying isolated small bones (Gasser). Total BMD, Trabecular BMD, total cross-sectional area, trabecular cross-sectional area and stress-strain index (SSI) in the proximal and distal region, cortical BMD and SSI in the mid-shaft region were obtained from in-house pQCT programme.

3.1.4 Biomechanical Measures of Tibia Mid-Diaphysis

After bone scanning, the left tibia was preserved immediately in phosphate-buffered saline (PBS) and stored at 4 ^{o}C until the performance of the 3-point bending test. A three-point bending machine (Hounsfield test equipment, UK) was used to perform a mechanical strength on the mid-shaft of the left tibia. The anterior side, which was the point receiving compression, was placed upward. A load was applied on the mid-shaft tibia until fracture occurred. All the specimens were pressed at a displacement of 5mm/min and a load-deformation curve (Fig. 3.2) was plotted simultaneously. Structural properties (including maximal load and stiffness) were determined from the load-deformation curve. The material properties were determined based on the

calculations of the curve with beam bending theory such as flexural modulus.

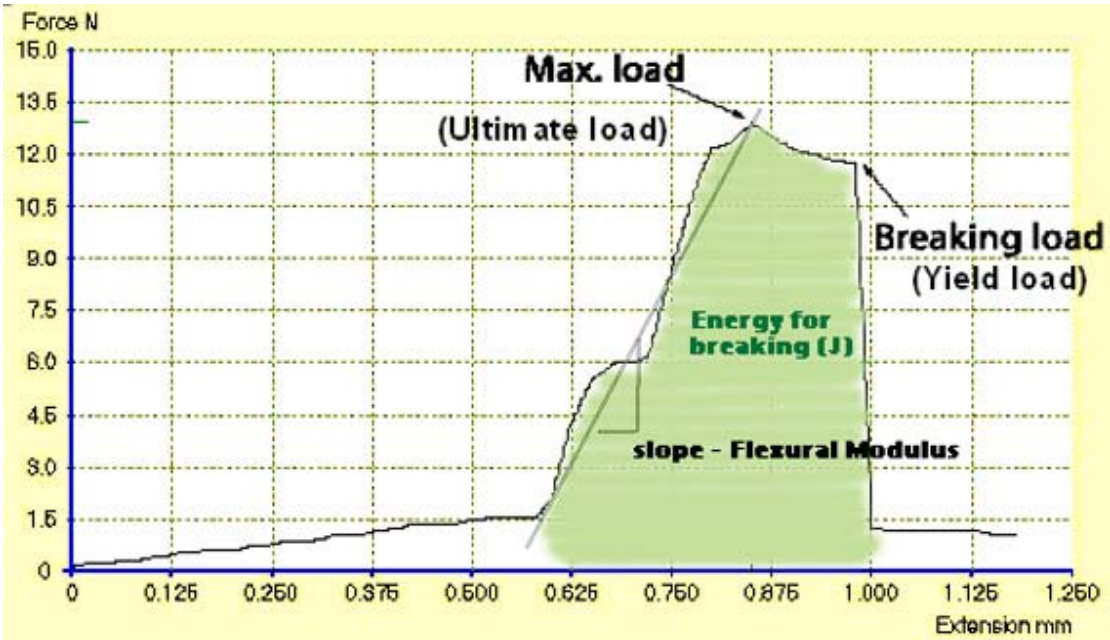


Figure 3.2 The load-deformation curve generated from Hounsfield test equipment. The graph indicates the force (N) applied on the tested tibia mid-shaft versus the distance of extension (mm). Maximal load (force), breaking load (force), flexural modulus (MPa) and energy for breaking (J) are deduced from this curve. *(Adopted from (Olsen 2007)*

3.1.5 Statistical Analysis

Data of the *in vivo* study are reported as mean ± SEM. Each set of data was combined from seven to eight individual measurements. Data was statistically analyzed by one-way analysis of variance (ANOVA). Critical differences between means were evaluated by Tukey's multiple comparison test set at $p < 0.05$.

3.2 Materials and Methods: *In Vitro* Study

3.2.1 Culture of Rat Osteoblast-like UMR-106 Cells

UMR-106 cells (Rat Osteosacroma, ATCC no. CRL-1661) were routinely cultured in Dulbecco's modified Eagle medium (DMEM) (Invitrogen, USA #12800-017) supplemented with 10% fetal bovine serum (FBS) (Gibco^{TM}, #16000-044), penicillin 100 U ml^{-1} and streptomycin 100 µg ml^{-1}. At about 80% confluence, cells were trypsinzied and seeded in a 96-well microtiter plate, 24-well or 12-well plates at a density of 3500, 25000 and 50000 cells per well, respectively. Cells were incubated at 37 ^{o}C in a 5% CO_2/95% air humidified atmosphere (Xie, Wu et al. 2005; Wong and Rabie 2006). After incubation for 48 hours, the culture medium was switched to phenol-red free DMEM supplemented with 1% charcoal stripped FBS for another 24 hours to remove steroids. Cells were then treated with Rhizoma Drynariae total flavonoids fraction, Naringin or other single compounds extracted from RD with four

to five concentrations, in order to study the dose-dependent effects. In terms of RD TF, the drug concentrations varied from 0.002 to 200 μg ml^{-1} were treated into the cells for 24 or 48 hours. RD total flavonoids fraction was dissolved in absolute ethanol and each final concentration was achieved by serial dilution. On the other hand, 10^{-9} to 10^{-5} M of Naringin was chosen to perform molecular assays. Other active compounds were selected for the examination of their molecular activities from 10^{-11} to 10^{-5} M. All single compounds were dissolved in absolute ethanol with serial dilutions.

3.2.2 Colorimetric Tetrazolium (MTS) Assay for Cell Proliferation

The CellTiter 96® AQ_{ueous} Non-Radioactive Cell Proliferation Assay (Promega, #G3580) is a colorimetric method for determining the number of viable cells in proliferation assay. The assay is composed of a novel tetrazolium compound (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H -tetrazolium), innier salt, MTS and an electron coupling reagent (phenazine methosulfate.; PMS). Cells were rinsed with PBS once, followed by the addition of 0.2 mg ml^{-1} MTS with 50μl ml^{-1} PMS). Viable cells would react with MTS and PMS to change the colorless MTS into orange or brown formazan. The absorbance of the formazan was then observed in a microplate reader at 490nm after two hours of incubation.

3.2.3 Cytoplasmic Total Protein Assay

Total protein is an indicator of the proliferative and biosynthetic capacities of bone cell cultures. Each well of the 96-well plate was lysed and the cellular material was transferred into 20µl/well of passive lysis buffer (Wong and Rabie 2006). The cellular protein concentration was determined by using specified Bradford assay (BioRad, #500-0006). The absorbance of Bradford reagent was observed in a microplate reader at 595nm after 10 minutes of incubation.

3.2.4 Biochemical Detection of the Alkaline Phosphatase Activity

The protocol of ALP assay which was developed by Spreafico A. and colleagues was followed (Spreafico, Frediani et al. 2006). Alkaline phsophatase activity was measured directly on the monolayer of cell cultures. After medium removal, cells were washed twice with PBS and dried for 5 minutes. The enzyme activities of the cell were inactivated by a freeze-and-thaw cycle. 100 µl/well of PBS containing 10mM *p*-nitrophenylphosphate (PNP; Sigma-Aldrich #N4645) was added and shaken for 30 minutes at 37 ^{0}C. The absorbance of colour change was measured at 405nm in a microplate reader. The alkaline phosphatase activity values were adjusted by the normalization to their relative cytoplasmic total protein concentration.

3.2.5 Estrogen Receptor (ER) Antagonist Assay

ICI 182, 780 purchased from Tocris bioscience (# 1047) was used to investigate the effects of RD total flavonoids, Naringin and other single compounds on regulating osteoblastic proliferation and differentiation with the ER mediated pathway. The chemical structure of ICI 182, 780 could mimic estrogen to antagonize ER effects in the study of estrogen functions (Figure 3.3) (Howell, Osborne et al. 2000). The cells were co-treated with the RD TF, Naringin and its other single compounds of optimal concentrations and 1μM of ICI 182, 780 for 24 or 48 hours. The blocking effects on cell proliferation and differentiation were determined according to the procedures described in 3.2.2 and 3.2.4.

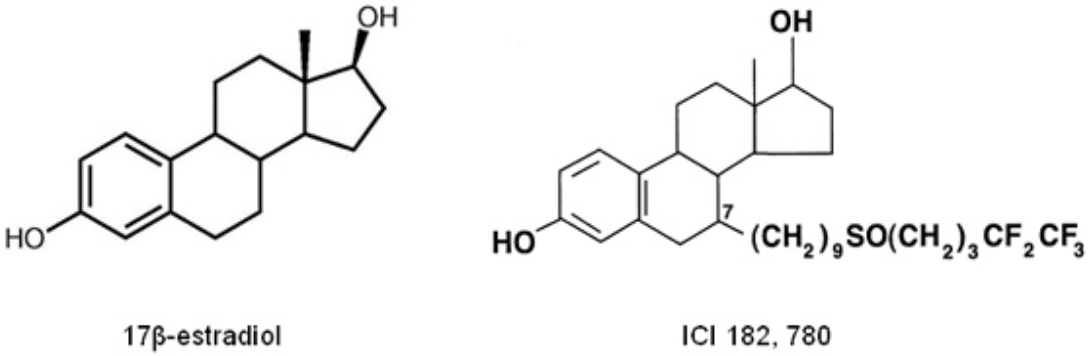


Figure 3.3 The chemical structures of 17β-estradiol and the estrogen antagonist, ICI 182, 780. The ICI blocker structurally resemble to 17β-estradiol with an additional bulky side chain at C-7. *(Adopted from (Howell, Osborne et al. 2000)*

3.2.6 Total RNA Extraction and Real-time PCR Analysis

Total RNA was isolated from cell cultured in a 12-well plate using TRIzol® reagent according to the manufacturer's instructions (Invitrogen, Rockville, MD, USA #15596-018). The concentration and purity of the RNA were determined by measuring the absorbance at 260 nm and 280 nm (Xie, Wu et al. 2005). 2 μg of total RNA was reversely transcripted to cDNA using a high capacity cDNA reverse transcription kit purchased from Applied Biosystems (#0707026). The mRNA of receptor activator of NF-κB ligand (RANKL) and osteoprotegerin (OPG) was determined by quantitative real time PCR using 7900HT Fast Real-time PCR system (Applied Biosystems). The cDNA was amplified with 600nM of gene specific sense and antisense primers described in Table 3.3. The PCR program was carried out as follows: denaturation 95 ^{o}C for 20 seconds, amplification for 40 cycles (95 ^{o}C for 1 sec.; 53/60 ^{o}C for 20 sec and 72 ^{o}C for 20 sec). Post-PCR dissociation curves were chosen to confirm the specificity of single-target amplification. The amount of cDNA of each amplification was calculated by using a standard curve which was generated from the negative control group (Franklin, Bu et al. 2006). Expression levels of RANKL and OPG were normalized to the expression of glyceraldehyde-3-phosphaste dehydrogenase (GAPDH), a housekeeping gene. Modulation of osteoclastogenesis was presented as the ratio between OPG and RANKL.

Table 3.3 Primers used for reverse transcription-polymerase chain reaction (RT-PCR)

Primer	Orientation	Sequence	Tm (^{o}C)
OPG	sense	5’ GTT CTT GCA CAG CTT CAC CA 3’	53
	antisense	5’ AAA CAG CCC AGT GAC CAT TC 3’	
RANKL	sense	5’ ACC AGC ATC AAA ATC CCA AG 3’	53
	antisense	5’ TTT GAA AGC CCC AAA GTA CG 3’	
GAPDH	sense	5’ TAC ATT TTG CTG ATG ACT GG 3’	60
	antisense	5’ TGA ATG GTA GGA GCT TGA CT 3’	

3.2.7 Transient Transfection of UMR-106 Cells for ER-α and ER-β-Mediated Luciferase Assay

The estrogen-like activity of RD total flavonoids was observed in an ERE-luciferase gene reporter assay. Plasmids containing ER-α and ER-β were amplified, which was followed by plasmid purification using Wizard® Plus Minipreps DNA Purification System (Promega, #A7500). The plasmids were further confirmed by cutting them with restriction enzyme, EcoR1 and HotE1, respectively. The concentrations of the plasmids were measured in the absorbance of 260/280nm. The products were then stored in -20 ^{0}C until use.

UMR-106 cells were seeded in a 24-well plate at a density of 25000 cells/well for 48 hours. The medium was switched to phenol-red free DMEM with 1% charcoal stripped FBS (Sigma-Aldrich, Germany) to remove steroid compounds. The cells were transfected by Lipofectamine^{TM} 2000 reagent (Invitrogen, Callsbad, CA) 24 hours later. 0.4 μg/well ERE-containing luciferase reporter plasmid vERETkluc, 0.4 μg/well of ER-α / ER-β expression construct (both were obtained from Dr. Vincent Gigurere of the McGill University), together with 0.1 μg/well of an inactive control plasmid pRL-TK (Promega, Madison, WI, USA), a *Renilla* luciferase control vector, were cotransfected in the cells (Figure 3.4). After 6 hours of transfection, the medium was changed and indicated amount of 17β-estradiol, RD total flavonoids, Naringin

were added. The cells were lysed in the passive lysis buffer for 15 min after 24 hours of incubation. The luciferase activity of the cell lysates was measured with the Dual-Luciferase® reporter assay system (Promega, #E1910, Madison, WI, USA) according to the manufacturer's guidelines. Estrogen receptor promoter (vERE) activity was expressed as *Firefly* luciferase levels while the pRL-TK *Renilla* luciferase assay was determined to correct for variations in transfection efficiencies (Kuiper, Lemmen et al. 1998). Both *Firefly* and *Renilla* luciferase signals were detected by TD-20/20 Luminometer (Turner Design, Sunnyvale, CA, USA)

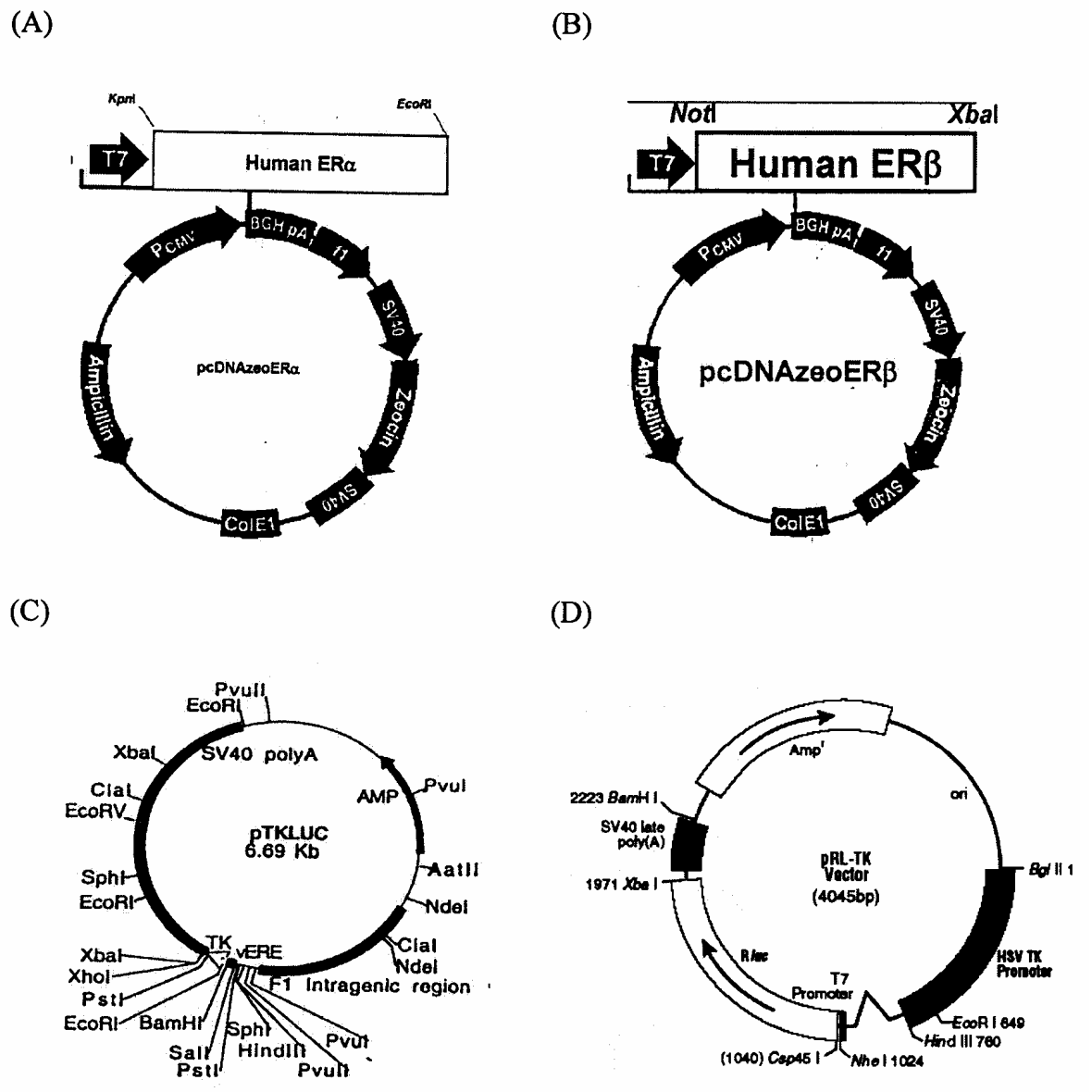


Figure 3.4 The circle maps of (A) ER-α plasmid, (B) ER-β plasmid, (C) ERE-containing luciferase reporter vector and (D) pRL-TK vector.

3.2.8 Immunoblotting

Cell lysates obtained from the above assays were kept for immunobloting. 20 µl proteins were separated by SDS-PAGE on 10% reducing gels at a constant voltage (200V) for 45 minutes. The protein gel was then transferred onto PVDF membrances (Immoblilin-P, Millipore Corp., MA, USA) in 2-8 ^{o}C. Immuno-detection was performed after blocking non-specific binding sites on the membrane with 5%-skimmed milk. The blots were probed with monoclonal rabbit anti-rat phospho-ER-α (1:2000), rabbit anti-ER-α (1:2000) and mouse anti-β-actin, an internal control (1:5000), as the primary antibodies for two hours. The goat anti-rabbit secondary antibodies which conjugated with horseradish peroxidase were incubated to probe phospho-ER-α and ER-α for one hour while goat anti-mouse secondary antibody was used to probe β-actin. 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)

3.2.9 Statistical Analysis

Data for *in vitro* study are reported as mean $\pm$ SEM. Each set of data was combined with three to five individual experiments. They were statistically analyzed by an unpaired t-test with the level of significance set at $p < 0.05$.

Chapter 4

Characterization of the osteoprotective effect of active fractions of Rhizoma Drynariae *in vivo* and *in vitro*

4.1 Background

Osteoporosis is a condition characterized by low bone mineral density, microarchitectural deterioration of bone tissue, and a consequent increase of fracture risk (Lau and Cooper 1996). It is estimated that over 200 million people worldwide have osteoporosis (Reginster and Burlet 2006). Liu Z et al. reported that, there are about 77 million women were suffering from primary osteoporosis in China. It is about 6 in 100 women in the whole population (Liu, Piao et al. 2002). Guidelines for the diagnosis and treatment of osteoporosis are available in many countries; however, the implementation is generally poor despite the availability of treatments with proven efficacy (Reginster and Burlet 2006). Traditional Chinese medicines have been used in the Chinese population for the treatment of bone diseases and to promote bone healing for thousands of years (Wong and Rabie 2006). In Chinese medicinal herbs, Rhizoma Drynariae has been reported as a good enhancer for bone healing. Its total extracts has shown promoting effects on bone cell activities and promising therapeutic effects on animal studies (Dong Fu-hui 2003; Xie, Ju et al. 2004; Wong and Rabie 2006). It is reasonable to postulate that Rhizoma Drynariae somehow affect the process of bone metabolism in osteoporosis. Recently, Jia et al. demonstrated that Rhizoma Drynariae restored trabecular structure from OVX-induced bone loss, similar to the effects of estrogen, by inhibiting bone turnover of postmenopausal

osteoporosis (Jia, Wang et al. 2006). The optimal dose and its active fractions of Rhizoma Drynariae, however, have not yet been identified. The mechanisms of actions on bone metabolism and bone formation has also yet been elucidated. The aim of this chapter is to demonstrate the osteoprotective effect of the active fractions of Rhizoma Drynariae and its mechanisms of actions in promoting bone health. In the first part of the study, the optimal dose of Rhizoma Drynariae administration is determined by using osteoporotic mice model. In the second part, its mechanisms of actions on treating osteoporosis are characterized by using bone cell culture. The results provide a detailed explanation on Rhizoma Drynariae in treating bone diseases.

4.1.1 Animal Study in the Efficacy of Rhizoma Drynariae in Treating Osteoporosis

In our previous study, the effects of different fractions of RD extracted by different solvents on bone mineral densities were studied using mice. RD crude extracts were divided into water-extract (RDA), 30% ethanol extract (RDB), 50% ethanol extract (RDC) and 95% ethanol extract (RDD). Chemical analysis showed that most of the extracts contained flavonoids compounds, except RDA. Preliminary animal study also revealed that total flavonoid fractions of RD could restore ovariectomized-induced bone loss in 3-month old female mice. Figure 4.1 indicates both non-flavonoid (RDA)

and total flavonoid mixture (RDB+RDC; RDD) can increase mice total bone mineral densities in proximal femur. RDB+RDC also significantly stimulated cortical BMD compared with OVX Vehicle group (Dosage used could be found in Appendix 2). It is postulated that components in RD might have positive effect on treatment of osteoporosis.

On the basis of the preliminary study, it is suggested that RD contains active components which favors bone health. RDB, RDC and RDD fractions effectively reduce OVX-induced bone loss, which are regarded as the active ingredients of RD in treating osteoporosis.

C57 BL/6J is one of the most common strains which is used in the research of bone biology. They are characterized as low bone mineral densities and high tolerant to alcohol, which allow us to provide drug with alcoholic content without harming the mice. In the study, all the mice, except sham-operated group, were undergone ovariectomy (OVX) (Remove of ovary). This procedure disrupts usual generation of endogenous estrogens and results in estrogen deficiency. It can mimic the situation of postmenopausal symptoms. Herbal treatment was initiated in animal upon recovery at two week.

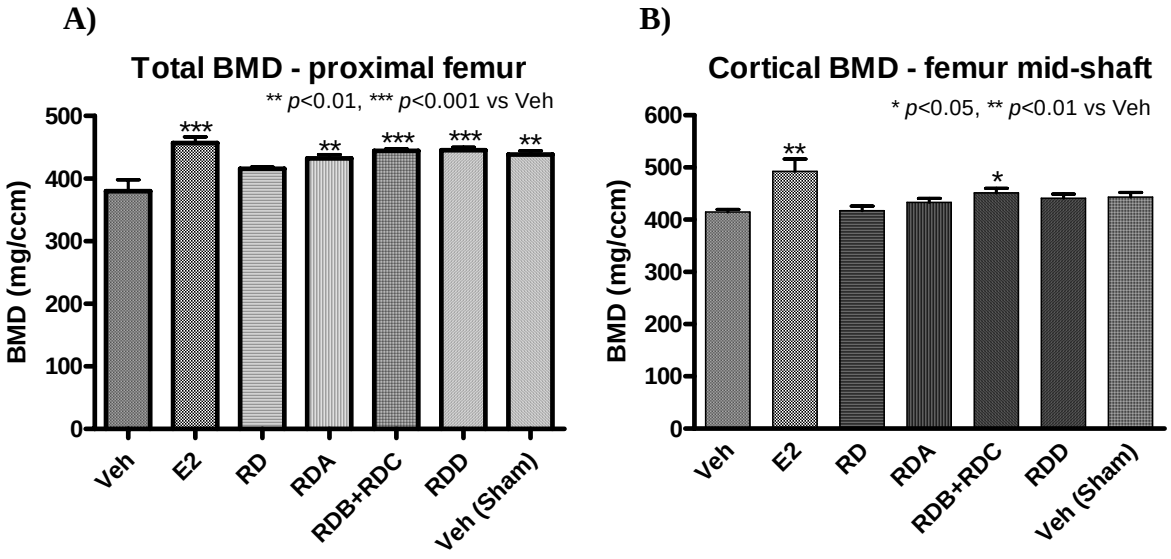


Figure 4.1 The effects of different fractions of Rhizoma Drynariae on bone mineral densities (BMD) at mice femur. A) The total bone mineral densities of proximal femur. Sham (p<0.01), 17β-estradiol (E2) (p<0.001), non-flavonoids fraction (RDA) (p<0.01), 30-50% EtOH extract (RDB+RDC) (p<0.001) and 95% EtOH extract (RDD) (p<0.001) increased BMD significantly, vs OVX control group (Veh). **B)** The cortical BMD of femur mid-shaft. 17β-estradiol group (E2) (p < 0.01) and flavonoids fraction I (RDB+RDC) (p< 0.05) increased cortical BMD significantly compared with OVX control group (Veh). The results suggested that flavonoids fraction RDB+RDC was the most active fractions in promoting bone formation in mice model. Results were analyzed by one-way ANOVA and data shown as mean $\pm$ SEM. (n = 7)

Several bio-markers were chosen to determine the effectiveness of drug in treating osteoporosis, including serum and urinary bone formation / resorption markers, uteri weight, bone mineral density and bone strength. Generally, serum and urinary calcium, phosphorus and Dpd are the commonly used biochemical markers for the assessment of the effect of drugs. Uteri weight is an indicator of estrogenic effect on uterus. Estrogen maintains uterus in function, thus a reduction in size of the uterus is the physical marker for the indication of estrogen lost. Bone mineral density (BMD) is the most common bio-marker for the diagnosis of osteoporosis. In this study, left femur, tibia and lumbar spine BMD were measured by pQCT. At last, bone strength was observed by using three-point bending machine.

4.1.2 *In vitro* Study of Rhizoma Drynariae in Bone Cell Activities

Apart from the *in vivo* study, *in vitro* study was also proceeded simultaneously to delineate the mechanisms of actions of Rhizoma Drynariae total flavonoids (RD TF) in treating osteoporosis. Lin CY et al. (2002) reported that Rhizoma Drynariae had a universal beneficial effect on bone cell metabolism which was probably mediated by the induction of apoptosis of the osteoclast cell population. Jeong JC et al. (2005) also demonstrated that RD performed stimulative effects on osteoblastic MC3T3-E1 cells proliferation and differentiation. The active ingredients of RD and its mechanism of

actions, however, were rarely been elucidated.

4.2 Methodology

For the animal study, the concentrations of RD TF used for the animal feeding were 0.087, 0.173 and 0.347 mg/g/day respectively. For the *in vitro* study, rat osteoblastic-like UMR-106 cells were cultured and the effect of herbal extract on bone cell proliferation and cell differentiation were studied and regarded as *in vitro* marker of osteoprotective effects. In addition, the estrogenic effect of RD total flavonoids in stimulating bone cell activities was determined by co-treating the cell with anti-estrogen ICI 182, 780. To define the modulating effect of herbal extract on osteoclastogenesis, the ratio of OPG/RANKL mRNA expression were studied. We also examined the effect of herbal extract on estrogen responsive element (ERE) dependent gene transcription in ER-α and ER-β transfected UMR-106 cells, using a luciferase gene reporter assay, followed by the immuno-detection of phospho-ERα. UMR-106 cells were treated with RD TF with concentrations varied from 0.002, 0.02, 0.2, 2, 20 and 200 μg ml^{-1}. 0.02 to 0.2 μg ml^{-1} was selected in the molecular studies of mRNA expression and transient transcription of UMR-106 cells.

4.3 Results

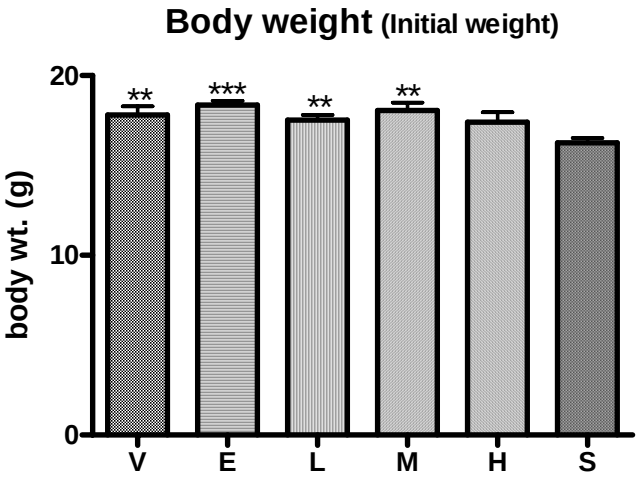
4.3.1 *In Vivo* Study

To investigate the effectiveness of Rhizoma Drynairae total flavonoids in osteoporotic model, serum, urine and bone specimens were collected for quantitative measurement. The following chapters include the results of the *in vivo* study.

4.3.1.1 Effects on Body and Uterine Weights

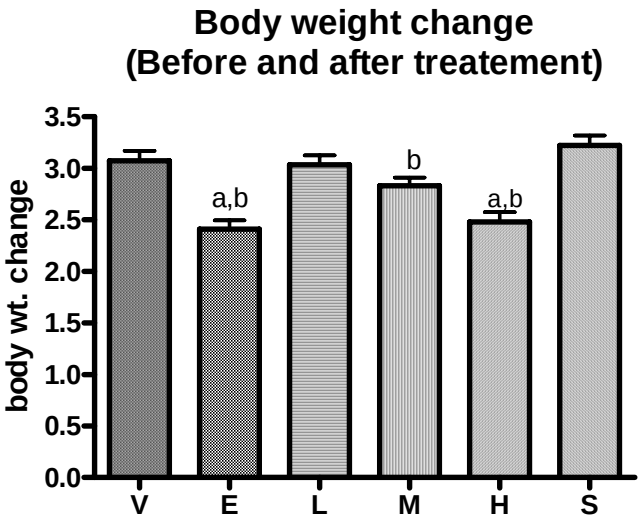
After two weeks of operation, the body weight of the OVX mice was significantly higher than sham-operated mice, despite the fact that they were pair-fed (Figure 4.2A). This phenomenon indicated that the OVX operation was successful as estrogen depletion induces body weight gain apart from treatment. Besides, OVX mice treated with E2 and high dose of RD TF significantly decreased body weight gain after treatment, compared with vehicle-treated OVX mice (Figure 4.2B, $p<0.05$). The uteri were also collected in harvesting period. Figure 4.2C displays that OVX significantly diminished uteri weights compared with sham (S) group ($p<0.001$ vs V). OVX mice fed with E2 significantly stimulated the growth of the atrophic uterus. On the other hand, RD TF-treated OVX mice did not increase uteri weight. The results suggested that RD TF did not exhibit the estrogenic side effect on uterus.

A)



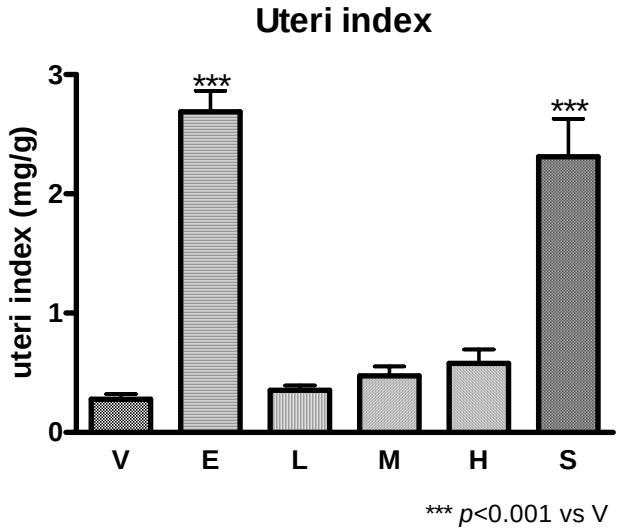
** $p<0.01$, *** $p<0.001$ vs V

B)



a $p<0.05$ vs V (OVX, veh)
b $p<0.05$ vs S (Sham, veh)

C)



*** $p<0.001$ vs V

Figure 4.2 The effects of Rhizoma Drynariae total flavonoids on body and uteri weights in Sham and OVX mice Six groups of mice were subjected to different treatments, ***V***: Vehicle-treated, OVX; ***E***: 17β-estradiol-treated, OVX; ***L***: low-dose RDTF-treated, OVX; ***M***: medium-dose RD TF-treated, OVX; ***H***: high-dose RD TF-treated; ***S***: Vehicle-treated, Sham-operated for 6-weeks. Results were analyzed by one-way ANOVA and data shown as mean $\pm$ SEM (n = 7). **A)** The effects of OVX operation on body weights. Except group H, OVX mice increased body weights significantly, compared with Sham-operated group (p<0.01). **B)** The body weight change before and after treatment. **C)** The uteri index, presented as uteri weights per corresponding body weights, were measured (mg/g). The uteri index in group S reflects normal weight of uteri. Uteri weights diminished dramatically after removing ovaries (group V to Group H). Upon treatment, estrogen-feeding group (E) greatly increased their uteri weight, showing 17β-estradiol exert uterotrophic effect. On the other hand, herbal treatment group did not promote this effect.

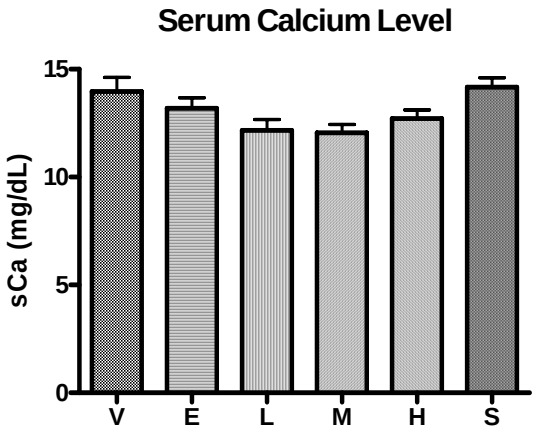
4.3.1.2 Effects on Biochemical Markers

In this chapter, serum and urinary biochemical markers were presented to conclude the efficacy of drug in terms of bone remodeling. Serum and urinary calcium and phosphorus levels are common biochemical markers indicating mineral balances. Urinary Dpd is a bone resorption marker which reflects the osteoclastic activity in bone remodeling process. Increased in urinary Dpd levels increase bone resorption and bone loss, which result in increasing fracture risk of patients.

(I) Serum and Urinary Calcium and Phosphorus Levels

Calcium and phosphorus are the essential minerals which regulate bone mineral absorbtion and resorption. Figure 4.3 A and B show the concentration changes of serum markers, sCa and sP. In contrary to vehicle-treated Sham or OVX mice, sCa was not greatly affected among the treatment. sP was fluctuated among groups with the tendency to be decreased by OVX and increased by estrogen. Figure 4.3 C and D show the levels of urinary calcium and phosphorus and they are expressed as uCa/Cr and uP/Cr. 17β-estradiol (E) group, medium and high doses of RD treatment groups (M and H) significantly suppressed OVX-induced uCa loss ($p<0.05$ vs vehicle-treated OVX group). These results suggested that RD total flavonoids extracts encourage bone mineral reservation by minimizing urinary calcium excretion while the sCa is maintained.

A)



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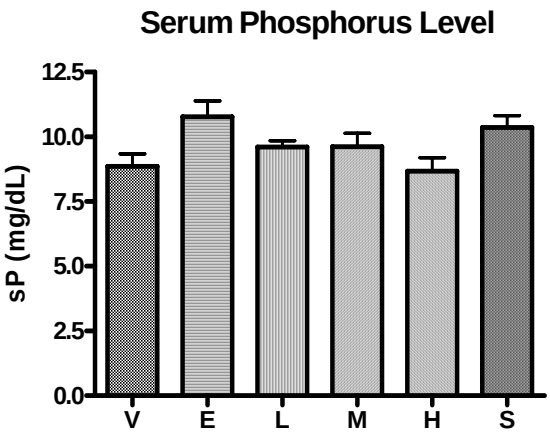
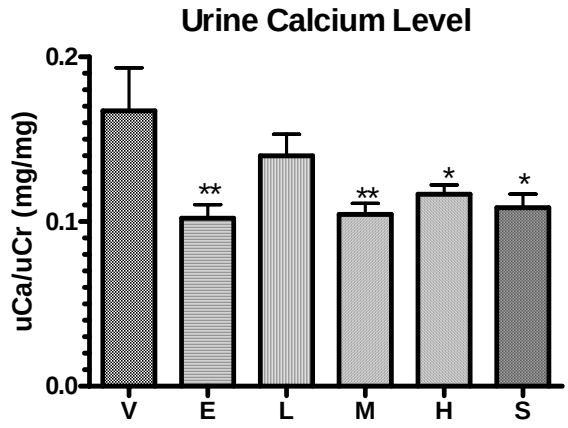
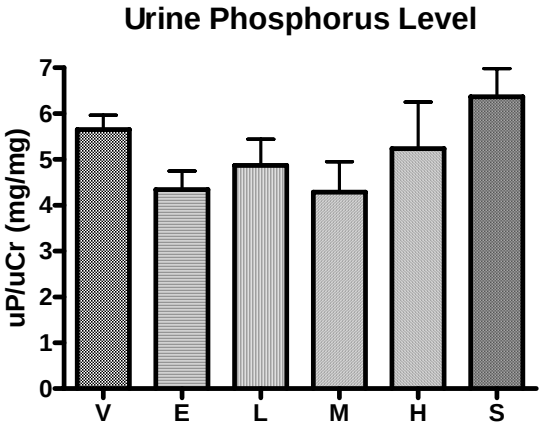


C)



D)



* / ** p <0.05, p <0.01 vs V

Figure 4.3 The effect of Rhizoma Drynariae total flavonoids on serum and urinary calcium and phosphorus levels in Sham and OVX mice. Six groups of mice were subjected to different treatments for 6 weeks and they are abbreviated as ***V***: Vehicle-treated OVX; ***E***: 17β-estradiol-treated OVX; ***L***: 0.087 mg/g/day RDTF-treated OVX; ***M***: 0.173 mg/g/day RDTF-treated OVX; ***H***: 0.347 mg/g/day RDTF-treated OVX; and ***S***: Vehicle-treated Sham-operated. Results were analyzed by one-way ANOVA and data shown as mean $\pm$ SEM (n = 7). Serum calcium **(A)** and phosphorus **(B)** levels. There was no significant change among groups in sCa and sP levels. Urinary calcium **(C)** and phosphorus **(D)** levels. Group V expressed the highest calcium excretion rate, followed by group L. Group E, M, H significantly reduced calcium loss induced by OVX. uP level varied in among groups but there was no statistical difference between them.

(II) Urinary Dpd

Urinary Dpd reflects the level of osteoclastic activity in the bone-remodeling process (McCormick 2007). According to the results show in Figure 4.4, 17β-estradiol (E) and medium dosage of RD total flavonoids (M) markedly diminished collagen degradation in bone. Both groups suppressed the increase of Dpd/Cr by OVX for about 80% ($p< 0.05$), to the level similar to that of the sham-operated group (S). Low dosage of RD TF treatment also decreased urinary Dpd formation by 24% but without statistically significance. These findings indicated that RD total flavonoids exert preventive effects on OVX-induced collagen degradation.

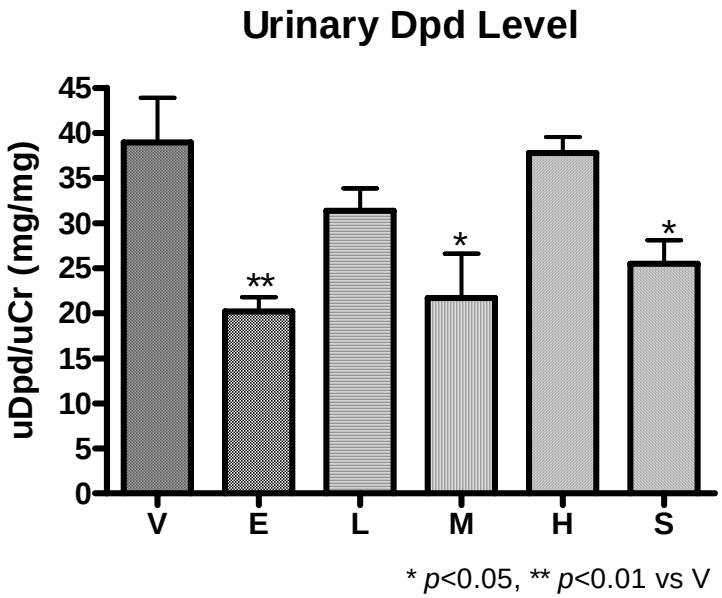


Figure 4.4 The effect of Rhizoma Drynariae total flavonoids on urinary Dpd level in Sham and OVX mice. Results were analyzed by one-way ANOVA and data shown as mean ± SEM. Urinary Dpd concentration significantly decreased in group E and M; compared with OVX vehicle group (V). Concentration of Dpd was expressed as uDpd / uCr. Creatinine was the internal control. (n = 7) (Group ***V***: Vehicle-treated OVX; ***E***: 17β-estradiol-treated OVX; ***L***: 0.087 mg/g/day RDTF-treated OVX; ***M*****:** 0.173 mg/g/day RDTF-treated OVX; ***H***: 0.347 mg/g/day RDTF-treated OVX; and ***S***: Vehicle-treated Sham).

4.3.1.3 Effects of Bone Quality and Bone Strength

Left femur and tibia were collected for the assessment of bone quality and bone strength. The total, trabecular and cortical bone mineral densities (BMD); cross-sectional area of bone were observed by pQCT, while the determination of bone strength was measured by three-point bending method.

(I) Bone Mineral Densities (BMD) of Mice Femur, Tibia and Lumbar Spine

There are three scanning sites for BMD measurement including proximal region, mid-shaft and distal-end. Proximal region is the growth plate region of femur or tibia head, mid-shaft is the position of the middle bone, distal-end is another growth plate region of femur and tibia end. Cross-sectional area and SSI-Polar provided supporting evidences for bone strength. BMD was measured in mg/ccm while cross-sectional area expressed in mm^2.

Proximal Femur

Figure 4.5A shows the BMD change in each treatment group. 17β-estradiol exerted universal beneficial effect on bone quality by elevating total, trabecular BMD and widens total cross-sectional area. Among different dosages of RD total flavonoids treatment, medium dosage of RD TF significantly stimulated total bone mass by about

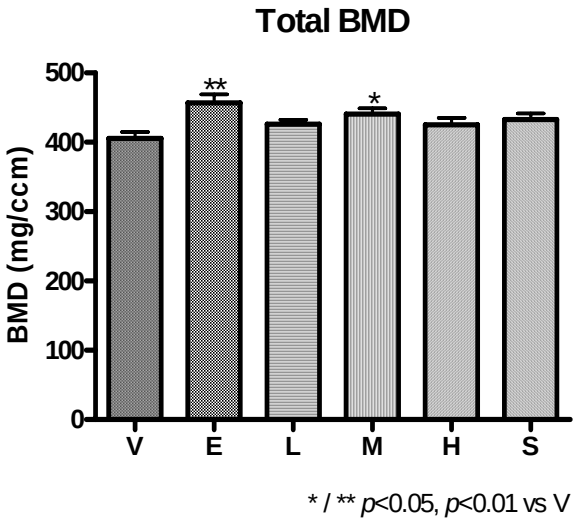
9% (p<0.05). There is no significant change of trabecular BMD measurement, while total cross-sectional area was increased only in OVX 17β-estradiol-treated group (E) (p<0.001 vs OVX vehicle-treated group).

Femur Mid-shaft

Cortical BMD was determined in femur mid-shaft. Figure 4.6 shows that 17β-estradiol markedly increased cortical BMD by 10% (p<0.01) and medium dosage of RD total flavonoids treated group also significantly induced cortical BMD by 6% (p<0.05). Group L and H performed slightly positive effects while the results did not reach statistical significance. The results suggested that 0.173 mg/g/day of RD (group M) total flavonoids have beneficial effect on increasing cortical BMD after ovariectomy.

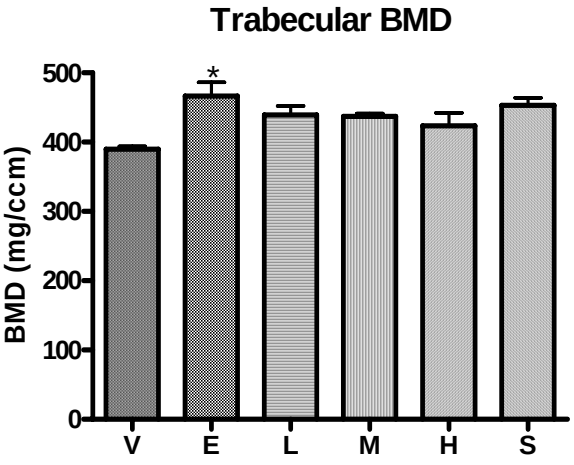
Proximal Femur

A)



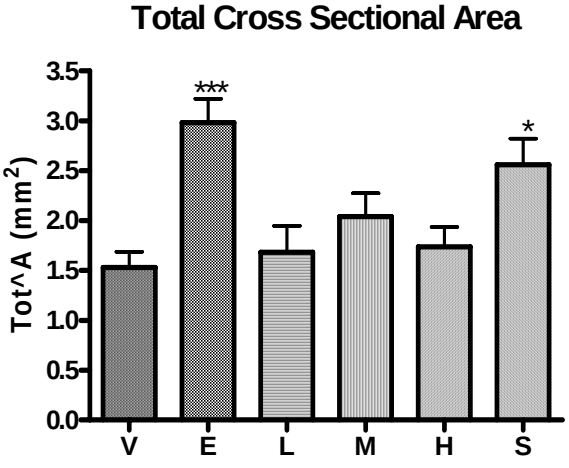
* / ** $p<0.05$, $p<0.01$ vs V

B)



* $p<0.05$ vs V

C)



* / *** $p<0.05$, $p<0.001$ vs V

Figure 4.5 The effect of Rhizoma Drynariae total flavonoids on bone mineral densities (BMD) at proximal femur in Sham and OVX mice Six groups of mice were subjected to different treatments for 6 weeks and they are abbreviated as ***V***: Vehicle-treated OVX; ***E***: 17β-estradiol-treated OVX; ***L*:** 0.087 mg/g/day RDTF-treated OVX; ***M***: 0.173 mg/g/day RDTF-treated OVX; ***H*:** 0.347 mg/g/day RDTF-treated OVX; and ***S***: Vehicle-treated Sham-operated. Results were analyzed by one-way ANOVA and data shown as mean $\pm$ SEM (n = 7). **A)** The total BMD of proximal femur. Group E and M had significant increase in BMD compared with group V (p<0.05). **B)** The trabecular BMD of proximal femur. Only group E can exert the beneficial effect to bone mass. **C)** The total surface area of the proximal femur. 17β-estradiol-treated group improved OVX-induced decrease in cross-sectional area.

Femur Mid-shaft

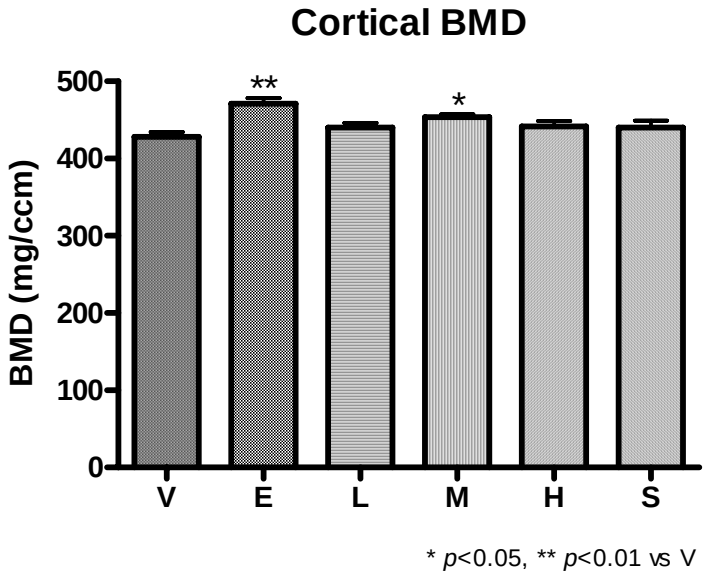


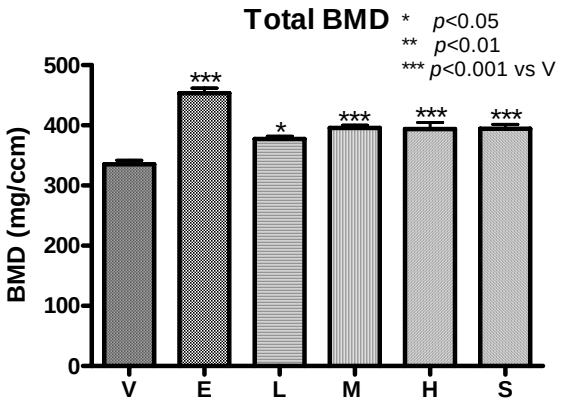
Figure 4.6 The effect of Rhizoma Drynariae total flavonoids on cortical bone mineral densities (BMD) at femur mid-shaft in Sham and OVX mice Six groups of mice were subjected to different treatments and they are abbreviated as ***V***: Vehicle-treated OVX; ***E***: 17β-estradiol-treated OVX; ***L***: 0.087 mg/g/day RDTF-treated OVX; ***M***: 0.173 mg/g/day RDTF-treated OVX; *H*: 0.347 mg/g/day RDTF-treated OVX; and ***S***: Vehicle-treated Sham-operated. Results were analyzed by one-way ANOVA and data shown as mean ± SEM (n = 7). Mice fed with 17β-estradiol (E) significantly increased BMD in OVX rats, compared with vehicle-treated OVX group (V) (p<0.01). Medium dose of RD TF significantly minimized OVX-induced cortical bone loss by increasing BMD, compared with vehicle-treated OVX group (p<0.05). Other RD treatment groups tended to increase BMD but the result did not reach statistical significance.

Distal Femur

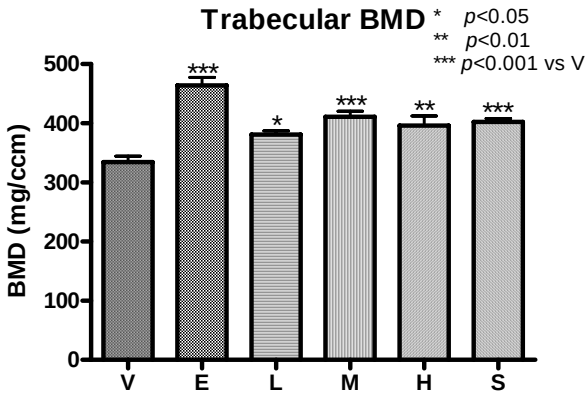
Figure 4.7A-D demonstrates the effect of herbal treatment on BMD and cross-sectional area. Figure 4.7A shows that all the treatment groups significantly restored BMD which were induced by OVX. RD at low (group L), medium (group M) and H (group H) dose increased total BMD by 13% ($p<0.05$), 18% ($p<0.001$) and 17% ($p<0.001$), respectively. Similarly, the effectiveness of RD total flavonoids in increasing trabecular BMD was shown in Figure 4.7B. Group L, M and H markedly induced trabecular BMD by 14% ($p<0.05$), 23% ($p<0.001$) and 18% ($p<0.01$), respectively. In terms of total cross-sectional area, low (group L) and medium (group M) dose of RD total flavonoids increased bone surface area significantly by 31% and 34%, compared with vehicle-treated OVX group (V) ($p<0.01$). Both treatments can also increase trabecular cross-sectional area significantly. Low (group L) and medium (group M) dose of RD TF increased trabecular cross-sectional area by around 46%, compared with vehicle-treated OVX group (V) ($p<0.05$), as well as restored cross-sectioned area to the level in sham-operated group (S). The above data indicated that RD total flavonoids could effectively restore OVX-induced bone loss at distal femur.

Distal Femur

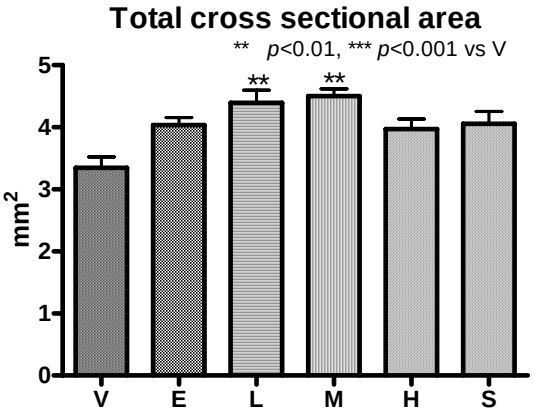
A)



B)



C)



D)

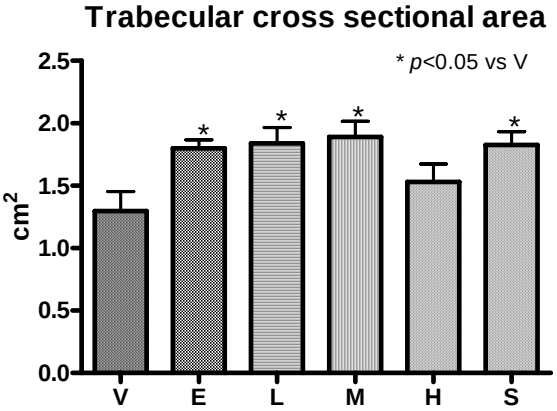


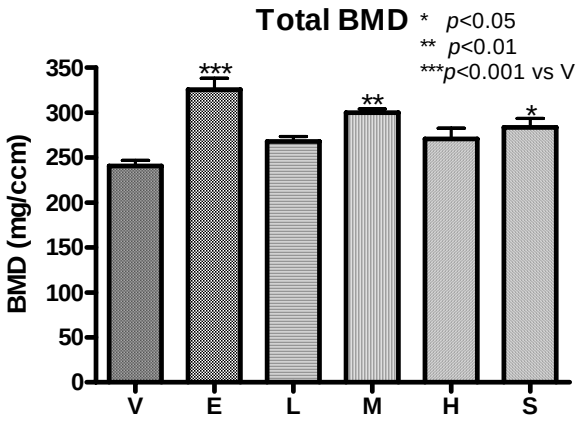
Figure 4.7 T**he effect of Rhizoma Drynariae total flaovnoids on bone mineral densities (BMD) at distal femur in Sham and OVX mice** Six groups of mice were subjected to different treatments for 6 weeks and they are abbreviated as ***V***: Vehicle-treated OVX; ***E***: 17β-estradiol-treated OVX; ***L***: 0.087 mg/g/day RDTF-treated OVX; ***M***: 0.173 mg/g/day RDTF-treated OVX; ***H***: 0.347 mg/g/day RDTF-treated OVX; and ***S***: Vehicle-treated Sham-operated. **A)** The total BMD of femur distal end. All the treatment groups significantly increased BMD in OVX mice ($p<0.001$). **B)** The trabecular BMD of femur distal end. Group E, L and M restored OVX-induced bone loss significantly ($p<0.001$). **C)** Total cross sectional area of femur distal end. **D)** The trabecular cross sectional area of distal femur. Results were analyzed by one-way ANOVA and are expressed as mean $\pm$ SEM. ($n = 7$)

Proximal Tibia

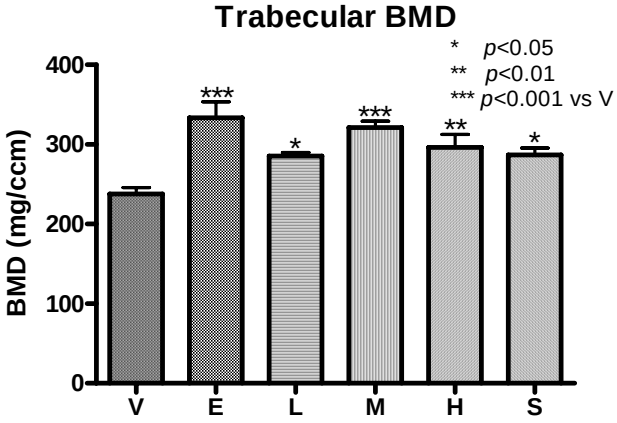
As shown in Fig. 4.8A, estradiol-treated (E) and medium dose of RD TF (M) significantly prevented the decrease in total BMD in OVX mice. Medium dose of RD TF increased total BMD by 24.5% ($p < 0.01$ vs vehicle-treated OVX group). Fig. 4.8B also shows that RD total flavonoids treatment is successful in restoring trabecular BMD in OVX mice. RD total flavonoids at low, medium and high doses increased trabecular BMD for 20% ($p < 0.05$), 35% ($p < 0.001$) and 24.5% ($p < 0.01$), respectively. Medium (group M) and high (group H) doses of RD TF treatment induced total and trabecular cross-sectional areas markedly. Figure 4.8C and 4.8D displays that medium dose of RD TF (group M) increased total area and trabecular area in proximal tibia by about 90% ($p < 0.01$) and 1-fold ($p < 0.01$), respectively, whereas high dose of RD TF treatment (group H) increased total and trabecular areas by 1-fold ($p < 0.001$). The results suggested that RD TF has exerted stimulatory effects on bone mineral density, especially in the region of distal femur and proximal tibia.

Proximal Tibia

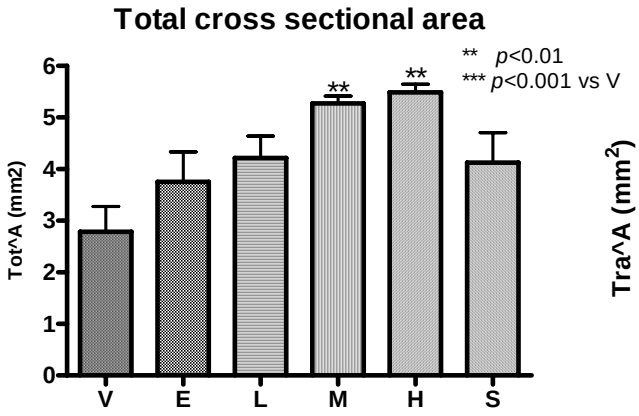
A)



B)



C)



D)

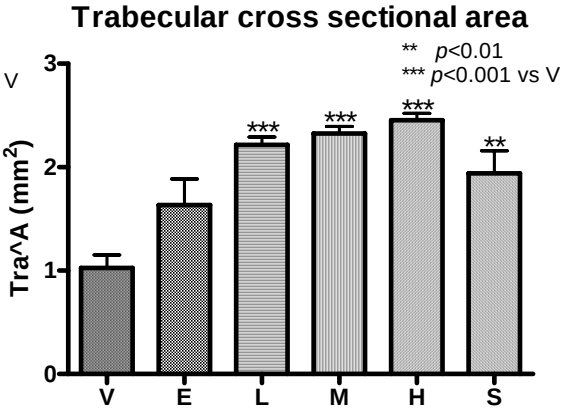


Figure 4.8 The effect of Rhizoma Drynariae total flavonoids on bone mineral densities at proximal tibia in Sham and OVX mice Six groups of mice were subjected to different treatments for 6 weeks and they are abbreviated as ***V***: Vehicle-treated OVX; ***E***: 17β-estradiol-treated OVX; ***L***: 0.087 mg/g/day RDTF-treated OVX; ***M***: 0.173 mg/g/day RDTF-treated OVX; ***H***: 0.347 mg/g/day RDTF-treated OVX; and ***S***: Vehicle-treated Sham-operated. Results were analyzed by one-way ANOVA and were expressed as mean $\pm$ SEM (n = 7). **A)** Total BMD at proximal tibia. Total BMD were significantly increased in group E and M as compared to V (p<0.05, p<0.01, p<0.001, respectively). **B)** Trabecular BMD at proximal tibia. Trabecular BMD were significantly increased in all treatment groups, compared with vehicle-treated group (p<0.05). Group E and M increased trabecular BMD (p<0.001). **C)** Total cross-sectional area. Group M and H increased total cross-sectional area in proximal tibia (p<0.01). **D)** Trabecular cross-sectional area. Group L, M, H had increased the trabecular cross-sectional area (p<0.01).

Tibia Mid-shaft

As shown in Fig. 4.9, RD total flavonoids treatment did not alter cortical bone mineral density in mid-shaft tibia. There was no significant change between OVX control group (V) and Sham group (S). It is postulated that there is no OVX-induced bone loss in cortical region within the period of the experimental treatment. Our result indicated that OVX for eight weeks did not alter cortical BMD at the site of tibia metaphysic in young mice.

Tibia Mid-shaft

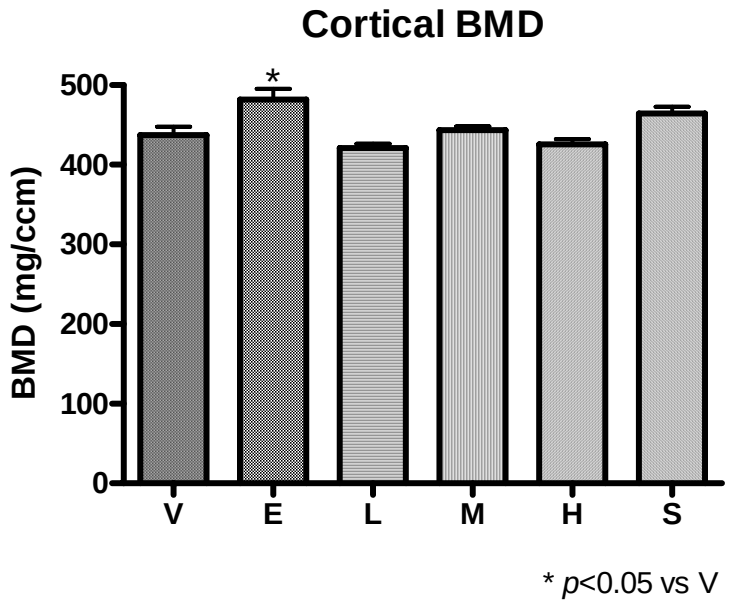


Figure 4.9 The effect of Rhizoma Drynariae total flavonoids on bone mineral densities at tibia mid-shaft in Sham and OVX mice Six groups of mice were subjected to different treatments abbreviated as ***V***: Vehicle-treated OVX; ***E***: 17β-estradiol-treated OVX; ***L***: 0.087 mg/g/day RDTF-treated OVX; ***M***: 0.173 mg/g/day RDTF-treated OVX; ***H***: 0.347 mg/g/day RDTF-treated OVX; and ***S***: Vehicle-treated Sham-operated. Results were analyzed by one-way ANOVA and were expressed as mean ± SEM (n = 7). There was no significant difference among treatment groups, except group E. 17β-estradiol raised cortical BMD above baseline.

Lumbar spine L1

According to Fig. 4.10A, medium and high doses (group M and H) of Rhizoma Drynariae total flavonoids significantly inhibited OVX-induced bone loss in lumbar spine region L1. Group M and H increased total bone mineral density by about 16% ($p < 0.001$) and 12% ($p < 0.01$), respectively, compared with vehicle-treated OVX group (V). In Fig. 4.10B, all the groups treated with RD TF prevent the decrease in total cross-sectional area induced by OVX. Medium dose of RD TF (group M) significantly increased total cross-sectional surface area of lumbar spine by about 90% (vs vehicle-treated OVX group, $p < 0.001$). Group L and H also increased the cross-sectional area by about 65% ($p<0.05$). The results suggested that RD TF was effective in increasing bone mass in lumbar spine in a dose-dependent manner.

Lumbar Spine L1

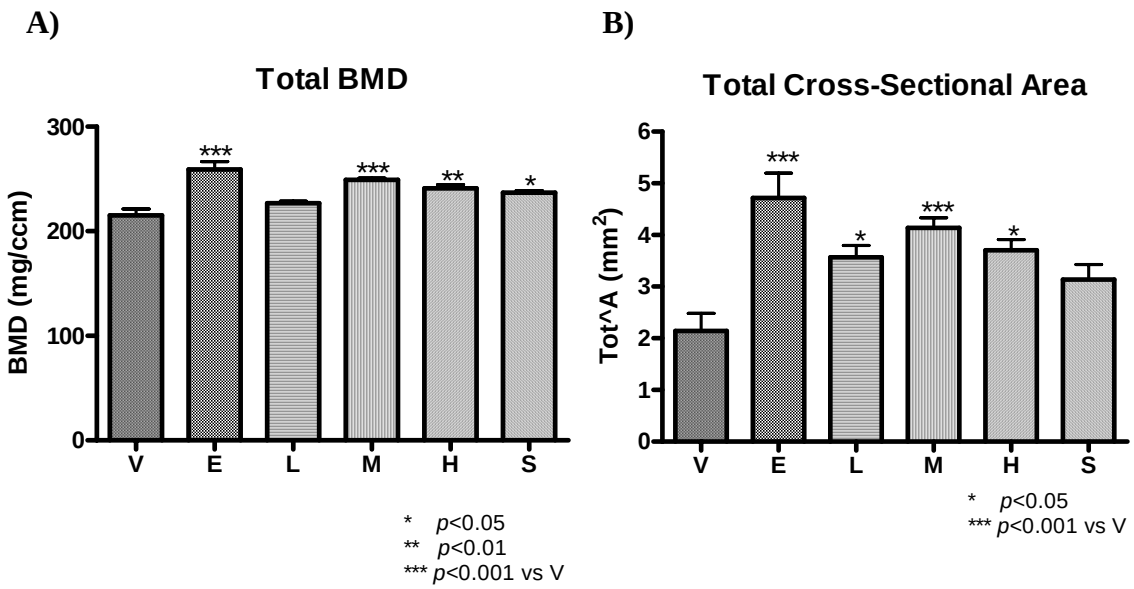


Figure 4.10 The effect of Rhizoma Drynariae total flavonoids on bone mineral densities at lumbar spine region L1 in Sham and OVX mice Six groups of mice were subjected to different treatments which are abbreviated as ***V***: Vehicle-treated OVX; ***E***: 17β-estradiol-treated OVX; ***L***: 0.087 mg/g/day RDTF-treated OVX; ***M***: 0.173 mg/g/day RDTF-treated OVX; ***H*****:** 0.347 mg/g/day RDTF-treated OVX; and ***S***: Vehicle-treated Sham-operated. Results were analyzed by one-way ANOVA and were expressed as mean ± SEM (n = 7). **A)** Total BMD of lumbar spine L1. BMD in Group E, M, H and S were significantly higher than group V. Mice fed with 17β-estradiol and medium dose of RD total flavonoids increased BMD most effectively (p<0.001). **B)** Total cross sectional area of L1. Treatment of OVX mice with estradiol and all doses of RD TF increased total cross-sectional area of lumbar spine significantly (p<0.05).

(II) Simulated Bone Strength (expressed as SSI)

Simulated bone bending strength is reported as stress-strain index (SSI). Table 4.1 demonstrated that Rhizoma Drynariae total flavonoids (RD TF) treatment groups significantly increased bone strength in distal femur and lumbar spine L1. Group M increased bone strength in distal femur and lumbar spine by 2-fold ($p< 0.001$) and 75% ($p< 0.05$), respectively. Group M also considerably restored bone strength in tibia mid-shaft similar to the level of Sham (S). Group L and H though improved mechanical strengths in distal femur and lumbar spine but they did not affect much in tibia mid-shaft. The above results suggested that RD TF provides certain protective effect in bone strength in dose-dependent manner.

Table 4.1 The effect of Rhizoma Drynariae total flavonoids on stress-strain index (SSI) at femur, tibia and lumbar spine in Sham and OVX mice Six groups of mice were subjected to different treatments for 6 weeks and they are abbreviated as ***V***: Vehicle-treated OVX; ***E***: 17β-estradiol-treated OVX; ***L***: 0.087 mg/g/day RDTF-treated OVX; ***M***: 0.173 mg/g/day RDTF-treated OVX; ***H***: 0.347 mg/g/day RDTF-treated OVX; and ***S***: Vehicle-treated Sham-operated. Results were analyzed by one-way ANOVA and were expressed as mean ± SEM (*n* = 7). Femur, tibia and lumbar spine were collected and subjected to pQCT scanning. Stress-strain indexes (SSI) were derived from pQCT scan using Hounsfield in-house programme.

			Rhizoma Drynaria Total Flavonoids			
Regions	V	E	L	M	H	S
Femur						
Proximal	0.467 ± 0.032	0.676 ± 0.053 **	0.554 ± 0.02	0.566 ± 0.017	0.498 ± 0.037	0.66 ± 0.025 **
Mid-shaft	0.196 ± 0.004	0.279 ± 0.019 **	0.219 ± 0.018	0.219 ± 0.009	0.233 ± 0.017	0.229 ± 0.010
Distal	0.380 ± 0.049	0.714 ± 0.055 **	0.700 ± 0.069 **	0.763 ± 0.038 ***	0.615 ± 0.046 *	0.738 ± 0.032 ***
Tibia						
Proximal	0.200 ± 0.037	0486 ± 0.120	0.344 ± 0.041	0.550 ± 0.033 *	0.438 ± 0.068	0.400 ± 0.065
Mid-shaft	0.121 ± 0.01	0.153 ± 0.010	0.122 ± 0.005	0.144 ± 0.011	0.129 ± 0.008	0.143 ± 0.014
Lumbar spine	0.400 ± 0.053	0.888 ± 0.089 ***	0.580 ± 0.040 *	0.700 ± 0.024 ***	0.650 ± 0.038 **	0.563 ± 0.042

* / ** /*** p<0.05, p<0.01, p<0.001 vs V

4.3.1.4 Biomechanical Testing of Mice Tibia

The above studies demonstrated that Rhizoma Drynariae TF exerted positive effects on SSI, the simulated bone strength (section 4.3.1.3) indicator. Further study of using three-point bending method in tibia was applied to confirm the effectiveness of RD total flavonoids in maintaining bone strength.

Table 4.2 shows the results as recorded by three-point bending analysis. The ultimate load and yield load were the maximal and breaking force required to fracture tibia. The results indicate that E2 increased ultimate load and yield load of tibia diaphysis in OVX mice ($p<0.001$). Moreover, our result indicated that medium (group M) and high dose (group H) of Rhizoma Drynariae total flavonoids protected bone strength by restoring ultimate load to the level similar to Sham (S) ($p<0.05$). The energy for breaking was only significantly up-regulated by high dose of RD TF in OVX mice. Both results demonstrated that the osteoprotective effect of RD TF was dose-dependent. Group H significantly increased in energy for breaking by 64% ($p<0.05$). A similar trend was found for the stiffness and flexural modulus of tibia diaphysis. Flexural modulus, in another word, is the modulus of elasticity. Both measurements were improved by applying medium and high doses of RD TF ($p<0.01$).

Table 4.2 The effect of Rhizoma Drynariae total flaovnoids on biomechanical bone strengths at tibia diaphysis in Sham and OVX mice

Sham (group S) or OVX mice were treated either with E2 (group E), RD total flavonoids with low (group L), medium (group M) and high (group H) dose; or its vehicle (group V) for 6 weeks. Tibia was collected for three-point bending analysis. Results were analyzed by one-way ANOVA and data was shown as mean ± SEM. (n = 7)

Treatment	*Ultimate load (N)*	*Yield load (N)*	*Energy for breaking ($x10^{-3}$ J)*	*Stiffness (N/mm)*	*Flexural Modulus (MPa)*
V	11.73 ± 0.34	11.36 ± 0.26	4.69 ± 0.59	14.89 ± 1.71	1.31 ± 0.11
E	16.46 ± 0.76 ***	16.37 ± 0.66 ***	6.93 ± 0.51	18.83 ± 1.53	1.84 ± 0.17
L	13.79 ± 0.66	13.60 ± 0.78	6.92 ± 0.93	27.27 ± 1.50 **	1.99 ± 0.19
M	14.80 ± 0.76 *	12.99 ± 0.59	6.19 ± 0.64	22.44 ± 3.40	2.24 ± 0.15 **
H	14.64 ± 0.53 *	13.05 ± 0.48	7.68 ± 0.40 *	29.94 ± 1.87 **	2.02 ± 0.24
S	14.71 ± 0.69 *	12.45 ± 0.84	8.63 ± 1.18 **	23.28 ± 4.90	1.74 ± 0.23

* / ** / *** p <0.05, p <0.01, p <0.001 vs V

4.3.2 *In Vitro* Study

As discussed in Chapter 4.3.1, RD total flavonoids are effective in promoting the increase in bone mass and suppressing bone resorption. Although numerous studies demonstrate the efficacy of this herb (Sun, Theriault et al. 2004; Zhang, Wang et al. 2005; Jia, Wang et al. 2006), research studies about its mechanisms of the actions are limited. To tackle this problem, a series of cell culture studies have been carried out and reported in the following sections.

4.3.2.1 Proliferative Effects in UMR-106 Cells

UMR-106 cells were treated with different concentrations of Rhizoma Drynariae total flavonoids fractions (RD TF) for 24 and 48 hours. Estrogen dependent assay was also applied by co-treating the cells with estrogen antagonist, ICI 182,780. Figure 4.11 shows that RD TF stimulated UMR-106 cell proliferation in time- and dose-dependent manner. In 24-hour treatment, 0.02 to 0.2 μg ml^{-1} of RD TF induced cell proliferation by about 33% ($p < 0.001$) to 25% ($p < 0.001$) respectively. While in 48-hour treatment, RD TF stimulated cell proliferation by about 50 to 33% at the concentration from 0.002 to 0.2 μg ml^{-1} ($p <0.001$). The optimal dosage was 0.02 μg ml^{-1}. This dosage promoted cell growth by 33% ($p < 0.001$). Same dosage of RD TF also stimulated cell proliferation by about 50% at 48 hours of treatment ($p < 0.001$). It

is obvious that high doses of RD TF (20 and 200µg ml^{-1}) promoted cell death at both time points. Figure 4.12 displays the effects of RD TF on cell proliferation when co-treated with ICI blocker, an estrogen receptor antagonist. It demonstrates that the proliferative effects of RD TF were completely blocked by the addition of ICI ($p < 0.01$). According to the results, the increase in cell proliferation by Rhizoma Drynariae total flavonoids fractions at low concentrations were mediated via estrogen receptor (ER) dependent pathway.

Time-Dependent and Dose-dependent Cell Proliferative Assay

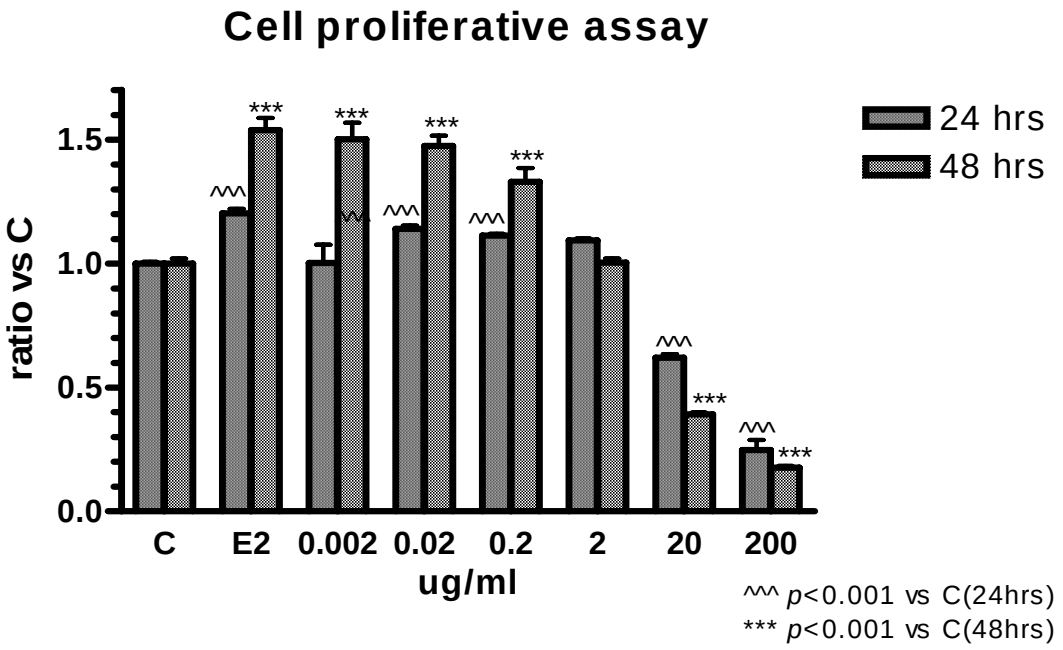


Figure 4.11 The effect of Rhizoma Drynariae total flavonoids on UMR-106 cell proliferation at 24 and 48 hours UMR-106 cells were treated with E2 (10 nM), RD total flavonoids (RD TF) (0.002 to 200 μg ml^{-1}) or its vehicle for 24 and 48 hours. Results were analyzed by unpaired t-test are expressed as mean ± SEM. Results are obtained from 3 independent experiments (n = 6) ^^^ p<0.001 (vs C at 24 hrs), *** p<0.001 (vs C at 48 hrs). Cell proliferation rate was determined by using MTS assay. RD TF increased cell proliferation in a dose- and time-dependent manner.

Estrogen receptor (ER) antagonist assay

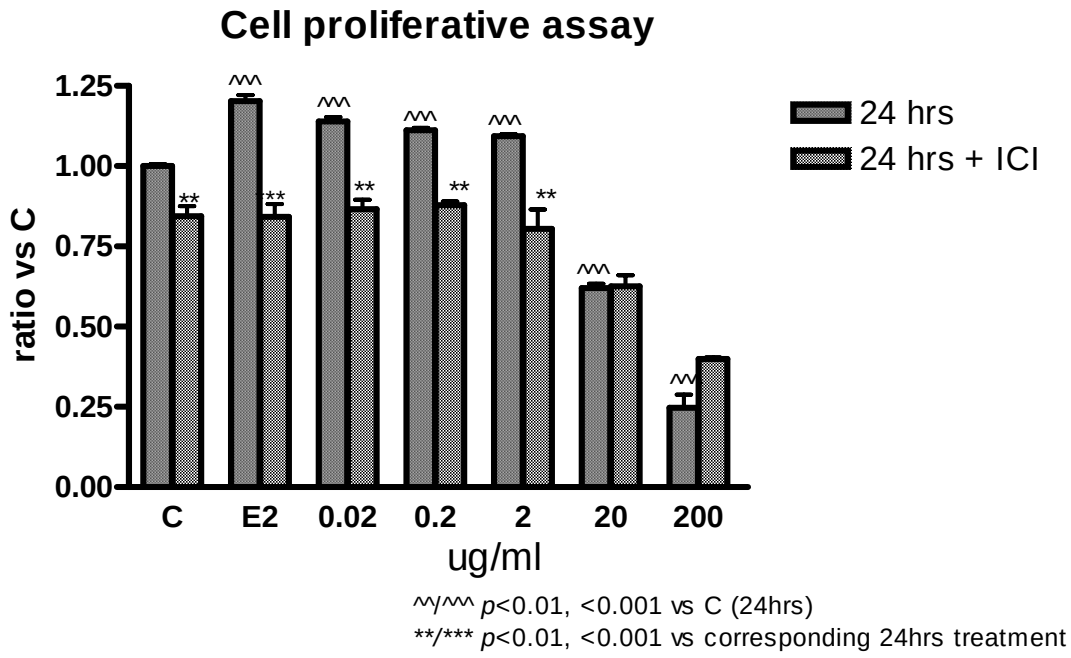


Figure 4.12 The effect of Rhizoma Drynariae total flavonoids on UMR-106 cell proliferation co-treated with estrogen antagonists. UMR-106 cells were treated with E2 (10nM), different doses of RD TF (0.002 to 200 μg ml^{-1}) or its vehicle (C) in the presence or absence of ICI blockers (10μM) for 24 hours. Results were analyzed by unpaired t-tests. They were obtained from 3 independent experiments and expressed as mean ± SEM (n = 6). ^^/^^^ p<0.01/p<0.001 (vs C at 24 hrs), ** / *** p<0.01/p<0.001) (vs C at 48 hrs). Cell proliferation stimulated by RD TF at a range from 0.002 to 0.2 μg ml^{-1} were completely inhibited by the addition of blocker (p<0.05), suggesting that the proliferation action of RD TF was mediated by estrogen receptor.

4.3.2.2 Differentiation Effects in UMR-106 Cells

Treatment of UMR-106 cells with Rhizoma Drynariae total flavonoids for 24 hours stimulated cell differentiation in a dose-dependent manner (Figure 4.13). RD TF, at 0.002, 0.02 and 0.2 μg ml^{-1}, significantly increased cell differentiation by 28%, 32% and 29% respectively (p<0.001). The increase in ALP activity in UMR-106 cells by RD TF fraction was significantly blocked by the co-treatment of ICI 182,780. The results suggested that RD TF promoted cell differentiation in an ER-dependent manner.

Cell differentiation assay (including ER-antagonist assay)

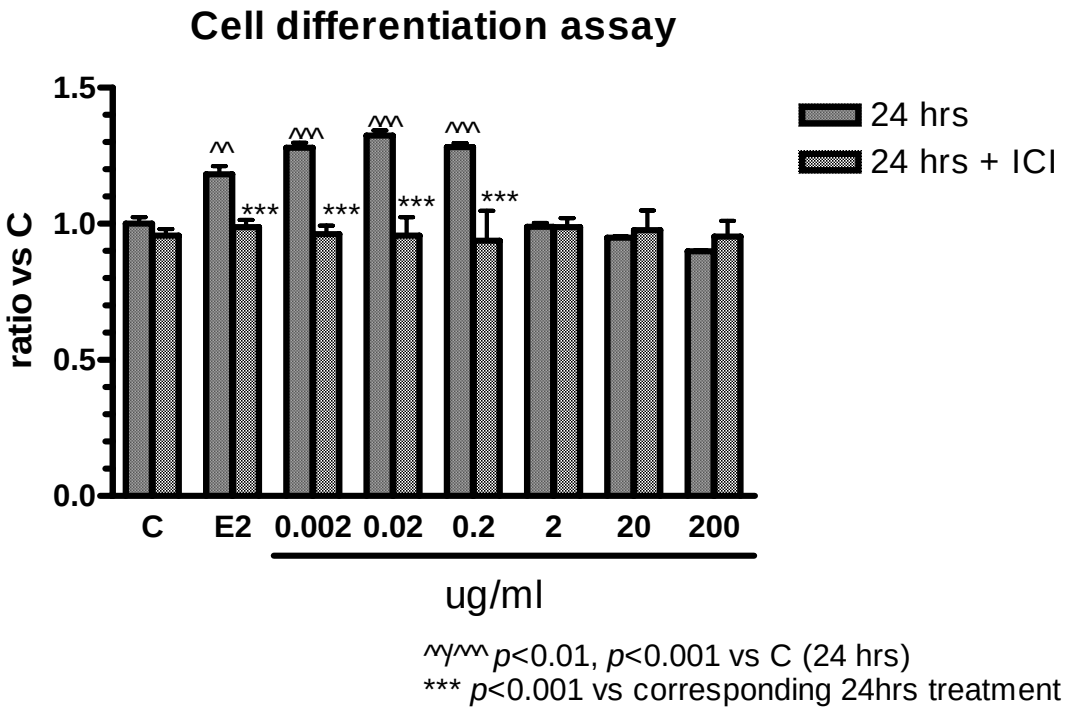


Figure 4.13 The effect of Rhizoma Drynariae total flavonoids on UMR106 cell differentiation, in the presence or absence of estrogen antagonists. UMR-106 cells were treated with E2 (10 nM), RD TF (0.002 to 200 μg ml^{-1}) or its vehicle for 24 hours in the presence or absence of ICI 182, 780 (10 μM). Cell differentiation rate was determined by ALP activity, and the results were normalized by the amount of total protein. Results were obtained from 3 independent experiments and were expressed as mean ± SEM (n = 6). Results were analyzed by unpaired t-test. The increase in alkaline phsphatase activities by 0.002 to 0.2 μg ml^{-1} of RD TF at 24 hours were blocked by co-treatment of UMR-106 cells with ICI 182, 780.

4.3.2.3 mRNA Expression of OPG to RANKL in UMR-106 Cells

Based on the results from cell proliferation and differentiation study, 0.02 to 0.2 µg ml^{-1} of Rhizoma Drynariae total flavonoids was selected to study the effects of RD TF on osteoclastogenesis in UMR-106 cells. Osteoprotegrin (OPG) is decoy receptor protein secreted by osteoblast that binds to RANK on osteoclast surface and prevents its interaction with RANKL on osteoblast. RANKL is expressed by osteoblast and is directly involved in inducing osteoclast activity. By the expression of OPG and RANKL, osteoblast is directly involved in modulating the process of osteoclastogensis. As shown in Fig. 4.14A, 10 nM E2 and 0.02 µg ml^{-1} RD TF significantly increased OPG mRNA expression by 2.2-fold and 55%, respectively (p<0.05 vs C). On the contrary, the expression of RANKL mRNA was considerably down-regulated by RD TF by 1-fold (Fig. 4.14B). The ratio of OPG to RANKL was calculated to demonstrate their effects on osteoclastogenesis. As shown in Figure 4.14C, it is obvious that both 10 nM E2 and 0.02µg ml^{-1} RD TF significantly increased OPG/RANKL ratio by 2-fold and 3-fold, respectively (p<0.05 vs C). The results suggested that 0.02 µg ml^{-1} RD TF might inhibit the process of osteoclastogenesis through the modulation of OPG and RANKL mRNA expression.

To determine of estrogen receptor (ER) is involved in the action of RD TF on osteoclastogenesis, UMR-106 cells were co-treated with 10 μM of estrogen antagonist, ICI 182, 780 and E2 or RD TF for 24 hours. As shown in Fig. 4.14A, the effect of RD TF on OPG/GAPDH mRNA expression was totally blocked by the estrogen antagonist significantly (p<0.05) while RANKL/GAPDH level did not alter greatly. The level of OPG/RANKL ratio was completely diminished by co-treating with ICI blocker (p < 0.05). The results suggested that the inhibitory effect of RD TF on osteoclastogenesis was at least in part mediated by estrogen receptor.

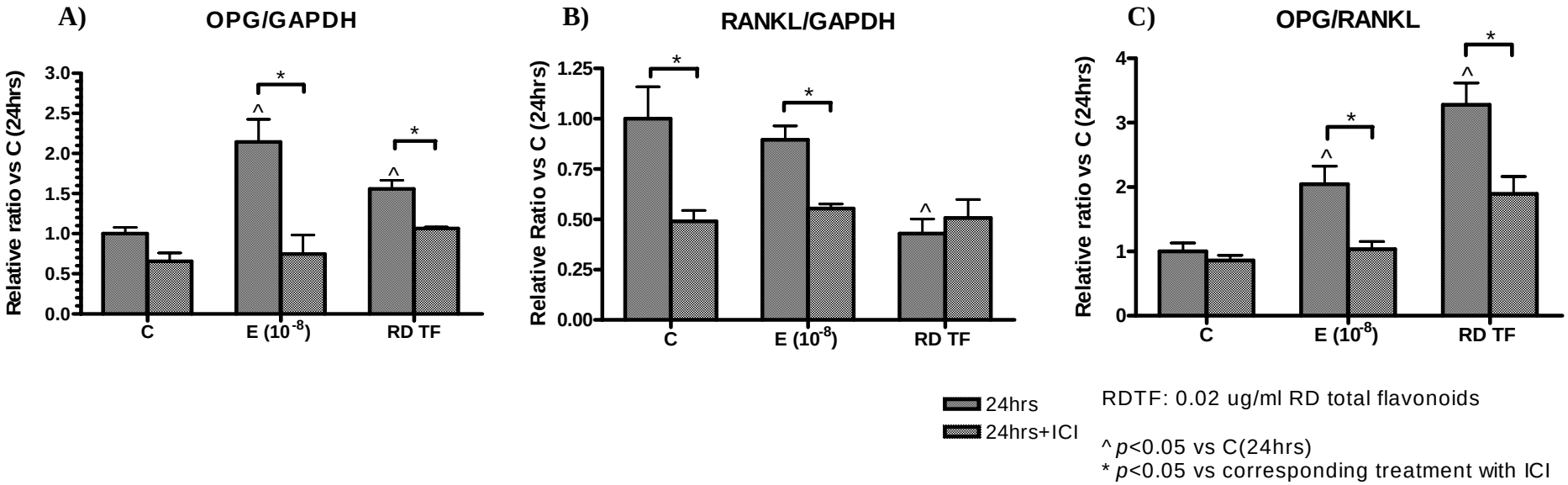


Figure 4.14 The effect of Rhizoma Drynariae total flavonoids on OPG and RANKL mRNA expression in UMR-106 cells UMR-106 cells were treated with 10 nM E2, 0.02 μg ml^{-1} RD TF or its vehicle (C) in the presence or absence of ICI 182780 (10 μM). The samples were subjected to quantitative real-time PCR analysis of OPG and RANKL mRNA expression. The ratio of OPG/RANKL was normalized by an internal control gene, GAPDH. Results were analyzed by unpaired t-tests and were expressed as mean ± SEM (n = 3). The above results indicate that 0.02 μg ml^{-1} RD TF significantly induced OPG mRNA expression and the activity was diminished by ICI 182,780 blocker. It is suggested that the stimulation of OPG expression is mediated through ER-pathway.

4.3.2.4 Effect of RD TF on ER-α or ER-β-mediated Luciferase Gene Expression in UMR-106 cells

The effects of the ER-α / ER-β-mediated luciferase gene expression stimulated by RD TF were demonstrated through its ability to induce ERE-dependent luciferase activity. As shown in Figure 4.15 A and B, E2 induced both ER-α and ER-β mediated estrogen response element (ERE) dependent luciferase activity by 2-fold and 2.7-fold respectively. 0.2 μg ml^{-1} of RD total flavonoids showed promoting effect on did not ERE transcription expression mediated by ER-α although it did not reach statistical significance. In addition, it significantly stimulated ER-β-mediated ERE-luciferase activity by 94% (p<0.01 vs C). The results suggested that 0.2 μg ml^{-1} RD TF may selectively increase ERE-transcription mediated by ER-β.

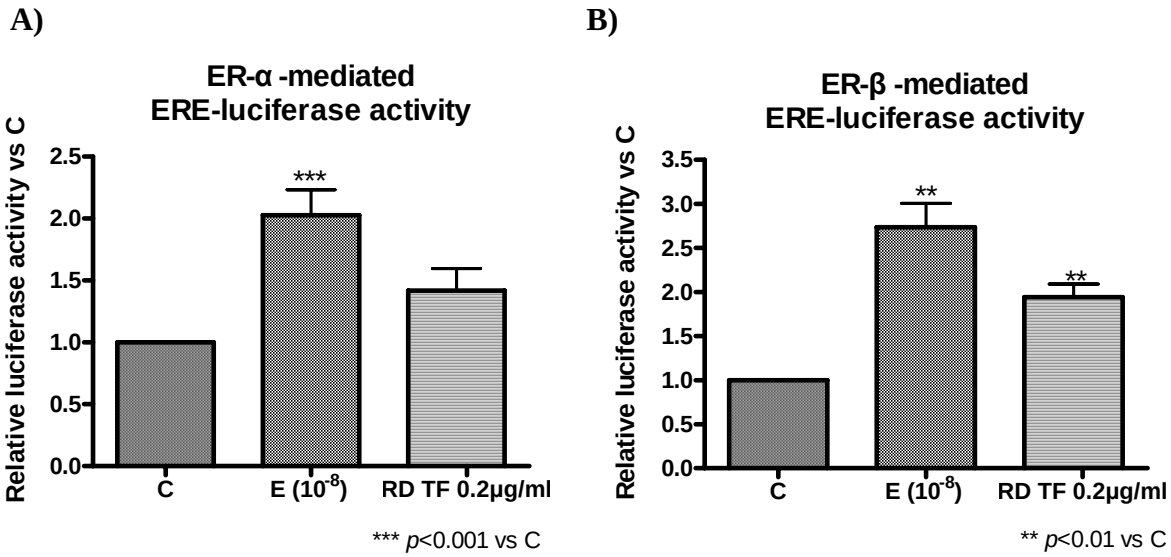
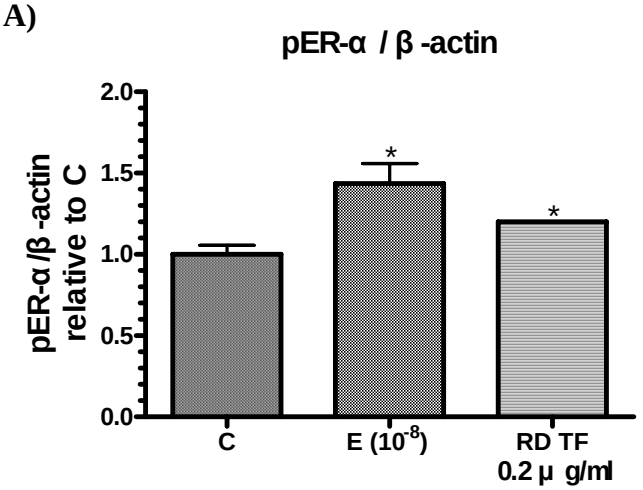


Figure 4.15 The effect of Rhizoma Drynariae total flavonoids on ER-α- or ER-β-mediated ERE-dependent luciferase activity in UMR-106 cells ER-α / ER-β and ERE DNA plasmids were co-transfected in UMR-106 cells 24 hours prior to the drug treatment. The cells were then treated with 10 nM E2, RD TF (0.2 µg ml^{-1}) or its vehicle (C) for 24 hours. Data represented as a ratio of ERE *Firefly* to TK *Renilla* luciferase activities, which are shown as mean ± SEM (n = 3). TK was the internal control for the monitoring of transfection efficiency. **A)** ER-α-mediated luciferase activity. 10 nM E2, but not 0.02 µg ml^{-1} of RD TF, induced ER-α-mediated ERE-dependent luciferase activity by 2-fold (p<0.001 vs C). **B)** ER-β-mediated luciferase activity. 10 nM E2 and 0.02 µg ml^{-1} of RD TF, induced ER-β-mediated ERE-dependent luciferase activity by 2.7-fold and 94% respectively (p<0.001 vs C).

4.3.2.5 Effect of Rhizoma Drynariae TF on ER-α Expression and its Phosphorylation in UMR-106 Cells

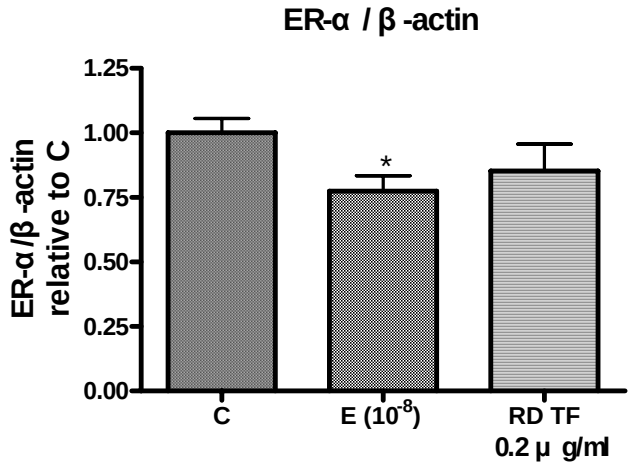
To determine whether RD total flavonoids can activate ER-α in the UMR-106 via ligand independent manner, the level of ER-α and phospho-ER-α expression were studied. Figure 4.16A shows that 10 nM of E2 and 0.2 μg ml^{-1} of RD TF significantly induced phospho-ER-α expression in UMR-106 cells by about 44% and 20%, respectively ($p < 0.001$ vs C). Figure 4.16B shows that 10 nM E2, but not RD TF (0.02 μg ml^{-1}), significantly decreased ER-α expression by 25% ($p<0.05$ vs C). To determine the modulating effect of RD TF on ER-α phosphorylation, the ratio of phospho-ER-α to ER-α was determined. Our results indicated that both E2 (10 nM) and RD TF (0.2 μg ml^{-1}) stimulated pER-α/ER-α expression by 85% ($p<0.001$) and 41% ($p<0.01$), respectively (Figure 4.16C). The results suggest that RD TF might activate ER-α through ligand independent pathway. In addition, the action of RD TF appears to be different from E2 as it did not significantly induce negative feedback of ER-α expression.

A)



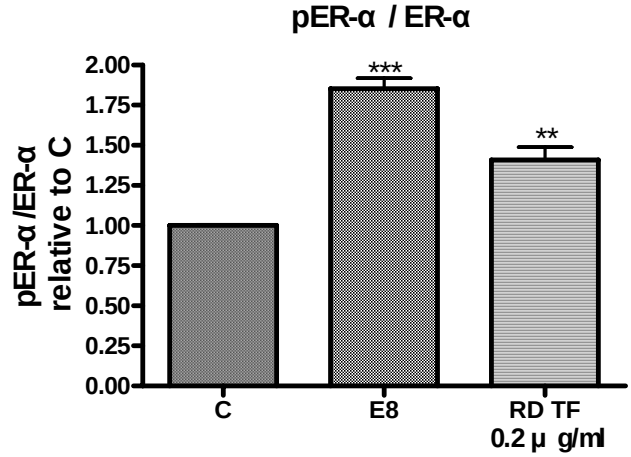
* / *** $p<0.05$, $p<0.001$ vs C

B)



* $p<0.05$ vs C

C)



** / *** $p<0.01$, $p<0.001$ vs C

D)

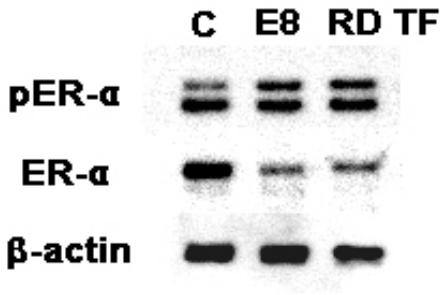


Figure 4.16 The effect of Rhizoma Drynariae total flavonoids on the expression of phosphorylation of ER-α in UMR-106 cells UMR-106 cells were treated with 10 nM E2, 0.2 μg ml^{-1} of RD TF or its vehicle for 24 hours. Cells lysates were subjected to western blotting analysis (detailed method were described in chapter 3). Data are shown as mean ± SEM (n = 3). **A)** Phospho-ER-α expression. The protein expression of pER-α was normalized by internal control, β-actin. 10 nM E2 and 0.2 μg ml^{-1} RD TF effectively induced the phosphorylation of ER-α (p<0.001 vs C). **B)** ER-α expression. The protein expression of ER-α was normalized by internal control, β-actin. E2, but not RD TF, significantly reduced ER-α expression (p<0.05). **C)** The relative ratio of pER-α to ER-α. Both E2 (10 nM) and RD TF (0.02 μg ml^{-1}) markedly increased the induction of phosphorylation of ER-α by 85% (p<0.001) and 41% (p<0.01), respectively. **D)** Illuminant images from Western immunoblotting.

4.4 Summary

Rhizoma Drynariae (RD) has been reported as a good enhancer of fracture healing. There are many findings demonstrated that RD flavonoids fractions have beneficial effects in treating osteoporosis (Gu min 2002; Xie 2004). However, its optimal dose and mechanisms of the actions are yet to be determined.

In summary, we confirmed that Rhizoma Drynariae total flavonoids exhibited beneficial effects on preventing OVX-induced bone loss in C57BL/6J osteoporotic female mice, which was evidenced by maintaining BMD in RD TF-treatment group compared to a significantly decreased BMD shown in OVX Vehicle group (versus OVX vehicle-treated group). Table 4.3 summarized the effect of different treatment on BMD, cross-sectional areas and SSI of bones obtained from sham- and OVX-operated mice. The results suggested that RD TF could restore the loss of bone properties induced by OVX in mice and that the osteoprotective effect of RD TF was dose-dependent. Based on the indicators of urinary calcium and collagen degradation, (Dpd/Cr levels), BMDs and bone strengths, they were found that 0.173 mg/g/day of RD TF was the most effective dosage *in vivo*. We also found that RD TF did not induce hyperplasia in uterus, suggesting RD TF might exert tissue selectivity with partial estrogen antagonist property (Carthew, Edwards et al. 1999).

In addition, we have demonstrated the *in vitro* osteoprotective effect of RD TF in rat

osteoblastic-like UMR-106 cells. Similarly, the stimulatory effects of RD TF on cell proliferation and cell differentiation under steroid-free culture conditions were dose-dependent. The proliferation and differentiation induced by RD TF were totally blocked by co-treating UMR-106 cells with ICI 182,780 estrogen antagonist, suggesting that the action of RD TF in promoting cell activities were mediated through estrogen receptor (ER) dependent pathway. mRNA expression of OPG and RANKL ratio suggested that RD TF might inhibit osteoclastogenesis by increasing the OPG expression from osteoblastic cells. Transient transfection study indicated that RD TF fractions significanly stimulated ERE-dependent luciferase activity in UMR-106 cells via ER-β but not ER-α. The results suggest that RD TF might activate ER-β in a ligand dependent manner but not in ER-α. Moreover, 0.2 μg ml^{-1} of RD TF significantly increased phospho-ER-α expression in UMR-106 cells. The latter results suggest that the osteoprotective effects of RD TF might also involve ligand independent activation of ER-α by phosphorylation. Thus, RD TF exerts ER dependent actions in UMR-106 cells in a manner similar from those of well actions of estrogen. Our results suggest that it might regulate osteoblastic activities through the activation of ERE transcription.

In lieu of these promising findings related to Rhizoma Drynariae active fractions and bone health in female mice and cell culture, the question that remains to be answered

is the identification of its bioactive single compounds responsible for the osteoprotective effect. In the next chapter, we will focus on one of the RD TF single compound responsible for bone-protective effect.

Table 4.3 Summarized data of the effects of Rhizoma Drynariae on BMD, cross-sectional area and SSI in femur, tibia and lumbar spine. Data was shown as mean ± SEM. (n = 7)

Group L 0.087 mg/g/day RD TF		Femur			Tibia		Lumbar spine L1
		Head	Mid-shaft	End	Head	Mid-shaft	
BMD (mg/ccm)	Total	426.2	440.3	377.4	267.9	421	226.9
	Trabecular	439.3	N/A	381.1*	285.6*	N/A	N/A
Cross Sectional Area	Total	1.68	N/A	4.40**	4.22	N/A	3.57*
	Trabecular	N/A	N/A	1.84*	2.22***	N/A	N/A
SSI polar		0.55	0.22	0.70**	0.34	0.12	0.58*

* p <0.05, ** p <0.01, *** p <0.001 vs V

Group M 0.173 mg/g/day RD TF		Femur			Tibia		Lumbar spine L1
		Head	Mid-shaft	End	Head	Mid-shaft	
BMD (mg/ccm)	Total	440.6*	453.7	395.6***	300**	443.4	249.1***
	Trabecular	437.2	N/A	410.9***	321.2***	N/A	N/A
Cross Sectional Area	Total	2.04	N/A	4.50**	5.27**	N/A	4.14***
	Trabecular	N/A	N/A	1.89*	2.33***	N/A	N/A
SSI polar		0.57	0.22	0.76***	0.55*	0.14	0.70***

* p <0.05, ** p <0.01, *** p <0.001 vs V

Group H 0.346 mg/g/day RD TF		Femur			Tibia		Lumbar spine L1
		Head	Mid-shaft	End	Head	Mid-shaft	
BMD (mg/ccm)	Total	425.1	441.6	393.8***	270.9	425.6	241.1**
	Trabecular	423.5	N/A	396.2**	296.0**	N/A	N/A
Cross Sectional Area	Total	1.74	N/A	3.975	5.49**	N/A	3.70*
	Trabecular	N/A	N/A	1.53	2.453***	N/A	N/A
SSI polar		0.5	0.23	0.62*	0.44	0.13	0.65**

* p <0.05, ** p <0.01, *** p <0.001 vs V

Key: N/A: Not Available

Chapter 5

Characterization of the osteoprotective effect of Naringin, the active component of Rhizoma Drynariae *in vivo* and *in vitro*

5.1 Background

Phytoestrogens are natural compounds found in plants and foods with biological activity like estrogen. A traditional Asian phytoestrogen-rich diet is associated with a lower incidence of breast cancer and postmenopausal symptoms (Chiechi 1999). The possibility of obtaining similar preventive effects of hormone replacement therapy arouses worldwide interests in alternative interventions with phytoestrogens (Chiechi 1999). Naringin (Figure 5.1A) is a yellowish flavonoid compound; a conjugate of a sugar molecule with naringenin (Figure 5.1B). Several studies have demonstrated the beneficial effects of Naringin on bone cell activities, including osteoblasts proliferation and differentiation (Wong and Rabie 2006). *In vivo* study was also attempted to demonstrate its positive effects on bone formation (Wong and Rabie 2006). Its backbone molecule, naringeinin, is able to bind to estrogen receptor (ER) with a higher binding affinity to ER-β than to ER-α. This estrogenic-like property suggested that Naringin might have potential beneficial effects on the prevention of postmenopausal illness without associated risks of the development of hormone-related cancers (Kuiper, Lemmen et al. 1998).

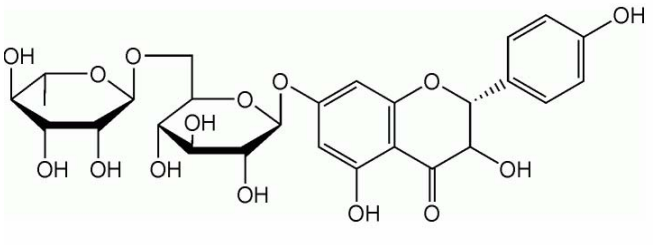
Rhizoma Drynariae (RD) is a traditional Chinese medicinal herb which is commonly prescribed in healing bone fracture (Wong and Rabie 2006). In the previous chapter, we have demonstrated the osteoprotective effect of Rhizoma Drynariae total

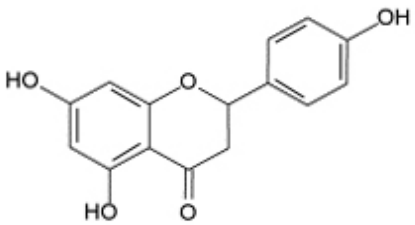
flavonoids *in vivo* and *in vitro*. It is worth investigating the bioactive compounds of this fraction. Naringin is the most abundant compound isolated from RD total extracts. Our collaborator has found that the RD total extract we obtained contains about 53% of Naringin, as shown in the HPLC profile (Figure 1.3). Combined with the previous studies on Naringin, Naringin is believed to be the most active ingredient in Rhizoma Drynariae. Similar to the study reported in chapter 4, the osteorprotective effects of Naringin were studied in osteoporotic mice model and rat osteoblastic-like UMR-106 cells. It is aimed to investigate the beneficial effect of Naringin on bone metabolism and study its molecular mechanism of actions.

5.1.1 Animal Study on the Effect of Naringin in Treating Osteoporosis

In this study, 50 one-month old C57 BL/6J female mice were kept for six weeks with daily Naringin treatment. Similar to the animal model described in section 4.1.1, mice were OVX or sham-operated before herbal treatment. Urine, serum, uterus, femur, tibia and lumbar spine were stored at -20^{o}C or -80^{o}C for further biochemical analysis. Serum and urinary bone resorption markers, uteri weight, bone mineral densities and bone strength were determined to characterize the osteoprotective effects of Naringin in mice models.

A) **B)**





Naringin

Molecular formula: $C_{27}H_{32}O_{14}$

Molecular weight: 580.53

Naringenin

Molecular formula: $C_{15}H_{12}O_5$

Molecular weight: 272.26

Figure 5.1 The chemical structures of Naringin and Naringenin. A) The chemical structure of Naringin. **B)** The chemical structure of Naringenin. Naringin has a conjugate sugar molecule on a naringenin backbone.

5.1.2 Naringin: The Molecular Mechanism of Actions in Rat Osteoblastic-like UMR 106 Cells

The molecular mechanisms of Naringin in bone cell activities were characterised in rat osteosacroma UMR-106 cells. Beneficial effects of Naringin on bone formation were assessed by studying osteoblastic cell proliferation and differentiation. The involvement of estrogen receptor (ER) in the action of Naringin was determined by co-treating cells with estrogen antagonist, ICI 182,780. The modulation of osteoprotegerin (OPG) and RANKL mRNA expression, transient transfection of estrogen responsive element (ERE) with luciferase construct as well as ER-α or ER-β plasmids; and the stimulative effect of phospho-ER-α protein expression were studied to elucidate its mechanism of actions involved in UMR-106 cells.

5.2 Methodology

Methods and materials were described in Chapter 3. For the animal study, 50 one month-old of C57BL/6J mice were divided into five groups (four of them were OVX-operated and one of the groups was sham-operated) and were administered orally with 17β-estradiol (0.2 μg/g/day), different doses of Naringin (0.2 and 0.4 mg/g/day) and its vehicle for six weeks (For detailed procedures please refer to Figure 3.1). For the *in vitro* study, 10^{-10} to 10^{-5} M of Naringin were used to treat UMR-106

cells for 24 or 48 hours. Naringin was purchased from Sigma-Aldrich, USA (# N1376) and dissolved in absolute ethyl alcohol.

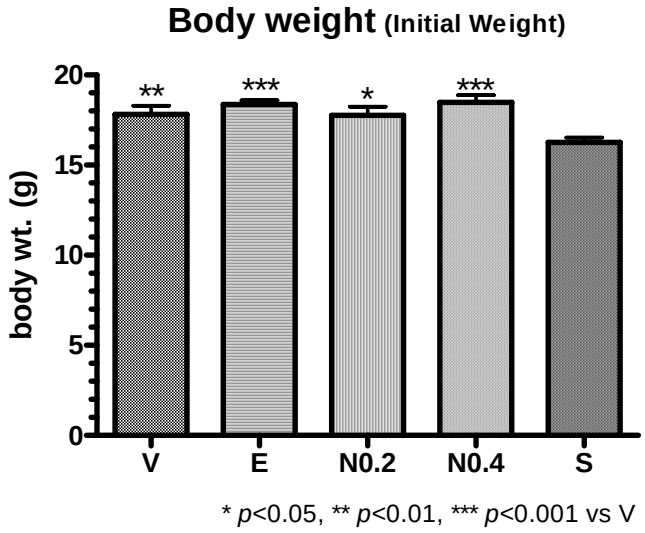
5.3 Results

5.3.1 Animal study

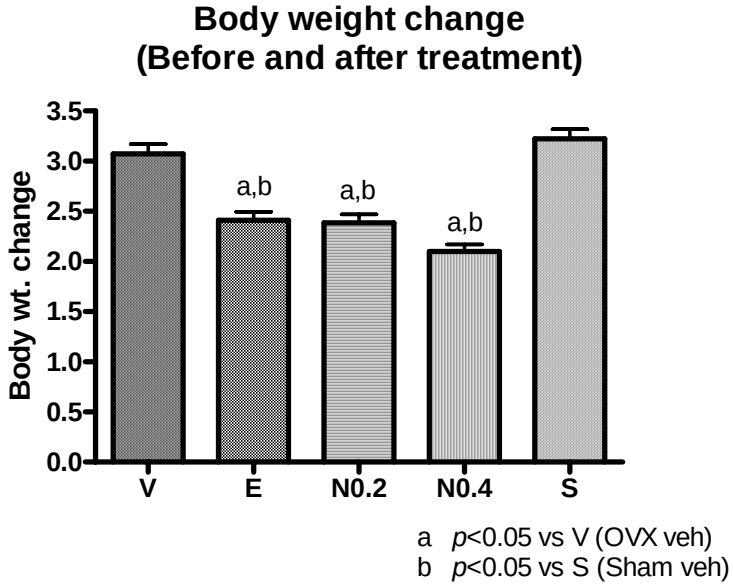
5.3.1.1 Effects on Body and Uterine Weights

OVX lead to an increase body weight in animals after 2-weeks operation despite the fact that the mice were subjected to pair feeding (Figure 5.2A) These results clearly indicate that depletion in endogenous estrogen lead to weight gain in animals. OVX mice treated with E2 and Naringin decreased body weight gain, compared with vehicle-treated OVX mice after treatment (Figure 5.2B, $p<0.05$). Uterine wet weight was significantly increased in group E (17β-estradiol) (Figure 5.3C). Comparing with OVX-vehicle (V) group, Naringin treatment groups did not increase uterine weight. The result suggested that Naringin did not exert estrogen-like activity in uterus.

A)


Body weight (Initial Weight)
body wt. (g)
20
15
10
5
0
**
***
*
***
V
E
N0.2
N0.4
S
* p<0.05, ** p<0.01, *** p<0.001 vs V

B)


Body weight change
(Before and after treatment)
Body wt. change
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
a,b
a,b
a,b
V
E
N0.2
N0.4
S
a p<0.05 vs V (OVX veh)
b p<0.05 vs S (Sham veh)

C)

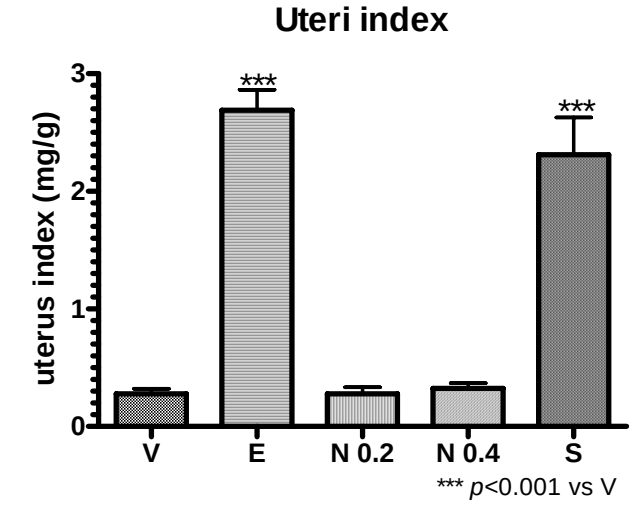

Uteri index
uterus index (mg/g)
3
2
1
0
***
***
V
E
N 0.2
N 0.4
S
*** p<0.001 vs V

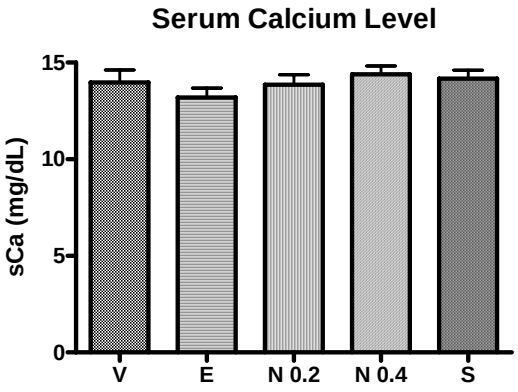
Figure 5.2 The effect of Naringin on body and uteri weights in Sham and OVX mice. Sham and OVX mice were subjected to the following treatments for 6 weeks: **V**: vehicle-group (OVX); **E**: E2 (0.2 μg/g/day); **N 0.2**: Naringin (0.2 mg/g/day); **N 0.4**: Naringin (0.4 mg/g/day) and **S**: vehicle (sham). Results were analyzed by one-way ANOVA and expressed as mean ± SEM. (*n*=8). **A)** Initial body weights after two weeks of ovariectomy. It is obvious that OVX leads weight gain than sham-operated mice. **B)** The body weight change before and after treatment. **C)** The uterine wet weight of sham and OVX mice. They were expressed as uterus weight to corresponding body weight (mg/g). It is found that, estrogen, but not Naringin, exert uterotrophic effect in OVX mice.

5.3.1.2 Effects on Biochemical Markers

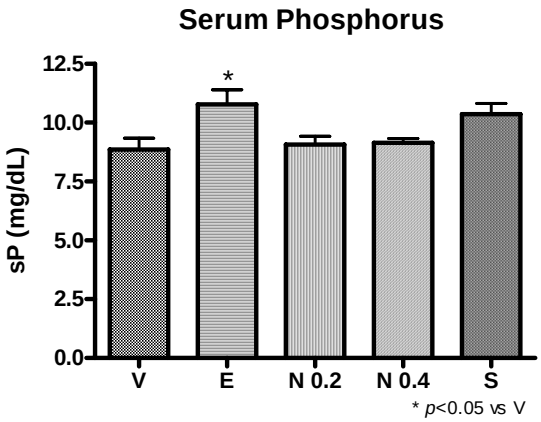
(I) Serum / Urinary Calcium and Phosphorus Levels

As shown in Figure 5.3A and 5.3B, treatment of OVX mice with E2 for six weeks alter serum P, but not serum Ca ($p<0.05$ vs vehicle-treated OVX group). Treatment of OVX mice with 0.2 mg/g/day or 0.4 mg/g/day Naringin for six weeks neither altered serum Ca nor P. Urinary Ca (uCa), but not urinary P (uP), excretion increased in response to OVX ($p<0.001$ vs vehicle-treated sham-operated) in mice (Figure 5.3C and 5.3D) However, treatment of OVX mice with E2 suppressed uCa and uP excretion ($p<0.05$ compared with vehicle-treated OVX mice) (Figure 5.3C and 5.3D). We also found that high dose of Naringin treatment (N 0.4) significantly inhibited uCa and uP loss induced by OVX in mice ($p<0.05$ compared with vehicle-treated OVX mice). Low dose of Naringin treatment (N 0.2) also diminished the increase of urinary calcium excretion considerably but the result did not reach statistical significance. The results suggested that Naringin might be useful for maintaining calcium and phosphorus balance.

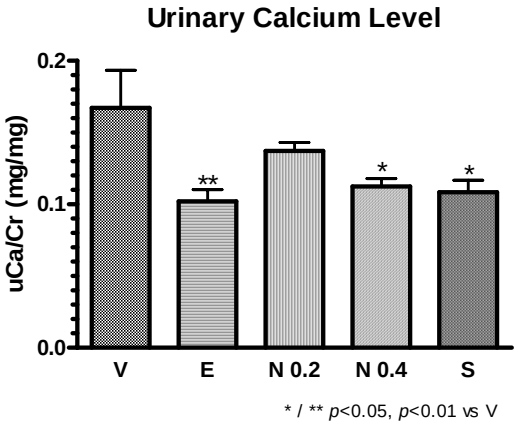
A)


Serum Calcium Level
sCa (mg/dL)
15
10
5
0
V
E
N 0.2
N 0.4
S

B)


Serum Phosphorus
sP (mg/dL)
12.5
10.0
7.5
5.0
2.5
0.0
*
V
E
N 0.2
N 0.4
S
* p<0.05 vs V

C)


Urinary Calcium Level
uCa/Cr (mg/mg)
0.2
0.1
0.0
**
*
*
V
E
N 0.2
N 0.4
S
* / ** p<0.05, p<0.01 vs V

D)

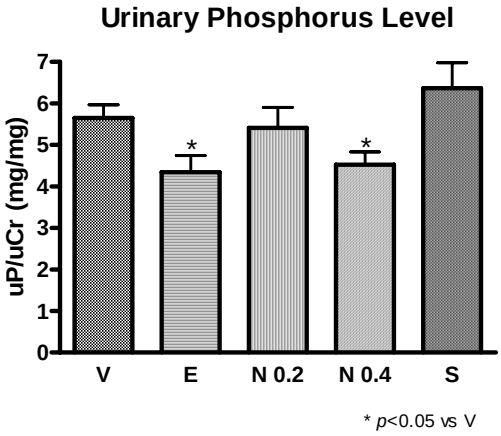

Urinary Phosphorus Level
uP/uCr (mg/mg)
7
6
5
4
3
2
1
0
*
*
V
E
N 0.2
N 0.4
S
* p<0.05 vs V

Figure 5.3 The effect of Naringin on serum and urinary calcium and phosphorus in Sham and OVX mice. Sham and OVX mice were subjected to the following treatments for 6 weeks: **V**: vehicle-group (OVX); **E**: E2 (0.2 μg/g/day); **N 0.2**: Naringin (0.2 mg/g/day); **N 0.4**: Naringin (0.4 mg/g/day) and **S**: vehicle (sham). Results were analyzed by one-way ANOVA and expressed as mean ± SEM. (n = 8). **A)** Serum calcium and **B)** Serum phosphorus levels in each treatment group. **C)** Urinary calcium and **D)** Urinary phosphorus levels in each treatment group. It is significant that 0.4 mg/g/day Naringin is effective in suppressing OVX-induced increase in uCa level (p<0.05) and reached to the level similar to E2 (group E) and sham-operated mice (group S).

(II) Urinary Dpd/Cr

Urinary Dpd/Cr is a biochemical marker for assessing bone resorption. Figure 5.4 shows that OVX increase urinary Dpd/Cr (uDpd/Cr) level ($p<0.05$, compared with vehicle-treated, sham-operated mice) while E2 suppressed OVX induced increase in Dpd/Cr level ($p<0.01$ vs vehicle-treated, OVX mice). Neither 0.2 nor 0.4 mg/g/day Naringin treatment significantly reduced uDpd/Cr level in OVX mice although there is a trend of decrease in uDpd/Cr in these groups.

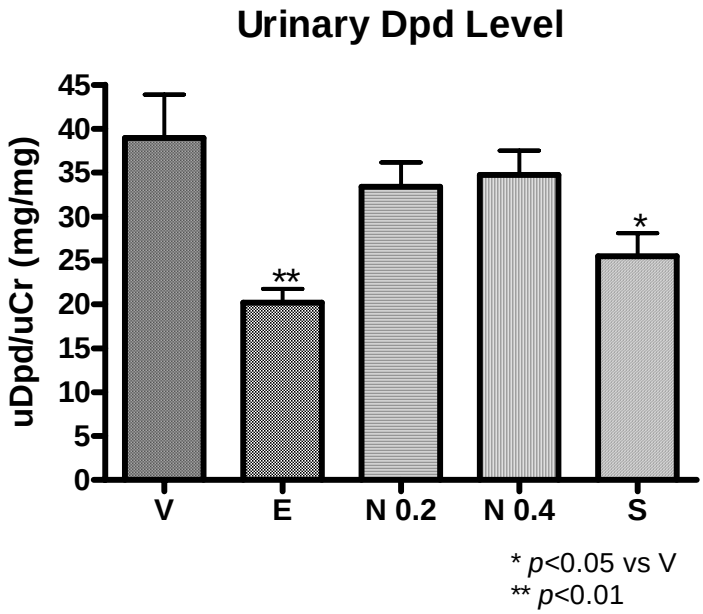


Figure 5.4 The urinary Dpd per unit of creatinine upon Naringin treatment in Sham and OVX mice Sham (**S**) and OVX mice were subjected to treatment with E2 (**E**, 0.2 μg/g/day); Naringin (**N0.2**, 0.2 mg/g/day and **N0.4**, 0.4 mg/g/day) and its vehicle (**V**) for 6 weeks. Urine samples were collected for 24 hours before the day of harvest. Naringin did not alter urinary Dpd level in OVX mice. Data was shown as mean ± SEM and results were analyzed by one-way ANOVA (n = 7).

5.3.1.3 Effects on Bone Quality and Bone Strength

Left femur and tibia were collected for the assessment of bone quality and bone strength. The total, trabecular and cortical bone mineral densities (BMD); cross-sectional area of bone and stress-strain index were measured by pQCT, while the bone strength of tibia mid-shaft was measured by three-point bending method.

(I) Bone Mineral Densities (BMD) of Mice Femur, Tibia and Lumbar Spine

Table 5.1 summarized the effect of different treatments on bone mineral density (BMD) at different regions of femur in sham and OVX mice. OVX mice treated with E2 significantly restored total BMD ($p<0.01$), trabecular BMD ($p<0.05$) and total cross-sectional area ($p<0.001$) at proximal femur, compared with vehicle-treated OVX mice. Besides, low and high dose of Naringin treatments did not alter their BMD and total cross-sectional area greatly, compared with vehicle-treated OVX mice.

The cortical BMD was detected in femur mid-shaft. Table 5.1 shows the cortical BMD changes in each treatment group. It shows that OVX-operation did not alter greatly in femur cortical bone region; there was even no significant difference between group V and S (Sham-operated). In contrast, OVX mice treated with E2 significantly increased cortical BMD from OVX-induced bone loss ($p<0.01$, compared with vehicle-treated OVX mice). As for Naringin treatment groups, varied

dosages of Naringin did not increase the decrease of cortical BMD at femur mid-shaft due to OVX-operation.

Table 5.1 The effect of Naringin on BMD and total cross-sectional area in different regions of femur. Femur was subjected to pQCT measurement. Data shown as mean ± SEM and results were analyzed by one-way ANOVA. (*n* = 7)

Proximal Femur

	V	E	N 0.2	N 0.4	S
Total BMD (mg/ccm)	405.74 ± 9.16	456.70 ± 12.44 **	419.03 ± 6.83	425.93 ± 6.58	432.73 ± 8.59
Trabecular BMD	389.80 ± 4.05	466.36 ± 19.94 *	395.87 ± 7.08	427.98 ± 13.67	453.00 ± 10.84 *
Total cross sectional area (mm^2)	1.53 ± 0.16	2.98 ± 0.24 ***	1.50 ± 0.18	1.35 ± 0.11	2.56 ± 0.26 **

** / *** p <0.01 / p <0.001 vs V

Femur Mid-shaft

	V	E	N 0.2	N 0.4	S
Cortical BMD (mg/ccm)	428.10 ± 6.39	470.85 ± 7.26 **	437.40 ± 6.30	433.53 ± 4.36	440.17 ± 8.83

** p <0.01 vs V

Distal Femur

Distal femur is the major scanning region for the assessment of efficacy on BMD in pre-clinical models. As shown in Figure 5.5A and 5.5B, total and trabecular BMD were decreased in vehicle-treated OVX mice (V), $p<0.001$ compared with vehicle-treated sham (S). OVX mice treated with E2 significantly restored total and trabecular BMD from OVX-induced bone loss by 35% and 39%, respectively ($p<0.001$ vs group V). Treatment of OVX mice with Naringin (0.2 and 0.4 mg/d/day) also significantly increased total BMD of distal femur by 12% ($p<0.01$ vs V) and 15% ($p<0.001$ vs V), respectively (Figure 5.5A). High dose of Naringin treatment (0.4 mg/g/day) increased trabecular BMD by 17% (Figure 5.5B, $p<0.01$ vs V). As shown in Fig. 5.5C, total cross-sectional areas of distal femur were not significantly altered in response to OVX mice treated with E2 or Naringin, compared with vehicle-treated Sham (group S) mice. However, OVX decreased trabecular cross-sectional area of distal femur ($p>0.05$, compared with group S, Fig. 5.5D). Treatment of OVX mice with E2 and 0.4 mg/g/day Naringin, but not 0.2 mg/g/day Naringin, restored trabecular cross-sectional area to the level similar to sham-operated mice (group S) ($p<0.05$, compared with vehicle-treated OVX mice). The results suggested that Naringin was effective in improving bone quality by increasing BMD and cross-sectional area at distal femur.

Distal Femur

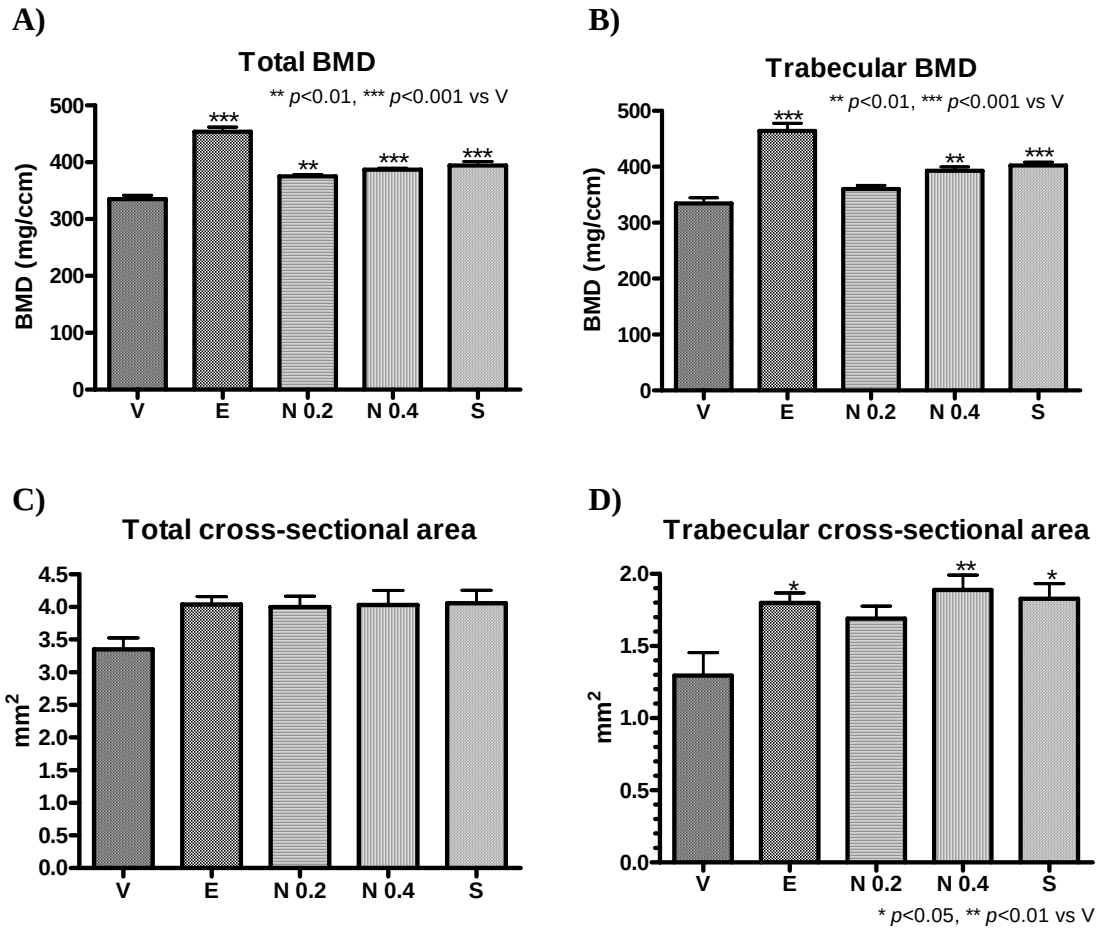


Figure 5.5 The effect of Naringin on total BMD, trabecular BMD, total cross-sectional area and trabecular cross-sectional area at distal femur in Sham and OVX mice Sham (**S**) and OVX mice were subjected to different treatments including E2 (**E**), two doses of Naringin (**N0.2** and **N0.4**) and its vehicle (**V**) for 6 weeks. Femur was subjected to pQCT measurement. Data was shown as mean ± SEM and were analyzed by one-way ANOVA (n = 7). The data demonstrated that higher dose of Naringin was more effective than its low dose in terms of trabecular BMD and trabecular cross-sectional area at distal femur.

Proximal Tibia

Proximal Tibia is another important scanning region for the detection of change in bone mineral density (BMD). Figure 5.6 shows the BMD and cross-sectional area of proximal tibia in mice in response to different treatment. OVX vehicle-treated mice significantly decreased total BMD, trabecular BMD and trabecular cross-sectional area, compared with vehicle-treated sham-operated mice ($p<0.05$), but no considerable decrease in total cross-sectional area. OVX mice treated with E2 significantly restored total and trabecular BMD from OVX-induced bone loss by 35% and 40%, respectively ($p<0.001$ vs vehicle-treated OVX (V) group). In contrast, E2 treatment did not exhibit promoting effect in cross-sectional area at proximal tibia. As compared with OVX vehicle group, 0.4 mg/g/day Naringin-treated group significantly induced total BMD, trabecular BMD and trabecular cross-sectional area in 19.3% ($p<0.05$), 20.1% ($p<0.01$) and 84.2% ($p<0.05$) respectively. It suggests that high dose of Naringin could normalize the above parameters back to the sham-level. Treatment with half dose of Naringin also promoted trabecular BMD obviously in 26.4% ($p<0.001$ vs V). In addition, the total cross-sectional areas of Naringin treatment groups were considerably increased although the increase did not reach statistical significance. The results suggested that Naringin was effective in preventing bone loss at proximal tibia which was induced by OVX.

Proximal Tibia

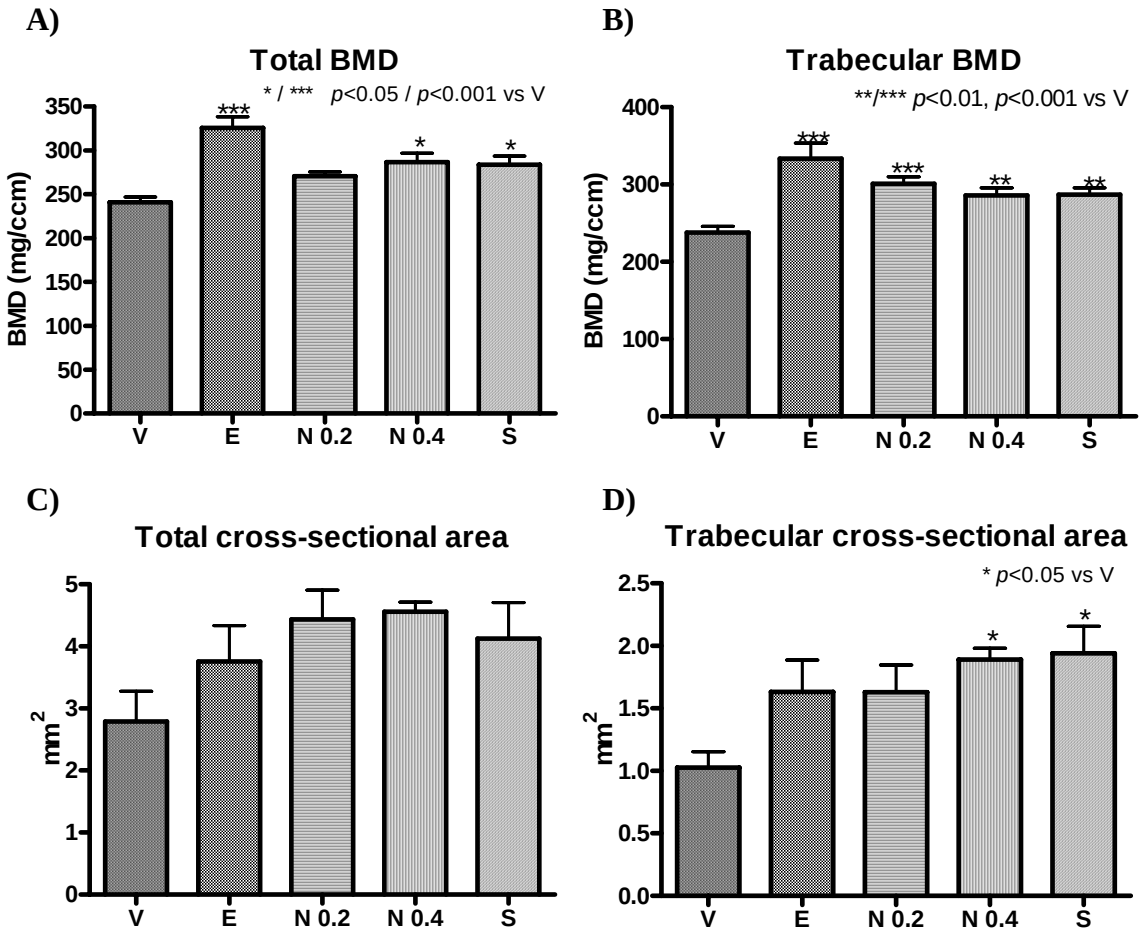


Figure 5.6 The effect of Naringin on total BMD, trabecular BMD, total cross-sectional area and trabecular cross-sectional area at proximal tibia in Sham and OVX mice Sham (**S**) and OVX mice were subjected to different treatments including E2 (**E**), two doses of Naringin (**N0.2** and **N0.4**) and its vehicle (**V**) for 6 weeks. Tibia was subjected to pQCT measurement. Data was shown as mean ± SEM and were analyzed by one-way ANOVA (n = 7). Results show that 0.4 mg/g/day Naringin treatment had universal benefits to bone quality at proximal tibia in mice (p<0.05 vs vehicle-treated OVX group). The BMD and trabecular cross-sectional area were increased significantly compared with OVX vehicle (V).

Tibia mid-shaft

To detect the effect of different treatments on cortical bone, the mid-shaft region of tibia were subjected to pQCT scanning. As shown in Figure 5.7, OVX mice (V) did not alter cortical BMD greatly, compared with vehicle-treated sham-operated mice (S). It seems that OVX could not induce the loss of bone in mid-shaft of tibia within the period of treatment. OVX mice treated with E2 significantly increased cortical BMD ($p<0.05$ vs V). Besides, both treatments with high and low doses of Naringin (N0.2 and N0.4) did not increase cortical BMD at mid-shaft tibia.

Tibia mid-shaft

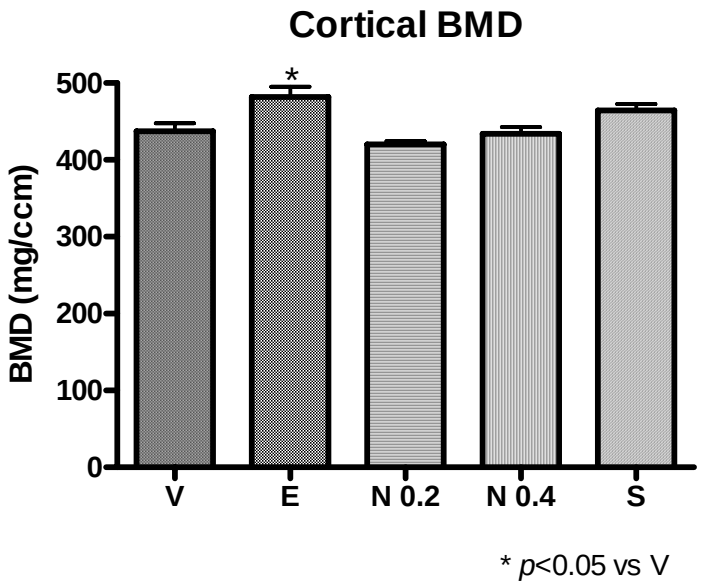


Figure 5.7 The effect of Naringin on cortical BMD at tibia mid-shaft in Sham and OVX mice Sham (**S**) and OVX mice were subjected to different treatments including E2 (**E**), two doses of Naringin (**N0.2** and **N0.4**) and its vehicle (**V**) for 6 weeks. Tibia was subjected to pQCT measurement. Data was shown as mean ± SEM and results were analyzed by one-way ANOVA (n = 7). Results indicate that OVX did not greatly affect the loss of cortical BMD at tibia mid-shaft, compared with sham-operated mice. However, OVX mice treated with E2 significantly increased cortical BMD at mid-shaft tibia (p<0.05 vs V).

Lumbar Spine L1

To assess the effect of Naringin on axial skeleton, the BMD of lumbar spine was measured by pQCT. As shown in Figure 5.8A and 5.8B, there were no significant differences in total BMD and total cross-sectional area between vehicle-treated OVX (V) and Sham-operated (S) mice at lumbar spine region L1. In contrast, OVX mice treated with E2 (E) significantly increased total BMD and cross-sectional area at lumbar spine region by 20.5% and 2.2-fold, respectively ($p<0.001$ vs V). 0.4 mg/g/day (N0.4), but not 0.2 mg/g/day (N0.2), Naringin treatment improved OVX-induced loss of BMD and total cross-sectional area (Figure 5.7). N0.4 significantly stimulated bone mineral density (BMD) by 15.8% ($p<0.001$ vs V) and total cross-sectional area by 66.6% ($p<0.05$ vs V). N0.2 appeared to raise BMD and cross-sectional area to the level attained by sham (S) mice at lumbar spine region L1 although the result did not reach statistical significance.

Lumbar Spine L1

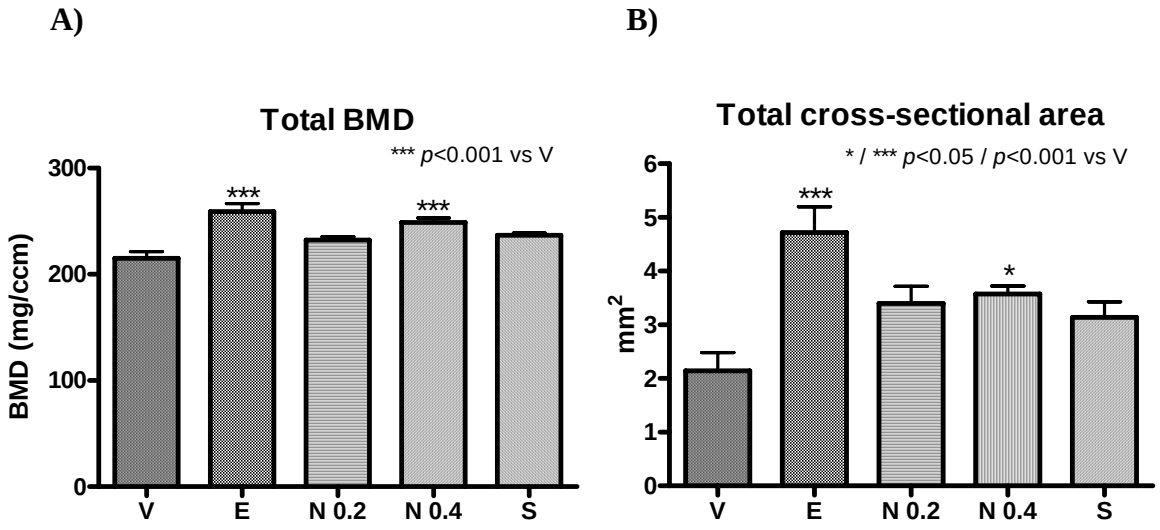


Figure 5.8 The effect of Naringin on total BMD and total cross-sectional area at lumbar spine region L1 in Sham and OVX mice Sham (**S**) and OVX mice were subjected to different treatments including E2 (**E**), two doses of Naringin (**N0.2** and **N0.4**) and its vehicle (**V**) for 6 weeks. Tibia was subjected to pQCT measurement. Data was shown as mean ± SEM and results were analyzed by one-way ANOVA (*n* = 7). **A)** Total BMD and **B)** total cross-sectional area of lumbar spine region L1 in Sham and OVX mice.

(II) Simulated Bone Strength (Expressed as SSI, Stress-Strain Index)

SSI is a parameter for measurement of bending bone strength. According to the results from bone strength simulative programme, the effects of E2, Naringin and its vehicle on bone strengths have been calculated. As shown in Table 5.2, ovariectomy weakened bone strengths significantly at proximal, mid-diaphysis and distal regions of femur (group V) (p<0.05, compared with vehicle-treated, sham-operated mice). This treatment also seemed to weaken mechanical strengths at lumbar spine and proxmal and mid-shaft regions of tibia but the results did not reach stastistical significance. Besides, OVX mice treated with E2 significantly compensated OVX-induced weakening bone strengths at all three regions of femur and lumbar spine by 46.7%, 42.3%, 88% and 2.2-fold, respectively (p<0.01 vs group V). Table 5.2 also indicates that N 0.2 and N 0.4 significantly strengthened the bone strengths in the region of distal femur and lumbar spine region L1. Group N0.2 significantly improved bone strengths on distal femur and lumbar spine by 61% (p<0.05) and 47% (p<0.01) respectively, while N0.4 promoted better bone strength on the same scanning regions by 88% (p<0.001) and 53.3% (p<0.001). The results confirmed that Naringin has potential in maintaining BMD, as well as bone strengths after OVX-operation, especially at the regions which are rich in trabecular bone.

Table 5.2 The effect of Naringin on stress-strain index (SSI) at femur, tibia and lumbar spine in Sham and OVX mice Sham (**S**) and OVX mice were subjected to different treatments including E2 (**E**), two doses of Naringin (**N0.2** and **N0.4**) and its vehicle (**V**) for 6 weeks. Tibia was subjected to pQCT measurement and SSI was calculated by in-house programme. Data was shown as mean ± SEM and results were analyzed by one-way ANOVA (n = 7). It is obvious that Naringin-treated groups strengthened bone strengths in distal femur and lumbar spine L1 region in OVX mice.

			Naringin		
Regions	V	E	N 0.2	N 0.4	S
Femur					
Proximal	0.467 ± 0.032	0.676 ± 0.053 **	0.489 ± 0.022	0.572 ± 0.017	0.660 ± 0.025 **
Mid-shaft	0.196 ± 0.004	0.279 ± 0.019 ***	0.201 ± 0.004	0.212 ± 0.010	0.229 ± 0.010 *
Distal	0.380 ± 0.049	0.714 ± 0.055 ***	0.613 ± 0.048 *	0.714 ± 0.055 ***	0.738 ± 0.032 ***
Tibia					
Proximal	0.200 ± 0.037	0.486 ± 0.120	0.325 ± 0.073	0.300 ± 0.038	0.400 ± 0.065
Mid-shaft	0.121 ± 0.010	0.153 ± 0.010	0.136 ± 0.009	0.154 ± 0.010	0.143 ± 0.014
Lumbar Spine	0.400 ± 0.053	0.888 ± 0.089 ***	0.588 ± 0.035 **	0.613 ± 0.044 ***	0.563 ± 0.042

* / ** / *** p <0.05, p <0.01, p <0.001 vs V

5.3.1.4 Biomechanical Measures of Tibia Mid-diaphysis

Biomechanical strengths of the cortical bone were measured by using 3-point bending theory. As shown in Table 5.3, ovariectomy (group V) weakened bone strengths at tibia mid-shaft by decreasing ultimate load and energy for breaking ($p<0.05$, compared with vehicle-treated, sham-operated mice). However, OVX did not alter breaking force, stiffness and flexural modulus greatly in mice tibia. In contrast, OVX mice treated with E2 significantly increased ultimate load from OVX-induced bone loss by 40% ($p<0.001$ vs group V) in mice. The maximum force that needed to break the bone was relatively stronger than those in sham-operated mice but E2 seems did not exhibit promoting effects on breaking force, stiffness and flexural modulus. Table 5.3 also indicates that N0.2 and N0.4 significantly strengthened bone strength at tibia mid-shaft in terms of increasing ultimate load (Max. Force), yield load (Breaking Force) and energy for breaking. OVX mice treated with 0.2 mg/g/day Naringin increased ultimate load by 29.4% ($p<0.01$ vs group V), breaking force by 22.6% ($p<0.05$ vs group V) and energy for breaking by 2.5-fold ($p<0.01$ vs group V). 0.4 mg/g/day Naringin increased ultimate load and energy for breaking by 6% and 84%, respectively in OVX mice ($p<0.01$vs group V). However, none of the treatment significantly altered stiffness and flexural modulus of tibia. Out results indicate that Naringin may exert beneficial effects to the structural and materials properties of tibia.

Table 5.3 The effect of Naringin on biomechanical bone strengths at tibia diaphysis in Sham and OVX mice Sham (**S**) and OVX mice were subjected to different treatments including E2 (**E**), two doses of Naringin (**N0.2** and **N0.4**) and its vehicle (**V**) for 6 weeks. Tibia was subjected to 3-point bending machine and the parameters were calculated by in-house programme. Data was shown as mean ± SEM and results were analyzed by one-way ANOVA (n = 7). It shows that N0.2 and N0.4 strengthed mechanical strengths at tibia mid-shaft in terms of increasing ultimate load and energy for breaking (p<0.01, compared with OVX vehicle-treated mice, group V).

	Ultimate load (N)	*Breaking Force (N)*	*Energy for breaking ($\times 10^{-3}$ J)*	*Stiffness (N/mm)*	*Flexural Modulus (MPa)*
V	11.734 ± 0.34	11.36 ± 0.26	4.69 ± 0.59	14.89 ± 1.71	1.31 ± 0.11
E	16.46 ± 0.76 ***	16.37 ± 0.66 ***	6.93 ± 0.51	18.83 ± 1.53	1.84 ± 0.17
N 0.2	15.19 + 0.57 **	13.93 + 0.50 *	11.68 + 2.1 **	18.87 + 2.39	1.79 + 0.13
N 0.4	15.61 + 0.39 ***	13.89 + 0.73	8.7 + 0.74 **	20.15 + 2.58	2.02 + 0.24
S	14.71 ± 0.69 *	12.45 ± 0.84	8.63 ± 1.18 *	23.28 ± 4.90	1.74 ± 0.23

* / ** / *** p <0.05, p <0.01 , p <0.001 vs V

5.3.2 *In Vitro* Study

The molecular mechanisms involved in the action of Naringin on bone were studied by using rat UMR-106 osteosacroma cells. Its effects on osteoblastic cell proliferation, differentiation, estrogen-receptor (ER) dependent assay and modulation of osteoclastogenesis were characterised. The effects of Naringin on ER-dependent pathway are demonstrated by using UMR-106 cells transfected transiently with both ER-(α and β) and ERE luciferase constructs, as well as studying its effect on ER-α phosphorylation. Detailed results are reported in the following sections:

5.3.2.1 Proliferative Effects of Naringin in UMR-106 Cells

UMR-106 cells were treated with 10^{-10} to 10^{-5} M of Naringin for 24 and 48 hours. Figure 5.8 shows the effects of Naringin on osteoblastic cell proliferation. At 24 hour, 10^{-8} M to 10^{-6} M of Naringin increased UMR-106 cell proliferation ($p<0.05$ vs its vehicle). In contrast, by 48 hours, Naringin at all concentrations (10^{-10} to 10^{-5} M) increased osteoblastic cell proliferation. Specifically, 10^{-10} to 10^{-8} M of Naringin effectively increased cell numbers by 1.3 to 1.4-fold ($p<0.01$ and $p<0.001$). The results suggested that the proliferative effects of Naringin on osteoblastic cell were time- and dose-dependent. To determine if the proliferation effect of Naringin require estrogen receptor (ER), cells were co-treated with estrogen receptor antagonist, ICI

182,780. As shown in Fig. 5.10, the stimulatory effect of 10 nM E2 on cell proliferation could be abolished by co-treatment with ICI, similarly, the positive effects of 1 to 10 nM of Naringin treatment on UMR-106 cell proliferation could be abolished by ICI co-treatment ($p<0.05$, $p<0.01$). The above results suggested that the stimulatory effect of 1nM and 10nM of Naringin on cell proliferation were mediated through the estrogen-receptor pathway.

Dose- and Time-dependent Cell Proliferative Assay

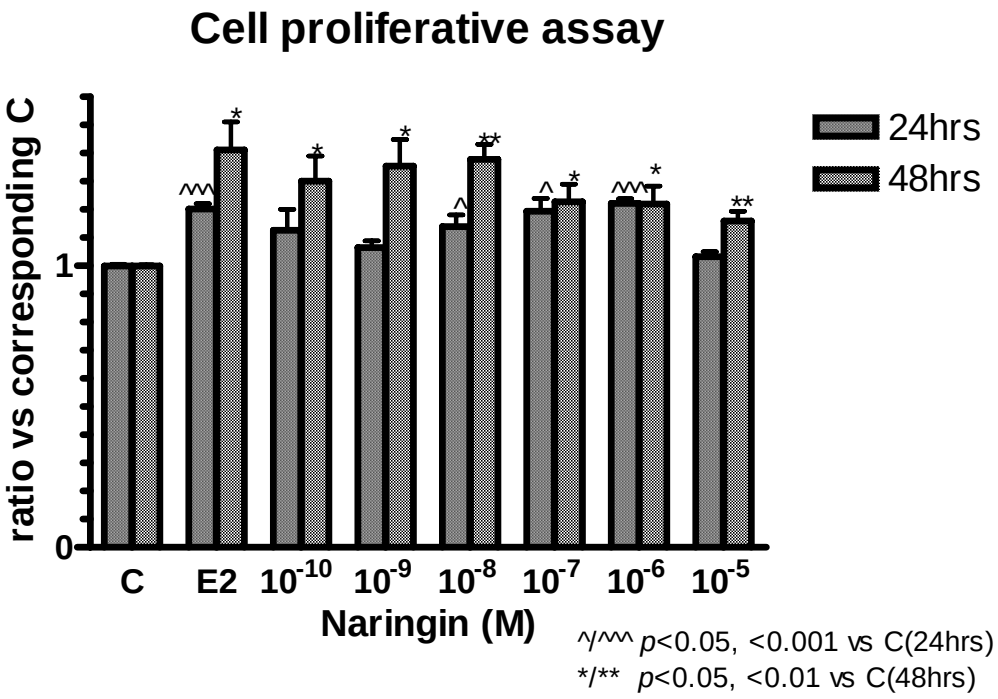


Figure 5.9 The effect of Naringin on cell proliferation in 24 and 48 hours in UMR-106 cells UMR-106 cells were treated either with E2 (10 nM), Naringin (10^{-10} to 10^{-5} M) or its vehicle for 24 or 48 hours. Their effects on cell proliferation were measured by MTS assay. Results were analyzed with unpaired t-test and data shown as mean $\pm$ SEM (n = 5). It shows that 10nM of E2, as well as 10^{-8} and 10^{-7} M of Naringin significantly increased cell proliferation in both 24 and 48 hours of treatment.

Estrogen-receptor (ER) antagonist assay (48 hours treatment)

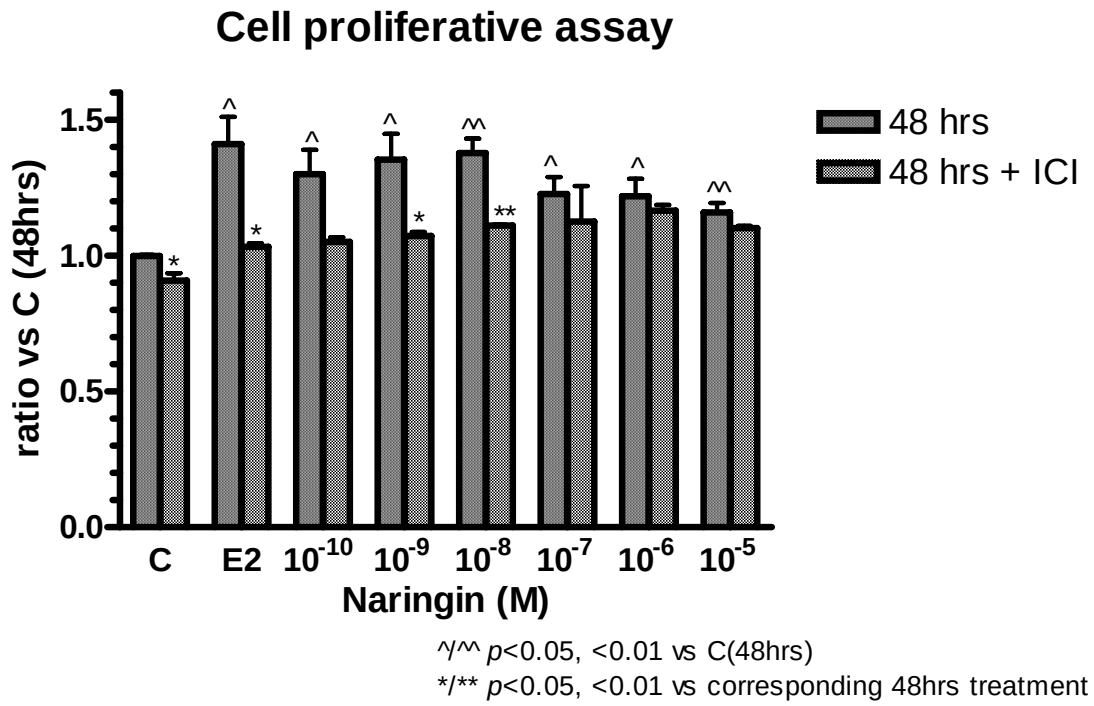


Figure 5.10 The effect of Naringin on cell proliferation, co-treated with estrogen antagonists in UMR-106 cells UMR-106 cells were treated either with E2 (10 nM), Naringin (10^{-10} to 10^{-5} M) or its vehicle in the presence or absence of 10 μM ICI 182,780 for 48 hours. Their effects on cell proliferation were measured by MTS assay. Results were analyzed with unpaired t-test and data shown as mean ± SEM (n = 5). It shows that proliferative effects of 1 and 10 nM of Naringin in UMR-106 cells were completely abolished by the presence of estrogen antagonist, ICI blocker (p<0.05 vs its vehicle).

5.3.2.2 Differentiative Effects of Naringin in UMR-106 Cells

Alkaline phosphatase activity is a common marker for assessment of osteoblastic cell differentiation. Treatment of UMR-106 cells with Naringin for 24 hours stimulated cell differentiation in a dose-dependent manner (Figure 5.11). Naringin, varied from 10^{-8} to 10^{-6} M, as well as 10 nM E2, significantly increased cell differentiation by 10% ($p<0.01$), 13% ($p<0.001$), 12% ($p<0.001$), and 15% ($p<0.001$), respectively. Their effects could be completely abolished in the presence of ICI 182,780, suggesting that the stimulatory effect of Naringin require the presence of ER (Fig. 5.11, $p<0.01$ vs its vehicle).

Cell Differentiation Assay (including Estrogen Antagonist Assay)

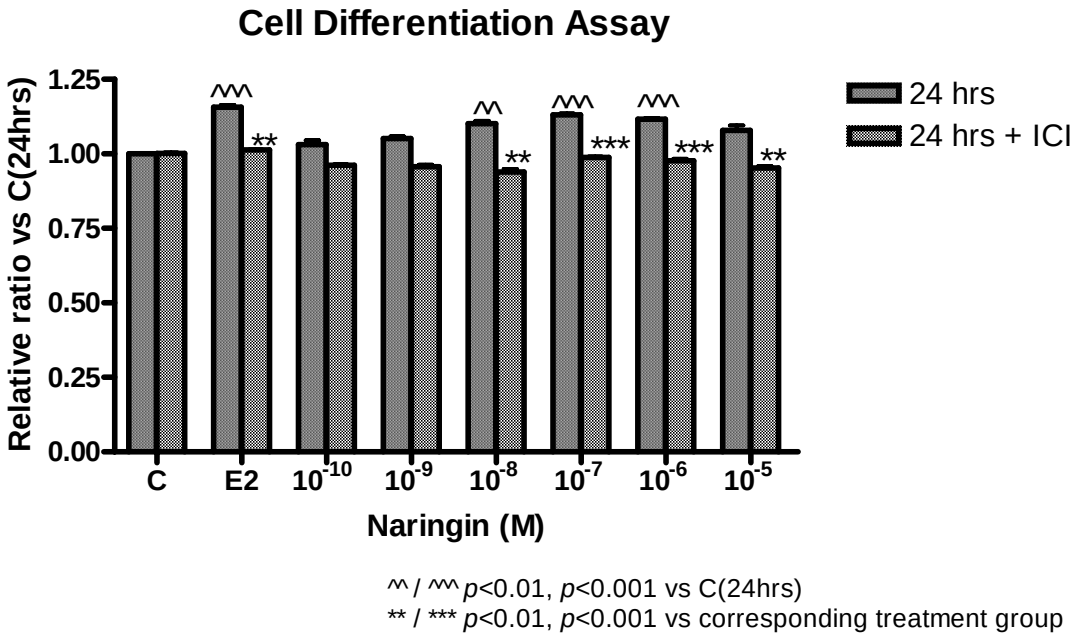


Figure 5.11 The effect of Naringin on cell differentiation in UMR-106 cells

UMR-106 cells were treated either with E2 (10 nM), Naringin (10^{-10} to 10^{-5} M) or its vehicle in the presence or absence of 10 μM ICI 182,780 for 24 hours. Their effects on cell differentiation were measured by ALP assay. Results were analyzed with unpaired t-test and data shown as mean ± SEM (n = 5). It shows that differentiative effects of 10 nM to 1 μM of Naringin in UMR-106 cells were completely abolished by the presence of estrogen antagonist, ICI blocker (p<0.01 vs its vehicle).

5.3.2.3 mRNA Expression of OPG to RANKL in UMR-106 Cells

Based on the proliferative and differentiative activities of Naringin in UMR-106 cells (Results could be found in 5.3.2.1 and 5.3.2.2), 10 nM was chosen for the study of its effect on osteoclastogenesis. As shown in Fig. 5.12A, 10 nM of E2 significantly increased osteoprotegerin (OPG) mRNA expression by about 2.2-fold (p<0.05 vs its vehicle) and this effect was completely abolished in the presence of ER antagonist, ICI 182,780 (p<0.05). UMR-106 cells treated with E2 increased OPG/RANKL mRNA expression ratio by 2-fold (Fig. 5.12C, p<0.05 vs its vehicle). Similarly, the expression was fully abolished in the presence of ICI (p<0.05). UMR cells treated with 10nM Naringin slightly increased OPG mRNA expression by 22% but the result did not reach statistical significance (Fig. 5.12A). On the contrary, the expression of RANKL mRNA was significantly down-regulated by 1-fold (Fig. 5.12B, p<0.05 vs its vehicle). The ratio of OPG to RANKL was calculated to demonstrate its effect on osteoclastogenesis. As shown in Figure 5.11C, it is obvious that 10nM Naringin significantly increased OPG/RANKL ratio by 2.2-fold (p<0.05 vs its vehicle) and the expression was also abolished in the presence of ICI (p<0.05). The above results suggested that the inhibitory effect of Naringin on osteoclastogenesis was at least in part mediated through estrogen receptor.

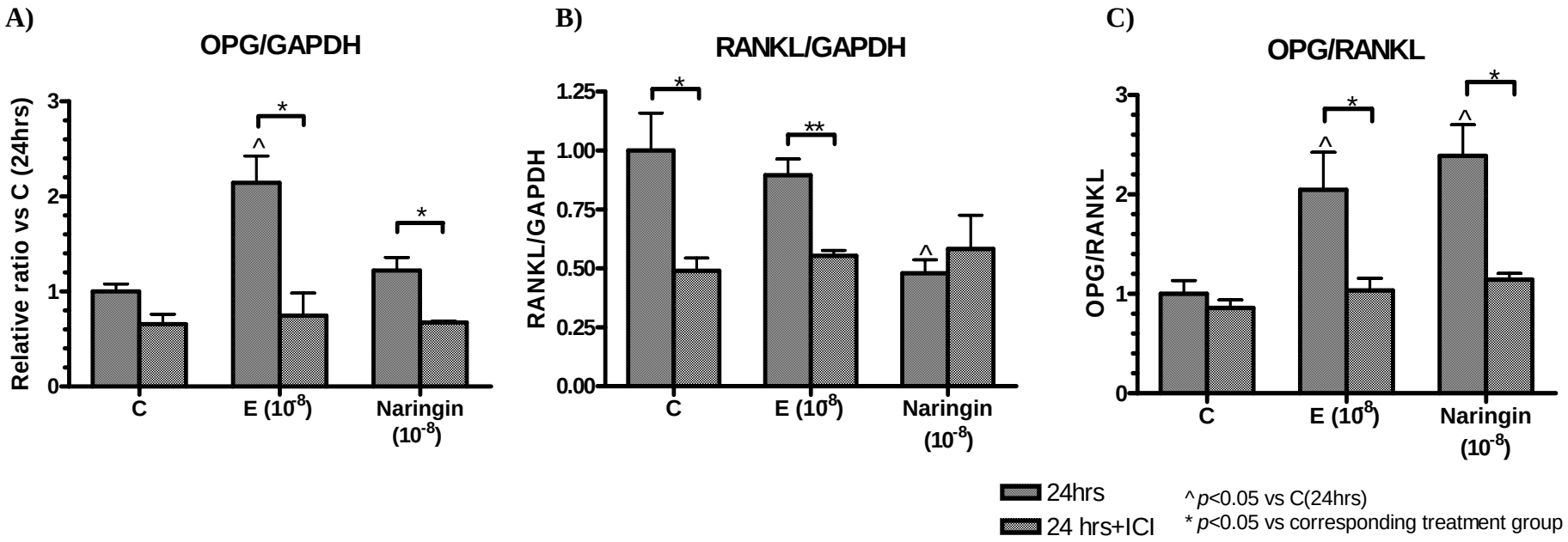


Figure 5.12 The effect of Naringin on OPG and RANKL mRNA expression in UMR-106 cells. UMR-106 cells were treated with 10 nM of E2, 10 nM of Naringin or its vehicle for 24 hours. Total RNA samples were extracted and then subjected to quantitative real-time PCR analyses for each target gene. The OPG and RANKL mRNA expression was normalized by an internal control gene, GAPDH. Results were expressed as mean $\pm$ SEM. (n = 3)

5.3.2.4 Effect of Naringin on ER-α or ER-β-mediated Luciferase Gene Expression in UMR-106 Cells

To determine if Naringin activate estrogen response element (ERE)-dependent transcription via estrogen receptor (ER) α and β, UMR-106 cells were co-transfected with ERE-luciferase and ER-(α or β) constructs and treated with either E2 or Naringin. Our results indicated that 10 nM E2 can activate ER-α (Figure 5.13A, $p<0.001$ vs its vehicle), as well as ER-β (Figure 5.13B, $p<0.01$ vs its vehicle) and induced the transcription of ERE-dependent luciferase gene. 0.1μM of Naringin induced ERE-dependent luciferase expression through both ER-α and ER-β in UMR-106 cells by 21% and 48%, respectively (Figure 5.13A and 5.13B, $p<0.05$ vs C). The result suggests that 0.1μM of Naringin stimulated ERE-dependent transcription in UMR-106 cells.

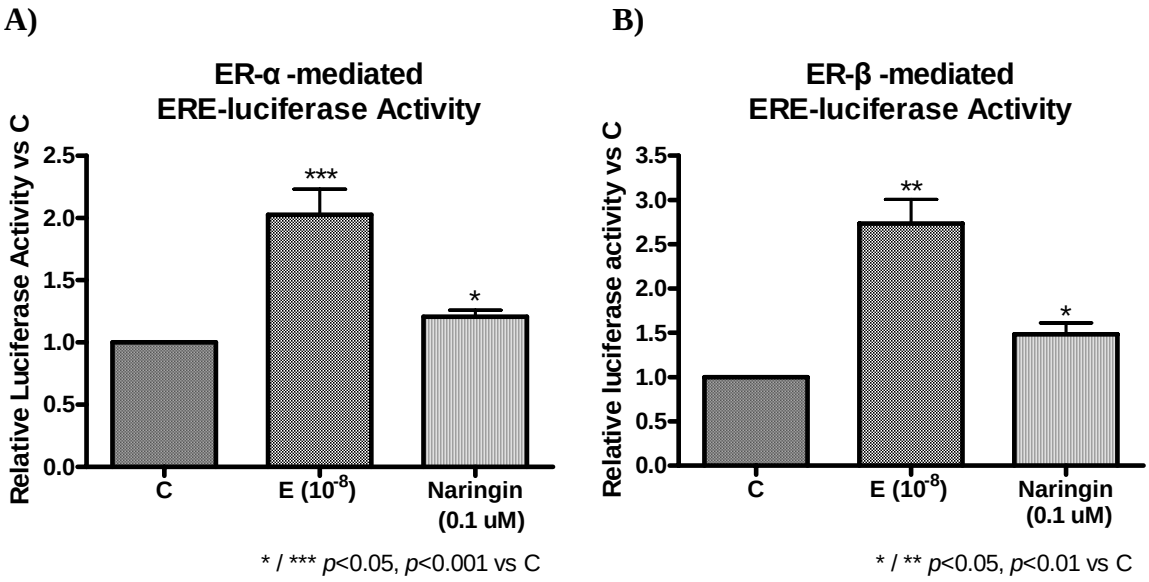
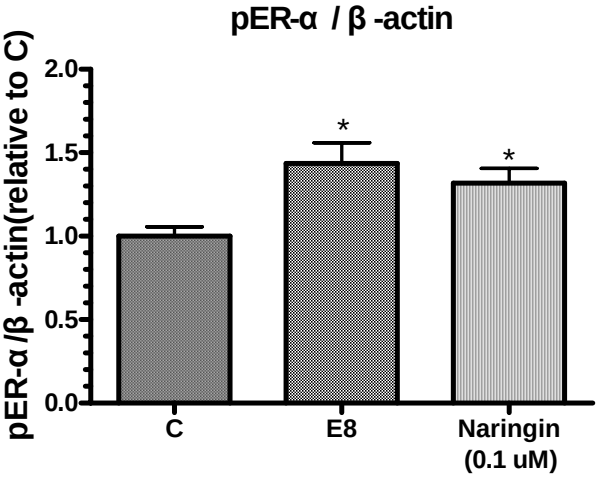


Figure 5.13 The effect of Naringin on ER-α- or ER-β-mediated ERE-dependent luciferase activity. UMR-106 cells were transfected with ERE-luciferase construct and ER-(α and β) and treated with either E2 (10 nM), Naringin (0.1μM) or its vehicle for 24 hours. Cells were lyzed and assay for luciferase activity. Data represented as a ratio of ERE *firefly* to TK *Renilla*. TK was the internal control for the monitoring of transfection efficiency. Data shown as mean ± SEM (n = 3). **A)** The ERE-luciferase activity mediated by ER-α. 10nM E2, but not 10nM Naringin, induce ERE- dependent luciferase activity (p<0.001 vs C. **B)** The EREluciferase activity mediated by ER-β. 10nM E2, but not 10nM Naringin, induce ERE-dependent luciferase activity (p<0.001 vs C).

5.3.2.5 Effect of Naringin on ER-α Expression and its Phosphorylation in UMR-106 Cells

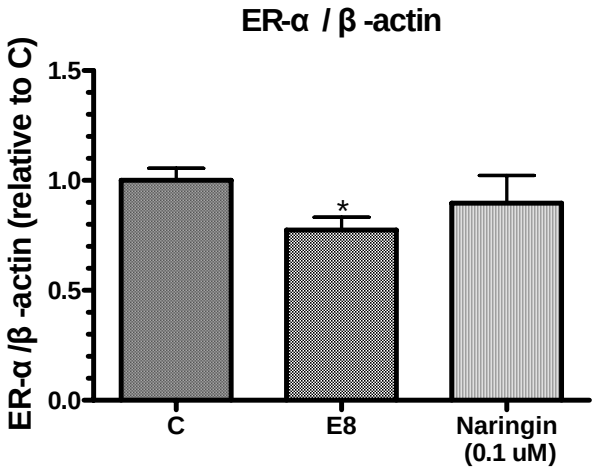
To determine if Naringin could alter ER expression and activate ligand-independent activation of ER, the level of ER-α and phospho-ER-α (pER-α) expression were studied. Serine 118 is the phosphorylation site of ER that can be triggered by ligand-independent pathway. UMR-106 cells were transfected with ERE-luciferase construct and ER-(α and β) for 24 hours. Transfected UMR-106 cells treated with 10nM of E2 significantly induced pER-α protein expression by 50% (Fig. 5.14A, $p<0.05$ vs its vehicle) and suppressed ER-α expression by 23% simultaneously (Fig. 5.14B, $p<0.05$ vs its vehicle). 0.1μM Naringin also significantly induced pER-α expression by about 32% (Fig. 5.14A, $p<0.05$ vs its vehicle). However, unlike E2, Naringin did not significantly down-regulate ER-α expression (Fig. 5.14B). To assess the overall effect on ER phosphorylation, the relative ratio of pER-α versus ER-α expression were calculated. As shown in Fig. 5.14C, both E2 (10nM) and Naringin (0.1μM) markedly increased pER-α / ER-α by 85% and 47%, respectively ($p<0.05$ vs its vehicle). The results suggested that the estrogenicity of Naringin may be also due to the activation of ER-α phosphorylation in a ligand-independent manner.

A)



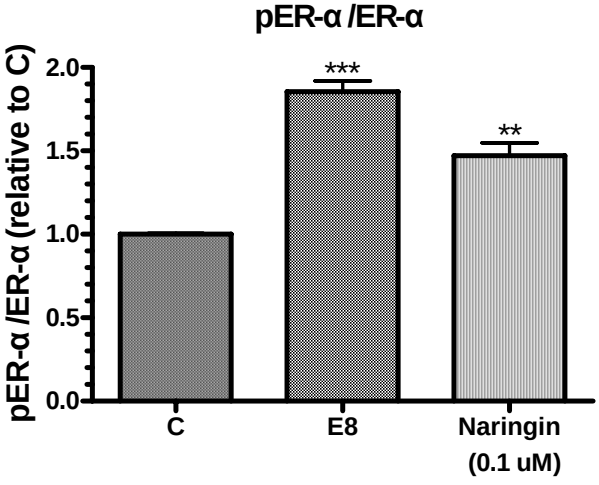
* p<0.05 vs C

B)



* p<0.05 vs C

C)



** / *** p<0.01, p<0.001 vs C

D)

Figure 5.14 The effect of Naringin on the expression of phosphorylated ER-α and ER-α in UMR-106 cells UMR-106 cells were transfected with ERE-luciferase construct and ER-(α and β) and were treated either with E2 (10nM), Naringin (0.1μM) or its vehicle for 24 hours. Cells lysate were subjected to Western blotting analysis. **A)** The phospho-S118-ER-α expression normalized by internal control, β-actin. **B)** The ER-α expression normalized by internal control, β-actin. **C)** The relative ratio of phospho-ER-α to ER-α. **D)** Illuminant images from Western immunoblotting. Results were analyzed with unpaired t-test and data were expressed as mean $\pm$ SEM. (n = 3)

5.4 Summary

Naringin has been reported as an effective single compound of bone formation (Deng, Zhang et al. 2005; Wong and Rabie 2006a; Wong and Rabie 2006b). It is a polymethoxylated flavonoid which was shown to have a HMG-CoA reductase inhibiting effect (Wong and Rabie 2006b). Wong and Rabie (2006b) also reported that rat osteoblastic-like UMR-106 cells treated with 0.1 μM of Naringin can increase cell proliferation by 43.6% ($p<0.007$) in 48 hours, as well as cell differentiation by 15% ($p=0.007$) in 24 hours. It is possible to postulate that Naringin may activate osteoblastic activities, as well as be beneficial for treatment of osteoporosis. However, animal studies for the efficacy of Naringin in treating osteoporosis is rare and the mechanisms of the actions of Naringin involved in estrogen receptor (ER)-dependent pathway has not yet been elucidated.

In this study, our *in vivo* study clearly demonstrated that Naringin, the most active component of Rhizoma Drynariae, effectively prevented OVX-induced deterioration in bone mineral density and bone strength in OVX female mice. We found that Naringin did not exert hyperplasia effect on uterus, suggesting that it possesses tissue selectivity toward bone. Two doses of Naringin were being administrated to OVX mice and 0.4 mg/g/day was found to be more effective. It appeared that Naringin can decrease urinary calcium excretion but did not decrease bone resorption marker level

(uDpd/Cr). For the effect of Naringin of animal bone properties, Table 5.4 summarized the BMD, cross-sectional areas and SSI of Naringin-treated OVX mice measured by pQCT. The results obtained suggested that the osteoprotective effect of Naringin was dose-dependent and the effects were more prominent at site rich in trabecular bone. Naringin exert similar osteoprotective effects compared with the treatment of RD total flavonoids in OVX mice (Chapter 4) although Naringin could not decrease urinary Dpd/Cr level.

In the *in vitro* study, we demonstrated that Naringin stimulated rat osteosacroma UMR-106 cells proliferation and differentiation in a dose-dependent manner. Cells treated with 10nM to 1μM of Naringin significantly stimulated cell growth and cell differentiation under steroid-free culture conditions. The proliferation and differentiation were totally abolished by co-treating cells with ICI 182,780 estrogen antagonist. The results suggested that the potential anabolic effects of Naringin might be mediated through ER-dependent pathway. The study of mRNA expression on OPG to RANKL ratio also concluded that 10nM of Naringin might inhibit osteoclastogenesis possibly by up-regulating OPG mRNA expression, as well as down-regulating RANKL mRNA expression from osteoblastic cells. This phenomenon suppresses the interaction between RANKL on the surface of osteoblastic cells and RANK expression on osteoclastic cells. Transient transfection

study indicated that 0.1μM of Naringin activated ERE-dependent gene transcription through the activation of ER-α or ER-β, suggesting that classical ER-dependent pathway might be involved. On the other hand, our results showed that tranfected UMR-106 cells treated with 0.1μM of Naringin significantly increased the phosphorylation level of ER-α at serine 118 without down-regulation of ER-α expression. It is postulated that the osteoporotive effects of Naringin was also mediated through the phosphorylation of ER-α without directly binding onto ER-α or -β. Thus, it appears that Naringin exert its bone protective effect in a manner similar to the typical action of estradiol as well as growth factors.

In lieu of these promising findings related to Naringin, the active ingredient of Rhizoma Drynariae (RD), on bone health in female mice and cell culture, the question remains to be answered is that if Naringin is the only active single compound of RD responsible for the prevention and treatment of osteoporosis. In the next chapter, we will discuss about the molecular activities of other single compounds extracted from RD total flavonoids fraction. It is postulated that there are more than one RD compounds that can exert similar osteoprotective effects in osteoblastic cells.

Table 5.4 Summarized table on the effect of 0.2 mg/g/day (N0.2) and 0.4 mg/g/day (N0.4) of Naringin on BMD, cross-sectional areas and SSI at femur, tibia and lumbar spine. Data shown as mean. (n = 7)

Group N 0.2 0.2 mg/g/day naringin		Femur			Tibia		Lumbar
		Proximal	Mid-shaft	Distal	Proximal	Mid-shaft	spine L1
BMD (mg/ccm)	Total	419.0	437.4	375.0 **	270.5	420.4	232.3
	Trabecular	395.9	N/A	386.8 ***	300.75 ***	N/A	N/A
Cross Sectional Area	Total	1.5	N/A	3.99	4.44	N/A	3.40
	Trabecular	N/A	N/A	1.69	1.63	N/A	N/A
SSI		0.49	0.20	0.63 *	0.33	0.14	0.588 **

* / ** / *** p <0.05, p <0.01, p <0.001 vs V

Group N 0.4 0.4 mg/g/day naringin		Femur			Tibia		Lumbar
		Proximal	Mid-shaft	Distal	Proximal	Mid-shaft	spine L1
BMD (mg/ccm)	Total	425.9	433.5	360.0	286.7	434.0	249.0 ***
	Trabecular	428.0		392.5**	285.7 **	N/A	N/A
Cross Sectional Area	Total	1.35	N/A	4.03	4.56	N/A	3.57 *
	Trabecular	N/A	N/A	1.89**	1.89	N/A	N/A
SSI		0.57	0.21	0.71***	0.30 *	0.154	0.61 ***

* / ** / *** p <0.05, p <0.01, p <0.001 vs V

Key: N/A: Not Available

Chapter 6

Screening of osteoprotective activities of single compounds isolated from Rhizoma Drynariae in UMR-106 cells

6.1 Background

We believed that, apart from Naringin, other constituents of Rhizoma Drynariae might be potent phytoestrogens that give benefits in treating osteoporosis. As mentioned in Chapter 1, four more single compounds from RD were selected for the bio-activity screening *in vitro*: *1)* Flavone #1, *2)* Phenylpropanoic acid #2, *3)* Phenylpropanoic acid #3, *4)* Chromone #4. The selection was based on their abundance in RD flavonoids-riched fraction, RDB (Figure 1.2). Flavone #1 and Chromone #4 had flavonoids backbone with other organic side chains; the other two were known as non-flavonoid compounds. The compound names used in this thesis were referable to their own chemical structures.

The bio-activities of each single compound were demonstrated by assaying their cell proliferative and differentiative effects in UMR-106 rat osteoblastic-like cells. Their estrogenicities were studied by co-treating with estrogen antagonist, ICI 182,780. Furthermore, their effects on modulating osteoclastogenesis were studied by observing the difference in mRNA OPG/RANKL expression.

6.2 Materials and Methods

UMR-106 cells were treated with different concentrations of each single compound with varied final concentrations (0, 10^{-14}, 10^{-12}, 10^{-10}, 10^{-8} and 10^{-6} M), and their

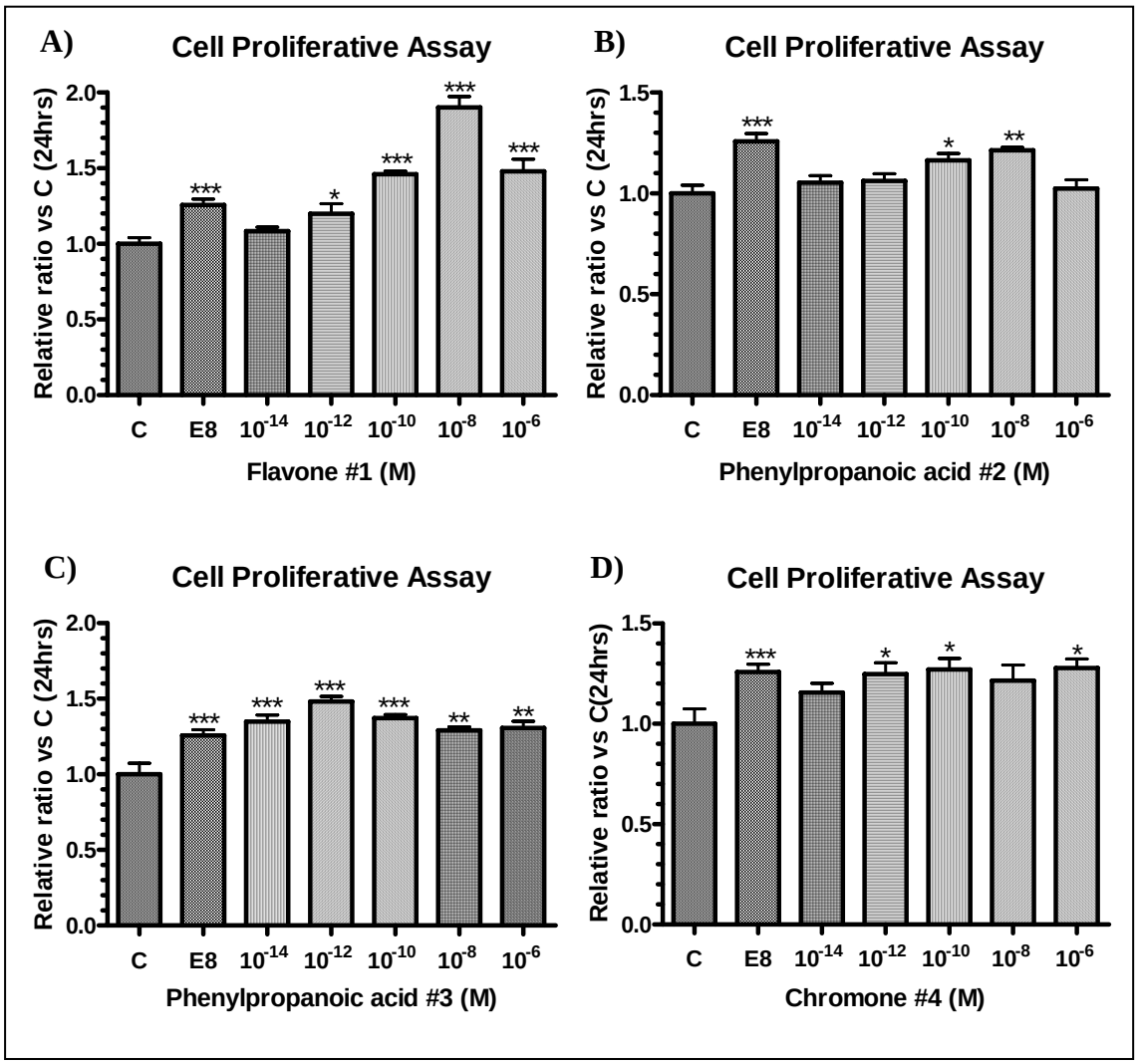
effects on cell proliferation and differentiation were examined. Upon selection of the optimal dose, the estrogen antagonist assay and the mRNA expressions were studied. The optimal concentrations were determined based on their activities in cell proliferation and differentiation assay. Detailed methodology could be referring to Chapter 3, section 3.2.1 to 3.2.6.

6.3 Results

6.3.1 The Proliferative Effects of 4 RD Single Compounds in UMR-106 cells

As shown in Figure 6.1, Flavone #1, Phenylpropanoic acid #2 and Phenylpropanoic acid #3, but not Chromone #4, significantly increased UMR-106 cell proliferation at 24 hours of treatment in a dose-dependent manner. Flavanone #1 dramatically increased cell proliferation at 10 nM by about 90% (Fig. 6.1A, $p<0.001$ vs its vehicle). It also stimulated cell growth in 0.1 nM and 1μM by about 45% (Fig. 6.1A, $p<0.001$ vs its vehicle). For Phenylpropanoic acid #2, it could significantly stimulate cell proliferation at 0.1 and 10nM (Fig. 6.1B). They increased cell proliferation by 16% ($p<0.05$) and 21% ($p<0.01$), respectively (Fig. 6.1B). As for Phenylpropanoic acid #3, it could stimulate UMR-106 cell proliferation at all concentrations tested (10^{-14} to 10^{-6}M) in a dose-dependent manner, and increase proliferation by 50% at 1ρM (Fig. 6.1C, $p<0.001$ vs its vehicle). Chromone #4 could significantly stimulated cell

proliferation at 1ρM, 0.1nM and 1μM by 25%, 27% and 28%, respectively (Fig. 6.1D, $p<0.05$ vs its vehicle).



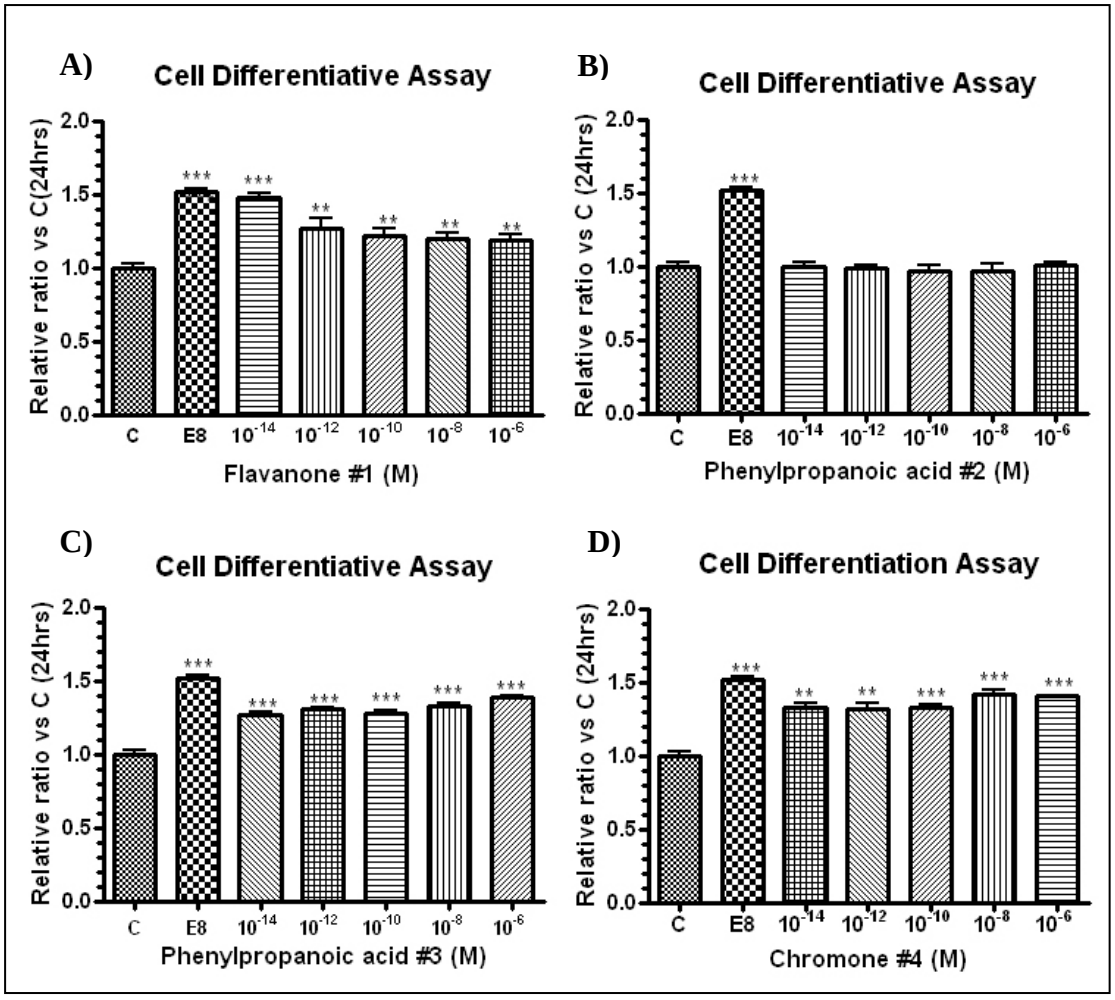
*/ ** / *** $p<0.05$, $p<0.01$, $p<0.001$ vs C (24hrs)

E8 = 10 nM 17β-estradiol

Figure 6.1 The proliferative effects of 4 isolated single compounds of Rhizoma Drynariae in UMR-106 cells. UMR-106 Cells were seeded in 96-wells plate and incubated with different concentration (10^{-14} to 10^{-6}M) of **A)** Flavone #1; **B)** Phenylpropanoic acid #2; **C)** Phenylpropanoic acid #3 and **D)** Chromone #4. Their effects on cell proliferation were measured by MTS assay. Results were analyzed by unpaired t-test and data were expressed as mean ± SEM. (n = 5)

6.3.2 The Differentiative Effects of 4 RD Single Compounds in UMR-106 cells

To determine if the four isolated compounds can stimulate osteoblastic differentiation in UMR-106 cells, the response of ALP activities to different concentrations of the compounds were studied. Fig. 6.2A, 6.2C and 6.2D indicate that all the concentrations of Flavone #1, Phenylpropanoic acid #3 and Chromone #4 (10^{-14} to 10^{-6}M) stimulated ALP activities. Flavanone #1 induced cell differentiation by about 48% ($p<0.001$) and 27% ($p<0.01$) at 10^{-14} and 10^{-12}M, respectively. Phenylpropanoic acid #2 could not stimulate cell differentiation at all concentrations tested (10^{-14} to 10^{-6}M) (Fig 6.2B). UMR-106 cells treated with Phenylpropanoic acid #3 increased cell differentiation by 40% at 1μM (Fig. 6.2C, $p<0.001$ vs its vehicle). Similarly, Chromone #4 also increased cell differentiation by 40% at 10nM (Fig. 6.2D, $p<0.001$ vs its vehicle).



*/ ** / *** $p<0.05$, $p<0.01$, $p<0.001$ vs C (24hrs)

E8 = 10 nM 17β-estradiol

Figure 6.2 The Differentiative Effects of 4 isolated RD compounds in UMR-106 cells. UMR-106 Cells were seeded in 96-wells plate and incubated with different concentrations (10^{-14} to 10^{-6}M) of **A)** Flavone #1; **B)** Phenylpropanoic acid #2; **C)** Phenylpropanoic acid #3 and **D)** Chromone #4. Their effects on cell differentiation were measured by ALP assay. Results were analyzed by unpaired t-test and data were expressed as mean ± SEM. (n = 5)

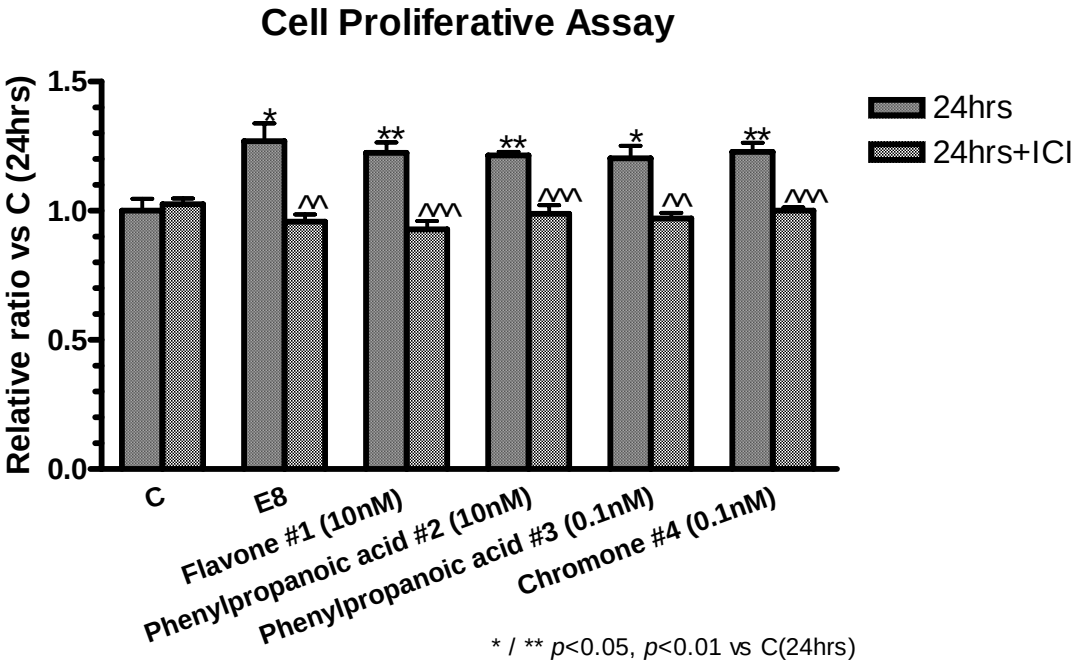
6.3.3 The Estrogen Antagonist Assay (ICI 182, 780)

For the estrogenicity study, only one concentration of each single compound was selected to co-treat cells with estrogen antagonist. Their optimal doses were chosen upon the results obtained from their UMR-106 cell proliferation (Figure 6.1). According to the proliferation assays, 10 nM of Flavone #1 and Phenylpropanoic acid #2; 0.1 nM of Phenylpropanoic acid #3 and Chromone #4 were selected for further investigation. ICI 182,780 blocker was added to the incubated cells associated with corresponding amount of RD isolated compounds. MTS and ALP assays were processed to observe if the stimulatory effects were mediated through estrogen receptor (ER)-dependent pathway.

As shown in Figure 6.3, UMR-106 cells treated with vehicle (C) did not decrease cell proliferation in the presence of ICI 182,780. E2 increased UMR-106 cell proliferation at 24 hours by 27% ($p<0.05$ vs C) while the promoting effect was completely abolished by co-treating cells with ICI ($p<0.01$). Moreover, ICI 182,780 inhibited the proliferative effects of all four isolated compounds from RD in UMR-106 cell proliferation ($p<0.01$ vs corresponding treatment with ICI absent). The results suggested that the induction of cell proliferation by RD isolated compounds was mediated through ER.

On the other hand, ALP activities were conducted for the cell differentiation assays.

UMR-106 cells treated with its vehicle did not alter the differentiative effects greately (Figure 6.4). Cells treated with E2 increased differentiation by about 50% at 24 hours, however, the effect was completely blocked by the presence of estrogen antagonist, ICI 182,780. The differentiative effects of all four isolated RD compounds were not abolished by ICI blocker (Figure 6.4). The results suggested that the up-regulation of ALP activities by these compounds might not be mediated through ER.



E8 = 10nM 17β-estradiol

Figure 6.3 The proliferative effects of 4 isolated single compounds of Rhizoma Drynariae in UMR-106 cells, in the presence or absence of estrogen antagonist

Cells were seeded in 96-well plate, treated with ICI blocker (1μM) associated with the corresponding concentrations of RD single compounds for 24 hours. The effects on cell proliferation were assessed by MTS assay. This demonstrated that the estrogen antagonist completely abolished the stimulatory effects induced by each single compound. Results were analysed by unpaired t-test and data shown as mean ± SEM. (n = 5)

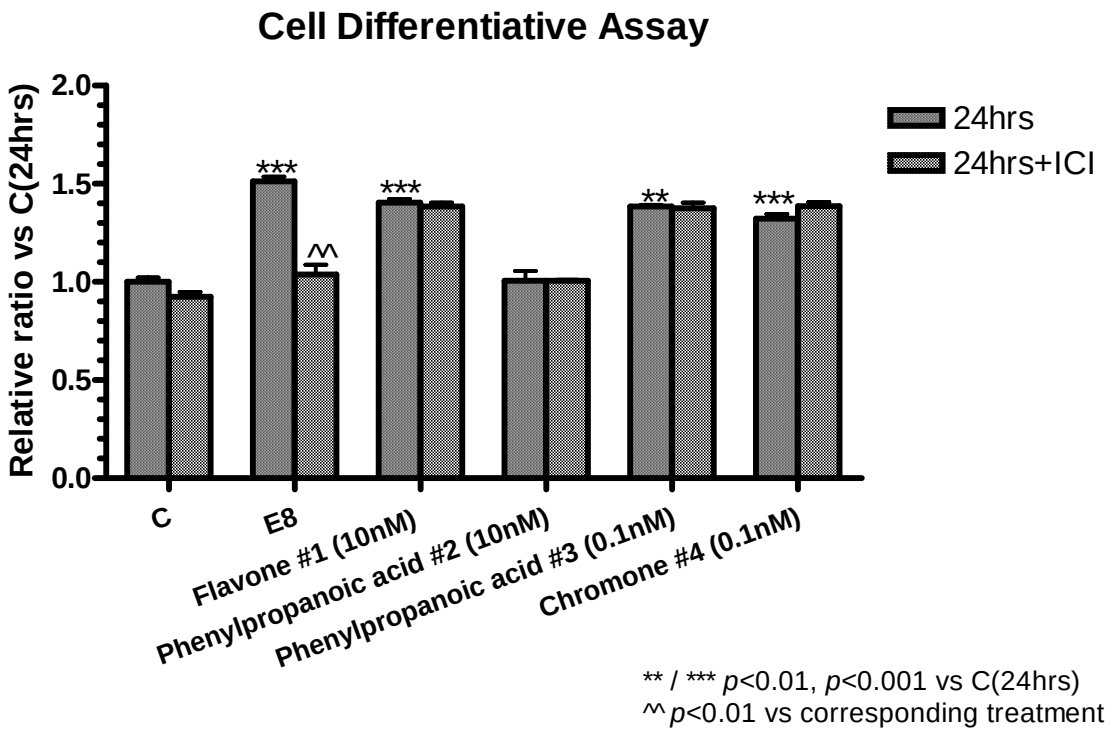


Figure 6.4 The differentiative effects of 4 single compounds isolated from Rhizoma Drynariae in UMR-106 cells, in the presence or absence of estrogen antagonist Cells were seeded in 96-well plate, treated with ICI blocker (1μM) associated with the corresponding concentrations of RD single compounds for 24 hours. The effects on cell differentiation were assessed by ALP assay. Results show that the estrogen antagonist could not inhibit RD single compounds stimulated UMR-106 cell differentiation. Results were analysed by unpaired t-test and data shown as mean ± SEM. (n = 5)

6.3.4 The mRNA Expression of OPG to RANKL in UMR-106 Cells

Based on the proliferative activities of 4 single compounds isolated from RD, the preferred dose of each single compound was chosen and the mRNA expression of OPG and RANKL in UMR-106 cells was checked after 24 hours of treatment (Results could be referred to 6.3.1). Similar to the estrogen antagonist assay (section 6.3.3), 10 nM of Flavone #1 and Phenylpropanoic acid #2; and 0.1 nM of Phenylpropanoic acid #3 and Chromone #4 were selected for this investigation.

As shown in Fig. 6.5A, 10 nM of E2 significantly increased osteoprotegerin (OPG) mRNA expression by about 2.2-fold ($p<0.05$ vs its vehicle) and this effect was completely abolished in the presence of ER antagonist, ICI 182,780 ($p<0.05$). UMR-106 cells treated with E2 increased OPG/RANKL mRNA expression ratio by 2-fold (Fig. 6.5C, $p<0.05$ vs its vehicle). Similarly, the expression was fully abolished in the presence of ICI ($p<0.05$).

UMR-106 cells treated with 10nM Flavone #1 dramatically increased OPG mRNA expression for 2.74-fold significantly (Fig. 6.5A, $p<0.001$ vs its vehicle) and the expression was significantly abolished by the presence of ICI ($p<0.01$) although the blocking effect was not completed (Fig. 6.5A). On the contrary, 10nM Flavone #1 increased the expression of RANKL mRNA increased by 40% (Fig. 6.5B) but the result did not reach statistical significance. The up-regulation of RANKL was

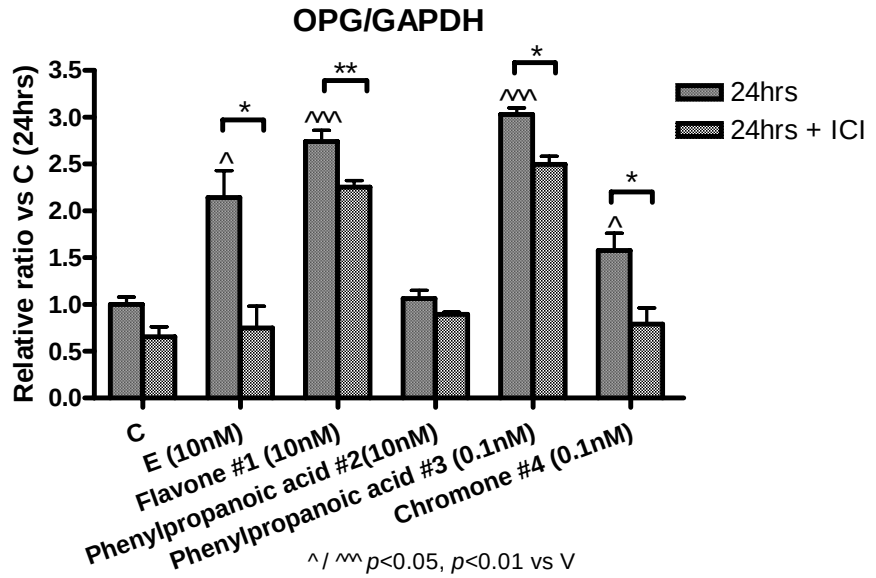
blocked in the presence of ICI (p<0.05). The ratio of OPG to RANKL was calculated to demonstrate its effect on osteoclastogenesis. As shown in Figure 6.5C, it is obvious that 10nM Flavone #1 significantly increased OPG/RANKL ratio by 55% (p<0.05 vs its vehicle) and the expression was also abolished in the presence of ICI (p<0.05).

10nM of Phenylpropanoic acid #2 did not induce mRNA expression of OPG and RANKL (Fig. 6.5A & 6.5B). As the results, the overall OPG/RANKL ratio did not show significant difference when it is compared with its vehicle (Fig. 6.5C). In contrast, Phenylpropanoic acid #3 dramatically up-regulated OPG mRNA expression by 3-fold (p<0.001 vs its vehicle) and the expression was down-regulated by about 33% in the presence of ICI blocker (Fig 6.5A, p<0.05). This compound did not alter RANKL mRNA expression in UMR-106 cells (Fig. 6.5B). As shown in Fig. 6.5C, Phenylpropanoic acid #3 increased OPG/RANKL ratio by 3.1-fold (p<0.01 vs its vehicle) but the action was not abolished by the presence of ICI. The results suggest that ER is not involved in mediating the effect Phenylpropanoic acid #3 on osteoclastogenesis.

Chromone #4 also significantly increased OPG mRNA expression by about 60% (Fig. 6.5A, p<0.05 vs its vehicle) and the up-regulation was totally abolished by the presence of ICI (p<0.05). On the other hand, Chromone #4 could induce RANKL

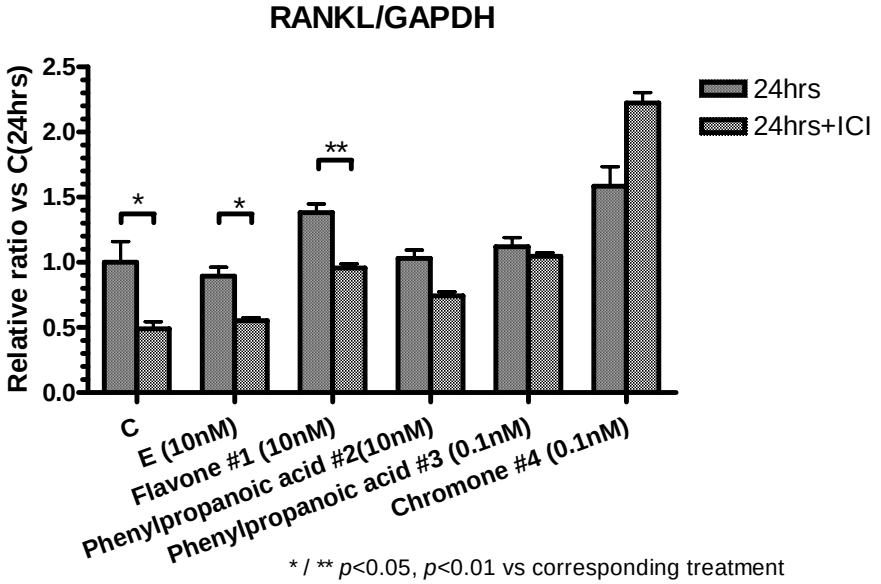
mRNA expression both in the presence and absence of ICI by 1.58- and 2.2-fold, respectively although the increase did not reach to statistical significant (Fig. 6.5B). Overall, Chromone #4 increased OPG/RANKL ratio in UMR-106 cells by 84% (p<0.05 vs its vehicle) and the promoting effect was abolished in the presence of ICI (p<0.05). The results suggested that the inhibitory effect of three of four isolated RD compounds on osteoclastogenesis was at least in part mediated through estrogen receptor.

A)



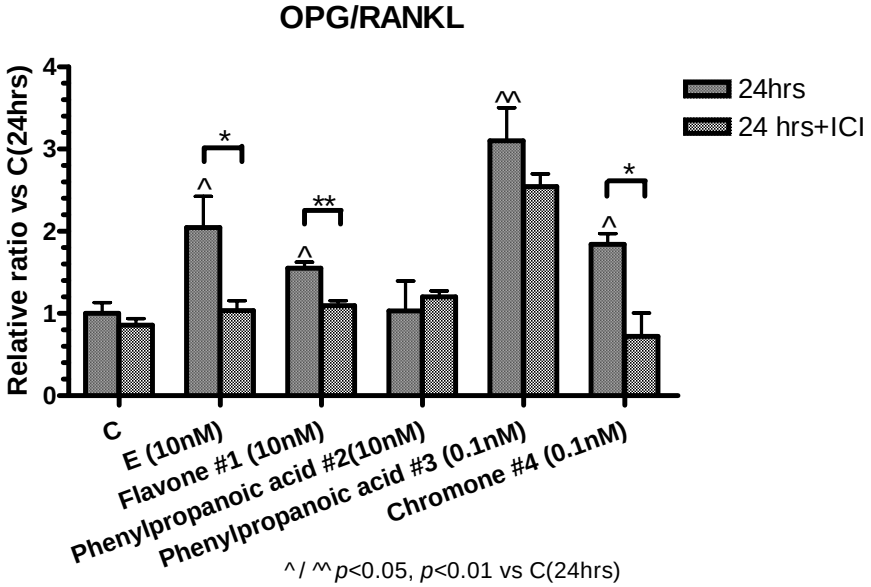
^ / ^^ $p<0.05$, $p<0.01$ vs V
* / ** $p<0.05$, $p<0.01$ vs corresponding treatment

B)



* / ** $p<0.05$, $p<0.01$ vs corresponding treatment

C)



^ / ^^ $p<0.05$, $p<0.01$ vs C(24hrs)
* / ** $p<0.05$, $p<0.01$ vs corresponding treatment

Figure 6.5 The effect of 4 single compounds isolated from RD on OPG and RANKL mRNA expression in UMR-106 cells. UMR-106 cells were treated with 10nM of Flavone #1, 10nM of Phenylpropanoic acid #2, 0.1nM of Phenylpropanoic acid #3, 0.1nM Chromone #4 or its vehicle for 24 hours. Total RNA samples were extracted and subjected to quantitative real-time PCR analyses for each target gene. The OPG and RANKL mRNA expression was normalized by an internal control gene, GAPDH. Results were expressed as mean $\pm$ SEM and analyzed by unpaired t-test. (*n* = 3)

6.4 Summary

According to the promising osteoprotective effects that we demonstrated for RD and its active ingredient, Naringin, we believed that other constituents of RD might have potential to be phytoestrogens that give benefits in treating osteoporosis. In this chapter, we demonstrated the *in vitro* osteoprotective effects of 4 single compounds isolated from RD by observing their cell proliferations, differentiations, and mRNA expressions of OPG and RANKL. Table 6.1 summarized the osteoprotective effects of 4 single compounds *in vitro*. Flavone #1 significantly induced UMR-106 cells proliferation and differentiation in a dose-dependent manner, and its proliferatory effect was completely abolished by the presence of ICI 182,780, the estrogen antagonist. On the other hand, the OPG/RANKL ratio indicates that Flavone #1 might modulate osteoclastogenesis from osteoblast by up-regulating OPG mRNA expression and the action might mediate through estrogen receptor (ER)-dependent pathway as the OPG and RANKL mRNA expression was blocked with the presence of ICI. Phenylpropanoic acid #2 is regarded as non-flavonoid compound and the results showed that it is weak in promoting UMR-106 cell proliferation and differentiation in steroid-free condition. Moreover, Phenylpropanoic acid #2 did not involve in the OPG and RANKL mRNA regulation. The results suggest that Phenylpropanoic acid #2 might not benefit in treating osteoporosis. Phenylpropanoic acid #3 is another

non-flavonoid compound but the results indicated that it can stimulate UMR-106 cell proliferation and differentiation. The proliferative effect was completely abolished by the presence of ICI, indicates that this effect was mediated through ER. OPG/RANKL ratio also demonstrated that Phenylpropanoic acid #3 was able to increase OPG mRNA expression without altering RANKL expression. However, the effect was partially abolished in the presence of ICI, indicates that only part of its effect was mediated through ER. Chromone #4 is a flavonoid compound which shows significant proliferative effects in UMR-106 cells at several concentrations tested. It also stimulated ALP activities significantly. Similarly, the proliferative effect of Chromone #4 was blocked by the presence of ICI but the differentiation did not. The results suggested that the differentiation effect of all four compounds might not be mediated through ER. Chromone #4 increased OPG mRNA expression but also increased RANKL expression simultaneously. After the calculation, the overall OPG/RANKL ratio increased significantly when it is compared with its vehicle. This indicates that Chromone #4 still has potential to modulate osteoblast-induced osteoclastogenesis by balancing OPG and RANKL in UMR-106 cells.

In conclusion, we found that flavonoid compounds, Flavone #1 and Chromone #4, but not non-flavonoid compounds, have more potential to be alternative therapeutic agents that might be favorable in treatment of osteoporosis. Although

Phenylpropanoic acid #3 induced cell proliferation, differentiation and mRNA expression of OPG/RANKL, the effects were partially abolished in the presence of ICI. In contrast, two selected flavonoid compounds significantly stimulated proliferation, differentiation and mRNA expression of OPG/RANKL and the effects were completely blocked in the presence of ICI.

Our study serves as preliminary screening for newly developed single compounds isolated from medicinal herb. Further investigations will be needed to study their efficacies and their mechanisms of the actions *in vivo*.

Table 6.1 Summarized table on the effect of 4 single compounds isolated from RD on cell proliferation, differentiation and OPG/RANKL mRNA expression in UMR-106 cells. Results were analysed by unpaired t-test and data shown as percentage change, compared with its vehicle. (n = 3-6)

	UMR-106 cells		
Single compound (Conc.)	Proliferation	ALP activity	OPG / RANKL ratio
Flavone #1 (10nM)	90% ***	22% **	55% *
Phenylpropanoic acid #2 (10nM)	21% **	--	–
Phenylpropanoic acid #3 (0.1nM)	50% ***	31% ***	310% **
Chromone #4 (0.1nM)	27% *	32% ***	84% *

*/**/*** p <0.05, p <0.01, p <0.001 vs its vehicle

The stimulatory effects were completely abolished in the presence of ICI (p <0.05)

Chapter 7

Discussion and Conclusion

7.1 Discussion

Osteoporosis is one of the most common disorders associated with estrogen deficiency and aging. Menopause results in elevated bone turnover rate and net bone loss. Recently, flavonoids-riched Chinese medicinal herbs (Wang, Wu et al. 2003; Zhang, Qin et al. 2007) have been reported to maximize the benefits in treating osteoporosis with minimize side effects. Rhizoma Drynariae, a Chinese *kidney*-tonifying herb which is rich in flavonoids compounds (Xie 2004), has been reported to enhance bone formation *in vivo* (Wong and Rabie 2006c) and *in vitro* (Lin, Sun et al. 2002). Besides, the RD total extract has been demonstrated that it might carry estrogen-like activities *in vivo* in maintaining trabecular structure and connection similar to sham-level and inhibited the increase of bone turnover in OVX rats (Jia, Wang et al. 2006). However, studies about estrogenic activities and the dose-dependent effect of RD total flavonoids (RD TF) *in vivo* and *in vitro* are rare. In addition, the active ingredients of RD have not been identified. Here, we demonstrated RD total flavonoids and its active ingredient, Naringin, show estrogen-like effects in maintaining bone mineral densities (BMD) in trabecular rich regions in OVX mice (Breen, Loveday et al. 1998), as well as increasing cell proliferation and differentiation in rat osteoblastic-like UMR-106 cells under steroid-free conditions (Xie, Wu et al. 2005). On the other hand, RD TF and Naringin

performed some anti-estrogenic activities *in vivo* such as the inhibition of inducing uterotrophic side effect on mice uterus (Carthew, Edwards et al. 1999). Both RD TF and Naringin carry partial estrogenic and partial anti-estrogenic character which might be favorable to develop as alternative therapeutic agents in prevention and treatment of osteoporosis, provided that they may maximize the benefits in treating osteoporosis with minimized side effects.

The present study clearly demonstrates RD total flavonoids (RD TF) and its isolated single compound, Naringin, not only prevented OVX-induced bone loss but also somehow significantly increased BMD at the optimal dosage (RD TF:0.173 mg/g/day and Naringin: 0.4 mg/g/day). They completely prevented bone loss in OVX mice models without exhibiting estrogenic side-effect in the uterus. There were selective changes in BMD in different scanning regions. Distal femur and proximal tibia were more sensitive in trabecular growth while lumbar spine is less sensitive. Thus, the BMD changes were more significant in distal femur and proximal tibia rather than in lumbar spine. Xie at al. (2004) reported the dose-dependent effect of RD TF in 6-mth old OVX rats. Results found that 0.108 g kg^{-1} day^{-1} of RD TF significantly increased trabecular bone volume and decreased trabecular resorption surface at lumbar spine region, but they did not measure uteri weight. Scientific evidence to support the use of active ingredients of RD TF and Naringin *in vivo* is absence. Similar results have also

been reported by using other well-known Chinese medicinal herbs like Puerariae Raix (Wang, Wu et al. 2003) and Herba Epimedii (Xie, Wu et al. 2005). Wang et al. (2003) and Xie et al. (2005) demonstrated that flavonoids-riched Chinese herbs prevent OVX-induced bone loss without uterotrophic effect in mice and rat models, respectively. In the present study, we demonstrated the optimal dose of RD TF and Naringin used in treating OVX-induced symptoms in mice and we also proved that RD TF and Naringin did not exert uterotrophic side effect. Both were found to be effective in osteoprotection, while compared with the degree of BMD change and other marker levels, RD TF was more favourable than just applying with Naringin.

In human and experimental animals, bone loss caused by estrogen deficiency is primarily due to an increase in osteoclastic bone resorption (Wang, Wu et al. 2003). Previous study shows one of the traditional Chinese medicines, Sambucus Williamsii Hance (SWH), can effectively suppress urinary calcium and Dpd levels in OVX rats (Xie, Wu et al. 2005). Recently, Zhang et al. (2007) reported the osteoprotective effect of Herba Epimedii in increasing BMD, as well as inhibiting Dpd excretion in late postmenopausal women. The result suggested that Chinese medicinal herbs may benefit in treating osteoporosis by promoting bone formation, and by inhibiting bone loss as well. In our study, we found that RD total flavonoids and Naringin may exert certain inhibitory effects on bone resorption by down-regulating the urinary calcium

excretion. Moreover, 0.173 mg/g/day of RD TF significantly decrease the increase of Dpd levels in OVX mice. The results suggest that RD TF maybe effective in preventing bone resorption by regulating urinary calcium loss and Dpd levels.

To investigate the molecular effects of RD total flavonoids, Naringin and other single compounds isolated from RD on bone metabolism, we employed rat osteoblastic-like UMR-106 cells culture system. MTS is the most common method for the detection of viable cells whereas ALP is the most widely recognized biochemical marker for osteoblastic differentiative activity (Wong and Rabie 2006b). In the present study, we examined the effects of RD total flavonoids (0.002 to 200 μg ml^{-1}), Naringin (10^{-10} to 10^{-5} M) and other four single compounds (10^{-14} to 10^{-6} M) on the cell proliferation, differentiation and the mRNA expression of OPG and RANKL. OPG/RANKL ratio is widely recognized as a powerful marker for modulation of osteoclastogenesis (Simonet, Lacey et al. 1997). In our study, the results supported that RD total flavonoids and its related single compounds involved in the stimulation of osteoblastic cells functions. RD TF, Naringin, Flavone #1 triggered cell proliferation in a dose-dependent manner. Phenylpropanoic acid #3 and Chromone #4 also stimulated cell proliferation significantly. RD TF and almost all of tested single compounds, but not Phenylpropanoic acid #2, increased cell differentiation significantly. Similar studies of RD TF and Naringin have been demonstrated by Xie

et al. (2005) and Wong et al. (2006b), respectively. They have demonstrated that both RD TF and Naringin exerted stimulatory effects on UMR-106 cells proliferation and differentiation. However, the mRNA expressions on OPG and RANKL have not been reported. Compared with previous studies, several traditional Chinese herbs exhibited similar osteoprotective effects, such as SWH (Xie, Wu et al. 2005) , Herba Epimdeii (Xie, Wu et al. 2005) and Gu Ling Pian (GLP)(Zhao, Li et al. 2007). All the herbal extracts promote bone protective effects by both increasing OPG and decreasing RANKL mRNA expression. In the present study, RD TF, Naringin, Flavone #1, Phenylpropanoic acid #3 and Chromone #4 significantly up-regulated OPG and OPG/RANKL mRNA expression. Similar to the studies of other medicinal herbs, RD TF and Naringin also suppressed RANKL mRNA expression significantly. The results suggested that RD TF and its active ingredients might modulate osteoclastogenesis by stimulating OPG mRNA expression in osteoblasts.

The estrogen-like activities of RD TF and Naringin, as well as other isolated compounds *in vitro* were rarely reported since the culture conditions commonly contain steroid-compounds that would interfere with the osteoblastic functions. In our study, the culture medium was changed to phenol-red free DMEM with 1% stripped serum one-day prior to drug treatment. It is aimed to deplete steroids, including estrogens. UMR-106 cells were treated with the steroid-free medium in the presence

or absence of the estrogen antagonist, ICI 182, 780. Presence of ICI could block the *in vitro* activities that are mediated through estrogen-receptor (ER) (Howell, Osborne et al. 2000). On the other hand, the ER-α- /ER-β-mediated estrogen response element (ERE) transcription activities of RD TF and Naringin were investigated. It is a standard method for assaying estrogen-like substances regulate gene expression by directly binds onto ERE. According to our findings, the stimulatory effects of RD TF, Naringin, Flavone #1 and Chromone #4 on UMR-106 cell proliferation, differentiation and mRNA expression of OPG/RANKL were completely abolished in the presence of ICI 182,780. The proliferative and differentiative effects of Phenylpropanoic acid #3 in UMR-106 cells were also blocked by the ICI although ICI did not fully blocked the OPG/RANKL mRNA expression. The results indicate that the stimulatory effects of RD TF and its active ingredients in UMR-106 cells might mediate through ER-dependent pathway, which is very similar to the actions of estradiol. RD TF and Naringin were then subjected to further investigation of ER-α-/ER-β-mediated ERE transcription activities. Our results found that, similar to estradiol, they could activate ERE transcription through ER-α or ER-β. They activated osteoblastic activity through classical ERE-binding transcription. In addition, they up-regulated ER-α phosphorylation significantly although they did not suppressed ER-α expression simultaneously. Some biologists believed that the

ligand-independent activity of the ER is a result of phosphorylation of the ER and creates cross-talk between the ER and other signalling pathways (Pearce and Jordan 2004). The argument was based on the findings that many effects induced by estrogen occur within a short time frame, which is faster than transcriptional events. These rapid effects may be mediated in part by plasma membrane associated forms of ER, and those activities would not activate ERE-transcription. Based on this hypothesis, the osteoprotective effects of RD TF and Naringin might also involve ligand-independent activation of ER-α by phosphorylation, which is distinct from those of estrogen. However, to confirm this hypothesis, further investigation such as the membrane phosphorylation of ER and the binding activities of ER-α or ER-β, should be conducted to elucidate the molecular actions involved in one of the possible signaling pathways.

Taken together, our study gives new insights to the use of RD and its active ingredients for the prevention and treatment of postmenopausal osteoporosis. Rhizoma Drynariae total flavonoids and Naringin treatment, like estrogen, can effectively suppress the OVX-induced imbalance bone remodeling possibly by both an increase in osteoblastic activities and a decrease in osteoclastogenesis in an ER-dependent manner. Apart from this, their partial anti-estrogenic effects might provide evidence for the use RD and its isolated single compounds as an alternative

medicine for the treatment of osteoporosis as they may be associated with a lower risk of cancer. By further investigating the mechanisms of the actions of each RD compound, it is not difficult to develop RD as an internationally accepted therapeutic agent for treating postmenopausal osteoporosis.

7.2 Conclusion

In conclusion, the present study provides the evidence to show that Rhizoma Drynariae total flavonoids and its isolated single compounds, Naringin, had partial estrogen agonist/ antagonist activities, via the estrogen receptor dependent pathway to mediate estrogen-like activities and activated ERE transcription. The molecular actions of RD total flavaonoids and Naringin should be further investigated.

The present study of other single compounds isolated from RD total flavonoids fraction also provide the evidence that they can act like estrogen to induce osteoblastic cell proliferation, differentiation and the modulation of osteoclastogenesis.

It is hoped that further investigations of mechanism of actions could support the development of RD total flavonoids, Naringin, and other four single compounds as a new class of phytoestrogen for treatment of postmenopausal osteoporosis.

Appendix 1 – Composition of experimental diet TD 98005 (Harlan Teklad, New Zealand). The experimental diet was used to place the normal diet during the herbal treatment.

Normal Ca, Control Diet (0.6% CA, 0.65% P)	
TD 98005	**g/kg**
Casein	110.0
Egg White Solids, spray-dried	97.9
DL-Methionine	3.0
Sucrose	551.1
Corn Starch	100.0
Corn Oil	50.0
Cellulose	20.0
Vitamin Mix, Teklad (40060)	10.0
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
Zine Carbonate	0.056
Chromium Potassium Sulfate	0.0193
Cupric Carbonate	0.011
Potassium Iodate	0.0004

Appendix 2 – Dosages used in the preliminary drug treatment. Sham and OVX mice were subjected to different treatments for 6 weeks and they are abbreviated as follows: Veh: vehicle-treated OVX, E2: 17β-estradiol, RD: Rhizoma Drynariae Crude Extract, RDA: RD water extract, RDB+RDC: RD 30% and 50% ethanol extract, RDD: 95% ethanol extract, Veh (Sham): vehicle-treated Sham.

Treatment group	Drug
Veh	2% Ethanol
E2	4 μg / g / day
RD	0.88 mg / g / day
RDA	0.567 mg / g / day
RDB+RDC	0.155 mg / g / day
RDD	0.018 mg / g / day
Veh (Sham)	2% Ethanol

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