CHAPTER II

LITERATURE REVIEW

Interest in plant derived estrogens, namely phytoestrogens, has recently increased due to the fact that hormone replacement therapy is not safe. Phytoestrogens have diverse physiological effects on cardiovascular system and reproductive system and can cause cancer (Cornwell et al., 2004). At present, the particular attention has been especially paid to its effects on bones. Aging and bone loss have become a major problem of the world. Based on this threat, many researches have been conducted. There are some possibilities that bone loss can be cured by phytoestrogens with less (or without) side effects of such as induction of breast cancer.

This chapter first summarizes what should be known about bones, for example bone structure, bone growth and bone loss, osteoporosis risk factors, bone homeostasis, influence of sex steroid hormones on bone and osteoporotic rat model. Secondly, it focuses on phytoestrogens, especially phytoestrogens in *P. mirifica*, in aspects of its source, biotransformation and metabolism in animals, phytoestrogen content in animal feed and in *P. mirifica* and estrogenic activity of phytoestrogens on reproduction and bone.

1. Bone

Bone is a specialized form of connective tissue. Its matrix is mineralized to lend the tissue to be extremely hard and tough. The mineral content is calcium phosphate in form of hydroxyapatite crystals ($Ca_{10}(PO_4)_6(OH)_2$). Both calcium and phosphate can be mobilized from bone matrix by specific stimuli, resorbed and circulated in blood. Generally, bone has the structure of cortex at the outer surface and trabecular bone inside (Figure 2.1 and 2.2). The diaphysis (shaft) of long bone contains a thick cortical bone and a small amount of trabecular bone. The proximal

and distal ends (epiphyseal part) and metaphyseal part of the long bone consist chiefly of trabecular bone with a thin outer shell of cortical bone (Figure 2.1). The vertebra is also composed chiefly of trabecular bone.

The bone tissue is composed of bone cells (osteocyte, osteoblast and osteoclast) and bone matrix. The major structural component of bone matrix is type I collagen in which deoxypyridinoline (Dpd) and pyridinoline (Pyr) form the pyridinium crosslink to tie collagen fibers together and to produce the matrix rigidity and strength. When the collagen is degraded during bone resorption process, Dpd and Pyr are released into circulation and excreted in urine. Thus, Dpd and Pyr have been used as biomarkers of the bone resorption. The matrix also contains ground substance, including glycoproteins, osteocalcin, osteopontin and osteonectin. Osteocalcin is a small noncollagenous protein which is specific for bone, and it is predominantly synthesized by osteoblasts and is incorporated into the extracellular bone matrix, but a fraction of newly synthesized osteocalcin is released into the blood circulation. Thus, osteocalcin is used as a biomarker of the bone formation.

Bone remodeling comprises of bone formation and resorption processes, and bone is on the balance of these two opposite processes to conform with its physical stresses and calcium demands in the body (Figure 2.3). Osteoblast is a bone cell responsible for bone formation and derived from osteoprogenitor cells. During proliferative phase, osteoprogenitor cells undergo DNA synthesis and cell division resulting in a rapid increase in cell number. Proliferation of cells is down-regulated and expression of osteogenic phenotype (alkaline phosphatase, osteopontin, bone sialoprotein and osteocalcin) is observed to increase, indicating the presence of mature osteoblasts (Mundy, 1996). During differentiation, osteoblast phenotypic markers are appeared. Osteoblasts produce alkaline phosphatase which processes procollagen to collagen. The collagen is deposited in the extracellular matrix containing additional proteins (e.g. osteopontin, bone sialoprotein and osteocalcin). The collagenous matrix is subsequently mineralized to calcified matrix (Aubin et al., 1995; Qu et al., 1998; Wada et al., 1998).

In contrast, an osteoclast is a bone cell responsible for bone resorption. It originates from bone marrow cell. Osteoclasts rest on the bone tissue where

resorption is taking place. They release proteolytic enzymes including collagenase, matrix metalloproteinases and cysteine proteinases which digest organic components of the bone matrix. They also create the local acidic environment in an extracellular space. Accordingly, calcium is dissolved and a shallow bay can be observed under the osteoclast (Vaananen et al., 1998).

After this resorption process, osteoblasts secrete both collagen and ground substance, including glycoproteins and osteocalcin, that constitute the initial unmineralized bone. The ground substance plays a role in binding calcium in this process and finally the collagen and ground substance components become mineralized to form a new bone.

Imbalance of bone remodeling can occur if osteoclasts produce an excessively deep resorption space and/or if osteoblasts fail to completely refill the resorption space. The imbalance can also produce losses of bone mass (Oursler et al., 1997).

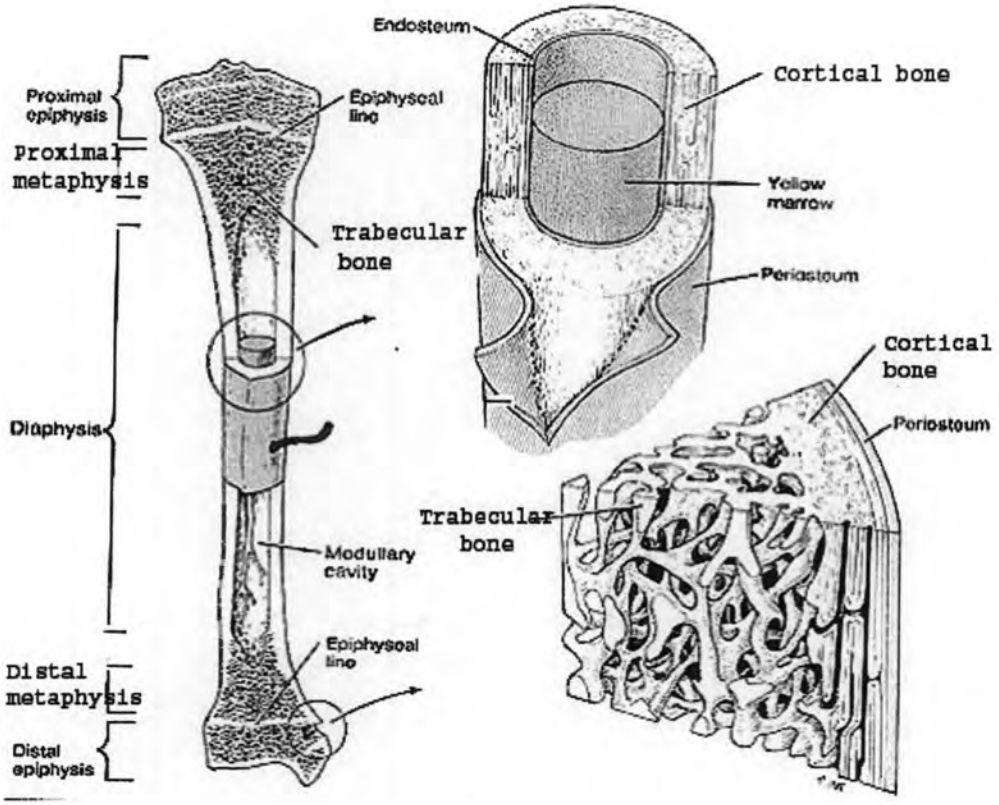


Figure 2.1 Bone structure. It comprises of cortical and trabecular compartments. Cortical bone is mainly in diaphysis sites, and trabecular bone is mainly in metaphysis and epiphysis sites

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

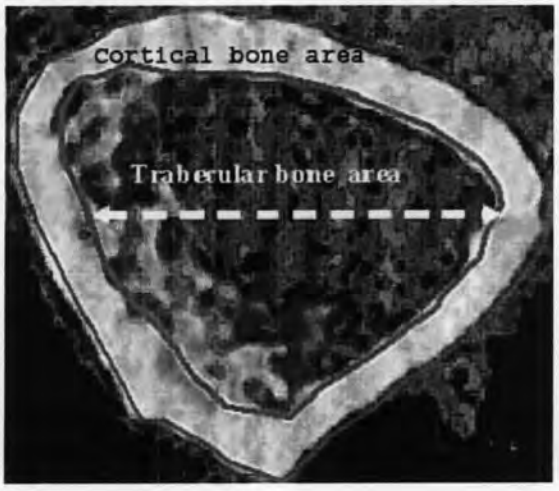


Figure 2.2 Cortical and trabecular bone areas in the cross-sectionally metaphyseal part of the rat distal femur taken by peripheral Quantitative Computed Tomography (pQCT).

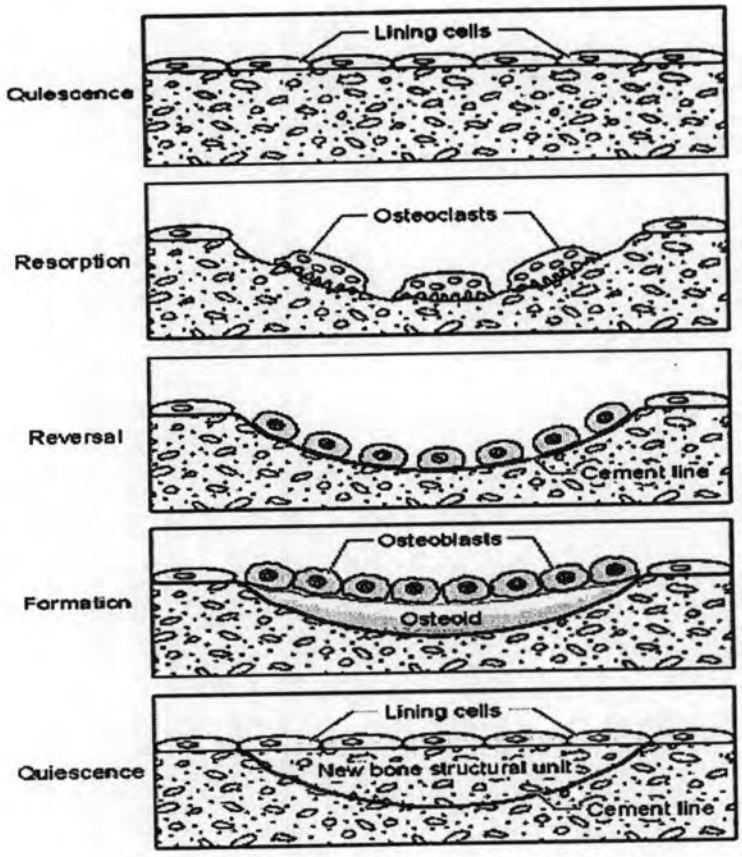


Figure 2.3 The bone remodeling sequence is initiated by osteoclasts responding for bone resorption process. Subsequently, osteoblasts appear within resorption sites and synthesize the matrix which is mineralized later
(Available from: http://www.fleshandbones.com/readingroom/pdf/113.pdf).

1.1 Bone growth and bone loss

From infancy until adolescence, bone formation occurs at a higher rate than bone resorption. Regardless of the site, humans achieve peak bone mineral density (BMD) at around 20 years of age. Women achieve peak BMD earlier than men but BMD in women is lower than that in men at the end (Lu et al., 1994; Williams et al., 1996; Code and Aronson, 2003). The peaks of total bone mineral content (BMC) and total BMD are achieved 1-2 years later than inflection ages for height and weight growth curves. It is well established that the growth spurt occurring at puberty is

driven largely by sex steroid secretion of the awakening gonads. Gradually rising of hormone levels causes the growth spurt and attainment of the higher steroid levels (approximately at adult levels) causing termination of vertical growth in linear dimension by closure of the growth plate (Sharpe, 1998).

The peak bone mass is maintained up to about the age of 40 years in both women and men. After 40 years old, bone mass begins to decrease, at the rate of about .0.4 to 1.3% per year (Code and Aronson, 2003). In women, two most frequently cited factors that contribute to development of the osteoporosis are aging and menopause. While the BMD declines with age in both women and men, women start to have the BMD lower than in men and show the accelerated loss during menopause by a sudden decline in estrogen production (Williams et al., 1996). In men, androgens are important for skeletal growth, especially during linear growth, in adolescence and are also responsible for maintenance of the skeletal mass and bone density during mature stage of life (Bertelloni et al., 1995; Vanderschueren and Bouillon, 1995; Behre et al., 1997). In contrast to women who have a precipitous decrease in serum estrogen levels around the time of menopause, the age-related decrease in serum bioavailable testosterone and estrogen levels in aging men is much more gradual. This can suggest that a dramatic decrease in serum estrogen levels at the time of menopause in women, which is absent in men, triggers a rapid bone loss (Maas et al., 1997; Khosla et al., 1998). However, men with acute hypogonadism which is caused by orchidectomy also have a rapid bone loss (Stepan et al., 1989). Therefore, rate of bone loss in men is substantially slower than that in women, particularly at appendicular sites (Orwoll et al., 1990). In most countries, the incidence of osteoporosis in women is about 2–4 times higher than in men. A sharp decrease in estrogen production by ovary is the predominant cause of rapid, hormone-related bone loss during the first decade after menopause (Annie et al., 2006). Nevertheless at some skeletal sites, there is an obvious increase in fracture frequency with advancing age in men. For example, the rate of increase in incidence of hip fractures with age is as dramatic as that in women, although its onset occurs somewhat later in life (Orwoll et al., 1990). The previous information also suggests that bone loss in women and men is associated with age (Figure 2.4). The lumbar spine and proximal femur show a significant negative linear regression of BMD with age (Orwoll et al., 1990; Marcus et al., 1994).

Bone loss seems to occur in axial and appendicular skeletons and at trabecular and cortical compartments in both sexes (Orwoll et al., 1990; Marcus et al., 1994). Different regions of the skeleton respond differently to age and hypogonadism (Lindsay et al., 1992; Salamone et al., 1995), and the degree of bone loss varies widely from one skeletal site to another (Melton, 2001; Wang et al., 2001). Thus, for a general evaluation of the skeletal response to a given chemical substances, BMD and BMC at several skeletal sites should be examined.

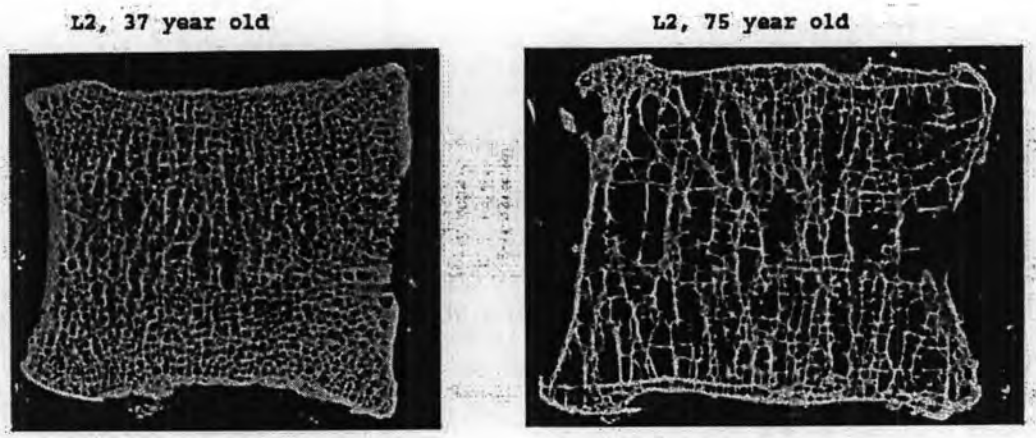


Figure 2.4 Trabecular bone density and structure in the second lumbar vertebra (L2). Trabecular bones are high density and connection (A), Trabecular bones lose density and connection (B),
(Available from: http://www.engr.iupui.edu/~turnerch/biomech.htm).

1.2 Osteoporosis and risk factors

Osteoporosis is a bone disorder characterized by low BMD and microarchitectural deterioration in bone tissue. There is a standard for a low BMD which is considered to osteoporosis. The World Health Organization (WHO) defines an osteopenia when a BMD being between 1 and 2.5 of standard deviation below the mean of young healthy adults. Osteoporosis is defined as the BMD of 2.5 or more of the standard deviation below the mean (Kanis et al., 1994). Low BMC, BMD and structural deterioration of the bone tissue lead to an increased bone fragility and an

increased susceptibility to fractures of the hip, vertebra, wrist and others. Bone fractures may lead to a disability, a decreased quality of life, and an increased morbidity and mortality (Ray et al., 1997).

Osteoporosis can be classified as either primary or secondary. Primary osteoporosis is a reduction in bone mass that is unrelated to other chronic illnesses and is primarily related to aging and decreased gonadal function (Harper and Weber, 1998). Thus, menopausal estrogen reductions in women and lower levels of testosterone and/or estrogen in men increase the risk of primary osteoporosis. Secondary osteoporosis is a consequence of chronic conditions that contribute to an accelerated bone loss. Some of these chronic conditions include the endogenous and exogenous thyroxin excess, hyperparathyroidism, gastrointestinal diseases, medications, connective tissue diseases, renal failure and a variety of other conditions (see Table 2.1) (Moyad, 2003).

Table 2.1 A partial list of shared important risk factors for osteoporosis found in men as well as in women (Moyad, 2003).

Advanced age
White or Asian race
Endocrine disorders (gonadal failure, hyperparathyroidism, hyperthyroidism, etc.)
Excessive alcohol intake
Family history of osteoporosis
High/excessive intake of some vitamins and minerals (e.g. mega-doses of vitamin A may inhibit bone growth)
History of a previous fracture
Impaired calcium and/or vitamin D absorption
Lack of weight-bearing exercise
Long-term use of specific drugs/hormones (corticosteroids, excess thyroid hormone, LHRH agonists, hyperprolactinemia, etc.)
Low calcium and/or vitamin D intake or other minerals/vitamins (e.g., inadequate vitamin K intake)
Low exposure to sunlight (sunlight activates the endogenous production of vitamin D)
Low levels of testosterone/estrogen
Propensity for falls (postural instability, neuromuscular impairment, poor vision, lower limb weakness, drugs that affect blood pressure, etc.)
Renal disease
Sedentary lifestyle/immobilization
Small body frame or low weight
Smoking

1.3 Bone homeostasis and sex steroid hormones

It is well established that estrogens and androgens are involved in bone metabolism in humans (Benz et al., 1991; Marcus, 1996; Goh and Ratnam, 1997). Androgen and estrogen receptors have been found in bone cells in both sexes of humans and rats (Benz et al., 1991; Orwoll et al., 1991; Vanderschueren and Bouillon, 1995; Goh and Ratnam, 1997).

The importance of estrogens in homeostatic regulation of many cellular and biochemical events is well illustrated by pathophysiologic changes that occur with estrogen deficiency. Postmenopausal women or bilateral OVX individuals, who remained in a prolonged state of estrogen deficiency, experience a progressive loss of bone mass with a result of increase in the risk of osteoporosis (Insogna et al., 1981; Riggs et al., 1982; Orwol and Meier, 1986). By studying in women at age of 21-94 years, serum bioavailable estrogen levels correlated positively with BMD at the total body, spine, proximal femur, and distal radius and negatively with bone resorption marker, urinary *N*-telopeptide of type I collagen (NTx) excretion (Khosla et al., 1998). Estrogen plays an important role in maintaining bone mass in adult women by exerting a tonic suppression of bone remodeling and maintaining remodeling balance between osteoblast and osteoclastic activity. Therefore, the onset of menopause, or a sudden loss of estrogen, is the point in life, where every woman loses BMC. The BMD becomes significantly lower and bone resorption markers are increased (Ohta et al., 2002).

The most effective way to prevent osteoporosis in women is estrogen replacement (Kalu, 1995). Estrogen replacement therapy is associated with a significantly increased age and an adjusted BMD in postmenopausal women (Marcus et al., 1994). The long-term estrogen treatment also reduces the incidence of fracture (Kalu, 1995). Studies in animals have demonstrated that estrogen deficiency in females can produce bone loss (Wronski et al., 1988; Wronski et al., 1989; Omi and Ezawa, 1995; Fanti et al., 1998; Deyhim et al., 2003; Devareddy et al., 2006).

In men, a body of evidence supports the involvement of sex steroids in the skeletal maintenance after the bone mass peak has been attained. Primary

hypogonadism caused by the anorchia, previous orchitis or unknown reasons or bilateral ORX, and secondary hypogonadism with idiopathic hypogonadotropic hypogonadism or Kallmann's syndrome, and pituitary tumor including macroprolactinoma or panhypopituitarism in men has a progressive loss of the bone density. The biochemical markers of bone resorption and bone formation are significantly increased in hypogonadism patients compared with healthy controls (Baran et al., 1978; Stepan et al., 1989; Behre et al., 1997). Male hypogonadism causes both trabecular and cortical osteoporosis and alters trabecular architecture (Francis et al., 1986).

Testosterone therapy increases osteoid volume, osteoid surface, bone formation and bone mineralization. The BMD can be normalized and maintained in the normal range regardless of age by continuous, long-term testosterone substitution at physiological level (Francis et al., 1986; Behre et al., 1997). Additionally, the clinical and animal studies have demonstrated that hypogonadism in males is a risk factor for osteoporosis (Verhas et al., 1986; Turner et al., 1989; Vanderschueren et al., 1992; Prakasam et al., 1999). Thus, sex steroid hormone deficiency is associated with accelerated bone loss which confirms the importance of androgens in bone modeling and remodeling (Baran et al., 1978; Stepan et al., 1989). Presently, some accumulated evidence have shown that the loss of bone mass over time in men related much to estrogen deficiency. In men, the formation of estrogens from androgens is catalysed by aromatase cytochrome P450, the product of the *CYP*19 gene. Aromatase converses androstenedione and testosterone to estrone and 17 β-estradiol, respectively (Murata et al., 2002). The enzyme is expressed in several tissue, including adipose tissues, chondrocytes and also osteoblasts (Sasano et al., 1997). These extragonadal sites of estrogen biosynthesis possess several fundamental features that differ from those of ovaries. Principally, the estrogen synthesized within these compartments is probably most biologically active only at the local tissue level in a paracrine or intracrine fashion (Labrie et al., 1997). Thus, these sources of estrogens play an important, but hitherto largely unrecognized, physiological and pathophysiological role (Murata et al., 2002).

The influence of estrogen on bone in men has been realized ever since defects either in the estrogen receptor gene or in the P450 aromatase gene were described. A man with a disruptive mutation of the estrogen receptor gene is associated with a

complete resistance to the action of estrogen. Although his testosterone and dihydrotestosterone (DHT) level are normal, and estradiol and estrone levels are 2 - 2.5 times greater than the normal man, bone density is still lower than the mean by 2 standard deviation (Smith et al. 1994). Morishima et al. (1995) described a man with a mutation in the aromatase gene. The three major androgens, androstenedione, testosterone and DHT, were all greatly raised, however, estradiol and estrone levels were undetectable and bone mass was low. The bone mass was increased after treatment with estrogens (Morishima et al., 1995). A similar case was reported by Carani et al. (1997).

Estrogen therapy in aromatase-deficient men is shown to lead a dramatic increase in apparent bone density (Rochira et al., 2000). Several cross-sectional studies have examined the relations between estrogen, testosterone, and bone mass in men. The results seem to agree; most authors conclude that plasma estrogen levels correlate better with BMD than plasma testosterone levels (Greendale et al., 1997; Slemenda et al., 1997; Khosla et al., 1998; Center et al., 1999; Gillberg et al., 1999; Szulc et al., 2001). Bioavailable estrogen levels decline significantly with age and are important predictors of BMD in men as well as in women (Khosla et al., 1998). The similar result is also obtained when an estrogen deficient model is carried out in animals. The aromatase deficient male mouse model (ArKO) is created through disrupting the *CYP*19 gene. The male ArKO mice have elevated levels of testosterone, but decreased trabecular bone volume, trabecular thickness, and reduced osteoblastic, osteoid and mineralizing surface (Murata et al., 2002).

Estrogen receptors, ERα and ERβ, are present in osteoblasts (Eriksen et al., 1988; Hoyland et al., 1997; Onoe et al., 1997), osteocytes (Hoyland et al., 1997), osteoclasts (Hoyland et al., 1997) and the progenitors of these cells (Onoe et al., 1997) in human bones. Remarkably, however, the level of expression of ERα and ERβ in osteoblasts and osteoclasts is at least 10-fold lower compared to that in reproductive organs (Kousteni et al., 2001). ERα and ERβ were also found in cortical and trabecular compartments of rat bones. The ERβ is highly expressed in both primary and mature osteoblasts more than ERα in rat bones (Onoe et al., 1997; Swindahl et al., 2000).

The androgen receptors (AR) also are found in osteoblasts, osteoclasts and the progenitors of these cells. In addition, the distribution of receptors does not vary by gender, as similar levels of ER and AR have been found in bone cells of males and females (Kousteni et al., 2001). As expected, androgen treatment of osteoporotic men and women is effective in increasing BMD and also estrogen treatment can prevent bone loss in both sexes (Rochira et al., 2000). Androgens including testosterone and dihydrotestosterone (DHT) act directly on osteoblasts, stimulating growth and differentiate of osteoblastic cells *in vitro*, and inhibit osteoclast functions by binding to an androgen receptor (Chen et al., 2001). However, testosterone has a relatively low affinity for the androgen receptor and is less active without conversion into more active metabolites by 5 alpha-reductase into DHT within target tissues (Oursler et al., 1997). The conversion rate of testosterone to DHT is very low in osteoblast. Moreover, the dose of DHT for prevention of bone loss is always high because of its fast metabolization rate *in vivo* (Vanderschueren et al., 1996). The alternative way of androgen expression is aromatization. Testosterone is aromatized to estrogen in osteoblast and expresses estrogenic activity via estrogen receptor in bone cells (Shozu and Simpson, 1998).

Vaananen and Harkonen (1996) and Vaananen et al. (1998) reported that estrogen inhibits differentiation of osteoclasts thus decrease their numbers and bone turn over. The possible ways of estrogen regulation are a direct expression via estrogen receptor at osteoclast cells to suppress the bone resorption and/or via estrogen receptor in osteoblast cells to increase synthesis and release of osteoblast derived osteoclast inhibition factors, including osteoprotegerin (OPG). OPG decreased osteoclast formation. Thus, the effect of estrogen to antagonize osteoclast mediated bone resorption appears to be the most important action to preserve bone. These changes are consistent with the effect of estrogen on osteoclasts and on bone resorption revealed in experimental animals and humans (Oursler et al., 1997).

As described above, estrogen is the most important factor for bone mass maintenance, however, estrogens have also clearly emerged as the predominant factor involved in breast cancer. Since estrogens can induce genomic damage (initiation) and increase cellular proliferation (promotion), it is likely that estrogen functions as both carcinogen and tumor promoter. Estrogens increase the number of mutations,

and these mutations give rise to a malignant phenotype (Henderson and Feigelson, 2000; Gruber et al., 2002).

Therefore, use of estrogen as hormonal therapy is considered to be the main risk factor for the development of breast cancer (Kenemans and Bosman, 2003; Fontanges et al., 2004) and endometrial cancer (Sulak, 1997; Canavan and Doshi, 1999) in women. Breast cancer is the most common type of hormone-dependent neoplasm in women (Santen, 1992). Use of testosterone or other androgens which has been considered to prevent bone loss in men are also able to stimulate prostate cancer (Janssens and Vanderschueren, 2000; Crawford, 2005; Gaylis et al., 2005).

1.4 Use of rats as experimental models for osteoporosis study

Rat is a useful model for osteoporosis study. The gonadectomized rats are determined as a standard model for studying bone loss caused by sex hormonal deficiency. Keeping in mind, the response of rat bone is site- and compartment-specific. The regions of predominantly trabecular compartments respond rapidly to intervention and cortical compartments are, as it lacks remodeling, relatively non-responsive (Thompson et al., 1995; Bloomfield et al., 2002). Thus, the bone site which is preferably used as an indicator of the study of bone loss is proximal tibial metaphysis (Wang et al., 2001), proximal and distal femoral metaphysis (Omi and Ezawa, 1995), and lumbar spine which are mainly consisted of the trabecular bone (Dempster et al., 1995; Wang et al., 2001) (Figure 2.5).

Although rats have a continuous growth throughout their life-span, at 6-9 months old most indices of bone mass in cortical and trabecular compartments of longitudinal bone growth are reaching a plateau or slowly (or insignificantly) increase. Ke et al. (1996) reported that total bone mass rapidly increases until male Sprague-Dawley rats are 6 months old, and then it slightly increases up to 10 months old. Age-related decrease in bone mass starts to occur in 12 months old (Ke et al., 1996). Adult female rats which have a regular 4-5 days of estrus cycle, during the second year of life, the fraction of rats found in constant diestrus stage rises gradually, and trabecular bone loss is frequently observed (Kimmel, 1996). From these reasons,

the 6-9 months old rats should be a good model for the study of bone loss induced by estrogen deficiency. This model is supposed to have no confounding effects by rapid bone growth as found in younger rats or have lesser effect of bone loss caused by aging. Other advantages of using rats for bone study are that, rats have a short life-span (2.5-3 yrs), not expensive, and convenient to handle and manage.

There have been many researches on bone loss in rats. In female rats, the progressive loss of proximal tibial metaphyses becomes more pronounced with a period up to 100 days of postovariectomy, after which trabecular bone loss and bone turnover both subside. These findings are consistent with concepts of skeletal dynamics in estrogen-deficient women. A rapid phase of bone loss occurs during the early postmenopausal period and an increased bone turnover also occurs at this time and later the rates of bone loss and bone turnover both diminish. Therefore, in the term of pattern of bone loss, the association between rapid bone loss and maximally increased bone turnover is qualitatively similar in estrogen-deficient rats and women (Wronski et al., 1988; Wronski et al., 1989). The effects of OVX in 6-month-old rats are the same as the effects of menopause in women, in regard to bone mass and renal handling of calcium and phosphorus. The OVX rats lose significantly more bone than sham-operated rats and increase urine calcium at 1 and 3 weeks after OVX, before returning to sham-operated levels by 6 weeks. OVX results in an increase in filtered load of calcium and phosphorus. There is an increase in maximal renal tubular reabsorption of phosphorus, but no clear change in renal calcium handling. As similar to the postmenopausal women, after OVX in female rats, there is a significant and generalized bone loss and a negative calcium balance. This is associated with an initial rise in urine calcium due to a rise in the filtered calcium load; plasma phosphorus and renal tubular reabsorption of phosphorus also rise. The rat model has many similarities of bone metabolism to that seen in menopausal women including calcium and phosphorus homeostasis (Dick et al., 1996). Similarly to previous reports, use of OVX aged or growing mice and rats as a model of osteoporosis, the increase in bone loss, bone turnover and urinary calcium are observed (Omi and Ezawa, 1995; Deyhim et al., 2003).

In male rats, bone mass is significantly decreased after 1 month of ORX and maintained at lower levels than that in normal rats (Ke et al., 1996; Prakasam et al.,

1999). Androgen deficiency occurring in aged rats can induce the intracortical porosis and significant decreases in BMD and BMC of both cortical and trabecular compartments (Vanderschueren et al., 1993; Prakasam et al., 1999). Testosterone treatment completely counteracts these effects. Testosterone replacement also suppresses bone remodeling and increases the BMD and BMC (Vanderschueren et al., 1993; Prakasam et al., 1999). Seidlova-Wuttke et al. (2005) reported that after three months of ORX male rats showed a more than 30% loss of the BMD at tibial metaphysis when compare to values prior to the ORX (Seidlova-Wuttke et al., 2005). Also, treatment of estradiol can completely prevent bone loss in ORX rats (Vanderschueren et al., 1992).

In conclusion, use of gonadectomized rats (or a sex-hormonal deficient rats) as a model for bone loss study the bone loss should be observed within three months after gonadectomy.

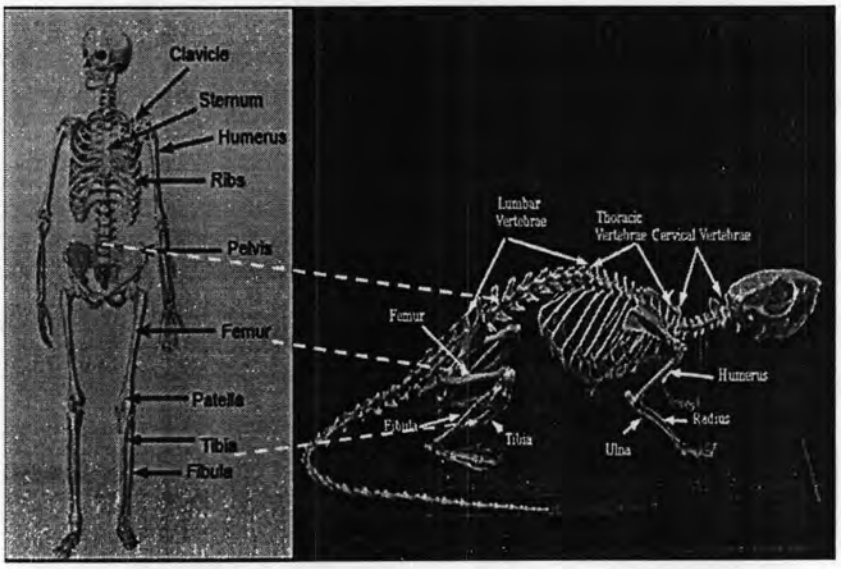
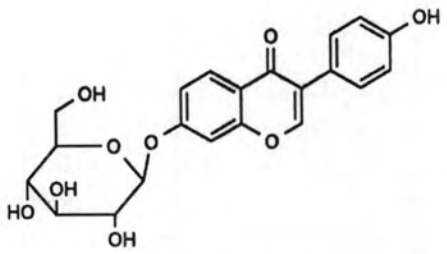


Figure 2.5 Comparing between human and rat bones at lumbar vertebra, femur and tibia (Available from: http://www.stayinginshape.com/3osfcorp/libv/i14.shtml).

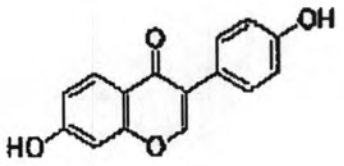
2. Phytoestrogens

Many major diseases are hormone dependent and epidemiologic data have shown a strong association between their incidences and diets. Particularly, the current dietary recommendations to consume a high proportion and amount of fruits and vegetables are announced. Although interpretation of the epidemiology based on an individual dietary consumption is difficult, it is recognized that there are many plant-derived bioactive non-nutrient phytochemicals that can have significant health benefits. Among these phytochemicals, there is a broad class of non-steroidal estrogens called phytoestrogens. Within the past decade considerable interests are paid to the role of isoflavone phytoestrogens which are present in high concentration in soy and soy products (Setchell, 1998).

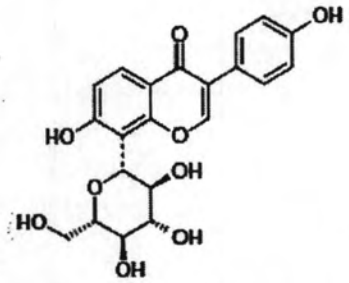
Phytoestrogenes are plant compounds that have estrogen-like biological activities. They have 2-phenylnaphthalene-type chemical structures similar to those of estrogens and have been found to bind to ERs. Currently, three different families of phenolic compounds produced by plants are considered as phytoestrogens: isoflavones (i.e., genistein, genistin, daidzein, daidzin, biochanin A, formononetin, puerarin and pratensein) (Figure 2.6), lignans and coumestans (i.e., coumestrol and 4'-o-methylcoumestrol). Isoflavones are a large chemical class of flavonoids. The isoflavones from legumes, including genistein and daidzein, are the most extensively studied phytoestrogens (Cornwell et al., 2004).



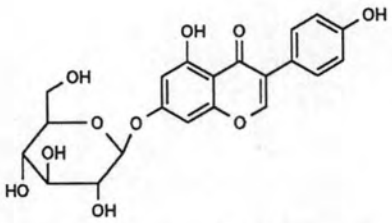
daidzin (daidzein glycoside form)



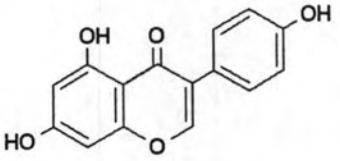
daidzein



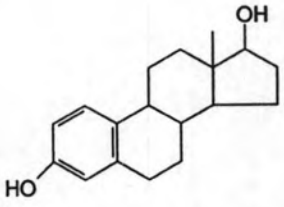
puerarin (daidzein-8-*C*-glycoside)



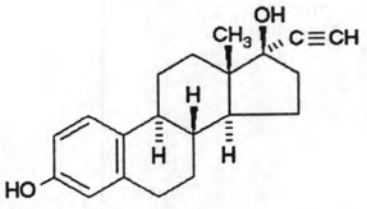
genistin (genistein glycoside form)



genistein



17 β-estradiol



17 α-ethinylestradiol

Figure 2.6 Chemical structures of major isoflavone phytoestrogens (daidzin, daidzein, puerarin, genistin and genistein), 17 β-estradiol (the most potent naturally occurring estrogen), and the synthetic 17 α-ethinylestradiol.

2.1 Sources of phytoestrogens

Phytoestrogens are produced by numerous Legummosae herbs and grasses, including plants commonly consumed by man and livestock. The estrogenic components are found in differing amounts in all parts of the plant, including seeds, flowers, leaves, roots and fruits. They are found either as the aglycone (unconjugated form) or various types of sugar conjugates. Concentrations in each tissue depend on plant type (Kaldas and Hughes, 1989). The major source of isoflavonoids in the diet is from soy-based foods. In Asia, the intake of soy can be as high as 30–50 g a day (Adlercreutz et al., 1993). The phytolignans appear in high amounts in flaxseed, whole grain breads, vegetables and tea. The main dietary source of coumestrol is clover and soybean sprout, however, low levels have been reported in brussels sprout and spinach (Cornwell et a., 2004).

2.2 Biotransformation and metabolism of phytoestrogens

Most isoflavones exist in the plant as glycosidic conjugates, generally located in the cell vacuoles. Bioavailability of these dietary components depends on relative uptake rates of conjugated and free forms, or hydrolysis of glycosides by intestinal bacteria or intestinal-glycosidase enzymes. The free aglycones, but not the glycosides, are absorbed from rat stomach. However, once in the small intestine, brush border lactase can effectively hydrolyze isoflavone glycosides (Steensma et al., 1999; Dixon and Ferreira, 2002). Germ-free rats fed a soy-isoflavone containing diet excrete large quantities of daidzein and genistein (a glycoside form of daidzin and genistin, respectively) in urine indicating that the gut microflora is not required for the absorption of aglycoside form of isoflavones. It seems likely that mammalian glycosidase activity associated with the small intestinal mucosa is involved in glycoside hydrolysis prior to absorption. The equol and *O*-desmethylangolensin which are isoflavone metabolites from daidzin and daidzein are not detectable in urine from the germ-free rats, but are present in human flora associated rats, indicating that they are produced by the gut microflora activity. Colonization of germ-free rats with a faecal flora from a human subject, which has the capacity to convert daidzein to equol, causes the excreting of substantial amounts of the metabolite in rats (Wong,

2002; Bowey et al., 2003). Nevertheless, in humans, isoflavones appear in blood plasma at a more rapid rate, and at higher levels, following oral administration of aglycones as compared to glycosides, genistein and daidzein, but not their glycosides, are readily transported across human intestinal epithelial cell monolayers to blood circulation (Steensma et al., 1999; Dixon and Ferreira, 2002). After absorption in the small and probably large intestines, isoflavones require phase II metabolism and form sulfate and glucuronide conjugates in the liver, and are then excreted through the kidney as urine and through the intestine as the bile (Wong, 2002).

After the daidzin is orally administered to rats its metabolites including daidzein-4′,7-di-*O*-sulfate, daidzein-7-*O*-*β*-D-glucuronide, daidzein-4-*O*-sulfate, and daidzein are found in plasma and urine. These metabolites appear in plasma as early as 30 minutes after oral administration. The concentration of daidzein-4′, 7-di-*O*-sulfate is significantly higher than those of other metabolites. The same metabolites are also found in rat bile after oral administration of daidzin or daidzein. In addition, a new major metabolite, daidzein-4′, *O*-sulfate-7-*O*-*β*-D-glucuronide is also found in bile. The compositions of metabolites found in plasma, urine and bile of rats administered with daidzin or its aglycone daidzein are not different. Daidzein is detected along with a trace amount of the unchanged compound in feces from rats to which daidzein is orally administered. In the feces of rats receiving daidzein, however, only the unchanged compound but no metabolite is detected. Other metabolites of daidzin such as equol that have been found to have estrogenic effect are detected as the minor metabolites (Ohsawa and Yasuda, 2002).

Genistin orally administered to rats is hydrolyzed to genistein and similar compounds in the intestine before being absorbed. Only metabolites and a trace amount of unchanged genistin are detected in the 48-h feces. The metabolites of genistin, including genistein-4′-*O*-sulfate, genistein-7-*O*-*β*-D-glucuronide, genistein-4′-*O*-sulfate-7-*O*-*β*-D-glucuronide and genistein are found in urine, and those including genistein-7-*O*-*β*-D-glucuronide, genistein-4′-*O*-sulfate-7-*O*-*β*-D-glucuronide are found in biliary excretion (Ohsawa and Yasuda, 2002).

Puerarin is a major phytoestrogenic compound found in the *Pueraria lobata* and *P. mirifica* (Cherdshewasart et al., 2007a). Plasma samples collected from rats

which were orally given puerarin, contain only the unchanged compound. However, its metabolites including daidzein-4′,7-di-*O*-sulfate, daidzein-7-*O*-*β*-D-glucuronide, daidzein-4-*O*-sulfate, and daidzein are found in urine, though the major urine compound is the unchanged puerarin. The unchanged compound as well as the major metabolites, puerarin-4′-*O*-sulfate and puerarin-7-*O*-*β*-D-glucuronide, are detected in biliary exretions in rats after oral administration of puerarin. Only the unchanged compound and no metabolites are detected in the 48-hour feces (Ohsawa and Yasuda, 2002).

In summary, the *O*-glycosides, e.g., daidzin and genistin, are mainly hydrolyzed, and then absorbed and excreted in the form of their aglycone metabolites. On the other hand, the *C*-glycosides, e.g., puerarin, appears in the plasma, urine and feces mainly in intact form. This could be attributed to the fact that the *C*-glycosides have the *C-C* linkage between the sugar and isoflavone moiety which is more resistant to the hydrolytic actions of enzymes found in the intestinal flora than the glycosidic linkage in the *O*-glycosides (Ohsawa and Yasuda, 2002).

2.3 Phytoestrogens in animal feed

Commercially available rodent dietary used in the US and European laboratories is reported to contain variable but significant levels of phytoestrogens, especially daidzein and genistein, which are known to have estrogenic properties (Murphy et al., 1982; Boettger-Tong et al., 1998; Casanova et al., 1999; Thigpen et al., 1999a, 1999b; Degen et al., 2002). It is because most commercial rodent diets are formulated with soybean product; the diets deliver large daily doses of isoflavones to experimental animals. This is shown by the high steady-state isoflavone levels in serum of adult rats (2,613 ± 873 ng/ml) and mice (2,338 ± 531 ng/ml), exceeding animals' endogenous estrogen levels by 30,000 to 60,000 folds (Brown and Setchell, 2001). From experiments evaluating the genistein effect on the relative uterus weight of OVX rats, Dagen et al. (2002) reported that the soybean diet enhanced the genistein effect compared with those of rats fed the phytoestrogen-free diet. Omi et al. (1994) and Arjmandi et al. (1996) also reported that rats fed soy derivatives had greater BMD than did control rats fed casein. It is, therefore, suspected that the dietary background

phytoestrogens may have influenced on the exogenously administered estrogens and phytoestrogens (Boettger-Tong et al., 1998; Thigpen et al., 1999a, 1999b). Thus, a standardized formula for diets in which phytoestrogens substances have been reduced to minimal levels is recommended for feeding to rodents in which study of the estrogen-like substances are carried out.

2.4 Estrogenic activity of phytoestrogens

The chemical structure of phytoestrogens are strikingly similar to that of mammalian estrogens and many of them have estrogenic potency (Wong, 2002, and see Figure 2.6). The study on the interaction of phytoestrogens with ERs in human breast cancer cell line, MCF7, shows that both genistein and coumestrol can bind ERs in the cytoplasm and be translocated to the nucleus (Martin et al., 1978; Mäkelaä et al., 1994; Scarlata and Miksicek, 1995). The ability of genistein, daidzein, coumestrol, biochanin A and naringenin to bind to ERs and to elicit transcriptional activation has been demonstrated by finding that luciferase-reporter-gene transfected cell line is responsive to the exposure of the cells to estrogen or estrogen agonists (Jefferson et al., 2002). Estrogenic property of phytoestrogens varies widely depending on the kinds of phytoestrogens and target tissues (Gao and Yamaguchi, 1999; Jefferson et al., 2002). Phytoestrogens may either elicit weak estrogenic responses or block estrogenic actions in estrogen responsive tissues. Whether they act as agonists or antagonists depend on many factors including receptor numbers and concentrations and binding affinities of competing estrogens. Isoflavones have a weak estrogenic effect in absence or low concentration of estrogen, but they exert an antagonistic effect when estrogen concentration is high (Messina et al., 1994; Hwang et al., 2006). Phytoestrogens are found to compete with estrogen for uterine receptors (Shutt and Cox, 1972; Scarlata and Miksicek, 1995). Equol, a potent-more isoflavone converted from daidzein, is antagonistic to estradiol by competition for ERs. Accordingly, these phytoestrogenic substances can compete for ERs and decrease a full estrogenic response by binding to ERs (Tang and Adams, 1980).

Phytoestrogens, such as genistein, coumestrol, apigenin, naringenin, and kaempferol have a higher affinity for ERβ than ERα, and the former two phytoestrogens could stimulate the transcriptional activity in the cells with both ERs

(Kuiper et al., 1998; Hwang et al., 2006). Genistein has one-third of the binding potency of estradiol with ERβ, and one-thousandth with ERα as determined by expression of luciferase reporter gene constructed in kidney cells that have been cotransfected with ERβ and ERα (Kuiper et al., 1998). Thus, ERβ is believed to be important for phytoestrogens' action. The ERβ is found mostly in bone and prostate gland, and ERα is found mainly in testes while both receptors are found mainly in breast and uterus (Wong, 2002). Slight amount of ERβ mRNA is detected in the primary osteoblastic cells (bone formation cells), and the concentration of ERβ mRNA gradually increases during the maturation of osteoblast in rat bones (Onoe et al., 1997). There is a marked difference between genistein dosages that protect against bone loss and those that induce uterine hypertrophy. Subcutaneous injection of genistein at 0.7 mg/day prevents trabecular bone loss in OVX mice without hypertrophic effects on the uterus, while subcutaneous injection of 5 mg/day of genistein induces uterine hypertrophy and prevent bone loss (Ishimi et al., 2000).

The estrogenic potency of phytoestrogens is significant, especially for ERβ, and they may trigger many biological responses as those of physiological estrogens trigger (Kuiper et al., 1998). The coumestrol, a coumestan phytoestrogen, binds more strongly to ERs than the isoflavones, and triggers adverse effects on the reproductive system in female rats (Whitten et al., 1995). Coumestrol has a strong hypertrophic effect on uterus, which is comparable to or more effective than that of estradiol, and has an additive effect when combined with estradiol (Pocock et al., 2002). Coumestrol does not antagonize the uterotrophic action of estradiol when given either prior to, or jointly with estradiol treatment, and when administered either orally or parenterally (Whitten et al., 1994).

2.4.1 Estrogenic effects of phytoestrogens on reproduction

Phytoestrogens have been shown to influence many aspects of the mammalian reproductive process. In female, the increase in uterine weight in OVX experimental animals (or so-called a uterotropic assay) by phytoestrogen treatment is remarkable. Genistein, cumestrol, miroestrol and equol significantly increase uterine weights in OVX as well as immature mice and rats (Pop et al., 1958; Breinholt et al.,

2000; Ishimi et al., 2000; Jefferson et al., 2002; Diel et al., 2006; Power et al., 2006). Genistein, daidzein, coumestrol, biochanin A and naringenin increase uterine epithelial cell heights and uterine gland numbers in immature mice. However, the estrogenic effects of phytoestrogens are varied and dependened on compounds and target organs (Jefferson et al., 2002).

Miroestrol known as the most potent phytoestrogenic compound exhibits its estrogenic activity in immature mouse uterine weight and rat vaginal cornification assay (Pop et al., 1958). It has been found that subcutaneous injection of miroestrol to OVX rats has one-quarter activity of 17 β-estradiol and double activity of estrone on vaginal cornification assay.

Besides miroestrol, kwakhurin, a prenylated isoflavonoid, has been reported to exhibit a moderate estrogenic activity when tested in MCF-7 human breast cancer cells (Chansakaow et al., 2000). Kwakhurin has an estrogenic potency on promoting growth of human breast cancer cells in comparable with that of daidzein (Chansakaow et al., 2000).

Dietary genistein (0.375 mg or 0.75 mg/g of feed) significantly increases uterine weight, inhibits mammary gland regression and increases plasma prolactin level in OVX rats. The effect of phytoestrogens on uterine weight is depended on the kinds and doses of treatment (Ishimi et al., 2000; Power et al., 2006). Subcutaneous injection of genistein (0.7 mg/day) does not alter the histological phenotype of epithelial uterine cells in OVX mice. Genistein administration at 2 mg/day in OVX mice slightly increases the uterine weight, while the dosage of 5 mg/day markedly increases the uterine weight twice of that of the sham-operated mice (Ishimi et al., 2000). Dietary supplementation of soy isoflavones (isoflavones 0.575-1.15 mg/g of dietary protein or isoflavones 0.96-1.92 mg/rat/day) does not increase the uterine weight in OVX rats (Deyhim et al., 2003). Subcutaneous injections of lignans, enterodiol and enterolactone at 10 mg/kg BW/day do not show a hypertrophic effect on uterus in OVX mice, while genistein does (Power et al., 2006). The dietary genistein does not antagonize the action of estradiol in estradiol-supplemented OVX or intact rats (Santell et al., 1997).

The dietary puerarin at 3,000 mg/kg of feed significantly increases uterine weight in OVX rats. Puerarin administration upregulates the expression of three estrogen responsive genes, that is, insulin-like growth factor 1 (IGF-1), progesterone receptor (PR) and complement protein 3 (C3), in uterus (Rachoń et al., 2007).

Besides the effects on reproductive organs, phytoestrogens administered orally in women can modulate the consequences of the postmenopausal estrogen deficiency state. So far, the modulation has been particularly observed in Asiatic populations who consume food products of phytoestrogen-rich plants, such as soy beans. A soy rich diet is efficacious in increasing maturation indices of vaginal cells in postmenopausal women (Chiechi et al., 2003). This consistency of results suggests that reproductive effects of phytoestrogens are the result of "typical estrogenic mechanisms," implying that it is mediated through the estrogen-receptor (Kaldas and Hughes, 1989).

In male, there is very little information on effects of phytoestrogens on reproduction. In theory, excessive exposure to phytoestrogens, as to any other exogenous estrogens, could induce structural and functional alteration in the developing and adult reproductive tract of males. Subcutaneous injection of 2.5 mg/kg BW/day of genistein for nine days in male mice induces the typical estrogenic effects comparable to that of 17 β-estradiol. Genistein reduces testicular and serum testosterone levels, pituitary LH-contents and ventral prostate gland weights in adult intact male mice (Strauss et al., 1998). A marked decrease of seminal vesicle weights in male mice by ORX could not be recovered by subcutaneous injection of 0.8 mg/day of genistein for three weeks or by treatment with 0.03 μg/day of estradiol (Ishimi et al., 2002). Genistein fed to adult male rats for two weeks reduces mRNA expression of AR, ERα and ERβ in the dorsolateral prostate gland. The expressions of AR, ERα and ERβ in rat prostate glands are down-regulated when genistein treatment is at the concentration that is comparable to those found in human soy diets. Down-regulated sex steroid receptor expression may cause a consequence of the lower incidence of prostate cancer in population where their diets contain high levels of phytoestrogens (Fritz et al., 2002).

2.4.2 Estrogenic effects of phytoestrogens on bone

The potential beneficial effects of phytoestrogens on prevention of osteoporosis has been the subject of intensive investigation. The anabolic effect of phytoestrogens on bone is different and dependent on specific kinds and doses of phytoestrognes (Gao and Yamaguchi, 1999; Ishimi et al., 2000; Power et al., 2006). Subcutaneous injection of 0.1 mg/day of genistein in OVX mice does not increase BMD while at a dose of 0.4 mg/day significantly enhances BMD and completely restores bone loss at a dose of 0.7 mg/day (Ishimi et al., 2000). Dietary supplementation of soy isoflavones (isoflavones 0.57-1.15 mg/g of dietary protein or isoflavones 0.96-1.92 mg/rat/day) does not prevent bone loss in lumbar vertebra, tibia, and femur of OVX rats when compares with those of sham control rats (Deyhim et al., 2003). Subcutaneous injections of lignans, enterodiol and enterolactone at 10 mg/kg BW/day do not exert a preventive effect on OVX-induced bone loss in female mice, while genistein at the same dose does (Power et al., 2006). In OVX rats, subcutaneous injection of genistein at 5 μg/day for three weeks can reduce bone loss. Administration of genistein is associated with a higher bone formation rate per tissue volume and trends toward a higher number of osteoblasts (Fanti et al., 1998). Subcutaneous injection of genistein (0.4-0.8 mg/day) for 3 weeks in ORX mice can prevent bone loss in trabecular bone of the distal femoral metaphysis (Ishimi et al., 2002). It suggests that treatment of isoflavone in males can prevent bone loss induced by sex hormonal deficiency. The previous report indicates that genistein has a direct inhibitory effect on bone resorption in rat bone culture (Yamaguchi and Gao, 1998). Levels of bone resorbing factors (parathyroid hormone (PTH), prostaglandin E2, and lipopolysaccharide), which cause a significant decrease in bone calcium content, are reduced completely by genistein (Yamaguchi and Gao, 1998). The inhibitory effect of genistein on PTH-stimulated bone resorption is clearly prevented by tamoxifen, an anti-estrogen reagent (Yamaguchi and Gao, 1998). Genistein has also been demonstrated to cause a significant increase in alkaline phosphatase activity, DNA and calcium contents in cortical bone tissues, and its effect is equal to that of daidzein (Gao and Yamaguchi, 1999).

Consumption of daidzein (10 μg/g BW/day) can also prevent bone loss in OVX rats. When daidzein is given orally for three months to OVX rats, BMDs in

lumbar vertebrae and femur at the metaphyseal and diaphyseal sites (rich in trabecular and cortical bone, respectively) are maintained to the level of sham control rats (Picherit et al., 2000). Daidzein significantly increases the alkaline phosphatase activity, DNA content and cellular protein content in osteoblastic cells, and osteocalcin synthesis by osteoblastic cells. The fact that DNA content in osteoblastic cells is increased significantly by the presence of daidzein, it suggests that the isoflavone stimulates cell proliferation. Alkaline phosphatase and osteocalcin are phenotypic markers for osteoblasts in early and terminal stages, respectively. Thus, daidzein may have a stimulatory effect on the proliferation and differentiation of osteoblastic cells (Sugimoto and Yamaguchi, 2000; Jia et al., 2003). Daidzein has also been demonstrated to cause a significant increase of alkaline phosphatase activity, DNA and calcium contents in cortical bone tissues and its effective dose is higher by about 1,000 times than that of 17 β-estradiol (Gao and Yamaguchi, 1999). The effect of daidzein in elevating cellular protein content and alkaline phosphatase activity is inhibited completely by the anti-estrogen tamoxifen, suggesting that the effect of isoflavone is mediated partly on osteoblasts via the ER (Sugimoto and Yamaguchi, 2000; Jia et al., 2003).

It is well known that the osteoclast originates from bone marrow cell and is responsible for bone resorption (Vaananen et al., 1998). The estrogen/androgen deficiency enhances bone marrow hemopoiesis, resulting in the selective accumulation of pre–B-lymphocytes in bone marrow, and that an increased lymphopoieisis is closely related to bone resorption in the trabecular bone in mice. In OVX female mice as well as ORX male mice, treatment with genistein or estrogen can normalize B-lymphopoiesis to the level that prior to the suppression of bone resorption (Ishimi et al., 2002). These results suggest that estrogen has a crucial role in the regulation of B-lymphopoiesis and bone metabolism in female and male mice, and also that genistein acts on bone and bone marrow, at least in part, by a mechanism similar to that by estrogen in both genders (Ishimi et al., 2002).

2.5 *Pueraria mirifica*, a Thai indigenous herb

Pueraria mirifica (Airy Shaw & Suvatabandhu) is an indigenous herb of Thailand, known as the "White Kwao Krua". Other dialects of *P. mirifica* are Tan-

Jom-Tong, Po-Ta-Goo, and Jan-Krua. It belongs to the Family Leguminosae, subfamily Papilionoidae as for soybean and pea. It is a liana with tuberous roots. Its tuberous root has a round or ellipse-shape and contains whitish starch granules. Flower color is purple-blue (Ingham et al., 2002; van der Maesen, 2002) (Figure 2.7). *P. mirifica* distributes throughout Thailand. The woody perennial climber *P. mirifica* is commonly and abundantly found in forests in the North, West and Northeast of Thailand. The *P. mirifica* is mainly found growing particularly in the deciduous forests of Chiang Mai Province.

For many years, its globular or pear-shaped tuberous roots has been collected in large quantities by local tribesmen, either in Chiang Mai or Bangkok, for processing into various traditional Thai medicines. After being sliced and sun-dried, the tuber powder is typically mixed with honey to give peppercorn-size pills and is taken once a day at night time for 3-6 months to repute a range of remarkable properties, including the ability to induce menstruation in elderly women, and to cause the regrowth of hair in bald men. Use of *P. mirifica* as a possible rejuvenator appears to drawn attention of scientists (Ingham et al., 2002).

Many chemicals possessing estrogenic activities contained in the tuberous root of this plant are determined by HPLC technique (Ingham et al., 2002; Cherdshewasart et al., 2007a). Tuberous root of *P. mirifica* contains at least 13 known phytoestrogens which comprise isoflavone (daidzin, daidzein, genistin, genistein, puerarin, kwakhurin and mirificin) and others such as miroestrol and its derivatives, beta-sitosterol, stigmasterol, puemiricarpene, coumestrol and mirificoumestan (Table 2.2) (Pope et al., 1958; Chansakaow et al., 2000; Ingham et al., 2002). Daidzin, puerarin and puemiricarpene are the main compounds, and other remaining compounds are found at low concentration (Ingham et al., 2002). Chansakaow et al. (2000) reported that 100 g of *P. mirifica* dry powder contains 46.1 mg of daidzein and 2-3 mg of miroestrol and deoxymiroestrol. Muangman and Cherdshewasart (2001) analyzed phytoestrogen contents in *P. mirifica* Cultivar Wichai III by HPLC technique and found that this cultivar contains 169.1 mg total isoflavones/100 g of the dry powder. Cherdshewasart et al. (2007a) also analyzed phytoestrogen content in *P. mirifica* roots collected from 28 out of 76 provinces of Thailand by HPLC technique and reported that the total five isoflavones (puerarin, daidzin, daidzein, genistin, genistein) ranging

over 18.1-198.3 mg/100 g of the dry powder. The high variability of isoflavone contents in *P. mirifica* roots is probably influenced by genetic and environmental factors.

Table 2.2 Phytoestrogen contents in *P. mirifica* (Trisomboon, 2003)

Category	Phytoestrogens	References
Coumestans	Coumestrol	Ingham et al., 1986, 1988
	Mirificoumestan	Ingham et al., 1988
	Mirificoumestan glycol	Ingham et al., 1988
	Mirificoumestan hydrate	Ingham et al., 1988
Isoflavones	Puerarin (daidzein-8-glycoside)	Ingham et al., 1986, 1989
	Daidzein	Ingham et al., 1986
	Daidzin (daidzein-7-o-glycoside)	Ingham et al., 1986
	Genistein	Ingham et al., 1986
	Genistin (daidzein-7-o-glycoside)	Ingham et al., 1986, 1989
	Kwakhurin	Ingham et al., 1986
	Kwakhurin hydrate	Ingham et al., 1989
	Mirificin (puerarin 6′-o-β-apiofuranoside)	Ingham et al., 1986
	Puerarin 6′-monoacetate	Ingham et al., 1989
Lignans	Miroestrol	Jones and Pope, 1960
	Deoxymiroestrol	Chansakaow et al., 2000
	β-sitosterol	Hoyadom, 1971
	Stigmasterol	Hoyadom, 1971

2.5.1 Effects of *P. mirifica* and other plants on reproduction

In female pigeons, *P. mirifica* administration reduces the follicular development and ovulation (Smitasiri and Sakdarat, 1995). *P. mirifica* feeding at doses of 50 and 100 mg/kg BW during midterm of pregnancy in female rats interrupts a pregnancy and has a toxic effect on fetuses (Songkaew and Smitasiri, 1985). It could also prevent a pregnancy in dams when given on the time of embryo transport, but it does not affect the embryo itself during implantation or post implantation periods in rats (Smitasiri et al., 1986). Feeding of 100 mg/kg BW/day of *P. mirifica* reduces mating efficiency in female mice (Jaroenporn et al., 2007). Feeding of crude *P. mirifica* at doses of 100 and 1,000 mg/kg BW/day in OVX rats induces a cornification of the vaginal epithelium, and the effect depends on dosages (Malaivijitnond et al., 2004, 2006; Cherdshewasart et al., 2007b). A single dose of 1,000 mg of *P. mirifica* can significantly prolong the menstrual cycle length in adult cyclic cynomolgus monkeys (Trisomboon et al., 2004). Treatment of lower doses of 10 or 100 mg/day is able to significantly increase the menstrual cycle length if the treatment period is extended to 3 menstrual cycles or 90 days. The menstrual cycle disappears completely when monkeys are fed with 1,000 mg/day of *P. mirifica* for 90 days. Levels of serum follicle stimulating hormone (FSH), luteinizing hormone (LH), estradiol (E2), progesterone, and inhibin are lower during the treatment period, in a dose-dependent manner (Trisomboon et al., 2005). The daily feeding of *P. mirifica* at doses of 100 and 1,000 mg/day also dose-dependently suppresses serum and urine FSH levels (Trisomboon et al., 2006a; 2007) and serum LH levels (Trisomboon et al., 2007) in aged menopausal monkeys. Moreover, the reduced LH and FSH levels can be restored to the pretreatment levels in regard to dosage of the treatment and cessation period (Trisomboon et al., 2006a). *P. mirifica* feeding of 10-1,000 mg/kg BW/day induces sex skin reddening in aged female monkeys (Trisomboon et al., 2006b). Daily intake of *P. mirifica* at the dose of 200 mg alleviates menopausal symptoms in women (Muangman and Cherdshewasart, 2001).

In male pigeons, *P. mirifica* administration inhibits mating behavior and testicular development (Smitasiri and Sakdarat, 1995). *P. mirifica* feeding at doses of 100 and 200 mg/kg BW in male rats significantly decreases epididymal sperm numbers and sperm motility (Langkalichan and Smitasiri, 1985). *P. mirifica*

P. mirifica feeding at the dose of 100 mg/kg BW for eight weeks also reduces mating efficiency in male mice (Jaroenporn et al., 2006). Administration of 1,000 mg/kg BW/day of *P. mirifica* in male rats reduces serum LH levels (Malaivijitnond et al., 2004).

From these numerous reports, it can be concluded that *P. mirifica* has an estrogenic effect on reproductive organs in females as well as in males. However, it has been reported that consumption of phytoestrogen-rich herbs at the uterothophic dose also has an osteoporotic preventive effect (Wang et al., 2003; Zhang et al., 2006; Yin et al., 2006).

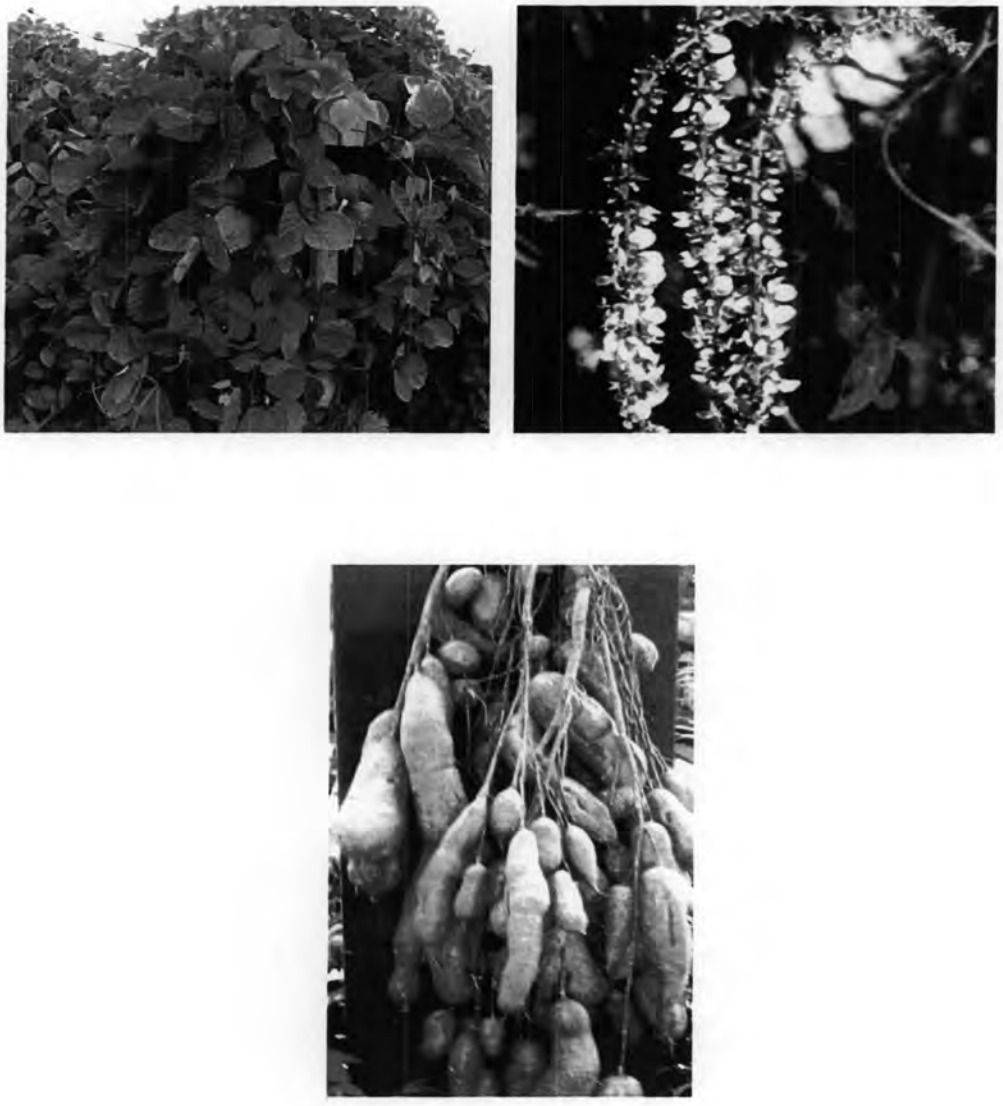


Figure 2.7 The *Pueraria mirifica*: woody climbers, flowers and tuberous roots in which estrogenic compounds are richly contained.

2.5.2 Effects of other phytoestrogen-containing plants on bone

There are many phytoestrogen-containing plants other than the *P. mirifica* which have been studied on their estrogenic effects on bone. The *Pueraria lobata* (Willd.) Ohwi, a wild creeper leguminous plant, is one of the earliest and most important herbs used as a traditional medicine in China for various purposes. Dry powder of the *P. lobata* contains a high amount of isoflavonoids, such as daidzein and genistein, which are known to prevent bone loss induced by estrogen deficiency. The decrease in BMD in OVX mice is significantly prevented by intake diet containing 5% of *P. lobata* for 4 weeks and completely inhibited after intake diet containing 10% of *P. lobata*. Moreover, high dose of *P. lobata* (20% of *P. lobata*) containing in diet fed to OVX mice remarkably increases BMD by 8.2% compared with that in sham mice, without effect on uterine weight (Wang et al., 2003). The ethanol extraction of *P. lobata* has no effect on viability of osteoblastic cells. The *P. lobata* can play an important role in osteoblastic bone formation through dose-dependent increase of alkaline phosphatase activity, marked increase of mRNA expression for osteocalcin, osteopontin, and type I collagen, and induction of mineralization in the culture of human osteoblast-like SaOS-2 cells (Huh et al., 2006).

The *Epimedium Brevicornum* Maxim has been traditionally used as a medicinal herb to treat fractures, bone and joint diseases, and gonad dysfunctions in Asia people for thousands of years. The herb mainly contains three flavonoids: phytoestrogenic compounds (icariin, genistein and daidzein). The previous report shows that oral administration of 10 mg/kg BW/day of flavonoids derived from *E. Brevicornum* (10 mg flavonoids preparation containing 5,000 μg icariin, 250 μg genistein, 1,250 μg daidzein, and 3,500 μg vehicle) for 12 weeks significantly decreases bone resorption and prevents bone loss in OVX rats. The level of urine deoxypyridinoline (Dpd), a bone resorption marker, is significantly decreased and the decrease of BMD of femoral neck is prevented, without hyperplastic effect on uterus (Zhang et al., 2006).

The *Wedelia calendulacea* Less. is found in Uttar Pradesh, Assam, Arunachal Pradesh and Tamil Nadu of India. The plant is used traditionally as cholagogue and deobstruent for hepatic enlargement and jaundice. A decoction of the

herb is used for uterine hemorrhage and menorrhagia. The leaves contain, as its principal constituents, isoflavonoids and wedelolactone, which are analogous in structure to the coumestrol. The findings, assessed on basis of biomechanical (loading, bending and compressing) and biochemical bone testing, and histopathological bone studies, show that the ethanol extract of this plant at doses of 500 and 750 mg/kg BW/day for 90 days has a definite protective effect on bone loss in OVX rats (Annie et al., 2006).

The *Rehmannia glutinosa* Libosch has been used widely as an herbal medicine in Eastern Asian countries for more than 2000 years. Many constituents of this herb have been isolated from the root. The major constituents of the herb are a lignan phytoestrogen, β-sitosterol and mannitol. *R. glutinosa* extraction shows a significant increase in both proliferation and alkaline phosphatase activity of osteoblasts *in vitro*. Osteoprotegerin secretion is markedly increased by *R. glutinosa* treatment. In addition, *R. glutinosa* treatment decreases the tartrate-resistant acid phosphatase-positive [TRAP(+)] multinucleated cell (MNC) formation and the resorption areas after culturing osteoclast precursors. *In vivo* studies using ovariectomy-induced osteoporotic rats reveals that intake of *R. glutinosa* extraction for four weeks alleviates the decrease in trabecular BMD, and increases the cortical bone thickness and trabeculation in bone marrow spaces (Oh et al., 2003).

The *Taxus yunnanensis* Cheng et L.K. Fu (Taxaceae) distributes in Yunnan Province, China. The main constituent of the water extract of this wood is isotaxiresinol, an aryltetralin-type lignan. After oral administration of isotaxiresinol at doses of 50 and 100 mg/kg BW/day for 6 weeks, the BMC and BMD in total and cortical bones are increased as compared to those of OVX control rats, and decreases in bone strength indices induced by OVX surgery are prevented without any side effect on uterine tissue (Yin et al., 2006).

From the reports mentioned above, the phytoestrogen-rich herbs exhibit an anti-osteoporotic effect.