CHAPTER V

DISCUSSION

Osteoporosis is increased dramatically as the life expectancy of humans has been prolonged. Elderly women and men suffering from osteoporosis have been considerable number (Henry et al., 2000; Melton, 2001; Olszynski et al., 2004). Osteoporosis causes pain, diminishes quality of life, decreases physical mobility and independence, inability to work, and increases burden on caregivers who must care for the patient with fractures(Ray et al., 1997; Melton, 2001; Olszynski et al., 2004). Hypogonadism is a well-established risk factor of osteoporosis in women (Insogna et al., 1981; Riggs et al., 1982; Orwoll and Meier, 1986; Harper and Weber, 1998) and men (Francis et al., 1986; Smith et al. 1994; Morishima et al., 1995; Harper and Weber, 1998; Code and Aronson, 2003). Hypogonadism causes both trabecular and cortical osteoporosis and alters trabecular architecture by loss of trabecular numbers, trabecular thickness and trabecular connectivities (Francis et al., 1986).

In women, estrogen deficiency has been recognized as a key factor of osteoporosis development. Estrogen plays an important role in maintaining bone mass in adult women by exerting a tonic suppression of bone remodeling and maintaining the balance between bone formation and bone resorption. Thus, entering the menopause in accompanying with the sudden loss of estrogen could result in a decrease of BMD and BMC (Ohta et al., 2002). The estrogen replacement therapy was proposed to prevent bone loss in postmenopausal women (Turner et al., 1994). However, the higher and the longer exposure to estrogen after menopause is considered as 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 men, age-related reductions in the number of Leydig cells and testosterone levels are associated with bone loss in elderly men. An estimated 5-fold increased risk of hip fractures was seen among elderly men with age-related hypogonadism

(Code and Aronson, 2003; Olszynski et al., 2004). Primary hypogonadism caused by the orchitis or bilateral ORX, and secondary hypogonadism with idiopathic hypogonadotropic hypogonadism or pituitary tumor in men has a progressive loss of the bone density (Baran et al., 1978; Stepan et al., 1989; Behre et al., 1997). Although androgens are effective in bone loss treatment for men (Olszynski et al., 2004), there were reports on side effects such as prostate cancer induction (Soronen et al., 2004). Prostate cancer is one of the most common cancers among men and androgens are involved in controlling the growth of androgen-sensitive malignant prostatic cells (Soronen et al., 2004).

Presently, some accumulated evidence have shown that the loss of bone mass in men related much to estrogen deficiency. In men, the formation of estrogens from androgens is catalyzed by aromatase. Aromatase responsibly converses androstenedione and testosterone to estrone and 17 β-estradiol, respectively (Murata et al., 2002). The enzyme is expressed in several tissues including adipose tissues, chondrocytes and also osteoblasts (Sasano et al., 1997). 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 man with a disruptive mutation of the estrogen receptor gene is associated with a lower bone mass (Smith et al. 1994). In addition, the distribution of the receptors does not vary by gender, as similar levels of ER have been found in bone cells of males and females (Kousteni et al., 2001).

Phytoestrogens, such as genistein, coumestrol, apigenin, naringenin, and kaempferol have a higher affinity for ERβ than ERα (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 (Kuiper et al., 1998). 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 administration of 5 mg/day of genistein induces uterine hypertrophy (Ishimi et al.,

2000). Thus, phytoestrogens are possible to be SERMs (selective estrogen receptor modulators) and ERβ is 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). Additionally, genistein also inhibits the autophosphorylation of the epidermal growth factor receptor, a receptor which is overexpressed in most cancers; acts as antioxidant, thus preventing oxidative DNA damage; and causes changes characteristic of apoptosis, a protective mechanism induced in damaged mutated, or cancerous cells (Mishra et al., 2003).

Our research team have previously analyzed the phytoestrogen contents in *P. mirifica* Cultivar Wichai-III by high performance liquid chromatography techniques (Malaivijitnond et al., 2004; Cherdshewasart et al., 2007a; Trisomboon et al., 2007; present study). The majority of the phytoestrogens analyzed are puerarin (daidzein-8-c-glucoside), daidzin (daidzein glycoside form), daidzein, genistin (genistein glycoside form) and genistein. Puerarin (Li and Yu, 2003) as well as other phytoestrogens including daidzin (Ishida et al., 1998) and daidzein (Gao and Yamaguchi, 1999; Picherit et al., 2000), genistin (Ishida et al., 1998) and genistein (Gao and Yamaguchi, 1999; Ishimi et al., 2002) were proven to have an anabolic effect on bone metabolism and prevented bone loss. However, it is probable that other phytoestrogens contained in *P. mirifica*, which are not mentioned here, also have preventive effects on bone loss. According to the latest information, at least 13 phytoestrogen substances were reported in *P. mirifica* (Chansakaow et al., 2000; Ingham et al., 2002; Cherdshewasart et al., 2007a). Thus, further study on the effects of other phytoestrogens on bone loss is necessary.

5.1 Effects of *P. mirifica* on bone loss in ovariectomized rats

5.1.1 The effects of *P. mirifica* on body weights

In agreement with previous studies reported that (Deyhim et al., 2003; Devareddy et al., 2006; McMillan et al., 2007) the OVX in rats can induce weight gain and treatment of isoflavone or 17 β-estradiol prevented excess weight gain

(Blum et al., 2003; Devareddy et al., 2006). In the present study, the rat body weights after three months of OVX (or P0 group) were significantly higher than that of the SH rats although all rats were fed the same amount of diet as did for the intact rats. Feeding of *P. mirifica* and EE could prevent the OVX-induced weight gain, thus body weights of P10, P100 and P1000 rats were lower than that of P0 rats and the effects were dependent on dosages of *P. mirifica* treatment. The prevention of body weight gain in rats after feeding with *P. mirifica* at the dose 100 mg/kg BW/day is comparable with that of 0.1 mg/kg BW/day of EE feeding. The results indicate that phytoestrogens contained in *P. mirifica* exhibit estrogen-like effect on rat body weights. Fanti et al. (1998) reported that subcutaneous injection of genistein dose-dependently prevented the excess weight gain in OVX rats. Dang et al. (2002) reported that the estrogen deficient state (or OVX) induced body fat accumulation and subsequently caused an increase in body weight and estrogen treatment can lessen its effects. Thus, it can conclude that phytoestrogens in *P. mirifica* involve directly in energy metabolism of rats by binding to ERs within the abdominal and subcutaneous fat (Tchernof and Poehlman, 1998; Joyner et al., 2001; present study).

5.1.2 The effects of *P. mirifica* on serum estradiol levels and uterine weights

Reduction in serum estradiol levels in OVX animals, which were significantly lower than that of the SH rats, indicates that the ovaries were completely removed (McMillan et al., 2007). In this study, serum estradiol levels were decreased after three months of OVX (in P0 rats), and feeding of *P. mirifica* or EE did not change the estradiol levels. Although estradiol levels in OVX rats were detectable, that estradiol hormone did not secreted from ovaries, it was aromatization of androgens to estradiol by peripheral tissues (Simpson, 2003). The low estradiol levels in EE rats indicate no cross-reactivity between the human-estradiol antiserum from the Estradiol RIA kit (DSL-4400, Diagnostic Systems Laboratories, Inc.) used in this study and the synthetic EE fed to the rats.

The uterine weight changes reflect the serum level of endogenous estrogen or its derivatives in animals. OVX significantly produces an estrogen deficient state and consequently causes a decrease in uterine weight in rats (Kippo et

al., 1995; Fanti et al., 1998; Malaivijitnond et al., 2004, 2006; present study). The increase in uterine weight after treatment of chemicals is considered to be a useful index to evaluate the estrogenic activity of those chemicals, so-called a uterotropic assay. The administration of EE was found to increase the uterine weight in OVX rats in the previous reports (Ke et al., 1997; Narayama et al., 2006). The increase in uterine weights of OVX rats in this study indicates that an oral route of EE replacement was effective. Feeding of *P. mirifica* in OVX rats obviously increased uterine weights which depended on dosages and duration of administration. Ishimi et al. (2000) also reported that administration of genistein significantly increased uterine weights in OVX mice which was dependent on the dosages. Malaivijitnond et al. (2006) reported that feeding of 100 or 1,000 mg/kg BW/day of *P. mirifica* for 2 weeks increased uterine weights in OVX rats, but the 10 mg/kg BW/day of *P. mirifica* did not. However, the present study, found that feeding for a longer period (three months) of 10 mg/kg BW/day of *P. mirifica* can significantly increase the uterine weight. On the other hand, feeding of *P. lobata* as high as 1,000 mg/kg BW/day for 2 weeks was not able to observe the increased uterine weight in OVX rats (Malaivijitnond et al., 2006). This can reflect the lower concentration of phytoestrogens in *P. lobata* than in *P. mirifica* used in the present study.

The administration of *P. mirifica* at the highest dose (1,000 mg/kg BW/day) increased the uterine weight to a level higher even than that in the SH rats. From this result, we therefore should be aware of the undesirable side effects on the reproductive organs after the use of *P. mirifica* to prevent bone loss in women if high doses and long-term consumption are used. Thus, further study is necessary to be conducted to determine the appropriate dose of *P. mirifica* for bone loss therapy in humans.

5.1.3 The effects of *P. mirifica* on BMD and BMC

Peak levels of BMD and BMC are used as indicators of the fully bone mature. Trabecular compartments in axial bone and in metaphysis of long bone are fully mature in 4 and 9 months old in rats, respectively (Wang et al., 2001). In the present study, effects of the OVX-induced estrogen deficiency and the

phytoestrogen treatment on bones are found to depend on bone types (axial bone: fourth lumbar vertebra or long bone: tibia and femur), bone sites (metaphysis or diaphysis), and bone compartments (trabecular or cortical). The decreases in trabecular BMDs and BMCs in any bone types varied from 15.15 to 40.9% and 11.99 to 35.72%, respectively. The decreases in trabecular BMDs and BMCs were greater in the metaphysis of long bone than that in the axial bone. Thompson et al. (1995) reported that trabecular BMDs and BMCs at the same site in OVX young rats are decreased greater than that in OVX aged rats. Thus, the growing bone seem to respond to OVX by losing BMDs and BMCs more rapidly than do the aged bone. Different bone sites reach the fully mature stage differently in relation to the advancing age. This may make bones response differently to the sex hormonal deficiency in rats. In the present study, the effects of three month OVX on BMD and BMC were found to be much smaller in cortical compartments than in trabecular compartments. The cortical BMDs and BMCs decreased by as little as 0.68% or less. This confirms that the response of trabecular bones to deficiency or supplement of estrogens is great, while on the other, that in cortical bones is relatively small (Thompson et al., 1995). The mechanism of cortical bone loss is thought to be an increased activation of Haversian remodeling systems accompanied by an increased Haversian canal diameter (Sietsema, 1995). Cortical bone loss is obviously occurred in Haversian remodeling in humans. However, the rat cortical bone displays a low level of Haversian remodeling that is impractical to use for bone loss study (Mosekilde, 1995; Kimmel, 1996). The lower response of rat cortical BMD to OVX was also reported at the tibial diaphysis (Yeh et al., 1996; Yoshitake et al., 1999) and the femoral diaphysis (Ke et al., 1997; Yoshitake et al., 1999).

Changes of BMD and BMC with age also considerably differ at different local bone tissues of rats. BMD and BMC in cortical compartments, both at the metaphyseal and diaphyseal sites of long bones, kept increasing over the age of 7 to 10 months. On the other hand, age-related changes in trabecular compartments of long bones were significant decrease in BMD while those in BMC did not significantly decrease. The continuous growth of cortical bone at the age of 7 to 10 months are a limitation of using rat as an osteoporotic model (Kimmel, 1996). On the other hand, the trabecular bone has an adequate amount of remodeling and it reaches

the maturing stage earlier than the cortical bone. Thus, the use of changes of trabecular for the study of bone loss should be a good indicator (Kimmel, 1996). Furthermore, the rats at 6 to 9 months old rats are a good model for the study of bone loss induced by estrogen deficiency. This model is supposed to have no confounding effects by a rapid bone growth which can be found in young rats (Zhang et al., 1999).

In agreement with the above statement, the strong counteracting effects between *P. mirifica* and OVX on bone loss were found at trabecular compartments in this study. A similar result was obtained for *P. lobata*, another phytoestrogen-containing herb, tested in OVX mice (Wang et al., 2003). In the present study, *P. mirifica* also increased cortical BMD and BMC in OVX rats. The anabolic effects of daidzein and genistein on cortical compartments were previously reported in cortical bone culture of female rats (Gao and Yamaguchi, 1999).

The preventive effect of *P. mirifica* on bone loss is depended on dosages. At middle and high doses (P100 and P1000), a complete prevention was found in both BMD and BMC. The effect of 100 mg/kg BW/day of *P. mirifica* is, as expected, comparable to that of 0.1 mg/kg BW/day of EE. An increase in BMD and BMC found in a high dose of *P. mirifica* was also reported for *P. lobata* in an equal degree of response (Wang et al., 2003).

5.2 Effects of *P. mirifica* on bone loss in orchidectomized rats

5.2.1 The effects of *P. mirifica* on body weights

Compared to day 0, the body weight at day 90 of SH rats significantly increased and were higher than that of the other groups. Although rats with three month ORX (or P0 rats) exhibit weight gain, the body weight was still lower than that of SH rats which is similar to previous reports (Iwamoto et al., 2002; Venken et al., 2005). Androgens, including testosterone, increase the food intake and it is a potent anabolic steroid (Earley and Leonard, 1979). Thus, ORX causes a slow increase in body weight (Earley and Leonard, 1979) and ORX also decreases the lean body mass (Venken et al., 2005). In contrary, estrogen is a potent depressor of food intake, body

weight and body weight gain in ORX rats (Earley and Leonard, 1979; Iwamoto et al., 2002) and SH rats (Brewster et al., 1997; Hossaini et al., 2003). Estrogen also exhibits some catabolic activities (Earley and Leonard, 1979). Suppression of body weight gain in intact rats by estrogen administration depends on dosage (Brewster et al., 1997). Therefore, treatment with EE and *P. mirifica* for three months to the ORX rats in the present study decreased the rat body weight, comparing between day 0 and day 90.

5.2.2 The effects of *P. mirifica* on serum testosterone and estradiol levels, seminal vesicle and prostate gland weights

In the present study, serum testosterone levels before ORX (at day 0), ranging 2.0 - 4.9 ng/ml were similar to the previously reported levels in young rats, ranging 1.5 - 3.6 ng/ml (Brewster et al., 1997). Serum testosterone levels at day 90 in SH rats were lower than at day 0, however, this low level was still significantly higher than those of other five groups of ORX rats. It indicates that testosterone levels decrease with advancing age, from 7 to 10 months old, in male Sprague Dawley rats. The undetectable testosterone levels of all ORX rats indicate the successful orchidectomy.

The significant level of estradiol determined in ORX rats indicates the extragonadal sites of estradiol synthesis (Simpson, 2003). Androgen is one of steroid hormones which can be converted in peripheral tissues to estradiol before releasing into the blood circulation (Simpson, 2003). In the present study, serum estradiol level in 10 months old SH male rats (16.8 pg/ml) was comparable to the OVX rats (15.7-21.5 pg/ml) ($p > 0.05$), but serum estradiol levels in ORX rats (10.6-17.1 pg/ml) was slightly lower ($p = 0.004$). These comparable levels of estradiol were also found in humans, that is, estradiol levels in adult men (0.1 nmol/l) were similar to the postmenopausal women (0.04 nmol/l) (Simpson, 2003). Serum estradiol levels at day 90 in SH rats were lower than at day 0. It indicates that estradiol levels decrease with advancing age, from 7 to 10 months old, in male Sprague Dawley rats. The low estradiol levels in EE rats indicate no cross-reactivity between the human-estradiol

antiserum from the Estradiol RIA kit (DSL-4400, Diagnostic Systems Laboratories, Inc.) used in this study and the synthetic EE fed to the rats.

Feeding of *P. mirifica* and EE did not recover sex organ weights, seminal vesicles and ventral prostate glands, which were greatly decreased by ORX (Vanderschueren et al., 1992; Venken et al., 2005). Similar results were reported in ORX mice treated with *P. lobata* (Wang et al., 2005) or genistein (Ishimi et al., 2002) or in ORX rats treated with estrogen (Seidlov'a-Wuttke et al., 2005). These results suggest that the use of *P. mirifica* for curing osteoporosis caused by hypogonadism in men is unlikely to increase the risk of prostate cancer. Moreover, some previous researchers reported that phytoestrogens have anti-prostate cancer effect (Wang et al., 2002).

5.2.3 The effects of *P. mirifica* on BMD and BMC

Changes of BMD and BMC in relation to age was examined in the subject rats, ranging over 7 to 10 months old. In this study, age change patterns were depended on bone types (axial bone and long bone) and bone compartments (trabecular and cortical). The cortical compartment of long bones at both metaphyseal and diaphyseal sites showed an increase of both BMD and BMC with advancing age of rats. Change of long bone in relation to age was also reported in rats aged less than 10 months (Wang et al., 2001; Banu and Kalu, 2004). On the other hand, the trabecular BMD of L4 and tibial metaphysis were stable, neither increased nor decreased, except that in the femoral metaphyseal site decreased. The axial bone, the 4th lumbar vertebral body, did not change during the experimental period, in both BMD and BMC, and both in trabecular and cortical compartments (Wang et al., 2001; present study). Although rats have a continuous growth throughout their life-span, at 6-9 months old most indices of bone mass reach a plateau or slowly change, especially in trabecular bone (Ke et al., 1996). Using rats in this age is supposed to have no confounding effects from a rapid bone growth as found in young rats (Zhang et al., 1999).

The responses of bone to ORX are depended on bone types, bone sites, and bone compartments. Although the methods for measurement of BMD and BMC

are different between studies, the results obtained in the present study using pQCT could differentiate the BMD and BMC values between trabecular and cortical compartments. The trabecular BMD was severely decreased at the metaphyseal site of tibia (Seidlova-Wuttle et al., 2005; present study) and femur (Venken et al., 2005; present study) in ORX rats. However, much smaller effects of ORX were found on cortical BMD and BMC in various bones. The decreases of trabecular BMD measured at metaphyseal and diaphyseal sites in tibia, femur, and lumbar vertebra body ranged 27-34% in the present study. On the other hand, those of cortical BMD ranged as small as 0–2.8%. Venken et al. (2005) also reported a diminutive decrease in cortical BMD at the metaphyseal site in femur. The mechanism of cortical bone loss is thought to be an increased activation of Haversian remodeling system accompanied by an increased Haversian canal diameter (Sietsema, 1995). Cortical bone loss are clearly occurred in Haversian remodeling in humans. However, rats are less satisfactory species for bone loss study in cortical bone because they do not exhibit much Haversian remodeling (Mosekilde, 1995; Kimmel, 1996). Thus, trabecular bone, which has an adequate amount of bone remodeling, is a useful for osteoporotic study (Kimmel, 1996).

The preventive effect of *P. mirifica* on BMD and BMC at both trabecular and cortical compartments of ORX rats was significant and comparable to that of EE. Although the ORX procedure induced an androgen-deficient stage as reported by Vanderschueren et al. (1992), the direct substance controlling the bone metabolism seems to be estrogen. The preventive effect of *P. mirifica* on bone loss in ORX rats was also found in *P. lobata* treated ORX mice as well (Wang et al., 2005).

The preventive effect of *P. mirifica* on bone loss induced by ORX was dose-dependent; ranging from prevention, maintenance, and increase the BMD and BMC. The highest dose of *P. mirifica* did not only completely prevent bone loss but it also increased the BMD to a value higher than that of the SH rats, which was similar to the *P. lobata* effects (Wang et al., 2003, 2005). The dose of *P. mirifica* on the prevention of bone loss, which is comparable to the preventive effect of 0.1 mg/kg BW/day of EE, is 100 mg/kg BW/day.

The effect of *P. mirifica* in the prevention of bone loss depends on quantities and kinds of phytoestrogens. Based on the high performance liquid chromatography technique, Malaivijitnond et al. (2004), Trisomboon et al. (2007) and the present study determined phytoestrogen contents in *P. mirifica* Cultivar Wichai-III and found puerarin (daidzein-8-c-glucoside), daidzin (daidzein glycoside form), daidzein, genistin (genistein glycoside form) and genistein. The puerarin was the major isoflavone in *P. mirifica* as found in *P. lobata* (Prasain et al., 2004). The isoflavones, which acquired anabolic effects on bone metabolism, were not only puerarin (Li and Yu, 2003), but other phytoestrogens, such as daidzin (Ishida et al., 1998), daidzein (Gao and Yamaguchi, 1999; Picherit et al., 2000), genistin (Ishida et al., 1998), and genistein (Gao and Yamaguchi, 1999; Ishimi et al., 2002) also did. It is probable that some other phytoestrogens which do not mention above and contain in *P. mirifica* may be attributable in bone loss prevention, because there are at least 13 phytogenic substances identified in *P. mirifica* (Chansakaow et al., 2000; Inghamet al., 2002). Thus, further studies on what is the effective contents in *P. mirifica* on bone are needed.

5.3 The effect of *P. mirifica* on TbBMD reduction comparing between OVX and ORX rats

In the present study, the preventive effect of *P. mirifica* on bone loss is particularly observed at trabecular compartments in both sexes of gonadectomized rats. Only the preventive effect of the lowest dose (P10) of *P. mirifica* on decrease of TbBMD depends on sexes. In ORX rats, P10 could not prevent the decrease of TbBMD on the long bone (tibial metaphysis and femoral metaphysis), while it prevented a decrease in BMD by 50.8% in L4 of ORX rats. In contrast, the preventive effects of P100 and P1000 on decreases of TbBMD did not differ between OVX female and ORX male rats.

5.4 Isoflavone contents in rodent diets and *P. mirifica*

Extraction of phytoestrogens from plants and rodent diets has been done by water (Rostagno et al., 2004), 30-80% methanol (Rostagno et al., 2004;

Cherdśhewasart et al., 2007a) and 30-80% ethanol (Rostagno et al., 2004). Rostagno et al. (2004), however, reported that 70% ethanol was the proper extraction solvent not only because of its superior efficiency but also because of its lower cost and toxicity (superior environmental compatibility) than methanol. Thus, 70% ethanol was used here and could obtain a high efficiency in the first extraction (>70% efficiency). However, a better result with a reasonable cost was obtained after two time extractions (91.2-96.4% of efficiency).

The standard rodent diet that is widely used in Thailand (C.P. 082) does not contain puerarin, a specific isoflavone of *Pueraria* herbs; however, it has considerably high amounts of other isoflavones: daidzin, genistin, daidzein and genistein. The total amount of these four isoflavones varied from 38.6-72.4 mg/100 g of diet. This concentration range was comparable to those reported in the US and German rodent diets (10-54 mg/100 g of diet) (Degen et al., 2002). The major isoflavones found in soybean-based rodent diets (C.P. 082) were glycoside isoflavones, daidzin and genistin, accounting for 82-86% of the total isoflavones. The high contents of glycoside isoflavones were also reported for the US rodent diet, at 70-72% of total isoflavones (Brown and Setchell, 2001).

The high isoflavone contents in standard rodent diets should be taken into consideration when studying the estrogenic effects of chemicals using rats as experimental animals. For example, if the effects of genistein were examined, the soybean-free diet (C.P. 082/SBF), with no detectable genistein as in the present study, should be used. On the other hand, if the researchers want to assess the effects of puerarin, the standard rodent diets (C.P. 082), with no puerarin could be used to feed the rodents.

With regard to our results, the daily isoflavone intakes of adult rats fed with the standard rodent diets (C.P. 082) were considerably high. Fully mature adult female rats (100 days of age) are 230-270 g in body weight (Malaivijitnond et al., 2004), and are generally fed 15 g of rodent diet per day (Kijkuokool et al., 2006). Thus, they receive 21.5-40.2 mg/kg BW/day of isoflavones (or 6.8-14.6 mg/kg BW/day of daidzin and 11.5-21.4 mg/kg BW/day of genistin). These amounts of

daidzin and genistin content in daily consumption of laboratory rat diets are high compared with the effective oral dose in preventing bone loss of 50 mg/kg BW/day of daidzin or genistin (Brown and Setchell, 2001). Likewise, the amounts of daidzein and daidzin (glycoside form of daidzein) content in rat diets, which are consumed daily by laboratory rats at 9.3-20.2 mg/kg BW/day, were typically higher than the effective oral dose of daidzein in preventing bone loss, 10 mg/kg BW/day, in OVX rats (Picherit et al., 2000). In addition to daidzein, the amount of genistein (0.2-0.8 mg/kg BW/day) from the standard rodent diets was considerably higher than the dose of genistein, 0.1 mg/kg BW/day, that caused a significant increase of the bone calcium content in aged female rats (Gao and Yamaguchi, 1998). Therefore, the standard rodent diet available in Thailand contains enough amount of isoflavones to prevent the bone loss caused by endogenous estrogen deficiency. It should also be emphasized that OVX rats, the preferable animal model for bone loss study, are hyperphagia (Breitman et al., 2003) and consume a larger daily amount of isoflavones in general. In the present study, the soybean-free diet was found to contain a very low concentration of isoflavones (6.1 mg/100 g of diet), and therefore, is a recommended diet for experimental rodents when estrogenic effects are studied.

P. mirifica Cultivar Wichai-III is a standard cultivar that has been thoroughly examined for its estrogenic effects on reproductive organs (Malaivijitnond et al., 2004, 2006; Trisomboon et al., 2004, 2005, 2006a, 2006b; Cherdshewasart et al., 2007b), fertility (Jaroenporn et al., 2006) and bones (Urasopon et al., 2007). The total isoflavone concentration in *P. mirifica* Wichai-III obtained in the present study (123.2 and 157.3 mg/100 g of *P. mirifica*) was comparable to that reported by a previous study (Malaivijitnond et al., 2004; 187.1 mg/100 g of *P. mirifica*) and in the range of other cultivars of *P. mirifica* collected in Thailand (18.61-198.29 mg/ 100 g of *P. mirifica*, Cherdshewasart et al., 2007a). Cherdshewasart et al. (2007a) reported that *P. mirifica* collected from different locations (so-called different cultivars) during March – April showed a high variation in those five isoflavone contents, which was considered to be influenced by climate and genetics. This study confirms that the two lots of *P. mirifica* Cultivar Wichai-III, although having the same genetic background and cultivated in the same location (Chiang Dao District, Chiang Mai Province) but collected on different days, still shows a difference in isoflavone contents. This

difference can be interpreted as the intra-cultivar (or inter-individual roots) variation. However, the variation between individual roots is lower than the variation between the cultivars (or inter-cultivar variation). Therefore, the products made of *P. mirifica* collected from different locations, i.e., different cultivars, or even from different roots should be calibrated for their isoflavone content.

It was reported that *P. mirifica* at the dose of 100 mg/kg BW/day showed a significant estrogenic effect, that is, inducing a cornification of the vaginal epithelium and an increase of uterine weight (Malaivjitnond et al., 2004, 2006; Cherdshewasart et al., 2007b) in OVX rats fed with a standard rodent diet (C. P. 082). This effective dose of *P. mirifica* is equivalent to 0.12 – 0.19 mg/kg BW/day of the five isoflavones measured in the present study. The isoflavone intake (21.5-40.2 mg/kg BW/day) from the standard rodent diet found in the present study is therefore much higher than that from *P. mirifica* treatment. However, the OVX rats fed only a standard rodent diet (C. P. 082) did not show the vaginal cornification or increase in uterine weight (Malaivjitnond et al., 2004, 2006; Cherdshewasart et al., 2007b). Thus, it is possible either that the estrogenic effects that occurred are due to the isoflavones in *P. mirifica* combined with the isoflavones in the rodent diet, or due to the puerarin isoflavone found in *P. mirifica* which is undetectable in the rodent diet in the present study or in the previous report (Cherdshewasart et al., 2007a). It is also probable that other phytoestrogens that we have not determined here, e.g., miroestrol (Jones et al., 1960) could play a role. It was reported that the *P. mirifica* tuberous root contains at least 13 known substances classified as phytoestrogens (Pope et al., 1958; Chansakaow et al., 2000). Although other phytoestrogens, e.g., puemiricarpene, tuberosin and coumestrol are not quantified here, they were reported to be of a low level (Chansakaow et al., 2000).

In conclusion, the findings from the present study illustrate the importance of control of diet back-ground phytoestrogens in the researches of phytoestrogens and/or estrogens, which could be a confounding factor, especially, in research on estrogenic effects of phytoestrogen-rich herb in Thailand.