Chapter II

Literature Review

2.1 Phytoestrogens

Phytoestrogens are a broad group of plant-derived substances that behave as endogenous estrogens. There are three major classes of phytoestrogens: isoflavones, coumestans, and lignans (Knight and Eden, 1996; Murkies et al., 1998) (Table 2.1).

2.1.1 Sources of Phytoestrogens

Phytoestrogens are found in plants and plant products. Lignans are highest detected in oilseed such as flaxseed and lower in cereal bran, whole cereals, vegetables, legumes, and fruits. Isoflavones are predominantly found in soybeans, chick peas, and other legumes. Coumestans occur at high concentrations in clover, alfalfa, and soy sprout and at lower concentrations in sunflower seeds, soybeans, soy flour, soy flakes, and tofu (Franke et al., 1995; Colborn, Dumanaski, and Myers, 1996; Murkies et al., 1998). Each plant, however, often contains more than one type of phytoestrogens (Murkies et al., 1998).

2.1.2 Biotransformation of Phytoestrogens

The major isoflavones, genistein and daidzein, are commonly found in the bound form as glycosides, genistin and daidzin, which are biologically inactive. They are derived from precursors, biochanin A and formononetin, which are broken down by intestinal bacteria into glucosides and then, converted to genistein and daidzein, respectively. Genistein is further partially metabolized to estrogenically inactive p-

Table 2.1 Phytoestrogens contents in *P. mirifica* (Adapted from Panriansaen, 2000)

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	Daidzein	Ingham et al., 1986
	Daidzin (daidzein-7-o-glucoside)	Ingham et al., 1986
	Genistein	Ingham et al., 1986
	Genistin (genistein-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 (daidzein-8-glucoside)	Ingham et al., 1986, 1989
	Puerarin 6"-monoacetate	Ingham et al., 1989
Lignans	Miroestrol	Bound and Pope, 1960; Jones and Pope, 1960
	Deoxymiroestrol	Chansakaow et al., 2000
	β-sitosterol	Hoyadom, 1971
	Stigmasterol	Hoyadom, 1971

ethylphenol. Daidzein, is also metabolized to estrogenically inactive substances, equol and O-desmethyllangdensin (O-DMA), respectively. The estrogenically active lignans, enterodiol and enterolactone, are derived from the precursors, secoislariciresinol and matairesinol, found in the aleuronic layer of the grain close to the fiber layer (Knight and Eden, 1996; Head, 1997; Murkies et al., 1998; Liggins, Grimwood, and Bingham, 2000). In humans, after the consumption of lignans and isoflavones, complex enzymatic conversions occur in the gastrointestinal tract resulting in the formation of heterocyclic phenols with a close similarity in structure to estrogens. Absorbed phytoestrogen metabolites undergo enterohepatic circulation and may be excreted in the bile and deconjugated by the intestinal flora, reabsorbed and reconjugated with glucuronic acid in the liver as in the case of endogenous estrogens, and then excreted in urine (Knight and Eden, 1996; Head, 1997; Murkies et al., 1998). After consumption of soy, free and total genistein and daidzein are rapidly cleared up from plasma. Half-lives of genistein and daidzein are 7 and 4 hour in women and 4 and 3 hour in men, respectively (Lu and Anderson, 1998; Busby et al., 2002). Phytoestrogens and their metabolites are detected in urine, feces, blood, and amniotic fluid (Xu et al., 1995; King, 1998; King and Bursill, 1998; Adlercreutz et al., 1999; Liggins et al., 2000), and their levels are depended on the amount of phytoestrogen consumption (Karr et al., 1997; Setchell et al., 1980; Lampe, 2003).

2.1.3 Binding of Phytoestrogens on Estrogen Receptors

Phytoestrogens have the chemical structures and functions similar to those of endogenous estrogens (Petrakis et al., 1996). Chemical structure of estrogens; estrone, estradiol, and estriol, is a C-18 steroid with four fused and non-polar rings (rings A – D) and has an aromatic ring A and a phenolic hydroxy group at position 3 (Smith et al., 1983; Voet, Voet, and Pratt, 1999). The nonsteroidal structures of phytoestrogens, which present phenolic ring structure, are closely related to estrogens (Figure 2.1). When both structures of phytoestrogens and estradiol are

superimposed, the distance between the hydroxy groups of both molecules is identical. Based on the structural similairties, phytoestrogens can bind to estrogen receptor (ER) (Setchell, 1998; Hopert et al., 1998) and act as a weak estrogen (Setchell, 1998).

Many biological effects of phytoestrogens occur by the direct interaction with ER at the target sites. The study on the interaction of phytoestrogens with ER in human breast cancer cell line, MCF7, found that both coumestrol and genistein can bind ER in the cytoplasm and translocate to the nucleus (Martin et al., 1978). Phytoestrogens were found to inhibit the binding of estrogen to uterine receptor (Shutt and Cox, 1972). From the study of the binding affinity of the receptors in sheep uterine cytosol, miroestrol and coumestrol, which had the highest estrogenic potency *in vivo*, had the relative molar binding affinity of only about one third of estrone and five percent of estradiol (Shutt and Cox, 1972). This evidence indicated that phytoestrogens exhibit biological effects by binding to ER but have less affinity than endogenous estrogens (Shutt and Cox, 1972; Santell et al., 1997). Tang and Adams (1980) also found that equol, a more potent isoflavone that is converted from daidzein, is antagonistic to estradiol by competition for ER complex. Accordingly, these phytoestrogenic substances can compete for ER and fail to stimulate a full estrogenic response after binding to the receptor.

Estrogen receptors have been classified into two types (ERα and ERβ). Genistein and daidzein are complete agonists with estrogens at rat ERα and ERβ *in vitro*, are more potent binding with ERβ than ERα (Kuiper et al., 1997; Casanova et al., 1999). Both receptor types (α and β) express in many tissues with different contents. ERα expresses mainly in the uterus, testis, ovary, kidney, epididymis, and adrenal, whereas ERβ expresses in the prostate, ovary, lung, bladder, brain, uterus, testis, kidney, mammary gland, and bone cells (Kuiper et al., 1997; Gustafsson,

1999; Weihua et al., 2000). Onoe et al (1997) studied the expression of ERβ mRNA in rat osteoblastic cells using PCR technique, and found that ERβ mRNA was slightly detected in the primary osteoblastic cells, and the concentration of ERβ mRNA gradually increased during the differentiation of immature osteoblast into mature osteoblasts.

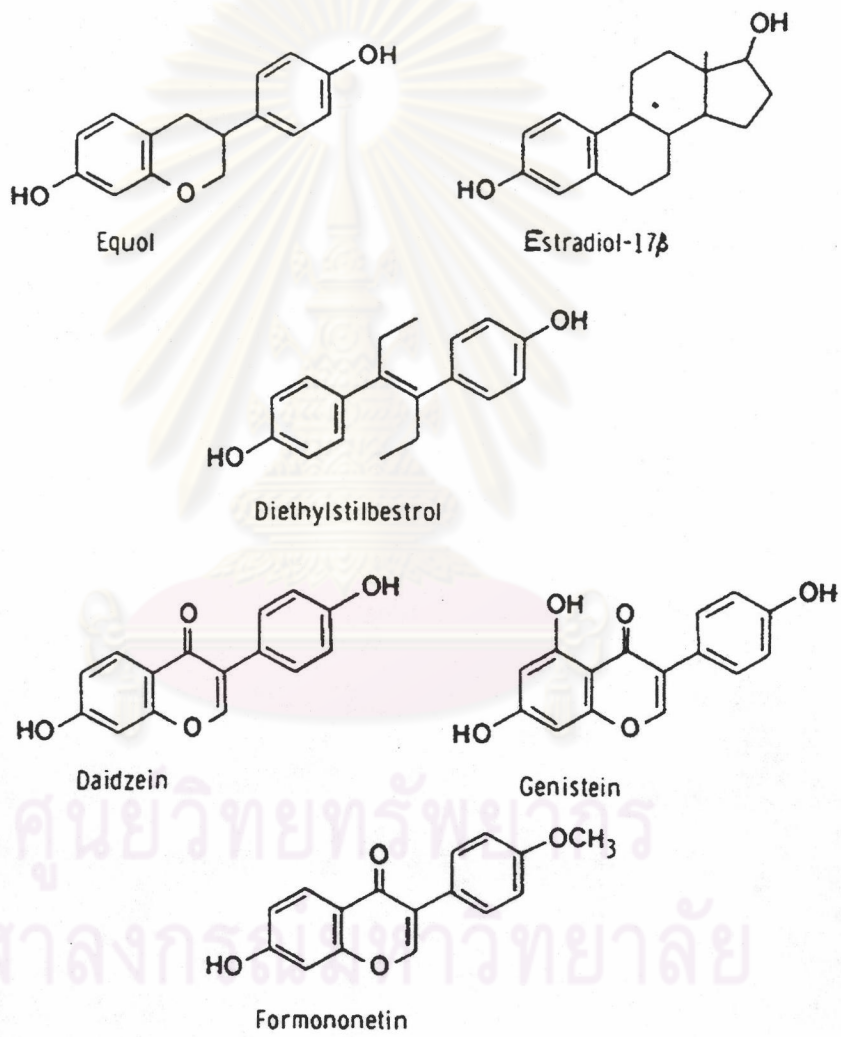

Figure 2.1 A comparison of the chemical structures of phytoestrogens, estradiol, and diethylstilbestrol, synthetic estrogen (Murkies et al., 1998)

2.2 Estrogen

Estrogen is a group of substances, which is a female hormone that mainly regulates reproductive systems. Estrogen cooperates with pituitary gonadotropins, both FSH and LH, to stimulate and regulate reproductive organs and their functions throughout the reproductive life.

2.2.1 Role in the Menstrual Cycle

In humans as well as in non-human primates, the reproductive cycle or so-called menstrual cycle can be divided into 2 phases: follicular and luteal. In follicular phase, after the cessation of previous menstruation, a particular follicle begins to enlarge in the ovary under the influence of FSH. FSH stimulates growth and development of the follicle. Estradiol levels are low during the early follicular phase, but, in response to the stimulation by FSH, the granulosa cells that surround the follicle synthesize estrogen. The increased synthesis of estradiol by the granulosa cells results in a rise of serum estradiol levels. Estradiol reaches to the maximal level 24 hours before the peak level of LH. The high level of estradiol stimulates further GnRH secretion from the hypothalamus in a positive feedback mechanism and then, the latter increases the secretion of FSH and LH from the pituitary gland. Serum progesterone levels are very low during the follicular phase. The LH rise and peak herald the end of the follicular phase and precedes the ovulation by 16 - 18 hours. The follicular phase length is approximately 12 $\pm$ 2 days in humans (Johnson and Everitt, 1995).

In luteal phase, immediately following the release of ovum from the follicle or ovulation, the granulosa cells around the ruptured follicle drive luteinization and form the corpus luteum. LH is necessary for the formation and maintenance of the corpus luteum. Corpus luteum secretes a large amount of progesterone and a smaller amount of estrogen, increasing serum levels of these hormones. The high levels of

serum estradiol and progesterone turn to inhibit both GnRH secretion from the hypothalamus and FSH and LH secretion from the anterior pituitary gland. Serum levels of FSH and LH fall thereafter. As serum FSH and LH levels are low during the luteal phase, the follicular growth diminishes and no new follicle begins to grow in the ovary. Progesterone is required for preparation and maintenance of the secretory endometrium that provides early nourishment for the implanted blastocyst. In the absence of implantation, the corpus luteum regress and menstruation ensues; after the endometrium is shed called menstruation, a new cycle commences. The luteal phase is always 14 $\pm$ 2 days in length. Variations in menstrual cycle length are almost always due to an altered follicular phase (Smith et al., 1983; Granner, 1988; Rhoades and Pflanzer, 1996).

2.2.2 Role in Bone Metabolism

Estrogens act to conserve bone mass and have an anabolic effect on bone. At the tissue level, estrogens tonically suppress bone turnover and maintain balance rates of bone formation and bone resorption. At the cellular level, estrogens affect the generation, lifespan, and functional activity of both osteoclasts and osteoblasts. Estrogen deficiency induces bone loss and osteoporosis in postmenopausal women. Although the exact mechanism has not been elucidated yet, one mechanism of bone loss induced by estrogen deficiency is the increase secretion of Parathyroid hormone (PTH), Mckenna and Frame, 1987; Granner, 1988). PTH, a bone-resorbing factor, has multiple effects on bone and apparently influences several types of bone cells. However, the net effect of PTH is bone destruction, with the concomitant release of calcium, phosphate, and organic matrix elements, including collagen breakdown products from osteoclasts. In addition, low concentration of PTH stimulates the differentiation of osteoclast cells. However, PTH also has an anabolic effect (bone formation). PTH can increase osteoblast cells and increase the alkaline phosphatase

activity that reflects new bone formation (Mckenna and Frame, 1987; Granner, 1988; Riggs, Khosla, and Melton, 2002).

2.3 Estrogenic Effect of Phytoestrogens

2.3.1 Effects on Reproduction

Miroestrol, which has a nonsteroidal structure with a heterocyclic fused ring, is phytoestrogenic substance that found only in the root of *P. mirifica* (Pope et al., 1958; Bounds and Pope, 1960). Pope and his coworkers (1958) found that miroestrol has a functional similarity to endogenous estrogens in intact ovaries rats and mice. This isolated substance prevented the implantation of the blastocyst in the normal inseminated female rats, promoted uterine and vaginal growth, increased uterine weight as estradiol and augmented the amount of fluid in the uterine of immature mice. It also produced cornification of the vaginal epithelium in the ovariectomized and ovariectomized-adrenalectomized rats (Jones and Pope, 1960). It did not, however, stimulate the secretion of endogenous estrogen by the ovaries or adrenal glands (Jones and Pope, 1960).

Besides, in rats, miroestrol had about half the activity of estradiol in reducing the body weight gain after ovariectomy. It exhibited mammogenic potency in both the ovariectomized rats and mice by restoring the mammary duct growth in the same manner as estradiol did. When miroestrol was given by subcutaneous injection, its potency is about 0.7 times of estradiol in ovariectomized rats, 2.2 times of estrone in mice on mammogenic activity (Benson, Cowie, and Hosking, 1961), and about 1.2 and 1.25 times of estradiol on the mouse uterine-weight and rat vaginal smear tests, respectively (Kashemsanta et al., 1957). Miroestrol given subcutaneously in multiple doses was as potent as estradiol and given orally was potent three times higher than

stilboestrol, one type of phytoestrogens in increasing the uterine weight in the immature female mice (Jones and Pope, 1960).

Other phytoestrogens from soy, soy diet, and other legumes also have been reported about the estrogenic activities (Fredricks et al., 1981, Phipps et al., 1993; Drapper, 1997). In the 1940s, the estrogenic effect of plant phytoestrogens was first described as crucial importance with the outbreak of infertility sheep grazing on pasture rich in subterranean clover (*Trifolium subterraneum L.*), an annual plant in Western Australia, later known as "clover disease" (Bennetts, Underwood, and Shier 1946). Prolonged exposure to such pasture, ewes ate great amount of coumestans and isoflavones, which caused a permanent infertility and reduced the ovulation rate in ewes because of a reduction of the viscoelasticity of cervical mucus, morphological changes of the cervix, and increased amount of mucus production. These phytoestrogens also caused a spermatozoa loss from the cervix and reduced the chance of conception (Smith et al., 1979; Adams, 1995). Offspring male rats receiving coumestrol via mother's milk during the critical period reduced the frequency of sexual behavior. Although the testicular weight or plasma testosterone levels wee not affected (Whitten et al., 1995), they showed a reduction of frequency both in mount and in ejaculation and a prolonged latency to mount and ejaculation. With the same protocol, female rats exhibited a persistent cornification of vaginal epithelia resembling the premature anovulatory syndrome and failed to elicit the LH elevation after estradiol stimulation (Whitten, Lewis, and Naftolin, 1993; Whitten et al., 1995). Coumestrol injection during the neonatal period reduced the primary and primordial follicles and increased the number of large maturing follicles and graffian follicles in rats (Sheehan, Medlock, and Burroughs, 1996). When the trial of phytoestrogenic effect was conducted in female mice, coumestrol isolated from alfalfa induced the reduction of ovulation rate as well as increased the incidence of embryo degeneracy in a dose dependent manner (Fredricks et al., 1981).

Phytoestrogenic substances also play a role in disturbing hormonal levels in various vertebrates. Both male and female goldfishes exposed to β-sitosterol had a reduction of plasma steroids concurrence with an increase in GtH-I, gonadotropin II, which has a high degree of homology with LH, similar to the result of estrogenic effect. An increase in GtH-I after reduction of plasma steroids may be due to a decrease in gonadal steroid biosynthesis capacity rather than to an inhibition of gonadotropin secretion from the pituitary (Maclatchy and Kraak, 1995). From the study in mammals, coumestrol diet from lucerne decreased the amplitude of LH pulses in sheep during the breeding season (Montgomery et al., 1985). All doses of β-sitosterol and coumestrol, except a high dose of coumestrol (10 μg), significantly increased basal LH and GnRH-induced LH releases in both castrated male and female rats that receiving these substances during neonatal period (Register et al., 1995). This phenomenon was concomitant with the adult castrated female rats that received genistein during prenatal period (Levy et al., 1995).

There has recently been a great deal of interest in the hormonal effect of soy phytoestrogens in human. Most studies have been done in women, especially premenopausal women, but only a few studies in postmenopausal women. The interest in hormonal effects of soy on premenopausal women has centered mainly in the potential benefits on estrogen-dependent cancers such as breast cancer. It has been believed that a low level of endogenous estrogen correlated with a low incidence of breast cancer in premenopausal women. Estrogen replacement treatment on postmenopausal women are regarded as the risk factor of such cancers. Outcome of soy phytoestrogen consumption on endogenous estrogen levels have been inconsistent among those reports, even though there are trend toward the notion that it decrease the estrogen concentrations (Wu et al., 2000). Previous reports demonstrated that isoflavones from a soy diet altered gonadotropin levels after a daily consumption and increased the length of menstrual cycle in normally

cycling women, although there have been conflicting data (Cassidy et al., 1994, 1995; Duncan et al., 1999; Lu et al., 1996, 2000; Kurzer et al., 2000). Consumption of soy isoflavones caused a statistically significant reduction in serum luteal estradiol levels in women, but there were no significant changes in concentration of follicular phase estradiol, luteal phase progesterone, sex hormone binding globulin (SHBG), or length of menstrual cycle (Wu et al., 2000).

2.3.2 Effects on Bone

Osteoporosis is an important metabolic bone disease affecting women with ovarian deficiency. Hormonal replacement therapy (HRT) with estrogen should theoretically be the best choice for the prevention and treatment of postmenopausal osteoporosis. Previous investigation was demonstrated that HRT is effective not only in prevent the rapid bone loss in women, but also in retarding the loss of bone mass occurring in menopause (Gennari et al., 1987). However, many women concern about the side effect of estrogen, mostly breast and endometrial cancers. Many women interest in a nonhormonal therapy, particularly the phytoestrogens, for the prevention of osteoporosis.

Phytoestrogens have a potential role in preventing bone fracture by the preventing bone loss and an increase in bone mineral content (BMC). From *in vitro* study, the low dose (0.5 mg/day) of genistein retained bone mass and lost less trabecular bone than that in control, while the higher dose (5.0 mg/day) is less effective and may even have adverse effects on bone cells. This finding is a biphasic effect of genistein on preventing bone loss (Anderson et al., 1998). By the studies using rat femoral-metaphyseal tissue and mouse marrow culture, there were found that the decrease in bone calcium content induced by bone-resorbing factors such as PTH, and prostaglandin E_2 was inhibited completely by genistein. The inhibitory effect of genistein on the bone resorption stimulated by PTH was clearly prevented

by the administration of tamoxifen, an anti-estrogen reagent. Genistein did not further enhance the inhibitory effect of estrogen on the bone resorption stimulated by PTH (Yamaguchi and Gao, 1998; Gao and Yamaguchi, 1999a). Coumestrol and estradiol treatments inhibited the bone resorption stimulated by PTH without affecting basal bone resorption in fetal rat limb bone. Coumestrol also increased calcium content of 9-day-old embryonic chick femur culture. This effect was not found in estradiol. The effective concentration of coumestrol for inhibiting bone resorption and that for stimulating bone mineralization was about the same (Smith et al., 1983; Tsutsumi, 1995).

In vivo study, genistein from soy could restore the increase of B-lymphopoiesis and bone loss caused by estrogen deficiency, without a substantial effect on uterus in ovarictomized mice (Ishimi et al., 1999). Aged ovariectomized rats treated with soy diet had a greater bone mineral density (BMD) and represented a trend for greater rate of periosteal bone formation than that of control (Blum et al., 2003). From the study in humans, intake of isoflavones for six months significantly increased BMC and bone mineral density (BMD) in the spine of postmenopausal women (Potter et al., 1998). Japanese postmenopausal women consumed daily capsules of soy isoflavone extract (61.8 mg of isoflavones) for four weeks reduced significantly urinary excretion of bone resorption markers, pyridinoline and deoxypyridinoline (Uesugi, Fukui, and Yamori, 2002). It was indicated that the reduction in bone resorption was associated with the intake of soy isoflavone in postmenopausal women. The continuous dietary intake of soy isoflavone may inhibit bone fracture or osteoporosis (Yamori et al., 2002). It can be concluded that isoflavones and coumestrol have a beneficial effect on protection of bone loss caused by estrogen deficiency (Barnes, 1998; Murkies et al., 1998; Setchell, 1998).

2.4 Estrogenic Effects of *P. mirifica*

The estrogenic activities of phytoestrogens were reported not only on the isolated phytoestrogens from soy, legumes, and their products but also on crude extract of *P. mirifica*. Sukhavachana (1941) firstly reported that the administration of crude extract of *P. mirifica* produced cornified cells, growth of the endometrium gland, and the proliferation of the spiral arteries in ovariectomized rats, in addition to these, increased breast tenderness and leucorrhea in ovariectomized women. Muangdet and Anuntalabhochai (1985) showed that a low dose of *P. mirifica* increased size and number of the oviduct cells in female Japanese quails fed for 10 and 20 days, while a higher dose or a longer period (60 days) of treatment decreased the survival rate and tended to inhibit the increased body weight in quails. Langkalichan and Smitasiri (1985) showed that *P. mirifica* at the high doses (100 and 200 mg/kg BW) significantly decreased the sperm numbers in the epididymis and the percentage of sperm motility but had no effect on the sperm length in male rats. The number and size of fetal implantation in the uterine horns were significantly reduced, and gestation length was prolonged in female rats. Songkaew and Smitasiri (1985) indicated that *P. mirifica* at the doses of 50 and 100 mg/kg BW treated female rats for 4 to 6 days during mid term of pregnancy could interrupt pregnancy and had toxic effect on the fetuses. It could also prevent pregnancy when it was given during the embryo transport, but did not affect the embryo during implantation or post-implantation periods (Smitasiri et al., 1986). The beneficial effect of *P. mirifica* as a contraceptive drug was also reported (Smitasiri and Sakdarat, 1995). It absolutely controlled pigeon reproduction (100%) by the inhibition of mating behavior and testicular development in the male pigeon and the reduction of the follicular development and ovulation in female pigeon (Smitasiri and Sakdarat, 1995). Besides, the administration of *P. mirifica* increased total blood protein and cholesterol levels in quails (Anuntalabhochai and Jesrichai, 1986), which led to increase serum

calcium concentrations, and to reduce width of the epiphyseal cartilage plates at the proximal end of the tibia in both sexes of gonado-parathyroidectomized rats (Bulintarathikul, 1978). From these numerous reports, it can be concluded that *P. mirifica* have an estrogenic effect on reproductive systems and others (Smitasiri, Pangjit, and Anuntalabhochai, 1989). It is also suggested that phytoestrogens from *P. mirifica* have an estrogenic effect on reproductive system, hormonal levels, and bone in both animals and humans.