CHAPTER II

LITERATURE REVIEWS

Pueraria mirifica

Pueraria mirifica Airy shaw and Suvatabandhu, white "Kwao-Keur", is an indigenous herb of Thailand that classified into family *Leguminosae* and subfamily *Papillionoideae* (เต็ม, 1980). This climbing plant found and grows in association with the moderate size tree timber wood in the deciduous rain forest of northern Thailand and Burma at the altitude of 300-800 meters above sea level (ช., 1995). *P. mirifica* has been used in Thai traditional medicine and knowing well as "rejuvenating" folk medicine for over a hundred year. It's recommended for both aged men and women for improvements of complexion, blood circulation, energy balance and vigor leading to more reflexive movement, grow and strengthen hair (หลวงอนุสารสุนทร, 1931).

The usages of *P. mirifica* in traditional medicine and folklore due to its estrogenic activity of active substances found in tuberous root. Natural compounds found in tuberous root of *P. mirifica* can be classified on the basis of their chemical structures (วันชัย และ ชาลี, 2001) into five categories; (i) chromenes including deoxymiroestrol, miroestrol, isomiroestrol; (ii) isoflavones including daidzein, genistein, kwakhurin, kwakhurin hydrate; (iii) isoflavones glycosides including daidzin, genistin, mirificin, puerarin, puerarin-6"-monoacetate; (iv) coumestans including coumestrol, mirificoumestan, mirificoumestan glycol, mirificoumestan hydrate; and (v) pterocarpenes including tuberosin, puemiricarpene.

The chemical structures of natural compounds found in tuberous root of *P. mirifica* were summarized in Table 2.1.

Table 2.1 **Chemical structures of natural compounds found in tuberous root of *P. mirifica*.**

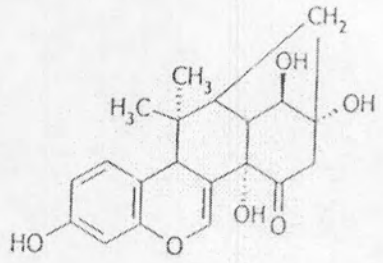
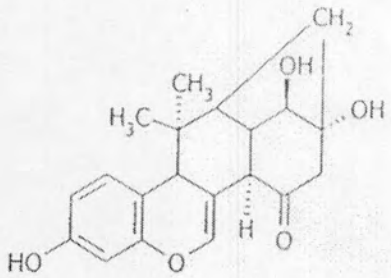
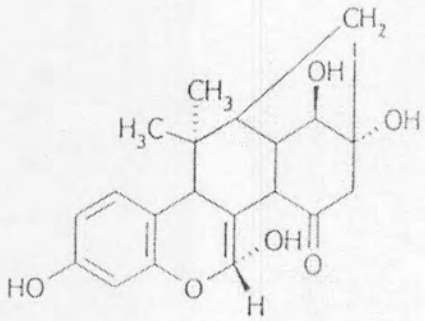
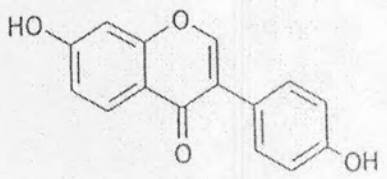
Compounds	Chemical structures
1. Chromenes	
1.1 Miroestrol	
1.2 Deoxymiroestrol	
1.3 Isomiroestrol	
2. Isoflavones	
2.1 Daidzein	

Table 2.1 Chemical structures of natural compounds found in tuberous root of *P. mirifica.* (Continue)

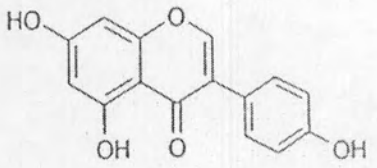
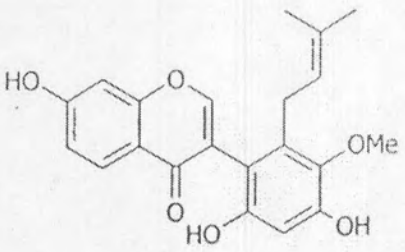
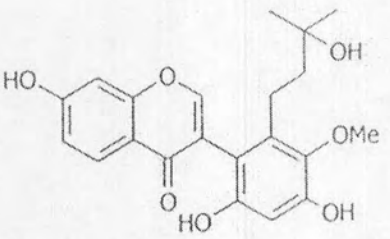
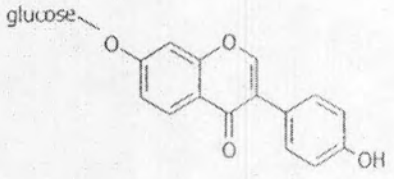
Compounds	Chemical structures
2.2 Genistein	
2.3 Kwakhurin	
2.4 Kwakhurin hydrate	
3. Isoflavone glycosides	
3.1 Daidzin	

Table 2.1 Chemical structures of natural compounds found in tuberous root of *P. mirifica.* (Continue)

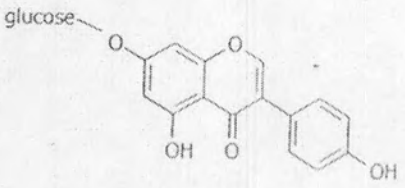
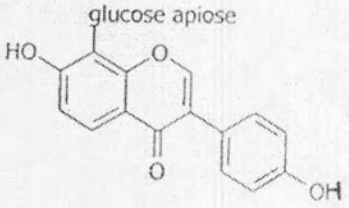
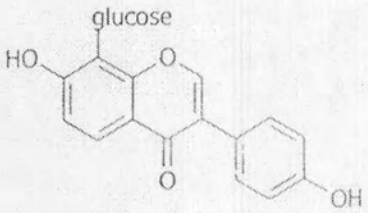
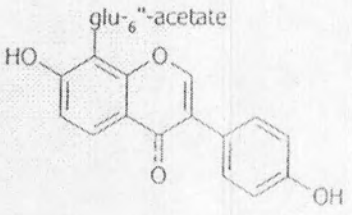
Compounds	Chemical structures
3.2 Genistin	
3.3 Mirificin	
3.4 Puerarin	
3.5 Puerarin-6”-monoacetate	

Table 2.1 **Chemical structures of natural compounds found in tuberous root of *P. mirifica.*** (Continue)

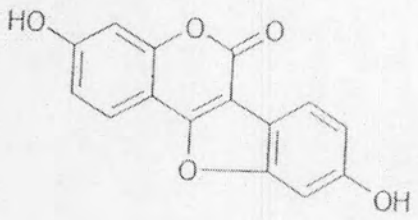
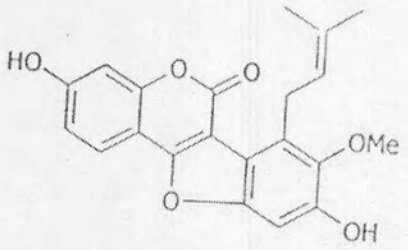
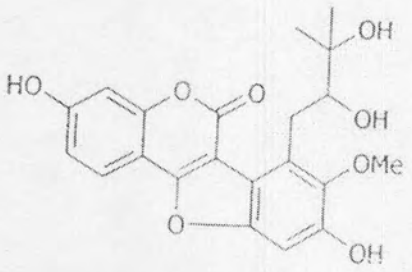
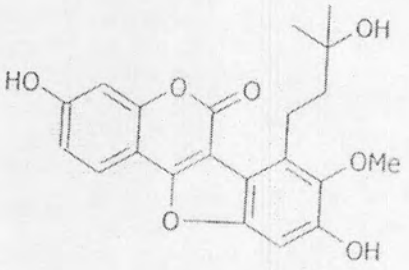
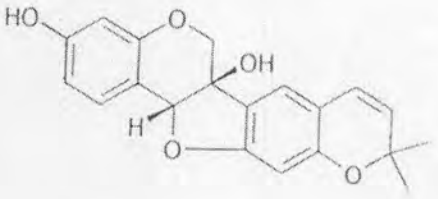
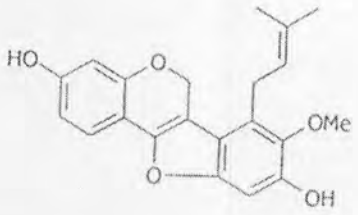
Compounds	Chemical structures
4. Coumestans	
4.1 Coumestrol	
4.2 Mirificoumestan	
4.3 Mirificoumestan glycol	
4.4 Mirificoumestan hydrate	

Table 2.1 **Chemical structures of natural compounds found in tuberous root of *P. mirifica*.** (Continue)

Compounds	Chemical structures
5. Pterocarpenes	
5.1 Tuberosin	
5.2 Puemiricarpene	

The recent study suggests that the actual phytoestrogen and possesses the highest estrogenic activity in tuberous root of *P. mirifica* is deoxymiroestrol, the phytoestrogen in chromenes group (Chansakaow et al., 2000a). Moreover, deoxymiroestrol is easily converted to miroestrol, the known active compound, and isomiroestrol, non-estrogenic compound, by air oxidation during the isolation. Thus, the known miroestrol may be an artifact.

The study of estrogenic activity based on growth promoting effects on MCF-7 human breast cancer cells of natural compounds found in tuberous root of *P. mirifica* were showed that deoxymiroestrol possesses the highest estrogenic activity and miroestrol also possesses high estrogenic activity almost as strong as deoxymiroestrol. Moderate estrogenic activity compounds were consisted of coumestrol and genistein. Whereas, daidzein and kwakhurin were the weaker estrogenic activity substances (Chansakaow et al., 2000b). The details were presented in Table 2.2.

Table 2.2 **The estrogenic activity based on growth promoting effects on MCF-7 human breast cancer cells of natural compounds found in tuberous root of *P. mirifica*** (Chansakaow et al., 2000b).

Compounds	Contents (mg / 100 g powder)	Growth promoting effects on MCF-7 (Minimal concentration*)
17ß-estradiol	-	$< 10^{-12}$
Chromenes		
Miroestrol	3.0	10^{-8}
Deoxymiroestrol	2.0	10^{-10}- 10^{-9}
Isomiroestrol	2.2	no activity
Isoflavones and Glycosides		
Daidzein	46.1	10^{-6}
Genistein	0.6	10^{-7}
Kwakhurin	0.6	$> 10^{-6}$
Daidzin	8.5	no activity
Genistin	data not showed	data not showed
Coumestans		
Coumestrol	0.07	10^{-7}
Pterocarpenes		
Tuberosin	0.3	no activity
Puemiricarpene	1.8	no activity
Acids		
Tetracosanoic acid	15.3	-

* Minimal concentrations of compounds that caused 50% MCF-7 human breast cancer cells growth when compared to the control

Pharmacological effects of *Pueraria mirifica*

1. Antifertility effects

Tuberous root extracts of *P. mirifica* showed the antifertility and contraceptive effects on both male and female animals (มยุรา และคณะ, 1987; อำพา และคณะ, 1998). *P. mirifica* at the dosages of 100 and 200 mg/kg decreased spermatogenesis and sperm movement due to the infertilization and reduction of embryonic implantation. Thus, the fertilization from these sperms was decreased but no congenital abnormalities or cripples occurred (ยุพดี, 1984; ยุพดี และ ยุทธนา, 1985; กนกพร และคณะ, 1994). *P. mirifica* treated male pigeons showed the reduction of sexual frequency and testis growth, beside that the inhibition of egg laying was also found in treated female pigeons (ยุทธนา, 1998). Moreover, *P. mirifica* showed a reversible inhibition on egg laying and crowing in female and male quails, respectively.

2. Induction of abortion

The study showed the complete abortion in pregnant rats but no early birth occurred in *P. mirifica* treated female rats at a dosage of 100 mg/kg/day for seven days (ยุทธนา, 1998).

3. Inhibition of lactation

P. mirifica treated lactating rats showed the decreases of mammary gland weight and milk secretion. The results from this treatment were similar to the effects of estrogen on lactating rats (ยุทธนา และคณะ, 1989).

4. Breast enlargement and reproductive organ growth

Mammary duct growth and breast enlargement were promoted in *P. mirifica* treated rats and mice (ยุทธนา และ ศุภชัย, 1997). The increases of size and weight of uterus were found in *P. mirifica* treated puppies (พูนศิลป์ และคณะ, 1987).

5. Lipid lowering effects

Cholesterol lowering effects were found in male rats treated with *P. mirifica* at the dosages of 10, 100 and 1,000 mg/kg/day for 90 days, and also in female rats that treated with *P. mirifica* at the dosages of 100 and 1,000 mg/kg/day for 90 days. Whereas, triglyceride lowering effect was found only in male rats treated with 1,000 mg/kg/day of *P. mirifica* for 90 days (ทรงพล และคณะ, 2000). Administration of *P. mirifica* at a dosage of 100 mg/kg/day for 90 days in high cholesterol feed male rats was showed the decreasing levels of HDL-cholesterol, LDL-cholesterol, triglyceride and also the improvement HDL-cholesterol / LDL-cholesterol ratio was exhibited (Auttapongpaiboon, 2002).

6. Cardiovascular protective effects

Treatments with *P. mirifica* at a dosage of 100 mg/kg/day for 90 days in male rats and ovariectomized rabbits showed the improvement of vascular response to acetylcholine, endothelium-dependent vascular relaxation, and also the integrity of endothelial cells was preserved (Auttapongpaiboon, 2002). The improvements of lipid profiles especially the decrease in LDL-cholesterol level and increase in HDL-cholesterol / LDL-cholesterol ratio were also considered to be the cardiovascular protective effect of *P. mirifica* due to the prevention of atherosclerosis.

Toxicity profiles of *Pueraria mirifica*

Acute toxicity of *P. mirifica* in animals has been previously reported. Median lethal dose, LD_{50}, of this plant in mice was greater than 16 g/kg.

Subchronic toxicity study was performed by orally administration of dried root powder of *P. mirifica* at various dosages (10, 100 and 1,000 mg/kg/day) in male rats for 90 days (ทรงพล และคณะ, 2000). The results showed that *P. mirifica* at the dosages of 100 and 1,000 mg/kg/day were caused the decrease in growth rate and feed intake comparing with control group.

Treatment of *P. mirifica* at the dosage of 1,000 mg/kg/day resulted in the decrease of hematocrit, RBC, hemoglobin and increase of percentage of reticulocytes in both male and female rats. Whereas, WBC, percentage of basophils and platelet were decreased in male rats. These were recovered after two weeks since *P. mirifica* treatment was terminated. However, RBC, hemoglobin in female rats and WBC, platelet in male rats were not recovered. Moreover, bilirubin, serum creatinine and uric acid were decreased at the dosages of 100 and 1,000 mg/kg/day in male rats. Hepatic function indicating enzymes, alkaline phosphatase (ALP) and alanine aminotransferase (ALT), were increased at the dosage of 1,000 mg/kg/day.

Beside that, treatment with *P. mirifica* at the dosages of 100 and 1,000 mg/kg/day resulted in uterine enlargement according to the increase of uterine weight, actual weight and relative weight. Treatment with *P. mirifica* at the dosage of 1,000 mg/kg/day, the incidences of testicular hyperemia and kidney tubular casts were found.

Phytoestrogens

Phytoestrogens are plant substances that may or may not be structurally similar to gonadal 17ß-estradiol and produce varying degrees of estrogenic activity. There are three mainly groups of phytoestrogens including i) isoflavones and lignans ii) coumestans and iii) resorcylic acid lactones (Lotke, 1998).

The contents of this part are confined to the first category, isoflavones and lignans, and the part of chromenes, natural compounds found in *P. mirifica*, are also described.

Isoflavones

Active isoflavones including genistein, from biochanin A, and daidzein, synthesized form formononetin via equol, are belong to the group of flavonoids (Murkies et al., 1998). The isoflavone glycosides are metabolized by bacterial enzymes in gut lumen to their active metabolites. The glycosides, genistin and daidzin, are glucose conjugated form of their corresponding aglycones, genistein and daidzein. Daidzein is partially further metabolized by bacteria to form the isoflavan, equol (estrogenic activity) and O-desmethylangolensin (O-DMA) (non-estrogenic activity). Genistein is metabolized to P-ethylphenol (non-estrogenic activity) and also metabolized by CYPs to isoflavone orobol (estrogenic activity). The conjugated phytoestrogens then pass through the enterohepatic circulation and may be excreted in bile or deconjugated, reabsorbed, reconjugated and excreted in the urine.

Isoflavones are found in soybeans, chickpeas, legumes, bluegrass and clover. Most isoflavones found in plants are in bound forms as glycosides and are biologically inactive. The others phytoestrogens in flavonoids group are flavonols (such as quercetin, kaempferol), flavones (such as apigenin, luteolin), flavonones (such as narigenin) and coumestans (such as coumestrol).

Lignans

The active metabolites of lignans are enterolactone and enterodiol that converted by gut bacteria from precursors in plants, secoisolariciresinol and metairesinol, respectively. Lignans are found in flaxseed (also known as linseed), whole cereals and legumes.

Chromenes

Chromenes, deoxymiroestrol and miroestrol, are phytoestrogens that found in tuberous roots of *P. mirifica*. Deoxymiroestrol, the actual active ingredients, is rapidly oxidized to miroestrol and isomiroestrol (non-estrogenic activity). Miroestrol possesses high estrogenic activity. Subcutaneous injected animals with miroestrol were found the equal results to 17ß-estradiol in mouse uterine growth and the one-quarter of the potency to 17ß-estradiol in rat vaginal cornification. Miroestrol subcutaneous injection exhibited 70 percents of the 17ß-estradiol activity and 2.2 times as active as estrone in promotion of mammary duct growth in rat and mice, respectively. Orally administration of miroestrol was exhibited three times potency to stillbestrol in immature mice uterine growth and two-thirds of stillbestrol in rat vaginal cornification. Adverse effects of miroestrol were malaise, headache, nausea and vomiting (Caine, 1960).

Pharmacological effects of phytoestrogens

The similar chemical structures of phytoestrogens to 17ß-estradiol, the endogenous estrogen, especially the presence of phenolic rings are a prerequisite for binding to estrogen receptors (ER) (Kuiper et al., 1998; Setchell, 1998; Wiseman and Duffy, 2001). The binding of phytoestrogens to estrogen receptors can exhibit as

either estrogen agonists (estrogen-like activity) or estrogen antagonists (anti-estrogenic activity). The estrogen antagonists, anti-estrogenic activity of some phytoestrogens, can be used as cancer chemopreventive compounds in estrogen-related cancerous events such as breast cancer, endometrial or ovarian cancers etc. The actions of phytoestrogens at cellular and molecular level are influenced by many factors including estrogen receptor subtypes (ER-α or ER-ß), the presence or absence of endogenous estrogens and types of target organs or cells (Setchell, 1998).

Dietary estrogens are weakly estrogenic substances (10^{-2} to 10^{-3}–fold depending on the system examined) when compared with estradiol or estrone. The affinity of non-steroidal estrogens to the novel estrogen receptor, ER-ß, suggests that they may be act through distinct and separate pathway from classical steroidal estrogens (Kuiper et al., 1998; Setchell, 1998). The study suggests that the estrogenic potency of phytoestrogens for both estrogen receptor subtypes, ER- α and ER-ß, is different as summarized in Table 2.3 (Kuiper et al., 1998).

Table 2.3 The ranking of estrogenic potency of phytoestrogens for estrogen receptor subtypes (ER- α and ER-ß) (Kuiper et al., 1998).

Estrogen receptor α	Estrogen receptor ß
Estradiol >> Coumestrol > Genistein > Daidzein > Apigenin = Phloretin > Biochanin A = Kaempferol = Naringenin > Formononetin = Ipriflavone = Quercetin = Chrysin	Estradiol >> Genistein = Coumestrol > Daidzein > Biochanin A = Apigenin = Kaempferol = Naringenin > Phloretin = Quercetin = Ipriflavone = Formononetin = Chrysin

Phytoestrogens supplement and their benefits

Many studies suggest that phytoestrogens have the beneficial effects on menopause-related abnormalities especially on cardiovascular diseases (CVDs) and osteoporosis, and also the preventive effects on estrogen-related cancerous events. The main contents of this part are described about the relationships of phytoestrogens on cardiovascular diseases and osteoporosis. The preventive effects of phytoestrogens on cancerous events are also described briefly here.

Phytoestrogens can decrease the incidences of estrogen-related cancers including breast cancer, endometrial cancer, ovarian cancer and also other types of cancer including colon cancer. The mechanisms of anti-carcinogenic properties of phytoestrogens were considerable related to anti-estrogenic activity of estrogen antagonists. Whereas, many non-estrogenic mechanisms have been reported. These include inhibition of tyrosine kinase, induction of differentiation, inhibition of DNA topoisomerases, inhibition of specific cell cycle events, induction of apoptosis, inhibition of angiogenesis, antioxidant activity and inhibition of hydrogen peroxidase (Barnes et al., 1995). Soybeans have been showed to contain protease inhibitors. There are at least two different inhibitors found in soybeans including Kunitz trypsin inhibitor and Bowman-Birk protease inhibitor (BBI) that can prevent conversion of normal cells to malignant cells. BBI has been showed the prevention of experimentally induced colon, oral, lung, liver and esophageal cancers in animals due to the inhibition of chymotrypsin.

Phytoestrogens and cardiovascular diseases

Effects of phytoestrogens on cardiovascular diseases have been reported that phytoestrogens can improve vascular function, endothelial integrity and also the anti-atherosclerotic effects were identified. The relationships of phytoestrogens on

cardiovascular diseases are due to the favorable effects on lipid profiles, antioxidants, vascular reactivity, thrombosis and cellular proliferation.

The hypocholesterolemic effects of phytoestrogens include the decreasing levels of total cholesterol, LDL-cholesterol, triglyceride and the increases in HDL-cholesterol level, HDL-cholesterol / LDL-cholesterol ratio. The possible mechanisms of lipid lowering effects have been proposed that phytoestrogens can increase the excretion of bile acid and enhance LDL-cholesterol removal or initiate the hyperthyroid stage that increase metabolic rate. However, the best supposed mechanism of phytoestrogens lipid-lowering effects is the alteration of hepatic metabolism with augmented LDL-cholesterol and VLDL-cholesterol removal by hepatocytes. Thus, the effects of phytoestrogens such as isoflavones on LDL receptor activity were implicated. The study in LDL receptor deficiency transgenic mice, C57BL/6J mice, suggests that isoflavones may reduce lipid levels by increasing the activity of the LDL receptor (Sirtori et al., 1995).

Phytoestrogens such as isoflavones also inhibit LDL-cholesterol oxidation. The performed studies were observed that isoflavones, genistein and daidzein, inhibit the formation of thiobarbituric acid-reactive substances in either cell free or endothelial cell systems. They were also found to protect against cytotoxic effects of oxidized LDL-cholesterol as assessed by cellular morphologic features and lactate dehydrogenase released by cultured endothelial cells (Kapiotis et al., 1997). Genistein has been shown the inhibition of hydrogen peroxide production and increase the activity of antioxidant systems such as catalase, superoxide dismutase, glutathione peroxidase and glutathione reductase.

Phytoestrogens such as genistein inhibits the expression of adhesion molecules, intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1), on human endothelial cells co-cultured with monocytes (Takahashi et al., 1996). The adhesiveness of endothelial cells is due to lipid-induced, oxidant-sensitive transcription of adhesion molecules and chemokines that promote monocytes binding.

Atherosclerosis is initiated by monocytes binding to the endothelium and migrating into the intimal layer or subendothelial space to develop into foam cells.

The anti-platelet activity of phytoestrogens was identified. Isoflavones, genistein and daidzein, decreased platelet aggregation by collagen and thromboxane (Nakashima et al., 1991; McNicol, 1992), which may be due to the inhibition of thromboxane receptor binding or diminished tyrosine phosphorylation.

The improvements of vascular reactivity due to phytoestrogens have been reported. Phytoestrogens can improve vasodilation in response to locally-administered acetylcholine, endothelium-dependent vascular relaxation, in isolated vessels. Estrogen (17ß-estradiol) and phytoestrogens (genistein and daidzein) were all found to relax mesenteric arterial rings of rats in a dose dependent manner. Consistent with receptor assays, estradiol was the most potent vasodilator followed by genistein, then daidzein. Moreover, phytoestrogens can also improve the endothelial integrity due to the increases of endothelial differentiation and proliferation.

Phytoestrogens and osteoporosis prevention

Osteoporosis is a condition of low bone mass and microarchitectural disruption that results in fractures with minimal trauma. Osteoporosis causes a generalized skeletal fragility and pathological bone fractures including vertebral bodies, distal radius, proximal femur, ribs and long bones etc. (Marcus, 2001)

Osteoporosis is described generally as primary or secondary. Secondary osteoporosis is due to systemic illness or medications such as glucocorticoids etc. Primary osteoporosis is composed of two separate causes including menopausal estrogen loss and aging. The primary osteoporosis represents two different conditions including type I osteoporosis, loss of trabecular bone due to estrogen lack at menopause, and type II osteoporosis, loss of cortical and trabecular bone (due to long-

term remodeling inefficiency, dietary inadequacy and activation of the parathyroid axis with age). (Riggs et al., 1982)

Osteoporosis primarily affects elderly women and its incidence increase with increasing length of estrogen deficiency. One of important health benefits of phytoestrogens is the prevention of osteoporosis. Phytoestrogens such as ipriflavone, the isoflavones-derived flavonoid, can prevent osteoporosis and osteopenia. It exerts bone protective actions by interfering with bone resorption, controlling preosteoclast recruitment, decrease the number of osteoclasts and mononuclear cells.

Moreover, other isoflavones such as genistein and daidzein treatment in ovariectomized rats showed an increase in bone mineral density (Fanti et al., 1998) due to its affinity to ER in bone tissue (Onoe et al., 1967) which exert bone protective effect via inhibit the production of osteoclast-stimulating cytokine, TNF-α (Bertolini et al., 1986). Treatment with *Cissus quadrangularis* extract which contains phytogenic steroids exhibited an increase in bone mineral density in ovariectomized rats (Shirwaikar et al., 2003).

Accordingly, Black cohosh (*Cimicifuga racemosa*) extract containing phytoestrogens exhibited protective effects on bone due to osteoclast-inhibiting cytokines production (Hostanska et al., 2004).

Estrogen and endothelial functions

Endothelium, a layer of cells lining in vascular lumen, plays an important role in cardiovascular homeostasis especially in vascular tone regulation (Maturana et al., 2007). Endothelium exerts its main action as a modulator of vascular tone through the production of vasoactive substances including vasodilator factors (nitric oxide (NO), prostacyclin (PGI_2), acetylcholine, bradykinin etc.) and vasoconstrictor factors (endothelin (ET), constrictor prostanoids (PGH_2, TXA_2), Angiotensin (AII) etc.) (Luz et al., 2003).

The regulation of vascular tonus is mediated by the balance of vasodilation and vasoconstriction. Nitric oxide is the main mediator of vasomotor tonus in physiological situations and various stimuli such as pressure of blood against vascular wall and shear stress etc. stimulate the basal generation of NO (Furchgott and Zawadzki, 1980; Yang and Loscalzo, 2000).

Moreover, previous studies suggested the other actions of endothelium including regulation of vascular wall selective permeability (Simionescu et al., 1975), maintenance of a balance between thrombosis and fibrinolysis (Moncada et al., 1977; Anderson et al., 2004), inhibition of vascular smooth muscle cell proliferation (Caramori and Zago, 2000; Andrews et al., 2001) and active participation in immune response.

Estrogen has many effects on cardiovascular functions. The hormonal changes that accompany menopause, particularly the decreased levels of endogenous estrogen, have a great physiological impact.

Accordingly, the evidence of an association between endothelial dysfunction and reduced endogenous estrogen after natural or surgical menopause has been reported (Taddei et al., 1996). Endothelial dysfunction is mainly associated with the reduction of endothelium-dependent vasodilation due to the attenuated biological vasodilating factors, especially nitric oxide. In addition, the impairments of other normal reactivities of endothelium including interaction with leukocytes, platelets and regulatory substances are involved in endothelial dysfunction (Suwaidi et al., 2000).

Many studies have suggested that endothelial dysfunction is the initial event in development of atherosclerosis (Anderson, 1998; Najemnik et al., 1999; Shimokawa, 1999; Davignon and Ganz, 2004).

The actions of estrogen on cardiovascular system are mediated directly on vessels or indirectly through the modulation of cardiovascular risk factors such as the improvement of lipid parameter levels (Bush et al., 1987) and the prevention of LDL-C oxidation etc. (Subbiah, 2002).

The direct effects of estrogen on vascular system are consist of the acute vasodilation due to NO synthesis (Khalil, 2005), long-term modulation of vascular tonus due to the regulation of PG production and expression of eNOS and endothelin gene (Hermenegildo et al., 2006), inhibition of endothelin-induced vasoconstriction (Nilsson and Gustafsson, 2000) and inhibition of sympathetic activity (Mercuro et al., 1999).

Moreover, estrogen exerts an antiproliferative effect on vascular smooth muscle layer (Barchiese et al., 2002), inhibits the proliferation of the inner vascular layer after injury (Pare et al., 2002) and increase the expression of contractile proteins in myocardium (Scheuer et al., 1987).