CHAPTER II

LITERATURE REVIEWS

Pueraria mirifica

The native plants, White Kwao Keur, *Pueraria mirifica* Airy Shaw and Suvantabandhu is one of the Thai folk medicinal plants (เต็ม สมิตินันท์, 2523). It belongs to the Family Leguminosae, subfamily Papilionoideae or the soy bean and pea subfamily. The herbal plants is grown in association with the moderate size tree timber wood in the deciduous rain forest of northern Thailand at the altitude of 300-800 meters above sea level. Active principles in this plant are found the tuberous root, which looks like a chain of round shaped bulbs of various sizes connected to the next one via small root throughout the entire length of the root (Fig.2.1). The shape and size of the tuberous root are diverse depending on the environment in which it exists (สมภพ ประธานธุรารักษ์ และคณะ, 2543).

Women in the rural communities in Thailand where this herb grows have used the tuberous roots of *P. mirifica* effectively as "rejuvenating" folk medicine for well over a hundred years.

According to Thai traditional medicine, this rejuvenating herb is recommended for both aged men and women for its efficacy to grow hair, strengthen and darken existing ones, help improve complexion, increase blood circulation , increase energy and vigor leading to more reflexive body movements (หลวงอนุสารสุนทร, 2474). These uses of *P. mirifica* in traditional medicine may be ascribed to its estrogenic activity (Murkies *et al.*, 1998).

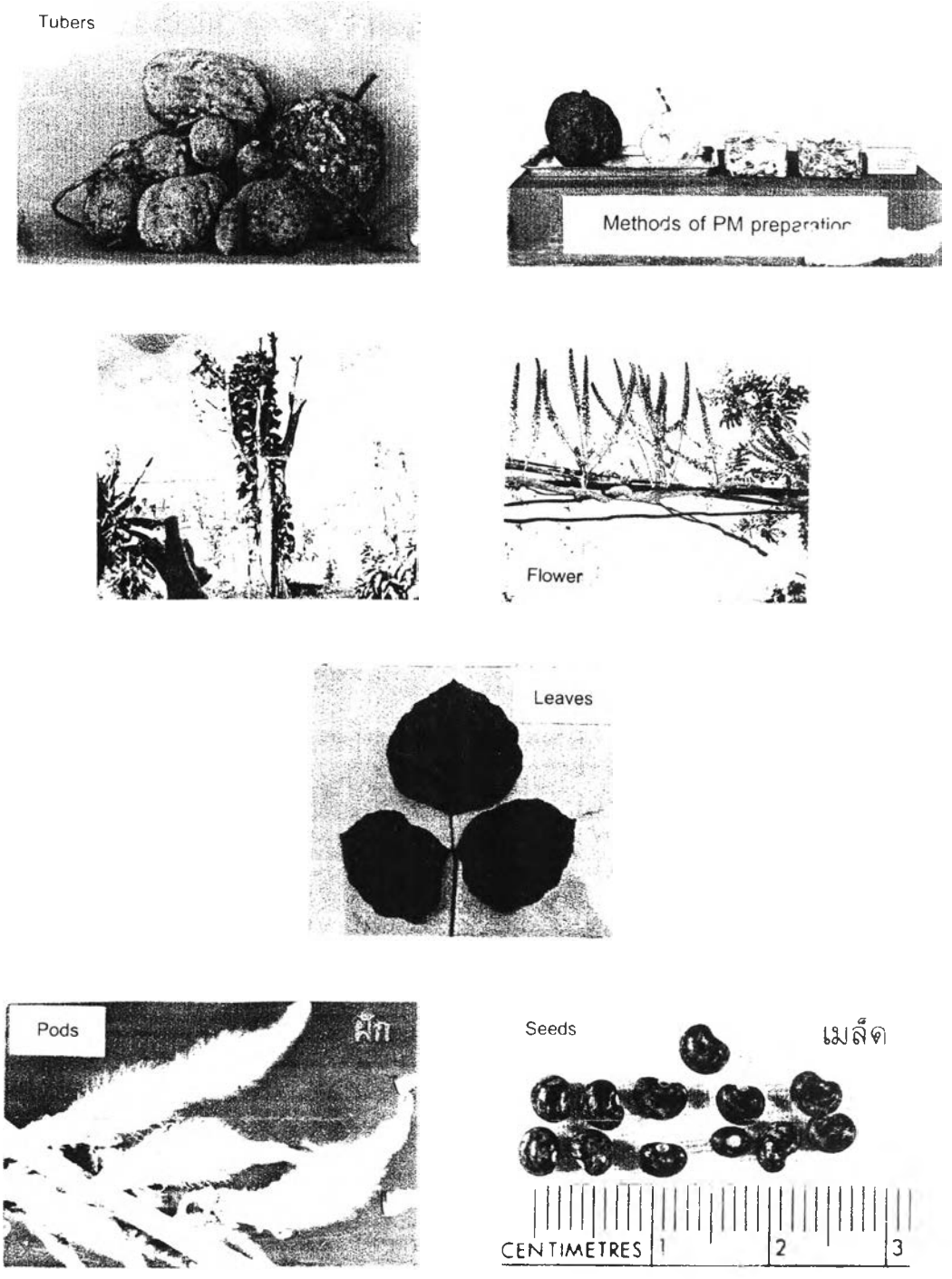


Fig. 2.1 **Diagrams of various parts of *Pueraria mirifica*. (Smitasiri et al., 2003)**

The enlarge underground tuber accumulate "phytoestrogens". The compounds that make *P. mirifica* different from any other phytoestrogens containing plants are miroestrol and deoxymiroestrol, which possess highest estrogenic activity among the known phytoestrogens due to structural similarity to estradiol (Fig. 2.2).

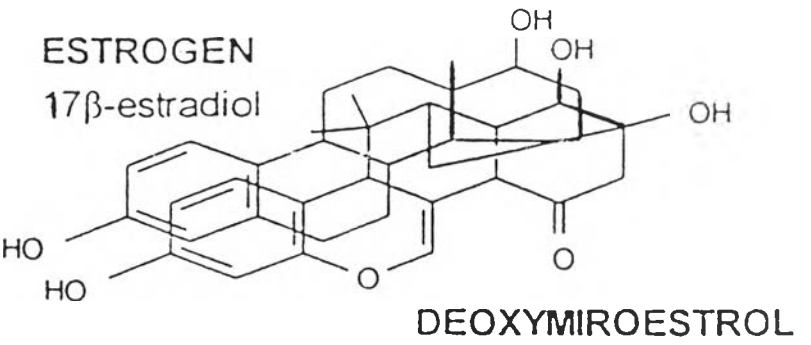


Fig 2.2 Chemical structure of 17β-estradiol compared with deoxymiroestrol.

The isolation and identification of deoxymiroestrol from the root of *P. mirifica* has just been reported (Chansakaow et al.,2000a). The authors proposed that since deoxymiroestrol is easily oxidized to miroestrol and isomiroestrol by air oxidation during the isolation. Thus deoxymiroestrol is more to be the actual chemical constituent of *P. mirifica.*

Moreover, deoxymiroestrol was found to possess the strongest growth promoting effect on MCF-7 human breast cancer cells compared to those of other phytoestrogens in this plant. Miroestrol also possesses high estrogenic activity, almost as deoxymiroestrol. Coumestrol and genistein possess moderate estrogenic activity whereas daiazein and kwakhurin possess a weaker estrogenic activity (Table 2.1) (Chansakaow et al., 2000b).

Table 2.1 **Natural compounds found in tuberous roots of *P. mirifica*. Their contents and growth-promoting effects were based on an effect on MCF-7 human breast cancer cells (Chansakaow et al., 2000b).**

Compounds	Content (mg/100 g power)	Growth-promoting effect on MCF-7 (Minimal concentration*)
17β-estradiol	-	$<10^{-12}$
Chromenes group		
Miroestrol	3.00	10^{-8}
Deosymiroestrol	2.00	10^{-10} –10^{-9}
Isomiroestrol	2.00	no activity
Isoflavones group		
Daidzein	46.10	10^{-6}
Genistein	0.60	10^{-7}
Kwahurin	0.60	$>10^{-6}$
Isoflavones glycosides group		
Daidzin	8.50	no activity
Puerarin	6.90	no activity
Coumestan group		
Coumestrol	0.07	10^{-7}
Pterocarpens group		
Tuberosin	0.30	no activity
Puemiricarpene	1.80	no activity

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

In addition to Miroestrol and Deoxymiroestrol, *P. mirifica* also contains other chemicals that belong to isoflavone, isoflavone glycoside and coumestans groups of phytoestrogens, e.g., genistein, daidzein, daidzin, genistein and coumestrol that are usually found in soybeans. However, the estrogenic activity of miroestrol and deoxymiroestrol is much more potent than that of soy isoflavone. Natural compounds found in tuberous root of *P. mirifica* can be classified on the basis of their chemical structure are presented in Table 2.2 (วันชัย ดีเอกนามกูล และ ชาลี ทองเรือง 2544).

Table 2.2 Chemical structure of natural compounds found in tuberous root of *P. mirifica*.

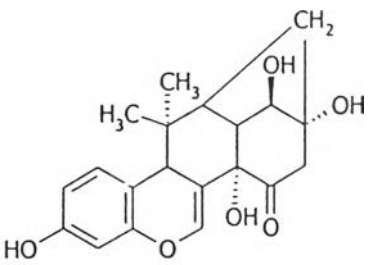
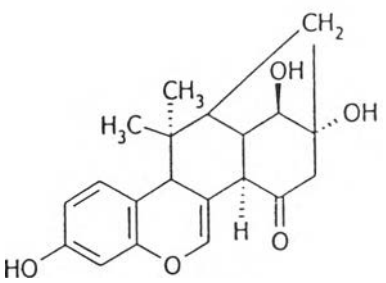
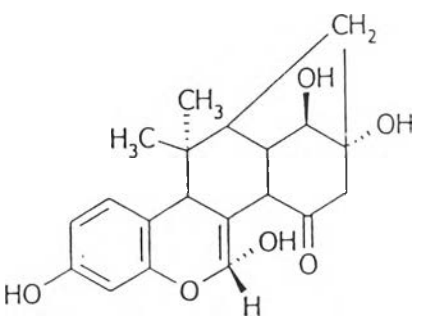
Compounds	Structures
1. Chromenes group	
1.1 Miroestrol	
1.2 Deoxymiroestrol	
1.3 Isomiroestrol	

Table 2.2 Chemical structure of natural compounds found in tuberous root of *P. mirifica.* (Continued)

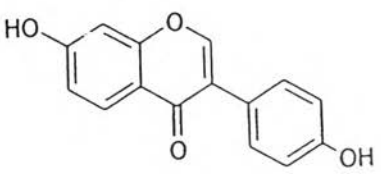
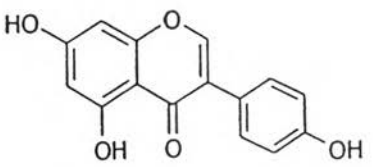
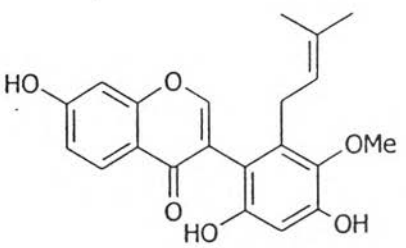
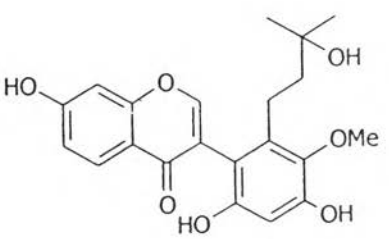
Compounds	Structures
2. Isoflavones group	
2.1 Daidzein	
2.2 Genistein	
2.3 Kwahurin	
2.4 Kwakhurin hydrate	

Table 2.2 Chemical structure of natural compounds found in tuberous root of *P. mirifica.* (Continued)

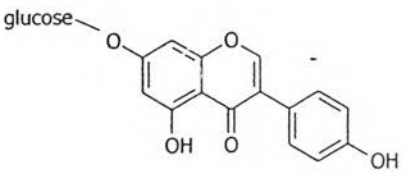
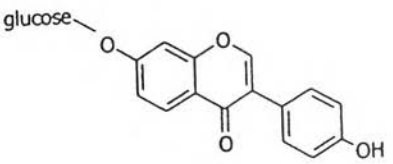
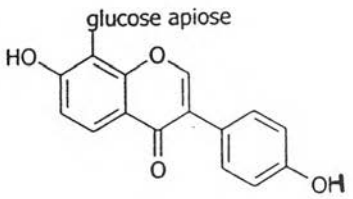
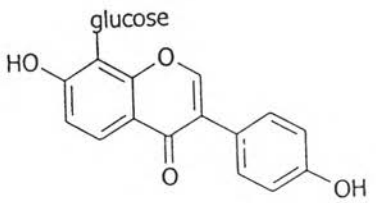
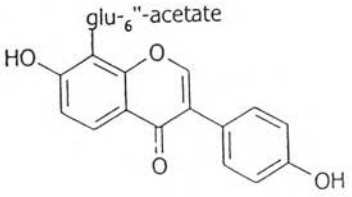
Compound	Structures
3. Isoflavones glycosides group	
3.1 Genistin	
3.2 Daidzin	
3.3 Mirificin	
3.4 Puerarin	
3.5 Puerarin-6"-monoacetate	

Table 2.2 Chemical structure of natural compounds found in tuberous root of *P. mirifica.* (Continued)

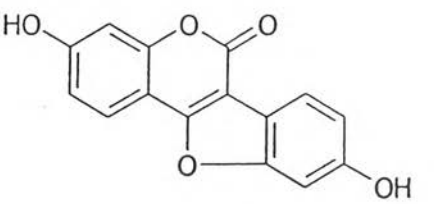
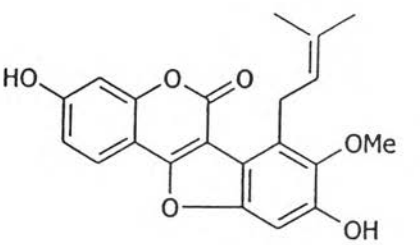
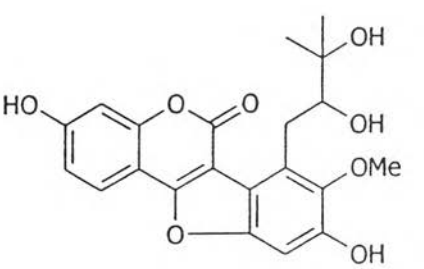
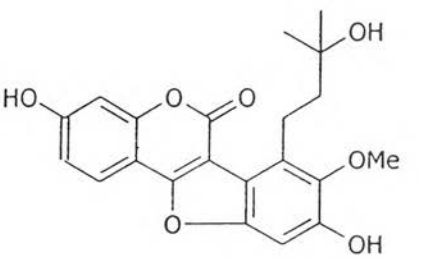
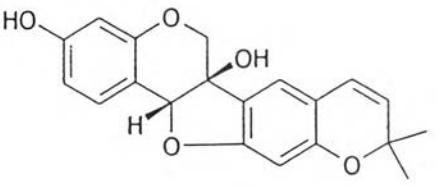
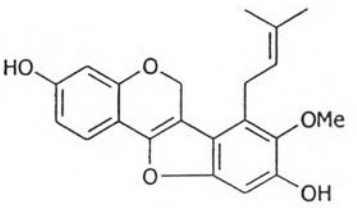
Compound	Structures
4. Coumestans group	
4.1 Coumestans	
4.2 Mirificoumestan	
4.3 Mirificoumestan glycol	
4.4 Mirificoumestan hydrate	

Table 2.2 Chemical structure of natural compounds found in tuberous root of *P. mirifica.* (Continued)

Compound	Structures
5. Pterocarpene group	
5.1 Tuberosin	
5.2 Puemiricarpene	

Pharmacological effect of *P. mirifica.*

1. Antifertility and induction of abortion.

P. mirifica was potent antifertility agent. Female rats receiving *P. mirifica* at concentration of 1 g/rat/week significantly decreased ovulation and embryo implantation. Therefore, the number of neonatal rats were decreased but their organs were not cripple (กนกพร กวีวัฒน์, 2537). *P. mirifica* caused an inhibition of spermatogenesis resulted in a reduction of fertilization. (อำพา เหลืองภิรมย์ และคณะ, 2541) *P. mirifica* was found to be an antifertility agent according to its potent estrogenic effect in rats (Smitasiri et al.,1986). Administration of *P. mirifica* at a concentration of 100 mg/kg/day resulted in a complete abortion in pregnant rats but did not induce early birth (ยุทธนา สมิตะสิริ, 2541)

2. Cholesterol lowering effects.

Phytoestrogens in *P. mirifica* could decrease blood lipid parameter, therefore reduce the risk of atherosclerosis. Cholesterol levels of male rats administered orally with *P. mirifica* at the concentration of 10, 100 and 1000 mg/kg/day for 90 days were significantly lower than those of the control groups. These changes were also observed in female rats given *P. mirifica* at the dosages of 100 and 1000 mg/kg/day. Triglyceride level in male rats administered with *P. mirifica* orally at dose of 1000 mg/kg/day was significantly lower than the control group. No significant in triglyceride level was found at any doses given to female rats (ทรงพล ชีวะพัฒน์ และคณะ, 2543). Moreover, treatment with *P. mirifica* reduced plasma total cholesterol, low density lipoprotein and triglyceride significantly, but no altered high density lipoprotein level was found in hypercholesterolemia rabbits (Ratanachamnong et al., 2000).

3. Inhibition of lactation.

Administration of *P. mirifica* to lactating rats resulted in a decrease of milk secretion by inhibition of mammary gland growth and milk production. This effect were similar to estrogen (ยุทธนา สมิตะสิริ และคณะ, 2532).

4. Reproductive organ growth and breast enlargement.

P. mirifica promoted mammary duct growth and breast enlargement in both mice and rats similar to estrogen. Female puppies fed with diet supplemented with *P. mirifica* for 26 days demonstrated an increase of size and weight or uterus comparing to the control group (พูลศิลป์ ไวทยะโชติ และคณะ, 2530)

Toxicity of *Pueraria mirifica.*

So far, acute toxicity of *P. mirifica* in animals has not been reported. Median lethal dose (LD_{50}) of this plant in mice was greater than 16 g/kg (ทรงพล ชีวะพัฒน์ และคณะ, 2543). A subchronic toxicity study was performed in Wistar rats by administration orally with dried root powder of *P. mirifica* at various doses (10,100 and 1000 mg/kg/day) for 90 consecutive days. The results revealed that growth rate and food consumption of rat receiving *P. mirifica* at the doses of 100 and 1000 mg/kg/day were significantly lower than the control group. Administration of *P. mirifica* at the dose of 1000 mg/kg/day resulted in decreasing of hematocrit, RBC and hemoglobin and increasing of % reticulocyte in both sexes of animals. Moreover, WBC, % basophil and platelet in male rats were also decreased. Two weeks after a cessation of *P. mirifica* administration, most of these parameters return to their normal levels. RBC, hemoglobin in female rats, WBC and platelet in males were still not recovered. That study concluded that prolonged

administration of high doses of *P. mirifica* could affect the haemopoetic systems of the treated rats and cause anemia in rats. The uterus of female rats receiving *P. mirifica* at the dose of 100 and 1000 mg/kg/day appeared swollen. The actual uterine weight and % relative uterine weight of these two groups of *P. mirifica* administration were significantly higher than those of the control group. Histopathological examinations indicated that male and female rats receiving the root powder of this plant at the dose of 1000 mg/kg/day demonstrated a significantly higher incidence of testicular hyperemia and kidney tubular cast, respectively compared to their corresponding control groups (ทรงพล ชีวะพัฒน์ และคณะ, 2543).

Male rat receiving 5% and 10 % of *P. mirifica* mixed with diet also demonstrated a decrease of RBC count, hemoglobin and lymphocyte (วราภรณ์ พงษ์คำ และคณะ, 2530). In addition, quails treated with *P. mirifica* had inflammation in some parts of the body such as head, under wings and legs (อารี ช่วยชู และคณะ, 2529).

Phytoestrogens.

Phytoestrogens are plant-derived compounds that mimic the effects of estrogens and may be consumed as part of a normal diet. They are present in various food, herbs and legumes (Sahelian et al., 2001). The classes of interest to human health can be classified as following : (Murkies et al., 1998).

1. Flavonoids.

1.1 Isoflavones.

Isoflavones are found mainly in legumes such as soy, lentils, beans and chickpeas. They exist in many forms and their effect on human health has been investigated to some extent particularly genistein and daidzein are presented in soybeans (Sun et al., 2002). Most of the isoflavones in soy exist primarily as glucose conjugates or glycones. Genistein is the aglycone that results from the enzymatic cleavage of genistin. Likewise, daidzein is the aglycone that results from the removal of glucose from daidzin. These enzymatic cleavages result from the actions of intestinal bacteria and must occur before isoflavones can be absorbed. In terms of dose, for every 100 mg of glycones genistein and daidzein consumed, approximately 62 mg of the aglycones will absorbed (Potter et al., 1998). Isoflavones have similar structure to estrogen and have the capacity to exert both estrogenic and antiestrogenic effects, they may block the effects of estrogen in some tissues e.g. the breast and womb lining but act like an estrogen in providing possible protection against bone loss and heart disease (Nestel et al., 1997).

1.2 Flavone

Flavone are found in cereals and fruit (Potter et al., 1998).

1.3 Flavanone

Flavanone are found mainly in grapefruit (Sirtori et al., 1996).

1.4 Flavonol

Flavonol are found in most olives and onions (Knight et al., 1996).

1.5 Coumestans

Coumestans are found in high concentrations in clover sprouts. Coumesterol is an example of the group which occurs predominantly during germination of bean sprout (Murkies et al., 1998).

2. Lignans.

Most mammalian lignans are known by the common names of enterodiol and enterolactone, which are converted by gut bacteria from precursors in plants, secisolariciresinol and matairesinol, respectively. These lignan precursors occur in the aleuronic layer of the grain close to the fiber layer. Lignans occur in high concentration in flaxseed which is also known as linseed. They are found in lesser concentration in whole grain cereals, vegetables, fruits and seeds (Knight et al., 1996).

3. Other.

Chromenes are natural compounds found in tuberous root of *P. mirifica.* Miroestrol and Deoxymiroestrol have been investigated regarding their therapeutic potential, particularly in disease prevention (Brzezinski et al., 1997). Especially, Miroestrol possesses high potency of estrogenic activity. Subcutaneous injection to animals, it was found to be equal to 17β-estradiol in mouse uterine growth test, and to have one-quarter of the potency to 17β-estradiol in the rat vaginal cornification test (Schoeller et al., 1940). Given to animals via subcutaneous injection, miroestrol exhibited 70 percent of the activity of 17β-estradiol, in

promoting rat mammary duct growth and 2.2 times as active as estrone in a similar test in mice (Benson, in press). Given orally, miroestrol was approximately three times potent to that of stilbestrol in immature mice uterine growth test and two-thirds of stilbestrol in rat vaginal cornification test (Jones and Pope, 1960).

Pharmacological effects

Phytoestrogens are non-steroidal plant molecules whose structure differ from gonadal hormone, with an estrogen-type bioactivity. They are capable of interacting with estrogen receptor, showing both agonist and antagonist mode of action (Sahelian, 2001).

Over 150 herbs traditionally used by herbalist for treating a variety of health problems were extracted and tested their relative capacity to compete with estradiol binding to intracellular receptors for estradiol (estrogen receptor) (Tikkanen et al., 2000). A conspicuous feature of the chemical structure of phytoestrogens is the presence of phenolic rings that, with few exceptions, is prerequisite for binding to the estrogen receptor (Wiseman and Duffy, 2001). For this reason, phytoestrogens can act either as estrogen agonist or antagonist. The actions to the compounds in specific cells are determined by many factors, including the levels of α and β estrogen receptor. Therefore, effects vary according to phytoestrogens studied, cell line, tissue, and species of the animal (Rajan et al., 2003). Dietary estrogens are weakly estrogenic (10^{-2} to 10^{-3} – fold depending on the system examined) when compared with estradiol or estrone (Setchell, 1998). Estrogenic potency of phytoestrogens for both estrogen receptor subtypes 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 (Kuiper et al., 1998).**

Estrogen receptor α	Estrogen receptor β
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

Phytoestrogen offer a profile of hormonal actions distinct from steroidal estrogens and are unlikely to replace steroidal estrogens in hormonal replacement therapy but instead may improve hormonal replacement therapy safety and efficacy by enhancing some benefits of hormonal replacement therapy while mitigating some of the undesirable risks of steroidal estrogens (Clarkson et al., 1997).

Phytoestrogen and Cardiovascular Disease Relationships.

Accumulating evidence from molecular and cellular biological experiments suggests that phytoestrogens may potentially health benefits related to cardiovascular diseases (Rajan et al., 2003).

In animal studies, phytoestrogen in soybean appear to reduce atherosclerosis and improved the blood fat levels, both of which affect the risk of heart attacks and strokes (Anthony et al., 1997). Soy intake has been to be associated with favorable changes in women's lipid profiles. Subjects consuming 50 g of soy protein in combination with a low fat diet and low cholesterol (less than 300 mg/day) diet experienced a 12 % decrease in LDL levels. A practical and achievable goal of consuming only 50 g of soy protein per day would be beneficial in the treatment and prevention of high blood cholesterol and coronary disease Consumption of soy protein significantly decreased serum

concentrations of total cholesterol LDL and triglyceride. As it has demonstrated some antiproliferative of phytoestrogens. It appears reasonable that women should consume soy in order to reduce the risk of cardiovascular disease (Rajan et al., 2003).

The possible mechanism of cholesterol lowering effects of phytoestrogens has been extensively discussed but the conclusion is still not clear. Phytoestrogens may modify plasma lipids and lipoprotein by upregulating the LDL receptor, increasing LDL catabolism, increasing the excretion of bile acid and neutral steroids into the bile (Potter et al., 1998). They may also have effects on arterial walls, either through their inhibitory effect on vascular smooth muscle cell proliferation and migration or through an effect on vascular reactivity (Tikkanen and Adlercreutz, 2000). Furthermore, clinical study regarding miroestrol was carried out by Dr.P.M.F. Bishop and his collaborates at the Chelsea Hospital for Women, London. Miroestrol was administered at doses of 5 mg or 1 mg daily to ten women suffering from amenorrhea or artificial menopause. Marked estrogen response was noted for both doses during the second or third week of the treatment. When the treatment was discontinued, withdrawal bleeding happened. Actually, these patients had been previously treated with oral estrogen. The withdrawal interval from discontinuing of miroestrol was much longer than that from estrogen. Hot flush was diminished in frequency and severity but recurred by the fifth day after the treatment was stopped. Adverse effects of miroestrol were malaise, headache, nausea and vomiting (Caine, 1960).

Changes of the endothelium in atherosclerotic blood vessels.

Endothelial cells play numerous physiological roles including : (1) a permeability barrier through which there is exchange and active transport of substances into the artery wall and maintenance of vascular tone by release of small molecules such as NO. (Ross, 1993).

In 1980, Furchgott and Zawadzki demonstrated that the relaxation induced by acetylcholine are dependent on the presence of functional vascular endothelium. Subsequently, these endothelium-dependent relaxation have been demonstrated in large arteries of most mammalian species and also in resistance vessels (Rosenblum, 1986). Pharmacological evidence suggests that the vasodilator substance released under these conditions has characteristics similar to the relaxing factor relaxed by acetylcholine. In isolated arterial rings with endothelium, studied in organ chambers under no-flow conditions, acetylcholine causes relaxation which are inhibited by the muscarinic antagonist atropine. Increases in intracellular calcium in endothelial cells, possibly through activation of a Ca^{2+} -dependent enzyme, appear to play a key role in either the production and release of endothelium-derived relaxing factor. In the rabbit and rat aorta, depletion of extracellular calcium inhibits endothelium-dependent relaxation to acetylcholine, but not those to the endothelium-independent vasodilator sodium nitroprusside (Winquist et al., 1985). Similar effects have been observed in the human coronary artery (Thom et al., 1987). Griffith et al., (1984) were the first to report a half-life of endothelium-derived relaxing factor released by acetylcholine from the rabbit aorta. Similar results were obtained in the canine femoral artery with acetylcholine and in cultured bovined endothelial cells on microcarrier beads with bradykinin (Cocks and Angus,1985).

In various experimental models of atherosclerosis a substantial decrease in endothelium-dependent relaxation is obvious in response to a variety of stimuli. In the regenerated endothelium model if the animals are fed a high-cholesterol diet following endothelial denudation, endothelium-mediated responses steadily disappear followed by an ultra-rapid form of atherosclerosis. The most important mechanism in the decrease in endothelium-dependent response is reduced release of EDRF; nevertheless, as the disease progresses and the artery thickens and stiffens, it becomes increasingly difficult for NO-EDRF to react smooth muscle that is still able to relax. It is tempting to hypothesize that endothelial dysfunction is a fundamental initial step on the progression of atherosclerosis. As a result, larger sections of the endothelium become unable to resist platelet adhesion and aggregation and respond less well to thrombin formation. The feedback effect of EDRF on platelet aggregation steadily decreases, while vasoconstrictor factors (serotonin and thromboxane) are released in increasingly greater amounts, together with growth factor (PDGF) which is probably responsible for the characteristic morphological changes in atherosclerosis.

The pathogenesis of atherosclerosis

Atherosclerosis is the principal cause of heart attack, stroke and unstable angina. The lesions result from an excessive, inflammatory fibroproliferative response to various forms of insult to the endothelium and smooth muscle of the artery wall.

The earliest recognizable lesion of atherosclerosis is the so called 'fatty streak', an aggregation of lipid-rich macrophages and T lymphocytes within the innermost layer of the artery wall. Animal observations have shown that fatty streaks precede the development of intermediate lesions, which are composed of layers of macrophages and smooth

muscle cells and, in turn, developed into the more advanced, complex, occlusive lesions by projecting into the arterial lumen, may impede the flow of blood. The fibrous plaques contain monocyte - derived macrophages, smooth muscle cells and T lymphocyte, many of which are activated. Recent data have shown that most of the sudden deaths from myocardial infarcts are due to ruptures or fissures, particularly in the margins of the fibrous cap where there are more macrophages, resulting in hemorrhage into the plaque, thrombosis and occlusion of the artery (Ross, 1993).

Atherosclerosis is a complex multifactorial process involving various types of cell and numerous systems. The following cellular processes implicated in the pathogenesis of the atherosclerosis lesion can be observed in the sequence of events (Fig. 2.3).

Vascular injury which leads to endothelial cell dysfunction results from several different causes such as hypertension, hypercholesterolemia, or chemical irritants (Badimon et al.,1993) It is evidenced that the normal vascular release of endothelium-derived relaxing and contracting factors (EDRF) in response to circulating agonists may be altered, reducing the local luminal cross-sectional area, further altering the endothelium and increasing the local conditions of the interactions between circulating cells and the endothelium. Once the endothelium is damaged, platelets adhere, aggregate and release many vasoactive factors, including platelet-derived growth factor (PDGF), which stimulates cell proliferation and thromboxne A_2, which causes vasoconstriction and further platelet aggregation (Hollenberg, 1991). After that, there are aaccumulation of monocytes, macrophages and lipid into the arterial wall. Adherence of monocytes to the endothelium is one of the earliest observable abnormalities of the vessel wall in hypercholesterolemic animals which occurs in parallel with an impaired relaxation mediated by EDRF. Monocytes-derived macrophages that are trapped in the

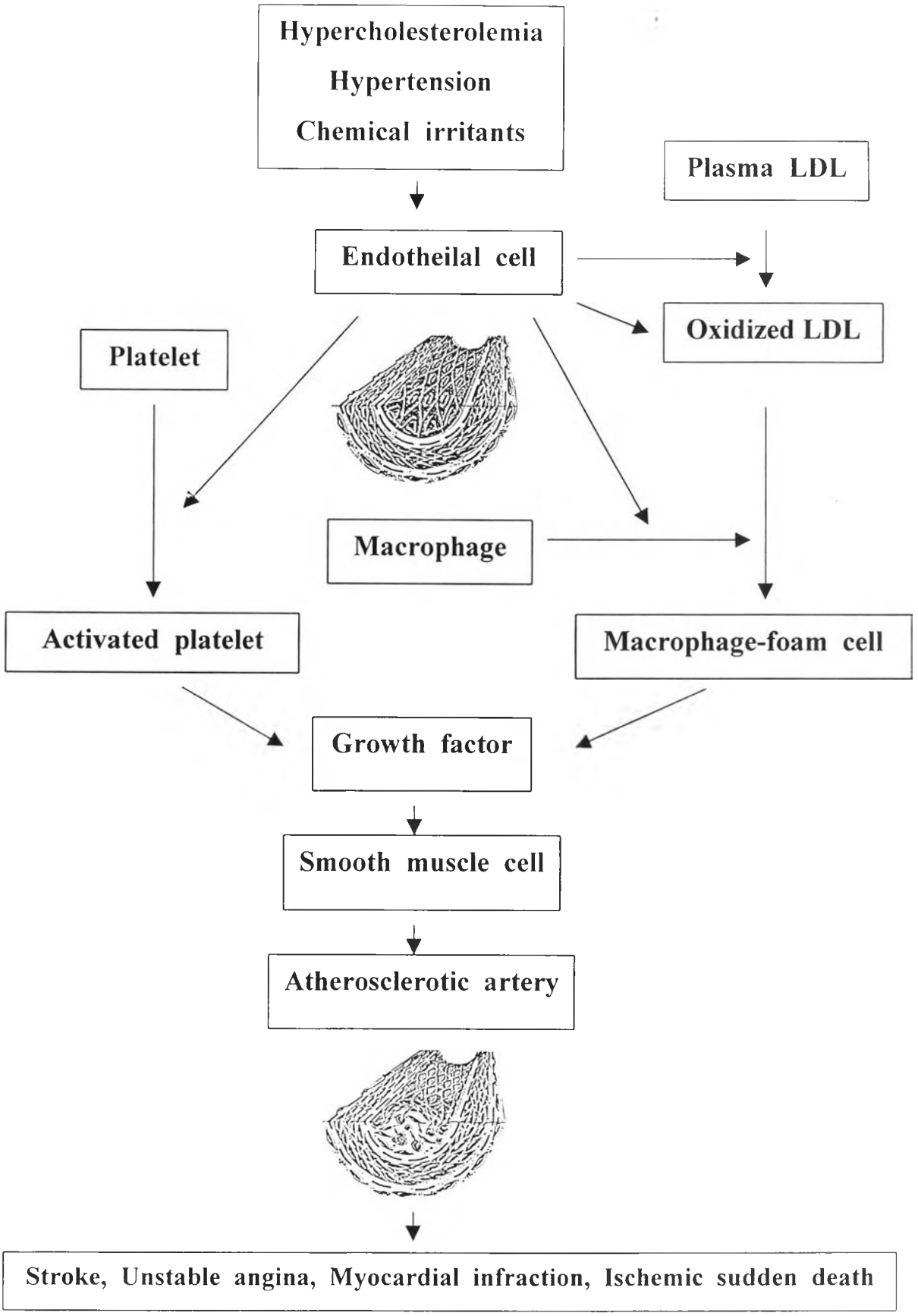


Fig. 2.3 Schematic illustration of pathophysiology of atherosclerosis. (Phivthong-ngam, 1997 and Ross, 1993)

intima actively ingest lipid and produce numerous cytokines and growth factors (Selke et al., 1990).

Modification and deposition of lipids are the main sectors involved in atherosclerosis. Clinical and experimental studies have firmly established that the lipids deposited in the atherosclelotic lesions are mostly derived from plasma LDL, which has been either conjugated with malondialdehyde, an aldehyde product of lipid peroxidation (Steiberg et al., 1989) by endothelial cells, smooth muscle cells or macrophages (Haberland et al., 1990 And Naito et al., 1995). The oxidatively modified LDL is taken up rapidly by scavenger receptors on macrophages. Oxidized LDL (oxLDL) is highly cytotoxic (Witztum and Steinberg, 1991). Once formed within the vascular wall, oxLDL may directly injure the endothelium. It induces functional changes in the endothelial cells that favor the penetration of circulating monocytes and the movement of LDL into the subendothelial space and thus accelerates the formation of the fatty streak (Steinberg et al., 1989). In regard to mitogenisis, macrophages can secrete a mitogenic factor similar to PDGF, thus has an influence on the vascular smooth muscle cell proliferation and stimulation of plaque formation (Prieto et al., 1988). Migration of smooth muscle cells from the media to intima and proliferation of connective tissue resulting in the formation of a sub-endothelial fibro-muscular cap which may compromise the integrity of the arterial lumen to a considerable degree. Necrosis of connective tissue at the plaque base leading to the formation of a soft, deformable atheromatous pool. If this is of massive proportions in relation to the fibro-muscular cap, plaque rupture may occur (Ross,1993) .