CHAPTER II
LITERATURE REVIEW

2.1 Phytoestrogens; chemical approach

2.1.1 Origin and classification

A phytoestrogen is any plant derived compound that induces biological responses in vertebrates and can mimic or modulate the actions of endogenous estrogens usually by binding to estrogen receptors. It was firstly found in the outbreak of infertility in sheep grazing on pastures rich in subterranean clover (Bennets et al., 1946). Phytoestrogen is subdivision of flavonoids and classified as isoflavonoids, lignans and coumestans, which distributed in various plants (Table 2.1). Isoflavonoids are found predominately in soybeans (*Glycine max* L.), whereas coumestans have been preliminary identified by genus of *Trifolium* such as clovers (Franke et al., 1994) and species of *Pueraria* and Glycyrrhiza (Dewick, 1993). Genistein and daidzein is the major isoflavonoids found in soybeans and their products. The available data especially in epidemiological study are examined the relationship between the consumption of natural food containing phytoestrogens and the reduced risk of cardiovascular symptoms, cancer and osteoporosis.

Chemical structure of phytoestrogen is diphenolic (better bisphenolic) compounds that comprised of 2 benzene rings (A and B) linked through a heterocyclic pyran or pyrone ring (C) in the middle (Figure 2.1). The basic structure of phytoestrogens is closely similar to natural and synthetic estrogens and antiestrogens such as resorcyclic acid lactones (e.g. zearalenone). When both structures of phytoestrogen and estradiol are superimposed, the distance between the hydroxyl groups is identical (Figure 2.1). Isoflavonoids are often present as glucoside conjugates (glycones) such as genistin, daidzin and glycitin. These glycosides can be further metabolized in gut to aglycone such as genistein, daidzein and glycitein. Based on the structural similarities, phytoestrogens can bind to estrogen receptors (ERs) (Setchell, 1998 and Hopert et al., 1998) and act as a weak estrogen (Setchell, 1998).

Table 2.1 Classification and sources of phytoestrogens (adapted from Krazeisen et al., 2001 and Cornwell et al., 2004)

Class	Examples	Sources
Flavonoid		
Isoflavonoids	• Genistein	• Soybeans, beer
	• Daidzein, Equol	• Clover, beer, soybeans
	• Glycitein	• Soybeans
	• Biochanin A	• Red clover, beer, bourbon
	• Formonoetin	• Red clover
Coumestans	• Coumestrol	• Clover, alfalfa, beans, *Pueraria* sp.
Prenyl flavonoids	• 8-Prenylnarigenin	• Grapefruit
	• 6-Prenylnarigenin	• Grapefruit
Non-flavoniods		
Lignans	• Isolaricriresinol	
	• Matairesinol	• Flaxseed, black gram, tomato, strawberries
	• Secoisolariciresinol	
	- (Enterodiol)	• Oilseed, flaxseed, black gram, tomato, strawberries
	- (Enterolactone)	• Linseed, flaxseed, cereal bran, whole cereals, vegetable, fruits, legumes

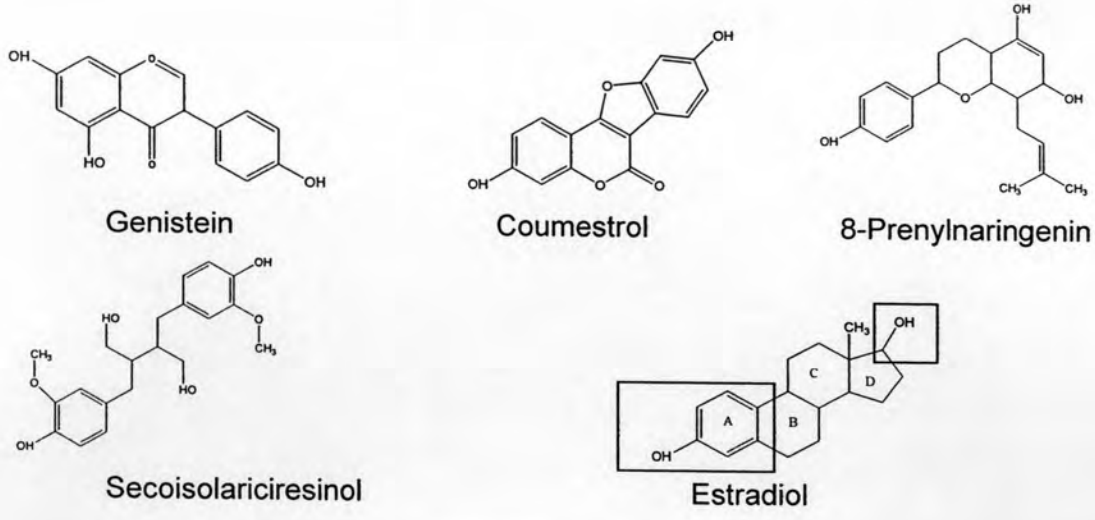


Figure 2.1 The similarity of the structure of the human hormone, estradiol and phytoestrogen examples. Outlined in boxed on estraidol structure of phenolic and hydroxyl moieties. The distance between the two groups in each compound is similar.

2.1.2 Analysis of phytoestrogens

Generally, phytoestrogens and their metabolites are present in part per billion to part per million in plants, foodstuff as well as in biological fluids such as urine, plasma and feces. Initially, phytoestrogens were analyzed using simply techniques such as thin-layer and paper chromatography. However, the development of increasingly sensitive technologies has advanced phytoestrogen analysis considerably. The most widely used methods for quantify of phytoestrogens are high performance liquid chromatography with ultraviolet detection (HPLC-UV) (Wang et al., 1990 and Thomas et al., 2001), gas chromatography with mass spectrometric detection (GC-MS) (Mazur et al, 1996; Tekel et al., 1999 and Nesbitt, Lam and Thompson, 1999) and liquid chromatography with mass spectrometric detection (LC-MS) (Coward et al., 1996; Cimino et al., 1999 and Doerge, Churchwell, and Delclos et al., 2000).

These developments have also been useful in pharmacological and toxicological studies. Prior to analysis and their metabolites, phytoestrogens must be isolated from matrices. The extraction of phytoestrogens is required. During extraction, phytoestrogen might be lost; an appropriate internal standard must be added prior to extraction. A general schematic of the steps involved in extraction and anyalyse of phytoestrogens is shown in Figure 2.2

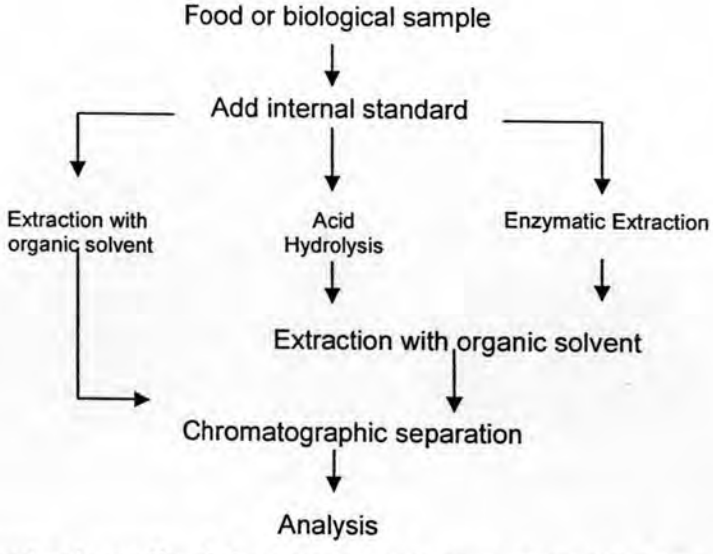


Figure 2.2 A general schematic of the steps involved in extraction and analysis of phytoestrogens (Adlercreutz et al., 1986 and Franke et al., 1994).

2.2 Phytoestrogens; physiological approach

2.2.1 Phytoestrogen action

Numerous studies have shown that phytoestrogens can exert multi-functions through genomic and non-genomic mechanisms of cellular regulation by competing to estrogen receptor binding or interfering to estrogen biosynthesis and metabolism.

2.2.1.1 Cellular and molecular mechanisms

According to the classic concept, estrogens are steroid hormones, which involved in the important functions for the sexual processes and act through protein, estrogen receptors which distributed in reproductive tissues such as ovary, mammary, uterus and vagina. Until recently, ER has been identified into two subtypes, ERβ and ERα. ERβ, discovered only a few years ago, is heterodimers in DNA binding domain (over 95% amino acid identity) and splicing variants of ERα (Kuiper et al., 1996 and Mosselman et al., 1996). ERs are predominately present in nucleus where is formed the complex with heat shock proteins when received the stimulating (Figure 2.3 and 2.4). Bound ERs are activated to the specific DNA-binding sites called estrogen receptor response elements (ERE) or AP-1 site. After binding, the target gene transcription in initiated or repressed which ultimately elicits biological responses as agonist or antagonist characters (Clark, et al., 1996; Fitzpatrick, 1999 and Diel, Smolnikar, and MIchna, 1999) depending on the phytoestrogens concentration and target organ (Setchell et al., 1998). If phytoestrogens induce biological effect as estradiol, they are considered as agonist, however, the potency is too weak that require much higher concentrations to boost the responses as well as estradiol. On the other hand, phytoestrogens can act as antagonists by block the binding of estrogen that caused an interrupted hormone response.

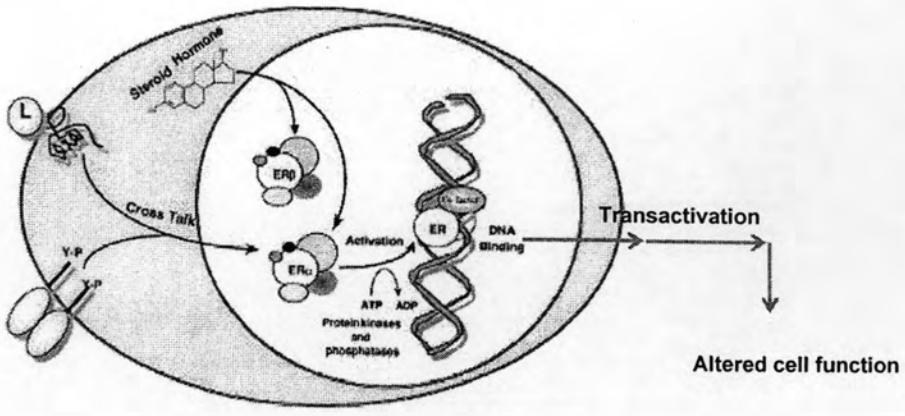


Figure 2.3 Mechanisms of estrogen receptor activation on target cells (Adapted from Diel, Schmidt, and Vollmer, 2002)

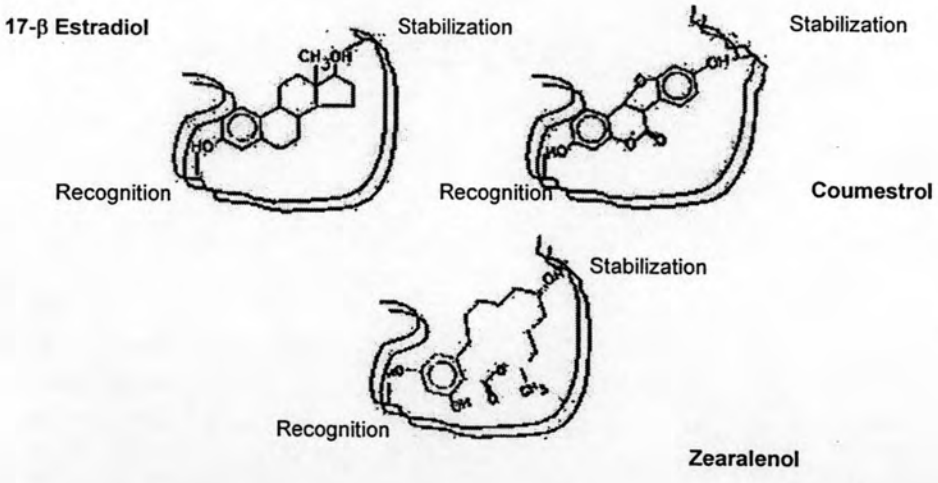


Figure 2.4 Structure-activity relationships of estrogen and some phytoestrogens (coumestrol and zearalenol) on estrogen receptor lignands (Clarke et al., 1995)

2.2.1.2 Biotransformation and metabolism

The metabolisms of isoflavonoids and lignans show similar patterns in animal (Price and Fenwick, 1985) and human (Adlercreutz et al., 1991) whereas coumestan have not been identities. After consumption, isoflavone and lignan glycosides are probably hydrolyzed within gastrointestinal tract by gastric acid (Xu et al., 1995) and intestinal microflora hydrolysis enzymes. The precursor of genistein and daidzein are biochanin A and formononetin, respectively (Figure 2.5). After absorption, isoflavonoids are transported to the liver, reconjugated and then excreted in urine and bile. The reconjugation of aglycone with glucuronic acid and sulfuric acid is function by hepatic phase II enzymes (Morton et al., 1994 and Adlercreutz et al., 1993). However, genistin was partly absorbed without previous cleavage (Andlauer, Kolb, and Fürst, 2000). In human, aglycones were absorbed faster and in greater amounts than their glycosides (Izumi et al., 2000). The maximum peak of isoflavonoids is range at 7- 8 hr after consuming a single soy meal (King and Bursill, 1998). Those isoflovones have been detected in biological fluid including plasma (Adlercreutz et al., 1994), amniotic fluid (Adlercreutz et al., 1999), urine (Adlercreutz et al., 1991), feces (Adlercreutz, et a., 1995), milk (Franke and Custer, 1996), saliva, breast aspirate (Hargreaves et al., 1999) and prostatic fluid (Finlay et al., 1991).

Biochanin A and formononetin are metabolized by gut microflora to genistein and daidzein, respectively. Genistein can be further metabolized to 4-ethylphenol and daidzein to equol, dihydrodaidzein and *O*-desmethylangolensin (Anderson and Garner, 1997). The data suggest that equol has a greater antioxidant effect than other phytoestrogens, which often found in highest level in biological matrices and exert significant biological effects (Hodgson et al., 1996).

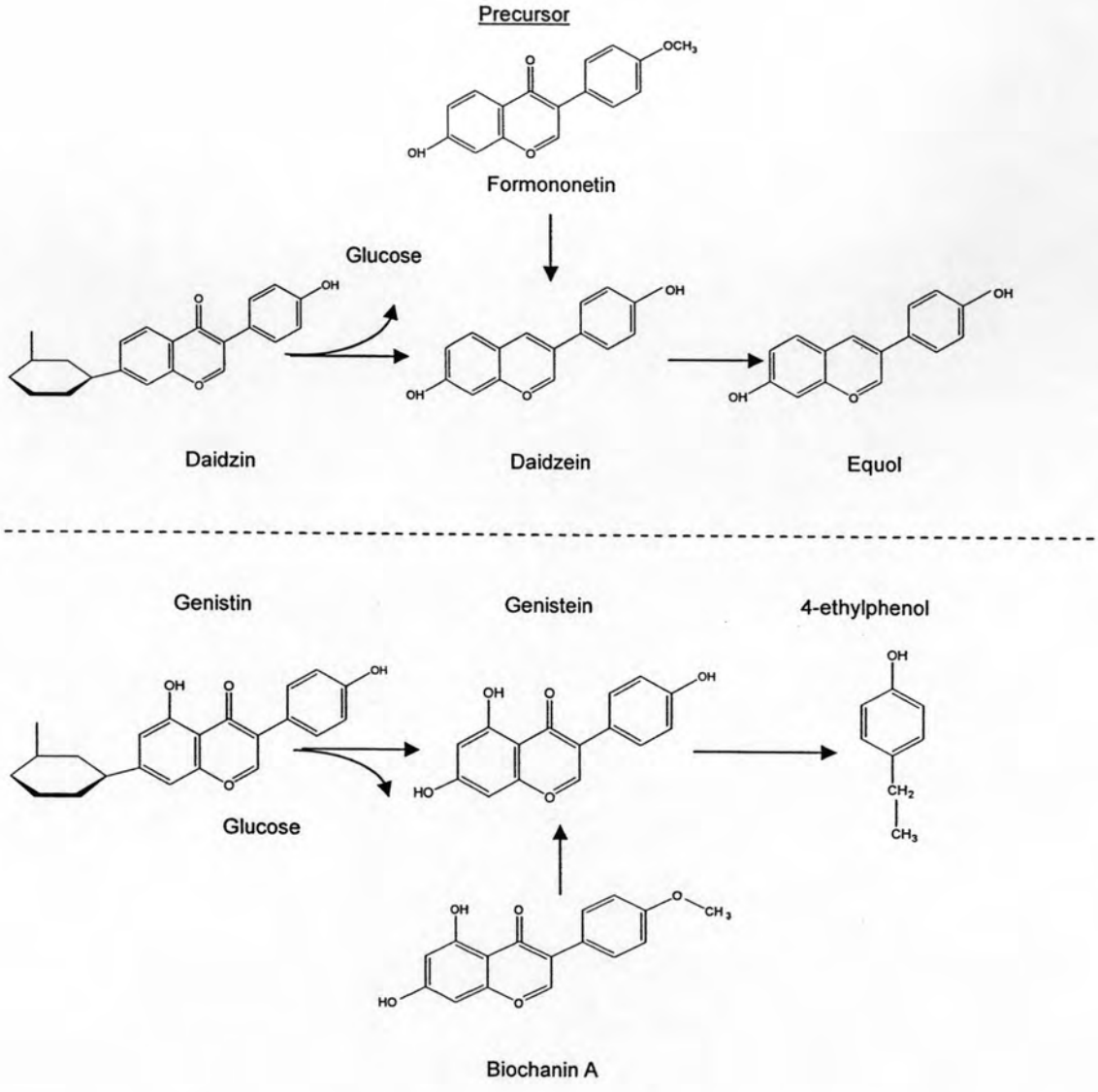


Figure 2.5 Schematic of some phytoestrogen metabolism in intestine (Anderson and Garner, 1997).

2.2.1.3 Phytoestrogens potency and active concentration

Bound compound to both type of ERs can stimulate transcription of ERE, with different tissue expression patterns. The splicing variants lead to different relative binding affinities in different compounds. Assessment of estrogenic potency varies across assays such as receptor binding affinity, transcriptional activation, cell proliferation and *in vivo* assays. It is derived from the difference in copies of EREs in used cells. Generally, a low concentration of phytoestrogen (1-100 nM) stimulates

cell growth, whereas, a high concentration (5-100 μM) shows suppression. This is called a biphasic pattern.

Phytoestrogens can bind with higher affinity to ERβ on transcriptional level than to ERα (in which amount %, should mention) (Kuiper, et al., 1997 and 1998 and Casanova et al., 1999). Phytoestrogens can affect endogenous hormonal production by inhibiting the key enzyme of hormonal biosynthesis (Evans, Griffiths and Morton, 1995; Kao et al, 1998 and Makela et al., 1998) or stimulating of sex hormone binding globulin (SHBG) (Adlercreutz et al., 1987), however, the estrogenic potency is less than estradiol. The proliferation action occurs at nanomolar concentration level. *In vitro* study, the concentration of phytoestrogens to elicit a response on ERα and ERβ site are 0.145 and 0.0084 μM, respectively (IC_{50} values for competition of estradiol-recepter binding) (Kuiper et al., 1998). Compared with all phytoestrogens, coumestrol has the highest estrogenic potency (Kuiper et al., 1997), however, an affinity to ERα is ten times less than estradiol. It is noticed that the methoxy derivative of genistein and biochanin A does not bind ER, but they can show estrogenic activity *in vivo*. This might be the role of hydroxyl substituents at 4' and 7' positions in phytoestrogen groups (Figure 2.4). The ranking of phytoestrogens potency as compared with estradiol is estradiol > coumestrol > 8-prenylnaringenin > equol ≥ genistein > biochaninA > daidzein > genistin > glucoronide > daidzin > glucoronide > formononetin (Kuiper et al., 1997 and Milligan et al., 2000) (Table 2.2).

Table 2.2 Relative binding activity of estrogen and various phytoestrogens binding to ERα and ERβ

Compound	**Binding at ERα**	**Binding at ERβ**
17- β Estradiol[a]	100	100
Estrone[a]	60	37
Estriol[a]	14	21
Progesterone[a]	<0.001	<0.001
Testosterone[a]	<0.01	<0.01
Coumestrol[a]	94	185
Genistein[a]	5	36
Daidzein[a]	0.2	1
8-Prenylnaringenin[b]	10	10
β-Sitosterol[a]	<0.001	<0.001
Tamoxifen[a]	7	6

([a]Kuiper et al., 1997 and [b]Milligan et al., 2000)

2.2.2 Biological and pharmacological effects

2.2.2.1 Hormonal effects

The hormonal effects have been shown in both animal and human, however, the results have been inconsistency depending on the analytical methods. Phytoestrogens are quite weak according to both *in vitro* and *in vivo* assays, which possessing less than activity of estradiol between 1,000 and 10,000 (Folman and Pope, 1966 and Markiewicz et al., 1993). The phytoestrogen-rich plant, red clover demonstrated hyperestrogenize and infertility in grazing animals (Bennets, Underwood and Shier 1946 and Shutt and Braden, 1968). In uterine growth of female mice, subcutaneous injection of genistein could inhibit the stimulation of estrone (Folman and Pope, 1966). The length of the follicular phase in pre-menopausal women is increased when administered within isoflavone-rich diet (Cassidy, Bingham and Setchell, 1995 and Lu et al., 1996). On the other hand, progesterone (Lu et al., 1996 and Lu et al., 2000) and testosterone could be decreased (Strauss et al., 1998). Estradiol-17β in serum is also affected (Cassidy, Bingham, and Setchell, 1995). At high dosages of isoflavonoids, the feedback-regulating system of the hypothalamus-pituitary gland axis is interrupted and affected to hormonal status.

Genistein has been shown to exert both estrogenic and antiestrogenic activity in human cell lines (MCF-7) (Wang, Sathyamoorthy, and Phang, 1996 and Sathyamoorthy, Wang, and Phang, 1994). In cell culture, the low concentration of those compounds to stimulate estrogen-dependent receptor gene activity is 10-1000 nM (Kuiper et al., 1998). Moreover, some reports have shown that soy isoflavonoids could increase nerve growth factor mRNA and brain-derived neurotropic factor mRNA in rats (Pan, Anthony and Clarkson, 1999) and also affect the cell signaling conduction on receptor expression. At high concentration of genistein, EGF receptor expression is inhibited in rat uterus and vagina (Akiyama et al., 1987 and Brown and Lamartiniere, 2000). Phytoestrogens may involve in estrogen synthesis and metabolism through interfering and/or inhibiting estrogen related enzyme such as 17-β-hydroxysteroid oxidoreductase; the conversion catalyst of estrone to estradiol (Makela et al, 1995), aromatase (Kellis and Vickery, 1984 and Pelissero et al., 1996) and steroid sulphatase (Murkies, Wilcox, and Davis, 1998 and Krazeisen et al., 2001). Aromatase converts the androgens, dehydroepiandosterone and testrone to estrogen and estradiol, repectively. Inhibition of these enzymes would alter the

balance between estradiol and the less potent of estrone. Genistein and daidzein also suppressed glucocorticoid and stimuated androgen production in human adrenal cortical cells cultures (Mesiano et al., 1999).

In HepG2 liver cancer cells culture, isoflavonoids have been reported to stimulate the biosynthesis of SHBG. It is noticed that the change of SHBG concentration affect in relatively large changes in amount of free and bound hormones whereas the change of total hormone concentration affect in relatively small changes (Loukovaara et al., 1995 and Mousavi and Adlercreutz, 1993). To sum up, the action of phytoestrogen depends on the hormonal status of animal and human.

2.2.2.2 Anticarcinogenic effect

There are evidences in human studies, pointing to the potency of soy products or phytoestrogen such as genistein or daidzein to inhibit and prevent on various cancers such as endometrial (Goodman et al., 1997), prostate, (Jacobsen, Knutsen and Fraser, 1998; Herbert et al., 1998; Kolonel et al., 2000 and Strom et al., 1999), stomach (Nagata, 2000 and Nagata et al., 2002), colon (Nagata, 2000), thyroid (Horn-Ross, Hoggatt and Lee, 2002), lung (Seow et al., 2002) and mammary (Lee et al., 1991; Yuan et al., 1995; Hirose et al., 1995; Wu et al., 1996; Ingram et al., 1997; Witte et al., 1997; Key et al., 1999; Dai et al., 2001 and Shu et al., 2001). It is noticed that all these cancers are hormone-dependent.

Understandably, anticancer effects of soybean have primarily attracted attention on breast cancer. The low incidence of breast cancer in East Asia was found associated with consumption of typically Asian/oriental diet (Tham, Gardner and Haskell, 1998). Animal studies support the notion that phytoestrogen have also been found to exert the inhibition of chemically induced mammary cancer (Barnes et al., 1990; Lamartiniere et al., 1995a, Murrill et al., 1996 and Gotoh et al., 1998a and 1998b). It was confirmed *in vitro* studies, isoflavonoids inhibit cancer cell growth including prostate cancer cell line (Davis et al., 1998 and Hillman et al., 2001) and MCF-7 human breast cancer cell line (Hsu, Ying, and Chen, 2000).

There is a correlation of reduced risk of breast cancer with a high plasma level of phytoestrogens such as mammalian lignan, enterolactone (Pietinen et al.,

2001). Several reports have shown that genistein can inhibit the growth of both hormone-dependent and hormone-independent cancers with IC_{50} at 5 - 100 μM/L (2-25 ug/mL) (reviewed in Messina, 1999). However, genistein can stimulate those cancers at physiologically concentrations (<5-6 μmol/L) (Zhang et al., 1999 and Ren et al., 2001).

The numerous mechanisms may be involved such as the inhibition of angiogenesis (Fotsis et al., 1993), protein tyrosine kinases (Akiyama et al., 1987) and related hormonal enzymes. The preventive effect of isoflavonoids may involve the decreasing synthesis and altering metabolism form of estrogen (Xu et al., 1998 and 2000). In addition, genistein has been shown to inhibit the metastatic activity of breast cancer (Scholar and Toews, 1994) and prostate cancer cells (Santibanez, Navarro and Martinez, 1997). Cell cycle progression at G_2-M is arrested by genistein that result to the differentiation and apoptosis of various cancer cell lines including human gastric cancer (Yanagihara et al., 1993), human breast carcinoma (Shao et al., 1998), leukemia (Spinozzi, et al., 1994), melanoma (Rauth, Kichina, and Green, 1997) and colon (Kuo, 1996).

In vitro and *in vivo* studies, at micro molar concentration level of phytoestrogens exert various non-hormonal related effects. Genistein was shown to inhibit DNA topoisomerase I and II (Yamashita, Kawada, and Nakono, 1990; Ji et al., 1999; Martin et al., 2000 and Salti et al., 2000) that effected to DNA damage and epidermal growth factor-induced phosphatidylinositol turnover (Imoto et al., 1988). Biochanin A is linked to increase nitric oxide level that later induction of cell apoptosis (Hsu et al., 1999). In addition, phytoestrogens may exert their effects by decreasing the activity of enzymes that activate procarcinogens, such as cytochrome P450 (CYPs) (Roberts-Kirchhoff et al., 1999).

2.2.2.3 Other effects

Epidemiological observations and laboratory animals and *in vitro* investigations have revealed a number of biological properties suggesting a prevention of western diseases such as cardiovascular, atherosclerosis, hypercholesterolemia, menopausal symptoms and osteoporosis (review in Kurzer and Hu 1997; Bingham et al., 1998 and Tham et al., 1998).

In vivo and epidemiologic studies have demonstrated that soy protein reduced risk of coronary heart disease and atherosclerosis (Anderson et al., 1999; Anthony, 2000, and van der Schouw et al., 2000). The effects result from a reduction of plasma low-density lipoprotein (LDL) (Tovar-Palacio et al., 1998; Crouse et al., 1999 and Ashton and Ball, 2000) cholesterol and triglycerides (Anderson, Johnstone, and Cook-Newell, 1995 and Ho et al., 2000). Soy could reduce absorption of dietary (Greaves et al., 2000), arterial permeability, concentration and delivery of LDL (Wagner et al., 2000) and increased LDL receptor quantity and activity (Baum et al., 1998).

Many studies suggest that phytoestrogens play role in maintaining bone density in postmenopausal women (Dalais et al., 2003; Alekel et al., 2000 and Kim et al., 2002). In animal studies, isoflavonoids could prevent bone loss that occurs as a result of estrogen deficiency in ovariectomized rats (Fanti et al., 1998; Vincent and Fitzpatrick, 2000; Picherit et al., 2000 and Uesugi et al., 2001). Postmenopausal woman seems to benefit the most from consumption of soy phytoestrogens. Bone mineral density (BMD) of the lumbar spine is increased with the treatment of 90 mg. isoflavonoids per day for 24 weeks (Potter et al., 1998). Osteoclastic bone resorption is inhibited by genistein and daidzein (Ono, Ma, and Yamaguchi, 2000) but stimuted osteoblastic bone formation (Yamaguchi, Gao, and Ma, 2000). However, data available in human about the effect of isoflavonoids on osteoporesis is limited.

Since estrogen has an important effect on the immune system. A changing of estrogen level result to autoimmune diseases that commonly occurs in women (Enmark and Gustafsson 1998 and 1999). Isoflavonoids have also exerted an anti-inflammatory potential in various animal models. High dosages of daidzein (20 and 40 mg/kg) can enhance several immonologic function (Zhang, Li, and Wang, 1997) and is proven to increase the activation of murine lymphocytes (Wang, Higuchi, and Zhang, 1997). Moreover, isoflavone glucuronides are able to activate natural killer cells to increase the immune defenses of the body against cancer (Zhang et al., 1999).

2.3 The phytoestrogens of rich plant; *Pueraria mirifica*

2.3.1 *Pueraria mirifica*

Ethnobotany and application: *P. mirifica* have been long recorded as domestic consumption to promote youth in both male and female (Suntara, 1931). Traditionally, women in some area of Thailand consume a traditional Thai remedy that includes both tuberous powder of *P. mirifica* and Tripala; *Terminalia bellerica*, T*erminalis chebula* and *Phyllanthus emblica* to relieve vasomotor symptoms (hot flashes and night sweats) associated with menopause. Recently, *P. mirifica* powder is extracted and used in cosmetic industry such as breast cream, eye gel and skin moisturizer. The beneficial of skin application is used for breast firming and anti-wrinkle. Moreover, the plant powder packed in capsule was manufactured and distributed as food supplement for aging (Dweck, 2002).

Botanical characteristics and habitats: *Pueraria mirifica* Airy Shaw et. Suvatabandhu (synonym: *Pueraria candollei* Wall. ex Benth var. *mirifica* Airy Shaw et Suvatabandhu), is classified in the family Leguminosae, subfamily Papilinoideae, the same as soy, bean and pea (Ridley, 1967 and Suvatti, 1978). The plant is known as "Kwao Krua" or "Kwao Krua Kao (White Kwao Krua)". *P. mirifica* was previously referred as *B. superba* until February 1947, it was identified as a new species of *Pueraria* and was named *Pueraria mirifica* Airy Shaw et Suvatabandhu (Lakshnakara and Suvatabandhu, 1952).

The plant is a long-living twinning wood. The leaves were pinnately three foliate stipulate; terminal leaflet. The tuberous roots were varied in sizes and shapes. The flower was bluish-purple legume shaped, blooming during late January to early April. The length of the inflorescence of certain flowers was approximately 15-40 cm. The flower contained five sepals and the petals were one standard with two keels. The pod was slender typically short or elongate, smooth or hairy, including 1-10 single seeds when fully matured and dried which turned into various color. (Smitasiri and Wungjai, 1986 and Cherdshewasart unpublished) (Figure 2.10).

P. mirifica is found in the deciduous forest of the northern, western and northeastern parts of Thailand at the 80-800 meters level (Panriansaen, 2000 and Lakshnakara and Suvatabandhu,1952). Recent survey revealed that the plant is

distributed in at least 28 provinces (Subtaeng and Cherdshewasart, 2003). Variation within province is also found. The co-habitated plant of *P. mirifica* was typically teak and bamboo. The plant was not found in the forest with high-density trees. The vine of *P. mirifica* elongated for climbing over the trees while spreads on the ground in an open area (Panriansaen, 2000 and Panriansaen and Cherdshewasart, 2003). Attempts had been made to establish *in vitro* multiplication and plantation of *P. mirifica*. It was found that the plant tissue was responsed to plant hormones and plantlets could initiate from *in vitro* (Cherdshewasart et al., 1996). The derived plants could initiate tubers (Sompornpailin et al., 2003).

Chemical constituents: *P. mirifica* had been found to contain at least 20 chemicals in the group of phytoestrogen with similar effects to estrogen. Miroestrol was the first isolated chemical and found in the amount of approximately 1.5 mg/ 100 g dry weight (Bound and Pope, 1960) and also shown estrogenic activity in rat vaginal cornification test (Jones and Pope, 1961). The chemical structure was not classified as steroid (Benson, Cowie and Howsking, 1961). The other compounds, mainly found in *P. mirifica* were isoflavonoids, chromenes, coumarins, sterols (Table 2.4 and figure 2.11) and macromolecule such as protein, lipid and starch (Appendix C). Deoxymiroestrol was isolated and found to be the compound with higher estrogenic potency than miroestrol, approximately 10-folds. However, it was easily oxidized by the air and converted to miroestrol and isomiroestrol (Chansakaow et al., 2000[a]).

The determination of isoflavone content, puerarin, daidzin, genistin, daidzein and genistein in the extracts of *P. mirifica* root from various location of Thailand by HPLC fingerprint assay revealed a great diversity of both total and individual assayed-isoflavone (Subtang, 2002 and Subtaeng and Cherdshewasart, 2003). The five isoflavonoids and isoflavone glycosides, daidzein, genistein puerarin, daidzin, and genistin had been used as markers. Whereas miroestrol cannot be used for quantitative standardization of *P.mirifica* root extract because no available commercialized standard.

Toxicity of *P. mirifica*: Several animal toxicology studies had been completed on *P. mirifica* using both crude powders and standardized extracts. *In vitro* study, *P. mirifica* root extract was not mutagenic by AMES test. (Julsiri and Cherdshewasart, 2003). LD_{50} of *P. mirifica* root extract, a single dose of 40 g/kg BW failed to induce signs of acute or subacute toxicity in mice (Chivapat et al., 2000). In long-term feeding experiments, a chronic toxicology study in rats treated orally with *P. mirifica* root extract at daily doses of 10, 100 and 1,000 mg/kg for 90 consecutive days revealed that at doses of 10 mg/kg BW or 100 mg/kg BW exhibited no toxic effects. However, in one study, a daily dose of 1000 mg/kg BW for 90 days induced reversible anemia and pathologic changes in the kidneys and testicles (Chivapat et al., 2000). The later study was found that plant powder and extract were evaluated and no toxicity was found (Cherdshewasart et al., 2000 and Cherdshewasart, 2003).

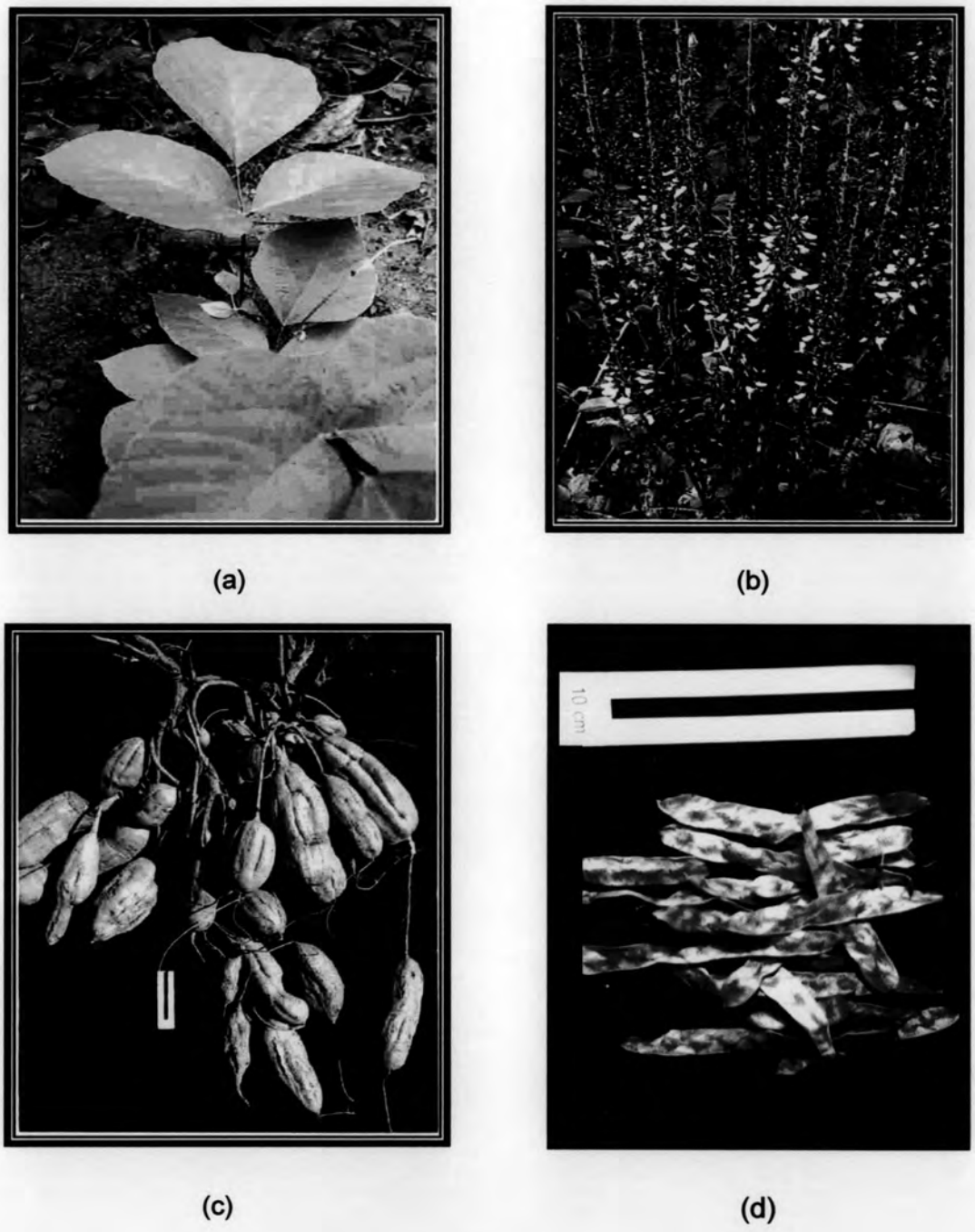
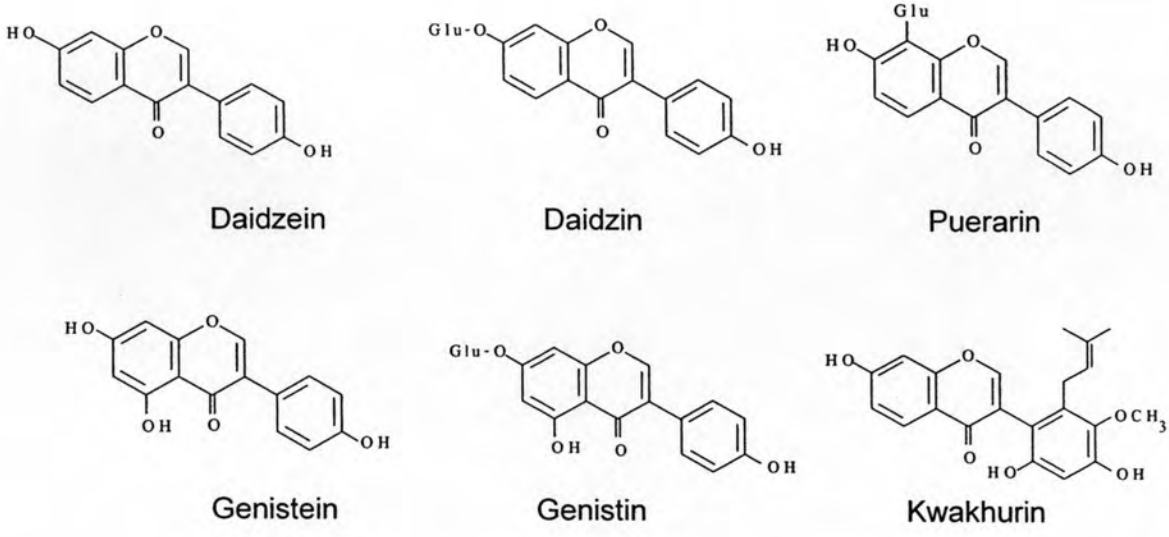


Figure 2.6 Leaves (a), flower (b), tuberous root (c) and pod (d) of *P. mirifica*

Table 2.3 Summary of the chemical constituents of *P. mirifica* (adapted from Panriansaen, 2000)

Category	Chemical	Reference
Isoflavonoids	Daidzein	Ingham et al., 1986
	Genistein	Ingham, Tahara and Dziedzic, 1986
	Kwakhurin	Ingham, Tahara and Dziedzic, 1986
	Kwakhurin hydrate	Ingham, Tahara and Dziedzic, 1989
Isoflavone	Daidzin	Ingham, Tahara and Dziedzic, 1986
glycosides	(daidzein-7-o-glucoside)	
	Genistin (genistein-7-o-glucoside)	Ingham, Taharaand Dziedzic, 1986 and 1989
	Mirificin	Ingham, Tahara and Dziedzic, 1986 and
	(puerarin 6"-o-β-apiofuranoside)	Ingham et al., 1986
	Puerarin (daidzein-8-glucoside)	Nilandihi et al., 1957
		Ingham, Tahara and Dziedzic, 1986 and 1989, Ingham et al., 1986
	Puerarin 6"-monoacetate	Ingham et al., 1989
Chromenes	Miroestrol	Schoeller, Dohrn and Hohweg, 1940
		Bound and Pope, 1960
		Jones and Pope, 1960
	Deoxymiroestrol	Chansakaew et al., 2000[a]
	Isomiroestrol	Chansakaew et al., 2000[a]
Coumestans	Coumestrol	Ingham, Tahara and Dziedzic, 1986 and 1988
	Mirificoumestan	Ingham, Tahara, and Dziedzic, 1988
	Mirificoumestan glycol	Ingham, Tahara, and Dziedzic, 1988
	Mirificoumestan hydrate	Ingham, Tahara, and Dziedzic, 1988
Sterols	β- sitosterol	Hoyodom, 1971
	Stigmasterol	Hoyadom, 1971
Pterocapans	Pueriicapene	Chansakaew et al., 2000[b]
	Tuberosin	Chansakaew et al., 2000[b]
Acid	Tetracosanoic acid	Chansakaew et al., 2000[b]

Isoflavone and Isoflavone glycosides



Coumestans

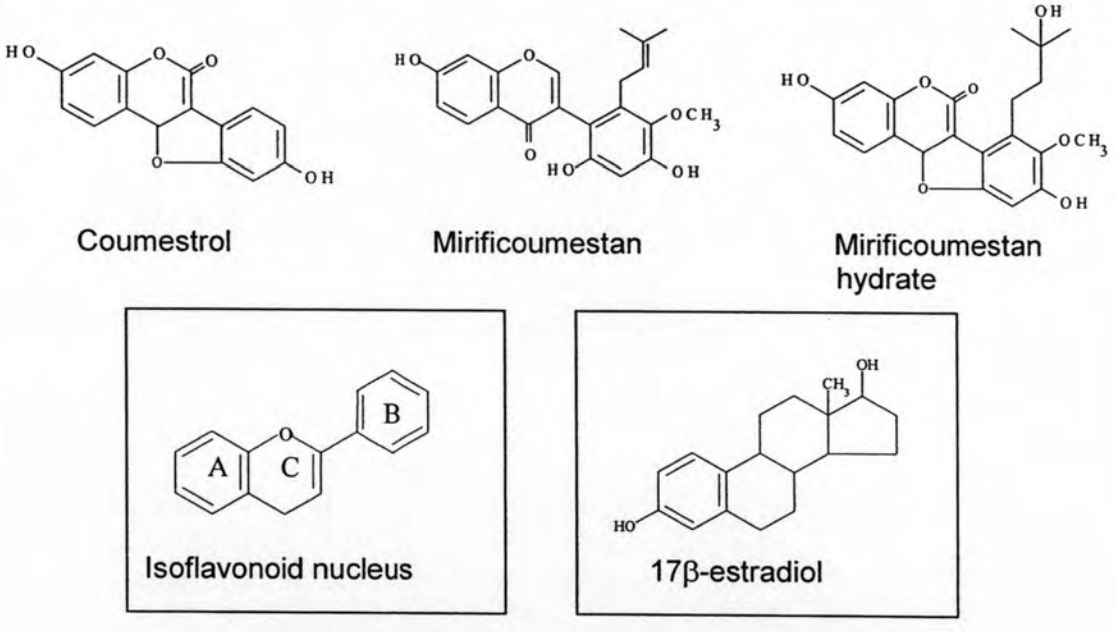
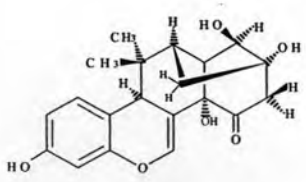
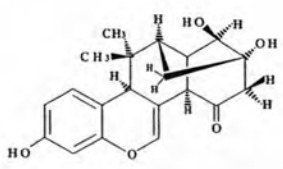


Figure 2.7 The structure of chemical compounds in *P. mirifica* and isoflavonoid nucleus compare with estrogen

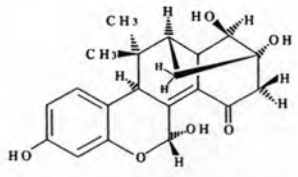
Chromenes



Miroestrol

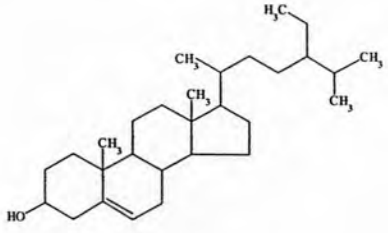


Deoxymiroestrol

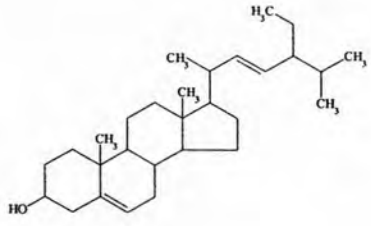


Isomiroestrol

Sterols

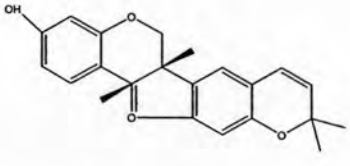


β-sitosterol

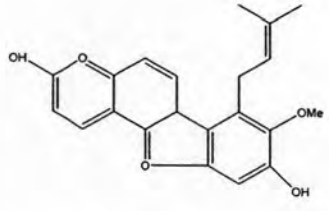


Stigmasterol

Pterocarpans



Tuberosin



Puemirificarpene

Figure 2.7 The structure of chemical compounds in *P. mirifica* and isoflavonoid nucleus compare with estrogen (continued)

Hormonal effect of *P. mirifica*: Many studies on *P. mirifica* were mostly evaluated in estrogenic activity on the reproductive system. In animal model, *P. mirifica* showed many effects depend on the method of bioassay such as dosage and period of exposure. The biological effects have been identified in Table 2.4. It was revealed that Miroestrol had 0.7 time of Estradiol on mammary gland proliferation activity (Pope et al., 1958). In ovariectomized rat, *P. mirifica* root extraction induced proliferation of the cornifined cell, increased uterus weight (Malaivijitnond, 2006) and exhibited strong estrogenic activity in uterotrophic assay (Kim et al., 2003). Uterus and vagina weight of *P. mirifica*-treated immature rat was

significantly increased (Sawatdipong, 1981). *P. mirifica* could influence the reproductive functions in both sex of rats, but the response was greater in male than female (Malaivijitnond et al., 2004; 2003a; 2003b and Kaitthaipipat, 2001). In monkey, a single dose of 1,000 mg/kg BW of *P. mirifica* disrupted ovarian function, menstrual cycle and also decreased the Parathyroid hormone and serum calcium level (Trisomboon, et al. 2004), *P. mirifica* greatly influences menstrual cycles and may suppress ovulation by lowering serum levels of gonadotropins (Trisomboon, et al. 2005), *P. mirifica* had estrogenic action by increasing reddish sexual skin coloration in aged menopausal monkeys (Trisomboon, et al. 2006b). In clinical trial, miroestrol (Cain, 1960) and the crude drug showed the effectiveness in treatment of menopausal symptom (Muangman and Cherdshewasart, 2001; Lamlertkittikul and Chandeying, 2004)

Estrogenic activity of *P. mirifica* was responded in dose-dependent manner on MCF-7 cells and HepG2 cells. The mechanism of action of the plant extract was evaluated. It was found that the chemicals needed a metabolic activation to promote their actions within human cells. Recombinant yeast exhibited no estrogenic activity because it lacked metabolic enzyme (Lee et. al., 2002). Crude extract of *P. mirifica* showed biphasic effect on the growth of MCF-7 as well as 17β-estradiol with proliferative effect at low concentration and antiproliferative effect at high concentration with ED_{50} value of 642.83 μg/mL (Cheewasopit, 2001; Cheewasopit et al., 2003; Trisap et al., 2003 and Cherdshewasart, Cheewasopit, and Picha, 2004a). While the crude extract of *P. mirifica* indicated no proliferative and anti-proliferative effect in HeLa cells at 100 and 1,000 μg/mL (Cherdshewasart, Cheewasopit and Picha, 2004b)

To compare the estrogenic activity in each compound on MCF-7 system, it was found that those compounds had different degree of estrogenic activity (17β-estradiol (lower 10^{-12}) > deoxymiroestrol (10^{-10}-10^{-9})>miroestrol (10^{-8})>coumestrol (10^{-7}) ≈ genistein (10^{-7}) > daidzein (10^{-6}) ≈ kwakhurin (10^{-3})). Whereas daidzin, puerarin, puermicapene, tuberosin and isomiroestrol had no estrogenic activity (Chansakaow et al., 2000a and 2000b) as shown in table 2.5.

Table 2.4 Summary of the recent reports of the biological effects of *P. mirifica* on animal model

Effects	References
1. Reproductive system	
1.1 Reproductive organ development	
• Promoted mammary duct and breast enlargement in mice, rat, pig	• Pope et al., 1958, Smitasiri, Pangjit , Anatalabhochai, 1986 and Panriansaen, 2005
• Proliferated the uterus , vaginal cornification in rat	• Sukhavachana, 1940, Sawatdipong, 1981and Malaivijitnond et al., 2006
• Estrogenic effect on Gonadotrophin levels ,sexual skin coloration in monkeys	• Trisomboon et al.,2006
1.2 Fertilization and birth control	
• Increased mating behavior	• Smitasiri, 1988
• Anti-fertilization	• Smitasiri and Pangjit, 1986, Smitasiri, 1988
• Induce abortion	• Sangkaew and Smitasiri, 1985, Smitasiri et al., 1986
• Reduction of sperm	• Langkalichan and Smitasiri, 1985
2. Others	
• Cholesterol level	• Thaiyanun, Trakulboon and Anuntalabhochai, 1992, Chivapat et al., 2000
• Calcium level	• Anuntalabhochai and Jersrichai, 1986, Bulintanthikul, 1978, Trisomboon, 2004

Table 2.5 The growth-promoting effects of chemical compound extracts of *P. mirifica* on MCF-7 human breast cancer cells. (Chansakaow et al., 2000a and 2000b)

Compounds	Content (mg/100 g powder)	Growth-promoting effects on MCF-7 (Minimal concentrations)
Isoflavone and glycoside		
Genistein	0.6	10^{-7}
Genistin	Data not shown	Data not shown
Daidzein	46.1	10^{-6}
Daidzin	8.5	No activity
Kwakhurin	0.6	$>10^{-6}$
CHROMENES		
Miroestrol	3.0	10^{-8}
Deoxymiroestrol	2.0	10^{-10}-10^{-9}
Isomiroestrol	2.2	no activity
Coumestrol	0.07	10^{-7}
PTEROCARPENS		
Tuberosin	0.3	No activity
Puemiricarpene	1.8	No activity
ACID		
Tetracosanoic acid	15.3	-
17β-estradiol (control)	-	$<10^{-12}$

* Minimal concentration of compounds that caused 50% MCF-7 breast caner cells growth when compared to the control

2.4 Reproductive cycle in rats

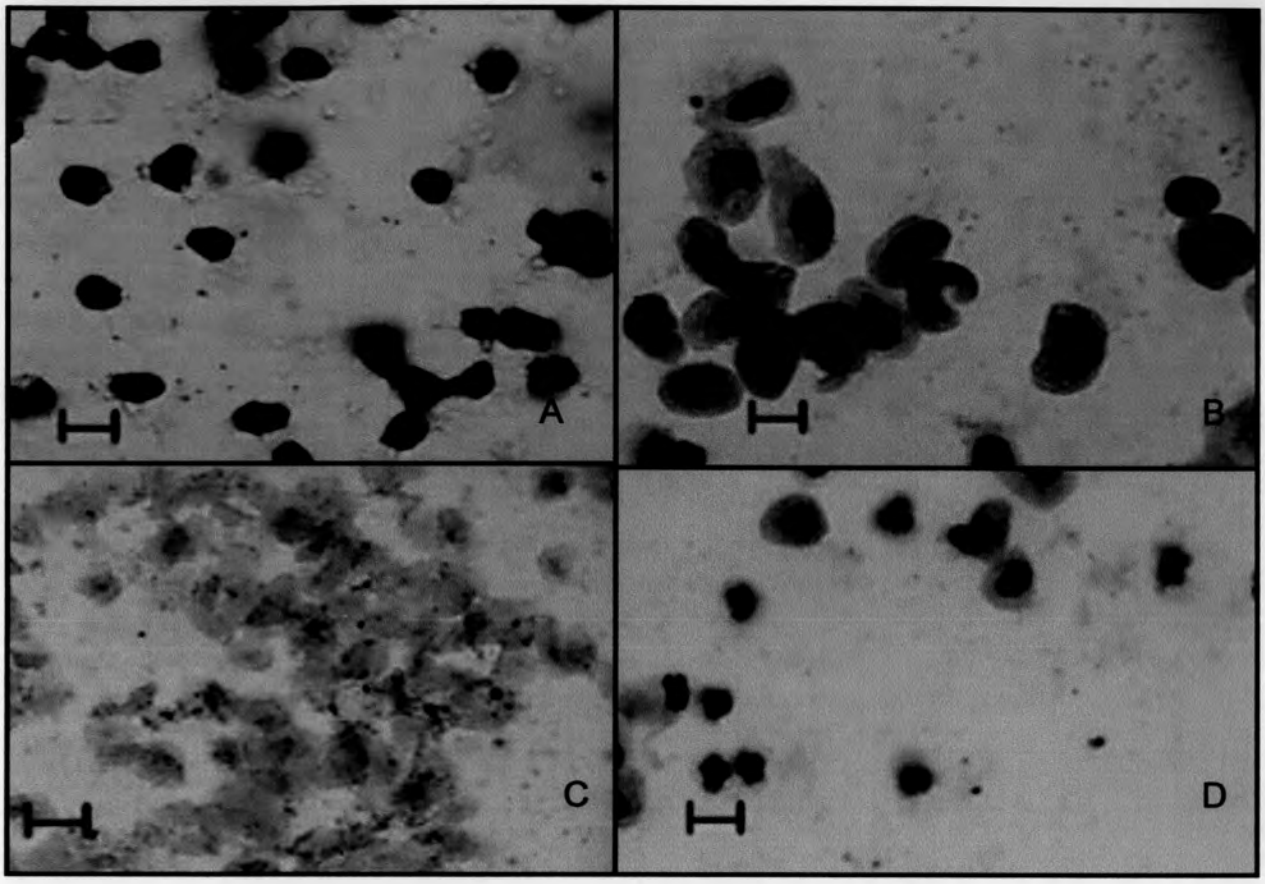
Reproductive cycle in rat is called estrous cycle which is exhibited in most mammals. The female animals showing estrous cycle are sexually receptive to males only around the time of ovulation (Johnson and Everitt, 1995). The rat estrous cycle is very short, only 4-5 days, although the timing of the cycle may be influenced by external factors such as light, temperature, nutritional status and social relationships. The cycle consists of 4 stages (Norris, 1997; Turner and Bagnara, 1976) as follows;

2.4.1 Estrous. The period of heat and copulation are permitted only at this time. The condition lasts from 9 to 15 hours and characterized by a high rate of running activity. Under the influence of FSH, a dozen or more ovarian follicles grow rapidly. Behavioral changes including quivering of the ears and lordosis, or arching the back in response to handling or that approaches by the male are found. The uteri undergo progressive enlargement and become distended owing to the accumulation of luminal fluid. Much mitosis occur in the vaginal mucosa and, as new cells accumulate. The superficial layers become squamous and cornified. The latter cells are exfoliated in the vaginal lumen, and their presence in vaginal smears is indicative of estrous. During late estrous, there are cheesy masses of cornified cells (Co) with degenerate nuclei present in the vaginal lumen, but few if any leucocyte are found during estrous. Ovulation occurs during estrous and is preceded by histologic changes in the follicle suggestive of early lutinization. Much of the luminal fluid in the uteri is lost before ovulation.

2.4.2 Metestrous. This occurs shortly after ovulation and intermediate between estrous and diestrous. The period lasts for 10 to 14 hours and mating is usually not permitted. The ovaries contain corpora lutea and small follicles. The uteri have diminished in vascularity and contractibility. Many leucocytes (L) appear in the vaginal lumen along with few cornified cells.

2.4.3 Diestrous. The period lasts 60 to 70 hours, during which functional regression of the corpora lutea occurs. The uteri are small, anemic, and only slightly contractile. The vaginal mucosa is thin, and leucocytes migrate through it. Vaginal smear appears entirely of leucocytes (L)

2.4.4 Proestrous. The next heat characterized by functional invulution of the corpura lutea and preovalutory setting of the follicles. Fluid accumulates in the uteri and they become highly contractile. The vaginal smear is dominated by nucleated epithalial cells (O) which occur singly or in sheets.



A. Diestrous is characterized by the prominence of leucocytes.These cells are small, round and can occur in large quantities

B. Proestrous, the smear is characterized by a prominence of nucleated epithelial cells, which are large, round and bear an easily visible nucleus.

C. Estrous is characterized by cornified cells, which are large and irregular. No leucocyte or nucleated cells are visible this time.

D. Metestrous consists of leucocytes, interspersed with nucleated and cornified cells.