CHAPTER II

LITERATURE REVIEW

1.Pueraria mirifica

1.1 Botanical background

White Kwao Krua, one of the indigenous Thai herbs with numerous tuberous roots, is classified into family Leguminocae, subfamily Papilionoidae, the same as soy. It was first discovered and classified as *Butea superba*. The plant was later recognized as a new species and reclassified as *Pueraria mirifica* (Kashemsanta *et al.*, 1952). Other Thai dialects of *P. mirifica* are Tong-krua, Tan-jom-tong, Po-ta-goo, Tan-krua and Jan-krua.

The plant is widely found in Thailand, particularly in the deciduous forests. The plant with twinning apperance is commonly found in abundant in the forests in the north, west and northeast of Thailand at the altitude of 300 -800 meters above sea level. The tuberous roots are varied in sizes (Figure 2-1). The flower is bluish-purple butterfly shaped, flowering during February-April. The leaf shape is closely similar to that of red Kwao Krua (*Butea superba*) that is tri-foliate, but thinner and smaller. Its tuberous root with white starch granules has a round or eclipse-shape (Kashemsanta *et al.*, 1957; Pisetpakasit, 1976) or other shapes (Cherdshewasart, 2003[a]). The length of the catkin in fluorescences is approximately 15-40 cm. It contains five sepals. The petal is one standard with two keels. The pod is slender typically short, hairy, including 1-10 single seeds when fully matured and dried which turned into brown color. The mature seed is varied from green to purple pattern (Cherdshewasart, 2003[a]).

The plant was found in 28 provinces in Thailand (Cherdshewasart, 2003[a]) with varied amount of isoflavone content (Subtang and Cherdshewasart, 2003). The plantlets could be initiated from *in vitro* culture (Cherdshewasart *et al.*, 1996) and could produce tubers in a field trial test (Cherdshewasart, 2003[a]).

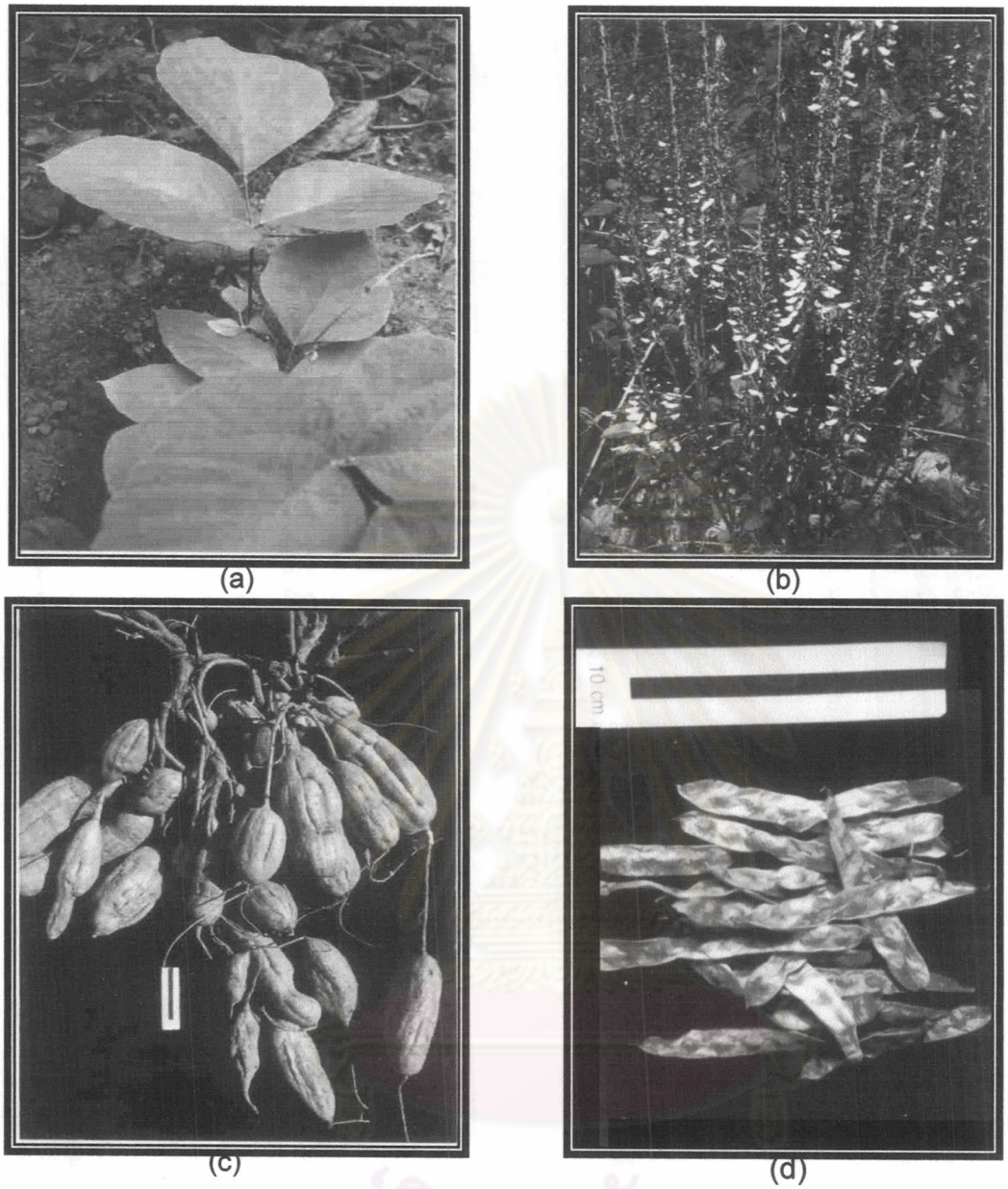


Figure 2-1. Leaves (a), flowers (b), tuberous roots (c) and pods (d) of *P. mirifica*

1.2 Traditional consumption of *P. mirifica*

From the plant tubers, a drug reputedly to have miraculous properties is made. The first account of the drug was in the form of a single leaflet, printed on one side only in Yuan (Northern Thai) characters without date, author, printer or place of printing. This leaflet pointed out that there are three kinds of the "Kwoa Krua"; Black, red and white. The White Kwao Krua (*Pueraria mirifica)* had the weakest effect. In the direction given, the tuber had to be sliced, dried and crushed into powder and mixed with honey. Of this mixture, a pill was made in the size of a peppercorn and use. Only one pill was taken daily at bed time. People under forty years of age or maturing stage were forbidden to take the *P. mirifica*. Taken for three to six months, the written leaflet said that these pills would cure all the 96 diseases, give long life and protect from danger (Kerr, 1932).

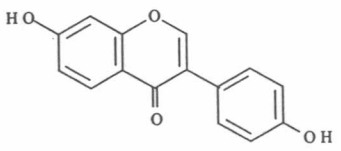
1.3 Chemical constituents in *P. mirifica*

P. mirifica has been found to contain at least 13 chemicals in the group of phytoestrogen with similar effects to estrogens (Figure 2-2). By its chemicals structure, these phytoestrogens are not steriod, however, the nucleus of the chemical structure is similar to that of steroid hormones (Benson *et al.*, 1961).

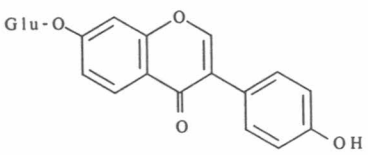
Table 2-1. Phytoestrogen contents in *P. mirifica* (Adapted from Panriansaen, 2000)

Category	Phytoestrogens	References
Coumestrol	Coumestrol	Ingham *et al.*, 1986,1988
	Mirificousmestan	Ingham *et al.*, 1988
	Mirificoumestan glycol	Ingham *et al.*, 1988
	Mirificoumestan hydrate	Ingham *et al.*, 1988
Isoflavones	Daidzein	Ingham *et al.*, 1986
	Daizin	Ingham *et al.*, 1986
	(daizein-7-o-glucoside)	
	Genistein	Ingham *et al.*, 1986
	Genistin	Ingham *et al.*, 1989
	(genistein-7-o-glycoside)	
	Kwakhurin	Ingham *et al.*, 1986
	Kwakhurin hydrate	Ingham *et al.*, 1989
	Mirificin	Ingham *et al.*, 1986
	(puerarin 6"-o-β-	Ingham *et al.*, 1986
	apiofuranoside)	
	Puerarin	Ingham *et al.*, 1986, 1989 and
	(daizein-8-glucoside)	Nilandihi *et al.*, 1957
	Puerarin 6"-monoacetate	Ingham *et al.*, 1989
Lignans	Miroestrol	Schoeller *et al.*, 1940
		Jones and Popes,1960
	Deoxymiroestrol	Chansakaow *et al.*, 2000[a]
	β-sitosterol	Hoyadom,1971
	Stigmasterol	Hoyadom,1971

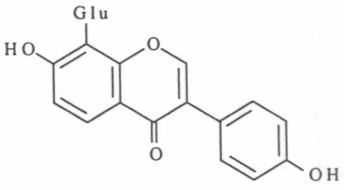
Isoflavone and Isoflavone glycosides



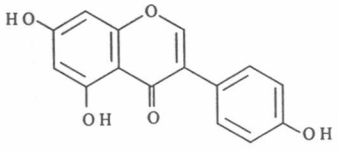
Daidzein



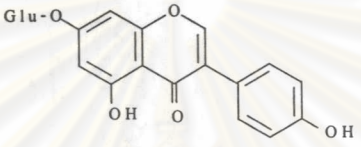
Daidzin



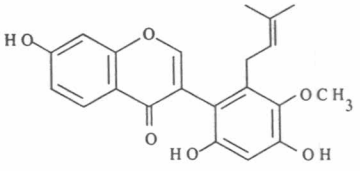
Puerarin



Genistein

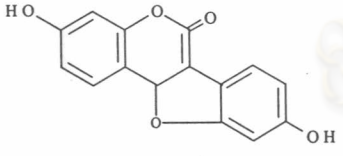


Genistin

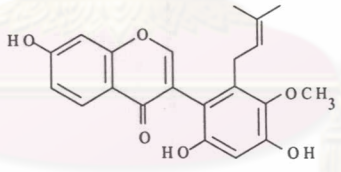


Kwakhurin

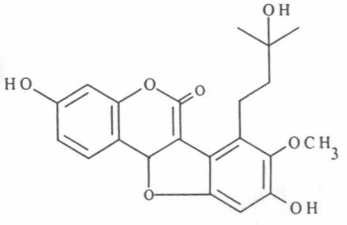
Coumestans



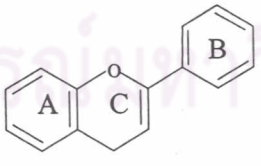
Coumestrol



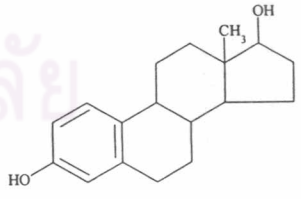
Mirificoumestan



Mirificoumestan hydrate



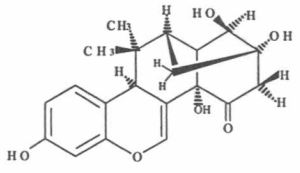
Isoflavonoid nucleus



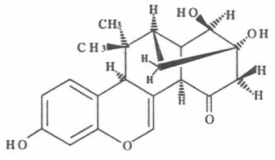
17β-estradiol

Figure 2-2. The structure of chemical compounds in *P. mirifica* and the isoflavonoid nucleus compared to estrogen hormone(17β- estradiol).

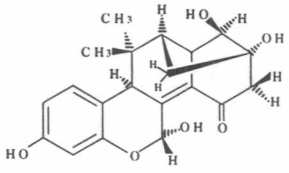
Chromenes



Miroestrol

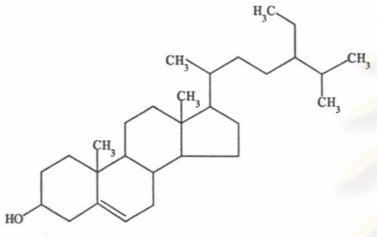


Deoxymiroestrol

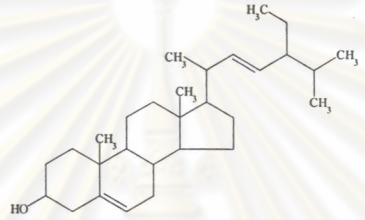


Isomiroestrol

Sterols

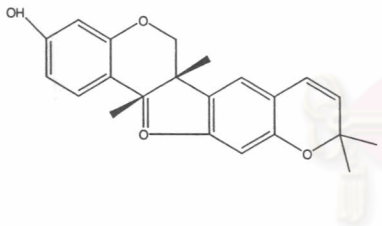


β-sitosterol

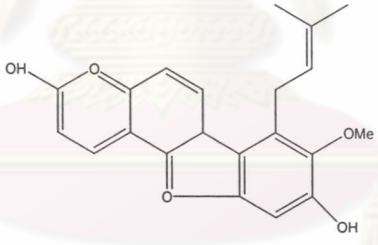


Stigmasterol

Pterocarpans



Tuberosin



Puemirificarpene

Figure 2-2. The structure of chemical compounds in *P. mirifica* and the isoflavonoid nucleus compared to estrogen hormone(17β- estradiol), (continued).

1.4 Toxicity tests of *P. mirifica*

The toxicity of the *P. mirifica* powder and extracted form were evaluated and no toxicity was found (Cherdshawasart, 2003[b]). *P. mirifica* produced no signs and symptoms of acute toxicity in mice. It was found that the doses of 10 and 100 mg/kg/day given orally in Wistar rats did not cause either abnormallities of hematological and biochemical parameters or dose-related histopathological changes of the visceral organs (Chivapat *et al.*, 2000). Mutagenic and anti-mutagenic tests in HeLa cells revealed that the plant exhibited non-mutagenic effect but the anti-mutagenic was found (Julsiri and Cherdshewasart, 2003; Sriwatcharakul *et al.*, 2003). The skin test was conducted in 52 normal Thai volunteers using 5% *P. mirifica* extract (in 50% propylene glycol and 50% deionized water). The result showed neither allergic nor irritating reactions (Kullavanijaya *et al.*, 2003).

1.5 Studies of estrogenic activities in crude powder or extract

The estrogenic activities of *P. mirifica* was firstly determined in ovariectomized rats that the administration of the crude extract induced cell proliferation, growth of the endometrium glands and the proliferation of the spiral arteries of uterus and vagina (Sukhavachana, 1949). Breast tenderness and leukorrhea in ovariectomized women was also recorded (Sukhavachana, 1949). Treatment of low dose of *P. mirifica* for 10 and 20 days increased size and number of the oviduct cells in female Japanese quails, while a treatment of higher dose or a longer period (60 days) decreased the survival rate and tended to inhibit the increased body weight in quails (Muangdet and Anuntalabhochai, 1985).

Gonadectomized male and female rats treated for 14 days with the plant powder showed a decrease in LH and FSH levels (Malaivijitnond *et al.*, 2004). It is confirmed that phytoestrogens from *P. mirifica* have an estrogenic effect on reproductive system, hormonal levels and bone in cynomolgus monkeys (Trisomboon *et al.*, 2004[a], Trisomboon *et al.*, 2004[b]). A single dose of 1,000 mg/day of *P. mirifica* treated to cynomolgus monkeys prolonged lengths of follicular phase and menstrual cycle without changes in FSH, LH, estradiol and progesterone levels (Trisomboon *et al.*, 2004[a]). The clinical trial in Thai menopausal women showed a successive improvement of all evaluated menopausal symptoms; sleep deprivation, hot flashes, mood swings, forgetfulness and difficulty

concentrating (Muangman and Cherdshewasart, 2001). The results were confirmed with phase II human clinical study that relatively alleviated the climacteric symptoms in perimenopausal women (Lamlertkittikul and Chandeying, 2004). It found that *P. mirifica* can influence the reproductive functions in both sexes of rats, but the response in females is greater than in males (Malaivijitnond *et al.*, 2004).

2. Phytoestrogens and their estrogenic activities

Phytoestrogen consumption is becoming of interest in the nutrition and public health sector. Due to the rapid increasing on awareness of the side effect to human health after the long-term consumption of synthetic hormones, most phytoestrogens from legumes and beans were chosen as an alternative choice (Price and Fenwick, 1985; Axelson *et al.*, 1984; Knight and Eden, 1995). Phytoestrogens are a group of naturally occurring chemicals derived from plants with estrogen-like biological activity. These compounds can bind to the estrogen receptors and are thought to exert their estrogenic effects through mechanisms similar to that of estrogen (Santell *et al.*, 1997). Most of the results were concluded that routine consumption of phytoestrogen-rich plant products would benefit for all ages and sex, mostly in terms of cancer protection (Murkies *et al.*, 1998).

Phytoestrogens are considered to be an effective remedy for the treatment of various symptoms of estrogen deficiency occuring during menopausal state. Menopause is defined as the end of menstruation, a state of failure in ovarian function and resulting in low rate of estrogen production. Consequently, it causes a loss of negative feedback mechanism on the secretion of gonadotrophins at the pituitary levels; accordingly, the levels of gonadotrophin progressively increased during this time and kept elevated throughout the menopause (Gill *et al.*, 2002). Moreover, the low level of endogenous estrogen is considered to be the main cause of bone loss or osteoporosis. The osteoporosis mainly occurs during the first two decades after the natural menopause. Menopausal state was found associated with a state of negative calcium balances (Khosla *et al.*, 1998).

There has been tremendous interest in the possibility that dietary phytoestrogens may be an alternative postmenopausal hormone therapy because of concerns about side effects and long term health consequences that prevent many women from using hormone

therapy for amelioration of the discomforts and increase risk associated with the menopausal transition (Kurzer, 2003).

The physiological and pharmacological effects of phytoestrogens found in *P. mirifica* reported and summerized in Table 2-2.

Table 2-2. Physiological and pharmacological effects of phytoestrogen in *P. mirifica*

Phytoestrogen	Effects
Genistein	Inhibit ovarian cancer cell growth from Stage IIIC disease (Gercel-Taylor *et al.*, 2004). Inhibit and act on the inactivated state of L-type calcium channel in guinea pig ventricular myocytes (Ji *et al.*, 2004). Directly block glycine receptors of rat neurons freshly isolated from the ventral segmental area (Zhu *et al.*, 2003).
Genistin	Stimulate estrogen-dependent breast cancer cell growth *in vivo* (Allred *et al.*, 2001).
Daidzein	Prevent ventricular fibrillation in rats (Ye *et al.*, 2003).
Daidzin	Prevent bone loss in ovariectomized rat (Ishida *et al.*, 1998).
Miroestrol	Exhibited mammogenic potency in ovariectomized rats and increase uterine weight in immature female mouse (Benson and Pope, 1961; Jones *et al.*, 1961; Jones and Pope ,1960).
Deoxymiroestrol	Stimulate the proliferation of MCF-7 cells (Chansakaow *et al.*, 2000[a]).
Coumestrol	Cause an atypical threefold induction of

	cytosolic ER without corresponding cytosolic depletion and nuclear accumulation of ER (Markaverich *et al.*, 1995). Increase the calcium content of 9-d-old chick embryonic femurs in organ culture (Tsusumi, 1995). Decrease the binding capacity of liver insulin receptors (Nogowski, 1999).
Puerarin	Therapeutic effect on sudden deafness effective in dilating the blood vessels and promoting microcirculation of the inner ear (Liu *et al.*, 2002). Improve the clinical heart function, increase the left ventricular ejection and decrease the level of ox-LDL in the patients with chronic cardiac failure (Duan *et al.*, 2000). Increase the superoxide dismutase activity, decrease lipid peroxidation level and enhance the activity of fibrinolysis in patients with coronary heart disease(Chen *et al.*, 1999). Against glutamate excitotoxicity on cultured mouse cerebral cortical neurons. protective effects on neurons damaged by sodium glutamate, N-methyl-D-asparate , or Kainic acid(Dong *et al.*,1998). Influence to alcohol pharmacokinetics and alcohol-drinking behavior in rats(Lin *et al.*,1998).

	Stimulate renin activity and angiotensin II in patients with acute myocardial infraction (Li *et al.*, 1997). Stimulate activity of endothelial progenitor cells from peripheral blood and enhance endothelial progenitor cells (EPCs) functional activity (Zhu *et al.*, 2004). Increase learning-memory and amino acid transmitters of brain in ovariectomized mice (Xu *et al.*, 2004). Restore neural function and histopathological damages after transient spinal cord ischemia in rabbits (Sang *et al.*, 2004). Protective effect on diabetic nephropathy, partly through inhibition of excessive deposition of glomeruli extracellular matrix by up-regulating MMP-2 and down-regulating TIMP-2 expressions besides reducing the blood glucose (Duan *et al.*, 2004).

P. mirifica was analyzed and found the high amount of isoflavone phytoestrogens. *P. mirifica* collected from 28 provinces, being determined the estrogenic activity in the present study, exhibited high variation in isoflavone content including puerain, daidzin, genistin, daidzein and genistein. The maximum and minimum amounts of isoflavone were found in the samples collected from Kanchanaburi (198.29 mg / 100 g powder) and Nan provinces (18.85 mg / 100 g powder), respectively (Subtang, 2002).

3. Assay for estrogenic activities of phytoestrogens

One of the most extensively used *in vivo* assays to characterise the estrogenic potency of the phytoestrogens is the rodent uterotrophic assay in which the ability of chemicals to stimulate uterine growth is determined (Reel *et al.*,1996 and Connor *et al.*, 1996). The assessment of estrogenic activity by the induction of vaginal cornification in ovariectomized rats has long-term used (Cook *et al.*, 1933). They first evaluated the two tetrahydrophenathrene compounds, THP-1 and THP-4, the natural estrogen, and found that a 100 mg/rat of THP-1 induced vaginal cornification in 100% of subject rats. Subsequently, a vaginal cytology assay was used to determine an estrogenic activity of biphenol A in ovariectomized rats. The protocols for this assay used ovariectomized rats and oral administration of test compound. Actually the increase of the uterine weight and the cornification of vagina epithelium were used as an indicator. The vaginal epithelium in rodents has been demonstrated to be a more sensitive endpoint for estrogenicity than the uterus. In many cases, the vaginal epithelium proliferation occured at low doses which no stimulation on the uterus was detectable at all (Diel *et al.*, 2001). In the rat vaginal cornification test, miroestrol was the most important active compound (Benson *et al.*,1961) . The estrogenic activity was previously estimated to be about 0.25 times that of 17β–estradiol (E_2) (Jones and Pope, 1960).

Another assessment of estrogenic activities is MCF-7 proliferation assay. *P. mirifica* showed either proliferation or antiproliferation effect (biphasic) in MCF-7 cells, ERα-positive human breast adenocarcinoma cells (Trisap *et al.*, 2003; Cherdshewasart *et al.*, 2003[b]). The plant also showed the anti-proliferation effect in HeLa cells, ERα-negative human cervical adenocarcinoma cells (Cherdshewasart *et al.*, 2004[b]; Trisap, 2003). The response pattern of HeLa cells after the administration of *P. mirifica* was similar to that of phytoestrogen such as genistein and daidzein (Wang and Kruzer, 1997; Zava and Duwe, 1997)

The recombinant yeast system, YES assay, can also accurately predict the estrogenic activity of various phytoestrogens and xenoestrogens in the mammalian cell system. It is useful for testing and detecting of novel estrogenic substances in the environment and natural specimens (Breithofer *et al.*, 1998). *P. mirifica* did not induce estrogenicity in recombinant yeast cells, but it was in MCF-7 cells, human breast

adenocarcinoma cells and HepG2 human hepatoma cells. Thus, it was proposed that *P. mirifica* in itself may neither bind estrogen receptor nor show estrogenic effect, but may require metabolic activation for estrogenic activity that may not be observed properly by yeast system (Lee *et al.*, 2002).

From the above mention, it is therefore necessary to compare the estrogenic activity of *P. mirifica* by those 3 methods. YES and MCF-7 proliferation assays are a rapid method, however, it could not transform the data to human directly. Because the absorption,distribution and biotransformation (or metabolizations) was different between that two assays and human organism. Using the vaginal cornification assay in ovariectomized rats was assumed to be the best candidate to resolve that problem.

4. Estrogens: Regulation and mechanism of actions

The steroid hormones influence the growth, differentiation and function of many target tissues. Estrogens are a class of steroid hormones made primarily in the ovary. They can be classified as a developmental hormone, responsible for the normal maturation of the females. They stimulate the development of female reproductive organs and the secondary sex characteristics, and play an important role in the adolescent growth. They are essential in maintaining the health and integrity of the skin and blood vessels. They also contribute indirectly to the health of bone tissue by opposing hormones that cause calcium depletion. They affect platelet aggregation and alter plasma lipid concentration in a manner now considered protective of the heart. They affect brain function and are known neuromodulators of emotion and memory. There are three types of estrogens; estrone, estradiol, and estriol. The major estrogen secreted by the ovary is 17β-estradiol, converted to estrone in the blood. Estriol is the principal estrogen formed by placenta during pregnancy. These three compounds, 17β-estradiol, estrone, and estriol, account for most of the estrogenic activity in humans. Estrone and estriol are largely products of estradiol metabolism. During the reproductive year, the daily secretion of estrogen varies cyclically throughout the quasi-monthly menstrual cycle. Estrogen production is governed by two pituitary gonadotrophins; follicle stimulating hormone (FSH) and lutinizing hormone (LH). Estrogen cooperated with FSH and LH regulates the growth and development of follicle and stimulates an ovulation. Estrogen and other ovarian hormones, including progesterone and

inhibin, regulates FSH and LH secretion from the anterior pituitary gland by both the negative and positive feedback mechanisms (Rhoades and Pflanzer, 1996).

The changes in the vagina epithelium of the normal animals are believed to be due to the fluctuation and interconversions of female sex hormones, estrogen and progesterone. The level of these hormones, however, is controlled by the pituitary gonadotrophins and hypothalamus releasing hormones. A feedback mechanism also operates whereby the pituitary releases gonadotrophins which are in turn controlled by estrogen and progesterone. The cornification in the vagina is mainly due to the level of stimulation of estrogen which acts directly on the vaginal epithelium. It is also known that only estrogen consistenly stimulates the proliferation of vaginal epithelium in adult female animals (Mandl, 1951; Boettiger, 1946)

4.1 Estrogen receptor

Recent studies have revealed the existence of two distinct estrogen receptors (ERs) in our bodies: ERα and ERβ. While they both bind estrogen as well as other agonists and antagonists, the two receptors have distinctly different localizations and concentrations within our body. Structural differences also exist between the two receptor allowing for a wide range of diverse and complex processes to take place (Gustafsson,1999).

ERβ is localized on human chromosome 14, in contrast to ERα which sites on chromosome 6 (Enmark *et al.*, 1997). ERβ and ERα thus represent two separate gene products and share a relationship to one another that is similar to those between, for instance, the glucocorticoid receptor and the mineralocorticoid receptor, or the glucocorticoid receptor and the progesterone receptor, which show a homology between their ligand-binding domains that corresponds to those of ERβ and ERα. The following diagram, slightly modified from Gustafsson (1999), shows the distribution of ERα and ERβ.

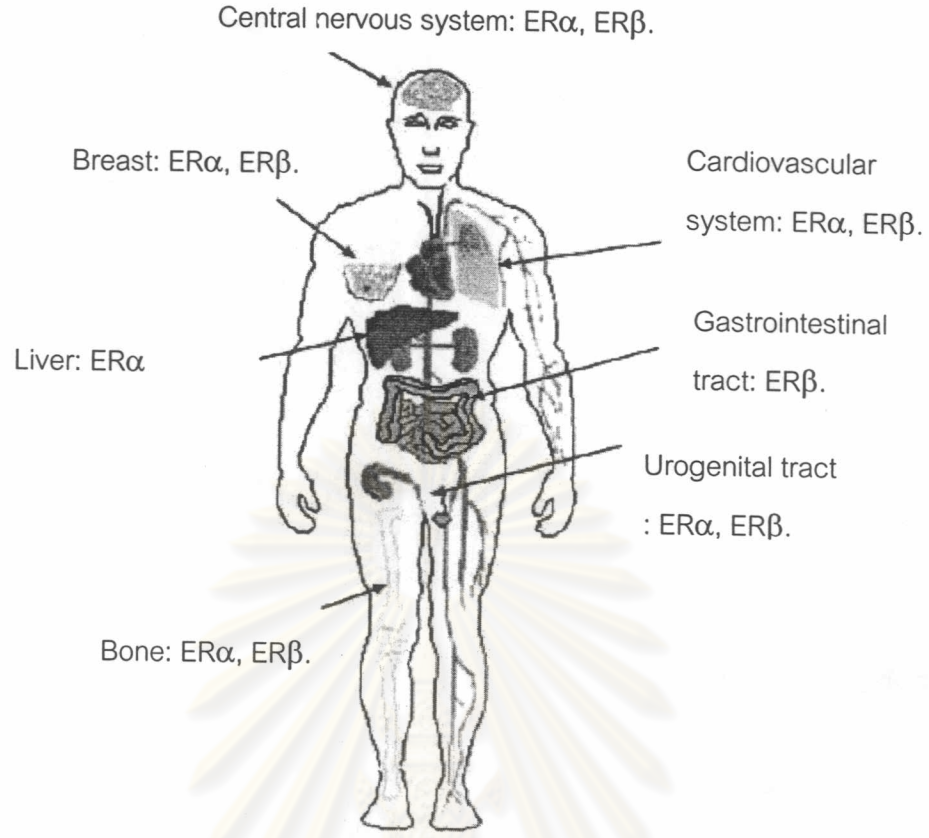


Figure 2-3. Overall distribution of ERα and ERβ in different tissues.

Most recently, the relative binding affinity of phytoestrogens to ERα and ERβ has been determined relative to the binding affinity of estradiol. It showed that phytoestrogens have a lower binding affinity to ER than estradiol, and have a stronger affinity to ERβ than ERα (Kuiper *et al.*, 1998).

4.2 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;

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. Many 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. **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.

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) as illustrated in Figure 2-3

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.

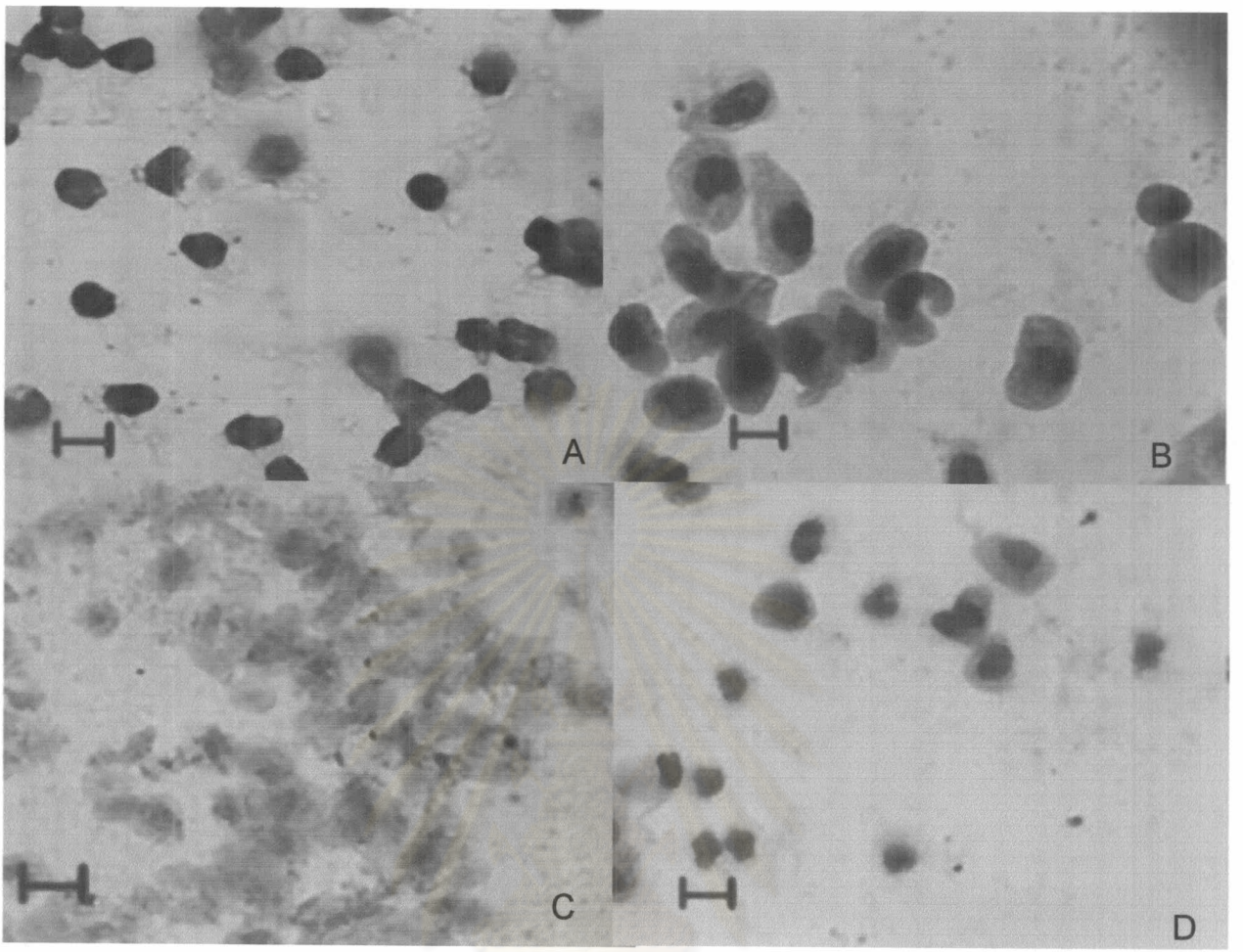


Figure 2-4. The vaginal cytology during rat estrous cycle (The scale bar represented 10 μm)

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.