CHAPTER II

THEORY AND LITERATURE REVIEW

1. Botanical background and chemical constituents of *P. mirifica*

Pueraria mirifica Airy Shaw and Suvatabandhu is an indigenous herb and known in Thai as White Kwao Krua. It belongs to the Family Leguminosae, subfamily Papilinoioideae or soy, bean and pea subfamily. It 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. Active principles in this plant are found in the tuberous root, which looks like zero chain of round-shape bulbs of various sizes connected to the next one via small root throughout the entire length of the root **(Figure 1)**. The shape and size of the tuberous root are diverse depending on the environment in which it exists (Chawalit, 1995).

Pueraria mirifica enable to enlarge and accumulate at least 17 known chemicals classified of phytoestrogens shown in **Table1.** Miroestrol has been known to be a potent estrogenic principle when assayed by the immature mouse uterine-weight and rat vaginal-cornification tests (Jones and Pop, 1960). Besides miroestrol, the previous reports show that kwakhurin, a prenylated isoflavonoid, exhibits moderate estrogenic activity when tested using MCF-7 human breast cancer cells (Chansakaow, *et al.*, 2000).

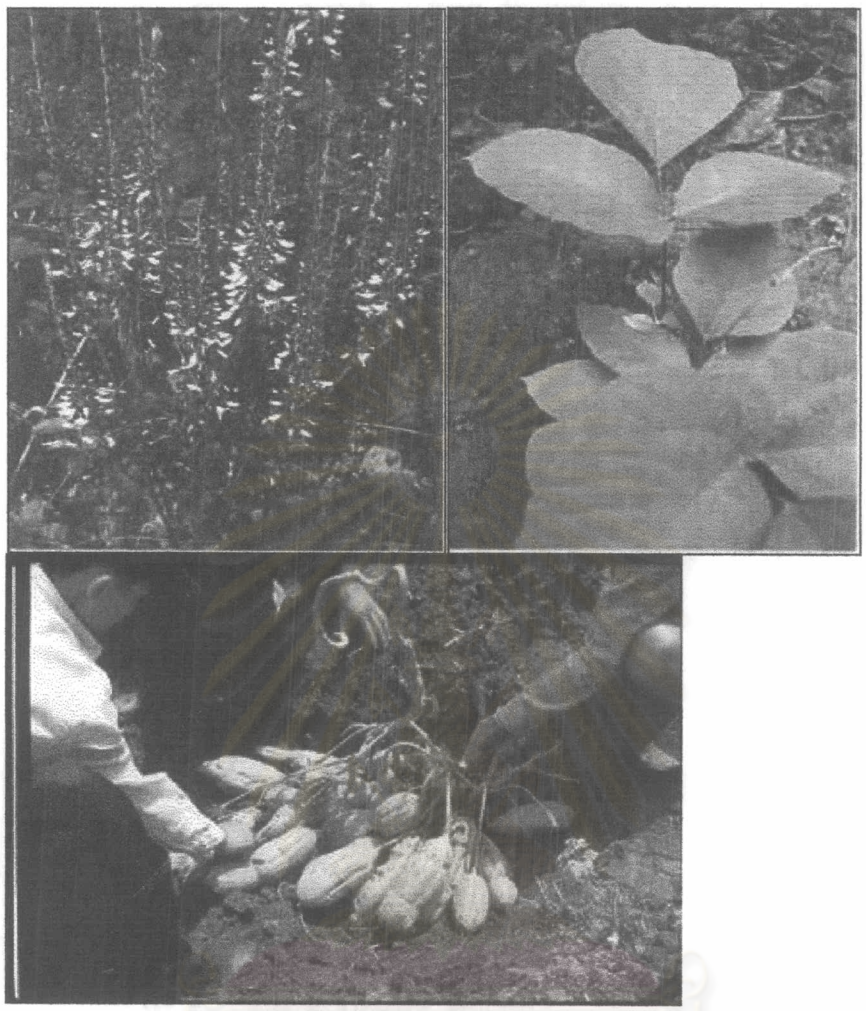

Figure 1. Characteristics of flowers, leaves and roots of *P. mirifica*.

Table1. Summary of the chemical constituents of *P. mirifica*. (modified from Panriansaen, 2000)

Category	Chemical	References
Coumarin	Coumestrol	Ingham,Tahara and Dziedzic,1986, 1988
	Mirificoumestan	Ingham, Tahara and Dziedzic, 1988
	Mirificoumestan glycol	Ingham, Tahara and Dziedzic, 1988
	Mirificoumestan hydrate	Ingham, Tahara and Dziedzic, 1988
Isoflavones	Daidzein	Ingham *et. al.*, 1986
	Daidzin (daidzein-7-o-glucozide)	Ingham, Tahara and Dziedzic, 1986
	Genistein	Ingham, Tahara and Dziedzic, 1986
	Genistin (genistein-7-o-glycozide)	Ingham, Tahara and Dziedzic, 1986, 1989
	Kwakhurin	Ingham, Tahara and Dziedzic, 1986
	Kwakhurin hydrate	Ingham, Tahara and Dziedzic, 1989
	Mirifirin (puerarin 6"-o-β-apiofuranoside)	Ingham, Tahara and Dziedzic, 1986 Ingham *et. al.*, 1986
	Puerarin (daidzein-8-glucoside)	Nilandihi *et. al.*, 1957 Ingham,Tahara and Dziedzic,1986, 1989 Ingham *et. al.*, 1986
	Puerarin 6"-monoacetate	Ingham *et. al.*, 1989
lignans	Miroestrol	Schoeller, Dohrn and Hohweg, 1904 Bound and Pope, 1960 Jones and Pope, 1960
	Deoxymiroestrol	Chansakaow *et. al.*, 2000
	β - sitosterol	Hoyadom, 1971
	Stigmasterol	Hoyadom, 1971

2. Estrogens

Estrogens refer to a substance that promotes female reproductive development. Estrogens activities have traditionally been quantified by bioassay, such as the ability to cause cornification of rat vaginal epithelium. Many structurally different compounds have estrogenic activity. Estradiol is the most potent endogenous human estrogen, with a high affinity for the estrogen receptor (ER).

2.1. Effects of estrogens on Hypothalamo-pituitary-gonadal axis

Estrogens exert both tonic negative and episodic positive feedback on the human female hypothalamic-pituitary axis, but probably only negative feedback on male axis. The positive feedback induces luteinizing hormone (LH) and follicle stimulating hormone (FSH) surge, and then ovulation will be occurred (Johnson and Everitt, 1995). In male, a major component of the negative feedback action of androgens on gonadotropin (Gn), LH and FSH, secretion is mediated via aromatization to estrogen (Hadley, 2000; O'Donnell, *et al.*, 2001). There are many studies of a role for estrogen in the regulation of Gn secretion. During pubertal, a single high dose of estradiol benzoate to 1-day-old male rats causes a reduction in both gonadotrophin releasing hormone (GnRH) secretion and pituitary responsiveness to GnRH (Pinilla *et. al.*, 1992). Adult male rats given increasing doses of estradiol for 10 days showed significant decreases in circulating concentration of LH and FSH, which leads to subsequent reductions in serum and testicular T levels (De Jong *et al.*, 1975).

2.2. Effects of estrogens on reproductive organs

The ovary is an important target of estrogen action. Estrogens have acknowledged as local ovulatory follicles actions. Estrogens act via two types of receptor and have direct poliferative and differentiative influences on follicle development, depending on the stage of folliculogenesis (Alonso and Rosenfield, 2002). The actions of estrogens are intimately to the actions of FSH, is a key driver, in folliculogenesis. It is essential for the final growth of antral follicles and small growing follicles are responsive to but not dependent on FSH for growth. FSH, together with insulin-like growth factor-I (IGF-I) and estradiol, stimulates the proliferation and differentiation of granulosa cells (Johnson and Everitt, 1995; Britt and Findlay, 2002). The specific genes induced in granulosa cells by estradiol have not been identified, but it is clear that estradiol exerts a supporting role on FSH action.

The female genital tract undergoes cyclical related to serum estradiol levels. In the absence of estrogens, the uterus hypotrophies and the stratified squamous epithelium of the vagina is thin. In response to estrogens, epithelial proliferation occurs, leading to the formation of additional layers of cells. The cells also become thicker, with expanded cytoplasm and increased glycogen synthesis. In many mammals, including humans, estrogens induce an increased mitotic activity in the columnar epithelium of the vagina, with a tendency to keratinize. This change is particularly marked in rodents, in which the stage of the estrous cycle can be assessed quite accurately by examination of the different cell types present in smears from the vaginal epithelium. Vaginal smears are therefore an effective way

to measure the degree of estrogens effect on the vaginal epithelium in rodents (Johnson and Everitt, 1995).

Now, it has been discovered that estrogens as well as androgens are necessary for normal reproductive function in the male (Knight, 1996; Hadlley, 2000). In estrogen receptor knockout (ERKO) mice, the testes undergo progressive atrophy associated with decreased epididymal sperm counts and motility and viability. Estrogens regulate the reabsorption of luminal fluid in the head of the epididymis. Disruption of estrogens function therefore causes sperm to enter the epididymis diluted rather than concentrated, resulting in infertility (O'Donnell *et. al.*, 2001; Hadlley, 2000).

3. Estrous cycle in the mice (Green, 1975)

The estrous cycle of the mice is completed in four to five days, although the timing of the cycle may be influenced by exteroceptive factors such as light, temperature, nutritional status, and social relationships. Individual cycles are actually complexes of related secretory, anatomical and behavioral cycles in which the rhythmic interaction of pituitary and ovarian hormones is fundamental and which, in their totality, have the function of insuring fertilization. The mouse estrous cycle has been divided into as few as four phases: proestrus, estrus, metestrus and diestrus. The cycle may be conveniently charted by examining vaginal smears. Cellular characteristics of vaginal smears reflect changes in the structure of the vaginal epithelium which, in turn, are dependent upon estrogen and follow a regular and predictable course during the cycle. Three types of cells are found in vaginal smears:

leukocytes (L), cornified epithelial cells (Co), and nucleated epithelial cells (O). Relative abundance of the various types of cells during the different stages of the cycle are shown in **Table 2** and **Figure 2.**

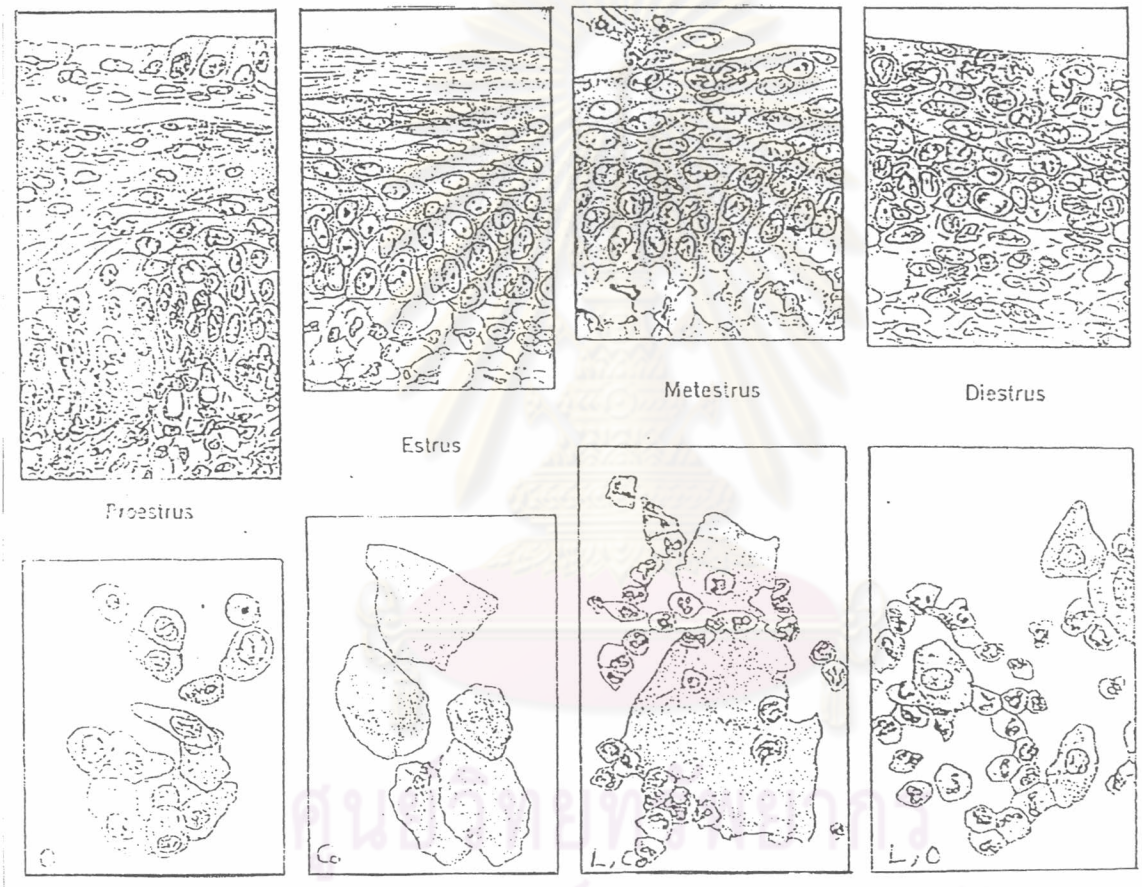

Figure 2. Types of cells in the different stages of estrous cycle in mice by vaginal smear.

O represented the nucleated cells in proestrous period.

Co represented the cornified cells in estrous period.

L represented the leukocyte cells in both metestrous and diestrous periods

Table 2 Schematic outlines of changes in the reproductive organs of the mice during the estrous cycle.

Stage	Smear[a]	Histology of the vaginal epithelium	Uterus	Ovary and oviduct
Proestrus	O to OCo or OCoL to OCo	Many cell layers (10 to 13). Outer 4 or 5 nucleated, stain lightly eosin. Under these, granulosa layer showing cornification. Active mitoses. Few leukocytes.	Increasing hypothermia and distension. Active mitoses in epithelium, few leukocytes.	Follicles large and distended with considerable liquor follicull. Few mitoses in germinal epithelium and in follicular cells.
Estrus	OCo to Co+	Superficial nucleated layer lost. Cornified layer now superficial. About 12 layers of nucleated cells under this. Mitoses decreasing.	Distension and mitotic activity reach miximum during estrus, and then decrease. No leukocytes.	Ovulation occurs followed by distension of the upper end of oviduct. Active mitoses in germinal epithelium and in follicular cells.
Metestrus-1	Co++	Cornified layer delaminated. Leukocytes begin to appear under epithelium.	Distension decreased. Leukocytes begin to penetrate epithelium.	Early corpora lutea present. Eggs in oviduct. Many follicles undergoing atresia.
Metestrus-2	Co++ OL++	4 to 7 layers of epithelial cells, with very many leukocytes in outer layers.	Walls collapsed. Epithelium shows degeneration. Mitoses rare. Leukocytes numerous.	Growing corpora lutea. Eggs in oviduct. Few mitoses in germinal epithelium and in follicular cells.
Diestrus	OL, more or less mucus	4 to 7 layers of epithelial cells, with leukocytes in outer layers. Growth commences towards end of diestrus.	Anemic, walls collapsed. Epithelium healthy but contains many leukocytes. Some secretion by uterine glands.	Follicles begin rapid growth toward end of period.

[a]O = nucleated cells, Co = cornified cells, L = leukocyte cells, + indicates many cells, ++ indicates very many cells.

4. The structure of ovary, uterus and testis in mice

4.1. Ovary (Zhang, 1999)

The ovary consists of two zones: cortex in the peripheral zone and medulla in the core. There is no distinct boundary between the two zones. The stroma of the cortex is composed of networks of reticular fibers and numerous spindle-shaped cells, arranged in irregular whorls. The cortex contains a great number of follicles in all stages of development. According to the stage of development, the follicles can be distinguished as: primordial follicles, primary follicles, secondary follicles and mature or Graafian follicles. The primary and secondary follicles are called growing follicles.

A primordial follicle consists of an immature ovum, the primary oocyte, surrounded by a single layer of flattened granulosa cells. The primary oocyte has a little cytoplasm or so-called ooplasm, and a large nucleus with a prominent nucleolus and finely dispersed chromatin. When the follicles are stimulated to be developed, a primordial follicle enlarges to form a primary follicle. Entering the stage of primary follicle, the primary oocyte enlarges because of an increase in the volume of the ooplasm. The flattened follicular cells become cuboidal shape and increase in number. The primary oocyte continues to enlarge until it reaches as size that is about three times that of the primordial oocyte. The granulosa cells proliferate to form the epithelium around the oocyte. Between the oocyte and the granulosa cells there is the homogeneous glycoprotein layer, zona pellucida, which it is synthesized by the granulosa cells.

With further development, the primary follicle gradually changes into a secondary follicle. Spaces between granulosa cells occur, widen, and eventually merge forming the antrum. The follicle becomes a secondary follicle. The antrum is filled with follicular fluid. Membrana granulosa, which it is formed by the arrangement of granulosa cells in a stratified manner along the basement membrane, occurs while the antrum enlarges. The granulosa cells surrounding the oocyte form the corona radiata and the cumulus oophorus, which it is, resulted form the continuous membrana granulosa surrounding the oocyte is presented. Early in the stage of the secondary follicle, the adjacent stromal cells organize into a capsule or the theca folliculi, which it is separated from the membrena granulosa by the basement membrane. Then the theca folliculi differentiates into two layers; an inner layer, the theca interna, and an outer layer, the theca externa. The theca interna contains enlarged stromal cells and numberous capillaries. The theca externa is composed of closely packed collagen fibers and small fusiform cells.

The mature (Graafian) follicle is a transparent vesicle that protrudes from the surface of the ovary. As a result of the accumulation of follicular fluid, the antrum increases greatly in size, and the membrana granulose becomes thinner. The oocyte adheres to the follicular epithelium through a pedicle by granulosa cells.

After ovulation, the follicular wall is thrown into folds and transformed into a tempory endocrine gland, the corpus luteum. The corpus luteum is surrounded by a layer of connective tissue. The parenchyma of the gland consists mainly of two cell types; granulosa lutien cells and theca lutien cells.

4.2. Uterus (Green, 1975)

The uterus undergoes a series of anabolic and catabolic changes during the estrous cycle, but they are somewhat less striking **(Table 2).** In general appearance, the uterus is distended because of the activity of the uterine glands in proestrus and estrus. The distension starts to diminish in late estrus, and in diestrus the uterine wall is collapsed and anemic. The wet and dry uterine weights are lowest at diestrus and heaviest at proestrus; the uterus is relatively hydrated at proestrus and estrus. Glycogen content is greatest at proestrus. The uterine epithelium is composed of low columnar cells in estrus. In metestrus, degenerative processes become apparent. The basement membrane fades into a pink-staining bland which includes the basal slides of the epithelial cells and the superficial stroma. The epithelium loses its definite organization and shows vacuolar degeneration. Leukocytes appear in the region of the basement membrane. Cell walls at this stage are no longer recognizable and leukocytes are numerous. The uterine glands show minimum activity. The onset of diestrus is marked by the beginning of regenerative processes. The ultrastructure of the mouse uterine epithelium and maximum secretory activity occurs at estrus.

1.3. Testis (Zhang, 1999)

The testes are paired organs lying within the scrotum, and are responsible for the production of sperm and male hormones. The parenchyma of the testis is composed of closely packed coils of seminiferous tubules within a stroma of interstitial connective tissue that contains blood vessels and groups of interstitial cells (Leydig cells). The seminiferous

tubules are lined by a stratified epithelium, composed of two distinct population of cells: spermatogenic series and supporting cells.

The spermatogenic series contains spermatogonia, primary spermatocytes, secondary spermatocytes, spermatid and spermatozoa. These cell types are arranged in layers that show their differentiation sequentially from the basal layers of the epithelium to the lumen.

Spermatogonia are stem cells; they are found in the basal layer of the germinal epithelium, resting upon the basement membrane.

Primary spermatocytes are the large germ cells found within the seminiferous tubules, present in the middle layers of the germinal epithelium. These cells are readily recognized by their spherical outline and large nucleus with either thin threads or coarse clumps of chromatin.

Secondary spermatocytes are smaller than primary spermatocytes. They rapidly undergo the second meiotic division (a regular miosis) to produce spermatids that contain 23 single chromosomes. Therefore, they are rarely seen in routine preparation.

Spermatids undergo a long metamorphic phase known as spermiogenesis to become spermatozoa, which have a head and a long tail. The spermiogenesis is a cytological transformation consisting of several steps, which include condensation of the nucleus to form the head of the spermatozoan, formation of the head cap from the Golgi complex, formation

of the tail from the centrioles and aggregation of mitochrondria around the middle piece of the tail, and shedding of the superfluous part of the spermatid cytoplasm.

5. Phytoestrogens

Phytoestrogens are estrogenic compounds naturally present in plant materials. They consist of numerous classes, including isoflavones (e.g. genistein, daidzein) coumestans (e.g. coumestrol) and ligans (e.g. enterolactone) **(Table 1)**. The structural similarity of phytoestrogens to endogenous estrogens has promoted the hypothesis that phytoestrogens exert hormonal or anti-hormonal effects relevant to the risk of hormone-dependent disease and for their suitability as a dietary alternative to hormone replacement therapy (Duncan *et. al.*, 2003).

5.1. Effects of phytoestrogens on ER binding properties

Most recently, the relative binding affinity of phytoestrogens to ER-α and ER-β 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 ER-β (Kuiper *et. al.*, 1997; Kuiper *et. al.*, 1998). Using human embryonic kidney cells, Kuiper *et al.* (1998) have shown the affinity ranking to the both ER subtypes at concentrations of 1-10 n*M*. The ranking of their estrogenic potency depends upon the ER subtype: 17 β-estradiol >> zearalenol = coumestrol > genistein > daidzein > biochanin A > quercitin for ERα and 17 β-estradiol >> genistein = coumestrol > zearalenol > daidzein > biochanin A > quercitin for

ER-β. Despite their lower binding affinities for ER than estradiol, phytoestrogens exhibit estrogenic activities in transactivation assays with ER-α and ER-β (Benassayag *et. al.*, 2002). At higher concentrations (100 nM), phytoestrogens are able to generate a response of the same magnitude as that induced by the physiological hormone, and at 1000 nM (a concentration reached in human serum after the consumption of food containing large amounts of soybean protein extracts) the estrogenic potency of genistein was greater than that of estradiol (Kuiper *et. al.*, 1998).

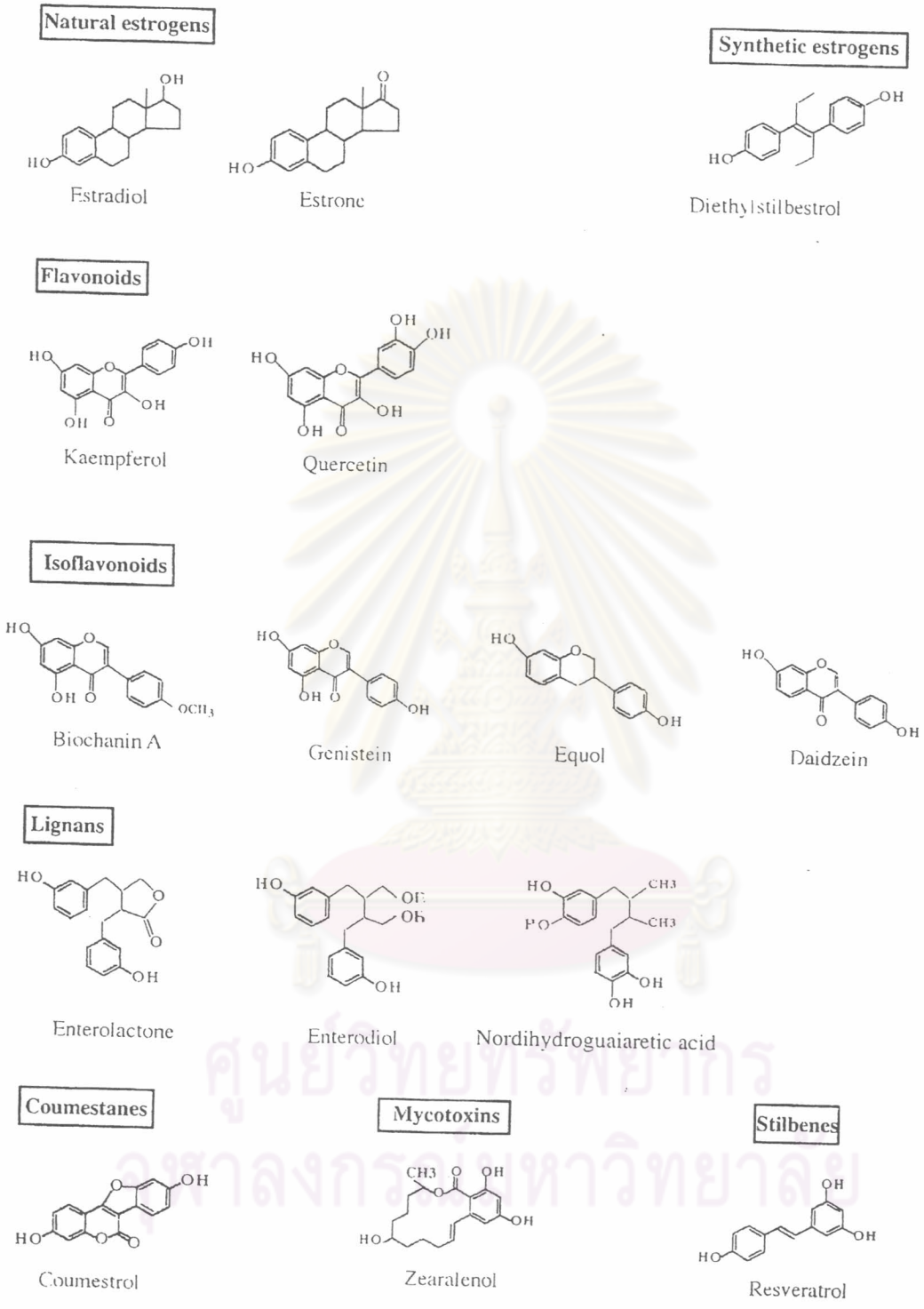

Figure 3. Comparison of molecular structures of phytoestrogens and natural or synthetic estrogens.

5.2. Effects of phytoestrogens on reproductive organs and fertility

Phytoestrogens are particularly common in the human diet and they may act as estrogens or antiestrogens, and harm the reproductive health of females and males. Therefore, there are many studies of the phytoestrogen on the fertility and reproductive tract.

The ability of phytoestrogens to disrupt reproductive function is well established in a number of species. In the neonatal rat, which exposure to coumestrol results in a premature and persistent anovulatory state and failure to show LH surges in response to estrogen (Whitten *et. al.*, 1993). In addition, the coumestrol consumption by rat pups suppressed testicular T concentration and resulted in abnormal sexual behavior in adulthood (Whitten and Naftolin, 1992). High doses of genistein are shown to alter pituitary responsiveness and basal LH secretion in castrated postpubertal rats. Hughes *et al.*, (1991) found that orally administered genistein at 1 and 10 mg/kg BW had no effect on LH in ovariectomized rats, but the low dose genistein of 0.1 mg/kg BW administered intravenously suppressed LH concentrations.

In addition to producing the disturbances on hormonal levels, phytoestrogens can affect the estrous cycle and cause infertility. Neonatal rats exposed to a low dose phytoestrogens (0.01% dietary levels of coumestrol) in the first 5 days of postnatal life exhibit persistent cornification of vaginal epithelium cells evidence of a persistent estrous state, on cytological examination by 4 months of age (Whitten *et al.*, 1993). Acyclicity with vaginal cornification is also seen in mice treated with coumestrol in the neonatal period.

The consumption of phytoestrogens leds to infertility, which were lower marked in males than in females. A recent study evaluating the long-term reproductive effects of genistein (0-10mg/kg/day) during gestation and lactation in mice showed no significant effect on sperm count, the number of motile sperm, or sperm motility in male offspring on postnatal days 105 and 315 (Fielden *et. al.*, 2003). However, Kurzer (2002) reviewed studies on adult men and found no adverse effects on sperm quality with the consumption of soy isoflavones.

The effect of phytoestrogens on fertility in sheep and cattle is well described with low birth rates, uterine prolapes, and uterin edema (Burton and Wells, 2002). Phytoestrogens mediated infertility in sheep may be either temporary or permanent. Temporary infertility occurs when adult ewes grazed on estrogenic pasture at the time of mating, with reduced ovulation and conception rates. Coumestrol concentration as low as 25 ppm may be sufficient to reduce fertility, but fertility returns to normal within 4 to 6 weeks of removal of the ewes to non-estrogenic pasture (Adams, 1995). Prolonged exposure to estrogenic pasture may cause permanent infertility (Burton and Wells, 2002).

6. Effect of *P. mirifica* on reproductive organs and fertility

Several researches have evaluated *P. mirifica* on the fertility and reproductive organs. Study by Langkalichan and Smitasir (1984) showed that high doses of *P. mirifica* (100 and 200 mg/kg/time for 3 time a day for 14 days) could decrease the number of sperm in epididymis and percentage of sperm motility in male albino rats. When high doses treated male rats were mated with normal females, the number and size of implantation of both

uterine horns were significantly reduced. Number and body weight of their offspring were also reduced. No congenital malformations of the pups were found.

Muangdet and Anuntalabhochai (1986) investigated the effects of *P. mirifica* mixed with commercial food at 0.5%, 1.5% and 4.5% (w/w) on reproductive organs and survival rate of female Japanese quails. The results showed that *P. mirifica* of all doses decreased the ovary weight but increased the number of follicles, and size and number of oviduct cells. In addition, the high dose of *P. mirifica* (4.5%) would decrease the survival rate and tended to inhibit the body weight increase of these birds.

Smitasiri *et al.* (1987) investigated the postcoital antifertility effects of *P. mirifica* at the doses of 50 and 100 mg/rat/day for 10 days in pregnant rats. The results showed that *P. mirifica* could prevent pregnancy in all rats when given orally from day 1-10 of pregnancy and when given during embryo transport period (day 1-3 of pregnancy).

Smitasiri *et al.* (1989) investigated the effects of *P. mirifica* at the dose of 100 mg/rat/day in lactating rat for 14 days. The results showed that the body weight of both sexes of fetuses reared by *P. mirifica* treated lactating rats were lower than the control group. Moreover, *P.mirifica* treated rats caused the fetal deaths. The mammary gland weights of *P. mirifica* treated rats were lower than the control group.

From this study, we comparatively investigated whether *P.mirifica* can affect the fertility and sex hormone levels in female and male mice after the long-term treatment with

the suggested doses for human consumption of 10 and 100 mg/kg BW/day and the malformation of the offspring born from *P.mirifica*-treated parents.