CHAPTER II

LITERATURE REVIEW

2.1. Estrogenic action

2.1.1. Estrogen

2.1.1.1. Regulation and mechanism of actions

The steroid hormones influent 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)

2.1.2 Estrogen receptor

Estrogen receptor (ER) was firstly found in the rat uterine. ER was categorized into a group of nuclear hormone receptor, the group of hormone-activated transcription factor that could initiate or enhance the transcription of genes containing specific hormone response element (Green *et. al.* 1986; Greene *et. al.*, 1986). The first type of ER was named ER-α, consisted of 595 amino acids that separated into six different function regions (Kuma *et. al.*, 1987).

The second type of ER was cloned from a rat prostate complementary DNA (cDNA) library and was named ER-β (Kuiper *et. al.*, 1996). The ER-β protein consisted of 485 amino acids and separated into six function regions.

Both ER subtype played many important roles in the reproductive system (Gorodeski and Pal, 2000), cardiovascular system (Makela *et. al.*, 1999), development (Cassanova *et. al.*, 1999), reproduction-related behaviors (Ogawa *et. al.*, 1998) as well as the regulation of the Na^+/H^+ exchanger (Ediger *et. al.*, 1999).

2.1.2.1. The distribution of ER

Both ER subtypes could be found through out the body including cardiovascular system, central nervous system, reproductive system, gastrointestinal system, breast and bone. The amount of each ER subtype was depended on the tissue type. For example, ER-α was found predominately in the reproductive system (Gustafsson, 1999) whereas the gastrointestinal tract was bared only ER-β (Foley *et. al.*, 2000)

The ratio of ER-α and ER-β might be important in determining the susceptibility of a tissue to estrogenic-induced carcinogenesis (Gustafsson, 1999). The changes in the ratio of ER subtypes could help to predict the pathological state of the tissue such as colon

(Foley et. al., 2000), myometrium (Benassayag *et. al.,* 1999) and breast (Gustafsson, 1999).

2.1.2.2. Estrogen action on ER

Estrogen penetrated through the cell membrane and bound to ER (Headley, 1996), which resulted in the dissociation of the heat shock protein. The ER conformation was then altered into active form by phosphorylation. The hormone receptor dimerization was subsequently occurred. The dimmer bound to estrogen responsive elements (EREs) represented in the promoter region of the estrogen-activated genes and resulted in activation of the transcription (Norris, 1997).

2.1.2.3. ER binding/affinity

Different estrogenic compounds have different binding affinities for alpha and beta ERs. While 17β-estradiol binds equally well to both receptors, estrone and raloxifene bind preferentially to the alpha receptor, and estriol and genistein to the beta receptor. The concept of selective estrogen receptor modulators (SERMs) is based on the ability to selectively activate (or block) one type of ER or to promote ER interactions with different proteins such as transcriptional co-activator or co-repressor proteins. Additionally, the different estrogen receptor combinations respond differently to various antagonists, and some compounds have partially agonistic and antagonistic effects, depending on the tissue (Kansra *et al.*, 2005). Tamoxifen, for example, is an ER agonist in bone and uterus, but antagonist in breast tissue, and is therefore used as a breast cancer treatment (Deroo and Korach, 2006)

2.1.3. Estrogen antagonist; Tamoxifen

The first SERM to be investigated extensively for its anticancer properties is a drug called tamoxifen. Tamoxifen blocks the action of estrogen in breast tissue. Tamoxifen exerts this antiestrogenic effect by binding to the estrogen receptors of breast cells, thereby preventing estrogen molecules from binding to these receptors. But unlike the normal situation, when estrogen binds to its receptor, the binding of tamoxifen to the receptor does not cause the receptor molecule to acquire the changed shape that allows it

to bind to coactivators. As a result, the genes that stimulate cell proliferation cannot be activated. By interfering with estrogen receptors in this way, tamoxifen blocks the ability of estrogen to stimulate the proliferation of breast cells.

Although tamoxifen has been useful both in treating breast cancer patients and in decreasing the risk of getting breast cancer in women at high risk, it also has some serious side effects. These side effects arise from the fact that while tamoxifen acts as an antiestrogen that blocks the effects of estrogen on breast cells, it mimics the actions of estrogen in other tissues such as the uterus. Its estrogen-like effects on the uterus stimulate proliferation of the uterine endometrium and increase the risk of uterine cancer. (Clemons, Danson and Howell, 2002).

2.1.4. Estrogenic assays

2.1.4.1. Uterotrophic assay

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 ; Connor *et al.*, 1996 ; Wade *et. al.*, 2003).

2.1.4.2. Vaginal cornification assay

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 occurrence of vaginal cornification after treatment and the recovery after the cessation was depend on dosages and cultivar of *Pueraria mirifica* (Malaivijitnond *et. al.*, 2006; Cherdshewasart, Kitsamai and Malaivijitnond, 2007). The estrogenic activity was previously estimated to be about 0.25 times that of 17β–estradiol (E_2) (Jones and Pope, 1960).

2.1.4.3 MCF-7 proliferation/antiproliferation assay

Another assessment of estrogenic activities is MCF-7 proliferation assay. *P. mirifica* showed either proliferation or anti-proliferation effect (biphasic) in MCF-7 cells, ERα-positive human breast adenocarcinoma cells (Trisap, Cherdshewasart and Picha., 2003; Cherdshewasart, 2003 ; Cherdshewasart, Cheewasopit and Picha, 2004[a]) and the same activities in *Butea superba* (Trisap, Cherdshewasart and Picha., 2004). The plant also showed the anti-proliferation effect in HeLa cells, ERα-negative human cervical adenocarcinoma cells Cherdshewasart, Cheewasopit and Picha, 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)

2.1.4.4. YES

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 Hep-G2 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 mention above, it is therefore necessary to compare the estrogenic activity of *P. mirifica* by those 4 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) is different between

that two assays and human organism. Using the vaginal cornification and uterotrophic assay in ovariectomized rats was assumed to be the best candidate to resolve this problem.

2.2. Estrogen replacement/disruption

2.2.1. Xenoestrogen

Xenoestrogens are synthetic substances that differ from those produced by living organisms and imitate or enhance the effect of estrogens. The estrogenic stimulation is an unintended side-effect of these agents or their metabolites. Xenoestrogens are part of a heterogeneous group of chemicals that are hormone or endocrine disruptors. They differ from phytoestrogens (estrogenic substances from plants), mycoestrogens (estrogenic substances from fungi), and pharmacological estrogens (estrogenic action is intended). External estrogens from a variety of sources may have a cumulative effect upon living organisms, and xenoestrogens may be part of a larger picture of a process of estrogenization of the environment. Xenoestrogens have only been recently (less than 70 years) introduced into the environment, as produced by industrial, agricultural, and chemical companies (Sonnenschein and Soto, 1998).

Xenoestrogens have been implicated in a variety of medical problems. Foremost is the concern that xenoestrogens as false messengers disrupt the process of reproduction. Studies have implicated observations of disturbances in wildlife with estrogenic exposure. Reproductive issues which are of concerns in humans are fetal exposure (perhaps leading to hypospadia) and decreased reproductive ability in men (i.e. decrease in sperm numbers). Another issue is the potential effect of xenoestrogens as oncogenes, specifically in relation to breast cancer (Vidaeff and Sever, 2005).

2.2.2. Phytochemicals

Plant chemical or phytonutrients. Phytochemicals naturally occur in vegetables and fruit. In broad terms, they are said to be any chemical or nutrient derived from a plant source. However, in common usage, they have a more limited definition. They are usually used to refer to compounds found in plants that are not required for normal functioning of the body but that nonetheless have a beneficial effect on health or an active role in the amelioration of disease. Thus, they differ from what are traditionally termed nutrients in that they are not a necessity for normal metabolism, and their absence will not result in a

deficiency disease; at least not on the timescale normally attributed to such phenomena. A minority claims that many of the diseases afflicting the people of industrialized nations are the result of those people's lack of phytonutrients in their diet. What is beyond dispute is that phytonutrients have many and various salubrious functions in the body. For example, they may promote the function of the immune system, act directly against bacteria and viruses, reduce inflammation, and are associated with the treatment and/or prevention of cancer, cardiovascular disease and any other malady affecting the health or well being of an individual. There is abundant evidence from epidemiological studies that the phytochemicals in fruits and vegetables can significantly reduce the risk of cancer, probably due to polyphenol antioxidant and anti-inflammatory effects. But studies of supplementation with large doses of beta-carotene in smokers have shown an increase in cancer risk (possibly because excessive beta-carotene results in breakdown products that reduce plasma Vitamin A and worsen the lung cell proliferation induced by smoke) (Murray, 1996).

Phytochemicals could be classified into primary and secondary metabolites, depending on harboring the essential role in plant metabolism and the presence in the plants. Primary metabolites, including common sugar, protein amino acid, purines and pyrimidines of nucleic acids, chlorolphyll, were the compounds necessary for plant survival. Secondary metabolites were the compounds with a restricted occurrence in taxonomic groups, not necessary for vitality of a cell (organism), but played a role in the interaction of the cell (organism) with its environment, ensuring the survival of the organism in its ecosystem (Verpoorte and Alfermann, 2000). The majority of the biologically-active compounds isolated from plants were secondary metabolites that might play an important function for example, defense against herbivores, bacteria and fungal infections or played an important role in protecting the plant from environmental damage due to UV radiation (Harborne, 1999)

Secondary metabolites could be classified in different ways: based on chemical characteristics and biosynthetic origin. From a chemical point of view, the compounds could be divided in a number of groups based on typical characteristic, such as alkaloids, characterized by a basic nitrogen function, or phenolic, which were characterized by aromatic ring system having a phenolic hydroxyl group. Other groups or subgroups were based on the presence of a certain type of basic skeleton. The classification based on biosynthetic origin had three main biogenetic classes: terpenoids, alkaloids, and related nitrogen compounds and phenolics (Verpoorte and Alfermann, 2000). The terpenoids or

isoprenoids were characterized by their biosynthetic origin from isopentenyl and dimethylallyl pyrophosphates and their board lipophyllic properties. They were mainly cyclic unsaturated hydrocarbons with varying degrees of oxygenation in the substituent groups attached to the basic carbon skeleton. Alkaloids, the nitrogen containing plant metabolite, were organic bases with a nitrogen atom usually linked with a five or six carbon cyclic system. Phenolic compounds were aromatic structure bearing one or more hydroxyl group substituents, one or more of which may be substituted by methyl or glycosyl groups such as flavonoids. The presence of secondary metabolites was strongly dependent on the plant species and plant part (flowers, leave, fruit, seeds, stems, bark, wood) but was normally below 10% (Van Beek, 1999)

Family of Phytochemical

Many phytochemicals are polyphenol antioxidants that impart bright colors to fruits and vegetables. Lutein makes corn yellow, lycopene makes tomatoes red, carotene makes carrots orange and anthocyanin makes blueberries blue, for example. Both the bright colors and the antioxidant activities are due to alternating single-bonded and double-bonded carbons.

Table 2.1 Groups or families of related phytochemicals and common sources of phytochmicals arranged by family.

Group	Family	Example	Source
I.	Flavonoids	quercetin, epicatechin	Berries, herbs, vegetable
II.	Isoflavonoids (Phytoestrogen)	genistein, daidzein	Soy, red clover, kudzu root
III.	Isothiocyanates	sulforaphane	Cruciferous vegetables
IV.	Monoterpenes	myrcene, ocimene	Citrus peels, essential oils
V.	Organosulfur compounds	methanethio,	Chives, garlic, onions
VI.	Saponin		Beans, cereals, herbs
VII.	Capsaicinoids	capsaicin	Chile peppers
VIII.	Phytosterols	stigmasterol,ergosterol	Vegetable oils

2.2.2.1. Flavonoid

The term flavonoid refers to a class of plant secondary metabolites. According to the IUPAC nomenclature, they can be classified into: flavonoids, derived from the 2-phenylchromone (2-phenyl-1,4-benzopyrone) structure, isoflavonoids, derived from the 3-phenylchromone (3-phenyl-1,4-benzopyrone) structure and neoflavonois, derived from the 4-phenyl-1,2-benzopyrone) structure.

Flavonoids are most commonly known for their antioxidant activity. Flavonoids are also commonly referred to as bioflavonoids in the media - these term are equivalent and interchangeable, since all flavonoids are biological in origin.

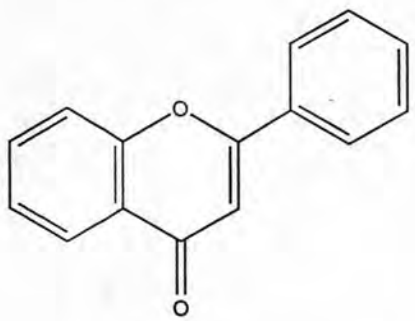


Figure 2.1 Molecular structure of the flavone backbone (2-phenyl-4*H*-1-benzopyran-4-one)

Organic compound, any member of a class of biological pigments containing no nitrogen that are found in many plants. They include anthoxanthins, which give yellow colours, often to flower petals, and anthocyanins, largely responsible for the red colouring of buds and young shoots and the purple and purple-red colours of autumn leaves. Their biological function is unknown; they may attract pollinators and seed dispersers.

A large category of natural plant products that derive from β-pyrone. All flavonoid compounds, which are derive from either 2-phenyllbenzopyrone or 3-phenylbenzopyrone, can be classified into 10 groups. Chalcone, flavanones, flavones, flavonoles, anthocyanidins (flavylium cations), flavan-3-ols (catechins), flavan 3,4-diols (proanthocyanidins), biflavonoids and oligomeric flavonoids, isoflavonoids, and the aurones. They differ in the oxidation level or substitution pattern of their heterocyclic ring.

More than 1300 different flavonoid compounds have been isolated from plants. Individual flavonoids in a group differ from each other by the number and position of the

hydroxy, methoxy, and sugar substituents. As a rule, flavonoid compounds occur in plants as glycosides, with hexoses such as glucose, galactose, and rhamnose, and pentoses such as arabinose and xylose as the most commonly found sugars. The sugars can be attached sigly or in combination with each other. Glycosylation renders these compounds water-soluble and permits their accumulation in the vacuoles of cells.

The few reports available indicate that flavonoids accumulate in epidermal tissues, with approximately 70% in the upper and 30% in the lower epidermis. Vacuoles are probably the only site of flavonoid accumulation in the cells, but synthesis of flavonoids takes place in the cytoplasm.

Flavonoid compounds were once regarded as stray end products of metabolism, but some are now known to be physiologically active. For example, a number of flavonoid compounds were discovered to be the host-specific signal molecules in the formation of nitrogen-fixing root modules. In addition, flavonoids have been linked to protection from ultraviolet radiation. The enzymatic machinery for flavonoid production is induced by ultraviolet irradiation. Flavonoids accumulate in the vacuoles of epidermal cells and absorb light strongly in the critical range of 280–380 nm, where damage caused by ultraviolet radiation occurs. Finally, many plant species synthesize phytoalexins upon invasion by microorganisms. The majority of phytoalexins produced by legumes are isoflavonoids, and each plant species seems to produce a specific compound.

Because of their strikingly vivid color, ranging from deep red through purple to deep blue, anthocyanins represent the most visible class of flavonoid compounds. Anthocyanins are most obvious in flowers and fruits, but they are also present in roots, stems, leaves, seeds, and other parts of the plant. The accumulated anthocyanins, together with carotenes, provide the varied colors characteristic of autumn. Anthocyanins are also produced when plants are subjected to other stress, such as ultraviolet radiation, injury by insects, malnutrition, or unusual concentrations of metal (Murray, 1996).

2.2.2.2. Isothiocyanates

Isothiocyanate is the chemical group ¯N=C=S, formed by substituting sulfur for oxygen in the isocyanate group. Allyl isothiocyanate is also called mustard oil. Isothiocyanates are largely found locked in the form of glucosinolates and are found largely in cruciferous vegetables such as broccoli and Brussels sprouts, as well as in wasabi (domestic Japanese horseradish) and watercress. Such plants contain an enzyme

termed myrosinase which can induce a rearrangement of the glucosinolates leading to the generation of the free isothiocyanate.

2.2.2.3. Monoterpenes

Monoterpenes consist of two isoprene units and have the molecular formula $C_{10}H_{16}$. Monoterpenes may be linear (acyclic) or contain rings.

2.2.2.4. Organosulfur compounds

Organosulfur compounds are organic compounds that contain sulfur. They are often associated with foul odours, but ironically many of the sweetest compounds known are organosulfur derivatives. Nature abounds with organosulfur compounds - sulfur is essential for life. Two of the 20 common amino acids are organosulfur compounds. Fossil fuels, coal, petroleum, and natural gas, which are derived from ancient organisms, necessarily contain organosulfur compounds, the removal of which is a major focus on oil refineries.

Sulfur shares the chalcogen group with oxygen, and it is expected that organosulfur compounds have similarities with carbon-oxygen compounds, which is true to some extent. (Sato *et. al.,* 2006)

2.2.2.5. Saponins

Saponins are glycosides of steroids, steroid alkaloids (steroids with a nitrogen function) or triterpenes found in plants, especially in the plant skins where they form a waxy protective coating. Some authors ditinguish a third class of saponin the alkaloid saponins. They dissolve in water to form a stable soapy froth, this is thought to be due to their amphiphillic nature. Saponins are believed to be useful in the human diet for controlling cholesterol, but some (including those produced by the soapberry) are poisonous if swallowed and can cause urticaria (skin rash) in many people. Any markedly toxic saponin is known as a sapotoxin.

Saponins are also mild detergents and are used commercially aswell as for research. They are used in the British museum as a mild detergent to gently clean ancient manuscripts. In laboratory studies saponins can be used at 0.04%-0.2% to permeabilize the plasma membrane as well as the membranes of internal organelles such as ER and

Golgi but does not penetrate the nuclear membrane. Therefore it is used in intracellular histochemistry staining to allow antibody access to intracellular proteins.

Because of its reversible nature on cells and its ability to permeabilize cells without destroying cell morphology,it is used in laboratory applications to treat live cells in order to facilitate peptide or reagents such as antibodies to enter cells instead of the harsher detergent triton X-100. It is also done on whole cell preparations such as cell smears and cytospins where the cell membrane is intact. It can also be done on frozen sections but is not used on fixed tissue sections. To preserve the permeabilizing effect, saponin has to be used in all processes involved in the staining steps or otherwise removed after reagent of interest has reached the cell.

Any of various plant glucosides that form soapy lathers when mixed and agitated with water, used in detergents, foaming agents, and emulsifiers.

2.2.2.6. Capsaicinoids

Capsaicin is the active ingredient in chili peppers, the substance that gives chili and cayenne its heat. In its purified form it is a crystalline alkaloid; a colorless, pungent, crystalline compound, $C_{18}H_{27}NO_3$, that is derived from capsicum and is a strong irritant to skin and mucous membranes. (Nasrawi and Pangborn, 1990)

2.2.2.7. Phytosterols

Phytosterols (also called plant sterols) are a group of steroid alcohol, phytochemicals naturally occurring in plants. They are white powders with mild, characteristic odor, insoluble in water and soluble in alcohols. They have many applications as fodd additives, and in medicine and cosmetics.

Plants contain a range of phytosterols. They act as a structural component in the cell membrane, a role which in mammalian cells is played by cholesterol (Li, Beveridge and Drover, 2007).

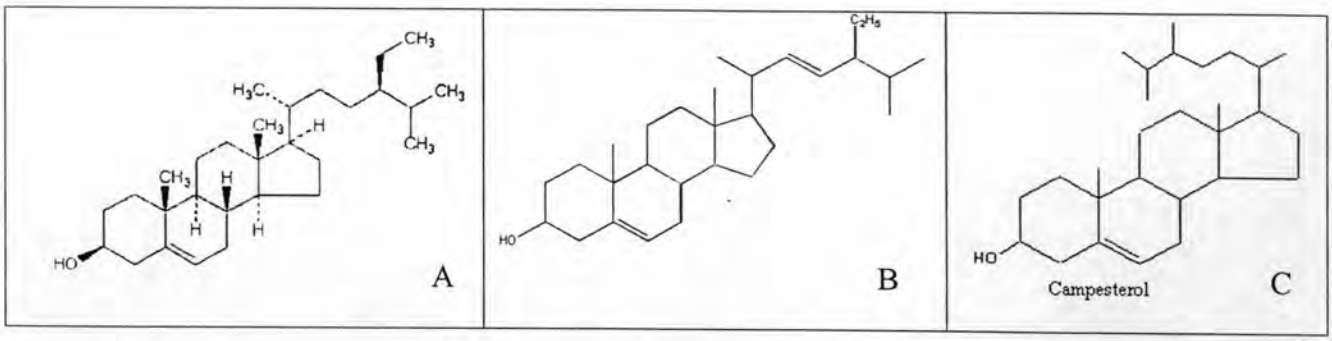


Figure 2.2 Chemical structure of (A) β-sitosterol, (B) Stigmasterol and (C) Campesterol

2.2.2.8. Isoflavonoids

Isoflavonoids are a class of organic compounds and biomolecules related to the flavonoids. They act as phytoestrogens in mammals. They are also very strong antioxidants. Isoflavonoids are thought of by many as useful in treating cancer. Isoflavonoids (3-phenyl-4*H*-1-benzopyr-4-one) differs from flavonoids in the following: in isoflavonoids, the position of the phenyl group on the 4*H*-1-benzopyr-4-one skeleton is in position 3 relative to the oxygen of the ring, whereas in flavones it is in position 2. Isoflavonoids are polyphenolic compounds produced almost exclusively by the members of the *Fabaceae/Leguminosae* (bean) family.

View on the inclusion of isoflavones in food differs radically. Supporters tout studies which provide evidence of significant cholesterol-reducing effects and protection against certain types of cancers, as well as other benefits. Critics claim that isoflavonoids increase the incidence of epithelial hyperplasia, which precedes cancerous tumors, and that they cause goiter and hyperthyroidism. Phytoestrogens may actually promote cancer.

Early evidence that plants produced estrogen-like compounds was observed in the infertility among sheep eating large amounts of clover in Australia. Similarly, Californian Quails were thought to feed on high-isoflavone legume seeds during periods of food shortage to reduce their fertility.

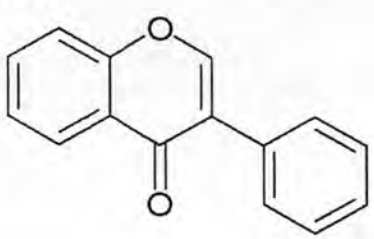


Figure 2.3 Chemical structure of the isoflavonoids backbone (3-phenyl-4*H*-1-benzopyr-4-one)

Isoflavonoids compounds, such as genistein and daidzein, are found in a number of plants, but soybeans and soy products like tofu and textured vegetable protein are the primary food source. Soy isoflavonoids are a group of compounds found in and isolated from the soybean. Besides functioning as antioxidants, many isoflavonoids have been shown to interact with animal and human estrogen receptors, causing effects in the body similar to those caused by the hormone estrogen. Soy isoflavonoids also produce non-hormonal effects.

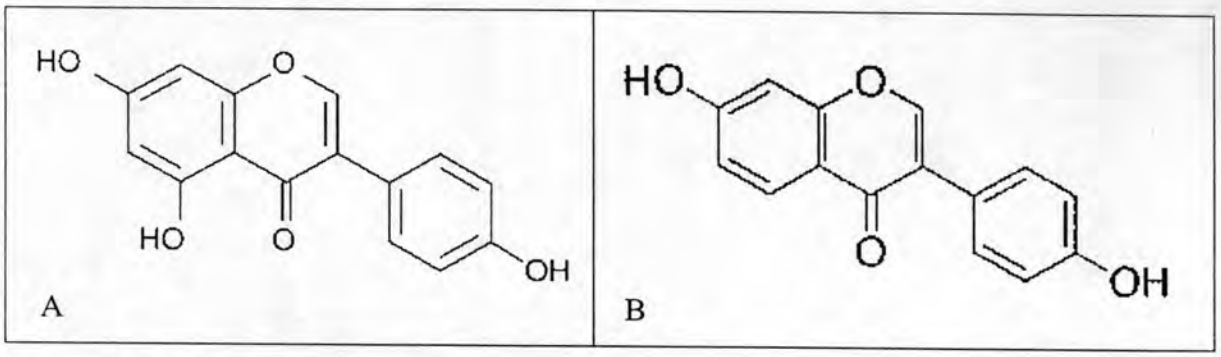


Figure 2.4 Chemical structures of (A) genistein and (B) daidzein

Isoflavonoids acts as antioxidants to counteract damaging effects of free radicals in tissues. Isoflavonoids can act like estrogen in stimulating development and maintenance of female characteristics or they can block cells from using other forms of estrogen. Isoflavonoids also have been found to have antiangiogenic effects (blocking formation of new blood vessels), and may block the uncontrolled cell growth associated with cancer, most likely by inhibiting the activity of substances in the body that regulate cell division and cell survival (growth factors).

Studies show that groups of people who eat large amounts of soy-based products have lower incidences of breast, colon, endometrial, and prostate cancers than the general (US) population. Initial studies of soy isoflavone mixtures containing genistein, daidzein, and glycitein have found them safe for human use. Laboratory studies using animals models have shown that both soy and isoflavonoids can be protective against cancer when given during early life but can stimulate response to cancer-causing chemicals when given during fetal development or when circulating levels of estrogen are low (menopause) (Kaufman *et. al*, 1997).

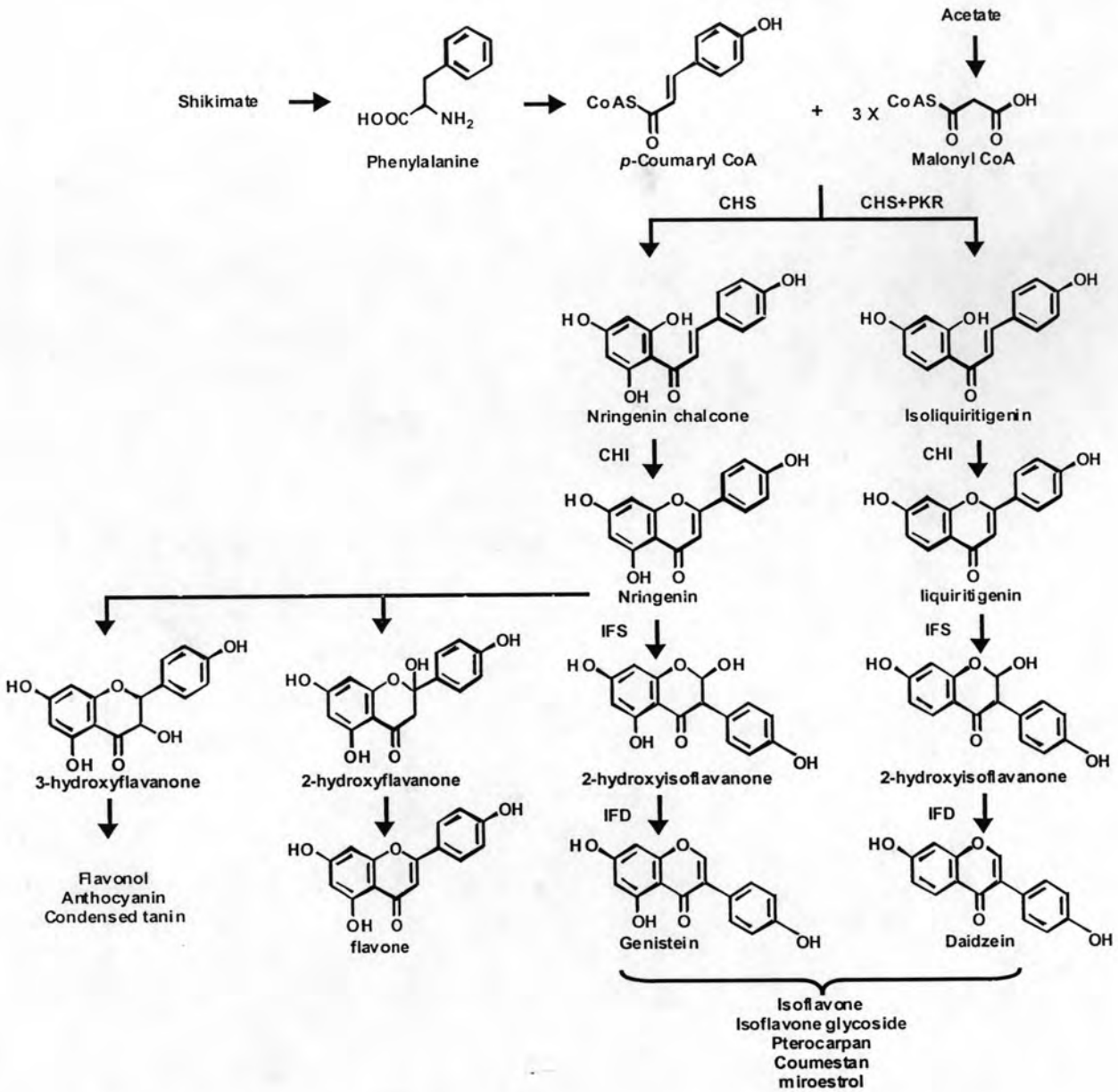


Figure 2.5 Biosynthesis of isoflavonoid enzyme is CHS, Chalcone synthase; PKR, Polyketide reductase; CHI, Chalcone isomerase; IFS, 2-hydroxy isoflavone synthesis; IFD, 2-hydroxy isoflavone synthase.

2.2.3. Phytoestrogen

Phytoestrogen are plant-derived compounds with estrogen-like bioactivity. The compounds could regulate gene expression mediated by an estrogen responsive element (ERE), in a manner either agonistic or apparently antagonistic to 17β - estradiol, as a result of binding to estrogen receptor (ER) (Clarke *et. al.* 1996; Murkies, Wilcox and Devis, 1998). 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). The use of some plants in traditional medicine and remedy could show evidence on their estrogenic effect. As describe above, *P. mirifica* was used as a rejuvenator and aphrodisiac purpose (Murkies, Wilcox and Devis, 1998) as well as crude drug for menopause symptom treatment (Muangman and Cherdshewasart, 2001). 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 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 consumption of diets containing large amount of phytoestrogens, such as soybean and its products, were associated with lower risk of cancer and cardiovascular disease (Messina *et. al.*, 1994).

Phytoestrogens were classified into 3 main classes; isoflavones (e.g. daidzein, genistein) coumestans (e.g. coumestrol) and lignans (e.g. enterolactone). A single plant often contained more than one class of phytoestrogens for example soybean was found to be rich in isoflavone (daidzein and genistein) while its sprout was a potent source of coumestan (coumestrol) (Murkies, Wilcox and Devis, 1998)

2.2.3.1. 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 2.6). 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 ; Adlercreutz *et al.*, 1993). However, genistin was partly absorbed without previous cleavage (Andlauer *et al.*, 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 al.*, 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).

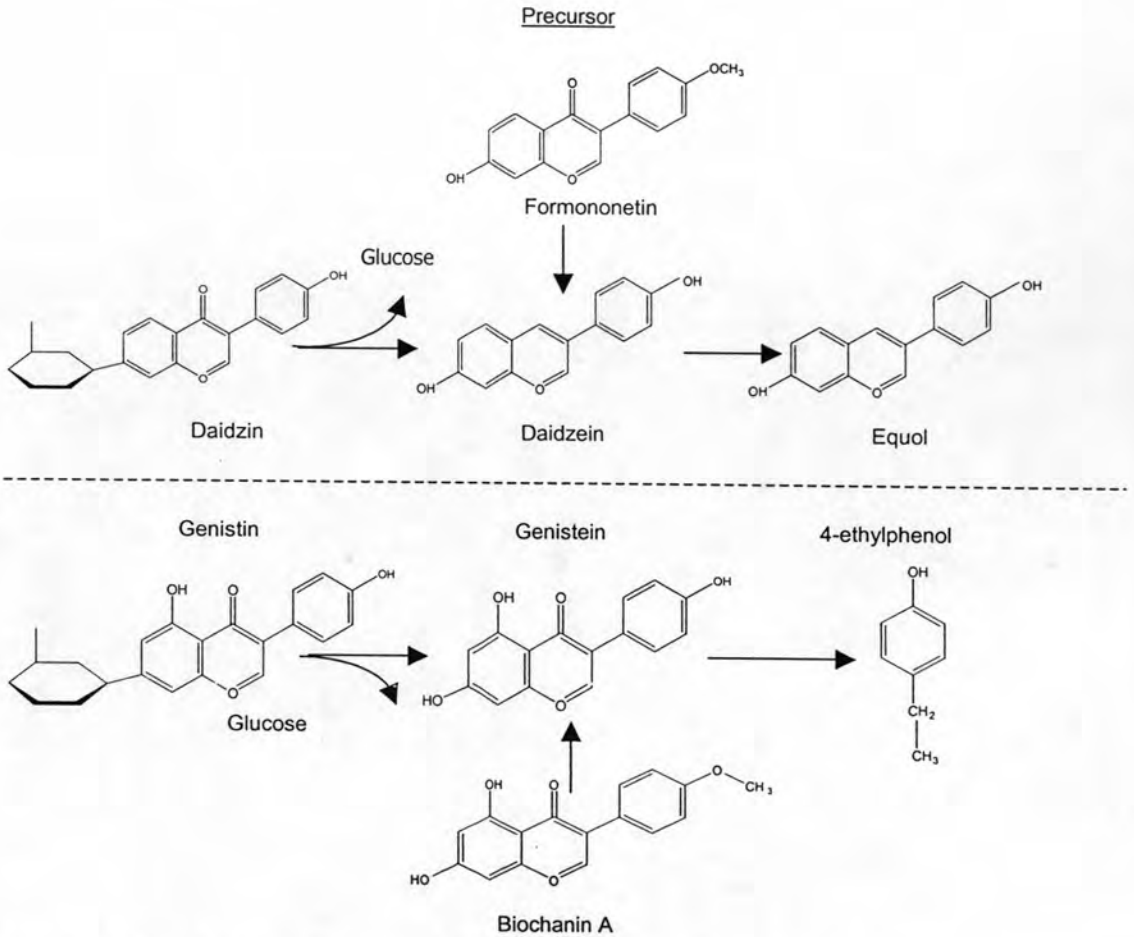


Figure 2.6 Schematic of some phytoestrogen metabolism in intestine.

2.2.3.2. Physical factors and genetic influence on isoflavonoid synthesis and storage

The role of environmental significant that effects on isoflavone concentration in soybean has been reported. The result showed that isoflavone content was significantly lower in seeds that developed in high temperature during seed fill than in seeds exposed to low temperature (Tsukamoto *et. al.*, 1995 ; Carrao-Panizzi *et. al.*, 1999 ; Carrao-Panizzi, Simao and Kikuchi, 2003). Total isoflavone content varied from 1160 to 3090 μg/g among four soybean cultivars grown in the same environment and from 460 to 1950 μg/g among four locations (Eldridge and Kwolek, 1983). Total isoflavone content of single cultivar ranged from 1176 to 3309 μg/g among years and from 1176 to 1749 μg/g among location within the same year (Wang and Murphy, 1994). There were significant differences among location in one or more year for total and individual isoflavone content. The year x location interaction was significant due to changes in rank and magnitude among the location. The genotype, genotype x year, genotype x location, and

genotype x year x location interaction were significant for total and individual isoflavone contents (Hoeck *et. al.*, 2000).

2.2.3.3. Source of phytoestrogens

Phytoestrogens could be found in plant to varying degrees including beans, peas, alfalfa seeds, clover sprouts and tea or even in cabbage (Ju *et. al.*, 2000). The most famous source of phytoestrogens was soybean with high content of daidzein and genistein. *P. mirifica* was also reported to contain high amount of isoflavonoid phytoestrogens including puerarin, daidzin, genistin, daidzein and genistein (Cherdshewasart, Subtang and Dahlan, 2007).

Table 2.2 Classification and sources of phytoestogens.

Class group	Examples	Food Sources
Flavonoid		
Flavone	• Tangeretin	Tangerine rind, juice
	• Apigenin	Grapefruit rind, juice, Flower petals
Flavonol	• Quercetin	All green leaves, onions, grapes
Flavanone	• Naringenin	Citrus peel, juice
	• Hesperitin	Grapefruit peel, juices
Isoflavone	• Genistein	Soybean
	• Daidzein	Red clover, soybean, kudzu
Catechin	• Epicatechin	Tea leaves
Coumestans	• Coumestrol	Red clover, alfalfa, beans
Non-flavonoids		
Ligan	• Isolaricriresitol	Flaxseed, black gram, tomato
	• Matairesinol	Straberries
	• Secoisolariciresinol	Oilseed, tomato, whole cereals

Ref: Modified from Hendrich *et. al.*, 1999; Krazein *et. al.*, 2001; Cornwell *et. al.*, 2004

2.2.3.3.1. *Trifolium pratense*

Red Clover is a species of clover, native to Europe, western Asia and northwest Africa. It is an herbaceous perennial plant, very variable in size, growing to 20-80 cm tall. The leaves are trifoliate (with three leaflets), each leaflet 15-30 mm long and 8-15 mm broad, green with a characteristic pale crescent in the outer half of the leaf; the petiole is 1-4 cm long, with two basal stipules. The flowers are dark pink with a paler base, 12-15 mm long, produced in a dense inflorescence 2-3 cm diameter.

Isoflavones from Red Clover have been used to treat the symptoms of menopause. Women who are pregnant and breastfeeding should avoid ingesting Red Clover. It has also been reported that red clover can be used for therapeutic purposes for coughs, bronchitis, eczema, sores, scrofula and can be gargled for mouth (Howes *et. al.*, 2002 ; Moyad, 2002 ; Beck, Rohr and Jungbauer, 2005).

2.2.3.3.2. *Glycine max*

The soybean is a species of legume native to Eastern Asia. It is an annual plant that may vary in growth habit and height. It may grow prostrate, not growing higher than 20 cm (7.8 inches), or even stiffly erect up to 2 meters (6.5 feet) in height. The pods, stems, and leaves are covered with fine brown or gray pubescence. The leaves are trifoliate (sometimes with 5 leaflets), and the leaflets are 6-15 cm (2-6 inches) long and 2-7 cm (1-3 inches) broad; they fall before the seeds are mature. The small, inconspicuous, self-fertile flowers are borne in the axil of the leaf and are either white or purple. The fruit is a hairy pod that grows in clusters of 3-5, with each pod 3-8 cm (1-3 inches) long and usually containing 2-4 (rarely more) seeds 5-11 mm in diameter.

Economically the world's most important bean, the soybean provides vegetable protein for millions of people and ingredients for hundreds of chemical products, including paints, adhesives, fertilizers, insect sprays, and fire-extinguisher fluids. Unlike other legumes, the soybean is low in carbohydrates and high in protein. Soy products are also a good source of iron and contain vitamins B_1 and B_2 and an essential oil-linoleic acid, one of the Omega-3 fatty acids (Liu, 1997).

Soybeans also contain isoflavones, a type of phytoestrogen, that are considered by some nutritionists and physicians to be useful in the prevention of cancer and by others to be carcinogenic and endocrine disruptive. Soy's high levels of isoflavone phytoestrogens,

being up to 3mg/g dry weight, are the subject of heated debate and controversy. They are also blamed for some thyroid and reproductive health problems. Isoflavones are polyphenol compounds, produced primarily by beans and other legumes, including peanuts and chickpeas. Isolated phytoestrogen-like isoflavones are an active research area. The effect of the isolated soy isoflavones genistein and daidzein (commonly found in dietary supplements and infant formulas) on adult mice with their ovaries removed. The study found the mice had thymic and immune system abnormalities and reduction in immune system activity. The article suggests further research into human phytoestrogen response is warranted (Srikanth *et. al.*, 2002). Soybeans contain isoflavones called genistein and daidzein. Isoflavones (isoflavonoids) are one of two primary groups of phytoestrogen, plant-based estrogen mimicking organic chemicals with antioxidant; free radical scavengers, properties. The other group is lignan. Plant lignans associated with high fiber foods such as cereal brans and beans are the principal precursor to mammalian lignans which have an ability to bind to human estrogen sites. The best source of lignans is flax seed. Soybeans are a significant source of mammalian lignan precursor secoisolariciresinol containing 13-273 μg/100 g dry weight (Adlercreutz *et. al.*, 2000). Another phytoestrogen, in the human diet, with estrogen activity is coumestans but much less well studied which are found in beans, split-peas, with the best sources being alfalfa, clover, and soybean sprouts. Coumesterol, an isoflavone coumarin derivative is the only coumestan in foods (de Kleijn *et. al.,* 2002; Valsta *et. al.*, 2003). There are some controversies on soy effects, because of the phytoestrogen content; some studies indicate that there is a correlation between a soybean-rich diet and a decrease in the level of testosterone in men (Dillingham *et. al.*, 2005). There have been studies that indicate that the soy isoflavones may be promotion or inhibition of breast cancer (Lorraine, 2003; Kenneth, 1998). Determined the women with current or past breast cancer should be aware of the risks of potential tumor growth when taking soy products (de Lemos, 2001). To summarize, the research recommendation is that the impact of isoflavones on breast tissue needs to be evaluated at the cellular level in women at high risk for breast cancer (Messina *et. al.,* 2006).

2.2.3.3.3. *Pueraria lobata*

Pueraria lobata (Kudzu) is classified in the genus *Pueraria*, Family Leguminosae and subfamily Papilionoideae. It is one of the earliest medicinal plants to be used in China. Root of this plant was used as an antipyretic, antidiarrhetic, diaphoretic and antiemetic agent (Yan *et al.*, 2004). The fruit is a legume, a pod, resulting from a superior ovary developing into a bivalved usually dehiscent structure, such as beans and pea pods. The pod is known in a multitude of shapes, from tiny rounded structures containing one seed, to large woody legumes up to 2 m long and 10-20 cm wide, the latter in fact the longest fruit structures in the world. Kudzu is widely spread over China and Japan (Keung, 2002) (Fig. 2.7).

Figure 2.7 (A) Flowers (B) pods and (C) tuberous roots of *P. lobata*.

The isolated constituents in ethanolic extracts of *P. lobata* are flavonoids, coumarins and especially isoflavones, such as daidzein, daidzin, puerarin and daidzin-4',7-diglucoside (Guerra *et al.*, 2000). But the crude aqueous extracts of *P. lobata* are daidzein, daidzin, puerarin, 5-hydroxypuerarin, 3'-hydroxypuerarin and 3'-methoxypuerarin (Jiang *et al.*, 2005).

2.2.3.3.4. *Pueraria mirifica*

2.2.3.3.4.1. Botanical background

Kingdom:	Plantae
Subkingdom:	Tracheobionta
Division:	Magnoliophyta
Class:	Magnoliopsida
Subclass:	Rosidae
Order:	Rosales
Family:	Leguminosae
Subfamily:	Papilionoideae
Genus:	*Pueraria*
Species:	*P. mirifica*

P. mirifica Airy Shaw & Suvatabundhu or "white Kwao Krua" is a Thai indigenous herb with a long history of domestic consumption as a rejuvenating herb to promote of youth in both male and female (Suntara, 1931). Other Thai dialects of *P. mirifica* are Tong-krua, Tong-kwao, Hua-kwao Tan-jom-tong, Po-ta-goo, Tan-krua and Jan-krua. The plant was a long-living twinning wood, which found in abundant in the forests of the north, west and northeast region of Thailand in 28 provinces (Cherdshewasart, Subtang and Dahlan, 2007) at the altitude of 300-800 meters above sea level. The leaves were pinnately three foliate stipulate; terminal leaflet. The tuberous root with white starch granules was varied in sizes and shapes. The flower was bluish purple legume shaped, flowering occurred during late January to early April. The length of the inflorescence of certain flowers was approximately 15-100 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 colors (Cherdshewasart, unpublished).

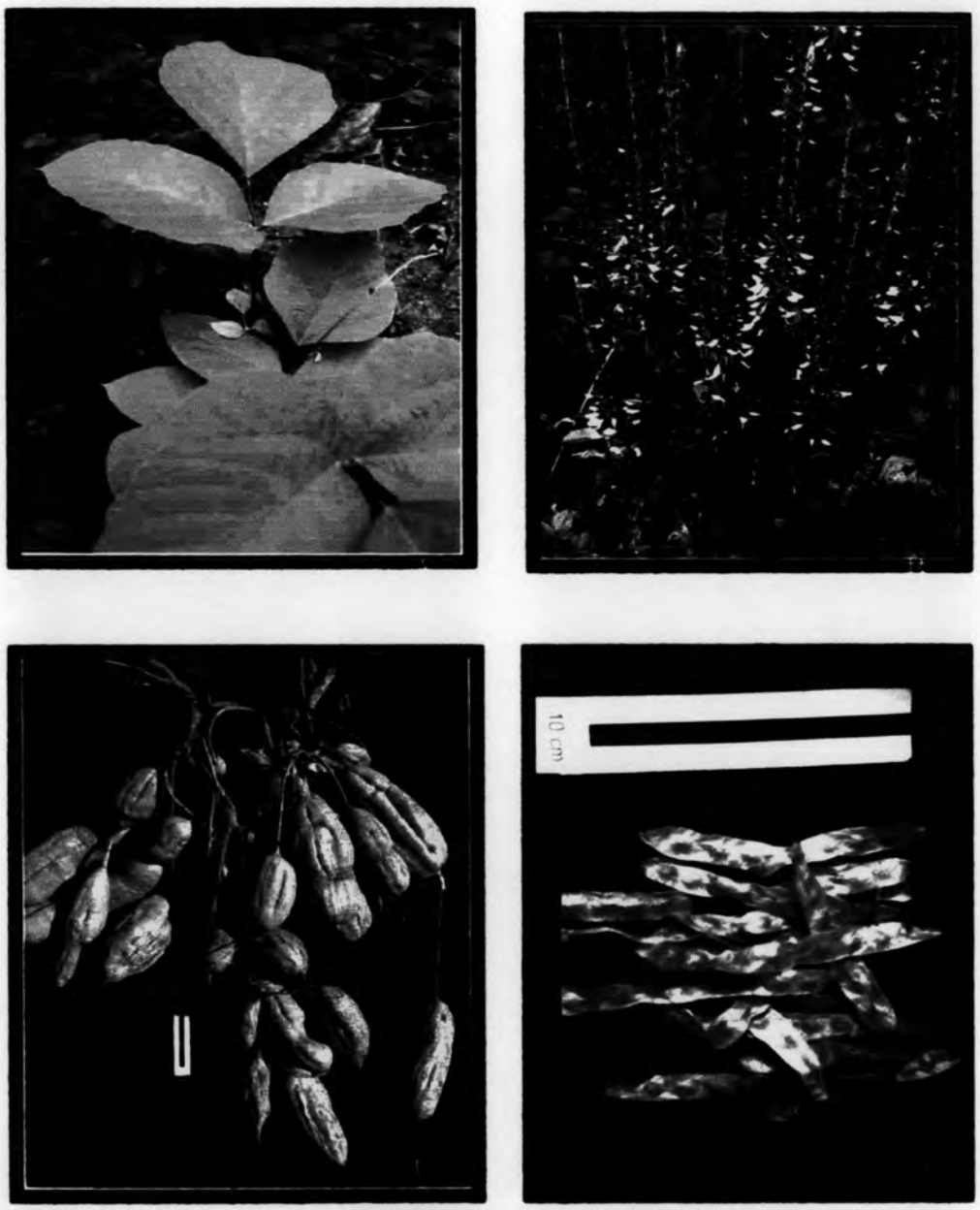


Figure 2.8 (A) Leaves, (B) flowers, (C) tuberous roots and (D) pods of *P. mirifica* from Chiang Mai Province, photos courtesy by W. Cherdshewasart

2.2.3.3.4.2. Chemical constituents

P. mirifica extracts were characterized into classes of compounds (Table 2.3) with some defined biological function and the chemical structures of these compounds were shown in Table 2.4.

Table 2.3 Summary of the chemical constituents of *P. mirifica*.

Categories	Chemical constituents	References
Isoflavonoids	Daidzein	Ingham *et al.*, 1986
	Genistein	Ingham *et al.*, 1986
	Kwakhurin	Ingham *et al.*, 1986
	Kwakhurin hydrate	Ingham *et al.*, 1989
Isoflavone glycosides	Daidzin (daidzein-7-o-glucoside)	Ingham *et al.*, 1986
	Genistin (genistein-7-o-glucoside)	Ingham *et al.*, 1986 ; Ingham *et* al., 1989
	Mirificin (puerarin6'-o-β-apiofuranoside)	Ingham *et al.*, 1986
	Puerarin (daidzein-8-glucoside)	Nilandihi *et al.*, 1957 Ingham *et al.*, 1986 ; Ingham *et* al., 1989
	Puerarin 6''- monoacetate	Ingham *et al.*, 1989
Chromenes	Miroestrol	Schoeller *et al.*, 1940 Bound and Pope, 1960 Jones and Pope, 1961
	Deoxymiroestrol	Chansakaew *et al.*, 2000[a]
	Isomiroestrol	Chansakaew *et al.*, 2000[a]
Coumestans	Coumestrol	Ingham *et al.*, 1986 ; Ingham *et* al., 1988
	Mirificoumestan	Ingham *et al.*, 1988
	Miricoumestan glycol	Ingham *et al.*, 1988
	Miricoumestan hydrate	Ingham *et al.*, 1988
Sterols	β-sitosterol	Hoyodom, 1971
	Stigmasterol	Hoyodom, 1971
Pterolcapans	Pueriicapene	Chansakaew *et al.*, 2000[b]
	Tuberosin	Chansakaew *et al.*, 2000[b]
Acid	Tetracosanoic acid	Chansakaew *et al.*, 2000[b]

Modified from Panriansaen, 2005; Subtang, 2002

Table 2.4 The Bioactivity and Pharmacological effects of chemical constituents in *P.mirifica*.

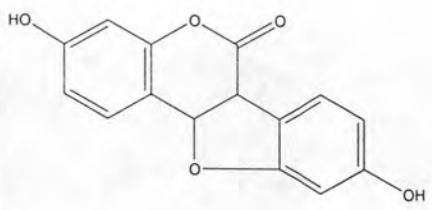
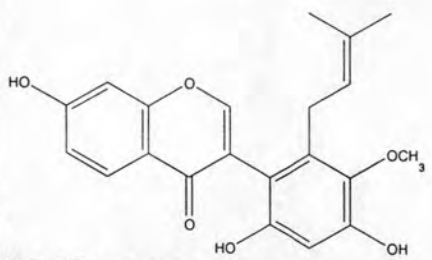
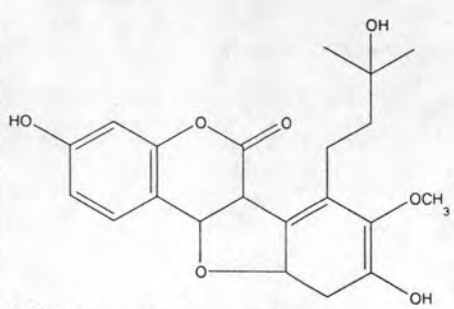
Category	Chemical compound structures	Bioactivity Effects
Coumestans /Coumarins	 Coumestrol MW. 268.2 (3,9-dihydroxycoumestan)	• Exhibit antiestrogenic properties on cell proliferation and apoptosis induction in human MCF-7 breast cancer cells (Schmidt, Michna and Diel,2005) • Suppress the functions of ovulation-inducing mechanisms and the induction of lordosis in female rats (Kouki *et. al.*, 2005) • Inhibit topoisomerase-II activity (Domon *et. al.*,2001) • Decrease the binding capacity of liver insulin receptors (Nogowski, 1999) • 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)
	 Mirificoumestan (3,9-dihydroxy-8-methoxy-7-(3,3 dimethyallyl)-coumestan)	
	 Mirificoumestan hydrate (3,9-dihydroxy-8-methoxy-7-(3-hydroxy-3-methylbutyl)-coumestan)	

Table 2.4 The Bioactivity and Pharmacological effects of chemical constituents in *P.mirifica* (continued)

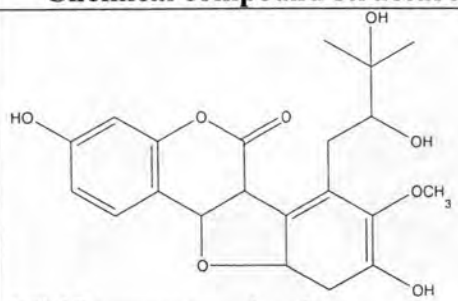
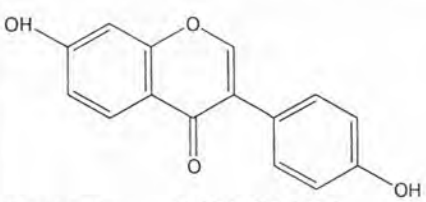
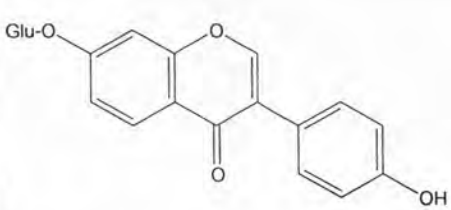
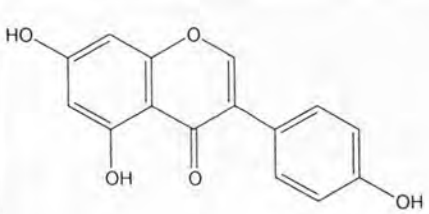
Category	Chemical compound structures	Bioactivity Effects
	 Mirificoumestan glycol (3,9-dihydroxy-8-methoxy-7-(2,3-dihydroxy-3-methylbutyl)-coumestan)	
Isoflavonoids	 Daidzein MW. 254.24 7-hydroxy-3-(4-hydroxyphenyl)-4H-1-benzopyran-4-4',7-dihydroxyisoflavone	• Decreased the levels of resting heart rate, blood pressure, fasting plasma glucose, blood lipids and inflammatory factors in 40 senile patients (Liu, Zhao and Zhang, 2006) • Shown to reverse scopolamine-induced amnesia in mice which an important factor in the treatment of Alzheimer's disease (Heo *et. al.*, 2006)
	 Daidzin MW. 416.4	• Stimulated glucose uptake in mice (Meezan *et. al.*, 2005) • Promoted the osteogenesis proliferation and inhibited the adipogenesis of primary mouse bone marrow stromal cells (Li *et. al.*, 2005) • Prevent bone loss in ovariectomied rat (Ishida *et. al.*,1998)
	 Genistein MW. 270.23	• Represses telomerase activity in prostate cancer cells (Jagadeesh, Kyo and Banerjee, 2006) • Decreased nicotine metabolism were investigated with 7 healthy Japanese homozygotes of CYP2A6 (Nakajima *et. al.*, 2006) • 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)

Table 2.4 The Bioactivity and Pharmacological effects of chemical constituents in *P.mirifica* (continued)

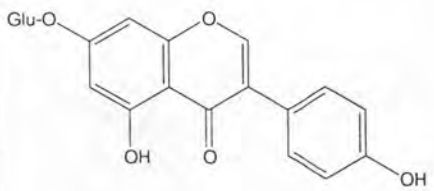
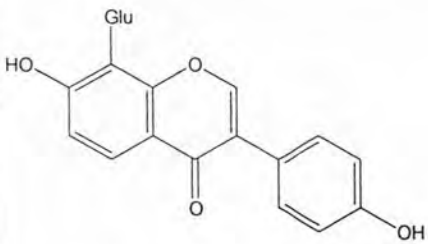
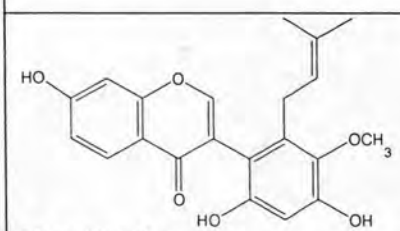
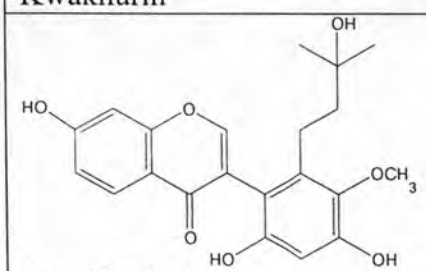
Category	Chemical compound structures	Bioactivity Effects
	 Genistin MW.432.4	• Shown to arrest the growth of malignant melanoma in vitro and to inhibit ultraviolet light-induced oxidative DNA damage in human melanoma cells (Russo *et. al.*, 2005) • The potent prevention regimen for bladder cancer progression in mice (Singh *et. al.*, 2006) • Stimulate estrogen-dependent breast cancer cell growth *in vivo* (Allred *et. al.*, 2001)
	 Puerarin MW.416 (daizein-8-glucoside)	• Inhibited glucose uptake into tissues and incorporation into glycogen in mice (Meezan *et. al.*, 2005) • Improved the neurological functions in male Sprague-Dawley rats (Xu *et. al.*,, 2005) • Anti-proliferation effect on vascular smooth muscle cell (Han, *et. al.*,, 2004) • Therapeutic effect on sudden deafness effective in dilating the blood vessels and promoting microcirculation of the inner ear (Liu et. al., 2002) • Restore neural function and histopathological damages after transient spinal cord ischemia in rabbits. (Sang et. al., 2004)
	 Kwakhurin	• Exhibited rejuvenating activity (Iwasaki *et. al.*, 2004)
	 Kwakhurin hydrate	

Table 2.4 The Bioactivity and Pharmacological effects of chemical constituents in *P.mirifica* (continued)

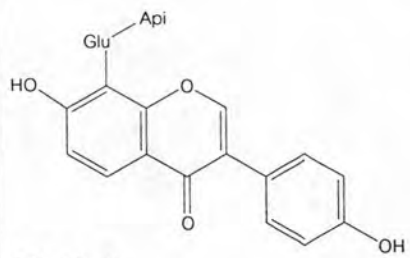
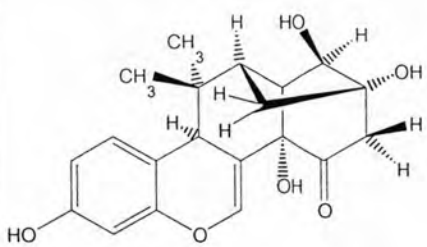
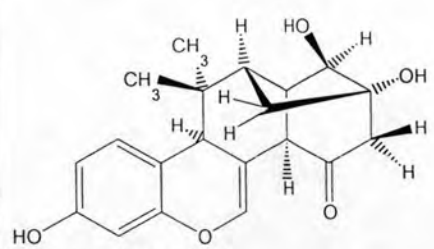
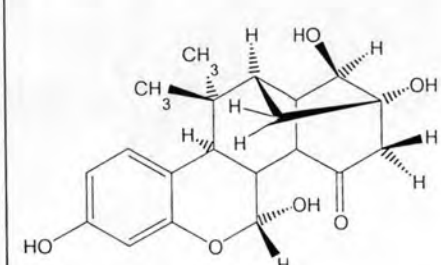
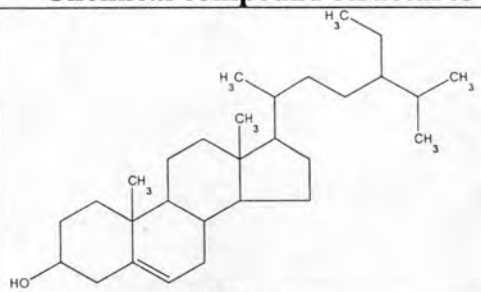
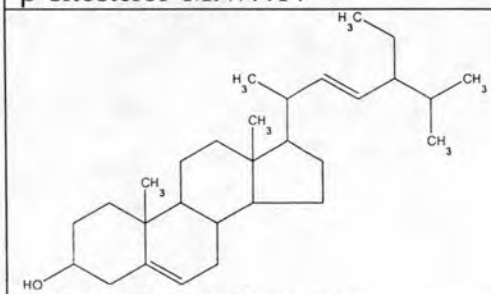
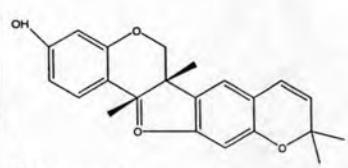
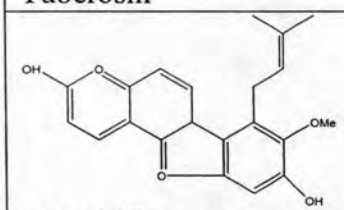
Category	Chemical compound structures	Bioactivity Effects
	 Mirificin (puerarin 6''-o-B-apiofuranoside)	
Chromenes	 Miroestrol	• Shown to oestrogenic properties in MCF7 human breast cancer (Chansakaow *et. al.,* 2000[a]; Matsumura *et. al.,* 2005) • Exhibited effect on vaginal cornification, pituitary function and pregnancy in the rat (Jones *et. al.,* 1961) • Exhibited mammogenic potency in ovariectomized rat and increase uterine weight in immature female mouse (Benson and Pope,1961; Jones and Pope, 1961; Jones and Pope, 1960)
	 Deoxymiroestrol	• Shown to oestrogenic properties in MCF7 human breast cancer (Chansakaow *et. al.,* 2000[a]; Matsumura *et. al.,* 2005) • Stimulate the proliferation of MCF-7 cells (Chansakaow *et. al.,* 2000[a])
	 Isomiroestrol	

Table 2.4 The Bioactivity and Pharmacological effects of chemical constituents in *P.mirifica* (continued)

Category	Chemical compound structures	Bioactivity Effects
Phytosterols/ Sterols	 β-sitosterol M.W.414	• Exhibited cytotoxicity to BC cell line and antituberculosis activity (Kanokmedhakul *et al.*,2005) • Decreased secretion of apolipoprotein B48 from Caco2 human intestinal cell (Ho and Pal, 2005)
	 Stigmatosterol M.W. 413	• Exhibited strong inhibition on the dRP lyase activity of DNA polymerase β (Shi-Sheng, *et al.*, 2004)
Pterocarpans	 Tuberosin	• Exhibited inhibition of the formation of advaced glycation end products (AGEs) (Kim, *et. a*l., 2006)
	 Puemirificarpene	

2.2.3.3.4.3. Pharmacological effects of *P. mirifica*

P. mirifica crude extract showed estrogenic effect on human, Hep-G2 cell and MCF-7 cells. The plant chemicals needed metabolic activation to promote their activity (Lee, *et. al.*, 2002), high concentration of the plan crude extract showed anti-proliferation to MCF-7 cells (Cherdshewasart *et. al.*, 2004[a]) and HeLa cells (Cherdshewasart *et. al*, 2004[b]). The plant tuberous powder showed influence on FSH and LH level in gonardectomized female and male rats (Malaivijitnond *et. al.*, 2004) and age monkeys (Trisomboon et. al., 2006[a]). In ovariectomized rat, *P. mirifica* root extraction induced

proliferation of the cornifined cell (Cherdshewasart, Kitsamai and Malaivijitnond, 2007 ; Malaivijitnond *et. al.*, 2006) and exhibited strong estrogenic activity in uterotrophic assay (Kim *et al.*, 2003). In orchidectomized rats, *P. mirifica* treatment may useful to prevent an osteoporosis in elderly hypogonadism (Urasopon *et. al.*, 2006). Uterus and vagina weight of *P. mirifica*-treated immature rat was significantly increased (Sawatdipong, 1981). In adult male mice treated with *P. mirifica* increased the mating efficiency after stop treatment (Jaroenporn *et. al.*, 2006), *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; 2003[a] and 2003[b]). 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[a] and 2004[b]), *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.* 2006[b]). In ovariectomized rabbits treated with *P. mirifica* exhibited that *P. mirifica* has potential source of phytoestrogens for menopausal woman to improve cardiovascular function or reduce cardiovascular risks (Wattanapitayakul, Chularojmontri and Srichirat, 2005). Isoflavonoids isolated from *P. mirifica* at the concentration of 0.1-1 μM exhibited inhibition the growth of MCF-7 human breast cancer at about 80% in the presence of tormifene, as compared with 17β-estradiol (Chansakaow *et. al.*, 2000[b]). Miroestrol had 0.7 time of Estradiol on mammary gland proliferation activity (Pope *et al.*, 1958).

2.2.3.3.4.4. Safety test of *P. mirifica*

After treatment of *P. mirifica* powder in mice, no symptom of acute toxicity was found with LD_{50}> 16 g/kg BW (Chivapat *et. al.*, 2000). The acute toxicity with LD_{50} was found over 2 g/kg BW in female mice (Cherdshewasart, 2003). The male and female rat treated with *P. mirifica* powder suspension for 3 months showed no any abnormality to the main organs and blood cells at the dose of 10 mg/kg BW (Chivapat, *et. al.*, 2000). *P. mirifica* root extract was not mutagenic by Ames test. (Chulasiri and Cherdshewasart, 2003). The formation of micronuclei in polychromatic erythrocytes was induced by oral administration of an aqueous extract of *P. mirifica,* resulted that the extracts of *P. mirifica* at the dose of 600 mg and 800 mg/kg might act as a mutagenic agent by inducing higher frequencies of micronuclei as compared to the control (Saenphet *et. al.*, 2005). The

aqueous and ethanolic extract of affected the red blood cell formation and acted as a mutagenic agent (Sanchanta *et. al.*, 2006.)

2.2.3.3.4.5. Clinical trial of *P. mirifica*

The tuberous root powder and the crude drug derived from *P. mirifica* powder could improve symptoms related to menopause (Sukhavachana, 1949; Muangman and Cherdshewasart, 2001). Evaluation of the preliminary efficacy and safety of *P. mirifica* powder with dose of 50 and 100 mg / day for 6 month in 48 enrolled patients at the age 17 to 37 resulted in decreasing of lipoprotein level on blood and increased on FSH and LH level (Lamlertkittikul and Chandeying, 2004). The clinical trial at Chelsea Hospital London with miroestrol, the key plant chemical, exhibited estrogenic response on amenorrhoea patients with no side effect (Cain, 1960).

2.3 Chemical analysis of phytoestrogens

2.3.1 HPLC

Chromatography was the most useful technique for the separation of phytochemicals. The chromatographic analysis were distributed between two phases, one was a stationary phase one while the other moved. The separation occurred because under an optimum ser of condition, each component in the mixture would interact with the two phases differently relative to the other components in the mixture. The methods including paper chromatography, thin layer chromatography (TLC), gas chromatography (GC) and high-performance liquid chromatography (HPLC). Paper chromatography and TLC is basically and widely used chromatographic technique available for the analysis of plant constituents, especially for preparation purposes. There are very simple, no special apparatus needed. GC and HPLC are more convenience, efficiency and resolution technique than TLC. The mobile phase of TLC and HPLC are liquid while the mobile phase of GC is gaseous. TLC and HPLC are thus the mail analytical techniques for analysis of non-volatile compounds whereas GC is a technique of choice for volatile compounds. GC is limited to secondary metabolites which has boiling point below 450 C and which are stable at the temperature of the separation. Therefore, HPLC has the advantage over GC in that it is not necessary to prepare volatile derivatives.

In isocratic HPLC the analyte is forced through a column of the stationary phase (usually a tube packed with small round particles with a certain surface chemistry) by

pumping a liquid (mobile phase) at high pressure through the column. The sample to be analyzed is introduced in a small volume to the stream of mobile phase and is retarded by specific chemical interactions with the stationary phase as it traverses the length of the column. The amount of retardation depends on the nature of the analyte, stationary phase and mobile phase composition. The time at which a specific analyte elutes (comes out of the end of the column) is called the retention time and is considered a reasonably unique identifying characteristic of a given analyte. The use of pressure increases the linear velocity (speed) giving the components less time to diffuse within the column, leading to improved resolution in the resulting chromatogram. Common solvents used include any miscible combinations of water or various organic liquids (the most common are methanol and acetonitrile). Water may contain buffers or salts to assist in the separation of the analyte components, or compounds such as trifluoroacetic acid which acts as an ion pairing agent.

A further refinement to HPLC has been to vary the mobile phase composition during the analysis; this is known as gradient elution. A normal gradient for reverse phase chromatography might start at 5% methanol and progress linearly to 50% methanol over 25 minutes, depending on how hydrophobia the analyte is. The gradient separates the analyte mixtures as a function of the affinity of the analyte for the current mobile phase composition relative to the stationary phase. This is a partitioning process is not unlike that which occurs during a liquid-liquid extraction but is continuous, not step-wise. In this example, using a water/methanol gradient, the more hydrophobic components will elute (come off the column) under conditions of relatively high methanol; whereas the more hydrophilic compounds will elute under conditions of relatively low methanol. The choice of solvents, additives and gradient depend on the nature of the stationary phase and the analyte. Often a series of tests are performed on the analyte and a number of generic runs may be processed in order to find the optimum HPLC method for the analyte - the method which gives the best separation of peaks.

2.4 Antioxidant activity studies

2.4.1. Free radicals

A free radical is a molecule that contains an unpaired electron in its outer orbit and that can exist independently. Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are various forms of activated oxygen and nitrogen, which include free radicals

such as superoxide ions ($O\bullet^{-}_{2}$), hydroxyl ($OH\bullet$) and nitric oxide radicals ($NO\bullet$) as well as non-free-radical species such as hydrogen peroxide (H_2O_2) and nitrous acid (HNO_2) (Clarkson and Thompson, 2000). In living organisms, various ROS and RNS can form by different ways, which these free radicals are toxic by-products from normal functions in the body. For normal aerobic respiration, stimulated polymorphonuclear leukocytes and macrophages, and peroxisomes as biochemical reactions appear to the main endogenous sources of most of the oxidant produced by cells. For exogenous sources of free radicals, which enter the body from environment, include tobacco smoke, ionizing radiation, certain pollutants, organic solvents and pesticides. The role of free radicals and active oxygen in the pathogenesis of certain human diseases including cancer, aging and atherosclerosis is becoming increasingly recognized (Hailliwell, 1994)

2.4.2. Preventing the free radicals

To fight free radicals, all aerobic organisms, including human, have antioxidant defense that protect against oxidative damages and repair enzymes to remove or repair damaged molecules (Hertog *et. al.*, 1993). This natural antioxidant mechanism can be inefficient although produces natural antioxidant enzymes, however, when we age, the activity of the antioxidant enzymes decrease and the absorbing function of the intestines reduce. Thus, access free radicals are accumulated in the body and dietary intake of antioxidant compounds will become important (Espin, Soler-Rivas and Wichers, 2000). Although, there are some synthetic antioxidant compounds such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA), which are commonly used in processed foods, it has been reported that these compounds have some side effects (Branien *et. al.*, 1975).

Some plant-derived phenolic compounds have marked antioxidant capacity due to their ability to donate H atom/electrons from their hydroxyl groups to free radicals. They may therefore protect cellular components from oxidative damage and prevent the development of diseases such as cancers because of phytoestrogens have phenolic hydroxyl groups on the A and/or B rings and thus could act as antioxidants in cellular systems. Isoflavonoid from soybean could inhibit LDL oxidation *in vitro* and LDL oxidative modification by J774 monocyte/macrophages to LDL, including inhibit of superoxide radical production (Hwang *et. al.*, 2000). Soybean isoflavones and their glycosides possess antioxidant activity but they were ineffective antioxidants compared with tea epicathechins and α-tocopherol (Lee *et. al.*, 2005). Genistein from soybean had

the highest activity of the isoflavonoids; the isoflavonoids were relatively poor hydrogen donors compared with the other estrogenic compounds, however, they inhibit DNA strand breaks in plasmid DNA mediated by reactive oxygen species and inhibit MDA-MB-231 breast cancer cells (Magee, McGlynn and Rowland, 2004), MCF-7 cells (Chen *et. al.*, 2003), negative result in Ames test for mutagenesis and acted as a specific inhibitor of tyrosine kinase (Akiyama *et. al.*, 1987) whereas daidzein do not (Dixon and Ferreira, 2002). Genistein and daidzein extract from soy exhibited antioxidant activity *in vivo* and inhibitor among TPA-induced H_2O_2 formation byHL-60 cells (daidzein is second), inhibit O_2- generation by xanthine/xanthine oxidase while daidzein showed a moderate inhibitory effect. These results suggest that the antioxidant properties of isoflavonoids are structurally related and the hydroxyl group at position 4′ is crucial in both system, which genistein's antioxidant properties and antiproliferative effects may be responsible for its anticarcinogenic effect, which a promising candidate for the prevention of human cancers (Wei *et. al.*, 1993). Quercetin at low concentration could scavenge the stable free radical DPPH, moderate effectively but genistein and daidzein did not (Johnson et. al., 2000). Quercetin inhibiting LDL oxidation *in vivo*, which exhibited antioxidant properties (Morand *et. al.*, 1998). Coumestrol was more effective antioxidants than genistein but had relatively limited activity in comparison with trolox; however, kaempferol was only estrogenic compound as significant antioxidant activity, which is better known as a dietary antioxidant than phytoestrogen (Mitchell *et. al.*, 1998). Flavonoids showed a dose dependent protecting activity to α-tocopherol in low-density lipoprotein (LDL) and kaempferol being less effective than quercetin against depletion of α-tocopherol in LDL (Zhu, Huang and Chen, 2000). Rotenoids from *Sarcolobus globosus* inhibited 15-lypoxygenase (Wangensteen *et. al.*, 2006)

2.4.3. Screening the antioxidant of plants

Several methods have been developed to measure the free radical scavenging capacity; regardless of the individual. Methods for measuring antioxidants and appraising antioxidant activity appear to be of two general types. If the chemical nature of the antioxidant is known, one may strive for a test specific for the compound of interest. Alternatively one may observe the inhibition of some natural oxidative process (Choi *et. al.*, 2002). Many different methods have been proposed for the evaluation of oxidant power. Most of them are based on the measurement of the relative abilities of antioxidant

to scavenge radicals in comparison with the antioxidant potency of a standard antioxidant compound. Preliminary studies, independently of the chosen method, suitable reference antioxidants should be tested for comparison, including rapid, sensitive and reproducible methods, preferably requiring small sample amounts (Parejo *et. al.*, 2002).

Diphenylpicrylhydrazyl (DPPH) assay is easy to use, with a high level of sensitivity, able to determine the most active components directly thus provides a fast, and allow for analysis of a large number of samples in a timely fashion and was provide preliminary information about screening antioxidant in phytochemical (Mitchell *et. al.*, 1998 ; Parejo *et. al.*, 2002), flavonois (Okawa *et. al.*, 2001), isoflavone aglycones (Murota *et. al.*, 2002). The mechanism of this method is based on the reduction of DPPH, a stable free radical. Because of its odd electron, DPPH gives a strong absorption maximum at 517 nm by visible spectroscopy (purple color). As the adding electron of the radical, the radical becomes parried off in the presence of a hydrogen donor, that is, a free radical scavenging antioxidant, the absorption strength is decreased, and the resulting decolorization is stoichiometric with respect to the number of electrons captured. The stable free radical 2,2-diphenyl-2-picrylhydrazyl) the violet color of which is modified to yellow, α-tocopherol (Vitamin E) was reference antioxidants in order to indicate the range of activity (Blois *et. al.*, 1958; Molyneux, 2004) as show the structure of DPPH in Figure 2.9.

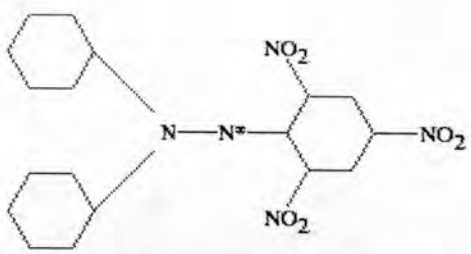


Figure 2.9 Structure of chromogen DPPH

2.5 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, 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.

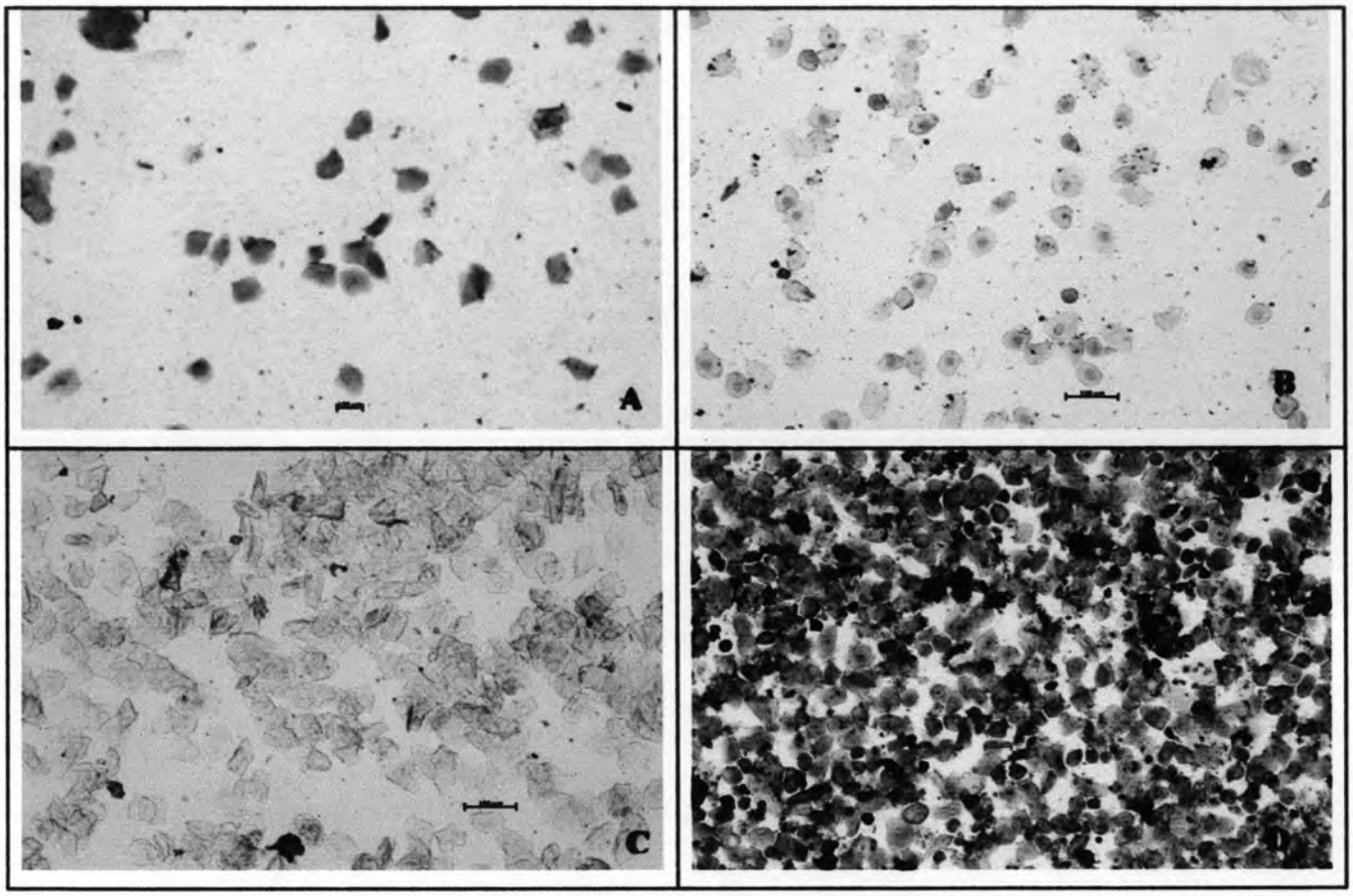


Figure2.10 The vaginal cytology during rat estrous cycle (The scale bar represented 100 μm)

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.