CHAPTER II

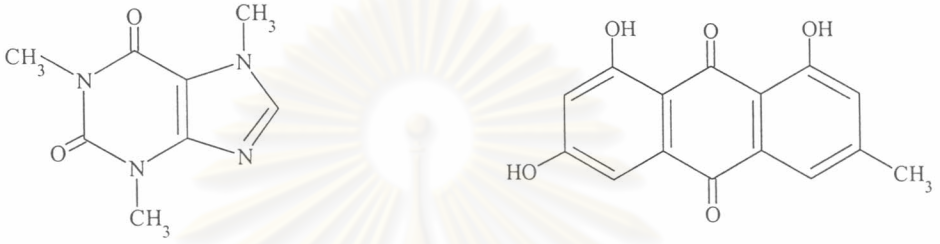
Literature Review

I. Phytochemicals

Plant chemicals or 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 sugars, protein amino acid, purines and pyrimidines of nucleic acids, chlorophyll, 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, 2000). The majority of the biologically-active compounds isolated from plants were secondary metabolites that might play an important function for example, defence against herbivores, bacteria and fungal infections or played an important role in protecting the plant from environmental damage due to UV radiation (Harborne, 1999).

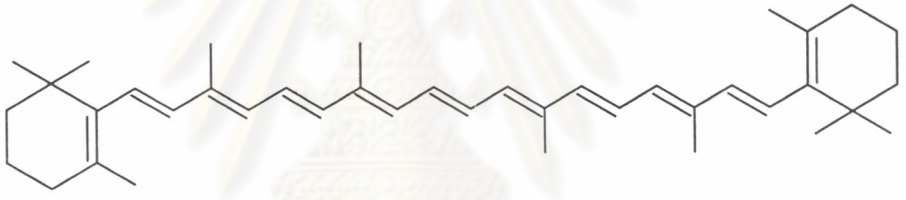
Secondary metabolites could be classified in different ways: based on chemical characteristics and biosynthetic origin. From a chemical point of view, the compounds could be divided in a number of groups based on typical characteristic, such as alkaloids, characterized by a basic nitrogen function, or phenolics, which were characterized by aromatic ring systems 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, 2000). The terpenoids or isoprenoids were characterized by their biosynthetic origin from isopentenyl and dimethylallyl pyrophosphates and their broad lipophillic 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 metabolites, 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 substitutents, one or more of which may be substitued by methyl or glycosyl groups such as flavonoids. The presence of secondary metabolites was strongly dependent on the plant species and plant part (fruits, seeds, stems, bark, wood, flowers, leaves) but was normally below 10 % (Van Beek, 1999).



Caffeine (Alkaloid)

Emodin (Phenolic)



β - carotene (Terpenoid)

Figure 1 The example of the 3 main plant secondary metabolite structures; terpenoids, alkaloids and phenolic compounds.

II. Phytochemical fingerprint establishment

The studies of phytochemicals focused on three major steps namely, extraction, separation and analysis. The most common and universal extraction technique was the solvent extraction. This step could be distinguished to two types;

(I) a pre-extraction either to remove impurities from the plant materials or to make the analytes available for further extraction, or (II) extraction of the compounds of interest. In a type I extraction, the solvent should not dissolve the analyze to an appreciable extent while the impurities should be readily soluble. In contrast, in a type II extraction the solvent should be sufficiently "strong" to dissolve

all metabolites of interest and to disrupt any matrix effects. Generally the selectivity should decreased from hexane (a "weak" solvent) to methanol (a "strong" solvent) (Van beek, 1999). The extraction served not only to remove the compounds of interest from the insoluble high molecular weight parts of the plants but also from other extractable which could interfere with later steps. The extraction time could vary from several minutes up to weeks. Other variables include amount of agitation, plant-solvent ratio, moisture content, temperature and the number of times that the extraction was repeated.

In the quality control of the herb, it was necessary to apply the appropriate analytical method to separate the major chemical components in the mixture. The fractionation of a crude extract was desirable in order to separate the main classes of constituent from each other, prior to chromatographic analysis.

If the active ingredients were not known or were presented in complex forms, the quality of the extracts could be assessed by the aid of a "fingerprint" chromatogram. The HPLC fingerprint analysis had became a useful and suitable tool for quality control and standardization of plant materials. The fingerprint should demonstrate identity and purity of a drug or an extract and should guarantee the therapeutic equivalence of the extract from the same herbal drug. Single marked constituents could be quantified. An exact chemical characterization of all major constituents of an extract could only be achieved by specific and selective analytical methods. If a preparation contained more than four or five drug or extracts, it was more reliable to characterize the individual drugs or extracts by HPLC before mixing. These profiles should give enough information to reach a reasonable conclusion for quality and could be used for comparative and relative quality assessment of samples (Wagner, 1999).

III. Botanical characteristics of the 3 Kwao Krua plants

Pueraria mirifica, *Butea superba* and *Mucuna collettii* were classified into the family of Leguminosae (Ridley, 1967; Pengklai, 1977; Suvatti, 1978). The name of Kwao Krua was commonly applied to plants in different genus. At least three kinds of Kwao Krua, the White, Red and Black Kwao Krua, were commonly found and long term consumed in Thailand (Santara , 1931).

3.1 *Pueraria mirifica*

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

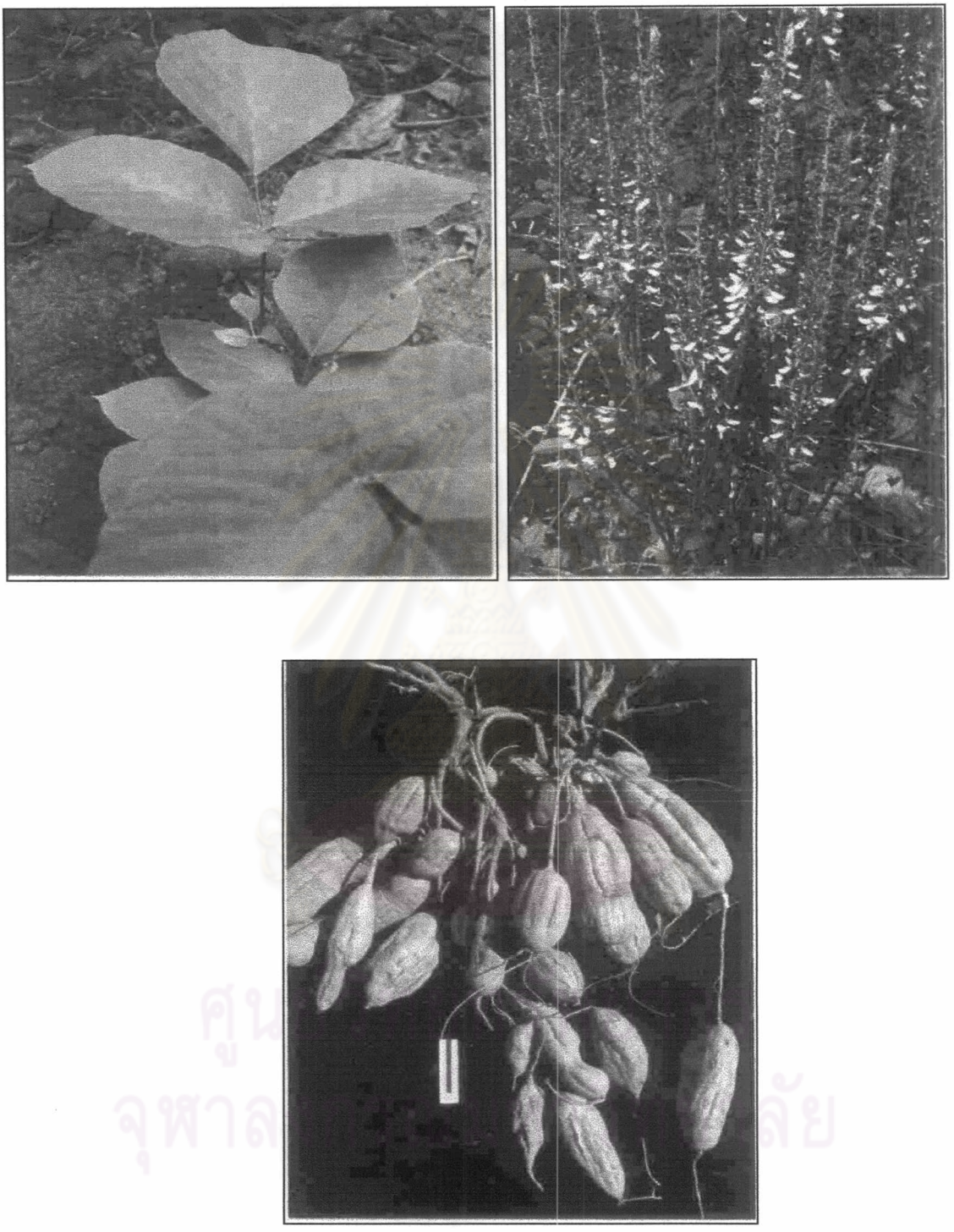
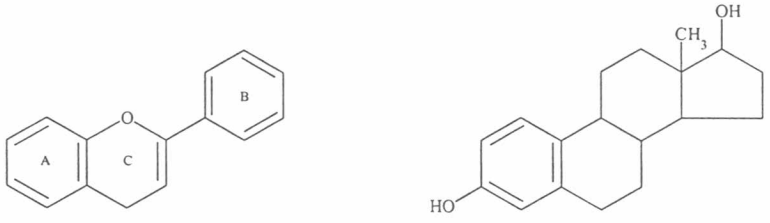


Figure 2 Leaves, flower and tuberous root of *P. mirifica*
(Photograph kindly provided by Wichai Cherdshewasart)

Chemical constituents of *P. mirifica*

P. mirifica had been found to contain at least 20 chemicals in the group of phytoestrogen with similar effects to estrogen. The chemical structure was not classified as steroid (Benson, Cowie and Howsking, 1961). The chemical constituents mainly found in *P. mirifica* were coumarins, isoflavones, chromenes, sterols and others such as alkane alcohol. Miroestrol was the first to be isolated and found in the amount of approximately 15 mg / kg dry weight (Bound and Pope, 1960). It was studied and believed to be the most important active compound. The estrogenic activity of miroestrol was previously estimated to be about 2.5 x 10^{-1} times that of 17β-estradiol (E_2) in the rat vaginal cornification test (Jones and Pope, 1961). Recently, deoxymiroestrol was isolated and found to be the compound with the highest estrogenic potency among the known phytoestrogens, approximately 10-fold more than miroestrol. However, it was easily oxidised by the air and converted to miroestrol and isomiroestrol (Chansakaow *et. al.*, 2000).

P. mirifica had proved to be extremely rich in isoflavonoid compound. The structure of isoflavone was fifteen carbon (C_6 - C_3 - C_6) backbone, a 1,2- diphenylpropane skeleton (isoflavonoid nucleus) (Fig3). The isoflavone was the most abundant isoflavonoids with different structures. The diversity was caused by the various substituents, for example methoxy, prenyl, methylenedioxy, that could occur on many different positions of the A and B rings (Dixon *et. al.*, 2000). Furthermore, it had structure features with the potent estrogen 17β-estradiol, particularly the phenolic ring and the distance between the two hydroxyl groups. These features determine their ability to bind estrogen receptors. Isoflavonoids could thus exert both estrogenic and anti-estrogenic activity, the latter by competing for receptor binding by estradiol.



isoflavonoid nucleus 17β-estradiol

Figure 3 The structure of isoflavonoid nucleus compare with estrogen

Isoflavone in soybean were reported to perform a number of important physiological functions involved in the growth and development of soybean for example soybean isoflavones induce *nod* genes in *Bradyrizobium japonicum*, and they are associated with the response of soybean to infection by *Phytothora megasperma* (Dixon *et. al.*, 1999). Isoflavonoids could act as stimulating, as well as inhibitory, factors in interactions of legumes with fungi. For example, the isoflavones daidzein and genistein, released in soybean root exudates, act as chemoattractant for zoospores of *Phytophthora sojae*, and also induce their encystment and germination (Morris and Ward, 1992). Likewise, biochanin A and several pterocarpan phytoalexin, including medicarpin and pisatin, stimulate germination of *Fusarium solani* spores. Isoflavone was one group of the potential anticarcinogenic soy compound such as daidzein and genistein that have been intensively studied which was reported to reduced incidence of breast cancer or mortality from prostate cancer (Adlercreutz *et. al.*, 1991). Daidzein was reported to create immune enhancing activity (Zhang, Li and Wang, 1988) inhibitory action on induced lung metastasis (Masson *et. al.*,1998) and on specific mutagenicity (Weisberger *et. al.*,1998). Genistein was shown to exhibit a negative result in Ames test for mutagenesis and acted as a specific inhibitor of tyrosine kinase (Akiyama *et. al.*,1987), an inhibitor of human breast cancer cell proliferation (Peterson and Barnes ,1991; Verma *et. al.*,1997; Wang and Kurzer, 1997), as well as reducing bone loss (Fanti *et. al.*,1998). Evidence had shown that isoflavones possess anticarcinogenic properties by acting as antiestrogen (Adlercreutz *et. al.*,1986), antioxidants (Naim *et. al.*,1976) and tyrosine kinase inhibitors (Akiyama *et. al.*,1987)

Other isoflavones, daidzin, from soy source was shown to prevent bone loss (Ishida *et al.*, 1998). Coumestrol was shown to be an estrogen supplement (Markaverich *et. al.*, 1995) and anti-oesteoporosis agent (Tsusumi, 1995). Stigmasterol was reported to exhibit anti-hypercholesterolemia (Westrate, Meijer, 1998), β-sitosterol was shown to reduce benign prostate hyperplasia (Klippel *et. al.*, 1997; Von Holtz *et. al.*, 1998) and inhibit human colon cancer growth (Awad *et. al.*, 1998), as well as mirificoumestan and its derivatives (Hoyodom, 1971; Ingham, Tahara, and Dziedzic, 1988). The list of chemical constituents and the structural formula found in *P. mirifica* tuberous root was summarized as shown in Table 1.

Table 1 The structure formulae of the chemical constituents in *P. mirifica*

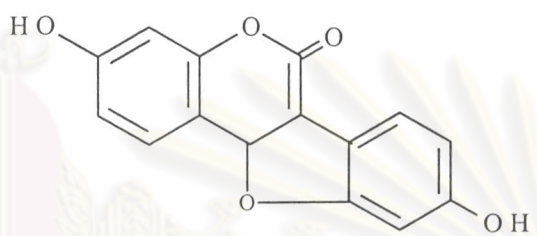
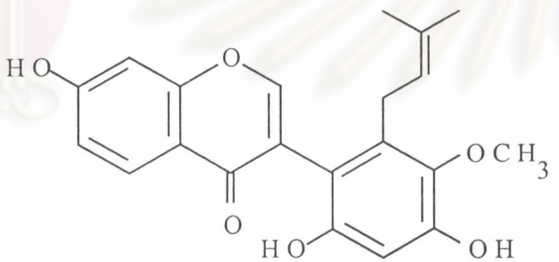
Category	Chemical	Structure	Reference
Coumarins	Coumestrol , (3,9 dihydroxycoumestan)		Ingham, Tahara and Dziedzic, 1986, 1988
	Mirificoumestan, (3,9- dihydroxy-8-methoxy- 7-(3,3-dimethylallyl)-coumestan)		Ingham, Tahara and Dziedzic, 1988

Table 1 (continued)

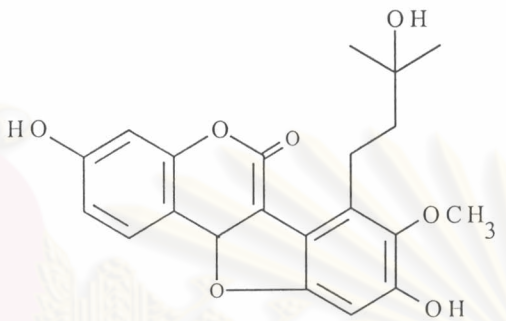
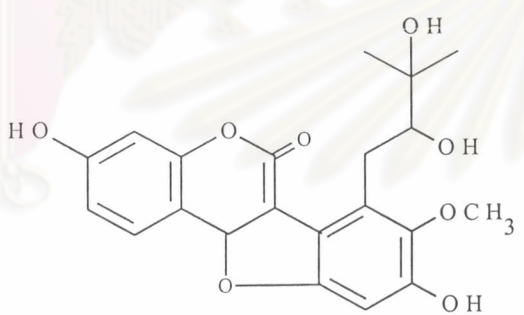
Category	Chemical	Structure	Reference
	Mirificoumestan hydrate (3,9- dihydroxy-8-methoxy-7-(3-hydroxy-3-mehtylbutyl)-coumestan)		Ingham, Tahara and Dziedzic, 1988
	Mirificoumestan glycol (3,9- dihydroxy-8-methoxy-7-(2,3-dihydroxy - 3-methylbutyl)-coumestan)		Ingham, Tahara and Dziedzic, 1988

Table 1 (continued)

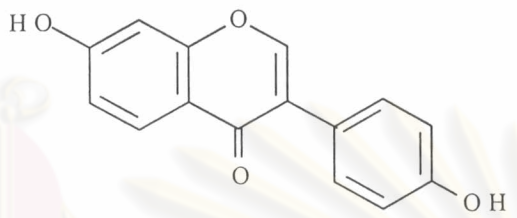
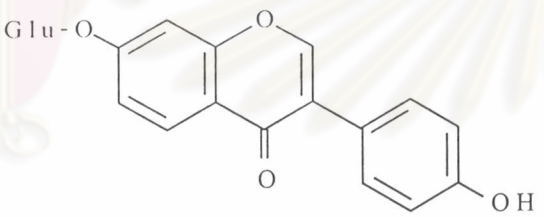
Category	Chemical	Structure	Reference
Isoflavone	Daidzein		Ingham Tahara and Dziedzic, 1986
	Daidzin (daidzein-7-o-glucoside)		Ingham, Tahara and Dziedzic, 1986

Table 1 (continued)

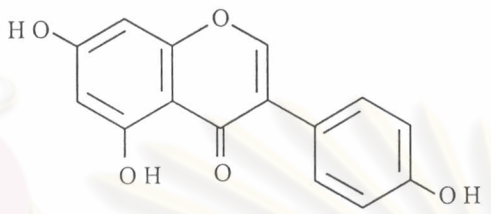
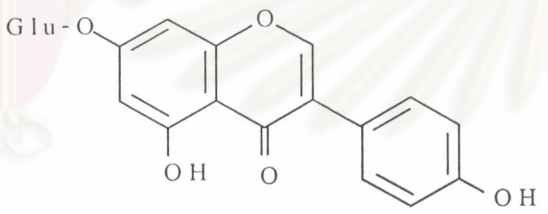
Category	Chemical	Structure	Reference
	Genistein		Ingham, Tahara and Dziedzic, 1986
	Genistin (genistein-7-o-glucoside)		Ingham, Tahara and Dziedzic, 1989

Table 1 (continued)

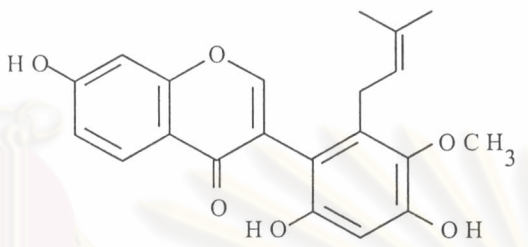
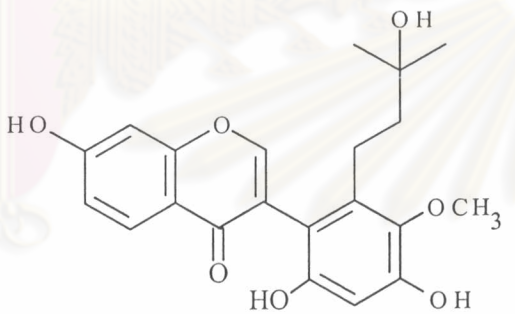
Category	Chemical	Structure	Reference
	Kwakhurin		Ingham, Tahara and Dziedzic, 1986
	Kwakhurin hydrate		Ingham, Tahara and Dziedzic, 1989

Table 1 (continued)

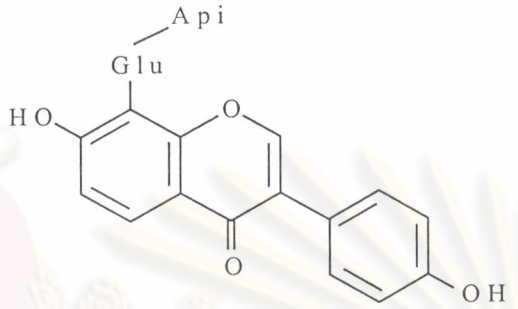
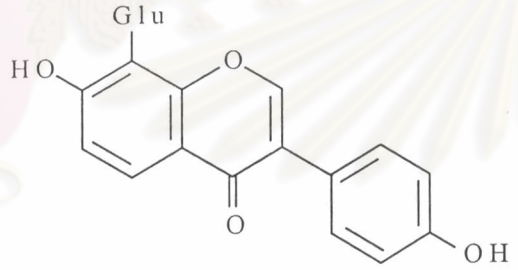
Category	Chemical	Structure	Reference
	Mirificin (puerarin 6"-o-β -apiofuranoside)		Ingham, Tahara and Dziedzic, 1986
	Puerarin (daidzein-8-glucoside)		Nilandihi *et al.*, 1957, Ingham, Tahara and Dziedzic, 1986

Table 1 (continued)

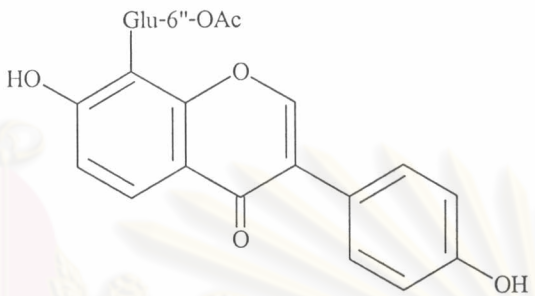
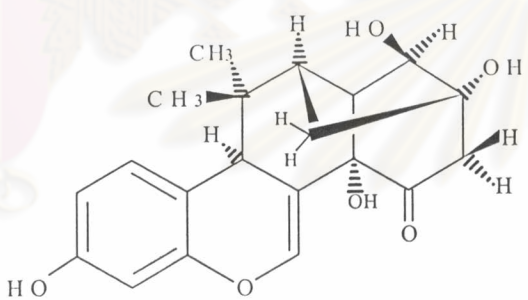
Category	Chemical	Structure	Reference
	Puerarin 6"-monoacetate		Ingham, Tahara and Dziedzic, 1989
Chromene	Miroestrol		Schoeller, Dohrn and Hohweg, 1940 Bound and Pope, 1960 Jones and Pope, 1960

Table 1 (continued)

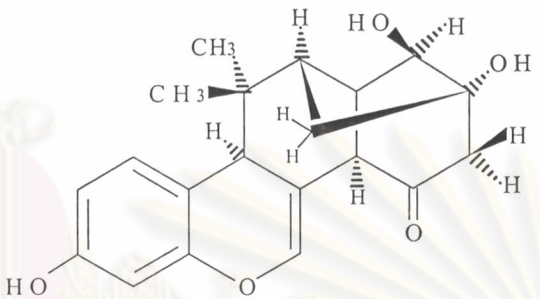
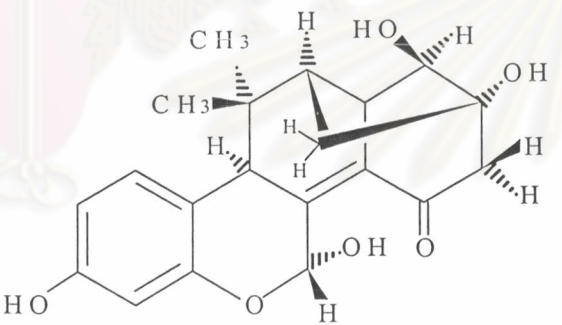
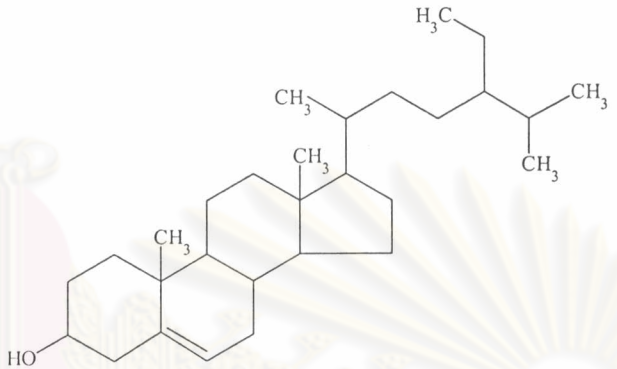
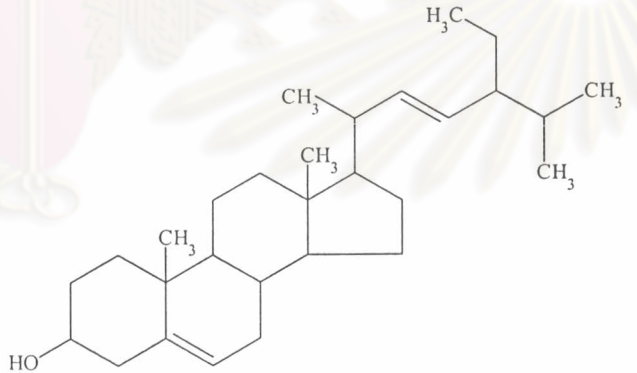
Category	Chemical	Structure	Reference
	Deoxymiroestrol		Chansakaew *et al.*, 2000.
	Isomiroestrol		Bound and Pope, 1960 Jones and Pope, 1960

Table 1 (continued)

Category	Chemical	Structure	Reference
Sterol	β-sitosterol		Hayodom, 1971
	Stigmatosterol		Hoyodom, 1971

2.2 *Butea superba*

B. superba or red Kwao Krua was a large size climber. The leaves were pinnately three foliate, acuminate leaflet and long leafstalk. The flowers were large with yellowish orange color. The petals were three times longer than the calyx. The pods were 3-4 inches long, oblong shaped with silvery silky short hair (Kruz, 1877; Brandis, 1990) but only one seed present. (Cherdshewasart, unpublished data)

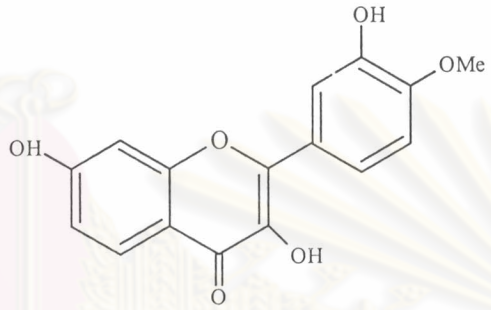
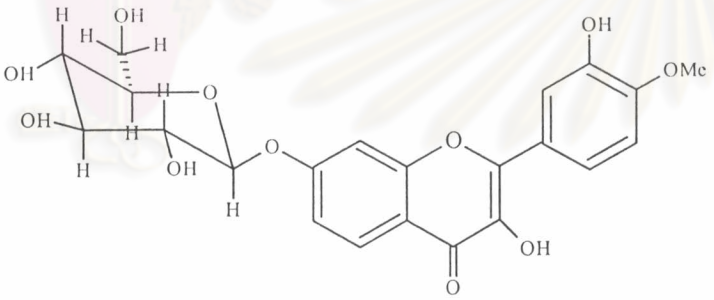
Figure 4 Tuber cross section and plant of *B. superba*
(Photograph kindly provided by Wichai Cherdshewasart)

Chemical constituents of *B. superba*

B. superba tuberous root was found to contain 5 groups of chemical constituents namely: carboxylic acid, steroid, steroid glycoside, flavonoid and flavonoid glycoside (Rugsilp, 1999). The stem of *B. superba* was found flavonoid glycoside (3, 7 – dihydroxy – 8 – methoxyflavone 7 – O - α - L - rhamnopyranoside) (Yadava and Reddy, 1998). The chemical constituents and their structural formula found in the tuberous root was summarized and shown in Table 2.

The flavonoid (3,7,3'- trihydroxy - 4'- methoxyflavone) and flavonoid glycoside (3, 5'- dihydroxy - 4'- methoxyflavone – 7 - β - D – glucopyranoside) in the tuber were found to be the inhibitor of cAMP phosphodiesterase at IC_{50} value of 190 and 58 μg/ml, respectively, which were the capable of stimulating the functioning of the central nervous system, the cells and the aldosterone hormone which attributes in increasing the male sexual performance (Roengsamran *et. al.*, 2000). β-sitosterol, Campesterol and Stigmasterol were presented in the plant. The chemical were offer protection and treatment for the common cancers (Awad and Fink, 2000) such as colon (Awad *et al*, 1998), prostate (Von Holtz, Fink and Awad, 1998; Awad *et al.*, 2001) and breast cancer (Awad, Downie and Fink, 2000; Awad *et al.*, 2000; Awad, Williams and Fink, 2001)

Table 2 The structure formulae of chemical constituent in *B. superba*

Category	Chemical	Structure	Reference
Flavone	3,7,3'- trihydroxy-4'-methoxyflavone		Roengsumran *et al.*, 2000
	3,5'-dihydroxy-4'methoxyflavone-7-O-β -D-glucopyranoside		Roengsumran *et al.*, 2000

2.3 *Mucuna collettii*

M. collettii or black Kwao Krua is a large woody climber, 30-40 m height scattered by stems in evergreen forest. The leaves were trifoliate; leaflets 4-8 by 2-4 inches sparsely hairy, entire margin; petiole 5-10 cm long, base stout. The flowers were hanging on the stem up to 12 inches long with 5 sepals covered with brown rough hair and unite into a bell-shaped tube. The petals were blackish-purple pea-like shaped. The stamens were two bundles. The pods were linear-oblong shaped up to 16 inches long. The seeds were hard and flattened. The flowers were blooming during January to March (Pengklai, 1977).

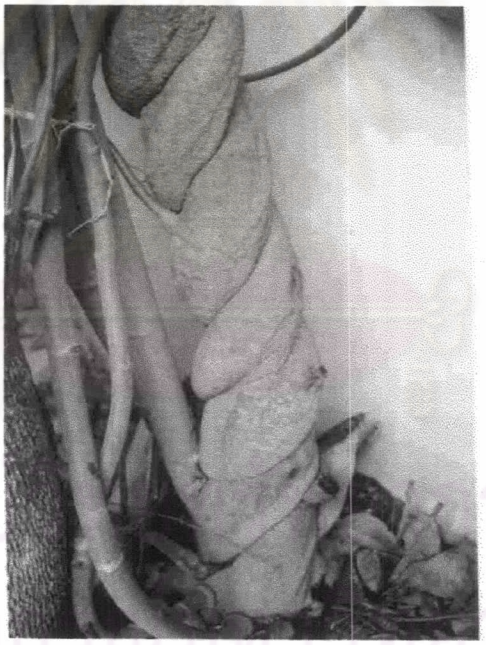


Figure 5 The stem of *M. collettii*
(Photograph kindly provided by Wichai Cherdshewasart)

Chemical constituents of *M. collettii*

The whole stem of *M. collettii* was found to contain 3 interested chemical constituents in ethyl acetate crude extract namely: Kaempferol Quercetin and Hopeaphenol. The chemical constituents and their structural formula found in the *M. collettii* was summarized and shown in Table 3.

The median inhibitory concentration (IC_{50}) for cAMP phosphodiesterase inhibiting effect of Kaempferol Quercetin and Hopeaphenol were found to be 281.83, 80.91 and 22.75 μg/ml, respectively (Roengsamran *et al.*, 2000). The effect of phytoestrogens, kaempferol and quercetin, on mammary cancer cell culture was analyzed. Hopeaphenol was reported to be highly cytotoxic on KB cell line (an epidermal carcinoma of the mouth) with IC_{50} value of 1.2 μg / ml (Ohyama *et al.*, 1999). The *in vivo* study indicated that *M. collettii* affected the reproductive system by causing the abnormality of spermatozoa (Wutteeraphon *et al.*, 2001).

Table 3 The structure formulae of chemical constituents in *M. collettii*

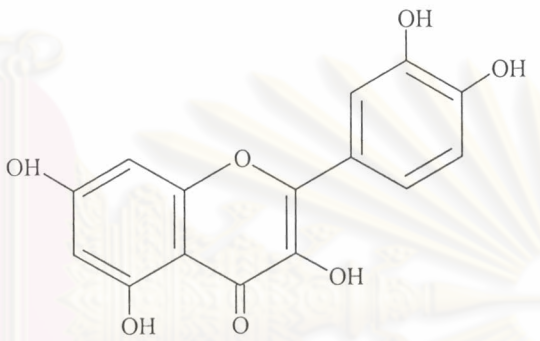
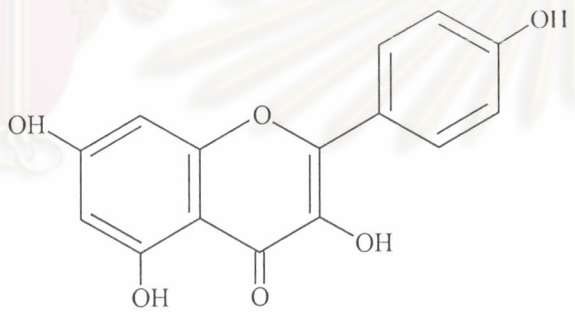
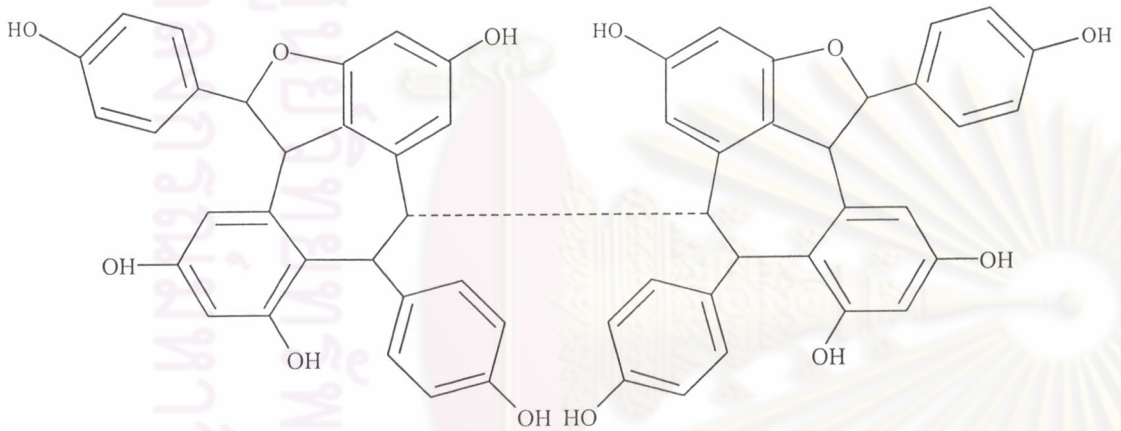
Category	Chemical	Structure	Reference
Flavone	Quercetin		Roengsumran *et al.*, 2000
	Kaempferol		Roengsumran *et al.*, 2000

Table 3 (continued)

Chemical	Structure	Reference
Hopeaphenol		Roengsumran *et al.*, 2000

IV . Factors affecting isoflavone content in *P. mirifica*

Traditional medicines are very important in primary health care, where they could be used instead of expensive western medicines. Their potential value is in the possibility that they may contain new biologically active compounds, which could be further developed into drugs for the international market. It had been established that various biological factors were responsible for the variability of chemical constituents in medicinal plant such as genetic, the stage of plant's development, seasonal variation, location.

Isoflavonoids were restricted primarily to the Leguminosae, although they occurred rarely in other families such as the Apocynaceae, Pinaceae, Compositae and Moraceae. (Dixon, 1999) *P. mirifica* was a traditional medicine which mostly contained isoflavones group. Therefore, the factors affecting isoflavone content of soybean seeds were studied including genetic and environmental factors. There are reported that the total isoflavone content in the hypocotyl of soybean seed was 5.5 to 6.0 times higher than that of the cotyledons; the glycitin forms occurred only in the hypocotyl part and no isoflavones were observed in the seed coat. The accumulation of isoflavones occurred during seed filling (between 35-60 days after flowering), with genistin and malonyl genistin content increasing at the end of the seed development stage, while daidzin and malonyl daidzin increasing at the entire period (Kodou *et al.*, 1991). Isoflavone concentration was observed among soybean cultivars grown in different location and crops years, found that significantly difference in location interaction for individual and total isoflavones (Eldridge and Kwolek, 1983) and in crop years in terms of individual and total isoflavones (Wang and Murphy, 1994). Early maturing soybean cultivars or "summer type soybeans", had a stable low concentration of isoflavones (Kitamura *et al.*, 1991). Furthermore, climatic was the one factor affecting to isoflavone concentration, high temperature during the seed filling stage was related as a major factor in determining the levels of isoflavones in seeds. (Tsukamoto *et al.*, 1995) Soybean cultivars with a reduced isoflavone concentration occur in location of warmer temperature (low latitude) (Carrao-panizzi *et al.*, 1999). Isoflavone contents of seeds of varieties grown at different location, under different temperature and different crop year had significantly difference in the concentration of isoflavone.

(Wang and Murphy, 1994; Carrao-panizzi *et al.*, 1999) Since a quality of *P. mirifica* is based directly on the isoflavone contents then it is interesting to determine the levels of those compounds, particularly *P. mirifica* obtained from various areas of Thailand and some environmental factors affecting to isoflavone concentration in *P. mirifica*.

V. Standard phytoestrogen in Leguminosae plant

The isoflavonoids were restricted primarily to the Leguminosae. Most of the leguminous plants contained genistein, daidzein, genistin and daidzin as common isoflavones. *P.lobata* and *P. mirifica* particularly contained a large amount of puerarin. Eventhough miroestrol and deoxymiroestrol were separated to be potent phytoestrogen with a comparable effect to that of estrogen (Jones and Pope, 1960) but they existed in a little amount and deoxymiroestrol could easily converted into miroestrol and isomiroestrol by arial oxidation (Chansakaow *et al.*,2000) thus was not practical to be assigned as marker for HPLC analysis in leguminous plant, especially in *P. mirifica*. Marker compound should be easily available and commercialize reference standards and had existed in plant extract at least 2 substances. This study thus emphasized the five isoflavones namely puerarin, genistein, daidzein, genistin and daidzin.