CHAPTER II
LITERATURE REVIEWS

1. *Pueraria candollei* Wall. ex Benth.

Pueraria candollei or White Kwao Krua is a well-known Thai herbal medicine belonging to family Leguminosae, subfamily Papilionoideae. White Kwao Krua is a climbing with tuberous roots widely distributed in deciduous or sparse forests in the north, west and northeast of Thailand (Van der Maesen, 2002). In Thailand there are two varieties of *P. candollei*, *P. candollei* Wall. ex Benth var. *mirifica* (Airy Shaw & Suvat.) Niyomgham and *P. candollei* Wall. ex Benth var. *candollei* (Smitinand, 2001). These two varieties have similar botanical characteristic but both vary in chemical component containing (Yusakul et al., 2011).

1.1 Botanical characteristics

Pueraria candollei is a climbing shrub preferring dimly sunlight. Their botanical characteristics are shows in figure 1(Van der Maesen, 2002). The leaves are pinnately trifoliate stipulate; terminal leaflet. Flowers are bluish-purple (Papilionaceous form) with a 30 cm. long inflorescences. The flower contains five sepals and the petals are one standard with two keels. Flowering occurs during February to March and produces seed in April. The pods are slender typically short or elongate, with hairs (*P. candollei* var. *mirifica*) or no hair (*P. candollei* var. *candollei*), including 1-10 single seeds when fully mature and dry turning into brown color and flat. Their tuberous roots look like a chain of round shape bulb of various sizes and shapes depending on the environment in which it is grown and their genetics (Suwanvijitr et al., 2010).

Figure 1 (A) *P. candollei* var. *mirifica* (PM) and (B) *P. candollei* var. *candollei* (PC)

1.2 Chemical constituents

The tuberous roots of *P. candollei* containing various chemical compounds, known as phytoestrogens, then categorized into five groups as shown in table 1.

(1) Major isoflavonoid and their glycoside group as daidzein/daidzin, genistein/genistin, kwakhurin, kwakhurin hydrate, mirificin, puerarin and puerarin-6" monoacetate.

(2) Coumestan group as coumestrol, mirificoumestan, mirificoumestan glycol and mirificoumestan hydrate.

(3) Minor chromene group as miroestrol, deoxymiroestrol and isomiroestrol.

(4) Sterol group as stigmasterol and β-sitosterol.

(5) Pterocarpan group as tuberosin and puemiricarpene.

Chansakaow et al. (2000) reported that 100 g of dry powdered *P. candollei* var. *mirifica* tubers contains major isoflavonoid/their glycoside (0.046% of daidzein) and minor chromene (0.002-0.004% of miroestrol and deoxymiroestrol). The amount of each phytoestrogens in tuberous roots were difference level depend on varieties and cultivation (Yusakul et al., 2011).

Table 1 Chemical constituents containing in the tuberous roots of *P. candollei* with their structure

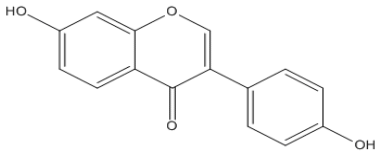
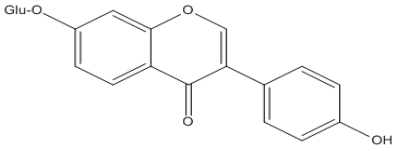
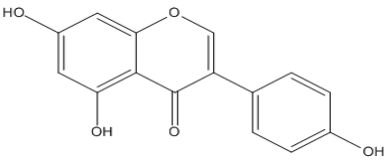
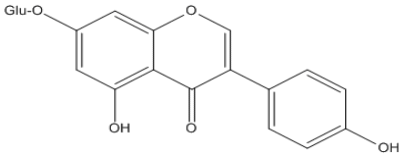
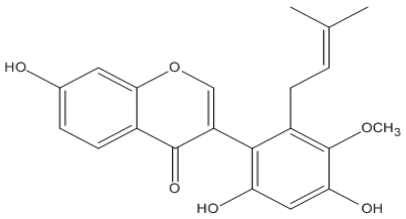
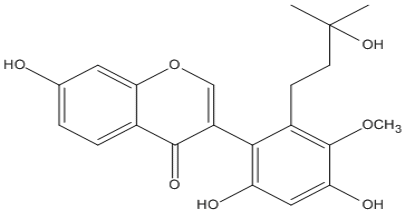
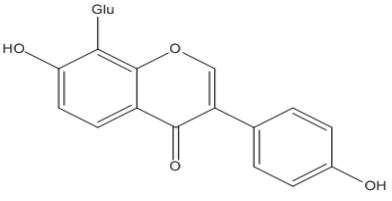
Chemical constituents	Structure	Reference
1. Isoflavone/Isoflavone glycoside		
Daidzein		Ingham et al., 1986
Daidzin		Ingham et al., 1986
Genistein		Ingham et al., 1986
Genistin		Ingham et al., 1986
Kwakhurin		Ingham et al., 1986
Kwakhurin hydrate		Ingham et al., 1989
Mirificin		Ingham et al., 1989

Table 1 Chemical constituents containing in the tuberous roots of *P. candollei* with their structure (Cont.)

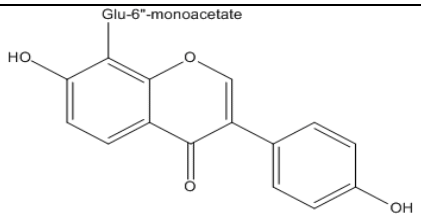
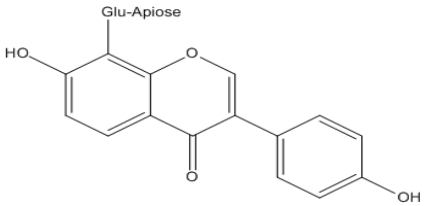
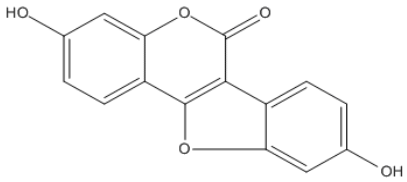
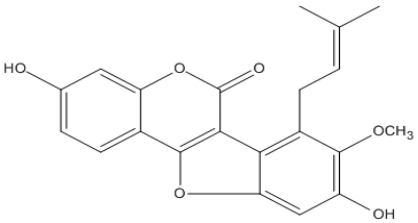
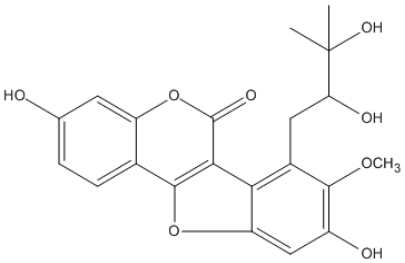
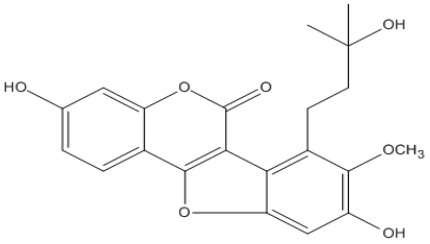
Chemical constituents	Structure	Reference
Puerarin		Ingham et al., 1989
Puerarin-6" monoacetate		Ingham et al., 1989
2. Coumestans		
Coumestrol		Ingham et al., 1986
Mirificoumestan		Ingham et al., 1988
Mirificoumestan glycol		Ingham et al., 1988
Mirificoumestan hydrate		Ingham et al., 1988

Table 1 Chemical constituents containing in the tuberous roots of *P. candollei* with their structure (Cont.)

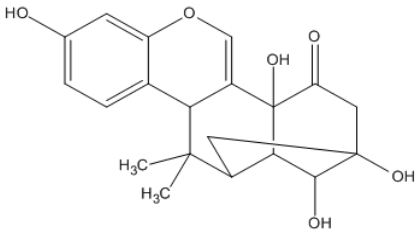
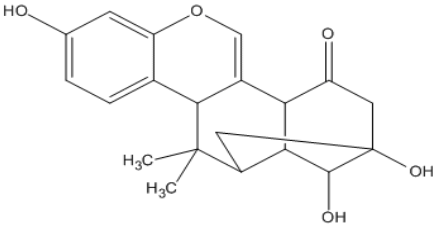
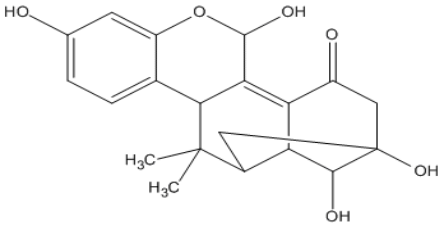
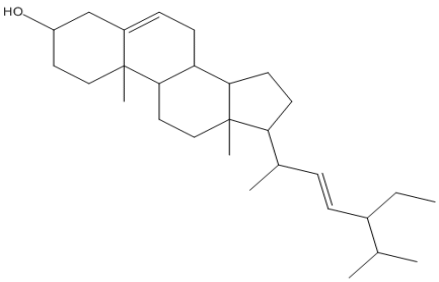
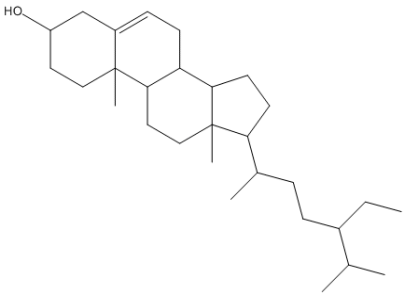
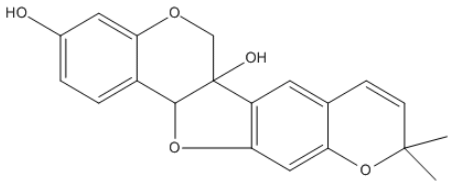
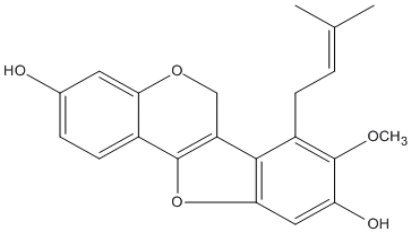
Chemical constituents	Structure	Reference
3. Chromenes		
Miroestrol		Bounds and Pope, 1960; Jones and Pope, 1961
Deoxymiroestrol		Chansakaow et al., 2000a
Isomiroestrol		Chansakaow et al., 2000a
4. Sterols		
Stigmasterol		Hayodom, 1971
β-sitosterol		Hoyodom, 1971

Table 1 Chemical constituents containing in the tuberous roots of *P. candollei* with their structure (Cont.)

Chemical constituents	Structure	Reference
5. Pterocarpans		
Tuberosin		Chansakaow et al., 2000b
Puemiricarpene		Chansakaow et al., 2000b

1.3 Pharmacological activities

For ethnobotanic use, local communities in Thailand have used White Kwao Krua for well over one hundred years, specifically for its rejuvenating qualities. It is stated in the pamphlet (Suntara, 1931) that *P. candollei* can serve as skin moisturizer, improve growth of hair, improve body flexibility and sexual performance, and firm and enlarge the breasts. Some of the rejuvenating effects reported by Kerr (1932) and Wanandorn (1933) for preparations containing powdered *P. candollei* root are consistent with the presence of one or more estrogenic compounds. Wanandorn (1933) found a gradual swelling and soreness of the breasts, with the symptoms disappearing slowly over several weeks when the medicine was discontinued.

According to the various phytoestrogens containing in tuberous roots of *P. candollei***,** the estrogenic activities were investigated in cell line, animals and in clinical trial. The studies of estrogenic activities of this plant affected on both female and male animals sexual reproductive organs, prevention of bone lose and reducing postmenopausal symptoms. Furthermore the antioxidant and antihyperglycemic activities were also investigated.

In uterotropic and vaginal cornification assay, *P. candollei* root extracted were fed on animals models (such as immature female and male mice, ovariectomized rats, male rats and female monkeys) (Sukhavachana, 1941; Jones et al., 1961; Malaivijitnond et al., 2006; Trisomboon et al., 2007a, 2007b; Cherdshewasart et al., 2007a, 2007b). Sukhavachana (1941) found that ethanol extracts of powdered *P. candollei* stimulated the proliferation of vaginal and uterus epithelium in ovariectomized rat. These results were subsequently supported by other studies (Jones et al., 1961; Malaivijitnond et al., 2006; Cherdshewasart et al., 2007a, 2007b). The estrogenic activities of *P. candollei* on reproductive organs were confirmed by suppression of pituitary gonadotropin and inhibit INH secretion in both female and male mice and rats, and in female monkeys but the response is greater than in female. (Trisomboon et al., 2004a, 2004b, 2005, 2006a, 2006b, 2007a, 2007b; Jaoenporn et al., 2006) The *in vitro* MCF-7 cell line proliferation assay used for determination of estrogenic bioactivity found the phytoestrogens from *P. candollei* could effective only binding to ERα of MCF-7 cell (Cherdshewasart et al., 2008). Matsumura et al. (2005) comparative study estrogenic potency of eight isoflavonoid isolated from *P. candollei* on this assay, showed variation in their bioactivity levels. In rank order of potency for the phytoestrogens of these plants, deoxymiroestrol > miroestrol > coumestrol > genistein > daidzein based in MCF7 human breast cancer cells. Strong estrogenic activity of deoxymiroestrol is equivalent to 17β-estraldiol, inducing of expression of aryhydrocarbon receptor (AhR) and ER related genes (Udomsuk et al., 2011b) and the regulation of the testicular gene related sex hormone synthesis pathway (Udomsuk et al., 2011c).

In sex-hormone-deficient male rats, treatment *P. candollei* with root powder completely inhibited bone loss in long bones and axial bones. At higher doses, it increased bone density without affecting accessory sex organs (Urasopon et al., 2007). In a similar study, *P. candollei* completely inhibited bone loss in long bones and axial bones in estrogen-deficient female rats (Urasopon et al., 2008). In bone cell culture, genistein induced significant increase in the calcium content and alkaline phosphatase activity (Yamaguchi and Gao, 1998). And in other study genistein and daidzien also increase in the proliferation of the mouse osteoblastic cell line MC3T3-E1 (Sugimoto and Yamaguchi, 2000).

Phase 1 to 3 trials in Thailand compared the estrogenic effect of *P. candollei* to conjugated equine estrogen in relieving climacteric symptoms in perimenopausal women (Lamlertkittikul and Chandeying, 2004; Chandeying and Sangthawan, 2007). In small clinical trials, the administration of *P. candollei* crude extract improved hot flushes, frustration, sleep disorder, skin dryness, high blood cholesterol, amenorrhea, and other menopause-related symptoms in women. There was no change in blood cells or liver and renal function (Muangman and Cherdshewasart, 2001; Hosoyama et al., 2007).

The antioxidant activity and antihyperglycemic effect were evaluated and found that the activity is correlated with puerarin (Cherdshewasart and Sutjit, 2008; Khitkal et al., 2009). The crude of *P. candollei* decreased neuronal cell death in a time- and dose-dependent manner against neurotoxic agents in an Alzheimer disease model in vitro (Sucontphunt et al., 2007; Sawatsri et al., 2004).

1.4 *P. candollei* with trend in market

Commercial products from *P. candollei* are continually introduced into the world market and have become popular in Thailand, Korea, and Japan. Most commercial products are available as topical rejuvenating, antiaging, or skin-lightening creams/gels, soaps, capsules or tablets for increasing appetite, enlarging breasts, modulating hair growth or regrowth, and other rejuvenating purposes. Until now domestic and global demand for the raw materials from *P. candollei* roots or tubers has increased, resulting in intense harvesting of the plant from the forests of Thailand.

2. Isoflavonoid

Isoflavonoid are phytoestrogens commonly found in family Leguminosae or soy bean and pea subfamily. In White Kwao Krua or *P. candollei* also found isoflavonoid, the major compounds containing in tubers roots of this plant. Ingham et al. (1986) isolated and identified by chemical and spectroscopic method then reported isoflavonoid and their glycoside in *P. candollei* as daidzein/daidzin, genistein/genistin, kwakhurin, kwakhurin hydrate, mirificin, puerarin and puerarin-6” monoacetate.

The pharmacological activity of isoflavonoid reported decreased the incident of the breast, prostate and intestine cancer (Watanabe et al., 2002), improved hot flushes, frustration in postmenopausal women (Taylor, 2003), preventive cardiovascular disease in term of decrease LDL and increase HDL level (Davis et al., 1998) and also reduce blood sugar level in STZ-diabetic rats (Khitkal et al., 2009).

2.1 Isoflavonoid biosynthesis pathway

Isoflavonoid are synthesized by a legume via a branch of phenylpropanoid pathway (fig. 2). Enzyme chalcone synthase is the first step in the production of flavonoid and isoflavonoid. In legume plants produce two kinds of chalcone, tetrahydroxy chalcone (naringenin chalcone) and trihydroxy chalcone (isoliquiritigenin chalcone) whereas nonlegume plants only produce tetrahydroxy chalcone (naringenin chalcone). The Both naringenin and isoliquiritigenin chalcone get converted into flavanones by chalcone isomerase (CHI). These chalcone isomerases convert naringenin chalcone to naringenin or convert isoliquiritigenin to liquiritigenin. The key enzyme that introduces isoflavonoid specific branch in the phenylpropanoid pathway is 2-hydroxyisoflavanone synthase (isoflavone synthase, IFS). IFS is a microsomal cytochrome P450 monooxygenase enzyme that catalyzes a 2, 3 aryl ring migration of flavanones to flavones and subsequent hydroxylation of the resulting C-2 radical. The reaction product of IFS, 2 hydroxyisoflavanone, is extremely unstable and undergoes dehydration by forming a double bond between C-2 and C-3 by a dehydratase to form genestein or daidzein (Dhaubhadel, 2011).

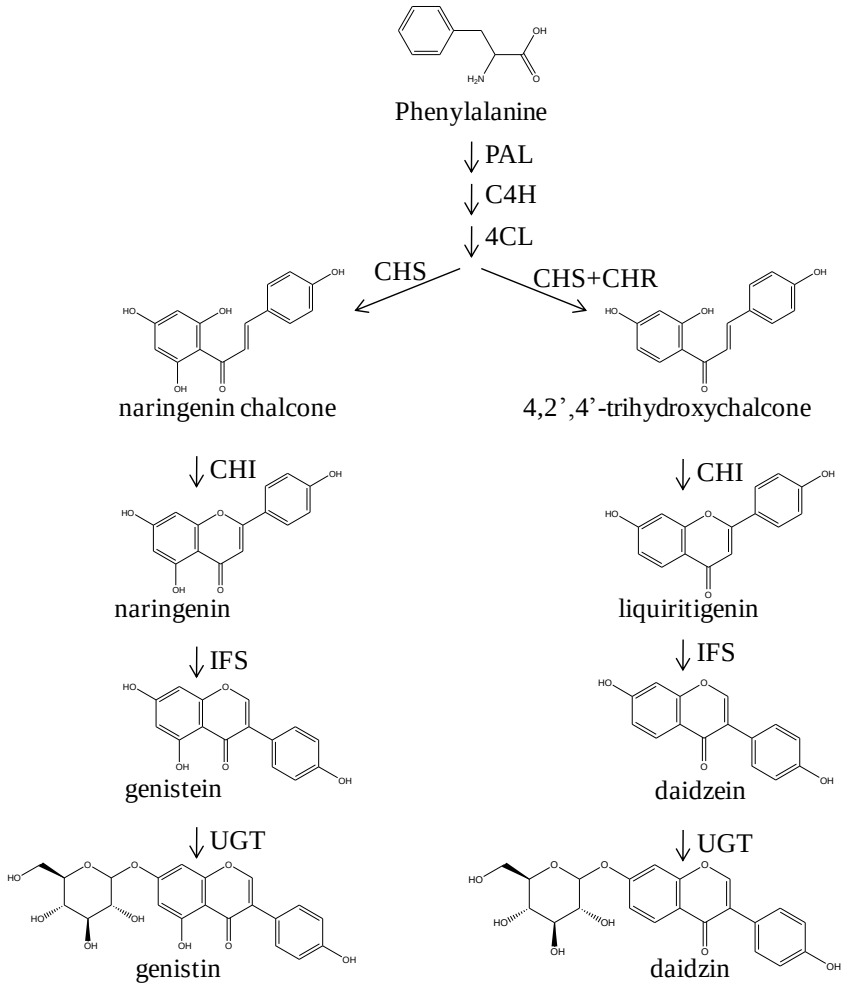


Figure 2 Isoflavonoid biosynthesis pathways in soybean (Dhaubhadel, 2011)

2.2 Analysis and identification

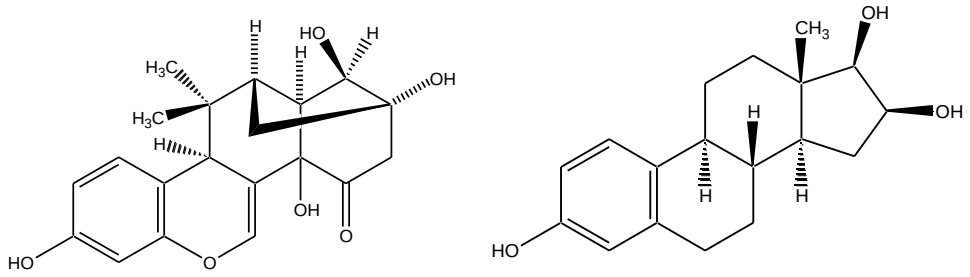
The alternative method (Pongkitwitoon et al., 2010) to analyze these isoflavonoid is an enzyme- linked immunosorbent assay (ELISA), using polyclonal antibody (PAb) against puerarin and daizin. An ELISA applied for analyses of the major active compounds, total isoflavonoid in *Pueraria* spp. plants and products distributed worldwide. The advantages of the ELISA are its effective cost performance, increasing sample throughput, rapidity, and sensitivity.

3. Chromene

Miroestrol and its derivative like deoxymiroestrol and isomiroestrol are a chromene compounds which possess highest estrogenic activity among the known phytoestrogens due to structural similarity to 17β-estradiol (E2), the main estrogen in the human body (fig. 3). The strong estrogenic activity of miroestrol can be assumed to reflect the non-planar nature of the molecule in which the distance between the 3-OH and the 18-OH (and the 3-OH and 17-OH) is similar to that between the 3-OH and 17β-OH of 17β-estradiol, thereby allowing miroestrol to effectively attach to estrogen receptor sites (Jones and Pope, 1961).

Chansakaow et al. (2000a) found deoxymiroestrol can be oxidized to give miroestrol, a process which might occur naturally in this plant as part of a biosynthetic sequence, or take place when the sliced tubers are being air-dried, powdered and processed into traditional medicines.

As reported previously (Matsumura et al., 2004), the estrogenic activity of deoxymiroestrol and miroestrol when compared to 17β-estradiol in proliferation of MCF-7 cell was estimated to be about 0.3 and 0.05 fold after 7 days, respectively. While in proliferation of MCF-7 cell after 14 days, estrogenic activity of deoxymiroestrol and miroestrol was about 1 and 0.25 fold of 17β-estradiol, respectively. In human trial (Cain, 1960), ten women with postmenopausal symptom were treated orally with 1 mg or 5 mg of pure miroestrol for 14 days. Both dose levels caused significant vaginal cornification as well as effects such as an enlargement but even small quantities of miroestrol cause unacceptable side effects (malaise, headaches, nausea/vomiting) in the majority of patients. Although the previously reported Chansakaow et al. (2000b) found very low content of miroestrol and its derivative in *P. candollei* var. *mirifica*, each compound estimated to be about 0.002% dry weight compare with other isoflavones like genistein to be about 0.040 % dry weight but both miroestrol and deoxymiroestrol having potent estrogenic activity. Thus when claim to *P. candollei* var. *mirifica* the most estrogenic activity was affected from predominance of miroestrol derived from deoxymiroestrol or, more probably, the combined effects of both compounds.



Miroestrol 17β-estradiol

Figure 3 Chemical structure of miroestrol and 17β-estradiol

3.1 Possible hypothesis of chromene biosynthesis in *P. candollei*

When consider the structure of miroestrol, we hypothesize that its structure come from the biosynthetic reaction coupling process (prenylation by prenyltransferase enzyme) of the shikimate or polyketide pathway providing an aromatic moiety (isoflavone) and the isoprenoid pathway (dimethylallyl diphosphate (DMAPP)) derived from the mevalonate or MEP (methyl erythritol phosphate) pathways (Fig. 4). Prenyltransferase transfer of a DMAPP to the B-ring of isoflavan, then modifications of the prenyl moiety become deoxymiroestrol and its oxidized to miroestrol. The prenyltransferase enzyme was commonly found in plastid site, it is possible that prenylation of chromene may take place in chloroplast. Thus plant tissue culture of *P. candollei* with ability cell producing chloroplast is one of interesting ways to increase the production of chromene in this plant.

Until recently, biochemical and genetic knowledge on this plant-enzyme was very limited, only 12 species were reported and no research on White Kwao Krua has been reported. Furthermore the hypothesis of biosynthesis of miroestrol and deoxymiroestrol, the potent estrogenic activity, is possible to relate with this enzyme.

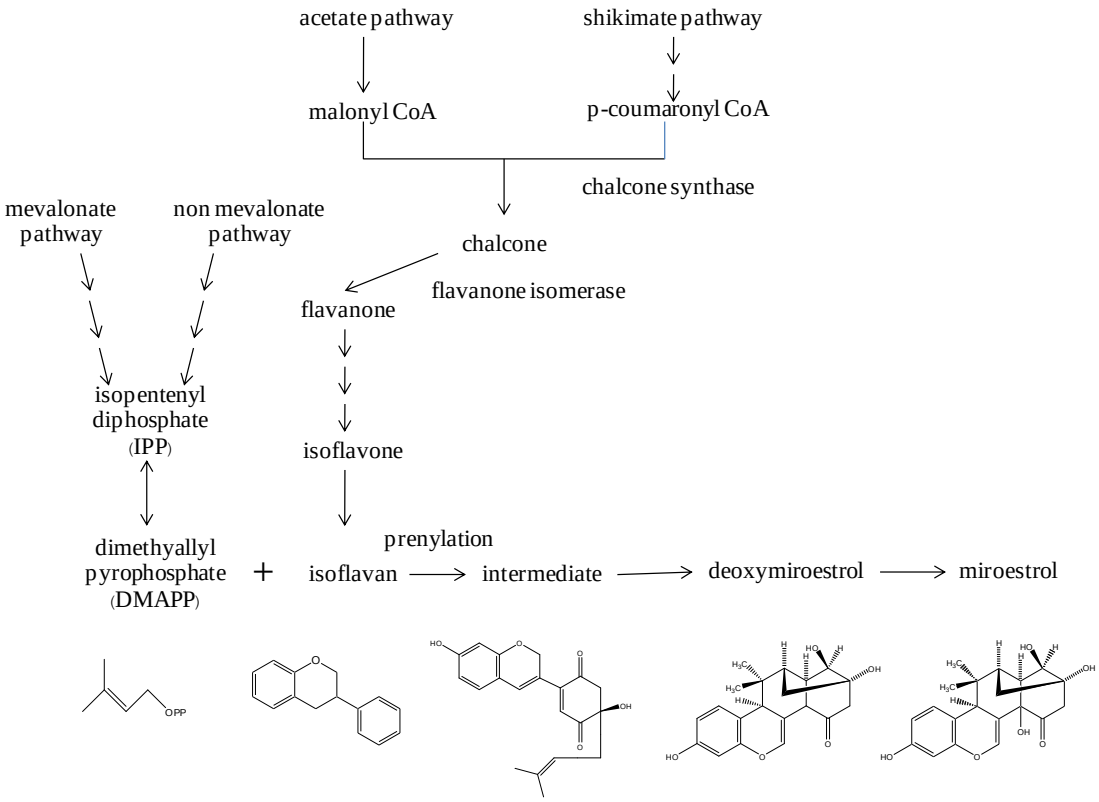


Figure 4 The possible hypothesis of chromene biosynthesis in *P. candollei*

3.2 Analysis and identification

Yusakul et al. (2011) was developed a high-performance liquid chromatography (HPLC) method to determine the contents of miroestrol and deoxymiroestrol from tuber of *Pueraria candollei* var. *mirifica* and *P. candollei* var. *candollei*. The mobile phase consisted of 20% acetonitrile containing 1.5% acetic acid at a flow rate of 1.0 ml/min. The detection wavelength was 254 nm. HPLC was performed using a PerkinElmer Series 200 LC pump connected with a PerkinElmer 785A UV/VIS detector. An RP-18 column was used.

4. Plant tissue culture

Plant cell, tissue, and organ culture is a set of techniques designed for the growth and multiplication of cells and tissues using nutrient solutions in an aseptic and controlled environment. This technology explores conditions that promote cell

division and genetic reprogramming in *in vitro* conditions. Until now plant tissue culturing techniques represent a potential renewable source of valuable medicinals, flavours, essences and colourants that cannot be produced by microbial cells or chemical syntheses.

Consequence of Cherdshewasard et al. (2007b) founding the variation of isoflavonoid containing in each *Pueraria* spp. depend on their cultivation and harvesting season. Nowadays the application of plant tissue culture techniques in herbal medicines is extensively, in case of enhancing pharmacologically active compounds in medicinal plants. Due to getting a highly and continuously production of plant secondary metabolite with quality control, many techniques were used improving active compounds accumulation in plant such as elicitation, immobilization, permeabilization and genetic modification or controlling biosynthesis pathway.

Elicitation was a popular techniques enhancer of plant secondary metabolites production by treatment called elicitors. Elicitors are divided in to (1) biotic elicitors from fungi, bacteria, viruses, plant cell wall component and (2) abiotic elicitors such as inorganic compounds. The mechanism of abiotic elicitation on overproduction of secondary metabolites in plants is poorly understood. A general mechanism for biotic elicitation in plants may be summarized on the basis of elicitor-receptor interaction. When a plant or plant cell culture is challenged by the elicitor an array of biochemical activities occur (Angelova et al., 2006).

Furthermore using of plant hormones as elicitors to increase flavonoid and terpenoid production in various plants have been studied (Cristiane et al., 2009; Inthima et al., 2009; Nagira et al., 2006; Gagne' et al., 2010; Sun et al., 2011). Recently, production of isoflavonoid in callus culture of *P. candollei* var. *mirifica* (PM) was reported to be influenced by plant hormone, thiadiazuron (Udomsuk et al., 2010). Therefore using plant hormone to enhance isoflavonoid production in this plant is interesting.

The production of isoflavonoid in *in vitro* culture of various plants has been reported. The reports of plant tissue culture studies in *P. candollei* were summarized as following.

Ormking (2003) studied *in vitro* multiplication of White Kwao Krua tissue culture, founding callus in woody plant media with NAA 0.1 mg/L and kinetin 1.5 mg/L were highest growth rate.

Wungsintaweekul (2003) had been established cell culture of *P. candollei* with MS medium supplemented with plant hormones. The result showed that callus and cell suspension of this medicinal plant had produce daidzein and genistein. The yield of isoflavone production in cell suspensions (daidzein 30.7 mg/g dry wt), genistein 4.9 mg/g dry wt), respectively) is higher than callus culture (daidzein 7.6 mg/g dry wt), genistein 0.5 mg/g), respectively)

Thanonkeo and Panichajakul (2006) were established callus cultures of *P. candollei* var. *mirifica* from various parts of explants with the objective of isoflavone, daidzein and genistein production. The result revealed that callus of *P. candollei* var. *mirifica* was capable of producing high level of both isoflavone consistently. The culture temperature played an important role in the growth and isoflavone production. The callus established from the stems produced the highest yield of daidzein (5.12 mg/g dry wt) and genistein (2.77 mg/g dry wt), which was remarkably higher than the intact plants.

Thanonkeo (2006) studied effect of duration time and types of elicitors on growth and production of daidzein and genistein in *P. candollei* var. *mirifica* cell suspension culture. The result showed methyl jasmonate could elicit daidzein and genistein higher than control group 6-fold, with the optimal duration 12 days of elicitation.

Khitkal et al. (2009) studied effect of salicylic acid, $CuCl_2$ and chitosan on *in vivo* culture of *P. candollei* var. *mirifica*. The six months plants were sprayed elicitors every 7 days for two months. The result found co-culture of $CuCl_2$ and chitosan increasing the highest puerarin and genistein content ((0.02 mg/g, 0.4 mg/g dry wt), respectively)

Udomsuk et al. (2009a, 2009b) reported total isoflavonoid content in *P. candollei* var. *candollei* hairy root culture higher than that found in native root about 5-fold (36.48 mg/g dry wt). Other study showed production of isoflavonoid in callus culture of *P. candollei* var. *mirifica* was influenced by plant hormone, thiadiazuron. Callus culture with 0.5 mg/l thiadiazuron produced the highest total isoflavonoid (50.39 mg/g dry wt).

Boonsnongcheep et al. (2010) found both variety of *P. candollei* cell suspension with 0.56 mM BA and 4.52 mM 2,4-D could produce the highest isoflavonoid (23.8 mg/g dry wt).

Korsangruang et al. (2010) studied effect of elicitors on isoflavonoid accumulation in both variety of *P. candollei* cell suspension culture. 0.2 mM methyl jasmonate could elicit the highest isoflavonoid production (40.5 mg/g dry wt).

Udomsuk et al. (2011) studied effect of elicitors on total isoflavonoid production in *P. candollei* var. *candollei* hairy root culture. Resulting to yeast could elicit the highest isoflavonoid accumulation in hairy root culture (60 mg/g dry wt, 4-5 folds than control group).