CHAPTER II

LITERATURE REVIEW

2.1 Kwao Krua plants

The consumption of Kwao Krua plants as traditional medicines has been long-time popular in Thailand. The plants in this group comprises of the white (*P. mirifica*), the red (*B. superba*) and the black (*M. collettii*) Kwao Krua. They are classified into the family Leguminosae (Ridley, 1976; Pengklai, 1977; Suvatti, 1978). Phytoestrogens are naturally occurring compounds derived from plant. Plants synthesis primary metabolites (sugar, protein, amino acid, purines, pyrimidines and chlorophyll) and secondary metabolites (phenolic and terpenoids, etc.) for plant growth and prevent itself from environment. However, the biologically active were mostly secondary metabolites (Verpoorte *et. al.*, 2000). The concentrations of phytoestrogens are found in different amounts and in different parts of the plant and seed, with the final concentration dependent upon variety, geographical location and age (Knight *et. al.*, 1995).

2.2 Botanical characteristics of Kwao Krua plants

2.2.1 *Pueraria mirifica*

2.2.1.1 Botanical Characteristics of *P. mirifica*

The tubers of *Pueraria mirifica* (Airy Shaw & Suvatabandhu) are commonly found in abundant in the forests of the north, west and northeast region of Thailand. The plant is a long living twinning wood and the leave are pinnately three foliate stipulate; terminal leaflet. The tubers are varied in sizes and shapes (Figure 2.1). The flower is bluish purple shaped, flowering occurred during late January to early April. The length of the inflorescence of flowers was approximately 15-40 cm. The flower contained five sepals and the petals are one standard with two keels. The pod is slender typically short or elongate, smooth or hairy, including 1-10 single seeds when

fully matured and dried which turned into brown color (Smitasiri and Wungjhai, 1986; Cherdshewasart unpublished data)

Figure 2.1 Tuberous roots of *P. mirifica*

2.2.1.2 Chemical constituents of *P. mirifica*

The chemical constituents of *P. mirifica* is in the group of phytoestrogen, comprising of isoflavone aglycones (daidzein, genistein, kawakhurin and kawakhurin hydrate), isoflavone glycosides (daidzin, genistin, puerarin and mirificin) (Ingham *et. al.*, 1986), coumestans (coumestrol, mirificoumestan, mirificoumestan hydrate and mirificoumestan glycol) (Ingham *et. al.*, 1988), chromenes (miroestrol and isomiroestrol) (Bound and Pope, 1960; Jones and Pope, 1960) and deoxymiroestrol (Chansakaow *et. al.*, 2000[a]), sterols (β-sitosterol, stigmatosterol) (Hoyodom, 1971) and isoflavone (Chansakaow *et. al.*, 2000[b]).

Table 2.1 Chemical constituent of *P. mirifica**

Types	Compounds	References
Isoflavone	Genistein	Ingham *et al.*, (1986)
	Daidzein	Ingham *et al.*, (1986)
	Kawakhurin	Ingham *et al.*, (1986)
	Kawakhurin hydrate	Ingham *et al.*, (1989)
	Daidzin	Ingham *et al.*, (1986)
	Mirificin	Ingham *et al.*, (1986)
	Genistin	Ingham *et al.*, (1989)
	Puerarin	Ingham *et al.*, (1986)
	Puerarin-6-monoacetate	Ingham *et al.*, (1989)
Chromene	Miroestrol	Bound and Pope, (1960) Johns and Pope, (1960)
	Deoxymiroestrol	Chansakaew *et al.*, (2000[a])
	Isomiroestrol	Jones and Pope, (1960)
Sterol	β-sitosterol	Hayodom, (1971)
	Stigmasterol	Hayodom, (1971)
Coumarins	Mirificoumestan	Ingham *et al.*, (1988)
	Coumestrol	Ingham *et al.*, (1986,1988)
	Mirificoumestan glycol	Ingham *et al.*, (1988)
	Mirificoumestan hydrate	Ingham *et al.*, (1988)

*(Panriansaen, 2000)

Table 2.2 Chemical structures in the constituent of *P. mirifica*

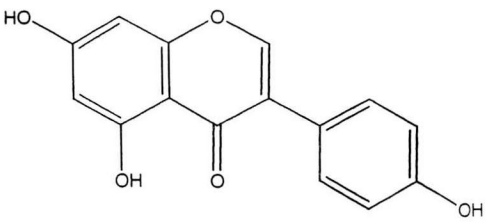
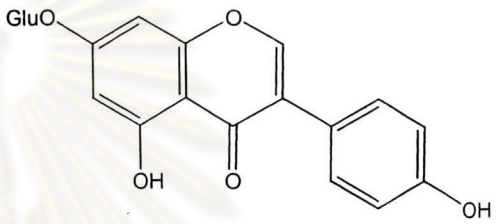
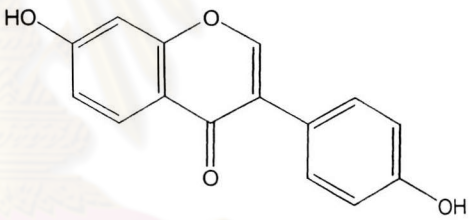
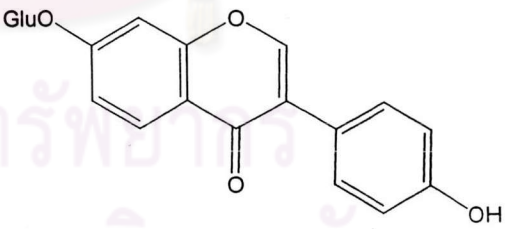
Types	Compounds	Structures
Isoflavone	Genistein (MW. 270.2)	
	Genistin (genistein-7-O-glucoside) (MW. 432)	
	Daidzein (MW. 254)	
	Daidzin (daidzein-7-o-glycoside) (MW. 416)	

Table 2.2 (continued)

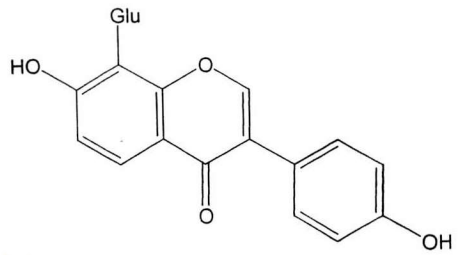
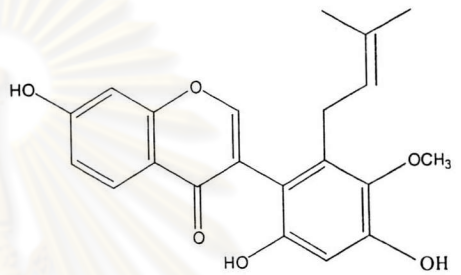
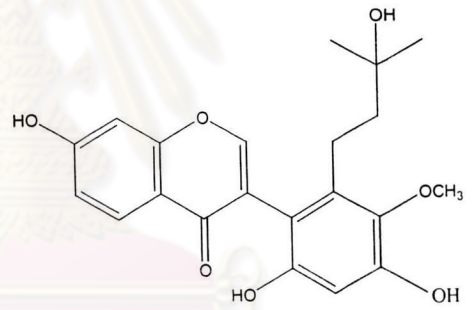
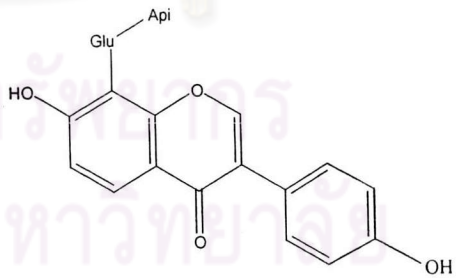
Types	Compounds	Structures
	Puerarin (daidzein-8-glucoside) (MW. 416)	
	Kwakhurin	
	Kawakhurin hydrate	
	Mirificin	

Table 2.2 (continued)

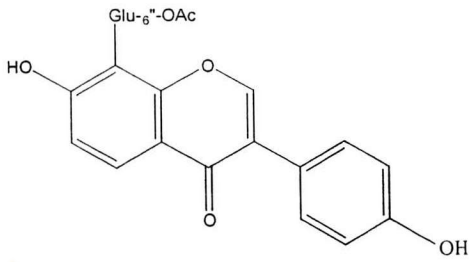
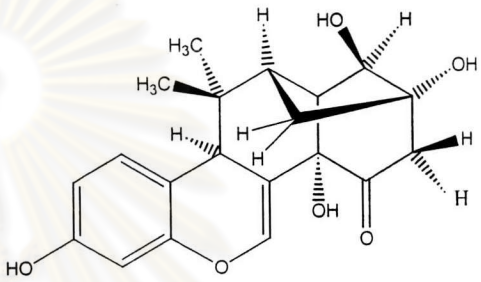
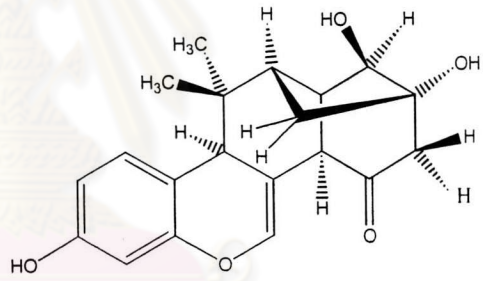
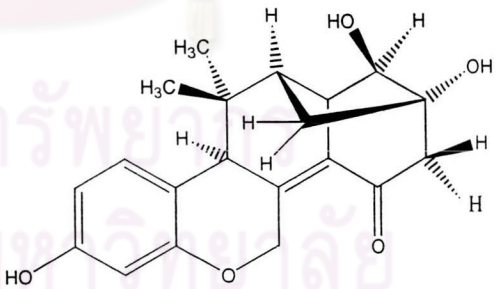
Types	Compounds	Structures
	Puerarin-6-monoacetate	
Chromene	Miroestrol	
	Deoxymiroestrol	
	Isomiroestrol	

Table 2.2 (continued)

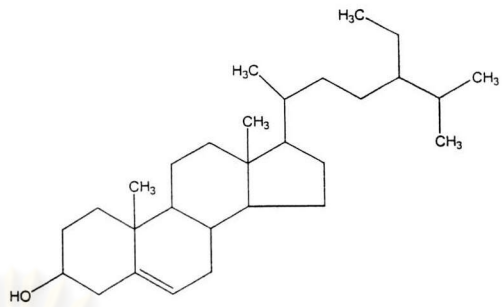
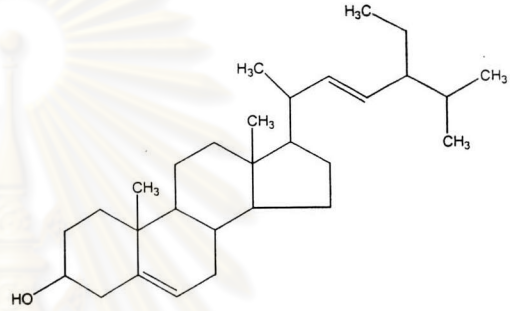
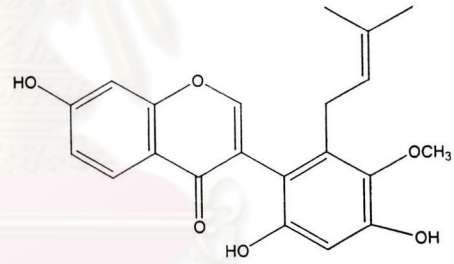
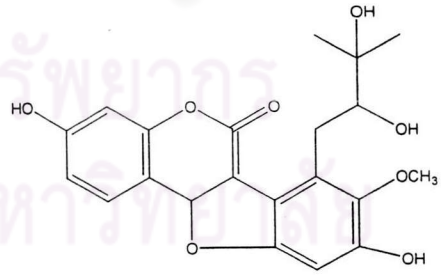
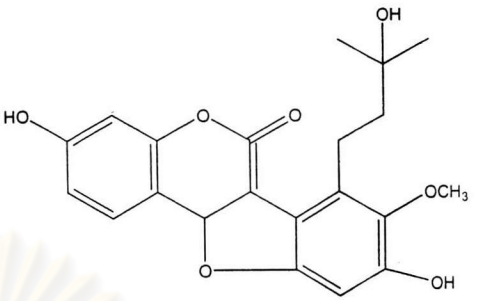
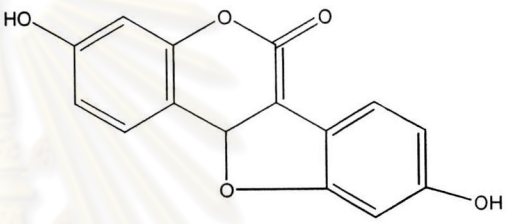
Types	Compounds	Structures
Sterol	β-sitosterol	
	Stigmasterol	
Coumarins	Mirificoumestan	
	Mirificoumestan glycol	

Table 2.2 (continued)

Types	Compounds	Structures
	Mirificoumestan hydrate	
	Coumestrol	

2.2.1.3 Pharmacological effects of *P. mirifica*

Powder and crude extract of *P. mirifica* was extremely investigated both *in vitro* and *in vivo*. *P. mirifica* could be applied as birth control agent in pigeon (100 %). Application at high dose could alter immunity in Japanese Quails (Smitasiri, 1995). *P. mirifica* showed effectiveness in the treatment of symptoms in menopausal women (Muangman and Cherdshewasart, 2001). *P. mirifica* showed estrogenic effect on human hepatoma cell line, HepG2 cells and MCF-7 cells (Lee *et. al.*, 2002). Isolate isoflavonoids from *P. mirifca* at the concentration of 0.1-1 μM could inhibit the growth of MCF-7 human breast cancer at about 80% in the presence of toremifene, as compare with 17β-estradiol (E_2) (Chansakaow *et. al.*, 2000[b]).

2.2.1.4 Toxicity effects of *P. mirifica*

Toxicity effects of *P. mirifica* at high dose have been tested in Japanese Quails receiving the plant powder for 15, 30 and 76 days. The toxicity effect was depended on the quantity and duration of feeding substance (Smitasiri, 1995). The powder and extract of *P. mirifica* were investigated in animal and human. *P. mirifica* exhibited no toxicity on skin and eye of rabbit, skin of guinea pigs, as well as human skin (Cherdshewasart, 2003).

2.2.1.5 Acute toxicity effects of *P. mirifica*

After receiving *P. mirifica*, no symptoms of acute toxicity in mice was found. LD_{50} value was more than 16 g/kg B.W. The plant showed no any toxicity in rats also (Chivapat *et. al.*, 2000). In recent report *P. mirifica* showed no acute toxicity with LD_{50} interval range of 2 g/kg B.W. only in female mice (Cherdshewasart, 2003)

2.2.1.6 Sub-chronic toxicity effects of *P. mirifica*

The sub-chronic toxicity on male and female rats treated orally with *P. mirifica* powder suspension for three months. It was found that *P.mirifica* at the dosage of 10 mg/kg B.W. showed no toxicity to blood cells (Chivapat *et. al.*, 2000).

2.2.1.7 Clinical effects of *P. mirifica*

The clinical trial at Chelsea Hospital London reported that miroestrol exhibited estrogenic response on amenorrhoea patients with no side effects (Cain, 1960). The clinical trial of crude drug in human revealed that *P. mirifica* could improve the symptoms related to menopause in female volunteers (Muangman and Cherdshewasart, 2001).

2.2.2 *Butea superba*

2.2.2.1 Botanical characteristics of *B. superba*

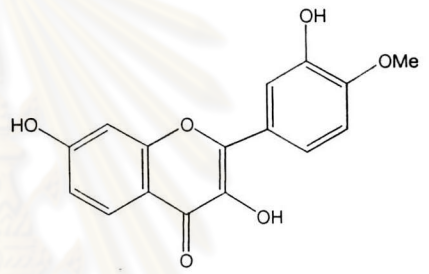
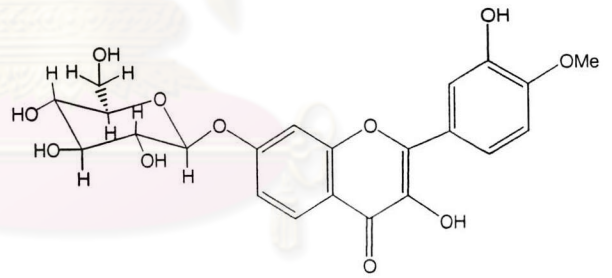
The tuber and stem of *B. superba* (Figure 2.2) are used in traditional medicines with a belief that it could give strength and increase male sexual performance. It is a woody climbing plant; the tuberous root is elongated, with red sap *B. superba* is a large size crawler and wraps itself around large trees. The leaves are pinnately three foliate, acuminate leaflets and long leafstalk. The flowers are yellowish orange colors. The petals are three times longer than the calyx. The pods are 3-4 inches long, oblong shaped with silvery silky short hair but only one seed present (Cherdshewasart, unpublished data).

Figure 2.2 Tuberous of *B. superba*

2.2.2.2 Chemical constituents of *B. superba*

The tubers of *B. superba* contained carboxylic acid, steroid, steroid glycoside, flavonoid (3,7,3'-trihydroxy-4-methoxyflavone) and flavonoid glycoside (3,5'-dihydroxy-4'-methoxyflavone-7-β-D-glucopyranoside) (Rugsilp, 1999; Roengsumran *et. al.*, 2000). The stem of *B. superba* contained flavonoid glycoside (3,7-dihydroxy-8-methoxyflavone 7-O-α-L-rhamnopyranoside) (Yadava and Reddy, 1998).

Table 2.3 Chemical structures of the flavone in the constituent of *B. superba* (Rugsilp, 1999; Roengsumran *et. al.*, 2000)

Types	Compounds	Structures
Flavone	3,7,3'-trihydroxy-4'-methoxyflavone (flavonoid)	
	3,5'-dihydroxy-4'methoxyflavone-7-O-β-D-glucopyranoside (flavonoid glycoside)	

2.2.2.3 Pharmacological effects of *B. superba*

B. superba flavonoid and flavonoid glycoside could inhibit cAMP phosphodiesterase enzyme, stimulate the function of the central nervous system, the cells and the aldosterone hormone which attributes in increasing male sexual performance, thus, able to control the numerous diseases (Roengsumran *et. al.*, 2000).

2.2.2.4 Sub-chronic toxicity of *B. superba*

The sub-chronic toxicity test of *B. superba* in mice and rats exhibited no abnormality in physiology, blood chemistry and histology of the liver, kidneys and testis (Cherdshewasart unpublished data). Furthermore, *B. superba* (1 g powder/kg/day) showed induction of the formation of micronucleus in polychromatic erythrocytes more than the control group ($p<0.01$) but none of the toxic effect on male rats (Pongpanparadorn *et. al.*, 2002).

2.2.2.5 Clinical trial of *B. superba*

Clinical trial of *B. superba* revealed that the ingredient of *B. superba* could improve the erectile function in males with no toxicity apparent (Nimsakul and Cherdshewasart, 2003).

2.2.2.6 Anti-cancer properties of *B. superba*

B. superba showed antiproliferative effects to MCF-7 and HeLa cells (Cherdshewasart *et. al.*, 2004[a,b]).

2.2.3 *Mucuna collettii*

2.2.3.1 Botanical characteristics of *M. collettii*

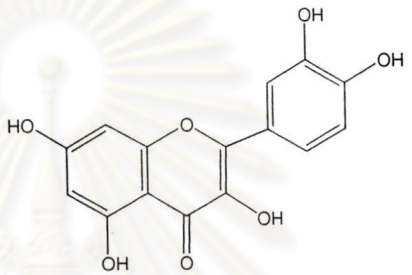
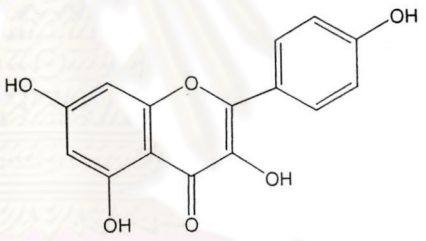
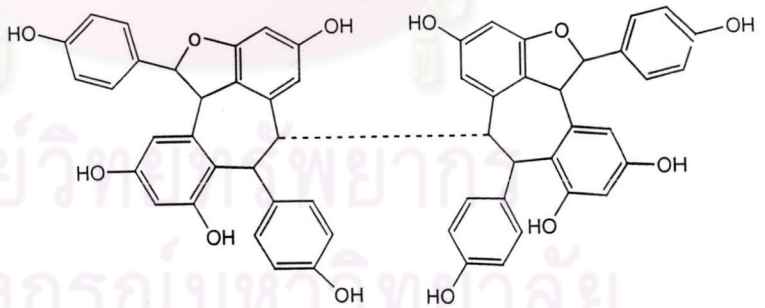
M. collettii (Figure 2.3) is a large woody climber, 30-40 m height scattered by stems in evergreen forest, the cut surface turns black soon after cutting. The leaves were trifoliate; leaflets 4-8 by 2-4 inches sparsely hairy, entire margin; petiole 5-10 cm long, base stout. The flowers were hanging on 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; Cherdshewasart, unpublished data).

Figure 2.3 Stem of *M. collettii*

2.2.3.2 Chemical constituents of *M. collettii*

The whole stem of *M. collettii* contained kaempferol, quercetin and hopeaphenol (Roengsumran *et. al.*, 2001) (Table 2.4).

Table 2.4 Chemical structured of the flavone in *M. collettii* (Roengsumran *et. al.*, 2001)

Compounds	Structures
Quercetin	
Kaempferol	
Hopeaphenol	

2.2.3.3 Pharmacological effects of *M. collettii*

The activity of *M. collettii* has more effects than *B. superba* and *P. mirifica,* respectively. *M. collettii* is believed to obtain for aphrodisiac effect. The ingredient of *M. collettii*, kaempferol, quercetin and hopeaphenol could inhibit the activity of cAMP phosphodiesterase (Roengsumran *et. al.*, 2001).

2.3.4 Toxicity of *M. collettii*

Powder of *M. collettii* could increase testes weight and depress the white blood cell formation, including abnormality of spermatozoa of the rats (Wutteeraphon *et. al.*, 2001). Furthermore, hopeaphenol was reported to be highly cytotoxic to KB cell line or mouth epidermal carcinoma (Ohyama *et. al.*, 1999).

2.2.4 *Pueraria lobata* (Kudzu)

2.2.4.1 Botanical characteristics of *P. lobata*

P. lobata is an edible vine used widely in China, Korea, and Japan for various medicinal purposes. The tubers contain isoflavonoids as puerarin, daidzin, and daidzein (Guerra *et. al.*, 2000). *P. lobata* is a high-climbing perennial wine with an immense root which was considered medicinal (Cao *et. al.*, 1999). The stem of *P. lobata* was showed in Figure 2.4.

Figure 2.4 Stem of *P. lobata*

2.2.4.2 Chemicals constituents of *P. lobata*

P. lobata tuber contained flavonoids, coumarins, isoflavones (daidzein, daidzin, puerarin and daidzin-4',7-diglucoside) and puerarin (3'-hydroxy-4'-O-β-D-glucosylpuerarin) as those present in the leaves and roots (Hirakura *et. al.*, 1997).

2.2.4.3 Pharmacogical of *P. lobata*

Certain glycosides in *P. lobata* are responsible for its effect on the heart and circulatory system. Two of these substances; daidzin and daidzein, have also been shown, in an animal study, to inhibit the desire for alcohol. The antioxidant properties of the extract from *P. lobata* have drawn a considerable amount of attention. They could act as a suppressive effect on gene expression of the SOS response in S. Typhimurium TA 1535 against the mutagen (Miyazawa *et. al.*, 2001). *In vitro*, *P. lobata* and puerarin could inhibit the steady state chemiluminescent reaction as had antioxidant activity. *P. lobata* (1,400 mg/kg B. W.) and puerarin (200 mg/kg B.W.) showed altered hepatic to cytochrome P450-linked monooxygenase in male rats. *P. lobata* is also used for treatment of allergies, migraine headache, measles, and diarrhea, but its effectiveness for these disorders has not been clinically verified. Puerarin as isoflavone isolated from *P. lobata* could inhibit the relaxation response to isoproterenol *in vitro* (Guerra *et. al.*, 2000).

2.3 Antioxidant activity studies

2.3.1 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 *et. al.*, 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 oxidants 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).

2.3.2 Preventing the free radicals

To fight free radicals, all aerobic organisms, including human, have antioxidant defenses 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 *et al.*, 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. Isoflavone 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). Genistein from soy bean had the highest activity of the isoflavones; the isoflavones 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 *et. al.*, 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 by HL-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 isoflavones are structurally related and the hydroxyl group at position 4' is crucial in both systems, 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 exhibit 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 a phytoestrogen (Mitchell *et. al.*, 1998). Flavonoids showed a dose-dependent protecting activity to α-tocopherol in low-density lipoprotein (LDL) and kampherol being less effective than quercetin against depletion of α-tocopherol in LDL (Zhu *et al.*, 2000).

2.3.3 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 antioxidant 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).

DPPH assay (spectrophotometric methods) 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; Perejo *et al.*, 2002), flavonoids (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 decolorizatoin is stoichiometric with respect to the number of electrons captured. The stable free radical 2,2-diphenyl-B-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 (Bolis *et al.*, 1958) as show the structure of DPPH in Figure 2.5

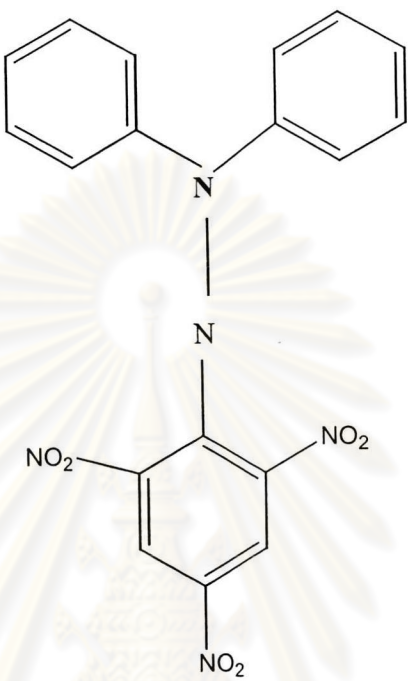


Figure 2.5 Structure of chromogen DPPH

2.4 Mutagenicity and antimutagenicity studies by Ames' test

2.4.1 Mutation

The term "mutation" refers both to the change in the genetic material. Since the genetic material is usually DNA, a mutation may be the result of any detectable, unnatural change that affects DNA's chemical or physical constitution, its replication, its phenotypic function, or the sequence of one or more DNA base pairs (Sirirat, 1995).

2.4.2 Types of mutation

A mutation that occurs at the level of a gene is called a gene mutation, which may result from the substitution of one nucleotide pair for another (a base-pair substitution mutation) or from the addition or deletion of one or more base pairs (frameshift mutation). A change in the organization of a chromosome is called a chromosomal mutation. These are recognized as morphological alterations in the gross structure of chromosomes (breaks, deletions, rearrangements) or changes in the number of chromosomes (gain or loss of single chromosomes or sets of chromosomes) (Amdur *et al.*, 1991).

2.4.3 Screening for mutagenicity

Testing of the mutagenic activity of chemical substances has been studies in various systems, both *in vitro* and *in vivo* with difference model such as microorganisms, plants, insect, mammalian and human cell. In addition, the mutagenic effect detectable in different tissues of the same system depends on aims of studies (Anderson *et al.*, 1992).

2.4.3.1 Salmonella/Mammalian microsomal assay (Ames'test)

Ames' test is a method for genotoxicity test, which use of the mutation is now firmly established, both for fundamental studies in mutagenesis and carcinogenesis properties. Reverse mutation assays using bacteria are by far the most widely used tests in primary screening such as the complex environmental and biological mixtures. Furthermore, safty-registration committee around the word accepts Ames test as the

primary screening test in evaluation of the mutagenicity in chemicals Ames and marons, 1983) because of identify quickly and inexpensively chemicals with mutagenic and carcinogenic potential (Mortelmans and Zeiger, 2000). Daidzein and genistien are isoflavones and coumestrol is a coumestan showed no mutagenic effect to S. Typhimurium strains TA1538, TA98 or TA100 at concentration 1-500 μg/plate (Bartholomew and Ryan, 1980). Quercetin suppressed the mutagenicity induced by MNNG mutagen in the Ames Salmonella assay and a good inhibition of mutagenicity for the micronuclei and chromosome aberration in rats (Taj *et al.*, 1996), and decreased the reduction of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium) test on MCF7 human breast cancer cells (Rodgers and Grant, 1998).

2.4.3.1.1 Principle of the mutagenicity test

The theoretical basis of the Ames' test is the sample attacked to DNA of bacterial reverse mutation, which bacteria already mutant at an easily detectable locus are treated with the test material to determine extremely it induces a second mutation as reverse the effect of the pre-existing mutation. Normally, these recombinant strains are histidine-auxotroph (His^-). When they expose chemical mutagens, the revertants will occur which grow of colonies or histidine prototroph (His^+) testing mutagenicity, tester stains were tested on a solid growth medium (minimal agar plate) as lack of histidine, the stains could not grow to form visible colonies because they required histidine for growth. Contrary, when the bacteria were exposed to a chemical mutagen on a solid growth medium that lack of histidine also; the mutation will occur then restore the ability to synthesize histidine so the bacteria can grow to form viable colonies again (called "Histidine revertant colonies). Reverse mutation from amino acid auxotrophy to prototrophy has proved to be the most useful type of genetic marker. Amino acid auxotrophs carry mutations, which prevent them, synthesizing one, or more amino acids from inorganic sources of nitrogen, unlike "wild-type" protrophic bacteria which grow and divide in minimal (inorganic salts) medium supplemented with a carbon source such as glucose (Parry, 1984). For bacterial reverse mutation assay was that developed, named Salmonella/ Mammalian Microsome assay (Ames' test) (Ames and marons 1983). Result obtained from Ames' test can be taken as an important consideration in evaluation of mutagenicity

as comparison with standard mutagen such as benzo (a) pyren (B(a)P) and 2-[2-furyl]-3-[5-nitro-2-furyl]acrylamide (AF_2).

2.4.3.1.2 Enzymatic activation (S9 fraction)

Unlike mammals, these bacteria lack the necessary oxidative enzyme systems for metabolizing foreign compounds to electrophilic metabolites capable of reacting with DNA. The bacteria are treated with the test compound in the presence of a post-mitochondrial supernatant (S9 fraction) prepared from the livers of mammals. Rats, mice, hamsters, guinea pigs etc. Moreover, S9 fraction preparations can be made from various tissues and species such as lung of rats and autopsy liver (Ames *et al.*, 1975).

Efficient detection of a wide variety of carcinogens requiring metabolic activation (predominantly mono-oxygenase activity mediated via the cytochrome P450 system), that is enzyme from liver animals (usually rats) by treating the animal with potent inducer of drug-metablising enzymes before they are killed and their livers are removed. There are various induction procedures. The report that liver from Aroclor 1254 induced rats was efficient for detecting different classes of carcinogens (Ames and marons, 1983). Liver homogenates from rats induced with a polychlorinated biphenyl (PCB) mixture was recommended (Alvares *et al.*, 1973). But Aroclor is a carcinogen of great stability and PCB is a toxic and environmental pollutant. Thus, it has been report that the combination treatment of two inducers, sodium phenobarbital and 5,6 –benzoflavone that could produced a similar to Aroclor 1254 or PCB (Matsushima *et al.*, 1976).

2.4.3.1.3 Method for mutagenicity test

For initial screening, chemical was tested in concentrations over a three-log dose range in the presence and absence of the S9 mix. A positive result should be confirmed by demonstrating a dose-response relationship using a narrow range of concentrations.

2.4.3.1.3.1 Plate incorporation method

This method is the standard method that has been used to test the mutagenicity of sample. The method consists of combine the test sample, the bacterial tester strain and S9 mix or buffer in molten top agar (45^0) which is poured onto a minimal glucose agar plate and incubated at 37 C^0 for 48 hours, the plates then are scored for number of histidine revertant colonies and for the presence of a background lawn of the bacteria. Positive and negative controls are also included in each assay. If samples that are negative by this test can be retested using preincubation method (Yahagi *et al.*, 1975).

2.4.3.1.3.2 Pre-incubation method

In the preincubation assay, the tester strains are exposed to the chemical for a short period (20 to 30 min) in a small volume (0.5 ml) of either buffer or S9 mix. (Mortelmans and Zeiger, 2000). This method was developed from the plate incorporation test, which has been used to detect the mutagenicity of carcinogenic nitrosamines, several carcinogenic alkaloids (Yamanaka *et al.*, 1979). Condition of this method consists of the sample, S9 mix and bacteria were incubated for 20-30 min at 37 C^0 and then the top agar was added. The report that the sensitivity of pre-incubation method was equal or greater than the plate incorporation method when determined the mutagenic activity of aflatoxin B_1, benzidine, benzo (a) pyrene and methylmethane-sulfonate using both methods (Matsushima *et al.*, 1976). The increased in sensivity may be due to the fact that the test chemicals, S9 and bacteria were incubated at higher concentration in the preincubation method than in the plate in corporation method (Prival *et al.*, 1979).

2.4.3.1.4 Toxicity test

The trace of histidine added in the S9 mix or buffer solution allows all the bacteria present to undergo a few divisions, and result in the appearance of a background lawn of bacteria. Examination of this background lawn, which may be accompanied by a decrease in the number of revertant colonies, absence of

background lawn or presence of pinpoint non-revertant colonies (Mortelmans and Zeiger, 2000)

2.4.3.1.5 Criteria for positive and negative response (Kier *et al.*, 1986):

1. Criteria for designing a chemical mutagenic in the Ames' test were as follows

(a). The chemical produced a positive response either with or without metabolic activation in any of the five designated tester strains.

(b). An qualified positive response was indicated by the presence of at least two dose levels showing greater revertant counts than the control and a response increasing with dose, with at least one dose giving a response greater three times than the control for TA1535, TA1537, TA1538 or greater two times than the control for TA98 and TA100.

(c). A reproducible effect at a single dose level was identified as a qualified positive test.

2. Criteria for designing a chemical not mutagenic in the Ames' test were considerably more restrictive.

(a) The chemical must have been tested under both activation and non-activation condition.

(b) The activation system must have been considered adequate.

(c). The test had at least two appropriate spaced nontoxic dosed in addition to (i) an apparent toxic dose, (ii) a dose equal to or greater than 1 mg/plate (iii) an insoluble dose.

2.5 Genotoxicity studies in micronucleus test

Little is known about the genotoxicity of these natural compounds. Genistein proved to be strong inducers of DNA strand breaks and micronuclei containing acentric fragments as shown with antikinetochore antibodies, but daidzein was not found. In addition, the clastogenicity of genistein may be due to its non-intercalative inhibitory effect on topoisomerase II, whereas coumestrol was a clear inducer of hypoxanthine phosphoribosyltransferase (HPRT) mutations in V79 cells; genistein was only marginally active and daidzein inactive (Kulling and Metzler, 1997). Coumestol at higher concentration (10 μM) were toxic to human pancreatic aenocarcinoma cells in vitro, however, coumestrol at low concentration (1 μM) inhibited the growth of the female of these cells by 95%, but they stimulated the growth of the male of pancreatic tumor cells as same as genistein (Lyn-Cook *et. al.*, 1999).

2.5.1 The micronucleus formation

Micronuclei are small extranuclear bodies, formed in mitosis from acentric chromosomal fragment or chromosomal fragments (chromosomes that lag behind in anaphase) and are not integrated into the daughter nuclei. Thus, micronucleus contains either chromosomal fragments or whole chromosomes. In anaphase, acentric chromatid and chromosome fragments lag behind when the centric elements move towards the spindle poles. After telophase, the undamaged chromosomes as well as the centric fragment, give rise to regular daughter nuclei. A considerable proportion is transformed into several nuclei, which much smaller than the principal nucleus, hence, called micronucleus (Heddle *et al.*, 1983; Schmid *et al.*, 1975).

2.5.2 Screening the genotoxicity by micronucleus test

After animals are exposed either acutely or chronically to a test substance, animals are sacrificed at interval time as depend on design experiment. The bone marrow is extracted, spreader on slides and stained. The frequency of micronucleus

in PCEs is determined as compared among control groups. Condition of experimental as follow by;

(a) **Number of animals**: For screening that used both sexes, at least 5 animals/sex/group for each sampling time (Macgregor *et al.*, 1987).

(b) **Selection the dose**: For acute toxicity that the high, intermediate and low doses are 1/2, 1/6 and 1/20 of the LD_{50}, respectively (Brusick *et al.*, 1989)

(c) **Sampling time**: since micronuclei are formed during division of the nucleated erythrocyte, however, micronucleus did not appear earlier after treatment than the interval between completions of the final erythroblast mitosis and enucleating. For most chemicals increases in the micronucleus frequency have not been found earlier than 9-12 hours after treatment, because of the subsequent of PCE stage last in the bone marrow for 12-24 hour, any micronucleated PCEs formed will remain in the bone marrow for at least 12 hours. Differences between tests agents may be two or more samples be taken if only one or two treatments are given. Data indicate that peak frequency usually occurs between 24-48 hours after treatment, but it may occur as late as 72 hours (Temcharoen, *et al.*, 2002: Salamone and Heddle, 1983).

(d) **Score of micronucleus**: the micronucleus can occur in any cell type of proliferating tissue. They are, the most easily recognized in cell lacking main nucleus, namely erytrocytes. Cell population to be scored, the frequency of micronucleus can be most easily evaluated in young erythrocytes shortly after the main nucleus is expelled. PCEs are convenient population for study because they represent the first a nucleate stage of erythrocyte development, their polychromatic staining characteristic enables distinction from mature erythrocytes (NCEs) by their different staining properties. With a combination of May-Grunwald and Giemsa staining, the PCEs stain bluish to purple due to their high content of RNA in the cytoplasm. In contrast, the NCEs stain reddish to yellow and are also slightly smaller than PCEs (Temcharoen, *et al.*, 2002)

(E) **Interpret data for micronucleus test**: scoring for micronucleated PCEs, an area for optimal cell morphology, spacing and staining should be chosen. True micronucleus is round, on rare occasion's oval or half-moon shaped, always with a sharp contour and evenly stained. At least 1,000 PCEs should be scored per animals to determine the number of micronucleated PCEs. In order to quantity the proliferative state of the bone marrow, the ratio of PCE to NCE should be determined by counting number of PCE among 100-200 total erythrocytes. The normal PCEs : NCEs ratio for bone marrow is approximately 0.4-1.0. A marked reduction in this ratio ($\leq$0.1) indicated a cytotoxic effect. Such a reduced proportion of PCEs arises from either a cessation of PCEs production (division and maturation of the nucleated erythropoietic cells have been inhibited) or massive invasion of the marrow by peripheral blood as a result of marrow depletion (Heddle *et al.*, 1983).