Chapter 2
Literature Review

2.1 *Boesenbergia pandurata* (Roxb.) Schltr.

2.1.1 Plant description

Boesenbergia pandurata (Roxb.) Schltr. (Syn. *Kaempferia pandurata*, Figure 2-1), a perennial herb of the family Zingiberaceae, has been known as 'kra-chai' in Thailand. It typically possesses cylindrical, 6-10 cm long, fascicled, acute tip, light brown outside, yellow inside and scented root. Leafy shoot is very short, consisting of 3-4 leaved; petioles 12-25 cm long, tincted red; blade elliptic or oblong, 10-30 cm long, 5-10 cm wide; apex acute; base cuneate to obtuse; margin entire. Inflorescences are terminal, subsessile, enclosed by the leaf sheaths; bearing 2-ranked bracts each subtending a single flower. Bracts are linear to lanceolate up to 5 cm long. Bracteoles are as long as bracts but narrower. The uppermost flower opens first. Calyx is about 2 cm long, bifid. Corolla is pink, tube exceeding the bracts; lobes about 1.5 cm, oblong. Labellum is bag-shaped about 2.5 cm long, 2 cm wide. Lateral staminodes are slightly shorter than corolla lobes and mottled purple. Fruits are ellipsoid capsule (Farnsworth and Bunyapraphatsara, 1992).

A

B

C

Figure 2-1 *Boesenbergia pandurata* (Roxb.) Schltr.

A: Whole plant

B: Flowers

C: Rhizome and roots

2.1.2 Ethnomedical uses

Kra-chai is generally utilized as a folk medicine in Southeast Asia. In Thailand, the fresh rhizome and roots are used in cooking, also in folk medicine as an aphrodisiac, dysentery, anti-inflammatory, the treatment of colic disorder and for health promotion (Farnsworth and Bunyapraphatsara, 1992). In Indonesia, the plant is used for thrush by chewed with areca, cough and throat (Hirschhorn, 1983). In Malaysia, it is used as a stomachic and decoction given to women after childbirth (Ilham *et al*., 1995).

2.1.3 Biological activities

2.1.3.1 Antibacterial activity

A hot water extract of the fresh roots at 0.5 ml/disc had no antibacterial activity against two strains of *Bacillus subtilis*, H-17 (rec^+) and M-45 (rec^-). The root juice also had no antibacterial activity (Ungsurungsie *et al*., 1982).

A dried root extract was obtained by maceration with ether, petroleum ether and distilled water for 48 hours and filtered. It was found that all these extracts possessed no antibacterial activities against *B. subtilis*, *Escherichia coli*, *Staphylococcus aureus* and *Pseudomonas aeruginosa* (Iamthammachard, 1982).

An ethanolic extract of the roots at a concentration of 200 mg/ml, tested by using the disc-paper technique, was reported to be effective against *β*-streptococcus gr A but not active against *S. aureus*, *P. aeruginosa* and *Klebsiella pneumoniae* (Laorpaksa, 1988).

2.1.3.2 Antifungal activity

Water, alcohol and chloroform extracts have been tested against *Microsporum gypseum*, *Trichophyton rubrum*, *Epidermophyton floccosum*, *Candida albicans*, *Cryptococcus neoformans*, and *Saccharomyces* sp. The water extract showed very low activity but the alcohol and chloroform extracts exhibited moderate activity (Achararit *et al*., 1983).

2.1.3.3 Mutagenic activity

Water extract of *B. pandurata* rhizome showed no mutagenic activity against two strains of *B. subtilis*, H-17 (rec^+) and M-45 (rec^-) at a concentration of 0.5 ml/disc. The root juice also had no mutagenic activity (Ungsurungsie *et al*., 1982).

Pinocembrin chalcone, pinocembrin, cardamonin, panduratin A and pinostrobin were isolated from fresh rhizomes of *B. pandurata* as strong antimutagens toward 3-amino-1,4-dimethyl-5H-pyrido[4,3-b]indole (Trp-P-1) in *Salmonella typhimurium* TA98. They also similarly inhibited the mutagenicity of 3-amino-1-methyl-5H-pyrido[4,3-b]indole (Trp-P-2) and 2-amino-1-methyl-6-phenyllimidazo[4,5-b] (PhIP). Antimutagenic activity was mainly due to the inhibition of the first step of enzymatic activation of heterocyclic amines. (Trakoontivakorn *et al*., 2001).

2.1.3.4 Anti-inflammatory activity

5,7-Dimethoxyflavone (5,7-DMF), a compound extracted from *B. pandurata* rhizomes, was tested for its anti-inflammatory activity using carrageenin-induced rat pedal edema model and was compared with aspirin, indomethacin, hydrocortisone and prednisolone. 5,7-DMF at a single oral dose of 300 mg/kg decreased the edema induced by carrageenin and kaolin by 16.0-48.0 % and 43.7-80.9 %, respectively. At a single oral dose of 150 mg/kg edema was significantly reduced by 9.8 %. By the granuloma pouch model, 5,7-DMF also inhibited inflammation significantly 3.9, 4.3 and 8.7% when using this compound at doses of 200, 400 and 800 mg/kg/day, respectively. When rat pleurisy was induced,

5,7-DMF at a single dose of 300 mg/kg inhibited the exudation by 18.0-56.7 %, decreased white blood cells by 15.5-67.7 % and inhibited prostaglandin biosynthesis over 86-97% (Tasneeyakul, 1984).

Morever, panduratin A and hydroxypanduratin A showed significantly topical anti-inflammatory activity in the assay of TPA-induced ear edema in rats (Tuchinda *et al*., 2002)

2.1.3.5 Antipyretic activity

In an antipyretic activity test using the yeast-induced pyrexia model, 5,7-DMF at dose of 37.5-150.0 mg/kg showed antipyretic activity better than aspirin at a dose of 300 mg/kg (Tasneeyakul, 1984).

2.1.3.6 Antispasmodic activity

A study on the antispasmodic activity of *B. pandurata* on isolated guinea pig ileum was carried out. Four spasmogens including acetylcholine, histamine, barium chloride and dimethyl-4-phenyl-piperazinium iodide (BMPP) were used. The results indicated that *B. pandurata* exhibited, statistically significant, antispasmodic effect by all spasmogens tested. The log dose-response curves showed non-competitive antagonism. Moreover, the degree of inhibition was dependent on the strength, amount of the plant solution and the time of immersion of the ileums in the solution before the application of spasmogens (Thamaree, 1985).

2.1.3.7 Insecticidal activity

Fresh rhizomes of *B. pandurata*, prepared by cold-rolling either with water, alcohol, acetone or hexane, were slightly to moderately toxic to adult oriental fruit flies (Areekul, 1987).

2.1.3.8 Toxicity assessment

From Hippocratic screening, 5,7-DMF at a dose of 3 g/kg (ten times of the anti-inflammatory dose) had low toxicity and rat mortality was not found within 7 days. At doses higher than 1.26 g/kg, it depressed respiration and decreased body temperature that might be caused by CNS depression and prostaglandin biosynthesis inhibition by 5,7-DMF (Tasneeyakul, 1984).

2.1.3.9 Miscellaneous

B. pandurata extracts showed inhibitory effects on tyrosinase and melanin formation (Naomi *et al*., 1997).

The essential oil from the rhizome of *B. pandurata* has ability to solubilize calcium kidney stone. It is apparent that essential oil at 1.2% has the maximum ability to solubilize calcium kidney stones (Ediati, 1994).

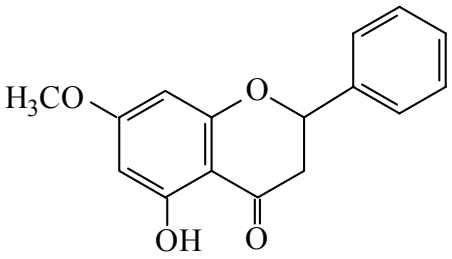
2.1.4 Phytochemistry

Boesenbergia pandurata (Roxb.) Schltr. is a plant indigenous in Thailand. It occurs in four varieties described by colours presented in the rhizome, as yellow, white, black or red. The chemical constituents found in yellow rhizomes are given in Table 2-1. Their chemical structures are shown in Figure 2-2. The white rhizome was found to contain crotepoxide, (+)-zeylenol, boesenboxide, isopimaric acid and 2′-hydroxy-4,4′,6′-trimethoxychalcone (Pancharoen *et al*., 1984; Tuntiwachwuttikul *et al*., 1987). Eleven flavonoids were reported to be the constituents of the black rhizome variety; 5-hydroxy-7-methoxyflavanone, 5,7-dimethoxyflavanone, 5-hydroxy-7-methoxyflavone,5-hydroxy-7,4′-dimethoxyflavone, 5,7-dimethoxyflavone, 5,7,4′-trimethoxyflavone, 5,7,3′,4′-tetramethoxy-flavone, 5-hydroxy-3,7-dimethoxyflavone, 5-hydroxy-3,7,4′-trimethoxyflavone, 3,5,7-trimethoxyflavone and 5-hydroxy-3,7,3′,4′-tetramethoxyflavone (Jaipetch *et al*., 1983). Chemical investigation of the hexane extract of the red rhizome of *B. pandurata* resulted in the isolation of panduratin A, pinostrobin, boesenbergin A and rubranine (Tuntiwachwuttikul *et al*., 1984), sakuranetin and dihydro-5,6-dehydrokawain (Tuchinda *et al*., 2002).

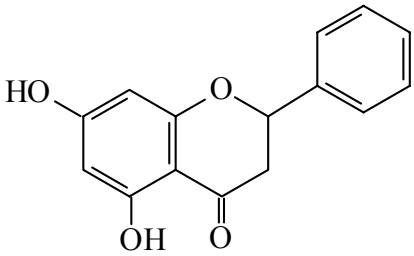
Table 2-1 Chemical constituents reported in *B. pandurata* (yellow rhizome) from literatures

Chemical groups	Chemical constituents	References
Flavanone	Pinostrobin (1)	Mongkolsuk and Dean, 1964
	Pinocembrin (2)	Mahidol *et. al*., 1984
	Alpinetin (3)	Mongkolsuk and Dean, 1964
	5,7-dimethoxyflavanone (4)	Jaipetch *et. al*., 1983
Flavone	Dimethoxyflavone (5)	Jaipetch *et. al*., 1983
	3 □□, 4′, 5, 7-tetramethoxyflavone (6)	Jaipetch *et. al*., 1983
Chalcone	2 ′ , 6 ′ , - d i h y d r o x y - 4 ′ - methoxychalcone (7)	Jaipetch *et. al*., 1982
	Cardamonin (8)	Mahidol *et. al*., 1984
	Panduratin A (9)	Tuntiwachwuttikul *et. al*., 1984
	Panduratin B (10)	Pancharoen *et. al*., 1987
	Boesenbergin A (11)	Jaipetch *et. al*., 1982
	Boesenbergin B (12)	Mahidol *et. al*., 1984
	Rubranine (13)	Tuntiwachwuttikul *et. al*., 1984
Monoterpene	Geranial (14)	Panji *et al*., 1993
	Neral (15)	Panji *et al*., 1993
Diterpene	Pimaric acid (16)	Tuntiwachwuttikul *et. al*., 1984
Alicyclic	Boesenboxide (17)	Tuntiwachwuttikul *et. al*., 1984

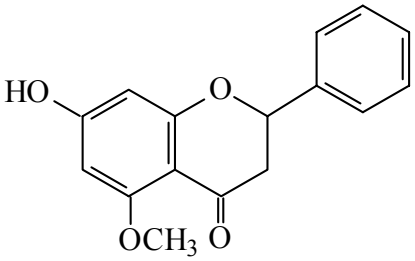
Flavanones



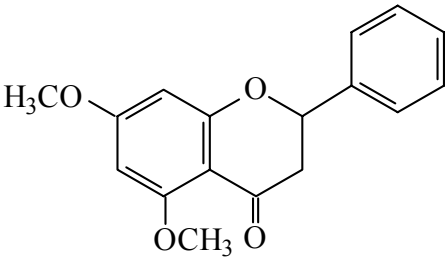
Pinostrobin (1)



Pinocembrin (2)



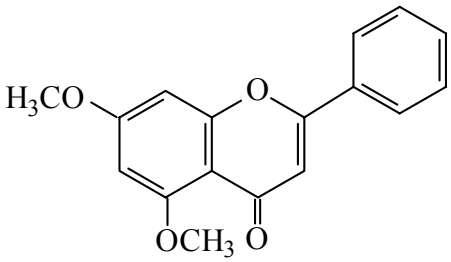
Alpinetin (3)



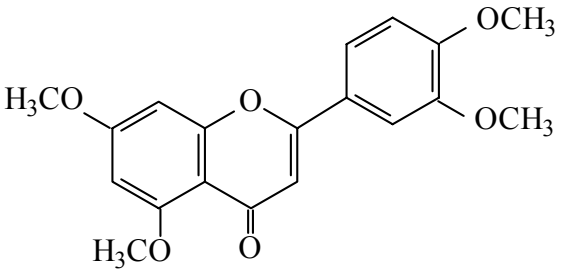
5,7-dimethoxyflavanone (4)

Figure 2-2 Structure of compounds from *B. pandurata* (yellow rhizome)

Flavones

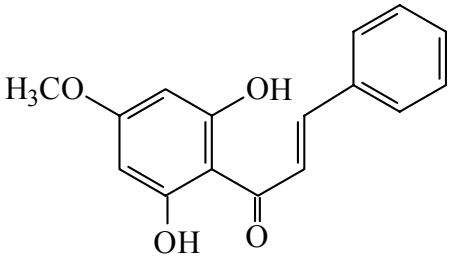


Dimethoxyflavone (5)



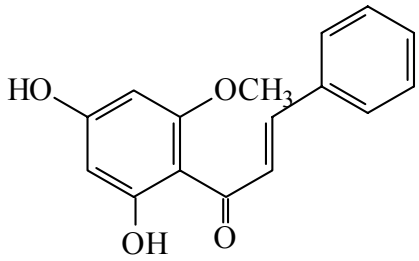
3′, 4′, 5, 7-tetramethoxyflavone (6)

Chalcones

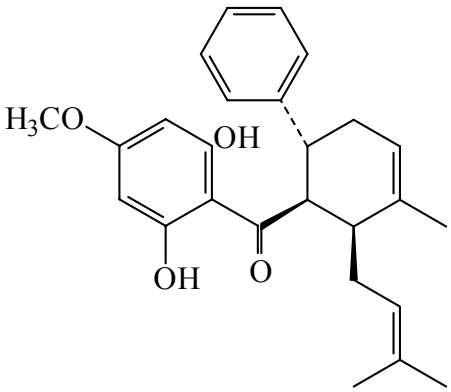


2′, 6′-dihydroxy-4′-methoxychalcone

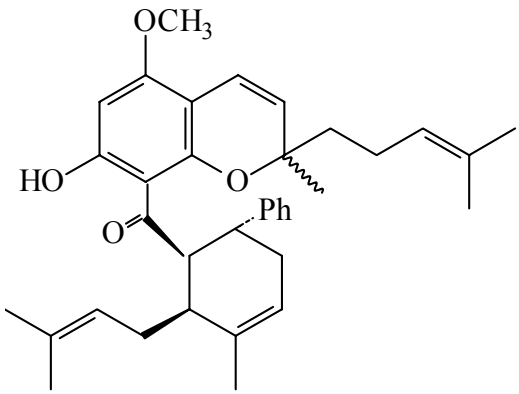
Figure 2-2 Structure of compounds from *B. pandurata* (yellow rhizome) (continued)



Cardamonin

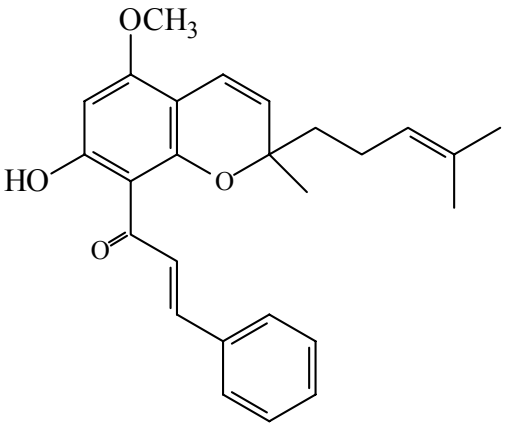


Panduratin A (9)

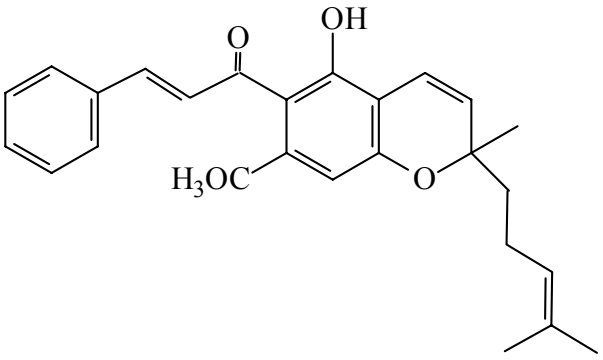


Panduratin B (10)

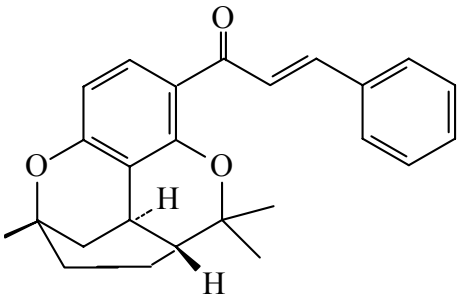
Figure 2-2 Structure of compounds from *B. pandurata* (yellow rhizome) (continued)



Boesenbergin A (11)



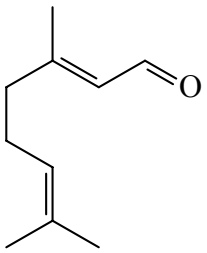
Boesenbergin B (12)



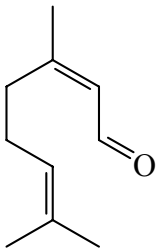
Rubranine (13)

Figure 2-2 Structure of compounds from *B. pandurata* (yellow rhizome) (continued)

Monoterpene

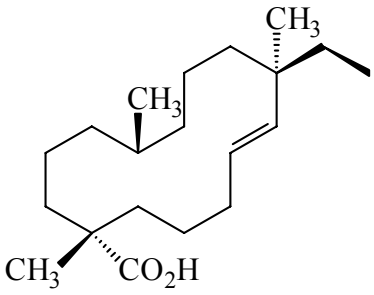


eranial (14)



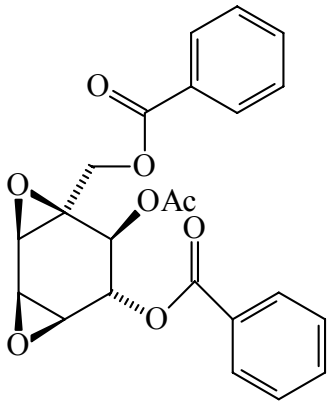
Neral (15)

Diterpene



Pimaric acid (16)

Alicyclic



Boesenboxide (17)

Figure 2-2 Structure of compounds from *B. pandurata* (yellow rhizome) (continued)

2.2 Oxidation and Antioxidants

2.2.1 Oxidation and free radicals

Most stable molecular species have electrons within their outer orbitals arranged in pairs. Each electron of this pair has an opposite "spin", an arrangement that stabilizes the molecule. A free radical is a molecule with one or more unpaired electrons in its outer orbital. Such unpaired electrons make these species very unstable and therefore quite reactive, as the free radical tends to react with other molecules to pair this electron and thereby generate a more stable species (Papas, 1998).

The most abundant radical in biologic systems is molecular oxygen (O_2) itself, or dioxygen ($^3O_2$), which has two unpaired electrons. The superoxide ($O_2^{\bullet -}$) and hydroxyl radical ($^{\bullet}OH$) are both species with one unpaired electron in their outer shells. The hydroxyl radical is particularly unstable, more reactive, and short-lived (10^{-6} seconds). Although hydroperoxides, such as hydrogen peroxide (H_2O_2), are themselves not true free radical species, they constitute an important secondary class of reactive oxygen metabolites that can also be quite toxic to tissue components (Reilly *et al*., 1991). Table 2-2 summarize the active oxygen and nitrogen species, which are relevant to lipid peroxide and oxidative stress *in vivo*.

Table 2-2 Reactive oxygen and nitrogen species

Radicals	Non-radicals
Reactive oxygen species (ROS)	
Superoxide, $O_2^{\bullet -}$	Hydrogen peroxide, H_2O_2
Hydroxyl, $HO^{\bullet}$	Hypochorous acid, HOCl
Peroxyl, $ROO^{\bullet}$	Ozone, O_3
Alkoxyl, $RO^{\bullet}$	Singlet oxygen
Reactive nitrogen species (RNS)	
Nitric oxide, $NO^{\bullet}$	Nitrous acid, HNO_2
Nitrogen dioxide, $NO^{\bullet}_2$	Dinitrogen trioxide, N_2O_3
	Peroxynitrite, $ONOO^-$

2.2.2 Physiological function and effects

Oxygen radicals are capable of reversibly or irreversibly damaging compounds of all biochemical classes, including nucleic acids, proteins and free amino acids, lipids and lipoproteins, carbohydrates, and connective tissues. These species may be have an impact on cell activities such as membrane function, metabolism, and gene expression. Oxygen radicals are formed in tissues by many processes as shown in Table 2-3 (Halliwell and Gutteridge 1999).

Table 2-3 Some of the clinical conditions in which the involvement of ROS/RNS has been suggested (Cross, *et al*., 1987)

Primary single organ involvement

- Lung
  - Cigarette-smoke effects
  - Emphysema
  - Hyperoxia
  - Bronchoplumonary dysplasia
  - Oxidant pollutants
  - Acute respiratory distress syndrome
  - Mineral dust pneumoconiosis
  - Bleomycin toxicity
  - Paraquat toxicity
- Heart and cardiovascular system
  - Alcohol cardiomyopathy
  - Keshan disease (selenium deficiency)
  - Atherosclerosis
  - Doxorubicin toxicity
- Kidney
  - Nephrotic antiglomerular basement membrane disease
  - Aminoglycoside nephrotoxicity
  - Renal graft rejection
- Gastrointestinal tract
  - Endotoxin liver injury
  - Carbon tetrachloride liver injury
  - Diabetogenic action of alloxan
  - Free-fatty-acid-induced pancreatitis
  - Nonsteroidal-antiinflammatory-drug-induced lesions
- Joint abnormalities
  - Rheumatoid arthritis
- Brain
  - Hyperbaric oxygen
  - Neurotoxins
  - Senile dementia
  - Parkinson disease-MPTP
  - Hypertensive cerebrovascular injury; cerebral truama
  - Neuronal ceroid lipofuscinoses
  - Allergic encephalomyelitis and other demyelinating diseases
  - Ataxia-telangiectasia syndrome
  - Potentiation of traumatic injury
  - Aluminium overload
  - A-β-lipoproteinemia
- Eye
  - Cataractogenesis
  - Ocular hemorrhage
  - Degenerative retinal damage
  - Retinopathy of prematurity
  - Photic retinopathy
- Skin
  - Solar radiation
  - Thermal injury
  - Porphyria
  - Contact dermatitis
  - Photosensitive dryness
  - Bloom syndrome
- Erythrocytes
  - Phenylhydrazine
  - Primaquine
  - Lead poisoning
  - Protoporphyrin photo-oxidation
  - Malaria
  - Nickle cell anemia
  - Favism
  - Fanconi anemia

Multiorgan involvement

- Inflammatory-immune injury
  - Glomerulonephritis (idiopathic, membranous)
  - Vasculitis (hepatitis B virus, drugs)
- Ischemia-reflow states
- Drug and toxin-induced reactions
- Iron overload
  - Idiopathic hemochromatosis
  - Dietary iron overload (red wine; beer brewed in iron pots)
  - Thalassemia and other chronic anemias
- Nutritional deficiencies
  - K washiorkor
  - Vitamin E deficiency
- Alcohol
- Radiation injury
- Aging
  - Disorders of “premature aging”
  - Immune deficiency of age
- Cancer
- Amyloid diseases

2.2.3 Antioxidant defences

Aerobic organisms are protected from oxidative stress induced by free radicals and active oxygen species by an array of defense systems. As summarized in Table 2-4, various kinds of antioxidants with different functions play an important role in these defense systems. The preventive antioxidants acting in the first defense line suppress the formation of free radicals and active oxygen species. The radical scavenging antioxidants are responsible in the second defense line and inhibit chain initiation and/or break the chain propagation. The antioxidant enzymes such as phospholipases, proteases, DNA repair enzymes, and transferases act as the third line defense. In addition, the appropriate antioxidant is generated and transferred to the right site at the right time and at the right concentration when the oxidative stress takes place. This adaptation mechanism is also important in the total defense systems (Papas, 1998).

Table 2-4 Defense systems *in vivo* against oxidative damage (Papas, 1998)

1. Preventive antioxidant: suppress the formation of free radicals	
1.1 Non-radical decomposition of hydroperoxides and hydrogen peroxide	
Catalase	Decomposition of hydrogen peroxide $2H_2O_2 \longrightarrow 2H_2O + O_2$
Glutathione peroxidase	Decomposition of hydrogen peroxide and free fatty acid hydroperoxides $H_2O_2 + 2GSH \longrightarrow 2H_2O + GSSG$ $ROOH + 2GSH \longrightarrow ROH + H_2O + GSSG$
Peroxidase	Decomposition of hydrogen peroxide and lipid hydroperoxides $ROOH + AH_2 \longrightarrow ROH + H_2O + A$ $H_2O_2 + AH_2 \longrightarrow 2H_2O + A$
Glutathione *S*-transferase	Decomposition of lipid hydroperoxides
1.2 Sequestration of metal by chelation	
Transferrin, lactoferrin	Sequestration of iron
Haptoglobin	Sequestration of hemoglobin
Hemopexin	Stabilization of heme
Ceruloplasmin, albumin	Sequestration of copper
1.3 Quenching of active oxygen species	
Superoxide dismutase (SOD)	Disproportionation of superoxide $2\ O_2^{\bullet -} + 2H^+ \longrightarrow H_2O_2 + O_2$
Carotenoids, vitamin E	Quenching singlet oxygen

Table 2-4 Defense systems *in vivo* against oxidative damage (continued)

2. Radical-scavenging antioxidants: scavenge radicals to inhibit chain initiation and break chain propagation
Hydrophilic: vitamin C, uric acid, bilirubin, alblumin
Lipophilic: vitamin E, ubiquinol, carotenoids, flavonoids
3. Repair and *de novo* enzymes: Repair the damage and reconstitute membranes
Lipase, protease, DNA repair enzymes, transferase
4. Adaptation: Generate appropriate antioxidant enzymes and transfer them to the right site at the right time and in the right concentration

2.2.4 Phytochemical antioxidants

The studies have shown that dietary phytochemical antioxidants are capable of removing free radicals. Among them, phenolic and polyphenolic compounds, such as flavonoids and catechin in edible plants, exhibit potent antioxidant activities.

2.2.4.1 Phenolic compounds

Phenolic compounds are a large, heterogeneous group of secondary plant metabolites that are widespread in the plant kingdom. Phenolics display a vast variety of structures; here only flavonoids, tannins and phenolic acids are reviewed (Figure 2-3). The structural basis for all flavonoids is the flavone nucleus (2-phenyl-benzo-γ-pyrane) but, depending on the classification method, the flavonoid group can be divided into several categories based on hydroxylation of the flavonoid nucleus as well as the linked sugar. Essential structures are divided into

eleven classes as presented in Figure 2-4 (Bors, *et al*., 1998). "Tannins" is a general name for phenolic substances capable of tanning leather or precipitating gelatin from solution. They can be divided into condensed proanthocyanidins, where the fundamental structural unit is the phenolic flavan-3-ol (catechin) nucleus, and into galloyl and hexahydroxydiphenoyl esters and their derivatives, gallotannins and ellagitannins (Haslam, 1996), as shown in Figure 2-3b. The essential two groups of phenolic acids are hydroxybenzoic acids and hydroxycinnamic acids (Figure 2-3c), both of which are derived from nonphenolic molecules benzoid and cinnamic acid, respectively (Macheix and Fleuriet, 1998).

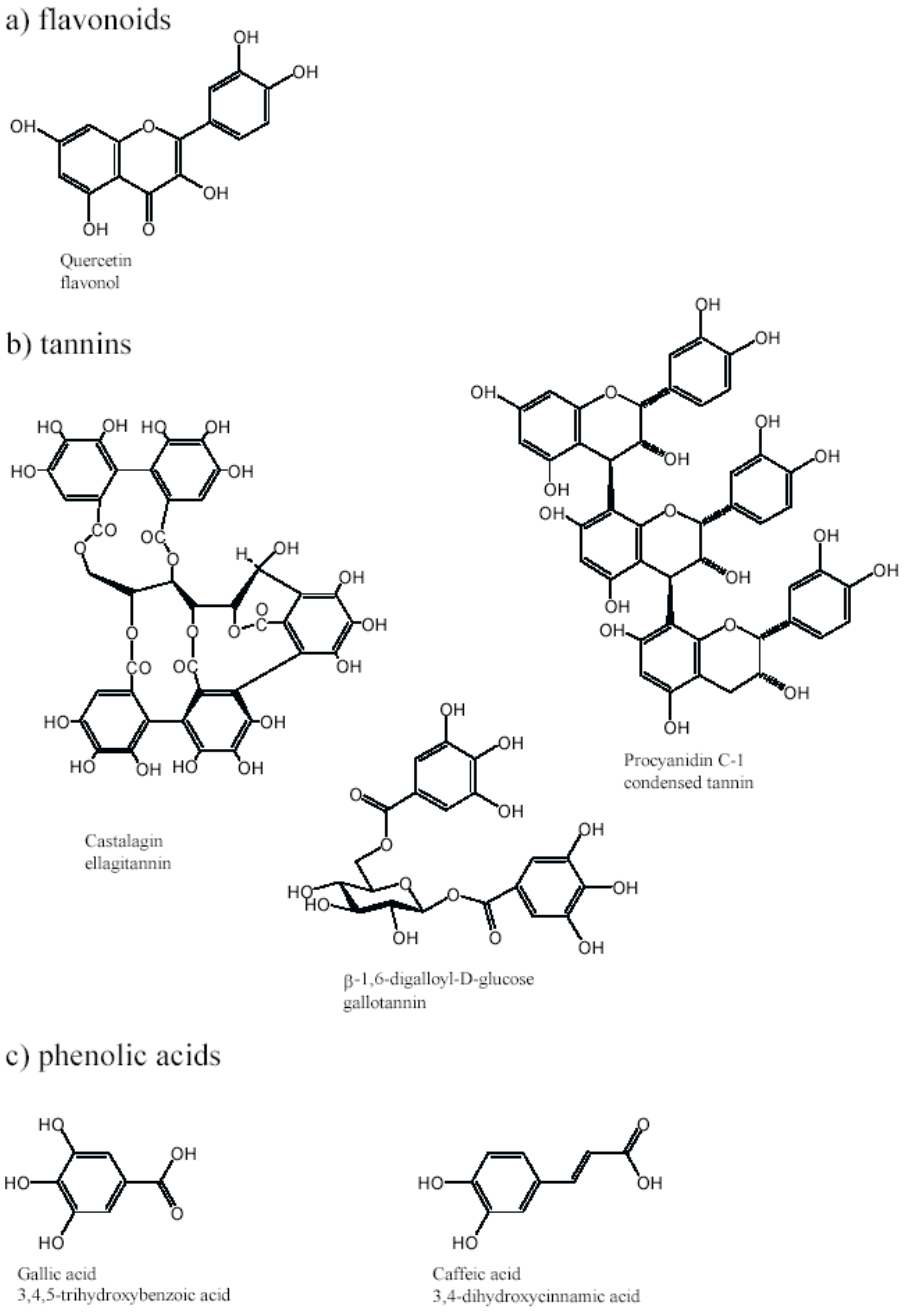


Figure 2-3 Typical structure of different groups of plant phenolics:

a) flavonoids

b) tannins

c) phenolic acids

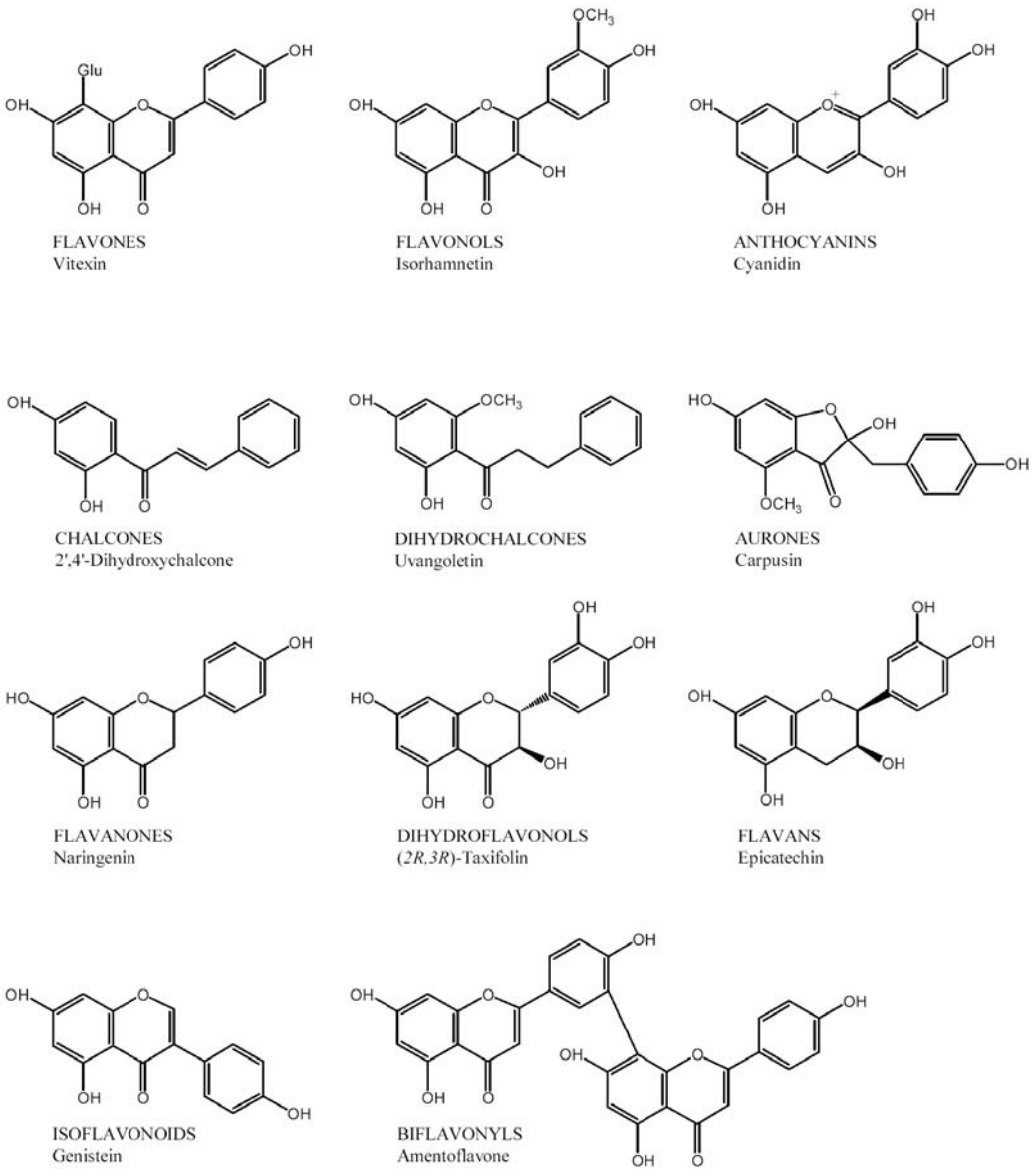


Figure 2-4 Typical structure of flavonoids divided into the different subgroups

2.2.4.2 Spices and herbs

Antioxidants from spices and herbs have the potential for large-scale applications. Spices have been used not only for their flavoring properties but also for their food-preserving ability. Over the past few years, a number of medicinal plants such as *Echinacea purpurea*, *Ginkgo biloba* (ginkgo) and pine bark extract (Oligopin) have been investigated for their quenching activity of specific reactive oxygen species (ROS), such as the hydroxyl radical, the superoxide anion, singlet oxygen, and lipid peroxides (Masaki *et al*., 1995). The seeds of *Cassia tora* Linn. have been conventionally used in traditional Chinese medicine for several centuries. The methanolic extracts from *Cassia tora* Linn. exhibited a strong antioxidant activity on lipid peroxidation (Yen and Chuang, 2000). This herb has been reported to contain many active components, including chrysophanol, emodin, aloe-emodin, rhein, physcion and obtusin. Spices are known for both its antioxidative and antimicrobial properties. The aqueous extracts of *Alpinia galanga* inhibited lipid oxidation and microbial growth in minced beef (Cheah and Gen, 2000). An extract from a mixture of aromatic spices (rosemary, sage, thyme and oregano), called Spice Cocktail Provencal effectively stabilized animal fats and frozen fish products (Aruoma *et al*., 1996). Rosemary and sage had very high antioxidative activity in lard, while clove was the most effective spice in oil-in-water emulsion. In French dressing and mayonnaise (oil-in-water emulsion), oregano was found to be the most potent spice in retarding oxidative deterioration (Chipault *et al*., 1995). The presence of tocopherols, mainly γ–tocopherol, in the non-polar fraction obtained from oregano by hexane extraction (Lagouri and

Boskou, 1996). Tocopherols are best known as efficient naturally occurring liposoluble antioxidants and its defense against reactive oxidative species (Diplock, 1992).

2.2.5 Measurement of antioxidant activity

The interpretation of results obtained from *in vitro* measurements of antioxidant activity of a compound or of a crude plant extract, must be dealt with caution as the antioxidative effect of a tested compound may vary considerably with the method and conditions used. Thus, selection of the appropriate assay to be used should be based on the intended application of the antioxidant.

Methods to examine antioxidant activity of a sample can be divided in principle into two major categories:

1. Measuring its ability to donate an electron (or hydrogen atom) to a specific ROS or to any electron acceptor.

2. Testing its ability to remove any sources of oxidative initiation, e.g., inhibition of enzymes, chelation of transition metal ions and absorption of UV radiation.

2.2.5.1 Scavenging effect on 1,1-diphenyl-2-picrylhydrazyl radical

Radical scavenging is the main mechanism by which antioxidants act in herbal. Several methods have been developed in which the antioxidant activity is assessed by the scavenging of synthetic radicals in polar organic solvents, e.g. methanol, at room temperature. Those used include 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals (Yamaguchi *et al*., 1998).

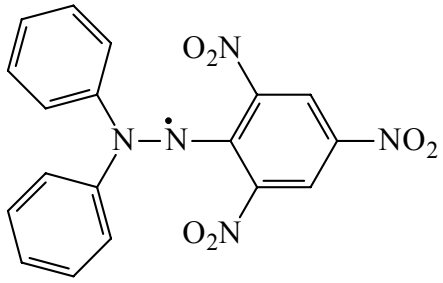


Figure 2-5 structure of 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical

In the DPPH test, the scavenging of DPPH radicals is followed by monitoring the decrease in absorbance at 520 nm which occurs due to reduction by the antioxidant (AH) or reaction with a radical species ($R^{\bullet}$) as the following reactions.

$$DPPH^{\bullet} + AH \longrightarrow DPPH\text{-}H + A^{\bullet}$$

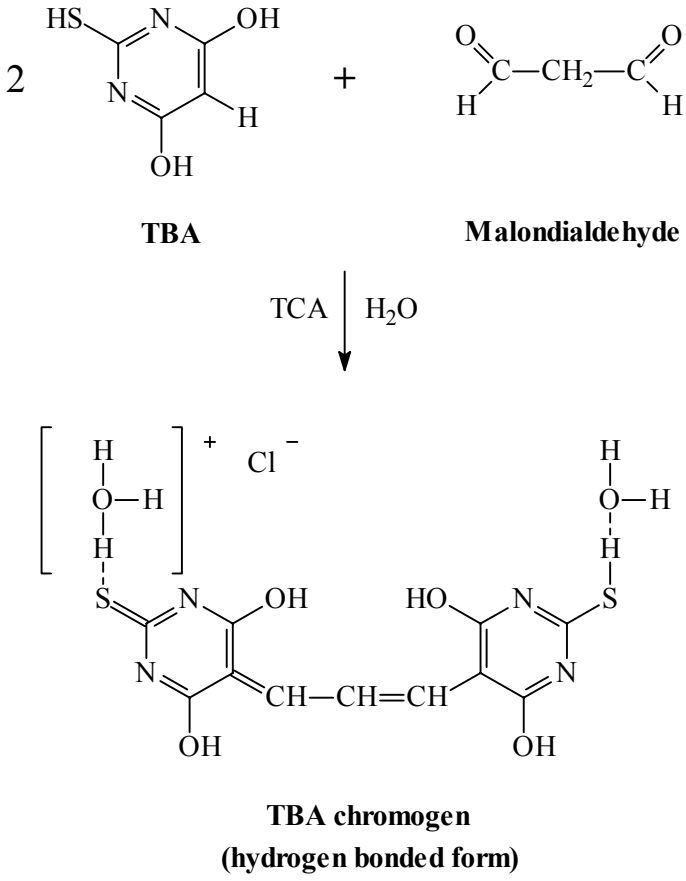
$$DPPH^{\bullet} + R^{\bullet} \longrightarrow DPPH\text{-}R$$

Fast reaction of DPPH radicals occurs with some phenols e.g. α–tocopherol, but slow secondary reactions may cause a progressive decrease in absorbance, so that the steady state may not be reached for several hours. Most

papers in which the DPPH method has been used report the scavenging after 15 or 30 min reaction time. The data is commonly reported as EC_{50}, which is the concentration of antioxidant required for 50 % scavenging of DPPH radicals in the specified time period.

2.2.5.2 Thiobarbituric acid value (TBA)

Malonaldehyde may be formed from polyunsaturated fatty acids with at least three double bonds. The concentration of this product may be assessed by reaction with thiobarbituric acid which reacts with malonaldehyde to form red condensation products (Figure 2-6) that show absorption at 532-535 nm. However, the reaction is not specific, and reaction with a wide variety of other products may contribute to the absorbance. 2,4-Alkadienals such as 2,4-decadienal also react with TBA to show strong absorption at 532 nm. Saturated aldehydes normally absorb at low wavelengths after reaction with TBA. Several herbal components including proteins, Maillard browning products and sugar degradation products affect the determination. In order to emphasise the lack of specificity, the values obtained in the test are commonly described as TBARS (TBA reactive substances). The TBA test has recently been reviewed (Rice-Evans, *et al*., 1998).



TBA chromogen
(hydrogen bonded form)

Figure 2-6 Formation of a chromogen by reaction of TBA with malondialdehyde

2.3 Chromatographic analysis

As long as the active principle is known, quality control and characterization of the extract have to be based on these compounds. Qualitative chromatographic fingerprint is not sufficient and must be supplemented by quantitative determination of the active constituents to obtain a correlation between the quantity of the relevant constituents and its pharmacological or therapeutic efficacy. When the active principle of the plant extract is not known, lead compounds are usually used for chemical characterization. These compounds may not be related to their activity, but they should be typically chemical markers of the extracts (Bauer and Tittel, 1996).

The types of chromatography useful in qualitative and quantitative analysis are column, gas, paper, thin layer and pressurized liquid chromatography. Paper and thin layer chromatography are ordinary useful for identification purposes, because of their convenience and simplicity. Column chromatography offers a wide choice of stationary phases and is useful for the separation of individual compounds from mixtures. Both gas and pressurized liquid chromatography require more elaborate apparatus and usually provide high resolution methods that will identify and quantify very small amounts of material. HPLC has distinct advantages over gas chromatography for the analysis of organic compounds. Compounds to be analyzed are dissolved in a suitable solvent and most separations take place at room temperature. Thus most drugs, being nonvolatile or thermally unstable compounds can be analyzed without decomposition or the necessity of making volatile derivatives.

HPLC is the method of choice for the analysis of most natural products. This form of chromatography is fast, reproducible, requires little sample, and can be used for both qualitative and quantitative analysis as well as for preparative work.

2.3.1 High pressure liquid chromatography

High performance liquid chromatography (HPLC) is a separation technique based on a solid stationary phase and a liquid mobile phase. The HPLC system comprises of a solvent delivery pump, an injector, a column, and a detector. The scientific literature contains numerous examples of the use of HPLC for flavonoid analysis, although several different types of column and/or detector can be used. Comment here will be confined to the most commonly used system, that of a reversed-phase (RP-C18) column and a UV/visible detector (Table 2-5).

Most analysis are performed with a pumping system capable of producing at least a binary gradient, a RP-18 column (particle size 5 μm or smaller, column size 4.6 mm x 125 or 250 mm), and a UV detector. The detector should be variable wavelenght, and in newer instruments, multichannel, fast scanning, or photodiode array detectors have now become the norm. The latter two are extremely useful for flavonoid analysis as they are capable of producing a UV/visible absorption spectrum for each peak. For detection of anthocyanins, the detector range should extend into the visible region (to at least 600 nm).

The solvent mixture pumped through the column can remain the same throughout the chromatographic run (an isocratic system), or the mixture may change in a predetermined way (a gradient system). Isocratic elution is unsuitable for most natural extract as the wide variety of components means unacceptably short

or long elution times for many of the components. Using gradient elution with two or more solvents, it is possible to optimize the chromatography for most components.

Table 2-5 HPLC condition for plant

Sample	Stationary phase	Mobile phase	Detector	Reference
Barosma betulina	LiChrosorb RP18	MeOH:H_2O 60:40 (isocratic)	UV 345	Azza *et al*., 2001
Betula pendula and *Betula pubescens*	Hypersil ODS	2.4%THF+0.25%orthophos-phoric acid:MeOH (gradient)	UV	Keinanen and Julkunen, 1998
Human urine containing *Gingko biloba*	Spherisorb ODS	H_2O:ACN:MeOH:AcOH (gradient)	DAD	Pietta *et al.,* 1997
Hypericum perforatum	RP-18	H_2O:85%phosphoric acid:ACN:MeOH (gradient)	UV 270	Broils *et al*., 1998
Standard flavonoid	Zorbax Eclipse XDB-C18	H_2O:MeOH: ACN containing 0.05% THF	UV 210	Marken and Beecher, 2000
Echinacea spp.	ODS Hypersil	MeOH: H_2O:AcOH (25:75:1)	UV	Glowniak *et al*., 1996
Standard flavonoid	Phenomenex RP-C18	MeOH: H_2O:1%formic acid (gradient)	DAD 220-450	Ulta *et al*., 1998
Catharanthus roseus	Lichrospher60 RP	MeOH : 1%HOAc at pH 4 (40:60)	UV 280	Retno *et al*., 2001

2.3.2 Optimization of condition for HPLC

Due to the variation of the phytochemical substances, an HPLC may not be applicable to all compounds. Thus optimization of HPLC conditions for separation of phytochemical substances has to be identified.

Method optimization is one of the essential steps in chromatographic analysis. Most optimization strategies are improved or maximized in terms of resolution, peak shape, plate counts, asymmetry, capacity and elution time. The system suitability parameters including on resolution (Rs) equation, in which the three basic parameters, capacity factor (k′), separation factor (α) and theoretical plate number (N), can be optimized separately (Outinen *et. al*., 1996). The US Food and Drug Administration (FDA) acceptable values of these parameters are tailing factor less than 2, N not more than 2000, Rs more than 2, and repeatability not more than 1%, while USP25/NF20 requirement is not more than 2%.

2.3.3 Calculations for chromatographic parameters

Capacity factor, **k′**, is calculated from the retention data by the following equation:

$$k' = (t_R - t_0)/t_0$$

where t_R is the retention time of the solute and t_0 is the retention time of an unretained substance

Relative retention, **α**, which calculated by the following equation:

$$\alpha = t_2\text{-}t_0 \,/\, t_1\text{-}t_0$$

where t_2 and t_1 are the retention times, measured from the point of injection, of the test and reference substances, respectively, determined under identical experimental conditions on the same column, and t_0 is the retention time of an unretained substance.

The separation of two components in a mixture, the resolution, **Rs**, is determined by the following equation:

$$Rs = 2(t_2-t_1)/w_2+w_1$$

in which t_2 and t_1 are retention times of the two components, and w_2 and w_1 are the corresponding widths at the bases of the peaks obtained by extrapolating the relatively straight sides of the peaks to the baseline.

Number of theoretical plates, **N**, is a measure of column efficiency. It is calculated by the equation:

$$N = 16\ (t/w)^2$$

where t is the retention time of the substance and w is the width of the peak at its base, obtained by extrapolating the relatively straight sides of the peak to the baseline.

The tailing factor, $\mathbf{T_f}$, a measure of peak symmetry, is unity for perfectly symmetrical peaks and its value increases as tailing becomes more pronounced.

$$T_f = w_{0.05}\ /2f$$

f distance from the peak maximum to the leading edge of the peak, the distance being measured at a point 5% of the peak height from the baseline $w_{0.05}$ width of peak at 5% height.

2.3.4 Markers in plant

Markers are chemically defined constituents of a herbal drug which are interest. It has been used for quality regardless of their therapeutic activity. Quantitative of the constituents by marker can be performed by determine marker content.