CHAPTER 1

INTRODUCTION

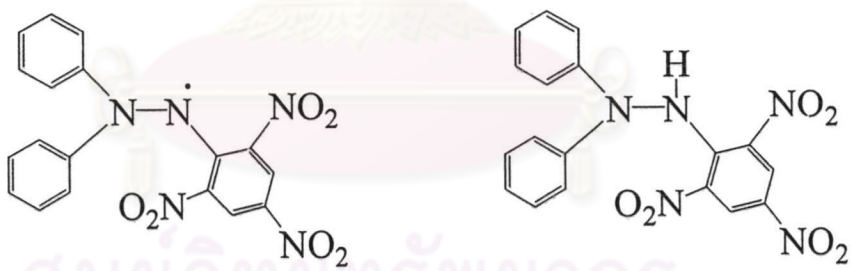
Nowadays, many diseases have no drug for treatment while new diseases still occur everyday. Therefore, searching for new drugs is necessary to continuously proceed. Secondary metabolites in plant have involved in nature as biologically active compounds with particular effects on other organisms. Thus, the study of chemical constituents from medicinal plants together with their pharmacological activities are important to find and develop new medicinal drugs.

Many studies have implicated reactive oxygen radicals and other oxygen derived species as an important causative agents in a wide range of diseases such as alzheimer, arteriosclerosis, ischemia and cancer.[1-4] Reactive oxygen species are generated in specific organelles of cells.[5,6] The phagocytic cells ingest and kill invading pathogens with free radicals including superoxide anion, hydrogen peroxide, nitric oxide and hypochlorite. The reduction of molecular oxygen (O_2) to water (H_2O) in mitochondria proceeds by a series of single electron transfers. Therefore, highly reactive intermediates such as superoxide anion (O_2^-), hydrogen peroxides (H_2O_2) and hydroxyl radical ($HO^{\cdot}$) are generated. Furthermore, these radicals can induce another radical such as lipid peroxyl radical too. The defense mechanisms against these reactive oxygen species include radical scavenging enzyme, cellular antioxidants and some kind of vitamin. For example, superoxide dismutase catalyzes the dismutation of O_2^- to O_2 and H_2O_2. Catalase and peroxidase scavenge H_2O_2 to metabolize O_2 and H_2O. Vitamin E serves to minimize $HO^{\cdot}$ concentration in cell membranes.

Under normal condition balance exists between the generation and detoxification of reactive oxygen species in cells. However, diseases, aging, sunlight and chemical environments such as drugs, pesticides, herbicides and various pollutants can disrupt this balance by inhibition of the cellular antioxidant defense or by stimulation of the formation of reactive oxygen species. These reactive oxygen species can damage macromolecules in cell such as proteins, lipids, amino acids and DNA to cause serious diseases, as mention before. The intake of antioxidants has been seen as a very attractive method of preventing these diseases. A number of synthetic

antioxidants such as butylate hydroxyanisole (BHA) and butylate hydroxytoluene (BHT) have been developed, but they have restricted to use due to their toxicity. Although vitamin E (α-tocopherol) is an effective natural antioxidant, it has limited usage about its accumulation in body[7]. As a result, it is interesting to search for new antioxidative agents that may lead to new medicinal drugs for treatment or prevention of these diseases.

In this study, we have primary screening test with some medicinal plants based on radical scavenging activity (radical scavenger has received as chain-breaking antioxidant). 2,2-Diphenyl-1-(2,4,6-trinitrophenyl)hydrazyl (DPPH) radical was used as radical source in this test model. DPPH is a kind of nitrogen-centered radical and stable with their resonance system so it long-lived enough to detect any change that occur when reacted with test compounds. For this detection we use the property of DPPH which has different color between radical form (DPPH, purple color) and non radical form (DPPHn, colorless). The structures of DPPH and DPPHn are shown in **Figure 1.1** This model was selected for primary screening test because it is rapid, inexpensive, convenient, requires a little material and reliable. Moreover, this method can be modified to use for both qualitative and quantitative analyses.



DPPH (purple color, λ_{max} 517 nm) DPPHn (colorless)

Figure 1.1 Structure of DPPH and DPPHn

From the primary screening test, crude extract of *Carallia brachiata* Merr. showed the attractive result for scavenging activity on DPPH. This plant was therefore chosen for further investigation of the chemical constituent and their scavenging activity. Besides the scavenging test with DPPH radical, we attempted to use the same radical source as radical inside the body.

Superoxide dismutase and ferric thiocyanate assays which use superoxide anion radical ($O_2^{\cdot -}$) and lipid peroxyl radical ($ROO^{\cdot}$) as radical source were selected for secondary test.

Xanthine Oxidase (XO) is a key enzyme that catalyzes the oxidation of hypoxanthine or xanthine to uric acid **Figure 1.2**. During the reoxidation of XO, molecular oxygen acts as an electron acceptor, producing superoxide anion radical and hydrogen peroxide.[8] Superoxide anion radical ($O_2^{\cdot -}$) was used as radical source in scavenging test.

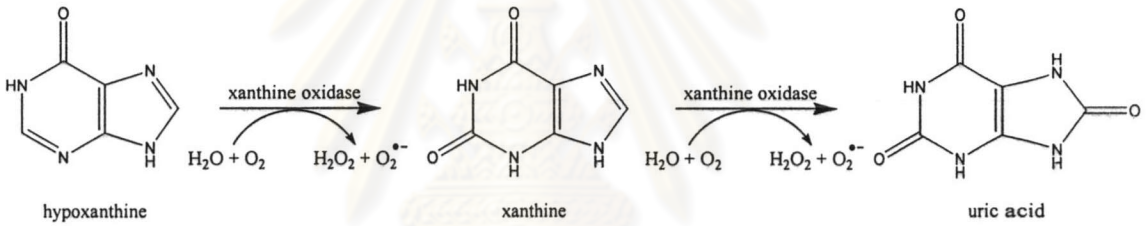


Figure 1.2 The oxidation of hypoxanthine to uric acid

Superoxide anion radical ($O_2^{\cdot -}$) is very reactive radical and cannot be separated from the reaction mixture, so testing compounds were added into the reaction mixture and the amount of $O_2^{\cdot -}$ was compared with blank. Decreasing of $O_2^{\cdot -}$ indicated a scavenging activity of testing compound. However decreasing of $O_2^{\cdot -}$ may occur with another reason, testing compound may react to xanthine oxidase so the $O_2^{\cdot -}$ could not be generated. For a definite proof of the cause of $O_2^{\cdot -}$ decrease compound of interest was tested by xanthine inhibition assay that showed the effect of testing compounds to xanthine oxidase.

The last radical that we chose for scavenging test using ferric thiocyanate assay is lipid peroxyl radical ($ROO^{\cdot}$) . The main principle of this assay is to oxidize fatty acid to lipid peroxyl radical and compare the amount of this radical between test compounds and blank. The auto-oxidation of unsaturated fatty acid is a chain process occurring autocatalytically through free radical intermediate.[9]

Initiation

$$LipH + initiator \longrightarrow Lip^{\cdot} + H^{\cdot}$$

Propagation

$$Lip^{\cdot} + O_2 \longrightarrow LipOO^{\cdot}$$

$$LipOO^{\cdot} + LipH \longrightarrow LipOOH + Lip^{\cdot}$$

Termination

$$Lip^{\cdot} + Lip^{\cdot} \longrightarrow Lip\text{-}Lip$$

Test compounds may act as initiator and/or scavenger that we known by observe the difference result between sample and blank. The positive value indicated prooxidant activity and the negative value showed antioxidant activity.

The isolated compounds from *Carallia brachiata* were not only tested for scavenging activities but also performed for KB cell lines.

Carallia brachiata Merr. is in the list of Thai Folk Medicine. Its bark used as an antipyretic, cardiotonic and used for fainting.[10, 11]

1.1 Botanical Aspect and Distribution

Carallia brachiata Merr. is in the Rhizophoraceae, a small family which have 16 genera and about 120 species.[12] Mostly, plants in this family have been known as mangroves, but *C. brachiata* is not a mangrove.

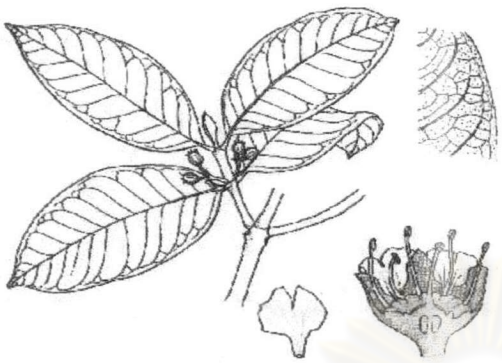
Carallia brachiata Merr. or *Carallia integerrima* DC. is a medium size tree, 15-20 meters high, widespread in tropical region of the world. In Thailand it has been known as "Chiang phraa naang ae" (central), "Seefan naang ae" (northern), "Khiang phraa" (Trat, Prachuap Khiri Khan) etc.[13] *C. brachiata* is an evergreen tree.

Bark : pale creamy brown to warm red-brown, quite smooth with many lenticels.

Leaf : 4-17 x 2.5-8 cm, simple, opposite-planar, oval to broadly obovate with blunt or abrupt tip & slightly pointed base, untoothed or with scattered fine teeth.

Flower : ±0.6 cm, white or pale yellow-green, bisexual, in head-like clusters (cymes) at leaf axils. Calyx bell-shaped with 5-8 short teeth, 5-8 free petals with short stalks, 10-16 slender stamens, petals & stamens attached to top of calyx tube around a thin disc, 1 slender style with 3-4 lobed stigma, all parts completely smooth.

Fruit : 0.5-1.8 cm, pale reddish-orange to dark red-purple, globose with persistent calyx teeth at top, slightly grooved, fleshy with 1(2) large kidney-shaped seeds surrounded by a thin orange coating (aril).



(a)

(b)

(c)

(d)

Figure 1.3 Leaf (a), flowers (b), tree (c) and bark (d) of *C. brachiata*

1.2 Chemical constituents studies on Rhizophoraceae

Up to now, only one literature was found on the study of chemical constituents of *C. brachiata*. It was reported that (+)-hygroline[14] **(Figure 1.4)** is a major alkaloid from the leaf of this plant, but is absent in the bark.

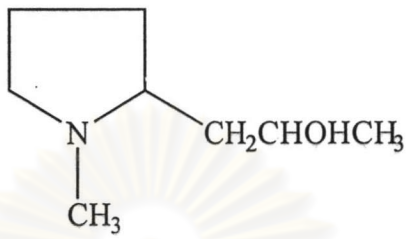
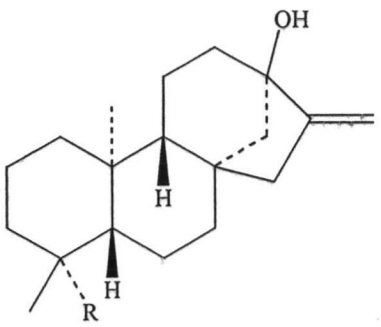


Figure 1.4 Structure of (+)-hygroline

For this reason, we have searching for chemical constituents study of plants belonging to Rhizophoraceae family. It was found that various types of organic compounds have been isolated. They can be classified as diterpenoid, steroid, triterpenoid, alkaloid, and a little of other compounds. Many types of them are reported in **Table 1.1** and the structures of some of these compounds are exhibited in **Figure 1.5 - 1.10**

Table 1.1 Chemical constituents studies on Rhizophoraceae family

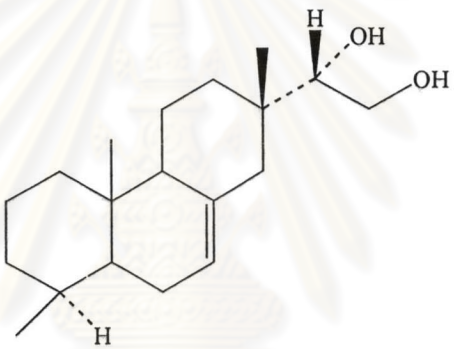
Type	Name of compounds	Parts of plant
Diterpene[15]	sterviol *ent*-kaur-16-en-13-hydroxy-19-al *ent*-kaur-16-en-13,19-diol 15(*S*)-isopimar-7-en-15,16-diol 1β,15(*R*)-*ent*-pimar-8(14)-en-1,15,16-triol	Root bark
Steroid[16,17]	β-sitosterol, stigmasterol campesterol, chloresterol 28-isofucosterol stigmast-7-en-3β-ol	Leave
Triterpenoid[17,18]	α-amyrin, β-amyrin taraxerol, gymnorhizol lupeol, oleanolic acid ursolic acid, friedelin betulin, betulinic acid β-amyrone	Leave
Alkaloid[14,19-22]	brugine, (+)-hygroline tropine cassipoureamide-A and -B	Stem and bark
	(+)-hygroline, cassipourine gerradine, gerradamine gerradoline, rhizophorine	Twig and leave
Tannin[23]	3-O-α-L-rhamnopyranosry-(+)-catechin-(4α→2)-phloroglucinol	Bark
Other compounds[16,24-26]	brugierol, isobrugierol, leucocyanidin 4-hydroxy-1,2-dithiolane	Stem and bark
	triacontanol, α-catechin 2,6-dihydroxy-*p*-benzoquinone 2,6-dimethoxy-*p*-benzoquinone 1-hydroxy-5-oxobicyclo[6.4.0]dodecane	Leave



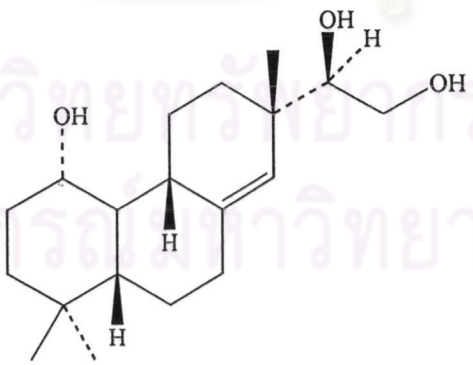
Sterviol (R = CO_2H)

ent-kaur-16-en-13-hydroxy-19-al (R = CHO)

ent-kaur-16-en-13,19-diol (R = CH_2OH)

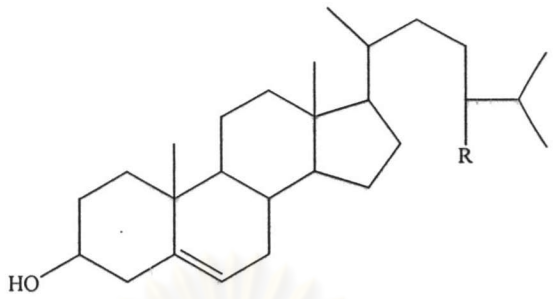


15(*S*)-isopimar-7-en-15,16-diol



1β,15(*R*)-*ent*-pimar-8(14)-en-1,15,16-triol

Figure 1.5 Diterpenoids found in Rhizophoraceae family

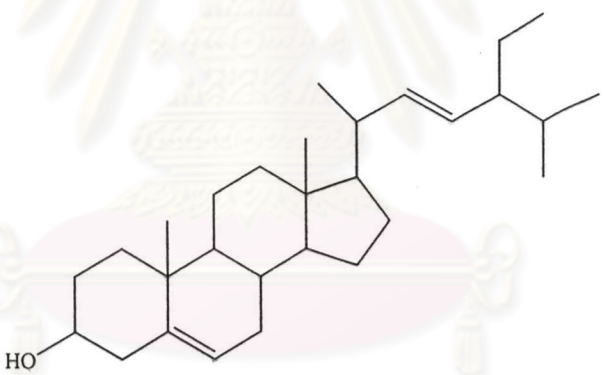


Cholesterol (R = H)

Campesterol (R = Me)

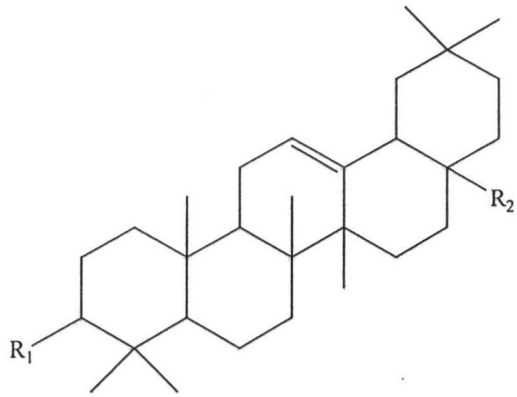
β-Sitosterol (R = Et)

28-Isofucosterol (R = CH_3CH=)



Stigmasterol

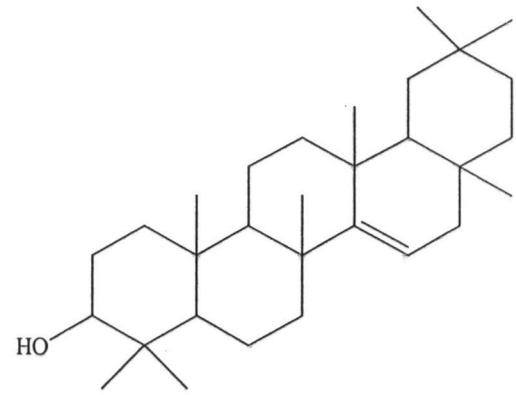
Figure 1.6 Steroids found in Rhizophoraceae family



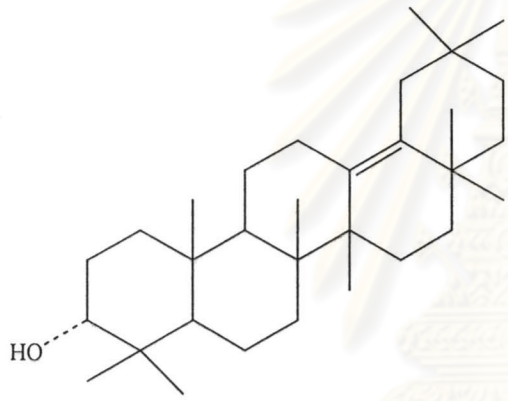
β-Amyrin (R_1 = OH(H), R_2 = CH_3)

β-Amyrone (R_1 = O, R_2 = CH_3)

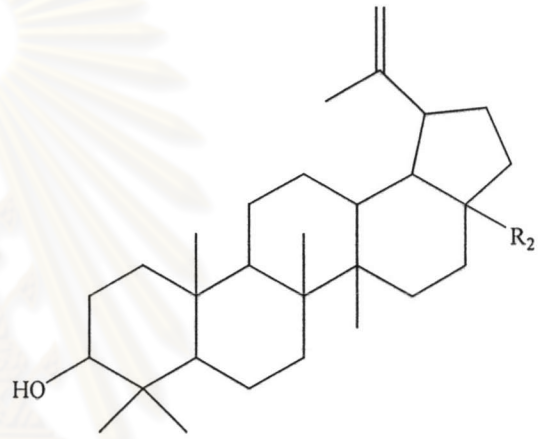
Oleanolic acid (R_1 = OH(H), R_2 = CO_2H)



Taraxerol



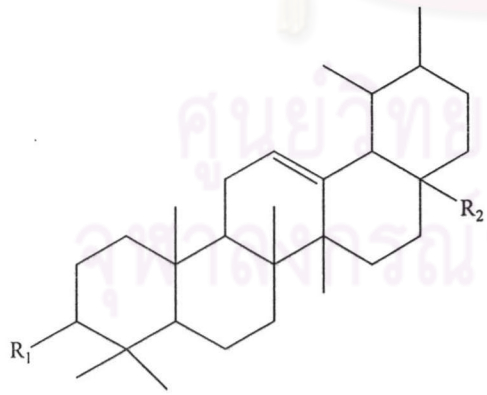
Gymnorhizol



Lupeol (R = CH_3)

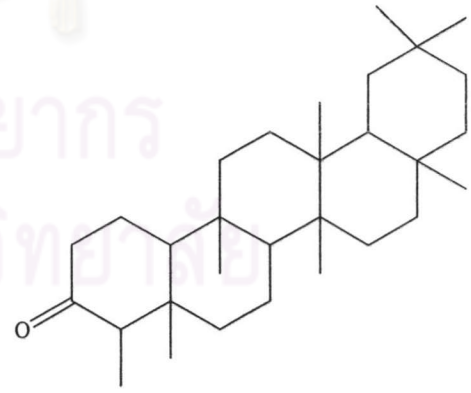
Betulin (R = CH_2OH)

Betulinic acid (R = CO_2H)



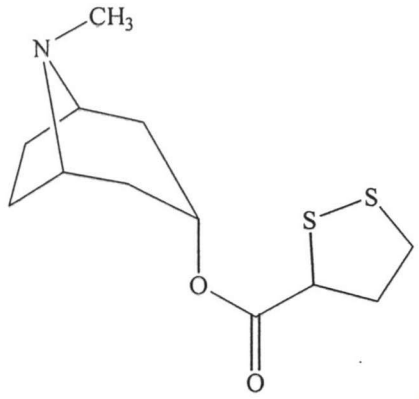
α-Amyrin (R_1 = OH(H), R_2 = CH_3)

Ursolic acid (R_1 = OH(H), R_2 = CO_2H)

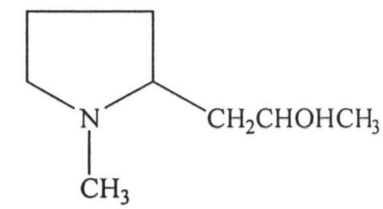


Friedelin

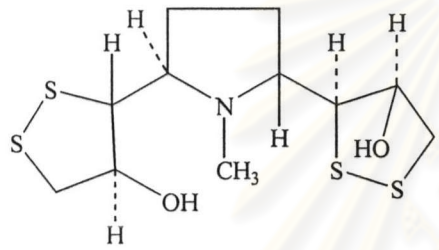
Figure 1.7 Triterpenoids found in Rhizophoraceae family



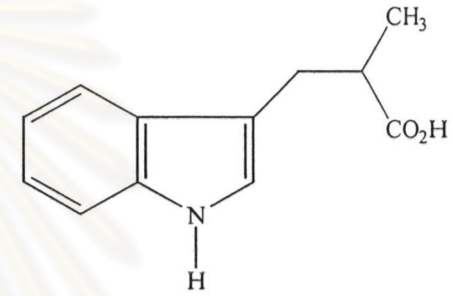
Brugine



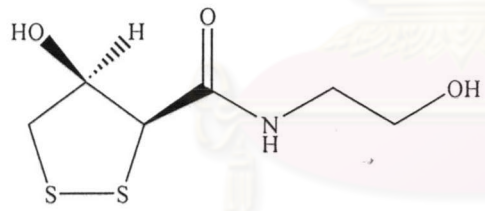
(+)-Hygroline



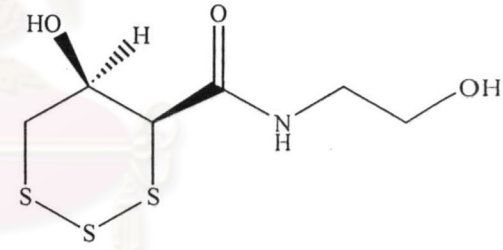
Gerrardine



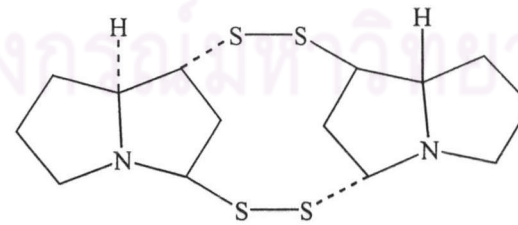
Rhizophorine



Cassipoureamide-A

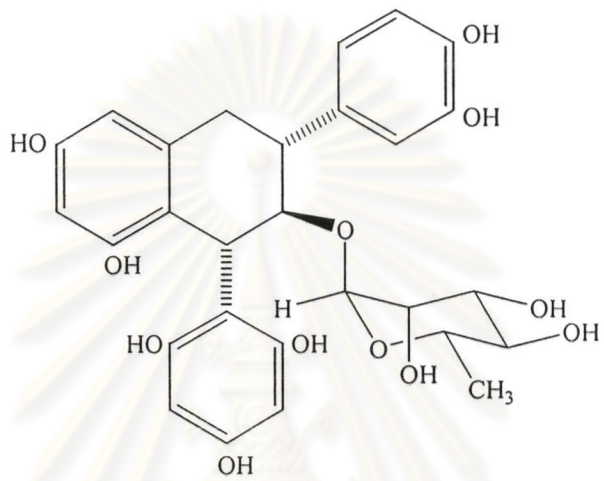


Cassipoureamide-B



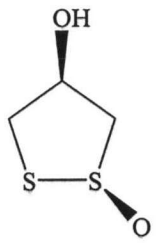
Cassipourine

Figure 1.8 Alkaloids found in Rhizophoraceae family

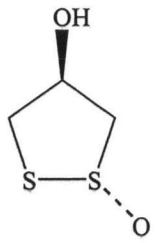


3-O-α-L-rhamnopyranosry-(+)-catechin-(4α→2)-phloroglucinol

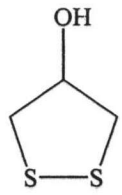
Figure 1.9 Tannins found in Rhizophoraceae family



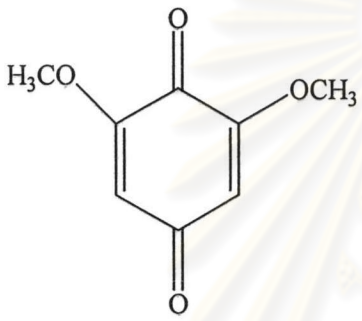
Brugierol



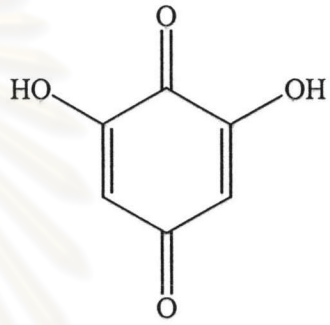
Isobrugierol



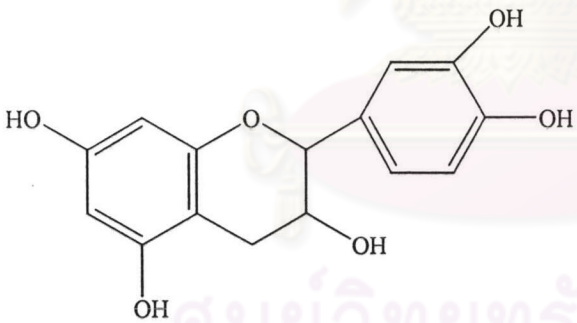
4-Hydroxy-1,2-dithiolane



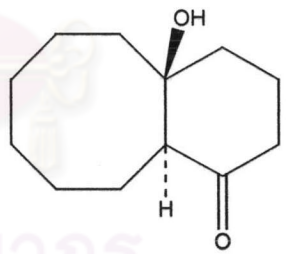
2,6-Dimethoxy-*p*-benzoquinone



2,6-Dihydroxy-*p*-benzoquinone



α-Catechin



1-Hydroxy-5-oxobicyclo[6.4.0]dodecane

Figure 1.10 Other compounds found in Rhizophoraceae family

1.3 The Goal of this Research

From the result of primary screening, base on scavenging activity of Thai medicinal plants, *C. brachiata* was selected for further investigation on chemical constituents which is the first study in the bark part of this plant. The goal of this study can be summarized as follows:

1. To extract and isolate the organic constituents from the bark of *C. brachiata*
2. To elucidate the structural formulae of the isolated compounds.
3. To test, the scavenging activities of the isolated compounds.

Separation and structure elucidation will be carried out by chromatographic techniques and spectroscopic techniques, respectively.