CHAPTER I

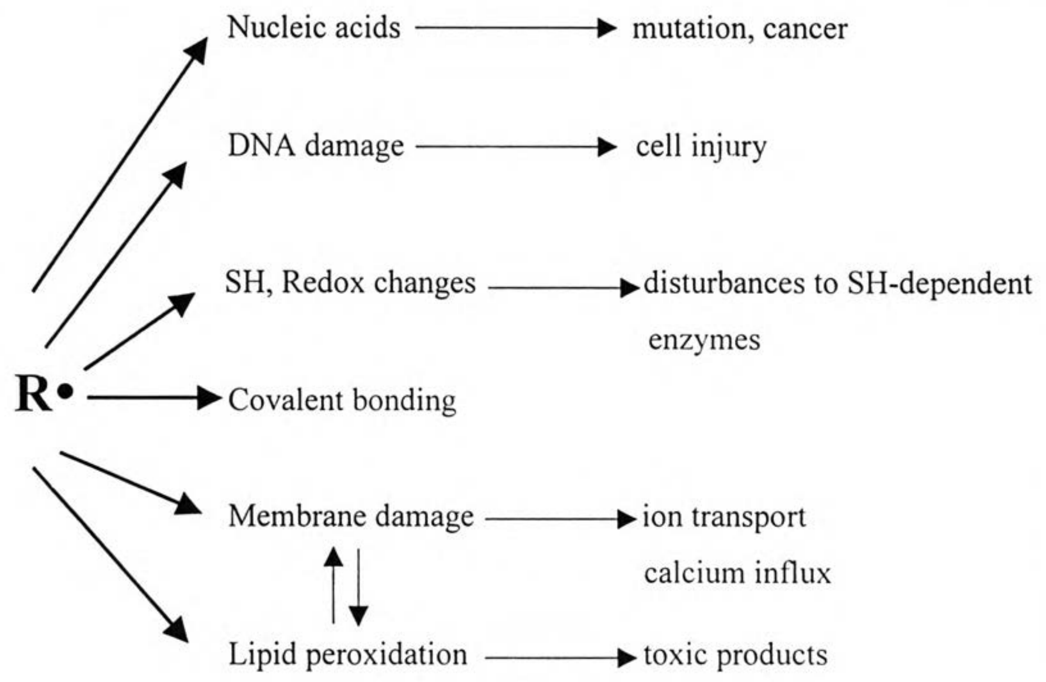
INTRODUCTION

Recently, there is a rapid growing movement back to the nature in the search for health and longevity. Traditional medicines, herbal-based medicines, and organic food have long been a part of worldwide culture for many centuries. Their importance to the world today is spreading due to increased awareness of the limited horizon of synthetic pharmaceutical products to control major diseases and the need to discover new molecular structures as lead compounds from plant kingdom. Therefore, today's herbal pharmacopoeia can frequently offer relief and prevention while avoiding the potential harmful side effects from many great pharmaceutical drugs.

Many medical researchers and health practitioners consent that the biggest challenge to long–term vibrant health is excessive free radicals damage or oxidative stress. The instantly growing of evidence in the scientific literature about the nature and role of free radicals has led to an increasing consciousness of their importance in health and disease. Free radicals have been implicated in a great number of human diseases such as cancer, atherosclerosis, aging, Alzheimer's and Parkinson's diseases (Martinez, 1995).

Free radicals are molecules with unpaired electron, which are produced in normal aerobic metabolism. Since oxygen, which is the ultimate electron acceptor in the electron transfer system, is used to generate the energy in the form of adenosine triphosphate (ATP). However, problems may arise when the electron becomes uncoupled, generating free radicals. In addition, environment insults, infections, smoking, ionizing radiation, and sunlight can also cause the formation of free radicals. The examples of free radicals are reactive oxygen species (ROS), various forms of activated oxygen, which include superoxide anion radical ($O_2^{-\cdot}$), peroxyl radical (ROO·), alkoxyl radical (RO·), hydroxyl radical (HO·), and nitric oxide radical (NO·) as well as nonfree-radical species such as hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), and hypochlorous acid (HOCl) (Halliwell, 1995).

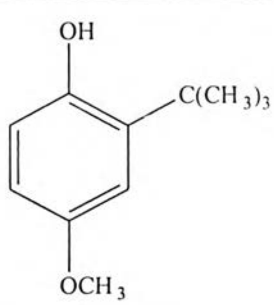
Free radicals are an important part of biological functions such as energy production, phagocytosis, regulation of cell growth and repair, and synthesis of biological compounds (Halliwell, 1995). Despite, free radicals can have negative effects when they are excessive due to environmental insults, diseases or malnutrition. They cause oxidative damage to biological macromolecules such as lipids in cell membrane resulting in lipid peroxidation, proteins in tissues or enzymes leading to cell inactivation (Dean *et.al.*, 1994), DNA cause mutation inducing to cancer (Cerutti, 1994; Diplock *et.al.*, 1994). These oxidative damages are considered to play a causative role in aging and several degenerative diseases as mentioned before. Major disturbances induced by the free radical reaction are shown in Scheme 1.1.



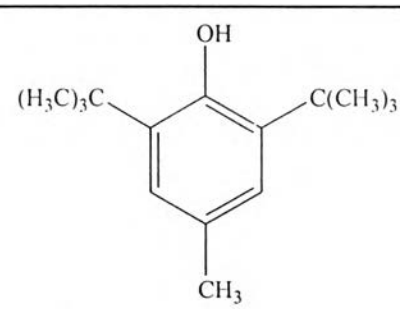
Scheme 1.1 Major disturbances induced by free radical to cause cell injury

In spite of that, all aerobic organisms, including human, have antioxidant defenses that protect against oxidative damages. These defense mechanisms are produced during normal cell aerobic respiration and act in concert in cell of differentiation and growth, immune response, cell membrane integrity, and normal DNA repair. These systems include some antioxidants produced in the body (endogenous) and others obtained from the diet (exogenous). The endogenous antioxidants include enzymatic defenses (catalase and superoxide dismutase) and non-enzymatic defenses (glutathione, reduce CoQ_{10}). However, when natural defenses are overwhelmed by an excessive generation of free radicals, which can be dealt with external factors (environmental insults, smoking). Hence, dietary intake of antioxidant compounds becomes an importance to maintain an adequate antioxidant status (Halliwell *et.al.*, 1995).

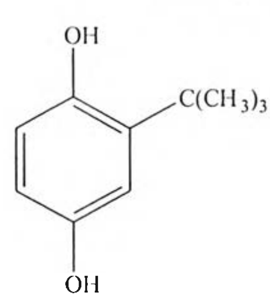
Antioxidants are compounds that inhibit or delay the oxidation of other molecules by inhibiting the initiation or propagation of oxidizing chain reactions. There are two basic categories of antioxidants, namely, synthetic and natural antioxidants. In general, synthetic antioxidants are compounds with phenolic structures of various degrees of alkyl substitution. Synthetic antioxidants (**Figure 1.1**) such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), and tertiary butylhydroxyquinone (TBHQ) have been used as antioxidants since the beginning of this century. Restrictions on the use of these compounds, however, are being imposed because of their carcinogenicity (Branen, 1975; Ito *et.al.*, 1983). Consequently, the importance of the search for and exploitation of natural antioxidants, particularly on plant origin, has greatly increased. Natural antioxidants can be phenolic compounds (tocopherol, flavonoids, and phenolic acid), nitrogen compounds (alkaloids, chlorophyll derivatives, amino acids, and amines), or carotenoids as well as ascorbic acid (Larson, 1988).



Butylated hydroxyanisole (BHA)



Butylated hydroxytoluene (BHT)

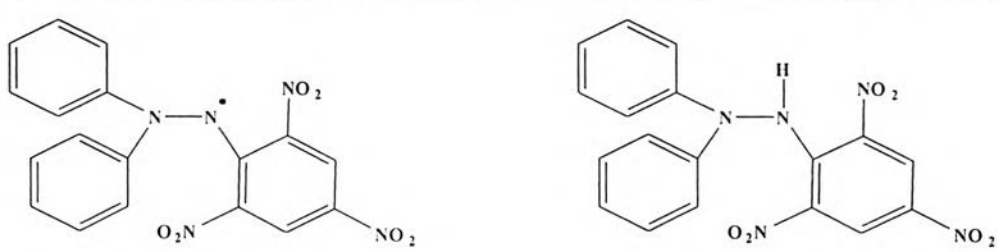


Tertiary butylhydroquinone (TBHQ)

Figure 1.1 Synthetic antioxidants

In this research, screening for the antioxidant-related activity on such medicinal plants has been investigated. A number of methods have been developed to determine the antioxidant activity of pure compounds from plant extracts. Principally, we selected 2,2-diphenyl-1-(2,4,6-trinitrophenyl)hydrazyl (DPPH) as stable radical source for primary screening because it is convenient, rapid, cheap, and reliable. Besides, this method requires a little amount of sample and can be altered to apply for both qualitative and quantitative examinations. In addition to other radical sources, we chose two important in vitro models, specifically, xanthine oxidase-related activity and lipid peroxidation that they generated superoxide anion and peroxyl radicals, respectively.

2,2-Diphenyl-1-(2,4,6-trinitrophenyl)hydrazyl (DPPH) radical is a class of nitrogen-centered radical and stable with its resonance system. It is a purple color solid with λ_{max}= 517 nm. We utilized the property that DPPH has different colors in radical form (DPPH, purple color) and non-radical form (DPPHn, colorless). The structures of DPPH and DPPHn are revealed in Figure 1.2.

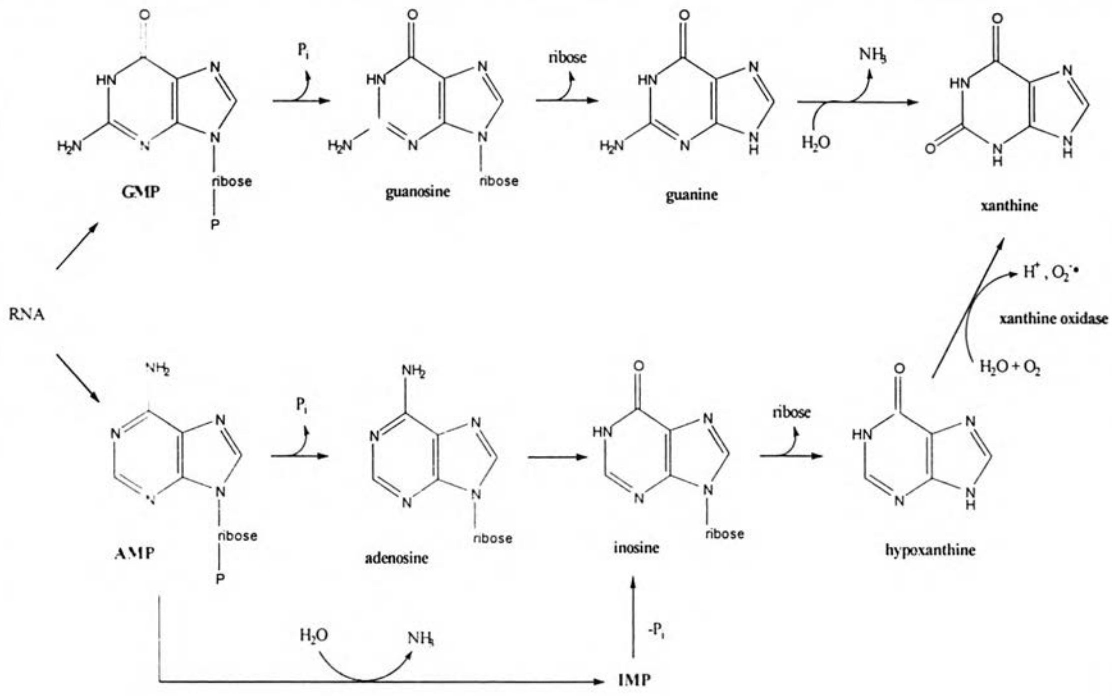


DPPH (purple color, λ_{max} 517 nm.) DPPHn (colorless)

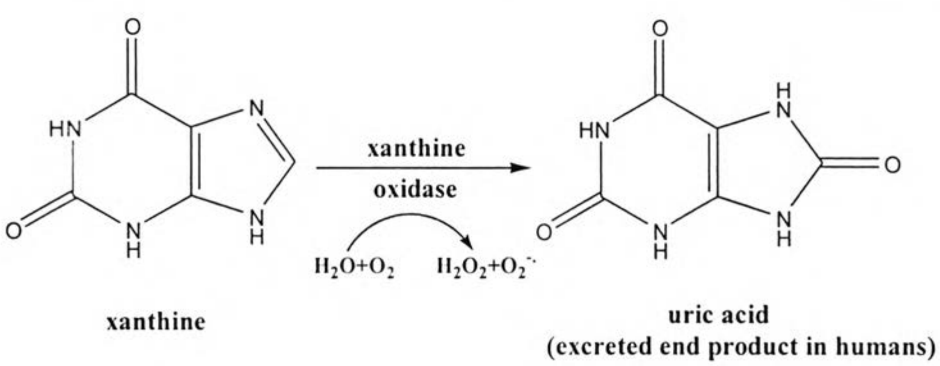
Figure 1.2 Structures of DPPH and DPPHn

Xanthine oxidase (EC.1.2.3.2) is a complex enzyme containing flavins, molybdenum, iron and sulfide cofactors. It is a highly versatile enzyme that is widely distributed from bacteria to human and within the several tissues of mammals. Xanthine oxidase is an important source of oxygen free radicals. The enzyme catalyzes the degradation of purines to yield uric acid, which causes gout condition, and produces the highly toxic superoxide anion radical ($O_2^{\cdot-}$) as a by-product. The purine degradation is shown in Scheme 1.2 and 1.3. This enzyme has been proposed as a central mechanism of oxidative injury such as heart attack, stroke, and arthritis.

Hence, the main principle of our assay was to determine the inhibition of xanthine oxidase and the superoxide anion radical scavenging activity of test compounds.

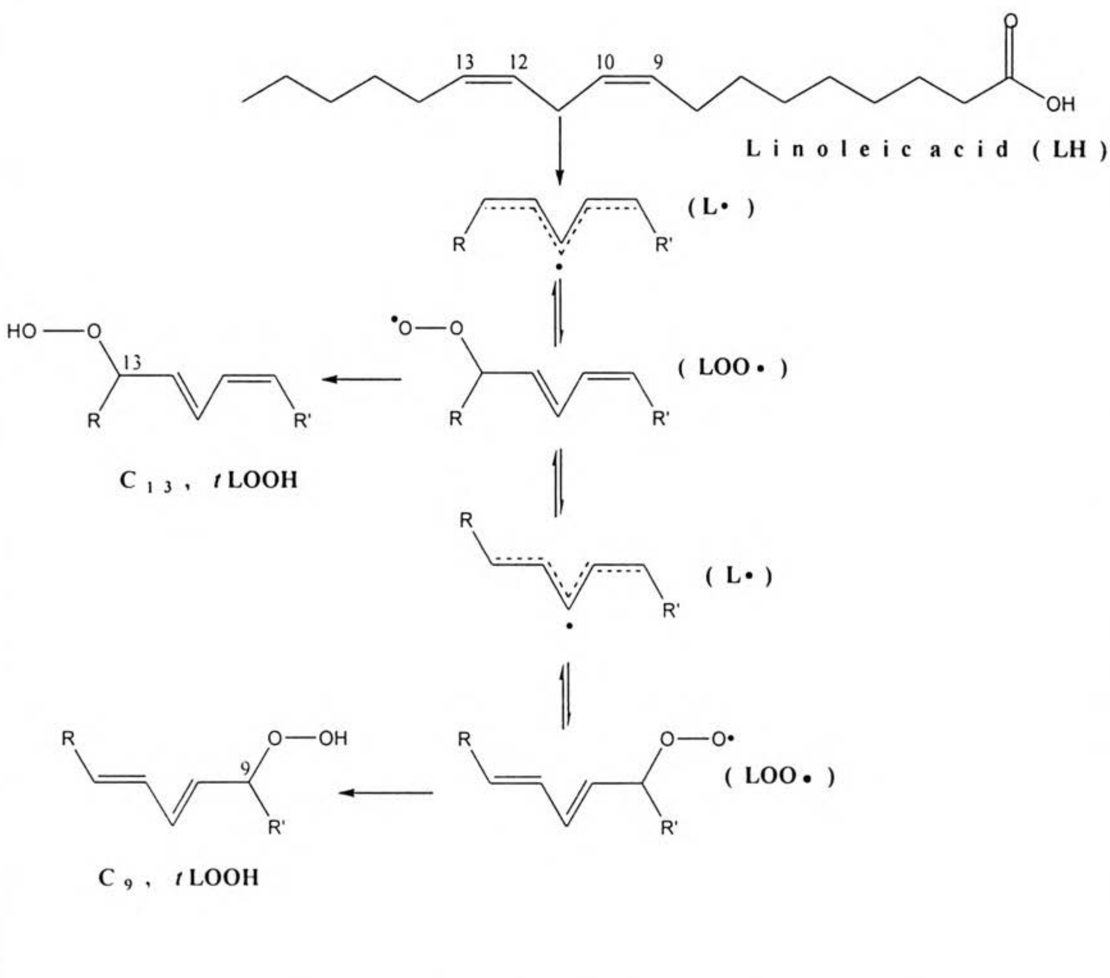


Scheme 1.2 The first stage of purine nucleotide degradation, which yield xanthine



Scheme 1.3 The second stage of purine degradation.

Lipid peroxidation is the role of oxidative injury in pathophysiological disorders. This oxidation is initiated by a reaction between ROS and unsaturated fatty acid side chains. The ROS abstracts a hydrogen atom, forming a fatty acid side chain peroxyl radical (LOO•). Scheme 1.4 illustrated the oxidation of linoleic acid (LH). This radical can cause destabilization and disintegration of the cell membrane, leading to liver injury, atherosclerosis, kidney damage, aging, and susceptibility to cancer (Rice-Evans and Burdon, 1993).



Scheme 1.4 Partial mechanism of oxidation of linoleic acid

From ferric thiocyanate (FTC) assay, lipid peroxidation is quantified by measuring hydroperoxide, which is the end product of this mechanism. Hydroperoxides are highly unstable. Therefore, hydroperoxides were readily reacted with ferrous ions to produced ferric ions. The resulting ferric ions are detected using thiocyanate ion as the chromogen. The reactions of this assay are shown in Scheme 1.5.

$$LOOH + Fe^{2+} \longrightarrow LOO\bullet + Fe^{3+}$$

$$LOO\bullet + Fe^{2+} + H^{+} \longrightarrow LOOH + Fe^{3+}$$

$$Fe^{3+} + 5SCN^{-} \longrightarrow Fe(SCN)_5^{2-}$$

λ_{max}: 500 nm.

Scheme 1.5 Reduction/oxidation reaction of ferric thiocyanate assay

The Zingeberaceae is an attractive family. This family is widely distributed throughout the tropics, principally in Southeast Asia. It is estimated that there are 1400 species in 47 genera. In the Southeast Asian region, various species of Zingeberaceae are utilized as spices, medicines, flavoring agents, and as the source of certain dye. In Thailand, the Zingeberaceae is a part of the herbaceous ground flora of the rainforest. They grow naturally in damp, shaded parts on the hill slopes, as scattered plants (Burkill, 1966).

From the primary screening test of 8 plants in Zingeberaceae, Waan Ma Lueang (*Curcuma* spp.) demonstrated the interesting result for DPPH radical scavenging activity. Hence, this plant was selected for further investigation of the chemical constituents and their antioxidant activity.

Waan Ma Lueang (*Curcuma* spp.) is in the list of Thai Folk Medicine. In general, this plant is known as "journey plant". Its rhizome can relieve ache and pain symptoms, including body, joint, tendon and bone (Petchsiri; in thai, 1978).

1.1 Botanical Aspects and Distribution

Waan Ma Lueang (*Curcuma* spp.) is in the Zingiberaceae. It is the same genus as *Curcuma longa* Linn. Botanists still do not previously identify its specie.

Waan Ma Lueang is a stemless rhizomatous herb, widely distributed in Thailand, especially Northeast and eastern regions. It has been well known as "Waan Ma Lueang" (Central), and "Waan Ma Thong" (Eastern).

Leaves: emerge directly from the underground stem with overlapping petioles 8-15 cm. long or more, light green, 30-40 cm, having thin ellipse-shaped or elongate lance-shaped blades.

Flower: light pink, yellow pollen, cylindrical inflorescene about 10-15 by 5-7 cm, appears with the leaves and develops in their centre.

Rhizome: small,long, horse-neck like, bright yellow, bitter taste

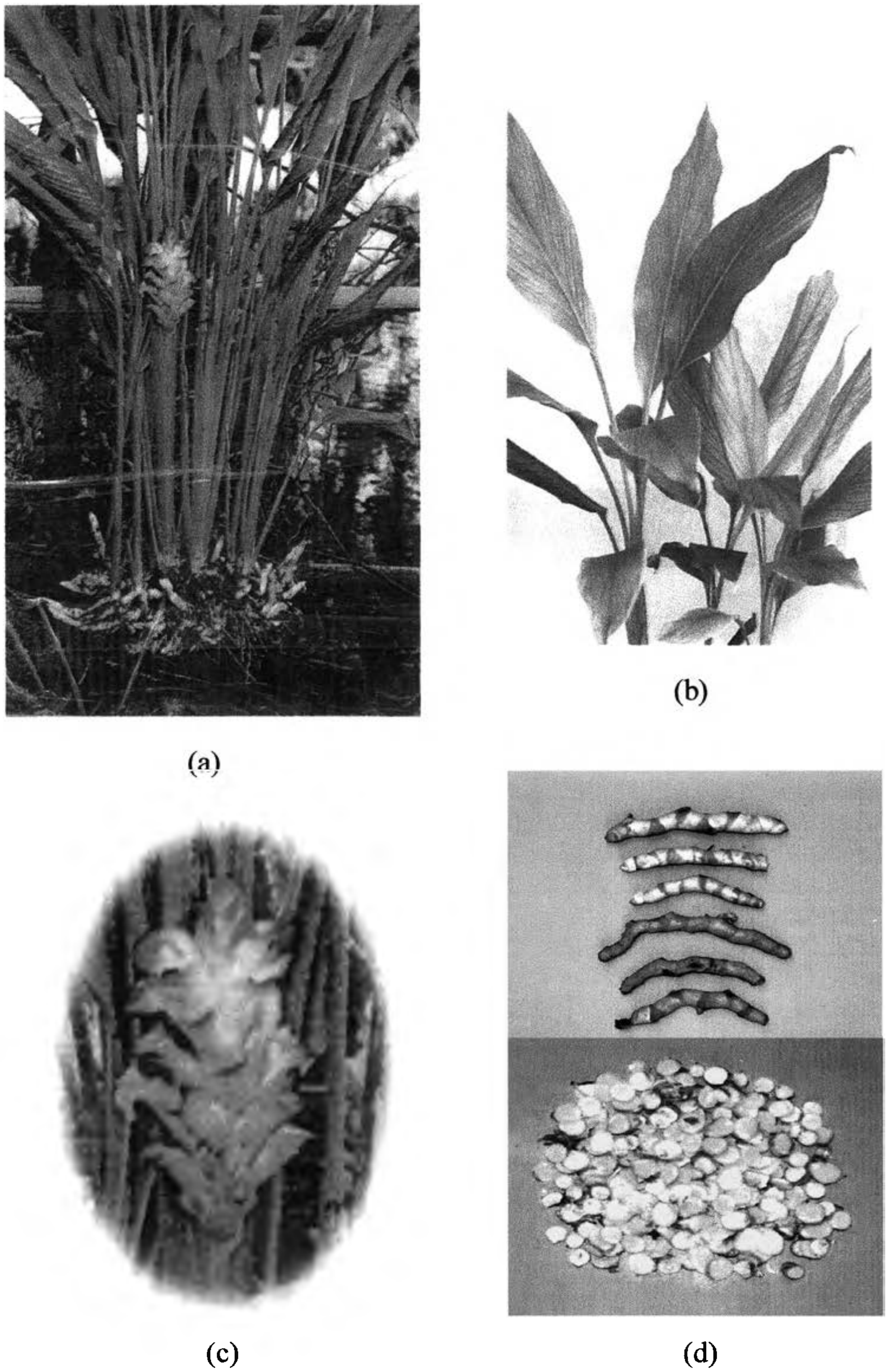
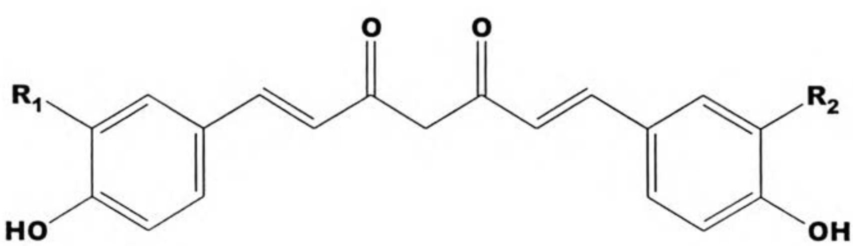


Figure 1.3 Whole plant (a), leaves (b) flower (c), and rhizomes (d) of Waan Ma Lueang (*Curcuma* spp.)

1.2 Chemical constituents study on *Curcuma* spp.

In the past, the researches on chemical constituents of the genus *Curcuma* were widely investigated. The numerous types of organic compounds have been isolated, mainly sesquiterpenoids. Table 1.1 reported the isolated compounds from the rhizomes of the genus *Curcuma*.

Until now, there is a little information about constituents of Waan Ma Lueang (*Curcuma* spp.). Only one literature was found in Thailand. It was noted that curcuminoids (**Figure 1.4**), which have the potent antioxidant activity, are the major components from the rhizomes of this plant (Plianbangchang and Manowat; in Thai, 1992). However, there is apparently no information in the literature on the antioxidant activity of some isolated compounds from Waan Ma Lueang. Therefore, searching for antioxidative components of Waan Ma Lueang (*Curcuma* spp.) was carried out in this research.



Curcumin: $R_1 = R_2 = OMe$
Demethoxycurcumin: $R_1 = OMe$, $R_2 = H$
Bisdemethoxycurcumin: $R_1 = R_2 = H$

Figure 1.4 Structures of curcuminoids

Table 1.1 Chemical constituents study on the genus *Curcuma*

Plant names	Isolated compounds	Type
Curcuma longa (Nakayama *et.al.*,1993) **(Figure 1.4)** **(Figure 1.5)**	curcumin demethoxycurcumin bisdemethoxycurcumin cyclocurcumin	Curcuminoids
Curcuma aromatica (Itokawa *et.al.*,1981) **(Figure 1.6)**	germacrone (4*S*, 5*S*)-germacrone-4,5-epoxide curdione neocurdione dehydrocurdione zedoarondiol procurcumenol	Sesquiterpenoids
Curcuma zedoaria (Hikino *et.al.*,1975) **(Figure 1.7)**	furanodienone isofuranodienone curzerenone	Furanosesquiterpenoids
(Shiobara *et.al.*,1985) **(Figure 1.8)**	curcumanolide A curcumanolide B	Spirolactones
Curcuma wenyujin (Gao *et.al.*,1989) **(Figure 1.9)**	wenjine curcumol curcumalactone	Sesquiterpenoids
Curcuma xanthorrhiza (Itokawa *et.al.*, 1985) (Uehara *et.al.*,1989) **(Figure 1.10)**	α-curcumene *ar*-turmerone β-atlantone xanthorrhizol bisacurone biscumol	Sesquiterpenoids
Curcuma comosa (Jurgens *et.al.*, 1994) **(Figure 1.11)**	1,7-diphenyl-3-acetoxy-(6*E*)-heptene	Diarylheptanoid
Curcuma heyneana (Firman *et.al.*, 1988) **(Figure 1.12)**	oxycurcumenol	Sesquiterpenoid

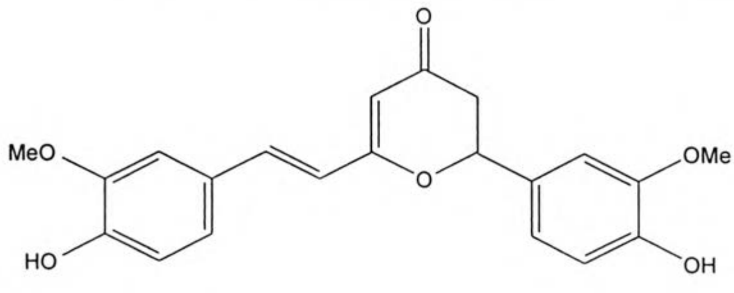


Figure 1.5 Cyclocurcumin from *Curcuma longa*

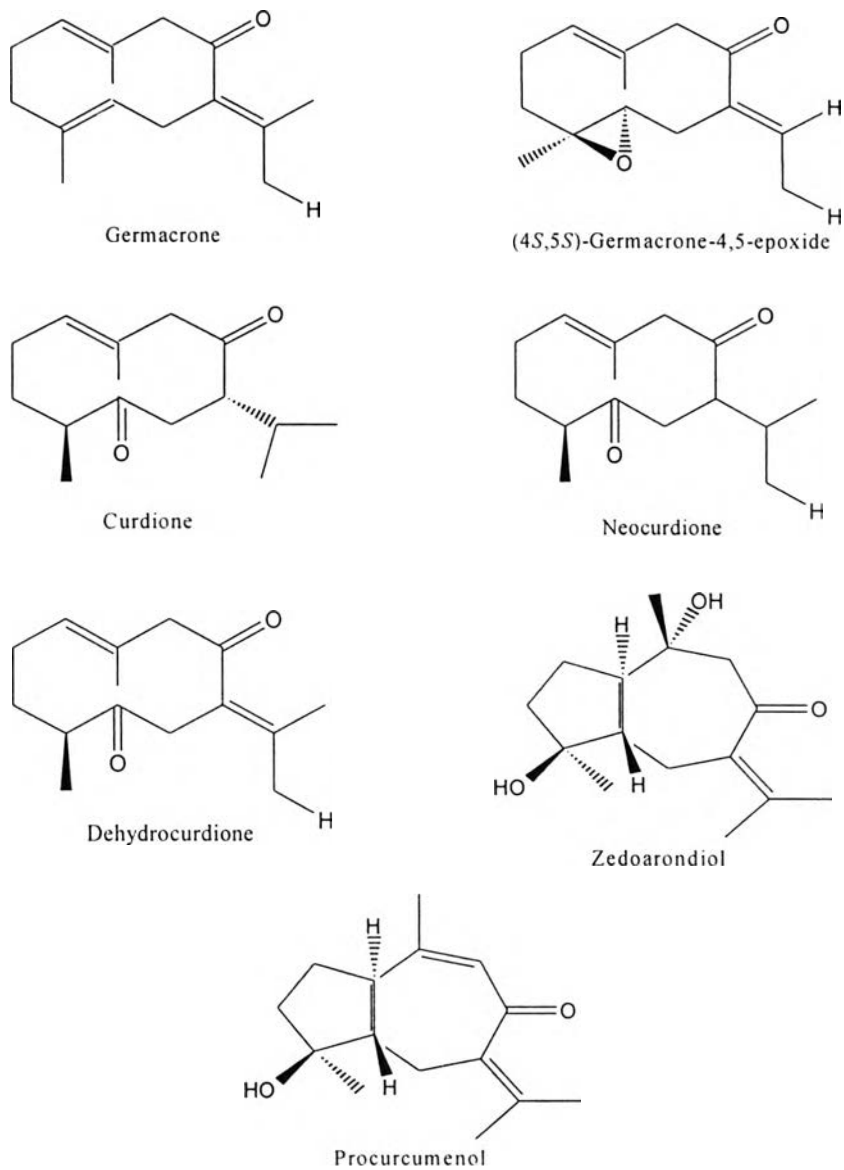
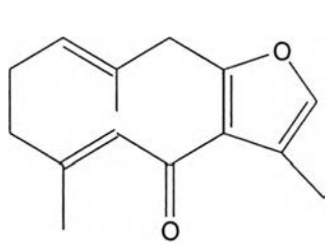
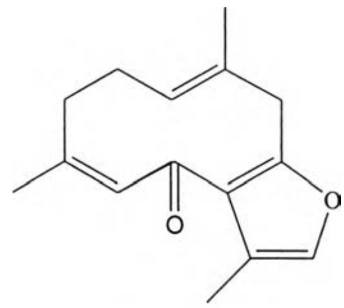


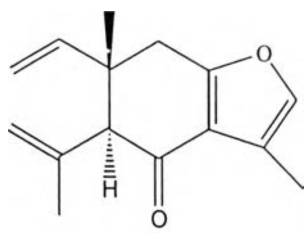
Figure 1.6 Sesquiterpenoids from *Curcuma aromatica*



Furanodienone

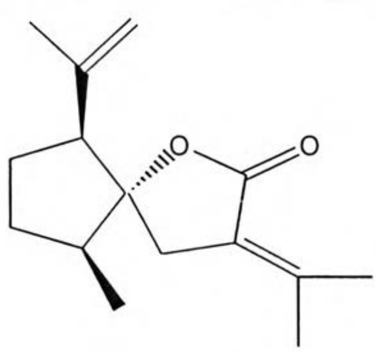


Isofuranodienone

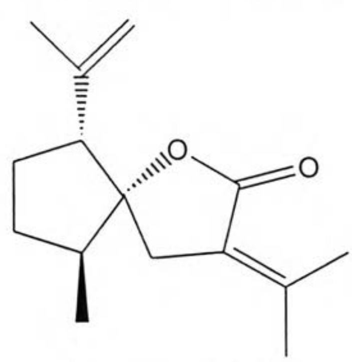


Curzerenone

Figure 1.7 Furanosesquiterpenoids from *Curcuma zedoaria*

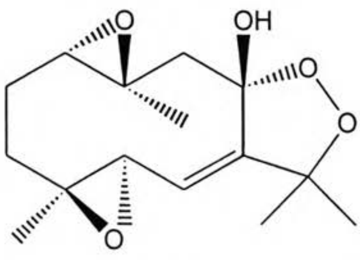


Curcumanolide A

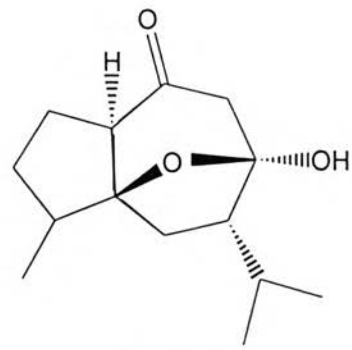


Curcumanolide B

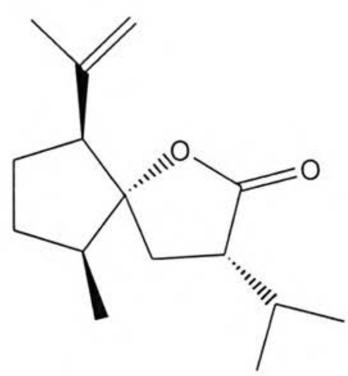
Figure 1.8 Spirolactones from *Curcuma zedoaria*



Wenjine

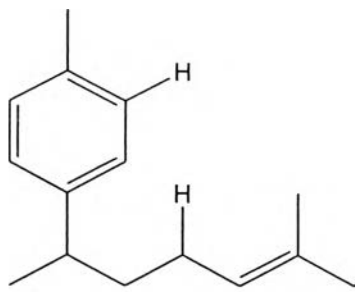


Curcumol

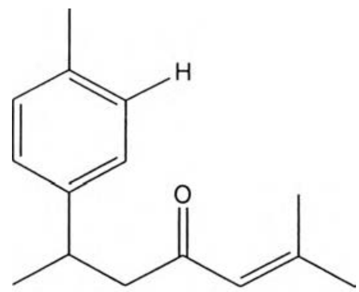


Curcumalactone

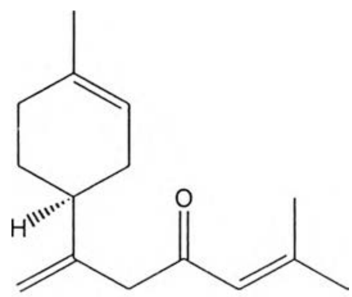
Figure 1.9 Sesquiterpenoids from *Curcuma wenyujin*



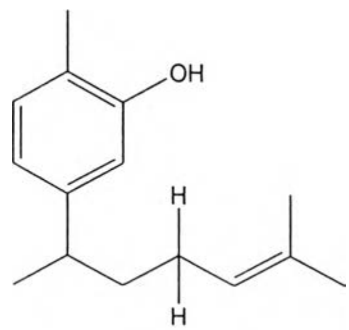
α-Curcumene



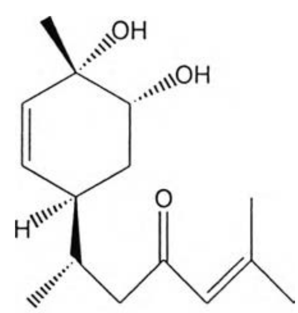
ar-Turmerone



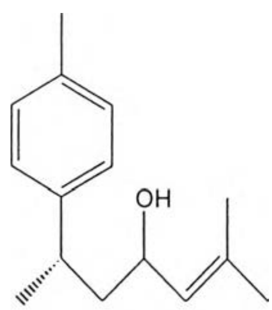
β-Atlantone



Xanthorrhizol

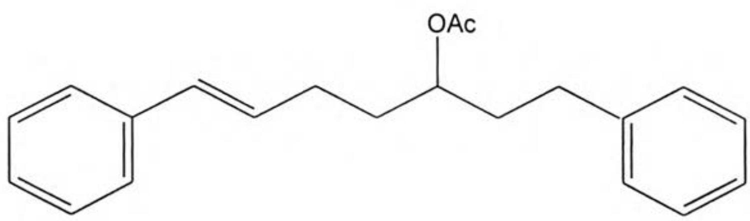


Bisacurone



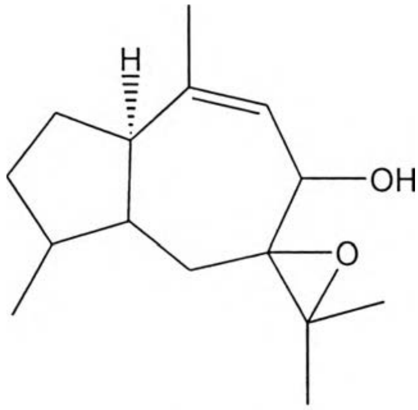
Biscumol

Figure 1.10 Sesquiterpenoids from *Curcuma xanthorrhiza*



1,7-Diphenyl-3-acetoxy-(6*E*)-heptene

Figure 1.11 Diarylheptanoid from *Curcuma comosa*



Oxycurcumenol

Figure 1.12 Sesquiterpenoid from *Curcuma heyneana*

1.3 The goal of this research

From the attractive result from primary screening test based on DPPH radical scavenging activity, Waan Ma Lueang (*Curcuma* spp.) was selected for further investigation of the chemical constituents and their antioxidant activity. The goal of this research can be tightly summarized as follows:

1. To extract and isolate the antioxidative constituents from the rhizomes of *Curcuma* spp. (Waan Ma Lueang)
2. To elucidate the structural formulae of the isolated compounds
3. To determine the antioxidant activity of the isolated compounds

Chromatographic and spectroscopic techniques will undertake separation and structure elucidation, respectively.