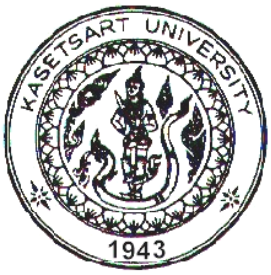

THESIS

EVALUATION OF ANTIOXIDANT POTENTIAL AND PHENOLIC CONSTITUENTS IN SELECTED THAI INDIGENOUS PLANT EXTRACTS

PITCHAON MAISUTHISAKUL

GRADUATE SCHOOL, KASETSART UNIVERSITY

2006

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Plants, which are sources of phytochemicals with strong antioxidant activity, have attracted a great deal of attention in recent years. This study was conducted to investigate the potential of Thai indigenous plants with respect to their phenolic content, total flavonoid content and antioxidant activity to provide new sources of natural antioxidants. Ethanolic extracts from various parts of twenty-six Thai plants were evaluated. Total phenolic compounds and flavonoid content were higher in seed extracts of berries used in wine production while the level in extracts obtained from herbs and vegetables were lower. Chewing plants which have an astringent taste gave significantly high total phenolic and flavonoid contents. Antiradical activity determined by the 2,2-diphenyl-1-picrylhydrazyl radical (DPPH$^{\bullet}$) scavenging method was highest in wine production seeds and chewing plants. *Cratoxylum formosum* Dyer. (Teaw), *Careya sphaerica* Roxb. (Kradonbok), and *Leaucaena glauca* Benth (Kratin) were selected to study phenolic radical scavenging components by high-performance liquid chromatography (HPLC) fitted with a diode array detector (DAD), and by electrospray ionization mass spectrometry (ESI-MS) due to their high antioxidant capacity, their safety for use as food additives, available sources, and cost. The main antioxidant component of Teaw leaf extract was chlorogenic acid, which was present at 60 % of the extract. The potent DPPH$^{\bullet}$ scavenging phenolic components of the Kradonbok and Kratin extracts correspond to the gallic acid derivatives and flavonoidal glycosides. Teaw extract showed higher antioxidant activity than α-tocopherol, Kradonbok extract and Kratin extract when assessed by the DPPH$^{\bullet}$ and 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS$^{\bullet+}$) assays. The antioxidant activity of extracts from Teaw, Kradonbok and Kratin leaves were also studied in stripped soybean oil and soybean oil-in-water emulsions. The Teaw extract was more effective than α-tocopherol, Kradonbok extract and Kratin extract in inhibiting lipid oxidation in bulk oil but was less effective than α-tocopherol in an oil-in-water emulsion in accordance with the polar paradox. There was only a small synergistic effect between α-tocopherol and ascorbyl palmitate in the emulsion and oil model systems. The Teaw extract was effective in inhibiting lipid oxidation when used alone in a stored rice snack assessed by peroxide value (PV), thiobarbituric acid reactive substances (TBARS) and sensory analysis. The sensory odor attributes of rice snack were related to TBARS more than PV values. In addition, Teaw extract was more effective than α-tocopherol due to metal ion presenting in snack, which made α-tocopherol less effective as an antioxidant. Consequently, Teaw leaf extract is a promising source of a natural food antioxidant.

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Student's signature

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28 / March / 2006

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Pitchaon Maisuthisakul

March 2006

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EVALUATION OF ANTIOXIDANT POTENTIAL AND PHENOLIC CONSTITUENTS IN SELECTED THAI INDIGENOUS PLANT EXTRACTS

INTRODUCTION

The oxidative deterioration of fats and oils in foods is responsible for rancid odours and flavours, discoloration, with a consequent decrease in nutritional quality and safety caused by the formation of secondary, potentially toxic compounds (Dillard and German, 2000). The addition of antioxidants, including polyphenolic compounds, vitamin E, and vitamin C, in food products is required to preserve flavour and colour and to avoid vitamin destruction. Among the synthetic types, the most frequently used to preserve food are butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and tert-butyl hydroquinone (TBHQ) due to their low cost and high stability and effectiveness. However, use of such compounds has been related to health risks resulting in strict regulation of their use in foods (Hettiarachchy *et al*., 1996). BHA has been shown to cause lesion formation in the rat forestomach. Moreover, several studies have shown that BHT may cause internal and external haemorrhaging at high doses that is severe enough to cause death in some strains of mice and guinea pig (McCarthy *et al*., 2001). Besides, the higher manufacturing costs and lower efficiency of natural commercial antioxidants such as tocopherols as well as increased consumer consciousness of food safety create a need for identifying alternative, natural and probably safer sources of food antioxidants (William *et al*., 1999).

The replacement of synthetic antioxidants by natural ones may have benefits due to health concerns and functionality such as solubility in oil, water and emulsions in food systems. However, some of them such as those from spices and herbs (oregano, thyme, dittany, marjoram, lavender, rosemary) have limited applications in spite of their high antioxidant activity, as they impart a characteristic herb flavour to the food, and deodorization steps are required

(Madsen and Bertelsen, 1995). Antioxidants have thus become a topic of increasing interest. A literature search revealed that the number of publications on antioxidants had nearly quadrupled in the past decade (565: 1994; 1820: 2004 (Sciencedirect database, 2005).

Recently, the food industry has been using natural antioxidants from plant materials. The use of naturally occuring antioxidants extracted from plants has been widely studied (Wong *et al*., 1995). Plant materials contain many compounds having antioxidant activity, most of them being phenolic compounds. These plant phenolics give antioxidant properties, which are proposed for protection against lipid oxidation (Hagerman *et al*., 1998). Some components of extracts isolated from fruits and vegetables have been proven in model food systems, to be as effective as synthetic antioxidants (Al Saikhan *et al*., 1995). Due to the complexity of the composition of foods, separating each antioxidant compound and studying it individually is costly and insufficient, since it ignores the possible synergistic interactions among the antioxidant compounds in the mixture (Huang *et al*., 2005). Multicomponent antioxidant systems can inhibit oxidation by many different potential mechanisms and at different phases of oxidation (Decker, 1998).

In Thailand, there are many possible plants, which have potential as sources of antioxidants for application in food such as Kradonbok (*Careya sphaerica* Roxb.), Phak Whan Ban (*Sauropus androgenus* Merr.), Teaw (*Cratoxylum formosum* Dyer.), Ceylon spinash (*Basella alba* Linn.) and Tubtaw (*Mimulus orbicularis* Benth) (Trakoontivakorn *et al*., 2001). However there is little literature reporting studies of the antioxidant activity of local plants in Thailand, and the characterization of phenolic compounds as well as their application in food is very scarce. Thai industries mostly import antioxidants for use in their companies at high cost. Rational and sustainable use of plant antioxidants as a source of high value-added food antioxidants could represent a potential source of income for Thailand. Therefore, extensive research on potential Thai local plant sources, knowledge of phenolic constituents and optimum use in food, are still required.

The present study is to evaluate antioxidant potential and phenolic compounds of indigenous plants in Thailand and to compare the antioxidant activity with commercial antioxidants in oil and emulsion models. The synergistic effects of plant antioxidants will be studied in oil and emulsion systems. Finally, the study will include the investigation of antioxidant effectiveness under accelerated oxidation condition for application to food products for controlling lipid oxidation in food systems.

The purpose of this study is as follows:

1. To investigate the potential of Thai indigenous plants with respect to their phenolic content, total flavonoid content and antioxidant activity to provide new sources of natural antioxidants.
2. To determine phenolic radical scavenging components in selected plants by high – performance liquid chromatography (HPLC).
3. To compare the antioxidant activity with commercial antioxidants in oil and emulsion model systems.
4. To study the synergistic effect of the plant antioxidants with ascorbyl palmitate and citric acid in oil and emulsion systems.
5. To investigate the potential use of the selected plant extract as antioxidants in rice snack.

LITERATURE REVIEWS

The development of rancidity in edible oils is a serious problem in some sectors of the food industry because of increasing emphasis on the use of polyunsaturated vegetable and fish oils, discontinuing the use of synthetic antioxidants and fortification of cereal food with iron. Oxidation of lipids not only produces rancid odors and flavors, but can decrease the nutritional quality and safety by the formation of secondary products in foods. Normally, rancidity consists of hydrolytic and oxidative rancidity. Hydrolytic rancidity is an enzyme-catalyzed reaction, leading to formation of free fatty acids (Narwar, 1996). Oxidative rancidity is mainly caused by lipid oxidation. Therefore, attention will be focused on oxidative rancidity, its mechanisms, prevention and measurement, in lipids and lipid containing food products.

1. Lipid Oxidation

Lipids occur in almost all foodstuffs, and most of them (more than 90 %) are in the form of triacylglycerols, which are esters of fatty acids and glycerol. Two major components involved in lipid oxidation are unsaturated fatty acids and oxygen.

1.1 Basic Chemistry of Lipid Oxidation

The two major components involved in lipid oxidation are unsaturated fatty acids and oxygen. In this process, oxygen from the atmosphere is added to certain fatty acids, creating unstable intermediates that eventually break down to form unpleasant flavor and aroma compounds. Although enzymatic and photochemical oxidation may play a role, the most common and important process by which unsaturated fatty acids and oxygen interact is a free radical mechanism characterized by three main phases:

Initiation:	$In\cdot + RH \rightarrow InH + R\cdot$	(1)
Propagation:	$R\cdot + O_2 \rightarrow ROO\cdot$	(2)
	$ROO\cdot + RH \rightarrow R\cdot + ROOH$	(3)
Termination:	$2ROO\cdot \rightarrow O_2 + ROOR$	(4)
	$ROO\cdot + R\cdot \rightarrow ROOR$	(5)

Initiation occurs as hydrogen is abstracted from unsaturated fatty acids, resulting in a lipid free radical, which in turn reacts with molecular oxygen to form a lipid peroxyl radical. The propagation phase of oxidation is fostered by lipid-lipid interactions, whereby the lipid peroxyl radical abstracts hydrogen from an adjacent molecule, resulting in a lipid hydroperoxide and a new lipid free radical. Interaction of this type may proceed 10 to 100 times before two free radicals combine to terminate the process. Further magnification of lipid oxidation, however, may occur through branching reactions (also know as secondary initiation): $Fe^{2+} + ROOH \rightarrow RO\cdot + OH\cdot$. The radicals produced will then proceed to abstract hydrogens from unsaturated fatty acids.

By themselves, lipid hydroperoxides are not considered harmful to food quality; however, they are further degraded into compounds that are responsible for off-flavours. Most of these compounds are responsible for oxidised flavours. The off-flavour properties of these compounds depend on the interactions, concentrations, threshold values, and food systems. Therefore, autoxidation of lipid molecules can be summarised as shown in Figure 1.

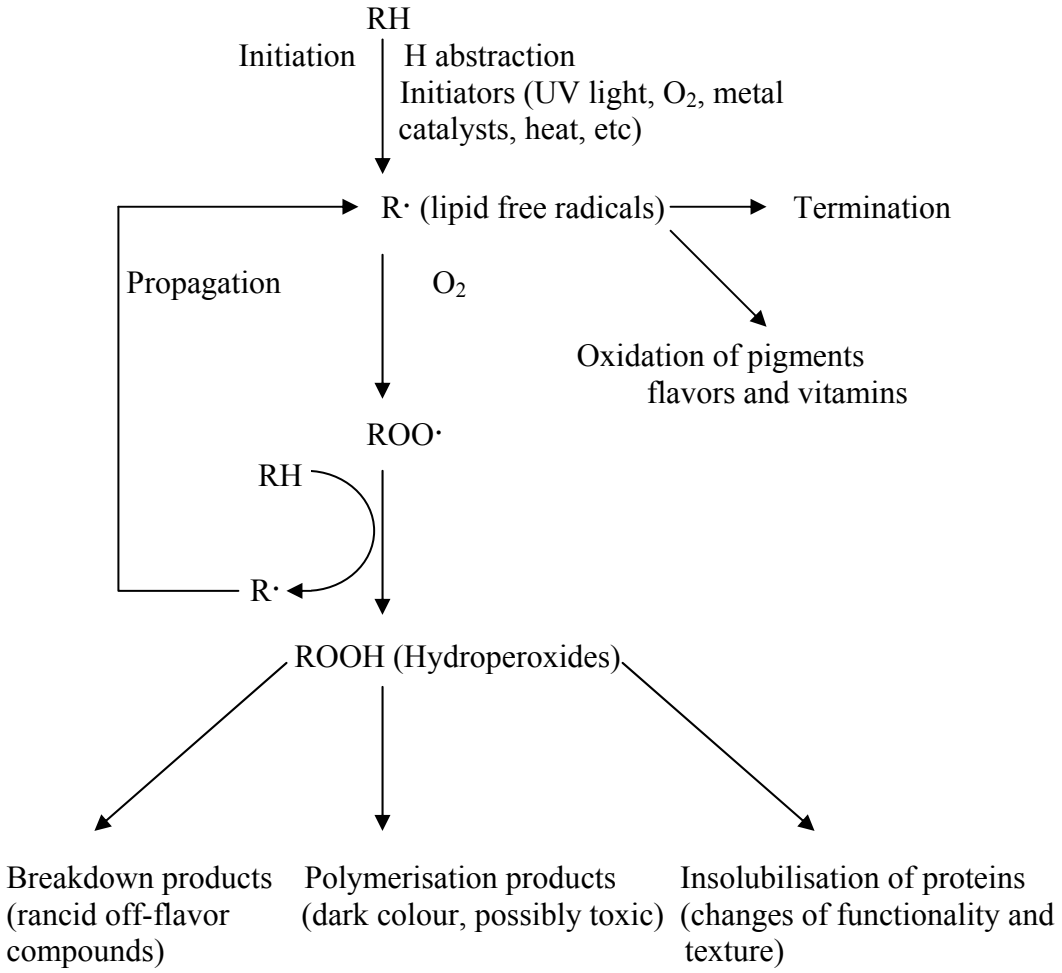


Figure 1 Autoxidation of polyunsaturated fatty acids of lipids and their consequences.

Source: Turkan (2000)

As lipid peroxides are very unstable, they break down to alkoxy free radicals which decompose via cleavage of the bond to the carbon atom bearing the oxygen atom to form a number secondary oxidation products such as alcohols, aldehydes, ketones etc. (Abdala and Roozen, 1999). The most likely decomposition pathway of hydroperoxide is the cleavage between the oxygen and oxygen of the R-O-O-H, that is, R-O-O-H → RO· + ·O-H instead of R-O-O-H → R-O-O· + ·H. The activation energy of the cleavage of -O-O- was 44 kcal/mol, compared to the cleavage between the oxygen and hydrogen of R-O-O-H, which

has high activation energy (≈90 kcal/mol). Therefore, the hydroperoxide groups are cleaved by homolysis to yield an alkoxy and a hydroxy radical.

The predominant volatiles for linoleate are hexanal, 2, 4-decadienal and heptanal, whilst the hydrocarbon pentane is most abundant. Methyl linolenate undergoes oxidation of its 9, 12 diene system to produce the 9- and 13-hydroperoxides and oxidation of its 12, 15 diene system to produce the 12- and 16-hydroperoxides. The marker volatiles produced from linolenate are unsaturated aldehydes, 2, 4, 7- decatrienal, 2, 4-heptadienal, propanal/propenal and 2-butenal together with hydrocarbons, ethane/ethanol.

1.2 Factors Affecting Lipid Oxidation

The extent to which oxidation of fatty acids and their esters occur in food depends on the chemical structure of the fatty acids and minor constituents present in the oxidizing system, e.g. on the substrate factors, as well as on the conditions of food processing and storage. Oxygen pressure, surface area with oxygen, temperature and irradiation are included in the physical factors. (Yanishlieva-Maslarova, 2001)

Basic to any discussion of oxidation is the concentration of oxygen in the affected system, which depends on the oxygen pressure. If the surface area in contact with the oxidizing substrate is ‘small’, even at sufficiently high oxygen pressure the diffusion of oxygen alone will be significant in starting oxidation.

Increasing the temperature is undesirable because of its accelerating effect on lipid oxidation and in fact on every stage of the process. That is why storage at low temperatures may be recommended. Nevertheless, there is a danger that when water freezes, all water present becomes frozen so that the food material becomes dry under these conditions, the protective layer of hydrated proteins is damaged and the lipid fraction leaks from the natural emulsions or liposomes, so that lipids become exposed to oxygen in the air (Pokorny, 2001).

Irradiation increases formation of free radicals. The shorter the wavelength of the radiation, the higher the energy and the more harmful the irradiation effect. Numerous microcomponents may influence the rate and products of lipid oxidation. The primary oxidation products, the hydroperoxides, are chain initiators and their presence in the lipid system causes an acceleration of the process.

Free fatty acids also exert a pro-oxidative effect. This effect is due to a complex formation between hydroperoxides and carbonyl groups through a hydrogen bond which results in an accelerated decomposition of hydroperoxides into free radicals (Miyashita and Takagi, 1986). The chemical structure of the lipid substrate and the oxidation conditions also affect lipid oxidation.

During oxidation, the hydrogen that is most weakly bond will be the one abstracted from an unsaturated fatty acid. Allylic hydrogens (those attached to a carbon atom adjacent to a double bond) are easier to abstract than vinylic hydrogens (those attached to carbons involved in a double bond), and hydrogens on doubly allylic methylene carbons (those attached to a carbon atom located between double bonds) are even more labile. Thus, in oleic acid the weakest bonds are the allylic positions C8 and C11. The most reactive site in linoleic acid is the doubly allylic position at C11, while linolenic acid has two reactive double allylic sites, at C11 and C14. These influence the rate of their oxidation (Love, 1992).

Transition metal ions catalyse hydroperoxide decomposition producing free radicals which initiate further reaction chains. Although metals are not soluble in oil, hydroperoxide complexation of metals can occur and is responsible for a major proportion of metal contamination of refined oils (Hu *et al*., 2004). Transition metals such as manganese, ferric, aluminum, which possess two or more valency states with a suitable oxidation-reduction potential act as pro-oxidants. Therefore once a small amount of hydroperoxide is formed, the transition metals can catalyse decomposition of pre-formed lipid hydroperoxides due to their unpaired electron (s) in 3d and 4s orbitals. The metal can readily lose or gain an

electron so they serve as excellent catalysts for the lipid hydroperoxide decomposition reactions (Hu *et al*., 2004).

The relationship between rate of lipid oxidation and water is complex. The amount of water, the water activity and the state of water in food along with other factors must be considered (Maltini *et al*., 2003). The non-lipidic components present in foods, such as proteins, sugars and minerals, mostly in the presence of water, may also have a strong influence on the rate and mechanism of lipid oxidation (Hu *et al*., 2004).

1.3 Inhibiting Oxidation

Optimum oxidative stability can be achieved by minimizing exposure of lipids and lipid-containing food products to air, light and higher temperatures during processing and storage. Theoretically, the most elegant way of preserving fatty foods from oxidative spoilage is to remove all oxygen from the food during manufacture and from the packaging container.

Besides, some chemicals can be used to protect food against lipid oxidation. They are called inhibitors or antioxidants. Antioxidants not only extend product shelf life, but also reduce raw material waste, reduce nutritional losses, and widen the range of fats that can be used in specific products (Reische *et al*., 1997).

1.4 Analytical Methods for Determining Lipid Oxidation

In common with all other olefinic compounds, oils and fats react with oxygen. The whole process is complex but it is sufficient to recognize at this point that the primary products of oxidation-even when produced by different mechanisms-are olefinic hydroperoxides which break down to a variety of compounds of lower molecular weight (secondary oxidation products). These are mainly aldehydes largely responsible for the development of the undesirable taste

and flavor which characterizes rancid fat. At higher temperatures these products are degraded further to short-chain acids (tertiary oxidation products). These changes are summarized in Figure 2.

The most important mechanism of oxidation is autoxidation. This is characterized by an induction period during which oxidation is relatively slow. At the end of the induction period oxidation is quicker and the oil/fat then deteriorates quickly. The length of this induction period is important in estimating the shelf life of oil and fats and of fatty foods. Because the induction period may be very lengthy at ambient temperature, accelerated tests have been developed in which the oil is held at elevated temperatures and the changes monitored. There are methods for measuring all these parameters: the primary, secondary, and tertiary products of oxidation and the induction period (Figure 2).

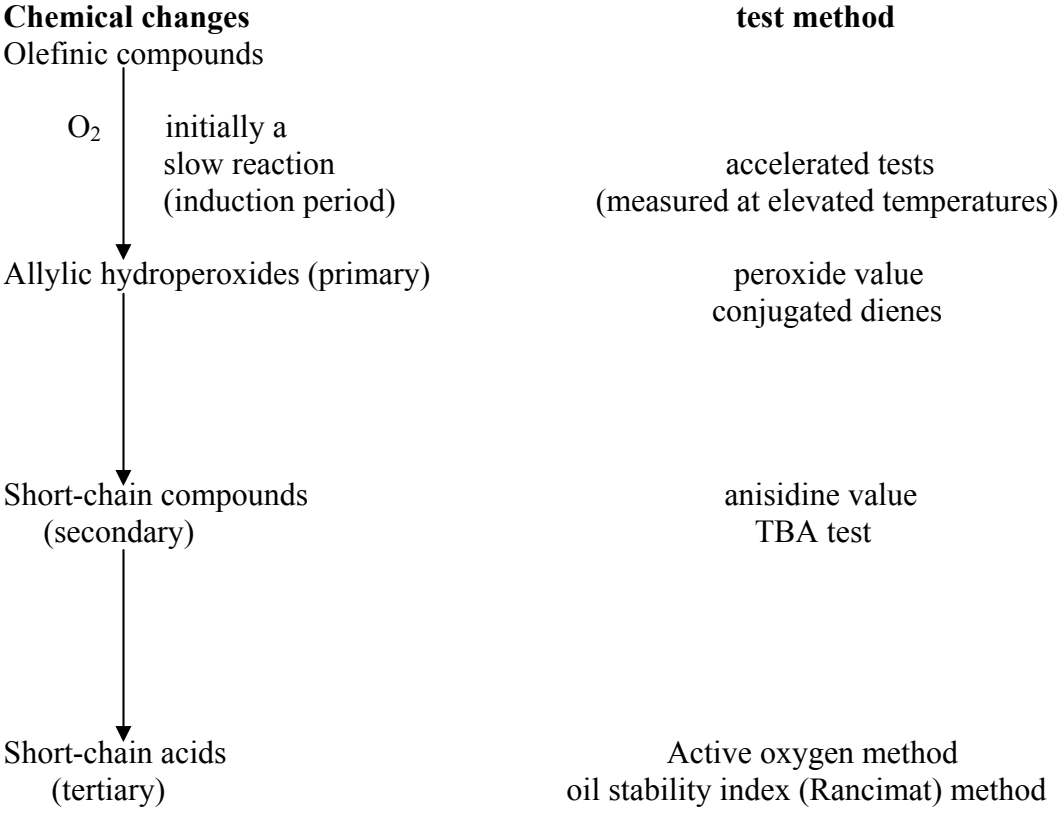


Figure 2 Lipid oxidation: chemical changes and test method.

Source: Gunstone (1996)

Peroxide value: The most commonly used method of assessing oxidative status is the (hydro) peroxide value. This is based on the fact that hydroperoxides react with potassium iodide to liberate iodine which can be measured by its reaction with thiosulphate, or electrochemically.

Chemical reactions involved in PV determination are given as follows:

$$2ROOH + 2H^{+} + 2KI \longrightarrow I_2 + 2\,ROH + H_2O + K_2O \qquad (6)$$

$$I_2 + 2Na_2S_2O_3 \longrightarrow Na_2S_4O_6 + 2\,NaI \qquad (7)$$

Results may also be affected by the structure and reactivity of peroxides as by reaction temperature and time. The iodometric method for the determination of PV can be applied to all normal fats and oils; it is highly empirical, however, and any variation in procedure may affect the results. This method also fails to adequately measure low PV because of difficulties encountered in determining the titration end point. In an attempt to increase the sensitivity for determination of low PV, therefore, this method has been modified by replacing the titration step with an electrochemical technique in which the liberated iodine is reduced at a platinum electrode maintained at a constant potential.

Conjugated dienes: Oxidation of polyunsaturated fatty acids is accompanied by an increase in the ultraviolet absorption of the product. Lipids containing methylene-interrupted dienes or polyenes show a shift in their double-bond position during oxidation that is due to isomerization and conjugated bond formation. The resulting conjugated dienes exhibit intense absorption at 234 nm; similarly conjugated trienes absorb at 268 nm. Shahidi *et al.* (1994) and Wanasundara *et al.* (1995) have found that conjugated dienes and PV of edible oils correlate well during their oxidation. These authors concluded that the conjugated diene method method might be used as an index of stability of lipids in place of, or in addition to, PV. The presence of double in the conjugated structure of carotenoids, however, may cause carotenoid-containing oils to give high absorbance values at 234-236 nm. The conjugated diene method is faster than PV

determination, is much simpler, does not depend on chemical reactions or color development, and requires a smaller sample size. However, the presence of compounds absorbing in the region of conjugated diene formation may interfere with such determinations.

Aldehyde products as secondary products of oxidation can be measured through determination of the anisidine value or by the thiobarbituric acid test.

Anisidine value; Anisidine value determines the amount of aldehydes (principally 2-alkenals and 2, 4-alkadienals) in animal fats and vegetable oils. Aldehydes in oil react with the anisidine reagent under acidic conditions. The reaction of anisidine with aldehydes affords yellowish products, as show in Figure 3. List *et al*. (1974) have reported a highly significant correlation between anisidine values and flavor acceptability scores of salad oils processed from undamaged soybeans.

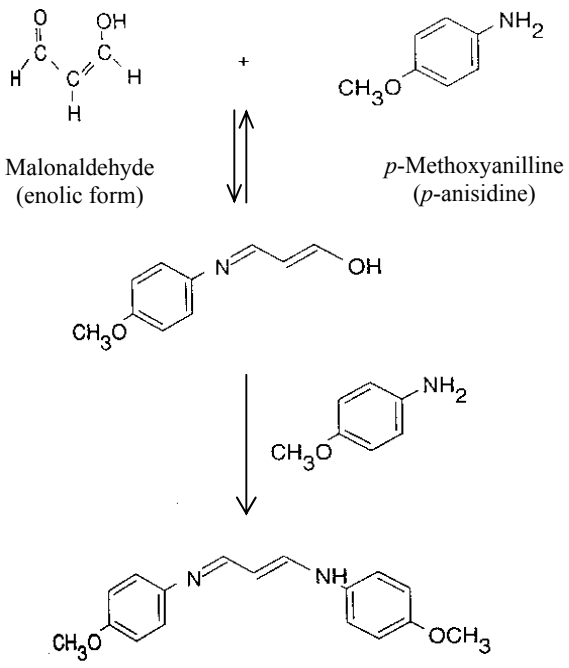


Figure 3 Possible reactions between anisidine reagent and malonaldehyde (MA).
Source: Shahidi and Wanasundara (1998)

TBA test; One of the oldest and most frequently used tests for assessing lipid oxidation in foods and other biological systems is the TBA test. The extent of lipid oxidation, reported as the TBA value, is expressed as milligrams of malonaldehyde (MA) equivalents per kilogram of sample or as micromoles of MA equivalents per gram of sample. Malonaldehyde, a relative minor product of oxidation of polyunsaturated fatty acids, reacts with the TBA reagent to produce a pink complex with absorption maximum at 530 to 532 nm. The adduct ion is formed by condensation of 2 molecules of TBA with 1 molecule of MA (Figure 4). Other products of lipid oxidation such as 2-alkenals and 2, 4-alkadienals also react with the TBA reagent. However, the exact mechanism of their reaction with the TBA reagent is not well understood.

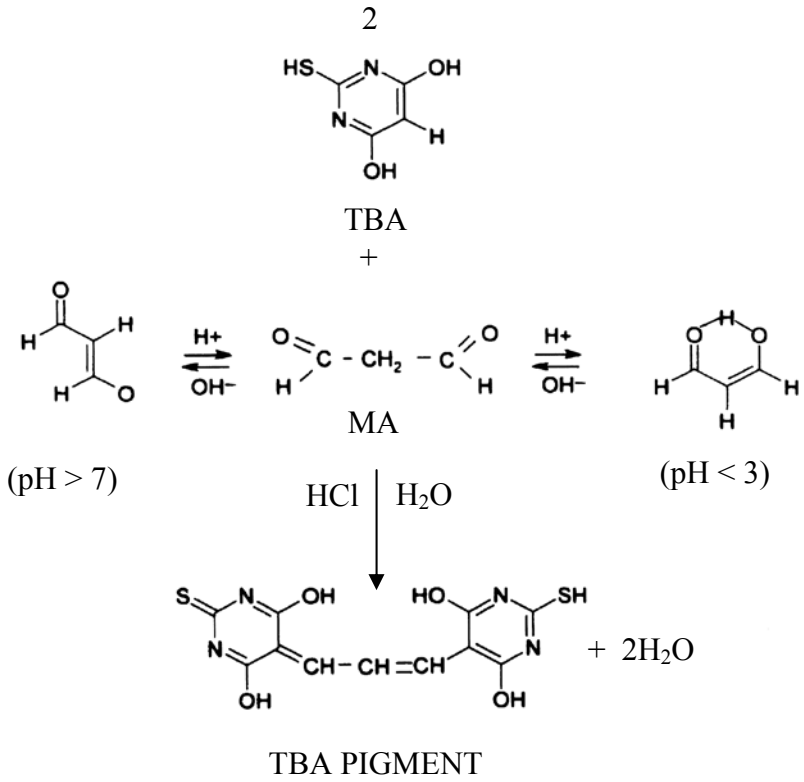


Figure 4 Reaction of 2-thiobarbituric acid (TBA) and malonaldehyde (MA).
Source: Shahidi and Wanasundara (1998)

There are several procedures for the determination of TBA value. The TBA test may be performed directly on the sample, its extracts or distillate. In case

of the distillation method, volatile substances are distilled off with steam. The distillate is then allowed to react with the TBA reagent in an aqueous medium. The advantage of the distillation method is the absence of interfering substances. In the extraction method, TBA reactive substances (TBARS) are extracted from material into an aqueous medium (e.g., aqueous trichloroacetic acid) prior to color development with the TBA reagent. The main disadvantages of both these methods are long assay time and possibility of artifact formation. In the direct assay method, lipid sample (oil) reacts with the TBA reagent, and the absorbance of the coloured complex so prepared is recorded. The direct assay method is simple, and sample preparation time is shorter.

The chemical complexity of food and biological samples imposes certain limitations on the use of the TBA test for evaluating their oxidative state. Dugan (1955) has reported that sucrose and some compounds in wood smoke react with the TBA reagent to give a red color that interferes with the TBA test. Modification of the original TBA test have been reported by Macuse and Johansson (1973); Shahidi *et al.* (1987); Thomas and Fumes (1987); Schmedes and Holmer (1989). However, it has been suggested that TBARS values provide an excellent means of evaluating the relative oxidative state of a system as affected by storage conditions or process variables. Nonetheless, it is preferable to verify the results by quantifying the extent of lipid oxidation by means of a complementary analytical procedure. Several attempts have been made to establish a relationship between TBA values and the development of undesirable flavors in fats and oils. It has been show that flavor threshold values correlate well with the TBA results of vegetable oils such as those of soybean, cottonseed, corn, safflower (Gray, 1998).

The Rancimat apparatus and the Oxidative Stability Instrument provide a way of measuring short-chain acids (C_1-C_3) produced during oxidation at temperatures up to 100-120°C. There are several procedures for measuring the time for a fat held at an elevated temperature (up to 100°C) to reach a peroxide

value that indicates rancidity. Results of accelerated tests (Schaal, Active oxygen) are assumed to give indication of the induction period and hence of shelf life under normal storage conditions. Results obtained at these higher temperatures must always be interpreted with care since it is known that the mechanism of the oxidation reaction changes with temperature.

2. Antioxidants

The broader definition of antioxidant (Halliwell, 2002) is any substance that when present at low concentrations compared with those of an oxidizable substrate significantly delays or prevents oxidation of that substrate.

2.1 Antioxidant Mechanisms

The basic mechanisms by which antioxidants influence oxidative reactions include: inactivation of free radicals, control of oxidation catalysts, inactivation of oxidation intermediates, and interactions between antioxidants and secondary lipid oxidation products or other antioxidants.

2.1.1 Inactivation of Free Radicals

Antioxidants can slow lipid oxidation by inactivation or scavenging free radicals, thus inhibiting initiation and propagation reactions. Free radical scavengers (FRS) or chain breaking antioxidants are capable of accepting or donating an electron to a radical from an oxidizing lipid species such as peroxyl (ROO·) and alkoxyl (RO·) radicals by the following reactions.

$$ROO\cdot \text{ or } RO\cdot + FRS \longrightarrow ROOH + FRS\cdot \qquad (8)$$

Any compound that has a reduction potential lower than the reduction potential of a free radical (or oxidized species) is donating hydrogen to that free

radical unless the reaction is kinetically unfeasible. For example, FRS including α-tocopherol ($E°'$ = 500 mV), catechol ($E°'$ = 500 mV), and ascorbate ($E°'$ = 282 mV) all have reduction potentials below peroxyl radicals ($E°'$ = 1000 mV) and are therefore capable of donating a hydrogen atom to the peroxyl radicals to form peroxide. Standard reduction potentials can also be used to predict the ease with which a compound can donate its hydrogen to a radical.

The efficiency of the FRS is also dependent on the energy of the resulting free radical scavenger radical (FRS·). If the FRS· is a low energy radical, then the likelihood that the FRS· will catalyze the oxidation of other molecules decreases. The most efficient FRS has low energy radicals as a result of resonance delocalization (Figure 5).

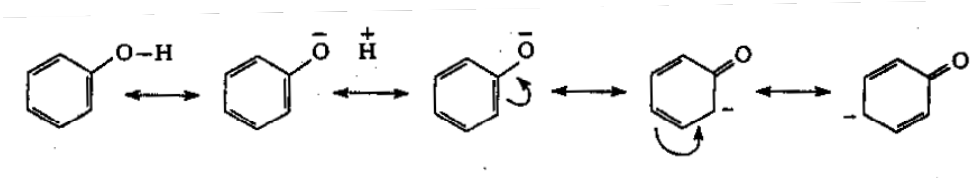


Figure 5 Resonance stabilization of a free radical by a phenolic.
Sourse: Akoh and Min (1998)

Phenolic compounds possess many of the properties of an efficient FRS. Hydrogen donation generally occurs through the hydroxyl group, and the radical subsequently formed is stabilized by resonance delocalization throughout the phenolic ring structure. The effectiveness of phenolic FRS can be increased by substituents. Alkyl groups in the ortho and para positions enhance the reactivity of the hydroxyl hydrogen toward lipid radicals; bulky groups at the ortho position increase the stabilisation of the phenoxy radical through steric hindrance and molecules with additional hydroxyl groups may stabilise the radical by an intramolecular hydrogen bond (Shahidi and Wanasundara, 1992).

2.1.2 Control of Lipid Oxidation Catalysts

Lipid oxidation rates in foods often depend on catalyst concentrations and activity, control of lipid oxidation catalysts can therefore be a very important factor in controlling oxidative rancidity. Both endogenous and added antioxidants help control the activity of transition metals, singlet oxygen, and enzymes.

a. Control of Pro-oxidant Metals

Transition metals accelerate lipid oxidation reactions by hydrogen abstraction and peroxide decomposition, resulting in the formation of free radicals. The activity of prooxidative metals is influenced by chelators or sequestering agents. Transition metals such as iron exhibit low solubility at pH values near neutrality (Dunford, 1987). Therefore, in food systems, transition metals often exist chelated to other compounds. Many compounds will form complexes with metals, resulting in changes in catalytic activity. Some metal chelators increase oxidative reactions by increasing metal solubility and/or altering the redox potential (Mahoney and Graf, 1986). Chelators that exhibit antioxidative properties inhibit metal-catalyzed reactions by one or more of the following properties: prevention of metal redox cycling, occupation of all metal coordination sites, formation of insoluble metal complexes, and steric hindrance of interactions between metals and lipids or oxidation intermediates (e.g., peroxides).

The most common metal chelators used in foods contain multiple carboxylic acid (e.g., EDTA and citric acid) or phosphate (e.g., polyphosphates and phytate) groups. Chelators are typically water soluble but some will exhibit solubility in lipids (e.g., citric acid), thus allowing the chelator to inactivate metals in the lipid phase. Chelator activity depends on pH, since the chelator must be ionized to be active.

b. Control of Singlet Oxygen

Singlet oxygen is an excited state of oxygen in which two electrons in the outer shell of orbitals have opposite spin directions. Initiation of lipid oxidation by singlet oxygen is due to its electrophilic nature, which leads to the formation of lipid peroxides from unsaturated fatty acids (Abdel-Shafi and Worrall, 2005).

Singlet oxygen can be inactivated by both chemical and physical quenching. Chemical quenching of singlet oxygen by β-carotene will lead to the formation of carotenoid breakdown products containing aldehyde and ketone groups as well as β-carotene-5, 8-endoperoxide. Tocopherols can chemically quench singlet oxygen in reactions that lead to the formation of tocopherol peroxides and epoxide. Other compounds, including amino acids, peptides, proteins, phenols, urate and ascorbate, can chemically quench singlet oxygen, but little is know about the resulting oxidation products (Pizarro-Urzúa and Núñez-Vergara, 2005).

c. Control of Lipoxygenases

Lipoxygenases are active lipid oxidation catalysts found in plants and some animal tissues. Lipoxygenase activity can be controlled by heat inactivation and plant breeding programs that decrease the concentrations of these enzymes. Phenolic compounds are capable of indirectly inhibiting lipoxygenase activity by acting as free radical inactivators, but also by chelating the iron or reducing it in the active site of the enzyme to the catalytically inactive ferrous state (Raghavenra *et al*., 2005).

2.1.3 Inactivation of Oxidation Intermediates

a. Superoxide Anion

Superoxide anion is produced by the addittion of an electron to molecular oxygen. Because superoxide anion participates in oxidative reactions, biological systems contain superoxide dismutase (SOD).

Two forms of SOD are found in eukaryotic cells, one in the cytosol and other in the mitochondria. Both forms of SOD catalyze the conversion of superoxide anion to hydrogen peroxide by the following reaction:

$$O_2^{\cdot -} + 2H^+ \longrightarrow H_2O_2 \qquad (9)$$

b. Peroxides

Peroxides are important intermediates of oxidative reactions because they decompose via transition metals, irradiation, and elevated temperatures to form free radicals. Hydrogen peroxide exists in foods as a result of direct addition and formation in biological tissues. Hydrogen peroxide is rapidly decomposed by the reduced state of transition metals (e.g., Fe and Cu) to the hydroxyl radical. The hydroxyl radical is an extremely reactive free radical that can oxidize most biological molecules at diffusion-limited reaction rates. Therefore, removal of hydrogen peroxide from biological materials is critical to the prevention of oxidative damage.

c. Photoactivated Sensitizers

In food, light is capable of activating sensitizers such as chlorophyll, riboflavin, and heme-containing proteins to an excited state. These photoactivated sensitizers can promote oxidation by directly interacting with an oxidizable substrate to produce free radicals, by transferring energy to triplet

oxygen to from singlet oxygen, or by transfer of an electron to triplet oxygen to form superoxide anion. Carotenoids inactivate photoactivated sensitizers by physically absorbing their energy to form the excited state of the carotenoid, which then returns to the ground state by transfer of thermal energy into the surrounding solvent (Stahl and Helmut, 2003).

2.1.4 Alterations in Lipid Oxidation Breakdown Products

Oxidation of fatty acids eventually leads to formation of breakdown products. These reactions lead to a multitude of different oxidation products, known as secondary lipid oxidation products, which affect both the sensory characteristics and the functional properties of foods. Rancid odors arise from the production of secondary products such as aldehydes, ketones, and alcohols. Secondary lipid oxidation products, and in particular aldehydes, also impact food quality and nutritional composition through interaction with the amino groups of proteins and vitamins, secondary products arising from lipid oxidation have been found to alter the function of proteins, enzymes, biological membranes, lipoproteins, and DNA (Suja *et al.,* 2003; Fiedor *et al*., 2005).

Since aldehydes and other secondary products arising from lipid oxidation are potentially damaging, biological systems seem to have developed mechanisms to control their activity. Sulfur- and amine-containing compounds have the ability to interact with aldehydes. This may help explain why many proteins, peptides, amino acids, phospholipids, and nucleotides display antioxidant activity when secondary products are used to measure lipid oxidation.

2.1.5 Surface-Active Antioxidants and Physical Effects

Food systems often have interfacial surfaces at which lipid oxidative reactions are prevalent. Examples include oil-in-water emulsions, water-in-oil emulsions, the air-lipid interface of bulk oils and solid fats, and the water-lipid interface of biological membranes. Oxidation is prevalent at these interfaces as a

result of increased contact with oxygen, the presence of aqueous phase free radicals, the presence of reactive oxygen generating systems and prooxidative metals, and possibly the migration of the more polar lipid peroxides out of the hydrophobic lipid core toward the more polar interface.

The effectiveness of phenolic antioxidants is often dependent on their polarity. Decker (1998) used the term "antioxidant paradox" to describe how polar antioxidants are most effective in bulk lipids while nonpolar antioxidants are most effective in dispersed lipids. In bulk tocopherol-stripped corn oil, Trolox (a water-soluble analog of α-tocopherol) more effectively inhibited lipid peroxide formation than α-tocopherol. However, when tocopherol-stripped corn oil was emulsified with Tween 20, α-tocopherol inhibited peroxide formation more effectively than Trolox. The observed increase in activity of α-tocopherol compared to Trolox in emulsified oil was attributed to its retention in the oil and possibly to its ability (due to its surface activity) to concentrate at the oil-water interface. The lower activity of Trolox in the emulsion was due to its partitioning into the water phase, where it was not effective at inhibiting autoxidation of the corn oil because of its dilution in the aqueous phase (Huang *et al.,* 1996).

Lipid oxidation can be inhibited by encapasulation. Potential mechanisms of inhibition include physical inhibition of oxygen diffusion into the lipid, chemical (e.g., free radical scavenging) and physical (e.g., chelation) antioxidant properties of the encapsulating agents, and possibly interaction of lipid oxidation products with the encapsulating material. Both protein and carbhydrate encapsulating agents have been found to retard oxidation rates (Iwami *et al*., 1988).

Another factor that may influence oxidation rates is the physical state of the lipids. Lipids in foods often exist as combination of both liquid and crystalline states, a condition that depends on both fatty acid composition and temperature. The increase in oxidation rates was attributed to phase separation of the most unsaturated fatty acid, which increases the concentrations of oxidizable

substrate. Little is known about how transition temperatures influence oxidation rates in food lipids (Orlien *et al*., 1999).

2.1.6 Antioxidant Interactions

Biological food systems usually contain multicomponent antioxidant systems. The numerous existing antioxidants have different potential functions, including inhibition of pro-oxidants of different types (e.g., metals, reactive oxygen species, enzymes); inactivation of free radicals and pro-oxidants in aqueous, interfacial, and lipid phases; and inactivation of compounds at different stages of oxidation [e.g., initiating species (·OH), propagating species (peroxides), lipid oxidation decomposition products (aldehydes)]. In addition, multicomponent antioxidant systems are beneficial because direct interactions occur between antioxidants.

Combinations of chelators and FRS often result in synergistic inhibition of lipid oxidation. Synergistic interaction most likely occurs by a "sparing" effect provided by the chelator. Since the chelator will decrease oxidation rates by inhibiting metal-catalyzed oxidation, fewer free radicals will be generated in the system (Saito *et al.*, 2003.).

Since multicomponent antioxidant systems can inhibit oxidation at many different phases of oxidation, the resulting antioxidant activity can be synergistic. This suggests that the most effective antioxidant systems for foods would contain antioxidants with different mechanisms of action and/or physical properties (Decker, 1998).

2.2 Synthetic Antioxidants

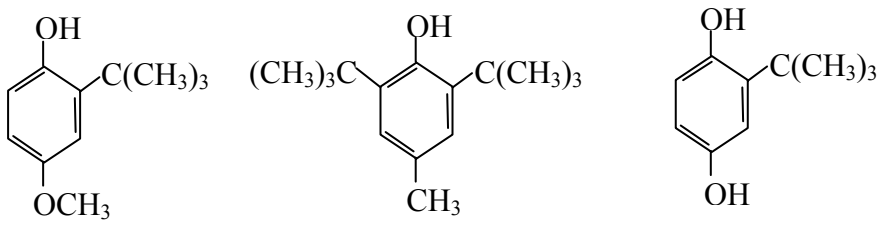
Most of the synthetic antioxidants are of the phenolic type. The differences in their antioxidant activities are related to their chemical structures,

which also influence their physical properties such as volatility, solubility, and thermal stability. The commercially available and currently used synthetic antioxidants are butylated hydroxyanisole (BHA, *tert*-butyl-4-hydroxyanisole), butylated hydroxytoluene (BHT) and tert-butyl hydroquinone (TBHQ).

BHA, which is highly fat-soluble, is a mixture of 2-BHA and 3-BHA (> 90%). BHA (Figure 6a) is widely used in lard, vegetable oils, cereals, packaging materials, dairy and meat products. The antioxidant effect is carry-through potent and synergistic when used in combination with other primary antioxidants such as BHT, TBHQ and propyl gallate (PG). BHT effectively retards lipid oxidation and discoloration at 0.01% level in meat and meat products (Madhavi *et al.*, 1996).

Butylated hydroxytoluene (BHT: 2, 6-di-*tert*-4-methylphenol), which is highly fat-soluble, contains two *tert*-butyl groups contributing steric hindrance to the reactions of the hydroxyl group, and the radical intermediate formed by hydrogen donation to a lipid radical. BHT (Figure 6b) is more volatile at high temperature and less carry-through potent than BHA. BHT and BHA are similar regarding their antioxidant properties in that they have synergistic effects with TBHQ and citric acid. The combination of BHT and BHA more effectively inhibits lipid oxidation at 0.02% in meat and meat products. Unlike BHA, the metabolism of BHT is more complicated and slower in reaction (Madhavi *et al.*, 1996).

TBHQ, a fat-soluble but slightly water-soluble compound, has two *para* hydroxyl groups contributing to its antioxidant activity. When compared to BHA, BHT, and PG, TBHQ (Figure 6c) more effectively enhances the oxidative stability at 0.02% level in foods. THBQ has a synergistic effect with other antioxidants and chelators. BHA, BHT, and TBHQ are classified as hindered-phenols due to the presence of a tertiary butyl group next to the hydroxyl group (Madhavi *et al.*, 1996).



BHA (a) BHT (b) TBHQ (c)

Figure 6 Structure of synthetic antioxidants: (a) BHA, (b) BHT, and (c) TBHQ.
Source: Madhavi *et al.* (1996)

2.3 Antioxidant Properties of Naturally Occurring Phenolic Compounds

Many of the natural components of plant materials have antioxidant activity. Tocopherols, for example, are important antioxidants occurring naturally in vegetable oils. Their effectiveness, however, is diminished at higher concentrations. Tocopherols may function both as free radical terminators and as scavengers for singlet oxygen (Houlihan and Ho, 1985).

Polyphenols are secondary plant metabolites and confer on fruits and vegetables both desirable and undesirable food qualities. Polyphenols account for the majority of antioxidant activity in plants. The antioxidant properties of phenolic compounds are mainly because of their redox potential, which allow them to act as reducing agents, hydrogen donators, metal chelators and singlet oxygen quenchers (Rice-Evan *et al*., 1996). It is known that the degree of glycosylation significantly affects the antioxidant properties of the compounds, for example, aglycons of quercetin and myricetin were more active than their glycosides (Marchand, 2002).

Flavonoids are naturally occuring phenolic compounds which largely include anthoxanthins (flavones, flavonols, flavanones, flavanols, chalcones and isoflavones), anthocyanins, leucoxanthins and flavonoidal alkaloids (Houghton,

2002). These compounds are found in a variety of plant materials (Kong *et al*., 2003). It is well known that flavonoids possess antioxidant properties *in vitro* and *in vivo*. The flavonoids contain a number of phenolic hydroxyl groups attached to ring structures, which confer the antioxidant activity. Catechins and their epimers serve as powerful antioxidants for directly eliminating superoxide anion radicals (Chen and Chan, 1996). Proanthocyanidins from grape seeds are apparently responsible for the action on the cardiovascular system (Pekić *et al*., 1997). Kaempferol 3–*O*-α-rhamnoside from *Licania licaniaeflora* exhibited DPPH radical scavenging activity and quercetin derivatives from this plant showed strong antiradical activity (Braca *et al*., 2002). Epicatechin, epigallocatechin, epicatechin gallate and procyanidin B1 and B2 from grape seed extract showed strong DPPH radical scavenging activity (Guendez *et al*., 2005).

The antioxidant activity of phenolic acids and their esters depends on the number of hydroxyl groups in the molecule and the activity can be strengthened by steric hindrance. The electron withdrawing properties of the carboxylate group in benzoic acids has a negative influence on the H- donating abilities of the hydroxy benzoates. Hydroxylated cinnamates are more effective than benzoate counterparts (Rice-Evans *et al*., 1996). Neochlorogenic acid and cryptochlorogenic acid isolated from prunes can scavenge superoxide anion radicals and inhibit oxidation of the methyl linoleate system (Nakatani *et al*., 2000).

Herbs and spices contain important natural antioxidants. Their antioxidant activity has been attributed to the presence of polar phenolic compounds and essential oils (Demo *et al*., 1998).

2.4 Analytical Methods for Determining Total Antioxidant Activity

Numerous methods are used to evaluate antioxidant activities of natural compounds in foods or biological systems with varying results. The assays commonly used to assess antioxidant activity are the Trolox-equivalent antioxidant capacity (TEAC) (Sellappan *et al.,* 2002), heat-induced oxidation of an aqueous

emulsion system of β-carotene and linoleic acid (Fukumoto and Mazza, 2000; Yan *et al.*, 2002) and the most widely used method for assessing total free radical scavenging activity is the DPPH method (Fukumoto and Mazza, 2000).

2.4.1 Trolox- Equivalent Antioxidant Capacity (TEAC) Assay

This assay is based on the inhibition by antioxidants of the absorbance of the radical cation of 2, 2′-azinobis (3-ethylbenzothiazoline 6-sulfonate) (ABTS). The ABTS radical is a quite stable radical. It has a characteristic long wavelength absorption spectrum (660, 734 and 820 nm). The TEAC assay measures the ability of a compound to reduce the ABTS radical. The TEAC assay was first reported by Miller *et al*. (1993). To date, the method has generated useful information regarding the antioxidant activities of various samples such as onions (Sellappan and Akon, 2002), blackberries, blueberries (Sellappan *et al*., 2002), lycopene (Bohm *et al*., 2002), phenolic components in extracts of *Cassia fistula* (Luximon-Ramma *et al*., 2002), and interactions between flavonoids and proteins (Arts *et al.,* 2002).

2.4.2 *β-Carotene* Bleaching Assay

In this method, antioxidant activity is measured by the ability of samples (plant extracts or phenolic compounds) to protect *β-carotene* from bleaching in the presence of linoleic acid (Marco, 1968). The oxidation of linoleic acid is usually induced by heat. This method can be used in measuring pure compounds with different structures or samples containing different compounds because this method is basically based on the inhibition of linoleic acid oxidation reactions by samples. An aqueous emulsion system of β-*carotene* and linoleic acid is very important. A successful assay must have a good emulsion system. Usually, Tween 20 is used as the emulsifier. Marco (1968) used this method to rank compounds for their antioxidant activity. Many researchers have used this method to find the antioxidant activities of polyphenols in carob pods. This method has been also utilized to measure antioxidant activities of phenolic compounds

(Fukumoto ans Mazza, 2000; Burda and Oleszek, 2001), fruits, vegetables, grain products (Velioglu *et al*., 1998; Makris and Rossiter, 2001), and *in-vitro* antioxidant and *ex-vivo* protective activities of green and roasted coffee (Daglia *et al.,* 2000).

2.4.3 DPPH Method

The 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical is a relatively stable and alcohol soluble free radical with absorption maximum at 517 nm. The DPPH method is one of the most studied methods to determine free radical scavenging activity (Brand-Williams *et al.,* 1995; Fuhrman *et al.*, 2001; Kumazawa *et al*., 2002; Saura-Calixto, 1998). In this method, some parameters have been defined such as percentage of remaining DPPH, EC_{50} and antiradical efficiency (AE) (Sánchez-Moreno *et al*., 1998). The EC_{50} is the concentration of antioxidant needed to decrease by 50% the initial substrate concentration in a given reaction time (the lower the EC_{50}, the higher the antioxidant power). This is a widely used parameter to measure antioxidant power (Sánchez-Moreno *et al*., 1998; Larrauri *et al*., 1997).

3. Phenolic Compounds in Plants

Phenolic compounds are a group of chemical compounds that are widely distributed in nature. They are simple compounds present in most fresh fruits and vegetables, or complex compounds present in bark, roots and leaves of plants. A group of polyphenols, responsible for the color of many fruits, vegetables, and flowers, are known as anthocyanins. There are several important classes, listed in Table 1, of phenolic compounds. According to the basic skeleton, the structure of natural polyphenols varies from simple molecules, such as simple phenols (volatile phenols), to highly polymerize compounds, such as condensed tannins (Waterman and Mole, 1994).

Table 1 The most important classes of phenolic compounds in plants

Basic skeleton	Class	Examples
C_6	Simple phenols	Phenol, guaiacol
	Benzoquinones	2,6- Dimethoxybenzoquinone
C_6-C_1	Hydroxybenzoic acids	Gallic, p-hydroxybenzoic, salicylic
C_6-C_2	Acetophenones	3-Acetyl-6-ethoxybenzaldehyde
	Phenylacetic acids	p-Hydroxyphenylacetic
C_6-C_3	Hydroxycinnamic acids	Caffeic, ferulic, p-coumaric
	Phenypropenes	Myristicin
	Coumarins	Aesculetin
	Isocoumarins	Bergenon
	Chromones	Eugenin
C_6-C_4	Naphthoquinones	Juglone
C_6-C_1-C_6	Xanthones	Mangiferin
C_6-C_2-C_6	Stilbenes	Resveratrol
	Anthraquinoids	Emodin
C_6-C_3-C_6	Flavonoids	Quercetin, catechin
	Isoflavonoids	Genistein
$(C_6$-$C_3)_2$	Lignans	Pinoresinol
	Neolignans	Eusiderin
$(C_6$-C_3-$C_6)_2$	Biflavonoids	Amentoflavone
$(C_6$-$C_3)_n$	Lignins	
$(C_6)_n$	Catechol melanins	
$(C_6$-C_3-$C_6)_n$	Condensed Tannins	

Souce: Waterman and Mole (1994)

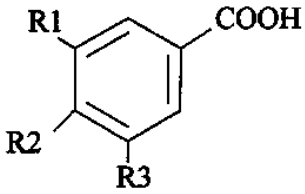
3.1 Volatile Phenols

Simple phenols such as phenol, *o*-cresol, 4-ethylphenol, guaiacol, 4-vinylguaiacol and eugenol have been found in the volatiles of fruits and vegetables (Tatum *et al.*, 1975; Buttery *et al*., 1976). The 4-vinylguaiacol is a major flavor component of citrus fruits and contributes a “rotten” flavor to orange

juice (Tatum *et al*., 1975). The 4-ethylguaiacol, eugenol, and 4-ethylphenol, a group of odorants with high flavor dilution (FD) factors, are a key difference between young and aged red wines (Margarita *et al*., 2001).

3.2 Phenolic Acids

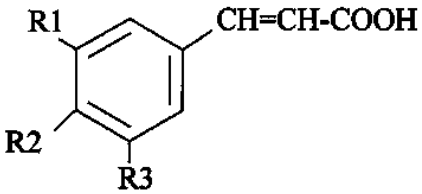
Hydroxybenzoic and hydroxycinnamic acids are predominant phenolic acids found in plants. Differences between their derivatives consist in the different patterns of hydroxylations and methoxylations of their aromatic rings. The structures of some of these compounds are shown in Figures 7 and 8.



R1	R2	R3	Compound
H	OH	H	*p*-Hydroxybenzoic acid
OH	OH	OH	Gallic acid
H	OH	OH	Protocatechuic acid

Figure 7 Basic structure of benzoic acid skeleton

Source: Schuster and Hemann (1985)



R1	R2	R3	Compound
H	OH	OH	Caffeic acid
CH_3O	OH	OH	Ferulic acid
H	OH	H	*p*-Coumaric acid

Figure 8 Basic structure of cinnamic acid skeleton

Source: Schuster and Hemann (1985)

3.2.1 Hydroxybenzoic Acids

Hydroxybenzoic acids have a general structure of C6-C1 (Figure 7). Hydroxybenzoic acids are commonly present in bound form. They are components of complex structures such as hydrolyzable tannins and lignins. Hydroxybenzoic acids are also found in the form of sugar derivatives (Schuster and Hemann, 1985). The hydroxybenzoic acid content in foods of plant origin is generally low. Gallic acid is one of the common hydroxybenzoic acids. Its dimeric condensation product and related dilactone, ellagic acid, are commonly found in plants. There is a particular interest in ellagic acid in fruits because of the increasing evidence of its anticarcinogenic and antioxidant effects (Meyer *et al.,* 1998).

3.2.2 Hydroxycinnamic Acids

Hydroxycinnamic acids (Figure 8) are also found commonly in foods of plant origin. *p*-Coumaric, caffeic, ferulic and sinapic acids are major hydroxycinnamic acids found in fruits. Among these, caffeic acid is the predominant hydroxycinnamic acid in many fruits. Caffeic acid represents over 75% of the total hydroxycinnamic acids in fruits. Caffeic acid has been found in plums, apples, apricots, blueberries and tomatoes (Kono *et al*., 1995).

Hydroxycinnamic acids are mainly present in bound form and are rarely found in the free form. Hydroxycinnamic acids usually occur in various conjugated forms. The conjugated forms are esters of hydroxyacids such as quinic, shikimic and tartaric acid, and their sugar derivatives. The free hydroxycinnamic acids can be released from chemical or enzymatic hydrolysis during tissue extraction (Schuster and Hemann, 1985).

3.3 Flavonoids

Flavonoids represent the most common and widely distributed group of plant phenolics. Their common structure (C6-C3-C6) consists of two aromatic

rings (A ring and B ring) linked through a three carbon bridge that is usually an oxygenated heterocycle (C ring). Figure 9 shows the basic structure and the system used for the carbon numbering of the flavonoid nucleus. The major flavonoid classes include anthocyanidins, chalcones, flavanols, flavanones, flavones, flavonols, and isoflavones. The variability of the flavonoids is based on the hydroxylation of the pyrone ring, absence or presence of double bond, the number of hydroxyls in the A ring and B ring, and/or a double bonded oxygen atom attached to position 4 of the C ring. Flavonoids may be monomeric, dimeric, or oligomeric. Polymeric flavonoids, known as tannins, are divided into two groups, condensed and hydrolysable. Condensed tannins are polymers of flavonoids while hydrolysable tannins contain gallic acid.

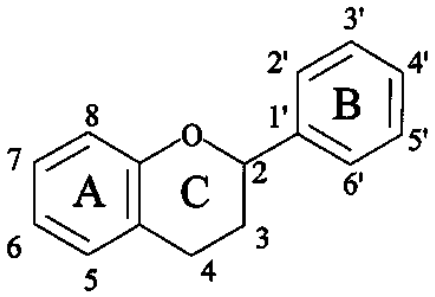
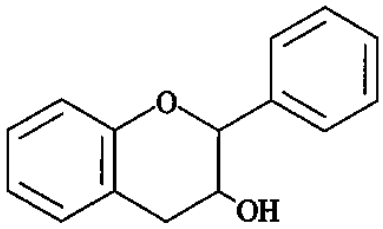


Figure 9 Basic structure of flavonoid skeleton
Source: Pietta (2000)

3.3.1 Flavanols and Flavonols

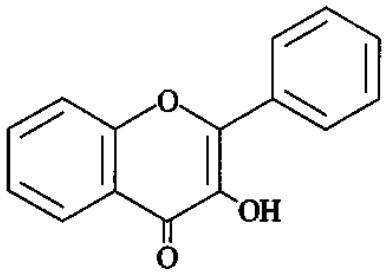
Flavanols are known as flavan-3-ols (Figure 10), and they are the subunits of proanthocyanidins, which have a hydroxyl group attached to the 3 position of the C ring, no positive charge on the oxygen atom and no double bond in the C ring. The structures of flavonols (Figure 11) are very similar to those of flavanols, except that there is a double-bonded oxygen atom attached to position 4 of the C ring and a double bond in the C ring.



Positions 5, 7, 3′, 4′: OH → Catechin
Positions 5, 7, 3′, 4′, 5′: OH → Epicatechin

Figure 10 Basic structure of flavan-3-ol skeleton

Source: Pietta (2000)



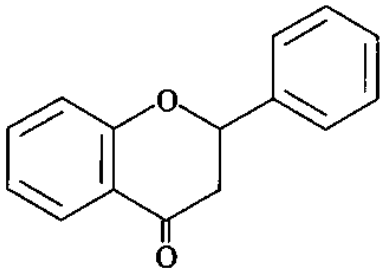
Positions 5, 7, 4′: OH → Kaempferol
Positions 5, 7, 3′, 4′: OH → Quercetin

Figure 11 Basic structure of flavonol skeleton

Source: Pietta (2000)

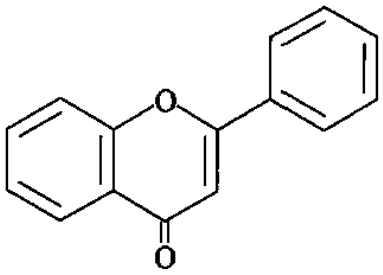
3.3.2 Flavanones and Flavones

Flavanones (Figure 12) and flavones (Figure 13) have structures similar to those of flavanols and flavonols, respectively. But, in each case, there is no longer a hydroxyl group attached to the 3 position of the C ring.



Positions 5, 7, 4′: OH → Naringenin
Positions 5, 7, 3′, 4′: OH → Eriodictyol

Figure 12 Basic structure of flavanone skeleton
Source: Pietta (2000)



Positions 5, 7, 4′: OH → Apigenin
Positions 5, 7, 3′, 4′: OH → Luteolin

Figure 13 Basic structure of flavone skeleton
Source: Pietta (2000)

3.3.3 Anthocyanins

Anthocyanins are widely distributed among fruits and vegetables. They are one of the main classes of flavonoids. They contribute significantly to the antioxidant activities of the flavonoids (Lapidot *et al*., 1999). Anthocyanins are water soluble pigments responsible for red, blue and violet colors. Anthocyanins (Figure 14) are glycosylated anthocyanidins with sugars generally attached to the 3-hydroxyl position of the anthocyanidin (Figure 15). In some cases the sugar residues are acylated by *p*-hydroxybenzoic, *p*-coumaric, caffeic, ferulic, sinapic, acetic acid, oxalic acid, malic acid, or succinic acid. Anthocyanidin is an aglycone.

This means that there is no sugar group or other functional group attached to the flavan nucleus. Also, the oxygen atom on the C ring has a positive charge on it, and there are two double bonds in the C ring. In addition to hydroxylated anthocyanidins, such as delphinidin, cyanidin, and pelargonidin, there are also methylated anthocyanidins (malvidin, peonidin, and petunidin).

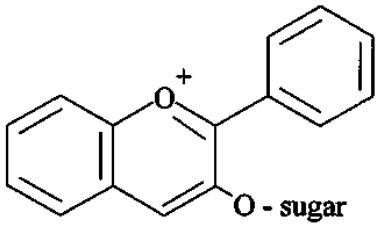
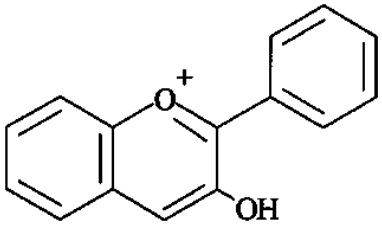


Figure 14 Basic structure of anthocyanin skeleton
Source: Pietta (2000)



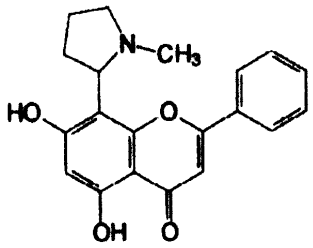
Positions 5, 7, 4′: OH → Pelargonidin
Positions 5, 7, 3′, 4′: OH → Cyanidin

Figure 15 Basic structure of anthocyanidin skeleton
Source: Pietta (2000)

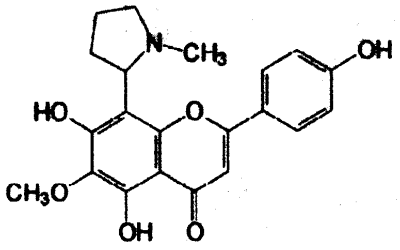
3.3.4 Flavonoidal Alkaloid

Flavonoid alkaloids are of interest because of the biological activities which they have been found to possess. These include antiviral properties and tyrosine kinase inhibition, the latter being the basis of their potential use in treating inflammatory conditions and acting as anti-neoplastic agents. The classification of flavonoidal alkaloids is somewhat unusual since most alkaloids are classified

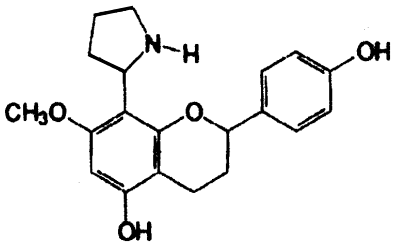
chemically according to the nitrogen-containing ring system. However, the class under consideration is typified by part of the molecule to which the nitrogenous moiety is attached (Houghton, 2002). Some naturally occurring flavonoidal alkaloids are shown in Figure 16.



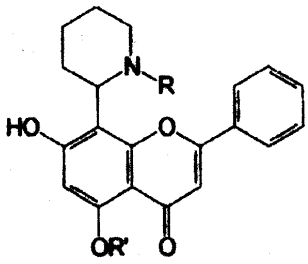
(1) Ficine



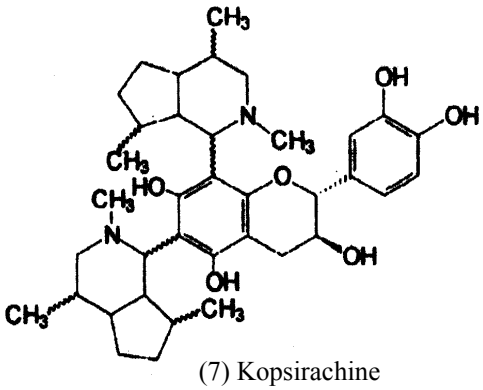
(2) Phyllospadine



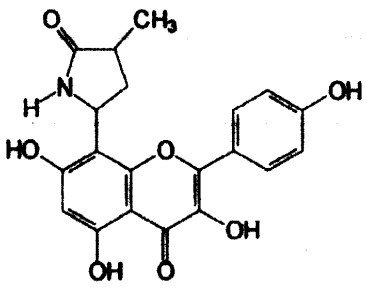
(3) Vochysine



	R	R′	
(4)	CH_3	CH_3	Buchenavianine
(5)	CH_3	H	*O*-Demethylbuchenavianine
(6)	H	H	N, *O*-Didemethylbuchenavianine



(7) Kopsirachine



(8) Lilianine

Figure 16 Some flavonoidal alkaloids

Source: Houghton (2002)

3.4 Thai Plants Which are Potential Antioxidant Sources

Many researchers have reported the antioxidant capacity of several Thai plants (Jirajariyavej *et al*., 1993; Sakthong *et al*., 1993; Darot *et al*., 1995; Kaweewong, 2001; Siengdee, 2003). Unfortunately, the wide variety of oxidation systems and ways to measure activity used in antioxidant evaluations make it difficult to compare results from different studies. However, eighty-three species of Thai plants have been studied for antioxidant properties (Trakoontivakorn and Saksitpitak, 2000). They were collected from local markets in Northern, Northeastern and Southern of Thailand. Methanol extracts of the plants were analysed by the β-*carotene* bleaching method. The antioxidative potential was classified based on antioxidant content reported as butylated hydroxyanisole equivalents (table 2).

Table 2 Antioxidative potency of some Thai indigenous vegetables

Families/Species	Local name	Antioxidant Potency
Mushroom		
Agaricaceae		
Lintinus praerigidus Break.	Hed lom	D
Amanitaceae		
Termitomyces albuminosus (Berk.) Heim	Hed pluak, Hed kone	C
Amanita vaginata	Hed la ngok	B
Other vegetables		
Aizoaceae		
Glinus herniarioides Tard.	Khi siat	B
Anacardiaceae		
Anacardium occidentale Linn.	Yod mamuamg Himmaphan	A
Bouca macrophylla Griff.	Yod maprang	A
Spandias pinnata Kurz.	Yod makok thai	A

Table 2 (Continued)

Families/Species	Local name	Antioxidant Potency
Asclepladaceae		
Dregea volubilis Stapf.	Phak huan	A
Gymnema inodorum Decne.	Dok phak chiengda	A
Barringtoniaceae		
Barringtonea acutangula (Linn.)	Kradon nam	A
Careya sphaerica Roxb.	Dok kradon bok	A
Combretaceae		
Tetminalia tripterordes Craib	Luk phab	B
Compositae		
Eupatorium triplinerve Vahl	Ya fan (kiengpa)	B
Cruciferae		
Brassica juncea Czern. & Coss.	Phak kad hin	B
Cucurbitaceae		
Cucurbita moschata Decne.	Dok phag tong	A
Citrullus vulgaris Schad.ex	Taeng mo on	B
Momomdica charantia Linn.	Bai mara khi nok	A
Euphorbiaceae		
Sauropus androgynus Merr.	Phak whan ban	B
Guttiferae		
Cratoxylum formosum Dyer.	Phak teaw	A
Garcinia cowa Roxb.	Cha muang	B
Leguminosae- Caesalpinioideae		
Caesalpinia mimosoides Lamk.	Phak pooya (Cha luad)	A
Cassia siamea Lamk.	Dok khi lhek	A
Tamarindus indica Linn.	Bai makham on	A
Leguminosae-Minosoideae		
Acacia rugata Merr.	Yod sompoy	A
Parkia speciosa Hassk.	Yod sator	A

Table 2 (Continued)

Families/Species	Local name	Antioxidant Potency
Leguminosae-Papilionoideae		
Cajanus cajan Millsp.	Tua mahae	A
Pachyrhizus erosus Urban	Yod munkaew	A
Psophocarpus tetragonolobus D.C	Tua pu	A
Sesbania grandiflora Desv.	Dok khae ban	A
Meliaceae		
Azadirachta indica juss. Var.	Phak wan	B
Siamensis valeton	Bai sadao	A
Mimosaceae		
Archidendron bubalium Nielsen	Luk nieng nok	A
Moraceae		
Ficus hispida Linn. F.	Maduea plang	A
Ficus fistulosa Reinw.	Luk ching	A
Moringaceae		
Moringa oleifera Lamk.	Dol marum	A
Myrtaceae		
Eugenia sp.	Phak sompong	A
Musaceae		
Musa acuminata Colla.	Kluai thuen	B
Musa sp.	Yhuak kluai	A
Nymphaeaceae		
Nymphaea nouchali Burm.	Boa puen	A
Ongraceae		
Jussiaea repens Linn.	Phangphuai nam	A
Opiliaceae		
Meliantha suavis Pierre.	Phak wan pa	A
Passifloraceae		
Passiflora foetida Linn.	Tamlueng tong	A
Rhamnaceae		
Colubrina asiatica Brongn.	Phak kantong	A

Table 2 (Continued)

Families/Species	Local name	Antioxidant Potency
Rutaceae		
Aegle marmelos (L.) Corr.	Mapin	A
Feroniella lucida Swing.	Doksang	A
Micromelum minutum	Yod mhui	A
Toddalia asiatica (Linn.) Lamk.	Leb rok	A
Solanaceae		
Solanum sanitwongsei Craib	Mawaeng	A
Sphenocleaceae		
Sphenoclea zeylanica Gaertn.	Kum pla	A
Stilaginaceae		
Antidesma acidum Retz.	Som mao	A
Antidesma acidum Retz.	Bai mamao	A
Tiliaceae		
Corchorus sp.	Bai poh	A
Umbelltferae		
Centella asiatica (linn.) Urban	Phak nhok	A
Zingiberaceae		
Boesenbergia sp.	Ton krachai	B
Curcuma domestica Valeton	Khamin chan	A
Languas galanga SW.	Ton kra on	B
Languas galanga SW.	Dok kha	A
Zygnermataceae		
Spirogyra sp.	Tao	A

Source: Trakoontivakorn and Saksitpitak (2000)

* calculated from 5 g dwb.

A = very high potency, contains substances >> 100 mg. BHA eq. in 100 g. fresh vegetable

B = high potency, contain substances > 100 mg. BHA eq. in 100 g. fresh vegetable

C = medium potency, contain substances < 100 mg. BHA eq. in 100 g. fresh vegetable

D = low potency, contain substances << 100 mg. BHA eq. in 100 g. fresh vegetable

Thai indigenous plants included samples which showed high potential as antioxidant sources (Table 2). Owing to the number of species studied in the present work, it would be impossible to characterize each plant in depth. The phenolic profile of three of the thai indigenous plants investigated; *Cratoxylum formosum* Dyer., *Careya sphaerica* Roxb. and *Leucaena glauca* Benth have been selected for detailed study because of their considerable biological activity and commercial value.

3.4.1 *Cratoxylum formosum* Dyer.

This plant family is Guttiferae. The name of this plant differs in different local areas, for instance, Teawkon (Central), Teawdang (North) and Tao (South). Thai people traditionally eat shoots and young leaves of this plant. It tastes sour and a little astringent due to the phenolic phytochemicals present. Harvesting season of *Cratoxylum formosum* Dyer. is during March to May of each year. Trees of this plant are planted commercially in Sakolnakhon, Kalasin, Yasothorn, Mahasarakham, Bureerum, which are the provinces in North – East Thailand. The production rate of this plant in 2003 was about 200 – 500 kgs/rai and the price varies from about 5 – 60 baht/kg depending on the area of production. The chemical composition of 100 grams of Teaw leaves is 58 Kcal for energy, 1.5 g of fiber, 67 mg of calcium, 19 mg of phosphorus, 205 mg of iron, 4500 μg of β-*carotene*, 750 μg vitamin A as retinol, 10.04 mg of thiamin, 0.67 mg of riboflavin, 3.1 mg of niasin and 58 mg of vitamin C (Nutrition division, 1992).

Relatively little work has been done on the phytochemicals in *Cratoxylum* sp. From 1960 to 2005 reports on phenolic compounds in *Cratoxylum formosum* Dyer. are limited. The only study concerning the phytochemistry of *Cratoxylum* sp. was published by Kitanov and Assenov (1988), and Kumar *et al.* (2004) reported the phenolic compounds in *Cratoxylum pruniflorum* Kurz were quercetin ($C_{15}H_{10}O_{12}$), hyperoside ($C_{21}H_{20}O_{12}$), 1, 3, 6, 7-tetrahydroxyxanthone, mangiferin ($C_{19}H_{18}O_{11}$) and isomangiferin ($C_{19}H_{18}O_{12}$), (Kitanov and Assenov, 1988).Whereas, phenolic constituents in *Cratoxylum neriifolium* Kurz were

biflavonol GB-2, pentahydroxyflavanone chromone and stigmasterol (Kumar *et al*., 2004).

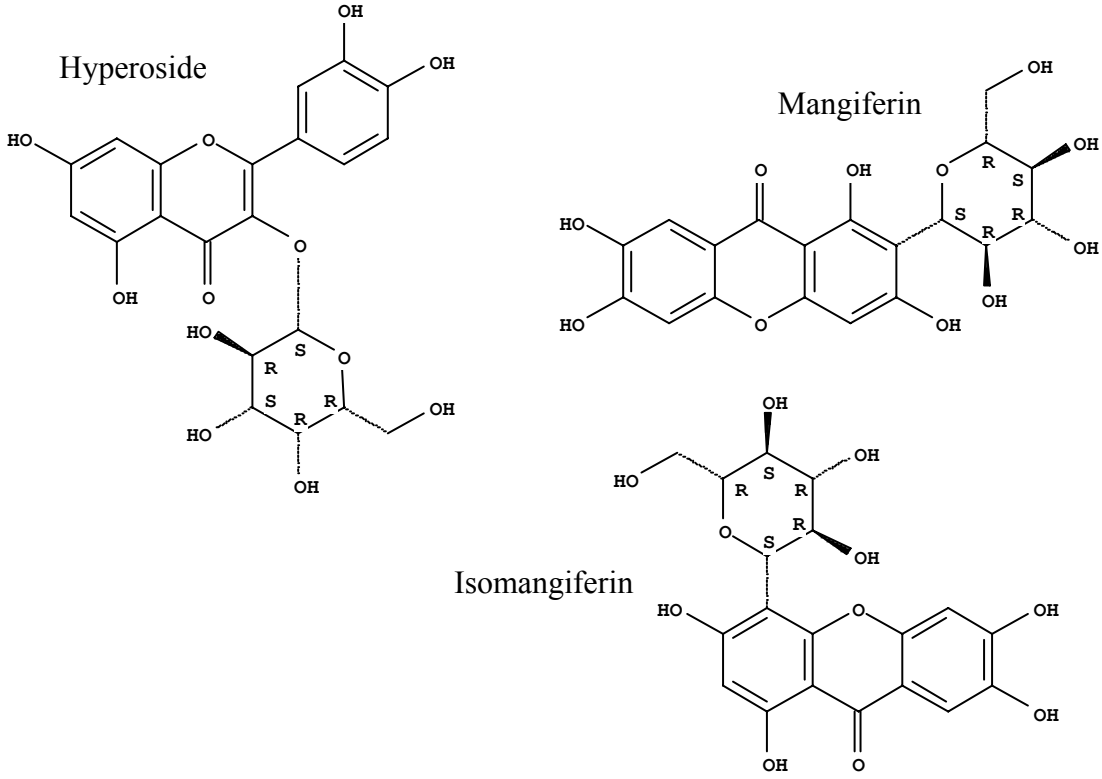


Figure 17 Some phenolic compounds found in *Cratoxylum* sp.

3.4.2 *Careya sphaerica* Roxb.

This plant family is Barringtoniaceae and the plant is also known as *Careya arborea* Roxb. This plant is normally consumed fresh and mostly found in North – East of Thailand. Its name is different in different areas, for instance, Kradon, Phak–Kradon, Kradonbok, Kradonkhon (North – East), Khui (Khanchanaburee), Phuk-Pui (North), Puikradon (South), Pui - khao (Chiangmai). Thai people traditionally eat shoots, young leaves and young flowers of this plant. It tastes a little astringent due to the phenolic compounds present. The harvesting season of Kradonbok is during March to May of each year. Kradonbok trees are planted commercially in Sakolnakhon, Kalasin, Yasothorn, Mahasarakham, and Bureerum which are provinces in North – East of Thailand. The production rate of this plant in 2002 was about 200-400 kgs/rai and the price is about 5-40 baht/kg

depending on area of production. Kradonbok has some health benefits such as use of Kradonbok leaf for healing a wound and flower for remedying a cough. The characteristics and chemical composition of 100 grams of Kradonbok leaves are 83 Kcal for energy, 1.9 g of fiber, 13 mg of calcium, 18 mg of phosphorus, 17 mg of iron, 3958 IU of riboflavin, 1.8 mg of niacin and 126 mg of vitamin C (Nutrition division, 1992).

Data on the phenolic compounds of *Careya sphaerica* Roxb. is relatively scarce during the last 30 years. Gupta *et al.* (1975) reported that the phenolic consituents present in the leaves when extracted with petroleum ether at room temperature were lupeol, hexacosanol ($C_{26}H_{54}O$), α-spinosterol ($C_{29}H_{48}O$), teraxerol ($C_{30}H_{50}O$), β-sitosterol ($C_{29}H_{50}O$), quercetin ($C_{15}H_{10}O_7$), taraceryl acetate ($C_{32}H_{52}O_2$) and ellagic acid ($C_{14}H_6O_8$) In addition, careaborin, β-amyrin, careyagenolide, maslinic acid and α-hydroxyursolic acid were also found in the leaves (Talapatra *et al.*, 1981; Das and Mahato, 1982).

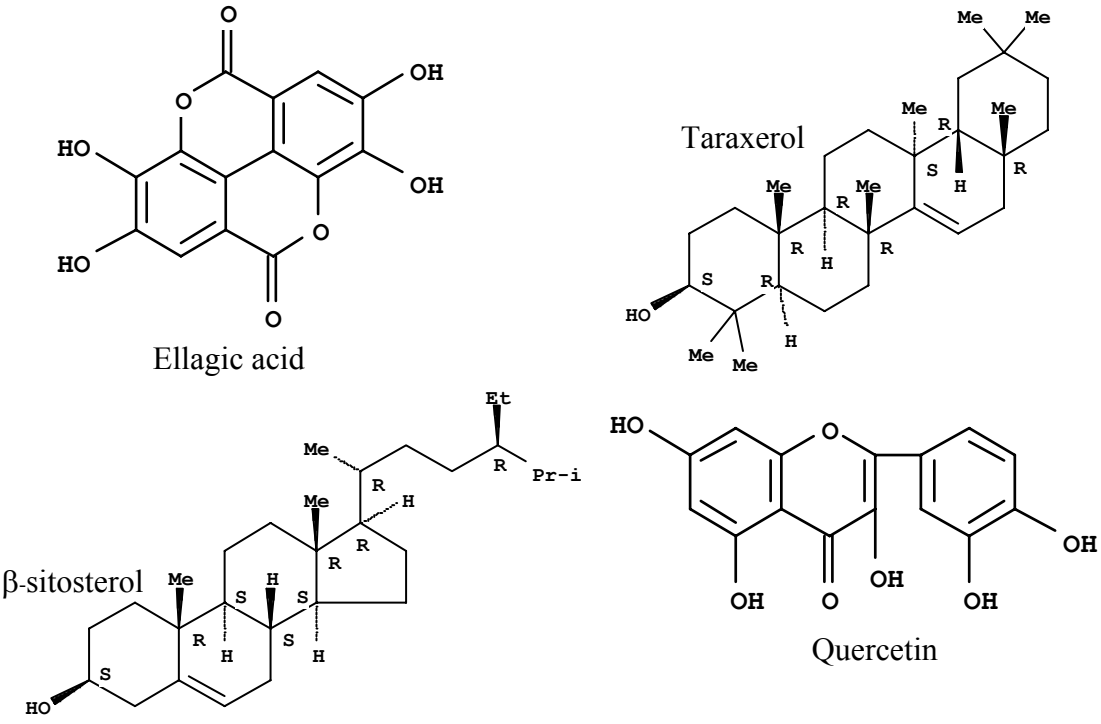


Figure 18 Some phenolic compounds found in *Careya sphaerica* Roxb.

3.4.3 *Leucaena glauca* Benth

This plant family is Leguminosae and the plant is found throughout Thailand in the settled areas at low and medium altitudes. It occurs widely and is abundant. Its name is different in different areas, for instance, Kratin-Thai (Central), Satorban (South), Katong and Kratin. Thai people traditionally eat young leaves and the young pod of this plant. The young leaf is found in all seasons, however, it is most abundant during March to May of each year. Kratin trees are planted commercially in Roi-ed, Amnat Charoen, Pichit, Nakhonsawan, Songkla, Krabi, Pattani and Trang. The production rate of this plant 2002 is about 300-1,500 kg/rai and the price is about 3-25 baht/kg depending on area of production. Kratin leaves contain leucine which can absorb selenium. The charcateristics and chemical composition of 100 grams of Kratin leaves are 62 Kcal for energy, 8.4 g of protein, 3.8 g of crude fiber, 137 mg of calcium, 11 mg of phosphorus, 9.2 g of iron, 7883 IU of total vitamin A, 0.33 mg of thiamin, 0.09 mg riboflavin, 1.7 mg of niacin and 8 mg of vitamin C (Nutrition division, 1992).

Relatively little information on the phenolic constituents of *Leucaena glauca* Benth has been published during the last 30 years. Chen (1979) found foeniculin ($C_{14}H_{18}O$) and kaempferol-3-xyloside ($C_{20}H_{18}O_{10}$) in the leaves. In addition, Guaijaverin ($C_{20}H_{18}O_{11}$), juglania ($C_{20}H_{18}O_{10}$), kaempferol-3-*O*-β-xyloside and quercitrin ($C_{21}H_{20}O_{11}$) were also found in the leaves (Morita *et al*., 1977).

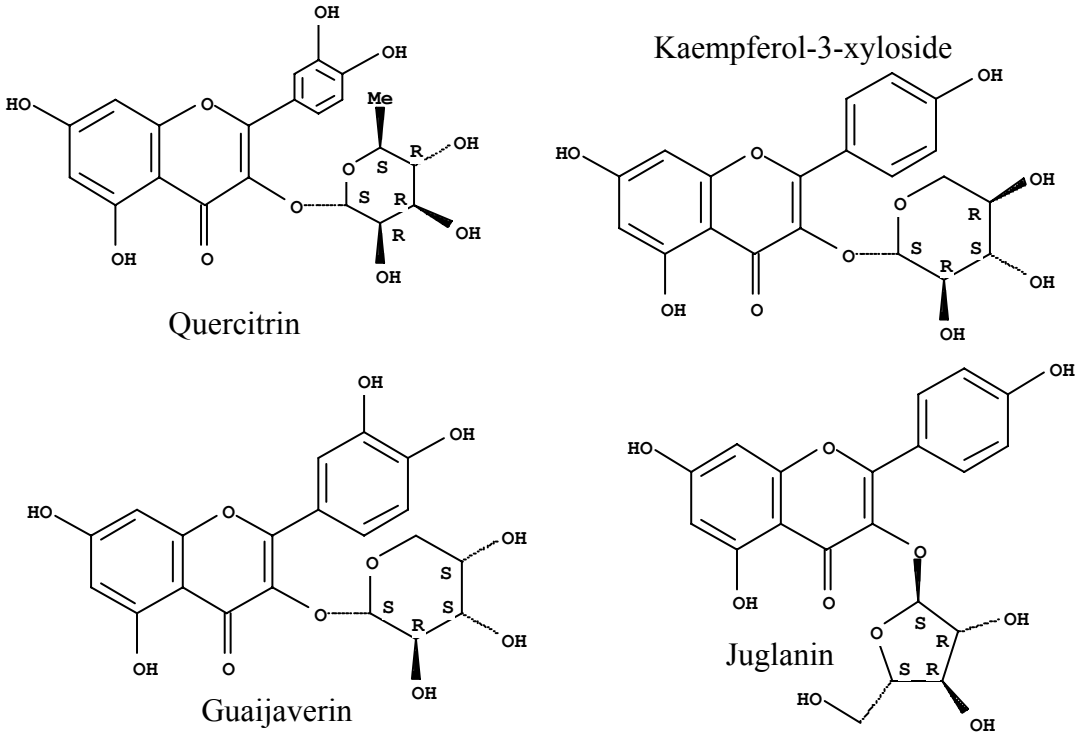


Figure 19 Some phenolic compounds found in *Leucaena glauca* Benth

3.5 Factors Affecting Preparation of Plant Antioxidants

There is a big difference between the preparation of synthetic antioxidants and natural antioxidants for application in food products and processing. Synthetic antioxidants are produced as pure substances of constant composition, and are applied as such or in well defined mixtures with other pure substances. Application is thus relatively easy, requiring no substantial modifications of the recipe and processing conditions (Moure *et al*., 2001).

On the contrary, natural antioxidants are available from raw materials of variable composition. Both the content of active substances and the content of various other compounds may vary. The quality of natural extracts and their antioxidative activity depends not only on the quality of the original plant, date and storage, but also the extraction conditions which affect the plant phenolic compounds extracted (Moure *et al.*, 2001).

However most natural plant antioxidants are usually limited to a harvesting season. The preliminary processing of plants is necessary for storage. The content of active antioxidants in natural materials is usually low, therefore it is often useful to prepare more concentrated materials by using extraction (Pokorny and Korczak, 2001).

3.5.1 Sample Preparation for Storage

Temperature and light are the major factors influencing changes in antioxidant activity during storage (Moure *et al*., 2001). From numerous publications, the main preservative processes before storage include drying plants with air in an oven at 40 ºC and keeping at room temperature (Bocco *et al*., 1998; Miean & Mohamed, 2001) or drying samples by air at 25ºC and keeping at room temperature (Habsah *et al*., 2000; Siddhuraju *et al*., 2002) or keeping the fresh plants in a still air freezer at -25 to -30ºC (Velioglu *et al*., 1998; Amakura *et al*., 2000).

Normally, temperature affects the compounds stability due to chemical and enzymatic degradation. These latter have been suggested to be the main mechanism causing the reduction in polyphenol content (Larrauri *et al.*, 1997). The reduction in antioxidant activity was higher than that expected from the reduction in polyphenol contents, probably due to the synergistic effect of natural phenols (Moure *et al*., 2001). In addition, phenols can react with other plant components. Besides, prolonged exposure at moderate temperatures can also cause phenolic degradation. Drying at room temperature (20ºC) for 2 - 4 days may enhance enzymatic degradation, and an oven with good ventilation at 48ºC for 5 - 10 h. gave higher antioxidant activity of willow leaves (Julkunen-Tiitto, 1985). Therefore, sample preparation conditions including temperature and time before storage should be controlled. Sample preparation before storage affects the antioxidant activity, but relatively little information about the effect of preservative temperature and time is available especially with regards to Thai indigenous plants.

3.5.2 Extraction Conditions

Recently, antioxidant studies have focused on the improvement of phenol extraction techniques from some natural products (Azizah *et al*., 1999; Zhou and Yu, 2004; Lapornik *et al*., 2005; Jerez *et al*., 2005). Solvent extraction is more frequently used for isolation of antioxidants and both extraction yield and antioxidant activity of extracts are strongly dependent on the solvent, due to the different antioxidant potential of compounds with different polarity (Marinova and Yanishlieva, 1997). Apolar solvents are among the most common solvents for removing polyphenols from water. Ethyl acetate and diethyl ether have been used for extraction of low molecular weight phenols from oak wood (Fernández de Simón *et al*., 1996). Ethanol and water are the most widely employed solvents for reasons of hygiene and abundance, respectively.

The temperature of extraction affects the compounds stability due to the decomposition of phenolic compounds. The effect of temperature has been studied in extraction of anthocyanins. They were shown to be degraded since the visible spectrum showed both a reduction in the peak at 400-500 nm and reduction in the red color. The temperature during extraction can affect extractable compounds to different extents; boiling and resting increases the total phenol content extracted from *Quercus suber cork* (Conde *et al.,* 1998). Milder extraction temperatures are desirable in those cases where some compounds can be degraded, e.g. carnosic acid (Ibañez *et al*., 1999).

The time of extraction can affect the antioxidant activity. Wang *et al.* (1996) found a significant increase in extractable polyphenols in strawberry and white grapes when they were extracted for times between 2 minutes and 4 hours. Lapornik *et al.* (2005) reported that extraction time (the time of extraction 1 h, 12 h and 24 h) affected the yield of extracted antioxidants from grapes, black and redcurrant marc. The antioxidant activity and total phenolic content increased with the time of extraction (Lapornik *et al.*, 2005). Extraction times between 15 minutes and 7 days have been reported (Fernández de Simón *et al.*, 1992; Kim *et al*., 1994;

Cao *et al.*, 1996; Bocco *et al.*, 1998; Masuda *et al.*, 1999; Zielinski and Kozlowska, 2000; Birch *et al.*, 2001; Martinez-tome *et al.*, 2001; Krings and Berger, 2001; Kähkonen *et al.*, 2001; Sellappen *et al*., 2002).

Other factors, such as extraction pH, the particle size of materials, and extraction methods were reported to affect the antioxidant activity and concentrations of phenolic compounds extracted. Sheabar and Neeman (1988) reported the maximum solubility of polyphenols from olive rape at pH 4 in the organic phase. The yield of extracted phenols was correlated with plant cell wall breakdown. Particle size reduction significantly increased the antioxidant activity as a result of both increased extractability and enhanced enzymatic degradation of polysaccharides (Weinberg *et al*., 1999). Various process conditions (refluxing, shaking and ultrasonic extraction) affected the concentrations of antioxidants in extracts from balm leaves (Herodež *et al*., 2003).

3.6 Analytical Method for Phenolic Compound Characterization

There are many ways to evaluate phenolic substances. The simplest and most common method of quantitative analysis is by spectrophotometric assays. The components are reported in the form of total phenolics (Table 3). In most of the assays, phenolics are quantified by first making measurements on solutions of known concentration of standard substances. Such measurements are simple in concept. Technical difficulties usually arise because in ecological and biochemical work in general, impurities and other contaminants (such as ascorbic acid) occur in the solution being measured. Therefore, the other way to quantify phenolic compounds requires extraction, separation and purification. Selection of an extraction strategy is always challenging and depends upon the type of herb, the accessibility of plant materials as well as the content of analytes. However, there is an enormous variation in the complexity of the analytical problems. Some problems are simple others will clearly require expert assistance as the skills involved and the equipment needed are highly specialized. Equipment costs will also vary markedly. For example, sometimes the necessary chromatographic

separation can be achieved with thin-layer chromatography (TLC). For other samples, a gradient high performance liquid chromatography (HPLC) system with a photo-diode array detector (DAD) may be adequate or, if you are dealing with unknown and perhaps novel compounds, you may need sophisticated mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectroscopy techniques.

Table 3 Examples of methods used for analysis of total phenolics

Method	Standard substrance	Reference
1. Folin-Ciocalteu method	Gallic acid	Ehlenfeldt and Prior (2001)
	Gallic acid	Kähkonen *et al*. (1999)
	Gallic acid	Yang *et al.* (2002)
	Gallic acid	Oktay *et al*. (2003)
	(+)-Catechin	Amarowicz *et al.* (2004)
	Chlorogenic acid	Kuti and Konuru (2004)
	Gallic acid	Chung *et al.* (2005)
	Tannic acid	Abdille *et al.* (2005)
	(+)-Catechin	Lima *et al.* (2005)
2. Folin-Dennis method	Tannic acid	Hyder *et al*. (2002)
	(+)-Catechin	Matthäus and Angelini (2005)

The first step in the isolation of phenolic components from a new material will probably involve either paper chromatography or TLC. The compounds can be separated or spread over the plate or paper and those that are phenolic can then be detected by a number of different methods. The most generally useful of these is examination under UV light. Phenols are by definition aromatic compounds and will as a consequence always either fluoresce or quench UV light.

Gas chromatography is a technique that brings additional dimensions and possibilities for the quantitative and qualitative analysis of phenolics, particularly where their structure or structural class is known. It will rarely, if ever, be of use in

isolating “unknown” compounds, or for their subsequent identification unless coupled with mass spectroscopy.

The most frequently used analytical technique for the separation of phenolic compounds is reversed-phase high-performance liquid chromatography (RP-HPLC) (Merken and Beecher, 2000) Detection techniques for HPLC methods are various, but diode array detection (DAD) is currently the most widely available and commonly used technique for routine qualitative and quantiative analysis of phenolic compounds (Häkkinen, 1999; He, 2000; Merken and Beecher, 2000). The UV-visible absorption spectra of phenolic compounds enable identification and classification of chromatographic peaks into classes, but the combination of these data with mass spectra (MS) data and information from the respective literature can be used for tentative identification of the conjugated forms (Gioacchini *et al.*, 1996; Schieber *et al.*, 2001).

3.6.1 High-Performance Liquid Chromatography (HPLC)

The phenolic compounds of natural origin have the positive property of being soluble in polar solvents. This leads to the possibility of using reversed phase HPLC (RP-HPLC) in their analysis, sufficient retention being achieved by using acidic conditions in order to avoid the presence of ionised forms of the analytes (Waksmundzka-Hajnos, 1998). Octadecylsilane (ODS, C18, RP-18) is by far the most popular of the stationary phases, both generally and for phenolics (Weston and Brown, 1997). Other RP materials (*e.g.* C8) are chosen only seldom, which is at least partly due to the fact that these products are to a lesser extent available on the market. However, polymeric condensed tannins cannot be analysed using RP-HPLC because of too strong adsorption to the stationary phases. Condensed tannins therefore have to be analysed using the normal phase HPLC technique, which is not so widely used since it has much poorer resolution compared to RP-HPLC (Waksmundzka-Hajnos, 1998).

The eluents used in the RP-HPLC analysis of phenolics are mixtures of aqueous pH modifiers with a polar, water-soluble organic solvent: methanol (MeOH) or acetonitrile (ACN). pH is a major factor especially in the separation of ionisable compounds such as phenolic acids. The most frequently used pH modifiers in RP-HPLC methods for phenolic compounds are the acids, such as formic, acetic, trifluoroacetic and phosphoric acid, as well as phosphate buffers adjusted to acidic pH in order to achieve the unionised form of the phenolic analytes (Robards and Antolovich, 1997; Waksmundzka-Hajnus, 1998).

Another positive property of phenolic compounds is their conjugated C-C double bonds which act as chromophores. Thanks to them, a combination of RP-HPLC and an ultraviolet/visible light (UV/Vis) detector is commonly used in both qualitative and quantitative analysis of nature-derived samples containing phenolics (Waterman and Mole, 1994). The UV wavelength of 280 nm has proved to be suitable for the universal detection of all phenolic compounds, ranging from simple hydroxybenzoic acids to larger tannins (Waksmundzka-Hajnos, 1998; Salminen *et al*. 1999). However, other wavelengths are frequently used for more selective detection of certain subgroups of phenolic compounds. On the basis of their absorption maxima, ellagic acid and *p*-hydroxybenzoic acid can be detected at 260 nm, gallic acid and catechins at 280 nm, hydroxycinnamic acids and their derivatives at 320 nm, flavonols at 360 nm, and anthocyanins at 530 nm (Häkkinen *et al*. 1998; Phippen and Simon, 1998).

In most cases, the spectrum of phenolics is preferably measured by HPLC-DAD. The spectrum can be used for qualitative and selective determination of the principal constituents of the plant phenolics. Therefore, much attention has been paid to the description of the spectra. Most publications show the spectrum of some flavonoids and phenolic acids since flavonoids and phenolic acids are the main groups of phenolic compounds, which are found in natural plants.

The retention times, UV maxima and structural assignment of some flavonoids and flavonoid glycosides (Llorach *et al*., 2003; Määttä *et al*., 2003;

Vvedenskaya *et al*., 2004; Yao *et al*., 2004) are shown in Table 4. The UV spectrums of some groups such as flavonol aglycones and glycosides, proanthocyanidins and anthocyanidins have similar spectra. These data allow spectrum analysis to be used for qualitative and selective evaluation of the classification of plant phenolics.

Table 4 Retention times, UV spectral characteristics and structure assignment of flavonoids and flavonoid glycosides.

No.	R_t (min)	UV spectral characteristics (nm)	Structure assignment	References
1.	12.3	255, 268sh, 351	Quercetin 3-diglucoside-7-glucoside	Llorach *et al*. (2003).
2.	14.0	267, 395sh, 286sh, 348	Kaempferol 3-triglucoside-7-glucoside	
3.	15.5	267, 293sh, 318sh, 348	Kaempferol 3-diglucoside-7-glucoside	
4.	16.0	268, 298sh, 318sh, 334	Kaempferol 3-caffeoyldiglucoside 7-glucoside	
5.	17.4	267, 294sh, 317sh, 347	Kaempferol 3-triglucoside-7-diglucoside	
6.	17.6	267, 294sh, 317sh, 347	Kaempferol 3-diglucoside-7-diglucoside	
7.	18.0	252, 269sh, 301sh, 338	Quercetin 3-sinapoylsiglucoside-7-glucoside	
8.	21.5	269, 333	Kaempferol 3-sinapoyldiglucisside-7-glucoside	
9.	22.1	249, 269, 331	Kaempferol 3-feuloydiglucoside-7-glucoside	
10.	22.7	252, 269, 321, 356sh	Kaempferol 3-sinapoyltriglucoside-7-glucoside	
11.	23.4	253sh, 269, 300sh, 333	Kaempferol 3-sinapoyltriglucoside-7-diglucoside	

Note : sh means maximum of the shoulder in the spectrum.

Table 4 (Continued)

No.	R_t (min)	UV spectral characteristics (nm)	Structure assignment	References
12.	23.7	253sh, 269, 300sh, 333	Kaempferol 3-feruloyldiglucoside-7-diglucoside	
13.	28.0	267, 294sh, 317sh, 347	Kaempferol 3-glucoside-7-glucoside	
14.	33.8	265, 299sh, 347	Kaempferol 3-triglucoside	
15.	34.2	251sh, 267, 333	Kaempferol 3-sinapoyltriglucoside	
16.	34.4	249, 267, 333	Kaempferol 3-sinapoyldiglucoside	
17.	34.9	268, 298sh, 346	Kaempferol 3-diglucoside	
18.	38.1	255sh, 265, 319sh, 367	Kaempferol 7-glucoside	
19.	38.2	255sh, 266, 319sh, 367	Kaempferol 7-diglucoside	
20.	47	253sh, 294sh, 322sh	Kaempferol	
21.	16.4	254, 300sh, 354	Quercetin hexoside-malonate	Määttä *et al.* (2003).
22.	17.1	264, 290, 348	Kaempferol hexoside	
23.	18.2	264, 348	Kaempferol hexose-deoxyhexoside	
24.	18.8	264, 348	Kaempferol hex	
25.	8.6	236, 278	oside-malonate	
26.	11.3	236, 278	(+)-catechin (-)-epicatechin	
27.	13.0	254, 300sh, 354	Myricetin hexose-deoxyhexoside	
28.	13.3	254, 300sh, 354	Myricetin hexose	
29.	14.2	254, 300sh, 354	Myricetin hexose-malonate	
30.	14.7	254, 262sh, 300sh, 35	Rutin	
31.	15.4	254, 262sh, 300sh, 35	Quercetin hexoside	
32.	29.0	261.5, 356.5	Myricetin 3-β-galactoside	Vvedenskaya *et a* (2004).
33.	29.8	261.5, 356.5	Myricetin 3-α-xylopyranoside	
34.	32.5	261.5, 356.5	Myricetin-3-α-arabinofuranoside	
35.	34.3	256.8, 356.5	Quercetin 3-β-galactoside	
36.	35.1	256.8, 356.5	Quercetin 3-β-glucoside	

Note : sh means maximum of the shoulder in the spectrum.

Table 4 (Continued)

No.	R_t (min)	UV spectral characteristics (nm)	Structure assignment	References
37.	36.3	256.8, 356.5	Quercetin 3-α-xylopyranoside	
38.	37.6	256.8, 356.5	Quercetin 3-α-arabinopyraside	
39.	39.8	256.8, 356.5	Quercetin 3-α-arabinofuranoside	
40.	40.4	256.8, 351.7	Quercetin 3-rhamnopyranoside	
41.	41.2	256.8, 356.5	3′-methoxyquercetin-3-β-galactoside	
42.	41.7	256.8, 356.5	*D*-methoxymyricetin-hexoside	
43.	42.9	256.8, 356.5	Mehoxyquercetin-pentoside	
44.	44.0	256.8, 351.7	Methoxyquercetin-pentoside	
45.	45.6	256.8, 356.5	Quercetin-3-O-(6′-benzoyl)-β-galactoside	
46.	46.8	256.8, 356.5	Methoxykaempferol derivatives	
47.	47.4	256.8, 290sh, 364.6	Methoxykaempferol derivatives	
48.	2.43	236, 271	Theogallin	Yao *et al*. (2004).
49.	4.53	233, 272	Theobromine	
50.	5.60	234, 275	Gallocatechin	
51.	6.30	235.5, 269	Epigallocatechin	
52.	7.58	233, 263	Catechin	
53.	11.78	236, 272	Epigallocatechin gallate	
54.	12.50	234, 277	Epicatechin	
55.	16.00	235, 273	Gallocatechin gallate	
56.	18.06	257, 306sh, 354	Quercetin 3-rhamnosylglucoside	
57.	18.77	237, 275	Epigallocatechin 3,5-digallate	
58.	20.67	234, 275.5	Epicatechin gallate	
59.	21.75	233, 277	Catechin gallate	
60.	22.75	255, 265sh, 353	Quercetin 3-glucoside	
61.	23.76	254, 262sh, 352.5	Quercetin glycoside	
62.	27.44	266, 346	Kaempferol 3-rhamnosylglucoside	

Note : sh means maximum of the shoulder in the spectrum.

The retention time, UV spectral characteristics and structure assignment of some phenolic acids and their derivatives (Cuvelier *et al*., 1996; Määttä *et al*., 2003; Yao *et al*., 2004) are shown in Table 5. The UV spectra of the derivatives of the same parent compound are similar. The results allow classification in the same way as the characteristic of flavonoids.

Table 5 Retention times, UV spectral characteristics and structure assignment of phenolic acids and their derivatives.

No.	R_t (Min)	UV spectral characteristics (nm)	Structure assignment	References
1.	6	260, 292	Vanilic acid	Cuvelier *et al.* (1996)
2.	7	242, 296, 324	Caffeic acid	
3.	14	244, 298, 324	Ferulic acid	
4.	19	290sh, 328	Rosmarinic acid	
5.	77	228sh, 282	Carnosic acid	
		*Hydroxybenzoic acid deriv*atives		Määttä *et al.* (2003)
6.	5.3	262	*p*-hydroxybenzoylhexose	
7.	6.3	264, 296	Vanilloyhexose	
		Hydroxycinnamic acid vative		
8.	6.5	*derivatives*	*p*-coumaric acid 4-*O*-glucoside	
9.	6.9	234, 296	Caffeoylhexose	
10.	9.7	244, 2300sh, 330	*p*-coumaroylhexose	
11.	10.1	236, 300sh, 314	Feruloylhexose	
12.	17.5	244, 296sh, 330]	*p*-coumaric acid hexose	
13.	17.8	236, 300sh, 314	derivatives	
		234, 296, 330	Ferulic acid hexose derivatives	
14.	3.09	232, 273	Gallic acid	Yao *et al*. (2004)
15.	4.97	235, 298sh, 323	Isochlorogenic acid	
16.	9.14	235, 301sh, 325	Chlorogenic acid	
17.	13.42	234, 310	*p*-coumaric acid	

Note: sh means maximum of the shoulder in the spectrum

Advances in chromatographic and spectroscopic techniques now permit the isolation and structural analysis of potent active constituents. There are also comprehensive electronic sources of separation information, which in larger part includes the major phenolics and their derivatives. ACD/Chromatography applications database is a summary of submissions such as Agilent Technologies, GL Science, Hamilton Company, Argonaut Technologies, Phenomenex, and Regis technologies. The ACD/Chromatography applications database brings "live" chromatograms with all necessary raw and elucidation data with peak to structure assignment in fully searchable format.

3.6.2 HPLC/Mass Spectrometry (LC/MS)

Mass spectrometric detection (MS) combined with chromatography provides, in addition to mass selective detection, a wealth of structural information (Covey *et al*, 1986). Older hyphenated MS techniques were associated with a number of problems in the analysis of phenolic compounds, such as the need of derivatisation for GC/MS, the technical complexity of moving belt (MB) and continuous-flow fast atom bombardment (CF-FAB), and the strong fragmentation of electron impact mass spectrometry (EI-MS) (Hedin and Phillips, 1992; Wolfender *et al*., 1993; Game and Martínez, 1989). Following the introduction of atmospheric pressure ionisation (API) techniques, electrospray ionisation (ESI) and atmospheric pressure chemical ionisation (APCI), these problems have been resolved and the hyphenated technique of HPLC and mass spectrometry (HPLC/MS) has become a powerful tool in the chemical characterisation of plant extracts and other samples of biological origin (Covey *et al*., 1986).

In ESI, the molecular mass of a compound is equal to the *m/z* of the molecular ion, however, many compounds do not give (abundant) molecular-ion peaks in ESI. Molecular ion peaks are, for instance, normally not detected for aliphatic alcohols, nitro-compounds and strongly branched compounds (Gall *et al.* 2003). Other experiments, involving ESI spectra generated at lower electron

energies, derivatization, and/or soft ionization methods, can be performed to further confirm the assignment. However, the selection of the soft ionization method must be done with some care since these methods show considerable selectivity (Tomer, 2001). The presence of the peaks in addition to the protonated molecule, e.g., at *m/z* +18, +23 and/or +39 due to adduct of ammonium (NH_4^+), sodium (Na^+) and/or potassium (K^+), respectively, are often helpful in assigning the correct molecular mass. The negative-ion mode, results in a peak at *m/z* +59 $[M+OAc]^-$. Furthermore, the use of both the positive-ion mode, resulting in the *m/z* of the $[M+H]^+$ ion/ M+1, and the negative-ion mode, resulting in the *m/z* of the $[M-H]^-$ ion/ M-1, also helps in unambiguously assigning the molecular mass. Negative ions generally have low abundance under soft ionization methods, hence analytical ESI-MS is restricted to the analysis of positive ions (Linscheid and Westmoreland, 1994).

4. Phenolic Antioxidant Effectiveness in Food

4.1 Edible Oils

The antioxidant activities of phenolic compound extracted from different sources have been studied in several bulk oil systems. Some natural antioxidants showed higher antioxidant activity than synthetic ones. For instance, the ethanolic extract of roasted wheat germ possessed stronger antioxidative properties in stripped corn oil than BHA and ascorbyl palmitate when measured by peroxide value and conjugated diene hydroperoxides (Krings *et al.,* 2000). In addition, the methanolic extracts of soybean seeds and soybean oil showed the potential to inhibit lipid oxidation in linseed oil (van Ruth *et al*., 2001). Besides, some phenolic compounds extracted from plant materials showed high antioxidant capacity on oil substrates such as alkanin, shikonin from *Alkanna tinctoria* root extracts (Assimopoulou *et al*., 2004), xanthophylls from Taiwanese orange peels (Yen and Chen, 1995), epicatechin (EC), epigallocatechin gallate (EGCG), epigallocatechin (EGC), gallic acid (GA) and epicatechin gallate (ECG) from cocoa (*Theobroma cacao* L.) leaves (Osman *et al*., 2003). It has been shown

recently that some phenolic substances isolated from natural sources can have pro-oxidant properties depending on the system employed and the presence of lipophilic or hydrophilic substances. Shahidi and Wanasundara (1998) found that green tea extract had a pro-oxidant offect on the oxidation of marine oils examined under Schaal oven condition at 65ºC. Also, the antioxidant activities of combinations of some plant extracts and other antioxidants and /or chemical substances were studied. Methanol extracts of oregano, thyme, marjoram, dittany, rosemary and sage combined with BHA, BHT, ascorbyl palmitate, and citric acid showed an additive antioxidant effect in lard (Banias *et al*., 1992). A synergistic effect of rosemary extract with BHT (Basaga *et al*. 1997) as well as with β-*carotene* and citric acid (Azeredo *et al*., 2004) on preventing oxidation of soybean oil was found.

4.2 Oil-in-Water Emulsions

An emulsion consists of two immiscible liquids (usually oil and water), one dispersed in the other in the form of small spherical droplets (Coupland and McClements, 1996). Some phenolic compounds or crude extracts from plants or plant foods such as from some Spanish and California wines prevented lipid oxidation in corn oil emulsions (Moreno-Sánchez *et al*., 2000). However, soy isoflavone exhibited pro-oxidant activity in sucrose fatty acid ester emulsions (Osborn-Barnes and Akoh, 2003). Phenolic compounds such as rosemary extracts showed different antioxidant effectiveness in bulk oil and in emulsion systems (Frankel *et al*., 1996; Kiokias and Gordon, 2003). This was attributed to interfacial phenomena, where hydrophilic antioxidants remained oriented in the air-oil interface, thus affording better protection against oxidation in oils. In contrast, non-polar antioxidants are more effective in emulsions because they form a protective membrane around the droplets. Some phenolic compounds, such as norbixin which is the annatto carotenoid, showed synergistic effects with ascorbic acid or ascorbyl palmitate in an emulsion system (Kiokias and Gordon, 2003).

4.3 Snack Foods

Snacks, such as deep fat fried, quick fried, roasted and extruded products, belong to a highly competitive, innovative and growing sector of the food market (Reilly and Man, 1994). The world snack food market has continued to grow reaching an estimated $66 billion in 2003. The United States continues to be the largest market, accounting for about a third of the world's total and Japan and the United Kingdom, together, another quarter of the world's total. The spread of western eating habits to other parts of the world continues as lifestyles in those parts of the world become busier, and traditional family meal times become a thing of the past. As a result, the demand for extruded snack foods continues to increase (Reilly and Man, 1994).

Extruded foods are susceptible to lipid oxidation. Lipid oxidation is a major cause of food deterioration, but limited success has been achieved in preventing this group of reactions from occurring in extruded foods. However, conventional control methods are often ineffective or not practical for extruded foods. Many manufacturers resort to more frequent rotation of retail stock to prevent consumers from purchasing rancid products (Campbell, 1992).

If extruder use is to increase for food and feed production, prevention or reduction of lipid oxidation must be improved in order to retain consumer acceptability (Grosso and Resurreccion, 2002). Composition, particularly degree of unsaturation and linoleic acid content, are important factors for determining the likelihood of oxidation for all foods. Factors that favour oxidation in extruded foods specifically include low moisture content, increased surface area due to expansion, and higher levels of iron, a catalyst for oxidation, caused by wearing of the screw and barrel during extrusion. Potato peel iron content increased 130-180% after extrusion (Camire *et al*., 1992). Transition metals can act as a pro-oxidant catalyst. Lipase and other enzymes that may contribute to oxidation are usually denatured during extrusion. Another factor that may retard oxidation is the formation of lipid amylose complexes during extrusion (Viscidi *et al*., 2004).

Consumers generally perceive natural antioxidants as better than synthetic additives. Phenolic compounds are one of the most important groups of natural antioxidants. Natural phenolic compounds added to snack food are widely used to retard lipid oxidation. The ways to add these compounds into products include direct addition of plants or extracts as ingredients (Lee *et al*., 2002; Reddy *et al*., 2005).

The addition of spices and herbs to foods limited due to their aroma (Karpińska *et al*., 2001). Some snacks include addition of natural phenolic compounds to grain prior to extrusion cooking because they may synergize and protect the endogenous antioxidants (Camire and Dougherty, 1998). However, most antioxidants are usually added in oil which is the medium for the flavoring step in commercial snack foods.

4.4 Analytical Methods for Determining Antioxidant Effectiveness in Foods

4.4.1 Edible Oils

Many new *in vitro* methods have been developed to evaluate antioxidant activity. Unfortunately, these *in vitro* methods often do not correlate with the ability of compounds to inhibit oxidative deterioration of foods. This is because the activity of antioxidants on food systems depends not only on the chemical reactivity of the antioxidant (e.g. free radical scavenging and chelation) but also on factors such as physical location, interaction with other food components, and environmental conditions (e.g., pH). One of the major factors affecting the activity of antioxidants that scavenge free radicals in foods is their partitioning behavior in lipids and water. Differences in the effectiveness of the antioxidants in bulk oils and emulsions are due to their physical location in the two systems. To accurately evaluate the potential of antioxidants in foods, models must be developed that have the chemical, physical, and environmental (e.g., pH and ionic strength) conditions expected in food products. Because these factors are not consistent throughout all food systems, individual models must be developed.

However, Decker *et al*. (2005) recommended general considerations for evaluation of food antioxidants for easy comparison of literature reports as follows;

a. Avoid high oxidation temperatures (>60°C) during storage studies because the mechanisms and kinetics of oxidation are not the same as at lower temperatures.

b. Ensure that the starting lipid does not contain high levels of oxidation products. Some oxidation products such as hexanal, 2,4-decadienal, free fatty acids act as pro-oxidants (Osborn-Barnes and Akoh, 2003). Thus, high levels of pre-existing lipid-oxidation products could result in the rapid decomposition of antioxidants. An AOCS collaborative study reported that corn, sunflower, canola and soybean oils oxidized to peroxide valves of 17.2-17.9 meq.kg^{-1} had significant levels of rancid and /or painty flavors (Warner and Nelson, 1996).

c. In order to effectively analyze the antioxidant activity, both primary (e.g., hydroperoxides, conjugated dienes) and secondary oxidation (TBARS, volatile compounds) products should be measured. Tocopherols can increase hydroperoxide levels by donating hydrogen to a peroxyl radical to form lipid hydroperoxides while simultaneously decreasing formation of secondary oxidation products. In addition, transition metals can decrease lipid hydroperoxides while increasing the secondary oxidation products that cause rancidity. Therefore, if one was only measuring either primary or secondary products as an index of lipid oxidation, we may reach the incorrect conclusion.

d. Types of fatty acid decomposition products formed during oxidation are related to the fatty acid composition of the oil. For example, hexanal is derived from the oxidation of omega-6-fatty acids such as linoleic acid. Therefore, it is essential to know the fatty acid composition of the oil being oxidized when a method for measuring volatile secondary oxidation products is chosen.

e. Use either crude extracts of biological materials or pure compounds as the source of antioxidants. If phenolic compounds are expected to be the major antioxidants in a crude extract, the total phenol content and composition of the crude extract should be reported in order to compare samples. Include a reference compound in the study such as BHA, BHT or tocopherol. Use the same molar concentration of tested components (e.g., phenolics) as the reference compound.

f. Use a food lipid source with a consistent source of natural endogenous antioxidants or pro-oxidants for all analyses because the antioxidant being tested can interact with endogenous antioxidants and pro-oxidants to alter oxidation kinetics. In some cases, variations in antioxidant concentrations can be minimized by isolating purified oils (triacylglycerols) that have been stripped of all minor oil constituents.

g. pH can affect oxidative reactions by influencing pro-oxidant (e.g., iron solubility increases with decreasing pH) and antioxidant (the pH can alter the charge of antioxidants, which can affect solubility and chelation capacity) activity. The pH of oxidation models should therefore be similar to the food of interest.

h. Standardize time and conditions to determine if the antioxidant is effective. Express antioxidant effectiveness as differences in induction period, percent inhibition of compound formation at a set time, rates of compound formation or decomposition, or percent loss or retention of antioxidant. These calculations should be conducted on data obtained from the early stages of oxidation because the concentrations of oxidation products in later stages will cease to increase or can even decrease. Statistical evaluation should be used to determine differences between controls (no added antioxidants) and samples.

4.4.2 Oil-in Water Emulsions

When antioxidant activity in emulsions is evaluated, it is important to utilize emulsion systems similar to those found in foods. Emulsions are

thermodynamically unstable because of the positive free energy needed to increase the surface area between oil and water phase. To form emulsions that are kinetically stable for a reasonable period, chemical substances known as emulsifiers must be added prior to homogenization. (In addition, the size of emulsion droplet will affect the physical stability of the emulsion. Emulsions should be prepared with similar droplet sizes (<1.0 mm), which can be verified by laser light scattering. The type of lipid used in emulsion studies should be triacylglycerols. Use of free fatty acids should be avoided because their acid group will migrate to the emulsion interfacial region. Many antioxidants in emulsions will partition into both the lipid and water phases of emulsions. Measurement of partitioning behavior can provide important insight into antioxidant behavior and can help predict if an antioxidant will be effective in oil from an emulsion.

4.4.3 Snack Foods

Methods for the evaluation of antioxidant effectiveness in snack foods can be classified into two groups; chemical and sensory analysis.

a. Chemical analysis; numerous analytical methods have been used to measure changes in fatty acid composition, peroxide (PV) value, thiobarbituric acid and reactive substances (TBARS) value, carbonyl compounds, oxygen absorption, redox potential, volatile secondary oxidation compounds, and fluorescence products which related to the extent of lipid oxidation (Wong *et al*., 1995; Aruoma *et al*., 1996; Baublis *et al*., 2000; Karpińska *et al*., 2001; McCarthy *et al*., 2001; Calligaris *et al*., 2004; Nissen *et al*., 2004).

For lipid oxidation in snack food, PV is one of the most widely used methods (Sharma *et al*., 1997; Viscidi *et al*., 2004). This method is used to evaluate hydroperoxides which are the primary oxidation products. Normally, the secondary oxidation products, such as malonaldehyde or hexanal, are related to sensory perception. Hwang *et al.* (1990) suggested that measurement of volatile components contributing to off-flavors should provide results that may more

closely correlate with sensory assessment. Hexanal, a decomposition product from 9- or 13-linoleic acid hydroperoxides has been used successfully for determination of the oxidative state of extruded food during storage (Viscidi *et al*., 2004). Besides, other carbonyl compounds (formaldehyde, acetaldehyde, propanal, butanal, pentanal, hexanal, heptanal, octanal, nonanal, decanal, butanone, octanone, pentenal, heptenal, octenal, decenal, decadienal) have been reported to be associated with the autoxidation of oleic, linoleic and linolenic acids in snack samples during storage (Sharma *et al*., 1997).

b. Sensory analysis; oxidation of the polyunsaturated fatty acid (PUFA) produces a complex mixture of volatile secondary oxidation products, which cause particularly objectionable off-flavors. Quantitative descriptive analysis (QDA) has been used to elucidate the mechanism of flavor development as well as to evaluate the efficiency of other analytical methods for measuring rancidity (Ravi *et al*., 2005). However, a wide variation exists among the reported methods that have been used such as variations in terminology and type of scale, evaluation of either aroma or flavor, or both, and use of 3-12 trained panelists (Ravi *et al*., 2005; Larsen *et al*., 2005; Ritvanen *et al*., 2005).

Terms used in descriptive analysis for extruded oat products were developed by Larsen *et al*. (2005). The descriptors used for describing odor attributes were oat odor, grass odor, hay odor, stearic odor, paint odor and off-odor, and those for describing flavor were oat flavor, sweet taste, bitter taste, grass flavor, hay flavor, stearic flavor, paint flavor, off-flavor, and aftertaste. They found a high correlation between odor and flavor scores (correlation coefficients in the range from 0.96 to 0.99). The intensity of oat odor and flavor was negatively correlated to the rancid odors and flavors.

MATERIALS AND METHODS

Materials

Soybean oil known to lack added antioxidants was provided by Thai Oil Industry, Bangkok, Thailand. The oil contained 115.7 $mg \cdot kg^{-1}$ of α-tocopherol; 918.7 $mg \cdot kg^{-1}$ of γ-tocopherol; 228.1 $mg \cdot kg^{-1}$ of δ-tocopherol and the peroxide value (PV) determined by AOCS official method cd 8-53 was 0.70 ± 0.12 $meq \cdot kg^{-1}$ oil; the thiobarbituric acid reactive substances (TBARS) value determined according to McDonald and Hultin (1987) was 0.0001 $mmol \cdot kg^{-1}$ oil.

Folin-Ciocalteu reagent, 2, 2-diphenyl-1-picrylhydrazyl (DPPH), sodium carbonate, hexamethyltetramine, aluminium chloride, rutin, ethanol, 2-propanol, potassium persulfate, and HPLC grade of chlorogenic acid, gallic acid, protocatechuic acid, hydroxybenzoic acid, vanillic acid, syringic acid, *p*-coumaric acid, *o*-coumaric acid, ellagic acid, trans-cinnamic acid, luteolin, myricetin, quercetin, apigenin and kaempferol were purchased from Sigma (Milwaukee, USA.). Butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), α-tocopherol, formic acid, 6-hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid (Trolox), chloroform, butanol, ascorbyl palmitate, acetic acid, sodium thiosulfate, potassium iodide were purchased from Fluka (Buchs, Switzerland). HPLC grade of methanol and water were purchased from Fisher Scientific (Leicestershire, United Kingdom). Acetonitrile (HPLC grade) was purchased from BDH (Poole, United Kingdom). 2, 2′-Azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS), 1-octanol, citric acid, 1, 1, 3, 3-tetraethoxypropane, Tween 20, isooctane and the other reagent - grade quality chemicals and solvents used in this experiment were purchased from Sigma-Aldrich (Gillingham, UK).

Methods

1. Preliminary Study to Investigate the Effects of Sample Storage, Extraction Conditions and Test Methods on the Determination of Antioxidant Activity

From literature reviews, there are many different sample storage conditions before extraction, extraction conditions and test methods reported, therefore a preliminary study to identify suitable conditions was conducted using Kradonbok leaves (*Careya sphaerica* Roxb.), Teaw leaves (*Cratoxylum formosum* Dyer.) and Phak whan ban leaves (*Sauropus andrugynus* Merr.) for analysis as follow;

1.1 Effect of Sample Storage before Extraction

Five different sample preparation methods were investigated for storage prior to extraction. These sample preparation methods were (1) hot air drying by tray dryer at 40°C for 18 h (Bocco *et al*., 1998) with air velocity about 0.5 $m \cdot s^{-1}$; (2) vacuum drying by vacuum dryer at 40°C for 10 h, 100 mm Hg (EYELA, model VOS-300SD, Japan); (3) air drying at room temperature 25°C for 12 h with air velocity about 3.2 $m \cdot s^{-1}$; (4) slow freezing by cooling fresh leaves in a freezer at -30°C after packing in an HDPE bag; (5) fast freezing by contacting with dry ice and further freezing at -30°C. All dried samples were stored at room temperature in PE bag while frozen samples were stored in the freezer at -30°C until extraction for the yield, total phenolic content and DPPH radical scavenging activity determination. The suitable storage condition was chosen by considering the condition that retained a high antioxidant activity.

All samples were sampled every 10 days. The samples prepared by drying methods were analyzed for their water activity using a thermoconstanter (Novasina, Zurich, Switzerland) at 25°C (Pongsawatmanit *et al*., 2002) during the storage period. The plant extracts were obtained by extracting the ground leaves (60 g) with 95% ethanol (300 mL) for 30 min at room temperature with shaking to

ensure the complete extraction (modified from Velioglu *et al*., 1998), then filtering through a Whatman No.4 filter paper. Samples were centrifuged (15 min, 1500g). The supernatant was evaporated under reduced pressure (50°C, 50 mmHg), kept in airtight amber bottles after flushing with nitrogen gas for 30 s (Azizah *et al*., 1999). The extracts were stored in a freezer at -20°C until they were analyzed for total phenolic compounds and DPPH radical activity (EC_{50}). The fresh sample leaves were also investigated for comparison. Extraction was replicated at least 3 times.

1.2 Effect of Extraction Time

The extract obtained in highest yield and with highest antioxidant activity obtained from the optimal sample preparation method (from part 1.1) was selected to study the effect of extraction time (0.25, 0.5, 1, 3, 4.5, 6, 24, 48, 96 and 168 h). The extraction procedure was carried out according to 1.1 by varying only the extraction time. The extracts were evaluated for their yield, total phenolic content and DPPH radical scavenging activity (EC_{50}).

1.3 Effect of Solvent Ratio on Antioxidant Activity

The appropriate extraction time was selected to study the effect of solvent ratio (80, 95 and 99% ethanol in water) on antioxidant activity assessed by the *β-carotene* bleaching and DPPH radical scavenging methods. The extraction procedure was carried out according to 1.1 by using the appropriate extraction time and varying only the solvent ratio. The extracts were evaluated for their yield, total phenolic content and DPPH radical scavenging activity as well as antioxidant activity assessed by the *β-carotene* bleaching method.

1.4 Assessment Methods

1.4.1 Determination of Plant Extract Yield

The yield of evaporated extracts on a dry weight basis was calculated from equation (10) shown below:

$$\text{Yield (\%)} = (W_1 \times 100)/W_2 \quad (10)$$

Where W_1 was the weight of extract after evaporation of ethanol and W_2 was the dry weight of the fresh plant sample.

1.4.2 Determination of Total Phenolic Content

The total phenolic content of ethanolic extracts was determined using the Folin-Ciocalteu reagent (Kähkonen *et al*., 1999). Each evaporated thick and viscous extract (~0.8-0.9 g ± 0.01 mg) was diluted with 5 mL methanol. The sample of each plant extract solution (200 μL) was transferred into a test tube and then mixed with one mL of Folin-Ciocalteu reagent thoroughly. After mixing for 3 min, 0.8 mL of 7.5% (w/v) sodium carbonate was added. The mixtures were agitated with a vortex mixer, then allowed to stand for a further 30 min in the dark, and centrifuged at 3300 g for 5 min. The absorbance of plant extracts and a prepared blank were measured at 765 nm using a spectrophotometer (UV-Vis model 1601, Shimadzu, Japan). The concentration of total phenolic compounds in all plant extracts was expressed as milligrams of gallic acid equivalents per gram dry weight of plant using the linear equation (11) derived from equation (12) which was determined from known concentrations of gallic acid standard analyzed similarly. Data were reported as a mean ± standard deviation for three replications.

$$\text{Absorbance (at 765 nm)} = \text{constant} \times (\text{gallic acid concentration}) \quad (11)$$

$$\text{Gallic acid equivalents} = \text{Absorbance (at 765 nm)}/\ 0.0508 \quad (12)$$

1.4.3 Determination of Free Radical Scavenging Using DPPH

The free radical scavenging activity of plant extracts was evaluated using the stable radical DPPH according to the method of Masuda *et al*., (1999). A series of extract concentrations with different ratios of extract to methanol, i.e. 1:10, $1:10^2$, $1:10^3$, $1:10^4$, $1:10^5$, $1:10^6$, $1:10^7$, were prepared. Then, 4.9 mL of each diluted plant extract was mixed with 100 µl of 5 mM DPPH in methanol. The mixtures of different extract concentrations and DPPH were placed in the dark at 37°C for 30 min. The absorbance of each sample of plant extract containing DPPH (A_1) was read at 517 nm using a spectrophotometer (UV-Vis model 1601, Shimadzu, Japan). The absorbance of each sample of plant extract dilution without DPPH (A_s), and only DPPH solution without plant extract (A_o, called control) were also recorded to determine the DPPH radical scavenging activity (modifying the method of Tachibana *et al*., 2001). All determinations were performed in triplicate. The percentage of DPPH radical scavenging activity of each plant extract determined at these seven concentrations within the range of dose-response (at least 10-90% reduction in absorbance) was calculated as shown in equation (13)

$$\text{DPPH radical scavenging activity (\%)} = [A_o - (A_1 - A_s)] / A_o \times 100 \quad (13)$$

Where A_o is the absorbance of the control solution (containing only DPPH), A_1 is the absorbance in the presence of the plant extract in DPPH solution and A_s, which is used for error correction arising from unequal color of the sample solutions, is the absorbance of the sample extract solution without DPPH.

The percentage of DPPH radical scavenging activity was plotted against the plant extract concentration ($\mu g \cdot mL^{-1}$) to determine the amount of extract necessary to decrease DPPH radical concentration by 50% (called EC_{50}). The EC_{50} value of each extract was estimated by sigmoid non-linear regression using SigmaPlot 2000 Demo (SPSS Inc., Chicago, IL, USA).

1.4.4 Determination of Antioxidant Activity by the *β-Carotene* Bleaching Method

The antioxidant activities of extracts were determined by modifying the method of Makris and Rossiter (2001). In a round-bottom flask, 0.02 mL of linoleic acid, 0.2 mL of Tween 20, 1.0 mL of *trans-β-carotene* (0.2 g·mL^{-1} in chloroform), were added, and the mixture was taken to dryness under reduced pressure (50°C, 50 mm Hg). To the resulting residue, 50 mL of oxygenated distilled water and 0.2 mL of each sample (2 mg·mL^{-1} in 99% ethanol) were added. Pure ethanol (0.2 mL) was used as the control, and the blank contained all the earlier chemicals except *β-carotene*. All these mixtures were then vigorously shaken to form an emulsion. The emulsion was then incubated in a water bath at 50°C to induce autoxidation. The absorbance at 470 nm (UV-VIS 1601 spectrophotometer, Shimadzu, Japan) was monitored at 10-min intervals. Flasks were covered with aluminum foil throughout experiments to avoid light-induced oxidation of *β-carotene*. All samples were assayed in duplicate. BHT (2 mg·mL^{-1} in 99% ethanol), BHA (2 mg·mL^{-1} in 99% ethanol) and α-tocopherol (2 mg·mL^{-1} in 99% ethanol) were used for comparison. The bleaching rate (R) of *β-carotene* was calculated by;

$$R = ln\ (a/b) / t \qquad (14)$$

Where: *ln* = natural log, a = absorbance at time 0, b = absorbance at time *t,* and t = 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, or 120 min. The antioxidant activity (A_A) was calculated in term of percent inhibition relative to that of sample without leaf extracts (control) by using the equation:

$$A_A = [(R_{control} - R_{sample}) / R_{control}] \times 100 \qquad (15)$$

Where $R_{control}$ and R_{sample} represent the bleaching rates of *β-carotene* without and with the addition of antioxidant leaf extract samples, respectively. The antioxidant rate ratio (R_{OR}) was calculated by:

$$R_{OR} = R_{sample} / R_{control} \quad (16)$$

In addition, an antioxidant activity coefficient (C_{AA}) was calculated using:

$$C_{AA} = [(A_{s(120)} - A_{c(120)})/A_{c(0)} - A_{c(120)})] \times 100 \quad (17)$$

Where $A_{s(120)}$ is the absorbance of the sample containing antioxidant at t = 120 min, $A_{c(120)}$ is the absorbance of the control at t = 120 min, and $A_{c(0)}$ is the absorbance of the control at t = 0 min.

1.5 Statistical Analysis

The data are mean values of at least three replicate experiments. Analysis of variance was carried out and significant difference among treatments was determined by Duncan's multiple range test ($P < 0.05$).

2. Determination of the Antioxidant Potential of Thai Indigenous Plants

The appropriate sample, extracting condition and testing method were used to screen antioxidant potential of 26 Thai indigenous plants.

2.1 Preparation of Plant Extracts

Twenty-six plant materials of various varieties and plant parts, classified into 3 groups as berries and fruits, herbs and vegetables, and chewy plants (Table 6), were selected for study. One batch of each plant material was obtained (at least

30 kg) from a wholesale market in March to June 2002 except for the seed of fruits and berries, which were obtained from food processing factories as by-products. The plant parts included fruit, fruit peel, fruit flesh, seed, seed skin, bud, flower, leaf, as well as tree parts such as stem core and bark. The moisture content of fresh plant material was determined according to AOAC (1995). The collected plant samples were frozen immediately after arrival and stored at -30°C until extraction time with less than two months storage. From preliminary tests, these extracts from frozen samples gave a free radical scavenging capacity and total phenolic content comparable to those of extracts prepared from fresh plants. The frozen plants were ground in a blender for 1 min. Ground sample (60 g) was mixed with 95% ethanol (300 mL) in the dark at 25°C for 4.5 h and shaken during the extraction time to ensure complete extraction (method modified from Velioglu *et al.,* 1998). The extracts were filtered through Whatman No. 4 paper and centrifuged (15 min, 1500g). Ethanol was evaporated from the supernatants on a rotary evaporator at 50 mm Hg pressure and 50°C. The evaporated plant extracts were thick and viscous materials and were kept in air-tight amber bottles after flushing with nitrogen gas for 30 s (Azizah *et al.,* 1999) and stored in a freezer at -20°C until they were analyzed. Extraction was repeated on a fresh batch of plant material until at least 50 g of extract was collected for each plant material.

2.2 Assessment Methods

2.2.1 Determination of Ethanolic Plant Extract Yield

The yields were determined according to 1.3.1

2.2.2 Determination of Total Phenolic Content

The total phenolic content were determined according to 1.3.2

Table 6 Scientific name and common name of various plant parts from Thai indigenous plants

Scientific name	Common name	Plant part
Berries and fruits		
Antidesma velutinum Tulas.		Seed
Cleistocalyx operculatus var *paniala* (Roxb.)		Seed
Diospyros kaki L.	Persimmon	Whole fruit
		Fruit peel
		Fruit flesh
Eugenia siamensis Craib.	Jambolan Plum	Seed
Garcinia mangostana Linn.	Mangosteen	Fruit peel
Leucaena glauca Benth	Leadtree	Seed
Mangifera indica Linn.	Mango	Seed
Nephelium lappaceum Linn.	Rambutan	Fruit peel
		Seed
Nephelium hypoleucum Kurz	Pulasan	Whole fruit
Piper nigrum Linn.	Pepper	Seed
Spondias pinnata Kurz	Hog plum	Fruit flesh
		Seed
Tamarindus indica Linn.	Tamarind	Seed
		Seed skin
Herbs and vegetables		
Basella alba Linn.	Ceylon spinach	Young leaf
Careya sphaerica Roxb.	Tummy wood	Young leaf
Cratoxylum formosum Dyer.		Young leaf
Hydrocharis dubia (Bl.) Back.	Frogs bit	Young leaf
Hydrolea zeylanica (L.) Vahl.		Young leaf
Lasia spinosa(Linn.) Thw.		Young leaf
Leucaena glauca Benth	Leadtree	Young leaf
Limnocharis flava Buch.		Young leaf and flower
Momordica charantia Linn.	Balsum pear	Young leaf and leaf
Sesbania grandiflora Desv.	Cork wood	Flower
Spondias pinnata Kurz	Hog plum	Young leaf
Syzygium gratum (Wight) S.N.Mitra var. gratum		Young leaf
Chewing plants		
Acacia catechu (L.F) Willd.	Black catechu	Bark
Areca catechu Linn.	Betel nut	Whole fruit
		Kernel
Cassia fistula Linn.	Golden shower	Stem core
Piper betel Linn.	Betel leaf	Leaf

2.2.3 Determination of Total Flavonoid Content

The total flavonoid content of plant extracts was evaluated by a colorimetric assay according to the method of Bonvehí *et al.*, (2001). One milliliter of 0.5% (w/v) hexamethyl tetramine, 20 mL of acetone, and 2 mL of 0.1 M HCl were added to each finely ground thawed-frozen plant sample (5 g) and boiled under reflux for 30 min. The resulting solution was filtered through Whatman paper No. 4 and the residue was further washed with 20 mL of acetone. The filtrate volume was finally adjusted to 100 mL with acetone. Ten milliliters of filtrate from each plant extract was pipetted into a separating funnel, along with 20 mL of H_2O and then the aqueous phase was extracted with 25 mL of ethyl acetate. Further extraction with 25 mL ethyl acetate was carried out at least twice. The extraction was repeated twice using another 50 mL of H_2O each time. The total amount of extract in the ethyl acetate layer collected from the separating funnel was subsequently made up to 100 mL with ethyl acetate. To determine the total flavonoid content, ten milliliters of extract in ethyl acetate was pipetted into a test tube and mixed with 1 mL of 2% (w/w) $AlCl_3$ in methanol solution containing 5% acetic acid using a vortex mixer. The absorbance was read immediately at 425 nm using a spectrophotometer (UV-Vis model 1601, Shimadzu, Japan). The absorbance of a prepared blank was also recorded. Total flavonoid content expressed as rutin equivalents in milligrams per gram dry weight of plant was also determined using the linear equation (18) from a standard curve of rutin standard. Data were reported as mean ± standard deviation for three replications.

$$\text{Rutin equivalents} = \text{Absorbance (at 425 nm)}/\ 13.33 \qquad (18)$$

2.2.4 Determination of Free Radical Scavenging Using DPPH

The DPPH radical scavenging activity was determined according to 1.3.3. The unit of EC_{50} was later converted to $\mu g \cdot \mu g^{-1}$ DPPH. These values were

changed to antiradical activity (A_{AR}) defined as $1/EC_{50}$: the higher antioxidant activity, the higher the value of the antiradical activity.

2.2.5 Statistical Analysis

Each of the measurements described above was carried out in at least three replicate experiments, and the results are reported as the mean and standard deviation.

The three most active plant materials were selected to identify and quantify major phenolic compounds which act as antioxidants.

3. Evaluation of Phenolic Radical Scavenging Components in Selected Plants by HPLC-DAD and HPLC-ESI-MS

The ethanol extracts from the 3 varieties of plant which showed the strongest antioxidant potential from part 2 were selected to study the acute toxicity and the phenolic DPPH radical scavenging constituents in the extracts

3.1 Preparation of Plant Extracts

The fresh plant leaves (80 g) were blended for 1 min with ethanol (400 mL) at -20°C and the containers were then flushed with nitrogen and shaken for 4.5 hours in the dark at 25°C. The supernatant, after filtration through cheesecloth and Whatman No 4 filter paper, was evaporated under vacuum. Sample was dried in a freeze dryer and stored in aluminum foil after flushing with nitrogen at -20°C.

3.2 Toxicity of Plant Extracts

The acute toxicity of plant leaf extracts was investigated in mice by the Medicinal plant research institute, Department of Medicinal Science, Ministry of Public Health, Thailand. The extracts were dissolved in distilled water and the concentration was adjusted to 0.8 $g \cdot mL^{-1}$. Acute toxicity was investigated in ten mice (five of each sex) by oral administration of two doses of 16 $g \cdot kg^{-1}$ body weights in two equal amounts, 6 hours apart.

3.3 Analysis of Radical Scavenging Components by HPLC-DAD

Each dry plant extract (1 mg) was dissolved in methanol (3 mL) and passed through a Sep-Pak C18 cartridge (Waters, Milford, MA.). The C-18 cartridge was first conditioned by suction with 1 column volume of methanol followed by 2 column volumes of a 3% HCl solution (v/v) in HPLC grade water. The cartridge was not allowed to dry out during conditioning. The aqueous sample extract was then transferred to the cartridge. The cartridge bed was then rinsed with HCl (3%, 5 mL) and air-dried under vacuum for ~10 min. Phenolic compounds were eluted with HPLC grade methanol (2 mL).

The analytical solution (100 μL) of eluted phenolic compounds was mixed with freshly prepared 5 mM DPPH methanol solution (100 μL). The mixture was well stirred and allowed to stand at 25°C for 5 min. Samples were filtered through a 0.20 μm Millipore filter (type HA) into a 2 mL autosampler vial for subsequent analysis by HPLC. The solution (10 μL) was injected into the HPLC and analyzed according to the following conditions: column, Synergi Hydro RP column (150 × 4.6 mm id., 4μm, Phenomenex), fitted with a Allsphere ODS-2 guard column (10 × 4.6 mm id., Alltech). The HPLC system was equipped with diode array detector (Dionex PDA 100 photodiode array, USA) controlled by Chromeleon software version 6.60 Build 1428 (Dionex Corporation, Sunnyvale, USA). Chromatograms were recorded at 260, 280 and 320 nm.

For the Teaw extract, solvent A was 1% formic acid in water and solvent B was 100% acetonitrile. The flow rate was 0.5 $mL \cdot min^{-1}$. The gradient applied was as shown in Table 7;

Table 7 HPLC condition for analysis of the Teaw extract

Time (min)	Solvent A (%)	Solvent B (%)
0	90	10
10	90	15
39	60	40
49	90	10
55	90	10

For the Kradonbok extract, solvent A was 1% formic acid in water, solvent B was 100% acetonotrile and solvent C was 100% methanol. The flow rate was 0.5 $mL \cdot min^{-1}$. The gradient applied was as shown in Table 8;

Table 8 HPLC condition for analysis of the Kradonbok extract

Time (min)	Solvent A (%)	Solvent B (%)	Solvent C (%)
0	95	0	5
5	90	5	5
60	85	5	10
90	90	10	0
180	75	35	0
185	95	0	5
190	95	0	5

For the Kratin extract, solvent A was 1% formic acid in water and solvent B was 100% acetonotrile. The flow rate was 0.5 $mL \cdot min^{-1}$. The elution gradient was as shown in Table 9;

Table 9 HPLC condition for analysis of the Kratin extract

Time (min)	Solvent A (%)	Solvent B (%)
0	90	10
5	85	15
40	60	40
45	90	10
55	90	10

3.4 Identification of Extract Components by HPLC-ESI-MS

Phenolic identification of potent antiradical peaks was determined by comparison of the retention time and UV spectrum and also by HPLC-ESI-MS chromatographic separation. The HPLC system consisted of a Shimadzu HPLC, Model LC-10ADvp two pumps, DGU-14A degasser, SIL-10ADvp autosampler, CTO-10ASvp column heater, and a SPD-10Avp detector controlled by SCL-10Avp for LC. The mass detector was an LCMS-2010A trap equipped with an electrospray ionization (ESI) system and controlled by LCMS solution software. Nitrogen was used as nebulizing gas at a pressure of 6 bars, and the flow was adjusted to 1.5 $L \cdot min^{-1}$. The heated capillary and voltage were maintained at 230°C and 1.7 kV, respectively. The full scan mass spectra of phenolic compounds were measured from m/z 650 up to m/z 1000. Mass spectrometry data were acquired in the positive and negative ionization mode. The HPLC conditions were the same as described above for each plant extract.

3.5 Assessment of Free Radical Scavenging Activity

The total free radical scavenging capacity of each plant extracts was determined and compared to that of α-tocopherol, BHT and chlorogenic acid by using the DPPH and ABTS methods.

3.5.1 Determination of Free Radical Scavenging Using DPPH

The DPPH radical scavenging activity was determined according to 2.2.4

3.5.2 Determination of Free Radical Scavenging Using ABTS

The ABTS radical scavenging activity was determined according to Re *et al.*, (1999). This method is based on the ability of antioxidants to quench the long-lived ABTS radical cation, a blue/green chromophore with characteristic absorption at 734 nm, in comparison to that of Trolox, a water soluble vitamin E analogue. The ABTS radical cation was prepared by reacting an aqueous solution of ABTS (7 mM) with potassium persulfate (2.45 mM, final concentration), which was kept in the dark at 25°C for 12-16 hours. The solution was diluted in ethanol to an absorbance of 0.70 (± 0.20) at 734 nm before use. Aliquots of Trolox or sample in water (20 μL) were added into 2 mL of this diluted solution, and the absorbance at 734 nm was determined at 30°C exactly 6 min after initial mixing. Appropriate solvent blanks were run in each assay. The antioxidant solution reduces the radical cation to ABTS which reduces the color. The extent of decolorization is calculated as percentage reduction of absorbance, and this is determined as a function of concentration and calculated relative to the equivalent Trolox concentration. The activity of each antioxidant was determined at three concentrations, within the range of the dose-response curve of Trolox, and the radical-scavenging activity was expressed as the Trolox equivalent antioxidant capacity (TEAC), defined as mmol of Trolox per gram of sample.

3.6 Statistical Analysis

Each experiment, from sample preparation to analysis, was repeated in triplicate, and the data were then analyzed by SPSS software program (SPSS Inc., Chicago, IL, USA). The general linear model procedure was applied and Duncan's

multiple range test was used to compare the mean values at $P < 0.05$. Mean values and standard error of the mean (SEM) were calculated.

4. Comparison of the Antioxidant Activity with Commercial Antioxidant in Oil and Emulsion Models

The oxidation of oil and emulsions, which contained the three selected varieties plant material as described in part 3 and BHT, α-tocopherol, ascorbyl palmitate, citric acid and chlorogenic acid was studied to compare the antioxidant activity.

4.1 Preparation of Plant Extracts

The extracts were prepared according to 3.1

4.2 Preparation of Oil and Oil-in-Water Emulsions

The tocopherols were removed from soybean oil by column chromatography using alumina with some modifications to the method described by Yoshida (1993). Refined oil (200 g) was passed through a column containing activated aluminium oxide (140 g) dried at 200°C for 8 hours before use. The column was wrapped in aluminium foil to avoid oxidation. The oil was drawn through the column by suction without a solvent. The oil collected was again passed through fresh alumina (140g) to complete the removal of the tocopherols. The oil was analyzed by HPLC to confirm the removal of the tocopherols. The oil was stored at –70°C until required for use.

Antioxidants were added to stripped soybean oil or 10% oil-in-water emulsion in the following quantities: 100 $mg \cdot kg^{-1}$ of crude Teaw extract, crude Kradonbok extract, crude Kratin extract, α-tocopherol, BHT, ascorbyl palmitate or citric acid; and 60 $mg \cdot kg^{-1}$ of chlorogenic acid.

Oil-in-water emulsion (300 g, 10% oil) was prepared by mixing a solution of sodium acetate buffer (267 g, 0.1 M, pH = 5.5) containing Tween 20 (3 g, 1%) and sodium azide to inhibit microbial growth (0.0195 g, 1mM). Stripped soybean oil (29.97 g) containing required antioxidants was added dropwise, as the sample was cooled in an ice bath and homogenized with a sonicator (Branson model 5210, Germany). Sonication was continued for 5 min after the oil had been added. The emulsion was then homogenized with a high-pressure homogenizer (Armfield model FT9, UK) in three cycles at 3000 psi. The droplet size distribution of the emulsion was determined by a static light-scattering technique, with a Malvern Instruments particle and droplet sizer (S version 2.19, He-Ne laser source; wavelength 633 nm, beam length 2.40 mm). The mean droplet diameter (μm) of the emulsion was characterized by d_{32} where:

$$d_{32} = \sum n_i d_i^3 / \sum n_i d_i^2 \qquad (19)$$

Where n_i is the number of droplets with diameter d_i. It was found that the mean d_{32} value for the emulsions was 0.42 ± 0.00 μm.

4.3 Oxidation and Analysis

Stripped soybean oil and oil-in-water emulsion samples with or without added antioxidant were transferred to screw-capped sample vials with aluminium foil wrapping and held in an oven at 60°C for 12 days and 9 days, respectively. The lids were only screwed loosely on the vials, therefore the air could pass in and out of the headspace above the samples. Aliquots (10 g) were removed every 3 days for analysis. The oxidative state of each sample was monitored by analysis of the peroxide value (PV) and thiobarbituric acid reactive substances (TBARS).

4.3.1 Determination of Peroxide Value

Peroxide value of stripped soybean oil was determined according to AOCS official method Cd 8-53 with an automatic titrator (Mettler-Toledo model DL 5X, Switzerland) equipped with stirrer and redox electrode. The solution was titrated against standard sodium thiosulfate (0.01 M). PV was calculated and expressed as milliequivalents peroxide per kg of sample:

$$PV\,(\text{meq}\cdot\text{kg}^{-1}) = \frac{(S-B)\times N\times F}{W}\times 1000 \qquad (20)$$

Where S is the titre (mL) for the sample; B is the titre (mL) for the blank, M is the molarity of the sodium thiosulfate solution, F is the factor from standardization with potassium dichromate and W is the sample weight (g).

Peroxide value of stripped soybean oil-in-water emulsion was determined by adding emulsion (0.3 mL) to isooctane: 2-propanol (3: 2 v/v, 1.5 mL) followed by vortexing three times for 10 s each. After centrifugation for 2 min at 1000 g, the clear upper layer (0.2 mL) was collected, and peroxides were quantified using a method based on that of Diaz *et al*., (2003). The sample extract (0.2 mL) was mixed with methanol/1-butanol (2:1 v/v, 2.8 mL) and thiocyanate/Fe^{2+} solution (30 μL) and then vortexed. The thiocyanate / Fe^{2+} solution was made by mixing thiocyanate solution (3.94 M) and Fe^{2+} solution (the supernatant of a mixture of equal volumes of 0.144 M $FeSO_4$ and 0.132 M $BaCl_2$ in 0.4 M HCl, 0.072 M). After 20 min of incubation at room temperature, the absorbance was determined at 510 nm. Lipid peroxide concentration was determined using a cumene hydroperoxide standard curve.

4.3.2 Determination of TBARS Value

TBARS of oil and emulsion were determined according to Mcdonald and Hultin (1987). Oil (0.1 mL) or emulsion (1 mL) was mixed with water (0.9

mL) and TBA reagent (2.0 mL, 15% w/v trichloroacetic acid and 0.375% w/v thiobarbituric acid in 0.25 M HCl) in test tubes and placed in a boiling water bath for 15 min. The tubes were cooled to room temperature for 10 min and then centrifuged (1000 × g) for 15 min. The absorbance was measured at 532 nm. Concentrations of TBARS were determined from a standard curve prepared using 1, 1, 3, 3-tetraethoxypropane.

4.4 Effects of Ferric Ions on Antioxidant Activity

The chelation of ferric ions by chlorogenic acid, α-tocopherol and citric acid was also investigated. Metal ions were removed from stripped soybean oil (100 g) by washing with 5% citric acid (100 mL) in a separating funnel. This was repeated three times and the oil was then washed with water (100 mL) twice and saturated sodium chloride solution (100 mL). The oil was then dried with anhydrous sodium sulfate (5 g). Ferric chloride (5 mM) and each antioxidant were added into the stripped, deionised soybean oil. Each sample was stored in a screw-capped sample vial wrapped in aluminium foil in an oven at 60°C for 48 hours. Aliquots (10 g) were removed every 2 hours for analysis. The oxidative state of the stripped soybean oil sample was monitored by analysis of the peroxide value (PV) and thiobarbituric acid reactive substances (TBARS). The storage was performed in triplicate for each sample.

4.5 Determination of Oil-Water Partition Coefficient

The partition coefficients of the Teaw extract, α-tocopherol, BHT and chlorogenic acid partitioned between soybean oil and water were determined. Soybean oil (2 g) was weighed into a screw-capped centrifuge tube containing test samples dissolved in HPLC water (2 g) for plant extract and chlorogenic acid. The sample was vortexed three times for 20 s with a 20 s interval. The samples were then centrifuged at 10000 ×g for 30 min at 20°C in a refrigerated centrifuge (Sorvall model RC5B, USA). The lower layer was removed from the centrifuge

tube with a syringe. The concentrations of the phenolic components in the aqueous phase were determined quantitatively by reversed-phase HPLC. Samples were filtered through a 0.20 μm Millipore filter (type HA) into a 2 mL autosampler vial for subsequent analysis by HPLC. The solution (10 μL) was injected into the HPLC and analyzed according to 3.3 for the HPLC-DAD condition and system. Chromatograms were monitored at 280 nm. The concentration of each phenolic compound in the plant extract was quantified using gallic acid as an external standard. The concentration of each phenolic compound in the oil phase (C_{oil}) was calculated as the difference between the total amount of antioxidant in the water before mixing and the amount in the water after mixing with oil (C_{water}). The Partition Coefficient (log *P*) was calculated as log (C_{oil}/ C_{water}).

BHT and α-tocopherol were dissolved in oil instead of water before mixing of the phases. After separation of the water and oil layers by centrifugation as described above, the concentration of these substances were analyzed in the oil phase by normal phase HPLC. The analysis of α-tocopherol was carried out on an HP series 1100 chromatograph (Hewlett-Packard, Avondale, PA) with detection at 292 nm. A normal phase silica column (250 × 8 mm id., 5 μM, Spherisorb 5 Silica 5U) was used with the mobile phase, hexane/isopropanol (99.5: 0.5) at a flow rate of 1 $mL \cdot min^{-1}$. Peaks were recorded and integrated using the HP Chemstation Chromatography Data System. Partition coefficients (log *P*) were calculated as above.

4.6 Assessment of Free Radical Scavenging Activity

The total free radical scavenging capacity of the aqueous layer of each antioxidant after partition was determined by using the ABTS method according to 3.5.2.

4.7 Statistical Analysis

Each experiment, from sample preparation to analysis, was repeated in triplicate, and the data were then analyzed by SPSS software program (SPSS Inc., Chicago, IL, USA). The general linear model procedure was applied and Duncan's multiple range test was used to compare the mean values at $P < 0.05$. Mean values and standard error of the mean (SEM) were calculated.

5. Determination of Synergistic Effects of the Plant Antioxidants in Oil and Emulsion Systems

The selected plants as described in part 3 which showed the strongest antioxidant activity were used to study synergistic effects with ascorbyl palmitate and citric acid.

5.1 Preparation of Plant Extracts

The extracts were prepared according to 3.1

5.2 Preparation of Oil and Oil-in-Water Emulsions

Oils and oil-in-water emulsions were prepared according to 4.2. Mixtures of antioxidants were added to stripped soybean oil or 10% oil-in-water emulsion in the following quantities: 100 $mg \cdot kg^{-1}$ of crude plant extracts (Teaw extract, Kradonbok extract and Kratin extract), α-tocopherol, BHT and 60 $mg \cdot kg^{-1}$ of chlorogenic acid with 100 $mg \cdot kg^{-1}$ of ascorbyl palmitate or citric acid.

5.3 Oxidation and Analysis

Oxidation and analysis of oil and emulsion systems were performed as described in 4.3

5.4 Synergy

Percent synergy between plant extracts, α-tocopherol, BHT, chlorogenic acid and ascorbyl palmitate, or citric acid was calculated using the equation:

$$\%Synergy = \frac{IPmixture - (IPantioxidant + IPadditive)}{(IPantioxidant + IPadditive)} \times 100 \qquad (21)$$

Where *IP* = induction period of sample to reach the specific oxidation level of PV equal to 50 meq·kg^{-1} oil.

5.5 Statistical Analysis

Each experiment, from sample preparation to analysis, was repeated in triplicate, and the data were then analyzed by SPSS software program (SPSS Inc., Chicago, IL, USA). The general linear model procedure was applied and Duncan's multiple range test was used to compare the mean values at $P < 0.05$. Mean values and standard error of the mean (SEM) were calculated.

6. Application of the Selected Plant Antioxidant in Food

Rice snack was selected to study the efficiency of antioxidant activity for the selected plant (Teaw extract and dried Teaw leaf powder), BHT, tocopherol and control (no antioxidant).

6.1 Rice Snack

The sticky rice snack was donated by Kanom Sakol Co., Ltd., Prathumthanee province, Thailand.

6.2 Preparation of dried Teaw Leaf Powder

Teaw leaves were purchased from the market in October, 2005. The plant was dried by air drying at room temperature 25°C for 12 h with air velocity about 3.2 $m \cdot s^{-1}$ with electric fan. Dried Teaw leaves were blended to be powder before mixing in oil to coat onto a rice snack.

6.3 Preparation of Rice Snack Coated with Antioxidants

Antioxidants were added to refined soybean oil, which had natural tocopherol and lacked added antioxidants, in the following quantities: 100 $mg \cdot kg^{-1}$ of crude Teaw extract, α-tocopherol, and BHT. The oil containing 2.44 $g \cdot kg^{-1}$ dried Teaw leaf powder, giving the same concentration of Teaw extract as 100 $mg \cdot kg^{-1}$, was included to compare.

The sticky rice snack was coated with 10% oil containing antioxidants without any seasoning. After that, 3 pieces (~10 g) of the rice snack were packed into OPP/PE/LLDPE, 80 μm thickness with packaging size 11 x 13 cm^2. The plastic film contains three layers of oriented polypropylene, OPP, (moisture permeability = 0.26 $g \cdot mm \cdot m^{-2} \cdot d^{-1}$ at 38°C/90% of relative humidity (RH) /20μm/20°C, oxygen permeability = 2000 $cc \cdot m^{-2} \cdot d^{-1} \cdot atm^{-1}$ at 85%RH), polyethylene, PE, (moisture permeability = 0.14 $g \cdot mm \cdot m^{-2} \cdot d^{-1}$ at 38°C/90%RH/20μm/20°C, oxygen permeability = 1500 $cc \cdot m^{-2} \cdot d^{-1} \cdot atm^{-1}$ at 85%RH) and linear low density polyethylene, LLDPE, (moisture permeability = 0.10 $g \cdot mm \cdot m^{-2} \cdot d^{-1}$ at 38°C/90%RH/20μm/20°C, oxygen permeability = 1200 $cc \cdot m^{-2} \cdot d^{-1} \cdot atm^{-1}$ at 85%RH) (Giles and Bain, 2000). They were stored in accelerated oxidation condition at 40°C, 80%RH in the dark for 18 days. Samples (40 packs for each treatment) were removed every 3 days for analysis. The oxidative state of each sample was monitored by analysis of the peroxide value (PV) and thiobarbituric acid reactive substances (TBARS) as well as by sensory analysis. The water activity was analyzingd every 6 days.

6.4 Determination of Peroxide and TBARS Values

Rice snack samples (200 g) were blended for 1 min and extracted with hexane (400 mL) three times by gently swirling for about 1 min each time. The combined hexane from the three extractions was evaporated from the oil extracted from the rice snack samples by using a rotary evaporator at 100 mm Hg pressure and 40ºC. The peroxide value was determined using a method based on AOCS official method Cd 8 – 53 according to 4.3.1. The TBARS value was analyzed according to Mcdonald and Hultin (1987) as described in 4.3.2.

6.5 Evaluation by Sensory Analysis

6.5.1 Panelists

A total of 10 panelists participated in the study. Prior to being allowed to participate in the study, panelists were screened by an odor recognition test (ASTM manual series: MNL 13-Manual on descriptive analysis testing for sensory evaluation) and trained to assess samples on an anchor scale. In addition, panelists who passed the screening test had to commit themselves to the full time required for the study. They were required to attend one session per day. Each session was one hour to two hours in length, at a specific time. The sensory quality was assessed by the odor since this avoided any possible concerns about the toxicity of the antioxidants, and panelists were trained with reference materials to evaluate the intensity of the attributes.

6.5.2 Panel Training

The first weeks of the study were devoted to panel training. One training session was conducted approximately 30 min in length. The first part of the panel training was generation of the terms to describe the odor related to the quality of the rice snack samples. Approximately 10 grams of each sample was

presented in 60 mL odor-free plastic cups with lids and labeled with three digit random numbers and equilibrated at 30ºC for a minimum 30 min prior to serving. Samples presented were fresh rice snacks and 7 as well as 14 day stored snacks. At the end of term generation for odor attributes, a total of 6 terms were collected. The next part of the panel training consisted of reduction in the number of terms so that only the terms that were significantly different were used to assess the samples. After the sessions was conducted, 3 attributes were selected from the results. These terms consisted of baked rice odor, vegetable oil odor and rancid odor. The consensus definitions and references were determined from the panelists. The final terms used to describe the attributes and their definitions are listed in Table 10. The reference samples used in this experiment are listed in Table 11, and once the lists of terms and reference samples were finalized, panelists started rating the intensity of the reference samples for each attribute. There were three references for each attribute. To obtain a well represented average for the intensity of the reference sample values, the references were rated a total of twice. The panelists also practiced rating the intensity using a 15–point scale with increments of 0.5 (Appendix A), each with anchors of 0 (none) and 15 (very much) and were also trained by using control sample which was the snack stored at 40°C for 10 days. The PV and TBARS values of the control sample were 19.25 ± 0.28 meq peroxide·kg^{-1} oil and 9.37±0.05 mmol·kg^{-1} oil, respectively.

Table 10 The attributes and their definitions used in QDA of the different antioxidant samples

Attribute	Definition
Baked rice odor	The amount of baked odor identified in the sample.
Rancid odor	The amount of odor associated with old or oxidized oil.
Vegetable oil odor	The amount of typical vegetable oil odor

Table 11 The reference samples used throughout QDA experiments

Attribute	Reference samples	Quantity	Quality*	Intensity
Baked rice odor	Baked rice cracker brand name; Shinmai	5 g		3
	Baked rice cracker brand name; Dozo	5 g		6
	Baked rice snack; Kanom sakol Co., Ltd.	5 g		11.5
Rancid odor	Refined soybean oil heated at 110 ºC 5 h.	20 g	PV=21.2±0.21 TBARS=9.36±0.05	5.5
	Refined soybean oil heated at 110 ºC 15 h.	20 g	PV=55.60±0.05 TBARS=29.88±0.10	7.5
	Refined soybean oil heated at 110 ºC 24 h.	20 g	PV=115.74±0.58 TBARS=95.33±0.03	14
Vegetable oil odor	Vegetable oil brand name; Kesorn	20 g	PV=0.49±0.00 TBARS=0.49±0.00	2.5
	Vegetable oil brand name; Aa–ngun	20 g	PV=0.74±0.02 TBARS=2.12±0.00	4
	Vegetable oil brand name; Dok–Poy–Sien	20 g	PV=1.29±0.32 TBARS=3.23±0.06	6

*Peroxide value in term of meq peroxide·kg^{-1} oil
TBARS value in term of mmol·kg^{-1} oil

6.5.3 Sample Testing

Each antioxidant-containing oil sample was coated onto a sticky rice snack in order to study the antioxidative effectiveness. After storage at 40ºC for 0, 3, 6, 9, 12, 15, and 18 days, snacks were analyzed by the sensory panel with two repetitions and the samples were presented with identification by three digit numbers and in a randomized order. The samples were stabilized at 30ºC for a minimum 30 min prior to serving and three pieces of snacks (~ 10 g) which were packed in OPP/PE/LLDPE bag were served for each treatment. Evaluations were

carried out under fluorescent light. Responses of panelists were calibrated and thus were reproducible (standard deviation less than 0.1). A warm–up sample was provided to each panelist one hour before testing for calibration. The PV and TBARS value of the warm up sample were 15.03 ± 0.16 meq peroxide·kg^{-1} oil and 7.71±0.05 mmol·kg^{-1} oil, respectively. Studies have shown that panelist performance is enhanced when a warm–up sample is used (Drake, 2005).

6.6 Determination of Water Activity

Water activity of samples was determining using a thermoconstanter (Novasina, Zurich, Switzerland) at 25°C (Pongsawatmanit *et al*., 2002) during the storage period.

6.7 Statistical Analysis

The results of water activity, PV, TBARS and the sensory profile test (QDA) including the repeated sensory profile tests were analysed by 2–way ANOVA using SPSS (version 10) at a significance level of 0.05. The relationships between chemical factors and sensory results were evaluated by regression was computed using SPSS (version 10) at a significance level of 0.05.

RESULTS AND DISCUSSION

1. Preliminary Study as Influenced by Sample Storage, Extracting Conditions and Test Methods, for Determination of Antioxidant Activity

1.1 Effect of Sample Storage before Extraction

1.1.1 Comparison of the Effects of Sample Preparation Method on Yield

The yields of extracts from Kradonbok (*Careya sphaerica* Roxb.), Teaw (*Cratoxylum formosum* Dyer.) and Phak whan ban (*Sauropus andrugynus* Merr.) leaves prepared by various methods were about 2, 3, 4% (db), which can be compared with yields of extracts from leaves reported in the literature which ranged between 2 to 15% (db) (Demo *et al.,* 1998). These yields were not significantly different during storage for each preparation method (Figure 20). The freezing methods gave a higher yield compared with those obtained from the other drying methods.

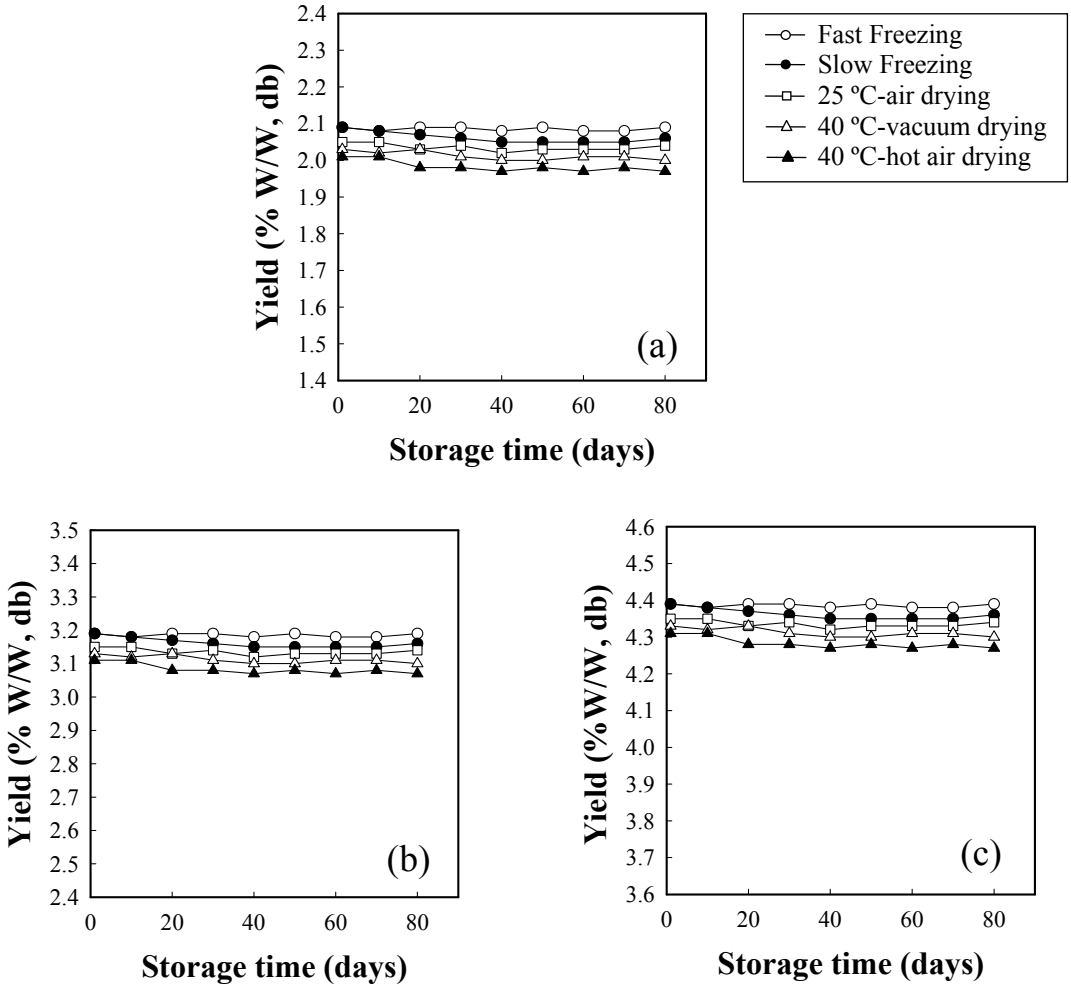


Figure 20 Yields of the extracts from (a) *Careya sphaerica* Roxb. (Kradonbok), (b) *Cratoxylum formosum* Dyer. (Teaw) and (c) *Sauropus andrugynus* Merr. (Phak whan ban) leaves obtained from various sample preparation methods.

1.1.2 Comparison of the Effects of Different Sample Preparation Methods on Total Phenolic Content and Antioxidant Activity

Sample preparation was necessary in order to keep samples for a certain period of time before analysis since the total phenolic content was reduced by storage compared with the fresh leaves. The total phenolic content of dried

extracts were significantly different ($P < 0.05$). For Kradonbok leaves, the total phenolic contents from different sample preparations (fast freezing, slow freezing, air drying at 25°C, vacuum drying at 40°C and hot air drying at 40°C) were reduced by about 3, 9, 12, 20 and 25%, respectively. For Teaw leaves, total phenolic contents of samples treated by these sample preparation methods were reduced by about 7, 10, 14, 20 and 22%, respectively and the corresponding values for Phak whan ban leaves were reduced by about 10, 26, 38, 60 and 72%, respectively. Total phenolic content and antioxidant activity determined as EC_{50} of extracts prepared from frozen samples showed higher values than those prepared by drying methods (Figure 21 and 22). The total phenolic content obtained from samples treated by the slow freezing method was lower than that obtained from samples cooled by fast freezing because larger ice crystals grew during slow freezing which would damage plant cells and cause loss of antioxidant activity. Enzymes from plant cells such as lipoxygenase can oxidize polyphenols (Akoh and Min, 1997). The total phenolic content of samples treated by air drying was higher than that of samples treated by vacuum drying and hot air drying (Figure 21) because some phenolic components may degrade at higher temperatures (Moure *et al*., 2001). Reported data for extracts from fresh Mulberry leaves has also shown that the amount of flavonoids was higher for air-dried than for oven-dried leaves, probably due to decomposition after storage (Zhishen *et al*., 1999). The total phenolic content during storage of dried samples decreased during storage due to decomposition of the phenolic compounds, which is increased at higher water activity (a_w) of the dried samples (Figure 23) from 0.3 to 0.6. Usually water activity values higher than 0.4 enhance deterioration from oxidation (Akoh and Min, 1997). The water activity was increased because the moisture content could penetrate through the PE plastic bags (Kadoya, 1990) during storage.

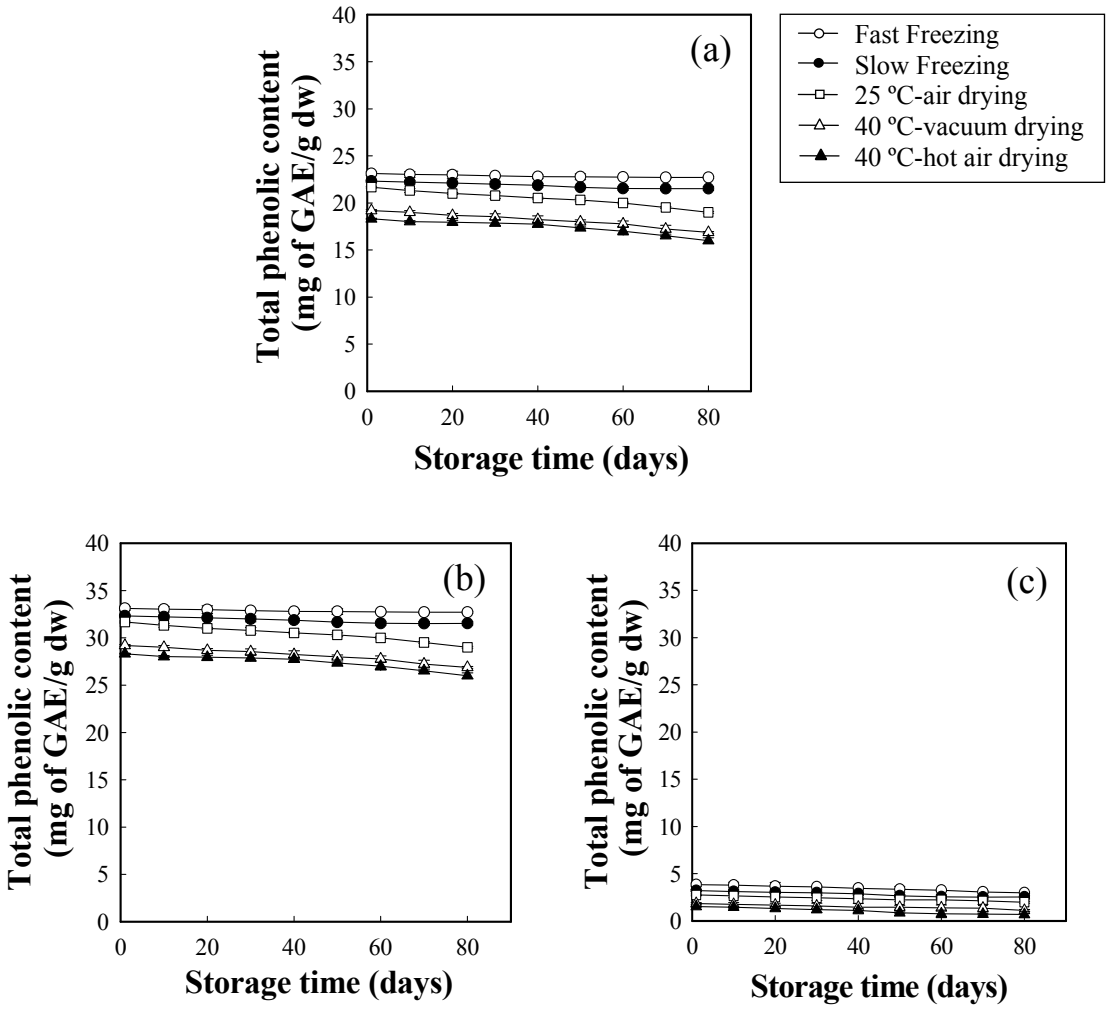


Figure 21 Total phenolic content of the extracts from (a) *Careya sphaerica* Roxb. (Kradonbok), (b) *Cratoxylum formosum* Dyer. (Teaw) and (c) *Sauropus andrugynus* Merr. (Phak whan ban) leaves obtained from various sample preparation methods.

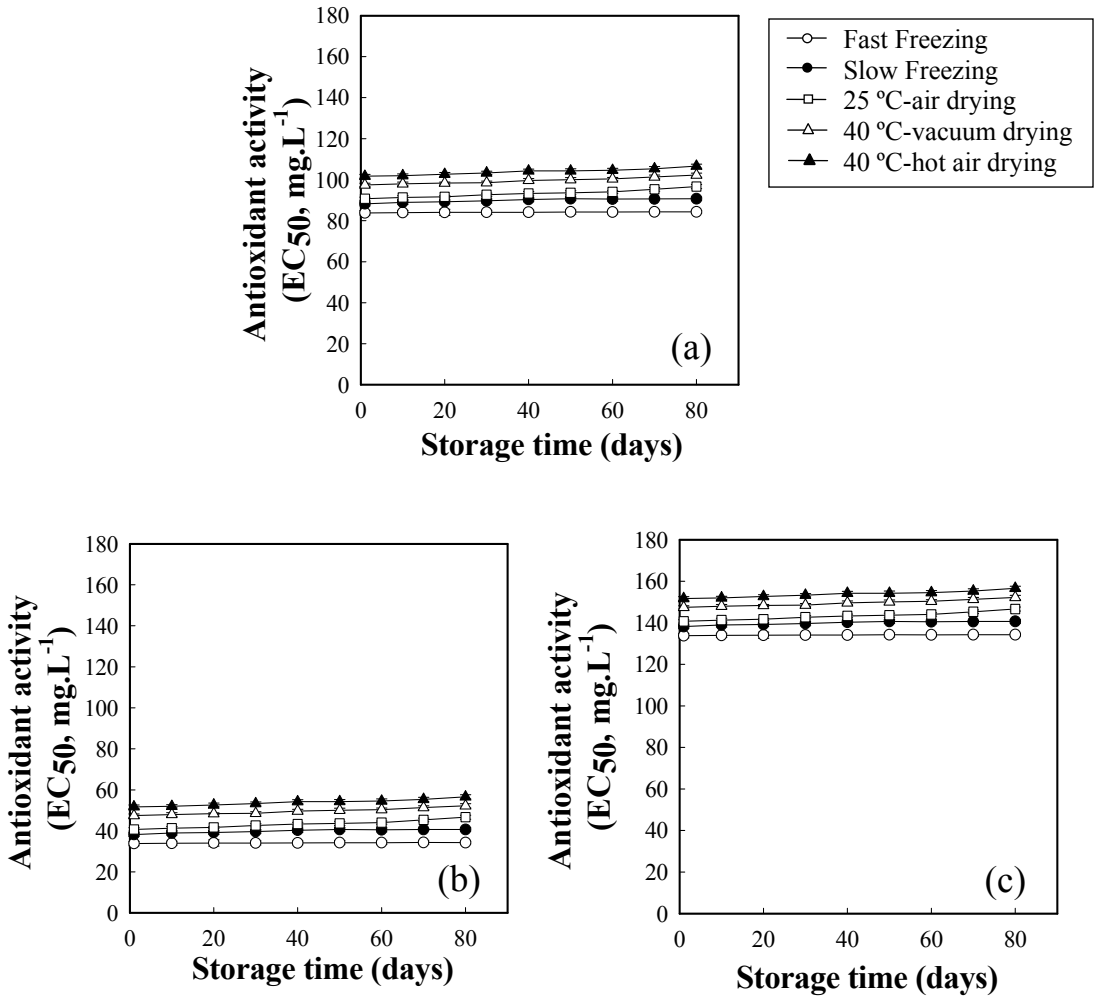


Figure 22 Antioxidant activity (EC_{50}) of the extracts from (a) *Careya sphaerica* Roxb. (Kradonbok), (b) *Cratoxylum formosum* Dyer. (Teaw) and (c) *Sauropus andrugynus* Merr. (Phak whan ban) leaves obtained from various sample preparation methods.

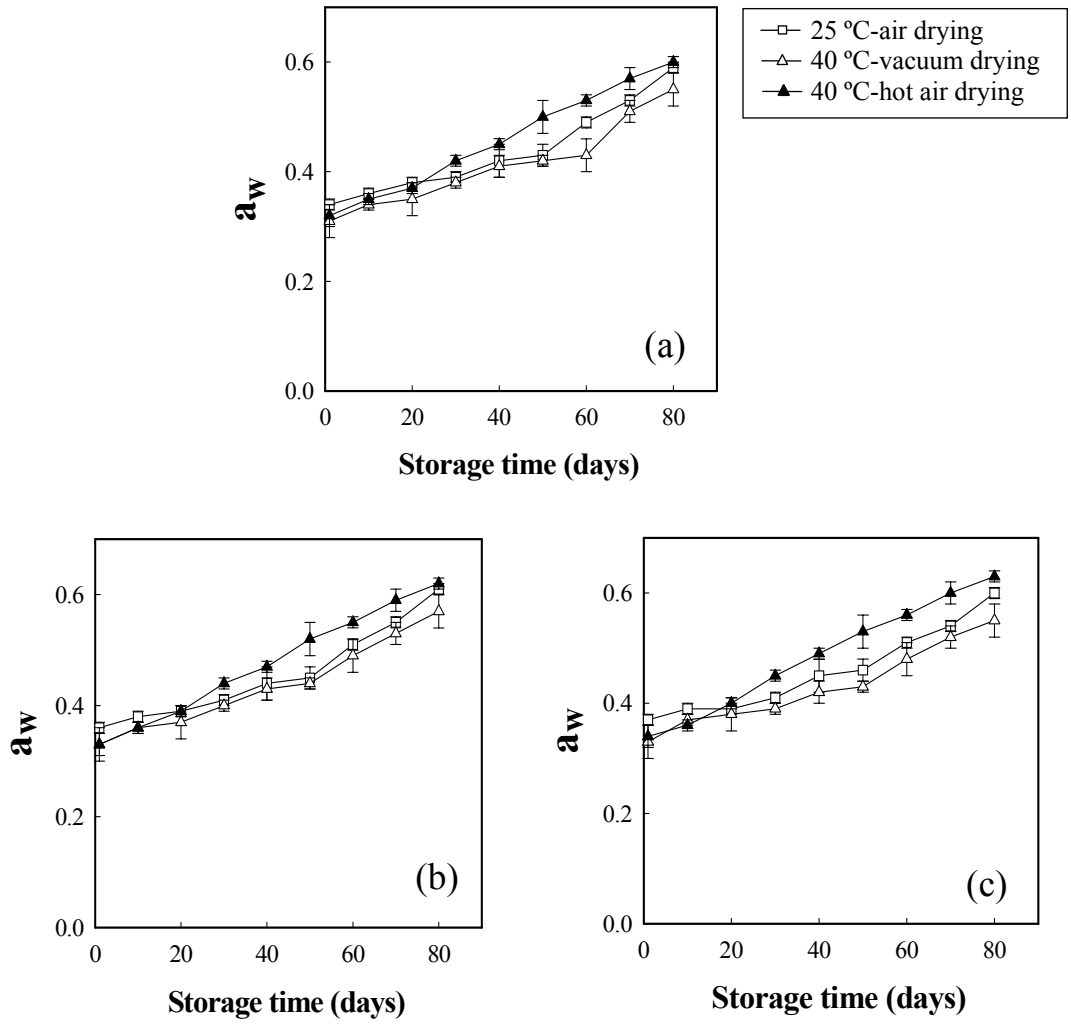


Figure 23 Water activity of the dried leaves from (a) *Careya sphaerica* Roxb. (Kradonbok), (b) *Cratoxylum formosum* Dyer. (Teaw) and (c) *Sauropus andrugynus* Merr. (Phak whan ban) obtained from various sample preparation methods.

1.2 Effect of Extraction Times

1.2.1 Comparison of Extraction Times on Yield

Extraction time affected the yield. For the first stage of extraction, the yield (Figure 24) increased with increasing extraction time. The yield remained almost constant after 3 h extraction at room temperature.

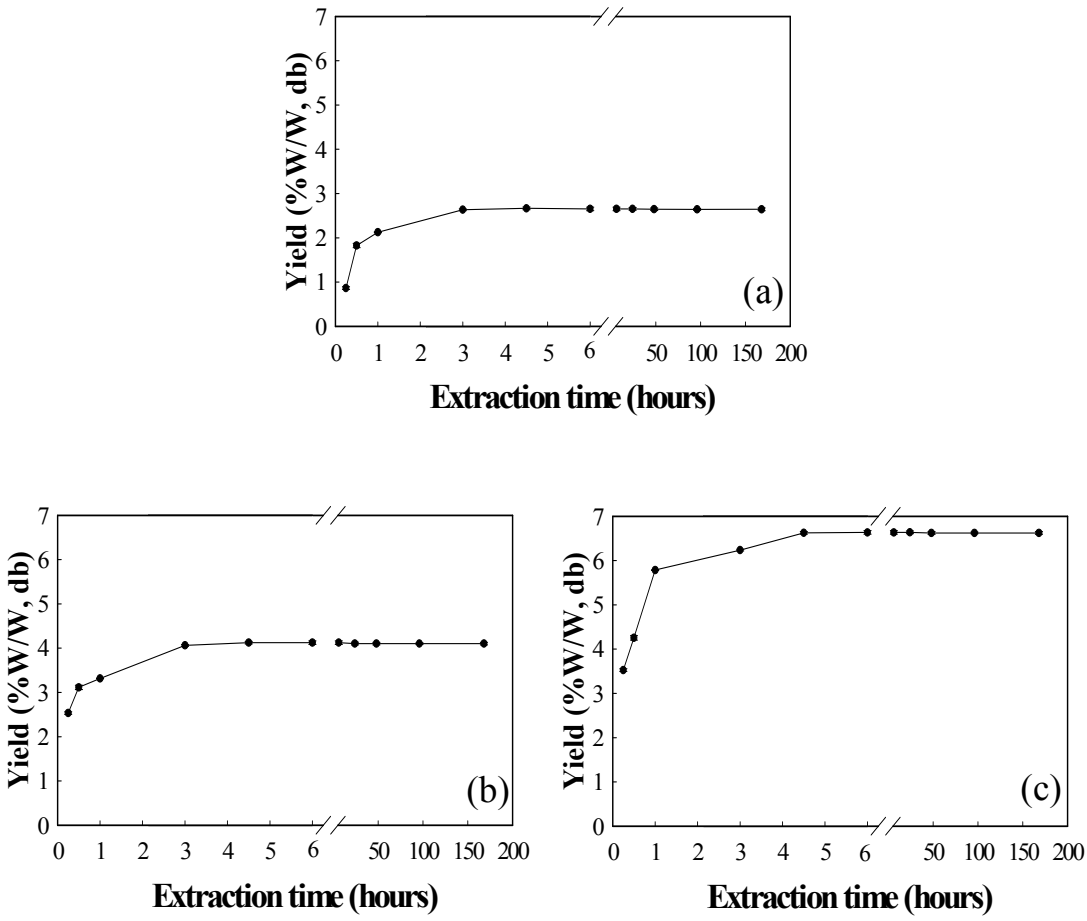


Figure 24 Yield as a function of extraction time of (a) *Careya sphaerica* Roxb. (Kradonbok), (b) *Cratoxylum formosum* Dyer. (Teaw) and (c) *Sauropus andrugynus* Merr. (Phak whan ban) leaves kept in the frozen state before analysis.

1.2.2 Comparison of Extraction Times on Total Phenolic Content and Antioxidant Activity

Extraction time affected the total phenolic content and antioxidant activity of extracts. The total phenolic content (Figure 25) increased with increasing extraction times for the first stage of extraction. The total phenolic content was almost constant after 3 h extraction. However, extraction times from 4.5 to 6 h gave the lowest EC_{50} value (highest antioxidant activity) compared with those obtained using extraction times less than 4.5 h or longer than 6 h. The EC_{50} value increased with increasing extraction time (Figure 26) when the extraction time was more than 6 h. This may result from chemical or enzymatic degradation which is suggested as the main mechanism causing the reduction in polyphenol content (Larrauri *et al*., 1997). In this study, the total phenolic content did not change whereas the antioxidant activity decreased. These results suggest that polyphenols can react with other plant components and decompose to form other less active phenolic components (Gordon, 2001).

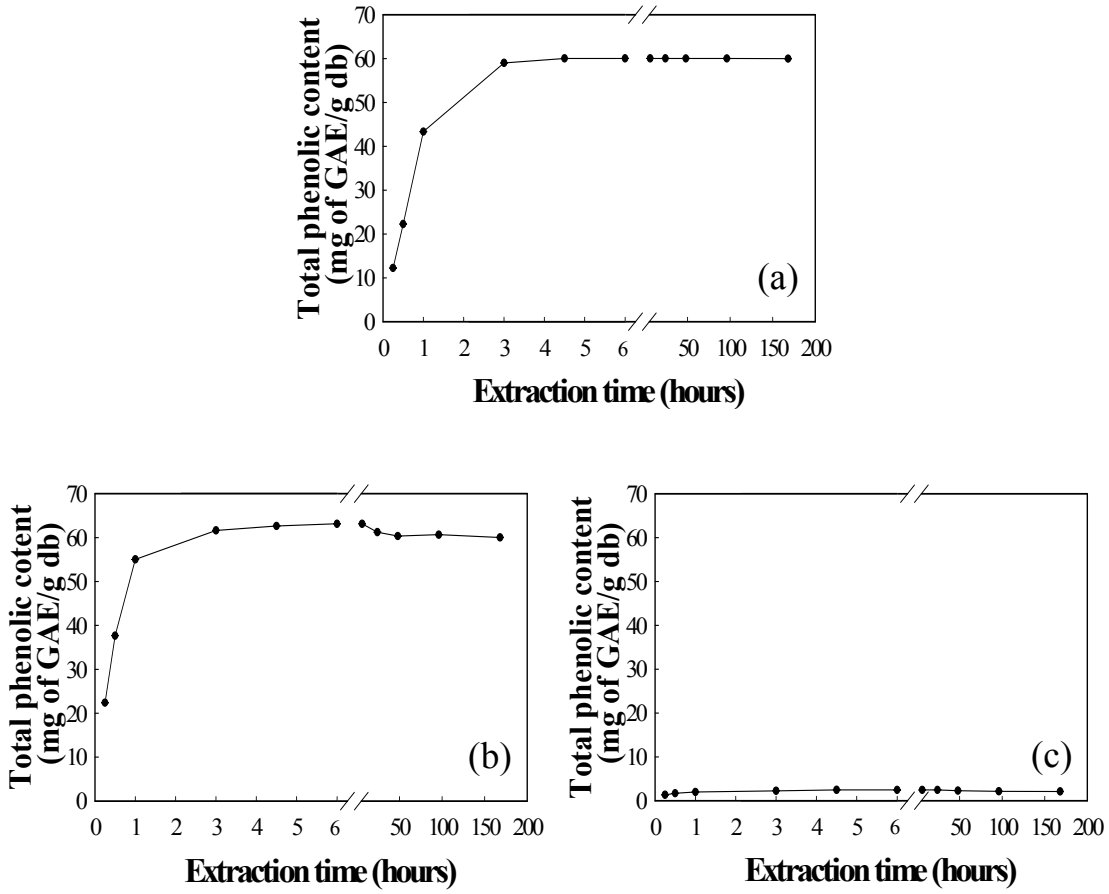


Figure 25 Total phenolic content as a function of extraction time of (a) *Careya sphaerica* Roxb. (Kradonbok), (b) *Cratoxylum formosum* Dyer. (Teaw) and (c) *Sauropus andrugynus* Merr. (Phak whan ban) leaves kept in the frozen state before analysis.

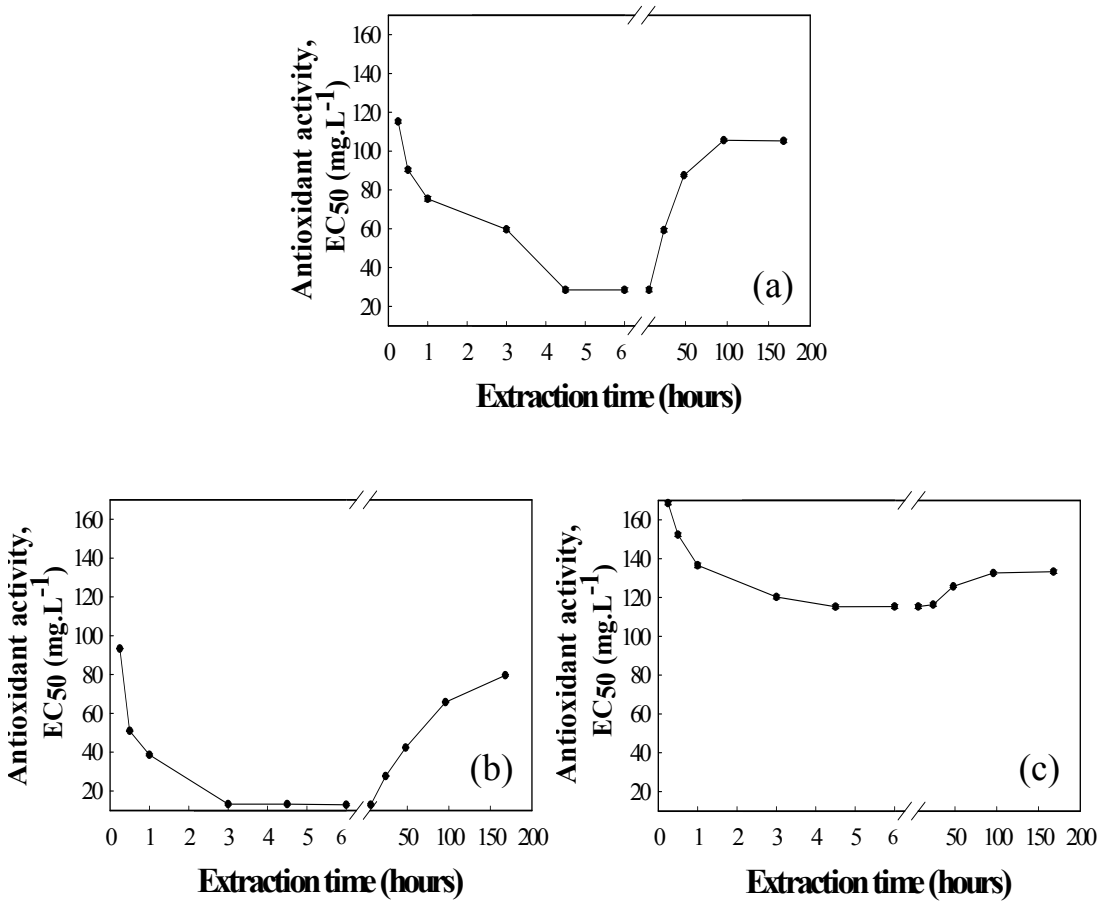


Figure 26 Antioxidant activity, (EC_{50}) as a function of extraction time of (a) *Careya sphaerica* Roxb. (Kradonbok), (b) *Cratoxylum formosum* Dyer. (Teaw) and (c) *Sauropus andrugynus* Merr. (Phak whan ban) leaves kept in the frozen state before analysis.

1.3 Effect of Solvent Ratio

1.3.1 Effect of Solvent Ratio on Yield

The yields of extracts from Kradonbok, Teaw and Phak whan ban leaves with different ethanol concentrations are shown in Figure 27 and were about 1.86-2.35, 3.98-4.30 and 5.86-5.93% which compares with yields of leaf extracts reported in the literature which ranged between 2 to 15% (db) (Demo *et*

al., 1998). The yields of Teaw and Kradonbok extracts were increased more than that from Phak whan ban, which had lower total phenolics, when ethanol concentration was increased.

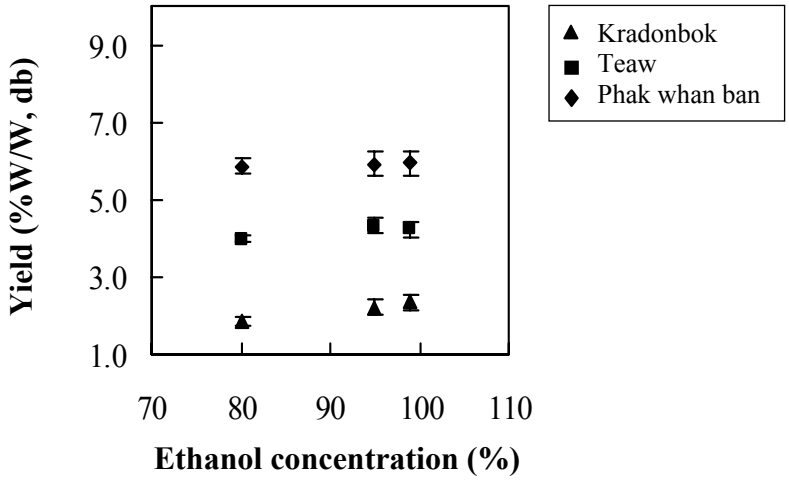


Figure 27 Yields of the extracts from *Careya sphaerica* Roxb. (Kradonbok), *Cratoxylum formosum* Dyer. (Teaw) and *Sauropus andrugynus* Merr. (Phak whan ban) leaves obtained with various ethanol concentrations.

1.3.2 Effect of Solvent Ratio on Total Phenolic Contents and Antioxidant Activity Assays

a. Total Phenolic Content

Since plant phenolics constitute one of the major groups of compounds acting as primary antioxidants or free radical terminators, it was reasonable to determine their total concentration in the selected plant extracts.

The concentration of phenolics in the extracts expressed as mg of GAE per g of extract (dry weight basis), was dependent on the ethanol concentration used in the extraction, as shown in Figure 28. The total phenolic content of plant extracts readily increased with increasing concentration of ethanol

from 80% to 95%, however the total phenolics of these plant extracts were not significantly different between the extracts using ethanol concentrations of 95% and 99% for each plant. These results showed the same effect of ethanol concentration as the results from assessment of the antioxidant activity by two methods.

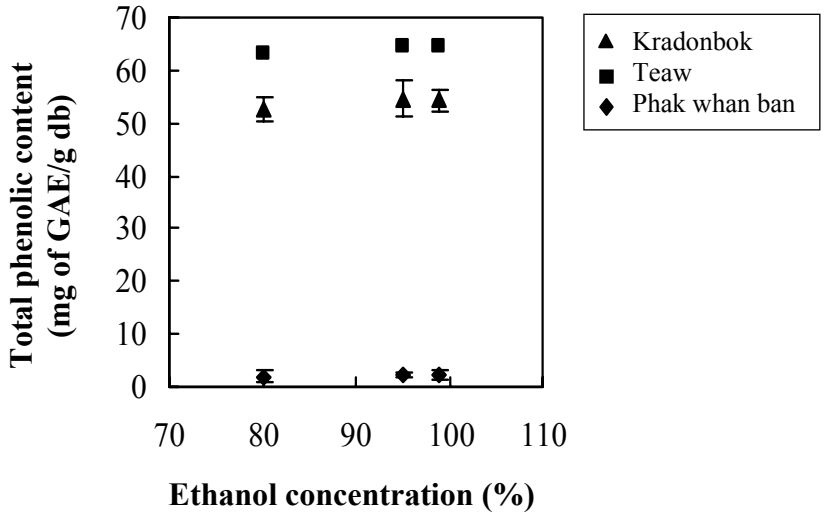


Figure 28 Total phenolic contents of the extracts from *Careya sphaerica* Roxb. (Kradonbok), *Cratoxylum formosum* Dyer. (Teaw) and *Sauropus andrugynus* Merr. (Phak whan ban) leaves obtained with various ethanol concentrations.

b. *β-Carotene Bleaching* Method (BCB)

The *β-Carotene bleaching* method is based on the loss of the yellow color of *β-Carotene* due to its reaction with radicals, which are formed by linoleic acid oxidation in an emulsion. The rate of *β-Carotene bleaching* can be slowed down in the presence of antioxidants. The BCB method is sensitive due to the strong absorption of *β-Carotene* but it is slower (2 hours per sample) than the DPPH method. Furthermore, we can see the high variation of absorbance values shown in Figure 29. Therefore, it was clear that this method gave worse reproducibility than the DPPH method. The poor reproducibility arises because

there are many complicated steps in the preparation process before the determination. For instance, solvent evaporation (under vacuum and at a temperature of 50°C) and preparation of an emulsion with reproducible composition and droplet size was essential for achieving reproducible results. Consequently, this method was relatively complicated and this made it difficult to achieve reproducible results. However, some literature has shown that the BCB method can be helpful especially for investigations of lipophilic antioxidants and it is appropriate for the investigation of the antioxidant activity of essential oils. On the other hand if polar compounds (ascorbic acid, rosmarinic acid, caffeic acid etc.) were tested only by the BCB method, they would be considered as weak antioxidants. However, the strong antioxidant activity of these compounds can be proven by other tests (Koleva et al., 2002; Kulisic et al., 2004).

This method is used for evaluation of the antioxidant activity of the Kradonbok, Teaw and Phak whan ban leaves by different ethanol concentrations in comparison with well known, synthetic and natural antioxidants, namely BHT, BHA and α-tocopherol. The bleaching rates of *β-Carotene* in the presence of plant extracts (Figure 29) were related to antioxidant power. Lower bleaching rates corresponded to higher antioxidant power. We found that the rates were less when using extracts obtained at increasing ethanol concentration from each plant. Furthermore, the bleaching rate obtained from Phak whan ban extract was faster than those from Kradonbok and Teaw. These data indicated that extracts from Kradonbok and Teaw were more effective antioxidants than the extract from Phak whan ban leaves. This was similar to results in terms of A_A and C_{AA}, which are measures of the inhibition of linoleic acid oxidation relative to that of a control, where higher values showed stronger antioxidant activity. From the results shown in Figure 30, the A_A and C_{AA} values of Kradonbok and Teaw were higher than those of Phak whan ban and they gradually increased with increasing concentration of ethanol used in the extraction from 80% to 99%. However the A_A and C_{AA} values of these plant extracts were slightly increased between extracts prepared using concentrations of ethanol 95% and 99% for each plant. If R_{OR}, a

measure of antioxidative strength, is larger than 1, oxidation proceeds faster in the presence of an additive than in its absence. Thus, the lower the R_{OR} value, the stronger the inhibitor (Oomah and Mazza, 1996). These results showed that R_{OR} values from these three plant extracts were less than 1, therefore these extracts showed effective antioxidant activity. In addition, R_{OR} values of extracts from Kradonbok and Teaw were less than those from Phak whan ban, which shows that Kradonbok and Teaw extracts were stronger antioxidants in the BCB test. The activity of the extracts was less than that of the well known antioxidants, BHA and BHT, which were the strongest antioxidants (Table 12). Teaw leaf extract showed a slightly higher antioxidant effect to α-tocopherol, while Kradonbok and Phak whan ban extract showed lower antioxidant power than α-tocopherol.

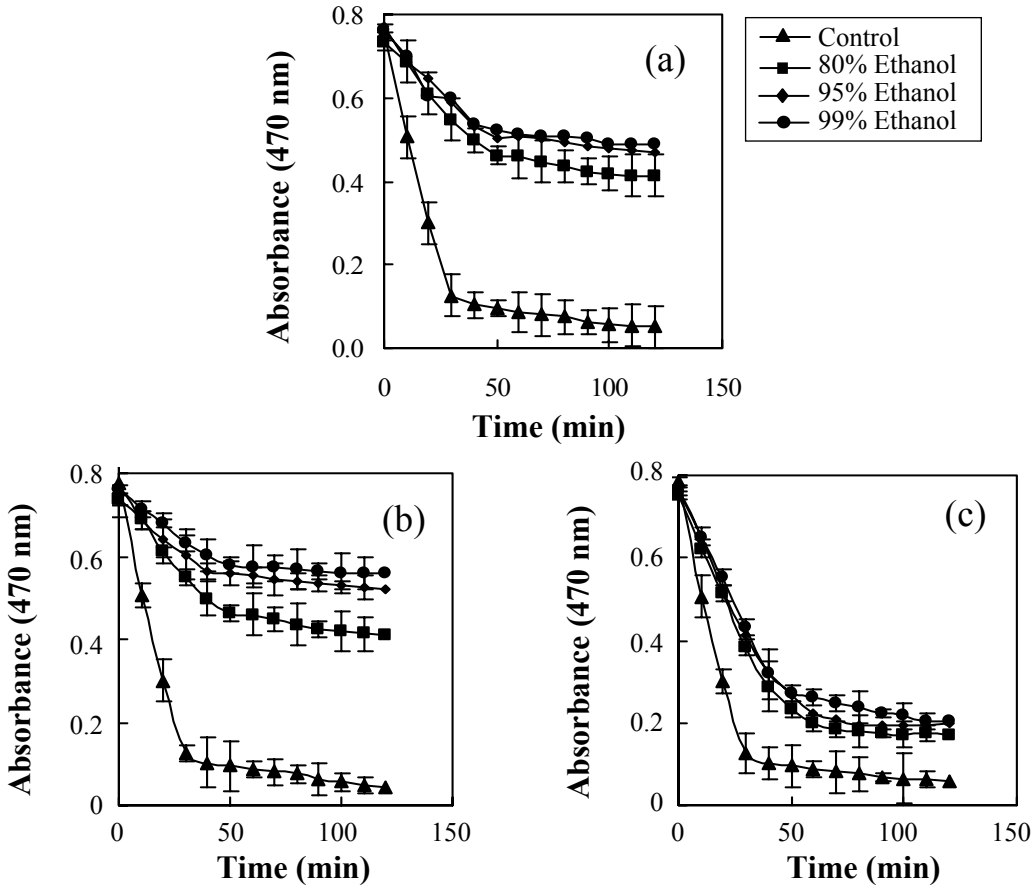


Figure 29 Bleaching rates of *β-carotene* as affected by (a) *Careya sphaerica* Roxb. (Kradonbok), (b) *Cratoxylum formosum* Dyer. (Teaw) and (c) *Sauropus andrugynus* Merr. (Phak whan ban) leaves obtained from various ethanol concentrations.

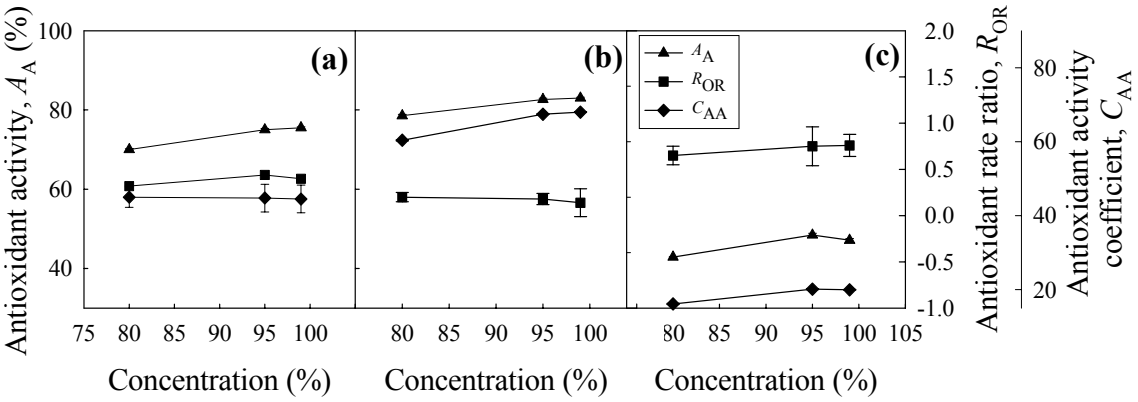


Figure 30 Parameters used to evaluate the antioxidant properties of (a) *Careya sphaerica* Roxb. (Kradonbok), (b) *Cratoxylum formosum* Dyer. (Teaw) and (c) *Sauropus andrugynus* Merr. (Phak whan ban) leaves obtained from various ethanol concentrations.

Table 12 Parameters used to evaluate the antioxidant properties of BHA, BHT and α- tocopherol

Sample	Parameters*		
	A_A	R_{OR}	C_{AA}
BHA	92.36±0.11[a]	0.09±0.10[a]	78.36±0.24[b]
BHT	93.53±0.16[c]	0.08±0.11[a]	80.32±0.23[c]
α-tocopherol	83.20±0.13[b]	0.19±0.12[b]	64.32±0.09[a]

Note: *Values expressed are means±standard error of duplicate measurements. Means in the same row with different letters were significantly different ($P < 0.05$).

c. 2, 2-Diphenyl-1-Picrylhydrazyl (DPPH) Radical Scavenging Activity Method

The relatively stable organic radical DPPH has been widely used in the determination of the antioxidant activity of single compounds and plant extracts (Yen *et al*., 1993). The method is based on the reduction of alcoholic

DPPH radical solutions in the presence of a hydrogen donating antioxidant. DPPH radical solutions show a strong absorption band at 517 nm which appears as a deep violet color. The absorption vanishes and the resulting decolorization is stoichiometric with respect to degree of reduction. The remaining DPPH radical, measured after a certain time, corresponds inversely to the radical scavenging activity of the antioxidant (Kulisic *et al.,* 2004). The sensitivity of this method is determined by the strong absorption of the DPPH radical. The method is rapid, a sample analysis takes 40 min in total and little manpower, no expensive reagents or sophisticated instrumentation are required (Koleva *et al*., 2002). The DPPH method is faster than the BCB method and it can be helpful in investigation of novel antioxidants for a rapid estimation and preliminary information about radical scavenging ability. This method is sensitive and requires a small amount of sample (Kulisic *et al.,* 2004).

This method was used to evaluate the antioxidant properties of extracts from Kradonbok, Teaw and Phak whan ban leaves prepared with different ethanol concentrations in comparison with BHA, BHT and α-tocopherol. Figure 31 shows antioxidant activity in term of EC_{50} of the plant extracts prepared with different ethanol concentrations (80%, 95% and 99%). The lower EC_{50} means the higher antioxidant activity. The antioxidant activity of plant extracts gradually increased with increasing concentration of ethanol from 80% to 99%, however the antioxidant activity of these plant extracts were slightly increased between extracts prepared using concentrations of ethanol 95% and 99% for each plant which is similar to results from the BCB test. Therefore, 95% ethanol should be selected for extraction because it is much cheaper than 99% ethanol for commercial applications and it was found that the antioxidant activity of these extracts prepared with 95% ethanol was not significantly different from those of 99% ethanol when determined by two methods.

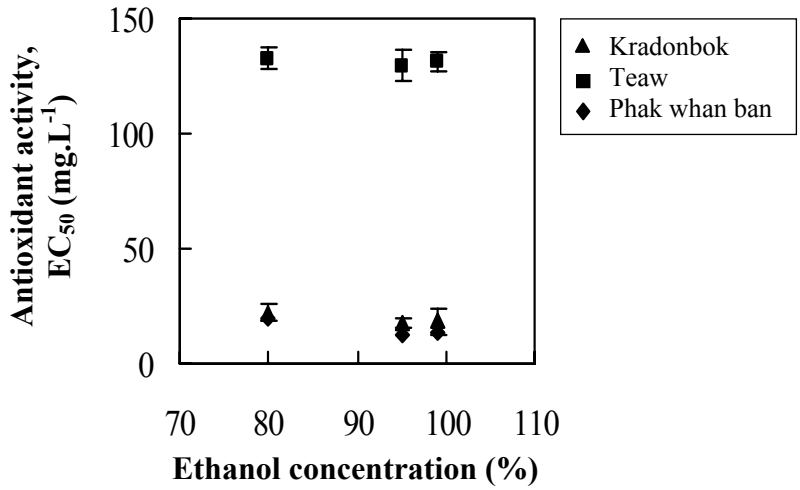


Figure 31 Antioxidant activity of the extracts from *Careya sphaerica* Roxb. (Kradonbok), *Cratoxylum formosum* Dyer. (Teaw) and *Sauropus andrugynus* Merr. (Phak whan ban) leaves.

The EC_{50} values of BHA, BHT and α-tocopherol were 11.95±0.03, 11.8±0.06 and 15.94 $\mu g \cdot ml^{-1}$, respectively. BHA and BHT were the strongest antioxidants. Teaw leaf extract showed slightly higher antioxidant activity than α-tocopherol, whereas Kradonbok leaf and Phak whan ban extract showed lower antioxidant power than α-tocopherol. These conclusions are similar to those from the BCB method.

In conclusion, the present study showed that freezing, especially fast freezing, appropriate for using prior to storage of the plant leaves until analysis gave higher yield, total phenolic content and antioxidant activity (lower EC_{50}) than the values obtained after treating samples by drying methods. When plant leaves treated by fast freezing were extracted with ethanol to study the effect of extraction times. The results showed that yield and total phenolic content were almost constant after 3 h extraction time at room temperature. However, extraction of the plant leaves for times of 4.5 to 6 h gave the lowest EC_{50} values which indicated the highest antioxidant activity. The appropriate ethanol concentration to extract plant leaves was 95%. It gave a high yield and high total phenolic content as well as

strong antioxidant activity assessed by the BCB and DPPH methods. The BCB test could be relatively complicated for achieving reproducible results. The DPPH method is not only as sensitive as the BCB test, but it is also faster than the BCB test. Consequently, the method using for screening plant antioxidant potential should be the DPPH method because it is easy and rapid, hence it can be helpful in investigations of novel antioxidants for a rapid estimation and for providing preliminary information about radical scavenging ability.

2. Determination of the Antioxidant Potential of Thai Indigenous Plants

Since the ethanolic extracts from 26 Thai indigenous plants were obtained from various varieties and plant parts (fruit, peel, flesh, seed, seed skin, young leaf, leaf, flower, stem core, bark), we classified the plants into 3 groups: (1) berries and fruits, (2) herbs and vegetables and (3) chewing plants for characterizing the selected plant parts in terms of the total phenolic compounds, total flavonoids and free radical scavenging activity. For leadtree and hog plum, we used both parts of seed and young leaf. Therefore, each part of both plants was analysed and classified according to the parts that were used within the groups of berries and fruits, and herbs and vegetables. The seed is defined as the plant part containing the embryo for the group of berries and fruits. In the case of tamarind, the seed skin refers to a thin brown skin layer covering the seed. For the herbs and vegetables, the young leaf includes outgrowths on a stem or branch consisting of a shortened stem and immature leaves. The young leaves as a plant part were selected for study because the young leaves of vegetables and herbs are consumed frequently in Thailand. The group of chewing plants was selected for studies of the antioxidant activity because the highly astringent taste reflects a high phenolic content (Llaudy *et al*., 2004). Betel nut in the group of chewing plants classified as a seed for chewing is one of the dark red seeds (kernels) of the betel palm that is wrapped in betel leaves with lime and chewed by some people in Asia. Some people use the whole fruit of the betel nut for chewing but others use only the

kernel. The difference between the whole fruit and kernel is that the kernel is obtained by removal of a fibrous husk surrounding the kernel.

Differences in polarity (and thus different extractability) of the antioxidative components are obviously the reason why extraction yields and antioxidant activity of the extracts differ (Julkunen-Tiito, 1985; Marinova and Yanishlieva, 1997). Ethanol was used as extraction medium in this work because it is the most widely used solvent and safe to apply in foods.

2.1 Ethanolic Plant Extract Yield

Among the 26 plant extracts studied, the moisture content of the fresh plant depends on the plant group and the type of plant part (Table 13). The yield of the ethanolic extract from all groups of plants was calculated based on a dry weight basis in order to eliminate the influence of the different moisture content of the plants.

For most berries and fruits, the moisture content of the whole fruit or fruit flesh was higher than 90% but it was lower in the fruit peel and seed (ranging from 36 to 86%). The tamarind seed skin showed the lowest moisture content (12.3%) in this group due to roasting being applied for 15 min during sample preparation prior to extraction in order to separate the thin layer of skin from the tissue inside the seed. The extract yields of fruit seeds, such as rambutan (3.7%), mango (3.2%) and tamarind (3.0%), were higher than those obtained from berry seeds (0.3 to 1.2%). The higher yield from fruit seeds may be due to extraction of the carbohydrate component in the seed as reported for mango kernel seed by Kabuki *et al.,* (2000). The extract yields from other fruit parts such as peel and fresh were 1.7 to 11.8%.

For the group of herbs and vegetables, the moisture content of the plants varied from 75 to 95% except for the young leaf of leadtree (50%) due to the

characteristic of this plant having low moisture content. Yields of extracts from this herb and vegetable ranged from 0.2 to 4.1%. Considering the chewing plants, the moisture content depends on the plant part: leaf and whole fruit or bark. The moisture content of fruit and leaf were high (80 to 91%) while those of bark and stem core were low (11 to 16%). However, the yields of ethanolic extracts from these chewing plants were lower than 1.5%.

2.2 Total Phenolic Content and Total Flavonoid Content in the Studied Plant Tissues

Phenolic compounds are very important plant constituents because they exhibit antioxidant activity by inactivating lipid free radicals or preventing decomposition of hydroperoxides into free radicals (Pokorny, 2001). Flavonoids are phenolic compounds, which are very effective antioxidants (Yanishlieva-Maslarova, 2001). The Folin-Ciocalteu method is a rapid and widely used assay to investigate the total phenolic content but it is known that different phenolic compounds have different responses in the Folin-Ciocalteu method (Kähkonen *et al*., 1999). Therefore, in this work, we calculated the total phenolic contents in units of mg gallic acid equivalent of phenolic compound in one gram of plant extracts based on the dry weight of the original plant sample as shown in Table 14. The total phenolic content differed among the different types and parts of plants and each plant extract contained a lower total flavonoid content than the total phenolic content since other compounds besides flavonoids are phenolic substances in plants (Pietta, 2000).

Table 13 Moisture content and yield of ethanolic extracts obtained from various plant parts of Thai indigenous plants with scientific and common name[a]

Scientific name	Common name	Plant part	Moisture Content (%)	Yield (%,db)[b]
Berries and fruits				
Antidesma velutinum Tulas.		Seed	38.4±0.0	0. 5±0.0
Cleistocalyx operculatus var *paniala* (Roxb.)		Seed	55.1±0.0	0.3±0.0
Diospyros kaki L.	Persimmon	Whole fruit	92.4±0.1	3.2±0.1
		Fruit peel	86.4±0.1	5.3±0.0
		Fruit flesh	94.4±0.1	3.2±0.1
Eugenia siamensis Craib.	Jambolan Plum	Seed	50.3±0.0	0.8±0.0
Garcinia mangostana Linn.	Mangosteen	Fruit peel	62.5±0.0	11.8±0.0
Leucaena glauca Benth	Leadtree	Seed	76.5±0.0	0.4±0.0
Mangifera indica Linn.	Mango	Seed	52.3±0.1	3.2±0.0
Nephelium lappaceum Linn.	Rambutan	Fruit peel	71.3±0.0	1.7±0.0
		Seed	36.3±0.2	3.7±0.0
Nephelium hypoleucum Kurz	Pulasan	Whole fruit	76.2±0.0	1.4±0.0
Piper nigrum Linn.	Pepper	Seed	61.0±0.5	0.6±0.0
Spondias pinnata Kurz	Hog plum	Fruit flesh	76.4±0.1	2.3±0.0
		Seed	52.3±0.1	1.2±0.0
Tamarindus indica Linn.	Tamarind	Seed	49.5±0.1	3.0±0.0
		Seed skin	12.3±0.0	0.5±0.0
Herbs and vegetables				
Basella alba Linn.	Ceylon spinach	Young leaf	91.9±0.1	0.8±0.0
Careya sphaerica Roxb.	Tummy wood	Young leaf	75.4±0.0	2.3±0.0
Cratoxylum formosum Dyer.		Young leaf	79.8±0.1	4.1±0.1
Hydrocharis dubia (Bl.) Back.	Frogs bit	Young leaf	95.0±0.0	0.8±0.0
Hydrolea zeylanica (L.) Vahl.		Young leaf	83.6±0.1	1.1±0.0
Lasia spinosa (Linn.) Thw.		Young leaf	94.2±0.3	1.4±0.1
Leucaena glauca Benth	Leadtree	Young leaf	49.9±0.1	3.0±0.0
Limnocharis flava Buch.		Young leaf and flower	94.7±0.3	1.9±0.0
Momordica charantia Linn.	Balsum pear	Young leaf and leaf	87.0±0.0	1.3±0.0
Sesbania grandiflora Desv.	Cork wood	Flower	91.1±0.1	2.6±0.0
Spondias pinnata Kurz	Hog plum	Young leaf	88.4±0.3	0.2±0.0
Syzygium gratum (Wight) S.N.Mitra var. gratum		Young leaf	90.4±0.1	1.2±0.0
Chewing plants				
Acacia catechu (L.F) Willd.	Black catechu	Bark	16.3±0.1	0.9±0.0
Areca catechu Linn.	Betel nut	Whole fruit	90.2±0.0	0.2±0.0
		Kernel	91.2±0.1	0.3±0.0
Cassia fistula Linn.	Golden shower	Stem core	11.4±0.0	1.0±0.0
Piper betel Linn.	Betel leaf	Leaf	82.6±0.4	1.5±0.0

[a] Values are the mean ± standard deviation (n = 3).

[b] dry weight basis of the original sample of plant parts.

For the group of berries and fruits, seeds from *Antidesma velutinum* Tulas., *Cleistocalyx operculatus* var *paniala* (Roxb.) and *Eugenia siamensis* Craib. were obtained as by-products of wine production in Thailand. The total phenolic content of these seeds was very high (123 to 180 mg GAE/g dry weight of plant extract) compared to that obtained from other fruit and berry seeds (20 to 54 mg GAE/g dry weight of plant extract). The reason for the lower total phenolic content of fruit seeds may be the contribution of carbohydrates in the extracts (data not shown). The total phenolic content of fruit peel ranged from 13 to 42 mg GAE/g dry weight of plant extract. In the case of tamarind, seed skin gave a high total phenolic content (134 mg GAE/g dry weight of plant extracts). The total flavonoid content of the berry and fruit group was low compared to the total phenolic content (Table 13). The total flavonoid content of seeds of *Antidesma velutinum* Tulas., *Cleistocalyx operculatus* var *paniala* (Roxb.) and *Eugenia siamensis* Craib. was remarkably high and was the highest in the group of berries and fruits (44 to 50 mg RE/g dry weight of plant extracts) compared to those obtained from other plant seeds (5 to 23 mg RE/g dry weight of plant extract). However, extracts with higher phenolic content did not always have a higher flavonoid content, as was evident for the seed of *Antidesma velutinum* Tulas. which had a higher total flavonoid content (50 mg RE/g dry weight of plant extract) compared with that of *Cleistocalyx operculatus* var *paniala* (Roxb.) (44 mg RE/g dry weight of plant extract) although the total phenolic content was lower (123 and 174 mg GAE/g dry weight of plant extract, respectively). The results suggest that different plant extracts contain different levels of total flavonoids as a proportion of the total phenolic compounds. The total flavonoids of fruit peel ranged from 2 to 10 RE/g dry weight of plant extract. The skin layer of tamarind also gave a high total flavonoid content (41 RE/g dry weight of plant extracts).

The most selected and studied plant part in herbs and vegetables was the young leaf which comprises the outgrowth on a stem or branch consisting of a shortened stem and immature leaves. Based on total phenolic content in the extracts from herbs and vegetables, the selected parts can be divided into 3 ranges

of GAE values. The lower, middle and higher ranges of total phenolic compounds were below 10, 10 to 20 and higher than 40 mg GAE/g dry weight of plant extract, respectively. Plants with the lower total phenolic content included the young leaves of *Hydrolea zeylanica* (L.) Vahl., *Lasia spinosa* (Linn.) Thw., and *Limnocharis flava* Buch. (5 to 7 mg GAE/g dry weight of plant extract), which are mostly vegetables consumed fresh with chilli paste in Thailand and most commonly found in rice fields as weeds. Plant from herbs and vegetables with a higher phenolic content also contained a higher flavonoid content (13 to 26 mg RE/g dry weight of plant extract).

The phenolic content of selected chewing plant extracts was higher than that in extracts from other studied herb and vegetable extracts (53 to 178 mg GAE/g dry weight of plant extracts) except that of some herbs and vegetables such as *Cratoxylum formosum* Dyer. or *Careya sphaerica* Roxb. In addition, the total flavonoid content of extracts from chewing plants was in the range 13 to 43 RE/g dry weight of plant extract. However, since these plant parts are used as chewing materials but without being swallowed, the toxicity of extracts from this group should be further investigated before they are used in food products.

We can observe that there are five plant young leaves in the group of herbs and vegetables containing remarkably high phenolic contents (51 to 63 mg GAE/g dry weight of plant extract) and flavonoid contents (20 to 26 mg RE/g dry weight of plant extract): *Careya sphaerica* Roxb*., Cratoxylum formosum* Dyer*., Leucaena glauca* Benth*, Momordica charantia* Linn*. and Syzygium gratum* (Wight) S.N. Mitra var gratum. These plants may be considered suitable for further investigation of their potential antioxidant activity in foods because usually they can be consumed fresh without toxicity.

Table 14 Total phenolic content, total flavonoid content and DPPH radical scavenging activity of Thai indigenous plants [a]

Scientific name	Plant part	Total phenolics (mg GAE/g dw)[c]	Total flavonoids (mg RE/g dw)[b]	DPPH radical scavenging activity (EC_{50},μg·μg^{-1} DPPH)[c]
Berries and fruits				
Antidesma velutinum Tulas.	Seed	123.3±0.3	50.3±0.0	0.07±0.01
Cleistocalyx operculatus var *paniala* (Roxb.)	Seed	173.6±1.9	44.2±0.2	0.09±0.00
Diospyros kaki L.	Whole fruit	17.8±0.7	2.5±0.0	0.98±0.00
	Fruit peel	12.9±1.2	1.6±0.0	1.70±0.01
	Fruit flesh	22.8±1.6	4.1±0.1	0.58±0.00
Eugenia siamensis Craib.	Seed	180.5±1.3	50.4±0.3	0.15±0.02
Garcinia mangostana Linn.	Fruit peel	24.9±0.7	10.9±0.1	1.09±0.00
Leucaena glauca Benth	Seed	20.4±0.0	5.3±0.0	7.01±0.09
Mangifera indica Linn.	Seed	51.6±0.1	14.6±0.1	0.34±0.01
Nephelium lappaceum Linn.	Fruit peel	42.3±0.1	9.6±0.0	1.46±0.04
	Seed	43.5±0.4	13.3±0.1	0.46±0.00
Nephelium hypoleucum Kurz	Whole fruit	89.6±0.2	11.1±0.7	0.40±0.00
Piper nigrum Linn.	Seed	53.1±0.4	22.8±0.1	0.33±0.00
Spondias pinnata Kurz	Fruit flesh	47.2±0.2	12.7±0.1	0.62±0.00
	Seed	50.7±0.1	17.8±0.3	0.43±0.00
Tamarindus indica Linn.	Seed	40.7±0.1	23.2±0.1	0.49±0.01
	Seed skin	134.4±0.1	41.3±0.1	0.14±0.00
Herbs and vegetables				
Basella alba Linn.	Young leaf	15.5±0.1	6.2±0.0	1.48±0.00
Careya sphaerica Roxb.	Young leaf	54.5±0.3	20.5±0.1	0.43±0.04
Cratoxylum formosum Dyer.	Young leaf	63.4±0.5	25.5±0.1	0.23±0.00
Hydrocharis dubia (Bl.) Back.	Young leaf	20.4±0.2	8.9±0.0	0.82±0.00
Hydrolea zeylanica (L.) Vahl.	Young leaf	7.4±0.0	3.6±0.0	6.14±0.05
Lasia spinosa (Linn.) Thw.	Young leaf	6.4±0.1	4.4±0.1	7.49±0.02
Leucaena glauca Benth	Young leaf	52.2±1.6	22.3±0.0	0.68±0.01
Limnocharis flava Buch.	Young leaf and flower	5.4±0.1	3.7± 0.2	7.42±0.04
Momordica charantia Linn.	Young leaf and leaf	50.9±0.9	21.6±0.1	0.59±0.00
Sesbania grandiflora Desv.	Flower	50.6±0.6	13.1±0.1	0.58±0.00
Spondias pinnata Kurz	Young leaf	42.6±0.2	14.8±0.1	1.48±0.02
Syzygium gratum (Wight) S.N.Mitra var gratum	Young leaf	57.3±0.1	23.6±0.1	0.55±0.00
Chewing plants				
Acacia catechu (L.F) Willd.	Bark	177.7±0.2	41.8±0.2	0.05±0.00
Areca catechu Linn.	Whole fruit	52.5±0.2	12.6±0.1	0.47±0.00
	Kernel	137.3±0.3	42.8±0.1	0.18±0.00
Cassia fistula Linn.	Stem core	103.6±0.2	25.4±0.2	0.16±0.01
Piper betel Linn.	Leaf	57.5±0.6	14.9±0.1	0.32±0.00

[a] Values are the mean ± standard deviation (n = 3).
[b] dry weight basis of the original sample of plant parts.
[c] calculated by using dry weight of the ethanolic plant extract.

2.3 Free Radical Scavenging Activity of Plant Extracts Using DPPH

Since the main mechanism of antioxidant action in foods is radical scavenging, many methods have been developed in which the antioxidant activity is evaluated by the scavenging of synthetic radicals in polar organic solvents such as methanol at room temperature. The radicals used include 2, 2-diphenyl-1-picrylhydrazyl (DPPH) and 2, 2′-azinobis-3-ethylbenzthiazoline-6-sulphonic acid (ABTS) radicals (Gordon, 2001). In this study, the DPPH method was selected to evaluate the antioxidant activity of plant extracts because it is one of the most effective methods for evaluating the concentration of radical scavenging materials active by a chain-breaking mechanism (Niki, 1987). The DPPH radical is a stable free radical and the DPPH radical scavenging activity was determined by the decrease in absorbance at 517 nm due to reduction by the antioxidant (AH) or reaction with a radical species as shown in the equations (22) and (23) (Gordon, 2001).

$$DPPH\cdot + AH \rightarrow DPPH - H + A\cdot \quad (22)$$

$$DPPH\cdot + R\cdot \rightarrow DPPH - R \quad (23)$$

The DPPH radical scavenging capacity in the studies was reported after 30 min reaction time for each diluted plant extract. All the plots of the plant extracts showed sigmoid non-linear curves (data not shown). EC_{50} value, defined as the concentration of antioxidant required for 50% scavenging of DPPH radicals in this specified time period, is a parameter widely used to measure antioxidant activity; a smaller EC_{50} value corresponds to a higher antioxidant activity of the plant extract. The EC_{50} value of various plant extracts shown in Table 14 was determined based on scavenging activity per unit mass of DPPH in μg.

For the group of berries and fruits, the DPPH radical scavenging activity (EC_{50}) values of ethanolic extracts of seeds from *Antidesma velutinum* Tulas., *Cleistocalyx operculatus* var *paniala* Roxb. and *Eugenia siamensis* Craib.

containing notably high levels of both phenolics and flavonoids were found to be very low (about 0.07 to 0.15 $\mu g \cdot \mu g^{-1}$ DPPH) compared to those obtained from other seed extracts (0.33 to 0.49 $\mu g \cdot \mu g^{-1}$ DPPH) except that of *Leucaena glauca* Benth which was 7.0 $\mu g \cdot \mu g^{-1}$ DPPH (Table 13). The antiradical activity (A_{AR}) defined as $1/EC_{50}$ was plotted for extracts from various berries and fruits as shown in Figure 32. As expected, extracts with higher antiradical activity were obtained from the three seed extracts mentioned above as well as from extracts from the tamarind seed skin (0.14 $\mu g \cdot \mu g^{-1}$ DPPH) (Table 13).

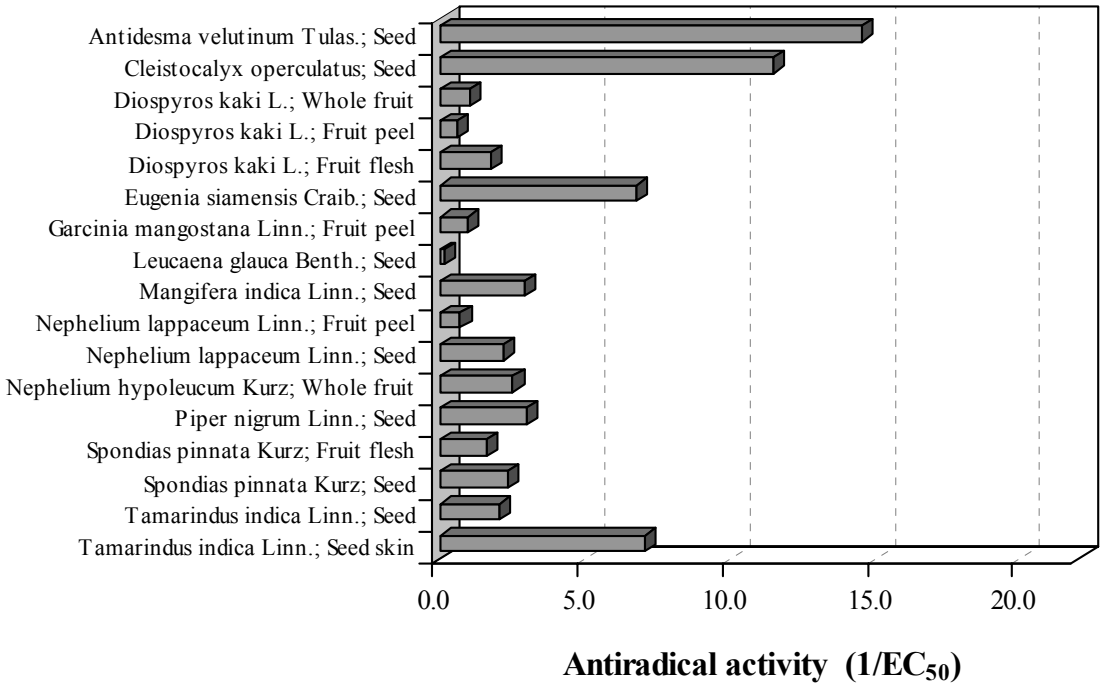


Figure 32 Antiradical activity ($1/EC_{50}$) of ethanolic extracts from various plant parts of berries and fruits.

As discussed previously, in the group of herbs and spices, samples with a low total phenolic content namely the young leaves of *Hydrolea zeylanica* (L.) Vahl., *Lasia spinosa* (Linn.) Thw., and *Limnocharis flava* Buch. (5 to 7 mg GAE/g dry weight of plant extracts) also exhibited a low total flavonoid content, and a high EC_{50} value (higher than 6 $\mu g \cdot \mu g^{-1}$ DPPH) as shown in Table 13 which indicated a very low antiradical activity (Figure 32). Extracts from other herbs and

vegetables also appeared to have lower antiradical activity compared with the activity of extracts from the seeds. Amongst the herbs and vegetables, the highest antiradical activity was found in the extract of *Cratoxylum formosum* Dyer. (young leaf) which can be easily found in North-East Thailand. However, this antiradical activity was still lower than that of extracts of seeds obtained from wine production and tamarind seed skin. Similar findings that seed extracts exhibited a much higher antioxidant activity than those from the edible portions were also reported by Soong and Barlow (2004).

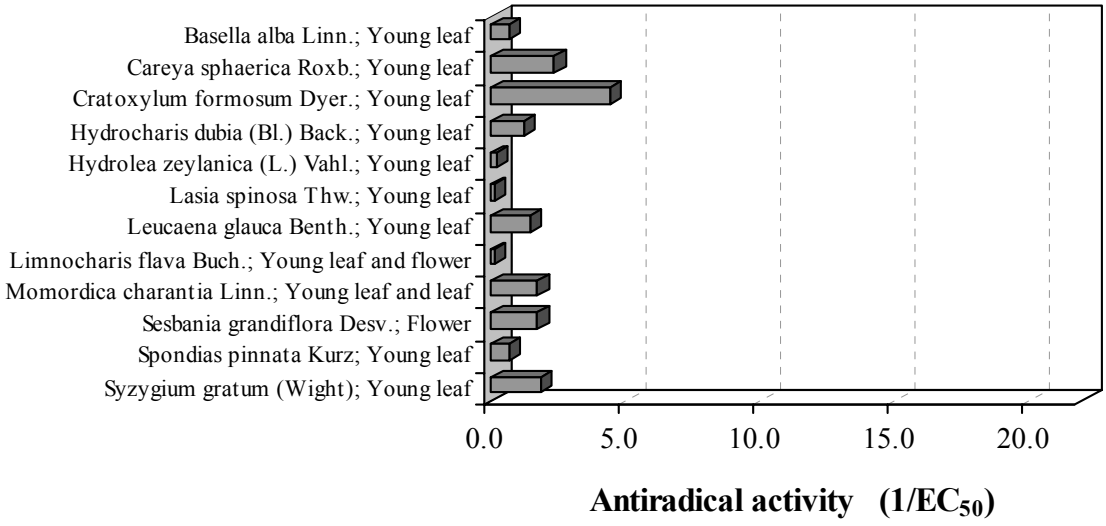


Figure 33 Antiradical activity ($1/EC_{50}$) of ethanolic extracts from young leaf, leaf and flower of herbs and vegetables.

In chewing plants, the EC_{50} value of bark extract from *Acacia catechu* (L.F) Willd. was lowest (0.05 $\mu g \cdot \mu g^{-1}$ DPPH) and gave the highest antiradical activity among all studied plant parts (Figure 33). The highest antiradical activity of bark extract (*Acacia catechu* (L.F) Willd.) reflects the high phenolic content (178 mg GAE/g dry weight of plant extract) and flavonoid content (42 mg RE/g dry weight of plant extract) (Table 14). However, it should be noted that the seed extract from *Eugenia siamensis* Craib had both the highest phenolic content (180 mg GAE/g dry weight of plant extract) and flavonoid content (50 mg RE/g dry weight of plant extract) among all the extracts investigated, but the antiradical

activity was lower than that of the bark extract from *Acacia catechu* (L.F) Willd. When considering the extracts of *Areca catechu* Linn. (kernel) and *Cassia fistula* Linn. (stem core) in the group of chewing plants, the extracts appeared to contain high antioxidant activity (EC_{50} values = 0.18 and 0.16 $\mu g \cdot \mu g^{-1}$ DPPH, respectively) with high phenolic content (137 and 104 mg GAE/g dry weight of plant extracts) and flavonoid content (43 and 25 mg RE/g dry weight of plant extracts), respectively (Table 12).

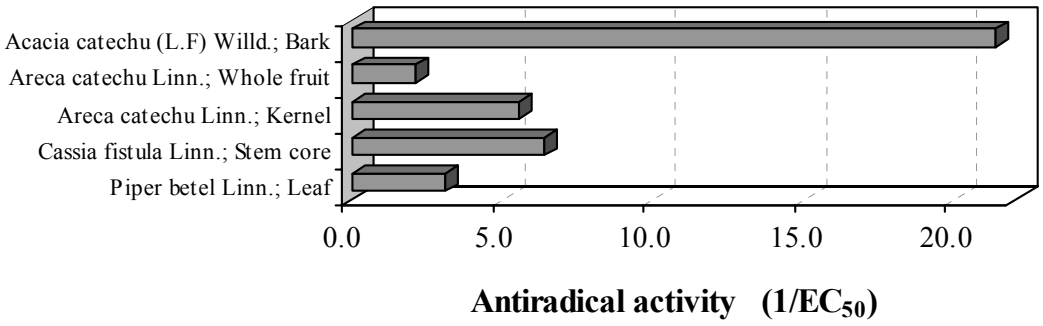


Figure 34 Antiradical activity ($1/EC_{50}$) of ethanolic extracts from plant parts of chewing plants.

2.4 Correlation between Total Phenolic and Flavonoid Content and Antiradical Activity

It is interesting to observe the correlation between the phenolic content and antioxidant activity between the plant extracts since phenolic compounds contribute directly to antioxidant activity (Duh *et al.*, 1999). In this study, there was a distinct correlation between studied parameters (total phenolic content, total flavonoid content and antiradical activity) in selected Thai indigenous plant parts. The antiradical activity ($1/EC_{50}$) as a function of total phenolic content and total flavonoid content are shown in Figure 35a and 35b, respectively and correlation coefficients (*r*) of those plots calculated from linear regression analysis were about 0.8 for all ethanolic extracts of plant parts. However, the literature includes studies reporting a weak correlation between antioxidant activity and total phenolics

(Kähkonen *et al*., 2001; Velioglu *et al.,* 1998). When the relationship between total phenolic content and total flavonoid content of all ethanolic extracts was plotted as shown in Figure 35c, the correlation coefficient (*r*) between these two parameters was higher than 0.9 indicating that there is a significant positive relationship between the total phenolic and flavonoid contents of all plant extracts selected in this study.

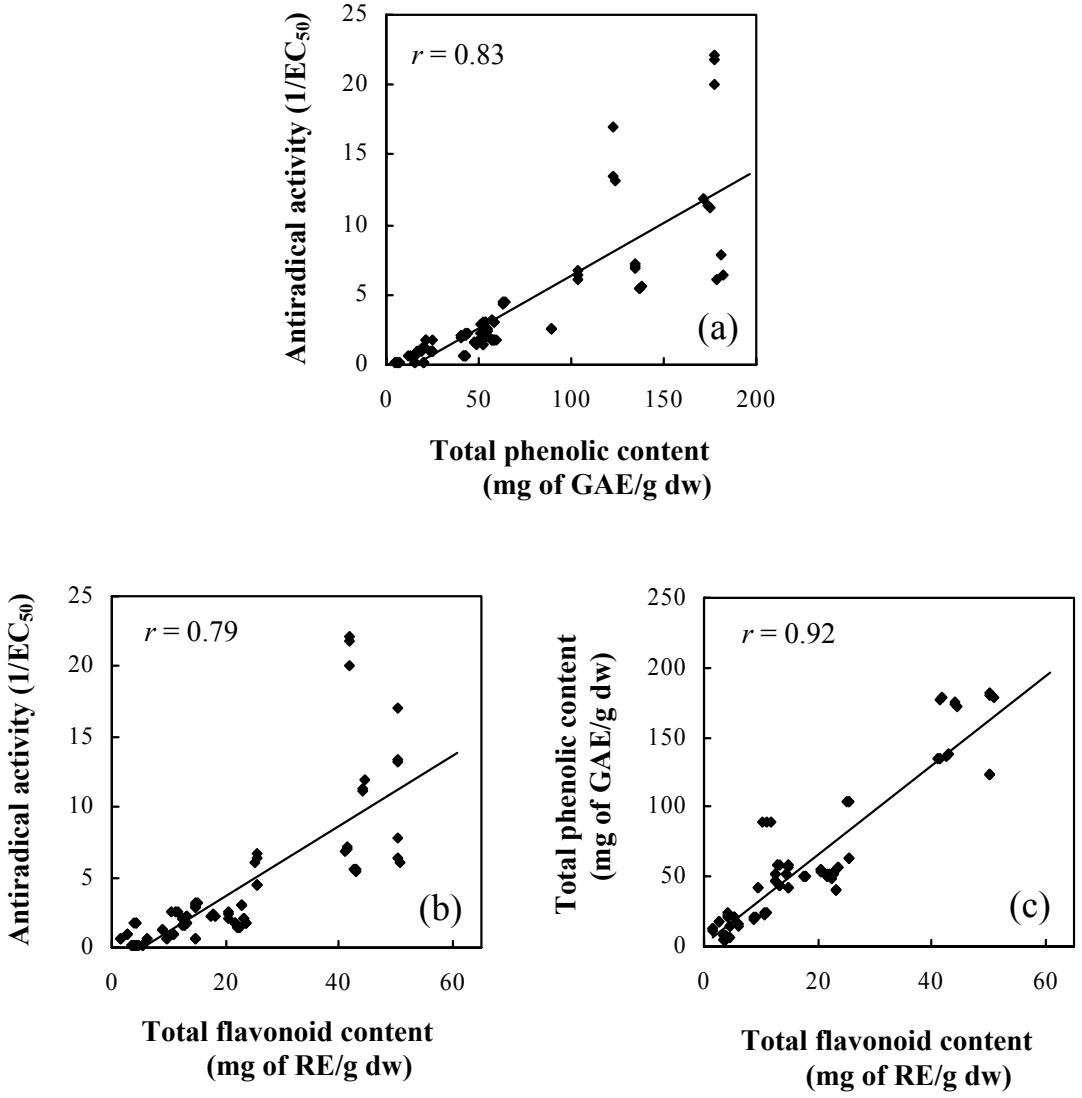


Figure 35 Correlation between (a) total phenolic content and antiradical activity, (b) total flavonoid content and antiradical activity and (c) total phenolic content and total flavonoid content of ethanolic extracts from Thai indigeneous plants.

In conclusion, according to the evaluation of phenolic constituents and free-radical scavenging capacity from ethanolic extracts from various parts of twenty-six Thai indigenous plants, the extracts of berries used in wine production were found to have a higher antiradical activity than those obtained from herbs and vegetables whereas chewing plants with an astringent taste had a high level of total phenolic content and flavonoid content. The correlation coefficients exhibited a high positive relationship between total phenolic and flavonoid contents in the plant extracts and antiradical activity. The present study suggests that ethanolic extracts of these Thai indigenous plants are a potential source of natural antioxidants. For selecting three plant extracts which should be investigated further, we have to consider their high antioxidant capacity, their safety for use as food additives, available sources, and cost. Therefore, *Cratoxylum formosum* Dyer. (Teaw), *Careya sphaerica* Roxb. (Kradonbok), and *Leaucaena glauca* Benth (Kratin) were selected to study phenolic radical scavenging components.

3. Evaluation of Phenolic Radical Scavenging Components in Selected Plants by HPLC-DAD and HPLC-ESI-MS

3.1 Toxicity of Plant Extracts

Each plant extract; *Cratoxylum Formosum* Dyer. (Teaw); *Careya sphaerica* Roxb. (Kradonbok) and *Leucaena glauca* Benth (Kratin) was replaced by distilled water for the control group (10 mice). After consuming the first dose, the mice showed decreased respiration and spontaneous motor activities. Following the second dose, the animals suffered from dyspnea and five of the ten mice showed signs of somnolence. These signs continued to be observed for the test group until day 10 of the experiment whereas the control group showed no abnormality. At the end of the 14-day-observation period, all animals survived and necropsy revealed no abnorminality of visceral organs. Hence LD_{50} of the Teaw, Kradonbok and Kratin extracts are > 32 $g \cdot kg^{-1}$, > 24 $g \cdot kg^{-1}$, > 20 $g \cdot kg^{-1}$, respectively.

3.2 Analysis of Radical Scavenging Components by HPLC-DAD and Identification of Extract Components by HPLC-ESI-MS

3.2.1 *Cratoxylum formosum* Dyer. (Teaw) Extract

The HPLC chromatogram of the Teaw extract is shown in Figure36. Four main peaks were detected and the area of these peaks was greater at 320 nm than at 260 or 280 nm. The component with the greatest peak area eluted at 11.84 min (peak 1) in the more hydrophilic region (short retention time). The other three main components eluted at 30.98 min (peak 2), 34.07 min (peak 3) and 37.66 min (peak 4) in the more hydrophobic region (long retention time).

The identity and purity of the main peak (peak 1) in the HPLC chromatogram of the extract of *Cratoxylum formosum* Dyer. was determined by comparison of the retention time and UV spectrum (Figure 37) with that of pure chlorogenic acid. The identification of the main peak as chlorogenic acid was confirmed by co-injection of chlorogenic acid with the plant extract. HPLC-ESI-MS confirmed the identification of this compound. Negative-ion MS gave a high mass peak with m/z 353 $[M - H]^-$ (parent peak). Positive-ion MS showed a base peak of m/z 377, due to the sodium adduct ion $[M + Na]^+$. A potassium adduct ion was present at m/z 393 $[M + K]^+$ (Appendix B), confirming the molecular weight of 354 for chlorogenic acid (Table 15, Figure 38a). The compound from peak 2 had a similar UV spectrum to that of peak 1 (Figure 37), characteristic of molecules with the caffeic acid moiety such as chlorogenic acid. Compound 2 had a negative-ion mass spectrum with a peak at m/z 515.1 (M + H) indicating a molecular weight of 514.1(Appendix B). This is consistent with the compound being dicaffeoylquinic acid (diCQA). The mass spectrum of 1, 3 dicaffeoylquinic acid (1, 3 diCQA) was reported as showing only the peak m/z 515.1 at high mass number in the negative-ion mode. Parejo *et al*. (2004) reported that chlorogenic acid showed a shorter retention time (8.22 min) than 1, 3 diCQA (21.09 min) under similar HPLC conditions to those used in the study. In this study, the

retention time of chlorogenic acid was 12 min and that of peak 2 was 31 min, and these values are consistent with the provisional identification of peak 2 as due to dicaffeoylquinic acid (Figure 38b). The negative ion mass spectra of 1, 3; 1, 4; and 1, 5 - diCQA isomers are similar, so the positions of the caffeic acid substituents cannot be identified from this data (Clifford *et al*., 2005). The wavelengths for maximum absorbance (λ max) in the spectrum of peak 2 at 325 and 296-298 nm are consistent with this identification.

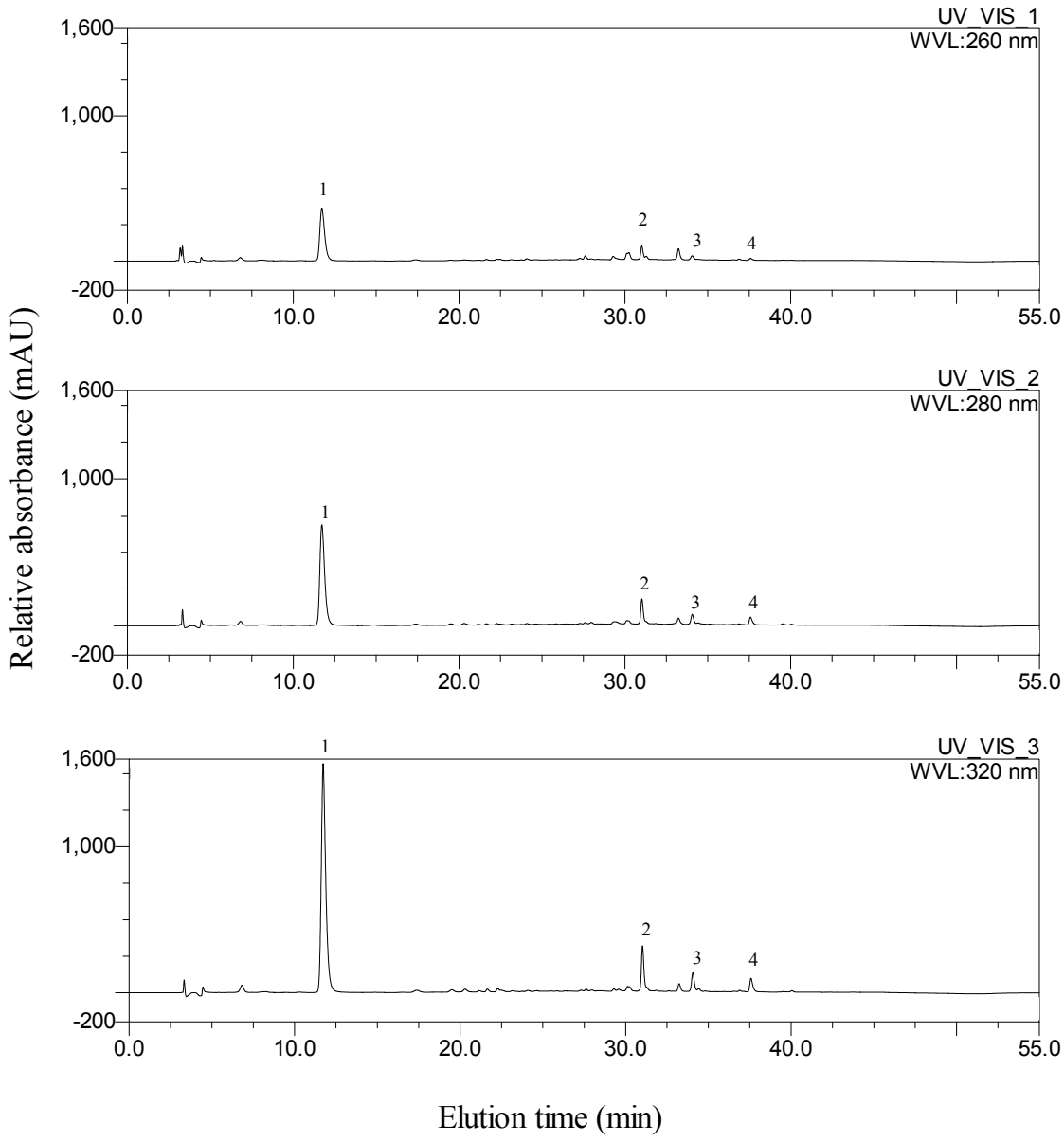


Figure 36 HPLC chromatogram for the extract from *Cratoxylum formosum* Dyer. (Teaw) detected at 260, 280 and 320 nm.

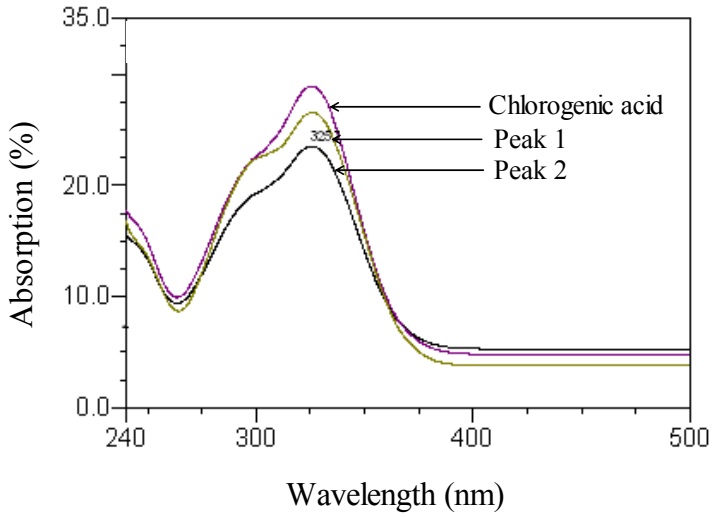


Figure 37 UV spectrum of peaks 1 and 2 of *Cratoxylum formosum* Dyer. (Teaw) extract and chlorogenic acid.

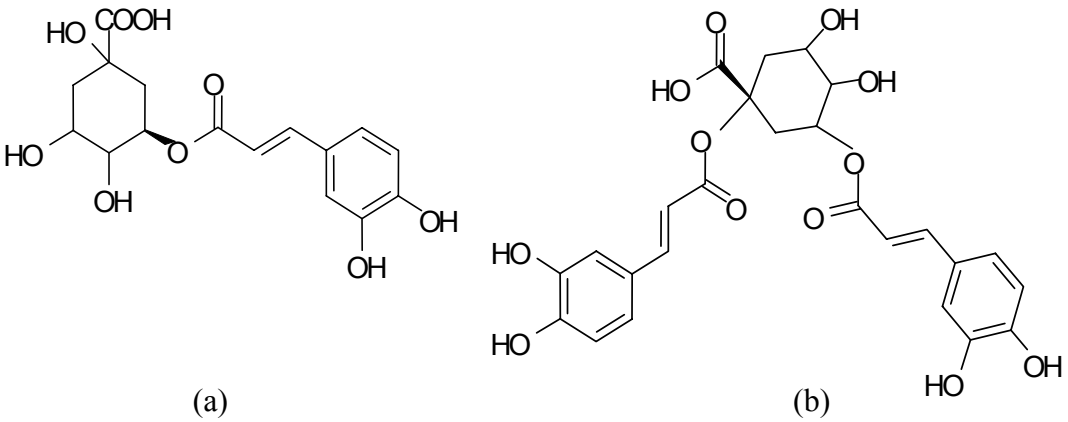


Figure 38 Structure of (a) chlorogenic acid and (b) dicaffeoylquinic acid (shown as 1, 3 diCQA).

Compounds 3 and 4 had similar UV spectra, characteristic of ferulic acid structures (λ max at 312-315 and 298 nm) (Figure 39). Only positive-ion mass spectra of compounds 3 and 4 were determined. A match for the mass spectrum of compound 3 (Figure 40) could not be found from the literature. The mass spectrum of compound 4 is consistent with a molecular weight of 452 since it has [M + H]+

ion at m/z 453.55 (Appendix B). This compound could be identical with the ferulic acid hexose derivative with a molecular weight of 452 reported in the literature as a component of berries of the *Ribes* species (Mááttá *et al*., 2003).

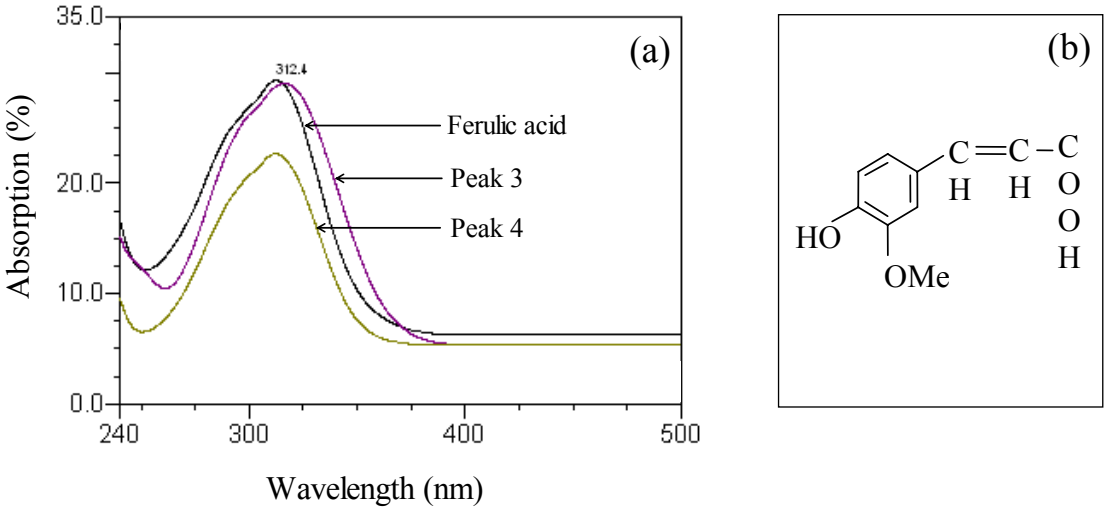


Figure 39 UV spectrum of (a) peaks 3 and 4 of *Cratoxylum formosum* Dyer. (Teaw) extract, ferulic acid and (b) structure of ferulic acid.

Table 15 Retention times, UV spectra and ESI mass spectra of phenolic radical scavenging components from *Cratoxylum formosum* Dyer. (Teaw) extract

Compound	Time (min)	UV-Peak maxima (nm)	UV-Peak maxima (nm) from Literature	ESI Mass spectra	Molecular weight	Identity	ESI-mass spectra from literature
1	11.84	235, 295 sh, 325	235, 301 sh, 325 (Yao *et al*., 2004)	$[M+H]^+$at m/z 355 $[M+Na]^+$ at m/z 377 $[M+K]^+$ at m/z 393 $[M-H]^-$ at m/z 353	354	Chlorogenic acid	$[M-H]^-$ at m/z 352.9 (Clifford *et al*., 2005)
2	30.98	235,295sh, 325		$[M+H]^+$at m/z 515.1	514.1	Dicaffeoyl-quinic acid	$[M-H]^-$ at m/z 515.1 (Clifford *et al*., 2005)
3	34.07	234, 298sh, 318				Unknown compound	
4	37.66	234, 293sh, 312	324, 296sh, 330 (Määta *et al*., 2003)	$[M+H]^+$at m/z 453.55	452	Ferulic acid hexose derivative	$[M+H]^+$at m/z 453 (Määta *et al*., 2003)

Note : sh mean shoulder

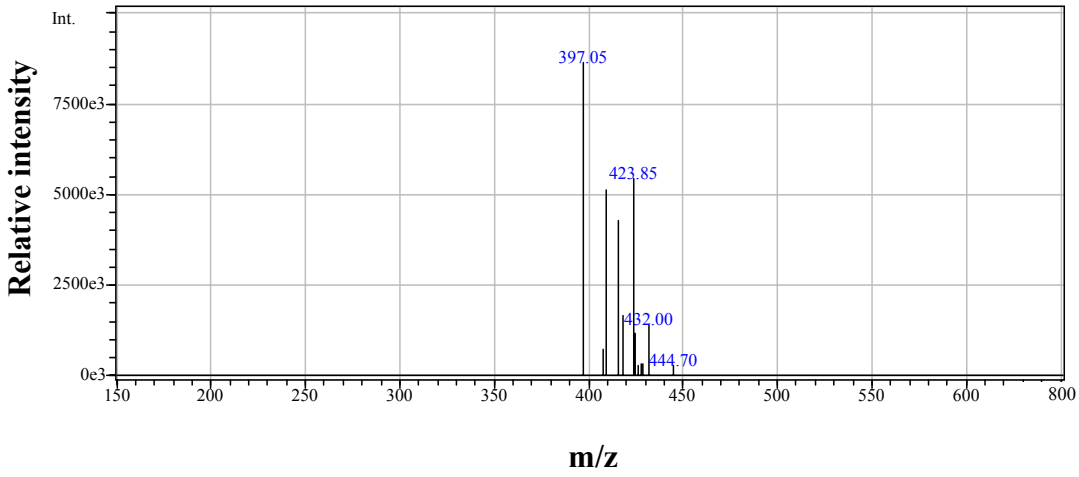


Figure 40 Positive ion LC-MS of peak 3 from *Cratoxylum formosum* Dyer. (Teaw) extract.

The main antioxidant component (peak 1) was chlorogenic acid, which was present at 60 % of the extract. Three minor components (peak 2, 3 and 4) were present at 7 %, 3 % and 2 %, and other components that were present at lower concentrations were also observed. These areas were calculated according to total peak areas from the HPLC chromatogram. The four phenolic compounds were about 72 % of the total phenolic compound of Teaw extract.

Conventional separation of all constituents of plant extracts to identify the radical scavenging components is very time-consuming and may cause decomposition of phenolic components during repeated fractionation. Addition of DPPH solution to the Teaw extract was used to detect which components reacted readily with the DPPH radical. The reduction in area of peaks 1, 2, 3 and 4 when reacted with DPPH was 58.1 %, 55.2 %, 56.7 % and 58.0 %, respectively with detection at 320 nm, and detection at 260 and 280 nm showed similar changes (Figure 41). The similarity in the wavelength of maximum absorbance of peaks 1-4 and the similar change in peak area due to 5 mins reaction with DPPH indicates that chlorogenic acid and components eluting as peaks 2-4 share common structural features.

Chlorogenic acid is a caffeic acid derivative that is reported to posess antibacterial, antimutagenic, antitumor and antiviral properties, as well as acting as an antioxidant by radical scavenging and metal chelation (Tudela *et al*., 2002; Armesto, *et al*., 2003).

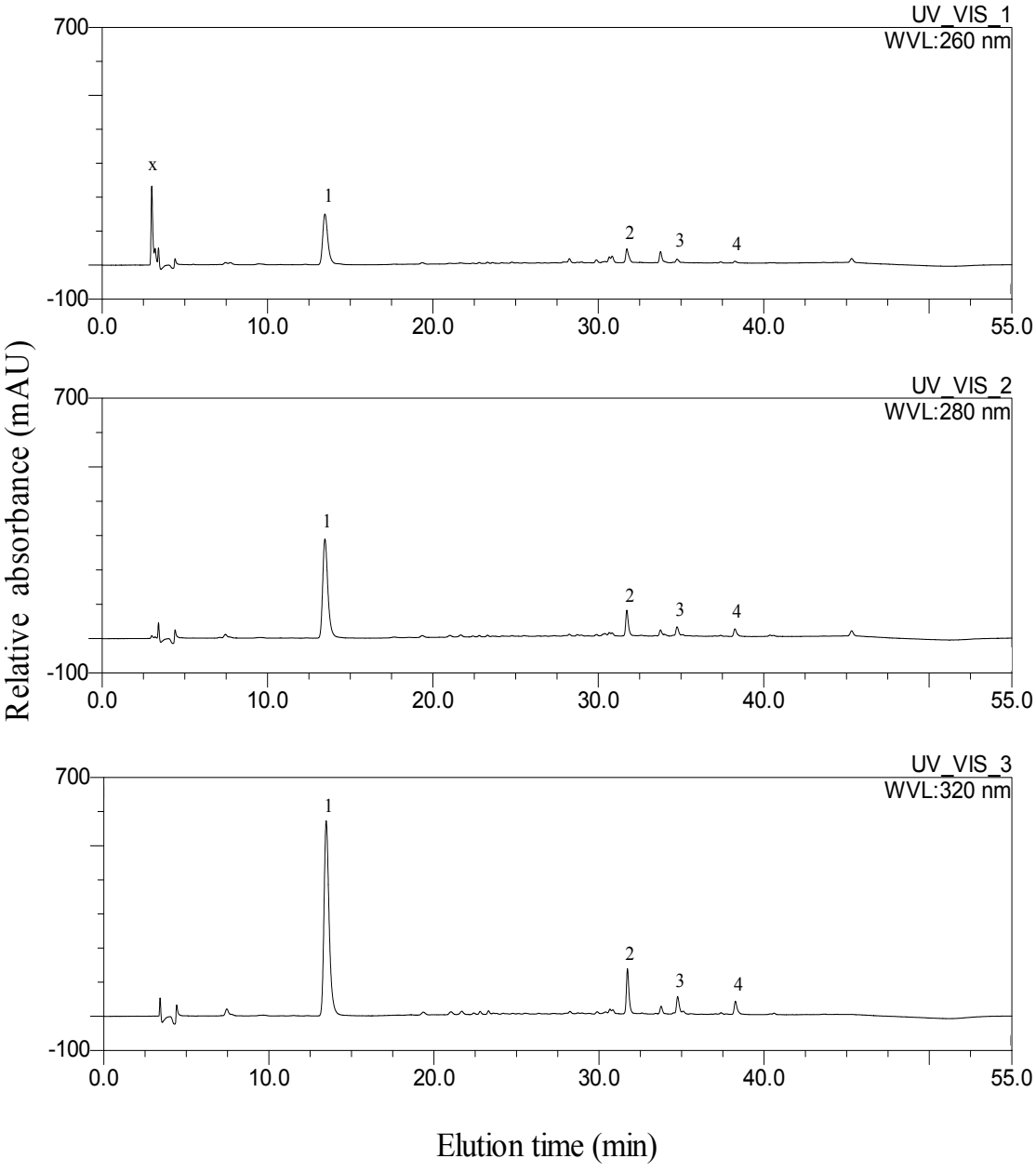


Figure 41 HPLC chromatogram for the extract from *Cratoxylum formosum* Dyer. (Teaw) after reaction with DPPH.

Treatment of pure chlorogenic acid with DPPH was compared with treatment of the Teaw leaf extract. The results showed that 1 ml of 5 mM DPPH solution reacted with 0.598 mg of pure chlorogenic acid and with 0.593 mg chlorogenic acid in the Teaw extract. These values are identical within experimental error and they confirm that chlorogenic acid is the main antioxidant in the Teaw extract.

Chlorogenic acid (3-caffeoyl-D-quinic acid) is an ester of caffeic acid and quinic acid, and has an *o*-diphenolic structure. In general, *o*-diphenolic compounds are oxidized to *o*-quinones by free radicals such as DPPH radical (Dufour *et al*., 2002). A new HPLC peak (peak X) appeared with a retention time of 3.05 min after reaction of the Teaw extract with DPPH for 5 min (Figure 41). This oxidation product absorbed more strongly at 260 nm than at 280 or 320 nm (Figure 41). The UV spectrum of the oxidation product showed an absorbance maximum at 255.6 nm (Figure 42). A previous study has shown that oxidation of chlorogenic acid with phenolase or with the reactive oxygen species hypochlorous acid produces a product with an absorption maximum at about 250 nm which was identified as an *o*-quinone (Kono *et al*., 1995). The oxidation of an *o*-diphenol, such as caffeoylquinic acid, to reactive *o*-quinones is well recognised (Le Bourvellec *et al*., 2004). *o*-Quinones are not stable but may polymerise, react with an *o*-diphenol to yield a condensation product, or oxidize other phenols by coupled oxidation with reduction of *o*-quinone back to *o*-diphenol (Le Bourvellec *et al*., 2004). The mechanism for the oxidative degradation of chlorogenic acid during trapping of DPPH radicals is shown in Figure 43. The peak at 3.05 min disappeared when the reaction between DPPH and the Teaw extract was allowed to continue for more than five minutes (data not shown). This is consistent with the known reactivity of *o*-quinones.

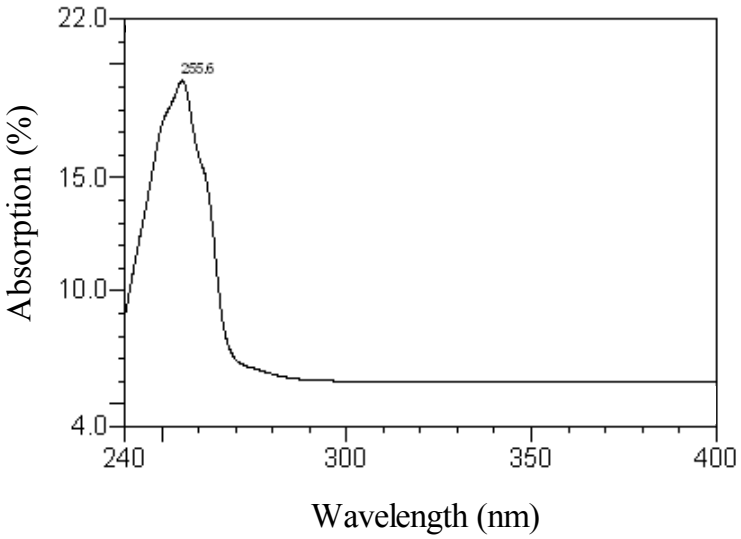


Figure 42 UV spectra of oxidation product of *Cratoxylum formosum* Dyer.(Teaw) extract after reaction with DPPH.

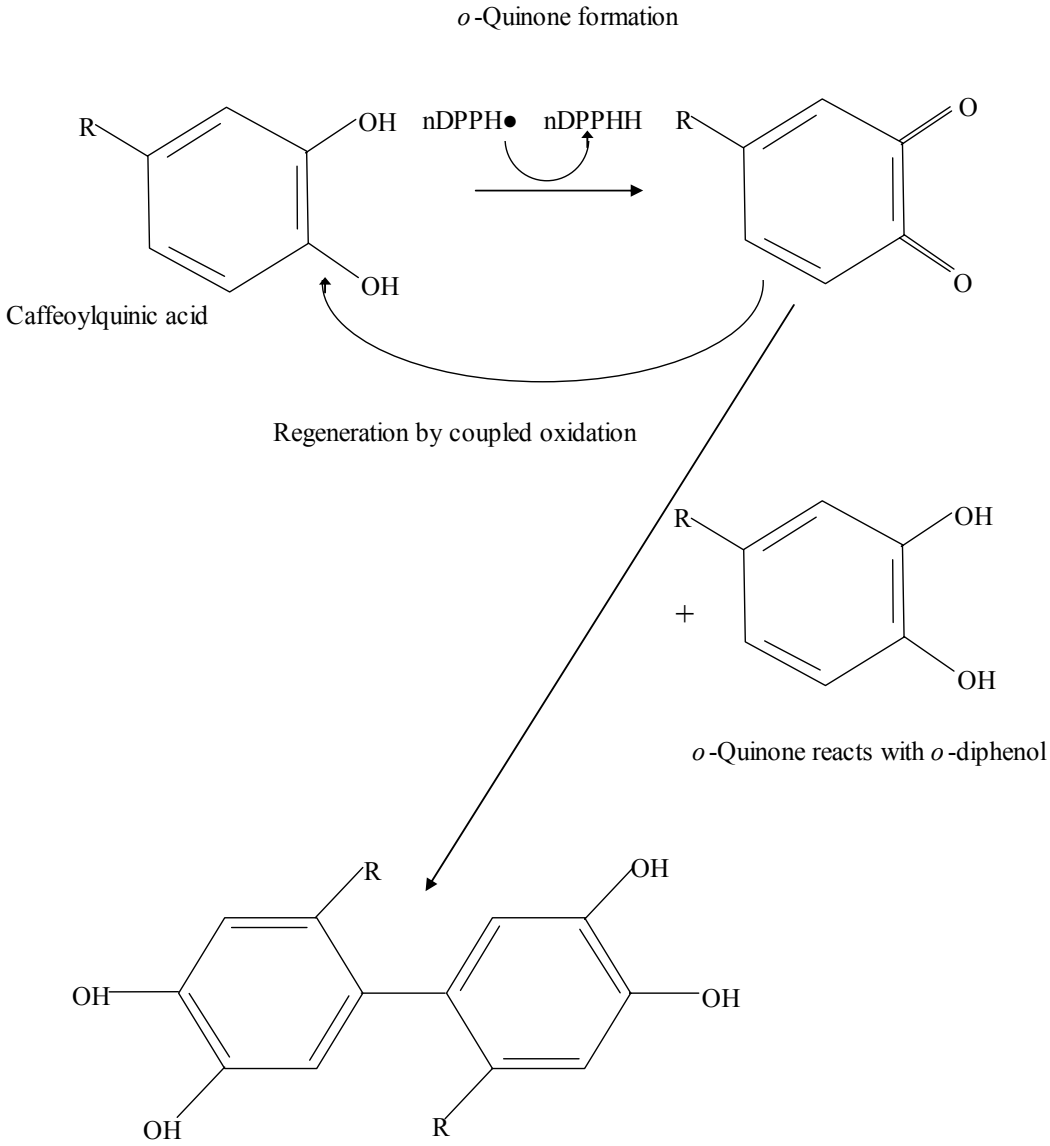


Figure 43 Possible pathway for the oxidative degradation of caffeoylquinic acid due to reaction with the DPPH radical.

3.2.1 *Careya sphaerica* Roxb. (Kradonbok) Extract

The HPLC chromatogram of the Kradonbok extract is shown in Figure 44 Thirteen main peaks were detected and the area of peaks 1-7 was greater at 260 nm than at 280, or 320 nm. However, the area of peaks 8-13 was greater at 320 nm than at the other wavelengths. The components corresponding to peaks 1, 2, 3, 4, 5, and 6 eluted at 11.06, 33.91, 41.79, 48.35, 51.91 and 62.36 min in the more

hydrophilic region (short retention time). The other main components eluted at 100.19 min (peak 7), 134.71 min (peak 8), 139.27 min (peak 9), 160.93 min (peak 10), 164.63 min (peak 11), 170.44 min (peak 12) and 174.90 min (peak 13) in the more hydrophobic region (long retention time).

The identity and purity of peak 1 in the HPLC chromatogram of the extract of *Careya sphaerica* Roxb. was determined by comparison of the retention time and UV spectrum (Figure 45) with that of pure gallic acid. The identification of the peak 1 as gallic acid was confirmed by co-injection of gallic acid with the plant extract. HPLC-ESI-MS confirmed the identification of this compound (MS $[M + H]^+$ at m/z 171, MS $[M + Na]^+$ at m/z 193 and MS $[M+4Na]^+$ at m/z 262), confirming the molecular weight of 170.12 for gallic acid (Appendix C). The compounds corresponding to peaks 2, 3, 4, 5 and 6 had a similar UV spectrum to that of peak 1, characteristic of molecules containing the gallic acid moiety. The ESI mass spectrum of peak-13 is shown in Table 16. Compound 2 had a positive-ion mass spectrum with a peak at m/z 229 $[M + 3H]^+$, m/z 272 $[M + 2Na]^+$, m/z 295 $[M + 3Na]^+$, m/z 382 $[M+4K]^+$ and m/z 456 $[M+10Na]^+$ indicating a molecular weight of 226 (Appendix C). Compound 3 had a negative-ion mass spectrum with a peak at m/z 307 $[M - H]^-$, m/z 367 $[M+Oac]^-$ indicating a molecular weight of 308 (Appendix C). Compound 4 had a positive-ion mass spectrum with a peak at m/z 374 $[M + H]^+$, m/z 396 $[M + Na]^+$, m/z 419 $[M + 2Na]^+$, m/z 442 $[M+3Na]^+$ indicating a molecular weight of 373 (Appendix C). Compound 5 had a positive-ion mass spectrum with a peak at m/z 205 $[M + H]^+$, m/z 243 $[M + K]^+$, m/z 360 $[M + 4K]^+$, m/z 438 $[M+6K]^+$ indicating a molecular weight of 204 (Appendix C). Compound 6 had a positive-ion mass spectrum with a peak at m/z 332 $[M + H]^+$, m/z 377 $[M + 2Na]^+$, m/z 538 $[M + 9Na]^+$, indicating a molecular weight of 331 (Appendix C). A match for the mass spectra of compounds 2-6 could not be found from the literature.

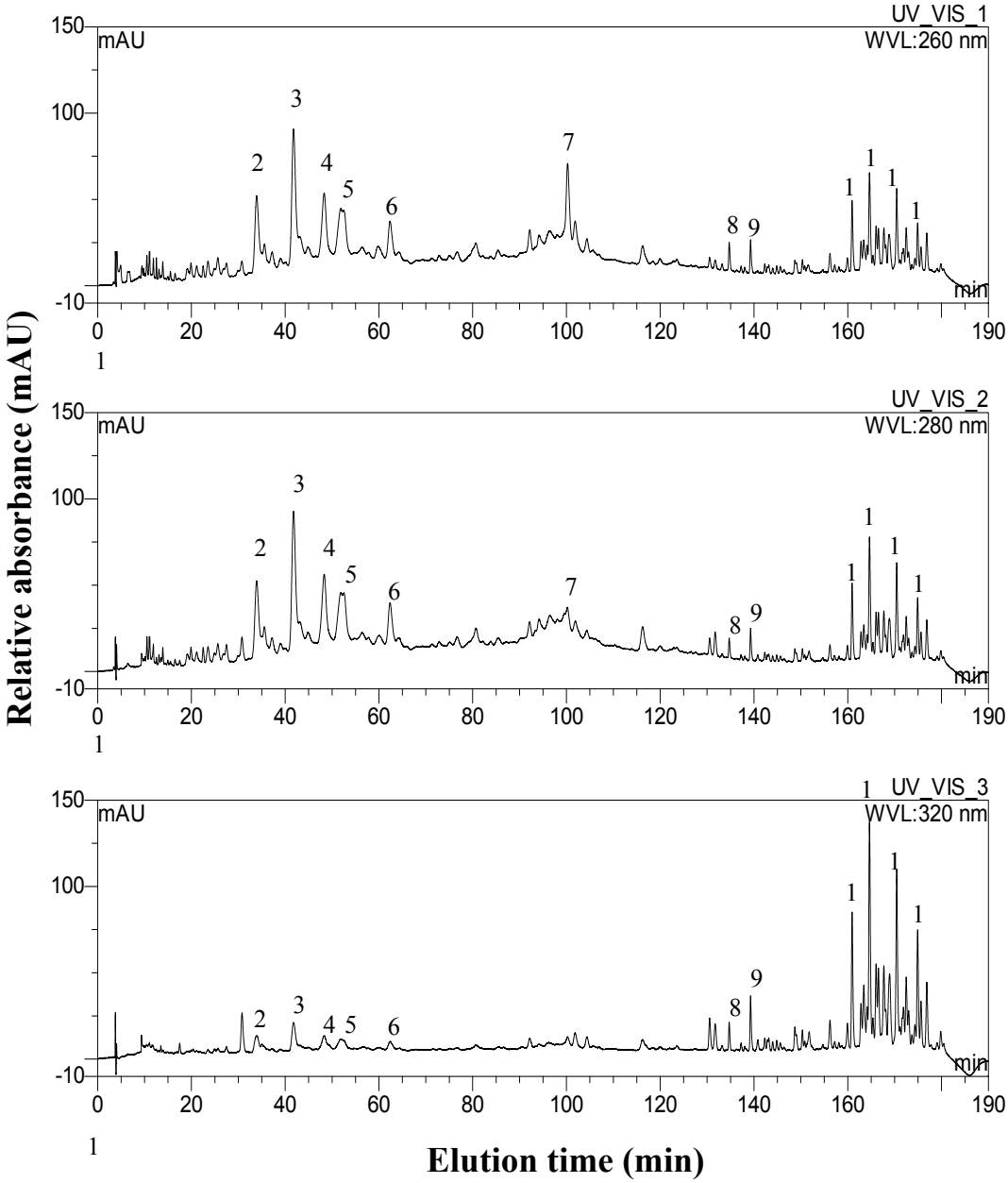


Figure 44 HPLC chromatogram for the extract from *Careya sphaerica* Roxb. (Kradonbok) detected at 260, 280 and 320 nm.

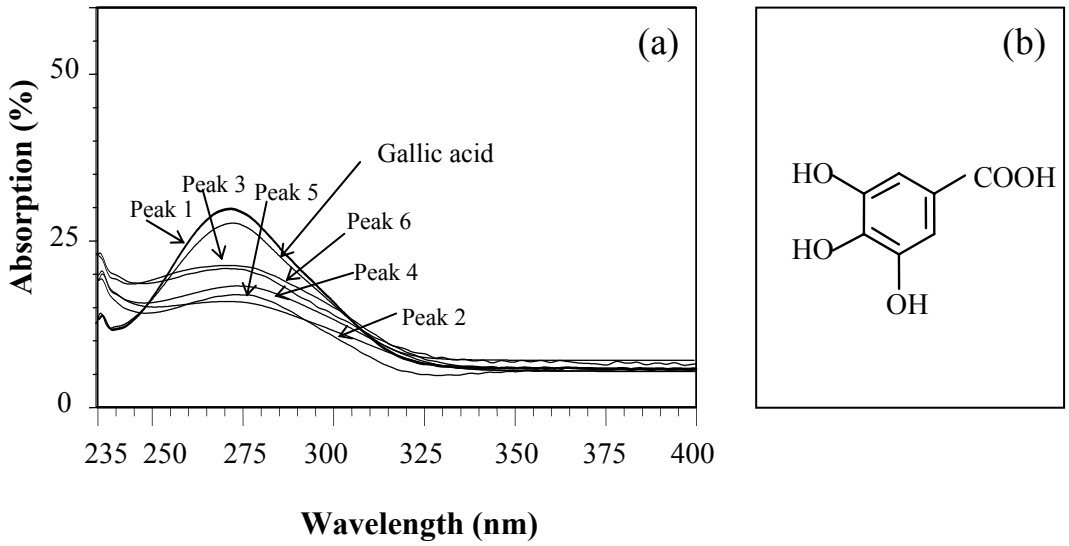


Figure 45 UV spectrum of (a) peaks 1, 2, 3, 4, 5 and 6 of *Careya sphaerica* Roxb. (Kradonbok) extract, gallic acid and (b) structure of gallic acid.

Compound 7 was identified by comparison of the retention time and UV spectrum (Figure 46) with that of pure ellagic acid. The identification of peak 7 as ellagic acid was confirmed by co-injection of ellagic acid with the plant extract. HPLC-ESI-MS confirmed the identification of this compound (MS $[M + H]^+$ at m/z 303, MS $[M + Na]^+$ at m/z 325), confirming the molecular weight of 302.19 for ellagic acid (Appendix C). Talapatra *et al*. (1981) reported that ellagic acid was isolated from the leaves of *Careya arborea,* which is the synonym of *Careya sphaerica* Roxb.

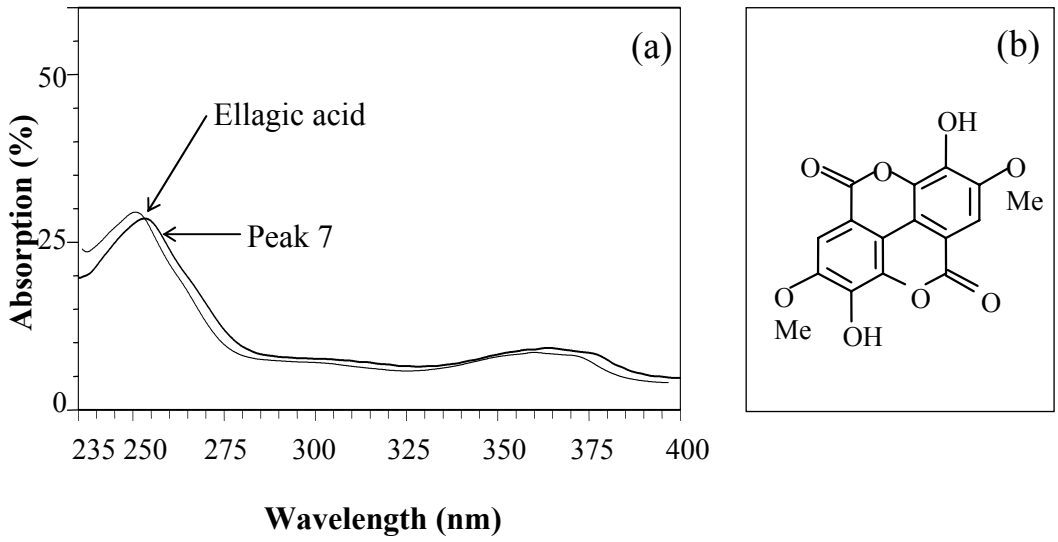


Figure 46 UV spectrum of (a) peak 7 of *Careya sphaerica* Roxb. (Kradonbok) extract, ellagic acid and (b) structure of ellagic acid.

Compounds 8-13 had similar UV spectra, characteristic of flavone glycoside structures at λ max at 320-330 and 260-270 nm (Wang *et al*., 2004) or flavanone type showed absorption maxima at 275 and 316 nm (Iwashina *et al*., 2005) (Figure 47). Only positive-ion mass spectra of compounds 8-13 were determined (Table 16). The mass spectrum of compound 8 is consistent with a molecular weight of 566 since it has $[M + H]^+$ ion at m/z 567, $[M + Na]^+$ ion at m/z 589, $[M + K]^+$ ion at m/z 605 (Appendix C). The mass spectrum of compound 9 is consistent with a molecular weight of 392 since it has $[M + H]^+$ ion at m/z 393, $[M + Na]^+$ ion at m/z 415, $[M + 2Na]^+$ ion at m/z 438 (Appendix C). The mass spectrum of compound 10 is consistent with a molecular weight of 394 since it has $[M + H]^+$ ion at m/z 395, $[M + Na]^+$ ion at m/z 417, $[M + 2Na]^+$ ion at m/z 440 and $[M + 3Na]^+$ ion at m/z 463(Appendix C). The mass spectrum of compound 11 is consistent with a molecular weight of 339 since it has $[M + H]^+$ ion at m/z 340, $[M + Na]^+$ ion at m/z 362, $[M + K]^+$ ion at m/z 378 (Appendix C). The mass spectrum of compound 12 is consistent with a molecular weight of 396 since it has $[M + H]^+$ ion at m/z 397, $[M + 13H]^+$ ion at m/z 409, $[M + Na]^+$ ion at m/z 419 and $[M + K]^+$ ion at m/z 435. The mass spectrum of compound 13 is consistent with a molecular weight of 448 since it has $[M + H]^+$ ion at m/z 449, $[M + Na]^+$ ion at m/z 471,

$[M + K]^+$ ion at m/z 487 (Appendix C). The molecular weight of compound 13 is that expected for luteolin glucoside or an isomer of this compound (Rauter *et al*., 2005), quercitrin (Liu *et al.,* 2000), quercetin-3-glycoside (Atoui *et al*., 2005), kaempferol-3-glycoside (Atoui *et al*., 2005), naringenin glycoside (Iwashina *et al*., 2005) and flavanone glycoside (Mansouri *et al*., 2005). Considering the UV spectra of each compound, this compound should be naringenin glycoside or flavanone glycoside because it has a maximum UV absorbance at 270-277 nm and 314-320 nm which was similar to the maximum UV absorbance from our experiments (Table 16). A match for the mass spectra of compounds 8-12 could not be found from the literature. The molecular weight of compounds 4,6 and 11 was an odd number, which would mean the compounds do not just contain carbon, hydrogen and oxygen but also nitrogen. They should be flavanoid alkaloids. Flavanoid alkaloids which are compounds containing C, H, O and N belong to a very small class of natural products, which have been reported only rarely in the literature. Some flavonoid alkaloids such as lilaline ($C_{20}H_{17}NO_7$, M_R 383, λ_{max} at 275, 329) showed absorption characteristics similar to flavonoids (Hougton, 2002).

The known antioxidant components 1 (gallic acid) and 7 (ellagic acid) were present at 0.93 % and 2.37 % of the extract. The other components (peaks 2, 3, 4, 5, 6, 8, 9, 10, 11, 12 and 13) were present at 6.09%, 11.64 %, 7.10%, 9.58%, 3.86%, 0.67%, 1.14%, 2.97%, 4.16%, 3.69% and 1.99% and other components that were present at lower concentration were also observed. These concentrartions were calculated according to the peak areas from the HPLC chromatogram. The thirteen phenolic compounds were about 56.16% of the total phenolic compound of Kradonbok extract.

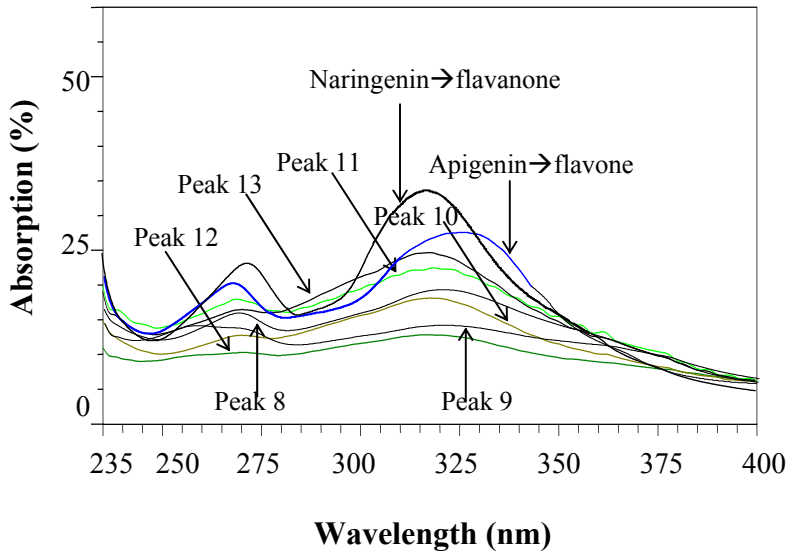


Figure 47 UV spectrum of peaks 8, 9, 10, 11, 12 and 13 of *Careya sphaerica* Roxb. (Kradonbok) extract, apigenin and naringenin (for comparing the UV spectrum of samples and reference chemicals).

Table 16 Retention times, UV spectra and ESI mass spectra of phenolic radical scavenging components from *Careya sphaerica* Roxb. (Kradonbok) extract

Compound	Time (min)	UV-Peak maxima (nm)	UV-Peak maxima (nm) from Literature	ESI Mass spectra	Molecular weight	Identity	ESI-mass spectra from literature
1	11.06	232, 273	232, 273 (Yao *et al*., 2004) 212,265 (Braca *et al*., 2003) 272 (Chen *et al*., 2001) 232,272 (Määta *et al*., 2003)	$[M+H]^+$at m/z 171 $[M+Na]^+$ at m/z 193 $[M+ 4Na]^+$ at m/z 262	170.12	gallic acid	$[M-H]^-$ at m/z not detect (Schieber *et al*., 2000)
2	33.91	230, 275		$[M+3H]^+$at m/z 229 $[M+2Na]^+$ at m/z 272 $[M+ 3Na]^+$ at m/z 295 $[M+ 4K]^+$ at m/z 382 $[M+10Na]^+$ at m/z 456	226	gallic acid derivative	$[M+H]^+$ at m/z 227 $[M-H]^-$ at m/z 226 (Chen *et al*., 2003)

Table 16 (Continued)

Compound	Time (min)	UV-Peak maxima (nm)	UV-Peak maxima (nm) from Literature	ESI Mass spectra	Molecular weight	Identity	ESI-mass spectra from literature
3	41.79	230, 278		$[M-H]^-$at m/z 307 $[M+OAc]^-$ at m/z 367	308	gallic acid derivative	
4	48.35	230, 278		$[M+H]^+$at m/z 374 $[M+Na]^+$ at m/z 396 $[M+ 2Na]^+$ at m/z 419 $[M+ 3Na]^+$ at m/z 442	373	gallic acid derivative	
5	51.91	230, 278		$[M+H]^+$at m/z 205 $[M+K]^+$at m/z 243 $[M+4K]^+$at m/z 360 $[M+6K]^+$at m/z 438	204	gallic acid derivative	

Table 16 (Continued)

Compound	Time (min)	UV-Peak maxima (nm)	UV-Peak maxima (nm) from Literature	ESI Mass spectra	Molecular weight	Identity	ESI-mass spectra from literature
6	62.36			$[M+H]^+$ at m/z 332 $[M+2Na]^+$ at m/z 377 $[M+9Na]^+$ at m/z 538	331	gallic acid derivative	
7	100.19	250, 367	254, 368 (Määta-Riihinen *et al*., 2004)	$[M+H]^+$at m/z 303 $[M+Na]^+$ at m/z 325	302	ellagic acid	$[M-H]^-$at m/z 301 (Määta, 2003)
8	134.71	270, 325		$[M+H]^+$at m/z 567 $[M+Na]^+$at m/z 589 $[M+K]^+$at m/z 605	566	Flavone or flavanone derivative	

Table 16 (Continued)

Compound	Time (min)	UV-Peak maxima (nm)	UV-Peak maxima (nm) from Literature	ESI Mass spectra	Molecular weight	Identity	ESI-mass spectra from literature
9	139.27	272, 325		$[M+H]^+$ at m/z 393 $[M+Na]^-$ at m/z 415 $[M+2Na]^+$ at m/z 438	392	Flavone or flavanone derivative	
10	160.93	260, 319		$[M+H]^+$at m/z 395 $[M+Na]^+$ at m/z 417 $[M+2Na]^+$ at m/z 440 $[M+3Na]^+$ at m/z 463	394	Flavone or flavanone derivative	
11	164.63	269, 317		$[M+H]^+$at m/z 340 $[M+Na]^+$at m/z 362 $[M+K]^+$at m/z 378	339	Flavone or flavanone derivative	

Table 16 (Continued)

Compound	Time (min)	UV-Peak maxima (nm)	UV-Peak maxima (nm) from Literature	ESI Mass spectra	Molecular weight	Identity	ESI-mass spectra from literature
12	170.44	270, 317		$[M+H]^+$ at m/z 397 $[M+BH]^+$ at m/z 409 $[M+Na]^+$ at m/z 419 $[M+K]^+$ at m/z 435	396	Flavone or flavanone derivative	
13	174.90	270, 317	270, 320 (Mansouri *et al*., 2005) 277,314 (Iwashina *et al*., 2005)	$[M+H]^+$at m/z 449 $[M+Na]^+$ at m/z 471 $[M+2Na]^+$ at m/z 487	448	Flavanone glycoside Naringenin glycoside	$[M+H]^+$ at m/z 449 (Mansouri *et al*., 2005) $[M+H]^+$ at m/z 449 (Iwashina *et al*., 2005)

Addition of DPPH solution to the Kradonbok extract was used to detect which components reacted readily with the DPPH radical. The reduction in area of peaks 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12 and 13 when reacted with DPPH was 98.8%, 93.1%, 78.8%, 67.0%, 78.3%, 83.6%, 74.8%, 40.6%, 42.1%, 41.9%, 40.7%, 42.3% and 41.4 %, respectively with detection at 280 nm, because detection at 260, and 320 nm could not show all the peaks (Figure 48). The similarity in the wavelength of maximum absorbance of peaks 8-13 and the similar change in peak area due to 3 mins reaction with DPPH indicate that the flavone glycosides eluting as peaks 8-13 share common structural features. When these compounds reacted with DPPH for more than 3 mins, all of the components decreased in concentration by approximately 100% (data not shown). However, peaks 8-13 were still present after reaction with DPPH for 3 mins but they continued to be oxidised as the Kradonbok extract was allowed to react with DPPH for 4 mins (data not shown). Compounds 3 and 5 were the main components of Kradonbok extract in concentration and they showed similar potent DPPH scavenging activities due to the reduction in area of more than 65% which was similar to other compounds in the groups corresponding to peaks 1-7 respectively.

Gallic acid and its dimeric derivative, known as ellagic acid, are present in a rich variety of plants. Gallic acid is considered to be a generally regarded as safe (GRAS) food additive and ellagic acid has been allowed for use as a food additive, functioning as an antioxidant in some countries, including Japan. Gallic acid is known to have anti-inflamatory, antimutagenic, anticancer and antioxidant activity. Ellagic acid has been found to exhibit antimutagenic, antiviral, anticancer, antitumour and antioxidant properties, along with causing whitening of the skin (Soong and Barlow, 2004). However, the antioxidant activity of phenolic acids and their derivatives depends on the number and position of hydroxyl groups bound to the aromatic ring, the binding site and mutual position of hydroxyl groups in the aromatic ring, and the type of substituents. The highest antioxidant potency was found for ortho-diphenols (Andjelković *et al*., 2005).

Flavones such as apigenin and luteolin represent one of the largest groups within the flavonoids, and show antioxidant properties. Apigenin and luteolin were reported as effective antioxidants acting by a one-electron-transfer mechanism (Leopoldini *et al*., 2004). However, flavones are less effective antioxidants than flavonols (such as myricetin, quercetin and kaempferol) since they lack a hydroxyl group in position three (3-OH) of the C-ring (Cook and Samman, 1996). Flavanones such as naringenin and flavanone glycosides such as naringin and prunin showed antioxidant activity (Zhao *et al*., 2005). Chalcones have greater antioxidant activity than flavanones (Zhao *et al*., 2005).

The oxidation of phenols can produce semiquinone radicals or quinones (Barbehenn *et al*., 2003). Moreover, the oxidation of gallic acid and its derivatives is irreversible and a two electron oxidation scheme leads to the production of quinonoid structures (Strlic *et al*., 2002). Flavonoid oxidation by either hydrogen atom abstraction or electron donation results in the formation of carbon-or oxygen-centered radical species. The subsequent secondary oxidation of the initially formed radical species either by hydrogen atom abstraction or by disproportionation is known to produce quinones or quinone methide intermediates (Awad *et al*., 2001). A new HPLC peak (peak X) appeared with a retention time of 3.36 min after reaction of the Kradonbok extract with DPPH for 4 min (Figure 48). This oxidation product absorbed more strongly at 260 nm than at 280, or 320 nm (Figure 48). The UV spectrum of the oxidation product showed an absorbance maximum at 236.6 nm (Figure 49). The UV spectrum of the oxidation product did not match with other oxidized phenolic compounds reported in the literature. A previous study has shown that oxidation of apigenin, naringenin, luteolin and quercetin with glutathione oxidase oxidation produces a product with an absorption at 260 nm which was identified as an oxidized glutathione conjugate (SG) such as luteolin SG conjugate (Galati *et al*., 2001). Moreover, the oxidation product of Kradonbok extract has a different UV spectrum from the UV spectrum of the oxidation product of the Teaw extract (Figure 49) due to the different phenolic components present. The peak at 3.36 min disappeared when the reaction between DPPH and the

Kradonbok extract was allowed to continue for more than four minutes (data not shown).

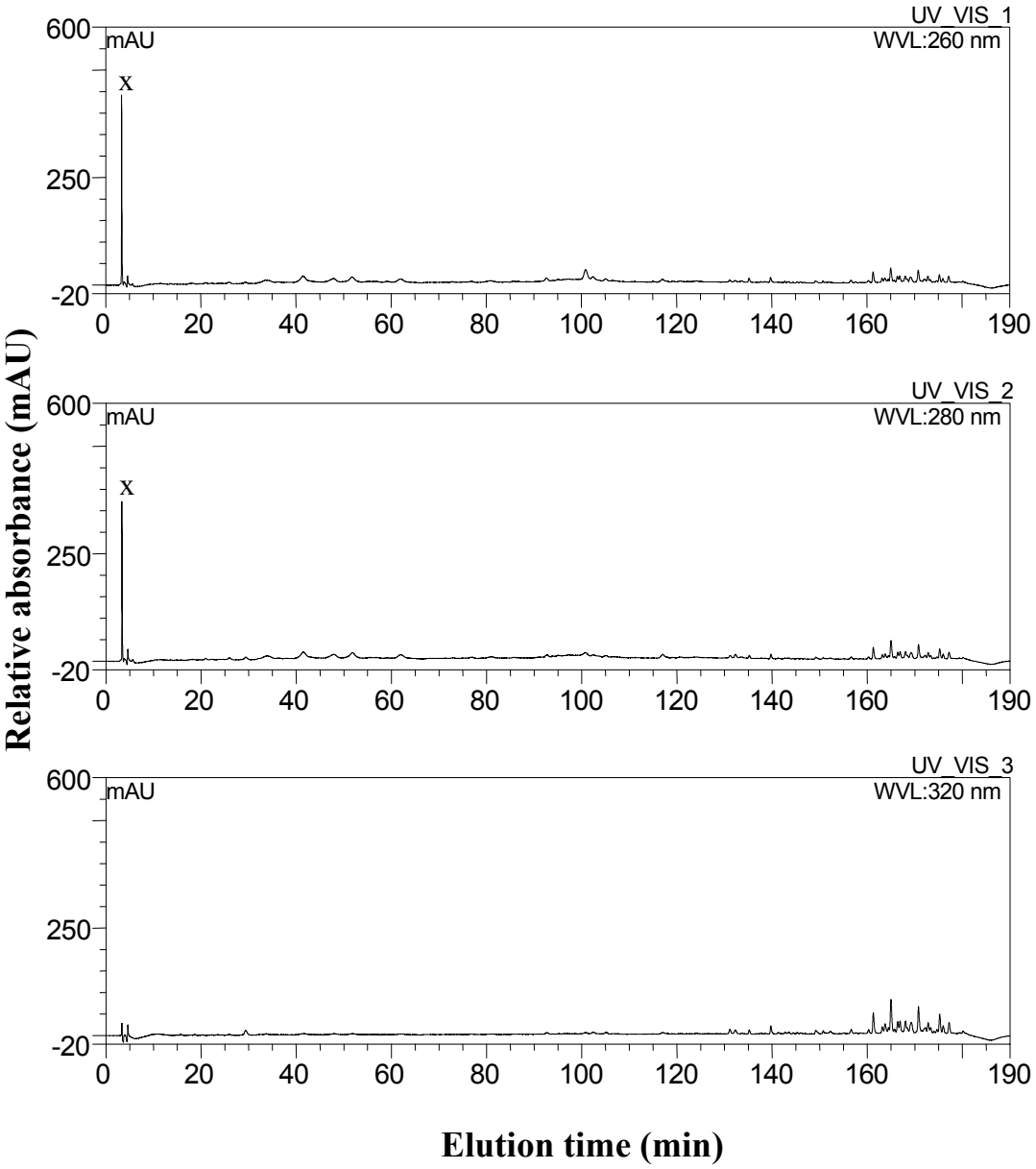


Figure 48 HPLC chromatogram for the extract from *Careya sphaerica* Roxb. (Kradonbok) after reaction with DPPH for 3 minutes.

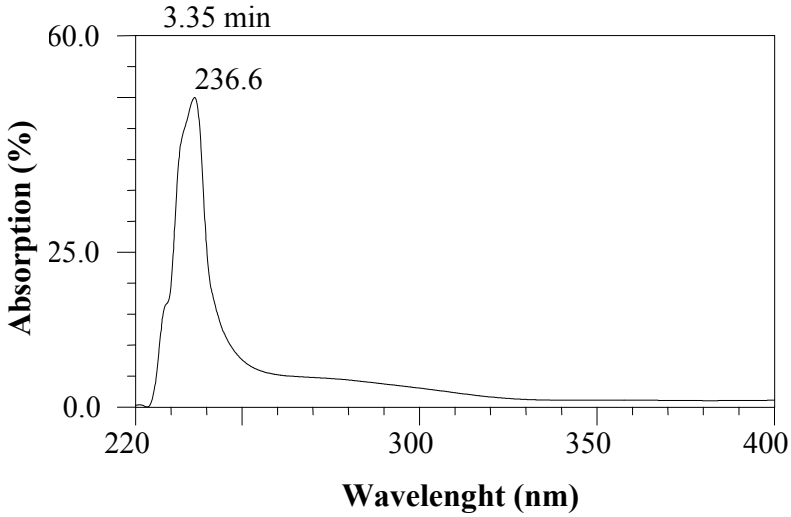


Figure 49 UV Spectrum of oxidation product of *Careya sphaerica* Roxb. (Kradonbok) extract after reaction with DPPH.

3.2.3 *Leucaena glauca* Benth (Kratin) Extract

Comparisons of their UV spectra and LC-MS in both positive and negative mode were used in order to obtain more information on the structural features of the conjugated forms of phenolic compounds. Normally, the masses of the sugars bound to the aglycons and the specific fragmentation patterns of the compounds were used for identification of the chromatographic peaks. The bound sugar moieties consisted of hexoses with a mass unit of 162 (glucose or galactose), or 146 (rhamnose), or deoxyhexoses with a mass unit of 132 (xylose or arabinose). When the conjugated phenolic forms present shifted in the UV-visible absorption spectra compared to their respective aglycons (shifts to longer wavelength), this indicated esterification of the aglycon with sugar, and when they shifted to shorter wavelengths, this indicated glycosidation. Whenever possible, chromatographic retention was used to support the tentative identification of some peaks. However, this was sometimes complicated by the fact that retention is governed not only by the polarity of the molecules but also by their size (Määtta *et al*., 2003).

The HPLC chromatogram of the Kratin extract is shown in Figure 50. Four main peaks were detected and the area of peaks 1-4 was greater at 260 nm

than at 280, or 320 nm. The components corresponding to peaks 1, 2, 3, and 4 eluted at 17.99, 19.18, 21.92 and 22.59 min in the more hydrophilic region (short retention time). In the more hydrophobic region (long retention time), we could not detect the other main components.

The compounds corresponding to peaks 1, 2, 3, and 4 had a similar UV spectrum to that of flavonoids such as quercetin, and kaempferol which are flavonols and luteolin which is a flavone (Figure 51). The identification of peak 1 was confirmed by using HPLC-ESI-MS. Negative-ion MS gave a high mass peak with m/z 463 $[M - H]^-$ (parent peak), fragment m/z 301 $[M - H - 162]^-$. Positive-ion MS showed a base peak of m/z 465, due to the hydrogen adduct ion $[M + H]^+$ (Appendix D), and m/z 510 $[M + 2Na]^+$, confirming the molecular weight of 464 (Table 17). This molecular weight is as expected for quercetin glycoside (Liu *et al*., 2000; Määtta *et al*., 2003; Braca *et al*., 2003; Atoui *et al*., 2005) and 6-hydroxyluteolin glycoside (Albach *et al*., 2005; Atoui *et al*., 2005). Quercetin glycoside showed the same as UV spectrum (maxima at 254-262 and 354-357), whereas 6-hydroxyluteolin glycoside showed maxima in the UV spectrum at 247-258 and 315-330 nm. So this compound, when compared with chromatographic behaviour and UV spectra from the literature, corresponded to quercetin glycoside which was found in St. John's Wort dietary supplement (Liu *et al*., 2000), extracts from *Sclerocarya birrea* (Anacadiaceae) leaves (Bracaz *et al.,* 2003), extracts of currants in *Ribes* species (Määtta *et al*., 2003) and chinese green tea (Atoui *et al.,* 2005).

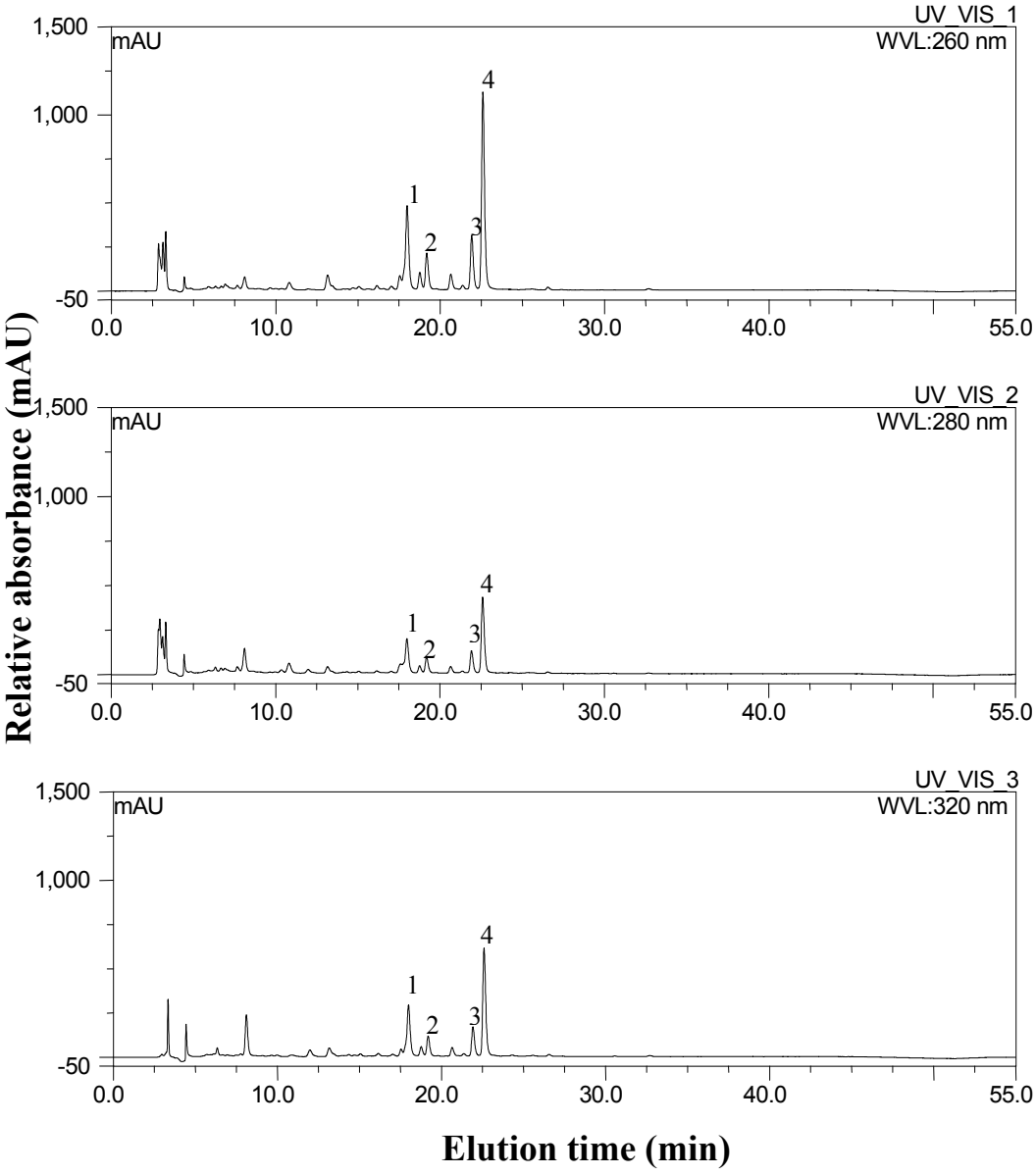


Figure 50 HPLC chromatogram for the extract from *Leucaena glauca* Benth (Kratin) detected at 260, 280 and 320 nm.

Table 17 Retention times, UV spectra and ESI mass spectra of phenolic radical scavenging components from *Leucaena glauca* Benth (Kratin) extract

Compound	Time (min)	UV-Peak maxima (nm)	UV-Peak maxima (nm) from Literature	ESI Mass spectra	Molecular weight	Identity	ESI-mass spectra from literature
1	17.99	263,355	254, 354(Atoui *et al*., 2005)	$[M-H]^-$at m/z 463 $[M+H]^+$ at m/z 465 $[M+ 2Na]^+$ at m/z 510	464	Quercetin glycoside	$[M+H]^+$ at m/z 465 (Atoui *et al*., 2005)
			262, 354(Braca *et al*., 2005)	fragment m/z 301 $[M-H-162]^-$			$[M-H]^-$ at m/z 463 (Braca *et al*., 2005)
			257, 357(Liu *et al*., 2000)				$[M+H]^+$ at m/z 465 $[M+Na]^+$ at m/z 487 (Liu *et al*., 2000)
			254, 354(Määta *et al*., 2003)				$[M+H]^+$ at m/z 465 $[M-H]^-$ at m/z 463 (Määta *et al*., 2003)

Table 17 (Continued)

Compound	Time (min)	UV-Peak maxima (nm)	UV-Peak maxima (nm) from Literature	ESI Mass spectra	Molecular weight	Identity	ESI-mass spectra from literature
2	19.18	256,350	257, 357 (Atoui *et al*., 2005) 255, 349 (Braca *et al*., 2005)	$[M+H]^+$at m/z 449 $[M+Na]^+$ at m/z 471 $[M-H]^-$ at m/z 447 fragment m/z 303 $[M-H-146]^+$ fragment m/z 301 $[M-H-146]^-$	448	Quercitrin (guercetin-3-*O*-rhamnopyranoside)	$[M+H]^+$ 449 fragment m/z 303 (Liu *et al*., 2000) $[M-H]^-$ 447 fragment m/z 301 (Braca *et al*., 2005)
3.	21.92	264, 345	263, 343	$[M-H]^-$ at m/z 431 fragment at m/z 285 $[M-H-146]^-$	432	Kaempferol 3-*O*-rhamnopyranoside	$[M-H]^-$ at m/z 431 fragment at m/z 285 $[M-H-146]^-$

Table 17 (Continued)

Compound	Time (min)	UV-Peak maxima (nm)	UV-Peak maxima (nm) from Literature	ESI Mass spectra	Molecular weight	Identity	ESI-mass spectra from literature
4	22.59	260,360		$[M+H]^+$at m/z 454 $[M+10H]^+$ at m/z 463 $[M+Na]^+$ at m/z 476 $[M+K]^+$ at m/z 492	453	Unknown compound	

The mass spectrum of compound 2 was consistent with a molecular weight of 448 since it had a positive-ion mass spectrum with a peak at m/z 449 $[M + H]^+$, m/z 471 $[M + Na]^+$ and a negative-ion mass spectrum with a peak at m/z 447 $[M - H]^-$, fragment m/z 301 $[M – H - 146]^-$ (Appendix D). This data was as expected for kaempferol hexoside (Määta *et al*., 2003), quercitrin or quercetin-3-*O*-rhamnopyranoside (Liu *et al*., 2000; Braca *et al*., 2003), luteolin glycoside (Rauter *et al*., 2005; Wang *et al*., 2005; Atoui *et al*., 2005), naringenin glycoside (Iwashina *et al*., 2005) and flavanone glycoside (Mansouri *et al*., 2005). When considering the UV maxima and MS spectra of each compound, differences as evident; kaempferol hexoside (UV maxima at 246, 290, 348 nm; MS peaks $[M+H]^+$ at m/z 449 and $[M-H]^-$ at m/z 447), quercitrin (UV maxima at 255-257 and 349-357 nm; MS peaks $[M+H]^+$ at m/z 449, $[M-H]^-$ at m/z 447, fragment ion at m/z 303 in positive mode and m/z 301 in negative mode), luteolin glycoside (UV maxima at 254-277 and 346-347 nm, MS peaks $[M+H]^+$ at m/z 449, $[M+Na]^+$ at m/z 471 and fragmentation at m/z 287 in negative mode), naringenin glycoside (UV maxima at 277, 314 nm and MS peak $[M+H]^+$ at m/z 449) and flavanone glycoside (UV maxima at 270, 320 nm and MS peak $[M+H]^+$ at m/z 449). The fragments at m/z 301 and 303 showed that this compound was rhamnose. In addition, Braca *et al*., (2003) reported quercitrin, which is present in the berries of *Sclerocarya birrea* (Anacardiaceae) as compounds 2 (30.8 min) which eluted after compound 1 (28.9 min) using methanol and water as the mobile phase. From our results, compound 2 (19.01 min) eluted after compound 1, (17.79 min) when eluted with 1 % formic acid and 100 % acetonitrile as the mobile phase. The maxima absorbance from our results at 256 and 350 nm of this compound were similar to those in the UV spectrum of quercetin (Figure 51a), and this is consistent with that of quercitrin. Moreover, Morita *et al.,* (1997) also found quercitrin in *Leucaena glauca* Benth leaves.

Only the negative-ion mass spectrum of compound 3 was determined. The mass spectrum of compound 3 is consistent with a molecular weight of 432 since it has [M - H]- ion at m/z 431 and fragment m/z 285 $[M– H –146]^-$ (rhamnose) (Appendix D). This compound could be identical with kaempferol 3-*O*-

rhamnopyranoside which has UV peaks (MeOH) at λ_{max} 263, 343 nm and was reported in the literature as a component of *Sclerocarya birrea* (Anacardiaceae) leaves (Braca *et al*., 2003). Moreover, compound 3 (retention time 33.3 min) eluted after compound 2 (retention time 30.8 min) when methanol and water were used as the mobile phase. The wavelengths for maximum absorbance (λ max) in the spectrum of peak 3 at 345 and 264 nm and the retention time of peak 3 equal to 21.74 min which eluted after peak 2 (19.01 min) when 1% formic acid and 100% acetonitrile were used in the mobile phase are consistent with this identification.

Compound 4 had a positive-ion mass spectrum with a peak at m/z 454 $[M + H]^+$, m/z 463 $[M + 10H]^+$, m/z 476 $[M + Na]^+$, m/z 492 $[M + K]^+$ indicating a molecular weight of 453 (Appendix D). A match for the mass spectrum of compound 4 could not be found from the literature. However, the UV spectrum of compound 4 was similar to that of luteolin which is one of the flavones (Figure 51c). Since this compound had an odd-numbered molecular weight it would be a nitrogen-containing compound such as flavonoidal alkaloid (Chen *et al*., 2001; Kanchanapoom *et al*., 2002; Houghton, 2002).

The antioxidant components 1, 2, 3 and 4 were present at 15.28%, 6.50%, 9.71% and 36.91%, respectively, and other components that were present at lower concentrations were also observed. These concentrations were calculated from each area by dividing by the total peak area from the HPLC chromatogram. The four phenolic compounds were about 68.4% of the total phenolic content of Kratin extract.

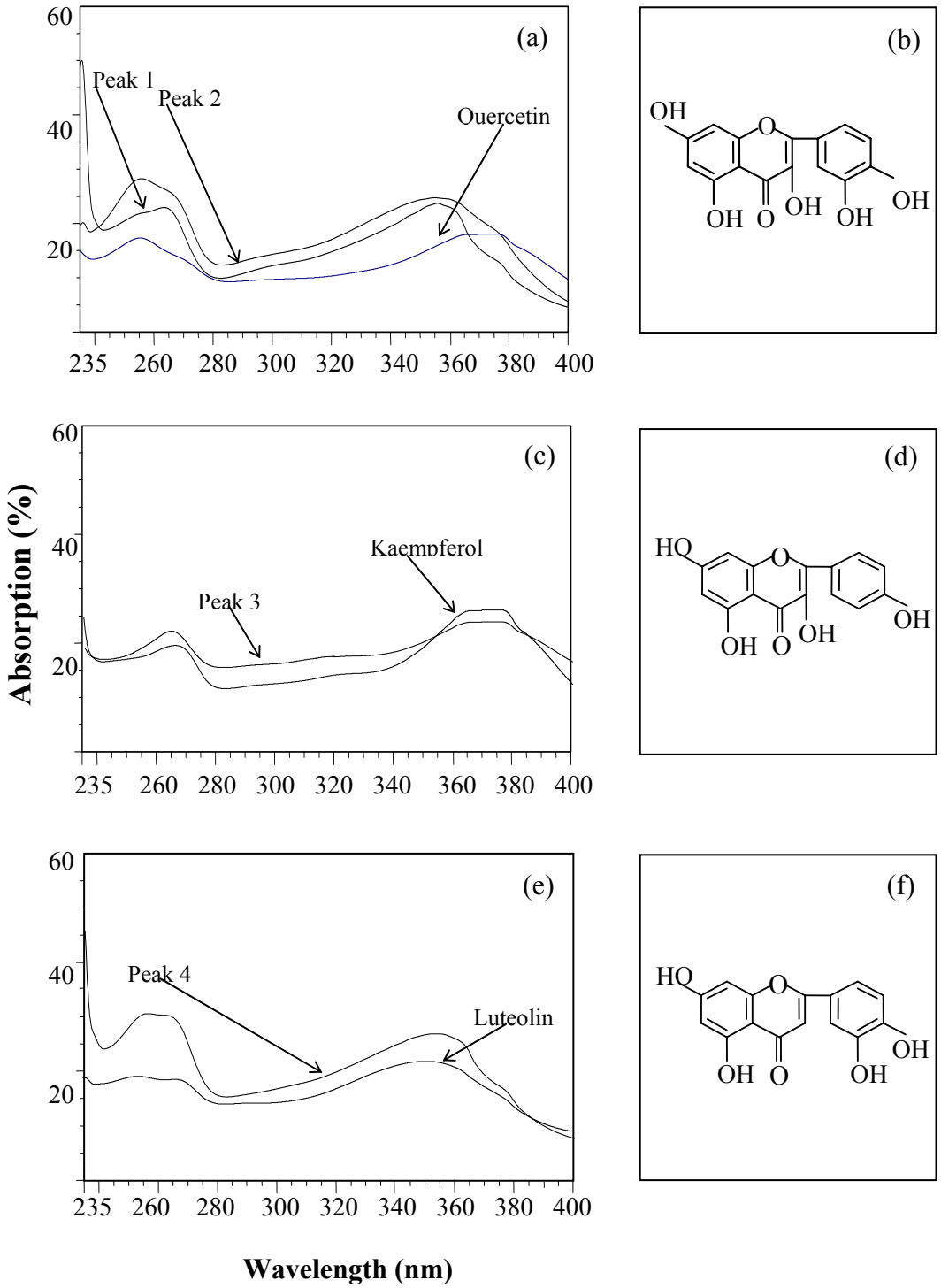


Figure 51 UV spectrum of (a) peaks 1, 2 of *Leucaena glauca* Benth (Kratin) extract, quercetin, (b) structure of quercetin, (c) UV spectrum of peaks 3 of Kratin extract, kaempferol, (d) structure of kaempferol, (e) UV spectrum of peaks 4 of Kratin extract, luteolin and (f) structure of luteolin.

Addition of DPPH solution to the Kratin extract was used to detect which components reacted readily with the DPPH radical. The reduction in area of peaks 1, 2, 3, and 4 when reacted with DPPH was 43.3 %, 41.3 %, 39.8 % and 32.3 %, respectively with detection at 260 nm, and detection at 280 and 320 nm showed similar changes (Figure 52). The similarity in the wavelength of maximum absorbance of peaks 1 and 2 and the similar change in peak area due to 5 mins reaction with DPPH indicates that flavonol glycosides eluting as peaks 1, 2 and 3 share common structural features. Moreover, compounds 1, 2 and 3 were similar in antioxidant activity measured by the TEAC assay (Braca *et al*., 2003).

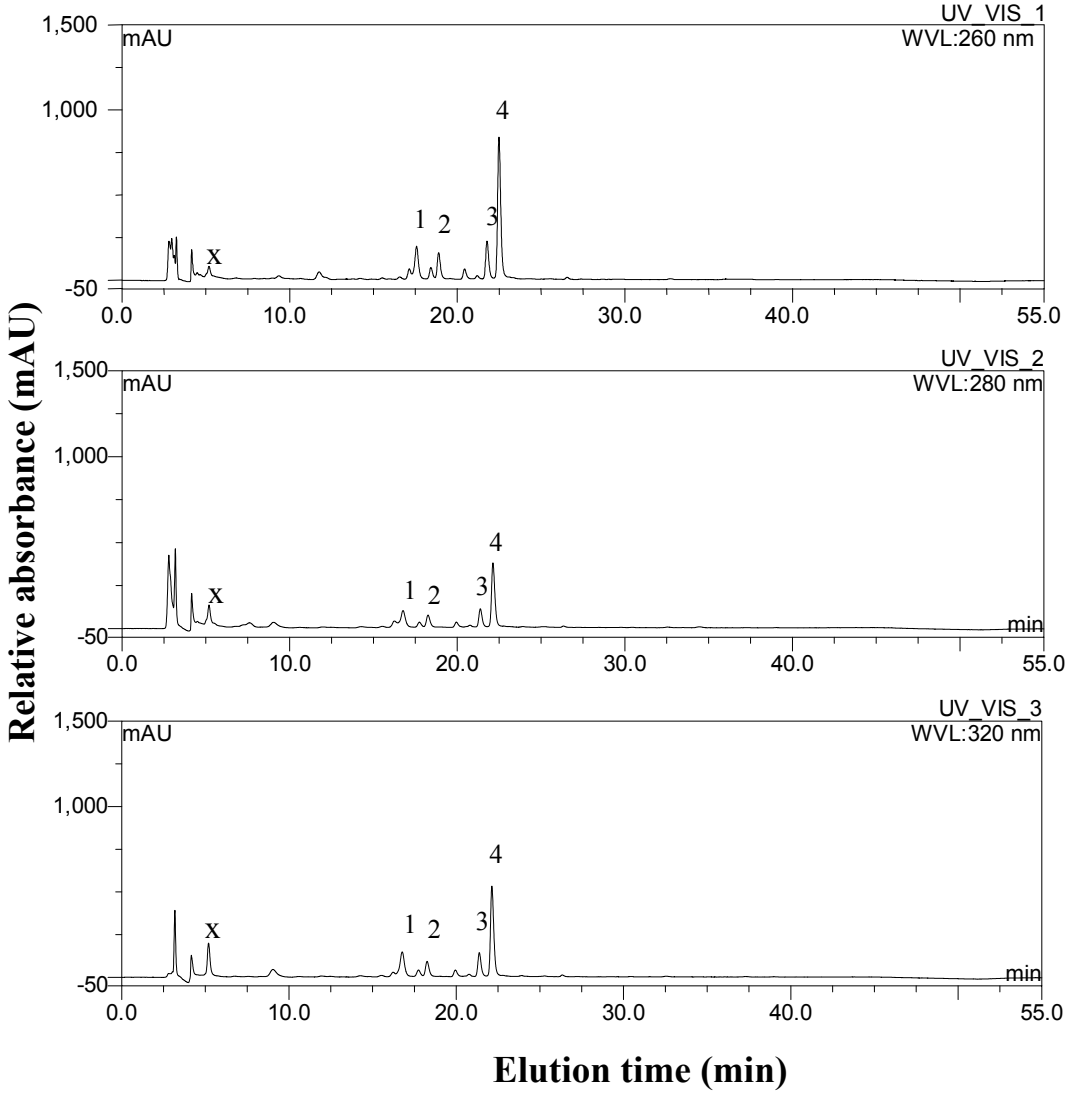


Figure 52 HPLC chromatogram for the extract from *Leucaena glauca* Benth (Kratin) after reaction with DPPH 5 minutes.

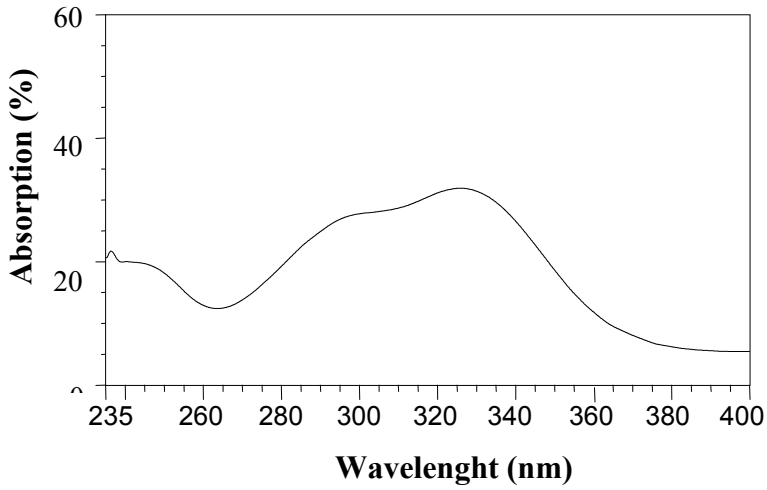


Figure 53 UV spectrum of oxidation product of *Leucaena glauca* Benth (Kratin) extract after reaction with DPPH

Flavonoids are diphenylpropanes that commonly occurs in plants and are frequently components of the human diet. It has been found that some flavonoids and other polyphenols possess antitumour, anti-allergic, anti-platelet, anti-ischemic, and anti-inflamatory activities. The antioxidant activity of flavonoids depends on their chemical structure. Normally, there are three structural groups that affect the free radical scavenging and/or antioxidative potential of flavonoids: (a) the catechol moiety of the B-ring, (b) the 2, 3 - double bond in conjugation with a 4 - oxofunction of a carbonyl group in the C-ring and (c) presence of hydroxyl groups at the 3 and 5 positions. Therefore, flavonoid aglycones showed higher antioxidant activity than flavonoid glycosides because of the blockage of the C-3 hydroxyl group in the glycosides (Shi, 2001).

The oxidation of phenols can produce semiquinone radicals or quinones (Barbehenn *et al*., 2003). Moreover, flavonoid oxidation by either hydrogen atom abstraction or electron donation results in the formation of carbon- or oxygen-centered radical species. The subsequent secondary oxidation of the initially formed radicals either by hydrogen atom abstraction or by disproportionation is known to produce quinones or quinone methide intermediates (Awad *et al*., 2001). A new

HPLC peak (peak X) appeared with a retention time of 6.57 min after reaction of the Kratin extract with DPPH for 5 min (Figure 52). This oxidation product absorbed more strongly at 320 nm than at 260, or 280 nm (Figure 52). The UV spectrum of the oxidation product showed an absorbance maximum at 335 nm (Figure 53). A previous study has shown that oxidation of flavonols such as quercetin and kaempferol with hydrogen peroxide and peroxidase in the presence of glutathione (GSH) produced a product with an absorption maximum at 335 nm which was identified as a flavonol-GSH conjugate such as quercetin-GSH conjugate (Galati *et al*., 2001). Moreover, the oxidation product of the Kratin extract has a different UV spectrum from that of the oxidation product of Teaw and Kradonbok extracts indicating a different structure. The peak at 6.57 min disappeared when the reaction between DPPH and the Kratin extract was allowed to continue for more than five minutes (data not shown).

3.2 Assessment of Free Radical Scavenging Activity

Free radical scavenging is the main mechanism by which antioxidants inhibit lipid oxidation. Two common methods, DPPH and ABTS radical scavenging, were used to assess the antioxidant activity of the Teaw extract compared to that of the major component, chlorogenic acid, and α-tocopherol and BHT as reference standards.

The loss of absorbance of the $DPPH^{\bullet}$ at 517 nm in the presence of the selected antioxidants was studied in methanol and the calculated EC_{50} values are presented in Table 18. Chlorogenic acid, which is the main phenolic compound in the Teaw extract showed the greatest radical scavenging activity but the Teaw extract was more active than α-tocopherol and BHT. According to Brand-Williams *et al*. (1995), the antiradical power of caffeic acid is ~2.2 times that of BHA and BHT, and chlorogenic acid has similar activity to caffeic acid.

Table 18 Antioxidant activity of extracts and reference compounds determined by the DPPH method ($1/EC_{50}$) and the ABTS decolorization assay[a]

Extract or Compound	EC_{50} ($\mu g \cdot mL^{-1}$)	EC_{50} ($\mu g \cdot \mu g^{-1}$ DPPH)	Antiradical activity ($1/EC_{50}$)	TEAC (mmol of Trolox/g sample)
Teaw extract	8.96±0.07 [b]	0.23±0.00 [b]	4.42±0.04 [e]	2.67±0.03 [e]
Kradonbok extract	17.03±0.48 [e]	0.43±0.04 [d]	2.33±0.20 [b]	2.15±0.06 [c]
Kratin extract	27.04±0.51 [f]	0.68±0.01 [c]	1.46±0.03 [a]	0.85±0.08 [b]
Chlorogenic acid	6.26 ±0.06 [a]	0.16±0.00 [a]	6.31±0.07 [f]	3.06±0.04 [f]
α- tocopherol	14.95±0.23 [c]	0.38±0.01 [c]	2.68±0.04 [d]	2.30±0.03 [d]
BHT	15.41±0.21 [d]	0.39±0.01 [c]	2.57±0.04 [c]	0.73±0.01 [a]

Note : [a] Data followed by different letters within each column are significantly different according to Duncan's multiple range test at $P < 0.05$. Data obtained from at least three replicates for the DPPH method and nine replicates for the ABTS assay.

The Kratin extract was less effective than Teaw and Kradonbok extracts due to lower concentration of phenolic components in the Kratin extract (considering the total area of all peaks in the HPLC chromatogram). Moreover, the Kradonbok and Kratin extracts were less active than α-tocopherol.

The antioxidant activity of the extract and pure compounds was also determined by the ABTS radical scavenging method. The advantages of this radical are its water solubility and high absorption coefficient at long wavelengths, allowing the determination of its rate of consumption with minimal interferences (Campos and Lissi, 1997). In addition, scavenging of the ABTS radical is less susceptible to steric hindrance when bulky antioxidants are studied. In the present study, it was found that the relative ranking of the additives using the ABTS radical was the same as in the DPPH assay except for Kratin and BHT (Table 18). However, the differences between chlorogenic acid, the Teaw extract and α-tocopherol were less than in the DPPH assay. The relative order of activity of different samples

assessed by the two radical scavenging methods is more relevant than absolute values for comparing activities. Generally, the two methods (ABTS and DPPH) correlated strongly with each other (Leong and Shui, 2001; Awika *et al*., 2003). In this study, a correlation was found ($r = 0.71$, $P < 0.01$) between the values from the two methods (Figure 54). The fact that α-tocopherol and BHT are not water soluble contributes to their low value in the ABTS assay, and reduces the correlation coefficient.

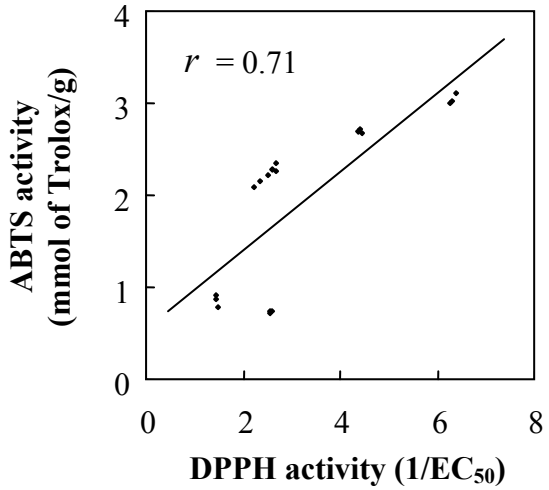


Figure 54 Correlation between radical scavenging activities determined by the ABTS and DPPH methods for *Cratoxylum formosum* Dyer. (Teaw) extract, *Careya sphaerica* Roxb. (Kradonbok) extract, *Leucaena glauca* Benth (Kratin) extract, chlorogenic acid, α-tocopherol and BHT.

The DPPH radical scavenging activity of the Teaw extract was about 70% of that of chlorogenic acid, whereas the TEAC value of the Teaw extract determined by the ABTS method was about 87% of that of chlorogenic acid. The HPLC analysis showed that the four main peaks in the Teaw chromatogram corresponded to phenolic compounds with concentrations of 0.60, 0.07, 0.03 and 0.02 CAE/g respectively when expressed as grams of chlorogenic acid equivalents per gram of plant extract (CAE/g). This indicated that the Teaw extract contained ~0.72 CAE/g from the main peaks. Since the radical scavenging activity of the

extract was found to be 70 – 87 % of that of chlorogenic acid, it appears that the compounds eluting as peaks 2-4 had similar activity to chlorogenic acid. Peak 2 has been identified as dicaffeoylquinic acid, and the chlorogenic acid isomers, 3, 5-dicaffeoylquinic acid and 4, 5-dicaffeoylquinic acid, were reported to have almost the same antioxidant activity as chlorogenic acid (Chun *et al*., 2003). The small difference in antioxidant activity of the Teaw extract obtained from the ABTS and DPPH assays when compared to the value for pure chlorogenic acid reflects differences in the structure and reactivity of the radical species.

In conclusion, the present study demonstrates for the first time to characterize of Cratoxylum formosum Dyer. (Teaw), Careya sphaerica Roxb. (Kradonbok), and Leucaena glauca Bentht (Kratin) extracts. We found that chlorogenic acid is the main antioxidant component in the ethanolic extract of Teaw leaves. Dicaffeoylquinic acid, and two ferulic acid derivatives were identified as minor components with similar radical scavenging activity to chlorogenic acid that were present in small concentrations in the extract. Moreover, gallic acid and ellagic acid were found as minor components in the Kradonbok extract. The gallic acid derivatives with molecular weight equal to 308 and 204 were the DPPH radical scavenging phenolic components present at highest concentration in the extract. The phenolic antioxidant constituents in the ethanolic extract of Kratin leaves were quercetin glycoside, quercetin – 3 – *O* - rhamnopyranoside, kaempferol – 3 – *O* - rhamnopyranoside and an unknown compound with molecular weight equal to 453 which is probably a flavonoidal alkaloid were the potent DPPH radical scavenging phenolic components in the extract. In addition, Teaw extract showed higher antioxidant activity than α-tocopherol, Kradonbok extract and Kratin extract measuring by DPPH and ABTS assays.

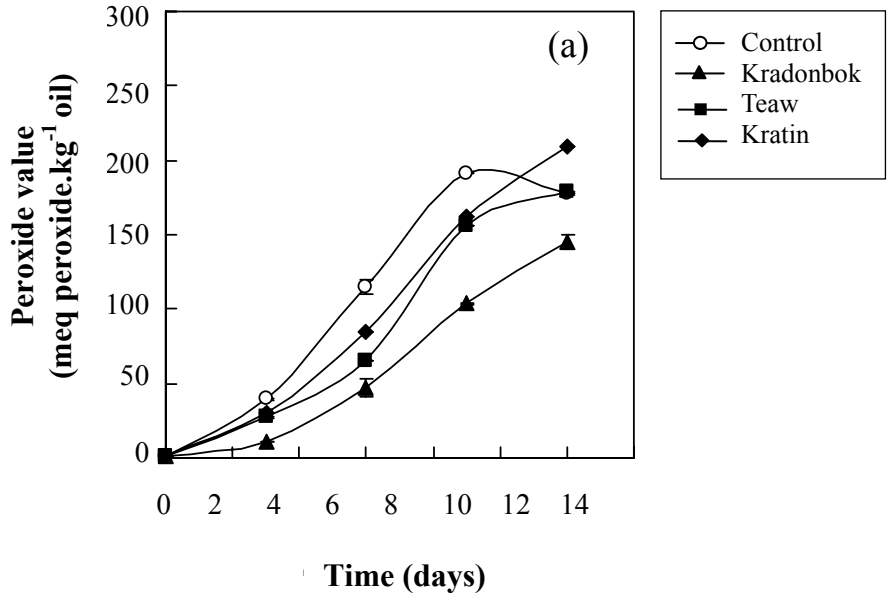
4. Comparison of the Antioxidant Activity with Commercial Antioxidants in Oil and Emulsion Models

4.1 Antioxidant Activity of Phenolic Antioxidant in Stripped Soybean Oil

Soybean oil was used to investigate the antioxidant activity of the Teaw extract and the standard antioxidants, since it contains a significant concentration of α-linolenic acid which makes it quite sensitive to oxidation (Naz *et al*., 2004). Tocopherols were removed from the soybean oil before use to get a consistent source of oil and to allow primary antioxidant effects of additives to be studied without interference from the tocopherols. Stored samples were analyzed periodically for PV and TBARS to allow both hydroperoxides and hydroperoxide degradation products to be monitored. Volatile hydroperoxide degradation products contribute oxidative off-flavours to foods, and consequently it is important to monitor both precursors of these off-flavours as well as the degradation products themselves. Secondary oxidation products include aldehydes, ketones, hydrocarbons and alcohols, among others. The detection of malonaldehyde (MA), a volatile dialdehyde, has been widely used as a measure of the oxidation of polyunsaturated fatty acids in foods and animal tissues. It is a secondary product of autoxidation of fatty acids having three or more double bonds, and the TBAR values that are a measure of malonaldehyde have been shown to correlate well with flavor threshold values for some vegetable oils (Gray, 1978).

Comparison of the peroxide values of stripped soybean oil containig Teaw, Kradonbok and Kratin was studied (Figure 55a). This is the first study on the effect of the plant extracts on both hydroperoxide formation and hydroperoxide decomposition during oxidation in the dark at 60°C. For each treatment, peroxide values were fitted to an exponential curve as a function of storage time. On the basis of the time to a PV of 50 meq·kg^{-1} (Table 19), antioxidant efficiency for 100 mg·kg^{-1} addition decreased in the following order: Teaw extract > Kradonbok extract > Kratin extract > control. The TBARS determinations confirmed the order of antioxidant activity (Figure 55b). The ratio of PV: TBARS for different plant

extracts is shown in Figure 56. The ratio decreased during storage but it was higher for the sample containing Teaw extract than for the sample which contained Kradonbok and Kratin extracts. This result suggested that the Teaw extract was more effective for inhibiting lipid oxidation than Kradonbok and Kratin extracts because of inhibition of hydroperoxide decomposition, since the sample had a lower TBARS value. Comparing the data to DPPH and ABTS radical scavenging activity, the relative ranking of the activity of different extracts assesed by radical scavenging methods and the lipid oxidation in oil model showed the same order. However, the differences in the effectiveness of plant extracts in oil were less than in the free radical scavenging assays. Consequently, Teaw extract showed the highest antioxidant potential to use as food lipid antioxidant. A further experiment was performed in order to compare the antioxidant activity of the Teaw extract with that of the commercial antioxidants.



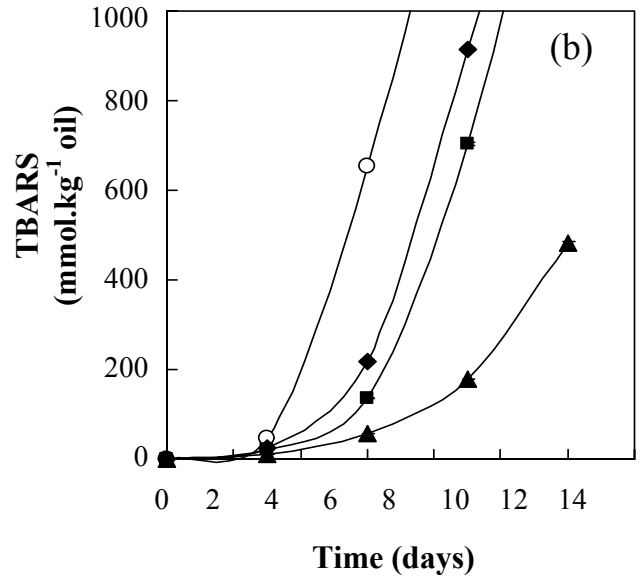


Figure 55 Effect of antioxidants (100 mg·kg^{-1}) on oxidative stability of stripped soybean oil at 60°C, assessed by determination of (a) PV and (b) TBARS. Data points represent mean ± standard deviation (n=3).

Table 19 Time for stripped soybean oil and oil-in-water emulsion samples containing antioxidants (100 mg·kg^{-1}) to reach a peroxide value of 50 meq·kg^{-1} at 60°C*

Compound	Bulk oil			10% oil-in-water emulsion		
	Time (days)	r^2	°PF	Time (days)	r^2	°PF
Control	3.27±0.01 [a]	0.99	1.00±0.00 [a]	1.56±0.11 [a]	1.00	1.00±0.00 [a]
Teaw extract	6.06±0.01 [d]	0.99	1.86±0.00 [d]	4.55±0.02 [d]	0.96	2.92±0.02 [d]
Kradonbok extract	4.11±0.01 [c]	0.96	1.26±0.00 [c]	3.66±0.01 [c]	0.97	2.35±0.00 [c]
Kratin extract	3.74±0.00 [b]	0.99	1.14±0.00 [b]	2.31±0.01 [b]	1.00	1.48±0.01 [b]

* Data followed by different letters within each column are significantly different according to Duncan's multiple range test at $P < 0.05$. Data are shown as means ± standard deviation from three replicate determinations.

° PF = days to reach PV 50 meq·kg^{-1} for each antioxidant / days to reach PV 50 meq·kg^{-1} for control (no antioxidant).

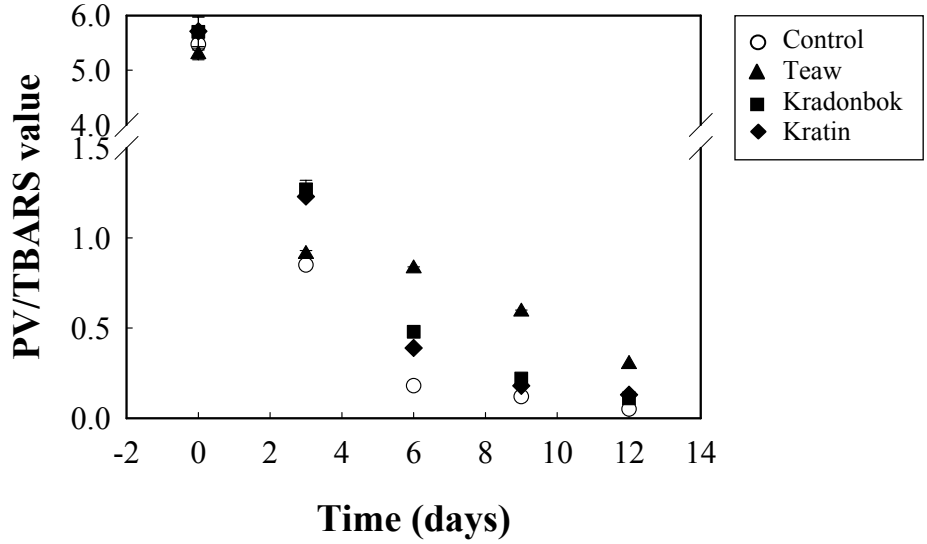


Figure 56 The ratio of peroxide and TBARS values (PV/TBARS value) of stripped soybean oil containing antioxidants (100 mg·kg^{-1}) during storage at 60°C. Data points represent mean ± standard deviation (n=3).

Changes during storage in the peroxide values of stripped soybean oil containing the Teaw extract were compared with oil containing individual antioxidants (Figure 57a). Since the Teaw extract has been shown to contain about 60% chlorogenic acid as the main antioxidant component, the concentration of chlorogenic acid used for comparison was 60 mg·kg^{-1}. On the basis of the time to a PV of 50 meq·kg^{-1} (Table 20), antioxidant efficiency for 100 mg·kg^{-1} addition decreased in the following order: citric acid > BHT > ascorbyl palmitate > chlorogenic acid (60 mg·kg^{-1}) > Teaw extract > α-tocopherol > control. The TBARS determinations (Figure 57b) confirmed that α-tocopherol was the weakest antioxidant in oil, but BHT was less effective at inhibiting formation of TBARS than chlorogenic acid, whereas it was more effective at reducing the rate of hydroperoxide formation.

The ratio of PV: TBARS for the different samples is shown in Figure 58. The ratio decreased during storage but it was higher for the samples containing chlorogenic acid, Teaw extract and citric acid than for the samples which contained BHT, α-tocopherol or ascorbyl palmitate throughout the storage period. Satue *et al*.

(1995) reported that antioxidants showed different activities toward hydroperoxide decomposition. For the antioxidants used in this study, the differences in the relative values for hydroperoxides and TBARS values are expected to relate to the metal chelating ability of the antioxidant. Metals are known to catalyse hydroperoxide decomposition, which leads to the formation of the aldehydes and related compounds that are determined in the TBARS assay. Chlorogenic acid is known as a metal chelator (Luzia *et al*., 1998), and the Teaw extract contained 60% chlorogenic acid. Consequently, it is consistent with the known metal chelating activity of chlorogenic acid that the PV: TBARS ratio for oil samples containing chlorogenic acid should be higher than for α-tocopherol and BHT, which do not have the *o*-diphenol structure necessary for molecules to chelate metal ions. A further experiment was performed in order to investigate whether this explanation was valid.

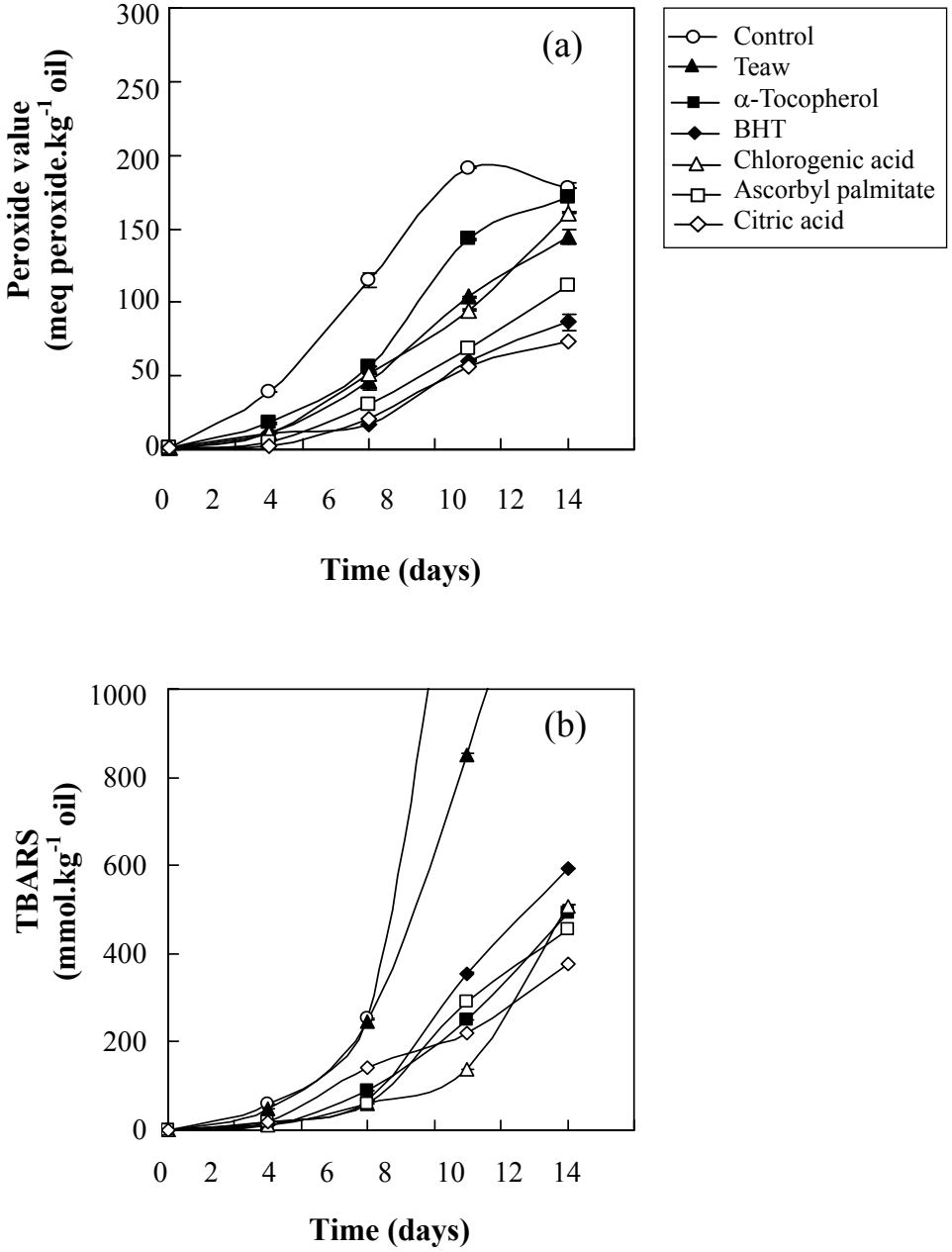


Figure 57 Effect of studied antioxidants on oxidative stability of stripped soybean oil at 60°C, assessed by determination of (a) PV and (b) TBARS. The concentration of each antioxidant was 100 mg·kg^{-1} except chlorogenic acid (60 mg·kg^{-1}). Data points represent mean ± standard deviation (n=3).

Table 20 Time for stripped soybean oil and oil-in-water emulsion samples containing studied antioxidants (100 mg·kg^{-1}) to reach a peroxide value of 50 meq·kg^{-1} at 60°C*

Compound	Bulk oil			10% oil-in-water emulsion		
	Time (days)	r^2	°PF	Time (days)	r^2	°PF
Control	3.27±0.01 [a]	0.99	1.00±0.00 [a]	1.56±0.11 [a]	1.00	1.00±0.00 [a]
Teaw extract	6.06±0.01 [c]	0.99	1.86±0.00 [c]	4.55±0.02 [d]	0.96	2.92±0.02 [d]
Chlorogenic acid♦	6.18±0.02 [c]	0.99	1.89±0.01 [c]	4.44±0.10 [c]	0.98	2.84±0.04 [c]
∝-tocopherol	4.81±0.01 [b]	0.97	1.47±0.00 [b]	6.52±0.07 [f]	0.97	4.19±0.07 [f]
BHT	8.80±0.02 [e]	0.97	2.69±0.01 [e]	14.73±0.01 [g]	0.98	9.45±0.07 [g]
Ascorbyl palmitate	7.84±0.01 [d]	0.99	2.40±0.01 [d]	5.00±0.06 [e]	0.97	3.21±0.09 [e]
Citric acid	9.29±0.00 [f]	0.98	2.84±0.01 [f]	4.25±0.03 [b]	0.97	2.73±0.01 [b]

* Data followed by different letters within each column are significantly different according to Duncan's multiple range test at $P < 0.05$. Data are shown as means ± standard deviation from three replicate determinations.

♦ Concentration of chlorogenic acid was 60 mg·kg^{-1}.

° PF = days to reach PV 50 meq·kg^{-1} for each antioxidant / days to reach PV 50 meq·kg^{-1} for control (no antioxidant).

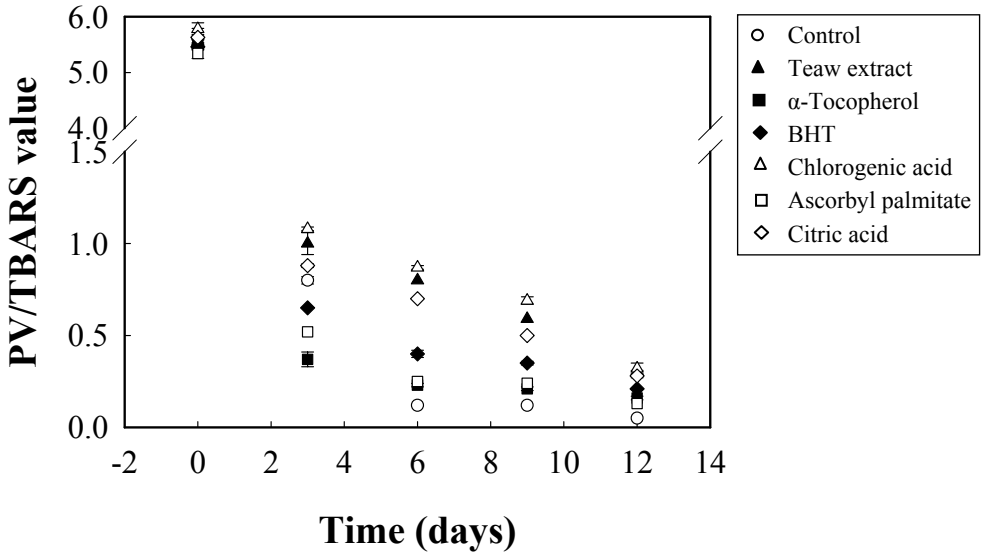


Figure 58 The ratio of peroxide and TBARS values (PV/TBARS value) of stripped soybean oil containing antioxidants during storage at 60°C. The concentration of each antioxidant was 100 mg·kg^{-1} except chlorogenic acid (60 mg·kg^{-1}). Data points represent mean ± standard deviation (n=3).

Citric acid-washed, stripped soybean oil with and without added ferric chloride was used to study the effect of antioxidants (α-tocopherol, chlorogenic acid and citric acid) on hydroperoxide and TBARS formation in oil with a low and a high metal ion content. The ratio of PV: TBARS for the metal-free samples is shown in Figure 59a. In the oil without added iron, the ratio remained about the same for the citric acid and control samples during storage or tended to increase with longer storage times for the chlorogenic acid and α-tocopherol samples. This indicates that the radical scavenging antioxidants were inhibiting hydroperoxide decomposition and causing some increase in the PV: TBARS ratio during storage if metal ions were absent. The PV values of the samples at 0, 12, 24, 36 and 48 hours are given in Table 21. There was no significant difference ($P \geq 0.05$) in the PV: TBARS ratio for samples containing chlorogenic acid at 60 and 100 mg·kg^{-1}. The PV: TBARS ratio was higher for chlorogenic acid than for α-tocopherol.

The ratio of PV: TBARS during storage was quite different in the oil containing added ferric ions (Figure 59b) from that in the metal-free oil. The ratio

was high initially but it fell progressively during storage. The ratio was highest in the order chlorogenic acid > citric acid > tocopherol > control. Antioxidants can promote oxidation in the presence of ferric ions under certain conditions by reducing ferric to the ferrous ion, which is more active in catalyzing decomposition of hydroperoxides, but this is an ionic reaction that occurs mainly in aqueous systems (Paiva-Martins and Gordon, 2002). In oils antioxidants may chelate the metal and thereby retard oxidation. In this experiment, the PV and TBARS values were higher for the control and antioxidant samples in the oil containing added ferric ions than for the oil lacking ferric ions (Table 21). This shows that the metal was catalyzing both hydroperoxide formation and hydroperoxide decomposition.

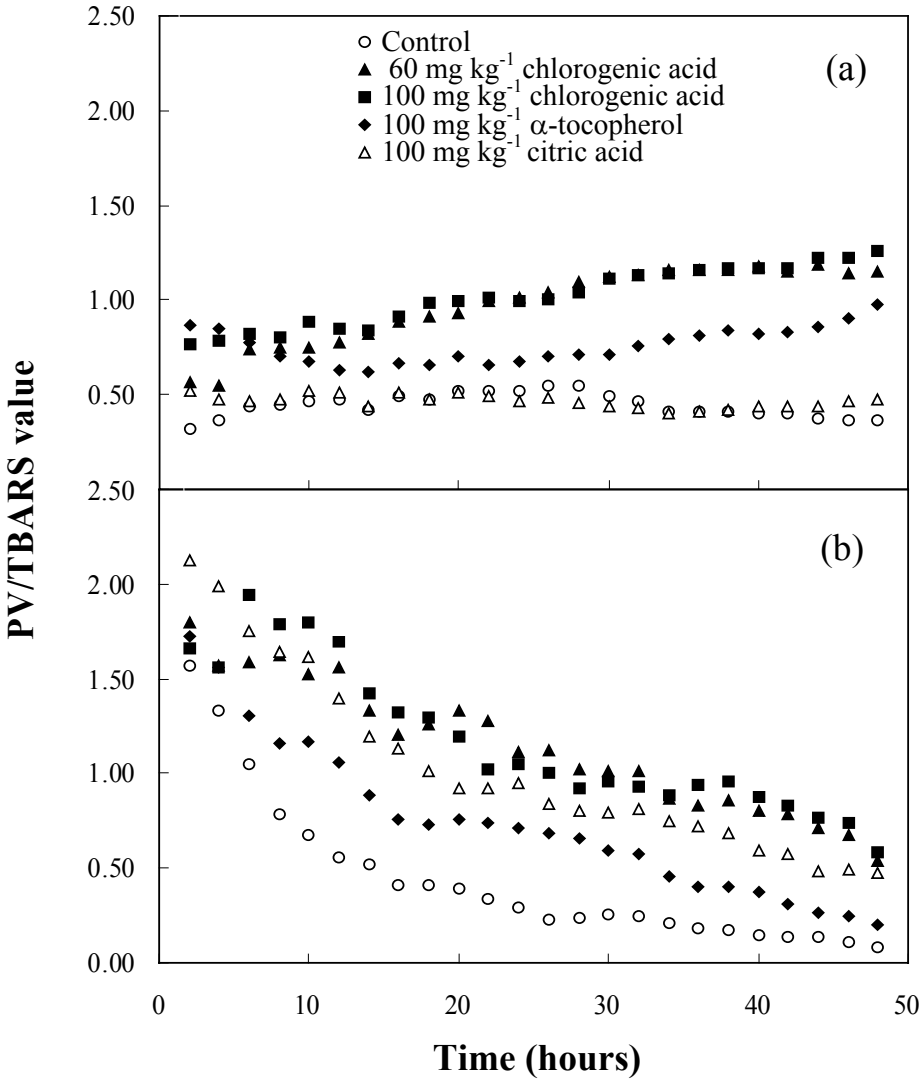


Figure 59 The ratio of peroxide and TBARS values (PV/TBARS value) of studied antioxidants on oxidative stability of (a) citric acid washed stripped soybean oil and (b) stripped soybean oil containing added ferric ions (5 mg·kg^{-1} $FeCl_3$) at 60°C. Data points represent mean ± standard deviation (n=3).

Table 21 Peroxide and TBARS values for samples of citric acid washed oil and oil containing added ferric ions plus antioxidants (100 mg·kg^{-1}) at 60°C*

Compounds	Time (hours)	Oil with no added ferric ions		Oil with added ferric ions	
		PV(meq·kg^{-1})	TBARS	PV (meq·kg^{-1})	TBARS
Control	0	1.48±0.05 ^{a}	0.24±0.03 ^{a}	1.48±0.05 ^{a}	0.24±0.03 ^{a}
	12	6.80±0.11 ^{c}	14.25±0.14 ^{e}	8.93±0.05 ^{f}	25.57±0.29 ^{h}
	24	11.39±0.07 ^{h}	21.76±0.54 ^{h}	15.42±0.04 ^{ij}	52.01±0.29 ^{l}
	36	15.82±0.07 ^{j}	40.01±0.47 ^{j}	33.84±0.66 ^{o}	183.44±0.92 ^{o}
	48	22.72±0.34 ^{k}	46.40±0.15 ^{l}	59.41±2.12 ^{q}	748.87±1.76 ^{q}
∝-tocopherol	0	1.48±0.05 ^{a}	0.24±0.03 ^{a}	1.48±0.05 ^{a}	0.24±0.03 ^{a}
	12	5.09±0.08 ^{b}	8.12±0.07 ^{cd}	6.79±0.04 ^{c}	6.42±0.01 ^{b}
	24	8.01±0.47 ^{de}	11.93±0.58 ^{ef}	14.35±0.04 ^{i}	20.31±1.35 ^{c}
	36	10.37±0.25 ^{h}	12.74±0.00 ^{fg}	25.49±0.50 ^{m}	63.38±0.10 ^{n}
	48	14.20±0.25 ^{i}	14.51±0.08 ^{gh}	41.43±0.63 ^{p}	211.09±1.25 ^{p}
Chlorogenic acid♦	0	1.48±0.05 ^{a}	0.24±0.03 ^{a}	1.48±0.05 ^{a}	0.22±0.00 ^{a}
	12	5.41±0.27 ^{b}	6.94±0.01 ^{b}	5.08±0.03 ^{b}	3.25±0.03 ^{b}
	24	7.70±0.19 ^{d}	7.62±0.03 ^{c}	10.09±0.03 ^{gh}	9.03±0.01 ^{c}
	36	9.38±0.16 ^{fg}	8.11±0.00 ^{c}	15.67±0.30 ^{j}	18.91±0.38 ^{h}
	48	10.78±0.14 ^{h}	9.41±0.18 ^{c}	29.66±1.08 ^{n}	54.87±1.41 ^{m}
Chlorogenic acid	0	1.48±0.05 ^{a}	0.24±0.03 ^{a}	1.48±0.05 ^{a}	0.23±0.01 ^{a}
	12	4.66±0.07 ^{b}	5.97±0.03 ^{b}	4.80±0.22 ^{b}	2.83±0.03 ^{b}
	24	7.30±0.52 ^{cd}	7.33±0.04 ^{c}	9.91±0.33g ^{h}	9.43±0.02 ^{c}
	36	9.13±0.29 ^{f}	7.86±0.01 ^{c}	15.01±0.29 ^{ij}	16.00±0.03 ^{h}
	48	10.82±0.11 ^{h}	8.60±0.01 ^{c}	24.04±0.88 ^{l}	43.50±1.23 ^{k}
Citric acid	0	1.48±0.05 ^{a}	0.24±0.03 ^{a}	1.48±0.05 ^{a}	0.24±0.03 ^{a}
	12	6.94±0.14 ^{c}	13.65±0.41 ^{de}	4.51±0.24 ^{b}	3.24±0.03 ^{b}
	24	10.69±0.20 ^{h}	22.97±0.54 ^{h}	8.66±0.28 ^{ef}	9.13±0.02 ^{c}
	36	15.51±0.25 ^{j}	37.58±0.51 ^{i}	14.68±0.29 ^{i}	19.50±0.14 ^{h}
	48	21.50±0.21 ^{k}	45.18±0.15 ^{l}	22.14±0.14 ^{k}	41.91±1.17 ^{j}

* Data followed by different letters within each column are significantly different according to Duncan's multiple range tests at $P < 0.05$. Data were represented as means from three replicates.
♦ Concentration of chlorogenic acid was 60 mg·kg^{-1}.

Chlorogenic acid can function both as a radical scavenger and as a metal chelator due to the presence of an *ortho*-dihydroxy grouping in its chemical structure (Luzia *et al*., 1998), whereas, citric acid can only act as a metal chelator. However, citric acid is a more effective metal chelator than chlorogenic acid, and hence citric acid is more effective at inhibiting an increase in the TBARS value due to metal-catalysed hydroperoxide decomposition as seen in the lowest TBARS value for citric acid in oil with added ferric ions. This experiment confirms that the metal-chelating activity of chlorogenic acid plays an important role in the ability of the antioxidant to inhibit decomposition of hydroperoxides in oils. Hence, the antioxidant is effective in retarding development of off-flavours not only because of its radical scavenging activity, which retards formation of hydroperoxides, but also because of its metal - chelating activity, which retards decomposition of hydroperoxides.

In a previous study, we found that the Teaw extract contained 0.60 g chlorogenic acid equivalent per gram (g of CAE/g) and other caffeic acid derivatives, including dicaffeoylquinic acid, at a level of approximately 0.12 g of CAE/g. In this study, the antioxidant effect of Teaw extract in stripped soybean oil at 100 $mg \cdot kg^{-1}$ was similar to that of chlorogenic acid at 60 $mg \cdot kg^{-1}$. This study confirmed that chlorogenic acid was the main antioxidant in the Teaw extract and the other components do not contribute significantly to the antioxidant activity of the extract in oil. The minor components in the Teaw extract elute later than chlorogenic acid in reversed phase HPLC and hence are less polar. The poor antioxidant activity of α-tocopherol and the minor components of the Teaw extract compared to chlorogenic acid is in agreement with the polar paradox, which concludes that less polar antioxidants are less effective than polar antioxidants in an oil medium (Porter *et al*., 1989).

4.2 Antioxidant Activity of Phenolic Antioxidants in Stripped Soybean Oil-in-Water Emulsions.

The antioxidant activity of Teaw, Kradonbok and Kratin extracts was evaluated in 10% stripped soybean oil-in-water emulsion during storage at 60°C in the dark by monitoring the PV and TBARS values. The emulsion was physically stable during a day of incubation. The size of the oil droplets in the emulsion was 0.42±0.00 μm, and no creaming, flocculation, coalescence or oil separation was observed. This is within the recommended range of < 1.00 μm (Decker *et al*., 2005). Since the droplet size of the emulsion was related to the lipid oxidation rate. The formation of hydroperoxides increased significantly more in the control emulsion than in the emulsion with individual plant extract added and stored at 60°C (Figure 60a). Oxidation was more rapid in stripped soybean oil-in-water emulsion than in stripped soybean oil as is commonly found when comparing oxidation in oil and emulsions (Schwarz *et al*., 2000). The order of antioxidant activity in the oil-in-water emulsion for the plant extracts was similar to that in bulk oil. On the basis of the time to a PV of 50 meq·kg^{-1}, the order of antioxidant activity (Table 19) was Teaw extract > Kradonbok extract > Kratin extract > Control. The TBARS values confirmed the order of activity (Figure 60b). The changes in the ratio of the PV: TBARS values with time are shown in Figure 61. The PV: TBARS ratio of the control sample was highest due to the high level of hydroperoxide formation in the emulsion. Teaw extracts showed the lowest PV: TBARS ratio due to the lowest peroxide value (Figure 60a). Consequently, comparing the antioxidant capacity of the plant extracts in the emulsion model, the Teaw extract showed the highest antioxidant activity. A further experiment was performed in order to compare the antioxidant activity of the Teaw extract with standard antioxidants including α-tocopherol, BHT, ascorbyl palmitate, citric acid and the main phenolic component of the Teaw extract which is chlorogenic acid by monituring the PV and TBARS values during storage at 60°C in the dark.

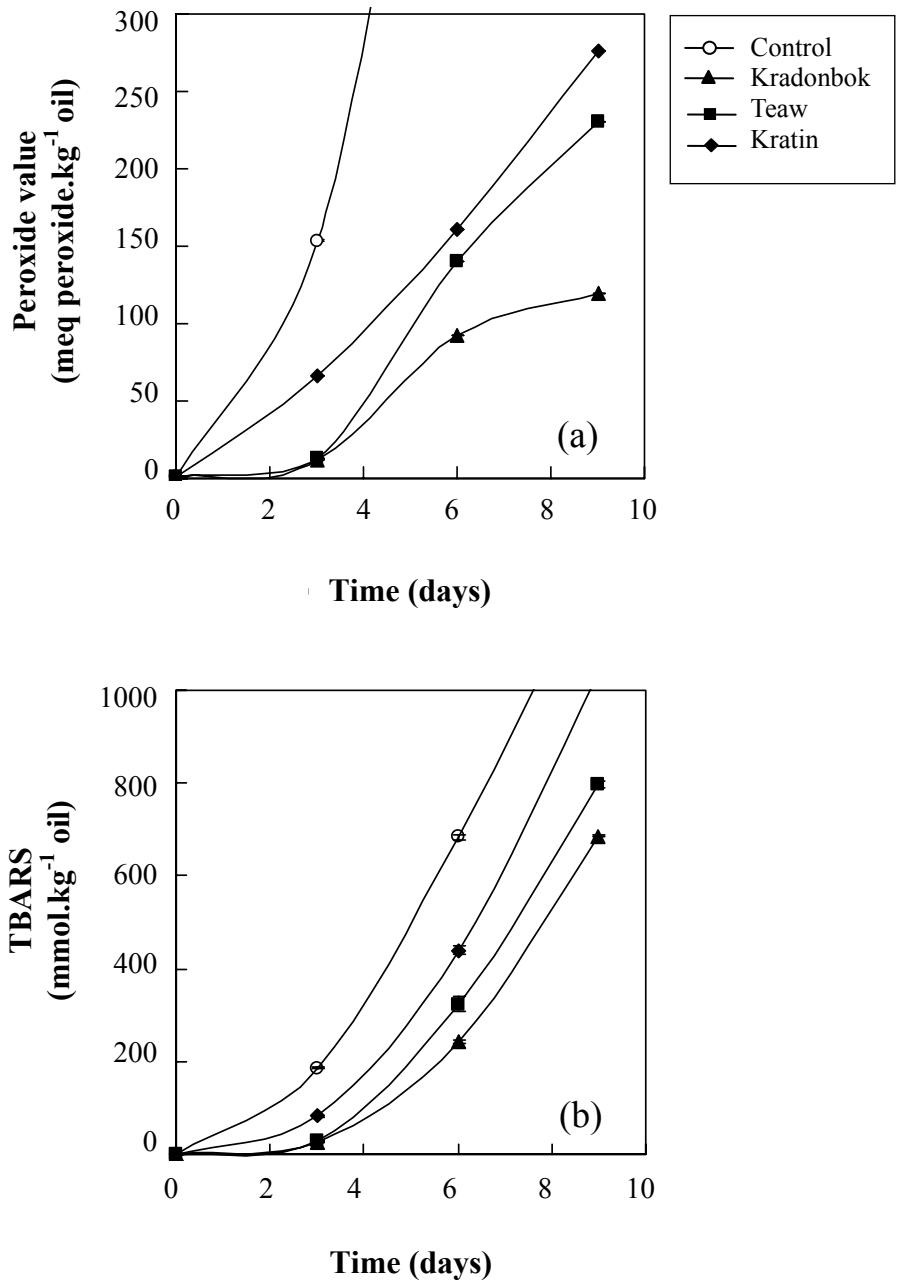


Figure 60 Effect of studied antioxidants (100 mg·kg^{-1}) on oxidative stability of stripped soybean oil-in-water emulsion at 60°C, assessed by determination of (a) PV and (b)TBARS. Data points represent mean ± standard deviation (n=3).

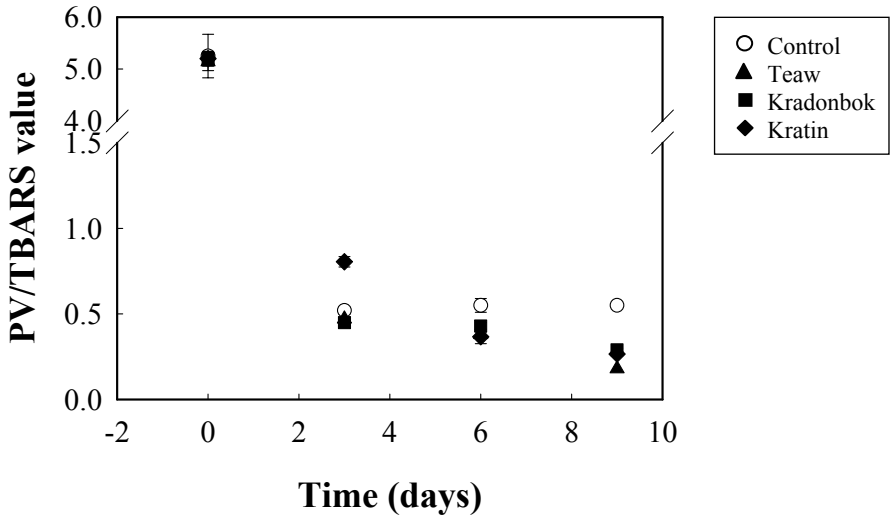


Figure 61 The ratio of peroxide and TBARS values (PV/TBARS value) of stripped soybean oil-in-water emulsions containing antioxidants (100 mg·kg^{-1}) during storage at 60°C. Data points represent means (n=3) ± standard deviation.

The order of antioxidant activity in the oil-in-water emulsion was different from that in bulk oil. On the basis of the time to PV of 50 meg·kg^{-1}, the order of antioxidant activity (Table 20) was BHT (100 mg·kg^{-1}) > α-tocopherol (100 mg·kg^{-1}) > ascorbyl palmitate (100 mg·kg^{-1}) > Teaw extract (100 mg·kg^{-1}), chlorogenic acid (60 mg·kg^{-1}) > citric acid (100 mg·kg^{-1}) > control. The TBARS value confirmed the order of the activity (Figure 62b).

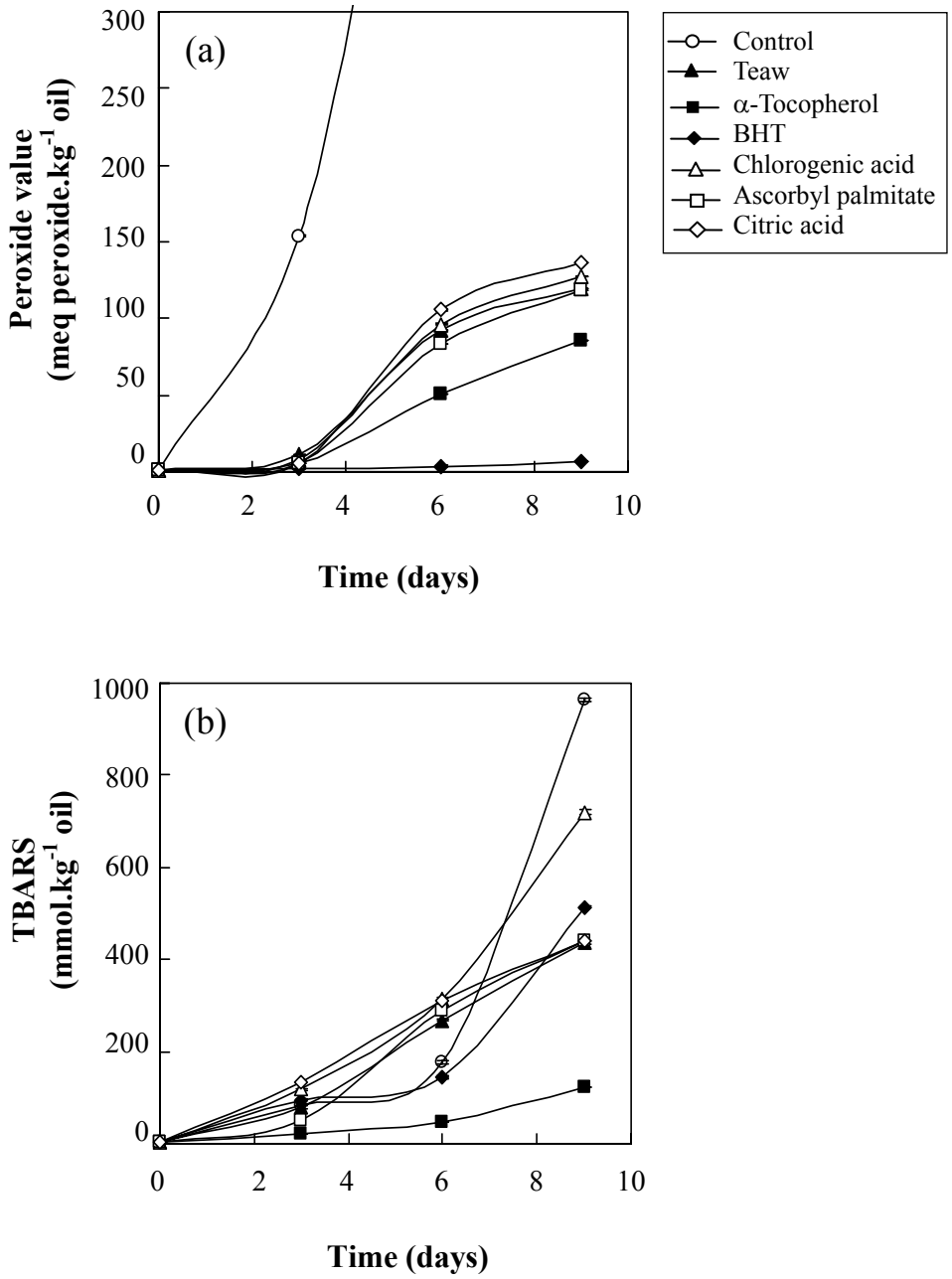


Figure 62 Effect of studied antioxidants on oxidative stability of stripped soybean oil-in-water emulsion at 60°C, assessed by determination of (a) PV and (b) TBARS. The concentration of each antioxidant was 100 mg·kg^{-1} except chlorogenic acid (60 mg·kg^{-1}). Data points represent mean ± standard deviation (n=3).

The range of TBARS values was much higher in the emulsion system (Figure 62b) than in the oil system (Figure 57). It appears likely that when hydroperoxides are formed in an emulsion they are concentrated at the oil-water interface where they are subject to further degradation by radicals generated in the aqueous phase or by metals. The PV: TBARS ratio of citric acid was the lowest ration (Figure 63). BHT was the most effective antioxidant in both oil and emulsion. The tertiary butyl group of BHT is effective in slowing reactions at the active -OH group by steric hindrance and thereby increasing the stability of the antioxidant radical and extending the active life of the antioxidant. The PV: TBARS ratios for the samples containing antioxidants fell rapidly to lower values (< 0.3) in the emulsion (Figure 63) than in the oil (Figure 58). Trace levels of metals are naturally present in oil and water. Metals are more active at catalyzing hydroperoxide decomposition in emulsions and this can partly explain the rapid fall in PV: TBAR ratio in the emulsion. It appears likely that when hydroperoxides are formed in an emulsion they are located at the oil-water interface where they are subject to further degradation by radicals generated in the aqueous phase or by metals. This leads to a more rapid increase in TBARS than in PV. Chlorogenic acid was relatively ineffective as an antioxidant in the emulsion because of its polarity.

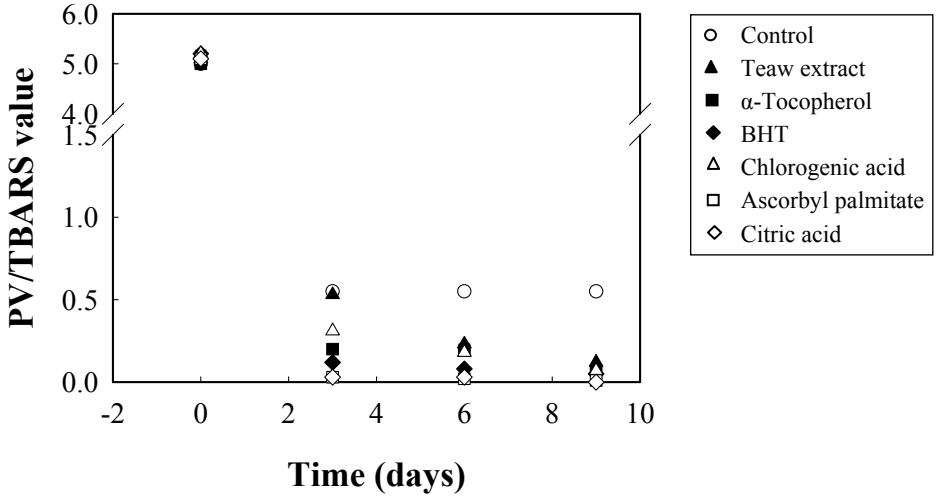


Figure 63 The ratio of peroxide and TBARS values (PV/TBARS value) of stripped soybean oil-in-water emulsions containing antioxidants during storage at 60°C. The concentration of each antioxidant was 100 mg·kg^{-1} except chlorogenic acid (60 mg·kg^{-1}). Data points represent means (n=3) ± standard deviation.

The polarity of the antioxidants was assessed by determination of the oil-water partition coefficient by HPLC. For the Teaw extract, the oil-water partition coefficient was calculated by summing the areas of the five phenolic peaks (approximately 94% of total peak area) in the HPLC chromatogram of an aqueous solution of the Teaw extract compared with the areas of the five peaks in the aqueous phase after preparation and breaking of an emulsion. The relative areas of the HPLC peaks of the Teaw extract in the aqueous phase before and after partitioning with oil changed as shown in Figure 64. The oil-water partition coefficients of the Teaw extract and chlorogenic acid were very similar as shown in Table 22. This was expected since a previous study showed that chlorogenic acid was the main component in the Teaw extract at a concentration of 60% of the extract and three minor phenolic components were present at a level of 12% expressed as chlorogenic acid equivalents (CAE). The Teaw extract had a slightly higher partition coefficient than chlorogenic acid because of the presence of the minor components, including dicaffeoylquinic acid and ferulic acid derivatives, which were less polar than chlorogenic acid. α-Tocopherol and BHT were

completely insoluble in water, since they are much less polar. Partition coefficients (log *P*) of ascorbyl palmitate (Kristl *et al*., 2003) and citric acid (Verchueren, 1996) were reported as 0.719 and -1.64, respectively, compared with values of -0.68 for the Teaw extract and -0.77 for chlorogenic acid. Therefore, citric acid is more polar than the other studied antioxidants. Comparing the order of activity as antioxidants, the finding that chlorogenic acid and the Teaw extract were more effective than α-tocopherol in inhibiting lipid oxidation in bulk oil but were less effective in an oil-in-water emulsion is consistent with the previous literature. This phenomenon was described as the "polar paradox" (Porter *et al*., 1989). This has been explained as being due to the non-polar antioxidant being concentrated at the oil-water interface, where they are effective at preventing reaction of triacylglycerols with radicals generated in the aqueous phase (Frankel *et al*., 1994). In the emulsion, polar antioxidants would tend to partition into the aqueous phase where they would not be able to protect the lipid effectively. The Teaw extract at 100 $mg \cdot kg^{-1}$ is more effective than chlorogenic acid (60 $mg \cdot kg^{-1}$) in an emulsion but it is less effective in oil (Table 20), which is also consistent with the Teaw extract containing some less polar antioxidants which contribute particularly in the emulsion system.

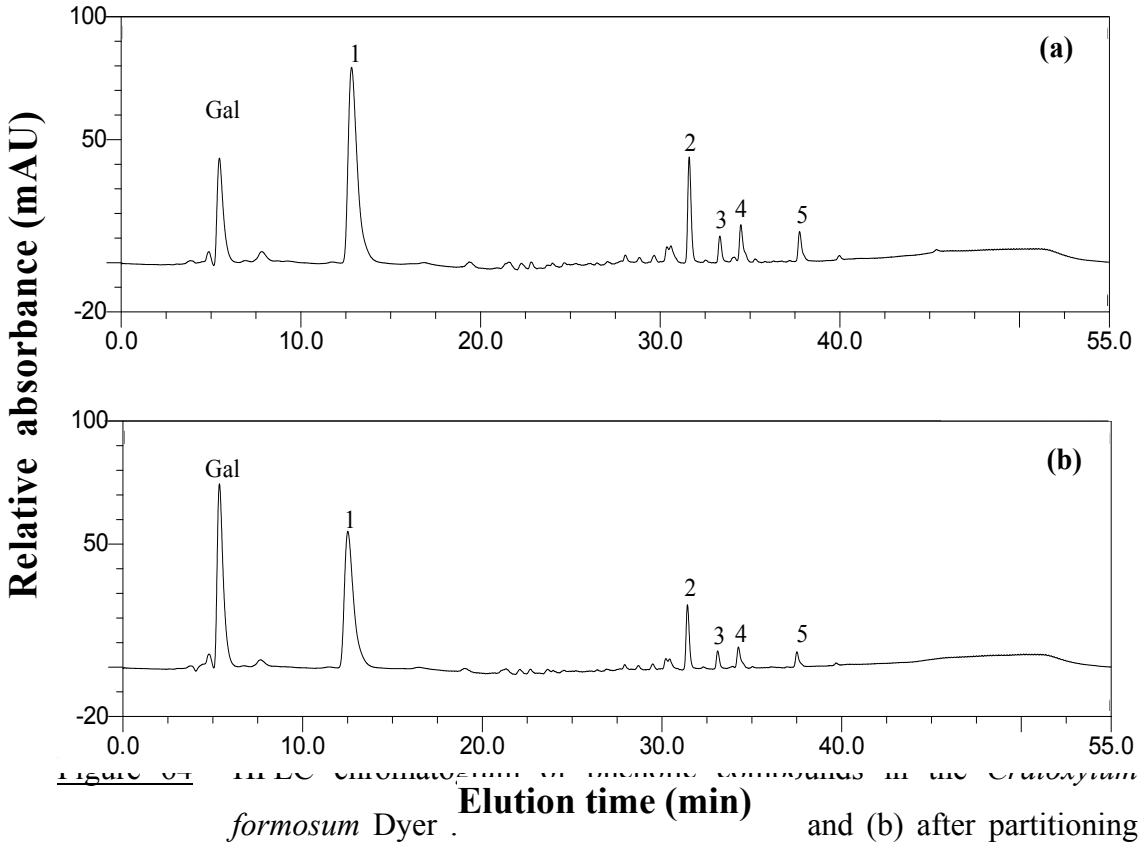


Figure 64 HPLC chromatogram of antioxidant compounds in the *Cratoxylum formosum* Dyer (a) before and (b) after partitioning into oil (Gal = gallic acid added as an external standard).

The change in antioxidant activity of the aqueous phase analysed directly without any oil addition and after breaking the emulsion was determined by the $ABTS^{+\cdot}$ radical scavenging assay (Table 20). The fall in the radical scavenging capacity of the aqueous phase after emulsion preparation was greater for the Teaw extract than for the chlorogenic acid solution, which confirms that the less polar components of the Teaw extract contribute to the radical scavenging activity in water, but partition more effectively into the oil phase than chlorogenic acid in the emulsion.

Table 22. Oil-water partition coefficient (log *P*) and $ABTS^{+\cdot}$ radical scavenging activity of *Cratoxylum formosum* Dyer .(Teaw) extract and chlorogenic acid*

compound	Log *P* ns	$ABTS^{+\cdot}$ radical scavenging activity	
		Stock solution	Aqueous phase after breaking emulsion
Teaw extract	-0.67±0.00	2.71±0.06 a	2.33±0.16 a
Chlorogenic acid	-0.77±0.00	3.06±0.04 b	2.92±0.04 b

* Data followed by different letters within each column are significantly different according to Duncan's multiple range test at $P < 0.05$. ns means non-significance within each column. Data obtained from at least ten replicates for the oil-water partition coefficient and three replicates for the $ABTS^{+\cdot}$ assay.

In conclusion, Teaw extract was more effective in oil and emulsion than Kradonbok and Kratin extract. Chlorogenic acid and the Teaw extract were more effective than α-tocopherol in inhibiting lipid oxidation in bulk oil but were less effective in an oil-in-water emulsion. The importance of the metal chelating activity in retarding hydroperoxide decomposition was confirmed by studying the decomposition of oil samples containing added ferric ions. The PV: TBARS ratio was higher for citric acid than for α-tocopherol in the presence of added ferric chloride, but the order was reversed in samples washed with citric acid to remove metal ions. Samples containing added chlorogenic acid gave the highest PV: TBARS ratios both in the presence and absence of ferric ions. The PV: TBARS ratios for the samples containing antioxidants fell rapidly to lower values in the emulsion than in the oil. This was due to a more rapid increase in TBARS than in PV due to increased hydroperoxide decomposition in the emulsion. The Teaw extract contained 12% oil-soluble components which contributed to a slightly higher oil-water partition coefficient than that of chlorogenic acid. The antioxidant activity of the aqueous phase of the Teaw extract was reduced more than that of

chlorogenic acid by partitioning of the oil-soluble components into oil, which showed that the less polar components could contribute to the antioxidant activity of the Teaw extract.

5. Determination of Synergistic Effects of the Plant Antioxidants in Oil and Emulsion Systems

In stripped soybean oil, a combination α-tocopherol with ascorbyl palmitate showed the synergistic effect (Table 23). The positive percent synergism corresponds to significant synergistic effect which generally observed when chain-breaking antioxidants are used together with preventive inhibitors. Ascorbyl palmitate can function by oxygen scavenging. An entirely different mechanism from that of phenolic antioxidants, are used as antioxidants to remove oxygen in solution. Ascorbyl palmitate cans synergist α-tocopherol. α-tocopherol act as the primary antioxidant and resulting α-tocopherylquinone radical and then reacting with ascorbyl palmitate to regenerate α-tocopherol. The reduction potential of ascorbate to form ascorbate radical (282 mV) was lower than α-tocopherol to form α-tocopherylquinone radical (500 mV). That is the reason why ascorbyl palmitate can regenerate α-tocopherol. The lipophilic ascorbyl palmitate has been proved not only to be synergist for α-tocopherol, but also to act as a radical trapping inhibitor which can observe from the inhibitory effectiveness of ascorbyl palmitate alone in oil and emulsion model. In addition, if the percent synergism was more than -50, it meant the combination effect of the mixture. However, if the synergism value was less than -50, it meant the antagonism effect of the combination. The combination of Kradonbok extract or BHT and ascorbyl palmitate provided a negative synergism. It is suggested that the mechanism behind the antagonistic interaction was due to hydrogen bonding between carbonyl and hydroxyl group (Yanishilieva-Maslarova, 2001). All combinations of citric acid plus phenolic antioxidants provide combination effects. Citric acid is more hydrophilic than phenolic antioxidants. The antioxidant mechanisms of these compounds are different. Citric acid can act as metal chelator. Synergistic and combination interaction most likely occur by a "sparing" effect provided by the chelator. Since the chelator will decrease oxidation

rates by inhibiting metal-catalyzed oxidation, fewer free radicals will be generated in the system (Decker, 1998).

In the emulsion system, a combination of α-tocopherol or Teaw extract with ascorbyl palmitate showed the synergistic effect. Synergism of all mixture of ascorbyl palmitae and the phenolic antioxidants in emulsion were higher than that of in oil (Table 23). Ascorbyl palmitate can protect oxidation by trapping the peroxyl radicals which can dissolve in the aqueous phase before peroxyl radicals can get into the lipid droplet. Besides, ascorbyl palmitate can act as hydrogen donator to primary antioxidants. Moreover, the advantage of solubility of ascorbyl palmitate in oil and aqueous phase make it can act as antioxidant both in lipid phase and aqueous phase. These reasons can explain why ascorbyl palmitate had higher additive effect in emulsion more than in oil. In contrast, most combination of citric acid and the studied antioxidant in emulsion were less combination effect than that in oil except combination of Teaw extract, Kratin extract or BHT and citric acid. Due to the stripped soybean oil technique, oil still contained a small amount of metal compounds. Metals interact with hydroperoxides and promote oxidation. Moreover, it is thought that a metal-hydroperoxide complex forms and subsequently decomposes to produce free radicals. Carboxyl group of citric acid is though to be responsible for binding with metals and forming complex. The reaction of metal with chelator is less significant in aqueous phase. Since metals in their lower oxidation states accelerate hydroperoxide degradation more than metals in their higher oxidation states. These results support that metal chelator was able to inhibit oxidation in emulsion less than in oil.

Table 23 Synergy (%) of *Cratoxylum formosum* Dyer. (Teaw), *Caraya sphaerica* Roxb. (Kradonbok) and *Leucaena glauca* Benth (Kratin) extract, α-tocopherol and BHT mixtures with ascorbyl palmitate (AP) or citric acid (CA) in stripped soybean oil and emulsion during oxidation at 60°C. The concentration of each sample was 100 mg·kg^{-1}[#]

Compound	Bulk oil	10% oil-in-water emulsion
Teaw extract + AP	-29.24±0.07 [c]	-12.01±0.97 [k]
Teaw extract + CA	-33.24±0.02 [g]	-33.02±0.75 [d]
Kradonbok extract + AP	-76.29±0.08 [a]	-45.16±0.50 [a]
Kradonbok extract + CA	-43.13±0.09 [d]	-45.70±0.42 [a]
Kratin extract + AP	-40.09±0.12 [e]	-16.53±0.77 [g]
Kratin extract + CA	-40.55±0.04 [e]	-27.26±1.98 [e]
α-tocopherol + AP	2.34±0.10 [k]	8.06±0.63 [j]
α-tocopherol + CA	-14.36±0.06 [j]	-20.73±1.07 [f]
BHT + AP	-72.21±0.16 [b]	-36.36±0.37 [c]
BHT + CA	-46.60±0.21 [c]	-11.72±1.75 [h]
Chlorogenic acid* + AP	-30.94±0.24 [h]	-9.87±1.94 [i]
Chlorogenic acid* + CA	-35.51±0.14 [f]	-42.57±2.25 [b]

Note: [#] Data followed by different letters within each column are significantly different according to Duncan's multiple range test at $P < 0.05$. Data were represented as means from three replication measurement.

[*] Concentration of chlorogenic acid equaled to 60 mg·kg^{-1}.

In conclusion, there was only slightly synergistic effect between α-tocopherol and ascorbyl palmitate in emulsion and oil model. Therefore, the selected Thai plant antioxidant is Teaw extract due to the highest activity in oil and emulsion model comparing with Kradonbok and Kratin extracts. In addition, the extract should be use in food with no combination with ascorbyl palmitate or citric acid.

6. Application of the Selected Plant Antioxidant in Food

Snack foods are susceptible to lipid oxidation because they are low moisture food. Autoxidation occur rapidly at the range of water activity between 0.01-0.15. In addition, cereal foods contain iron, a catalyst for oxidation. From previous study, Teaw extract contained chlorogenic acid which acts as free radical scavenger and metal chelator. Hence, rice snack was selected to study the antioxidant effectiveness of crude Teaw extract comparing with commercial antioxidant including α-tocopherol and BHT.

6.1 Changes in Chemical Properties

The progression in chemical change of rice snack with studied antioxidants (crude Teaw extract, α-tocopherol, BHT and dried Teaw leaf powder) was monitored in terms of peroxide values (PV) and TBARS values in rice snack during storage at 40°C for 18 days. The total extractable lipid in samples averaged about 11 % of the mass of samples (data not shown). Water activity of the rice snack sample was about 0.08-0.20 during storage (data not shown). Oxidative changes in snack were initiated by the formation of free radicals, the precursors for the hydroperoxides, which were normally regarded as primary oxidation products. Free radicals may be formed enzymatically, by transition metal catalyst (Kristensen *et al.,* 2000). Storage time was a significant factor ($p < 0.001$) for PV and TBARS values (Table 24). Each antioxidant including freeze-dried Teaw extract, dried Teaw leaf powder, α-tocopherol and BHT was also significant effectiveness to lipid oxidation in term of PV and TBARS (Figure 65). For sample without antioxidants increased PV and TBARS value markedly. The PV value increased slowly at the first period of storage and then increased more rapidly over longer times. This type of behavior is an indication that later stages of lipid oxidation have been reached (Abegaz *et al*., 2004). Sample with BHT was the significant lowest of PV and TBARS values. Teaw extract appeared to possess stronger antioxidant activity than α-tocopherol and dried Teaw leaf powder.

Typically, the iron in polished glutinous rice, which was the raw material in this snack, is 2.5 $mg \cdot g^{-1}$ edible portion (Nutririon Devision, 2001). In a previous study, we found that the Teaw extract contained 0.60 g chlorogenic acid equivalent per gram (g of CAE/g) and other caffeic acid derivatives. Chlorogenic acid can function both as a radical scarvenger and as a metal chelator. Consequently, the antioxidant effect of Teaw extract in rice snack at 100 $mg \cdot kg^{-1}$ was higher than that of α-tocopherol. The TBARS value (Figure 65 b) confirmed this reason. The TBARS values of sample containing Teaw extract was lower that of samples containing α-tocopherol. In addition, from previous study (section 1.1), the lower antioxidant activity of Teaw leaf was obtained from drying treatment, hence, the antioxidant effectiveness of Teaw extract was more than dried Teaw leaf powder.

Table 24 *P*-values of PV, TBARS, baked rice odor, rancid odor and vegetable oil odor for the tests of each antioxidant and storage time differences

	Factors	*P*-values
PV	days	$P < 0.001$
	antioxidants	$P < 0.001$
TBARS	days	$P < 0.001$
	antioxidants	$P < 0.001$
Backed rice odor	days	$P < 0.001$
	antioxidants	$P < 0.001$
	panel	$P = 0.112$
	replications	$P = 0.965$
Rancid odor	Days	$P < 0.001$
	antioxidants	$P < 0.001$
	panel	$P = 0.272$
	replications	$P = 0.841$
Vegetable oil odor	days	$P < 0.001$
	antioxidants	$P < 0.001$
	panel	$P = 0.118$
	replications	$P = 0.711$

Note: a low *P*-value means a highly significant difference.

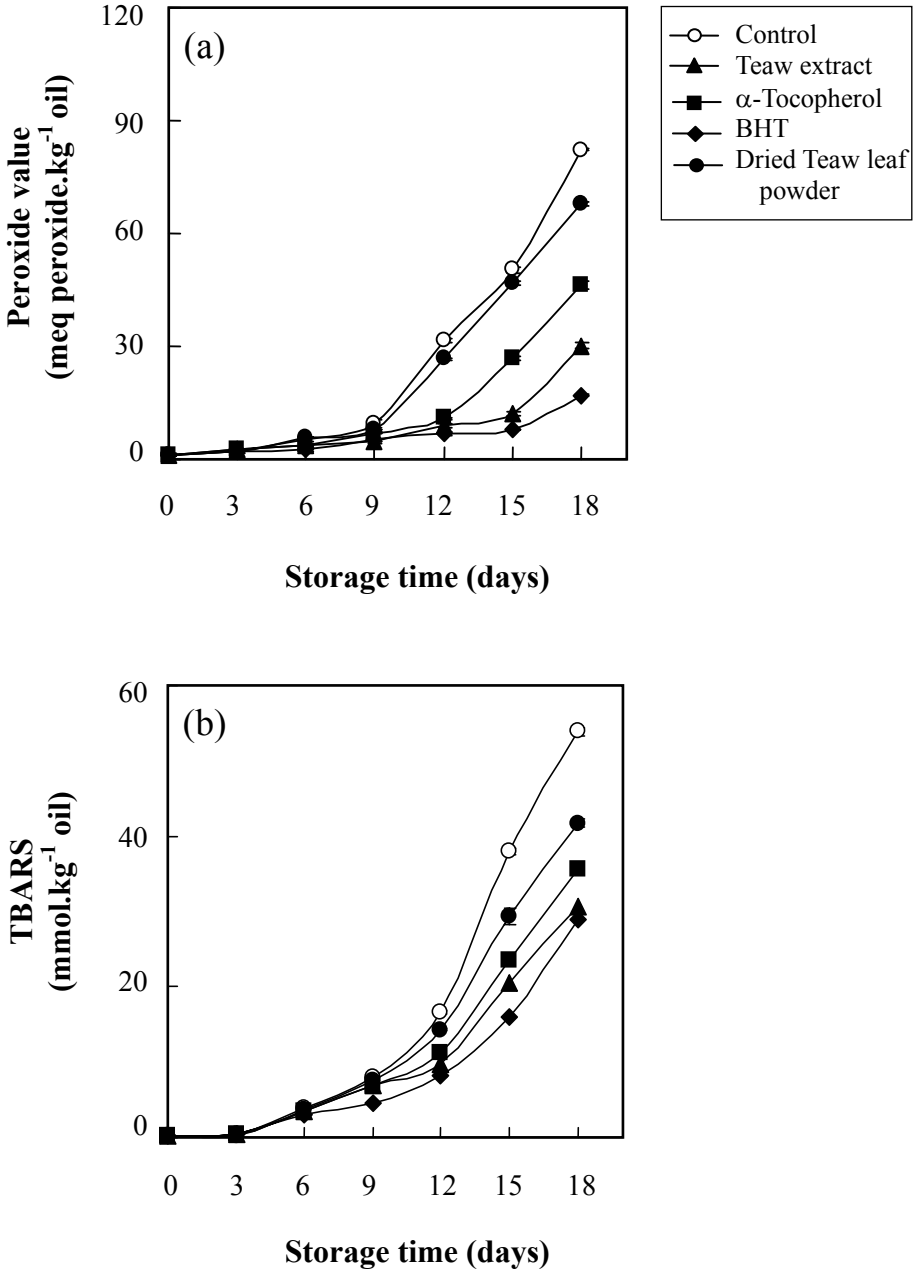


Figure 65 Effect of studied antioxidants on the oxidative stability of rice snack at 40°C, assessed by determination of (a) PV and (b) TBARS. The concentration of each antioxidant was 100 mg·kg^{-1} except dried Teaw leaf powder (2.44 g·kg^{-1}). Data points represent mean ± standard deviation (n=4).

6.2 Changes in Sensory Properties

Change in the odors of rice snack, namely, baked rice, rancid, and vegetable oil odor after storage for 18 days at 40°C were shown in Figure 66. It was conclude that each panel and the repetitions from sensory analysis did not differ significantly (Table 24). Normally, the replicates were used to check panelists' accuracy and reliability. This result showed that calibration with warm-up sample, balancing serving with random three digit codes could reduce classical psychology errors. Storage time and antioxidants significantly effected for backed rice, rancid, and oil odor (Table 24). A significant storage time effect to rancid odor more than baked rice ordor and oil odor (Figure 66). The sample with BHT gave the significant lowest rancid odor scores from sensory analysis. Moreover, the samples containing BHT showed the highest baked rice odor. The intensity of baked rice for control (no antioxidant) and samples containing dried Teaw leaf powder increased in the first period (6 days) and then decreased with the rapidly rancid development (Figure 66). Whereas, the baked rice odor was slightly increased for the samples containing Teaw extract. These samples showed the low value of rancid odor scores comparing to control. These results implied that samples which had higher rancid odor score, showed lower baked rice scores. Increasing rancid or oxidation-related warmed-over odor is often accompanied by decreasing the other flavors (Olsen *et al*., 2005), and this was observed in the present study as well. The decrease might be caused by a loss of components contributing to these desirable attributes or by masking due to increasing amount of lipid oxidation products. The changes in oil odor during storage showed the same scores in the low range between 2.8 - 3.5. This implied that natural vegetable oil had small amount of odor. Typically, the odor of oil come from products of numerous reaction between fat and other food components such as proteins and carbohydrates as well as oxidation products. Consequently, the oxidized oil gave higher odor than the fresh one. Moreover, it was difficult for human sense to detect the odor in a low intensity so the odor scores were slightly different.

The rancid odor scores of samples were in agreement with the degree of rancidity measured by PV and TBARS values. After 6 days of storage at 40°C, the snack samples had developed a relatively high degree of rancidity not only rancid odor scores from sensory analysis but also PV and TBARS from chemical analysis. Lipid oxidation leads to formation of volatile aldehydes and other low molecular compounds with low sensory thresholds. Such components can give odors and flavors typically associated with rancidity. Lipid oxidation products and compounds from other degradation reactions might also contribute to increased scores of other nondesirable sensory attributes (Olsen *et al*., 2005). A sensory rancid odor descriptors such as painty, grassy are attributed indicating production of lipid degradation products such as hexanol, heptanol, 2-hexanol, 2-octenal and (E, E)-2,4-decadienal. These compounds were detected in extruded oat flours (Viscidi *et al*., 2004). However, the susceptibility of various fatty acids toward oxidation is highly dependent on the number of doubly allylic hydrogen atom presented. Long chain fatty acids with more double bonds are therefore expected to be major contributors of lipid oxidation products in matrix where they are present. Soybean oil contained unsaturated acid about 80.7% which are palmitoleic acid (<0.5%), oleic acid (20-50%), linoleic acid (35-60%), linolenic acid (2-13%) and eicosenoic acid (<1.0%) (Hui, 1996). Normally, type of fatty acid related to oxidation volatile products, such as hexanal is derived from the oxidation of omega-6-fatty acid such as linoleic acid, and propanal is derived from the oxidation of omega-3-fatty acids such as linolenic acid. For oleic acid rich oils, nonanal may be used as a marker of oxidation (Decker *et al*., 2005). These results indicated that there was a relationship between sensory and chemical analysis.

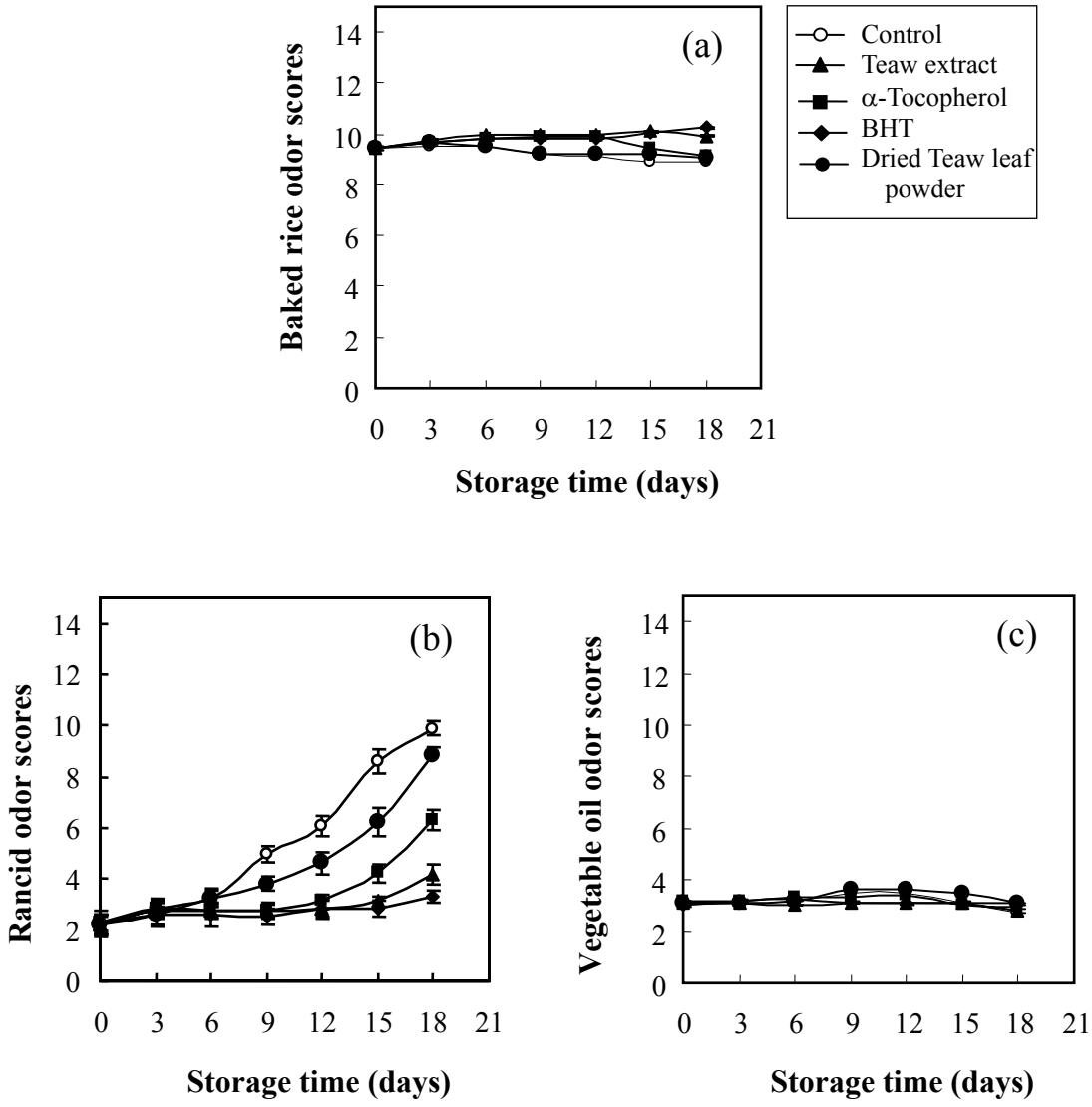


Figure 66 Sensory (a) baked rice, (b) rancid and (c) vegetable oil odor characterization of rice snack with different antioxidants at 40°C, 80% RH for 18 days, assessed by Quantitative Descriptive Analysis (QDA) using 10 trained panelists with two replications. Data points represent mean ± standard deviation (n=20).

6.3 Relationship between Chemical and Sensory Properties

The chemical analysis PV and TBARS values were significantly positively correlated with rancid odor sensory terms, However, TBARS value had closely relationship more than PV values (Figure 67). The TBARS method is widely used as indicator of lipid oxidation and has repeatedly been demonstrated to correlate with sensory term although most frequently reported as an exponential relationship of sensory terms versus log TBARS in meat (Nissen *et al*., 2004). In these results, we found the linear relation between PV or TBARS value and rancid odor was better than the exponential relationship (Table 25). These could be explained by the ingredients in meat and snacks are different. The presence of protein in meat affects the flavor release from foods. Lipid oxidation products might thus be differently formed and perceived in the food systems because of the influence of other compounds more than lipids (Olsen *et al*., 2005).

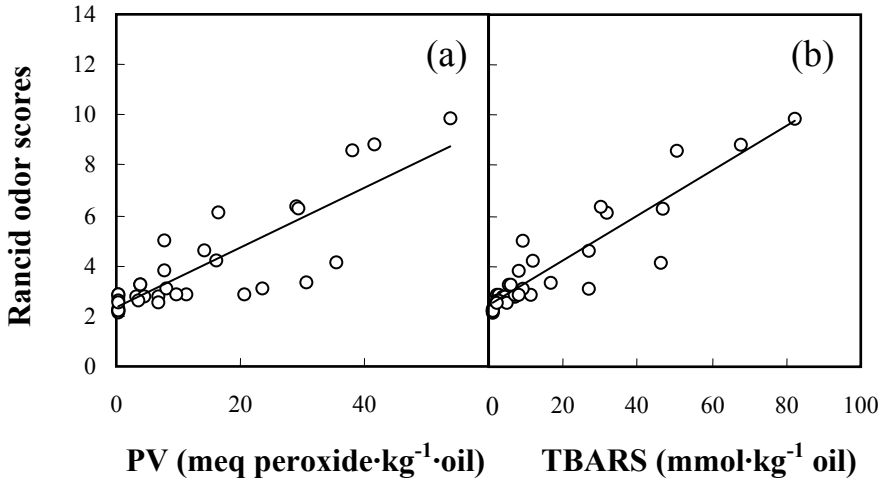


Figure 67 Regression analysis between (a) PV and (b) TBARS values with sensory rancid odor attribute of rice snack storage at 40°C.

Table 25 Relationship between PV or TBARS values and rancid odor scores of rice snack containing various antioxidants

Relationship	Regression equation	r^2
PV and rancid odor scores	linear	0.7238
	Exponential	0.7142
TBARS and rancid odor scores	linear	0.8546
	Exponential	0.8082

Our results indicated that dried Teaw leaf powder possessed antioxidant effectiveness in inhibiting lipid oxidation but less activity than Teaw extract in stored rice snack. In addition, Teaw extract was more effect than α-tocopherol due to metal presenting in snack, which made α-tocopherol less effective as an antioxidant. Sensory odor attributes of rice snack were related to TBARS more than PV values in form of linear regression. In addition, this study showed that the basic scientific principles from previous sections (section 3 and 4) can be applied to improve the oxidative stability of the selected food model by retarding the rancidity of rice snack food during storage at 40°C for 18 days. The understanding of the mechanism and phenolic constituents of crude plant extract can be benefit to apply for future food antioxidant.

CONCLUSION

Thai indigenous plants are consumed throughout the year as food and medicine. The contribution of these plants to health improvement can be related to their antioxidant capacity. Thai Indigenous plants are a potential source of natural antioxidants and the phenolic components and free-radical scavenging capacity of ethanolic extracts from various parts of twenty-six plants were determined. The extracts from berries used in wine production were found to have a higher antiradical activity than those obtained from herbs and vegetables, whereas chewing plants with an astringent taste had a high total phenolic content and flavanoid content. There was a high positive correlation between total phenolic and flavanoid contents in the plant extracts and antiradical activity. Three plant extracts were selected for further investigation based on their high antioxidant capacity, their safety for use as food additives, available sources, and cost. Therefore, Cratoxylum formosum Dyer. (Teaw), Careya sphaerica Roxb. (Kradonbok), and Leaucaena glauca Benth (Kratin) were selected to study the composition and properties of phenolic radical scavenging components.

The present study has characterized the phenolic antioxidant constituents in the Teaw extract for the first time. We found that chlorogenic acid is the main antioxidant component (60%) in the ethanolic extract of Teaw leaves. Dicaffeoylquinic acid (7%), and two ferulic acid derivatives (5%) were identified as minor components with similar radical scavenging activity to the same concentration of chlorogenic acid. Moreover, the potent DPPH radical scavenging phenolic components of the Kradonbok extract were found to be gallic acid derivatives with molecular weight equal to 308 (11.6%) and 204 (9.6%) which were present at higher concentration than other active components in the extract. Gallic acid (0.9%) and ellagic acid (2.4%) were found as minor components in the Kradonbok extract. The potent DPPH radical scavenging phenolic components in the ethanolic extract of Kratin leaves were quercetin glycoside, quercetin–3–*O*-rhamnopyranoside, kaempferol-3-*O*-rhamnopyranoside and an unknown

compound with molecular weight equal to 453 which was provisionally identified as a flavonoidal alkaloid. The Teaw extract showed higher antioxidant activity than α-tocopherol, Kradonbok extract and Kratin extract when assessed by the DPPH and ABTS assays.

The Teaw extract was more effective in oil and emulsion than Kradonbok and Kratin extracts. Chlorogenic acid and the Teaw extract were more effective than α-tocopherol in inhibiting lipid oxidation in bulk oil but were less effective in an oil-in-water emulsion. The importance of the metal chelating activity in retarding hydroperoxide decomposition was confirmed by studying the decomposition of oil samples containing added ferric ions. The PV: TBARS ratio was higher for citric acid than for α-tocopherol in the presence of added ferric chloride, but the order was reversed in samples washed with citric acid to remove metal ions. Samples containing added chlorogenic acid gave the highest PV: TBARS ratios both in the presence and absence of ferric ions. The PV: TBARS ratios for the samples containing antioxidants fell rapidly to lower values in the emulsion than in the oil. This was due to a more rapid increase in TBARS than in PV due to increased hydroperoxide decomposition in the emulsion. The Teaw extract contained 12% oil-soluble components which contributed to a slightly higher oil-water partition coefficient than that of chlorogenic acid. The antioxidant activity of the aqueous phase of the Teaw extract was reduced more than that of chlorogenic acid by partitioning of the oil-soluble components into oil, which showed that the less polar components could contribute to the antioxidant activity of the Teaw extract.

There was only a small synergistic effect between α-tocopherol and ascorbyl palmitate in the emulsion and oil model systems. Therefore, the Thai plant selected as a good source of antioxidants was the Teaw extract which had higher activity in oil and emulsion models compared with Kradonbok and Kratin extracts. In addition, the extract should be used in food without addition of ascorbyl palmitate or citric acid.

The Teaw extract was effective in inhibiting lipid oxidation when used alone in a stored rice snack. In addition, the Teaw extract was more effective than α-tocopherol due to metal ions present in the snack, which made α-tocopherol less effective as an antioxidant. The sensory odor attributes of the rice snack were related more closely to the TBARS than to PV values in the linear regression analysis. In addition, this study showed that the basic scientific principles from model system studies (section 3 and 4) can be applied to improve the stability of rice snack based food models stored at 40°C for 18 days. The understanding of the mechanism of action and phenolic constituents of crude plant extracts can be beneficial for future food antioxidant product development.

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APPENDIX

APPENDIX A

SENSORY TEST

BALLOT

Ballot for rice snack study

Panelist name:……..…….Sample……..……..Date……..

Procedure: Please cut the bag of sample and sniff immediately to give the rancid odor score and then sniff again to give the bake rice and oil odor by comparing with reference samples.

Odor: none very much

Rancid	0	0.5	1	1.5	2	2.5	3	3.5	4	4.5	5	**5.5**	6	6.5	7	**7.5**	8	8.5	9	9.5	10	10.5	11	11.5	12	12.5	13	13.5	**14**	14.5	15
Bake rice	0	0.5	1	1.5	2	2.5	**3**	3.5	4	4.5	5	5.5	**6**	6.5	7	7.5	8	8.5	9	9.5	10	10.5	11	**11.5**	12	12.5	13	13.5	14	14.5	15
Veg.Oil	0	0.5	1	1.5	2	**2.5**	3	3.5	**4**	4.5	5	5.5	**6**	6.5	7	7.5	8	8.5	9	9.5	10	10.5	11	11.5	12	12.5	13	13.5	14	14.5	15

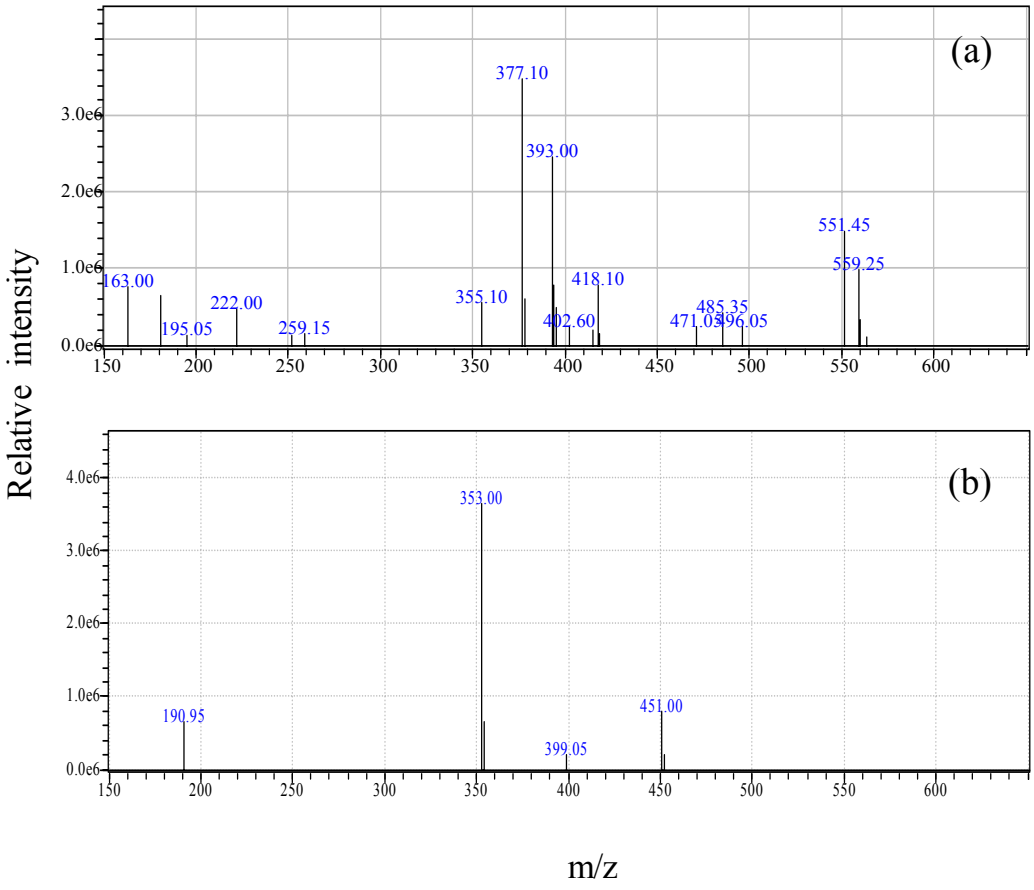
References

attribute	score								
	719	328	445	459	134	213	128	417	323
Rancid	5.5	7.5	14						
Bake rice				3	6	11.5			
Veg.Oil							2.5	4	6

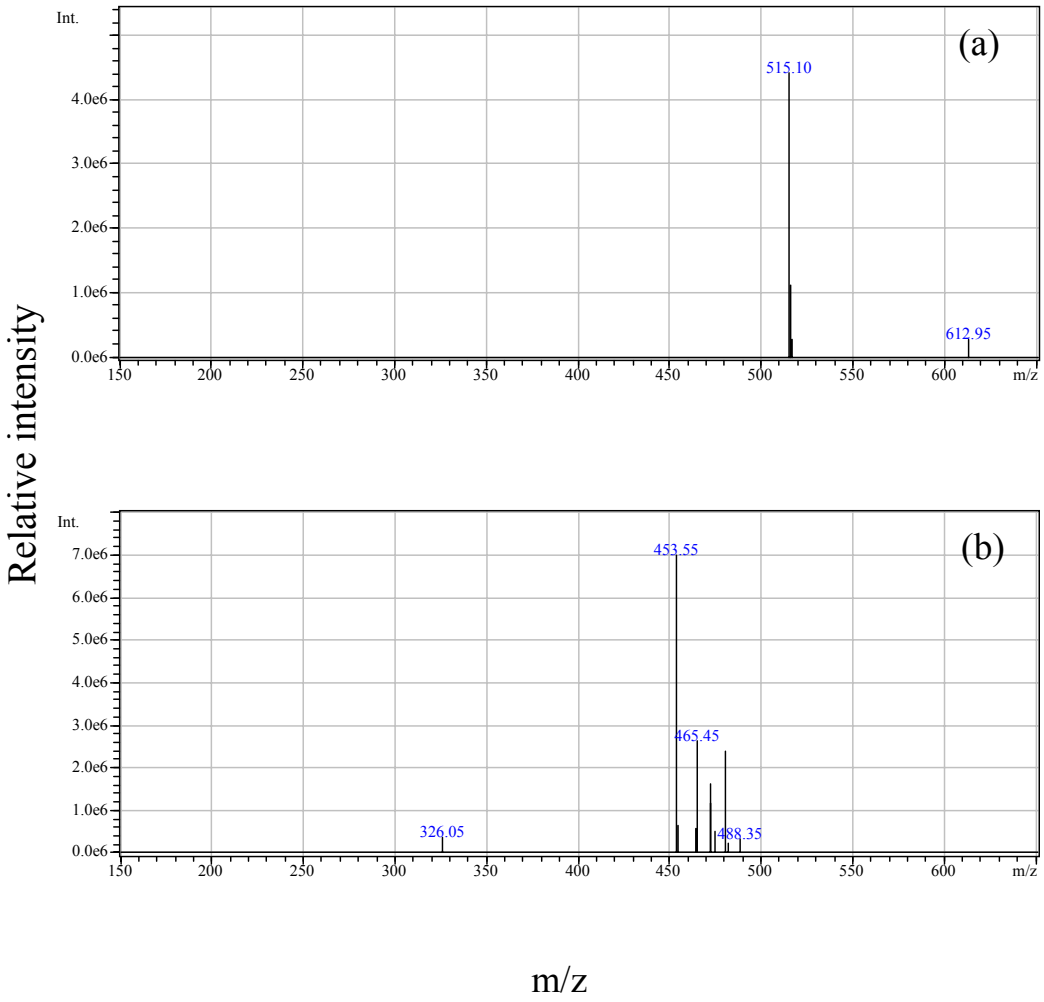
APPENDIX B

HPLC-ESI-MS spectrum of

Cratoxylum formosum Dyer.



Appendix Figure B1 LC-MS (a) positive ion and (b) negative ion spectra of peak 1 of *Cratoxylum formosum* Dyer.

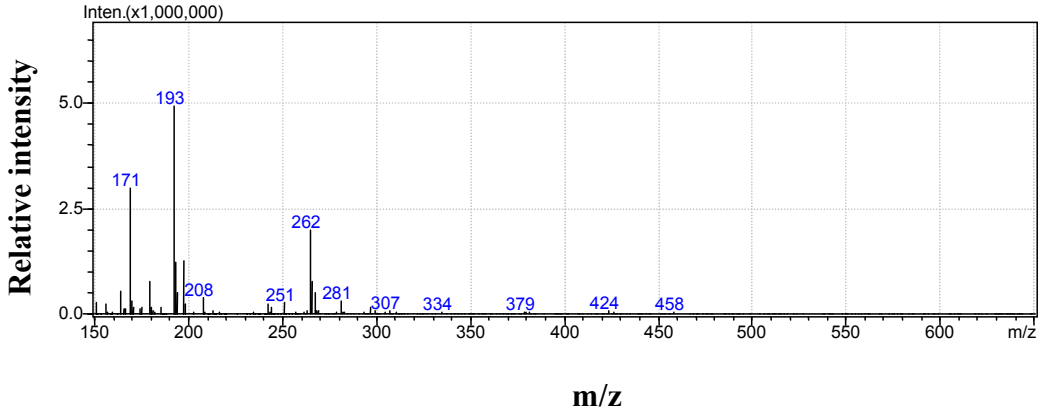


Appendix Figure B2 LC-MS negative ion spectra of (a) peak 2 and positive ion spectra of (b) peak 4 from *Cratoxylum formosum* Dyer.

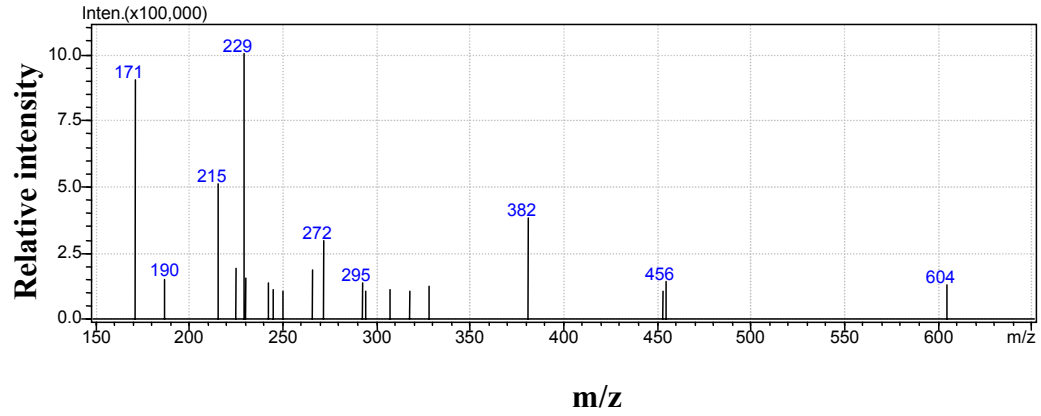
APPENDIX C

HPLC-ESI-MS spectrum of

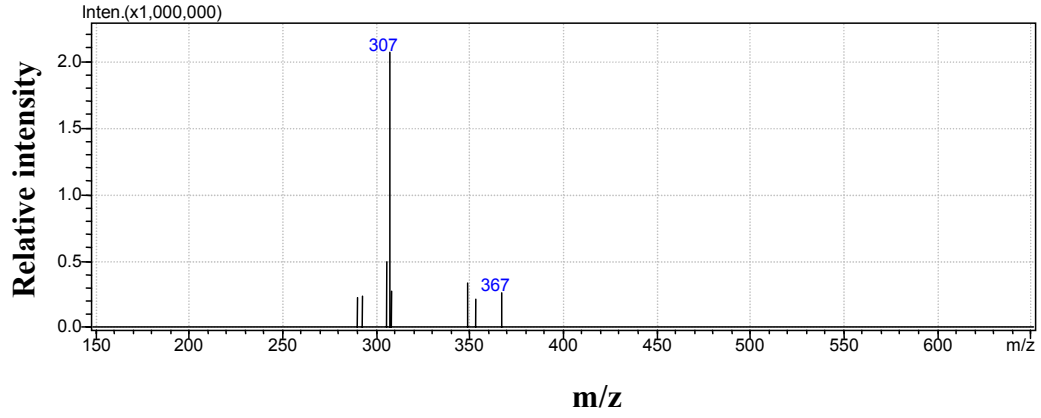
Careya sphaerica Roxb.



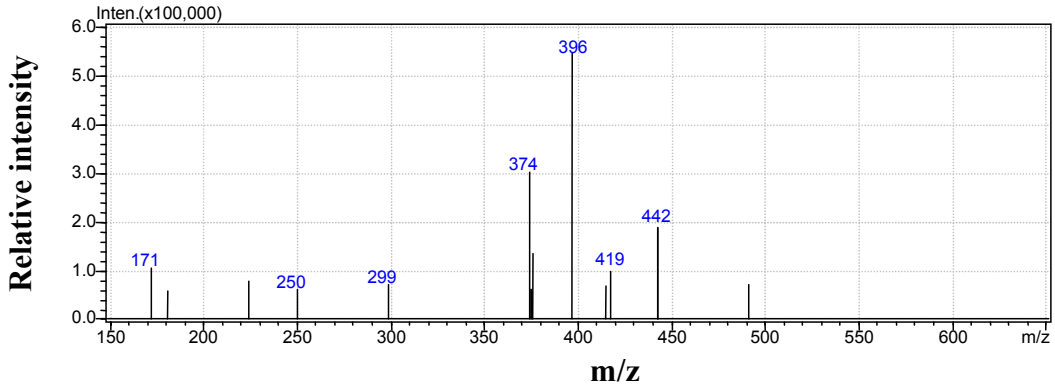
Appendix Figure C1 LC-MS positive ion spectra of peak 1 of *Careya sphaerica* Roxb.



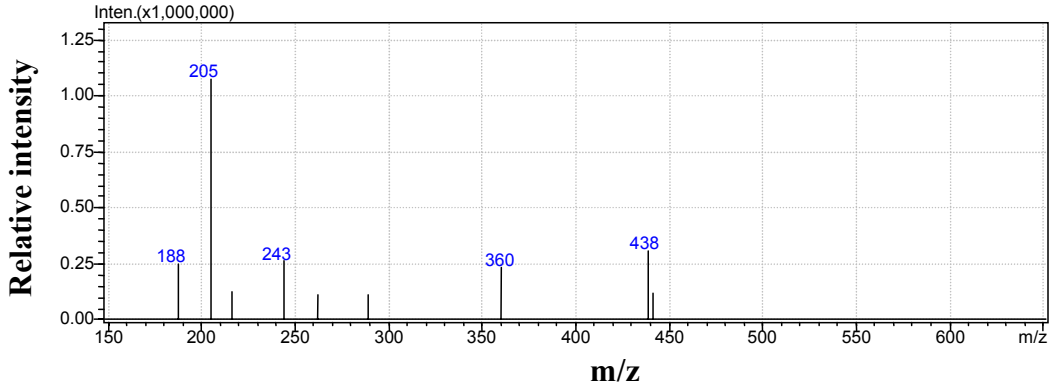
Appendix Figure C2 LC-MS positive ion spectra of peak 2 of *Careya sphaerica* Roxb.



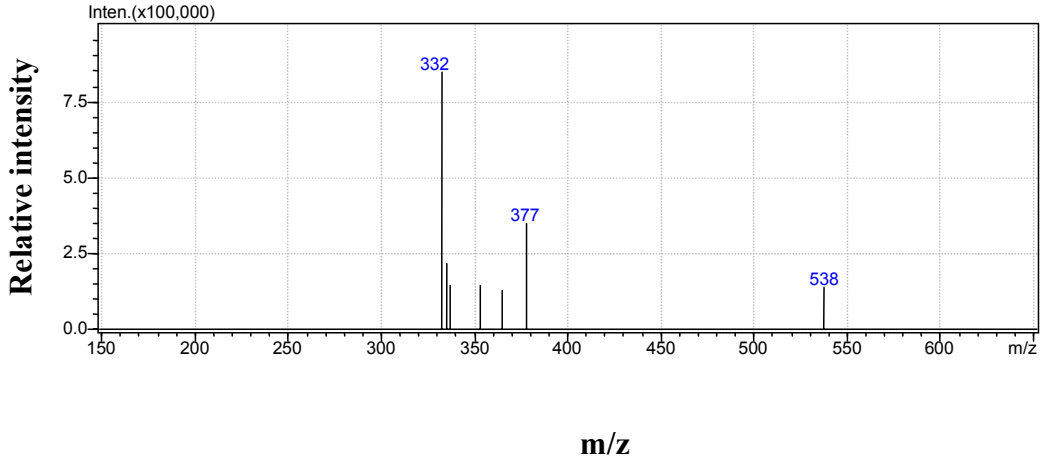
Appendix Figure C3 LC-MS negative ion spectra of peak 3 of *Careya sphaerica* Roxb.



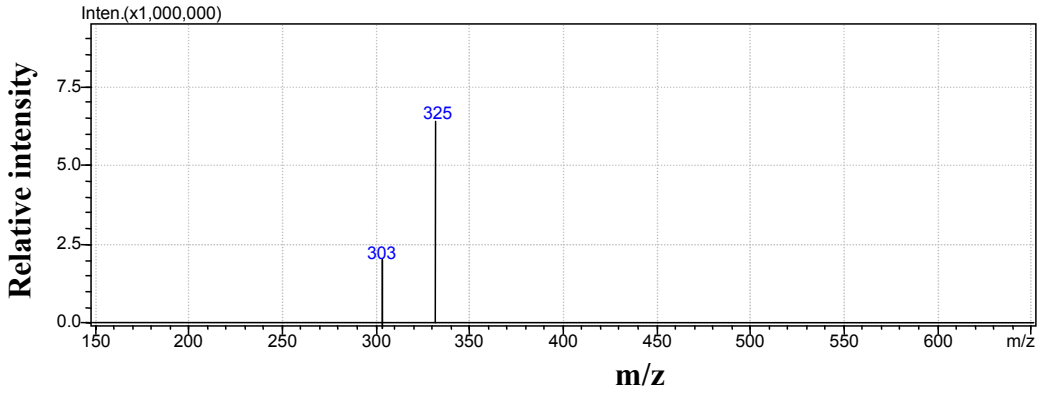
Appendix Figure C4 LC-MS positive ion spectra of peak 4 of *Careya sphaerica* Roxb.



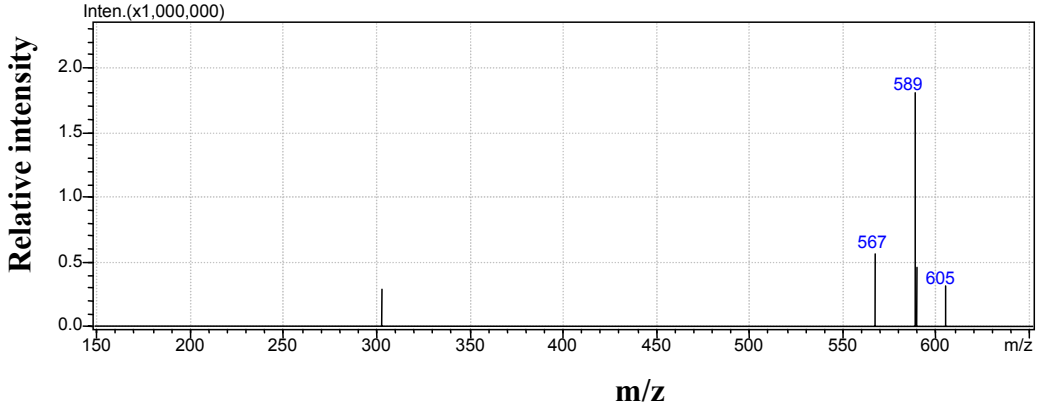
Appendix Figure C5 LC-MS positive ion spectra of peak 5 of *Careya sphaerica* Roxb.



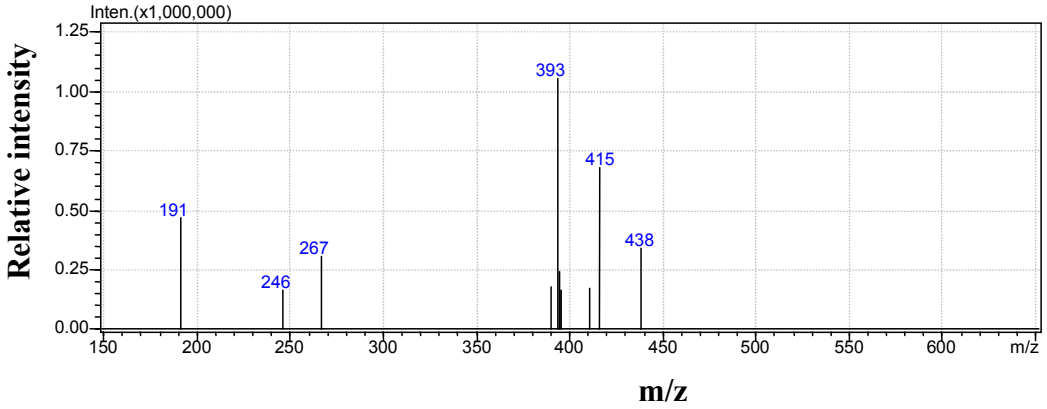
Appendix Figure C6 LC-MS positive ion spectra of peak 6 of *Careya sphaerica* Roxb.



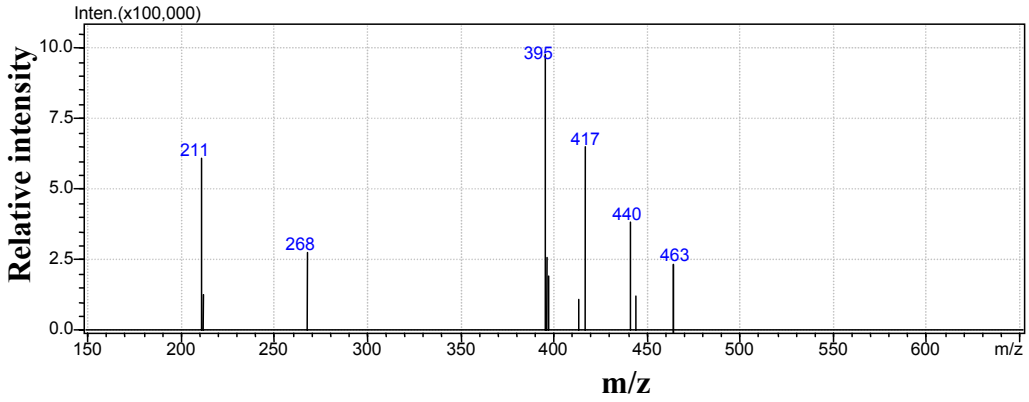
Appendix Figure C7 LC-MS positive ion spectra of peak 7 of *Careya sphaerica* Roxb.



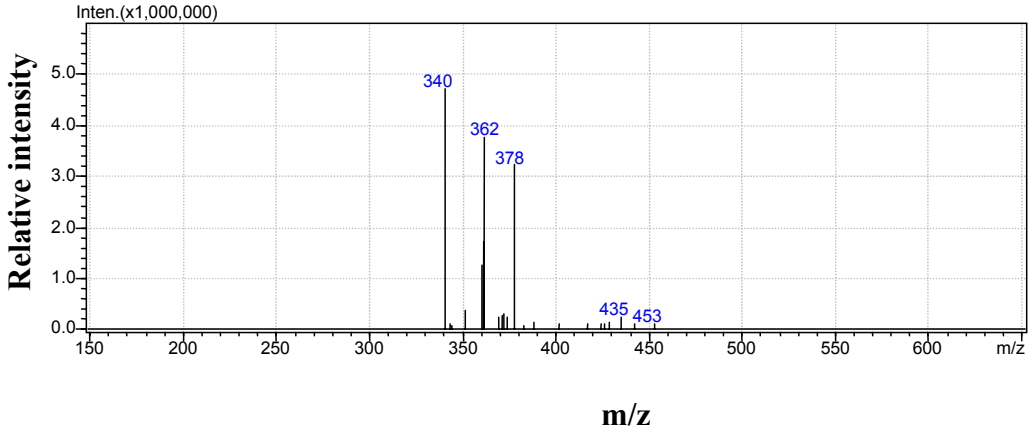
Appendix Figure C8 LC-MS positive ion spectra of peak 8 of *Careya sphaerica* Roxb.



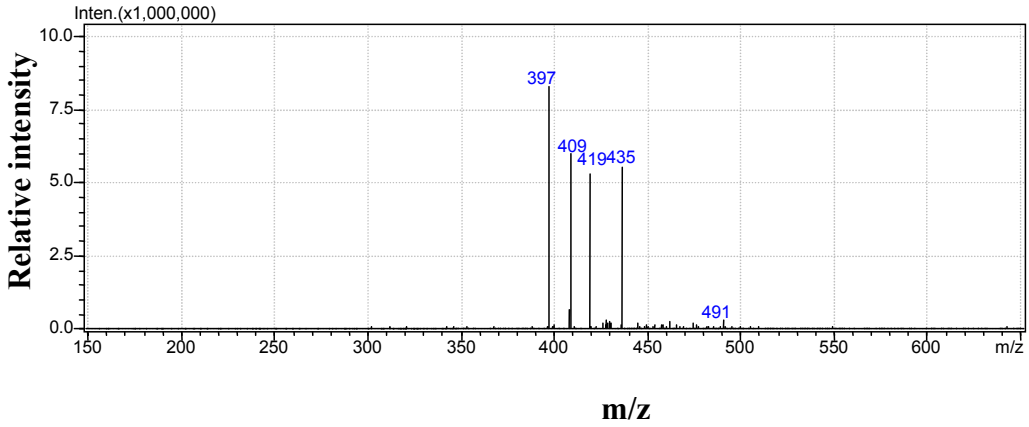
Appendix Figure C9 LC-MS positive ion spectra of peak 9 of *Careya sphaerica* Roxb.



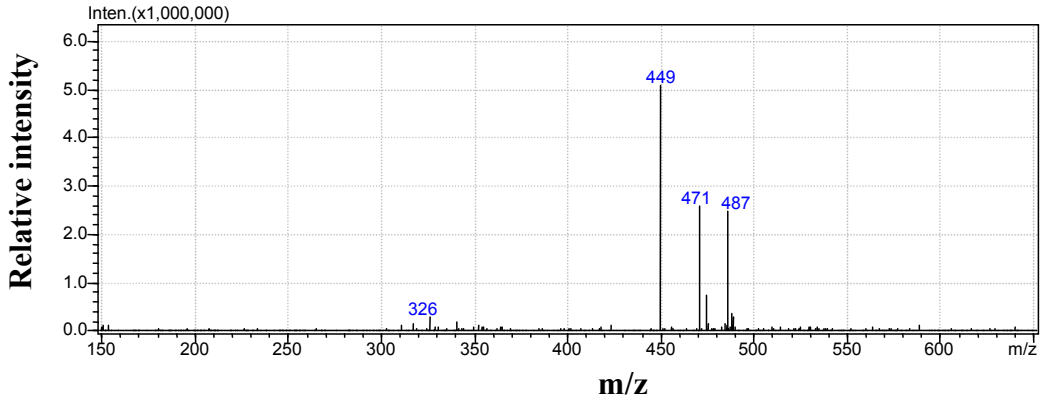
Appendix Figure C10 LC-MS positive ion spectra of peak 10 of *Careya sphaerica* Roxb.



Appendix Figure C11 LC-MS positive ion spectra of peak 11 of *Careya sphaerica* Roxb.



Appendix Figure C12 LC-MS positive ion spectra of peak 12 of *Careya sphaerica* Roxb.

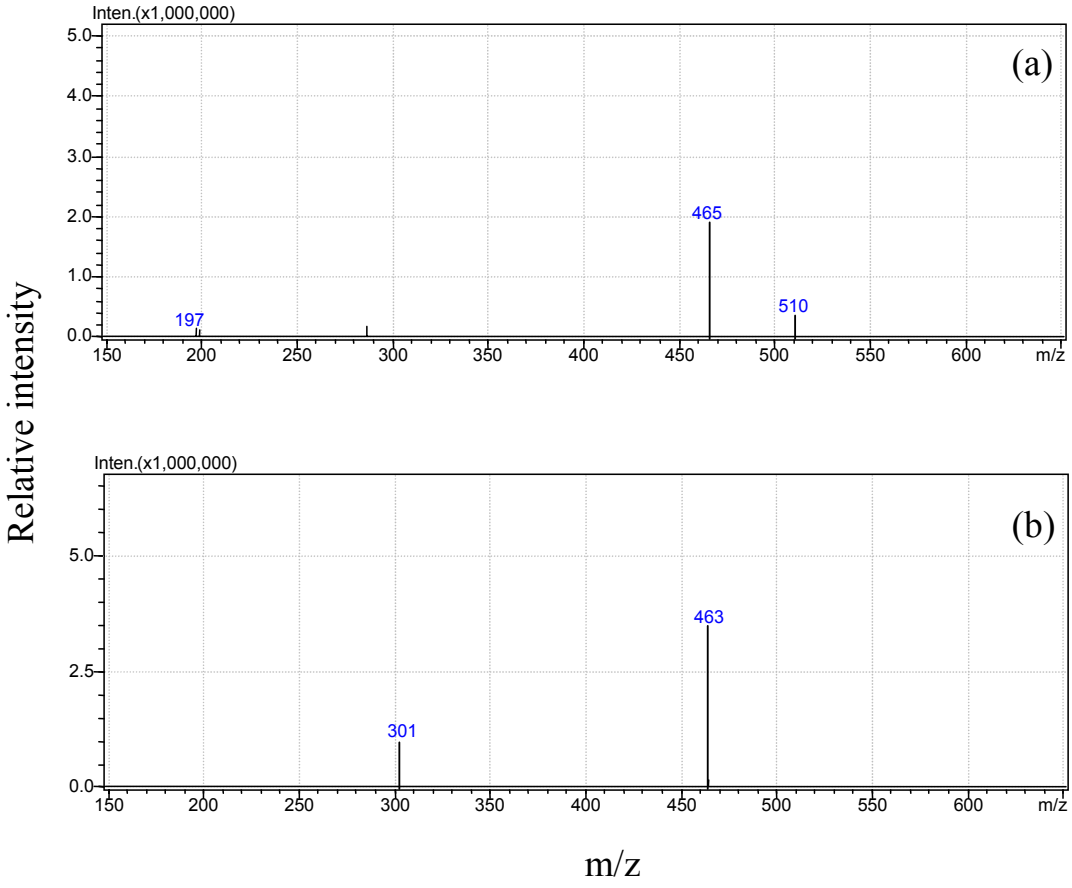


Appendix Figure C13 LC-MS positive ion spectra of peak 13 of *Careya sphaerica* Roxb.

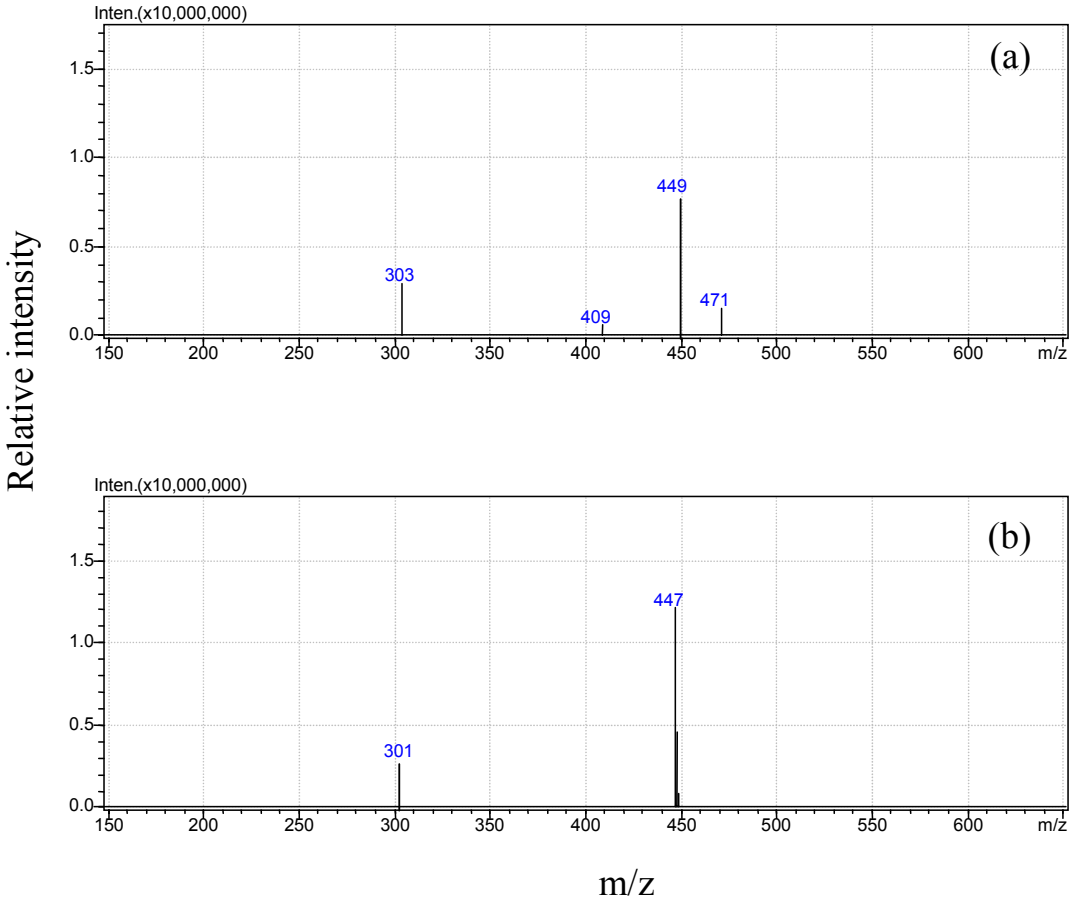
APPENDIX D

HPLC-ESI-MS spectrum of

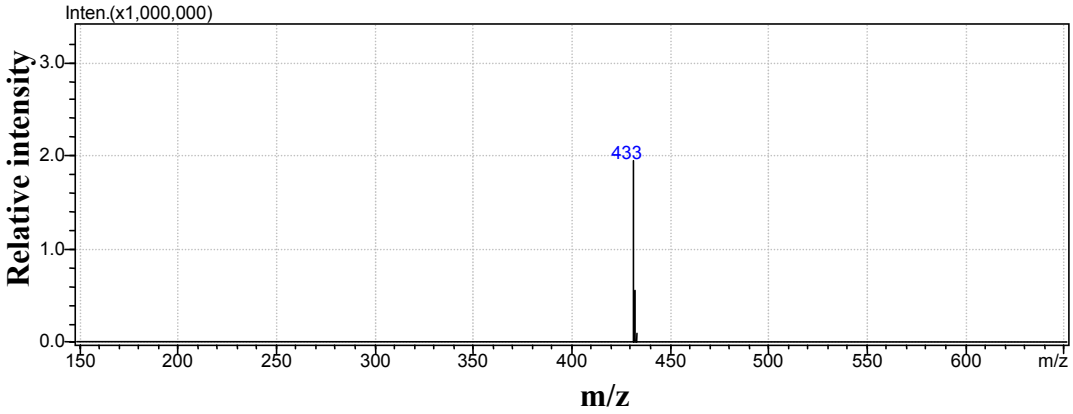
Leucaena glauca Benth



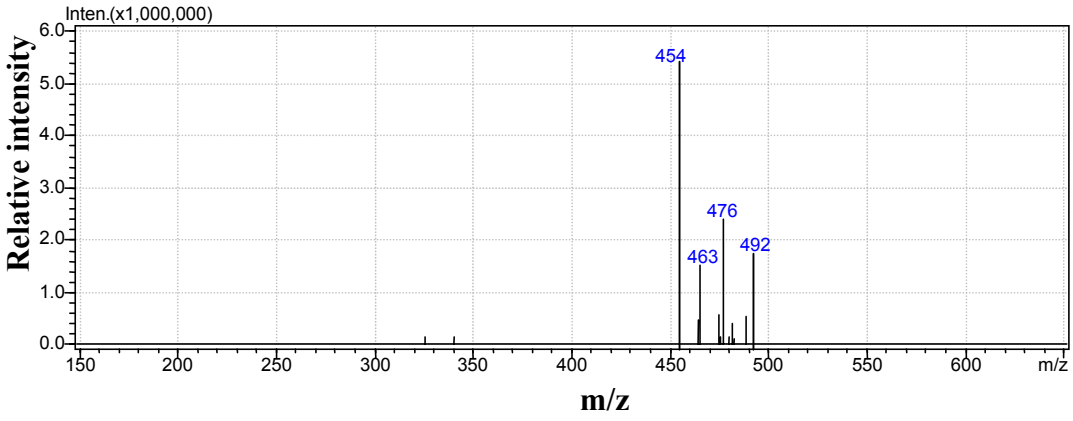
Appendix Figure D1 LC-MS (a) positive and (b) negative ion spectra of peak 1 of *Leucaena glauca* Benth.



Appendix Figure D2 LC-MS (a) positive and (b) negative ion spectra of peak 2 of *Leucaena glauca* Benth.



Appendix Figure D3 LC-MS negative ion spectra of peak 3 of *Leucaena glauca* Benth.



Appendix Figure D4 LC-MS positive ion spectra of peak 4 of *Leucaena glauca* Benth.

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