CHAPTER 1

INTRODUCTION

1.1 General introduction

In recent years, there has been increasing evidence that free radicals are associated with pathological conditions such as atherosclerosis, carcinogenesis and aging. Basically, free radicals and other reactive oxygen species including hydroxyl radical, superoxide radical and other singlet oxygen are continually formed in the human body since they are by products of a variety of pathways in aerobic metabolism. In addition, free radicals derived from the environment, especially ultraviolet radiation are important extrinsic factors accelerating aging (Ames *et al.*, 1993; Rieger, 1993; Halliwell and Gutteridge, 1989; Wickens, 2001 and Bokov *et al.*, 2004). They also cause damage to connective tissue components of the dermis, particularly collagen, leading to premature skin age and deepening wrinkles (Jenkins, 2002). Generally, the defense mechanisms of the skin against oxidative damage need antioxidant compounds such as ascorbic acid, tocopherols, selenium and antioxidant enzymes such as glutathione peroxidase and glutathione reductase. Such compounds can, therefore, diminish with aging. Currently, various antioxidants such as ascorbic acid, tocopherols and coenzyme Q_{10} have been added to products of body and skin care cosmetics touted as anti-aging to provide the skin directly with antioxidant nourishment.

Today, medicinal plants are gaining more popularity as ingredients in cosmetic formulation because of their antioxidant properties. Several Thai medicinal

plants have been reported for their antioxidant activities including *Curcuma longa* (Motterlini *et al.*, 2000), *Aloe barbadensis* (Lee *et al.*, 2000) and *Hibiscus sabdariffa* Linn. (Wang *et al.*, 2000).

Hibiscus sabdariffa Linn. (Roselle) is an annual herb belonging to Malvaceae family. It is one of the Thai medicinal plants which used for making cold drink and jams. In folk medicine, Roselle has been used as a diuretic, mild laxative and in treatment of cardiac and nerve diseases and cancer (Dermarderosian and Beutler, 2002). It has been reported that anthocyanins and protocatechuic acid extracted from Roselle showed antioxidant bioactivities to quench DPPH free radicals and protected against hepatoxicity in rats (Tseng *et al.*, 1996; Tseng *et al.*, 1997; Wang *et al.*, 2000 and Liu *et al.*, 2002). However, there is no report on the possibility of using Roselle as an antioxidant or ingredient in cosmetic preparations. In our preliminary studies, extractions of both fresh and dried Roselle calyxes were prepared as an ethanolic extract and the water extracts dried by different methods including freeze dry, vacuum dry and spray dry. All extracts were determined for their antioxidant activities using the DPPH free radical scavenging assay. It was found that all water extracts showed higher antioxidant activity than that of the ethanolic extract and, compared with the same extraction method, the fresh calyxes gave extracts with about two-fold higher activity than that of the dried calyxes. In addition, the highest yield was obtained from the vacuum dry method (46% w/w of dried calyxes). In this study, the water extract of fresh Roselle calyxes were prepared and dried using vacuum dry. The preformulation studies were carried out for its antioxidant capacity and contents of active ingredients (total phenolics and total antocyanins) as well as its

stability in both liquid and solid states. In addition, creams containing Roselle extract were formulated and evaluated for their stability.

The aims and objective of the present study were therefore on follow

1. To determine the antioxidant activity of Roselle extract by the DPPH radical scavenging assay and the liposome model system.
2. To measure the contents of total phenolics and total anthocyanins of the extract and examine the correlations between these contents and the antioxidant activity.
3. To study the stability of Roselle extract in aqueous solutions, effect of pH on its color stability and antioxidant capacity.
4. To study on the stability of Roselle extract in solid state under accelerated conditions.
5. To prepare creams containing Roselle extract and evaluate their stability and antioxidant activity of the formulations.

1.2 Literature review

1.2.1 Free radicals and skin aging

The idea that free radicals can cause aging was offered by Denham Herman in 1956. He proposed that aging and age-related disease might be due to the long term effect of oxidative damage by free radicals which, in turn, are modified by genetic and environmental factors (Wickens, 2001).

According to the free radical theory of aging, cellular senescence is a cumulative oxidative damage by free radicals, a causative factor in aging. Skin is

constantly exposed to various environmental insults such as exposure to UV and ionizing radiation, oxygen, ozone and pollutants that may deleteriously augment the normally occurring intracellular oxidative stress. Skin is also a major candidate and target of oxidative damage by free radicals. Lipids, proteins and DNA are biological sites of the skin which are candidates for oxidative damage (Kohen, 1999). The signs of skin aging are commonly associated with increased wrinkling, sagging and increased laxity (Jenkin, 2002)

Free radicals are normal biochemical intermediates of any metabolic reactions. They consist of any chemical species (atom, ions or molecules) that contain one or more unpaired electrons in their outer atomic or molecular orbital. This makes them highly unstable and violently reactive (Benedetto, 1998 and Wickens, 2001). The most important free radicals found are reactive oxygen species (ROS) which include oxygen free radicals or oxygen-centered free radicals and non radical species as illustrated in Table 1.1.

ROS are one of the major and important contributions to skin aging, skin disorders and skin diseases (Benedetto, 1998 and Kohen, 1999). They are mostly formed in mitochondria where oxygen is reduced in four sequential steps to produce water. This chain reaction produces a number of short-lived intermediates including superoxide anion ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^{\bullet}$) (Ames *et al.*, 1993). In addition, the intracellular formation of these free radicals can be stimulated by the environmental sources, especially UVA and UVB radiation (Halliwell and Gutteridge, 1989). H_2O_2 may initiate a peroxidation process in either lipids or proteins. This lipid peroxidation process may lead to a change in the fluidity of the plasma membranes resulting in a molecule leakage and a subsequent

dysfunction in its essential roles. In addition, ROS may also directly inactivate enzymes and cause protein and DNA degradation. Damage to DNA may result in deleterious process, aging, as well as onset of cancer and other pathological disorders (Kohen, 1999 and Vendemiale, *et al.*, 1999).

Table 1.1 Reactive oxygen species (ROS)

Oxygen radical		Non-radical oxygen	
Superoxide anion	$O_2^{\bullet-}$	Hydrogen peroxide	H_2O_2
Hydroxyl	$OH^{\bullet}$	Hypochlorous acid	HOCl
Peroxyl	$ROO^{\bullet}$	Ozone	O_3
Alkoxyl	$RO^{\bullet}$	Singlet oxygen	
Hydroperoxyl	$HOO^{\bullet}$		

1.2.2 Defense mechanism of skin against oxidative damage

The epidermis of the skin possesses an extremely efficient antioxidant activity that is superior to most tissues (Jenkins, 2002). There are two types of antioxidants, the enzymatic and non enzymatic antioxidants (Benedetto, 1998).

The enzymatic antioxidants including superoxide dismutases (SOD), catalase, glutathione peroxidase (GSHP) and glucose-6-phosphate dehydrogenase (G-6-PD), protect cells by hastening biochemical reaction. Moreover, thioredoxin reductase, catalase and GSHP/reductase are the main antioxidant enzymes which are involved in the protection of the epidermis against UV-radiation-generated ROS (Benedetto, 1998).

The nonenzymatic antioxidants, including ascorbic acid (vitamin C), α-tocopherol (vitamin E), retinol (vitamin A), β-carotene and glutathione, help dissipate intracellular oxidants or ROS by acting as free radical scavengers, thereby maintaining intracellular redox homeostasis and reducing the potential for cellular oxidative damage (Benedetto, 1998). However, UV radiation exposure (both acute and chronic) causes a decrease of these nonenzymatic antioxidants in cell diminishing their role as oxidant quenchers and free radical scavengers. Hence, the rationale for the suggestion that vitamins A, C, E and their derivatives might be effective in the prevention of skin damage caused by ROS that are induced by UV radiation (Benedetto, 1998).

1.2.3 *Hibiscus sabdariffa* Linn. (Roselle)

Roselle is a potential source of antioxidant due to being high in phenolic compounds, especially anthocyanins. This plant belongs to Malvaceae family where it is known by different synonyms and vernacular names including roselle, sorrel, red sorrel, Jamaica sorrel and karkade. It is an annual herb that grows up to 150 cm in height or more. Flowers are red to yellow with a dark center containing short-peduncles as present. The calyx enlarges at maturity as shown in Figure 1.1 (Morton, 1987). Roselle is a folk remedy for abscesses, heart ailments and hypertension (Akindahunsi and Olaleye, 2003). In Thailand, its red calyxes are used for making cold drinks and jam and also as traditional medicines including astringent, antihypercholesterolemic, antidiabetic, diuretic, digestive, expectorant, stomachic, antihypertensive, and for treatment of gallstones (Farnsworth and Bunyapraphatsara, 1992 and Cheeptham and Tower, 2002). The chemical constituents found in Roselle

are demonstrated in Table 1.2 along with the structures of those compounds which were of interest in Figure 1.2.

Figure 1.1 Calyxes of *Hibiscus sabdariffa* Linn.

Table 1.2 Chemical constituents of various parts of Roselle.

Plant part	Chemical substance	Yield (%)	Reference
Calyx	Anthocyanins:		
	Delphinidin-3-sambubioside (1)[#]	1.5%[a]	Du and Francis, 1974
	Delphinidin-3-glucoside (2)		Du and Francis, 1974
	Cyanidin-3-sambubioside (3)		Du and Francis, 1974
	Cyanidin-3-glucoside (4)		Du and Francis, 1974
	Flavonoids:		
	Hibiscetin (5)	N/A	Abbas *et al.*, 1993
	Gossypetin (6)	N/A	Hirunpanich *et al.*, 2005
	Quercetin (7), Myricetin (8)	N/A	Abbas *et al.*, 1993

[#] Structure indicated in Figure1.2.

[a] expressed as delphinidin-3-glucoside.

N/A : data not available

Table 1.2 (continued).

Plant part	Chemical substance	Yield (%)	Reference
Calyx	Flavoniod glycoside:		
	Hibiscitrin (9)[#]	N/A	Abbas *et al.*, 1993
	Organic acids:		
	Ascorbic acid	0.014 %	Duke and Atchley, 1984
	Hibiscus acid	N/A	Griebel, 1942
	Citric acid, Malic acid, Tartaric	N/A	Reaubourg and Monceaux, 1940
	acid, Oxalic acid, Gallic acid and	N/A	
	Lactic acid	N/A	
	Phenolic acids:		
	o-Coumaric acid,	N/A	Abbas *et al.*, 1993
	p-Coumaric acid and Feruric acid	N/A	Abbas *et al.*, 1993
	Polysacharides:		
	Hemicellulose	N/A	Abbas *et al.*, 1993
	Pectic substances	N/A	Abbas *et al.*, 1993
	Mucopolysaccharide	N/A	Hirunpanich *et al.*, 2005
Flower	Flavonoid:		
	Gossypetin	0.04 %	Mounnissamy *et al.*, 2002
	Flavonoid glycosides:		
	Gossytrin (10)	N/A	Seshadri and Thakur, 1961
	Gossypitrin (11)	N/A	
	Gossypin (12)	N/A	

[#] Structure indicated in Figure1.2

N/A : data not available

Table 1.2 (continued).

Plant part	Chemical substance	Yield (%)	Reference
Flower	Phenolic acid:	N/A	Tseng et al., 1996
	Protocatechuic acid (13)[#]		
	Organic acids:		
	Hibiscus acid (14)	N/A	Ahmad *et al.*, 2000
	garcinia acid (15)	N/A	Ahmad *et al.*, 2000
	Glycolic acid, Citric acid, Malic	N/A	Fransworth and
	acid, Tartaric acid, L-Ascorbic	N/A	Bunyapraphatsara, 1992
	acid, Aspartic acid and Oxalic acid	N/A	
	Mucopolysaccharides:		
	Rhamnogalacturonan,	N/A	Muller and Franz, 1992
	Arabinogalactan and arabinan	N/A	Muller and Franz, 1992
	Vitamins:		
	β-carotene, Niacin, Riboflavin	N/A	Duke, 1992
	Thiamine	N/A	Duke, 1992
Leave	Flavonoid glycosides:		
	Kaempferol-3-O-glucoside (16)	N/A	Akiyoshi *et al.*, 2005
	Kaempferol-3-O-rutinoside (17)	N/A	Akiyoshi *et al.*, 2005
	Quercetin-3-O-rutinoside (18)	N/A	Akiyoshi *et al.*, 2005
	Glycosides:		
	Citrusin C (19)	N/A	Akiyoshi *et al.*, 2005
	Corchoionoside C (20)	N/A	Akiyoshi *et al.*, 2005

[#] Structure indicated in Figure1.2

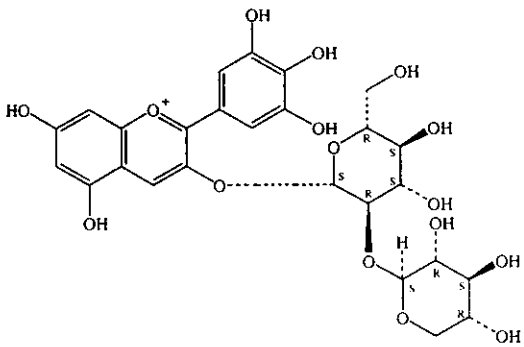
N/A: data not available

Table 1.2 (continued).

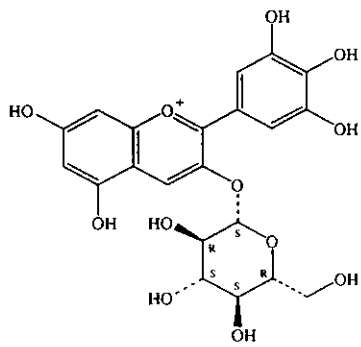
Plant part	Chemical substance	Yield (%)	Reference
Leave	Phytosterol:		
	β-Sitosterol-1-3-β-D galactoside	N/A	Osman *et al.*, 1975
	Vitamins:		
	β-carotene, Niacin, Riboflavin and Thiamine	N/A	Duke, 1992
Seed	Fatty acids:		
	Linoleic acid	14.6 %	Ahmad *et al.*, 1979
	Malvalic acid	1.3 %	Ahmad *et al.*, 1979
	Myristic acid	2.1 %	Ahmad *et al.*, 1979
	Oleic acid	34.0 %	Ahmad *et al.*, 1979
	Palmitic acid	35.2 %	Ahmad *et al.*, 1979
	Palmitoleic acid	2.0 %	Ahmad *et al.*, 1979
	Stearic acid	3.4 %	Ahmad *et al.*, 1979
	Sterculic acid	2.9 %	Ahmad *et al.*, 1979
	Phytosterols:		
	Campesterol	16.5 %	Salama and Ibrahim, 1979
	Cholesterol	5.1 %	Salama and Ibrahim, 1979
	Ergosterol	3.2 %	Salama and Ibrahim, 1979
	β-Sitosterol	61.3 %	Salama and Ibrahim, 1979
	α-Spinasterol	10 %	Salama and Ibrahim, 1979
	Stigmasterol	4.1 %	Salama and Ibrahim, 1979
	Terpene:		
	Gossypol	25.2 %	Wandawi *et al.*, 1984

N/A: data not available

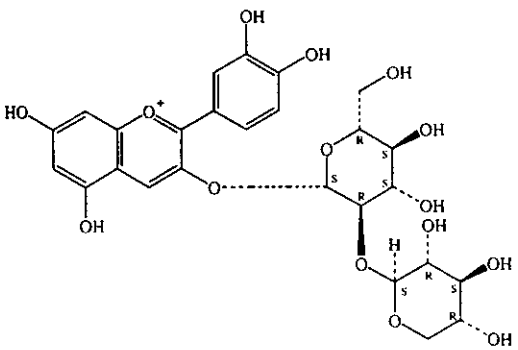
Anthocyanins



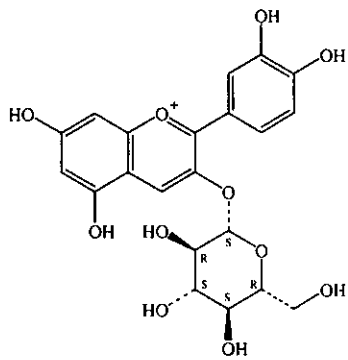
Delphinidin-3-sambubioside (1)



Delphinidin-3-glucoside (2)

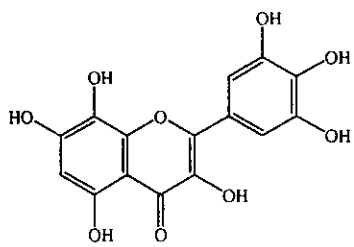


Cyanidin-3-sambubioside (3)

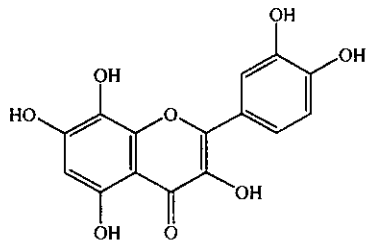


Cyanidin-3-glucoside (4)

Flavonoids



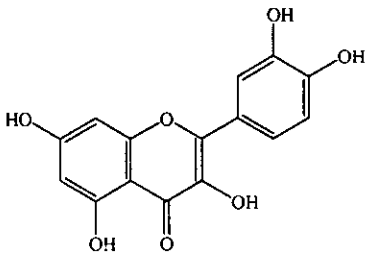
Hibiscetin (5)



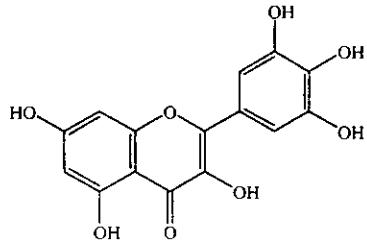
Gossypetin (6)

Figure 1.2 Structure of compounds which were of interested in Table 1.2.

Flavonoids (continued)

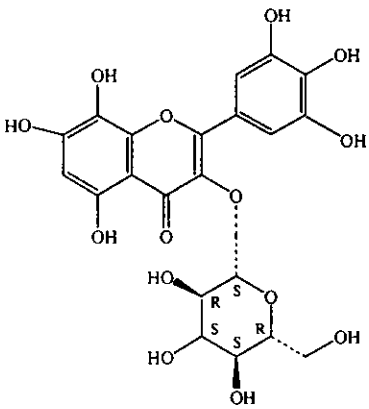


Quercetin (7)

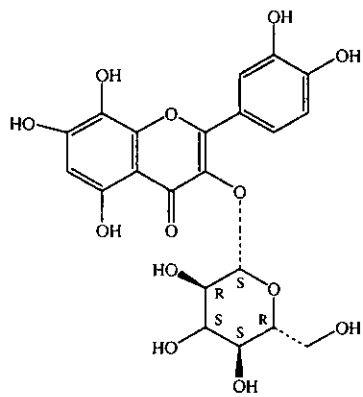


Myricetin (8)

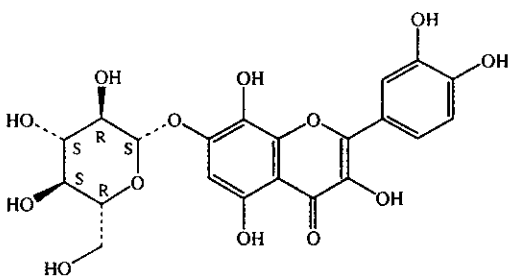
Flavonoid glycosides



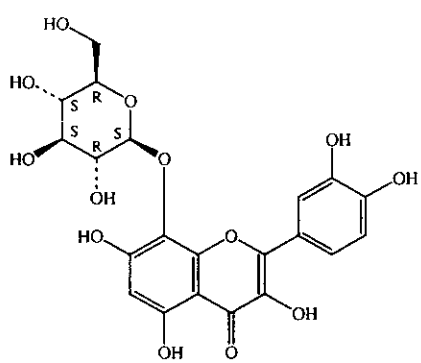
Hibiscitrin (9)



Gossytrin (10)



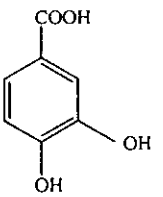
Gossypitrin (11)



Gossypin (12)

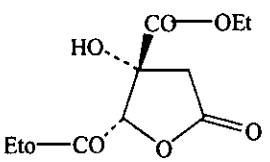
Figure 1.2 (continued).

Phenolic acid

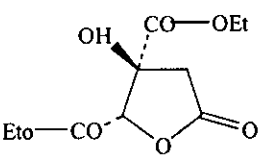


Protocatechuic acid (13)

Acids



Hibiscus acid (14)



Garcinia acid (15)

Figure 1.2 (continued).

Since the phenolic compounds such as anthocyanins, flavonoids and phenolic acids are attributed with antioxidant activity and chelating ability (Heim *et al.*, 2002), the significance of phenolic compounds in Roselle has been of considerable interest. There were many reports on pharmacological activities of these components including antihypertensive activity (Onyenekwe *et al.*, 1999; Odigie *et al.*, 2003; Haji-Faraji and Haji Tarkhani, 1999), antiatherosclerotic activity (Chen *et al.*, 2003; Lee *et al.*, 2002 and Hirunpanich *et al.*, 2005), angioprotective activity (Jonadet, *et al.*, 1990), antihyperlipidemic activity (Aboutabl *et al.*, 1999, Chen *et al.*,

2004, Hirunpanich *et al.*, 2005), antileukemia (Chang *et al.*, 2005), antitumor promotion effect (Tseng *et al.*, 1998), antimutagenic activity (Chewonarin *et al.*, 1999), antiinflammatory activity (Mounnissamy *et al.*, 2002), antimicrobial activity (Cheeptham and Towers, 2002), heapatoprotective in rat (Amin and Hamza, 2005) and protective effect on CCl_4-induced rat liver fibrosis (Liu *et al.*, 2005).

1.2.4 Antioxidant activities of Roselle extract

Hibiscus anthocyanins isolated from dried Roselle flowers showed antioxidant bioactivities to quench DPPH free radicals (EC_{50} = 0.20 mg/mL) *in vitro* experiment. These anthocyanins exhibited protective effects against cytotoxicity of hepatocytes at concentration 0.10-0.20 mg/mL. In addition, the *in vivo* investigation showed that the oral pretreatment of hibiscus anthocyanins (100 and 200 mg/kg) for 5 days before a single dose of *tert*-butyl hydroperoxide (*t*-BHP) significantly reduced oxidative liver damage in rat (Wang *et al.*, 2000).

Hibiscus protocatechuic acid (PCA), a simple phenolic compound isolated from dried flowers of Roselle, showed antioxidant activities in several models. At concentration of 0.01 and 0.10 mg/mL, it scavenged about 58% ($p < 0.01$) and 82% ($p < 0.01$) of DPPH radicals in solution, respectively. In addition, it has been reported that PCA exhibited an effective ability to quench DPPH free radicals (EC_{50} = 0.01 mg/mL). In addition, it has been reported that PCA at concentrations of 0.05 and 0.10 mg/mL exhibited antioxidant activity to protect primary cultured rat hepatocytes against hepatotoxicity induced by *t*-BHP (Tseng *et al.*, 1996). With its antioxidant and anti-inflammation characteristics, PCA showed *in vivo* protective

effect on *t*-BHP induced rat hepatotoxicity after pretreatment at concentration of 50-100 mg/kg for 5 days (Liu *et al.*, 2002).

Three fractions of the ethanol crude extract (HS-C: chloroform-soluble fraction; HS-E: ethyl acetate soluble fraction and HS-R: residual fraction) from dried flowers of Roselle were found to possess antioxidant activities. HS-E showed the greatest capacity of scavenging free radicals (EC_{50} = 0.017 mg/mL) while HS-C showed the strongest inhibitory effect on xanthine oxidase (XO) activity (EC_{50} = 0.742 mg/mL). Furthermore, HS-C and HS-E showed antioxidant bioactivities by protecting against *t*-BHP induced hepatic cytotoxicity and genotoxicity, whereas HS-R showed only efficient protective action against *t*-BHP induced hepatic genotoxicity (Tseng *et al.*, 1997)

The water crude extract isolated from dried calyx of roselle showed markedly antioxidative activity. Duh and Yen (1997) reported that the decolorized water extract of Roselle at concentration of 25 mg/mL showed 88.6 % inhibition of the DPPH radical in solution and the water extract (0.1 mg) showed about 62.8% inhibition of peroxidation of lecithin. Chen (2003) showed that the feeding the water extract of Roselle (0.5 and 1% in the diet, for 10 weeks) to rabbits significantly reduced severe atherosclerosis in the aorta. In addition, it has been reported that the water extract of dried Roselle flowers exhibited the protective effect against liver fibrosis induced using carbon tetrachloride in rats after dosing 1-5% extract for 9 weeks (Liu *et al.*, 2005)

1.2.5 Toxicological studies of Roselle extract

The aqueous fraction of aqueous-alcoholic extract of Roselle calyx was orally given to Wistar albino rats as drugs as a study for toxicity. It was found that the prolonged usage of the extract at 15-dose level (250 mg/kg, once per day) could cause liver injury while the effect was mild at small dose levels (1-10). Though the average consumption of 150-180 mg/kg per day appears safe, the extract should be taken with caution bearing in mind that a higher dose could affect the liver (Akindahunsi and Olaleye, 2003). In addition, the sub-chronic effect of the aqueous extract of Roselle given at dose levels of 1.15, 2.3 and 4.6 g/kg for 12 weeks showed evidence of nephrotoxic in rats (Orisakwe *et al.*, 2003) and testicular toxicity (Orisakwe *et al.*, 2004). However, with the same dosing regimen, it did not cause cardiotoxicity in rats (Hussaini *et al.*, 2004).

1.2.6 Stability studies of Roselle extract

In Roselle extract, most anthocyanins exist as monomer (Tsai and Huang, 2004). However, these pigments are quite unstable during both processing and storage. Light, temperature and pH, oxygen, ascorbic acid and sugar may enhance their decomposition rates and promote polymerization (Morais *et al.*, 2002 and Tsai and Huang, 2004).

A study by Tsai *et al.* (2002) showed that the anthocyanin contents were decreased due to conversion of some monomeric anthocyanins into polymerized phenolics after drying at 75 °C and storage at 40 °C for 15 weeks. However, its antioxidant level did not substantially decrease. In addition, the works of Tsai and Huang (2004) found that monomeric anthocyanins changed into polymeric forms after

heating at 90 °C for 147 hours and these polymeric forms promoted antioxidant activity.

The kinetic studies on the thermal degradation of isolated Roselle anthocyanins, which are delphinidin-3-sambubioside and cyaniding-3-sambubioside, were investigated by Gradinaru *et al.* (2003) in both solutions and dry states. In solution, the degradation kinetics of Roselle anthocyanins followed first-order kinetics after examining at four different temperatures (55, 70, 85 and 90 °C). In dry state, it was found that their degradation rate constants increased with the water activity, particularly above 0.53. Free anthocyanins stored at higher relative humidity level showed faster degradation. Despite color fading after storage at different water activity (a_w) environments, the breakdown products of anthocyanins still exhibit significant antiradical power. These results suggest that anthocyanins presented in food products may continue to provide their antioxidant even after some color loss has occurred during processing and storage.